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CHROMATOGRAPHY OF GLYCOGENS ON AGAROSE GELS

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SUMMARY

The chromatographic behavior of several glycogens on the agarose gels Sepharose 2B and Sepharose 4B has been investigated. These are shown to be convenient media for examination of the polydispersity and relative molecular weights of different glycogens. Sepharose 2B is useful for the separation of artificial mixtures of glycogens and the components of glycogens occurring naturally as mixtures of distinct populations of molecules.

INTRODUCTION

Gel chromatography on dextran (Sephadex) and polyacrylamide (Bio-Gel) gels has been widely used for the separation of proteins, nucleic acids and certain carbohydrates on the basis of molecular weight[1]. However, the extremely high molecular weights of polysaccharides, such as glycogens, rendered them non-susceptible to fractionation by this technique since they are excluded from all grades of Bio-Gel and Sephadex. The recent development and commercial availability of agarose gels of low degree of cross-linking has resulted in successful fractionation of other high-molecular-weight polysaccharides, *e.g.* plant gums[2], bacterial lipopolysaccharides[3] and certain mucopolysaccharides[4,5]. In this publication the use of 2% and 4% agarose gels (Sepharose 2B and 4B) for column chromatography of a number of glycogen samples from various sources is described.

EXPERIMENTAL

Materials

Rabbit-liver glycogen was extracted from the livers of both well-fed and two-days starved animals using the mercuric chloride procedure of MORDOH *et al.*[6]. After purification by three reprecipitations, followed by dialysis and treatment with mixed-bed ion-exchange resin (Bio-Rad Laboratories) to remove residual mercuric chloride, the polysaccharides were isolated by freeze-drying. Normal human-liver glycogen was the sample examined by MERCIER AND WHELAN[7]. Human-muscle glycogen, prepared by the KOH procedure[8], was supplied by Prof. D. J. MANNERS. Glycogen from the muscle of Type II glycogen storage disease patients

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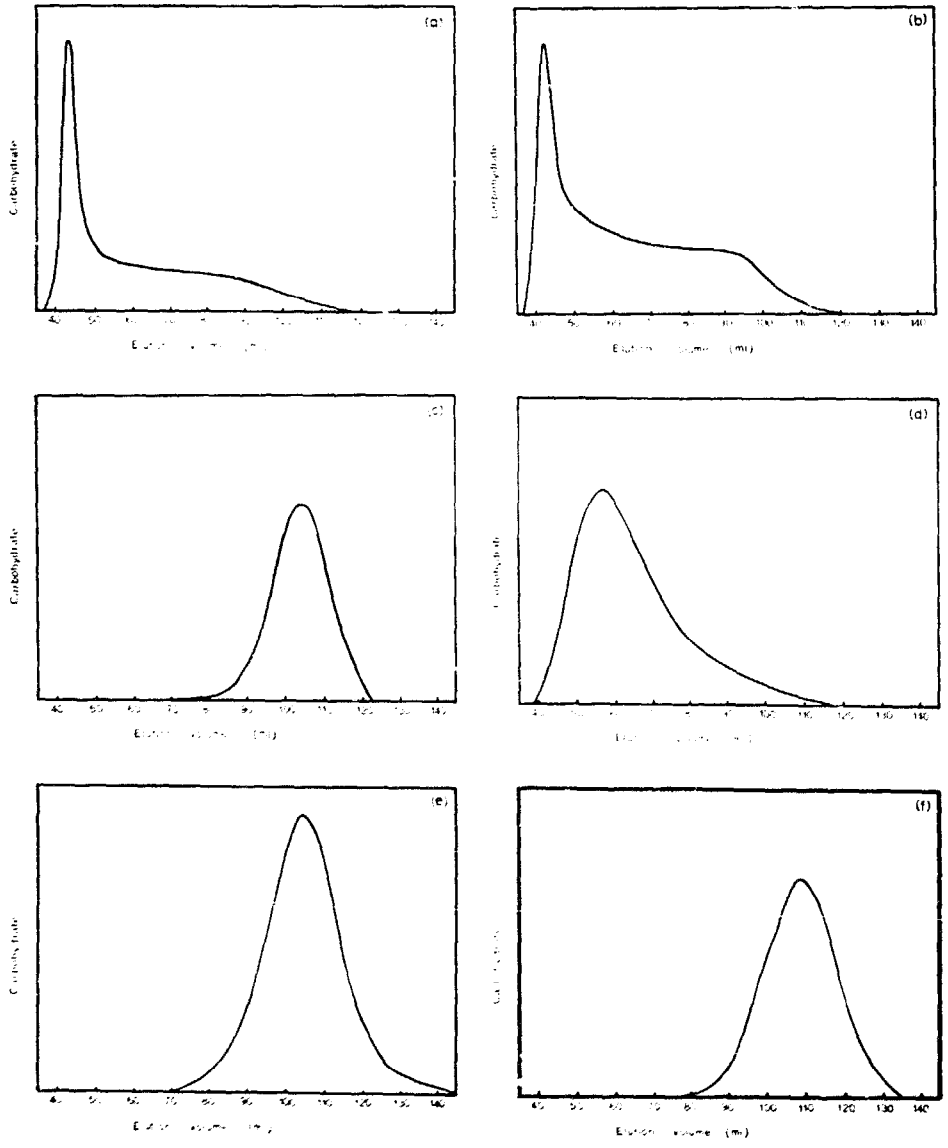
was prepared by cold-water extraction of the tissue and kindly provided by Prof. BRENDA E. RYMAN. *Ascaris* and tape-worm glycogens were donated by Dr. S. A. ORRELL. Shellfish glycogen was purchased from Mann Research Laboratories and was three times reprecipitated before use. Phytoglycogen A was prepared from immature sweet corn as by PEAT *et al.*[9]. Unfractionated sweet-corn glycogen was prepared by homogenization of sweet corn kernels in mercuric chloride solution as in the initial stage of phytoglycogen A preparation[9]. After centrifugation to remove grain residues the solution was dialyzed, then treated with mixed-bed ion-exchange resin. No attempt was made to isolate the polysaccharide from the solution, this being subjected directly to chromatography.



Sepharose 2B (Lot 50-C-0630), Sepharose 4B (Lot 30-C-1560-1) and Blue Dextran 2000 were products of Pharmacia Fine Chemicals. Glucoamylase from *Aspergillus niger* was prepared by QURESHI[10], using the method of FLEMING AND STONE[11]. Glucose oxidase (Grade II) and horseradish peroxidase were obtained from Boehringer Mannheim GmbH.

Molecular-sieve chromatography

Chromatography was performed in columns of dimensions 90 × 1.5 cm with gel beds approximately 87.5 cm high. Elution was with 1 % sodium chloride solution under a head of 12–15 cm of eluent or with an LKB Varioperpex peristaltic pump, the flow-rate being approximately 5 ml/h. Under the conditions used no variation in the bed volume took place during the period of several weeks continuous use. Samples containing polysaccharide (approximately 10 mg) dissolved in eluent (1 ml) were applied to the top of the gel bed and fractions of constant volume (2.5–3.5 ml) collected automatically. Concentrations of glycogen in column fractions were determined enzymically by quantitative conversion into glucose using *Aspergillus niger* glucoamylase and α-amylase[12], followed by measurement of glucose

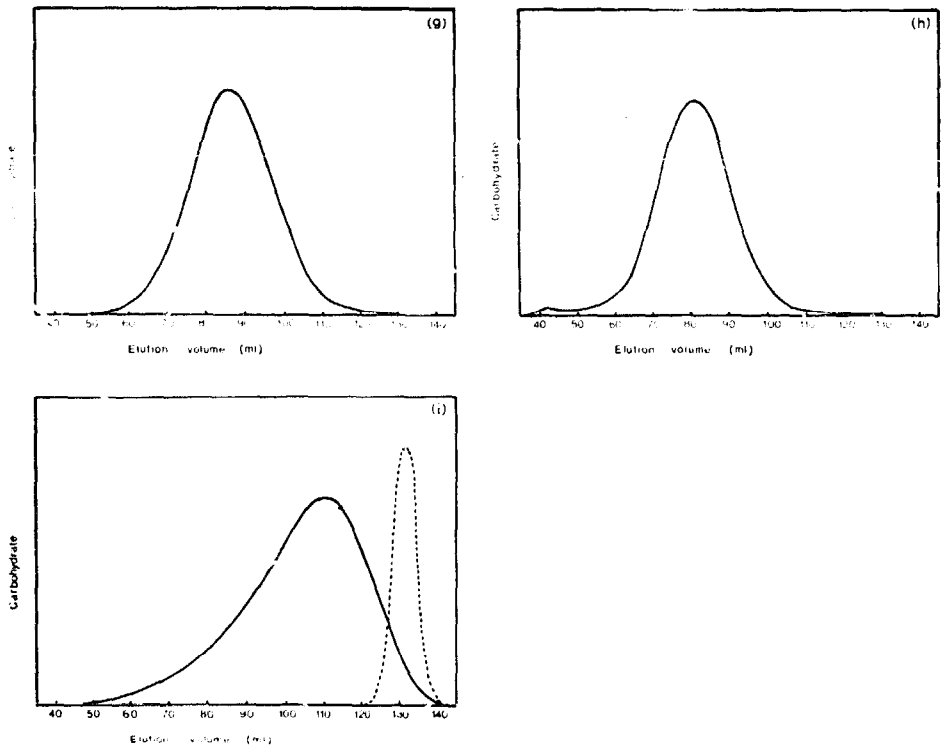


Fig. 1. Distribution of carbohydrate in the effluent from a column of Sepharose 2B after chromatography of: (a) fed rabbit-liver glycogen; (b) starved rabbit-liver glycogen; (c) shellfish glycogen; (d) human-liver glycogen; (e) human-muscle glycogen; (f) Type II glycogenosis muscle glycogen; (g) phytoglycogen A; (h) sweet-corn juice; (i) Blue Dextran + glucose. For experimental details see the text. For convenience, carbohydrate concentrations have been shown in purely relative terms.

with glucose oxidase as by LLOYD AND WHELAN[13]. Blue Dextran was detected by measurement of the absorption of fractions at 600 nm.

Ultracentrifugation

Analytical ultracentrifugation was carried out in a Spinco Model E ultracentrifuge at speeds of the order of 10,000 r.p.m.

RESULTS

Figs. 1a–h show the results obtained on chromatography of individual glycogen samples on Sepharose 2B. Fig. 1i shows the distribution of Blue Dextran from the same column and the elution of glucose (total column volume). The results of chromatography of shellfish glycogen, phytoglycogen, Blue Dextran and glucose on Sepharose 4B are given in Figs. 2a–c. Fig. 3 illustrates the results

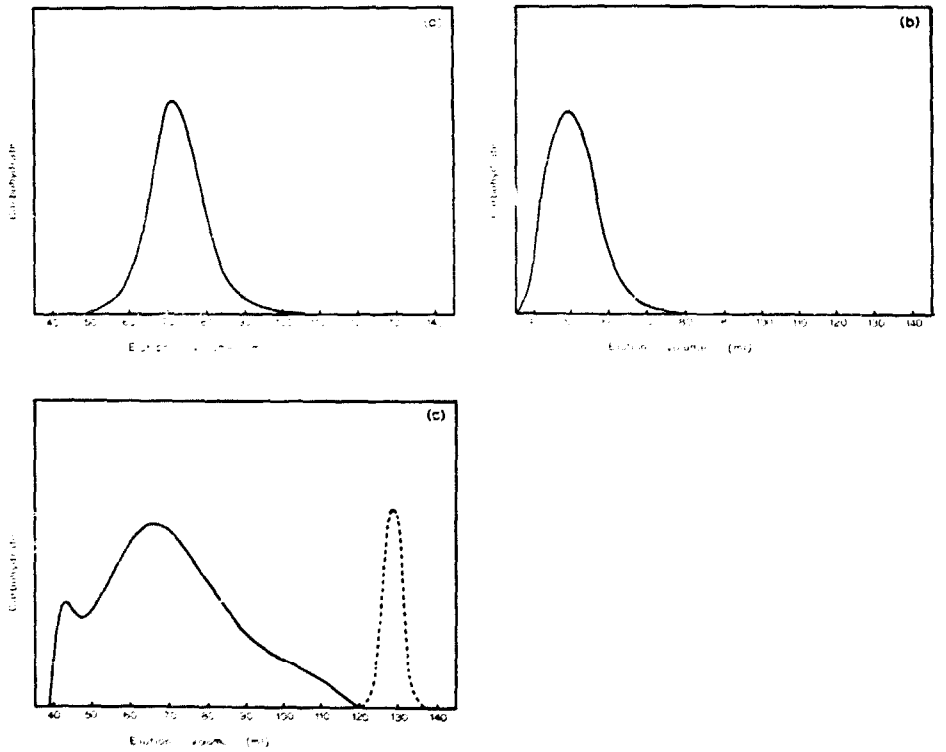


Fig. 2. Distribution of carbohydrate in the effluent from a column of Sepharose 4B after chromatography of: (a) shellfish glycogen; (b) phytoglycogen A; (c) Blue Dextran + glucose.

of successful fractionation experiments using Sepharose 2B; Fig. 3a shows the separation of a mixture of approximately equal amounts of (fed) rabbit-liver glycogen and shellfish glycogen; Figs. 3b and 3c show the separation of the high- and low-molecular-weight components of *Ascaris* and tape-worm glycogens on the same

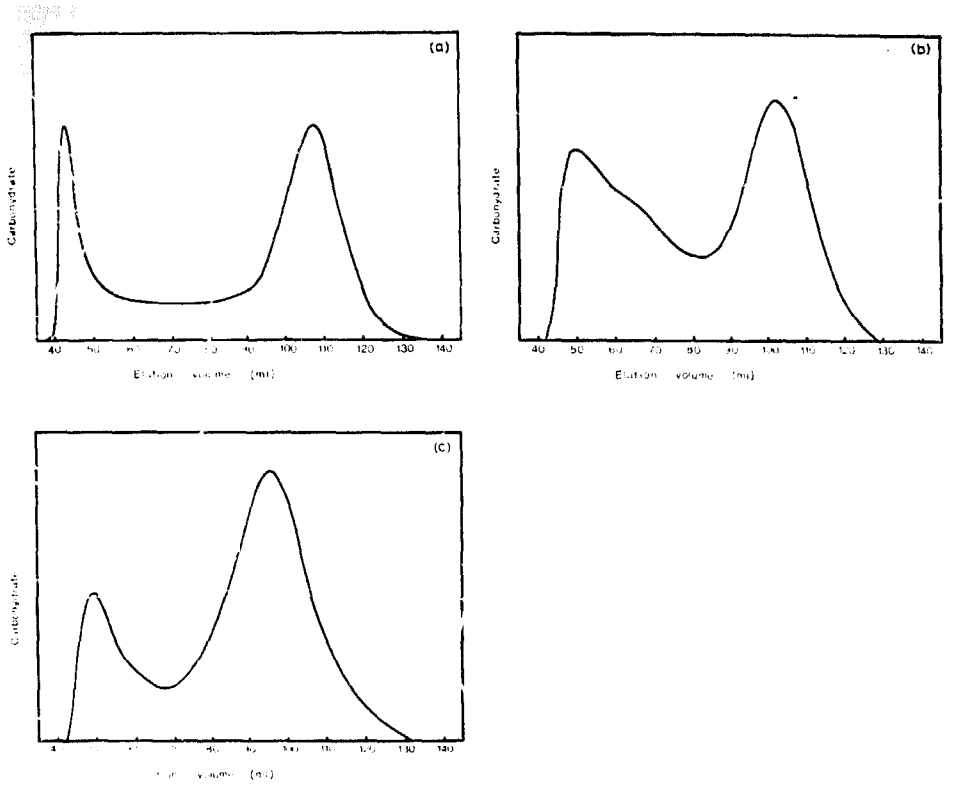


Fig. 3. Separation of glycogen-components using Sepharose 2B: (a) mixture of fed rabbit-liver glycogen and shellfish glycogen; (b) *Ascaris* glycogen; (c) tape-worm glycogen

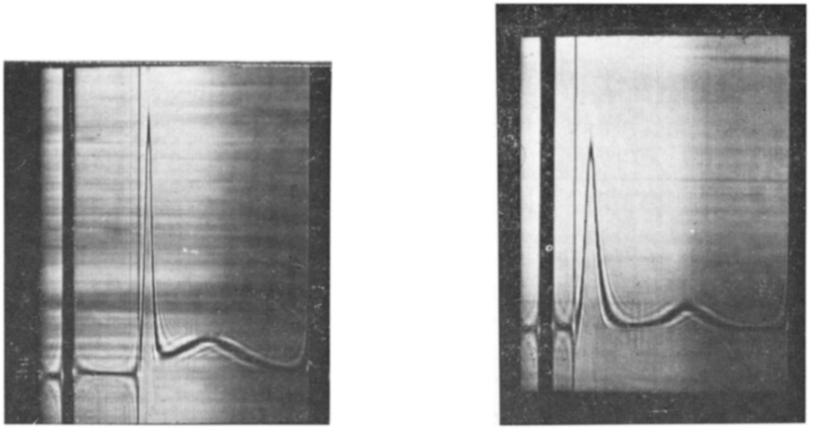


Fig. 4. Sedimentation of: (a) *Ascaris*; and (b) tape-worm glycogens in the analytical ultracentrifuge. The photographs were taken 12 min after reaching maximum speed (9,341 r.p.m.). Sedimentation is from left to right. (Courtesy of Dr. S. A. ORRELL.)

column. The sedimentation behavior of *Ascaris* and tape-worm glycogens in the analytical ultracentrifuge are shown in Figs. 4a and 4b

DISCUSSION

Chromatography of mercuric chloride extracted (fed) rabbit-liver glycogen on Sepharose 2B (Fig. 1a) shows a large initial peak eluted at a volume considered to be the void volume of the column. This indicates that a large proportion of the population of molecules in this glycogen sample is of molecular weight exceeding the exclusion limit of the gel. In addition, however, a significant amount of material penetrating the gel is present, indicating a high degree of polydispersity, the significance of which is not presently understood. It is of interest to find that chromatography of liver glycogen from a starved rabbit (Fig. 1b) shows a closely similar carbohydrate distribution pattern on chromatography under the same conditions. Thus, although starvation results in a considerable decrease in liver glycogen content, it is clear that there is little change in the molecular weight of the molecules remaining. This is in direct contrast with the results of DE WULF *et al.*[14], who have suggested that the number of glycogen molecules remains constant (*i.e.* the molecular weight decreases) during depletion of liver glycogen by starvation. Further evidence, based on analysis by enzymic methods, to support the contention that these glycogens are closely similar, will be presented elsewhere.

Hot-water-extracted human-liver glycogen is seen (Fig. 1d) to be of considerably lower molecular weight and is much less polydisperse than mercuric-chloride-extracted rabbit-liver glycogen. It is not yet known whether this difference can be related to the different methods of purification (see *Materials*), although we would see the technique presently described as being an ideal method for comparing the effect of different methods of glycogen preparation on the molecular weight of the product obtained. Human-muscle glycogen (Fig. 1e) is seen to be of low molecular weight and to be one of the least polydisperse of all the glycogens examined. This may, however, simply reflect a rather drastic degradation during the KOH extraction procedure[8] used in its preparation[31]. The low molecular weight and low degree of polydispersity of Type II glycogenosis muscle glycogen (Fig. 1f) is in agreement with the results of other workers[15,16]. The distribution of shellfish glycogen in the effluent from the column (Fig. 1c), where it is eluted at a volume approaching the total volume of the column (indicated by the glucose peak in Fig. 1i), shows it to be of low molecular weight, although again the possibility that this is the result of degradation[31] during the preparation of this (commercial) sample must be borne in mind.

Phytoglycogen A was eluted as a single major peak (Fig. 1g). However, in view of reports[18,19] suggesting the presence of two water-soluble polysaccharides in sweet-corn, a solution of the unfractionated water-soluble polysaccharides from this cereal was chromatographed. Again a single component appeared to be present (Fig. 1h), this having a molecular weight almost identical to that of phytoglycogen A. Thus it is considered that earlier suggestions[18,19] of two water-soluble polysaccharides in this *Zea mays* mutant are incorrect, and that the separation into phytoglycogens A and B is of little significance, in agreement with previous conclusions[9]. The alternative explanation, namely that there are two water-soluble polysaccharides present with identical molecular weights, seems unlikely.

It will be clear from our results that Sepharose 2B is an ideal medium for chromatography of glycogens, allowing comparison of relative molecular weights

and polydispersity. Sepharose 4B may also be of use for this purpose, although its use will be restricted to the lower-molecular-weight glycogens. Even then, it does not appear to offer any major advantages over Sepharose 2B. Thus, chromatography of phytoglycogen A and shellfish glycogen (Figs. 2a and 2b) still does not reveal any inhomogeneity in these glycogens. In the latter case particularly this is of some significance. One of the reasons for the present work was to discover whether the macrodextrins left after treatment of this glycogen with α-amylase[20–23] do arise from shellfish glycogen molecules per se, or whether they exist as a separate population of molecules whose presence only becomes detectable after α-amylase action. Our results would, however, appear to be inconsistent with this latter idea and we must therefore accept that macrodextrins do arise from highly branched regions of the shellfish glycogen molecules as suggested by SCHRAMM and co-workers[20–23].

From Figs. 1a and 1c it is clear that, in the absence of any intermolecular interactions, a mixture of rabbit-liver and shellfish glycogens should be largely separated by chromatography on Sepharose 2B. This was found to be the case when these glycogens were co-chromatographed (Fig. 3a), the resultant carbohydrate elution profile being what would be expected on the basis of Figs. 1a and 1c. In the past the standard technique for examining the heterogeneity of glycogens has been analytical ultracentrifugation[17]. BUEDING and his co-workers have in this way examined a large number of glycogen samples prepared in different ways and from different sources[15,16,24,25]. In some cases clear indication of populations of molecules of two different sizes has been obtained (Figs. 4a and 4b) and these have been partly separated by zonal ultracentrifugation[24]. Chromatography of such samples on Sepharose 2B also results in separation of the two components (Figs. 3b and 3c). It is anticipated that, in view of its facility, the present technique will be useful for fractionation of multicomponent glycogens on a preparative scale, thus allowing a detailed comparison of the constituent fractions.

It is not possible at the present time to use the method described for the determination of absolute molecular weights, in view of the absence of suitable standards for calibration purposes. This possibility does, however, exist. The distribution of Blue Dextran 2000, a colored polysaccharide with a reported average molecular weight of $2 \cdot 10^6$, is shown on columns of both types but it is likely that the elution volume of this near-linear polysaccharide will correspond to that of a branched polysaccharide of much higher molecular weight, in view of the much more compact nature of the glycogen molecule. Some idea of the mean molecular weights of the glycogens chromatographed may, however, be obtained by using a value of $19–20 \cdot 10^6$ for that of sweet-corn glycogen[26]. On this basis it is difficult to see the molecular weights of the two components of *Ascaris* glycogen being 450 and $50 \cdot 10^6$ as suggested by ORREL *et al.*[24].

Further uses of this techinque are for the examination of the mechanism of action of glycogen synthesizing and degrading enzymes. Thus examination of the carbohydrate elution profiles after different extents of hydrolysis may be useful in determining the action pattern (*i.e.* endo or exo attack) of debranching enzymes such as isoamylase[27,28] on glycogen. It should also be a convenient method for detecting whether higher- or lower-molecular-weight molecules are preferentially utilized as substrates for various enzymes such as glycogen synthetase and phosphorylase[29].

Additionally it is likely to be useful for examining glycogens from organisms subjected to different metabolic stresses, and particularly for studying the distribution of radioactive label in glycogen molecules of different sizes during metabolic studies. The application of the method to the separation of the components of various starches will be described elsewhere[30].

ACKNOWLEDGEMENTS

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