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# An h.p.l.c. method for determining chain-length distribution in some glycogens

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## ABSTRACT

Human, oyster, *Streptococcus mitis*, and phyto-glycogen samples were debranched using *Pseudomonas amylodermosa* isoamylase (EC 3.2.1.68). The distribution of chain lengths was studied by high-performance liquid chromatography on reversed-phase columns, with water as eluent. Quantitative data was obtained over the degree of polymerisation range three to eighteen (d.p. 3–18), and oligosaccharides up to d.p. 26 were detected. No single column was found suitable for the resolution of the complete range of oligosaccharides, two columns being necessary for the quantitative analysis. The resulting "fingerprints" of chain lengths are characteristic of the glycogen source and should be useful for both comparison purposes among glycogens and for monitoring procedures of glycogen isolation.

## INTRODUCTION

Glycogen is a highly branched polymer consisting of relatively short -(1→4)-α-D-glucan chains joined by -(1→6)-α-branch points[1]. As with starch, the structure is deceptively simple. By the 1930's Haworth and others had established, by methylation analysis, the basic structure[2]. Biochemical interest in glycogen was stimulated by the Coris' discovery of liver phosphorylase[3-5] and the subsequent discovery of phosphorylase in potatoes[1]. A vast amount of effort has been spent on the study of the structure of glycogen and its metabolism since then. Much effort has been directed towards the study of average chain length ($\overline{\text{CL}}$)[1,6]. A number of extraction procedures have been devised. As glycogen is a labile material, its levels *in vivo* are susceptible to the nutritional state of the organism[1,7,8], temperature[9,10], time of day[1], and season[1]. For glycogen extraction, one must, therefore, choose conditions which are reproducible and which do not impose unwanted variations on the normal biological ones[1].

The method described herein allows, in principle, monitoring of the distribution of chain lengths as a function of the conditions. Whether such is feasible in practice is likely to be determined by the feasibility of isolating truly "native" glycogen. Many of the extraction methods used since the early work of Bernard[11] are now known[1] to have caused extensive degradation of the glycogen molecule.

The most common extraction methods for glycogen include the following[1]: (*i*) Alkaline extraction. Minced liver is heated in 30% potassium hydroxide for three h on a boiling water bath, and the glycogen is precipitated by the addition of four volumes of

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ethanol[12]. (*ii*) Methyl sulfoxide ($Me_2SO$) extraction[13]. (*iii*) Water extraction[14]. (*iv*) Trichloroacetic acid (TCA) extraction[15]. Purification of the isolated sample may be carried out by its dissolution in distilled water, removal of any precipitate by centrifugation, and precipitation of glycogen by excess ethanol.

The effect of the four extraction methods listed above on the molecular weight distribution of final product has been assessed by Geddes and Greenwood[16,17]. The hydroxide-extracted glycogen had the narrowest molecular weight distribution profile, but it also contained the material of lowest molecular weight. The TCA and $Me_2SO$-extracted glycogens had similar molecular weight distributions, being polydisperse, with more than one distribution peak. The water-extracted glycogen showed extreme polydispersity, with much high molecular weight material being present. The method described herein has the potential to monitor individual chain lengths of either the two distributions of molecular weight or of degraded samples.

The effect of alkali treatment on the sedimentation coefficients of glycogen isolated by each of the above methods has been studied by Stetten and Katzen[18]. Within experimental error, all glycogens were degraded to similar products, which were closely related to those obtained from the respective extraction of each sample by alkali alone. Useful information on the distribution of individual chain lengths could be gained using the method described herein.

Models of the fine structure of glycogen usually describe the various types of (1→4)-α-D-glucose chains as A, B, or C chains. A chains are found near the surface of the particle and have no substituent chains, while B chains carry substituent A and B chains linked by (1→6)-α-D-glucosidic bonds. A single chain with a reducing group is called the C chain.

The A:B chain ratio is determined by the use of isoamylase (EC 3.2.1.68) and pullulanase (EC 3.2.1.41) on (*i*) β-limit dextrins prepared by the action of β-amylase on glycogen or (*ii*) on glycogen previously subjected to exhaustive treatment with rabbit-muscle phosphorylase[19]. Most glycogens have an A:B chain ratio of close to unity[5]. Two important models are those of Meyer and Bernfeld[20] and Whelan and coworkers[21]. The major differences between these are (*i*) the variation in the lengths of B chains in the former model as compared with the latter, and (*ii*) the single, highly extended C chain in the Meyer–Bernfeld model as opposed to the short chain of the Whelan model, which has only two branch points[19]. Total debranching of glycogen by isoamylase from *Cytophaga*, followed by gel-permeation chromatography, indicated that the chains had a fairly symmetric distribution of chain lengths, *i.e.*, a value that is approximately the average $\overline{CL}$[21]. The method described in this work could lead to the development of a much more detailed account of chain-length distribution in glycogens.

## EXPERIMENTAL

*Materials and Methods.* — Oyster glycogen was obtained from BDH Chemicals, Poole (U.K.). Human, *Streptococcus mitis*, and phyto-glycogen were kindly provided by Dr. G. J. Walker. Isoamylase (EC 3.2.1.68) from *Pseudomonas amyloderm osa* was purchased from the Sigma Chemical Company, St. Louis, MO (U.S.A.) as a suspension in 2.0M ammonium sulfate (1000 U/0.05 mL; 1.9 mg protein/mL). Standard individual malto-oligosaccharides were also gifts from Dr. G. J. Walker. A mixture of malto-oligosaccharides ($\overline{\text{d.p.}}$ 17), obtained from the enzymic hydrolysis of amylose, was a product of Hayashibara Biochemicals, Tokyo (Japan).

The h.p.l.c. system consisted of a Waters Associates M6000 pump, an Erma R7510 refractive index detector (Erma Optical Works, Tokyo), a Rheodyne 7125 injector, and an SIC Chromatocorder 12 integrator (SIC Instrument Co., Tokyo). The columns used were the following: Waters Nova-Pak C18 cartridge, 10 × 0.8 cm; a Waters Dextro-Pak cartridge, 10 × 0.8 cm; a Lichrosorb RP-8 steel column, 25 × 0.4 cm (E. Merck); a TSK-Gel G 5000PW steel gel-permeation column 60 × 0.7 cm (Toyo Soda Co.). The Nova-Pak and Dextro-Pak columns were used in conjunction with a Waters Radial Compression Module RCM100. The eluting solvent was water, which had been passed through a Milli-Q purification system (Millipore Corp., U.S.A.).

*Hydrolysis of branching linkages in glycogens by* Pseudomonas *isoamylase.* — Glycogen (100 mg) was incubated with isoamylase (0.0027 mg; ~ 1.4 μL) in ammonium acetate buffer (0.05M, pH 3.5) in a total volume of 10 mL. A drop of toluene was added to inhibit growth of microorganisms, and the sample was incubated for 50 h at 40°. Aliquots (10 μL) were drawn at intervals of 0.3, 21, and 26 h, heated to boiling for 5 min, and injected on to the TSK-Gel column, which was run at 0.6 mL/min.

*Reduction of the glycogen hydrolysates.* — Glycogen hydrolysate (1 mL) was treated with sodium borohydride (10 mg) for 90 min at 40° to reduce the end groups to alditols. The reaction mixture was treated with acetic acid until effervescence had ceased, and it was then passed through a short column of Dowex-50W [$H^+$] ion-exchange resin to remove excess sodium ions and then freeze-dried. The resulting solid was treated with methanol (3 × 3 mL), which was repeatedly evaporated to dryness under reduced pressure at 40° in a rotary evaporator. Reduced maltotriose, maltohexaose, and maltooctaose were tested for chromatographic purity using the Dextro-Pak column. These were stored *in vacuo* for two days over phosphorus pentoxide and then dissolved in the mobile phase to a concentration of 1% (w/v). Each oligosaccharide sample (20 μL) was injected onto the Dextro-Pak column at a flowrate of 1 mL/min, and the peak areas were calculated by the integrator. Each sample was injected three times, and the mean area was determined. The sample procedure was repeated for the Nova-Pak column.

## RESULTS AND DISCUSSION

The oligosaccharides were reduced with borohydride to their glycosylalditols to avoid the problems of two anomers arising for each d.p. Refractive index response factors for the three standard oligosaccharides were shown to be the same on a w/w basis. Completeness of enzymic hydrolysis was shown to have taken place in 21 h for each sample. There was no residual high molecular weight material, as shown by gel-permeation chromatography on the TSK Gel column, and the reducing power after

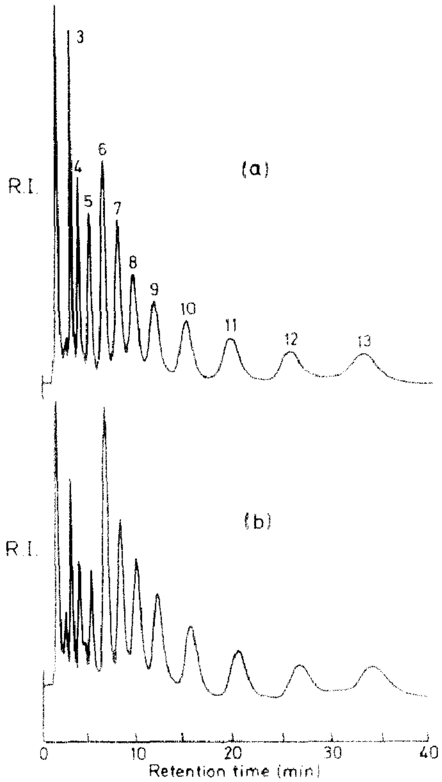

Fig. 1. Elution profile of glycogen chains on a Dextro-Pak column. Solvent: water. Flowrate: 1 mL/min. (a) *S. mitis* glycogen. (b) Oyster glycogen.

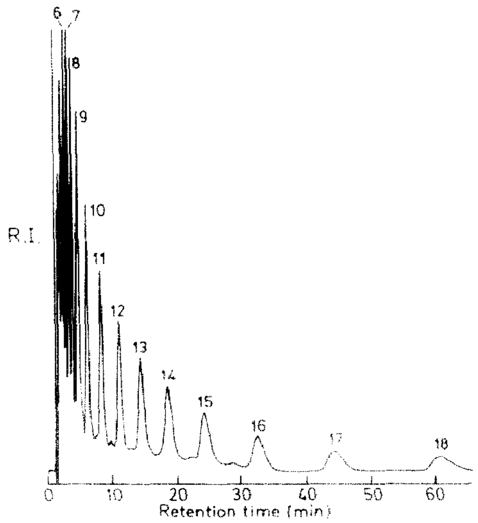

Fig. 2. Elution profile of chains from human glycogen on a Nova-Pak column. Solvent: water. Flowrate: 1 mL/min.

21 h remained essentially constant. Incubation of maltotriose under the same concentration, pH, and temperature showed no detectable change in reducing power or h.p.l.c. profile on the Dextro-Pak column, showing that no reversion or hydrolysis had occurred. Incubation of maltotriose and maltohexaose with the isoamylase also failed to reveal any transglycosylation activity in the enzyme preparation.

Fig. 1 shows the elution profile of the debranched oyster and *S. mitis* glycogen samples on a Dextro-Pak column at a flowrate of 1 mL/min. Resolution and peak-shapes from these chromatograms were deemed to be suitable for quantitative analysis for oligosaccharides up to d.p. 13. Higher oligomers were detected, but their retention times and peak shapes were unsatisfactory for accurate analysis. Fig. 2 shows the elution profile for debranched human glycogen on the Nova-Pak column at a flowrate of 1 mL/min. Resolution and peak shape from d.p. 11–18 are satisfactory for quantitative analysis.

In order to calculate the weight distribution over the d.p. range 3–18, three peaks (d.p. 11, 12, and 13), which were well resolved on both the above columns, were chosen,

TABLE I

The composition of debranched oligosaccharides of glycogens[a]

| *d.p.* | *% Area of each d.p.* | | | |
|---|---|---|---|---|
| | *Oyster* | *Human* | *Phyto* | S. Mitis |
| 3 | 3.64 | 9.29 | 4.63 | 6.95 |
| 4 | 3.43 | 3.56 | 2.08 | 5.51 |
| 5 | 3.06 | 3.90 | 4.43 | 5.82 |
| 6 | 11.20 | 6.14 | 5.45 | 10.10 |
| 7 | 7.20 | 7.40 | 6.57 | 7.99 |
| 8 | 6.21 | 7.02 | 6.77 | 5.89 |
| 9 | 6.28 | 7.24 | 8.01 | 5.84 |
| 10 | 6.35 | 7.45 | 8.53 | 6.42 |
| 11 | 6.43 | 7.92 | 8.76 | 6.72 |
| 12 | 5.46 | 6.94 | 8.12 | 5.55 |
| 13 | 7.00 | 6.65 | 8.60 | 7.64 |
| 14 | 6.91 | 6.79 | 7.98 | 6.27 |
| 15 | 6.59 | 6.33 | 6.47 | 4.92 |
| 16 | 5.63 | 4.90 | 5.41 | 5.03 |
| 17 | 7.02 | 4.24 | 4.43 | 5.44 |
| 18 | 4.10 | 4.23 | 3.76 | 3.91 |
| 19 | 3.49 | ***[b] | ***[b] | ***[b] |
| 20 | ***[b] | *** | *** | *** |
| 21 | *** | *** | *** | *** |
| 22 | *** | *** | *** | *** |
| 23 | ** | ** | *** | *** |
| 24 | ** | ** | *** | *** |
| 25 | * | * | ** | ** |
| 26 | | | | ** |

[a] The relative percentage of each oligosaccharide was the average figures obtained from the integrator. [b] The *, etc. are indicative of the relative sizes of the broader peaks of high d.p. These data are included to show that such peaks are observable on chromatograms.

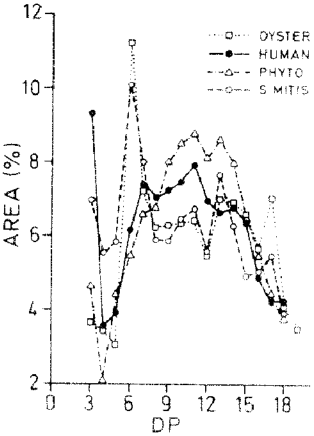

Fig. 3. Weight-distribution of chains for some glycogens. d.p. (degree of polymerisation).

and factors were calculated to allow normalisation of peak areas. Each factor used was the mean of those calculated for d.p. 11, 12, and 13. For example, for oyster glycogen, the mean factor used was 0.82, where

$$\text{factor} = \frac{\text{area of oligosaccharide on Dextro-Pak}}{\text{area of oligosaccharide on Nova-Pak}}$$

In this manner the weight distribution of the oligosaccharides over the range d.p. 3–18 was calculated for each glycogen sample using the SIC Chromatocorder area values. (Table I and Fig. 3). For detection of oligosaccharides in the range 19–26, it was necessary to use the Lichrosorb RP-8 column (Fig. 4). The resolution and peak shape are not satisfactory for quantitative analysis of d.p. 19–26 oligosaccharides, so their relative amounts in Table I are only estimates. The weight distributions in Fig. 3 give a fingerprint reflecting the fine structure of each glycogen. Conversion to relative numbers

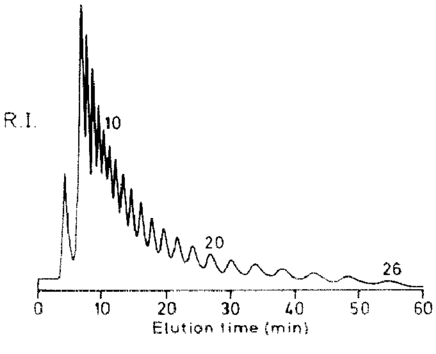

Fig. 4. Elution profile of chains from *S. mitis* glycogen on a Lichrosorb RP-8 steel column. Solvent: Water. Flowrate: 1 mL/min.

of chains may be determined[6] by division of relative areas by d.p. Thus the number of longer chains above d.p. 20 is quite low. The fingerprint of each glycogen is useful for comparison purposes, and the results allow some interesting comparisons, *e.g.*, *S. mitis* and oyster glycogens are similar, and each has a large proportion of maltohexaose. Human, phyto-, and *S. mitis* glycogens all have high levels of maltotriose. The significance of the similarities is uncertain. Before any conclusions can be drawn, one must consider the isolation treatment employed, as this could affect the structure to some extent. (The practise of alkali-extraction could modify in some way the native glycogen structure). In fact the method could be used to monitor extraction and isolation procedures. Other applications include analysis of the isoamylase hydrolysis products of glycogens at various times to follow the release pattern of glycogen chains, and a similar approach to follow the action of isoamylase on starch or glycogen $\beta$-limit dextrins. The fact that oligosaccharides of every chain-length between three and twenty-six D-glucose units appear to be present is interesting and has possible biochemical significance. It is hoped that work that is in progress will allow the analysis of oligosaccharides up to d.p. 30. This will enable fingerprints of the lower-size group of starch amylopectin chains to be produced. The short time required for the analysis of oligosaccharides by the quantitative method described herein, compared to the many hours needed for a non-quantitative gel-permeation run[22], is a distinct advantage.

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