

DEXTRANS AND GLYCOGENS AS PARTICULATE TRACERS FOR STUDYING CAPILLARY PERMEABILITY

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ABSTRACT

Commercially available glycogens and dextrans can be used as biological particulate tracers in work on capillary permeability. These polysaccharides are well tolerated in intravenous injection and induce no vascular leakage when applied topically (cremaster test) in mice and in Wistar-Furth rats. The particles stain adequately with lead after aldehyde-OsO₄ fixation in phosphate buffer and provide a relatively wide set of probes (~45 A–300 A) for work on the large and small pore systems.

The permeability of capillary walls for water and water-soluble molecules is currently explained by assuming the existence of two pore systems: one of small pores with diameters of ~90 A and relatively high frequency, and the other of large pores with diameters of up to 700 A and considerably lower frequency (1, 2). Electron-opaque tracers have been used in attempts to identify these pores in different types of capillaries (3–5), but the results so far obtained can not be considered definitive without further checking by more diversified tracers. To this intent it would be highly desirable to: (a) extend the dimensional spectrum of available tracers, (b) use tracer particles of biological, rather than inorganic (metal colloids), nature, and (c) rely on particles which can be visualized individually and directly, rather than through the intermediary of a reaction product (cf. 3, 5).

The results we report in this paper show that polysaccharide particles, i.e. glycogens¹ and dextrans², satisfy in part the requirements indicated above.

¹ Branched polysaccharide made up of chains of D-glucopyranose units linked by α -1:4 glucosidic bonds with branching points arising from additional α -1:6 linkages.

² Long chains of D-glucopyranose units linked by α -1:6, α -1:4, and α -1:3 glucosidic bonds, the majority of them being α -1:6.

MATERIALS AND METHODS

Animals

Since capillary permeability can be affected by histamine liberated from the tissues by the tracers, we selected for these experiments animals known to be resistant to histamine-release by dextrans (6–10), e.g., mice (40 adult males, 25–30 g, of The Rockefeller University noninbred strain), and rats of the Wistar-Furth strain (6, 7) (60 adult males, 130–165 g, obtained from Microbiological Associates, Inc., Bethesda, Md.). Additional experiments were carried out on Sprague-Dawley rats (68 adult males, 130–145 g).

Tracers

PREPARATION OF SOLUTIONS

GLYCOGEN: Shellfish glycogen and rabbit liver glycogen, both homogeneous by paper chromatography, were obtained from Mann Research Labs., Inc., New York, as powders and dissolved at 15% concentration (w/v) in 0.9% NaCl, just before use. Dissolution was accelerated by sonication in an ultrasonic tank-model Dr. 50H, Acoustica, Los Angeles, Calif., operated at 42 kc for 25–30 min at 20°–25°C.

DEXTRANS: The following dextrans were similarly prepared as 10% w/v solutions, except that sonication was carried out for 40–50 min: (a) Dextran grade A (clinical), mol. wt., 200,000–300,000, hereafter referred to as "D₂₅₀" (Mann Research Labs,

Inc.), (b) Dextran (clinical grade), mol. wt., 37,000–50,000, "D₄₀" (Nutritional Biochemicals Corporation, Cleveland, Ohio), and (c) Dextran grade D (not clinical), mol. wt., 15,000–20,000, "D₂₀" (Mann Research Labs, Inc.). A fourth dextran, mol. wt., 75,000, "D₇₅", was obtained as a 6% w/v solution in 0.9% NaCl (Abbott Laboratories, North Chicago, Ill.).

In some experiments, samples of D₂₅₀, D₇₅, and D₄₀ solutions were dialyzed at 4°C for 24–36 hr in cellulose tubes (dialyzer tubing no. 4465-A₂, obtained from Scientific Apparatus, Arthur H. Thomas Co., Philadelphia, Pa.), first against three changes, each of 100 × volumes, of 0.01 M ethyleneglycol bis (β-aminoethyl ether) N,N'-tetraacetic acid (EGTA), Sigma Chemical Co., St. Louis, Mo.) in distilled water adjusted to pH 7.2 with 0.1 N NaOH, and second against two changes of 0.9% NaCl. Before injection, the temperature of the solutions was brought up to room temperature (~25°C) or animal body temperature (~39°–40°C). In addition to the dextrans mentioned, iron-dextran (Imferon, containing 50 mg of elemental iron in each cc, Lakeside Laboratories, Inc., Milwaukee, Wis.) was used only in experiments designed to test effects on vascular leakage.

DETERMINATION OF DISPERSION AND PARTICLE SIZE

Droplets of tracer solutions placed on carbon-coated Formvar grids were stained by either 2% potassium phosphotungstate (pH 7.4) (11) or 2.5% uranyl acetate (pH 4.2). The preparations were examined in a Hitachi-11C electron microscope provided with a 200 μ condenser II aperture, a 50 μ objective aperture, and operated at a 75 kv accelerating voltage. Micrographs were taken at 50,000 × initial magnification and enlarged photographically to 150,000 ×. The microscope was calibrated with a carbon grating replica, cross lines, 2160 lines/mm (Fullam Co., Schenectady, N.Y.). The preparations were examined for the state of dispersion of the particles (presence or absence of aggregates) and for their size distribution. For the latter, ~3000 particles were measured with the micrometer and the measuring magnifier, at 150,000 ×. Dispersion and size distribution were also studied on positively-stained particles, usually in the plasma of sectioned vessels.

CONTROLS FOR VASCULAR LEAKAGE

Since the tracers used (or impurities associated with them) may affect capillary permeability by liberating histamine and serotonin from the wall of blood vessels or from tissues, dextrans and glycogens were tested for their ability to form intramural deposits in the small venules of the cremaster (12, 13) and the scrotal skin. For this test, 0.1 ml colloidal carbon (Günther-Wag-

ner Pelikan Werke, Hanover, Germany-Batch #C11/1431a) per 100 g body weight, was administered intravenously. Immediately after, small amounts of tracers in isotonic saline alone (negative control) or histamine in isotonic saline (positive control) were injected subcutaneously on the anterior face of the scrotum. The dose of tracer topically administered approximated the concentration reached in the blood plasma under usual experimental conditions. After 1 hr, the animals were killed, the skin of the scrotum and the cremaster were excised, stretched-fixed in 10% formaldehyde, and examined by transillumination under a dissecting microscope at 50 ×. The preparation were scored for the presence or absence of "vascular tattoo" at the site of the injection.

TRACER INJECTION-TISSUE FIXATION IN SITU

Under ether anesthesia, 1.5 ml/100 g body weight of tracer solution was slowly (1 min) injected into the saphenous vein of the animal. At selected intervals after the injection, the tissues were fixed *in situ*, with or without concomitant vascular ligation. We chose organs whose blood capillaries are provided with either a fenestrated endothelium (jejunum, thyroid, adrenals), a continuous endothelium (diaphragm), or a discontinuous endothelial layer (liver). For the jejunum, an isolated loop was filled with the fixative solutions and, in some cases, its vessels were ligated a few minutes later. For the diaphragm, the fixative was injected into both the pleural and peritoneal cavities (4) and for the liver into the peritoneal cavity only. For the thyroid and adrenals, the fixative solution was injected into the surrounding loose connective tissue. Fixation *in situ* generally allowed the retention of blood plasma in the lumina of the small vessels, even without concomitant vascular ligation. This paper is illustrated only with results obtained on jejunum.

Tissue Processing

Since it is known already that intracellular glyco-gen particles are stained intensely by lead after fixation in either osmium tetroxide (14, 15) or glutaraldehyde (16) in phosphate buffer, we carried out experiments to find out whether dextran molecules are also stainable by lead and to determine optimal staining conditions for both types of polysaccharide particles.

The fixatives used were: (a) OsO₄ (1%, 2 hr), (b) glutaraldehyde (5%, 2 hr), and (c) formaldehyde (4%, 2 hr), prepared beforehand from paraformaldehyde and used either alone or mixed with glutaraldehyde (17). Aldehyde-fixed specimens were postfixed in OsO₄ (1%, 2 hr). The fixatives were prepared in either Na phosphate-, or Na cacodylate-HCl, or *s*-collidine-HCl buffers, each of them at 0.1 M and pH 7.4. The specimens were rapidly dehydrated in

graded ethanols (18) and embedded in Epon. Washing between fixation and postfixation and between the latter and dehydration was found unnecessary. Some specimens were stained for 1 hr in either uranyl acetate (0.5% in Na acetate-Na Veronal, pH 5.2) (19), or in magnesium uranyl acetate (0.5% in 0.9% NaCl, at pH 5.0). All specimens were sectioned on a Porter-Blum MT2 ultratome with Dupont diamond knives, at ~ 600 A thickness (silver-gray sections). The sections were stained with either lead hydroxide or lead citrate (20), alone or after a previous staining in uranyl acetate or in magnesium uranyl acetate (21), modified by reducing the time of staining to 1 hr at 40°C. Subsequently, the preparations were examined by electron microscopy as indicated above.

Thick sections ($\sim 1 \mu$) from the same tissue specimens were stained for glycogen (22) or dextran (23).

RESULTS

Glycogen

DISPERSION AND SIZE DISTRIBUTION

These parameters were studied by negative staining in the solutions to be injected and by positive staining in the blood plasma of the vessels of fixed tissues. In both cases the dispersion was satisfactory, particles being present mostly as monomers, rather than as dimers or trimers, and only occasionally as larger aggregates (Figs. 2-7). In the solutions to be injected (Figs. 2, 3; Table I), the average diameter was 302 A for rabbit liver glycogen and 253 A for shellfish glycogen. The latter particles were reasonably homogeneous; the former showed a wider size distribution (Figs. 1, 2). Both were slightly asymmetrical (Figs. 2, 3) (axial ratio ≈ 1.2 -1.6). In tissue sections, the par-

TABLE I
Size of Glycogen Particles in Injected Solution and in Blood Plasma

Glycogen	In injected solutions		In blood plasma*
	Particle diameter mean \pm SD	Percentage of main groups of particles	
Shellfish	253 \pm 62 A	~ 90 †	241 \pm 81 A
Rabbit liver	302 \pm 98 A	~ 75 §	315 \pm 72 A

* 1-10 min after intravenous injection.

† 233-266 A.

§ 266-333 A.

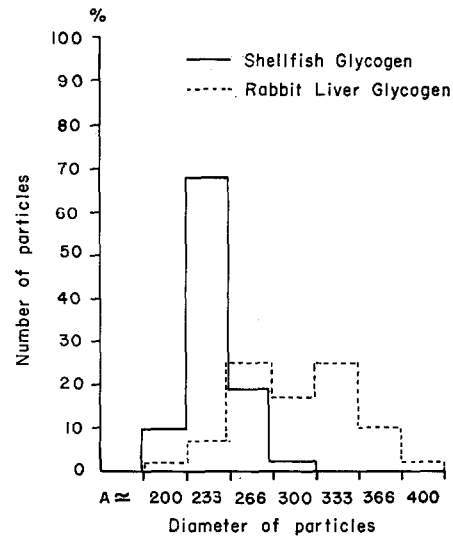
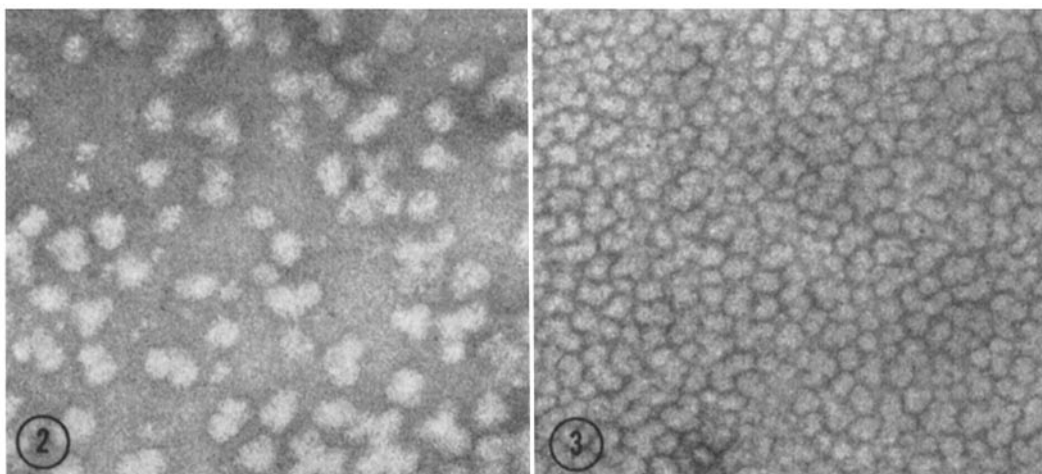


FIGURE 1 Size distribution of glycogen particles. The histogram gives the size distribution in a population of 3000 particles of both shellfish glycogen and rabbit liver glycogen measured, as given under Materials and Methods, in negatively-stained preparations of the solutions to be injected.

ticles retained their dimensions (Figs. 4, 5; Table I) and appeared compact, well outlined, and evenly stained (Figs. 4-7).

FIXATION AND STAINING

As is known, glycogen particles cannot be "fixed" by the reagents used; they are only immobilized by the fixation of the proteins in their environment. After all combinations of fixative and buffer tested, the particles could be stained by lead, but the intensity of the staining varied. The best preparations (characterized by even distribution of the tracer in the blood plasma, high contrast staining of tracer particles, and well defined cell structure) were obtained by glutaraldehyde-formaldehyde followed by OsO_4 , both in phosphate buffer. For the staining of the tracers, the presence of phosphate in the OsO_4 solution was found to be more important than in the aldehyde solution. The contrast of the tracers was further enhanced by lead staining of the sections. For better visualization of cytological details, the preparations can be doubly stained without substantial loss in relative particle density (Figs. 6, 7).



FIGURES 2-3 Fig. 2, Rabbit liver glycogen; Fig. 3, Shellfish glycogen. Glycogen particles negatively stained with 2.5% uranyl acetate (pH 4.2) in samples of tracer solutions prepared for intravenous injection. $\times 150,000$.

Dextrans

DISPERSION AND SIZE DISTRIBUTION

The procedures used gave a satisfactory dispersion of all the dextrans tested. The results are illustrated only for D_{75} and D_{40} in the solutions to be injected (Figs. 9, 10) and in the blood plasma (Figs. 11-14). Fig. 8 and Table II give data for a series of four dextrans of graded molecular weight and show that their size distribution is relatively broad, although each includes a substantial fraction of the expected size. The particles generally retain their dimensions in the plasma. The average diameters measured in negatively-stained preparations (~ 200 A for D_{250} , ~ 125 A for D_{75} , ~ 80 A for D_{40} , and ~ 45 A for D_{20}) are in close agreement with the corresponding effective diffusion radii (see Table II) calculated on the basis of molecular weight and diffusion coefficients (24, 25).

FIXATION AND STAINING

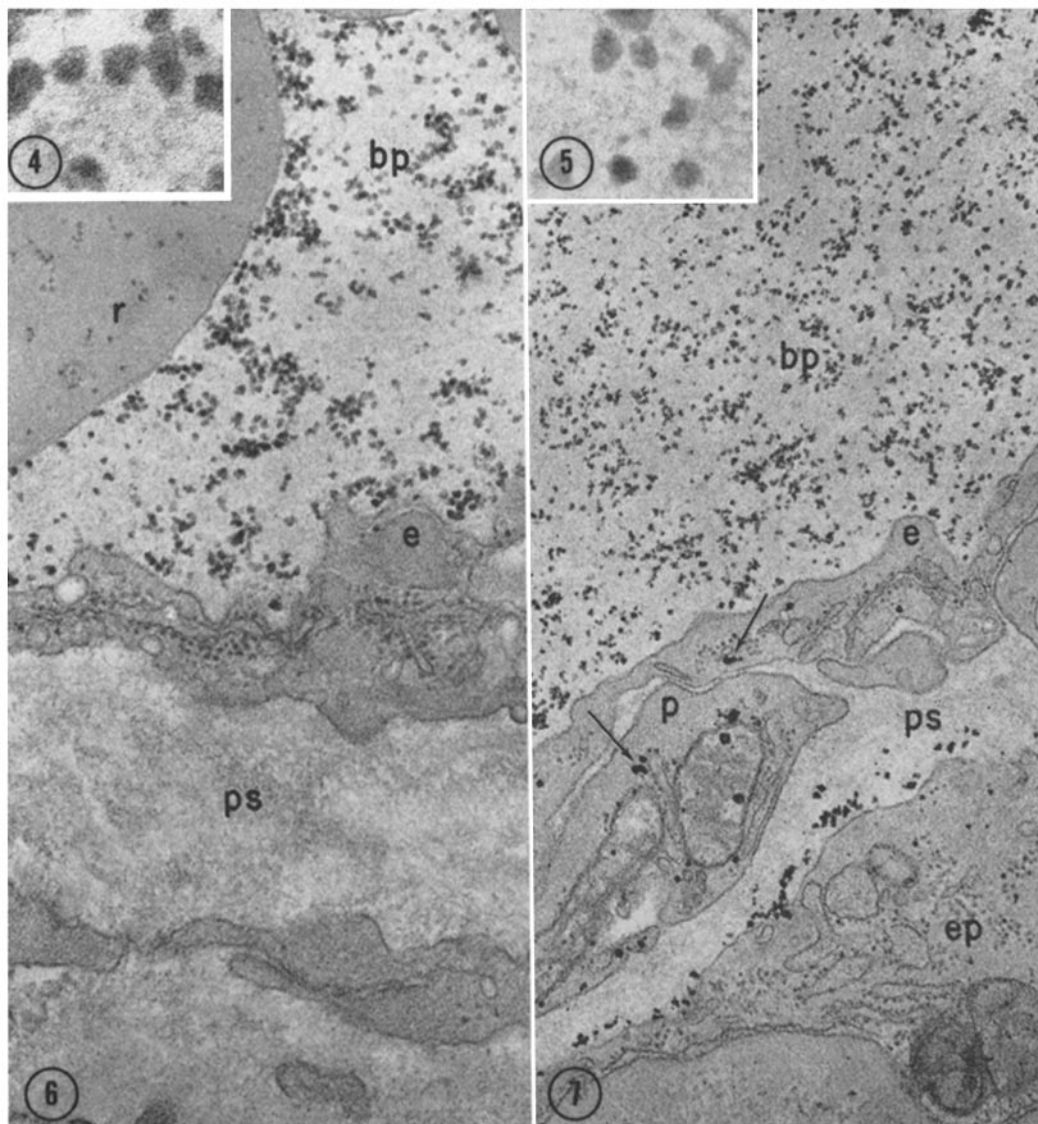
Dextran particles are retained in reasonably homogeneous distribution in the plasma and tissue interstitia presumably by the fixation of the surrounding proteins (Figs. 13, 14). For retention, the best results were obtained by fixation with 5% glutaraldehyde-4% formaldehyde in 0.1 M phosphate buffer at pH 7.4 (2 hr at 0°C) rapid dehy-

dration in ethanol at 0°C, and usual Epon embedding. As in the case of glycogen, the presence of phosphate during postfixation is required for optimal staining. The particles can be adequately stained in sections with 7.5% magnesium uranyl acetate (1 hr at 40°C) followed by lead hydroxide (15 min at 20°C). Dialyzed particles appear to stain more intensely than their nondialyzed counterparts. Staining in block with 0.5% uranyl acetate increases the contrast of most structural details of the tissue, but interferes, to some extent, with the visualization of the particles because of the heavy staining of proteins and probably other macromolecules in the plasma and interstitial fluid.

In tissue specimens, the dextran particles appear less compact and more irregular in outline than glycogen particles³; presumably, due to their structure (longer, less branched chains than in glycogen), they are more sensitive to stresses developed in their environment during the fixation of proteins.

At the concentrations reached in the plasma, a positive reaction for dextrans was not obtained by the Mowry-Millican method (23).

³ Dextran particles have less regular profiles in fixed tissue than in negatively-stained preparations.



FIGURES 4-5 Fig. 4, Rabbit liver glycogen; Fig. 5, Shellfish glycogen. Glycogen particles positively stained with 2.5% uranyl acetate (1 min) followed by 0.4% lead citrate (10 min). Sections of rat intestinal mucosa; small fields in the plasma of blood capillaries. $\times 150,000$.

FIGURE 6 Field in the jejunal mucosa (rat) including part of a blood capillary. Specimen fixed 2 min after an intravenous injection of rabbit liver glycogen solution. The glycogen particles appear intensely stained and relatively evenly distributed, singly or in small clusters in the blood plasma (*bp*). At this time point, there are no tracer particles in the pericapillary space (*ps*). The endothelium is marked *e*, and a reticulocyte in the capillary lumen, *r*. $\times 35,000$.

FIGURE 7 Specimen similar to the one in Fig. 6 except that the tracer used was shellfish glycogen and the tissue was fixed 15 min after the intravenous injection. The particles stain intensely and appear evenly dispersed in the blood plasma (*bp*). A relatively large number of tracer particles has already reached the pericapillary spaces (*ps*). The endothelium is marked *e*, a pericyte *p*, and an epithelial cell *ep*. Note the difference in size and density between the tracer and the intracellular (endogenous) glycogen (arrows). $\times 31,000$.

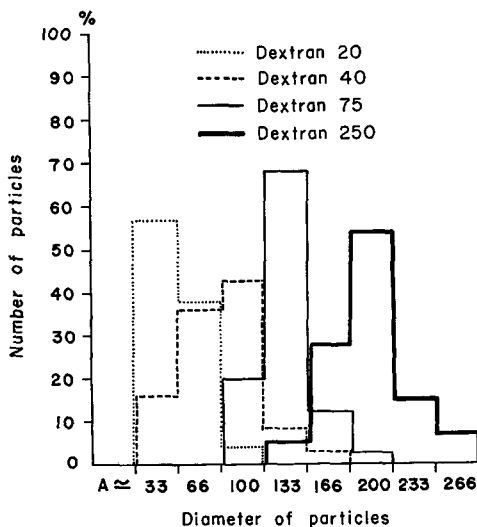


FIGURE 8 Size distribution of dextran particles. Three thousand particles of each type of dextran have been measured, as indicated under Materials and Methods, in negatively-stained preparations of the solutions to be injected.

TESTS FOR VASCULAR LEAKAGE

Table III shows that the glycogen injected locally induces no leakage in the vascular bed of the cremaster and scrotal skin of the Wistar-Furth rats. Sprague-Dawley rats occasionally give a slight labeling of the cremaster vessels for both

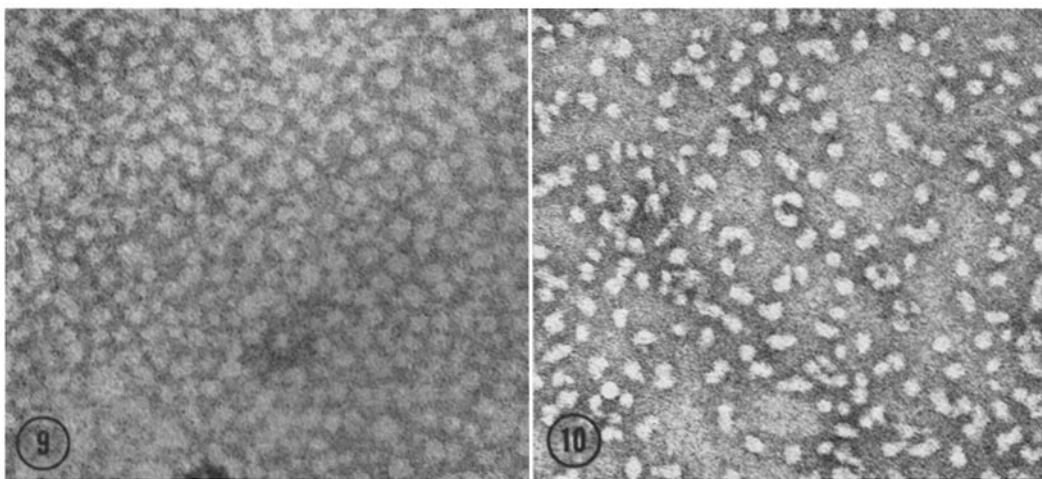
glycogens, and Rockefeller University mice react in the same way to shellfish glycogen. The dextrans are well tolerated by Wistar-Furth rats, except for occasional slight positive reactions in the skin evasculature. D₇₅ induces permeability changes in Rockefeller University mice. Iron dextran causes extensive vascular leakage in all animals used.

These findings were confirmed by electron microscope observations which showed that in tissue sections the small vessels were generally free of intramural deposits.

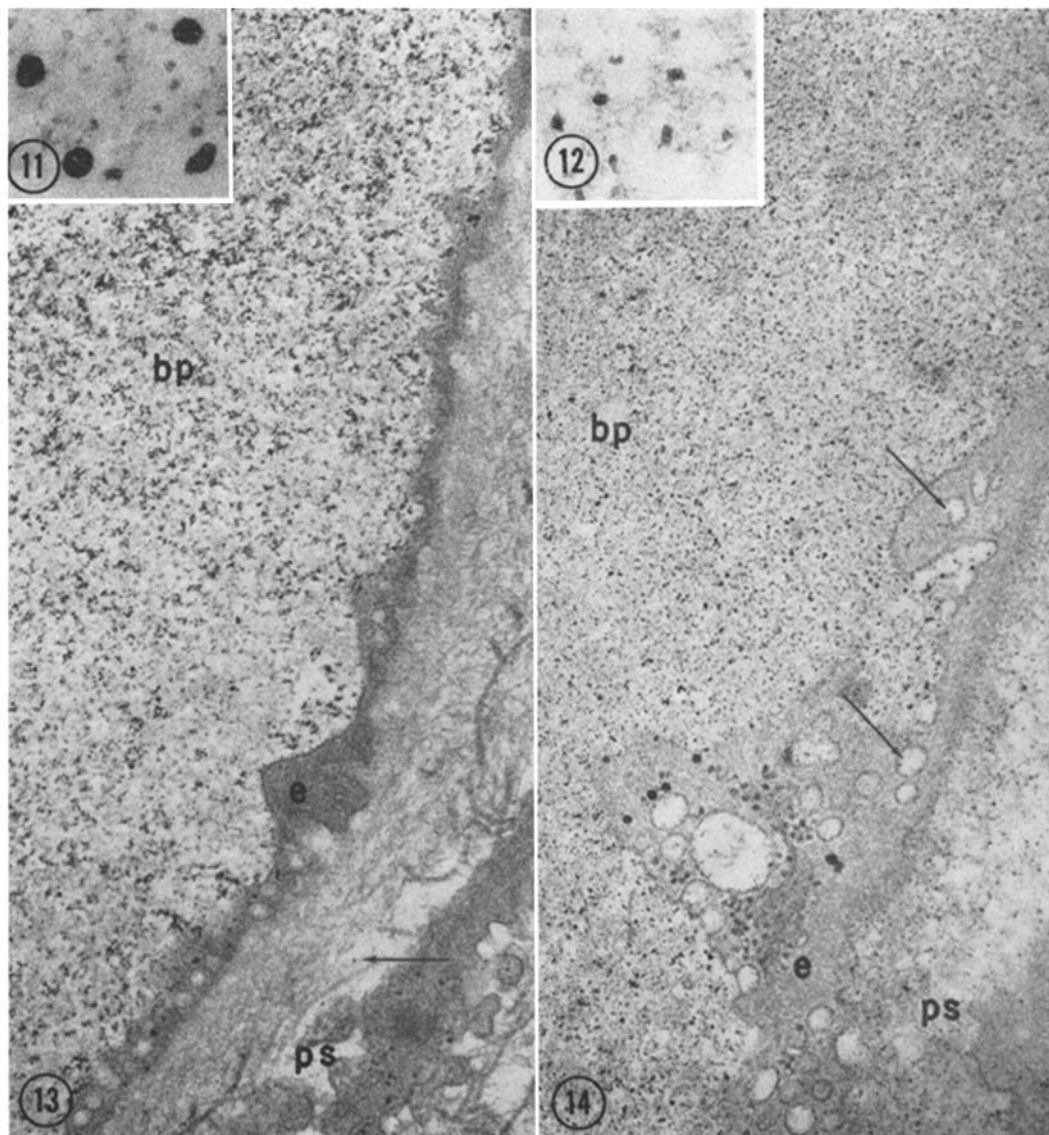
DISCUSSION

Our findings indicate that glycogen and dextran particles from commercially available preparations can be used as tracers to explore structural aspects of capillary permeability. They can be suspended, mostly as individual particles, at high concentrations, in 0.9% NaCl solutions; they are well tolerated upon intravenous injection; they retain their state of dispersion and size distribution in the blood plasma, and there is no morphological evidence of particle degradation within the intervals examined in our experiments. These tracers induce no or negligible vascular leakage (of histamine type) in mice and especially in Wistar-Furth rats.

Both particles can be intensely stained by lead after fixation in the presence of inorganic phosphate and thereby both can be individually visualized in the plasma and interstitial fluid.



FIGURES 9-10 Fig. 9, Dextran-75; Fig. 10, Dextran-40. Dextran particles negatively stained with 2.5% uranyl acetate in samples of the solutions prepared for intravenous injection. $\times 150,000$.



FIGURES 11-12 Fig. 11, Dextran-75; Fig. 12, Dextran-40. Same dextran particles positively stained in the blood plasma of rat intestinal capillaries. Sections stained with 7.5% magnesium uranyl acetate (60 min at 40°C) followed by 2.5% lead hydroxide (15 min at 25°C). $\times 150,000$.

FIGURE 13 Blood capillary in the jejunal mucosa (rat). Specimen fixed 1 min after an intravenous injection of Dextran-75. The dextran particles appear intensely stained and relatively evenly scattered in the plasma (*bp*). There are few particles (arrow) in the pericapillary space (*ps*). The endothelium is marked *e*. $\times 29,000$.

FIGURE 14 Specimen similar to the one in Fig. 13 except that the tracer (injected 1 min before fixation) was Dextran-40. The particles are well stained and appear evenly dispersed in the blood plasma (*bp*). Some particles are seen in plasmalemmal vesicles (arrows) and many have already reached the pericapillary spaces (*ps*). Note the variation in particle size. The endothelium is marked *e*. $\times 30,000$.

TABLE II
Some Physical Data Concerning the Injected Dextrans

Dextrans	Mol wt	R _{es} *	Theoretical particle diameter ‡	Particle diameter of injected dextran §	Particle diameter of dextran in blood capillaries
			<i>A</i>	<i>A</i>	<i>A</i>
D ₂₅₀	200,000-300,000	95-120	190-240	197 ± 37	191 ± 51
D ₇₅	75,000	58	116	124 ± 29	136 ± 33
D ₄₀	37,000-40,000	40-50	80-90	82 ± 14	71 ± 19
D ₂₀	15,000-20,000	27-32	55-65	48 ± 11	41 ± 14

* R_{es}: effective diffusion radius, taken from Grotte (24).

‡ Estimated as the double of R_{es}.

§ Mean value ± SD calculated from measurements made on negatively-stained specimens.

|| Mean value ± SD calculated from measurements made on positively-stained specimens.

TABLE III
Permeability Changes Induced by Hypodermal Injection of Dextran or Glycogen Solutions

Animal, Test substance	Dose*	Intensity of "vascular labeling"	
		in cremaster	in scrotal skin
	μg		
Mice (Rockefeller University, noninbred)			
NaCl, 0.85%	85	-	-
Histamine, 0.25%	25	++	++
Shellfish glycogen, 1%	100	-‡	-‡
Rabbit liver glycogen, 1%	100	-	-
Dextran-75, 0.6%	60	+	+
Rats (Sprague-Dawley)			
NaCl, 0.90%	450	-	-
Histamine, 0.25%	100	++	++
Shellfish glycogen, 1%	500	-‡	-
Rabbit liver glycogen, 1%	500	-‡	-
Dextran-75, 0.6%	300	-	-‡
Rats (Wistar-Furth)			
NaCl, 0.90%	450	-	-
Histamine, 0.25%	100	++	++
Shellfish glycogen, 1%	500	-	-‡
Rabbit liver glycogen, 1%	500	-	-
Dextran-20, 1%	500	-	-‡
Dextran-40, 1%	500	-	-‡
Dextran-75, 0.6%	300	-	-
Dextran-250, 1%	500	-	-‡
Iron-dextran, 0.5%	250	++	++

-, negative; +, moderately positive (spot of vascular labeling, ≈ 5-6 mm diameter); ++, intensely positive (spot of vascular labeling, ≈ 10-12 mm diameter; reaction spread controlaterally).

* Volume injected: 0.01 ml for mice; 0.05 ml for rats.

‡ Occasionally slight labeling limited to the site of injection (spot of vascular labeling, ≈ 1-2 mm diameter).

Because of their relatively broad dimensional spectrum (glycogen, ~200–300 Å diameter; dextrans, ~45–200 Å diameter), these particles should be useful in further work on all types of blood capillaries. They provide a relatively wide set of probes for the large pores and a more restricted, but still useful set for the small pore system.

Other features which make the new type of tracers particularly attractive are their biological origin, and the fact that the dextrans have been extensively used in the past as probe particles in physiological experiments. In fact, the current concept of the two pore systems is derived primarily from the work done by Grotte and his collaborators (24, 25), and Mayerson et al. (26) with graded dextrans.

Our results indicate that it is now possible to explore with the same tools the physiological as well as the structural aspects of capillary permeability.

Further results will be reported in subsequent publications.

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