COLOUR REACTIONS BETWEEN SUGARS AND DIPHENYLAMINE–UREA AND DIPHENYLAMINE–*p*-ANISIDINE ON PAPER CHROMATOGRAMS

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INTRODUCTION

The development of spray reagents, for use in paper chromatography, which give an indication of the position of the glycosidic links in oligosaccharides has made some progress in the past few years. Probably one of the most useful sprays of this type is acidic diphenylamine–aniline[1] which gives a characteristic bright blue colour with aldohexose oligosaccharides containing a glycosidic link on C-4 of the reducing monosaccharide unit (*e.g.* maltose, cellobiose and panose). In a detailed study of the reaction of this spray reagent on paper chromatograms BAILEY AND BOURNE[2] defined the conditions for several other specific colour reactions with sugars, particularly monosaccharides. They suggested that the colour reactions given by the mixed spray were due to diphenylamine in some way modifying the reaction of aniline with the sugars. This modifying action of diphenylamine might be a general effect and occur with the other amino compounds which, like aniline, are used alone in acid solution for locating sugars on chromatograms. It seemed likely, therefore, that further spray reagents, giving colour reactions dependent on the position of the glycosidic links in oligosaccharides, could be obtained by mixing these compounds with diphenylamine. A number of amino-compounds have, therefore, been tested in mixed sprays against a range of sugars and two promising spray reagents containing diphenylamine plus urea and diphenylamine plus *p*-anisidine were found. This paper contains a description of the colour reactions of these sprays with mono- and di-saccharides.

EXPERIMENTAL

Materials

Isomaltose, xylobiose (4-O-β-D-xylopyranosyl-D-xylose) and laminaribiose were isolated, by ethanol fractionation on charcoal, from partial acid hydrolysates of dextran, xylan and laminarin respectively. Leucrose was isolated from a *Streptococcus bovis*–sucrose culture[3]. Lactulose, maltulose, cellobiulose and melibiulose were prepared from the corresponding aldoses by treatment with lime water[4]. Isomaltulose, sophorose, mannoketoheptose and 1-O-β-D-glucopyranosyl-D-fructose were provided by Dr. F. H. STODOLA (U.S. Department of Agriculture), Dr. M. J. CLANCY (The Agricultural Institute, Thurles, Ireland), Dr. D. A. L. DAVIES (Microbiological

Research Establishment, Porton, Wilts., England) and Dr. D. H. HUTSON (Royal Holloway College, University of London) respectively. Other sugars were obtained from the usual commercial sources. Most of the sugars were chromatographically pure. In cases where they were not the solvents employed for developing papers clearly separated the required sugar from any contaminant sugar.

The components of the spray reagents were B.D.H. or L. Light and Co. reagent grade chemicals.

Chromatograms

The preparation, development and heating of papers, and the solvents used, have already been described[2]. Except where stated otherwise, 25–40 μg of each sugar was applied to the papers.

Spray reagents

For a preliminary survey of available amino-compounds, sprays were prepared by mixing equal volumes of solutions of diphenylamine (4 % w/v) and the amino-compound (4 % w/v) in acetone or butanol-1 containing conc. (80 %) orthophosphoric acid (10 % v/v). The phosphates of several of the compounds tested were very insoluble; in these cases the papers were first sprayed with acidic diphenylamine, then dried and resprayed with a solution of the amino-compound acidified with HCl. The following gave poor results and were not investigated further: α-naphthylamine, β-naphthylamine, benzidine, *m*-phenylenediamine and *p*-phenylenediamine. Two promising mixed spray reagents were encountered containing diphenylamine with urea and *p*-anisidine respectively. With these mixtures the effect of varying factors such as the concentration of each component, heating temperatures and heating times was investigated in more detail and the sprays and conditions described below were selected as being the most useful.

(*a*) *Diphenylamine–urea.* The spray reagent for *ketoses* consisted of a mixture of equal volumes of a solution of diphenylamine (1 %, w/v) in acetone containing conc. phosphoric acid (10 %, v/v) and of a solution of urea (3 %, w/v) in water-saturated butanol-1 also containing phosphoric acid (10 %, v/v). Sprayed dried papers were heated for 2–3 min at 90° and the developed colours noted immediately and again 20–30 min after heating; after this time the colours remained stable for at least 1 h.

For *aldoses* the spray reagent was of the same composition as that for ketoses except that a 2 % (w/v) diphenylamine solution was used. With this spray the papers were heated for 4–5 min at 95–100°.

(*b*) *Diphenylamine–p-anisidine.* The spray reagent consisted of a mixture of equal volumes of a solution of diphenylamine (2 %, w/v) in acetone containing conc. phosphoric acid (10 %, v/v) and of a solution of *p*-anisidine (2 %, w/v) in ethanol also containing phosphoric acid (10 %, v/v). The one spray was suitable for both aldoses and ketoses. The anisidine phosphate was not completely soluble in the acetone–ethanol solvent but the mixture could be sprayed quite easily after first shaking to disperse the insoluble material. The sprayed, dried papers were heated for 2–3 min at 90–95° and the developed colours were stable for several hours.

As there was evidence that ageing of the solutions affected some of the colour reactions all of the spray solutions were always prepared immediately before use and discarded after 8–12 h.

RESULTS

Colour reactions of diphenylamine–urea spray reagents with sugars

The appropriate spray reagents were tested against a range of mono- and reducing di-saccharides and the colour reactions obtained are listed in Table I. Acid labile non-reducing disaccharides, *e.g.* sucrose, reacted as a mixture of their hydrolysis products; more stable non-reducing compounds, *e.g.* α,α-trehalose did not react.

TABLE I

COLOUR REACTIONS ON PAPER CHROMATOGRAMS OF SUGARS AND DIPHENYLAMINE–UREA REAGENTS

Sugars	*Colour immediately after heating*	*Colour 20–30 min after heating*
(1) *Reducing ketoses*		
D-Fructose	red-brown	brown-black
L-Sorbose	grey-black	grey-blue
Mannoketoheptose	purple-blue	purple-blue
1-O-β-D-Glucopyranosyl-D-fructose (1 → 1)	pink	pink
Turanose (1 → 3)	bright purple	bright purple
Maltulose, lactulose, cellobiulose (1 → 4)	pink-red	brown
Leucrose (1 → 5)	pale orange	pale orange
Isomaltulose, melibiulose (1 → 6)	red-brown	green
(2) *Reducing aldoses*		
L-Arabinose, D-ribose, D-lyxose, D-xylose	bright red	bright red
Xylobiose (4-O-β-D-xylopyranosyl-D-xylose)	bright red	bright red
L-Rhamnose, L-fucose	pink	pink
D-Glucose, D-galactose, D-mannose	pink	bright pink
D-Glucoheptose (L. Light & Co.)	purple-blue	purple-blue
D-Galacturonic acid, D-glucuronic acid	deep red-brown	deep red-brown
Sophorose (1 → 2)	no reaction	no reaction
Laminaribiose (1 → 3)	pink	bright pink
Maltose, lactose, cellobiose (1 → 4)	pink	orange-brown
Isomaltose, melibiose (1 → 6)	pink	bright pink

All of the results in Table I were obtained with papers carrying 25–40 μg of each sugar. Sensitivity tests with various amounts of sugars (1–20 μg) showed that with ketoses satisfactory results could be obtained with only 5 μg of sugar. The reaction with aldoses was, however, much less sensitive and for good colour development at least 15–20 μg of sugar was required. With both classes of sugar the nature of the developed colours was independent of sugar concentration over the range 5–40 μg. When both aldoses and ketoses were co-chromatographed, sprayed with the ketose spray reagent and heated at 90° for 2 min, the ketoses could be distinguished from the aldoses, not only by the different colours produced, but also by the much more rapid appearance of the intense ketose spots.

With diphenylamine–aniline additional colour reactions were obtained with some sugars by the use of split reagents[2]; *i.e.* papers were sprayed with diphenylamine, heated, sprayed with aniline and reheated, and *vice-versa*. When this procedure was used with diphenylamine and urea there was no improvement on the colour differentiation obtained with the mixed spray.

Colour reactions of the diphenylamine–p-anisidine spray reagents with sugars

The diphenylamine–*p*-anisidine spray reagent was tested against the same mono- and di-saccharides and the colour reactions obtained are listed in Table II. As with diphenylamine–urea acid labile non-reducing disaccharides reacted as a mixture of hydrolysis products but stable compounds such as α,α-trehalose did not react.

Sensitivity tests with ketoses showed that this spray reagent gave distinctive

TABLE II

COLOUR REACTIONS ON PAPER CHROMATOGRAMS OF SUGARS AND DIPHENYLAMINE–*p*-ANISIDINE

Sugars	*Colour produced*
(1) *Reducing ketoses*	
D-Fructose	yellow-brown
D-Sorbose	bright yellow
Mannoketoheptose	bright red-brown
1-O-β-D-Glucopyranosyl-D-fructose (1 → 1)	pink-red
Turanose (1 → 3)	dark red-brown
Maltulose, lactulose, cellobiulose (1 → 4)	orange
Leucrose (1 → 5)	bright yellow
Isomaltulose, melibiulose (1 → 6)	bright yellow
(2) *Reducing aldoses*	
L-Arabinose, D-ribose, D-lyxose, D-xylose, xylobiose	green to blue-green
L-Rhamnose, L-fucose	pale yellow-brown
D-Glucose, D-galactose, D-mannose	blue-green
D-Glucoheptose	blue-green
D-Galacturonic-, D-glucuronic acid	deep red-brown
Sophorose (1 → 2)	orange
Laminaribiose (1 → 3)	blue-green
Maltose, cellobiose, lactose (1 → 4)	blue-green to blue
Isomaltose, melibiose (1 → 6)	blue-green

colour reactions with as little as 2 μg of sugar. Reaction with aldoses was generally less sensitive and a little slower. Although aldoses could be clearly detected at the 2–5 μg level, for the production of intense colours the presence of at least 10 μg of sugar was preferable. The colour reactions with all of the sugars were independent of sugar concentration over the range 2–40 μg of sugar. The only exception to these results so far noted was sophorose whose colour reaction appeared to be weaker than that of the other aldoses tested.

When split reagents were tested, diphenylamine followed by *p*-anisidine gave the same end results as the mixed spray. *p*-Anisidine followed by diphenylamine with ketoses, but not with aldoses, gave slightly more intense colours than the mixed reagent and better differentiation between sorbose and fructose.

DISCUSSION

Both diphenylamine–urea and diphenylamine–*p*-anisidine give sensitive colour reactions which distinghuish between reducing disaccharides substituted at different

position on a reducing ketohexose unit (Tables I and II). Colour reactions obtained with D-glucoheptose and mannoketopheptose suggest that diphenylamine–*p*-anisidine but not diphenylamine–urea can clearly distinguish between aldo- and keto-heptoses. The different colours produced are quite distinct and have been obtained in this laboratory consistently during several months of testing.

With reducing aldohexose disaccharides, diphenylamine–urea distinguishes between 1 → 4 and 1 → 3 or 1 → 6 linked compounds and thus provides a confirmation of the diphenylamine–aniline identification of the 1 → 4 linked compounds. The failure of this reagent to react with sophorose may also provide negative evidence to support the identification of this type of sugar by its specific colour reaction (orange) with diphenylamine–aniline[2] or –*p*-anisidine (Table II). Apart from its reaction with sophorose, diphenylamine–*p*-anisidine does not give clear cut colour differences between aldohexose disaccharides. However, the colour given by 1 → 4 linked disaccharides tends to be a more definite blue than the colour given by the other compounds. This tendency to give a more distinct blue colour with 1 → 4 linked reducing aldose disaccharides, compared with other disaccharides, was also observed with spray reagents containing diphenylamine and the other amino-compounds which were not further investigated.

In addition to their reactions with disaccharides both spray reagents give a strong specific colour with uronic acids while diphenylamine–urea gives a stable and specific colour with pentoses. The reaction of diphenylamine–*p*-anisidine with reducing aldoses provides a reasonably sensitive identification of reducing aldose units. This reaction, particularly with disaccharides, appears to be more sensitive than that obtained with diphenylamine–aniline[2] or the conventional *p*-anisidine[5] and aniline hydrogen phthalate[6] sprays.

The colour reactions obtained between sugars and various diphenylamine–aniline spray reagents[2] indicated that the reactions were extremely complex. In many cases they could not be explained in terms of conversion of the sugar, in the presence of acid, to a furfural derivative which then reacted with the amino-compounds. Studies with split reagents suggested that the diphenylamine modified the reaction of aniline with the sugars. Although the present results do not permit any further conclusions to be drawn they do suggest that the modifying effect of diphenylamine is a general one so far as the reaction of acidified amino-compounds and sugars is concerned. It may, however, be confined to amino-compounds. Thus the specific colours produced on chromatograms with ketoses by the acidified phenols, resorcinol and napthoresorcinol, were in no way affected by the addition of diphenylamine to the phenol spray.

The spray reagents described in the present paper should be of value for the identification of disaccharides and for the prediction of the position of the glycosidic links in many of these compounds. A spray reagent is still required, however, to distinguish clearly between 1 → 3 and 1 → 6 aldohexose disaccharides.

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SUMMARY

Conditions have been defined in which mixed acidic diphenylamine–urea or diphenylamine–*p*-anisidine spray reagents give, on paper chromatograms, sensitive, specific colour reactions which distinguish between 1 → 1, 1 → 3, 1 → 4, 1 → 5 and 1 → 6 linked disaccharides containing a reducing ketose unit.

The diphenylamine–*p*-anisidine spray reagent gives different colour reactions with aldo- and keto-heptoses and is a sensitive locating spray for disaccharides with a reducing aldose unit.

A less sensitive diphenylamine–urea spray reagent has been developed which gives specific colour reactions with pentoses and uronic acids, and differentiates between 1 → 2, 1 → 4 and 1 → 3 or 1 → 6 linked reducing aldose disaccharides.

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