

rapid communication

Capillary endothelial surface layer selectively reduces plasma solute distribution volume

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Vink, Hans, and Brian R. Duling. Capillary endothelial surface layer selectively reduces plasma solute distribution volume. *Am. J. Physiol. Heart Circ. Physiol.* 278: H285–H289, 2000.—We previously reported that a 0.4- to 0.5- μm -thick endothelial surface layer confines Dextran 70 (70 kDa) to the central core of hamster cremaster muscle capillaries. In the present study we used a variety of plasma tracers to probe the barrier properties of the endothelial surface layer using combined fluorescence and brightfield intravital microscopy. No permeation of the endothelial surface layer was observed for either neutral or anionic dextrans ≥ 70 kDa, but a neutral Dextran 40 (40 kDa) and neutral free dye (rhodamine, 0.4 kDa) equilibrated with the endothelial surface layer within 1 min. In contrast, small anionic tracers of similar size (0.4–40 kDa) permeated the endothelial surface layer relatively slowly with half-times (τ_{50}) between 11 and 60 min, depending on tracer size. Furthermore, two plasma proteins, fibrinogen (340 kDa) and albumin (67 kDa), moved slowly into the endothelial surface layer at the same rates, despite greatly differing sizes ($\tau_{50} \approx 40$ min). Dextran 70, which did not enter the glycocalyx over the course of these experiments, entered at the same rate as free albumin when it was conjugated to albumin. These findings demonstrate that for anionic molecules size and charge have a profound effect on the penetration rate into the glycocalyx. The equal rates of penetration of the glycocalyx demonstrated by the different protein molecules suggests that multiple factors may influence the penetration of the barrier, including molecular size, charge, and structure.

intravital microscopy; capillaries; endothelium; glycocalyx; solute barrier

EXCHANGE OF SUBSTRATE from blood to tissue entails solute transport across the endothelial lining of blood vessels. A molecule diffusing from plasma to interstitium must traverse both the endothelial cell surface glycocalyx and the pericellular junctions of the endothelium. Although considerable attention has been paid to passive and active mechanisms that enable molecules

to traverse endothelial cells, little is known about the permeability characteristics of the endothelial surface layer, i.e., the endothelial cell glycocalyx including associated plasma proteins. Functional studies and electron-microscopic observations of endothelial surface structures suggest that the endothelial surface layer is an important determinant of solute exchange (3, 4, 9, 11, 14, 15). However, measurement of permeation of plasma solutes into the endothelial surface layer was not made until recently when we (15) demonstrated that the *in vivo* barrier properties of the endothelial surface layer can be studied using intravital microscopy. By observing labeled tracer molecules and the capillary endothelium using a combination of fluorescence and brightfield microscopy, estimates can be obtained of the luminal distribution of circulating solutes relative to the location of the luminal endothelial cell membrane. Injected solute initially fills a core plasma volume and then invades a circumferential annulus. The rate at which solute approaches the luminal endothelial surface can be accurately determined by repeated observations over time following intravenous injection of a bolus of fluorescent tracer (Fig. 1).

MATERIAL AND METHODS

In this study we used a variety of molecules of different size, charge, and molecular structure to probe the permeability characteristics of the endothelial surface layer. Free fluorescent dyes and fluorescently labeled dextrans of various molecular masses (4–2,000 kDa) were administered to establish a size and charge dependence of solute permeation rates into the endothelial surface layer. To test for a charge dependence of permeability, neutral dextrans were labeled with either Texas Red (Texas Red, neutral) or FITC (anionic). Finally, two critical plasma proteins (albumin and fibrinogen) as well as a protein-dextran complex (albumin-Dex 70) were injected, and permeation rates were compared with dextrans of similar molecular mass and charge.

Animal Preparation

All procedures and the care of animals were in accordance with institutional guidelines. Male Syrian golden hamsters

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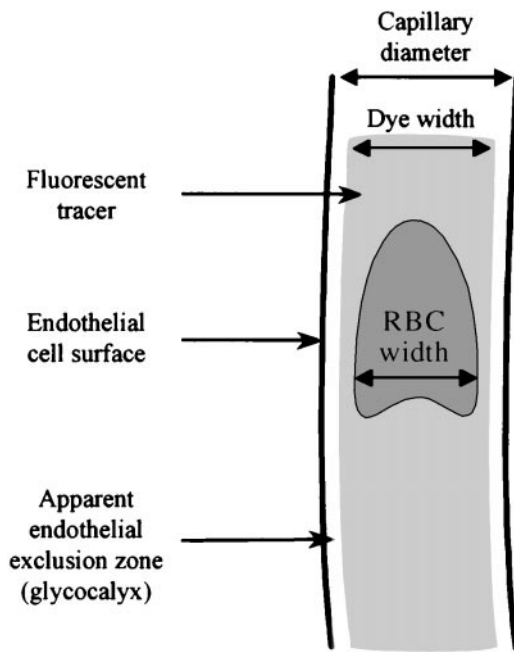


Fig. 1. Schematic illustration of method for measuring permeation of fluorescently labeled plasma solutes into endothelial surface layer (glycocalyx). Method is based on exclusion of fluorescent tracers and red blood cells (RBC) from a space adjacent to capillary endothelial cell surface. Measurements of anatomical capillary diameter, dye column width, and RBC width were compared to obtain estimates of the apparent endothelial exclusion zone (see Ref. 15 for details). Depending on molecular size, charge, and structure, some fluorescent tracers are able to permeate into endothelial surface layer over time, which leads to a decrease in apparent endothelial exclusion zone for these tracers.

(934 capillaries observed in 46 animals, mean weight \pm SD = 112 ± 12 g, mean age \pm SD = 6.6 ± 0.7 wk) were anesthetized with pentobarbital sodium (70 mg/kg body wt ip), and the trachea was cannulated to ensure a patent airway. The left femoral vein was cannulated for continuous infusion of 0.9% saline (0.5 ml/h) containing 10 mg/ml pentobarbital sodium and for bolus injection of fluorescent tracers (see *Fluorescent Tracers*). The hamster was placed on a Plexiglas platter, and the right cremaster muscle was prepared for visualization of the microcirculation as previously described. The cremaster muscle was continuously superfused at 5 ml/min with a bicarbonate-buffered physiological salt solution consisting of (in mM) 131.9 NaCl, 4.6 KCl, 2.0 CaCl₂, 1.2 MgSO₄, and 20 NaHCO₃. The superfusion solution was gas equilibrated with 5% CO₂-95% N₂ to obtain a pH of 7.35–7.45, and the solution was maintained at 34°C. Succinylcholine (10⁻⁵ M, Sigma) was added to the superfusion solution to reduce spontaneous skeletal muscle contractions. The body temperature of the hamster was maintained at 37–38°C with conducted heat.

Fluorescent Tracers

To determine the permeability of the endothelial surface coat, fluorescently labeled tracer molecules were injected via the femoral venous cannula. All tracers were dissolved in normal saline (20 mg/ml) and injected as a bolus (0.3–0.4 ml) 45–60 min after completion of the surgical preparation of the cremaster muscle.

Anionic dextrans. Anionic dextrans, i.e., dextrans conjugated with FITC, were used to probe the porosity of the endothelial surface layer. FITC-dextrans of 4 (34 capillaries in 2 animals), 17 (45 capillaries in 2 animals), 39 (95

capillaries in 2 animals), 70 (42 capillaries in 9 animals), 580 (75 capillaries in 3 animals), and 2,000 kDa (88 capillaries in 2 animals) were used. All FITC-dextrans were supplied by Sigma. The net charges on these molecules varies from batch to batch, but according to the distributor there are roughly 0.01 FITC molecules/glucose residue. Accordingly, the net charge on the dextrans would be proportional to molecular mass ranging from slightly less than 1/dextran to slightly more than 100 for the 2,000-kDa molecule. Therefore, the variation in penetration rate of the glycocalyx for these molecules represents changes in both size and charge.

Neutral dextrans. Neutral dextrans (i.e., dextrans labeled with Texas Red) of 40 (57 capillaries in 3 animals) and 70 kDa (35 capillaries in 1 animal), respectively, were also used. Dextrans conjugated with Texas Red dye were obtained from Molecular Probes.

Proteins. Bovine serum albumin [Sigma A-2934, molecular mass (mol. mass) 67 kDa] and fibrinogen (Sigma F-8630, mol. mass 340 kDa) were labeled with the fluorescein derivative dichlorotriazinylaminofluorescein dihydrochloride (DTAF). These were used in 91 capillaries in four animals and 104 capillaries in five animals, respectively. Two additional fibrinogen experiments were performed using FITC-fibrinogen (69 capillaries in 2 animals) that was kindly provided by the American Red Cross. DTAF was obtained from Research Organics Albumin conjugated with Dextran 70 (FITC-labeled albumin-Dex 70 complex, mol. mass ~136 kDa, 50 capillaries in 1 animal) was custom made by Molecular Probes.

Free dyes. Two anionic dyes, fluorescein (mol. mass 376 Da, 52 capillaries in 3 animals) and DTAF (mol. mass 568 Da, 54 capillaries in 3 animals), and one neutral dye, rhodamine (mol. mass 479, 10 capillaries in 3 animals), were injected systemically as free dyes to test for possible (charge dependent) exclusion of these small molecules by the endothelial surface coat. In three animals, two different dyes were present together, allowing for the simultaneous observation of rhodamine (3 capillaries) in the presence of fluorescein, rhodamine (2 capillaries) in the presence of DTAF, and Texas Red-labeled Dextran 40 (9 capillaries) in the presence of DTAF-albumin.

Intravital Microscopy

Microvessels of the cremaster muscle were observed with an intravital microscope (Zeiss ACM) and an SIT video camera (model 66, DAGE MTI). For brightfield observations, the hamster cremaster muscle was transilluminated with a 150-W xenon lamp. Epifluorescence observations were made using a xenon high-pressure lamp (75 W). All preparations were examined at $\times 90$ [Leitz, water immersion, numerical aperture (NA) 1.20]. A $\times 20$ objective (Leitz, NA 0.33) acted as a long-working distance transillumination condenser. Brightfield measurements were made with a 450- to 490-nm band-pass interference filter (blue light) in the light path; fluorescent FITC- and DTAF-associated molecules were observed using Zeiss fluorescence packages for fluorescein and rhodamine. Fluorescein samples were observed with an excitation filter (450–490 nm), a dichroic beam splitter (FT 510), and a long-pass filter (LP 520) in the excitation/emission pathway. Rhodamine and Texas Red were observed using an excitation filter (510–560 nm), a dichroic mirror (FT 580), and a long-pass filter (LP 590). The image was displayed on an MTI video monitor (Dage), and experiments were recorded on videotape using a Panasonic S-VHS videocassette recorder for further image analysis. Measurements were made as described previously (15).

Data Analysis

Video images were captured from videotape using a Matrox capture board and Image-1 software (Universal Imaging, West Chester, PA). An onscreen caliper using a $100 \times 0.01 = 1$ mm stage micrometer (Graticules) was used for all calibrated dimensional measurements. Estimates of the anatomical capillary diameter were obtained from brightfield images using the imaging techniques described previously (6, 15). Measurements are dependent on careful selection of the vessel midplane, using care to neither underfocus nor overfocus the image of the capillary (6). The anatomical capillary diameter was then estimated by positioning digital calipers at the inside of the capillary wall (see Fig. 1). To correct for the thickness of the caliper bars, one of the calipers was positioned on the capillary wall with the luminal surface against the capillary lumen. The other caliper was positioned in the capillary lumen with its abluminal surface against the capil-

lary wall. These estimates were compared with measurements of the fluorescent dye column after injection of the tracer. Estimates of the apparent thickness of the endothelial surface layer were obtained by subtracting the diameter of the dye column from the anatomical capillary diameter and dividing the difference by two. Prior measurements using this technique had shown good agreement between brightfield and fluorescence measurements made on beads of known size (6). Precision in these measurements was enhanced by repeated, paired measurements at a given site and by always referencing the brightfield measurement to the fluorescence determination. All values in Fig. 2 are means \pm SE.

Experimental Protocols

All experimental protocols were started 45–60 min after completion of the hamster cremaster preparation. Measure-

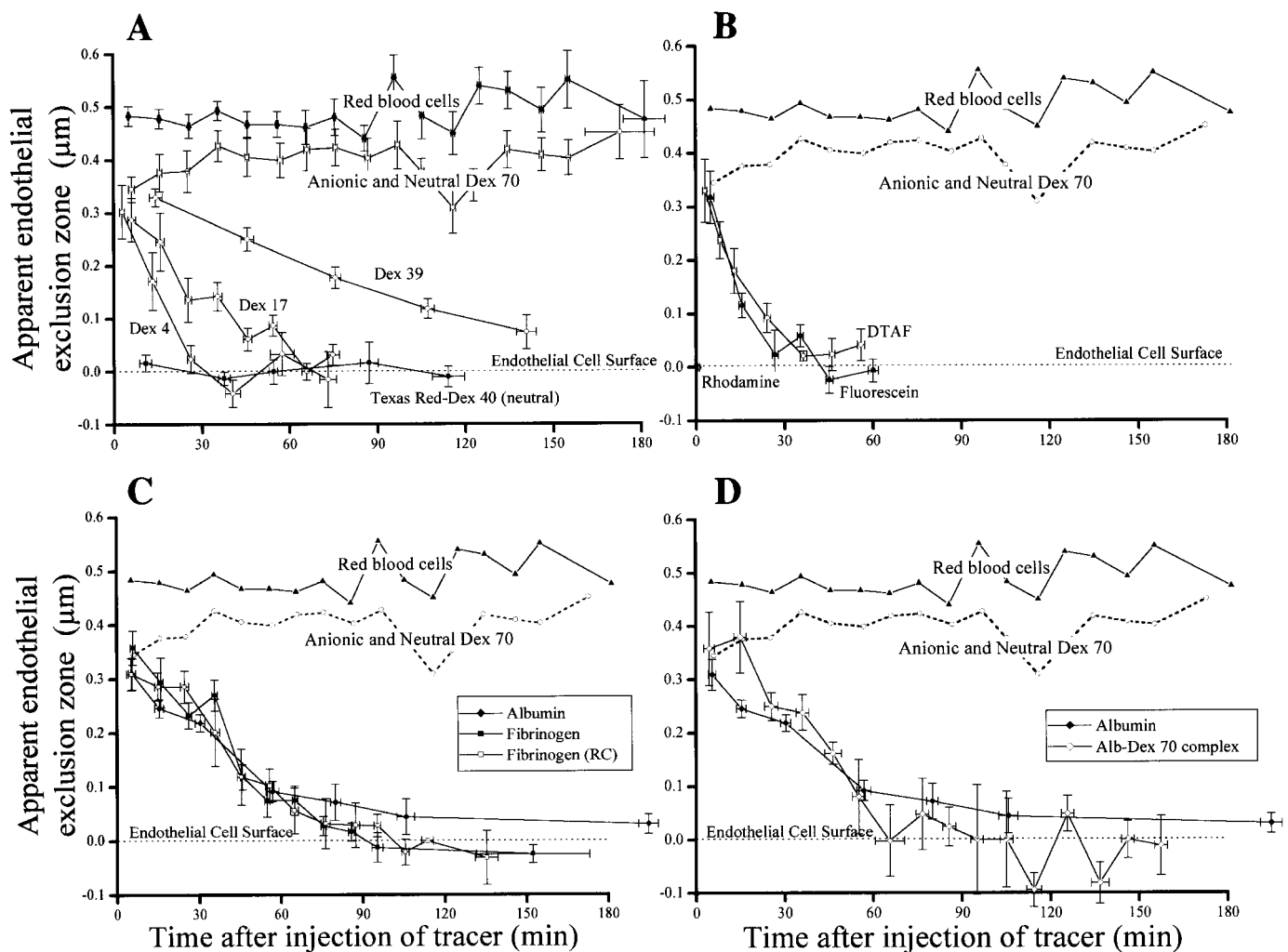


Fig. 2. Equilibration of various fluorescent molecules with endothelial surface layer. Large dextran molecules (≥ 70 kDa) remain excluded from apparent endothelial exclusion zone for >3 h, regardless of whether they are labeled with anionic (FITC) or neutral (Texas Red) fluorescent dyes. *A*: smaller anionic dextrans (mol. mass 4, 17, and 39 kDa) invade endothelial glycocalyx with size-dependent half-times of 12, 22, and 60 min, respectively. No apparent exclusion could be detected 1 min after injection of neutral (Texas Red) Dextran 40. *B*: free fluorescent dyes [FITC and dichlorotriazinylamino-fluorescein dihydrochloride (DTAF)] approached endothelial surface with half-times of ~ 11 min. Free rhodamine (neutral) approached endothelial surface within 1 capillary transit time (<1 min). *C* and *D*: proteins and conjugated proteins, respectively. Entry of albumin, fibrinogen, and albumin-Dextran 70 (alb-Dex 70) complex into endothelial cell-exclusion zone. Despite dissimilar sizes, these protein-related molecules invaded endothelial glycocalyx at similar rates, i.e., with half-times of ~ 40 min (RBC fibrinogen provided by the American Red Cross).

ment of capillary dimensions was made starting immediately after injection of the fluorescently labeled tracers.

Images of randomly selected cremaster muscle capillaries were recorded on videotape during transillumination and epi-illumination (measurements made in random order). Care was taken to ensure that epi-illumination of an individual capillary was completed within a few seconds to minimize photo damage (15). As illustrated schematically in Fig. 1, the diameter of capillaries during transillumination was taken as an estimate of the anatomical capillary diameter. The diameter of the fluorescent tracer column in capillaries was measured during epi-illumination as an estimate of the functional capillary diameter available to the tracer molecules. Repeated, paired measurements of anatomical and functional capillary diameters were made for randomly selected capillaries at intervals of ~ 3 min for >3 h or until tracer intensity had equilibrated between the vascular lumen and the extravascular space.

RESULTS

Figure 2 shows time courses of movement of various tracers into the endothelial surface layer. Data are shown for several dextrans and free dyes (Fig. 2, *A* and *B*), for selected proteins (Fig. 2*C*), and for a protein-dextran complex (Fig. 2*D*). Neutral and anionic dextran molecules with a molecular mass of ≥ 70 kDa remained fully excluded from the glycocalyx for >3 h. Smaller anionic dye molecules and dextrans (mol. mass 0.4–40 kDa) invaded the coat with size-dependent half-times between 11 and 60 min (Fig. 2, *A* and *B*). For clarity, data on the 2,000- and 580-kDa molecules are not shown because these did not penetrate the glycocalyx during the time of observation and they followed a pattern indistinguishable from that of the 70-kDa dye. Small neutral dye molecules and neutral dextrans equilibrated within the glycocalyx within a single capillary transit time (Fig. 2, *A* and *B*). This made adequate quantitation of the rate of penetration impossible using the current methodology. To ensure that the measurements of fluorescein- and rhodamine-based dyes were comparable, in a few measurements the two dyes were injected together. The patterns of dye movement were identical to those shown in Fig. 2, *A* and *B*.

The dextrans are rather long, extended polymers with uniform charge groups occurring on $\sim 1\%$ of the glucose molecules. We also examined the movement into the glycocalyx of physiologically more relevant plasma proteins. Albumin [mol mass 67 kDa, isoelectric point (pI) 5.6] entered with a half-time of ~ 40 min. Surprisingly, fibrinogen (mol mass 340 kDa, pI 5.5) entered the endothelial surface layer at the same rate as albumin, though the molecular mass ratio would predict an entrance rate for fibrinogen 44% that of albumin (Fig. 2*C*). To more directly compare the entry patterns of proteins and dextrans we obtained a protein-dextran complex (albumin-Dex 70, Molecular Probes). Remarkably, the complex penetrated the endothelial surface layer with a half-time equivalent to that of albumin (Fig. 2*D*). This rate belies the fact that unbound Dextran 70 itself did not penetrate the glycocalyx for more than 3 h (Fig. 2*A*).

DISCUSSION

Our findings demonstrate that large dextran molecules (mol mass ≥ 70 kDa) remain restricted to the central core of hamster cremaster capillaries for over 3 h, indicating that the endothelial surface layer (glycocalyx) is relatively impermeable to polysaccharides with effective radii ≥ 5.7 nm (10). Smaller dextrans and free dyes permeated the endothelial surface layer at rates that depended on molecular size and charge; half-times for anionic solutes (0.5–4.7 nm) were 11–60 min. It is important to recognize that charge and molecular weight of the dextrans vary in proportion to one another. There are roughly 0.6 fluorescein molecules per sugar in the dextrans (Sigma, unpublished data). The net charge on the proteins used to probe the glycocalyx was not determined, and the variability in labeling efficiency could be expected to cause significant variation in charge in the labeled molecules. Thus although there is an obvious correlation between both size and charge in these experiments, its exact nature at this time cannot be specified because charge and size were not systematically and independently varied.

Small neutral tracers penetrated the endothelial surface layer within a single capillary transit time. In contrast with the expected size-dependent behavior of anionic dextrans, protein entry rates did not follow predictions on the basis of their molecular mass. Uptake rates for two anionic plasma proteins, albumin (67 kDa) and fibrinogen (340 kDa), were similar despite an almost sixfold difference in molecular mass. Charge may have contributed to this unexpected similarity, though the pI for the two is reported to be the same. After labeling was completed, both would have had additional anionic groups added, but pIs of the labeled molecules were not determined because all the material was used in the animal experiments. The dextran-albumin complex (137 kDa) entered the endothelial surface layer at the same rate as native albumin (Fig. 2, *C* and *D*). This suggests that the passage of macromolecules into this space involves processes other than simple diffusion and highlights the need for systematic studies of the molecular structure of the glycocalyx. One interpretation of the observation is that the albumin can facilitate the entry of the large-molecular-weight dextran into the endothelial surface layer, a fact of major significance to our understanding of the transcapillary movement of macromolecules.

Size- and Charge-Dependent Hindrance of Macromolecular Penetration

Current transvascular exchange models predict an important role for the endothelial surface layer as a selective solute barrier (5, 13). These studies have shown that the classic pore model of exchange (12) must be extended to include polymer structures similar to those of the glycocalyx to allow consistent descriptions of transvascular pathways available to both water and solutes. It has been noted by Curry and Michel (5) that the presence of a matrix in or over the entrance to interendothelial gaps would hinder transport of solutes

relatively more than water. The effect of this would be to reduce the apparent dimension of the solute exchange pathway from an equivalent pore radius of 8.0 to 5.5 nm (5). Our experimental data on mammalian capillaries in vivo show that dextrans with radii ≥ 5.7 nm do not appear to penetrate the endothelial surface layer, which is consistent with the theoretical prediction of the equivalent pore size.

In addition to the size-dependent hindrance of solute exchange, anionic electrostatic charge distributions within the endothelial surface layer are likely to further impair exchange of anionic solutes (7, 8, 11, 16). This is evidenced here by the slower diffusion rate of anionic dextrans with radii ≤ 4.7 nm compared with neutral ones. These data are also consistent with numerous previous findings on the solute charge dependence of microvascular wall permeability (1, 10). We emphasize, however, that our measurements are made on a structure extending well into the vessel lumen, not one confined tightly to the cell surface. The physiological significance of this barrier can be appreciated by calculating the root-mean-square diffusion time for entry of albumin by free diffusion into a 0.4- μm space ($c^2 = -4Dt$; where d is the distance, D is the diffusion coefficient, and t is the time). For a molecule with a diffusion coefficient of 8.5×10^{-7} , a value roughly equal to that for albumin, the predicted diffusion time is 0.5 ms rather than the roughly 40 min observed. In any case, both theoretical predictions and our experimental data strongly support the idea that there is an important role for the endothelial glycocalyx in creating a size- and charge-selective exchange barrier associated with the microvessels.

Protein Interaction With Endothelial Surface Layer

The endothelial surface layer does not appear to be simply a size exclusion matrix, however. Although dextrans penetrated the matrix with a simple dependence on charge size, permeation by albumin (an albumin-dextran complex) and fibrinogen took place at indistinguishable rates; i.e., entry for these molecules does not follow a pattern that is predictable by size or charge alone. Although these solutes are all known to bear a net negative charge at physiological pH, their estimated radii are quite different, ranging between 3.7 and 10.8 nm for albumin and fibrinogen, respectively (2). Despite these differences in size, each of the solutes permeated the endothelial surface layer with a roughly the same half-time. We hypothesize that the similarity

in uptake times reflects some specific interaction between the diffusing molecule and structures within the endothelial surface layer.

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Received 22 March 1999; accepted in final form 28 September 1999.

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