954. *Esterification of the Primary Alcoholic Groups of Carbohydrates with Acetic Acid: a General Reaction.*

By R. B. Duff.

The primary alcoholic group of various carbohydrates is selectively esterified by 50% acetic acid at 100°. The crystalline acetylglucose obtained in this way is shown to be identical with the 6-*O*-acetyl-D-glucopyranose obtained from cultures of *Bacillus megaterium* grown on glucose media.[1] Crystalline 6-*O*-acetyl-D-galactopyranose, an unstable syrupy xylopyranose acetate, and a syrupy di-*O*-acetylglucose have also been prepared.

An estimate is made of the " total esterification " (*i.e.* including di- and tri- as well as mono-acetates) obtained with various carbohydrates including laminarin, glycogen, and amylose.

A preliminary note [1] reported the isolation of 6-*O*-acetylglucose from cultures of *Bacillus megaterium* (N.C.I.B. No. 8508). In view of the activity of the primary alcoholic group of a carbohydrate [2] an attempt was made to prepare the ester by preferential acetylation *in vitro*.

Acetic acid (50%), used at 100° for about 24 hr., was more satisfactory than the conventional reagents and the excess of acid could conveniently be removed *in vacuo* at a low temperature, to give a syrup containing unchanged glucose, 6-*O*-acetylglucose, and a di-*O*-acetylglucose as the principal components. The proportions of glucose and 6-*O*-acetylglucose were determined by quantitative paper chromatography and of the diacetate by difference, since under the conditions used the last travelled as a diffuse spot. Crystalline monoacetylglucose and syrupy diacetylglucose were separated by partition of the mixture on a cellulose column, and the monoacetate was shown to be identical with the ester from *B. megaterium*. Crystalline 6-*O*-acetylgalactose was prepared similarly; its structure follows by analogy. Moreover, 6-*O*-acetyl-glucose and -galactose failed to form

[1] Duff, Webley, and Farmer, *Nature*, 1957, **179**, 103.
[2] Sugihara, *Adv. Carbohydrate Chem.*, 1953, **8**, 1.

formaldehyde on periodate oxidation, indicating participation of the primary alcoholic groups in ester formation. In this work periodate oxidation was carried out by a modification of Reeves's method [3] in which the mixture was kept at pH 7·0 throughout the estimation (see p. 4732).

A syrupy monoacetylxylose was obtained which gave 1 mol. of formaldehyde on periodate oxidation, and the infrared spectrum indicated a pyranose structure in the ester as in the parent sugar. It seems clear that in this case the acetoxyl group is secondary. The acetylxylose was appreciably less stable than the 6-*O*-acetylhexoses. The periodate consumption of all these esters was 1 mol. less than that of the parent sugar.

A small-scale procedure was devised to examine the effect of acetic acid on sugars at various concentrations and at various temperatures. Hestrin's [4] colorimetric assay for esters was used and hence the results represent " total esterification," *i.e.*, include the proportion of di- and tri-acetyl-sugar formed as well as the monoacetyl derivative. The results (Table, p. 4734) show that appreciable amounts of esters are formed even with low concentrations of acetic acid and at lower temperatures. Hexitols give about twice the " total esterification " obtained with the parent sugars. Ketohexoses form monoacetates (presumably 1-acetates) since the primary alcoholic group on $C_{(6)}$ takes part in ring formation. These observations support the view that the primary hydroxyl group is principally concerned in these esterifications.

Some polysaccharides were examined by the above technique. In laminarin, glycogen, and amylose, about one sugar residue in every four or five was acetylated. The polysaccharides, however, appeared to be degraded, as periodate oxidation of the product from laminarin indicated an increased number of end groups. The acetylated amylose had no " blue value." During isolation by conventional methods some polysaccharides and sugars are exposed to relatively concentrated acetic acid (*e.g.*, glycogen, acidic polysaccharides such as chondroitinsulphuric acid, turanose). If acetylation without degradation takes place under these conditions the periodate consumption of the polysaccharides would be expected to be less than the true value.

Experimental

Paper Chromatography.—This was carried out by the descending technique on Whatman No. 1 paper with butan-1-ol saturated with water as a solvent. Normally a cabinet thermostatically controlled at 30° was used, but R_G values [5] were determined at room temperature (15—20°). Sugars and their esters were revealed by sprays of aniline phthalate,[6] resorcinol and hydrochloric acid,[7] aniline–diphenylamine–phosphoric acid,[8] or ammoniacal silver nitrate.[9]

Colorimetric Estimation of Acetylated Sugars.—The reaction with alkaline hydroxylamine and colour development with ferric chloride were carried out essentially as described by Hestrin,[4] and the results were calculated by comparison with a standard curve obtained with the recrystallised ester (6-*O*-acetyl-glucose and -galactose) or the chromatographically purified syrup (acetylxylose). When an E.E.L. colorimeter with small (4 ml.) tubes and a green filter was used the hexose esters and acetylxylose (0·5 mg. each) in water (1 ml.) gave colour densities of 3·48 and 3·20 respectively. The determination extended to about 1 mg. in the conditions used.

Estimation of Acetic Acid.—Acetates were hydrolysed with toluene-*p*-sulphonic acid, and the acetic acid distilled and titrated with 0·1N-alkali essentially as described by Kuhn and Roth.[10]

6-O-*Acetyl*-D-*glucopyranose.*—Glucose (1000 g.) and 50% acetic acid (100 ml.) were heated overnight in a water-bath at 100°. The excess of acid was removed at 35° *in vacuo* and the

[3] Reeves, *J. Amer. Chem. Soc.*, 1941, **63**, 1476.
[4] Hestrin, *J. Biol. Chem.*, 1949, **180**, 249.
[5] Hirst, Hough, and Jones, *J.*, 1949, 928.
[6] Partridge, *Nature*, 1949, **164**, 443.
[7] Forsyth, *ibid.*, 1948, **161**, 239.
[8] Buchan and Savage, *Analyst*, 1952, **77**, 401.
[9] Partridge, *Nature*, 1946, **158**, 270.
[10] Kuhn and Roth, *Ber.*, 1933, **66**, 1276.

resulting syrup separated in four lots on a powdered cellulose column (5 × 85 cm.) with water-saturated butan-1-ol. Owing to overlap it was necessary to pass some of the fractions through the washed column a second time to obtain the maximum yield of monoacetate. The ester (29 g.) crystallised on removal of solvent *in vacuo* at room temperature and recrystallised on removal of solvent from a concentrated aqueous solution. The crystals and syrupy mother-liquor were separated on a porous tile.

Four small-scale experiments were set up to determine the relative proportions of products. The syrups were dried (P_2O_5) *in vacuo* at room temperature to constant weight. Quantitative paper chromatography with the Somogyi reagent [11] (12 minutes' heating) indicated that the syrups contained 52—55% of uncombined glucose and 26—30% of 6-*O*-acetylglucose. By difference the di- and tri-acetylglucose together appeared to be 15—22%. Direct determination of the higher acetylated material was difficult because of " streaking " of the paper in that region, and the amount present will be smaller than that indicated above as some reversion products were also seen on the chromatogram.

Five recrystallisations of the crude acetylglucose from 95% ethanol gave a product with m. p. 135° alone or mixed with 6-*O*-acetyl-D-glucopyranose from *B. megaterium* cultures, $[\alpha]_D^{20}$ +48° (equil.; *c* 4·0 in H_2O), R_G 0·28 (Found: C, 42·6; H, 6·35; Ac, 18·4. Calc. for $C_8H_{14}O_7$: C, 43·2; H, 6·35; Ac, 19·4%). The infrared spectrum of the ester shows variations due to differences in the proportions of the α- and β-anomer. A description of these variations will be published later. After five recrystallisations from water the ester prepared *in vitro* gave a spectrum which was virtually identical with that of the biologically prepared material.

Periodate Oxidation of 6-O-*Acetylglucose*.—(a) *Uptake*. The periodate consumption (determined by the usual methods) was 1·7 (1 day), 3·5 (3 days), 3·7 (11 days), 3·7 (18 days) mol. The ester prepared from *B. megaterium* cultures similarly consumed 2·9 (1 day), 3·5 (7 days), 3·65 (11 days), and 3·65 (18 days) mol.

(b) *Formic acid production with hot periodate solution*. Oxidation was effected by Hirst and Jones's method,[12] and the formic acid formed was titrated potentiometrically.[13] The ester, prepared as described above, gave 4·0 mol. of formic acid in good agreement with the amount obtained from the naturally occurring ester (4·1 mol.). An unsubstituted hexose gives 5 mol. of formic acid, so it appears that the ester is oxidised without much elimination of the acetyl residue under these conditions.

(c) *Formaldehyde production by Reeves's* [3] *procedure*. (1) Unmodified. Formaldehyde–dimedone compound (m. p. 188—189°) produced (moles per mole of ester) was 0·24 (natural ester), 0·24, 0·25 (ester prepared *in vitro*).

(2) With less oxidant. The material was oxidised in separate portions with 2, 3, 4, 5, and (for comparison) 6 mol. of oxidant. The ester and glucose respectively gave 0, 0, 0, 0·26, 0·27, and 0, 0, 0, 1·0, 1·0 mol.

(3) At an acid pH. The ester was oxidised in a buffer solution of the usual composition but with the pH adjusted to 5·0 instead of 7·0. Yields were 0·20 and 0·23 mol.

(4) Removal of unused periodate with lead acetate.[14] The ester (40 mg.) was oxidised as usual with the appropriate quantities of reagents. Excess of M-lead acetate (8 ml. of aqueous solution) was added and insoluble salts were removed on the centrifuge. An aliquot part (10 ml.) of the liquid was made just acid to methyl-red with 2N-acetic acid, and buffer added (10 ml. of 1 : 1 v/v 2N-sodium acetate–N-hydrochloric acid). Addition of dimedone solution gave no immediate precipitate but a small amount of amorphous, sticky material with a low m. p. (90—110°) was slowly obtained.

For comparison, glucose (36 mg.) was treated similarly: the theoretical yield was obtained (1·0 mol.; m. p. 189°).

(5) Removal of excess of periodate with lead acetate and separation of formaldehyde by distillation. The method described in (4) was followed but an aliquot part of the periodate-free solution (10 ml.) was treated with water (60 ml.), and formaldehyde was distilled from the solution generally as described by Boyd and Logan.[15] The buffer and dimedone were added to the distillate (70 ml., including 5 ml. of water added to the receiver initially) and the compound

[11] Somogyi, *J. Biol. Chem.*, 1945, **160**, 61.
[12] Hirst and Jones, *J.*, 1949, 1659.
[13] Anderson, Greenwood, and Hirst, *J.*, 1955, 225.
[14] Akiya, *J. Pharm. Soc. Japan*, 1951, **71**, 865.
[15] Boyd and Logan, *J. Biol. Chem.*, 1942, **146**, 279.

collected as before. In three determinations 0—0·05 mol. was obtained. Glucose gave almost the theoretical yield (0·95 mol.).

Experiments (2) and (3) show that the proportion of formaldehyde given in (1) cannot be due to over-oxidation either by the large excess of periodate present in the initial stage of the oxidation (6 mol.) or by the use of an oxidation medium buffered at pH 7·0 instead of pH 5·0 (where over-oxidation should be at a minimum). In Reeves's procedure excess of periodate is destroyed with arsenite in alkaline solution (pH 8) and the reaction is slow. It seems that either acetylglucose or its oxidation product is partly deacetylated in these conditions and is then oxidised to formaldehyde by the remaining periodate. When excess of periodate is removed rapidly and at pH 7·0 with lead acetate, and the formaldehyde is separated from the mixture as in (5), glucose, containing a free primary hydroxyl group, gave a theoretical yield of formaldehyde while acetylglucose gave none.

Experiment (4) shows that reliable results are not always obtained when formaldehyde is estimated directly in an oxidation medium containing lead acetate.

Di-O-acetylglucose.—Crude diacetylglucose, accumulated from several separations, was further purified by chromatography and obtained as a syrup, apparently homogeneous on the paper chromatogram (R_G 0·58) (Found: Ac, 30·4. Calc. for $C_{10}H_{16}O_8$: Ac, 30·6%), had $[\alpha]_D^{25}$ $+50·4°$ (*c* 1·94 in H_2O) and gave 1·39 times the optical density given by an equal weight of 6-*O*-acetylglucose in the colorimetric assay. It seems likely that this is a 4 : 6-diacetate.[16]

*6-O-Acetyl-*D*-galactopyranose.*—Galactose (100 g.) was acetylated and the products were separated as described above. The yield of ester obtained was 33% (estimated on the crude reaction mixture by the colorimetric method). *6-O-Acetyl-*D*-galactopyranose* formed prismatic rods (from aqueous alcohol), m. p. 138°, $[\alpha]_D^{20}$ $+66°$ (equil.; *c* 2·0 in H_2O), R_G 0·27 (Found: C, 42·9; H, 6·1; Ac, 18·9. $C_8H_{14}O_7$ requires C, 43·2; H, 6·3; Ac, 19·4%). The infrared spectrum did not clearly indicate the type of ring structure.[17]

Periodate Oxidation of Acetylgalactose.—The methods used were as described for acetylglucose. 4 Mol. of formic acid were produced by hot periodate solution. 6-*O*-Acetylgalactose and galactose gave 0·08 and 0·94 mol. respectively of formaldehyde–dimedone compound.

Acetylxylose.—Xylose (25 g.) was acetylated and the products were separated as described above. Only 17% of esterified material was obtained (estimated colorimetrically). The syrupy product had $[\alpha]_D^{20}$ $+20·6°$ (equil.; *c* 1·94 in H_2O) (Found: Ac, 21·5. $C_7H_{12}O_6$ requires Ac, 22·4%). The material was homogeneous on the paper chromatogram, with R_G 0·47. It was appreciably less stable than the hexose acetates and was decomposed to some extent if the aqueous solution was heated, *e.g.*, when dried on a paper chromatogram with warm air.

Infrared examination of this acetate showed absorption bands at 941, 905, and 755 cm.$^{-1}$, close to those of D-xylopyranose (935, 905, and 762 cm.$^{-1}$) and other xylopyranose derivatives.[17] Periodate oxidation as for acetylglucose [(5) above] gave 0·95 mol. of formaldehyde. Oxidation with hot periodate gave 2·7 mol. of formic acid.

Esterification of Carbohydrates with 50% Acetic Acid.—The substance (10 mg.) with 50% acetic acid (0·5 ml.) was heated at 100° for 18 hr. in a sealed tube. Acetic acid was removed *in vacuo* over solid potassium hydroxide at room temperature, and water (1 ml. or 5 ml.) added. In aliquot parts (0·1 ml. or 0·5 ml.) ester was determined as hydroxamic acid. In the Table

Substance	" Esterification units "	R_G of products	Substance	" Esterification units "	R_G of products
Glucose	100	0·28, 0·6	3-*O*-Methylglucose	130	0·30
Sorbitol	215	0·11, 0·29	Fructose	103	0·32, 0·40
Mannitol	225	0·12, 0·32	Sorbose	78	0·35, 0·41
Erythritol	187	0·27, 0·55	Arabinose	55	0·41, 0·54
Glucosamine hydrochloride	83	0·1, 0·17, 0·22	Ribose	38	0·48
Acetylglucosamine	90	0·2, 0·43	Rhamnose	62	0·63
Dihydroxyacetone	9	0·41 (Trail)	Fucose	65	0·52
Glyceraldehyde	6	Trail	Sucrose	207	0·30

the values (molar) are expressed on the basis that the colorimeter reading for 1 mol. of glucose was equivalent to 100 " Esterification Units." A blank determination was performed with the parent sugar and the reading (normally small) on the E.E.L. colorimeter subtracted from that

[16] Cf. Gottlieb, Caldwell, and Hixon, *J. Amer. Chem. Soc.*, 1940, **62**, 3342; Tarkow and Stamm, *J. Phys. Chem.*, 1952, **56**, 262.

[17] Barker, Bourne, Stephens, and Whiffen, *J.*, 1954, **3468**; Barker and Stephens, *J.*, 1954, **4550**.

given by the acetylated product. The remainder of the solution was examined on the paper chromatogram and the R_G value(s) of the product(s) measured.

Amount of Acetylation with Variation of Acetic Acid Concentration, Temperature, and Time.—Determinations were carried out as above except that the tubes were kept at 100° for 24 hr. or as indicated. A standard of the appropriate monoacetate was used. The results, expressed in the Table as monoacetate, include higher acetylated material and are actually proportional to the total amount of acetylation.

(*a*) Acid concn. (%)	10	20	30	40	50	60	70	80	90	100%
Acetylglucose formed (%)	6·6	17·8	18·3	38	41	50	64	91	127	172
Acetylgalactose formed (%)		6·7		13·8		26·4		61		94
Acetylxylose formed (%)		4·5		14·5		28		64		180
(*b*) Time (hr.) of heating with 50% acid at 100°				8		18		48		
Acetylglucose formed (%)				43		46		52		
(*c*) Temperature				50°		75°		100°		
Acetylglucose formed (%) (after 24 hr. with 50% acid)				18·6		38·5		46·0		

Acetylation of Polysaccharides with Acetic Acid.—(*a*) Laminarin (5 g., provided by Dr. E. T. Dewar of the Scottish Seaweed Research Institute) was treated with 50% acetic acid as usual. The filtered solution was concentrated *in vacuo* at 30°, the acetylated polysaccharide was precipitated by pouring of the syrup into alcohol (400 c.c.) and purified by three further precipitations. The "acetylglucose content" (27%, constant) determined colorimetrically with the crystalline ester as a standard, agreed with the amount of acid obtained by hydrolysis (Found: Ac, 5·3%).

(*b*) The polysaccharide (0·5 g.) was treated with glacial acetic acid. The product was a glass, insoluble in water. It was precipitated with ether from alcoholic solution and washed with dry ether containing 5% of dry acetone (Found: Ac, 16·4%).

(*c*) Laminarin and acetylated laminarin (prepared with 50% acetic acid) were oxidised with potassium periodate in the usual way.[13] The formic acid liberated (in mmoles per anhydroglucose unit) was: laminarin, 60 (30 min.), 112 (4 days), 135 (10 days), 163 (25 days); acetyl-laminarin, 39 (30 min.), 113 (4 days), 240 (10 days). Iodine was liberated in the latter case after 10 days. The results correspond to the liberation of 1 mole of formic acid from about 6·15 and 4·2 anhydroglucose units for normal and acetylated laminarin respectively.

(*d*) Glycogen (from *Mytilus edulis*; Light & Co. Ltd.) had 4·4 and 9·6% of Ac after treatment with 50 and 100% acetic acid respectively.

(*e*) Amylose (from potato starch; blue value 1·19; supplied by Dr. J. S. D. Bacon of this Institute) had 6·5% of Ac after treatment with 50% acetic acid (the acetylated material had no blue value).

(*f*) Cellulose (Whatman's standard grade ashless powder) had 0·8% of Ac after treatment with 50% acetic acid.

The yields of acetylated polysaccharides were generally poor (25% or less, except for cellulose which was recovered practically unchanged in weight), supporting the view that degradation takes place.

The author thanks Dr. V. C. Farmer of this Institute for recording and interpreting the infrared spectra and to Professor E. L. Hirst, F.R.S., for his interest.

The Macaulay Institute for Soil Research,
Craigiebuckler, Aberdeen.

[*Received, July 3rd,* 1957.]