129. *Polysaccharides. Part XXIII. Determination of the Chain Length of Glycogen.*

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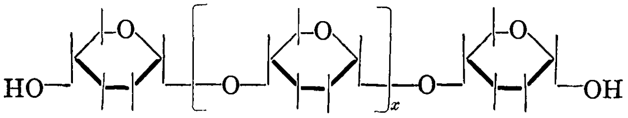
IN Part XI (Haworth and Percival, J., 1932, 2277) a study of the molecular structure of glycogen provided evidence of the structural relationship of this polysaccharide to starch. Whereas for starch the chemical assay of the end group has revealed in every case hitherto examined a chain of 24—30 glucose units, we had found for glycogen a value corresponding to 12 glucose units. Recently, in examining specimens of glycogen from a variety of sources, we encountered (from rabbit liver) glycogen which gave values for the chain length differing from those we had previously observed for this polysaccharide. The results now described are concerned with these specimens, which are exceptional in that they reveal by the end-group method the presence of approximately 18 glucose units in the chain. A preliminary account of these observations was given by one of the authors (Haworth, Pres. Add. Chem. Sec. Brit, Association, 1935, p. 41) where it was pointed out that glycogen had been shown to possess, in different specimens, a chain length of 12 or 18 glucose units. The experimental data which form the basis of these results are now appended. It will be seen that control experiments were instituted in the endeavour to expose any source of error in the chain length determination. It is now clear that there exist at least two types of glycogen which display no essential differences in structure or general properties excepting only the chain length as determined by the method of end-group assay. Since the above conclusions were recorded by one of us a similar specimen of glycogen containing also 18 glucose units has been described by Bell (*Biochem. J.*, 1936, **30**, 1612).

On the subject of the particle size of glycogen and starch, experiments have been conducted in this laboratory by Carter and Record (*J. Soc. Chem. Ind.*, 1936, **55**, 218) which suggest that glycogen and starch may exist in a very highly aggregated form, amounting in the case of glycogen to 3000—5000 glucose units. A general discussion and interpretation of these results is deferred, but reference may be made to a preliminary statement of the facts recorded by one of us (Haworth, *Monatsh.*, 1936, **69**, 314).

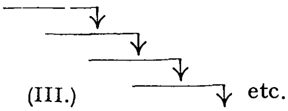
It is significant that from the purified methylated glycogen which had been exhaustively methylated we were able to isolate an amount of dimethyl glucose which is about equivalent to that of the tetramethyl glucose isolated as the end group. Despite every effort to effect complete methylation of the available hydroxyl groups a very small proportion may escape protection. Methylated glycogen is not of such a nature that it can be purified as rigidly as a substance which is capable of recrystallisation. The origin of the dimethyl glucose may therefore be adventitious. The alternative view, however, has been advanced in our earlier paper on methylated xylan (Haworth, Hirst, and Oliver, J., 1934, 1917). In the latter paper various hypotheses were suggested which apply equally to other polysaccharides which we have investigated and these may again be expressed and emphasised in relation to both glycogen and starch.

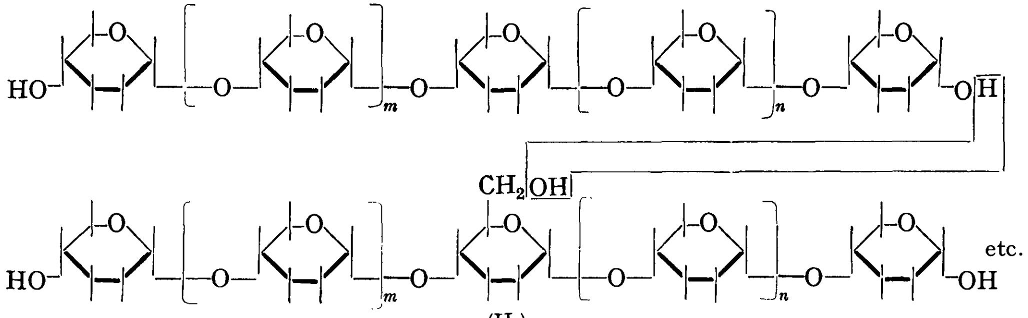
On the interpretation suggested in the xylan paper glycogen is represented as groups of 18 (and alternatively of 12) α-glucopyranose units linked at positions 1 and 4 (I) in the form of chains which are themselves joined by a type of union which links the reducing end of one chain with a hydroxyl of an adjoining chain. This occurs through a hydroxyl group, other than that at 1 and 4, of a non-terminal glucose residue. This glucose residue would therefore be united at three points of its ring with adjoining glucose members, leaving only two exposed hydroxyl groups, which appear therefore as a dimethyl glucose member in the methylated glycogen. This conception is expressed by (II) and diagrammatically by the laminated formula (III), which are similar to those given by Haworth, Hirst, and Oliver (*loc. cit.*).

(I.)



(III.)





(II.)

The expression (III) reproduces formula (I) as the line ⟶, and the arrow head gives the position of the reducing member which engages an adjoining chain. This indicates the linking of consecutive chains, whether in the manner of an ordinary covalency bond produced by dehydration, or by a co-ordinated or hydroxyl bond connecting the potential reducing end of one chain with another glucose unit of a succeeding chain. At this stage it is not possible to present a precise picture of the nature of this bond or of the position of the intermediate glucose unit involved in this union, but further experiments may make this clear. Until the orientation of the groups in the dimethyl glucose fragment, both from glycogen and from starch, has been decided, this and other questions are in abeyance, assuming that the molecular structures (II) and (III) are ultimately found to be the best expression of the experimental facts.

Yet another formulation which must equally be considered is that glycogen (and starch) consists of a continuous main chain of α-glucopyranose units linked through the positions 1 and 4 in the form of a loop and having other glucopyranose residues attached as side chains at intervals. This is merely a special case of the expression (III) and has equally been advanced in the earlier paper (Haworth, Hirst, and Oliver, *loc. cit.*).

EXPERIMENTAL.

Properties of Glycogen.—The specimens of glycogen used in this work were prepared from rabbit's liver and had been subjected to the action of 15% aqueous potassium hydroxide during the process of isolation. The glycogen was a white powder, soluble in cold water, giving opalescent solutions. Colour with iodine, deep red. Non-reducing towards boiling Fehling's solution. Iodine number (Bergmann and Machemer's method, *Ber.*, 1930, **63**, 316) 4·4. $[\alpha]_{D}^{22^\circ}$ + 188° in water (*c*, 0·3) (Found : P_2O_5, 0·10; ash on incineration, 1·3%) (all data refer to dry material). By electrodialysis the ash content of the glycogen was reduced to 0·3% without appreciable alteration of the other properties.

Acetylated Glycogen.—Alcohol (1750 c.c.) was added gradually to an aqueous solution (250 c.c.) of glycogen (25 g.). The precipitated glycogen was washed rapidly with alcohol and immediately mixed with pyridine (285 c.c.) and acetic anhydride (250 c.c.). The temperature rose slightly and after an hour the glycogen had dissolved almost entirely. After 3 hours the clear solution was poured into cold water and the glycogen acetate was collected, washed, and dried in the usual way. Yield, 90% of the theoretical. This sample of glycogen acetate was a non-reducing (Fehling's solution) white powder, $[\alpha]_{D}^{22^\circ}$ + 170° in chloroform (*c*, 1·0) (Found : $CH_3{\cdot}CO$, 45%). Attempts to divide the acetate into fractions with different properties were unsuccessful. For example, 20 g. of the acetate, dissolved in chloroform, were fractionally precipitated by light petroleum, giving four fractions (5, 3·5, 3·5, and 8 g.) with identical properties, including specific viscosity (Found : $\eta_{sp.}^{20^\circ}$ 0·10 for a solution in *m*-cresol containing 0·05 g. in 5 c.c.; apparent M.W. 3,000, using Staudinger's formula with $K_m = 10^{-3}$). The acetate gave no colour with iodine, but the glycogen regenerated from the acetate by the action of alkali gave the usual deep red colour. Most of the phosphorus in the original glycogen was removed during the acetylation process, the mean of many concordant estimates on the acetate indicating a phosphorus content (calc. as P_2O_5) of 0·02%. In view of the retention of combined phosphorus during the acetylation of starch it is likely, therefore, that most of the phosphorus present in the original glycogen was contained in the adherent inorganic impurities.

Methylated Glycogen.—Glycogen acetate in portions of 10 g., dissolved in acetone (100 c.c.), was methylated at 55° with methyl sulphate (50 c.c.) and 30% aqueous sodium hydroxide (130 c.c.). The methylated glycogen separated as a solid at the end of the experiment and was immediately remethylated. After eight such treatments it was dissolved in chloroform and on evaporation of the solvent was obtained as a pale yellow glass, which was extracted with boiling ether and triturated with light petroleum. Methylated glycogen (yield, 85% of the theoretical) was then obtained as a fine white powder soluble in cold water and in chloroform, insoluble in hot water. The aqueous solution gave a deep red colour with iodine. $[\alpha]_{D}^{20^\circ}$ + 209° in chloroform (*c*, 1·0) (Found : OMe, 43·3%). A considerable quantity of methylated glycogen was prepared in this way and subjected to fractional precipitation from chloroform solution by light petroleum. For instance, from 40 g. of methylated glycogen six fractions were obtained weighing severally 2, 4, 15, 10, 7, and 1 g. The first fraction (which contained about 1% of mineral matter) and the last (which was obtained by evaporation of the mother-liquors) were neglected. The other four had identical properties, $[\alpha]_{5780}^{19^\circ}$ + 220° in chloroform (*c*, 1·0); $\eta_{sp.}^{20^\circ}$ 0·10 in *m*-cresol solution (0·04 g. in 5 c.c.) corresponding to a chain length of 13 glucose units, using Staudinger's formula with $K_m = 10^{-3}$. All fractions in cold aqueous solution gave a dark red colour with iodine.

Hydrolysis of Methylated Glycogen.—Powdered methylated glycogen (with the properties of fractions 2—5 referred to above) (20 g.) was boiled for 8 hours with 1·2% methyl-alcoholic hydrogen chloride, the progress of the reaction being followed polarimetrically. When the rotation had diminished to a constant value, the hydrochloric acid was removed by silver carbonate and the neutral solution was evaporated to a thin syrup. This was dissolved in ether, and the filtered solution evaporated under diminished pressure, giving a syrup (A) (22·4 g.), which was fractionally distilled, the distillation being stopped when the refractive index of the distillate had reached $n_{D}^{16^\circ}$ 1·4580. This fraction (5 g.) was redistilled through a column, giving (B) 1·265 g., bath temp. 108—110°/0·04 mm., $n_{D}^{16^\circ}$ 1·4480, and (C) 1·378 g., bath temp. 120°/0·04 mm., $n_{D}^{16^\circ}$ 1·4525. Fractions (B) and (C) were mixed and redistilled, giving (D) 1·153 g., bath temp., 104°/0·01 mm., $n_{D}^{17^\circ}$ 1·4444, and (E) 0·46 g., $n_{D}^{18^\circ}$ 1·4555. The remainder of (A) and the still residue from the last distillation were joined and redistilled, giving a distillate (F) and an undistillable portion (3·5 g.). The latter was boiled with 1% methyl-alcoholic hydrogen chloride and the product was isolated as before and distilled, giving a distillate (G) and a small undistillable portion. The latter was again boiled with 1% methyl-alcoholic hydrogen chloride and the product gave on distillation (H) together with an undistillable portion which was now negligible in amount. These complications appear to be due to condensation of trimethyl glucose under the influence of methyl-alcoholic hydrogen chloride rather than to incomplete hydrolysis of the methylated glycogen. Similar phenomena are observed when 2 : 3 : 6-trimethyl glucose is boiled with 1% methyl-alcoholic hydrogen chloride. (F), (G) and (H) were now joined and fractionally distilled through a column. No material of refractive index less than $n_{\mathrm{D}}^{20^\circ}$ 1·456 was detected and after exhaustive fractionation the following were obtained: (J) 18 g., $n_{\mathrm{D}}^{18^\circ}$ 1·4565, (K) 0·2 g., $n_{\mathrm{D}}^{20^\circ}$ 1·4580, and (L) 1·85 g., bath temp. 170—175°/0·01 mm., $n_{\mathrm{D}}^{20^\circ}$ 1·4710 (Found: OMe, 41·8%).

Fraction (D) consisted entirely of tetramethyl methylglucoside and gave on hydrolysis tetramethyl glucopyranose, m. p. 88°, $[\alpha]_{\mathrm{D}}^{20^\circ}$ + 82° in water (*c*, 1·2) (Found: C, 51·0; H, 8·8; OMe, 51·8. Calc. for $C_9H_{18}O_6$: C, 50·8; H, 8·5; OMe, 52·5%). Fraction (E) was estimated to contain 0·08 g. of tetramethyl methylglucoside. The total yield was therefore 1·233 g. from 20 g. of methylated glycogen. After correction (addition of 10%) to compensate for experimental losses this amounts to 1·35 g. (6·7%), corresponding to a chain length of 18 units for the methylated glycogen.

Fraction (J) was 2 : 3 : 6-trimethyl methylglucoside. Part of it crystallised, giving 2 : 3 : 6-trimethyl β-methylglucopyranoside, m. p. 58°. On hydrolysis both syrup and crystals gave in good yield 2 : 3 : 6-trimethyl glucose, m. p. 117°.

(L) consisted mainly (85—90%) of dimethyl methylglucoside. The yield of dimethyl methylglucoside was therefore approx. 1·6 g. from 20 g. of methylated glycogen. On oxidation by nitric acid (*d* 1·4) at 90° for 8 hours the dimethyl glucose (1 g.) gave a mixture of acids, which were converted into the methyl esters in the usual way. On distillation a fraction (0·3 g.), b. p. 80°/0·01 mm., $n_{\mathrm{D}}^{24^\circ}$ 1·4360, $[\alpha]_{\mathrm{D}}^{20^\circ}$ + 70° in methyl alcohol (*c*, 1·0), was obtained. This was mainly methyl *d*-dimethoxysuccinate, recognised as the corresponding diamide, m. p. 278° (decomp.), $[\alpha]_{5780}^{20^\circ}$ + 94° in water (*c*, 0·23) (Found: OMe, 34·6. Calc. for $C_6H_{12}O_4N_2$: OMe, 35·2%). A considerable proportion of the dimethyl glucose must therefore be the 2 : 3-derivative, but the yield of oxidation product was not sufficiently high to warrant conclusions concerning the homogeneity of the dimethyl methylglucoside.

Control Experiments.—(1) The whole of the above series of operations was repeated with another sample of rabbit liver glycogen. The acetylated glycogen and methylated glycogen were indistinguishable from the corresponding substances obtained in the experiments previously described. After hydrolysis of the methylated glycogen (20 g.) by 1% methyl-alcoholic hydrogen chloride and exhaustive fractional distillation of the product the following yields were recorded, the identity and purity of the substances being established as described above:

(a) Tetramethyl methylglucoside, 1·25 g. (corrected value, 1·38 g.). Chain length of methylated glycogen, 18 units.

(b) Dimethyl methylglucoside, 1·2 g.

(2) Another portion of the glycogen used in the first control experiment was acetylated and thereafter converted into the methyl derivatives by the method already described with the single exception that in this case no less than thirty successive methylations were given. The purified methylated glycogen thus obtained had $[\alpha]_{5780}^{20^\circ}$ + 223° in chloroform (*c*, 1·0), $\eta_{\mathrm{sp.}}^{20^\circ}$ 0·130 in *m*-cresol (0·05 g. in 5 c.c.), corresponding to an apparent chain length 13 (Found: C, 52·9; H, 8·1; OMe, 44·8. Calc. for $C_9H_{16}O_5$: C, 52·9; H, 7·8; OMe, 45·6%). This methylated glycogen (13 g.) was hydrolysed by boiling 1·2% methyl-alcoholic hydrogen chloride. Fractional distillation of the product gave 0·84 g. of tetramethyl methylglucoside (yield, 6·5%), corresponding, after correction, to a chain length of 17 units for the methylated glycogen. The amount of dimethyl methylglucoside in the hydrolysis product was approx. 0·75 g.

(3) In another series of experiments a sample of the methylated glycogen used in the first control series was hydrolysed by a different method. Finely powdered methylated glycogen (20 g.) was added to cold hydrochloric acid (100 c.c.) which had been saturated at 0° with hydrogen chloride. After 44 hours at 15° the solution was diluted, neutralised with barium carbonate, and evaporated to a volume of 150 c.c. This solution (X) was extracted six times with chloroform and the chloroform (1,000 c.c.) was concentrated to a syrup, which was boiled with 1% methyl-alcoholic hydrogen chloride for 8 hours. The methylglucosides (9 g.) were isolated in the usual way and fractionally distilled, giving finally (a) 0·951 g., bath temp. 132—135°/0·07 mm., $n_{\mathrm{D}}^{20^\circ}$ 1·4420 (Found: OMe, 60·9%), and (b) 0·424 g., bath temp. 143—144°/0·07 mm., $n_{\mathrm{D}}^{22^\circ}$ 1·4490. The remainder consisted entirely of 2 : 3 : 6-trimethyl methylglucoside. (a) and (b) together contained 1·2 g. of tetramethyl methylglucoside. The solution (X) was evaporated to dryness, and the organic material extracted with boiling chloroform and converted into the methylglucosides. Fractional distillation showed that no tetramethyl methylglucoside was present, the material not removed from the aqueous solution by the chloroform extraction being 2 : 3 : 6-trimethyl glucose together with a little dimethyl glucose. The total yield of tetramethyl methylglucoside was therefore 1·2 g. from 20 g. of methylated glycogen (chain length, 20 units). The identity of the products was established by the methods given above.

(4) *Experiments with artificial mixtures.* (a) The efficiency of the fractionating column used in the preceding experiments was tested by distilling through it a mixture of 2 : 3 : 6-trimethyl methylglucoside (3·50 g.) and 2 : 3 : 4 : 6-tetramethyl methylglucoside (0·43 g.). The procedure was exactly similar to that used in the experiments with glycogen and the yield of tetramethyl methylglucoside recovered was 0·395 g. (92%). The quantity used in the control experiment was less than that distilled in the course of the experiments with glycogen and in this respect the test of the apparatus was more stringent. In other experiments it was demonstrated that no tetramethyl methylglucoside escaped during the distillation, since no trace of the latter was detectable in a receiver, cooled in acetone–solid carbon dioxide, placed between the pump and the distillation apparatus.

(b) A set of experiments similar to (a) but commencing with 2 : 3 : 6-trimethyl glucose (5·69 g.) and 2 : 3 : 4 : 6-tetramethyl glucose (0·42 g.) was then carried out. The mixed sugars were transformed into the methylglucosides and the mixture of the latter was distilled through the column. The yield of tetramethyl methylglucoside was 0·40 g. (90% recovery). Here again the choice of small quantities was deliberate in order to test the accuracy of the experimental procedure under the least favourable conditions. There is therefore no possibility of serious experimental loss of tetramethyl glucose at any stage of the work on methylated glycogen.

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