22. GLYCOGEN

7. THE GLYCOGEN OF *HELIX POMATIA*

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EARLY work by Claude Bernard, Hammarsten and others [see von Fürth, 1903; Biedermann, 1911 for references] left little doubt that in the metabolism of *Helix pomatia* the part played by glucose and glycogen is analogous to that which they play in the mammal. These earlier workers demonstrated the presence of glycogen in the hepatopancreas and in the tissues generally, its increase in feeding and its diminution in starvation. It is therefore interesting to find [Baldwin, 1938] that the metabolic behaviour of the hepatopancreas ("liver") of *Helix* is similar, in a general kind of way, to that of mammalian liver, an observation which might be regarded as giving some biochemical support to the view of Dorman [1928] that the hepatopancreas of molluscs and the liver of vertebrates are homologous. But the carbohydrate metabolism of *Helix* is peculiar in several respects. In common with certain other herbivorous gastropods such as *Pterocera* and *Strombus* [Yonge, 1932], *Helix* secretes a powerful extracellular cellulase which enables this snail to derive quantities of glucose from cellulose. This enzyme has been much studied by Karrer [e.g. Karrer & Illing, 1925]; its occurrence among Metazoa appears to be uncommon, for even among the highly specialized wood-eating insects it is by no means universally present [Mansour & Mansour-Bek, 1934]. Most remarkable, perhaps, is the presence in the organism of a laevorotatory, galactose-containing polysaccharide discovered by Hammarsten [1885] and named "tierisches Sinistrin" by him. This compound has been extensively studied from a predominantly biological viewpoint by May [1931; 1932; 1934], while more strictly chemical investigations of its structure have been carried out by Schlubach & Loop [1937] and by Baldwin & Bell [1938]. Both the glycogen and the galactogen of the snail presumably owe their origin largely to the products of the enzymic digestion of cellulose.

Biedermann [1911] gives references to early investigations in which glycogen was isolated from the hepatopancreas of *H. pomatia* and the finding of abnormally low values for its specific rotation. These were attributed at the time to admixture with the glycogen of the laevorotatory galactogen. May [1934] however found that galactogen is confined to the albumin gland, a conclusion which our present observations have confirmed; for, working with snails from which the albumin glands had previously been removed as a source of galactogen, we obtained typical preparations of glycogen which gave no evidence of containing or of being contaminated with galactose or galactogen. Our preparations gave a specific rotation of $+192^\circ$ as compared with May's [1934] value of 196.6° . May [1934] observed also that while the albumin gland is rich in galactogen, it contains no glycogen and this too has been confirmed by our own work [Baldwin & Bell, 1938].

May [1934] showed that galactogen accumulates in the albumin gland during feeding and is transferred to the eggs when these are laid and that it must be

synthesized, in all probability, from glucose. If, during the winter sleep, the animals' reserves of glycogen become exhausted, it seems that the stores of galactogen can be drawn upon so that probably glucose (or glycogen) and galactose (or galactogen) are interconvertible in the organism. Baldwin [1938] has shown that the Q_{O_2} of sliced hepatopancreas tissue is increased by about one-third at most times of year by the addition of galactose or of galactogen though not, apparently, during the period which is normally spent in hibernation. Glucose and glycogen do not influence the Q_{O_2} .

In view of these various peculiarities and the somewhat confused state of the earlier literature it seemed to us desirable to isolate and study glycogen from *H. pomatia* with a view to finding out whether or not the unusual character of the carbohydrate metabolism of this form might be in some way reflected in peculiarities in the structure of its glycogen reserves. This seemed the more desirable in view of the fact [Bell, 1936, 1] that the glycogen of the liver of normal rabbits has a chain length of 12 units, while that from the livers of fasted rabbits fed with galactose by stomach tube has a chain length of 18 units. The only other molluscan glycogen of which the chain length has been determined is that from the whole tissues of *Mytilus edulis*, the edible mussel, and in this case a value of 18 units was found [Bell, 1936, 2]: other glycogens agree in having a chain length of only 12 units [cf. Bell, 1935, for fish liver glycogen, and 1937, for horse muscle glycogen: see also Haworth & Percival, 1932, and Haworth *et al.* 1937, who found values of 12 and 18 respectively for commercial samples of rabbit liver glycogen].

EXPERIMENTAL

Material used and isolation of glycogen. Our material consisted of the bodies of about 300 hibernating specimens of *H. pomatia* from which the albumin glands had been removed as a source of galactogen. The material was collected in alcohol, its total live weight, including shells, amounting to approx. 5 kg. After expressing most of the alcohol the material was boiled with 30 % KOH for 5 hr., centrifuged to remove insoluble material, and precipitated with 2 vol. alcohol. The insoluble matter was again treated with hot KOH, centrifuged, precipitated with alcohol, and added to the main product. The combined crude products were dissolved in 50 % acetic acid, the solution was passed twice through the Sharples centrifuge to remove some insoluble matter, and the polysaccharide precipitated by the addition of 1 vol. alcohol. The product was then dissolved in 200 ml. very dilute acetic acid, centrifuged at high speed, and reprecipitated by the addition of 4 vol. glacial acetic acid. The precipitate was washed with 80 % acetic acid on the centrifuge, again dissolved in very dilute acetic acid, and once more precipitated from 80 % acetic acid. After dissolving in water and being precipitated by alcohol the product separated as a slightly brown powder which dissolved freely in water to give a very opalescent solution; yield 27 g. This, which corresponds very roughly to 0.5 % of the live weight of the original material, may be compared with the yields obtained by Bafurth and other workers [see von Fürth, 1903].

Purification and properties of the glycogen. 3 g. of the glycogen were reprecipitated four times from 80 % acetic acid, and finally from alcohol [cf. Bell & Young, 1934]. When thoroughly dried the product was a white powder which dissolved freely in water to give an opalescent solution. This gave a faint brown coloration with excess of iodine [cf. *Mytilus* glycogen, Bell, 1936, 2]: $[\alpha]_D^{20} = +192^\circ$ (water, $c=1$).

Acid hydrolysis. A 3.1% solution in 2.5% HCl was hydrolysed on a boiling water bath and gave the following polarimetric data, calculated in terms of polysaccharide:

t (min.) =	0	15	30	45	60	90	120	180	300
$[\alpha]_D$	+192°	+143°	+106°	+83°	+72°	+62°	+59.5°	+58°	+57°

Expressed in terms of glucose the final $[\alpha]_D$ value is thus +51.7°, or 98% of the theoretical value for an equilibrium mixture of α - and β -glucose. 20 ml. of the hydrolysate were neutralized with Ag_2CO_3 , filtered, treated with H_2S and again filtered after treatment with charcoal, and finally evaporated to dryness. The product weighed 0.70 g. (theory 0.69 g.) and this was dissolved in hot 90% alcohol and allowed to crystallize. Several crystalline fractions were obtained, totalling 0.64 g. (yield 93%): m.p. 142–144° (uncorr.), mixed m.p. with an authentic sample of α -glucose showed no depression: $[\alpha]_D$ = +52.1° at equilibrium (water, $c=2$). No sugar other than glucose was detected.

Acetylation. 22 g. of the crude glycogen were dissolved in 10 parts of water, precipitated by the addition of 3 vol. alcohol and at once collected on a Büchner funnel. After washing rapidly with alcohol and ether the now slightly moist precipitate was transferred to a flask and stirred mechanically with 400 ml. pyridine. Vigorous mechanical stirring was maintained during the addition of 350 ml. acetic anhydride and continued until a clear solution was obtained. After standing overnight at room temperature the solution was passed through a G 3 sintered Jena glass funnel into an excess of cold water. The precipitate was washed with cold water and dried; yield 35 g. (90%). Found: COCH_3 = 44.3%; $[\alpha]_D$ +160°, +161° (chloroform, $c=2$). It may be pointed out that the specific rotation of this acetyl compound is about 8° lower than most of those in the literature.

Methylation. 33 g. of the triacetate were dissolved in 200 ml. acetone, warmed to 50° and treated with 400 ml. 30% NaOH and 130 ml. dimethyl sulphate, these reagents being added in 10 equal portions at 10 min. intervals with violent mechanical stirring. After four such treatments, OMe = 39.0%. After 8 methylations, OMe = 42.4%. After each methylation the reaction mixture was heated till the acetone just began to boil and the aqueous layer then sucked off to leave the supernatant solution of the product in acetone. The acetone layer was then treated again with the methylating reagents. Finally, the product was obtained by boiling off the acetone, heating the whole on a boiling water bath for 40 min. to decompose dimethyl sulphate, and the precipitate was collected on a G 3 sintered Jena glass funnel and washed once with boiling water.

The crude methylated product was dissolved in chloroform, dried with anhydrous Na_2SO_4 , filtered and evaporated to give a thin syrup from which a somewhat brown precipitate was separated by the addition of light petroleum. Further purification was effected by dissolving the material in acetone and adding light petroleum. After several such precipitations a slightly yellow powder was obtained with the following properties: OMe = 44.1%; $[\alpha]_D$ +213° (chloroform, $c=1$): soluble in acetone and chloroform, insoluble in hot water. Yield 16.8 g. (72%). With excess of iodine this substance gave a faint brown coloration.

Hydrolysis of the methylated glycogen. 16.2 g. of the methylated glycogen were boiled for 8 hr. with 500 ml. methyl alcohol containing 1% by weight of dry HCl [cf. Haworth *et al.* 1937]. The hot solution was then neutralized with PbCO_3 , filtered when cold, and evaporated to dryness. The residue was extracted with chloroform and the resulting solution, now free from mineral matter, was

evaporated to dryness to give 17.6 g. of material. This was distilled at a pressure of 0.005 mm. Hg from a flask fitted with a vacuum-jacketed column, and we obtained the fractions described in Table 1.

Table 1

Fraction	Bath temp.	$n_D^{18^\circ}$	$[\alpha]_D$ in water	% OMe	Weight, g.
I	101°	1.4425	+ 21.8	61.3	1.073
II	101–110°	1.4442	+ 69.0	58.0	0.312
III	110–130°	1.4552	+ 33.8	53.7	2.396
IV	130°	1.4581	+ 49.0	51.5	6.089
V	130–140°	1.4586	+ 120.7	51.5	2.966
VI	140–170°	1.4711	—	43.7	3.097
Residue	—	—	—	44.0	1.734

Employing the criteria of purity established by Hirst & Young [1938] for homogeneous fractions of 2:3:4:6-tetramethyl-methylglucosides and 2:3:6-trimethyl-methylglucosides we found that Fraction I consisted entirely of the former and Fractions IV and V of the latter. The distillation residue, a hard glass, consisted of a condensation product of 2:3:6-trimethyl glucose [cf. Haworth *et al.* 1937] and corresponded in amount to 1.85 g. of the methyl glucoside. By a method of approximate estimation based on Hirst & Young's [1938] procedure Fractions II and III were found to consist of

	II	III
Tetramethyl-methylglucoside	97.5 %	13 %
Trimethyl-methylglucoside	2.5 %	87 %

respectively. Fraction VI contains approximately 24.7 % dimethyl-methylglucoside (calculated from methoxyl determinations), the remainder corresponding to trimethyl-methylglucoside. The amounts of the various fractions are tabulated in Table 2, and the total yield represents an approximately 90 % recovery of the methylated glycogen. The chain length corresponds to 11–12 units, a value which differs from that of the only other specimen of molluscan glycogen which has been examined in this way [Bell, 1936, 2].

Table 2

Fraction	g. "Tetra"	g. "Tri"	g. "Di"
I	1.073	—	—
II	0.304	0.008	—
III	0.311	2.085	—
IV	—	6.089	—
V	—	2.966	—
VI	—	2.322	0.775
Residue	—	1.850	—
Totals	1.688	15.320	0.775
Total products		17.783	

Identification of the methylated sugars. Fractions I and II, hydrolysed by *N* HCl in the usual way, yielded 2:3:4:6-tetramethyl glucose, M.P. 88°, unchanged by admixture with an authentic sample.

Fractions III, IV and V, hydrolysed by *N* HCl in the usual way, yielded crystalline 2:3:6-trimethyl glucose, M.P. 102–105°, not depressed by admixture with an authentic sample. The syrupy mother liquors from these fractions were dissolved in methyl alcohol containing 1 % by weight of dry HCl (*c* = 8.5) and showed polarimetric behaviour characteristic of pure 2:3:6-trimethyl glucose; initial $[\alpha]_D + 74^\circ$, falling to a value of -27° in 20 hr. at room temperature.

SUMMARY

1. Glycogen has been isolated from the bodies of specimens of *Helix pomatia* from which the albumin glands had been removed. No indications of the presence of any contaminating polysaccharide were found. This confirms the belief that galactogen is confined to the albumin gland.

2. The glycogen has been acetylated and methylated, and by the method of end-group assay the chain length has been estimated at 11–12 units, compared with the only other known value for molluscan glycogen (18 in the case of *Mytilus edulis* glycogen).

3. The glycogen of *Helix*, its acetate and the corresponding methylated compound, showed no significant differences from similar products obtained from other sources.

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