283. *Glycogen. Part II. Methylation and Acetylation.*

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THE complete methylation of glycogen has been accomplished by Haworth, Hirst, and Webb (J., 1929, 2479) by the action of methyl sulphate and sodium hydroxide on an acetone solution of glycogen triacetate. Inasmuch as the procedure was parallel to that followed

in the case of starch by the same workers (J., 1928, 2681) and practically identical constants were observed for trimethyl glycogen and trimethyl starch, one may fall into the error of assuming from those records that no difference is presented in the general behaviour of starch and glycogen towards methylating agents. In our previous paper (J., 1924, **125**, 1513) such a difference was emphasised, and further experiments carried out prior to and since the publication of Haworth's complete methylation have confirmed these results. Irvine and Macdonald (J., 1926, 1502) first succeeded in obtaining a trimethyl derivative by direct methylation of starch, but we have never completely methylated glycogen under similar conditions even after 25—30 treatments with the reagents. The methoxyl content (*ca.* 41%) of our final product accounts for some eight out of every nine hydroxyl groups; but it cannot on such evidence be contended that the product is homogeneous or that there is any structural reason for the arrest of methylation. Over 75% of the theoretical amount of trimethyl methylglucoside has been isolated after hydrolysis of this methylated glycogen, but the conversion cannot be expressed on a quantitative basis in view of the fractionation necessary on account of the presence of dimethyl methylglucoside.

We have repeated, and fully confirm, the results obtained by Haworth and his co-workers on the methylation of glycogen triacetate. The efficiency of the method seems partly to lie in the solvent power of acetone for acetylated and partially methylated glycogens. In a parallel series of experiments, however, glycogen which had been methylated to the 36% methoxyl stage without the intervention of acetylation was completely methylated only after many more treatments than are sufficient when the initial material is glycogen triacetate: a behaviour which would indicate that acetylation effects in the glycogen molecule or micelle some change, which may be more superficial than profound, since both starch and glycogen are undoubtedly composed of chains of glucopyranose units (Haworth and Percival, J., 1931, 1342; compare Karrer and Nageli, *Helv. Chim. Acta*, 1921, **4**, 263).

Other differences in the behaviour of starch and glycogen have been recorded (Pringsheim and Lichtenstein, *Ber.*, 1916, **49**, 364; Gatin-Gruzewska and collaborators, *Compt. rend.*, 1909, **149**, 359; *Bull. Soc. chim.*, 1910, **7**, 744). Pictet and Jahn (*Helv. Chim. Acta*, 1922, **5**, 640) depolymerised potato starch by heating it with glycerol, but we have failed to resolve glycogen or methylated glycogen into simpler products by such means. It is difficult to reconcile this behaviour with the view that the difference between starch and glycogen is merely one of size of the micelles. Examination of

trimethyl and partially methylated starch along similar lines is not yet completed.

We have also observed differences in the behaviour of starch and glycogen in acetylations which do not involve heat treatment. Glycogen is acetylated readily, but "treated" glycogen only with extraordinary difficulty; on the other hand, treated starches are all acetylated more readily than the original polysaccharides. This may mean much or little, for it may only involve a surface change such as perhaps accounts for Bayer's observation (*Biochem. Z.*, 1921, **124**, 97) that glycogen when exposed to sunlight becomes insoluble in water—a behaviour which he attributes either to polymerisation or to physical alteration of the surface. The various starches themselves present points of difference, potato starch, for example, reacting much more slowly than rice, wheat or maize starch unless previously treated by solution in boiling water and precipitation by alcohol.

Acetolysis is observed in acetylations by this method, depolymerisation of the acetates rendering them soluble in alcohol, the change being accompanied by some degradation to sugar acetates. By repeated treatment of the alcohol-soluble acetates it is possible to obtain depolymerised products, m. p. 151—154°, which are practically without reducing action: and by conversion of these quantitatively into α- and β-methylglucosides they can be shown to consist essentially of glucose units. A similar product is obtained from glycogen. There is no definite evidence, however, that the products are homogeneous derivatives and not mixtures.

Pregl (*Monatsh.*, 1901, **22**, 1049) by acetolysis of starch isolated a product which molecular-weight determinations indicated was a triacetate of a glucose anhydride $C_6H_{10}O_5$. Pringsheim (*Annalen*, 1926, **450**, 255; *Ber.*, 1926, **59**, 3008), Hess (*Annalen*, 1926, **448**, 99), and other workers have shown that cellulose and lichenin acetates are derived from cellosan or other anhydroglucoses. Molecular-weight determinations on our products in boiling benzene or ethyl acetate by the Menzies–Wright method revealed a molecule of great complexity (*M*, *ca.* 20,000), but it is possible for polymerisation to occur under such experimental conditions.

In view of the differences in reactivity now recorded between starch and glycogen one will await with interest the result of the application to these substances of the large-scale hydrolysis of the methylated compounds and the quantitative fractionation of the products described in the case of cellulose by Haworth (*Nature*, 1932, **129**, 365). This method, if further work shows it to be valid, will supply information as to the sizes of the respective molecules, but there will still remain some room for doubt as to its rigid

application in the deduction of the actual complexities of the polysaccharides themselves while the acetates are taken as initial materials, since our experiments indicate that the process of acetylation is not without influence on the character of these molecules.*

EXPERIMENTAL.

Methylation of Glycogen in Absence of Acetone.—Purified glycogen was dissolved in 15% NaOH aq. (150 c.c.), 35% NaOH aq. (200 c.c.) and Me_2SO_4 (80 c.c.) added slowly and simultaneously at 35°, and the temp. then raised to 100° for ½ hr. The cooled mixture was neutralised with dil. H_2SO_4, and the partially methylated glycogen was collected after addition of $(NH_4)_2SO_4$ and further methylated in presence of MeOH. The product was freed from Na_2SO_4 and $NaMeSO_4$ by extraction in $CHCl_3$ under reflux, dried (Na_2SO_4), filtered, recovered, and refluxed with Et_2O to remove any methylated glucoses present (Found: OMe, 20, 28, 32, and 36% after 1, 2, 4, and 6 methylations respectively). $[\alpha]_D^{20°}$ + 183·2° in $CHCl_3$ (c = 1·51).

Part of this product was further methylated in the absence of Me_2CO, since by such treatment Irvine and Macdonald (*loc. cit.*) prepared trimethyl starch: after 13 methylations with Me_2SO_4 and NaOH, OMe 38·1%; after 16 such methylations, OMe 38·9% and $[\alpha]_D^{20°}$ + 185·7° in $CHCl_3$ (c = 1·56); after 3 methylations with Ag_2O and MeI, OMe 39·4% and $[\alpha]_D^{20°}$ + 186·2° in $CHCl_3$ (c = 1·52); after 5 methylations with Me_2SO_4 and NaOH, OMe 40·8% and $[\alpha]_D^{20°}$ + 186·3° in $CHCl_3$ (c = 1·5) [Found: C, 52·0; H, 7·85; OMe, 40·8. $C_{18}H_{22}O_7(OMe)_8$ requires C, 52·2; H, 7·7; OMe, 41·5%]. Four further methylations with Me_2SO_4 failed to raise the methoxyl content.

Methylation of Glycogen in Presence of Acetone.—When confirming Haworth's work on the methylation of glycogen triacetate we found it essential to remove $CHCl_3$ completely from the extracted glycogen, as otherwise chloretone was produced in subsequent methylation treatment and stuck with great tenacity to the product.

Some of the above methylated glycogen (36% OMe) was further treated with Me_2SO_4 in presence of Me_2CO as in the immediately preceding work (Found: OMe, 41% after 4 methylations and 44% after repeated methylation).

We found it a convenience to dissolve trimethyl glycogen in Me_2CO after the usual purification and remove the solvent under reduced pressure: the substance was left as a froth which broke down to an easily removable white powder.

Hydrolysis of Methylated Glycogens.—A solution of methylated glycogen (OMe, 40·8%) (17·5 g.), in dry MeOH (500 c.c.) containing HCl (1·5%) was heated under reflux for 6 hrs. (the rotation had then the minimum value); the mixture was thereafter cooled and neutralised with Ag_2CO_3. The syrup (18·9 g.) obtained after filtration and removal of the solvent was distilled, yielding first runnings (1·9 g.), fraction A (10·7 g.), b. p. 119—123°/0·16 mm., a transitional fraction B (1·9 g.), fraction C (2·9 g.), b. p. 140—145°/0·2 mm., consisting essentially of a dimethyl glucose, and residue (1·5 g.). Fraction A

* The communication by Irvine (*Nature*, 1932, **129**, 471) has just come to hand which suggests that this method is not applicable in the light of work carried out at the St. Andrews laboratories: his experiments indicate the disturbing effect of acetylation on the amyloses.

was identified as 2 : 3 : 6-trimethyl methylglucoside; on hydrolysis with 5% HCl aq. it yielded 2 : 3 : 6-trimethyl glucose, m. p. 110—112° after recrystallisation from Et_2O.

Attempted Depolymerisation of Glycogen and Partially Methylated Glycogen.—Glycogen (5 g.) was dissolved in glycerol (50 g.) at 190° and after some time poured into 90% EtOH. The precipitate, washed with spirit, had physical constants agreeing closely with those of glycogen.

Methylated glycogen (*ca.* 36% OMe) (6 g.) was heated with glycerol (60 g.) at 200—210° for 4 hrs.; none appeared to dissolve. The glycerol was decanted, and the solid dissolved in H_2O, pptd. in $(NH_4)_2SO_4$, and purified as in the methylation experiments; it (upwards of 5 g.) then had OMe 35·7% and $[\alpha]_D^{17°}$ + 179·2° in $CHCl_3$ (c = 1·54) and showed in general the solubilities of, and was as resistant to further methylation as, the original material.

Acetolysis of Glycogen and the Starches.—Experiments were carried out with rice, maize, wheat, and potato starch, with glycogen, and with treated samples of these materials. The treated materials were obtained by grinding the polysaccharide (20 g.) with H_2O to a smooth paste, which was poured into H_2O (500 c.c.), boiled for ½ hour, and pptd. by EtOH; the settled ppt. was sucked off, washed with EtOH and Et_2O, and dried in a vacuum oven.

The general procedure in the acetylation was as follows. Starch (10 g.) was shaken during the reaction period with Ac_2O (100 g.) containing conc. H_2SO_4 (5%) at room temp., and the filtered solution poured slowly into much H_2O. The pptd. acetates were washed to remove anhydride and acid, dried, and extracted with EtOH under reflux; the alcohol-soluble acetate separated on cooling. After several such treatments the alc. solution of the acetate was poured into much Et_2O, and repetition of this removed the sugar acetates. A depolymerised starch or glycogen acetate was thus obtained practically free from reducing action, giving an Ac value indicating an average of three Ac groups per $C_6H_{10}O_5$ unit, and having m. p. within the range 150—155° and $[\alpha]_D$ in $CHCl_3$ within the limits + 146° and + 159°. A few typical experiments are summarised below.

Potato starch (untreated) was acetylated very slowly in all cases, approx. 80% remaining unchanged after 6 days' shaking.

Potato starch (treated) had practically all gone into solution after 1 day, and 16 g. of acetate were isolated after pptn. and washing. Rigorous purification raised the m. p. from 125° to 153—154°, and the alcohol-soluble acetate (4 g.) thus obtained was practically without reducing action. It had $[\alpha]_D^{25°}$ + 159° in $CHCl_3$ (c = 1·0284) (Found: Ac, 44·9. $C_6H_7O_5Ac_3$ requires Ac, 44·8%). Six other experiments gave similar results.

Experiments in which the reaction had proceeded for 2 or more days gave crude acetates which were more difficult to work up on account of their sticky nature: the yields of alcohol-soluble acetate were lower, and from the "mother-liquors" a syrup was isolated which crystallised slowly, was soluble in Et_2O, and after deacetylation gave a mixture of osazones in which no sign of glucosazone crystals could be detected on microscopic examination. Experiments continued for longer than 2 days gave still greater amounts of reducing substances.

Wheat starch (untreated) was acetylated in 1 day, 10 g. yielding 15 g. of crude acetate, from which 8 g. of purified alcohol-soluble acetate were obtained, m. p. 151—154°, $[\alpha]_D^{20°}$ + 149° in $CHCl_3$ (c = 0·876), Ac val. 44·3%.

Maize starch (treated) behaved similarly, the acetate having m. p. 152—153°, $[\alpha]_D^{20°}$ + 146° in $CHCl_3$ (c = 1·106), Ac val. 44·5%.

Rice, wheat, and maize starch (untreated) dissolved in the acetylating mixture more slowly than the treated materials, but, in contrast to potato starch (untreated), were almost completely dissolved after 3 days.

Glycogen (untreated) (5 g.) went into solution in less than a day and by the usual procedure 2·5 g. of purified alcohol-soluble acetate, m. p. 152—154°, $[\alpha]_D^{20°}$ + 147° in $CHCl_3$ (c = 1·08), Ac val. 44·9%, were obtained. In these respects there is no marked difference between the product obtained from glycogen and the starches.

In several experiments with glycogen (treated), reaction was remarkably slow: one sample remained undissolved after 6 days, and another had only partly dissolved even after 19 days: a behaviour which is the exact reverse of that observed in the case of the starches.

Conversion of Alcohol-soluble Triacetates into Glucose.—All the samples of alcohol-soluble triacetate examined may be quantitatively converted into methylglucoside. Rice starch triacetate (4 g.) was heated in a sealed tube at 120° for 30 hrs. with MeOH (50 c.c.) containing HCl (2%). After neutralisation by Ag_2CO_3, the solution was refluxed with charcoal, filtered, and evaporated. The syrup (2·5 g.) obtained crystallised on nucleation and consisted of α- and β-methylglucosides. After a $CHCl_3$ solution had been heated with a few drops of acid MeOH in a sealed tube, the equilibrium value $[\alpha]_D^{20°}$ + 108° (c = 1·530) was obtained. After crystallisation from abs. EtOH the glucoside had m. p. 160°. These values are in good agreement with the constants observed in the case of α-methylglucoside and the equilibrium mixture of the α- and the β-compound.

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