

University of Nottingham

**FACTORS INFLUENCING
THE REMOVAL OF
IMMUNE COMPLEX DEPOSITS
FROM THE RENAL GLOMERULUS**

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ABSTRACT

This thesis describes a study of the mechanisms involved in the elimination of established, morphologically identifiable electron dense deposits from the glomeruli of rats with chronic serum sickness. The initial experiments established the experimental model and evaluated the effects of variations in the method of administration, the antigen dose and charge, and the route of administration. The model was further characterized in terms of the progression of the morphological, functional and biochemical abnormalities induced.

Methods were developed to measure the amount of radio-labeled antigen in isolated glomeruli, and to measure the volume fraction of the electron dense deposits in glomeruli. Study was then limited to the first two weeks of recovery from chronic serum sickness, after injections of antigen have ceased, to ascertain the rates at which the antigen and deposits were removed.

The influence of a variety of forms of intervention during this recovery period was then assessed.

Manoeuvres which increased the level of circulating anti-BSA antibody were found to inhibit the removal of antigen and deposits from glomeruli. This effect was confirmed by passive immunization with immune rat serum. Manoeuvres which increased the number or activity of

glomerular macrophages had no detectable effect on the removal of antigen or deposits.

In the absence of plasma complement, the removal of antigen virtually ceased after four days. Cobra venom factor treated rats had lower proteinuria than controls, despite having a heavier antigen load.

Manoeuvres which altered the glomerular filtration rate and renal haemodynamics did not alter the rate of removal of antigen or deposits; nor did the administration of highly cationic molecules. The administration of large doses of heparin enhanced the rate of removal of antigen from the glomerulus, though an effect on deposit volume was not detected.

ABBREVIATIONS

BSA	Bovine serum albumin
C3 (C4, etc.)	Third (Fourth) component of complement
CR1	Complement receptor 1
CVF	Cobra venom factor
FCA	Freund's complete adjuvant
FIA	Freund's incomplete adjuvant
GFR	Glomerular filtration rate
HSP	Heparan sulphate proteoglycan
Ig	Immunoglobulin
PAN	Puromycin aminonucleoside
PVA	Polyvinyl alcohol
SEM	Scanning electron micrograph
SLE	Systemic lupus erythematosus
TEM	Transmission electron micrograph

PART ONE

INTRODUCTION

Immune complex glomerulonephritis in Man

This thesis describes a study of the factors which influence the removal of immune complex deposits from within the glomeruli of rats with a form of chronic serum sickness glomerulonephritis. Glomerulonephritis in man is most commonly a chronic progressive disease, so the existence of mechanisms by which glomerular deposits are removed has rarely been considered. The purpose of this chapter is therefore to review some of the main forms of immune complex glomerulonephritis in man, the current theories of how glomerular deposits are formed, and, of major relevance to this thesis, the evidence that mechanisms exist which can remove them. This is not intended to be an exhaustive review but rather to select and emphasize those aspects of human disease which are of relevance to the experimental work to follow. Glomerular diseases in which morphologically identifiable immune complex deposits do not characteristically accumulate in the glomerulus, such as Goodpasture's syndrome, will not be discussed.

The second part of this introduction will then describe the main forms of experimental immune complex glomerulonephritis which are available for study, and will assess the contributions which these models have

made to our understanding of the pathogenesis of immune complex glomerulonephritis. The third chapter will discuss the limited experimental data available relating to the mechanisms by which glomerular immune complex deposits are altered and removed.

The mechanisms by which immune complexes accumulate in the glomerulus are controversial. There are three main theories, which are not mutually exclusive. At the risk of oversimplification they may be summarized as follows:-

- 1) Circulating complexes of antigen and antibody become trapped in the glomerulus. Such preferential deposition is presumably a consequence of the function of the glomerulus as a filter.
- 2) Circulating antibodies bind to antigens, foreign or autologous, which have for some reason (for example electrical charge) become trapped within the glomerulus. This is commonly referred to as 'in situ' complex formation.
- 3) Autoantibodies develop against an intrinsic glomerular antigen which has (or can develop) a discontinuous localization along the glomerular basement membrane.

These theories have evolved largely as a result of work in experimental animals, which will be discussed

later. All three are capable of causing the accumulation of electron dense deposits, but in the absence of perfect animal models the exact pathogenesis of human glomerulonephritis can only be confirmed by studies in humans with that disease. Such evidence is circumstantial.

The commonest form of human glomerulonephritis in which electron-dense deposits are found is **membranous glomerulonephritis**, a disease which bears several similarities to the form of experimental glomerulonephritis described in this work; notably the presence of deposits in a subepithelial location, the paucity of cellular proliferation and the development of a nephrotic syndrome. Immunofluorescence or immunoperoxidase studies indicate the presence of immunoglobulins and complement components, mainly IgG and C3, in a granular pattern around the capillary loops, correlating within the location of the electron dense deposits. This co-localization is the principal evidence in man that the electron-dense deposits which are identified morphologically are actually composed of 'immune complexes' - antibody, complement and antigen - though the presence in the deposits of other material, such as basement membrane components, has not been excluded. The antigen content of these deposits is usually unidentifiable, a problem which has considerably

hindered research into the aetiology and treatment of the condition.

For many years the theory that membranous deposits accumulate by trapping of circulating immune complexes was unchallenged. In man, the main evidence against this theory is the observation that immune complexes frequently can not be found in the circulation, even using a battery of sensitive techniques. Abrass et al. (1980) found such complexes in 22 of 55 patients after exhaustive investigation. This is not the case with the membranous pattern of glomerulonephritis which is a common complication of systemic lupus erythematosus (SLE). The role of DNA-containing immune complexes in SLE nephritis has recently been reviewed by Fournie (1988). Circulating immune complexes are common in SLE; the amount correlates with the activity of the renal and systemic disease, and one of the defects in SLE is a reduced ability to clear immune complexes from the circulation (Frank et al. 1979). Nuclear antigens can often be found in the glomerulus in SLE nephritis and anti-DNA antibodies can be eluted (Koffler et al. 1971). However, the membranous pattern of glomerulonephritis in SLE differs from the idiopathic form in that deposits are also found in subendothelial and mesangial locations. Animal models in which trapping of circulating complexes is the only possible mechanism generally produce mesangial deposits (*vide infra*) and it

is likely that more than one mechanism is operative in SLE. In the development of capillary loop deposits in SLE, circulating nucleoproteins, being cationic, might bind to the anionic sites of the glomerular basement membrane, with secondary binding of antibody; indeed, such an affinity has been reported (Izui et al. 1976). The evidence supporting this theory again comes almost entirely from animal experiments (*vide infra*). Similarly, the suggestion that autoantibodies could be binding to discontinuous glomerular antigens arises from the similarities between human membranous glomerulonephritis and Heymann nephritis (Heymann et al. 1959) in the rat, which is induced by immunization against an autologous antigen found in the proximal tubule brush border and on glomerular epithelial cells.

The arguments in favour of membranous glomerulonephritis being an organ-specific auto-immune disease have been proposed by Pusey et al. (1988) as follows:-

- 1) There is a strong association with HLA-DR3 (Klouda et al. 1979)
- 2) There is an absence of features such as hypergammaglobulinaemia and hypocomplementaemia which are common in known immune complex diseases.
- 3) There is an association with auto-immune diseases. (Pusey et al. quote Ploth et al. 1978, Horvath et

al. 1979 and Jordan et al. 1981; though these are all single case reports of association with thyroid disease, and in each case the glomerular deposits are attributed with good evidence to thyroglobulin-antithyroglobulin complexes in the serum).

- 4) Membranous glomerulonephritis tends to recur in grafts (though why this makes an auto-immune aetiology more likely is not explained by the authors).

It seems that the auto-immune theory will remain unproven until the responsible antigen system is identified in normal glomeruli. In a few cases it has been claimed that renal tubular epithelial antigens are present in glomeruli in human membranous glomerulonephritis (Naruse et al. 1973, 1974). The analogy with Heymann nephritis in the rat might support an auto-immune aetiology, but the presence of such antigens has been denied by others (Thorpe & Cavallo 1980, Collins et al. 1981). In some cases membranous glomerulonephritis is clearly associated with Hepatitis B infection, with surface and/or core antigen present in circulation and glomeruli (Nagy et al. 1979, Takekoshi et al. 1978). This would support either trapping of complexes or *in situ* formation but is conclusive for neither.

Membranous glomerulonephritis is usually a chronic disease with an inexorable progression, but it is important to emphasize that the deposits can be removed. Spontaneous resolution is quite common in children (Ramirez et al. 1982). In the terminal stages, when the glomerulus is scarred beyond further use, the deposits become electron-lucent and disappear (Ehrenreich & Churg 1968). Many different patterns of glomerulonephritis have been reported in association with neoplasia; membranous glomerulonephritis is the most common pattern, and tumour-associated antigens have frequently been reported in the affected glomeruli (Reviewed by Eagen & Lewis 1977, Heptinstall 1983). Successful excision of the tumour has occasionally been reported, with subsequent resorption of the deposits (Couser et al. 1974). In a detailed study of patients suffering from cancer, but without overt renal disease, 55% were found to have small amounts of IgG, C3 or electron dense deposits in the glomeruli (Pascal et al. 1976). Subepithelial deposits may also complicate syphilis (Yuceoglu et al. 1974), hydatid disease (Ibarrola et al. 1981) and treatment with drugs such as penicillamine (Jaffe et al. 1968) captopril (Hoorntje et al. 1980) or gold salts (Ainsworth et al. 1981). In most cases successful treatment of the disease or withdrawal of the drug has resulted in the disappearance of the deposits, and in some the relevant exogenous antigen was demonstrated in the glomeruli. These points suggest a

role for an exogenous antigen in the pathogenesis of membranous glomerulonephritis, and serve to emphasize that although membranous glomerulonephritis is a progressive disease, its progression depends on the rate of continuing deposition of immune substances exceeding the rate of removal. Consequently, to enhance the rate of deposit removal is theoretically as laudable a therapeutic aim as to inhibit deposition.

Acute proliferative glomerulonephritis is morphologically and clinically distinct from membranous glomerulonephritis, though subepithelial electron dense deposits are found in both. The disease usually follows an acute streptococcal infection and typically produces a nephritic picture, rather than nephrotic syndrome. The glomeruli are swollen, almost bloodless and hypercellular, with an influx of neutrophils and mononuclear cells. Ultrastructural examination reveals subepithelial deposits or 'humps', which are characteristically larger but fewer in number than those seen in membranous glomerulonephritis. Smaller subendothelial and mesangial deposits are seen in some cases. It is occasionally possible, with suitable staining, to visualize faint concentric lines in these deposits, suggesting growth by accretion on the surface (Furness et al. 1986).

The three theories of pathogenesis suggested for membranous glomerulonephritis may again be invoked but the strength of evidence for each differs. Immune complexes are reported to be present in the circulation in most cases of active post-streptococcal glomerulonephritis (Border 1979, Yoshizawa et al. 1983) but the nature of the antigen remains undefined. Indeed, the presence of streptococcal antigens in the glomeruli, though confirmed by some (Treser et al. 1969, 1971) has been denied by others (Feldman et al. 1966). The finding of immune complexes in the circulation does not prove that they are deposited in the glomerulus; they might dissociate or be present intermittently, and the possibility of 'in situ' binding of antibody to streptococcal antigen which is trapped in the glomerulus has been supported by the elution of cationic streptococcal antigens from affected kidneys (Vogt et al. 1983). Antisera from convalescent cases can be used to stain the glomeruli in biopsies taken early in the disease, but reactivity appears to be independent of the type of streptococcus involved (Treser et al. 1971). Finally, the possibility of a cross-reaction between streptococcal and intrinsic glomerular antigens has been reported (Fillit et al. 1985) but is not widely accepted, even though this would readily explain the specificity of this disease as a sequel to streptococcal infections.

The clinical course of acute proliferative glomerulonephritis differs markedly from that of membranous glomerulonephritis. Like the infection which precedes it, acute proliferative glomerulonephritis usually resolves spontaneously; the electron dense deposits become lucent and disappear (Tornroth 1976) though the glomerular damage caused in the acute phase may in a minority of cases result in functional renal impairment and progression to renal failure. It is tempting to suggest that the influx of cells leads to this more effective removal of the deposits. In addition to the neutrophils, macrophages infiltrate the mesangium. However, in uncomplicated cases none of the inflammatory cells are found in the urinary space, in the compartment in which most of the deposits are found. Neutrophils may be seen closely apposed to the glomerular basement membrane, even having replaced the endothelium (Furness et al. 1986) but they do not appear to pass through, despite the facility with which inflammatory cells penetrate basement membranes at other sites. Phagocytosis of deposits is therefore not seen and the case for the direct involvement of cells in the removal of deposits remains unproven. The influx of neutrophils could be an epiphenomenon to the process of removal; they are probably attracted by fragments of activated complement. Complement components co-localize with immunoglobulins at the site of the subepithelial deposits, and serum C3 is often reduced (Baldwin et al.

1974). This is of relevance to the potential of complement to remove immune complex deposits (*vide infra*).

Mesangiocapillary glomerulonephritis is a clinically heterogeneous disease which has been divided into at least two groups on morphological grounds. The glomeruli present similar appearances on light microscopy, but electron microscopy reveals subendothelial and mesangial electron dense deposits in type I and a peculiar ribbon-like accumulation of electron dense material within the glomerular basement membrane in type II. The complement system seems to be intimately involved; C3 is characteristically identified within glomeruli by immunofluorescence, often in the absence of immunoglobulins in type II disease, which may not be an immune complex glomerulonephritis (Galle & Mahieu 1975). Serum complement levels are depressed in 70% of cases, especially in type II disease (Cameron et al. 1970) and the circulation often contains an autoantibody to the C3 convertase of the alternative complement pathway (C3bBb), known as C3 nephritic factor (Schreiber & Muller-Eberhard 1979, Whaley et al. 1979). This autoantibody stabilizes C3bBb, resulting in persistent complement activation. It has been suggested that complement depletion might predispose to infection and consequent antigenaemia, which with a concurrent deficit in complement-mediated clearance of circulating

immune complexes might precipitate glomerulonephritis (Peters & Lachmann 1974). Such a theory cannot explain those cases with normal complement, nor the absence of immunoglobulin in type II disease. The relevance of complement depletion is also undermined by the finding that glomerulonephritis is not induced in mice made immunologically tolerant to cobra venom factor then chronically complement depleted by that agent (Simpson *et al.* 1978).

The role of complement in disrupting circulating immune complexes has been extensively studied in recent years (Reviewed by Schifferli *et al.* 1986). The precipitation of antigen-antibody complexes, *in vitro* and in the circulation, has been shown to be due largely to interactions between the Fc components of the antibodies, rather than by extension of the Fab-antigen lattice (Moller 1979, Moller & Steengaard 1979). Activation of complement by the classical pathway leads to the binding of activated C1 and C3 to the Fc component, which inhibits precipitation (Schifferli *et al.* 1980). If precipitation does occur, then activation of complement by the alternative pathway can also lead to dissolution, by disrupting the Fc-Fc bonds (Takahashi *et al.* 1978, 1980; Takata *et al.* 1984). The formation of small soluble complexes might appear to be counter-productive, as experimental work has shown such complexes to have the greatest nephritogenic potential.

(*vide infra*); but after fixation of complement the complexes are rapidly taken up by binding of the attached C3b to the complement receptor CR1 on erythrocytes (primates: Siegel et al. 1981, Cornacoff et al. 1983) or platelets (other species; Taylor et al. 1985). These are then carried to the liver, where the complexes are stripped from the circulating cells and degraded by mononuclear phagocytes (Cornacoff et al. 1983). CR1 also plays a role in the feedback inhibition of complement activation (Medof et al. 1982, 1984) and this fascinating molecule is also found in human glomeruli (Gelfand et al. 1975) on the epithelial cells (Burkholder et al. 1977). An imbalance in this system clearly could have the potential to precipitate or exacerbate the deposition of immune complexes at sites such as the glomerulus. The possible involvement of complement in the dissolution of glomerular deposits has been the subject of relatively little study.

Further insight into the role of complement in human glomerulonephritis is gained by study of inherited deficiencies of complement components. A partial or complete absence of several of the early complement components is associated with the development of an SLE-like disease, often with an immune complex glomerulonephritis. This has again been attributed to an increased incidence of infections and antigenæmia, or to decreased removal of circulating immune complexes. The

latter theory is supported by the observation that inherited deficiencies of components after C3 (affecting microbial killing but not involved in immune complex clearance) are not associated with an increased incidence of SLE (Ammann & Fudenberg 1982), and by reports that deficiency of the complement receptor on human red cells (CR1) is associated with SLE (Miyakawa et al. 1981), though this is probably secondary to the SLE (Walport et al. 1985). The presence of CR1 on human glomerular epithelial cells was originally cited as a possible reason for glomerular localization of immune complexes (Gelfand et al. 1975) but as the function of CR1 on human red cells and macrophages appears to be the removal of immune complexes, it seems more likely that there is a role for glomerular epithelial cells in the removal of electron dense deposits, and/or the inhibition of complement activity in the glomerulus. These possibilities do not appear to have been explored, probably because of the absence of CR1 on the glomerular epithelial cells of experimental animals (Moran et al. 1977) and the rapid loss of CR1 expression on human podocytes in culture (Ross & Medof 1985).

Several categories of human glomerulonephritis characteristically show electron dense deposits which are confined to the mesangium. One of these, **Berger's disease** or IgA glomerulonephritis, is a common form of

glomerulonephritis with a characteristic clinical presentation of intermittent gross hæmaturia in adult males. It appears to be closely related to Henoch-Schönlein purpura. The major morphological abnormalities are limited to the mesangium and include increased cell numbers, mesangial electron dense deposits, and mesangial IgA and C3 on immunofluorescence, with smaller amount of IgG. The levels of plasma IgA, both monomeric and polymeric, are often increased and circulating IgA immune complexes are reported in many cases (Woodroffe et al. 1980, Stachura et al. 1981). No consistent antigen has been identified but the association with IgA and the common development of mesangial IgA deposits in chronic liver disease has led to the suggestion that gut-associated antigens are involved, possibly with a defect in the hepatic processing of gut-associated antigen-IgA immune complexes (Kalsi et al. 1983). Experimental work (*vide infra*) lends credence to the theory that deposits in a mesangial location, such as those which are found in Berger's disease, are most likely to be produced by intact circulating immune complexes. Berger's disease characteristically has a good prognosis, with exacerbations and remissions; it is of interest that in one study, re-biopsy two months after the documentation of Berger's disease with numerous deposits, IgA deposits were absent (Bergstein 1978) emphasizing again that deposits will be removed if deposition ceases.

It is evident that the investigation of pathogenetic mechanisms in human immune-complex glomerulonephritis has been curtailed not only by obvious ethical limitations, but by the inability to identify an antigen in the majority of cases. This may be due to a variety of factors. If, as seems likely, many different antigens can cause a single pattern of disease, identification will be difficult. It is likely that further deposition of antibody masks antigenic determinants, or perhaps those determinants are altered in some way while trapped in the glomerulus. It is generally believed that only a small proportion of the morphologically identifiable electron dense deposits is actually made up of antigen. This is supported by the experimental evidence to follow.

Most of our understanding of human immune-complex glomerulonephritis has come through comparison with a variety of animal models, of variable relevance to human disease. These will be the subject of the next chapter. However, several important points are evident from the discussion above. Human immune complex glomerulonephritis is usually a chronic disease which involves not only the deposition but also the concurrent removal of antibody, complement and a variety of antigens within the glomerulus. Several factors can be identified which one might expect to influence either deposition or removal. The nature and amount of antigen

and antibody are obvious possibilities, along with the form in which they are presented; complexed or not, continuously or in pulses, etcetera. Glomerular factors might be important; for example the glomerular blood flow and filtration rate, the activities and types of the cells in the glomerulus and the state of the glomerular basement membrane, in particular its electrical charge. Finally, systemic factors could be involved: the ability of the body to effectively eliminate circulating antigens and immune complexes is likely to be particularly important. There have been considerable advances in recent years in our understanding of the factors which can influence the deposition of electron dense deposits in the glomeruli of experimental animals, but our understanding of the mechanisms of removal has not kept pace. These subjects will be discussed in the next two chapters.

Experimental models of immune complex

glomerulonephritis:

Factors influencing deposition

In the absence of clearly understood pathogenetic mechanisms of human disease, experimental models of glomerulonephritis can only be an approximation to human glomerulonephritis; the validity of a model can only be assessed by crude parameters such as its morphological similarity to a human disease. Theories derived from disease in animals should be confirmed in man. Experimental models of glomerulonephritis have produced almost all our evidence of how immune complex deposits can develop in glomeruli, but this can only imply how they do develop in man.

The purpose of this chapter is to review the major animal models of immune complex glomerulonephritis (excluding anti-glomerular basement membrane disease) and to evaluate the resultant theories of how immune complexes may be deposited in glomeruli. The question of how such complexes then cause damage to the glomerulus will not be addressed. This chapter will provide a necessary preface to a discussion of our understanding of the mechanisms by which the deposits are removed, which forms the final part of this Introduction.

Acute serum sickness

The group of models known as serum sickness has contributed more data to this field than any other. Serum sickness was originally identified as a human disease, the result of the treatment of infectious disease with large amounts of serum from an animal which was immune to the relevant organism. Von Pirquet (1917) recognized that the disease developed as the animal protein was cleared from the circulation, and correctly suggested that it was the result of a combination of the foreign protein with factors elaborated by the body which produced a toxic substance. Hawn & Janeway (1947) showed that purified foreign proteins are also effective in producing a rabbit model of the disease. Purified proteins have been used almost exclusively since then, making the title 'serum sickness' a time-hallowed misnomer. Hawn & Janeway reported that pure bovine gamma globulin tended to produce glomerular lesions, whereas bovine serum albumin (BSA) tended to produce an arteritis. In the light of current theories of the importance of the anionic charge of the glomerular basement membrane (*vide infra*), this might relate to the greater anionic charge of BSA but nevertheless pure BSA has been the antigen most widely used to induce glomerulonephritis since that time.

Acute serum sickness is classically induced by a single intravenous injection of a large dose (about

500mg/Kg) of BSA in the rabbit. Rabbits treated thus may develop an acute diffuse glomerulonephritis 10 to 14 days later, sometimes with endocarditis and vasculitis. The glomeruli show diffuse uniform hypercellularity, due mainly to an influx of macrophages from the circulation (Holdsworth et al. 1980). The lesions heal in a few weeks, usually with no residual deficit. On the day at which the histological changes are most intense, electron dense deposits are said to be few and mainly subendothelial and mesangial in location, but numerous subepithelial deposits develop a little later in the course of the disease (Fish et al. 1966). The model in this form is unpredictable; results vary between groups but typically only a third of rabbits develop glomerulonephritis and that is of variable severity. This unpredictability caused obvious problems, but it also provided an opportunity to study the circumstances under which glomerulonephritis may develop. The severity of the glomerular damage was found to relate to the animal's production of anti-BSA antibody. Animals with a very poor or no response produced no lesions, but more surprisingly animals with a very high antibody titre also had no disease (Germuth & Rodriguez 1973). This was attributed to very rapid clearance of antigen from the circulation in the latter group. Germuth (1953) described the kinetics of antigen removal in this model. After an initial equilibration, the level of BSA in the serum falls slowly for a few days. It is then rapidly

eliminated, presumably due to the initiation of antibody production, and for a day or two immune complexes are detectable in the circulation. These are then cleared and free antibody is found. Glomerular lesions develop only at the time of elimination of the antigen, when circulating immune complexes are present. Dixon et al. (1958) reported that antigen is not detectable in the glomeruli until immune complexes are present in the circulation. These findings led to the theory that intact circulating immune complexes are trapped in the glomeruli, a theory which precluded any alternative or additional suggestions for some years.

Some further interesting studies were carried out by correlating the intensity of disease with immune complex size. Dreesman & Germuth (1972) reported that severe glomerular lesions were only found in animals with a fairly low antibody response, in association with complexes in the circulation of weight 300,000 to 500,000 Daltons, corresponding to IgG complexes of Ag_2Ab to Ag_3Ab_2 . Germuth & Rodriguez (1973) claimed that small complexes produce severe glomerulonephritis with subepithelial deposits, but animals with a slightly better antibody response have larger complexes in the circulation (above 10^6 Daltons), which are associated with exclusively mesangial deposits. The importance of the size of circulating complexes was supported by the observations that serum sickness induced a high

molecular weight antigen (thyroglobulin: 670,000 Daltons) produced only mesangial deposits (Germuth et al. 1978).

There is a theoretical objection to a correlation between the nature of a glomerular disease and the immune complexes present in the circulation. Only a tiny proportion of injected antigen is deposited in the kidney (Wilson & Dixon 1970, 1971). Complexes in the circulation may therefore not be representative of those trapped in the kidney; indeed, they may be detectable in the circulation because they are not being trapped in the tissues (McCluskey 1983). In the light of subsequent discoveries it is relevant that small immune complexes, which were found to be associated with severe disease, are also likely to be associated with the persistence of free antigen in the circulation.

Chronic serum sickness

Chronic serum sickness glomerulonephritis was first described by McLean et al. (1951). This model is usually produced by daily intravenous injection of smaller amounts of antigen in an immune animal: typically 12.5mg daily in the rabbit. This produces more severe glomerular disease than acute serum sickness, with larger, more numerous electron dense deposits. The chronicity of the disease is closer to most forms of

human glomerulonephritis. This model initially suffered from the same lack of reproducibility as acute serum sickness, but Dixon et al. (1961) produced glomerulonephritis more consistently in the rabbit by matching the daily antigen dose to the amount of antibody produced by the animal. The success of this manoeuvre again emphasized the importance of the antibody response and, by inference, the immune complex size. Unfortunately this technique is very laborious and animals with a very low or very high response still did not develop glomerulonephritis.

Correlation of glomerular lesions with the properties of circulating immune complexes is open to even more objection in chronic serum sickness than in the acute form. It is to be anticipated that at the time of daily injection, there will be a circulating bolus of blood containing a considerable antigen excess. With mixing, the antigen will bind to circulating immunoglobulin and produce small immune complexes. Diffusion of antibody from the extravascular space and continuing antibody production will gradually reduce the antigen excess, producing larger complexes which will be more effectively cleared from the circulation (Mannik & Arend 1971). At the end of the day free antibody may again be found in the circulation. Thus the results of serum analysis for immune complexes may be found to correlate better with the time of day than with the

glomerular lesion induced! Nevertheless it was reported that given a constant daily antigen dose, animals with a low level of antibody production develop a membranous pattern of electron dense deposits with little cellular proliferation; a moderate response is associated with severe glomerulonephritis, whereas a higher antibody response is associated with only mesangial deposits (Germuth et al. 1972, 1977; Holdsworth et al. 1980).

In the early work of Hawn & Janeway (1947) it was suggested that the injected antigen was trapped in the glomerulus, and deposits formed when circulating antibody bound to antigen *in situ*. This theory was subsequently discounted for reasons outlined above. However, the possibility that *in situ* complex formation could occur in some circumstances had not been excluded. The evidence cited above indicates strongly that large (but soluble) complexes can be trapped in the mesangium and create deposits there. This is not surprising as plasma macromolecules are known to pass through the mesangium in the normal state (Takamiya et al. 1979, Latta & Fligiel 1985). But how can large complexes pass intact through the glomerular basement membrane? It was perhaps not fully appreciated at the time, but in the circumstances described above in which subepithelial deposits were found, it was usually the case that free antigen was either known or was likely to be present in the circulation. This raises the possibility of *in situ*

formation as a mode of development of subepithelial deposits. Early evidence that antigens could be trapped in the glomerulus, at least in the mesangium, in the absence of antibody was provided by Mauer et al. (1973), who gave intravenous injections of heat-aggregated human IgG. The subsequent administration of antibody against human IgG produced glomerular lesions. The validity of this alternative mechanism was supported by the findings of Steward (1979). He induced chronic serum sickness glomerulonephritis in two strains of mice, selected for their tendency to produce either high avidity or low avidity antibodies to protein antigens. The former, whose immune complexes would not be expected to dissociate in the circulation, formed mesangial deposits. The latter, whose complexes tended to dissociate, formed subepithelial deposits. Further evidence of the importance of local dissociation of complexes was provided by Iskandar and Jennette (1983), who induced chronic serum sickness glomerulonephritis in mice, and found that animals producing predominantly mesangial deposits had circulating antibodies of high avidity, but the development of subepithelial deposits was associated with low avidity antibodies.

Passive serum sickness

The serological complexity of acute and chronic serum sickness models made it difficult to resolve the situations in which trapping of immune complexes or *in situ* aggregation was occurring. This problem was partly solved by the development of passive serum sickness models of glomerulonephritis, in which lesions are induced by the infusion of immune complexes (or their components) into the circulation (McCluskey & Benacerraf 1959, McCluskey et al. 1960, 1962). This can cause deposits to form within hours, too rapidly for the animal's own immune system to be activated. Such infusions still invariably contain complexes of various sizes and the possibilities of alteration in the circulation and selective deposition of a small subgroup still persist, but the situation is undeniably better controlled than in active forms of serum sickness.

Large insoluble complexes do not cause glomerulonephritis unless they are infused directly into the arterial system (Gabbiani et al. 1975, Shigematsu et al. 1979), when they cause a focal segmental proliferative glomerulonephritis with deposits confined to the capillary loop and mesangium. This pattern is probably analogous to that produced by subacute bacterial endocarditis. This is presumably because large complexes are effectively removed in the lungs, liver

and spleen. Very small complexes (in considerable antigen excess) are not trapped at all, but persist in the circulation (Mannik & Arend 1971). Infusion of fairly small soluble complexes usually produces mesangial deposits, but studies which paralleled that of Steward described above showed that infused complexes formed with high avidity antibodies tend to localize in the mesangium (Germuth et al. 1979a) but complexes formed with low avidity antibodies can form subepithelial deposits (Germuth et al. 1979b; Koyama et al. 1978). This finding has more recently been confirmed using monoclonal antibodies of carefully defined characteristics, thus avoiding the problem of antibody heterogeneity (Lew et al. 1984). The possibility that subepithelial deposits form by disaggregation and *in situ* re-aggregation in the glomerulus is again implied; indeed, it has been suggested that spontaneous glomerulonephritis occurs when the increase in antibody affinity normally seen with progression of an immune response does not occur (Devey et al. 1984).

Variations of the passive serum sickness model are well suited to test the validity of *in situ* immune complex formation. Sequential perfusion of the kidney with antigen followed by antibody, without mixing, was shown to be capable of producing subepithelial deposits, particularly if the antigen concerned has some affinity which leads it to bind to the glomerular capillary wall.

Golbus and Wilson (1979) gave concanavalin A as an infusion to one renal artery. This lectin binds to saccharide components of the glomerular capillary. Subsequent systemic administration of anti-concanavalin A antibody induced glomerulonephritis in the perfused kidney only, eliminating the possibility of involvement of circulating immune complexes. Other investigators have since produced similar results using a variety of antigens (Batsford et al. 1980, Adler et al. 1983a), usually producing subepithelial deposits. This confirms that *in situ* formation can occur, and the contrast with other forms of passive serum sickness supports the view that this is at least a possible mechanism by which subepithelial deposits may form. However, immune complex trapping and *in situ* formation are not mutually exclusive. They could occur simultaneously and even where whole complexes are trapped in the mesangium there is evidence that dissociation and rearrangement are essential for persistence, as complexes which were covalently bound together were rapidly removed (Mannik et al. 1983). This rearrangement may be dependent upon the presence of plasma complement (Sawtell et al. 1988).

The amount and avidity of the antibody have thus been shown to influence the pattern and severity of the glomerular lesions in serum sickness, but other properties of the antibodies have received less attention. Pincus et al. (1968) suggested that non-

precipitating antibodies produced a worse glomerulonephritis than precipitating antibodies, presumably because they are cleared from the circulation more slowly: though the large size of precipitating IgM antibody might also inhibit subepithelial deposition (Dreesman & Germuth 1972). Haakenstad (1976) showed that immune complexes prepared with reduced and alkylated antibodies accumulate more in glomeruli and persist longer than complexes formed with untreated antibodies. Such altered antibody does not interact normally with complement or Fc receptors on phagocytes, factors which are likely to cause persistence in the circulation. It has since been shown that the aggregation of immune complexes occurs at least partly through interaction of the Fc components of the antibodies (Moller & Steengaard 1979), rather than by formation of a lattice with antigen and Fab, so the degree of aggregation of the complexes may also have been altered in a way which the author was not able to assess.

The influence of antibody class has received little attention, though it has been reported that glomerulonephritis in man where the only immunoglobulin class present is IgM is associated with deposits in an exclusively mesangial location (Cohen et al. 1978, Lawler et al. 1980, Hsu et al. 1984), and it was suggested that the large molecular weight of IgM was the cause of this pattern of localization. Similarly, the

characteristic nature of Berger's disease in man indicates that antibody class might be important; one may suggest here that the ability of IgA to adopt a polymeric form might predispose to the development of large complexes and hence the observed mesangial deposits. Models of IgA disease have been described; Rifani et al. (1979) inoculated mice with a myeloma cell line which produced an IgA antibody against dinitrophenol (DNP). Subsequent administration of BSA linked the DNP produced mesangial deposits alone. Passive serum sickness using the same monoclonal antibody also produced mesangial deposits, but only if polymeric IgA was used. Monomeric IgA complexes did not cause glomerular deposition. Isaacs et al. (1981) used neutral dextrans to induce chronic serum sickness. This antigen elicits a predominantly IgA response, and only mesangial deposits were produced; but here it is unclear whether it is the nature of the antibody or the large size of the antigen which causes this localization. In a recent experiment hybridoma technology was used to create two monoclonal antibodies with identical binding sites but different heavy chain types (IgE and IgG₂). Passive serum sickness was induced in mice using these antibodies and no differences could be discerned in the pattern or intensity of glomerular damage induced (Chen et al. 1987). Given the intimate involvement of the complement system in the handling of immune complexes in the circulation and in the glomerulus, one might perhaps

expect that the ability of an antibody to activate complement might have profound effects on the localization and fate within the glomerulus; IgA, D, E and G4 do not activate complement. Regrettably, with regard to the work of Chen et al. described above, IgG₂ only fixes complement weakly. This aspect of complement class does not appear to have been studied further.

It is apparent that the nature and quantity of both antigen and antibody have a profound influence on the extent to which deposition in the glomerulus will occur. However, the characteristics of the host are also important in this respect.

Hæmodynamics

The fact that the glomerulus is a high pressure ultrafiltration system may be assumed to contribute to the trapping of immune complexes (Germuth et al. 1967). It has been demonstrated that hæmodynamic factors can influence the localization of complexes in glomeruli. Stenosis of the artery to one kidney inhibits the subsequent development of immune complex glomerulonephritis in that kidney in experimental animals (Germuth et al. 1967) and in man (Salzer & Salzer 1974). Benacerraf et al. (1959) first showed that

the injection of vasoactive amines can enhance the glomerular accumulation of colloidal carbon. Conversely, antagonists of serotonin and histamine decrease the severity of acute serum sickness glomerulonephritis in the rabbit (Kniker & Cochrane 1968). The injection of preformed immune complexes with colloidal carbon was also shown to enhance glomerular localization of the carbon (Benacerraf et al. 1959), providing the interesting suggestion that immune complexes might produce hæmodynamic effects which enhance their own localization in the glomerulus. Camussi et al. (1982) have provided some experimental evidence that immune complexes in the circulation can produce hæmodynamic effects by causing release of platelet activating factor from basophils; this in turn causes the release of vasoactive amines from platelets. It is reasonable to suggest that alterations in glomerular hæmodynamics might also be responsible for the increased glomerular trapping of immune complexes which has been reported when the glomerulus already contains immune complex deposits (Ford & Kosathka 1980).

Other properties of the normal glomerulus may contribute to the trapping of circulating complexes or free antigen. The most important of these appears to be **electrical charge** (Gallo et al. 1982).

The observation that the glomerular filter is selective on the basis of molecular charge as well as molecular size was first reported by Bohrer et al. (1978), on the basis of studies of the glomerular rejection coefficients of various macromolecules including dextrans of similar molecular weight but different isoelectric point (pI). Cationic macromolecules were found to reach the urinary space much more readily than anionic ones. 'Anionic sites' were subsequently demonstrated on the laminae rarae of the glomerular basement membrane and on the plasma membranes of the epithelial cells by several groups, by the use of cationic dyes such as polyethyleneimine (Schurer et al. 1978), Alcian blue (Caulfield 1979) and colloidal iron (Hirsch et al. 1981). Digestion with selective enzymes suggested that the anionic charge of the epithelial cells is created predominantly by sialic acid residues (Quatacker et al. 1985), whereas that on the basement membrane is created by the presence of heparan sulphate proteoglycan (HSP), as it is abolished by heparitinase (Kanwar et al. 1980, Rosenzweig & Kanwar 1982, Kanwar 1984). Biochemical analysis of isolated glomerular basement membranes indicates that carboxyl groups are much more numerous than the sulphate groups of HSP but perhaps the localization of the latter renders its charge more functionally important.

There has been much speculation and conflicting evidence over the importance of these charged layers in maintaining normal glomerular filtration, and over whether the charge is lost in human glomerular disease. A discussion of these controversies is outside the scope of this thesis, but the evidence that the glomerular anionic charge can influence the localization of antigen will be reviewed.

Border *et al.* (1982) studied the chronic BSA serum sickness model of glomerulonephritis in the rabbit. Using native BSA under the conditions of their experiments, the animals developed mainly mesangial deposits. The antigen was then modified by the covalent linkage of ethylenediamine by the method of Hoare and Koshland (1967), altering the pI of the BSA from 4.6 (anionic) to above 9 (cationic). Animals receiving this antigen under otherwise identical conditions rapidly developed subepithelial deposits. (In a similar experiment in the rat, the same group had previously reported deposit localization reversed from that described above for the rabbit; native BSA produced mesangial deposits and cationic BSA produced subepithelial accumulation (Border *et al.* 1981). This report has not been confirmed and the anomaly remains unexplained). No differences could be detected in the immune responses of the animals or in the circulating immune complex load, so it was assumed that the

difference was caused by attraction between the anionic basement membrane and the cationic antigen. This was supported by the finding that simultaneous administration of protamine (another cation) with the cationic BSA decreased the numbers of subepithelial deposits and the proteinuria in this model (Adler et al. 1983b). It was assumed that this was due to neutralization of the glomerular anionic charge by the protamine. The possibility that the protamine might have this effect through haemodynamic alterations was not adequately excluded but this seems unlikely as polyethyleneimine (which is also cationic) was reported to enhance the deposition in the glomerular capillary wall of pre-formed immune complexes containing native BSA (Barnes & Venkatachalam 1984). The pI of these complexes was not measured but it is not likely that they were cationic.

These experiments did not prove whether it is cationic complexes or free cationic antigen which initially binds to the glomerular anionic sites but it is tempting to suggest that the subepithelial localization is the result of binding of free antigen, with secondary attachment of antibody and *in situ* immune complex formation. Adler et al. (1983b) showed that this mechanism is at least a possibility; they gave cationic BSA to non-immune rabbits, then removed the kidneys and perfused them *in vitro* with sheep anti-BSA serum.

Capillary wall deposits of IgG and C3 were formed. There appear to be efficient mechanisms for the removal of cationic substances from the glomerular basement membrane if antibody is not present; Gauthier et al. (1984) reported initial rapid glomerular localization of cationic antigen but complete removal within 12 hours in non-immune animals. However, the events following binding of antigen in the glomerulus of an immune animal are probably quite complex. Vogt et al. (1982) suggested, on the basis of careful sequential electron microscopic studies, that complexes may form first in the subendothelial space, but then move (presumably by dissociation and re-aggregation) to the subepithelial space. This suggestion has been supported by a recent report which suggests that transport of complexes across the basement membrane may be complement dependent. Sawtell et al. (1988) reported that mice given cationized bovine gamma globulin would develop subepithelial deposits only if complement was present; after depletion of C3 with cobra venom factor only subepithelial deposits formed. A related but C5 deficient strain could form subepithelial deposits, indicating that, as with immune complex disaggregation *in vitro*, the later complement components are not required.

The electrical charge of the antibody is also likely to affect glomerular localization (Feintzeig et

al. 1986). Cationic antibodies appear to gain more ready access to the subepithelial space than anionic antibodies (Madaio et al. 1984), and anti-DNA antibodies eluted from the kidneys of mice suffering from a model of SLE glomerulonephritis were found to be more cationic than those in the circulation (Ebling & Hahn 1980). Gauthier et al. (1984) reported that immune complexes prepared with anionic antibodies tended to produce mesangial deposits, whereas complexes containing cationized antibodies localized initially in the subendothelial space, with subsequent transfer to the subepithelial location (Gauthier et al. 1982).

The effect of charge is clearly of great interest but its importance is not exclusive; although some form of *in situ* aggregation is probably always involved in the formation of subepithelial deposits, the antigen does not have to be cationic. Fleuren et al. (1980) perfused the isolated rat kidney alternately with native (anionic) BSA and anti-BSA. This produced subepithelial deposits, whereas pre-formed BSA-anti-BSA complexes in the same preparation did not. Furthermore, if the antigen is very large it may be excluded from the subepithelial space despite bearing a cationic charge. (Vogt et al. 1982). Other factors may cause an antigen to bind to the glomerular wall; the example of concanavalin A has been mentioned above and DNA has been found to have an affinity for and bind to the glomerular

basement membrane, an observation of great potential significance to the nephritis of SLE (Izui et al. 1976). Finally, it should be borne in mind that the relevance of antigen charge has not been shown in man, with the possible exception of cationic streptococcal antigens and acute proliferative glomerulonephritis (Vogt et al. 1983).

Circulating antigen/antibody levels

It has been stressed above that the ratio of antigen to antibody in the circulation was found to be an important factor in the development of immune complex glomerulonephritis; but in addition it is reasonable to assume that the glomerular deposition of circulating immune complexes or antigen will be related in some way to the overall amount of the complexes or antigen in the circulation. The rate of elimination of such substances from the circulation is therefore likely to be important. In normal circumstances the formation of circulating immune complexes can be seen as a protective mechanism, as it leads to the rapid elimination of the antigen concerned; the involvement of complement in the clearance of immune complexes was outlined in the previous chapter. It has already been noted that large complexes formed in antibody excess are rapidly cleared and are not nephritogenic (McCluskey et al. 1962, Mannik

& Arend 1971). If the efficiency of this clearance is impaired, the complexes might persist in the circulation and accumulate in the glomeruli. Some evidence that this effect might be important in man has already been discussed, in relation to the increased incidence of SLE and glomerulonephritis in cases of inherited deficiency of complement components. In experimental animals the presence of large quantities of circulating immune complexes seems to saturate the antigen clearance system, decrease the rate of elimination and increase glomerular localization (Haakenstad & Mannik, 1974, Bourne et al. 1983). This was attributed to blockage of macrophage function but depletion of complement and complement receptors could also have played a role. Conversely, stimulation of the phagocytes by inoculation with *Corynebacterium parvum* increases the uptake and degradation of complexes by the mononuclear phagocyte system and decreases glomerular localization (Barcelli et al. 1981), a finding of direct relevance to this thesis.

Heymann nephritis

It has been shown that immune complexes can be deposited in glomeruli by trapping of circulating complexes or by assembly *in situ*, after an antigen has been trapped in the glomerulus, or a combination of

both, with dissolution and re-assembly in another (subepithelial) site. It was thought until recently that the presence of auto-antibodies against components of the glomerular capillary wall could not give rise to morphologically identifiable electron dense deposits, but would instead produce a 'linear' pattern of immunoglobulin deposition, as demonstrated by immunofluorescence. Studies of the form of auto-immune glomerulonephritis in rats known as Heymann nephritis (Heymann et al. 1959) have now shown this view to be false and have established a third mechanism by which glomerular immune complex deposits can form. In Heymann nephritis, administration of homologous kidney homogenate in Freund's complete adjuvant produces a nephrotic syndrome and chronic glomerulonephritis with a striking morphological similarity to human membranous glomerulonephritis (Alousi et al. 1969). It was subsequently shown that the same disease can be induced by immunization with microgram amounts of a fraction of renal tubules known as Fx1A (Edgington et al. 1968, Glassock et al. 1968). The antigen has been characterized as a glycoprotein of molecular weight 330,000 (gp330) which is localized in the brush border of the proximal tubules (Keraschki & Farquhar 1983, Makker & Singh 1984). Similar deposits can be produced by transfer of serum from diseased rats (Sugisaki et al. 1973) or immune rabbits (Barabas & Lannigan 1974); the latter is known as heterologous Heymann nephritis, as

the most severe glomerular damage results from the development of immunity against the foreign antibody deposited in the glomerulus.

The amount of antigen given at immunization is so small that any directly produced circulating immune complexes would be present in insufficient quantity to produce disease. Consequently it was initially thought, by analogy with the concepts of serum sickness current at that time, that the disease was due to the formation of circulating immune complexes with the trace amounts of gp330 normally present in the circulation (Glasscock *et al.* 1968, Singh & Makker 1985). Initial evidence that this was not necessarily the case came first from *in vitro* perfusion experiments (Couser *et al.* 1978). Antibody against Fx1A, from immune rabbits or eluted from diseased rat kidneys, was shown to produce subepithelial deposits in the absence of any external antigen. Furthermore, antibody from diseased kidneys was found to bind to sections of normal rat glomeruli, indicating the constitutive presence of the appropriate antigen (Neale & Wilson 1982). Glomerular reactivity was eliminated by prior adsorption with Fx1A. More recent studies indicate that antibody binds to gp330 which is localized in coated pits on the glomerular epithelial cells, but instead of producing antigen elimination or a linear pattern of deposition, relocation and aggregation takes place, leading to the formation of subepithelial

deposits (Kerjaschki & Farquhar 1983, Camussi et al. 1985, Cybulski et al. 1988). There is thus good evidence that antibodies against Fx1A can produce immune complex deposits which are indistinguishable by electron microscopy or immunohistochemistry from those produced by other means. It is possible that a mechanism analogous to this produces membranous glomerulonephritis in man - some authors refer to the model as 'experimental membranous glomerulonephritis' - but there is no direct supporting evidence. Indeed, Heymann nephritis has not been adequately duplicated in any other species, and Collins et al. (1981) were unable to detect a comparable tubular antigen in human glomeruli, whether normal or affected by membranous glomerulonephritis. The main factor controlling the formation of immune complex deposits by this mechanism is the maintenance of immunological tolerance; this is outside the scope of this thesis and has recently been discussed in this context by Pusey et al. (1988).

Many other models of immune complex glomerulonephritis have been described but although they have contributed valuable information to the field in general, no other basic mechanisms of immune complex deposition have been demonstrated beyond the three outlined above; the trapping of circulating immune complexes, the formation of immune complexes *in situ*

after trapping of antigen in the glomerulus, and the formation of complexes after binding of antibody to a normal component of the glomerulus. It is appropriate to stress again that glomerulonephritis is usually a chronic disease and the deposition of immune complexes, by whatever mechanism, is only the first of a complex sequence of events. Deposits in the glomerulus may be modified, made more stable or removed, even as fresh deposition continues. These processes will be discussed in the next chapter.

Experimental models of immune complex

glomerulonephritis:

The alteration and elimination of immune complexes

Much of the experimental work relating to the deposition of immune complexes in the glomerulus has utilized acute animal models, where the nature and distribution of the complexes are studied almost as soon as they form. This approach successfully limits the possibility of confusion due to alteration of the deposits after they form, but the specific study of the nature and effects of such alterations has consequently been neglected. It has been emphasized that human glomerulonephritis is usually a chronic disease and therefore such alterations are likely to be of importance; indeed it is theoretically possible that enhancement of the removal of immune complexes could be as valuable a therapeutic manoeuvre as inhibition of deposition. The purpose of this chapter is to examine the evidence that immune complexes are modified after deposition in the glomerulus, and the theories of how modification and removal might occur.

Evidence for the modification of deposits

Much of the evidence that the deposits seen in human glomerulonephritis can be modified over time has already been discussed. It has long been evident that the electron dense deposits seen in long-standing immune complex glomerulonephritis differ in their morphology and content from those found in more acute forms of the disease. Ehrenreich & Churg (1968) described the morphological alterations in human membranous glomerulonephritis; the deposits become surrounded by what appears to be basement membrane matrix, and as the glomerulus becomes non-functional, the deposits become electron-lucent. Similar changes can be seen in chronic animal models such as murine SLE. These changes occur over weeks or months, but modification of immune complex deposits also occurs much more rapidly than this. As has been stressed in the previous chapter, cationic immune complexes and antigens can bind to the glomerular anionic sites. Such immune complexes can be displaced from the glomerulus if another cation is administered within a few minutes, but by one hour a change has occurred such that removal is no longer possible by this method (Gauthier & Mannik 1986). In chronic serum sickness, alteration of the location of deposits has been reported during the early course of the disease (Vogt et al. 1982) but although the authors suggested that this was due to dissolution and re-localization of

the same molecules, the possibility that further deposition of new complexes was responsible was not excluded.

The importance of re-equilibration of immune complexes after deposition was demonstrated more convincingly by Mannik et al. (1983). They developed a passive model of serum sickness using an antigen which could be induced (by irradiation with light) to form covalent bonds to the complexed antibody, thus precluding any possibility of further rearrangement. Covalent and non-covalent complexes accumulated in the mesangium in similar amounts at 1 hour, but subsequently only the non-covalently linked complexes persisted. The conclusion, that rearrangement of immune complexes is necessary for persistence, illustrates the importance of processes which occur after the initial deposition (As was noted in the previous chapter, this work is also of significance to the argument about the relative importance of trapping of circulating complexes versus 'in situ' formation).

There is further direct evidence that the composition of immune complexes changes with time; rheumatoid factors have been found both in the serum and in glomerular deposits in chronic serum sickness (Penner et al. 1979) and in long-standing human glomerulonephritis, where the presence of anti-globulins may indicate a worse prognosis (Rossen et al. 1975).

More recently, globulin/antiglobulin complexes in serum and glomeruli have been shown to represent idiotype/anti-idiotype antibodies (Zanetti & Wilson 1983), as might be expected from current theories of regulation of the immune system (Jerne 1975). Free antibody and even immune complexes of the same antigen system can bind to deposits already present in glomeruli (Ford & Kosatka 1980), which supports the widespread view that the deposits grow by a process of 'molecular accretion' (Pusey *et al.* 1988).

Finally, apparently unrelated substances such as horseradish peroxidase and catalase (both small macromolecules) can accumulate within established deposits in Heymann nephritis (Schneeberger *et al.* 1974, 1979). No explanation of this phenomenon was proposed other than passive trapping. Given the stimulus which immune deposits appear to provide to the synthesis of basement membrane material by epithelial and mesangial cells in some forms of chronic glomerulonephritis, it would also seem likely that with time, intrinsic basement membrane components such as type IV collagen could become enmeshed in the deposits. This might confer increased stability but such an event has not been experimentally confirmed.

Removal by phagocytosis

There is in normal glomeruli a continuous flow of plasma through the mesangium (Takamiya et al. 1979, Latta & Fligiel 1985). It has been known for many years that apparently inert particles such as colloidal carbon and ferritin can become trapped in the glomerular mesangium (Farquhar & Palade 1961, Elema et al. 1976). The particles may emerge at the vascular pole (Elema et al. 1976, Michael et al. 1979) and leave via the distal tubules, the efferent arteriole or the lymphatics, but such particles can frequently also be seen within the cytoplasm of cells in the mesangium, presumably having been phagocytosed. The cells involved were initially assumed to be the intrinsic mesangial cells, and this may indeed be the case with some substances (Sterzel et al. 1982, Mancilla-Jimenez et al. 1982) but the discovery that glomeruli normally contain a sub-population of macrophage-like cells (Schreiner et al. 1981, Schreiner & Unanue 1984) which are recruited from blood monocytes (Gurner et al. 1986) has led to a re-appraisal. Numerous studies have assessed the importance of these glomerular phagocytes in the trapping and processing of a variety of materials which accumulate in the mesangium (Sterzel et al. 1982, Goode et al. 1985, Sedor et al. 1987). The effect of polyvinyl alcohol (PVA) has been much studied in this context. Repeated injections of this inert polymer cause accumulation of

PVA in the mesangium. It is found to be inside the phagocytic vacuoles of Ia⁺ positive mesangial macrophages, which are increased in number and activity, although glomerular hemodynamics appear to be almost unaltered (Sterzel et al. 1983, Seiler et al. 1983). Such treatment causes increased accumulation of colloidal carbon (Mauer et al. 1979) and iron dextran (Seiler et al. 1986); in the latter example the PVA-induced macrophages processed the iron dextran to ferritin more rapidly than controls. These findings with particulate tracers would appear to be of relevance to the mesangial processing of immune complexes. Striker et al. (1979) showed that mesangial macrophages are involved in the phagocytosis and degradation of immune complexes in a passive serum sickness model. In this important work, the mice studied had received bone marrow transplants from Chediak-Higashi mice, whose phagocytic cells have recognizable giant lysosomes; this allowed reliable confirmation that the intrinsic mesangial cells were not involved. Batsford et al. (1985) have studied some of the characteristics of the antigen which influence the uptake and processing by mesangial macrophages: cationic charge and 'biologic activity' ('increased' by covalent attachment of glutaraldehyde to the antigen) were found to enhance uptake and removal.

It is thus reasonably well established that mesangial macrophages are involved in the uptake and processing of material, presumably including immune complexes, as it arrives in the mesangium and soon after. But what of the capillary loop deposits, to which macrophages do not have ready access? Even in the mesangium, is a mechanism which deals with immune complexes as they arrive in the glomerulus necessarily involved in the processing or removal of established, morphologically identifiable deposits, which are unlikely to have the same content or structure as circulating immune complexes? Mesangial deposits are capable of persisting for some time without further deposition of the original antibody/antigen system, and although Striker *et al.* (1979) described material morphologically resembling electron dense deposits within phagocytic vacuoles, this was seen within hours of administering pre-formed immune complexes. Phagocytosis of electron dense deposits has been assumed to occur later than this: in the NZB/NZW model of SLE in the mouse, Kimura *et al.* (1987) commented that mononuclear phagocytes are "presumably contributing to the scavenging of the mesangium" and observed that "mononuclear cells, extending their cytoplasmic protrusions towards the dense (immune complex) deposits, were frequently noticed". Phagocytosis of established electron dense deposits does not appear to have been observed. It is of interest in this context to note the

effect of a modification in the number of glomerular macrophages on the course of experimental glomerulonephritis. Lavelle et al. (1981) gave anti-macrophage serum to rabbits with acute serum sickness at the point of immune elimination of the antigen from the circulation (i.e. the time of glomerular deposition). The amount of glomerular damage and proteinuria was reduced, confirming the importance of macrophages in the development of glomerular injury in this model. If macrophages are involved in the degradation of immune complexes, one would expect treated animals to have heavier deposits in their glomeruli. The reverse was reported. The authors suggest that this was due to "the absence of the deleterious effect of macrophage enzymes". These results are confirmed by Holdsworth et al. (1981), but again the authors ignore the inference that macrophages are not removing the deposits. Furthermore, pre-existing mesangial complexes, which might be expected to enhance phagocytic function if this system is involved, are reported to cause a decrease the efficiency of removal (Keane & Raj 1980). Boyce & Holdsworth (1986) showed that administration of anti-macrophage serum reduced the subsequent glomerular trapping of heat-aggregated IgG: conversely, they also observed that in glomeruli with an increased number of macrophages due to anti-basement membrane glomerulonephritis, the mesangial uptake of heat-aggregated IgG was greater than in controls (Boyce &

Holdsworth 1986). Similarly, in nephrotic syndrome induced by puromycin aminonucleoside, the mesangial uptake of heat-aggregated IgG was increased (Keane & Raij 1985). This increase is probably caused by altered glomerular haemodynamics, as it was blocked by infusion of an inhibitor of Angiotensin II, but it is remarkable that in none of these studies was the expected inverse correlation between glomerular macrophage numbers and mesangial deposits reported. This implies that the presence and activity of macrophages in the glomerulus does not enhance deposit removal.

If, despite the above evidence, macrophages do degrade established glomerular immune complex deposits, then it is likely that the proteolytic enzymes they contain will be utilized. The importance of such enzymes is given some support by the observation that administration of proteolytic enzymes can enhance deposit removal, whether given acutely in large doses (Nakazawa et al. 1986a) or more chronically in smaller doses (Nakazawa et al. 1986b) but such studies are highly artificial, and macrophages are not the only potential source of proteolytic enzymes. Jennette et al. (1987) concluded that the administration of amidine-type protease inhibitors could decrease glomerular injury in a chronic serum sickness model, but despite studying numerous inhibitors no change was seen in the amount or site of immune complex localization.

It is therefore open to question whether macrophages are involved in the removal of mesangial immune complexes after the initial phase of accumulation. The involvement of macrophages in the removal or modification of subepithelial deposits seems even less likely, as macrophages do not normally gain access to this space unless glomerular crescents are formed. The relevance of macrophages to deposit removal will be considered further in this thesis.

Removal by complement

The involvement of the constituents of the immune deposits in their own removal has been the subject of some study. The frequent observation of complement components co-localizing with other constituents of electron dense deposits has led to the belief that complement becomes intimately enmeshed in the deposits and contributes to their bulk. This has been reinforced by the observation that the deposits in acute serum sickness may contain copious amounts of C3 when the known antigen is barely detectable (Fish et al. 1966). Although complement presumably does contribute to the mass of the deposits, activated C3 is unlikely to be an inert component. There is abundant evidence that complement can dissolve immune complexes in the circulation (reviewed by Schifferli et al. 1986) and

there is a single report in the literature indicating a similar function with immune complexes in glomeruli. Bartolotti & Peters (1978) depleted complement by giving cobra venom factor to rabbits with acute BSA serum sickness, four days after immune elimination of the antigen from the circulation. The rate of removal of BSA from the glomeruli of these rabbits was significantly reduced, though proteinuria was not affected. Bartolotti and Peters (1979) subsequently reported that sections of kidney from rabbits with acute serum sickness at the time of glomerular deposition could activate complement by alternative or classical pathways, but that a few days after deposition this capacity was lost, again indicating a change of the properties of the immune complexes with time.

Removal by antigen and antibody

Despite the observation that the deposits appear to grow by 'molecular accretion', it has been known for some time that intravenous administration of large amounts of antigen can lead to the removal of deposits (Valdes et al. 1969). It has been claimed that a large excess of antibody can have the same effect (Pusey et al. 1988); such an excess might inhibit deposition but there is no published evidence that removal is enhanced. By analogy with the observations on immune complex size

described in the previous chapter, the effect of excess antigen was presumed to be due to the interference with the immune lattice and the generation of small soluble complexes, a theory supported by the observation that this can occur after the production of deposits by administration of pre-formed immune complexes, where the host immune system is unlikely to be involved (Mannik & Striker 1980). Penner et al. (1979) studied this phenomenon *in vitro*, by incubating frozen sections of kidney from rabbits with chronic BSA serum sickness with excess BSA. In early lesions this caused the removal of the deposits, as assessed by immunofluorescence, but after glomerulonephritis had been present for several weeks the deposits were only removed by incubation with excess BSA followed by heat-aggregated IgG. This supports the importance of the later accumulation of anti-idiotypic or rheumatoid antibodies mentioned above. Haakenstad et al. (1983) found a similar time dependency of dissolution by antigen *in vivo*, again using chronic serum sickness; dissolution became only partial after chronic serum sickness had persisted for six weeks. Dissolution by antigen appears to be a phenomenon largely confined to mesangial deposits but it has recently been shown that this may be due to the limited access of excess antigen to subepithelial deposits; an excess of cationic antigen, which crosses the basement membrane more readily, can remove subepithelial deposits (Agodoa & Mannik 1987). These interesting observations

may have therapeutic implications in cases where the antigen can be identified but their relevance to the unmodified pathophysiology of glomerular disease is less clear.

It is evident that recent advances in our understanding of the mechanisms by which glomerular immune complex deposits form have not been matched by advances in understanding of how they may be removed; this is particularly true in the case of subepithelial deposits. Even where advances have been made, the possibility that deposits in different sites, of different age and in different models may be very different has rarely been addressed. These observations prompted the work described in this thesis.

PART TWO

EXPERIMENTAL WORK

Required characteristics of the model

There are two main prerequisites for a study of the factors involved in the removal of antigens and deposits from the glomerulus; an appropriate animal model, and a method for measuring the quantities of antibody and deposits in glomeruli.

The ideal model should have the following characteristics:

- 1) The pathogenesis, morphological changes and functional abnormalities should closely resemble a form of human disease.
- 2) Disease of comparable severity should be induced in all the animals treated.
- 3) The induction of disease should be reasonably easy and rapid.
- 4) The morphological and functional changes induced should be well characterized.
- 5) There must be a method of stopping deposit accumulation at a known time, so that removal can

be studied without interference from continuing deposition.

- 6) After antigen/antibody deposition has stopped, the rate of removal of antigen and deposits from the glomerulus should be measurable and should be of a magnitude such that an increase or decrease in the rate which is induced by experimental manipulation can be of a size which is detectable.

The requirement of sudden cessation of deposition makes many of the available models unsuitable. In autoimmune models such as Heymann nephritis, the only way to end deposition suddenly would be to transplant the kidney into a syngeneic, non-immune rat. Even this may not be appropriate or possible in virus-induced or inherited forms of animal glomerulonephritis. Transplantation to a 'clean' host has several disadvantages; the kidney suffers a variable degree of ischaemia and trauma and the time-consuming nature of the procedure would severely limit the number of animals which could be studied, thereby limiting this investigation to relatively few variables. The serum sickness models provide an alternative approach; the injections of antigen or immune complexes can be stopped, but there then remains a problem of continued antigen deposition for an unknown time, and potential relocation of intact antigen from other organs to the kidney. Transplantation would again avoid these problems

but at the expense of the difficulties outlined above. An alternative approach is to wait after the last antigen dose until all measurable parameters indicate that antigen deposition in the kidney has ceased before attempting to influence the rate of antigen removal. This approach has been used before (Bartolotti and Peters 1978) and was chosen for the work to follow.

The model should resemble a human disease in morphology and pathogenesis. This requirement is unavoidably difficult to fulfil, because so little is known about the pathogenesis of human glomerulonephritis. One can only choose a model which appears to resemble human disease in morphology and in the functional and biochemical abnormalities induced. These factors favour a chronic model which induces glomerular electron dense deposits and either nephritic or nephrotic syndrome.

I therefore decided to study chronic serum sickness glomerulonephritis. The main problem with this model is its unpredictability, described in Chapter 2, unless antigen doses are matched to antibody response, which would again be laborious and limit the number of animals available for study.

The unpredictability of chronic serum sickness appeared to be potentially subject to improvement by two other routes. The first was the choice of experimental

animal. Rabbits have been most widely used: these are outbred animals and individuals are known to produce unpredictable antibody responses to identical stimuli. Rats from a single local colony might produce a more homogeneous antibody response. Chronic serum sickness has been described in rats (Fennel & Pardo, 1967, Arisz et al. 1979, Yamamoto et al. 1978, 1983, Miyazaki et al. 1985 a & b) but the rat is more resistant to glomerulonephritis, and unusual immunization and dosage regimens have been employed, often in spontaneously hypertensive rats. The second potential area of improvement was in the choice of antigen. The recently proposed theory of *in situ* binding of cationic antigens to the glomerular wall, with subsequent binding of antibody, might if correct reduce the critical dependency on antibody response by eliminating the importance of immune complex size. The reported increased nephritogenicity of cationic antigens might also be expected to facilitate the development of a model in the rat. I therefore decided to study chronic serum sickness in the rat, initially using either native or chemically cationized bovine serum albumin as antigen.

The second prerequisite of this study was a method of measurement for antigen and deposits in glomeruli. The identification of a known antigen in glomeruli has usually been achieved by immunofluorescence or a related

technique. Such methods lend themselves at best only to semi-quantitative evaluation. Administration of radio-labeled antigen would permit more precise quantitation. Detection could then be achieved by a variety of means:

- 1) Autoradiography. Counting of grains over glomeruli might permit accurate quantitation but as only a tiny fraction of antigen would localize in glomeruli, low isotope levels might make this impractical.
- 2) Gamma counting of whole renal cortex. This would not discriminate between isotope in glomeruli and that elsewhere in the kidney.
- 3) Gamma counting of isolated glomeruli. This would require measurement of numbers of glomeruli.

An isotope was therefore required with the following characteristics:

- 1) An available method of binding or incorporation of the isotope into the protein antigen.
- 2) An emission detectable by autoradiography.
- 3) An emission detectable by gamma counting. (Liquid scintillation would be inappropriate with tissue fragments or homogenates).

- 4) A half life at least as long as the duration of the experiments.

The isotope best fulfilling these criteria is iodine-125, which has a half life of 60 days, is detectable by autoradiography and gamma counting, and can readily be attached to protein antigens.

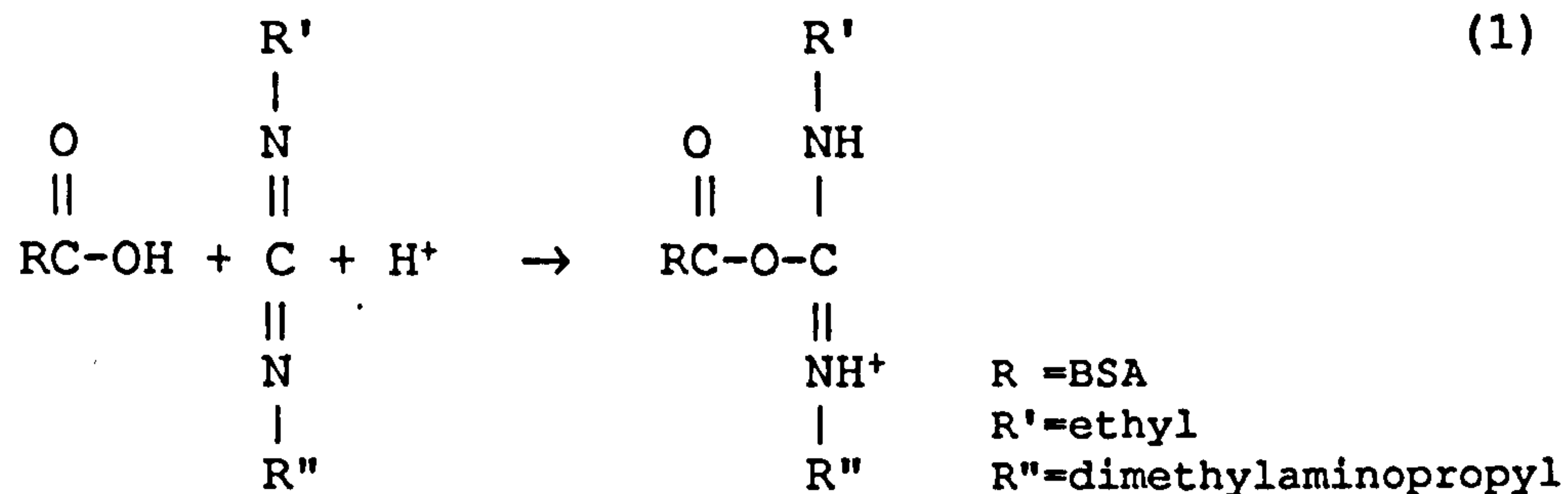
Electron dense deposits can only be observed by electron microscopy. There is no reported method of quantitative extraction or staining; the only available technique is morphometry at electron microscopy level. Though laborious, this has the advantage of permitting separate analysis of deposits in different locations in the glomerulus.

Establishment of the experimental modelPreparation of the antigen.

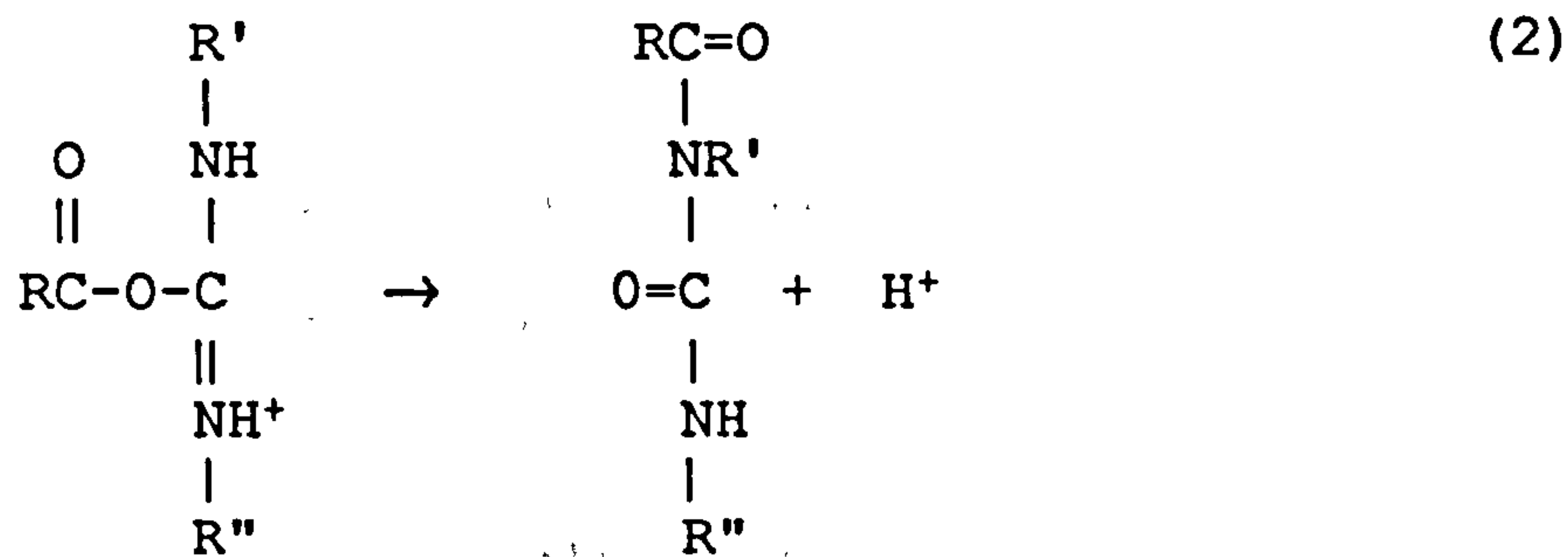
Native bovine serum albumin was obtained from Sigma (A7030: essentially fatty acid and globulin free).

Cationization of BSA Native BSA is anionic at physiological pH (pI 4.6). To make this substance cationic without substantially altering its antigenicity, the method of Hoare and Koshland (1967) was used, as suggested by Border et al. (1982). A water soluble carbodiimide (1-ethyl-3(3-dimethylaminopropyl) carbodiimide) is used to create a covalent bond between carboxyl groups of the protein and ethylenediamine, a small, strongly cationic molecule. Hoare and Koshland suggested the reaction to be as follows:

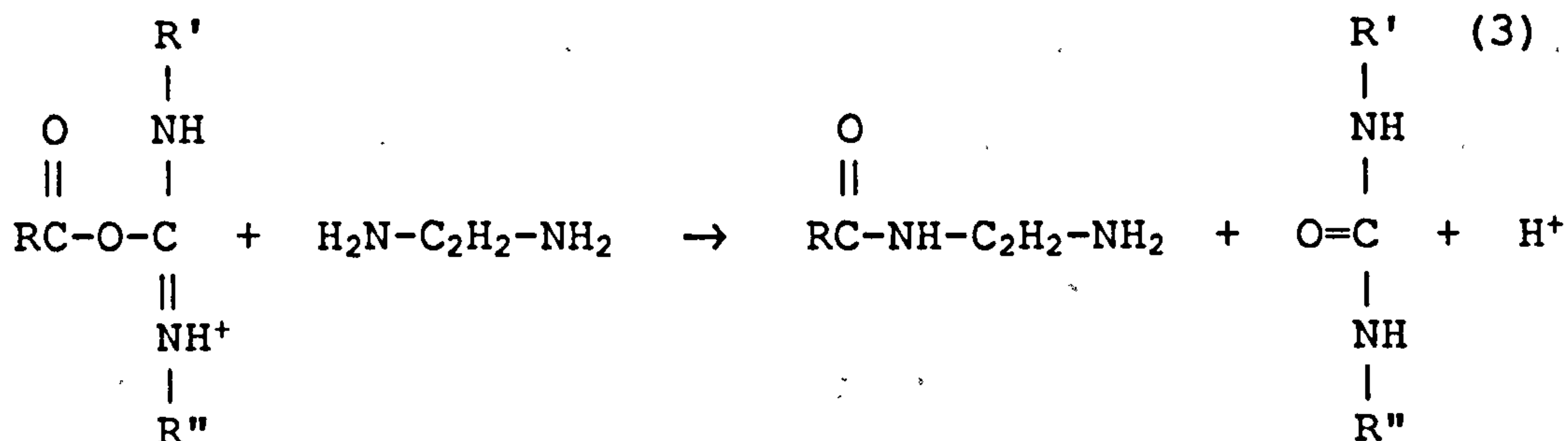
Initial reaction of the carbodiimide group with a carboxyl group of the protein produces an O-acylisourea:



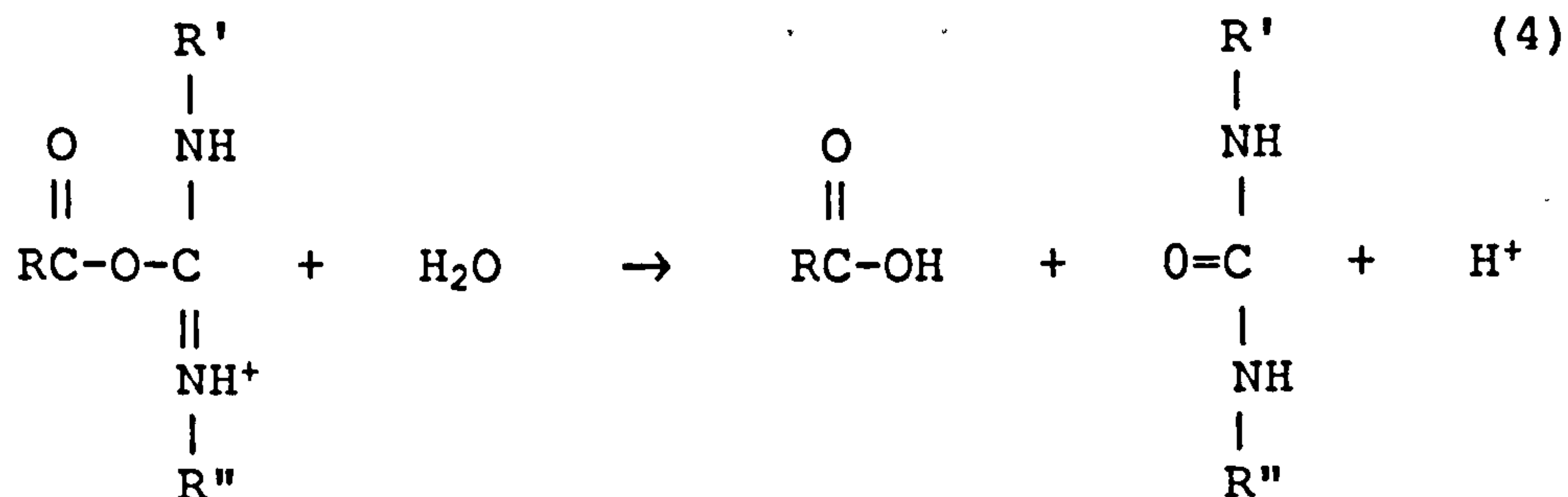
This may then re-arrange to produce an N-acylurea:



or react with a nucleophile such as ethylenediamine:



or water:



which regenerates the carboxylic acid.

The reaction is stopped by adding an excess of an acetate buffer, which reacts with and eliminates any remaining carbodiimide.

This reaction was proposed by Hoare and Koshland as a quantitative method to substitute the nucleophile to all available carboxyl groups of the protein. Of the reactions (2) to (4), only (3) has this effect. Reaction (3) is favoured over reaction (4) at more acid pH, and was reported to be the main reaction at pH 4.75. In my hands, this reaction resulted in a product with a pI of over 11 (*vide infra*) which was toxic, despite careful aseptic technique, passage through a 0.2 μ m filter and confirmation by thin layer chromatography that none of the original reagents remained after the final dialysis.

The reaction conditions were therefore modified to give a slightly less cationic product, and carried out at pH 5.5, to enhance reaction (4) at the expense of (3), as described in Appendix 1.

Characterization of the antigen

The pI of each batch of cationic BSA was measured by isoelectric focussing on thin cellulose acetate membranes (Ambler & Walker 1970) using ampholines with a pH gradient of 2 to 11 (Serva). The batch of BSA was rejected if the main band had a pI outside the limits of 8 to 10. (Appendix 2)

To exclude the presence of aggregates, an iodine-125 labeled sample from each of the first two batches was layered on to a 12-38% sucrose gradient and spun for 8 hours at 50,000 r.p.m. in a Sorvall OTD-65

ultracentrifuge in an AH650 head. Seventeen fractions were collected and counted. Only one peak was identified; there was therefore no evidence of dimers or larger aggregates (Appendix 3).

Radioiodination of the antigen

When an experiment was planned which would require the use of radiolabelled antigen, Iodine-125 was obtained from Amersham (EMS30) at an activity of 74MBq per experiment. Cationic and/or native BSA was labeled by a Chloramine T method, as described in Appendix 4.

Samples taken before and after dialysis indicated that the efficiency of iodination was never less than 85%. Precipitation of the protein from the final product with trichloroacetic acid left less than 2% of the activity in the supernatant.

The radio-labeled protein was mixed with sufficient unlabeled antigen to complete the experiment, at a concentration of 100mg/ml. This was then separated into an appropriate number of aliquots (usually seven) and stored at -20 C until required.

Immunization and dosage regimens**Protocol**

Male Wistar rats, initial weight 100-125g, were used throughout the experimental work. In the experiments designed to establish and characterize the model, a single untreated rat was kept in each cage, to confirm variation from the normal and to exclude possible effects of infection in the colony.

To establish an immune response, three different adjuvant regimes were assessed, using 1mg of either native or cationic BSA as antigen in every case:

- 1) Antigen with 1µg of *Escherichia coli* endotoxin (Sigma), intravenously, seven days before starting regular injections of antigen.
- 2) Antigen in 0.25ml saline with 0.25ml of Freund's incomplete adjuvant (FIA) subcutaneously to the back of the neck, 22 days and again 10 days before starting regular injections.
- 3) Antigen in 0.25 ml saline with 0.25ml of Freund's complete adjuvant (FCA), subcutaneously to the back of the neck, 22 days and again 10 days before starting regular injections.

Each group contained six rats immunized with each of the antigens (a total of 36 rats). Pairs of rats were

then given the appropriate antigen by thrice weekly tail-vein injection for two months, in doses of 0.1mg, 1mg or 10mg, in every case in 0.1ml of saline. After eight weeks the rats were killed by exsanguination under anaesthetic (Hypnorm® and diazepam). Samples of renal tissue were taken into formalin for light microscopy (Appendix 5) and into 2.5% glutaraldehyde for electron microscopy (Appendix 6). Paraffin sections were stained conventionally with hæmatoxylin and eosin. Tissue for electron microscopy was post-fixed in osmium tetroxide and processed into Epon resin. Thin sections (0.5µm) were cut and stained with Toluidine Blue to identify a suitable area for study, then ultrathin sections were cut, stained with uranyl acetate and lead citrate, and examined in a JEOL 1200EX transmission electron microscope at 80KV.

Serum samples were taken from each rat at the end of the experiment. Ouchterlony plates were used to identify the presence of an antibody response and to assess the degree of identity between antibodies raised against native or cationic BSA (Appendix 7).

Results

One rat which had been immunized with FCA died within minutes of receiving its first intravenous dose, which was 10mg of cationic BSA. This was presumably due to hypersensitivity in an animal with high circulating

antibody levels. There were no other unexpected complications.

None of the rats showed any detectable alteration in glomerular morphology as assessed by light microscopy of paraffin sections. The results of the electron microscopy study are shown in Table 1. Rats immunized with endotoxin or with FCA developed no lesions detectable by electron microscopy. In animals immunized with FIA, administration of native BSA produced only mesangial electron dense deposits. These were present in every glomerulus of rats given 1mg. (Fig. 1) A dose of 10mg produced only occasional small deposits, and after 0.1mg no deposits were found at all. In contrast, cationic BSA produced subepithelial and mesangial deposits. The lowest dose produced no deposits but they were readily found in both locations in rats given 1mg and they were large and very numerous in rats given 10mg (Fig. 2).

Table 1. The effect of variations in immunization schedule and antigen dose on glomerular morphology.

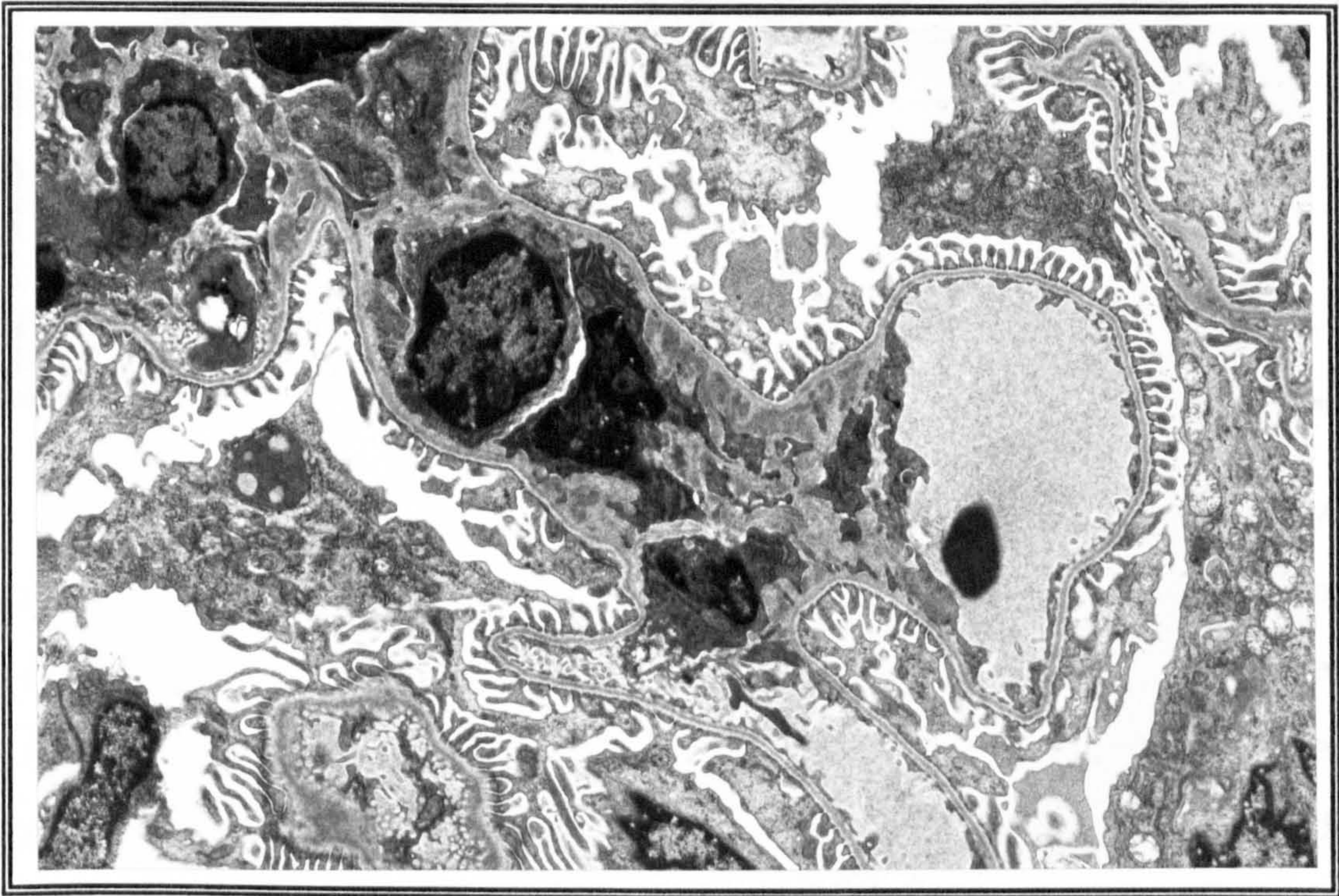
Antigen: *Native bovine serum albumin*

Antigen dose	Endotoxin	FIA	FCA
0.1mg	No deposits	No deposits	No deposits
1mg	No deposits	Numerous mesangial deposits	No deposits
10mg	No deposits	Few mesangial deposits	No deposits

Antigen: *Cationic bovine serum albumin*

Antigen dose	Endotoxin	FIA	FCA
0.1mg	No deposits	No deposits	No deposits
1mg	No deposits	Occasional subepithelial and mesangial deposits	No deposits
10mg	No deposits	Numerous subepithelial and mesangial deposits Foot process effacement	No deposits

Figure 1. Mesangial deposits in a rat which had received 1mg doses of native BSA.

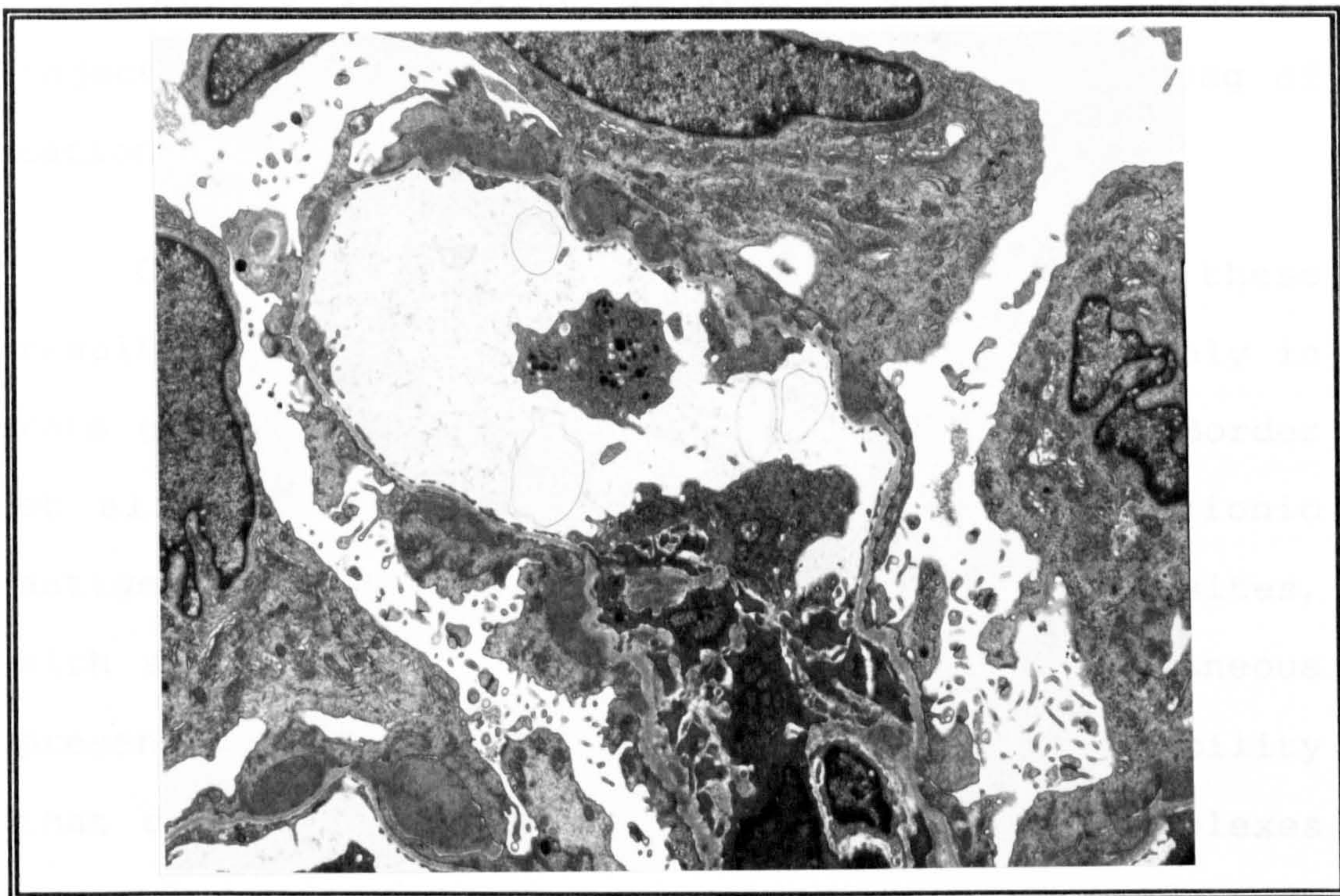


...were evident, with a single line of identity between samples from rats immunized with either antigen.

Conclusions

The results of this experiment were used to assess the best conditions to induce chronic serum sickness.

Figure 2. Numerous subepithelial and mesangial deposits in a rat which had received 10mg doses of cationic BSA.



The Ouchterlony diffusion plates did not detect any antibody in rats immunized with endotoxin or in rats given 10mg of either antigen - presumably because these large doses had caused consumption of most of the antibody produced. In rats given FCA or FIA and subsequent doses of 1mg or 0.1mg of antigen, lines of precipitate were evident, with a single line of identity between samples from rats immunized with either antigen.

Conclusions

The results of this experiment were used to assess the best conditions to induce chronic serum sickness

glomerulonephritis. In every subsequent experiment, the rats were therefore immunized twice with 1mg of antigen in Freund's incomplete adjuvant, and thrice weekly injections were given of 1mg of native BSA or 10mg of cationic BSA, in 0.1ml of saline.

Other conclusions can also be drawn from these results. The presence of subepithelial deposits only in rats given cationic BSA confirms the findings of Border et al. (1982) and supports the theory that cationic antigens can bind directly to glomerular anionic sites, with subsequent binding of antibody. The simultaneous presence of mesangial deposits suggests the possibility that deposition of whole circulating immune complexes may also be occurring in the cationic BSA model.

The dependence of the native BSA model on antigen dose is well known and is described in Chapter 2. The number of rats studied here is small, but the apparent absence of an optimal nephritogenic dose for cationic BSA also supports the theory of '*in situ*' binding of antigen, as it implies that the size of any circulating immune complexes is not critical.

The absence of any deposits in rats which received only endotoxin as adjuvant is explained by the apparent absence of an antibody response in those animals. The absence of deposits in rats which received FCA is more complex. Antibody levels were not measured but if these

were higher after FCA, one would have expected the higher dose of native BSA to restore the balance; with cationic BSA the antibody level is probably less important. The alternative explanation is that FCA has caused increased activity of the mononuclear phagocytes, with more rapid antigen clearance. Such clearance could occur before the antigen reach the glomerulus, but there could also be more rapid removal from the glomerulus immediately after deposition. Other studies were planned to investigate these points but it was first necessary to further characterize the experimental models.

Characterization of the experimental models

Introduction

Having established conditions under which rats would develop glomerular deposits after eight weeks of antigen injections, it was necessary to document the speed with which such deposits formed, and any changes in the composition of blood or urine which were induced during that period. A parallel experiment was performed to evaluate the intramuscular administration of antigen. This route would, if successful, reduce the 'bolus' effect whereby an intravenous injection causes a brief but considerable circulating antigen excess. It would also increase the speed with which experiments could be performed.

Protocol

Animals were immunized as described above, using either native BSA (15 rats) or cationic BSA (15 rats). Thrice weekly intravenous injections were then given of the appropriate antigen at a dose of 1mg (native) or 10mg (cationic). Five rats from each group were killed after two weeks, four weeks and eight weeks. The last antigen dose was in every case given 24 hours before death and was labeled with iodine-125. At sacrifice, samples were taken of blood, renal cortex, liver, spleen and lung. The tissue samples were weighed and submitted to a gamma counter. Renal tissue was processed for

electron microscopy; all tissues were processed for light microscopy and autoradiography (Appendix 8). Full blood counts were performed using a Coulter counter with the assistance of the Department of Hæmatology. Serum urea, albumin, cholesterol and triglyceride were measured on a Hitachi 712 Autoanalyzer with the assistance of the Department of Clinical Chemistry. Samples from six normal rats were included to establish a normal range. All the animals were weighed before and after the course of injections. Three rats from each of the two groups surviving for eight weeks were kept in metabolic cages and 24 hour urine collections were made to assess proteinuria at 0, 2, 4 and 8 weeks. Protein concentrations were measured by the sulphosalicylic acid technique (Appendix 9).

To assess the effect of intramuscular administration of antigen, three groups of six rats each were immunized with FIA and native BSA, cationic BSA or saline. Each group of six was then divided into two groups of three, and given the appropriate antigen by tail-vein or intramuscular administration (native 1mg, cationic 10mg) thrice weekly for four weeks. Intramuscular injections were given to the musculature of the hind leg on alternate sides. Renal tissue was then taken for light and electron microscopy.

To confirm that the observed difference in the effects of the two antigens was due to the regular injections rather than the initial immunization, two groups of three rats each were immunized with FIA and antigen as before, but were then given the other antigen for three weeks before death and sampling of renal tissues.

To assess the influence of antigen dose on the size of any circulating immune complexes, pairs of rats were immunized as above against native or cationic BSA, then given 0.1mg, 1mg or 10mg of the appropriate antigen by intramuscular injection thrice weekly for two weeks. The last dose was labeled with iodine-125 and the rats were killed by exsanguination under anaesthetic three hours later. The blood was allowed to clot at 38 C; serum was collected and stored in liquid nitrogen. Samples were layered on to sucrose gradients (12%-28%) and spun for 6 hours at 55,000 r.p.m. in a Sorvall OTD-65 ultracentrifuge in an AH650 head (Appendix 3). Samples of labeled native BSA and human IgG were also spun as molecular weight markers. The tubes were cut into 17 fractions and submitted to the gamma counter.

Results

Morphology. No changes were detected by light microscopy of the glomeruli of any of the rats at any time, irrespective of the antigen dose. Electron microscopy showed that all the rats receiving 10mg of cationic BSA had subepithelial and mesangial electron dense deposits by two weeks, with foot process effacement. An impression was gained that these changes were more severe at four and eight weeks but as quantitation was not attempted this could not be confirmed. One of the rats receiving 1mg of native BSA had occasional mesangial deposits at two weeks; the others remained normal. At four weeks three of the five rats killed in this group had mesangial deposits. By eight weeks mesangial deposits could be found in all five rats, though they were infrequent in one. In all the rats given native BSA the capillary loops remained morphologically normal, with no deposits and no foot process effacement.

Tissue antigen load. The amount of radio-labeled antigen remaining in renal cortex 24 hours after the last dose was higher than in any other tissue and was higher for cationic BSA than native BSA. The amount of antigen deposited in the kidney appeared to be fairly constant over the course of the experiment (Table 2).

Table 2 Percentage of one dose of radio-labeled BSA in each gram of renal cortex 24 hours after administration.

	<u>Duration of disease</u>		
	2 weeks	4 weeks	8 weeks
Native BSA (%/g) ($\pm 95\%$ limits)	0.108 (± 0.018)	0.111 (± 0.013)	0.107 (± 0.027)
Cationic BSA (%/g) ($\pm 95\%$ limits)	0.226 (± 0.044)	0.155 (± 0.023)	0.206 (± 0.017)

Autoradiography An exposure of two months was required before adequate autoradiographs were produced. Even then, the number of grains over each glomerulus varied considerably and it was obvious that this method was not suitable for the quantitation of glomerular antigen load. Nevertheless, some information was gained. The only structures with detectable activity by this method were the glomeruli, which indicated that measurement of the isotope content of whole renal cortex would give at least an approximation of the isotope levels in glomeruli. The pattern of silver grains reflected the diffuse mesangial and capillary loop localization of the deposits after cationic BSA, and the more localized mesangial pattern of the deposits after native BSA.

Haematology Rats receiving cationic BSA for eight weeks developed low haemoglobin and raised reticulocyte counts, indicating a hæmolytic anaemia (Table 3). Numbers of leukocytes and platelets showed no significant change.

Table 3. Haemoglobin and reticulocyte count of control and serum sickness rats at eight weeks.

	Control	Native BSA	Cationic BSA
Haemoglobin (g/dl) (\pm 95% limits)	14.3 (\pm 0.3)	13.5 [†] (\pm 0.46)	10.7* (\pm 1.02)
Reticulocytes (%) (\pm 95% limits)	1.6 (\pm 0.54)	2.1 (\pm 0.61)	6.3* (\pm 1.8)

[†]: $p < 0.05$

*: $p < 0.01$ by Student's t test

Biochemistry Changes in serum urea, albumin, cholesterol and triglyceride are illustrated in Figures 3 to 6. None of the groups showed any significant difference in urea, but by two weeks the rats receiving cationic BSA had low serum albumin and high cholesterol and triglyceride. These abnormalities persisted for the rest of the experiment.

Figure 3

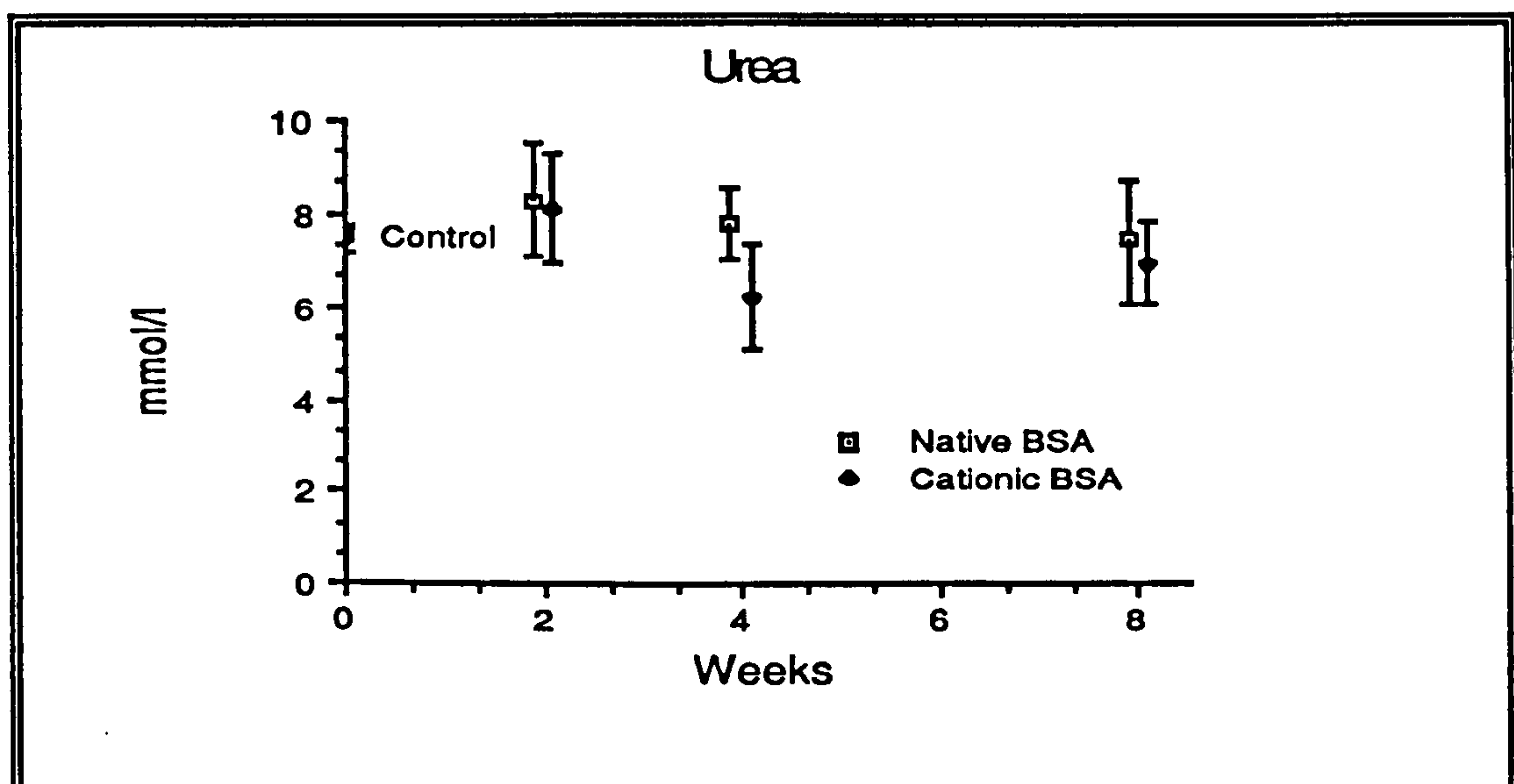


Figure 4

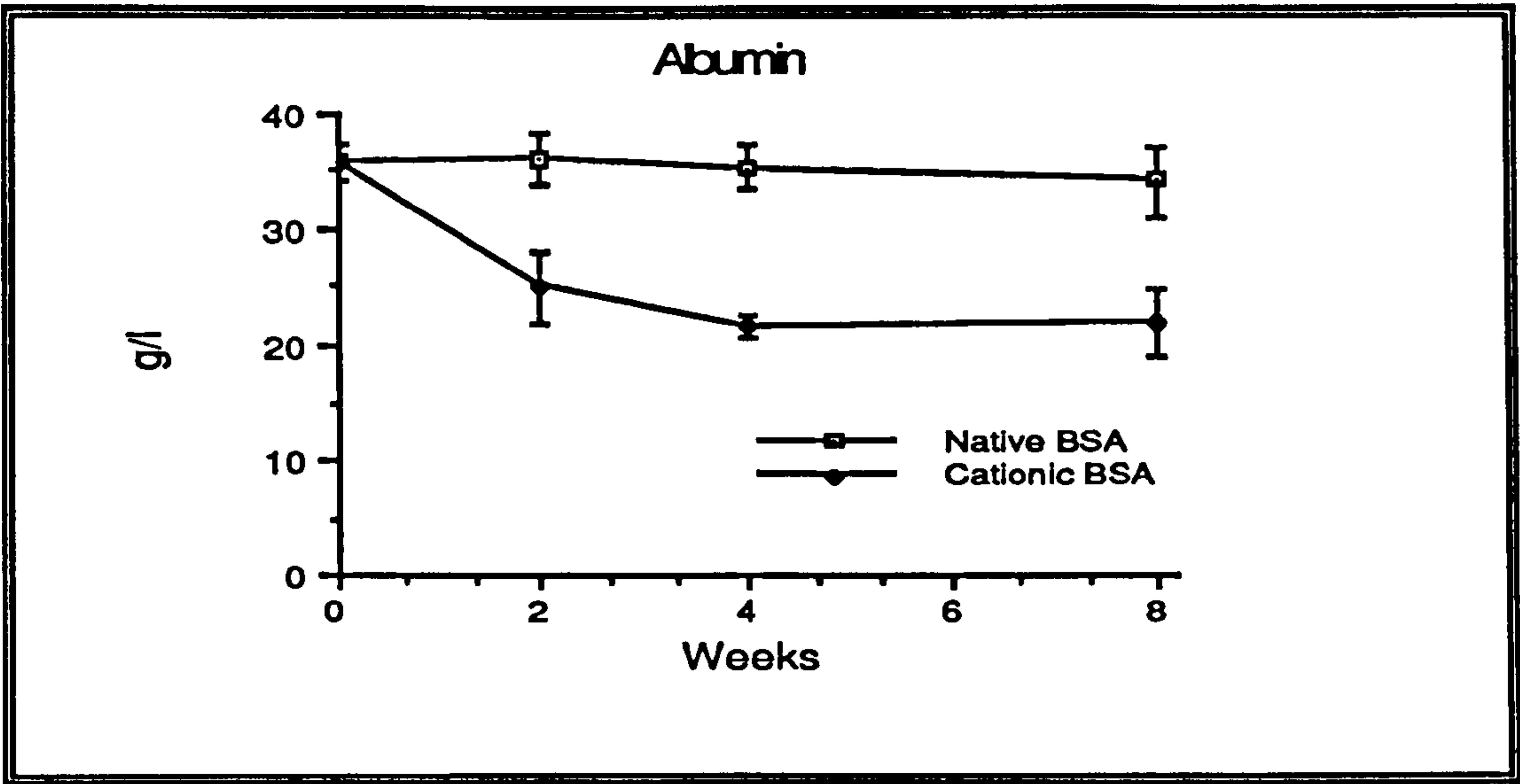


Figure 5

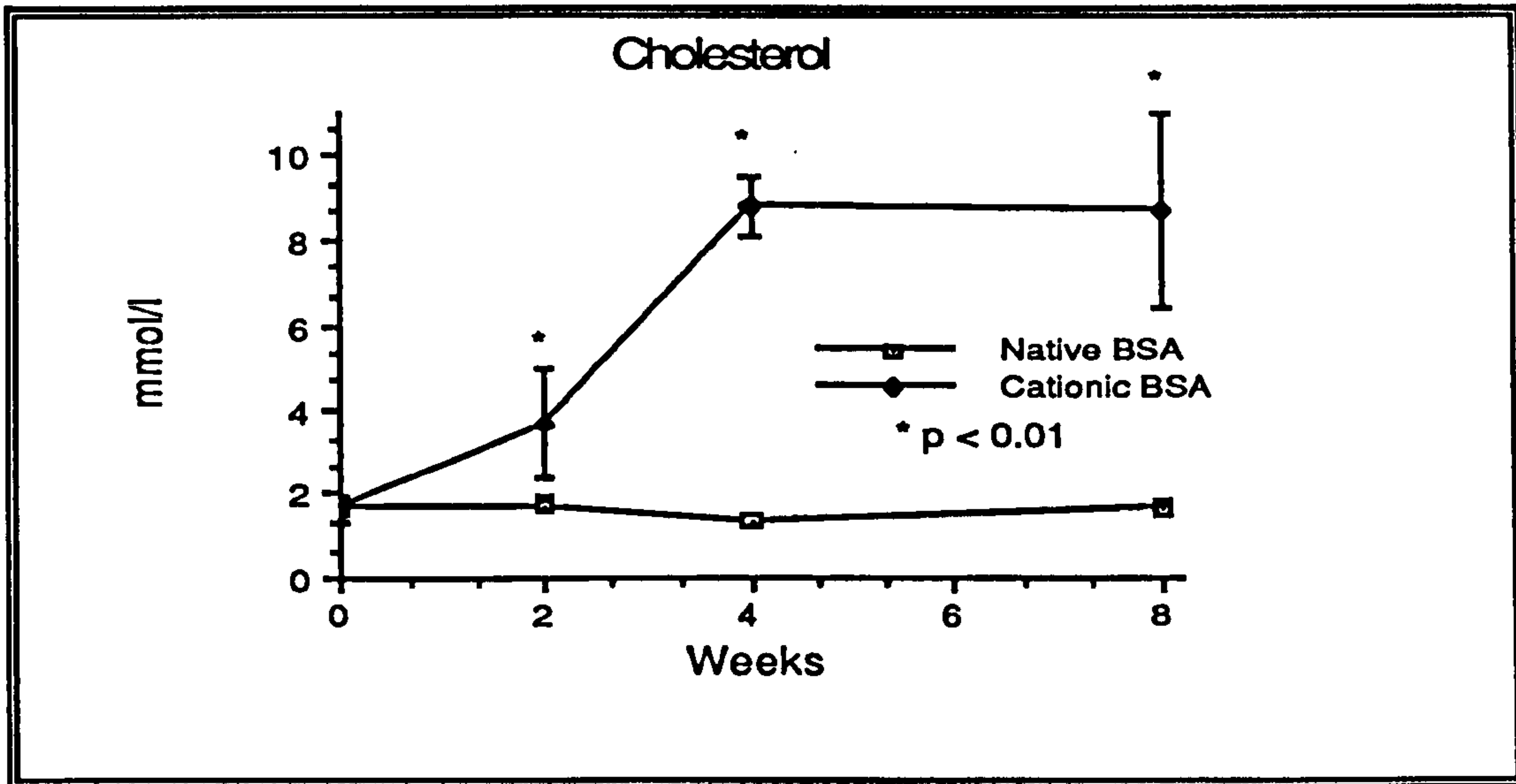
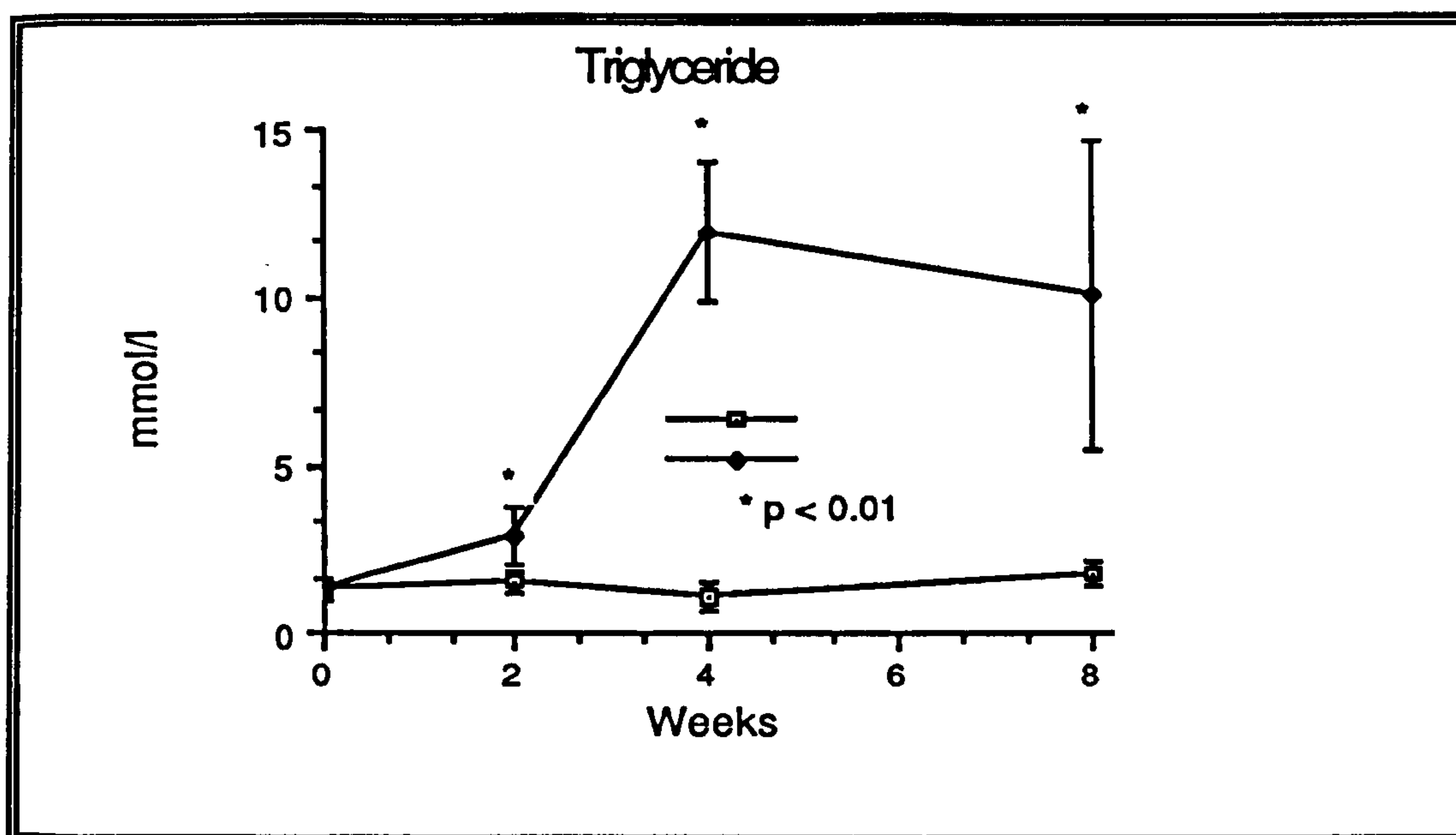
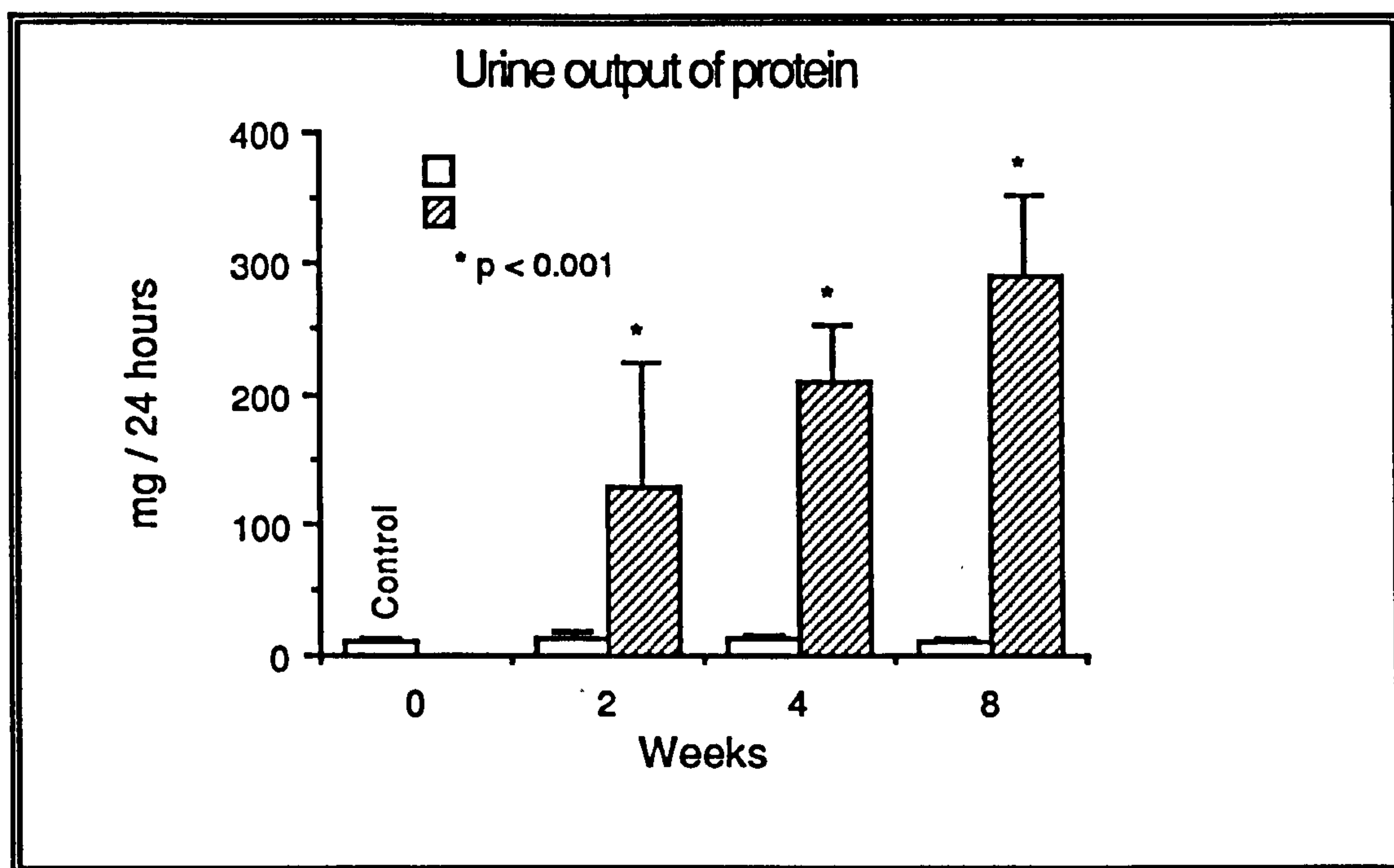


Figure 6



Proteinuria. The protein output of the groups of rats is shown in Fig. 7. By two weeks, cationic BSA serum sickness had caused proteinuria in all the rats examined. This persisted and became heavier throughout the experiment. None of the rats receiving native BSA developed proteinuria.

Figure 7



Weight. The rats used in this study had not attained their final adult weight, but rats with cationic BSA serum sickness virtually stopped gaining weight, despite having ascites (Table 4). Native BSA serum sickness also inhibited normal weight gain, despite the absence of any detectable renal functional impairment.

Table 4. Weight gain of rats per week

	Control	Native BSA	Cationic BSA
Average weight gain per week ($\pm 95\%$ limits)	24g ($\pm 3.2g$)	11g ($\pm 2.0g$)	1g ($\pm 3.1g$)

Intramuscular administration. The rats which had received cationic BSA by intramuscular injection for

four weeks had glomeruli which showed abnormalities at electron microscopy which were indistinguishable from those seen after intravenous injection. The rats given native BSA by intramuscular injection had no detectable deposits, and only one of the three rats given native BSA by intravenous injection had mesangial deposits.

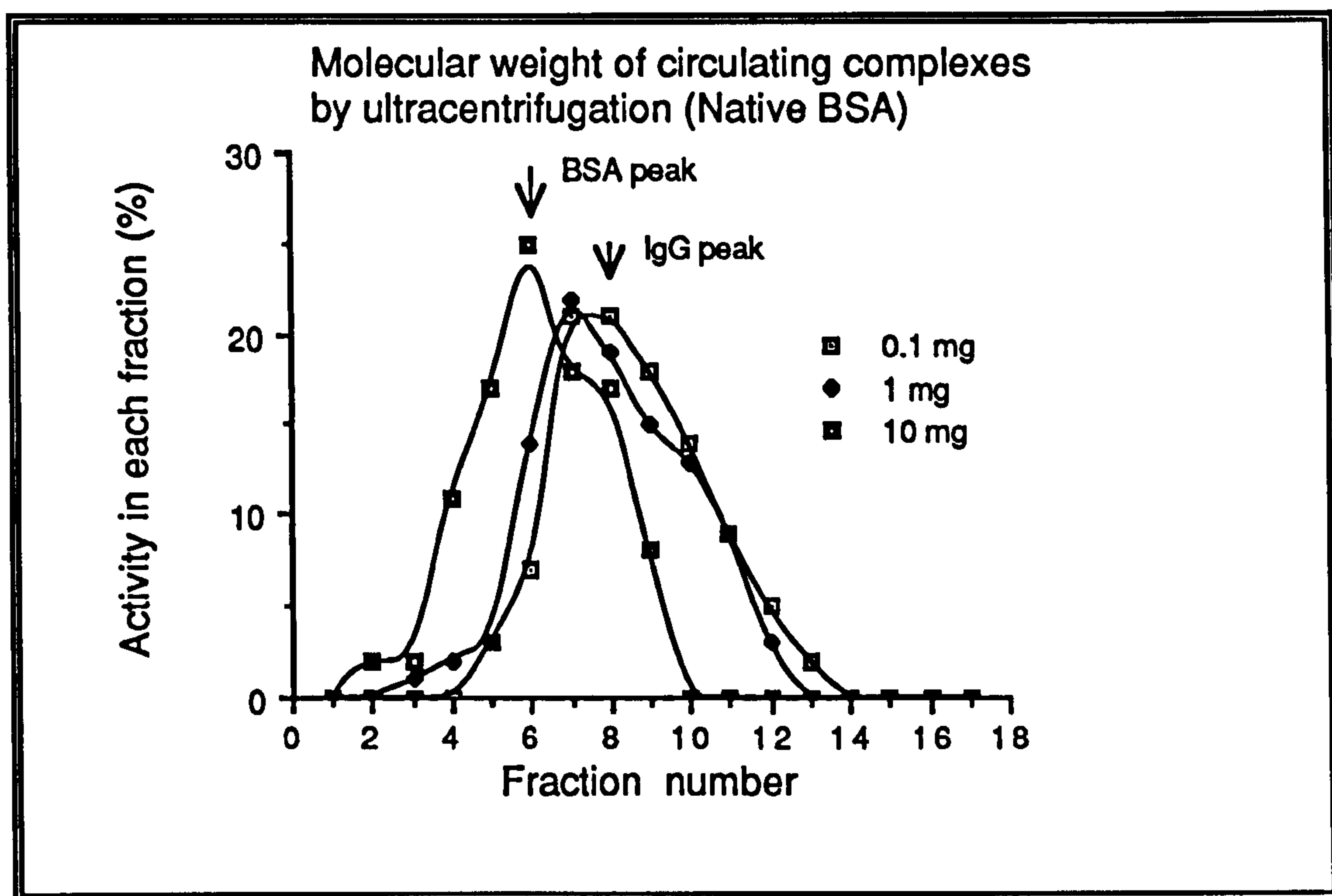
Tissue damage at the injection sites in the legs was not detected on macroscopic examination.

Effect of initial immunization. The rats which were initially immunized with one antigen then given regular doses of the other developed glomerular lesions consistent with the antigen given by regular injection, not the antigen given initially in FIA.

Effect of dose on circulating immune complexes

The results of the ultracentrifugation study for animals receiving native BSA are shown in Fig. 8. They indicate that three hours after the optimum dose of 1mg, most of the isotope is present in the serum in a form at or above the weight of one molecule of IgG: that is, as small immune complexes. A dose of 10mg produced mainly free BSA at this point.

Figure 8



An attempt to perform a similar analysis using cationic BSA failed; contrary to previous reports (Border et al. 1982) only a trace amount of isotope was present in the serum and this had the mobility of free iodine. The finding of an autoimmune hæmolytic anaemia described above suggested that at least some of the cationic BSA might be binding to erythrocytes. To test this, about 1 μ g of radio-labeled cationic or native BSA was injected into two non-immune rats. After 30 minutes blood samples were taken and allowed to clot. Serum and clot were separated and counted. The results (Table 5) indicate that cationic BSA does tend to remain in the clot, presumably due to charge interactions with the erythrocytes.

Table 5. Separation of native and cationic BSA with serum or blood clot.

	Native BSA	Cationic BSA
Percentage of counts in blood clot	13	92
Percentage of counts in serum	87	8

Conclusions

These results indicated that biochemical and renal abnormalities which are characteristic of nephrotic syndrome can be reliably and relatively rapidly induced by pre-immunization with antigen in FIA followed by seven doses of cationic BSA, spread over two weeks. This result can be achieved by either intravenous or intramuscular administration of antigen. Intramuscular administration reduces the 'bolus' effect described above, and it is more convenient and less distressing to the animals. This mode of induction of glomerulonephritis was therefore adopted for all the subsequent experiments. The more classical native BSA model was studied in parallel but this antigen was much less reliable in inducing deposits, and did not induce any detectable abnormality in renal function. Its use was therefore dropped.

The studies of antigen location indicated that although the kidney was a preferred site of antigen accumulation, only a tiny proportion of the injected dose was deposited there; and although most of this was deposited in glomeruli, autoradiography was not a suitable tool to assess any alterations in glomerulus antigen load and other methods would be required.

The morphology of the development and recovery of
chronic serum sickness glomerulonephritis

Introduction

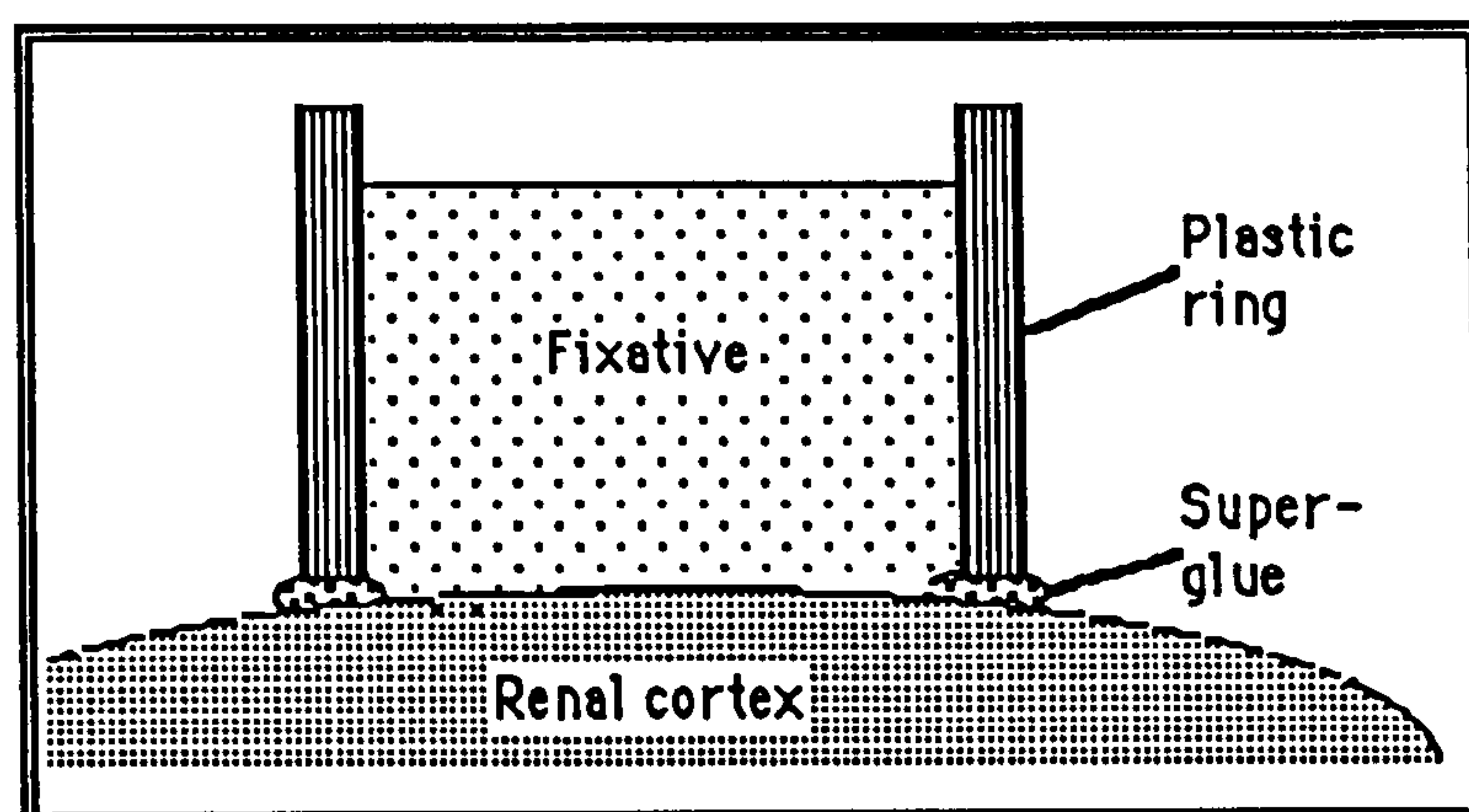
To assess further the development of this form of chronic serum sickness glomerulonephritis, a sequential study was performed to correlate the morphological changes in the glomerulus with the level of proteinuria, during the development of and recovery from glomerulonephritis. To obtain optimal ultrastructural detail, the kidneys were fixed by a modification of the surface fixation technique of Griffith et al. (1967).

Protocol

Chronic serum sickness glomerulonephritis was induced in 24 male Wistar rats using cationized bovine serum albumin and an immunization schedule with Freund's incomplete adjuvant as described in Chapter 4. Intramuscular injections of 10mg of cationized BSA were given thrice weekly for two weeks (seven injections). During the induction of glomerulonephritis and thrice weekly during recovery, one of the rats was placed in a metabolic cage and a 24 hour collection of urine was made, for estimation of proteinuria by the sulphosalicylic acid method (Appendix 9). After that collection the rat was anaesthetized (Hypnorm® and

Diazepam), the abdomen was opened and the left kidney exposed. The renal capsule was peeled back. A 1cm long section of plastic tubing, 1cm in external diameter, was attached to the surface of the kidney using 'Super Glue' (Cyanoacrylate ester; 'Loctite Super-Glue Extra'®). This created a watertight well on the surface of the kidney, which was filled with Karnovsky's fixative (Fig.9).

Figure 9. Method of surface fixation of the kidney in the live, anaesthetized animal.



This modification of previously described surface fixation methods (Griffith et al. 1967) eliminated the exposure of other abdominal organs to the fixative, allowing a fixation period of two hours in the live anaesthetized animal. By this method good fixation of glomeruli was achieved in the Wistar rat, without having to resort to the more superficial glomeruli of the Munich-Wistar strain.

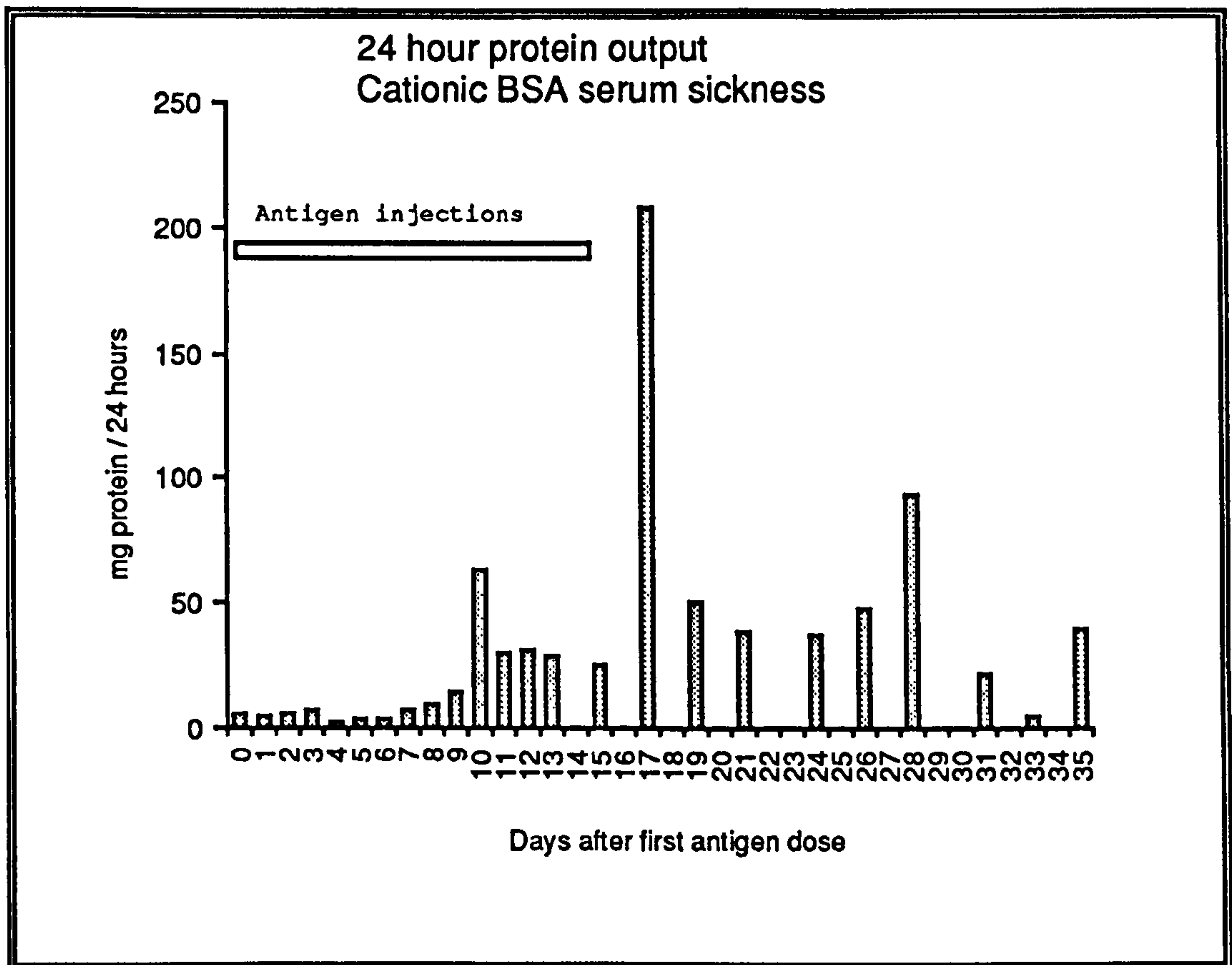
After two hours of surface fixation the kidney was excised and the animal killed by exsanguination. The

surface fixed area of kidney was sliced off and placed in Karnovsky's fixative for a further period of up to 24 hours. Small samples (1mm) were taken and processed routinely into Epon resin for transmission electron microscopy (Appendix 6) and examined in a JEOL TEM 800X at 80KV. A slice approximately 0.5mm thick was then removed by hand from the surface of the rest of the specimen, to expose surface-fixed glomeruli. The tissue was processed for scanning electron microscopy (Appendix 10) and examined in a JEOL JSM 35 at 20KV.

Results

Proteinuria. The normal low level of proteinuria in this species (less than 5mg/24 hours) was not exceeded until the 9th day of induction (5 injections), when a value of 9.3mg/24h was recorded. The subsequent values are shown in Fig. 10. There is great variation, probably because each bar represents a different experimental animal, but after day 10 proteinuria was never found to be below 20mg/24h until the animal killed on day 33, after 18 days of recovery.

Figure 10



Morphology. The earliest abnormal morphological findings were seen in the rat killed on day 3, 1 hour after the second antigen injection. Small electron dense deposits were occasionally seen in subepithelial and mesangial locations (Figure 11), but foot process effacement was not evident on scanning or transmission electron microscopy. Deposits were not seen in the rat killed on day 4, but were present in all subsequent animals except on day 33. Scanning electron microscopy revealed short microvillous processes on the external surfaces of the epithelial cells on day 3; these became more numerous as the disease progressed and never

completely resolved in the course of the experiment (Fig. 12). Foot process effacement was seen from day 7 onwards, two days before the onset of proteinuria (Fig. 13). The development of proteinuria correlated best with the appearance of large swellings or 'balloons' beneath the epithelium (Fig. 14). This phenomenon could only be seen in surface fixed glomeruli; in deeper glomeruli the 'balloons' had presumably collapsed. Balloons have been described before in Puromycin nephrosis in the rat (Messina *et al.* 1987), where they also correlate well with the onset of proteinuria. Near-normal foot processes could be seen in the base of many of the balloons. In contrast to the features described in Puromycin nephrosis, areas of basement membrane without an epithelial covering were not detected.

Figure 11. Day 3. Small subepithelial deposits are present. Transmission electron micrograph, x 25,000.

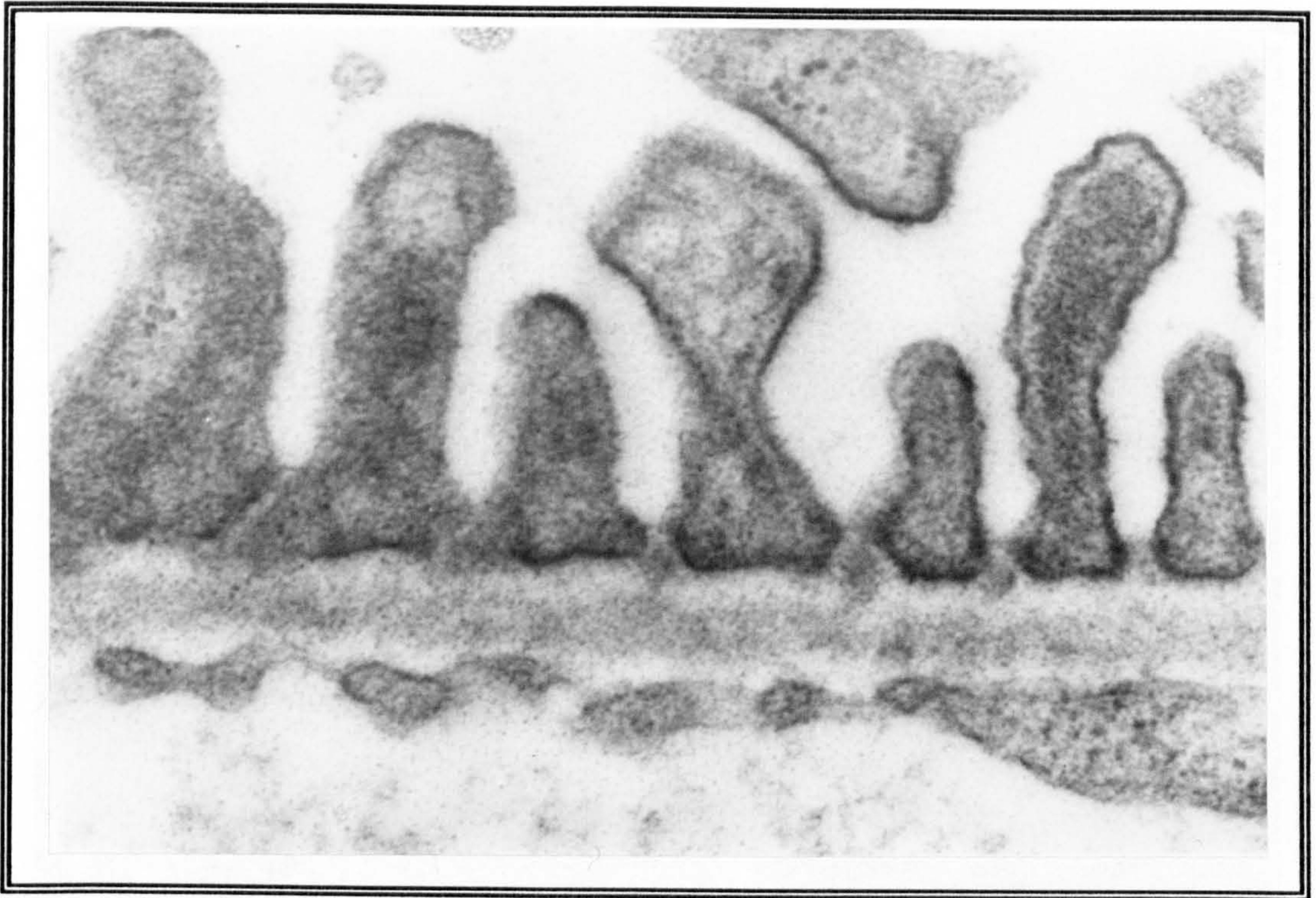


Figure 12. Microvillous processes on the external surface of the epithelial cells. Scanning electron micrograph, x 4,300. (Foot process effacement is advanced in this rat).

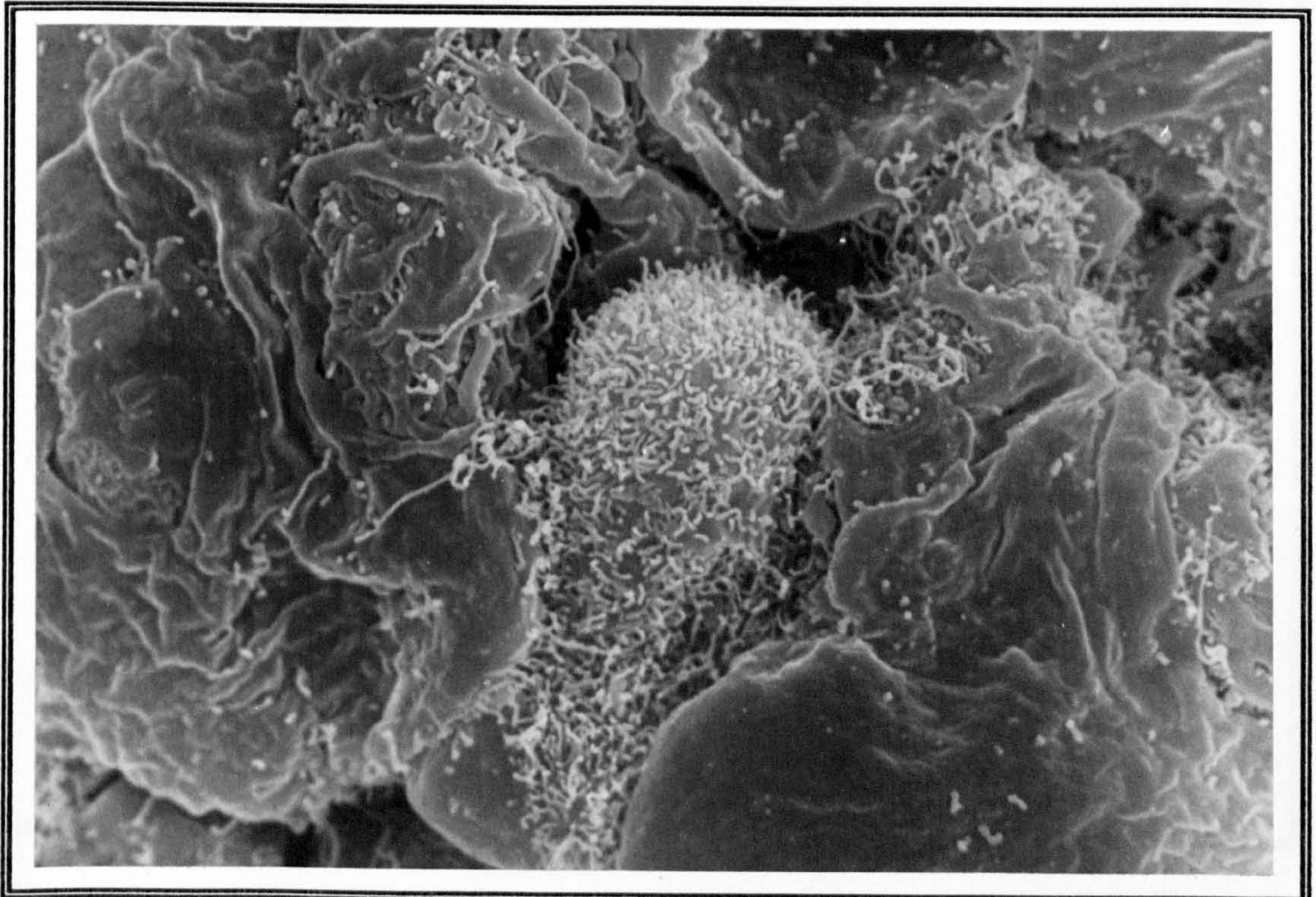


Figure 13. Day 7. The first evidence of foot process effacement. Scanning electron micrograph, x 4,600.

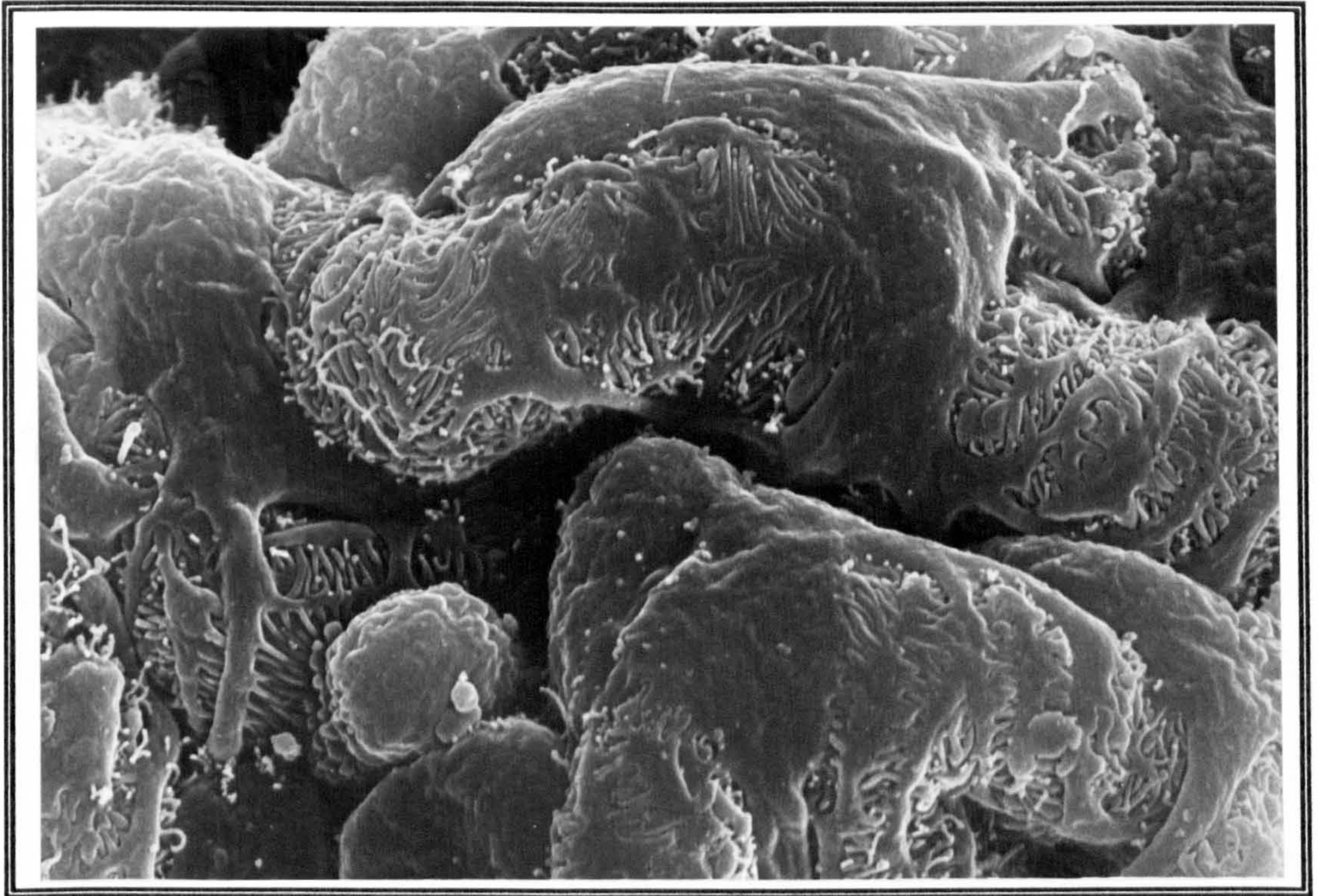
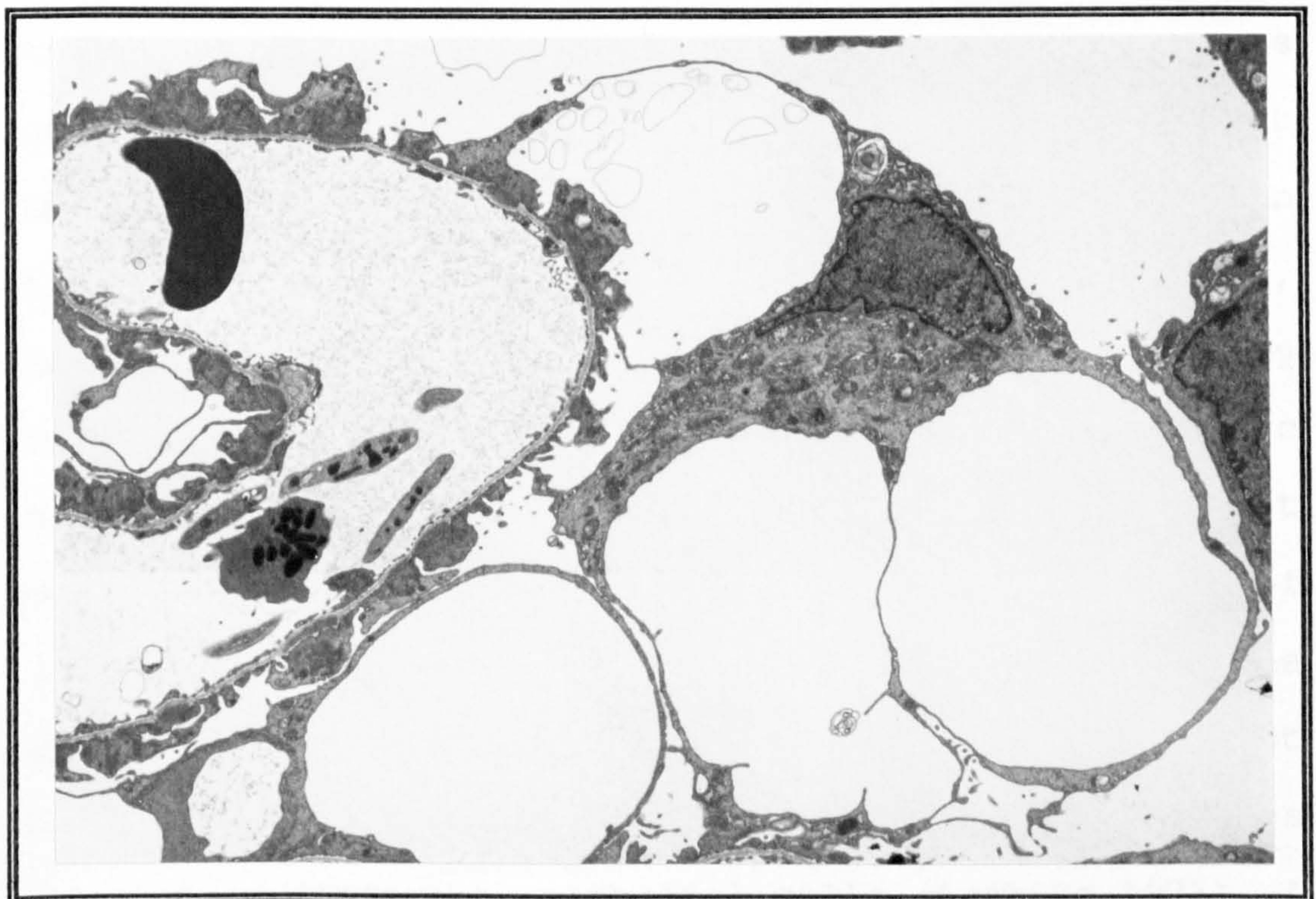


Figure 14. Epithelial 'balloon'. Transmission electron micrograph, x 8,000.



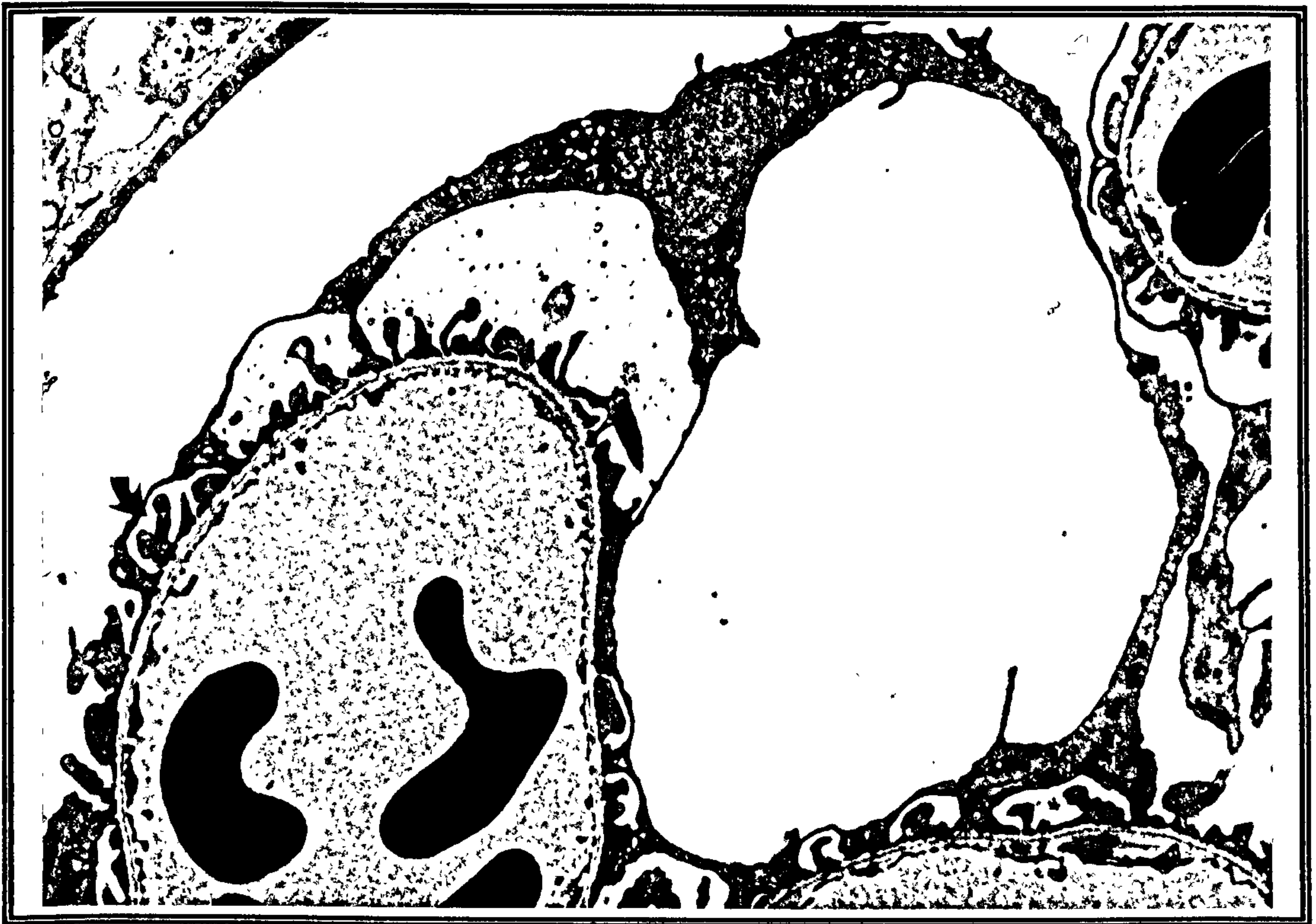
Conclusions

These results indicated that there is a rather poor temporal correlation between the development of electron dense deposits and the onset of proteinuria. The immunization regimen used produced deposits after only two injections. Proteinuria was not seen for another six days, but continued to get worse after the injections ceased. This finding, and the considerable variation of proteinuria between animals, suggested that urinary protein levels would not provide a useful index of glomerular antigen deposition in the work to follow.

A mechanism has been proposed to explain the formation of the balloons in Puromycin nephrosis. Messina et al. (1987) suggested that foot process effacement first leads to the formation of a continuous sheet of epithelial cell cytoplasm over the basement membrane. A focal detachment of this sheet permits an increased flux of fluid across the basement membrane, and a blow-out of the epithelial cytoplasm results. My results do not support a similar mechanism in chronic serum sickness, because: i) foci of bare basement membrane could not be found, and ii) the presence of interdigitating foot processes at the base of the balloons means that a single cytoplasmic sheet could not have been present, because alternate foot processes arise from different epithelial cells (Arakawa 1971). A study of balloons of different sizes suggests that in

chronic serum sickness glomerulonephritis the balloons arise as a result of containment of ultrafiltrate between the epithelial cells (Fig. 15 and legend).

Figure 15. Epithelial balloons of various sizes. The suggestion is made that if the derangement of podocyte structure which is associated with proteinuria causes obstruction of the escape of filtrate from the space marked with an arrow, then a progressive dilatation of the space will result, possibly producing the balloons seen at the top of the picture. Transmission electron micrograph, x 7.500.



Methods for the quantitative study of
antigen location

Introduction

The results of the experiments described above indicated that an immune complex glomerulonephritis and nephrotic syndrome could reliably be induced in immune rats after only seven intramuscular injections of cationic BSA. A basic protocol was therefore devised for the induction of glomerulonephritis and the assessment of the load of antigen and deposits in glomeruli. The following procedures were followed in all subsequent investigations unless varied as stated in the text.

Induction of glomerulonephritis

Male Wistar rats, initial weight 100-125g, were used throughout.

Initial immunization was achieved by subcutaneous injection of an emulsion of 0.25ml of Freund's incomplete adjuvant and 0.25ml of saline containing 1mg of cationic BSA, 22 days and again 10 days before starting regular injections. A batch of cationic BSA was labeled with Iodine-125 as described above (Appendix 4), divided into seven aliquots at a concentration of 100mg/ml, and stored at -20°C. Aliquots were thawed as required and administered to the rats as 0.1ml injections to the musculature of the hind leg on

alternate sides. Each group of rats received seven such injections over a two-week period.

Procedure at sacrifice

After the induction of glomerulonephritis and a period of recovery appropriate to the experiment being performed, the rats were anesthetized with Hypnorm® (0.2ml i.m.) and diazepam (0.2ml i.p.); the abdomen was opened and a small piece of renal cortex was taken, cut into 1mm cubes and fixed in 2% glutaraldehyde for processing for transmission electron microscopy. If necessary, a blood sample was taken from the inferior vena cava. The aorta was clamped just below the diaphragm and cannulated just above its bifurcation. The inferior vena cava was opened and the abdominal organs were flushed with a minimum of 20ml of saline via the aortic cannula. The kidneys were rapidly removed, and samples (50 - 100mg) of renal cortex, liver spleen and lung were taken, weighed and fixed in individual tubes of 10% formalin. These samples were subsequently submitted to the gamma counter for assessment of isotope content, then the tissues were recovered and processed to paraffin wax for sectioning. If required, samples of tissue were frozen in freezing isopentane and stored at -70°C. The rest of the kidneys was placed in saline at 4°C for subsequent isolation of glomeruli.

Isolation and counting of glomeruli

A technique was developed by modification of the method described by Raij et al. (1979). The renal cortex was roughly cut from the medulla, and the latter discarded. The cortex was disrupted by pushing through a coarse nylon mesh, with copious phosphate buffered saline, pH7.4. Sieves had previously been prepared by sawing off the ends of 60ml plastic syringes and covering the opening with stainless steel wire mesh (Greenings Ltd), 100 or 200 mesh, attached by a resin adhesive. The fragmented cortical tissue was poured into a 100 mesh sieve, which permits glomeruli to pass through, and was washed through with saline and gentle action of the plunger. The suspension which penetrated this sieve was poured into a 200 mesh sieve (which traps glomeruli) and washed several times with saline. Glomeruli were freed from the mesh by sucking saline into the syringe, removing the plunger and pouring the contents into a conical test-tube. The glomeruli settled to the bottom and the overlying saline was discarded to a volume of approximately 0.7ml. This suspension was then agitated and a 10 μ l sample was placed on a glass slide with a cover-slip, for counting of numbers of glomeruli by conventional transmitted light microscopy, unstained. 500 μ l was then rapidly pipetted to a tube for measurement of isotope content in a gamma counter.

Initial evaluation of this technique by performing multiple replicate counts of glomeruli indicated an unacceptably high variation. This appeared to be due to adherence of glomeruli to glassware and pipette tips. Several substances were added in an attempt to reduce adherence. The addition of a few drops of a strong solution of (native) BSA before pipetting was found to be effective, and variation was thus reduced to less than 5%. The method typically produced a yield of 5,000 to 10,000 glomeruli; the preparations were invariably in excess of 90% glomeruli, and usually in excess of 95%. The remainder appeared to be mainly small blood vessels.

Morphometric assessment of deposit volume fraction

Samples of renal cortex fixed in glutaraldehyde were processed for transmission electron microscopy by routine methods (Appendix 6). Each rat was identified by a code number in the electron microscopy unit to eliminate subsequent observer bias. Photographs were taken at a magnification of x5,000, locating the aperture over a glomerulus at the intersection of the grid bars to provide a random selection of areas. No glomerulus was photographed more than once.

Electron dense deposits were outlined by pen on the resultant photographs, making a distinction between subepithelial and mesangial deposits. A transparent grid with points 0.2cm apart was laid over each photograph,

and points overlying the deposits were counted. This process was continued until the relative standard error of the measurement was 5% or less (Aherne & Dunnill 1982); this required between 4 and 8 photographs from each rat. Volume fractions of mesangial and subepithelial deposits were calculated. In the experiments described after Chapter 8 a SeeScan image analyser became available so the method was modified to take advantage of this. Clear acetate sheets were laid over the photographs and the outlines of the deposits were drawn on to the sheets, using a separate sheet for mesangial and subepithelial deposits. The image analyser was calibrated using an acetate sheet with a black square of known dimensions, then the acetate sheets were submitted and volume fractions calculated. All the measurements for one experiment were made in one session; reproducibility within one session was greater than 98%, but variations in illumination made comparison of measurements between sessions much less precise.

To allow for any variation in glomerular size which might develop between experimental groups, paraffin sections stained with Hæmatoxylin and Eosin were examined with a light microscope using an eyepiece graticule, and the diameter of ten glomeruli was measured for every rat. If a significant difference was detected the fractional alteration in glomerular volume was calculated and the volume fraction results for the

relevant experimental group were corrected to the volume of its control group. This manoeuvre only proved to be necessary after the administration of polyvinyl alcohol (Chapter 12).

Calculations

Calculations were performed using an Amstrad PCW8256 microcomputer, using programs written for the purpose in *BASIC* or using the *AMSTAT* statistics package. The differences between means were assessed using Student's 't' test where the data was suitable, and Mann Whitney's 'U' test where it was non-parametric.

Error bars on the histograms indicate 95% confidence limits.

The potential problem of antigen relocation:Renal transplantation

The intention of these experiments was to attempt to modify the rate at which antigen and deposits are removed from the glomerulus. However, if some form of intervention is applied immediately after the last antigen dose, its effects could be attributed just as readily to an influence on the deposition of antigen in the glomerulus as to an influence on its removal.

Renal transplantation has been mentioned as a potential way of avoiding this problem. If an antigen-laden kidney is transplanted into a syngeneic antigen-free host, then the potential for late deposition of antigen is eliminated. To assess the feasibility of this approach, I learned the technique of orthotopic renal transplantation in the rat, with the assistance of members of the Nuffield Department of Surgery in Oxford. Preliminary experiments indicated that the inbred rat strain F344 is susceptible to the cationic BSA form of chronic serum sickness glomerulonephritis. Unfortunately, although transplantation of the normal kidney was achieved, re-perfusion of nephrotic kidneys was never established, despite intact anastomoses and attempts to supplement the standard method of anticoagulation by systemic heparin by perfusion of the

kidney with ice-cold heparinized 'kidney perfusion fluid' (formulated for human renal transplantation). As a result, and bearing in mind the other disadvantages of transplantation (ischaemia and the limited number of animals which could be studied), this technique was abandoned and experiments were carried out to assess the kinetics of antigen levels in the glomerulus after the last antigen dose, and to estimate how long after the last antigen dose relocation of antigen might still be occurring.

The rate of removal of antigen from the glomerulus

Introduction

An experiment was performed to evaluate the rate of removal of antigen from the glomeruli after the cessation of injections, in the absence of any form of intervention.

Protocol

Chronic serum sickness glomerulonephritis was induced in 18 rats, using cationic BSA over 2 weeks as described above. The last injection only was radio-labeled. Groups of 3 rats were killed 3 hours, 24 hours, 2 days, 4 days, 7 days and 14 days after the last antigen injection. The three rats killed after 14 days were housed in metabolic cages throughout, to permit daily 24 hour urine collections for assessment of proteinuria. To determine the proportion of the isotope in the urine which was free iodine-125 (presumably released after degradation of antigen), 500 μ l of each sample was mixed with an equal volume of 10% trichloroacetic acid, to precipitate protein. The supernatant was removed, the precipitate was washed, and supernatant and precipitate were submitted to the gamma counter. The volume fraction of the deposits and the amount of isotope in isolated glomeruli, renal cortex,

liver, spleen and lung were measured by the methods described in Chapter 7. In addition, whole blood samples were taken for assessment of isotope content.

To confirm that the observed changes were indeed due to interactions between the antigen and the immune system, and were not due to the properties of the cationic BSA itself, a further 18 rats were given six injections of 10mg of cationic BSA followed by one of radiolabelled BSA, over two weeks, but without initial immunization. Samples of tissue were taken at intervals as in the above group, although urine was not collected and blood samples were used to confirm (by Ouchterlony immunodiffusion) that these rats had not made a significant antibody response.

Results

The amount of isotope in isolated glomeruli and whole tissues are shown in Figures 16 and 17. Each point represents the mean of values from the three rats killed at that time.

Figure 16

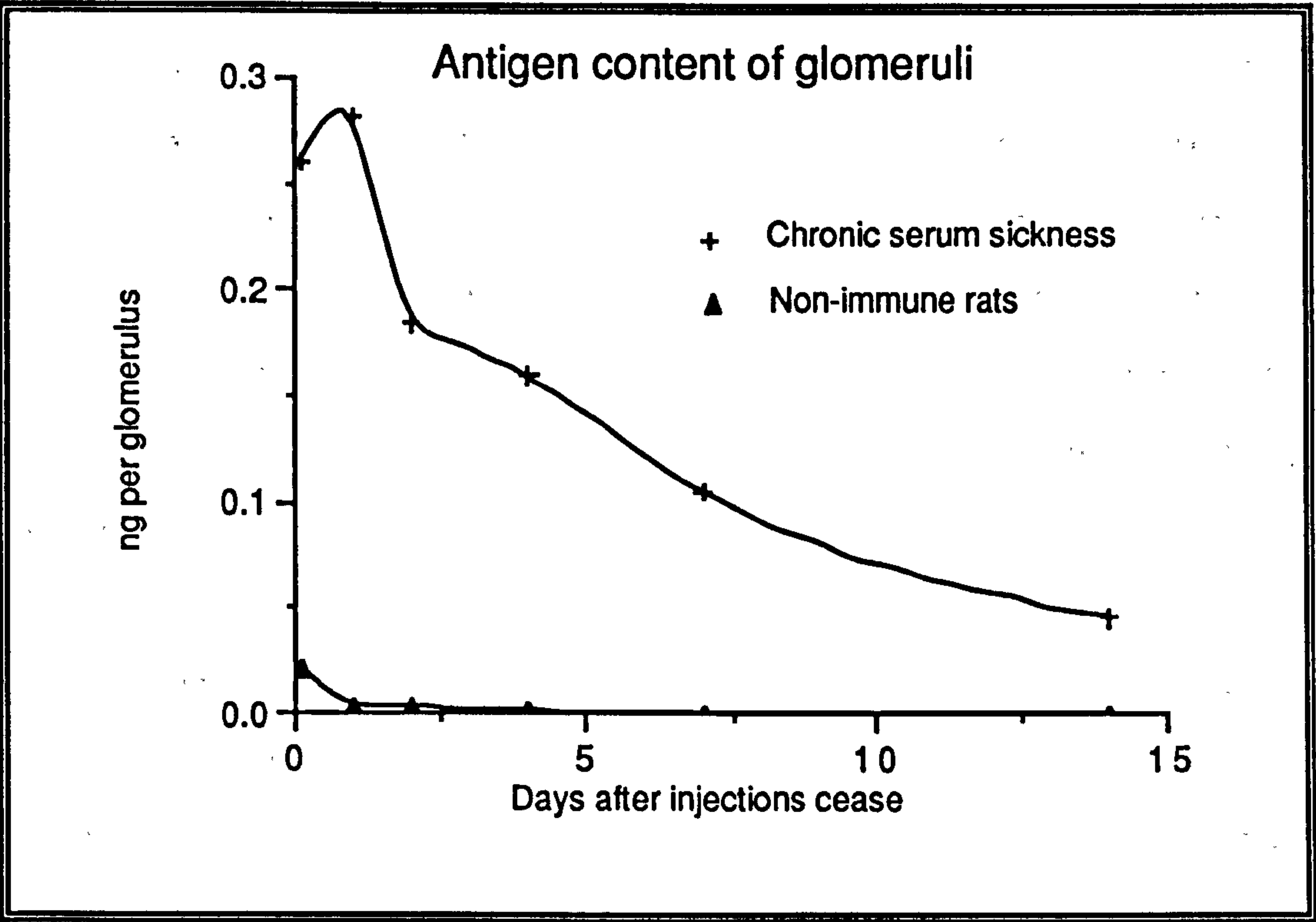
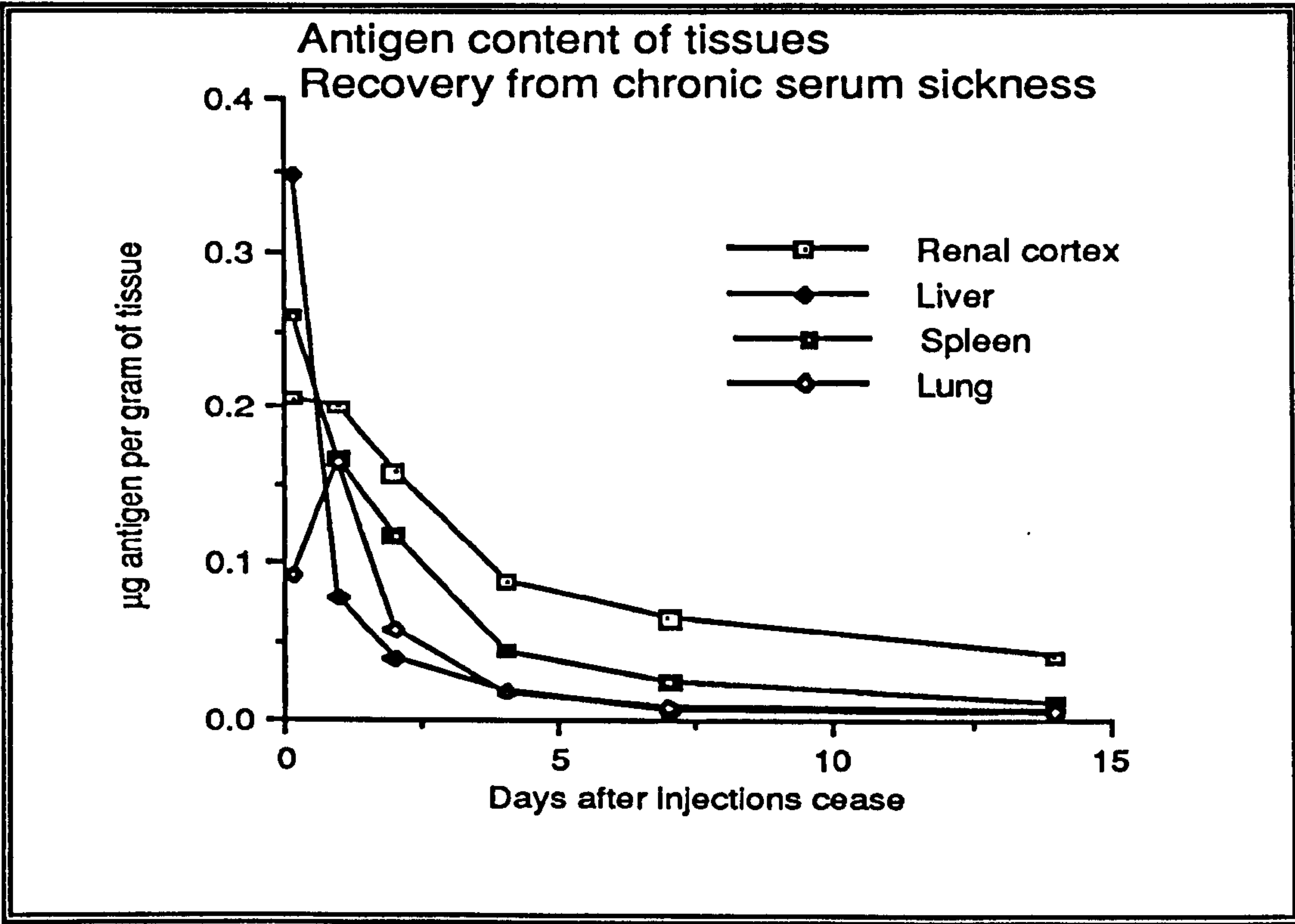
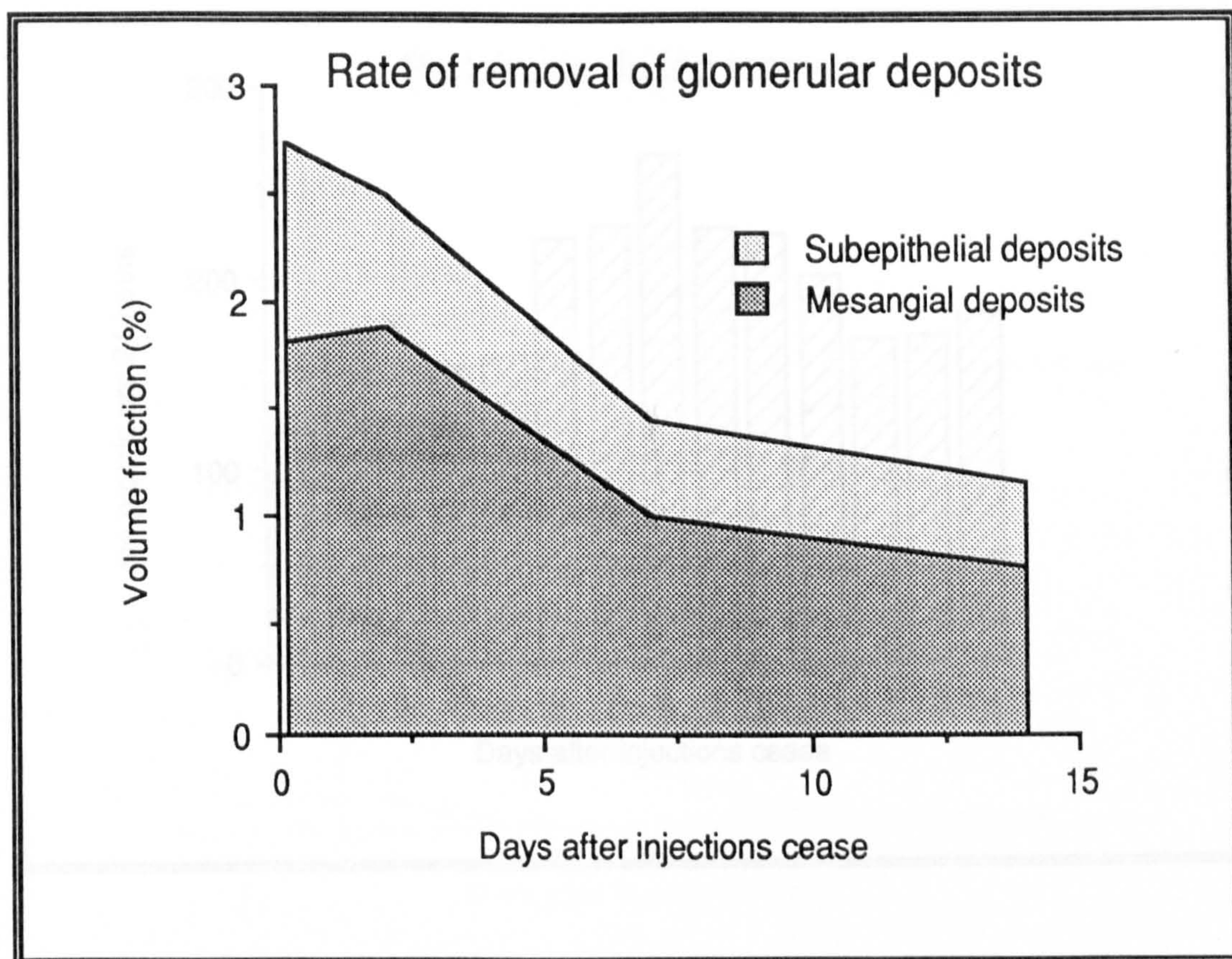


Figure 17



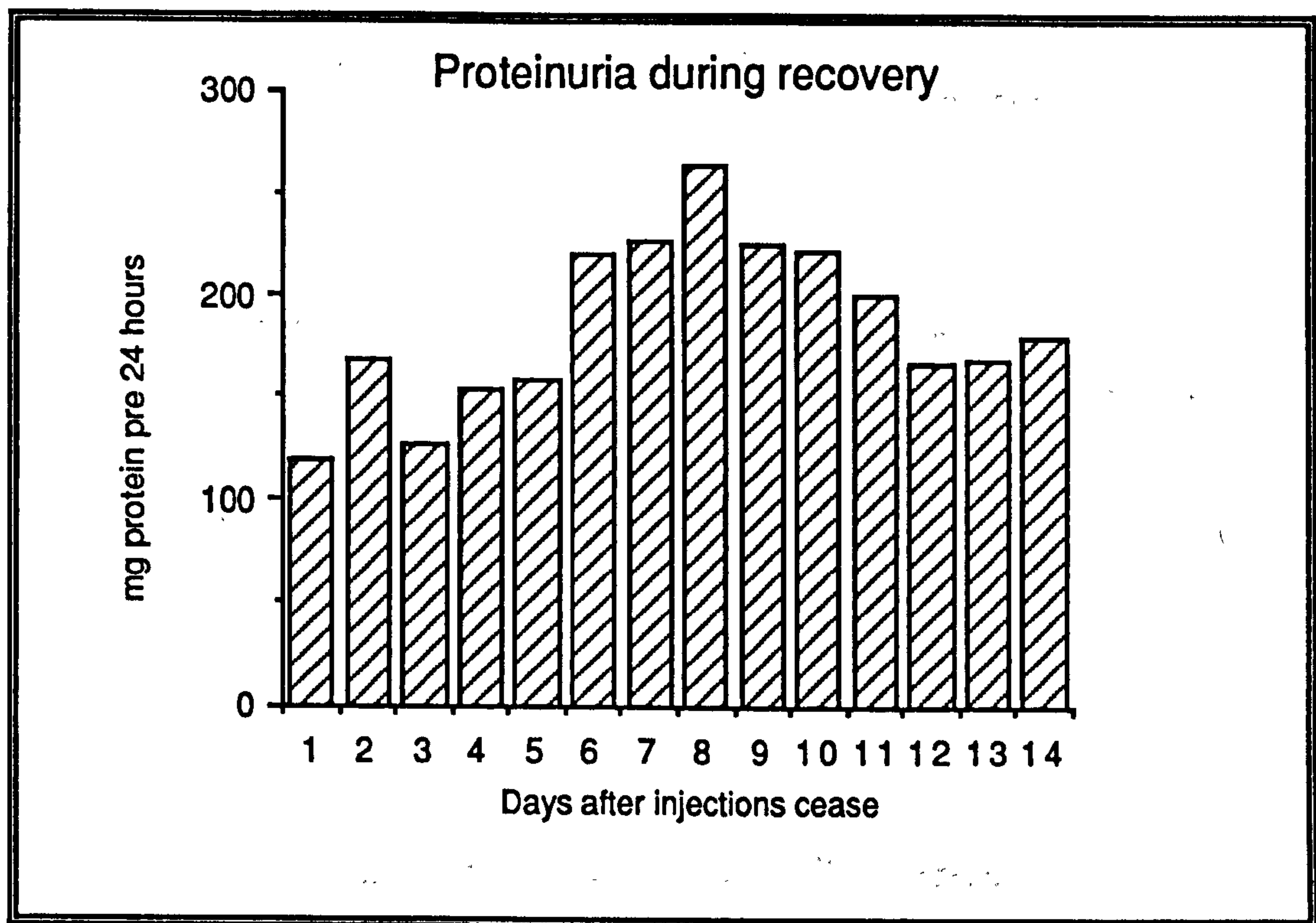
In non-immune animals, the antigen is rapidly eliminated, with little evidence of binding in glomeruli (Fig. 16) or in other tissues. In chronic serum sickness it appears that antigen accumulation in glomeruli and in lung is continuing between 3 and 24 hours after the last antigen dose, but after this time all the tissues examined showed a rapid fall in isotope levels (Fig. 17). This fall was least marked in glomeruli and renal cortex. Morphometric assessment of deposit volume fraction confirmed that subepithelial and mesangial deposits were being removed, though the changes were more rapid in the mesangial deposits (Fig. 18).

Figure 18



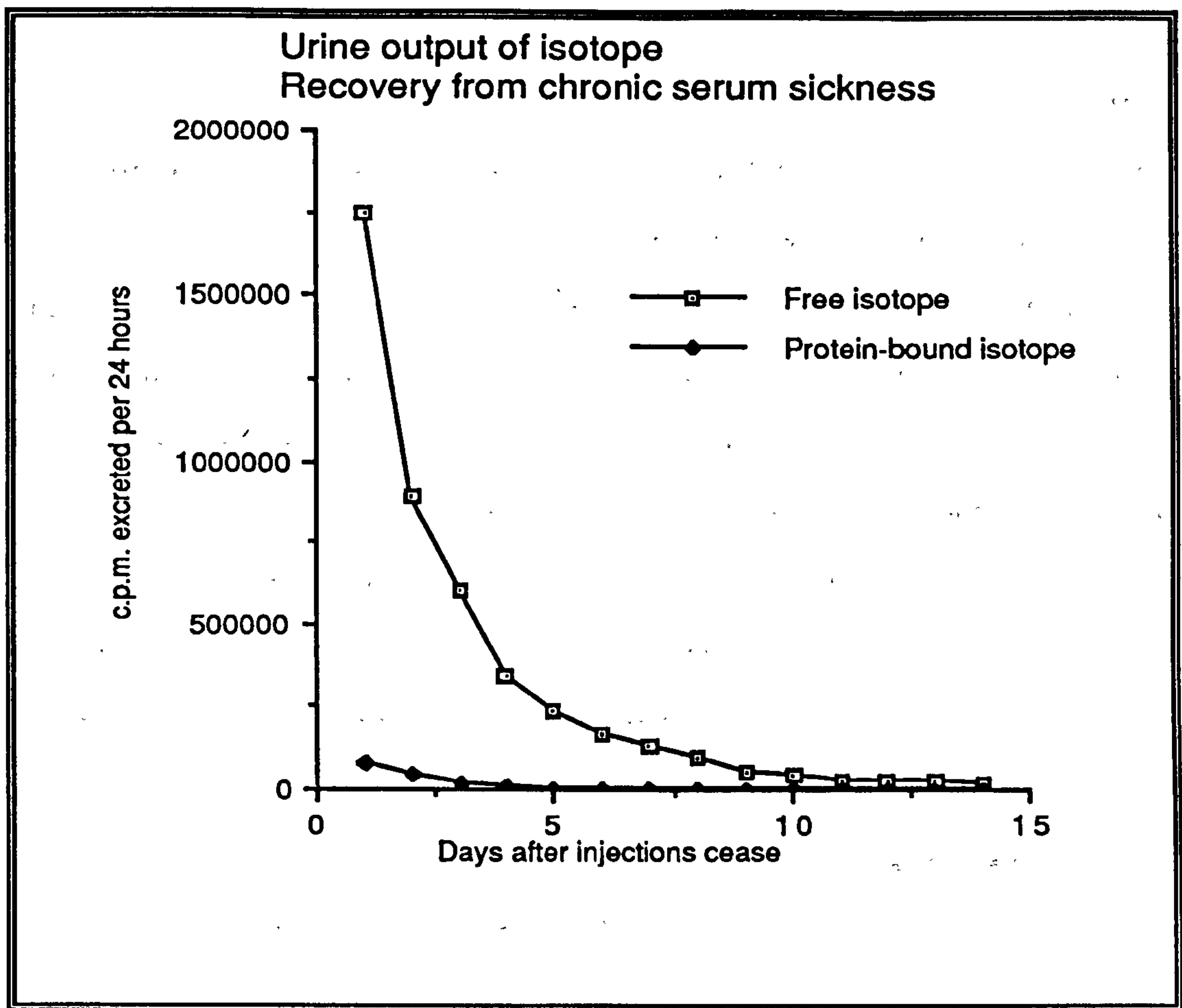
Iodine-125 proved to be almost undetectable in the blood after two days. The level of proteinuria was found to continue to increase after the antigen injection ceased (Fig. 19), confirming the impression given by the results of Chapter 6.

Figure 19



The rate and pattern of isotope excretion was of particular interest (Fig. 20). Most of the isotope was excreted very rapidly, and most was excreted as free isotope. Protein-bound isotope was undetectable in the urine after 4 days of recovery, despite continuing heavy proteinuria.

Figure 20



Conclusions

The finding that isotope levels in immune rats decreased in all tissues examined after 24 hours does not of itself prove that deposition of antigen has ceased by that time, merely that the rate of removal is exceeding the rate of deposition. The urine analysis provided more insight into the speed at which the antigen is disposed of by the whole body. The vast excess of free over protein bound isotope indicated that

most of the antigen is rapidly degraded.¹ The absence of detectable protein-bound iodine-125 in the urine after 4 days indicated the latest point at which there was evidence of free antigen which could still bind in the kidney.^{2,3} Subsequent experiments were designed with this result in mind; it was assumed that any form of intervention applied four or more days after the last antigen dose could not influence the deposition of antigen in the kidney, but could only affect its removal. The very rapid removal of BSA from the glomeruli of the non-immune rats confirmed that the observed pattern of movement of cationic BSA was genuinely due to the immune complex glomerulonephritis and was not due to direct binding of the antigen to the anionic sites of the glomerulus.

This experiment also provided information on the rate of removal of glomerular antigen and deposits which was of value in designing subsequent experiments. If an intervention at four days was to have a detectable

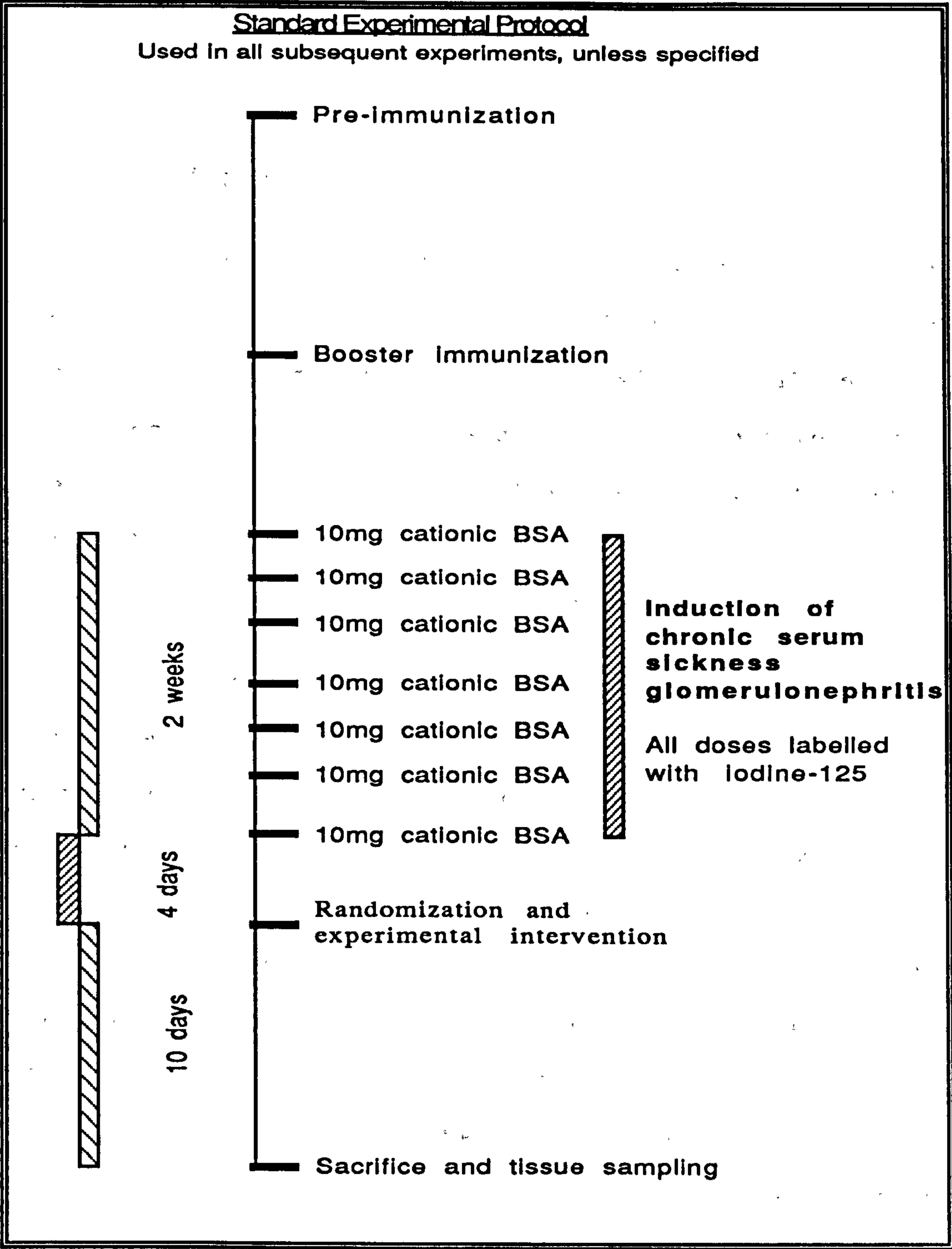
¹The standard diet on which the rats are fed contains ample iodine, so it is reasonable to assume that the rate of excretion of iodine-125 in the urine reflects the rate of its release from the BSA complex. Isotope excretion in the faeces was almost undetectable.

²The greater sensitivity of detection of iodine-125 in the urine rather than the blood is presumably due to concentration of the urine, and the larger volumes available for study.

³Iodine-125 is covalently bound to the protein, so it is reasonable to assume that release of free isotope only occurs when the whole molecule is degraded.

effect, the assessment of isotope and deposits in glomeruli must be delayed until a large proportion of the antigen and deposits would normally have been removed, but not for so long that the amount remaining was too small to detect with any accuracy. Figure 16 indicates that the continuing loss of antigen from glomeruli is quite rapid after 4 days, such that an increase or decrease in the rate should be detectable at two weeks. The deposit volumes show a less rapid fall, and it might be anticipated that even a proportionately large drop in the rate of removal of subepithelial deposits might not be detectable at two weeks. Nevertheless, as the amounts of isotope and deposits in glomeruli after two weeks of recovery were quite small, it was decided to design subsequent experiments with division into experimental groups occurring after 4 days of recovery, and evaluation of the results after 14 days.

Figure 21.



The effect of inhibition or stimulation of the immune
response

Introduction

It has been amply demonstrated that the status of the immune system can have a profound effect on the deposition of immune complex deposits in glomeruli. Having established a model and a protocol to investigate the removal of immune complex deposits, it seemed reasonable to investigate first the effects of crude stimulation or inhibition of the immune system.

The observation that pre-immunization with Freund's complete adjuvant inhibited the development of glomerulonephritis (Chapter 4) raised the possibility that stimulation of the mononuclear phagocytes might enhance the removal of deposits from glomeruli, either by recruitment of activated macrophages to the glomerulus or by activation of the resident population. The contrary effect, immunosuppression, might be achieved by administering large doses of corticosteroids. Finally, depletion of anti-BSA antibody might be achieved by administering native BSA, as large doses of this agent appeared to be non-nephritogenic, and antibodies raised against native or cationic BSA produced a single line of identity on Ouchterlony plates. It was appreciated that such crude forms of

intervention would have complex results; an excess of antigen, for example, has been shown of itself to facilitate deposit removal (Mannik & Striker 1980, Agodoa & Mannik 1987). This first experiment was therefore performed in the hope of identifying effects for further investigation, rather than with the intention of providing specific answers.

Protocol

Chronic serum sickness glomerulonephritis was induced in 40 rats by the methods already described, using seven intramuscular injections of radio-labeled cationic BSA over two weeks. The rats were left for four days after the last antigen injection then randomized to five groups of eight rats each. The groups were treated as follows:

- 1) Control. No treatment.
- 2) A single subcutaneous injection of 0.5 ml of Freund's complete adjuvant and saline.
- 3) A single subcutaneous injection of 0.5 ml of Freund's complete adjuvant with 1 mg of cationic BSA in saline.
- 4) Daily subcutaneous injections of 5mg of prednisolone for ten days.

- 5) Daily intraperitoneal injection of 100mg of native BSA in saline for ten days.

Ten days after the randomization the rats were killed and tissue samples taken and processed as described in Chapter 7 to assess the content of antigen and the volume fraction of deposits. In addition, blood samples were taken for assessment of the level of circulating anti-BSA antibody, using the ELISA technique described in Appendix 11.

Results

The mean amount of antigen in isolated glomeruli for each of the five groups at the end of the experiment is shown in Figure 22. It is reasonable to assume that at the time of randomization the glomeruli of all the groups of rats had the same mean antigen content. The mean antigen content at the end of the experiment therefore reflects the inverse of the average rate of antigen removal over the last ten days. It is evident that the administration of Freund's complete adjuvant, with or without added antigen, produced a significant decrease in the rate at which the antigen was being removed from the glomerulus ($p < 0.01$). Administration of prednisolone had no detectable effect, but the administration of native BSA produced a significant enhancement of the rate of antigen removal ($p < 0.01$). A similar result was seen after the analysis of whole

renal cortex (Fig. 23), although here the differences from the control group were statistically significant only in the groups given Freund's complete adjuvant with antigen or given native BSA.

Figure 22

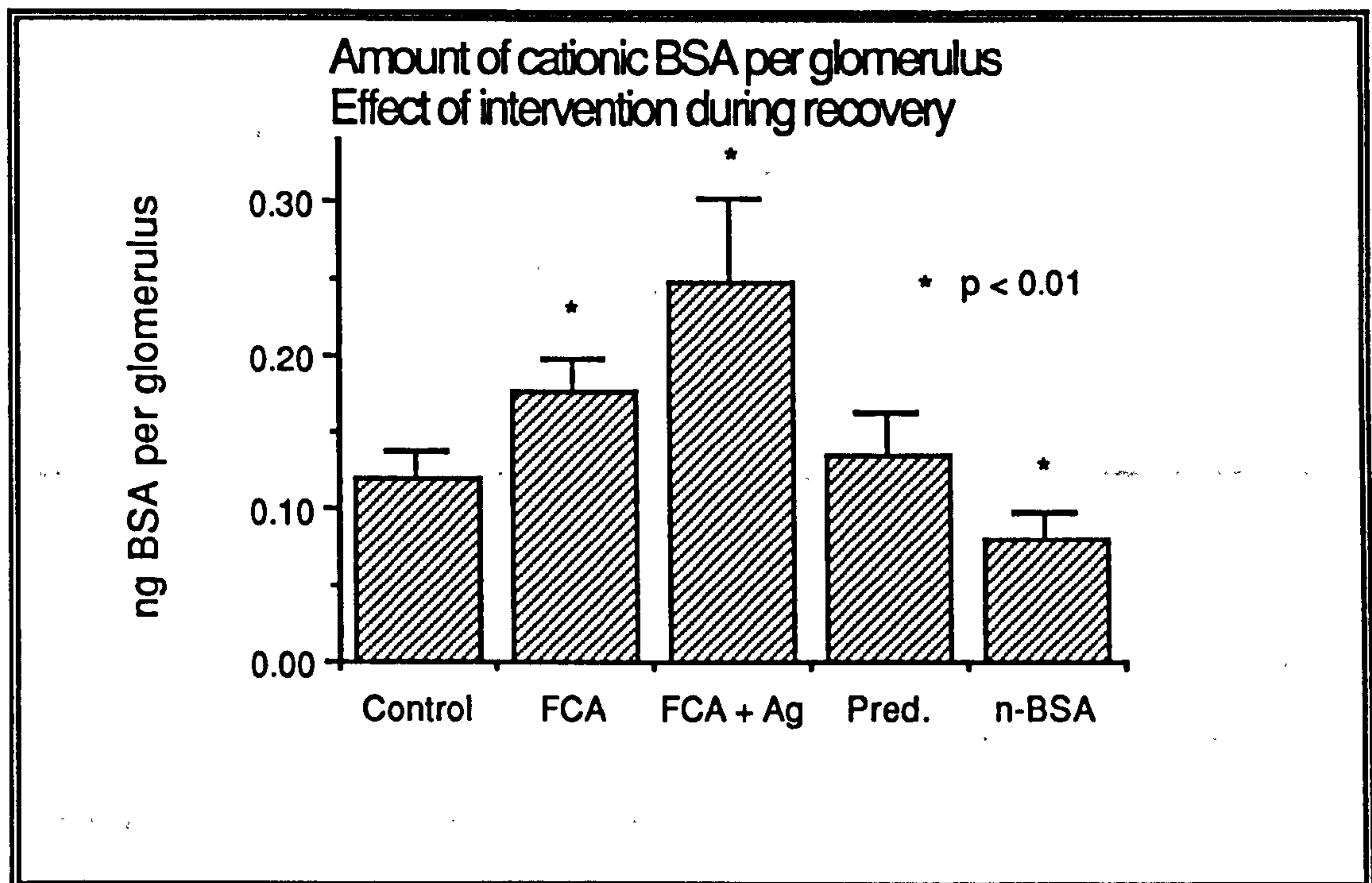
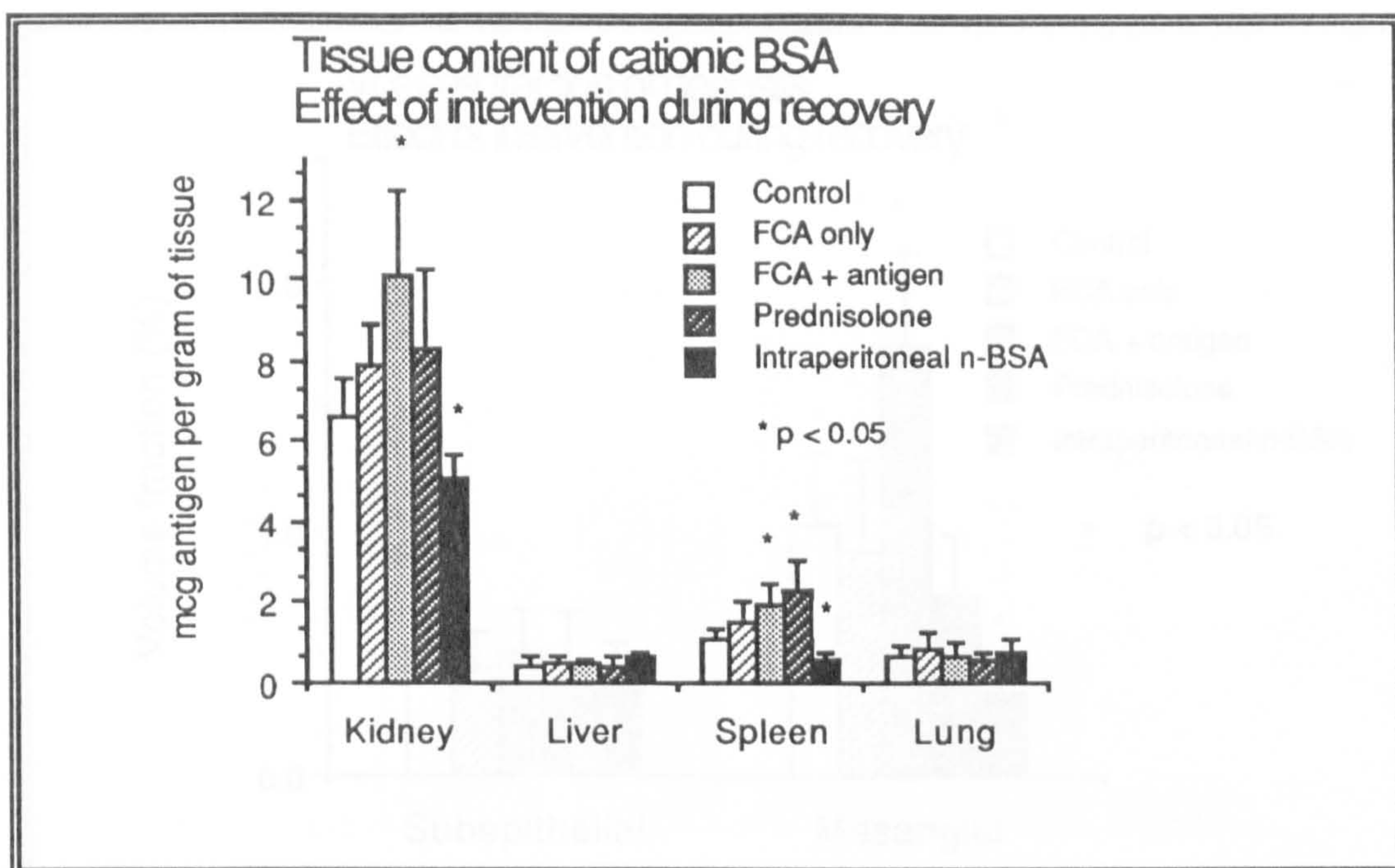


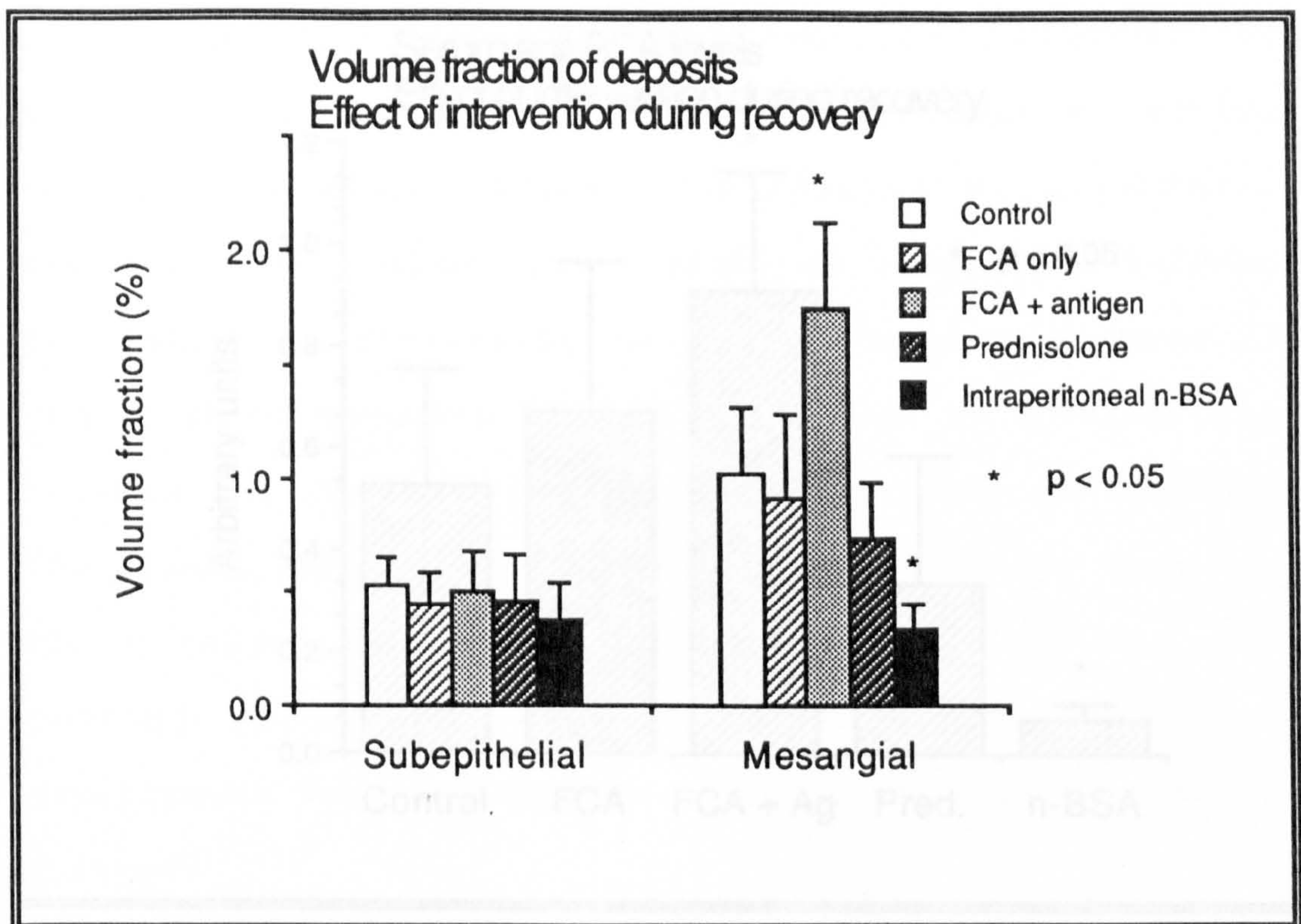
Figure 23



Morphometric analysis of deposit volume again provided similar results; rats give Freund's complete adjuvant and antigen were found to have a greater residual volume of mesangial deposits than controls, whereas the administration of native BSA enhanced the removal of mesangial deposits ($p < 0.05$). A significant difference could not be demonstrated after Freund's complete adjuvant alone, nor after prednisolone, and no differences could be demonstrated in the volume fraction of subepithelial deposits between any groups (Fig 24).

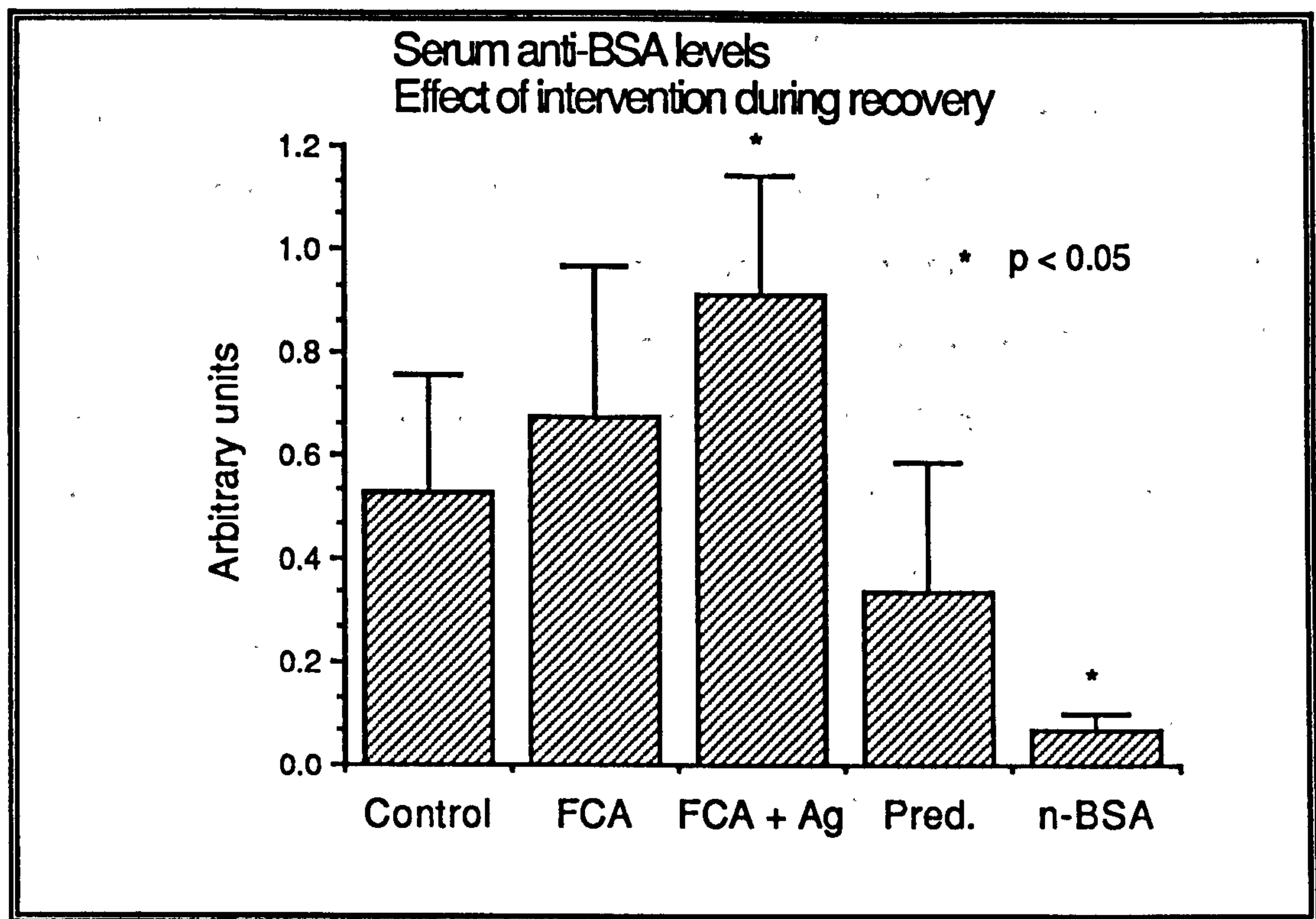
(comparable to a 'titre') because a standardized source of rat anti-BSA antibody was not available. However, the specificity and linearity of the assay were confirmed (see Appendix 1) and the measurements suffice to make comparisons between the groups.

Figure 24



The antibody levels showed rather more variation within each group than did the glomerulus antigen load, and perhaps for this reason a statistically significant difference from the control group was only demonstrated in the groups given Freund's complete adjuvant and antigen or native BSA (Fig. 25). The measurement of anti-BSA antibody was made in arbitrary units (comparable to a 'titre') because a standardized source of rat anti-BSA antibody was not available. However, the specificity and linearity of the assay were confirmed (see Appendix 11) and the measurements suffice to make comparisons between the groups.

Figure 25



Conclusions

This experiment was initiated with the suspicion that stimulation of the mononuclear phagocytes by Freund's complete adjuvant might enhance the removal of antigen from the glomerulus. The reverse was demonstrated. Since it was shown in Chapter 4 that rats which had received Freund's complete adjuvant did not develop glomerulonephritis, one must conclude that less antigen reached the glomeruli of those rats, rather than they were better able to remove it after deposition.

If the administration of Freund's complete adjuvant did succeed in stimulating the glomerular phagocytes, it seems inherently unlikely that the presence of active macrophages would inhibit the removal of glomerular deposits. What other system might be responsible? There is a striking similarity in pattern between Figures 22 and 25 which suggests the possibility of an inverse link between the rate of removal of a glomerular antigen and the plasma level of the corresponding antibody; that is, the presence of specific antibody might inhibit the removal of antigen from the glomerulus. Subsequent experiments were designed to investigate this hypothesis further.

Another conclusion may be drawn from these results. As the antigen load per glomerulus and the volume fraction of deposits are known, and the mean glomerular volume can be readily measured (Aherne & Dunnill 1982), one can calculate a crude estimate of the ratio of antigen mass to deposit volume. Such a calculation makes several very dubious assumptions, such as the assumption that all the deposits have the same composition, and the assumption that all the antigen is within the deposits. Nevertheless the result is of interest. The ratio increases with the course of recovery, presumably due to continued accretion of materials other than antigen, but a figure of 1:100 is typical. This indicates the paucity of antigen within the deposits, and supports the view

that some of the difficulty experienced in the identification of antigen in human glomerulonephritis may be due to 'masking' by other components.

The effect of passive immunization against bovine serum
albumin

Introduction

To test directly the hypothesis that antibody against BSA inhibits the removal of BSA deposited in the glomerulus, passive immunization with rat anti-BSA antibody was employed.

Protocol

To produce the anti-BSA immunoglobulin, seven adult (over 250g) male Wistar rats were each given two subcutaneous injections of 1mg of cationic BSA in 0.25ml of saline, emulsified with 0.25ml of Freund's complete adjuvant, three weeks apart. Two weeks after the second dose the rats were killed by exsanguination under anaesthetic. To maximize the yield of serum, blood was collected from a cannula in the abdominal aorta while 5ml of saline was infused into the inferior vena cava. Seven non-immune rats of similar weight were also exsanguinated to provide control serum. The blood was allowed to clot, the serum collected and the two groups pooled. A gamma globulin fraction of each was prepared by ammonium sulphate precipitation (Appendix 12). The precipitates were re-dissolved, dialysed, centrifuged and passed through a 0.2µm filter to remove particulate

matter, lyophilized and stored at -20°C . A simple ELISA assay was used to assess the ability of the immune globulin to bind to BSA; compared to control globulin, activity was still present at a titre of 100,000 (Appendix 11).

Cationic BSA chronic serum sickness was induced in 20 rats by the standard protocol. Four days after the last antigen injection the rats were divided into two groups of ten. Rats in one group received a single intraperitoneal injection of 75 mg of non-immune rat gamma globulin in 0.75ml of saline, while the other group received 75mg of anti-BSA rat gamma globulin. The rats were all killed ten days later and tissues were sampled as described in Chapter 7 for the assessment of antigen content and deposit volume fraction.

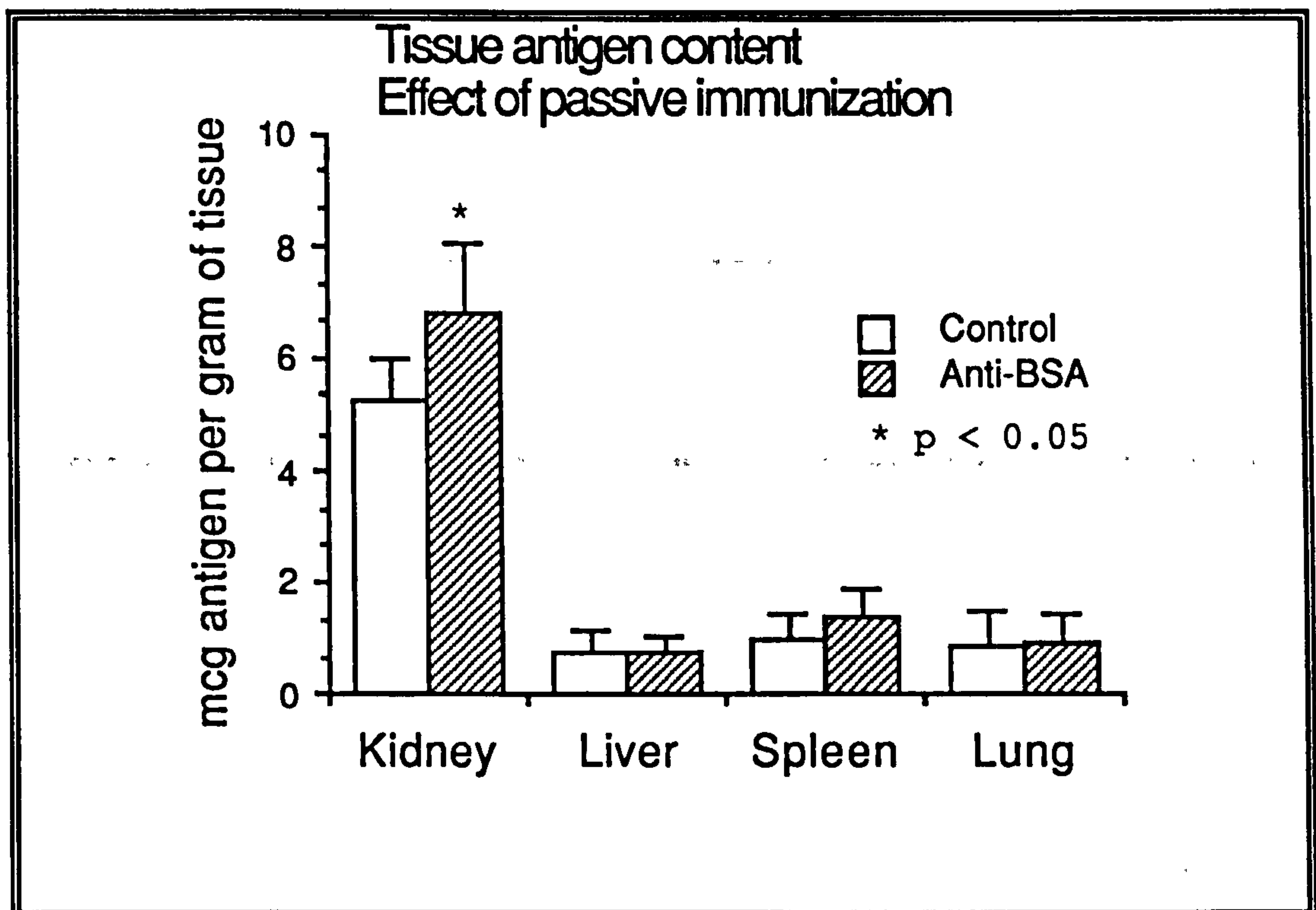
Results

Assessment of the mean amount of antigen per glomerulus at the end of the experiment showed that the control group had a mean of 76pg of antigen per glomerulus (95% confidence limits ± 10 pg), whereas the group which had received immune globulin had a mean of 107pg per glomerulus (± 17 pg). The administration of anti-BSA antibody had therefore inhibited the removal of cationic BSA from the glomerulus (t-test: $p < 0.005$).

The amount of antigen remaining in whole tissues at the end of the experiment is shown in Figure 26. This

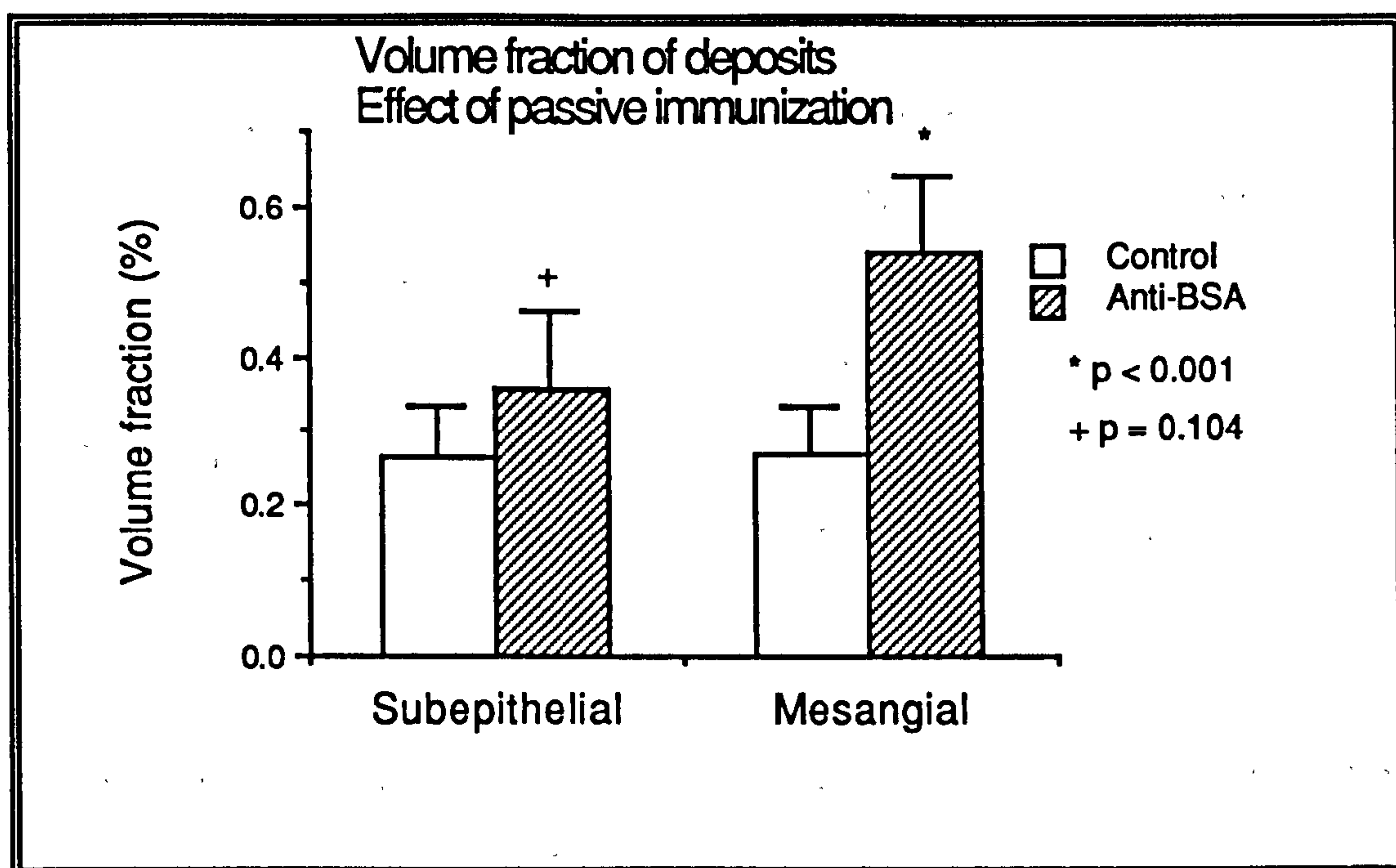
confirmed that passive immunization inhibits the removal of antigen from the renal cortex ($p < 0.05$), though a significant effect was not found in any of the other tissues studied.

Figure 26



Morphometric evaluation of the volume fraction of the glomerular deposits showed that the rats which had received anti-BSA globulin had significantly more mesangial deposits at the end of the experiment than the control group (Fig 27: $p < 0.001$). A similar but smaller result is suggested for the subepithelial deposits, but the difference did not reach statistical significance (t-test: $p = 0.104$).

Figure 27



Conclusions

These results confirmed that specific immunoglobulin can inhibit the removal of antigen and mesangial electron dense deposits from the glomerulus. The significance of this will be discussed later, but it is worth noting here that the opposite view is widely held (Pusey et al. 1988), without supporting evidence, although there are some reports in the literature which indirectly support my findings (Goode et al. 1985).

It may be noted that the mean glomerular antigen content of the control group after ten days of recovery differed in this experiment from the last, despite the fact that the control groups were apparently treated in

identical manner. This observation was repeated in subsequent experiments. There is no clear explanation for this. The largest differences were seen when a different batch of cationic BSA was used; the cationization reaction is somewhat capricious, so this may be due to minor differences in the pI between batches, even though batches with a pI which was detectably different by isoelectric focussing were discarded. This would not influence the results within one experiment, as the BSA was always pooled before the experiment started. Some variation in the results of the control groups of different experiments was noticed even where the same batch of cationic BSA was used. This could be due to variation in the immunization protocol (e.g. the degree of emulsification of the adjuvant) or to different batches of rats. This effect was not considered to have any influence on the conclusions because, as the results indicate, the degree of variation within each group was quite small. It does provide grounds for excluding historical control groups.

The effect of depletion of complement
by cobra venom factor

Introduction

The major known constituents of most electron dense deposits are antigen, antibody and complement components. After having demonstrated the influence of one component, antibody, on the rate of removal of the deposits, it was a logical progression to test another, complement. Depletion of plasma complement was achieved by administration of cobra venom factor (CVF).

Protocol

Chronic serum sickness glomerulonephritis was induced as before in 20 male Wistar rats, using cationic BSA over two weeks. Four days after the last antigen dose the rats were divided into two groups of ten. The animals in one group received 25 μ g of CVF (Sigma) in 0.1ml of saline by intraperitoneal injection every three days for the following ten days. The control group received no treatment. To confirm that the CVF of itself did not produce glomerular deposits or proteinuria, three normal, non-immune, age-matched rats were also given CVF by the same regimen. Blood samples were taken daily from the tail tip and serum hæmolytic complement

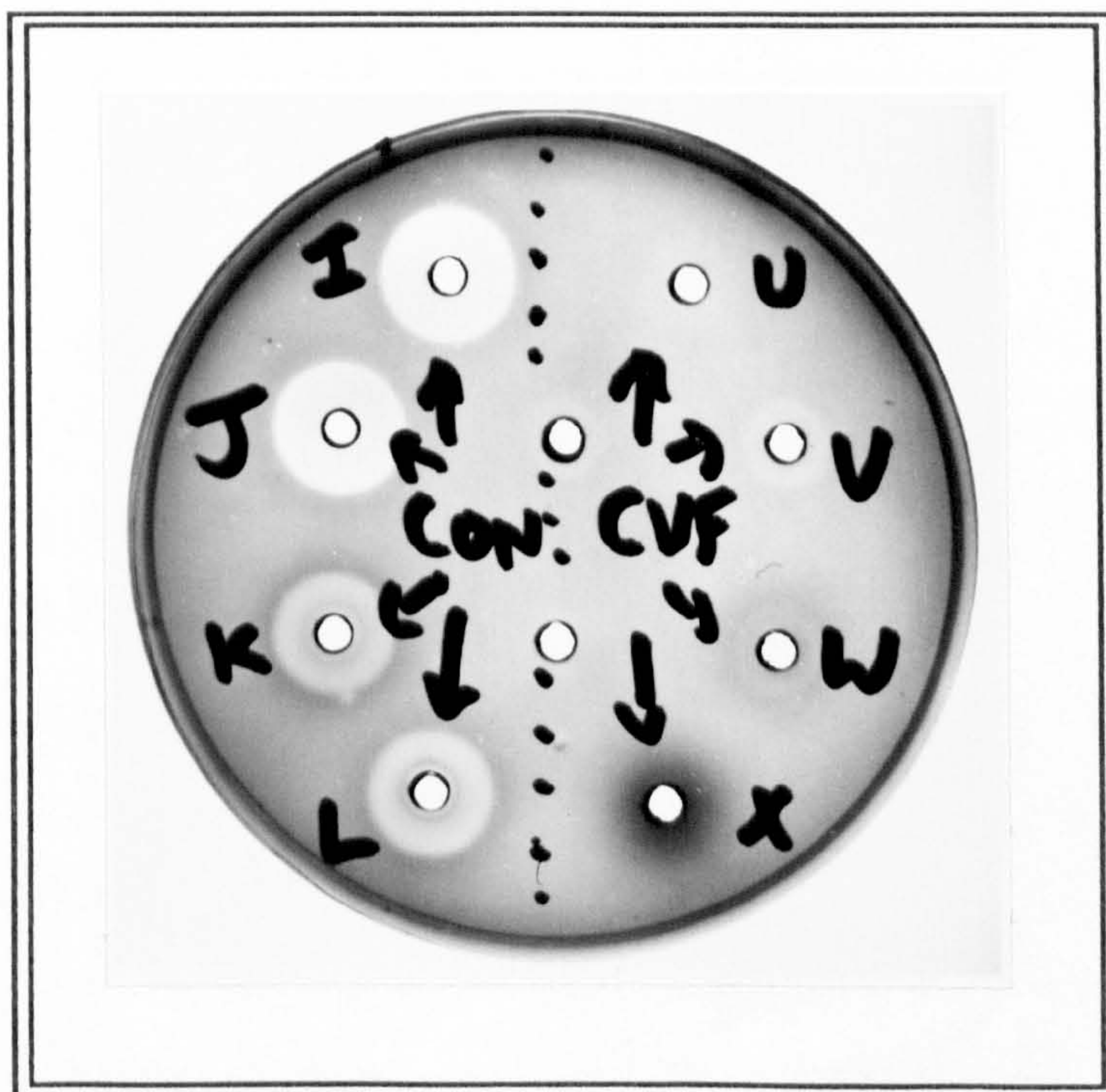
was estimated by a simple hæmolytic plate assay (Appendix 13).

At the end of this period the rats were killed and tissues sampled by the routine method.

Results

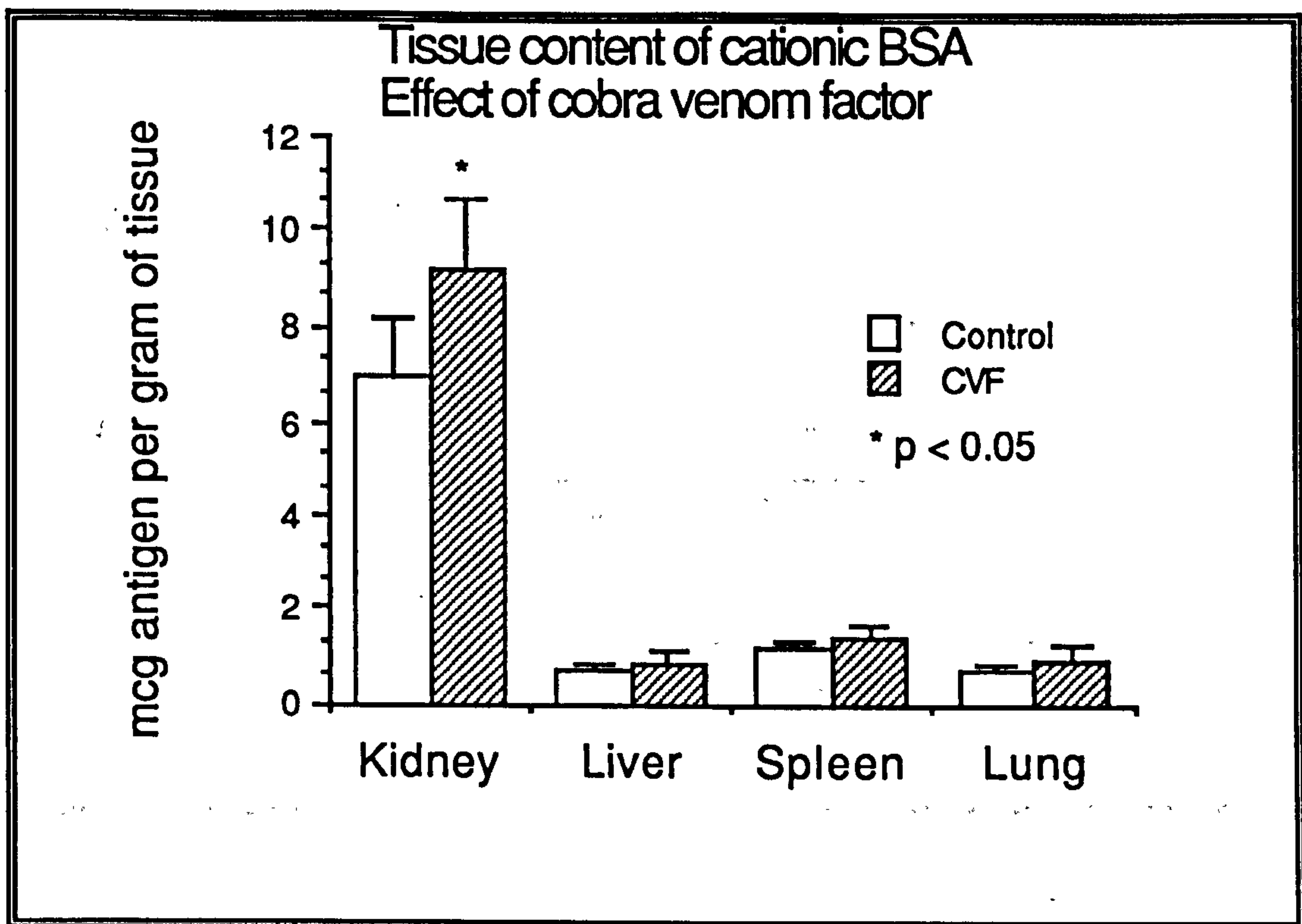
Complement activity was not detectable by hæmolytic plate assay in any of the rats receiving CVF. Figure 28 shows one of the plates produced at the end of the ten day period of de complementation; there are clear zones of hæmolysis around the wells containing serum from the control rats, whereas serum from rats given CVF has produced no hæmolysis.

Figure 28. Hæmolytic plate assay for complement. Samples on the left (I to L) are from rats with chronic serum sickness alone. Those on the right (U to X) are from rats which also received CVF. Samples taken on the last day of the experiment. Sample X was partly hæmolysed.



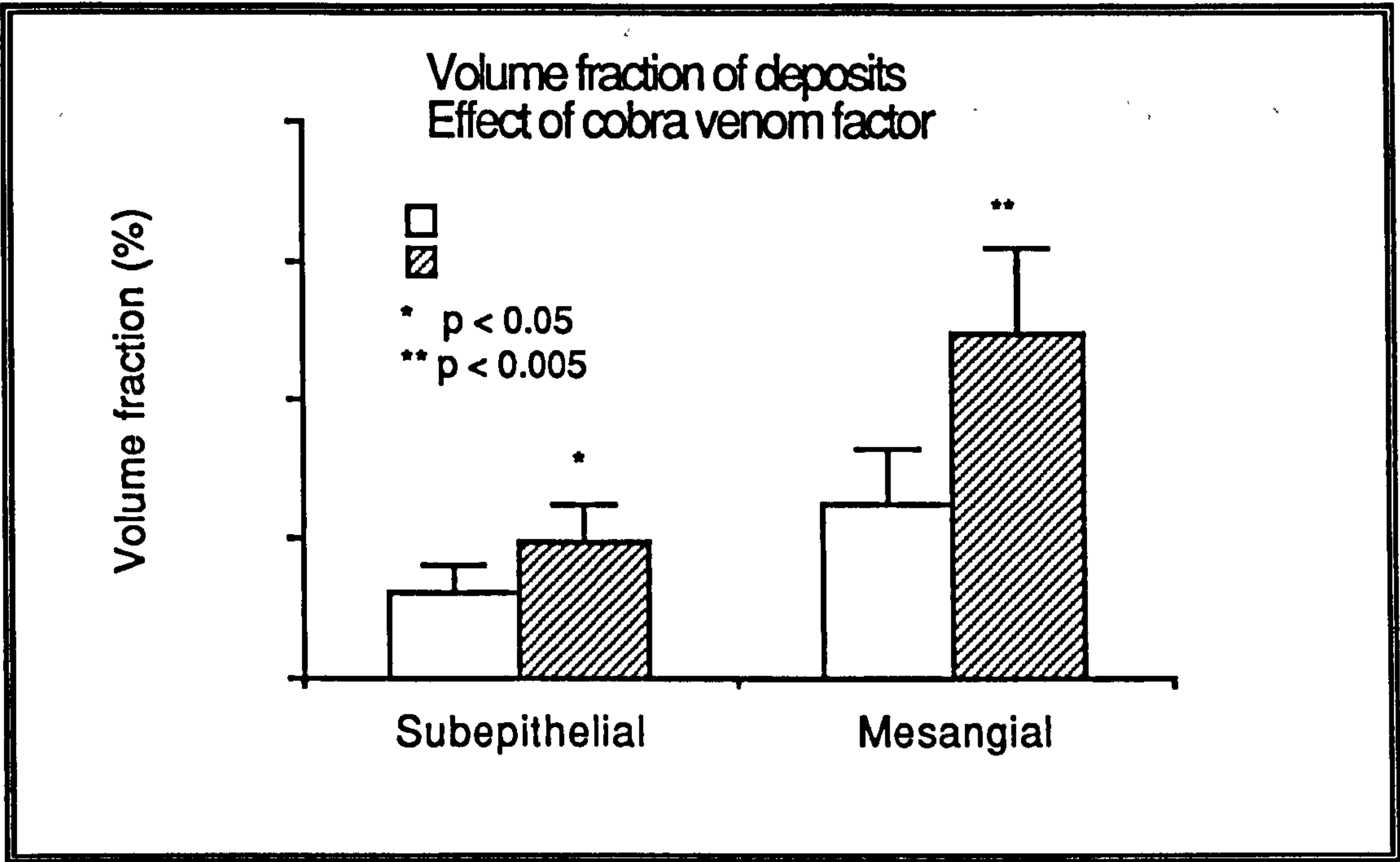
The mean glomerular antigen content of the control group was 123pg (95% limits \pm 20pg) per glomerulus. In contrast to the results after passive immunization described in the previous chapter, the rats which had received CVF had more antigen remaining in their glomeruli at the end of the experiment: 205pg (\pm 30pg) per glomerulus. This is a highly significant difference; $p < 0.001$ by t test. It indicates that the presence of plasma complement enhanced the removal of glomerular antigen. If whole renal cortex was assessed, the same result was found (Fig. 29). The amounts of antigen remaining in liver, spleen and lung all appeared to show minor alterations in the same direction, but the differences were not statistically significant.

Figure 29



Morphometric analysis of the volume fraction of deposits in the glomeruli of these rats indicated that the rate of removal of both subepithelial and mesangial deposits is reduced in the absence of complement (Fig. 30).

Figure 30



Conclusions

Administration of cobra venom factor inhibits the removal of antigen from glomeruli and of both subepithelial and mesangial electron dense deposits. The role of complement in glomerulonephritis is usually considered to be deleterious, and proteinuria is abolished by the elimination of complement in many animal models. There is a single report in the literature indicating that complement depletion inhibits antigen removal in acute serum sickness in the rabbit (Bartolotti and Peters 1978). More recent work in this field has concentrated on the *in vitro* demonstration of the ability of the complement system to inhibit immune

complex aggregation and to dissolve immune complexes once formed (Schifferli et al. 1980, 1986). The effect described here is presumably an *in vivo* reflection of this capability.

The kinetics of the CVE effectIntroduction

The inhibitory effect of decomplementation with cobra venom factor appeared to be large. Comparison of the difference between the control and experimental groups with the uninterrupted rate of antigen removal shown in Chapter 8 suggested that the elimination of plasma complement might actually have caused cessation of removal of antigen, but as has already been noted, comparisons between groups of experimental animals studied at different times are not valid with this model. A further experiment was therefore performed to assess changes in the rate of antigen removal with or without circulating complement.

Protocol

Chronic serum sickness glomerulonephritis was induced in 32 rats by the standard protocol, but instead of allowing 14 days of recovery, small groups of rats were killed at intervals after the last antigen dose. Four were killed 24 hours after the last dose and four at four days. At this point the remaining 24 rats were divided into two groups. One group was treated with CVF, 0.25 μ g, every three days as in the last experiment. Four rats from each group were killed 3, 6 and 10 days after

the start of CVF treatment. Tail-tip blood samples were taken daily after the start of CVF treatment and complement activity assessed by hæmolytic plate assay as before.

Samples of tissue were taken as before, except that morphometric analysis of deposit volume was not attempted with these small groups of animals, and urine samples were taken by bladder puncture for the assessment of proteinuria (Sulphosalicylic acid method: Appendix 9).

Results

Sequential assessment of the glomerular antigen load of rats recovering from chronic serum sickness glomerulonephritis provided the results shown in Figure 31. The elimination of plasma complement had very little effect on the rate of antigen elimination for a few days, but after one week elimination appeared to have virtually ceased in the CVF group. Not surprisingly, the evaluation of whole renal cortex showed the same effect, and again liver, spleen and lung showed little change (Fig. 32).

Figure 31

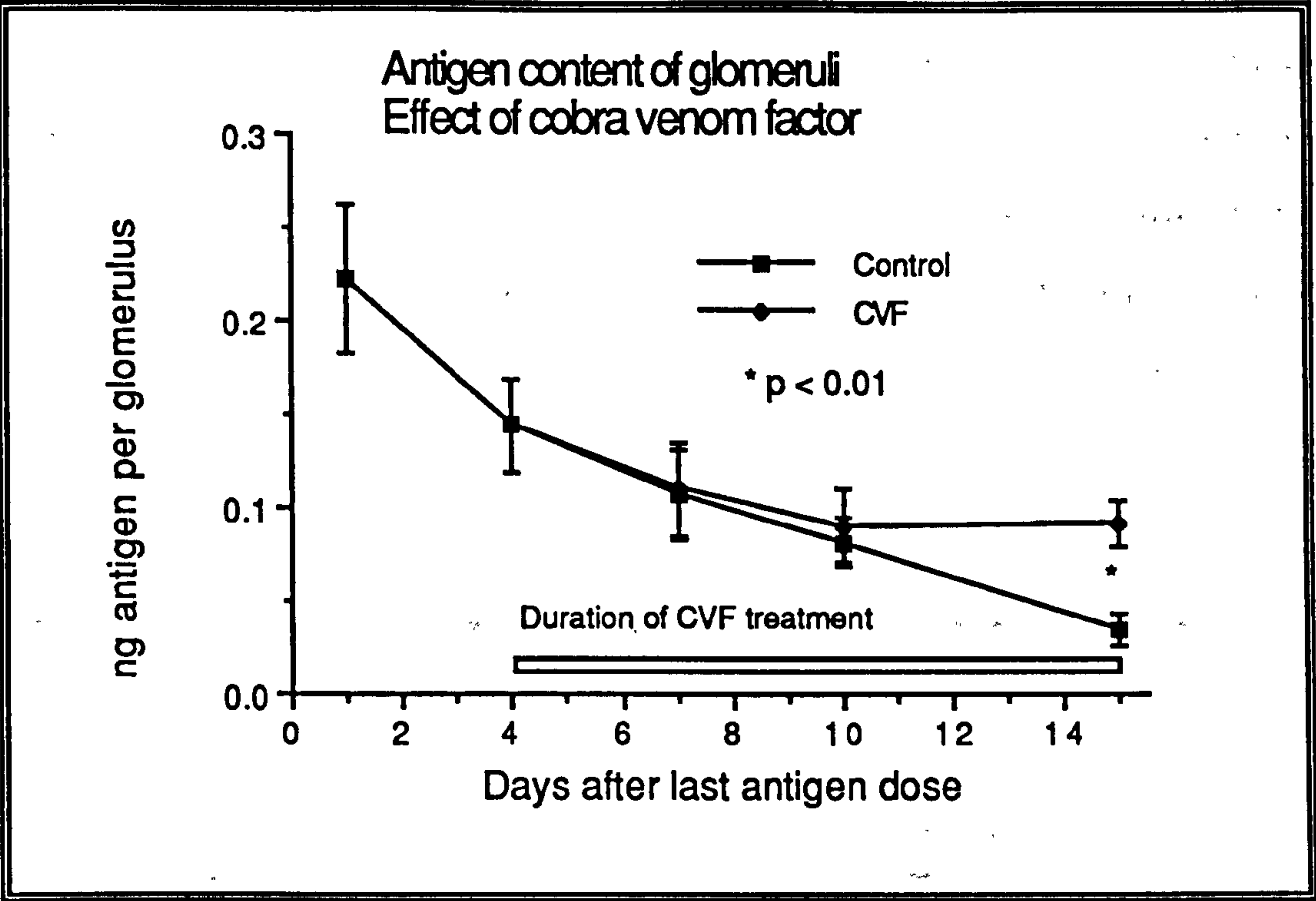
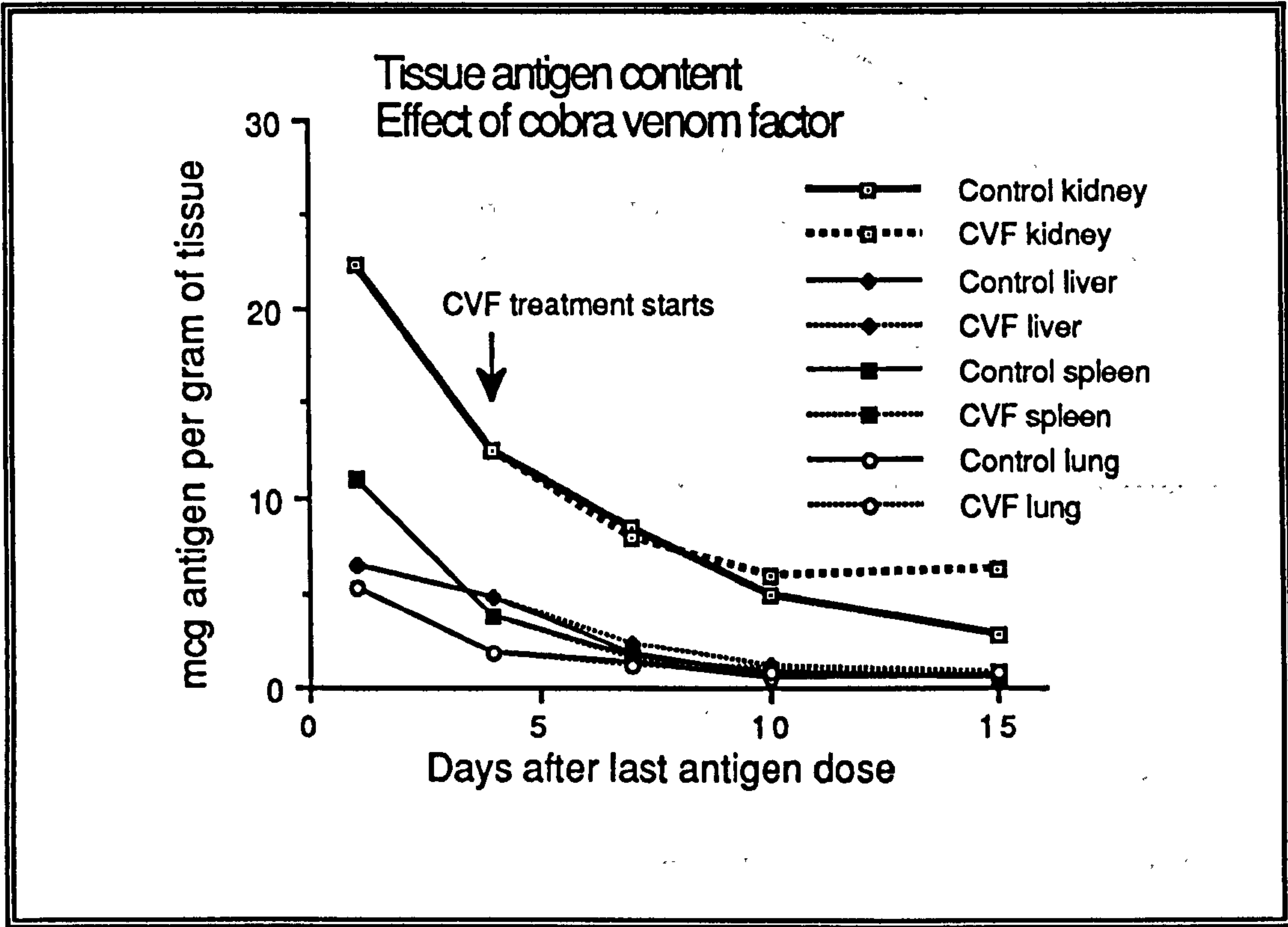
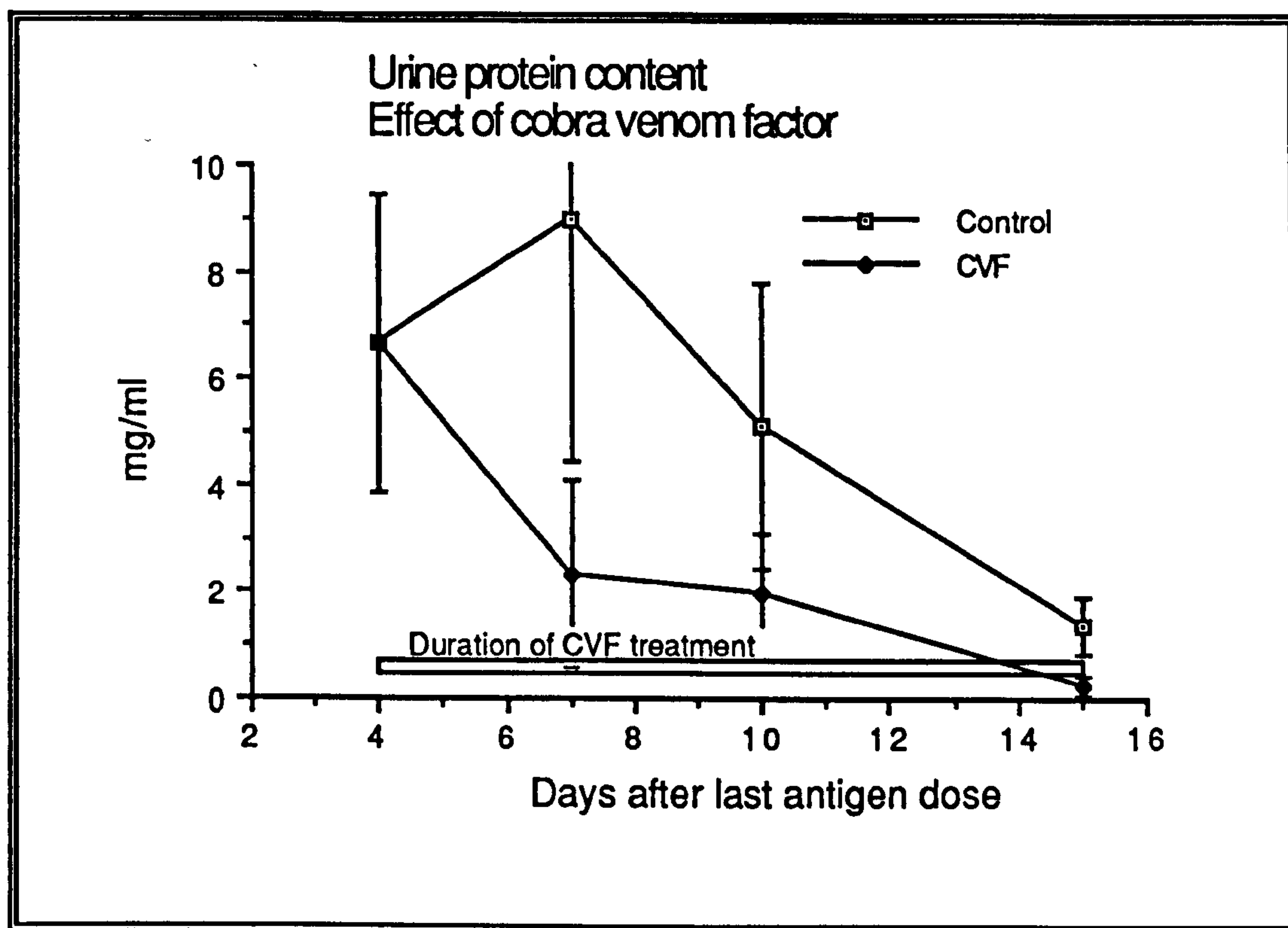


Figure 32



The level of proteinuria induced by this model has been shown to be more variable than the glomerulus antigen content, but nevertheless the rats given CVF during the recovery phase were found to have significantly less protein in the urine than the control group (Fig. 33).

Figure 33



The hæmolytic plate assays again confirmed successful complete elimination of complement activity during the whole of the ten day period of CVF treatment.

Conclusions

The elimination of complement was found to have a profound effect on the rate of antigen removal, but not an immediate one. One may suggest that perhaps activated C3 bound in the glomerulus continues to have an effect for a few days, but our understanding of the mechanism by which complement dissolves immune complexes is too incomplete to press this hypothesis further. It is interesting to note that rats treated with CVF have less protein in the urine, despite having more antigen and larger deposits in their glomeruli.

Are mononuclear phagocytes involved?

The effect of Polyvinyl Alcohol
and Puromycin Aminonucleoside

Introduction

If the elimination of complement causes cessation of removal of antigen from the glomerulus, one might suggest that any other mechanisms of antigen removal must work in co-operation with and be dependent upon complement. Resident glomerular mononuclear phagocytes have been held to be responsible for antigen removal; if so, does their action require 'opsonization' by complement? There is good evidence for the involvement of glomerular macrophages in the processing of immune complexes and other particulate matter as it arrives at the glomerulus, but direct evidence of the involvement of macrophages in the removal of established, morphologically identifiable deposits is not available. The results of experiments using anti-macrophage serum were discussed in Chapter 3; they do not appear to support a role for macrophages. Furthermore, the observation that the administration of Freund's complete adjuvant *inhibits* the removal of deposits (*vide supra*) suggests that macrophages are probably not involved in this process.

To investigate this question further, methods were required to stimulate or inhibit macrophage activity. Three agents were used, selected on the basis of their likely efficacy and selectivity, and their availability.

- 1) Polyvinyl alcohol (PVA). This inert polymer has recently been shown to accumulate in the glomerulus after subcutaneous injection, causing an increase in the activity and number of glomerular macrophages (Seiler et al. 1983) with very little effect on glomerular blood flow or filtration rate (Sterzel et al. 1983). The uptake into the glomerulus of particulate tracers such as colloidal carbon or iron dextran is consequently increased (Mauer et al. 1979, Seiler et al. 1986).
- 2) Puromycin aminonucleoside (PAN). This agent is widely used to induce a model of minimal change nephrotic syndrome. It probably has a toxic effect on glomerular epithelial cells, but in addition it appears to increase the numbers and activity of mesangial phagocytic cells (Schreiner et al. 1984), with increased trapping of particulate tracers (Grond & Elema 1981, Hoyer et al. 1976). If mesangial macrophages remove mesangial deposits, one might therefore expect the administration of PAN to enhance their removal. Macrophages do not normally gain access to subepithelial deposits, but if the epithelial cells are involved in the removal

of such deposits the use of Puromycin aminonucleoside might alter the rate of their elimination, as assessed by morphometry.

- 3) Colchicine. This drug is known to act by blocking microtubular function (Borisy & Taylor 1967), and has been used in the past to inhibit macrophage activity (Von Figura et al. 1978, Cain et al. 1983); it appears to inhibit their motility and phagocytic function. Colchicine also alters the shape of glomerular podocytes *in vitro* (Andrews 1981) and decreases the proteinuria in Heymann nephritis (Milner et al. 1987). The problem with its use is the narrow dose range between therapeutic efficacy and toxicity. In this study it proved impossible to detect any change in the glomeruli of rats given colchicine, so although the results for this group are presented, no conclusions are drawn from them.

Protocol

Chronic serum sickness glomerulonephritis was induced in 32 male Wistar rats, by the standard protocol. Four days after the last antigen dose, the rats were divided into four groups of eight, and treated as follows:

- 1) Control. No treatment.

- 2) Polyvinyl alcohol (Sigma: M.W. 40,000 to 60,000), 1ml of a 5% solution in saline by daily subcutaneous injection for ten days.
- 3) Puromycin aminonucleoside 100mg by a single intraperitoneal injection.
- 4) Colchicine 20µg in water, by daily gavage for ten days.

To confirm alterations in glomerular macrophage function the rats received an intravenous injection of 100 mg of iron dextran (Imferon®) 24 hours before sacrifice.

At the end of the ten day intervention period the rats were killed and samples taken according to the standard protocol. In addition, blood samples were taken from the control and Puromycin groups to assess the severity of the nephrotic syndrome. Samples of frozen renal cortex were also taken.

Results

An attempt was made to assess the numbers of glomerular macrophages using an immunoperoxidase technique on frozen sections with the monoclonal antibodies OX3 and OX4 (Sera-lab), which recognize rat macrophages. This was not successful because the secondary antibody (rabbit anti-mouse IgG) bound to the rat IgG which was in the glomeruli as a result of the

glomerulonephritis, causing heavy granular staining even in control sections, and masking any cellular staining. Fortunately other methods were available to confirm the effect of the treatment given. In the case of polyvinyl alcohol the morphological changes were obvious (Fig. 34). Globules of amorphous material (PVA) accumulated in the mesangium; on electron microscopy these were seen to be mainly inside vacuoles in cells with the morphological characteristics of macrophages. Puromycin aminonucleoside caused the nephrotic syndrome to become considerably worse (Table 6). Very little of the injected iron dextran had localized in the glomeruli, but a Perl's Prussian Blue stain of paraffin sections of kidney showed patchy blue staining of some of the glomeruli of rats given PAN, whereas iron could not be detected in any of the glomeruli of the control group. Colchicine did not produce any detectable effect.

Figure 34. A glomerulus from a rat which had received PVA, showing an influx of PVA-laden foamy macrophages. H&E, x 1,200.

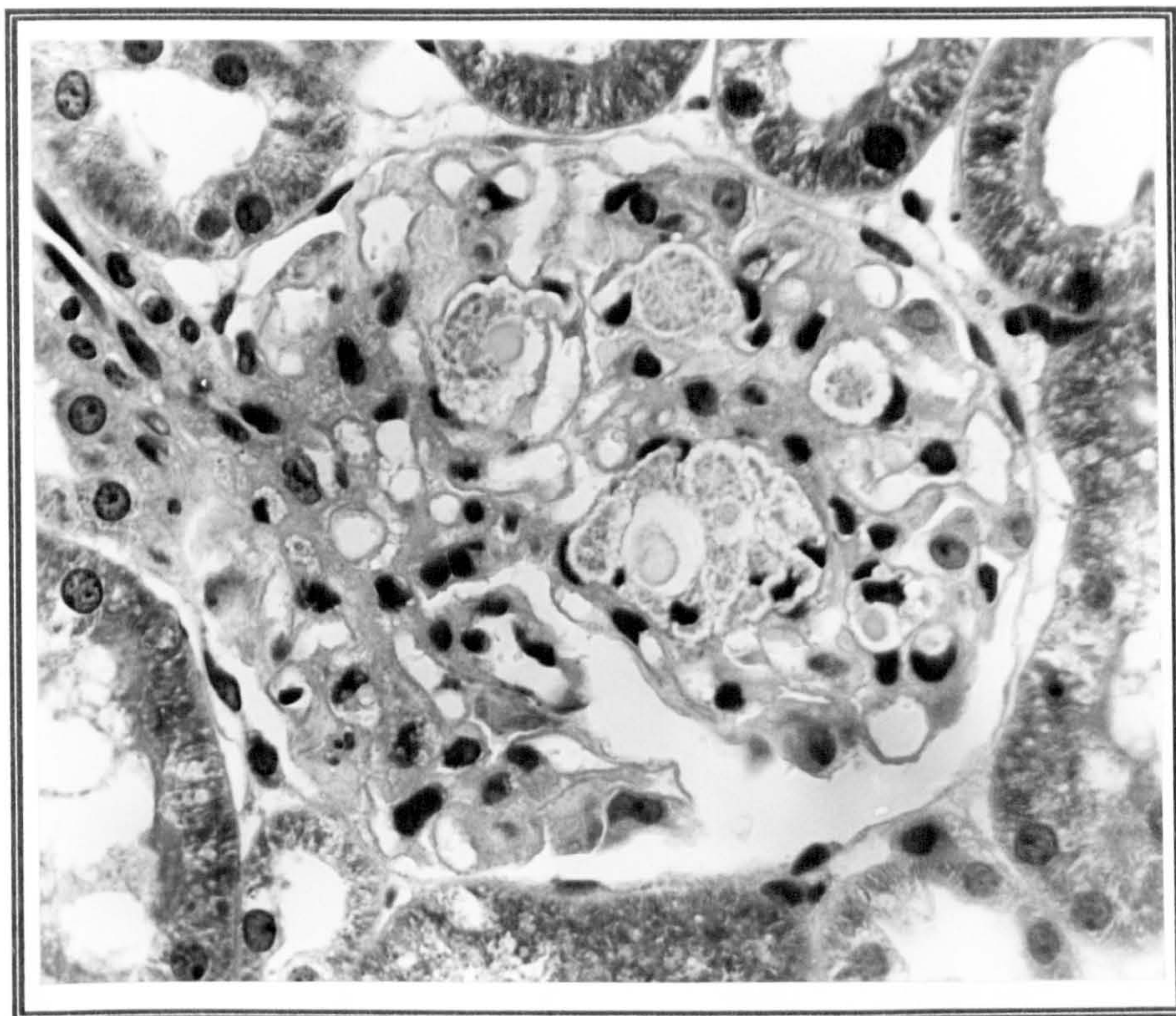


Table 6. Serum evidence of enhancement of nephrotic syndrome due to Puromycin aminonucleoside (Mean \pm 95% confidence limits)

	Albumin (mg/ml)	Cholesterol (mmol/l)
Normal rats (Data from Ch.5)	35.8 \pm 1.66	1.62 \pm 0.175
Control (Chronic serum sickness)	25 \pm 3.62	3.7 \pm 1.47
Puromycin aminonucleoside (CSS + PAN)	17.9 \pm 3.16 <i>p</i> < 0.01 cf chronic serum sickness alone	11.5 \pm 1.65

A significant difference could not be demonstrated between the amount of antigen in the glomeruli of any of

the experimental groups (Fig. 35). Analysis of whole renal cortex provided the same result (Fig. 36). The glomeruli from rats which had received PVA were found to be increased in volume by a factor of 1.25 compared with the control group; after adjustment of the volume fractions of the deposits by this amount as described in Chapter 7, a difference could not be demonstrated between any of the groups (Fig. 37).

Figure 35

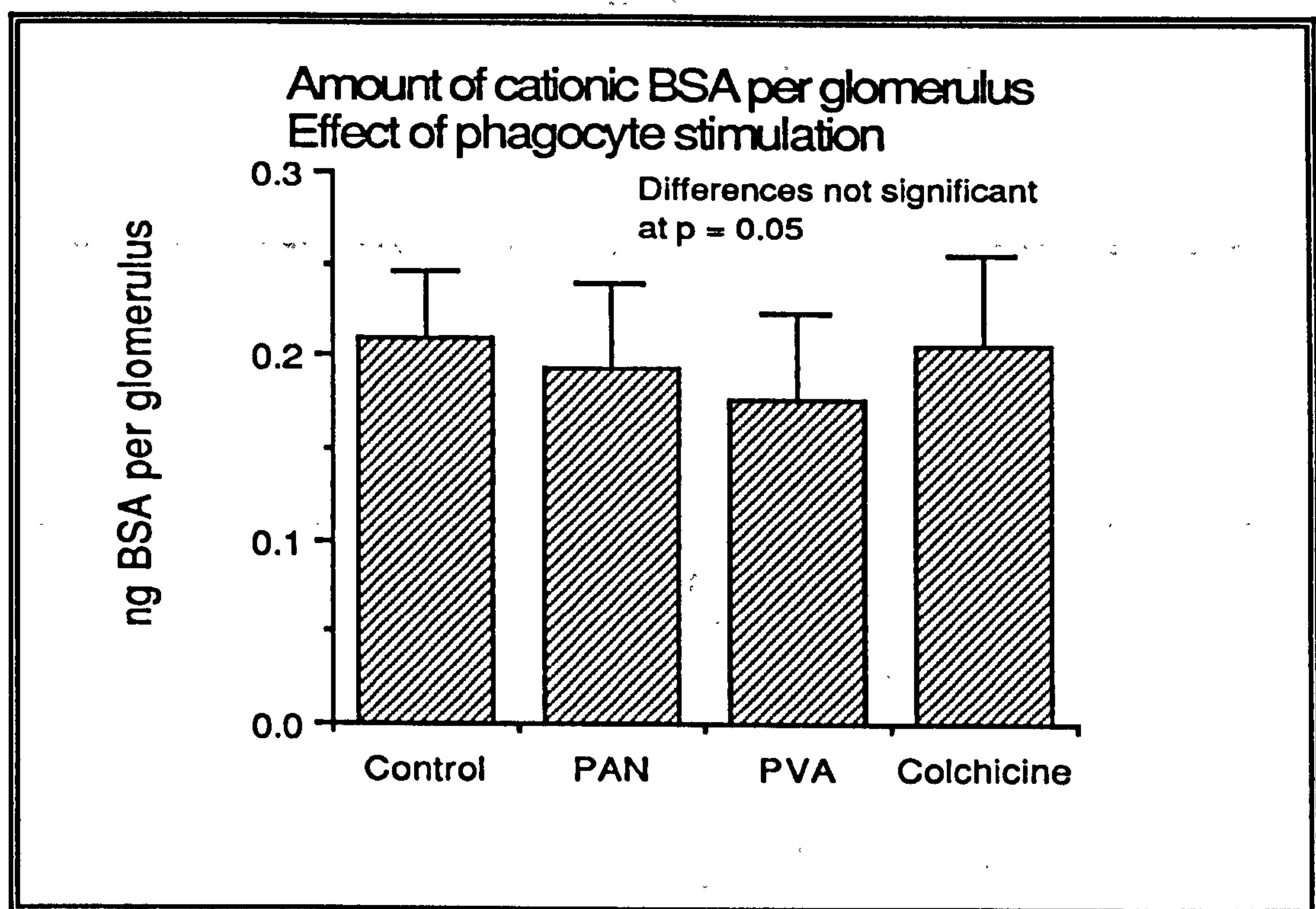


Figure 36

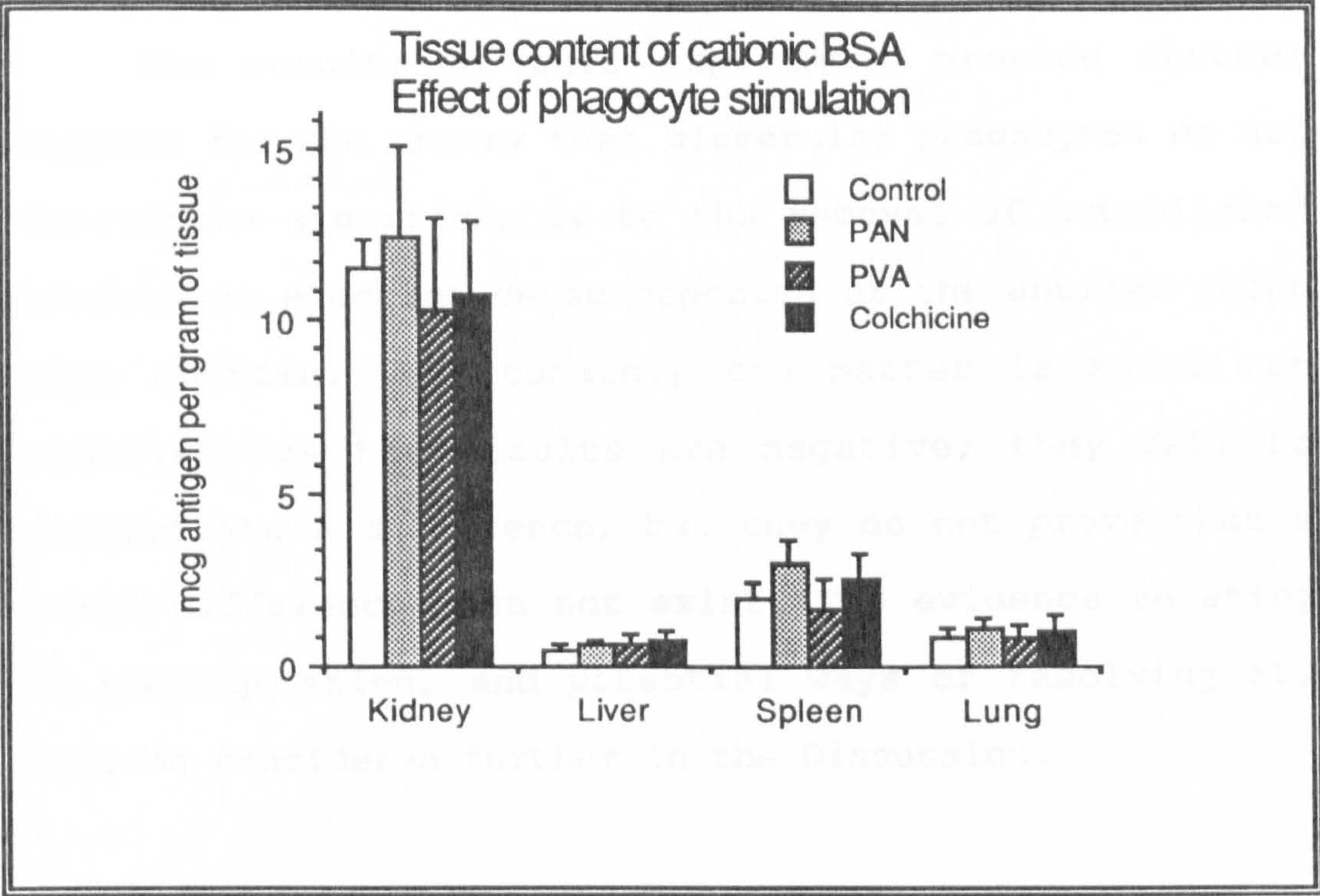
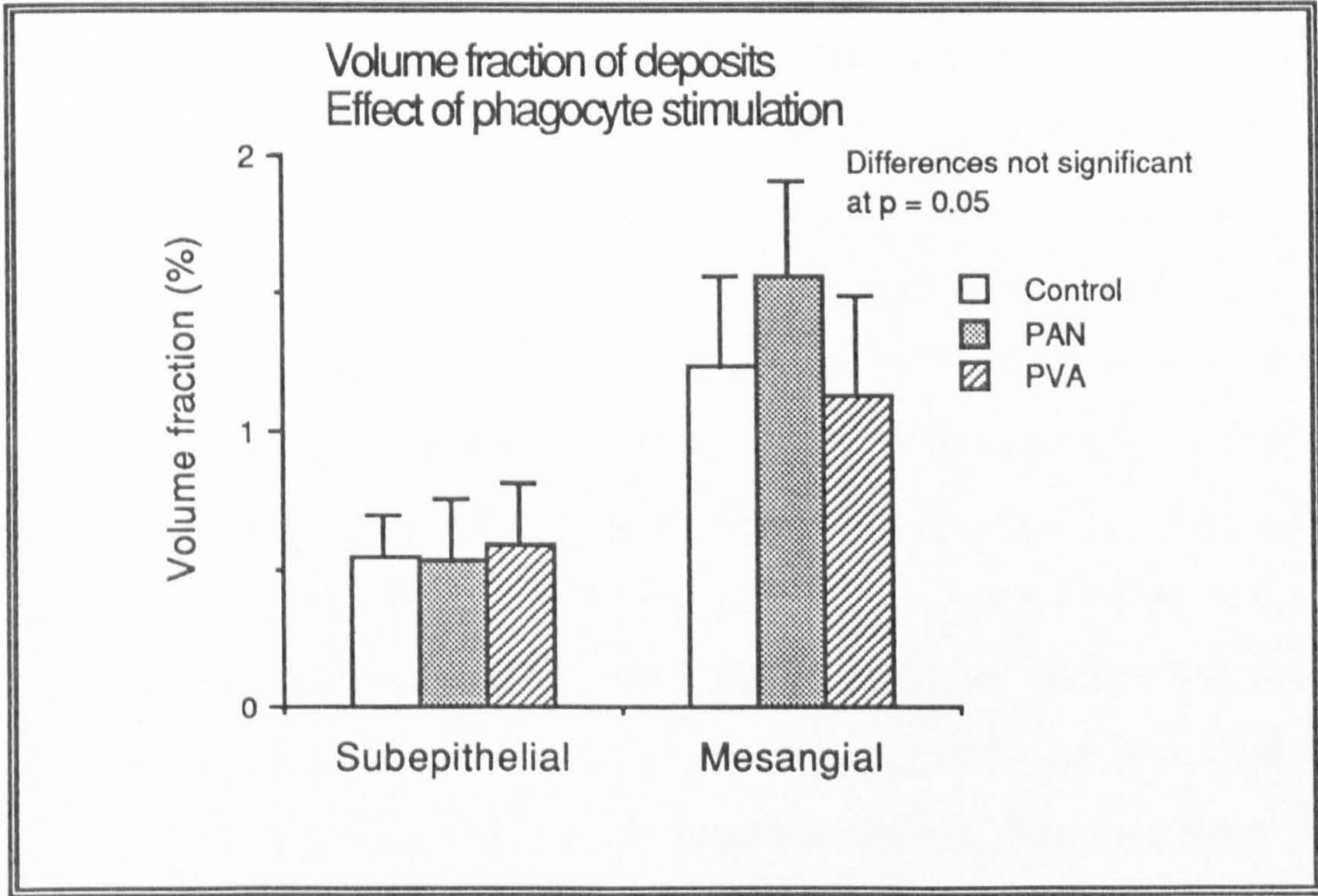


Figure 37



Conclusions

The results of this experiment provide further support for the theory that glomerular phagocytes do not contribute significantly to the removal of established glomerular electron dense deposits or the antigen which they contain. Unfortunately the matter is still not resolved, as the results are negative; they fail to demonstrate a difference, but they do not prove that a small difference does not exist. The evidence relating to this question, and potential ways of resolving it, will be considered further in the Discussion.

The effect of charged molecules

Introduction

The importance of the electrical charge of an antigen in determining its localization in the glomerulus has already been discussed. Neutralization of the glomerular charge by simultaneous infusion protamine, a non-antigenic cationic substance, has been reported to influence this localization (Adler *et al.* 1983b). It is obviously of interest to investigate whether such interference can alter the rate of removal of antigen and deposits. This question has already been addressed using an acute, passive form of serum sickness. Gauthier and Mannik (1986) reported that an infusion of protamine would enhance the removal of cationic immune complexes from the glomerulus only if given within an hour of localization of the complexes. After that time no effect was detectable, indicating a change in the nature of binding of the complexes in the glomerulus, and suggesting that charge interactions are only of importance in the early stages of deposit development. However, the model used in that experiment is very different to the chronic serum sickness model of these investigations. In view of the considerable potential for therapeutic intervention in Man if a charge effect could be demonstrated, it was decided to

proceed with an attempt to influence deposit removal by the chronic administration of protamine (cationic), polyethyleneimine (PEI; cationic) or heparin (anionic).

Protocol

Chronic serum sickness glomerulonephritis was induced in 24 rats by the standard protocol, using seven 10mg doses of radiolabelled cationic BSA. After four days of recovery the rats were divided into four groups and treated as follows:

- 1) Control group. No further treatment.
- 2) 500 units of heparin were given by intraperitoneal injection, twice daily (daily at weekend).
- 3) 5mg of protamine was given by intraperitoneal injection, twice daily (daily at weekend).
- 4) 0.4ml of a 2% solution of PEI (Polysciences) was given daily by tail vein injection.

After ten days of this treatment the rats were killed according to the standard protocol.

Results

The administration of heparin enhanced the removal of glomerular antigen (Fig. 38). This effect was confirmed by analysis of whole tissues (Fig. 39), but

measurement of deposit volume fraction did not detect any significant differences (Fig. 40).

Figure 38

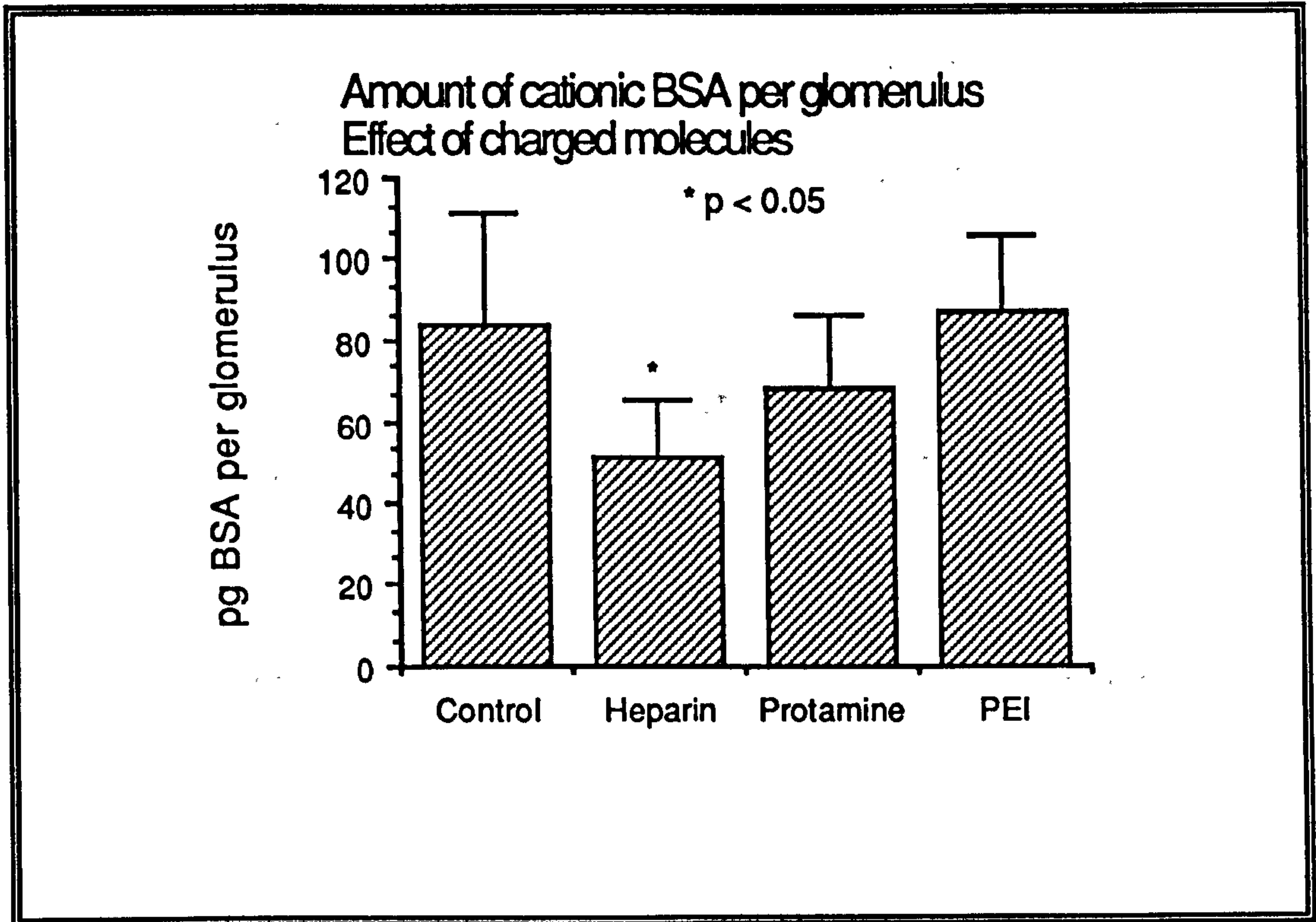


Figure 39

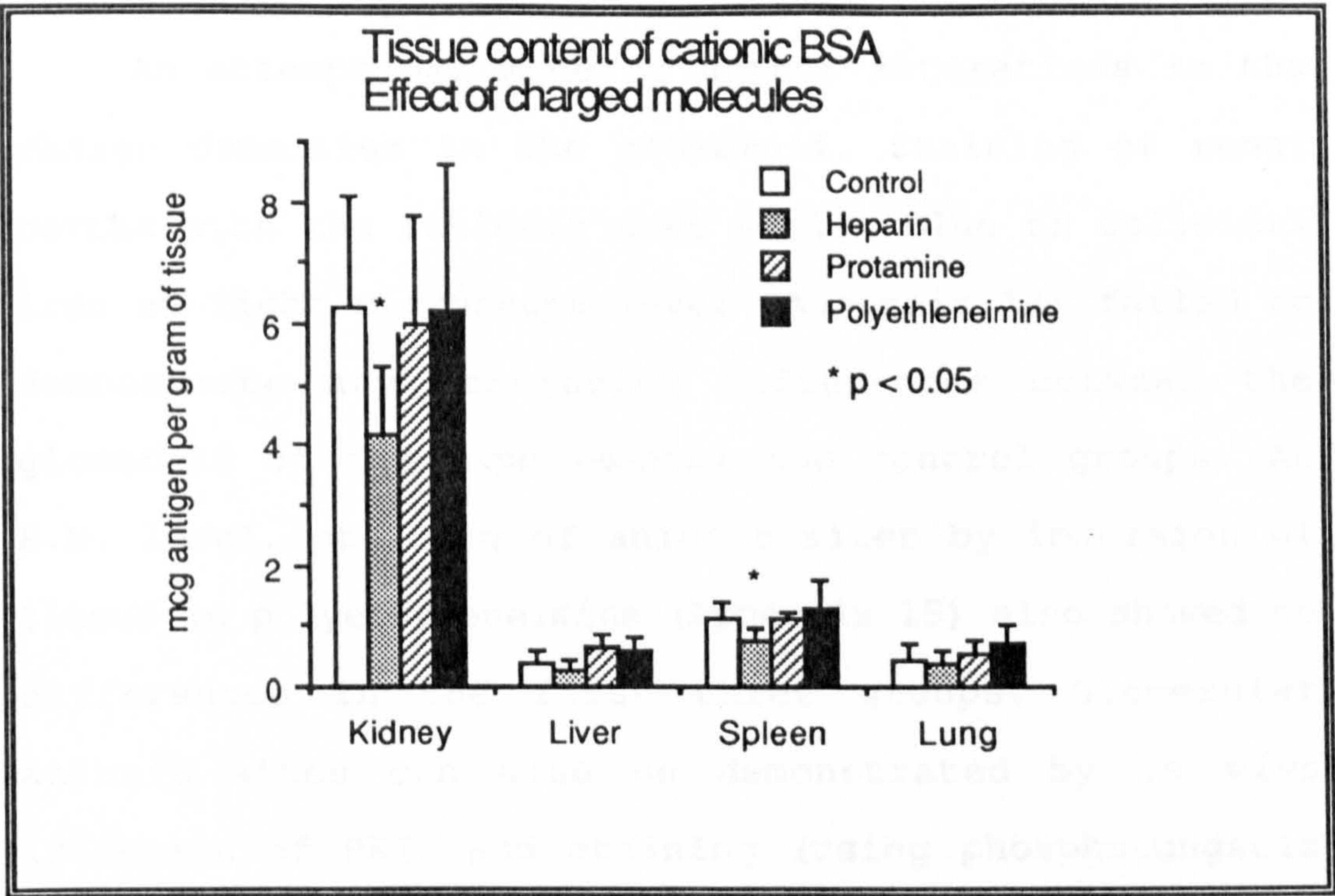
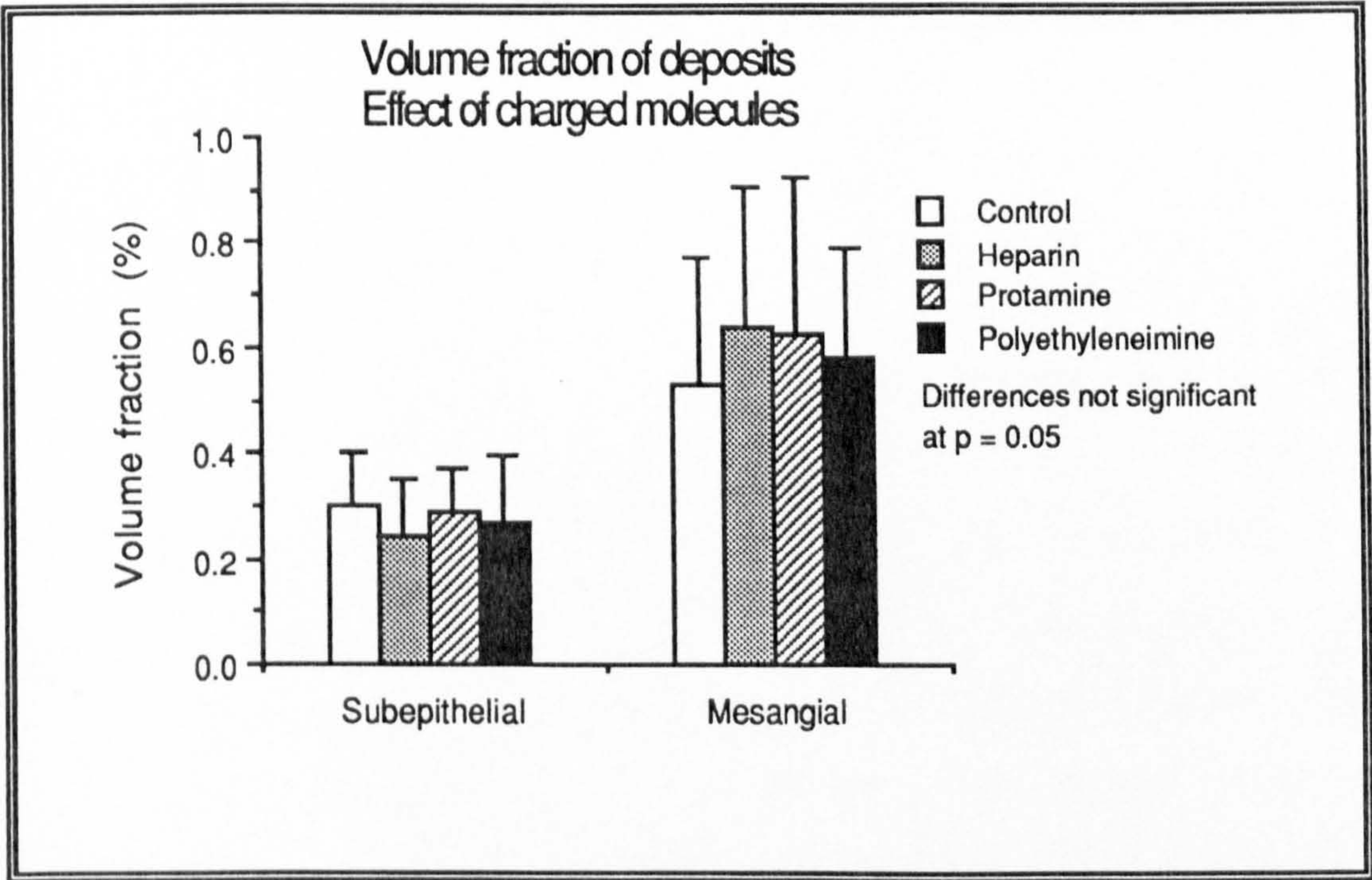
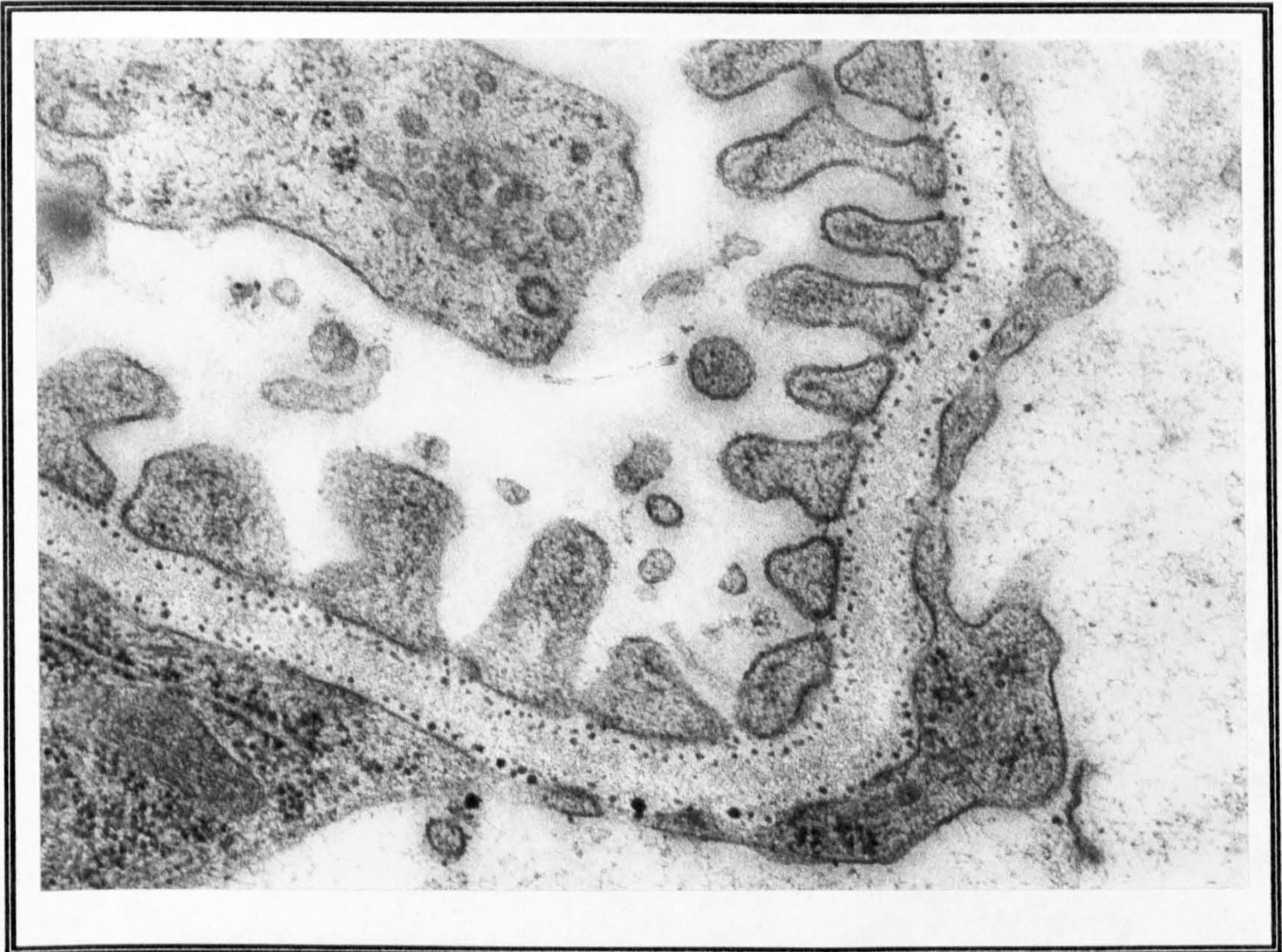


Figure 40



An attempt was made to detect alterations in the charge densities in the glomeruli. Staining of renal cortex with the cationic dyes Alcian Blue or colloidal iron at light microscope level (Appendix 14) failed to demonstrate any convincing differences between the glomeruli of the experimental and control groups. At E.M. level, staining of anionic sites by immersion of tissue in polyethyleneimine (Appendix 15) also showed no differences in the first three groups. Glomerular anionic sites can also be demonstrated by *in vivo* injection of PEI, and staining (using phosphotungstic acid; Appendix 15) of tissues from the rats which had been given intravenous PEI did indeed show the anionic sites, confirming that the PEI given was indeed reaching the glomerulus (Fig. 41).

Figure 41. Glomerular basement membrane of a rat treated with PEI. The anionic sites of the basement membrane are demonstrated as black dots, confirming that the PEI was reaching and adhering to the glomerular basement membranes. Transmission electron micrograph: PEI given *in vivo*, tissue immersed in 2% phosphotungstic acid, then processed routinely. x 30,000.



Conclusions

It is difficult to know what weight should be attached to these findings in view of the absence of evidence that the charged molecules administered were producing any alteration in the glomerulus. A search of the literature failed to provide any guidance for appropriate dosage for the drugs when given over a period of several days. The dose of heparin given was approximately twice that necessary to cause complete

anticoagulation of blood samples taken two hours after administration. Complications due to spontaneous haemorrhage were not seen. The dose of protamine was sufficient to neutralize an equal amount of heparin, had the two been administered to the same animal. The dose of PEI was half of an approximate LD₅₀ for this substance, determined before the experiment. It is sixteen times the amount given intravenously to produce ultrastructural staining of the glomerular anionic sites (Schurer et al. 1978); doses of this magnitude have been shown to penetrate the full thickness of the basement membrane and bind to anionic sites on the epithelial cells (Andrews & Bates 1985). In the light of this, the absence of any alteration in deposit removal in the group given PEI does give some support to the claim that charge interactions are not of great importance after the initial binding of antigen (Gauthier & Mannik 1986). Polyethyleneimine is cationic, as is the antigen. Heparin is anionic; the mechanism by which heparin enhances the removal of antigen from the glomerulus could perhaps be a simple charge effect, but it is more likely to be related to the complex biological effects of this substance. This will be considered further in the Discussion.

The effect of alterations in glomerular filtration rate

Introduction

Hæmodynamic changes can profoundly alter the function and indeed the structure of the glomerulus. A wealth of evidence can be quoted to support this point; it is particularly relevant to note that stenosis of one renal artery, with consequent diminution in blood flow and glomerular filtration rate (GFR), provides protection for that kidney against the development of immune complex glomerulonephritis (Germuth et al. 1967, Salyer & Salyer 1974). Other evidence of the influence of hæmodynamic factors has been reviewed in Chapter 2. It follows that a decrease in the blood supply must either reduce the rate of deposition of immune complexes or facilitate their removal. It seems likely that a decreased blood flow would decrease delivery of the components of the deposits, but this may be simplistic, and other effects are not excluded. The foregoing work suggests that plasma complement is an important agent in the removal of deposits; could the rate of delivery of complement influence the rate of removal? To investigate this area, two experiments were performed in which the GFR of one group of rats was altered during the course of recovery from chronic serum sickness glomerulonephritis. This was achieved by unilateral

nephrectomy in one experiment, and extreme alteration in the salt and protein content of the diet in the other. Pharmacologic methods of altering the GFR were avoided. The GFR of each rat was measured using an accepted modification of the ^{51}Cr -EDTA method, as described by Layzell and Miller (1975: Appendix 16).

Protocol

Unilateral nephrectomy. Chronic serum sickness glomerulonephritis was induced in 25 rats by the standard protocol. After four days of recovery the rats were divided into two groups. All were anaesthetized (Hypnorm® and diazepam). Twelve rats received a sham operation in which the abdomen was opened in the midline, the left kidney was briefly palpated, and the abdominal wall was closed in two layers. A left nephrectomy was performed on the remaining 13 rats. All the animals made an uneventful recovery and were killed ten days later for routine tissue sampling.

Diet. Two special rat diets were obtained from Special Diet Services (Stepfield, Witham, Essex). Both had a low salt content; one (designated LP) had a protein content of less than 2.5%, the other (designated HP) had a protein content of 40%, made up of casein, soya and egg protein. Chronic serum sickness glomerulonephritis was induced in 32 rats by the standard protocol. After four days of recovery the rats

were divided into four groups of eight rats each and provided with food and water as follows:

Table 7

	Food	Drink
LP,LS	LP diet	Water
LP,HS	LP diet	1.8% saline
HP,LS	HP diet	Water
HP,HS	HP diet	1.8% saline

After ten days of this regimen, the rats were killed and tissues sampled.

In both these experiments the procedure at sacrifice was modified to permit measurement of the glomerular filtration rate, which was achieved by the ^{51}Cr -EDTA method. The rats were first given a small dose of anaesthetic (0.1ml of Hypnorm i.m.) to facilitate the accurate intravenous injection of 0.1ml (10 μCi) of ^{51}Cr -EDTA (Amersham); the time of the injection was noted. Approximately an hour later a full dose of anaesthetic was given (Hypnorm and diazepam) and the abdomen opened. After the sample of renal cortex had been removed for electron microscopy, a blood sample was taken from the inferior vena cava and 500 μl of heparinized plasma was submitted to the gamma counter. The weight of each rat and the time of blood sampling were also noted, and the GFR was calculated from these data (Appendix 16).

Results

Unilateral nephrectomy produced the expected rise in the GFR of the remaining kidney, as shown in Table 8.

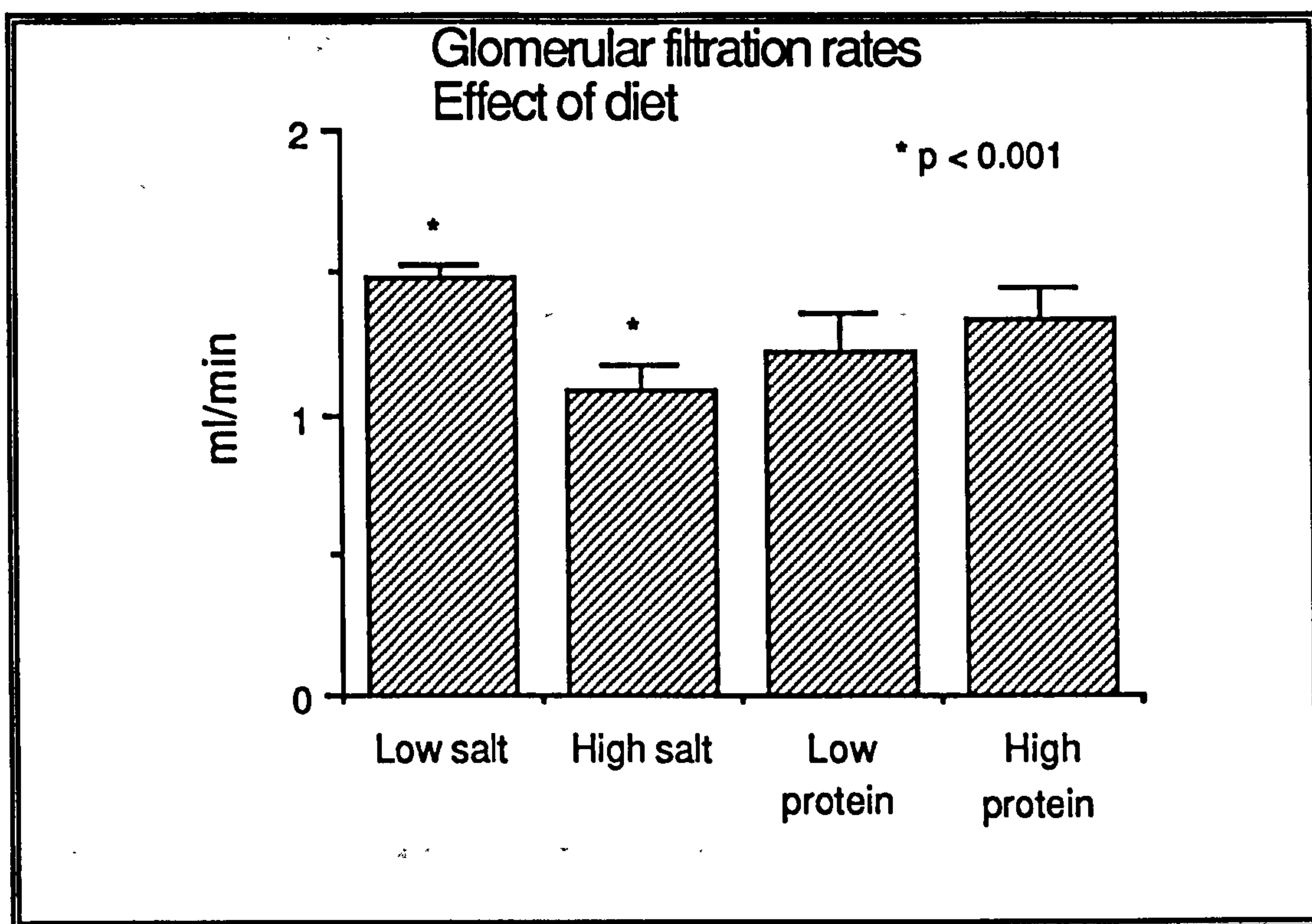
Table 8. Single kidney GFR (ml/min)

	GFR	95% limits
Sham operated	0.58	0.04
Unilateral nephrectomy	0.95	0.06

$p < 0.001$

Alteration in diet produced smaller changes in GFR. Because of this the results were pooled to give groups of 16 rats each, receiving low salt (LP,LS & HP,LS), high salt (LP,HS & HP,HS), low protein (LP,LS & LP,HS) or high protein (HP,LS & HP,HS). The results of this manoeuvre are shown in Figure 42. A high salt intake produced a significantly lower glomerular filtration rate. The high protein diet produced only a slightly higher mean GFR; the difference was not statistically significant. It should also be noted that the different groups had significantly different mean body weights at the end of the experiment; the high protein group weighed more (261g vs 243g; $p < 0.05$) as did the low salt group (263g vs 241g; $p < 0.05$), indicating that the high salt intake had not caused increased oedema.

Figure 42



The results of the standard investigations are shown in Figures 43 to 46. None of the manoeuvres to alter the GFR, whether successful or not, produced any detectable differences in the parameters measured, with the exception of a lower antigen content of whole renal cortex in the group receiving a high salt diet (Fig. 46). This difference was just significant at $p = 0.05$, but in view of the absence of a parallel effect on the antigen content of isolated glomeruli (Fig. 44) it is likely that this result has either arisen by chance or relates to antigen in renal structures other than the glomeruli.

Figure 43

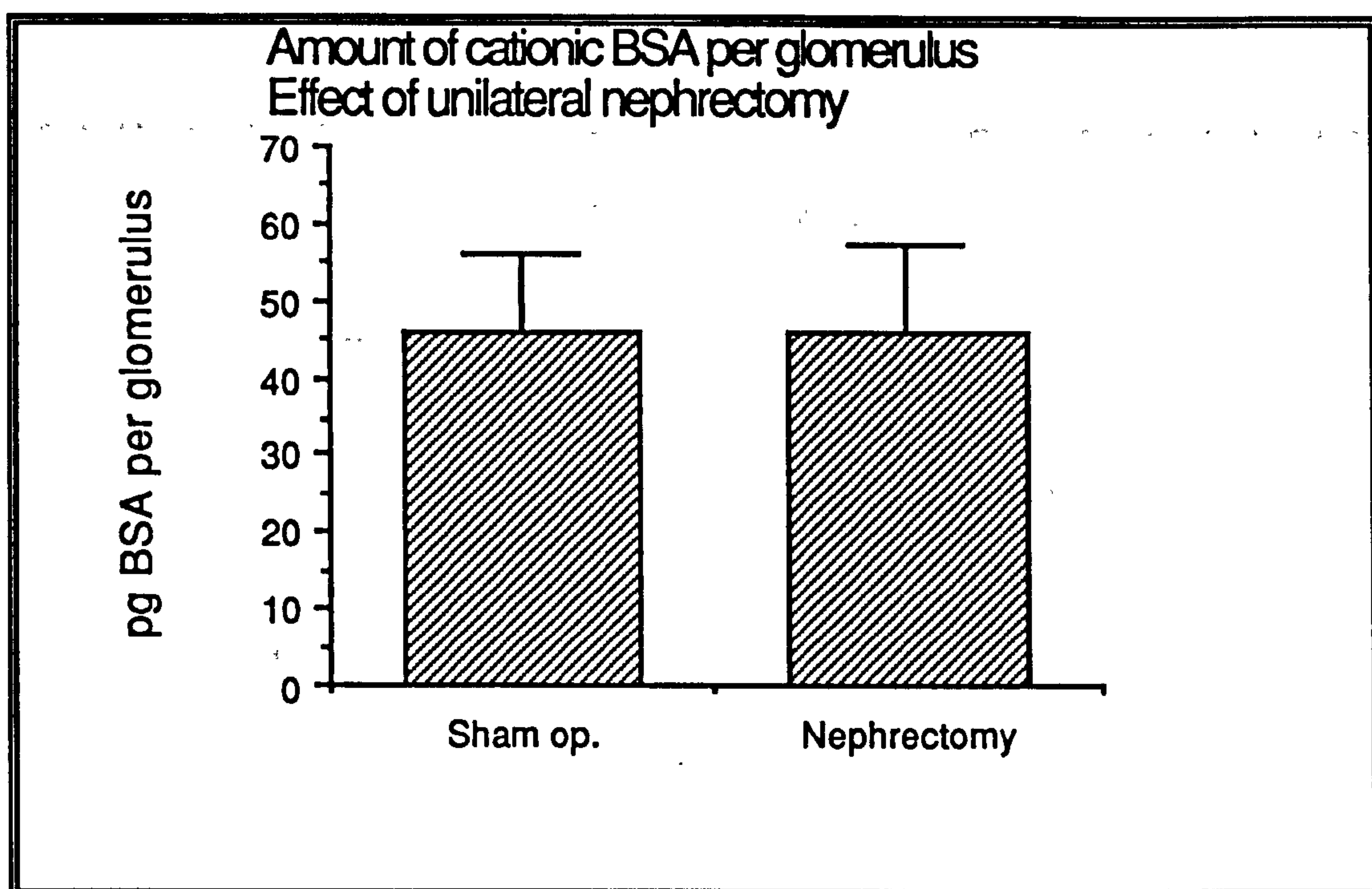


Figure 44

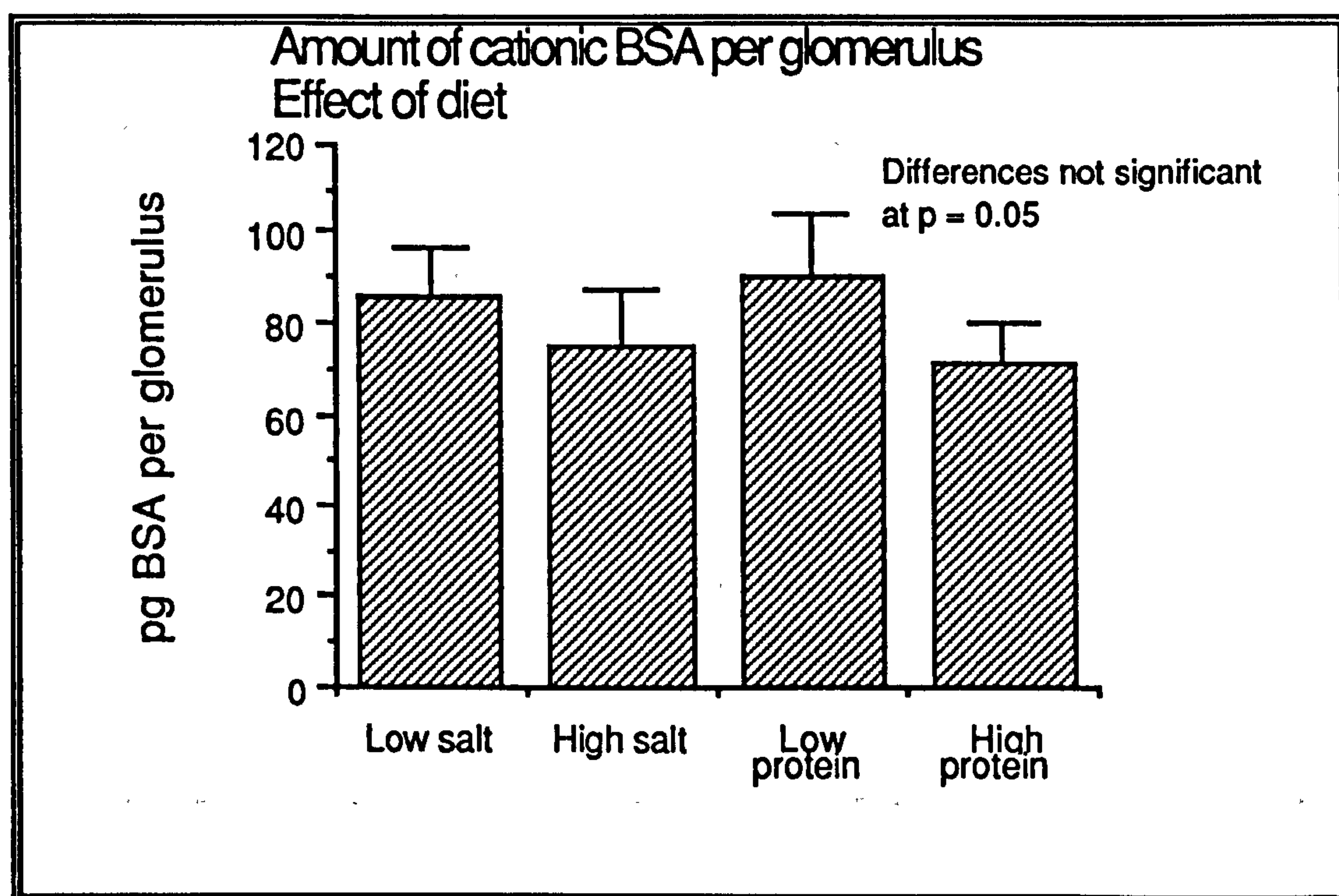


Figure 45

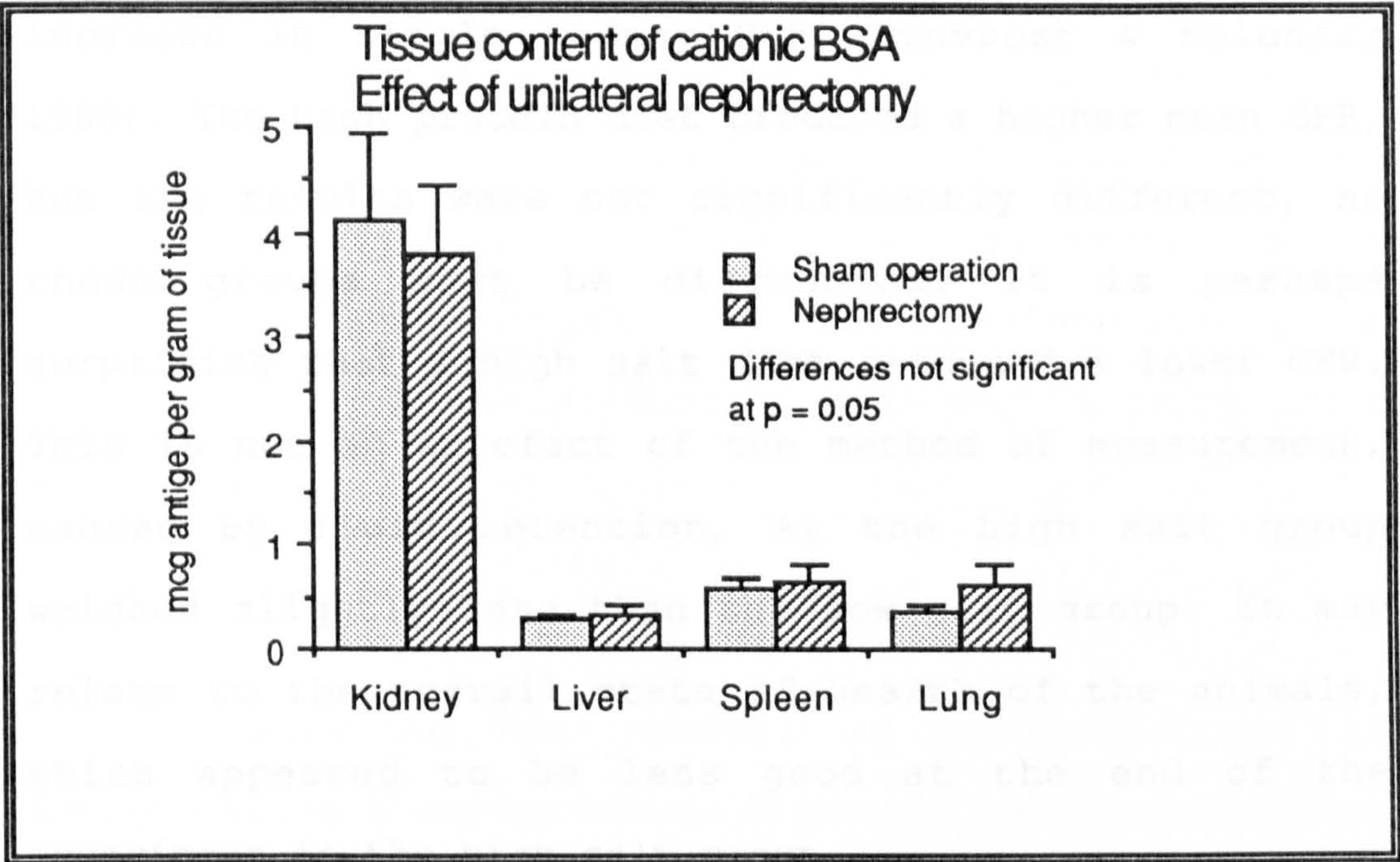
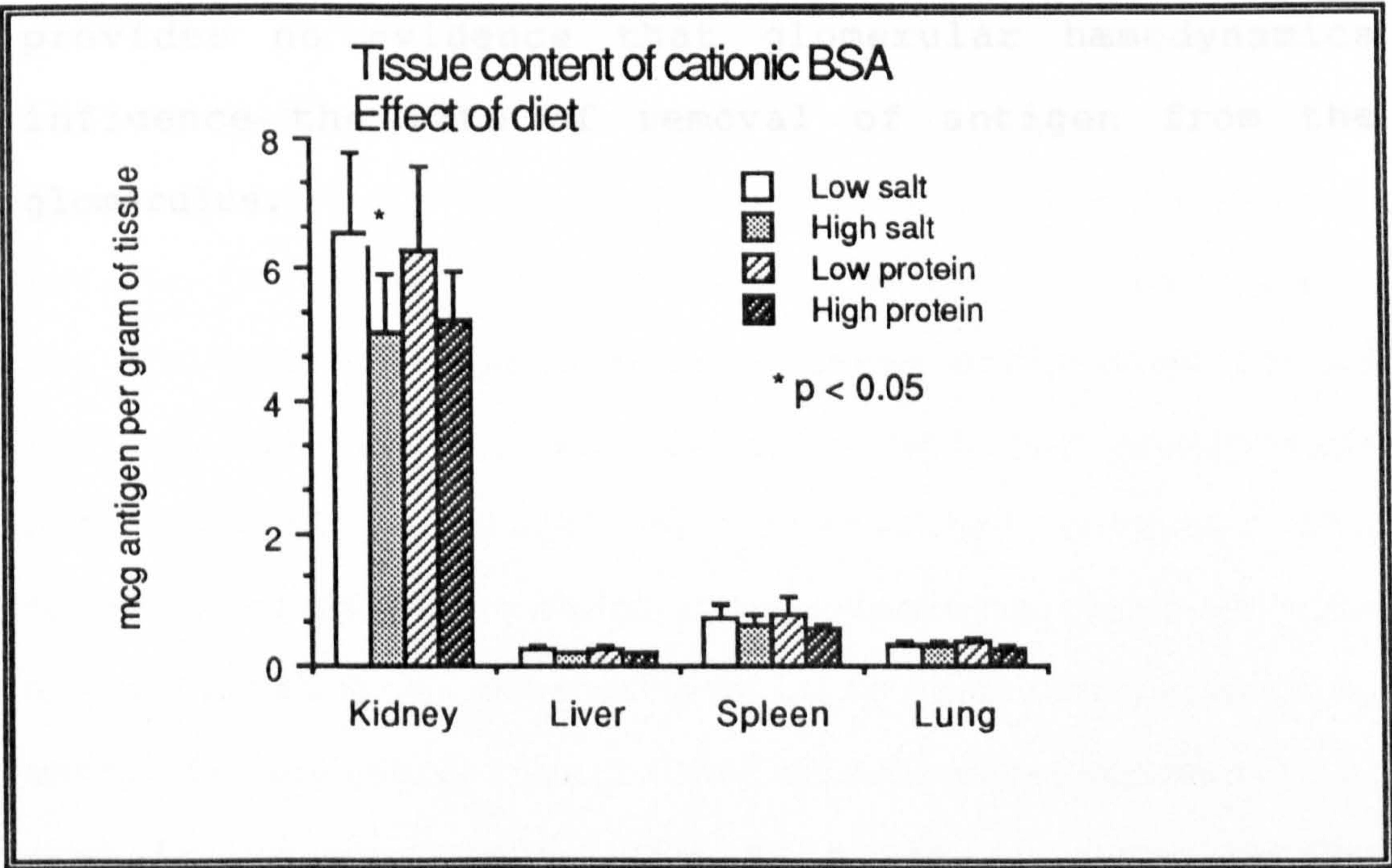


Figure 46



Conclusions

Unilateral nephrectomy produced the expected large increase in single kidney GFR (Provoost & Molenaar 1980). The high protein diet produced a higher mean GFR, but the results were not significantly different, so these groups must be discounted. It is perhaps surprising that a high salt diet produced a lower GFR. This is not an artefact of the method of measurement, caused by fluid retention, as the high salt group weighed slightly less than the low salt group. It may relate to the overall state of health of the animals, which appeared to be less good at the end of the experiment in the high salt group.

Within the range of alteration achieved, this study provides no evidence that glomerular haemodynamics influence the rate of removal of antigen from the glomerulus.

PART THREE

DISCUSSION

This work has attempted to elucidate some of the mechanisms involved in the removal of immune complex deposits from the glomeruli of rats with chronic serum sickness glomerulonephritis. It is appropriate to examine some of the problems which such a study must acknowledge.

In studying the removal of deposits, one is investigating a system which is poorly understood acting upon a material of unknown and variable composition. It is not necessary to stress here how little is known about the functional capabilities of the various cells of the glomerulus, but it is worth emphasizing the heterogeneous nature of the deposits. The causative antigen is rarely known in human disease, but even where the antigen is known, the associated antibodies are of varying classes and affinities. Complement components may bind by classical or alternative pathway. The proportions of these three main components vary; in some diseases, such as mesangiocapillary glomerulonephritis, antibody may occasionally be almost undetectable. The proportions may change with time, as is shown by the disproportionate changes in glomerular antigen content and deposit volume fraction during recovery from chronic

serum sickness reported in Chapter 8. In most glomerulonephritides no other major constituents of the deposits are known. This thesis has provided crude evidence that the proportional content of antigen in the deposits induced in this model is very small, despite large doses of antigen. The proportional content of antibody and complement is unknown, and it is likely that other constituents are incorporated into and modify the properties of the deposits. Other components of the plasma may be included. Epithelial, endothelial and intrinsic mesangial cells all synthesize basement membrane components, so it is likely that these substances will be enmeshed in the deposits as they grow. In the chronic serum sickness model described in this thesis it can be demonstrated that the anionic layer of the lamina rara externa re-forms over subepithelial deposits within four weeks of their formation (Furness & Turner 1987), and the manner in which the deposits of human membranous glomerulonephritis become surrounded by basement membrane material is well known. It is likely that such surface changes as these also influence the mechanisms which remove the deposits. Deposits at different sites in the glomerulus are likely to have different characteristics and are certainly exposed to different conditions which might cause their removal. These conditions will also vary in different diseases; it cannot be assumed that mechanisms which lead to deposit

removal in acute proliferative glomerulonephritis will be of significance in, say, membranous glomerulonephritis.

In view of these problems, what justification can be given for an attempt to elucidate the mechanisms of deposit removal in an artificial form of glomerulonephritis in the rat? I would propose the following arguments.

- 1) Despite the differences, the protean forms of immune-complex glomerulonephritis have many features in common, the most notable being the near-universal presence of antibody, complement and antigen (if identifiable), and the morphological similarities of the deposits formed in different diseases, as visualized by electron microscopy. These similarities suggest that though the diseases differ, similarities in the properties of the deposits may exist, and may even be necessary for their formation and persistence. This argument provides hope that features of an artificial model may be relevant to at least some forms of spontaneous human disease.
- 2) There are differences in the conditions at the various locations at which deposits may develop, but these locations number only three;

subepithelial, subendothelial and mesangial. Two of these are represented in the model used in this work, and the recognition that their properties may differ led to the attempt to distinguish between the two by morphometric analysis of their volume fractions. Subendothelial deposits are not seen in this model, but they are the least common form of deposit in human glomerulonephritis.

- 3) Differences in the conditions in the glomerulus in different forms of glomerulonephritis lie mainly in the cells infiltrating the glomerulus and in haemodynamic alterations. Differences in the composition of the plasma perfusing the glomerulus are probably slight. If a glomerulus is capable of recovery from an immunological insult, that is, if crescents have not formed and the glomerulus is not sclerosed, then subepithelial deposits (which are probably most relevant to glomerular dysfunction) are separated by the glomerular basement membrane from the infiltrating cells, and are in contact only with the podocytes and any material which can cross the filtration barrier. This point is valid irrespective of disease or species, and again argues for a wide applicability of any removal mechanism, at least in the subepithelial location.
- 4) The final argument is one of practical necessity. Most forms of human glomerulonephritis are not

susceptible to therapeutic intervention at present. Any improvement in our understanding of the natural mechanisms of deposit removal may permit the manipulation of those mechanisms to the benefit of patients with glomerulonephritis. Experiments with relevant animal models provide the best hope for gaining insight into such complex systems. The arguments that the model used here is appropriate for this purpose were proposed in Chapter 4.

The problem may be approached by considering what mechanisms could in theory be influencing the removal of deposits, then assessing the evidence for each. This is best done initially according to the location of the deposit in the glomerulus.

Mesangial deposits are exposed to all the soluble constituents of plasma, irrespective of molecular size. This is evident from the ease with which particulate tracers pass from the capillary into the mesangial channels (Farquhar & Palade 1961, Elema et al. 1976). For the same reason, soluble substances administered therapeutically are likely to gain ready access. Hemodynamic changes induced by the disease or by therapy may alter the rate of delivery of soluble material. Mesangial deposits are exposed to the intrinsic mesangial cells, which probably have some

phagocytic capacity (Sterzel et al. 1982) but appear to have a mainly structural and contractile role. The cells which are of most interest in this area are clearly the mesangial macrophages, which are normally present in small numbers (Schreiner et al. 1981, Schreiner & Unanue 1984, Gurner et al. 1986) and whose numbers and activity can be considerably enhanced by various forms of glomerular damage.

The situation of **subepithelial deposits** is clearly different. Soluble constituents of the plasma can only gain access by crossing the basement membrane. The fact that IgM can localize in this space (at least in diseased glomeruli) indicates that this is not as formidable a barrier as a study of normal glomerular function might suggest, but the rate of access of a relevant substance might be limited. The glomerular basement membrane is probably more important in limiting the access of cells. The podocytes are in intimate contact with subepithelial deposits, but there is no evidence that these highly specialized cells influence deposit removal. No other cell type has ready access to the subepithelial deposits unless the glomerulus is very severely damaged. One might suggest that the appearance of macrophages in crescents represents an ineffective attempt to remove the deposits; but as the development of crescents appears to relate to the presence of fibrin

in the urinary space (Silva et al. 1984) rather than the presence of deposits, this does not seem likely.

Subendothelial deposits are subject to conditions which resemble those of mesangial deposits: ready access to plasma constituents and macrophages. It has been suggested that the process of mesangial interposition seen in mesangiocapillary glomerulonephritis represents an attempt by the mesangial cells to reach and dissolve the deposits. In addition these deposits are exposed to the endothelial cells, and to circulating neutrophils.

A final possibility has received very little attention; the basement membrane itself. It is surely no coincidence that deposits at all locations (including most mesangial deposits) can be shown to be in apposition to a basement membrane. It has been suggested that these structures contain up to fifty separate components, of which only a handful have been isolated (Timpl et al. 1984). The properties of the basement membrane presumably influence the initial accumulation of deposits, but the possibility that some of the unknown constituents are involved in deposit removal has not been studied.

The evidence provided in this thesis will now be related to the theoretical possibilities.

Humoral agents

The three known constituents of the deposits in the model studied are all derived from the plasma, and these will be considered first.

Antigen. The Ouchterlony immunodiffusion plates indicated that antibody raised against cationic BSA recognized cationic or native BSA with a line of identity. The excess of native BSA given in the experiment described in Chapter 9 would therefore interact with anti-BSA antibody in the circulation or the glomerulus just as would cationic BSA, but without the tendency (at such high doses) to produce further glomerular deposition. The administration of excess antigen has been shown in a chronic mouse model of glomerulonephritis to enhance antigen removal (Haakenstad et al. 1983), and this effect was confirmed here. The mechanism is believed to involve disruption of the antigen-antibody lattice, as can be demonstrated by addition of excess antigen to immune complexes generated *in vitro*. Mannik and Striker (1980) confirmed that this can happen in an acute, passive serum sickness model which was not complicated by involvement of the host's own immune system. The apparent limitation of this effect to mesangial deposits (described in the above reports and in this thesis) may be due to limitation of

access of antigen to the subepithelial deposits, as an excess of cationic antigen has been claimed to remove acute subepithelial deposits (Agodoa & Mannik 1987). But is this lattice disruption theory necessarily relevant to the removal of long-standing deposits? Penner *et al.* (1979) found that antigen-mediated dissolution of deposits from tissue sections became less effective as the deposits became older. Haakenstad *et al.* (1983) reported a comparable effect *in vivo*. The deposits studied in this thesis were found to have an approximate mean deposit volume : antigen mass ratio of 100 : 1, which is presumably much higher than the pre-formed complexes of passive serum sickness. In the model studied here, the administration of a large antigen excess produces a considerable depletion of specific circulating antibody. The results described here indicate that this effect may provide an additional mechanism of enhancing deposit removal.

Antibody. The inability of cationic BSA to persist in the glomeruli of non-immune rats indicates that specific antibody is necessary for the initial formation of the deposits. The results described in Chapter 9 suggest that the presence of specific antibody also inhibits the removal of mesangial deposits; this is confirmed by the results of passive immunization in Chapter 10. The results suggest but do not prove a similar effect on subepithelial deposits; the negative

result is certainly not proven, as the slower natural rate of removal of the subepithelial deposits makes the morphometric detection of changes more difficult. In some respects these results are not surprising, as the deposits are generally believed to grow by surface accretion of material, including antibody. Goode *et al.* (1985) showed that if BSA-coated latex beads are caused to lodge in the mesangium, the administration of anti-BSA antibody inhibits their removal, a phenomenon which parallels the effect of antibody described here. Nevertheless it has been claimed, by analogy with the effect of excess antigen, that specific antibody enhances the removal of the deposits (Pusey *et al.* 1988). This is clearly not the case, but it is very different from the effect of antibody in other situations, where opsonization and enhanced removal of the antigen is the usual effect of the binding of antibody. The deleterious effect of specific antibody provides some theoretical justification for therapeutic attempts to reduce the titre in cases of human disease, but the benefits of the non-specific forms of immunosuppression which are currently available are doubtful in most forms of glomerulonephritis (Achiari-Rey & Pollack 1982, Glasscock 1982).

Complement. There is an extensive literature on the role of complement in the prevention of the formation of immune complexes, and the dissolution of

such complexes once formed. (Reviewed by Schifferli et al. 1986, Whaley 1987). The probable mechanism of these effects has been summarized in Chapter 1. The emphasis of research in this area has been on immune complexes *in vitro* and in the circulation, and an effect on immune complexes trapped in the tissues has been largely overlooked. The single exception is a report by Bartolotti and Peters (1978) that the removal of glomerular antigen by rabbits with acute serum sickness is inhibited if complement is depleted by cobra venom factor. My findings confirm this result, and also indicate that although the effect of de complementation is not immediate, after a few days the removal of antigen virtually ceases.¹ This implies that either the activity of complement is the only important mechanism by which deposits are removed, or other mechanisms are dependent upon the activity of complement. Removal of complement was the only form of intervention attempted which produced an unequivocal effect on subepithelial as well as mesangial deposits. As with complex dissolution *in vitro*, this effect probably only requires activation of complement components up to C3; depletion of C5 was recently reported to have no effect on the morphology of a comparable form of glomerulonephritis, though

¹This delay suggests that it is complement already bound to the deposit which is having this effect, rather than complement in the circulation.

assessment of deposit load was only semi-quantitative and C5 depletion was incomplete (Iida et al. 1987).

The action of complement in glomerulonephritis has previously been viewed by most authors as exclusively deleterious, causing proteinuria probably largely by the action of the membrane attack complex on the basement membrane and podocytes (Biesecker 1983, Couser et al. 1985, Perkinson et al. 1985). Proteinuria in the cationic BSA model studied here is also complement dependent, and complement is seen to be a 'two-edged sword': the rats given CVF during recovery had less severe proteinuria despite having more antigen and larger deposits in their glomeruli than the control group. A recent publication in this area suggests further involvement of complement. In a chronic serum sickness model in the mouse, Sawtell et al. (1988) found that animals given CVF could produce subendothelial but not subepithelial deposits. Mice with circulating C3 produced subepithelial deposits, whether or not C5 was present. The authors suggest that complement is necessary for the dissociation of subendothelial immune complexes (probably formed *in situ*) prior to translocation across the basement membrane and re-assembly at the subepithelial site. If dissolution of deposits was the only effect of complement then one might hope that the therapeutic administration of an excess of C3 (perhaps produced by recombinant DNA

techniques) might be beneficial in human glomerulonephritis; but the other functions of complement make this hope unlikely to be fulfilled, even if such a reagent became available.

Other constituents of plasma. There is little evidence to implicate any other normal constituent of plasma in the removal of deposits, and none was studied here. The effects of administration of proteases has been described in Chapter 3; the biological relevance of this remains in doubt, and the administration of protease inhibitors has been claimed to reduce glomerular damage but have no effect on the glomerular deposits (Jennette et al. 1987). C-reactive protein has recently been claimed to be involved in the elimination of DNA from the circulation (Robey et al. 1985); there is no evidence that C-reactive protein is involved in deposit removal. This question was not addressed in this thesis, but since an acute phase response would presumably have been induced by several of the manoeuvres employed, notably the administration of Freund's complete adjuvant, it is probably not relevant.

Other soluble agents. The only attempt made in this thesis to influence deposit removal by the direct action of a foreign agent is described in Chapter 13, relating to the effect of charged molecules. The failure of high doses of polyethyleneimine to have a detectable effect supports the contention of Gauthier and Mannik

(1986) that electrical charge is not involved in the persistence of deposits beyond the first few hours after deposition. The apparent effect of heparin in enhancing the removal of glomerular antigen remains difficult to explain. This was the only experiment where an effect on the glomerulus antigen load was not paralleled by a significant effect on the deposit volume fraction. The result might therefore be called into question, but it is supported by the finding of a significant decrease in the antigen content of whole renal cortex in the same group. Heparin is a heavily sulphated mucopolysaccharide. It is strongly anionic, a strong acid. Its biological effects are numerous and most are poorly understood. In addition to its anticoagulant properties it facilitates the breakdown of chylomicra by activation of lipoprotein lipase; an action of potential relevance to the nephrotic state but without obvious relevance to the removal of immune complexes. Heparin inhibits the aggregation of platelets and has a slight vasodilator effect (O'Reilly 1980). *In vitro* it inhibits the action of complement, but such an action *in vivo* would be expected to inhibit deposit removal rather than enhance it. The natural function of heparin is not known; it is limited in distribution to the mast cells and is absent in some species (e.g. the rabbit). However it has close structural homology with the heparan sulphate proteoglycan of the basement membrane; it is even more highly sulphated (Hovingh et al. 1986). This

implies a potential interaction between heparin, the basement membrane and the deposits. If this effect is genuine, its mechanism is unknown, but it could have therapeutic potential and is clearly worth further investigation.

Hæmodynamics. Hæmodynamic alterations are known to influence the localization of immune complexes in the glomerulus, and it seemed possible that the rate of delivery of components of the plasma might influence the rate of deposit removal. Attempts were made to influence glomerular hæmodynamics by unilateral nephrectomy and modulation of salt and protein intake. The glomerular filtration rate was chosen to confirm that changes were being induced; this is easily measurable, though it is perhaps of more relevance to subepithelial deposits than mesangial ones. Unilateral nephrectomy produced the greatest rise in single kidney GFR, but none of the methods applied produced any detectable change in the rate of deposit removal. Of course, it remains a possibility that larger alterations might have an effect; to take the extreme case, a complete cessation of perfusion could be expected to stop antigen removal, but the use of unilateral nephrectomy did produce a considerable alteration in GFR without altering antigen removal. It therefore seems unlikely that the changes in GFR seen in response to physiological stimuli would have any effect. The possibility that an extreme fall in

perfusion and GFR caused by severe glomerulonephritis itself might inhibit antigen removal has not been addressed.

The importance of one other potential barrier to access was investigated by the administration of puromycin aminonucleoside. This agent increased the permeability of the filtration barrier (Table 6) and therefore presumably improved access of macromolecules to the subepithelial deposits. The extent of this improvement was not quantified, but the absence of any detectable alteration in deposit or antigen removal suggests that the permeability of the glomerular basement membrane is not rate-limiting.

Degradation by cells

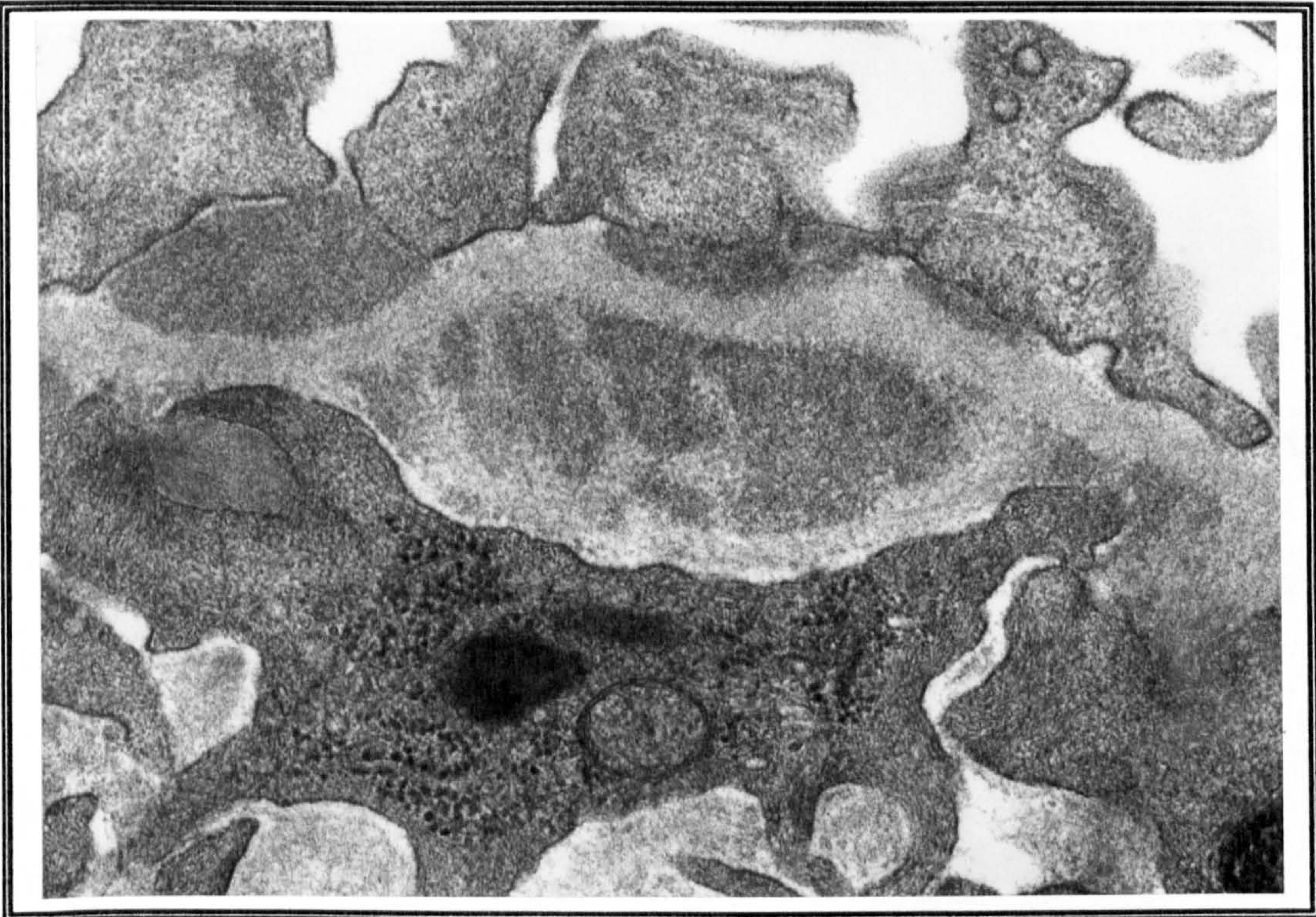
It has already been stressed that the cell population available to degrade deposits depends on the location of the deposits. There is widespread belief that in the mesangium, cells of the macrophage lineage are responsible for removal of the deposits. The apparent importance of complement in the removal of the deposits does not disprove this, as complement might be

necessary for opsonization.¹ If this is so, one might expect mesangial deposits to be removed more rapidly than subepithelial ones, and this has been shown to be the case. The number and activity of glomerular macrophages increases in many forms of glomerulonephritis. Macrophages have been seen having phagocytosed material resembling deposits in an acute passive serum sickness model (Striker et al. 1979), but there is evidence in the literature which casts doubt on the importance of macrophages in the removal of deposits which have become established in the glomerulus over a period of days or longer. Most notable is the failure of anti-macrophage serum to cause an increase in the amount of antigen left in the glomerulus (Lavelle et al. 1981, Holdsworth et al. 1981) although in these experiments the antiserum was administered to assess its effect on glomerular damage, and the protocols were not ideally suited to detect an effect on deposit removal. Stimulation of the mononuclear phagocyte system with *Corynebacterium parvum* (Barcelli et al. 1981) or, as reported here, with Freund's complete adjuvant, will diminish the glomerular antigen load if performed while antigen is still being deposited. If performed during recovery this effect is not seen, indicating that the stimulated phagocytes reduce the delivery of antigen to

¹Such a requirement would be strikingly at variance with the ability of macrophages to phagocytose immune complexes in other locations.

the glomerulus, but do not enhance its removal. The failure of PVA and puromycin to enhance removal also suggests that glomerular macrophages do not have an important role in this respect. I have seen no convincing morphological evidence of phagocytosis of deposits by macrophages in the electron microscopic studies described in this thesis; on the contrary, after two weeks without antigen administration the mesangial deposits often appear to be fragmenting, as if dissolving *in situ* (Fig. 47). Unfortunately it is difficult to prove the negative. It will be necessary to repeat studies of the type described here with complete elimination of glomerular macrophages. Suppression of macrophages by irradiation will probably not be appropriate because of concurrent immunosuppression and direct damage to the kidneys. Administration of anti-macrophage serum perhaps provides the best hope of achieving this goal, but the preparations available commercially are not cytotoxic and production of a suitable antiserum will be difficult.

Figure 47. A typical mesangial deposit after two weeks of recovery, showing irregular, fragmented outlines and variation in density. There is no evidence of phagocytosis. Compare this with the firm outlines of the smaller subepithelial deposits above the basement membrane. x 45,000



In concentrating on the mesangial macrophages one should not forget the more numerous intrinsic mesangial cells. It does not seem likely that these cells are involved, but it is difficult to assess their role as there is no tool reliably to enhance, inhibit or measure this activity. These cells can be damaged by the administration of anti-Thy.1 antibody (Bagchus et al. 1986) but the structural changes induced are so massive as to render meaningless any data on this role of mesangial cells.

The discussion of degradation by cells has so far considered only cells in the mesangium. These are clearly irrelevant to the removal of subepithelial deposits, which are in a space where the only cell type is the podocyte, an epithelial cell not known to have any capacity to degrade immune complex deposits. One may nevertheless consider the possibility of involvement of these cells. Subepithelial deposits are invariably covered by a sheet of podocyte cytoplasm. The deposits appear to influence the podocytes, causing foot process effacement and in some circumstances synthesis of an excess of basement membrane material. Phagocytosis is not seen, but could the podocytes influence deposit removal at the surface? Rearrangement of immune complexes on the podocyte surface is seen in Heymann nephritis (Kerjaschki & Farquhar 1983, Camussi et al. 1985, Cybulski et al. 1988), but perhaps the persistence of the deposits in this model is evidence of the inability of the podocytes to remove them. The evidence presented here of the lack of an effect of puromycin aminonucleoside, which appears to be toxic to podocytes, is also indirect evidence against their involvement. What then is the function of the complement receptor CR1 on the surface of the podocytes (Gelfand et al. 1977, Shin et al. 1977, Burkholder et al. 1977)? It was originally suggested that its presence enhances the accumulation of subepithelial deposits by binding complement (Gelfand et al. 1977), but this detrimental

effect, even if true, does not provide any biological justification for the synthesis of these receptors at this site. The importance of complement in removing subepithelial deposits and the known functions of other CR1-bearing cells in disposing of immune complexes suggests a similar role for podocytes, but is far from conclusive. CR1 also stimulates the degradation of the active forms C3b (Medof et al. 1982) and C4b (Medof et al. 1984), which might limit the destructive effects of complement activation at this site. The absence of CR1 on the podocytes of laboratory animals (Moran et al. 1977) suggests that its presence in man is perhaps not of vital importance, but CR1 has been reported to be present on human podocytes at a greater density than on any other cell type (Hogg et al. 1984), and the possibility that CR1 is induced in other species in the presence of immune complex disease does not appear to have been investigated. It would also be of considerable interest to measure the rate of breakdown of pre-formed immune complexes by cultured human podocytes in the presence or absence of complement.

CONCLUDING REMARKS

This thesis has provided evidence that the immune complex deposits formed in the cationic BSA model of chronic serum sickness glomerulonephritis are removed mainly by a physico-chemical process of dissolution *in situ*. This process is largely dependent on the presence of complement but is inhibited by specific antibody, which is also essential for initial deposition. No evidence could be found of the direct involvement of any of the various cell types of the glomerulus. Tentative evidence is offered that the administration of heparin enhances the removal of deposits; the mechanism of this effect is unclear.

Further investigation is required to elucidate the role of complement and in particular the involvement of CR1 on the glomerular epithelial cells. It will be necessary to confirm the effect of heparin tentatively identified here and to investigate its mechanism. This work has also provided a format by which any existing or new therapeutic substance may be tested for an effect on deposit removal. An improved understanding of the mechanism by which deposits are removed provides a potential avenue of therapy for the numerous forms of human glomerulonephritis which do not at present respond adequately to treatment.

APPENDICES

APPENDIX 1

Preparation of cationic BSA

The difficulty of toxicity encountered in the preparation of the antigen has been described in Chapter 4. The published protocols (Hoare & Koshland 1967, Border et al 1981, 1982) in my hands resulted in a product with an extremely high pI (>11) which was toxic. The following protocol results in a more modest cationization, and a product with a pI which is usually between 8 and 10.

All equipment was sterilized before use.

100ml of water was added to 15ml of ethylenediamine (Sigma) in a large beaker with stirring and cooled to 25°C. Hydrochloric acid was added to pH 5.5 and the mixture cooled again to 25°C. 5g of BSA (dissolved in a minimum of water) and 0.5g of 1-ethyl-3(3-dimethylaminopropyl) carbodiimide (Sigma) was then added as a dry powder, with stirring. The reagents were maintained at pH 5.5 and 25°C for one hour, then the reaction was stopped by adding 30ml of 4M acetate buffer, pH 5.5. The liquid was passed through a 1.2µm filter into dialysis tubing and dialysed against distilled water for 48 hours at 4°C with frequent changes. Excess water was extracted

using Aquacide III (Calbiochem) to a volume of about 50ml. This concentrate was divided into vials (of known weight) for lyophilization and stored under vacuum at -20°C .

APPENDIX 2Isoelectric focussing

The pI of each sample of cationic BSA was checked by isoelectric focussing on modified thin cellulose acetate membranes as described by Ambler & Walker (1979), with the assistance of Dr Ambler in the Department of Clinical Chemistry, University Hospital, Nottingham. Briefly, thin cellulose acetate membranes ('Cellogel', Whatman) were methylated by immersion in boron trifluoride in methanol. The modified membrane was soaked in a 50-80g/L solution of ampholines (pI 2-12; Serva) with 5% glycerol. It was placed in a Shandon electrophoresis tank with tap-water cooling and moist chamber, and run at 600V for 90 minutes. Samples (0.5µl) were applied 10 minutes after separation started, using a double wire applicator. After separation the membrane was fixed in 5% trichloroacetic acid, washed and stained with Coomassie Blue R. A mixture of protein markers of known pI was included in each run to permit quantitation of the changes in pI.

APPENDIX 3Ultracentrifugation

The method is modified from that of Martin and Ames (1961).

Preparation of gradients

250ml of 37.5% sucrose in gelatin-veronal buffer was prepared and dilutions made as follows:

Final strength (%)	ml 35% sucrose	ml buffer
12.5	25	50
18.5	37	38
25	50	25
31	62	13
37.5	75	0

The solutions were cooled to 4°C then 0.8ml of each was layered by hand into Beckman polyallomer ultracentrifuge tubes, starting with the most dense. Equilibration was allowed to proceed for 1 hour then 100μl of the sample was layered on to the surface, followed by 300μl of liquid paraffin to minimize surface vibration. The tubes were spun for 6 hours at 4°C in a Sorvall OTD-65 ultracentrifuge in an AH650 head. The bottom of each tube was then pierced and each sample collected into 17 fractions for gamma counting.

APPENDIX 4

Radioiodination: Chloramine T method

Reagents

^{125}I : 74MBq, pH 7-11. (Amersham)

Protein solution, 50mg/ml, in 0.05M phosphate buffer

Sodium metabisulphite, 1.2mg/ml, in 0.05M phosphate buffer, pH7.2

Potassium iodide solution, 1mg/ml, in 0.05M phosphate buffer, pH7.2

Chloramine T solution, 5mg/ml, in 0.05M phosphate buffer, pH7.2

Protocol

20 μl of 0.5M phosphate buffer, pH7.2 was added to the ^{125}I , mixed, and 20 μl of protein solution was added followed by 20 μl of Chloramine T. The reaction was allowed to proceed for two minutes. 200 μl of sodium metabisulphite solution was added to stop the reaction. Two minutes later, 620 μl of potassium iodide solution and 100 μl of protein solution was added, to make up to 1 ml. 10 μl was removed to subsequently assess labeling efficiency and the rest was dialysed against phosphate buffered saline for 24 hours. A further 10 μl sample was removed. Both samples were counted, then the protein in these samples was precipitated with trichloroacetic acid

and the precipitate was re-counted, permitting calculation of the efficiency and loss at dialysis.

The labeled protein was added to sufficient unlabeled protein (at 100mg/ml in saline) to complete the experiment. It was mixed thoroughly and duplicate 10 μ l samples were taken for counting to measure the specific activity in cpm/mg of the antigen as injected. The remainder was separated into aliquots and stored at -70°C until use.

APPENDIX 5

Light Microscopy

Paraffin sections. Tissue samples were fixed in 10% buffered formalin, placed in TissueTek® cassettes and processed to paraffin wax in a TissueTek® VIP automatic tissue processor. Sections 5µm thick were cut on a Leitz sledge microtome and stained conventionally with Hæmatoxylin and Eosin.

Frozen sections. Fresh tissue was mounted on cork discs in OCT embedding medium (TissueTek®) and frozen by immersion in freezing isopentane (-170°C). Samples were stored where necessary over liquid nitrogen. Sections were cut using a Slee cryostat.

Sections were examined using a conventional Leitz SM Lux microscope.

APPENDIX 6Transmission electron microscopy

Tissue samples were cut into cubes approximately 1mm across and fixed for a minimum of 4 hours in 0.25% glutaraldehyde (TAAB) in 0.1M cacodylate buffer, pH7.4, adjusted to 300mOsm. with sucrose. They were then washed in buffer and post-fixed in 1% aqueous osmium tetroxide for 1½ hours, dehydrated through six grades of alcohol/water, infiltrated with EMIX® resin (EMSCOPE) and polymerized at 60°C for 16 hours.

Preliminary 0.5µm sections were cut with 25mm glass knives on a Reichert ultracut E ultramicrotome and stained on glass slides with 1% toluidine blue. After selection of an appropriate area the block was trimmed to size and 80nm sections were cut, mounted on 200 mesh copper grids, flattened with chloroform vapour and stained with uranyl acetate (Stempak & Ward 1964) and lead citrate (Reynolds 1963). The grids were examined in a JEOL 1200EX electron microscope at 80KV. Photographs were taken using Kodak 4489 electron microscope film and printed on Agfa Rapitone paper.

APPENDIX 7Ouchterlony diffusion plates

Plates were prepared by pouring 3.5ml of 1% agarose in barbitone buffer pH 8.5 into 5cm diameter Petri dishes. After the gel had set, holes were punched 0.8cm apart, 0.5cm in diameter, using a template and cutter. The wells created were filled with serum or antigen solutions and left in a moist chamber at room temperature overnight. The plates were viewed by dark ground illumination.

APPENDIX 8

Autoradiography

The dipping film technique was used throughout, with Ilford K5 emulsion. Sections were prepared on slides coated with chrome gelatin. Blank slides and slides bearing non-radioactive rat kidney were used as controls.

Equipment

Coplin jar

50ml measuring cylinder

Glass stirring rod

Slides (at least 6 from each block, in a plastic rack)

Water bath & thermometer

Sterile plastic forceps

Light-proof boxes

Silica gel bags (dry)

Ice tray and cooling plate

Paper tissues

Protocol

Glassware was cleaned in 5% chromic acid overnight, with thorough rinsing and a final rinse in distilled water. The water bath was pre-heated to 40°C, with the Coplin jar and measuring cylinder in, and 20ml of water in the

measuring cylinder. The sections were thoroughly dewaxed in xylene and graded alcohols, then allowed to dry.

Under appropriate illumination, emulsion shreds (20ml) were added to the water in the measuring cylinder, stirred and poured into the Coplin jar. This was stirred gently for about 15 minutes in the dark, avoiding bubbles, until dipping a blank slide produced an even emulsion. The section slides were then dipped, allowed to drain for 30 seconds, wiped on the back and placed on the cold plate for 5 minutes to set. They were then placed in light-proof boxes with silica gel bags to dry at 4°C. The bags were changed after 24 hours, then left until developing. In each batch a small number of sections were fogged deliberately by exposure to light, as a control against negative chemography.

Developing

Slides were developed at intervals in Ilford Phenisol, diluted 1:4 with distilled water, at 20°C for 5 minutes in the dark. They were transferred to a 0.5% acetic acid stop bath for 30 seconds, then to Ilford IF23 fixer, diluted to 30%, for twice the time taken to clear the slides (about 30 seconds). They were washed for 30 minutes in distilled water, dried and stained lightly with Toluidine blue.

APPENDIX 9

Protein estimation in urine

Urine samples were centrifuged to remove sediment. 0.5ml was mixed with 3.5ml of 3% sulphosalicylic acid in a clean glass test tube, and placed in a calibrated EEL nephelometer. If the reading was over 100 the sample was diluted and the test repeated.

A calibration curve for this protocol was constructed by dilution of a sample of rat plasma of known total protein concentration, to provide a range of 0.01 to 1 mg/ml.

APPENDIX 10Scanning electron microscopy

Tissue was fixed using Karnovsky's fixative (5% glutaraldehyde, 20% paraformaldehyde) using the surface fixation method described in Chapter 6. After a minimum of 4 hours of fixation the superficial cortex was removed with a scalpel to a depth of approximately 0.5mm, to expose surface fixed glomeruli. The sample was trimmed, washed in distilled water and dehydrated through graded alcohols with ultrasonication (Mettler electronics) to clean the surface. It was subjected to critical point drying under carbon dioxide using a Samdri pvt-3 and mounted on 1cm aluminium stubs using colloidal graphite in isopropanol (TAAB). After drying, this assembly was placed in a Balzers SCD 030 sputter coater and coated with gold in an atmosphere of argon.

The samples were examined in a JEOL JSM-35 scanning electron microscope at 20KV. Photographs were taken using Ilford Pan F 120 film and printed on Agfa Rapitone paper.

APPENDIX 11ELISA method for anti-BSA antibody

The assay was performed using Titertek 280µl flat-bottomed PVC microplates. 0.01M phosphate buffered saline, pH7.2, with 0.1% Tween was used unless specified otherwise.

The wells were coated with BSA by soaking in 1% BSA in buffer for 1 hour. They were washed three times with buffer and allowed to dry.

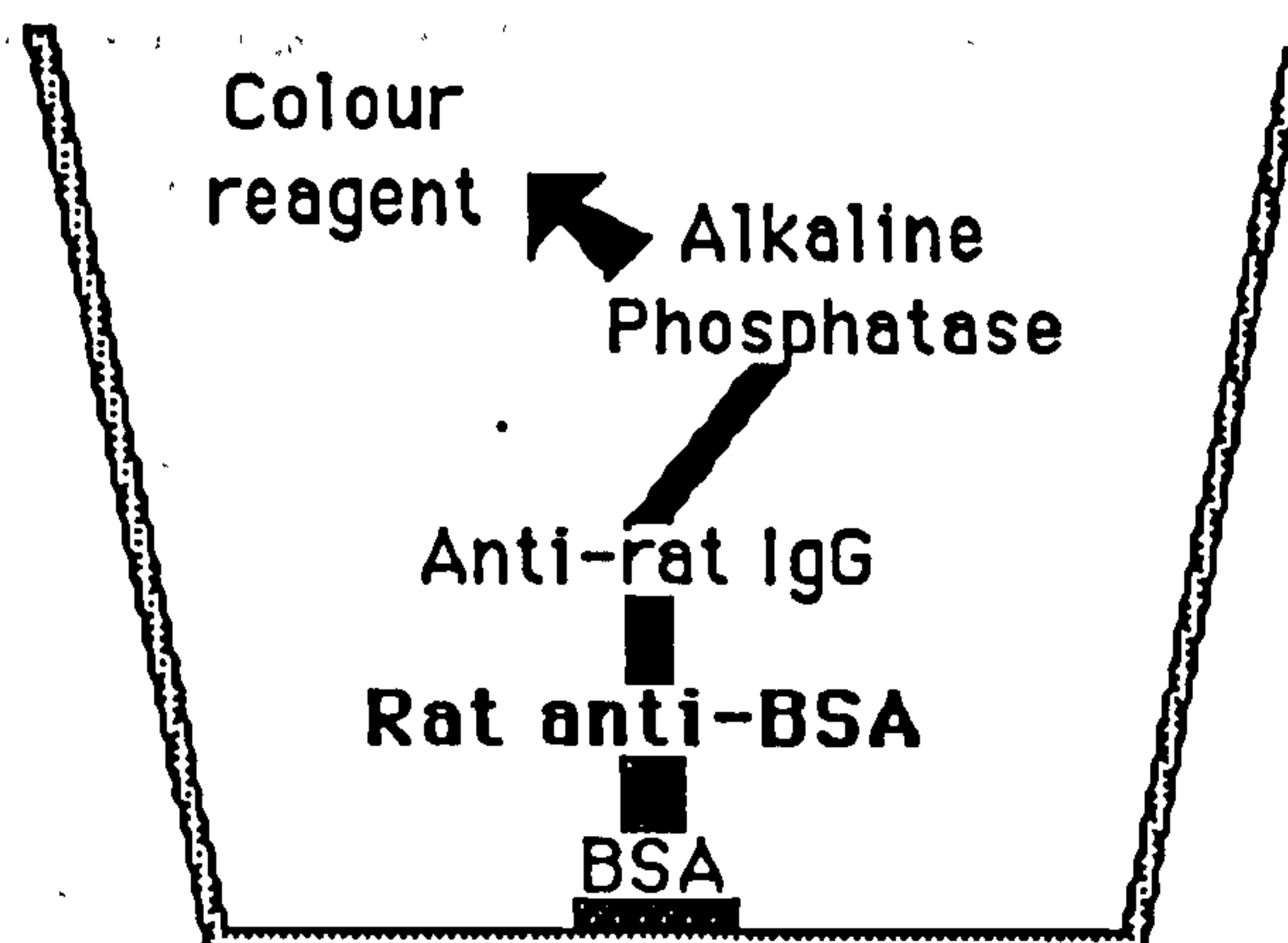
Serum samples were added, initially at a dilution of 1:1000, 200µl per well and incubated at 37°C for 1 hour, washed three times and allowed to dry.

Alkaline phosphatase-conjugated anti-rat IgG was purchased from Sigma and applied at a dilution of 1:10⁶, 200µl per well, incubated at 37°C for 1 hour, washed three times and allowed to dry.

Alkaline phosphatase colour substrate (p-Nitrophenyl phosphate disodium; Sigma 104 phosphatase substrate) was added at a concentration of 1mg/ml, 200µl per well, in carbonate buffer, pH 9.0. This was incubated until colour production was obvious (about 30 minutes). The reaction was stopped by adding 50µl of 3M sodium hydroxide to each well, and the colour was read in a Titertek multiscan plate reader.

If rat anti-BSA was present in the serum, the resultant 'sandwich' is shown in Figure 1A.

Figure 1A



The results were recorded as arbitrary 'optical density' units, because a sample of rat anti-BSA of known concentration was not available for use as a standard. A single sample of serum with a high titre of anti-BSA antibody was diluted up to 20-fold and replicate assays performed. This confirmed the linearity of the assay over the range studied, justifying the comparison of readings between samples on a single plate. Controls included the use of non-immune rat serum and the omission of each of the reagent steps.

APPENDIX 12

Isolation of a gamma globulin fraction by salt precipitation

Aseptic precautions were observed as far as was practical. A saturated solution of ammonium sulphate was prepared by heating with stirring, then cooling overnight. The pH was adjusted to 7.2. This solution was added to the serum, drop by drop with vigorous stirring, to a concentration of 82ml of ammonium sulphate to 100 ml of serum. This was allowed to stand at room temperature overnight. The precipitate was washed twice in 45% saturated ammonium sulphate solution, then re-dissolved in a minimum volume of saline and dialysed for 24 hours against distilled water at 4°C. The product was lyophilized and stored at -20°C. Reconstituted samples were passed through a 0.2µm filter before administration to animals.

APPENDIX 13Hæmolytic plate assay for complement

All solutions were prepared in complement fixation diluent (CFD: Flow laboratories).

Sheep red blood cells were washed three times and a 10% suspension prepared. 2ml of this was mixed with 2ml of a 1:500 dilution of rabbit hæmolytic serum (Wellcome) and incubated at 4°C for 15 minutes. The cells were washed three more times and diluted to a 1% suspension.

2% agarose was melted then cooled to 52°C in a water bath. The temperature of 4.5ml of the sensitized cells was also raised to 52°C then mixed with 4.5ml of the agarose. 3ml aliquots were immediately poured into three level 5cm Petri dished and allowed to cool. Holes 0.3cm in diameter were cut using a template and cutter and the plates were stored at 4°C for a maximum of four days before use.

Samples of serum were added to fill the wells. The plates were kept at 4°C overnight in a moist chamber to allow diffusion of complement components into the gel, then developed for 1 hour at 37°C. Each plate included at least one sample of normal rat serum as control.

APPENDIX 14

Colloidal iron method for anionic sites

200-300ml of distilled water was brought to a vigorous boil and 10ml of a 30% solution of ferric chloride was added rapidly. The resultant dark red liquid was cooled and dialysed for 24 hours against distilled water. 5ml of this stock solution was diluted before use with 9ml of distilled water and 6ml of glacial acetic acid, resulting in a pH of about 1.8.

The working solution was applied to de-waxed paraffin sections for 5 minutes, then washed in 5% acetic acid. The localization of the cationic colloidal iron was visualized by Perl's stain (2% potassium ferrocyanide).

APPENDIX 15Polyethyleneimine method for anionic sites

This method was adapted from Schurer et al. (1978). All solutions were used at 4°C.

Tissue was lightly fixed in 0.5% glutaraldehyde for 1 hour, then washed in three changes of cacodylate buffer, pH 7.3.

The samples were then immersed in a 0.5% solution of low molecular weight (1,800) polyethyleneimine (PEI) in saline, pH 7.3, for 30 minutes. Some of the animals described in Chapter 13 had already received systemic high molecular weight PEI (40,000-60,000); this step was omitted in these cases.

The tissue was washed three times in buffer again then immersed in 2% phosphotungstic acid in cacodylate buffer, with the pH corrected to 7.3, for 1 hour. Washing was again performed three times and the tissue was processed routinely for transmission electron microscopy as described in Appendix 6, though the times in uranyl acetate and lead citrate were reduced to enhance the contrast of the PEI staining.

Appendix 16

Determination of glomerular filtration rate using $^{51}\text{Cr-EDTA}$

This method is essentially that described and characterized by Layzell & Miller (1975), who demonstrated a close correlation between the results gained by this method and the conventional inulin clearance technique. $^{51}\text{Cr-EDTA}$ was obtained from Amersham. Rats were lightly anaesthetized (0.1ml Hypnorm® i.m.) to facilitate an accurate intravenous injection of $^{51}\text{Cr-EDTA}$ (0.1ml: 10 μCi , 740KBq). The time was noted and the animal weighed. After an accurately timed interval of about one hour the animal was heavily anaesthetized (0.2ml Hypnorm®, 0.2ml diazepam), the abdomen opened and a blood sample taken from the inferior vena cava into a heparinized tube. The usual procedure of tissue sampling was then performed as described in Chapter 7.

The clearance of $^{51}\text{Cr-EDTA}$ was then calculated using the formula derived by Layzell and Miller:

$$C = V \ln (P_0/P_t) / t$$

where C = the clearance in ml/min

V = the distribution volume of $^{51}\text{Cr-EDTA}$

P_0 = the plasma concentration of isotope at time zero, assuming instant homogeneous mixing. This is calculated as $P_0 = I/V$, where I is the initial dose.

P_t = the plasma concentration of isotope at time t , determined by direct measurement.

t = the time of sampling in minutes.

To gain an accurate absolute value of GFR the volume of distribution V should be determined experimentally, using $V = I/P_{\text{stable}}$, where I is the initial dose and P_{stable} is the steady-state concentration of isotope reached in rats with bilateral nephrectomy. As the aim of this study was to compare groups this experiment was not performed, but the figure of 25% of body weight was taken from the literature ($24.3\% \pm 0.9\%$, Layzell & Miller 1975; $26.9\% \pm 1.7\%$, Bryan et al. 1972). The values of GFR obtained should therefore not be assumed to be 'correct', but any error applies equally to all groups and the data is adequate for comparison between groups.

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