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Research Note

Use of scanning absorption optics for sedimentation equilibrium analysis of labelled polysaccharides: molecular weight of Blue Dextran

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The utility of using the absorption optical system on an analytical ultracentrifuge for the measurement of molecular weight of a polysaccharide labelled with a suitable chromophore is described. The potential of the method is illustrated, as are the limitations, by application to a polysaccharide widely used in gel permeation chromatography: Blue Dextran.

INTRODUCTION

The analytical ultracentrifuge is undergoing something of a renaissance (see, e.g. Schachman, 1989) after many years of being considered as a 'Cinderella' technique for the characterisation of biological macromolecules in solution — particularly polysaccharides. One of the reasons for this Cinderella status in the polysaccharide field has been the complexity of the technique and time-consuming nature of the analyses involved, particularly with regard to the classical method of recording distributions of solutes at sedimentation equilibrium: Rayleigh interference optics.

Use of scanning ultraviolet absorption optics has, for proteins (many of which have satisfactory extinction properties at a wavelength of 280 nm) provided a considerably easier route for recording solute distributions (either off- or on-line), provided that allowances for anomalous adsorption of solute onto cell windows is taken (see, e.g. Lloyd, 1974; Spragg, 1980; Rowe, 1984). Most polysaccharides, however do not possess a useful chromophore absorbing in the 'usable' ultraviolet (i.e. away from significant extinction caused by solvent molecules).

Some polysaccharides do possess a useable chromophore, usually synthetically attached. For example, 'Blue Dextran', solutions of which have been very popular in aqueous gel permeation chromatography, for identification of the void volume. Acosta *et al.* (1981) have demonstrated the utility of the absorption optical system for recording sedimentation transport of this labelled polysaccharide. For the first time, to the authors' knowledge, this paper demonstrates the utility of this optical system for determining the molecular weights of polysaccharides from sedimentation equilibrium.

MATERIALS AND METHODS

Blue Dextran (Cibacron Blue F3GA-dextran) was obtained from Sigma Chemicals Ltd. (Poole, UK), and fractionated using gel permeation chromatography on a Sepharose CL-6B gel, the excluded high molecular weight fraction being retained for sedimentation analysis. This had an absorption maximum at 630 nm (Fig. 1(a)). and an extinction coefficient (Fig. 1(b)) of $(79{\cdot}3 \pm 2{\cdot}7)$ ml g^{-1} cm^{-1} at this wavelength. This ex-

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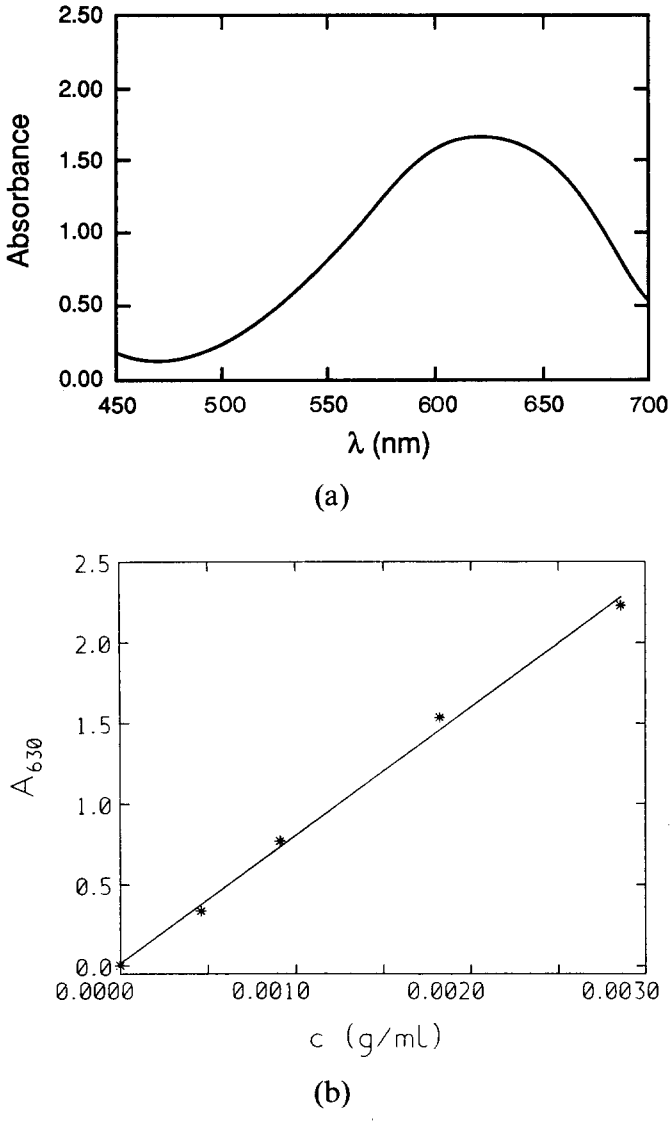


Fig. 1. (a) Absorption spectrum for blue dextran recorded using an LKB 4010 spectrophotometer. (b) Corresponding extinction coefficient plot of absorbance at 630 nm versus concentration, c (g ml^{-1}).

tinction coefficient was used to define the loading concentrations for both the sedimentation velocity and sedimentation equilibrium experiments. Blue Dextran preparations were dialysed against a standard phosphate-chloride buffer, pH 6·5 containing Na_2HPO_4 and KH_2PO_4 made up to a combined ionic strength of 0·30 by adding sodium chloride in the relevant proportions according to Green (1933).

An MSE Centriscan-75 (MSE Instruments, Crawley, UK) analytical ultracentrifuge, equipped with a 6-place aluminium rotor (Model No. 43111-104), scanning absorption optics and appropriate filters, was used. Insufficient signal was produced by the mercury arc light source at 630 nm; a wide band-pass filter was used instead (415–530 nm). This instrument was used for both sedimentation velocity (evaluation of sample homogeneity and sedimentation coefficient) and low-speed sedimentation equilibrium (direct determination of weight average molecular weight). Rotor speeds were 20 000 rev min^{-1} for sedimentation velocity experiments and 3700 rev min^{-1} for sedimentation equilibrium. Sedimentation velocity experiments were performed at 20·0 °C in standard 10-mm optical path length cells, and a column length of 10 mm. Sedimentation equilibrium experiments were performed under the same conditions with a column length of 1·6 mm. The value for the partial specific volume for Blue Dextran was determined by precision densimetry in the standard way (Kratky *et al.*, 1973): a value of (0·58 ± 0·01) ml g^{-1} was obtained.

Equilibrium distributions were captured off-line by a Graphics Tablet interfaced to an Apple IIE computer. A baseline was obtained by overspeeding (to 20 000 rev/min) and pelleting the solute. This did not differ significantly from the 'electrical' zero on the chart recorder output, indicating an absence of free dye. The time required for equilibrium was taken as the time at which there was no change in the absorption trace after a significant passage of time (in this case 40 h).

RESULTS

The sedimentation velocity profiles for Blue Dextran at a loading concentration of (1·04 ± 0·03) mg/ml^{-1} are given in Fig. 2. Despite prior fractionation by GPC, some degree of sample polydispersity was still evident. An estimate for the weight average $s_{20,w}$ of (29·1 ± 0·3) S was obtained.

The solute distribution at sedimentation equilibrium, as recorded using scanning absorption optics, at the same loading concentration as used for sedimentation velocity, is given in Fig. 3(a); Fig. 3(b) gives the corresponding plot of $\ln A$ versus r^2, where A is the solute absorbance at a given radial position, r, in the cell. From these data an (apparent) weight average molecular weight of (2·03 ± 0·08) × 10^6 was obtained. This is an *apparent* value (i.e. at a finite concentration) but due to the relatively low loading concentration used, the error due to non-ideality is likely to be within the precision (approximately ±5%) of the measurement (see Table 2·2 of Harding *et al.*, 1991). The value obtained is in close agreement with the manufacturer's own stipulation, albeit for the unfractionated sample.

DISCUSSION

Sedimentation equilibrium using the absorption optical system may represent a useful addition to the suite of absolute methods currently available {conventional sedimentation equilibrium recorded using Rayleigh

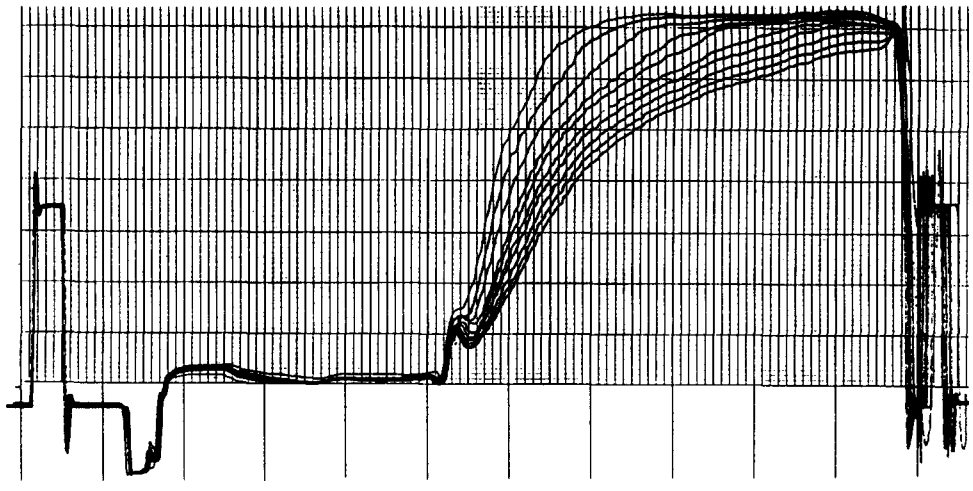
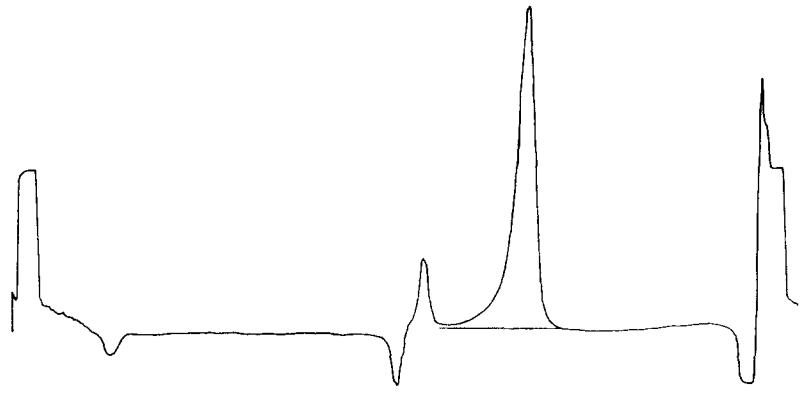
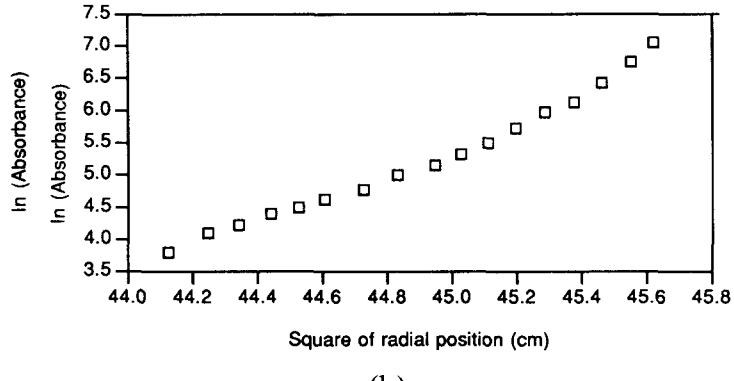


Fig. 2. Sedimentation velocity diagram for labelled dextrans in standard phosphate–chloride buffer (pH 6·5, I = 0·30) recorded using scanning absorption optics (λ = 415–530 nm) in an MSE Centriscan Analytical Ultracentrifuge. Temperature = 20°C. Loading concentration = (1·04 ± 0·03) mg ml^{-1}; rotor speed = 20 000 rev min^{-1}; scan interval = 8 min. The direction of sedimentation is from left to right.



(a)



(b)

Fig. 3. Sedimentation equilibrium of Blue Dextran. (a) Solute distribution recorded using scanning absorption optics (λ = 415–530 nm). Temperature = 20°C; rotor speed = 3700 rev min^{-1}; initial loading concentration = (1·04 ± 0·03) mg ml^{-1}. (b) Corresponding plot of ln A versus r^2.

interference optics; sedimentation velocity/diffusion coefficient analysis ('Svedberg Equation'); total intensity light scattering; osmotic pressure} for the determination of molecular weights of polysaccharides.

Provided that (1) incorporation of the chromophore does not appreciably affect the molecular weight of the polysaccharide being measured; (2) after dialysis, negligible amounts of free chromophore are present in

the solution, and the usual criteria for the partial specific volume used are satisfied (see, e.g. Creeth and Pain (1967) for a discussion on this) the method would appear to offer some advantage over other methods as a tool for polysaccharide molecular weight measurement. As with other analytical ultracentrifuge methods (see, e.g. Harding (1988)) the method described here does not suffer from the problems of dust/supramolecular aggregates that can seriously affect the interpretation of results from light scattering.

It has been demonstrated that if criterion (2) above is not satisfied, serious error can result: for example experiments on a preparation of 3,5-di-iodo-tyrosinated T50 dextran gave results 30–40% lower than the expected value, with residual dye apparent even after overspeeding to pellet.

The principal advantage of using the absorption optical system in this way is the ease of data capture and measurement compared to conventional Rayleigh optical methods of recording solution distributions at sedimentation equilibrium. Data can be relatively rapidly processed using a computer graphics tablet arrangement as has been used here, or the analogue data from the photomultipliers can be fed on-line into a computer. This development would appear to be particularly timely with the scheduled appearance of the new Optima XL Analytical Ultracentrifuge from Beckman Instruments (Palo Alto, CA, USA) which will possess this facility (Schachman, 1989).

Some polysaccharides are extremely non-ideal in the thermodynamic sense (see, for example Table 2.2 in Harding *et al.* (1991)), and for substances like guar and alginate loading concentrations less than 0·2 mg ml^{-1} are necessary to render these effects negligible. If this is not possible, a series of measurements of apparent molecular weights at different concentrations is necessary: in this case, the use of absorption optics with appropriate (and routine) multiplexing for different concentrations would appear to offer an additional advantage compared to other techniques.

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