Visualization and Quantification of Transmural Concentration Profiles of Macromolecules Across the Arterial Wall

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Transport parameters that describe a macromolecule entering the arterial wall from plasma can be obtained from concentration profiles of the labeled macromolecule entering the tissue. A new technique has been developed for measuring such concentration profiles, which offers spatial resolution superior to methods that measure profiles of radiolabeled macromolecules by serially sectioning tissue in planes parallel to the endothelium. In addition, this new method preserves cellular organization and tissue structure and permits measurement of concentration profiles underlying focal endothelial injuries or vascular lesions. The technique quantifies the concentration of a protein by measuring associated peroxidase activity. Although the present study was performed using horseradish peroxidase (HRP), the same principles can be applied to other macromolecules linked to HRP or microperoxidase. The colored reaction product of HRP was detected in transverse aortic sections using an image processing system. In the present study, profiles obtained by this new method were validated by comparison with HRP concentration profiles in rat aortas obtained by a serial slicing technique using radiolabeled HRP. We used the technique to measure high-resolution HRP concentration profiles in the intima and media of normal animals. These concentration profiles suggest that the internal elastic lamina acts as a major barrier to transport of macromolecules across the wall of the normal rat aorta. The new method should allow concentration profiles for macromolecules to be quantified in tissue surrounding vessels in the microcirculation, within the thickened intima of large vessels, and across coronary artery walls. (Circulation Research 1990;67:11–22)

One of the characteristics of atherosclerosis is the accumulation of low density lipoprotein (LDL) in the intima and inner media of the arterial wall. In early atherosclerosis, interstitial LDL accumulation in arteries accompanies the formation of lipid-laden foam cells.1–5 This accumulation of LDL in the interstitial space is believed to be associated with abnormal transport, or abnormal local catabolism, of LDL.

Various mathematical approaches have been used to model this accumulation. Schwenke and Carew,6 Carew et al,7 and Finkelstein et al8 have used lumped kinetic models to represent the transport of macromolecules through the arterial wall, whereas others have depicted the arterial wall using distributed models.9–12 To evaluate a distributed model of macromolecular transport, arterial concentration profiles of labeled macromolecules entering from the plasma are measured across the intima and media, and the distributed model is used to fit the profiles and estimate transport parameters.10,13 Profiles can be obtained from animals fed normal or high-cholesterol diets to examine changes in transport believed important to understanding atherosclerosis. The macromolecule used is often LDL itself or another protein from plasma such as albumin labeled with a radioactive nuclide.14,15 By measuring the profile of both LDL and albumin, one can determine if a particular change in a transport property is specific for LDL or shared with other proteins.

A new technique has been developed for measuring the arterial concentration profiles of macromolecules using horseradish peroxidase (HRP). For many years, HRP has been used as a marker for macromolecular transport,16–18 but it has not typically been used quantitatively. We chose HRP as the macromolecule of interest because of its native per-
oxidase activity, but the technique may be expanded to measure the concentration profile of HRP- or microperoxidase-conjugated macromolecules.

The concentration of HRP is quantifiable because the transmission of light through the tissue containing peroxidase reaction product is a predictable function of the concentration of the peroxidase present. Therefore, measurement of the transmission of light through the tissue is an indirect means of determining macromolecular concentration. The measurements are made by means of an image processing system offering increased resolution through computer enhancement and optical magnification. This approach to measuring concentration profiles does not destroy the cellular and structural features of the tissue, and the spatial resolution is superior to many techniques used previously. The technique offers enhanced resolution of macromolecular concentration profiles across large arteries, and it also opens possibilities for quantifying concentrations in smaller vessels, in the tissue surrounding microcirculation networks, and in focal regions of arteries, such as within an arterial intima or underlying a damaged endothelial cell.

**Methods**

**Quantification of the Horseradish Peroxidase Reaction Product**

*Tissue samples prepared in vitro as standards of known horseradish peroxidase concentrations.* Male Sprague-Dawley rats (250–300 g) were killed to obtain aortic tissue for incubation with known concentrations of HRP. Each animal was anesthetized with sodium pentobarbital (80 mg/kg body wt i.p.), and the descending thoracic aorta from the aortic arch to the diaphragm was excised. Each vessel was rinsed in phosphate buffered saline (PBS), the adventitia was teased away, and the vessel was sectioned into 10–15 cylindrical “rings” that were immediately placed into an incubation bath of known HRP concentration. The incubation solutions consisted of HRP (Sigma type II, Sigma Chemical Co., St. Louis) dissolved in PBS with 10 units/ml penicillin, 0.025 mg/ml Fungizone, and 10 μg/ml streptomycin (GIBCO Laboratories, Grand Island, N.Y.). All tissue incubations were carried out at room temperature for at least 35 hours, unless otherwise indicated. After incubation, the tissue was immersed in 2.5% glutaraldehyde at 4 °C for 4 hours, followed by two washes, one with PBS and one with distilled water, each for 30 minutes at room temperature.

After the tissue samples were washed, they were placed in a reaction mixture containing the substrate for the HRP reaction, 3,3′-diaminobenzidine (DAB) and hydrogen peroxide, dissolved in imidazole (0.1N, pH 7.6).19 The concentrations of the substrates used to determine the relation between time of incubation and the relative gray scale (RGS, see below) of the tissue were 5 mg/ml DAB and 0.015% H₂O₂. For all other cases, the DAB concentration used was 1 mg/ml. All peroxidase reactions were allowed to continue for 30 minutes.16 It was demonstrated that after 30 minutes in the reaction mixture, no increase in reaction product signal was obtained in tissue equilibrated with 0.2 mg/ml HRP (data not shown).

**Embedding and sectioning of tissue samples.** All tissues were dehydrated in sequential solutions of increasing ethyl alcohol content. The tissues were then infiltrated with Spurr’s resin via propylene oxide. Dehydration and infiltration were carried out under vacuum. Each sample was sectioned (2 μm) using glass knives on an LKB Huxley microtome (Rockville, Md.).

**Measurement of the horseradish peroxidase signal.** The HRP reaction product in tissue sections was measured as RGS by means of an IBAS II image processor (Carl Zeiss, Thornwood, N.Y.). RGS of the tissue was equal to the gray scale (GS) of the adjacent Spurr’s resin in the lumen of the vessel (GS) minus the GS of the tissue of interest (GS′). Using neutral density filters to evaluate our camera, we determined that over the differences of transmission we were interested in measuring, GS was linear with the natural log of transmission. The Beer-Lambert law relates transmission to the concentration of the species:

\[ \frac{I}{I_0} = \exp(-\varepsilon CL) \]  

where \(I_0\) is the intensity of the incident light source, \(I\) is the intensity of light that passes through the tissue, \(\varepsilon\) is the molar absorption coefficient, \(L\) is path length, and \(C\) is the concentration of the species.

The natural log of Equation 1 yields

\[ \ln(I) - \ln(I_0) = -\varepsilon CL \]  

Equations 3 and 4 depict the Beer-Lambert law for our measurement of the Spurr’s resin (superscript s) and our measurement of the tissue infiltrated with Spurr’s (superscript t), respectively:

\[ \ln(I^s) - \ln(I^t) = -\varepsilon CsL \]  

\[ \ln(I^t)-\ln(I^s)=-\varepsilon C^t L - \varepsilon C^s L \]  

Subtraction of Equation 4 from Equation 3 yields Equation 5:

\[ \ln(I^s)-\ln(I^t)=\varepsilon C^s L \]  

Because GS is approximately linear with the log of transmission over the range of our measurements, we defined RGS (i.e., \(GS - GS'\)) as equal to \(\ln(I^s) - \ln(I^t)\). The derivation above and this definition results in RGS being independent of the intensity of the incident light from the microscope, \(I_0\). For consistency, the intensity of the incident light in practice was set equal to a GS value near 200 each time the imaging system was turned on. This precaution was taken in an attempt to make the system behave as similarly as possible each day it was used.

The image was presented to the IBAS by means of a ×40 objective (unless otherwise noted) on a Zeiss
microscope via a video camera. The IBAS offers an eight-bit gray scale (0, absolute black; 255, absolute white) and an image array of 512x512 pixels. The measured RGS value was equal to the gray scale of the Spurr’s resin adjacent to areas of the intima and media of interest minus the gray scale of the tissue. The RGS of the Spurr’s resin was lighter than the tissue (i.e., a higher gray scale value); therefore, all measured RGS values were positive except for elastic layers (see below). To account for any variations in the characteristics of the light source and video camera from one day to the next, each time the video camera and microscope were turned on, they were allowed to warm up for 15–30 minutes, and then a standard curve was measured to calibrate RGS with HRP concentration.

When infiltrated with Spurr’s resin, elastin layers appear lighter (a higher gray scale value) than Spurr’s resin alone. This lightness is due to the high scatter coefficient of elastin and results in negative RGS values for a pixel representing elastin. These pixels could not be ignored because the volume of the artery wall that they occupied had to be included for comparison to the serial slicing technique; therefore, these negative RGS values were set equal to the RGS value of the zero HRP standard. By setting the negative RGS values equal to the zero HRP standard, the contribution of the elastin layers to the RGS values most accurately portrayed how the elastin layers actually exist: tissue that has volume, but contains no HRP.\(^\text{18}\)

The IBAS was programmed such that the operator could delineate a section of media to be measured by drawing a rectangle from the endothelium to the medial-adventitial border. This rectangle could be divided into an operator-selected number of rectangles to obtain the desired resolution in the radial direction. Thus, the RGS value of each small rectangle was representative of the density of HRP reaction product a known radial distance from the lumen of the vessel. For measuring the RGS value of a standard piece of tissue (one that was incubated in a known concentration of HRP), the rectangle used extended from the lumen to the medial-adventitial border.

**Characterizing the System for In Vivo Use**

**Purification and labeling of horseradish peroxidase.** For labeling with \(^{125}\)I, HRP was obtained from ICN Immunobiologicales, Lisle, Ill. (HRP, labeling grade), and was further purified by gel filtration (Ultrigel Aca 44, Pharmacia LKB Biotechnology, Piscataway, N. J.) to remove possible contaminants.\(^\text{20}\) Fractions containing HRP were identified by measuring the absorbance at 405 nm. HRP was concentrated and dialyzed against borate buffer (0.03 M, pH 9.0) in a Micro-ProDiCon dialysis unit (BioMolecular Dynamics, Beaverton, Ore.) under vacuum.

The HRP was iodinated with sodium \(^{125}\)I using the iodinating agent 1,3,4,6-tetrachloro-3\(α\),6\(α\)-diphenylglycouril, (Iodo-gen, Pierce Chemical Company, Rockford, Ill.) with approximately 2 mCi \(^{125}\)I per milligram of HRP. The labeled solution was then dialyzed against borate buffer to remove free iodide. The fraction of bound \(^{125}\)I was determined using a Centricon 30K filter (Amicon, Danvers, Mass.). The sample was spun twice at 5,000g with buffer added before each spin to dilute unbound \(^{125}\)I. The fraction of unbound \(^{125}\)I was equal to the ratio of radioactivity that passed through the filter to the radioactivity in the original sample. Radioactivity was assayed in all samples using a Packard MINAXI Gamma Counter (Downers Grove, Ill.) until the standard deviation reached 0.2% up to a maximum of 20 minutes.

The purity of the \(^{125}\)I-labeled HRP solution was evaluated by autoradiography after sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on a Phast System (Pharmacia LKB Biotechnology). Verification that HRP was the major \(^{125}\)I-labeled component was by the appearance of a single band on the SDS-PAGE autoradiograph from an overloaded gel.

**Measurement of labeled horseradish peroxidase in aortic tissue.** The concentration of \(^{125}\)I-HRP solutions for these experiments was 1.25% of the concentration of unlabeled HRP. Cylindrical aorta samples were incubated in the HRP solution to determine equilibrium tissue concentrations. After incubation, each tissue sample was placed in an ice-cold solution of 4.0% bovine serum albumin for approximately 10 seconds to remove adsorbed HRP. The tissues were then fixed and washed as described above.

Each cylindrical tissue sample was cut longitudinally and placed on a glass slide with the endothelium on the glass surface. The sample was then transferred, endothelium down, onto the microtome chuck for frozen sectioning.\(^\text{21}\) The microtome chuck was previously prepared by coating it with tissue mounting medium (O.C.T., Miles Scientific, Naperville, Ill.) and allowing the medium to freeze. The frozen mounting medium was then sliced to create a surface parallel to the plane of the microtome knife. A piece of filter paper (filter type GS, pore size 0.22 \(\mu\)m, Millipore Corp., Bedford, Mass.), previously equilibrated in a dilute solution of Evan’s blue, was then frozen onto the level surface by applying pressure evenly across the surface. The filter paper was made level by retracting the knife and slicing through the filter in increments of 4 \(\mu\)m until a uniform blue surface resulted. The slide covering the luminal surface of the tissue was removed by light thawing of the surface, the surface was lightly cleaned with a Kimwipe (Kimberly-Clark, Roswell, Ga.), and the luminal surface of the tissue was frozen to the filter paper by applying pressure to the adventitial side. The frozen tissue was covered with mounting medium, which was allowed to freeze; tissue and medium were sliced through until the medial-adventitial border was reached.\(^\text{14,15}\) The edges of the remaining intima and media were trimmed, and the trimmed intima and media sample was removed, thawed, and weighed. Unbound \(^{125}\)I was removed by...
twice washing with trichloroacetic acid. The sample was then assayed for protein bound radioactivity.

Comparison of Transmural Horseradish Peroxidase Concentration Profiles Using Horseradish Peroxidase Reaction Product With Those Using Labeled Horseradish Peroxidase

Five Sprague-Dawley rats were anesthetized with sodium pentobarbital (80 mg/kg body wt i.p.) and injected via the femoral vein with approximately 0.5 ml containing 10 mg HRP type II per 200 g body weight and 0.080 mg 125I-labeled HRP per 200 g body weight. The HRP was allowed to circulate for 15 minutes. During the final 90 seconds of the circulation time, a 3–5-ml blood sample was taken through the iliac artery bifurcation. At the end of the circulation time, the descending thoracic aorta was excised and divided into three “rings” and two “cylinders.” The rings were referred to as A, upper thoracic aorta, distal to the aortic arch; B, midthoracic aorta; and C, lower thoracic aorta, immediately proximal to the diaphragm. These rings were immediately placed in ice-cold 2.5% glutaraldehyde and processed as described above. The two cylinders were the tissue remaining between rings A and B, and B and C. They were sectioned longitudinally between each pair of intercostal osea and opened into a sheet of tissue. This tissue was then sandwiched between two glass slides, frozen (Cryowik, Damon IEC Division, Needham Heights, Mass.), and stored at −70°C to await sectioning.

Serial tissue sections of the media from adventitia to lumen were obtained on a frozen microtome. The microtome chuck was prepared as described above. Any plasma contaminating the luminal surface of the tissue piece was removed as a thin sheet of ice with a Kimwipe before the luminal surface of the tissue sample was frozen to the filter paper. The tissue was covered with mounting media, allowed to freeze, and sliced through until the medial-adventitial border was reached. The edges of the tissue were trimmed to remove the intercostals and any blood contamination, and in the process, an outline of the tissue piece was made in the underlying blue filter paper using a scalpel. Slicing began through the media at 10 μm until the blue filter was reached. After each slice, the microtome blade was wiped clean. Slices were placed in precooled test tubes to which three drops of a solution containing 5.0% glutaraldehyde and 10% potassium iodide in PBS were added. The glutaraldehyde fixed the HRP in the tissue slice; the potassium iodide displaced any free residual or adsorbed iodide. The tissues were allowed to incubate in this solution overnight at 4°C, and were then twice washed with trichloroacetic acid.

After the serial sectioning was completed, three tracings of the tissue outline left in the filter paper were made on thin plastic sheets. The areas of these tracings were determined by comparing their weights with the weights from tracings of known area. This area multiplied by the thickness of the tissue slices gave the volume of each tissue slice; thus, the data are portrayed as counts per minute 125I-HRP per volume of tissue.

Determination of final plasma concentration. The 125I-HRP plasma concentration was determined by measuring the fraction of bound isotope as outlined above. The HRP plasma concentration was determined by equilibrating rings of tissue not exposed to HRP with solutions of plasma diluted 1:5 with 4% albumin in PBS. These tissue pieces were then compared with the HRP standard tissues.

Preparation of standard tissue. For preparation of tissue standards, tissue samples were equilibrated in solutions of known HRP concentrations containing 4% albumin as described above. Added to each HRP incubation solution was 125I-labeled HRP in a concentration equal to 0.8% of the unlabeled HRP concentration. After incubation, these tissue samples were fixed and processed as described above. Both the tissues from the rats injected with HRP and the tissues equilibrated in vitro with known HRP concentrations were reacted with the same DAB and H2O2 mixture at the same time to eliminate possible variations from mixture to mixture.

Evaluation of horseradish peroxidase reaction product density. Each profile used to determine the HRP concentration profile and total HRP accumulation in the tissue for a given animal was the average of 36 individual, randomly chosen profiles. Averaging was necessary to eliminate profile-to-profile variation, which, although real, would not allow comparison with the lower resolution of the 125I-labeled HRP method. In other words, the enhanced resolution offered by the HRP technique was ignored for the purpose of comparison with the labeling technique. Total accumulation for both methods was obtained by integrating the appropriate concentration profiles using the trapezoidal rule. The distance from the endothelium to the medial-adventitial border in the glutaraldehyde-fixed tissue samples was 63.7 ± 3.8 μm (n = 5). By necessity, these tissues were immersion fixed; therefore, the tissue was relaxed, and this distance does not represent in vivo dimensions. We have found in parallel experiments (using 21 rats) that the distance from the endothelium to the medial-adventitial border is less by a factor of two in aortas that have been fixed in situ. The distances given in the figures have been corrected to reflect the in situ–fixed dimensions, which more closely represent the in vivo case (i.e., 31.8 ± 1.9 μm, n = 5).

Results

Quantification of the Horseradish Peroxidase Reaction Product

Cylindrical samples of rat thoracic aorta were removed from incubation in 0.2 mg/ml HRP at different times to determine when the RGS value in the tissue became constant. Figure 1 shows the average transmural RGS of the intimal-medial samples versus incubation time. These data indicated
that an incubation of at least 30 hours allowed sufficient time for equilibration.

To determine substrate concentrations required to maximize HRP reaction product in the tissue, the optimal ratio of DAB to H₂O₂ and subsequently the optimal concentration of DAB at that ratio were determined. Each sample was reacted with a known

![Figure 1](image1.png)

**FIGURE 1.** The reaction product of horseradish peroxidase (HRP) as relative gray scale (RGS) in samples of thoracic aortic intima and media as a function of the time of incubation in 0.2 mg/ml HRP. Error bars represent standard errors of data from five cross sections of tissue. The RGS value of each cross section was the average of four separate measurements of intima and media.

![Figure 2](image2.png)

**FIGURE 2.** The effect of varying the 3,3'-diaminobenzidine (DAB) concentration of the horseradish peroxidase (HRP) reaction mixture on the average relative gray scale (RGS) measured in aorta samples after incubation in solutions of two different HRP concentrations (0.1 mg/ml [○] and 0.4 mg/ml [●]) for 30 hours at room temperature. The H₂O₂ concentration was constant at 0.015%.

![Figure 3](image3.png)

**FIGURE 3.** The effect of including albumin in the incubation solutions on ¹²⁵I-labeled horseradish peroxidase (HRP) accumulation in the intima and media of aorta samples. Incubations were carried out in solutions containing 0.2 mg/ml HRP for 30 hours at room temperature.

concentration of DAB in the presence of 0.015% hydrogen peroxide. Figure 2 shows that 1 mg/ml DAB with 0.015% H₂O₂ (a molar ratio of 0.64) yielded a maximal reaction product. The concentration of DAB in the reaction mixture was also varied, holding the molar ratio of DAB to H₂O₂ constant at 0.64. The RGS value could not be appreciably enhanced by the presence of additional substrate at the optimal ratio (data not shown).

**Characterizing the In Vivo System**

Because the aortic tissue in vivo has a component of plasma protein, predominantly albumin, in the interstitial space, the effect of albumin on aortic HRP accumulation was examined in vitro. Figure 3 shows that the presence of albumin in the incubating medium markedly decreased the accumulation of HRP in the tissue, but that a further decrease did not occur above an albumin concentration of 0.5 mg/ml. The decrease in the accumulation of HRP in the presence of albumin may be due to the fact that the albumin competed for adsorption or binding sites within the tissue space.

We quantified the amount of HRP that diffused out of the tissue during the processes of fixing and washing. In Table 1 the amounts of ¹²⁵I-labeled HRP lost from the tissue during the fixation procedure and subsequent washes are presented. The data suggest that approximately 22% of the protein that entered the tissue during incubation was lost from the tissue.

**Table 1. Losses of ¹²⁵I-Horseradish Peroxidase Radioactivity During Arterial Tissue Processing Following In Vitro Incubation**

<table>
<thead>
<tr>
<th>Sample</th>
<th>Label displaced by dip in 4°C albumin</th>
<th>Tissue total before fixation</th>
<th>Label removed during</th>
<th>Fixation in glutaraldehyde</th>
<th>Washes in PBS</th>
<th>Washes in Water</th>
<th>Fraction removed</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>10,058</td>
<td>1,858</td>
<td></td>
<td>395</td>
<td>42</td>
<td>19</td>
<td>0.25</td>
</tr>
<tr>
<td>2</td>
<td>7,196</td>
<td>2,623</td>
<td></td>
<td>450</td>
<td>61</td>
<td>19</td>
<td>0.20</td>
</tr>
<tr>
<td>3</td>
<td>2,628</td>
<td>1,121</td>
<td></td>
<td>206</td>
<td>25</td>
<td>9</td>
<td>0.21</td>
</tr>
</tbody>
</table>

Values in columns 2 through 6 represent counts per minute of ¹²⁵I-labeled horseradish peroxidase (i.e., after elimination of unbound ¹²⁵I). PBS, phosphate buffered saline.
during fixation and washing. It is likely, however, that the protein lost was HRP that was adsorbed to the surface of the tissue. This is suggested by the fact that the total amount lost in fixation and washing (Table 1, column 7) was always less than 10% of the amount of adsorbed HRP that was removed by dipping the fresh tissue in cold albumin solution for 10 seconds (column 2) as described in “Methods.”

To determine the fraction of the tissue volume available to HRP, aorta samples were incubated in solutions containing 4.0% albumin and varying concentrations of 125I-HRP. If we express HRP in tissue in terms of the equivalent water space in the tissue equilibrated with the bulk phase HRP concentration, then the slope of the line relating the equilibrium concentrations of label in tissue to the bulk phase concentration (Figure 4) is the effective fraction of the tissue volume available to HRP. These data indicate that 32% of the tissue volume is available to HRP when measured in equivalents of the bulk phase concentration.

Figure 5 depicts the range for which RGS value is linearly related to bulk phase HRP concentration in the presence of 4% albumin. The data indicate that in designing experiments for this level of resolution, one should choose plasma HRP concentrations that will not load the tissue with HRP above a concentration of 0.2 mg/ml.

Comparison of Transmural Horseradish Peroxidase Concentration Profiles Obtained by Using Horseradish Peroxidase Reaction Product and by Assessing Labeled Horseradish Peroxidase in Serial Slices

To test whether measurements using the RGS of the HRP reaction product yielded similar results to those using the labeled macromolecule, results obtained with the peroxidase method were compared with those obtained by evaluating samples of tissue for radioactive HRP. Radiolabeled HRP was injected intravenously and allowed to circulate for 15 minutes in five rats. Three samples from each aorta were then processed for peroxidase activity and two samples for labeled HRP accumulation. The standard curve in Figure 6 was used to relate the RGS profiles obtained from rings of tissue from the descending thoracic aorta, distal to the aortic arch (A), from the mid-descending thoracic aorta (B), and from the descending thoracic aorta, proximal to the diaphragm (C) to HRP concentrations. This curve was obtained by converting the HRP concentration in bulk phase (Figure 5) to milligrams HRP per milliliter tissue using the known relation between the tissue HRP concentration and the bulk phase HRP concentration.

**Figure 4.** Horseradish peroxidase (HRP) standard curve relating 125I-labeled HRP accumulation in the media and intima to 125I-labeled HRP in the incubating solution. Incubations were carried out for 30 hours at room temperature. The slope of this standard curve (0.32 ml/g) is the interstitial volume fraction available to HRP if expressed at an HRP concentration equivalent to the bulk phase. Error bars represent standard deviations of data from three pieces of tissue.

**Figure 5.** Horseradish peroxidase (HRP) standard curve relating relative gray scale (RGS) of the intima and media to the HRP concentrations of the incubating solutions. Incubations were carried out for 30 hours at room temperature in the presence of 4 g albumin/100 ml. Error bars represent standard errors of data from five cross sections of tissue. The RGS value of each cross section was the average of four separate measurements of intima and media from that cross section.

**Figure 6.** Horseradish peroxidase (HRP) standard curve relating milligrams HRP per milliliter to relative gray scale (RGS) value of the tissue. This curve is obtained by first converting the abscissa of Figure 5 from HRP concentration in the bulk phase to mg HRP/ml tissue by taking into account that only 32% (Figure 4) of the tissue is available for HRP equilibration, and then solving for mg HRP/ml tissue as a function of RGS. This is the curve used to convert the measured RGS profiles to HRP concentration profiles.
tion (Figure 4). Once an RGS value was obtained as a function of HRP concentration (milligrams HRP per milliliter tissue), the equation was rearranged to obtain milligrams HRP per milliliter tissue as a function of RGS.

By using the known relation between RGS and bulk phase HRP concentration, plasma concentrations were also determined. The goal was to determine what bulk phase HRP concentration would yield the RGS values measured in the tissue samples equilibrated with plasma solutions.

Figure 7 is an example of the data obtained from a representative animal. The data using the RGS value of the HRP reaction product represent the averages of the 12 profiles taken from each of the three rings of tissue, or 36 points. Figure 7 also shows data obtained from the radiolabeled HRP in tissue slices. Half of the data is from the tissue cylinder between rings A and B, and the other half is from the tissue between rings B and C; there are 16 data points per animal.

Figure 8 presents the grand average of the profiles from all of the five animals obtained by each of the two techniques. This figure demonstrates that the two techniques yield results with no significant difference between them. Figure 9 shows the total aortic accumulations of HRP in each of the five animals and the average total accumulation of the group. The accumulation values were obtained by integrating the areas under the transmural concentration profiles by the trapezoidal rule. The average value obtained by our new technique is not statistically different from the value obtained by the radiolabel technique.

Figure 9. The total aortic HRP accumulation relative to final HRP concentration (Cpf) in each of the five experimental animals and the average accumulation of the group obtained by the new technique and the radiolabel technique. Total accumulation was determined by integrating the appropriate concentration profile by the trapezoidal rule. The error bars represent standard deviations of the mean.
obtained with the width of the user-defined rectangle equal to its minimum (that of a single pixel) with a

×100 lens. The arrows above Figures 10a and 10b mark the location of elastin layers in the HRP image (Figure 10a) and in the methylene blue–stained image (Figure 10b). The arrows within Figure 10b indicate the stained nuclei.

High-resolution data were obtained to examine the concentration profiles in the intima and the medial layers between the elastic laminae. To construct Figure 11a, the first four elastin layers were aligned from 12 RGS profiles from each of five animals (total of 60 concentration profiles), and the medial layers between pairs of successive elastin layers were divided into 20 rectangles of equal area. The endothelium and intima were divided into five boxes of equal area. Distances from the endothelium shown are averages from the 60 transmural profiles. Each data point in Figure 11 represents approximately 0.5 μm of radial distance. For the purposes of determining background peroxidase activity, the data in Figure 11b were obtained from a single animal that was not injected with HRP but was processed identically to that of Figure 11a. For the purpose of evaluating regular spatial variations in light transmission in the media, the data in Figure 11c are from an aorta of an animal that was not injected with HRP, nor was the tissue exposed to DAB·H₂O₂ substrate, but it was otherwise processed identically to that of Figure 11a. Figure 11a also depicts the RGS profile in terms of relative concentration (right-hand ordinate scale). The data in Figure 11a demonstrate one type of information that can be obtained by the new technique used at relatively high resolution.

**Discussion**

A new technique to quantify transmural macromolecular concentration profiles in arterial tissue has been developed that relates peroxidase reaction product density to macromolecule concentration. Figures 7, 8, and 9 show that this new technique, when used at lower resolution, yields results comparable to those obtained by assessing radiolabeled HRP concentration in transmural serial sections.

The close agreement between concentration profiles obtained using ¹²⁵I-HRP and those using peroxidase reaction product suggests that the new technique is as reliable as those used previously. The HRP lost in the processing of the in vitro tissue standards (Table 1) may have been from surface contamination rather than from diffusion out of the deeper layers of the tissue. Consistent with this speculation is the fact that the amount lost during fixation and washing (Table 1) was less than 10% of the adsorbed protein removed by a 10-second dipping of the fresh tissue in cold albumin solution. Furthermore, if the lost HRP had come from deeper in the tissue, the in vivo concentration profiles measured by the two techniques would not have been similar in shape and would not have been of the same magnitude.

The new technique offers spatial resolution superior to that of Bratzler et al.¹⁴,¹⁵ which consisted of
labeling albumin or LDL with radioactive iodine and tracking the label into serial sections of rabbit aorta. In the present study, the latter method was performed in parallel for comparison using labeled HRP in rat aorta. This method has offered insight into the behavior of proteins in arterial tissue, but it has certain limitations. It offers relatively limited spatial resolution across the vessel wall because a slice thickness of 10 μm is usually required to contain sufficient amounts of radioactivity. As demonstrated in Figures 7 and 8, an aorta excised from a rat would be expected to yield no more than eight to 10 such data points across the intima and media. This small number of data points reduces the precision with which transport parameters can be estimated from the concentration profile. In addition, concentration profile data nearest the endothelium are the least accurate. This inaccuracy can lead to difficulty in estimating certain parameters; for example, data nearest the endothelium are important for obtaining reliable estimates of endothelial permeability. The new technique has no diminished accuracy near the endothelium.

Another limitation of the serial slicing technique is that a relatively large luminal area of the aorta must be included in each tissue slice (>0.5 cm²). The need to integrate the tissue in both the longitudinal and circumferential directions precludes assessment of concentration profiles underlying important focal physical features such as damaged endothelium, replicating endothelial cells, and focal intimal thickening. This level of resolution is particularly important in studies of the large arteries in which increased permeability has been suggested as a contributing factor in atherogenesis due to the entry of lipoproteins, fibrinogen, and growth factors that influence the vessel wall. Although data exist on the average permeability of the aorta to certain molecules, attempts to correlate morphological features with enhanced transport have been hindered by focal variations in permeability. Such focal variation in permeability at different sites on the aorta have been documented. Freeze fracture studies of the aorta show variations between sites with extensive arrays of tight junctions and those where the space between cells appears open. Other studies using transmission electron microscopy, cell kinetics, or immunocytochemistry show that the endothelium contains focal areas of cell injury or cell death where one might expect to see focal increases in permeability. These focal changes are increased in experimental hypertension and may be increased in atherosclerosis. Because of this variability, it has been difficult to use permeability data based on the whole vessel to model transendothelial pathways. Mathematical models have been proposed to account for this variability, but experimental data verifying these simulations are limited.

Figure 10a demonstrates that as the magnification of the optics used to input the image into the processor increases and the area of the user-defined rectangle decreases, the morphological details of the tissue become more evident. Fluctuations in the concentration profile depicted in Figure 10a reflect...
the various inhomogeneities present in the arterial wall. Stained nuclei of medial smooth muscle cells are indicated in Figure 10b. As would be expected after only 15 minutes of HRP circulation, these smooth muscle cells contained little or no peroxidase activity. Figure 10 further demonstrates that the elastic layers were devoid of HRP, an observation consistent with that of Backwinkel et al.\textsuperscript{18} This figure emphasizes the extent to which transmural concentration profiles such as those published previously are smoothed functions because of lower resolution.

The average of 60 HRP profiles in Figure 11a offers an example of what the increased spatial resolution achievable with this new technique allows one to visualize. High-resolution HRP concentration profiles were averaged across the intima and first three medial layers of five aortas with the aid of a ×100 objective lens. In particular, these figures show that the technique allows arterial wall concentration to be correlated with the morphology (e.g., cellularity, occurrence of elastin layers) of the vessel wall. This figure shows that a precipitous drop in HRP concentration occurs across the internal elastic lamina. The concentration profiles are direct evidence that the internal elastic lamina acts as a major barrier to the transport of macromolecules across the wall of the normal aorta as predicted by Fry.\textsuperscript{35} Data such as these will, for the first time, allow mathematical models that take into account the inhomogeneities of the arterial wall to be fit to data.\textsuperscript{35} In addition, because the vessel is analyzed in cross section, these data can be obtained under various conditions to determine if, and to what degree, changes in the physiology or morphology of the vessel wall affect the macromolecular transport parameters that occur in homogeneous and in inhomogeneous models. Such conditions of interest could be mechanical denudation\textsuperscript{36} or chemical injury\textsuperscript{37} of the endothelial monolayer, or hypercholesterolemia. By estimating the transport parameters that govern macromolecular transport under various conditions, one could quantify the effects of various layers of the aorta on the movement of macromolecules. By estimating permeability after denuded endothelium has regenerated, one could determine the degree to which new endothelium is similar to the original that it replaced.

To verify that the morphological variations in HRP concentration measured in the various layers (Figure 11a) were not due to background peroxidase activity, we measured high-resolution transmural profiles in an animal that was not injected with HRP before death (Figure 10b). A transmural HRP profile is depicted in Figure 11a in terms of RGS for comparison with Figures 11b and c. The data in Figure 11b show that the variations in RGS observed in Figure 11a are not the result of background peroxidase activity. Interestingly, the data in Figure 11b indicate that the background peroxidase activity in a medial layer has some of the same spatial characteristics as those observed in HRP-injected animals, but the magnitude of background peroxidase signal was 10- to 20-fold less. To evaluate the effect of variations in the absorption coefficients among interstitium, smooth muscle cells, or other tissue elements on the measured RGS signal, we measured high-resolution profiles in the aorta of an animal that was not injected with HRP before death, and whose tissue was not exposed to DAB: H\textsubscript{2}O\textsubscript{2} (Figure 11c). In all other ways, the animal in Figure 11c was treated identically to the experimental animals that received HRP (Figure 11a). We were concerned that the variations we observed in the transmural concentration profiles at high magnifications were in part the result of differences in the absorption coefficients between heterogeneous tissue components such as interstitium and smooth muscle cells. The measured optical density in an inhomogeneous medium can be written as:

\[ I(x) = I_0 \exp(-\sum u_i(x)L_i) \]

where \( u_i \) is the absorption coefficient of the \( i \)th material.\textsuperscript{38,39} We can rewrite this equation as:

\[ I(x) = I_0 \exp(-[u_{HRP}(x) + \sum u_i^T(x)]L) \]

where \( u_{HRP} \) is the absorption coefficient of HRP and is a function of concentration, C(x), and the molar absorption coefficient of HRP, \( \varepsilon \) (see "Methods"); and \( u_i^T \) is the absorption coefficient of the \( i \)th tissue element. The validity of the new technique requires that the spatial dependence of \( I(x) \) is solely a function of \( u_{HRP}(x) \). For this to be true, either or both of the following conditions must hold: 1) \( u_{HRP}(x) \gg u_i^T(x) \), and 2) \( u_i^T \) must be spatially invariant. If the latter condition is met, it does not mean that the absorption coefficients of the different tissue elements are virtually the same; rather, it indicates that they were not sufficiently different to cause a measurable change in light transmission through a 2-μm cross section embedded in Spurr's resin. Figure 11c depicts an RGS profile constructed as those in Figures 11a and b, but from cross sections of tissue that were not exposed to the peroxidase substrate (DAB plus H\textsubscript{2}O\textsubscript{2}). The data clearly show that absorption in this tissue is orders of magnitude less than that when HRP is present, indicating that condition 1 pertains. These data further demonstrate that there is no predictable spatial variation in absorption across the medial layers; rather, variations in RGS because of tissue elements appear to represent noise in the system, indicating that condition 2 is also met. Thus, we conclude the spatial variations in HRP concentration that we observe across medial layers are not artifactually influenced by the possibly different absorption coefficients present in the arterial wall.

High-resolution techniques have been reported by others. Autoradiographic methods for measuring transmural concentration profiles have been developed by Fry et al.\textsuperscript{40,41} and Morrel et al.\textsuperscript{42} A fundamental difference between the autoradiography techniques and the peroxidase technique is that autoradiography methods measure macromolecular concentration by quantifying an energy (radiation) that originates from within.
the tissue, whereas the peroxidase method measures the ability of an external source of energy (white light from the microscope) to pass through the tissue. On the other hand, autoradiography techniques have certain similarities to the peroxidase technique. Both techniques preserve the vessel structure and tissue organization by measuring transmural profiles across transverse sections of the vessel wall. The spatial resolution of the autoradiographic methods is determined by the method invoked to measure the signal. The spatial resolution of either of these two techniques can be significantly greater than that offered by the measurement of radioactivity, as in the serial slicing technique. An advantage of the peroxidase technique is that it avoids the high levels of radioactivity required to obtain a reliable autoradiographic signal in vivo. An advantage of autoradiography is that it can be applied to a variety of proteins for which the labeling does not interfere with biological activity. It must be demonstrated for each protein of interest whether linking microperoxidase or HRP to the protein alters its biological function.

The new technique is an alternative to existing methods used to measure transmural concentration profiles. It offers increased spatial resolution, preserved tissue structure, and the convenience of avoiding use of high levels of radioisotopes. As outlined, it may also offer a unique opportunity for the quantitative examination of a variety of questions regarding macromolecular transport in arteries. In addition, the technique offers new possibilities to quantify tissue concentrations surrounding microvessels and across the intima and media of small arteries.

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References


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