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Note

Preparation and properties of fluorescein-labelled dextrans

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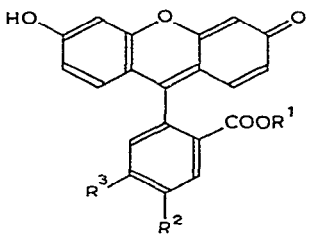
Fluorescein-labelled dextrans are valuable materials for studying permeability and microcirculation *in vivo*[1,2]. Labelling by means of fluorescence offers considerable advantages over conventional dyeing procedures[3], particularly the very much lower degree of substitution (d.s.) at which fluorescence measurements can be made.

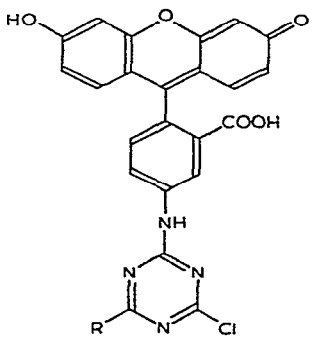
Thus, isothiocyanatofluorescein (mixed 5- and 6-isomers; **1**) reacts with dextran in methyl sulphoxide at elevated temperatures to give *O*-(fluoresceinylthiocarbamoyl)dextrans (FITC-dextran, **2**). The reaction is catalysed by dibutyltin dilaurate[4], and the d.s. of the products is in the range 0.01–0.001. Depolymerisation during the reaction is negligible, but care must be taken that no acidic impurities are introduced with the reagents.



1 $R^1 = R^2 = H$; $R^3 = NCS$
$R^1 = R^3 = H$; $R^2 = NCS$

2 $R^1 = R^2 = H$; $R^3 = NHCSODextran$
$R^1 = R^3 = H$; $R^2 = NHCSODextran$

4a $R^1 = R^2 = H$; $R^3 = NHCSOCH_3$
b $R^1 = R^3 = H$; $R^2 = NHCSOCH_3$



3 R = Cl

Although FITC-dextrans are satisfactory for most purposes, an alternative procedure, using the fluoresceinyltriazine derivative[5] (**3**), was also effective. This reagent could be coupled to dextran in aqueous solution at pH 8–10.

The d.s. of the FITC-dextrans was determined spectrophotometrically. For this purpose, methyl *N*-fluoresceinylthiocarbamate (**4a,b**) was synthesised as a model compound. Its absorption maximum (493 nm) coincided with that of FITC-dextran.

The chromatographic properties of **4** have previously been described[6], but the compound was not further characterised. The n.m.r. spectrum of **4** affords convincing evidence for the structure—particularly the *O*-methyl peaks at τ 5.9 and 6.02 assigned to the 5- and 6-isomers, respectively. No SMe signal, which would be expected higher upfield, was observed. The larger peak (τ 5.9) was assigned to the 5-isomer after inspection of the n.m.r. spectrum for the 6-isomer of **4** prepared from a pure sample of 6-aminofluorescein. The 5-isomer is also the preponderant isomer in the isothiocyanatofluorescein used.

The thiocarbamoyl linkage is stable at pH 4 and 20–30° for at least 1 month. At pH 9 (20°), little hydrolysis could be detected even after 1 month. However, at 35°,

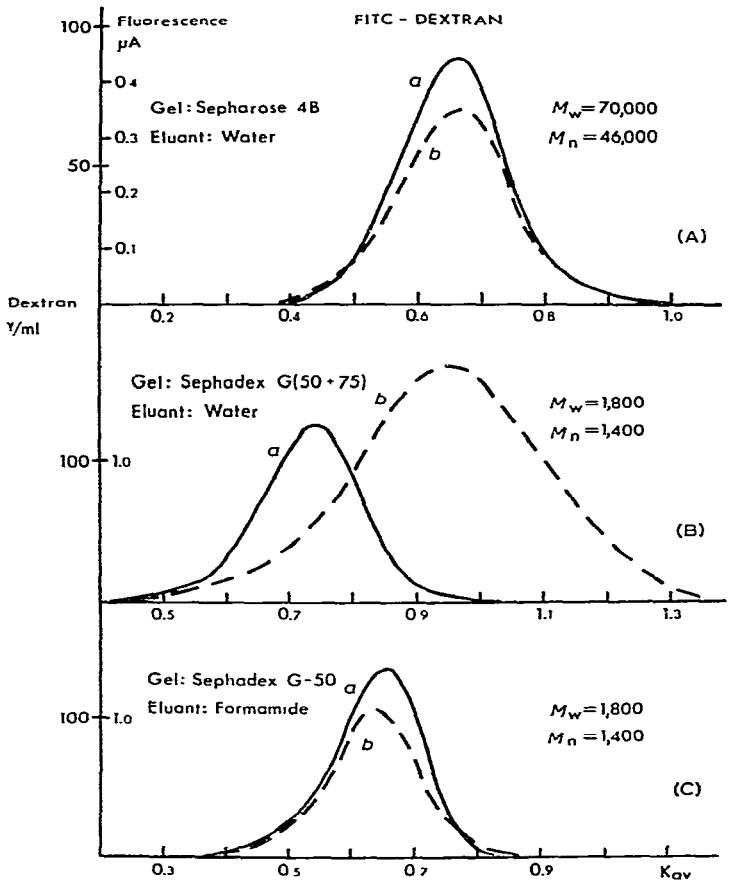


Fig. 1A. Elution curves of FITC-dextran ($\bar{M}_w = 70{,}000$) detected by (*a*) anthrone reaction and (*b*) fluorescence. Gel: Sepharose 4B; eluant: water.
Fig. 1B. Elution curves of FITC-dextran of low molecular weight ($\bar{M}_w = 1{,}800$): (*a*) anthrone reaction, (*b*) fluorescence. Gel: Sephadex G (50+75); eluant: water. The molecular weights were computed according to curve (*a*).
Fig. 1C. Elution curves of FITC-dextran as in B. (*a*) Anthrone reaction, (*b*) fluorescence. Gel: Sephadex G-50; eluant: formamide.

hydrolysis after 1 month was appreciable. Extensive studies *in vivo* have revealed that the linkage is stable for at least 24 hours under physiological conditions[2], and presumably remains intact for a longer period.

The distribution of the fluorescein label was examined by gel chromatography[7]. It was found that, for molecular weights ($\overline{M}_w$) exceeding 10,000, both traces coincided, indicating a uniformly distributed label (Fig. 1A). However, when a FITC-dextran ($\overline{M}_w$ <10,000) was chromatographed on Sephadex G-75, a distinct retardation of the fluorescence trace was observed (see Fig. 1B). This phenomenon could be overcome by eluting the gel (Sephadex or cross-linked agarose[8]) with formamide. Under these conditions, it was necessary to perform the carbohydrate analysis manually because of the steady evolution of gas during the anthrone reaction. The carbohydrate and fluorescence traces for a FITC-dextran ($\overline{M}_w$ 1,800) are shown in Fig. 1C and indicate an even distribution of the fluorescein label.

From these results, we conclude that interaction between the gel and the fluorescein moiety in aqueous media becomes increasingly important as the gel used becomes more densely cross-linked and as the carrier molecule becomes smaller.

The procedures described here have been used for labelling other polysaccharides, *e.g.*, starches, starch derivatives, and glycosaminoglycuronoglycans.

EXPERIMENTAL

Dextran B-512, in fractions of various molecular weights, was obtained from Pharmacia AB, Uppsala. Isothiocyanatofluorescein (mixed 5- and 6-isomers) was prepared from aminofluorescein by established procedures[9]. The fluoresceinyltriazine derivative (3) was prepared according to Barskii *et al.*[5]. Thin-layer chromatography was performed on Merck Kieselgel F_{254} with chloroform–methanol (3:1).

Gel filtration. — The gel filtrations were carried out as described earlier[7]. The fluorescence was measured at 520 nm (excitation 493 nm) after dilution of the fractions with 50mM borate (pH 8.5). For the formamide runs, the formamide was first passed through a short column of Celite to prevent discolouration of the Sephadex gel. A sample applicator, rather than an adaptor, was used in order to reduce the risk of bubble formation at the surface. The column was calibrated and eluted as for the aqueous runs. Carbohydrate was analyzed in an Auto-Analyser by means of the anthrone reagent, except in the case of the formamide fractions.

Preparation of FITC-dextran. — Dextran ($\overline{M}_w$ 70,000; 1 g) was dissolved in methyl sulphoxide (10 ml) containing a few drops of pyridine. Isothiocyanatofluorescein (1) (0.1 g) was added, followed by dibutyltin dilaurate (20 mg), and the mixture was heated for 2 h at 95°. After several precipitations in ethanol to remove free dye, the FITC-dextran was filtered off and dried *in vacuo* at 80°. Yield, 0.9 g; d.s. 0.001.

Reaction between the fluoresceinyltriazinyl derivative (3) *and dextran.* — The fluorescein reagent (3, 200 mg) was added portionwise to a solution of dextran ($\overline{M}_w$ 5,000; 1 g) in water (30 ml). The pH was maintained at 10 by means of a pH-stat

coupled to M sodium hydroxide. After 2 h, the product was precipitated with ethanol, re-precipitated 3 times from aqueous solution with ethanol, filtered off, and dried to give the fluorescent dextran. Yield, 0.7 g; d.s. 0.025.

Preparation of methyl N-*fluoresceinylthiocarbamate* (**4**). — Isothiocyanatofluorescein (mixed isomers, 1 g) was dissolved with warming in methanol (25 ml), and the solution was kept for 4 days at room temperature. Evaporation of the methanol, with recrystallisation of the residue from methanol–water, afforded **4** (0.4 g), m.p. >360°, which was a mixture of isomers. N.m.r. data (60 MHz, methanol-d_3): τ 2.1–3.5 (9 protons, fluorescein ring system), 5.9 and 6.02 (3 protons; 2 OMe singlets, ratio of intensities 2:1). U.v. data: λ_{max} 493 nm (25mM borate, pH 9).

Anal. Calc. for $C_{22}H_{15}NO_6S$: C, 62.7; H, 3.56. Found: C, 62.4; H, 3.80.

Preparation of methyl N-*fluoresceinylthiocarbamate* (**4a**, 6-isomer). — 6-Isothiocyanatofluorescein was prepared from 6-aminofluorescein[9], and treated as described above to give **4a**, m.p. >360° (dec.). N.m.r. data (60 MHz, methanol-d_3): τ 2.1–3.5 (9 protons, fluorescein ring system), 6.02 (3 protons, OMe).

Measurement of d.s. of FITC-dextran. — For **4**, a standard curve was constructed of absorption (493 nm) against concentration [$1–10 \times 10^{-9}$ mole of **4** per ml of 25mM borate (pH 9)]. The FITC-dextran 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 was calculated.

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