Transport properties of alveolar epithelium measured by molecular hetastarch absorption in isolated rat lungs

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Conhaim, Robert L., Kal E. Watson, Stephen J. Lai-Fook, and Bruce A. Harms. Transport properties of alveolar epithelium measured by molecular hetastarch absorption in isolated rat lungs. J Appl Physiol 91: 1730–1740, 2001.—To evaluate the transport properties of the alveolar epithelium, we instilled hetastarch (Het; 6%, 10 ml, 1 – 1 × 104 kDa) into the trachea of isolated rat lungs and then measured the molecular distribution of Het that entered the lung perfusate from the air space over 6 h. Het transport was driven by either diffusion or an oncotic gradient. Perfusate Het had a unique, bimodal molecular weight distribution, consisting of a narrow low-molecular-weight peak at 10–15 kDa (range, 5–46 kDa) and a broad high-molecular-weight band (range 46–2,000 kDa; highest at 288 kDa). We modeled the low-molecular-weight transport as (passive) restricted diffusion or osmotic flow through a small-pore system and the high-molecular-weight transport as passive transport through a large-pore system. The equivalent small-pore radius was 5.0 nm, with a distribution of 150 pores per alveolus. The equivalent large-pore radius was 17.0 nm, with a distribution of one pore per seven alveoli. The small-pore fluid conductivity (2 × 10−5 ml·h−1·cm−2·mmHg−1) was 10-fold larger than that of the large-pore conductivity.

Our understanding of the resolution of edema could be improved by more quantitative information about the permeability characteristics of the alveolar epithelium to macromolecules. To address this, Matsukawa and colleagues (13) measured rates of dextran flux across cultured lung epithelial cell monolayers. Their results suggested that the monolayers were perforated by pores with radii of 56 Å. Such pores would be relatively impermeable to albumin molecules, which have a radius of 35 Å and which are the main macromolecular component of edema liquid. The data of Matsukawa et al. additionally suggested that the epithelial cells also transferred dextran across the monolayers by endocytosis, a process that would allow the transport of molecules the size of albumin and larger (7).

Our approach was similar to that of Matsukawa and colleagues (13), except that we used hetastarch (Het) as a tracer instead of dextran. Furthermore, we measured Het flux across the alveolar epithelium of intact lungs that were partially inflated with Het, rather than across cell monolayers. The advantage of using Het is that it consists of a continuous spectrum of molecular masses that range from 104 to 107 Da. Inflating the lung with Het exposed the air space epithelium to macromolecules. To address this, Matsukawa and colleagues (13) measured rates of dextran flux across cultured lung epithelial cell monolayers. Their hypothesis was that each of these components represented a different route of molecular transfer across the epithelium. We hypothesized that the narrow peak represented Het flux through small pores in the epithelium, whereas the broad band represented Het flux through a large-diameter pore. We applied pore-modeling techniques to both the narrow peak and the broad band to determine the porosity and conduc-

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tivity of the alveolar epithelium on the basis of a two-pore population. Our results suggest that macromolecules are cleared from the alveolar air space by diffusion through pores of two different radii.

MATERIALS AND METHODS

We anesthetized retired male breeder rats (442 ± 25 g; n = 9) with intraperitoneal ketamine (40 mg/kg), xylazine (6 mg/kg), and acepromazine (1 mg/kg), and tied them supine. We infused heparin (750 U/kg) through a cannula (PE-190) that was tied into a femoral vein. After 10 min, to allow the heparin to circulate, we cut the femoral artery and allowed the animals to die from exsanguination. We infused Ringer lactate into the venous cannula during exsanguination (1 ml/ml of shed blood; total 20 ml) to facilitate the removal of residual red blood cells from the pulmonary circulation. Once breathing ceased, we tied a cannula into the trachea, set the tracheal pressure to 5 cmH₂O with air, and opened the chest using a sternum-splitting incision. After tying cannulas (PE-190) into the pulmonary artery and left atrium, we removed the lungs from the chest and placed them into a styrofoam perfusion chamber.

The lungs were perfused with albumin in phosphate-buffered saline (see concentration below) and ventilated with air using a piston pump (25 breaths/min). Inflation and deflation pressures were set to 15 and 5 cmH₂O, respectively. We used a recirculating perfusion system. The perfusate that dripped from the left atrial cannula was pumped to a perfusion chamber set 10 cm above the base of the lung. These airway and vascular pressures were chosen to obtain an air space-vascular pressure gradient near zero in a ventilated lung. Thus the transport of Het from the air space to the vasculature was mainly due to concentration gradients. Perfusion flow was measured by using a drop counter placed beneath the left atrial cannula, and vascular and inflation pressures were measured via transducers (Statham) connected to an oscillograph (Grass). After 10 min, to allow the pulmonary circulation to clear of any remaining red blood cells, we stopped the perfusion and introduced 10 ml of Het (6%; oncotic pressure, 40.0 ± 0.6 mmHg) into the tracheal cannula, using a reservoir placed 10 cm above the lung.

We prepared two sets of lungs. In the first (n = 5), the perfusion solution contained 10% albumin in phosphate-buffered saline (oncotic pressure, 57.3 ± 1.7 mmHg) (oncotic gradient). The second (n = 4) contained 7.2% albumin (measured oncotic pressure, 39.7 ± 1.0 mmHg), to match the oncotic pressure of Het filling the air space (oncotically balanced). Oncotic pressures were measured by use of an oncometer (Wescor) equipped with a 10-kDa membrane (Fig. 1). Before reestablishing perfusion, we filled the perfusion system with fresh solution using a minimal volume (27 ml) to minimize dilution of Het entering the perfusate from the air space. Perfusion and ventilation with air were reestablished by using the baseline (pre-Het inflation) pressures (above).

Once each hour during perfusion, 2 ml of the perfusion solution were withdrawn for Het analysis and replaced with 2 ml of fresh solution. After the last perfusate sample was collected (hour 6), the lung was homogenized and a sample of the lung supernatant was collected after centrifugation.

Het concentrations and molecular distributions in the homogenate and in the hourly perfusate samples were measured by use of high-performance size-exclusion chromatography (HPSEC). Details of our HPSEC system have been published elsewhere (10). Briefly, the perfusate Het samples were pumped through three columns, arranged in series, each of which was packed with inert particles of specific size.

The rate at which Het polymers flowed through the spaces between the particles was inversely proportional to the polymer molecular weight. Polymer emergence from the columns was measured by use of a refractance detector, the output of which was proportional to polymer concentration. Het consists of polymers ranging from 10^3 to 10^7 kDa, and refractance detector output for a Het stock solution produces a peak in which retention time is shown on the horizontal axis and concentration on the vertical axis. The retention times were converted to molecular mass on the basis of the results of a calibration plot consisting of the retention times of dextran samples of known molecular mass (2,000, 580, 71, and 11 kDa). The peak produced by a 6% Het sample consists of ~600 individual retention times (1 s apart), each of which represents a unique Het polymer weight. The area enclosed by such a peak is proportional to the Het sample concentration. Peak areas were converted to concentration by using a calibration plot composed of peak areas produced by Het samples of known concentration. Before lung perfusate samples were introduced into the HPSEC, trichloroacetic acid was added to each sample to precipitate all proteins and prevent protein contamination of the size-exclusion columns. After centrifugation to remove the precipitate, the pH of the supernatant was restored to neutral by using 5 N KOH before analysis of the supernatant by HPSEC (10).

Electron Microscopy

Analysis of our Het data suggested that some large-molecular-weight Het entered the perfusate by way of a nonpore mechanism. We speculated that epithelial cell vesicles might have been responsible for this transport (see Discussion). To investigate the potential for vesicular transport, we used electron microscopy to examine the lungs of two anesthetized rats into which a colloidal gold solution had been instilled. The gold instillate was prepared by centrifugation of 100 ml of a commercial gold solution (Aurion, 3-nm radius, Electron Microscopy Sciences) at 10^6 g for 1 h. The pellet was resuspended in 0.2 ml of 5% bovine serum albumin labeled with Evans blue (albumin-to-Evans blue molar ratio of 4:1). The resulting solution (0.25 ml) was instilled into the trachea of each spontaneously breathing rat, which had been anesthetized by the methods described above (n = 2). The rats were returned to their cages and allowed to recover from anes-
sia. Six hours later, each rat was anesthetized again and killed by exsanguination, and its lungs were removed. The pulmonary circulation was flushed briefly with saline to remove residual blood, and the lungs were then perfused with 30–40 ml of 2.5% glutaraldehyde in Sorenson’s phosphate buffer. Tissue blocks were harvested from Evans blue-stained areas and postfixed in Caulfield’s fixative. The blocks were dehydrated through increasing concentrations of ethanol, embedded in EM bed-812 (Electron Microscopy Sciences), and thin sections were cut by using a diamond knife (100 nm) on a Reichert-Jung microtome. The sections were stained with uranyl acetate and lead citrate and were examined by use of a Hitachi H600 transmission electron microscope. Epithelia in six to eight alveoli of each rat were examined.

Statistics

Data are expressed as means ± SE. Data between treatment groups (oncotic gradient, oncotically balanced) were compared by using Student’s unpaired t-test. Comparisons within treatment groups were completed by using paired t-tests. The comparisons were made by commercially available software (StatView v.5.0.1, SAS Institute). This software was also used for regression analysis of the data. Differences were considered significant at P ≤ 0.05.

RESULTS

Baseline perfusate flows before Het inflation averaged 2.4 ± 0.9 ml/min in both groups. One hour after Het inflation, flows were 2.2 ± 1.1 ml/min in the oncotically balanced group and 2.9 ± 0.9 in the oncotic gradient group (not significant). After 6 h, flows in the two groups were, respectively, 1.7 ± 0.5 and 2.2 ± 0.9 ml/min (P = 0.03).

Examples of the molecular distribution in Het tracheal instillation solution and lung homogenate are shown in Fig. 2. Molecular masses in the instillation solutions for both groups ranged from 4.2 ± 2.9 to 14,095 ± 5,410 kDa with a peak at 228 ± 38 kDa that had an average height of 13,224 ± 230 (HPSEC refractance detector units) (Fig. 2). The lung homogenates, which did not differ significantly between the two groups, ranged from 5.0 ± 3.2 to 10,408 ± 2,120 kDa, with a peak at 304 ± 46 kDa that had an average height of 6,811 ± 3,301.

An example of HPSEC analysis from a lung inflated with 6% Het and perfused with 10% albumin (oncotic gradient) is shown in Fig. 3. The most striking finding is the bimodal shape of the Het molecular distribution in the hourly perfused samples. Each sample is composed of a narrow, low-molecular-weight peak followed by a broader but lower high-molecular-weight band. This profile is distinctly different from that of the infusate and the homogenate (peak tops not shown), suggesting possible mechanisms by which Het crossed the alveolar epithelium to enter the perfusate (see DISCUSSION). Values used to characterize the perfusate Het molecular distributions are noted (Table 1). The vertical axis is expressed in units of HPSEC detector output, which is proportional to concentration. m.w., Molecular weight; max., maximum; min., minimum; Homog., homogenate.

Fig. 2. Het molecular distributions in a 6% Het lung instillate solution and in lung homogenate after perfusion for 6 h (oncotic gradient study). The height of the homogenate peak is lower than that of the instillate because of dilution of the instillate concentration by the lung tissue. That is, the Het concentration in a mixture of Het + lung is lower than in the Het solution alone. There is some loss of the smallest Het fractions in the homogenate (<10^4 Da), perhaps due to tissue amylase activity.
Table 1. Values of Het peaks in perfusate of lungs inflated with 6% Het

<table>
<thead>
<tr>
<th>Oncotic Gradient</th>
<th>Low MW Min, kDa</th>
<th>Low MW Max, kDa</th>
<th>Low MW Peak height, HPSEC detector output</th>
<th>Weight at Low MW Peak, kDa</th>
<th>High MW Band Max Height</th>
<th>Het Mass at High MW Max, kDa</th>
<th>High MW Max, kDa</th>
</tr>
</thead>
<tbody>
<tr>
<td>Oncotic gradient</td>
<td>6.3 ± 3.3</td>
<td>46.0 ± 6.9</td>
<td>448 ± 395</td>
<td>16.0 ± 1.5</td>
<td>63 ± 41†</td>
<td>288 ± 85</td>
<td>2,120 ± 522</td>
</tr>
<tr>
<td>Oncotically balanced</td>
<td>5.4 ± 0.6</td>
<td>43.3 ± 3.4</td>
<td>70 ± 32†</td>
<td>10.4 ± 1.0†</td>
<td>24 ± 8††</td>
<td>287 ± 156</td>
<td>1,887 ± 2,111</td>
</tr>
</tbody>
</table>

Values are means ± SE. Het, hetastarch; Min, minimum; HPSEC, high-performance size exclusion chromatography. *Significantly different from low-molecular-weight (MW) maximum (Max) height; †significantly different from oncotic gradient value (*P < 0.05).

significantly less than that for the oncotic gradient group (16.0 ± 1.5 kDa; *P = 0.0001) (Table 1). The heights of the peaks for the 7.2% albumin group were also significantly lower than those of 10% albumin-perfused lungs (Table 1). The maximum height of the low-molecular-weight peak was only 15% of that in the oncotic gradient group, whereas the maximum height of the high-molecular-weight band was 38% of the gradient group (Table 1).

To estimate the fraction of the air space Het that entered the perfusate, we compared areas enclosed by the highest (hour 6) perfusate sample with those enclosed by the homogenate. These areas are proportional to the total amount of Het found in the perfusate and air space, respectively, after 6 h of perfusion. We summed these two areas, on the basis of the assumption that this represented the total amount of Het in the air space at the start of perfusion. We then divided the area enclosed by the hour 6 perfusate sample by this sum to obtain the percentage of Het initially in the air space that entered the perfusate after 6 h. The percentage of air space Het that entered the perfusate after 6 h increased to 3.9 ± 2.9% (*P = 0.04). The low-molecular-weight peak accounted for about three-fourths of the total.

Modeling the Small Pore System

We hypothesized that the small-molecular-weight Het peaks in the perfusate samples of the oncotically balanced studies were due to passive restricted diffusion of Het out of the alveolar space through liquid-filled cylindrical pores. We assumed that the diffusive flux was proportional to the increase in the peak heights over the 6-h perfusion period. We determined diffusion coefficients (D) for specific Het fractions (15–40 kDa) from the slopes of linear regression lines plotted through peak heights of each of these fractions over hours 3–6 (Fig. 5). Diffusion was immeasurable before hour 3. The reason for this is speculative, but it might have been due to the growth of perivascular fluid cuffs that offset diffusive flux into the perfusate (12).

To determine the mass of each fraction diffusing, we multiplied each slope by the perfusate volume (27 ml) and divided by the height of each fraction in a 6% Het sample to obtain the diffusive mass flow (Q) per unit of Het concentration difference (ΔC), Q/ΔC (Fig. 6). We assumed that the perfusate Het concentration was negligible compared with that of the instillate.

We attributed the monotonic decrease in Q/ΔC with increasing molecular mass (M) to the diffusion of Het molecules of different size through cylindrical pores of constant radius (r). The diffusion of a spherical solute molecule through a cylindrical pore is given by (18)

\[ D = D_{\text{free}} \phi/k \]  

(1)

Here \( D_{\text{free}} \) is the free diffusion coefficient of the solute in an unbounded solution; \( \phi \) is the solute distribution function, a measure of the steric exclusion of the solute from a region within one solute radius of the pore wall.

Table 2. Percentage of air space Het appearing in perfusate after 6 h

<table>
<thead>
<tr>
<th>Oncotic Gradient</th>
<th>Low MW Peak, % of Homogenate</th>
<th>High MW Band, % of Homogenate</th>
<th>Total (Low + High), % of Homogenate</th>
</tr>
</thead>
<tbody>
<tr>
<td>Oncotic gradient</td>
<td>2.9 ± 2.8</td>
<td>1.2 ± 1.3</td>
<td>3.9 ± 2.9</td>
</tr>
<tr>
<td>Oncotically balanced</td>
<td>0.30 ± 0.22</td>
<td>0.24 ± 0.39</td>
<td>0.53 ± 0.57*</td>
</tr>
</tbody>
</table>

Values are means ± SE calculated from ratio of perfusate and homogenate peak areas. *Significantly different from value for oncotic gradient value (*P < 0.05).

Fig. 4. HPSEC peaks from a lung inflated with 6% Het (\( \pi = 35.7 \) mmHg) and perfused with an Alb solution of equal osmotic pressure (7.2%). The bimodal Het mass perfusate distributions seen in Fig. 3 are evident, although the peak heights are only \(-[1/5]\) as great.
\( \phi \) is equal to \((1 - a/r)^2\) where \(a\) is the solute radius. The parameter \(k\) is the hydrodynamic drag coefficient of a sphere moving along the pore centerline, obtained as a function of \(a/r\) from tables (17). Application of the Starling equation to the excluded region near the wall and the remaining pore region results in the reflection coefficient \((\sigma)\) of the solute (1)

\[
\sigma = [1 - (1 - a/r)^2]^2
\]

where \(a\) is the solute radius and \(r\) is the pore radius.

We obtained the pore radius and pore number required to fit the \(\dot{Q}/\Delta C\)-vs.-\(M\) data (Fig. 6) as follows. We used Eq. 1 to compute \(D\) vs. \(M\) for a single pore using a chosen value of \(r\). We assumed that the solute radius \((a)\) of each molecular weight fraction was proportional to \(M^{1/3}\), which was scaled to albumin, for which \(M = 66\) kDa and the radius is 3.5 nm. Molecular radii for molecular weight fractions of 15, 20, 25, 30, 35, and 40 kDa were 2.14, 2.35, 2.53, 2.69, 2.83, and 2.96 nm, respectively. We assumed that \(D_{\text{free}}\) was proportional to \(M^{-1/2}\) and that \(D_{\text{free}}\) for albumin was \(6 \times 10^{-7} \text{ cm}^2/\text{s}\) (21). We chose the value of \(r\) so that the computed values of \(D\) multiplied by a constant provided a good fit to the \(\dot{Q}/\Delta C\)-vs.-\(M\) data. The constant \((A_d/L)\) is given by Fick’s equation for steady-state diffusion

\[
\dot{Q}/\Delta C = DA_d/L
\]

Here \(A_d\) is the total diffusive area and \(L\) is the diffusion distance from the alveolar space to the capillary lumen. The constant \(A_d/L\) that converts \(D\) to \(\dot{Q}/\Delta C\) provides an estimate of pore number if \(L\) is known. A pore radius of 5 nm with an \(A_d/L\) value of 71 provided a good fit to the data (curve, Fig. 6). Using an \(L\) value of \(5.0 \times 10^{-5} \text{ cm}\) (22) yielded a value for \(A_d\) of \(3.6 \times 10^{-3} \text{ cm}\). This resulted in a total of \(4.5 \times 10^9\) pores per lung. Assuming \(3 \times 10^5\) alveoli per rat lung, this equates to 150 pores per alveolus (20) (Table 3).

The reflection coefficients \((\sigma)\) for each molecular weight fraction, calculated using Eq. 2, are shown in Fig. 7. The estimated \(\sigma\) for albumin is 0.83.

**Modeling Small-Pore Membrane Conductivity**

We used the data from the oncotic gradient studies to estimate membrane conductivity \((K)\), on the basis of the Starling equation, as follows

\[
\dot{Q}_b = KA_d(dP - \sigma\Delta\pi)
\]

where \(\dot{Q}_b\) is the bulk (convective) liquid (Het) flux, \(K\) is the membrane conductivity per unit area (total alveolar surface area, \(A_i\)), \(dP\) is the hydrostatic pressure difference between the circulation and the air space, and \(\Delta\pi\) is the albumin-Het oncotic pressure difference, which in our studies was 17 mmHg. We used a value for \(\sigma\) equal to the mean value calculated for the six Het fractions (Fig. 7). We assumed \(dP\) to be zero, on the basis of the airway pressure (5–15 cmH\(_2\)O) and vascular (0–10 cmH\(_2\)O) pressures used. The mean airway air pressure (10 cmH\(_2\)O) was higher than the mean vascular pressure (5 cmH\(_2\)O). However, alveolar liquid pressure could be 5 cmH\(_2\)O with an airway air pressure of 10 cmH\(_2\)O, reducing the airway liquid-to-vascular pressure gradient to zero (2). In any case \(dP\) was small compared with \(\sigma\Delta\pi\).

A value for \(\dot{Q}_b\) can be obtained from the solute flux equation (19)

\[
\dot{Q}_s = (1 - \alpha)\dot{Q}_b A_d\Delta C/L
\]

where \(\dot{Q}_s\) is the solute flux, \(C_m\) is the mean solute concentration in the membrane, and \(\Delta C\) is the concentration difference. The first term on the right-hand side of Eq. 5 is the solute flux due to osmotic flow that arises from the bulk flow due to the osmotic pressure difference in the Starling equation (4). Rewriting Eq. 5

\[
\dot{Q}_s/C_m = -\dot{Q}_b\alpha + \dot{Q}_b + DA_d\Delta C/(C_mL)
\]

**Fig. 6.** Diffusive Het mass flow (\(\dot{Q}\)) per unit of Het concentration difference (\(\Delta C\)) for specific Het fractions. The squares represent measured values (means ± SE). The curve, which represents a pore with a radius of 5 nm and a total diffusive area-to-diffusion distance ratio \((A_d/L)\) of 71, provides a good fit to the data.
We assumed that the last term of Eq. 6 was negligibly small relative to Qb. That is, the diffusive flux was small compared with the bulk flow. This assumption is verified after the calculation for Qb. With this assumption, Qb is equal to either the slope magnitude or the intercept of the linear equation fit to the Qs/Cm-vs.-σ data.

Qs/Cm was obtained from the oncotic gradient data (Fig. 3) as follows. First, we calculated the slope of the linear regression fit to the peak heights of each of the six Het fractions (15–40 kDa) over hours 1–6 (Fig. 8). Qs/Cm for each molecular fraction was equal to the six Het fractions (15–40 kDa) over a linear regression fit to the peak heights of each of the data (Fig. 9) was calculated for specific Het fractions (Fig. 8). The resulting Qs/Cm was obtained from the oncotic gradient data (Fig. 9) was calculated for specific low-molecular-weight Het fractions in perfusate samples of the oncotic gradient studies (means ± SE). The equation for the 15-kDa regression line is y = 108.9 ± 23.6 h, n = 6, R² = 0.89. The equation for the 40-kDa regression line is y = 1.22 ± 8.06 h, n = 6, R² = 0.83.
equation for each molecular fraction with different values for $K$ and $s$ was not justified.

**Modeling the Large-Pore System**

In the oncotically balanced experiments (Fig. 4), the high-molecular-weight fractions showed no systematic increase with time, indicating a nondiffusive transport process. Thus those data could not provide estimates of a pore radius and pore number by the method used to model the small-pore system (Eqs. 1–3). By contrast, the transport of the large-molecular-weight fractions increased linearly with time in the oncotic gradient experiments (Fig. 3), and we applied the solute flux equation (Eq. 6) to those data to model an equivalent large-pore system.

First, we calculated the slope of the linear regression fit to the peak heights of each of five Het fractions (100, 200, 400, 800, and 1,600 kDa) over hours 1–6 (Fig. 10). Molecular radii for these molecular weight fractions were 4.02, 5.06, 6.38, 8.04, and 10.1 nm, respectively. $Q_b/C_m$ for each molecular fraction was equal to the slope divided by one-half of the height of the corresponding peak in the 6% Het sample ($C_m$), then multiplied by the perfusate volume (27 ml). We used a trial-and-error procedure to fit the $Q_b/C_m$ data to obtain the osmotic flow ($Q_b$). We chose a value for the large pore ($R_l$), and from the $a/R_l$ values for the five molecular weight fractions we computed the values for $s$ (Eq. 2). The value $R_l$ was adjusted until the y-intercept equaled the slope magnitude of the linear regression equation fit to the $Q_b/C_m$ data. The resulting equation satisfied Eq. 6 for solute flux without the diffusion term: $Q_b/C_m = 0.080 - 0.082 Q_b$, $R^2 = 0.98$. The equivalent large-pore radius was 17 nm (Table 3). The osmotic flow through the large pores ($Q_b$) was 0.082 ml/h, which is 8% of the total flow through both sets of pores (Fig. 11).

In the absence of diffusive transport of the large-molecular-weight fractions in the oncotic balanced data, the diffusion area and number of the large pores were estimated as follows. The large-to-small pore number ratio ($N_l/N_{sm}$) was obtained by using Poiseuille’s law for osmotic flow through cylindrical pores

$$
N_l/N_{sm} = (Q_b/Q_{b_{sm}})(R_{sm}/R_l)^4(\sigma_{sm}/\sigma_l)
$$

Here the subscripts l and sm denote large-pore and small-pore variables, respectively. We used the molecular mass fractions of 16 and 288 kDa at the peak signals (oncotic gradient data, Table 1) to represent the transport of low- and high-molecular-weight fractions through the small (5 nm) and large (17 nm) pores. The large and small solute radius of 5.7 nm and 2.2 nm produced a value of $\sigma_{sm}/\sigma_l$ of 0.46/0.31 (Eq. 2). With $Q_b/Q_{b_{sm}}$ of 0.082 and $R_{sm}/R_l$ of 5/17, $N_l/N_{sm}$ was 1/1,100. For $N_{sm}$ of 150 pores per alveoli, $N_l$ was one pore for every seven alveoli (Table 3). The area ratio ($A_l/A_{sm}$), given by ($N_l/N_{sm})(R_l/R_{sm})^2$, was 1/95. On the basis of the estimated $A_{sm}/L$ of 71 cm, $A_l/L$ was 0.75 cm.

From the estimates of $R_l$ and $A_l/L$, we justified omitting the diffusive term from Eq. 6 to calculate $Q_b$, as follows. The magnitude of the diffusive term $D_l(A_l/A_{sm})$ was 4.02, 5.06, 6.38, 8.04, and 10.1 nm, respectively. $Q_b/C_m$ for each molecular fraction was equal to the slope divided by one-half of the height of the corresponding peak in the 6% Het sample ($C_m$), then multiplied by the perfusate volume (27 ml). We used a trial-and-error procedure to fit the $Q_b/C_m$ data to obtain the osmotic flow ($Q_b$). We chose a value for the large pore ($R_l$), and from the $a/R_l$ values for the five molecular weight fractions we computed the values for $s$ (Eq. 2). The value $R_l$ was adjusted until the y-intercept equaled the slope magnitude of the linear regression equation fit to the $Q_b/C_m$ data. The resulting equation satisfied Eq. 6 for solute flux without the diffusion term: $Q_b/C_m = 0.080 - 0.082 Q_b$, $R^2 = 0.98$. The equivalent large-pore radius was 17 nm (Table 3). The osmotic flow through the large pores ($Q_b$) was 0.082 ml/h, which is 8% of the total flow through both sets of pores (Fig. 11).

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$$

Here the subscripts l and sm denote large-pore and small-pore variables, respectively. We used the molecular mass fractions of 16 and 288 kDa at the peak signals (oncotic gradient data, Table 1) to represent the transport of low- and high-molecular-weight fractions through the small (5 nm) and large (17 nm) pores. The large and small solute radius of 5.7 nm and 2.2 nm produced a value of $\sigma_{sm}/\sigma_l$ of 0.46/0.31 (Eq. 2). With $Q_b/Q_{b_{sm}}$ of 0.082 and $R_{sm}/R_l$ of 5/17, $N_l/N_{sm}$ was 1/1,100. For $N_{sm}$ of 150 pores per alveoli, $N_l$ was one pore for every seven alveoli (Table 3). The area ratio ($A_l/A_{sm}$), given by ($N_l/N_{sm})(R_l/R_{sm})^2$, was 1/95. On the basis of the estimated $A_{sm}/L$ of 71 cm, $A_l/L$ was 0.75 cm.

From the estimates of $R_l$ and $A_l/L$, we justified omitting the diffusive term from Eq. 6 to calculate $Q_b$, as follows. The magnitude of the diffusive term $D_l(A_l/A_{sm})$ was 4.02, 5.06, 6.38, 8.04, and 10.1 nm, respectively. $Q_b/C_m$ for each molecular fraction was equal to the slope divided by one-half of the height of the corresponding peak in the 6% Het sample ($C_m$), then multiplied by the perfusate volume (27 ml). We used a trial-and-error procedure to fit the $Q_b/C_m$ data to obtain the osmotic flow ($Q_b$). We chose a value for the large pore ($R_l$), and from the $a/R_l$ values for the five molecular weight fractions we computed the values for $s$ (Eq. 2). The value $R_l$ was adjusted until the y-intercept equaled the slope magnitude of the linear regression equation fit to the $Q_b/C_m$ data. The resulting equation satisfied Eq. 6 for solute flux without the diffusion term: $Q_b/C_m = 0.080 - 0.082 Q_b$, $R^2 = 0.98$. The equivalent large-pore radius was 17 nm (Table 3). The osmotic flow through the large pores ($Q_b$) was 0.082 ml/h, which is 8% of the total flow through both sets of pores (Fig. 11).

In the absence of diffusive transport of the large-molecular-weight fractions in the oncotic balanced data, the diffusion area and number of the large pores were estimated as follows. The large-to-small pore number ratio ($N_l/N_{sm}$) was obtained by using Poiseuille’s law for osmotic flow through cylindrical pores

$$
N_l/N_{sm} = (Q_b/Q_{b_{sm}})(R_{sm}/R_l)^4(\sigma_{sm}/\sigma_l)
$$

Here the subscripts l and sm denote large-pore and small-pore variables, respectively. We used the molecular mass fractions of 16 and 288 kDa at the peak signals (oncotic gradient data, Table 1) to represent the transport of low- and high-molecular-weight fractions through the small (5 nm) and large (17 nm) pores. The large and small solute radius of 5.7 nm and 2.2 nm produced a value of $\sigma_{sm}/\sigma_l$ of 0.46/0.31 (Eq. 2). With $Q_b/Q_{b_{sm}}$ of 0.082 and $R_{sm}/R_l$ of 5/17, $N_l/N_{sm}$ was 1/1,100. For $N_{sm}$ of 150 pores per alveoli, $N_l$ was one pore for every seven alveoli (Table 3). The area ratio ($A_l/A_{sm}$), given by ($N_l/N_{sm})(R_l/R_{sm})^2$, was 1/95. On the basis of the estimated $A_{sm}/L$ of 71 cm, $A_l/L$ was 0.75 cm.

From the estimates of $R_l$ and $A_l/L$, we justified omitting the diffusive term from Eq. 6 to calculate $Q_b$, as follows. The magnitude of the diffusive term $D_l(A_l/A_{sm})$ was 4.02, 5.06, 6.38, 8.04, and 10.1 nm, respectively. $Q_b/C_m$ for each molecular fraction was equal to the slope divided by one-half of the height of the corresponding peak in the 6% Het sample ($C_m$), then multiplied by the perfusate volume (27 ml). We used a trial-and-error procedure to fit the $Q_b/C_m$ data to obtain the osmotic flow ($Q_b$). We chose a value for the large pore ($R_l$), and from the $a/R_l$ values for the five molecular weight fractions we computed the values for $s$ (Eq. 2). The value $R_l$ was adjusted until the y-intercept equaled the slope magnitude of the linear regression equation fit to the $Q_b/C_m$ data. The resulting equation satisfied Eq. 6 for solute flux without the diffusion term: $Q_b/C_m = 0.080 - 0.082 Q_b$, $R^2 = 0.98$. The equivalent large-pore radius was 17 nm (Table 3). The osmotic flow through the large pores ($Q_b$) was 0.082 ml/h, which is 8% of the total flow through both sets of pores (Fig. 11).
L(ΔC/Cm) was calculated by using the molecular mass fraction of 100 kDa. Dl was 5.4 × 10⁻⁴ cm²/h, calculated from values for \( D_{\text{free}} \), \( \phi \), and \( k \) (Eq. 1), appropriate for the molecular radius of 4 nm. The diffusive term was 0.0014 ml/h or 2% of the calculated \( Q_{b\text{l}} \).

To determine the contribution of nondiffusive transport mechanisms to the transport of the large-molecular-weight band, we compared the mass flux of the large molecular fractions transported through the large pores by restricted diffusion to that measured in the oncotic balanced data (Table 2). The large-to-small pore mass flux ratio (\( Q_{s\text{l}}/Q_{s\text{sm}} \)) is related to the diffusion and area ratios as follows

\[
Q_{s\text{l}}/Q_{s\text{sm}} = (D_{l}/D_{\text{sm}})(A_{l}/A_{\text{sm}})
\]

We used the molecular weight fractions of 10 and 288 kDa at the peak signals to represent the low- and high-molecular-weight bands (oncotic balanced data, Table 1). Large- and small-pore diffusion coefficients \( D_{l} \) and \( D_{\text{sm}} \) were 1.6 × 10⁻⁴ and 7.0 × 10⁻⁴ cm²/h, calculated from values for \( D_{\text{free}} \), \( \phi \), and \( k \) (Eq. 1) appropriate for Het molecular radii of 5.7 and 1.9 nm, respectively. \( Q_{s\text{l}}/Q_{s\text{sm}} \) was 1/530. This ratio for diffusive mass flux through the large pores was considerably smaller than that measured at 6 h in the oncotic gradient experiments (Table 2). Thus half of the large molecular mass was transported by osmotic flow through large pores and half was transported by a nondiffusive mechanism.

To determine the contribution of this nondiffusive mechanism, the mass flux of the large molecular fractions transported by osmotic flow through the large pores was compared with that from the oncotic gradient data (Table 2). From the solute flux equation (Eq. 6)

\[
\frac{Q_{s\text{l}}}{Q_{s\text{sm}}} = \frac{1 - \sigma_{l}}{1 - \sigma_{\text{sm}}} \times \frac{Q_{b\text{l}}}{Q_{b\text{sm}}} \times \frac{\Delta C_{m\text{l}}}{\Delta C_{m\text{sm}}}
\]

For high- and low-molecular-weight fractions of 288 and 16 kDa at the signal peaks, \( \sigma \) was 0.31 and 0.46, respectively. \( \Delta C_{m\text{l}}/\Delta C_{m\text{sm}} \) was 2.5 ± 0.56 (Fig. 3). For \( Q_{b\text{l}}/Q_{b\text{sm}} \) of 0.082, \( Q_{s\text{l}}/Q_{s\text{sm}} \) was 0.26, which is half of the ratio of the perfusate large molecular mass (0.51 ± 0.37) measured at 6 h in the oncotic gradient experiments (Table 2). Thus half of the large molecular mass was transported by osmotic flow through large pores and half was transported by a nondiffusive mechanism. Of the half of the nondiffusive transport that occurred with the osmotic flow, ~10% would have occurred in the absence of osmotic flow (oncotically balanced data, Table 2), leaving ~40% that was associated with the oncotic gradient. Thus nondiffusive transport might have been increased by the presence of an oncotic gradient (Table 3).

The nature of this nondiffusive transport is unknown, but transport by cytoplasmic vesicles, which are known to be present in alveolar epithelial cells, is one possibility (5, 22, 23). To investigate the potential for vesicular transport, we used electron microscopy to examine alveolar epithelia in the lungs of rats into which colloidal gold had been instilled. We found gold particles to be present in epithelial cell vesicles of both type I and type II cells (Figs. 12 and 13). The number of particles present in each vesicle ranged from four to...
six to several dozen. We found no gold particles in intercellular junctions between alveolar epithelial cells. These results do not provide direct evidence for Het transport by epithelial cell vesicles, but they do demonstrate the capability of these vesicles to engulf particulates that are of approximately the same size as the largest Het molecules. We will return to this point in the DISCUSSION.

**DISCUSSION**

Results of our calculations are summarized in Table 3. The table shows the radii of the small and large pores, the number of pores of each type, their fluid conductivity, and the percentage of instilled Het that passed through each. Also shown is our estimate of the contribution of nondiffusive mechanisms.

Our results can be compared with those of Matsukawa and colleagues (13), who measured dextran transport across rat alveolar epithelial type II cell monolayers generated in culture. Using fluorescent dextran fractions ranging from 4 to 150 kDa, these authors concluded that the smaller fractions crossed the monolayers via diffusion-limited pores, whereas the larger fractions (>70 kDa) crossed by means of nondiffusional transcellular mechanisms such as pinocytosis. They calculated the pore radius to be 56 Å, which is near to our estimate of 50 Å obtained using Het. On the basis of a pore area-to-length ratio of 0.05 for their monolayers, Matsukawa and colleagues estimated the number of equivalent pores to be $2.5 \times 10^6$/$\text{cm}^2$. From our estimate of the number of pores per alveolus (150) and the surface area of a spherical alveolus with a radius 35 μm, the number of pores in the rat lung should be $1 \times 10^6$/$\text{cm}^2$. This is 2.5-fold less than the monolayer estimate. However, this difference is within the expected margin of error, considering the differences between a type II cell monolayer and the intact lung epithelium, which consists primarily of type I cells. Furthermore, our estimate for epithelial fluid conductivity is near that estimated from in vivo sheep studies (see below).

Our results also support the conclusions of a previous study by Conhaim and colleagues (4), who inflated lungs with solutes of various diameters and measured their concentrations in perivascular interstitial liquid-filled cuffs. They concluded that the air space epithelium could be characterized as if it were perforated by pores with radii of 10, 400, and 4,000 Å, which accounted for, respectively, 68, 30, and 2% of total liquid flux across the epithelium. Our present results, obtained using the continuous molecular distribution of Het, refine this estimate.

The rates of Het disappearance from the air space we measured (Table 2) can be compared with those of Matthay, Berthiaume, and colleagues (3, 15), who measured rates of albumin disappearance from the lungs of unanesthetized sheep into which 100 ml of autologous, radiolabeled serum had been instilled. They measured a protein disappearance rate of 1.6%/h. By comparison, we measured a disappearance rate of 0.53% after 6 h (0.09%/h) in the absence of an oncotic gradient and 3.9% after 6 h (0.65%/h) at an oncotic gradient of 17 mmHg. Oncotic gradients in the sheep experiments were initially absent because the instillate osmotic pressure (29 mmHg) was similar to that in the blood plasma (15). Thus the initial rapid increase in liquid clearance in the first 4 h with an increasing instillate protein concentration was attributed to active transport mechanisms. The progressively reduced liquid clearance from 12 to 24 h was attributed to passive osmotic reabsorption (see below). This is consistent with the results of the oncotic gradient experiments that modeled macromolecular transport as osmotic flow through water-filled pores. The higher protein disappearance rate in the sheep studies might have been due to the larger epithelial surface area in contact.
with an instillate volume (100 ml) that was larger than that used in the present studies (10 ml).

The sheep studies showed that, by 12 h, liquid clearance progressively slowed as instillate protein became more concentrated with time. These data are used to estimate epithelial fluid conductivity as follows. Specifically, the rate of liquid absorption of the 100 ml of serum albumin instilled into the airways decreased from 3.2 ml/h at 12 h to 1.4 ml/h at 24 h, whereas the instillate protein concentration increased from 9 g/dl to 12 g/dl at 12 h, the instillate volume was reduced to 30 ml (15). We assume that the reduction in absorption was due to a reduction in osmotic flow and estimated $K$ from the Starling equation (Eq. 4). At 12 h, when $Q_{\text{abs}}$ was 1.4 ml/h and instillate protein osmotic pressure ($\pi_i$) was 40 mmHg

$$-3.2 = K_A [\Delta P - \sigma(\pi_c - 40)] \quad (10)$$

Here $\pi_c$ is capillary protein osmotic pressure, and instillate $\pi_i$ was computed from Landis and Pappenheimer’s (11) relation between protein concentration and osmotic pressure. At 24 h, when $Q_{\text{abs}}$ was 1.4 ml/h and $\pi_i$ was 60 mmHg

$$-1.4 = K_A [\Delta P - \sigma(\pi_c - 60)] \quad (11)$$

Assuming no change in $\Delta P$ and $\pi_c$, subtraction of Eq. 10 from Eq. 11 results in a value of $K_A \sigma$ of 0.09. With a value for $\sigma$ (albumin) of 0.83 (Fig. 7), the calculated value for $K_A$ in the sheep lung was 0.11 ml h$^{-1}$ mmHg$^{-1}$, similar to that estimated for the rat lung. On the basis of mean alveolar diameters of 70 and 100 $\mu$m (20) and alveolar liquid volumes of 10 and 30 ml for the rat and sheep, respectively, the alveolar surface area in contact with alveolar liquid was twofold greater in the sheep than in the rat. Thus $K$ for the sheep was half that for the rat. The effects of lymphatic drainage and a significant vascular-to-airway hydrostatic pressure gradient may also contribute to liquid and protein clearance in vivo.

Our electron microscopy studies showed that vesicles of both type I and type II cells are capable of internalizing colloidal gold particles present within the air space. The radius of these particles (3 nm) corresponds to a protein molecular mass of 40 kDa. However, the particles were coated with albumin, which means that their effective radius was larger. The presence of several of these particles within each vesicle supports our speculation that Het molecules with masses up to 2,000 kDa (radius 11.5 nm) might have been transported out of the air space by this mechanism.

In a previous study, 5-nm-radius gold particles were not found to be present within either type I or type II cell vesicles in the lungs of rabbits (8). Possible explanations for these discrepant findings may be differences in the quantity of gold instilled, differences in the radius of the particles instilled, or differences between rabbit and rat lungs.

We did not find gold particles to be present in intercellular junctions between epithelial cells, which supports our estimate for the radius of the intercellular pores (5 nm radius). The reflection coefficient of a 40-kDa macromolecule by such a pore is 0.7 (Fig. 7). If one albumin molecule (3.5 nm) were attached to each gold particle (3 nm), its effective radius would be 6.5 nm and impermeable through a 5-nm radius pore.

Electron microscopic studies have shown that vesicular transport in rabbit lungs could be inhibited by using the pharmacological agents monensin and nocodazole (9). However, the authors of those studies also found that the clearance of albumin and IgG from rabbit lungs was not significantly decreased by these agents. They concluded that pathways other than endocytosis were responsible for the air space clearance of albumin and IgG measured. This is consistent with our conclusion that the large-molecular-weight Het fractions entered the perfusate through a combination of large-pore flux plus a nondiffusive mechanism that might have been vesicular transport.

Our results confirm the conclusions of others that the alveolar epithelium can be characterized as if it contains both small, diffusion-limited pores as well as larger transcellular pathways (4, 13). However, the continuous distribution of Het that we recorded allows the molecular weight limits of these pathways to be seen more readily and provides insight into the magnitude of these processes in intact lungs. The slow rate of Het removal from the air space that we measured confirms that the alveolar epithelium represents a significant impediment to the clearance of macromolecules in pulmonary edema. Our studies allowed us to quantitatively separate the transport of macromolecules by diffusion (and osmotic flow) through pores from that due to nondiffusive mechanisms. Vesicular transport may be such a mechanism.

The methods we have developed will facilitate the study of mechanisms that promote transcellular diffusion and vesicular transport and thereby enhance the resolution of this life-threatening condition.

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