Chapter 4

Preparation of Fluorescently Labeled Dextrans and Ficolls

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I. Introduction

Electroneutral, hydrophilic polysaccharides, such as dextran and Ficoll, are valuable and versatile tools in the biomedical sciences. Traditionally used as inert colloids in perfusion studies and in the isolation of cells and organelles by phase separation or density gradient centrifugation, dextran and Ficoll are also utilized in a variety of experimental strategies that require inert macromolecules. Two examples of this are the use of size-fractionated dextran for studying the permeability of capillaries and basement membranes (e.g., Grotte, 1956; Simionescu and Palade, 1971; Farquhar, 1982), and the use of Ficoll derivatives as carriers for eliciting antibodies to small haptens (Mosier *et al.*, 1974; Inman, 1975).

Dextran is a poly-D-glucose of very high molecular weight, with sparse, short branches that is produced by several strains of bacteria (Granath, 1958; Larm *et al.*, 1971). Commercially available dextran fractions of

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different average molecular weight ($\overline{M}_w$) are obtained from the natural product of *Leuconostoc mesenteroides* by partial acid hydrolysis, followed by size fractionation. The polydispersity of the commercial product prepared by Pharmacia (Pharmacia Fine Chemicals, Piscataway, New Jersey), defined as the ratio of $\overline{M}_w$ to the number average molecular weight ($\overline{M}_n$), is about 1.6–2.0 (Basedow and Ebert, 1979; Laurent and Granath, 1967). In solution, dextan molecules of $\overline{M}_w$ greater than 2000 are almost perfect statistical coils, and show no detectable self-association (Basedow and Ebert, 1979). Ficoll is a synthetic polymer of sucrose and epichlorohydrin (Holter and Moller, 1956). Its high degree of branching and internal cross-linking make it a more compact molecule than dextran on a molecular-weight basis (Laurent and Granath, 1967; Bohrer *et al.*, 1984). The polydispersity of Ficoll from Pharmacia is about 1.6 (Laurent and Granath, 1967; Bohrer *et al.*, 1984).

The addition of a fluorophore to dextran or Ficoll extends their usefulness to the realms of fluorescence spectroscopy and fluorescence microscopy. The focus may be on either the polysaccharide or the fluorophore. Thus, an environment-insensitive fluorophore may be used as a marker for detecting the polysaccharide, or the polysaccharide may be used as in a carrier for an environment-sensitive fluorophore in order to probe parameters of the chemical environment, such as pH, calcium ion concentration, and solvent polarity.

Fluorescein dextran was originally used by Arfors and Hint (1971) to visualize the microcirculation. The use of fluorescent dextrans in microcirculation studies has been reviewed recently by Thorball (1981).

Like the underivatized polysaccharides, size-fractionated, fluorescent derivatives of dextran and Ficoll can be used as "molecular rulers" in studies of permeability. For example, fluorescein dextran has been used to monitor changes in the permeability of the blood–brain barrier (Mayhan and Heisted, 1985) and to study the diffusion of macromolecules in the extracellular matrix of tumors (Nugent and Jain, 1984). Fluorescein dextran has also been used to estimate the size of nuclear pores in studies of the exchange of macromolecules between cytoplasm and nucleus (Lang *et al.*, 1986; Jiang and Schindler, 1986). By a similar rationale, narrowly size-fractionated fluorescein and rhodamine derivatives of dextran and Ficoll have been used as inert tracer particles to probe the submicroscopic structure of the cytoplasmic ground substance in living cells by fluorescence recovery after photobleaching (FRAP) and digital fluorescence microscopy (Luby-Phelps *et al.*,1986, 1987, 1988; Luby-Phelps and Taylor, 1988).

Using a technique originally developed by Okhuma and Poole (1978), several investigators have used fluorescein derivatives of dextran to measure intracellular pH (e.g., Heiple and Taylor, 1982; Rothenberg *et al.*,

1983; Tanasugarn *et al.*, 1984; Bright *et al.*, 1987; Paradiso *et al.*, 1987) and to follow the acidification of endosomes (e.g., Tycko and Maxfield, 1982; McNeil *et al.*, 1983; Murphy *et al.*, 1984). A modification of this approach has been used to monitor proton uptake by vesicles reconstituted with a Na^+/H^+-ATPase (Hara and Nakao, 1986).

The fact that dextran and Ficoll cannot cross biological membranes makes them ideal as markers and probes of specific subcellular compartments. Fluorescein dextran was first reported as a marker for fluid phase pinocytosis by Berlin and Oliver (1980). More recently, a red-fluorescent, cyanine derivative of dextran has been used to mark the pinosome compartment in a multiparameter study of living Swiss 3T3 cells (DeBiasio *et al.*, 1987). Because they do not appear to bind to intracellular components, fluorescent dextran derivatives have been microinjected into living cells as volume markers and as a control for fluorescent analogs of functional macromolecules (Lanni *et al.*, 1985; Gingell *et al.*, 1985; Luby-Phelps *et al.*, 1985; DeBiasio *et al.*, 1987). Dextrans small enough to penetrate the nuclear envelope can be used to mark the total accessible volume of the cell, exclusive of membrane-bounded organelles or vesicles, while dextrans larger than the size of nuclear pores can be microinjected into either the nucleus or the cytoplasm to mark those compartments separately. Internalized fluorescein dextran has recently been utilized in the isolation of endosomes from cell lysates by flow cytometry and fluorescence-activated sorting (Murphy, 1985). Fluorescent dextran or Ficoll trapped within membrane-bounded vesicles can be used to assay vesicle fusion, both *in vivo* (Goren *et al.*, 1984) and *in vitro* (Sowers, 1986; Stutzin, 1986). Fluorescein dextran has been used to assay the efficiency of methods for bulk-loading macromolecules into living cells (McNeil *et al.*, 1984; Fechheimer *et al.*, 1986) and has also been microinjected into embryos as a marker for single cells to obtain fate maps and cell lineages during development (e.g., Gimlich and Braun, 1985). Additional references on the properties and uses of dextran, Ficoll, and fluorescent derivatives of dextran are available in bibliographies furnished upon request from Pharmacia and from Molecular Probes, Inc. (Eugene, Oregon).

Fluorescein isothiocyanate derivatives of dextran (FTC-dextran)[1] are available from Pharmacia or Sigma (St. Louis, Missouri,), and Molecular Probes now offers dextrans labeled with several different fluorophores. Molecular Probes dextrans generally have high substitution ratios and are intended for studies where sensitive detection of small amounts of material

[1]Abbreviations: FITC, fluorescein isothiocyanate; FTC-, fluorescein thiocarbamoyl-; TRITC, tetramethylrhodamine isothiocyanate; TRTC-, tetramethylrhodamine thiocarbamoyl; BSA, bovine serum albumin; AECM, aminoethylcarboxymethyl.

is desired. However, at present, no fluorescent derivatives of dextran of $\overline{M}_w > 150{,}000$ are available commercially, nor are any fluorescent derivatives of Ficoll. In addition, many useful fluorescent probes have been developed recently, but are not yet available on dextrans (for review, see Waggoner, 1986). Fortunately, custom-tailored fluorescent derivatives can be prepared from commercially available dextran (e.g., T500, T1000, T2000 from Pharmacia or Sigma) and Ficoll (Ficoll 70, Ficoll 400 from Pharmacia) by the methods described below.

TABLE I

DEBELDER AND GRANATH METHOD

Materials

Anhydrous DMSO (10 ml)	Dextran or Ficoll (1 g)
Pyridine (0.3 ml)	FITC or TRITC (100 mg)
Dibutyl tin dilaurate (20 μl)	95% Ethanol (1 liter)

Procedure

1. Mix 10 ml anhydrous dimethyl sulfoxide (DMSO), 0.3 ml pyridine, and 20 μl dibutyl tin dilaurate in a 50 ml KIMAX screw top tube. After opening, DMSO should be stored with molecular sieve in a closed container with desiccant.
2. Add 1 g dextran. Place tube in 95°C water bath and allow dextran to dissolve. This may take several hours.
3. Add 100 mg FITC or TRITC, mix well, and incubate at 95°C for 2 hours.
4. Divide reaction mixture evenly into two or three 30-ml Corex tubes and add 15 ml ethanol to each tube while vortexing. This precipitates the dextran, leaving free dye in the supernatant.
5. Add an additional 10 ml ethanol to each tube.
6. Centrifuge at room temperature for 10 minutes at 10,000 *g* to collect labeled dextran.
7. Remove the supernatant and test it for unprecipitated dextran by adding ethanol. If none, discard supernatant. Otherwise, add more ethanol and centrifuge as before to collect precipitate.
8. Add 10 ml ethanol to each pellet. Resuspend the pellets as well as possible. They will be very gooey.
9. Centrifuge as before to collect precipitate.
10. Repeat steps 7–9 twice more. The pellets will become less gooey and more brittle each time.
11. After final spin, discard the supernatant and resuspend the pellets in 4 ml glass distilled water. Let sit at room temperature for 1 hour.
12. Transfer resuspended pellets to dialysis tubing and dialyze versus 2 × 4 liters distilled water overnight to redissolve labeled dextran. For dextrans of MW ≤ 20,000, low-molecular-weight dialysis tubing should be used to avoid loss of material to the dialysis medium.
13. Clarify dextran solution by centrifugation at 20,000 rpm in a Sorvall SS-34 rotor, or equivalent, for 30 minutes at room temperature.
14. Shell freeze and lyophilize the supernatant. Lyophilized dextran should be stored as a powder, desiccated, at −20°C for best results.

II. Methods

A. Preparation of Fluorescent Derivatives of Polysaccharides

Detailed procedures for preparing fluorescent derivatives of polysaccharides, such as dextran and Ficoll, are given in Tables I–III. Comments on the procedures are presented below.

TABLE II

INMAN METHOD: A. PREPARATION OF AECM-DERIVATIVES

Materials

Chloroacetic acid (6.44 g)	2 *M* NaH_2PO_4 (1 ml)
5 *N* NaOH (20 ml)	6 *N* HCl, for pH titration
10 *N* NaOH (5 ml)	1 *N* NaOH, for pH titration
Ficoll or dextran	Ethylenediamine dihydrochloride (1 g)

1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (1 g)

Procedures

1. Prepare a stock solution of 1.35 *M* sodium chloroacetate by dissolving 6.44 g of chloroacetic acid in a mixture of 30 ml of distilled water and 13.5 ml of 5.0 *N* NaOH. Cool to 25°C, adjust pH to 6.8–7.2 with either 5 *N* NaOH or 10% (w/v) chloroacetic acid, and dilute to 50 ml with distilled water. This should be made fresh each time.
2. Dissolve 1.33 g of Ficoll or dextran in 18.5 ml of 1.35 *M* sodium chloroacetate, and place in 25°C water bath.
3. Add 5 ml of 10 *N* NaOH, followed by 1.5 ml distilled water, to bring the total volume to 25 ml.
4. Incubate for 30 minutes.
5. Stop the reaction with 0.2 ml of 2 *M* NaH_2PO_4.
6. Titrate the mixture to pH 7.0 with 6 *N* HCl.
7. Dialyze extensively (4 × 2 liters) at 4°C against distilled water (saturated with toluene to retard microbial growth. Do not use azide). The dialyzed product may either be used directly, or lyophilized for storage.
8. Measure the volume of the dialyzed solution and add ethylenediamine dihydrochloride to 1.0 *M* (14.3 g/100 ml). If using lyophilized sample, dissolve 25 mg/ml in distilled water and add 5.7 mg ethylenediamine dihydrochloride per mg of sample.
9. Titrate to pH 4.7 with 1 *N* NaOH (approximately one drop).
10. Add 0.5 mg of 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride per mg of Ficoll or dextran (665 mg for 1.33 g), with stirring, over the course of 10 minutes, while maintaining the pH near 4.7 with 1 *N* NaOH.
11. React for 3.5 hours at room temperature with stirring, while maintaining the pH near 4.7.
12. Dialyze at 4°C versus 3 × 4 liters of toluene-saturated distilled water, followed by one change of 4 liters of distilled water without toluene.
13. Lyophilize and store desiccated.

TABLE III

INMAN METHOD: B. LABELING OF AECM-DERIVATIVES WITH FITC OR TRITC

Materials

AECM-Ficoll or -dextran (100 mg)
10 m*M* Carbonate–bicarbonate buffer, pH 9.2 (3 ml)
FITC or TRITC (15 mg)
0.1 *N* NaOH for pH titration
Sephadex G-25

Procedures

1. Dissolve 100 mg AECM-Ficoll or -dextran in 2 ml of carbonate–bicarbonate buffer at pH 9.2.
2. Dissolve dye in 1 ml of same buffer:
   a. Dissolve 15 mg of TRITC in 400 μl dimethyl formamide (DMF) and add dropwise, with vortexing, to 1 ml of carbonate–bicarbonate buffer. Adjust pH to ≥ 90 with 0.1 *N* NaOH.
   b. Dissolve 15 mg of FITC in 1 ml of buffer. Adjust pH to ≥ 9.0 with 0.1 *N* NaOH. FITC may not go into solution completely until pH has been adjusted.
3. Add dye solution dropwise with stirring to AECM-Ficoll or -dextran solution.
4. Incubate at 40°C for 30 minutes.
5. Desalt on 1 × 30 cm column of Sephadex G-25 to remove unreacted dye.
6. Dialyze versus 2 × 1 liter of distilled water and lyophilize for storage.

1. DIBUTYL TIN DILAURATE METHOD (DeBelder and Granath, 1973)

This procedure has the advantage that it is a one-step process, and is therefore quick and easy. It is the method by which Pharmacia labels the fluoresceinyl-dextrans that are currently available through them and through Sigma. The same method should be directly applicable to labeling Ficoll, although we have not used it for this purpose.

The product of the procedure, as described here, generally has a substitution ratio of ≥ 0.02 fluorophore molecules per sugar residue. The linkage is fairly stable in aqueous solution at pH 7.0 (DeBelder and Granath, 1973). This method is convenient and suitable for studies in which the degree of substitution doesn't need to be controlled accurately and detectability of small amounts is the limiting factor, for example, in microcirculation studies, or fluorescence activated cell sorting.

When the method is used to prepare tetramethylrhodamine (TRTC-) derivatives, a minor complication results, apparently due to the hydrophobicity of the fluorophore. TRTC-dextran is slightly soluble in ethanol, so that repeated washing in ethanol sometimes results in progressive loss of labeled material. After two washes, it is recommended that the labeled dextran be dialyzed against distilled water and then desalted on a short column of Sephadex G-25 to remove unreacted dye. If necessary, chroma-

tography on a hydrophobic resin can also be used to remove unreacted dye (for example, see Materials and Methods: Meigs and Wang, 1986).

2. AMINOETHYLCARBOXYMETHYL METHOD (Inman, 1975)

This procedure is preferable to DeBelder and Granath for preparing derivatives with a low degree of substitution for studies in which it is necessary to alter the charge and hydrophilicity of the polysaccharide as little as possible. We have used fluorescent Ficolls prepared in this way as inert tracer particles to probe the structure of cytoplasm in living cells by FRAP (Luby-Phelps *et al.*, 1987). Fluorescent dextrans prepared by this method have been used in a recent multiparameter fluorescence study of locomoting Swiss 3T3 cells (DeBiasio *et al.*, 1987).

The procedure involves conversion of hydroxyl groups to O-carboxymethyl ethers and subsequent amidation of these groups by ethylene diamine in the presence of a water soluble carbodiimide (Fig. 1). This aminoethylcarboxymethyl (AECM) derivative can then be reacted directly with amino-selective reagents, such as FITC and TRITC, to obtain fluorescent derivatives.

The number of carboxyls introduced into the polymer at the first step is a function of time and temperature (Fig. 2). The final degree of substitution can thus be controlled by varying these parameters at the first step and using excess ethylene diamine and excess fluorophore at subsequent steps. The procedure detailed in Table II introduces about 11 mol of amino groups/mol of Ficoll 400, or 0.01 mol/mol of sucrose (0.005/sugar residue). Adjustment of the duration of the carboxymethylation reaction may be necessary to obtain the desired degree of substitution when the method is applied to polysaccharides of molecular weights other than 400,000. Carboxymethylation can also be carried out at 40°C in order to introduce large numbers

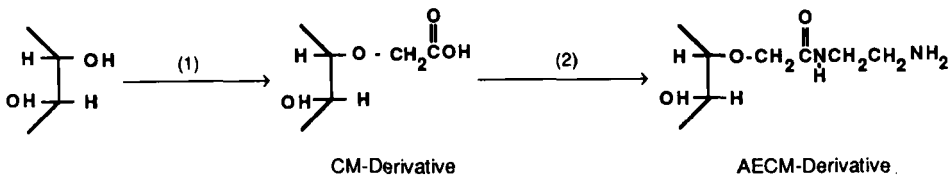

FIG. 1. Preparation of AECM-polysaccharides. Reaction (1): The polysaccharide is combined with chloroacetate in strongly alkaline aqueous solution with the elimination of NaCl and formation of a stable carboxymethyl ether linkage. Reaction (2): A monoamide is formed with ethylenediamine, present in large excess. Carboxyl groups are activated with a water-soluble carbodiimide in aqueous solution, pH-stated at 4.7. [Redrawn with permission from J. K. Inman. Thymus-independent antigens: the preparation of covalent, hapten-Ficoll conjugates. *J. Immunol.*, **114**, 704–709, © by American Association of Immunologists (1975).]

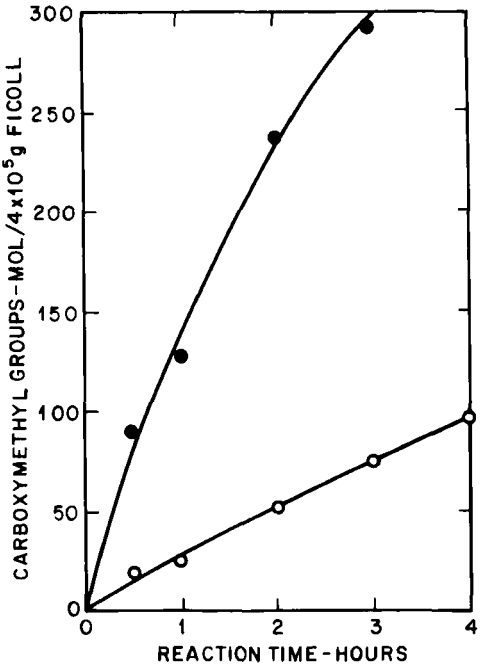

FIG. 2. Time course of the carboxymethylation reaction [Reaction (1), Fig. 1] at 25°C (○) and 40°C (●). Initial concentrations were as follows: Ficoll 53.2 g/liter, sodium chloroacetate 1 *M*, and NaOH 2 *M*; the solvent was water. Reaction was terminated at various times by neutralization of samples with 5 *N* HCl. Following exhaustive dialysis against water, samples were analyzed for carboxymethyl content by acid–base titration and calculation of hydrogen ion-binding capacity. [With permission from J. K. Inman. Thymus-independent antigens: the preparation of covalent, hapten-Ficoll conjugates. *J. Immunol.*, **114**, 704–709, © by American Association of Immunologists (1975).]

of carboxyl groups while minimizing exposure to harsh conditions. As pointed out by Inman (1975), this may be desirable when the polysaccharide is to be used as a carrier for immunization with a hapten.

The number of free carboxymethyl groups at any step can be determined by titrating a known dry weight of material between pH 2.2 and 7.0, since the carboxymethyl groups have a pK_a of 3.6. The number of free amino groups of either the AECM or the fluorescent derivatives can be determined by titration from pH 5.6 to 11.0, since the amino groups have a pK_a of 9.2 (Inman, 1975). When titrating heavily labeled material, the pK_a of the fluorophore must be taken into account. Free amines can also be determined by the ninhydrin method, outlined in Table IV (see also below).

The labeling procedure detailed in Table III, which uses a concentration of FITC or TRITC in 10-fold molar excess of the number of free amines on

TABLE IV

NINHYDRIN TEST FOR FREE AMINES

Materials

$SnCl_2 \cdot 2H_2O$ (80 mg)
Ninhydrin (2 g)
0.2 *M* citrate buffer, pH 5.0
50% aqueous *n*-propanol (500 ml)
1 m*M* glycine or glucosamine (10 ml)
Methyl cellulsolve (ethylene glycol monomethyl ether) (50 ml)

Procedure

1. Ninhydrin solution (100 ml):
   a. Dissolve 80 mg reagent grade $SnCl_2 \cdot 2H_2O$ in 50 ml of 0.2 *M* citrate buffer (4.3 g citric acid, 8.7 g Na_3 citrate $\cdot 2H_2O$ in 250 ml distilled water, pH to 5.0).
   b. Add this solution to 50 ml methyl cellusolve containing 2 g of dissolved ninhydrin. Store in cold.
2. Take 50 μl aqueous samples of material to be tested.
3. Add 150 μl ninhydrin solution with vortexing.
4. Heat in a boiling water bath for 20 minutes, then cool in a room temperature water bath.
5. Add 800 μl of 50% aqueous *n*-propanol while vortexing.
6. Let color develop for 10 minutes at room temperature. Blue color indicates a positive test.

the AECM-polysaccharide, produces a final fluorescent product with a negligible content of free carboxyls or free amines. These derivatives have a very low net surface charge, as shown by flat bed electrophoresis at pH 7.0 in agarose gels. Fluorescein- or rhodamine-labeled polysaccharides with a low degree of substitution show negligible migration in the electric field during a time in which both fluorescein isothiocyanate and fluorescein-labeled BSA migrate several centimeters toward the cathode.

In principle, AECM-Ficoll or -dextran can be labeled with any amino-selective reagent. However, we have found that lissamine rhodamine B sulfonyl chloride (Molecular Probes, Eugene, Oregon), apparently labels sites other than amino groups since we obtained very high substitution ratios before all the amino groups were blocked. This excess dye was not removed by boiling in SDS, suggesting that it was covalently bound to the polysaccharide.

While all the linkages involved in the synthesis are reported to be relatively stable (Inman, 1975), we have found that a low molecular weight, fluorescent contaminant appears when the labeled polysaccharides are stored in aqueous solution at 4°C for extended periods. We have not determined whether this is due to release of dye or to digestion of the polysaccharide by microorganisms. The labeled polysaccharides have proved to be quite stable when stored in aqueous solutions at ≤ -20°C or when lyophilized to a powder and stored with desiccant at ≤ 4°C.

B. Characterization of Fluorescent Polysaccharides

1. Degree of Substitution

The degree of substitution can be determined with reasonable accuracy by reading the absorbance of the fluorophore for a known concentration of fluorescent polysaccharide (dry wt/vol). For FTC-derivatives, the absorbance is read at 495 nm in a buffer of pH 8.0, and a molar extinction coefficient (ε) of $7.5 \times 10^4\ M^{-1}\ cm^{-1}$ (Haugland, 1985) is used to calculate the concentration of fluorescein. For TRTC-derivatives, absorbance is read at 554 nm at pH 7.2, and an ϵ of $5.5 \times 10^4\ M^{-1}\ cm^{-1}$ (Haugland, 1985) is used. The approximate molar ratio of fluorophore to polysaccharide can be determined by reference to the nominal $\overline{M}_w$ of the polysaccharide. If this is unknown, as, for example, after size-fractionation, or if greater precision is desired, the anthrone reaction (Table V) can be used to determine the glucose or sucrose content of a given dry weight of sample.

2. Determination of Free Amino Groups by Ninhydrin Assay (Spackman *et al.*, 1958)

Details of a method for determining the content of free amino groups on AECM-polysaccharides by the ninhydrin assay are given in Table IV. For

TABLE V

Anthrone Determination of Sugars (Jermyn, 1975)

Materials

Conc. HCl	Anthrone
90% Formic acid	80% (v/v) Sulfuric acid
Glucose or sucrose	

Procedure

1. Place a 1 ml aqueous sample of the material to be assayed in a 25-ml test tube.
2. Add 1 ml of concentrated HCl and 0.1 ml of 90% formic acid.
3. Add slowly to avoid excessive foaming, 8 ml of freshly prepared anthrone reagent (200 μg/ml anthrone dissolved at room temperature in 80% (v/v) sulfuric acid).
4. Mix thoroughly, heat tubes for 12 minutes in a boiling water bath, and then cool immediately in a cold-water bath.
5. When cool, mix on a Vortex mixer and allow to stand for 5 minutes to allow bubbles to disperse.
6. Read absorbance at 630 nm and compare to a standard curve (glucose for dextran, sucrose for Ficoll) to determine micrograms of monosaccharide. The assay is linear from 20 to 100 mg of total carbohydrate in a 1-ml sample. For assay of smaller amounts, the entire assay may be scaled down by a factor of ten. Under these conditions it is linear from 2 to 10 μg in 0.1 ml of sample.

quantitative tests, the absorbance of each sample at 570 nm can be read and compared to a standard curve of glycine or glucosamine. When performed as described here, the standard curve is linear over the range of 0–50 nmol of amino groups.

A complication arises when using the ninhydrin test to quantitate free amines on rhodamine derivatives because the absorbance maximum of the fluorophore (≈ 550 nm) is very close to the wavelength at which the assay is read (570 nm). In this case, it is better to quantitate free amines on the AECM-polysaccharide before reacting it with the fluorophore and then subtract the number of fluorophores (determined from the absorbance of a known dry weight of sample, as described above) to obtain the number of unreacted amino groups. Alternatively, the content of free amino groups may be determined by the fluorescamine assay (Udenfriend *et al.*, 1972; Weigele *et al.*, 1972). In cases where quantitation is not important, the ninydrin test can also be used qualitatively as a colorimetric indicator of the presence of unreacted amine groups on fluorescent polysaccharide derivatives. Fluorescein and rhodamine do not appear to interfere with the production of color in the ninhydrin test, or to give false positives. However, other fluorophores should be tested for these possibilities before applying the ninhydrin test in their presence.

3. Size-Fractionation

Polysaccharides can be size fractionated by gel permeation chromatography (Granath and Flodin, 1961; Laurent and Granath, 1967). Residual free dye is removed at the same time. In general, the best choice of a resin for size-fractionation can be determined based on the fractionation range of the resin (for polysaccharides) relative to the $\overline{M}_w$ of the unfractionated polysaccharide. This information can usually be obtained from the manufacturer of the chromatography resin. The column can be eluted with any dilute aqueous buffer, provided a small amount of salt (≥ 10 mM) is present to minimize interaction of the polysaccharide with the column resin.

To obtain adequate amounts of narrowly fractionated material, it is necessary to load a relatively large volume of fairly concentrated material on a long column. For example, we routinely fractionate FTC-Ficoll 400 by loading 100 mg of material at 25 mg/ml on a 3 × 150-cm column of Sepharose CL-6B (Pharmacia), and collecting 5-ml fractions (Luby-Phelps *et al.*, 1987). The upper limits of volume and concentration that can be applied to the column to maximize the yield will depend on the dimensions of the column and the incremental viscosity of the polysaccharide. As for gel permeation chromatography in general, a sample volume that is too large relative to the elution volume of the column, or a sample viscosity that

is too high relative to the viscosity of the eluant, will result in poor fractionation (Pharmacia, 1981). The relative viscosity for dextran and Ficoll at a given concentration can be estimated by reference to the product literature provided by Pharmacia for these polysaccharides. Selected column fractions should be dialyzed extensively against distilled water, lyophilized to a powder, and stored with desiccant.

4. Measurement and Meaning of Hydrodynamic Radius

Size-fractionated polysaccharides are not necessarily monodisperse. Although polydispersity will eventually become negligible with repeated chromatography (Laurent and Granath, 1967; Basedow and Ebert, 1979; Bohrer *et al.*, 1984), this is not recommended for applications where yield is important. Thus, polydispersity may have to be taken into account when using size-fractionated dextran or Ficoll molecules as "molecular rulers" to measure the size of pores or channels in permeability studies (for more extensive discussion, see Luby-Phelps *et al.*, 1988).

The average molecular size of the distribution of particles in each fraction can be described by a mean hydrodynamic radius ($\overline{R}_H$). The $\overline{R}_H$ of the particles contained in each column fraction can be determined from the aqueous diffusion coefficient, D, which, for fluorescent derivatives, is measured most easily by FRAP (Axelrod *et al.*, 1976; Yguerabide *et al.*, 1982). The Einstein equation is used to define $\overline{R}_H = kT/6\pi\eta D$, where k is Boltzmann's constant, T is absolute temperature, and η is the viscosity of the solvent. When 100 mg of FTC-Ficoll 400 at 25 mg/ml is fractionated on Sepharose CL-6B, as described above, it is found that $\overline{R}_H$ decreases by about 2 Å between successive 5-ml fractions. Thus, from unfractionated Ficoll 400, size-fractions ranging in $\overline{R}_H$ from 30- to 230 Å can be obtained. Size-fractions with a slightly larger $\overline{R}_H$ can be obtained by pooling and concentrating the void volumes from several column runs and chromatographing them together on Sepharose CL-4B.

For permeability studies, it is necessary to know how the hydrodynamic radius (R_H) of a monodisperse tracer particle is related to the size parameter that determines exclusion of the particle from a pore or channel. According to Tanford (1961) the radius of gyration, R_G, determines the distance of closest approach of a macromolecule to a barrier. Recently, Cassasa (1985) has proposed the mean external diameter ($\overline{X}$) as a more appropriate size parameter, based on thermodynamic considerations. The relationship of $\overline{X}$ to R_H will depend on the conformation and flexibility of the macromolecule in question. For a hard sphere, $\overline{X} \approx 2R_H$. For a random coil, $\overline{X}$ is only slightly larger (13%) than $2R_G$. Thus, for random coils, radius of gyration

remains a convenient and reasonably precise descriptor of molecular dimensions. In aqueous solution, dextran molecules larger than 2000 D appear to be almost ideal statistical coils in spite of their branching (Basedow and Ebert, 1979). According to the Kirkwood-Riseman result, for an ideal statistical coil, $R_H = 0.665\ R_G$. (Tanford, 1961). By including a correction for the degree of branching, Granath (1958) calculated that $R_H = 0.65\ R_G$ for the type of dextran provided by Pharmacia. This value was closely approximated by her experimental data. Thus, R_G probably best describes the dimensions of a dextran molecule, and can be approximated by $R_H/0.65$. In contrast to dextran, Ficoll has a much more compact configuration and a well-defined size that is more accurately described by R_H (Laurent and Granath, 1967; Deen *et al.*, 1981; Bohrer *et al.*, 1984).

A possible drawback to using dextrans in permeability studies is that they are highly flexible molecules. Thus, although their average configuration is described by the radius of gyration, their deformability may enable them to fit through pores or channels of diameter $\leq 2\ R_G$ (e.g., Bohrer *et al.*, 1984). In addition, dextrans of ≤ 2000 D apparently have a rigid, rodlike conformation (Granath, 1958; Basedow and Ebert, 1979) and should not be used in permeability studies unless this is taken into account.

III. Further Considerations in the Use of Fluorescent Dextrans and Ficolls

In working with polysaccharides, it should be remembered that dextran is susceptible to attack by the dextranases secreted by some microorganisms, and that the glycosidic linkages of polysaccharides are readily hydrolyzed at pH ≤ 3, especially at high temperature. If antimicrobial agents are employed during the preparation or storage of the fluorescent derivatives, they should be dialyzed out of the preparation before its use with living cells.

The procedures described in this chapter are aimed at obtaining fluorescently labeled polysaccharides with minimal net charge at pH 7.0. For studies where it is of interest to compare the behavior of electroneutral particles with the behavior of charged particles, these procedures can be adapted to leave some of the carboxyl or amino groups unmodified, thus giving a final product with either a net negative or a net positive charge.

The labeling procedures can also be adapted so that each polysaccharide molecule is lableled with two different fluorophores. This would be very useful, for example, for mapping intracellular pH by the ratio of fluorescein to rhodamine fluorescence. This method would have a better signal-to-noise ratio than the current method involving measurement of the ratio of

fluorescein fluorescence at two excitation wavelengths, and having the two fluorophores on the same carrier would eliminate the potential problem of differential partitioning of the fluorescein and rhodamine probes within the cell. Similarly, each polysaccharide molecule could be labeled with a fluorophore and a photoactivatable cross-linker in order to create a fluorescent derivative that could be fixed in place within cells.

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REFERENCES

Arfors, K.-E., and Hint, H. (1971). *Microvasc. Res.* **3,** 440.

Axelrod, D., Koppel, D. E., Schlessinger, J., Elson, E., and Webb., W. W. (1976). *Biophys. J.* **16,** 1005–1069.

Basedow, A. M., and Ebert, K. H. (1979). *J. Polym. Sci. Polym. Symp.* **66,** 101–115.

Berlin, J. M., and Oliver, J. M. (1980). *J. Cell Biol.* **85,** 660–671.

Bohrer, M. P., Patterson, G. D., and Carroll, P. J. (1984). *Macromolecules* **17,** 1170–1173.

Bright, G. R., Fisher, G. W., Rogowska, J., and Taylor, D. L. (1987). *J. Cell Biol.* **104,** 1019–1033.

Casassa, E. F. (1985). *J. Polym. Sci. Polym. Symp.* **72,** 151–160.

DeBelder, A. N., and Granath, K. (1973). *Carbohydr. Res.* **30,** 375–378.

DeBiasio, R., Bright, G. R., Ernst, L. A., Waggoner, A. S., and Taylor, D. L. (1987). *J. Cell Biol.* **105,** 1613–1622.

Deen, W. M., Bohrer, M. P., and Epstein, N. B. (1981). *Am. Inst. Chem. Eng. J.* **27,** 952–959.

Farquhar, M. G. (1982). *In* "Cell Biology of Extracellular Matrix" (E. D. Hay, ed.), pp. 335–378. Plenum, New York.

Fechheimer, M., Denny, C., Murphy, R., and Taylor, D. L. (1986). *J. Cell Biol.* **40,** 242–247.

Gimlich, R. L., and Braun, J. (1985). *Dev. Biol.* **109,** 509–514.

Gingell, D., Todd, I., and Bailey, J. (1985). *J. Cell Biol.* **100,** 1334–1338.

Goren, M. B., Swendsen, C, L., Fiscus, J., and Miranti, C. (1984). *J. Leukocyte Biol.* **36,** 273–292.

Granath, K. A. (1958). *J. Colloid Sci.* **13,** 308–328.

Granath, K. A., and Flodin, P. (1961). *Makromol. Chem.* **48,** 160–171.

Grotte, G. (1956). *Acta Chir. Scand. Suppl.* **11,** 1–84.

Hara, Y., and Nakao, M. (1986). *J. Biol. Chem.* **261,** 12655–12658.

Haugland, R. P. (1985) "Handbook of Fluorescent Probes and Research Chemicals," Molecular Probes, Inc., Eugene, Oregon.

Heiple, J., and Taylor, D. L. (1982). *In* "Intracellular pH: Its Measurements, Regulation and Utilization in Cellular Function," (R. Nuccitelli and D. W. Deamer, eds.), pp. 22–54. Liss, New York.

Holter, H., and Moller, K. M. (1956). *Exp. Cell Res.* **15,** 631–632.

Inman, J. K. (1975). *J. Immunol.* **114,** 704–709.

Jermyn, M. A. (1975). *Anal. Biochem.* **68,** 332–335.
Jiang, L.-W., and Schindler, M. (1986). *J. Cell Biol.* **102,** 853–858.
Lang, I., Scholz, M., and Peters, R. (1986). *J. Cell Biol.* **102,** 1182–1190.
Lanni, F., Waggoner, A. S., and Taylor, D. L. (1985). *J. Cell Biol.* **100,** 1091–1102.
Larm, O., Lindberg, B., and Svensson, S. (1971). *Carbohydr. Res.* **20,** 39–48.
Laurent, T. C., and Granath, K. A. (1967). *Biochem. Biophys. Acta* **136,** 191–198.
Luby-Phelps, K., Lanni, F., and Taylor, D. L. (1985). *J. Cell Biol.* **101,** 1245–1256.
Luby-Phelps, K., Taylor, D. L., and Lanni, F. (1986). *J. Cell Biol.* **102,** 2015–2022.
Luby-Phelps, K., Castle, P. E., Lanni, F., and Taylor, D. L. (1987). *Proc. Natl. Acad. Sci. U.S.A.* **84,** 4910–4913.
Luby-Phelps, K., and Taylor, D. L. (1988). *Cell Motil.* **10,** 28–37.
Luby-Phelps, K., Lanni, F., and Taylor, D. L. (1988). *Annu. Rev. Biophys. Chem.* **17,** 369–396.
McNeil, P. L., Tanasugarn, L., Meigs, J., and Taylor, D. I. (1983). *J. Cell Biol.* **97,** 692–702.
McNeil, P. L., Murphy, R. F., Lanni, F., and Taylor, D. L. (1984). *J. Cell Biol.* **98,** 1556–1564.
Mayhan, W. G., and Heistad, D. D. (1985). *Am. J. Physiol.* **248,**H712–H718.
Meigs, J. B., and Wang, Y-L. (1986). *J. Cell Biol.* **102,** 1430–1438.
Mosier, D. E., Johnson, B. M., Paul, W. E., and McMaster, P. R. B. (1974). *J. Exp. Med.* **139,** 1354–1360.
Murphy, R. F. (1985). *Proc. Natl. Acad. Sci. U.S.A.* **82,** 8523–8526.
Murphy, R. F., Powers, S., and Cantor, C. R. (1984). *J. Cell Biol.* **98,** 1757–1762.
Nugent, C. J., and Jain, R. K. (1984). *Cancer Res.* **44,** 238–24.
Okhuma, S., and Poole, B. (1978). *Proc. Natl. Acad. Sci. U.S.A.* **75,** 3327–3331.
Paradiso, A. M., Tsien, R. Y., and Machen, R. E. (1987). *Nature (London)* **325,** 447–450.
Pharmacia Fine Chemicals (1981). "Gel Filtration: Theory and Practice." Rahms i Lund, Stockholm.
Rothenberg, P., Glaser, L., Schlesinger, P., and Cassel, D. (1983). *J. Biol. Chem.* **258,** 12644–12653.
Simionescu, N., and Palade, G. E. (1971). *J. Cell Biol.* **50,** 616–624.
Sowers, A. E. (1986). *J. Cell Biol.* **102,** 1358–1362.
Spackman, D. H., Stein, W. H., and Moore, S. (1958). *Anal. Chem.* **30,** 1190–1206.
Stutzin, A. (1986). *FEBS Lett.* **197,** 274–280.
Tanasugarn, L., McNeil, P. L., Reynolds, G., and Taylor, D. (1984). *J. Cell Biol.* **98,** 717–724.
Tanford, C. (1961). "Physical Chemistry of Macromolecules." Wiley, New York.
Thorball, N. (1981). *Histochemistry* **71,** 209–233.
Tycko, B., and Maxfield, F. (1982). *Cell* **28,** 643–651.
Udenfriend, S., Stein, S., Böhler, P., Dairman, W., Leimgruber, W., and Weigele, M. (1972). *Science* **178,** 871–872.
Waggoner, A. S. (1986). *In* "Applications of Fluorescence in the Biomedical Sciences" (D. L. Taylor, A. S. Waggoner, R. F. Murphy, F. Lanni, and R. R. Birge, eds.), pp. 3–28. Liss, New York.
Weigele, M., De Bernardo, S. L., Tengi, J. P., and Leimgruber, W. (1972). *J. Am. Chem. Soc.* **94,** 5927–5928.
Yguerabide, J., Schmidt, J. A., and Yguerabide, E. E. (1982). *Biophys. J.* **39,** 69–75.