192. *Applications of Periodate Oxidation to Some Problems of Carbohydrate Chemistry.*

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A study has been made of the liberation of formaldehyde from primary hydroxyl groups of some partially methylated aldohexoses subjected to periodate oxidation in phosphate buffer at pH 7·5. While 2 : 3-dimethyl glucose, 2 : 3-dimethyl galactose, and 2-methyl glucose rapidly yield theoretical quantities of formaldehyde (determined by the dimedon method), 3-methyl glucose, 2 : 3 : 4-trimethyl glucose, 2 : 4- and 3 : 4-dimethyl galactose, and 3 : 4-dimethyl mannose react more slowly, and the yield of formaldehyde is always considerably below the theoretical. The method is therefore of useful qualitative application, but is not generally available for quantitative purposes.

An attempt has been made to analyse the dimethyl glucose fraction of the cleavage products from two samples of methylated glycogen. This has not been fully successful, although the analytical method employed has been proved to give reliable results on artificial mixtures of the appropriate sugars. The impossibility of effecting complete methylation of glycogen resulting in " extra " dimethyl glucoses appearing on hydrolysis renders the findings inconclusive although there is strong evidence of the presence of a 1 : 3-linkage uniting the " unit-chains ".

For some time the author has been attempting to elucidate the type of the glycosidic linkage uniting the " unit-chains " comprising the glycogen molecule. As these experiments have now reached a stage where, on the one hand, the available methods of attacking the problem have, in the author's experience, failed to yield absolutely conclusive evidence, while on the other, certain definite experimental observations have accrued, it is considered that a brief report should be made.

The fundamental difficulty experienced in this work lies in the apparent impossibility of effecting complete etherification of the polysaccharide. Although several instances have been encountered where repeated methylation has produced material displaying a methoxyl content closely approaching the expected maximum of 45·6%, analysis by the partition chromatogram (Bell, *J.*, 1944, 473) has revealed that the dimethyl glucose fraction of the hydrolysis products is always in excess of the expected proportion of one mole to every mole of 2 : 3 : 4 : 6-tetramethyl glucose arising from the end-group of each unit chain. Control experiments have shown that the 2 : 3 : 6-trimethyl glucose radical is, to a small extent only (2·2%), demethylated to dimethyl glucoses when subjected to the conditions of hydrolysis employed for methylated glycogen. The amounts of the dimethyl sugar formed in this way, however, do not account for the high yields from methylated glycogen. The origin of this " extra " dimethyl glucose is obscure. A possible explanation may be that some hydroxyl groups are masked by strong hydrogen-bonding. The average molecular weights of (vertebrate) glycogens are very large (Bell, Gutfreund, Cecil, and Ogston, *Biochem. J.*, 1948, **45**, 405), and, since the molecule is roughly spherical in shape, it may be that the methylating reagents are prevented by steric hindrance from penetrating its innermost recesses where the masked hydroxyl groups may lie. The results of experiments on two samples of rabbit glycogen are summarised below.

As it appeared likely that the dimethyl glucose fraction was not homogeneous, the necessity was felt for developing a small-scale method of analysis.

Reeves (*J. Amer. Chem. Soc.*, 1941, **63**, 1476) has described an elegant method of assay for 2 : 3-dimethyl glucose, using gravimetric determination of the formaldehyde liberated by periodate oxidation of the free primary alcoholic group on position 6. Reeves has indicated that it is necessary to carry out the oxidation in the neighbourhood of neutrality, since it has been observed that when using acid periodate 2 : 3-dimethyl glucose does not yield the theoretical

Source of material.	Mol. wt.[1]	OMe (%) after 12 Me_2SO_4–NaOH methylations.	OMe (%) after 3 MeI–Ag_2O methylations.	Hydrolysis products per mole of 2 : 3 : 4 : 6-tetramethyl glucose.[2] 2 : 3 : 6-Trimethyl glucose (moles).[3]	Dimethyl glucose (moles).[3]
Liver	$4{\cdot}4 \times 10^6$	44·9	44·9	9·7	1·3
Muscle	$2{\cdot}6 \times 10^6$	44·2	44·4	8·9	3·2

[1] See Bell, Gutfreund, Cecil, and Ogston (*loc. cit.*).
[2] Separated on chloroform–water partition column.
[3] Values corrected for 2·2% demethylation of 2 : 3 : 6-trimethyl glucose during hydrolysis.

amount of formaldehyde. Since the possible dimethyl glucoses from methylated glycogen must be, with a high degree of certainty, the 2 : 3-, 3 : 6-, and 2 : 6-derivatives, it was decided to investigate their estimation using Reeves's work as the starting point. While this work was in progress, the author learned of the experiments of Jeanloz (*Helv. Chim. Acta*, 1944, **27**, 1509) who had extended Reeves's original observations and had confirmed that a pH near 7 was necessary for quantitative yields of formaldehyde to be obtained from 2 : 3-dimethyl glucose. Both Reeves and Jeanloz have employed the carbon dioxide–bicarbonate buffering system at *ca.* pH 7·5; for the work now described it was decided to employ phosphate buffer of pH 7·5 as this appeared to possess certain manipulative advantages, and when 2 : 3-dimethyl glucose was oxidised alone or in presence of its 3 : 6-dimethyl isomer and of 2 : 6-dimethyl galactose (used in place of the syrupy glucose isomer) quantitative yields of formaldehyde (determined as the dimedon derivative) were obtained. 2 : 6-Dimethyl hexose was determined in the mixtures by first converting the sugars into the ethyl glycopyranosides by boiling ethanol containing hydrogen chloride and then measuring the uptake of periodate by the 3 : 4-glycol grouping of the 2 : 6-dimethyl component. The 3 : 6-dimethyl component could be then determined by difference. Experiments on the determination of formaldehyde by the distillation technique showed that when 2 : 6-dimethyl sugars were present a volatile bisulphite-binding compound was produced in addition to the formaldehyde. This, however, did not form an insoluble dimedon derivative.

Application of the above procedure to the dimethyl glucose fractions from the methylated samples of liver and muscle glycogens yielded the following results.

Source of glycogen sample.	Moles of Me_2 glucose per mole of Me_4 glucose found.	Me_2 glucoses expressed as molar fractions of the total. 2 : 3	2 : 6	3 : 6
Liver	1·3	0·22	0·91	0·17
Muscle	3·2	0·9	1·9	0·4

Since the report of Haworth, Hirst, and Isherwood (*J.*, 1937, 577), which these authors put forward with reserve, it appears to have been generally assumed that the linkage between the unit chains of glycogen concerned position 6 of one of the glucosyl radicals. The above results do not necessarily disprove this for the muscle glycogen specimen, but the very small amount of formaldehyde yielded by the dimethyl fraction from the liver glycogen is a clear indication of a 3-linkage predominating in that material. Until a method is devised for the complete methylation of glycogen it cannot be definitely assumed either that the cross linkages always concern the same position, or, alternatively, that these linkages occur at random on positions 2, 3, or 6.

Having shown that 2 : 3-dimethyl glucose can be easily determined by the formaldehyde-dimedon method, the author studied the possible extensions of the procedure to other partially methylated aldohexoses possessing a free primary alcoholic group. Jeanloz (*loc. cit.*) has found that 2 : 3 : 4-trimethyl glucose, oxidised in his bicarbonate buffer, could not be made to yield more than a fraction of the formaldehyde expected. Similar results were obtained in the present work, using the phosphate buffer, and with 2 : 4- and 3 : 4-dimethyl galactoses, 3 : 4-mannose, and 3-methyl glucose. On the other hand, 2-methyl glucose and 2 : 3-dimethyl galactose yielded the theoretical quantities of formaldehyde-dimedon at the same rate as 2 : 3-dimethyl glucose. The reason for these " anomalous " oxidations is not yet clear, but work in progress in this laboratory has indicated that the action of periodate on partially substituted sugars, and on free sugars themselves, can be modified by the presence of phosphate ion. Indeed, unpublished work (with A. T. Johns and Miss A. Palmer) has so far shown that, at pH 7·5 in phosphate buffer, secondary oxidations result in the production of carbon dioxide

and of steam-volatile acids (largely formic), where such would not be expected normally to be formed. A somewhat similar observation regarding phosphate buffer has already been made by Lindstedt (*Nature*, 1945, **156**, 448) although this author was working at a temperature above that of the laboratory.

No sugar having a free primary hydroxyl group on position 6 and a potential hydroxyl group on position 5 (*i.e.*, the sugar reacts with periodate ion in the open-chain form) failed to yield sufficient formaldehyde-dimedon for satisfactory identification when 10—20 mg. of material were oxidised during 6—12 hours. This procedure, therefore, affords a convenient small-scale method for qualitative detection of a free primary alcoholic group in a partly-methylated sugar. Hitherto the qualitative procedure generally applied has been that of Oldham and Rutherford (*J. Amer. Chem. Soc.*, 1932, **54**, 366) which requires substitution of free hydroxyl groups in the appropriate glycoside by the *p*-toluenesulphonyl radical followed by replacement of the latter by iodine, and, finally, detection of the halogen.

Experimental.

Determination of Formaldehyde from Primary Alcoholic Groups.—The procedure finally adopted owes much to the above-quoted work of Reeves and of Jeanloz. The sugar, or mixture of sugars, is dissolved in water and diluted to a suitable volume so that not more than 20 mg. of sugar having a free primary hydroxyl group are contained in 1—3 ml. The required volume is measured into 10 ml. of phosphate buffer (0·066—0·2M), pH 7·4—7·5, and 6 ml. of 0·1M-sodium metaperiodate (0·6 millimole of 10^{-4}) added. After being kept for the desired time at room temperature (15—17°), the solution is acidified by addition of 1·5 ml. of 2N-hydrochloric acid, and periodate and iodate reduced by the action of 4 ml. of 1·2N-sodium arsenite. When reduction is complete, 16 ml. of 2N-sodium acetate–acetic acid buffer (pH 4·5) are added followed by 160 mg. of dimedon in 2 ml. of ethanol. The mixture is then heated on the boiling water-bath for 10—15 minutes and kept at room temperature for 2 hours.

The crystalline precipitate of formaldehyde-dimedon (hereafter referred to as " FD ") is collected on a tared sintered filter, washed with 75 ml. of water, and weighed after drying for 30—60 minutes at 0·05 mm. over phosphoric oxide.

With dimedon obtained from B.D.H. it was found necessary to run a simultaneous control determination on the reagents as a small precipitate was formed. Material of German origin, however (Merck, " pro analysi "), gave no precipitate.

Experiments on single sugars. Results are tabulated below. In every instance the FD isolated had m. p. 189—190° (corr.).

Sugar.	Duration of oxidation (hrs.).	Sugar taken (mg.).	FD found (mg.).	Yield of FD (%).
2 : 3-Dimethyl glucose	2	9·92	13·8	98·8
,, ,,	2	26·1	35·5	97·0
2 : 3-Dimethyl galactose	2	22·4	30·1	95·8
,, ,,	6	22·4	30·3	96·5
2-Methyl glucose	2	26·4	39·0	98·2
,,	2	22·0	33·0	99·7
3-Methyl glucose	2	31·0	5·2	11·2
,,	4	24·7	6·4	17·2
,,	16	24·7	9·4	25·2
2 : 4-Dimethyl galactose	3	10·38	8·1	56·9
,, ,,	36	10·38	11·7	80·3
3 : 4-Dimethyl galactose	3	8·73	6·1	49·7
,, ,,	48	4·75	5·3	79·9
3 : 4-Dimethyl mannose	8	7·34	2·66	25·8
,, ,,	36	7·34	3·62	35·1
2 : 3 : 4-Trimethyl glucose	2	22·94	13·7	45·4
,, ,,	72	14·48	10·7	56·2

Determination of 2 : 3-*Dimethyl Glucose in the Presence of* 3 : 6-*Dimethyl Glucose and* 2 : 6-*Dimethyl Galactose.*—Experiments showed that syrupy 2 : 6-dimethyl glucose (Bell and Synge, *J.*, 1938, 833) was oxidised without producing formaldehyde; the galactose isomer (Oldham and Bell, *J. Amer. Chem. Soc.*, 1938, **60**, 323; Bell, *J.*, 1945, 692), being crystalline, was, however, used for convenience in the following work. 3 : 6-Dimethyl glucose was prepared by the method of Bell (*J.*, 1935, 175).

(a) *Gravimetric experiments.* 134·23 Mg. of mixed sugars (16·33 mg. of " 2 : 3 ", 87·3 mg. of " 3 : 6 ", and 30·6 mg. of " 2 : 6 "), dissolved in 2 ml. of water and 10 ml. of phosphate buffer, were oxidised for 2 hours with 6 ml. of 0·1M-sodium metaperiodate. After dilution of the mixture to 50 ml. with water, duplicate determinations of FD were carried out on 20 ml. samples. Found: 8·85 mg. (96·2%); 8·72 mg. (94·7%). A control experiment with 45·1 mg. of 3 : 6-dimethyl glucose and 18·0 mg. of 2 : 6-dimethyl galactose yielded no FD.

107 Mg. of mixed sugars (21·55 mg. of " 2 : 3 ", 67·00 mg. of " 3 : 6 ", and 18·50 mg. of " 2 : 6 ") were dissolved in 10 ml. of water, 20 ml. of phosphate buffer, and 24 ml. of 0·1M-periodate. After 2 hours, 40 ml. samples were taken for FD determinations of the 2 : 3-component. Found: 11·88 mg. (98%); 11·69 mg. (96·5%).

(b) *Volumetric experiments.* 84·0 Mg. of mixed sugars (10·0 mg. of " 2 : 3 ", 48·8 mg. of " 3 : 6 ",

and 25·2 mg. of " 2 : 6 ") were oxidised for 2 hours. The solution was diluted to 30 ml., and 15 ml. portions steam-distilled in the Pregl micro-Kjeldahl apparatus following the procedure of Rees (*Biochem. J.*, 1946, **40**, 632) for the determination of hydroxyamino-acids. The amounts of bound bisulphite required 13·38 and 13·09 ml. of 0·01N-iodine respectively. These results indicated that some volatile bisulphite-binding substance had been formed in addition to formaldehyde. Control experiments using 2 : 3-dimethyl glucose alone had previously shown that the formaldehyde formed could be satisfactorily estimated by distillation either titrimetrically, or gravimetrically as FD.

Analysis of a Mixture of the Three Dimethyl Sugars.—69·42 Mg. of mixed sugars (22·44 mg. of " 2 : 3 ", 20·85 mg. of " 3 : 6 ", and 26·13 mg. of " 2 : 6 ") were made up to a volume of 3·00 ml. with pure ethanol. (*a*) Determination of " 2 : 3 ". 1 Ml. of the solution was treated in the usual manner. FD found : 10·00 mg., corresponding to 7·12 mg. of 2 : 3 dimethyl glucose or 21·36 mg. (95·2%) in the original mixture. (*b*) Determinations of " 2 : 6 ". To each of two portions of the solution (1 ml. and 0·835 ml.) were added 2 ml. of pure ethanol containing 2% (w/v) of hydrogen chloride. The mixtures were boiled under reflux for 5 hours, a slight excess of saturated sodium bicarbonate added to each, and the alcohol evaporated on the water-bath. 2 Ml. lots of water were then added to the residues, the pH adjusted to 7 (bromothymol-blue) by dilute acetic acid, and 2 ml. of 0·3325N-sodium metaperiodate added. After 5 hours at room temperature, the periodate consumed was determined by titration with 0·05N-sodium arsenite after addition of 2 ml. of 0·5M-phosphate buffer (pH 7·5) and excess of potassium iodide. A control experiment, omitting the sugars, was run simultaneously. The results were as follows :

Sample taken (ml.).	0·05N-Arsenite consumed (ml.).	2 : 6-Me_2 glycoside in sample (moles).	2 : 6-Me_2 sugar in mixture (mg.).	Recovery (%).
1·00	1·70	0·0425	26·52	101·5
0·835	1·43	0·0358	26·70	102·1

The Demethylating Action of Aqueous Hydrochloric Acid on 2 : 3 : 6-*Trimethyl Methylglucoside.*—(a) *Preparation of the pure α/β methylglucoside.* A specimen of 2 : 3 : 6-trimethyl glucose which had been twice chromatographed by partition (Bell, *J.*, 1944, 473) was boiled for 4 hours in 5% concentration with methanol containing 2% (w/v) of dry hydrogen chloride. After neutralisation of the acid by addition of barium carbonate and filtration, the solvent was removed under reduced pressure and the residual syrup dried in a vacuum, dissolved in ether, and boiled with charcoal. The syrup obtained after filtration and evaporation of the ether was distilled at 0·05 mm. through a vacuum-jacketed fractionating column. The middle fraction was collected and redistilled, the middle fraction being again collected. This had $[\alpha]_D^{20°}$ + 18·1° (*c*, 7·0 in water; *l* = 4) and $n_D^{16°}$ 1·4566. These constants exactly fit the line given by Hirst and Young (*J.*, 1938, 1247) for the graphical relationships between $[\alpha]_D$ and $n_D^{16°}$ of mixtures of the α- and β-methylglucoside.

(b) *Hydrolysis of the glucoside and chromatography of the products.* The method of hydrolysis was identical with that used in the following experiments on methylated glycogen and has been described, with the partition procedure, by Bell (*J.*, 1944, 473). The following results were obtained :

2 : 3 : 6-Me_3 methyl-glucoside (mg.).	Dimethyl sugars found (mg.).	Methoxyl content of dimethyl sugars (%).	Dimethyl sugars formed (as % of trimethyl sugar expected).
2,330	47	29·6	2·14
2,405	54	29·9	2·34

The mean yield of dimethyl sugars is therefore 2·2%. Analysis showed that the material consisted mainly of the 2 : 6-dimethyl isomer.

Experiment on the Recovery of a Pure Dimethyl Sugar.—Experiments having shown that the recovery of tetramethyl and trimethyl glucose in the partition chromatogram was of the order of 94%, it seemed necessary that a control experiment should be performed using a pure dimethyl sugar. To this end a sample of pure 2 : 3-dimethyl α-methylglucoside (Irvine and Scott, *J.*, 1913, **103**, 575) (560 mg.) was subjected to hydrolysis followed by partition as for a dimethyl sugar. The final acetone solution was evaporated to dryness and the sugar extracted with dry ethyl acetate. Evaporation of the solvent left 496·3 mg. of a colourless glass (94·5% recovery) (OMe, 29·8; 2 : 3-dimethyl glucose by the periodate method, 100%).

Experiments on Glycogen.—(a) *Muscle.* As no previous investigation on rabbit muscle glycogen has been recorded, the following details are appended. (The isolation was performed in 1936.) Rabbits were given lethal doses of " Numal–Roche " and the carcases immediately frozen in ice and salt. 3·5 Kg. of muscle tissue were dissected into 3·5 l. of hot 30% potassium hydroxide. After the tissue had disintegrated the crude glycogen was precipitated by the addition of 4 l. of 95% ethanol. Thereafter purification followed the procedure of Bell and Young (*Biochem. J.*, 1934, **28**, 882). 11 G. of phosphate- and ash-free glycogen were finally obtained, after 4 treatments with acetic acid, representing a tissue-glycogen content of 0·31%. The material, in dilute solution, gave a distinct mauve coloration with iodine, and had $[\alpha]_D$ + 196·0° (*c*, 1·025 in water; *l* = 2).

A sample of this glycogen was acetylated and methylated after Haworth and Percival (*J.*, 1932, 2277). After 8 treatments the methoxyl content was 44·2%; this value was not increased by 4 additional methylations. 3 Methylations with Purdie's reagents, the methylated glycogen being dissolved in dry toluene, likewise failed to effect any further methylation.

Hydrolysis of 1·00 g. followed by partition on the silica–water column gave the following fractions : Me_4, 75 mg.; Me_3, 530 mg.; Me_2, 193 mg. Correcting for 2·2% of the Me_3 fraction appearing as Me_2 by hydrolytic demethylation the final figures become Me_3, 542 mg.; Me_2, 181 mg. Molecular proportions are therefore 1 : 7·7 : 2·4 giving an average unit chain of 11 radicals.

186 Mg. of the dimethyl fraction were dissolved in pure dry ethanol and the solution made up to 10 ml. 2 Ml. of this solution were taken for the determination of 2 : 3-dimethyl glucose. 14·75 Mg. of

FD were found corresponding to 10·5 mg. of the sugar in the sample, or 52·5 mg. (28·2%) in the total. A further 1 ml. of the ethanol solution was converted into the ethyl glucopyranosides as described above. The product consumed 2·15 ml. of 0·05N-periodate corresponding to 11·18 mg. (60%) of 2 : 6-dimethyl glucose. The 3 : 6-dimethyl glucose fraction therefore amounted to 11·8% of the total.

(b) *Liver.* This material had already been examined and reported on (Bell, *Biochem. J.*, 1935, **29**, 2031; *J.*, 1944, 473). 8 Methylations yielded material of OMe 44·7%, a value not raised by 4 further methyl sulphate treatments followed by three with Purdie's reagents in dry toluene. 2·0 G. were hydrolysed and the cleavage products separated by partition to yield the following fractions: Me_4, 199 mg.; Me_3, 1·773 mg.; Me_2, 261 mg. Correcting for 2·2% of the Me_3 fraction appearing as Me_2, by hydrolytic demethylation the final figures become Me_3, 1812 mg.; Me_2, 222 mg. Molecular proportions are therefore 1 : 9·7 : 1·3 giving an average chain length of 12 radicals.

260 Mg. of the dimethyl sugars (OMe, 29·7%) were dissolved in ethanol and the volume made up to 10 ml. 3 × 1 Ml. samples were used for the FD estimation. Found: 6·0, 6·2, and 5·9 mg. corresponding to 4·35 mg. of 2 : 3-dimethyl glucose in the sample of 26·0 mg. of mixed sugars or 16·7% of the total. 1-Ml. samples of the above ethanol solution were used for the 2 : 6-dimethyl glucose determination. Both consumed 3·50 ml. of 0·05N-periodate corresponding to 18·2 mg. (70%) of the sugar. The 3 : 6-isomer was therefore present to the extent of 13·3%.

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