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Physico-chemical studies on di-iodotyrosine dextran

Neil Errington, Stephen E. Harding

University of Nottingham, Department of Applied Biochemistry & Food Science, Sutton Bonington, LE12 5RD, UK

Lisbeth Illum

Department of Pharmaceutical Sciences, University of Nottingham, University Park, Nottingham, NG7 2RD, UK

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Etienne H. Schacht

Laboratorium voor Organische Chemie, Afdeling Polymeerchemie, Universiteit Gent, Krijgslaan 281-S4, B-9000 Gent, Belgium

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Dextran is currently being considered as a carrier biopolymer system for drug targeting. Di-iodotyrosine (DIT), and its radioactive derivative are useful markers for identifying the in-vivo route of these substrates. The performance of these substances *in vivo* is closely linked to their physico-chemical properties in solution. These properties have been investigated on a series of gel permeation chromatography fractionated DIT-dextrans using a combination of sedimentation, viscometric and laser-light scattering techniques. Weight average molecular weights, M_w, from sedimentation equilibrium and light scattering range from 0·0195 to 0·054 × 10^6. Double logarithmic representations of M_w versus sedimentation coefficient, $s^0_{20,w}$, and intrinsic viscosity $[\eta]$ suggest that these labelled dextrans have a more compact conformation in solution than do their unlabelled analogues — which behave as random coils. The implications of this for drug delivery are indicated.

INTRODUCTION

Dextran is being widely considered as a carrier biopolymer for drug targeting and for positioned or site-specific drug delivery at mucosal membranes such as the colon in the gastrointestinal tract (Harboe *et al.*, 1989). Dextran has also been used as a hydrophilic model drug for characterising the permeability of mucosal membranes such as the nasal membrane in terms of molecular weight cut-off (Maitani *et al.*, 1989).

It is important in such studies to have available dextrans of uniform properties such as well defined, narrow molecular weight ranges so as to avoid complications due to polydispersity for determining the maximum molecular size that can penetrate a membrane in sufficient quantities so as to be useful. In its commercially available forms dextran does not meet these criteria, being poorly characterised and very polydisperse: for example, a polydispersity index (ratio of highest to lowest molecular weight species present) as high as 1·6–2·6 is common for T70 (molecular weight ~70000 g mol^{-1}) dextran (Sigma Chemical Company Ltd, Poole, UK), making it of very limited use for the applications described above.

To overcome this problem the dextrans used in this study were fractionated using gel permeation chromatography to produce fractions having well defined, narrow molecular weight ranges.

In order to facilitate the detection of dextrans during in-vivo experiments, labels such as 3,5-di-iodo-L-tyrosine (DIT), covalently bound to the dextran chain, are commonly employed. In this way the dextran can be detected spectrophotometrically and can be radioactively labelled by replacement of the iodine with an isotope (^{125}I). The DIT also acts as a model ligand representing potential drugs that could be attached to the dextran (see e.g. Harboe *et al.*, 1989).

The purpose of this study is therefore twofold: to assess, using a combination of hydrodynamic and light scattering techniques, firstly the solution characteristics of a series of six such fractionated labelled dextrans having molecular weights from approximately 20 000 to 50 000 g mol^{-1}, and secondly, to determine any

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conformational changes in the dextran brought about by the conjugation.

MATERIALS AND METHODS

Materials

The dextran for labelling and fractionation was supplied by Sigma Chemical Company Ltd, Poole, UK. All other materials used were of the highest available grade.

Solvent for physical studies
The (aqueous) solvent used was a phosphate buffer, pH = 7·0, I = 0·30. Na_2HPO_4 and KH_2PO_4 were made up in the relevant proportions to give a combined solution ionic strength of 0·05 and then NaCl added to give the desired ionic strength in accordance with Green (1933).

Methods

DIT-dextrans
The DIT-dextran was synthesised by first activating the dextran [Fig. 1(a)] by reaction with 4-nitrophenyl chloroformate in DMSO/pyridine (1/1) at 5°C (Vandoorne *et al.*, 1985). The 4-nitrophenylcarbonate ester was then reacted with di-iodotyrosine (DIT) in the same reaction medium. The reaction product [Fig. 1(b)] was isolated and purified by preparative gel permeation chromatography on a Sephacryl S-200 gel (Pharmacia) with water as eluent. The DIT label is most likely to be attached to the C-2 hydroxyl groups of the dextran. NMR studies (Schacht, E.H., unpublished results) indicate that this position is activated first during the 4-nitrophenyl chloroformate reaction. However, the site of substitution is not of significant importance in this work. The percentage activation of the dextran (~2%) was found from the total carbonate content of the activated polymer. This was determined by hydrolysis with barium hydroxide according to the method of Kol'tsova *et al.* (1977).

Fractionation of the DIT-dextran
All DIT-dextrans were fractionated using preparative gel filtration on a 5 × 100 cm Sephacryl S-200 gel column using distilled water as the eluent at 3 ml min^{-1}. A refractive index detector was used to detect the elution profile of the dextran. The eluting polymer was divided over 10 fractions using an LKB (Bromma, Sweden) fraction collector and the fractions were freeze dried. M_n for each dextran fraction was determined by analytical GPC on a TSK column system G 2000 SW + G 3000 SW. The eluent was 0·1 M phosphate buffer (pH 7) at a flow rate of 1 ml min^{-1}. Peak position was used to calculate M_w from a calibration curve established using Pharmacosmos dextran standards in the molecular weight range 6000–180 000.

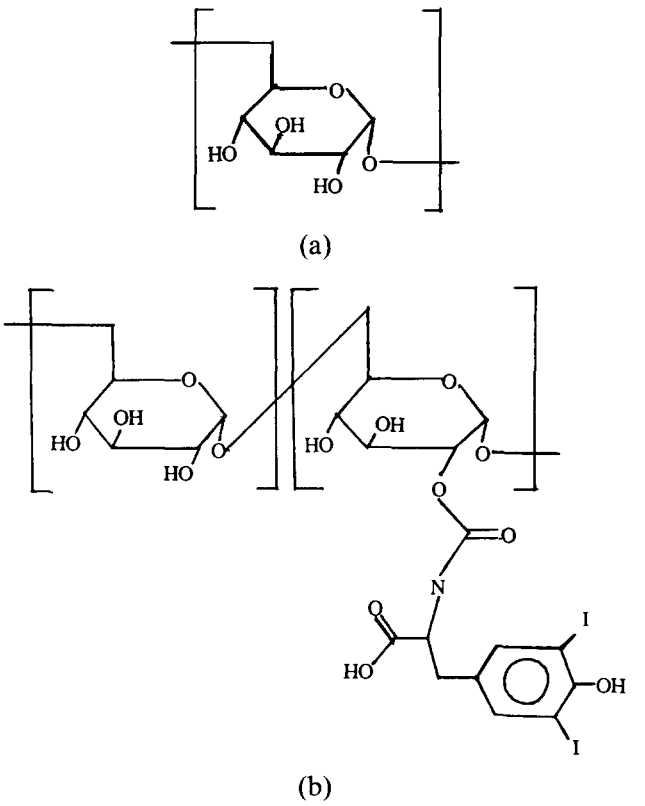

Fig. 1. Structure of (a) native dextran and (b) di-iodotyrosine labelled dextran.

Partial specific volume determination
An Anton-Paar precision density meter, coupled to a thermostatically controlled, refrigerated water bath was used to measure the densities of various solutions of one of the DIT-dextrans (T50). The instrument was calibrated using CsCl solutions (Crossley *et al.*, 1980), and deionised distilled water was used as a reference standard as its density is known over a wide range of temperatures.

The arithmetical mean of 10 values of density for each concentration of solution was taken and plotted against concentration (Fig. 2). The partial specific volume was calculated from this using the following equation (Kratky *et al.*, 1973):

$$\bar{v} = (1 - \partial\rho/\partial c)/\rho_o \quad (1)$$

where ρ_o is the density at infinite dilution and $\delta\rho/\delta c$ (the density increment) is obtained from the slope of the plot.

Sedimentation coefficient measurements
For measuring sedimentation coefficients for each of the fractions, an MSE Centriscan-75 (MSE Instruments,

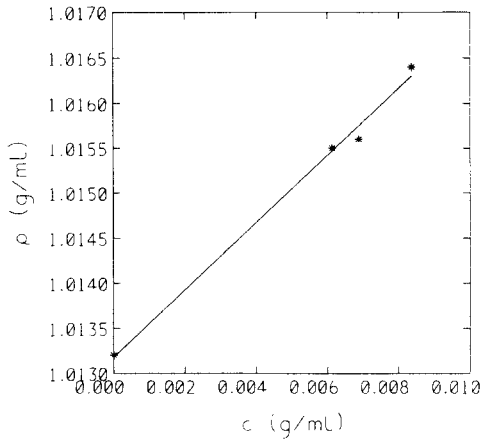

Fig. 2. Plot of the density of T50 DIT-dextran solution versus concentration. Temperature = 25 °C.

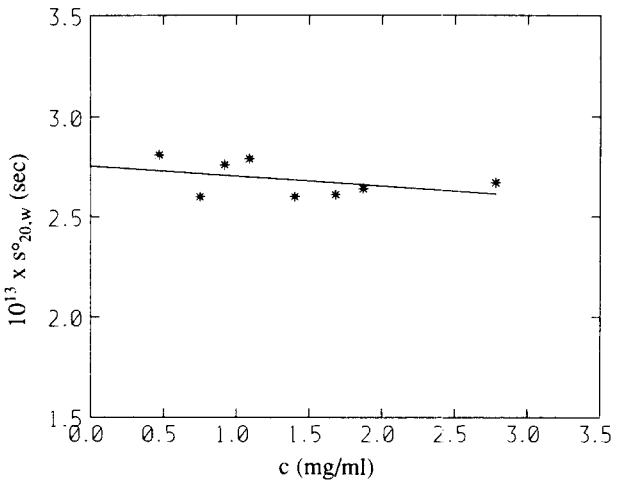

Fig. 4. Concentration extrapolation of the (apparent) sedimentation coefficient of T50 DIT-dextran.

Crawley, UK) analytical ultracentrifuge was used, equipped with an aluminium rotor, scanning absorption optics and a monochromator set to 295 nm. The instrument produces scans of absorbance versus radial distance at set time intervals (see, e.g., Fig. 3). Between 8 and 10 consecutive scans were used to determine each sedimentation coefficient.

The sedimentation coefficients obtained ($s_{T,b}$, corresponding to the buffer, *b*, at temperature *T*) were then corrected to standard conditions ($s_{20,w}$, corresponding to a temperature of 20 °C and water as the solvent). The equation below is used for this purpose (Tanford, 1961):

$$s_{20,w} = s_{T,b}\{\eta_{20,w} \cdot (1 - \bar{v}\rho)_{T,b}/\eta_{T,b} \cdot (1 - \bar{v}\rho)_{20,w}\} \quad (2)$$

where the ηs are the corresponding viscosities.

These $s_{20,w}$ values are then extrapolated to infinite dilution in order to obtain $s^{\circ}_{20,w}$ (see, e.g., Fig. 4).

Sedimentation equilibrium

For measurement of molecular weight on all fractions, low-speed sedimentation equilibrium analysis was employed. A Beckman model E analytical ultracentrifuge with an electronically controlled drive was used. Rayleigh interference optics (using both laser (5 mW, He-Ne) and mercury arc light sources) were employed as the method of recording the distribution of solute at sedimentation equilibrium. An Ultroscan XL enhanced laser densitometer (LKB Instruments, Bromma, Sweden) with appropriately modified software was used to capture the fringe data. A Fourier series algorithm 'analyser' was used to produce from the data the fringe concentration (relative to the meniscus) versus radial displacement (Rowe *et al.*, 1989). These data were then transferred to the mainframe IBM 3081/B computer at Cambridge for full molecular weight analysis (Creeth & Harding, 1982).

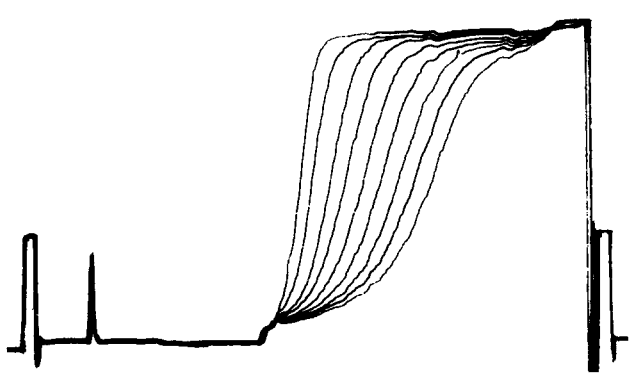

Fig. 3. Sedimentation velocity scans of T50 DIT-dextran. Scan interval = 960 s, temperature = 20 °C, rotor speed = 50 000 rpm, λ = 295 nm.

The concentration, in fringe units, of the dextran at the meniscus was finite at the rotor speeds used, and was obtained using mathematical analyses of the fringe data as described in, e.g., Creeth & Harding, 1982. (Apparent) whole cell weight-average molecular weights, $M_{w(app)}$, were obtained from the limiting value at the cell base of a particularly useful operational point average, M^* (Creeth & Harding, 1982).

The second virial coefficient, B, and the 'ideal' weight average molecular weight, M_w, were estimated from plots of $1/M_{w(app)}$ versus concentration, $(1/M_w)$ $(2BM_w - \bar{v})$ being equal to the slope of the plot (Ross & Minton, 1977). Double sector cells with an optical path length of 30 mm were used.

Determination of intrinsic viscosity

Experiments to determine intrinsic viscosity were performed in an Ostwald-type viscometer of 2 ml capacity upon all six fractions of the labelled dextran. This gives information on the degree of entanglement of the polysaccharide chain - the more entangled the chain then the slower the rate of passage through the capillary and thus the greater the viscosity.

The viscometer was suspended in a thermostatically controlled water bath (Schott-Geräte) which was maintained at approximately 25°C and within limits of ± 0·05°C. The flow times were recorded electronically using photoreceptors mounted on the viscometer stand capable of detecting the passage of the solution meniscus. From the solution : solvent flow time ratio the kinematic relative viscosity was obtained. Because of the low concentrations used, the density corrections for the different solutions were assumed to be very small and the kinematic viscosities were assumed to be approximately equal to the dynamic viscosities (Tanford, 1955). The intrinsic viscosity was found by extrapolation to infinite dilution of the reduced specific viscosities (see, e.g., Fig. 5).

Multi-angle laser light scattering

As an independent check on the molecular weights of four of the DIT-dextran fractions (DIT-D2, DIT-D3, DIT-D4 and DIT-D6), additional measurements were made using high-pressure liquid gel permeation chromatography coupled on-line to a DAWN-F (Wyatt Technology, Santa Barbara, USA) multi-angle laser light scattering (GPC/MALLS) photometer (equipped with a 5 mW He–Ne laser, λ = 632·18 nm) and a differential refractometer (Waters Instruments, UK). A full description of the operation of this instrument is given in Jackson *et al.* (1989). The photometer was calibrated using toluene (Stacey, 1956). A value of 0·147 ml g^{-1} was taken for the refractive index increment at 632·18 nm (Application note LS7 (1985), LDC/Milton Roy Ltd, Stone, UK).

This 'on-line' procedure offers two key advantages (see, e.g., Harding *et al.*, 1991). Firstly, sample polydispersity is resolved prior to light scattering analysis and secondly, the GPC assists in the on-line clarification of the solutions from supramolecular aggregates, thus removing the severe effects on the data that the latter can cause (see, e.g., Hourdet & Muller, 1987).

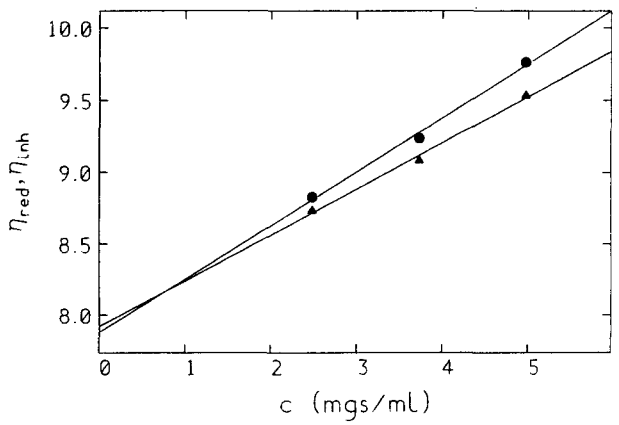

Fig. 5. Plot of reduced viscosity versus concentration for T20 DIT-dextran.

RESULTS

Partial specific volume

The plot of density versus concentration for the DIT-D1 dextran (Fig. 3), produced a slope ($\partial\rho/\partial c$) of (0·37 ± 0·02) and an intercept (ρ_0) of (1·013 ± 0·001) g ml^{-1}, corresponding to a partial specific volume $\bar{v}$ of (0·62 ± 0·02) ml g^{-1}. This value is consistent with the value for unlabelled dextrans [(0·611 ± 0·005) ml g^{-1}, (Grönwall, 1957)]. This value was assumed to be the same for all of the fractions.

Sedimentation velocity

The data given in Table 1 show the expected general increase of the sedimentation coefficient with molecular weight, from ~ 1·7 Svedbergs (for M_w ~ 20 000) to ~ 2·9 Svedbergs (for M_w ~ 50 000). The DIT-D5 fraction is the exception to this trend, having a higher sedimentation coefficient than would be predicted from the other results. The traces shown in Fig. 2 show the sedimenting boundary for the DIT-D1 fraction. The sharpness of the boundary is an indication (but not proof) of a very low polydispersity.

Molecular weights

In general, good agreement is shown between the molecular weights obtained from sedimentation equilibrium, GPC/MALLS and comparative GPC as shown in Table 1, although for one of the samples, DIT-D3, the result from GPC/MALLS was some 20% higher than that from sedimentation equilibrium, possibly due to effects of supramolecular contamination using the former technique.

Intrinsic viscosity

There is not as marked a trend in intrinsic viscosity with M_w as there is with the sedimentation coefficient, but there is still an increase with increasing molecular weight as would be expected (the longer chains will entangle to a greater extent).

DISCUSSION

Assuming the DIT-dextrans studied represent part of an homologous series (Tanford, 1961) it is possible to probe in more detail their gross conformation. This can be done by plotting the logarithm of their molecular weights against the logarithm of the intrinsic viscosity (Smidsrød & Andresen, 1979). The slope of this plot will give the value of the parameter a in the Mark-Houwink-Kuhn-Sakurada (MHKS) equation (see, e.g., Harding *et al.*, 1991):

Table 1. Molecular weights and hydrodynamic data for fractionated DIT-dextrans

Name of dextran	Molecular weights			Sedimentation coefficient	Intrinsic viscosity
	M_w^a (g mol^{-1})	M_w^b (g mol^{-1})	M_w^c (g mol^{-1})	$s^\circ_{20,w}$ (sec × 10^{-13})	$[\eta]$ (ml g^{-1})
DIT-D1	54 000 ± 1000	50 400	—	2·90 ± 0·2	17·6 ± 2·0
DIT-D2	41 000 ± 2000	45 840	52 300	2·84 ± 0·03	18·2 ± 0·1
DIT-D3	36 000 ± 1000	33 600	45 000	2·83 ± 0·05	18·6 ± 0·4
DIT-D4	27 000 ± 1000	27 100	22 100	1·77 ± 0·08	14·3 ± 0·6
DIT-D5	20 500 ± 500	23 000	—	2·10 ± 0·1	7·2 ± 0·3
DIT-D6	21 500 ± 1000	20 800	19 500	1·66 ± 0·27	15·4 ± 0·2

[a]Value determined from extrapolation of sedimentation equilibrium results to infinite dilution.
[b]Calculated from number average molecular weights as determined by peak elution volume from GPC by comparison with Serva dextran standards.
[c]From GPC/MALLS experiments.

$$[\eta] = K'M^a \quad (3)$$

where K' and a are empirical parameters, constant at a given temperature for a given polysaccharide/solvent system. A similar relation exists for $s^\circ_{20,w}$ versus molecular weight:

$$s^\circ_{20,w} = K'' M^b \quad (4)$$

Haug's triangle representation of macromolecular conformation (Fig. 6) can be used to relate a and b to the gross conformation of the molecule. In this representation, the three extremes of macromolecular conformation (sphere, rod and random coil) are placed at the corners of this triangle; the conformation of a given macromolecule can then be represented by a locus along the sides of the triangle.

The (MHKS) plots for the DIT-dextran are given in Figs 7 and 8. The values of a and b obtained for the DIT-dextrans (a = 0·23 ± 0·1, b = 0·56 ± 0·1 using M values from sedimentation equilibrium data, and a = 0·24 ± 0·07, b = 0·58 ± 0·05 using M values from GPC/MALLS data) correspond to a gross conformation part way between random coil and compact sphere.

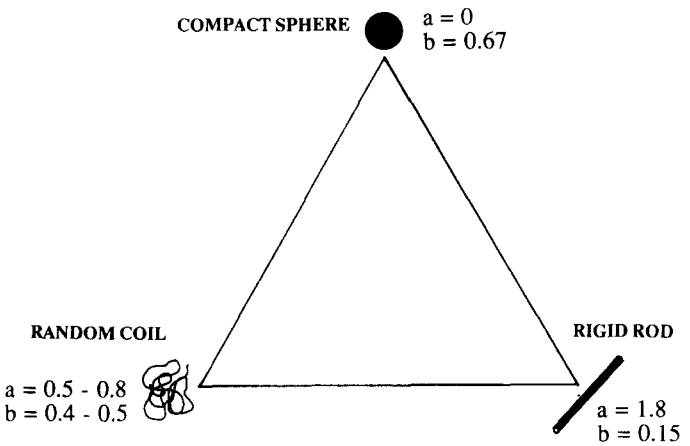

Fig. 6. Haug's triangle representation of macromolecular conformation. a and b are the MHKS parameters [see text, eqns (3) and (4)].

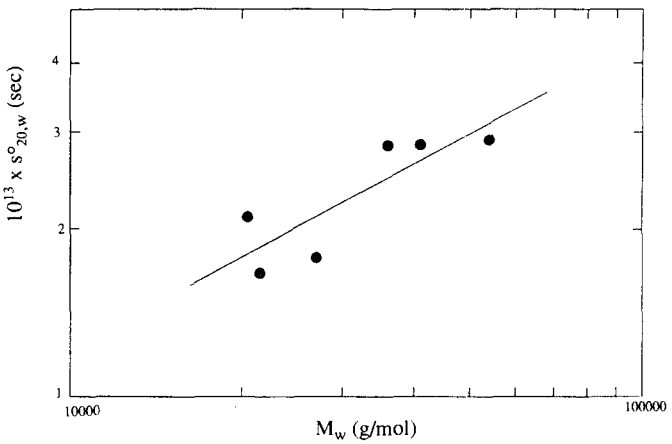

Fig. 7. Double log plot of $s^\circ_{20,w}$ versus (sedimentation equilibrium) molecular weight for the DIT-dextran series.

In Table 2 is shown data from the work of Senti *et al.* (1955) on a similar range of fractionated unlabelled dextran. The trends in intrinsic viscosity and sedimentation coefficient with molecular weight are similar to those found for DIT-dextran.

From logarithmic plots of molecular weight versus intrinsic viscosity and sedimentation coefficient for these dextrans the parameters corresponding to a and b above [0·51 ± 0·04 and 0·46 ± 0·03, respectively (Senti *et al.*, 1955)] correspond more closely to a random coil conformation. Table 3 shows the values of a and b for both labelled and unlabelled dextrans.

Our results suggest that addition of the label has caused the dextran to adopt a more compact conformation than its native counterpart, although the errors allow for a range of conformations between those of random coil and compact sphere for the DIT-dextran. A possible explanation for the more compact conformation of the DIT-dextran is that the dextran sugar

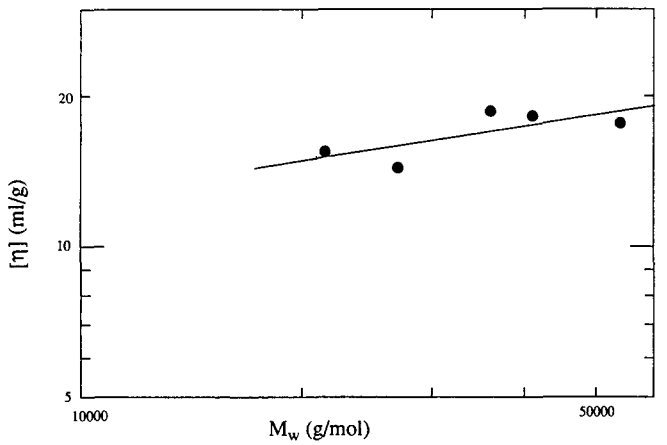

Fig. 8. Double log plot of intrinsic viscosity versus (sedimentation equilibrium) molecular weight for the DIT-dextran series.

Table 2. Molecular weight, sedimentation and viscosity data for native dextran

Fraction	Molecular weight $10^{-3} \times M_w$ (g mol^{-1})	Intrinsic viscosity $[\eta]$ (ml g^{-1})	Sedimentation coefficient $s^0_{20,w}$ (sec $\times$ 10^{-13})
1	17·7	12·9	1·73
2	18·4	13·3	1·86
3	31·4	18·2	—
4	37·7	19·7	2·55
5	49·9	21·4	—
6	65·6	25·2	3·24

From Senti *et al.* (1955).

Table 3. MHKS parameters for unlabelled and DIT-labelled dextran

	a	*b*	Reference
DIT-dextran	0·23 ± 0·10[a]	0·56 ± 0·10[a]	This study
	0·24 ± 0·07[b]	0·58 ± 0·05[b]	This study
Unlabelled dextran	0·51 ± 0·04	0·46 ± 0·03	Senti *et al.*

[a]Using molecular weights from sedimentation equilibrium.
[b]Using molecular weights from GPC/MALLS.

chain is being folded around the labelled regions due to the relatively hydrophobic nature of the label. This could be very important for pharmaceutical studies on, for example, site-specific release, as it could mean that if a hydrophobic 'drug' is attached to the dextran carrier, then the hydrolysis of the drug-dextran bond may be impeded due to the dextran chains screening the drug from the external medium. This would result in a reduced release of the drug and so reduce the amount that could penetrate the membrane. In studies upon the permeation of membranes by dextran conjugates, the effective maximum molecular weight for permeation could be increased slightly by conjugating a hydrophobic compound to the dextran, thus causing the adoption of a more compact conformation.

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