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PREPARATION AND PROPERTIES OF FLUORESCEIN-LABELLED HYALURONATE

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ABSTRACT

Hyaluronate has been labelled with fluorescein groups by two procedures. Products with degrees of substitution (d.s.) between 0.05 and 0.001 were obtained. Physico-chemical analysis (viscometry, gel chromatography, and measurements of sedimentation and diffusion coefficients) of the parent compound and the products showed that the labelling procedures caused only a limited degradation of the polysaccharide.

INTRODUCTION

Hyaluronate is a copolymer of 2-acetamido-2-deoxy-D-glucose and D-glucuronic acid, and it has been found extracellularly in various connective tissues[1]. Fluorescein labelled hyaluronate has been prepared by two methods for investigations on the biological function and the physico-chemical properties of the polymer. The methods of labelling and the properties of the products are now reported.

Fluorescent-labelled polymers have been widely used for studies on microcirculation, tissue permeability, and tissue distribution by fluorescence microscopy. The fluorescein-labelling of dextran has been described in an earlier note[2].

RESULTS AND DISCUSSION

It was found that isothiocyanatofluorescein (mixed 5- and 6-isomers; abbreviated to FITC) reacts with sodium hyaluronate dissolved in formamide and thereafter diluted with methyl sulphoxide. A small quantity of sodium hydrogen carbonate was added to prevent acidity, and dibutyltin dilaurate was added as catalyst. The reaction mixture may either be kept for 15 min at 95° or for 1 day at 35°. The d.s. attained by these procedures ranged from 0.05 to 0.001.

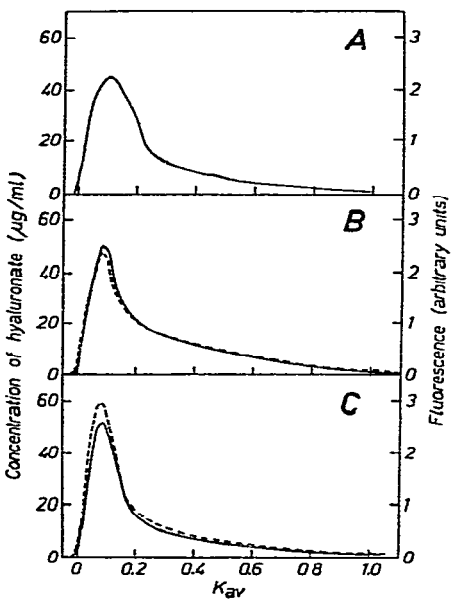
When a solution of the FITC-labelled hyaluronate was incubated at pH 7.48 at 37°, no hydrolysis of the fluorescein moiety could be detected even after one month.

Attempts to confirm the presence of the thiocarbamoyl linkage by means of u.v. spectroscopy were inconclusive, as the absorption of the hyaluronate at 260 nm

obscured the small spectral differences between the –NHCO– and –NHCNH– linkages. It was found, however, that FITC-labelled hyaluronate samples having significant protein content appear brown–orange, whereas those with insignificant contents are yellow. We have observed earlier that the fluorescein thiocarbamoyl chromophore is associated with a yellow colour, whereas the fluorescein thioureylene derivatives appear orange.

An alternative procedure using the isocyanide reaction[3,4] was also successful. The multicomponent condensation employing, in this case, fluorescein amine (abbreviated to FA), acetaldehyde, cyclohexyl isocyanide, and sodium hyaluronate proceeded under aqueous conditions at 22°, to give fluorescent hyaluronate (FA-hyaluronate). The new amide linkage was also stable when the product was incubated at pH 7.48 at 37° for one month.

Some depolymerization of the macromolecule is unavoidable during labelling, but the physico-chemical analysis reveals that this is not a serious problem. Although there is no suitable gel for fractionating the entire range of samples of hyaluronate of high molecular weight (M_w several millions), partial fractionation is effected by gel chromatography on Sepharose 2B, and this is particularly diagnostic of depolymerization of the molecule. Fig. 1 demonstrates the gel-chromatographic pattern of the



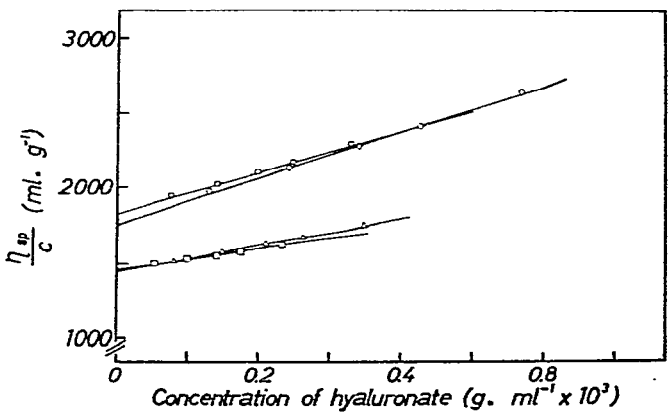


Fig. 1. Gel chromatography of unlabelled (*A*), FITC-labelled (*B*), and FA-labelled hyaluronate (*C*) on a column (1.6 × 100 cm) of Sepharose 2B. The chromatograms were followed by uronic acid (————) analysis and fluorometry (– – – –).

Fig. 2. Reduced viscosities plotted versus concentration for unlabelled (○), FITC-labelled (batch 1: △; batch 2: □), and FA-labelled hyaluronate (◇).

parent hyaluronate, a batch of FITC-labelled hyaluronate, and a batch of FA labelled hyaluronate. The labelled compounds contain more material at the tailing-end, indicating some degradation. The fluorescent label follows closely the distribution of uronic acid, indicating a uniform labelling of the material.

The viscosity data for the parent compound, two batches of FITC-labelled hyaluronate, and one batch of FA-labelled hyaluronate are presented in Fig. 2, and also indicate a slight degradation. The extrapolated values of the intrinsic viscosities are given in Table I, together with the molecular weights calculated from the viscosities.

TABLE I

PHYSICO-CHEMICAL DATA

	Viscosity data		*Ultracentrifuge data*[a]		
	Intrinsic viscosity ($ml.g^{-1}$)	*Molecular weight*	*Sedimentation coefficient* $s^0_{20,w} \times 10^{13}$ (s)	*Diffusion coefficient* $D^0_{20,w} \times 10^7$ ($cm^2.s^{-1}$)	*Molecular weight*
Unlabelled hyaluronate	1960	1.1×10^6			
FITC-labelled hyaluronate					
Batch 1	1460	0.78×10^6	4.9	0.34	1.0×10^6
Batch 2	1470	0.78×10^6			
FA-labelled hyaluronate	1860	1.0×10^6			

[a]The figures given represent the mean values for all samples.

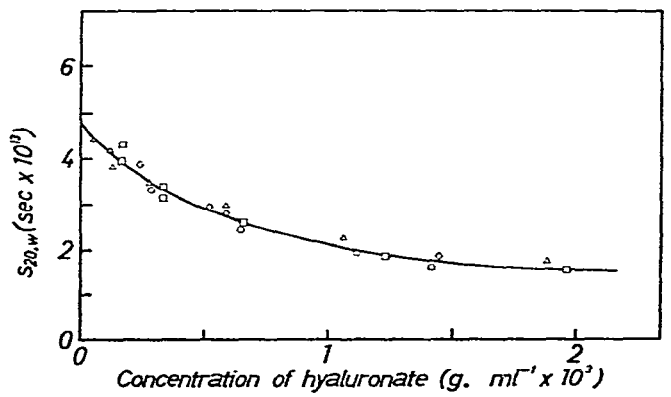


Fig. 3. Sedimentation coefficients for unlabelled (○), FITC-labelled (batch 1: △; batch 2: □), and FA-labelled hyaluronate (◇) plotted versus polysaccharide concentration.

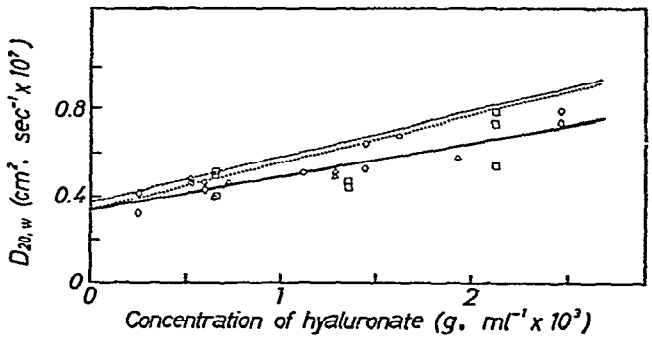


Fig. 4. Diffusion coefficients for unlabelled (○), FITC-labelled (batch 1: △; batch 2: □), and FA-labelled hyaluronate (◇). The data have been compared with the published concentration-dependence of the diffusion coefficient of hyaluronate preparations with molecular weights of 1.3×10^6 (– – – –) and 1.5×10^6 (·····)[5].

The sedimentation coefficients (Fig. 3) determined for these materials all extrapolate within experimental error to the same value of $s^0_{20,w}$. This corresponds[5,6] to a molecular weight in the order of 500,000. The determination of the diffusion coefficients by the techniques used involves a relatively larger error, but Fig. 4 demonstrates that the experimental values are close to, although below, the earlier published curve[5,6] for hyaluronate of a molecular weight of 1.5 million. The discrepancy in the values obtained with these two methods may be attributable to the fact that the earlier authors derived their data using hyaluronate from vitreous body, whereas the material in the present work was isolated from rooster comb. However, the molecular weight of the labelled and unlabelled hyaluronates described in this study, calculated from the sedimentation and diffusion data using the Svedberg equation[7], is in good agreement with the values derived by viscometry (Table I).

The physico-chemical analysis of the fluorescent hyaluronate thus shows that the degradation of the product during the labelling procedures is only slight and that the labelling is uniform. The material should therefore be well-suited for biological work. Investigations are also in progress to use the material for evaluating the motion of hyaluronate chains in concentrated hyaluronate chain-networks.

EXPERIMENTAL

General. — Hyaluronate prepared from rooster comb was obtained from Biotrics, Inc. (24 Beck Road, Arlington, Mass. 02174, U.S.A.). According to the manufacturer, the amount of protein was below the limit of detection. Aminofluorescein hydrochloride (mixed 5- and 6-isomers; Fluka, Switzerland) was converted to isothiocyanatofluorescein by established procedures[8,9]. Methyl sulphoxide was stored over Linde molecular sieves (Type 4A). Cyclohexyl isocyanide and dibutyltin diaurate were commercially available from Fluka.

Analytical techniques. — The concentration of hyaluronate was calculated from

the analysis of uronic acid using the carbazole reagent with D-glucurono-6,3-lactone as standard[10]. The fractions from gel chromatography were analyzed by a modification of the automated version of the same method[11]. Fluorescence was measured at 515 nm (excitation at 485 nm) in a Foci (Farrand Optical Co.) spectrofluorometer.

Measurement of d.s. of fluorescein hyaluronate. — A standard curve was constructed for methyl *N*-fluoresceinylthiocarbamate[2] by plotting concentration [$1–10 \times 10^{-9}$ mole per ml of 24mM borate (pH 9)] against absorption (493 nm). The fluorescein-hyaluronate samples (accurately weighed, ~50 mg) were dissolved in 25mM borate (pH 9, 100 ml), and the absorption was measured. The molar ratio of fluorescein to "anhydroglucose" units (mol. wt., 162) was then calculated (d.s.).

Preparation of FITC-hyaluronate. — Sodium hyaluronate (0.2 g) was dissolved in formamide (40 ml) by gentle agitation on a laboratory shaker for periods of 12–48 h. If dissolution was incomplete after two days, the residue was filtered off on a G-2 glass filter. Methyl sulphoxide (50 ml) was added together with sodium hydrogen carbonate (0.1 g), dibutyltin dilaurate (0.1 g), and isothiocyanatofluorescein (0.3 g), and the reaction mixture was stirred on a steam bath for 0.5 h. The product was diluted with water (50 ml) and precipitated in ethanol (2 l) containing a few drops of saturated, aqueous sodium chloride. The precipitate was allowed to sediment, and the supernatant was decanted. The precipitation was repeated until the product was free from non-bound fluorescein. This was readily checked by t.l.c. (Merck Kieselgel F_{254}) with chloroform–methanol (3:1). Inspection under u.v. light revealed any fast-moving, free fluorescent substances. The purified FITC-hyaluronate was dried *in vacuo* at 50° for 12 h. Yield, 0.17 g; d.s., 0.002.

Coupling of fluorescein amine to hyaluronate using isocyanide. — A solution of hyaluronate (50 mg) in water (40 ml) was diluted with methyl sulphoxide (20 ml), whereupon acetaldehyde (25 μl), cyclohexyl isocyanide (25 μl), and fluorescein amine (25 mg) dissolved in a small volume of methyl sulphoxide were added (the pH of the mixture should not exceed 5–7). After 5 h at 22°, the reaction mixture was poured into ethanol (~800 ml). The precipitated hyaluronate was collected and reprecipitated from water in ethanol containing a few drops of saturated, aqueous sodium chloride. The FA-hyaluronate was dried as above. Yield, 46 mg; d.s., 0.0013.

Stability of thiocarbamoyl and amido linkages. — Solutions (0.6%) of the FITC-labelled hyaluronate and the FA-hyaluronate in 0.1M phosphate (pH 7.48) were separately incubated at 37°. At weekly intervals, a sample of each solution was examined by t.l.c. (as in the FITC-hyaluronate synthesis above) for the presence of fluorescent compounds of low molecular weight.

Gel chromatography. — The hyaluronate samples (1.5 mg in 2 ml of 0.154M sodium chloride) were applied to a Sepharose 2B column (1.6 × 100 cm), which was eluted with 0.154M sodium chloride at 15 ml/h. Determination of void and total volumes was performed as described[12], and the position of the elution expressed[12] as K_{av}.

Viscometry. — An automated Ubbelohde viscometer (Viscomat 1 5200) with a shear stress of 16 $dyn.cm^{-2}$ and a shear rate in the order of 1,000 s^{-1} was used. The

reduced viscosity was measured at five concentrations of hyaluronate dissolved in 0.18M sodium chloride and 0.01M sodium phosphate (pH 7.4) at 20°. The intrinsic viscosity thus obtained was corrected for zero shear according to the method of Cleland and Wang[6]. The molecular weight was calculated according to the following equation for the dependence of intrinsic viscosity ($[\eta]$) on molecular weight (M) of hyaluronate in 0.2M sodium chloride[6]: $[\eta] = 0.0228 \times M^{0.816}$.

Ultracentrifugation. — Ultracentrifugation was carried out at 20° in a Spinco Model E Analytical Ultracentrifuge equipped with RTIC-unit, electronic speed control, Schlieren and interference optics, monochromatic light source, and a photoelectric scanning system. All runs were made in 0.18M sodium chloride and 0.01M sodium phosphate (pH 7.4). The value 0.66 ($cm^3.g^{-1}$) for the partial specific volume of hyaluronate was used[13].

Sedimentation runs were performed in 12-mm cells at 52,000 r.p.m. Unlabelled material was followed by Schlieren optics, and labelled material by absorption optics at 495 nm. Sedimentation coefficients were calculated by standard procedures; the values were corrected for the viscosity and density of the medium[14].

Diffusion coefficients were determined in synthetic boundary cells in the ultracentrifuge. Buffer was layered over hyaluronate solutions. The centrifuge was run at 10,000 r.p.m. and the boundary registered at regular intervals up to 3 h. Unlabelled material was followed by Schlieren optics and the diffusion coefficient calculated by the height-area method[15]. Labelled hyaluronate was followed by absorption optics and the diffusion coefficient determined from boundary spreading as described by Chervenka[7] and Laurent *et al.*[16]. The diffusion values were corrected for the viscosity of the medium[15].

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