THE USE OF DIPHENYLAMINE–ANILINE–PHOSPHORIC ACID REAGENT IN THE DETECTION AND DIFFERENTIATION OF MONOSACCHARIDES AND THEIR DERIVATIVES ON PAPER CHROMATOGRAMS

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INTRODUCTION

Diphenylamine–aniline–phosphoric acid reagent*** which is used for the detection of mono- and oligo-saccharides on paper, displays a wide range of colors with carbohydrates of different structures. This reagent has been used to advantage for distinguishing between various sugars, and some interrelationships between structure and the color produced have been described[1,2].

The aim of the present paper is to draw attention to another useful application of this reagent in the detection and differentiation of some monosaccharide derivatives. The reagent, as used successfully for several years in our laboratory, is a modification of the original reagent of BUCHAN AND SAVAGE[3], developed independently by VÍTEK[4,5].

EXPERIMENTAL

Materials

Most of the sugars and sugar derivatives used were prepared in our laboratory or were gifts from the laboratories acknowledged below. Some of the free sugars were commercial preparations.

Chromatograms

In the majority of cases, the sugars and their derivatives were applied to papers (Whatman No. 3) in 1 % aqueous or ethanolic solutions, in quantities of 10–50 μg, and, if not otherwise stated, were developed by downward irrigation in *n*-butanol–acetic acid–water (10:1:3). The dipping technique of detection was used as a rule, the chromatograms being dried at room temperature and finally heated at 95–100° in a thermostatic oven until the background was faintly gray. For the detection of acid-labile non-reducing derivatives, the time of heating depended on the stability of the hydrolysable bond and, if necessary, was prolonged until distinct spots appeared. In addition to the time of heating, the shades of the colors given in the tables depend more or less on the quantities of sugars applied; in this respect the most marked

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*** Abbreviation: DAPA reagent.

TABLE I

COLOR REACTIONS ON PAPER CHROMATOGRAMS OF SOME COMMON SUGARS WITH THE DIPHENYLAMINE–ANILINE–PHOSPHORIC ACID REAGENT IN VÍTEK'S MODIFICATION

System: *n*-butanol–acetic acid–water (10:1:3).

Sugar	R_F	*Color*
(*a*) *Aldopentoses*		
Arabinose	0.22	green-gray
Ribose	0.31	green-gray
Xylose	0.28	green-gray
(*b*) *Ketopentoses*		
Ribulose	0.32	orange-yellow
Xylulose	0.32	green-yellow
(*c*) *Aldohexoses*		
Altrose	0.15	blue-gray
Galactose	0.14	blue-gray
Glucose	0.14	blue-gray
Mannose	0.15	blue-gray
(*d*) *Ketohexoses*		
Fructose	0.16	brown-red*
Sorbose	0.18	yellow-brown**
Tagatose	0.16	brown-red
(*e*) *Disaccharides*		
Cellobiose	0.58***	blue-gray
Lactose	0.48***	blue-gray
Maltose	0.60***	bright blue
Sucrose	0.81***	green-brown
(*f*) *Uronic acids*		
Galacturonic acid	0.05	blackish brown
Glucuronic acid	0.07	blackish brown

* During the heating the color changes from orange-yellow to brown-red.
** During the heating the color changes from yellow-green to yellow-brown.
*** R_{Glc}.

variations of colors were observed with ketoses (Table I). When exposed to the atmosphere of the laboratory, the colors of the spots, as well the background, gradually change, the background acquiring a more blue or blue-green shade. In some cases the spots of otherwise undetectable sugars appear (higher concentrations of sugar alcohols) or those giving a weak reaction (1-thioaldoses) are intensified.

Reagent

Diphenylamine (0.15 g) is dissolved in about 20–25 ml of ethyl acetate from a total volume of 100 ml. Then 0.8 ml of aniline, the remainder of the ethyl acetate and 11 ml of 80 % (w/v) phosphoric acid are added. During the addition of phosphoric acid a voluminous precipitate of amine phosphates is formed, which disappears on further addition of the solvent, when gently shaken. Phosphoric acid must be added after the dissolution of the bases is complete, otherwise an insoluble precipitate can be formed.

When stored in the dark at 0–5° the reagent is stable for 2–3 weeks. After prolonged periods of storage changes in the color shades produced were observed.

RESULTS

Types of compounds examined

According to their reactivity or stability toward the DAPA reagent under the conditions given above, four principal groups of carbohydrates have been tested: *viz.* (1) free sugars, (2) sugar derivatives with acid-labile blocking groups, (3) sugar derivatives with acid-stable blocking groups and (4) sugar alcohols. While the compounds of the second group give, as a rule, the same color reactions as the corresponding free sugars, the colors produced by the derivatives of the third group may differ markedly from those of the parent sugars, due to the different chromogen formed by the action of the reagent. Free 1-thioaldoses, 1-selenoaldoses and 2-amino-2-deoxyaldoses give but non-characteristic weak yellow spots, while sugar alcohols do not react under the given standard conditions.

Owing to the slightly different composition of the reagent used in this work the colors differ, in some cases, from colors given by the reagents reported in previous papers. For comparison Table I shows colors given by some more important common sugars. The results summarized below are divided according to the type of carbohydrate compounds tested.

(*a*) *Derivatives without a secondary hydroxyl group adjacent to the carbonyl group or with an acid-stable blocked one* (*Table II*)

Absence or acid-stable blocking of the secondary hydroxyl group in the neighborhood of the carbonyl group results in a typical color change with DAPA reagent

TABLE II

DERIVATIVES WITHOUT A SECONDARY HYDROXYL GROUP ADJACENT TO THE CARBONYL GROUP OR WITH AN ACID-STABLE BLOCKED ONE, ALL GIVING VARIOUS SHADES OF RED-VIOLET WITH DAPA REAGENT

System: *n*-butanol–acetic acid–water (10:1:3), if not otherwise stated.

Sugar	R_F
(*a*) *Deoxy-sugars and derivatives*	
2-Deoxy-D-*erythro*-pentose (2-deoxy-D-ribose)	0.42
2-Deoxy-D-*lyxo*-hexose (2-deoxy-D-galactose)	0.33
2-Deoxy-D-*arabino*-hexose (2-deoxy-D-glucose)	0.34
2,6-Dideoxy-3-O-methyl-D-*ribo*-hexose (cymarose)	0.83
2^G-Deoxysucrose	0.14
2-Deoxy-D-ribohexonic (2-deoxygluconic) acid lactone	0.60
2-Deoxy-D-*lyxo*-hexose-1-phosphate	0.25*
2-Deoxy-D-*arabino*-hexose-6-phosphate	0.23*
(*b*) *Glycals*	
D-Galactal	0.54
D-Glucal	0.61
(*c*) *Ethers and α-D-glycopyranosyl derivative*	
2-O-*p*-Tolyl-D-altrose	0.85
2-O-Methyl-D-glucose	0.26
3-O-Methyl-D-fructose	0.34
3-O-α-D-Glucopyranosyl-D-fructose (turanose)	0.05

* System: *n*-propanol–ammonia–water (6:1:3).

to various gradations of violet or red-violet. Reactions of 2-deoxyaldoses, as well as glycals, are especially sensitive and the color predominates also in acid-labile oligosaccharides containing a 2-deoxyaldose moiety in addition to other monosaccharide units, as in 2^{G}-deoxysucrose or 2^{Gal}-deoxyraffinose[6]. A red-violet color is also given by 2-O-methyl-D-glucose and 3-O-methyl-D-fructose and even turanose which is 3-O-α-D-glucopyranosyl-D-fructose. The rule does not apply when the secondary hydroxyl group adjacent to the carbonyl function is replaced by a more polar group, *e.g.* an amino or sulfhydryl group in 2-amino-2-deoxyaldoses or 2-thioaldoses. Similarly, further acid-stable substitution on remaining free hydroxyl groups as in 2,4,6-tri-O-methyl-D-glucopyranose (see Table III) brings about the loss of this typical reaction.

(*b*) *Aldohexoses in which a hydroxyl group not adjacent to the carbonyl group is missing or is acid-stably blocked, or aldohexoses which besides the secondary acid-stably blocked hydroxyl group adjacent to the carbonyl function have additional acid-stably blocked hydroxyl groups* (*Table III*).

For the few compounds of this type available no definite regularity in the color reaction could be established. It seems likely that in aldohexoses the absence (or stable blocking) of the single hydroxyl group at C-3 or C-4 results in a change of the originally blue-gray color of the parent hexose to yellow or green. The blue-gray color given by the higher methylated glucoses is remarkable when compared with the red-violet given by 2-O-methyl-D-glucose.

TABLE III

ALDOHEXOSES IN WHICH A HYDROXYL GROUP NOT ADJACENT TO THE CARBONYL GROUP IS MISSING OR IS ACID-STABLY BLOCKED, OR ALDOHEXOSES WHICH BESIDES THE SECONDARY ACID-STABLY BLOCKED HYDROXYL GROUP ADJACENT TO THE CARBONYL FUNCTION HAVE ADDITIONAL ACID-STABLY BLOCKED HYDROXYL GROUPS

System: *n*-butanol–acetic acid–water (10:1:3).

Sugar	R_F	*Color*
3-Deoxy-D-*ribo*-hexose (3-deoxy-D-glucose)	0.26	dull yellow
4-Deoxy-D-*xylo*-hexose (4-deoxy-D-glucose, 4-deoxy-D-galactose)	0.25	dull yellow
4-O-Methyl-D-galactose	0.31	green-gray
2,4,6-Tri-O-methyl-D-glucopyranose	0.67	blue-gray
2,3,4,6-Tetra-O-methyl-D-glucopyranose	0.82	blue-gray
1,2-O-Isopropylidene-5,6-O-carbonyl-α-D-glucofuranose	0.80	blackish brown

(*c*) *Simple aliphatic, alicyclic and aromatic glycosides* (*Table IV*)

Most of the aliphatic glycosides are easily detectable in amounts of about 200 μg and produce the same color as the parent sugar within 1 to 10 min. The time necessary for splitting off the aglycone and hence for the color development is in good agreement with the general rules holding for the stability of the glycosidic linkage and can be used as a means of differentiation between glycosides of various structures. From the compounds examined the alkyl β-D-fructofuranosides are the most labile ones, whereas some of the methyl α-D-aldohexopyranosides show a considerable stability; methyl α-D-glucopyranoside fails to react at all.

The sensitivity limits lay at 100 μg for most aliphatic and at about 200 μg for

TABLE IV

SIMPLE ALIPHATIC AND AROMATIC GLYCOSIDES

System: *n*-butanol–acetic acid–water (10:1:3).

Glycoside	R_F	*Minimum time necessary for developing color (min)*	*Color*
(*a*) *Aliphatic glycosides*			
Methyl β-D-arabinopyranoside	0.34	< 5	gray-green
Methyl α-D-altropyranoside	0.32	> 5	blue-gray
Methyl α-D-galactopyranoside	0.22	< 10	blue-gray
Methyl β-D-galactopyranoside	0.21	< 10	blue-gray
Ethyl α-D-galactopyranoside	0.35	< 10	blue-gray
n-Propyl β-D-glucopyranoside	0.48	< 10	blue-gray
n-Butyl β-D-glucopyranoside	0.58	< 10	blue-gray
Methyl α-D-glucopyranoside	—	—	nil
Methyl β-D-glucopyranoside	0.24	5	blue-gray
Methyl 3-keto-β-D-glucopyranoside	0.25	> 5	blue-gray
Isopropyl β-D-glucopyranoside	0.47	< 5	blue-gray
Isobutyl β-D-glucopyranoside	0.61	< 5	blue-gray
tert.-Butyl β-D-glucopyranoside	0.51	< 5	blue-gray
Cyclohexyl β-D-glucopyranoside	0.59	< 5	blue-gray
Methyl α-D-mannopyranoside	0.35	10	blue-gray
Methyl β-D-fructofuranoside	0.35	< 1	brown-red
Ethyl β-D-fructofuranoside	0.45	< 1	brown-red
n-Propyl β-D-fructofuranoside	0.56	< 1	brown-red
n-Butyl β-D-fructofuranoside	0.65	< 1	brown-red
Amyl β-D-fructofuranoside	0.71	< 1	brown-red
Benzyl β-D-fructofuranoside	0.66	< 1	brown-red
(*b*) *Aromatic glycosides*			
o-Hydroxyphenyl β-D-glucopyranoside	0.40	> 10	faint yellow
m-Hydroxyphenyl β-D-glucopyranoside	0.30	> 10	faint yellow
p-Hydroxyphenyl β-D-glucopyranoside (arbutin)	0.24	> 10	yellow
Phenyl β-cellobioside	0.31	> 10	yellow-brown

aromatic glycosides. The weak spots of aromatic glycosides appear after an average of 10 min heating and have non-characteristic shades of dull yellow.

(*d*) *Glycosans* (*Table V*)

Being internal β-D-glycosides, glycosans react readily, developing, as a rule, the color of the parent sugar.

(*e*) *Alkylidene, arylidene and acyl derivatives* (*Table VI*)

The various acid-labile compounds listed in Table VI react smoothly. Increase in molecular weight caused by the various blocking groups attached, leads to a corresponding decrease in sensitivity, but, as a rule, not to a perceptible color change.

(*f*) *Thio and selenosugars* (*Table VII*)

1-Thioaldoses and 1-selenoaldoses give after prolonged heating non-characteristic light spots on a dark background. The use of the reagent for this class of sugars

TABLE V

GLYCOSANS, ANHYDROSUGARS, AND THEIR DERIVATIVES

System: *n*-butanol–acetic acid–water (10:1:3).

Glycosan	R_F	*Color*
1,6-Anhydro-β-D-glucopyranose	0.40	blue-gray
1,6-Anhydro-4-deoxy-β-D-altropyranose	0.47	yellow
1,6-Anhydro-4-deoxy-β-D-glucopyranose	0.53	yellow
1,6-Anhydro-2-O-*p*-tolyl-β-D-altropyranose	—	nil
1,6-Anhydro-2-O-*p*-tolylsulphonyl-β-D-altropyranose	—	nil
1,6:3,4-Dianhydro-β-D-altropyranose	0.62	blue-gray
1,6:3,4-Dianhydro-β-D-galactopyranose	0.71	blue-gray
1,6:2,3-Dianhydro-β-D-gulopyranose	0.75	green
1,6:2,3-Dianhydro-β-D-mannopyranose	0.62	blue-gray

TABLE VI

ALKYLIDENE, ARYLIDENE, AND ACETYL DERIVATIVES OF SUGARS

System: *n*-butanol–acetic acid–water (10:1:3).

Sugar derivative	R_F	*Color*
1,2:3,4-Di-O-isopropylidene-L-arabinopyranose	0.98	green-gray
1,2-O-Isopropylidene-α-D-glucofuranose	0.70	blue-gray
1,2:5,6-Di-O-isopropylidene-α-D-glucofuranose	0.97	blue-gray
1,2-O-Isopropylidene-5,6-anhydro-α-D-glucofuranose	0.78	green-gray
Methyl 2-deoxy-4,6-O-benzylidene-α-D-*arabino*-hexopyranoside	0.90	red-violet
1,2-O-Isopropylidene-3-deoxy-α-D-*ribo*-hexofuranose	0.75	yellow
6-O-Acetyl-D-galactose	0.29	blue-gray
6-O-Acetyl-D-glucose	0.29	blue-gray
1,6-Anhydro-3-O-acetyl-β-D-glucopyranose	0.96	blue-gray
3,4,6-Tri-O-acetyl-D-galactal	0.98	red-violet
Hepta-O-acetyl-2G-deoxysucrose	0.96	red-violet

TABLE VII

THIOSUGARS AND SELENOSUGARS

System: *n*-butanol–acetic acid–water (10:1:3).

Sugar	R_F	*Color*
1-Thio-D-glucose	0.25	nil*
6-Thio-D-glucose	0.35	green-gray
1,6-Dithio-D-glucose	0.62	nil*
1-Seleno-D-glucose	0.08	nil*
2-Thio-D-altrose	0.33	yellow-green

* Pale yellowish spots on deep gray or blue-gray background on prolonged heating.

is of no particular advantage. On the other hand, reducing thiosugars with secondary —SH groups (other than on the anomeric C-atom), as far as examined, give well-developed spots differing in shade from the corresponding —OH compounds.

(*g*) *Amino sugars*

Of the amino sugars only 2-amino-2-deoxy-aldohexoses were tested. They only produce non-typical dull yellow spots. DAPA reagent is not suitable for detection of this type of sugar.

(*i*) *Sugar alcohols*

In the usual concentrations sugar alcohols do not react with DAPA reagent. At higher concentrations (more than 500 μg) light spots appear after prolonged heating on the gray or blue-gray background which, on prolonged exposure to the atmosphere at room temperature, turn bright blue, acquiring a shade similar to that given by maltose.

DAPA reagent has been used with success for the detection and differentiation of sugar derivatives in plant extracts and in mixtures of enzymic transglycosylation products. Furthermore it has been proved valuable in following the course of reaction in synthetic carbohydrate chemistry. For example, the two different ethers (at C-2 or C-3) which arise, as a rule, by splitting 2,3-anhydro rings in aldohexose epoxy sugars by the action of alkali alcoholates or phenolates, can clearly be distinguished on paper, by the red-violet and yellow-green colors given by the respective ethers.

DISCUSSION

In this paper two advantages of practical application of DAPA reagent are demonstrated; they are (1) the specific color reactions given by the reagent with different types of reducing sugars and (2) the detection of various non-reducing sugar derivatives which are hydrolysed by the action of the reagent to the free sugars.

Due to the limited number of sugar compounds of a particular structure that were at our disposal, it was not possible to present a detailed study on the interrelationships between the structure and the corresponding color produced by the reagent. Nevertheless, a few groups of sugars of similar structure could be shown to give characteristic and more or less identical colors: (a) aldohexoses, blue-gray; (b) aldopentoses, green-gray; (c) hexuronic acids, dark brown; (d) derivatives with no secondary hydroxyl group adjacent to the carbonyl group, or with an acid-stable blocked one, red-violet.

On the other hand there are groups of sugars of very similar structure, *e.g.* ketoses like fructose and sorbose, or ribulose and xylulose which show clear-cut differences in color and thus bear evidence to the complexity of the color reaction. Another example is represented by the reactions given by 2-O-methyl-D-glucose and the higher methylated glucose derivatives. While 2-O-methyl-D-glucose follows the general rule found for derivatives summarized under (d) and gives a red-violet color, 2,4,6-tri-O-methyl-D-glucopyranose and 2,3,4,6-tetra-O-methyl-D-glucopyranose give, contrary to all expectations, the blue-gray color of free glucose.

These rather contradictory results make it difficult to suggest a mechanism for the chromogen formation involved. Apparently, the simple theory which ascribes

the color production to the preliminary conversion of the sugar by acid to furfural, or a derivative, which then reacts with the reagent to give a colored compound[7] cannot give a sufficient explanation for the variety of cases described. The practical aspects of the reaction should, however, stimulate its further investigation.

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SUMMARY

The diphenylamine–aniline–phosphoric acid reagent, as modified by VÍTEK, has been used for the detection of various monosaccharides and their derivatives. Under the defined conditions, the reagent gives specific color reactions and can be used (1) to distinguish between sugars of different structure and (2) to detect various non-reducing sugar derivatives with acid-labile blocking groups.

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