1119. *The Identification of Carbohydrates giving Derivatives of Malonaldehyde on Oxidation with Sodium Periodate.*

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The yellow colours produced by certain carbohydrates on paper chromatograms when treated with sodium periodate followed by Schiff's reagent have been found usually to be associated with derivatives of malonaldehyde. Some applications of this to the determination of carbohydrate structures are suggested. Reactions of benzyloxymalonaldehyde with Schiff-type reagents have been examined and compared with those of various aromatic hydroxy-aldehydes.

Sodium periodate and Schiff's reagent were first applied to the detection of carbohydrates and other α-glycols on paper chromatograms by Buchanan, Dekker, and Long.[1] The technique was later modified [2,3] and has proved of great value in this laboratory.[4] When Schiff's reagent prepared from Pararosaniline or its hydrochloride is used, almost every compound containing an α-glycol group gives a positive reaction although the sensitivity of the test varies. Carbohydrates containing glycol groups not easily oxidised by periodate, *e.g.*, 1,6-anhydroglucopyranose, cannot be detected in this way. When formaldehyde is a product of oxidation an immediate purple colour is observed on spraying with Schiff's reagent, whereas other aldehydes give blue colours more slowly.

During an investigation of the isomeric monophosphates of ribitol, however, it was found that one isomer, later identified as ribitol 3-phosphate, gave a yellow colour on paper chromatograms when treated with these reagents.[5] Also, when a mixture of two isomeric monoglucosylribitols (degradation products of the teichoic acid from *Lactobacillus arabinosus*) was examined by this technique, one of them (3-*O*-α-D-glucopyranosylribitol [6]) was found similarly to give a yellow colour. More recently 2-*O*-α-D-glucopyranosyl-D-galactose (from *Pneumococcus* type 34 specific substance [7]) and -D-glucose (kojibiose) (from the intracellular teichoic acid of *Streptococcus faecalis* strain 39 [8]) have been shown to give yellow colours.

In addition to these examples many other carbohydrates, listed in Table 1, have been shown, in these laboratories, to behave similarly. In some cases, particularly those where formaldehyde is one of the products of periodate oxidation, a yellow " halo " to a purple spot is observed instead of a yellow spot.

The carbohydrates in Table 1 have one property in common—oxidation with periodate would give malonaldehyde or a substituted malonaldehyde of type (I) or (III). In some cases, *e.g.*, 2-*O*-methyl-D-galactose (V), a hemiacetal of the substituted malonaldehyde, *e.g.*, (VI), is the product of periodate oxidation [9] but this would be rapidly hydrolysed during the reduction of the excess of periodate with sulphur dioxide. It was thought that these malonaldehydes might be responsible for the yellow colours produced on treatment with Schiff's reagent.

From Table 1 it can be seen that 2-*O*-benzyl-D-arabinose is one of the compounds giving a yellow colour under these conditions. Schwarz and MacDougall [10] had shown that oxidation of this compound with 1 mol. of sodium metaperiodate gave benzyloxymalonaldehyde (II). Consequently this aldehyde was prepared and its reactions with Schiff's

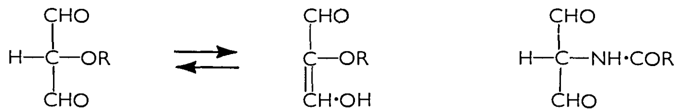
1 Buchanan, Dekker, and Long, *J.*, 1950, 3162.
2 Baddiley, Buchanan, Handschumacher, and Prescott, *J.*, 1956, 2818.
3 Baddiley, Buchanan, and Carss, *J.*, 1957, 4138.
4 Baddiley, *Proc. Chem. Soc.*, 1959, 177; *J. Roy. Inst. Chem.*, 1962, 366.
5 Baddiley, Buchanan, and Carss, *J.*, 1957, 1869.
6 Archibald, Baddiley, and Buchanan, *Biochem. J.*, 1961, **81**, 124.
7 Roberts, Buchanan, and Baddiley, *Biochem. J.*, 1963, **88**, 1.
8 Wicken and Baddiley, *Biochem. J.*, 1963, **87**, 54.
9 Cantley, Hough, and Pittet, *J.*, 1963, 2527.
10 Schwarz and MacDougall, *J.*, 1956, 3056.

TABLE 1.

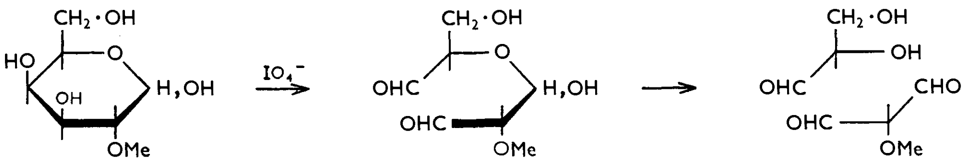
Carbohydrates giving yellow colours with the periodate–Schiff reagents on paper chromatograms.

Yellow spot	Yellow " halo "
2,5-Anhydro-D-mannose	Ribitol 3-phosphate
3,6-Anhydro-D-mannose	3-*O*-α-D-Glucopyranosylribitol
2-*O*-Methyl-D-galactose	3-*O*-Methyl-D-galactitol
2-*O*-α-D-Glucopyranosyl-D-galactose	Cellobiitol
2-*O*-α-D-Glucopyranosyl-D-glucose	Lactitol
3,6-Anhydro-D-glucose	3-*O*-Methyl-D-glucitol
D-Glucose 1,2-(methyl orthoacetate)	3,6-Anhydro-D-glucitol
2,5-Anhydro-D-talose	2-*O*-Benzyl-D-arabinose
N-Formyl-D-glucosamine	2-Deoxy-D-ribose
N-Acetyl-D-glucosamine	Me α- and β-D-galactofuranoside
	Me α- and β-D-glucofuranoside

reagent on paper were studied. Treatment with Schiff's reagent alone produced the normal purple colour, but if the paper was first sprayed with aqueous potassium iodate, treated with sulphur dioxide to reduce iodate to iodide, and then sprayed with Schiff's



(I) (II; R = CH_2Ph) (IV; R = CH_2Ph) (III)



(V) (VI) (VII)

reagent a yellow colour was produced. Since benzyloxymalonaldehyde and iodate ion are both products of the reaction of 2-*O*-benzyl-D-arabinose with periodate ion, this result showed that the yellow colour produced on treatment of this sugar with the periodate–Schiff reagents was due to the production of the malonaldehyde. It was then shown that it is the strong acid formed during the sulphur dioxide treatment which causes the aldehyde to give a yellow instead of the purple colour given with Schiff's reagent alone.

The formation of a yellow colour on treatment of a carbohydrate with the periodate–Schiff reagents is thus an indication that a derivative of malonaldehyde has been produced in the periodate oxidation. Such information has been useful in the determination of carbohydrate structures. Among the monosubstituted pentitols, only those substituted at the 3-hydroxyl group give a yellow colour, *e.g.*, ribitol 3-α-D-glucoside. In the case of the anhydrohexitols a yellow colour indicates the presence of a 1,4(3,6)-anhydro-compound, *e.g.*, 3,6-anhydro-D-glucitol. A distinction between the furanosides and pyranosides of the hexoses is also possible since the former, *e.g.*, methyl galactofuranosides, give purple spots which appear rapidly and develop yellow " halos," while the latter give blue spots which appear slowly. Useful information can also be gained about the structure of disaccharides. All the 2-*O*-substituted pentoses and hexoses so far examined, *e.g.*, 2-*O*-methyl-D-galactose (V), give yellow colours which are presumably due to the derivatives of malonaldehyde, *e.g.*, (VII), formed in the periodate oxidations. Similarly, among the hexopyranosylhexoses only those containing the 1,2-biose linkage give a yellow colour; the others give blue colours. However, when these disaccharides are reduced to the corresponding hexosylhexitols, those with 1,2- or 1,6-linkages give rapidly appearing, purple spots, while those with 1,3- or 1,4-linkages give purple spots with yellow " halos."

The structures of the dyes produced when aldehydes react with Schiff's reagent have not been fully elucidated.[11] Most experiments have been carried out with formaldehyde and it seems probable that the violet dye formed when this aldehyde reacts with a Schiff's

$$\mathrm{Cl^-\ \mathit{p}\text{-}{}^+H_3N{\cdot}C_6H_4{\cdot}\underset{\displaystyle SO_3H}{\underset{|}{C}}(C_6H_4{\cdot}NH_2\text{-}\mathit{p})_2 \xrightarrow{CH_2O} Cl^-\ \mathit{p}\text{-}{}^+H_2N{:}C_6H_4{:}C(C_6H_4{\cdot}NH{\cdot}CH_2{\cdot}SO_3H\text{-}\mathit{p})_2} \quad \text{(VIII)}$$

$$\mathrm{[2,4\text{-}(HO)_2C_6H_3{\cdot}CH(OH){\cdot}NH{\cdot}\underset{\mathit{p}}{C_6H_4}]_3C{\cdot}SO_3H} \quad \text{(IX)}$$

reagent prepared from Pararosaniline hydrochloride has a stucture of type (VIII).[12,13] The violet dyes produced from other aldehydes may have analogous structures although other types of structure have been proposed.[14]

In 1927 Shoesmith and his co-workers found that certain aromatic aldehydes gave yellow precipitates with Schiff's reagent instead of the expected soluble violet dyes.[15] Such precipitates were most readily given by aromatic 2-hydroxy-aldehydes, but if a Schiff's reagent containing only a small concentration of sulphur dioxide was used then aromatic 4-hydroxy-aldehydes also gave yellow precipitates; at higher concentrations of sulphur dioxide the normal violet dye was produced. Aromatic 3-hydroxy-aldehydes gave violet dyes at all sulphur dioxide concentrations. A study was made of the precipitate given by 2,4-dihydroxybenzaldehyde and structure (IX) was proposed.[15] The reason for this abnormal behaviour of the aromatic 2- and 4-hydroxy-aldehydes is not known.[11] In view of the present work it was thought that there might be a connection between these yellow precipitates and the yellow colour given by benzyloxymalonaldehyde with Schiff's reagent on paper in the presence of dilute sulphuric acid. In fact, benzyloxymalonaldehyde, which gives a strong violet colour with ferric chloride and can be titrated as a monobasic acid, appears to exist largely in the enolic form (IV) [10] which bears a structural relation to aromatic 2- and 4-hydroxy-aldehydes.

The colours given on paper by several aldehydes with Schiff's reagent alone and after treatment with potassium iodate–sulphur dioxide were examined and the results are summarised in Table 2.

TABLE 2.

Colours given on paper by aldehydes with Schiff's reagent (A) and potassium iodate–Schiff's reagent (B).

	A	B
Benzyloxymalonaldehyde	Purple	Yellow
p-Hydroxybenzaldehyde	Yellow	Yellow
Vanillin	Yellow	Yellow
o-Hydroxybenzaldehyde	Yellow	Yellow
m-Hydroxybenzaldehyde	Purple	—
p-Methoxybenzaldehyde	Purple	—
Glyceraldehyde	Purple	Purple

o-Hydroxybenzaldehyde, vanillin, and *p*-hydroxybenzaldehyde gave yellow colours under both sets of conditions. Thus these aldehydes and benzyloxymalonaldehyde behaved similarly when treated with potassium iodate–Schiff's reagent. *m*-Hydroxy- and *p*-methoxy-benzaldehyde, while giving purple colours with Schiff's reagent, gave no colour with potassium iodate–Schiff's reagent. This behaviour may be contrasted with that of glyceraldehyde, derivatives of which are often formed as products of the periodate oxidation of carbohydrates; this aldehyde gave a purple colour under both conditions although in the latter case there was a delay of half an hour after spraying before the colour appeared.

[11] Kasten, *Internat. Rev. Cytology*, 1960, **10**, 1.
[12] Hörmann, Grassman, and Fries, *Annalen*, 1958, **616**, 125.
[13] Nauman, West, Tron, and Gaeke, *Analyt. Chem.*, 1960, **32**, 1307.
[14] Wieland and Scheuing, *Ber.*, 1921, **54**, 2527.

Shoesmith and his co-workers [15] also found that aromatic 2- and 4-hydroxy-aldehydes gave yellow precipitates with sulphurous acid solutions of 4,4′,4″-triamino- or 4,4′-diamino-triphenylmethane. Benzyloxymalonaldehyde gives similar yellow products with these solutions and this fact has also been applied to the detection on paper chromatograms of carbohydrates giving derivatives of malonaldehyde on periodate oxidation. When Schiff's reagent is used for the detection of these carbohydrates, the other aldehydic products of periodate oxidation give purple or blue colours which may partly obscure the yellow colour from the malonaldehyde. If this happens, then a sulphurous acid solution of one of these bases may be used instead of Schiff's reagent. Only the yellow colour from the malonaldehyde is then observed.

Experimental

Pararosaniline hydrochloride was a technical dye (Messrs. Hopkin and Williams). 4,4′-Di-amino-triphenylmethane and -triphenylmethanol were prepared by Baeyer and Villiger's method.[16]

4,4′,4″-Triaminotriphenylmethane.—Pararosaniline hydrochloride (4·5 g.) was dissolved in boiling water (250 c.c.). Carbon (Norit A) (1 g.) was added and the mixture left for 3 min. After filtration through "Hyflo" silica the solution was left to cool overnight. Crystals (2·5 g.) were collected and dried. This purified material was dissolved in N-hydrochloric acid (50 c.c.). Zinc powder was added from time to time to the hot solution until the red colour disappeared. The mixture was filtered and concentrated hydrochloric acid (100 c.c.) added to the filtrate, which was then left overnight at 0°. The crystals were collected, dissolved in a small volume of warm water, and reprecipitated by the addition of concentrated hydrochloric acid (50 c.c.). The base was obtained by addition of dilute aqueous ammonia to an aqueous solution of the hydrochloride and was recrystallised from ethanol. The colourless product (1·0 g.) had m. p. 206—208° (Found: C, 78·8; H, 6·7; N, 14·4. Calc. for $C_{19}H_{19}N_3$: C, 78·9; H, 6·6; N, 14·5%). Fischer *et al.*[17] give m. p. 207°.

Detection of Carbohydrates on Paper Chromatograms by the Sodium Metaperiodate–Schiff's Reagent Technique.—As described elsewhere,[2] the dried chromatogram is sprayed with a 1% aqueous solution of sodium metaperiodate, left for 7 min., treated with sulphur dioxide, and lightly sprayed with a Schiff's reagent prepared by dissolving Pararosaniline hydrochloride (1 g.) in water (100 c.c.) which has been saturated with sulphur dioxide. An immediate purple spot indicates usually that periodate oxidation has produced formaldehyde; other aldehydes give blue colours which usually appear between 15 min. and 4 hr. after spraying. Yellow colours due to the presence of derivatives of malonaldehyde usually appear within 40 min. of spraying. Identical results were obtained if this Schiff's reagent was replaced by reagents prepared from purified Pararosaniline hydrochloride or 4,4′,4″-triamino- or 4,4′-diamino-tri-phenylmethanol. In all cases the most reliable results were obtained with reagents which were less than six weeks old. The chromatograms occasionally develop a pale brown background which can be removed by holding the paper over a steam-bath.

Carbohydrates giving yellow colours under the above conditions can also be detected by a modification of this technique. The dried chromatogram is sprayed with a 0·5% solution of sodium metaperiodate, left for 8 min., treated with sulphur dioxide, and sprayed with a 1% solution of 4,4′,4″-triaminotriphenylmethane (or the corresponding diamino-compound) in sulphurous acid. Yellow colours quickly appear although the sensitivity is not as high as when Schiff's reagent is employed. Aldehydes which give blue or purple colours with normal Schiff's reagent are almost undetectable, giving only faint brown colours after long periods.

Reaction of Benzyloxymalonaldehyde with Schiff's Reagent on Paper.—A purple coloration was observed. However, if the paper strip was first sprayed with a 1% solution of potassium iodate, then treated with sulphur dioxide, and sprayed with Schiff's reagent, a bright yellow colour was observed which after several hours became yellow-green. An identical colour was observed when the aldehyde on a paper strip was sprayed with 0·2N-sulphuric acid followed by Schiff's reagent.

[15] Shoesmith, Sosson, and Hetherington, *J.*, 1927, 2221.
[16] Baeyer and Villiger, *Ber.*, 1904, **37**, 2860.
[17] Fischer and Fischer, *Ber.*, 1904, **37**, 3355.

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