THE UNIVERSITY OF CALGARY

FIBRINOGEN AND ITS ROLE IN

GLOMERULAR PERMEABILITY

by

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A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES

IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE

DEGREE OF DOCTOR OF PHILOSOPHY
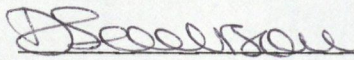

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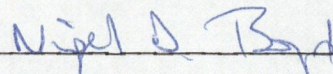

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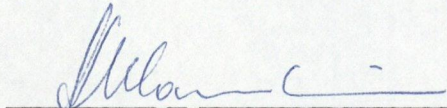
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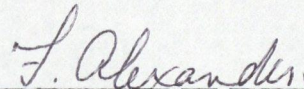

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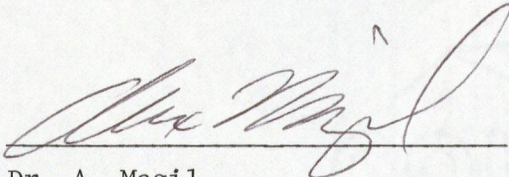(Department of Medical
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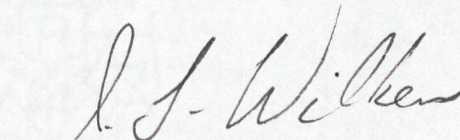(Departments of Medical Biochemistry and
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May 31, 1983

ABSTRACT

The role of fibrinogen in glomerular permeability of the isolated perfused dog kidney was investigated.

Dog kidneys were removed and flushed out, by gravity feed, with 500 ml of Collins solution and the kidneys were then perfused on a kidney pump with the perfusate containing inulin and/or dextrans of different molecular weights and charges. Two hours after the start of the perfusion, human fibrinogen was added (0.3 g/100 ml) to the perfusate. Urine and venous effluent samples were obtained at 30 minute intervals and estimation of protein, inulin, dextran, as well as enzyme content was carried out. In addition the concentration of inulin and dextran in the venous effluent was determined and the values obtained were used to calculate the plasma flow rates, glomerular filtration rate and fractional clearances of dextrans. Biopsies before and after the addition of fibrinogen were obtained for electron and immunofluorescence studies.

The urine of the dog kidneys maintained on the pulsatile perfusion pump using the perfusate that contained albumin, alpha 1 and alpha 2 globulins, but did not contain any fibrinogen contained a considerable amount of protein. However, the inclusion of fibrinogen in the perfusate resulted in a reduction in the degree of proteinuria, an increase in glomerular filtration rate, an increase in the renal

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plasma flow rate, and a decrease in urinary enzyme levels, without any reduction in the urine volume.

Electron and immunofluorescence microscopy revealed no detectable cellular damage in the kidneys perfused with or without fibrinogen, and that fibrinogen does not bind to the glomerular wall to any great extent. In the presence of fibrinogen the clearances of neutral, negatively and positively charged dextrans were unchanged increased and decreased respectively which indicate that the observed effects of fibrinogen are charge related.

These experiments required the use of pure and undegraded fibrinogen which was isolated using a polyethylene glycol 1,000 procedure. This procedure eliminates problems of denaturation, degradation and contamination encountered with other procedures. The technique is simple, rapid, gives a high yield and gram amounts of fibrinogen can be readily obtained.

Characterization of the fibrinogen that was isolated indicated that it had a molecular weight of 680,000, twice the currently accepted value. This could be explained on the basis of its tendency to dissociate on ultracentrifugation. The inability of the fibrinogen to cause rouleaux formation was found to be due to the fact that various clotting factors are also required to be present. These act to promote aggregation of fibrinogen molecules.

ACKNOWLEDGMENTS

I wish to thank the following people for their assistance during my graduate program.

My supervisor, Dr. Nigel Boyd, for his great knowledge, assistance, direction, concern and friendship.

Dr. Fred Alexander, for his help with the electron microscopy studies.

Dr. John Klassen, for providing the plasma that was used in the preparation of the perfusate.

Dr. Henry Mandin, for the use of the kidney pump and the equipment used in the inulin studies.

Dr. George Fritz Department of Physics University of Calgary for his help in light scattering studies.

Dr.s Patrick Lee and Saad Masri, Department of Microbiology, University of Calgary for the use of gel electrophoresis equipment.

Dr. C. Kay, Department of Biochemistry, University of Alberta for the use of The Model E ultracentrifuge.

The Department of Plant Science, University of Alberta for the performance of high pressure liquid chromatography.

My great friends Edward Chan, Kay Carter and Michael Chooi for their help with the animal work.

Mr Robert Carter, for donating his blood.

My wife Rima, for proof reading the thesis.

Medical vivarium staff for the help with the animals.

This thesis is dedicated to

my parents

and to

Rima and Wiam

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INTRODUCTION

1.01 Structural Components of the Glomerulus

The glomerulus, described by Bowman in 1843 as a cluster of capillaries, is the anatomic structure of the kidney responsible for the formation of an almost complete ultrafiltrate of plasma (Ludwig, 1843).

The hypothesis regarding fluid transfer across capillaries by a passive diffusion mechanism and the formation of urine by ultrafiltration of plasma was first suggested by Ludwig (1843) and then supported by Cushny (1917) and later experimentally confirmed by Richards (1936). Extensive investigation to determine the structural and functional basis of this filtration process has been going on over the past 20 years. Current views of the mechanism whereby the kidney is able to produce large quantities of plasma ultrafiltrate, from which plasma proteins of the size of albumin and larger are excluded, result from direct and indirect observations of the specific characteristics of the three structural layers of the glomerulus (endothelial, basement membrane and epithelial) (Venkatachalam and Rennke, 1978).

1.01.1 Endothelial Cells

The lumen of the glomerular capillaris is lined with endothelial cells in which there are irregulary spaced fenestrations where most of the glomerular filtration takes

place (Latta, 1973). The fenestrations measure up to 60 - 100 nm in diameter (Karnovsky, 1979) and are lined by a thin and highly permeable diaphragm composed of a protein-polysaccharide film (Clementi and Palade, 1969). The diaphragm seems to act as a barrier for high molecular weight (MW) molecules such as ferritin, MW 480,000, (Farquhar et al, 1961), myeloperoxidase, MW 160,000, (Groniowski et al, 1969) and catalase, MW 240,000 (Karnovsky et al, 1969).

1.01.2 The Glomerular Basement Membrane (GBM)

The basement membrane is composed of three layers, a central dense layer called the lamina densa, and two thinner layers, the lamina rara externa and lamina rara interna. The total reported mean width of the membrane is about 300 nm (Osawa et al, 1966). The lamina rara externa and lamina rara interna are 20 to 40 nm in thickness (Jorgensen, 1966).

Physical, enzymatic and chemical studies have shown two of the major constituents of the GBM to be collagen like polypeptides (Cohen and Surma, 1980) and glycoproteins (Kefalides and Winzler, 1966; Kefalides, 1974). The collagen like peptides are relatively non-polar, they contain many disaccharide units (Kefalides, 1974) and have a MW range of 30,000 to 220,000 daltons (Freytag et al, 1976). They form a fibrillar network which is closely packed in the lamina densa and much less so in the lamina rara interna and externa (Latta, 1973). However, the lamina

rara contains the more polar glycopeptides that are rich in dicarboxylic amino acids and oligosaccharides (Sato, and Spiro, 1976).

Fixed negatively charged sites have been demonstrated on the rara externa, and these may influence the filtration of macromolecules (Caulfield and Farquhar, 1976). Groniowski et al (1969) and Jones (1969) demonstrated that sialic and aspartic acid residues were components of these sites. Kanwar and Farquhar (1980) have observed that the perfusion of kidneys with the sialic acid specific enzyme neuraminidase, leads to detachment of the cellular elements from the GBM. This suggests that the sialic acid glycopeptides serve to anchor the epithelial and endothelial cells to the GBM.

Kanwar and Farquhar (1979), using ruthenium red and cationized ferritin as morphological probes, have demonstrated the presence of heparan sulphate proteoglycan, which is located in the lamina rara in clusters about 60 nm apart. More recently laminin, a high MW, and negatively charged glycoprotein rich in disulfide, has been found in the GBM matrix of human and mouse glomeruli (Timple et al, 1979; Scheinman et al, 1980). Immunofluorescence studies indicated, that in man, laminin is principally located in the lamina rara (Foidart et al, 1980), where it may associate with heparan sulphate proteoglycan (Sakashita et al, 1980). In this location, laminin seems

likely to promote adhesion of the cells to the basement membrane (Kleimman et al, 1981). The lamina rara interna and externa are not, however, identical, but they differ in their antigenic characteristics (Fish et al, 1979) and hence presumably in their chemical composition.

Based on the above, the matrix substance of the GBM appears to be made of a hydrated polyanionic gel with a branched intertwining network of glycopeptide chains and proteoglycans. The gel interior is made of water filled spaces between the chains that form the channels for water and solute transport. The size of the channels and net charge on the glycopeptide and proteoglycan chains would thus influence glomerular filter permeability (Venkatachalam and Rennke, 1969).

1.01.3 Epithelial Cells

Epithelial cells have cytoplasmic foot processes interdigitating with one another and anchored to the GBM by filaments that connect the lamina densa to both epithelial and endothelial cells. The distance between adjacent,foot processes that arise from different podocytes (Arkawa, 1970), termed the filtration slit (Latta, 1973) is about 25 to 60 nm wide at the side near the basement membrane (Farquhar et al, 1961). These slits are completely filled with highly polyanionic cell coats, which consist of a branching network of glycoproteins to which sialic acid residues are attached (Ranbourg and Leblond, 1976; Michael

et al, 1970). At their narrowest points, the slits are bridged by a flat, single-layered filtration slit diaphragm, 7nm thick. After fixation with tannic acid-glutaraldehyde, the diaphragms exhibit elongated central bars, which are connected to the plasma membrane of the foot processes by rod like subunits. Within the diaphragms, there appear to be shallow, repeating, rectangular "pores", approximately 4 X 14 nm (Rodwald and Karnovsky, 1974).

1.02 Filtraton of Macromolecules

It is possible to measure the clearance of a macromolecule relative to that of inulin in order to obtain an indication of the ability to pass across the glomerular permeability barrier, given the requirements that the macromolecule is not secreted or reabsorbed, i.e. the rate of excretion of the macromolecule must equal its rate of filtration. A test molecule that satisfies these requirements is dextran and its derivatives (Chang et al, 1975a; Chang et al, 1975b; and Bohrer et al, 1978). By using micropuncture and clearance techniques, it has been established that several factors influence the glomerular clearance of macromolecules (Brenner et al, 1970). These factors include:

1) Molecular size.

2) Molecular charge.

3) Molecular shape.

4) Plasma flow rate.

5) Initial glomerular protein concentration (CA).

6) Filtration pressure (P) = Transmembrane hydraulic pressure - Oncotic pressure.

7) Effective permeability coefficient (Kf) = Effective hydraulic permeability (K) X Surface area (S).

8) Filtration fraction (FF).

1.02.1 <u>Molecular Size</u>

The effect of molecular size on filterability has been determined by measuring the fractional clearances of uncharged molecules of graded sizes, such as polyvinyl pyrrolidone or dextran. The fractional clearance of dextran is expressed as urine-to-plasma concentration ratio of dextran divided by the same ratio for inulin. These tracers were administered to rats, together with inulin, and their clearances determined from plasma and urine samples, following gel filtration to separate molecules according to size. It was observed that the fractional clearances of the test molecules decrease progressively with increasing molecular radius (Renkin and Gilmore, 1973). The rat GBM does not restrict the clearance of neutral dextrans until effective dextran radii exceed about 2 nm. However, the clearance decreases progressively with increasing size and approaches zero at radii greater than 4.5 nm. The clearance of proteins of varying sizes similarly suggests an effect of molecular size on filtration. However, these results

are often not clear-cut, due to the effects of other molecular factors.

1.02.2 Molecular Charge

The charge of macromolecules has a great effect on filterability and has been investigated by using dextrans of identical sizes but of different charges (Chang et al, 1975c). It has been shown that the fractional clearance of positively charged dextrans of the same size is greater than that of negatively charged or neutral ones (Chang et al, 1975c). Similar results were obtained for horse radish peroxidase molecules, whose net charge had been modified (Rennke et al, 1978). It was also shown in ultrastructural tracer studies that ferritin molecules of increasing isoelectric point (pI) penetrate the GBM with progressively greater ease (Rennke et al, 1978; Rennke and Venkatachalam, 1977). Purtell et al (1979) has shown that the renal excretion in rats of cationized albumin (pI; 7.2 - 8.2) exceeds that of native unmodified albumin (pI; 4.9) by a factor of 300. However, filtration of unmodified rat albumin was increased as well, which could indicate an interaction between exogenously adminstered cationic albumin and the glomerular filter, resulting in the neutralization of glomerular polyanion. Similar effects have also been observed following the partial neutralization of rat glomerular anionic sites by the administration of the polycation hexadimethrine bromide (Hunsicher and Shearer,

1979) and with hydralazine and the positively charged N-methyl histamine (pI; 8.9; Masri et al, 1982). Related to this is the observation that in nephrotoxic serum nephritic rats, the fractional clearance of anionic dextran sulfate (DS) is increased over a wide size range; corresponding clearances of neutral and cationic dextran molecules are decreased (Bohrer et al, 1978; Bennett et al, 1976; Chang et al, 1976). The increase in the clearance of DS has been attributed to the net charge of the glomerulus becoming more positive, as demonstrated by the decreased staining of the glomeruli with colloidal iron (Kreisberg and Karnovsky, 1979). Thus, for negatively charged molecules, the channels in the GBM gel are functionally "narrowed", whilst entry of the positively charged molecules is facilitated (Bohrer et al, 1978).

1.02.3 Molecular Shape

Molecular shape and deformability also influence the glomerular filtration of macromolecules. The fractional clearance of neutral dextrans is greater than that of neutral horse radish peroxidase molecules of identical size by a factor of 7, even after correction for tubular reabsorption of the protein peroxidase (Rennke and Venkatachalam, 1979a). Such a finding could be explained by the different molecular structural characteristics of dextrans as compared to those of proteins. Dextrans are linear sugar polymers that assume coiled configurations

in free solution. However, since they are not internally bonded, they are susceptible to deformation and uncoiling when subjected to shearing effects. Proteins, on the other hand, are made up of polypeptide chains that are internally bonded by disulfide links. During the forced migration of filtered molecules, linear flexible polymers, such as dextrans, behave like molecules of smaller dimensions and move with ease, which accounts for their greater clearance as compared to proteins. Similar observations have been made with respect to the glomerular filtration of dextrans as compared to the more compactly structured ficoll molecules (Bohrer et al, 1978).

1.02.4 Effect of Plasma Flow Rate (QA)

According to models put forward by Deen et al (1972), increases in glomerular QA would be expected to result in a decreased fractional clearance of macromolecules due to a greater increase in the filtration of water as compared to solute. When the glomerular filtration rate (GFR) goes up, there is a concomitant increase in the filtration of the convected component of filtered molecules, but not in the fraction diffused (Deen and Satavat, 1981). This model was tested experimentally using uncharged dextrans as probe molecules. Iso-oncotic plasma volume expansion, which on the average caused an increase in renal QA from 70 - 220 ml/min, resulted in a reduction in the fractional clearance of all but the smallest (<2.2 nm radius) and the largest

(>3.6 nm radius) dextran molecules (Chang et al, 1975c).

The injection of angiotensin II into rats, which resulted

in a reduction in QA from 83 to 60 ml/min, was associated

with a significant increase in the fractional clearance

of dextrans (Chang et al, 1975c). Comparable results were

also obtained by Ryan and Karnovsky (1976), who found

that endogenous plasma globulin and albumin permeated the

glomerular barrier to a greater extent under low QA and

pressure, as compared with normal hemodynamic conditions.

These results can be attributed to the formation of a

concentration polarization layer made up of large MW plasma

proteins (Deen et al, 1974). Under good QA, the con-

centration polarization layer is well established and

filtration of macromolecules is prevented. However, under

reduced QA, the concentration polarization layer would

disperse, thus allowing macromolecules to diffuse into

the glomerular wall and traverse the GBM (Ryan and Karnovsky,

1976).

1.02.5 Glomerular Capillary Protein Concentration (CA)

The effect of CA on the clearance of macromolecules

is not fully understood, mainly due to difficulty in

assessing its effect on GFR. In theory, according to the

model of Deen et al (1972), an increase in CA from 5.7 to

8.0 g/100 ml should result in an increase in the fractional

clearance of macromolecules. Brenner et al (1977)

demonstrated that an acute reduction in CA below 3.4 g/100

ml by volume expansion in rats leads to decrease in QA and an increase in single nephron filtration fraction (SNFF). Deen et al (1972) predicted that CA should be able to vary independently of other determinants of SNFF and GFR. However, it has been shown by Baylis et al (1977) that this assumption is not valid and a reduction in CA would be accompanied by a reduction in filtration coefficient Kf. An increased clearance of macromolecules, due to an increase in CA, was obtained using isolated perfused kidneys. However, the increased clearance in this case resulted from high perfusion rates and low FF, which prevented filtration pressure equilibrium and, therefore, exposed the whole filtration area of the glomerular capillary tuft for filtration (Brenner et al, 1981).

1.02.6 Filtration Pressure

A selective decrease in filtration pressure enhances the fractional clearance of all but the smallest and largest macromolecules. An increase of filtration pressure above normal is predicted to have little or no effect on any molecular size. As filtration pressure is raised, the convective and diffusive components of solute transport are also enhanced, in proportion to the increase in voume flux (Chang et al, 1975b).

1.02.7 Effective Permeability Coefficient (Kf)

Occlusion of the capillary lumen or alterations of the capillary wall will reduce Kf, thus leading to decreased GFR. A number of factors are involved in the consideration of Kf:

a) The number and size of the fenestrations on the glomerular endothelium are thought to regulate the access of plasma to the GBM and thus exert a controlling influence on the glomerular barrier function.

b) The structure of the GBM, with its hydrated polyanionic gel, is involved in the consideration of Kf. The interior of the gel, is made of water-filled spaces between the chains and forms the channels for water solute transport. The size and internal geometry of these channels can be considered as properties that influence glomerular fiter permeability (Venkatachalam and Rennke, 1969).

1.02.8 Filtration Fraction (FF)

An increase in FF (FF = GFR/QA) is accompanied by an increase in the concentration of plasma proteins, averaged along the length of the glomerular capillary (Brenner et al, 1977). This increase leads to an increase in the transglomerular passage of proteins, as a result of an increase in the concentration gradient for diffusion and the greater average concentration of proteins in the fluid being convected across the glomerular capillary (Brenner et al, 1981).

1.03 The Nature of the Filtration Barrier

1.03.1 First Theory

According to the initial theory that was presented, structural pores exist in the GBM whose size does not allow the passage of albumin, except under abnormal conditions of blood flow and hemodynamics. Albuminuria wuld thus be prevented by molecular sieving (Pappenheimer, 1953). Support for this theory was obtained from experiments with rat kidneys in which the superficial glomeruli were fixed in situ by applying glutaraldehyde to the renal surface, albumin was detected in the lamina rara interna only in small amounts, under conditions of good blood flow. Although there are fibers in the basement membrane that could serve as channels for macromolecules, the existence of pores, has not been able to be established within the size limitation imposed by the electron microscope (Simpson, 1981).

1.03.2 Second Theory

Clearance studies with enzymatic tracers, such as myeloperoxidase and lactoperoxidase, led to the second theory, which suggests two successive glomerular filtration barriers in series:

1) The GBM which acts as a coarse filter to exclude very large molecules (diameter larger than 10 nm).

2) The epithelial slits to retard smaller molecules (Karnovsky and Ryan, 1961).

This theory was supported by studies in which the tracer formed a concentration gradient to the epithelial slit pores. However, the use of histochemically detectable enzymatic tracers presents major problems, such as:

1) The tracer used would adhere to negatively charged sites on the epithelial cells (Karnovsky and Ainsworth, 1973).

2) Diffusion of the tracer before fixation of specimens could have resulted in different localization patterns (Renkin and Robinson, 1974).

3) There was no direct confirmation of the size of the circulating tracer, which could have been changed due to metabolism (Schults et al, 1976).

4) Clearance values obtained were only specific to the particular tracer in use (Farquhar, 1975).

5) The rapid clearance of cationic myeloperoxidase relative to the anionic catalase is explainable on the basis of charge differences.

1.03.3 Third Theory

The third theory, based on results obtained by Farquhar et al (1976), postulates that the basement membrane, rather than the slit pores, acts as a main filter with the endothelium, due to its fenestrated surface, serving to regulate the access to the filter. According to this theory, the role of the epithelial slit diaphragms would be to serve as a further restriction to those molecules

that become able to penetrate the GBM, due to alterations in normal permeability characteristics. Protein accumulating at the epithelial slit diaphragms would then be taken up into the epithelial cells by endocytosis and degraded by lysozomal action. This has been observed for ferritin and dextran molecules that have penetrated the GBM (Caulfield and Farquhar, 1974; Farquhar et al, 1976; Rennke and Venkatachalam , 1977). In their experiments, Farquhar et al (1961) observed a sharp drop in the concentration of tracers (same size as albumin) between the lamina rara interna and lamina densa. Similar findings came from experiments performed by other investigators (Venkatachalam et al, 1970).

Although dextrans do not bind to the basement membrane, they have not been detected in the basement membrane, where they would be expected to occur at low concentrations during their transit. This appears to represent a major deficiency in this theory.

1.03.4 Fourth Theory

The effects of charge repulsion have had to be included in theories concerning the nature of the filtration barrier. The fractional clearance for the same size molecules has been shown to be less for negatively charged dextrans or proteins and greater for positively charged dextrans or proteins, than for neutral molecules (Chang et al, 1975c; Bohrer et al, 1977; Rennke et al, 1978). Also, the decrease

in the polyanionic content found in aminonucleoside nephrosis and nephrotoxic serum nephritis results in an increase in the fractional clearance of negatively charged, but not neutral dextrans (Bennett et al, 1976). The albuminuria associated with these conditions, and also that of human lipoid nephrosis (minimal change; foot processes loss disease), would then be consistant with the reduction in polyanion content that is observed (Blau and Haas, 1973). Kanwar and Farquhar (1979), who demonstrated the existence of heparan sulfate on the GBM, showed that the removal of these molecules will increase the clearance of macromolecules.

A corollary to the above is that the more positive a molecule, the greater the size of the molecule that is able to penetrate the GBM. In this regard, Rennke and Venktachalam, (1977) have observed that in perfused mice kidneys, cationized ferritin (MW 480,000; pI 8-9) is able to penetrate into the interior of the lamina rara interna, with some molecules reaching the lamina rara externa. This is in marked contrast to native ferritin (pI 4.1-4.7), which penetrates into the lamina rara interna to only a very limited extent.

1.03.5 Fifth Theory

An additional postulate that has been made to explain the failure of albumin and other similar sized molecules to pentrate the lamina rara interna under conditions of

good blood flow is that of a "concentration-polarization layer" that forms in the relatively unstirred zone beneath the endothelium (Deen et al, 1974). This layer would consist of plasma proteins that are of sufficiently large size that they are unable to penetrate the pores in the GBM. According to this hypothesis, the molecules comprising the concentration polarization layer would tend to become dispersed if slowing of blood flow occurred and ultrafiltration was reduced. Since molecules such as myeloperoxidase (MW 160,000, radius 4.4 nm, pI 10) and cationized ferritin (MW 480,000, pI> 8.8) have been observed to penetrate through to the subepithelial region, one would predict on the basis of this model that the molecules forming the concentration-polarization layer would have to be very large if they are neutral or positively charged, i.e. 480,000 and larger, since they would otherwise be filtered. However, molecules smaller than this might be effective if they are negatively charged, due to charge repulsion effects. Collagenase extracts of human GBM have been found to contain - 3.1 ug/mg of albumin, 2.0 - 7.5 ug/mg of immunoglobulin G(IgG), and 10.8 - 26.6 ug/mg of fibrinogen (Westberg and Michael, 1970). This order of concentration is directly opposite to that of plasma and would indicate selective trapping of these molecules and be consistent with IgG and particularly fibrinogen, being components of the concentration-polarization layer.

1.04 <u>Pathogenesis of proteinuria</u>

Normal glomerular barrier function is dependent on the interaction of many factors considered above; abnormalities of any of these factors may produce a breakdown of the barrier function, resulting in proteinuric disorders.

Experimental proteinuric disorders can be attributed to:

1) Structural alterations, as in:

a) Nephrotoxic serum nephritis (Galaske and Baldamus, 1978).

b) Aminonucleoside induced nephrosis (Vernier <u>et al</u>, 1959).

c) Experimental membranous nephropathy (Schneiberger and Grup, 1976).

2) Altered glomerular hemodynamics, as in:

a) Angiotensin induced proteinuria (Bohrer <u>et al</u>, 1977).

1.04.1 <u>Nephrotoxic Serum Nephritis</u>

Glomerular capillaries of rat kidneys injected with anti-GBM serum become permeable to albumin and globulins in a dose dependent manner (Galaske <u>et al</u>, 1978). Injection of anti-GBM serum results in thickening of the lamina rara interna and lamina densa regions. However, the epithelium does not exhibit any major alterations. Thickening of the GBM and loss of the endothelium lead to changes in the filtration characteristics of the membrane,

as evidenced by an increase in urinary protein loss, while the glomerular filtration of water and small molecules decreases. The increased protein loss is thought to be related to altered charge characteristics of the glomerular capillary wall (Bennett et al, 1976).

1.04.2 Aminonucleoside Induced Proteinuria

In this experimental model, the GBM often appears attenuated, suggesting an underlying molecular disorder. Focal epithelial detachment from the GBM is common (Venkatachalam et al, 1969). Ultrastructural tracer studies have also indicated that glomeruli injured by puromycin injection show increased permeability to uncharged dextran molecules (Caulfield and Farquhar, 1975). In 1979, Olson et al found out that in puromycin treated rats, fractional clearances of molecules below 4 nm in radius were either unchanged or lower than in controls. However, clearances of molecules larger than 4 nm in radius were increased. In the same study, it was also shown that the fractional clearances of negatively charged, neutral and positively charged horse radish peroxidase molecules were respectively greatly increased, moderately increased and decreased in nephrotic rats, when compared to controls. The results suggest that the puromycin treated glomerulus shows aberrations in the capacity to discriminate between macromolecules on the basis of both size and net electrical charge (Michael et al, 1970).

1.04.3 Experimental Membraneous Nephropathy

Immunization of rats with proximal tubular brush border antigen leads to a nephropathy with massive non-selective proteinura. Ultrastructural studies reveal loss of epithelial slit diaphragms, detachment of epithelial foot processes, and deposition of immune complexes. All three of these will lead to massive leakage of protein into the urinary space (Schneeberger and Grup, 1976).The glomerular filtration characteristics of differently charged horse radish peroxidase molecules have been studied in experimental membraneous nephropathy. The results show that the fractional clearances of negatively charged, neutral and positively charged horse radish peroxidase are increased above control values in this disease (Rennke and Venkatachalam, 1979b). The results are consistent with the development in the glomeruli of large defects that lead to gross derangement of size, discriminatory function and non-selective proteinuria.

1.04.4 Angiotensin Induced Proteinuria

Infusion of angiotensin II into rats causes proteinuria (Eisenback et al, 1975; Chang et al, 1975b). This proteinuria is attributed to an increase in the amount of protein filtered by the glomerulus (Eisenback et al, 1975). Clearance studies by Bohrer et al (1977) indicate that in angiotensin infused rats the fractional clearance of anionic dextran, neutral dextran, and albumin is increased

above control levels. In all the infused animals there was also an associated decrease in QA without a change in GFR. Bohrer et al (1977) demonstrated that the decrease in QA was also accompanied by an offsetting increase in filtration pressure and rise in FF. The observed increase in the fractional clearance of anionic dextran and albumin can be attributed to the rise in FF, and to the decrease in QA (Brenner et al, 1981). The increase in the fractional clearance of neutral dextran was attributed to a decrease in QA (Deen et al, 1972).

Thus the decrease in QA as well as the rise in filtration pressure and FF would cause an increase in the amount of protein that diffuses across the capillary as well as the amount convected with the filtered water (Brenner et al, 1981). The net result is an increase in the absolute amount of protein in the glomerular filtrate (Rennke et al, 1981).

1.05 Kidney Preservation

More than 60,000 (U.S.A., and Canadian) patients are on dialysis awaiting kidney transplantation, and increasingly human cadavre kidneys are utilized for this. In order to ensure the maximum degree of compatiblity a large organ sharing system is required. This necesitates having techniques available for preserving the kidney in a viable state during the time required for tissue typin, and matching with compatable recipients, preparation of

operating room, and personnel, preparation of recipient, and transport of the organ (when needed).

The preservation methods used affects the graft survival of renal transplants (Opelz and Terasaki, 1977). Cadaver human kidneys, at the present time, are preserved either by machine perfusion or cold storage, or a combination of both (Terasaki et al, 1977). Different methods have been used at different transplant centers, with different and often contradictory results. Some centers report that cold storage is easier and results in superior survival rates (Opelz and Terasaki, 1977). However, other investigators indicate that machine preservation is more advantageous and gives better survival rates (Belzer et al, 1972; Toledo-Pereyra, 1976).

Cold storage preservation by surface cooling was first reported by Ackermann and Barnard (1966). The method is simple and requires the storage of the kidney in an ice-saline slush, to keep the metabolic activity at a minimal level. Kidneys preserved in an ice-saline slush exhibit considerable glomerular and tubular cell damage which consists of gross necrosis of cortex and medulla, cellular destruction in the proximal tubules and massive small-vessel thrombosis (Ackermann and Barnard, 1966; Collin's et al, 1969; Belzer and Kountz, 1970;Collins, 1977). Collins et al (1966) attributed these damages to the use of saline that causes loss of intracellular cations.

In (1969) Collins et al successfully preserved human cadavre kidneys for up to 16 hours by flushing the kidney (to remove blood and to achieve rapid cooling) and then storing the kidney in ice cold-Collins solution. Collins attributed his success to the use of Collins solution (a solution of intracellular-like composition) which is rich in magnesium, potassium (to prevent cellular expansion) and dextrose (an energy source) (Collins et al, 1969). Better preservation with Collins solution was achieved especially after the addition of pharmacological agents such as vasiodilators, membrane stabilizers, and diuretic agents (Collins 1977; Kreis et al, 1977). However, survival rates of kidneys preserved by cold storage in ice cold-Collins solution decreases after 16 hours of storage, with acute tubular necrosis as the most demonstrable histological finding (Satiago-Delpin et al, 1972; Light et al, 1977, McCabe et al, 1977; Noble et al, 1980).

A reduction of acute tubuler necrosis is able to be achieved by pulsatile perfusion of kidneys at 4 C (McCabe et al, 1977). Pulsatile perfusion was initially developed at 37 C using whole blood to deliver the large quantities of disolved oxygen required by kidney cells (10 ml/min/g kidney) (Rosenfeld et al, 1959). However, embolization of glomerular capillary channels and irreversible vasoconstriction occurred, even when defibrinated blood was used (Waugh and Kubo, 1969; Nizet, 1975; Brandani

Pacin and Bocci, 1983). The development of a cell free perfusate enabled kidneys to be preserved for 2 hours at 37 C (Weiss et al, 1959; Fuller et al, 1977; Ross, 1978). However, these kidneys require a perfusion pressure of 400 mm Hg and a flow rate of 20 ml/min/g kidney to ensure an adequate supply of oxygen (Maack, 1980), and this results in edema, acute tubular necrosis and on transplantation their survival rate is very low (Ackremann and Barnard, 1966; Belzer et al, 1967; Belzer et al, 1968, Belzer et al, 1976).

The requirement for high oxygen flow in the absence of whole blood can be overcome by hypothermic pulsatile perfusion with a cell free solution at low pressure (Weiss et al, 1959; Belzer et al, 1976; Belzer et al, 1968; Belzer and Kountz, 1970; Cooperman et al, 1971; Nizet, 1975; Belzer, 1977; Anderson, 1977; Sampson et al, 1977). The oxygen requirement of kidneys preserved by hypothermic pulsatile perfusion is only a fraction (1/20) of an in vivo kidney (Maack, 1980). The perfusion at low pressure with a cell free perfusate reduces cellular damage, and the kidneys can be subjected to longer perfusion periods with less resistance and better flow rates (Steuber et al, 1968; Terasaki et al, 1977; Belzer et al, 1977; Teledo-Pereyra, 1977; Masri,1980; Masri et al, 1981). Moreover hupothermic perfusion retards microbial growth in the perfusate (Cooperman et al, 1971). Continuous

pulsatile perfusion is now the method of choice for long term kidney preservation (over 16 hours).

Structural and functional changes (high proteinuria, high enzyme levels, and low GFR) have been observed in kidneys preserved by hypothermic pulsatile perfusion (Maack, 1980). These have been attributed to mechanical and human errors (Terasaki et al, 1977) and also to the composition of the perfusion fluids that have been used (Filo et al, 1974; Maack, 1980). The most commonly used perfusate is cryo-precipitated plasma which has certain disadvantages:

1) Its preparation is time consuming.

2) There is a small risk of hepatitis transmission to the recipients (MRC report, 1954).

3) Its protein concentration varies from one preparation to the other.

4) There is a possibility of immunological damage to the kidney during perfusion (Filo et al, 1974).

5) High urinary protein content (Maack, 1980). Histologically recognizable damage, resembling that of hyperacute rejection has been reported in kidneys perfused with cryo-precipitated plasma (Clark et al, 1973), presumably due to the presence, in the perfusate, of antibodies directed against kidney cells (Collins, 1977).

Improvement in the perfusate has been achived by using plasma that has been filtered by passing through silica gel (Toledo-Pereyra et al, 1977) or by using a

reconstituted human plasma fraction one (Plasmonate) that contains the albumin, alpha 1 and alpha 2 fractions only (Johenson et al, 1972; Mendez-Picon et al, 1976). These solutions have a defined protein content and concentration and can be kept at room temperature for up to 2 years (Toledo-Peryera et al, 1977). However, kidneys perfused with these solutions still exhibit high levels of proteinuria (Maack, 1980; Bowman and Maack, 1980; Masri, 1980).

1.06 Fibrinogen

Extensive studies have been carried out to establish the physical and molecular properties of the plasma protein fibrinogen.

Different techniques have been deployed in the purification of human fibrinogen, which have included salt fractionation, ether precipitation (Kekwick et al, 1955), ethanol fractionation (Morrison et al, 1948), glycine and ethanol precipitation (Blomback and Blomback, 1956), glycine precipitation (Kazal et al, 1963; Mosesson and Sherry, 1966), polyethylene glycol fractionation (Lackner et al, 1970; Longas et al, 1980). However, most of these methods are time consuming and the final product is usually contaminated (Longas et al, 1980) degraded (Mosesson et al, 1972), or insoluble (Laki and Lorand, 1948). The difficulties in isolating a pure, soluble and undegraded product have affected the reported physical and molecular

properties of fibrinogen. The MW and shape are the most disputed.

Oncley et al (1947) calculated the MW of fibrinogen, purified by ethanol fractionation, to be 400,000 by viscosity and sedimentation constant measurements, and 580,000 with a length of 70 nm, using osmotic pressure measurements. A similar MW was also reported by (Shulman, 1953), for fibrinogen prepared by ethanol fractionation method. Steiner and Laki (1951) reported a MW of 550,000 as determined by light scattering studies, with a diffusion coefficient of 1.52 X 10 cm per sec and a length of 85 nm. A MW of 407,000 was obtained by Scheraga and Mandelkern (1953), using light scattering measurements on an 88 clottable fibrinogen. Similar results were also reported by Hocking et al (1952), using fibrinogen prepared by the Laki method. Kzal et al (1952), reported a MW of 340,000 and length of 65 nm. Shulman (1953), using the same fibrinogen preparation, determined the MW, sedimentation constant and diffusion coefficient from ultracentrifugation studies at 50,000 rpm to be 330,000, 7.95 X 10 sec and 2.01 X 10 cm per sec respectively. Caspary and Kekwick (1954), reported a MW of 330,000 for fibrinogen at concentrations of 1 g/100 ml and 130,000 at concentrations 0.05 g/100 ml, and a diffusion coefficient of 1.97 X 10 cm per sec and a sedimentation constant of 7.60 X 10 sec as determined by ultracentrifugation at 54,000 rpm, for fibrinogen prepared

by ether fractionation. Similar results were reported by Kekwick et al (1955), using ultracentrifugation for fibrinogen prepared by Cohn fractionation. The same results were obtained by Caspary and Kekwick (1957), using the same fibrinogen preparation and the same ultracentrifugation procedure. Palmer et al (1979) concluded that human fibrinogen obtained commercially (Connaught Laboratories), and bovine fibrinogen (Sigma) have a MW of 340,000 and a diffusion coefficient of 2.04 X 10 cm per sec. In 1966, McKee et al, determined the MW and amino acid sequence of human fibrinogen subunits purified from a commercially available preparation (Cutter Laboratories) and they reported a MW of 340,000 for the fibrinogen molecule, made up of 2906 amino acids.

Enzymatic and chemical cleavage of the fibrinogen molecule has indicated that fibrinogen is a dimer made up of two identical halves (Blomback and Yamashima, 1958). Each half is made up of three non-identical polypeptide chains: alpha, beta and gamma, which are held together by disulfide bonds which forms the disulfide knot (DSK). MW of the alpha, beta and gamma chains are 64,000, 57,000 and 48,000 respectively (McKee et al, 1970). Amino acid sequencing of the subunits indicated that the alpha, beta, and gamma chains are made up of 561, 506, and 386 amino acids respectively (Blomback et al, 1976; Doolittle et al, 1979; Watt et al, 1979).

Hydrodynamic, sedimentation and diffusion data prompted Shulman (1953) to propose an ellipsoid multinodular structure for fibrinogen. Electron microscopic observation of fibrinogen revealed a nodular structure, consisting of four linked globules measuring 6 - 8 X 50 nm (Siegel et al, 1953). The multinodular structure was supported by electron microscopy studies of Hall and Slayter, who in (1959) observed the multinodular structure in their specimens that were fixed by formaldehyde and shadowcasted, with platinum. Although most of their molecules appeared to have a trinodular structure, however, they also observed some single globules and dyads, as well as a few higher combinations. Based on the above, Hall and Slayter proposed a trinodular structure for fibrinogen, with two globular spheres at the ends that are 6.5 nm in diameter and one sphere in the center. Supporting evidence for the Hall and Slayter model was reported by Krawkow et al (1972) and by Fowler and Erickson (1979). Although the trinodular model is widely accepted, a sausage-like model with no nodules was suggested by Bachmann et al (1975), a cage-like structure by Kopel (1966), a linear nodular structure by Kay and Caddigan (1967), and a spherical globular model by Stewart (1971) and later supported by Mosesson et al (1979).

Although investigators were able to reveal most of the physical and chemical properties of fibrinogen subunits,

including amino acid sequence, the contradictions in the MW, as well as structure, still exist. These differences have been attributed to difference in purification techniques (Mosesson et al, 1979), contamination (Caspary, 1956), and intrinsic heterogeneity of the fibrinogen molecule as shown by Colvin et al (1954); Ogeston (1955); Finlayson and Mosesson (1963).

1.07 Purpose

The purpose of this thesis is to:

1) Purify and characterize the plasma protein fibrinogen.

2) Investigate the role of fibrinogen in glomerular permeability, and the function of the isolated perfused dog kidney.

1.07.1 Purification and Characterization of Fibrinogen

We have set out to develop a procedure for the purification of human fibrinogen which would be more rapid, and produce fibrinogen of higher purity and yield, than existing methods. The MW of the purified product and its effects on rouleaux formation have been examined. (rouleaux is french for column-like, used clinically to describe the stucture formed when red blood cells reversibly adhere to each other broadside against broadside).

1.07.2 Kidney Preservation

In our laboratory, we detected considerable amounts of protein in the urine of dog kidneys perfused with a

solution that contained albumin, alpha 1, and alpha 2 globulins(plasmonate), but which significantly did not contain any fibrinogen or the gammaglobulin fraction. On investigation, it was discovered that this was a characteristic and consistent phenomenon (Masri, 1980). The significance of this is:

1) Charge repulsion by the anions present in the GBM and/or filtration slit is insufficient to prevent the passage of albumin from the capillary lumen into the urinary space, in the perfused kidneys.

2) The presence of negatively charged molecules, MW> 800,000, is insufficient to prevent the passage of albumin through the GBM of perfused kidneys, since these molecules were demonstrated to be present in the perfusate (Masri, 1980).

We have investigated the significance of these observations by perfusing dog kidneys (dogs were used due to, size, ease of handling, volume of urine produced, ease of perfusion) and determining whether the addition of fibrinogen to the "Plasmafusate" containing perfusate would reduce the proteinuria that occurs. (plasmaafusate is a modified plasmonate containing the same amounts of albumin and alpha 1, and alpha 2 globulins, but contains no thrombin, prothrombin, or plasmin). If so, to determine the mechanism of action of fibrinogen, which may provide evidence for the theory that a protein concentration

polarization layer exists in the subendothelial space that limits the filtration of plasma proteins.

MATERIALS AND METHODS

2.01 Purification of Fibrinogen

2.01.1 Collection and Treatment of Plasma

Venous blood was obtained from volunteers and collected into 50 ml tubes each containing 2.5 ml (10,000 KIU/ml) of the plasmin inhibitor, Trasylol (Miles Pharmaceuticals), and 16.60 mg of sodium citrate (Fisher Scientific).

The plasma fraction was separated by centrifugation and then incubated for 1 hour at room temperature with 2.25 g of solid barium sulfate and 0.125 g of magnesium sulfate to absorb the thrombin (Surgenor and Noertker, 1952). Following centrifugation, the absorption step was repeated on the supernatant a further two times.

2.01.2 Isolation of Fibrinogen

Fibrinogen was isolated by three methods:

(1) Polyethylene glycol 1,000 fractionation.

(2) Column chromatography.

(3) Glycine precipitation.

(1) Polyethylene Glycol 1,000 Fractionation

A polyethylene glycol 1,000 (PEG, Fisher Scientific) fractionation procedure at room temperature was used to prepare fibrinogen from thrombin free plasma in which plasmin activity was inhibited.The plasma was made 10% weight/volume with respect to PEG, by adding PEG with stirring over a period of 10 minutes, and then

centrifuged on a Sorvall (RC2-B) centrifuge. The pre-cipiate, (PII), was dissolved in 50 ml of 0.06 M sodium citrate, 0.15 M sodium chloride buffer at pH 7.4 (citrate buffer), made 7% (W/V) with respect to PEG and centrifuged. The precipitate, (PIII), was again dissolved in 25 ml citrate buffer pH 5.4, made 5% (W/V) with respect to PEG and centrifuged. All centrifugations were carried out at 8,000 to 10,000 g at room temperature. The precipitate, (PIV), was dissolved in a minimum volume of citrate buffer, dialysed against citrate buffer to remove residual PEG, lyophilized and stored at -70 C (Figure 1).

(2) Column Chromatography

Freshly obtained thrombin and plasmin free plasma was fractionated by applying a 3 ml sample to a 2 x 100 cm Sepharose 6B column (Pharmacia), calibrated with thyroglobulin, ferritin, and catalase and eluted with 50 mM potassium phosphate/150 mM sodium chloride buffer, pH 7.4 (PBS). Two ml fractions were collected and tested for clottability. The clottable fractions were pooled and concentrated using an Amicon concentrator fitted with a XM100 membrane, and reapplied to the Sepharose 6B column. The elution profile was monitored at OD 280 nm on a LKB Ultraviolet Flow Analyzer and the molecular weights of the individual components determined from their elution volumes (Figure 2) .

PURIFICATION PROCEDURE

1 liter citrated plasma & 50 ml Trasylol
incubated with 90 g of BaSO$_4$:
5.3 g MgSO$_4$ then centrifuged.

Supernatant is made 10% W/V
with respect to PEG and then
centrifuged.

Precipitate (PI) discarded.

Supernatant discarded.

Precipitate (PII) is dissolved
in citrate buffer pH 7.4 and
then made 7% (W/V) with respect
to PEG and centrifuged.

Precipitate (PIII) dissolved in
0.06 M citrate buffer pH 5.4 and
then made 5% (W/V) with respect
to PEG and centrifuged.

Supernatant discarded.

Supernatant discarded.

Precipitate (PIV) pure fibrino-
gen dissolved in 0.06 M citrate
buffer pH 7.4, dialyzed and
lyophilized.

Figure 1: PEG fractionation of plasma to prepare
fibrinogen:

a. A 50% solution of PEG 1,000 was used at
room temperature.

b. All centrifugations were at 10,000 g at room
temperature.

Figure 2: Elution profile of the purified fibrinogen in PBS buffer pH 7.5 a calibrated Sepharose 6B column (2.5 cm X 100 cm).
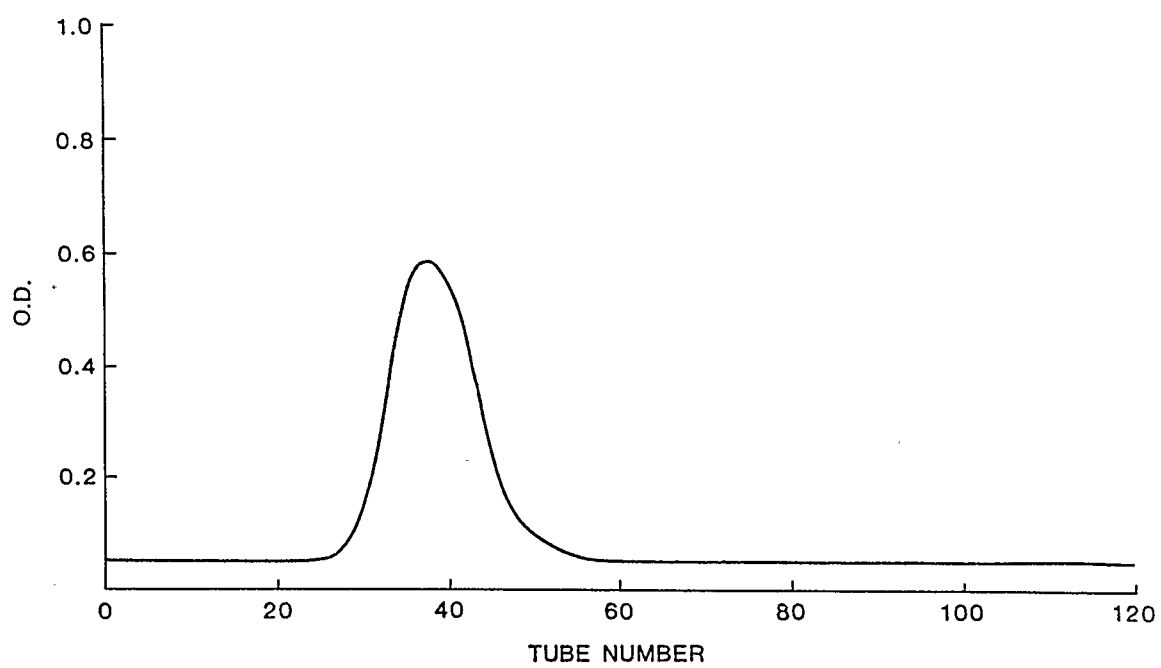
(3) <u>Glycine Precipitation</u>

Glycine precipitation was carried out according to the method of Mosesson and Sherry, (1966).

2.02 <u>Characterization of the Purified Fibrinogen</u>

2.02.1 <u>Recovery</u>

The quantity of the purified fibrinogen that was isolated was determined by the method of Lowry <u>et</u> <u>al</u>, (1951) and the over all yield calculated from the initial fibrinogen content of the plasma, was determined by the method of Reiner and Cheung,(1961).

2.02.2 <u>Purity of Fibrinogen</u>

The purity of fibrinogen was determined by three methods:

(1) Cellulose acetate electrophoresis.

(2) Sodium dodecyl sulfate (SDS) polyacrylamide electrophoresis.

(3) Immunodiffusion in two dimensions.

(1) <u>Cellulose Acetate Electrophoresis</u>

The purification procedure for the isolation of fibrinogen was monitored by performing cellulose acetate electrophoresis on the initial, intermediate and final products, according to the following procedure:

Samples were applied to a cellulose acetate membrane that had been equilibrated in pH 8.5 sodium barbital buffer of 0.075 ionic strength. The membrane was placed in a Beckman Microzone electrophoresis apparatus

and electrophoresis carried out for 20 minutes at 250 volts. The membrane was then stained in a solution of 0.2% Ponceau S, 3% trichloroacetic acid and 3% sulfosalicylic acid, destained in 5% glacial acetic acid, dehydrated in absolute ethanol, cleared in glacial acetic acid;ethanol (3:7) and finally mounted and scanned on a Beckman Microzone Densitometer with an attached integrator.

(2) SDS Polyacrylamide Gel Electrophoresis

Polyacrylamide gel electrophoresis was performed, to evaluate the purity of the isolated fibrinogen and to determine the molecular weight of the individual peptide chains.

To determine the purity of the fibrinogen, a 5% separating gel (Table 1), prepared from stock solution (Table 2), was poured into 0.5 X 12 cm glass tubes (Bio-Rad Modele 150A) and polymerized for 45 minutes. A 2.5% stacking gel, (Table 3), 1 cm thick was layered onto the gel and polymerized for 30 minutes. Seventeen ug of protein in 17 ul of citrate buffer was incubated with 2% SDS (Sigma) and applied to the stacking gel, together with bromophenol blue (0.01%).

For characterization of the peptide chains of fibrinogen, a 0.3 cm thick, 10% separating gel was poured into a 10 X 22 cm glass slab, (Bio-Rad Model 220), and polymerized for 45 minutes. A 2.5% stacking gel, 1cm

Table 1: Composition of the separating gel.

Solution A	10 ml
Solution B	6.67 ml
Water	10 ml
Ammoniumm persulfate	5%

Buffer:

Tris	375 mM
Cl	37.5 mM
SDS	0.1%
TEMED	0.06%

Table 2: Composition of stock solution for SDS gel electrophoresis.

Solution A: Tris   18.15 g

              TEMED   0.24 ml

              SDS   0.4 g

              HCl   To pH 8.8

              Water   To 100 ml

Solution B: Bis   0.8 g

              Acrylamide   30%

Solution C: Tris   3 g

              SDS   0.2 g

              HCl   To pH 6.8

              Water   To 100 ml

Solution D: Acrylamide   10 g

              Bis   0.8 g

              Water   To 100 ml

Solution E: Solution C   4 ml

              Sucrose   1.2 g

              SDS   0.3 g

              Water   1 ml

              Bromophenol blue   0.1 mg

AmmoniumPersulfate solution

              Ammonium persulfate   40 mg

              Water   20 ml

Table 3: Composition of the stacking gel.

Solution C	4 ml
Solution D	1 ml
Ammonium persulfate	2 ml
TEMED	5 ul
Water	1 ml

Buffer

Tris	124 mM
Cl	115 mM
SDS	0.1%
TEMED	0.062%
pH	6.8

thick was then layered onto the slab gel and polymerized for 30 minutes. A sample of fibrinogen was dissolved in a Tris-HCl buffer, pH 6.8, containing 2% SDS, 10% glycerol, 5% 2-mercaptoethanol and 0.001% bromophenol blue, and immersed in a boiling water bath for 2 minutes. A 17 ul aliquot containing 17 ug of the dissociated fibrinogen chains was layered onto the stacking gel.

Following application of the fibrinogen or its dissociated chains to the gel, electrophoresis was performed using a 0.025 M Tris, 0.192 M glycine buffer, pH 8.3 containing 0.1% SDS, and a current of 1 mA per sample for 12 hours. The proteins were then fixed and stained by immersing the gel in 50% trichloroacetic acid and 0.1% Coomassie blue solution for 1 hour at 38 C. The gel was then destained by repeated washing in 7% acetic acid/ 20% methanol at 38 C.

(3) Immunodiffusion

Immunodiffusion in two dimensions was performed according to the method of Ouchterlony, (1968), using goat antisera to human plasma and human fibrinogen (Cappel Laboratories).

2.02.3 Plasmin, Thrombin and Prothrombin Assay

The amount of plasmin, thrombin and prothrombin in the purified fibrinogen was determined using reagent kits supplied by General Diagnostics.

2.02.4 Clottability

The clottability of the purified fibrinogen was determined according to the method of Mosesson and Sherry, (1966). Ten ul of thrombin, (Park Davis) were added to 40, 60 or 100 ul samples of a 1 mg/ml solution of fibrinogen, in order to insure complete clotting, and the time to form a clot determined. The percent clottability was determined using the following formula:

% clottability = 100 (1-OD at 280 nm of the clot

.supernatant/OD at 280 nm of the unclotted

solution).

2.02.5 Sedimentation Coefficient

The sedimentation coefficient, S20,W of the purified fibrinogen was calculated with data obtained from ultra-centrifugation using the sedimentation velocity technique. This was performed using a Beckman Model E ultracentrifuge, fitted with a Schlieren optical system, and also with a Beckman Model L8-80 ultracentrifuge fitted with a UV optical analyzer. All runs were carried out in water at 54,000 rpm for 90 minutes at 20 C. The radial distance (r), in centimeters, of the sedimenting boundary was determined as a function of time (T), in minutes and the S20,W value obtained by inserting the slope, obtained from a plot of ln r versus T, in the equation:

S20,W = 1/w X d ln r/60 dT

where w = angular velocity in radians per second.

To determine the effect of ultracentrifugation on molecular size, fibrinogen samples obtained before and after each run were applied to the calibrated Sepharose 6B column and the elution volume determined.

2.02.6 Diffusion Coefficient

Diffusion coefficient of the purified fibrinogen, fibrinogen solutions containing clotting factors VIII and IX, and perfusate samples obtained before and after perfusion, were obtained from quasielastic light scattering studies that were carried out as described by Palmar et al, (1979).

2.02.7 Molecular Weight Determination

The molecular weight of the purified fibrinogen was determined by three methods:

(1) Column chromatography on a Sepharose 6B column.

(2) Sedimentation equilibrium ultracentrifugation.

(3) High pressure liquid chromatography.

(1) Column Chromatography

To a calibrated Sepharose 6B column, a 1 ml sample of 2 mg/ml fibrinogen in PBS solution was applied, followed by 2 ml of 10 % sucrose in PBS, and eluted with PBS at a flow rate of 20 ml/hour. Fractions of 2 ml were collected while the elution profile was monitored at OD 280 nm.

(2) <u>Sedimentation Equilibrium Ultracentrifugation</u>

MW of the PEG purified fibrinogen was calculated using data from sedimentation equilibrium, which was carried out on a Beckman Model E ultracentrifuge fitted with an Interference optical system. All runs were carried out at 2,800 rpm for 36 hours at 5.9 C.

The fringe displacement (Y) was measured as a function of the radial position (r) in centimeters and a plot of ln Y versus r was made, the slope of which was used to calculate the MW of fibrinogen according to the following:

$$MW = 2RT/(1 - vd) \ w \ X \ dlnY/dr$$

where R = gas constant

   T = absolute temperature

   v = partial specific volume

   d = density of the solution

   w = angular velocity in radians per second.

(3) <u>High Pressure Liquid Chromatography</u>

A 10 ul sample of purified fibrinogen (1 mg/100 ml) was applied to a high pressure liquid chromatography column (Varian) packed with a TS 3,000 type SW gel which had been calibrated with thyroglobulin, myosin and ferritin. The protein was eluted with PBS buffer at 800 p.s.i. and the flow rate of 0.5 ml per minute and the elution profile monitored at 280 nm.

2.02.8 Rouleaux Formation

The ability of the initial , intermediate and final products in the PEG fractionation procedure to cause rouleaux was determined using the following procedure:

Human red blood cells were isolated from 10 ml of fresh blood by centrifugation (I.E.C. Model centrifuge) at 10,000 g for 10 minutes. The plasma layer on top was carefully pipetted off. The red cells were gently shaken with an equal volume of PBS in order to wash the cells. The red cells were sedimented by centrifugation, as above, and the PBS solution carefully removed. The PBS wash was repeated once more and the resulting red cell concentration (4.5 ml) was termed 100% RBC solution. A 1:4 dilution of this stock allowed convenient 20 ul aliquots to be added to 5 ml samples of a 5 mg/ml fibrinogen solutions to yield 0.1% cell suspensions.

An index window was formed from a 22 X 40 mm glass slide, Corning high vacuum grease (silicone lubricant) and a 20 X 20 mm plastic cover slip. A square was edged with grease on the glass coverslip. A drop of sample was placed in the center, covered and sealed with a plastic coverslip. The "index window" was then inverted quickly such that the cells would fall onto the plastic coverslip during the 20 minutes required for the experiment. The inversion is necessary because glass seems to affect cell

shape, whereas plastic seems to have little or no effect on cell shape.

After 20 minutes the samples were observed under a Nikon Model M inverted microscope.

The same procedure was repeated on the initial, intermediate and the final products in the PEG procedure after the addition of 2 ul of the clotting factors VIII and IX to each sample.

2.03 Perfusion Studies

2.03.1 Preparation of the Perfusion Fluid 'Plasmafusate'

Plasmafusate was prepared by PEG 6,000 fractionation (Figure 3). Plasmin and thrombin free plasma was made 10% (W/V) with respect to PEG, with vigorous stirring over a period of 10 minutes and then centrifuged. The supernatant was recovered and pH adjusted to 5.0 with 0.1 N HCl and centrifuged. The precipitate was recovered, dissolved in a minimal amount of 1.45 M sodium acetate buffer containing 850 mM NaCl, and stabilized with 40 mM acetyltryptophan. Buffer was then added to bring the protein concentration to 4.5 g/100 ml and its pH adjusted to 7.0 with solid sodium carbonate, and then stored at + 4 C.

Figure 3: PEG fractionation of plasma to prepare Plasmafusate.

a. A 50% solution of PEG 6,000 was added at room temperature.

b. All centrifugations were at 10,000 g for 30 minutes at room temperature.

1 liter citrated plasma & 50 ml Trasylol
& 90 g BaSO₄ & 5.3 g MgSO₄ centrifuged.

Precipitate PI discarded.

Supernatant (SI) is made
10% W/V with respect to PEG
and centrifuged.

The pH of supernatant (SII) is
adjusted to 5.0 by diluted HCl
and centrifuged.

Precipitate PII discarded.

Precipitate III (plasmonate) is
dissolved in sodium acetate buffer
pH 7.0.

Supernatant (SIII) discarded.
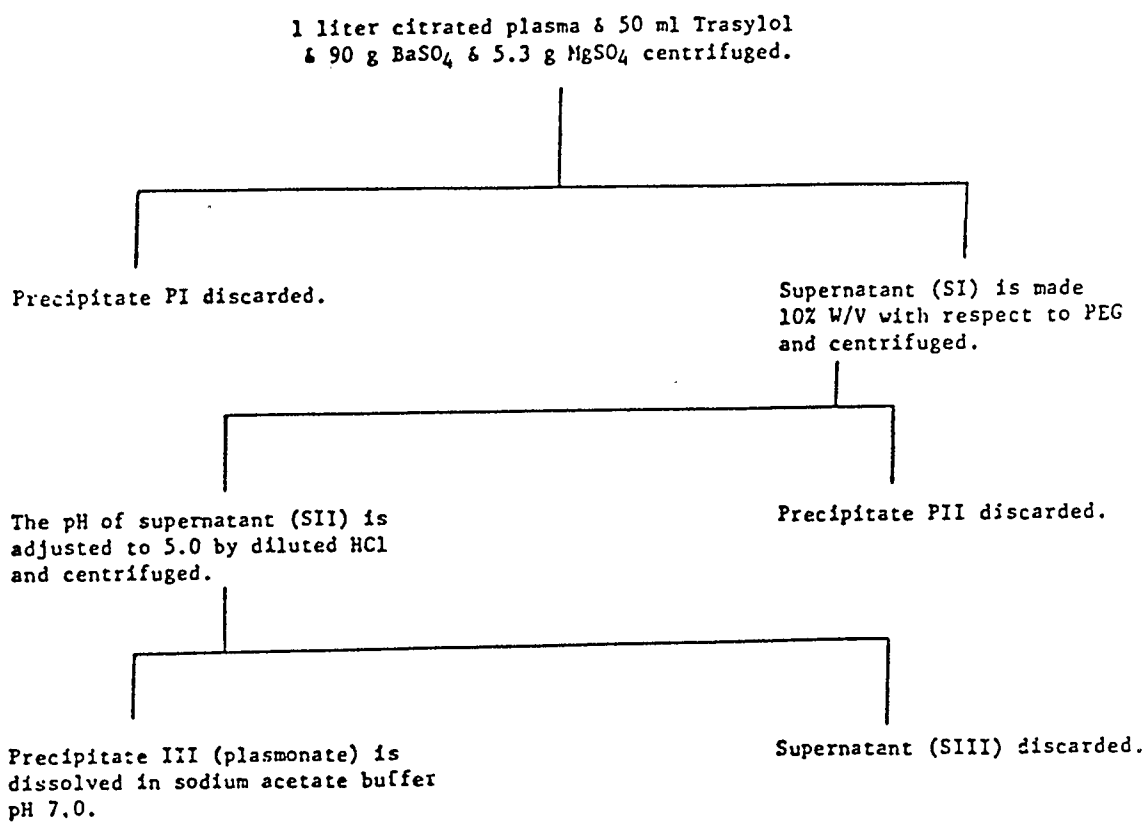
2.03.2 <u>Characterization of Plasmafusate</u>

2.03.3 <u>Protein Concentration</u>

Protein concentration was determined by the method
of Lowry <u>et</u> <u>al</u>, (1951).

2.03.4 <u>Protein Composition</u>

The composition of the final product was determined
by cellulose acetate electrophoresis as described above.

2.03.5 <u>Preparation of Kidneys</u>

Kidneys of 42 dogs, 10 of which served as controls,
were removed and placed in an ice-Collins slush. After
flushing out the kidney with 500 ml Collins solution (Table
4), catheters were introduced into each ureter and the
kidneys attached, two at a time, to a kidney pump (Waters
MOX-100). Ten of the kidneys were rejected, 4 due to flow
rates below 1 ml/min/g kidney and 6 due to very low GFR
values (0.06 ml/min/100 g kidney).

The kidneys were then perfused with a Plasmafusate
containing solution (Table 5) at a pressure of 60 mm Hg
and 60 pulses/minute. The temperature of the perfusate
set at 4 C and the actual observed temperature was 8 C.

2.03.6 <u>Proteinuria and Enzyme Studies</u>

20 kidneys were used for this study, which was
performed as follows:

Urine samples were collected from each kidney every 20
minutes for 4 hours, either without any further additions
to the perfusate or after addition of fibrinogen to give

Table 4: composition of Collins solution.

1) Potassium phosphate dibasic   42 mM

2) Potassium phosphate monobasic 15 mM

3) Potassium Chloride   15 mM

4) Sodium bicarbonate   9 mM

5) Inderal (vasodilator)   2 mg/L

6) Magnesium sulfate   7g/L

7) Dextrose   0.14 M

Table 5: Composition of perfusate.

1.	Plasmafusate	500 ml
2.	NaCl (3%)	40 ml
3.	Dextrose (50%)	5 ml
4.	Penicillin G (500,000 IU/ml)	0.5 ml
5.	Solu Medral (125 mg/2 ml)	1.0 ml
6.	KCl (2 M)	1 ml
7.	Calcium gluconate (0.05 M)	ml
8.	Heparin (1,000IU/ml)	2.5 ml
9.	Regular insulin (100 IU/ml)	0.5 ml
10.	Albumin (250/liter)	50 ml
11.	Mannitol (220 g/liter)	5 ml
12.	Lasix (10 g/liter)	4 ml
13.	NaHCO3 (50 mM)	To pH 7.0

a final concentration of 0.3/100 ml.

The volume of each of the urine samples was measured as well as protein content, percentage of albumin, alpha 1 and alpha 2 globulins present (the only ones present in the perfusate) and the activity of the enzymes, lactate dehydrogenase (LDH), creatinine phosphokinase (CPK) and alkaline phosphatase (ALK Phos).

The same procedure was repeated on 8 kidneys, except the perfusate contained inulin (0.03 g/100 ml).

2.03.7 Hemodynamic and Clearance Studies

The remaining 36 kidneys were used in this study which was carried out as follows:

The kidneys were perfused as above with the perfusate, to which inulin (0.03 g/100 ml) and dextrans (0.1 g/100 ml), of different molecular weights and charges were added.

Urine samples were collected every 30 minutes for 4 hours. Samples of the venous effluent were obtained every 30 minutes for 4 hours. Fibrinogen 0.3 g/100 ml, or dextran, MW 500,000, of different charges were added after the first hour of perfusion. The volume of each of the urine samples was measured and the inulin and dextran concentration determined on both urine and venous effluent.

Biopsy specimens for electron microscopy (EM) and immunofluorescence (IF) were obtained before, and 4 hours after, the start of perfusion.

2.03.8 Preparation of Dextrans of Narrow MW Range

In order to obtain dextrans of low MWs, large MW dextran (500,000) was hydrolyzed according to the method of Chang, et al (1975c) as follows:

Dextran sulphate (Pharmacia) was added to 18 ml of distilled water and 2ml 1N HCl, and the solution was then incubated at 100 C for 40 minutes. The solution was then applied to a S-200 superfine column (5 cm X 50 cm; Pharmacia), calibrated with ferritin, catalase and albumin. Fractions that corresponded to the desired MW were collected and concentrated using an Amicon ultrafiltration cell fitted with an XM 30 membrane (Sigma), which were also used as standards.

2.03.9 Dextran Concentration Measurements

Dextran concentrations were measured according to the method of Chang, at al (1975c) as follows:

To 0.1 ml of urine or perfusate sample, 0.1 ml of 5 % phenol and 50.1 ml of concentrated H_2SO_4 were added and the mixture was then read at 490 nm (Beckman DBGT Spectrophotometer).

2.03.10 Urine Protein Concentration Measurement

Urine protein concentration was measured using the method of Lowry, et al (1951) and the turbidimetric method, of Varley, et al, 1964. The turbidity of the urine-sulfosalicylic acid mixture was measured against standards prepared from human albumin (Figure 4).

2.03.11 Measurement of the Percentage of Urine

Albumin and Alpha1 and Alpha2 Globulins

The percentage of albumin alpha 1, and alpha 2 in the urine was determined using cellulose acetate electrophoresis.

2.03.12 Inulin Concentration Measurements

Inulin concentration was measured using the method of Walser, et al, (1955) as follows:

One ml of deproteinated perfusate or 1 ml of diluted urine (1/100 - 1/200) was added to 0.2 ml 4N NaOH and then incubated at 100 C for 15 minutes in a wax bath. The solutions were cooled and 4 ml of diphenylamine (Diph) reagent added. Diph reagent is made up of 3.5 g Diph + 150 ml of glacial acetic acid + 90 ml HCl. The solutions were then incubated in the wax bath for an additional 30 minutes, cooled, read at 620 nm (LKB Calculating Absorptiometer 7400) and compared to inulin standards.

2.03.13 Deproteination of Perfusate

The perfusate was deproteinated by adding 3.9 ml water, 1 ml $ZnSO_4$ reagent (made up of 100 g $ZnSO_4.7 H_2O$ + 40 ml of 6.25 N H_2SO_4 + H_2O to 120 ml) and 1 ml of 0.75 N NaOH to 0.1 ml of perfusate and the solution then centrifuged at 6,000 rpm in a clinical centrifuge (IEC) and the precipitate discarded.

Figure 4: Standard curve for urinary protein concentration
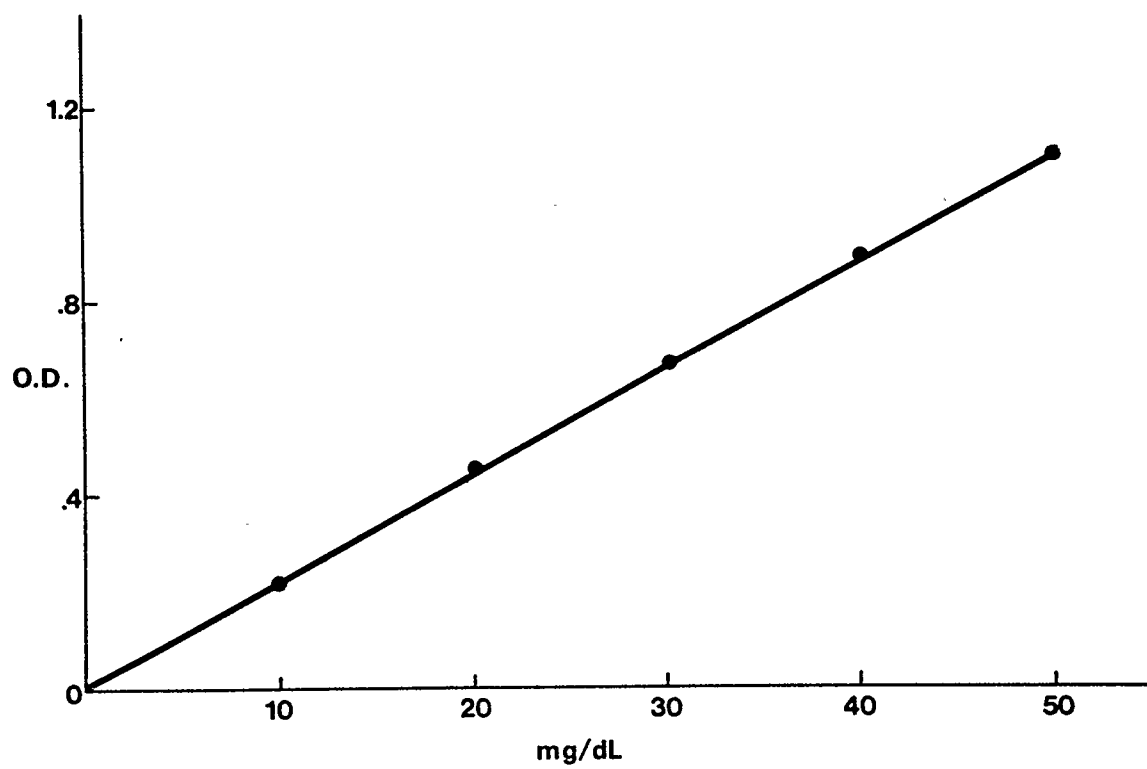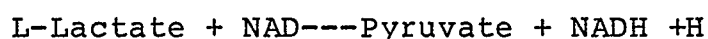
measurements.

2.03.14 Urinary Enzyme Measurements

Enzyme activity was measured using an automated procedure (Gemini II Electronculeonics instrument).

2.03.15 Measurement of Lactate Dehydrogenase (LDH)

LDH enzyme activity was measured using the method of Wacker et al, (1956) and Amador, et al, which is based on the reaction:
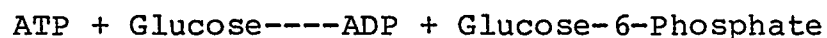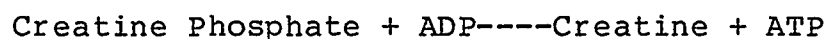
L-Lactate + NAD---Pyruvate + NADH +H

LDH activity is directly proportional to the increase in absorbance of NADH at 340 nm which was measured as follows:

2.7 ml of NADH and 100 ul of urine were incubated at 37 C for 5 minutes. Two hundred ul of sodium pyruvate was then added and the change/minute of the absorbance (A) of the solution was measured at 340 nm. Enzyme activity (E) was then calculated according to the formula:

E in international units/liter (IU/L) = A X 2410

2.03.16 Measurement of Creatine Phosphokinase (CPK)

CPK was measured according to the method of Oliver (1955) and Rosalki (1967), which is based on the reactions:

Creatine Phosphate + ADP----Creatine + ATP

ATP + Glucose----ADP + Glucose-6-Phosphate

Glucose-6-Phosphate + NAD---- 6-Phosphogluconate + NADH+H

The NADH produced in the final reaction is proportional to creatine produced in the initial

reaction. The increase in absorbance of NADH at 340 nm is proportional to CPK activity and is measured as follows:

2.7 ml of adenine triphosphate (ATP) and 100 ul of urine were incubated at 37 C for 5 minutes. Two hundred ul of creatine phosphate was then added and the change/minute of A of the solution was measured at 340 nm. Enzyme activity was then calculated according to the formula:

E = IU/L = A X 2410

2.03.17 Measurement of Alkaline phosphatase (ALk Phos.)

The concentration of ALK Phos. was measured according to the method of Bowers and McComb (1966) which is based on the following reaction:

P-nitrophenylphosphate-----Phosphate + P-nitrophenol

The increase in the A of P-nitrophenol measured at 405 nm is proportional to the ALK Phos. activity which was determined as follows:

2.9 ml of P-nitrophenylphosphate was added to 100 ul of urine and incubated at 37 C for 5 minutes. The change/minute of A of the solution was measured at 450 nm and enzyme activity calculated according to the following formula:

E = IU/L = A X 1600

2.03.18 <u>Renal Plasma Flow Rate (QA)</u>

Renal plasma flow rate was measured using an electromagnetic flow meter (Narco Biosystems Model RT-400).

2.03.19 <u>Glomerular Filtration Rate (GFR)</u>

GFR was determined using the inulin concentration of the perfusate and urine, and the volume of urine collected per minute according to the following equation:

GFR = urine inulin X urine volume/ perfusate inulin.

2.03.20 <u>Filtration Fraction (FF)</u>

FF was measured using QA and GFR values according to the following equation:

FF = GFR/QA.

2.03.21 <u>Fractional Clearance of Dextrans</u>

Fractional clearance of the various dextrans was determined using the concentration of dextrans in urine and perfusate as follows:

Fractional Clearance (C) =urine dextran X urine volume/perfusate dextran X 1/GFR

2.03.22 <u>Electron Microscopy</u>

Kidney cortex cubes of 1 cu mm were fixed in buffered glutaraldehyde and embedded in EPON. Thin sections containing glomeruli were stained with uranyl acetate and lead citrate and examined using a Philips 300 transmission electron microscope.

2.03.23 Immunofluorescence Microscopy

To ensure better sections, excess fluid was removed from the specimens, and the cryostat temperature was set at - 20 C. The specimens were cut and mounted on microscope slides, washed in PBS buffer, and fixed with ethanol/ether (1:1). All sections were then incubated for 30 minutes with flourescence labeled goat anti human fibrinogen (Cappal).

2.03.24 Statistical Analysis

The mean, standard deviation and the 1% test of significance were determined according to the following formulas:

Mean = $M = SX/n$

Standard deviation = $sd = S(X - M)/n$

1% Test of significance = $T = A/sx$

$sx = S(D - A)/n-1 \times 1/n$

Where: S = Sum of

X = Value of an item

n = Number of items or trials

$A = D/n$

$D = (X1 - X2)$

RESULTS

3.01 Isolation Of Fibrinogen.

3.01.1 Cellulose Acetate Electrophoresis.

Cellulose acetate electrophoresis (Figure 5) indicated that a high proportion of the albumin was removed from plasma by the initial treatment with 10% PEG. The remainder was removed with 7% PEG. Use of 5% PEG at pH 4.5 selectively precipitated the fibrinogen, with the other plasma proteins remaining in the supernatant.

3.01.2 Immunodiffusion.

Immunodiffusion in two dimensions confirmed the identity of the isolated fibrinogen (Figure 6) and did not reveal any contamination with other plasma proteins (figure 7).

3.01.3 Prothrombin, Thrombin And Plasmin.

Tests for prothrombin, thrombin and plasmin were negative. Clotting time, in the absence of thrombin, was over 6 hours.

3.01.4 Clottability.

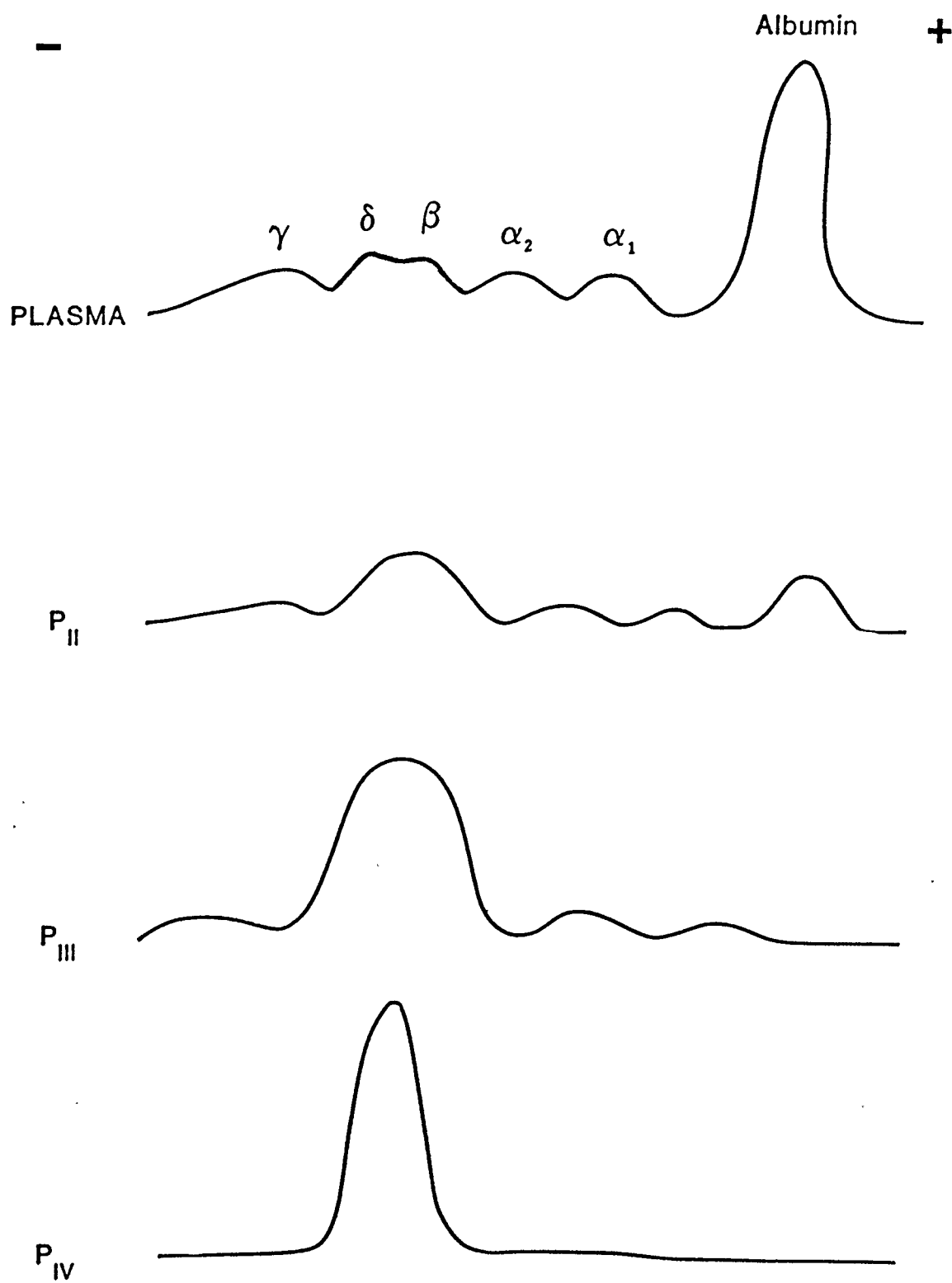The final purified product was 99-100% clottable.

3.01.5 Recovery

The overall yield of the purified fibrinogen was 65-75% of that found in plasma.

3.01.6 Solubility.

The purified fibrinogen was readily soluble in 0.06 M acetate buffer, pH 7.4.

Figure 5: Electrophoresis profile of precipitates obtained
during the PEG fractionation of human fibrinogen
from plasma.

Figure 6: Ouchterlony plate of purified human fibrinogen against goat anti-human fibrinogen with serial dilution of purified human fibrinogen ranging from 1:1 in well 1 to 1:100,000 in well 6, and anti-human fibrinogen in well 7.
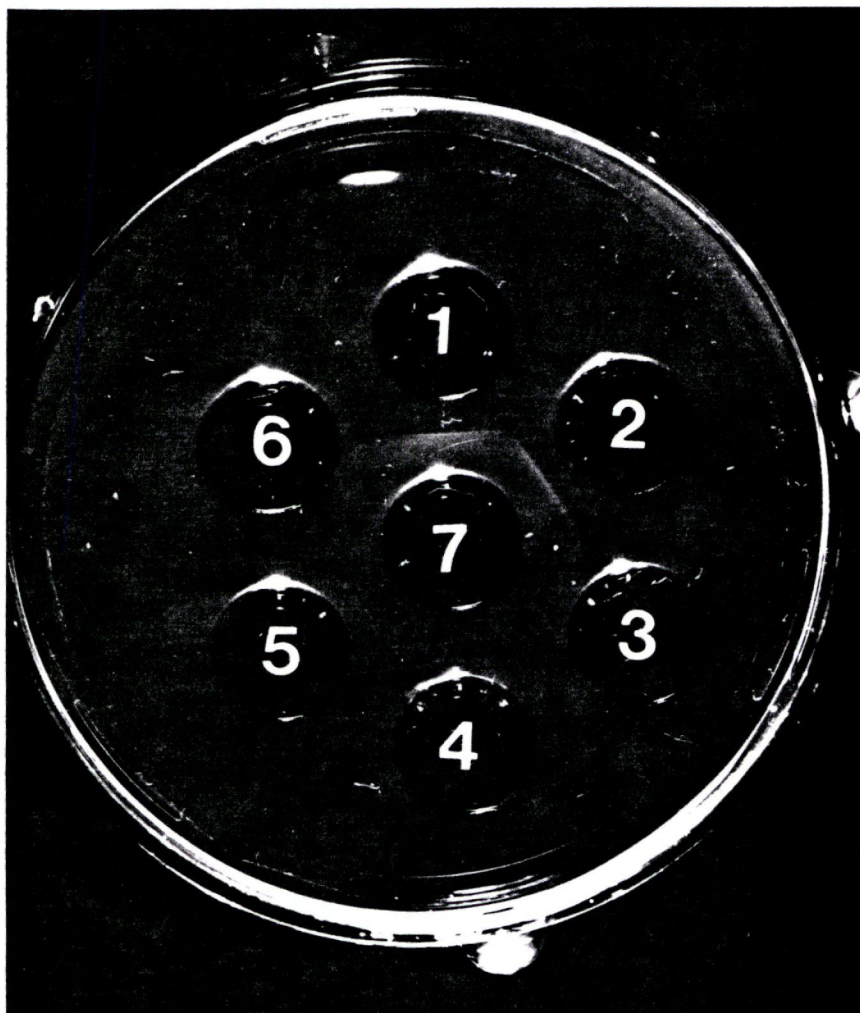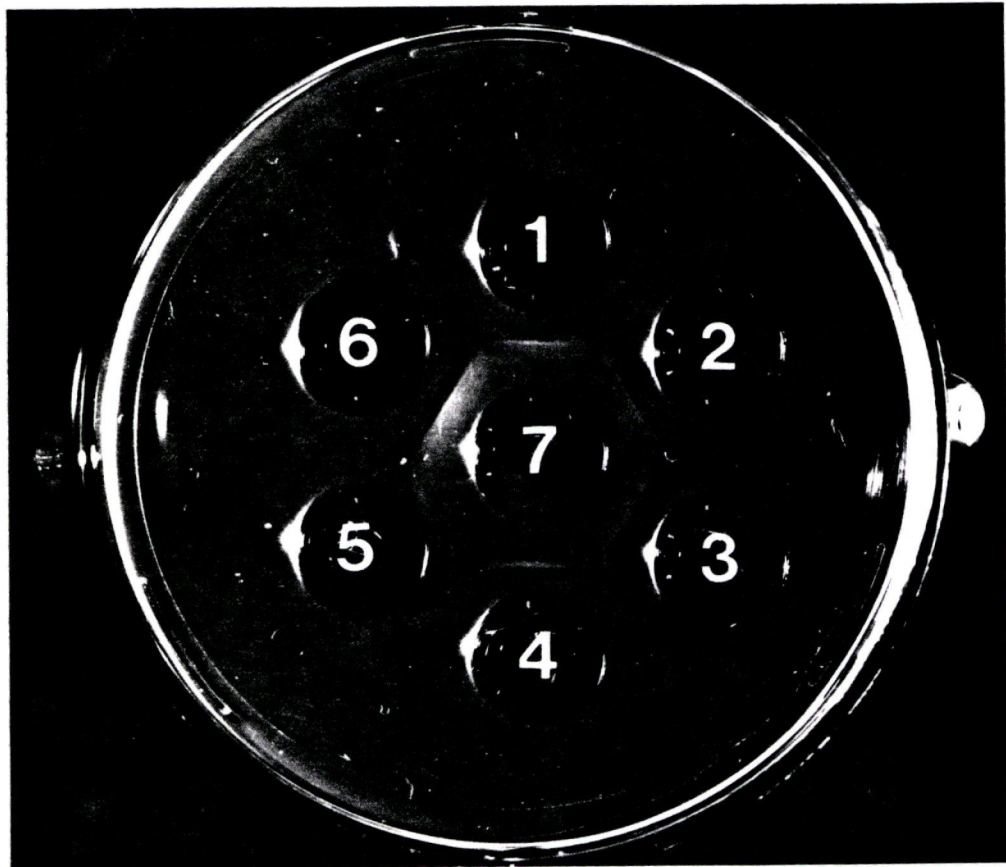
Figure 7: Ouchterlony plate of purified human fibrinogen against anti-human plasma with serial dilutions of purified fibrinogen ranging from 1:1 in well 6 to 1:100,000 in well 5, and anti-human plasma in well 7.

3.01.7 SDS Electrophoresis.

On SDS electrophoresis only a single band was detected, indicating a highly purified product (Figure 8). Exposure of the fibrinogen to mercaptoethanol resulted in its dissociation into 4 components (Figure 9) of MWs 67,000, 56,000, 47,000 and 32,000 respectively (Figure 10) which correspond to the MW of the alpha, beta, gamma and disulfide knot (DSK) chains.

3.01.8 Molecular Weight of fibrinogen

Determination of the MW of the purified fibrinogen from column chromatography (Figure 11), high pressure liquid chromatography (Figure 12), and sedimentation equilibrium gave a value of 680,000 + 10,000 over a concentration range of 1.8 mg/ml to 8.2 mg/ml (Table 6).

3.01.9 Sedimentation Coefficient

When sedimentation velocity measurements were made, it was found that the sedimentation coefficient value, $S_{20,w}$, that was obtained depended upon the protein concentration. At concentrations of 2.5 to 7 mg/ml, two components were observed (Figure 13), a small component of 15.9S and a major component of 7.3S. At concentrations of 1 to 2 mg/ml only one component was detected, with an S value of 7.3. Column chromatography of fibrinogen that had been centrifuged at 54,000 rpm, indicated that centrifugation dissociates the fibrinogen molecule into

Figure 8: SDS polyacrylamide gel electrophoresis of the

PEG purified human fibrinogen using a 5% gel.

1) PEG 1,000 purified fibrinogen.

2)   Commercial   fibrinogen   (Connaught

Laboratories).

3) Cryo-precipitate.
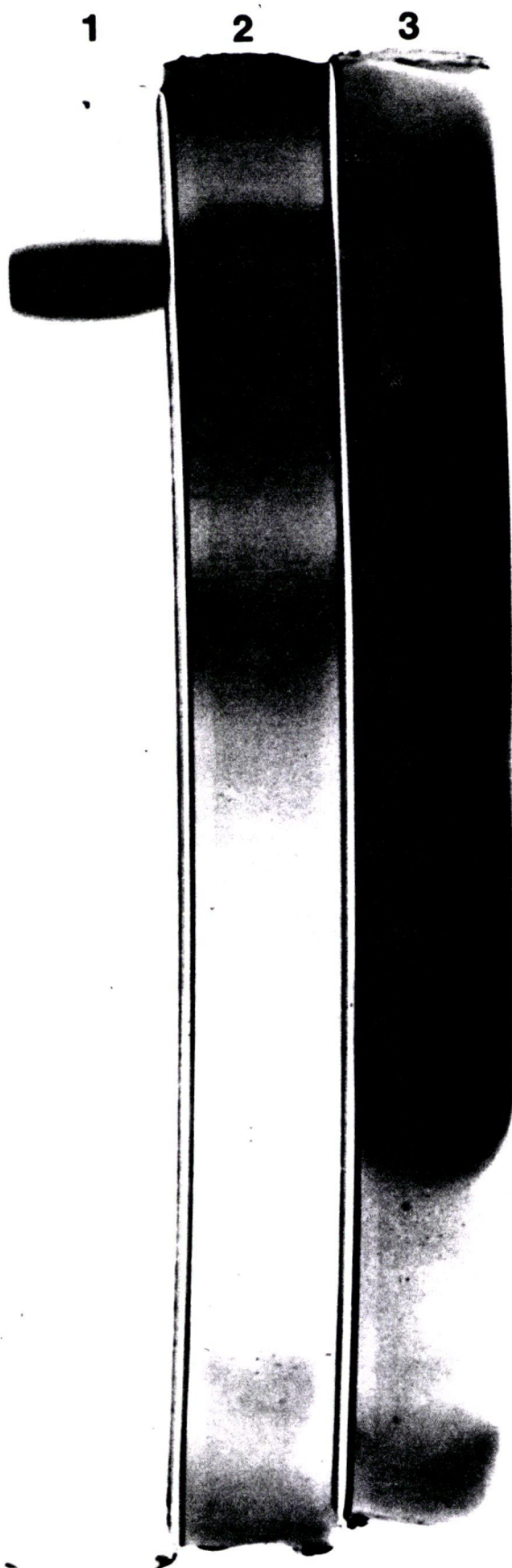
Figure 9: SDS polyacrylamide gel electrophoresis of purified human fibrinogen dissociated at 100 C with 5% mercaptoethenol. 1 and 2 are the fibrinogen subunits. 3 and 4 are MW standards (200,000; 116,000, 70,000 and 25,000).

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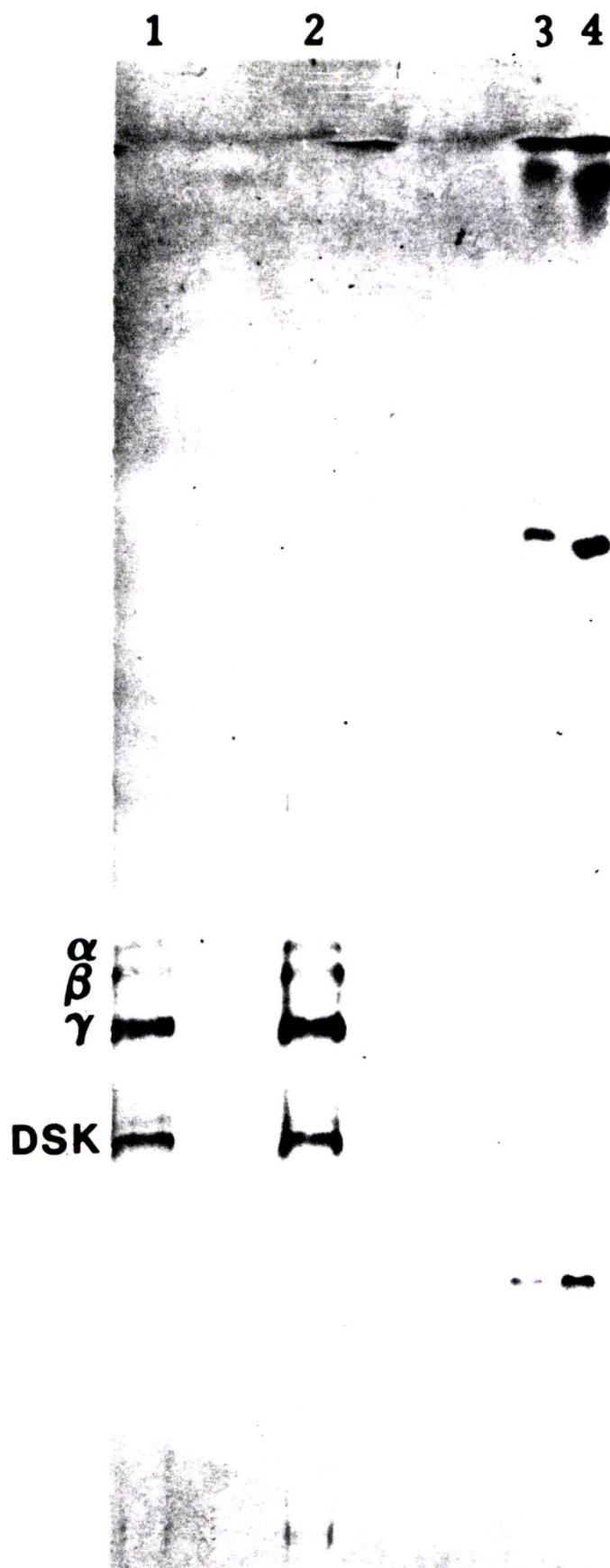
Figure 10: MW determination of the purified human fibrinogen subunits, using values obtained from 10% SDS polyacrylamide gel electrophoresis.
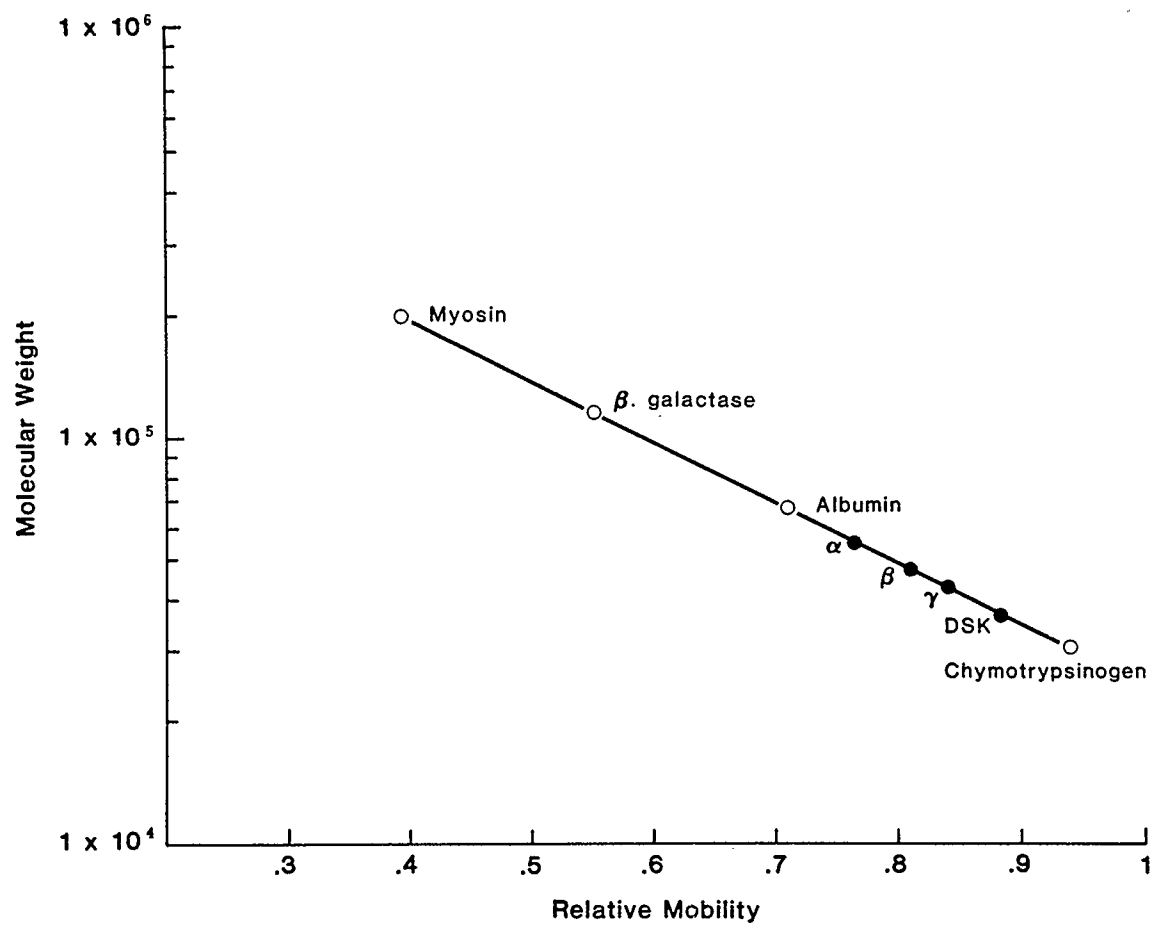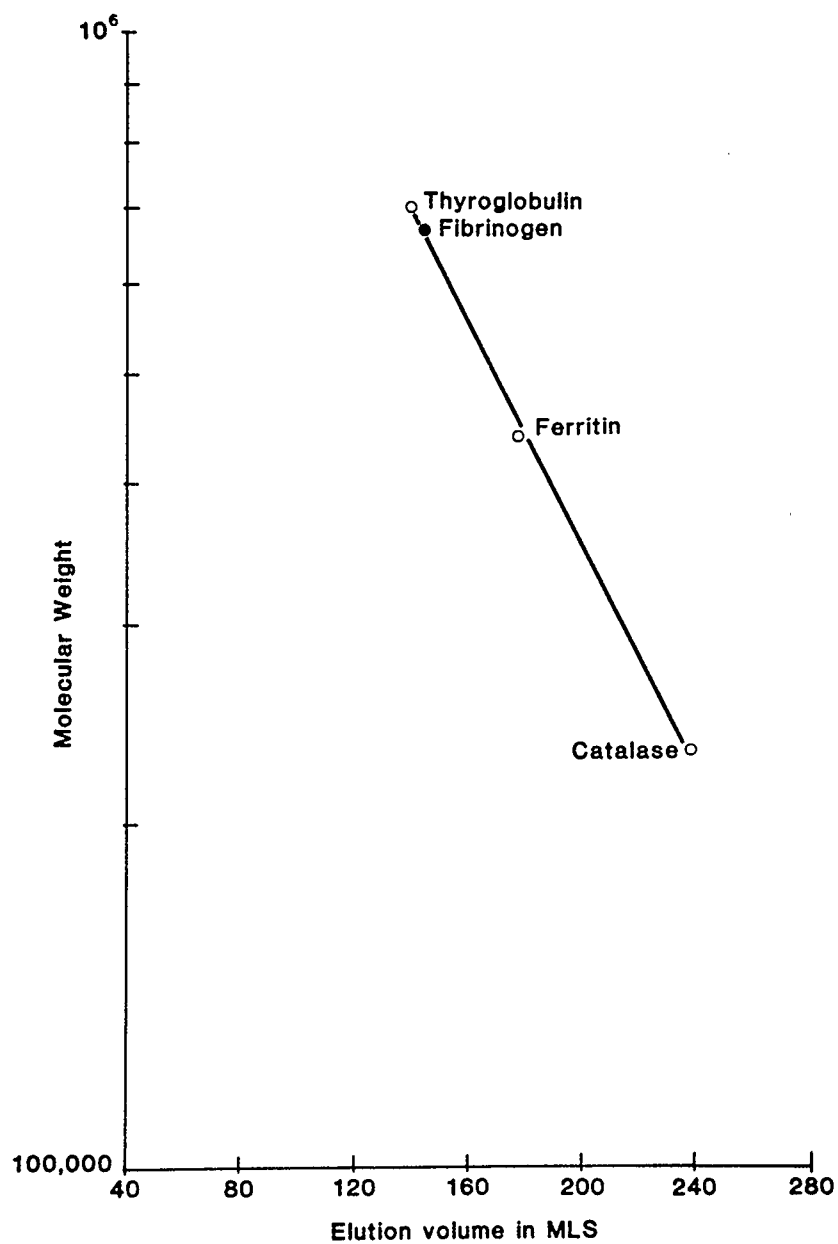
Figure 11: MW determination of the PEG purified fibrinogen
using data obtained from gel chromatography on
a calibrated Sepharose 6B column.

Ve fibrinogen = 97 ml; Ve thyroglobulin = 94 ml

Ve ferritin = 111 ml; Ve catalase = 132 ml

Figure 12: MW determination of the PEG purified human fibrinogen using values obtained by high pressure liquid chromatography on a calibrated TS 3,000 column.

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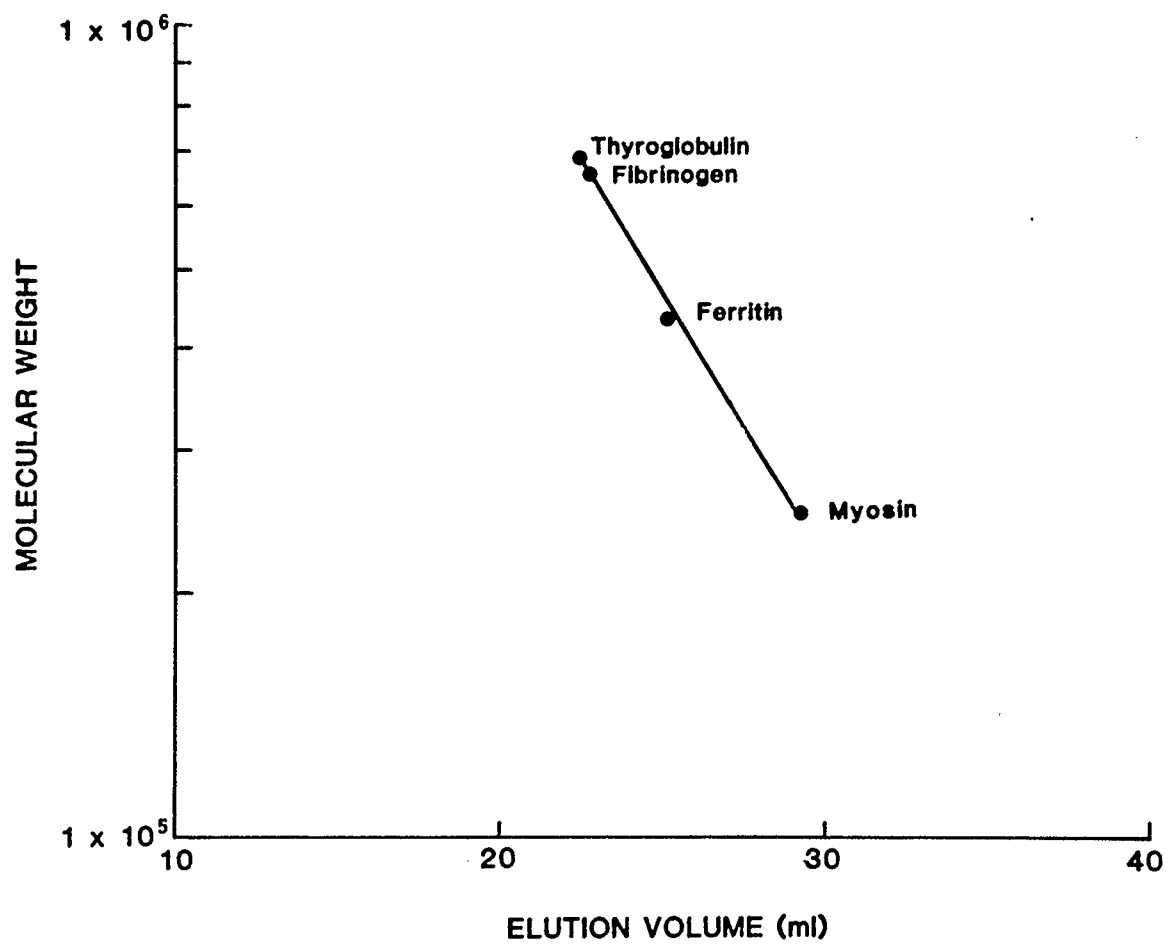
Table 6: Mw determination of the PEG purified human fibrinogen using sedimentation equilibrium data.

Concentration (mg/ml)	MW in Daltons
1.7	650123
2.2	659971
2.5	663465
2.9	668927
3.2	671286
3.7	676141
3.9	678121
4.2	680277
4.7	684355
4.9	686639
5.2	687456
5.8	690987
6.3	690377
6.8	691389
7.4	692224
7.9	692360
8.3	692862

Figure 13: Sedimentation velocity of the PEG purified fibrinogen as seen by means of Schlieren Optical system showing the light and heavy components (S = 15.9 and S = 7.3).

Photos taken at 0, 30, 60, and 90 mins, from 1 to 4 respectivly.

T = Top of cell.

B = Bottom of cell.

Concentration of fibrinogen 3 mg/ml.
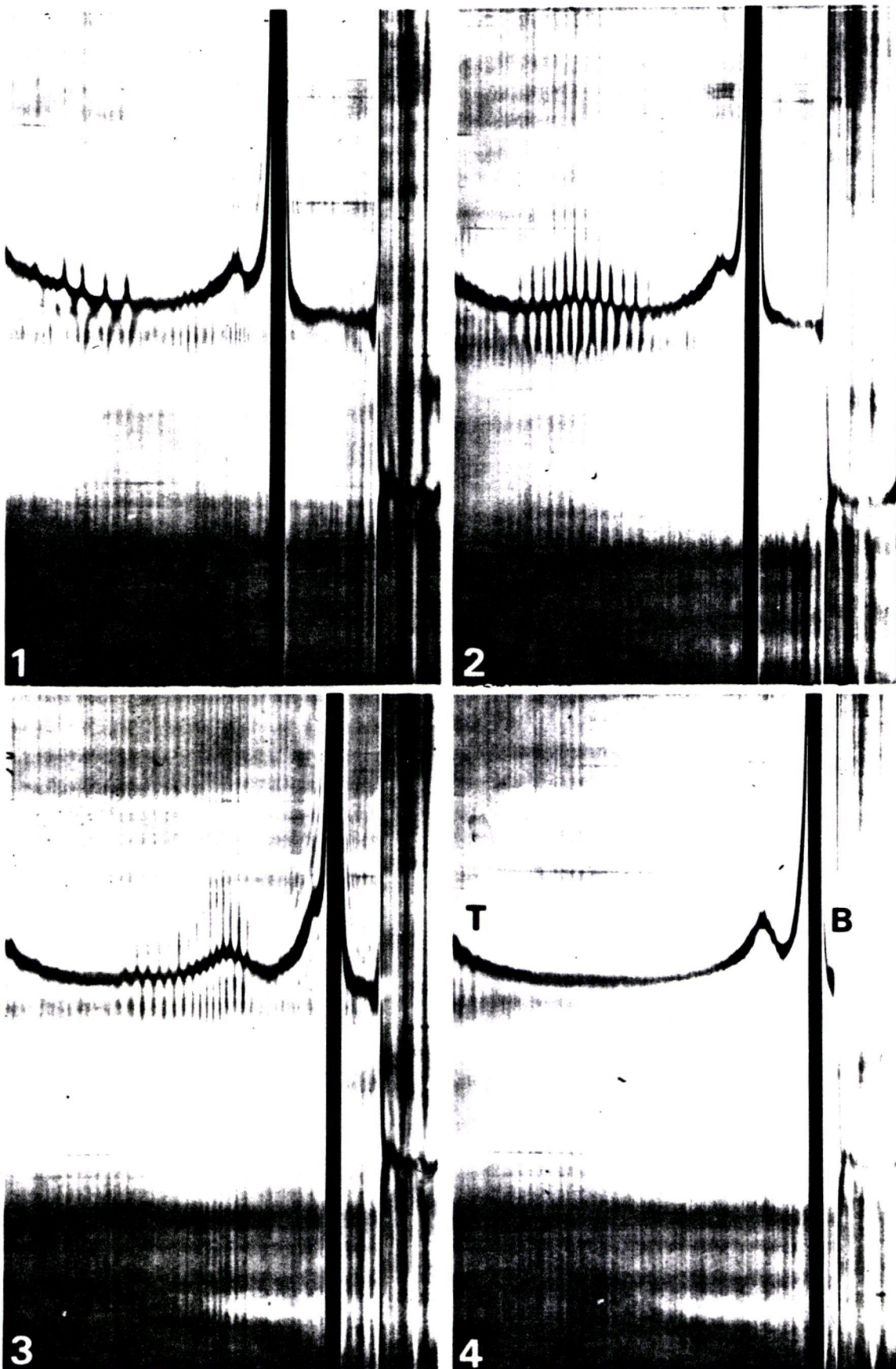
smaller fragments (Figure 14). It was also noted that the degree of dissociation was dependent upon the concentration used, with the greatest effect being observed below 2 mg/ml.

3.01.10 Diffusion coefficient

The diffusion coefficient value of the purified fibrinogen was found to be 2.068 X 10 cm per sec, if the fibrinogen was centrifuged at 54,000 rpm before measurements were made and 1.21 X 10 cm per sec, if the fibrinogen was n at centrifuged. The value of the diffusion coefficient for fibrinogen increased by a factor of 10 following addition of the clotting factors VIII and IX (Table 7).

3.01.11 Rouleaux Formation

The initial untreated plasma caused rouleaux (Figure 15). However, the treated plasma or the final purified product did not cause rouleaux to form (figures 16 and 17) unless the clotting factors, VIII and IX were added (Figures 18 and 19).

3.02 Kidney Perfusion Studies

3.02.1 Characterization of Plasmafusate

Electrophoresis of the plasmafusate indicated that albumin, alpha 1 and alpha 2, to be the only proteins present (Figure 20).

3.02.2 Light scattering studies

Light scattering measurements of the perfusate, were the same before and after perfusion.

Figure 14: Elution profile of the PEG purified fibrinogen, that has been centrifuged at 54,000 rpm for 1 hour on a calibrated Sepharose 6B column (2.5 cm X 100 cm) showing a peak corresponding to a MW of 340,000.

A = Fibrinogen centrifuged at 54,000 rpm.

B = Native PEG purified fibrinogen.

Concentration of fibrinogen = 1.5 mg/ml.
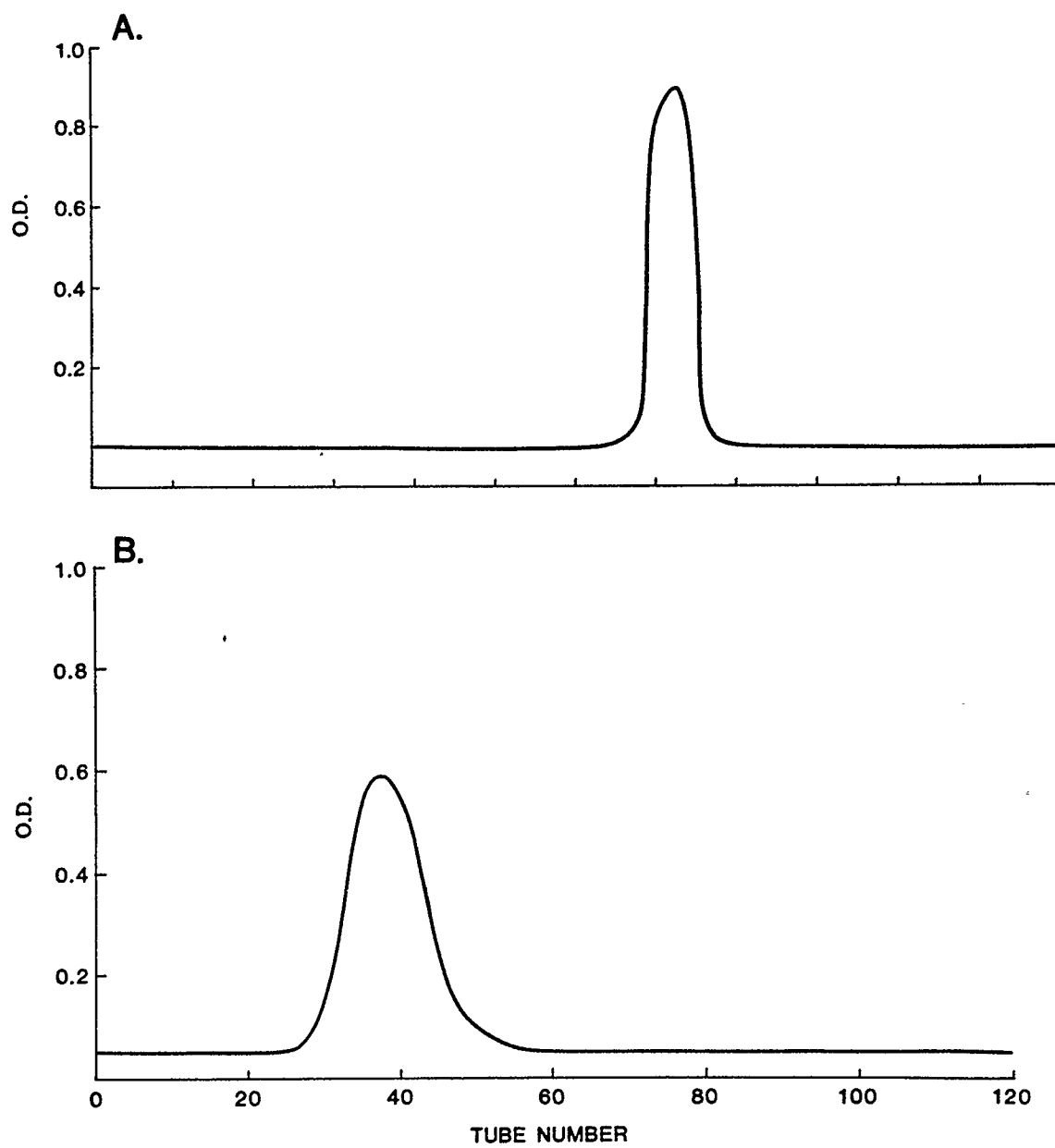
Table 7: Diffusion coefficient values of fibrinogen before
and after clarification at 54,000 rpm, and PEG
purified fibrinogen to which clotting factors
VIII, and IX were added.

Angle	30	40	50	60
A	1.02X10	1.06X10	1.01X10	1.23X10
B	2.09X10	2.06X10	2.08X10	2.10X10
C	1.8X10	1.9X10	3.1X10	3.5X10
D	2.3X10	2.7X10	2.5X10	2.9X10

a. A = Fibrinogen.

   B = Clarified fibrinogen.

   C = Fibrinogen and factor VIII.

   D = Fibrinogen and factors VIII and IX.

b. Values are expressed as per cm per sec.
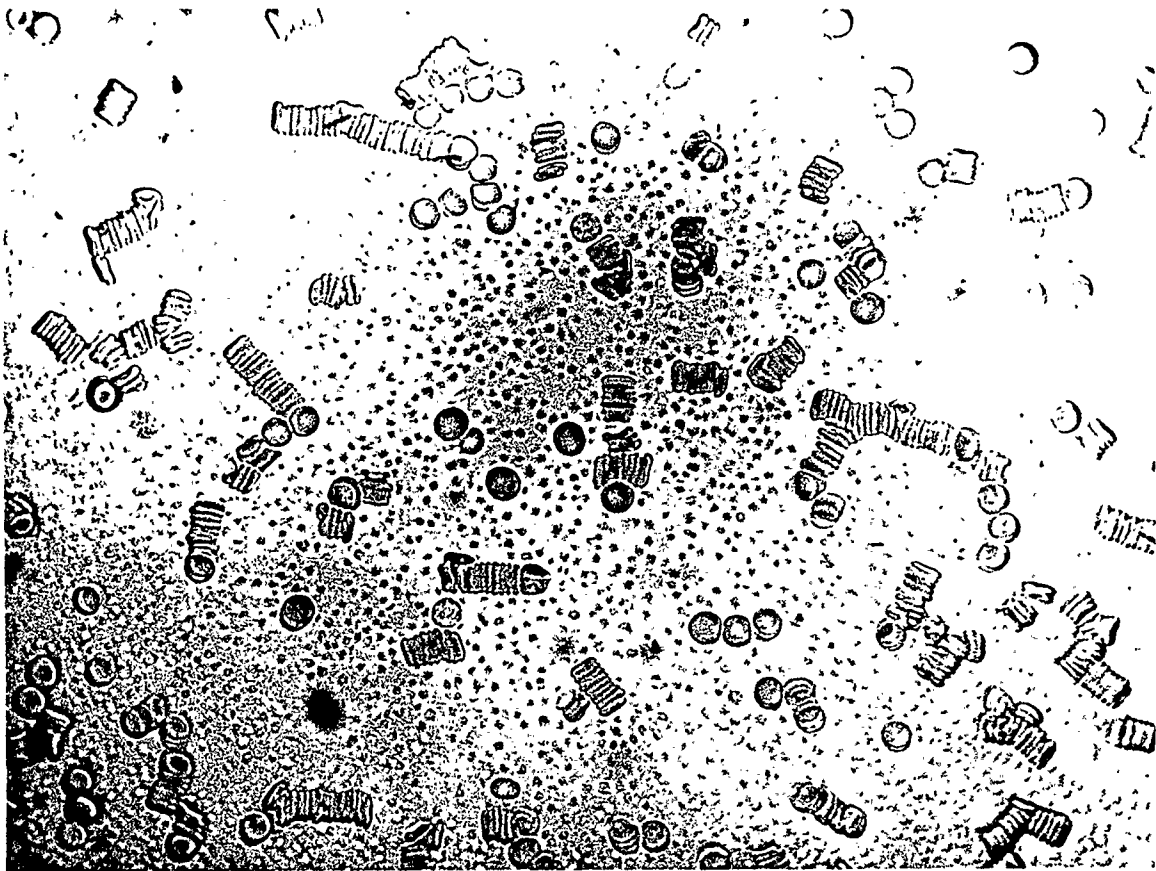
Figure 15:A photograph of red blood cells and untreated
plasma showing rouleaux formation (Mag. X 100).
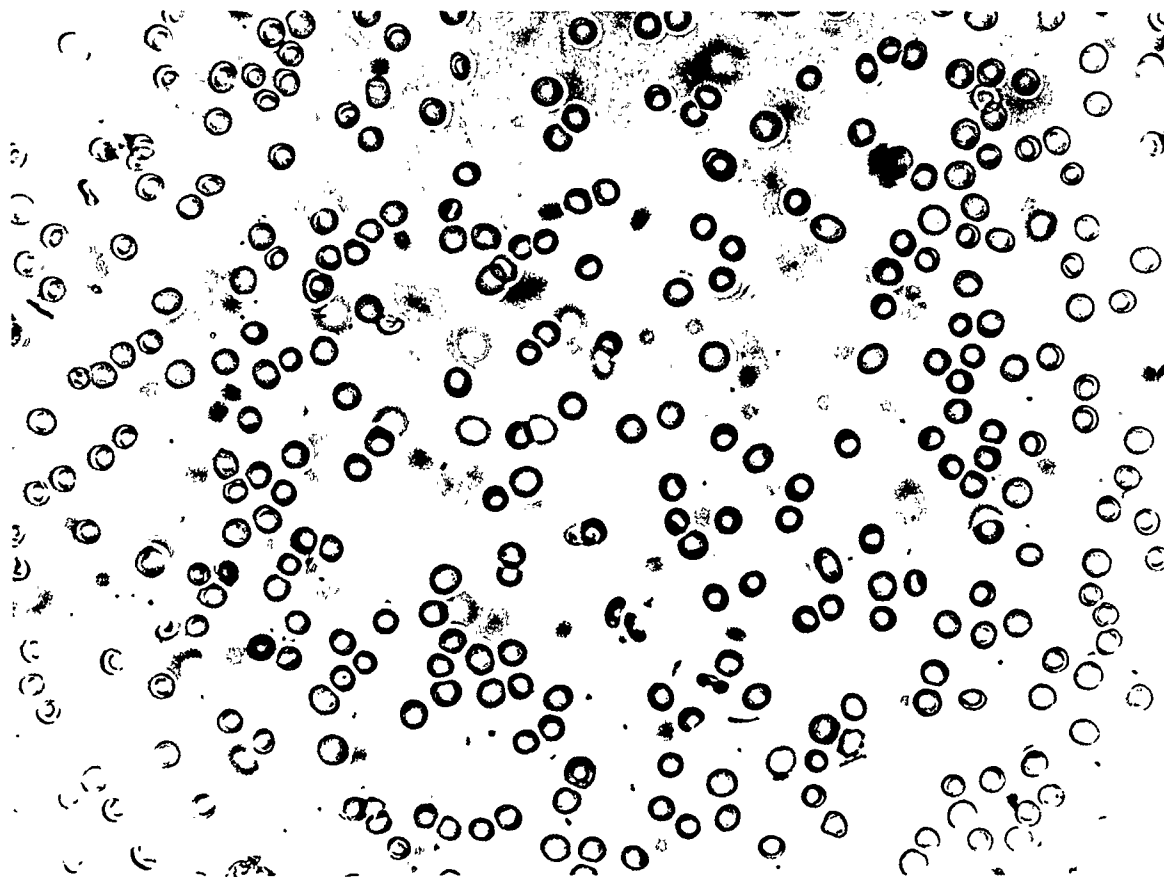
Figure 16: A photograph of red blood cells and treated plasma showing no rouleaux formation (Mag. X 100).
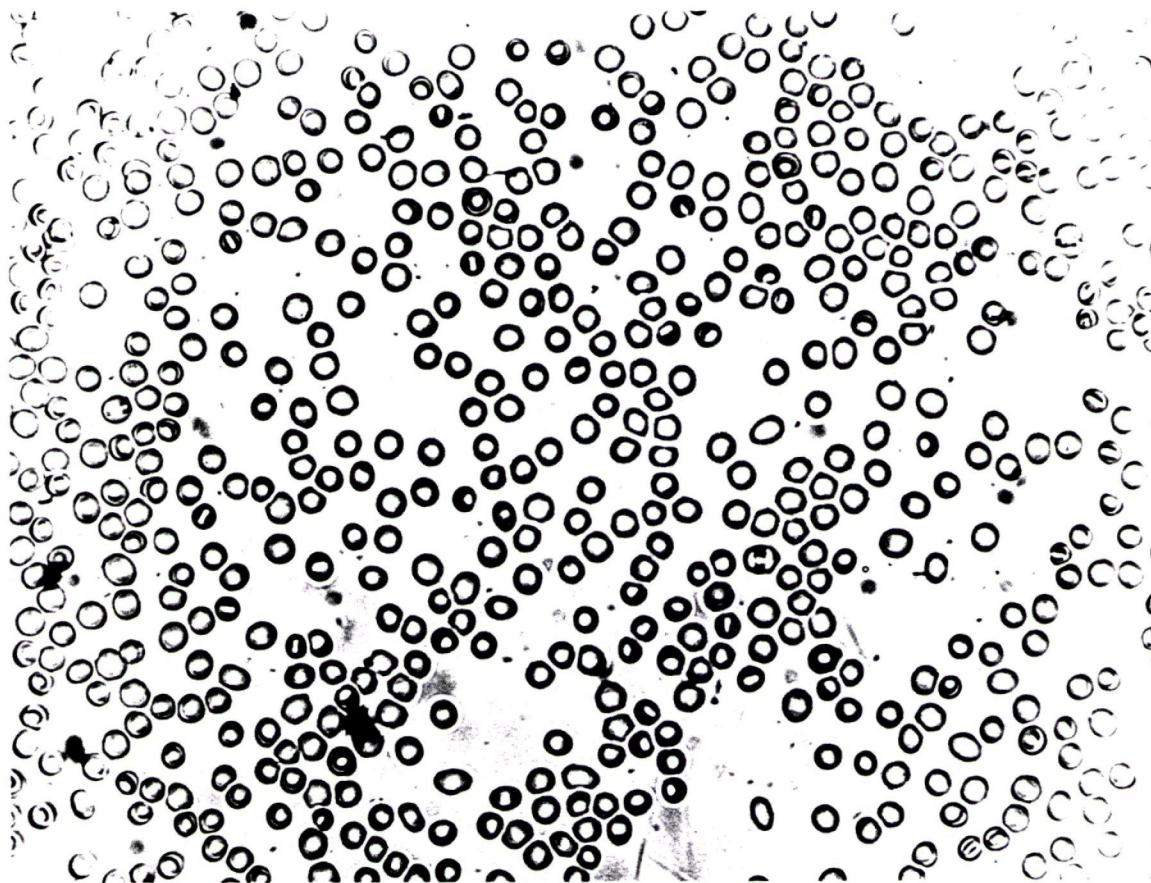
Figure 17: A photograph of red blood cells and purified human fibrinogen showing no rouleaux formation. (Mag. X 100).
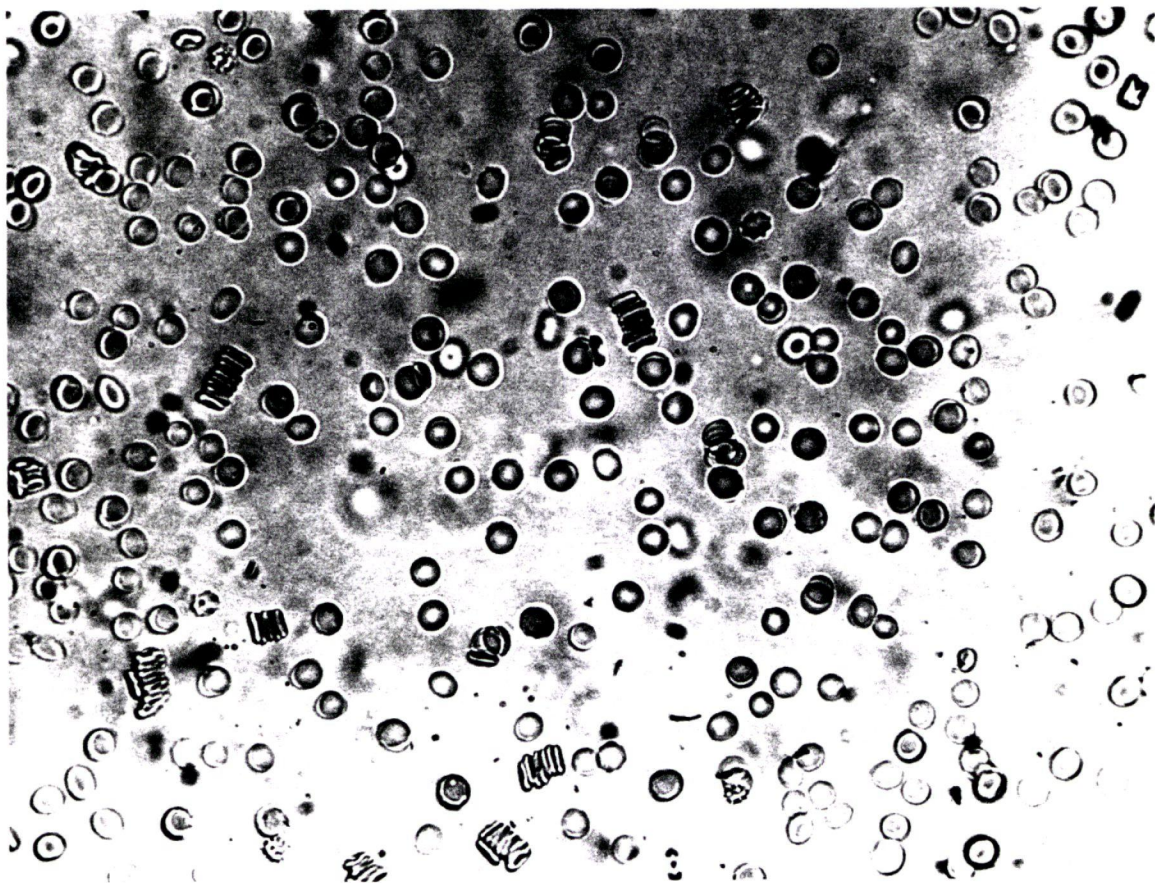
Figure 18:A photograph, indicating rouleaux formation, of
red blood cells and treated plasma to which
clotting factors VIII and IX were added (Mag.
X 100).
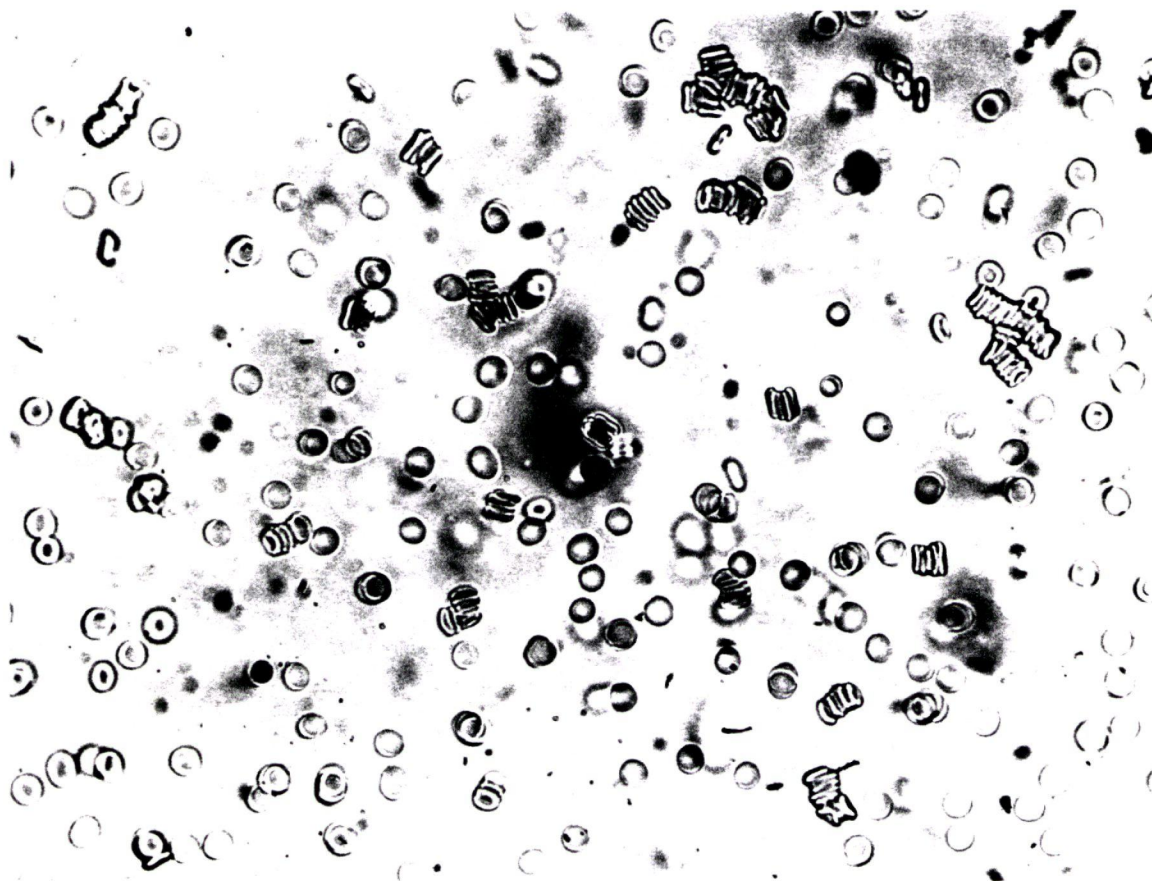
Figure 19: A photograph, indicating rouleaux formation, of red blood cells with fibrinogen to which clotting factors VIII and IX were added (Mag. X 100).

Figure 20:Densitometer   tracing   of   cellulose   acetate

electrophoresis   of   plasma   and   the   prepared

plasmafusate.

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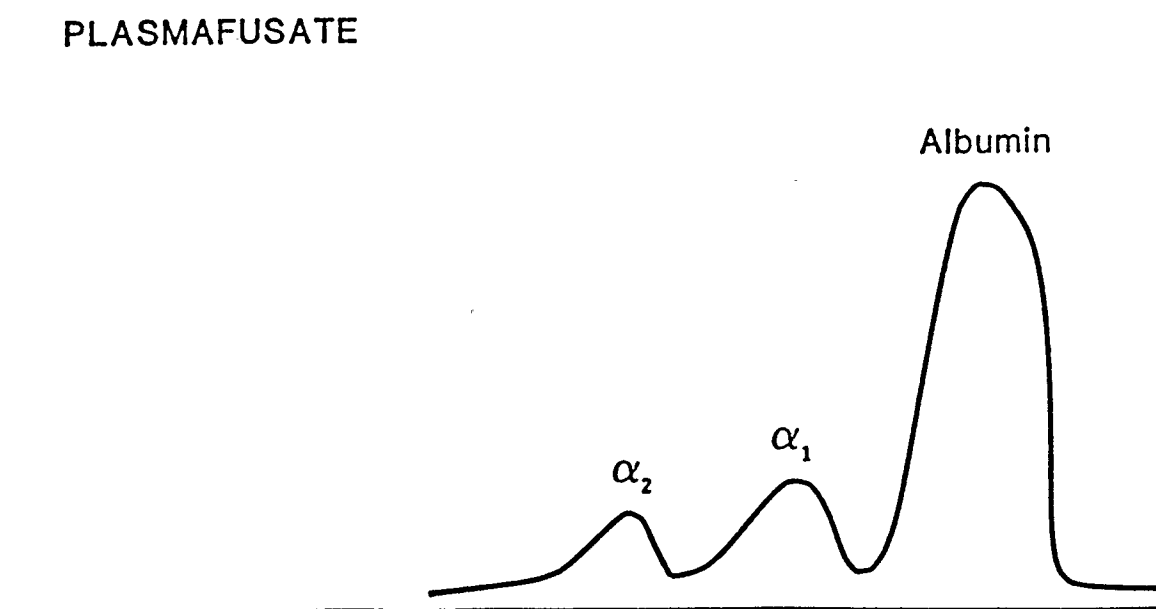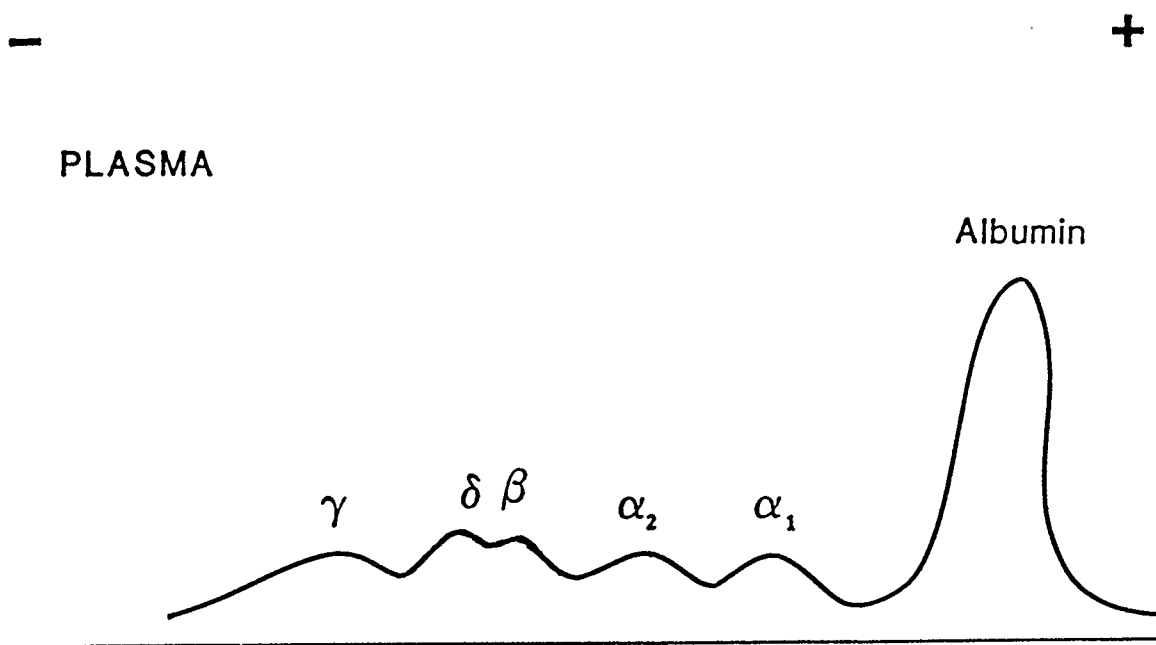
3.02.3 Proteinuria

A significant decrease in proteinuria was observed after the initial addition of fibrinogen. Further increases in the fibrinogen concentration up to a level of 0.3 g/100 ml (same level as in normal plasma) resulted in a further decrease in proteinuria (Figure 21).

3.02.4. Urine Enzymes

The level of urine LDH, CPK, and ALK Phos enzymes declined sharply with the increase in fibrinogen concentration. A further increase in fibrinogen concentration, up to a level of 0.3 g/100 ml, resulted in a further decrease in enzyme levels (Figures 22, 23, & 24).

3.02.5 Urine Flow

Urine flow rate increased significantly after the addition of fibrinogen to the perfusate (Figure 25).

3.02.6 Glomerular Filtration Rate

GFR was found to be 4 + 0.5 ml per minute/100 g kidney. This increased in the presence of fibrinogen to 7 + 0.4 ml/minute/100 g kidney (Figure 26).

3.02.7 Electrophoresis of the Urine

Electrophoresis of the urine indicates that in the presence of fibrinogen the proteinuria observed is selective, in contrast to what is observed in its absence, with albumin accounting for virtually all the protein present (Figure 27).

Figure 21:A plot of urine total protein vs. time for dog kidneys maintained on a kidney pump, with or without fibrinogen in the perfusate (values are expressed as per 100 g kidney; mean + standard deviation).

1% test of significance:

at 110 P < 0.001

at 140 p < 0.001

at 170 and up to 230 P < 0.001

degree of freedom = d.f. = n − 1 = 6
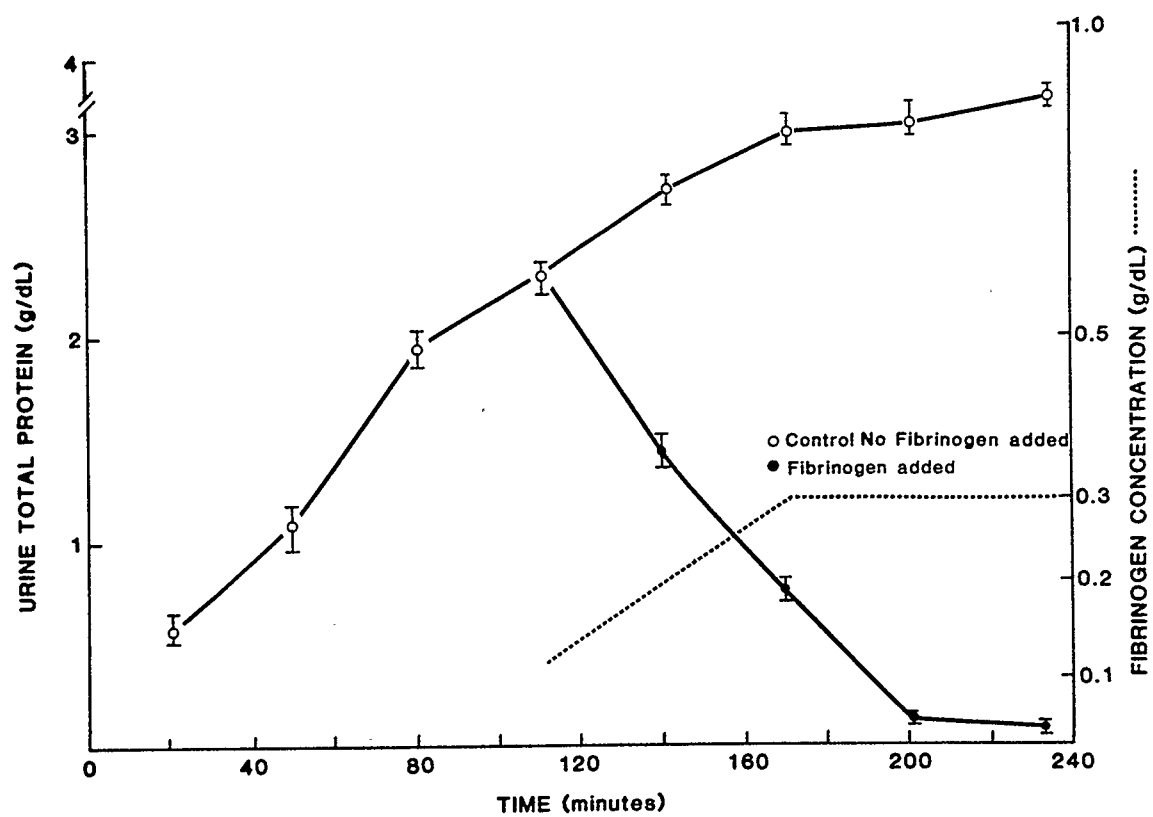
Figure 22:A plot of urine LDH enzyme levels vs. time for

dog kidneys maintained on a kidney pump, with

or without fibrinogen in the perfusate (values

are expressed as per 100 g kidney; mean + standard

deviation).

1% test of significance:

at 110 P < 0.001

at 140 P < 0.001

at 170 and up to 230 P < 0.001

d.f = 6
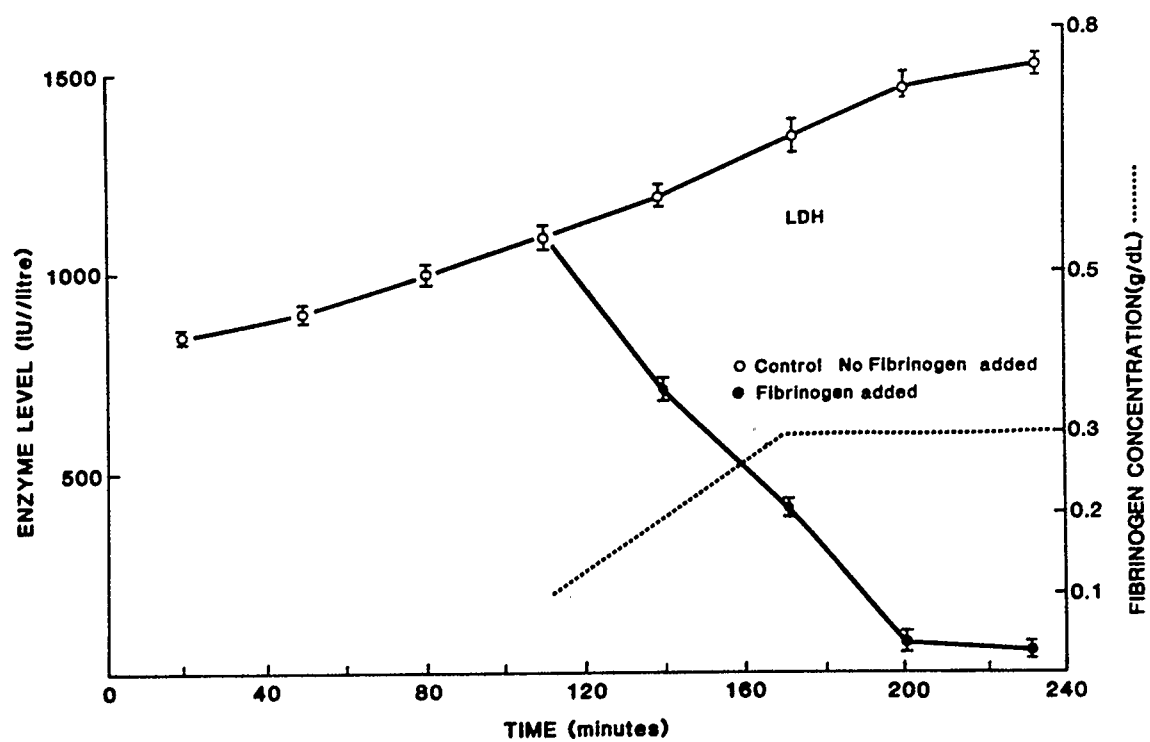
Figure 23:A plot of urine CPK enzyme levels vs. time for
dog kidneys maintained on a kidney pump, with
or without fibrinogen in the perfusate (values
are expressed as per 100 g kidney; mean + standard
deviation).

1% test of significance:

at 110 P < 0.001

at 140 P < 0.001

at 170 and up to 230 P < 0.001

d.f. = 6
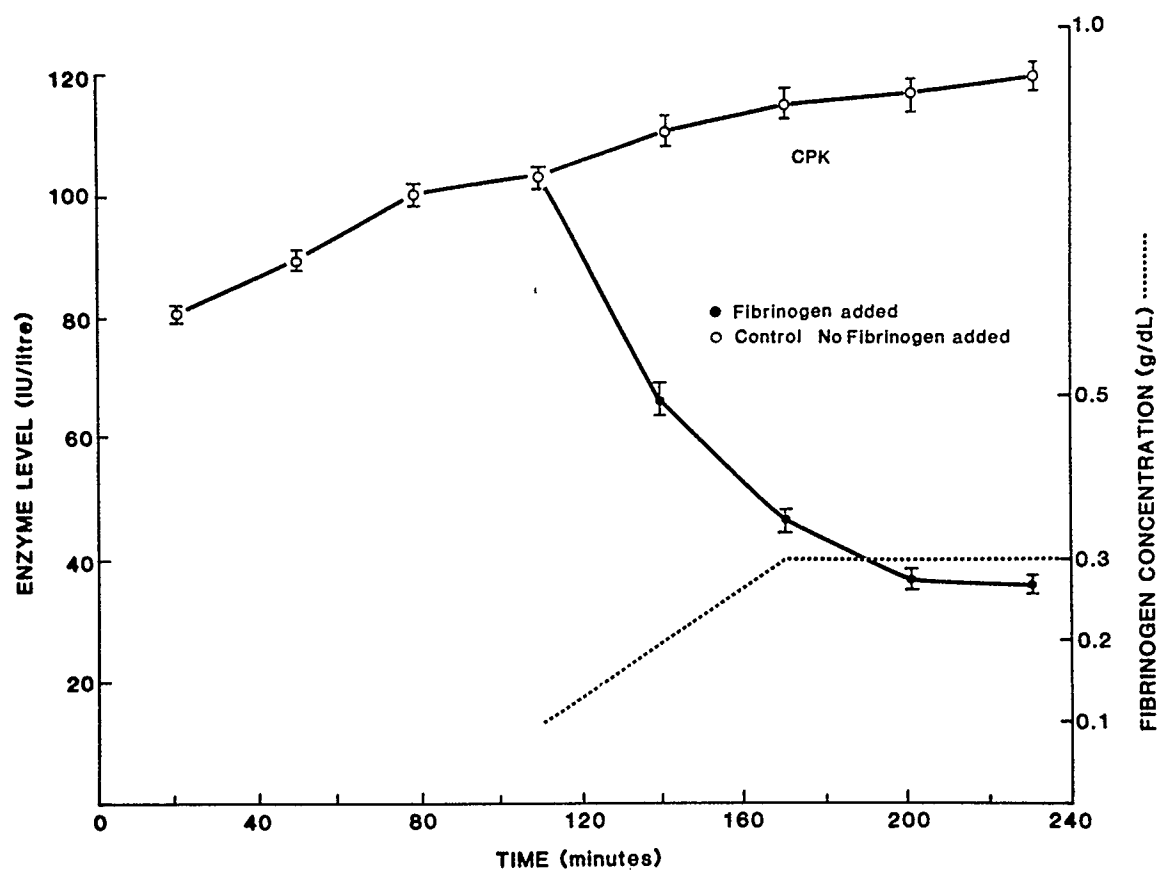
Figure 24:A plot of urine ALK Phos enzyme levels vs. time

for dog kidneys maintained on a kidney pump,

with or without fibrinogen in the perfusate

(values are expressed as per 100 g kidney; mean

+ stamdard divation).

1% test of significance:

at 110 P < 0.001

at 140 P < 0.001

at 170 and up to 230 P < 0.001

d.f. = 6
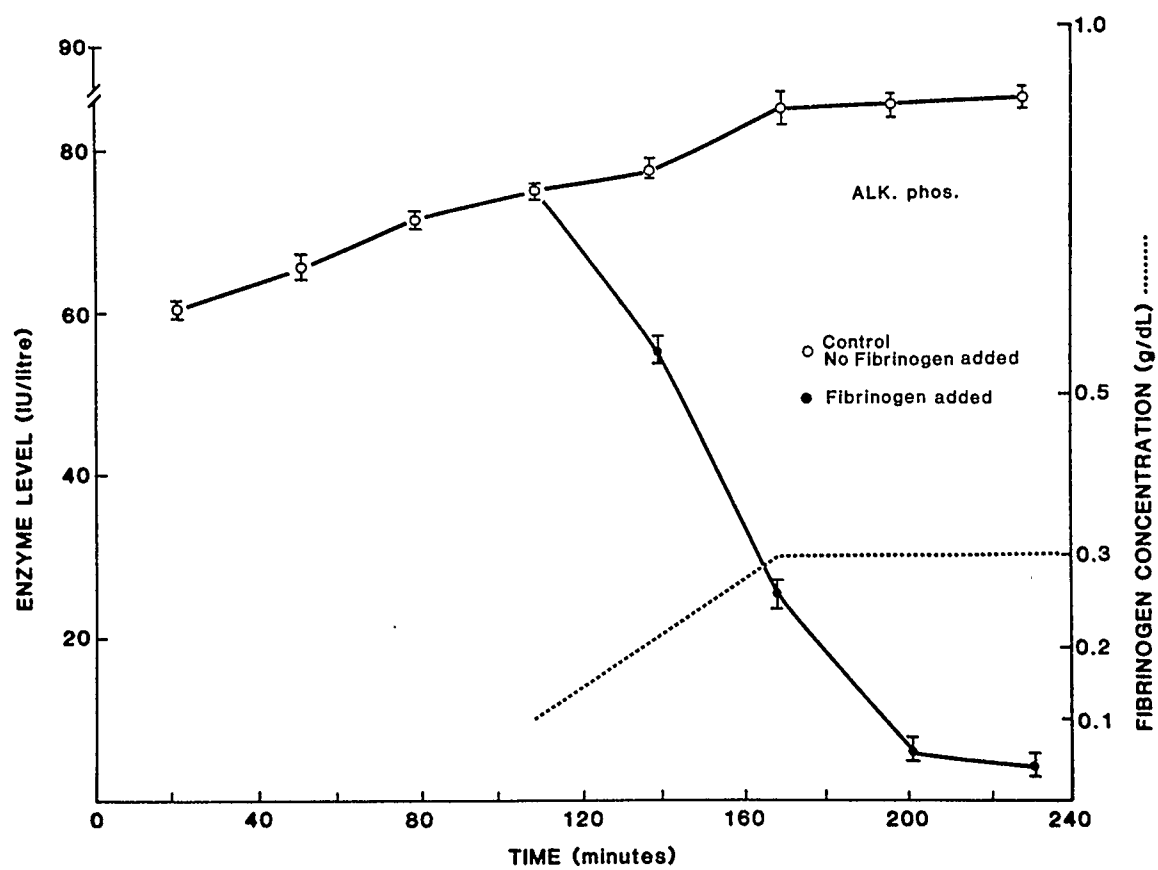
Figure 25: A plot of urine volume vs. time for kidneys
maintained on a kidney pump, with or without
fibrinogen in the perfusate (values are expressed
as per 100 g kidney; mean + standard divation).

1% test of significance:

at 110 $P < 0.001$

at 140 $P < 0.001$

at 170 and up to 230 $P < 0.001$

d.f = 6
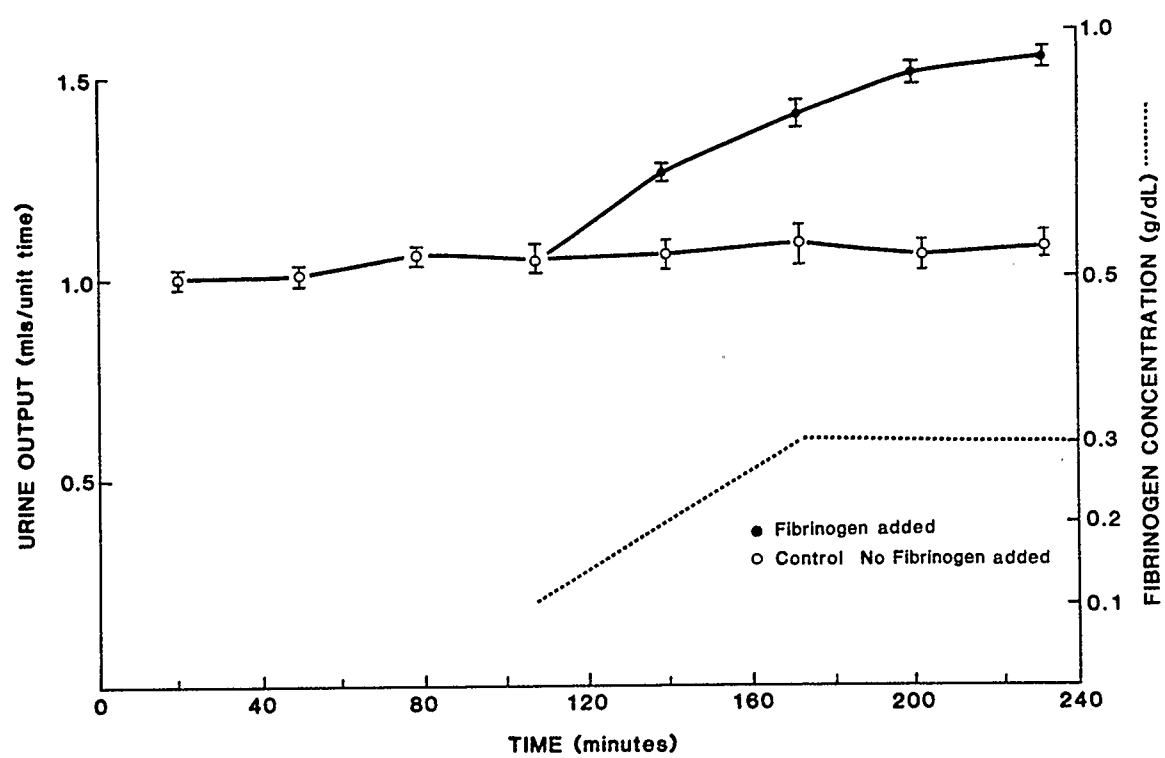
Figure 26: A plot of GFR vs. time for dog kidneys maintained
on a kidney pump, with or without fibrinogen in
the perfusate (values are expressed as per 100
g kidney; mean + standard deviation).

1% test of significance:

at 110 P < 0.001

at 140 P < 0.001

at 170 and up to 230 P < 0.001

d.f = 6
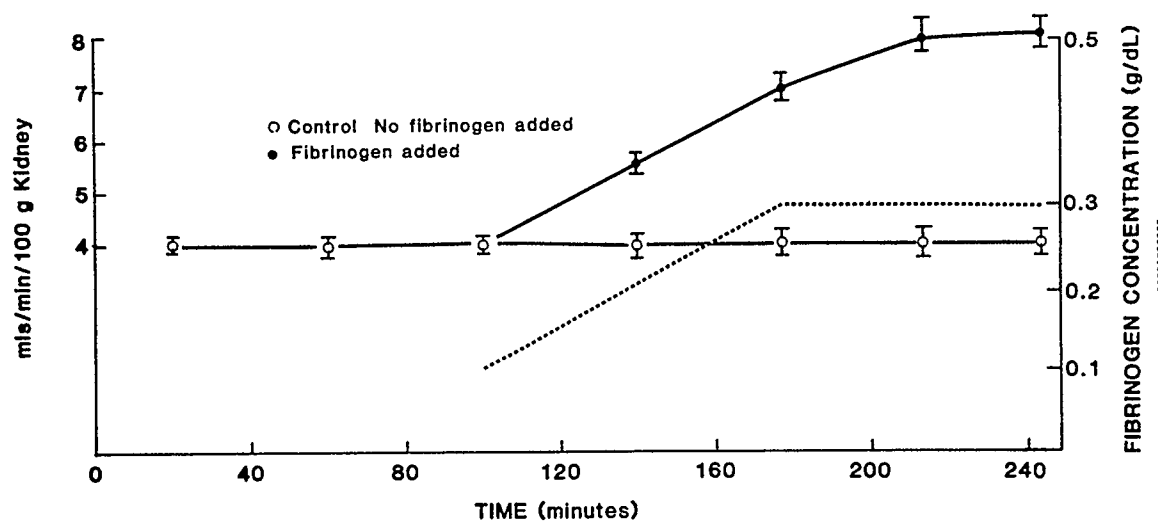
Figure 27: A plot of percentage urine albumin vs. time for dog kidneys maintained on a kidney pump, with perfusate, in the presence or absence of fibrinogen (values are expressed as per 100 g kidney; mean + standard deviation).

1% test of significance:

at 110 $P < 0.001$

at 140 $P < 0.001$

at 170 and up to 230 $P < 0.001$

d.f = 6
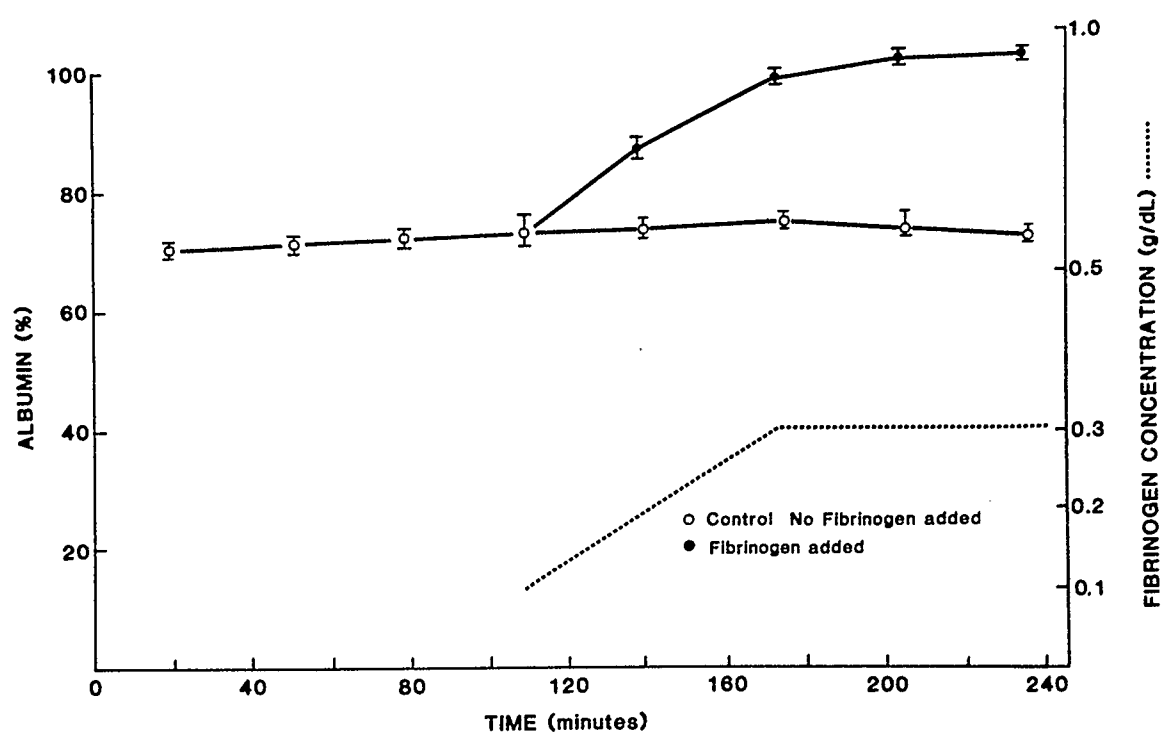
3.02.8 Renal Plasma Flow Rate

The renal plasma flow rate was found to be 150 + 6 ml/minute/100 g kidney, which increased in the presence of fibrinogen to 195 + 5 ml/minute/100 g kidney.

3.02.9 Dextran Clearance Fraction

The clearance of neutral dextrans MW, below 90,000 was unchanged in the presence or absence of fibrinogen. In the presence of fibrinogen there was a slight decrease in the clearance of neutral dextrans MW above 90,000 (Figure 28).

The clearance of positively charged dextrans increased in the presence of fibrinogen for MW below 90,000 and decreased slightly for those over 90,000 (Figure 29).

The clearance of negatively charged dextrans decreased in the presence of fibrinogen over the entire MW range tested (Figure 30).

Kidneys perfused with high molecular weight dextran (500,000) exhibited very high vascular resistance and perfusion pressure (over 90 mm Hg) and were discarded.

3.02.10 Glomerular Filtration Rate

GFR, after the addition of fibrinogen to the perfusate, increased significantly for neutral and negatively charged dextrans and decreased for positively charged dextrans (Table 8).

Figure 28: Fractional clearance of neutral dextrans (C D/IN) in kidneys maintained on kidney pump, with perfusate, in the presence or the absence of fibrinogen (values are expressed as per 100 g kidney; mean + standard deviation).

1% test of significance:

from 20 to 160 P > 0.5

d.f = 6

NEUTRAL DEXTRAN
□ with Fibrinogen
■ without Fibrinogen
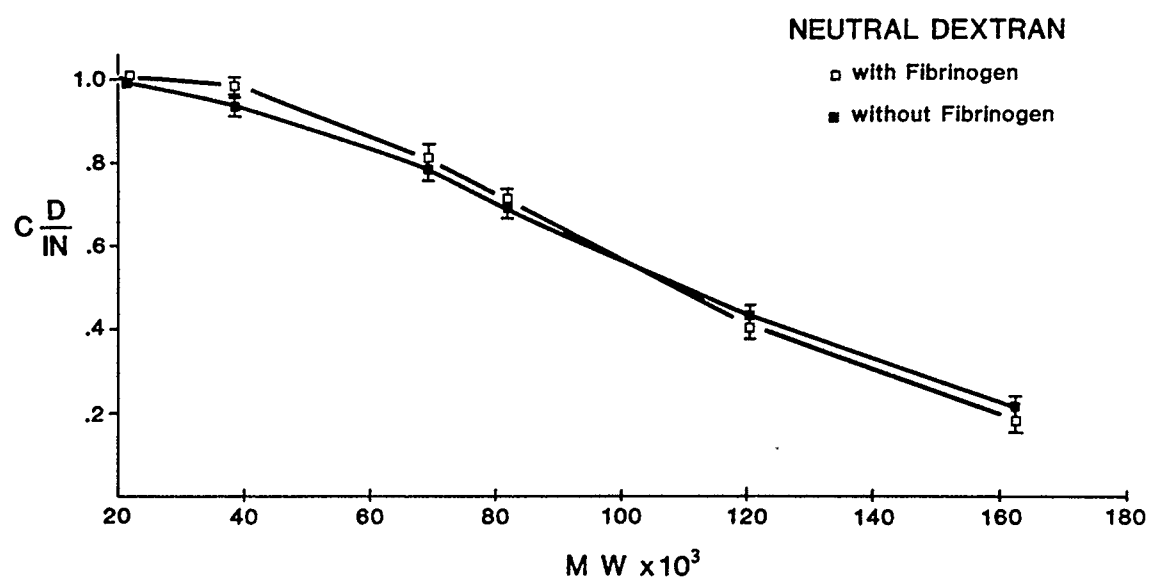
Figure 29: Fractional clearance of positively charged dextrans (C D/IN) by kidneys maintained on a kidney pump, with perfusate, in the presence or absence of fibrinogen (values are expressed as per 100 g kidney; mean + standard deviation).

1% test of significance:

at 30 $P < 0.1$

at 50 $P < 0.01$

at 70 $P < 0.001$

at 90 $P < 0.01$

at 130 and over P is between 0.4 and 0.2

d.f = 6

POSITIVE DEXTRAN
□ with Fibrinogen
■ without Fibrinogen
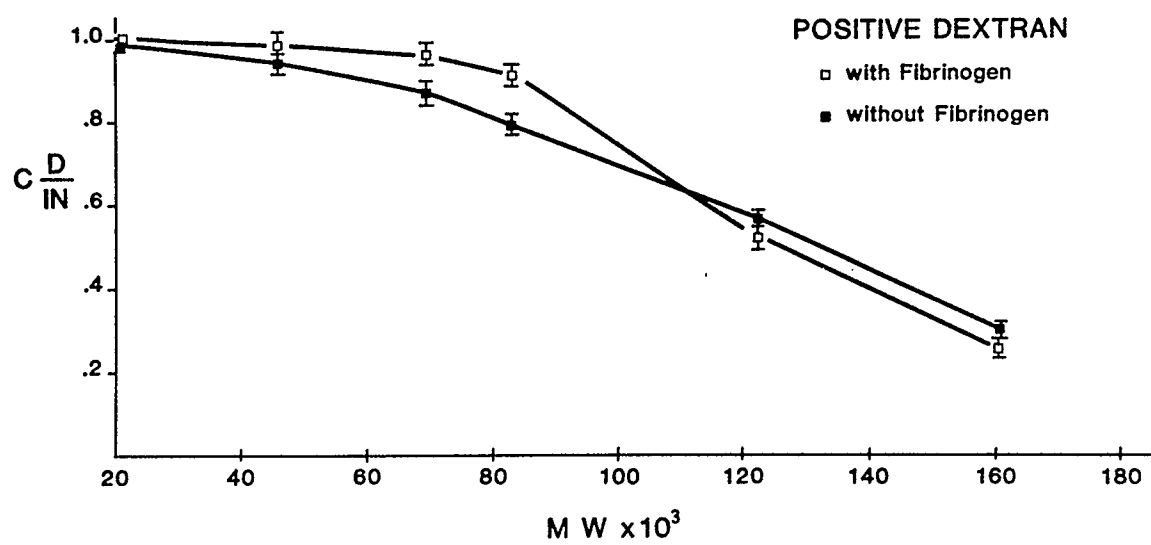
Figure 30: Fractional clearance of negatively charged
dextrans (C D/IN) by dog kidneys maintained on
a kidney pump, with the perfusate, in the presence
or absence of fibrinogen (values are expressed
as per 100 g kidney; mean + standard deviation).

1% test of significance:

at 30 P is between 0.01 and 0.005

at 70 P < 0.001

at 80 P < 0.001

at 120 P is between 0.005 and 0.001

at 150 P is between 0.4 and 0.5

d.f = 6

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NEGATIVE DEXTRAN

□ with Fibrinogen
■ without Fibrinogen
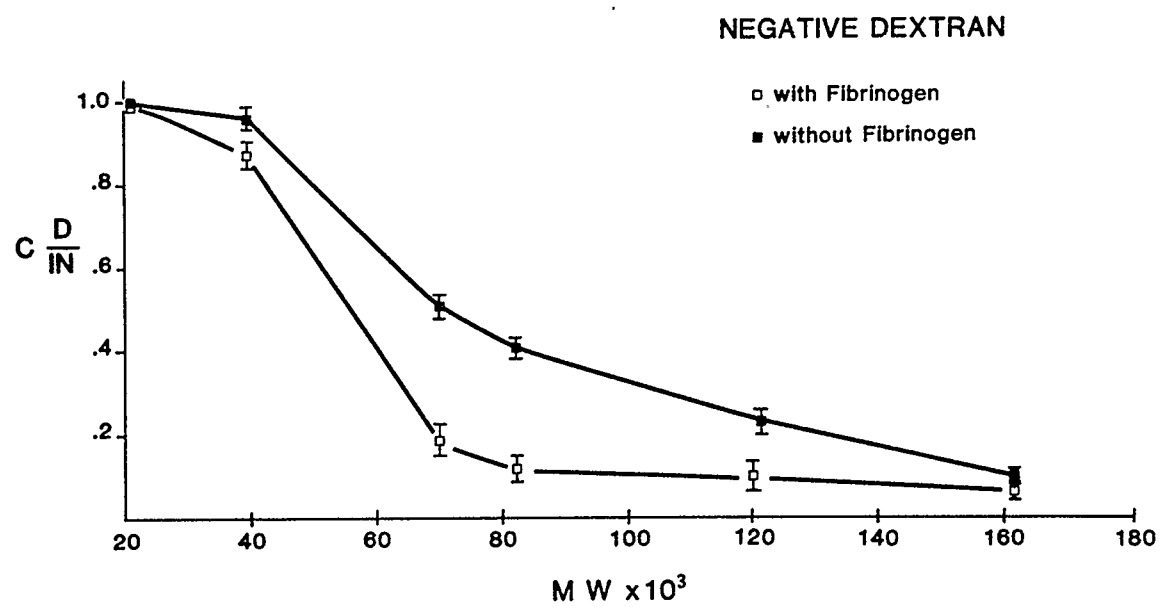
Table 8: GFR values of dog kidneys maintained on a kidney
pump, with perfusate to which dextrans of various
MW and charge were added, in the presence or
absence of fibrinogen (valeues are expressed as
per 100 g kidney; mean + standard deviation).

Tracer used	A	B
Neutral dextran	6.84 + 0.5 ml/min	7.8 + 0.6 ml/min
Negative dextran	8.10 + 0.8 ml/min	12.8 + 0.58 ml/min
Positive dextran	6.55 + 0.6 ml/min	5.97 + 0.6 ml/min

A = Without fibrinogen.

B = with fibrinogen.

values are expressed as mean + standard
deviation.

1% test of significance:

for neutral dextran P is between 0.005
and 0.001

for negative dextran P < 0.001

for positive dextran P is between 0.025
and 0.01

d.f = 6

3.02.11 Renal Plasma Flow

In the presence of fibrinogen, plasma flow rate was reduced for postively charged dextrans and increased for both neutral and negatively charged dextrans (Table 9).

3.02.12 Filtration Fraction

The FF was unchanged for positive and neutral dextrans, and increased significantly ($P < 0.001$) for negative dextran in the presence of fibrinogen .

3.02.13 Electron Microscopy

Electron microscopy studies revealed no detectable structural abnormalties in the glomeruli (Figure 31) and tubules (Figure 32) of the kidneys perfused with the perfusate containing fibrinogen.

3.02.14 Immunofluorescence

Immunofluorescence studies for the detection of fibrinogen on the glomerular capillary wall were negative (Figure 33).

Table 9: Plasma flow rates of dog kidneys maintained on a kidney pump, with perfusate to which dextrans of various MW and charge were added, in the presence or absence of fibrinogen (values are expressed as per 100 g kidney; mean + standard deviation).

Tracer used	A	B
Neutral dextran	160 + 4 ml/min	170 + 5 ml/min
Negative dextran	170 + 5 ml/min	220 + 6.5 ml/min
Positive dextran	150 + 5.5 ml/min	130 + 6 ml/min

A = Without fibrinogen.

B = With fibrinogen.

values are expressed as/100 g kidney; mean + standard deviation.

1% test of significance:

for neutral dextrans $P < 0.001$

for negative dextrans $P < 0.001$

for positive dextrans $P < 0.001$

d.f = 6

Figure 31: An electron micrograph of a glomerulus obtained from a dog kidney maintained on a kidney pump for 4 hours, with the perfusate containing fibrinogen, indicating no detactable structurall or cellular damage. (Mag. x 6,000).

CL = Capillary lumen.

EP = Epithelial cell.

ENF = Endothelial fenestrations.

GBM = Glomerular basement membrane.

FP = Foot processes.

P = Podocyte.

ME = Mesangial cell.

EN = Endothelial cell.

US = Urinary (also Bowman space).
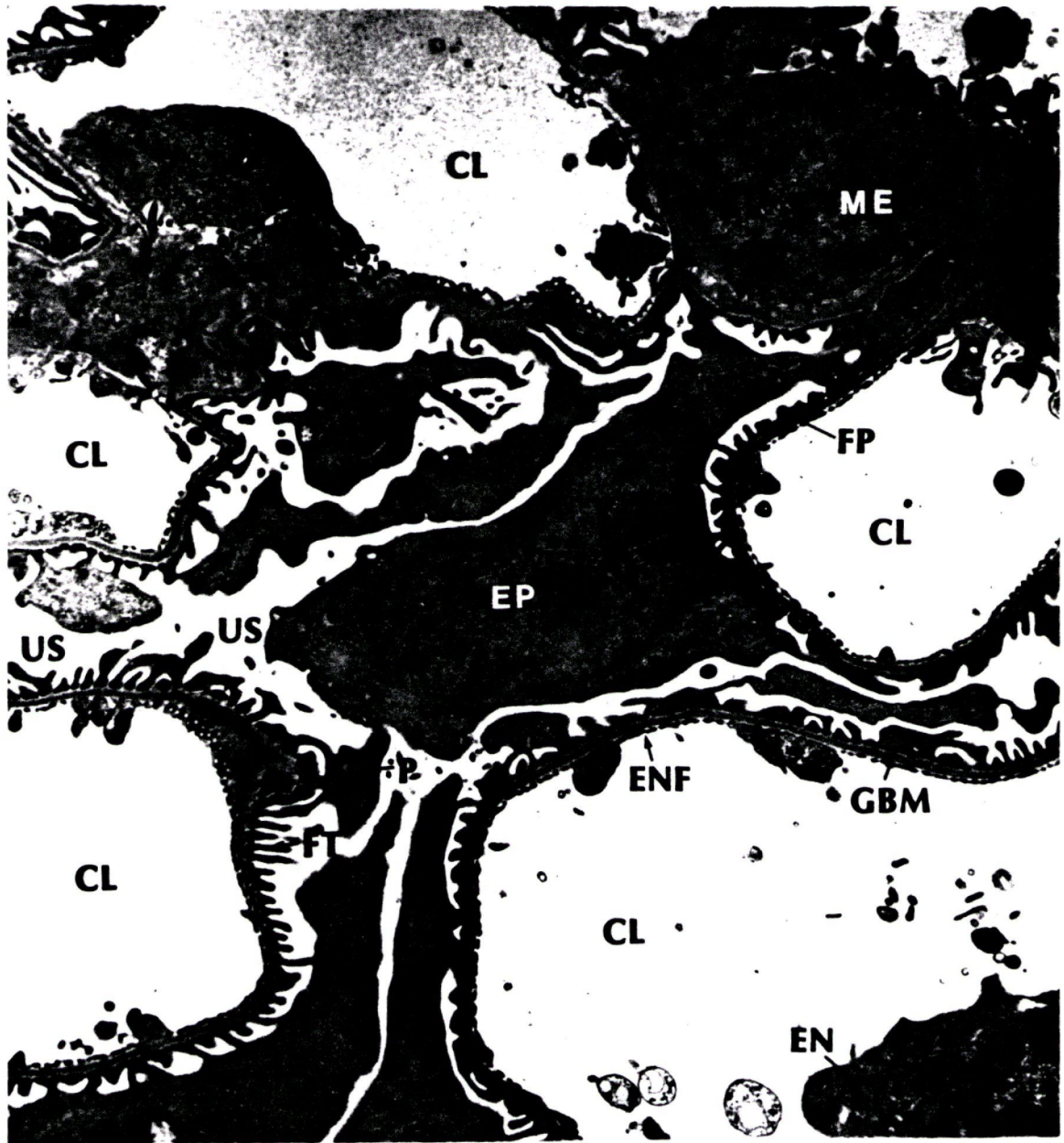
Figure 32: An electron micrograph of tubules obtained from a dog kidney maintained on a kidney pump for 4 hours, with the perusate containing fibrinogen, indicating no detactable cellular damage. (Mag. 8,000).

TL = Tubular lumen.

M = Mitochondria.

C = Cytosome.

N = Nucleus.

G = Golgi apparatus.

ER = Endoplasmic reticular spaces.

CL = Capillary lumen

BB = Microvilli forming the brush border.

BM = Basment membrane.

AV = Vacules.
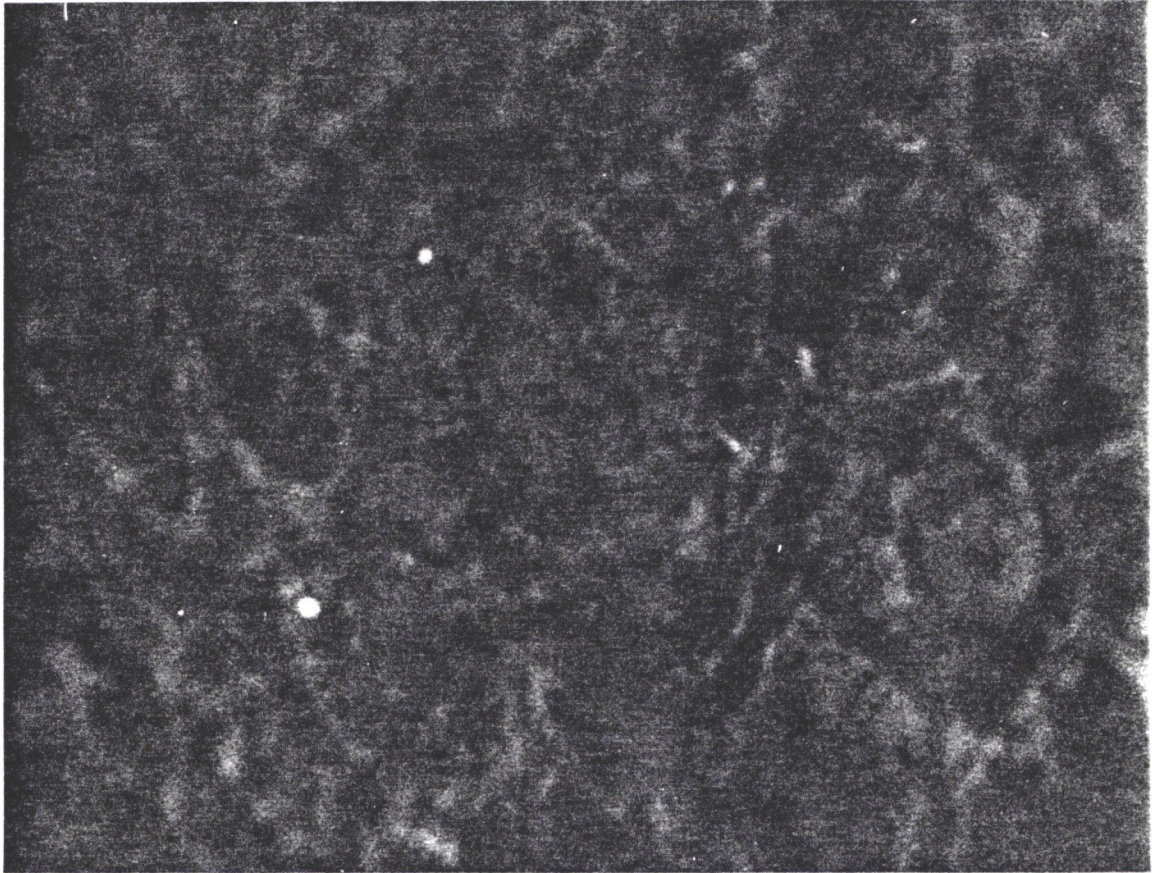
Figure 33: An immunofluorescence photograph of a glomerulus, obtained from a dog kidney maintained on a kidney pump for 4 hour, with the perfusate containing fibrinogen, indicating no specific binding of fibrinogen to the capillary wall. (Mag. 100).

DISCUSSION

4.01 Fibrinogen

The PEG fractionation procedure that we have developed to isolate fibrinogen from human plasma is rapid, gives a high yield of a pure product and is able to be used for the production of large amounts of fibrinogen.

The fibrinogen that was isolated by this technique had a MW of 680,000, which corresponded to the MW of the major clottable fraction isolated from plasma by column chromatography. However, the fibrinogen was observed to dissociate under the ultracentrifugation conditions used for sedimentation velocity and diffusion coefficient measurements, with the proportion of the small MW component increasing with decreasing protein concentration. This result is consistent with those obtained by Kazal et al,(1963), using fibrinogen isolated by glycine precipitation, and that obtained by Caspary and Keckwick (1957) using fibrinogen isolated by ether fractionation. In both cases a peak corresponding to a component of 18S was observed, in addition to the one of 7.3S. However, this was attributed to aggregation rather than dissociation (Caspary and Keckwick 1957). Subsequent workers failed to dispute the 340,000 MW that was assigned to fibrinogen, although analysis of data for the elution volume of purified fibrinogen on column chromatography (Donati, 1976) would suggest a value in the 600,000 MW range.

Exposure of the fibrinogen to mercaptoethanol resulted in its dissociation, with four bands appearing in the gel. The MW of three of the bands correspond to those of the alpha, beta, gamma chains (McKee et al, 1966; McKee et al, 1970). The fourth band have a MW corresponding to that of a DSK which has been observed in similar preparations (McKee et al, 1970; Mckee et al, 1972; McDonagh et al, 1972).

Based on our data it seems that in plasma, fibrinogen exists as a tetromer, consisting of 4a, 4b, and 4y chains. Such a model would be in agreement with a recently reported model of fibrinogen that is based on data obtained using electron microscopy and X-ray diffraction (Weisel et al, 1983). This model suggests that fibrinogen is made up of 7 globular domains rather than the three domains model of Hall and Slayter (1959).

It has previously been shown that fibrinogen is necessary for rouleaux to occur in normal plasma as defibrinated plasma fails to form rouleaux. (Van Haeringen and Glasius, 1970). However, our data indicates that the presence of fibrinogen is not by itself sufficient, as purified fibrinogen or the treated plasma that did not contain thrombin, and clotting factors VIII and IX failed to form rouleaux. The ability to form rouleaux was restored by the addition of the clotting factors IX, which was found to be a complex made up of factors II, VII, X and

IX, and factor VIII to the purified fibrinogen or the treated plasma. The addition of these factors had a profound effect on the MW of fibrinogen, which was increased by a factor of 5 if only one factor was added at a time or by a factor of 10 if both clotting factors were added together. This would indicate that rouleaux formation is a result of the interaction between fibrinogen and clotting factors to form large MW complexes. This is in agreement with the finding that rouleaux can be caused by dextrans of large MW and that rouleaux formation is increased with an increase in the MW of the dextran used (Chien and Jan, 1973).

4.02 Perfusion Studies

The perfusion experiments indicate that the inclusion of fibrinogen in the perfusate leads to a decrease in urinary enzyme, and protein levels, as well as to an increase in GFR, FF, QA and urine output. Moreover, the proteinuria that does occur is more selective for albumin.

Kidneys perfused with the perfusate that lacks fibrinogen had high levels of proteinuria. This was also observed by other investigators using similar perfusates (Toledo-Peryra et al , 1977; Ross, 1978; Toledo-Peryra and Candie, 1979; Maack, 1980). This proteinuria is unlikely to be the result of structural abnormalities in the filtration barrier since no alterations were detectable by electron microscopy. This is also supported by studies

of Toledo-Pereyra and Candie, 1979) and Masri et al (1981)
who found no detectable changes in the filtration barrier
even after 24 hours of perfusion. The observed proteinuria
has been attributed to the composition of the perfusate
(Maack, 1980). Kidneys perfused with plasmonate containing
7.5 g/100 ml protein have greater proteinuria levels (7
times) than those perfused with plasmonate containing 5
g/100 ml protein and 30 times greater than the in vivo
animals (Schurek et al, 1978; Maack, 1980; Masri et al,
1981). It would appear that the proteinuria that occurs
could be reduced by altering the protein composition of
the perfusate; by using albumin in combination with dextran,
by using plasmonate with low protein concentration (3 g/100
ml) or by using perfusate in which albumin is substituted
by dextrans. However, kidneys perfused with such perfusates
exhibit progressive edema and vascular spasm (Pegg, 1970),
acute tubular necrosis (Toledo-Pereyra et al (1977),
impaired sodium and water reabsorption (Bowman and Maack,
1974) and when transplanted they have very low survival
rates (Toledo-Pereyra and Candie, 1979).

The inclusion of fibrinogen in the perfusate resulted
in a significant decrease in the observed proteinuria without
any reduction in the GFR or QA. This decrease may be
clinically important, since it is now believed that fusion
of the foot processes, such as in minimal change disease,
is a reaction to the passage of large proteins (Zollinger

and Mihatsch, 1978) or to small positively charged substances (Hunsicker and Shearer, 1979). Upon significant reduction of proteinuria, fusion of the foot processes disappears (Zollinger and Mihatsch, 1978).

The inclusion of fibrinogen in the perfusate resulted in a reduction in the urinary enzymes levels which is of clinical significance. The levels of the enzymes LDH, CPK, and ALK Phos in the plasma and urine have been used as a measure of the extent of cellular damage in the in vivo kidney (West and Zimmerman, 1958; Rosalki and Wilkinson, 1959). Levels of these enzymes in the urine or perfusate have been also used to assess the viability of the isolated perfused kidney (Belzer et al, 1968; Kohn and Ross, 1971; Mcleish et al, 1980). Belzer et al (1968) and Toledo-Pereyra et al (1975) have indicated that high urinary or perfusate levels of LDH are indicative of acute tubular necrosis, and are highly suggestive of irreversible cellular damage. Kidneys that exhibit such high levels of LDH (800 IU/liter or higher) are not considered viable and if transplanted they have very low survival rates (Belzer et al, 1968). Kidneys that exhibit high levels of ALK Phos. and CPK (more than 110 IU/liter) are considered to be ischemic and when transplanted they have low survival rates (Belzer et al, 1968; Mcleish et al, 1980).

The GFR and QA of the kidneys perfused with the perfusate lacking fibrinogen were about 10% of the values

obtained for the in vivo dog kidney (70 ml/min/100 g kidney and 300 ml/min/100 g kidney) (Brenner et al, 1980). The inclusion of fibrinogen in the perfusates caused an increase in both GFR and QA. However, their values were still below the normal in vivo levels. Such low values are consistent with the values obtained from isolated kidneys perfused with similar perfusates under similar conditions (Wicklam et al, 1967; Nishiitsutsuji et al, 1967; Belzer et al, 1968; Bowman, 1970; Martin and Smith, 1970; Little and Cohen, 1972; Schurek et al, 1975; Maack, 1980). The observed reduced GFR and QA values have been attributed to:

a) Influence of hypothermia (Martin and Smith, 1970).

b) Reduced oxygen uptake by the perfused kidney (Wicklam et al, 1967).

c) Protein content of the perfusate (De Mello and Maack, 1976). Kidneys perfused with a perfusate containing 7.5 g protein/100 ml have lower GFR and QA than those perfused with perfusate containing 5 g protein/100 ml (De Mello and Maack, 1976; Maack, 1980).

d) Perfusion pressure, Kidneys perfused at high pressure (over 90 mm Hg) have higher GFR and QA than those perfused at low pressure (40 -60 mm Hg) (De Mello and Maack, 1976).

e) Length and diameter of the catheter used (Schurek and Alt, 1981). A small diameter catheter causes high

ureteral and tubular pressure and decreased GFR (Cortell et al, 1972; Vandelwalle and Bonvalet, 1976; Schurek and Alt, 1981).

Theoretically the observed low GFR and QA values could be raised by perfusing the kidneys with perfusate with low protein content (5 g/100 ml) under high pressure (over 90 mm Hg). However, kidneys perfused under these conditions exhibit irreversible cellular damage, acute tubular necrosis and when transplanted they have very low survival rates (Belzer et al, 1968, Toledo-Peryra et al, 1975; Toledo-Pereyra and Candi, 1979).

Clearance studies indicate that the addition of fibrinogen to the perfusate leads to a significant decrease in the fractional clearances of negatively charged dextrans over a MW range of 30,000 to 110,000, and to a significant increase in the fractional clearances of positively charged dextrans over a MW range of 30,000 to 90,000. However, there was no significant change in the fractional clearances of neutral dextrans over the entire MW range tested. These results suggest that the observed effects of fibrinogen in reducing proteinuria are more likely to be due to the molecular charge. The fractional clearance pattern of the differently charged dextrans obtained are similar to those reported for the normal in vivo animal, and would suport the charge repulsion theory (Chang et al, 1975b; Chang et al, 1975c; Brenner et al, 1977; Brenner et al,

1981). However, the addition of fibrinogen potentiates the reduction in the filtration of the negatively charged dextrans and would indicate that the negatively charged fibrinogen is contributing to the charge repulsion effects of the glomerular wall.

Due its large MW of 680,000, its large size (over 75 nm), its net negative charge (pI 5.5) and to the negative results obtained with immunofluorescence, fibrinogen would not be expected to specifically bind or to traverse the glomerular wall to any great extent. However, it would be expected to reach the subendothelium where it could form a concentration polarization layer in the relatively unstirred zone beneath the endothelium. This layer, could then restrict the access of negatively charged plasma proteins, such as albumin, to the GBM (Deen et al, 1974). Deen et al (1974) who postulated the existence of such a layer indicated that it would be expected to consist of plasma proteins, whose most important characteristic would be that they are of sufficiently large size that they are unable to penetrate the GBM. However, the clearance results, would indicate that this layer would be more effective in preventing the loss of negatively charged proteins such as albumin if it is made of large molecules that are negatively charged as well. Furthermore, since the urinary protein loss occured with kidneys perfused with plasmonate containing all the plasma proteins with the exception of

fibrinogen and the gamma globulin fractions and since gamma globulin failed to cause any major reduction in proteinuria when added to the perfusate (Masri, 1980), it would appear tht fibrinogen is the most important constituent of this concentration polarization layer.

It could be concluded that glomerular permeability is a complex process involving hemodynamic factors, charge and molecular structure of the glomerular wall, molecular charge, shape and size of the plasma proteins and in addition the concentration polarization layer formed by fibrinogen. The fact that proteinuria occurs in the absence of fibrinogen would suggest that this layer is a major factor in limiting the passage of negatively charged proteins across the glomerular wall. It would also appear that the addition of fibrinogen to the perfusate resulted in better kidney function.

4.03 Future Research

Suggested future research includes the following:

1) A study on the quantum interaction between PEG purified fibrinogen and red blood cells in the absence and presence of blood clotting factors.

2) A study to compare graft survival rates of dog kidneys that are perfused with the perfusate containing fibrinogen to those dog kidneys that are perfused with the perfusate that does not contain fibrinogen.

3) An immunohistochemistry study to determine the site of interaction of fibrinogen and the glomerular capillary loops.

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