395. *Polysaccharides. Part XXXVIII. The Constitution of Glycogen from Fish Liver and Fish Muscle.*

By W. N. Haworth, E. L. Hirst, and F. Smith.

Glycogen contains a repeating unit consisting of α-glucopyranose residues joined through positions 1 and 4 and the number of glucose residues in this repeating unit, *i.e.*, its chain length, is ascertained from the amount of tetramethyl glucose produced on hydrolysis of the methylated glycogen. A specimen of rabbit liver glycogen examined by Haworth and Percival (J., 1932, 2277) had a chain length of 12, and another specimen of rabbit liver glycogen was found to have a chain length of 18

(Haworth, Hirst, and Isherwood, J., 1937, 577). This communication shows that four specimens of glycogen of fish origin (three from fish liver and one from fish muscle) contain a repeating unit of 12 glucopyranose residues.

THE methyl derivative of the specimen of rabbit liver glycogen investigated by Haworth and Percival (J., 1932, 2277) yielded on hydrolysis 9% of 2 : 3 : 4 : 6-tetramethyl glucose and a large proportion of 2 : 3 : 6-trimethyl glucose. This evidence was taken to indicate the presence of a repeating unit in glycogen consisting of approximately 12 glucose residues joined by 1 : 4-glycosidic links. Another specimen of rabbit liver glycogen, however, examined by Haworth, Hirst, and Isherwood (J., 1937, 577) furnished a methyl derivative which gave 7% of tetramethyl glucose, corresponding to a repeating unit of 18 glucose residues. Similarly, glycogens investigated by Bell (*Biochem. J.*, 1935, **29**, 2031; 1936, **30**, 1612, 2144; 1937, **31**, 1683) have also been shown to have differing " chain-lengths " of 12 and 18 units, one of these specimens being from a fish liver (compare also Hassid and Chaikoff, *J. Biol. Chem.*, 1938, **123**, 755). In view of these results it seemed important to examine glycogen from other sources and to ascertain the size of their repeating units. Accordingly, glycogens from fish liver (dogfish, haddock and hake) and fish muscle (dogfish) have been investigated. Only the specimen of glycogen from haddock liver was soluble in cold water and behaved normally. The glycogens from dogfish liver, dogfish muscle, and hake liver were almost insoluble in both cold water and cold potassium hydroxide solution and only slightly soluble in hot water and hot potassium hydroxide solution. It was found, however, that these dissolved readily in cold dilute acetic acid or, better, in cold dilute mineral acid, from which solutions they could be precipitated with alcohol; the samples of glycogen treated in this way were now soluble in cold water and indistinguishable from the rabbit liver glycogen of Haworth and Percival. By the courtesy of Mr. Adrian Lumley, the specimens were prepared for us at the Torrey Research Station, Aberdeen, by Dr. J. G. Sharp, who used Pflüger's method of extraction. It is evident, therefore, that the specimens must have reverted to an insoluble form after their extraction, although they were not kept longer than 4 months. The reason for the insolubility in water of the glycogen specimens examined during the course of this work, and the nature of the transformation which takes place when these specimens are converted into the soluble form with mineral acid, are problems which, at present inexplicable, are under investigation.

To minimise the possibility of degradation of the fish glycogens, these were acetylated with pyridine and acetic anhydride under very mild conditions and the acetyl derivatives so formed were then converted into the corresponding methyl derivatives by the use of methyl sulphate and sodium hydroxide. All the specimens of methylated glycogen from the different sources mentioned above gave on hydrolysis an amount of tetramethyl glucose (average, 9·3%) which corresponds to a chain length of 12 glucose residues for the repeating unit (see table below).

Source of fish glycogen.		% OMe of sample hydrolysed.	% Yield of tetramethyl glucose.*	% Methylglucosides.†		
				Tetramethyl.	Trimethyl.	Dimethyl.
I Dogfish liver	(a)	43·95	9·0			
	(b)	43·85	9·0	8·3	74·7	17·0
	(c)	44·3	9·7			
II Dogfish muscle	(a)	43·9	9·6	8·8	73·1	18·1
III Haddock liver	(a)	44·6	9·1			
	(b)	44·9	9·3			
IV Hake liver	(a)	44·5	9·7	8·7	80·9	10·4

* Value after addition of correction to compensate for experimental losses.

† Observed values, not corrected.

In this investigation a closer study has been made of the yields of dimethyl methylglucoside; the amounts isolated appear to vary to some extent with the percentage of methoxyl in the methylated glycogen and hence a portion of this " dimethyl " may arise as a result of incomplete methylation. It is, however, significant that the amount of dimethyl methylglucoside is never less than that of the tetramethyl methylglucoside and, in the case of methylated hake liver glycogen, the amount of dimethyl methylglucoside approximates to that of the tetramethyl methylglucoside (see Haworth, Hirst, and Isherwood, *loc. cit.*).

Since this sample of methylated glycogen has been subjected to ten methylations, it would appear unlikely that any free hydroxyl groups remain unmethylated and hence any formula proposed for the structure of glycogen must take account of the occurrence of dimethyl glucose.

The interpretation put forward by Haworth, Hirst, and Isherwood (*loc. cit.*) is equally applicable in this case. Thus, glycogen as well as starch is represented as groups of α-glucopyranose units linked at positions 1 and 4 in the form of chains (Haworth, *Monatsh.*, 1936, **69**, 314), and these chains or repeating units are themselves joined to one another by a type of bond which connects the reducing end of one chain with a hydroxyl group of an adjoining chain of glucose units. From experiments recently conducted on methylated starch a value for the energy of activation of the bond which joins neighbouring chains of glucose units in starch has been furnished. Hirst and Young (this vol., p. 952) have concluded that this bond is of the primary valency type, and it is probable that in glycogen also the bond connecting adjoining chains of 12 glucose units has the nature of a primary valency. The linking of the chains occurs through a hydroxyl group, other than that at C_1 and C_4, of a non-terminal glucose residue; this glucose residue would clearly be the one which gives rise to the dimethyl glucose, since three out of five of its hydroxyl groups are involved in links with other adjoining glucose units. Such a formulation for glycogen is supported by its non-reducing character and by its large molecular size (3000—5000 glucose units, *i.e.*, *M* 500,000—800,000) as determined by osmotic pressure measurements (Carter and Record, this vol., p. 670) and, furthermore, it offers the necessary explanation for the cleavage fragments of the methyl derivative.

Experimental.

I. *Glycogen from the Liver of Dogfish.*

Glycogen from dogfish liver was a white amorphous powder, slightly soluble in cold water or aqueous potassium hydroxide; more dissolved on boiling, but complete solution could best be effected by treating the glycogen at 15° with dilute acetic or with N/10-hydrochloric acid. When precipitated by addition of alcohol to the clear solution so obtained and further purified by repetition of the procedure, the glycogen had $[\alpha]_D^{20°}$ + 195° (*c*, 0·31) in water; iodine number *ca.* 2·5 (Bergmann and Machemer).

Acetylation.—Glycogen (10 g.) freshly precipitated from aqueous solution by alcohol and washed with absolute alcohol was dissolved in pyridine (150 c.c.). Acetic anhydride (130 c.c.) was added slowly with stirring during 2 hours. The clear colourless solution was kept for 12 hours at room temperature and then poured into water. The acetate was filtered off, washed with water, alcohol, and ether, and dried in a vacuum (yield, 16·2 g.). The amorphous white acetate was insoluble in alcohol, ether, and light petroleum but readily soluble in acetone and chloroform; $[\alpha]_D^{21°}$ + 167° in chloroform (*c*, 0·2); $\eta_{sp.}^{20°}$ 0·048 in *m*-cresol (*c*, 0·4) corresponding to an apparent mol. wt. of 3460 (using Staudinger's formula with $K_m = 10^{-4}$) * (Found : $CO{\cdot}CH_3$, 44·6%). Fractional precipitation of the acetate from chloroform or acetone solution by addition of light petroleum and examination of the various fractions in respect of rotation in chloroform, acetyl content, and viscosity in *m*-cresol failed to disclose the presence of degraded material.

Regeneration of the glycogen was effected by dissolving the acetate (1 g.) in acetone (15 c.c.) and shaking the solution with 30% aqueous potassium hydroxide (15 c.c.) for 1 hour. The aqueous layer was separated, acidified with acetic acid, and poured into alcohol. The glycogen thus precipitated was dissolved in dilute acetic acid and reprecipitated with alcohol. After two further precipitations from an aqueous solution by alcohol the white amorphous powder was free from ash and was neutral to litmus. It did not reduce boiling Fehling's solution. It showed $[\alpha]_D^{20°}$ + 191° (*c*, 0·6) in water and its iodine number was *ca.* 2·7. Reacetylation by the above method gave an acetate which had $[\alpha]_D^{21°}$ + 173° in chloroform (*c*, 0·3) (Found : $CO{\cdot}CH_3$, 44·6%). The viscosity in *m*-cresol solution was the same as before.

Methylation of Glycogen Acetate.—The acetate (10 g.) was simultaneously deacetylated and methylated by methyl sulphate (150 c.c.) and 30% sodium hydroxide solution (450 c.c.) in the presence of acetone at 30—35°. The reagents were added in one-tenth quantities every 15 minutes and acetone was added from time to time to replace that lost by evaporation in order to

* Here and on all subsequent occasions in this paper where apparent mol. wts. from viscosity data are given they are derived from Staudinger's formula using $K = 10^{-4}$.

keep the methylated glycogen in solution. When the reaction was completed (2¼ hours), the solution was heated on the boiling water-bath to expel the excess of acetone, and the methylated glycogen, which separated as a pale yellow mass, was filtered off through linen, dissolved in acetone, and remethylated. After seven such methylations the crude material was taken up in chloroform and the solution was extracted twice with water to remove inorganic salts, dried over anhydrous magnesium sulphate, filtered, concentrated to a suitable volume (*ca.* 150 c.c.), and poured with stirring into excess of light petroleum. The amorphous white precipitate was filtered off, washed with light petroleum and dried (yield, 50 g. from 80 g. of acetate); $[\alpha]_D^{20°}$ + 209° in chloroform (*c*, 0·8); $\eta_{sp.}^{20°}$ 0·065 in *m*-cresol (*c*, 0·4), corresponding to an apparent mol. wt. 3300 (Found : OMe, 44·0%).

Fractional precipitation was carried out by the gradual addition of light petroleum to a solution of the methyl glycogen (50 g.) in chloroform (200 c.c.). Each fraction was dissolved in acetone and precipitated as a white powder by pouring the solution into light petroleum. After removal of solvent by drying in a vacuum at 100° the methoxyl content, specific rotation, and viscosity of each fraction were determined :

Fraction.	I.	II.	III.	IV.	V.
Weight (g.)	3·5	11·9	9·7	6·6	11·2
% OMe	43·0	43·7	44·2	43·6	44·1
$[\alpha]_D^{20°}$ in $CHCl_3$	+209°	+213°	+215°	+215°	+216°
M from viscosity in *m*-cresol	4000	2860	2650	2450	2720

Fraction I contained inorganic impurities and was therefore rejected.

Hydrolysis of Methylated Glycogen.—(a) *With dilute hydrochloric acid.* Fractions II and III combined (20·4 g.) were dissolved in a mixture of glacial acetic acid (100 c.c.) and 5% hydrochloric acid (200 c.c.), and the solution was heated on the boiling water-bath until the rotation became constant (*ca.* 7 hours) (see Bell, *Biochem. J.*, 1935, **29**, 2031). The amount of barium carbonate required to neutralise the hydrochloric acid was added and the solution was evaporated to dryness under diminished pressure at 45°, water being added from time to time to facilitate the removal of acetic acid. The dry residue was exhaustively extracted with alcohol. The syrup which remained on removal of the alcohol was dissolved in water (200 c.c.) and the solution (A) was extracted twenty times with chloroform (20 portions of 50 c.c.). Evaporation of the chloroform gave a syrup, which was dissolved in 1% methyl-alcoholic hydrogen chloride (300 c.c.). The solution was boiled for 7 hours, neutralised with silver carbonate, and filtered. The syrup obtained on removal of the solvent was distilled, giving :

	Fraction.	B. p. (bath temp.)/0·06 mm.	Weight, g.	$n_D^{15°}$.	% OMe.
I		105—125°	1·373	1·4450	61·0
II		125—132	0·603	1·4508	57·0
III		132—135	0·465	1·4564	53·1
				(last drop 1·4580)	

The next portion of the distillate consisted of 2 : 3 : 6-trimethyl methylglucoside, b. p. 135°/0·06 mm., $n_D^{15°}$ 1·4580 (Found : OMe, 51·5%). On the assumption that the refractive indices of tetra- and tri-methyl methylglucosides are $n_D^{15°}$ 1·4450 and 1·4580 respectively the estimated amount of tetramethyl methylglucoside is 1·94 g., corresponding to 9·0% of tetramethyl glucose in the hydrolysis products from methylated glycogen. In estimating the amount of tetramethyl methylglucoside a 10% correction is applied (cf. Haworth and Machemer, J., 1932, 2270).

The aqueous solution (A) (see above) after extraction by chloroform to remove tetramethyl glucose was concentrated to dryness and the reducing sugars were converted into their methylglucosides. These were isolated in the usual way and subjected to fractional distillation. No tetramethyl methylglucoside could be detected in the initial stages of the distillation and it appears therefore that the method employed above in removing tetramethyl glucose from an aqueous solution is fully effective.

(b) *With fuming hydrochloric acid.* Methylated dogfish liver glycogen (17·4 g.) from fractions IV and V was hydrolysed by the methods of Haworth and Percival (J., 1932, 2280). The methylated glycogen was dissolved in concentrated hydrochloric acid (100 c.c., *d* 1·2) and the solution, cooled to 0°, was saturated with hydrogen chloride. The viscous brown liquid was kept for 40 hours at — 5° and then for 20 hours at 20°. After aeration at 20° to remove as much hydrochloric acid as possible the solution was diluted to 1 litre, neutralised with lead carbonate, and filtered, the residue being well washed with hot 50% aqueous alcohol. The

combined filtrate and washings were evaporated to dryness under reduced pressure. The residue was exhaustively extracted with alcohol and on removal of solvent there was obtained a mixture of methylated glucoses; these were dissolved in water (200 c.c.) and the solution was extracted with chloroform as in (a). The chloroform extract was dried over anhydrous magnesium sulphate, filtered, and evaporated to dryness and the mixture of tetramethyl and trimethyl glucose was converted into the corresponding mixture of glucosides, which were distilled, giving :

Fraction.	B. p. (bath temp.)/0·04 mm.	Weight, g.	$n_D^{15°}$.	% OMe.
I	110°	1·063	1·4450	61·1
II	110—140	0·546	1·4520	56·0
III	140	0·788	1·4577	52·2
			(last drop 1·4580)	

Further distillation gave pure trimethyl methylglucoside, $n_D^{15°}$ 1·4580 (Found : OMe, 51·7%). The amount of tetramethyl methylglucoside existing in fractions II and III (estimated from the value of the refractive index) together with the pure tetramethyl methylglucoside of fraction I amounts to 1·65 g., corresponding to *ca.* 9·0% of tetramethyl glucose in the methyl glycogen subjected to hydrolysis.

The aqueous solution from which all tetramethyl and some trimethyl glucose had been extracted with chloroform was evaporated to dryness. The methylated sugars were separated from inorganic material by extraction with alcohol. The alcoholic solution gave a mixture of reducing sugars, which was converted in the usual way into the corresponding methylglucosides with 1% methyl-alcoholic hydrogen chloride. These were isolated, as in previous instances, and combined with the still residue from the first fractional distillation; fractionation was then continued, giving :

Fraction.	B. p. (bath temp.)/0·04 mm.	Weight, g.	$n_D^{15°}$.	% OMe.
IV	140°	0·482	1·4580	51·8
V	140	7·687	1·4580	51·7
VI	140—150	4·818	1·4610	49·4
VII	150—160	1·416	1·4680	45·16
VIII	above 160	1·30	1·4740	40·75

Since the dimethyl methylglucoside has a refractive index of $n_D^{15°}$ 1·4740 when prepared under these conditions, the total distillate of 18·1 g. is composed of 1·5 g. of tetramethyl, 13·5 g. of trimethyl, and 3·1 g. of dimethyl methylglucoside, corresponding respectively to 8·3%, 74·7%, and 17·0% of the weight of methylated glycogen submitted to hydrolysis.

(c) *With dilute sulphuric acid.* Another specimen of dogfish liver glycogen acetate was prepared as described above and methylated ten times. The crude methylated derivative (24 g.), $[\alpha]_D^{16°}$ + 209° in chloroform (*c*, 0·6), had apparent mol. wt. 2900 (from viscosity measurements in *m*-cresol) (Found : OMe, 44·7%). It was purified by fractional precipitation in the manner already described and the purified material then had $[\alpha]_D^{18°}$ + 210°, apparent mol. wt. 2900 (from viscosity data) (Found : OMe, 44·3%). This methylated glycogen (18·6 g.) was hydrolysed as under (a), 6% sulphuric acid being used instead of hydrochloric acid; the sulphuric acid was neutralised with barium carbonate and the solution was filtered and evaporated to dryness under reduced pressure. The whole of the hydrolysis product (consisting of tetra-, tri-, and di-methyl glucose) was extracted with alcohol and converted into the methylglucosides by boiling for 7 hours with 20 parts of 1% methyl-alcoholic hydrogen chloride. The solution was cooled, neutralised with silver carbonate, and filtered. The glucosides (21 g.) obtained on elimination of solvent were slowly distilled until the refractive index of the distillate was constant at that of pure trimethyl methylglucoside ($n_D^{15°}$ 1·4580). This distillate (10·6 g.) was then refractionated, giving :

Fraction.	B. p. (bath temp.)/0·07 mm.	Weight, g.	$n_D^{15°}$.	% OMe.
I	115°	1·21	1·4461	60·5
II	115—125	0·930	1·4516	56·45
III	125—135	1·490	1·4566	53·1
			(last drop 1·4580)	

The next fraction collected was pure trimethyl methylglucoside and had $n_D^{15°}$ 1·4580 (Found : OMe, 51·9%). From the refractive index, the estimated tetramethyl methylglucoside in fractions I, II, and III amounts to 1·9 g. (9·65% by weight of the methylated glycogen submitted to hydrolysis).

II. *Glycogen from the Liver of Haddock.*

This glycogen was soluble in cold water, giving an opalescent solution which had $[\alpha]_D^{18°}$ + 180° (*c*, 0·5), iodine number *ca.* 2·8. Like all the other specimens of glycogen examined in this investigation, it gave a deep red colour with iodine and did not reduce Fehling's solution.

Acetylation.—By the pyridine–acetic anhydride method described above, 60 g. of glycogen gave 95 g. of acetate, $[\alpha]_D^{18°}$ + 171° in chloroform (*c*, 0·7) (Found : CH_3·CO, 44·2%). Apparent mol. wt. 3100 from viscosity measurements in *m*-cresol. Fractional precipitation from chloroform solution by the addition of light petroleum failed to reveal the presence of degraded material. The acetate (3·3 g.), dissolved in acetone, was deacetylated with potassium hydroxide (see above) and regenerated glycogen (1·6 g.) was obtained. This gave a clear solution in water and showed $[\alpha]_D^{18°}$ + 189° (*c*, 0·9); iodine number *ca.* 2·9. It gave the characteristic deep red colour with iodine and did not reduce Fehling's solution.

Methylation of Glycogen Acetate.—The acetate (90 g.) was methylated in portions of 10 g. with methyl sulphate and sodium hydroxide in the presence of acetone as previously described. After nine methylations, the crude material (55 g.) was freed from inorganic impurity. It then had $[\alpha]_D^{19°}$ + 212° in chloroform (*c*, 1·0); apparent mol. wt. by viscosity measurements 2600 (Found : OMe, 44·5%). Precipitation of the methyl glycogen from chloroform solution by addition of light petroleum gave the following fractions :

Fraction.	I.	II.	III.	IV.
Yield, g.	10·0	20·0	11·1	8·9
% OMe	44·6	44·6	45·0	44·8
$[\alpha]_D$ in $CHCl_3$	+213°	+215°	+214°	+214°
Apparent mol. wt. from viscosity in *m*-cresol	2600	2500	2800	3000

Molecular-weight determinations carried out on fraction II by Rast's method gave values between 2300 and 2500 (cf. Haworth and Percival, *loc. cit.*).

Hydrolysis of Methylated Glycogen with Aqueous Acid.—(a) Fraction II (19·6 g.) was hydrolysed by heating with glacial acetic acid (100 c.c.) and 6% hydrochloric acid (200 c.c.). The whole of the tetramethyl glucose and some trimethyl glucose were extracted from the aqueous solution by chloroform and after removal of the solvent they were converted into the corresponding glucosides by boiling with 1% methyl-alcoholic hydrogen chloride and these were separated by fractional distillation :

Fraction.	B. p. (bath temp.)/0·08 mm.	Weight, g.	$n_D^{15°}$.	% OMe.
I	115—120°	1·199	1·4460	60·55
II	120—125	0·376	1·4480	59·0
III	125—135	0·831	1·4530	55·45
			(last drop 1·4580)	

A further fraction of trimethyl methylglucoside was obtained having $n_D^{15°}$ 1·4580 (Found : OMe, 51·7%). The estimated yield of tetramethyl methylglucoside present in the various fractions amounted to 1·89 g. (yield, 9·1% of the weight of methylated glycogen hydrolysed).

(b) In the same way as in (a) fractions III and IV of the methylated glycogen were combined (18·4 g.) and hydrolysed with a mixture of glacial acetic acid and dilute hydrochloric acid. Separation of the tetramethyl methylglucoside was similarly effected, giving the following fractions in the final distillation :

Fraction.	B. p. (bath temp.)/0·04 mm.	Weight, g.	$n_D^{15°}$.	% OMe.
I	112—120°	1·523	1·4460	60·1
II	120—125	0·504	1·4516	56·25
			(last drop 1·4580)	

Further fractional distillation gave pure 2 : 3 : 6-trimethyl methylglucoside (0·6 g.), $n_D^{15°}$ 1·4580 (Found : OMe, 51·5%). In the fractions I and II the estimated amount of tetramethyl methylglucoside is 1·82 g. (yield, 9·3% of the weight of methylated glycogen hydrolysed).

In the above hydrolyses, the aqueous solutions, after chloroform extraction to remove the tetramethyl glucose, were carefully examined for the presence of tetramethyl glucose, by converting the reducing methylated sugars (obtained on evaporation) into glucosides and subjecting the latter to fractional distillation. None could be detected.

III. *Glycogen from the Liver of Hake.*

Like the dogfish liver glycogen, this material was only partly soluble in cold water. The fraction (80%) insoluble in cold water could be dissolved with difficulty in boiling water (remaining soluble on cooling) or aqueous alkali, or with greater facility in cold dilute acetic or dilute hydrochloric acid. There appeared to be no difference between samples of glycogen prepared by cold water extraction or by dilute acid treatment. All specimens gave an intense red colour with aqueous iodine and had $[\alpha]_D^{18°}$ *ca.* + 190° in water (*c*, 0·5); iodine number *ca.* 2·8. They had no action on boiling Fehling's solution.

Acetylation.—The fraction of the crude glycogen soluble in cold water gave, on treatment with pyridine and acetic anhydride, an acetate having $[\alpha]_D^{20°}$ + 171° in chloroform (*c*, 0·8) and an apparent mol. wt. 3900 (by viscosity measurements in *m*-cresol) (Found : CH_3·CO, 44·5%). The fraction insoluble in cold water after conversion into the soluble form by boiling water gave an acetate identical with the previous one {$[\alpha]_D$ + 173° in chloroform (*c*, 0·8); apparent mol. wt. by viscosity determinations, 3100} (Found : CH_3·CO, 44·7%). Similarly the cold-water-insoluble fraction made soluble with 0·1N-hydrochloric acid gave an acetate which showed $[\alpha]_D^{20°}$ + 175° in chloroform (*c*, 0·7) and had an apparent mol. wt. 3300 by viscosity measurements in *m*-cresol (Found : CH_3·CO, 44·5%).

The main bulk of the acetate was prepared by boiling the glycogen (20 g.) in water (500 c.c.) for 2 hours, complete solution then being attained; the opalescent solution (1 vol.) was poured into alcohol (3 vols.), and the flocculent glycogen acetylated by the pyridine–acetic anhydride method previously described. The acetate (33 g.) thus obtained had $[\alpha]_D^{20°}$ + 171° in chloroform (*c*, 0·8) and an apparent mol. wt. 3300 by viscosity determinations in *m*-cresol (Found : CH_3·CO, 44·6%). Glycogen regenerated from a sample of this acetate had $[\alpha]_D^{18°}$ + 192° in water (*c*, 0·6) and iodine number *ca.* 2·7.

Methylation of Glycogen Acetate.—The acetate (33 g.) gave after ten methylations by the method already described 21 g. of methylated glycogen which had $[\alpha]_D^{20°}$ + 210° in chloroform (*c*, 1·0), and an apparent mol. wt. 3000 by viscosity measurement in *m*-cresol (Found : OMe, 44·4%). The methylated derivative, dissolved in chloroform, was precipitated in fractions by the addition of light petroleum. These were reprecipitated and dried in a vacuum at 100° :

Fraction.	I.	II.	III.	IV.
Weight (g.)	2·5	7·0	6·2	4·3
% OMe	44·5	44·5	44·1	44·8
$[\alpha]_D$ in $CHCl_3$	+212°	213°	212°	213°
Apparent mol. wt. from viscosity in *m*-cresol	2910	2960	3030	2890

Hydrolysis of Methylated Glycogen.—The above four fractions were combined and the material (19·5 g.) was hydrolysed with a mixture of glacial acetic acid (100 c.c.) and 5% hydrochloric acid (200 c.c.). By the procedure already outlined above, tetramethyl glucose was removed together with some trimethyl glucose from an aqueous solution by extraction with chloroform. The methylglucosides were then formed and fractionally distilled, giving :

Fraction.	B. p. (bath temp.)/0·05 mm.	Weight, g.	$n_D^{15°}$.	% OMe.
I	108—112°	1·640	1·4454	60·84
II	112—130	0·60	1·4530	55·2
			(last drop 1·4580)	
III	130	1·739	1·4580	51·5

The total amount of tetramethyl methylglucoside estimated in fractions I and II was 2·0 g., which corresponds to a yield of 9·7% of the methylated glycogen hydrolysed. The aqueous solution after chloroform extraction was evaporated to dryness and the methylglucosides were prepared and isolated in the way previously described. These were combined with the residual undistilled glucosides from the first distillation, and the process of fractional distillation continued :

Fraction.	B. p. (bath temp.)/0·05 mm.	Weight, g.	$n_D^{15°}$.	% OMe.
IV	130—140°	13·823	1·4580	51·3
V	140—150	1·44	1·4640	47·72
VI	above 150	1·122	1·4725	42·0
VII	,, ,,	0·646	1·4740	41·1

On the assumption that the refractive index of dimethyl methylglucoside is $n_D^{15°}$ 1·4740 it can be calculated that the fractions V, VI, and VII contain a total of 2·0 g. of dimethyl methylglucoside. Since fractions I and II contain 1·82 g. of tetramethyl methylglucoside, it follows that the amount

of trimethyl methylglucoside in the total distillate (21 g.) is 17·18 g. The yields, calculated on the weight of material hydrolysed, are 8·7, 80·9, and 10·4% for tetra-, tri-, and di-methyl methylglucoside respectively.

IV. *Glycogen from the Muscle of Dogfish.*

The hard white powder was indistinguishable from the dogfish liver glycogen. It partly dissolved in water, forming a neutral opalescent solution which gave a deep red colour with iodine and did not reduce Fehling's solution even on prolonged boiling. The fraction soluble in cold water had $[\alpha]_D^{18°}$ + 190° in water (*c*, 0·5), iodine number *ca.* 2·8. The insoluble fraction, after conversion into the soluble form by 0·1N-hydrochloric acid, gave a white powder on precipitation from aqueous solution by alcohol. It then dissolved in water, giving a clear solution, and had $[\alpha]_D^{18°}$ + 195° (*c*, 1·0), iodine number *ca.* 2·7.

Preparation of Glycogen Acetate.—Both the cold-water-soluble and the cold-water-insoluble portion of the glycogen furnished the same acetate and hence the main bulk of crude glycogen (17 g.) was dissolved in 200 c.c. of 0·1N-hydrochloric acid, precipitated with alcohol, freed from mineral acid by reprecipitation, and immediately acetylated with pyridine and acetic anhydride as described in previous cases. The acetate (24 g.) had $[\alpha]_D^{18°}$ + 170° in chloroform (*c*, 0·7) and an apparent mol. wt. of 3840 by viscosity measurements in *m*-cresol (Found : CH_3·CO, 44·5%). It gave on deacetylation an almost quantitative yield of regenerated glycogen, $[\alpha]_D^{20°}$ + 193° in water (*c*, 1·0), iodine number *ca.* 2·8.

Methylation of Glycogen Acetate.—The acetate (24 g.) gave after seven methylations by the method previously described 15 g. of methylated glycogen, which had $[\alpha]_D^{20°}$ + 210° in chloroform (*c*, 0·9) and an apparent mol. wt. of 2830 by viscosity measurements in *m*-cresol (Found : OMe, 44·0%). It was separated into two fractions by precipitation from chloroform with light petroleum. These were identical and had $[\alpha]_D^{18°}$ + 211° in chloroform (*c*, 1·0) and apparent mol. wt. 2850 by viscosity measurements (Found : OMe, 44·0%).

Hydrolysis of Methylated Glycogen with Aqueous Acid.—The methylated glycogen (13·6 g.) was hydrolysed with glacial acetic acid (80 c.c.) and 5% hydrochloric acid (160 c.c.) and the product was isolated as before. The syrupy mixture of all the tetramethyl glucose and some of the trimethyl glucose so obtained was boiled with 1% methyl-alcoholic hydrogen chloride for 7 hours. The methyl glucosides thus produced were isolated (15·2 g.) and subjected to fractional distillation, giving :

Fraction.	B. p. (bath temp.)/0·05 mm.	Weight, g.	$n_D^{15°}$.	% OMe.
I	120—125°	0·946	1·4470	59·9
II	125—135	1·046	1·4524 (last drop 1·4580)	55·7
III	135—140	6·725	1·4580 (last drop 1·4580)	51·3
IV	140—145	1·89	1·4590	50·5
V	145—155	1·332	1·4620	48·83
VI	155—170	1·13	1·4717	42·8
VII	above 170	1·162	1·4740	40·95

The estimated amount of tetramethyl methylglucoside in fractions I and II is 1·38 g., giving a yield of 9·6% of tetramethyl glucose. The total distillate of 14·23 g. therefore consists of 1·25 g., 10·4 g., and 2·58 g. of tetra-, tri-, and di-methyl methylglucoside respectively. The yields calculated on the weight of material hydrolysed were therefore tetramethyl methylglucoside 8·8%, trimethyl methylglucoside 73·1%, and dimethyl methylglucoside 18·1%. These figures are calculated from the refractive index.

Isolation of Crystalline 2 : 3 : 4 : 6-*Tetramethyl Glucopyranose.*—A portion of the tetramethyl methylglucoside obtained in each of the above hydrolyses was hydrolysed by heating with 6% sulphuric acid on the boiling water-bath. When the rotation of the solution became constant, the sulphuric acid was neutralised with barium carbonate and the solution was evaporated to dryness under diminished pressure. The residue was extracted with alcohol to remove a small amount of mineral impurity and the alcoholic solution was evaporated to dryness, giving a pale yellow syrup which crystallised spontaneously. After recrystallisation from ether–light petroleum the samples of tetramethyl glucose had m. p. 91—92° alone or in admixture with an authentic specimen, $[\alpha]_D^{20°}$ + 83° (equilibrium value in water).

Isolation of Crystalline 2 : 3 : 6-*Trimethyl Glucopyranose.*—Similarly a portion of the trimethyl methylglucoside from each hydrolysis experiment was converted into the corresponding trimethyl glucose. After recrystallisation from ether the 2 : 3 : 6-trimethyl glucose had m. p.

117° alone or in admixture with authentic 2 : 3 : 6-trimethyl glucopyranose, $[\alpha]_D^{20°}$ + 69° (equilibrium value in water).

We are greatly indebted to the Torry Research Station of the Department of Scientific and Industrial Research at Aberdeen for the specimens of glycogen mentioned in this paper.

THE A. E. HILLS LABORATORIES,
THE UNIVERSITY, EDGBASTON, BIRMINGHAM. [*Received, October 4th,* 1939.]