The electron microscopy of fluorescent dextrans (FITC-dextrans) in thin sections of tissue

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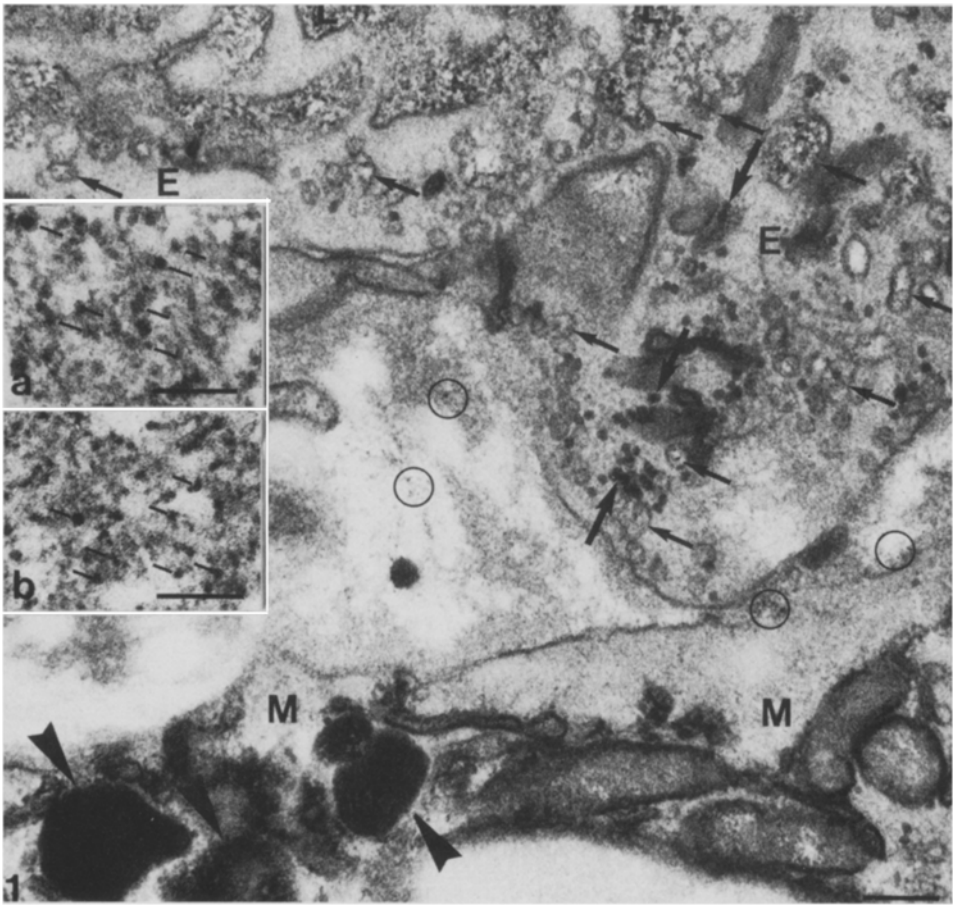
Summary. Fluorescein-coupled dextrans (FITC-Ds) were detected in sections in the electron microscope using 4 different contrasting methods when tissue was postfixed in osmium tetroxide. Endogeneous glycogen was also stained by the methods used, but FITC-D particles appeared smaller and more irregular, and in other locations. In dextranase treated specimens glycogen was still visible, while FITC-D was not.

The coupling of fluorescein-isothiocyanate to dextran extends the usefulness of dextrans as electron microscopic tracers[2] by permitting combined in vivo fluorescence stereo microscopy of the microcirculation, fluorescence stereo microscopy of fixed tissue and high-power fluorescence microscopy of sections of embedded tissue. For electron microscopy, dextrans as well as fluorescein-isothiocyanate coupled dextrans (FITC-D) have to be visualized by special staining methods. Excellent electron microscopic contrast of FITC-D was obtained by Hulström and Svensjö[3,4], who applied the one step fixation and contrasting technique (with glutaraldehyde, osmium tetroxide and lead) of Simionescu et al.[5]. Unfortunately the osmium tetroxide destroys the fluorescence, so that only in vivo fluorescence stereo microscopic and electron microscopic demonstration of the tracer are possible by that method. Aldehyde fixatives do not destroy the fluorescence, and to achieve complete utilization of FITC-Ds as tracers the methods employed should involve only a primary aldehyde fixation for tissue specimens designed for fluorescence light microscopy, and a secondary fixation with osmium tetroxide for tissue specimens designed for electron microscopy. The electron microscopic contrast of FITC-D can then be obtained using a modified osmium tetroxide fixation or by specific staining of thin sections.

Among different methods employed, the four giving the best results for contrasting FITC-D will be reported here and the results compared with controls.

Material and methods. Male Wistar Furth rats, 200-250 g, were anesthetized with mebumal 4 mg/100 g b.wt and i.v. injected with 3.8-8.2% (w/v) solutions of FITC-D (Pharmacia Fine Chemicals AB. Box 181. S 75104 Uppsala 1, Sweden) in 0.9% NaCl using 0.5 ml/100 g b.wt. Average molecular weights of the FITC-Ds used were 40,000, 70,000 and 150,000. Between 30 sec and 24 h later, the small intestine was fixed in situ by immersion for 5 min[6]. The intestine and other organ samples were excised and immersed in the fixative for an additional 18-24 h at 4 °C. The fixatives employed were a) 6% glutaraldehyde or b) 4% glutaraldehyde and 3% formaldehyde in a 0.08 M phosphate buffer, pH 7.2. The aldehyde fixed tissue was cut into small pieces and the specimens photographed using a



Figures 1 and 2. Electron micrographs of intestinal pericryptal venoles of a rat which was injected with FITC-D 150 24 h and again 15 min before immersion fixation in situ. Before post-fixation with modified osmium tetroxide fixative the tissue was either untreated (fig. 1) or treated with dextranase (fig. 2). Sections were stained with alkaline bismuth and lead citrate.

Figure 1. The section is slightly oblique to the endothelial cell. Dextran particles are seen in the lumen (L) of the venole, in vesicles (arrows) of the endothelium (E), in the paravascular connective tissue (circles) and in phagocytic vacuoles (arrow heads) of a macrophage (M).

fluorescence stereo microscope. Half of the specimens were treated with 2% osmium tetroxide in 0.08 M phosphate buffer, pH 7.2, for 2 h at 20 °C for electron microscopy. These specimens, and the other half, were then rinsed in 2 changes of 5.5% glucose in distilled water for 10 min and continuously dehydrated[7] in ethylene alcohol from 30% or in acetone from 25% and embedded in Epon 812. Guided by the fluorescence stereo micrographs and by 1 µm sections stained for dextran[6], pyramids for ultrathin sectioning were placed at desired levels of the osmium tetroxide treated specimens. Ultrathin sections 600–800 Å, were cut on a LKB Ultratome III ultramicrotome and examined in a Philips 300 or 400 T electron microscope.

Controls: a) Tissue specimens from rats which were not injected with FITC-D, and b) tissue specimens containing FITC-D, but treated with the highly specific enzyme dextranase (obtained from Pharmacosmos A/S. DK-4130 Viby, Denmark) in 1% solution in 0.1 M McIlvain phosphate buffer, pH 5.8, at 37 °C for 2–3 h following the primary fixation and dissection.

FITC-D contrasting for electron microscopy: a) Modified osmium tetroxide fixation: specimens designed for electron microscopy were treated with 1.5% osmium tetroxideein 0.08 M phosphate buffer which contained 0.05 M $K_3Fe(CN)_6$ (originally introduced by De Bruijn[8] for staining glycogen and with results very much like those reported by Ainsworth[9]). Thin sections were stained with lead citrate[10] or alkaline bismuth[11,12] and lead citrate.

b) Ultrathin sections of tissue treated with 2% osmium tetroxide collected on copper or nickel grids were stained in one of the following ways:

1. Modified alkaline bismuth: sections were treated with 0.25% Na_2SO_3 (10–15 min, 40 °C), rinsed in decreasing concentrations of ethylene alcohol, stained with alkaline bismuth (20–40 min, 40 °C) followed by lead citrate (3 min, 40 °C).
2. Nickel grids were treated in sequence with 1% periodic acid (5–15 min, 20 °C), 1% sodium chlorite in 5% acetic acid (10 min, 20 °C), 5% uranyl acetate (10 min, 40 °C) and lead citrate (1 min, 40 °C). Modified after Vye and Fischman[13].
3. Uranyl acetate at pH 9. The staining solution was produced by mixing 5 ml 0.1 M uranyl acetate with 10 ml 0.2 M K_2CO_3, both in double distilled water, and then adding 3 ml 1% uranyl acetate in absolute ethylene alcohol (40 min, 40 °C followed by lead citrate 1–3 min, 40 °C).

Results. The electron microscopic appearance of FITC-D granules in sections differed a little depending on the contrasting method employed. In all cases the granules varied a little in size, being about 170–190 Å, 110–120 Å and 80–90 Å for FITC-D 150, 70 and 40, respectively. The granules were slightly irregular or polygonal in shape, and sometimes showed small thread-like ramifications and a slight tendency to aggregation (insets a–d, figs 1 and 2). The particle density was highest where particles lay freely in the interstitial space or in vascular lumina (fig. 1). Depending on molecular weight and circulation time the FITC-Ds were found in one or more of the following locations: vascular lumina; caveolae, vesicles and fenestrae of endothelial cells (fig. 1); occasionally in vesicles of vascular smooth muscle; between the formed elements of the interstitial connective tissue (fig. 1); in vesicles, vacuoles and phagosomes of macrophages (fig. 1); in vesicles and junc-

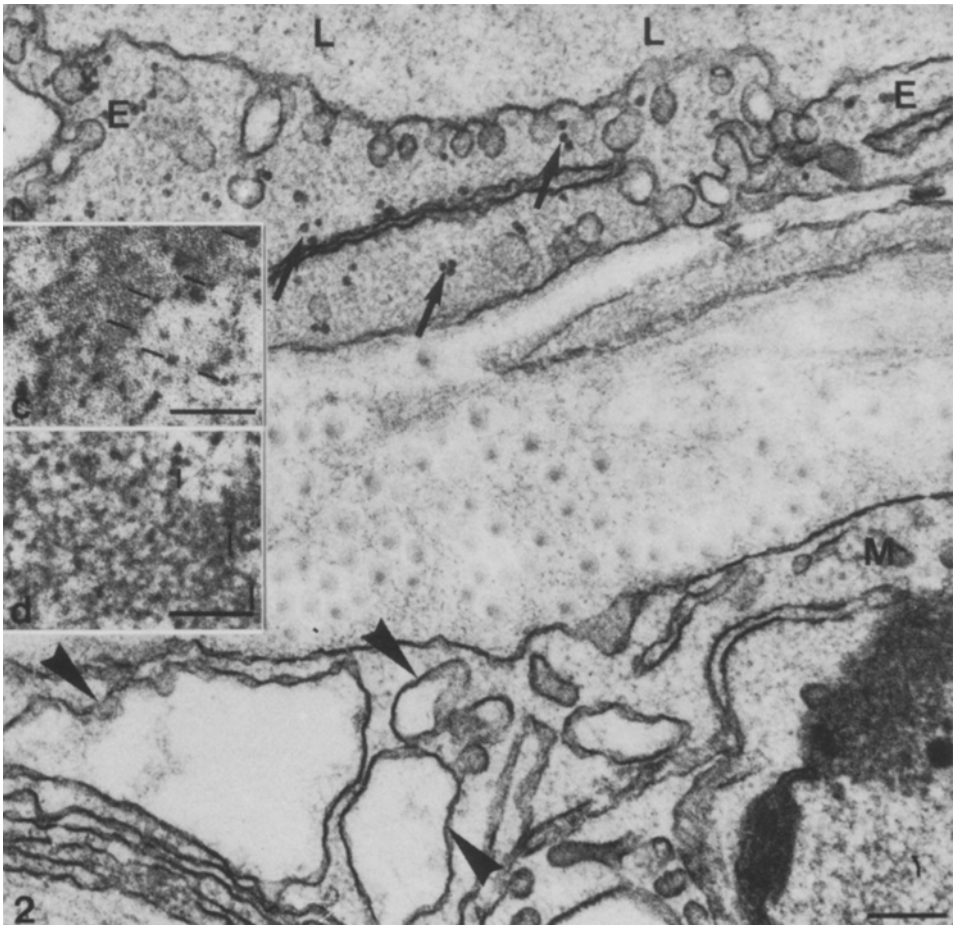


Figure 2. Dextran particles are not found contrary to the endogenous glycogen (thick arrows), which differ from the dextran particles with respect to size and shape. Insets a, b, c and d show the appearance of dextran particles in plasma when stained with alkaline bismuth following postfixation in modified osmium tetroxide fixative (a), and when stained with alkaline bismuth (b), uranyl acetate following periodic acid oxidation (c) and uranyl acetate at pH 9 (d) following postfixation in 2% buffered osmium tetroxide. The bars indicate 0.2 µm in figures 1 and 2 and 0.1 µm in insets a–d.

tions of lymphatic endothelium; in lumina of lymphatics and occasionally also in vesicles of other cell types. In sections of control experiments the FITC-D contrast was absent, was very weakened or had disappeared contrary to the contrast of intracytoplasmic glycogen (fig. 2).

Discussion. The 4 contrasting methods employed all showed an electron microscopic appearance of FITC-D which was in accordance with the findings on dextrans by Simionescu and Palade[2] and Simionescu et al.[5], but in most respects differed from the results of a recent paper on FITC-D by Heinzmann[14]. These differences concern the appearance, size and location of particles, since the FITC-Ds as described by Heinzmann[14] were non-aggregated, rounded particles observed obviously freely scattered in the cytoplasm and with a size of 310–400 Å as measured on the figures.

The 4 staining methods employed are all equally simple to use, but a somewhat grainy contrast is often the result of the alkaline bismuth staining methods, whereas the method involving periodic acid oxidation often results in contamination of sections. The observation of dextran in endothelial vesicles is unequivocal when using alkaline bismuth stain after postfixation with modified osmium tetroxide and when using uranyl acetate at alkaline pH. The latter method also yields good results in observing intercellular dextran particles in the connective tissue. The methods used were found to be complementary to each other, none of the methods being specific for dextran. Since glycogen is also a carbohydrate, it will be stained as well; however, the size, regular surface, form and localization of glycogen make it easily distinguishable from dextran. Ribosomes, among other structures, might also be stained, but differ from dextrans with respect to localization and regularity. When the amount of FITC-D in the tissue specimens is small or the content of FITC-D has been partly washed out by vascular perfusion, the particles might be difficult to detect, while glycogen, for example, will still be easily detected. In such cases the study of controls should enable one to distinguish between dextran and other particulate structures. In conclusion, FITC-Ds are very valuable tracers when used with the necessary precautions including controls.

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