324. *Polysaccharides. Part XI. Molecular Structure of Glycogen.*

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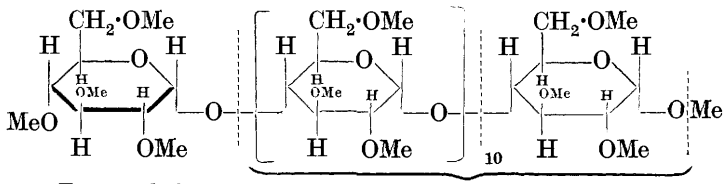
METHYLATED glycogen prepared as herein described is constituted as an extended chain of α-glucopyranose residues which are arranged as in maltose (compare Haworth, Hirst, and Webb, J., 1929, 2482; Haworth and Percival, J., 1931, 1342). Hydrolytic cleavage reveals the presence of one terminal unit which retains one more methyl group than the remainder. This can be assayed under the conditions described and is recognised as crystalline tetramethyl glucopyranose, which occurs to the extent of 9%.



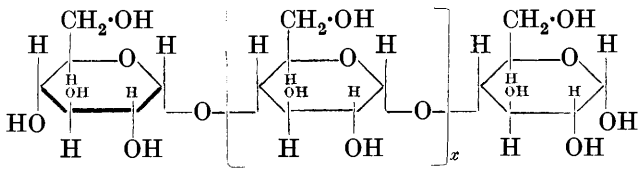
Tetramethyl Glucose.

Trimethyl Glucose and MeOH.

It follows that the number of glucose residues in the methylated polysaccharide is not more than about twelve, corresponding to a

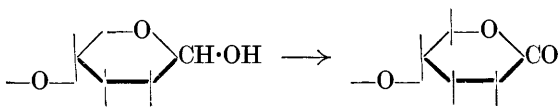
M.W. of approximately 2500, a value which is in agreement with that determined by Rast's method.

The glycogen was prepared from rabbit's liver by British Drug Houses Ltd. and was a good specimen of the commercial product. It was purified by the method previously described (Haworth, Hirst, and Webb, *loc. cit.*) and acetylated under a new procedure, and the glycogen acetate readily underwent methylation in aqueous acetone solution by eight treatments with methyl sulphate. Precautions were taken to test the homogeneity of the specimens of glycogen acetate and of methylated glycogen and no evidence of the presence of smaller degraded fragments was obtained. Under the treatment described, there is therefore nothing to suggest that the glycogen with which we started has suffered any breakdown by conversion into these derivatives. It is, however, clear that natural glycogen contains a minimum number of 12 glucose units in its molecular structure. The process adopted for the isolation of glycogen from livers involves the use of alkali and we have not studied the changes which may be involved in this procedure, although the fact is significant that the glycogen so extracted exhibits a marked stability towards alkali. As ordinarily obtained, the polysaccharide cannot contain many more units than the average value of twelve, and its general properties, such as the comparison of its reducing power with that of starch, suggest that glycogen is not a highly complex polysaccharide. The structure of native glycogen may be deduced from the expression



where the minimum value of x is 10, and in the commercial specimen this minimum is probably not much exceeded. The fate of the terminal reducing group in such a constitutional expression demands consideration. Our experience with cellodextrins, freshly prepared by the break-down of cellulose, shows that with a chain-length of 26 β-glucopyranose residues the reducing property of the terminal group on the right is preserved and leads to a stoicheiometric value for hypoiodite reduction. Glycogen which has a shorter chain-length in its methylated derivative shows only faint reduction with this reagent. It is recalled, however, that glycogen is derived from animal sources by extraction with fairly concentrated alkali, and either *in vivo* or by this subsequent treatment the terminal reducing

group must be affected. This is a problem remaining over for solution, in the cases of cellulose, glycogen and starch. It is possible that the terminal " aldose " component in each is modified by oxidation to the acid (or lactone) group.



EXPERIMENTAL.

The Acetylation of Glycogen.—Haworth, Hirst, and Webb (J., 1929, 2482) had shown that glycogen could be acetylated with pyridine and Ac_2O by heating for 20 hrs. at 80°. A new method has been developed during this investigation which is suitable for the acetylation of large quantities of glycogen, by treatment with the above reagents for only 3 hrs. without heating. This method of acetylation is extremely mild, and it is considered unlikely that degradation of the glycogen macromolecule occurs.

Glycogen (25 g.) was dissolved in H_2O (250 c.c.) and pptd. by the gradual addition of abs. EtOH (1750 c.c.), the glycogen being collected and washed once with EtOH. The dried product was added immediately to a mixture of pyridine (285 c.c.) and Ac_2O (250 c.c.). Heat developed; the mixture was agitated from time to time, nearly the whole of the glycogen dissolving in 1 hr. After 3 hrs., the faintly yellow solution was filtered into cold H_2O, and the white product washed with H_2O, EtOH, and Et_2O. Yield 39 g., *i.e.*, 87%. The " triacetate " was thus obtained as a white powder, sol. in acetone and $CHCl_3$. $[\alpha]_D^{20°} = +170°$ (*c*, 1·0 in $CHCl_3$) (Found: C, 50·0; H, 5·9; CH_3·CO, 44·4. $C_{12}H_{16}O_8$ requires C, 50·0; H, 5·6; CH_3·CO, 44·8%).

This *glycogen acetate* was homogeneous in a general sense, since careful fractional pptn. failed to reveal any differences in the properties of a wide range of fractions. For example, glycogen acetate (32 g.) dissolved in $CHCl_3$ and pptd. with Et_2O and light petroleum as well as by evaporation of the final residual solution yielded six fractions having identical solubilities, iodine values, specific rotations, and acetyl values. The same results were observed in a fractional pptn. of glycogen acetate (36 g.) from $CHCl_3$–acetone by means of EtOH, Et_2O, and light petroleum. Having regard to the large amount of polysaccharide acetate employed in these typical expts., it seems inconceivable that marked differences in molecular aggregation exist.

Simultaneous Deacetylation and Methylation of Glycogen Acetate.—The glycogen acetate (35 g.) was dissolved in acetone (350 c.c.) and treated at 50° during 90 mins. with Me_2SO_4 (170 c.c.) and 30% NaOH aq. (450 c.c.) with vigorous stirring. The reagents were admitted in 1/10th portions every 10 mins., acetone being added from time to time to replace that lost by evaporation. The operation was completed by heating at 80° for 30 mins. to remove acetone; the methylated product, which then separated as a solid, was removed and dissolved in acetone for remethylation. Eight successive methylations were carried out in this way except that two batches, each from 35 g. of the acetate, were combined for the second and subsequent treatments.

The final isolation of the methylated glycogen was accomplished by extracting the solid methylated product with boiling $CHCl_3$, the extract being dried

over $MgSO_4$ and concentrated to a pale yellow glass, which on trituration with light petroleum yielded a fine white powder. This was extracted with boiling Et_2O to remove traces of impurities. Yield from 140 g. of glycogen acetate, 83·5 g., *i.e.*, 82%.

As in the case of glycogen acetate, expts. were instituted to discover whether the methylated glycogen was uniform in character. To this end large amounts of the methylated compound were fractionally pptd. both from acetone and $CHCl_3$ solution by means of Et_2O and light petroleum.

In a typical expt. the initial fraction gave C, 52·7; H, 7·9; OMe, 45·0%, and the final fraction C, 52·5; H, 7·6; OMe, 45·0%; whereas a representative sample of the methylated polysaccharide used in the hydrolysis expts. gave C, 52·9; H, 8·0; OMe, 45·4 (Calc. for $C_9H_{16}O_5$: C, 52·9; H, 7·8; OMe, 45·6%).

The specific rotation of methylated glycogen, $[\alpha]_D^{20°} = +209°$ (*c*, 1·0 in $CHCl_3$), was observed for all the fractions obtained. Experience has shown that the determination of the specific rotation of methylated polysaccharides, as well as being more convenient, is more sensitive as a control of OMe content than Zeisel estimations.

Refractionation of the final fractions from a number of pptns. having failed to indicate any differences, the conclusion was reached that the methylated glycogen used in these expts. consisted of groups of products of similar molecular size.

The Hydrolysis of Methylated Glycogen.—Powdered methylated glycogen (80 g.) was added to cold HCl aq. (*d* 1·16; 400 c.c.), contained in a large distillation flask fitted with a Bunsen valve on the side arm. The methylated polysaccharide dissolved in 2 hrs.; the liquid was then cooled to − 15° and saturated with dry HCl. After a further 2 hrs. the liquid was again saturated with HCl and the mixture was kept for a total of 44 hrs. at room temp. The excess of HCl was then removed by aeration, and the solution diluted and neutralised with $BaCO_3$ in the presence of a little charcoal. After filtration the almost colourless liquid, together with the washings (1½ l.), was extracted with $CHCl_3$ (5 l.) in eight operations, the extracts being evaporated at 50°/15 mm. after drying over $MgSO_4$. The aq. solution was treated with an equal vol. of abs. EtOH, which eliminated a large quantity of $BaCl_2$, and the aq.-alc. filtrate was concentrated at 50° under diminished press., the residue being dried with EtOH–C_6H_6.

Examination of the material from the chloroform extract. The syrup (15 g.) was redissolved in $CHCl_3$ (75 c.c.), and light petroleum (b. p. 40—60°; 1500 c.c.) added slowly and with stirring. The petroleum was poured off after 30 mins., and the syrup redissolved in $CHCl_3$ and repptd. in an exactly similar manner, the entire operation being repeated a third time. The light petroleum was removed by distillation to yield a syrup (10 g.), part of which crystallised in the characteristic form of 2 : 3 : 4 : 6-tetramethyl glucose.

Preparation of the glucosides. This mixture was heated at 70° for 8 hrs. with 1% methyl-alc. HCl (150 c.c.), the acid neutralised with Ag_2CO_3, and the filtered solution evaporated to a thin syrup (10·5 g.) (C) and heated at 100°/15 mm. for several hrs. to remove solvent.

Treatment of the solid residues from the aqueous solution. The well-dried residues together with the pptd. $BaCl_2$ were extracted 10 times with boiling $CHCl_3$, the extracts being dried over $MgSO_4$ and concentrated. This treatment yielded a syrup which, on trituration with Et_2O and cooling in the refrigerator,

crystallised to yield 2 : 3 : 6-trimethyl glucose (25 g.). The crystals were removed by filtration and the ethereal solution of syrup was concentrated, the resulting syrup being added to that left over from the light-petroleum extractions of the material from the $CHCl_3$ extract. This syrup (46 g.) was dissolved in $CHCl_3$ (100 c.c.), and the above treatment with light petroleum repeated. The petroleum extracts on concn. yielded a syrup (1·5 g.), which was converted into the methylglucosides as before (1·5 g.) (D).

The isolation of 2 : 3 : 4 : 6-*tetramethyl methylglucopyranoside.* This was achieved by fractional distillation of (C) and (D) in a high vacuum from a Widmer flask fitted with a special fractionating column. Previous expts. (Haworth and Machemer, *Ber.*, 1932, **65**, [*A*], 43) had proved that an almost quantitative separation of the tetra- from the tri-methyl methylglucosides could be obtained.

Syrup C was distilled into the Widmer flask for the first fraction and into a collecting tube for the second.

(I) 7·5 g., b. p. 93°/0·03 mm. Bath temp. 108—110°. $n_D^{12°}$ 1·4462.
(II) 2·5 g., b. p. 93—97°/0·03 mm. Bath temp. 115—120°. $n_D^{12°}$ 1·4540. Still residue, 0·5 g.

Refractionation from the Widmer flask of fraction (I).

(III) 6·40 g., b. p. 87°/0·04 mm. Bath temp. 130°. $n_D^{13°}$ 1·4456. Distilled in 1½ hrs.
(IV) 0·95 g., b. p. 87°/0·04 mm. Bath temp. 140°. $n_D^{13°}$ 1·4460. Distilled in ½ hr.
(V) 0·02 g., b. p. 90°/0·04 mm. Bath temp. 150°. $n_D^{13°}$ 1·4511.
(VI) 0·05 g., b. p. 98°/0·04 mm. Bath temp. 150—160°. $n_D^{13°}$ 1·4575.

After fraction (III) had been collected, fraction (II) was added to the residue in the Widmer flask and the distillation was continued as above. After the collection of fraction (VI), the syrup D was distilled from the Claisen flask into the Widmer apparatus (bath temp. 90—110°/0·04 mm.). The fractional distillation was continued, and the following fractions collected.

(VII) 0·30 g., b. p. 85—90°/0·04 mm. Bath temp. 135°. $n_D^{13°}$ 1·4450, in 1 hr.
(VIII) 0·10 g., b. p. 95—100°/0·04 mm. Bath temp. 140—150°. $n_D^{13°}$ 1·4550.
(IX) 0·02 g., b. p. 95—100°/0·04 mm. Bath temp. 150—160°. $n_D^{13°}$ 1·4570.

From a consideration of the refractive indices, fraction (I) appeared to be in the main tetramethyl methylglucopyranoside, and fraction (II) the corresponding trimethyl derivative. A more detailed examination confirmed this, analyses being carried out for each fraction, the final fraction hydrolysed, and the resulting sugar weighed.

It was found that (III), (IV), and (VII) consisted of tetramethyl methylglucopyranoside (Found : C, 52·75; H, 8·9; OMe, 59·5. $C_{11}H_{22}O_6$ requires C, 52·8; H, 8·9; OMe, 62·0%), whilst the remainder was trimethyl methylglucopyranoside. The total yield of tetramethyl methylglucopyranoside was therefore 7·65 g., so the result, expressed as the percentage of tetramethyl glucose obtained from the trimethyl glycogen, is 9·0%. The expression connecting the number of anhydroglucose residues (x) with the percentage of tetramethyl glucose (y) derived from any fully methylated polysaccharide is $y = 236 \times 100/(204x + 46)$. The shorter expression, $x = 116/y$, gives the approximate result.

By the hydrolysis of 16·0 g. of methylated glycogen in another series of expts., 8·8% of tetramethyl methylglucopyranoside was obtained. This is seen to be in good agreement with the previous result when it is considered that the losses are relatively greater by working with smaller quantities.

The isolation of tetramethyl glucopyranose. Tetramethyl methylglucopyranoside (3 g.) obtained from fractions (III), (IV), and (VII) was hydrolysed during 8 hrs. with 5% HCl aq. (100 c.c.) and gave cryst. tetramethyl glucopyranose (2·65 g.), which was recrystallised from light petroleum and had m. p. 88—89° alone or mixed with an authentic specimen, $[\alpha]_D^{20°}$ + 83° (equilibrium value in H_2O; *c*, 1) (Found : C, 50·5; H, 8·6; OMe, 50·6. $C_{10}H_{20}O_6$ requires C, 50·8; H, 8·5; OMe, 52·5%).

The recovery of trimethyl glucose. The total yield was 73 g., being made up of 25 g. of cryst. material, 46 g. of syrup, and 2 g. as the methylglucoside. This, together with the 7 g. of tetramethyl glucopyranose, constitutes a recovery of 96% of products derived from the original methylated glycogen.

The Reducing Power of Glycogen.—It was observed that slight but definite reduction of Fehling's solution took place when pure glycogen (electrodialysed and pptd. twice with EtOH; ash = 0·15%) was heated at 100° for 10 mins. with the reagent.

The reducing value according to the method of Bergmann and Machemer (*Ber.*, 1930, **63**, 316) was investigated, and the iodine value, *i.e.*, the number of c.c. of *N*/10-I required to oxidise 1 g. of the substance, as the mean of several expts., is given as 1·95, whereas the value for starch, which is non-reducing to Fehling's solution, is *ca.* 0·7 depending on the source. Glycogen is therefore more reducing in character than starch, a fact which is readily explained if glycogen contains fewer anhydroglucose residues than starch.

Estimations of the Molecular Weights (Rast).—These gave the following mean values : Glycogen acetate, 2600; methylated glycogen, 2400. As in other cases, these are to be regarded as minimum values. The results are interpreted merely as an indication that no degradation occurs on methylation of the acetate.

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