617. *The Detection of Glycosides and Non-reducing Carbohydrate Derivatives in Paper Partition Chromatography.*

By J. G. Buchanan, C. A. Dekker, and A. G. Long.

Non-reducing sugar derivatives and 1 : 2-glycols may be detected on paper chromatograms by methods depending on their specific reactions with sodium metaperiodate or lead tetra-acetate. The Dische and the Feulgen reaction have also been adapted for use in paper chromatography. These methods have been used successfully to investigate mixtures of nucleosides and nucleotides.

The application of paper chromatography to the separation and identification of carbohydrates has been extensively investigated (Hirst and Jones, *Faraday Soc. Discussions*, 1949, No. 7, p. 268; Partridge, *Biochem Soc. Symposia*, 1949, No. 3, p. 52; Hough, Jones, and Wadman, *J.*, 1950, 1702). For the most part the methods adopted have involved location of the compounds on the chromatograms by chemical reactions dependent on their reducing properties. Such methods are clearly inapplicable to non-reducing carbohydrate derivatives save in the case of certain

disaccharides which undergo hydrolysis under the comparatively vigorous conditions employed. Attempts to meet this difficulty have been made by Pacsu, Mora, and Kent (*Science*, 1949, **110**, 446), who used alkaline permanganate as a spraying solution, and by Hough (*Nature*, 1950, **165**, 400), who exploited the surprising reactivity of certain non-reducing carbohydrates towards ammoniacal silver nitrate. We have observed that potassium mercuri-iodide is similarly reduced and the site of reaction is located by the appearance of a metallic zone.

Such reagents as the above are by no means specific and since a general method for detecting and identifying non-reducing carbohydrate derivatives was necessary for various investigations current in this laboratory it was decided to utilise for the purpose the peculiar properties of polyhydroxy-compounds. The ability of *cis*-diols to form boric acid complexes (Böeseken, *Ber.*, 1913, **46**, 2612 : *Rec. Trav. chim.*, 1921, **40**, 354, 553), which are stronger acids than boric acid itself, makes it possible to detect *cis*-diols by means of a borate-indicator spray. Unfortunately, such differences in acidity could only be detected if inconveniently large amounts of material were used in the chromatography, but nevertheless mannitol and sorbitol could be located by using tetrabromophenolphthalein ethyl ester as the indicator (cf. Feigl, " Qualitative Analysis by Spot Tests," Elsevier, 1939, p. 345).

Feigl (*op. cit.*, p. 272) had described a method for detecting 1 : 2-glycols, on a micro-scale, by periodic acid. This technique has now been adapted for use in paper chromatography. After oxidation, the iodate and excess of periodate were reduced with sulphur dioxide, and the aldehydes detected with Schiff's reagent. Dialdehydes gave stable blue or purple stains, but mono-aldehydes reacted more feebly, and precautions had to be taken to prevent their destruction by aerial oxidation. While this work was in progress we became aware that Hotchkiss (*Archiv Biochem.*, 1948, **16**, 131) had used a similar technique for staining certain cell constituents. This author's theory of the formation of dialdehyde–dye complexes receives some support from our work.

The method gave positive results with every 1 : 2-glycol tested (see Tables I and II, where R_F values of compounds which react positively are given), and was satisfactory for quantities of 10 μg. or, in many cases, less. As a means of identifying the components of mixtures of unknown reducing and non-reducing carbohydrates, it can be applied in conjunction with other spraying reagents (*e.g.*, aniline hydrogen oxalate, which is specific for reducing carbohydrates). In the case of 1 : 2 : 3-triols, formic acid occurs among the periodate oxidation products (Jackson, " Org. Reactions," 1944, Vol. II, p. 341). This too could be detected on the paper, even in the presence of weak organic acids or bases, by converting excess of periodate into iodate with ethylene glycol and subsequently applying potassium iodide solution. Zones containing formic acid were marked by an iodine stain, the acid being strong enough to promote reaction between the iodide and the iodate. The terminal system ·CH(OH)·CH_2·OH in a molecule yields formaldehyde during periodate oxidation. Attempts to detect this aldehyde by using chromotropic acid (Feigl, *op. cit.*, p. 327) were successful only when relatively large amounts of the original diol were used, because the accompanying iodide and sulphurous acid, or iodate, appeared to render the colour reaction less sensitive and very slow.

A benzene solution of lead tetra-acetate applied as a spray furnished an alternative method of detecting 1 : 2-glycols. When the solvent had evaporated from the paper, the unused tetra-acetate was hydrolysed to brown lead dioxide so that the glycols appeared as white zones on a brown background. This reagent was also applied successfully to α-hydroxy-acids, pyruvic acid, and dihydroxyacetone. In no case was the reaction sufficiently vigorous to effect any noticeable oxidation of the paper itself.

These methods are widely applicable. Thus, for example, by using either periodate or lead tetra-acetate it was a simple matter to follow the separation of 6 : 7- and 9 : 10-dihydroxystearic acids * and of the DL- and *meso*-forms of tartaric acid. We were particularly interested in the success of the periodate method with glycosides, especially nucleosides and nucleotides. The methods available for the detection of such compounds on a chromatogram have hitherto depended on the presence of a purine or pyrimidine nucleus (for summary, see Beale, Harris, and Roe, *J*, 1950, 1397), or of a phosphate residue (Hanes and Isherwood, *Nature*, 1949, **164**, 1107), and a technique for the detection of the carbohydrate component would clearly be of value. We have found that the oxidative methods described above are satisfactory for this purpose.

Periodate oxidation of *N*-glycosides was first used by Lythgoe and Todd to investigate the size of the lactol ring in natural and synthetic nucleosides (*J.*, 1944, 592; Davoll, Lythgoe, and Todd, *J.*, 1946, 833), and to establish the position of the phosphate residue in the naturally occurring nucleotides (*Nature*, 1945, **155**, 695). The furanoside nature of the deoxyribose com-

* Geneva numbering (CO_2H = 1).

ponent of four deoxyribonucleosides has also been confirmed by this method (Brown and Lythgoe, *J.*, 1950, 1990). The periodate spraying technique has now been applied to these compounds, and in every case the results have been in full agreement with those of Lythgoe and Todd. Purple or blue colours have been obtained with all the ribonucleosides and with muscle adenylic acid, but none of the nucleotides derived from yeast ribonucleic acid shows any reaction. The R_F values of a number of compounds giving a positive reaction under these conditions are given in Table II. Application of these methods to the purine glycopyranosides synthesised in this laboratory (see Todd, *J.*, 1946, 647) showed that, as expected, they were oxidised to dialdehydes which gave the characteristic colours with Schiff's reagent, and also gave formic acid which was detected as described above. When 2′ : 3′-*iso*propylidene adenosine was treated with neutral sodium metaperiodate in the usual way, no oxidation took place, but when acidic periodate was used as the spraying reagent, a blue-purple spot was obtained; the acidic reagent effected removal of the *iso*propylidene residue, and periodate oxidation followed. This may have an application in following reactions in the synthesis of nucleotides where the *iso*propylidene group is frequently used to protect $C_{(2)}$ and $C_{(3)}$ of the ribose unit.

The sensitivity of this technique for the detection of nucleosides varied considerably with the nature of the sugar. Ribofuranosides were detectable in quantities of 20 μg., but for some of the other synthetic glycosides the method was less sensitive. The oxidation of the *cis*-α-glycol system of the naturally occurring nucleosides takes place very rapidly on paper. Attempts to increase the sensitivity of the reaction for the synthetic nucleosides by varying the strength of the periodate solution and by lengthening the time of reaction have been unsuccessful. This failure was due, in all probability, to simultaneous oxidation of the filter paper, which contains glycopyranose units. 9-β-D-Glucopyranosyl- and 9-α-D-arabofuranosyl-adenine gave especially weak reactions, and neither 9-β-D-mannopyranosyl- nor 9-β-D-ribopyranosyl-adenine gave a colour equal to that of the ribofuranoside, adenosine. The reasons for this are not quite clear, since both of the latter pair of compounds have *cis*-hydroxyl groups, and, moreover, the mannoside gives the same dialdehyde on oxidation as does adenosine (Lythgoe, Smith, and Todd, *J.*, 1947, 355).

Another method of determining whether periodate oxidation has occurred is to treat the substance with periodate, destroy the excess of periodate with glycerol or ethylene glycol, and subject the resulting solution to paper chromatography. If the compound contains an α-glycol system, its oxidation product travels at a markedly different rate (cf. Buchanan, Johnson, Mills and Todd, *J.*, 1950, in the press). The lead tetra-acetate spray has also been applied to the location of nucleosides on paper. Ribosides were easily detected, but the method was less satisfactory for those compounds containing *trans*-hydroxyl groups.

The deoxyribonucleosides derived from deoxyribonucleic acids are not susceptible to periodate oxidation (Brown and Lythgoe, *loc. cit.*). Other methods for their detection on a paper chromatogram were therefore sought. It was considered practicable to adapt existing colorimetric methods for our purpose. Both the Dische reaction (Dische, *Mikrochemie*, 1930, **8**, 4), and the Feulgen reaction (Feulgen and Rossenbeck, *Z. physiol. Chem.*, 1924, **135**, 203) were found to be suitable, after difficulties caused by the destruction of the paper by the strong acids employed had been overcome (see Experimental). The R_F values of several natural deoxyribonucleosides are recorded in Table III. These two reactions have been re-investigated recently (Deriaz, Stacey, Teece, and Wiggins, *J.*, 1949, 1222; Overend and Stacey, *Nature*, 1949, **163**, 538) and their mechanism and specificity have been examined and clarified. It was observed that the great difference in the rate of acid hydrolysis of purine and pyrimidine deoxyribonucleosides enabled a distinction between these two types of glycoside to be made, using either the Dische or the Feulgen reagent. Indeed, the lability of the glycosidic linkage in the purine deoxyribonucleosides (cf. Chargaff, *Experientia*, 1950, **6**, 204) was such that, when the periodate spray technique was employed, a pink colour developed due to acid hydrolysis followed by a Feulgen reaction. This effect was not observed with the natural pyrimidine deoxyribosides. Some synthetic 2-deoxyglycopyranosides were available to us (Davoll and Lythgoe, *J.*, 1949, 2526), and these compounds, whether of the purine or pyrimidine series, were oxidised by sodium metaperiodate, and the products gave brownish-purple colours with Schiff's reagent.

Experimental.

The paper chromatography was carried out with a descending solvent in the manner of Consden, Gordon, and Martin (*Biochem. J.*, 1944, **38**, 224). Purified solvents were employed throughout. Unless otherwise stated, Whatman No. 1 filter paper was used.

Periodate Oxidation, followed by Treatment with Schiff's Reagent.—Two standard solutions were required : (A) 2% aqueous sodium metaperiodate, (B) Schiff's reagent, prepared by dissolving rosaniline (1 g.) in water (50 c.c.), followed by decolorisation with sulphur dioxide (charcoal) and dilution to 1 l. with water.

The paper chromatogram was dried for 5 minutes at 80° to remove the solvent. A thin spray of solution A was applied, and the oxidation was allowed to proceed for 7 minutes at 60° in an atmosphere of nitrogen. For the purpose of incubation, the paper was placed in a beaker on an edge perpendicular to the solvent front so that only lateral displacement of the substance could occur. Sulphur dioxide was passed into the beaker until most or all of the iodine liberated was consumed, and the paper was finally sprayed with solution B. Coloured zones usually appeared within 3 hours, and in all cases in less than a day. This process was accelerated considerably by heating the paper at 60° for 10 minutes.

In the case of the nucleosides, the procedure was modified slightly. A 1% aqueous solution of sodium metaperiodate was used as solution A and oxidation was allowed to take place for 10 minutes at room temperature in the open air. The colours with Schiff's reagent (solution B) were developed by heating the paper for about 10 minutes at 85—90°.

Periodate Oxidation : Detection of Formic Acid.—Two additional solutions were employed : (C) 10% aqueous ethylene glycol; (D) 5% aqueous potassium iodide.

The solvents were removed as before, and the periodate spray applied. After 7 minutes, solution C was sprayed on the paper. 10 Minutes were allowed for the destruction of the excess of periodate, the paper being kept in a nitrogen atmosphere as before. Solution D was applied and the positions of the 1 : 2 : 3-triols were indicated by iodine stains on the paper.

Lead Tetra-acetate Oxidation.—The reagent was prepared by dissolving lead tetra-acetate (1 g.) in benzene (100 c.c.). The solution was decolorised (charcoal) and filtered, if necessary.

The dry paper was first moistened with a thin spray of xylene. The reagent was applied and the solvents were allowed to evaporate at room temperature. Where oxidation had occurred white zones appeared on a brown background.

General.—All the compounds listed in Table I reacted with both periodate and lead tetra-acetate, except for citric acid, lactic acid, and pyruvic acid, which could be located only with the latter reagent. Formic acid was detected after the periodate oxidation of glucose, glycerol, mannitol, sorbitol, sucrose, and maltose.

TABLE I.

Compound.	R_F value. *n*-Butanol(4)–acetic acid(1)–water(5).	R_F value. *n*-Butanol(20)–acetic acid(5)–water(25)–conc. HCl(1).[1]
Glucose	0·19	—
Ethylene glycol	0·64	0·69
Glycerol	0·48	0·52
Mannitol	0·21	0·26
Sorbitol	0·19	0·25
Inositol	0·09	—
Lactose	0·04	—
Maltose	0·05	—
Raffinose	0·03	—
Sucrose	0·08	—
Trehalose	—	0·03 [2]
Potassium glucose-1 phosphate	0·03	—
α-Methylglucopyranoside	0·34	—
Gluconic acid	0 → 0·24 ; 0·41 [3]	0·18 ; 0·45 [3]
DL-Tartaric acid	—	0·48
*meso*Tartaric acid	—	0·41
erythro-9 : 10-Dihydroxystearic acid	—	0·52 → 0·94 [3]
erythro-6 : 7-Dihydroxystearic acid	—	0·91
Citric acid	—	0·45 [2]
Lactic acid	—	0·68 ; 0·78 [2, 3]
Pyruvic acid	—	0·77 [2]
Dihydroxyacetone	—	0·38 [2]

[1] Mineral acid was used to depress the ionisation of the carboxylic acids (cf. Lugg and Overell, *Nature*, 1947, **160**, 87; *Australian J. Sci. Res.*, 1948, *A*, **1**, 98). [2] Whatman No. 54 paper was used. In this case the solvent front moved very rapidly (cf. Kowkabany and Cassidy, *Analyt. Chem.*, 1950, **22**, 817). [3] Certain carboxylic acids gave diffuse, or even multiple, zones on the paper. Separate zones are given their respective R_F values, while the extent of the diffuse regions is indicated. Analogous effects have been noted by Lugg and Overell and by Hanes and Isherwood (*locc. cit.*).

Table II contains a list of nucleosides and nucleotides to which the periodate method was applied and which gave a positive reaction. The following gave no reaction : adenine, cytosine, hypoxanthine, thymine, uracil, benziminazole, cytosine deoxyriboside, thymidine, uracil deoxyriboside, adenylic

acid *a* *, adenylic acid *b* *, cytidylic acid, uridylic acid. Formic acid was detected after the periodate oxidation of 9-β-D-glucopyranosyl-, 9-β-D-mannopyranosyl-, 9-β-D-ribopyranosyl-adenine, 1-D-glucopyranosyl- and 1-D-arabopyranosyl-benziminazole.

TABLE II.

Compound.	R_F value. *n*-Butanol, saturated with water.	*s*-Collidine saturated with water.	*n*-Propanol(6)–ammonia (*d* 0·88) (3)–water (1).	*n*-Butanol(4)–water(5)–acetic acid (1).	Colour.
Adenosine	0·14	0·68	—	0·41	B
Cytidine	0·13	0·50	—	—	B
Guanosine	0·09	—	—	0·22	B
Uridine	0·21	0·81	—	—	B
Adenosine-5′ phosphate	—	—	0·20 [1]	—	B
Guanine deoxyriboside	0·17	0·66	—	—	P
Hypoxanthine deoxyriboside	0·19	—	—	—	P
9-α-D-Arabofuranosyladenine	0·14	—	—	—	B
9-β-D-Glucopyranosyladenine	0·08	0·36	—	0·28	BG
9-β-D-Mannopyranosyladenine	0·09	0·42	—	0·30	BG
9-β-D-Ribopyranosyladenine	0·12	0·42	—	0·36	BG
2′-Deoxy-D-glucopyranosyltheophylline	0·30	—	—	—	BrPu
2′-Deoxy-D-ribopyranosyltheophylline	0·40	—	—	—	BrPu
2′-Deoxy-D-glucopyranosyluracil [2]	0·22	—	—	—	BrPu
1-D-Arabopyranosylbenziminazole [2]	0·62	0·78	—	—	B
1-D-Glucopyranosylbenziminazole [2]	0·49	0·82	—	—	B
2′ : 3′-*iso*Propylidene adenosine	0·59	—	—	—	BPu

[1] This compound was chromatographed on acid-washed paper (Hanes and Isherwood, *loc. cit.*). [2] Synthesised by Dr. Irving Goodman of the University of Colorado. [a] Colours: B, blue; P, pink; G, grey; Br, brown; Pu, purple.

Dische Reaction: Detection of Deoxyribonucleosides.—A 1% solution of diphenylamine in glacial acetic acid, to which 2·75% by volume of concentrated sulphuric acid had been added, was diluted with one-half of its volume of water.

After the dried paper chromatogram had been sprayed with the reagent, it was clamped between two glass plates and placed in an oven at 90°. The purine deoxyribosides gave a purplish-blue colour in 5—10 minutes, whereas the pyrimidine deoxyribosides gave blue spots after 25—30 minutes. The glass covering prevented rapid dehydration and charring of the paper.

Feulgen Reaction: Detection of Purine Deoxyribonucleosides.—Schiff's reagent, as described above, was made 0·1N. with respect to sulphuric acid, by addition of N-sulphuric acid.

The paper chromatogram was freed from solvents and treated with the reagent. The purine deoxyribosides appeared as purple spots after the paper had been heated in an oven at 60° for 6 minutes and then kept at room temperature for 24 hours. Longer heating hastened the appearance of the colour, but it also rendered the test less sensitive by causing decomposition of the sugar. The pyrimidine deoxyribosides, being much more resistant to acid hydrolysis, showed weak spots after 10—15 days. Acidification of the Schiff's reagent with sulphuric acid was necessary to prevent basic substances, such as cytosine deoxyriboside, from giving an immediate colour by mere neutralisation of the sulphurous acid of the reagent. These false positive tests were easily distinguished from the true tests since the colour produced was that of the dye (red) rather than the aldehyde–dye complex (purple). Results obtained by application of the Dische and the Feulgen method are shown in Table III.

TABLE III.

Compound.	R_F value. *iso*Propanol(65)–2N-HCl(35).	*tert.*-Butanol(60)–pyridine(25)–water(15).	*n*-Butanol(86)–water(14).
Guanine deoxyriboside	*	0·56	0·12
Hypoxanthine deoxyriboside	*	0·59	0·16
Cytosine deoxyriboside	0·64	0·57	0·22
Thymine deoxyriboside	0·90	0·73	0·47
Uracil deoxyriboside	0·84	0·68	0·33

* The purine deoxyribosides are decomposed by the strongly acidic solvent.

Limits of Sensitivity.—The periodate oxidation techniques were generally suitable for quantities of 10 μg. in the simple carbohydrate series, and 20—50 μg. for ribonucleosides and ribonucleotides (50—100 μg. for other nucleosides). The limit with lead tetra-acetate appeared to be about the same.

* Kindly sent to Professor A. R. Todd by Drs. C. E. Carter and W. E. Cohn, cf. *Fed Proc.*, 1949, **8**, 190.

The Dische reaction required quantities of 10 μg. for the purine deoxyribosides, but 50—100 μg. for the pyrimidine deoxyribonucleosides. Smaller amounts of the latter compounds could be detected if the ultra-violet-absorbing spots on the chromatogram were cut out and treated with the reagent (*ca.* 1 c.c.) in small test-tubes. With 30 minutes' heating on a water bath, the characteristic blue colour was given by 20—30 μg. In the Feulgen reaction, only 10 μg. of the purine deoxyribonucleosides were required.

The R_F values of nearly all the compounds investigated were found to vary considerably on different chromatograms, owing chiefly to variations in temperature. As emphasised by other workers, it is always necessary to run standard substances on the same chromatogram when attempting to identify unknown compounds.

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