Neutral and DEAE dextrans as tracers for assessing lung microvascular barrier permeability and integrity

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Sanders, Jonathan R., N. Adrienne Pou, and Robert J. Roselli. Neutral and DEAE dextrans as tracers for assessing lung microvascular barrier permeability and integrity. J Appl Physiol 93: 251–262, 2002. First published March 22, 2002; 10.1152/japplphysiol.00635.2000.—Steady-state lymph-to-plasma concentration ratios (L/Ps) of neutral dextrans, cationic DEAE dextrans, and endogenous proteins were determined under normal and increased permeability conditions in six unanesthetized yearling sheep prepared with chronic lung lymph fistulas. Fluorescent dextrans with radii ranging from 1 to 30 nm were intravenously infused, and after 24 h, perilla ketone (PK) was given to alter permeability while the dextran infusion was maintained. Plasma and lymph samples were collected before and after PK administration and analyzed for dextran and protein concentrations after high-performance liquid chromatography size separation. Under both baseline and increased permeability conditions, DEAE dextrans had higher L/Ps than neutral dextrans of similar size but lower L/Ps than proteins of similar size. Comparison of L/Ps before and after PK revealed that the percentage change in permeability for neutral and DEAE dextrans was significantly larger than that for proteins. These results suggest that 1) the pulmonary microvascular barrier behaves as a net negative barrier, 2) some transport mechanisms for proteins and dextrans are different, and 3) neutral and cationic dextrans are more sensitive markers than proteins of the same size for assessing changes in pulmonary capillary permeability.

lymph-to-plasma concentration ratios; sheep; high-performance liquid chromatography; diethylaminoethyl

MAINTENANCE OF NORMAL CAPILLARY PERMEABILITY is essential for viable organ function. However, during certain pathological disease states such as acute lung injury or, in its worst form, adult respiratory distress syndrome, normal capillary permeability is compromised. Under these conditions, proteins leak through the capillary membrane and accumulate in the tissue spaces, increasing osmotic pressure that further contributes to edema formation. Although the associated increase in permeability has been studied for many years, the exact mechanisms through which acute lung injury develops and progresses remain unresolved.

Since the pioneering studies of Pappenheimer et al. (27), the permeability properties of microvessels and microvascular beds in intact preparations and of cell monolayers have been extensively investigated. Based on information obtained from these studies, it now appears that transvascular exchange is via the following mechanisms: 1) passive electrochemical gradients that develop across intercellular gaps (pores) and/or plasmalemmal invaginations in the membrane; 2) energy-dependent active-transport mechanisms associated with receptors in vesicles (39, 40); and 3) convection through large transmembrane pores, possibly resulting from vesicle fusion (35). Despite an abundance but perhaps insufficient amount of data, considerable debate still exists as to which mechanisms truly exist and/or dominate in vivo (11, 26).

It is widely accepted that fenestrated capillaries behave as size-selective sieves, allowing small neutral molecules to cross unhindered and larger molecules to cross as a function of their size. However, the mechanisms via which this sieving takes place are still under some debate. Before the early 1980s, it was believed by many that this sieving was associated with gaps located between adjacent endothelial cells, and many theoretical equivalent pore descriptions for describing this process were developed (27, 36). However, the observation that the bulk of these intercellular gaps were too large to fully account for the measured sieving properties (26) and the identification of a cell surface fibrous matrix (glycocalyx), which also extends into intercellular junctions, have led many to believe that molecules are sieved primarily by this structure (Ref. 12; for review, see Ref. 26). Furthermore, the fact that the glycocalyx is composed of membrane-anchored anionic glycoproteins and stabilized by adsorbed plasma proteins such as albumin and fibrinogen lends support to the hypothesis that solute and barrier charge are also determinants of solute flux.

Though the presence of these charged sites is recognized, the effects of charge and other solute properties on transvascular exchange are not well understood. In addition, it is not known how disruption of the integrity of the vascular barrier affects solute transport or...
what role specific elements, such as charge, play in maintaining normal barrier function. Modification of the cell surface charge in different vascular beds with polycations alters the permeability of charged species (3, 13, 20, 22), and various inflammatory mediators increase microvessel permeability (8, 32), although some of these latter effects may be due to direct cell damage and not via a charge-dependent mechanism (8).

Furthermore, the total barrier to solute flux is heterogeneous and consists of numerous components in addition to endothelial cells. Also, the barrier does not simply sieve solutes in a static manner but rather in a dynamic one with endothelial cells responding to their surroundings via signal-transduction mechanisms to regulate permeability through changes in cytoskeletal properties, junction protein content, and cell surface composition (26). Thus the transport of solutes across microvascular barriers is complicated and appears to be affected by any combination of the following: 1) solute size, charge, shape, and reactivity; 2) the concentration of fuel sources (such as ATP and GTP) available for active-transport processes (39); and 3) properties of the barrier itself, specifically electrical charge and steric properties that exclude components from reaching the cell surface (46), capillary basement membranes (6, 45), and collagen-supported interstitium (47).

If indeed transport mechanisms for proteins and other tracer molecules are different, it is likely that some test solutes could be more sensitive to different types of lung injury than others. Thus distinguishing between these different mechanisms will likely lead to a better understanding of the determinants of solute transport and will ultimately improve our ability to accurately diagnose various lung pathologies. In measurements made from the same sheep, it was the goal of this research to 1) examine the role that solute charge plays in regulating blood to lymph solute transport, 2) determine whether neutral and cationic dextrans are transported in a different manner than proteins or each other, and 3) determine whether fluorescent dextrans are more sensitive to changes in lung permeability than endogenous proteins. Thus we examined the steady-state lymph-to-plasma concentration ratios (L/Ps) of neutral dextrans, cationic DEAE dextrans, and anionic proteins under baseline permeability conditions and compared these ratios to those obtained after lung injury with perilla ketone (PK), a permeability increasing agent.

METHODS

Dextran Preparation

Neutral dextrans labeled with tetramethylrhodamine isothiocyanate (TRITC) and with peak molecular masses ranging from 4,400 to 155,000 g/mol were purchased from Sigma Chemical (St. Louis, MO). Positively charged DEAE dextrans with weight-average molecular masses of 70,000 (70K) and 500,000 (500K) g/mol and nitrogen contents of 1% were generously provided by Pfeifer and Langen (Dormagen) and were labeled with 5-(4,6-dichlorotiazin-2-ylamino)-fluorescein (DTAF; Sigma Chemical) according to the labeling protocol of deBelder and Granath (14). Briefly 1.0 g of 70K DEAE dextran was dissolved in 40 ml of water purified via reverse osmosis (0.5 g of 500K DEAE dextran in 30 ml of water). The pH of the resulting solution was adjusted to 10 by titration with 1 N NaOH. Two hundred milligrams of DTAF (50–100 mg for 500K) were added in portions of 30–50 mg with pH adjustment to 10 after each addition. After all DTAF was added, the solution was incubated at room temperature for at least 2 h. Free dye was removed by passing the mixture through disposable PD-10 columns packed with Sephadex G25M (Pharmacia Biotech, Piscataway, NJ) that had been equilibrated with five column volumes of 50 mM sodium phosphate buffer (pH 7.3). To remove any residual free dye, the resulting complex was dialyzed against ultrapure water for at least 36 h by using Spectra-Por tubing with a molecular weight cutoff for proteins of 1,000 (Spectrum, Houston, TX). To obtain DEAE of a smaller size than that obtained from Pfeifer and Langen, 0.5 g of the larger DEAE dextran was heated for 3 or 5 h in 10 ml of 1 N HCl at 70°C or for 25 min at 100°C. After the indicated time, the pH was brought to 10, and the resulting solution was labeled with DTAF as described above.

Size-Exclusion Column Calibration

Size-exclusion high-performance liquid chromatography (HPLC) was performed at room temperature (22°C) by using a TSKgel 4,000 polymer-based size-exclusion column (60 cm × 7.5 mm ID; Tosoh Haas, Montgomeryville, PA) and guard column (7.5 cm × 7.5 mm ID) equilibrated with 0.8 M sodium nitrate mobile phase as described previously (38). Briefly, known concentrations of dextran, polyethylene glycol, and polyethylene oxide standards were injected at the column inlet and allowed to elute in parallel outlet streams through a refractive index detector and a differential viscometer. Discrete refractive index data were converted into discrete concentration values on the basis of the known concentrations of the standards. Intrinsic viscosity was then calculated from these values and the viscosity measurements. Products of the manufacturer-determined molecular masses (M) and the calculated intrinsic viscosity (η1) were plotted on a log scale against solute elution coefficient K (Fig. 1), determined from the following expression: K = (Ve/V0)/((Ve/V0) − 1), where Ve represents solute elution volume, V0 represents column void volume, and Vt represents total column volume. The result was a universal calibration curve, fit with a third-order polynomial, from which the size of dextrans and proteins with unknown radii were predicted as described in Sample Concentration Analysis.

Animal Surgery

Six yearling sheep ranging in weight from 30 to 40 kg were prepared with chronic lung lymph fistulae under sterile conditions, as previously reported (31). Each sheep received a priming dose of thiopental sodium (20–25 mg/kg) to allow for intubation and was subsequently ventilated and maintained under halothane anesthesia for the duration of the surgery. A right thoracotomy was made at the fifth intercostal space to allow cannulation of the effenter lymphatic of the caudal mediastinal lymph node (CMLN). The lymph catheter was exteriorized and stabilized with silicon glue. A second thoracotomy was made at the ninth intercostal space and used to ligate the tail of the node at the level of the inferior ligament to help eliminate systemic contamination. Any observable lymphatics deemed not to be of pulmonary origin were li-
lymph samples were collected every 30 min to 1 h. After pressures were continuously monitored, and blood and at a constant rate of 42 ml/h over the course of 26 Swan-Ganz catheter by an IMED volumetric infusion pump saline and administered through the proximal port of the neutral dextrans (radii

within 48 h were frozen and thawed just before solute size analysis of dextrans and proteins. All samples not analyzed in gated, enabling collection of lymph from pulmonary sources only. After wound closure, incisions were then made on the right side of the neck, and the right carotid artery and jugular vein were cannulated for blood collection and placement of an introducer (used for insertion of a Swan-Ganz catheter), respectively. The sheep were allowed to recover for 6–10 days before experimentation.

Protocol

On the day of the experiments, unanesthetized sheep were transported via metabolic cages to the laboratory, where food and water were provided. A Swan-Ganz catheter was floated into the pulmonary artery, allowing for dextran infusion and measurements of pulmonary artery and wedge pressures. Systemic arterial pressure was measured from the carotid artery catheter. Approximately 1 g of both TRITC-labeled neutral dextrans (radii = 3–30 nm) and DTAF-labeled DEAE dextrans (radii = 1–30 nm) were filtered into 1,200 ml of saline and administered through the proximal port of the Swan-Ganz catheter by an IMED volumetric infusion pump at a constant rate of 42 ml/h over the course of 26–30 h. Pressures were continuously monitored, and blood and lymph samples were collected every 30 min to 1 h. After 16–20 h, small labeled dextrans (1–5 nm, 0.3–0.5 g) were added to the dextran-saline solution. After 24 h, barrier integrity was altered by administering PK (25 mg/kg) in equal parts of DMSO, as reported previously (2). Blood and lymph samples were centrifuged at 2,000 revolutions/minute for 10 min to remove red blood cells and/or any large particulate matter, and the plasma and lymph were recovered and injected into the HPLC system for size and concentration analysis of dextrans and proteins. All samples not analyzed within 48 h were frozen and thawed just before solute size separation and concentration measurements.

Sample Concentration Analysis

Concentrations and L/Ps from HPLC. Plasma and lymph samples were filtered by using a 0.2-µm Supor membrane filter, and 100 µl of each sample were injected into the calibrated size-exclusion column. The postcolumn eluant flow was again split as in the case for column calibration, but this time flow was passed through the refractive index detector, which measured protein concentration, and also through a fluorescence spectrophotometer F4500 (Hitachi) for fluorescent-dextran concentration measurements. This dual-detector approach was necessary for detection of the small quantities of dextran and for distinguishing between dextran and unlabeled endogenous proteins.

The fluorescence software that we employed (Multi-Wavelength Time Scan Program, Hitachi) enabled us to scan from one to eight different excitation-emission wavelength pairs. Because TRITC and DTAF emit at different wavelengths [excitation/emission = 555/580 nm (TRITC) and 490/520 nm (DTAF)] and with essentially no overlap in the concentrations present in the plasma and lymph samples, we were able to simultaneously measure the fluorescence of neutral and DEAE dextrans in plasma and lymph samples at each eluted from the column. An additional excitation-emission pair (377/433 nm) was monitored such that peaks from the intrinsic fluorescence of proteins could be used to account for small variations in pump flow rate. The duration of time required to scan through these three pairs of wavelengths once was 2 s.

Although many of these chromatograms revealed the presence of dextrans as early as 920 s after injection and as late as 1,800 s after injection, the exclusion properties of our size-exclusion column were such that we could accurately distinguish only between molecules that eluted in the range of 1,020–1,700 s, corresponding to an elution volume range of 12.75–21.25 ml under constant-flow conditions of 0.75 ml/min. To express the dextran and protein elution volumes in terms of molecular radius, dextrans were assumed to behave as random coils in plasma and lymph and the proteins as rigid spheres, such that their size could be described by using the following expressions (9, 38) for dextrans

\[ R_g = 0.288(\eta)M^{1/3} \]

and for proteins

\[ R_h = 0.254(\eta)M^{1/3} \]

where \( R_g \) represents radius of gyration (in nanometers), \( R_h \) is hydrodynamic radius (in nanometers), and \( \eta \) is equal to the product of solute hydrodynamic volume, Simha’s shape factor, and Avogadro’s number (44). After converting the range of elution volumes given above into exclusion coefficients and substituting these values into the universal calibration equation (Fig. 1) at each data-acquisition time after sample injection, L/Ps for dextrans and proteins at each of these sizes were determined by simply dividing values on the lymph chromatograms by those on the plasma chromatograms after subtraction of plasma and lymph protein autofluorescence in the respective wavelengths.

Electrophoresis. As a potentially more sensitive indicator of protein concentration in plasma and lymph, samples ac-
quired during baseline and altered permeability conditions were separated via electrophoresis on precast vertical slab polyacrylamide gels (4–20%, Novex), and concentrations of nine distinct protein fractions were determined. Briefly, each gel was placed in a Novex mini-cell II electrophoresis apparatus and the wells occupied with either a plasma sample, a lymph sample, or low- and high-molecular-mass protein standards (Pharmacia). The anode and cathode chambers were filled with a Tris-glycine buffer, and samples were allowed to migrate at 4°C for 16 h under a constant potential of 125 V. Staining was accomplished with a Coomassie blue dye allowed to react for 10 min, and destaining was accomplished with a mixture of methanol, water, and acetic acid. Gels were scanned in an electrophoresis ultrascanning XL scanner and analyzed for protein concentration by using Gelscale software (Pharmacia). From these calculations, L/Ps were determined for nine distinct protein fractions and compared with those obtained from protein analysis with HPLC.

Other Measurements

Multiple-indicator dilution. To quantify changes in membrane integrity, multiple-indicator dilution (MID) data were collected immediately before and ∼3 h after infusion of PK. Briefly, mixtures of 51Cr-labeled red blood cells, 125I-labeled albumin, tritiated water, and either [14C]-urea or [14C]-butanediol were prepared and injected via the Swan-Ganz catheter into the right atrium. Samples were collected every 0.5 s from the carotid artery catheter and analyzed for radioactivity by using gamma and beta detectors after correcting for spectral overlap. Data were plotted in the form of radioactivity vs. time curves, which were then analyzed for cardiac output, urea permeability-surface area product, butanediol surface area, and extravascular lung water, as previously described (21).

Wet-to-dry lung weight calculations. After post-PK MID data collection, each sheep was given a lethal dose of thiopental sodium, and the chest wall was opened and each lung excised and processed for blood-corrected wet and dry lung weights (10).

Statistical Analysis

L/P data are represented as means ± SE for 21 select fractions corresponding to dextran and protein sizes ranging from 1 to 20 nm. During conditions of normal permeability, six sheep were included in the analysis, whereas for measurements made during altered permeability conditions, five sheep were included since one sheep did not survive the PK treatment. To compare the L/Ps for neutral dextrans, DEAE dextrans, and proteins before or 2–3 h after PK, we employed the Student’s t-test for unpaired observations. A Student’s t-test for paired observations was employed to compare the effects of treatment (PK on 1) the L/Ps for each dextran and protein and 2) pulmonary arterial and venous pressures, systemic pressures, lymph flows, total protein concentrations in lymph and plasma, and total protein L/Ps. P values of <0.05 were taken as significant.

RESULTS

Dextran Characterization

Charge determination. Once the DEAE dextrans were labeled with DTAF, confirmation of charge was accomplished by using cation-exchange columns containing an SP Sepharose gel (Pharmacia). Briefly, columns were equilibrated with a 50 mM phosphate buffer (pH = 7.3), and 0.5-ml samples were injected and eluted through a Hitachi F-4500 fluorescence detector or a Rainin refractive index detector. The column was removed, and another 0.5 ml of the same sample was injected through the reconnected system at the same flow. The area under each chromatogram was determined and compared. On the basis of differences in areas, the percentage of DEAE dextran binding to the column was determined. The charge on DEAE dextrans was not affected by DTAF labeling, with ∼90% of both the unlabeled and labeled DEAE dextrans binding to the column. However, the degree to which the DEAE dextrans interacted with the cation-exchange gel was compromised when these dextrans were broken down over longer periods of time (3–5 h) and at a lower temperature (68°C). Comparisons of the concentrations of DEAE dextrans in urine to neutral dextrans of similar size (data not shown) indicated that these DEAE dextrans maintained enough positive charge to exhibit similar plasma-to-urine exclusion trends as those proposed by Bohrer et al. (5) for rat glomerular membranes. As expected, the charge of the TRITC-labeled dextrans was essentially neutral.

Dextran degree of substitution. With the use of various concentrations of DTAF dissolved in sodium tetraborate (25 mM, pH = 9.0), a calibration curve was constructed in which dye fluorescence vs. dye concentration was plotted. The fluorescence of a known amount of DTAF-labeled DEAE dextran was also determined, and this value was used to determine how much dye was present on the dextran in terms of a ratio of milligrams of dye to milligrams of dextran. Degree of substitution values ranged from 0.008 to 0.02 mg of dye/mg of dextran. However, it is important to note that buffer salts were also present in the dried conjugate, so these values represent underestimates of the true substitution ratios. The degree of TRITC-substitution on neutral dextrans was determined by the manufacturer and taken as accurate, ranging from 0.002 to 0.01 molecules of TRITC/molecule of glucose equivalent.

Linearity of Fluorescence vs. Concentration Curves

To determine the nature of the relationship between fluorescence and dextran concentration in a range corresponding to the experimental concentrations, the injectate from one study was serially diluted, and each diluted sample was separated by using size-exclusion chromatography. The respective chromatograms generated were then discretized into fractions, radii computed from Eq. 1, and fluorescence vs. concentration plots produced for each size neutral and DEAE dextran. From these data, it was determined that the fluorescence-concentration relationship was linear for all sizes of TRITC-neutral dextran and most sizes of DTAF-DEAE dextran, but there was a marked nonlinearity for DTAF-DEAE dextrans with $R_g \leq 2$ nm. However, this was not an issue in calculations of L/P since DEAE dextrans in this size range had similar
concentrations in lymph and plasma, and ratios of the two cancelled out any nonlinear effects.

Steady-State L/P

After data acquisition, fluorescence data for TRITC-neutral dextrans, and DTAF-DEAE dextrans, and refractive index data for endogenous sheep proteins, all obtained after size separation of lymph and plasma sample contents, were exported into Microsoft Excel for analysis. After baseline subtraction and conversion of elution volumes into radii, the ratios of the appropriate lymph and plasma data were calculated to generate L/Ps as a function of solute radius (Fig. 2). Substantial sieving of each dextran type was observed with neutral dextran L/Ps approaching 0.9 for small sizes and 0 when dextran size reached 5.5 nm. L/Ps for DEAE dextrans were nearly identical to those for neutral dextrans when $R_g \leq 2.5$ nm, but, in contrast to the larger neutral dextrans, DEAE dextrans as large as 20 nm were able to penetrate the plasma-lymph barrier. However, there was a bimodal distribution in DEAE dextran L/Ps as a function of solute radius, with the second peak corresponding to $R_g = 4.5$ nm. We present evidence below indicating that this second maximum was associated with DEAE dextran binding to plasma proteins.

Proteins with radii from 4 to 9 nm revealed barrier sieving characteristics with L/Ps of $\sim 0.8$ for proteins with $R_h = 4$ nm, closely corresponding to the hydrodynamic size of albumin, and dropping to 0.28 for those with radii of 8–9 nm. However, a plateau in L/Ps was observed for proteins with $R_h \geq 9$ nm, suggesting a different transport mechanism for these large proteins. Concentrations of proteins <2.5 nm were too low to allow for accurate characterization of their L/Ps by using our technique. As a potentially more sensitive indicator, the concentration of nine endogenous protein fractions in plasma and lymph were determined after electrophoretic separation and L/Ps were determined (data not shown). L/Ps calculated from the resulting data were nearly identical to those obtained from chromatography measurements with no statistical significance at an alpha value of 0.05.

Differences in L/Ps were significant for neutral and DEAE dextrans with $R_g$ between 3.5 and 20 nm and for neutral dextrans and proteins with radii in the range of 2.5–20 nm. The differences in L/Ps for DEAE dextrans and proteins were significant in the range of 2.5–19 nm (with the exception of 4.5–5.0 nm). These data appear to be consistent with a negative transport barrier, with active or convective transport of proteins explaining their higher values for L/P.

To examine whether the cause of the extra hump in the DEAE dextran L/P curves was associated with dextran-protein binding, we subjected the proteins in lymph and plasma samples to hydrolysis with proteinase K (Fig. 3). Optimal conditions were found by adding 1 ml of a 3 mg/ml solution of proteinase K to 1 ml of plasma or lymph and heating it at 60°C for 30 min. Activating the enzyme at 45°C resulted in substantially less hydrolytic activity and lowering the proteinase K concentration to 2 mg/ml and activating at 60°C also reduced the effect but only slightly. Increasing the proteinase K concentration to 5 mg/ml had no additional effect. The fact that proteinase K did not cause breakdown of neutral and DEAE dextrans was confirmed by subjecting a known volume of plasma DEAE dextran concentration levels, although there was a clear reduction in the lymph and plasma DEAE dextran concentration levels, although this reduction was not sufficient to remove all of the large DEAE dextrans, suggesting that a notable percentage of these larger DEAE dextrans were not bound to plasma proteins. To confirm that the binding was not an in vivo artifact, we added DTAF-labeled DEAE dextrans to a plasma sample, incubated the mixture for 30 min, and then measured fluorescence of the size-separated fractions. A distinct peak (not present in the infusate) was noted for fluorescence in the albumin range, suggesting that these dextrans do indeed bind to proteins and indicating that the binding was not associated with DEAE breakdown in vivo. Attempts to examine L/Ps after protein digestion proved unsuccessful due to difficulties associated with the resolving capability of our single HPLC column and a lack of adequate amounts of sample to sufficiently quantify the nature of the hydrolysis.

L/P After Injury With PK

After 22–24 h, PK was dissolved in DMSO and administered at a concentration of 25 mg/kg, as previously described (2). Sheep were closely monitored for 2–3 h after PK and allowed to breathe oxygen-enriched air if needed. Blood and lymph samples were taken every 15–30 min, and paired samples were injected into the column to determine L/Ps. At the end of this time, MID data were again collected to assess changes in permeability, and the sheep were killed and their lungs excised for postmortem processing. We noted
only transient increases in pressure that lasted no more than the 15- to 30-min period immediately after PK and stabilized at near-baseline values (Table 1). Lung lymph flow increased an average of fivefold within 1 h, after which it remained constant at an average rate of ~30 ml/h (Table 1). This increased lymph flow was associated with an increase in L/P for all tracers (Fig. 4) and slightly elevated (but not statistically different) total protein levels in lymph compared with steady-state values (Table 1). MID data revealed elevated permeability (Table 1) and a slight increase in blood-corrected wet-to-dry lung weight ratios compared with the normal data of Collins et al. (10). Electrophoretic data for proteins were consistent with data obtained from chromatography.

Differences in L/Ps before and after injury were found to be significant in the ranges of \( R_g = 1-15 \) nm for neutral dextrans, \( R_g = 1.5-20 \) nm for DEAE dextrans, and \( R_b = 4.5-15 \) nm for proteins, excluding 6.5–7.5 nm. Interestingly, the degree to which the permeability to each tracer changed in response to PK were such that neutral dextran L/Ps were elevated by as much as an astounding 2,500% (Fig. 5). This change was still significant, although less pronounced for DEAE dextrans (maximum of ~1,500%). Proteins proved to be much less sensitive to changes in permeability, with maximal changes of <100% and only ~10–30% for proteins <7 nm in size.

**DISCUSSION**

**Justification of Expressions for Estimating Solute Radii**

Since it was first proposed by Benoit et al. (4), evidence suggests that solutes elute from a size-exclusion column on the basis of their hydrodynamic volume, not their molecular weight or Stokes radius as previously believed. Thus an expression relating hydrodynamic volume to radius must be utilized to characterize solute size after size-exclusion chromatography, with the choice of this expression being dictated by solute shape in a given solution. In light of the fact that it is extremely difficult to be absolutely certain of the configuration and shape of a molecule in vivo, generaliza-
tions, on the basis of the behavior of test solutes in solutions that mimic biological fluids, are necessary. In the calculations of solute size presented herein, neutral and DEAE dextrans were assumed to behave as random coil molecules in plasma and lymph and endogenous proteins as spheres. On the basis of these properties, dextran sizes were described in terms of their radii of gyration (Eq. 1) and protein radii determined from the Einstein equation (Eq. 2), with both expressions being functions of hydrodynamic volume and proportional to solute elution volumes.

$L/P$ ratios of dextrans and proteins that eluted from the size exclusion column at the same time were compared. On the assumption that universal calibration applies to these solutes, their hydrodynamic volumes and hydrodynamic radii would be equal. However, the amount of dextran and protein in the lymph and plasma samples is partially governed by their size in vivo, which might be different than their size in the mobile phase used for solute elution. To examine the implications of using different expressions for radius when comparing $L/P$s, we calculated dextran radii by using the Einstein equation (Eq. 2), thereby assuming a spherical shape for the dextrans, and compared $L/P$ values as a function of this size parameter. The effect in these cases was a magnification of the differences in $L/P$ between dextrans and proteins. This effect would have been even more pronounced had the diffusivity-based Stokes equation been utilized. Thus we believe our use of different expressions for radius to be valid for interpreting differences in $L/P$s of like-sized solutes because these expressions should mimic dextran and protein size in vivo.

**Table 1. Effects of dextran and PK infusion on protein concentrations in lymph and plasma, lymph flow, hemodynamics, MID data, and postmortem lung weight calculations**

<table>
<thead>
<tr>
<th></th>
<th>Preinfusion</th>
<th>Initial</th>
<th>SS</th>
<th>Final</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lymph, g/dl</td>
<td>4.49 ± 0.25</td>
<td>4.62 ± 0.24</td>
<td>4.11 ± 0.12</td>
<td>4.23 ± 0.20</td>
</tr>
<tr>
<td>Plasma, g/dl</td>
<td>6.51 ± 0.25</td>
<td>6.51 ± 0.18</td>
<td>5.96 ± 0.12</td>
<td>5.48 ± 0.17*</td>
</tr>
<tr>
<td>$L/P$</td>
<td>0.69 ± 0.02</td>
<td>0.71 ± 0.03</td>
<td>0.69 ± 0.02</td>
<td>0.77 ± 0.03*</td>
</tr>
<tr>
<td>Flow, ml/h</td>
<td>5.9 ± 1.3</td>
<td>6.7 ± 1.3</td>
<td>6.0 ± 1.1</td>
<td>29.8 ± 3.5*</td>
</tr>
<tr>
<td>SA, mmHg</td>
<td>86 ± 2.9</td>
<td>88 ± 3.6</td>
<td>80 ± 2.6</td>
<td>30 ± 4.6</td>
</tr>
<tr>
<td>PA, cmH$_2$O</td>
<td>18 ± 0.8</td>
<td>22 ± 2.1</td>
<td>20 ± 0.7</td>
<td>21 ± 1.5</td>
</tr>
<tr>
<td>Wedge pressure, cmH$_2$O</td>
<td>7 ± 0.7</td>
<td>10 ± 1.0</td>
<td>9 ± 0.6</td>
<td>10 ± 2.3</td>
</tr>
<tr>
<td>CO, l/min</td>
<td>4.2 ± 0.4</td>
<td>4.5 ± 0.4</td>
<td>4.8 ± 0.09</td>
<td>4.91 ± 0.43</td>
</tr>
<tr>
<td>$PS_{urea}/PS_{but}$</td>
<td>0.33 ± 0.04</td>
<td>0.48 ± 0.09</td>
<td></td>
<td></td>
</tr>
<tr>
<td>$(W - D)/D$</td>
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</tbody>
</table>

Values are means ± SE. Steady-state (SS, time ≥ 22 h) values for each parameter were compared with those obtained at the end of the study (final, 2–3 h after perilla ketone (PK)). Preinfusion corresponds to the period of time between inserting the Swan-Ganz catheter and beginning the dextran infusion. Initial corresponds to the 1 h after initial infusion. MID, multiple-indicator dilution; $L/P$, lymph-to-plasma concentration ratio; SA, systemic arterial pressure; PA, pulmonary arterial pressure; CO, cardiac output. $PS_{urea}/PS_{but}$, ratio of urea permeability-surface area product to butanediol permeability-surface area product; $(W - D)/D$, blood-corrected wet-to-dry weight ratios. *Significant difference ($P < 0.05$).

Justification of Measurements

**Steady state.** All pre-PK (baseline) $L/P$s were calculated from lymph and plasma samples that were taken at least 22 h after initiation of the large dextran (3–30 nm) infusion and at least 3 h after the smaller dextrans (1–3 nm) were added to the infusate. Assurance that
baseline L/Ps represented steady-state values was confirmed with transient DEAE dextran L/P data obtained during these studies and by following the approach to steady state of a 5.0-nm TRITC-labeled neutral dextran. In contrast to the neutral dextrans, very small DEAE dextrans (1–3 nm) were infused into each sheep throughout the experiments from the beginning of the study until its termination. As a result, DEAE dextran L/P ratios could be determined at many different times for each of the sheep. The transient nature of these curves revealed that steady-state L/P values were achieved within ~16 h in all but one sheep, although lymph and plasma concentrations of infused solutes tended to increase even after 22 h of infusion. Due to the fact that small neutral dextrans were not infused until ~20 h into each experiment, we could not measure the transient nature of the neutral dextran L/P curves. However, the transient lymph concentration of a 5.0-nm neutral dextran revealed that steady state for this dextran was also reached within ~16 h.

Lanken et al. (24) reported that the L/Ps for different-sized titrated neutral dextrans reached steady state within 5.5–7.5 h during an injection protocol consisting of a bolus followed by a constant infusion. Initial attempts to inject our tracers in a similar manner were halted after 1–3 h after PK because these two sheep responded adversely to this rapid administration of fluorescent dextrans, with pulmonary arterial pressures climbing to ~30 cmH₂O during this time. Thus we infused at lower rates and extended our study to three times that reported to be of sufficient time to reach steady state in the study of Lanken et al. and believe that this is an adequate amount of time for steady state to be reached.

**Drawbacks of lymph fistula preparation.** A fundamental assumption in assessing barrier properties after analysis of samples collected from lung lymph fistulas is that the concentration of dextran in the lymph is the same as in the interstitium and that this represents lymph from pulmonary sources only. Observations by Henze et al. (23) indicate that ⁹⁹mTc-labeled neutral dextrans are not taken up in large quantities by lymph nodes in dogs and humans. In addition, the protein composition of lymph is not changed by the CMLN (41). Whether fluorescent DEAE dextrans are taken up by the CMLN has not been investigated, but we suspect that they are not since their L/Ps were significantly higher than those for neutral dextrans. Regarding possible lymph contamination, numerous precautions, as described by Parker (31), were taken during the initial surgery to prevent systemic contamination.

### Pulmonary Microvascular Transport

Studies designed to assess transport in the pulmonary microcirculation have employed two different experimental techniques: 1) measurements of dextran or other solute concentrations in lung lymph relative to that in plasma, with results taking the form of L/Ps and/or reflection coefficients (19, 24, 28, 29, 33), and 2) tissue-uptake studies (28, 43). These methods of presentation have proven extremely valuable, but precious few have examined the effects of solute charge on pulmonary microvascular transport. Pietra et al. (33) showed that neutral dextrans were sieved in the lung in a size-selective manner and that the L/P was higher for anionic dextran sulfate than for neutral dextrans of similar size. These data correlated well with measurements of protein L/P. However, only trace quantities of dextran were administered, and the L/P was only characterized for six discrete sizes. Contrary to these findings, McNamee (25) showed that the blood-lung lymph barrier was less restrictive to neutral dextrans than to proteins of similar size. These latter data may possibly be explained by the finding that lymph collected from sheep prepared with chronic lymph fistulas can have substantial contamination from systemic lymph sources when systemic lymphatics are not cauterized (15).

In addition, comparison of L/P data collected in similar sheep preparations but by different groups reveal that very large proteins are not sieved in a size-selective manner (30) but large neutral dextrans are (19, 24), suggesting that protein and dextran transport may be via mechanisms that are fundamentally different. Most proteins carry a net negative charge at physiological pH, and many also have specific membrane receptors to facilitate transport. Thus, from most previous studies, it cannot be determined whether it is the size and charge of proteins, the other available transport mechanisms, or the combination of each of these that leads to their elevated L/Ps. The studies presented herein allow some distinction between the different transport mechanisms available to proteins and dextrans as discussed below.

### Implications of L/P Curves During Baseline Permeability Conditions

Many implications can be drawn from the above data. The fact that both the cationic dextrans and predominantly anionic proteins had L/Ps greater than those found for the neutral dextrans appears at first to

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**Fig. 5.** Percent change in L/Ps for neutral dextrans (NEUT), DEAE dextrans, and proteins (PROT) when values obtained 2–3 h after PK are compared with those obtained during steady-state conditions.

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be contradictory; however, it is entirely possible that the mechanisms responsible for the enhanced transport are different. At least three different albumin receptors have been localized on endothelial cell surfaces and caveolae (40), and it was recently found that these caveolae have all the machinery needed for transport across microvascular endothelial cells (39). Because the neutral dextrans did not bind substantially to proteins, and by virtue of their electroneutrality, the L/Ps obtained for neutral dextrans likely reflect passive paracellular transport.

If this sieving pathway were the only one available to the dextran and protein molecules, molecules with radii above a certain value would be excluded from lung lymph. However, it is interesting that protein L/Ps approached a plateau for radii greater than ~9 nm, a finding consistent with data reported by Parker et al. (30). In that study, the authors used an intact dog lung lymph preparation to determine the osmotic reflection coefficients for several different proteins as a function of size. Data were fit with a two-pore model and indicated that the plateau in reflection coefficients could be associated with solvent drag via convective transport through a small population of large pores. However, because the large neutral dextrans examined in our study did not exhibit a similar plateau in their L/Ps, it seems that the transport of these large proteins is not via pores.

We present evidence in this study that suggests that much of the enhanced transport for the DEAE dextrans was due to cotransport with proteins, mainly albumin. As shown in Fig. 2, the DEAE dextran L/P curve was bimodal in nature, with a peak occurring at an $R_g$ of ~1.5 nm and a second peak at 4.5 nm. This extra hump occurred at a slightly larger elution volume than that which corresponded to albumin peak elution, suggesting that small cationic dextrans likely bind to albumin. To examine the effects of dextran size on dextran-protein binding interactions, we conducted a simple study in which we incubated dextrans in saline and in plasma for 30–40 min at 37°C and examined differences in the chromatograms. The underlying assumption made in these studies was that the true distribution of dextrans should be indicated by the fluorescence chromatogram obtained when the dextrans were dissolved in saline. Thus any differences between the chromatograms obtained for fluorescent dextrans in saline and those with the same dextran solution in plasma (after subtracting intrinsic protein fluorescence) would reflect binding of dextrans to the proteins. The data indicated that dextrans ranging in size from 1 to 8 nm bind to proteins to create much larger particles, with this effect being most pronounced for the larger dextrans in this range. This binding could also be the reason that the L/P values were <1 for the small neutral and DEAE dextrans.

Thus, in the in vivo model utilized in these studies, there would likely exist two distinct populations of dextran: 1) free, unbound dextran and 2) dextran complexed with protein. The transport of these complexes across the blood-lymph barrier might then be associated with an additional transport mechanism normally available only to proteins. Studies to more fully delineate the transport properties of bound and unbound dextran are currently underway in our laboratory. However, two points should be emphasized in regard to the studies presented in this manuscript: 1) the extent of neutral dextran binding to proteins was substantially less than that for the DEAE dextrans (Fig. 3) and 2) DEAE dextrans were found in the lymph even after protein digestion, suggesting that a large fraction of these dextrans was not bound and that an additional pathway is available for transport of these cationic molecules.

A couple of observations could be used to sufficiently explain the presence of the larger DEAE dextrans in lymph. 1) Numerous anionic sites have been identified throughout the pulmonary microvasculature (6, 45) that could act to facilitate the transport of cationic species, although these would also be expected to decrease the transport of anionic proteins. Previous studies have shown that charge modification of the glomerular basement membrane or pulmonary microvessels with polycationic agents, such as polyethyleneimine or protamine sulfate, resulted in loss of the selectivity of the barrier wall (3, 43). Thus it appears likely that these charged sites also play a role in regulating lung microvascular permeability. 2) It is also possible that the shape of DEAE dextrans is more compact than that of its neutral counterparts due to the polycationic nature of this molecule. However, recent experiments in our laboratory (38) indicated that neutral dextrans and DEAE dextrans containing 1% nitrogen have identical shapes in 0.3–0.8 M salt solutions. Because these dextrans are very similar in structure and should behave in a similar manner in terms of their hydrodynamic properties, it appears that any differences in L/P values not attributed to protein binding would have to be in some way attributed to electrostatic effects.

Implications of L/P Curves During Elevated Permeability Conditions

Under conditions of lung injury, the ratio of urea permeability-surface area product to butanediol permeability-surface area product increased to 0.48, as revealed by MID data (Table 1), indicating an ~50% increase in vascular permeability. This increased permeability was associated with a substantial increase in dextran L/Ps but only a slight increase in protein L/Ps. Thus it seems that PK causes changes in permeability that do not affect protein L/Ps to the same extent as dextrans, indicating that the transport mechanisms for dextrans and proteins are different and/or that proteins are transported at least in part via a mechanism not readily available to dextrans.

The change in permeability was also associated with a substantial increase in lung lymph flow, similar in magnitude to that observed in sheep after elevations in left atrial pressure (16), and a slight elevation in protein L/Ps, indicating an increase in net fluid and solute filtration across the blood-lymph lymph barrier. In con-
The increase in protein L/Ps in our studies was coincident to that lung, indicating that the injury was not via single lung causes unilateral increases in permeability protein associated with higher interstitial protein levels. However, these authors also indicated that the increased protein flux might be due to washout of interstitial proteins.

Abernathy et al. (1) reported that PK given to a single lung causes unilateral increases in permeability to that lung, indicating that the injury was not via some circulating mediator. Thus permeability in other organ systems probably remains unchanged after PK. The increase in protein L/Ps in our studies was coincident with a significant reduction in plasma protein levels rather than any changes in lymph protein levels and, as such, were likely not due to washout of interstitial proteins, as further discussed below.

Lymph flow after PK increased to 30 ml/h within 1 h and remained constant for the ensuing 2 h. At a 30 ml/h lymph flow rate and with a steady-state plasma total protein concentration of 5.96 g/dl (Table 1), protein flux from the pulmonary vascular space after 3 h would be ~5.4 g. Furthermore, Pou et al. (34) reported that the lung interstitial fluid volume available to albumin was 0.57 ml/g of blood-free dry lung weight. Multiplying this value by their measured blood-free dry lung weight of 66.04 g indicates a sheep lung protein concentration of 5.96 g/dl (Table 1), protein flux from the pulmonary vascular space after 3 h would be ~5.4 g. Furthermore, Pou et al. (34) reported that the lung interstitial fluid volume available to albumin was 0.57 ml/g of blood-free dry lung weight. Multiplying this value by their measured blood-free dry lung weight of 66.04 g indicates a sheep lung interstitial fluid volume of 37.6 ml. Assuming that this volume represents the fluid volume available to all proteins in the interstitium and that lymph contents are representative of interstitial fluid contents, the lymph protein concentration of 4.23 g/dl reported here indicates that the interstitial fluid contains ~1.6 g of protein. The amount of protein cleared from the tissue through the exteriorized lymphatic after PK would be the product of the lymph protein concentration and the volume of lymph that was collected. Thus we would expect to collect ~3.8 g of protein in 3 h at a lymph flow of 30 ml/h. Subtracting 3.8 g from the 5.4 g of protein cleared from the vascular space reveals a value of ~1.6 g, the same value calculated for the amount of protein in the interstitial fluid. This indicates that the protein collected in the lymph is not due to washout but rather to enhanced flux from the pulmonary vascular space to the interstitium.

After PK, the spaces between adjacent endothelial cells are thought to increase, as indicated by the elevated L/Ps and the permeability values determined from the indicator dilution measurements. These data further suggest that the predominant transport mechanism for proteins is not associated with pores and that injury with PK does not alter the normal protein transport mechanisms. Thus changes in pore size might not be reflected from measurements of protein L/P. In contrast, neutral and DEAE dextran L/Ps are markedly affected, suggesting that the transport mechanism for neutral and cationic dextrans is via pores. The difference between these two that is not attributable to protein binding is likely due to electrostatic interac-

tions. The difference between neutral and DEAE dextran L/Ps is less pronounced after injury, indicating that anionic charged sites on the cell surface or in intercellular pores are removed during injury with PK, which leads to an attenuation in the increase in transport of DEAE dextrans.

An additional point is that small dextrans (1–2 nm) are not sensitive to changes in permeability, probably because they are relatively unrestricted in their passage from vascular spaces to lung lymph under normal permeability conditions. Although transport of DEAE dextrans as large as 6–7 nm is highly restricted, these charged dextrans are substantially less sensitive than neutral dextrans of similar size to lung injury induced by PK. Application of a two-pore model of the lung microvascular barrier theory to the data obtained for the neutral dextrans indicates that the large pore is on the order of 7–8 nm (37). Taken together, these observations suggest that a nonpore pathway is responsible for the transport of proteins and DEAE dextrans that are larger than ~8 nm. By assuming the surface area for the large pores obtained from the indicator dilution measurements to be much smaller than that for cell surface vesicles, it seems likely that vesicles are an important transport pathway for larger, charged dextrans.

If PK caused alterations in cell surface glycocalyx anionic charge density, the difference between neutral and DEAE dextran L/Ps before permeability alteration would likely increase after injury. This was not the case. It appears that either an increase in pore size is offset by a decrease in anionic charge on cell surfaces or the transport of molecules >7–8 nm is via vesicles that are seemingly unaffected by PK treatment. Thus dextran probes would be useful in diagnosing lung diseases associated with increases in microvascular permeability, whereas use of radiolabeled or fluorescently labeled proteins might hold diagnostic value under conditions when vesicular transport is compromised.

Comparisons With Previous Studies

In contrast to histological data (see below) and the L/P data presented here, much of the data that has been gathered to determine the steric and electric properties of the plasma-lymph barrier in sheep have indicated that the pulmonary microvascular barrier exhibits a net positive charge. In three such studies (19, 24, 33), sheep have been prepared with chronic lung lymph fistulas, and the L/Ps of anionic dextran sulfates and/or neutral dextrans were determined. L/Ps for anionic dextran sulfates were found to be higher than those for similar-sized neutral dextrans, and the sieving nature of this barrier appeared to be less pronounced for the anionic dextrans. In addition, studies by Glauser et al. (17) indicated that the half times for clearance of anionic dextrans from plasma into lung lymph were substantially shorter than those for neutral dextrans of similar size. Experiments were conducted in intact anesthetized sheep, and measurements were made after a bolus injection of 2.5-, 3.5-,
and 4.5-nm neutral and anionic dextrans. However, mass balances were not reported and no corrections were made to account for the fact that different vascular beds might have cleared the anionic dextrans substantially faster than the neutral dextrans, thus affecting pulmonary transvascular concentration gradients.

Histological data indicate that the pulmonary microvascular is lined with anionic proteoglycans (6, 45). In addition, Swanson and Kern (43) found that the initial tissue uptake of cationic albumin was higher than that of native albumin and that this effect was attenuated after an inferred charge modification with protamine. Also, Parker et al. (28) found that the initial tissue uptake of cationic lactate dehydrogenase was elevated relative to that of anionic lactate dehydrogenase, but the L/P of the cationic isozyme was lower than that of the anionic isozyme. Furthermore, these conflicting data were described with a single mathematical model (29), and it was proposed that the pulmonary microvasculature acts as a cation-exchange barrier, hindering the equilibration of cationic species and enhancing that of anionic species. However, in light of the data presented here, it appears that the steric and electric properties of the pulmonary microvasculature remain elusive and are even more complicated than that presented in this model.

A strength of the approach utilized in the studies presented in this manuscript is that dextran is an exogenous molecule. Thus their transport would likely not involve specific active-transport mechanisms. Instead, dextran movement would be by virtue of passive electrochemical gradients and/or electrostatic interactions with endogenous species that are transported via specific mechanisms. Previous studies have utilized endogenous species, which likely are transported via selective pathways.

Sensitivity of Tracers for Assessing Changes in Permeability

It is interesting that neutral and DEAE dextrans were more sensitive to changes in permeability than endogenous proteins and that there were still substantial differences in L/Ps after injury. This suggests that some molecules might be better markers of barrier integrity than others and that techniques might be developed to exploit these solute-specific properties to access barrier damage. Gamma scanning is an underutilized experimental technique for assessing the development of pulmonary edema (2, 18). In this procedure, gamma-emitting molecular probes are injected into the bloodstream, and the accumulation of a diffusible tracer relative to that of a nondiffusible tracer is measured by placing scintillation probes over the heart and lungs. Typically, the diffusible tracer has been a protein such as albumin or transferrin. These probes are large, and, as such, the accumulation of sufficient quantities to give good signals takes 30–60 min. However, when adequate accumulation occurs, the data are expressed in the form of a normalized slope index that can be compared under normal and altered permeability conditions. From the data presented here, it appears that exogenous species such as neutral dextrans might prove better markers for use in gamma studies since these molecules are more sensitive to changes in permeability.

In conclusion, the size, charge, and type of molecular species affects the pulmonary transcapillary exchange of macromolecules. This was supported by the fact that both cationic DEAE dextrans and anionic endogenous proteins experienced enhanced plasma-lymph transport relative to neutral dextrans of similar size. We believe that the differences in transport of neutral and DEAE dextrans are probably due to electrostatic interactions of DEAE dextrans with proteins and the presence of anionic sites in the pulmonary microvasculature. The differences in the L/P of neutral dextrans and proteins of similar size are probably associated with different transport mechanisms, although these were not examined in this study. Assessment of lung microvessel permeability and sieving properties both before and after lung injury suggests that dextrans might be used to more accurately assess and quantify changes in permeability during disease than proteins. Determination of the effects of charge in plasma-lymph exchange will probably assist us in understanding many of the proposed mechanisms and in further developing diagnostic tools for treating lung disease.

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