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Studies on agaroses: 1. Specific refractive index increments in dimethyl sulfoxide and in water at various wavelengths and temperatures

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Specific refractive index increments of three agarose samples in dimethyl sulfoxide and in water were measured at 436, 546 and 633 nm, and at several temperatures. The wavelength and temperature dependences of the increments were determined, simultaneously correlated using the surface regression method, and presented in a form suitable for tabulation. The influence of differences in the structure of agaroses from various algal sources on the specific refractive index increment is discussed.

(Keywords: agarose; specific refractive index increment; algal species)

INTRODUCTION

Agarose is the fraction (with the lowest charge content) of cell-wall polysaccharides, known as agar, which is extracted from red seaweeds (*Rhodophyceae*, mainly *Gracilaria* and *Gelidium* spp.). The properties of agarose products vary from manufacturer to manufacturer and among samples, due to use of various species of seaweed[1], different harvesting seasons[2], and the different manufacturing processes applied[3].

The idealized structure of agarose can be described by an agarobiose repeating unit[4], [→ 3)-β-D-galactose-(1 → 4)-3,6-anhydro-α-L-galactose-(1]$_n$. In agarose, sulphate[5] (< 0.35%), methoxy[6] (0.4–2.5%) and pyruvate[7,8] (< 0.10%) groups are the dominating substituents, but also neutral sugars such as L-galactose, methylpentose, xylose[9,10] and 4-*O*-methyl-L-galactose have been shown to occur as branches on agar[11,12].

For our intended light-scattering studies on agaroses, reliable data on specific refractive index increments (dn/dc) of this polysaccharide are of great importance (c is concentration expressed in w/v units). Even an otherwise careful study on the molar masses of agaroses by Rochas and Lahaye[13] (based on size exclusion chromatography coupled with low-angle laser light scattering in 0.1 M $NaNO_3$) contains only a rough estimate of the dn/dc value at the wavelength used ($\lambda = 633$ nm). Their estimate (0.14) was obtained by extrapolation from experimental dn/dc measured at $\lambda = 940$ nm assuming that the slope of the dependence of dn/dc on λ^{-2} for agarose is the same as that for dextran. A similar approximate approach was applied also by other authors[14,15]. We have therefore decided to carefully determine the dn/dc values for three agarose samples, at several wavelengths (λ) and temperatures (T), in two single solvents, water and dimethyl sulfoxide (DMSO). Water is by far the most common solvent of agarose both in research and in practice; DMSO was chosen due to its ability to dissolve agarose even at room temperature (gelation temperature is below 0°C) and to break hydrogen bonding. Mixed solvents like various aqueous salt systems or DMSO/water mixtures will be the subject of a forthcoming study.

For a solution of a polymer (having sufficiently high molar mass M) in a single solvent, dn/dc depends only on T and λ. It is known that, for most practically important systems, dn/dc is a linear function of T and λ^{-2} (see, for example, ref. 16). However, below a certain limit (usually $< 10^3$ g mol^{-1} or, in some cases, $< 10^4$ g mol^{-1}), specific for every polymer/solvent system, M of a polymer can influence dn/dc, thus becoming a third independent variable[17]. The mass-average molar mass of the agarose samples under study has previously been estimated by viscosity measurements[18] (using an Ubbelohde viscometer at 80°C in 0.1 M $NaNO_3$ and adopting reported Mark–Houwink constants[13]) and, for one sample (MC-agarose, see Experimental), also by preliminary static light-scattering measurements. By both methods, M values of the order of 10^5 g mol^{-1} were found and,

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Table 1 Characteristics of agarose samples

Sample code	Content of substituents						Moisture (% w/w)	
	Sulfate[a]		Pyruvate[a]		Methoxy[c]			
	% (w/w)	mol%[b]	% (w/w)	mol%[b]	% (w/w)	mol%[b]	Manuf.[a]	This study
MC-agarose	0.060	0.2	0.12	0.5	0.6	6	8.5	9.4
HA-agarose	0.097	0.3	n.a.	n.a.	1.8	18	5.4	11.9
LI-agarose	0.152	0.5	0.01	0.04	1.6	16	4.3	11.2

[a] Data from manufacturers' product certificates
[b] Moles of substituent per 100 moles of agarobiose units
[c] Approximate values estimated using a relation between the gelation temperature and content of methoxy groups in agarose[6] (gelation temperatures of 36.5, 41.5 and 41.0°C for MC-, HA- and LI-agarose, respectively)
n.a. = not analysed

Table 2 The constants from equation (1) for agarose samples and both solvents. The constants k_1, k_2, k_3 and k_4 are given in , respectively, $cm^3\ g^{-1}$, $cm^3\ nm^2\ g^{-1}$, $cm^3\ (°C)^{-1}\ g^{-1}$ and $cm^3\ nm^2\ (°C)^{-1}\ g^{-1}$

Sample code	k_1		k_2		$10^4 k_3$		k_4	
	DMSO	water	DMSO	water	DMSO	water	DMSO	water
MC-agarose	0.0452	0.1368	−623	2735	2.30	1.3	−5.6	−19.9
HA-agarose	0.0543	0.1487	−1367	1867	2.38	0.86	7.8	−1.5
LI-agarose	0.0584	0.1517	−1821	1615	2.07	0.17	5.8	2.1

consequently, the effect of M on dn/dc could be neglected.

Since dn/dc is an important quantity in the characterization of any polymer/solvent pair, we have decided to measure dn/dc at several wavelengths and temperatures, correlate both variables simultaneously, and publish the results in a form suited for tabulation.

EXPERIMENTAL

Three commercial agarose samples, kindly supplied by Pharmacia Biotech, Uppsala, Sweden, were studied without any additional preparations or drying. Code symbols used in this study, manufacturers, and some specifications are as follows.

1) MC-agarose: Seakem CB agarose, lot no. 60784, FMC BioProducts, Inc. (formerly Marine Colloids, Inc.), Rockland, ME, USA;
2) LI-agarose: batch no. 130170-01, FMC BioProducts, Inc., Litex Co., Copenhagen, Denmark; and
3) HA-agarose: batch no. HO 300/93, Hispanagar, Pronadisa, Spain.

Selected characteristics of these samples (contents of important groups, etc.) are given in *Table 1*. The values of moisture content from manufacturer's thermogravimetric data are compared in *Table 1* with those measured by us (the Fischer method with DMSO as solvent).

Pure redistilled water and DMSO (analytical grade, 99.4% purity by gas chromatography, 0.37% water by the Fischer method) were employed as solvents.

Solutions of the individual agarose samples were prepared by weighing agarose and the chosen solvent into glass ampoules which were subsequently sealed. In the case of DMSO, the ampoules were shaken for a few days at room temperature. During this period they were heated several times to 100°C for a few minutes only, until solutions were obtained that were homogeneous also at room temperature. Extended heating was avoided to reduce the risk of thermodegradation. Aqueous solutions were shaken at 100°C for 30 min to make the system homogeneous. Hot aqueous solutions were then transferred into a thermostatted (pre-heated) measuring cell. Agarose concentrations were calculated taking into account both the moisture content in the sample and the density of the solvent at a given temperature. Each value of dn/dc was obtained by a linear regression from five points in the plot of Δn *versus* c, the range of c being ~ 0.4–2% (w/v) for DMSO and ~ 0.2–1% (w/v) for water.

The measurements were performed using a Brice-Phoenix model BP-2000-V differential refractometer. The instrument was calibrated by aqueous solutions of KCl, using data calculated by the manufacturer (according to ref. 19) and given in the Instruction Manual. We have found that the resulting calibration constant (relating measured readings to the differences between refractive indices of solvent and solution) is virtually independent of both T and λ in the given ranges. We measured at $\lambda = 436$, 546 and 633 nm, at temperatures of 50, 60 and 70°C for aqueous systems and at 25, 50 and 75°C for DMSO systems. The lower temperature limit for aqueous systems was chosen to avoid gelation of the agarose solution, which occurs at ~ 45°C or below. In DMSO, within the range of agarose concentration used by us, the system remains in solution state at temperatures down to at least 15°C. The upper temperature limits were given mainly by the construction of the glass refractometric cell.

RESULTS AND DISCUSSION

To minimize subjectiveness in treating the data, we correlated measured values of dn/dc simultaneously with two independent variables, T and λ^{-2}, using the method of three-dimensional ('surface') regression. We knew

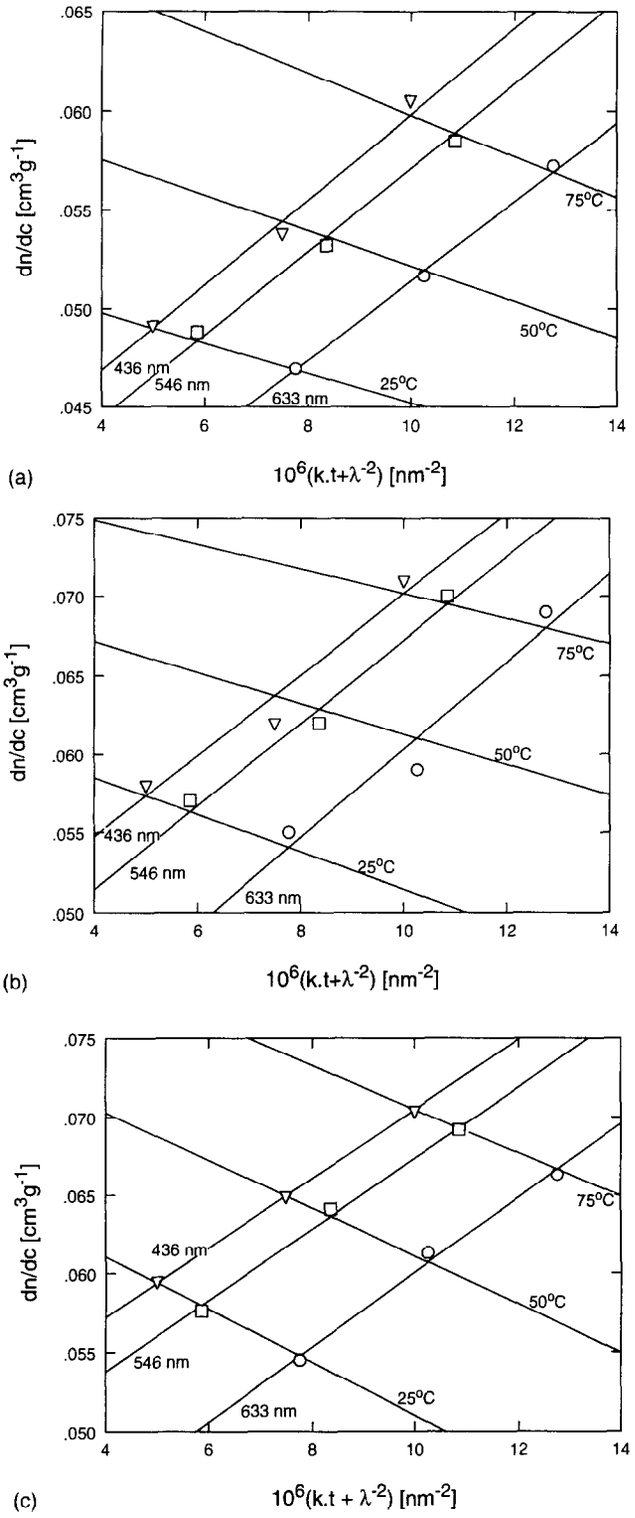

Figure 1 Experimental and calculated dependences of the specific refractive index increment dn/dc ($cm^3 g^{-1}$) of agaroses in dimethyl sulfoxide on temperature (°C) and wavelength λ of the primary beam *in vacuo* (nm); straight lines are denoted by the respective constant values of T and λ; k is an arbitrary constant (here, $k = 1 \times 10^{-7}$ nm^{-2} °C). Experimental points: ∇, 436 nm; □, 546 nm; ○, 633 nm. (a) MC-agarose; (b) HA-agarose; (c) LI-agarose

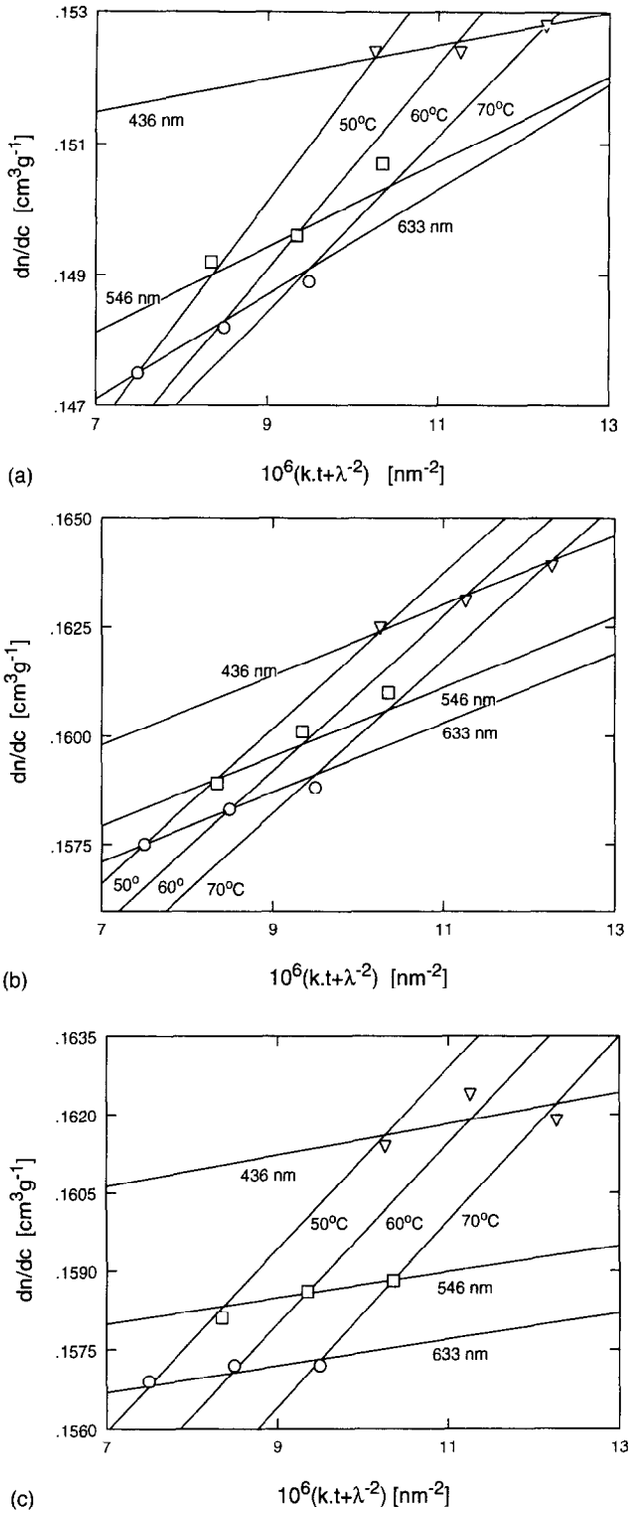

Figure 2 Experimental and calculated dependences of the specific refractive index increment dn/dc ($cm^3 g^{-1}$) of agaroses in water on temperature (°C) and wavelength λ of the primary beam *in vacuo* (nm); straight lines are denoted by the respective constant T and λ; k is an arbitrary constant (here, $k = 1 \times 10^{-7}$ nm^{-2} °C). Experimental points: ∇, 436 nm; □, 546 nm; ○, 633 nm. (a) MC-agarose; (b) HA-agarose; (c) LI-agarose

from preliminary experiments that both dn/dc *versus* T and dn/dc *versus* λ^{-2} dependences really are linear, as expected from published data[16]. The 'planar' regression (the least-squares fit of a plane through the experimental points in the three-dimensional plot) was therefore chosen. The quantity dn/dc can thus be expressed as a product of two first-order polynomials, which gives, after rearrangement,

$$dn/dc = k_1 + k_2\lambda^{-2} + k_3 T + k_4 T\lambda^{-2} \qquad (1)$$

in which k_1 to k_4 are coefficients, the values of which are to be calculated by the regression mentioned above. For

g cm $^{-3}$, λ in nm and T in °C. In graphical form, this dependence can be presented most conveniently as a plot of dn/dc *versus* $(kT + \lambda^{-2})$, where k is a constant[20], arbitrarily chosen so as to make the graph lucid. In *Figures 1* (*a*, *b*, *c*) and *2* (*a*, *b*, *c*), the individual straight lines are dependences of dn/dc on T and dn/dc on λ^{-2} calculated from k_1–k_4 at a constant value of the other variable. Experimental points have the same abscissae as the intersections of the 'network' lying below or above them.

In all cases, at constant λ, dn/dc increases with increasing T. It follows from the values of k_3 (*Table 2*) that the temperature dependence of dn/dc is substantially more pronounced for DMSO than for water. This is in accordance with the fact that, for most liquids including DMSO, the temperature coefficient of the refractive index is $\sim 5 \times 10^{-4}$ (°C)$^{-1}$, whereas for water it has the unusually low value of $\sim 1 \times 10^{-4}$ (°C)$^{-1}$.

At a constant T, however, the shape of the dn/dc *versus* λ^{-2} dependence differs for the solvents: with increasing λ^{-2}, dn/dc decreases for DMSO and increases for water.

The negative slope of the dn/dc *versus* λ^{-2} dependence obtained at constant T for DMSO and all agarose samples seems to be rather unusual (with most polymer/solvent systems, dn/dc increases with increasing λ^{-2}; see, e.g., ref. 16) but is perfectly reproducible.

For aqueous systems, there is a positive slope of the dn/dc *versus* λ^{-2} dependence for all samples, and, in the investigated range of T and λ, the dn/dc values vary from 0.148 to 0.164 cm^3 g^{-1} (with a mean value of 0.157 cm^3 g^{-1}). This (relatively small) variation in dn/dc is mainly caused by including the results for the agarose sample from Marine Colloids. The MC-agarose gives distinctly lower dn/dc values than the LI- and HA-agaroses. *Table 1* gives a comparison of the data on substitution patterns of the three samples, extracted from the manufacturers' production certificates. The content of the methoxy substituents was estimated from the dependence of the gelation temperature on methoxyl content using reported data[6]. Comparing the data from the three samples, the LI- and HA-agaroses share common features and differ from the MC-agarose. The MC-agarose has a high degree of substitution by pyruvic acid residues and a low calculated degree of substitution by methoxy residues, indicating that the algal species utilized is *Gelidium* sp. The contents of these substituents in the other two samples correspond to the utilization of *Gracilaria* species as the algal source. Agaroses from *Gelidium* and *Gracilaria* spp. give mean dn/dc values of 0.15 and 0.16 cm^3 g^{-1}, respectively, exhibiting little variation with T and λ in aqueous systems (*Table 2*).

For the DMSO systems, the variation of dn/dc with T and λ is larger than for aqueous systems. The dn/dc values for agarose from *Gelidium* sp. are again lower than those for agarose from *Gracilaria* sp., ranging from 0.057 to 0.070 cm^3 g^{-1}.

Table 2 giving coefficients k_1–k_4 from equation (1) has been applied here for various agaroses. However, thanks to its versatility, the proposed regression method of determination of these coefficients can be used quite systematically and generally for most (if not all) polymer/solvent systems. The coefficients for any system could thus be measured and tabulated in a form similar to *Table 2* and, subsequently, dn/dc could readily be obtained for any T and λ using equation (1). Obviously, care should always be taken when using the coefficients for calculating the dn/dc values far outside the investigated range of T and/or λ. For polymers of very low molar masses and for oligomers, the coefficients of molar-mass dependences should be included. The results also show that a difference in dn/dc is to be expected between agaroses originating from various algal species, and the tabulation is recommended when the algal source is known.

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