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### Original Citation

Morris, Gordon, Ang, S., Hill, S. E., Lewis, S., Schafer, B., Nobbmann, U. and Harding, S. E. (2008) Molar mass and solution conformation of branched alpha(1 - 4), alpha(1 - 6) Glucans. Part I: Glycogens in water. *Carbohydrate Polymers*, 71 (1). pp. 101-108. ISSN 0144-8617

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# Molar mass and solution conformation of branched $\alpha(1\rightarrow4)$ , $\alpha(1\rightarrow6)$ Glucans. I: Glycogens in water.

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## Abstract

Solution molar masses and conformations of glycogens from different sources (rabbit, oyster, mussel and bovine) were analysed using sedimentation velocity in the analytical ultracentrifuge, size-exclusion chromatography coupled to multi-angle laser light scattering (SEC-MALLS), size-exclusion chromatography coupled to a differential pressure viscometer and dynamic light scattering. Rabbit, oyster and mussel glycogens consisted of one population of high molar mass (weight averages ranging from  $4.6 \times 10^6 - 1.1 \times 10^7$  g/mol) as demonstrated by sedimentation velocity and SEC-MALLS, whereas bovine glycogen had a bimodal distribution of significantly lower molar mass ( $1.0 \times 10^5$  and  $4.5 \times 10^5$  g/mol). The spherical structure of all glycogen molecules was demonstrated in the slopes of the Mark-Houwink-Kuhn-Sakurada-type power law relations for sedimentation coefficient ( $s_{20,w}^0$ ), intrinsic viscosity ( $[\eta]$ ), radius of gyration ( $r_{g,z}$ ) and radius of hydration ( $r_{H,z}$ ) respectively and was further supported by the  $\rho$  ( $= r_{g,z}/r_{H,z}$ ) function, the fractal dimension and the Perrin function. The degree of branching was estimated to be  $\sim 10$  % from the shrinking factors,  $g'$  ( $= [\eta]_{\text{branched}}/[\eta]_{\text{linear}}$ ) and also  $h$  ( $= (ff_o)_{\text{branched}}/(ff_o)_{\text{linear}}$ ) respectively where  $(ff_o)$  is the translational frictional ratio, consistent with expectation.

*Keywords:* glycogen; molar mass; viscosity; sedimentation; friction; shrinking factors; branching.

## Introduction

Glycogen is the primary glucose storage molecule in animal cells and is deposited predominantly in the muscle and liver (Horton, Moran, Ochs, Rawn & Scrimgeour, 2002). It is found in two different forms: *macroglycogen* (molar mass  $M \sim 10^6 - 10^7$  g/mol (Ioan, Aberle & Burchard, 1999; Chebotareva, Andreeva, Makeeva, Livanova & Kurganov, 2004)) and *proglycogen* ( $M \sim 4 \times 10^5$  g/mol (Alonso, Lomako, Lomako & Whelan, 1995)) with the latter constituting approximately 3 wt% and 15 wt% of the total glycogen in liver and muscle cells respectively (Alonso et al., 1995).

The chemical repeat unit of glycogen is  $\alpha(1 \rightarrow 4)$ -linked glucose of which approximately 8 % are branched at the 6-position (Manners, Schutt, Stark & Thanbyrajah, 1971; Burchard, 2001; Hurley, Walls, Bennett, Roach & Wang, 2006). Glycogen or more precisely macroglycogen is structurally very similar to the high molar mass ( $\sim 10^7 - 10^8$ ) plant storage polysaccharide, amylopectin,  $\alpha(1 \rightarrow 4)$ -linked glucose containing approximately 5 % of additional  $\alpha(1 \rightarrow 6)$  branches (Burchard, 2001; Parker & Ring, 2001). Hyper-branched energy storage polysaccharides such as glycogen and amylopectin are expected to adopt compact structures, in order to most efficiently store a large numbers of glucose residues (Ioan et al., 1999). Although previous studies have shown that amylopectin does not fit into this model (Lelievre, Lewis & Marsden, 1986; Tongdang, 2001) at least not under the solution conditions studied (90 % dimethyl sulphoxide/ 10 % water).

As part of an ongoing study into the biophysical characterisation of the mass, conformation and possible interactions of energy storage polysaccharides we have revisited the solution properties of glycogen in aqueous solution demonstrating clear agreement between different biophysical characterisation techniques (sedimentation velocity, multi-angle laser light scattering, size exclusion chromatography coupled to differential pressure viscometer and dynamic light scattering.). Further papers in this series will consider other energy storage polysaccharides (*e.g.* amylopectin) and the effects of environmental conditions (*e.g.* solvent conditions) on their hydrodynamic properties.

## **Materials and Methods**

### **Samples**

Glycogens (Rabbit liver Type III; Bovine liver Type IX; Oyster Type II and Mussel Type VII) were purchased from Sigma (Poole, U.K.) and used without any further purification. Glycogens (80 mg) were dissolved in deionised distilled water (10 ml) with stirring for 30 minutes and the resultant solutions were diluted to the appropriate concentrations required for biophysical characterisations. The absence of any insoluble particulate matter as estimated by differential centrifugal sedimentation (Laidlaw & Steinmetz, 2005) (CPS Disc Centrifuge Model DC18000, CPS Instruments Europe, Oosterhout, The Netherlands) ensured complete (> 99 %) solubility.

### **Sedimentation Velocity in the Analytical Ultracentrifuge**

Sedimentation velocity experiments were performed using a Beckman Instruments (Palo Alto, U.S.A.) Optima XLI Analytical Ultracentrifuge. Glycogen solutions (380  $\mu$ l) of various concentrations (0.5 – 8 mg/ml) and distilled water (400  $\mu$ l) were injected into the solution and reference channels respectively of a double sector 12 mm optical path length cell. Samples were centrifuged at 15000 rpm (rabbit, oyster and mussel) and 50000 rpm (bovine) at a temperature of 20.0 °C. Concentration profiles and the movement of the sedimenting boundary in the analytical ultracentrifuge cell were recorded using the Rayleigh interference optical system and converted to concentration (in units of fringe displacement relative to the meniscus,  $j$ ) versus radial position,  $r$  (Harding, 2005). The data was then analysed using the ls-g(s) model incorporated into the SEDFIT (Version 9.3b) program (Schuck, 1998). This software based on the numerical solutions to the Lamm equation follows the changes in the concentration profiles with radial position and time and generates an apparent distribution of sedimentation coefficients in the form of  $g^*(s)$  versus  $s_{20,w}$ , where the \* indicates that the distribution of sedimentation coefficients has not been corrected for diffusion effects (although a correction procedure is available in the “c(s) model” of SEDFIT, this is not applicable to polydisperse systems such as glycogen).

To account for hydrodynamic non-ideality (co-exclusion and backflow effects), the apparent weight average sedimentation coefficients ( $s_{20,w}$ ) were calculated at each

concentration and extrapolated to infinite dilution using the standard equation (Rowe, 1977; Ralston, 1993; Gralén, 1944).

$$s_{20,w} = s_{20,w}^0(1-k_s c) \quad (1)$$

where  $k_s$  (ml/g) is the sedimentation concentration dependence or “Gralén” coefficient (Gralén, 1944).

### **Size Exclusion Chromatography coupled to Multi-Angle Laser Light Scattering (SEC-MALLS)**

Analytical fractionation was carried out using a series of SEC columns TSK G6000PW, TSK G5000PW and TSK G4000PW protected by a similarly packed guard column (Tosoh Bioscience, Tokyo, Japan) with on-line MALLS (Wyatt, 1992; Harding, Vårum, Stokke & Smidsrød, 1991) (Dawn DSP, Wyatt Technology, Santa Barbara, U.S.A.) and refractive index (Optilab rEX, Wyatt Technology, Santa Barbara, U.S.A.) detectors. The eluent (deionised distilled water at 25 °C) was pumped at 0.65 ml/min (PU-1580, Jasco Corporation, Great Dunmow, U.K.) and the injected volume was 100 µl (1.5 mg/ml) for each sample. Absolute molar masses and radii of gyration were calculated using the ASTRA<sup>®</sup> (Version 5.1.9.1) software (Wyatt Technology, Santa Barbara, U.S.A.).

### **Size Exclusion Chromatography coupled to a Differential Pressure Viscometer**

Analytical fractionation was carried out using a series of SEC columns 2 x ViscoGEL GMPW<sub>XL</sub> (Tosoh Bioscience, Tokyo, Japan) with an on-line Viscotek Tetra Detector TDA 302 (Viscotek Europe, Ltd., Crowthorne, U.K.) (Haney, 1995a,b; Harding, 1997). The eluent (phosphate saline buffer pH 7.4 at 25 °C) was pumped at 0.7 ml/min (Viscotek GPCMax sample/ solvent delivery module, Viscotek Europe, Ltd., Crowthorne, U.K.) and the injected volume was 100 µl (3.0 mg/ml) for each sample. Intrinsic viscosities were calculated using the OmniSEC<sup>™</sup> (Version 4.2) software (Viscotek Europe, Ltd., Crowthorne, U.K.).

## **Dynamic Light Scattering (DLS)**

Dynamic light scattering measurements were made on a fixed scattering angle ( $173^\circ$ ) Zetasizer Nano-S system (Malvern Instruments Ltd., Malvern, U.K.). Samples were measured at  $25.0^\circ\text{C}$ , scattered light was detected at  $173^\circ$  and data collected in automatic mode, typically requiring a measurement duration of 150 seconds. The resulting data was then analysed using the DTS (Version 4.2) software (Malvern Instruments Ltd., Malvern, U.K.) to obtain the z-average radius according to ISO 13321.

## **Results and Discussion**

### **Sedimentation Velocity in the Analytical Ultracentrifuge**

Sedimentation coefficient profiles (Figure 1) for rabbit, oyster and mussel glycogens each show the presence of one continuous population of polysaccharides (there is evidence of very minor amounts of aggregated material at high  $s$  values, which does not have a great effect on the sedimentation coefficient) with weight average sedimentation coefficients of 123 S, 89 S and 83 S (Table 1) after extrapolation to infinite dilution, where the sedimentation coefficient for rabbit glycogen is in good agreement with the previous estimate of 135 S found by Wanson and Drochmans (Wanson & Drochmans, 1968). However, in the case of bovine glycogen (Figure 1) we see the presence of two distinct populations of 14.6 S and 4.9 S (Table 1). This difference with the others may be due to species variation or alternatively may represent intermediate or degraded form of glycogen such as proglycogen (Alonso et al., 1995). As the  $ls-g(s)$  profiles in Figure 1 have not been corrected for diffusion the width of the distribution will be wider than the “true” distribution of sedimentation coefficients after correction for diffusion. This effect would be expected to be largest for the smaller faster diffusing bovine glycogen.

### **Size Exclusion Chromatography coupled to Multi-Angle Laser Light Scattering (SEC-MALLS)**

Weight average molar masses ( $M_w$ ) and z-average radii of gyration ( $r_{g,z}$ ) are shown in Table 1 and are consistent with the results from sedimentation velocity. We can again see (Figure 2) that there are two populations present in bovine glycogen, although. Because of the small size we are unable to estimate the z-average radius of gyration for the lower molar mass population.

The weight average molar mass and the z-average radius of gyration for mussel glycogen ( $4.6 \times 10^6$  g/mol and 13.7 nm) are lower than was previously estimated (Ioan et al., 1999)  $6.2 \times 10^6$  g/mol and 26 nm, although taking into account the intrinsic viscosity of this glycogen ( $[\eta] = 6.67$  ml/g) from the same paper the  $r_{g,z}$  can be estimated to be 14.6 nm based on the following approximation for spheres (Grubisic, Rempp & Benoit, 1996):

$$r_{g,z} = 4.22 \times 10^{-2} (M_w[\eta])^{0.333} \quad (2)$$

Having now calculated the weight average molar masses and z-average radii of gyration for each sample we can now construct “Mark-Houwink-Kuhn-Sakurada”- (MHKS)-type power law plots for both the sedimentation coefficient (Figure 3(a)) and the radius of gyration (Figure 3(b)). The slopes give the power law coefficients of  $b = 0.71$  and  $c = 0.33$  (Table 2) respectively which are in good agreement with the limits for spherical particles (Tombs & Harding, 1998) of 0.67 and 0.33.

### **Size Exclusion Chromatography coupled to a Differential Pressure Viscometer**

The values for intrinsic viscosity (Table 1) are consistent with those found previously (Ioan et al., 1999; Geddes, Harvey & Wills, 1977a) and show no molar mass dependency (Figure 3(c) and Table 2), which is typical for spherical macromolecules in solution (Tombs & Harding, 1998).

### **Dynamic Light Scattering (DLS)**

Dynamic light scattering allows the estimation of the radius of hydration,  $r_H$  of a macromolecule in solution. As with the radius of gyration we can construct a power-law relation (Figure 3(d) and Table 2) which is again consistent with a spherical particle, although the value of  $c = 0.32$  is slightly lower than the predicted value of 0.33. The slight difference may be due to the non-sphericity (or polydispersity) of the glycogen particles: measurement of  $r_H$  at a single fixed scattering angle  $\theta$  without extrapolation to  $\theta = 0$  assumes there are no complications though rotational diffusion effects, an assumption strictly valid only for monodisperse spherical particles (Harding & Johnson, 1985a,b; Pusey, 1974).



### **Tsvetkov, Eskin and Frenkel Relations**

The values MHKS-type power law exponents (Table 2) are all consistent with the solution properties of spherical macromolecules. The validity of these parameters can be further explored by the calculation of their corresponding Tsvetkov, Eskin and Frenkel (TEF) relations (Table 2).

$$a = 2 - 3b \quad (3)$$

$$b = 1 - c \quad (4)$$

$$c = (a + 1)/3 \quad (5)$$

This demonstrates clear consistency between each set of results and also reinforces the spherical model for glycogen.

We can also use the average value of  $c$  ( $0.32 \pm 0.03$ ) to estimate the fractal dimension ( $d_f = 1/c = 3.1 \pm 0.3$ ) which is close to the theoretical value for a hard sphere (Ioan et al., 1999) of 3.0 (see Table 3).

### **The $\rho$ Parameter**

A further estimate of molecular conformation can be obtained from the ratio  $\rho = r_{g,z}/r_H$  which has a theoretical limit of 0.78 for hard spheres (Burchard, 1992; Freire & Garcìa de la Torre, 1992) and has been previously estimated to be 0.77 for glycogen (Reiner, 1981). Our value of  $0.7 \pm 0.1$  (Table 3) is again in good agreement and there is no molar mass dependency, which is indicative of self-similar structures (Burchard, Schmitt & Stockmayer, 1980).

Having now established with some confidence shown that all studied glycogens adopt a spherical structure in solution - typical of hyper-branched polysaccharides (Galinsky & Burchard, 1995) - we can now take the analysis further with the estimation of the (time averaged) degree of hydration,  $\delta$  (g of solvent per g of polysaccharide) and make an estimate of the branching ratio (number of glucose residues per branching point).

### Hydration (time average) and the Perrin Function

Using the Einstein equation (Einstein, 1906, 1911, Harding, 1997) we can estimate the hydration of a spherical macromolecule in solution using the following relation (Tanford, 1961).

$$[\eta] = 2.5 (\bar{v} + \delta) \quad (6)$$

where  $\bar{v} = 0.63$  ml/g for glycogen (Geddes et al., 1977a; Geddes, Harvey & Wills, 1977b).

Using the intrinsic viscosity data (Table 1) we find  $\delta \sim (2.4 \pm 0.4)$  g of solvent/ g of polysaccharide (averaged over time). This value can then be combined with the translational frictional ratio,  $f/f_0$  (equations 7a and 7b) to estimate the Perrin (frictional ratio due to shape) parameter, P (equation 8).

$$\frac{f}{f_0} = \frac{M_w (1 - \bar{v} \rho_0)}{(N_A 6\pi\eta_0 s_{20,w}^0) \left( \frac{4\pi N_A}{3\bar{v} M_w} \right)^{-1/3}} \quad (7a)$$

$$\frac{f}{f_0} = \left( \frac{4\pi N_A}{3\bar{v} M_w} \right)^{1/3} r_H \quad (7b)$$

where  $N_A$  is Avogadro's number and  $\rho_0$  and  $\eta_0$  are respectively the density and viscosity of water at 20.0 °C.  $f$  is the friction coefficient of the molecule and  $f_0$  the corresponding value for a spherical particle of the same mass and (anhydrous) volume (Tanford, 1961).

$$P = \left( \frac{f}{f_0} \right) \left[ \frac{\bar{v}}{(\bar{v} + \delta)} \right]^{1/3} \quad (8)$$

Using the results in Table 1 we find a translational frictional ratio,  $f/f_0 = 2.0 \pm 0.2$  (Table 3), and insertion of a value for  $\delta = 2.4 \text{ g/g}$  into eq. 8 yields  $P = 1.2 \pm 0.2$ , consistent with a spherical conformation.

### Degree of Branching

The degree of branching of a hyper-branched macromolecule can be estimated from the shrinking factors (Ioan et al., 1999, Freire & Garcia de la Torre, 1992; Burchard et al., 1980; Zimm & Stockmayer, 1949; Galinsky & Burchard, 1995; Ioan, Aberle & Burchard, 2001) (Figure 4)  $g$ ,  $g'$  and  $h$ .

$$g = \frac{r_{g,z \text{ branched}}^2}{r_{g,z \text{ linear}}^2} \quad (9a)$$

$$g' = \frac{[\eta]_{\text{branched}}}{[\eta]_{\text{linear}}} \quad (9b)$$

$$h = \frac{f_{\text{branched}}}{f_{\text{linear}}} = \frac{(f/f_0)_{\text{branched}}}{(f/f_0)_{\text{linear}}} \quad (9c)$$

For our evaluations we take the radii of gyration, intrinsic viscosities and frictional ratios of a linear polymer of the same molar mass as that of pullulan, with appropriate interpolations (Kato, Tsunehisa & Takahashi, 1984; Kawahara, Ohta, Miyamoto & Nakamura, 1984; Nishinari, Kohyama, Williams, Phillips, Burchard & Ogino, 1991), and the values for  $h$  were calculated using the average frictional ratio from both sedimentation (equation 7a) and hydrodynamic radius (equation 7b).

According to Ioan and co-workers (Ioan et al., 1999) the molar mass dependency of  $g$ ,  $g'$  and  $h$  allow an estimation of the number of branch points per molecule,  $n$  and  $M_{BU}$  ( $= M_w/n$ ) the molar mass of the branching unit (equations 10a, 10b and 10c),  $M_{BU}$  from which we calculate the number of glucose residues per branch,  $M_{BU}/162$ .

$$g = \left[ \left(1 + \frac{n}{7}\right)^{0.5} + \frac{4n}{9\pi} \right]^b \quad (10a)$$

$$g' = \left[ \left( 1 + \frac{n}{7} \right)^{0.5} + \frac{4n}{9\pi} \right]^{b'} \quad (10b)$$

$$h = \left[ \left( 1 + \frac{n}{7} \right)^{0.5} + \frac{4n}{9\pi} \right]^{b''} \quad (10c)$$

where  $b = -0.55$ ,  $b' = -0.73$  and  $b'' = -0.17$  are the slopes of the molar mass dependencies of  $g$ ,  $g'$  and  $h$  respectively (Figure 4).

The fit of the experimental data for  $g$  with equation 10a suggests that 1 in every 2.5 glucose residues in the glycogen chain are branched, which is clearly different to the value of about 1 in 12 (Manners et al., 1971; Hurley et al., 2006), however the fit of the experimental data for  $g'$  and  $h$  with equations 10b and 10c results in the branching ratio of 1:12 and 1:11 respectively. We can therefore clearly confirm that glycogen is hyper-branched macromolecule with a high degree of branching. It is our opinion that the branching ratios of 1:12 and 1:11 calculated from  $g'$  and  $h$  are more valid estimates as the slopes of molar mass dependencies  $-0.73$  and  $-0.17$  are very close to the theoretical values of  $-0.75$  and  $-0.17$  respectively, whereas in the corresponding plot for  $g$  the slope ( $-0.55$ ) is further from the theoretical value (Zimm & Stockmayer, 1949) of  $-0.50$ . It must also however be noted that values for  $g$ ,  $g'$  and  $h$  are very sensitive to the choice of model for the linear polymer of the same molar mass and this may go some way to explaining the different estimations for the number of branches. It is also expected that these estimates are influenced by polydispersity (Ioan et al., 1999; Freire & García de la Torre, 1992).

### **Concluding remarks**

We have shown from four different types of measurement (light scattering, viscosity, sedimentation and translational diffusion) that the hyper-branched compact sphere model in aqueous solution for glycogens extracted from at least four different sources (rabbit, oyster, mussel and bovine) is valid, and that three of them have a similar unimodal type of molar mass distribution, the other (bovine) being bimodal and an order of magnitude smaller.

All four types of measurement give consistent results throughout for the size and size distribution (molar mass from light scattering, sedimentation coefficient from analytical ultracentrifugation) and conformation (light scattering, sedimentation, translational diffusion and intrinsic viscosity). And, despite the small number of data points (4 – i.e. the 4 different glycogens) it was possible to make an estimate for the branching ratio of ~10% (i.e. one in 10 residues having an  $\alpha(1\rightarrow6)$  branch point). It would be interesting to see how this picture for glycogens compares with other glucan storage polysaccharides, the subject of further papers in this series.

### **Acknowledgement**

We would like to thank Prof. Arthur Rowe of the NCMH Business Centre for his helpful comments and technical expertise and GAM would like to thank the BBBRC for their financial support.

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**Table 1.** Solution properties of glycogens from different sources in water

<b>Glycogen Type</b>	<b><math>M_w</math> (g/mol) <math>\times 10^{-6}</math></b>	<b><math>s_{20,w}^0</math> (S)</b>	<b><math>[\eta]</math> (ml/g)*</b>	<b><math>r_{H,z}</math> (nm)</b>	<b><math>r_{g,z}</math> (nm)</b>
<b>Rabbit</b>	$11.0 \pm 0.1$	$123 \pm 4$	$6.8 \pm 0.2$	$31 \pm 1$	$20 \pm 2$
<b>Oyster</b>	$5.90 \pm 0.06$	$89 \pm 2$	$6.5 \pm 0.7$	$22 \pm 1$	$15 \pm 2$
<b>Mussel</b>	$4.60 \pm 0.04$	$83 \pm 1$	$8.3 \pm 0.5$	$18 \pm 1$	$14 \pm 2$
<b>Bovine (fast)</b>	$0.45 \pm 0.01$	$14.6 \pm 0.6$	$8.5 \pm 0.4$	$10 \pm 1$	$7 \pm 5$
<b>Bovine (slow)</b>	$0.10 \pm 0.03$	$4.9 \pm 0.3$	-	-	-

\* measured in pH 7.4 phosphate saline buffer

**Table 2.** Experimental Mark-Houwink-Kuhn-Sakurada-type power relation values for glycogen and their corresponding Tsvetkov, Eskin and Frenkel relations and the theoretical values for spherical macromolecules

<b>Power Law Parameter</b>	<b>Value</b>	<b>Theoretical</b>
a	$-0.07 \pm 0.05$	0.00
2-3b	$-0.13 \pm 0.06$	
b	$0.71 \pm 0.02$	0.67
1-c	$0.67 \pm 0.03$	
c ( $r_{g,z}$ )	$0.33 \pm 0.03$	0.33
(a+1)/3	$0.31 \pm 0.02$	
c ( $r_{H,z}$ )	$0.32 \pm 0.05$	

**Table 3.** Experimental shape parameters for glycogen and their corresponding theoretical values for spherical macromolecules

<b>Shape Parameter</b>	<b>Value</b>	<b>Theoretical</b>
$d_f$	$3.1 \pm 0.3$	3.0
$\rho$	$0.7 \pm 0.1$	0.78
$f/f_0$	$2.0 \pm 0.2$	1.7*
P	$1.2 \pm 0.2$	1.0

\* $\delta = 2.4$  g of solvent per g of polysaccharide

## Legends to Figures

**Figure 1.** Normalised sedimentation coefficient distributions for rabbit (■), oyster (○), mussel (7) and bovine (◇) glycogens at 2 mg/ml. Figure normalised ( $ls-g(s)_{\max} = 1$ ) for clarity.

**Figure 2.** Normalised molar mass distributions for rabbit (■), oyster (○), mussel (7) and bovine (◇) glycogens at 2 mg/ml. Figure normalised ( $W_i M_{i(\max)} = 1$ ) for clarity.

**Figure 3.** Mark-Houwink-Kuhn-Sakurada-type power law plots for glycogens from different sources:

- (a) Molar mass dependency of the weight average sedimentation coefficient,  $s_{20,w}^0$  (slope = 0.71)
- (b) Molar mass dependency of the radius of gyration,  $r_{g,z}$  (slope = 0.33)
- (c) Molar mass dependency of the intrinsic viscosity,  $[\eta]$  (slope = -0.07)
- (d) Molar mass dependency of the radius of hydration,  $r_{H,z}$  (slope = 0.32).

**Figure 4.** Molar mass dependency of shrinking factors  $g$  (■)  $g'$  (●) and  $h$  (7) for glycogens from different sources. The slopes for  $g$ ,  $g'$  and  $h$  are -0.55, -0.73 and -0.17 respectively.

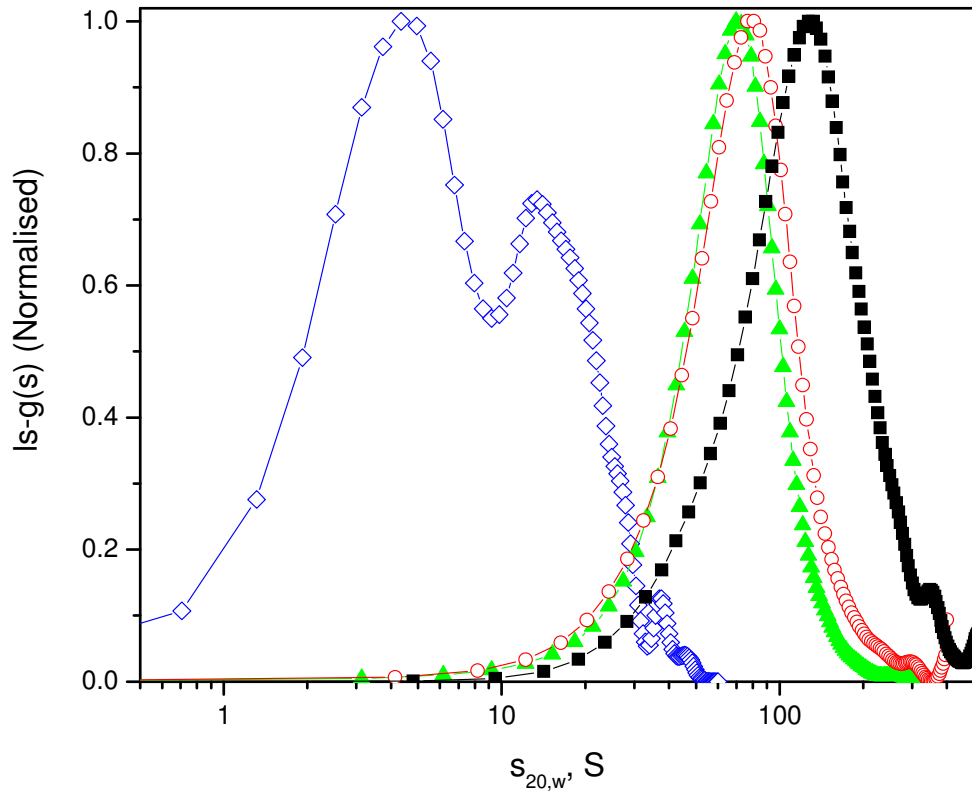


Figure 1.

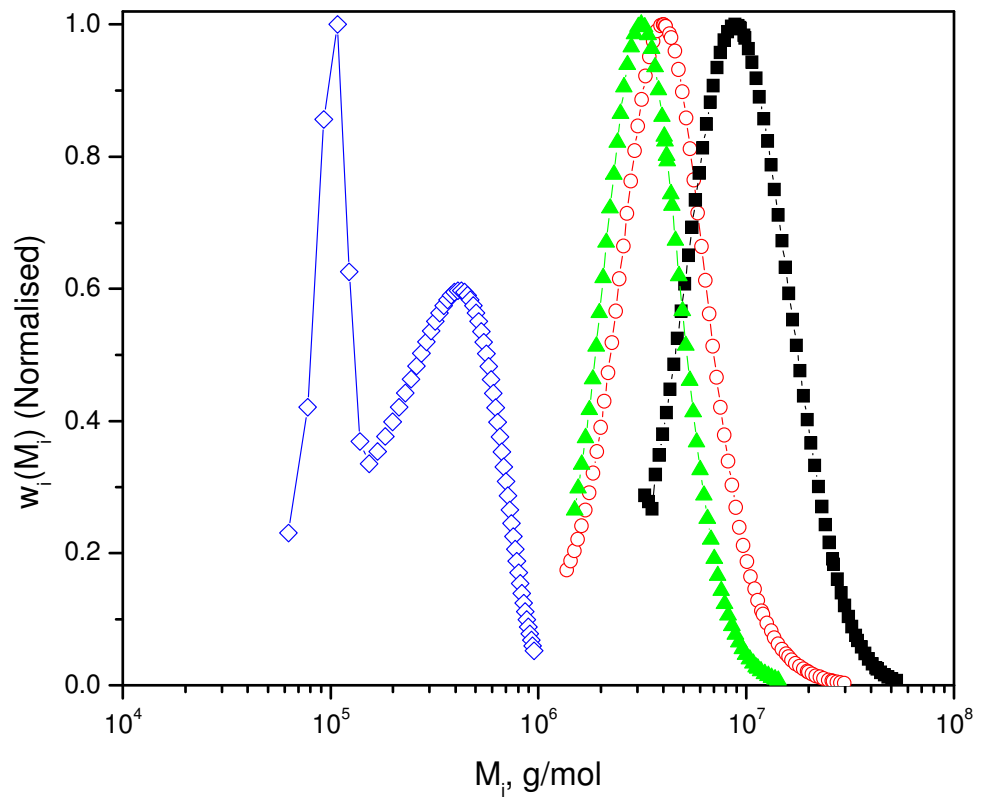
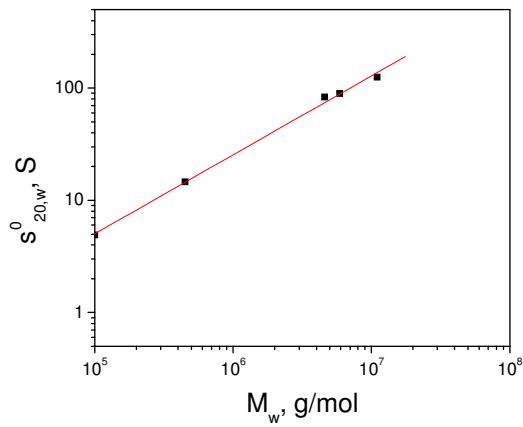
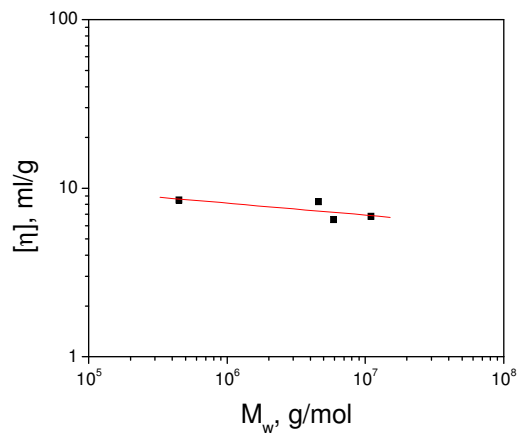


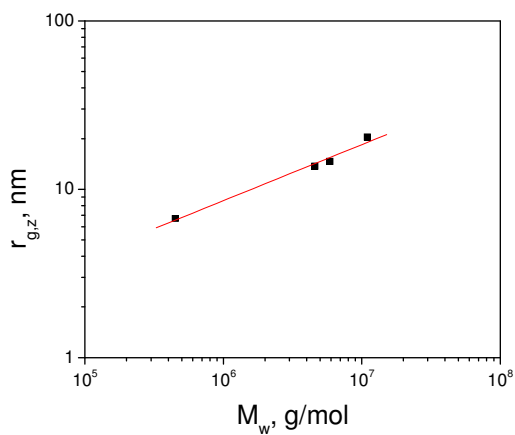
Figure 2.



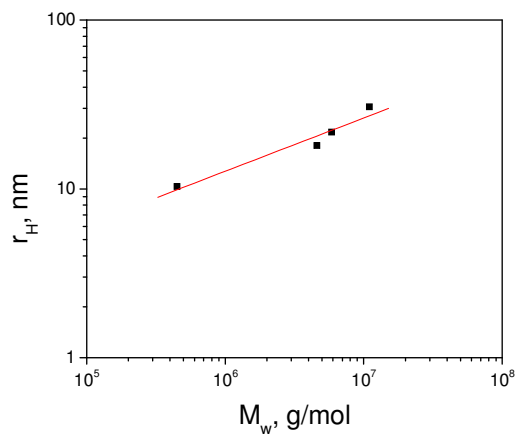
**Figure 3(a)**



**Figure 3(c)**

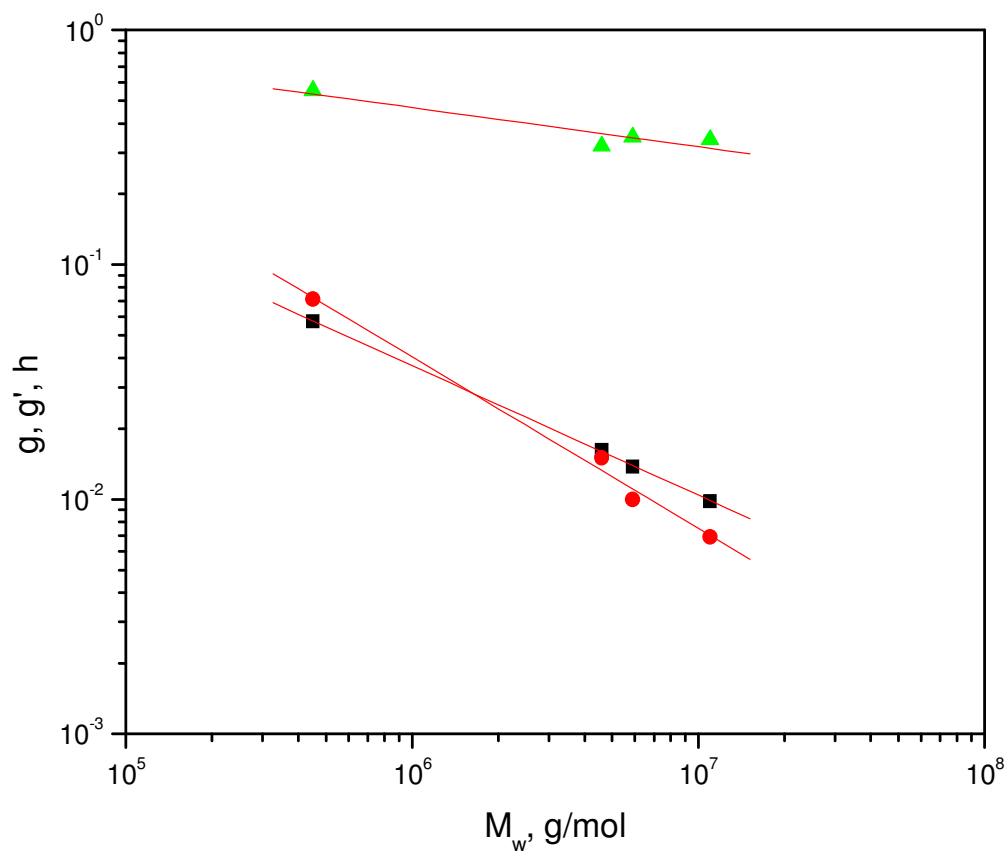


**Figure 3(b)**



**Figure 3(d)**





**Figure 4**