section was made at the level of the cerebral peduncles to separate the brainstem. A transverse section at the level of the optic chiasm passed through the anterior commissure and separated the forebrain rostral to this transection. The remaining tissue was divided by a horizontal section 1 mm above the anterior commissure. Tissue ventral to this cut, which included the hypothalamus and amygdala, formed the ventral forebrain, and tissue dorsal to this section, which included the hippocampus and most of the neocortex, formed the dorsal forebrain.

15. A washed suspension of rat red blood cells in Hanks buffered salt solution (HBSS) was incubated with approximately 0.75 mCi of ^{51}Cr for 1 hour at 37°C. Cells were washed repeatedly in HBSS and finally diluted with HBSS to form the injected material.
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17. Concentrations of sodium, potassium, and chloride in plasma were measured with an ion-selective electrode for sodium (Beckman E4A System).
18. The difference in the ratios of amount of label in brain tissue and in blood between Fig. 1c and Fig. 1, a and b, resulted from administration of 20 μCi of ^{125}I-labeled BSA (Fig. 1c) rather than 10 μCi (Fig. 1, a and b), which caused greater concentrations of label in the blood and increased the denominator of the ratio. Despite these differences between experiments, concentrations of ^{125}I-labeled BSA in blood did not differ between groups within an experiment.
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Receptor-Mediated Transport of Insulin Across Endothelial Cells

Abstract. *Hormones such as insulin are transported from the interior to the exterior of blood vessels. Whether endothelial cells, which line the inner walls of blood vessels have a role in this transport of hormones is not clear, but it is known that endothelial cells can internalize and release insulin rapidly with little degradation. The transport of iodine-125–labeled insulin was measured directly through the use of dual chambers separated by a horizontal monolayer of cultured bovine aortic endothelial cells. In this setting, endothelial cells took up and released the labeled insulin, thereby transporting it across the cells. The transport of insulin across the endothelial cells was temperature sensitive and was inhibited by unlabeled insulin and by antibody to insulin receptor in proportion to the ability of these substances to inhibit insulin binding to its receptor. More than 80 percent of the transported insulin was intact. These data suggest that insulin is rapidly transported across endothelial cells by a receptor-mediated process.*

The mechanism by which macromolecules such as polypeptide hormones are transported across nonfenestrated capillaries is not well understood. Endothelial cells probably have an important role in this process since they are connected by tight junctions and thus form a major barrier for the rapid diffusion of hormones to their target cells (*1–3*). The rapid onset of action after secretion of hormones is thought to be important for regulation of the metabolic homeostasis. Hence the control of hormonal transport across the vascular barrier may be a rate-limiting and regulating step for the mediation of hormonal actions in many tissues (*1–3*). To traverse the endothelial cell barrier, polypeptide hormones could diffuse between the endothelial cells or be transported through the cells. We and others have been studying the processing of insulin by endothelial cells (*4–7*). Insulin, like other polypeptide hormones, has a rapid onset of action and must therefore cross the endothelial cell barrier quickly in order to react with target muscle and adipose tissues.

We showed earlier that endothelial cells can internalize insulin rapidly (*4*). The internalized insulin is released from the cells in minutes with little degradation (*4*). We have now directly measured the transport of ^{125}I-labeled insulin across the endothelial cell barrier.

To determine whether insulin can be transported across endothelial cells, we used dual-chambered vessels, the upper chambers of which were prepared by attaching dialysis membranes to plastic cylinders (1 × 2 cm). The membrane used had a nominal cutoff of molecular weight 50,000, and within 2 hours, 44 ± 6 percent [mean ± standard error of the mean (S.E.M.)] of the ^{125}I-labeled insulin diffused from the upper to the lower chamber in the absence of cells. The culture apparatus was sterilized by exposure to ultraviolet light for 48 hours, and the cylinders were subsequently attached to the covers of 60 m*M* culture dishes—that is, to the lower chambers—with sterile vacuum grease. The membrane was coated with human fibronectin, and bovine aortic endothelial cells harvested from calf aorta (*4*) were densely seeded and allowed to form a confluent monolayer for 48 hours. The lower chambers contained Dulbecco's minimum essential medium with 10 percent calf serum and were in communication with the upper chambers, which contained the same growth medium.

For the transport studies, the medium was replaced with 0.1*M* Hepes binding buffer at *p*H 7.8 (*4*). Insulin labeled with ^{125}I, inulin labeled with ^{14}C, and other molecules were placed in the upper chamber, and the system was incubated in a shaker water bath set at 37°C. At various times, 1.0-ml samples were taken from the bottom chamber for measurement of radioactivity. The volume of the lower chamber was maintained at 9.0 ml with addition of fresh buffer.

When ^{125}I-labeled insulin (1 ng/ml) was added alone, 15 ± 2 percent of it was transported across the cellular and membrane barrier after 2 hours at 37°C (Fig. 1A). The specific transport rate for labeled insulin, calculated after subtracting the amount of labeled insulin transported in the presence of $10^{-6}M$ unlabeled insulin, was linear for the 2 hours. Addition of unlabeled insulin ($1.7 \times 10^{-9}M$ to $1.7 \times 10^{-6}M$) with the labeled insulin resulted in a progressive decrease in the amount of labeled insulin transported, indicating that the transport is a saturable process. Insulin concentrations of 10^{-9}, 10^{-8}, and $10^{-6}M$ inhibited the transport of labeled insulin by 43 ± 2, 44 ± 14, and 67 ± 7 percent, respectively (Fig. 1A). This correlated well with the ability of the unlabeled insulin to compete with labeled insulin for binding to the receptors on endotheli-

al cells (Fig. 2A). Fifty percent inhibition of specific binding and transport of labeled insulin was achieved with an insulin concentration of $10^{-9}M$. Unrelated polypeptides such as nerve growth factor (NGF) were ineffective in inhibiting the transport and the binding of labeled insulin by endothelial cells (Fig. 1B). The extent of intercellular leakage on nonspecific pinocytosis was estimated with the use of ^{14}C-labeled inulin, a molecule that is not taken up by cells and has a molecular weight similar to that of insulin (*2*). The fraction of ^{14}C-labeled inulin in the lower chamber was similar to the amount of ^{125}I-labeled insulin found in the presence of $10^{-6}M$ unlabeled insulin and accounted for 10 to 30 percent of the total labeled insulin transported (Figs. 1A and 2B). Thus, the data suggest that insulin is specifically transported through endothelial cells, and the portion of the ^{125}I-labeled insulin transported that was not inhibited by $1.6 \times 10^{-6}M$ unlabeled insulin probably represents intercellular leakage of the tracer. When aortic smooth muscle cells were grown on the porous membrane (*8*) instead of endothelial cells, the amount of labeled inulin traversing the cell layers was increased by a factor of 6 to 7 and equaled the amount of labeled insulin. Addition of unlabeled insulin at $1.6 \times 10^{-6}M$ did

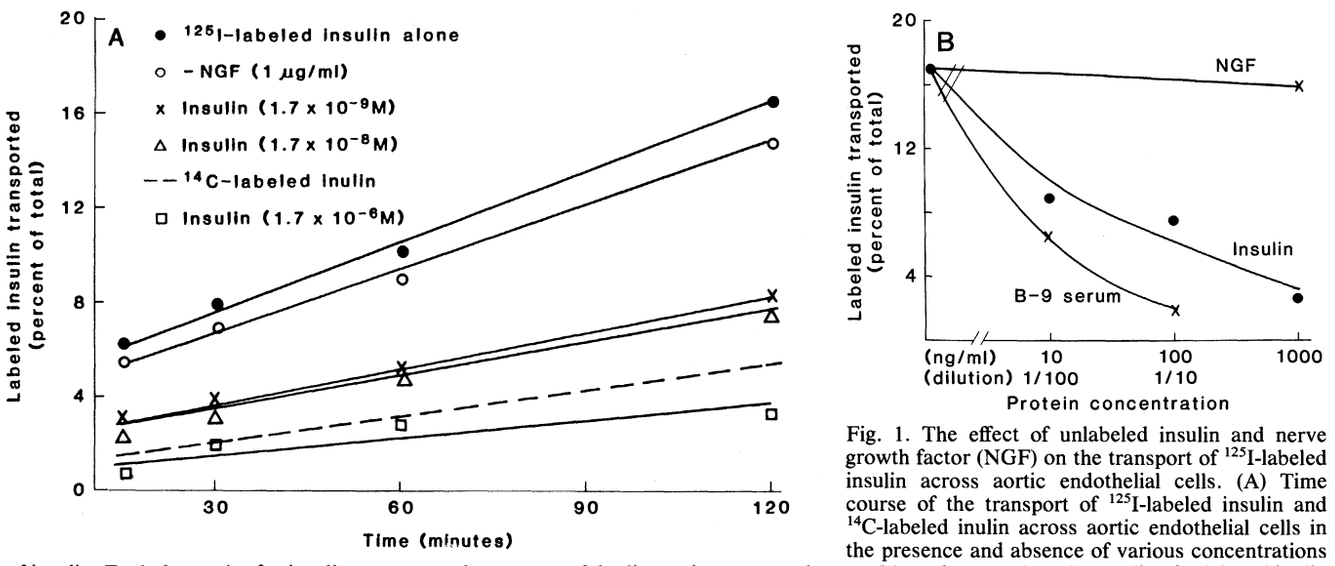


Fig. 1. The effect of unlabeled insulin and nerve growth factor (NGF) on the transport of ^{125}I-labeled insulin across aortic endothelial cells. (A) Time course of the transport of ^{125}I-labeled insulin and ^{14}C-labeled inulin across aortic endothelial cells in the presence and absence of various concentrations of insulin. Each data point for insulin represents the average of duplicates in two experiments. The values used to plot the line for labeled inulin were averages for inulin measured at all the points at which labeled insulin was measured at the noted time. The labeled inulin was placed in every chamber to provide an estimate of nonspecific transport at each point. For all of the studies shown, the inulin values were within 10 percent of each other. (B) Effects of insulin, NGF, and antiserum to the insulin receptor (B-9) on the inhibition of ^{125}I-labeled insulin transport after 2 hours. Bovine aortic endothelial cells were harvested and cultured from calf aortas as described (*8*, *18*). Cells of the 15th passage or earlier were used for the studies. The cells were identified by their characteristic morphology as judged with inverted–phase contrast light microscopy and by their positive response to antibody of bovine factor VIII antigen (*4*, *8*). The ^{125}I-labeled insulin (specific activity, 350) was iodinated by the chloramine method, and monoiodinated A14 insulin was purified by high-performance liquid chromatography (*4*, *19*, *20*). The ^{14}C-labeled inulin (specific activity, 200) was purchased from Amersham and added in all studies to estimate intercellular diffusion. Unlabeled porcine insulin was purchased from Elanco Production Company, Indianapolis, Indiana. The B-9 antiserum to insulin receptor was derived from a patient with type BB insulin resistance syndrome (*9–11*). Transport studies were performed at 37°C in 0.1*M* Hepes buffer, *p*H 7.8, containing 0.12*M* NaCl, 5 m*M* KCl, 1.2 m*M* $MgSO_4$, 8 m*M* glucose, and 0.5 percent bovine serum albumin. At 2 hours, 45 to 50 percent of the ^{125}I-labeled insulin or ^{14}C-labeled inulin had diffused across the dialysis membrane (exclusion size 50,000) in the absence of cells. The data shown in all the figures are from one of the experiments, and each point shown represents assays of duplicate or triplicate samples. The interexperimental variation was 10 percent or less.

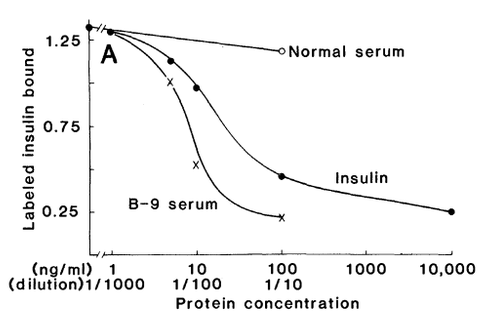
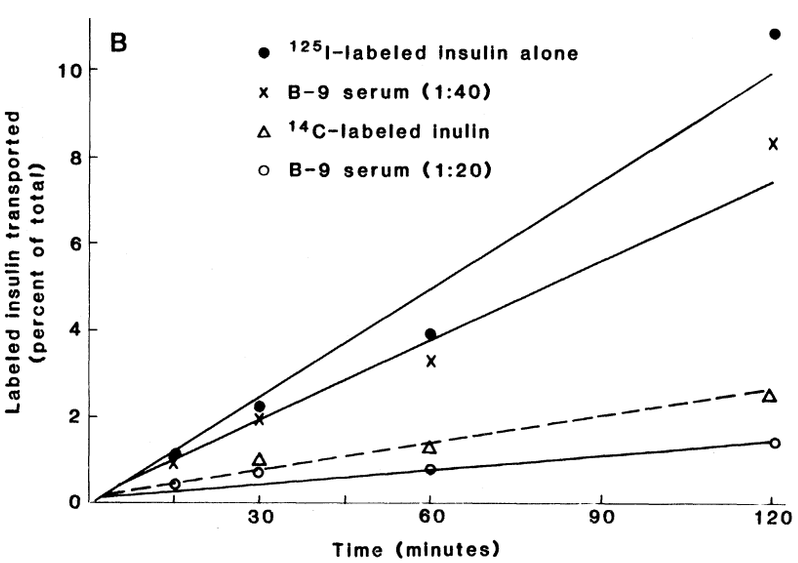


Fig. 2. Effect of antibodies to the insulin receptor on (A) binding and (B) transport of ^{125}I-labeled insulin in aortic endothelial cells. Binding studies were performed in 0.1*M* Hepes buffer as described (Fig. 1) at 15°C and normalized as percentage of binding to 10^6 cells. B-9 serum has been characterized (*9–11*) and was added in the dilutions indicated. The conditions for the transport studies have been described in the legend of Fig. 1.



not affect the appearance of either the labeled insulin or the labeled inulin crossing the layers of smooth muscle cells.

To further substantiate the involvement of insulin receptors in the transport of insulin across the endothelial cells, we determined the effect of antibodies to the insulin receptor on the transport of labeled insulin (Fig. 2B). The antiserum to the receptor (B-9) was derived from patients with insulin resistance; this antiserum has been characterized extensively (*9–11*). Antiserum to the receptor inhibited the binding of labeled insulin to the endothelial cells by 50 ± 12 and 90 ± 4 percent at serum dilutions of 1:100 and 1:20, respectively (Fig. 2A). Normal serum did not show any significant inhibition of insulin binding even at 1:10 dilution. When antibodies to the receptor (1:40 and 1:20 dilutions) were added with labeled insulin to the upper chamber for transport studies, transport was inhibited, on average, by 37 and 82 percent, respectively (Fig. 2B). The inhibition caused by antibodies to the receptor at these dilutions was comparable to their inhibitory effect on the binding of insulin to the endothelial cells.

The effect of temperature on the transport of ^{125}I-labeled insulin was also studied (Fig. 3). The total amount of labeled insulin crossing the cells and membrane was 2.1 ± 0.1 (S.E.M.) times higher at 37°C than at 15°C. When the amount of labeled insulin transported via the receptor was estimated (the difference between total transport and that in the presence of $10^{-6}M$ insulin), the amount of labeled insulin transported at 37°C was 2.7 ± 0.8 times that at 15°C. Thus, intercellular diffusion was affected less than receptor-mediated transport by the decrease in temperature. Receptor-mediated transport had a lag period of about 15 minutes at both 15° and 37°C.

The direction of the transport process was also studied. In simultaneous experiments, ^{125}I-labeled insulin and ^{14}C-labeled inulin were placed either inside or outside of the upper chamber, which corresponds to the interior of the blood vessel. When ^{125}I-labeled insulin was placed in the upper chamber, the transport of the labeled insulin was inhibited by unlabeled insulin at $1.6 \times 10^{-6}M$ (Fig. 4A). In contrast, when labeled insulin was placed in the lower chamber, which corresponds to the exterior of the blood vessel, the transfer of labeled insulin across the endothelial cell barrier was not inhibited by the addition of unlabeled insulin, and the amount transferred was equal to the amount of labeled inulin that diffused across the endothelial cells (Fig. 4B).

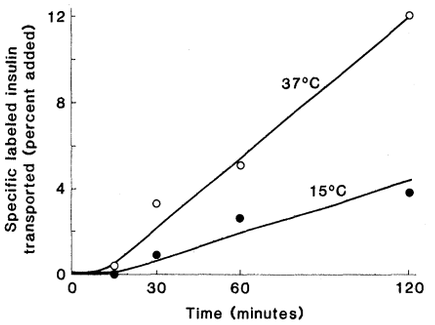


Fig. 3. Effect of temperature on the transport of ^{125}I-labeled insulin across bovine aortic endothelial cells. The conditions of the study have been described in the legend of Fig. 1. The percentage of ^{125}I-labeled insulin transported was derived by subtracting the amount of labeled insulin transported in the presence of $1.7 \times 10^{-6}M$ unlabeled insulin from the amount of labeled insulin transported in the absence of unlabeled insulin at each time point. The transport of ^{14}C-labeled inulin was also measured at every point and was found to be within 10 percent of the amount of ^{125}I-labeled insulin transported in the presence of $1.7 \times 10^{-6}M$ unlabeled insulin.

The integrity of the ^{125}I-labeled insulin was estimated by precipitation with 10 percent trichloroacetic acid before and after transport. More than 95 ± 2 percent of the initial tracer was precipitated with trichloroacetic acid. After 2 hours at 37°C, 85 ± 5 percent of the insulin in the upper chamber was intact and 80 ± 6 percent in the lower chamber was intact. These data indicate that insulin can be transported across the endothelial cells with only a small amount of degradation.

We have shown that insulin can be transported across a layer of endothelial cells. The process of transport appears to be mediated by insulin receptors and to be energy dependent. These properties suggest that the transport process is similar to the internalization mechanism for polypeptide hormones (*12, 13*). The signals and the mechanisms for the transport of insulin across the endothelial cells are not known, and whether the transport of hormones across the vascular barrier differs from that of other large molecules and proteins is unclear. Since endothelial cells, unlike other cells, do not degrade insulin to any extent (*4–6*), insulin is probably not channeled through a lysosomal pathway. In general, studies have shown that complex molecules and large proteins can exit the vessel by multiple ways, including penetrating the intercellular clefts between the cells or going through the cells via vesicles (*14–16*). The data from Simionescu *et al.* (*14*) and by Palade *et al.* (*15*) suggest that a large part of macromolecular transport across the capillary wall could be due to micropinocytosis with the formation of vesicles that shuttle between the luminal and the external sides of the vessel wall. Alternatively, multiple vesicles in series could become functional pores of different sizes that would provide a conduit for the macromolecules. It is possible that the endocytotic vesicles formed during the internalization of hormones are also involved in the vesicle transport process. It is not clear which pathway represents the main route of macromolecule transport across capillary walls (*16*). In our system, trans-

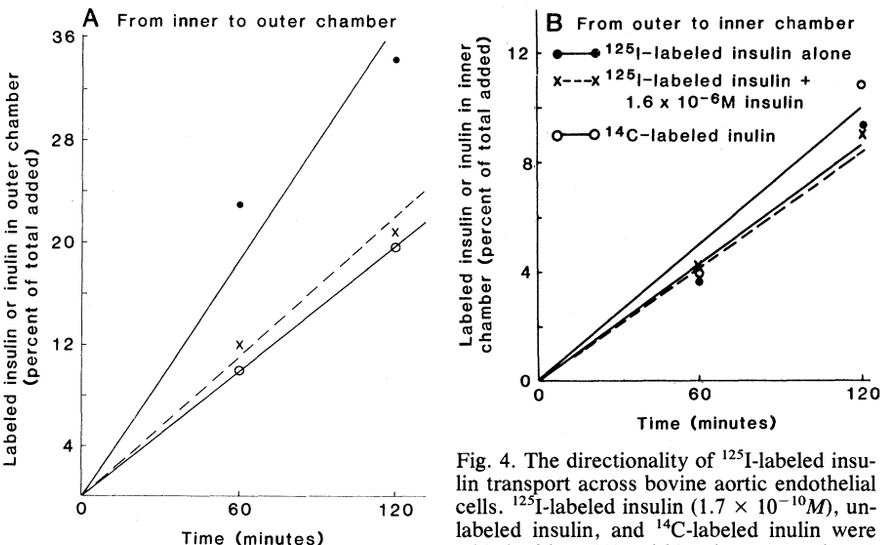


Fig. 4. The directionality of ^{125}I-labeled insulin transport across bovine aortic endothelial cells. ^{125}I-labeled insulin ($1.7 \times 10^{-10}M$), unlabeled insulin, and ^{14}C-labeled inulin were added either (A) within the upper (inner) chamber or (B) in the lower (outer) chamber. All experiments were performed at 37°C. At each time point, a fixed amount of the buffer from either the lower chamber (A) or the upper chamber (B) was used to determine the counts of both the labeled insulin and the labeled inulin.

port across the endothelial cells was always greater than intercellular diffusion. However, this system differs from physiological conditions in some important respects, including the absence of pressure differences and flow dynamics. In addition, the findings in this system are also dependent on the permeability and culture characteristics of the membrane used.

In summary, endothelial cells can transport insulin at physiological concentrations by a specific receptor-mediated process. Since endothelial cells have specific receptors for hormones and growth factors (*4–8, 17*), we suggest that receptors for hormones on vascular endothelial cells may be functioning as transporters of polypeptide hormones across the capillary wall. The regulation of hormone receptors on vascular endothelial cells could affect the concentrations of hormone in the extravascular space and provide an additional point for the modulation of hormone action.

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Separation Techniques Based on the Opposition of Two Counteracting Forces to Produce a Dynamic Equilibrium

Abstract. *Useful compounds, whether produced by chemical synthesis or biological synthesis, often need to be purified from complex mixtures. Biochemists and chemists thus recognize the need for efficient new preparative purification techniques for product recovery. Such fractionation techniques must have high capacity and high resolution. In a novel group of separation methods suited to the preparative fractionation of proteins, antibiotics, and other classes of compounds, the chromatographic flow of a solute down the column is opposed by solute electrophoresis in the opposite direction. Useful separation is achieved when these two counteracting forces drive the solute to a unique equilibrium position within the separation chamber. The properties of chromatographic matrices, for example, gel-permeation matrices of various porosities, provide a means of establishing the unique equilibrium points. Extraordinary resolution and capacity are attainable by these methods.*

In recent years, major improvements have been made in the development of analytical techniques for the separation of proteins and nucleic acids (*1–3*). However, methods for large-scale preparative fractionation have advanced more slowly and have relied heavily on the use of increased scale to adapt the analytical technologies (*4*). I describe here the results of initial investigations of a new group of separation methods that can provide the high capacity and high resolution needed for preparative fractionation. When solvent flow and electrophoresis directly oppose each other, their action on a particular solute can be precisely counterbalanced to achieve a steady state, but ordinarily there would be no restoring force moving the solute toward a particular equilibrium position. A chromatographic matrix can influence solute movement with a flowing solvent differently from the way it influences solute electrophoresis and thereby can bring about a balance between these opposing forces. With an appropriate configuration of different chromatographic matrices, movement driven by electrophoresis will dominate within part of the separation column while movement with the flowing solvent will dominate in another part of the column. These imbalances can produce a restoring force that concentrates the solute at a unique equilibrium position (*5*). The following example illustrates this principle and describes the approach used in the first experimental tests of this method.

A column (Fig. 1) was packed with a restrictive matrix, BioGel P-10 (Bio-Rad), on top of a bed of a much more porous matrix, BioGel A-50m. The colored protein ferritin is found to chromatograph rapidly downward through the upper part of this column (where it is excluded from the matrix beads) but more slowly through the lower part of the column (where it is included in the matrix beads). As illustrated in Fig. 1, at the appropriate applied voltage, the upward electrophoresis rate, R_E, will exceed the downward rate of movement with solvent flow, R_F, in the bottom matrix but will be insufficient to counteract the more rapid downward R_F in the top matrix. At this voltage, the net movement (R_N) of ferritin is downward in the top part of the column and upward in the bottom part of the column. The ferritin is thereby highly concentrated at an equilibrium position adjacent to the interface of the P-10 and A-50m gel beds.

This type of solute behavior provides the basis for a powerful separation method. Although I will illustrate the method with a specific example, the approach is very general and adaptable. I call this new group of separation methods counteracting chromatographic electrophoresis (CACE).

If CACE is to prove effective as a preparative procedure, it must have high capacity. In the two-component separation column described above, how much protein can be held in an equilibrium zone? A protein that is driven toward the interface of the two matrices is clearly not in equilibrium in either the upper or the lower matrix. Obviously, protein cannot accumulate indefinitely at the interface, yet, because the conditions in the upper and lower matrices trap the protein, it cannot escape. A concentration-dependent effect must cause R_E to equal R_F so that an equilibrium zone is established in an area of the column where the protein would otherwise (that is, at low concentration) still have a net mobility. One obvious way in which high protein concentration will alter electrophoretic mobility is by increasing the local electrical conductivity, thereby locally decreasing the voltage gradient and thus decreasing R_E. For example, as the ferritin begins to accumulate at the junction of the P-10 and A-50m matrices, its