CCXI. GLYCOGEN¹

VI. THE MOLECULAR STRUCTURE OF HORSE MUSCLE GLYCOGEN

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THE past 30 years have seen a multitude of publications concerned with glycogen breakdown in anaerobically contracting muscles. Few aspects of metabolic activity have attracted so much attention since the days of Claude Bernard when it became known that the muscle glycogen, discovered by Sanson [1857], broke down to give lactic acid. Nevertheless, the starting material itself, the glycogen of the muscle, had been subjected to no critical study until Young [1937] applied to a number of specimens the methods previously used by Bell & Young [1934] during a preliminary examination of some liver glycogens. Young's results failed to reveal any significant facts which might lead to the conclusion that his specimens were in any way different from one another or from liver glycogens. As Young has pointed out, it has tacitly been assumed, since their discovery, that the glycogens of liver and of muscle are identical, although on grounds of physiological function this need not be so. It therefore seemed to us of the utmost importance that mammalian muscle glycogen should be examined by the only method yet at our disposal which can yield definite, though not complete, information regarding the chemical structure of polysaccharides; namely, methylation followed by hydrolysis and examination of the cleavage-products by such a method as Haworth's end-group assay. Bell & Young [1934], Bell & Kosterlitz [1935], Bell [1935; 1936, 1, 2] and Oakley & Young [1936] have accumulated, by a variety of means, evidence that mere examination of glycogens with respect to such properties as rotatory power, reducing power, osmotic pressure of solutions or properties of acyl derivatives affords no clue as to the structure or magnitude of the chemical molecule. For example, the basic chemical molecule of the glycogen from rabbit liver can apparently exist in either of two magnitudes: (a) 12 glucose units [Haworth & Percival, 1932; Bell, 1935] or (b) 18 units [preliminary report by Haworth, 1935; Bell, 1936, 1; Haworth *et al.* 1937]. Yet the superficial properties of both varieties are not significantly distinguishable.

Since no preliminary data other than those compiled by Young [1937] were to hand, we decided, for our initial experiments in this field, to prepare a large sample from a single authentic source; to this end, therefore, from some 22 kg. of freshly dismembered horse muscle, we isolated 230 g. of polysaccharide. This, according to the criteria of Bell & Young, was not obviously different from samples of rabbit liver glycogen, nor, likewise, did the acetyl or methyl derivatives show unusual properties (cf. Table I).

¹ Abbreviation of the title of this series renders it more comprehensive. "The molecular structure of glycogen from the whole tissues of *Mytilus edulis*" [Bell, 1936, 2] is to be regarded as Part V.

Table I

Source of glycogen	No. of units	Reference*	$[\alpha]_D$ in H_2O	Organic P as P_2O_5 %	$[\alpha]_D$ of methyl derivative	$[\alpha]_D$ of acetyl derivative
Horse muscle	12	—	+197°	0.084	+208°	+169°
Rabbit muscle	—	Y	197	—	—	—
"	—	Y	192	0.02	—	—
Rabbit liver	12	B ₁	197	Nil	208	172
"	18	B ₂	200	Nil	210	170
"	12	HP	—	—	209	170
"	18	HH	188	0.10	209	170

* Y, Young [1937]; B₁, Bell [1935]; B₂, Bell [1936, 1]; HP, Haworth & Percival [1932]; HH, Haworth *et al.* [1937].

Our muscle glycogen was purified by the acetic acid technique of Bell & Young and contained no inorganic phosphorus, but the organic phosphorus content was rather higher than we have been accustomed to find in glycogens purified in this manner; the amount was low, and it bore no obvious relationship to the chain length as determined by end-group assay. The methylated glycogen retained phosphorus, though in diminished amount, 0.042 % as P_2O_5 .

Acetylation of the muscle glycogen gave an 87.5 % yield of an apparently homogeneous acetate, which, on simultaneous deacetylation and methylation (12 treatments) gave an 87 % yield of methylated glycogen showing no significant differences when compared with methylated liver glycogen.

End-group assay, by a procedure closely resembling that of Haworth *et al.* [1937], yielded 2:3:4:6-tetramethyl methylglucoside amounting to 10 % of the starting material, thus indicating, for the methylated glycogen, a minimum chain length of 11–12 glucose units. In addition to 2:3:6-trimethyl methylglucoside, free from isomerides, nearly 15 % of dimethyl methylglucoside, i.e. approximately 2 molecules per molecule of pentamethyl glucose, was found to compose the sole remainder of the cleavage products. The significance of the last fraction will be discussed below.

DISCUSSION

The foregoing results show that, as far as we can at present determine, there seems to be no difference between this muscle glycogen and the liver glycogens of 12 unit chains examined by ourselves and other workers. The fact that muscle extracts are able to effect the same transformations using liver glycogen as substrate is in favour of such an idea.

As is now well known, chemical evidence affords a picture of the "minimum glycogen molecule" as a chain of 12 or 18 glucopyranose units linked by 1:4- α -glycosidic links, thus:

Fig 1.

If this were the true structure, hydrolysis of completely methylated glycogen would give

(1) from A, 1 molecule of 2:3:4:6-tetramethyl glucose;
 (2) from B, 1 molecule of 2:3:6-trimethyl glucose and 1 molecule of methyl alcohol;

(3) from C, 10 or 16 molecules of 2:3:6-trimethyl glucose, and no other substances. As Haworth *et al.* have pointed out, it is extremely difficult to lay down "criteria of purity" for such a material as methylated glycogen and we therefore cannot be absolutely certain that the dimethyl glucose, first found to be a regular constituent of the hydrolysate of methylated glycogen by Bell [1935] and later by Haworth *et al.* [1937], has its origin in an intrinsic property of the chemical molecule of the polysaccharide and does not arise merely as a result of incomplete methylation attendant on mechanical causes. Table II shows the amounts of dimethyl glucoses so far isolated from methylated glycogens.

Table II

References (as Table I)	Source of glycogen	No. of units	No. of units of dimethyl sugar	OMe % of methyl glycogen
Present work	Horse muscle	12	2	44.7
B ₁	Rabbit liver	12	2	45.4
B ₁	"	12	2	45.5
B ₁	Fish liver	12	2	45.5
B ₂	Rabbit liver	18	2	45.6
B ₂	"	18	3*	44.8
HH	"	18	1	—
B ₃ †	<i>Mytilus</i>	18	2	45.1

* Probably due to incomplete methylation of glycogen. † B₃, Bell [1936, 2].

Solely on the grounds of viscosity measurements, which seem to indicate a "molecular weight" for glycogen enormously greater than is afforded by end-group assay, Staudinger & Husemann [1937, 1, 2] have suggested a macro-molecular structure of this type:

R = chain of 12 or 18 glucose units as in Fig. 1.

Fig. 2.

This idea does not commend itself to us for the following reasons:

(1) It does not explain the origin of the dimethyl glucose fraction, provided that the latter is significant of a property of the glycogen molecule.

(2) Such a condition of the "central" chain of glucose units is quite unknown where glycosidic linkages engage positions 1, 2, 3, 4 and 6 in one unit. This would lead, on hydrolysis of methylated glycogen, to the liberation of 1 molecule of unsubstituted glucose to every 36 or 54 molecules of methyl glucoses, an amount not impossible of detection. So far we have failed to find any evidence of free glucose after hydrolysis.

However, the acceptance or otherwise of this suggested structure must, at present, remain an open question.

Not only do viscosity measurements point to an extremely large "particle weight" for glycogens and their derivatives, but determinations of their osmotic properties [Oakley & Young, 1936; Carter & Record, 1936] give similar results. In an attempt to reconcile these measurements with the results of end-group assay, including the occurrence of dimethyl glucose among the cleavage products of methylated glycogen, Haworth *et al.* have logically suggested a "laminated" structure, consisting of chains of 12 (or 18) glucose units attached to other chains through occasional hydroxyl groups by some manner of union not as yet clear; possibly the links may be glycosidic in nature or they may be co-ordinate. Since they found 2:3-dimethyl glucose in the dimethyl fraction these authors have suggested the possibility of 1:6-linkage between chains, diagrammatically indicated below:

Fig. 3.

This structure is able to account for (a) lack of reducing power, and hence, the complete resistance to destruction by alkali evinced by glycogen; (b) the occurrence of dimethyl glucoses among the cleavage-products; (c) the relatively anomalous physical properties.

With regard to the question as to whether the dimethyl glucose has its origin in mere failure to methylate unengaged hydroxyl groups or is due to these groups being involved in "cross-linkages" between chains, we at one time thought that we had been able to detect, in methylated glycogen, unetherified hydroxyl groups [Bell, 1935]. This we now consider to be erroneous. Our experiments consisted in attempting to substitute such groups by either dichloroacetyl or by nitrate. The former method did indeed produce material containing approximately the expected amount of chlorine, but we have since found that the results are unreproducible, and that our original product contained nitrogen. We therefore conclude that the presence of chlorine was adventitious and was due to the presence of an inseparable by-product of the violent reaction between the reagents used, namely, dichloroacetyl chloride and pyridine. This was confirmed by examination of the "nitrated" methylated glycogen. At the time of conducting the original experiments we were unable accurately to estimate small amounts of nitrate-N; now we are able to state that the amount of nitrate introduced was less than 1/20th of a nitrate group per

12 C₆ units—a negligible amount. We therefore withdraw the statement that we were able to detect free hydroxyl groups in glycogen which had been methylated until no rise in methoxyl content could be observed.

We must therefore, for the time being, consider that our “fully methylated glycogens” are in fact fully etherified, and that the dimethyl glucose fraction invariably found in the cleavage-products, is due to certain hydroxyl groups of the glycogen molecule being involved in some kind of inter- or intra-molecular linkage.

In our own experience, we have come to the conclusion that the original formula of Fig. 1 does not fully express the properties of the unit chain of glycogen. Besides the invariable isolation of dimethyl glucoses (in two molecular proportions for every 12 or 18 unit chain) and the apparent absence of free OH groups from the fully methylated glycogen, we have been totally unable to detect the glycosidic methyl group which Fig. 1 postulates. For this examination we employed the principle of the method of Freudenberg & Soff [1932] in which the glycoside is hydrolysed by aqueous acid and the liberated methyl alcohol passed through a Zeisel apparatus and determined as silver iodide. In practice, we find that the actual procedure of the original authors is unsatisfactory in that the dilute mineral acid employed as hydrolytic agent brings about a not inconsiderable liberation of methyl alcohol from ether-linked methyl groups of methylated sugars. The use of *p*-toluenesulphonic acid as hydrolytic agent, however, obviated this error and no silver iodide was found in the receiver of the apparatus, even when several g. of different methylated glycogens were used. Actually we were unable to obtain the theoretical values for glycosidic methyl in this way, but several glycosides gave 85% of the calculated values, and methylated sugars gave complete blanks.

These findings are, to a certain extent, confirmatory of the structures of Figs. 2 and 3, but we feel that a slight modification (Fig. 4) of the suggested scheme of Haworth *et al.* would fit in better with our experience. We therefore suggest, with every reservation, that:

(1) The “beginning group” (as opposed to the “end-group”) has its reducing hydroxyl intramolecularly combined as a 1:2, 1:3 or 1:6 anhydride. This would account for 1 molecule of dimethyl glucose among the cleavage products, for

The above 1:2 anhydride structure is employed purely for convenience in setting out the formula.

Fig. 4.

the non-reducing properties of the free polysaccharide and for the absence of glycosidic methyl from methylated glycogen.

(2) One hydroxyl group in a unit-chain forms the link in a "molecular compound" through the residual affinity of an oxygen atom forming part of a cyclic ether ring belonging to another unit-chain, the anhydro-sugar unit accounting for the second molecule of dimethyl glucose.

Cyclic ethers have long been known to form "molecular compounds", in many cases, of considerable stability. Cineol behaves in this way, combining with phenols and also with formaldehyde and thymol [Henschke, 1905]. Bennett & Willis [1929] state that diethyl ether itself has been proved to exist in solution as a molecular compound with chloroform; and Bennett & Waine [1936] have shown that some simple cyclic ethers show a like tendency to form stable complexes with phenols. That true aliphatic alcohols can enter into the formation of molecular compounds has been found by Rheinboldt [1929] in the instance of cetyl alcohol, a stable compound of the alcohol with deoxycholic acid (8 molecules: 1 molecule) being formed. It must be noted, however, that a search of the literature has failed to reveal any instance of compound formation between aliphatic hydroxy-compounds and cyclic ethers, and that those anhydro-sugars which are at present known have not been shown to possess a tendency to form organic molecular compounds.

The author wishes it to be clearly understood that this modification of the laminated structure is intended as a purely speculative attempt to reconcile his experimental data with the findings of others, and we think it appropriate to recall the remarks of Haworth *et al.* [1934] anent the structure of xylan, to the effect that the whole problem of the chemistry of the polysaccharides is so fraught with such complications, that only as a result of prolonged and intensive investigation can we hope to arrive at a true interpretation of their molecular condition.

EXPERIMENTAL

Isolation of muscle glycogen

22 kg. of muscle tissue from a freshly killed horse were boiled, in large open pans, for 3-4 hr. with 22 l. of 40% commercial caustic soda solution. After allowing to cool, 22 l. of 97% alcohol were added and the resulting dark-coloured precipitate allowed to settle. As much as possible of the supernatant fluid was decanted, and the precipitate, containing much sodium carbonate, collected by filtration on a Büchner funnel, well drained and washed with alcohol. The solid was stirred up with 4 l. of water and acidified with acetic acid; a dark precipitate formed, which was allowed to settle and was then easily filtered off, the crude glycogen being obtained from the filtrate by precipitation by an equal volume of 97% alcohol. The precipitate was washed three times by decantation with several litres of 60% alcohol. Some protein was still present. To remove this the solid was dissolved in water and dilute hydrochloric acid added until no more precipitate formed. By passing the fluid twice through the Sharples centrifuge the protein precipitate was completely removed, and the glycogen was isolated by addition of an equal volume of 97% alcohol. The crude material was then dissolved in 30% KOH solution and the whole heated on the boiling water-bath for 3 hr. Isolation was effected by alcoholic precipitation; further purification was then carried out by the acetic acid technique of Bell & Young [1934]; yield, 230 g. In order further to purify the glycogen it was precipitated by 80% acetic acid three more times; it then displayed the following properties:

$[\alpha]_D$ in water, $+197.5^\circ$ ($l=2$: $c=0.61$). Reducing-power (Macleod-Robison) 1.4 (glucose=100). Phosphate (as P): inorganic, nil; organic, 0.05%. Colour with iodine: red-brown.

Acetylation. 50 g. of glycogen were acetylated by the technique of Haworth & Percival [1932]; yield 77 g. (87.5%); $[\alpha]_D$ in chloroform, $+169.2^\circ$ ($l=2$; $c=2$). Found: COCH_3 , 45.0%. Fractional precipitation failed to effect any kind of detectable separation into fractions.

Methylation. 76 g. of the above acetate were simultaneously deacetylated and methylated by Haworth & Percival's procedure. After 12 treatments the methoxyl content was 44.7% and no increase was found on further treatment. The material was purified by dissolving in cold water and then heating the solution; the methylated glycogen separated as an insoluble mass. This process was repeated, then the crude material was dissolved in chloroform and the solution dehydrated over sodium sulphate. The solvent was distilled off and the residue dissolved in acetone and precipitated by petroleum (B.P. $60-80^\circ$), the process being repeated thrice. After distilling off the solvent from an acetone solution, the methylated glycogen was obtained as a colourless, friable solid; yield 47 g. (87%): $[\alpha]_D$ in chloroform, $+208^\circ$ ($l=2$: $c=4.4$); in water, $+207^\circ$ ($l=2$: $c=2$). P: inorganic, nil; organic, 0.018%.

Hydrolysis of methylated glycogen. 30 g. of the above material were hydrolysed by the method of Bell [1935]. Polarimetric observation showed that the reaction was complete in 2.5 hr. The cleavage products were isolated as in the above paper, and were converted into the mixed methyl glucopyranosides by boiling with methyl alcohol containing 1% of hydrogen chloride. After neutralization of the acid by solid barium carbonate, the alcohol was distilled off, the residue extracted with acetone, the solvent distilled off from the acetone extract and the residue extracted with hot chloroform. The residue from the chloroform was distilled at 0.05 mm. from a Claisen flask into a flask fitted with a special fractionating column, until the n_D^{19} of the distillate was 1.4560 indicating that trimethyl methylglucoside was distilling over. The scheme of the fractionation at 0.05 mm. is represented on p. 1690.

Fraction B was pure pentamethyl glucose	
Fraction C contained 1/10 pentamethyl glucose	
	g.
Total tetramethyl methylglucoside	2.903
5% correction for losses	0.145
	3.048
Fraction C contained 9/10 tetramethyl glucose*	1.382
Fraction I was pure tetramethyl glucose	12.015
Fractions H and I were pure tetramethyl glucose	3.113
Fraction J contained 4/5 tetramethyl glucose*	5.360
Total trimethyl methylglucoside	21.870
Fraction J contained 1/5 trimethyl glucose*	1.34
Fraction K was pure trimethyl glucose	3.392
Total dimethyl methylglucoside	4.732

* Calculated from methoxyl content and refractive index.

The amounts of the above fractions correspond to a chain length for this glycogen, of 11-12 units, and for the presence of 2 "potential" hydroxyl groups which are not etherifiable and which manifest themselves as dimethyl glucose on cleavage of the methylated glycogen.

The tetramethyl methylglucoside was identified as derived from 2:3:4:6-tetramethyl glucose, aqueous acid hydrolysis yielding the crystalline sugar, M.P. 95–98° alone or mixed with authentic material. In the same way, the trimethyl methylglucoside was shown to be the 2:3:6-trimethyl derivative,

crystals of the free sugar melting at 115–117° alone or mixed with authentic material. The dimethyl methylglucoside fraction will be investigated.

Careful examination of the cleavage products failed to reveal any evidence of trimethyl glucoses other than the 2:3:6-derivative, and no traces of mono-methyl or free glucose itself could be detected.

SUMMARY

1. Purified horse muscle glycogen has been studied for the first time by the "end-group" assay method. The results show that the polysaccharide is built up of chains of 11–12 glucose units, and its properties do not distinguish it from 12-unit liver glycogens so far examined.

2. The significance of the occurrence of dimethyl glucoses among the cleavage products is discussed with a view to suggesting a reason for the aggregation of the relatively small "unit-chains" into large particles.

3. The evidence leading to a previous statement [Bell, 1935] that free hydroxyl groups (free, in the sense that they could be esterified) had been detected in fully methylated glycogen, has been shown to be unsound. The aforesaid statement is therefore withdrawn.

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