

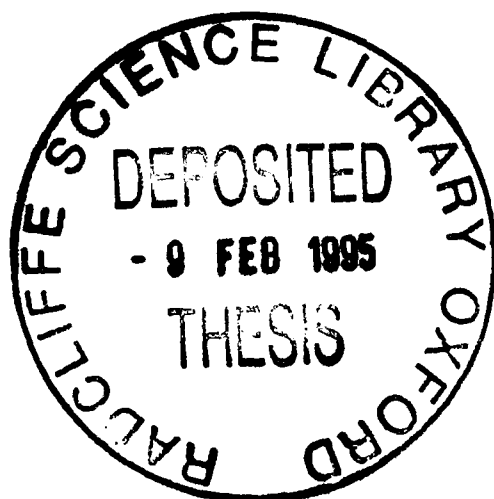
STUDIES ON BASEMENT MEMBRANE PERMEATION:
MODELS OF PATHOGENIC MECHANISMS OF
GLOMERULONEPHRITIS

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A thesis submitted for the degree of Doctor of Philosophy in the University of Oxford

October 1994



DEDICATION

This thesis is dedicated to Rachel and my mother.

ACKNOWLEDGEMENTS

I would like to thank Garth Robinson for his supervision. I am also indebted to Robert Cooke for his kind gifts of filtration cells, to Les Wood, Roger Woodward, Tony Temples, Raoul Heller for their stimulating friendship, to my mother for a loan to buy a lap top for thesis writing, and last but not least to Rachel for her unstinting support and encouragement.

I am grateful to the Science and Engineering Research Council for a research studentship, which enabled me to carry out my research.

ABSTRACT

The effects of the biological cross-linker transglutaminase, the neutrophil oxidant hydrogen peroxide, and neutrophil proteinases on glomerular basement membrane permeability have been examined using an *in vitro* model of glomerular ultrafiltration. The main focus of the study lies in determining whether any of the test agents were able to render glomerular basement membrane more permeable to protein.

Guinea pig liver transglutaminase was used as a model enzyme to test for the effect of biological cross-linkers on glomerular basement membrane permeability. It cross-linked glomerular basement membrane proteins, caused membrane contraction, and rendered glomerular basement membrane less permeable both to water and the low molecular weight protein marker myoglobin but had no effect on the membrane permeability to the high molecular weight marker protein bovine serum albumin or serum protein. The pathophysiological relevance of the effect is discussed.

Hydrogen peroxide increased glomerular basement membrane permeability to water and proteins but the effect depended on hydrogen peroxide concentration and incubation time. The minimum concentration needed to render glomerular basement membrane more permeable to bovine serum albumin and serum protein was 1 M and the minimum incubation time needed was 6 hrs. A respiratory burst analysis of activated neutrophils showed that the average concentration of hydrogen peroxide that could be generated by the neutrophils was less than 50 mM and the time taken for extracellular hydrogen peroxide concentration to fall off to zero was less than 1 hr. Therefore, neutrophils seemed unable to generate and sustain a sufficiently high hydrogen peroxide concentration to render glomerular basement membrane more permeable to protein *in vivo*.

Proteinases extracted from pig neutrophil granules were used to assess their effect on glomerular basement membrane permeability. The extract showed activity against glomerular basement membrane and the activity was primarily attributed to the serine proteinases elastase and cathepsin G, judged from substrate and inhibitor

analyses. The proteinase extract also contain latent metalloproteinases, activatable by the organomercurial 4-aminophenyl mercuric acetate and calcium ions. Once activated, they also showed activity against glomerular basement membrane. The extract rendered glomerular basement membrane more permeable to water, myoglobin, bovine serum albumin, and serum protein. The increase in membrane permeability to water and proteins was due to membrane thinning and an increase in the intrinsic porosity of the membrane. When the serine and metalloproteinases were allowed to act in concert, they synergistically degraded glomerular basement membrane and increased the membrane permeability to serum protein and water. The study provides the first direct evidence that pathophysiological amounts of serine and metalloproteinases are able to render glomerular basement membrane more permeable to protein and suggests they may be capable of promoting proteinuria in neutrophil-dependent forms of immune glomerulonephritis.

ABBREVIATIONS

$^1\text{O}_2$	= Singlet oxygen
4-APMA	= 4-Aminophenyl mercuric acetate
A	= Glomerular ultrafiltration area
A_{280}	= Absorbance at 280 nm
A_{525}	= Absorbance at 525 nm
AAPA	= N-tert-butoxycarbonyl-L-alanyl-L-alanyl-L-prolyl-L-alanine p-nitroanilide
ACE	= Angiotensin converting enzyme
BM-40	= 40 kda basement membrane protein or osteonectin
BOC-APN-SBzl(Cl)	= N-tert-butoxycarbonyl-L-alanyl-L-prolyl-L-norvaline benzylthioester
BOC-Gly-Leu-Phe-CH ₂ Cl	= N-benzyloxycarbonyl glycyl-L-leucyl-L-phenylalanine chloromethyl ketone
BSA	= Bovine serum albumin
Cb	= Protein concentration of the overstanding solution
Cf	= Protein concentration of the filtrate
CSPG	= Chondroitin sulphate proteoglycan
d.p.m.	= Disintegrations per minute
DEAE	= Diethylaminoethyl
DMSO	= Dimethyl sulphoxide
ΔP	= Filtration pressure
DTT	= Dithiothreitol
EDTA	= Ethylenediaminetetraacetic acid
EM	= Electron microscope
ER	= Endoplasmic reticulum
Fig.	= Figure
GBM	= Glomerular basement membrane

GFR	=	Glomerular filtration rate
GN	=	Glomerulonephritis
GP330	=	Podocyte glycoprotein of molecular weight 330k
GPV	=	L-pyroglutamyl-L-prolyl-L-valine p-nitroanilide
H ₂ O ₂	=	hydrogen peroxide
HBSS	=	Hank's balanced salt solution
HOCl	=	Hypochlorous acid
HPLC	=	High performance liquid chromatography
HSPG	=	Heparan sulphate proteoglycan
IgG	=	Immunoglobulin class G
INT	=	2-(<i>p</i> -iodophenyl)-3-(<i>p</i> -nitrophenyl)-5-phenyltetrazolium
J _s	=	Protein flux
J _v	=	Water flux
KCN	=	Potassium cyanide
kda	=	Kilodalton
KF	=	Potassium fluoride
kPa	=	Kilopascal
MAC	=	Membrane attack complex
μm	=	Micron
mmHg	=	Millimetres of mercury
MPO	=	Myeloperoxidase
MW	=	Molecular weight
NADPH	=	Reduced nicotinamide adenine dinucleotide phosphate
NBA	=	p-Nitrophenyl-N-tert-butoxycarbonyl-L-alanine
NC	=	Non collagenous
NSN	=	Nephrotoxic serum nephritis
O ₂ ⁻	=	Superoxide anions

OD	= Optical density
OH·	= Hydroxyl radical
pI	= Isoelectric point
PMA	= Phorbol 12-myristate 13-acetate
PMSF	= Phenyl methyl sulphonyl fluoride
R	= Rejection
RPGN	= Rapidly progressive glomerulonephritis
r.p.m.	= Revolutions per minute
RBM	= Renal basement membrane
RNA	= Ribonucleic acid
ROS	= Reactive oxygen species
SA ₃	= Succinyl-L-alanyl-L-alanyl-L-alanyl-L-prolyl-L-valine p-nitroanilide
SAAPAbu	= Succinyl-L-alanyl-L-alanyl-L-prolyl- α -aminobutyric acid p-nitroanilide
SAAPP	= N-Succinyl-L-alanyl-L-alanyl-L-prolyl-L-phenylalanine p-nitroanilide
SAAPV	= Methoxy-O-succinyl-L-alanyl-L-alanyl-L-prolyl-L-valine p-nitroanilide
SD	= Standard deviation
SDS	= Sodium dodecyl sulphate
SDS-PAGE	= Sodium dodecyl sulphate polyacrylamide gel electrophoresis
SEM	= Scanning electron microscopy
SLE	= Systemic lupus erythematosus
SPARC	= Secreted protein acid and rich in cysteine or osteonectin or BM-40
TBM	= Tubular basement membrane
TBS	= Tris buffered saline, pH 7.4

TBS	=	Tris buffered saline, pH 7.4
TCA	=	Trichloroacetic acid
TEM	=	Transmission electron microscopy
TGase	=	Transglutaminase
TLCK	=	N α - <i>p</i> -tosyl-L-lysine chloromethyl ketone
TTS	=	Tris tricine sodium dodecyl sulphate

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CHAPTER ONE

CHAPTER 1 **INTRODUCTION**

Glomerulonephritis (GN), as a family of glomerular diseases, continues to represent a major cause of end stage renal failure throughout the world and accounts for 1 - 2 % mortality (Couser, 1990), though the frequency of GN as a cause of chronic renal failure has decreased from over 60 % in the 1970s to less than 40 % in the 1990s (U.S. Renal Data System, 1992; Couser, 1993). Immune mechanisms underlie the majority of glomerular diseases and by far the most common mechanism underlying immune GN is antibody-mediated injury (Cotran et al., 1994). The pathological consequences of these immune induced conditions are the main subjects of this thesis. In introducing this work it will be necessary to survey a range of topics pertinent to the theme, most of them being large in scope and rich in information. Detailed all-embracing surveys are necessarily impossible and so this chapter will concentrate on those topics which are particular relevant to the work to be discussed.

1.1. The glomerulus

The functional unit of the kidney is the nephron and each human kidney contains approximately 1.4 million (Ellis et al., 1989). Each nephron is composed of a glomerulus, a proximal convoluted tubule, a loop of Henlé and a distal convoluted tubule which leads to the collecting duct (Burkitt et al., 1993). The glomerulus consists of capillary loops within the Bowman's capsule (Bloom and Fawcett, 1975) as illustrated in Fig. 1.1. The glomerulus is the site of ultrafiltration of the plasma and is the primary functional element of the nephron studied here.

The glomerular capillary wall filters the plasma, retaining most of the protein and all of the cellular elements of the blood, whereas low molecular weight solutes pass into the ultrafiltrate and are subsequently excreted into the urine or reabsorbed. During the 1940s and 1950s investigators demonstrated that renal clearance of macromolecules, relative to the filtration rate of water, decreased with increasing molecular size up to a molecular weight of approximately 70,000; proteins and dextrans

Fig. 1.1. The glomerulus



From Bloom, W. and Fawcett, D.W. (1975) A textbook of histology, 10th ed., p. 365,
Saunders, Philadelphia.

being used as the probe molecules (Maddox et al., 1992). Molecules with a molecular weight greater than this are effectively retained by the glomerulus. The glomerular capillary walls is comprised of endothelial cells lying on a basement membrane with specialised epithelial cells lying on the opposite face from the lumen (Chan et al., 1993) (Fig. 1.2.). In considering this barrier it is pertinent to examine each structural element separately.

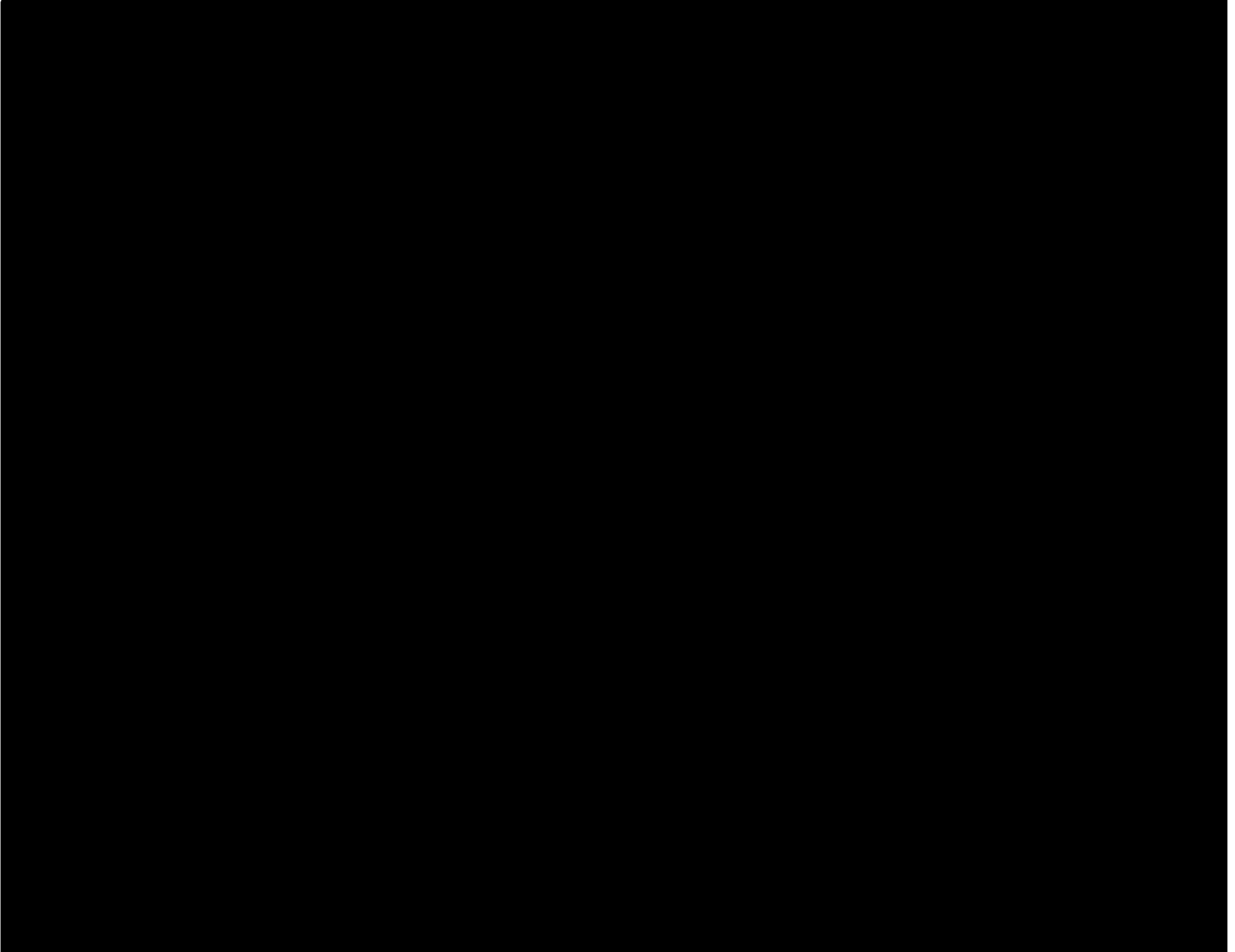
1.2. The endothelial cells

Endothelial cells are fenestrated cells whose cell surface is lightly coated with a negatively charged 140 kda sialoprotein called podocalyxin (Kerjaschki et al., 1984). Their fenestrae, or pores, have a mean diameter of ~60 nm (Lea et al., 1989). By using high-resolution scanning electron microscopy, Lea et al. (1989) showed that the walls of the fenestrae are curved (Fig. 1.3.) rather than vertical as commonly depicted in artist's renditions (e.g., Ham and Cormack, 1979, p. 766). The smallest diameter of the fenestra (60 nm) lies ~30 nm above the plane of the glomerular basement membrane (GBM), while at the bottom of the fenestra its diameter and that of the exposed basement membrane can approach 120 nm. This finding may have important implications regarding the actual filtration surface area of the GBM. Instead of having only 20% of the GBM area exposed by fenestrae, the actual area available for filtration at the base of the fenestrae has been calculated to be 83% (Lea et al., 1989). The glomerular endothelial fenestrae lack the diaphragms that are present in other fenestrated capillaries (Farquhar, 1991) so that the GBM is directly exposed to the blood plasma on its endothelial surface. This is a highly efficient arrangement to facilitate the filtration function, but it has the disadvantage of rendering the GBM vulnerable to toxic and immune injury (Farquhar, 1991).

1.3. The epithelial cell

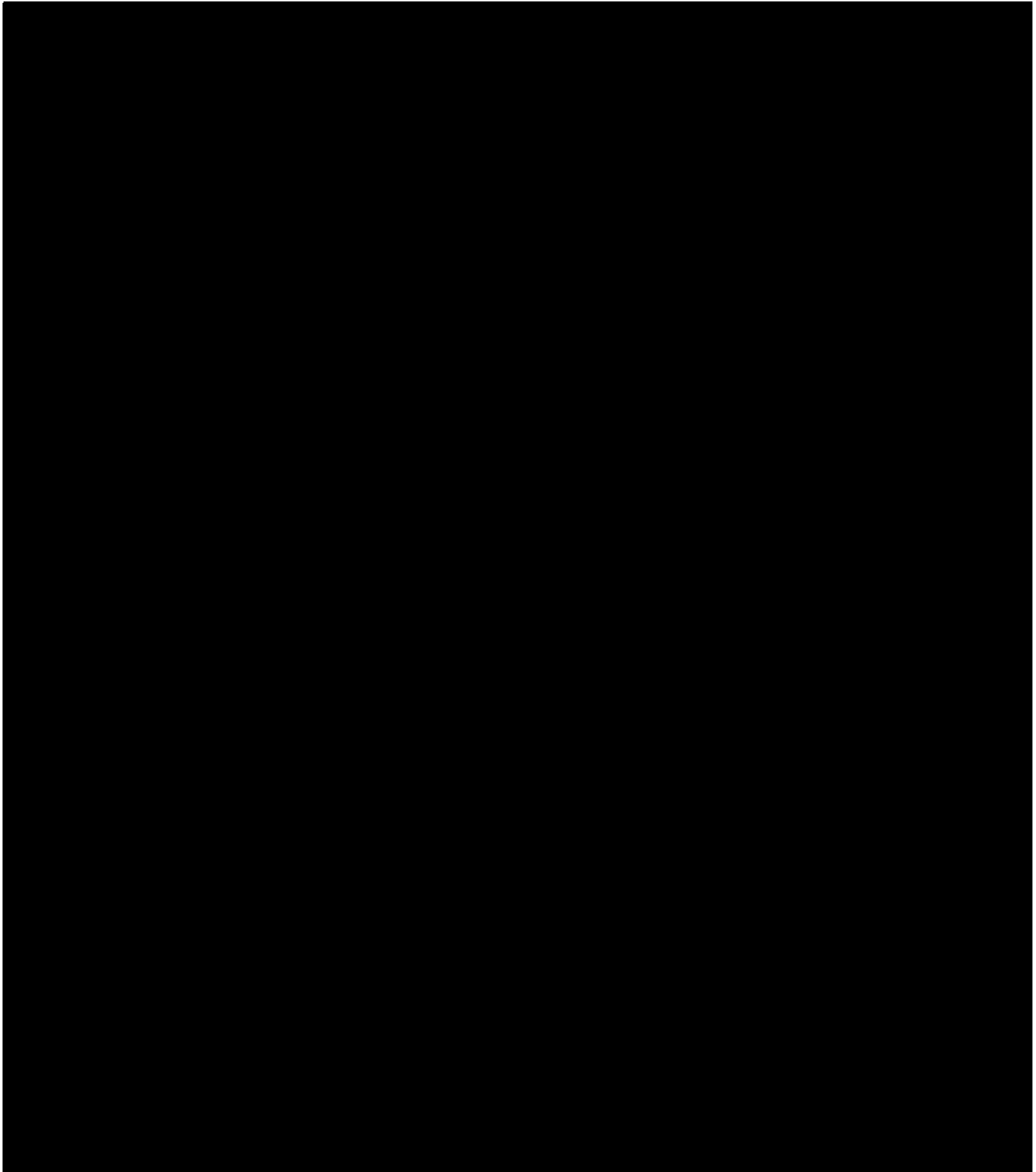
Epithelial cells or podocytes are the largest cell type found in the glomerulus extending out from the extra-luminal side of the GBM. Their surfaces are heavily

Fig. 1.2. The glomerular capillary wall



From Chan, F.L., Inoue, S., and Leblond, C.P. (1993) The basement membranes of cryofixed or aldehyde-fixed, freeze-substituted tissues are composed of a lamina densa and do not contain a lamina lucida. *Cell Tissue Res.* **273**, 47.

Fig. 1.3. The glomerular endothelium fenestrae



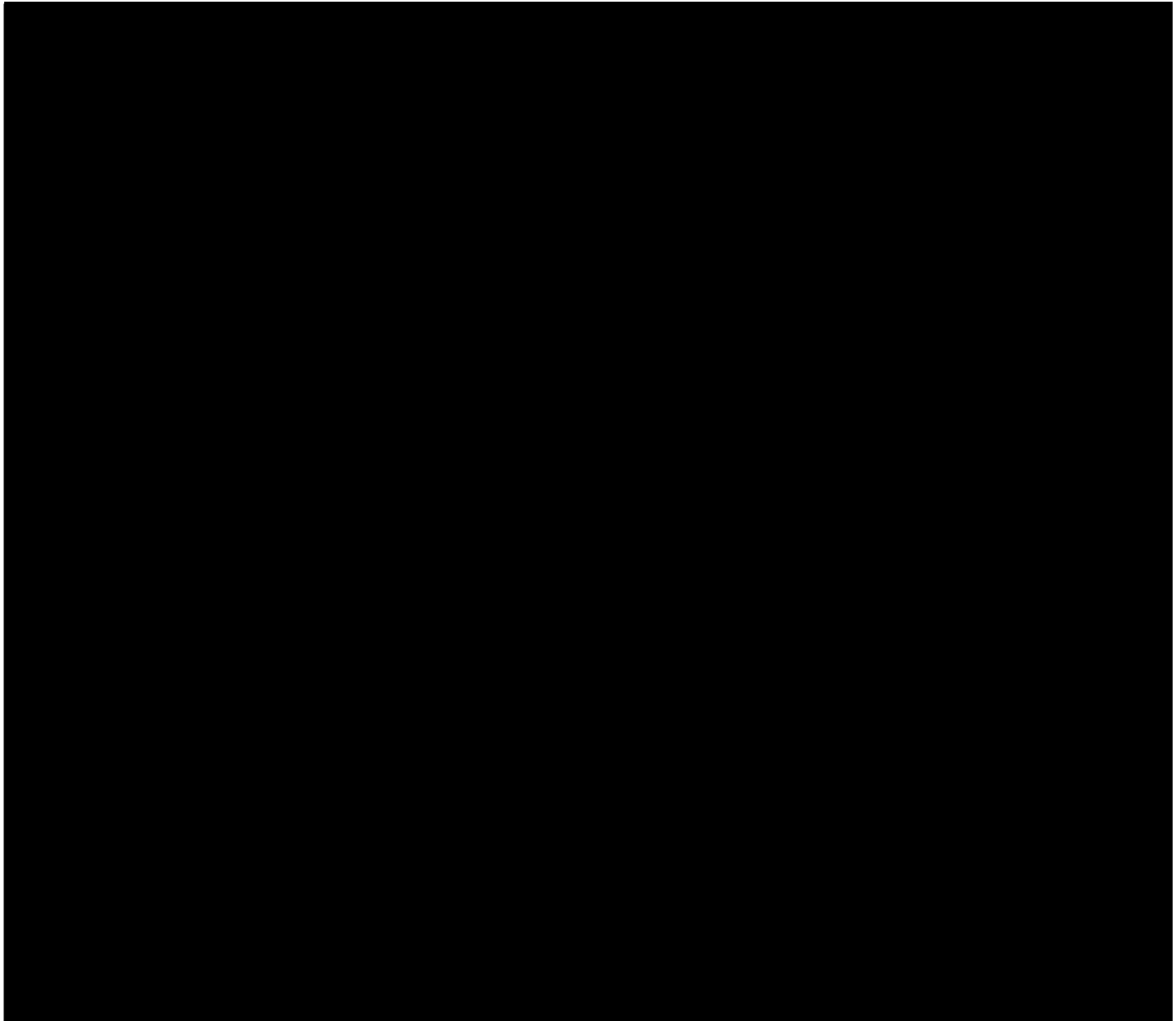
Adapted from Lea, P.J., Silverman, M., Hegele, R., and Hollenberg, M.J. (1989)
Tridimensional ultrastructure of glomerular capillary endothelium revealed by high-
resolution scanning electron microscopy. *Microvasc. Res.* **38**, 306.

coated with the anionic protein podocalyxin (Kerjaschki et al., 1984). Processes or trabeculae extend from the main body of the cell; these give rise to individual pedicles or foot processes which project onto the basement membrane (Burkitt et al., 1993). The podocalyxin's negative charge on the foot processes is thought to be important for the maintenance of their integrity (Kanwar, 1984). The distance between foot processes varies between 25-60 nm at the basement membrane (Kanwar and Venkatachalam, 1992). A thin membrane-like structure, termed the slit diaphragm (Kanwar, 1984), bridges the gap between adjoining foot processes. Rodewald and Karnovsky (1974) examined the structure of the slit diaphragm in detail and found it to exhibit a zipper-like structure with staggered rod-like units connected centrally to a linear bar and laterally to the foot process plasma membrane. Rectangular spaces between the rod-like units measure $\sim 4 \times 14$ nm. Although Rodewald and Karnovsky (1974) suggested that these spaces may restrict the passage of protein molecules, e.g., albumin and immunoglobulins, there is no direct experimental evidence supporting this possibility (Maddox et al., 1992).

1.4. The mesangial cells

The mesangial cells, together with the surrounding extracellular matrix, form the mesangium which is found in the intercapillary region of the glomerulus (Fig. 1.4.) (Cotran et al., 1994). The mesangial matrix is composed of similar biochemical components as the GBM (Couchman et al., 1994). Mesangial cells are irregular in shape and are contractile (Schlondorff, 1987). By contraction in response to neurohormonal agents they may modulate intraglomerular blood flow under physiological conditions (Johnson et al., 1992a). Mesangial cells are phagocytic and may ingest macromolecules that have leaked across the glomerular capillary wall (Farquhar, 1991) as judged by their uptake of colloidal gold and ferritin following intravenous injection of these tracers (Farquhar and Palade, 1962). The mesangial cell is known to proliferate in a number of renal diseases, most notably in acute proliferative GN (Churg and Grishman, 1975), membranoproliferative GN (MacAdam, 1970;

Fig. 1.4. The mesangium



From Cotran, R.S., Kumar, V., and Robbins, S.L. (1994) Glomerular diseases. In 'Robbins pathologic basis of disease' (R.S. Cotran, V. Kumar, and S.L. Robbins, eds.), 5th edition, Chap. 20, p. 928, W.B. Saunders Company, Philadelphia.

Couser, 1993), and IgA nephropathy (Couser, 1993). A detailed review of pathogenic mechanisms underlying these glomerulonephritides is given by Couser and Johnson (1994).

1.5. GBM

Between the endothelial and epithelial cells lies a continuous sheet of basement membrane which can be up to 350 nm thick in humans, much thicker than similar membranes found in other tissues (Farquhar, 1991). The basement membrane has traditionally been viewed as having three layers: a central dense layer (lamina densa) with two adjacent electron-lucent layers, the lamina rara interna on the endothelial side and the lamina rara externa on the epithelial side (Farquhar, 1978). Whether the two outer layers are fixation artefacts has been a subject of controversy (Reale et al., 1989). Whilst some workers, such as Farquhar (1991), insist that laminae rarae are real, others, such as Goldberg and Escaig-Haye (1986), have suggested that they are fixation artefacts due to GBM contraction during tissue fixation. By replacing the glutaraldehyde fixation step with cryofixation, i.e., sudden exposure to a -180°C pre-chilled copper block, and slowly dehydrating the tissue at -80°C in acetone (freeze-substitution), they found that the basement membrane was composed of a continuous homogeneous layer devoid of laminae rarae (Goldberg and Escaig-Haye, 1986). They proposed that basement membrane contraction occurred as a result of fixation in glutaraldehyde.

More recently, Reale and Luciano (1990) confirmed the artefactual nature of the laminae rarae. They further showed that it was not actually the glutaraldehyde that caused GBM contraction but the rapid dehydration in acetone at room temperature. By retaining the glutaraldehyde fixation step but slowing down the dehydration process in acetone at -80°C , no laminae rarae were seen (Reale and Luciano, 1990). Interestingly enough, a much earlier work on human GBM fixation by Jorgensen (1966) displayed no artefact. A closer analysis of his method revealed dehydration in acetone at $0-4^{\circ}\text{C}$, supporting the view that dehydration at low temperatures helped to prevent laminae

rarae formation. It has been demonstrated that using osmium tetroxide to stain tissues also helped to create the artefact; the stain extracted some GBM proteins (Reale and Luciano, 1993), particularly those of the fibrillar type (Lea et al., 1993). Since then, Chan et al. (1993) verified Reale and Luciano's (1990) results and demonstrated that if the tissue dehydration step was effected slowly at -80°C , then basement membranes of many other tissues (nephron proximal and distal convoluted tubules; ciliary, seminiferous, and epididymal epithelia; vascular endothelium; skeletal and smooth muscle fibres; thyroid follicular cells) also lacked the artefact.

Since GBM represents the only major morphologically continuous structure in the glomerular capillary wall separating blood from urinary space, it is presumed to be the principal permeation barrier (Farquhar et al., 1961). Tracer evidence reviewed in the next section showed that this is indeed the case.

1.6. GBM - the primary filtration barrier

Two similar approaches were used to determine the primary filtration barrier. The method consisted of injecting a tracer of known dimension into the blood stream with subsequent fixation of the tissue examined by electron microscopy to identify the barrier that prevented its passage across the glomerular capillary wall. Farquhar et al. (1961) presented evidence that the GBM was the primary barrier, based on a sharp drop in the concentration of tracer ferritin molecules seen along the inner or subendothelial surface of the basement membrane. Similar results have been obtained by others (Rennke and Venkatachalam, 1977; Schneenberger et al., 1974). Graham and Karnovsky (1966), in their efforts to determine the primary filtration barrier, used horse-radish peroxidase and myeloperoxidase which could be detected by their peroxidase activity. The tracers were injected intravenously and the reaction products subsequently located following fixation. These workers observed a sharp drop in the tracer concentration between the basement membrane and the epithelial slit pore and suggested that two barriers acted in series; the basement membrane serving as a coarse filter retaining molecules of the size of albumin with smaller molecules being retained at

the level of the slit pore. Further studies using lactoperoxidase and catalase seemed to confirm this hypothesis (Graham and Kellermeyer, 1968; Venkatachalam et al., 1970). The double-barrier model gained wide acceptance in spite of conflicting data.

It must be noted that the techniques used by Graham and Karnovsky presented a number of problems. Firstly, there was a fixation delay because tissue was excised from the kidney before fixation by immersion. The effect of cutting off blood flow through the kidney has a dramatic effect on capillary permeability. Secondly, the tracers used were positively charged and would be expected to bind to the highly negative coat of the epithelial cells, thus any electron microscope findings could be explained on this basis. Caulfield and Farquhar (1974) circumvented these problems by using neutral dextrans and by *in situ* fixation. By injecting dextrans of three different sizes, one approximately the same size as albumin (MW = 62,000), the second being larger (MW = 125,000) and the third smaller (MW = 32,000), they attempted to resolve the issue of whether the basement membrane was the primary filtration barrier or acted in series with the epithelial slit pore. If the slit pores were acting as a secondary barrier one would expect to see accumulation of the two smaller dextrans at the slit pore membrane, however, this was not observed. The three dextrans accumulated at the luminal side of the basement membrane with no accumulation occurring at the slit pore level. These findings were similar to those reported for ferritin and point to the basement membrane as the principal filtration barrier which accords with the structural evidence cited earlier.

1.7. Properties of GBM as an ultrafilter

The ability of GBM as the only structure in the glomerular capillary wall to retain macromolecules has led to the GBM being regarded as a molecular sieve or *ultrafilter* (Caulfield and Farquhar, 1974) which retains macromolecules in a size-dependent manner *in vivo*. Using graded dextran sulphate molecules, it was shown that as the Stokes-Einstein radius of macromolecules increased to ~ 3.6 nm, the size of plasma albumin, their permeability across the glomerular capillary wall approached

zero, as judged by their fractional clearance relative to the freely permeable inulin (Brenner et al., 1978; Deen et al., 1985). *In vitro* filtration studies on isolated GBM showed similar size-dependent discrimination among protein molecules (Robinson and Walton, 1989), supporting the notion that GBM of the glomerular capillary wall does act as an ultrafilter which exhibits size-dependent discrimination.

In addition to size, the glomerular capillary wall can discriminate between molecules according to their charge, allowing greater penetration of neutral and cationic molecules than of anionic molecules of the same size (Dworkin and Brenner, 1992; Kanwar and Venkatachalam, 1992; Maddox et al., 1992). Such property has been demonstrated between cationic and anionic ferritin molecules (Rennke et al., 1975), neutral and anionic albumin molecules (Bertolatus and Hunsicker, 1985), and among the cationic DEAE-dextran, neutral dextran, and the anionic dextran-sulphate molecules (Brenner et al., 1978; Deen et al., 1985) *in vivo*. *In vitro* studies based on ultrafiltration of the dextrans across GBM films also showed similar charge-dependent discrimination (Bray and Robinson, 1984), indicating that the GBM of the glomerular capillary wall alone can account for such behaviour. This charge-dependent restriction is important in the complete exclusion of the anionic albumin (pI 4.5) (Purtell et al., 1979) from the filtrate. Charge selectivity is dependent on the net negative charge on the GBM (pI 5.5); this charge was attributed mainly to carboxylate anions whilst HSPG contributed only ~13% of the net charge (Bray and Robinson, 1984).

In vivo clearance studies revealed that the glomerular capillary wall can also discriminate between molecules of different shapes, allowing greater permeability to neutral dextrans which behave as random coils in solution than rigid, spherical, and uncharged Ficoll molecules of the same effective molecular radii (Bohrer et al., 1979; Oliver et al., 1992). Cotter (1979) attributed such differences to the different deformability of the two molecular species when they were swept to the GBM filter surface by convective water flow. Whilst dextrans readily deformed upon impact and could subsequently 'wiggle' through the GBM in a reptate manner, Ficoll's non-

deformability allowed it to be retained on the filter surface until it found a large enough opening on the filter matrix to move into.

In vitro studies showed that the GBM behaves as a compressible ultrafilter (Robinson et al., 1985; Robinson and Walton, 1989). The thickness of the GBM decreases up to ~50% of its relaxed thickness (Robinson et al., 1985; Robinson and Walton, 1987) when the filtration pressure gradient across a single layer approximates to that *in vivo*, i.e., ~ 6 kPa (Cotter, 1979; Maddox et al., 1992). At this filtration pressure gradient, the GBM rejection of serum protein is effectively 100% (Robinson and Walton, 1989) which is consistent with the finding of single nephron filtration studies (Dworkin and Brenner, 1992; Maddox et al., 1992). As the filtration pressure across the GBM decreases below the normal physiological pressure, the GBM becomes more permeable to serum proteins, indicating that GBM does not have a fixed permeability but is pressure-dependent (Robinson and Walton, 1989).

The behaviour of GBM as a compressible ultrafilter has been attributed to its elastic three-dimensional meshwork of collagen IV fibres which Robinson and Walton (1989) referred to as a fibre matrix. They assumed that the fibres of the matrix is of a defined radius packed at a given density when compressed under a fixed pressure and that the movement of solute through the barrier is hindered by collision of solute molecules with flexing fibres (Robinson and Walton, 1987; 1989). This model of the GBM structure differs fundamentally from an earlier model which assumes that the membrane is pierced by cylindrical pores with a defined diameter and at a given frequency per unit area (Chang et al., 1975; Deen et al., 1985). Electron microscopy evidence of GBM and its protein structures indicated that the GBM is composed of a meshwork of fibres embedded in a granular matrix (Farquhar et al., 1961; Carlson and Audette, 1989; Shirato et al., 1991; Shikata et al., 1992; Hironaka et al., 1993) and no cylindrical 'pores' were ever seen (Yurchenco, 1994). *In vitro* evidence showed that GBM proteins can interact to form a meshwork structure very similar to GBM (Grant et al., 1989; Leblond and Inoue, 1989). Before we consider such interactions, it is necessary to survey GBM protein composition.

1.8. GBM composition

GBM is a complex biochemical structure which has been studied extensively in recent years. It is composed of glycoproteins and a proteoglycan (Yurchenco and Schittny, 1990). Known glycoproteins include collagen IV, laminin, nidogen (or entactin), and BM-40 (or osteonectin or SPARC). Fibronectin is another glycoprotein that is sometimes found associated with the GBM (Farquhar, 1991), though it is uncertain whether it represents an intrinsic component of GBM, is derived from blood (Courtoy et al., 1982; Oberley et al., 1979; Pettersson and Colvin, 1978; Scheinman et al., 1980) or from the mesangial matrix (Lee et al., 1993). Heparan sulphate proteoglycan (HSPG), alternatively termed perlecan (Noonan et al., 1991), represents the only known proteoglycan in GBM (Farquhar, 1991).

1.8.1. Collagen IV

The collagen found in GBM was first characterized by Kefalides (1968) who named it collagen IV. Following its purification (Kefalides, 1978), collagen IV was shown to be composed of 3 parallel polypeptide chains, two $\alpha 1(\text{IV})$ (MW 185 kda each) and one $\alpha 2(\text{IV})$ (MW 170 kda) (Brazel et al., 1988). This 3-chain structure constitutes a collagen IV monomer which is a flexible thread-like molecule measuring about 400 nm long and possesses a distinctive globular domain, termed NC1, at its carboxyl terminus and a cysteine-rich domain, termed 7S, at its amino terminus (Brazel et al., 1988). More recently, three new chains have been purified from GBM: $\alpha 3(\text{IV})$, $\alpha 4(\text{IV})$ (Butkowski et al., 1987; Saus et al., 1988; Hudson et al., 1989), and $\alpha 5(\text{IV})$ (Pihlajaniemi et al., 1990). Judging by the stronger immunofluorescence staining of $\alpha 3(\text{IV})$ and $\alpha 4(\text{IV})$ epitopes in the GBM than $\alpha 1(\text{IV})$ and $\alpha 2(\text{IV})$ epitopes, it has been suggested that the $\alpha 3(\text{IV})$ and $\alpha 4(\text{IV})$ isoforms of collagen IV monomers are more abundant than the $\alpha 1(\text{IV})$ and $\alpha 2(\text{IV})$ isoforms in the GBM (Butkowski et al., 1989; Kleppel et al., 1989; Sanes et al., 1990; Couchman et al., 1994). However, to what extent this was due to greater avidity of the anti- $\alpha 3(\text{IV})$ and $\alpha 4(\text{IV})$ antibodies for their epitopes than anti- $\alpha 1(\text{IV})$ and $\alpha 2(\text{IV})$ antibodies remains to be seen. The antigen

involved in the classic anti-GBM GN Goodpasture's syndrome resides in the NC1 region of the $\alpha 3(\text{IV})$ chain (Hudson et al., 1989). A $\alpha 6(\text{IV})$ chain has been predicted from its cDNA sequences located near the gene coding for the $\alpha 5(\text{IV})$ chain of collagen IV (Oohashi et al., 1994). Mutations in DNA sequence encoding the $\alpha 5$ and 6 chains may account for X-linked cases of Alport syndrome (Oohashi et al., 1994).

The $\alpha 1(\text{IV})$ and $\alpha 2(\text{IV})$ chains RNA transcripts were found only in the glomerular endothelial cells *in situ* (Lee et al., 1993), indicating that this is the only cell type that secretes collagen IV in the glomerulus. This challenges the previous belief that collagen IV is secreted only by podocytes, based on the capacity of cultured podocytes to secrete the protein (Scheinman and Fish, 1978; Killen and Striker, 1979; Foidart et al., 1980; Natori et al., 1992).

1.8.2. Laminin

Laminin, a major component of GBM, is a large flexible cross-shaped non-collagenous sialoglycoprotein (MW ~850 kda) consisting of three short arms (~37 nm) and one long arm (~77 nm) (Engel et al., 1981). Laminin is a complex of three different polypeptide chains, an A chain (~400 kda) and two smaller B chains (~200 kda) (Sasaki et al., 1988). Laminin has a fairly high carbohydrate content (12-15%), with nine forms of N-linked oligosaccharides, mostly of the complex variety (Fujiwara et al., 1988). Judging by the *in situ* location of laminin RNA transcripts, the A and B2 chains are synthesized by glomerular endothelial, epithelial, and mesangial cells whilst the B1 chain is synthesized exclusively by the mesangial cells (Lee et al., 1993). This is consistent with Natori et al.'s demonstration that cultured podocytes secreted laminin A and B chains (Natori et al., 1992).

1.8.3. Entactin

Entactin (Carlin et al., 1981) and nidogen (Timpl et al., 1983), discovered by Chung and co-workers (Carlin et al., 1981) are different names for the same macromolecule (Dziadek et al., 1985; Durkin et al., 1988; Mann et al., 1989); nidogen

being a fragment of entactin. This glycoprotein (~150 kda) contains about 5% carbohydrate (Fujiwara et al., 1988), is sulphated at tyrosine residues 262 and 267 (Mann et al., 1989), and is shaped like a dumbbell (Yurchenco and Shittny, 1990). Entactin RNA transcripts are expressed by glomerular endothelial, epithelial, and mesangial cells *in situ*, indicating that all three cell types synthesize entactin of the mature GBM *in vivo* (Lee et al., 1993).

1.8.4. BM-40

BM-40, osteonectin, and SPARC (secreted protein acidic and rich in cysteine) are different names for the same glycoprotein (MW 40 kda) found in basement membranes, bone, and other extracellular matrices, and even within platelets (Timpl, 1989). The glycoprotein has four domains, I-IV, with domain I at the amino terminus and domain IV at the carboxyl terminus (Lankat-Butgereit et al., 1988). Its domain I is glutamic acid-rich; domain II is disulphide bond-rich; domain III is an α -helix, and domain IV has two helices (E- and F-) linked by a loop, a 'EF-hand motif' (Engel et al., 1987). Domain I and IV has the ability to bind several Ca^{2+} ions (Engel et al., 1987; Bolander et al., 1988). Which glomerular cells synthesize this protein is unknown.

More recently, a further basement membrane glycoprotein has been identified - BM-90 or fibulin (MW 90k) (Pan et al., 1993). Fibulin was identified by Argraves et al. (1990) as a fibroblast-secreted extracellular matrix which is also present in plasma. It has since then been found in human placenta basement membrane and a BM-producing mouse tumour (Pan et al., 1993), though whether it is present in GBM remains to be established.

1.8.5. HSPG

HSPG (MW 620-720 kda), a GBM glycosaminoglycan, has an elongated protein core (MW 400-450 kda) subdivided into a tandem array of globular domains (Paulsson et al., 1987) with intervening connectors, resembling 'beads on a string'

(Noonan et al., 1991). Three heparan sulphate chains extend from the N-terminal domain (Timpl, 1986; Noonan et al., 1991; Noonan and Hassel, 1993; Timpl 1993, 1994). RNA transcripts for HSPG are primarily expressed by podocytes *in situ*, indicating that they are the main glomerular cells involved in secreting HSPG of the mature GBM (Lee et al, 1993). This view was supported by the evidence of Farin et al. (1980), Stow et al. (1989), and Yaoita et al. (1990) who showed that HSPG is the main proteoglycan synthesized by cultured podocytes.

1.9. GBM architecture

The GBM was first described as having a feltwork of fibres, 3-4 nm in diameter embedded in a granular matrix when examined under low resolution TEM (Farquhar et al., 1961). Since then the presence of such basic three-dimensional network of fibres were confirmed by high resolution TEM studies (Carlson and Audette, 1989; Shikata et al., 1992), high resolution SEM analysis (Shirato et al., 1991), and ultra-high resolution SEM studies (Hironaka et al., 1993). Interfibril spaces, termed 'pores' by all of these authors, were clearly evident. However, no transmembrane cylindrical pores, such as those postulated by Chang et al. (1975) and Deen et al. (1985) have ever been demonstrated in human GBM (Yurchenco and Schittny, 1990). On the grounds of electron microscopy evidence alone GBM is generally accepted as having a fibre-matrix structure rather than a solid sheet pierced by cylindrical pores (Yurchenco, 1994). Robinson and co-workers further showed that isolated GBM behaved like a fibre matrix and not a porous sheet when its permeation behaviour was mathematically modelled on both the fibre matrix hypothesis and the pore hypothesis (Robinson et al., 1985; Robinson and Walton, 1987; 1989).

Although informative, EM studies of GBM offered few clues of how different GBM proteins assemble into the matrix structure since individual protein types could not be easily discerned in electron micrographs. Much of what is known about how GBM proteins assemble into this structure was adduced from *in vitro* studies of protein binding either to themselves to form homologous oligomers and polymers or binding to

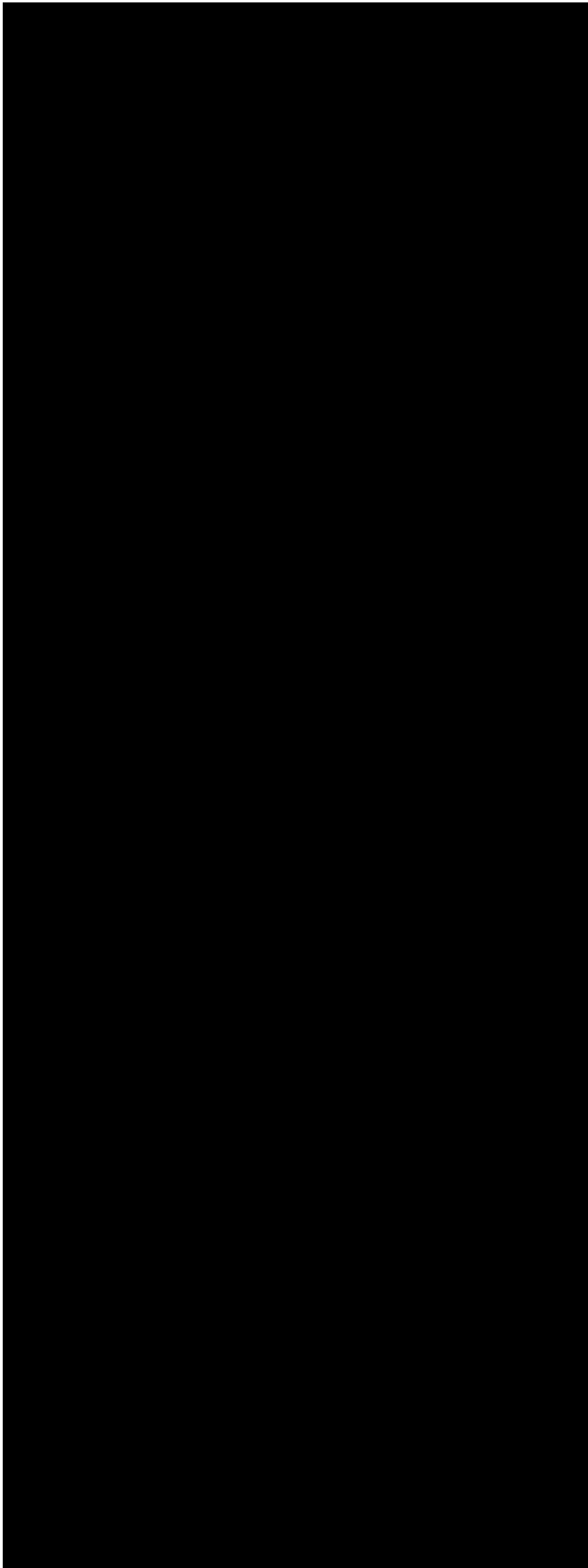
each other to form heterologous complexes (Yurchenco and Schittny, 1990, Yurchenco, 1994).

Collagen IV chains, using NH₂-terminal (Timpl et al., 1981; Weber et al., 1988; Siebold et al., 1988), COOH-terminal (Dunkan et al., 1982; Yurchenco and Furthmayr, 1984, 1986; Glanville et al., 1985; Siebold et al., 1987), and lateral association (Yurchenco and Furthmayr, 1984; Yurchenco and Ruben, 1987, 1988), form a covalently stabilized polygonal framework (Figs. 1.5.-1.6.) (Yurchenco and Schittny, 1990). Laminin self-assembles through terminal domain interactions to form a second polymer network (Fig. 1.7.) (Yurchenco et al., 1985; Charonis et al., 1986; Paulsson et al., 1988; Schittny and Yurchenco, 1989, 1990; Yurchenco, 1990). Entactin/nidogen binds laminin near its centre (Paulsson et al., 1987; Timpl, 1989) and interacts with collagen IV (Aumailley et al., 1989), bridging the two frameworks (Fig. 1.8.) (Yurchenco and Schittny, 1990). A large heparan sulphate proteoglycan is firmly anchored in the basement membrane and can bind itself through a core-protein interaction to form dimers and oligomers (Fig. 1.9.) (Yurchenco et al., 1987). It can also bind laminin (Timpl, 1989) and collagen IV (Fujiwara et al., 1984) through its glycosaminoglycan chains (Fig. 1.10.).

From these interactions, Yurchenco and Schittny (1990) proposed 3-D models of GBM architecture, starting with a simple collagen IV meshwork (Fig. 1.6.) which progresses in complexity to include laminin and entactin (Fig. 1.11.) and finally HSPG (Fig. 1.12.). How BM-40 and fibronectin fit into this model is unknown.

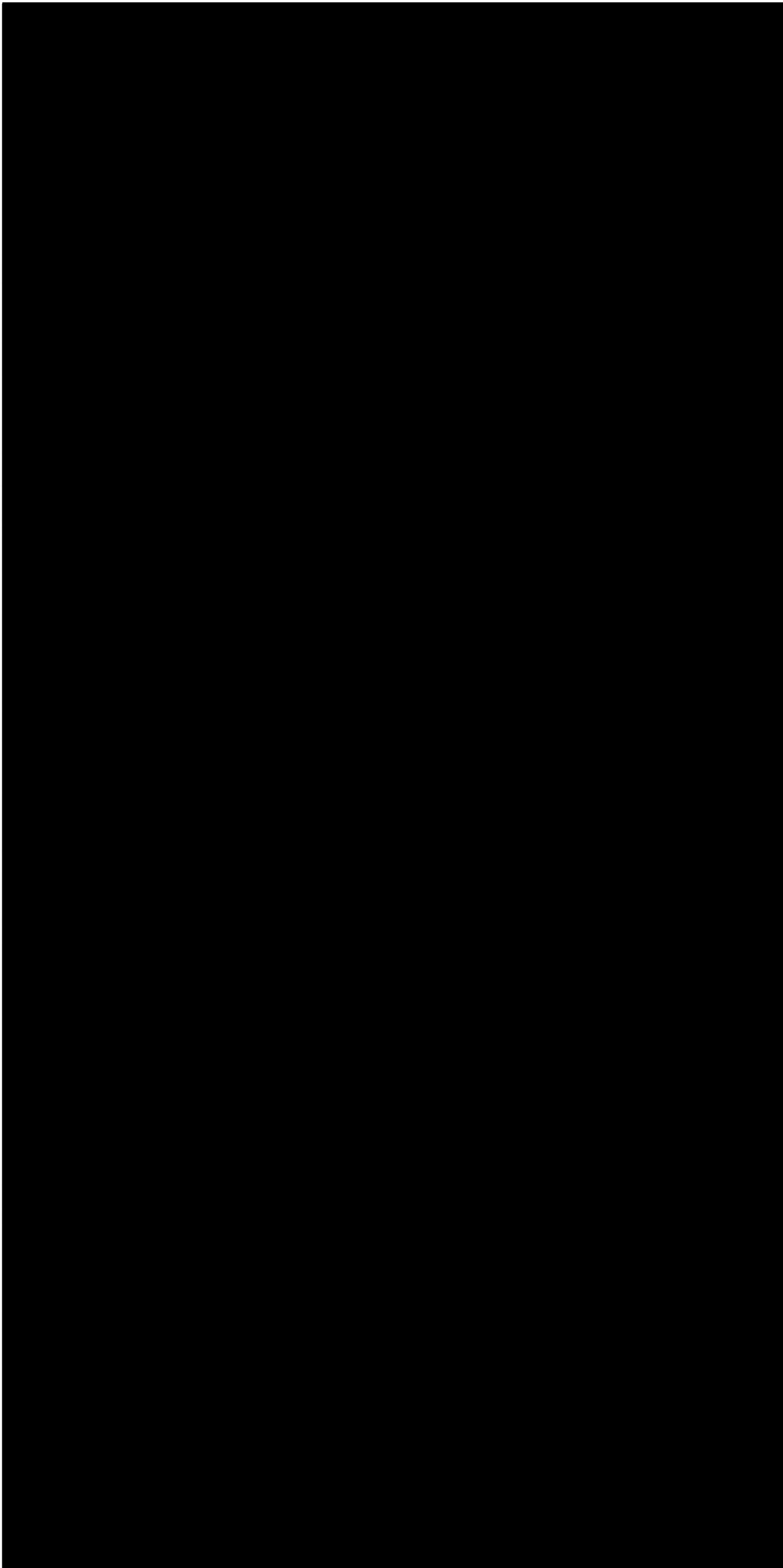
Yurchenco and Schittny's heteropolymeric meshwork model does not appear to have small enough polygonal interfibrillar spaces to explain near-complete rejection of albumin, even after a further reduction in the effective dimension of interfibrillar spaces due to the contribution of water structure surrounding the basement membrane proteins, particularly the HSPG (Yurchenco, 1990). However, the meshwork represents a 'relaxed' structure not under physiological filtration pressure. When compressed under physiological transcapillary hydrostatic pressure, GBM thickness can reduce to ~50% of its relaxed thickness with its fibre-packing density becoming high enough to give

Fig. 1.5. The collagen IV self-interactions



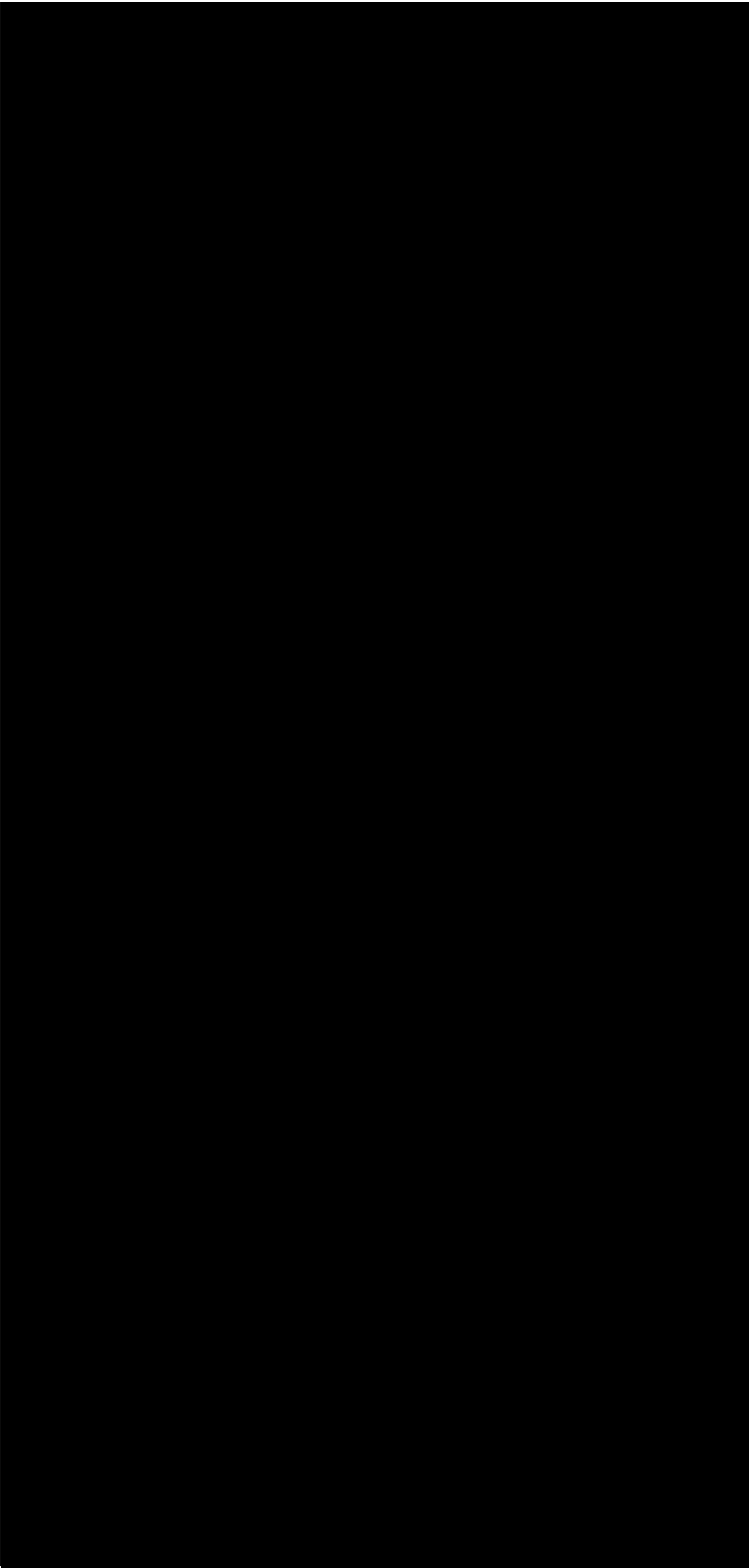
From Yurchenco, P.D. and Schittny, J.C. (1990) Molecular architecture of basement membranes. FASEB J. 4, 1587.

Fig. 1.6. Model of collagen IV framework



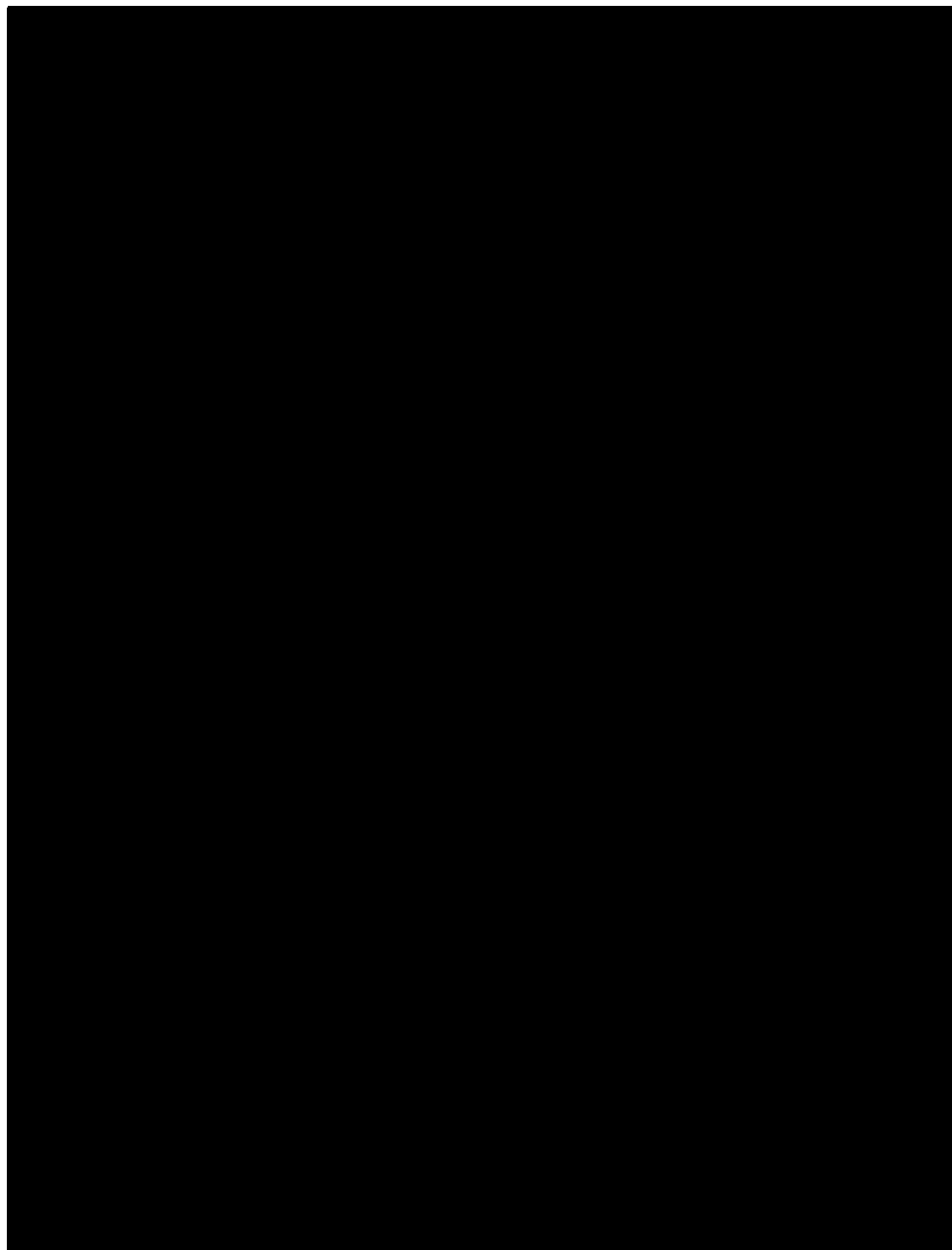
From Yurchenco, P.D. and Schittny, J.C. (1990) Molecular architecture of basement membranes. FASEB J. 4, 1588.

Fig. 1.7. Model of laminin polymer framework



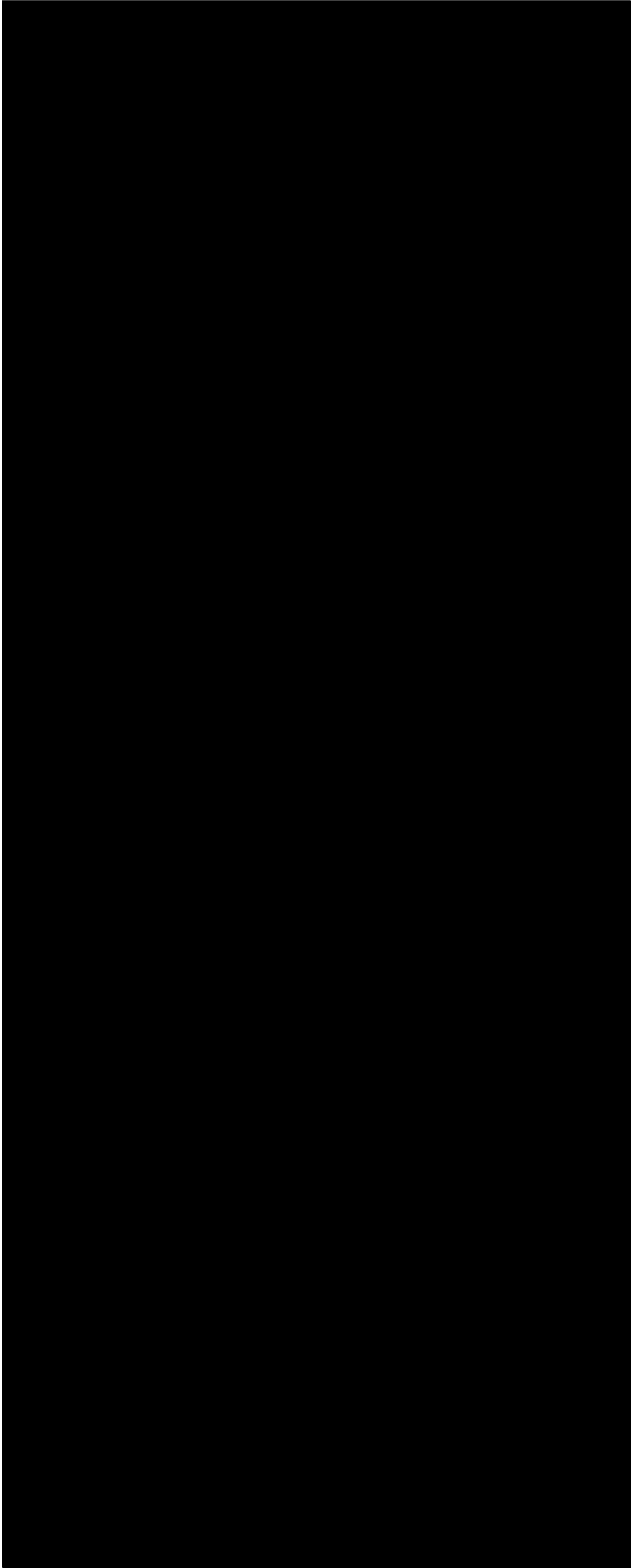
From Yurchenco, P.D. and Schittny, J.C. (1990) Molecular architecture of basement membranes. FASEB J. 4, 1587.

Fig. 1.8. Model of entactin-laminin-collagen IV interaction



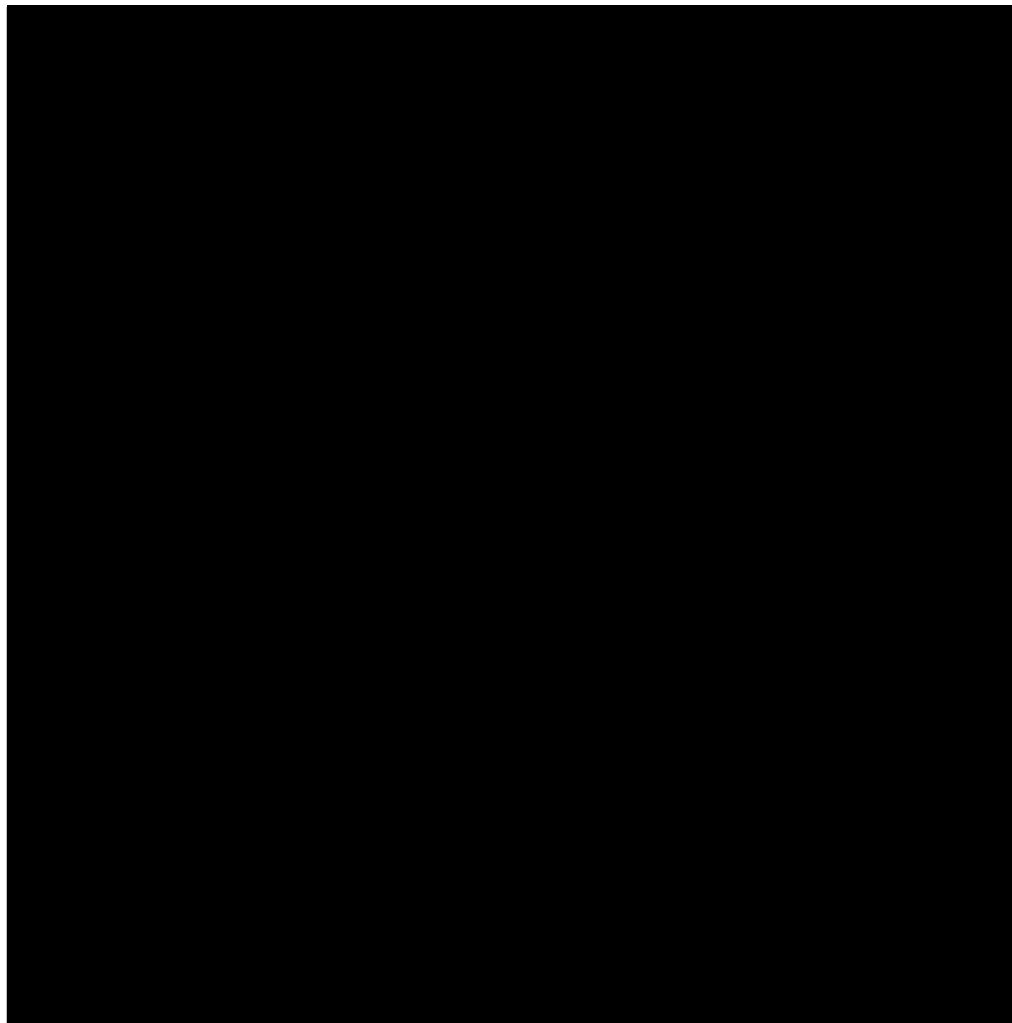
From Yurchenco, P.D. and Schittny, J.C. (1990) Molecular architecture of basement membranes. FASEB J. 4, 1587.

Fig. 1.9. Model of HSPG self-interaction



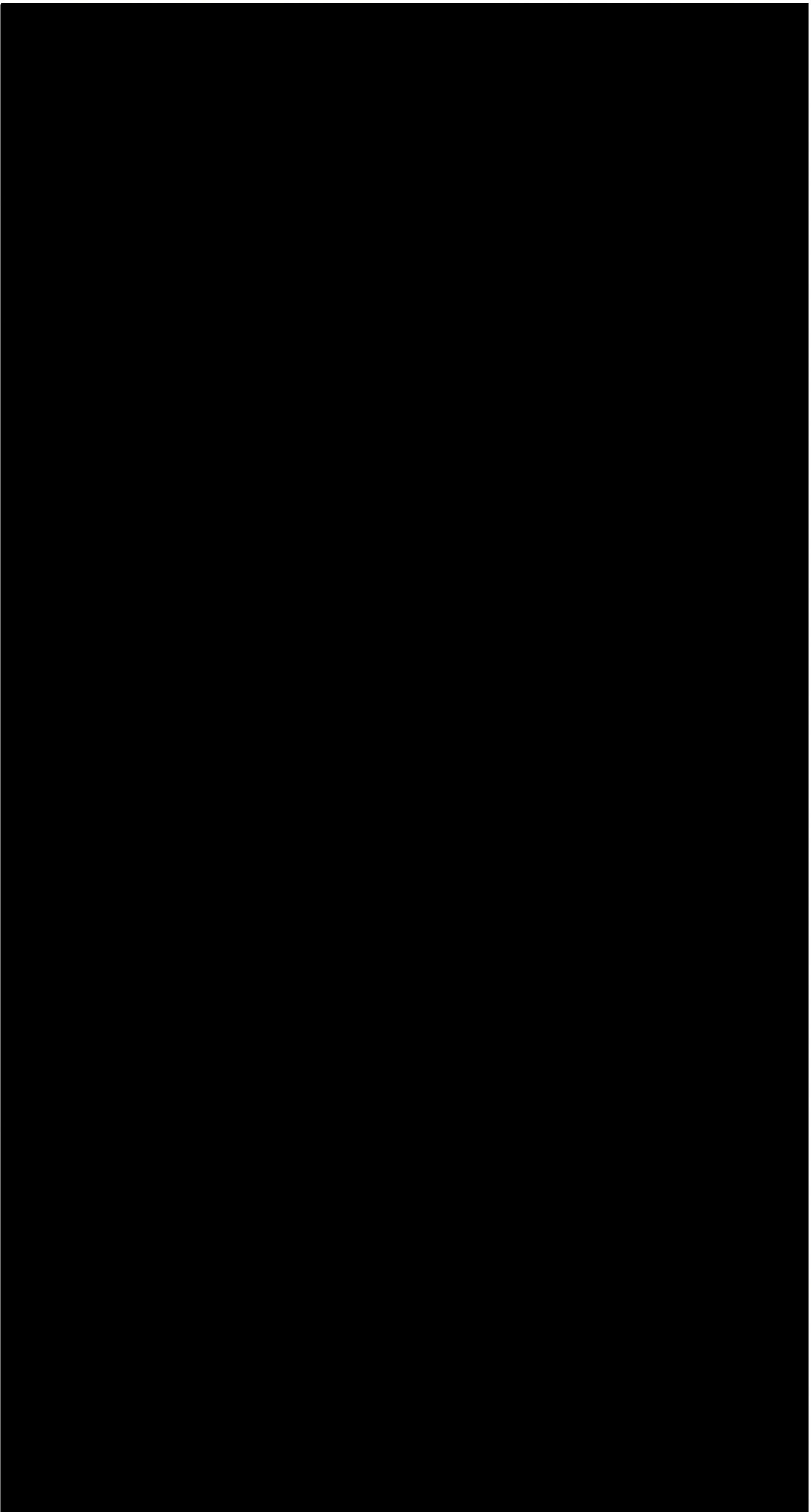
From Yurchenco, P.D. and Schittny, J.C. (1990) Molecular architecture of basement membranes. FASEB J. 4, 1587.

Fig. 1.10. Model of HSPG-laminin-collagen IV interactions



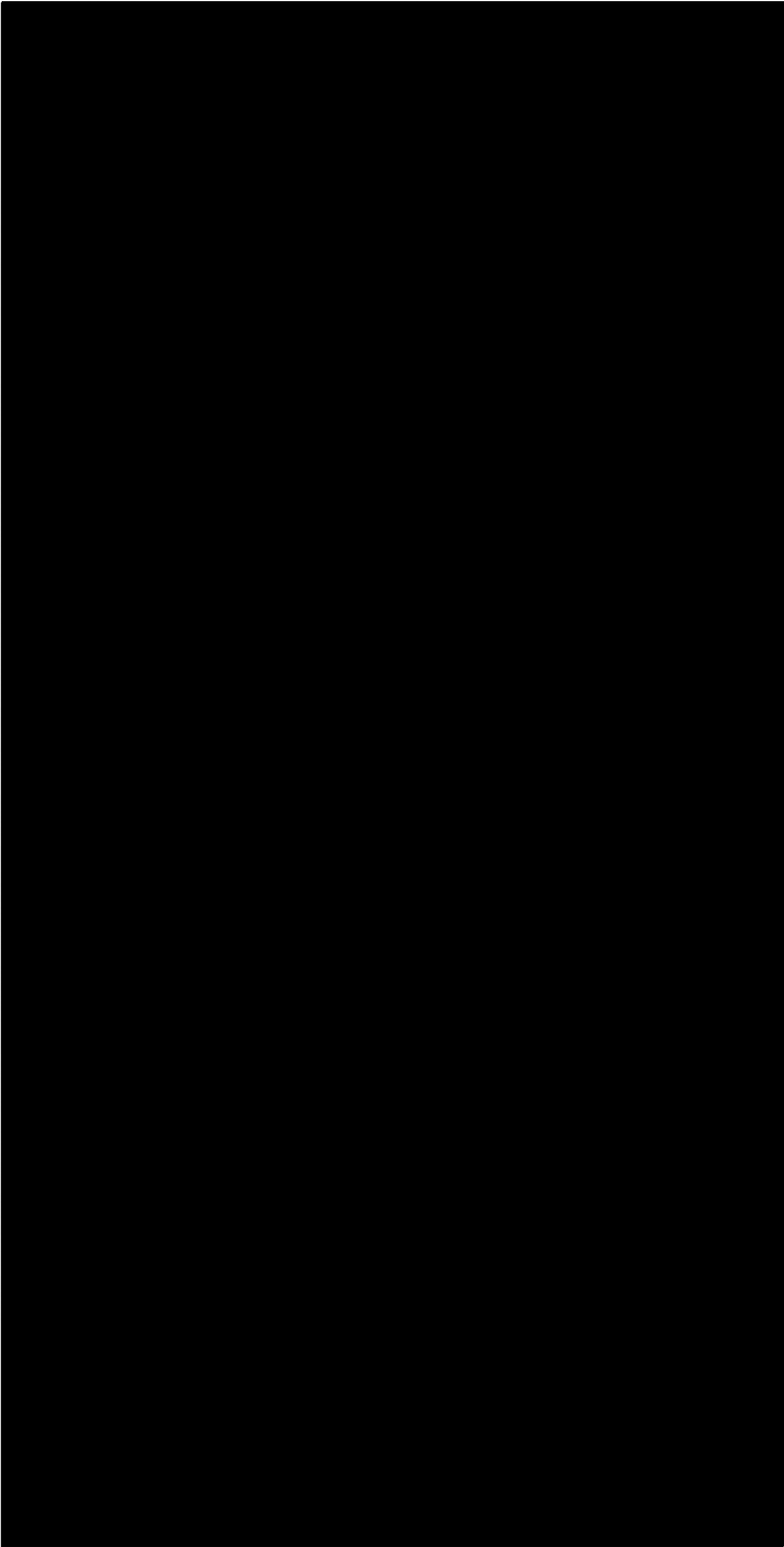
Adapted from Yurchenco, P.D. and Schittny, J.C. (1990) Molecular architecture of basement membranes. FASEB J. 4, 1587.

Fig. 1.11. Model of laminin-collagen IV polymer meshwork with incorporated entactin/nidogen



From Yurchenco, P.D. and Schittny, J.C. (1990) Molecular architecture of basement membranes. FASEB J. 4, 1588.

Fig. 1.12. Model of laminin-collagen IV polymer meshwork with incorporated entactin/nidogen and HSPG oligomers



From Yurchenco, P.D. and Schittny, J.C. (1990) Molecular architecture of basement membranes. FASEB J. 4, 1588.

near-complete rejection of albumin *in vitro* (Robinson and Walton, 1989) and *in vivo* (Maddox et al., 1992). Thus, it is possible to reconcile Yurchenco and Schittny's model structure and actual EM meshwork structure (Yamasaki et al., 1990; Hironaka et al., 1993), which have interfibrillar spaces (≥ 10 nm in width) too large to exclude albumin (7 nm in diameter), with the finding that the GBM rejects albumin.

1.10. Pathology of the GBM

Since GBM is the principal structure responsible for size discrimination amongst permeant plasma proteins (Farquhar et al., 1982), alterations in the structure of the GBM are central to the leakage of proteins (proteinuria) characteristic of glomerular injury (Farquhar, 1991). Indeed gross morphological damage to the GBM, such as gaps, have been seen in a large number of glomerular diseases, including severe acute poststreptococcal GN, rapidly progressive (or crescentic) GN, Goodpasture's syndrome, lupus nephritis, anaphylactoid purpura, and malignant nephrosclerosis (a form of renal disease, e.g., GN, associated with malignant or accelerated phase of hypertension) (Stejskal et al., 1973, Cotran et al., 1994). Such damage can explain haematuria and proteinuria diagnostic of these glomerulonephritides. Glomerular infiltration by neutrophils is frequently seen in these disorders and even at the gaps in GBM (Burkholder, 1969; Stejskal et al., 1973; Min et al., 1974) or lying adjacent to thinned GBM (Burkholder, 1969; Morrin et al., 1978).

In other forms of GN, morphological damage to the GBM is not evident and is not associated with neutrophil infiltration. Instead, thickening of GBM is seen in a wide range of glomerular diseases, including membranous nephropathy, diabetic nephropathy, and membranoproliferative GN (Cotran et al., 1994). Paradoxically, heavy proteinuria still occurs in these glomerular lesions in spite of basement membrane thickening, implying structural alterations had taken place which rendered the GBM more permeable to plasma proteins (Farquhar, 1991). To understand how proteinuria is brought about in all these different forms of GN and to explain the morphological

aberrations of the GBM associated with each lesion, it is necessary to briefly survey our current concept of the pathogenesis of glomerular diseases.

1.11. Pathogenesis of glomerulonephritis (GN)

Immune mechanisms of GN underlie the majority of glomerular diseases and by far the most common mechanism underlying immune GN is antibody-mediated injury (Cotran et al., 1994). Non-immune mechanisms of GN have been reviewed elsewhere as have non-antibody related mechanisms (Cotran et al., 1994). Two basic forms of antibody-associated injury have been established. First, injury by antibodies reacting *in situ* within the glomerulus, either with intrinsic glomerular antigens or with circulating antigens planted within the glomerulus (Couser and Salant, 1980). Intrinsic glomerular antigens include those of the GBM and on glomerular cells. Planted antigens can be DNA, lectin, IgG aggregates, bacterial antigens, etc. Second, injury resulting from deposition of soluble preformed circulating antigen-antibody complexes in the glomerulus (Couser, 1985; Andres et al., 1986; Wilson, 1988). The ensuing sections (1.11.1.-1.11.3.) introduce current concepts of how the various ways glomerular immune complexes form can lead to specific forms of immune GN.

1.11.1. Antibody binding to intrinsic glomerular antigens

A. GBM as source of intrinsic antigen

The intrinsic antigens involved in *in situ* immune complex formation could either be components of GBM or glomerular cells. Where GBM components serve as intrinsic antigens, anti-GBM GN results (Cotran et al., 1994), of which the best characterized example in man is Goodpasture's syndrome (Goodpasture, 1919). Here, the GBM antigen involved is the carboxyl terminal NC1 domain of collagen IV $\alpha 3$ chain (Butkowski et al., 1987). Goodpasture's syndrome accounts for ~2/3 of human anti-GBM GN in man (Weber, 1993) which, in turn, accounts for < 5% of human nephritis (Cotran et al., 1994). Goodpasture's syndrome is an autoimmune disease characterized by the presence of circulating anti-GBM antibodies which can bind to

normal human GBM (Donovan et al., 1994), indicating that the cause of this disease is not due to the existence of abnormal antigens but to abnormal production of autoantibodies. The autoantibodies bind to the GBM, forming a characteristic linear pattern when examined by fluorescence microscopy (Cotran et al., 1994).

The experimental prototype of human anti-GBM GN is Masugi or nephrotoxic serum nephritis (NSN) (Masugi, 1933) which can be induced in rats by injecting them with rabbit sera containing anti-rat kidney tissue antibodies. About 3 decades later, specific anti-GBM antibodies were used to elicit the glomerular disease (Cochrane et al., 1965) and this animal model has since been extensively studied to identify the mediators of the glomerular lesion, defined primarily by proteinuria. Neutrophil proteinases and oxidants appear to be responsible for the glomerular injury (Johnson et al., 1994). Serine proteinases secreted by activated neutrophils can digest and render GBM permeable to serum proteins *in vitro* (Cotter and Robinson, 1980b) and *in vivo* (Johnson et al., 1988). Neutrophil-derived hypochlorous acid can activate metalloproteinases (Weiss and Peppin, 1986; Shah et al., 1987) which then digest GBM (Vissers and Winterbourn, 1988a) and presumably also render GBM permeable to plasma protein. A more detailed review on the topic of neutrophil proteinases and oxidants in GN is given later.

B. Endothelial cells as source of intrinsic antigen

When glomerular cells provide the evocative antigens, different forms of animal models of GN become manifest, depending on which type of glomerular cells are affected. If the endothelial cell surface antigen angiotensin converting enzyme (ACE) is the target of antibody binding, then anti-endothelial nephrotoxic serum nephritis ensues, characterized by immune complex formation on the endothelial surface, neutrophil infiltration, and endothelial cell damage (Matsuo et al., 1985, 1987). There is no proteinuria in this glomerular disease (Matsuo et al., 1987). Unlike anti-GBM nephritis (Cochrane et al., 1965; Hawkins and Cochrane, 1968) neutrophils do not adhere to GBM (Matsuo et al., 1987), instead, they adhere to the endothelial cells

(Matsuo et al. 1987). This might account for the lack of proteinuria since any proteinases or oxidants released from immune complex activated neutrophils would not be directed onto the GBM.

C. Epithelial cells as source of intrinsic antigen

If epithelial cell surface antigen Fx1A (or GP330) serves as the site for antibody binding, then the so-called Heymann nephritis results (Heymann et al., 1959; Barabas and Lannigan, 1974; Feenstra et al., 1975). The human equivalent of Heymann nephritis is membranous glomerulonephropathy (Couser, 1993). Although the autoantibodies against the human GP330 antigen have been claimed to be found in the sera of patients with this disease (Makker and Kanalas, 1989), it has not since then been verified by other workers. However, in these glomerular lesions, anti-GP330 antibodies permeate across the GBM, bind to the antigen on the epithelial cell surface, and give the characteristic granular immune complex deposits on the sub-epithelial surface of GBM (Cotran et al., 1994). Basement membrane material is laid down between these deposits, appearing as irregular spikes which thicken to produce dome-like protrusions (Cotran et al., 1994). With progression of the disease, these eventually close over the deposits, burying them within a markedly thickened irregular membrane which can be 5 or 6 times thicker than normal GBM (Wheater et al., 1991). Since the immune complexes formed are separated from circulating inflammatory cells by the GBM, membranous nephropathy is non-inflammatory, characterized by total absence of neutrophil infiltration of the glomerulus (Rosen, 1971; Couser, 1993).

Antibody binding to the epithelial cell antigens results in foot process detachment from GBM surface (Cohen et al., 1977; Mendrick and Rennke, 1988a & b; Oriksa et al., 1988) and proteinuria (Salant et al., 1981; Mendrick and Rennke, 1988a & b; Oriksa et al., 1988). Detachment may cause proteinuria through loss of hydraulic conductivity barrier and increased water flux (Drumond and Deen, 1994) which produces bulk flow of macromolecules, including protein, through the capillary wall (Couser, 1993; Rennke, 1994). Indeed, in experimental nephritis where podocytes

were injured by aminonucleosides, e.g., puromycin, tracers (catalase and native ferritin) were seen to have leaked through the GBM into the urinary space at areas of detachment (Venkatachalam et al., 1970; Kanwar and Rosenzweig, 1982).

GBM may also be damaged by proteinases secreted by glomerular epithelial cells (Couser, 1993; Johnson et al., 1994). Watanabe et al. (1990a) demonstrated that Heymann nephritic rat glomerular epithelial secreted ~ 9 x more active gelatinase (Ca^{2+} -dependent; MW 92 k) than normal epithelial cells. Johnson et al. (1992b) had since then found that normal epithelial cells constitutively secreted an active 150 kda Ca^{2+} -dependent gelatinase capable of degrading GBM. Precisely how the epithelial cells are triggered to secrete these gelatinases is unknown, though Couser (1993) and Johnson et al. (1992b) speculated that antibody binding to podocyte antigens may elicit proteinase release. It is of interest to note that antigen binding to podocyte antigens can also result in the formation of membrane attack complex (MAC) which contributes to the development of proteinuria (Couser et al., 1985; Cybulsky et al., 1988; Couser, 1991). MAC itself may provide another means of triggering proteinase release (Couser, 1993; Johnson et al., 1992b).

D. Mesangial cells as source of intrinsic antigen

The involvement of a mesangial cell surface antigen in triggering off GN is best illustrated in an animal disease model called mesangial proliferative GN*, induced by injecting anti-mesangial antigen Thy 1.1 antibodies into rats (Johnson et al., 1990). The hallmark of this disease is mesangial cell proliferation and overproduction of mesangial extracellular matrix (Johnson et al., 1990). The human glomerular disease that most resembles mesangial proliferative GN is membranoproliferative GN (Couser and Johnson, 1994). It represents a major inflammatory type glomerular lesion that is not characterized by prominent glomerular neutrophil infiltration.

* Mesangial proliferative glomerulonephritis is strictly speaking a morphological pattern, not a disease entity, which does occur in man; for example in association with IgA nephropathy. However, it has been commonly used to describe that form of glomerulonephritis induced in animals by anti-mesangial Thy 1.1 antibodies.

The pathogenic mechanisms of mesangial proliferative GN have been intensively investigated. Following antibody binding to the mesangial antigen, the disease progresses in two phases. In the first phase (2-3 days) mesangial cell lysis, mesangial matrix dissolution (mesangiolysis), and glomerular platelet infiltration occur. In the second phase (2-3 weeks), prominent mesangial cell proliferation and matrix expansion with foci of matrix dissolution ensues (Yamamoto and Wilson, 1987; Johnson et al., 1990; Lovett et al., 1992). Heavy proteinuria started in the first phase and persisted for ~ 2 weeks (Bagchus et al., 1986). The mesangial cell lysis in the first phase is presumed to be mediated by MAC formed after antibody binding to Thy 1.1 antigen (Yamamoto and Wilson, 1987; Lovett et al., 1992). There is no evidence of active mesangial secretion of GBM-degrading proteinases at this phase (Lovett et al., 1992), though mesangial cells contain proteinases and their lysis may have leaked cytosolic proteinases into the extracellular matrix, resulting in mesangial matrix dissolution.

As for the cause of proteinuria underlying the second phase of the disease, a GBM-degrading 66-68 kda gelatinase secreted by proliferating mesangial cells is thought to be responsible (Lovett et al., 1992; Johnson et al., 1994). MAC has been shown to trigger off interleukin 1 release from mesangial cells (Lovett et al., 1984; 1987) which, in turn, has been demonstrated to stimulate gelatinase secretion (Martin et al., 1986). The secreted gelatinase is found along GBM, in high concentrations at points of GBM disruption, and at pockets of mesangial matrix dissolution in the expanding matrix (Lovett et al., 1992). Presumably the gelatinase had diffused from mesangial cells through its matrix to the GBM, digested the basement membrane, and rendered it permeable to plasma protein.

1.11.2. Antibody binding to planted antigens

In situ immune complex formation resulting from deposition of planted antigens has been reviewed in detail by Couser and Salant (1980). GN associated with systemic lupus erythematosus (SLE), a multisystem autoimmune disease of the connective tissue,

is thought to have resulted in part from deposition of DNA on GBM (Izui et al., 1976; Cotran et al., 1994). Concanavalin A is another antigen that was used as a planted antigen to trigger off a proliferative GN (Golbus and Wilson, 1979). The human equivalent of this disorder is post-infectious GN where bacterial lectin* antigens (Gilboa-Garber et al., 1972) or viral lectin-like antigens (Fenner et al., 1973) are thought to be deposited on the GBM, acting as planted antigens (Golbus and Wilson, 1979). When IgG aggregates are used as a planted antigen, acute nephritis results (Mauer et al., 1973). In all these forms of GN, glomerular infiltration by neutrophils is common (Couser and Salant, 1980). Neutrophils are attracted to the site of immune complex formation by the chemotactic attractant C5a (Cochrane et al., 1965), generated as a result of complement activation triggered by antibody binding to planted antigen (Reid, 1983). Upon binding to immune complexes on non-phagocytosable GBM, neutrophils have been shown to exocytose proteinases (Bray et al., 1983; Davies et al., 1984; Vissers and Winterbourn, 1984; 1988a) and generate oxidants (Bray et al., 1983; Vissers et al., 1985). The proteinases and oxidants are thought to be involved in GBM degradation and promoting proteinuria as will be discussed later.

1.11.3. Deposition of immune complexes at the glomerulus

Deposition of circulating immune complexes at the glomerulus can also induce GN. In animal models, circulating immune complexes form as a result of single or repeated intravenous injection of foreign antigens, giving rise to acute (Germuth, 1953; Germuth et al., 1955, 1957; Dixon et al., 1958, 1961; Cochrane and Koffler, 1973; Germuth and Rodriguez, 1973) and chronic serum sickness (Dixon et al., 1961; Dixon, 1969; Wilson and Dixon, 1971, 1976; Cochrane and Koffler, 1973; Germuth and Rodriguez, 1973), respectively; glomerular injury being common to both. Alternatively, preformed immune complexes can be injected into experimental animals, resulting in trapping of the circulating immune complexes on the glomerular wall and

* Lectins are proteins widely distributed in nature. Their presence is not confined only to plants but can be found on bacterial cell coat (Gilboa-Garber et al., 1972) and mammalian tissues (Ashwell and Morell, 1974).

manifesting acute serum sickness (Germuth and McKinnon, 1953; McCluskey and Benaceraff, 1959; McCluskey et al., 1960; 1962; Benaceraff et al., 1960; Miller et al., 1960). In man, certain post-infectious GN, e.g., poststreptococcal GN, circulating immune complexes, are thought to have formed as a result of antibody binding to streptococcal antigens* (Rossen et al., 1976; Ooi et al., 1977a & b; Tung et al., 1978; van der Rijn et al., 1978; Border, 1979), e.g., the cytoplasmic antigen endostreptosin and several cationic antigens (Vogt et al., 1983). In another human glomerular disease, IgA nephropathy (Berger's disease), circulating IgA aggregates (acting as immune complexes) become trapped in the mesangium and can also trigger off glomerular lesion (Cotran et al., 1994).

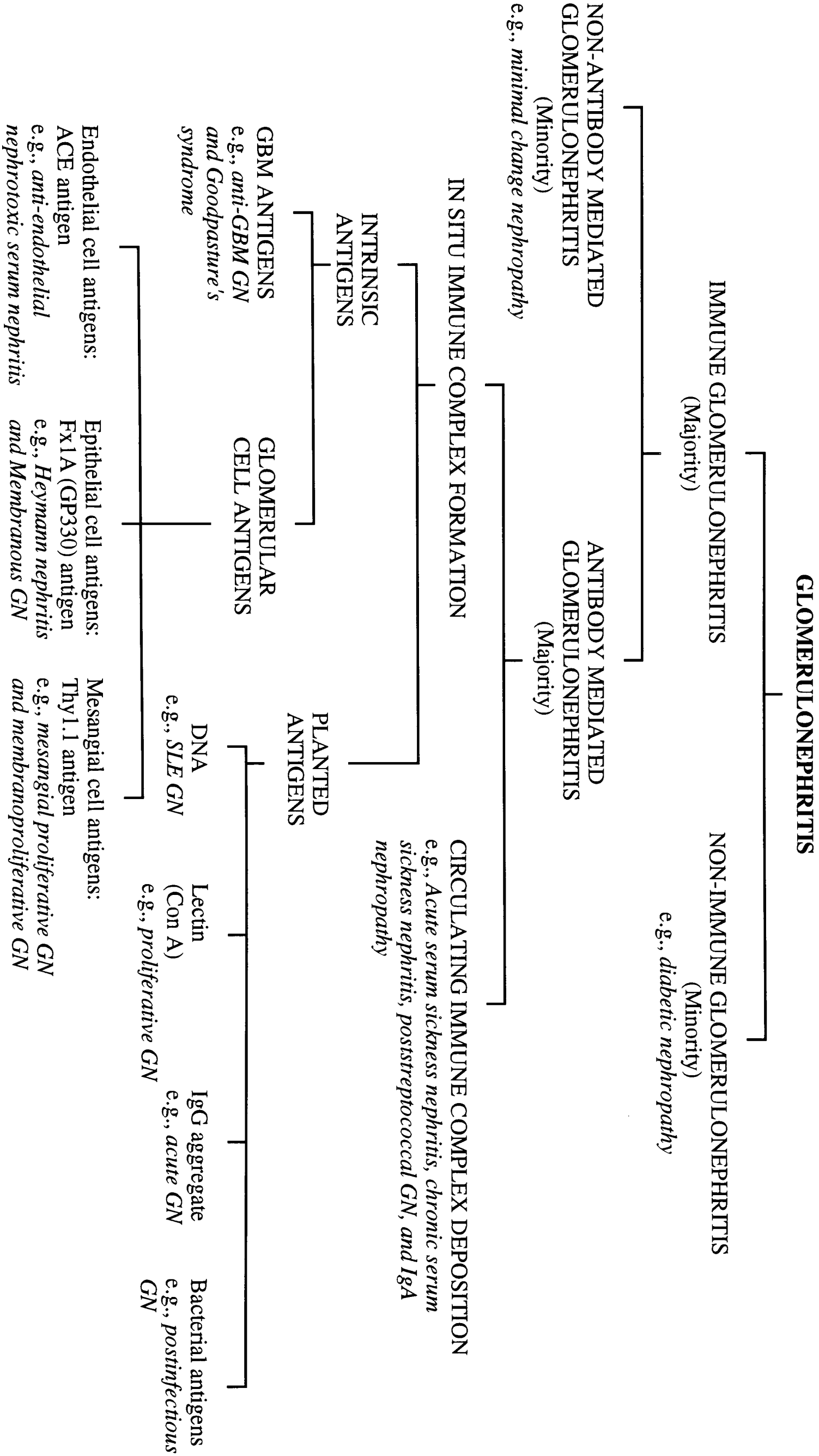
The foregoing account of how various forms of GN can be induced depending on the way glomerular immune complexes form is summarized in a family tree (Fig. 1.13.).

1.11.4. Mediators of glomerular injury

Regardless of how immune deposits form in glomerular disease, they cause injury, defined primarily as proteinuria, by two distinctly different mechanisms (Couser, 1993). One is an entirely non-inflammatory lesion in which a massive increase in glomerular permeability occurs in the absence of any glomerular infiltration by inflammatory cells or proliferation of resident cells. A second broad category of mediation mechanisms is that involving inflammatory hypercellular lesions resulting either from glomerular infiltration by inflammatory cells or proliferation of resident glomerular cells (Couser, 1993). The pathogenesis of non-inflammatory GN has been reviewed elsewhere (Couser, 1993). Our current understanding of pathogenesis of the inflammatory glomerular diseases will be discussed here since it is most relevant to this thesis.

* Streptococcal antigens are thought to have been bound to antibodies, forming immune complexes in plasma prior to deposition in the glomerulus. But they may become deposited in the glomerulus first prior to forming immune complexes with antibodies as suggested in Section 1.11.2. The two mechanisms are not mutually exclusive (Cotran et al., 1994).

Fig. 1.13. Mechanistic classification of the pathogenesis of glomerulonephritis



Glomerular infiltration by circulating inflammatory cells, particularly neutrophils and macrophages, has long been recognised as the major process which leads to inflammatory immune injury in glomeruli. This mechanism was extensively studied for decades before the non-inflammatory processes were identified (Cochrane et al., 1965; Hawkins and Cochrane, 1968). The participation of inflammatory cells in mediating GN depends largely on where immune deposits are formed in glomeruli (Salant et al., 1985; Fries et al., 1988). Deposits at subendothelial and mesangial sites are easily accessible to circulating cells which leads to their infiltration and activation. Deposits at subepithelial sites are separated from circulating cells by the GBM, and a non-inflammatory C5b-9 (MAC) mediated lesion results (Couser, 1985). The human equivalents of inflammatory cell mediated glomerular lesions would include diseases with predominantly mesangial and subendothelial immune complex deposits such as diffuse proliferative lupus nephritis, type I membranoproliferative GN, IgA nephropathy, and some type of acute post-infectious nephritis with mesangial and subendothelial deposits (Couser, 1993).

A. Neutrophil involvement in glomerular injury

Neutrophil infiltration of glomeruli was demonstrated by Cochrane et al. (1965) in NSN. Infiltration of the neutrophils is associated with heavy proteinuria and the severity is directly dependent upon the number of neutrophils present (Cochrane et al., 1965; Henson, 1972). The proteinuria can be prevented by neutrophil depletion using nitrogen mustard (Cochrane et al., 1965; Knicker and Cochrane., 1965; Henson, 1971b).

B. Neutrophil proteinases as injurious agents

The idea that neutrophil proteinases could damage GBM in immune complex GN and thereby promote proteinuria was proposed as early as 1968 by Hawkins and Cochrane. Using a rabbit model of experimental GN, they demonstrated neutrophil infiltration of the glomerulus and displacement of glomerular endothelial cells from

GBM by neutrophils. Neutrophil-dependent glomerular injury followed, as characterized by liberation of GBM fragments into the urine and a concomitant gross increase in the permeability of the GBM to plasma protein (based on renal clearance studies). They also detected neutrophil-derived acid proteinases (cathepsins) and cationic proteins in the urine along with the GBM fragments. Taking these observations together, they suggested that proteinases released from neutrophils are probable mediators of GBM damage leading to proteinuria.

Since then, a growing body of evidence supporting this hypothesis has accumulated. Neutrophil lysosomal proteinases (elastase and cathepsins G & D) and GBM fragments were found in urine of RPGN patients (Sanders et al., 1976; 1978). These proteinases degraded GBM collagen IV (Sanders et al., 1978). Neutrophil serine and metalloproteinases as well as GBM laminin and collagen IV were found in the urine of rats with experimentally induced anti-GBM nephritis (Davin et al., 1987). Neutrophils undergo exocytosis, releasing their granule proteinases to the exterior upon adherence to GBM coated with anti-GBM antibodies (Bray et al., 1983; Davies et al., 1984) or immune complexes (Vissers et al., 1984; Vissers and Winterbourn, 1988a). These proteinases included serine proteinases, elastase and cathepsin G (Bray et al., 1983; Vissers et al., 1984; Vissers and Winterbourn, 1988a), and metalloproteinases (Vissers and Winterbourn, 1988a) which degraded the GBM. Degranulated neutrophils lying in close apposition to GBM in the glomeruli of rabbits with NSN were also observed (Henson, 1971a & b).

These observations of neutrophil proteinase-mediated GBM damage were accompanied by evidence from Johnson et al. (1988a) who showed that kidneys infused with the human neutrophil serine proteinases elastase and cathepsin G, in amounts equivalent to *in vivo* release exhibited massive proteinuria. More direct evidence that GBM, damaged by neutrophil proteinases, becomes more permeable to protein came from an *in vitro* study by Cotter and Robinson (1980b). They showed that films, made from mixed rabbit GBM and TBM digested with rabbit neutrophil granule extract proteinases, became more permeable to serum protein, most of the effect

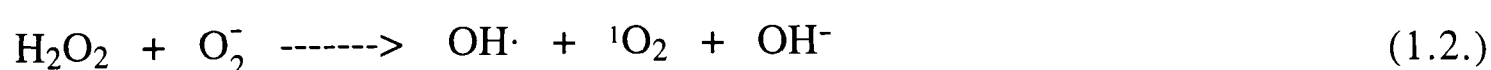
being mediated by an elastase-like enzyme. Although compelling, the evidence was based on a rabbit model of GN and rabbit neutrophil proteinases are quite different from human's. The rabbit neutrophils lack a true elastase (Cotter and Robinson, 1980a & b) and have no cathepsin G (Brown and Robinson, 1987). As yet there is no direct evidence to show that these particular serine proteinases are able to render GBM permeable to protein. In addition, it is not known whether neutrophil metalloproteinases are able to increase the permeability of GBM to protein. Moreover, the proteinases to BM ratio used by Cotter and Robinson for BM digestion was much higher than that generated by neutrophils were they to release all their proteinases. Thus, in spite of the impressive evidence of GBM degradation by neutrophil proteinases, that of GBM being rendered permeable to plasma protein remains circumstantial.

C. Neutrophil oxidants as injurious agents

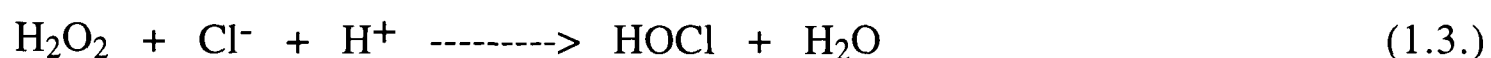
Neutrophil generation of oxidants has been under intensive investigation since the 1950s (Iyer et al., 1961) and has recently been reviewed in detail (Klebanoff, 1992). Briefly, neutrophils produce oxidants when stimulated by soluble immune complexes (Weiss and Ward, 1982; Ward et al., 1983) or by immune complexes immobilised on non-phagocytosable GBM (Vissers et al., 1985; Vissers et al., 1986). Superoxide anions (O_2^-) are generated by NADPH oxidase of neutrophil plasma membrane (Dewald et al., 1979). O_2^- then undergo spontaneous dismutation to form hydrogen peroxide (H_2O_2) (Root and Metcalf, 1977):



Several reactive oxygen species (ROS) can subsequently form depending on the fate of H_2O_2 (Shah, 1989; Klebanoff, 1992). If trace metals, such as iron (Fe^{3+}), are present then H_2O_2 can react with O_2^- to form hydroxyl radicals ($OH^\cdot$), singlet oxygen ($^1O_2$), and hydroxide ions (OH^-) via the so-called Haber-Weiss reaction (Aust et al, 1985; Halliwell and Gutteridge, 1986; Weiss, 1986):



If neutrophils release myeloperoxidase (MPO), then it can catalyse the oxidation of Cl^- by H_2O_2 , giving rise to hypochlorous acid (HOCl) - a potent oxidant (Klebanoff, 1980; Weiss and LoBuglio, 1982; Fantone and Ward, 1982; Lampert and Weiss, 1983):



The injurious effect of neutrophil-derived oxidants in NSN is well documented (Shah, 1989). Whether an oxidant is nephrotoxic has been largely inferred from oxidant depletion studies, usually involving the use of scavengers of specific oxidants. The therapeutic effect of catalase (H_2O_2 scavenger) administration on nephritic experimental animals indicates that H_2O_2 is responsible for the glomerular injury (Rehan et al., 1984; 1985a & b; 1986). By contrast, the administration of $\text{OH}\cdot$ scavengers, e.g., dimethyl sulphoxide (DMSO), mannitol, dimethyl thiourea (DMTU), sodium benzoate, and ethanol, confers no therapeutic benefits (Rehan et al., 1984; 1985a; 1986). Furthermore, $\text{OH}\cdot$ generation via the Fe^{3+} -catalyzed Haber-Weiss reaction is limited *in vivo* by the availability of free Fe^{3+} (Cohen et al., 1988; Ramos et al., 1992) and the source of Fe^{3+} remains unknown (Weiss, 1986). Taking this evidence together, $\text{OH}\cdot$ are thought unlikely to play a role in the pathogenesis of glomerular lesions (Shah, 1989).

Based on similar oxidant depletion studies, O_2^- does not appear to be important in mediating glomerular injury in experimental GN either, since superoxide dismutase (SOD) administration does not prevent the development of proteinuria or glomerular cell damage (Rehan et al., 1984; 1985a & b; 1986). As for the relevance of $^1\text{O}_2$ in GN, it was shown that $^1\text{O}_2$, generated by intrarenally perfused pheophorbide a (a plant pigment) following exposure to light, damaged glomerular endothelial and mesangial cells with evidence of glomerular coagulation (Ito et al., 1993). However, the formation of $^1\text{O}_2$ by activated neutrophils remains controversial (Steinbeck et al., 1992; Klebanoff, 1992) and it is not clear if substantial amounts are produced under physiological conditions (Foote et al., 1980a & b; Kanofsky et al., 1984). The physiological relevance of the findings of Ito et al. and the role of $^1\text{O}_2$ in the pathogenesis of GN remains questionable.

HOCl, as with the other oxidants, has been tested for its possible involvement in pathogenesis of proteinuria. Johnson et al. (1987a & b; 1988b) demonstrated the injurious effect of HOCl on the glomerulus using two experimental models. One involves the intrarenal perfusion of physiological amounts of myeloperoxidase, Cl^- , and H_2O_2 which results in *in situ* generation of HOCl in the glomerulus and glomerular injury (Johnson et al., 1987a; 1988b). The other model involves a demonstration of the activation of the MPO- H_2O_2 -halide system by intact neutrophils in a model of immune complex nephritis (Johnson et al., 1987b). Glomerular damage, such as endothelial cell swelling, lysis, or blebbing as well as podocyte foot process effacement, were clearly visible, particularly in the early part of the first disease model (Johnson et al., 1988b). Transient proteinuria, lasting 2-4 days, and glomerular infiltration by platelets, frequently found occluding the capillary lumen and degranulating onto denuded GBM, was also seen (Johnson et al., 1988b). Of particular interest was the demonstration of $^{125}\text{I}^-$ incorporation into the glomerular capillary wall, including the GBM, following activation of the cell-free MPO- H_2O_2 -halide system (Johnson et al., 1987a).

Using $^{125}\text{I}^-$ incorporation into the glomerulus as an indicator of the activation of MPO- H_2O_2 -halide system, Johnson et al. (1987b) further showed that the system is also activated by neutrophils in the immune complex nephritis model as judged by incorporation of intravenously injected $^{125}\text{I}^-$ into the glomerular wall. Together, the evidence from the two disease models suggested that HOCl generated by activated neutrophils may mediate glomerular injury, defined by glomerular cell damage and proteinuria. G.B. Robinson and co-workers showed that HOCl (0.3 - 1.5 mM) can render GBM films more permeable to serum protein following an incubation period of 1.5 hrs (G.B. Robinson, personal communication), suggesting that HOCl may have the potential directly to cause an increase in GBM permeability to protein *in vivo*, resulting in proteinuria. The finding may partly explain the proteinuria observed in Johnson et al.'s studies. An alternative explanation for the HOCl-dependent glomerular injury has been offered by Weiss and Peppin (1986) who suggested that neutrophil-

generated HOCl activates neutrophil gelatinase (Weiss and Peppin, 1986) which then degrades GBM (Shah et al., 1987; Baricos et al., 1988; Vissers and Winterbourn, 1988a), thereby rendering it more permeable to plasma protein.

Although glomerular injury mediated by H_2O_2 may be explained by the aforementioned mechanisms involving its reaction product HOCl, there is fragmentary evidence showing that it may directly damage the GBM and bring about proteinuria. For example, continuous intrarenal infusion of H_2O_2 alone at micromolar concentrations (5-500 μM) for 30 min (total amount = 0.12 - 12 μmol) induced endothelial and mesangial damage in the rabbit (Stratta et al., 1989). Stratta et al. (1989) suggested that such low concentrations of H_2O_2 may be produced by activated neutrophils and the results are likely to have pathophysiological relevance. Yoshioka et al. (1991) subsequently demonstrated massive reversible proteinuria without apparent glomerular ultrastructural abnormality in Munich-Wistar rats following a 1 hr continuous infusion of H_2O_2 at mM concentrations (18 - 100 mM) (total amount = 9 - 50 μmol). Incubation of soluble collagen IV with low concentrations of H_2O_2 (50 mM) at 37°C resulted in extensive degradation after just 8 hrs (Pipoly and Crouch, 1986). Similar concentrations of H_2O_2 (< 70 mM) fragmented mucus glycoproteins (Creeth et al., 1982; 1983). Incubation with much higher concentrations of H_2O_2 completely solubilized GBM (Fligiel et al., 1984). Thus H_2O_2 may directly affect GBM *in vivo*.

1.11.5. Glomerular coagulation and activation of plasma transglutaminase

Coagulation frequently occurs in nephritic glomeruli following injury of the glomerular capillary wall (Kanfer, 1989). Indeed, the presence of endproducts of coagulation, such as fibrin (McCutcheon et al., 1993), platelets, and activated enzymes of coagulation, such as plasma transglutaminase (Factor XIII) (Kamitsuji et al, 1983; Colasanti et al., 1987; Deguchi et al., 1989) have been demonstrated in nephritic glomeruli of patients with IgA glomerulonephropathy, membranous nephropathy, Lupus nephritis, non-IgA mesangial proliferative GN, minimal change nephrotic

syndrome, focal glomerular sclerosis, and diabetic glomerulosclerosis (Deguchi et al., 1989). These materials were identified by immunohistochemical methods in the glomeruli of renal biopsies from these patients as well as from animals with experimental GN (Deguchi et al., 1989).

Of interest is the nature of plasma transglutaminase (TGase) as a protein cross-linking enzyme capable of cross-linking fibrin molecules in coagulation to form tight clots (Greenberg et al., 1991). By virtue of its general ability to form cross-links between NH_2 group of lysine residues of one polypeptide and COOH group of glutamate residue of another (Greenberg et al., 1991), it might be able to cross-link proteins within the GBM. Indeed, cross-linking of fibronectin to extracellular matrix (Barry and Mosher, 1988) and α_2 -antiplasmin to fibrin (Tamaki and Aoki, 1985) mediated by plasma TGase has been demonstrated. TGase is also located in the cytoplasm and on the surface of monocytes and tissue macrophages (Henriksson et al., 1985) as well on platelet surface (Greenberg and Shuman, 1984) and cytosol (Folk and Chung, 1985). The TGase here is termed tissue TGase. During glomerular inflammation monocytes and platelet infiltration of the glomerulus is common (Cotran et al., 1994; Couser, 1993). Either platelets or dying monocytes may release their TGase onto GBM and thereby contribute to introducing cross-links. Aeschlimann and Paulsson (1991) have recently shown that guinea pig liver tissue transglutaminase cross-linked laminin-nidogen complexes.

Chemical cross-linkers such as glutaraldehyde and dimethyl malonimidate have been shown to cross-link GBM proteins and render the GBM more permeable to serum protein, particularly at high filtration pressures (Walton et al., 1992). It was proposed that by cross-linking GBM collagen IV fibres, the GBM contracted and became incompressible (Walton et al., 1992). Walton et al. (1992) argued that at high filtration pressures, the native GBM was compressed and had a smaller void volume through which protein could permeate than the rigid cross-linked membrane. Therefore, the protein flux across cross-linked matrix was higher than the native matrix. The possibility that plasma or tissue transglutaminase might also cross-link GBM proteins,

achieving similar results has not been explored. It may be that cross-links can be introduced into the GBM by activated plasma transglutaminase following coagulation or by tissue transglutaminase released from dying monocytes or platelets, rendering it permeable to plasma protein. Since cross-links in GBM are expected to be chemically stable (Walton et al., 1992) it may explain long term proteinuria which does occur in nephrotoxic nephritis in the presence of macrophages (Holdsworth, 1983) and following coagulation (Cotran et al., 1994; Couser, 1993).

1.12. Directions in the current study

Essentially three ideas have been discussed in Sections 1.11.4. and 1.11.5. where evidence was presented to indicate that neutrophil oxidants/proteinases and transglutaminases may cause proteinuria in immune GN. This prompted an *in vitro* analysis of whether they can render GBM more permeable to protein. Chapters 4 and 5 are devoted to analyses of the effect of guinea pig liver transglutaminase and H₂O₂ on the permeability of pig GBM to protein, respectively. Chapter 6 deals with the extraction and preliminary characterization of pig neutrophil proteinases prior to using them to study their effect on GBM permeability, the main subject of Chapter 7.

In vivo analysis of the effect of test agents on glomerular permeability, such as the intrarenal oxidant and proteinase perfusion studies of Johnson et al. (1987a & b; 1988a & b) has one distinct disadvantage, namely, that it is difficult to attribute proteinuria to GBM damage alone since these agents may also injure or perturb other components of the glomerular wall, e.g., endothelial cells and podocytes (Johnson et al., 1987a & b; 1988b). These may in turn damage GBM through secretion of their own oxidants and proteinases (Johnson et al., 1994). In addition, podocyte injury may result in foot process detachment from the GBM, causing proteinuria (Couser, 1993; Rennke, 1994). An alternative system of analysis is needed to eliminate these complications and allow a carefully controlled perturbation study of the effect of different agents (e.g., proteinases) on the permeability of GBM to protein. Such a

system has been developed in this laboratory (Robinson and Brown, 1977) and proved useful in determining whether a test agent can render the GBM more permeable to protein (Cotter and Robinson, 1980b; Walton et al., 1992). The set up of this filtration system is introduced in Chapter 3 where the normal filtration behaviour of GBM to protein and water under varying filtration pressures is outlined and explained.

CHAPTER TWO

CHAPTER 2 MATERIALS AND METHODS

2.1. Materials

Pig kidneys and blood were obtained from freshly killed abattoir pigs. Guinea pig livers were from freshly sacrificed guinea pigs of Oxford University Animal Farm. Hema-Gurr rapid differential staining set for haematology was purchased from BDH Diagnostics (Poole, England). BCA protein microassay kit, 3.5 x 10 cm DEAE-cellulose ion exchange chromatography column, 2.5 x 100 cm, 10 % fine agarose Biogel A-0.5m gel filtration chromatography column were from BioRad (Hertfordshire, U.K.). Amicon pressure filtration cells of 65 ml capacity were from Amicon Ltd., High Wycombe, Bucks., U.K. Precast SDS PAGE gels (3-40 % highly cross-linked; 3-13 % linear gradient, both 3 x 72 x 72 mm) were purchased from Flowgen Instruments Ltd., Kent, U.K. *Micrococcus lysodeikticus* capsules were from Sigma Chemical Co., London, U.K.

Chemicals were obtained commercially: hydroxylamine, carboxy-glutaminyglycine, tricine, sodium dodecyl sulphate, glycerol, dithiothreitol, bromophenol blue, Coomassie Brilliant Blue, *Clostridium histolyticum* collagenase (IV), 3,3',5,5'-tetramethylbenzidine, *p*-nitrophenol, *p*-nitrophenol disodium orthophosphate, tetrazolium salt of 2-(*p*-iodophenyl)-3-(*p*-nitrophenyl)-5-phenyltetrazolium, N-tert-butoxycarbonyl-L-alanyl-L-prolyl-L-norvaline benzylthioester, methoxy-O-succinyl-L-alanyl-L-alanyl-L-prolyl-L-valine *p*-nitroanilide, *p*-nitrophenyl-N-tert-butoxycarbonyl-L-alanine, and succinyl-L-alanyl-L-alanyl-L-alanyl-L-prolyl-L-valine *p*-nitroanilide, N-succinyl-L-alanyl-L-alanyl-L-prolyl-L-phenylalanine *p*-nitroanilide, *p*-nitroaniline, 5,5'dithiobis(2-nitrobenzoic acid), 4,4'-dithio-bis(pyridine), N-benzyloxycarbonyl glycyl-L-leucyl-L-phenylalanine chloromethyl ketone, phenyl methyl sulphonyl fluoride, elastin-orcein, molecular weight markers: subunit of myoglobin (205k), subunit of β -galactosidase, subunit of phosphorylase B (97.4k), bovine albumin (66k), ovalbumin (45k), subunit of glyceraldehyde-3-phosphate dehydrogenase (36k), carbonic anhydrase (29k), trypsinogen (24k), trypsin inhibitor (20k), and alpha

lactalbumin (14k), myoglobin (horse), bovine serum albumin (BSA), (ethylenediamine tetraacetic acid) EDTA, protamine, deoxyribonuclease I (Type DN25), 30 % H_2O_2 solution were from Sigma Chemical Comp. (London, U.K.).

N-tert-butoxycarbonyl-L-alanyl-L-alanyl-L-prolyl-L-alanine *p*-nitroanilide, L-pyroglutamyl-L-prolyl-L-valine *p*-nitroanilide, and succinyl-L-alanyl-L-alanyl-L-prolyl- α -aminobutyric acid *p*-nitroanilide were from Calbiochem (Nottingham, U.K.). Hank's balanced salt solution (Ca^{2+} , Mg^{2+} , NaHCO_3 , and phenol red-free) and 1 M Hepes buffer, pH 7.4 were from Gibco, Paisley, Scotland. Succinimidyl[2,3- ^3H]propionate was from Amersham (Buckinghamshire, U.K.). Ecoscint was from National Diagnostics (Buckinghamshire, U.K.). All other chemicals were purchased from usual commercial sources and were analytical reagent grade where possible.

2.2. Preparative methods

2.2.1. Pig GBM preparation

Pig GBM was prepared by a modification of the method of Ligler and Robinson (1977). Kidney cortices from 6-8 pig's kidneys were minced through a Clampex mincer fitted with a screen containing a ring of 6 mm apertures and the minced materials was smeared through a 250 μm sieve (Endecott Ltd., London) with the base of a beaker, accompanied by washing with large volumes of TBS (0.01 M Tris/HCl, pH 7.4, 0.15 M NaCl, 0.02 % NaN_3). This procedure fragmented the nephrons as they passed through the mesh. The fragmented nephrons were then washed through a 75 μm sieve which retained glomeruli but allowed tubules to go through. Repeatedly dipping the sieve back into the buffer yielded glomeruli > 90% pure. To dissolve cellular elements from the GBM, the glomeruli were vigorously homogenised in 0.5 % (w/v) of the detergent N-lauroyl sarcosine for 10 min. using a Silverson mixer homogeniser (Silverson Ltd., Chesham, Bucks., U.K.). The GBM was then sedimented by centrifugation at 2,000 x *g* for 10 min., resuspended and homogenised in fresh detergent for a further 10 min. to dissolve any cell residues. The GBM was sedimented as before and washed three times in TBS. To prepare fragments from the

GBM structure, GBM suspension was sonicated on ice for periods of 45 seconds with cooling intervals of 30 seconds for a total of 8 min. using a MSE transducer equipped with a 1 cm probe, peak to peak amplitude of 18 μm . To remove DNA contaminants from the preparation, GBM fragments were resuspended in 200 ml of unbuffered isotonic saline containing 0.01 % (w/v) deoxyribonuclease I (Type DN 25, Sigma Chemical Co., London) and left standing at room temperature for 30 min. Since the preparation still had some collagen fibre contamination, the suspension was washed through a 250 μm sieve and this removed virtually all of the fibres. The GBM was collected by centrifugation at 2,000 x g for 10 min., washed three times in TBS, and stored with 0.02 % NaN_3 at 0°C.

2.2.2. Pig GBM film preparation

Thin films of pig GBM, ~ 8 μm thick, were prepared by packing a suspension of 1.5 mg (dry weight) of GBM fragments onto Millipore membrane (0.45 μm exclusion HAWP membranes, Millipore, London, U.K.) under pressure in Amicon type 52 filtration cells (Amicon, High Wycombe, U.K.). Tris buffered saline was filtered through the resulting films for 30-40 min. under pressure (200 kPa). Films formed in this way have been shown to consist of closely interleaved fragments of membrane. They are free from leaks, mechanically contiguous, and show reproducible water fluxes (Robinson and Walton, 1989).

2.2.3. Guinea pig liver transglutaminase preparation

Guinea pig liver transglutaminase (TGase) was isolated using the method of Folk and Chung (1985). Briefly, guinea pig liver homogenate underwent DEAE cellulose chromatography (3.1 x 24 cm Cellex D column, Biorad), two differential precipitations involving protamine sulphate and ammonium sulphate, and gel filtration chromatography (2.5 x 100 cm Biogel A column). The TGase purified was assayed by following the formation of Z-L-glutamyl(γ -hydroxylamine)-glycine (A_{525}) from the substrates carboxy-glutamyl-glycine and hydroxylamine (Folk and Chung, 1985). One

unit of enzyme activity is defined as the amount of enzyme needed to form 1 μmol peptide derivative of γ -glutamyl hydroxylamine per min. at 37°C, pH 6.0. The yield of TGase is routinely ~ 40 units per 60 g liver (2 %) with a specific activity of ~ 20 units/mg.

2.2.4. Pig peripheral neutrophil preparation

Pig peripheral neutrophils were isolated using a slight modification of the method of Eggleton et al. (1989). Briefly, 4 litres of blood from freshly killed pigs, anti-coagulated with potassium salt of EDTA at a final concentration of 1.5 mg/ml, was mixed well with 16 litres of isotonic NH_4Cl at 4°C, and left for 15 min. to permit haemolysis. Leukocytes were then sedimented by centrifugation at $160 \times g$ for 10 min., gently resuspended in 100 ml Hank's balanced salt solution (HBSS), and recentrifuged at $55 \times g$ for 10 min. to deposit most of the neutrophils. The neutrophil rich pellet was subject to differential centrifugation once more to enrich the neutrophil fraction. The neutrophils were resuspended in 50 ml HBSS and stored at 4°C. The only difference between the modified and the original methods was that 4 litre of pig's blood was used instead of 4 ml of human blood. The neutrophils so prepared were generally 85 ± 8 % pure (mean $\pm$ SD). The yield was routinely $4.55 \pm 0.90 \times 10^9$ cells per litre of blood which, as a percentage of total neutrophils, was 65 ± 9.5 %.

2.2.5. Pig peripheral neutrophil proteinase preparation

The granules were isolated from pig neutrophils and the proteinases subsequently extracted from the granules by a modification of the method of Cohn and Hirsch (1960). 1×10^9 neutrophils were washed in 50 ml of ice cold 0.34 M sucrose solution, resuspended in 20 ml of fresh sucrose solution, and sonicated on ice using a MSE transducer (1 cm probe, amplitude 18 μm) until complete cell disruption resulted. This normally took 5-7 min. Sonication was an introduced protocol which significantly enhanced neutrophil breakage and granule release.

Intact nuclei and plasma membrane debris were sedimented by centrifugation at 400 x g, 4°C for 10 min, giving a supernatant rich in granules. The nuclear fraction was washed in sucrose, recentrifuged, and the resulting supernatant combined with that from the previous centrifugation. The granules were sedimented by centrifugation at 8,200 x g for 15 min. at 4°C, gently resuspended in sucrose by means of a Teflon pestle, recentrifuged as before, and resuspended in 10 mM Tris/HCl buffer, pH 7.4 instead of 0.1 M sodium phosphate buffer. The granules were lysed by freezing and thawing three times in -70°C dry ice/ethanol mixture to liberate proteinases and centrifuged at 30,000 x g for 20 min. to yield a supernatant containing soluble proteinases and granule membrane pellet containing solubilizable proteinases. The proteinases from the pellet was extracted three times in total with 10 mM Tris/HCl buffer, pH 7.4, containing 1.0 M NaCl and 0.5 % Triton X-100 (v/v) by vigorous syringing and pooled.

2.2.6. ³H-pig GBM preparation

³H-GBM was prepared according to a modification of the method of Bolton and Hunter (1973). Succinimidyl[2,3-³H]propionate was used to label protein instead of succinimidyl-3-(4-hydroxy-[¹²⁵I]iodophenyl)propionate. The labelling method was based on the reaction between the carbonyl group of propionate residues and the amino group of lysine residues in protein, giving rise to protein with tritiated propionate moieties. To label 50 mg of dry-weight equivalent of GBM, it was prewashed three times in 0.1 M borate buffer, pH 8.5, added to 0.1 mCi of air-dried succinimidyl[2,3-³H]propionate in a boiling tube, stirred for 15 min., and washed three times in Tris buffered saline, pH 7.4. The ³H-GBM was then ready for use but if stored for any length of time, slight spontaneous release of ³H-label occurred and washing with TBS, pH 7.4 was required to remove free label.

2.2.7. Pig serum preparation

Blood from freshly killed abattoir pigs were centrifuged at 500 x g for 20 min. to sediment blood cells. The plasma was allowed to clot in the presence of 5 mM CaCl_2 and 0.02 % NaN_3 at 37°C for 12 hrs. Serum was decanted over a 4 layer cheese cloth filter to strain off the clot and centrifuged at 20,000 x g for 15 min to sediment any contaminant clot in the filtrate. The serum in the supernatant was removed and used immediately for filtration studies.

2.3. Analytical methods

2.3.1. SDS-PAGE

SDS-PAGE was performed by the method of Laemmli (1970). For analysis of guinea pig liver TGase purity, gels (3-40 % highly cross-linked, 72 x 72 x 3 mm) were run in a TTS continuous buffer system at 100 V gel for 6 h at 4°C and stained with Coomassie Brilliant Blue (0.1 % w/v). Protein samples were boiled for 10 min. in TTS buffer (9.2 mM Tris, 20 mM tricine, 0.1 % SDS, pH 7.5), containing 10 % glycerol, 10 mM DTT, and bromophenol blue.

For demonstration of cross-linked GBM proteins catalysed by TGase, gels (3-13 % linear gradient, 72 x 72 x 3 mm) were run in a TTS continuous buffer system at 25 V per gel for 7 h and stained with Coomassie Blue. Since cross-linked GBM were insoluble, it had to be solubilized by a combination of collagenase treatment and heating at 100°C for 1 h in TTS buffer with 10 % glycerol, 10 mM DTT, and bromophenol blue. The collagenase treatment involved digestion of 6 mg of TGase-treated GBM with 195 units (0.5 mg) of *Clostridium histolyticum* collagenase IV (Sigma) in 2 ml of TBS, pH 7.4, containing 10 mM CaCl_2 with tumbling at 37°C for 9 h (1 unit = amount of enzyme needed to liberate peptides from bovine archilles tendon collagen type I equivalent in ninhydrin colour to 1.0 μmol of leucine in 5 hrs at pH 7.4, 37°C in the presence of Ca^{2+} ions (Seglen, 1976)). Controls with GBM treated with heat-denatured TGase were similarly prepared.

2.3.2. GBM film thickness measurement

Film thickness analysis was performed according to the method of Robinson and Walton (1989). Films of membrane were deposited on Millipore membranes marked with fine ink lines in Amicon filtration cells (65 ml volume) and film surfaces made visible by coating with *Micrococcus lysodeikticus* capsules. Film thickness was measured from the vertical displacement of a microscope, focused first on the capsules and then on the ink lines. Measurements at 20 disperse points were made for each film and averaged. Thicknesses of films made from native GBM with 1.5 mg dry weight were generally $7.9 \pm 0.6 \mu\text{m}$.

2.3.3. Filtration studies

These were conducted as described elsewhere (Robinson and Cotter, 1979; Walton et al., 1992). Briefly, protein solutions (myoglobin: 0.5 mg/ml; BSA: 2 mg/ml) were filtered through films made from pig GBM fragments (Section 2.2.2.) in Amicon pressure filtration cells. Filtration was started by applying gas (N_2) pressure and filtration continued until 8 ml of filtrate had accumulated; by this time the dead space below the filter had equilibrated with the filtrate. Small samples of filtrate were then collected for determination of buffer flux (J_v) and solute flux (J_s). Protein concentrations were determined from A_{280} measurements. When serum was filtered, protein in samples of serum and filtrate was precipitated with trichloroacetic acid (final concentration 5 % w/v), washed, and dissolved in 0.1 N NaOH for the measurement of absorbance values. The solute rejection, the fraction not passing through the filtration barrier ($1 - C_f/C_b$), was calculated from the protein concentrations in the filtrate (C_f) and in the overstanding solution (C_b). Usually, up to 6 cells were operated in parallel, and three consecutive samples were taken from each cell at each filtration pressure for the calculation of results. Filtrations were undertaken at a range of pressures to provide a pressure profile; the cells were stirred at 1,000 r.p.m. to minimise unstirred layer effects. Pressure was routinely varied from 10 up to 200 kPa, the results were unaffected by working from high to low pressure.

2.3.4. Hema-Gurr rapid differential staining

Blood cells were stained using Hema-Gurr rapid differential staining set for haematology (BDH Diagnostics, Poole, England). An air-dried smear was dipped five times for about one second each in a methanol fixative solution, followed by three to five times for a second each in a buffered (red) Eosin staining solution then in a buffered Methylene blue solution. The stained smear was rinsed with buffered solution pH 7.2 and allowed to dry. Stained neutrophils have multilobular purple nuclei, purplish-grey cytoplasm, and lilac-violet granules whilst monocytes have blue-grey cytoplasm devoid of granules and indented, eccentrically placed violet nuclei. Lymphocytes also have agranular blue-grey cytoplasm but the nuclei are large, round, and dark blue.

2.3.5. Protein estimation

Proteins from neutrophil sub-fractions were assayed using BCA microassay (BioRad) based on the method of Smith et al. (1985). Fifty volumes of Reagent A and one volume of Reagent B of the BCA protein assay reagents were mixed to give a working reagent. One volume of a protein sample was mixed with twenty volumes of the working reagent and the mixture incubated at 37°C for 30 min. before an A_{562} reading was taken against a reagent blank. Bovine serum albumin was used as a protein standard.

2.3.6. Nucleic acid assay

Subfractions of neutrophils were hydrolysed in 70 % (v/v) perchloric acid at 90°C for 15 min. with occasional mixing, left to cool, and centrifuged at 1,000 x g for 5 min. to precipitate denatured proteins. Supernatants containing nucleotides were assayed by A_{260} measurements (Sambrook et al., 1989) against a reagent blank. Calf thymus type I DNA was used as standard and A_{260} of 1.0 was found equivalent to 50 µg/ml nucleic acids after hydrolysis with perchloric acid (Marshak and Vogel, 1951).

2.3.7. Myeloperoxidase assay

Myeloperoxidase was assayed by a modification of the method of Suzuki et al. (1983). The substrate, 3,3',5,5'-tetramethylbenzidine, was dissolved in N,N-dimethylformamide (16 mM) and added to the assay mixture to give a final concentration of 1.6 mM in 0.08 M sodium phosphate buffer, pH 5.4, containing 0.2 M NaCl, 0.3 mM H₂O₂ and 8% (v/v) N,N-dimethylformamide. The reaction was started by the addition of 40 µl of sample to 1.0 ml of the assay mixture and the increase in A₆₅₅ measured at 37°C against a reagent blank. One unit of myeloperoxidase activity is defined as the amount of enzyme needed to bring about a change of 1.0 OD unit per min.

2.3.8. Alkaline phosphatase assay

Alkaline phosphatase activity was measured as described by Brown (1984). It is based on alkaline phosphatase catalysed liberation of *p*-nitrophenol from *p*-nitrophenol disodium orthophosphate. A neutrophil subfraction (200 µl) was added to 0.8 ml of 40 mM MgCl₂ and 1 mM KF. The mixture was incubated at 37°C for 30 min. and the reaction stopped by the addition of 2.5 ml of 0.1M NaOH. An A₄₅₀ reading of the supernatant was taken after brief centrifugation. One unit of enzyme activity is defined as the amount of enzyme needed to release 1 µmol of *p*-nitrophenol per min. A standard of *p*-nitrophenol in the assay buffer gave a molar extinction coefficient ($E_{450}^{1\text{ cm}}$) of 16,270.

2.3.9. NADPH cytochrome C reductase (E.R. marker) assay

The enzyme was assayed by a modification of the method of Sottocasa et al. (1967), all conditions were the same except that a reaction temperature of 37°C was used instead of 30°C. The enzyme was assayed by measuring the rate of reduction of cytochrome C by NADPH at 550 nm. A 100 µl aliquot of neutrophil subfraction was mixed with 0.8 ml of assay mixture containing 0.1 mM cytochrome C, 0.3 ml KCN, and 50 mM phosphate buffer, pH 7.5, and 100 µl of 1 mM NADPH was added to

start the reaction. One unit of enzyme activity is defined as the amount of enzyme needed to cause an increase in 1.0 OD unit per min.

2.3.10. Succinate dehydrogenase (mitochondrial marker) assay

Succinate dehydrogenase was assayed according to the method of Pennington (1961). The enzyme was assayed by measuring the rate of reduction of the tetrazolium salt of 2-(*p*-iodophenyl)-3-(*p*-nitrophenyl)-5-phenyltetrazolium (INT) by succinate. The reaction was started by adding 100 μ l neutrophil fraction to 900 μ l 50 mM potassium phosphate buffer, pH 7.4 with 0.05 M sodium succinate, 0.1 % INT, and 25 mM sucrose (37°C) and stopped after 15 min. with 1 ml of 10 % (w/v) TCA. The formazan released was extracted with 4 ml ethyl acetate and A_{490} of the supernatant measured. Formazan dissolved in ethyl acetate has a molar extinction coefficient of $201 \times 10^3 \text{ M}^{-1}\text{cm}^{-1}$. Controls containing no enzyme were also carried out. One unit of enzyme activity is defined as the amount of enzyme needed to produce 1 μ mol of formazan per min.

2.3.11. Synthetic substrate based elastase assays

2.3.11.1. SA₃ esterase assay

The hydrolysis of succinyl-L-alanyl-L-alanyl-L-alanyl-L-prolyl-L-valine *p*-nitroanilide (SA₃) was measured according to the method of Bieth et al. (1974). 7 μ l of 125 mM SA₃ in N-methylpyrrolidone was added to 833 μ l of 0.2 M Tris/HCl buffer, pH 8.0, containing 1 M NaCl. The reaction was started by the addition of 10 μ l of enzyme sample and the increase in absorbance at 410 nm measured at 37°C against a reagent blank. One unit of SA₃ esterase activity is defined as the amount of enzyme needed to release 1 μ mol of *p*-nitroaniline per min. A standard of *p*-nitroaniline in the assay buffer gave a molar extinction coefficient of $8,917 \text{ M}^{-1}\text{cm}^{-1}$.

2.3.11.2. NBA esterase assay

NBA esterase activity was measured according to the method of Visser and Blout (1972) by following the hydrolysis of *p*-nitrophenyl-N-tert-butoxycarbonyl-L-alanine (NBA). 33 μ l of enzyme was added to 0.833 ml of an assay mixture, containing 0.42 mM NBA in 10 mM sodium phosphate buffer, pH 6.5, 1 M NaCl, and 0.1 % (v/v) Triton X-100 to start the reaction. The change in absorbance at 347 nm was measured against a reagent blank with no enzyme. For convenience, the substrate was initially dissolved in acetonitrile having a concentration of 10 mM and 33 μ l of this solution was added to the buffer (0.833 ml). Assays were carried out at 37°C and the activity was expressed as μ mol of *p*-nitrophenol released per min.; *p*-nitrophenol was used as the standard. One unit of NBA esterase activity is defined as the amount of enzyme needed to release 1 μ mol of *p*-nitrophenol per min. A standard of *p*-nitrophenol in the assay buffer gave a molar extinction coefficient of 5,377 M⁻¹cm⁻¹.

2.3.11.3. GPV esterase

GPV esterase activity was measured by following the hydrolysis of L-pyroglutamyl-L-prolyl-L-valine *p*-nitroanilide (GPV) (Kramps et al., 1983). 80 μ l of 2 mM GPV in dimethyl sulphoxide was added to 800 μ l of 0.2 M Tris/HCl buffer, pH 8.0. The reaction was started by the addition of 10 μ l of enzyme and the increase in absorbance at 410 nm measured at 37°C against a reagent blank. One unit of GPV esterase activity is defined as the amount of enzyme needed to release 1 μ mol of *p*-nitroaniline per min. A standard of *p*-nitroaniline in the assay buffer gave a molar extinction coefficient of 8,917 M⁻¹cm⁻¹.

2.3.11.4. AAPA esterase

AAPA esterase activity was measured according to the method of Ashe and Zimmerman (1977). 80 μ l of 2 mM N-tert-butoxycarbonyl-L-alanyl-L-alanyl-L-prolyl-L-alanine *p*-nitroanilide (AAPA) in dimethyl sulphoxide was added to 800 μ l of 50 mM potassium phosphate buffer, pH 7.5 and the reaction started by an addition of 10 μ l of

enzyme. The increase in absorbance at 410 nm with time was measured at 37°C against a reagent blank. One unit of AAPA esterase activity is defined as the amount of enzyme needed to release 1 μmol of *p*-nitroaniline per min. A standard of *p*-nitroaniline in the assay buffer gave a molar extinction coefficient of $8,917 \text{ M}^{-1}\text{cm}^{-1}$.

2.3.11.5. SAAPAu esterase

SAAPAu esterase activity was measured according to the method of Del Mar et al. (1980). 80 μl of 2 mM succinyl-L-alanyl-L-alanyl-L-prolyl- α -aminobutyric acid *p*-nitroanilide (SAAPAu) in dimethyl sulphoxide was added to 0.8 ml of 200 mM Tris/HCl buffer, pH 8.0 and the reaction started by the addition of 10 μl of enzyme. The increase in absorbance at 410 nm was measured at 37°C against a reagent blank. One unit of AAPAbu esterase activity is defined as the amount of enzyme needed to release 1 μmol of *p*-nitroaniline per min. A standard of *p*-nitroaniline in the assay buffer gave a molar extinction coefficient of $8,917 \text{ M}^{-1}\text{cm}^{-1}$.

2.3.11.6. SAAPV esterase

SAAPV esterase activity was measured according to the method of Nakijima and Powers (1979). 80 μl of 17 mM methoxy-O-succinyl-L-alanyl-L-alanyl-L-prolyl-L-valine *p*-nitroanilide (SAAPV) in dimethyl sulphoxide was added to 0.8 ml of 50 mM Tris/HCl, pH 7.4, containing 1 M NaCl and 0.1 % (v/v) Triton X-100. The reaction was started by the addition of 10 μl of enzyme and the increase in absorbance at 410 nm measured at 37°C against a reagent blank. One unit of SAAPV esterase activity is defined as the amount of enzyme needed to release 1 μmol of *p*-nitroaniline per min. A standard of *p*-nitroaniline in the assay buffer gave a molar extinction coefficient of $8,917 \text{ M}^{-1}\text{cm}^{-1}$.

2.3.11.7. BOC-APN-SBzl(Cl) thioesterase

BOC-APN-SBzl(Cl) thioesterase activity was measured according to the method of Harper et al. (1984). The enzymatic hydrolysis of the thioester substrate

was carried out in the presence of 5,5'-dithiobis(2-nitrobenzoic acid) or Ellman's Reagent (Ellman, 1959). 80 μ l of 2 mM N-tert-butoxycarbonyl-L-alanyl-L-prolyl-L-norvaline benzylthioester (BOC-APN-SBzl(Cl)) and 9 μ l of 15 mM 5,5'-dithiobis(2-nitrobenzoic acid) were added to 0.72 ml of 0.1 M Hepes buffer, pH 8.1, containing 0.15 M NaCl. The reaction was started by the addition of 10 μ l of enzyme and the increase in absorbance at 412 nm measured at 37°C against a reagent blank. One unit of BOC-APN-SBzl(Cl) thioesterase activity is defined as the amount of enzyme needed to release 1 μ mol of 3-carboxy-4-nitrothiophenoxide anions (a product of the reaction between the released thiol from BOC-APN-SBzl(Cl) and 5,5'-dithiobis(2-nitrobenzoic acid)) per min. A standard of 3-carboxy-4-nitrothiophenoxide anion in the assay buffer gave a molar extinction coefficient of 13,600 M⁻¹cm⁻¹. Reactions were linear with time and rates proportional to concentration of enzyme.

2.3.12. Elastase assay

Elastase activity was measured by determining the amount of soluble dye released from orcein impregnated elastin. Elastin-orcein was suspended in 0.05 M Tris/HCl buffer, pH 8.3 at a concentration of 2 mg/ml, 100 μ l of enzyme was added to 1 ml of the substrate suspension, and incubation allowed to proceed with constant stirring for periods up to 12 hr at 37°C. The reaction was stopped by sedimenting undigested elastin-orcein. Supernatant containing digested elastin-orcein was removed for optical density measurements at 590 nm. The extinction coefficient of elastin-orcein was 1.138(mg/ml)⁻¹cm⁻¹. One unit of elastase activity is defined as the amount of elastase needed to release 1 μ g of elastin-orcein per hr.

2.3.13. Cathepsin G assay

The enzyme assay was carried out according to the method of Nakijima and Powers (1979), except that the assay temperature used was 37°C instead of 25°C. The enzyme was assayed by measuring the rate of production of p-nitroanilide resulting from the hydrolysis of the synthetic substrate N-succinyl-alanyl-alanyl-prolyl-phenyl

alanyl-*p*-nitroanilide (SAAPP). The assay was carried out in a reaction mixture containing 0.16 mM substrate, 0.1M Hepes buffer, pH 7.5, 0.5 M NaCl, and 10 % dimethyl sulphoxide. The reaction was started by the addition of 25 μ l of enzyme solution to 1.8 ml of the reaction mixture and the rate of change of absorbance at 410 nm with respect to time was measured. The molar extinction coefficient of *p*-nitroaniline is 8,800 M⁻¹cm⁻¹. One unit of cathepsin G activity is defined as the amount of enzyme needed to release 1 μ mol of *p*-nitroaniline per min.

2.3.14. GBM proteolysis assay

The rate of GBM proteolysis by proteinases is measured by following the release of ³H-peptides from ³H-GBM. An aliquot of neutrophil granule proteinases (100 μ l) containing ≤ 0.05 units of BOC-APN-SBzl(Cl) thioesterase activity was incubated with 0.6 mg/ml of ³H-GBM suspension at 37°C with tumbling in a final volume of 0.5 ml TBS, pH 7.4. The reaction was stopped at 10 min. intervals to up to 50 min. by centrifugation. Radioactivity from ³H in supernatants and precipitates was measured by liquid scintillation counting using a Beckman LS 1701 counter. Precipitates were solubilized in 2 N NaOH at 90°C for 15 min and neutralized in 2 N HCl prior to mixing with Ecoscint (National Diagnostics, Buckinghamshire, U.K.) (volume ratio of 1: 3.5). Supernatants were mixed directly with the scintillant. The radioactivity measurements were corrected for sample quenching by the sample channel ratio method. Controls with heat-inactivated or inhibitor-treated proteinases were carried out to correct for any background release of isotope. One unit of ³H-GBM degrading activity is defined as the amount of enzyme needed to release label equivalent to 1 μ g of ³H-GBM per min. at pH 7.4, 37°C. The amount of ³H-GBM solubilized was estimated from radioactivity released from known weight of ³H-GBM, assuming uniform labelling of GBM.

2.3.15. Student t-test

The student t-test (Box et al., 1989) was used to ascertain if two groups of data are significantly different. The standard error of difference between two groups of data (SE-diff) are multiplied by each value *s* in the significance table shown below (Pearson and Hartly, 1958) and the product (*y*) compared with the difference between the two group means (*z*). Where *y* is less than *z* the difference is said to be significant at that level, indicated by either % significance or p-value. The lower the p-value the more significance the difference between the two groups of data.

Significance table

Level of significance		<i>s</i>
5%	(p < 0.05)	1.66
1%	(p < 0.01)	2.33
0.1%	(p < 0.001)	3.09
0.05%	(p < 0.0005)	3.29

SE-diff is defined as follows.

$$SE\text{-}diff = \sqrt{SE\text{-}mean_1^2 + SE\text{-}mean_2^2}$$

where SE-mean₁ = standard error of the mean ($\frac{SD}{\sqrt{n}}$) of the 1stgroup

SE-mean₂ = standard error of the mean ($\frac{SD}{\sqrt{n}}$) of the 2ndgroup.

$$SD = \text{standard deviation} = \sqrt{\frac{\sum x^2 - \frac{(\sum x)^2}{n}}{n}}$$

n = number of measurements in a group.

$\sum x^2$ = sum of squares of measurements

$(\sum x)^2$ = square of sum of measurements.

CHAPTER THREE

CHAPTER 3 THE ULTRAFILTRATION SYSTEM AND NORMAL PERMEATION BEHAVIOUR OF ISOLATED GBM

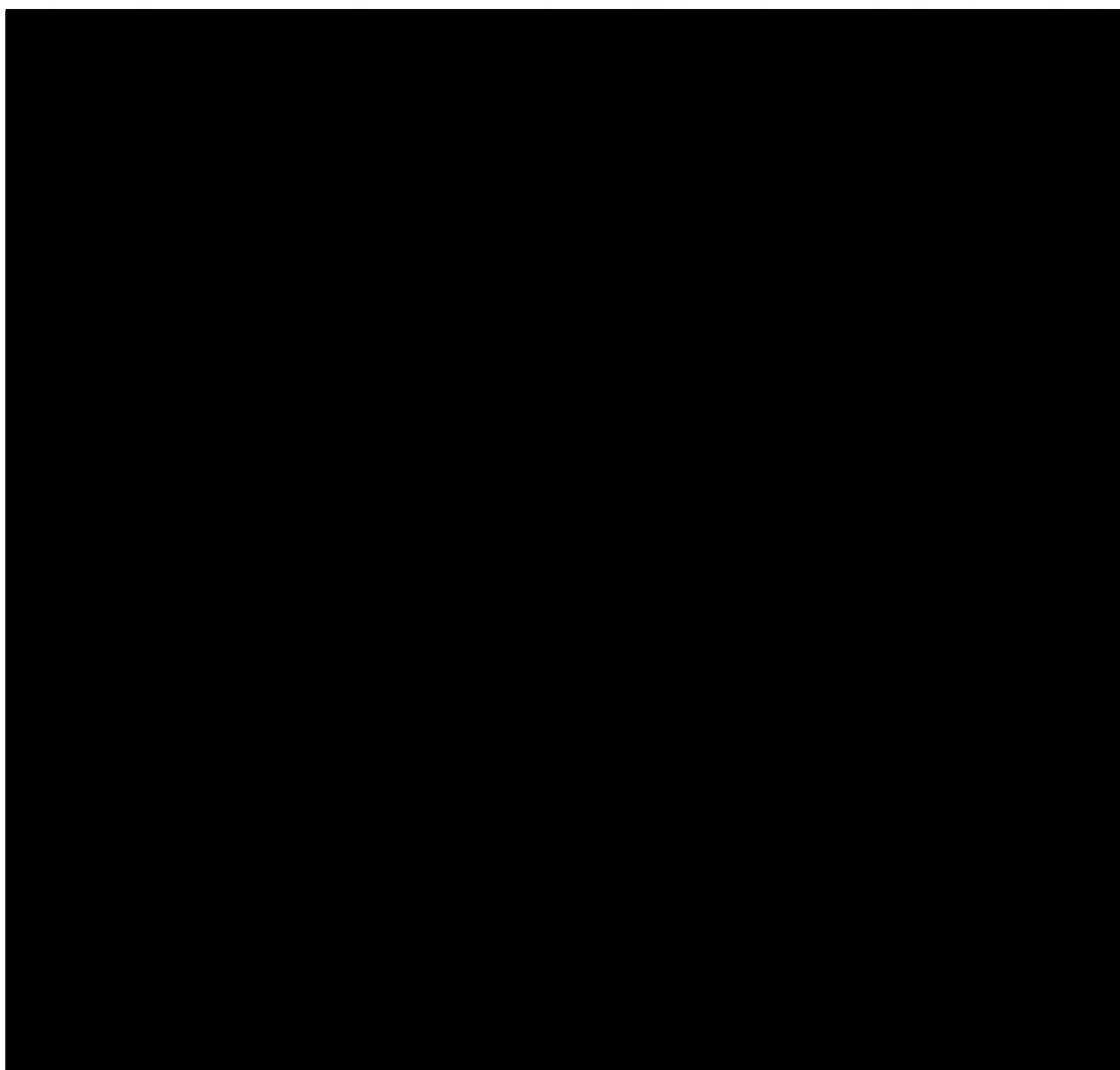
This chapter introduces the set up and the salient features of the ultrafiltration system used in the current study. The normal permeation properties of isolated pig GBM will also be discussed. This will serve as a reference against which altered permeation properties due to treatment with test agents are compared. Two aspects of the permeation behaviour will be discussed in particular: (1) the molecular sieving properties of the isolated GBM and (2) how the permeability of the GBM changes as the membrane is compressed under increasing filtration pressures.

3.1. The GBM ultrafiltration system

The system is essentially composed of a GBM filter film constructed from GBM fragments in an Amicon Type 52 filtration cell (Fig. 3.1.). The GBM fragments were prepared from pig glomeruli by detergent and sonication treatment and layered into a coherent film by filtering a suspension (containing 1.5 mg dry weight GBM) onto Millipore membranes in the filtration cell (Chap. 2). The film or filter pad so formed is transparent, mechanically contiguous, resistant to shear, and leak proof to high MW tracers, e.g., ferritin and IgG (Robinson and Walton, 1987; Walton et al., 1992). By adjusting the amount of membranes used, film thickness can be controlled. Routinely, 1.5 mg membrane (dry weight) produces films of $\sim 7.9 \mu\text{m}$ thickness, composing of ~ 25 layers of GBM (Bray, 1982), in cells with a filtration area of 13.8 cm^2 (Walton et al., 1992).

The GBM films are then used for ultrafiltration studies by filling the cells with protein solutions or buffer (50 ml), applying a pressure using N_2 , and measuring the flux of water and protein (Section 2.3.3.). Briefly, timed samples of filtrate were collected ($\sim 1.5 \text{ ml}$), the exact volume was measured, and the protein concentration determined by A_{280} measurements. These measurements permitted calculation of J_v

Fig. 3.1. Schematic view of the ultrafiltration cell



The cell has a volume of 65 ml and a bottom area of 13.8 cm². A coherent film of GBM is formed by blowing a suspension of GBM fragments onto a filter support in the filtration chamber under N₂ pressure. The filter support consists of a Millipore membrane (0.45 µm exclusion, Millipore, London, U.K.) and a hardened Whatman 50 filter paper resting on a porous polypropylene base. The properties of the GBM film are described in text. Diagram adapted from Westberg, N.G. (1982) Filtration properties of isolated glomerular basement membrane. In 'New Trends in basement membrane research' (R. Kuehn, H.-H. Schloene, R. Timpl, eds.) p. 50, Raven Press, New York.

(water flux, cm/min) and J_s (protein flux, mg/cm²/min). Protein rejections (R) were also calculated for each filtrate sample:

$$R = 1 - C_f/C_b$$

where C_f is filtrate protein concentration and C_b protein concentration in the overstanding solution. Typically, ~ 15 ml of the overstanding solution is filtered per filtration run. The cells are stirred, at 1,000 r.p.m., to prevent concentration polarization, the build up of rejected protein at the filter surface. The pressure (ΔP) used ranged from 10 - 200 kPa. At 150 kPa, the average pressure gradient across each layer of GBM is 6 kPa (45 mmHg) which is similar to the glomerular hydrostatic pressure (Lote, 1985; Maddox et al., 1992). Pressures above and below 150 kPa are used to take into account the possible variations in glomerular hydrostatic pressure in normal (Rosenberg and Hostetter, 1992) and nephritic conditions (Blantz and Wilson, 1976; Wilson, 1988; Wheeler et al., 1991).

3.2. Normal permeation behaviour of GBM

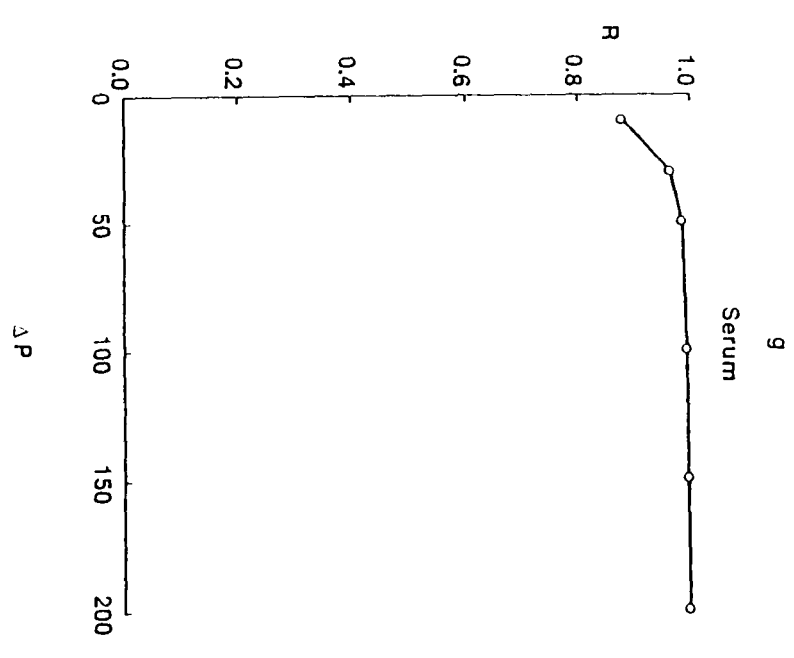
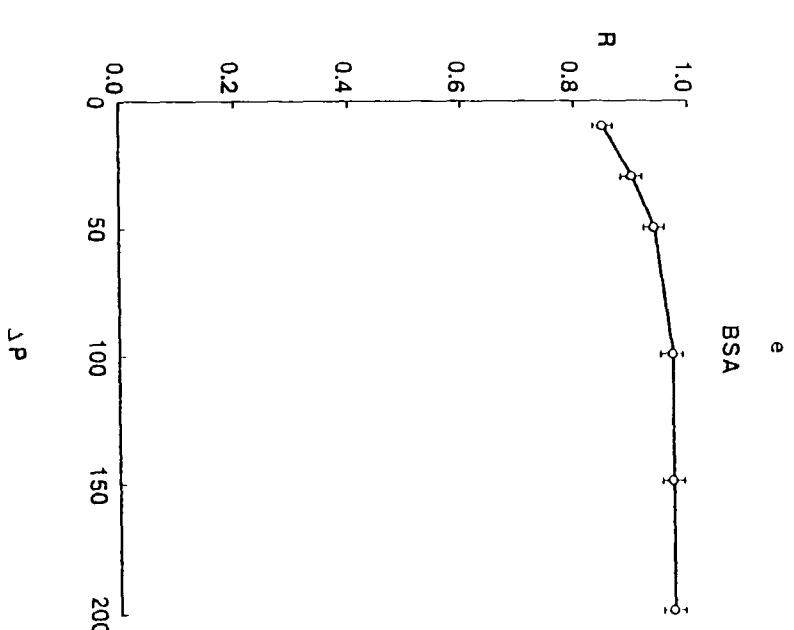
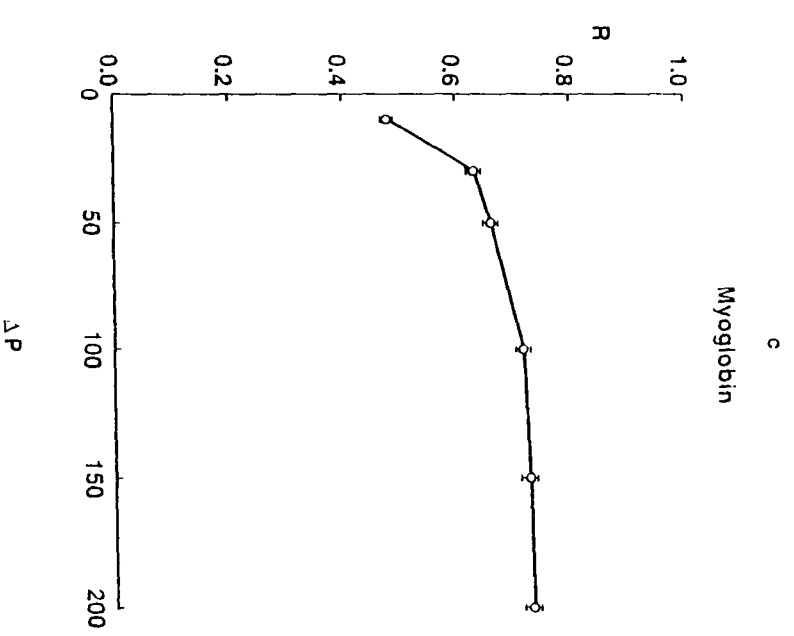
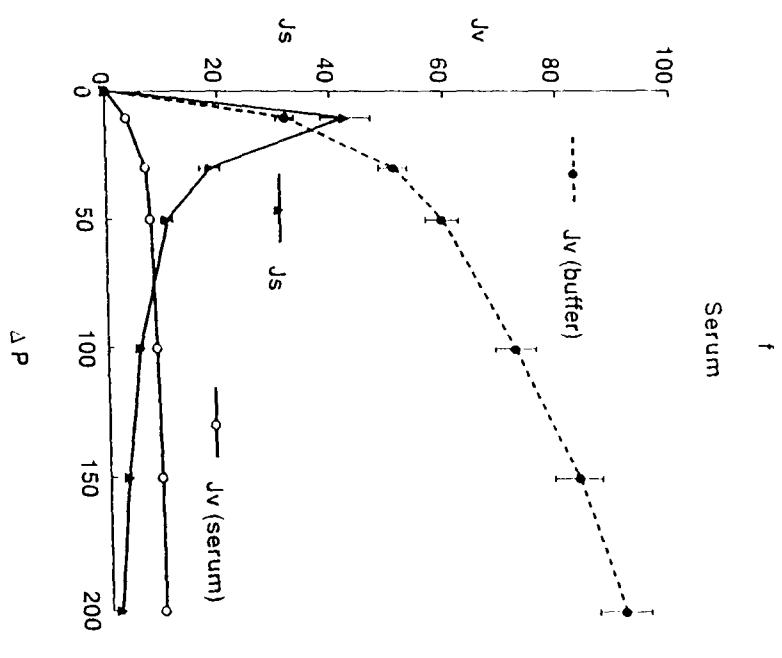
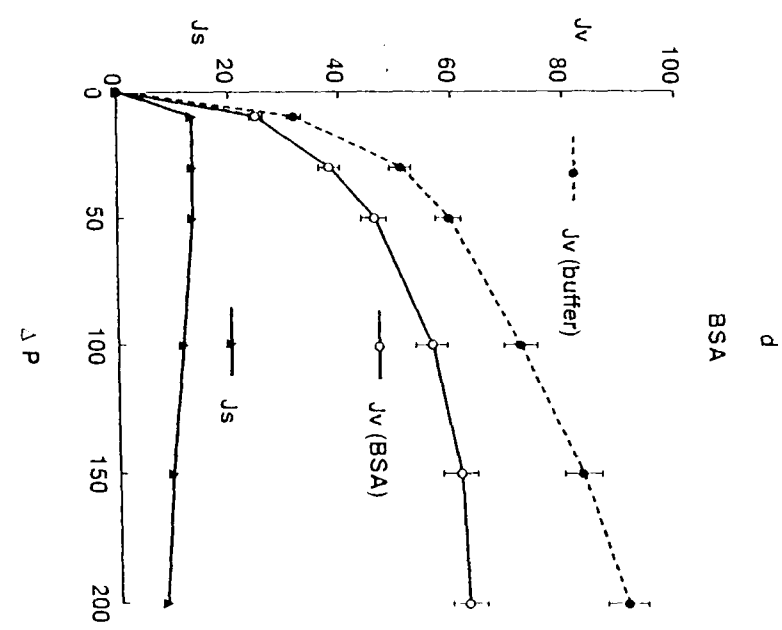
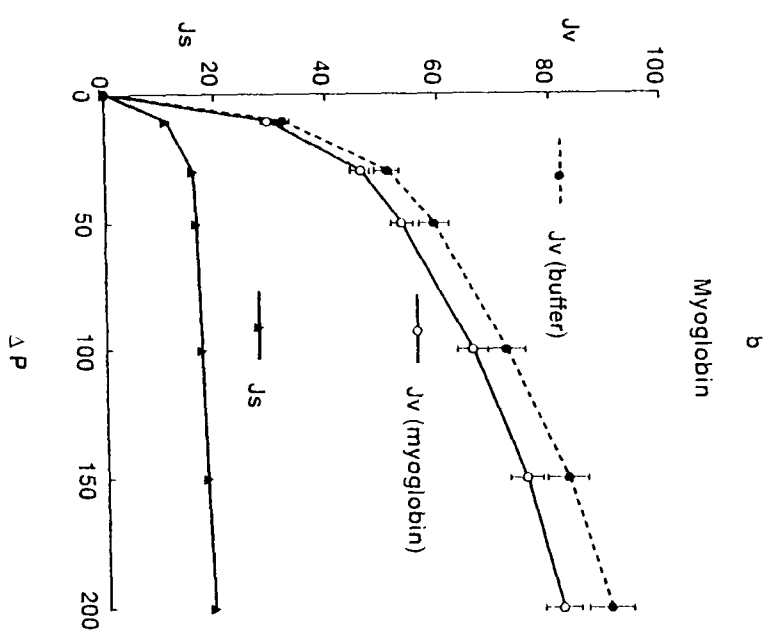
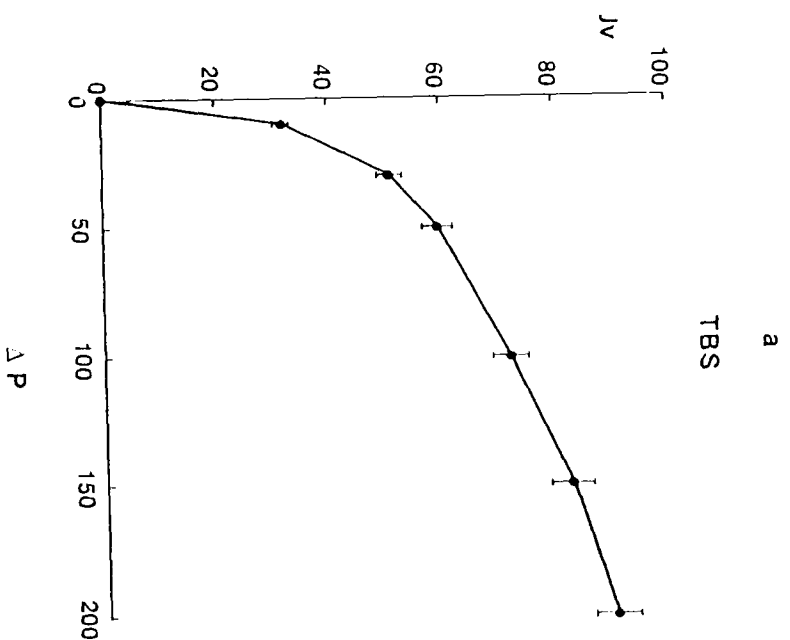
The ultrafiltration behaviour of GBM films, expressed in terms of J_s , J_v , and R as a function of ΔP in ultrafiltration of TBS, myoglobin and BSA solutions, as well as serum will be discussed.

A TBS filtration

The result for the filtration of TBS, pH 7.4 is shown in Fig. 3.2a. J_v was found to increase with increasing pressure tending toward a plateau. An ideal ultrafiltration membrane would be expected to show a linear increase in J_v (Robinson and Walton, 1989). The deviation of GBM film behaviour from ideality, suggested that the films were becoming compressed at increased pressures (Robinson and Walton, 1989). Indeed, film compression had been demonstrated by Robinson and Walton (1987; 1989) by thickness measurement of films under hydrostatic pressure. They showed that films of ~ 8 μ m average relaxed thickness decreased to ~ 4 μ m when pressure was increased to 150 kPa. They proposed that the non-ideality of J_v was due

Legend to Fig. 3.2.

The normal ultrafiltration behaviour of GBM for buffer (a), myoglobin (b-c), BSA (d-e), and serum (f-g). Films of native pig GBM were prepared in ultrafiltration cells and their permeation properties studied at different filtration pressures as described in text using buffer (TBS, pH 7.4), myoglobin (0.5 mg/ml in TBS, pH 7.4), BSA (2.0 mg/ml in TBS, pH 7.4), and serum (70 mg/ml protein estimated by BCA protein assay, using albumin as standard). Legends are: J_v = water flux (ml/min/cm² or cm/min x 10³), J_s = protein flux (μg/min/cm²), R = protein rejection, ΔP = filtration pressure (kPa). J_v (buffer) is included (dotted lines) in protein ultrafiltration graphs for comparison with J_v (protein). Three films were used for the ultrafiltration of each of the solutions. Each value on graphs indicates the mean of 9 measurements, 3 from each of 3 films. Error bars indicating SDs were shown but are omitted where they are smaller than the size of the symbols.



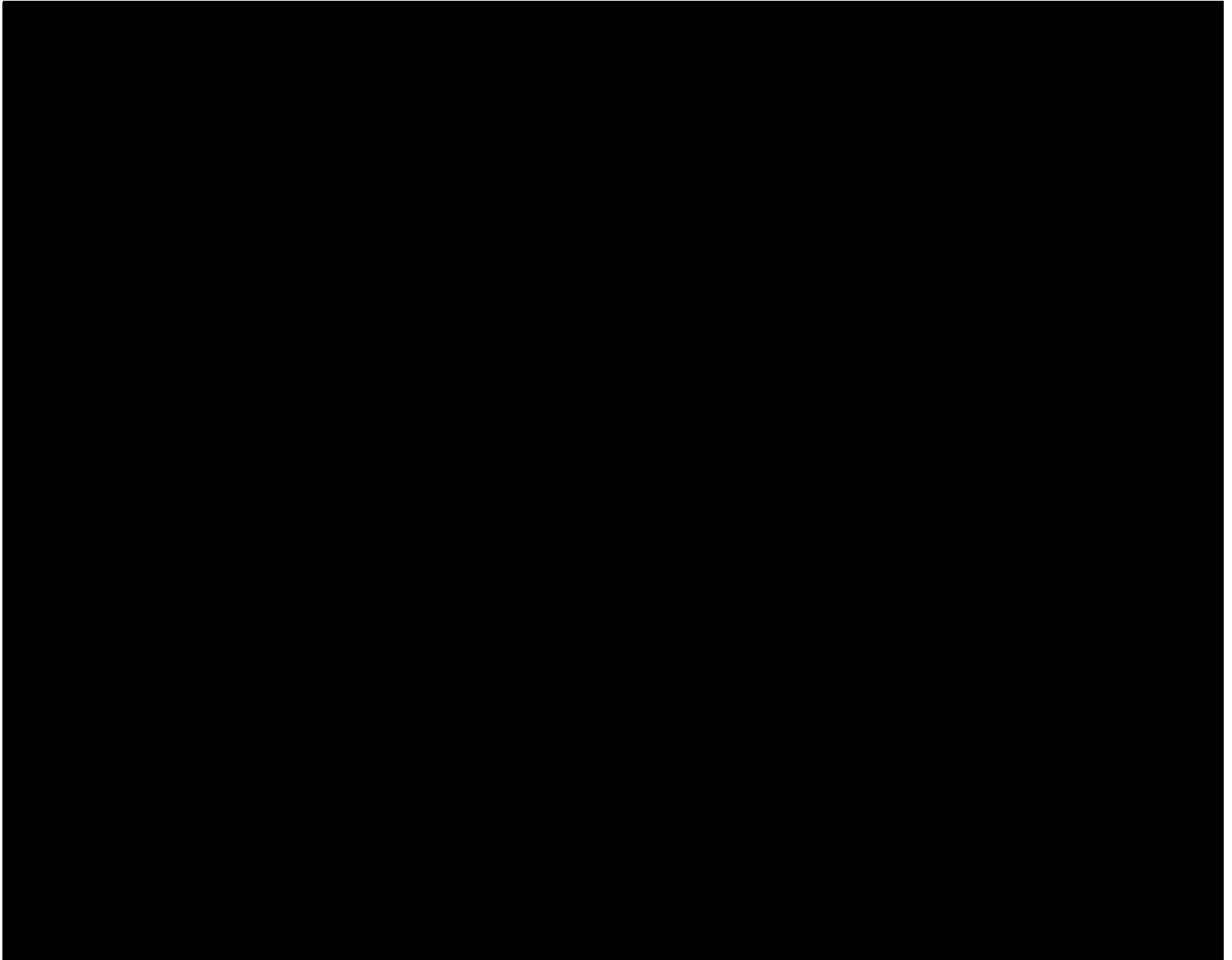
to the fibre matrix of the GBM being compressed which reduced the void space through which water could permeate.

B Myoglobin and BSA ultrafiltration

The results for the ultrafiltration of each protein solution were expressed in terms of $J_s \propto \Delta P$, $J_v \propto \Delta P$, and $R \propto \Delta P$ (Figs. 3.2b-c for myoglobin and 3.2d-e for BSA). In both cases, J_v increased but tended towards a plateau, indicating film compression. J_v (BSA) was lower than J_v (myoglobin) which was, in turn, lower than J_v (TBS). The presence of protein in the solution to be filtered seemed to have reduced J_v compared with buffer filtration. The larger protein BSA (MW 68 k) reduced J_v to a greater extent than did myoglobin (MW 18 k). Such behaviour has been independently observed by Cotter (1979) and Walton et al. (1992). Robinson and co-workers attributed the reduction of J_v to the accumulation of a layer of rejected protein on the filter surface which forms a secondary barrier - a phenomenon known as 'concentration polarization' (Cotter and Robinson, 1978; Robinson and Cotter, 1979; Robinson and Walton, 1987). The process of concentration polarization is illustrated in Fig. 3.3. The GBM film was not freely permeable to either myoglobin or BSA. These rejected protein molecules would concentrate on the filter surface, tending to obstruct water movement and would account for the lower J_v values than in TBS ultrafiltration. BSA, being a larger protein, was rejected more effectively than myoglobin as shown by the former having higher rejection values than the latter over the whole pressure range (Figs. 3.2c & 3.2e). The build up of BSA at the filter surface would be more severe than that of myoglobin, possibly giving rise to a thicker, more effective barrier. This would reduce water flux more effectively and account for the lower J_v (BSA) than J_v (myoglobin) seen.

J_s (myoglobin) increased with ΔP and then remained relatively constant (Fig. 3.2b). J_s (BSA) showed a similar pattern of change except that it decreased slightly with increasing ΔP (Fig. 3.2d). Such behaviour differs from that expected of an ideal membrane which would exhibit a linear increase in J_s with ΔP . The deviation from

Fig. 3.3. Concentration profile of protein in ultrafiltration of protein across a partially obstructive membrane



As protein is ultrafiltered across a partially obstructive membrane (from left to right), a polarizing layer of rejected protein is formed on the membrane surface. This phenomenon is known as concentration polarization. The diagram shows that the protein concentration is highest at the membrane surface (C_w , wall concentration) but decreases with increasing distance from the membrane until it becomes that of the bulk solution (C_b). The diagram is adapted from Blatt, W.F., Dravid, A., Michaels, A.S., and Nelsen, L. (1970) Solute polarization and cake formation in membrane ultrafiltration: causes, consequences, and control techniques. In 'Membrane science and technology. Industrial, biological, and waste treatment processes' (J.E. Flinn, ed.), p. 57, Plenum Press, New York.

linearity is most likely to be due to film compression which presumably has the effect of increasing the fibre packing density of the GBM fibre matrix and reducing the interfibril void space through which protein molecules traverse (Robinson and Walton, 1987; 1989).

R (myoglobin) and R (BSA) were found to increase with increasing ΔP (Figs. 3c & e), indicating that the films were becoming less permeable to protein which is consistent with the idea that the films were becoming compressed with increased ΔP . R (BSA) was higher than R (myoglobin), indicating that the films rejected BSA more effectively than it did myoglobin. The GBM films were thus behaving as a molecular sieve showing size-dependent discrimination, similar to the behaviour of the glomerular capillary wall *in vivo* (Brenner et al., 1978; Deen et al., 1985).

C Serum ultrafiltration

Figs. 3.2f-g show the results of serum ultrafiltration. J_v showed the characteristic deviation from linearity as with buffer and protein ultrafiltrations, except that the J_v was much lower. The non-linear increase in J_v was probably due to the effect of compression. The relatively low J_v could be attributed to the effect of very severe concentration polarization, resulting presumably from the sedimentation of a thick unstirred layer of proteins at the filter surface from the very concentrated mixture of high MW proteins in serum. This would be expected severely to obstruct H_2O flux through the film and result in the greatly reduced J_v seen.

J_s (serum protein) exhibited a complicated pattern of change with ΔP (Fig. 3f), characterized by a sharp rise and then a fall tending to a plateau. The high J_s at low ΔP probably reflected the high protein flux from a very concentrated protein solution through a relatively relaxed fibre matrix. As the films became progressively compressed with increasing ΔP , the fibre packing density of the membrane matrix increased, reducing the void space through which protein could permeate. This effect seems to be more important than the tendency of increasing pressures to drive more solute through the matrix, giving a fall in J_s . In this respect, the permeation behaviour

of the GBM is quite different from that in the filtration of simple protein solutions where the J_s tended to remain relatively constant. Also, the J_s (serum protein) at high ΔP was considerably lower than that for BSA or myoglobin in spite of the fact that the protein concentration of serum (~ 70 mg/ml) was ~ 35 x greater than the BSA concentration and ~ 140 x greater than the myoglobin concentration. A plausible explanation for this has been offered by Robinson and Cotter (1979) as well as Westberg (1982) who suggested that the accumulation of high MW serum protein molecules (e.g., IgG) at the filter surface blocked the movement of low MW serum protein molecules (e.g., albumin) across the membrane. This kind of blockade effect would not be seen in the filtration of simple protein solutions where only one species of protein is present. Robinson and Cotter (1979) arrived at this conclusion following a demonstration of a reduction in cytochrome C flux when a larger protein IgG was added to the cytochrome C solution. As ΔP increased the J_s (cytochrome C) decreased in a similar manner to J_s (serum protein) (Robinson and Cotter, 1979). However, if cytochrome C alone was filtered, then the pressure profile of J_s resembles that for myoglobin shown in Fig. 3.2b. Westberg (1982) demonstrated a similar effect using BSA instead of cytochrome C; the presence of IgG lowered the BSA flux. Also if the concentration of IgG was increased in the admixture of proteins, then BSA flux was significantly reduced (Westberg, 1982). It would seem that either increasing the filtration pressure or increasing the concentration of high MW protein in the admixture of proteins exacerbated the blockade effect, possibly because either would cause the formation of a thicker polarising layer of the high MW species, reducing the flux of lower MW proteins.

Moving now to a discussion of the effect of ΔP on serum protein rejection, R (serum protein) increased with increasing ΔP , indicating a progressive decrease in film permeability as the film became compressed. This further supported the findings based on myoglobin and BSA ultrafiltrations. The R (serum protein) was higher than that for BSA, suggesting that films are less permeable to serum protein than to albumin. This could be explained by the blockade effect discussed earlier where the formation of a

polarizing layer of high MW proteins, e.g., IgG, on the filter surface obstructed the movement of smaller proteins, e.g., albumin, across the films, reducing their flux and increasing their rejection.

3.3. Limitations and advantages of the ultrafiltration system

The ultrafiltration system used in this study was unphysiological since protein solutions were filtered across a dead-end, multiple-layer GBM filter surface under high pressure. Concentration polarization appeared to affect the filtration process in spite of vigorous stirring, particularly when BSA and serum was filtered. In the glomerulus, cross flow ultrafiltration occurs across a three layer capillary wall (endothelial cells, single-layer GBM and podocytes) under low hydrostatic pressure (6 kPa) presumably with efficient erythrocyte brushing of the capillary wall, minimising concentration polarization (Cotter and Robinson, 1978). Attempt have been made to overcome these shortcomings in this laboratory by setting up a thin channel cross flow system. No workable system has been achieved since it was impossible to coat a thin channel with a single layer of leak proof GBM. Furthermore, it was difficult to keep the filtration pressure constant along the entire length of the thin channel which does not appear to be a problem *in vivo*. Despite the drawbacks of the present system, some of which are unavoidable, an attempt has been made to mimic the *in vivo* filtration conditions. A filtration pressure of 150 kPa across ~ 25 layers of GBM was used which gave a pressure gradient of ~ 6 kPa across a single layer of GBM. This was similar to measured glomerular capillary hydrostatic pressure (Maddox et al., 1992).

This system does have several advantages over the *in vivo* filtration studies. (1) The permeabilities of isolated GBM can be quantitated directly and accurately, in terms of J_s , J_v , and R . This is impossible with *in vivo* filtration analysis since glomerular ultrafiltration occurs across a three layer composite capillary wall of which GBM is only one. (2) Chemical perturbation studies can be directly carried out on GBM without affecting other components of the glomerular capillary wall. Permeability analysis can then be carried out on the treated GBM under controlled conditions.

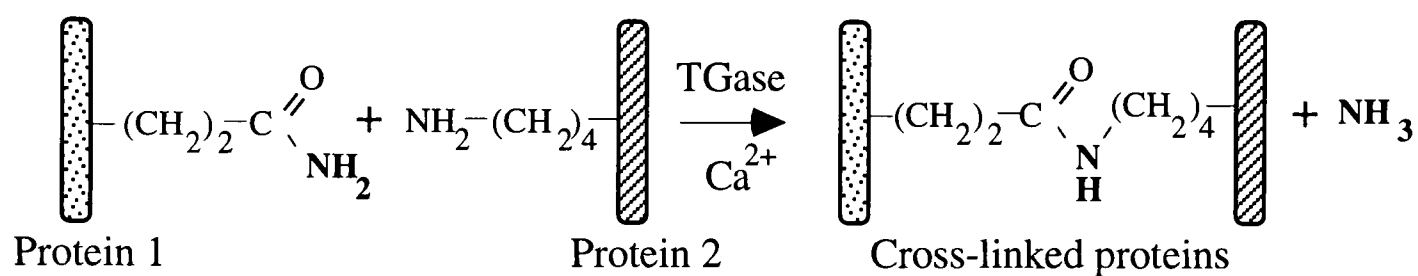
Attempts have been made to alter the permeability of the glomerular capillary wall *in vivo*, e.g., intrarenal perfusion of serine proteinases (Johnson et al., 1988a) and H_2O_2 (Yoshioka et al., 1991). Although proteinuria was seen in these studies, it was not known if GBM was the only structure affected. Moreover, it was unclear whether haemodynamic changes had contributed to the proteinuria as a result of perfusion of the test agents. There is evidence to indicate that the cells of the glomerular capillary wall contain metalloproteinases (Lovett et al., 1983; Martin et al., 1986; Davies et al., 1988; Martin et al., 1989; Watanabe et al., 1990a; Lovett et al., 1992; Marti et al., 1994) which may be involved in rendering the GBM more permeable to plasma protein when the cells are perturbed (Couser, 1993; Johnson et al., 1994). The aforementioned uncertainties highlight the potential complexities of the *in vivo* analysis. The *in vitro* perturbation studies side-stepped these complications and permitted a more controlled analysis of elements of a complicated problem.

CHAPTER FOUR

CHAPTER 4 THE EFFECT OF GUINEA PIG LIVER TRANSGLUTAMINASE ON THE PERMEATION BEHAVIOUR OF GBM

4.1. Introduction

Transglutaminases (TGases) are a family of enzymes capable of cross-linking proteins by forming ϵ -(γ -glutamyl)lysine cross-links between the γ -carboxamide group of a peptide-bound glutamine residue and the ϵ -amino group of a peptide-bound lysine (Greenberg et al., 1991):



Primary amines, such as hydroxylamine, putrescine, methylamine or glycine ethyl ester, may also provide the amino groups needed to form cross-links with peptide-bound glutamine residues (Folk and Finlayson, 1977; Folk and Chung, 1985). TGase can be of plasma, platelet, monocyte or liver origin (Harris et al., 1984; Folk and Chung, 1985). They are all able to cross-link fibrin molecules to form clot (Lorand et al., 1966; Tyler and Laki, 1966; Francis and Marder, 1987; Conkling et al., 1989) and to cross-link the polyamine putrescein to casein (Chung, 1972; Harris et al., 1984; Folk and Chung, 1985; Conkling et al., 1989), indicating a similarity in their substrate specificities. There is also a high amino acid sequence homology amongst these enzymes (Greenberg et al., 1991). As has been reviewed in Section 1.11.5., there is evidence to indicate that plasma TGase and possibly monocyte and platelet TGases are present in nephritic glomeruli. The ability of chemical cross-linkers (e.g., glutaraldehyde and dimethyl malonimidate) to introduce cross-links into the GBM and render it more permeable to protein (Walton et al., 1992) suggests that biological cross-linkers, such as TGases, may be able to do the same. If they do then cross-linking by TGases may provide a new mechanism of promoting proteinuria. This prompted the

pilot study here which uses guinea pig liver TGase as a model enzyme to investigate its effect on GBM permeability to protein. The liver enzyme was chosen because it is easily obtainable in large quantities and is similar to the other TGases in substrate specificity and sequence homology.

4.2. Purification of TGase

Guinea pig liver TGase was purified according to the method of Folk and Chung (1985). Guinea pig liver (~ 60 g) was homogenised in 0.25 M sucrose (Polytron PT 20 homogeniser) and centrifuged (150,000 x g, 1 hr). The TGase was purified from the supernatant by ion exchange chromatography (3.5 x 10 cm DEAE-cellulose; 0 to 1 M NaCl, 1.5 l linear gradient elution in 5 mM Tris/HCl, 2 mM EDTA buffer, pH 7.5), protamine extraction (1% protamine w/v in water), followed by gel filtration chromatography (2.5 x 100 cm, 10 % fine agarose Biogel A-0.5m; 10 mM Tris/acetate, 1 mM EDTA, 0.16 M KCl, pH 6.0 elution buffer). The activity of TGase was determined by measuring spectrophotometrically the concentration of cross-linked product Z-L-glutamyl(γ -hydroxylamine)-glycine at 525 nm wavelength. The substrates were hydroxylamine and carboxy-glutamyl-glycine (CBZ-Gln-Gly) (Folk and Chung, 1985). One unit of activity is defined as the amount of enzyme needed to catalyse the formation of 1 μ mol of Z-L-glutamyl(γ -hydroxylamine)-glycine per min at 37°C, pH 6.0. The purified TGase had a specific activity of 15 units/mg protein which was comparable to the published figure (Folk and Chung, 1985) with a yield of 2 % (Table 4.1.). The figures were found to be reproducible in a repeat experiment.

Protein extracts at key stages of purification were analysed by SDS-PAGE (Laemmli, 1970) for purity. Protein samples were boiled for 10 min in Tris-tricine-SDS (TTS) buffer (9.2 mM Tris, 20 mM tricine, 0.1% SDS, pH 7.5), containing 10% glycerol, 10 mM dithiothreitol (DTT), and bromophenol blue. They were then loaded onto a gel (3-40% highly cross-linked, 72 x 72 x 3 mm), electrophoresed in a TTS continuous buffer system (100 V, 6 h, 4°C) and stained with Coomassie Brilliant Blue (0.1% w/v in H₂O, 10% acetic acid). The number of protein bands decreased as

Table 4.1. Purification of guinea pig liver transglutaminase

STEP		PROTEIN (mg)	SPECIFIC ACTIVITY (units/mg)	YIELD (%)
1.	Homogenate	60,780	0.04	100 %
	Supernatant	23,502	0.09	87 %
2.	DEAE-Cellulose chromatography	1,007	1.00	41 %
3.	Protamine extract	50	1.40	3 %
4.	Agarose chromatography	2	15.00	2 %

Protein (mg) was assayed using BCA microassay method with BSA as a standard (Section 2.3.5.). TGase was purified from guinea pig liver as described in text where unit TGase activity is defined. Yield is expressed as activity recovered at each step of purification as a percentage of the total activity in the homogenate.

purification progressed until a dominant band (MW 75 k), corresponding to TGase (Folk and Chung, 1985), was obtained (Plate 4.1.). The band patterns were reproducible in a repeat experiment.

4.3. Incubation of GBM proteins with TGase

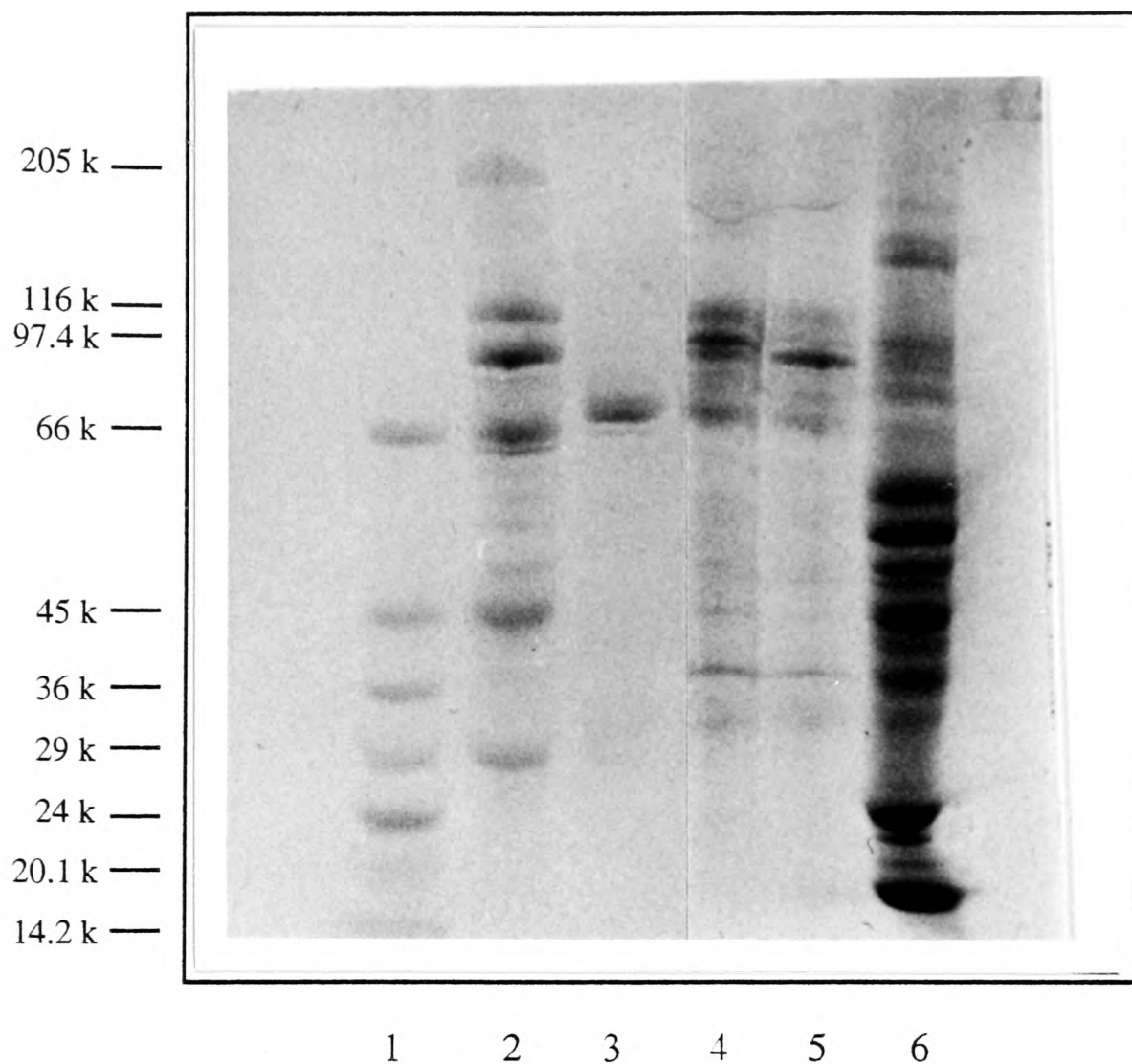
GBM (6 mg dry weight) was incubated with 10 units of TGase in 15 ml of TBS, pH 7.4, containing 5 mM CaCl_2 at 37°C for 24 hrs with gentle shaking. Controls were GBM incubated with heat-denatured TGase which lost all hydroxylamine cross-linking activity. GBM was recovered at the end of the reaction by centrifugation (2,000 x g, 10 min, 4°C) and was washed twice in TBS, pH 7.4 prior to being made into films in Amicon filtration cells and for cross-linking analysis.

4.4. SDS PAGE confirmation of GBM protein cross-linking by TGase

TGase-treated GBM was analysed for cross-linking using SDS-PAGE. Cross-linked membrane proteins are expected to appear on a gel as high MW bands which are absent in the control. Unlike the control, TGase-treated GBM remained insoluble following treatment with SDS (0.1%) and DTT (10 mM) (100°C, 12 hrs), making electrophoresis analysis impossible. To overcome this problem, the TGase treated membranes were solubilized using collagenase (IV) (*Clostridium histolyticum*, Sigma). 6 mg of the membranes were incubated with 195 units of the collagenase* in TBS buffer, pH 7.4 with 5 mM CaCl_2 (37°C, 9 hrs). The digest was then denatured (see Section 4.2.) for SDS PAGE analysis using a 3-13 % linear gradient gel (72 x 72 x 3 mm) in a TTS continuous buffer system (25 V, 7 hrs, 4°C). GBM treated with heat-denatured TGase (control) was similarly digested with collagenase and electrophoresed. Both digests gave reproducible band patterns in a repeat experiment. TGase-treated GBM showed high MW bands ~ 175 k and ~ 160 k, presumably representing cross-linked GBM proteins, which were absent in the control (Plate 4.2.). In addition, one

* 1 unit of the collagenase activity is the amount of enzyme needed to liberate peptides from bovine archilles tendon collagen type I equivalent in ninhydrin colour to 1.0 μmol of leucine in 5 hrs at pH 7.4, 37°C (Seglen, 1976).

Plate 4.1. Electrophoretic analysis of protein purity at various stages of TGase purification

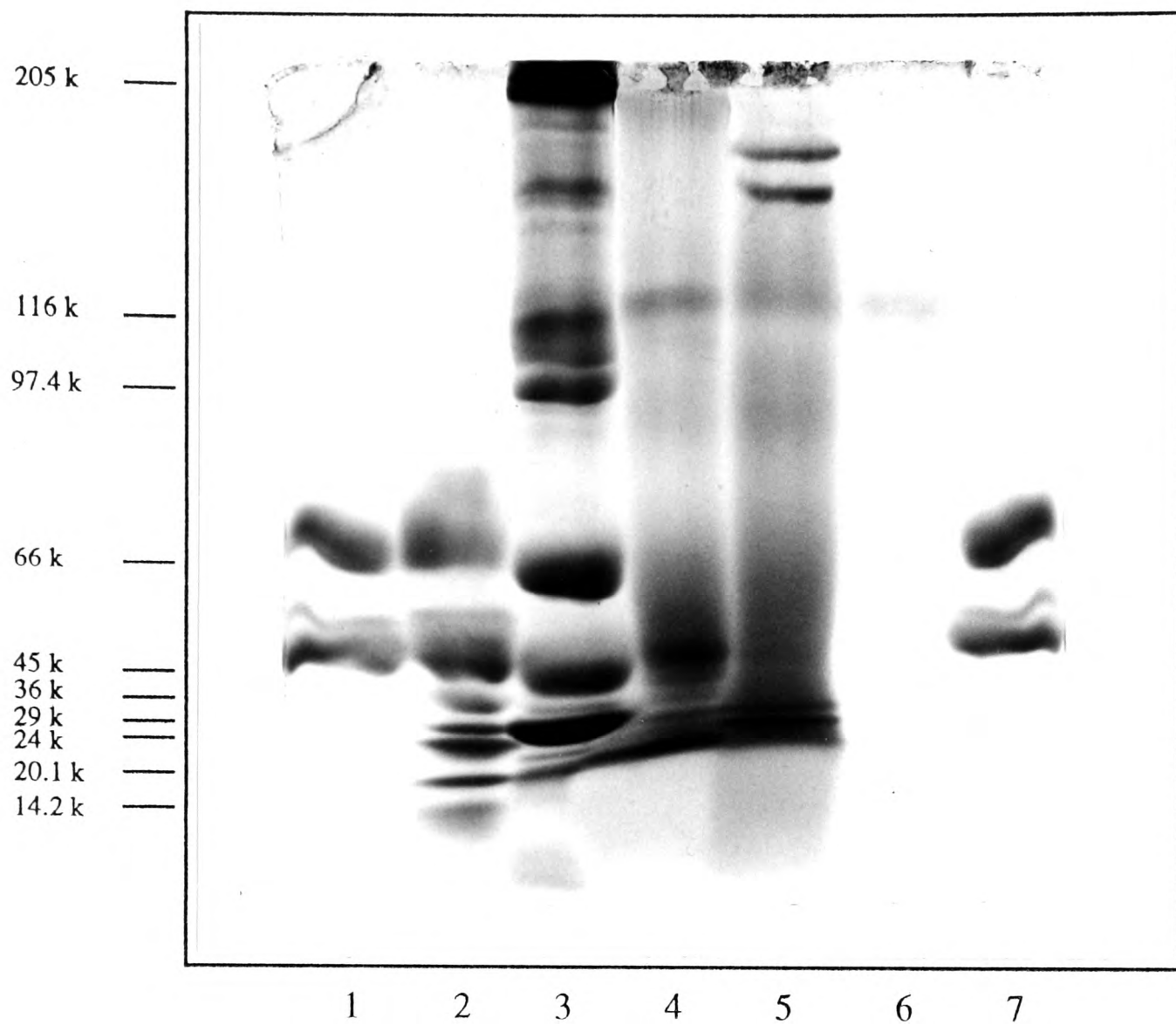


The gel tracks are:

1. Low MW markers (60 μ g) (14.2 k - 66 k)
2. High MW markers (60 μ g) (29 k - 205 k)
3. Purified TGase (10 μ g)
4. First $(\text{NH}_4)_2\text{SO}_4$ extract following protamine precipitation (10 μ g)
5. Second $(\text{NH}_4)_2\text{SO}_4$ extract following protamine precipitation (10 μ g)
6. Homogenate (120 μ g)

The MW markers are subunit of myoglobin (205 k), subunit of β -galactosidase (116 k), subunit of phosphorylase B (97.4 k), bovine albumin (66 k), ovalbumin (45 k), subunit of glyceraldehyde-3-phosphate dehydrogenase (36 k), carbonic anhydrase (29 k), trypsinogen (24 k), trypsin inhibitor (20.1 k), and α -lactalbumin (14.2 k).

Plate 4.2. Electrophoretic analysis of TGase-treated GBM proteins



The gel tracks are:

1. Flanking MW markers (10 μ g) (45 k & 66 k)
2. Low MW markers (60 μ g) (14.2 k - 66 k)
3. High MW markers (60 μ g) (20.1 k - 205 k)
4. Collagenase digest of GBM treated with heat-denatured TGase (control) (150 μ g)
5. Collagenase digest of GBM treated with TGase (150 μ g)
6. Collagenase autodigest (50 μ g) (prepared by incubation collagenase alone: 5 mM CaCl_2 , TBS, pH 7.4, 37°C, 24 hrs)
7. Flanking MW markers (10 μ g) (45 k & 66 k)

The MW markers used here has been listed elsewhere (Plate 4.1.).

band at ~ 46 k that was present in the control tract had disappeared in the tract containing digest of TGase-treated GBM, suggesting that a membrane protein was being used for cross-linking.

4.5. Thickness analysis of TGase-treated GBM films

Chemical cross-linkers have been shown to reduce the GBM film thickness (Walton et al., 1992). To determine if TGase was able to do the same, thickness analysis was carried out on TGase-treated GBM films and controls according to the method of Robinson and Walton (1989) (Section 2.3.2.). Each film was made by blowing a suspension (1.5 mg GBM dry weight) onto a filter support in an Amicon filtration cell (65 ml capacity) under N₂ pressure (Section 2.2.2.). The films made were not used for filtration studies. 20 disperse measurements taken per film for 3 TGase-treated films gave an average thickness of $7.3 \pm 0.6 \mu\text{m}$ ($\pm$ SD, $n = 60$). Measurements made on control films ($n = 60$) gave an average thickness of $7.9 \pm 0.6 \mu\text{m}$. The TGase-treated films were significantly thinner than the controls as assessed by student t-test ($p < 0.0005$ %).

4.6. Filtration studies on TGase-treated GBM films

To ascertain if the permeation behaviour of GBM had changed after TGase treatment, filtration analysis was performed on films made from the membranes. Three films of TGase-treated GBM were freshly prepared, each with 1.5 mg dry weight equivalent of GBM, as described in Section 4.5. Three control films were prepared accordingly from GBM incubated with heat-denatured TGase. TBS, pH 7.4, myoglobin solution (0.5 mg/ml in TBS, pH 7.4), BSA solution (2.0 mg/ml in TBS, pH 7.4), and serum were consecutively filtered through the films, each over a range of pressures (10 - 200 kPa) with stirring (1,000 r.p.m.). Water fluxes (J_v), protein fluxes (J_s), and protein rejections (R = fraction of protein unable to cross the filter) were determined as detailed in Section 2.3.3. The films were rinsed with TBS, pH 7.4 after filtering each protein solution under low pressure to remove any protein that might

have been entrapped in the membrane matrix. No decrease in J_v (TBS) was seen relative to when TBS, pH 7.4 was initially filtered, indicating no membrane fouling. The experiment was repeated once and comparable results were obtained for both experiments, indicating good reproducibility. Representative results were presented as follows.

4.6.1. TBS ultrafiltration

TGase-treated GBM films displayed lower water fluxes (J_v) than the controls during TBS ultrafiltration over the entire pressure (ΔP) range: 10 - 200 kPa ($p < 0.0005$ for every pressure point) (Fig. 4.1.). It appeared, therefore, that TGase had rendered GBM less permeable to water.

4.6.2. Myoglobin ultrafiltration

Protein fluxes (J_s) and water fluxes (J_v) across TGase-treated films were lower than those across the controls over all of the pressure points ($p < 0.001$, and $p < 0.0005$, respectively) (Figs. 4.2.-4.3.), indicating that GBM had become less permeable to water and myoglobin after TGase treatment. Surprisingly, protein rejection values (R) were lower for TGase-treated films than the controls ($p < 0.0005$ for all pressure points) (Fig. 4.4.), suggesting that TGase had rendered GBM more permeable to protein. The reduction in R must, however, be interpreted with caution since R is a function of filtrate protein concentration, C_f :

$$R = 1 - \frac{C_f}{C_b} \quad (4.1.)$$

where C_b = protein concentration of the bulk or overstanding solution.

C_f is, in turn, a function of J_s and J_v :

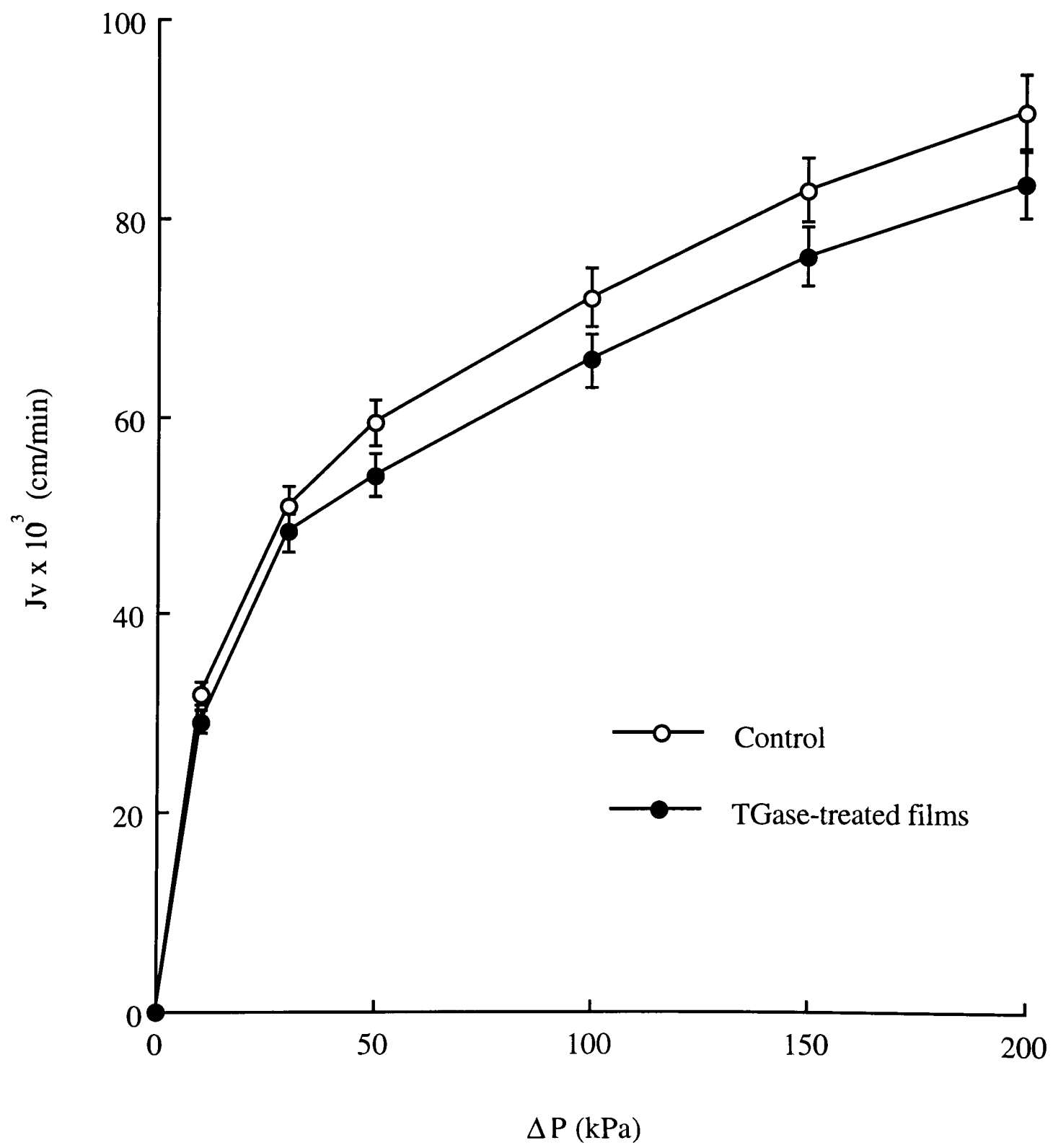
$$C_f = \frac{J_s}{J_v} \quad (4.2.)$$

such that

Legend to Fig. 4.1.

The effect of TGase treatment on the permeability of GBM films to TBS, pH 7.4. The films were formed and ultrafiltration studies carried out as described in text. Water flux (Jv) was measured over the ultrafiltration pressure range 0 - 200 kPa. Three films were made from GBM treated with TGase whilst the same number of control films were made from GBM treated with heat-denatured TGase. Each value on graphs indicates the mean of 9 measurements, 3 from each of three films. SDs are shown as error bars.

Fig. 4.1.



Legend to Figs. 4.2.-4.4.

The effect of TGase treatment on the ultrafiltration behaviour of GBM films to myoglobin solution (0.5 mg/ml in TBS, pH 7.4). The films were formed and ultrafiltration studies carried out as described in text. Protein flux (J_s), water flux (J_v), and protein rejection (R) were measured over the ultrafiltration pressure range 0 - 200 kPa and are shown on Figs. 4.2., 4.3., and 4.4., respectively. Three films were made from GBM treated with TGase whilst the same number of control films were made from GBM treated with heat-denatured TGase. Each value on graphs indicates the mean of 9 measurements, 3 from each of three films. SDs are shown as error bars.

Fig. 4.2.

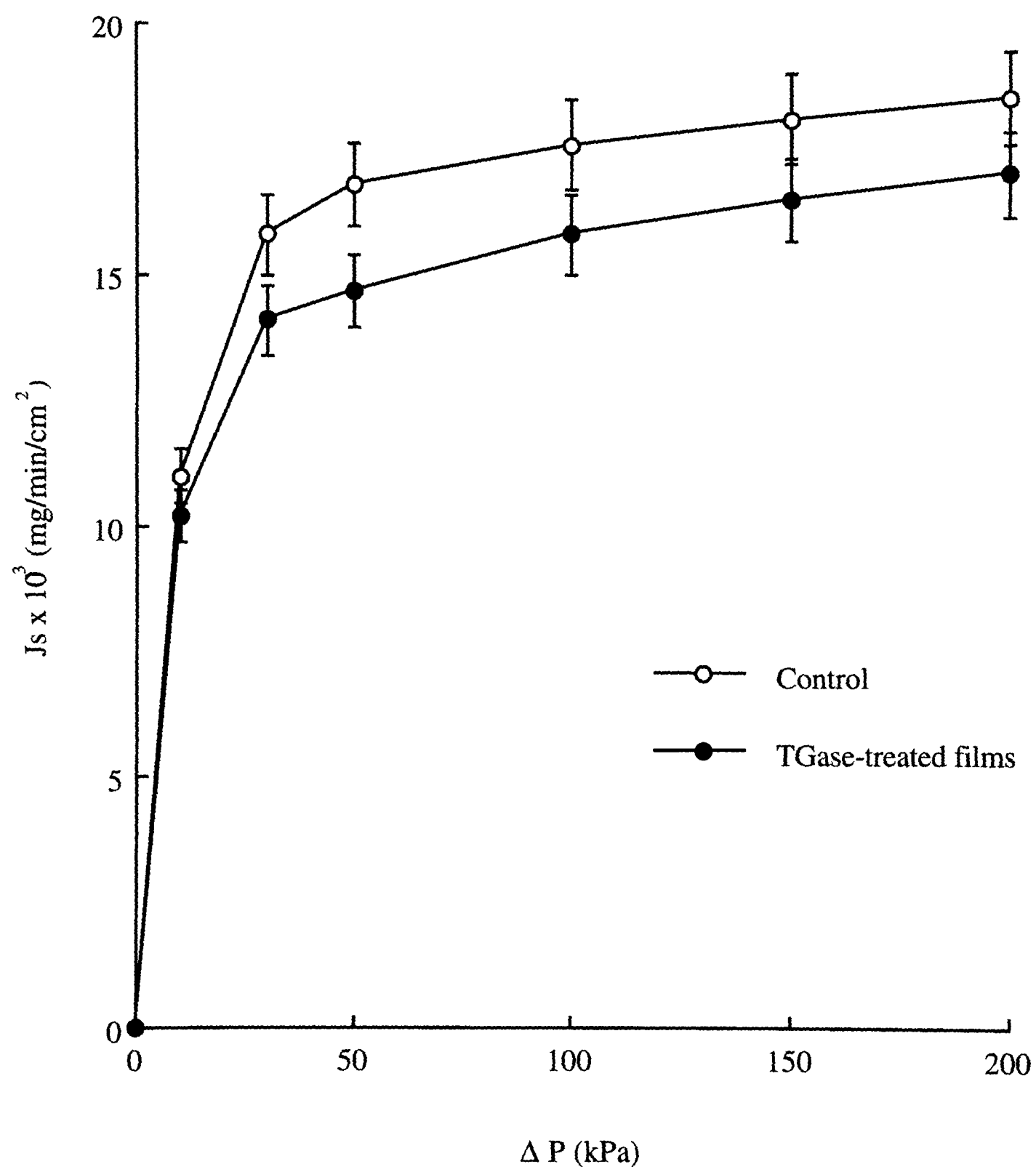


Fig. 4.3.

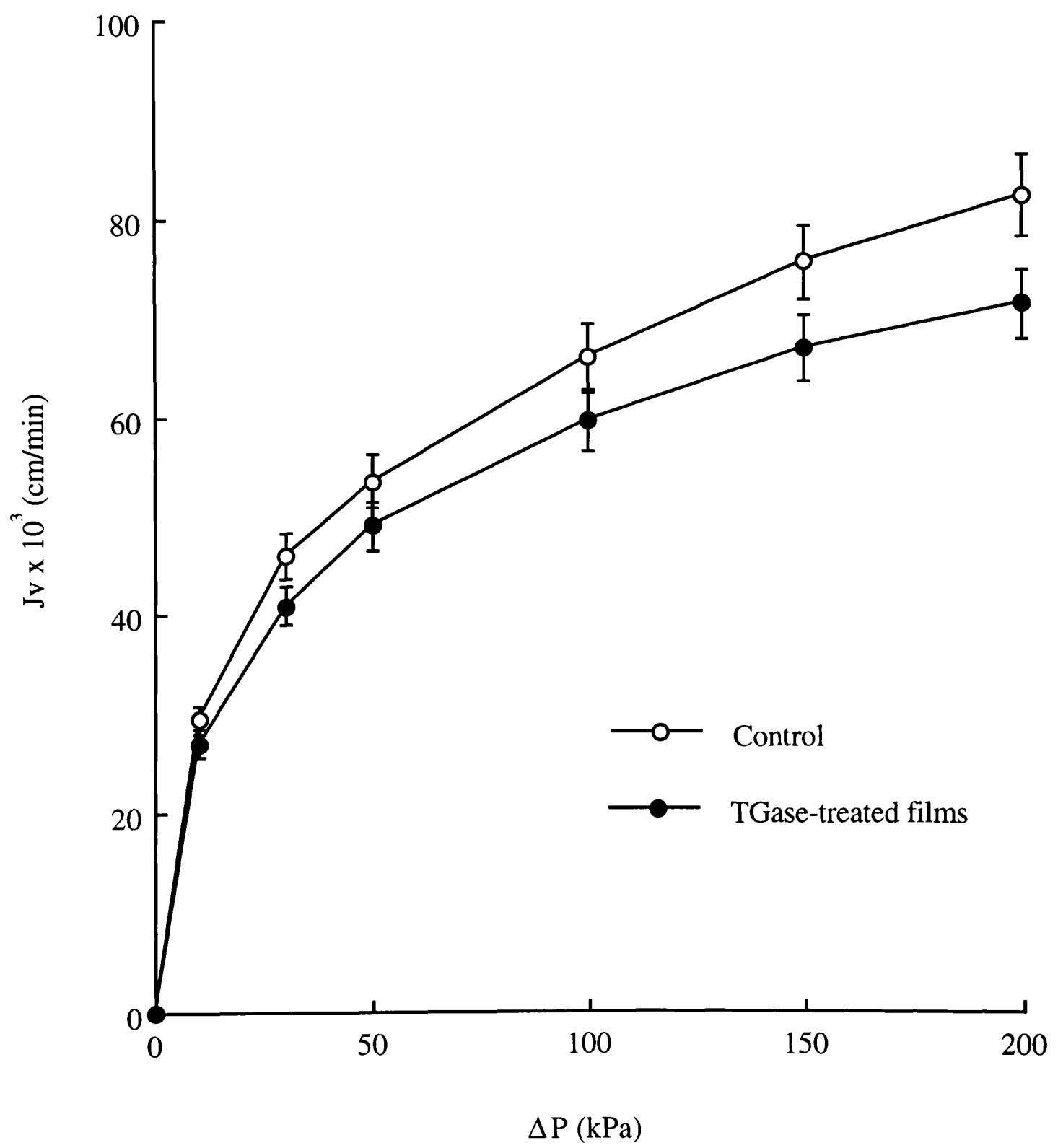
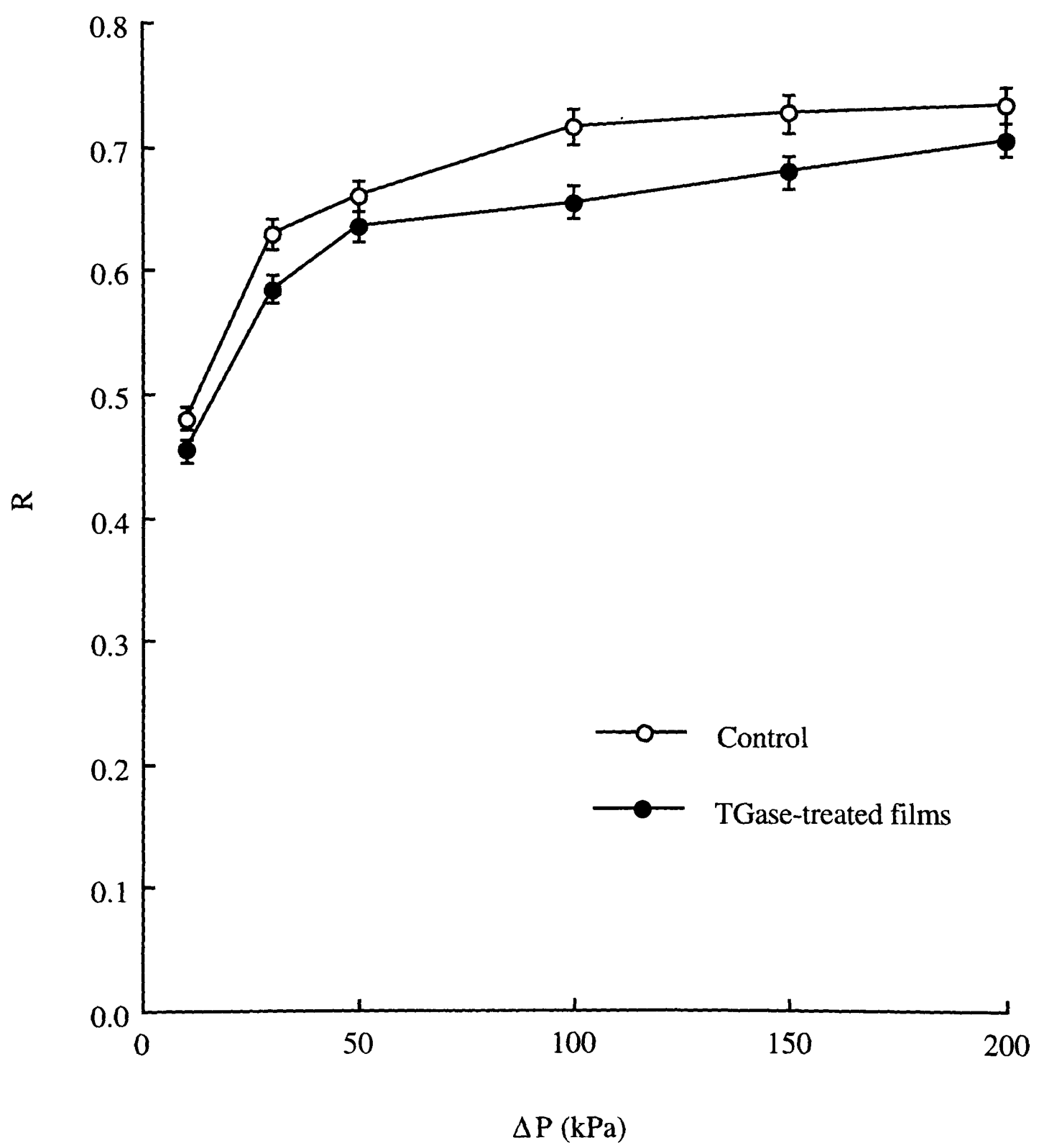


Fig. 4.4.



$$R = 1 - \frac{\left(\frac{J_s}{J_v}\right)}{C_b} \quad (4.3.)$$

Whether R changes or not depends on the relative magnitude of J_s and J_v . For example, J_s and J_v may have both decreased to the same degree, giving rise to no change in R. Alternatively, J_v may have decreased to a greater extent than J_s , giving rise to a higher J_s/J_v value and consequently a lower R. To determine if this had occurred following TGase treatment, J_s/J_v values were calculated and compared with those of the controls at each pressure point (Table 4.2.). J_s/J_v values for TGase treated films were marginally higher than the controls at every pressure point except at 50 and 100 kPa. This suggests that J_v had decreased more than had J_s following TGase treatment of GBM, causing a reduction in R values.

It must be noted that a decrease in C_b following TGase treatment of GBM could also have contributed to the decrease in R according to Eqn. 4.3. If J_v had decreased to a greater degree than had J_s , i.e., less water was being filtered across the film than protein, then the bulk solution would become more dilute. This meant that C_b ought to have decreased after TGase treatment which was in fact observed (Table 4.2.). The observation supported the idea that the reduction in R was caused by a reduction in C_b which was in turn caused by J_v having decreased more than had J_s . Therefore, the reduction in R following TGase treatment of GBM does not seem to be due to an increase in the permeability of the membrane to protein but appeared to be caused by an unequal reduction in J_s and J_v across the membrane as explained. This particular case of ultrafiltration illustrated the need to interpret changes in membrane permeability to protein by examining several filtration parameters simultaneously (J_s , J_v , and R). Using R as the only indicator may lead to the wrong conclusion being drawn.

4.6.3. BSA ultrafiltration

J_s across TGase-treated films fluctuated marginally above and below the control values, showing no consistent increase or decrease with respect to the control (Fig. 4.5.). A similar pattern was seen in a repeat experiment. It was thus concluded that

Table 4.2. The effect of TGase treatment on filtration parameters in myoglobin ultrafiltration

ΔP (kPa)	Js/Jv (mg/ml)		Cb (mg/ml)	
	Controls	TGase-treated films	Controls	TGase-treated films
10	0.373	0.376	0.72	0.69
30	0.343	0.344	0.93	0.86
50	0.314	0.300	0.92	0.82
100	0.267	0.265	0.94	0.85
150	0.240	0.247	0.87	0.82
200	0.226	0.239	0.84	0.81

Js/Jv values were calculated for each pressure point using data from Figs 4.2.-4.3. All values indicated are means.

Legend to Figs. 4.5.-4.7.

The effect of TGase treatment on the ultrafiltration behaviour of GBM films to BSA solution (2.0 mg/ml in TBS, pH 7.4). The films were formed and ultrafiltration studies carried out as described in text. Protein flux (J_s), water flux (J_v), and protein rejection (R) were measured over the ultrafiltration pressure range 0 - 200 kPa and are shown on Figs. 4.5., 4.6., and 4.7., respectively. Three films were made from GBM treated with TGase whilst the same number of control films were made from GBM treated with heat-denatured TGase. Each value on graphs indicates the mean of 9 measurements, 3 from each of three films. SDs are shown as error bars.

Fig. 4.5.

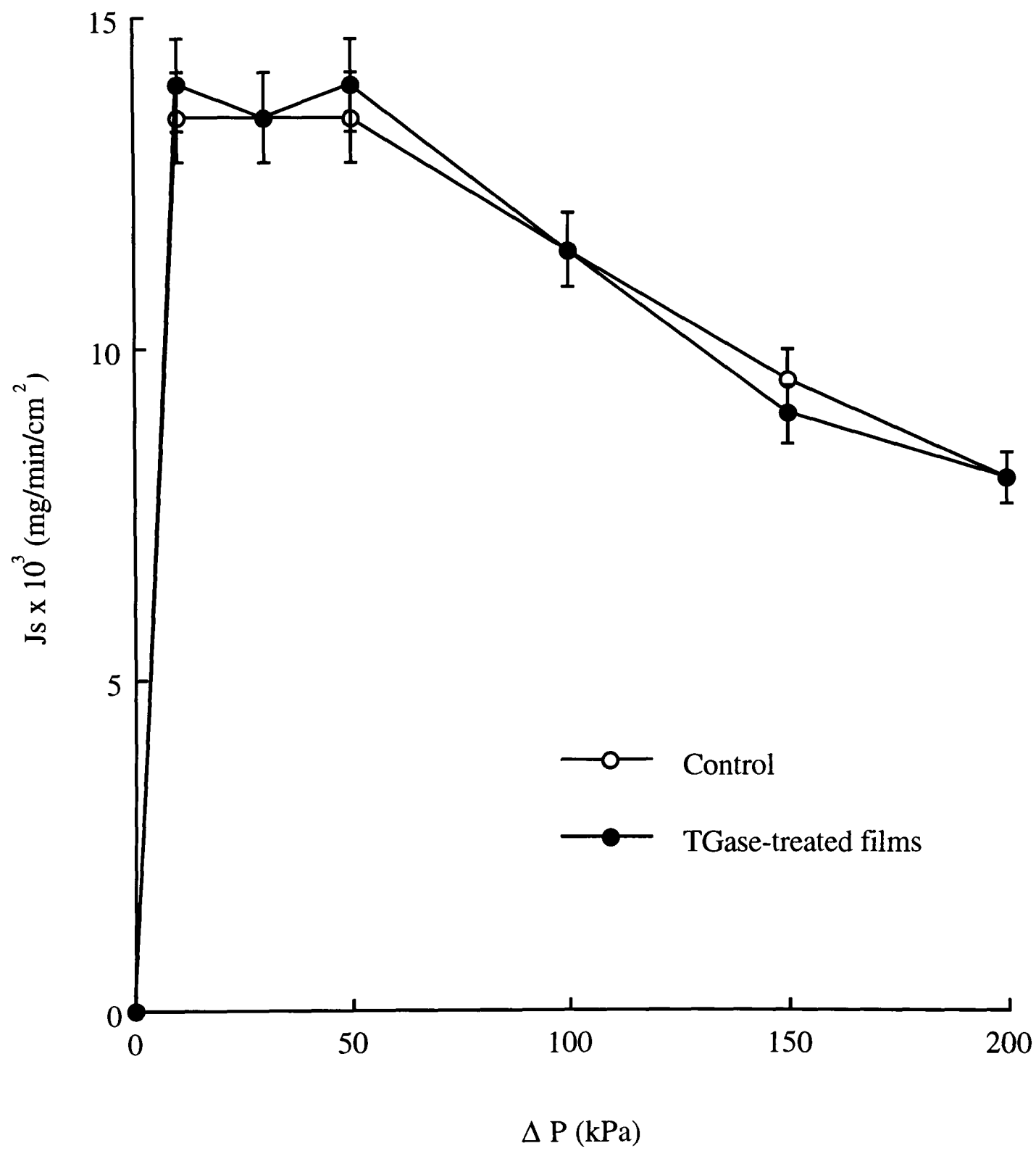


Fig. 4.6.

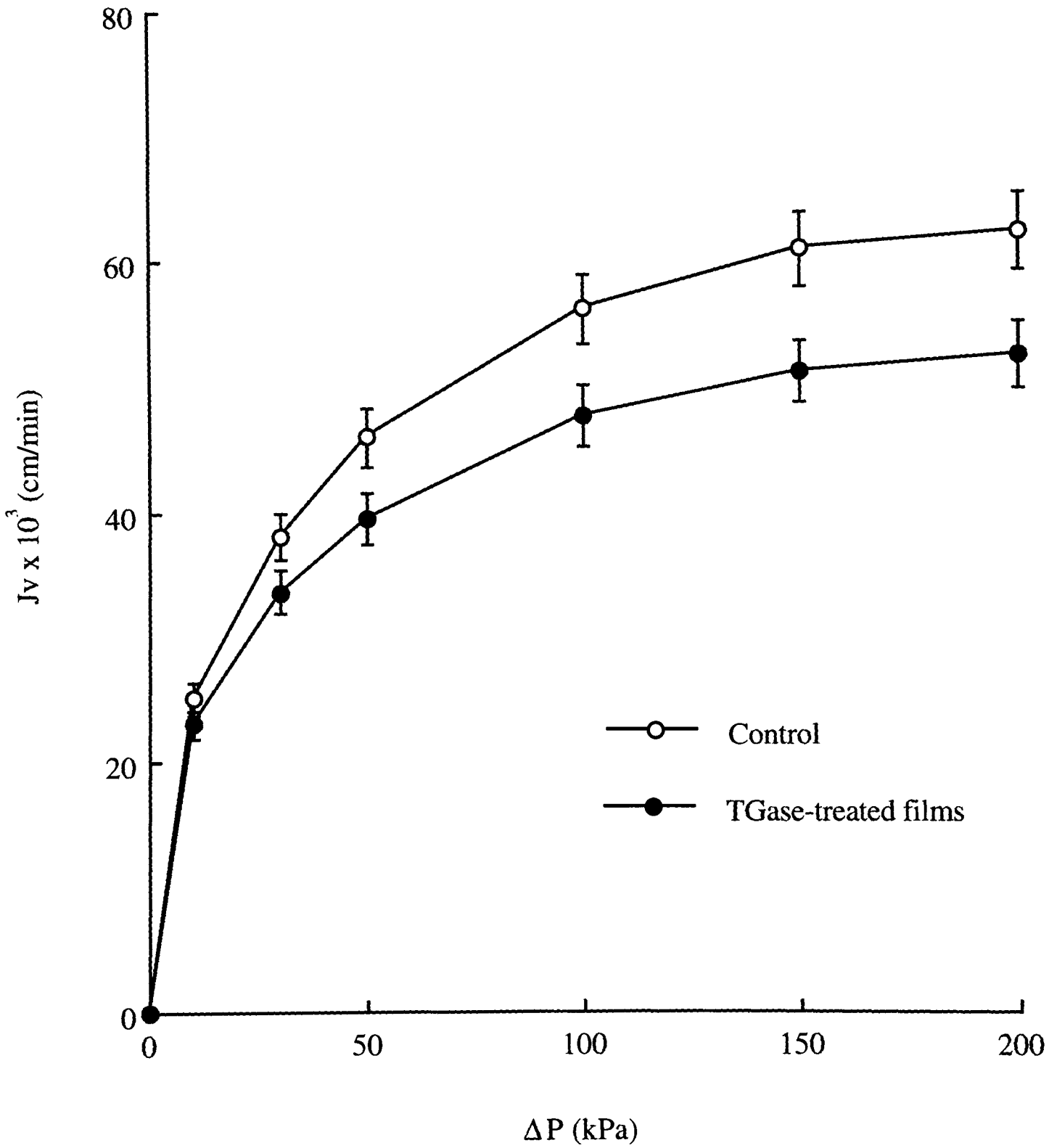
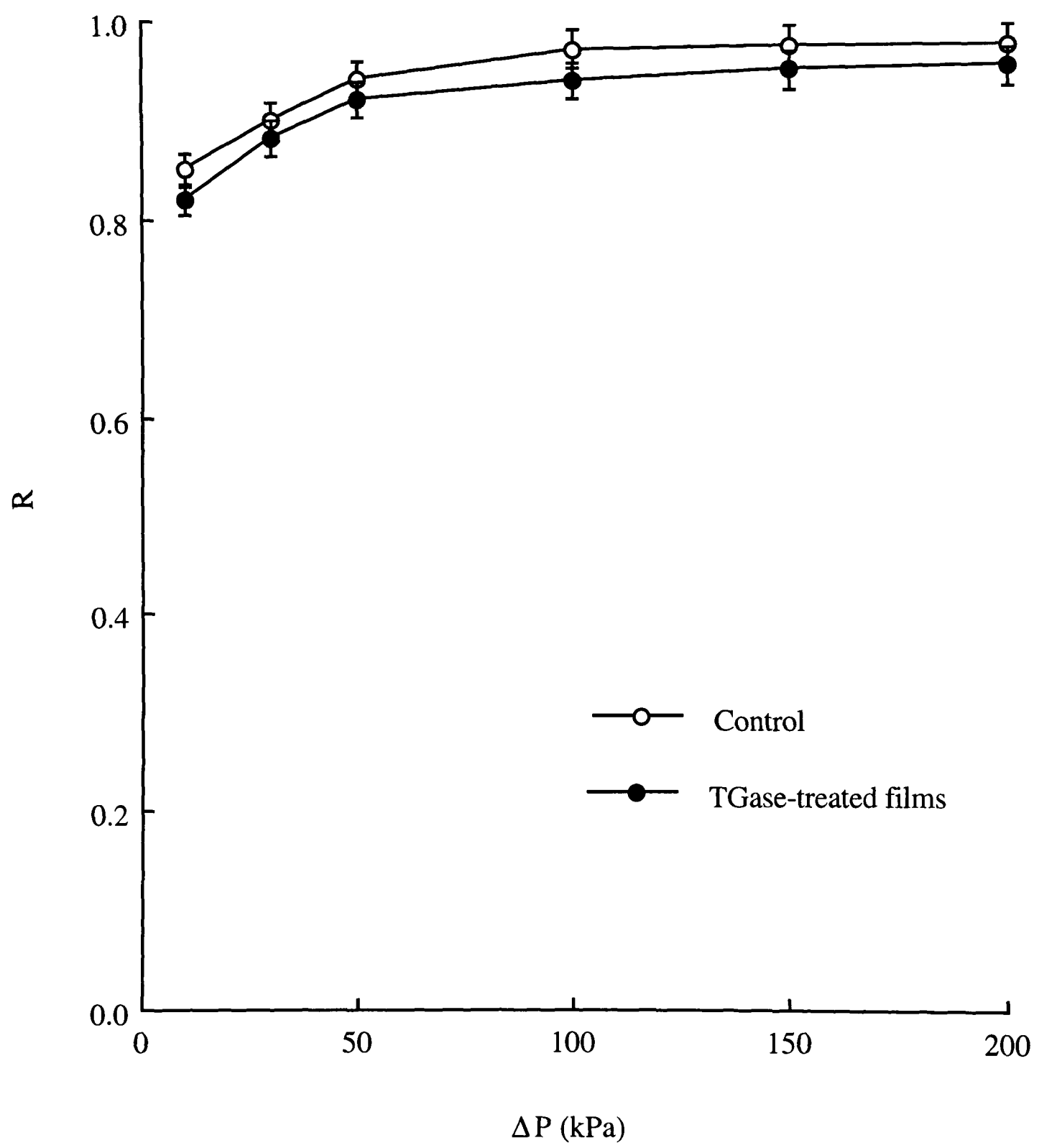


Fig. 4.7.



TGase treatment had no effect on membrane permeability to BSA. The change in J_v brought about by the TGase treatment was more clear cut: a consistent reduction in J_v was seen over the entire pressure range with respect to the control ($p < 0.0005$) (Fig. 4.6.), indicating a decrease in membrane permeability to water. R values for the TGase-treated films were consistently lower than the control (Fig. 4.7.) ($p < 0.01$ at each pressure point), suggesting a reduction in membrane permeability to BSA following TGase treatment. As has been argued in the previous section, the reduction in R does not necessarily mean that the membrane had become more permeable to protein. It could have resulted from unequal reductions in J_s and J_v values. In this case, J_v had decreased whilst J_s remained unchanged following TGase treatment. The effect this had on J_s/J_v and C_b , and consequently on R would be similar to that when myoglobin was filtered as explained below. The reduction in J_v coupled with no change in J_s would lead to an increase in J_s/J_v , reducing R according to Eqn. 4.3. The increase in J_s/J_v has actually been observed (Table 4.3.), supporting the foregoing reasoning. The reduction in J_v and no change in J_s could also have reduced C_b since the reduction in J_v meant that less water was being filtered across the membrane, making the bulk solution more dilute. This was in fact observed (Table 4.3.) and so could have caused a reduction in R according to Eqn. 4.3. This example of protein filtration illustrated the ability of J_s and J_v to vary independently, resulting in a reduction in R which does not necessarily indicate a decrease in membrane permeability to protein. The usefulness of R as a gauge of membrane permeability is thus questionable. The use of J_s seems to be a more useful gauge of a change in GBM permeability to protein.

4.6.4. Serum ultrafiltration

J_s values across TGase-treated films were not significantly different from those of controls over the entire pressure range (Fig. 4.8.), indicating that TGase did not alter the permeability of GBM to serum protein. J_v values for TGase-treated films were lower than the controls for all except the lowest pressure point (Fig. 4.9.) ($p < 0.05$ for

Table 4.3. The effect of TGase treatment on filtration paramters in BSA ultrafiltration

ΔP (kPa)	Js/Jv (mg/ml)		Cb (mg/ml)	
	Controls	TGase-treated films	Controls	TGase-treated films
10	0.540	0.609	3.60	3.58
30	0.355	0.402	3.55	3.16
50	0.293	0.354	4.89	4.54
100	0.204	0.241	6.81	5.48
150	0.156	0.176	5.56	4.29
200	0.128	0.153	4.59	3.72

Js/Jv values were calculated for each pressure point using data from Figs 4.5.-4.6. All values indicated are means.

Legend to Figs. 4.8.-4.10.

The effect of TGase treatment on the ultrafiltration behaviour of GBM films to serum (70 ± 4 mg/ml as quantitated using BCA protein assay (Section 2.3.5.) with BSA as a protein standard). The films were formed and ultrafiltration studies carried out as described in text. Protein flux (J_s), water flux (J_v), and protein rejection (R) were measured over the ultrafiltration pressure range 0 - 200 kPa and are shown on Figs. 4.8., 4.9., and 4.10., respectively. Three films were made from GBM treated with TGase whilst the same number of control films were made from GBM treated with heat-denatured TGase. Each value on graphs indicates the mean of 9 measurements, 3 from each of three films. SDs are shown as error bars. Where error bars are smaller than the diameter of the symbols, they are omitted.

Fig. 4.8.

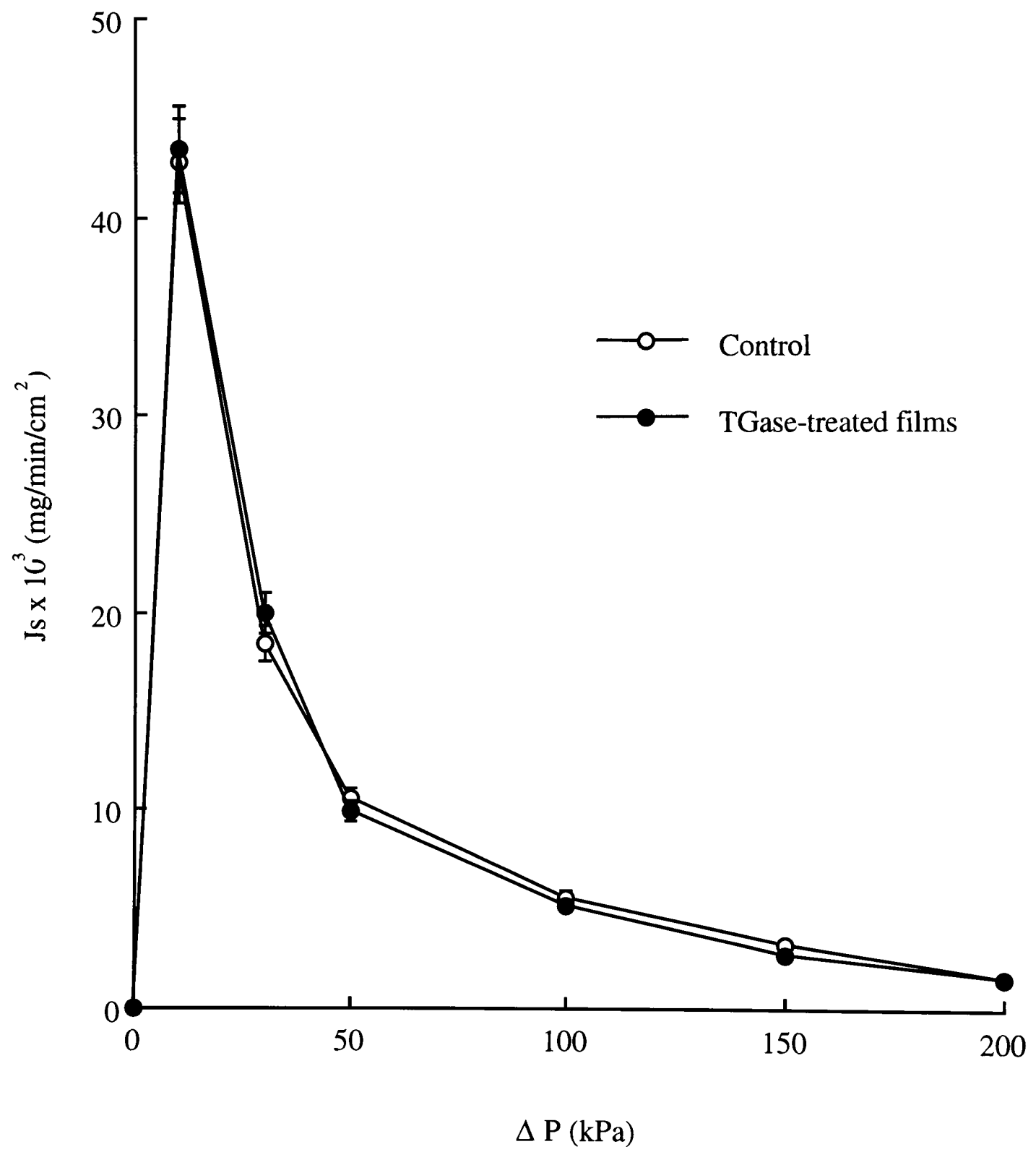


Fig. 4.9.

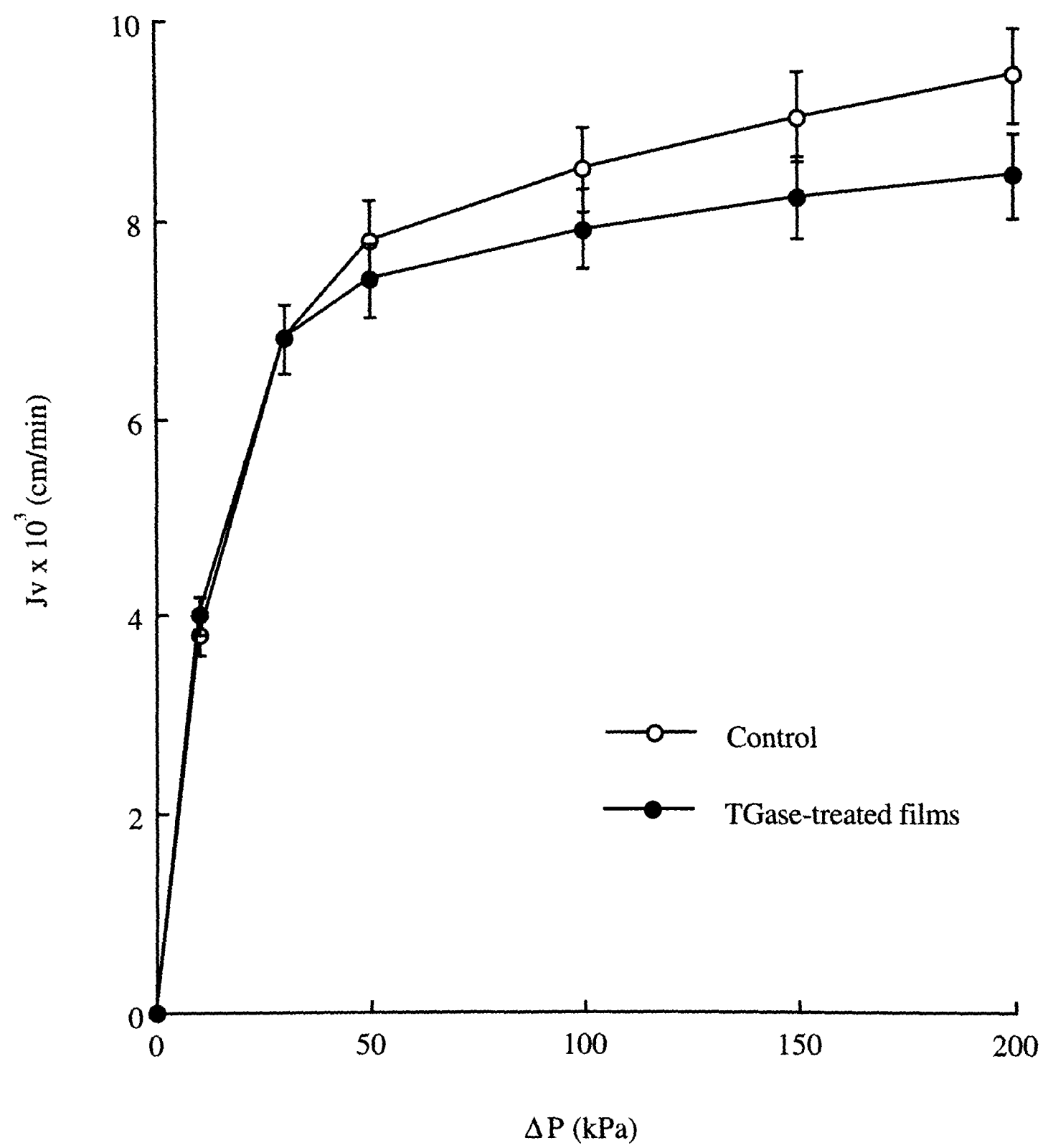
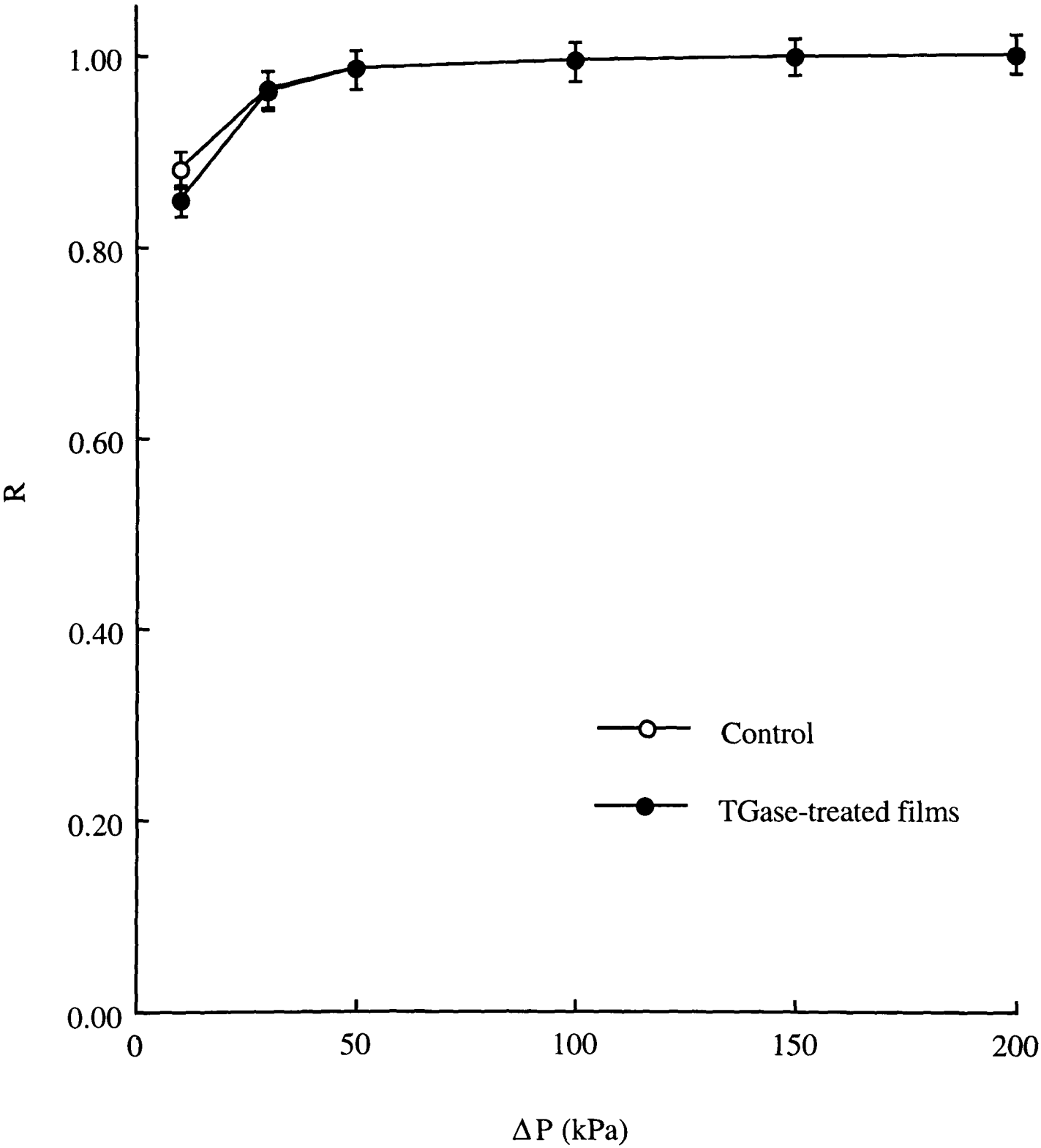


Fig. 4.10.



ΔP 50 - 200 kPa), indicating that TGase caused a decrease in the permeability of the GBM to water. R for TGase-treated films was generally not significantly different from the controls except at the lowest pressure point where R was lower than the control ($p < 0.0005$) (Fig. 4.10.).

Based on J_s and J_v alterations as indicators of membrane permeability change, it would seem that TGase treatment effected a decrease in GBM permeability to water but had little effect on membrane permeability to serum protein. The lack of change in R here also indicated no change in membrane permeability to serum protein following TGase treatment, concurring with the indication rendered by the lack of change in J_s . Curiously a decrease in J_v coupled with no change in J_s had led to a decrease in R in BSA filtration studies and yet the same pattern of change in J_v and J_s here did not result in a similar reduction in R . The reason for this seemed to lie in the huge difference between the magnitudes of J_v (~ 0.009 ml/min/cm² max.) and C_b (~ 120 mg/ml) such that a small decrease in J_v ($< 10\%$) following TGase treatment would not lead to a large reduction in R according to Eqn. 4.3. The decrease in R here was probably so small that it was masked by the larger intrinsic sampling errors and was thus undetectable. This illustrated another problem of using of R as a measure of changes in membrane permeability to protein, i.e., it could be an insensitive indicator when C_b was very high, as was the case with serum.

4.7. Discussion

TGase treatment appeared to have reduced the GBM permeability to water and to the low MW protein myoglobin (17.8 kDa) but had no effect on the larger protein BSA (MW 68 k). The treatment also had no significant effect on membrane permeability to serum protein. SDS PAGE analysis of collagenase digest of TGase-treated GBM showed high MW bands absent in the controls, suggesting that cross-linking of GBM proteins had occurred. The control was a collagenase digest of GBM pre-treated with heat-denatured TGase. The difference between the control and test band patterns was thus not due to the effect of collagenase digestion. If cross-linking

of GBM proteins had occurred, one would expect the GBM to have contracted and GBM film thickness to have been reduced. Indeed, thickness analysis of GBM films confirmed a significant reduction in film thickness following TGase treatment assessed by t-test ($p < 0.0005$). Electron microscopy evidence has shown that GBM has a fibre matrix structure (Farquhar et al., 1961; Carlson and Audette, 1989; Shirato et al., 1991; Shikata et al., 1992; Hironaka et al., 1993). It is conceivable that by cross-linking fibres of the matrix, TGase had drawn neighbouring fibres closer together, increasing the fibre packing density and reducing membrane void volume through which water and protein could permeate. This notion was supported by myoglobin filtration results where both J_v and J_s decreased following TGase treatment of the GBM (Section 4.5.2.). Surprisingly, chemical cross-linkers, e.g., glutaraldehyde, caused an increase in the membrane permeability to protein (Walton et al., 1992). Why such difference exists is discussed in detail in the general discussion.

That there was no decrease in the membrane permeability to BSA (MW 68 k) or to serum proteins (predominantly albumin, MW 68 k and immunoglobulins, MW > 150 k) may reflect the fact that BSA and serum proteins are not as sensitive a probe to small permeability changes as myoglobin or water. GBM shows low permeabilities to BSA and IgG as judged by their relatively small J_s across GBM films (particularly at high pressures) compared with J_s (myoglobin) (Chapter 3: Fig. 3.2.). Any small changes in membrane permeability to the already small BSA and serum protein fluxes across GBM may be masked by relatively large sampling errors. For example, TGase treatment caused a change in BSA flux of 3.7 % at 50 kPa compared with the 5.6 % SD in BSA flux at the same pressure. This may explain the insignificant changes in J_s in BSA and serum filtration studies. Myoglobin permeability across GBM is, by contrast, comparatively high and so changes in membrane permeability to myoglobin in terms of J_s would be proportionately greater and may not to be masked by sampling errors. GBM film permeability to water is higher still which makes water an even more sensitive probe to small changes in membrane permeability. This was supported by the

fact that irrespective of the solutions filtered, a significant reduction in J_v following TGase treatment was always seen (Figs. 4.1., 4.3., 4.6., and 4.9.).

The data presented here shows that TGase was unable to render GBM more permeable to serum protein. If anything, TGase rendered the membrane less permeable to low MW protein and water. The evidence from this study does not, therefore, substantiate the hypothesis that TGase is able to render GBM more permeable to plasma protein and give rise to proteinuria. It could be argued that the effect of liver TGase shown here is not representative of that of plasma, platelet or monocyte TGase which may behave quite differently and could have rendered GBM more permeable to protein. This is considered unlikely for two reasons. First, all of the aforementioned TGases share similar substrate specificities. They are all able to cross-link fibrin (Tyler and Laki, 1966; Lorand et al., 1966; Francis and Marder, 1987; Conkling et al., 1989) and to cross-link the polyamine putrescine to casein (Chung, 1972; Harris et al., 1984; Folk and Chung, 1985; Conkling et al., 1989). Thus, the effect of liver TGase on GBM permeation is likely to reflect that of the other enzymes from the same family. If this were the case, then plasma, platelet, and monocyte TGases are unlikely to mediate proteinuria in glomerulonephritis. Secondly, activated plasma, platelet, and monocyte TGases (MWs 142 k, 142 k, 77 k, respectively (Folk and Chung, 1985; Greenberg et al., 1991)) are generally larger than the liver TGase (MW 75 k (Folk and Chung, 1985)). These enzymes would not be able to permeate the GBM matrix as readily as the liver enzyme and so would not be able to introduce as many cross-links. They are thus less likely to have an effect on GBM permeability. This further weakens the argument that the TGases are able to cause proteinuria if exposed to GBM *in vivo*.

An analysis of changes in permeation parameters following TGase treatment of GBM were instructive in determining the relative usefulness of J_s , J_v , and R as indicators of membrane permeability changes. As discussed earlier, it was clear that J_s is the most direct measure of membrane permeability changes. R , by contrast, is not a useful indicator of changes in membrane permeability. It is more a reflection of the relative changes of J_s , J_v , and C_b governed by Eqn. 4.3. and could be misleading

when used alone to interpret changes in membrane permeability to protein. R has also been found to be an insensitive indicator of permeability changes when the difference between C_f and C_b is large, as was the case when serum was filtered. Thus R is of limited value in indicating permeability changes in this study.

CHAPTER FIVE

CHAPTER 5. THE EFFECT OF HYDROGEN PEROXIDE ON THE PERMEATION BEHAVIOUR OF GBM

5.1. Introduction

H_2O_2 is an oxidant generated by neutrophils following activation by immune-complexes (Fantone and Ward, 1982) and has been implicated as a mediator of glomerular injury in immune GN (Couser, 1993). The evidence came from the effect of catalase, a H_2O_2 scavenger, on the injury produced by circulatory inflammatory cells. Catalase infusion into rats with immune GN significantly reduced proteinuria and glomerular injury (Rehan et al., 1984; 1985a & b; 1986).

Glomerular injury, such as pronounced glomerular endothelial cell blebbing, damage and necrosis, as well as extensive focal fusion of the foot processes of podocytes, has been attributed to the cytotoxic effect of H_2O_2 . H_2O_2 cytotoxicity to a variety of normal cells, including endothelial cells, is well documented (Sacks, et al., 1978; Johnson and Ward, 1981; McCormick et al., 1981; Weiss et al., 1981). However, the mechanism by which H_2O_2 causes proteinuria is unknown. There is some evidence which suggests that H_2O_2 may be able to cause proteinuria by solubilizing GBM. For example, H_2O_2 (50 mM) degraded collagen IV, the major GBM protein after 8 hrs of incubation at 37°C (Pipoly and Crouch, 1986). Similar concentrations of H_2O_2 (< 70 mM) fragmented other proteins, e.g., mucus glycoproteins (Creeth et al., 1982; 1983). Molar concentrations of H_2O_2 were able to solubilized rat GBM completely (Fligiel et al., 1984). Perfusion studies by Yoshioka et al. (1991) showed that 1 hr of continuous intrarenal infusion of H_2O_2 at 18-100 mM resulted in massive proteinuria. Together, the evidence raises the possibility that activated neutrophils may be able to generate sufficiently high concentrations of H_2O_2 at the neutrophil-GBM interface to solubilize the basement membrane, rendering it more permeable to plasma protein and causing proteinuria.

The aim of the present study is to test this hypothesis directly. The concentration of H_2O_2 needed to render GBM more permeable to protein is determined

and compared with that which can be realistically generated by an activated neutrophil. From this, one can assess whether neutrophil-generated H_2O_2 can increase the permeability of the GBM to protein to account for the proteinuria observed. One additional parameter to be considered in this study is the minimum time needed for H_2O_2 to render GBM more permeable to protein. This will be compared with the length of exposure of GBM to neutrophil-generated H_2O_2 to determine if the *in vivo* exposure is sufficient to render the basement membrane more permeable to protein.

5.2. Incubation of GBM films with H_2O_2 and permeation analysis

GBM films (1.5 mg dry weight each), prepared as described in Section 2.2.2., were saturated with aqueous solutions of H_2O_2 at 37°C by percolating it through the films under low gas pressure (20 kPa) for 5 min. H_2O_2 (10 ml) of varying concentrations (0 - 4 M) was used; this volume was sufficient to saturate and cover the films with H_2O_2 . The films were then incubated at 37°C for varying time periods up to 12 hrs. The incubation was terminated by draining H_2O_2 off the films and washing them with copious TBS, pH 7.4 under low gas pressure to remove all traces of H_2O_2 . Controls were GBM films incubated with TBS, pH 7.4. Three films of GBM were used for each set of experimental conditions. Permeability studies on these films were then carried out in parallel by filtering TBS, pH 7.4, myoglobin solution (0.5 mg/ml in TBS, pH 7.4), BSA solution (2.0 mg/ml in TBS, pH 7.4), and serum through them consecutively, each under a range of filtration pressures (10 - 200 kPa) (Section 2.3.3.). The permeability of the films to protein and water, assessed by protein fluxes (J_s), protein rejections (R) and water fluxes (J_v), were compared with the controls to determine if H_2O_2 had altered the permeabilities of GBM.

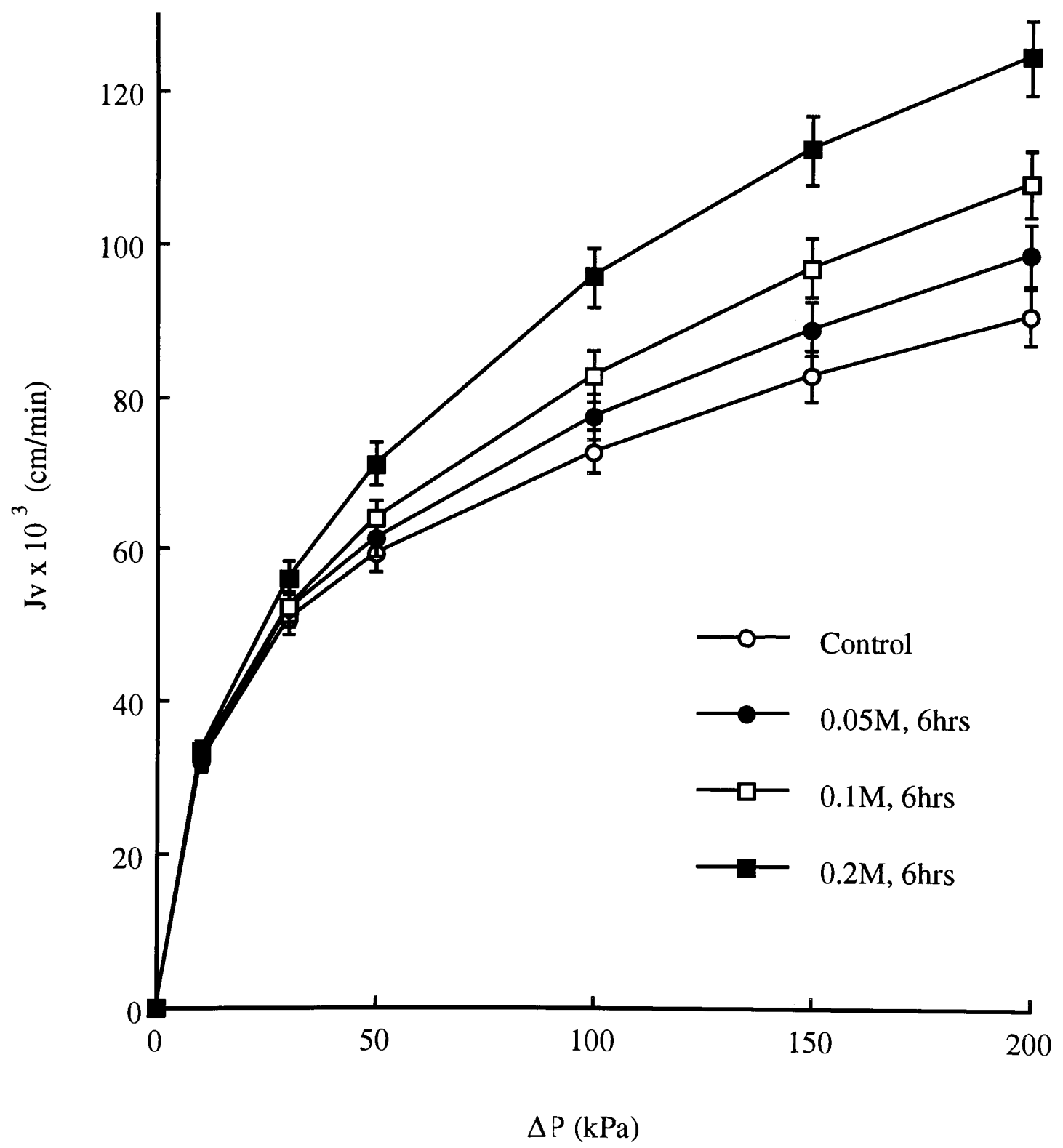
5.3. TBS ultrafiltration

The minimum H_2O_2 concentration needed to cause a significant increase in J_v across GBM films was 0.05 M (Fig. 5.1.) and the minimum incubation time required was 6 hrs (Fig. 5.2.). When the H_2O_2 concentration was increased above 0.05 M, J_v

Legend to Fig. 5.1.

The effect of H₂O₂ concentration on the permeability of GBM films to water. The films were incubated with aqueous solutions of H₂O₂ for 6 hrs at 37°C. Three films were treated with each concentration of H₂O₂ indicated whilst the same number of control films were treated with TBS, pH 7.4. TBS, pH 7.4 was ultrafiltered over the pressure range 0 - 200 kPa and water fluxes (J_v) measured as described in text. Each value on graphs indicate the mean of 9 measurements, 3 from each of three films. SDs are shown as error bars.

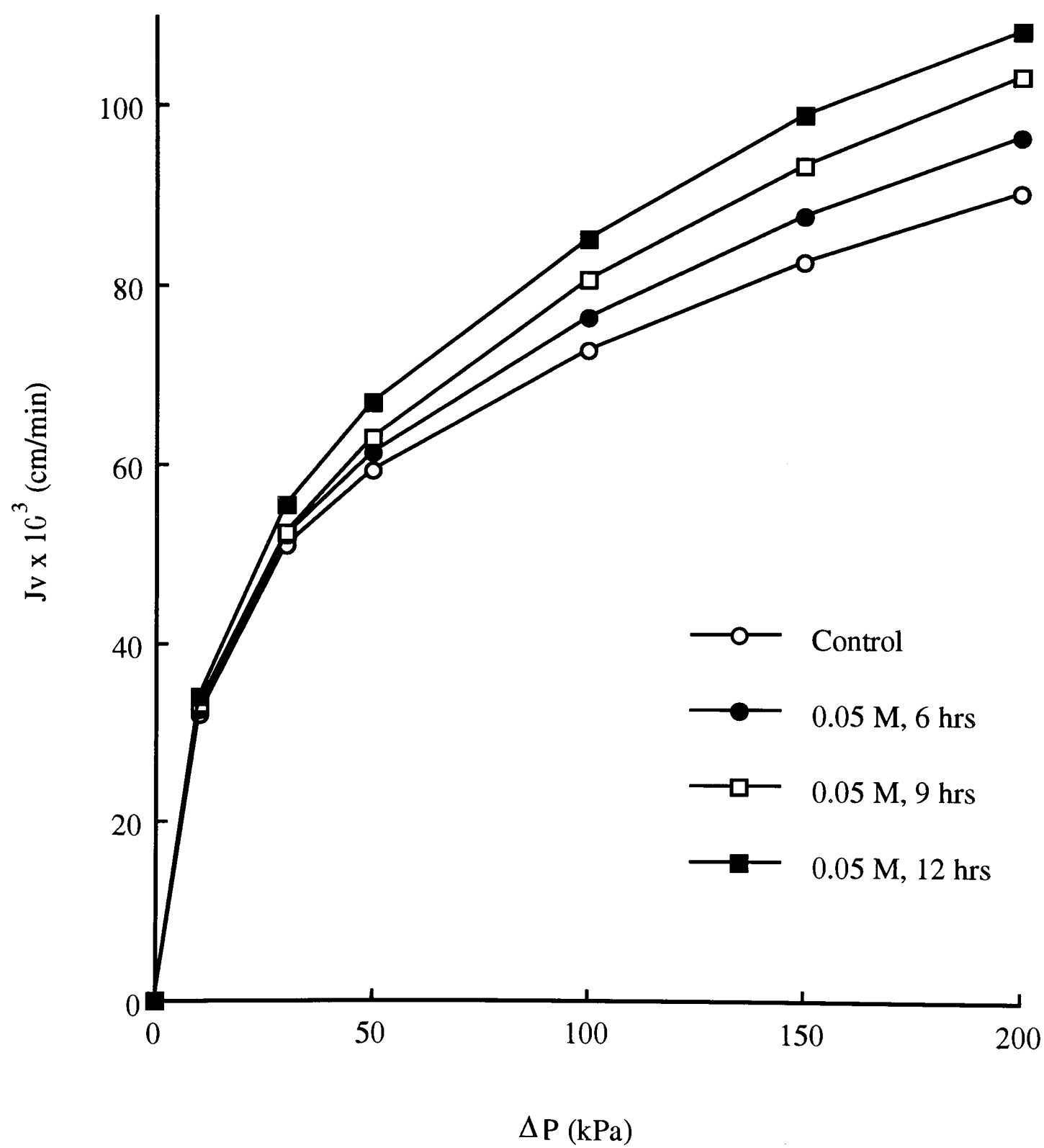
Fig. 5.1.



Legend to Fig. 5.2.

The effect of incubation time with H₂O₂ on the permeability of GBM films to water. 0.05 M H₂O₂ was used to incubate films up to 12 hrs at 37°C and filtration study carried out and water fluxes (J_v) across GBM films over the pressure range 0 - 200 kPa measured as described in text. A set of 3 films were treated for each length of time indicated. Sets of 3 control films were treated likewise except with TBS, pH 7.4. Each value on graphs indicate the mean of 9 measurements, 3 from each of three films. Error bars indicating SDs are omitted for clarity but are of similar magnitude as those shown on Fig. 5.1.

Fig. 5.2.



increased accordingly (Fig. 5.1.). If the incubation time was increased beyond 6 hrs, J_v also increased (Fig. 5.2.). It appeared that H_2O_2 was able to render GBM more permeable to water in a concentration- and time-dependent manner.

5.4. Myoglobin ultrafiltration

The minimum concentration of H_2O_2 needed to cause an increase in J_s (myoglobin) across GBM films was 0.1 M (Fig. 5.3.) and the minimum incubation time was 6 hrs (Fig. 5.4.). Increasing the concentration of H_2O_2 above 0.1 M resulted in a concentration-dependent increase in J_s (Fig. 5.3.). If the incubation time was extended beyond 6 hrs, J_s increased in a time-dependent manner (Fig. 5.4.). The same minimum concentration and time were needed to cause a decrease in R (myoglobin) (Figs. 5.5. & 5.6.). Also, concentration- and time-dependent decreases in R were seen with increasing H_2O_2 concentration above 0.1 M and increasing incubation time beyond 6 hrs (Figs. 5.5. & 5.6.). Judging from both J_s and R as indicators of GBM permeability, the minimum H_2O_2 and incubation time required to effect an increase in the permeability of the membrane to the MW probe myoglobin (MW 17.8k) were 0.1 M and 6 hrs, respectively.

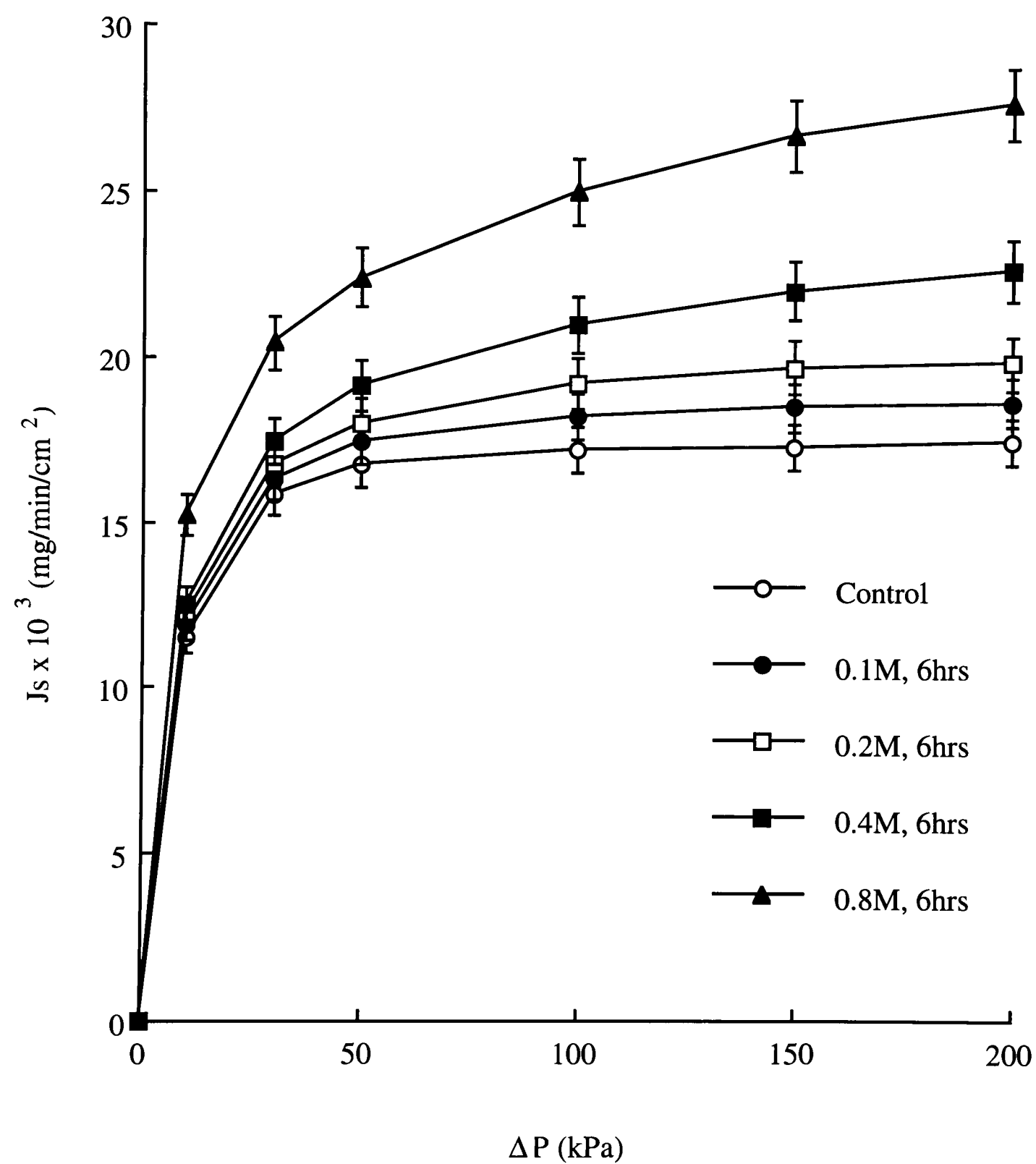
5.5. BSA ultrafiltration

The minimum H_2O_2 concentration needed to increase J_s and to reduce R of the larger probe BSA (MW 68 k) was 1 M (Figs. 5.7. & 5.9.). The minimum incubation time needed was 6 hrs (Figs. 5.8. & 5.10.). If higher H_2O_2 concentrations or longer incubation times were used, then J_s increased (Figs. 5.7. & 5.8.) and R decreased further (Figs. 5.9. & 5.10.). It appeared that considerably higher H_2O_2 concentration was needed here to render GBM more permeable to the larger protein BSA, suggesting that incubating the GBM with more concentrated H_2O_2 increased its porosity, allowing larger proteins to permeate across the membrane.

Legend to Fig. 5.3.

The effect of H₂O₂ concentration on myoglobin flux (Js) across GBM films. The films were incubated with aqueous solutions of H₂O₂ for 6 hrs at 37°C. Three films were treated with each concentration of H₂O₂ indicated whilst the same number of control films were treated with TBS, pH 7.4. Myoglobin solution (0.5 mg/ml in TBS, pH 7.4) was ultrafiltered and Js determined as described in text. Each value on graphs indicates the mean of 9 measurements, 3 from each of three films. Error bars indicating SDs are shown.

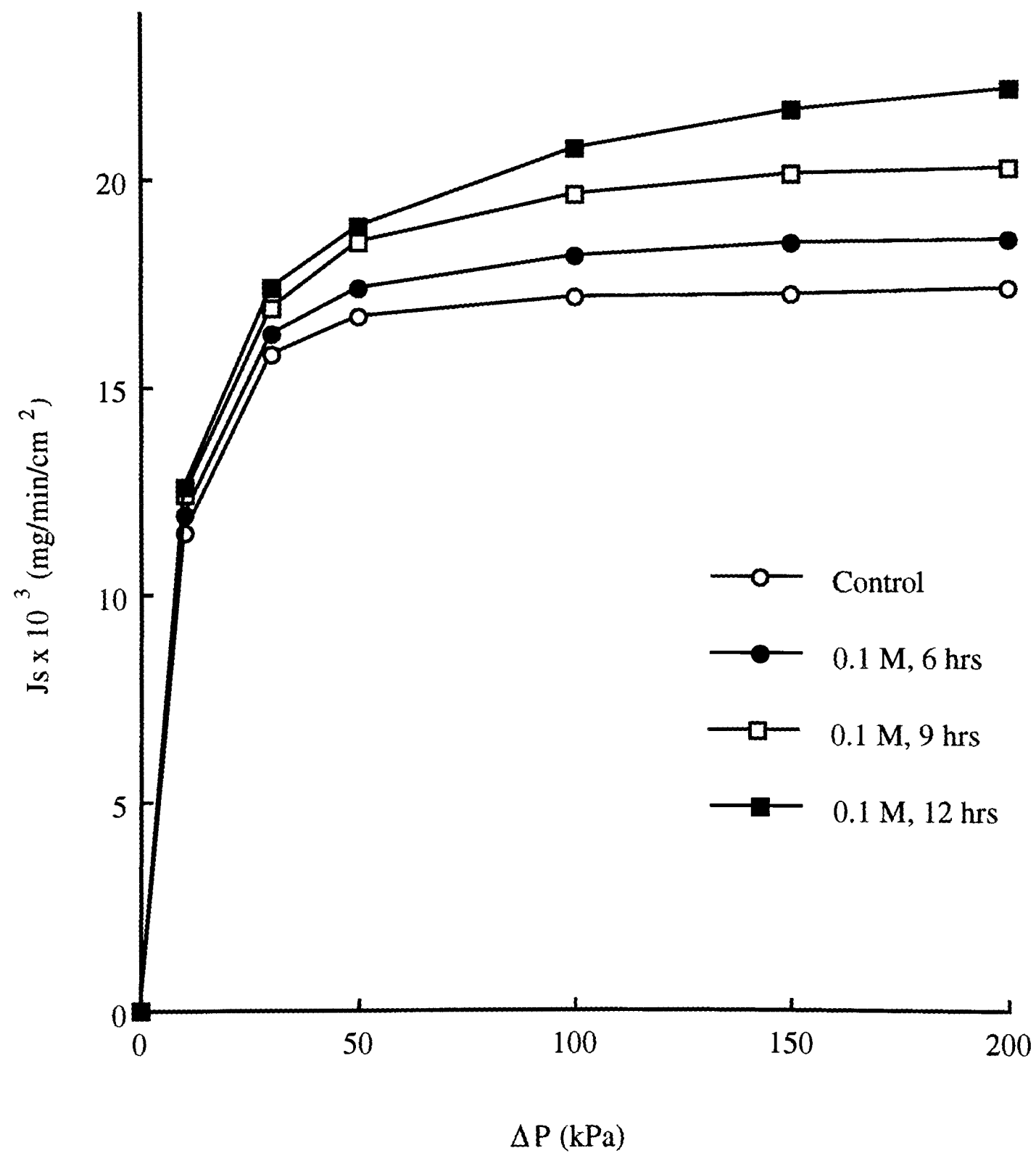
Fig. 5.3.



Legend to Fig. 5.4.

The effect of incubation time with H_2O_2 on myoglobin flux (J_s) across GBM films following treatment with a fixed concentration of H_2O_2 . The films were incubated with an aqueous solution of H_2O_2 for up to 12 hrs at 37°C . Each set of 3 films was treated for a specific length of time indicated. Sets of 3 control films were treated likewise except with TBS, pH 7.4. Myoglobin solution (0.5 mg/ml in TBS, pH 7.4) was ultrafiltered and J_s determined as described in text. Each value on graphs indicates the mean of 9 measurements, 3 from each of three films. Error bars indicating SDs are omitted for clarity but are of similar magnitude as those in Fig. 5.3.

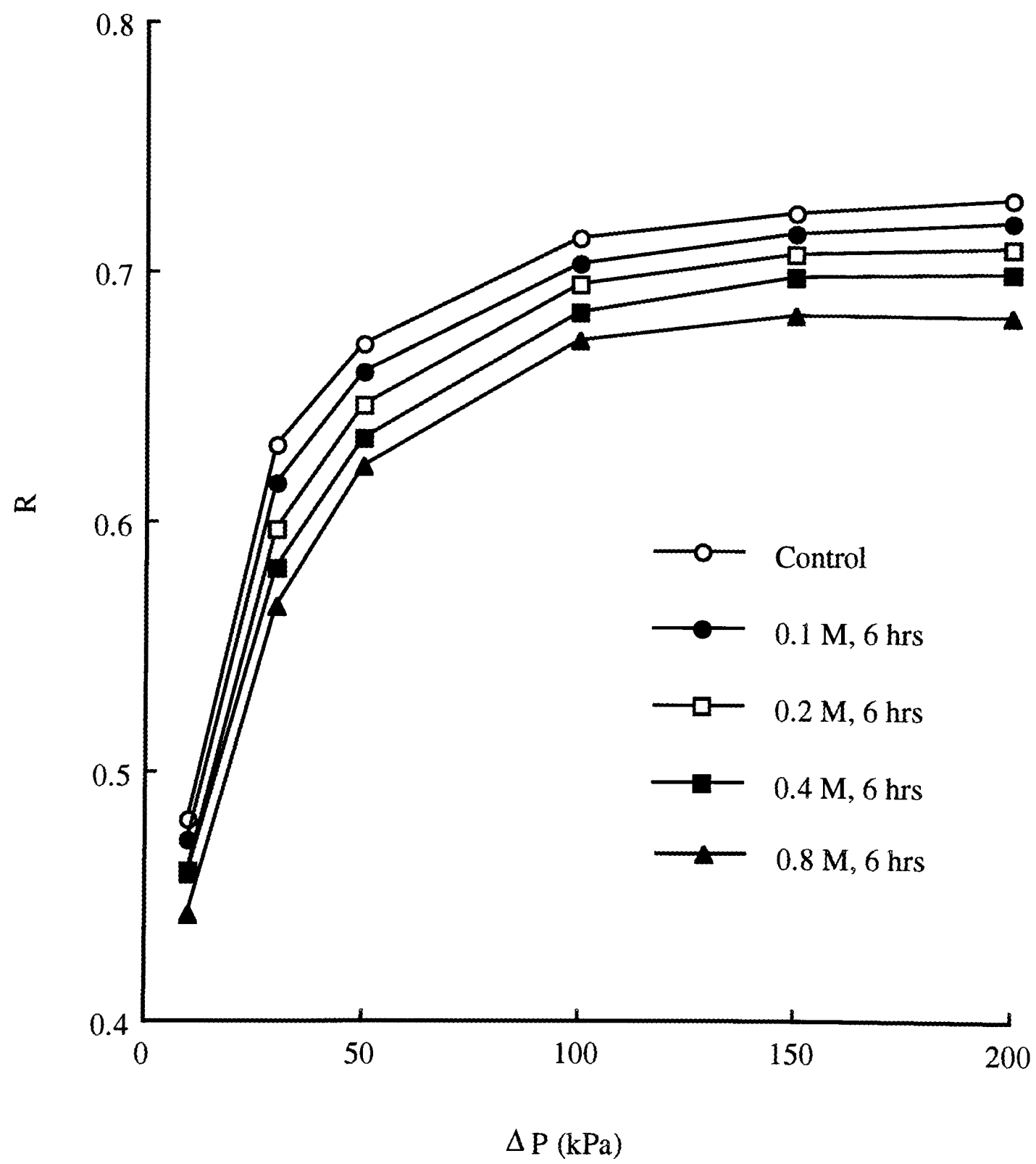
Fig. 5.4.



Legend to Fig. 5.5.

The effect of H₂O₂ concentration on myoglobin rejection (R). The films were incubated with aqueous solutions of H₂O₂ for 6 hrs at 37°C. Three films were treated with each concentration of H₂O₂ indicated whilst the same number of control films were treated with TBS, pH 7.4. Myoglobin solution (0.5 mg/ml in TBS, pH 7.4) was ultrafiltered and R determined as described in text. Each value on graphs indicates the mean of 9 measurements, 3 from each of three films. Error bars indicating SDs are omitted for clarity, though they were 2 - 4 % of R values.

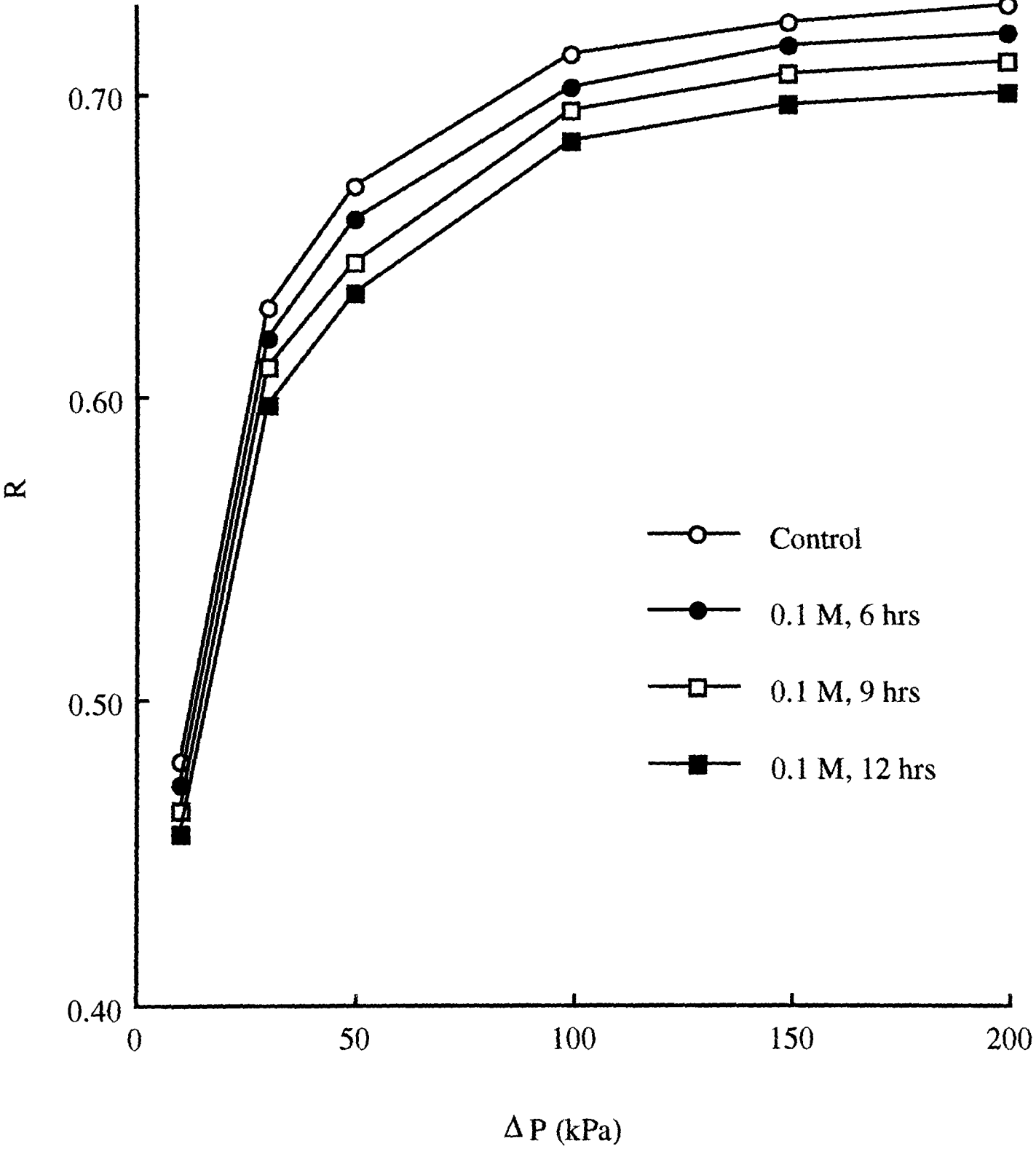
Fig. 5.5.



Legend to Fig. 5.6.

The effect of incubation time with H_2O_2 on myoglobin rejection (R) following treatment with a fixed concentration of H_2O_2 (0.1 M). The films were incubated with an aqueous solution of H_2O_2 for up to 12 hrs at 37°C. Each set of 3 films was treated for a specific length of time indicated. Sets of 3 control films were treated likewise except with TBS, pH 7.4. Myoglobin solution (0.5 mg/ml in TBS, pH 7.4) was ultrafiltered and R determined as described in text. Each value on graphs indicates the mean of 9 measurements, 3 from each of three films. Error bars indicating SDs are omitted for clarity but are of similar magnitude as those in Fig. 5.5.

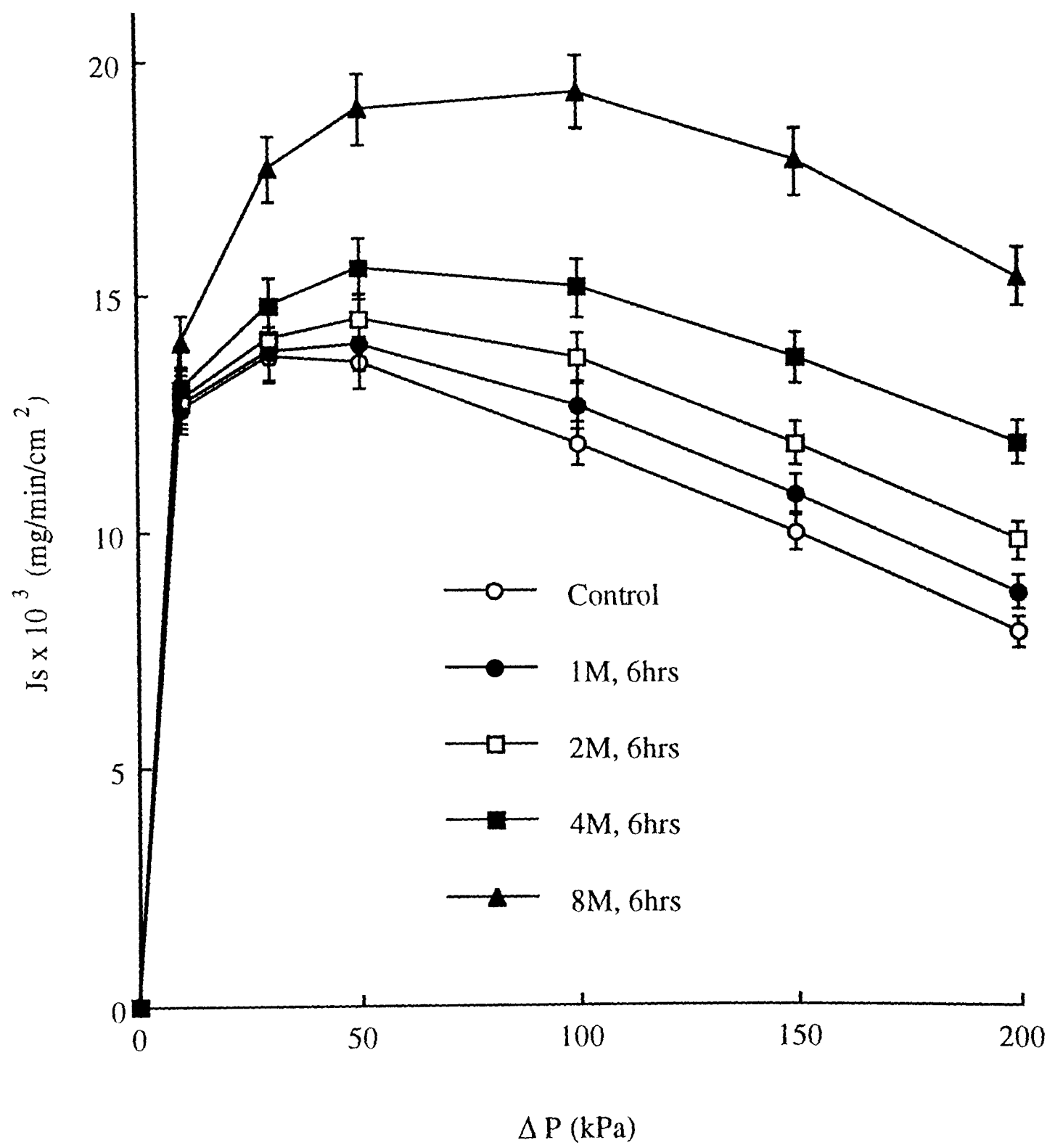
Fig. 5.6.



Legend to Fig. 5.7.

The effect of H₂O₂ concentration on BSA flux (Js) across GBM films. The films were incubated with aqueous solutions of H₂O₂ for 6 hrs at 37°C. Three films were treated with each concentration of H₂O₂ indicated whilst the same number of control films were treated with TBS, pH 7.4. BSA solution (2.0 mg/ml in TBS, pH 7.4) was ultrafiltered and Js determined as described in text. Each value on graphs indicates the mean of 9 measurements, 3 from each of three films. Error bars indicating SDs are shown.

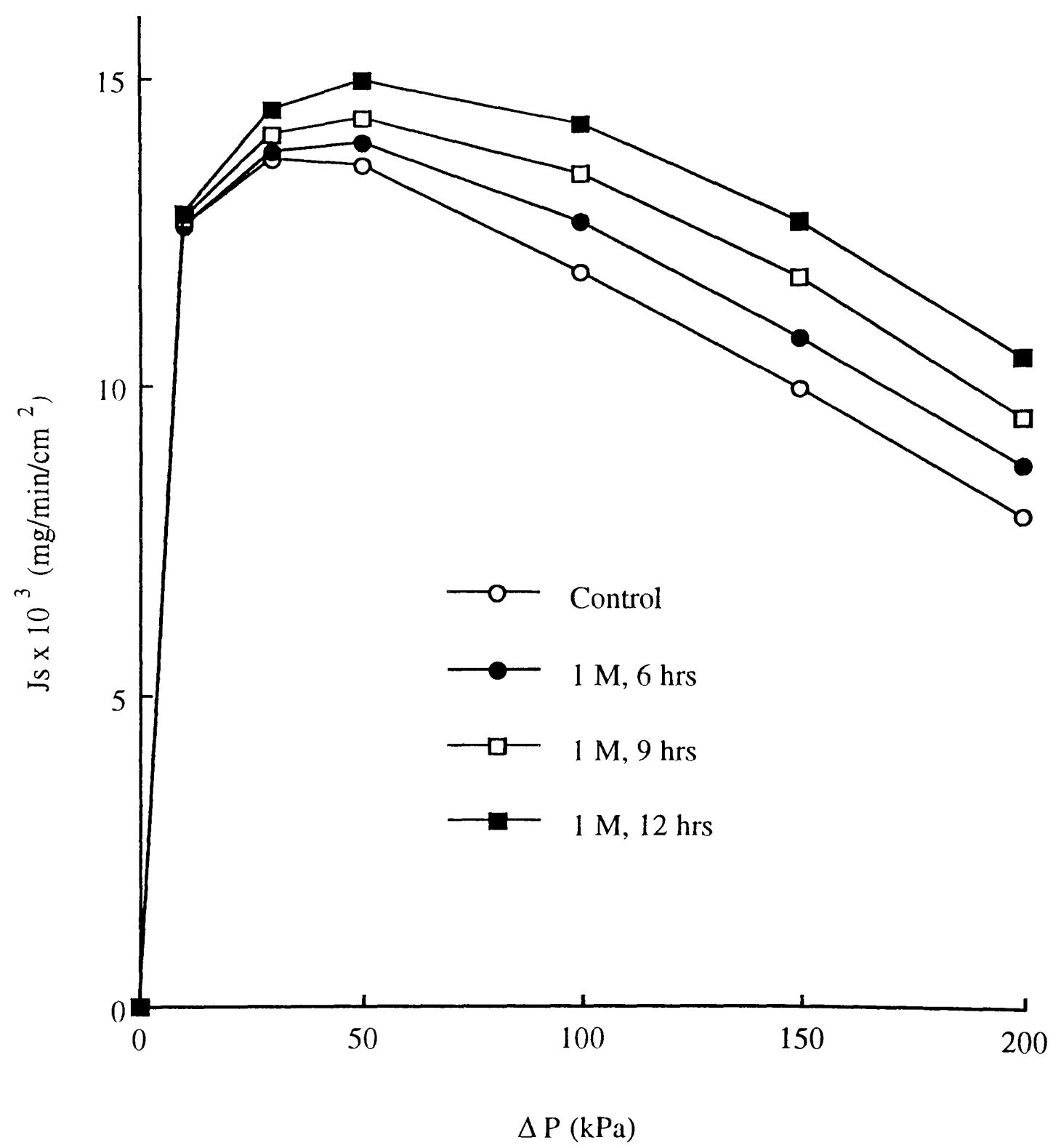
Fig. 5.7.



Legend to Fig. 5.8.

The effect of incubation time with H_2O_2 on BSA flux (J_s) across GBM films following treatment with a fixed concentration of H_2O_2 (1 M). The films were incubated with an aqueous solution of H_2O_2 for up to 12 hrs at 37°C. Each set of 3 films was treated for a specific length of time indicated. Sets of 3 control films were treated likewise except with TBS, pH 7.4. BSA solution (2.0 mg/ml in TBS, pH 7.4) was ultrafiltered and J_s determined as described in text. Each value on graphs indicates the mean of 9 measurements, 3 from each of three films. Error bars indicating SDs are omitted for clarity but are of similar magnitude as those in Fig. 5.7.

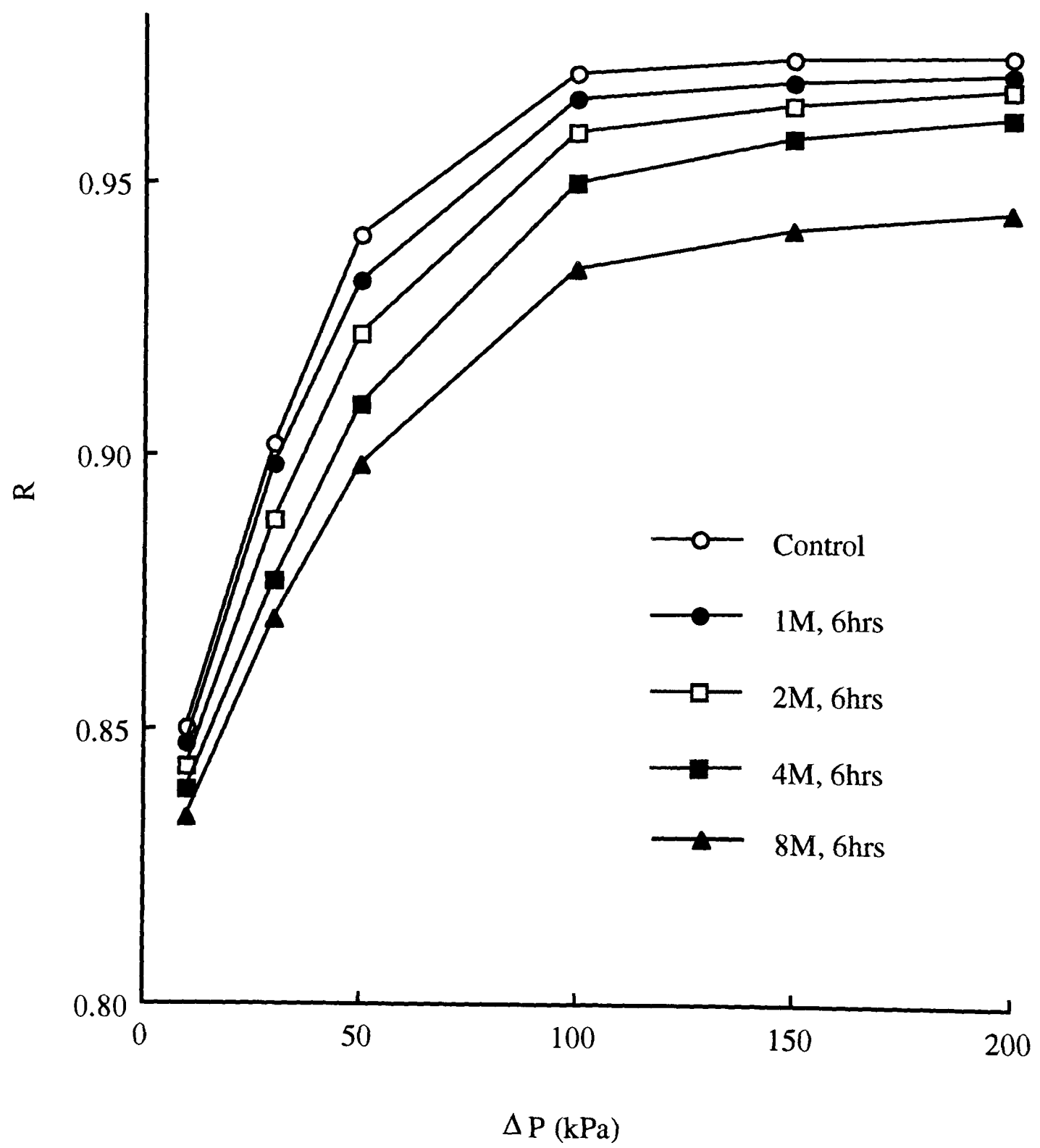
Fig. 5.8.



Legend to Fig. 5.9.

The effect of H₂O₂ concentration on BSA rejection (R). The films were incubated with aqueous solutions of H₂O₂ for 6 hrs at 37°C. Three films were treated with each concentration of H₂O₂ indicated whilst the same number of control films were treated with TBS, pH 7.4. BSA solution (2.0 mg/ml in TBS, pH 7.4) was ultrafiltered and R determined as described in text. Each value on graphs indicates the mean of 9 measurements, 3 from each of three films. Error bars indicating SDs are omitted for clarity, though they were 3 - 5 % of R values.

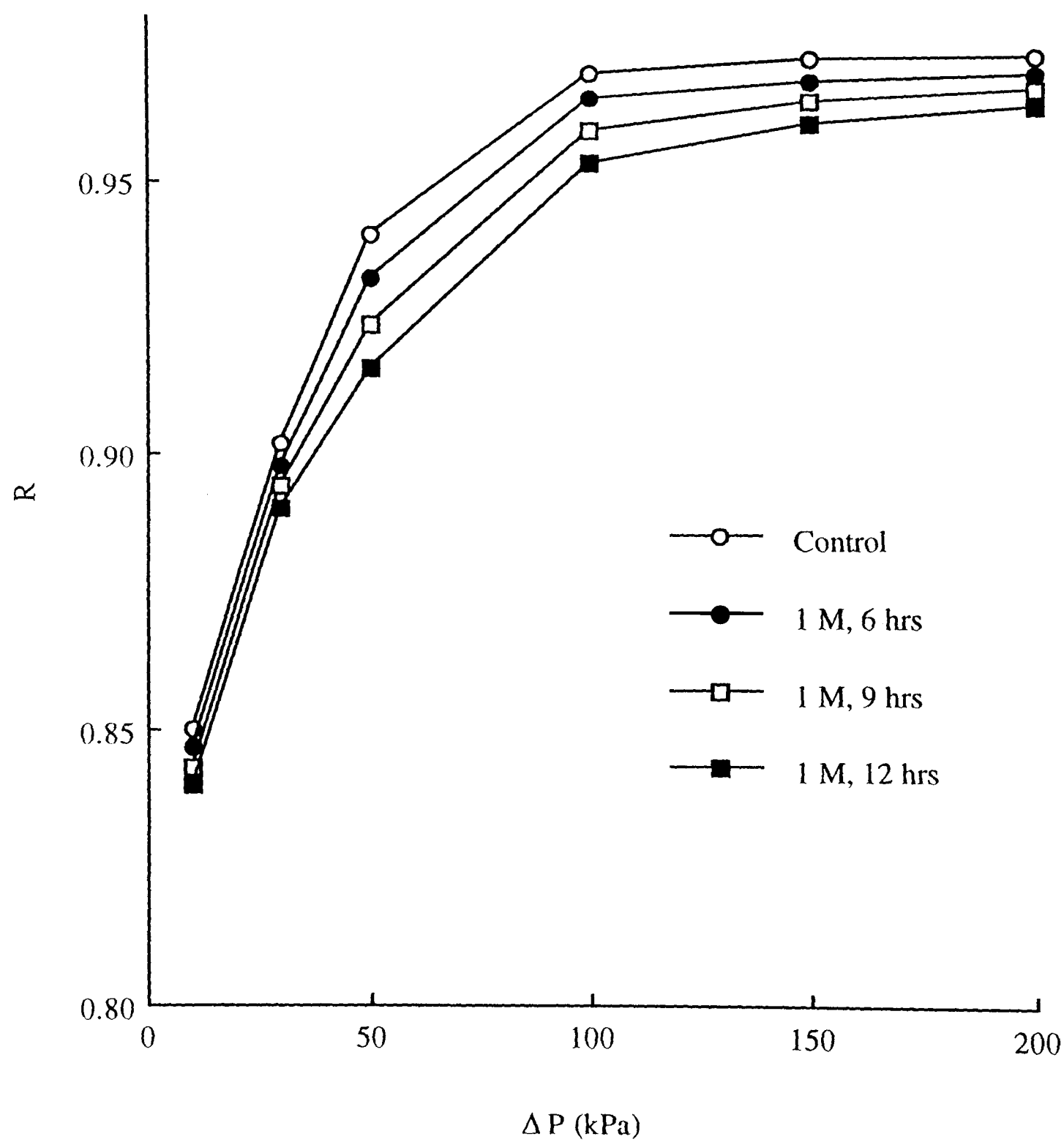
Fig. 5.9.



Legend to Fig. 5.10.

The effect of incubation time with H_2O_2 on BSA rejection (R) following treatment with a fixed concentration of H_2O_2 (1 M). The films were incubated with an aqueous solution of H_2O_2 for up to 12 hrs at 37°C. Each set of 3 films was treated for a specific length of time indicated. Sets of 3 control films were treated likewise except with TBS, pH 7.4. BSA solution (2.0 mg/ml in TBS, pH 7.4) was ultrafiltered and R determined as described in text. Each value on graphs indicates the mean of 9 measurements, 3 from each of three films. Error bars indicating SDs are omitted for clarity, though they were 3 - 5 % of R values.

Fig. 5.10.



5.6. Serum ultrafiltration

A similar pattern of results was seen when the permeation analysis was carried out using serum as a probe of permeability changes. The minimum H_2O_2 concentration and incubation time needed to render GBM films more permeable to serum protein, judged by J_s and R , were 1 M and 6 hrs, respectively (Figs. 5.11.-5.14.). Using concentrations < 1 M and incubation periods < 6 hrs had no detectable effect on J_s and R , relative the controls. If H_2O_2 concentration used was increased above 1 M, then J_s increased and R decreased with increasing concentration (Figs. 5.11. & 5.13.). Similarly, increasing the length of incubation beyond 6 hrs resulted in time-dependent increase in J_s and decrease in R (Figs. 5.12. & 5.14.).

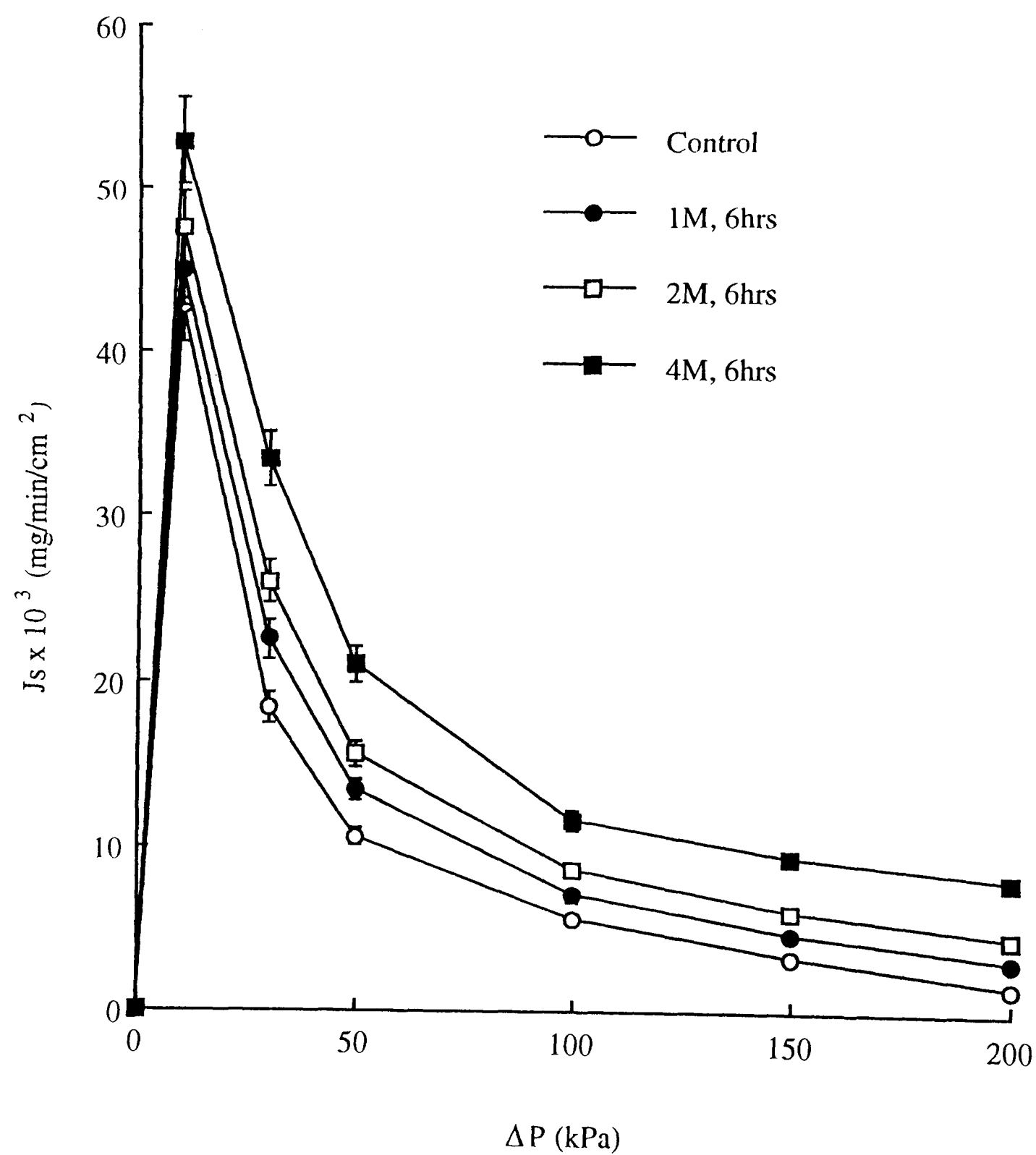
5.7. Summary of permeation analysis

The change in the porosity of GBM following treatment by H_2O_2 had been examined using buffer, purified proteins (myoglobin and BSA), and serum. A relatively low H_2O_2 concentration (0.05 M) rendered GBM more permeable to water. A higher concentration (0.1 M) was needed to render the membrane more permeable to the low MW protein myoglobin (MW 17.8). To cause an increase in the porosity of the membrane to the larger protein BSA (MW 68 k) and to serum proteins (predominantly albumin and immunoglobulins, MW > 150 k), a considerably higher H_2O_2 concentration (1 M) was required. The minimum incubation time necessary to increase membrane permeability to water and proteins was 6 hrs for the concentrations used. The results suggest that as the H_2O_2 concentration used was increased, the porosity of the membrane also increased, permitting larger protein molecules to permeate across the membrane matrix. A similar effect was achieved if the exposure time of GBM to H_2O_2 was increased. Having established the H_2O_2 concentration and exposure time needed to cause an increase in GBM permeability to protein, whether activated neutrophils can realistically generate such concentration over the exposure time was analysed.

Legend to Fig. 5.11.

The effect of H₂O₂ concentration on serum protein flux across GBM films. The films were incubated with aqueous solutions of H₂O₂ for 6 hrs at 37°C. Three films were treated with each concentration of H₂O₂ indicated whilst the same number of control films were treated with TBS, pH 7.4. Pig serum (70 mg/ml) was ultrafiltered and Js and R determined as described in text. Each value on graphs indicates a mean of 9 measurements, 3 from each of three films. Error bars indicating SDs are shown but are omitted where SDs are too small to be visible.

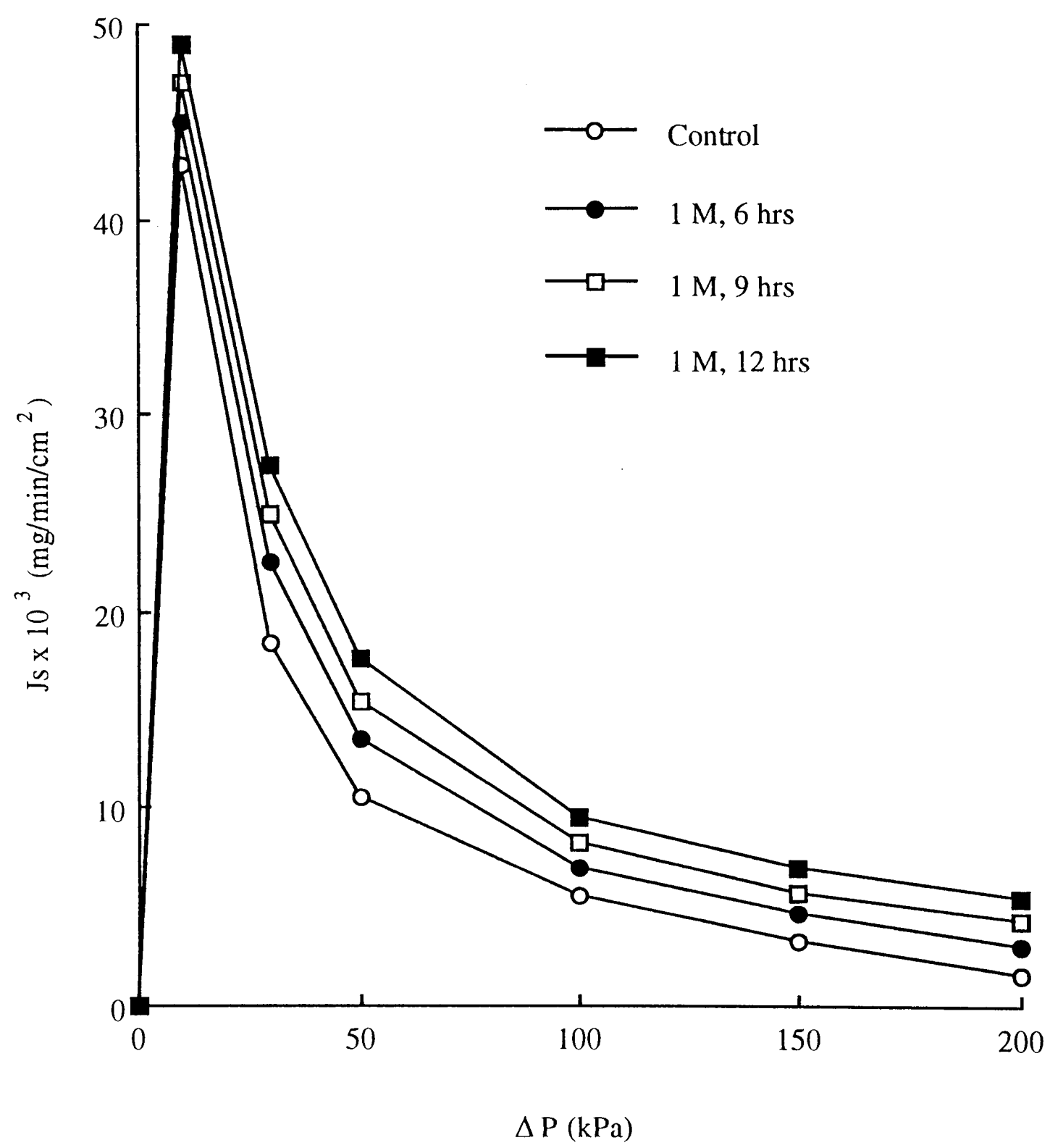
Fig. 5.11.



Legend to Fig. 5.12.

The effect of incubation time with H_2O_2 on serum protein flux (J_s) across GBM films following treatment with a fixed concentration of H_2O_2 (1 M). The films were incubated with an aqueous solution of H_2O_2 for up to 12 hrs at 37°C . Each set of 3 films was treated for a specific length of time indicated. Sets of 3 control films were treated likewise except with TBS, pH 7.4. Pig serum (70 mg/ml) was ultrafiltered and J_s determined as described in text. Each value on graphs indicates a mean of 9 measurements, 3 from each of three films. Error bars indicating SDs are omitted for clarity but are of similar magnitude as those in Fig. 5.11.

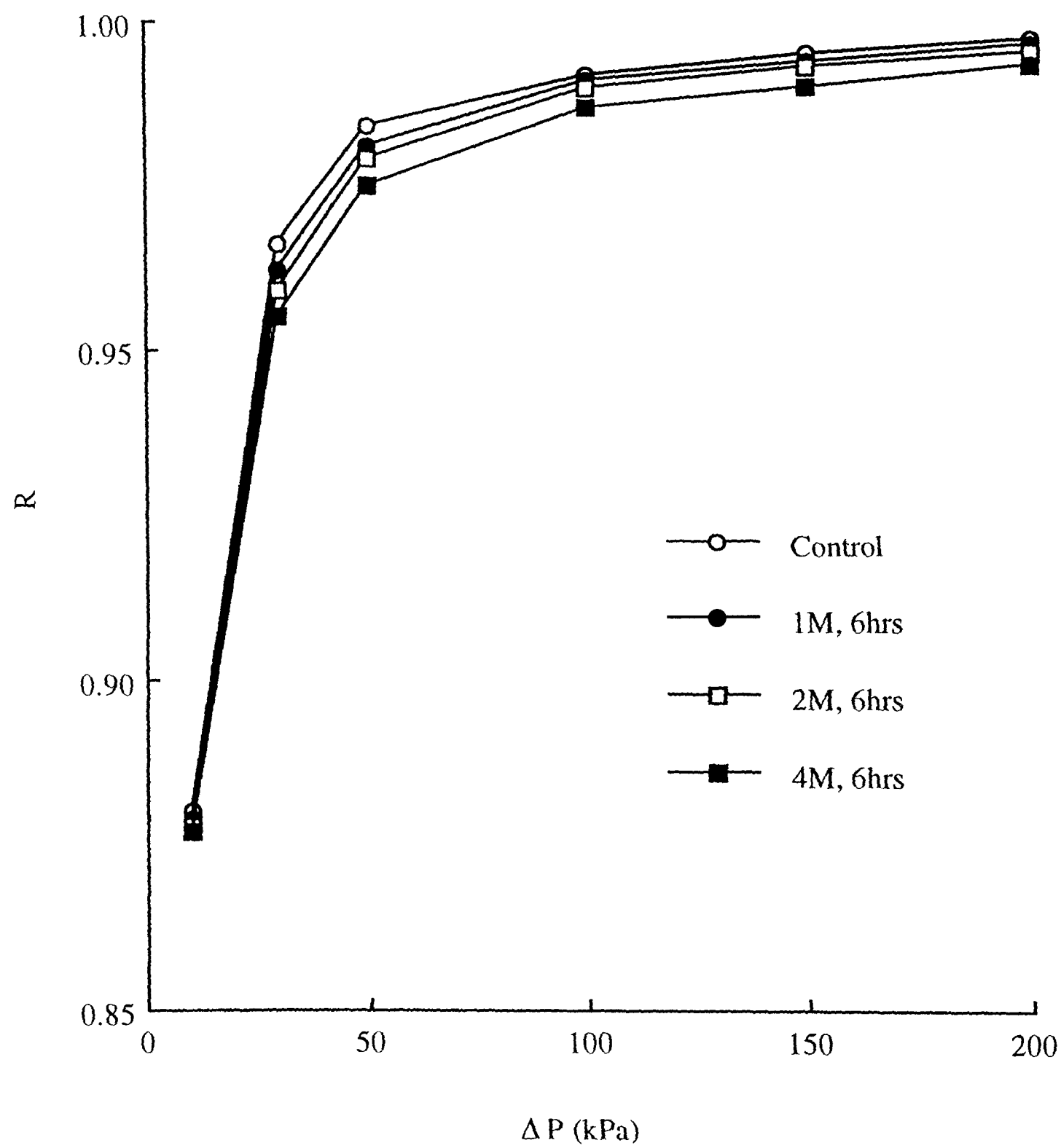
Fig. 5.12.



Legend to Fig. 5.13.

The effect of H_2O_2 concentration on serum protein rejection (R). The films were incubated with aqueous solutions of H_2O_2 for 6 hrs at 37°C . Three films were treated with each concentration of H_2O_2 indicated whilst the same number of control films were treated with TBS, pH 7.4. Pig serum (70 mg/ml) was ultrafiltered and R determined as described in text. Each value on graphs indicates a mean of 9 measurements, 3 from each of three films. Error bars indicating SDs are omitted for clarity, though they were 0.5 - 2 % of the R values.

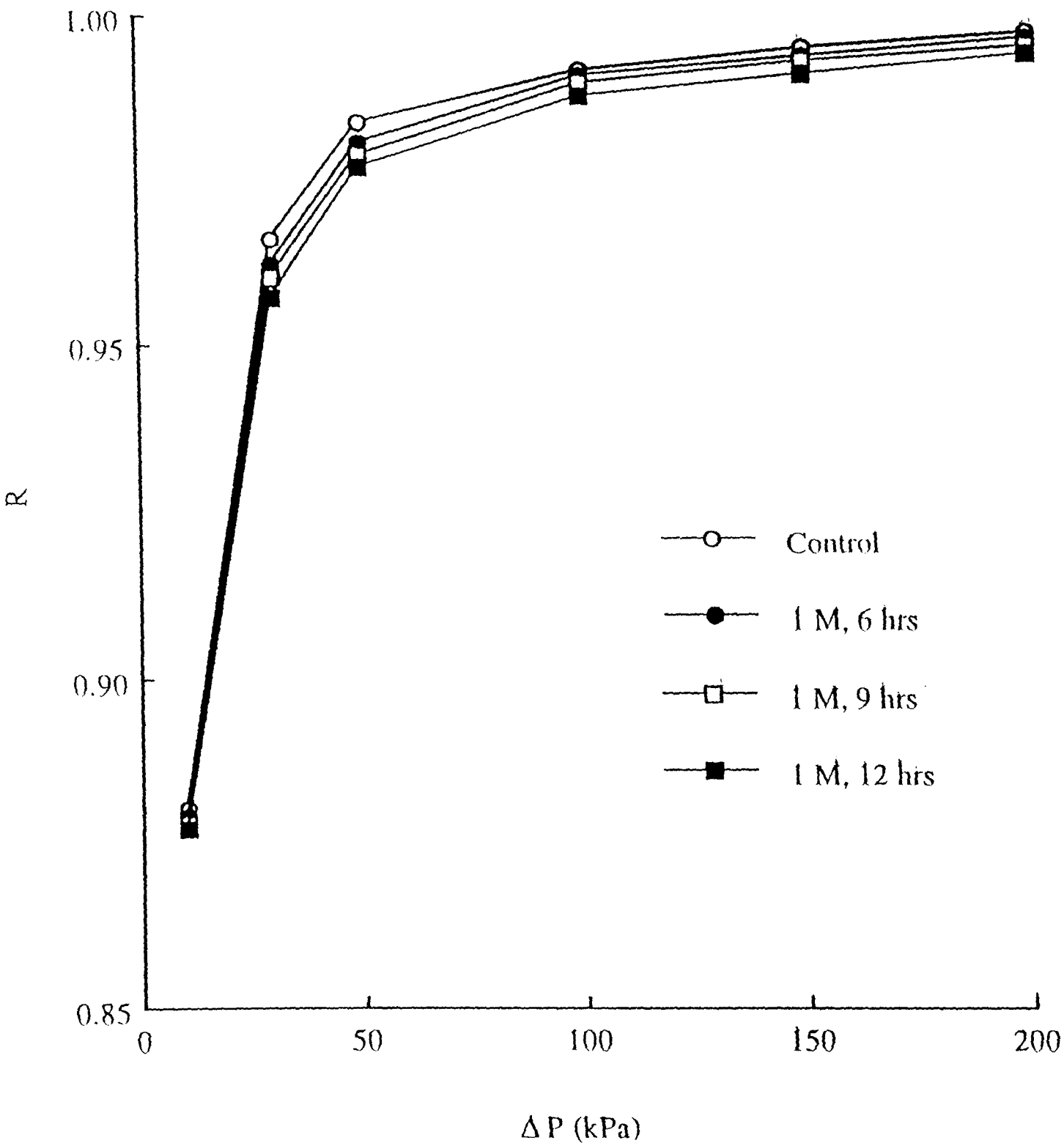
Fig. 5.13.



Legend to Fig. 5.14.

The effect of incubation time with H_2O_2 on serum protein rejection (R) following treatment with a fixed concentration of H_2O_2 (1 M). The films were incubated with an aqueous solution of H_2O_2 for up to 12 hrs at 37°C. Each set of 3 films was treated for a specific length of time indicated. Sets of 3 control films were treated likewise except with TBS, pH 7.4. Pig serum (70 mg/ml) was ultrafiltered and Js determined as described in text. Each value on graphs indicates a mean of 9 measurements, 3 from each of three films. Error bars indicating SDs are omitted for clarity, though they were 0.5 - 2 % of R values.

Fig. 5.14.



5.8. Respiratory burst analysis of activated neutrophils: concentration of H_2O_2 in the extracellular milieu and its duration

5.8.1. Introduction

Measured concentration of H_2O_2 as a function of time in the GBM subjacent to an activated neutrophil is unknown. However, it is possible to estimate this if (1) the variation of the amount of H_2O_2 generated by an activated neutrophil with time and (2) the volume of space into which the H_2O_2 is released are known. These parameters were evaluated here based on neutrophil respiratory burst data produced by Johnston and Lehmeyer (1976) and Test and Weiss (1984). Three models were examined here: activation of neutrophils (1) by immune complexes immobilized on a non-phagocytosable surface (Johnston and Lehmeyer, 1976), (2) by PMA (Test and Weiss, 1984), and (3) by opsonized zymosan particles (Test and Weiss, 1984). The first model was the closest to the situation in immune GN where GBM coated with immune complexes was thought to activate neutrophils. However, owing to a problem with its assay system, the measured extracellular H_2O_2 was higher than normal. The latter two models, though further removed from the nephritic situation, used a problem-free method of monitoring extracellular H_2O_2 and provided a logical basis for extrapolating H_2O_2 release in the first model.

5.8.2. Respiratory burst model based on activation of neutrophils by immune complexes immobilized on a non-phagocytosable surface and its shortcomings

In this model, neutrophils (1×10^6) steadily released H_2O_2 into the extracellular medium with time, giving ~ 30 nmol of H_2O_2 after 1 hr (Johnston and Lehmeyer, 1976). However, the amount of H_2O_2 measured was greater than normal owing to the fact that the normal H_2O_2 conversion to HOCl by myeloperoxidase (also released by activated neutrophils) in the extracellular fluid was inhibited by azide, a potent myeloperoxidase inhibitor. The inhibition of myeloperoxidase had a second undesirable effect. When respiratory burst is allowed to proceed normally, the HOCl

generated by myeloperoxidase would begin to inhibit plasma membrane NADPH oxidase (Jandl et al., 1978), limiting the duration of the respiratory burst. If myeloperoxidase was inhibited, no HOCl would be generated to effect the normal cessation of the respiratory burst (Sagone et al., 1977; Nauseef et al., 1983), resulting in more H_2O_2 being released into the extracellular medium.

The use of azide by Johnston and Lehmeyer (1976) was necessary for the H_2O_2 assay employed since H_2O_2 was quantitated based on a measurement of the rate of horse-radish peroxidase-catalyzed oxidation of fluorescent scopoletin by H_2O_2 to the non-fluorescent derivative (Andreae, 1955; Root et al., 1975). NaN_3 had to be included in the neutrophil incubation medium to prevent neutrophil-derived myeloperoxidase from interfering with horse-radish peroxidase-based H_2O_2 detection. Thus, the main problem with this model resided in the assay system where the normal catabolism of H_2O_2 was inhibited.

5.8.3. Respiratory burst models based on activation of neutrophils by PMA and opsonized zymosan particles

Test and Weiss (1984) side-stepped the problem by using a radically different method of assaying H_2O_2 . They used a H_2O_2 sensor (Yellow Springs Instrument Co., Yellow Springs, Ohio, USA) capable of accurately detecting temporal variations of H_2O_2 in the extracellular milieu without interfering with normal H_2O_2 catabolism. The two respiratory burst models they studied were activation of neutrophils (1) by PMA and (2) by opsonized zymosan particles. From the two models characterized, they proposed that the amount of H_2O_2 being released by activated neutrophils and its rate of conversion to HOCl depended on the relative potencies of the neutrophil activator at triggering H_2O_2 generation and myeloperoxidase release (Test and Weiss, 1984). PMA was more potent at triggering H_2O_2 generation than opsonized zymosan particles but was weaker at eliciting myeloperoxidase release. Consequently, PMA caused greater H_2O_2 release into the extracellular medium than the zymosan and the extracellular H_2O_2 took longer to decay to undetectable levels.

It is known that immune complexes immobilized on a non-phagocytosable surface are weaker at triggering H_2O_2 release than opsonized zymosan (Johnston and Lehmeyer, 1976; Test and Weiss, 1984) but are able to trigger greater myeloperoxidase release (Henson, 1971c; Estensen et al., 1974). From this, it is possible to estimate both the amount of H_2O_2 generated by a neutrophil activated by the immune complexes, relative to that from a zymosan activated neutrophil, as well as the time taken for the extracellular H_2O_2 to fall off to zero. The zymosan caused neutrophils (1.2×10^6) to generate a peak extracellular H_2O_2 concentration of $\sim 5 \mu\text{M}$ which was equivalent to $\sim 2 \times 10^{-8}$ mol (since the total incubation volume = 4 ml). The time taken for the extracellular H_2O_2 concentration to decay to zero was ~ 1 hr. This meant that non-phagocytosable immune complexes would be expected to trigger neutrophils to generate a peak extracellular $\text{H}_2\text{O}_2 < 1.7 \times 10^{-14}$ mol per neutrophil. Also, it would take < 1 hr for the extracellular H_2O_2 to fall off to undetectable levels.

The quantification of the relative potency of the immune complexes and zymosan to release H_2O_2 allows a more precise means of calculating the peak extracellular H_2O_2 of an immune complex activated neutrophil. Test and Weiss (1984) showed that zymosan-activated neutrophils were able to generate a peak extracellular H_2O_2 of $\sim 1.5 \times 10^{-13}$ mol per neutrophil after 1 hr when H_2O_2 conversion to HOCl was inhibited by azide. This amount was ~ 5 x greater than that generated by neutrophils activated by immune complexes immobilized on a non-phagocytosable surface where azide was also present ($\sim 3 \times 10^{-14}$ mol per neutrophil after 1 hr, calculated from figures cited in Section 5.8.2.). Therefore, judging by the relative ability of the two neutrophil activators to trigger H_2O_2 release, the amount of H_2O_2 released by an immune complex activated neutrophil would be ~ 5 x lower than that released by a zymosan stimulated neutrophil. If one also takes into account that an immune complex activated neutrophil is able to release more myeloperoxidase and hence can reduce the extracellular H_2O_2 more effectively, then the peak extracellular H_2O_2 of the neutrophil would be even lower than $1/5$ that of an zymosan activated neutrophil. Therefore, non-phagocytosable immune complexes would be expected to

cause neutrophils to generate a peak extracellular $\text{H}_2\text{O}_2 < 3.4 \times 10^{-15}$ mol per neutrophil.

5.8.4. Estimation of the volume of space into which neutrophil generated H_2O_2 is secreted

Since GBM is in close apposition to neutrophils in the diseased state, H_2O_2 secreted would quickly diffuse into the basement membrane structure. An infiltrating neutrophil obstructs glomerular capillary blood flow and stops glomerular ultrafiltration locally. In the absence of convective flow across the GBM, H_2O_2 is not expected to dissipate rapidly into the urinary space by convection. H_2O_2 generated on the surface of the neutrophil would, however, diffuse down a concentration gradient across the width of the basement membrane. The highest concentration would be at the surface of the GBM facing the neutrophil plasma membrane. At the GBM-podocyte interface, H_2O_2 would continue to diffuse into the urinary space through the podocyte filtration slits.

The precise H_2O_2 concentrations at the GBM-neutrophil interface during a neutrophil respiratory burst are unknown, nor is the concentration profile across the width of the GBM in the model examined here. However, it is known that the peak amount of H_2O_2 secreted by an activated neutrophil might be $< 3.4 \times 10^{-15}$ mol per neutrophil (Section 5.8.3.), achieved ~ 5 min after the start of the respiratory burst (Test and Weiss, 1984). This amount would decay to zero in < 1 hr (Section 5.8.3.) owing to conversion to HClO by neutrophil-derived myeloperoxidase (Test and Weiss, 1984). Let us consider that point in time when the extracellular H_2O_2 reaches a peak. Assuming that the peak amount of H_2O_2 is distributed evenly in that volume of GBM directly subjacent to an adherent neutrophil, it is possible to estimate the concentration of H_2O_2 in that space if the volume of GBM is known. In reality, the H_2O_2 would not be evenly distributed but would tend to be concentrated at the neutrophil face of the GBM. The H_2O_2 concentration to be estimated would represent an 'average'

concentration of an integral of concentrations within the membrane concentration gradient.

A neutrophil adherent to an immune complex-coated non-phagocytosable surface only produces oxidants at the site of attachment (Vissers et al., 1985). Electron micrographs showing neutrophil adherence to GBM in a nephritic glomerulus indicated that roughly 1/2 of the surface area of a neutrophil was in contact with GBM (Cochrane et al., 1965). Electron microscopy studies of neutrophils adherent to immune complex-bound GBM *in vitro* also indicated a similar fraction of attachment (Vissers et al., 1985). If the external surface area of a neutrophil and the thickness of the GBM are known then it would be possible to estimate the volume of GBM into which H_2O_2 is released from an activated neutrophil. One half of the surface area of a neutrophil, assumed spherical with a radius $\sim 5.5 \mu m$ (Burkitt et al., 1993), is $\sim 1.9 \times 10^{-10} m^2$. The average thickness of human GBM is $\sim 300 nm$ (Farquhar, 1991). Thus the volume of GBM subjacent to a neutrophil is $\sim 5.7 \times 10^{-14} l$.

Given that a neutrophil activated by non-phagocytosable immune complexes could only release a peak extracellular $H_2O_2 < 3.4 \times 10^{-15} mol$, the maximum average concentration of H_2O_2 in the GBM matrix would be $< 0.05 M$, assuming even distribution of H_2O_2 . Allowing for H_2O_2 diffusion away from the matrix into the urinary space, the maximum average concentration would be very much lower than $0.05 M$, perhaps in the order of a few mM. Since a membrane concentration gradient exists, the maximum H_2O_2 concentration at the neutrophil face of the membrane would be higher than the maximum average concentration. At the podocyte face of the basement membrane the maximum H_2O_2 would be lower than the maximum average concentration. The H_2O_2 concentrations would fall thereafter due to myeloperoxidase-mediated catabolism (Test and Weiss, 1984), tending to zero in $< 1 hr$.

5.9. Discussion

The minimum H_2O_2 concentration needed to render GBM more permeable to serum protein ($1 M$) was much higher than the highest 'average' H_2O_2 concentration in

GBM ($\ll 50$ mM), attained ~ 5 min after the start of the neutrophil respiratory burst. Given that a H_2O_2 concentration gradient exists across the width of the GBM, the concentration would be higher than the average at one face of the membrane and lower at the other. Whether H_2O_2 could render GBM more permeable at the high concentration end would depend on the concentration achieved and its duration. Suppose the concentration does reach 1 M, the concentration would have to be maintained for 6 hrs before that part of the membrane becomes more permeable to serum proteins. Given that this concentration starts to decrease after ~ 5 min, taking < 1 hr to fall to zero, it may be unlikely that even the neutrophil facing part of the GBM could become more permeable to serum proteins. The H_2O_2 concentration at the neutrophil face of the membrane would have to be very much higher than 1 M before it could cause that part of the basement membrane to become more permeable to serum proteins in < 1 hr. Being a small molecule, H_2O_2 would diffuse rapidly into a relatively porous basement membrane ($\sim 90\%$ water) (Robinson et al., 1987) away from the neutrophil surface and so H_2O_2 at the neutrophil face of the GBM may not be allowed to reach the kind of high concentrations needed to render the membrane more permeable to serum proteins in < 1 hr.

If physiological concentrations of H_2O_2 seems unlikely to render GBM more permeable to protein, then one has to consider other mechanisms by which H_2O_2 causes proteinuria. Yoshioka et al.'s demonstration that intrarenally infused H_2O_2 (18-100 mM) caused massive but reversible proteinuria (Yoshioka et al., 1991) proved very difficult to explain if one abandoned the idea that H_2O_2 is able to render GBM more permeable to protein. However, it is possible that H_2O_2 may have perturbed glomerular cells, causing them to release eicosanoids which subsequently altered the glomerular haemodynamics, resulting in an increased protein flux across the glomerular capillary wall. This notion is not entirely without basis since isolated glomerular cells have been shown to be capable of producing eicosanoids, e.g., thromboxanes and prostaglandins, when perturbed by H_2O_2 (Baud et al., 1981). These eicosanoids have been shown to exert important effects on glomerular haemodynamics (Schlondorff and

Ardaillou, 1986) and increased production has been demonstrated in various human and experimental glomerulonephritides (Rahman et al., 1987; Stahl et al., 1987). In particular, thromboxanes have been implicated as important mediators causing proteinuria in anti-GBM antibody disease (Lianos et al., 1983), adriamycin-induced nephrotic syndrome (Remuzzi et al., 1985) and complement-mediated glomerular injury (Cybulsky et al., 1987). The vasoconstrictive effects of thromboxanes may reduce the glomerular blood flow and consequently lower the glomerular hydrostatic pressure. The GBM may then be less compressed, increasing the matrix void space and allowing more protein to permeate across the membrane (Chapter 3), causing proteinuria. This idea is only a speculation based on circumstantial and somewhat fragmentary evidence. Clearly, more evidence is needed to substantiate or disprove this hypothesis.

The ability of neutrophil-derived H_2O_2 to cause persistent proteinuria, as demonstrated by Rehan et al., was easier to explain since there is direct evidence for the H_2O_2 reaction product HOCl to directly render GBM more permeable to serum protein (G.B. Robinson, personal communication). HOCl has also been shown to activate latent metalloproteinases released from neutrophils (Peppin and Weiss, 1986) which then digested GBM (Vissers and Winterbourn, 1986) and which may subsequently increase its permeability to serum protein. Indeed, the effect of neutrophil metalloproteinases on the permeability of GBM to protein was studied in Chapter 7. The mechanisms by which H_2O_2 may have indirectly mediated proteinuria were discussed in greater detail in Chapter 8 - the General Discussion.

CHAPTER SIX

CHAPTER 6 EXTRACTION AND PRELIMINARY

CHARACTERIZATION OF PIG NEUTROPHIL PROTEINASES

6.1. Introduction

As a prerequisite to investigating the effect of neutrophil proteinases on the permeability of GBM to protein, it is necessary to characterize the proteinases. Pig and human neutrophils appear to share similarity in their respective proteinase complements; both contain elastase and cathepsin G (Janoff and Scherer, 1968; Odeberg et al., 1973; Kraeva et al., 1988). Rabbit neutrophils, by contrast, lack cathepsin G (Brown and Robinson, 1987) and a true elastase (Cotter and Robinson, 1980a) but possess an 'elastase-like' enzyme capable of hydrolysing NBA but not elastin. The main interest of this chapter lies in determining the types and amounts of proteinases present in the pig neutrophil, and in assessing their ability to degrade pig GBM. The question of whether these proteinases, either individually or in concert, are able to render the GBM more permeable to protein will be addressed in the next chapter. Pigs are used as a disease model for studying the pathogenic mechanisms of glomerulonephritis because their neutrophil proteinases and GBM are readily available in quantities needed for these studies. In addition, the similarity shared by pig and human neutrophil proteinase complements makes pigs a useful disease model for human GN.

Serine proteinases from human neutrophils have been shown to degrade human GBM *in vitro* (Davies et al., 1978; Sanders et al., 1978; Vissers et al., 1984). Stimulated human neutrophils have also been shown significantly to degrade human GBM *in vitro* and the degradation was markedly reduced by EDTA, suggesting that a metalloproteinase(s) was responsible for the degradation (Shah et al., 1987). Both the serine proteinases and metalloproteinase(s) function at physiological pH but the latter requires activation by any one of the following: 4-aminophenyl mercuric acetate (4-APMA), trypsin or hypochlorous acid (HOCl). Ca^{2+} is also needed as a cofactor (Williams et al., 1975; Murphy et al., 1980) before metalloproteinase activities are fully manifest.

4-APMA is a thiol-blocking agent that has been commonly used to activate the latent neutrophil metalloproteinases collagenase and gelatinase (Murphy et al., 1980; Shah et al., 1987) as well as tissue metalloproteinases, e.g., fibroblast collagenase and stromelysin (Chin et al., 1985). 4-APMA belongs to a group of organomercurial compounds, including *p*-chloromercurial benzoate, which have been shown to activate human neutrophil collagenase (Robertson et al., 1972; Macartney and Tschesche, 1980; 1983a; 1983b) and gelatinase (Fedrowitz et al., 1983) via a thiol-disulphide exchange mechanism. The mechanism essentially involves the removal of a peptide linked to the catalytic site of metalloproteinases via a disulphide bond thereby activating the proteinases.

Trypsin is capable of activating neutrophil metalloproteinases (Murphy et al., 1980) via the same mechanism (Macartney and Tschesche, 1980; 1983a; 1983b). The surface accessible disulphide linkage in trypsin is thought to participate in the mechanism, releasing the single peptide from the metalloproteinase. Trypsin does not activate metalloproteinases by proteolysis since N α -*p*-tosyl-L-lysine chloromethyl ketone (TLCK)-inhibited trypsin still effected activation (Macartney and Tschesche, 1980; 1983a).

HOCl, a reactive oxygen species produced *in vivo* by the neutrophil during the respiratory burst (Johnson et al., 1987b; 1988b), has been shown to activate metalloproteinases (Peppin and Weiss, 1986; Shah et al., 1987). However, the mechanism of activation is, as yet, poorly understood (Weiss and Peppin, 1986).

The human neutrophil serine proteinases include elastase (MW 30 k) and cathepsin G (MW 30 k), both derived from the azurophil (primary) granules of the neutrophil (Baggiolini et al., 1978). Metalloproteinases from human neutrophils include collagenase (MW 61 k) and gelatinase (MW 150 k). The collagenase is localized in the specific (secondary) granules (Murphy et al., 1977) whilst the gelatinase is in tertiary granules or C-particles (Murphy et al., 1980).

Pig neutrophil proteinases have also been characterized, though not as completely as the human neutrophil proteinases. Pig neutrophil serine proteinases have

been purified and include elastase and cathepsin G, MWs 30-31 k and 28-29 k, respectively (Kraeva et al., 1988). Geiger et al. (1985) has purified an elastase from pig neutrophils (MW 27k). A gelatinase from pig neutrophils has been characterised (Murphy et al., 1989), though no collagenase from pig neutrophils has been reported.

6.2. Isolation of pig peripheral neutrophils

Neutrophils were isolated from pig's blood using a slight modification of the method of Eggleton et al. (1989) as detailed in Section 2.2.4. This economical and rapid method was used because it does not require the use of dextran or density gradient polymers which have been shown to impair neutrophil functions (Jarstrand et al, 1979; Haslett et al., 1985). The method is briefly outlined below. Following erythrocyte lysis in isotonic NH_4Cl , the leukocytes were sedimented by centrifugation and purified by differential centrifugation in Hank's balanced salt solution. The only modification of the original protocol was that 4 litres of pig's blood per preparation was processed instead of 4 ml of human blood. Such large volumes of blood were used in order to obtain neutrophils in numbers sufficient for proteinase extraction. In a series of eight preparations, the yield, expressed as mean $\pm$ SD, was $4.55 \pm 0.90 \times 10^9$ neutrophils per litre of pig's blood. The number of neutrophils per unit volume was determined in a haemocytometer. The yield, as a percentage of total neutrophils, was $65 \pm 9.5 \%$ (Table 6.1.) - a figure similar to the yield of human peripheral neutrophils (Eggleton et al., 1989): $63 \pm 9.9 \%$. The purity of the yield was $85 \pm 8 \%$ (Table 6.1.) as assessed by Hema-Gurr differential staining Section 2.3.4. which was, again, comparable to that by Eggleton et al. (1989): $83 \pm 8.8 \%$. It is of interest to note that the purity of the neutrophils obtained by this method was not greatly different from those obtained by the dextran and dextran/Lymphoprep techniques (78 % - 91 %) and so further justified its use. The highest purity achieved was 95 %. Only preparations whose purity exceeded 90 % were used for subsequent neutrophil granule extraction, though this meant discarding half the preparations.

Table 6.1. Yields and purities of pig neutrophils isolated by differential centrifugation in Hank's balanced salt solution

Batch	Neutrophil yield		Neutrophil purity (%)
	number/l (10 ⁹)	(%)	
1	5.49	67	84
2	4.50	55	95
3	3.64	53	76
4	3.46	74	86
5	5.61	77	78
6	4.60	63	91
7	5.65	76	94
8	3.45	54	76
Mean ± SD	4.55 ± 0.90	65 ± 9.5	85 ± 7.3

6.3. Isolation of neutrophil granules

Neutrophil granules were isolated by a modification of the method of Cohn and Hirsch (1960) as detailed in Section 2.2.5. The protocol involved neutrophil lysis by sonication in sucrose solution, removal of nuclei by low speed centrifugation, precipitation of granules by high speed centrifugation, washing and resuspension of the granules in Tris/HCl buffer, pH 7.4. The protocol differed from the original in two respects. Firstly, the neutrophils were lysed by sonication instead of by repeated syringing through a fine needle aperture of $\sim 160 \mu\text{m}$. Secondly, the harvested granules were resuspended in Tris/HCl buffer, pH 7.4 instead of in sodium phosphate buffer.

The reasons for the modifications were as follows. Sonication proved much more effective in cell breakage than syringing through a fine aperture as only 5-7 min. of sonication (MSE transducer, 1 cm probe, peak to peak amplitude: $18 \mu\text{m}$) was needed to effect total cell lysis but repeated syringing took five times longer only to achieve $\sim 95\%$ cell disintegration. To ascertain the relative purity of the granules obtained by the sonication protocol, granules from the two protocols were analysed for contaminants such as nucleic acids, mitochondria, and endoplasmic reticulum. The granule markers used were myeloperoxidase and alkaline phosphatase for the primary and secondary granules, respectively. Nucleic acids were assayed by A_{260} measurements. Endoplasmic reticulum and mitochondrial markers used were NADPH cytochrome C reductase (Sottocasa et al., 1967) and succinate dehydrogenase (Pennington, 1961), respectively. The enzyme and nucleic acid assays are detailed in the Sections 2.3.6. - 2.3.10. Assays were performed in triplicates for each of three different batches of neutrophils ($n = 9$). Table 6.2. gives a summary of the analysis. It appeared that sonication yielded somewhat purer granules than the syringing protocol as shown by higher specific activities of the primary granule marker myeloperoxidase and the secondary granule marker alkaline phosphatase. In addition, sonication gave rise to granules with consistently lower nucleic acid contamination than did repeated syringing. Granules from neither protocol had detectable mitochondrial (succinate

Legends to Table 6.2.

The figures in the table were based on three different batches of 1×10^9 pig peripheral neutrophils. For each batch, individual measurements were made in triplicates. Figures were given as means $\pm$ SD (n = 9). The units of enzyme activities were as defined in Section 2.3.6.-2.3.10. Protein estimation was based on BCA microprotein assays (Section 2.3.5.). Son. = Sonication; Syr. = Syringing.

Table 6.2. A comparison of granule purity between two methods of cell lysis

<u>Part 1</u>									
Fraction	Protein (mg)	Nucleic acids (mg)		Myeloperoxidase		Total activity (units)		Specific activity (units/mg)	
		Son.	Syr.	Son.	Syr.	Son.	Syr.	Son.	Syr.
Sucrose lysate	259 ± 36	262 ± 34	581 ± 75	586 ± 64	170 ± 12	174 ± 10	0.66 ± 0.05	0.66 ± 0.04	
Nuclear pellet	20.5 ± 2	53.7 ± 6	122 ± 12	168 ± 15	15 ± 1	45 ± 2	0.73 ± 0.05	0.84 ± 0.04	
Post-granule supernatant	187 ± 17	141 ± 11	453 ± 54	399 ± 32	25 ± 2	8.6 ± 0.8	0.13 ± 0.01	0.06 ± 0.01	
Granules	49 ± 4	63 ± 5	0.9 ± 0.09	12.2 ± 1.0	128 ± 16	115 ± 10	2.61 ± 0.33	1.83 ± 0.16	
Percentage recovery	99%	99%	99%	99%	98%	97%	——	——	

Part 2

Fraction	Alkaline phosphatase				NADPH Cytochrome C reductase (E.R. marker)			
	Total activity x 1,000 (units)		Specific activity x 1,000 (units/mg)		Total activity x 1,000 (units)		Specific activity x 1,000 (units/mg)	
	Son.	Syr.	Son.	Syr.	Son.	Syr.	Son.	Syr.
Sucrose lysate	4850 ± 388	4960 ± 397	18.7 ± 1.5	18.9 ± 1.5	1096 ± 110	1101 ± 140	4.23 ± 0.42	4.20 ± 0.53
Nuclear pellet	34 ± 2	13 ± 1	1.7 ± 0.1	0.2 ± 0.02	0	0	0	0
Post-granule supernatant	873 ± 87	299 ± 30	4.7 ± 0.5	2.1 ± 0.2	1046 ± 100	1056 ± 120	5.59 ± 0.53	7.49 ± 0.85
Granules	3880 ± 271	3470 ± 208	79.2 ± 5.5	55.1 ± 3.3	0	0	0	0
Percentage recovery	99%	98%	—	—	95%	96%	—	—

Part 3

Fraction	Succinate DH (Mitochondrial marker)			
	Total activity (units)		Specific activity x 1,000 (units/mg)	
	Son.	Syr.	Son.	Syr.
Sucrose lysate	12.29 ± 1.23	12.31 ± 1.35	47.5 ± 4.7	47.0 ± 5.2
Nuclear pellet	0	0	0	0
Post-granule supernatant	11.55 ± 0.92	11.69 ± 0.82	61.5 ± 4.9	82.9 ± 5.8
Granules	0	0	0	0
Percentage recovery	94%	95%	—	—

dehydrogenase) or endoplasmic reticulum (NADPH cytochrome C reductase) contamination.

Sonication was probably more effective at disintegrating nuclei (containing high nucleic acid and protein content) than syringing and so released more nucleic acids and proteins into the cytosol. This would account for the relatively high nucleic acid and protein contents in the post-granule supernatant fraction derived from sonicated cell lysate. Syringing, by contrast, probably gave rise to incompletely disintegrated nuclei, some of which might have co-precipitated with the granules. This would explain the relatively high nucleic acid contaminants present in the granule fraction as compared with those obtained using sonication.

Phosphate was excluded from the preparative buffer in the modified protocol because phosphate precipitated with Ca^{2+} (a cofactor for metalloproteinase activities) in metalloproteinase assay reaction mixtures. This caused co-precipitation of GBM fragments in the reaction buffer which interfered with measurements of enzyme activities. In summary, the improved protocol gave purer granules and eliminated an assay problem pertaining to the presence of phosphate.

6.4. Granule proteinase extraction

The granules were lysed by repeated freezing and thawing in an acetone/dry ice mixture. The lysate was centrifuged at 30,000 x g for 20 min and the supernatant was set aside for proteinase analysis. Membrane-bound proteinases were extracted from the pellet using an extraction optimized for salt and detergent concentrations. High salt and traces of detergent have been shown to facilitate proteinase extraction from rabbit neutrophil granule membranes (Cotter and Robinson, 1980a & b; Brown and Robinson, 1987). High ionic strength salt solution was used to release enzymes which were principally attached to the membrane by ionic interactions (Massoulié and Rieger, 1969) whereas detergents were required to release enzymes associated with membrane lipids (Panfili et al., 1971).

The proteinase extraction from the pellet was effected by resuspending the pellet in ice cold 10 mM Tris/HCl buffer solutions, pH 7.4 containing 0-2.0 M NaCl with a syringe until a smooth suspension was formed. The suspension was forced repeatedly through a needle aperture for a further 10 min. The extracted granule membranes were sedimented by centrifugation at 30,000 x g for 20 min and the pellet extracted twice more. The supernatants were combined to give the membrane extract and stored at -20°C for proteinase assays.

Elastase was used as a proteinase marker since it permitted rapid and sensitive colorimetric assays based on hydrolysis of its chromogenic substrates. This avoided cumbersome assays involving GBM-degradation followed by hydroxyproline analysis of the digests (Cotter, 1979). BOC-APN-SBzl(Cl) was found to be the most sensitive of the chromogenic substrates used since it gave rise to the highest rate of change of optical density with time (Table 6.3.). The assays were carried out as detailed in Section 2.3.11.

1M NaCl appeared to give maximum elastase release (Fig. 6.1.) while the concentration of Triton X-100 needed for optimal extraction was found to be 0.5 %; further increase in detergent concentration did not improve extraction efficiency (Fig. 6.2.).

6.5. Characterization of granule proteinases

6.5.1. Serine proteinase analysis: synthetic substrate specificity, inhibitor profiles, and distribution in the granules

All granule subfractions (from three separate batches of neutrophils) were analyzed for the presence of the two known neutrophil serine proteinases elastase and cathepsin G. Whether the granule proteinases exhibited any activity against synthetic and natural substrates of elastase was ascertained in the first instance. Synthetic chromogenic substrates of elastase used included BOC-APN-SBzl(Cl), SAAPV, AAPAbu, AAPA, GPV, NBA, and SA₃. The natural substrate of elastase used was

Table 6.3. Relative sensitivities of synthetic substrate-based elastase assays

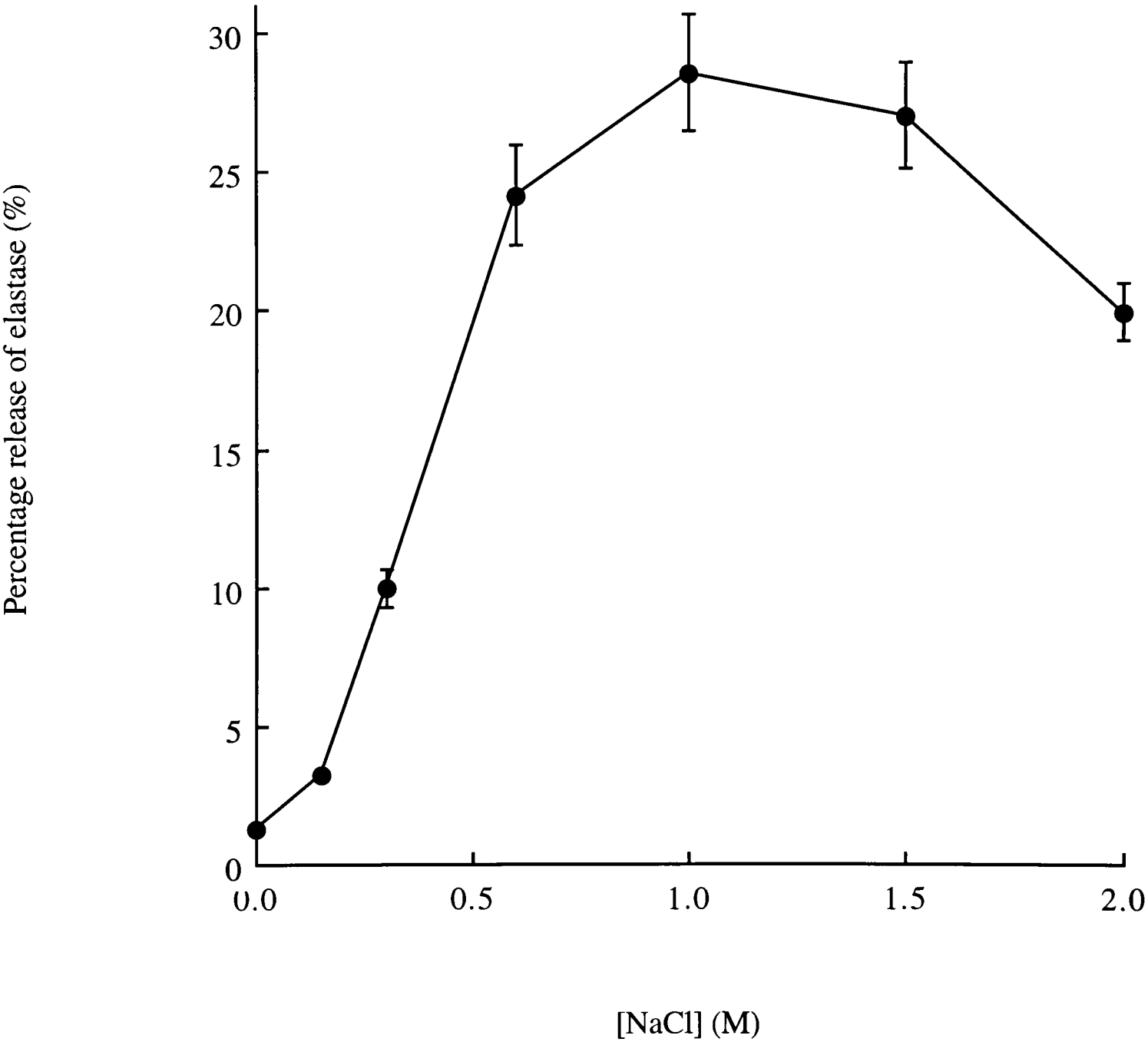
Synthetic substrate	$\Delta OD/t \times 10^5 \text{ (min}^{-1}\text{)}$
BOC-APN-SBzl(Cl)	5453 $\pm$ 399
SAAPV	119.7 $\pm$ 6.7
SAAPAu	59.2 $\pm$ 6.7
AAPA	30.6 $\pm$ 2.7
GPV	14.0 $\pm$ 1.3
NBA	12.0 $\pm$ 4.0
SA ₃	4.0 $\pm$ 0.1

The above results were based on assays carried out on triplicate samples of 10 μ l of membrane extract ($\sim 7 \mu$ g protein) from 3 different batches of granules. The only exception was the assay based on NBA where 33 μ l aliquots of enzyme were being used. Each batch of granules were derived from 1×10^9 pig peripheral neutrophils. All figures above were expressed as mean $\pm$ SD. $\Delta OD/t$ = rate of change of optical density with respect to time. The relative magnitude of assay sensitivities was the same when elastase from the 30,000 x g supernatant were assayed.

Legend to Fig. 6.1.

The effect of NaCl concentration on extraction of elastase from granule membranes. Percentage elastase release is defined as the elastase activity in membrane extract expressed as a percentage of elastase activity in the granule lysate from which the membrane extract was derived. The results indicate means $\pm$ SD and were based on triplicate measurements of each of three different samples of neutrophils processed to give the membrane extract and granule lysate. The granule lysate has 0.733 ± 0.063 units of BOC-APN-SBzl(Cl) thioesterase activity per mg protein.

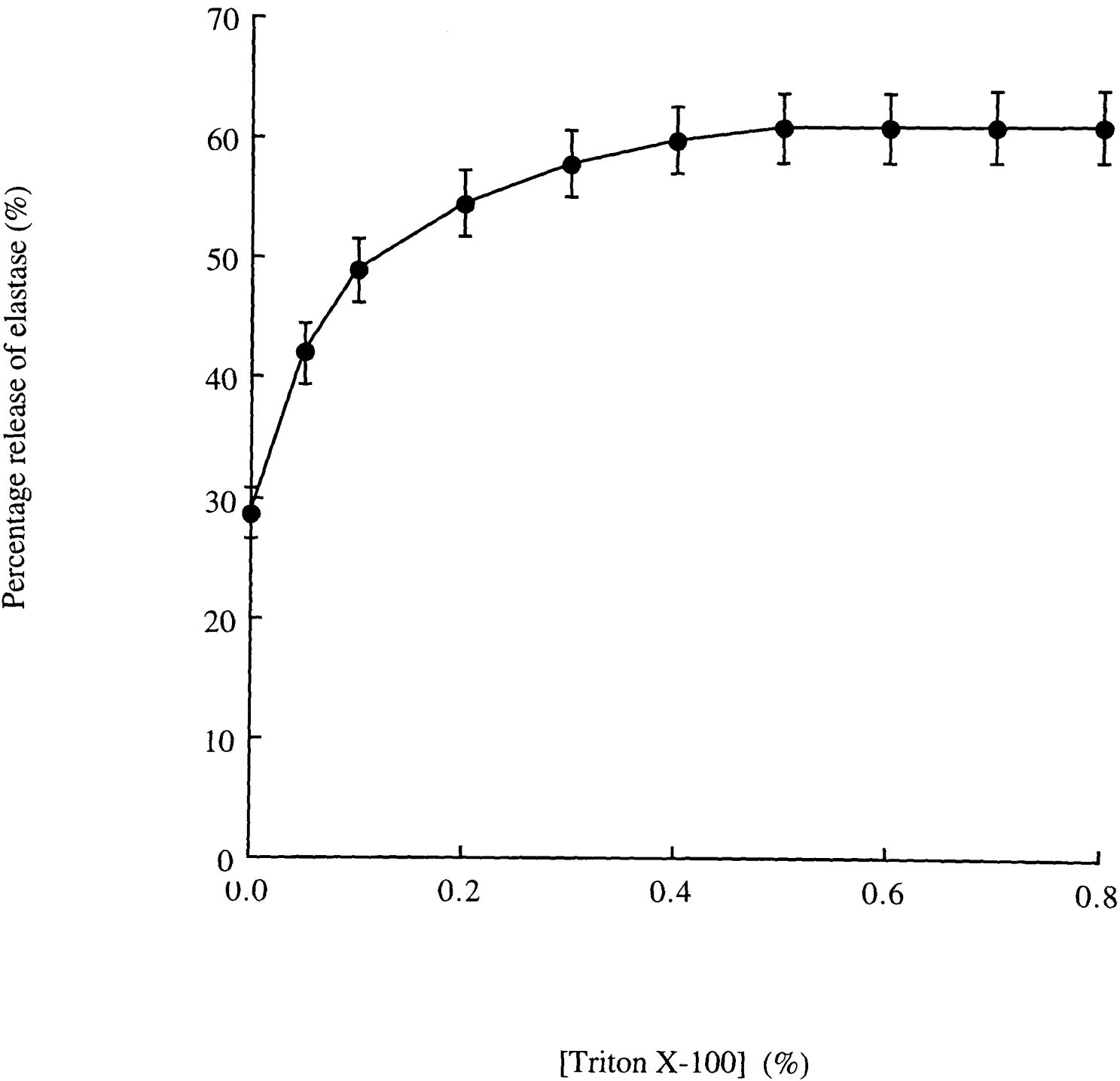
Fig. 6.1.



Legend to Fig. 6.2.

The effect of Triton X-100 concentration on extraction of elastase from granule membranes. The extraction buffer contained 1M NaCl previously shown to facilitate elastase release (Fig. 6.1.). Percentage elastase release is defined as the elastase activity in membrane extract expressed as a percentage of elastase activity in the granule lysate from which the membrane extract was derived. The results indicate means $\pm$ SD and were based on triplicate measurements of each of three different samples of neutrophils processed to give the membrane extract and granule lysate. The granule lysate has 0.733 ± 0.063 units of BOC-APN-SBzl(Cl) thioesterase activity per mg protein.

Fig. 6.2.



elastin impregnated with orcein. The protocols for measurements of activities against the above substrates are detailed in Sections 2.3.11. and 2.3.12.

The granule subfractions showed activities against the synthetic substrates of elastase (Table 6.3.) as well as elastin (Table 6.4.), indicating the presence of elastase. It is of interest to note that the percentage recovery of elastase was greater than 100 %. This illustrated the insoluble nature of membrane bound elastase and the need to solubilize it in a high salt and detergent containing extraction buffer before its activity was fully manifest. Thus, the total granule elastase activity ought to be taken as the sum of elastase activities from all granule fractions after complete proteinase extraction and not as that from the granule lysate. The distribution of elastase in the 30,000 x g supernatant and membrane bound (membrane extract + membrane residue) fractions was then found to be 56 % and 44 %, respectively.

The effect of the specific inhibitor of human neutrophil elastase SAAPV chloromethyl ketone on the activities of the granule subfractions against elastin and synthetic substrates of elastase was investigated. Aliquots of known amounts of enzymes from the 30,000 x g supernatant (with $1.40 \pm 0.13 \times 10^{-2}$ units BOC-APN-SBzl(Cl) thioesterase activity) and membrane extract (with $9.0 \pm 0.7 \times 10^{-3}$ units BOC-APN-SBzl(Cl) thioesterase activity) fractions were pre-incubated with SAAPV chloromethyl ketone for 5 min. at 37°C. The inhibitor was previously dissolved in dimethyl sulphoxide and the final inhibitor concentrations in the assay mixture were 1 and 10 mM. Positive controls using only enzymes were carried out as were negative controls where the enzymes were omitted. It was found that the activities of all granule subfractions against elastin and indeed against the most sensitive synthetic substrates of elastase BOC-APN-SBzl(Cl) were completely abolished at both concentrations of the inhibitor used. This provided further confirmation of the presence of elastase in pig neutrophil granules.

The next objective was to ascertain if cathepsin G activity could be detected in the granule proteinase extract. This was achieved by determining if granule proteinases showed activity against the synthetic substrate of cathepsin G SAAPP (Nakijima and

Table 6.4. Distribution of elastase in pig peripheral neutrophil granules

Fraction	Protein (mg)	Elastase			
		Elastinolytic activity		BOC-APN-SBzl (Cl) thioesterase activity	
		Total activity (units)	Specific activity (units/mg)	Total activity (units)	Specific activity (units/mg)
Granule lysate	49 ± 4.1 (100%)	1788 ± 160 (100%)	36.5 ± 3.3	35.9 ± 3.1 (100%)	0.733 ± 0.063
30,000 x g supernatant	24.5 ± 2.1 (50.0%)	1627 ± 150 (91.0%)	66.4 ± 6.1	32.3 ± 2.9 (90.0%)	1.318 ± 0.118
Membrane extract	17.6 ± 1.5 (35.9%)	1135 ± 100 (63.5%)	64.5 ± 5.7	21.9 ± 1.8 (61.0%)	1.244 ± 0.102
Membrane Residue	6.7 ± 0.6 (13.6%)	157 ± 15 (8.8%)	23.4 ± 2.2	3.0 ± 0.2 (8.4%)	0.448 ± 0.030
Percentage recovery	99.5%	163.3%	—	159.4%	—

The figures in the table were based on granules harvested from three different batches of 1 x 10⁹ pig peripheral neutrophils. For each batch, individual measurements were made in triplicates. Figures are given as means ± SD (n = 9). The units of enzyme activities are defined in Chapter 2. Protein estimation was based on BCA microprotein assays and elastinolytic activity was measured by determining the amount of soluble dye released from elastin impregnated with orcein (Chapter 2). The figures in parentheses are the total activities of each fraction expressed as a percentage of the granule lysate activity.

Powers, 1979). The assays were carried out according to the method of Nakijima and Powers (1979) by following the hydrolysis of SAAPP (Section 2.3.13.). All granule subfractions showed activities against SAAPP (Table 6.5.), indicating the presence of cathepsin G in pig neutrophil granules. The percentage recovery of cathepsin G was, like elastase, greater than 100 %, indicating latency of the activity in the insoluble membrane bound form. The distribution of cathepsin G in 30,000 x g supernatant and membrane bound fractions was 59.1 % and 40.9 %, respectively; the total granule cathepsin G activity being taken as the sum of activities in all granule subfractions.

The activities against SAAPP were completely inhibited by a specific inhibitor of human neutrophil cathepsin G, Z-Gly-Leu-Phe chloromethyl ketone (Powers et al., 1985) at 1 mM and 10 mM final concentrations. The use of an alternative inhibitor of cathepsin G chymostatin (Feinstein et al., 1976) at the same final concentrations also gave complete inhibition of the activities. The enzymes were pre-incubated with each inhibitor, previously dissolved in dimethyl sulphoxide, for 5 min. at 4°C, as per normal. These observations further supported the presence of cathepsin G in pig neutrophil granules.

Exhibiting activities against synthetic substrates of elastase and cathepsin G does not necessarily mean that the pig serine proteinases would degrade pig GBM. In order to demonstrate activities against GBM, a GBM proteolysis assay was developed.

6.5.2. Development of a quantitative GBM proteolysis assay

Cotter (1979) measured GBM degrading activities by following hydroxyproline release from the collagenous proteins in GBM. The drawbacks with this method were two-fold. It was time-consuming since degraded GBM had to be subject to 48 hr acid hydrolysis followed by a hydroxyproline analysis. Also the assay did not measure degradation of GBM proteins in general but only of the collagenous proteins. Brown (1984) measured GBM degrading activities by monitoring release of ³H-peptides from a solid layer of ³H-GBM in a microtitre well. This method was less time-consuming and was not restricted to measuring collagen degradation, but also did not give linear

Table 6.5. Distribution of cathepsin G activities in the granules of pig peripheral neutrophils

Fraction	Protein (mg)	Cathepsin G	
		Total activity (units)	Specific activity x 1,000 (units/mg)
Granule lysate	49 ± 4.1 (100%)	0.199 ± 0.018 (100%)	4.1 ± 0.4
30,000 x g supernatant	24.5 ± 2.1 (50.0%)	0.189 ± 0.019 (95.0%)	7.7 ± 0.8
Membrane extract	17.6 ± 1.5 (35.9%)	0.080 ± 0.006 (40.2%)	4.5 ± 0.3
Membrane Residue	6.7 ± 0.6 (13.6%)	0.051 ± 0.002 (25.6%)	7.6 ± 0.3
Percentage recovery	99%	160.8%	—

The figures in the table were based on granules harvested from three different batches of 1 x 10⁹ pig peripheral neutrophils. For each batch, individual measurements were made in triplicates. Figures are given as means ± SD (n = 9). The units of enzyme activities are defined in Chapter 2. Protein estimation was based on BCA microprotein assays (Chapter 2). The figures in parentheses are the total activities of each fraction expressed as a percentage of the granule lysate activity.

release of ^3H -labels over time and thus was not quantitative. The lack of linearity in product release with time was due to the substrate not being in solution. Furthermore, since the amount of ^3H -GBM in the microtitre plate varied greatly from well to well, there tended to be variations in the release of ^3H -labels over time and thus was not an accurate assay.

To achieve a quantitative assay, a suspension of ^3H -GBM was incubated with proteinases with shaking at 37°C under substrate excess conditions. To establish substrate excess conditions samples of membrane extract (100 μl) with activity equivalent to 0.05 units of BOC-APN-SBzl(Cl) thioesterase was mixed with a range of 0.2 - 1.0 mg/ml ^3H -GBM in TBS (0.01 M Tris/HCl, 0.15 M NaCl, 0.02 % NaN_3), pH 7.4 in a final volume of 0.5 ml and incubated at 37°C with thorough mixing by tumbling. The concentration of ^3H -GBM at and above which linear proteolysis was achieved was thereby determined.

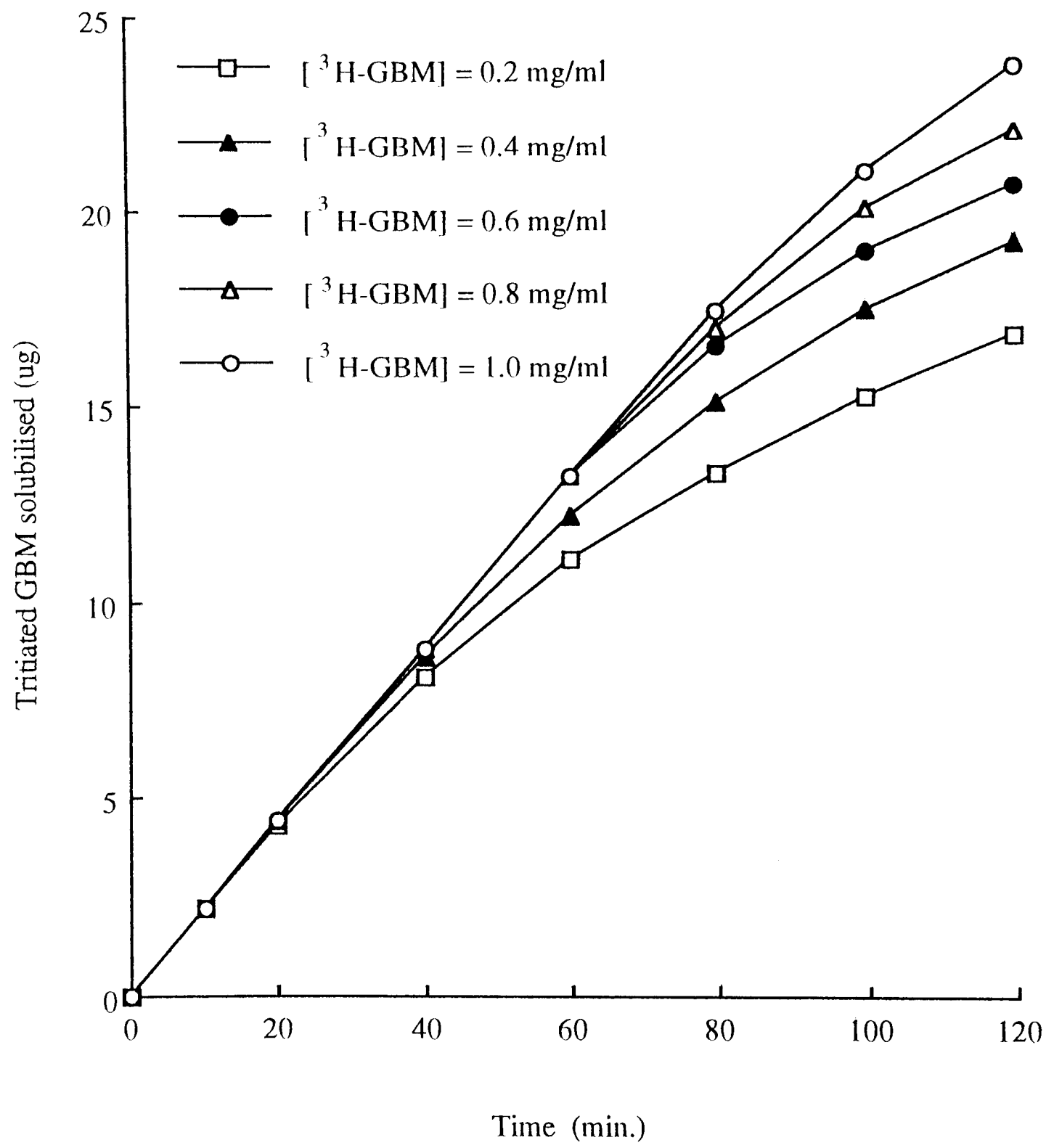
^3H -GBM was prepared by labelling GBM proteins with succinimidyl [2,3- ^3H] propionate (Bolton and Hunter, 1973) (Section 2.2.3.). The proteolysis was stopped by sedimenting the GBM by centrifugation at timed intervals for periods up to 2 hrs. The supernatants and pellets were analyzed for radioactivity by scintillation counting (Section 2.3.14.). Controls where enzymes were omitted were carried out in order to correct for any background release of labels. It was found that a ^3H -GBM concentration of ≥ 0.6 mg/ml was needed to effect linear release of ^3H -peptides with time and linearity was maintained for up to 1 hr (Fig. 6.3.). The initial rate of GBM solubilization by the membrane extract sample containing 0.05 units of BOC-APN-SBzl(Cl) thioesterase activity was 0.22 $\mu\text{g}/\text{min}$, estimated from the release of radioactivity from a known weight of ^3H -GBM, assuming uniform labelling of GBM.

When 30,000 x g supernatant was used as the source of enzyme (0.05 units of BOC-APN-SBzl(Cl) thioesterase activity, diluted to 100 μl with TBS, pH 7.4), it was found that a ^3H -GBM concentration of ≥ 0.6 mg/ml was required to achieve linearity (Fig. 6.4.) - the exact condition needed when membrane extract samples were used. The only difference between the two sets of results was that 30,000 x g supernatant

Legend to Fig. 6.3.

The effect of ^3H -GBM concentration on the rate of release of ^3H -label from ^3H -GBM suspension resulting from proteolysis of ^3H -GBM by membrane extract proteinases (0.05 units of BOC-APN-SBzl(Cl) thioesterase activity in 100 μl). The results indicate the mean and were based on triplicate assays of proteinases from each of three different batches of neutrophils processed to give granule membrane extract samples. The results shown had been corrected for background release of ^3H -labels. Bars representing SD ($n = 9$) were omitted for clarity but they varied from 3 - 7 % about the mean.

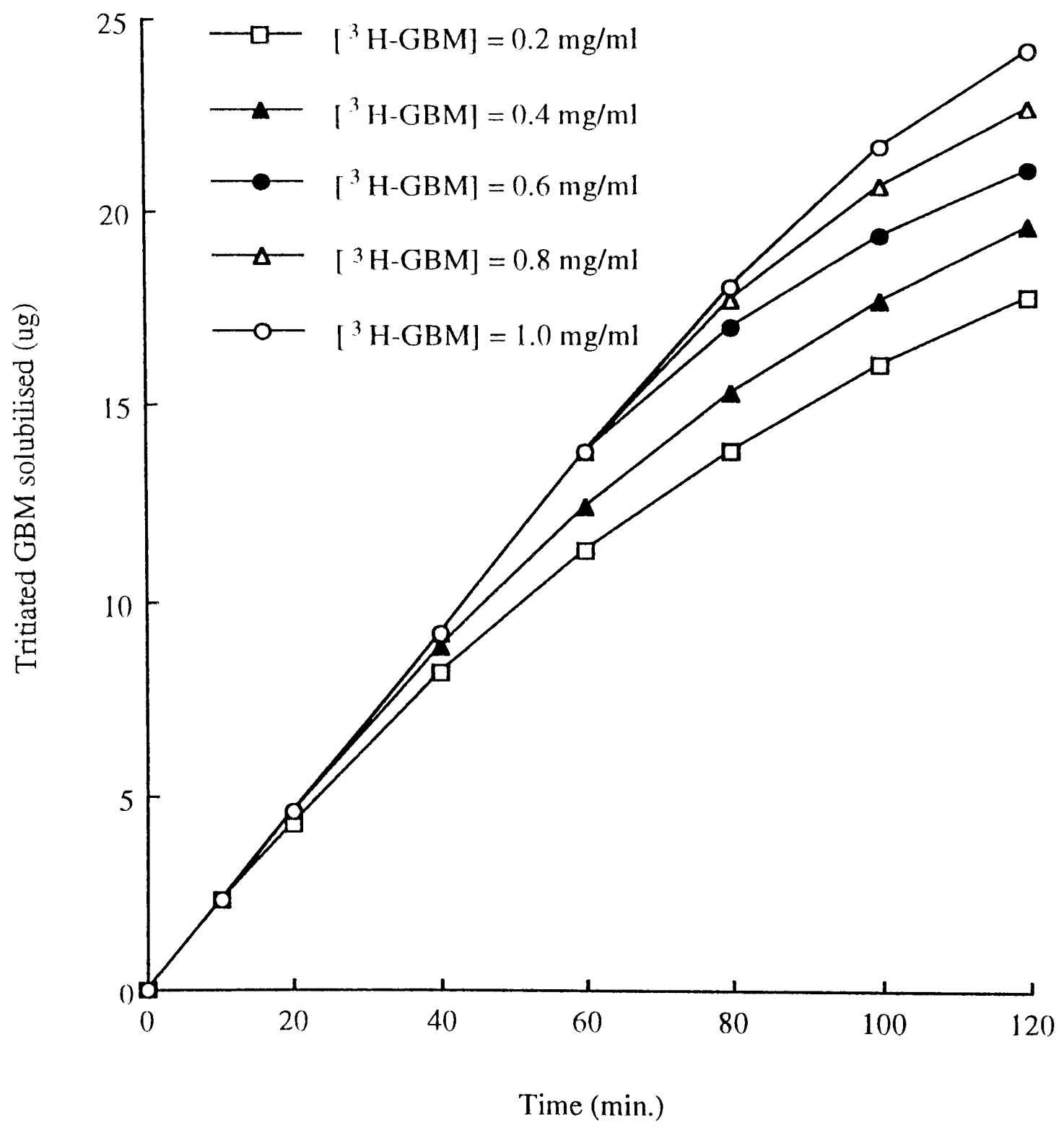
Fig. 6.3.



Legend to Fig. 6.4.

The effect of ^3H -GBM concentration on the rate of release of ^3H -label from ^3H -GBM suspension resulting from proteolysis of ^3H -GBM by 30,000 x g supernatant proteinases (0.05 units of BOC-APN-SBzl(Cl) thioesterase activity in 100 μl). The results indicate the mean and were based on triplicate assays of proteinases from each of three different batches of neutrophils processed to give 30,000 x g supernatant samples. The results shown had been corrected for background release of ^3H -labels. Bars representing SD ($n = 9$) were omitted for clarity but they varied from 3 - 7 % about the mean.

Fig. 6.4.



gave a fractionally higher initial rate of ^3H -label release than membrane extract samples : 0.23 $\mu\text{g}/\text{min}$.

Routinely, an amount of enzyme containing 0.05 units of BOC-APN-SBzl(Cl) thioesterase was incubated at 37°C with 0.6 mg/ml ^3H -GBM in a final volume of 0.5 ml TBS, pH 7.4 with the reaction stopped at lengthening intervals of up to 50 mins. One unit of ^3H -GBM degrading activity is defined as the amount of enzyme needed to solubilize 1 μg of ^3H -GBM per min at pH 7.4, 37°C . The enzyme activity was found to be proportional to the amount of enzyme used under the assay conditions (Figs. 6.5. & 6.6.).

6.5.3. Serine proteinase analysis: GBM degrading activity

Having established a quantitative GBM proteolysis assay, this was then used to quantify GBM degrading activities in granule subfractions. All subfractions appeared to contain GBM degrading activities (Table 6.6.). The percentage recovery of GBM degrading activities was greater than 100 % - an indication of the latency of proteinase activities owing to their insolubility in the membrane bound form. The distribution of GBM degrading activities in the 30,000 x g supernatant and membrane bound fractions were 60.0 % and 40.0 % respectively, the total GBM degrading activity was taken as the sum of activities in all subfractions. These figures were not dissimilar to the distribution of elastase activities (56 % + 44 %) or cathepsin G activities (59 % + 41 %) in the two respective fractions.

To elucidate which proteinases were responsible for the observed GBM degrading activities, PMSF (the inhibitor of all serine proteinases: elastase and cathepsin G), SAAPV chloromethyl ketone (inhibitor of elastase only), and z-Gly-Leu-Phe chloromethyl ketone or chymostatin (inhibitors of cathepsin G only) were separately included in the GBM proteolysis reaction mixture. The serine proteinase inhibitor PMSF was used first. PMSF, previously dissolved in propan-2-ol, had the problem of precipitating out when added to the aqueous reaction buffer TBS . To overcome this problem the reaction buffer containing PMSF was heated up to 80°C for

Legend to Figs. 6.5. & 6.6.

Linear relationship between GBM degrading activity and amount of granule proteinases used in GBM degrading assays. Each error bar indicates the SD for three measurements from each of three different samples of neutrophils processed to give membrane extract and 30,000 x g supernatant proteinases (n = 9). The membrane extract proteinases used contained 1.24 ± 0.10 units of BOC-APN-SBzl(Cl) thioesterase activity per mg protein whilst the 30,000 x g supernatant used contained 1.32 ± 0.12 units of BOC-APN-SBzl(Cl) thioesterase activity per mg protein. Serial dilutions of the sample indicated on the graph gave respective GBM degrading activities shown.

Fig. 6.5.

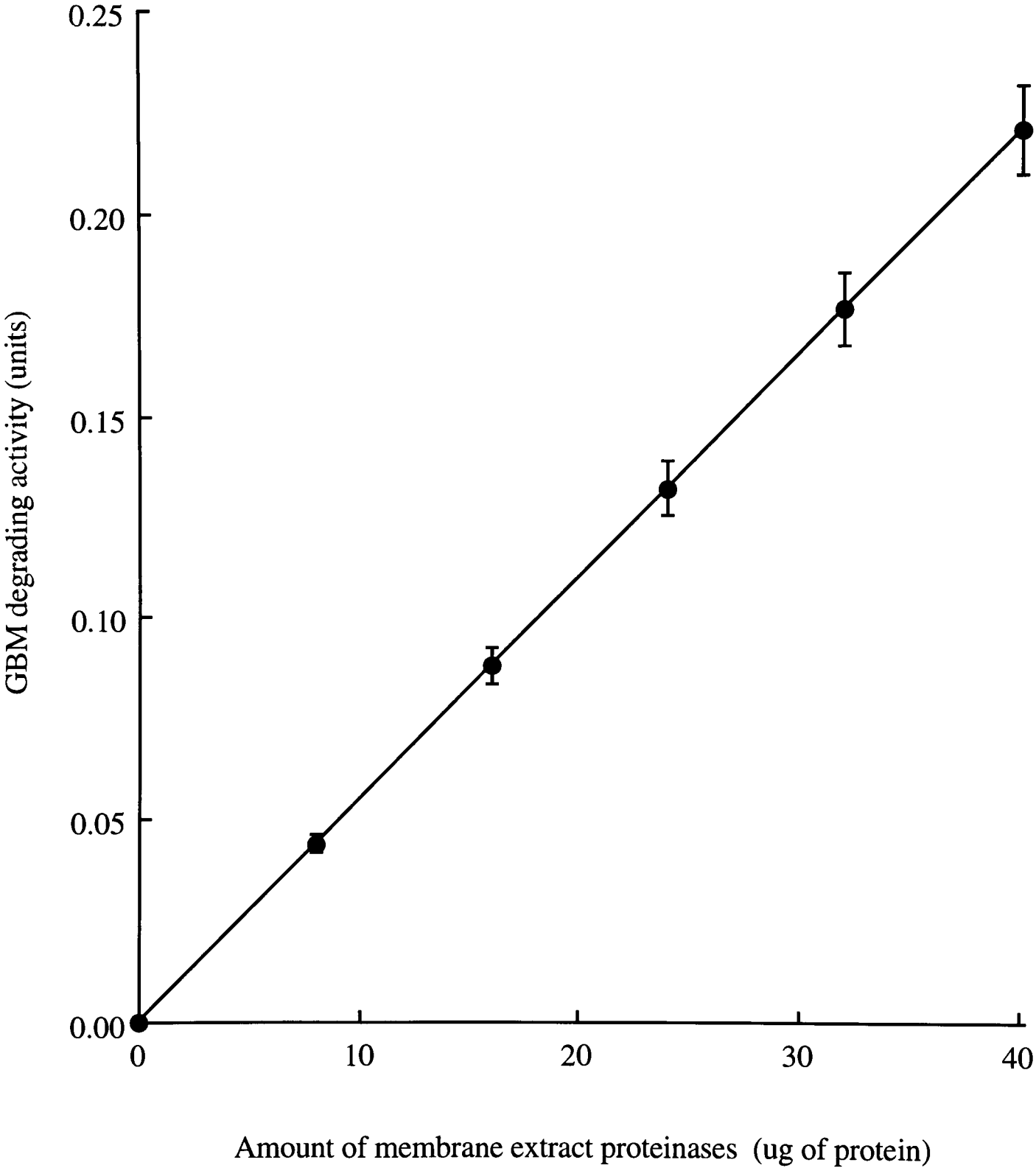


Fig. 6.6.

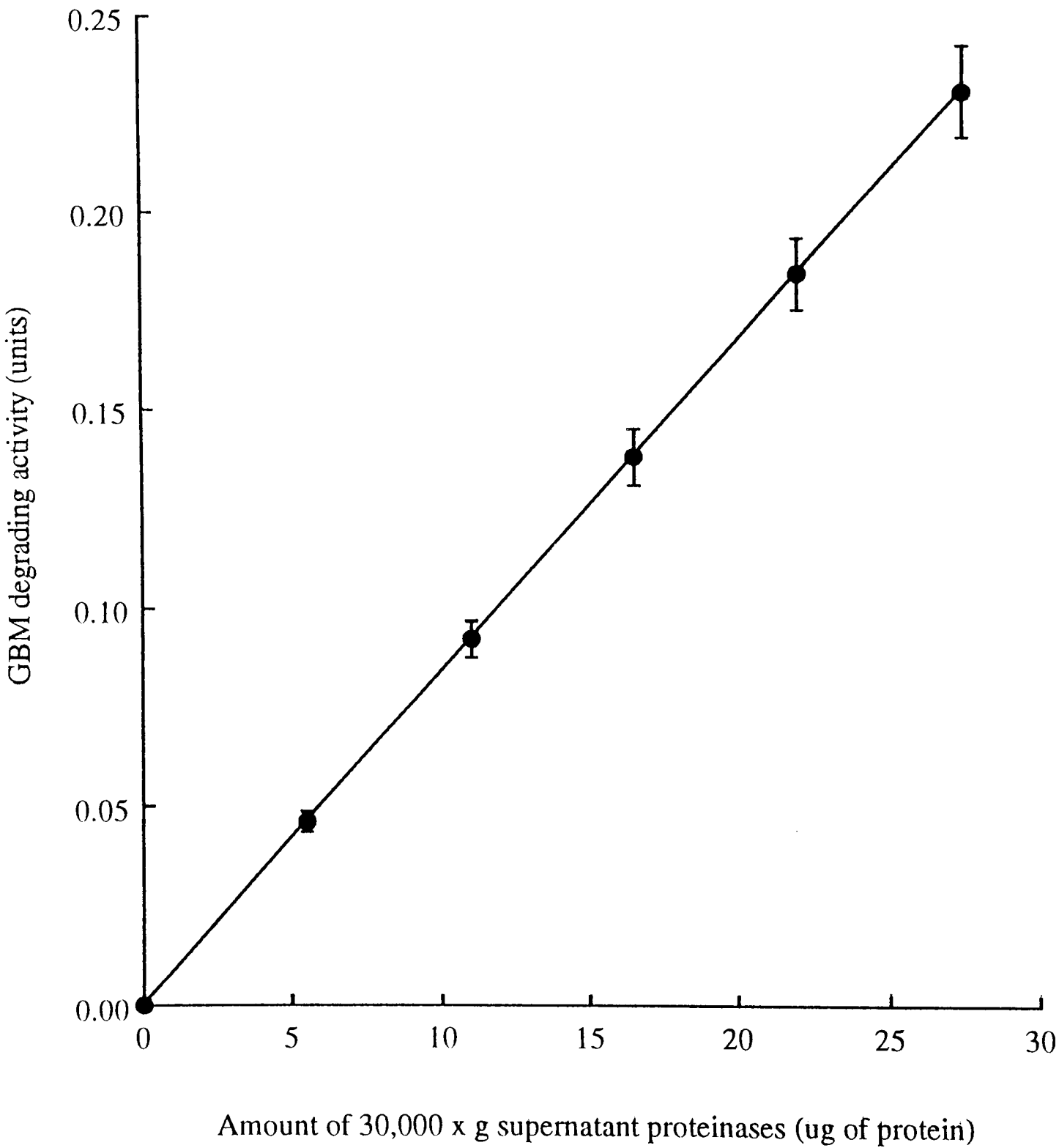


Table 6.6. Distribution of GBM degrading activities in the granules of pig peripheral neutrophils

Fraction	Protein (mg)	GBM degrading activity	
		Total activity (units)	Specific activity (units/mg)
Granule lysate	49 ± 4.1 (100%)	149 ± 10 (100%)	3.04 ± 0.20
30,000 x g supernatant	24.5 ± 2.1 (50.0%)	142 ± 9 (95%)	5.80 ± 0.37
Membrane extract	17.6 ± 1.5 (35.9%)	96 ± 5 (64%)	5.45 ± 0.28
Membrane Residue	6.7 ± 0.6 (13.6%)	2.8 ± 0.1 (1.9%)	0.42 ± 0.01
Percentage recovery	99%	161.6%	—

The figures in the table were based on granules harvested from three different batches of 1 x 10⁹ pig peripheral neutrophils. For each batch, individual measurements were made in triplicates. Figures are given as means ± SD (n = 9). The GBM proteolysis assays were carried out as described in text. The units of enzyme activities are defined in Chapter 2. Protein estimation was based on BCA microprotein assays (Chapter 2). The figures in parentheses are the total activities of each fraction expressed as a percentage of the granule lysate activity.

3 min. with shaking in order to solubilize the inhibitor. This did not appear to have destroyed PMSF's inhibitory function. Known amounts of enzyme from 30,000 x g supernatant and membrane bound fractions (0.22 ± 0.01 units of GBM degrading activity) from three different batches of neutrophils were pre-incubated with PMSF in the reaction buffer without the substrate ^3H -GBM at 37°C for 5 min. ^3H -GBM was then added to start the reaction. Final concentrations of PMSF used were 0.1 mM and 1 mM. Positive controls with only the enzymes added were carried out as were negative controls where only the inhibitor was included. The presence of PMSF at both concentrations reduced GBM degrading activities to zero, indicating that all of the activities were due to serine proteinases.

When only the elastase inhibitor SAAPV chloromethyl ketone (10 mM final concentration) was present in the digestion buffer, $90 \pm 5\%$ of the GBM-degrading activity in membrane extract enzyme samples was eliminated. If the cathepsin G inhibitor z-Gly-Leu-Phe chloromethyl ketone (10 mM final concentration) alone was added then $10 \pm 5\%$ of GBM-degrading activity was lost. The same result was achieved if the cathepsin G inhibitor chymotrypsin (10 mM) was used instead. If both inhibitors were added, then all of the GBM degrading activities were lost. Positive controls (enzyme only) and negative controls (Table 6.7a.) were carried out. Table 6.7a. gives a summary of the results.

When the same experiment was repeated with 30,000 x g supernatant proteinases containing the same amount of GBM degrading activity (0.22 ± 0.01 units of GBM degrading activity), it was found that the elastase inhibitor removed $86 \pm 5\%$ of the GBM degrading activity. Either of the cathepsin G inhibitors eliminated $14 \pm 5\%$ of the activity. A combination of both types of inhibitors abolished all of the activity (Table 6.7b.). Therefore it appeared that the serine proteinase activities were comprised of those of elastase and cathepsin G and that their combined actions on GBM degradation were additive. Also, it seemed that, in both fractions of the granule, most of the GBM degrading activities was attributable to the action of elastase. Only a minor part of the GBM degrading activities was due to the action of cathepsin G.

Table 6.7a. Inhibition of GBM degrading activities in membrane extract samples by elastase and cathepsin G inhibitors

Mixtures	GBM degrading activity (units)	
Enzyme (positive control)	0.22 ± 0.01	(100%)
Enzyme + SAAPV CMK	0.02 ± 0.01	(10%)
Enzyme + GLP CMK	0.20 ± 0.01	(90%)
Enzyme + chymostatin	0.20 ± 0.01	(90%)
Enzyme + SAAPV CMK + GLP CMK	0	(0%)
Enzyme + SAAPV CMK + chymostatin	0	(0%)
Negative controls:		
1. No enzyme	0	
2. SAAPV CMK	0	
3. GLP CCMK	0	
4. Chymostatin	0	
5. SAAPV CMK + GLP CMK	0	
6. SAAPV CMK + Chymostatin	0	

The figures in the tables were based on triplicate analyses for three different batches of neutrophils processed to give membrane extract proteinases. The figures were given as mean ± SD (n = 9). An aliquot of proteinases containing 0.22 units of GBM degrading activity, appropriately diluted with TBS to a final volume of 100 µl, was used for each assay. The assay was carried out as described in the text and unit of enzyme activity are defined in Chapter 2. The figures in parentheses represent GBM degrading activities expressed as a percentage of the uninhibited enzyme activity. CMK = chloromethyl ketone; GLP = z-Gly-Leu-Phe.

Table 6.7b. Inhibition of GBM degrading activities in 30,000 x g supernatant by elastase and cathepsin G inhibitors

Mixtures	GBM degrading activity (units)	
Enzyme (positive control)	0.22 ± 0.0	(100%)
Enzyme + SAAPV CMK	0.03 ± 0.01	(14%)
Enzyme + GLP CMK	0.19 ± 0.01	(86%)
Enzyme + chymostatin	0.19 ± 0.01	(86%)
Enzyme + SAAPV CMK + GLP CMK	0	(0%)
Enzyme + SAAPV CMK + chymostatin	0	(0%)
Negative controls:		
1. No enzyme	0	
2. SAAPV CMK	0	
3. GLP CMK	0	
4. Chymostatin	0	
5. SAAPV CMK + GLP CMK	0	
6. SAAPV CMK + Chymostatin	0	

The figures in the tables were based on triplicate analyses for three different batches of neutrophils processed to give 30,000 x g supernatant proteinases. The figures were given as mean ± SD (n = 9). An aliquot of proteinases containing 0.22 units of GBM degrading activity, appropriately diluted with TBS to a final volume of 100 µl, was used for each assay. The assay was carried out as described in the text and unit of enzyme activity are as defined in Chapter 2. The figures in parentheses represent GBM degrading activities expressed as a percentage of the uninhibited enzyme activity. CMK = chloromethyl ketone; GLP = z-Gly-Leu-Phe.

6.5.4. Metalloproteinase analysis: GBM degrading activity

Since Ca^{2+} was not included in the GBM digestion buffer, activated metalloproteinases in granules, if any, would not be detected in experiments carried out in the previous section. Therefore, Ca^{2+} at 5 mM final concentration was included in the digestion buffer with enzyme (0.22 units of GBM degrading activity) and substrate (0.6 mg/ml ^3H -GBM). No additional GBM degrading activity was detected, indicating a lack of detectable 'activated metalloproteinases' in pig neutrophils. Negative controls with enzyme omitted ($\pm \text{Ca}^{2+}$) were also carried out.

The same experiment was performed in the presence of 0.1 mM PMSF in order to eliminate serine proteinase activities. In this way only metalloproteinases, if any, could be detected. The presence of Ca^{2+} did not give rise to any GBM-degrading activity. The appropriate controls were carried out. This supported the previous conclusion that no detectable metalloproteinases capable of degrading GBM was present in pig neutrophil granules.

The lack of detectable metalloproteinases could have been due to their need for activation prior to full expression of their GBM degrading activities. Incubation of either 30,000 x g supernatant or membrane extract proteinases (each containing 0.22 units of serine proteinase mediated GBM degrading activity) with an activator of metalloproteinases, 4-APMA (0.4 mM final concentration), at 37°C for 1 hr resulted in positive GBM-degrading activities in the presence of 0.1 mM PMSF: 0.08 ± 0.005 and 0.175 ± 0.009 units of GBM degrading activity, respectively. The results were based on triplicate measurements from three different samples of neutrophils processed to give granule proteinases. This showed the presence of a latent metalloproteinase(s). Omission of Ca^{2+} resulted in loss of all GBM degrading activities. Inclusion of the Ca^{2+} chelator EDTA (5 mM final concentration) abolished all GBM degrading activity. These observations pointed to the presence of an activatable metalloproteinase(s) in pig neutrophil granules.

Since serine proteinase activities were abolished by the presence of PMSF in the digestion mixture, all of the observed GBM degrading activities were attributable to metalloproteinases alone. It would appear that the activated metalloproteinase(s) was capable of degrading GBM in the absence of serine proteinases. When all of the granule subfractions were analysed for metalloproteinase-mediated GBM degrading activities, the distribution of these activities in the 30,000 x g supernatant and membrane bound fractions of the neutrophil granules was 75.7 % and 24.3 %, respectively (Table 6.8.). The total GBM degrading activity was taken as the sum of the activities from all the subfractions.

6.5.5. Synergistic degradation of GBM by serine- and metalloproteinase(s)

As demonstrated in Sections 6.5.3. and 6.5.4., serine proteinases and metalloproteinases were able to degrade GBM, but their combined effect had not been investigated. It was not known whether they would degrade GBM additively or synergistically. If their combined action was additive then the rate of GBM degradation would be expected to equal the sum of their individual rates. If their combined effect was synergistic then their combined rate of GBM degradation would be expected to exceed the sum of their individual rates. To determine which of these occurs, the metalloproteinase inhibitor EDTA (5 mM) and the serine proteinase inhibitor PMSF (0.1 mM) were added to the GBM proteolysis digestion buffer separately and in combination.

An amount of enzyme equivalent to 0.22 units of GBM degrading activity from either the 30,000 x g supernatant or membrane extract fractions was treated with 4-APMA as described in section 6.5.4. The enzyme was then pre-incubated with the inhibitors (either separately or in combination) in the digestion buffer containing 5 mM Ca^{2+} at 37°C for 5 min. The reaction was started by adding ^3H -GBM suspension to the digestion buffer and GBM degrading activity measured in the usual way. Positive controls with both inhibitors omitted were carried out. Appropriate negative controls were also carried out as indicated in Table 6.9. which summarises the results.

Table 6.8. Distribution of metalloproteinase-mediated GBM degrading activities in the granules of pig peripheral neutrophils

Fraction	Protein (mg)	GBM degrading activity	
		Total activity (units)	Specific activity (units/mg)
Granule lysate	49 ± 4.1 (100%)	122.6 ± 12.3 (100%)	2.5 ± 0.3
30,000 x g supernatant	24.5 ± 2.1 (50.0%)	113.2 ± 6.0 (92.3%)	4.6 ± 0.2
Membrane extract	17.6 ± 1.5 (35.9%)	34.9 ± 1.7 (28.5%)	2.0 ± 0.1
Membrane Residue	6.7 ± 0.6 (13.6%)	1.5 ± 0.1 (1.2%)	0.2 ± 0.01
Percentage recovery	99%	122.0%	—

The figures in the table were based on granules harvested from three different batches of 1 x 10⁹ pig peripheral neutrophils. For each batch, individual measurements were made in triplicates. Figures are given as means ± SD (n = 9). The GBM proteolysis assays were carried out as described in text. The units of enzyme activities are defined in Chapter 2. Protein estimation was based on BCA microprotein assays described in the same chapter. The figures in parentheses are the total activities of each fraction expressed as a percentage of the granule lysate activity.

Table 6.9. The effect of PMSF and EDTA on GBM degrading activities of pig neutrophil granule proteinases

Reaction mixtures	GBM degrading activity (units)	
	30,000 x g supernatant	Membrane extract
Enzyme (positive control)	0.61 ± 0.04	0.34 ± 0.01
Enzyme + PMSF	0.18 ± 0.01	0.08 ± 0.01
Enzyme + EDTA	0.22 ± 0.01	0.22 ± 0.01
Enzyme + PMSF + EDTA	0	0
Negative controls:		
1. No enzyme	0	0
2. PMSF only	0	0
3. EDTA only	0	0
4. PMSF + EDTA only	0	0

The figures in the tables were based on triplicate measurements made from each of three different batches of neutrophils processed to granule proteinases. The figures were given as mean ± SD (n = 9). An aliquot of proteinases containing 0.22 units of serine proteinase mediated-GBM degrading activity, appropriately diluted with TBS to a final volume of 100 µl, was used for each assay. The enzyme samples were activated with 4-APMA (final concentration 0.4 mM) by incubation at 37° for 1 hr, 4-APMA being previously dissolved in dimethyl sulphoxide. In the final reaction mixture, Ca²⁺ (5 mM) was included in all samples. The assay was carried out as described in the text and the unit of enzyme activity is defined in Chapter 2. The proteolysis of GBM in the positive controls were not linear with time owing to the much heightened rates of proteolysis. The rates were thus taken as the initial rates at time = 0 min. If the overall enzyme activity was reduced to 0.22 units of GBM degrading activity by appropriate dilutions, then linear proteolysis resumed.

In 30,000 x g supernatant samples, the sum of GBM degrading activities owing to serine proteinases (0.22 units) and metalloproteinase(s) (0.18 units) amounted to 0.4 units. Compared with the combined effect (0.61 units), it was apparent that the combined effect was greater than the sum of individual effects by ~ 53 %. When the same analysis was applied to membrane extract samples, it was clear that the combined GBM degrading activity (0.34 units) was again greater than the sum of individual activities ($0.08 + 0.22 = 0.3$ units) by ~ 10 %. Therefore, it would seem that the pig neutrophil serine proteinases and metalloproteinase(s) degraded GBM synergistically.

6.6. Discussion

The data presented in this chapter show that pig neutrophil proteinases can be extracted from their granules in almost the same way as their rabbit counterparts as outlined by Cohn and Hirsch (1960). The granules were lysed by the conventional method of freezing and thawing to release soluble proteinases found in the supernatant following centrifugation at 30,000 x g. Any proteinases that remained membrane bound were insoluble and needed to be extracted with a high salt and detergent buffer to release the proteinases, giving the membrane extract.

The proteinases so obtained, were they from the soluble 30,000 x g supernatant or the solubilizable membrane extract, displayed activities against both the synthetic chromogenic substrates and the natural substrate of elastase elastin. These activities were inhibited by a specific elastase inhibitor of human neutrophil elastase SAAPV chloromethyl ketone. All these observations pointed to the presence of an elastase in pig neutrophils which is consistent with the findings of Geiger et al. (1985) and Kraeva et al. (1988) who independently isolated and purified a pig neutrophil elastase.

The granule proteinases also expressed activities against the synthetic substrate of cathepsin G SAAPP which were inhibited by specific inhibitors of cathepsin G (z-Gly-Leu-Phe chloromethyl ketone and chymostatin), indicating the presence of cathepsin G in pig neutrophils. This finding is in agreement with that by Kraeva et al. (1988) whom isolated and purified a pig neutrophil cathepsin G.

By successfully developing a quantitative GBM proteolysis assay, it was possible to assess accurately the ability of each of the pig neutrophil serine proteinases to degrade pig GBM *in vitro*. The pig neutrophil elastase and cathepsin G previously characterised by a combination of substrate and inhibitor profile analyses displayed activities against pig GBM using this assay. This finding is in keeping with those of Davies et al. (1978), Sanders et al. (1978), and Visser et al. (1984) who had earlier demonstrated the degradation of human GBM by human neutrophil serine proteinases. The results in this chapter also show that the combined activity of the two serine proteinases are additive - as Vissers and Winterbourn (1988a) found when human serine proteinases were used to degrade human GBM. These authors stated that the degradation was achieved primarily by elastase and that the two proteinases accounted for all of the serine proteinases activities, concurring with the present study (~ 90 % degradation by elastase and ~ 10% by cathepsin G).

It has also been possible to infer the presence of a latent metalloproteinase(s) in the granule extract of pig neutrophils. It appears that the metalloproteinase(s) required activation by 4-APMA before showing any GBM degrading activity and this activity was Ca^{2+} -dependent. Removal of Ca^{2+} by the divalent cation chelator EDTA abolished the activity. Similar findings were reported by Vissers and Winterbourn (1988b) when they analysed the GBM collagen IV degrading activity of the metalloproteinase gelatinase released from human neutrophils following stimulation with phorbol 12-myristate 13-acetate (PMA). No gelatinase activity was measured in the absence of a mercurial activator and so indicated that the enzyme was released in a latent form.

It is of interest to note that a latent pig neutrophil gelatinase has been characterized (Murphy et al., 1989), though no collagenase from the same source has yet been reported. It is possible, therefore, that the Ca^{2+} -dependent GBM degrading activity reported here is the gelatinase characterized by Murphy et al. (1989). It was difficult to attribute the metal-dependent GBM degrading activity to either gelatinase or collagenase (if it exists in pig neutrophils) because there are no known separate

inhibitors specific to each enzyme. By isolating collagenase and gelatinase separately from a degranulated fluid of stimulated human neutrophils and using them to degrade human GBM, Vissers and Winterbourn (1988a) have shown that only gelatinase was able to degrade human GBM, as assessed by hydroxyproline release, a marker for collagen IV breakdown. The current evidence therefore favours gelatinase as the only metalloproteinase in neutrophils capable of degrading GBM.

Not only is the pig neutrophil metalloproteinase activity able to degrade GBM on its own, it is also able to assist synergistically the proteolysis of GBM mediated by the serine proteinases - a novel feature that has not been reported previously in the literature. It is possible that the synergism was due to cathepsin G activation of gelatinase since purified human neutrophil cathepsin G has been shown to activate human neutrophil gelatinase (Murphy et al., 1980; Macartney and Tschesche, 1980 & 1983a). This is unlikely for the following reason. If cathepsin G had activated gelatinase in the proteinase extract, then any Ca^{2+} -dependent GBM degrading activity would have been observed prior to activation by 4-APMA but this was not so (Section 6.5.4.). Therefore, cathepsin G did not appear to have activated any metalloproteinases in the pig system. Elastase has also been implicated as a candidate capable of activating gelatinase (Vissers and Winterbourn, 1988b) but it did not seem to have done so in the system, for the same reason. It might also be argued that activated metalloproteinase(s) had activated the serine proteinases, giving rise to the observed synergism. This is again unlikely in since the serine proteinases showed no extra activity against the GBM after being incubated with the activated metalloproteinases for 4 hrs at 37°C, indicating that the metalloproteinases could not have activated the serine proteinases.

A more likely explanation for the observed synergistic degradation of GBM is as follows. When one class of proteinases degrade GBM proteins, it is conceivable that proteolytic sites for the other class of proteinases are being sterically uncovered and vice versa. This could explain the greater than additive GBM proteolytic effect when the two classes of proteinases are working in concert. Since our understanding of the three dimensional structure of GBM is far from complete, it is difficult to provide an

adequate molecular explanation. However, it is known that neutrophil elastase can degrade the GBM proteins collagen IV (Mainardi et al., 1980; Vissers and Winterbourn, 1988a; Watanabe et al., 1990b), laminin (Otto et al., 1982), and heparan sulphate proteoglycan (Barrett, 1981). Cathepsin G is able to degrade collagen IV (Vissers and Winterbourn, 1988a), laminin (Bruch et al., 1989; Otto et al., 1982), and entactin (Bruch et al., 1989). Gelatinase can degrade collagen IV (Murphy et al., 1982). Collagenase, by contrast, is unable to degrade collagen IV (Murphy et al., 1982). It is quite possible that the degradation of the aforementioned GBM proteins by one proteinase unmask proteolytic site(s) which then becomes accessible by another proteinase. Clearly, a more thorough study would be required to dissect the molecular mechanisms underpinning the purported concerted proteolysis of GBM.

A more immediate concern is to address the crucial question of whether these characterized pig neutrophil proteinases, either working separately or in concert, are able to render pig GBM more permeable to protein. It is only when studies such as these are carried out that the relevance of each of these proteinases in glomerulonephritis can be more fully understood and this forms the basis of next chapter's investigation.

CHAPTER SEVEN

CHAPTER 7 THE EFFECT OF PIG NEUTROPHIL PROTEINASES ON THE PERMEABILITY OF PIG GBM TO PROTEIN

7.1. Introduction

The evidence supporting the view that neutrophil proteinases can damage GBM and cause proteinuria in neutrophil-dependent forms of GN has been reviewed in detail in Sections 1.11.4A & B. There is very little doubt that neutrophils activated by immune complex bound GBM can exocytose their granule proteinases and degrade the subjacent GBM. However, the evidence that the GBM would become more permeable to protein following the degradation remains circumstantial. There are essentially two lines of evidence which suggest that neutrophil proteinases may be able to effect an increase in the permeability of the GBM to protein and thereby cause proteinuria. Firstly, rats that were intrarenally perfused with human neutrophil elastase and cathepsin G in amounts that could be provided by neutrophils *in vivo* developed massive proteinuria (Johnson et al., 1988a). Secondly, mixed rabbit GBM and TBM that were predigested with rabbit neutrophil proteinases in a crude granule extract became more permeable to serum protein (Cotter and Robinson, 1980b).

In Johnson et al.'s study, it was uncertain whether the proteinuria was due only to the effect of the perfused neutrophil serine proteinases on GBM. The proteinases might have also perturbed the glomerular cells which then damaged the basement membrane through secretion of their own proteinases or oxidants (Johnson et al., 1994). Cotter and Robinson's *in vitro* study eliminated this uncertainty and directly demonstrated neutrophil proteinases' ability to render basement membranes more permeable to protein. However, their evidence was inconclusive for the following reasons. Firstly, the use of mixed GBM and TBM meant that it was impossible to attribute the permeability change of the basement membrane to GBM proteolysis alone. It could be argued that the effect was due to TBM proteolysis. Secondly, the proteinase to basement membrane ratio was vastly in excess of what a neutrophil can provide if it

were to exocytose all of its proteinases onto the subjacent GBM, as will be discussed. It was uncertain that if a more physiological ratio were used, a detectable increase in membrane permeability would still be seen. Thirdly, the rabbit neutrophil proteinases used were quite different from human's. The rabbit neutrophils lack a true elastase (Cotter and Robinson, 1980a & b) and have no cathepsin G (Brown, 1984; Brown and Robinson, 1987). There is thus no evidence that a true neutrophil elastase and cathepsin G are able to render GBM more permeable to protein. It ought to be noted that there is no evidence that neutrophil metalloproteinases are able to do likewise.

Cotter and Robinson's study has been extended here by using highly purified pig GBM as proteinase substrate, instead of mixed GBM and TBM. A pathophysiological proteinase to GBM ratio was used instead of the excessive ratio previously employed (Cotter and Robinson, 1980b). Proteinases from pig neutrophils were used instead of from rabbit neutrophils since the former (elastase, cathepsin G, and gelatinase) are more like human's (Section 6.1.). Cotter and Robinson did not resolve the question of whether the increased GBM permeability to protein was due to a reduction in membrane thickness or an increased intrinsic membrane permeability (Cotter and Robinson, 1980b). This issue has also been examined here.

7.2. Estimation of pathophysiological neutrophil proteinase to GBM ratio to be used for GBM digestion

The pathophysiological neutrophil proteinase to GBM ratio can be estimated if (1) the proteinase activity released by a neutrophil activated by GBM coated with anti-GBM antibodies and (2) the dry weight of GBM subjacent to that neutrophil are known. 1×10^9 neutrophils contain ~ 57 units of BOC-APN-SBzl (Cl) thioesterase activity (Section 6.5.1., Table 6.4.). The extent of neutral proteinase release by human neutrophils undergoing exocytosis when they adhered to human GBM coated with rabbit anti-human GBM antibodies or with anti-GBM antibodies from serum of Goodpasture's syndrome patients was found to be 16 % and 11 %, respectively (Davies et al., 1984). Thus an activated neutrophil might release ~ 14 % of its total

proteinase activities, i.e., that equivalent to $\sim 8 \times 10^{-9}$ units of BOC-APN-SBzl (Cl) thioesterase activity. The thioesterase was used as a convenient marker for the general pool neutrophil proteinases. Using other proteinase markers makes no difference to the outcome of the ensuing calculation.

To calculate the dry weight of GBM onto which this amount of proteinases is released, it is necessary (1) to estimate the area of neutrophil contact with GBM and (2) to convert this area into the dry weight of GBM. Roughly one half of the surface area of a neutrophil might be in contact with the GBM in nephritic glomeruli as assessed by electron microscopy (Hawkins and Cochrane, 1968). This is equivalent to an area of $\sim 1.9 \times 10^{-10} \text{ m}^2$ given that a spherical neutrophil has a radius of $\sim 5.5 \mu\text{m}^{\text{@}}$ (Burkitt et al., 1993). Disc-shaped GBM films (1.5 mg dry weight) have been found to be ~ 25 layers thick by electron microscopy (Bray, 1982) and had a surface area of $1.38 \times 10^{-3} \text{ m}^2$. The dry weight of a single layer of GBM per unit area would be $\sim 43.5 \text{ mg/m}^2$. The dry weight of the area of GBM covered by a neutrophil ($\sim 1.9 \times 10^{-10} \text{ m}^2$) is thus $\sim 8 \times 10^{-9} \text{ mg}$. If a neutrophil were to release proteinases equivalent to $\sim 8 \times 10^{-9}$ units of BOC-APN-SBzl (Cl) thioesterase activity onto $\sim 8 \times 10^{-9} \text{ mg}$ of GBM, the proteinase to GBM ratio would be ~ 1 unit BOC-APN-SBzl (Cl) thioesterase activity : 1 mg GBM. This ratio was therefore chosen as approximating pathophysiological conditions *in vivo*.

7.3. Incubation of GBM with neutrophil proteinases

Proteinases pooled from the two neutrophil granule fractions (30,000 x g and membrane extract fractions) (Section 6.4.) were treated with 4-aminophenyl mercuric acetate (4-APMA) (0.5 mM final concentration) for 1 hr at 37°C. This has been shown to activate the metalloproteinases fully with no loss of elastase or cathepsin G activities (Murphy et al., 1989). The pooled proteinases equivalent to 6 units of BOC-APN-SBzl (Cl) thioesterase activity was incubated with 6 mg of GBM in a final volume of 6 ml of TBS, pH 7.4 containing 5 mM Ca^{2+} at 37°C with tumbling for two different

[@] The external surface area of a spherical neutrophil is $4\pi r^2$ where r = radius of the sphere.

incubation periods (6 hrs and 12 hrs). To study the separate effects of serine and metalloproteinases, their respective inhibitors, phenyl methyl sulphonyl fluoride (PMSF) (0.1 mM) and sodium EDTA (5 mM), were used to inactivate the proteinases (5 min, 37°C) prior to being added to reaction mixtures. The inhibition was complete as judged by the absence of GBM degrading activities for up to 12 hrs. To study the combined effect of the two families of proteinases, they were not inactivated by the inhibitors. The controls were enzymes which were either heat-inactivated or inhibited by both inhibitors. Following digestion, the GBM was recovered by centrifugation (2,000 x g, 5 min). It was washed three times in TBS before forming into films. Dry weight measurements were made in parallel on digested GBM after washing in distilled H₂O.

7.4. Release of material from GBM following digestion by neutrophil serine and metalloproteinases

The extent of GBM proteolysis by neutrophil serine and metalloproteinases was assessed by the loss of GBM dry weight and by the release of ³H-material from ³H-GBM. ³H-GBM was prepared as described in Section 2.2.6. GBM, either native and labelled, was digested with the proteinases as described in Section 7.3. The release of ³H-material from ³H-GBM was determined by liquid scintillation counting as detailed in Section 2.3.14. The results are summarized in Table 7.1. The serine proteinases released significantly more GBM material than did the metalloproteinases for each digestion period. The release of GBM material only increased slightly after 12 hrs as compared with 6 hrs irrespective of the family of proteinases used. The percentage release of GBM material, judged by loss of dry weight, was not significantly different from that shown by the release of isotope. Thus the labelling of GBM proteins seemed uniform. The combined effect of the serine and metalloproteinases exceeded the sum of their individual effects, indicating a synergistic degradation of GBM. This did not appear to be the result of metalloproteinase activation of serine proteinases or vice versa since preincubation of the granule extract for 1 hr resulted in no detectable increase in

Table 7.1. The release of GBM material following proteolysis by pig neutrophil serine and metalloproteinases

Proteinases	Digestion time (hrs)	loss of dry weight (%) (mg)	Release of ³ H-material (%) (d.p.m. x 10 ⁻⁴)
Metalloproteinases	6	0.47 ± 0.25 (7.8%)	14.9 ± 0.7 (7.1%)
	12	0.61 ± 0.21 (10.2%)	19.3 ± 1.2 (9.2%)
Serine proteinases	6	0.96 ± 0.24 (16.0%)	32.8 ± 2.0 (15.6%)
	12	1.22 ± 0.28 (20.3%)	40.3 ± 2.0 (19.2%)
Serine+ metallo-proteinases	6	2.11 ± 0.31 (35.2%)	73.1 ± 5.1 (34.8%)
	12	2.40 ± 0.35 (40.0%)	82.1 ± 5.7 (39.1%)

6 mg of ³H-GBM (activity 21.0 x 10⁵ d.p.m.) were incubated with granule extract (6 units of BOC-APN-SBzl(Cl) thioesterase) for 6 or 12 hrs. Loss of isotope and change in dry weight were deteremined as described in text.

either proteinase activities as assessed by degradation of native or ^3H -GBM. When both families of proteinases were inhibited, no GBM degradation could be detected.

7.5. Serum ultrafiltration

GBM fragments treated with serine and metalloproteinases separately or in combination (Section 7.3.) were made into thin films in Amicon Type 52 ultrafiltration cells (65 ml) according to the method of Cotter and Robinson (1980b). A suspension of GBM fragments with an amount equivalent to 1.5 mg dry weight prior to proteolytic digestion was packed down onto Millipore membrane under gas (N_2) pressure (200 kPa) in the filtration cells. The controls were films made from GBM treated with heat- or inhibitor-inactivated proteinases. Pig serum was filtered over a range of filtration pressures (0-200 kPa) and protein fluxes (J_s), water fluxes (J_v), and protein rejections (R) determined as described in Section 2.3.3.

GBM treated with pig neutrophil proteinase extract containing only active metalloproteinases appeared to be more permeable to pig serum compared with the controls over all of the filtration pressures used after 6 hrs of digestion. This was evident from the increases in protein fluxes, water fluxes, and reductions in protein rejections (Figs. 7.1.-7.3.). When only serine proteinases were active in the proteinase extract, an even greater increase in membrane permeability to protein and water than that mediated by metalloproteinases was seen (Figs. 7.1.-7.3.). Of the two families of neutrophil proteinases, serine proteinases were more effective at increasing the permeability of GBM to serum protein. When the full complement of proteinases were allowed to act on GBM, a marked increase in membrane permeability to protein and water was observed, far greater than the sum of the individual effects (Figs. 7.1.-7.3.).

Extending the membrane digestion period up to 12 hrs, caused a notably greater increase in membrane permeability (Figs. 7.4.-7.6.). The effect was mediated by either families of proteinases or both, indicating that the effects were time-dependent. The relative effect of the two families of proteinases was similar to that resulting from 6 hrs

Legend to Figs. 7.1.-7.6.

The ultrafiltration behaviour of GBM for pig serum (70 mg/ml) following digestion with porcine neutrophil proteinases for 6 hrs (Figs. 7.1.-7.3.) and 12 hr (Figs. 7.4.-7.6.). Each set of graphs shows the effect of the proteinases on serum protein flux (J_s), water flux (J_v), and serum protein rejection (R) in that order. Each value is the mean of 9 observations, 3 from each of 3 separate films. Each error bar represents SD ($n = 9$). The controls represent films constructed from GBM treated with either heat-denatured proteinases or proteinases inhibited with both PMSF and EDTA.

Fig. 7.1.

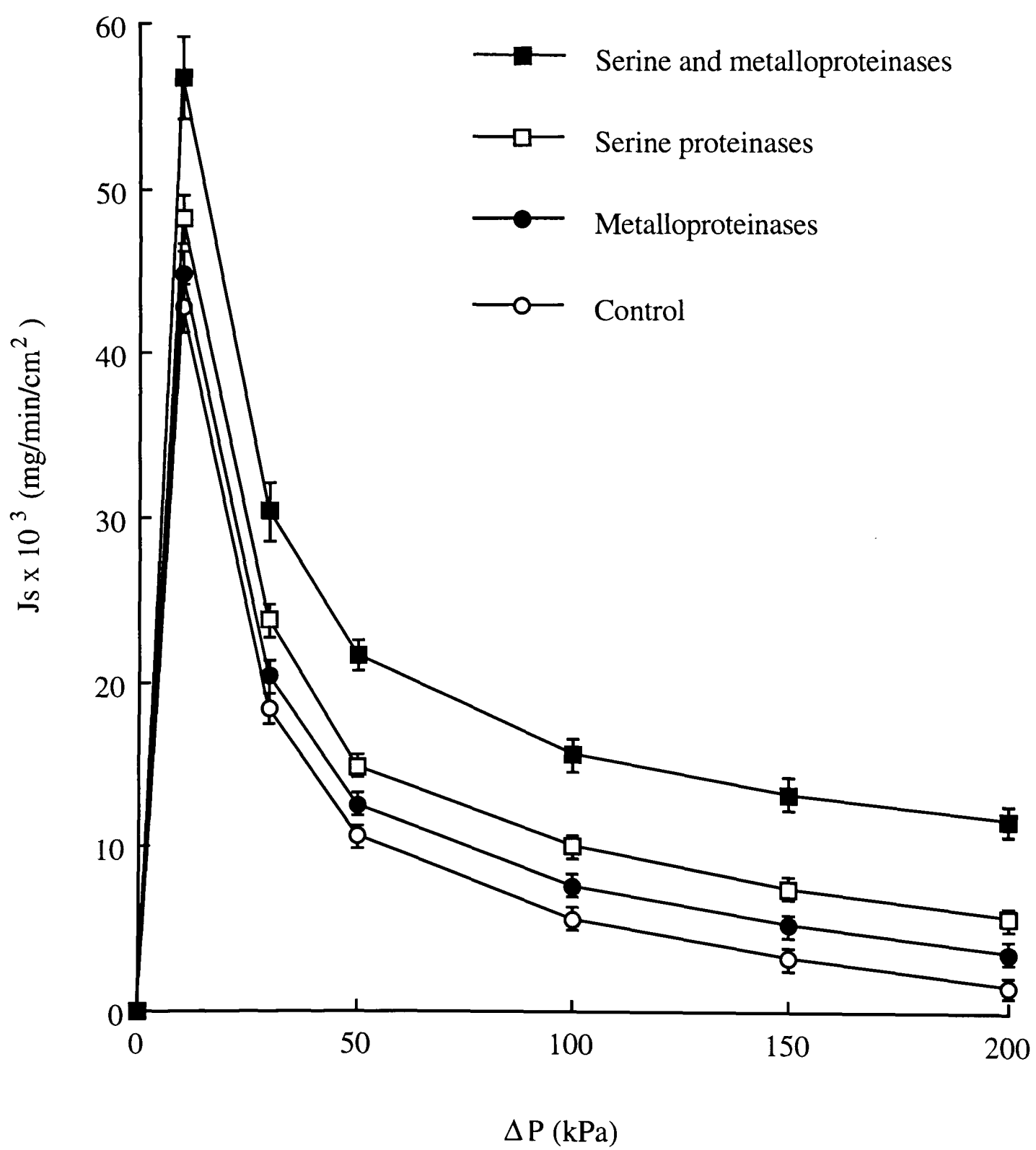


Fig. 7.2.

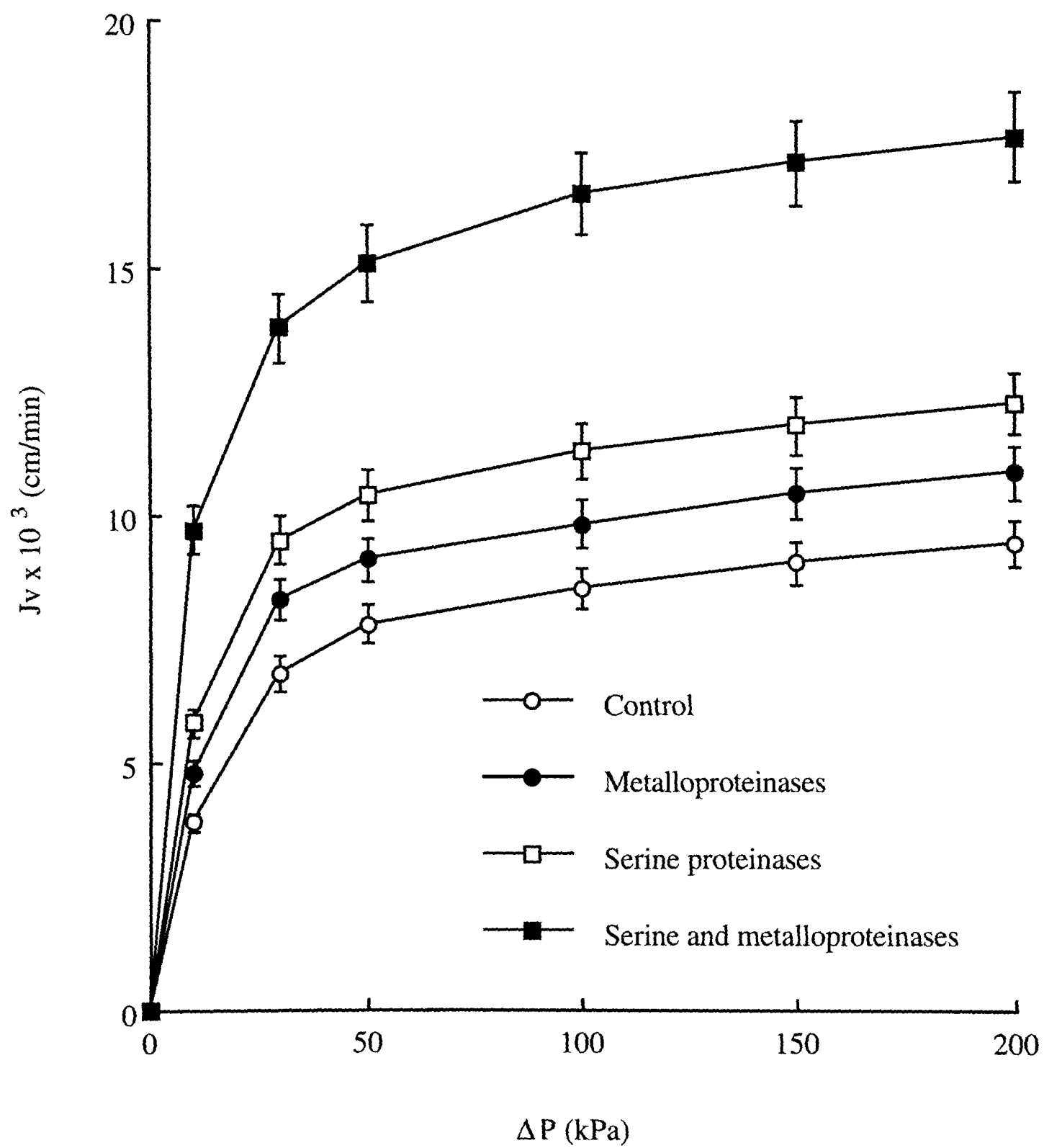


Fig. 7.3.

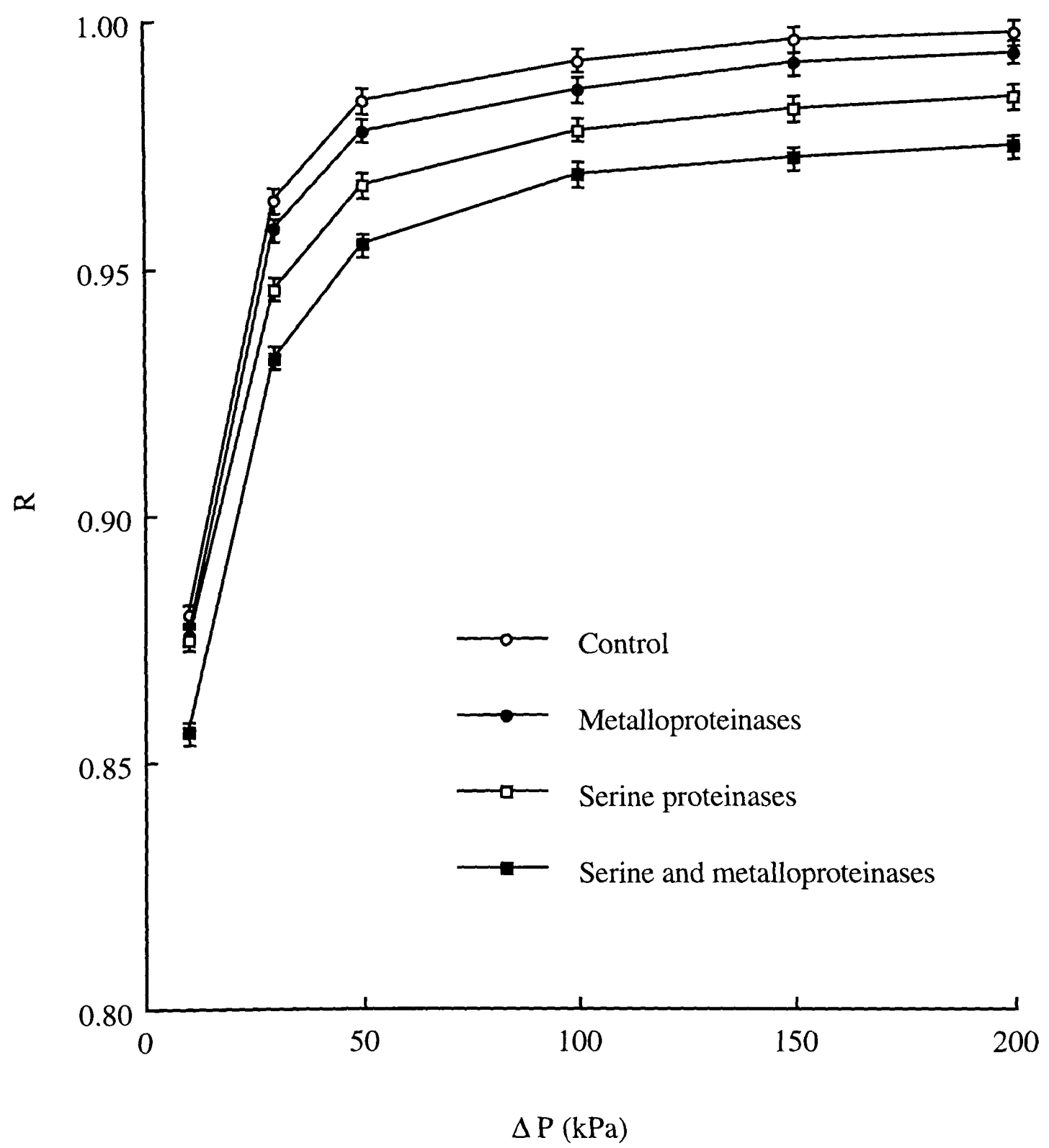


Fig. 7.4.

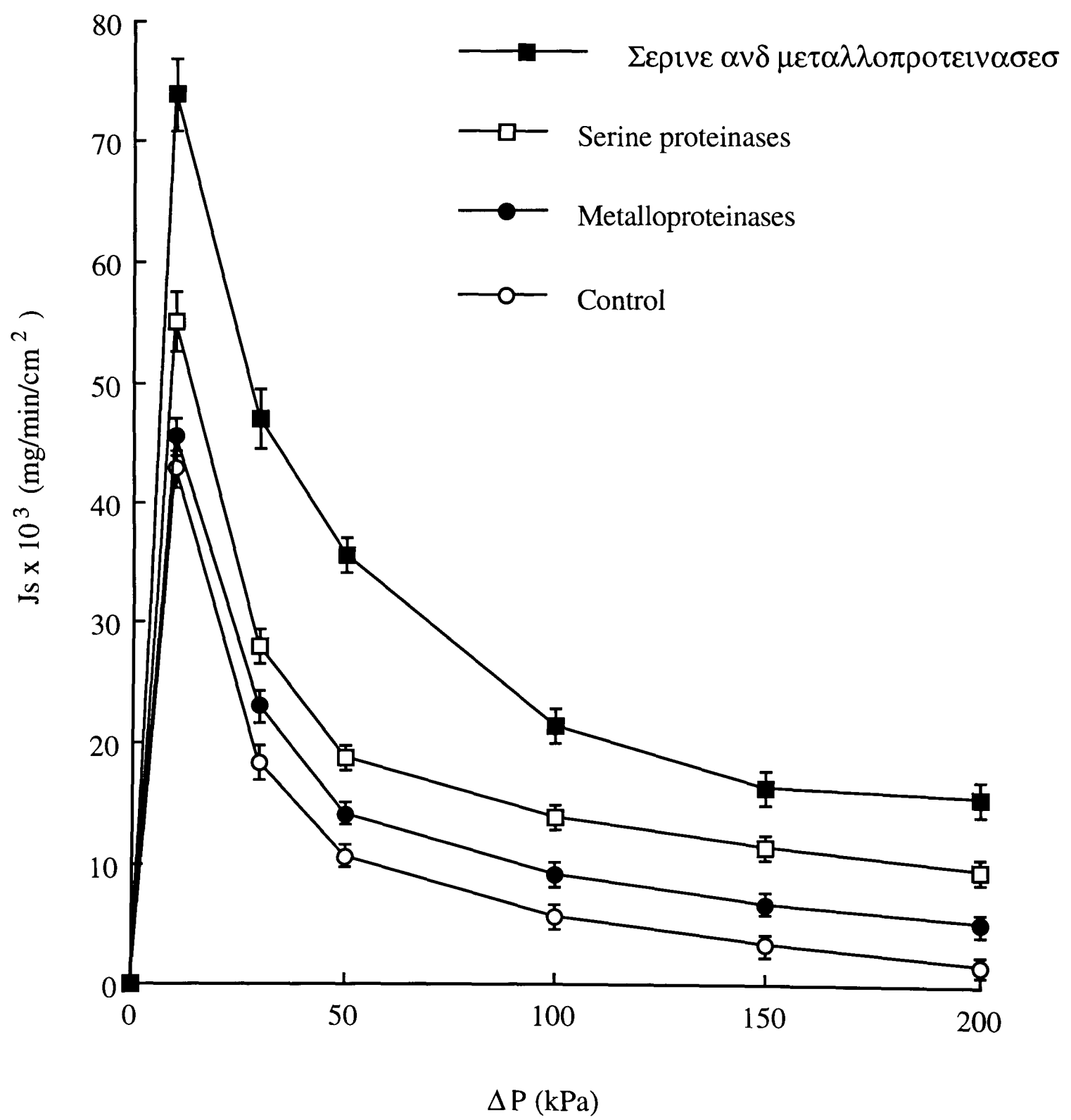


Fig. 7.5.

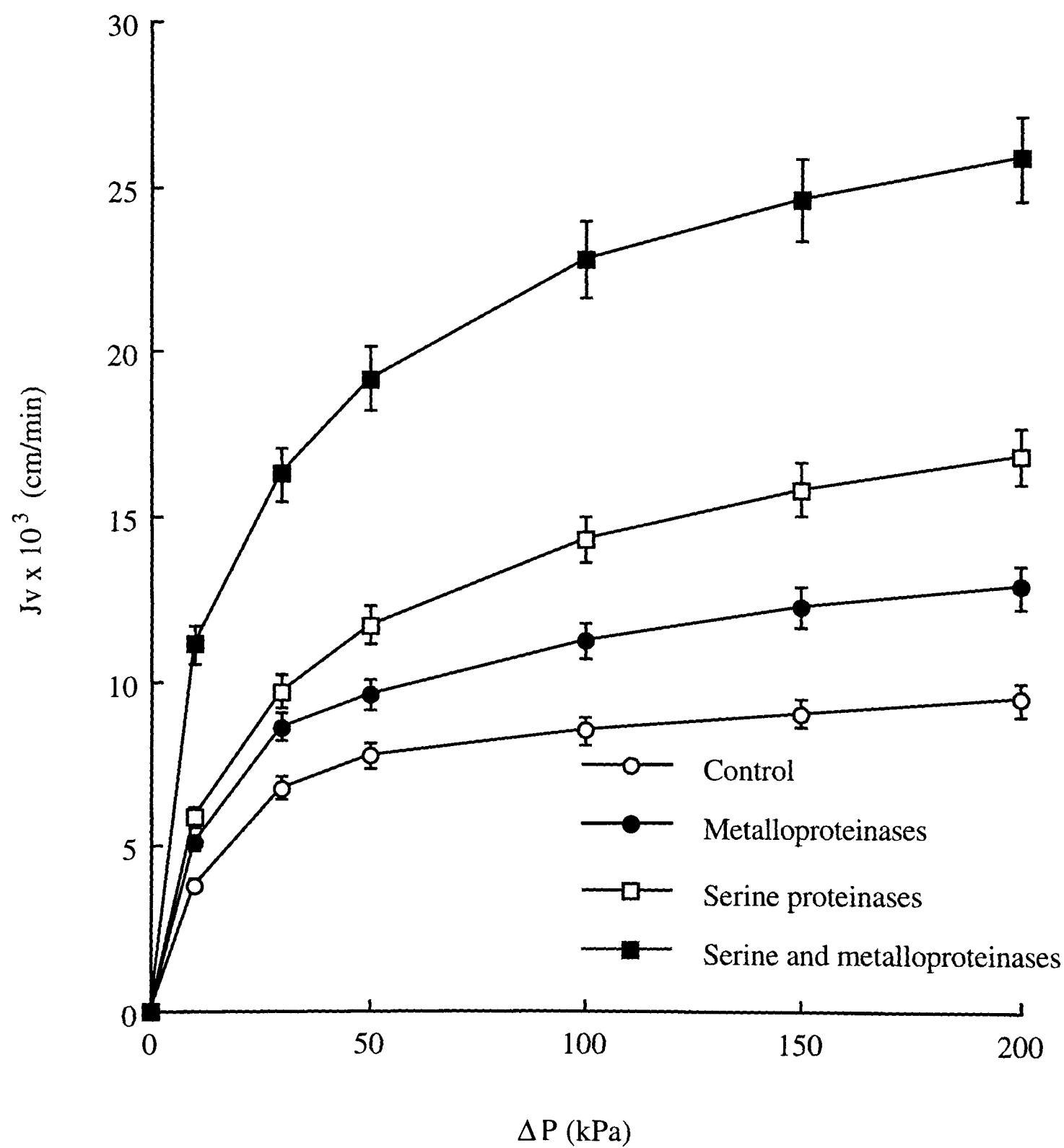
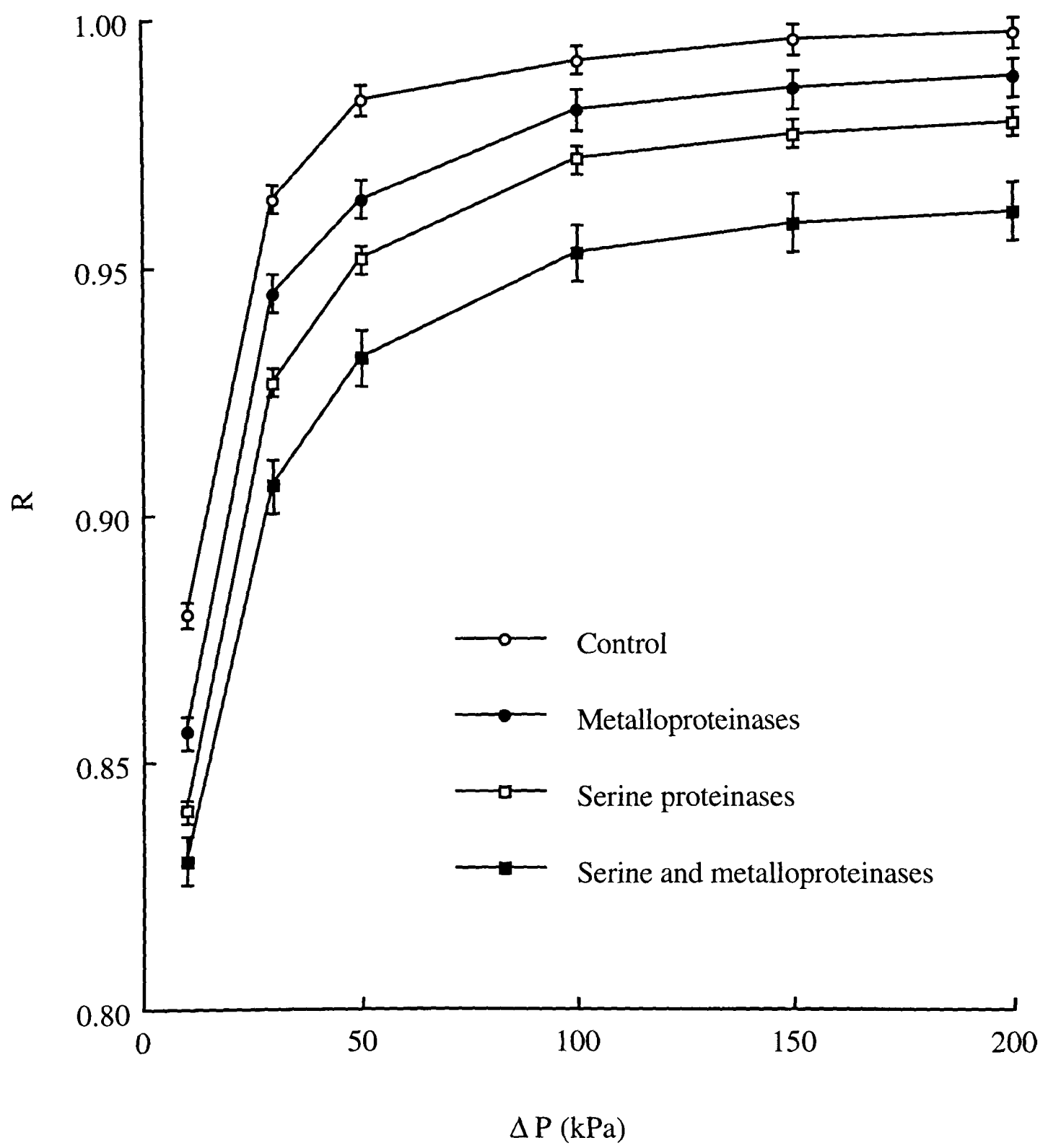


Fig. 7.6.



of digestion. A potentiative effect from the combined action of the two families of proteinases was once again apparent.

7.6. GBM film thickness analysis

The thickness of the GBM film influences the protein flux; the thinner the film the greater the protein flux (Cotter, 1979). If proteolysis results in membrane thinning, hence reduction in film thickness, then this might account for any increase in membrane permeability to serum protein. It was thus pertinent to ascertain whether the films used in filtration studies (Section 7.5.) suffered any thickness reductions. To do this, thicknesses of films were measured as described in Section 2.3.2. at the end of filtration experiments. Table 7.2. summarizes the results. Films made from GBM fragments digested for 6 hrs with metalloproteinases alone in the neutrophil granule extract had a thickness reduction of ~ 8 % compared with the controls ($p < 0.01$). Films made from membranes digested with only serine proteinases suffered a greater thickness reduction: ~ 16 % ($p < 0.001$). A combination of serine and metalloproteinases gave a ~ 35 % thickness reduction ($p < 0.001$). To monitor whether the protein filtrations *per se* had altered film thicknesses, an extra set of proteinase-treated and control films were prepared but were not subject to protein filtration. No difference in film thicknesses was detected between films subjected to protein filtration and those which were not, indicating that protein filtrations had no effect on film thicknesses.

It was possible that film thickness reduction was due to loss of GBM fragments and not to membrane thinning. To determine if this had occurred, the number of GBM fragments was counted in a haemocytometer before and after proteolysis. No loss of fragments was detected following digestion by any of the proteinases. Therefore, film thickness reduction appeared to have resulted only from membrane thinning.

When the digestion period was lengthened to 12 hrs, a similar pattern of results was seen but the respective thinning of the films were greater than those resulting from only 6 hrs of digestion (Table 7.2.). Films made from GBM treated with

Table 7.2. The effect of proteinase digestion on GBM film thickness

Proteinases	GBM film thickness (μm)	
	6h digestion	12h digestion
Metalloproteinases	7.3 ± 0.6	6.9 ± 0.6
Serine proteinases	6.6 ± 0.6	6.0 ± 0.5
Serine+ metalloproteinases	4.9 ± 0.7	4.1 ± 0.7
Heat inactivated proteinases	7.9 ± 0.5	7.9 ± 0.6
PMSF and EDTA inactivated proteinases	7.9 ± 0.6	7.9 ± 0.7

The results indicate averages of 60 random thickness readings, 20 per film for a set of 3 films. SDs (n = 60) are shown.

metalloproteinases, serine proteinases, and a combination of both had thickness reductions of 13 %, 24 %, and 48 %, respectively. No loss of GBM fragments was detected following proteolysis by any of the proteinases. It appeared therefore that proteolysis brought about a time-dependent reduction in GBM thickness. Given that protein flux through GBM films is dependent on film thickness, it is reasonable to assume that film thinning due to neutrophil proteinases was responsible for the increments in serum protein fluxes (Section 7.5.).

7.7. Analysis of changes in intrinsic permeability to protein independent of changes in membrane thickness

Although proteolysis-mediated basement membrane thinning could account for the observed increase in permeability to serum protein, it might not be the only means by which proteinases achieve this. Cotter and Robinson (1980b) pointed out that proteinases might be able to do so by altering the intrinsic permeabilities of GBM. It is conceivable that by diffusing into the membrane, proteinases can destroy internal structure and render it more permeable to macromolecules without reducing its thickness. To determine if neutrophil proteinases could effect this it was necessary first to eliminate the film thickness variable. Proteinase digested GBM films of a thickness equal to the thickness of undigested films were compared for their permeability to serum protein. If such films remained permeable to serum protein then this would indicate that neutrophil proteinases were able to increase the intrinsic permeability of GBM to serum protein.

The average thickness of a set of 3 films, each made from a known amount of proteinase-digested GBM, was measured and compared with the average thickness of a set of 3 undigested GBM films ($7.9 \pm 0.6 \mu\text{m}$). If the thickness was lower than the control films then larger amounts of GBM were used to prepare the next set of films whose average thickness was again measured and compared with the controls. This process was repeated until a set of proteinase digested GBM films with identical thickness as the control was made; the amount of GBM needed was noted. These films

could not be used for filtration experiments since they were coated with *Micrococcus lysodeikticus* capsules (Section 2.3.2.). A fresh set of films ($n = 3$), each made from the necessary amount of digested GBM was used for serum ultrafiltration experiments. Five sets of films were made in total: two from GBM separately treated with serine proteinases and metalloproteinases, one from GBM treated with both classes of proteinases, and two from GBM treated with heat- and inhibitor-inactivated proteinases (controls). GBM digestion conditions were as described in Section 7.3.

Serum protein fluxes through films made from metalloproteinase digested GBM (thickness $7.9 \pm 0.5 \mu\text{m}$) were the same as controls over the entire pressure range (Fig. 7.7.), indicating that metalloproteinases were not able to change the intrinsic permeability of the GBM to serum protein. Serine proteinase treated films (thickness $7.9 \pm 0.6 \mu\text{m}$), by contrast, had higher serum protein fluxes than the controls over the same pressure range, in spite of the lack of difference in their film thicknesses (Fig. 7.7.), indicating that serine proteinases were able to increase the intrinsic permeability of GBM to serum protein. When serum was filtered through films treated with both classes of proteinases, higher protein fluxes than the controls were again seen (Fig. 7.7.). This showed that the combined action of the two was also able to increase the intrinsic permeability of GBM to serum protein but to a greater extent than could serine proteinases alone.

When the films were analysed for serum protein rejection, it was found that rejection values were lower for serine proteinase treated GBM films than the controls over the whole filtration pressure range ($p < 0.005$) (Fig. 7.8.). Metalloproteinase treated films had no detectable changes in rejection values compared with the controls (Fig. 7.8.). Films made from GBM treated with both types of proteinases had lower rejection values than the controls and those of serine proteinase treated films ($p < 0.005$) (Fig. 7.8.). These findings further supported the view that serine proteinases but not metalloproteinases were able to bring about an increase in the intrinsic permeability of GBM to serum protein and that the two families of neutrophil proteinases in combination were able to do the same but to a greater extent.

Legend to Figs. 7.7.-7.9.

An analysis of intrinsic permeability of GBM to pig serum (70 mg/ml) following digestion with pig neutrophil proteinases for 6 hrs. Films made from GBM digested with proteinases were thinner than those treated with inactivated proteinases (controls) but had extra digested GBM added to give identical average thickness as the controls (see text). Figs. 7.7.-7.9. show the effects of proteinases on serum protein flux (J_s), rejection (R), and water flux (J_v), respectively. Each value is the mean of 9 observations, 3 from each of 3 separate films. Each error bar represents SD ($n = 9$). The control represents films constructed from GBM treated with either heat-denatured proteinases or proteinases inhibited with both PMSF and EDTA. Incubation with the metalloproteinases gave rise to no detectable difference in J_s , R , and J_v to the controls as indicated on the graphs.

Fig. 7.7.

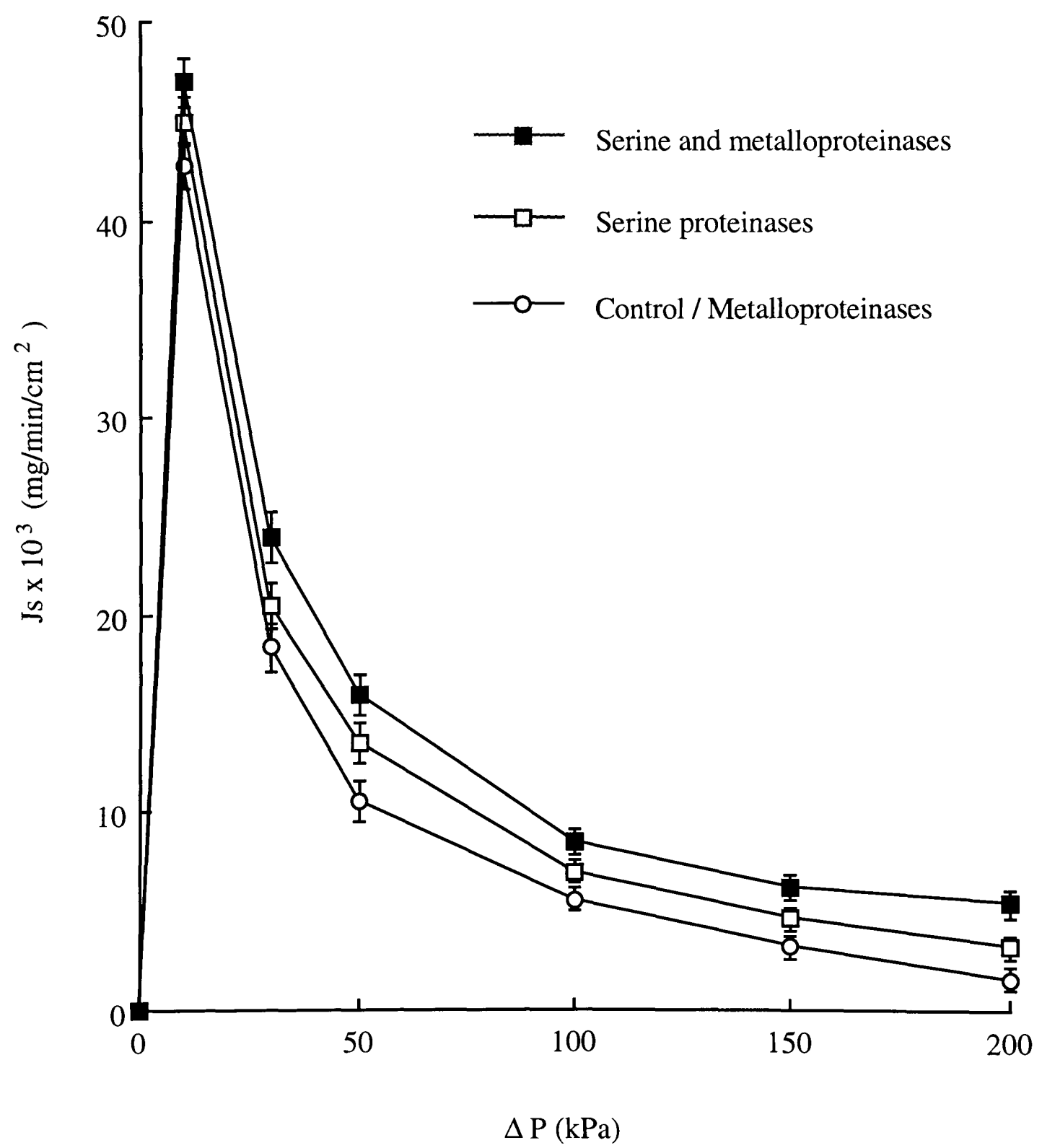


Fig. 7.8.

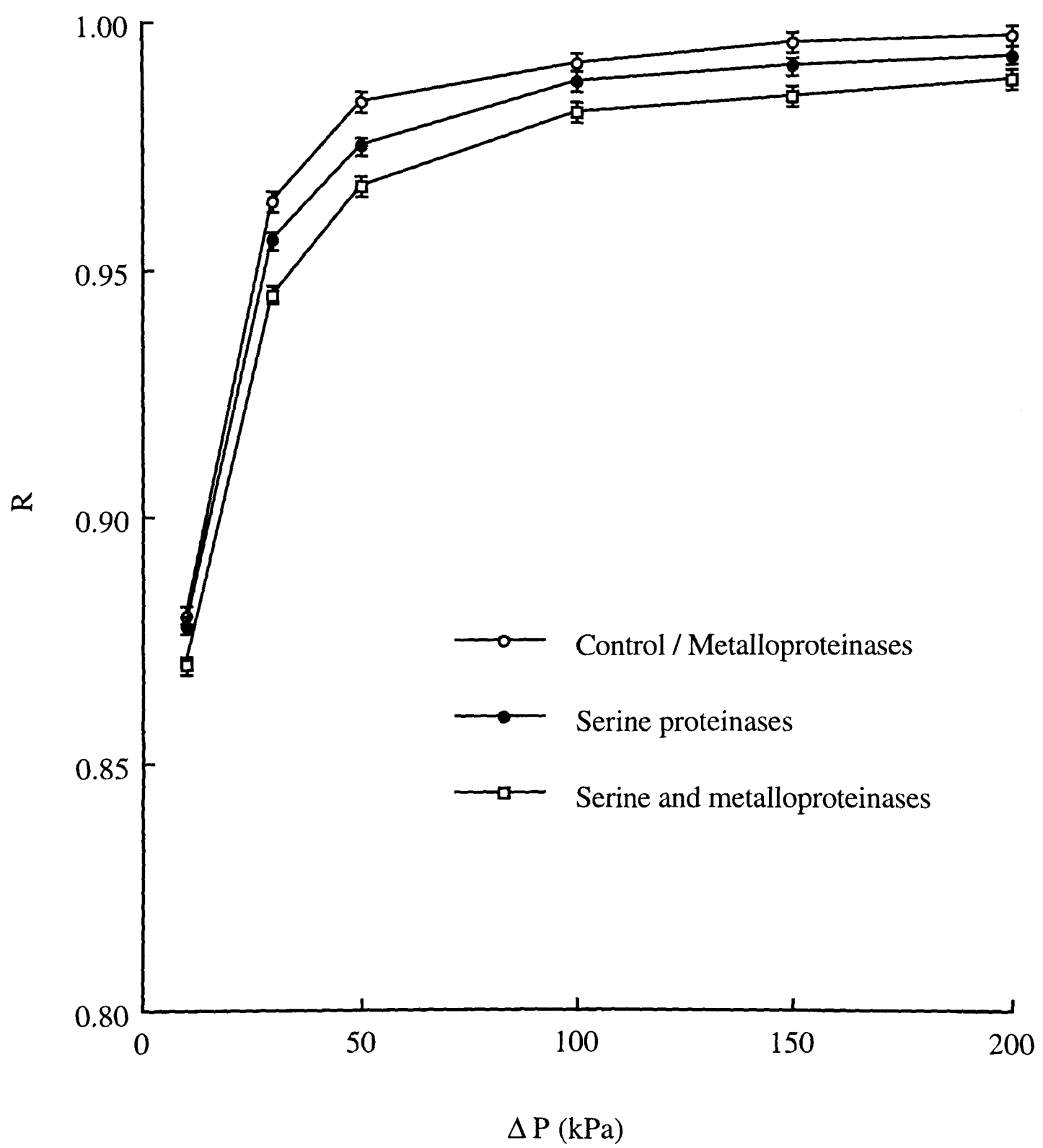
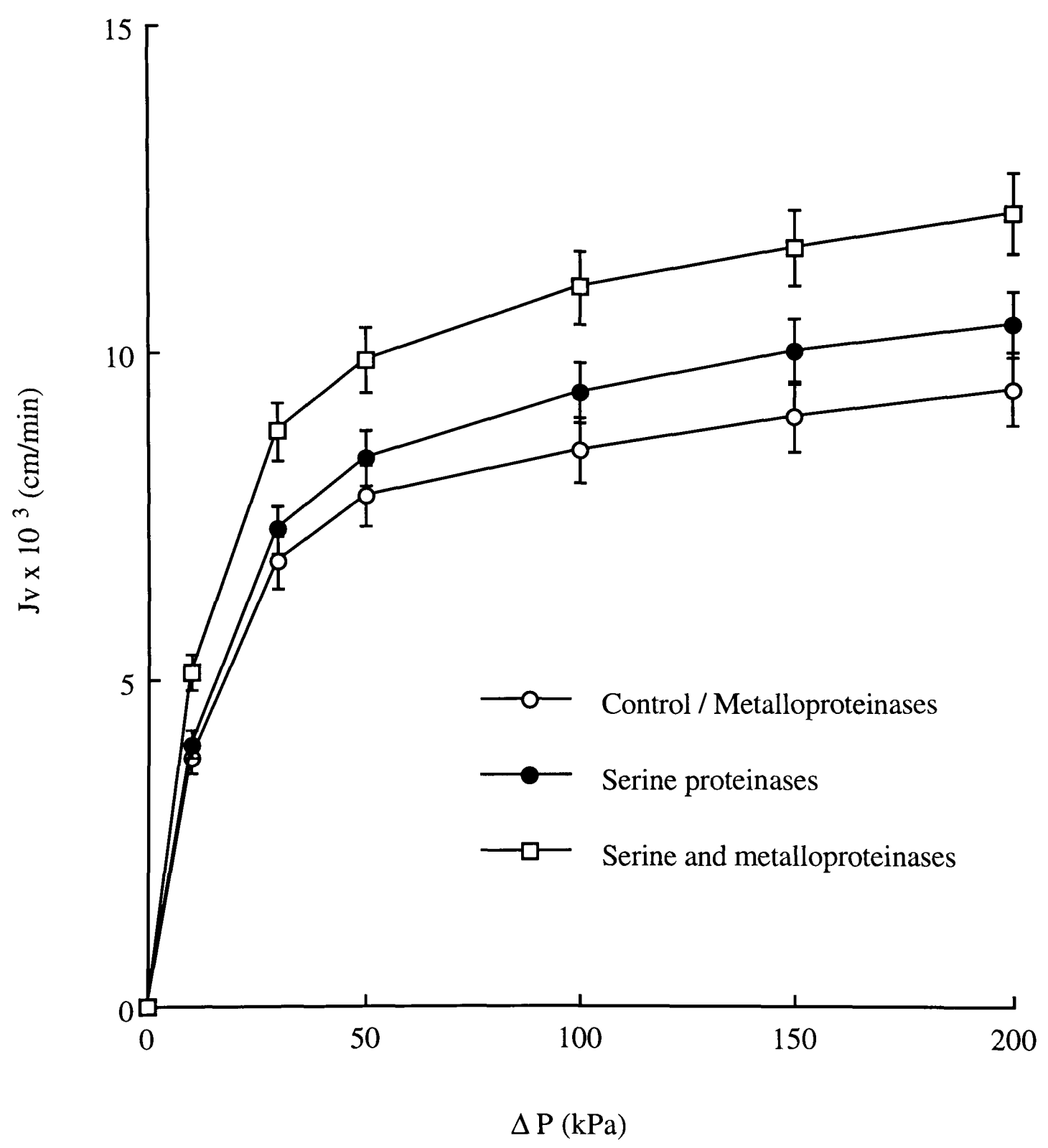


Fig. 7.9.



The intrinsic permeability of GBM films to water also increased following serine proteinase treatment, though metalloproteinases had no effect (Fig. 7.9.). Both types of proteinases working in concert rendered GBM films more permeable to water in spite of the lack of thickness difference to undigested GBM films (Fig. 7.9.).

Prolonged treatment with the various proteinases up to 12 hrs achieved a similar pattern of results except that the effects were more marked. This was evident from a comparison of serum protein fluxes, protein rejections, and water fluxes of proteinase treated films (Figs. 7.10.-7.12.) with those from the 6 hr digestions (Figs 7.7.-7.9.). A time-dependent increase in the intrinsic permeability of GBM to serum protein was effected by serine proteinases but not by metalloproteinases. The combined action of serine and metalloproteinases also resulted in a time-dependent increase in the intrinsic membrane permeability. This was greater than if serine proteinases were active alone.

In summary, a full complement of neutrophil proteinases are able to render GBM more permeable to serum protein by reducing its thickness (Section 7.6.) as well as by increasing its intrinsic permeability to serum protein.

7.8. Filtration studies involving BSA, myoglobin solutions, and TBS

Although an increase in membrane permeability to serum protein has been demonstrated following GBM proteolysis by neutrophil proteinases, it was not known precisely how the molecular sieving properties have changed. To shed light on this, dilute solutions containing proteins of different MWs were filtered. The proteins used were myoglobin (17.8k) and BSA (68k); they were dissolved in TBS at 0.5 and 2.0 mg/ml, respectively. TBS, pH 7.4 was also filtered. Digestion with the serine and metalloproteinases rendered GBM films more permeable to both proteins after 6 hrs of proteolysis as shown by increased myoglobin and BSA fluxes and reduced protein rejections (Figs. 7.13-7.16.). In addition, water fluxes were also increased (Fig. 7.17.), indicating increased film permeabilities to water. A similar pattern of results was observed after 12 hrs of proteolysis, though the increase in permeability to proteins and water was greater (Figs. 7.18.-7.22.), indicating that the effects were time-

Legend to Figs. 7.10.-7.12.

An analysis of intrinsic permeability of GBM to pig serum (70 mg/ml) following digestion with pig neutrophil proteinases for 12 hrs. Films made from GBM digested with proteinases were thinner than those treated with inactivated proteinases (controls) but had extra digested GBM added to give identical average thickness as the controls (see text). Figs. 7.10.-7.12. show the effects of proteinases on serum protein flux (J_s), rejection (R), and water flux (J_v), respectively. Each value is the mean of 9 observations, 3 from each of 3 separate films. Each error bar represents SD ($n = 9$). The control represents films constructed from GBM treated with either heat-denatured proteinases or proteinases inhibited with both PMSF and EDTA. Incubation with the metalloproteinases gave rise to no detectable difference in J_s , R , and J_v to the controls as indicated on the graphs.

Fig. 7.10.

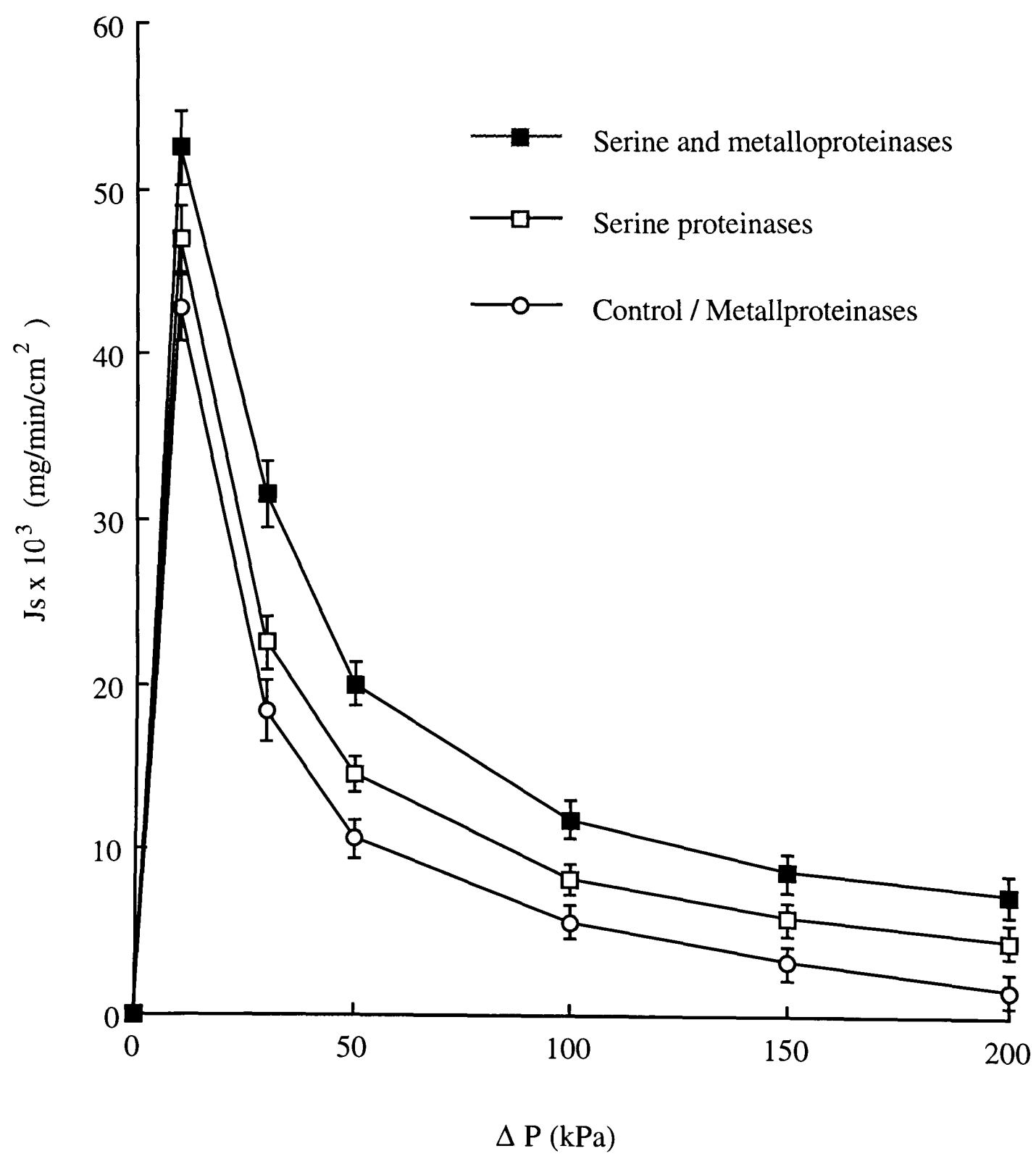


Fig. 7.11.

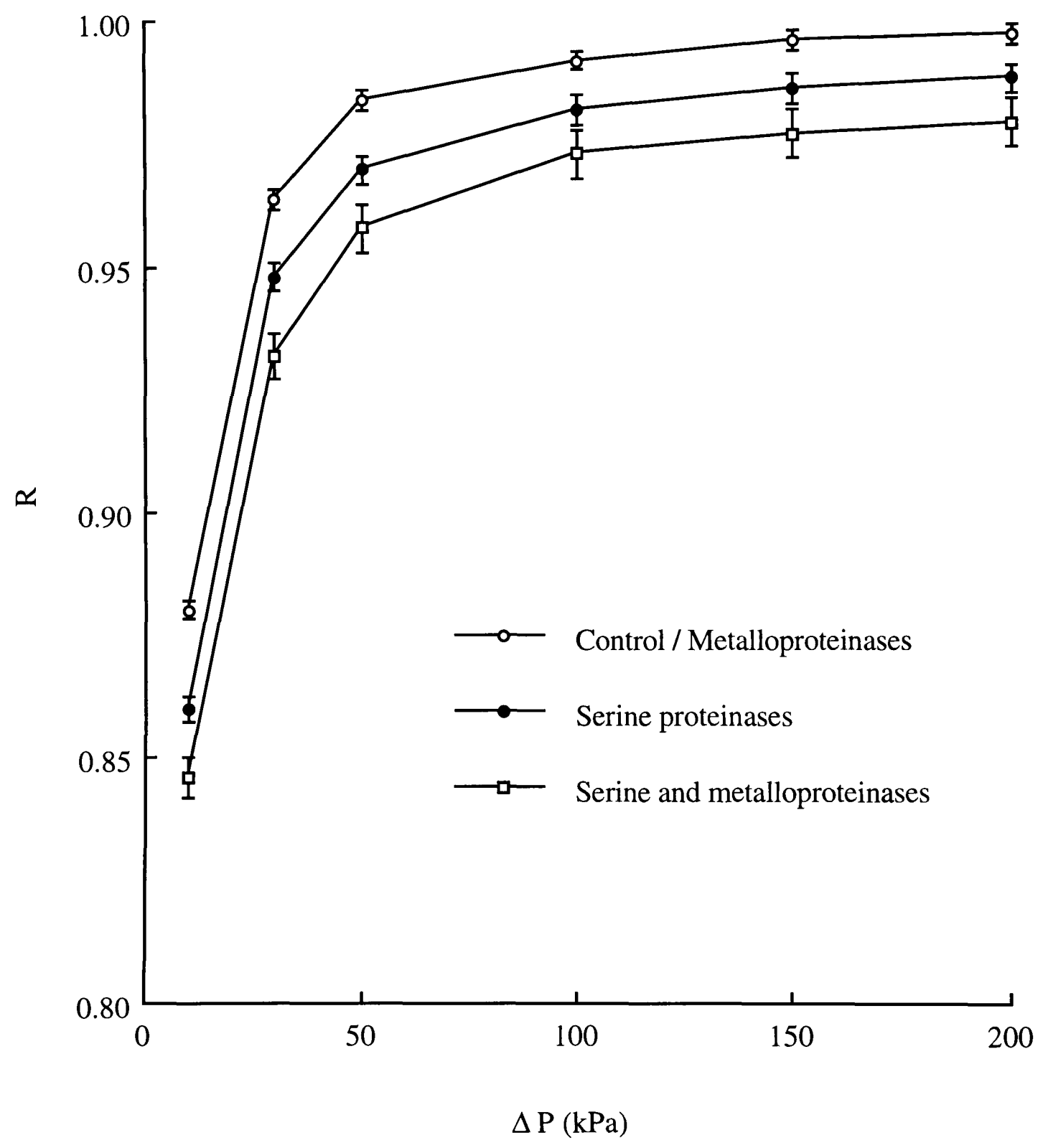
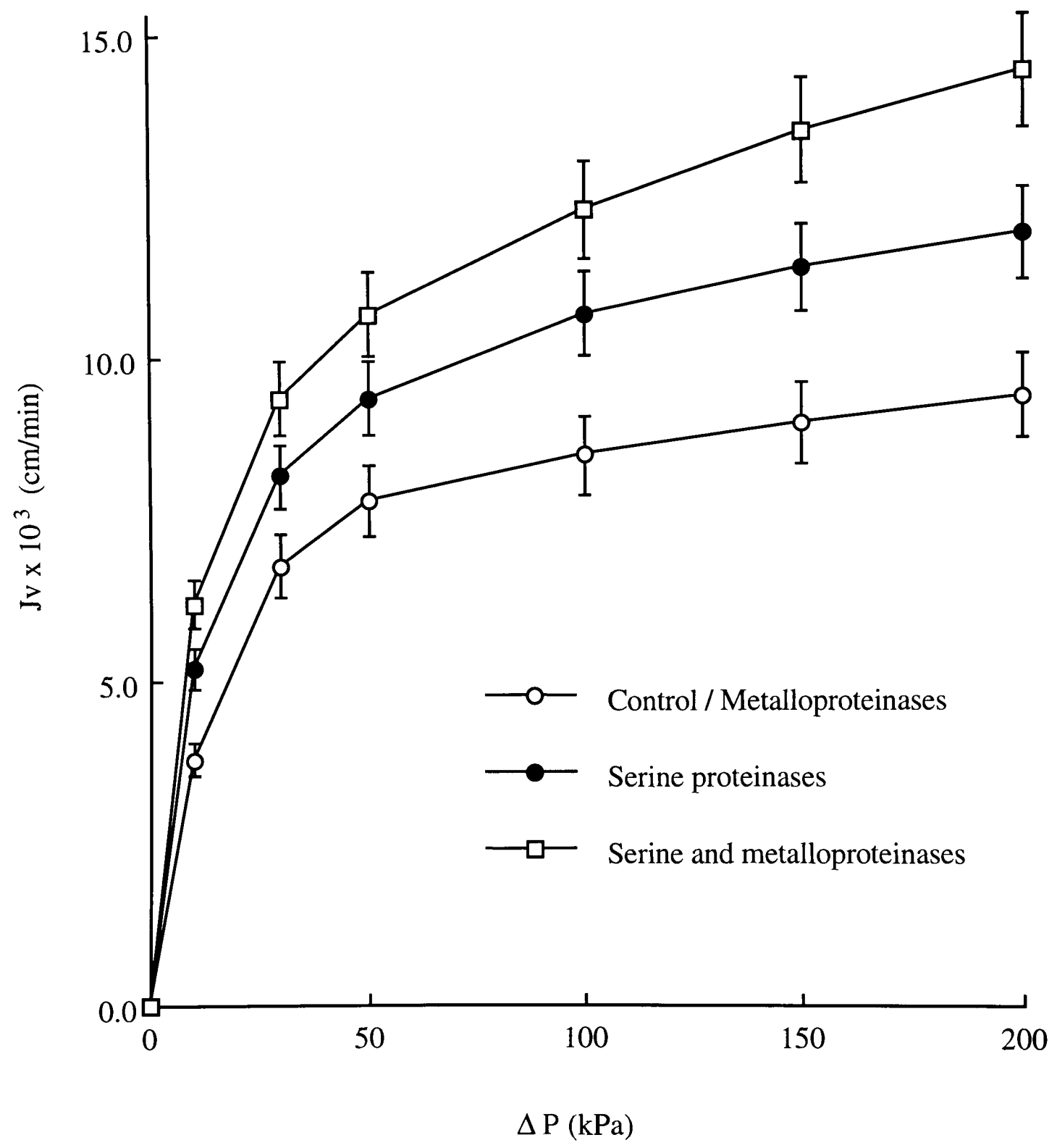


Fig. 7.12.



Legend to Fig. 7.13.-7.17.

The ultrafiltration behaviour of GBM for BSA, myoglobin, and water following digestion with pig neutrophil proteinases for 6 hrs . Figs. 7.13.-7.14. show the effect of proteinase digestion on myoglobin and BSA fluxes (J_s) across GBM films. Figs. 7.15.-7.16. show the effect of proteinase digestion on film rejection of myoglobin and BSA (R). Fig. 7.17. shows the effect of proteinase digestion on water flux (J_v) across GBM films in TBS ultrafiltration. The concentrations of BSA and myoglobin solutions filtered were 2.0 mg/ml and 0.5 mg/ml, respectively. Each value on the graphs is the mean of 9 measurements, 3 from each of 3 separate films. Each error bar represents SD ($n = 9$). The controls represent films constructed from GBM treated with either heat-denatured proteinases or proteinases inhibited with both PMSF and EDTA.

Fig. 7.13.

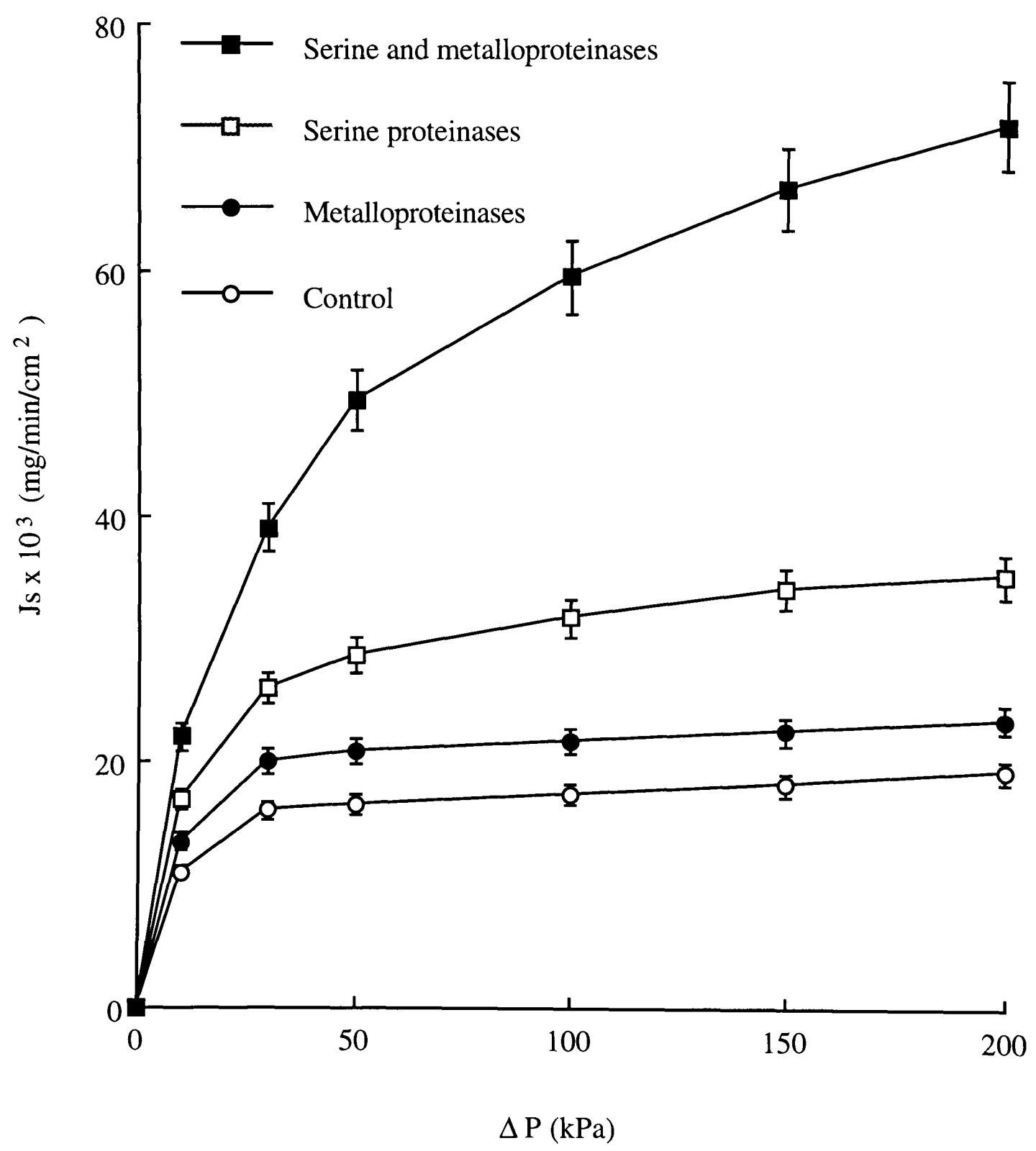


Fig. 7.14.

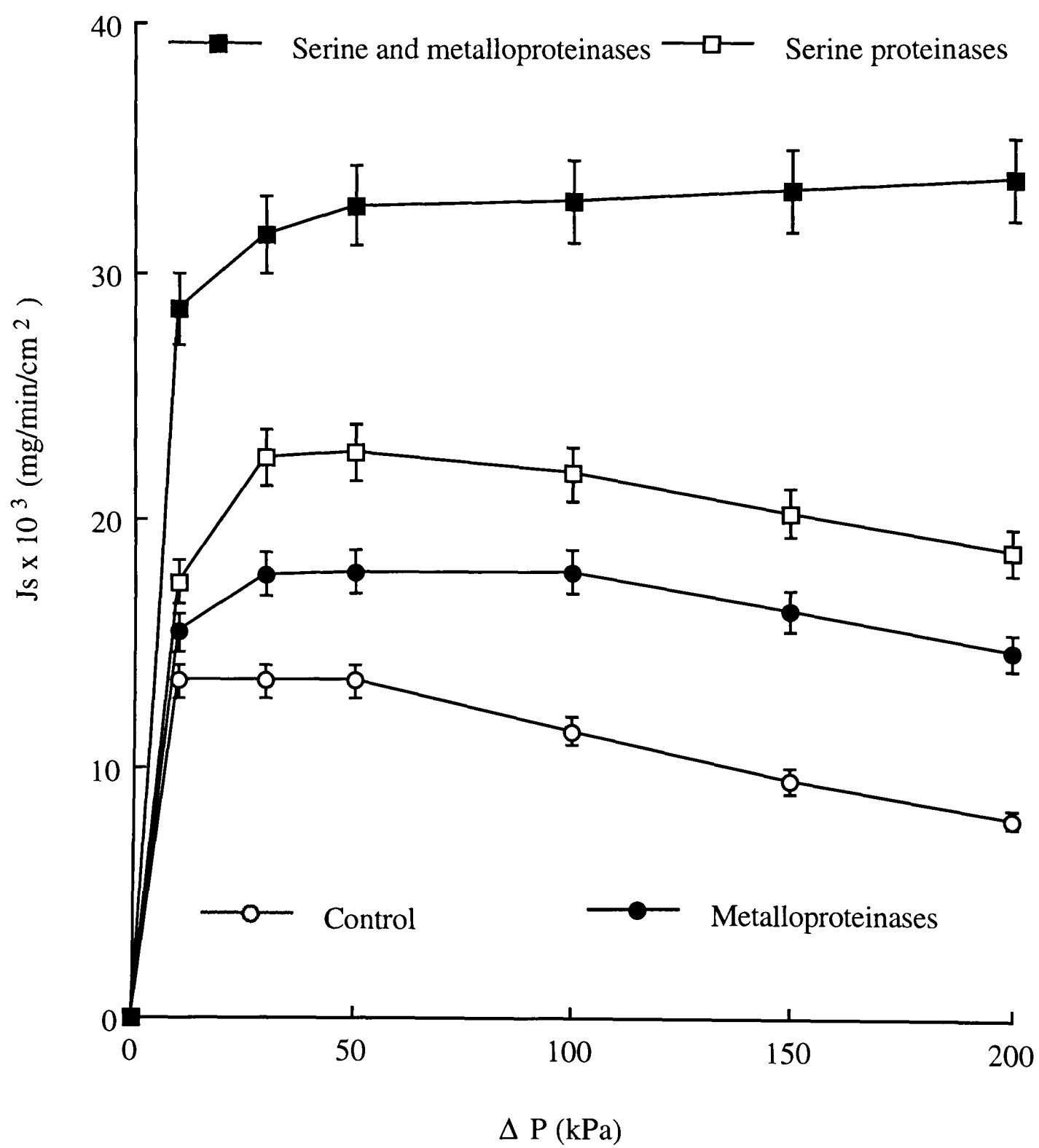


Fig. 7.15.

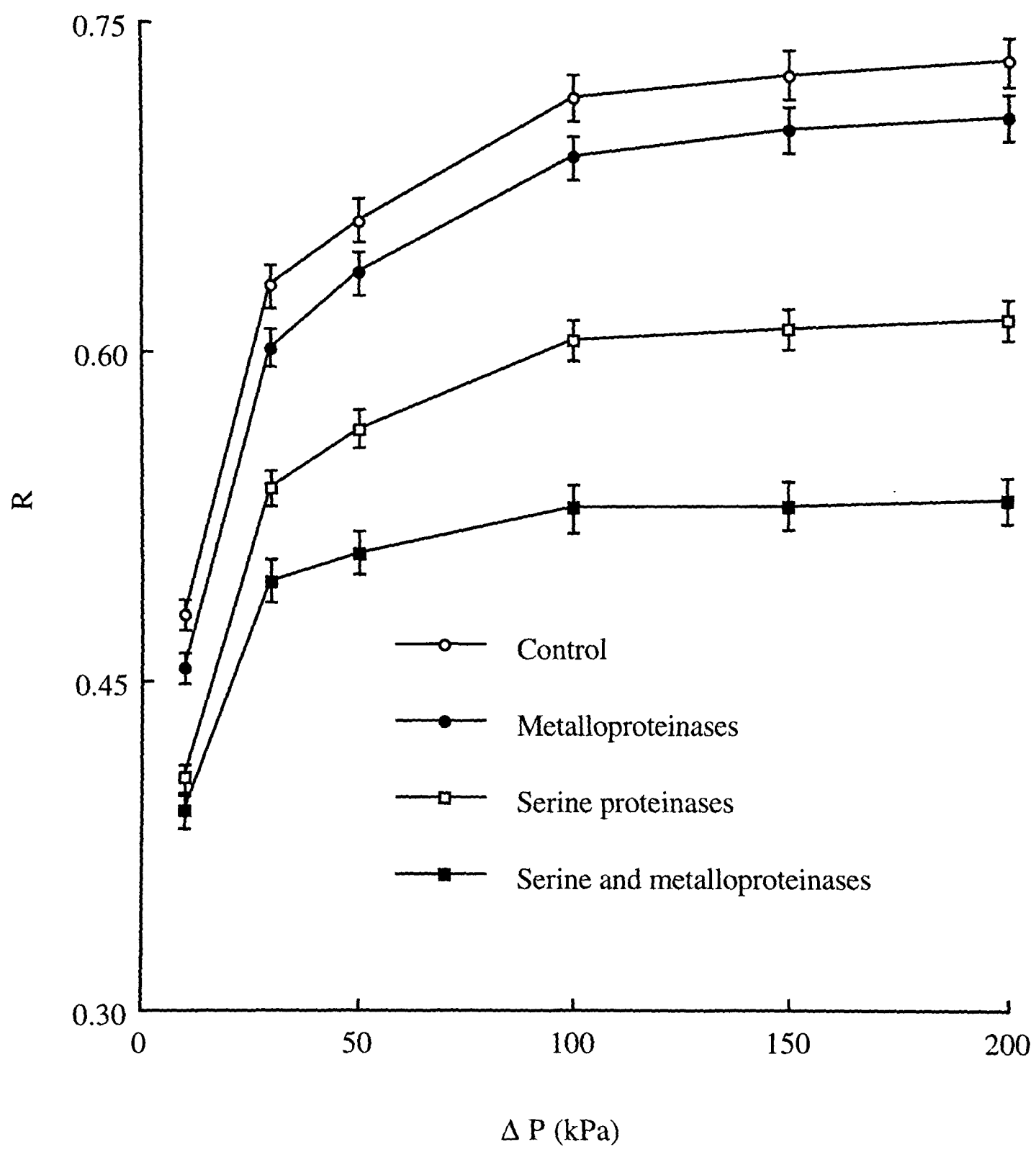


Fig. 7.16.

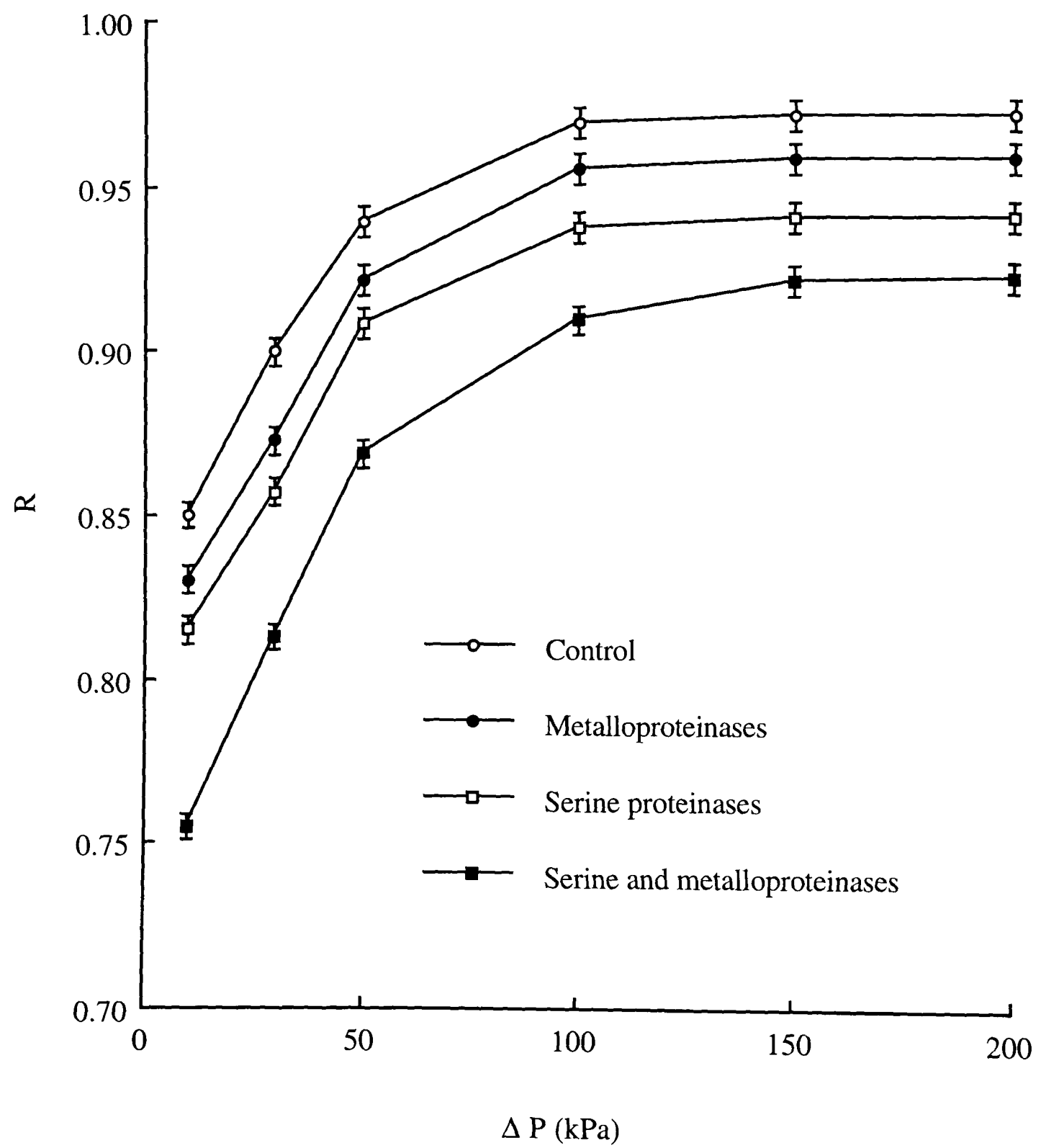
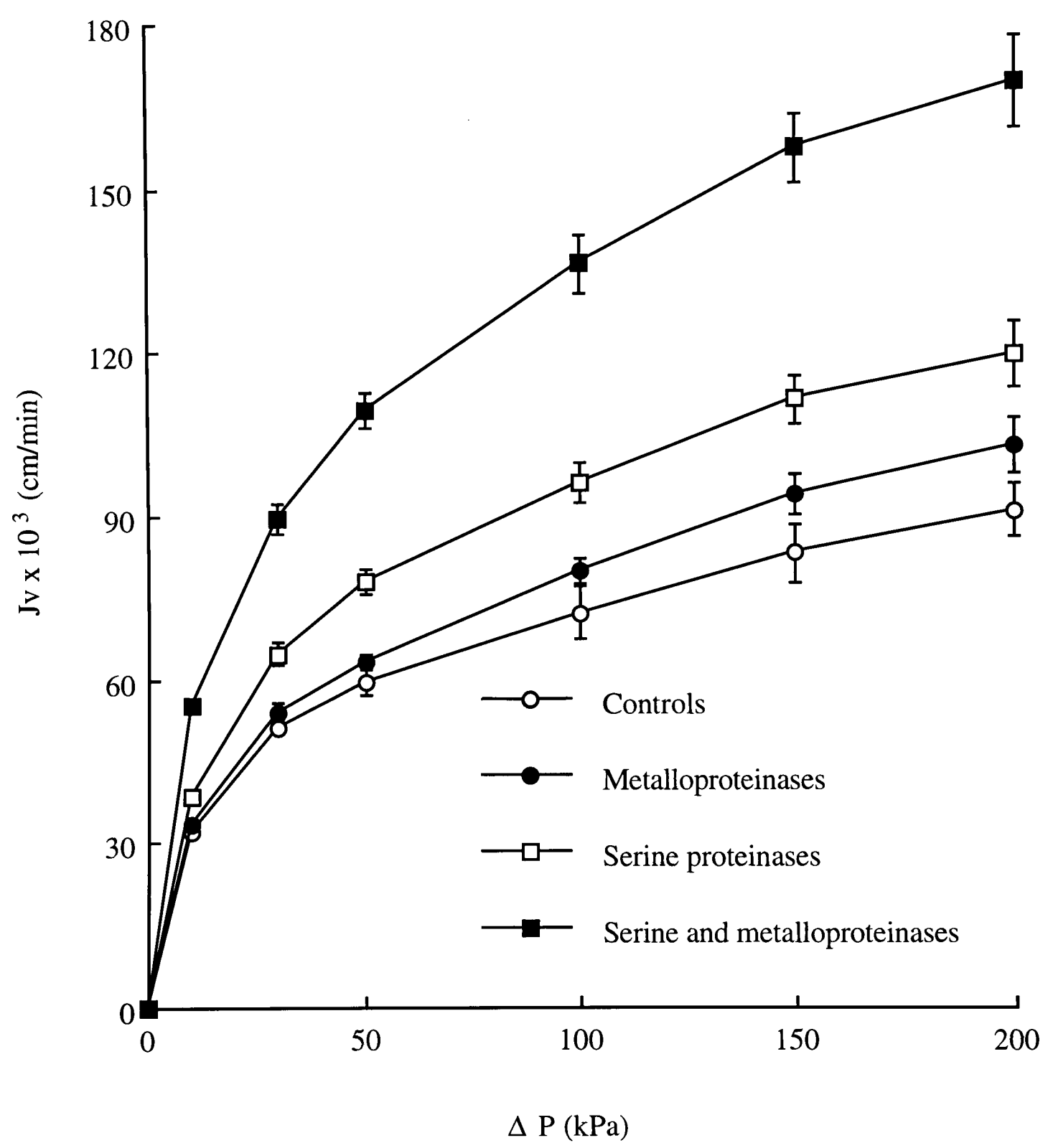


Fig. 7.17.



Legend to Fig. 7.18.-7.22.

The ultrafiltration behaviour of GBM for BSA, myoglobin, and water following digestion with pig neutrophil proteinases for 12 hrs . Figs. 7.18.-7.19. show the effect of proteinase digestion on myoglobin and BSA fluxes (J_s) across GBM films. Figs. 7.20.-7.21. show the effect of proteinase digestion on film rejection of myoglobin and BSA (R). Fig. 7.22. shows the effect of proteinase digestion on water flux (J_v) across GBM films in TBS ultrafiltration. The concentrations of BSA and myoglobin solutions filtered were 2.0 mg/ml and 0.5 mg/ml, respectively. Each value on the graphs is the mean of 9 measurements, 3 from each of 3 separate films. Each error bar represents SD ($n = 9$). The controls represent films constructed from GBM treated with either heat-denatured proteinases or proteinases inhibited with both PMSF and EDTA.

Fig. 7.18.

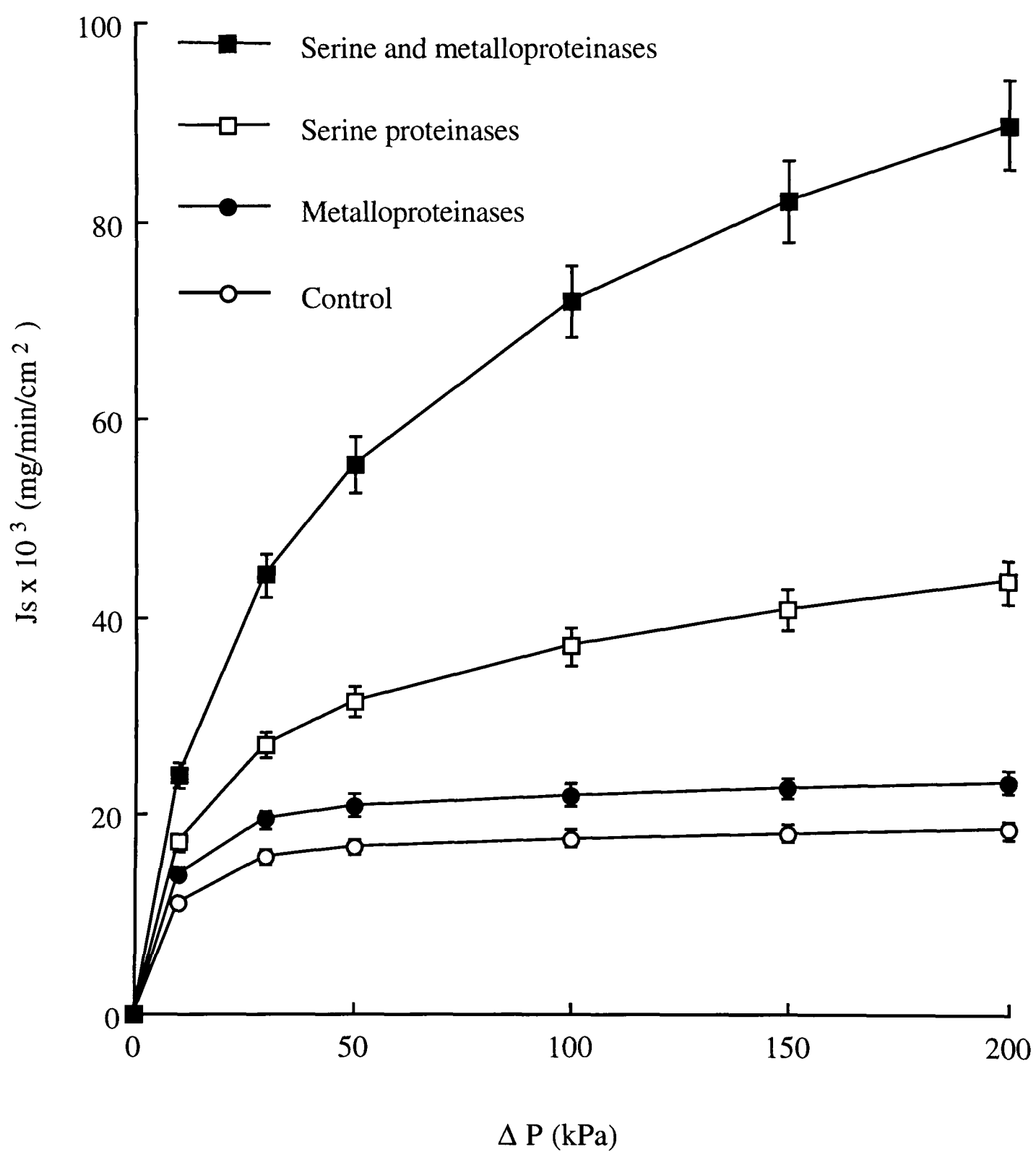


Fig. 7.19.

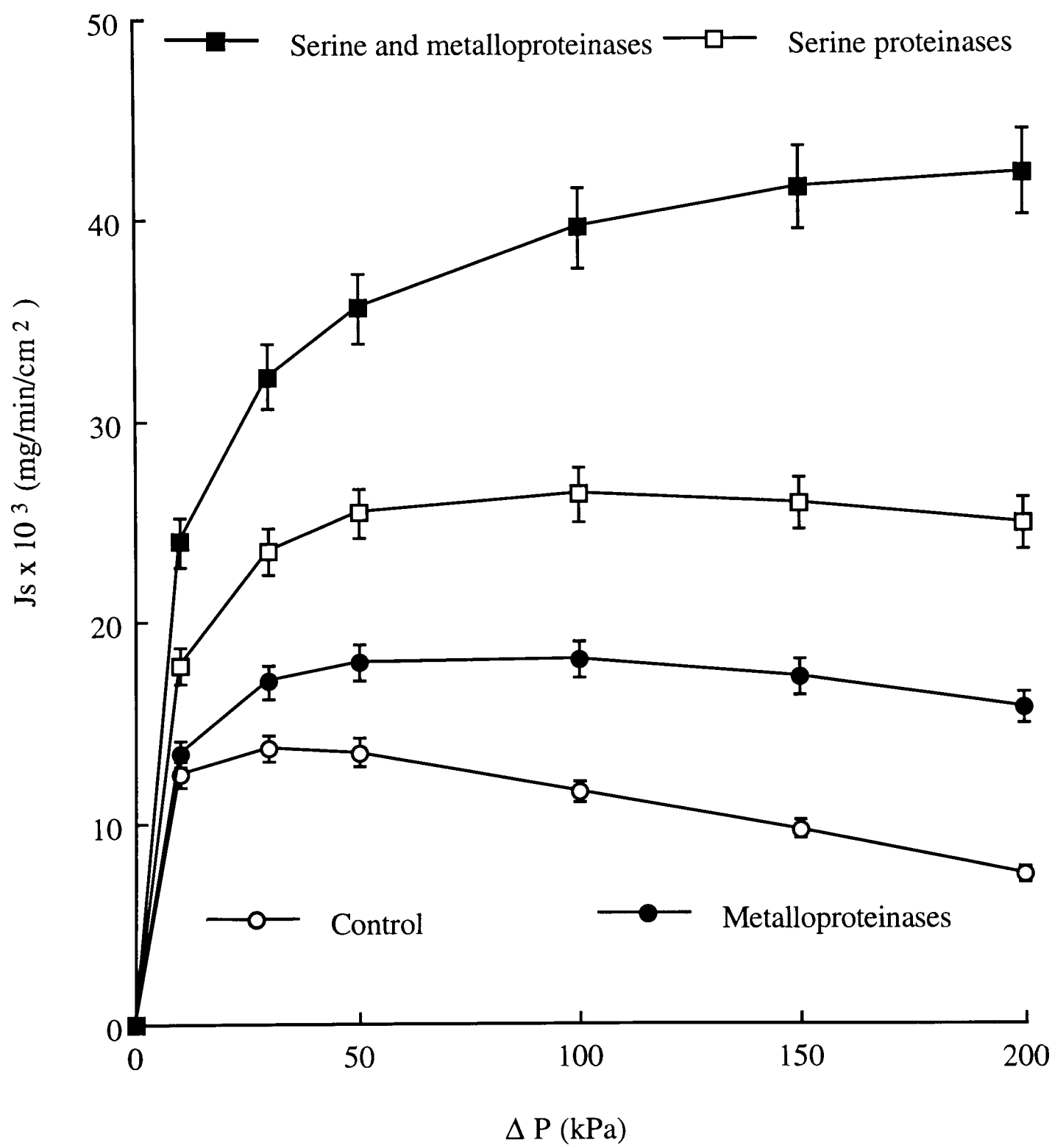


Fig. 7.20.

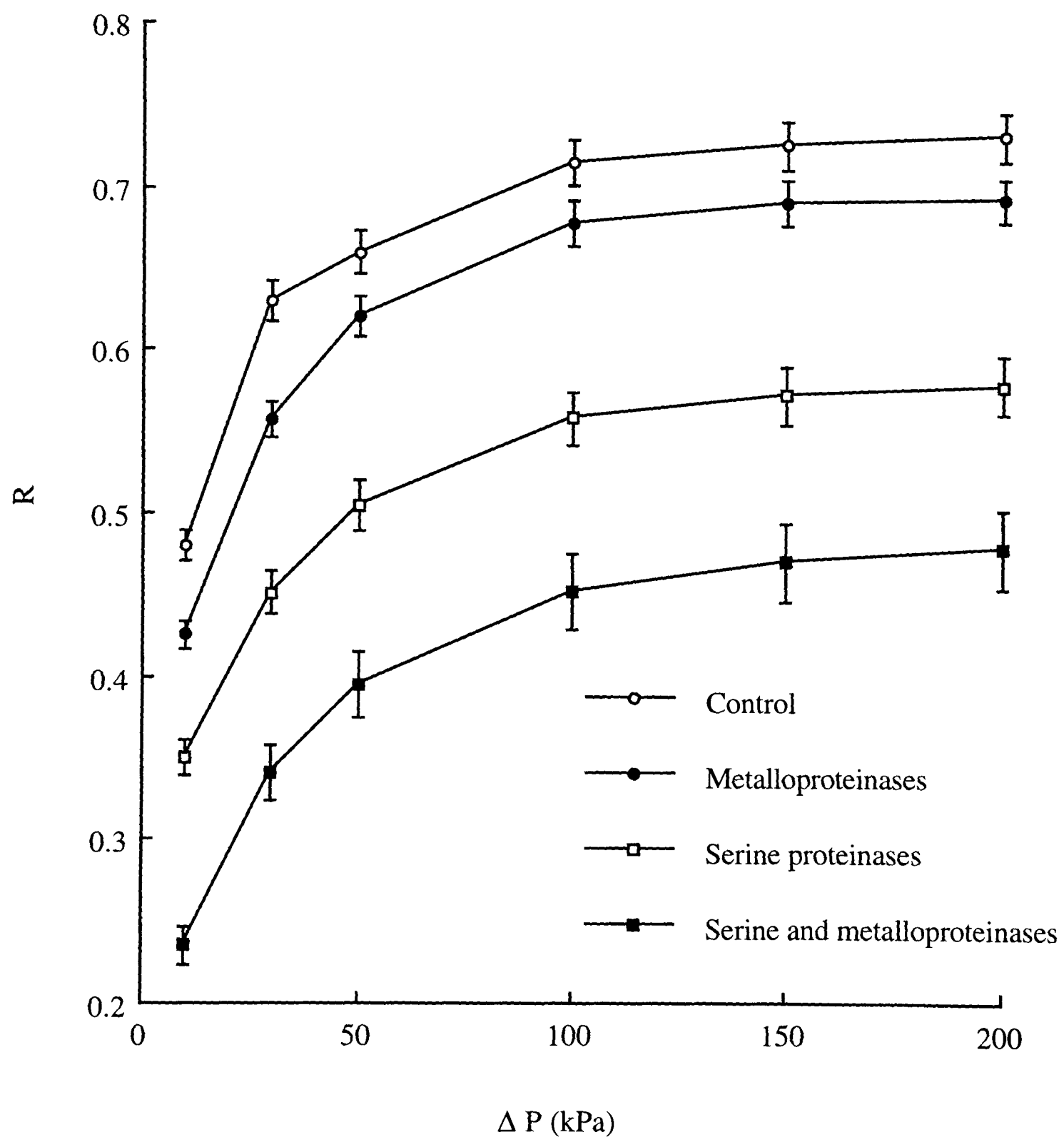


Fig. 7.21.

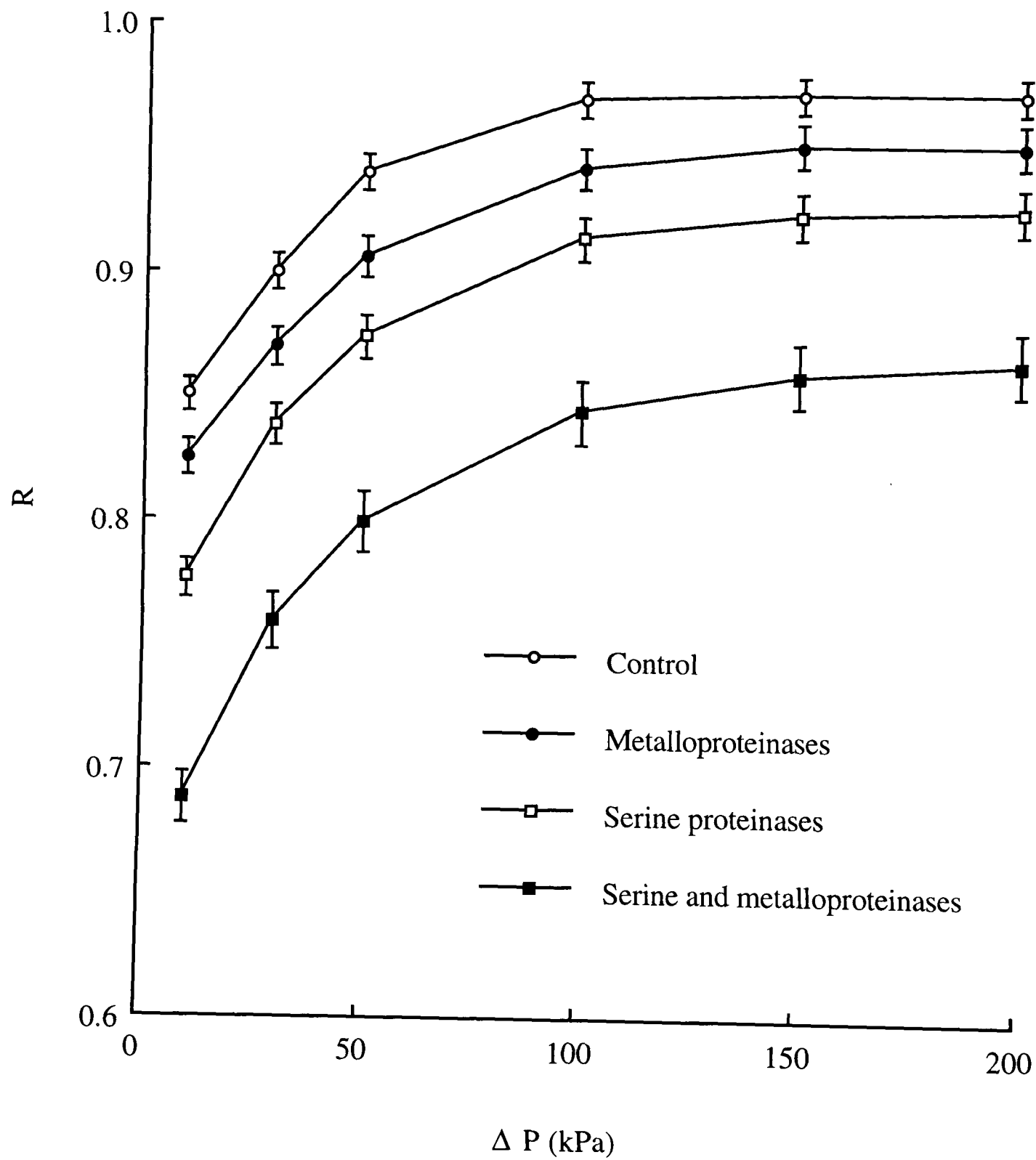
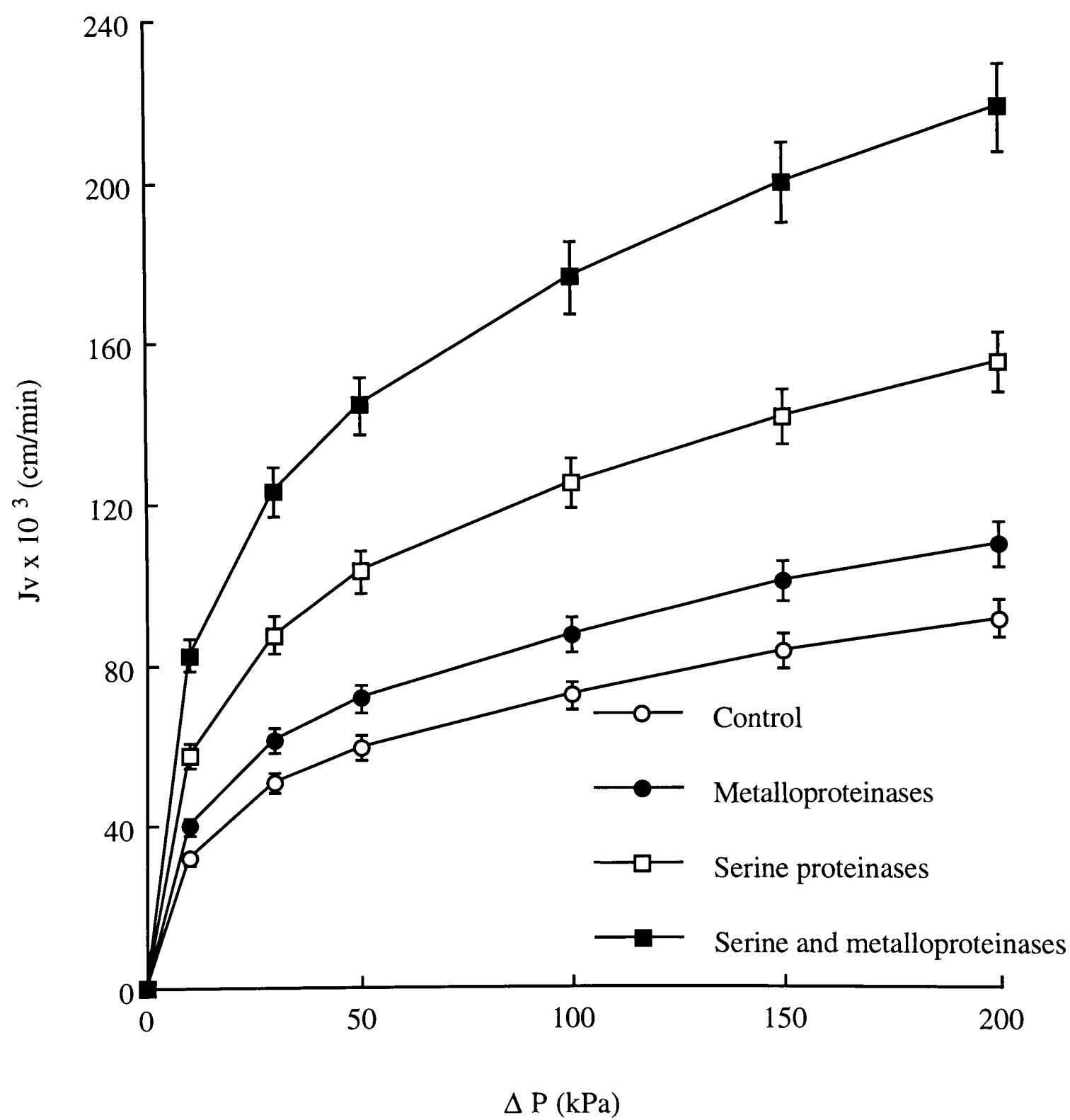


Fig. 7.22.



dependent. This showed that the changes in membrane permeability was the result of an increase in permeability to molecules of a wide range of MWs, ranging from H₂O to BSA. Potentiative effect was observed when both families of proteinases were working in concert.

7.9. Discussion

The data presented in this chapter provided the first direct evidence that both neutrophil metalloproteinases and serine proteinases analogous to human's were able to render GBM more permeable to serum protein. The use of high purity GBM in this study eliminated the possibility that the effect observed was due only to proteolysis of TBM, since the percentage of basement membranes degraded (~ 35 % after 6 hrs and ~ 40 % after 12 hrs) exceeded that of TBM impurity (< 10 %). That the use of a proteinase to GBM ratio approximating that likely to be achieved under pathophysiological conditions *in vivo* was able to effect a marked increase in the permeability of the GBM to serum protein, suggests that neutrophils could promote proteinuria in diseased states by releasing their proteinases onto the subjacent basement membrane. Permeation analysis using buffer and simple protein solutions showed that proteolysis resulted in an increase in the porosity of GBM to molecules of a wide range of MW ranging from water to BSA (68 kda). The increase in the permeability of the membrane to BSA was consistent with the idea that neutrophil proteinases digested GBM *in vivo*, causing albuminuria in immune GN (Cotran et al., 1994).

Cotter and Robinson showed a similar effect using rabbit neutrophil neutral proteinases (Cotter and Robinson, 1980b) but the proteinase to GBM ratio used was arbitrary. A proteinase to GBM ratio of 100 units of *p*-nitrophenol-*n*-tertbutoxycarbonyl-l-alaninate (NBA)-esterase activity : 6 mg GBM (dry weight) was employed where 1 unit of NBA-esterase was taken as the hydrolysis of 1 μ mol of the synthetic elastase substrate NBA per min. at pH 6.5, 37°C. Brown measured the NBA-esterase activity in rabbit neutrophils and found that 1 x 10⁹ neutrophils contained ~ 7 units of the activity (Brown, 1984). Hence were a neutrophil to exocytose all of its

proteinases onto $\sim 8 \times 10^{-9}$ mg of GBM at the zone of adhesion (calculated in Section 7.2.), the proteinase to GBM ratio would be ~ 5.25 units of NBA-esterase activity : 6 mg GBM. The ratio used was ~ 19 times greater than the estimated theoretical maximum. Thus there was a doubt of whether a more pathophysiological proteinase to BM ratio would still have resulted in an increase in membrane permeability to serum protein. The uncertainty was resolved here since a marked effect was still observed when a more pathophysiological proteinase to GBM ratio was used.

The question raised by Cotter and Robinson (1980b) relating to how the increase in membrane permeability was effected has been addressed here. When both families of proteinases were allowed to digest the GBM, the increase in the permeability of the membrane to serum protein appeared to be due to a combination of membrane thinning and an increase in the intrinsic permeability of the membrane. When only serine proteinases were allowed to digest the GBM, membrane thinning and an increase in the intrinsic permeability of the basement membrane was also seen. The metalloproteinases, by contrast, were only able to effect a thickness reduction but had no effect on the intrinsic permeability of the membrane. Conceivably, the serine proteinases were able to increase the intrinsic permeability of the GBM because they were small enough (MW ~ 30 k) (Barrett and McDonald, 1980) to diffuse into the membrane matrix and nick polypeptides, creating larger channels through which protein molecules could permeate. The activated metalloproteinases were larger (gelatinase, MW 88 k; collagenase, MW 64 k) (Murphy et al., 1989; Macartney and Tschesche, 1983a) and might not have been able to diffuse into the matrix and so their effect might only be limited to the matrix surface, causing only membrane thinning.

Another interesting feature to emerge from this study was the ability of the two families of proteinases synergistically to increase the permeability of the GBM to serum protein (Section 7.5.). The synergism was also seen in GBM proteolysis by these proteinases (Section 7.4.). This does not appear to be due to the result of serine proteinase activation of the metalloproteinases. One explanation for this is that one family of proteinases sterically uncovered proteolytic sites for the other proteinases.

Serine proteinases, being smaller than the metalloproteinases might have been more effective at opening up the matrix for the metalloproteinases. The cooperativity between these proteinases seemed effective in releasing proteins from the GBM structure, presumably creating large channels for serum protein permeation which probably accounted for the marked increase in serum protein flux across the GBM.

The important point demonstrated in this chapter was that the effect of neutrophil proteinases on the permeability of GBM can be quantitated using the filtration model. The use of a proteinase to GBM ratio approximating pathophysiological conditions *in vivo* was able to effect a marked increase in protein flux across the GBM, thus satisfying the requirement for being able to cause proteinuria. This effect appeared to be due to the release of proteins from the membrane matrix, reducing its thickness and increasing its porosity and hence its intrinsic permeability to protein.

CHAPTER EIGHT

CHAPTER 8 GENERAL DISCUSSION

8.1. The filtration model

The main aim of this thesis was to determine if certain test agents, suspected of being able to render GBM more permeable to protein, could do so and thereby promote proteinuria. An *in vitro* perturbation analysis of GBM was carried out and the permeability of the membrane assessed using an *in vitro* model of glomerular ultrafiltration. Although the ultrafiltration model is unphysiological, its use seems justifiable for two reasons. Firstly, under appropriate filtration conditions GBM films mimic physiological ultrafiltration. For example, the GBM film exhibits size-dependent discrimination of protein molecules of varying MW. The permeability of the film approaches zero as the MW of the protein molecule reaches ~ 70 k (Chapter 3; Robinson and Walton, 1989), a property which the glomerular capillary wall is known to display (Brenner et al., 1978; Deen et al., 1985).

Secondly, this method permits carefully controlled perturbation analysis of GBM to be carried out in a manner impossible to effect *in vivo*. For example, GBM may be treated with proteinases, and the effects of such treatments on membrane permeability examined. Such controlled experiments would be impossible to effect *in vivo* since test agents, e.g., proteinases, may also perturb cells of the glomerular capillary wall. These cells may then release their own proteinases or reactive oxygen species onto the GBM (Baricos and Shah, 1991) which could, in turn, increase the membrane permeability. The cells may also release autocrines which may alter the renal haemodynamics (Baricos and Shah, 1991) and hence the ultrafiltration conditions. This may subsequently alter the flux of plasma protein across the GBM (Cotter, 1979). The *in vitro* analysis side-stepped these complications and permitted a study of the direct effect of test agents on the permeability of the GBM.

8.2. The effect of TGase on the permeability of GBM

Following the demonstration that GBM could be rendered more permeable to serum protein by chemical cross-linkers (glutaraldehyde and dimethyl malonimidate), Walton et al. (1992) speculated that cross-links may be introduced into the GBM in GN, giving rise to proteinuria. The biological cross-linker guinea pig liver TGase was the first test agent used to determine if it could alter the permeability of GBM. It has been shown that activated plasma TGase (coagulation factor XIIIa) is present in nephritic glomeruli (Kamitsuji et al, 1983; Colasanti et al., 1987; Deguchi et al., 1989). Monocytes and platelets, rich in TGases (Henriksson et al., 1985; Greenberg and Shuman, 1984), have also been shown to be present in nephritic glomeruli (Hunsicker et al., 1979; Holdsworth and Neale, 1984; Watanabe and Tonaka, 1976; Miller et al., 1980). Monocyte death (Salin and McCord, 1975; Clark and Klebanoff, 1977; McCord and Salin, 1977) and platelet degranulation (Nachman and Polley, 1979) at site of inflammation may release TGases onto GBM. This study was prompted by the interesting possibility that the TGases may introduce cross-links into the GBM, rendering it more permeable to protein, and so providing a novel mechanism of mediating proteinuria.

In a pilot study, guinea pig liver TGase was used as a model enzyme for studying the effect of TGases on GBM permeabilities. Its use seemed justifiable for several reasons. Firstly, all of the TGases exhibit very similar substrate specificities (Tyler and Laki, 1966; Lorand et al., 1966; Francis and Marder, 1987; Conkling et al., 1989). The effect of guinea pig liver TGase on GBM is thus likely to reflect that of the other TGases. Secondly, the liver TGase is easily obtainable in large quantities (Folk and Chung, 1985). Finally, the liver enzyme has been found to be almost 18 x more active than plasma TGase, judged by cross-linking ^{14}C -putrescine to casein (Chung, 1972). Permeability studies showed that purified guinea pig liver TGase significantly reduced water flux and myoglobin flux but had no detectable effect on BSA flux or serum protein flux across GBM films. It would appear that, unlike the

chemical cross-linkers, the TGase was unable to render GBM more permeable to protein and water.

The reduction in J_s and J_v was unlikely to be due to concentration polarization being more severe on the test GBM films since the filtration conditions, e.g., stirring rate and filtration pressure, that could affect its severity were identical to the control. Moreover, if concentration polarization was more severe, an increase in protein flux (J_s) and a reduction in water flux (J_v) might be expected. In contrast, J_s decreased rather than increased following TGase treatment of GBM. Therefore, the decrease in J_s and J_v was more likely to reflect a change in the GBM permeability to protein (myoglobin) and water.

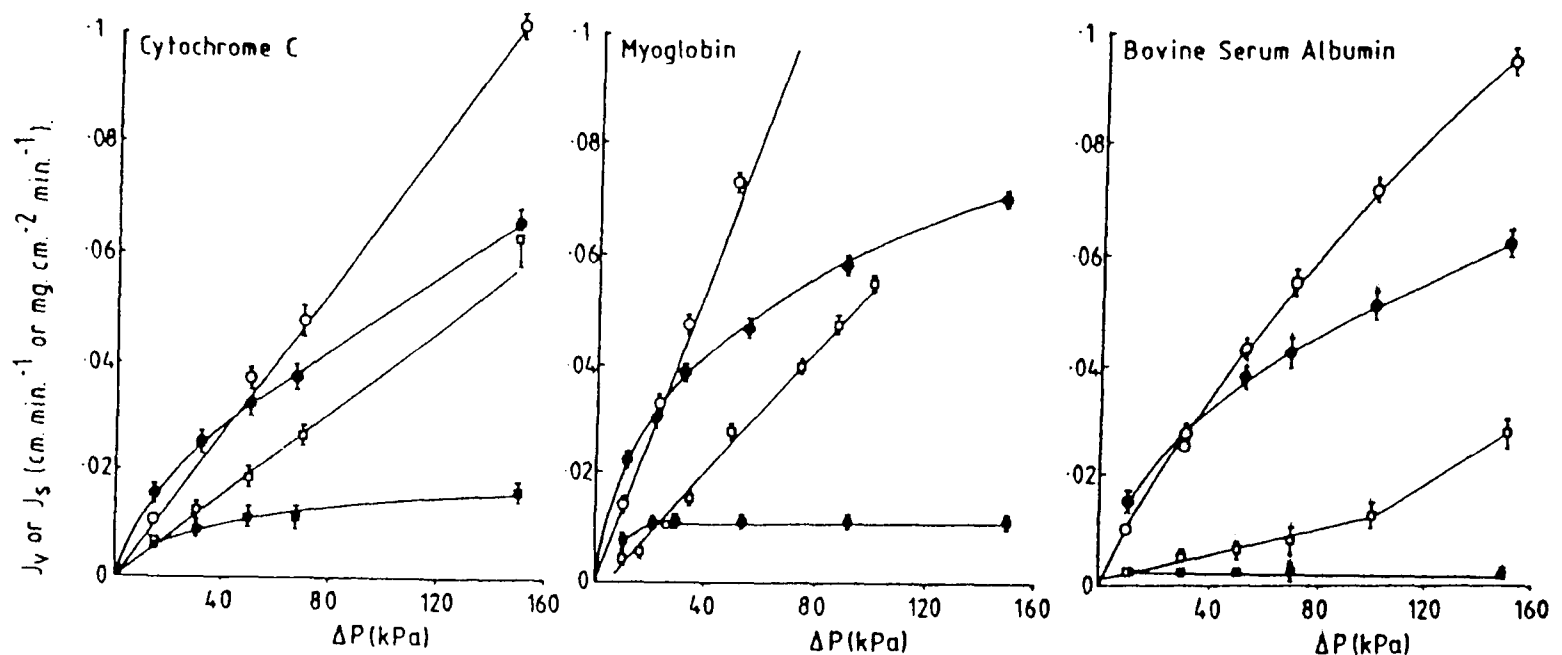
That the effects of liver TGase and chemical cross-linking on the permeability of GBM to protein and water are contradictory proved difficult to explain. However, the difference is most likely be due to the differing extent of cross-linking effected by the two families of cross-linkers, reasoned as follows. The liver TGase is a large enzyme (MW ~ 75 k) with high substrate specificity (Greenberg et al., 1991) as compared with glutaraldehyde, a small flexible molecule ($\text{CHO-CH}_2\text{-CH}_2\text{-CH}_2\text{-CHO}$, MW 100) with low substrate specificity (Walton et al., 1992). As such, the chemical cross-linker would diffuse more easily into the GBM fibre matrix, introducing more cross-links than the enzyme. If cross-linking is more extensive, one might expect the neighbouring fibres to be drawn closer together, causing the membrane to become more contracted and thinner. Indeed, this was observed; glutaraldehyde caused ~ 34 % reduction in GBM film thickness (Walton et al., 1992) as compared with ~ 7 % by liver TGase (Section 4.5.).

Robinson and co-workers postulated that cross-linking of the GBM fibre matrix draws neighbouring fibres closer together, contracting the matrix, increasing the fibre packing density, and effectively reducing the membrane void volume through which protein and water can permeate (Robinson et al., 1985; Robinson and Walton, 1987; Walton et al., 1992). However, if the cross-linking is so extensive that it renders the matrix rigid and incompressible, then this would have a second effect. As filtration

pressure increases, the rigid, heavily cross-linked matrix would resist compression, tending to maintain its void volume (Walton et al., 1992). At high pressures, the void volume may actually be larger than that of a compressed uncross-linked matrix, permitting more protein and water to permeate across the structure. This supposition was supported by thickness measurements and permeability analysis on glutaraldehyde-treated and native GBM films at high pressures. At 150 kPa, for example, the cross-linked GBM films ($4.9 \pm 1.0 \mu\text{m}$) were thicker than native films ($3.3 \pm 0.9 \mu\text{m}$) (Walton et al., 1992) and so the former would have larger void volumes. Protein and water fluxes were greater across glutaraldehyde-treated GBM films than across the native GBM films at high pressures (Fig. 8.1.), consistent with what is expected of a matrix with a higher void volume. At low pressures, the situation is reversed: uncross-linked native GBM films are relatively uncompressed, and have greater void volumes than films heavily cross-linked by glutaraldehyde. This could be adduced from the fact that the native films have thicknesses $> 6.5 \pm 0.8 \mu\text{m}$ at low pressures ($\Delta P < 30 \text{ kPa}$), thicker than the cross-linked films which have thicknesses of $5.0 \pm 0.5 \mu\text{m}$ at the same pressures (Walton et al., 1992). Native GBM would be expected to be more permeable to protein and water under the conditions. This is in fact observed as J_s and J_v across native GBM films are somewhat higher than those across glutaraldehyde-treated films at low pressures (Fig. 8.1.).

Films made from GBM treated with the liver TGase seemed less heavily cross-linked since they are less condensed than glutaraldehyde-treated films. TGase-treated GBM would thus be expected to be more compressible. Robinson and Walton (1989) showed that compressible GBM films exhibited non-linear increases in water flux with increased pressure. TGase-treated GBM films exhibited a similar flux characteristics, indicating that they are also compressible. As they are not rigid like the glutaraldehyde-treated films, their matrix would tend to collapse as pressure increases. Their void volumes may be smaller than the native matrix at high pressures, permitting less water and protein to cross the structure. This was observed since water and protein

Fig. 8.1. Ultrafiltration behaviour of GBM for individual proteins before and after cross-linking with glutaraldehyde



Films of native pig GBM were prepared in ultrafiltration cells and their ultrafiltration properties studied at different filtration pressure using the proteins indicated at 0.5 mg/ml in TBS, pH 7.4 (except for bovine serum albumin: 2.0 mg/ml). The films were then cross-linked *in situ* with glutaraldehyde and ultrafiltration again studied.

● and ○ show water flux (J_v) for native and cross-linked films, respectively.
 ■ and □ show protein flux (J_s) similarly. The bars indicates the standard deviation for 3 measurements from each of 3 filters. Where standard deviations were very small the bars were omitted for clarity. The graphs are from Walton, H.A., Byrne, J., and Robinson, G.B. (1992) Studies of the permeation properties of glomerular basement membrane: cross-linking renders glomerular basement membrane permeable to protein. *Biochim. Biophys. Acta* **1138**, 173-183.

(myoglobin) fluxes are notably lower across films made from guinea pig liver TGase-treated GBM than the control (Figs. 4.1.-4.3.).

The results show that guinea pig liver TGase does not render GBM more permeable to serum protein. If anything, TGase treatment rendered the membrane less permeable to low MW protein and water. Thus TGase is unlikely to promote proteinuria. It could be argued that the liver TGase behaves differently from plasma, platelet or monocyte TGase. This is considered unlikely for two reasons. First, all of the aforementioned TGases share similar substrate specificities. They all show comparable mode of action in cross-linking fibrin (Tyler and Laki, 1966; Lorand et al., 1966; Francis and Marder, 1987; Conkling et al., 1989) and cross-linking the polyamine putrescine to casein (Chung, 1972; Harris et al., 1984; Folk and Chung, 1985; Conkling et al., 1989). Secondly, activated plasma, platelet, and monocyte TGases (MWs 142 k, 142 k, 77 k, respectively) are generally larger than the liver TGase (MW 75 k) (Folk and Chung, 1985; Greenberg et al., 1991). The former would be less able to diffuse into the GBM matrix and would be less effective in introducing cross-links. They are thus less likely to have an effect on GBM permeability. This further weakens the argument that the TGases are able to mediate proteinuria if exposed to GBM *in vivo*. The focus of the study was then switched to the second test agent H₂O₂.

8.3. The effect of H₂O₂ on GBM permeability

Neutrophil-derived H₂O₂ has been implicated as a mediator of proteinuria in neutrophil-dependent forms of GN (Couser, 1993) since the administration of catalase (H₂O₂ scavenger) significantly reduced proteinuria in nephritic animals (Rehan et al., 1984; 1985a & b; 1986). High concentrations of H₂O₂ have been shown to solubilize rat GBM *in vitro* (Fligiel et al., 1984). The evidence suggests that activated neutrophils may be able to generate sufficiently high concentrations of H₂O₂ at the neutrophil-GBM interface during respiratory burst to solubilize the GBM and render it more permeable to

protein, causing proteinuria. The effect of H_2O_2 on GBM permeability was analyzed here using the filtration system to test this hypothesis.

By incubating GBM films with a range of H_2O_2 concentrations at 37°C , over a range of incubation times, it was found that the minimum H_2O_2 concentration needed to render GBM films more permeable to serum protein and albumin was 1 M and the minimum incubation time was 6 hrs. To cause an increase in GBM permeability to the low MW marker protein myoglobin (17.8 k) and water, the minimum H_2O_2 concentrations were only 0.1 M and 0.05 M, respectively, though the minimum incubation time was still 6 hrs. An analysis of the respiratory burst characteristics of activated neutrophils based on a number of experimental models showed that the likely average concentration of H_2O_2 to which GBM is exposed in GN is lower than 50 mM (discussed in detail in Section 5.8.). Also, the time of exposure to H_2O_2 is likely to be less than 1 hr. Therefore, the conditions needed for H_2O_2 to render GBM more permeable to serum protein, assessed by the permeability analysis here, are unlikely to be attained *in vivo*.

If H_2O_2 cannot directly render GBM more permeable to serum protein, one still has to explain how it caused proteinuria in Rehan et al.'s study (Rehan et al., 1984; 1985a & b; 1986). It is known that H_2O_2 can give rise to several other reactive oxygen species, namely, $\text{OH}\cdot$, $^1\text{O}_2$, and HOCl (reviewed in detail by Klebanoff, 1992). These ROS are more reactive than H_2O_2 and may themselves react with GBM and render it more permeable to protein. However, it has been shown that neither the administration of the $\text{OH}\cdot$ scavenger dimethyl sulphoxide (DMSO) nor the iron chelator deferoxamine (removes iron needed for $\text{OH}\cdot$ synthesis via the Haber-Weiss reaction) ameliorated proteinuria in several models of neutrophil-dependent GN (Rehan et al., 1984; 1985a & b; 1986). Therefore, $\text{OH}\cdot$ seems unlikely to cause proteinuria.

Ito et al. (1992), in their effort to determine whether $^1\text{O}_2$ has any pathogenic relevance in GN, infused the plant pigment pheophorbide a into isolated kidneys and exposed it to light. The pigment generated $^1\text{O}_2$, resulting in severe glomerular cell injury but no perfusion experiment was carried out to determine if proteinuria had also

occurred. It is unclear if the amount of $^1\text{O}_2$ generated can be produced by neutrophils or indeed it is produced at all. The formation of $^1\text{O}_2$ by intact neutrophils was first proposed by Allen et al. (1972) based on the chemiluminescence of stimulated neutrophils. However, chemiluminescence merely indicates the formation electronically excited states and is not necessarily specific to the presence of $^1\text{O}_2$ (Klebanoff, 1992). No evidence of $^1\text{O}_2$ production by stimulated neutrophils was found using instruments which can detect $^1\text{O}_2$ emission at 1268 nm (Kanofsky and Tauber, 1983). When cholesterol, believed to specifically trap $^1\text{O}_2$, was used to detect $^1\text{O}_2$ generation by stimulated neutrophils, the expected product (3 β -hydroxy-5 α -cholest-6-ene-5-hydroperoxide) was not found (Foote et al., 1980a & b). It seems that the severe glomerular damage observed in Ito et al.'s study was likely to be due to exposure to an unphysiological amount of $^1\text{O}_2$ that would not be produced by activated neutrophils. Assuming that traces of $^1\text{O}_2$ were generated by activated neutrophils, it is doubtful if it has a chance to diffuse into the GBM matrix and react with the proteins as its half life is extremely short ($\sim 10^{-6}$ sec) (Kearns, 1979). Therefore, current evidence does not favour the involvement of $^1\text{O}_2$ in mediating proteinuria, if indeed, it is produced by activated neutrophils at all.

There is stronger evidence that HOCl, the metabolic product of H_2O_2 generated via the myeloperoxidase- H_2O_2 -halide system of activated neutrophils (Klebanoff, 1980; Weiss and LoBuglio, 1982; Fantone and Ward, 1982; Lampert and Weiss, 1983), is able to render GBM more permeable to serum protein (G.B. Robinson, personal communication), so promoting proteinuria (Johnson et al., 1987a; 1988a & b). Johnson et al. (1987a) perfused rat kidneys with pathophysiological amounts of myeloperoxidase, H_2O_2 , and Cl^- , and showed that the animals developed severe glomerular cell injury and proteinuria. Using $^{125}\text{I}^-$ incorporation into the glomerulus as an indicator of the activation of myeloperoxidase- H_2O_2 -halide system, they further showed that the system is activated by neutrophils in an immune complex model of GN (Johnson et al., 1987b). Together, the evidence suggests that HOCl generated by activated neutrophils caused glomerular cell injury and proteinuria. Robinson and co-

workers subsequently showed that HOCl (0.3 - 1.5 mM, room temperature) was directly able to render GBM films more permeable to serum protein (G.B. Robinson, personal communication). From the neutrophil respiratory burst analysis (Section 5.8.), the 1 mM H₂O₂ used for HOCl generation in Johnson et al.'s perfusion experiment may be judged physiological as might the HOCl concentration used by Robinson.

It must be noted that the above mechanism is one possible way by which H₂O₂ mediates proteinuria indirectly. The other possibility involves the HOCl-activation of the latent neutrophil metalloproteinases, gelatinase (Peppin and Weiss, 1986) and collagenase (Weiss et al., 1985). The activated metalloproteinases can degrade the collagen IV component of GBM in the presence of physiological concentrations of Ca²⁺ (Vissers and Winterbourn, 1988a) and thus have the potential to render GBM permeable to plasma proteins, giving rise to proteinuria. This prompted a detailed analysis of whether pathophysiological amounts of activated neutrophil metalloproteinases were able to render GBM more permeable to plasma proteins (Chapter 7).

In addition to the aforementioned mechanism by which H₂O₂ may indirectly mediate proteinuria, it has been suggested that H₂O₂ contributes to tissue damage by increasing the susceptibility of GBM to digestion by neutrophil proteinases (Fligiel et al., 1984). Pretreatment of rat GBM with 0.8-8 mM H₂O₂ for 1 hr at 37°C rendered the basement membrane more susceptible to degradation by trypsin. It would be interesting to establish whether the same effect is achieved with neutrophil proteinases. The ability of H₂O₂ to hydrolyse GBM at high concentrations (Fligiel et al., 1984) concurred with our observations: 2 M H₂O₂ solubilized up to 18 ± 3% of GBM following 12 hrs of incubation at 37°C. Since such a concentration cannot be achieved in a milieu outside neutrophils triggered by even the most potent known respiratory burst activator - PMA, the GBM solubilizing ability of H₂O₂ appears to have no physiological relevance.

8.4. Neutrophil proteinases and pathological proteinuria

Neutrophil proteinases are perhaps the earliest agents suspected of being able to cause proteinuria in immune GN (Hawkins and Cochrane, 1968). From the evidence reviewed previously (Sections 1.11.4 A, B, 6.1., and 7.1.), there is very little doubt that neutrophils, activated by an anti-GBM antibody bound GBM surface, exocytose proteinases onto the subjacent GBM. The exocytosed proteinases then digest the basement membrane, releasing membrane fragments. However, there is no direct evidence that such pathophysiological amounts of proteinases can render GBM more permeable to serum protein, causing proteinuria. Moreover, the relative effect of neutrophil serine and metalloproteinases on GBM permeability, if any, had not been examined. The present study aimed to resolve these issues. A homologous system was used whereby proteinases from pig neutrophils were used to digest pig GBM. The use of the pig system seems justifiable for two reasons. Firstly, the pig neutrophils have been shown to contain a proteinase spectrum similar to that of human's, i.e., consisting of elastase, cathepsin G, and gelatinase (Section 6.1.). Secondly, unlike human neutrophils and GBM, the pig materials are easily obtainable in large quantities.

The characteristics of the extracted proteinases are discussed first before moving onto their effects on GBM permeability. The proteinases were extracted from lysed pig neutrophil granules in an extraction buffer containing high salt and traces of detergent (Section 6.4.). The extract was then examined for the known serine and metalloproteinases, using a combination of substrate and inhibitor analyses. The extract contained activities against synthetic substrates of elastase and cathepsin G which were inhibited by known inhibitors of these proteinases, establishing the presence of these serine proteinases. This is consistent with the findings of Geiger et al. (1985) and Kraeva et al. (1988) who had independently isolated pig neutrophil elastase and cathepsin G. These proteinases also displayed activities against ³H-GBM, in parallel with the findings of Davies et al. (1978), Sanders et al. (1978), and Vissers et al. (1984) who had earlier demonstrated the degradation of human GBM by human neutrophil serine proteinases. The GBM degrading activities of elastase and cathepsin

G were found to be additive with ~ 90 % of the activity being due to the action of elastase, concurring with the a previous finding by Vissers and Winterbourn (1988a).

The extract contained no detectable activity against ^3H -GBM when Ca^{2+} was present, indicating an absence of active metalloproteinase. However, incubation of the extract with 4-APMA (activator of metalloproteinases) revealed a Ca^{2+} -dependent GBM degrading activity and removal of Ca^{2+} by the divalent cation chelator EDTA abolished the activity, indicating the presence of a latent metalloproteinase(s). Similar findings were reported by Vissers and Winterbourn (1988b) when they analysed the GBM collagen IV degrading activity of the metalloproteinase gelatinase released from human neutrophils following stimulation with phorbol 12-myristate 13-acetate (PMA): no active gelatinase activity was detected in the exocytosed fluid unless treated with 4-APMA. Baricos et al. (1988) have shown that human neutrophils have two metalloproteinases: gelatinase and collagenase. Only the gelatinase was able to degrade ^3H -GBM; the collagenase had no activity against the substrate. A latent pig neutrophil gelatinase has been characterized (Murphy et al., 1989), though no collagenase from the same source has yet been reported. It was difficult to attribute the metal-dependent GBM degrading activity to either gelatinase or collagenase in the pig system because there are no known separate inhibitors specific to each enzyme. However, it is known that pig neutrophils do have at least one latent metalloproteinase, namely, gelatinase which is Ca^{2+} -dependent and is capable of degrading GBM (Murphy et al., 1989).

A novel feature previously unreported in literature emerged from this study: when both families of proteinases were allowed to degrade GBM, their combined activity was greater than the sum of individual activities, indicating synergism. Initially, it was thought that the synergism was due to cathepsin G activation of gelatinase since purified human neutrophil cathepsin G has been previously shown to activate human neutrophil gelatinase (Murphy et al., 1980; Macartney and Tschesche, 1980 & 1983a). However, this was unlikely for the following reason. If cathepsin G had activated gelatinase in the proteinase extract, then a Ca^{2+} -dependent GBM degrading activity would have been observed prior to activation by 4-APMA but this

was not seen (Section 6.5.4.). Elastase has also been implicated as capable of activating gelatinase (Vissers and Winterbourn, 1988b). Again, metalloproteinase activity should be detectable prior to 4-APMA activation but this was not observed. It might also be argued that activated metalloproteinase(s) had activated latent forms of the serine proteinases, giving rise to the observed synergism. This was again unlikely because if the activated metalloproteinase(s) had achieved this when incubated with the serine proteinases, then extra serine proteinase activity against GBM should have been evident, but this was not observed (Section 6.6.).

A conceivable explanation for the observed synergistic degradation of GBM is the nicking of polypeptides of the GBM fibre matrix by one family of proteinases, opening up the matrix and facilitating the entry of the other family of proteinases deeper into the structure to effect proteolysis. Without the help from one family of proteinases, the diffusion of the second family into the matrix might have been more difficult and some polypeptides might thus remain undigested. Neutrophil proteinases other than the serine and metallo-proteinases have been reported, such as neutrophil proteinases 3 (Bretz, 1976; Rao et al., 1991), 4 (Ohlsson and Olsson, 1973), and 5 (Wintroub et al., 1974). It is doubtful whether these proteinases have any relevance in our study since the serine and metallo-proteinases accounted for all of the GBM degrading activity.

Moving now to a discussion of the effect of the extracted serine and metalloproteinases on GBM permeability, results from Chapter 7 provided the first direct evidence of pathophysiological amounts of each family of proteinases being able to cause increases in serum protein flux and water flux across the GBM films. This suggests that the proteinases can act as direct mediators of proteinuria in neutrophil-dependent forms of immune GN. It ought to be noted that the increase in protein flux occurred over the entire pressure range, above and below 150 kPa, the pressure equivalent to glomerular hydrostatic pressure across a single layer of GBM (Cotter, 1979). This means that if the glomerular hydrostatic pressure changes in GN, then a relative increase in protein flux may still occur *in vivo*. Indeed, the glomerular

hydrostatic pressure has been known to vary in diseased states. For example, the glomerular hydrostatic pressure rises in NSN shortly after anti-GBM antibody administration (~ 1 hr) (Blantz and Wilson, 1976; Wilson, 1988). In acute proliferative GN, glomerular capillary lumen is narrowed by swollen proliferating glomerular endothelial cells. This would have the effect of reducing the glomerular blood flow and hydrostatic pressure (Wheater et al., 1991). The study here suggests that the proteinase treated GBM would still be more permeable to protein than the native membrane even if the glomerular hydrostatic pressure rises or falls in nephritis.

The incubation time used in this study (6 and 12 hrs) did not seem excessive since neutrophils adherent to a solid wall of anti-GBM antibody bound GBM were able proteolytically to degrade the GBM, releasing peptide fragments for up to ~ 24 hrs (Bray et al., 1983). In addition, neutrophils have been known to be present in nephritic glomeruli for at least 12 hrs in NSN (Cochrane et al., 1965). During that time, the neutrophils were in close contact with GBM as shown by electron microscopy. The exocytosed proteinases presumably had direct contact with the GBM and were able to digest the membrane over that time period which was comparable to the incubation periods used in the present study. Thus the incubation times used here were likely to be physiologically reasonable.

A significant finding to emerge from this study was that when the two families of neutrophil proteinases were allowed to digest the GBM in concert, the increase in serum protein and water fluxes was greater than the sum of the effects due to each family of proteinases, indicating synergism. The effects were also time dependent as increasing the digestion time increased the fluxes irrespective of the family of proteinases used. The increase in protein flux across the digested GBM filter did not appear to be due to the effect of concentration polarization being more severe than in the control, brought about by less efficient stirring since the stirring rate was kept constant for both the test and control filtration cells.

The increase in protein flux could be due to the GBM becoming thinner following proteolysis. Cotter (1979) and Robinson and Cotter (1979) showed that the

protein flux across GBM films depended on the film thickness; decreasing the film thickness resulted in an increase in protein flux across the film. Thickness analysis on films made from GBM pre-digested with proteinases showed that both families of proteinases caused a decrease in GBM thickness, the effect of serine proteinases being greater than the metalloproteinases. This seems to explain why protein flux across films made from GBM pre-digested with serine proteinases was greater than that for films constructed from GBM pre-treated with metalloproteinases. Membrane thinning has been observed in rapidly progressive GN (Burkholder, 1969; Morrin et al., 1978), frequently associated with neutrophil infiltration of the glomerulus. Neutrophil proteinase-caused GBM thinning may account for the membrane thinning observed in the GN.

Another possible way by which proteinases might cause an increase in protein flux across the membrane is if they increased the intrinsic porosity of the membrane to protein, a possibility that has been raised by Cotter and Robinson (1980b). To ascertain if proteinases have this effect, it is necessary to eliminate the film thickness variable. This was achieved by packing more proteinase digested GBM onto filtration cells such that the film thickness was identical to the films made from GBM pre-incubated with inactivated proteinases (Section 7.7.). It was found that metalloproteinases were unable to cause an increase in the GBM's intrinsic permeability to protein and water since the test and control films showed no difference in fluxes. Serine proteinases, by contrast, were able to do so since serum protein and water fluxes across test films were significantly greater than those across the controls. A similar result was achieved when both families of proteinases were used, though the effect was more marked. Conceivably, serine proteinases (MW ~ 30 k), being smaller than metalloproteinases (MW ~ 88 k, gelatinase) could diffuse into the GBM fibre matrix, nicking polypeptide deep within the GBM structure and creating bigger channels. The metalloproteinases might not be able to diffuse into the matrix and might only be able to cleave polypeptides at the membrane surface, thus unable to alter the intrinsic membrane porosity. In summary, the study showed that both families of pig neutrophil

proteinases working in concert are able to render GBM more permeable to serum protein and water. They did so by reducing the GBM thickness and increasing its intrinsic permeability to protein and water. The ability to increase the intrinsic permeability seemed to reside in membrane proteolysis by the serine proteinases and not by the metalloproteinases; the latter cause membrane thinning.

In an attempt to show to what extent the GBM's porosity to solute has increased following proteolysis, two protein MW markers have been used for permeability analysis: myoglobin (MW 17.8 k) and BSA (MW 68k). TBS was also filtered. Increased GBM permeability to myoglobin and BSA, shown by increased protein fluxes and decrease in protein rejections, was seen. An increase in membrane permeability to water was also observed, judged by increased water flux in TBS ultrafiltration. Therefore, it would appear that proteolysis has caused an increase in membrane porosity to molecules of a wide range of MW ranging from water to macromolecules, e.g., myoglobin and BSA.

It is pertinent to mention that the incubation medium used in this study contained no plasma proteinase inhibitors, e.g., α -1-proteinase inhibitor and α -2-macroglobulin - inhibitors of elastase and cathepsin G (Barrett and McDonald, 1980). These inhibitors are present *in vivo* and could conceivably inhibit neutrophil exocytosed serine proteinases. However, current evidence shows that the inhibitors appear to have little to no effect on the action of the neutrophil proteinases. The serine proteinases exocytosed by neutrophils has been shown to degrade subjacent extracellular matrices (Weiss and Regiani, 1984; Campbell and Campbell, 1988), including the GBM (Bray et al., 1983), in the presence of α -1-proteinase inhibitor and α -2 macroglobulin. The serine proteinases were judged to have been protected from their inhibitors by a combination of inhibitor exclusion from the zone of adherence by neutrophils (Campbell and Campbell, 1988) and inactivation by neutrophil-generated hypochlorous acid and N-chloramines (Weiss et al., 1986). It has also been shown that neutrophils may use non-oxidative means to inactivate the α -1-proteinase inhibitor since the serine proteinases still degraded some subendothelial matrices when the reactive oxygen species were removed by scavengers (Weiss et al., 1986).

Unlike serine proteinases, metalloproteinase activities are unaffected by α -1-proteinase inhibitor (Barrett and McDonald, 1980). However, the metalloproteinases are exocytosed in latent forms which must be activated before they can digest GBM. No organomercurials, e.g., 4-APMA, are present *in vivo* but HOCl generated by activated neutrophils has been shown to activate the latent metalloproteinases (Peppin and Weiss, 1986). Ca^{2+} ions are also naturally present in plasma as cofactor to the activated metalloproteinases which means the neutrophil metalloproteinases can conceivably damage GBM in a plasma milieu. This coupled with the direct evidence that they can render GBM permeable to serum protein (Section 7.5.) suggests that neutrophil metalloproteinases can act as mediators of proteinuria in GN.

Current evidence indicates that both serine and metallo-proteinases are active when exocytosed by neutrophils. This study showed that active serine and metallo-proteinases working in combination gave rise to marked increases in membrane permeability to serum protein; this far exceeded the sum of effects from individual proteinases. The disease model used here suggests that the two classes of proteinases working in concert *in vivo* may also show synergism and effect the severe proteinuria commonly seen in neutrophil-dependent forms of GN.

8.5. Conclusion

An *in vitro* model of glomerular ultrafiltration has been used in this study to analyse changes in GBM permeability to protein and water. Although the model was unphysiological, the direct effect of test agents on the permeability of the GBM can be quantified by flux changes. Such controlled analysis was impossible to achieve *in vivo* because the test agents may perturb cells in the glomerular capillary wall which might in turn secrete proteinases and oxidants, damaging the GBM (Johnson et al., 1994). A number of test agents, suspected of being able to cause proteinuria were studied, namely guinea pig liver TGase, H_2O_2 , and neutrophil proteinases. Only the latter two caused increases in serum protein flux across the GBM. The guinea pig liver TGase - a biological cross-linking agent was considered an unlikely candidate for promoting

proteinuria. The interest in H_2O_2 lies in whether it could cause proteinuria when released by activated neutrophils in GN. However, the concentration of H_2O_2 needed to cause a significant increase in serum protein flux was much higher than could be achieved by activated neutrophils. It was thus concluded that H_2O_2 is also an unlikely candidate for mediating proteinuria directly.

Neutrophil proteinases, by contrast, were able to render GBM more permeable to serum protein in pathophysiological amounts, providing the first direct evidence in support of the hypothesis that neutrophil proteinases can cause proteinuria in GN. Investigations into how they caused increased GBM permeability to protein showed that they did so by digesting GBM, causing membrane thinning, as well as increasing the intrinsic permeability of the membrane, achieved presumably by enlarging channels in the membrane fibre matrix. Further analysis of the nature of the increase in membrane porosity indicated that an increase in membrane porosity to proteins up to the MW of albumin has been achieved, which can explain the frequent clinical finding of albuminuria in immune GN (Cotran et al., 1994). Further studies are needed to complete our understanding of how the neutrophil proteinases altered the molecular architecture of GBM matrix. Perhaps ultrahigh resolution electron microscopy study of GBM matrix and immunoblotting analysis of GBM proteins released following proteolysis may prove a fruitful way forward.

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