4. Glycogen 8. The Glycogen of *Ascaris lumbricoides* from the Pig

By Ernest Baldwin and Hugh Kirkman King, *From the
Biochemical Laboratory, Cambridge*

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Recent years have seen a number of chemical investigations of the polysaccharides of invertebrates. This is a field of considerable interest for, apart from chitin, the only invertebrate polysaccharide that has received much attention, there exists an animal cellulose in the body wall of the tunicates as was first shown by C. Schmidt [1845] while *Aplysia*, according to Röhmann [1900], contains a polyrhannose. Recent work by Baldwin & Bell [1938] and by Bell & Baldwin [1941] has done something to elucidate the structure of the polygalactose, 'sinistrin', which occurs in the albumin gland of *Helix pomatia*, while a start has been made on the investigation of the glycogens and the various 'modified glycogens' and 'animal starches' that have been reported from time to time. Thus Bell [1936] found that the glycogen of *Mytilus edulis* differs from the glycogen typical of mammals in possessing a chain-length of 18 instead of 12 units. The glycogen of *H. pomatia*, studied by Baldwin & Bell [1940], probably has a chain-length of 12 units.

We have here to report the results of a brief study of the polysaccharide of *Ascaris lumbricoides* from the small intestine of the pig. Until the recent work of Campbell [1936; 1937] there seemed no good reason to think that this was anything other than a typical glycogen, though it was nevertheless a substance of rather special interest if only because it occurs in this nematode in such remarkable quantities. Weinland [1900] prepared from *Ascaris* a polysaccharide with a specific rotation of $+188^\circ$ in yields corresponding to 4.2–7.1 % of the live weight, or 20–34 % of the dry weight of his material. Yields of the same order have since been reported by various other workers and our own observations add to the ample confirmation already available on this point. It will be remembered that Weinland [1901; 1902] was much struck by the presence of some lower fatty acids, among which a valeric acid appeared to predominate, in the contents of saline media in which the worms had been living, and sought to show that these acids originated by the anaerobic breakdown of glycogen. This problem has been repeatedly re-investigated, but without leading to any conclusions that can be regarded as satisfactory (see the review by Slater [1928]). But the possibility that the polysaccharides of *Ascaris* and other intestinal parasites might give rise to such peculiar metabolic end-products adds a little more to their considerable inherent interest.

More recently Campbell [1936] isolated from *A. lumbricoides* a polysaccharide which, to judge from his description, closely resembles glycogen, but at the same time possesses marked antigenic properties: it is not uninteresting to find that sensitization to this worm is a fairly common phenomenon among workers who are much concerned with it. Campbell investigated the possibility that the immunological properties of his preparations might be due to a contaminating protein, but found that they were not destroyed by boiling in neutral solution or by digestion with proteolytic enzymes, while no nitrogen could be detected in the active material. The antigenic potency was however destroyed by boiling in alkaline solution. There can be little doubt that the polysaccharide material, which Campbell obtains by the deproteinization of aqueous extracts of the worm, does indeed contain a potent antigen of some kind, but he appears to believe that the whole of his product consists of an antigenic polysaccharide.

In his second paper Campbell [1937] reports that he was able to isolate from seven different parasitic helminths, seven immunologically distinct polysaccharide fractions which nevertheless resembled each other very closely in chemical properties. He was even able, by immunological tests, to distinguish between products obtained from *A. lumbricoides* parasitic upon pigs, and from the corresponding parasite of the human intestine. These two forms resemble each other so closely that they cannot be morphologically distinguished. In this second paper, Campbell still appears to be of opinion that the whole, or at any rate the main bulk, of his polysaccharide fractions consists of the antigenic substances. A modified method is described in this second paper by means of which yields of the order of 4-5 % of the wet weight of the worm may be obtained. From Campbell's chemical data, as well as from the magnitude of his yields, it seems highly probable that the bulk of his polysaccharide must consist of glycogen, and that the specific antigen, whether protein in nature or not, represents only a small part of the total product.

Although we have not been able to go very far into the many interesting problems which these observations suggest, it seemed to us well worth while to study glycogen from *A. lumbricoides*, which happened to be available to us in large quantities from time to time, in the hope that other workers more fortunately situated may be stimulated to pursue the enquiry further.

EXPERIMENTAL

Material used: isolation of the polysaccharide. Our starting material consisted of specimens of *A. lumbricoides* freshly brought from the slaughterhouse. These were kept in the refrigerator for 12 hr. before being worked up. They were then digested on the boiling water bath for 3 hr. with 30 % aqueous KOH in the proportion of 1 ml. for each g. of tissue. After cooling the digest the polysaccharide was precipitated by adding 1.5 vol. alcohol. When the precipitate had settled, the supernatant was decanted and the somewhat slimy product treated with 97 % alcohol until hard enough to be filtered off at the pump. After washing with a little more alcohol the material was dissolved in the same quantity of KOH as before and digested for a further 2 hr. on the boiling water bath. To the cooled mixture an equal bulk of water was added and the whole was then centrifuged at high speed. This removed a quantity of brown gelatinous material. The polysaccharide was again precipitated by adding 1.5 vol. alcohol and after settling the precipitate was treated with alcohol to 80 % concentration and the product collected at the pump, washed with more 80 % alcohol and sucked as dry as possible. The product, now almost colourless, was dissolved in about 500 ml. water, the solution being faintly acidified with acetic acid. After filtration through a G2 sintered glass funnel an opalescent fluid was obtained from which the crude polysaccharide was precipitated by 2 vol. alcohol. It was allowed to settle, hardened with alcohol as before, filtered off and dried *in vacuo* over calcium chloride.

In this way we obtained from 484 g. of *Ascaris* 33 g. (or 6.8 %) of crude glycogen in one experiment, and in another 55.5 g. (or 7.7 %) from 724 g. of worms.

Purification and properties of the glycogen. 5 g. of the crude product were dissolved in 100 ml. water and the solution was centrifuged. Acetic acid was added to 80 % concentration and the precipitate was collected by centrifuging. This acetic acid precipitation was repeated and the product, dissolved again in 100 ml. water, was centrifuged at very high speed on an Ecco 'Blitz' machine without, however, giving any sediment. Another precipitation was carried out with acetic acid and the material again dissolved in water and precipitated from 67 % alcohol. The precipitate was again collected and dissolved in water, the solution being neutralized with ammonia and again precipitated with alcohol. After washing with alcohol the product was filtered off and dried *in vacuo* over calcium chloride; yield 4 g. of the purified polysaccharide.

With an excess of iodine a rich reddish brown coloration was obtained. The substance itself was a white, slightly hygroscopic powder, freely soluble in water to give a somewhat opalescent solution.

Acid hydrolysis of the glycogen. A solution containing 2.935 % of the polysaccharide in 2.5 % HCl was hydrolysed on the boiling water bath and gave the following polarimetric data, calculated in terms of the polysaccharide:

$t(\text{min}). =$	0	15	30	45	60	90	120	180	300
$[\alpha]_D^{16^\circ} =$	+196°	+153°	+114°	+87.0	+74.3	+62.8	+60.2	+58.3	+57.0

The initial value agrees well with those given for glycogen in the literature and the final figure, calculated in terms of glucose, corresponds to +51.3° as against +52.5° for equilibrated $\alpha \rightleftharpoons \beta$ -D-glucoses.

Acetylation. 33 g. of the crude polysaccharide were dissolved in 330 ml. water, precipitated with 3 vol. alcohol, and the precipitate washed on to a suction filter with 97 % alcohol. After rapid washing with ether the substance was transferred to a flask and stirred mechanically with 600 ml. pyridine, to which 15 ml. water had been added; 450 ml. acetic anhydride were now slowly added with vigorous stirring at room temperature. Stirring was continued until the reaction mixture became homogeneous and the whole was then left overnight. The solution was then filtered through a G3 sintered glass funnel into an excess of cold water, the resulting precipitate being allowed to settle. After repeated washing with tap water the precipitate was filtered off, washed well with tap water on the filter, sucked as dry as possible, and finally transferred to a vacuum desiccator over conc. H_2SO_4 . Yield 45 g. (77 % of theoretical, calc. on assumption that the starting material was pure).

A sample of the acetate was purified by dissolving it in acetone and chloroform, filtering through a hardened paper and evaporating to dryness. The product, a brownish scaly solid, had the following properties: $[\alpha]_D^{16^\circ} = +168^\circ$, $+169^\circ$ (chloroform, $c=1$): COCH_3 found = 41.2 %, 41.4 %.

Methylation. 40 g. of the acetate were dissolved in 400 ml. acetone. 480 ml. 30 % NaOH and 160 ml. dimethyl sulphate were then added in one-tenth portions at 10 min. intervals at 50°, vigorous mechanical stirring being maintained throughout. After boiling off the acetone the product which separated was filtered off and washed with a little boiling water. The reaction mixture itself was worked up in order to see what losses, if any, were incurred by this method of separation. After cooling to room temperature the mixture was extracted 6 times with chloroform, but after drying the extracts, filtering and evaporating to dryness, only a negligible residue remained.

The solid substance, which thus represented the entire yield, was accordingly dissolved in 400 ml. acetone and again methylated, the whole process being repeated until 15 such treatments had been given. After 5 methylations $\text{OMe} = 41.6\%$ and after 10 methylations $\text{OMe} = 42.2\%$. After the fifteenth methylation the acetone was boiled off and the whole heated on the boiling water bath for 40 min. to decompose excess of dimethyl sulphate. The solid was collected on a hot G3 sintered glass funnel and washed once with boiling water. The crude product was now exhaustively extracted with chloroform, dried with anhydrous sodium sulphate, filtered and evaporated *in vacuo* to a thin syrup from which the methylated polysaccharide was precipitated by the addition of light petroleum (B.P. 50–60°). The precipitate was allowed to settle and the supernatant decanted, the solid dissolved in chloroform and again precipitated with light petroleum to yield a supernatant which was only faintly yellow in colour. The precipitate was again dissolved in chloroform, the solution dried and filtered, and evaporated to dryness *in vacuo* to yield a slightly brownish solid. Yield 26.9 g. (95.5 %).

The methylated compound had the following properties: easily soluble in cold water, acetone and chloroform, insoluble in boiling water: $\text{OMe} = 43.0\%$, 43.2% , 43.7% : $[\alpha]_D^{16^\circ} = +209^\circ$ (chloroform, $c=1$): brown coloration with excess of iodine.

Methanolysis. 25.7 g. of the methylated substance were heated under a reflux condenser for 8 hr. with 1 l. methyl alcohol containing 1% by weight of dry HCl. The hot solution was neutralized with lead carbonate, allowed to cool, filtered and evaporated to dryness *in vacuo*. The product was extracted with chloroform, the solution filtered and evaporated to dryness *in vacuo* to give 27.5 g. of mixed glucosides (92.6% of the calculated yield).

Fractionation of the mixed glucosides. The glucosides were now distilled into a flask provided with a vacuum-jacketed fractionating column, from which the substances were fractionally distilled in a high vacuum into tared receivers. In this way we obtained fractions A–F. A still residue of about 8 g. which remained was treated with methyl alcoholic HCl as before and again fractionated to give fractions G–I (see Table 1).

Employing the criteria established by Hirst & Young [1938] we concluded that fractions A and B consisted entirely of 2:3:4:6-tetramethyl-methylglucosides, fractions E, F and H of 2:3:6-trimethyl-methylglucosides, while fractions C, D and G were mixtures of 2:3:4:6-tetra- and 2:3:6-tri-methyl-methylglucosides (referred to as 'tetra'- and 'tri'- respectively in what follows. The compositions of these fractions were approximately calculated from the observed values of refractive index and specific rotation, making use of the data given by Hirst & Young [1938]. Fraction I corresponded to dimethyl-methylglucosides ('di') and must for purposes of calculation be regarded as derived from potentially 'tri'-yielding material which has escaped full methylation for some reason, probably through being involved in cross-linkages between adjacent molecules of the parent polysaccharide [cf. Hirst & Young, 1939]. The still residue, a hard glass, appeared to consist of a condensation product of 2:3:6-trimethyl-methylglucoside [cf. Haworth *et al.* 1937]. Taken altogether, these products amount to 25.3 g., representing a 92% recovery of the mixed glucosides taken for fractionation. Details of the properties of the various fractions are summarized in Table 1 and the compositions in Table 2.

Table 1

Fraction	Bath temp. °C.	mm. Hg	$n_D^{16^\circ}$	$[\alpha]_D^{16^\circ}$	% OMe	Wt. (g.)
A	102	0.02	1.4412	—	61.5	0.133
B	105	0.01	1.4436	+19.1	59.5	0.568
C	105–115	0.02	1.4452	+31.8	59.7	0.960
D	125	0.02	1.4539	+13.7	51.3	1.392
E	125–135	0.015	1.4574	+25.6	51.2	10.189
F	135–145	0.015	1.4592	+64.0	50.6	4.878
G	115–130	0.001	1.4546	+19.0	52.7	1.027
H	130–150	0.001	1.4607	+60.0	52.0	2.132
I	165–170	0.006	1.4738	+61.4	38.8	1.421
Residue	—	—	—	—	39.8	2.665
					Total	25.365

Table 2

Fraction	g. 'tetra'	g. 'tri'	g. 'di'
A	0.133	—	—
B	0.568	—	—
C	0.807	0.153	—
D	0.223	1.169	—
E	—	10.189	—
F	—	4.878	—
G	0.119	0.908	—
H	—	2.132	—
I	—	—	1.421
Totals	1.850	19.429	1.421

Isolation of the methyl glucoses: 2:3:6-trimethylglucose. Fraction E was hydrolysed by refluxing for 2 hr. with $N H_2SO_4$. The free sugar thus liberated was isolated and recrystallized from a large volume of ether: M.P. 113° , not depressed on admixture with an

authentic sample: $[\alpha]_D^{16} = +62.0^\circ$, falling in 24 hr. to -38.0° ($c=5$, in methanol containing 1% dry HCl).

2:3:4:6-Tetramethylglucose. Fractions B and C were hydrolysed in the usual way, the free sugar being recrystallized from ligroin: m.p. 92° , not depressed by admixture with an authentic sample.

Calculation of the chain length. In order to calculate the chain length it is usual [cf. Haworth *et al.* 1937] to add 10% to the amount of 'tetra' material. In the present case the corrected amount is therefore 2.035 g. The yield of 'tri' material includes 19.429 g. to which must be added 1.510 g. corresponding to the 'di' material, and 2.832 g. corresponding to the material of the still residue. The total of 'tri' products is thus to be taken as 23.771 g. Calculating in the usual way we arrive at a 'tetra'/'tri' molecular ratio of 1/12.4 and at a chain length of 13.4 units accordingly. Since the accuracy of this method is stated by Hirst & Young [1938] to correspond to an error of 3 units in a chain of 25 radicles, this figure is consistent with the supposition that *Ascaris* glycogen conforms to the common 12-unit type.

DISCUSSION

The data presented above indicate that the presumptive glycogen of *Ascaris*, prepared by digestion with strong alkali, consists entirely of glucose units and does not differ significantly from other glycogens of the widely distributed 12-unit type. At no stage did we find reason to believe that *Ascaris* glycogen is in any way unusual, and our observations support the view that the bulk of the antigenic polysaccharide material obtained by Campbell [1936; 1937] must consist of glycogen, a view to which, though Campbell himself appears not to subscribe, his chemical data would certainly seem to point.

The immunologically active component of Campbell's material would, of course, have been destroyed by our method of preparation, but all the information available suggests that it cannot account for more than a small proportion of his yields. The antigen might thus be present simply as a contaminant of the glycogen proper, in which case it should be possible, after digesting away the glycogen from suitable preparations, to concentrate and isolate the antigen itself. Alternatively it is conceivable that the antigenic properties of Campbell's preparations might be attributable to the attachment to the glycogen proper of a prosthetic group of some kind which, though it might be immunologically inert in the free state, confers upon the complex the antigenic properties. If such a grouping exists it may well be, if we judge from Campbell's observations, that the linkage involved is thermostable in neutral, though not in alkaline solutions, and is stable moreover to proteolytic enzymes.

In either case it seems that there is here a field of no small interest and potential importance from the standpoint of immunochemistry, and it is hoped that the data reported here may be of some assistance in the further pursuit of the problem.

SUMMARY

1. Glycogen has been prepared from *Ascaris lumbricoides*, parasitic upon the pig, and shown to have a probable chain-length of 12–13 units.
2. Neither the polysaccharide itself nor the products obtained from it by acetylation, methylation and subsequent methanolysis gave any evidence of containing significant amounts of any radicles other than those present in any typical 12-unit glycogen.
3. The significance of these observations is discussed in relation to the immunological properties of certain polysaccharide preparations obtained from *Ascaris* by other workers.

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