Structural Studies of the Cellulose Synthesised by Acetobacter acetigenum.*

By K. S. Barclay, E. J. Bourne, M. Stacey, and M. Webb.

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The nitrogen-free polysaccharide synthesised from D-glucose by cultures of *Acetobacter acetigenum* (T. K. Walker's strain) has been proved to be a cellulose, in agreement with Kaushal and Walker (*Biochem. J.*, 1951, **48**, 618). Methylation and end-group assay revealed the average chain length of its trimethyl ether to be of the order of 600 glucose units. The relative merits of the Barnett and the acetic acid–trifluoroacetic anhydride method of acetylation have been compared. Membranes produced by resting cells of the organism from D-glucose and from D-mannose have been shown to contain only D-glucose residues in the polysaccharide component.

Although it has been known for more than twenty years that the polysaccharide constituent of the membrane produced by *Acetobacter xylinum* is a cellulose (cf. Eggert and Luft, *Z. physikal. Chem.*, 1930, **7**, *B*, 468; Hibbert and Barsha, *Canad. J. Res.*, 1931, **5**, 580; 1934, **10**, 170), it was not until 1951 that Kaushal and Walker (*Biochem. J.*, 1951, **48**, 618) reported the first structural studies of the water-insoluble polysaccharide of the membrane of *Acetobacter acetigenum*. These authors showed that acetolysis yielded cellobiose octa-acetate and α- and β-D-glucose penta-acetate, while acetylation afforded a triacetate, and

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they concluded that the polysaccharide concerned was a cellulose. We now present more detailed evidence which confirms this conclusion.

Acetobacter acetigenum (T. K. Walker's strain) grows well on media containing ammonium sulphate 0·3%, potassium dihydrogen phosphate 0·3%, magnesium sulphate 0·2%, ethanol 2·0%, D-glucose 2·0%, and either Marmite 0·5% (Medium A), or calcium pantothenate and riboflavin (10^{-5}%, each) (Medium B). In most of the experiments described, medium (A) was employed. The membranes from many such cultures, after being pulped and extracted with 5% sodium hydroxide solution, gave a fibrous white polysaccharide, which was free from nitrogenous contaminants and gave analyses correct for a hexosan. Acetolysis with acetic anhydride–sulphuric acid afforded a mixture of acetates; on deacetylation of this and filter-paper chromatography, glucose and cellobiose were the only sugars detected. Crystallisation of the bulk of the acetate mixture gave crystalline α-cellobiose octa-acetate.

The polysaccharide was methylated with sodium and methyl iodide in liquid ammonia at −70°, essentially as described by Freudenberg and Boppel (*Ber.*, 1938, **71**, 2505). The methyl ether (OMe, 44·3%, compared with 45·6% for a tri-*O*-methylhexosan) had $[\alpha]_D$ −5·75° in chloroform, which agreed well with the values (−5·0°, −4·0°, −8·0°) recorded by Haworth, Hirst, Owen, Peat, and Averill (*J.*, 1939, 1885) for various tri-*O*-methylcelluloses. Paper-chromatographic analysis of a hydrolysate showed only one spot; this had an R_F value and staining reactions identical with those of 2 : 3 : 6-tri-*O*-methyl-D-glucose. Indeed, this sugar was subsequently isolated in crystalline form from the hydrolysate. Repeated extractions of the hydrolysate with chloroform under carefully controlled conditions yielded a syrup, in which 2 : 3 : 4 : 6-tetra-*O*-methylglucose also could be detected. Quantitative analysis of this syrup on filter-paper (Hirst, Hough, and Jones, *J.*, 1949, 928) disclosed 0·88 mole of tetraether per mole of triether. From the known partition coefficients of these components between chloroform and water (Macdonald, *J. Amer. Chem. Soc.*, 1937, **59**, 1503), the average chain length of the tri-*O*-methylpolysaccharide was calculated to be of the order of 600 glucose units. If the polysaccharide ether were unbranched, this would represent also the number-average degree of polymerisation. Thus the bacterial polysaccharide was proved to be a cellulose, in which β-D-glucopyranose units were linked through positions 1 and 4 to give long, essentially unbranched, chains, probably having an average molecular weight somewhat higher than was found, after methylation, in the trimethyl ether.

A sample of the bacterial cellulose was acetylated by Barnett's method (*J. Soc. Chem. Ind.*, 1921, **40**, 8), and another by treatment with acetic acid–trifluoroacetic anhydride (Bourne, Stacey, Tatlow, and Tedder, *J.*, 1949, 2976; Bourne, Randles, Tatlow, and Tedder, *Nature*, 1951, **168**, 942). Each acetate was deacetylated catalytically with sodium methoxide, and then re-acetylated by the method employed initially. The molecular weights of the four acetates thus obtained were compared by viscosity measurements (see Table). It was confirmed that very extensive degradation had occurred during

Molecular weights of cellulose acetates

Method of acetylation	$[\eta]$	D.P.
Barnett	0·11	21
Barnett (repeat)	0·09	17
$CH_3{\cdot}CO_2H$–$(CF_3{\cdot}CO)_2O$ (2 expts.)	4·75, 2·00	900, 380

acetylation by Barnett's process, whereas only about one bond per thousand was broken by acetic acid–trifluoroacetic anhydride. Such a result was not unexpected since it had been shown already (Bourne, Stacey, Tatlow, and Tedder, *loc. cit.*) that the latter process gives a good yield of the octa-acetate from so acid-labile a carbohydrate as sucrose. The acetates produced by acetic acid–trifluoroacetic anhydride were fibrous, whereas those resulting from the Barnett process were powders. However, the acetyl contents of the products from the trifluoroacetic anhydride procedure were *ca.* 2% lower than the theoretical figure, possibly owing to the introduction of a few trifluoroacetyl groups, which would be removed subsequently during the purification stage.

During the two years which have elapsed since the above work was completed, it has become possible to distinguish between α- and β-linked polyglucosans by determinations of infra-red spectra over the frequency range 730—960 cm.$^{-1}$ (Barker, Bourne, Stacey, and Whiffen, *Chem. and Ind.*, 1953, 196; *J.*, 1954, 171). Bacterial cellulose showed absorption peaks at 766, 894, 914, and 933 cm.$^{-1}$, and identical peaks were given by cotton cellulose. The absorption peak at 894 cm.$^{-1}$ is given by all β-anomers examined in the glucose series, including the cello-dextrins; there was no peak at *ca.* 840 cm.$^{-1}$, at which frequency the α-anomers show absorption.

The abilities of *Acetobacter acetigenum* and *A. xylinum* to grow on various substrates have been compared; these substrates were used instead of D-glucose in the synthetic medium (B). Whereas *Acetobacter acetigenum*, at the fourth sub-culture, could utilise ammonium lactate, methyl α-D-glucopyranoside, and dipotassium α-D-glucose 1-phosphate, none of these compounds supported the growth of *Acetobacter xylinum*. It follows that the former organism has a more complete cellulose-synthesising enzyme system than the latter. This confirms Kaushal and Walker's finding (*loc. cit.*), that *A. acetigenum*, but not *A. xylinum*, can utilise methyl α- and β-D-glucopyranoside, three pentoses, erythritol, and ethylene glycol.

Resting cells of *A. acetigenum* produced membranes when incubated with either D-glucose or D-mannose in phosphate buffer. After purification, each membrane was submitted to acetolysis, and the product was deacetylated, before being examined on a paper chromatogram. In each case, spots were observed which corresponded to reference spots of glucose and cellobiose. Thus the cells have the ability to convert D-mannose into derivatives of glucose.

When a suspension of the cells in phosphate buffer was shaken with glass beads, it was rendered non-viable and 95—99% of the cells were ruptured. The supernatant liquid produced no cellulose from D-glucose, whereas there was synthesis when a suspension of the cell debris in fresh phosphate buffer was incubated with D-glucose. It seems, therefore, that the enzyme(s) responsible for cellulose synthesis remained bound to the cell debris and were not extra-cellular.

Experimental

Nutrient Media.—Medium (A) contained ammonium sulphate (3·0 g.), potassium dihydrogen phosphate (3·0 g.), magnesium sulphate heptahydrate (2·0 g.), Marmite (5·0 g.), glucose (20 g.), ethanol (20 ml.), and water (1000 ml.) (cf. Henneberg, " Handbuch der Gärungsbakteriologie," Parey, Berlin, 1926). In medium (B), the Marmite was replaced by calcium pantothenate (0·10 mg.) and riboflavin (0·10 mg.).

Synthesis and Purification of Cellulose.—The membranes formed in 7 days by numerous cultures of *Acetobacter acetigenum* (T. K. Walker's strain), grown on medium (A) at 30°, were pulped with water in a Waring blender, and the insoluble residue, collected on cheese-cloth, was treated with 5% sodium hydroxide solution at 30° for 18 hr. The cellulose was collected, washed with water, and suspended in water, to which acetic acid was added at intervals until the solution remained acid to litmus for 12 hr. After being dialysed against distilled water, the stock cellulose sample (18 g.) was freeze-dried [Found: C, 44·1; H, 7·0; N, 0. Calc. for $(C_6H_{10}O_5)_n$: C, 44·5; H, 6·2%].

Infra-red Analysis.—The infra-red spectrum of the stock cellulose sample, determined over the frequency range 730—960 cm.$^{-1}$, by the " Nujol "-mull technique, showed absorption peaks at 766, 894, 914, and 933 cm.$^{-1}$ (cf. Barker, Bourne, Stacey, and Whiffen, *loc. cit.*).

Acetolysis of the Stock Cellulose Sample.—The cellulose (1·00 g.) was added in small portions, with vigorous stirring, to a mixture of acetic anhydride (3·80 ml.) and concentrated sulphuric acid (0·40 ml.), the temperature being kept below 5° during the addition and for several hours afterwards. Then, acetic anhydride (1·00 ml.) was added, and the mixture was kept at room-temperature for 24 hr., and subsequently at 85° for 14 min. It was cooled and poured into ice-water (100 ml.). The precipitate (X; 1·50 g.) was collected, washed, and dried. The bulk of the material (1·40 g.) was extracted with hot ethanol, and the filtered extract was evaporated under diminished pressure. The residue, crystallised from chloroform–methanol, gave α-cellobiose octa-acetate (0·63 g.), m. p. 224°, $[\alpha]_D^{20}$ +41·6° (*c*, 0·96 in $CHCl_3$) (Found: C, 49·7; H, 5·8:

Ac, 50·5. Calc. for $C_{28}H_{38}O_{19}$: C, 49·6; H, 5·6; Ac, 50·7%). Hudson and Johnson (*J. Amer. Chem. Soc.*, 1915, **37**, 1276) reported m. p. 229°, $[\alpha]_D^{20}$ +42·0° (in $CHCl_3$).

The remainder (0·10 g.) of precipitate (X) was treated, in chloroform (5 ml.)–methanol (2 ml.), with a trace of sodium methoxide at room temperature for 24 hr. The solvents were removed at 20°/12 mm., and the residue was dissolved in water, freed from ions, and freeze-dried. A paper chromatogram of the product, irrigated with the upper layer of a mixture of *n*-butanol (40%), ethanol (10%), water (49%), and ammonia (1%), and sprayed with Partridge's aniline hydrogen phthalate reagent (*Nature*, 1949, **164**, 443), showed two components, having R_F values identical with those of cellobiose and glucose reference spots.

Methylation of the Stock Cellulose Sample.—The cellulose (6·30 g.) was methylated with sodium and methyl iodide in liquid ammonia at −70°, essentially as described by Freudenberg and Boppel (*loc. cit.*). The treatment was repeated thrice more, the product being isolated at each stage. The product obtained finally when the ammonia was removed was suspended in water, dialysed against distilled water until free from iodide, and freeze-dried to give the crude polysaccharide ether (3·66 g.) (Found: ash, 3·4; OMe, 40·3. Calc. for tri-*O*-methylcellulose: OMe, 45·6%). This material was extracted (Soxhlet) with ether for 3 hr., and then dissolved in chloroform. The solution was clarified in the centrifuge, and evaporated under diminished pressure at room temperature from beneath a layer of water. The cellulose ether (2·46 g.), recovered when the aqueous suspension was freeze-dried, had $[\alpha]_D^{20}$ −5·75° (*c*, 0·69 in $CHCl_3$) [Found: ash, 2·7; OMe, 43·1% (44·3% when corrected for ash)].

End-Group Assay.—The methylated cellulose (1·004 g.) was dissolved in chloroform (15 ml.), mixed with 8% methanolic hydrogen chloride (15 ml.), and heated at 100° (sealed tube) for 8 hr. The solvents were removed under diminished pressure at 20°, and the residue was dissolved in 4% hydrochloric acid (85 ml.). The solution was kept at 100° for 5 hr., neutralised with silver carbonate, and filtered; the last traces of silver were removed with hydrogen sulphide. The syrup obtained when the solution was evaporated at 40°/12 mm. was dissolved in water (10 ml.), and the solution was extracted with chloroform (9 × 10 ml.); each extraction was continued for 30 min. to ensure equilibration. The combined chloroform extracts were shaken with an equal volume of water at intervals during 45 min. The chloroform layer was separated, and concentrated at 30° to a syrup, which was redissolved in water (0·5 ml.).

Paper-chromatographic examination of the final aqueous solution showed only two components; these had R_F values identical with those of reference spots of 2 : 3 : 6-tri-*O*-methyl- and 2 : 3 : 4 : 6-tetra-*O*-methyl-glucose. Quantitative paper-chromatographic analysis, essentially as described by Hirst, Hough, and Jones (*loc. cit.*), but with minor modifications introduced by Bebbington, Bourne, and Wilkinson (*J.*, 1952, 246), revealed the molar ratio of "tetra" to "tri" to be 0·88. On the basis of the partition coefficients between chloroform and water given by Macdonald (*loc. cit.*), the factors 1·62 and 825 were applied to give the molar ratio of "tetra" to "tri" in the hydrolysate of the tri-*O*-methylcellulose; this corresponded to an average chain length of the order of 600 glucose units.

Isolation of 2 : 3 : 6-*Tri*-O-*methylglucose.*—The aqueous layer remaining from the above chloroform extractions was concentrated to a syrup, which crystallised from ethyl acetate to give 2 : 3 : 6-tri-*O*-methyl-α-D-glucose (0·63 g.), m. p. and mixed m. p. 115°, $[\alpha]_D^{20}$ +70·2° (equil.; *c*, 0·97 in H_2O) (Found: C, 48·7; H, 8·2; OMe, 41·3. Calc. for $C_9H_{18}O_6$: C, 48·6; H, 8·2; OMe, 41·9%).

Acetylation of the Stock Cellulose Sample.—(a) *By Barnett's method.* The dry cellulose (1·00 g.), treated with acetic acid, acetic anhydride, chlorine, and sulphur dioxide, as described by Irvine and Hirst (*J.*, 1922, **121**, 1585), gave the acetate (1·59 g.) (Found: Ac, 44·6. Calc. for tri-*O*-acetylcellulose: Ac, 44·8%). A portion (0·80 g.) of the acetate was deacetylated with a trace of sodium methoxide in chloroform–methanol at room temperature for 24 hr. Acetone was added to diminish the density of the liquid phase, and the cellulose was collected in the centrifuge, washed with acetone, and then with water, and dried. Reacetylated as before, it gave an acetate (0·80 g.) (Found: Ac, 44·7%).

The acetyl contents were determined by Clark's method (*Ind. Eng. Chem. Anal.*, 1936, **8**, 487; 1937, **9**, 539).

(b) *By acetic acid–trifluoroacetic anhydride.* The dry cellulose (0·86 g.) was stirred with dry acetic acid (13·3 ml.) and trifluoroacetic anhydride (21·0 ml.) at room temperature for 4 hr. (cf. Bourne, Stacey, Tatlow, and Tedder, *loc. cit.*). Dry light petroleum (30 ml.; b. p. 60—80°) was added, and stirring was continued for 15 min., before the precipitate was collected and washed repeatedly with light petroleum. Chloroform (50 ml.) was added and removed by distillation under diminished pressure, a procedure which was repeated six times to remove any residue of

the acetylating reagents. A solution of the product in chloroform (100 ml.), clarified in the centrifuge, was poured into dilute sodium hydrogen carbonate solution, and the chlorofrom was removed by distillation under diminished pressure at room temperature. The precipitate was filtered off, thoroughly washed with water, and dried, to give the acetate (1·40 g.) (Found : Ac, 42·9%).

A portion (0·80 g.) of this material, deacetylated as above, and then re-treated with acetic acid–trifluoroacetic anhydride, gave an acetate (0·80 g.) (Found : Ac, 42·6%).

Viscosity Measurements on the Cellulose Acetate Samples.—A modified Ubbelohde viscometer of the type described by Davis and Elliott (*J. Colloid Sci.*, 1949, **4**, 313) was used to measure viscosities of chloroform solutions of the acetates. The procedure was as described by Gilbert, Graff-Baker, and Greenwood (*J. Polymer Sci.*, 1951, **6**, 585). The intrinsic viscosities $[\eta]$, obtained by extrapolation to infinite dilution of the graph of $\eta_{sp.}/c$ against c (in g. per 100 ml.), are shown in the Table. Staudinger and Werner's constant ($K = 5{\cdot}3 \times 10^{-4}$ base mole $l.^{-1}$; *Ber.*, 1937, **70**, 2140) was used to give the degrees of polymerisation.

Acetolysis of Membranes produced by Resting Cells of Acetobacter acetigenum.—(a) *From glucose.* Membranes from cultures of *A. acetigenum*, which had been grown for 7 days on medium (A), were washed thoroughly with water, suspended in water, and pulped in a Waring blender. The suspension was filtered through cheese-cloth, which retained the bulk of the cellulose but allowed the cells to pass through; they were collected in the centrifuge and washed again with water (cf. Aschner and Hestrin, *Nature*, 1946, **157**, 659). The resting cells were suspended in 0·2M-phosphate buffer (pH 5·6; 30 ml.), and a portion (1 ml.) of the suspension was added to each of 25 Petri dishes containing a 2% solution (20 ml.) of glucose. After incubation at 37° for 24 hr., the membranes produced were washed, purified by treatment with sodium hydroxide, and submitted to acetolysis, as described above. The crude product obtained on pouring the acetolysis mixture into water was deacetylated and examined on a paper chromatogram; two spots appeared, having R_F values identical with those of glucose and cellobiose.

(b) *From mannose.* The experiment was repeated with mannose instead of glucose in the Petri dishes. The deacetylated product showed glucose and cellobiose, but no mannose, on a paper chromatogram; after hydrolysis with acid, it showed only glucose.

Cellulose Synthesis by Cell Debris.—A thick suspension of resting cells in 0·2M-phosphate buffer (pH 5·6; 5 ml.) was shaken with glass beads (0·5 mm. diameter) in a Mickle shaker for 10 periods of 2 min., with intervals of 3 min. (to avoid over-heating). Staining tests indicated that 95—98% of the cells were disrupted. The cell debris was suspended in 0·2M-phosphate buffer (pH 5·6; 5 ml.), and when a test portion (1 ml.) of the suspension was incubated with a 2% solution (10 ml.) of glucose at 37° for 20 hr. cellulose was produced. On the other hand, the supernatant liquid (2 ml.) gave no cellulose when tested in the same way.

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CHEMISTRY DEPARTMENT, THE UNIVERSITY,
EDGBASTON, BIRMINGHAM, 15.

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