936. *Quantitative Microestimation of Formaldehyde in the Presence of Periodate and its Application to the Structural Examination of Carbohydrate Phenylosazones.*

By L. HOUGH, D. B. POWELL, and B. M. WOODS.

Colorimetric estimation of formaldehyde by means of phenylhydrazine and ferricyanide has been adapted for use in periodate oxidations.

A semimicro procedure for determining the positions of substituted groups in reducing monosaccharides has been devised by studying the periodate oxidation of phenylosazones of deoxy- and *O*-methyl-monosaccharides, and of disaccharides. Oxidations of unsubstituted hexose and pentose phenylosazones each yielded mesoxalaldehyde **1** : 2-bisphenylhydrazone and **1** mol. of formaldehyde. However, substitution of one of the hydroxyl groups in these phenylosazones resulted in the absence of one of these products.

A CONVENIENT method was required for studying the production of micro-quantities of formaldehyde during periodate oxidations of carbohydrates under varying conditions of pH. Schryver's method,[1] whereby formaldehyde is allowed to react with phenylhydrazine and ferricyanide reagents giving a mauve colour, has been improved by Dowse and Saunders,[2] but the use of this method for periodate studies demanded removal of all the periodate ion owing to interference with the colour reaction. The usual reagents for removal of periodate such as lead, arsenite, hydrogen sulphite, and stannous ions also interfered. Complete removal of periodate was effected by precipitation as the barium salt in the presence of sodium hydrogen carbonate, and subsequent estimations of formaldehyde were satisfactory.

Erythritol was used as a source of standard amounts of formaldehyde since periodate oxidations of erythritol in either unbuffered or buffered sodium hydrogen carbonate solution gave 2·0 mol. of formaldehyde as determined by the dimedone method,[3] and under both of these conditions the colorimetric method gave the same linear relation between erythritol concentration and intensity of colour. In agreement with Dowse and Saunders,[2] this direct relation was found to hold only over the range 1—45 μg. of formaldehyde. Oxidations of galactitol, D-mannitol, D-glucitol, D-glucose, and D-galactose in the presence of sodium hydrogen carbonate afforded theoretical quantities[4] of formaldehyde (±2%). Under unbuffered conditions, however, the hexitols gave low yields of formaldehyde

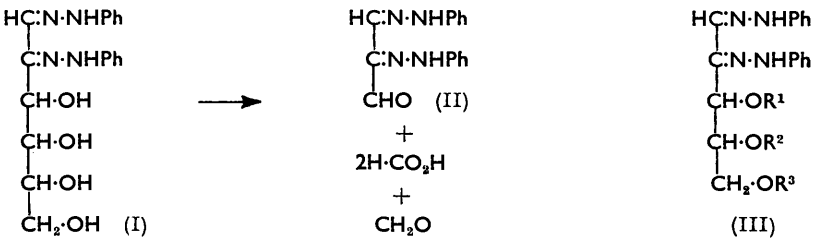
[1] Schryver, *Proc. Roy. Soc.*, 1910, *B*, **82**, 226.
[2] Dowse and Saunders, *Biochem. J.*, 1955, **60**, xxi.
[3] Bell, *J.*, 1948, 992.
[4] Reeves, *J. Amer. Chem. Soc.*, 1941, **63**, 1476.

(*ca.* 1·75 mol.), which was probably due to the presence of relatively stable formyl esters formed from the oxidation of intermediate cyclic hemiacetals.

Structural and enzymic studies of polysaccharides have necessitated the identification of disaccharide fragments, frequently in small yield, which are sometimes difficult to crystallise but often form crystalline phenylosazones. Purity of these phenylosazones can be determined for microquantities by paper chromatography [5] and by ultraviolet absorption data.[6] Periodate oxidations of various monosaccharide phenylosazones were studied for use as a semimicro procedure in subsequent investigations of the interglycosidic linkages present in phenylosazone derivatives of disaccharides.

In agreement with previous observations,[7] D-*arabino*hexose phenylosazone (cf. I) consumed the theoretical 3 mol. of periodate when oxidised in 50% alcohol at room temperature. No " over-oxidation " was observed because the cleavage product, mesoxalaldehyde 1 : 2-bisphenylhydrazone (II), was precipitated during the reaction. Simultaneously, 1 mol. of formaldehyde was formed, as determined by the colorimetric procedure, together with 2 mol. of formic acid.

Under the same conditions, D-*threo*pentose phenylosazone consumed 2·0 mol. of periodate with the formation of the product (II) and 0·9 mol. of formaldehyde. Oxidations of various deoxy- and *O*-methyl derivatives of monosaccharides (see Table) have shown that the production of the insoluble material (II) is characteristic for free hydroxyl groups at $C_{(3)}$ and $C_{(4)}$ of phenylosazone derivatives and that the liberation of formaldehyde requires the presence of a terminal glycol system. Similarly, phenylosazones derived from 1→4- and 1→3-linked dihexosaccharides (*e.g.*, maltose, cellobiose, lactose, and turanose) afforded 1 mol. of formaldehyde and none of insoluble material (II), whereas 1→6-linked dihexosaccharides (*e.g.*, melibiose) gave rise to the characteristic precipitate (II), but no formaldehyde. Thus, reaction under these conditions provides a quantitative method for differentiating between these two classes of dihexosaccharides, as little as 2 mg. of phenylosazone being required. Analogous results were obtained by Courtois, Wickstrom, and Le Dizet,[8] who estimated formaldehyde by the dimedone method, although their yields were not quantitative.



Maltose, cellobiose, and lactose phenylosazones rapidly consumed the theoretical 3 mol. of periodate (30 min.), which was followed by a slower over-oxidation, presumably involving the phenylhydrazone groups. The initial uptake of reagent could not be attributed entirely to glycol cleavage since triose phenylosazone consumed 1·3 mol. of periodate (constant value in 30 min.) and no formaldehyde or formic acid was detected. Likewise, the above workers [8] reported that glyoxal bisphenylhydrazone was also oxidised. Melibiose and turanose consumed 3 mol. of reagent rapidly, whereas the Malaprade theory requires that each should consume 4 mol. The reason for this behaviour is obscure, but it might perhaps be attributed to intramolecular interaction between the liberated reducing groups and the phenylhydrazone groups. In all cases, the disaccharide osazones gave less than theoretical yields of formic acid.

Application of the above procedure to disaccharides containing a pentose residue as the reducing group should provide a distinction between those which contain 1→5-, 1→4-,

[5] Barry and Mitchell, *J.*, 1954, 4020.
[6] Barry, McCormick, and Mitchell, *J.*, 1955, 222.
[7] Chargaff and Magasanik, *J. Amer. Chem. Soc.*, 1947, **69**, 1459.
[8] Courtois, Wickstrom, and Le Dizet, *Bull. Soc. chim. France*, 1952, 1006.

or 1→3-interglycosidic linkages. Periodate oxidation of the first (III; R^3 = glycosyl; $R^1 = R^2 = H$) would give rise to mesoxalaldehyde 1 : 2-bisphenylhydrazone (II), but not formaldehyde, whereas the last (III; R^1 = glycosyl; $R^2 = R^3 = H$) would yield formaldehyde but none of the hydrazone (II), whilst a 1→4-linked disaccharide (III; R^2 = glycosyl; $R^1 = R^3 = H$) would produce neither formaldehyde nor the hydrazone (II). Small quantities of 5-*O*-L-*arabino*furanosyl-L-arabinose and 3-*O*-L-*arabino*furanosyl-L-arabinose have been isolated [9] from the selective hydrolysis products of the araban component of sugar-beet pectin and this technique has been utilised in determining the interglycosidic linkages present in these disaccharides.

In all cases where periodate oxidation of a phenylosazone gave formaldehyde, the rate of formation was concomitant with the uptake of reagent and is consistent with the acyclic formulation suggested by Barry, McCormick, and Mitchell [6] and by Mester [10] for phenylosazones.

Experimental

Determination of Formaldehyde.—(i) *Reagent solutions.* " AnalaR " potassium ferricyanide was recrystallised from water, and then to a 2% aqueous solution concentrated hydrochloric acid (*d* 1·16) was added (2 : 5 v/v respectively).

" AnalaR " phenylhydrazine hydrochloride was dissolved in water (1 : 3 w/v respectively), and the solution was boiled for 30 min. with a little charcoal, filtered through a hot funnel, mixed with concentrated hydrochloric acid (1 : 3 v/v respectively), and cooled. The colourless crystals were collected, washed with acetone, and dried over phosphoric oxide under reduced pressure. A 1% solution of this purified material in 0·2N-sodium acetate–acetic acid buffer (pH 3·5) was prepared.

A saturated solution of barium chloride was added to a saturated solution of sodium hydrogen carbonate (4 : 1 v/v respectively), and the precipitate removed on the centrifuge. This reagent was prepared as required.

(ii) *Method.* The appropriate amount of polyhydroxy-compound (*e.g.*, 4·50 mg. of aldohexose) was dissolved in water (*ca.* 20 ml.), freshly prepared 0·3M-sodium metaperiodate solution (2 ml.) added, and the mixture made up to 25 ml. with water and stored in the dark at room temperature. At intervals, aliquot parts (2 ml.) were mixed with the barium chloride–sodium hydrogen carbonate reagent (2 ml.) contained in centrifuge tubes (10 ml.). [When barium chloride solution was used alone, periodate was not completely precipitated from solution. Oxidations carried out at pH >7·0 were buffered by the addition of saturated sodium hydrogen carbonate solution (2 ml.) and after withdrawal of aliquot parts (2 ml.) excess of periodate was removed by the addition of saturated barium chloride solution (2 ml.). During such oxidations [11] the pH rose from 7·85 to 8·75 in 24 hr.] After being kept for 10 min., the solutions were clarified on the centrifuge, aliquot parts (2 ml.) of the supernatant liquids were mixed with the phenylhydrazine reagent (2 ml.) and set aside in the dark for 30 min. Potassium ferricyanide reagent (7 ml.) was then added, and after 3 min. the mixture diluted with water to 50 ml. The clear solutions were thoroughly mixed and the absorptions at 518 mμ measured against a blank determination, either an " EEL " Portable Colorimeter (Filter No. 623) or a " Unicam " SP500 Photoelectric Absorptiometer being used. A calibration graph was constructed over the range 1—45 μg. of formaldehyde (from erythritol). Galactitol, D-mannitol, and D-glucitol gave after 3 hours' oxidation at room temperature under unbuffered conditions 1·76, 1·80, and 1·70 mol. of formaldehyde respectively, and in the presence of sodium hydrogen carbonate 1·96, 2·02, and 2·04 mol. of formaldehyde.

D-Galactose and D-glucose afforded 1·03 and 1·01 mol. of formaldehyde (constant values) respectively after oxidation for 35 min. in the presence of sodium hydrogen carbonate.

Phenylosazones.—These were prepared by Haskins, Hann, and Hudson's method; [12] the crude products were washed with benzene and recrystallised twice from ethanol–water. Each osazone gave a single zone when examined by chromatography [5] on Whatman No. 1 filter-paper circles, with toluene–ethanol–water (270 : 30 : 1 v/v) as mobile phase and ammoniacal silver nitrate as spray reagent. The molecular weights of the osazones were determined by Barry, McCormick and Mitchell's procedure [6] and were within ±2% of the theoretical values.

[9] Hough and Powell, unpublished results.
[10] Mester, *J. Amer. Chem. Soc.*, 1955, **77**, 4301.
[11] Personal communication from M. B. Perry, Queen's University, Canada.
[12] Haskins, Hann, and Hudson, *J. Amer. Chem. Soc.*, 1946, **68**, 1766.

Periodate Oxidation of Phenylosazones.—The osazone derivative (*ca.* 20 mg. accurately weighed) was dissolved in 50% ethanol (*ca.* 40 ml.), sometimes by heating and subsequent cooling, 0·3M-sodium metaperiodate (2 ml.) was added, and the whole made up to 50 ml. with 50% ethanol. After thorough mixing, the solution was stored in the dark and, at intervals, the periodate uptake and the liberated formic acid and formaldehyde were determined as follows. A blank containing no osazone was worked concurrently. The periodate uptake [13] was estimated on portions (5 ml.) which were transferred into a mixture of 0·2N-phosphate buffer (pH 7·0; 25 ml.) and 20% potassium iodide (2 ml.). Liberated iodine was titrated with 0·01N-sodium thiosulphate solution with 1% starch solution as indicator.

Phenylosazone of	Wt. (mg.)	Time (min.)	IO_4^- consumed (mol.)	$H{\cdot}CO_2H$ liberated (mol.)	CH_2O liberated (mol.)	1 : 2-Bisphenylhydrazone of mesoxalaldehyde [a]
D-Glucose	18·4	5	2·6	1·6	0·94	+
		15	2·9	1·7	1·01	
		160	3·0	1·9	1·01	
		1440	3·0	1·9	0·87	
3-*O*-Methyl-D-glucose	21·5	5	2·0	0·5	1·12	—
		15	2·2	0·6	1·15	
		160	2·3	0·8	1·08	
		1440	2·6	1·0	0·96	
D-Xylose	20·0	15	2·0	0·5	0·86	+
		60	2·0	0·6	0·91	
		120	2·0	0·6	0·89	
		1440	1·9	0·7	—	
Maltose	21·2	30	2·9	0·2	0·98	—
		240	3·5	0·3	0·94	
		360	3·7	0·6	0·95	
		1440	4·5	0·5	—	
Cellobiose	20·1	60	2·6	0·5	1·01	—
		180	3·0	0·6	1·01	
		300	3·2	0·6	0·93	
		1440	4·1	0·8	—	
Lactose	19·0	60	2·8	0·0	0·98	—
		180	3·4	0·3	1·17	
		480	3·7	0·6	1·09	
		1440	4·3	0·7	—	
Melibiose	22·0	30	2·8	0·6	0	+
		180	3·7	0·9	0	
		300	3·8	1·4	0	
		1440	4·6	1·0	0	
Turanose	21·6	30	2·8	0·6	1·00	—
		180	3·6	1·1	1·00	
		300	3·8	1·5	0·95	
		1440	4·7	0·9		
3-*O*-L-*arabino*Pyranosyl-L-arabinose	23·6	30	2·9	0·1	1·04	—
		180	3·6	0·3	1·06	
		360	3·9	0·5	1·06	
		1440	4·7	0·7	—	
3-*O*-α-D-*xylo*Pyranosyl-L-arabinose	3·0 [b]	30	—	—	1·02	—
		180	—	—	1·03	
		360	—	—	1·02	
		1400	—	—	0·88	
4-Deoxy-D-*erythro*hexulose	3·5 [b]	60	—	—	0·90	—
D-Glyceraldehyde	12·7 [c]	30	1·3	—	0	—
		120	1·3	—	0	
		180	1·3	—	0	

[a] M. p. and mixed m. p. 198° ± 1° and ultraviolet absorption identical with that of an authentic specimen. [b] In a total volume of 10 ml. [c] In a total volume of 25 ml.

Formic acid was determined in another sample (5 ml.), after addition of ethylene glycol (2 ml.), storage for 15 min., and dilution with 50% ethanol (5 ml.), by potentiometric titration to pH 8·2 with 0·01N-sodium hydroxide. The equivalence point was determined [14] by potentiometric titration of standard solutions of formic acid in 50% ethanol. Addition of 0·01N-formic

[13] Neumüller and Vasseur, *Arkiv Kemi*, 1953, **5**, 235.
[14] Anderson, Greenwood, and Hirst, *J.*, 1955, 225.

acid (5 ml.) to a solution of the phenylosazone of maltose in ethanol (5 ml.) followed by back-titration after 3 hr. with 0·01N-sodium hydroxide showed that the osazone did not consume any formic acid.

Formaldehyde determinations were carried out on samples (2 ml.) as described above, a calibration graph being obtained by oxidising erythritol in 50% ethanol. The results are given in the Table. When only small quantities of phenylosazone derivatives were available, any insoluble mesoxalaldehyde 1 : 2-bisphenylhydrazone produced in the oxidation mixtures was isolated for characterisation by separation on the centrifuge, and the supernatant liquors were used for formaldehyde determinations. The phenylhydrazone was thoroughly washed with water and recrystallised from aqueous ethanol.

Phenylosazones derived from 5-deoxy-L-arabinose, 5-deoxy-D-xylose, 5 : 6-dideoxy-D-*xylo*-hexose, L-rhamnose, and L-fucose gave mesoxalaldehyde 1 : 2-bisphenylhydrazone and no formaldehyde; L-*glycero*tetrose phenylosazone gave both formaldehyde and the hydrazone (II).

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