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Anionic content of basement membranes

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ANIONIC CONTENT OF BASEMENT MEMBRANES

by

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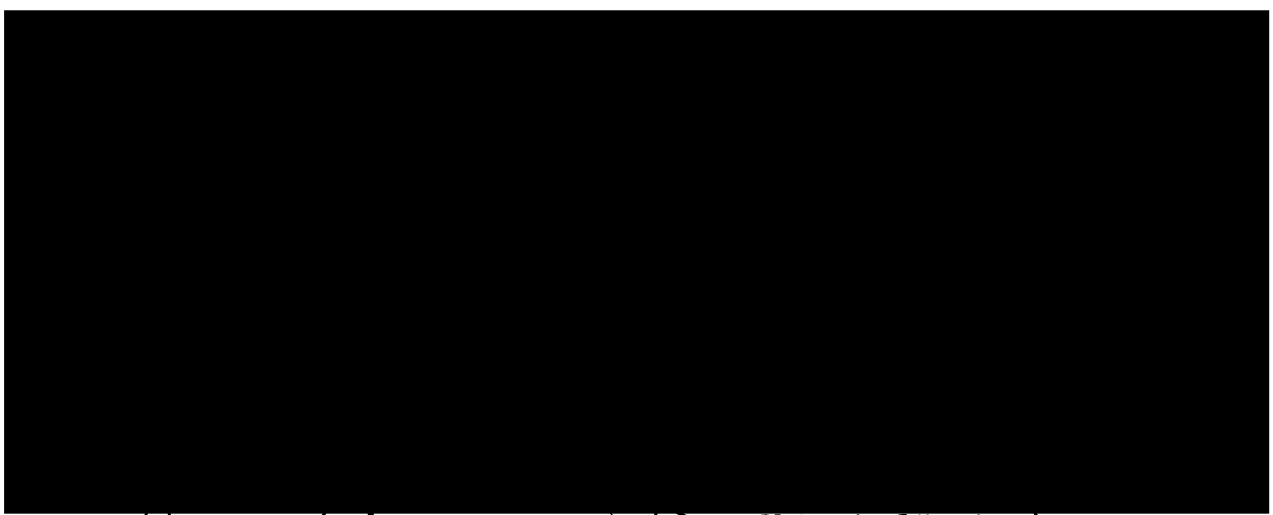

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ANIONIC CONTENT OF BASEMENT MEMBRANES

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ABSTRACT

We have used ruthenium red, a cationic tracer, to detect at the electron microscopic level the presence of anionic sites in various murine basement membranes, with emphasis on those of the microvasculature. We have observed anionic (ruthenium red staining) sites in all continuous and fenestrated capillaries examined. Terminal lymphatics (known to have a patchy basement membrane) had regionally anionic sites. Anionic sites were not present under the sinusoidal lining of the liver where a basement membrane is totally lacking. Basement membranes of epithelial cells and those surrounding striated and smooth muscle cells, pericytes, fat cells and Schwann cells also exhibited anionic sites. We have compared the electrostatic properties of two capillary basement membranes: those from continuous capillaries of the muscular portion of the diaphragm with those from renal cortical tubule fenestrated capillaries. The comparison was done by determining the salt concentration (critical electrolyte concentration, Scott and Dorling, 1965) required to displace ruthenium red from its binding sites. Our results suggest that the fenestrated capillary basement membrane is more strongly negatively charged than the continuous capillary basement membrane, because 0.5M Na^+ displaced ruthenium red to a large extent from the continuous capillary whereas 1.3 M Na^+ was required to displace ruthenium red from the fenestrated capillary. We conclude from this study that first, anionic sites are a general characteristic of basement membranes and second, their properties differ in different vascular basement membranes. We speculate that the anionic content of basement membranes and its variability might be

of physiological significance. We have applied the double antibody immunocytochemical technique on chopped sections from mouse diaphragm briefly prefixed with a weak fixative. Our first antiserum was affinity purified rabbit anti-heparan sulfate core protein (Hassel et al, 1980). Our second antiserum was ferritin-coupled goat anti-rabbit IgG. We have observed, at the electron microscopic level the presence of ferritin molecules in the basement membrane of endothelial cells and muscle cells. This finding indicates that heparan sulfate proteoglycan is a component of these basement membranes.

I. GENERAL PART: CURRENT KNOWLEDGE ON BASEMENTMEMBRANES--AIMS AND CONCLUSIONS OF THIS STUDY

Introduction

The basement membrane is a specific type of extracellular matrix. It is found under the basal surface of cell types such as simple epithelia, the basal cell layer of stratified epithelia, mesothelia and endothelia. It completely surrounds other cell types including smooth muscle cells, striated muscle cells (both skeletal and cardiac), pericytes, fat cells and Schwann cells. In this way, the basement membrane forms the outer limit of the interstitial compartment and surrounds certain cell types inside the interstitial compartment (Vracko, 1974). From these anatomical considerations it can be concluded that every substance, no matter how large or small, has to cross a basement membrane in order to gain access or leave the interstitial compartment. Two exceptions to this rule should be stressed: first, terminal lymphatics, where the basement membrane is discontinuous and second, the space of Disse, where the sinusoidal lining cells of most species do not possess a basement membrane.

Historical Review

Todd and Bowman (1857) can be considered pioneers in the field of basement membrane structure and function. By examining the rabbit peritoneum they found that "the epithelium rests immediately on a continuous transparent basement membrane of excessive tenuity" (p. 129). In discussing the digestive track, they state that "the basement membrane in the best marked examples is distinctly homogenous and transparent, but in some situations it is finely fibrous and not easily separable from the tissues below", (p. 523),

that "the basement membrane cannot be everywhere traced, for example, in the interior of the hepatic lobules," (p. 523) and that "in most mucous membranes, the basement membrane is placed between them (capillaries) and the epithelium" (p. 660). As for the function of the basement membrane, they state that "the office of the basement membrane seems to be in all cases to sustain the epithelium and to shut in or cover over those tissues which may be considered as internal to the element of the simple mucous membrane" (p. 523).

With the development of histochemistry, light microscopists observed that basement membranes stained with the periodic acid-Schiff reagent (Ramburg et al, 1966), indicating the presence of carbohydrate. Basement membranes also stained with periodic acid-silver (Ramburg and Leblond, 1967) indicating the presence of reticular fibers.

The use of the electron microscope allowed us to visualize the ultrastructure of the basement membrane. Rhodin (1955) and Yamada (1955) described for the first time the ultrastructure of the glomerular basement membrane, and Bruns and Palade (1968a) described for the first time the ultrastructure of capillary basement membrane in muscle.

Because "membrane" is a term referring to lipid bilayers, and because the basement membrane of light microscopy does not correspond exactly to that observed at the electron microscope, Fawcett (1962) suggested the term "basal lamina" to designate the lamina rara interna and the lamina densa (see below). However, not many investigators have adopted this terminology. For this reason, in our work we use the term basement membrane instead of basal lamina.

The use of appropriate model systems for isolation of basement membrane material in high yield and purity and the use of sophisticated biochemical techniques have led to the isolation, characterization, and analysis of macromolecular constituents of basement membranes. Furthermore, cytochemical and immunocytochemical techniques have contributed to our knowledge about the localization of macromolecules inside the basement membrane. Most of these developments took place during the last ten years and have produced a wealth of information, to be briefly reviewed in the following paragraphs.

Current Knowledge

A. Morphology

With the electron microscope the basement membrane is usually observed as a feltwork of fine fibrillar material, 50-100nm thick, consisting of three layers: a central electron dense layer, called lamina densa; an electron lucent layer between the lamina densa and the cell surface, called lamina rara interna or lamina lucida; and an electron lucent layer between the lamina densa and the surrounding connective tissue, called lamina rara externa or lamina diffusa (Katsuyama et al, 1977).

In certain areas where two basement membranes are fused (renal glomerular basement membrane, renal tubular basement membrane), the overall thickness of the basement membrane exceeds 100nm.

With the combination of quick-freezing, deep etching and rotary shadowing, the basement membrane can be visualized

three-dimensionally as a meshwork of interconnecting filaments, mainly localized in the lamina densa (Heuser and Salpeter, 1979).

B. Macromolecular Components

Biochemical analysis of basement membranes has produced a list of macromolecular components, which is probably not a final one. Basement membrane macromolecules can be classified in three groups: collagenous glycoproteins, non-collagenous (glyco)proteins and glycosaminoglycans (proteoglycans).

B1-Collagenous Glycoproteins

Among the collagenous glycoproteins we can list collagen type IV, collagen type V and Acetylcholinesterase.

Collagen type IV, first described by Kefalides (1975) is a basement membrane-specific macromolecule. It has three similar α-chains and the macromolecule can be represented by the empirical formulas $[\alpha 1(IV)]_3$ or $[\alpha 2(IV)]_3$, where α1 and α2 are two slightly different α chains. Each chain has a molecular weight of 140,000-160,000 daltons (Kleinman et al, 1981; Orkin et al, 1977). Compared to the other types of collagen it contains more carbohydrate and more 3-hydroxyproline and hydroxylysine.

Collagen type V, first described by Burgeson et al (1976), seems to be present only in some basement membranes, especially those of blood vessels and smooth muscle cells (Kleinman et al, 1981). It contains three α-chains and is designated as $[\alpha 1(V)]_2\alpha 2(V)$. An alternative terminology for α1(V) and α2(V)

is αA and αB respectively. Type V collagen has a high hydroxylysine and low alanine content.

Acetylcholinesterase is the enzyme that degrades acetylcholine. It is found in synaptic clefts of the neuromuscular junction, tightly bound to the basement membrane of the cleft (McMahan et al, 1978), but not in the rest of the basement membrane surrounding the muscle cell (Sanes and Hall, 1979). Chemical analysis of acetylcholinesterase from the electric organ of the eel Torpedo californica has shown that it is a pentamer consisting of four globular catalytic subunits and a fifth elongated subunit; the elongated subunit is rich in glycine, hydroxyproline and hydroxylysine and is susceptible to collagenase treatment (Lwebuga-Mukasa et al, 1976), thus making it collagen-like in its properties.

B2-Non-collagenous (Glyco)proteins

The non-collagenous glycoproteins of basement membranes include laminin, fibronectin, entactin and bullous pemphigoid antigen.

Laminin was discovered similtaneously by two groups of investigators (Timpl et al, 1979; Chung et al, 1979). It is now known to be a tetrameric glycoprotein consisting of three small subunits, each 200,000 daltons and a large subunit 400,000 daltons in molecular weight (Martin, personal communication). It has been demonstrated in vitro that laminin is a molecule involved in epithelial cell adhesion to the substratum (Carlsson et al, 1981; Johansson et al, 1981) and it has been suggested that cell surfaces contain receptors for the large

subunit of laminin (Martin, personal communication). Laminin has been detected immunocytochemically in the lamina lucida of human epidermal and mouse esophagus basement membranes (Foidart et al, 1980).

Fibronectin is a new name for a long-known glycoprotein previously called cold insoluble globulin. It is a dimer of two rod-like chains, each about 200,000 daltons in molecular weight (Yamada and Olden, 1975). Each chain of fibronectin has several globular and non-flexible domains separated by linear and very flexible domains which give the molecule the ability to deform (Yamada, personal communication). Fibronectin is a component of the interstitial matrix and it is also present in plasma, but it is debatable whether it is or is not a basement membrane component. For example, at the electron microscopic level, there are conflicting reports about its presence in the glomerular basement membrane (Madri et al, 1980; Courtoy et al, 1980).

Recently, a new glycoprotein has been isolated from the basement membrane-like matrix of an endodermal cell line (Carlin et al, 1981). It has been given the name "entactin." Entactin has a molecular weight of 158,000 daltons and is sulfated. Antibodies raised against entactin react specifically with various epithelial basement membranes. At the EM level, the staining is localized very close to or at the surface of tubular epithelial cells (Carlin et al, 1981). That localization has led to the suggestion that entactin might be involved in the interaction between cell surfaces and basement membranes.

Another recently characterized macromolecule and a candidate as a basement membrane component, is the bullous pemphigoid antigen. It is a high molecular weight protein, consisting of disulfide-linked subunits, each with an apparent molecular weight of 220,000 daltons (Stanley et al, 1981). It is found almost exclusively in the basement membrane of stratified squamous epithelia and it is produced by epidermal cells in culture (Woodley et al, 1980). In patients suffering from bullous pemphigoid, antibodies reacting specifically with the bullous pemphigoid antigen have been detected in their sera (Stanley et al, 1981). In such patients, a deposition of IgG has been observed at the EM level in the lamina lucida of their epidermal basement membrane (Holubar et al, 1975).

B3-Glycosaminoglycans (Proteoglycans)

The term glycosaminoglycan (GAG) refers to linear, (unbranched) macromolecules composed of repeating dissacharide units consisting of one hexuronic acid and one hexosamine, appropriately modified. However, all glycosaminoglycans (with the exception of hyaluronic acid) are covalently linked to a polypeptide core, from which they sprout, like branches of a tree. For this reason, the less frequently used term proteoglycans (PG) seems to more appropriately describe their macromolecular composition.

Glycosaminoglycans found in basement membranes include heparan sulfate, chondroitin sulfate and hyaluronate.

Heparan sulfate has been found to be the main glycosaminoglycan of most adult basement membranes studied so far. Hassel

et al (1980) have isolated and analyzed the basement membrane heparan sulfate from the matrix of EHS (Engelbreth-Holm-Swarm) sarcoma, a tumor that produces exclusively basement membrane material (Orkin et al, 1977). It has an approximate molecular weight of 500,000-1,000,000 daltons; half of it is protein and half carbohydrate. Six to twelve polysaccharide side chains are associated with each protein molecule. The carbohydrate chains have an approximate molecular weight of up to 70,000 daltons.

Choindroitin sulfate and hyaluronate have been detected in renal basement membranes, in experiments studying their biosynthesis with radioactive tracers (Lemkin and Farquhar, 1981). The chondroitin sulfate glycosaminoglycan chains from glomerular basement membranes seem to be attached to a different core protein than the heparan sulfate glycosaminoglycan chains (Kanwar et al, 1981). Information about the protein core of basement membrane chondroitin sulfate proteoglycan is not available so far. Chondroitin sulfate has been implied as the main glycosaminoglycan found in embryonic corneal epithelium basement membrane (Trelstad et al, 1974).

The information presented above about the macrmolecular constituents of basement membranes is summarized in Table I.

C. Spatial Arrangement of Basement Membrane Components

How are all these molecules held together and arranged in space? The data so far indicate that many of these macromolecules have, as part of their structure, sites of high affinity for the other macromolecules.

TABLE I

CHEMICAL CLASS	NAME	LOCALIZATION	CHEMICAL CHARACTERISTICS Subunits	Total MW	Other Comments	ISOLATION FROM	REFERENCES
Collagenous Glycoproteins	Collagen Type IV	Present in all basement membranes	3 identical subunits (each of MW 140-160GOUD)-~ 450KD $[\alpha 1(IV)]_3$ or $[\alpha 2(IV)]_3$		more CHO high 3-OH-proline and CH-lysome	Lens capsule glomerular basement EHS sarcoma	Kefalides, 1975 Orkin et al, 1977 Kleinman et al, 1981
	Collagen type V	Blood vessel and and smooth muscle cell basement membrane	2 types of subunits $[a^1(V)]_2$ $\alpha 2(V)$ formerly αA and αB		high-OH-lysome low alanine	fetal membranes	Burgeson et al, 1976 Kleinman et al, 1981
	Acetylcholinesterase	Only in basement membranes of synaptic clefts of neuro-muscular junctions	4 globular catalytic subunits 1 elongated tail structure structure collagen-like		tail structure high glycine OH-proline OH-lysine susceptible to	Torpedo californica electric organs	Lwebuga-Mukasa et al 1976 McMahan et al, 1978 Sanes and Hall, 1979
Non-collagenous (Glyco)proteins	Laminin	present in all basement membranes	3 small subunits ~ 200KD each 1 large subunit ~ 400KD	1000 KD	glycoprotein in 3-D has shape of cross cell adhesion	EHS sarcoma embryonal carcinoma-derived line	Timple et al, 1979 Chung et al, 1979 Foidart et al, 1980 Madri et al, 1980
	Fibronectin	not established as a basement membrane component	2 subunits ~220KD each	440 KD	glycoprotein binding sites for several macromolecules	plasma cultured cells	Yamada and Olden, 1978 Madri et al, 1980 Courtoy et al, 1980
	Entactin	not enough data		158 KD	contains $-SO_4=$	embryonal carcinoma derived line	Carlin et al, 1981
	Bullous pemphigoid antigen	present (only) in the basement membrane of stratified epithelia	unknown number of subunits each of 220KD	-?-		cultured epidermal cells serum of patients	Stanley et al, 1981
ycosaminoglycans	Heparan sulfate	present in all adult basement membranes	50% protein 50% CHO 6-12 side chains	-0.5-1.0x10^6D-	high content of anionic groups	EHS sarcoma	Hassell et al, 1980
	Chondroitin sulfate	? present in embryonic basement membranes	?			glomerular basement membrane	Kanwar et al, 1981 Lemkin and Farquhar, 1981 Trelstad et al, 1974

Laminin has binding sites for cell surfaces (probably its large molecular weight subunit) and also binds collagen type IV (Kleinman et al, 1981) and heparan sulfate (Sakashita et al, 1980).

Fibronectin has binding sites for all types of collagen, including type IV and type V, sites for cell surface binding and sites for heparan sulfate and hyaluronate binding (Kleinman et al, 1981).

Studies on cartilage have shown that hyaluronate has the ability to bind many proteoglycan molecules and that this association is stabilized by two "link proteins" (Lindahl and Höök, 1978). It is not known whether a similar role is played by hyaluronate present in basement membranes. "Link proteins" have not been identified thus far in preparations from basement membranes.

The information available up to now, does not allow us to construct a three dimensional model of the arrangement of basement membrane components.

Physiological Significance

We will now discuss briefly the physiological role played by basement membranes as a whole or by their individual components. We will concentrate on their role as (a) supportive materials, (b) permeability barriers, (c) generators of non-thrombogenic surfaces and (d) hosts of functionally significant macromolecules.

A. Basement Membranes as Supportive Materials

This function for basement membranes has been suggested since 1857. It is of interest to discover which of the

components of basement membranes are involved in this process. Two candidates have been considered: laminin and fibronectin (Kleinman, 1981). In both of these molecules there are areas of high affnity for cell surfaces and areas of high affinity for other components of the basement membrane (e.g., collagen type IV).

Kidney perfusions with neuraminidase (an enzyme that specifically eliminates sialic acid residues) have resulted in various degrees of detachment of either the endothelial or the epithelial cells from the glomerular basement membrane (Kanwar and Farquhar, 1980). This finding suggests that sialic acid might play an important role in anchoring cells to basement membranes. Both laminin and fibronectin contain sialic acid; however, sialic acid residues from basal cell surface glycoproteins and/or glycolipids might also be involved.

The attachment of cells to the glomerular basement membrane is abolished in certain pathological conditions (as documented at the electron microscopic level): radiation nephritis (Madrazo et al, 1969), focal glomerulonephritis (Grishman and Chung, 1975), aminonucleoside nephrosis (Ryan and Karnovsky, 1975, Caulfield et al, 1976). However, there is no evidence that alternations of basement membrane components are responsible for these detachments.

B. Basement Membranes as Permeability Barriers

The basement membrane acts as a permeability barrier to macromolecules crossing it. One important parameter for this function is the geometry of the pores formed between its

constituents. As the molecular weight of a macromolecule increases, its ability to cross the basement membrane decreases (Renkin and Gilmore, 1973). In addition, the shape of the macromolecule influences this process: elongated macromolecules cross the basement membrane more easily than globular macromolecules of the same molecular weight (Rennke and Venkatachalam, 1977). Another important parameter for macromolecular permeability is the negative charge of the basement membrane. Sulfated glycosaminoglycans contribute a large number of highly anionic groups. These groups probably form a charge barrier that screens macromolecules and determines to some extent their ability to cross the basement membrane. Negatively charged macromolecules cross the basement membrane much less efficiently than the same macromolecules when positively charged (Chang et al, 1975; Rennke et al, 1978). In lupus nephritis, the anionic groups of the lamina rara externa of the glomerular basement membrane are drastically reduced in number (Kelley and Cavallo, 1980). The reduction of anionic sites might be a factor contributing to the observed proteinuria.

During intestinal absorption, lipoprotein particles cross the basement membrane underlying intestinal epithelial cells, and reach the lacteal of the villi (Tytgat, et al, 1971). The mechanism by which particles as big as 1μm in diameter cross this basement membrane is unknown.

Not only macromolecules and macromolecular complexes, but also cells cross the basement membrane, both under physiological and under pathological conditions. For neutrophils and mono-

cytes, it is essential for their function to cross the vascular wall and gain access to the interstitium. Malignant cells with the ability to metastasize are also able to cross the vascular wall. It has been shown that at the area of contact between a metastatic cell and a basement membrane, the basement membrane is locally absent (Chew et al, 1976). It has been suggested that cells invading the basement membrane have high levels of specific lytic enzymes. Both neutrophils and metastatic cells produce collagenase type IV, an enzyme which specificaly degrades collagen type IV (Liotta et al, 1980). Furthermore, the more metastatic a cell line is, the higher the levels of collagenase type IV produced (Liotta et al, 1980). Degradation of heparan sulfate in endothelial extracellular matrix occurs during invasion by metastatic cell in vitro (Kramer et al, 1980).

C. Basement Membranes as Generators of Non-thrombogenic Surfaces

Experimental evidence suggests that basement membranes form non-thrombogenic surfaces and that the type(s) of collagen present in them play an important role in this function.

When platelets come in contact with interstitium or collagens type I or III, they secrete serotonin and ADP, aggregate, change shape and finally form a thrombus. However, if platelets are incubated with collagen type IV or V, none of these changes is observed (Trelstad and Carvalho, 1979). If platelets are incubated with isolated glomerular basement membrane, they adhere to it but do not release ADP and do not aggregate (Huang et al, 1974). The adherence and spreading of

platelets might be a physiologically important response in situations where a disruption of the continuity of the endothelial layer occurs, without disruption of the integrity of the basement membrane.

D. Basement Membranes as Hosts of Functionally Significant Macromolecules

The presence of acetylcholinesterase as a component of the basement membrane of synaptic clefts in neuromuscular junctions (McMahan et al, 1978) indicates that this portion of the basement membrane carries on a physiologically important function, i.e., the degradation of a neurotransmitter. The fact that achetylcholinesterase is not present in exosynaptic basement membrane (Sanes and Hall, 1979) suggests that synaptic basement membrane is a highly differentiated structure.

Involvement of Basement Membranes in Pathological Situations

Basement membranes are affected in various pathological conditions. Changes can be classified as of two types: (a) thickening and (b) presence of immunoglobulins.

The most important situation where thickening of the basement membrane occurs is diabetes mellitus. Local thickening is observed, and is more pronounced in the glomerular basement membrane (Orci et al, 1970) and in certain small vessels where the subendothelial basement membrane is arranged in concentric layers (Vracko, 1970). An increase in microvascular permeability is observed (McMillan, 1975), the etiology of which is unknown. There is no accepted theory about the etiology of the thickening of the basement membrane. Thickening of the basement membrane has been observed at the electron

microscopic level in other pathological situations such as polymyositis (Gonzales-Angulo et al, 1968), and Alzheimer disease (Mancardi et al, 1980).

Presence of immunoglobulins in the glomerular basement membrane, is associated with a number of well studied nephropathies. Two pathophysiological mechanisms have been suggested in the accumulation of immunoglobulins in the glomerular basement membrane.

In the first mechanism, immunoglobulins are antibodies directed against elements of the glomerular basement membrane, as is the case in Goodpasture syndrome, subacute progressive or chronic glomerulonephritis and lipoid nephrosis (Jones, 1977). In the case of Goodpasture syndrome, it has been observed that circulating immunoglobulins have the ability to cross-react with the alveolar basement membrane (Botting et al, 1964).

In the second mechanism, circulating antigen-antibody complexes are deposited as such in the glomerular basement membrane. In this case, the antigen is not a component of the glomerular basement membrane. This is the case in lupus nephritis, poststreptococcal glomerulonephritis and membranous glomerulonephritis (Jones, 1977).

Aims of This Study

The overall aim of this study is the examination of various basement membranes, with emphasis on vascular basement membranes, to test for the presence of fixed anionic sites in them and to determine their characteristics. More specifically, we wanted:

1. To check for the presence of anionic sites in basement membrane of exchange vessels of both the continuous and the fenestrated type.

2. To examine the subendothelial anionic content of vascular beds that either totally lack or have an interrupted basement membrane.
3. To investigate the anionic content of non-vascular basement membranes, including epithelial basement membranes and basement membranes surrounding muscle cells, pericytes, fat cells and Schwann cells.
4. To compare certain characteristics (numerical density, extent of negativity) of anionic sites present in basement membranes of different types of capillaries (continuous versus fenestrated); possible differences in these characteristics may be of physiological significance.
5. In the specific case of continuous capillaries and skeletal muscle cells basement membranes, to determine whether heparan sulfate, a proteoglycan rich in anionic (sulfate) groups, is present.

Conclusions from This Study

The techniques used and the results obtained in this study are described and discussed in detail in the second and third part of this thesis. Our general conclusions are:

1. Anionic sites are present in every basement membrane examined, both vascular and non-vascular (epithelial, surrounding cells); therefore, they can be considered as a general characteristic of basement membranes.
2. Differences exist between the electrostatic properties of anionic sites in basement membranes of continuous and fenestrated capillaries, the sites in fenestrated capillaries being more strongly negatively charged.

3. The presence or absence and, when present, the differences in electrostatic properties of basement membrane anionic sites may be of physiological significance in determining the traffic of plasma proteins (mostly of low pIs) between various compartments, by (a) reducing their loss from the vascular lumen via fenestrae of fenestrated capillary beds and (b) once in the interstitium, channeling them into terminal lymphatics.

Future Trends

To uncover differences in the electrical charge between continuous and fenestrated capillaries basement membranes, we used the critical electrolyte concentration concept (Scott and Dorling, 1965). We compared only muscle capillaries and renal cortical capillaries. This comparison should be extended to other capillary beds. Of particular interest would be the examination of fenestrated capillaries exhibiting fenestrae only at one pole of their perimeter (the one facing epithelial cells) such as intestinal capillaries or capillaries of the choriocapillaris of the eye. Possible differences in the anionic sites between subfenestral basement membrane and the rest of the basement membrane would indicate that charge micro-environments exist in the basement membrane of the same capillary.

Heparan sulfate has been implied as the main contributor of anionic sites in certain basement membranes such as the glomerular basement membrane (Kanwar and Farquhar, 1979) and the alveolar basement membrane (Vaccaro and Brody, 1979). We do not know yet if this is the case for most types of vascular basement membranes, although heparan sulfate might be present in them. The clarification of this point awaits chemical purification and analysis of

these vascular basement membranes. Chemical analysis will also be a test of our hypothesis that continuous capillary basement membranes contain heparan sulfate with a low degree of sulfation.

Although studies using specific degradative enzymes have suggested that heparan sulfate might be the only contributor to anionic sites in the glomerular basement membrane (Kanwar and Farquhar, 1979b), this finding is not supported by findings from chemical analysis (Lemkin and Farquhar, 1981), indicating that small but detectable amounts of sulfated macromolecules other than heparan sulfate exist, e.g., entactin (Carlin et al, 1981) and chondroitin sulate (Lemkin and Farquhar, 1981). Studies using degradative enzymes for glycosaminoglycans have the drawback that the purity and the specificity of the enzymatic prepration is usually questionable (Morris Karnovsky, personal communication). In that regard, studies on the presence and distribution of chondroitin sulfate in basement membranes should follow the scheme: isolation of chondroitin sulfate from pure preparations of basement membranes (matrix of basement membrane-producing cultured cells should again be the source)--isolation of the protein core of this chondroitin sulfate--raising an antibody against the polypeptide--using this antibody in localization studies. The use of an antibody against chondroitin sulfate has been recently reported (Hayman et al, 1981) but information about this antibody is not yet available.

Provided that endothelial cells are the main source of capillary basement membrane, our studies suggest that capillary endothelial cells might be highly differentiated and might exhibit different biosynthetic capabilities. An attempt to culture capillary

endothelial cells from different types of vascular beds (continuous capillary-fenestrated capillary-terminal lymphatic) is fully justified. Of course, cultured endothelial cells might dedifferentiate in vitro. At present, cultured endothelial cells do not seem to form a basement membrane similar to the one seen beneath them in vivo (Kramer, personal communication). When these drawbacks are overcome, such a system would allow us to carefully analyze the differences between vascular basement membrane components from different sources and the turnover rates of each individual component, as well as isolating factors affecting their turnover rates.

Based on our data and on data from other studies, we speculate that the presence or absence, and when present, the differences in electrostatic properties of basement membrane anionic sites might be of physiological significance in determining the traffic of plasma proteins between various compartments. This hypothesis should be tested in experiments using both morphological and physiological approaches with the same tracer proteins chemically modified to exhibit either low or neutral isoelectric point (pI), and with all the other characteristics being exactly the same. In such experiments, isoelectric points higher than the physiological pH should be avoided, because the strong electrostatic attraction may substantially interfere and thus complicate the interpretation of the results.

Although our attention has been focused on basement membrane anionic sites, cell surface anionic sites should be considered. Heparan sulfate has been isolated from liver cell membranes. It differs both in the protein core and the number and length of

polysaccharide chains from the heparan sulfate isolated from basement membranes of EHS sarcoma cells (Oldberg et al, 1979).

Such diversity in the family of heparan sulfates, already mentioned (Linker and Hovingh, 1973), stresses the importance of further investigations of the role(s) played by heparan sulfate in various physiological processes. Fenestral diaphragms of fenestrated endothelial cells have been shown to contain heparan sulfate (Simionescu et al, 1981). Knowledge of the types of the heparan sulfate present in fenestral diaphragms might give us clues to the mode of formation of fenestral diaphragms; its similarity with cell surface heparan sulfate might suggest that certain components of diaphragms are contributed by cell surface macromolecules.

II. ANIONIC SITES IN BASEMENT MEMBRANES. DIFFERENCES IN THEIR ELECTROSTATIC PROPERTIES IN CONTINUOUS AND FENESTRATED CAPILLARIES

Abstract

We have used ruthenium red, a cationic tracer, to detect at the electron microscopic level the presence of anionic sites in various murine basement membranes, with emphasis on those of the microvasculature. We have observed anionic (ruthenium red staining) sites in all continuous and fenestrated capillaries examined. Terminal lymphatics (known to have a patchy basement membrane) had regionally anionic sites. Anionic sites were not present under the sinusoidal lining of the liver where a basement membrane is totally lacking. Basement membranes of epithelial cells and those surrounding striated and smooth muscle cells, pericytes, fat cells and Schwann cells also exhibited anionic sites. We have compared the electrostatic properties of two capillary basement membranes: those from continuous capillaries of the muscular portion of the diaphragm with those from renal cortical tubule fenestrated capillaries. The comparison was done by determining the salt concentration (critical electrolyte concentration, Scott and Dorling, 1965) required to displace ruthenium red from its binding sites. Our results suggest that the fenestrated capillary basement membrane is more strongly negatively charged than the continuous capillary basement membrane, because 0.5M Na^+ displaced ruthenium red to a large extent from the continuous capillary whereas 1.3 M Na^+ was required to displace ruthenium red from the fenestrated capillary. We conclude from this study that first, anionic sites are a general characteristic of

basement membranes and second, their properties differ in different vascular basement membranes. We speculate that the anionic content of basement membranes and its variability might be of physiological significance.

Introduction

The basement membrane is a specialized type of extracellular matrix. It has often been described as a trilaminar structure, exhibiting a central electron-dense layer, the lamina densa, sandwiched between two less electron dense layers which are (a) the lamina rara interna or lamina lucida between the cell surface and the lamina densa and (b) the lamina rara externa or lamina diffusa outside the lamina densa (Katsuyama, et al, 1977). The basement membrane is found underneath the basal surface of epithelial, mesothelial and endothelial cells and surrounds certain cell types: both smooth and striated muscle cells, pericytes, fat cells and Schwann cells. Thus, the basement membrane forms the boundary between these cells and the underlying or surrounding connective tissue (Vracko, 1974).

Anionic sites have been detected histochemically in selected basement membranes (Trelstad et al, 1974; Wight and Ross, 1975; Katsuyama et al, 1977; Vaccaro and Brody, 1979; Kanwar and Farquhar, 1979a, Ausprunk et al, 1981). In most of these studies, a cationic molecule (Alcian Blue, ruthenium red, cationic ferritin) has been used to detect anionic sites at the electron microscopic level. Although the physiological significance of the presence of negative charges is poorly understood, it has been suggested that they might play a role in determining the permeability properties of the

basement membranes (Kanwar et al, 1980).

Until now little information has been available about basement membranes of different types (continuous versus fenestrated) of capillaries. Difference in distribution of anionic sites is potentially important, because the endothelial barrier has different permeability properties in these two types of vascular beds (Bruns and Palade, 1968b; Clementi and Palade, 1969; Williams and Wissig, 1975). The anionic content of the basement membrane might play an important role in determining overall vascular permeability. In addition no systematic reports are available on adult epithelial basement membranes and none of these studies deals with basement membranes surrounding cells.

Furthermore, no effort has been made to compare the anionic properties of different basement membranes. For such a comparison, biochemical isolation and analysis is not an ideal approach because tissues contain more than one type of basement membrane and it is difficult to obtain each one in high purity and yield. However, such a comparison can be done in situ, using the critical electrolyte concentration (CEC) concept of Scott and Dorling (1965). This concept deals with the competition between a positively charged molecule and an inorganic cation for the anionic sites of a polyanionic macromolecule. According to the critical electrolyte concentration concept, the electrolyte concentration required to displace a positively charged molecule from a polyanion is characteristic for any particular polyanion and depends on several parameters, the most important being the chemical nature of the anionic groups and their number (see Appendix).

The aim of this study was twofold: first, to extend previous observations on anionic sites in basement membranes and second, to compare the anionic proerties of basement membranes from different types of capillaries. In order to extend previous observations we examined the anionic content of basement membranes from various types of exchange vessels, of subendothelial areas either totally or regionally lacking a basement membrane, and of non-vascular basement membranes associated with various cell types. In order to compare capillary basement membranes we applied the critical electrolyte concentration concept to a continuous (muscle) and a fenestrated (tubules of the renal cortex) capillary bed.

In our studies we used rutheniun red (Luft, 1971) as a cationic tracer and NaCl as the competing electrolyte.

Materials and Methods

Animals

Fifteen male adult (20-30 g) Swiss albino mice were used in this study. They were purchased from Simonsen (Simonsen, Gilroy, California) and were fed ad libitum. All specimens were taken from animals fixed by intravascular perfusion of solutions and fixatives as described below.

Perfusion Techniques and Solution

Animals were anesthetized with an intraperitoneal injection of sodium pentobarbital (85 mg per Kg of body weight) and the external jugular vein and abdominal aorta were exposed. The abdominal aorta was cannulated with a 23 gauge needle below the level of the renal

arteries. The perfusion was started after the jugular vein was cut for the outlet of the perfusate. The perfusion pressure was maintained at 80-100 mm Hg, which gave an average flow rate of 3-4 ml/min.

Two to four ml of Gey's balanced salt solution at room temperature, pH 7.4, was perfused first, to wash out blood. It contained 4% polyvinylpyrolidone (PVP, to mimic the osmotic effect of plasma proteins), 10 units per ml of heparin (as an anticoagulent) and 0.1% Na nitrate (as a vasodilator). This was followed by 30-40 ml of 2% glutaraldehyde-1% formaldehyde fixative in 0.1M Na cacodylate buffer at room temperature, containing 4% PVP, 7.3 mM KCl, 2 mM $CaCl_2$ and 0.2% ruthenium red (ruthenium red, Aldrich Chemical Company, Milwaukee, Wisconsin). The ruthenium red was added to the fixative just prior to use, the fixative filtered and its pH adjusted to 7.4. Two variations of the above described techniques were used. First, in a limited number of animals the second perfusing solution contained 1% acrolein instead of 1% formaldehyde. Second, in a limited number of animals, when we sought to create interstitial edema and thus separate closely apposed basement membranes, PVP was omitted from both perfusing solutions.

Tissue Processing

After the completion of the perfusion, specimens of diaphragm, heart, thyroid, intestine, liver and kidney cortex were removed for study.

The tissues were cut into small pieces and stored overnight in the same fixative at 4°C. The next day the specimens were washed

for 30 min with 0.15M Na cacodylate buffer, pH 7.4, containing 0.1% ruthenium red. They were then postfixed for 90 min in 1% OsO_4 (D.F. Goldsmith Chemical and Metal Company, Evanston, Illinois) in 0.1M Na cacodylate buffer, pH 7.4, containing 0.05% ruthenium red. The concentration of ruthenium red in the fixative, the washing solution and the OsO_4 were those recommended by Luft (1971). Specimens were then dehydrated for 30 min in cold acetone and propylene oxide. They were slowly infiltrated with EPON and embedded. Sections 60-80 nm in thickness, were stained with 5% aqueous uranyl acetate for 30-40 min and/or 0.6-0.8% lead citrate for 15 min, examined and photographed in a Siemens I electron microscope.

In control preparations, the aldehyde fixative, the washing solution and the OsO_4 solution did not contain ruthenium red.

Critical Electrolyte Concentration Experiments

For determination of critical electrolyte concentrations which abolished ruthenium red binding, the diaphragm and left kidney of animals perfused as described above were excised and chopped at a thickness of 100μm in a Sorvall tissue chopper (Sorval, Inc., Newtown, Connecticut). Groups of chopped slices were incubated overnight at 4°C in the glutaraldehyde-formaldehyde fixative solution without PVP, at pH 5.8, which contained in addition NaCl at concentrations from 0 to 1.8M in 0.1M or 0.2M steps. The sections were then placed in 1% OsO_4 at pH 5.8 buffered with 0.1M Na cacodylate and containing 0.05% ruthenium red and the appropriate concentration of NaCl. The pH of 5.8 was selected for these incubations in order to compare our results with those of Scott and Dorling. They point out that maximum specificity of the reaction occurs at pH 5.8. After osmication, the

sections were dehydrated, embedded and examined as described previously.

In order to explore the possibility that high salt treatment non-specifically extracts the molecules bearing the anionic sites, sections incubated overnight with the highest NaCl concentration (1.8M) were incubated for an additional 24 hrs in the same fixative, pH 5.8, containing 1% ruthenium red and no NaCl and processed as described above with no NaCl in the OsO_4 solution (restaining experiments).

Quantitation of Ruthenium Red Staining Sites

Sections 60 nm thick were stained with uranyl acetate and lead citrate solutions as described and photographed at 10,000X magnification in the electron microscope. Capillaries and small venules were photographed randomly. Only cross sections of endothelial cells were used in our measurements. Ruthenium red staining sites in vascular basement membranes were counted; the length of the basement membranes from which the counts of sites were made was measured by a map reader and the number of ruthenium red staining sites per unit length of basement membrane was calculated.

Results

With the exception of the renal glomerular and tubular basement membranes, distinction of the three layers of the basement membrane is not always easy or possible. In some instances, we are able to see the electron dense layer, the lamina densa, but the laminae rarae are not seen as clearly defined layers in our preparations. Therefore, we are using the terms "cellular surface" or "interstitial surface" of the lamina densa which are topographically equivalent to

the lamina rara interna and the lamina rara externa of previous descriptions. In many instances the basement membrane has the same electron density over its entire thickness and its limits with respect to the underlying interstitium are poorly visualized. In these instances the limits of the structures that we see are only indirectly suggested by the distances of these structures from the cell surface. In these instances we use the terms "cellular surface" or "interstitial surface" of the basement membrane to describe localization.

Control Versus Experimental Preparations--General Remarks

In control specimens, basement membranes exhibit a fine filamentous texture, as shown in Figure 1. This figure shows a basement membrane underlying an endothelial cell of a small venule from the diaphragm. When we fix tissue specimens in aldehyde solutions and subsequently in OsO_4 solutions both of which contain ruthenium red, punctate densities are observed in the basement membranes. These densities have a diameter of 10-20 nm and are arranged in a quasi-periodic way (Figs. 2-8). Because similar densities are not observed in our control preparations, we think that the term "ruthenium red staining sites" (RRss) is justified.

Continuous Capillary Basement Membranes

We examined the basement membrane of continuous capillaries in diaphragm (Fig. 2) and heart (Fig. 3). Ruthenium red staining sites are present and exhibit the characteristics described above. Figure 2 is from an animal perfused without PVP in the solutions, in order to create interstitial edema. Basement membranes of muscle and endothelium are widely separated. The staining sites are localized

at the interstitial surface of each basement membrane. In Figure 3, in areas where the endothelial and the cardiac muscle basement membrane appear locally fused, the sites are located equidistant from the two cell surfaces.

Fenestrated Capillary Basement Membranes

We examined the basement membrane of fenestrated capillaries in the intestine (Fig. 4), thyroid (Figs. 5, 6) and renal cortical tubules (Fig. 7). Ruthenium red staining sites are present in all of them, with diameters similar to those seen in basement membranes of continuous capillaries; however, size variation of staining sites is observed (Figs. 4, 5). In the intestinal and thyroid capillaries sites are localized predominantly at the interstitial surface of their basement membrane. Occasionally, sites are observed also at other levels across the thickness of the basement membrane (Figs. 4, 5). In renal cortical capillaries, the vascular basement membrane and the epithelial basement membrane are frequently fused. In these cases, sites are found subendothelially and subepithelially (Fig. 7). In places where the two basement membranes are not fused, ruthenium red staining sites can be seen in two rows mainly at the cellular surface but also at the interstitial surface of each separate basement membrane (Fig. 8).

Subendothelial Areas Lacking Basement Membranes

We examined subendothelial areas of liver sinusoidal lining cells and terminal lymphatics of the intestine. It is generally well recognized that hepatic sinusoids in the mouse lack a basement membrane, and we did not observe a row of staining sites immediately beneath the endothelium, running parallel with its adluminal surface

(Fig. 9). However, electron dense particles closely associated mainly with the surface of hepatic microvilli but also with a sparse fibrillar matrix-like material, are observed in the space of Disse (Fig. 9). Terminal lymphatics are known to have a discontinuous basement membrane (Casley-Smith, 1968). In the lacteals, we observed a discontinuous row of ruthenium red staining sites beneath the lymphatic endothelium (Fig. 10). The discontinuities in the row of staining sites appeared to be associated with gaps in the basement membrane.

Non-vascular Basement Membranes

We examined epithelial basement membranes from intestinal epithelium (Fig. 4) follicular epithelium of the thyroid (Fig. 6) and renal tubular epithelium (Figs. 7 and 8). All contain ruthenium red staining sites. In the intestinal and follicular epithelium of the thyroid, the sites are localized at every level across the thickness of the basement membrane, but their main localization is the interstitial suface and to a lesser extent the cellular surface of the basement membrane (Figs. 4, 6). In the renal tubular epithelium, sites are found in two rows at the cellular and interstitial surface of the lamina densa. This is the case for tubular epithelium of proximal tubules (Fig. 8), distal tubules, and collecting tubules (Fig. 11).

We also examined basement membranes that totally surround cells. Ruthenium red staining sites are detected in the basement membrane surrounding striated muscle cells, both skeletal (Fig. 2) and cardiac (Fig. 3), smooth muscle cells (Figs. 10, 12) pericytes (Figs. 2, 5), fat cells (Fig. 5) and Schwann cells (Fig. 13).

Comparison of Anionic Properties of Continuous and Fenestrated Capillary Basement Membrane

We compared the basement membrane of continuous capillaries from the diaphragm, with the basement membrane of fenestrated capillaries from the renal cortical tubules. We examined two parameters of the staining sites: their numerical density and the electrolyte concentration required for abolition of their staining with ruthenium red (CEC). Both parameters were determined in experiments using a pH of 5.8 (see Materials and Methods). The results of our calculations are presented in Table I for the continuous capillaries and Table II for the fenestrated capillaries.

The numerical density (number of sites per unit length of basement membrane measured) is of the same order of magnitude in the two types of capillaries examined (compare first line of the two tables, 0.1M Na^+ concentration).

In the continuous capillaries, a Na^+ concentration from 0.1M (Na^+ provided only from the buffer) to 0.4M (0.3M Na^+ added) did not alter significantly the numerical density of the sites. However, at a Na^+ concentration of 0.5M and higher, the numerical density of the sites decreased dramatically as shown in Table I and Fig. 14.

In the fenestrated capillaries, a Na^+ concentration from 0.1M to 0.9M did not alter significantly the numerical density of the sites, as shown in Table II. Concentrations of Na^+ from 1.0M to 1.2M slightly reduced the numerical density of the sites (not shown in Table II). A Na^+ concentration of 1.3M was required to dramatically decrease their numerical density, as shown in Table II and Fig. 15.

Tissues incubated with 1.8M NaCl did not exhibit any ruthenium red staining sites in basement membranes. However, if high salt treatment was followed by incubation in media containing a final Na^+ concentration of 0.1M, (see Materials and Methods), ruthenium red staining sites were again present (Fig. 16).

Discussion

The first aim of this study was to localize anionic sites in basement membranes. The emphasis was put on vascular basement membranes of small vessels because of their important role in the exchange of substances between the circulation and various tissues.

For this purpose, we selected ruthenium red as a cationic tracer for anionic sites because it has relatively high charge density (6 positive charges per molecule of molecular weight $\tilde{}$ 800) and it is relatively small (molecular diameter 1.13nm [Luft, 1971]). Ruthenium red staining sites were present in all basement membranes examined and absent from those subendothelial areas which lack a basement membrane.

The second aim of this study was to compare the anionic properties of endothelial basement membranes from continuous and fenestrated capillaries.

For this purpose, we used increasingly high salt concentrations to compete for ruthenium red binding at anionic sites. This was done in situ, on previously fixed tissue, in order to minimize possible extraction of macromolecules by high salt concentration. The finding that staining sites reappear after staining in solutions of low salt concentration following high salt treatment, indicates that little or no extraction of the sites has occurred. This

observation also confirms the ionic nature of the interaction between ruthenium red and the sites it stains. We observed that although the numerical density of the sites is comparable in the two types of capillaries examined, the two basement membranes exhibited drastically different anionic properties. Basement membranes of fenestrated capillaries required a much higher Na^+ concentration (1.3M) to be destained, compared to those of continuous capillaries (0.5M). According to the critical electrolyte concentration concept (see Appendix), this difference indicates that the basement membrane of fenestrated capillaries is much more strongly negatively charged compared to the basement membrane of continuous capillaries.

Chemical Nature of Ruthenium Red Staining Sites in Vascular Basement Membranes

There is strong evidence that the main contributor to ruthenium red staining sites, at least in the case of the glomerular basement membrane, is the glycosaminoglycan heparan sulfate. Kanwar and Farquhar (1979b) have shown that anionic sites in the rat glomerular basement membrane are removed by heparitinase and heparinase, enzymes which degrade heparan sulfate, but that these anionic stes are unaffected by other enzymes, such as hyaluronidases, chondroitinase ABC, and neuraminidase, which do not attack heparan sulfate. Furthermore, they purified glomerular basement membranes and determine chemically that the major glycosaminoglycan present was heparan sulfate (Kanwar and Farquhar, 1979c). Evidence from a tumor cell line producing basement membrane-like matrix* suggests that basement

*Engelbreth-Holm-Swarm (EHS) sarcoma cell (Orkin et al, 1977)

membrane heparan sulfate is a proteoglycan of molecular weight ~ 750,000 daltons and contains equal amounts of polypeptide and carbohydrate. With an antibody against this core protein (kindly provided to us by Dr. George Martin of NIH) we have demonstrated at the electron microscopic level the presence of this proteoglycan in the basement membrane of capllary endothelial cells and skeletal muscle cells of the mouse diaphragm (Charonis et al, 1981). Hassel et

al (1980) have detected by fluorescence microscopy the presence of this proteoglycan in various other basement membranes. Other sulfated glycosaminoglycans and sulfated glycoproteins (Lemkin and Farquhar, 1981; Carlin et al, 1981) may be components of basement membranes and may contribute to the formation of ruthenium red staining sites. However, studies using degradative enzymes in the lung (Vaccaro and Brody, 1979) and in the limbus of the eye (Ausprunk et al, 1981) suggest that heparan sulfate appears to be largely accountable for ruthenium red staining sites.

Chemical Basis for the Differences Observed Between Continuous and Fenestrated Capillaries Basement Membranes

In the absence of detailed chemical analysis of various vascular basement membranes, some insight about their anionic groups can be gained by using the concept of critical electrolyte concentration (Scott and Dorling, 1965). In biological systems, three types of anionic groups are by far the most commonly found: carboxyl, phosphate and sulfate. Scott and Dorling have obtained families of critical electrolyte concentrations, characteristic for each of these three groups. They used known substances on filter paper or

histological preparations and found that they could displace the cationic dye Alcian Blue (with NaCl) from carboxyl groups at a concentration 0.13-0.475M NaCl, from phosphate groups at 0.4-0.5M NaCl and from sulfate groups at 1.0-2.5M NaCl. Even in macromolecules having chemically homogenous anionic groups, not a single value but a family of values of salt concentration has been obtained. Other parameters such as the number and geometry of distribution of anionic groups affect the salt concentration required for successful competition. Furthermore, some macromolecules, including most glysaminoglycans, have a mixture of different types of anionic groups, a factor further complicating the situation. In conclusion, the results from salt competition experiments can give us qualitative answers and clues about the chemical nature of anionic sites of a macromolecule but not precise quantiative data, as would a chemical analysis.

In the basement membrane of fenestrated capillaries of the renal cortex a critical Na^+ concentration of 1.3M was required to displace ruthenium red from its staining sites. A comparison of this value with the values obtained by Scott and Dorling suggests that the anionic sites of this basement membrane are predominantly sulfate in nature. This corroborates the recent report that renal cortex predominantly synthesizes heparan sulfate (Lemkin and Farquhar, 1981).

In the diaphragm, a critical Na^+ concentration of 0.5M was required to displace ruthenium red from capillary basement membrane. This value suggests that the anionic sites of this basement membrane are predominantly carboxyl or phosphate groups (Scott and Dorling,

1965). Basement membrane components containing phosphate groups have not been detected so far, but macromolecules isolated from other basement membranes such as laminin, hyaluronate and sulfated glycosaminoglycans are rich in carboxyl groups (Timpl et al, 1979; Lindahl and Hook, 1978). No chemical analysis of basement membranes of continuous capillaries has been reported so far. However, we have detected heparan sulfate in the basement membrane of continuous capillaries of the diaphragm by immunoelectron microscopy, using the antibody previously described (Charonis, et al, 1981). This finding, however, does not exclude the possibility that hyaluronate or other sulfated glycosaminoglycans might be also present in basement membrane.

It is possible that the heparan sulfates in basement membranes of continuous and fenestrated capillaries may differ from each other chemically. Unlike polypeptides, glycosaminoglycans have a considerable structural heterogeneity; among glycosaminoglycans, heparan sulfate appears to be the most heterogeneous of all (Linker and Hovingh, 1973). The isolation by Linker and Hovingh (1973) of four different heparan sulfate fractions from beef lung supports this possibility.

Physiological Implications

The results of this and other studies suggest that basement membranes form a layer of strongly anionic sites between the interstitium and other compartments (cellular, vascular). In morphological studies using ruthenium red, anionic sites appear as electron dense granules, with a diameter of 10-20 nm and exhibit a quasi-periodic pattern with a center-to-center spacing of $\tilde{}$ 60 nm.

Are the negative charges clustered in vivo or is this appearance an artifact of processing the tissue? That the clustering might be an artifact should be seriously considered, because it is known that proteoglycans exist in vivo in a hydrated state (Comper and Laurent, 1978). Dehydration, as used in processing the tissue, may make them to shrink. No matter what the distribution of anionic sites is in vivo, they are encountered by any (macro)molecule moving across a basement membrane. This is illustrated in the diagram of Figure 17, modified after Vracko (1974). The basement membrane of a fenestrated capillary is shown with higher numbers of negative sites (-) than the basement membrane of a continuous capillary, to illustrate our finding that it is more strongly negatively charged. A terminal lymphtic is shown with sparse negative sites, because the basement membrane is regionally absent.

Does the negative charge of the basement membrane influence its physiological functions? This seems to be the case in the kidney. The charge of a macromolecule is a factor determining its permeability through the glomerular basement membrane, as demonstrated by Chang et al (1975) and Rennke et al (1978). Their findings may suggest that anionic sites of the glomerular basement membrane interact with charged macromolecules; this interaction might determine to some extent the ease with which macromolecules can cross this basement membrane, by resulting in rejection of similarly charged ones and in facilitation of the passage of oppositely charged ones. Treatment of the glomerular basement membrane with heparinase to remove anionic sites resulted in a pronounced increase in the permeability of this basement membrane to ferritin (Kanwar et al,

1980). Nevertheless, this result is not a clear demonstration of the participation of anionic sites in the permeability properties of the glomerular basement membrane, because enzymatic treatment and degradation of heparan sulfate may simply have increased the porosity of the basement membrane.

By extrapolation from these findings, we speculate that anionic sites of vascular basement membranes play an important physiological role by affecting the traffic of macromolecules across them, depending on the net electrical charge of each macromolecule. More negatively charged macromolecules present in the vascular lumen might therefore have more difficult access to the interstitium than more positively charged ones. This sieving effect may be enhanced by the fact that most plasma proteins are negatively charged at physiologic pH. For macromolecules in the interstitium, anionic sites of the vascular basement membrane might similarly affect their back transport to the vascular lumen (Johansson, 1978). However, electrostatic screening can be avoided in terminal lymphatics, where anionic sites are absent from some areas. The regional absence of anionic sites might be of physiological significance in determining the composition of the lymph.

Ultrastructural studies have revealed that the endothelium of fenestrated capillaries is more permeable to protein tracers than is the endothelium of continuous capillaries presumably owing to the presence of the fenestrae (Bruns and Palade, 1968; Clementi and Palade, 1969; Williams and Wissig, 1975). After having traversed the fenestrae, anionic plasma proteins encounter a porous barrier with a net negative charge which might repel them. Whether this

repulsion actually occurs is unknown. Renkin (1977) however has pointed out that capillaries of the dog's intestine are no more permeable to albumin that capillaries of the dog's paw. He suggested that the basement membrane, rather than the endothelium, may be responsible for limiting diffusion of this plasma protein. Fenestrated capillaries therefore may permit freer exchange of solutes between the plasma and the interstitium while restraining plasma proteins somewhat.

TABLE I

NaCl Competition for Ruthenium Red Bound to Basement Membranes of Continuous Capillaries (Muscle of Diaphragm)

Final Na^+ Concentrations*	Ruthenium red staining sites in the vascular basement membrane	Length of vascular basement membrane counted	Ruthenium Red staining sites per unit length of vascular basement membrane	Number of animals used
(Moles	(n)	(μm)	(n/μm)	(n')
0.1	1605	204	7.86	3
0.2	481	66	7.40	2
0.3	770	133	5.78	3
0.4	409	68	6.01	2
0.5	122	175	0.69	3

*Solution prior to addition of NaCl contains 0.1M Na cacodylate buffer pH 5.8

TABLE II

NaCl Competition for Ruthenium Red Bound
Bound to Basement Membranes of Fenestrated
Capillaries (Renal Cortical Tubules)

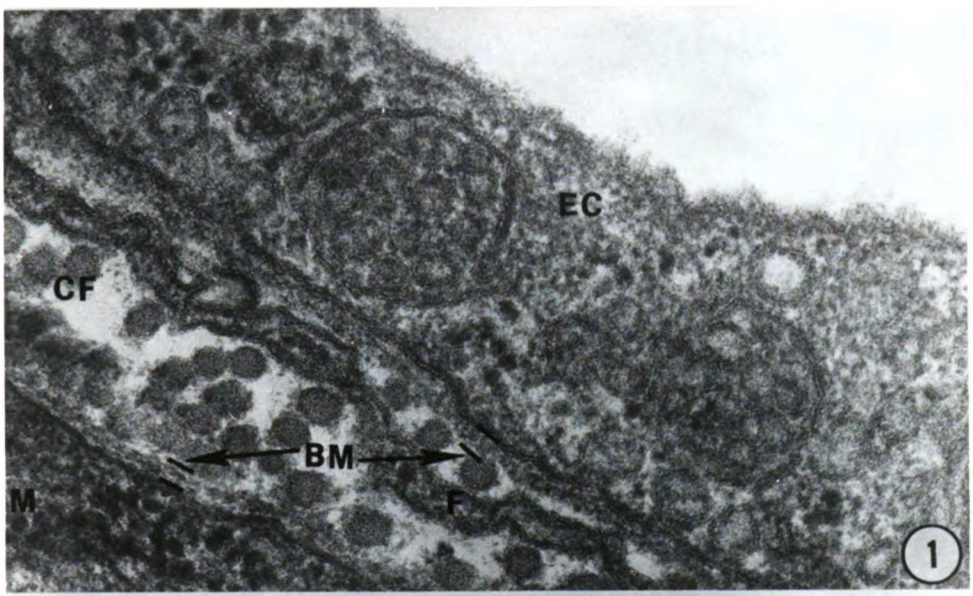
Final Na^+ Concentration*	Ruthenium red staining sites in the vascular basement membrane	Length of vascular basement membrane counted	Ruthenium red staining sites per unit length of vascular basement membrane	Number of animals used
(moles)	(n)	(μm)	(N/μm)	(n')
0.1	622	105	5.92	3
0.5	272	47	5.78	2
0.9	525	94	5.58	3
1.3	24	148	0.16	3

*Solution prior to addition of NaCl contains 0.1M Na cacodylate buffer pH 5.8

Figure Legends

Fig. 1 The basement membranes (BM) of a muscle cell (M) and an endothelial cell (EC) from a venule of the diaphragm have fibrillar texture. Between them, a fibroblast (F) and collagen fibers (CF) are seen. Ruthenium red was absent from all solutions. X98,000.

Fig. 2 The basement membranes of an endothelial cell (EC), a pericyte (P) and a muscle cell (M) from diaphragm exhibits ruthenium red staining sites (arrows). The animal was perfused without PVP. X92,000


EC
CF
BM
M
F
1

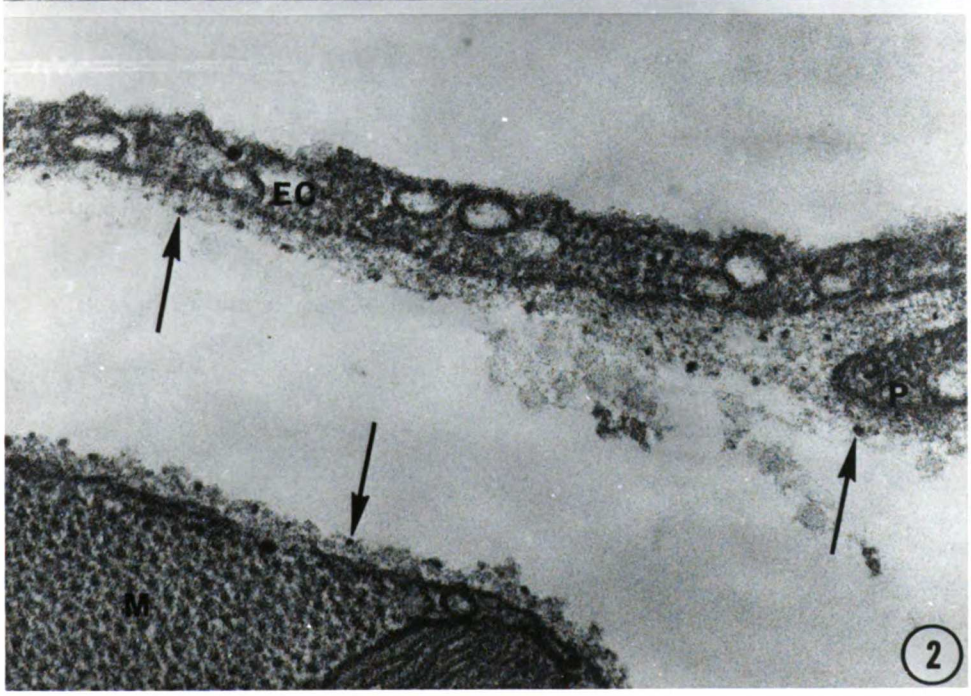
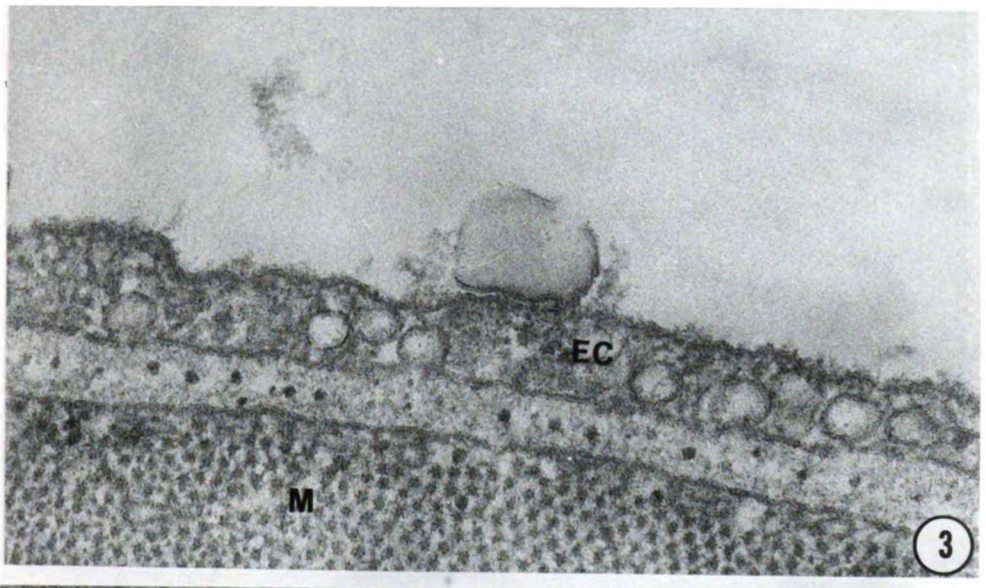

EC
P
M
2

Fig. 3 Capillary from cardiac muscle. In areas of fusion of the endothelial cell (EC) and the muscle cell (M) basement memrane, only one row of ruthenium red staining sites is observed. X90,000.

Fig. 4 Fenestrated intestinal capillary. Ruthenium red staining sites are seen at the interstitial surface of the endothelial (EC) basement membrane and in both the interstitial and the cellular surface of the basement membrane of an intestinal lining cell (ILC). Interstitial elements also bind ruthenium red. X48,000


EC
M
3

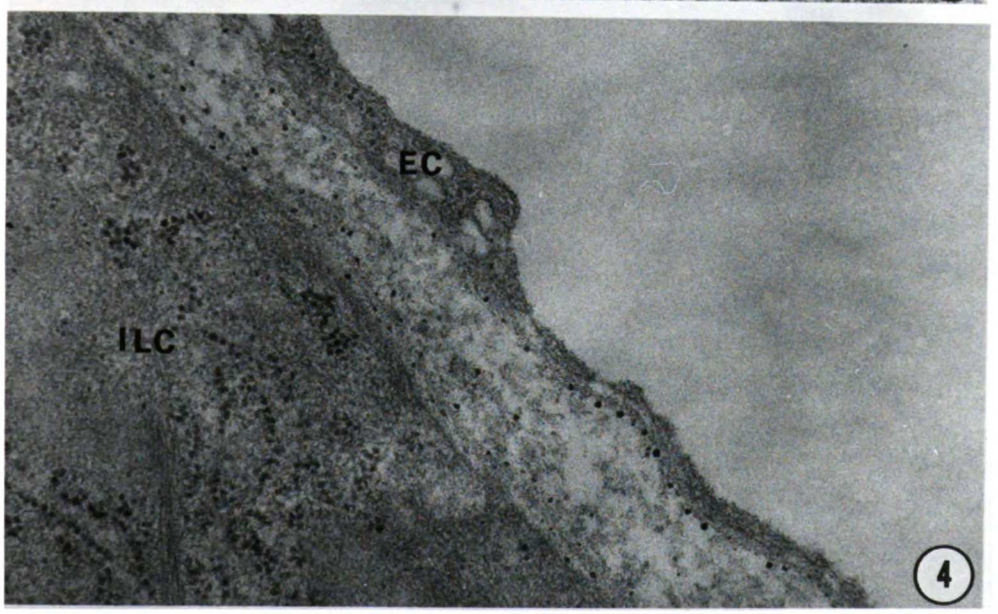
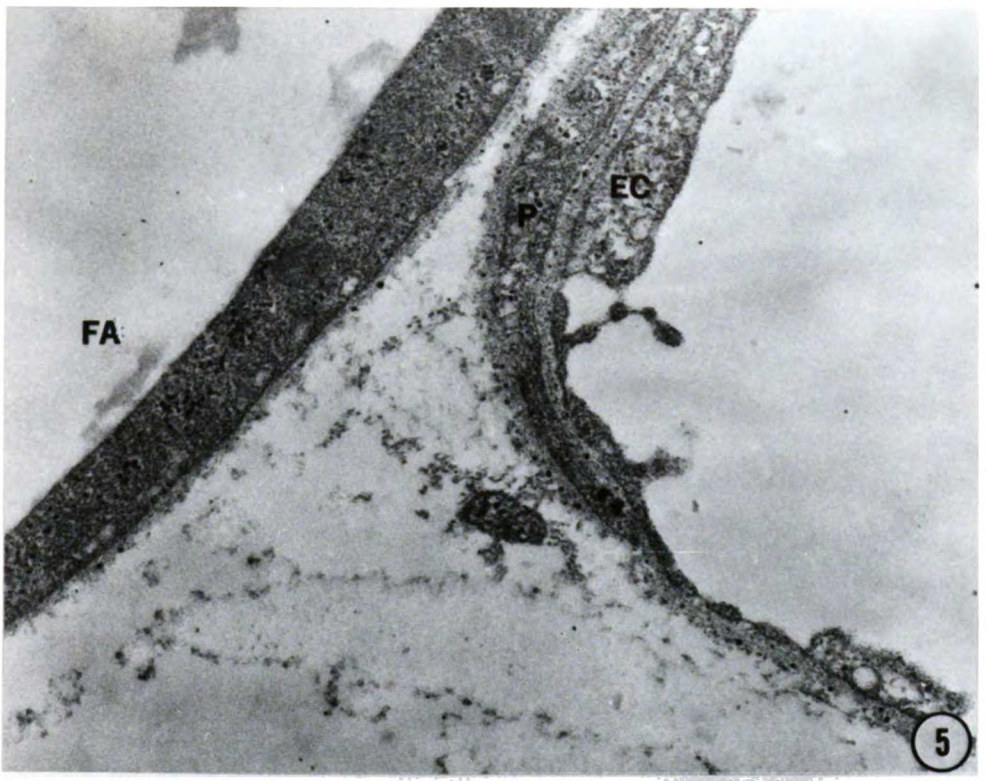

EC
ILC
4

Fig. 5 Endothelial cell (EC) and pericyte (P) from a fenestrated capillary of thyroid. Ruthenium red staining sites are seen in the endothelial and pericytic basement membranes and in smaller number in the basement membrane surrounding a fat cell (FA). X34,000

Fig. 6 Endothelial cell (EC) of a fenestrated capillary and follicular cell (FC) from thyroid. Ruthenium red staining sites are present in both basement membranes. X71,000


FA
P
EC
5

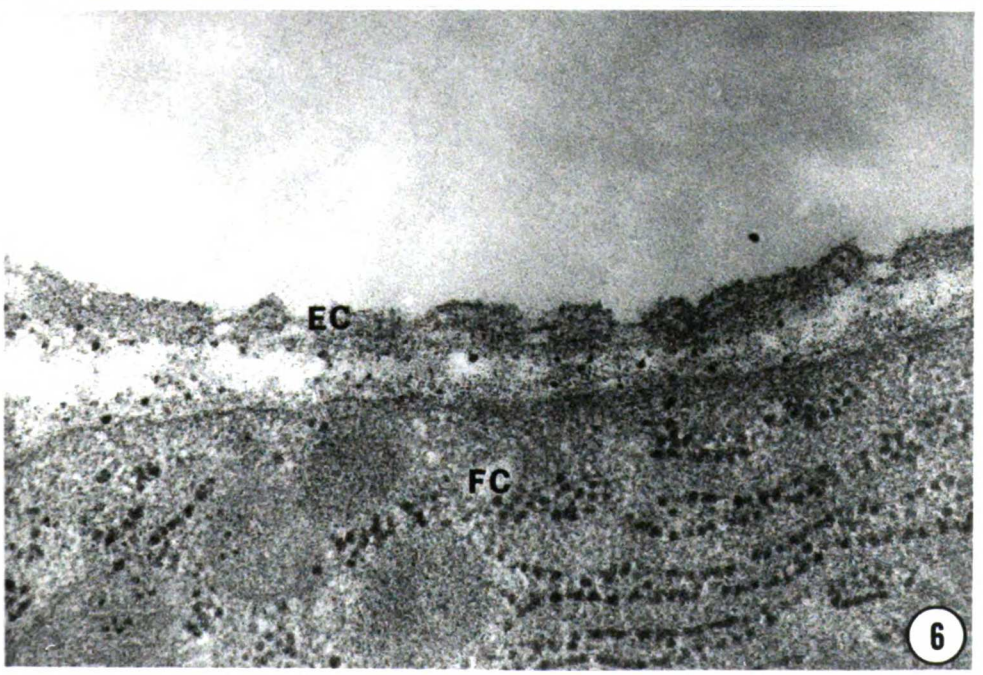
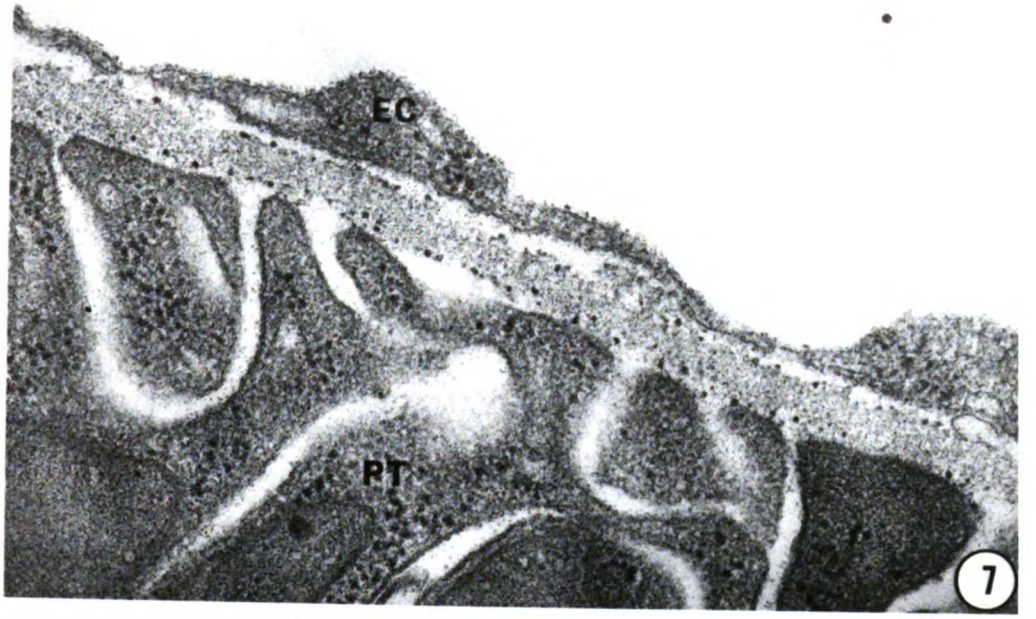
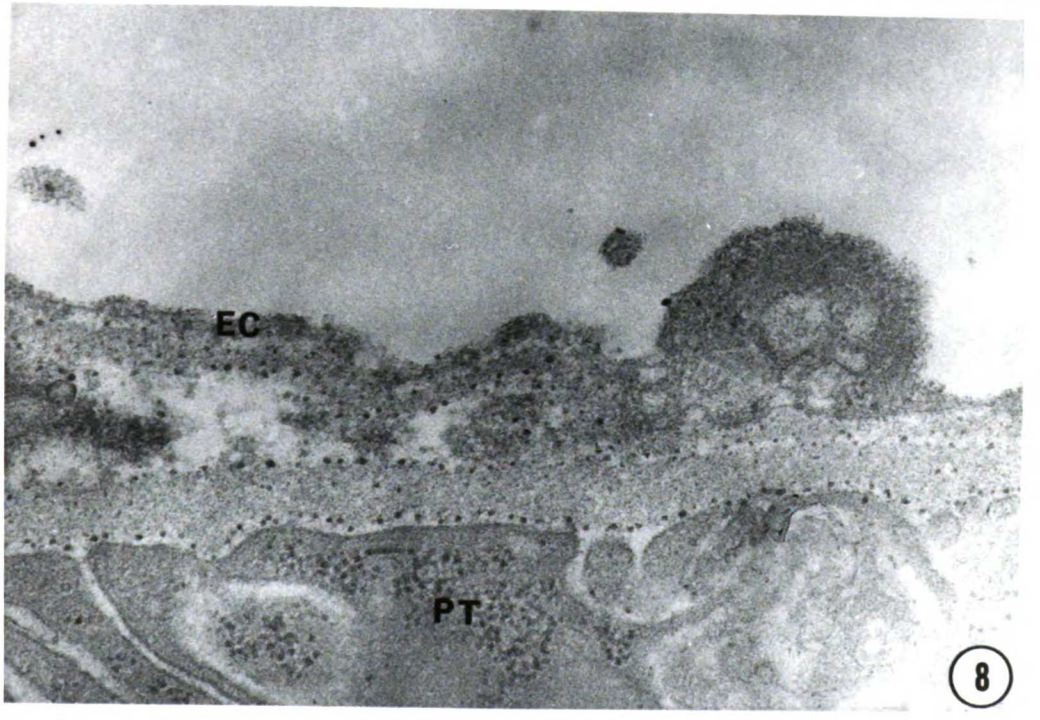

EC
FC
6

Fig. 7 Renal proximal tubules. The basement membranes of a fenestrated endothelial cell (EC) and a proximal tubule epithelial cell (PT) are fused. Ruthenium red staining sites are seen both subendothelially and subepithelially along the edges of the common lamina densa. X66,000

Fig. 8 Fenal proximal tubules. At the left part of the figure, the basement membranes of a fenestrated endothelial cell (EC) and a proximal tubule epithelial cell (PT) are separated. Each basement membrane has two rows of ruthenium red staining sites, one along its cellular and one along its interstitial surface. X50,000



7



8

Fig. 9 In the liver, no staining sites are observed underneath the endothelial cells (EC) forming the sinusoidal lining. However, electron densities are seen, mainly in close association with microvilli of hepatic cells (arrow). X43,000

Fig. 10 Intestinal lacteal. The arrow at the basal surface of the lymphatic endothelial cell (EC) indicates the transition from a subendothelial area with practially no sites (on the left of the arrow) to a subendothelial area with ruthenium red staining sites (on the right of the arrow). At the lower edge of the figure, a smooth muscle cell (M) exhibits ruthenium red staining sites in its basement membrane. X45,000


EC
9

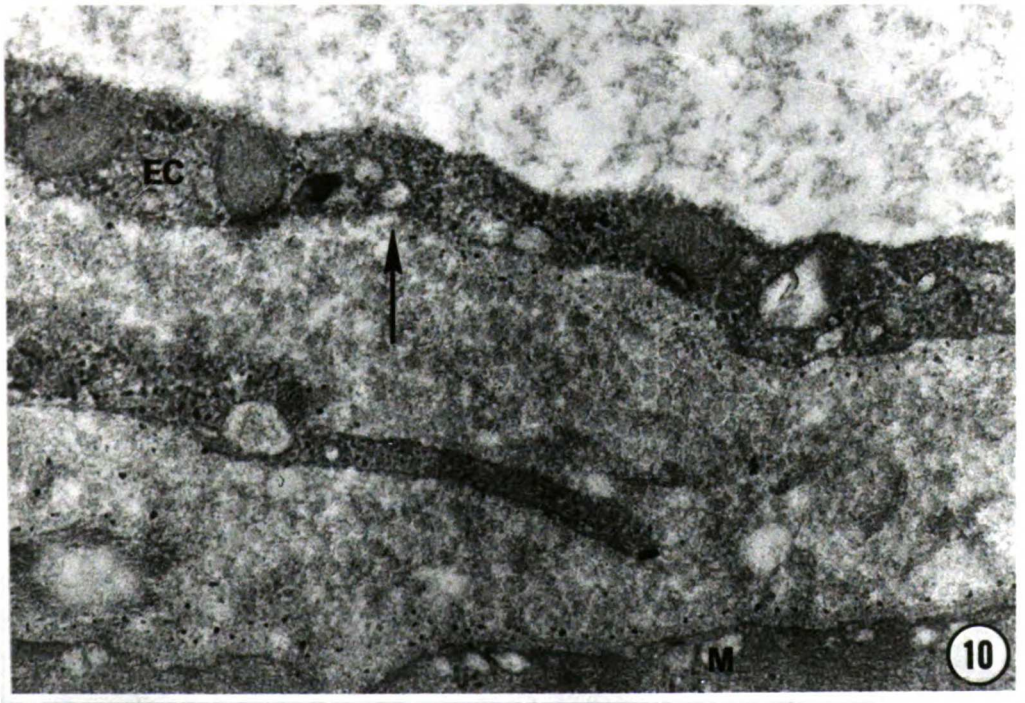
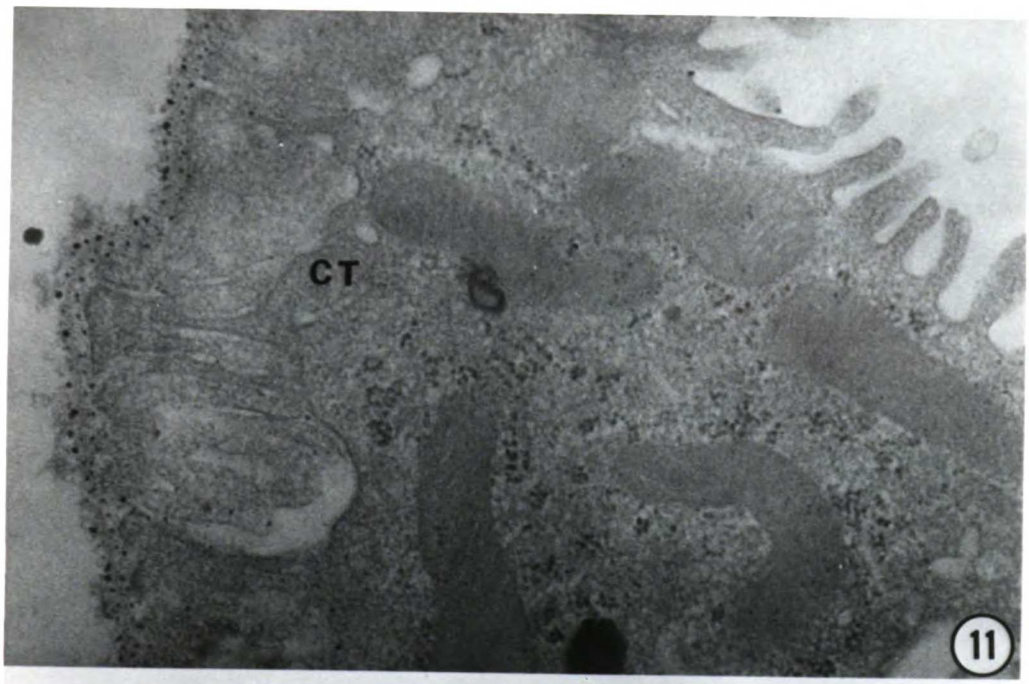

EC
M
10

Fig. 11 Collecting duct epithelial cell (CT) from the renal cortex. The basement membrane exhibits two rows of ruthenium red staining sites, one at the cellular and one at the interstitial surface of its lamina densa. X57000

Fig. 12 Smooth muscle cell (M) and endothelial cell (EC) from an arteriole. Ruthenium red staining sites are present in the basement membrane of both cells. EL: elastic tissue. X55,000


CT
11

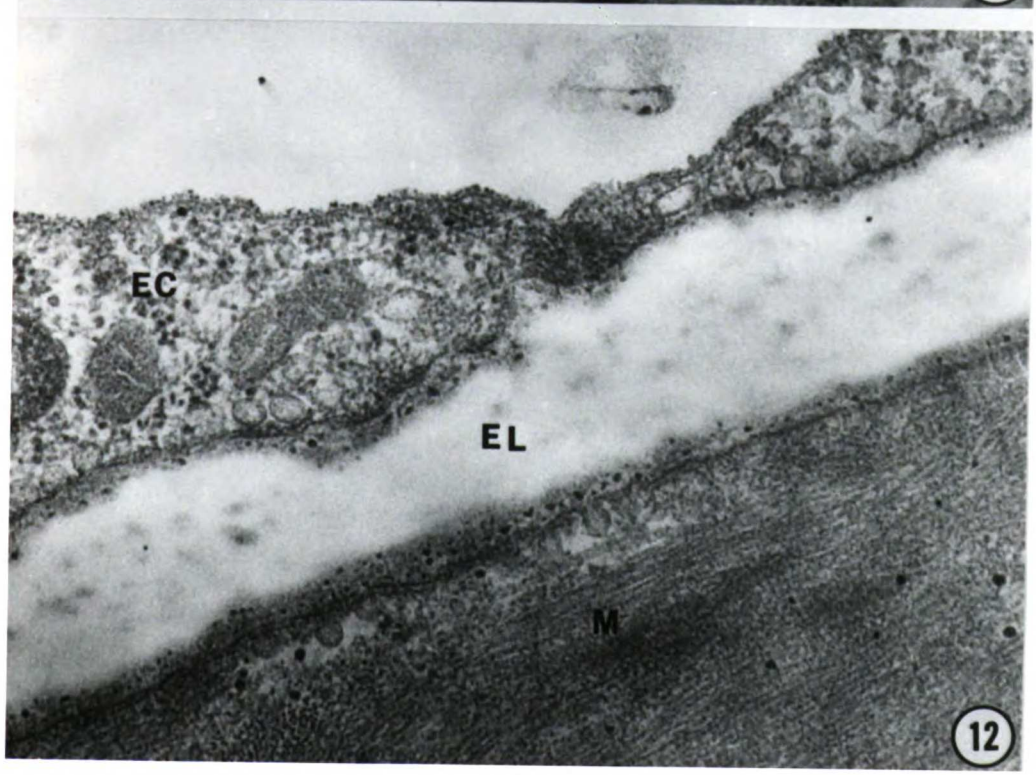
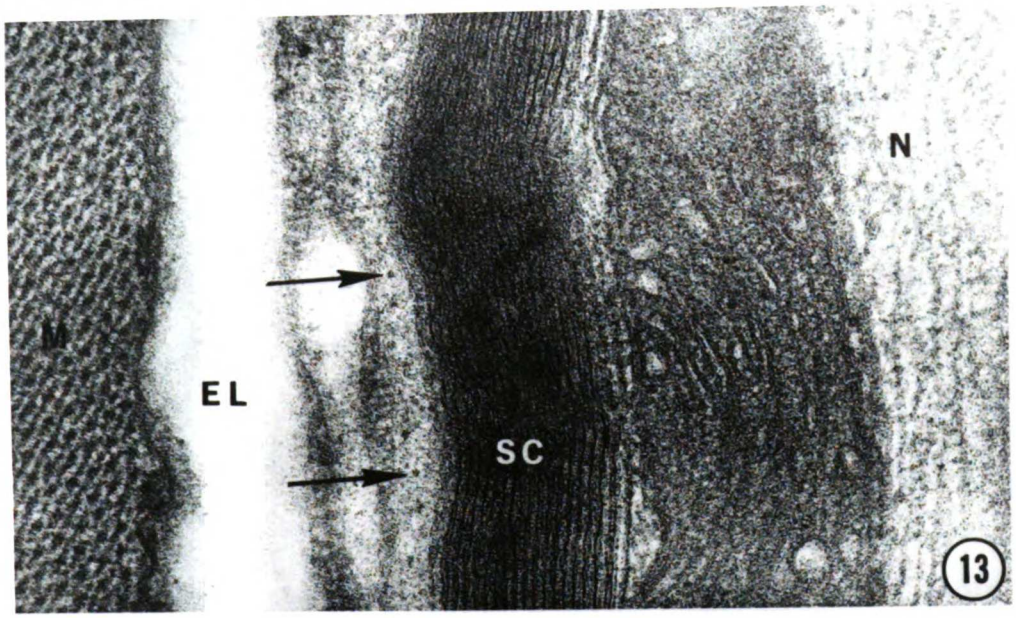

EC
EL
M
12

Fig. 13 Small nerve from diaphragm. The basement membrane around a Schwann cell (SC) has ruthenium red staining sites (arrows). Faint staining of these sites and absence of staining sites in the basement membrane of a muscle cell (M) are probably due to poor penetration. A mitochondrion is present in the nerve axon. EL: elastic tissue. X83,000

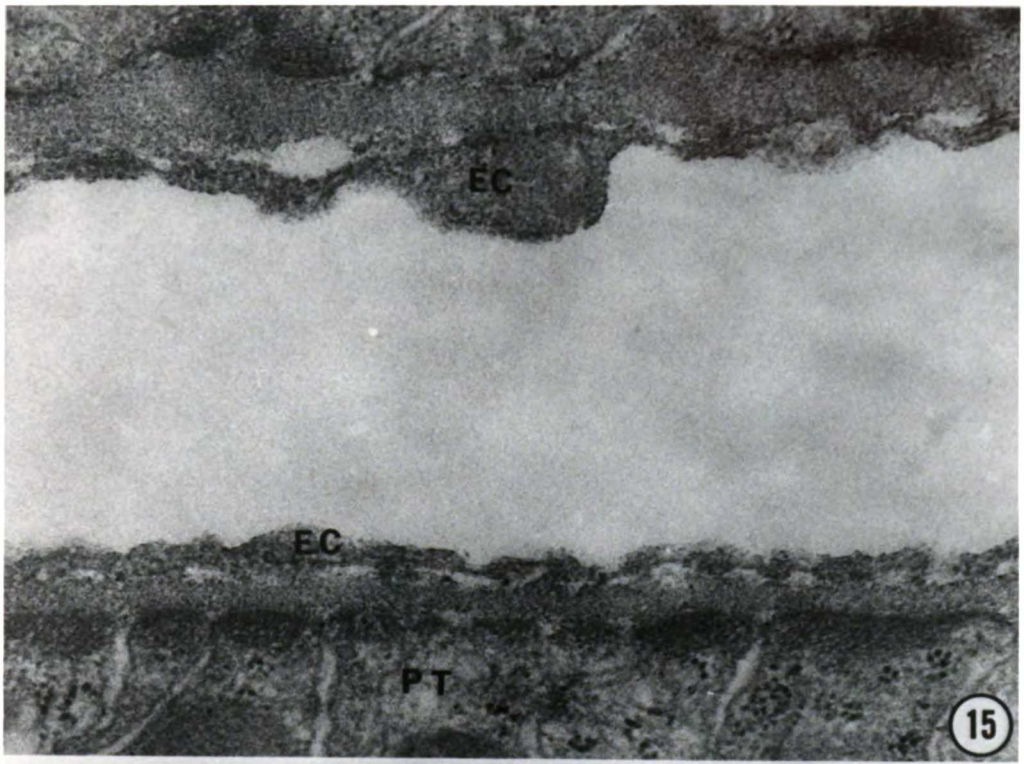
Fig. 14 Continuous capillary from diaphragm. At a total Na^+ concentraton 0.5M, ruthenium red staining sites have largely been abolished. EC: endothelial cell, M: striated muscle cell. X48,000


M
EL
SC
N
13


EC
M
14

Fig. 15 Fenestrated capillary from renal cortex. At total Na^+ concentration 1.3M, ruthenium red staining sites have been largely abolished. EC: endothelial cell(s), PT: epithelial cells from proximal tubule. X48,000

Fig. 16 Fenestrated capillary from renal cortex when Na^+ concentration is reduced from 1.8M to 0.1M, ruthenium red staining sites reappear. EC: endothelial cell, PT: epithelial cell from proximal tubule. X71,000


EC
EC
PT
15

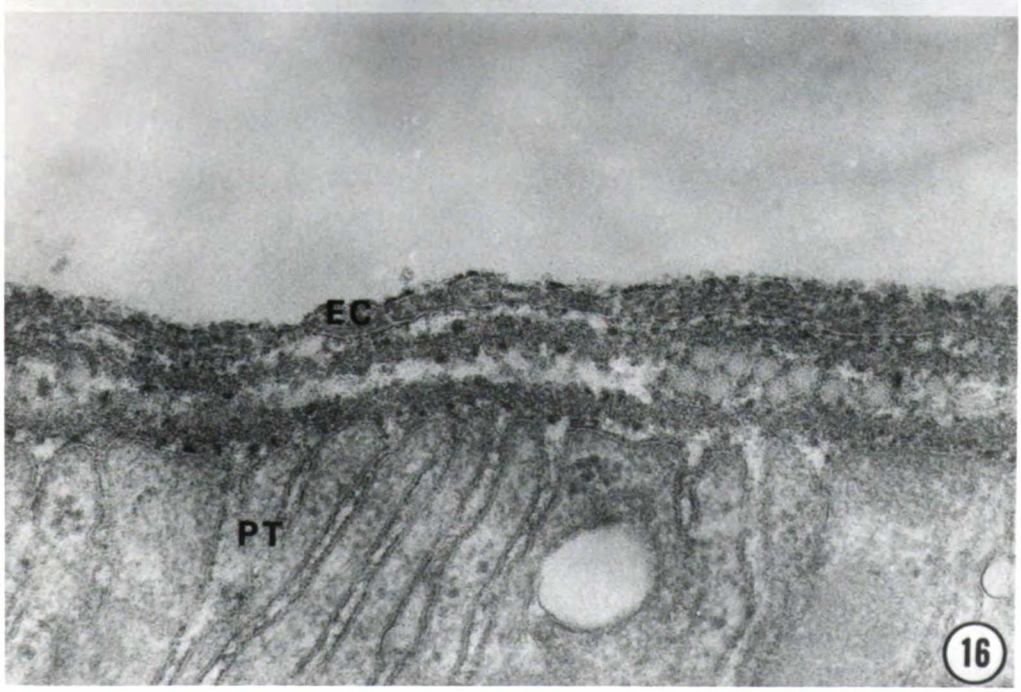
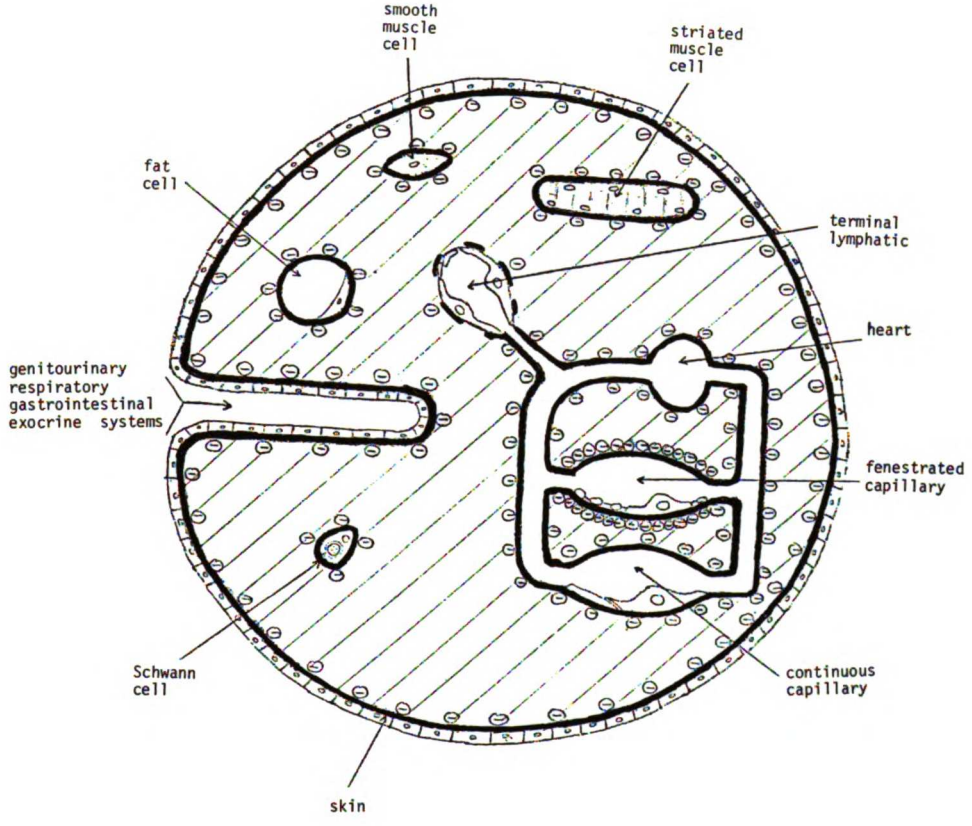

EC
PT
16

Fig. 17 Diagram of anatomic distribution of the basement membrane, represented as heavy black line. Shadowed area represents the interstitial space. Anionic sites are shown as ⊖, under each basement membrane. The microcirculation is depicted in more detail. A terminal lymphatic is shown with interrupted basement membrane and fewer anionic sites as a result. A fenestrated capillary is shown with a higher number of anionic sites to illustrate our finding that it contains stronger anionic groups than a continuous capillary (Modified after Vracko, Am. J. Path. 77:316, 1974).


smooth muscle cell
striated muscle cell
fat cell
terminal lymphatic
heart
genitourinary respiratory gastrointestinal exocrine systems
fenestrated capillary
Schwann cell
continuous capillary
skin

III. PRESENCE OF HEPARAN SULFATE CORE PROTEIN IN THE BASEMENT MEMBRANE OF CONTINUOUS CAPILLARIES AND SKELETAL MUSCLE CELLS

Abstract

We have applied the double antibody immunocytochemical technique on chopped sections from mouse diaphragm briefly prefixed with a weak fixative. Our first antiserum was affinity purified rabbit anti-heparan sulfate core protein (Hassel et al, 1980). Our second antiserum was ferritin-coupled goat anti-rabbit IgG. We have observed, at the electron microscopic level the presence of ferritin molecules in the basement membrane of endothelial cells and muscle cells. This finding indicates that heparan sulfate proteoglycan is a component of these basement membranes.

Introduction

The basement membrane is a specific extracellular component that lies under the basal surface of epithelia, mesothelia and endothelia and surrounds muscle cells, fat cells, and Schwann cells. Thus, it forms the boundary between the interstitium and these cell types (Vracko, 1974).

The chemical composition of basement membranes has been the subject of intensive investigation in recent years. As a result, the list of macromolecular components found in selected basement membranes now includes collagenous glycoproteins (Kefalides, 1975; Burgeson et al, 1976), non-collagenous glycoproteins (Timple et al, 1979; Chung et al, 1979; Carlin et al, 1981), and glycosaminoglycans (Kanwar and Farquhar, 1979b; Lemkin and Farquhar, 1981).

Heparan sulfate has been identified as the most abundant glycosaminoglycan present in the glomerular basement membrane (Kanwar and Farquhar, 1979b). In vivo this molecule, as most other glycosaminoglycans, is bonded covalently to a protein core; the whole complex is termed a proteoglycan. Heparan sulfate proteoglycan has been recently isolated from the matrix produced by Engelbreth-Holm-Swarm (EHS) sarcoma cells (Hassel et al, 1980). This matrix is basement membrane-like in molecular composition (Orkin et al, 1977). Analysis of the isolated heparan sulfate proteoglycan showed that it has an average molecular weight of 750,000 daltons and contains about 50% protein and 50% carbohydrate; it probably contains 6-12 heparan sulfate chains per molecule. Hassel and co-workers (1980) prepared an antibody against the protein core of this proteoglycan, and by immunofluorescence they have localized heparan sulfate specifically in some basement membranes of normal tissue (skin, cornea, glomerulus).

We decided to do further studies localizing the protein core of heparan sulfate for several reasons. First, no studies had yet been reported at the electron microscopic level. Second, studies using the antibody against the protein core of heparan sulfate were so far limited to basement membranes in a small number of specific sites. As basement membranes are known to exhibit some degree of diversity (Katsuyama et al, 1977; Carlson et al, 1981; Charonis and Wissig, 1981), further studies were required in order to determine whether heparan sulfate is a component of basement membranes in other locations. Finally, the possibility that heparan sulfate contributes to regulation of microvascular permeability to macromolecules and

cells called for examination of the basement membrane of various microvascular beds.

The general aim of our study was to examine by electron microscopic immunocytochemistry the presence and distribution of heparan sulfate in the basement membranes of endothelial of continuous capillaries and of skeletal muscle of the mouse diaphragm.

Materials and Methods

Four male adult (20-30g) Swiss albino mice were used in this study. All specimens were taken from animals fixed by intravascular perfusion of solutions and fixatives as described below. Animals were anesthetized with an intraperitoneal injection of sodium pentobarbital (86 mg per kg of body weight) and the external jugular vein and the abdominal aorta were exposed. The abdominal aorta was cannulated with a 23 gauge needle below the level of the renal arteries. The perfusion was started after the jugular vein was cut for the outlet of the perfusate. During the perfusion, the pressure of the perfusing solution was kept constant at 100 mm Hg. With that pressure, the average flow rate was 4 ml/min. We first perfused 3-5 ml 0.9% saline buffered to pH 7.4 with Na-Na phosphate buffer (PBS). The PBS contained 4% polyvinylpyrolidone (PVP), 10 units/ ml heparin and 0.1% Na nitrate. This was followed by 25-30 ml of 1% freshly prepared formaldehyde and 0.1% glutaraldehyde in PBS, pH 7.4. The fixative contained 4% PVP. The third solution was 8-10 ml PBS which contained 4% PVP.

The diaphragms were then excised and sectioned at 100 μm thickness on tissue chopper. The chopped sections were incubated first, for 60 min at room temperature in a solution containing

rabbit antibody directed against heparan sulfate proteoglycan. The heparan sulfate antiserum (50 µg/ml) was provided by Dr. George Martin, NIH, Bethesda, Maryland (Hassel et al, 1980) and was used in a final dilution 1:15 in PBS containing 0.1% bovine serum albumin (BSA) and 50% normal goat serum. This antibody was purified on an affinity column of heparan sulfate coupled to sepharose. It reacted strongly with heparan sulfate proteoglycan but did not cross-react with laminin, type IV collagen, or the proteoglycans from cartilage or the corneal stroma (Hassel et al, 1980). The sections were then rinsed (6 changes, 10 min each) at 4°C in PBS containing 0.1% BSA, and incubated for 60 min at room temperature in a solution containing ferritin-coupled goat anti-rabbit antibodies (Cappel, Cochranville, Pennsylvania). The antiserum was diluted 1:50 in PBS containing 0.1% BSA and 50% normal goat serum. The sections were then rinsed as previously described. During incubations with both antibodies, the solution contained goat serum in order to avoid non-specific binding of ferritin-coupled goat anti-rabbit antibodies.

Controls consisted of (1) substituting pooled normal rabbit serum for the first antiserum; (2) omitting the first antiserum or (3) substituting a 15 mg/ml ferritin solution for the second antiserum.

After the second rinse, the sections were fixed overnight in 2% glutaraldehyde and 1% formaldehyde in 0.1M Na cacodylate buffer, pH 7.4. The sections were then placed in 1% OsO_4 in 0.1M Na cacodylate buffer, pH 7.4, for 90 min, dehydrated in acetone and propylene oxide, infiltrated and embedded in Epon. Thin sections unstained or stained with 0.6% lead citrate for 30-40 min were examined and photographed in a Siemens I electron microscope.

Results

Control tissue sections incubated with pooled control rabbit serum (Fig. 1) omitting the first antiserum (Fig. 2) or substituting a ferritin solution for the second antiserum (Fig. 3) were uniformly lacking ferritin molecules. Figs. 1, 2 and 3 are unstained sections showing endothelial cells. Muscle cells were also uniformly lacking ferritin molecules (pictures not shown).

In tissue sections treated with rabbit anti-heparan sulfate proteoglycan and subsequently with ferritin-coupled goat anti-rabbit IgG, ferritin molecules were seen in the basement membranes underlying capillary endothelium (Fig. 4) and surrounding skeletal muscle cells (Fig. 5). In unstained sections, because of the low contrast of the tissue, ferritin molecules were very easily identified. Fig. 6 is an example of an unstained section. Ferritin is observed in the basement membranes of three different cell types: an endothelial cell, a pericyte and a skeletal muscle cell. However, the basement membrane between the endothelial cell and the pericyte does not contain any ferritin molecules.

Ferritin molecules were present exclusively in basement membranes. Their distribution appeared to be non-random: usually ferritin was seen in clusters of more than three molecules and only occasional single molecules were present between such clusters (Figs. 4, 5 and 6). Also ferritin tended to be localized along the interstitial surface of basement membranes (Figs. 4,5,6). Occasionally, however, ferritin molecules were seen at every level across the thickness of basement membranes.

With our experimental protocol, the tissue was briefly fixed with a weak fixative before being chopped and then incubated in various antisera and rinsing solutions for 5 hours. Even though the sections were subsequently fixed with a stronger dialdehyde mixture, the preservation of the tissue is not optimal.

The rabbit antibodies and especially the ferritin-coupled goat anti-rabbit complexes have a high molecular weight. Consequently, they penetrate poorly into the center of the tissue section. For this reason, successful staining with antibody was confined to the edges of each section. Although in most of these areas the interstitium appeared extracted, the basement membrane was present around skeletal muscle cells and beneath endothelial cells.

Discussion

Using immunocytochemical techniques, we have detected at the electron microscopic level the presence of heparan sulfate in the basement membrane of endothelial cells of continuous capillaries and skeletal muscle cells of the mouse diaphragm. In tissue treated with both antisera ferritin molecules were mainly localized at the interstitial surface of the basement membranes examined. This localization suggests that the polypeptide core of heparan sulfate is predominantly found at the outermost layer of these basement membranes. However, this distribution might be artifactually created by displacement of basement membrane components caused by prolonged handling of weakly fixed tissue.

The lack of ferritin molecules in the basement membrane between the endothelial cell and the pericyte (Fig. 6) may be interpreted in two ways: either heparan sulfate proteoglycan is not present, or the accessibility of the ferritin-coupled antibody is minimal.

In most cases, the few interstitial components (predominantly collagen fibers) found between muscle cells and blood vessels at the edges of chopped sections were extracted by our technique. However, in the cases where collagenous fibers were present, they were not stained with ferritin.

We did not observe ferritin molecules inside endothelial or muscle cells, the potential site of synthesis of the proteoglycan core protein. This observation does not imply that occasional heparan sulfate polypeptides should not be expected to be present intracellularly. It reflects the fact that in our protocol we did not include any treatment to make cell membranes permeable to molecules as large as the ferritin-IgG complex.

Heparan sulfate may be a physiologically very important macromolecule. It has a large number of highly anionic sulfate groups that have been identified as ruthenium red staining sites in the renal glomerular basement membrane (Kanwar and Farquhar, 1980) and in the alveolar basement membrane (Vaccaro and Brody, 1979). Anionic sites in the glomerular basement membrane have been implicated in the regulation of glomerular permeability to macromolecules according to the net electrical charge (pI) of each macromolecule (Kanwar et al, 1980). A similar phenomenon many occur in all microvascular beds. During invasion, metastatic cells have been shown to degrade heparan sulfate (Kramer et al, 1981). Degradation of heparan sulfate might be also taking place when blood elements (leukocytes, monocytes) cross the vascular wall, although this has yet to be demonstrated.

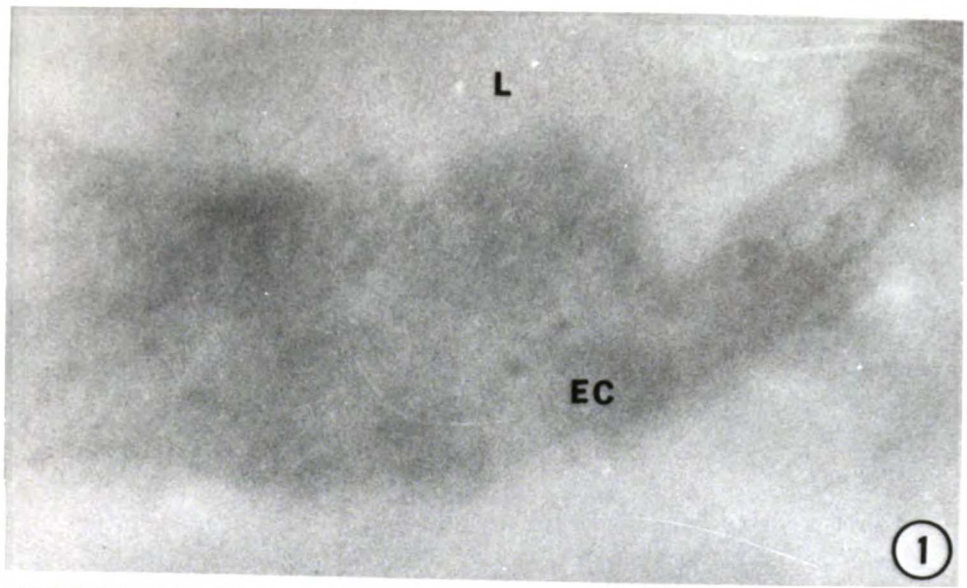
Although our study indicates the presence of heparan sulfate in basement membranes of the diaphragm, we do not presently know its precise molecular structure. In view of the extensive variability observed in the carbohydrate portion of heparan sulfate (Linker and Hovingh, 1973), it is possible that basement membrane heparan sulfates might have an antigenically similar protein core but chemically different sugar side chains, and thus have potentially different physiological roles.

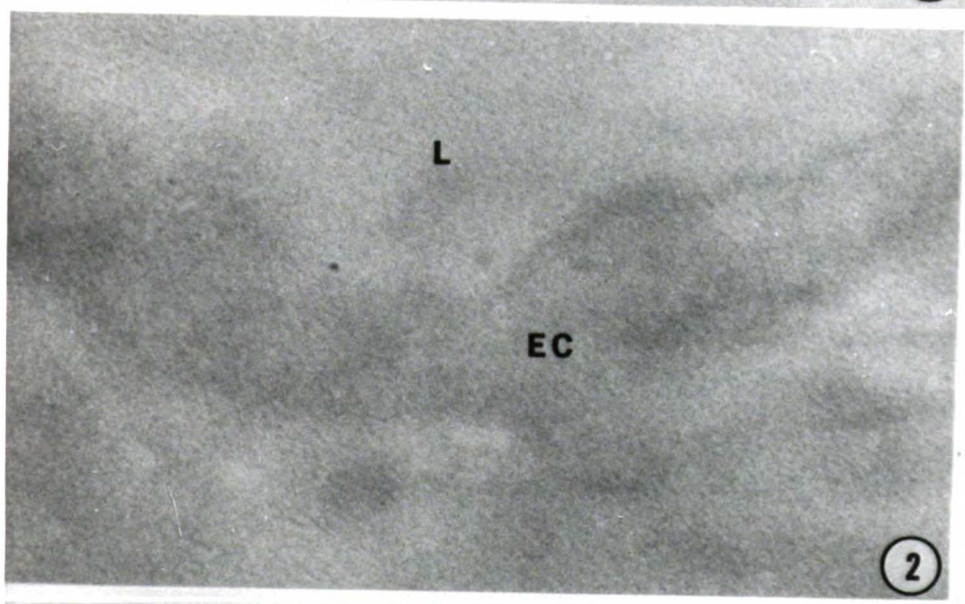
Figure Legends

Fig. 1 Incubation with pooled control rabbit serum instead of the first antibody. Unstained section. No ferritin molecules are seen. EC: endothelial cell, L: lumen. X102,000

Fig. 2 Incubation with saline instead of the first antibody. Unstained section. No ferritin molecules are seen. EC: endothelial cell, L: lumen. X102,000

Fig. 3 Incubation with ferritin solution instead of the second antibody. Unstained section. No ferritin molecules are seen. EC: endothelial cell, L: lumen. X102,000.


L
EC
1


L
EC
2

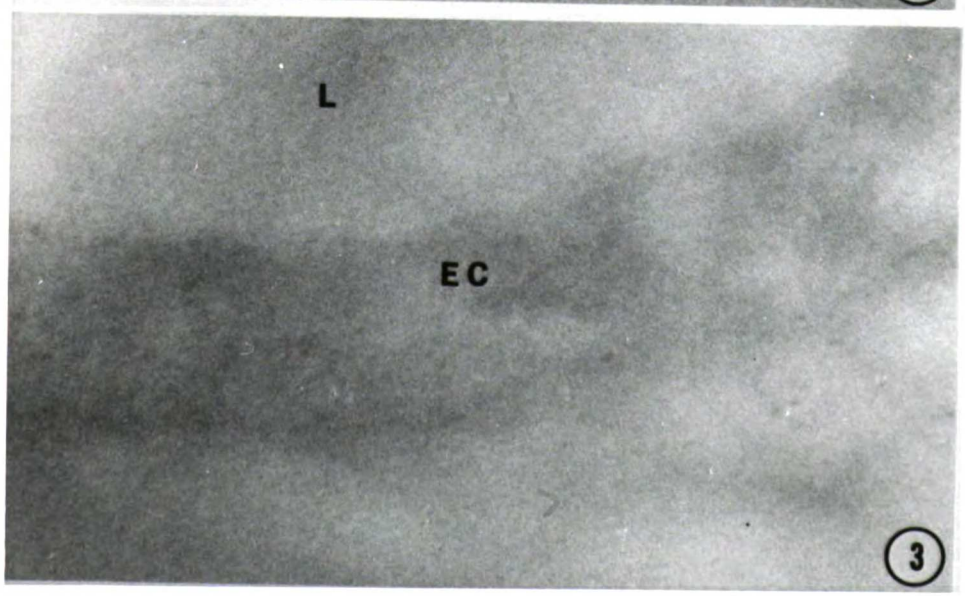
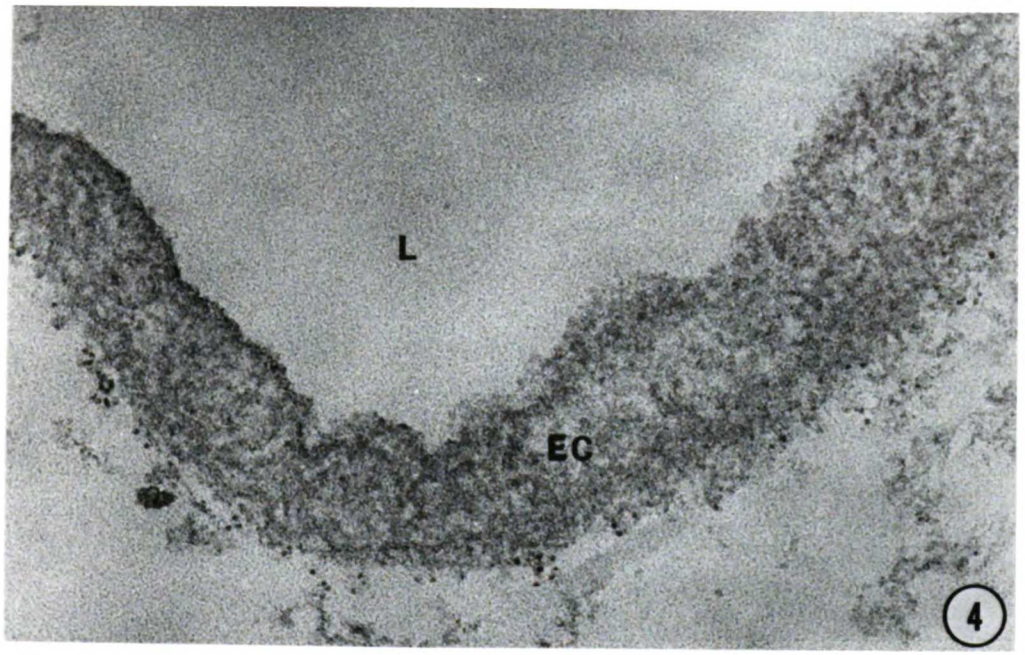

L
EC
3

Fig. 4 Experimenal. Ferritin molecules are seen subendothelially. Stained with lead. EC: endothelial cell, L: lumen. X102,000

Fig. 5 Experimental. Ferritin molecules are seen around the muscle cell (M). The cell membrane is not clearly delineated. Stained with lead. X102,000


L
EC
4

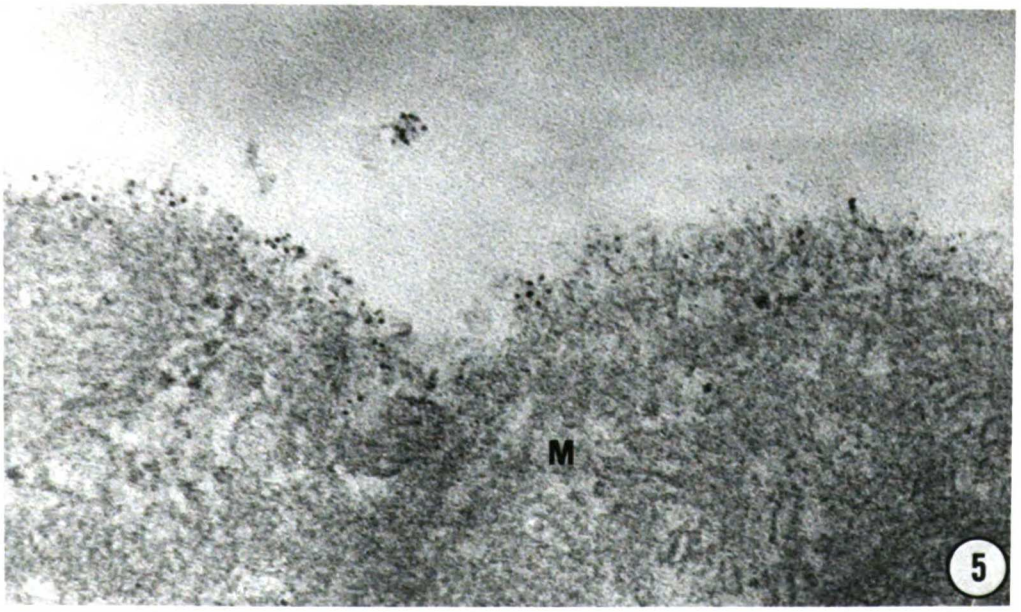
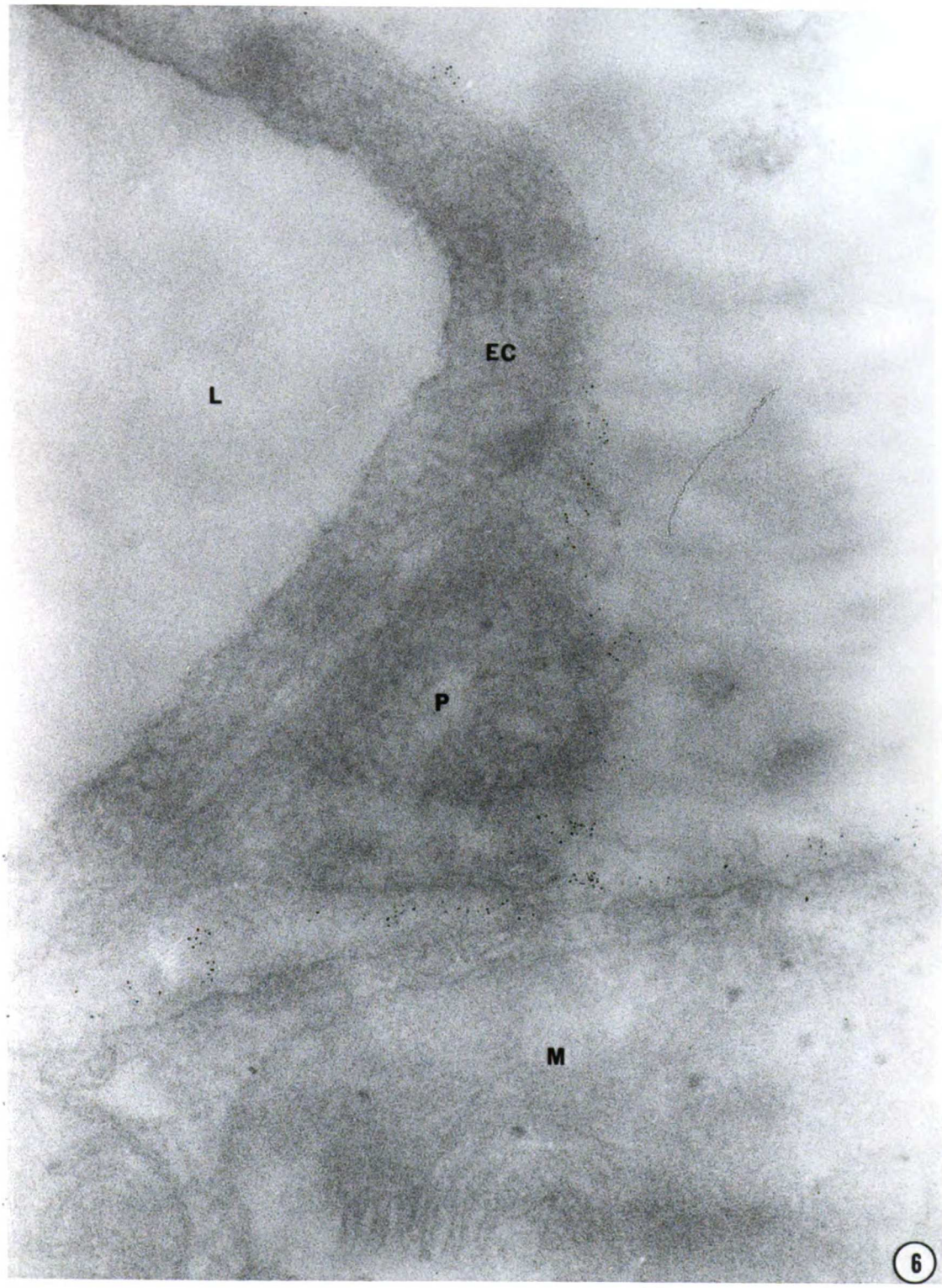

M
5

Fig. 6 Experimental, unstained. Ferritin molecules are seen in the basement membrane of an endothelial cell (EC), a pericyte (P) and a muscle cell (M). L: lumen. X102,000


EC
L
P
M
6

IV. APPENDIX: THE CRITICAL ELECTROLYTE CONCENTRATION (C.E.C.)

A cationic dye, interacting electrostatically with a polyanion can be effectively displaced by adding an inorganic cation in the solution. This cation can be provided by a fully ionizable electrolyte, such as NaCl, KCl, $MgCl_2$. The concentration of the electrolyte at which the binding of the cationic dye is completely abolished, is called the critical electrolyte concentration.

Several factors influence ths reaction and have to be considered in the determination of the critical electrolyte concentration. The most important of these factors is the chemical nature of the anionic groups of the polyanion. In biological systems, three types of anionic groups are by far the most commonly found: carboxyl groups, phosphate groups and sulfate groups. Scott and Dorling have obtained families of critical electrolyte concentrations, characteristic for each of these groups. In their measurements, they have used model experiments with known substances on filter paper or histological preparations at the light microscopic level and Alcion Blue as a cationic dye. When they used NaCl as the inorganic electrolyte, they found that they could displace Alcian Blue from carboxyl groups at a concentration of 0.13-0.475 M NaCl, from phosphate groups at a concentration of 0.4-0.5M, from phosphate groups at a concentration of 0.4-0.5M Nacl and from sulfate groups at 1.0-2.5M NaCl (from Table 6 in Scott and Dorling, 1965).

Even in an ideal polyanion that contains only one type of anionic groups (only carboxyl or only pohosphate or only sulfate) at least two other parameters have to be considered: first, the number of these anionic groups and second, the geometry of their distribution (the distance between the groups). Both these parameters affect the salt concentation required for a successful competition. Higher number of anionic groups and smaller distances between them lead to higher values of critical electrolyte concentration. Thus, even in macromolecules homogeneous in the chemical nature of their charged groups, not a single value but a family of values of salt concentration has been determined.

However, some macromolecules do not have only one type of anionic groups, but usually two types: for example, most glycosaminoglycans have both carboxyl and sulfate groups in their molecules. In these situations, the critical electrolyte concentration is found between the ones calculated for carboxyl groups and the ones calculated for sulfate groups, depending on the relative number of each type of groups. Also, in these situations, the pH at which the competition is done becomes a crucial factor. Extremely low pHs should be avoided because in these pHs carboxyl groups are neutralized and macromolecules having both carboxyl and sulfate groups demonstrate an erroneously high critical electrolyte concentration, similar to the one characteristic of macromolecules having only sulfate groups. For this reason, Scott and Dorling recommend as an optimal pH for competition experiments the pH of 5.8.

In conclusion, the results from salt competition experiments can give us qualitative answers and clues about the chemical nature of anionic sites of a macromolecule.

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