GLOMERULAR SELECTIVE PERMEABILITY TO PROTEIN, DEXTRAN, AND POLYVINYLPYRROLIDONE IN HEALTH AND DISEASE.

by

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SUMMARY

This thesis presents the results of six year's work on macromolecular clearance determinations.

This work is placed in context by an introductory review of literature relating to the physiology of protein excretion. Early concepts of the mechanisms involved in proteinuria are discussed. Among the studies reviewed are observations on micropuncture of the glomeruli and proximal tubules, clearance experiments, stop-flow analyses, histochemical findings, and electron microscopy studies. It is concluded from this review of the literature that some protein is filtered at the glomeruli, but that in health virtually all of this is reabsorbed by a process which, for albumin and molecules larger than albumin, is non-selective. Reasons are given for the opinion that tubular secretion of protein is unimportant.

The immunodiffusion method used in protein clearance studies is then described, along with the gel filtration technique used to determine protein, dextran, and polyvinylpyrrolidone clearances. The errors involved in these techniques are discussed.

Results are presented for protein clearance studies carried out in parallel by the two techniques. From an analysis of these results it is concluded that in patients excreting over 1.0G of protein daily, -K (the index of protein selectivity by the immunodiffusion technique) is related to $\triangle$ (the index of selectivity by the gel filtration technique) by the formula $\triangle = 0.73\ (-K)$.

Dextran and protein selectivity values are compared in normal subjects, in proteinuria induced by plasma infusion, and in a

variety of disease states. In minimal lesion glomerulonephritis, membranous glomerulonephritis and in induced proteinuria, dextran and protein selectivity values are in substantial agreement. In normal subjects under normal conditions, in proliferative glomerulonephritis, in postural proteinuria, and in acute ischaemic renal failure, dextran selectivity values are consistently and considerably higher than protein selectivity values. These findings are explained in terms of differences in the renal handling of protein and dextran and in terms of differences in the mechanisms involved in proteinuria in the various conditions described. From the experiments on induced proteinuria it is concluded that the filtration of protein at the normal glomerulus, like that of dextran, is highly selective.

The results of protein selectivity determinations in 207 patients with major proteinuria are presented and related to the histological diagnoses. Selectivity values in minimal lesion glomerulonephritis are consistently high, while in patients with renal failure proteinuria is uniformly unselective.

The relationship between protein selectivity and prognosis is assessed in 197 patients. A very high selectivity (-K over 2.6) is associated with a very good prognosis (3 year survival with functioning kidneys of 96%). A very low selectivity (-K less than 1.4) is associated with a poor prognosis (3 year survival of 27%).

The relationship between selectivity and responsiveness to steroid therapy is assessed in 82 treated patients. In our experience, a selectivity value of 2.0 or less is consistently

associated with unresponsiveness to steroid therapy. While prompt abolition of proteinuria following steroid therapy is to be expected only in patients with minimal lesion glomerulonephritis, comparison of the outcome at 8 weeks in 30 treated proliferatives with findings at 8 weeks in 26 untreated controls with similar histology shows a significant reduction in proteinuria in response to steroids in patients with proliferative histology and selective proteinuria.

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PREFACE

There have been major advances in the treatment of chronic renal failure over recent years. Our ability to halt or reverse deterioration in renal function, however, remains unimpressive, and we are equally impotent in the field of prevention. Despite the evolution of sophisticated diagnostic techniques in the field of nephrology, our understanding of first causes is sketchy in the extreme.

Some answers to the fundamental unsolved questions which still confront the renal physician may come from the correlation of data derived from a number of fields of study. Any technique which sheds light on the pathophysiology of renal disease is potentially of importance in this respect.

One of the many laboratory techniques which has received attention over the past decade is that of differential protein clearances, or protein selectivity determinations. Such determinations are said to give an index of glomerular permeability to macromolecules, and it has been claimed that selectivity results can be of help in the diagnosis of the aetiology of proteinuria, in assessing prognosis, and in selecting patients for steroid therapy.

Clearances studies of protein, dextran and polyvinylpyrrolidone have been carried out in Edinburgh over the past six years. This thesis gives an account of these studies. An attempt has been made to assess the value of selectivity determinations in the clinical situation, and also to indicate the light which studies on macromolecules shed on the function of the kidneys

in health and in a variety of disease states.

A glance at the table of contents may suggest that the introductory review of the literature is inordinately long. This preliminary discussion is detailed because selectivity studies cannot be discussed meaningfully except in the context of what is known of the pathophysiology of proteinuria. There are many tubular convolutions between the glomerular filter and the collection bottle, and it cannot be taken for granted that urinary phenomena reflect glomerular events. The very title of this thesis assumes that selectivity determinations measure glomerular permeability. An assertion of this kind must be justified, critically and in detail.

The work described in this thesis was carried out by a research team of which I have been a member since 1964. It was initiated by Dr. James Robson and financed by the Scottish Hospitals Endowment Research Trust. The immunodiffusion studies were carried out by Dr. Pamela Maclean and by technicians trained by her. The gel filtration technique was developed jointly by Dr. Maclean and myself, and estimations by this technique were carried out by one or other of us. Samples for protein selectivity determinations were obtained from patients by the staff of the medical renal unit. Clinical experiments involving the infusion of albumin, dextran or polyvinyl-pyrrolidone were carried out by myself. The various studies undertaken were planned jointly by Dr. Robson, Dr. Maclean and myself. The composition of this thesis represents my own unaided work.

SECTION I

INTRODUCTION

1. The Mechanisms involved in the Excretion of Protein by the Kidney

It is currently held that protein is filtered at the glomerulus in amounts depending on the size of the molecules concerned, and that the bulk of what is filtered is reabsorbed by the tubules. Some protein enters the urine as a result of desquamation of the cells lining the renal tract, but tubular secretion of protein is thought not to occur.

Since these concepts are central ones in the interpretation of the results of protein clearance studies, it is pertinent to give an outline of how they evolved, and of the experimental work on which they are based.

a. Early Concepts of the Mechanisms Involved in Proteinuria

Cushny (1926) propounded what became known as the "classical" theory of urine production. He held that the tubular fluid produced by the glomerulus was a true ultrafiltrate of plasma, and as such, was entirely free of protein.

In the nineteenth century Claude Bernard (1859) cited work by Berzelius and also experiments of his own which showed that egg white protein appeared in the urine of experimental animals following injection, and also in the urine of human subjects following the ingestion of raw eggs. Ascoli (1902) showed that the injection of egg white into rabbits resulted in proteinuria. Using an immunological technique, he proved that rabbit serum albumin appeared in the urine in addition to the injected egg protein. Ponfick (1875) found that haemoglobin appeared in the urine of certain patients following reactions to transfusion.

These facts were of course well known to Cushny. He explained them by stating that ovalbumin and extracellular haemoglobin were proteins foreign to the circulation, and although the normal glomerulus was impermeable to endogenous protein, renal mechanisms existed for the elimination of foreign protein. In their passage through the glomerulus, they caused damage to its structure, and the escape of normal proteins into the urine was the result of this damage.

The idea that proteinuria was the result of abnormal proteins rather than abnormal kidneys was a prevalent one in the 1920s. Munk, Benatt, and Flochenhaus (1925) suggested that in "lipoid nephrosis" there was a change in the physico-chemical characteristics of the body colloids, and that the renal changes were secondary to abnormal colloid metabolism. Epstein (1928) put forward the concept of "diabetes albuminuricus", where as a result of a generalised metabolic disease the serum proteins became waste products and were therefore excreted by kidneys which then became damaged as a secondary effect. Andrews et al (1929) stated that toxic nitrogenous substances became complexed with normal serum proteins in certain disease states. The protein-toxin complex, being foreign to the circulation, was then excreted by the kidneys.

Bayliss and his co-workers were, however, unimpressed by the concept of the kidney automatically excreting foreign protein and automatically retaining normal endogenous protein. They felt that since the glomerulus was a filter, molecular size would tend to have more bearing on the occurrence of proteinuria than whether the

protein was foreign to the circulation (Tookey-Kerridge and Bayliss, 1932; Bayliss, Tookey-Kerridge and Russell, 1933). Thanks to the work of Svedberg (1930) on the determination of the molecular weight of a number of proteins by ultracentrifugation, they were able to test their hypothesis. Protein excretion was measured in intact cats and rabbits, and in the isolated dog kidney-lung-pump preparation. It was found that the infusion of gelatin, ovalbumin, and Bence Jones protein (all substances with molecular weights of well under 60,000) resulted in the appearance of substantial amounts of protein in the urine. The infusion of albumin, globulin, casein, edetin, and haemocyanin (molecular weights over 60,000) did not result in proteinuria. Haemoglobin (M.W. 68,000) occupied an intermediate position, giving rise to haemoglobinuria only when its serum concentration was above a minimum "threshold" level. Their failure to demonstrate any proteinuria at all following the infusion of the larger molecules led them, Bayliss and his colleagues, to postulate a relatively sharp "cut off" in glomerular permeability at a molecular weight of about 60,000.

More recent studies indicate that the glomerulus filters minute amounts of quite large proteins (Dirks et al, 1964), but since they infused small amounts of protein, and carried out their estimations on unconcentrated urine, Bayliss, Tookey-Kerridge and Russell did not demonstrate this. Their work, however, indicated that the occurrence of proteinuria depended more on the molecular size of the protein concerned (and hence the permeability of the glomerulus) than on the endogenous or exogenous origin of that

protein.

Cushny (1926) had postulated that renal injury resulted from the excretion of foreign protein. Bayliss et al (1933) found no histological evidence of such injury. They admitted, however, that their work left this particular question open, as renal lesions resulting from proteinuria could well be submicroscopic.

The ingenious cross-circulation studies of Brull (1931, 1934) cast doubt on the idea of significant and lasting renal damage being a necessary consequence of the excretion of foreign protein. The renal circulation of a "test dog" was isolated in such a way that it could be perfused with blood from either of two "donor dogs". One donor dog served as a control; the second was rendered proteinuric by the injection of either egg white or haemoglobin. Blood from the proteinuric dog, when perfused through the kidneys of the test dog, produced proteinuria in the test dog. This procedure was followed immediately by perfusion of the test dog's kidneys by blood from the normal, non-proteinuric dog. Protein disappeared within minutes from the urine produced by the test dog's kidneys. The transient perfusion of the test dog's kidneys by blood containing ovalbumin or haemoglobin had clearly not resulted in renal damage of sufficient severity to give rise to persistent proteinuria.

These experiments, in conjunction with more recent work (Terry et al 1948; Petrie et al 1970) indicate that proteinuria per se does not give rise to major and lasting glomerular damage. It should however be noted that alterations in the epithelial cell

foot processes of Bowman's capsule can be demonstrated with the electron microscope in normal animals made proteinuric by the infusion of albumin or other proteins (Ashworth and James, 1961; Anderson and Recant, 1962; Post, 1960).

Since Cushny (1926) believed that the glomerulus was normally impermeable to serum proteins, it was not necessary for him to consider the question of tubular reabsorption of filtered protein. If the normal glomerulus is in fact sparingly permeable to plasma protein, however, it becomes necessary to postulate a tubular reabsorptive mechanism for protein. The volume of the glomerular filtrate is vast in comparison with the volume of urine excreted. In the absence of a reabsorptive mechanism, trace amounts of protein in the glomerular filtrate would lead to substantial concentrations of protein in the urine - in normal individuals. The demonstration of detectable quantities of protein in the glomerular filtrate, therefore, would imply the existence of a tubular reabsorptive mechanism for protein.

b) Evidence for the Presence of Protein in the Glomerular Filtrate

Cerebrospinal fluid, produced by filtration at the choroid plexus, contains between 10 mg% and 30 mg% of protein in health. By analogy, the glomerular filtrate might be expected to contain protein in concentrations of this order. More direct evidence on this point is to be found in micropuncture studies of the fluid in the glomerular capsular space and in the proximal tubule.

Wearn and Richards (1924) performed micropuncture studies in the frog and in nectarus, obtaining fluid from the capsular space around the glomerulus. The amounts of fluid obtained were minute, and methods of protein estimation at that time were lacking in sensitivity. They were, however, able to conclude that protein was present in the capsular fluid, but the concentration of this protein was less than 50 mg%.

Ekehorn (1931) had similar technical problems in relation to his micropuncture studies on the frog. His findings, however, supported the concept of tubular reabsorption of protein, since a semi-quantitative method showed that protein could be present in the glomerular filtrate while absent from simultaneously collected bladder urine.

The first mammalian micropuncture studies were carried out in 1941. (Walker et al, 1941; Bott et al, 1941). Walker and his colleagues demonstrated protein in 14 out of 41 specimens of capsular fluid obtained from normal rats and guinea pigs. Where protein was not found this did not mean that it was not present; the method used could not detect protein in concentrations of less

than 30 mg%. The protein concentration in the specimens in which protein was detected ranged from 80 mg% to 200 mg%.

Dirks, Clapp, and Berliner (1964) studied fluid obtained from the proximal end of the proximal tubule of normal dogs. They used an immunological method of protein assay with a sensitivity for albumin of 2 mg% and for globulin of 5 mg%. In 18 samples, no protein could be detected. In the 20 samples in which it was possible to measure the protein concentration, the mean albumin level was 5.9 mg%. Globulin was detected in two samples only. These figures are substantially lower than those obtained by other workers. Species difference may be part of the explanation; normal rats excrete considerably more protein in relation to body size than normal dogs. The site of micropuncture may also have had an important bearing on the results. Electron microscopy studies (Ashworth and James, 1961; Anderson and Recant, 1962; Graham and Karnovsky, 1966) indicate that substantial protein reabsorption occurs in the epithelium of Bowman's capsule. It is probably incorrect to assume that proximal tubular fluid represents glomerular filtrate. Carone and von Haeme (1965), for example, showed that while the capsular fluid of the rat contained a mean protein concentration of 73 mg%, the protein concentration in the fluid obtained from the proximal tubule was of the order of 10 mg%.

Bickford and Winton (1937) devised a method for obtaining glomerular filtrate which did not involve micropuncture. They studied the effect of varying the temperature of the perfusate in the isolated mammalian kidney. At temperatures of between 4°C and 15°C tubular reabsorption became abolished, and measurement of the

urea, creatinine and electrolyte content showed that the composition of the urine approached that of an ultrafiltrate of plasma.

Dock (1942) used a modification of this technique to determine the protein content of the glomerular filtrate. The haemoglobin clearance of his preparations was found to be 3% to 5% of the creatinine clearance, suggesting that glomerular permeability was altered little by the procedure. Dock found that the protein content of the urine obtained from rabbit kidneys perfused with ice cold saline was between 15 and 22 mg%.

The Japanese workers Ozawa and Yamauchi (1963) carried out similar experiments, and in addition performed protein electrophoresis on the perfusate and on the urine obtained from their chilled isolated rabbit kidney preparations. They noted a strong correlation between the perfusion pressure and the protein content of the urine obtained. At low perfusion pressures, the urine contained predominantly albumin; as the pressure increased the electrophoretic pattern of the urine obtained became more and more similar to that of the plasma perfusate. They decided that a perfusion pressure of between 35 and 40 mm of Hg represented approximately physiological conditions. The urine creatinine concentration was found to be slightly higher than the plasma concentration, indicating a degree of water reabsorption. Applying a correction based on the U/P creatinine ratio, they calculated from the protein concentrations found in the urine that the glomerular filtrate contained on average 26 mg% of protein.

It will be seen from the above account that estimates for the protein content of the glomerular filtrate vary considerably - depending in part on the methods used in the studies, and in part

on the experimental animal employed. The results of Dirks et al (1964) suggest a concentration of about 5 mg%. Carone and von Haeme (1963) obtained a figure of about 70 mg%. The concentrations obtained by other workers fall between these two extremes.

Caution must obviously be exercised in applying these results to man. Even where the architecture of the nephrons in different animals appears similar, important species differences in renal function are known to exist. These differences tend to be of degree rather than kind as far as mammals are concerned, however. It would appear overwhelmingly probable that some protein is present in the glomerular filtrate of man. Since the volume of this filtrate is about 180 litres per day, and the daily protein excretion in man is only about 40 mg, there must be tubular reabsorption of at least 1.8G per day, even if the protein concentration of the filtrate is as low as 1 mg%. If the protein level in the filtrate was 25 mg%, the daily level of protein reabsorption in the tubules would be about 45G per day.

c. Histological Observations Relating to Tubular Reabsorption and Tubular Secretion of Protein

Further evidence concerning the mechanisms involved in proteinuria is to be found in the results of histological and histochemical studies on the renal tubules. The nineteenth century pathologists - particularly those of the German school - were interested in discrete, spherical bodies observed within renal tubular cells. The nature and origin of these droplets gave rise to considerable controversy (Lehnert, 1912), but as a result of a review of the subject by Fahr (1925) it became accepted for a time that they were "degenerative" and reflected damage to renal tubular cells.

Terbrüggen (1931) challenged Fahr's conclusions following a detailed study which involved the examination of autopsy material from 1000 randomly selected cases, and also experimental studies on the rat. In 664 of the autopsied cases, droplets were noted in the proximal tubules. 88 of the patients had established renal disease, and of this group, 53 had droplets within their tubular cells. Since the presence or absence of renal disease did not appear to affect the incidence of droplets appreciably, Terbrüggen doubted the postulate that they were histological evidence of tubular cellular damage. He concluded that they represented tubular secretion of protein.

In cases of the nephrotic syndrome, however, many of the tubules contained large amounts of proteinacious material within their lumina. The cells lining these tubules contained large numbers of droplets. Terbrüggen suggested that in this particular situation droplets might be the result of tubular reabsorption rather than tubular secretion.

In his animal experiments Terbrüggen noted that droplets were a constant finding in the tubules of normal rats. Starvation led to a decrease in their number, while an increase resulted from the injection of sodium caseinate. Intraperitoneal sodium caseinate also led to an increase in the protein content of rat urine. Terbrüggen concluded that in the rat, as well as in man, droplets arose from the tubular secretion of protein.

Much earlier, Ponfick (1875) had noted that droplets giving the staining reactions of haemoglobin appeared in tubular cells in association with haemoglobinuria. Like Terbrüggen, he interpreted this finding in terms of the tubular secretion of protein.

The presence of protein-containing droplets in tubular cells, and an increase in the number of these droplets in situations associated with proteinuria, can be explained either on the basis of tubular secretion of protein or on the basis of tubular reabsorption. There is in fact a considerable body of evidence which suggests that tubular secretion of protein does not occur.

The first observations on this point date back to 1877, when Nussbaum carried out experiments on renal artery occlusion in the frog. The frog has a double blood supply to its kidneys. The glomeruli are supplied by blood from the renal arteries, but the tubules are perfused by a renal portal system arising from the iliac veins. A degree of anastamosis exists between the two systems, but glomerular filtration is suppressed by occlusion of the renal arteries (Randerath, 1937).

Nussbaum (1877) found that tying the renal arteries of the frog did not lead to suppression of urine formation; urine continued to be formed by a process of tubular secretion. Frogs made proteinuric by the injection of egg albumin, however, lost their proteinuria after ligation of the renal arteries, although the excretion of urea appeared to be relatively unimpaired. This finding suggested that proteinuria was dependant on the process of glomerular filtration.

Bieter (1931) studied proteinuria in the fresh water catfish, the europhyline eel, and the toadfish. The first two species mentioned possess glomeruli. The kidneys of the toadfish, however, are aglomerular; urine being produced entirely by a process of tubular secretion.

Bieter found that exposure of the eel or the catfish to asphyxia, poisoning with mercuric chloride, haemoglobin injection, or egg albumin injection gave rise, in every experiment, to readily demonstrable proteinuria. When the same stimuli were applied to the aglomerulate toadfish, proteinuria was never produced in detectable quantities. Bieter argued that proteinuria did not occur in the absence of glomeruli, and therefore the tubular secretion of appreciable quantities of protein did not occur.

Gerard and Cordier (1931) noted that droplets arose at the luminal (brush border) edge of the tubular cell, and migrated later to the basal border. Lambert (1932) made the same observation a year later. These workers pointed out that this localisation of the droplets within the tubular cells suggested

that they were evidence of reabsorption rather than secretion. Oliver (1950), Randerath (1937), Rather (1948) and Smetana (1942) confirmed these observations and agreed that droplets were indicative of tubular reabsorption of protein rather than tubular secretion.

Gerard (1936) and Gerard and Cordier (1932, 1933) also confirmed that in animals with a renal portal system similar to that of the frog, suppression of glomerular filtration led to the suppression of droplet formation - despite continued urine production by means of tubular secretion. This work almost clinched the reabsorptive origin of hyaline droplets, but it was open to one criticism. Interference with the renal arteries might have led to metabolic dysfunction of the tubular cells, rendering them incapable of droplet formation. This one remaining doubt was dealt with by Lison (1937). After ligation of the renal arteries of animals possessing a renal portal system, he injected protein into the lumen of the proximal tubule by a micropuncture technique. Droplet formation occurred. The tubular cells had obviously retained their ability to form droplets. The presence or absence of droplets depended on the presence or absence of protein in the tubular lumen. Droplets are therefore evidence of tubular reabsorption of protein and not of tubular secretion.

Dock (1942) reported studies on rats in which he employed the strongly protein-bound dye Evan's blue. Following the injection of this dye into normal rats, no blue coloration could be detected in the urine. Definite traces of the dye were however noted in vacuoles in the proximal tubular cells. Dock argued

that small quantities of protein, labelled with the blue dye, were escaping into the glomerular filtrate and being reabsorbed by the proximal tubular cells.

The demonstration that droplets resulted from the reabsorption of protein left a number of unanswered questions. From the micropuncture studies on the protein content of the glomerular filtrate discussed in section (b) it would appear that normal kidneys reabsorb several grams of protein daily. Yet droplets are observed only occasionally in normal kidneys under normal circumstances. The work of Smetana (1947) intensified this paradox.

He carried out studies on the salamanders and mice, using the protein-bound dye 2-naphthol 3:6 disulphonic acid. In both species, he demonstrated droplets of protein stained with this dye in the proximal tubular cells. In his mammalian experiments, he showed persistence within the renal tubular cells of the protein-dye complex for a period of several weeks. He concluded that the renal tubular cells did not reabsorb protein in the usually accepted sense of the word. They simply stored protein molecules in droplet form until the cells containing the droplets underwent desquamation.

It is clear that a storage mechanism of this kind would be incapable of removing many grams of protein daily from the glomerular filtrate.

Rather (1952) suggested that the dyes used by Smetana might have interfered with the metabolic pathways employed by the tubular cells for the elimination of reabsorbed protein. He performed experiments which involved the injection of rats with human

haemoglobin, and demonstrated breakdown of haemoglobin within the tubule cells and the disappearance of haemoglobin droplets within days rather than weeks.

Even on the basis of Rather's time-scale for protein disappearance from tubular cells, it is difficult to envisage the continued reabsorption of significant amounts of protein - as suggested by the previously cited micropuncture evidence. If the clearance of protein droplets from tubular cells is a slow process, and if reabsorbed protein consistently gives rise to droplet formation, in the presence of more than trace amounts of protein reabsorption, all normal proximal tubules should contain hyaline droplets at all times. The observed facts are that droplets occur only occasionally in normal renal tubular cells.

Oliver et al (1954 a and b) drew attention to this problem and attempted to resolve it. They studied the effect on the tubules of the albino rat of the infusion of a variety of proteins. Haemoglobin appeared in the urine of the rat some two minutes after being injected intravenously. Rats killed fifteen minutes after such an injection did not have haemoglobin droplets in their proximal tubular cells; the protein was instead distributed diffusely within these cells, and could be readily demonstrated by staining with benzidine. If larger amounts of haemoglobin were given, and the rats killed 45 minutes after injection, haemoglobin droplets were clearly visible in the proximal tubular cells.

Oliver and his colleagues showed that the ease with which droplets could be produced in rat tubular cells depended on the

protein injected. Small amounts of egg white protein gave large numbers of readily demonstrable droplets. Considerably larger quantities of rat serum protein produced droplets less readily. This finding was explained in part by the fact that egg white proteins pass the glomerular filter with considerably greater ease than rat serum proteins. This was, however, not the whole explanation. Rats were given a variety of proteins parenterally in quantities sufficient to ensure droplet formation, and killed at a time when this droplet formation was maximal. Thin sections of renal cortex were incubated in vitro, and the process of droplet lysis was then observed microscopically in living cells. The droplets derived from rat serum protein were dispersed much more rapidly than the droplets derived from foreign protein. Staining techniques indicated that mitochondrial enzymes were active in the process of droplet disintegration.

These experiments suggest that under normal circumstances, reabsorbed protein is distributed diffusely within the cell. When the rate at which protein entered the cell exceeds the rate at which the cell is cleared of protein, droplet formation occurs. Filtered colloidal material enters the tubular cells with relative ease, whatever its nature. The rate of its removal from the cell, however, depends on whether or not the cell possesses the appropriate enzymes for its degradation.

On the basis of this hypothesis, it can be seen that homologous serum protein can be reabsorbed in relatively large amounts without giving rise to droplet formation. Foreign protein, on the other hand (which appears to be stored rather than reabsorbed

by the tubule cells) can give rise to extensive droplet formation without having a detectable renal threshold; this is the situation with egg albumin. An analogous situation exists with respect to dextran. Following injection of this substance James and Ashworth (1961) demonstrated dextran particles within the cells of Bowman's capsule and the cells of the proximal tubule. A renal theshold for dextran, however, is not demonstrable, and it has been shown that the renal clearance of dextran molecules of molecular weight 10,000 or less is not appreciably different from the clearance of creatinine. (Brewer, 1951; Wallenius, 1954).

An ingenious attempt to quantitate tubular reabsorption of protein in the rat was made by Sellars et al (1954). Evan's blue, which is strongly protein-bound, was given intravenously in a dose of 20 mg. Blood was taken from the injected animals at intervals, and the amount of dye per gram of serum protein was estimated. This calculation of "specific activity" enabled concentrations of dye to be expressed in terms of their protein equivalent.

Rats were sacrificed at predetermined intervals following injection. The renal vessels and renal tubules were rinsed by in vitro perfusion and following this all the dye remaining was confined to the proximal tubular cells. The kidneys were then homogenised, and their content of Evan's blue assayed.

It was assumed that the transfer of the protein-dye complex from tubular lumen to tubular cell occurred at the same rate as that of unlabelled protein. This assumption would appear to be

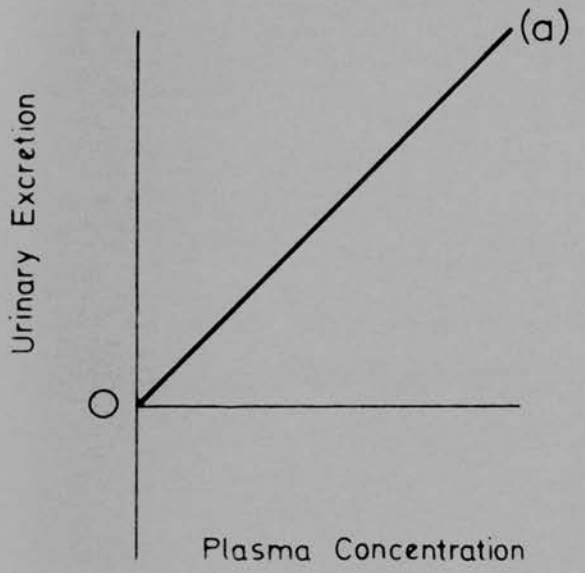
reasonably valid. The half life of an Evan's blue-protein complex in vivo is not demonstrably different from that of I^{131} labelled albumin (Gilson, 1949). Furthermore, the renal clearance of intravenously injected dose of Evan's blue is equal to the renal clearance of albumin. (Chinard et al, 1952; Allen and Orahoevets, 1948). It was also assumed that the persistence of Evan's blue within the tubular cells would be prolonged, at least in comparison with the time base of the experiment. There was a continued rise of dye concentration within the tubular cells over a 48 hour period, despite a simultaneous fall in plasma levels of Evan's blue.

From the tubular dye content of rats sacrificed 3 hours after injection, Sellars et al calculated that tubular reabsorption of protein was proceeding at the rate of 5 mg. per hour. The urinary excretion of protein was 0.5 mg per hour, indicating glomerular filtration of 5.5 mg hourly. Taking the figure of 72 ml per hour (1.2 ml per minute) as being the glomerular filtration rate for rats of the size and species used, this gave a protein content in the glomerular filtrate of 8 mg per 100 ml.

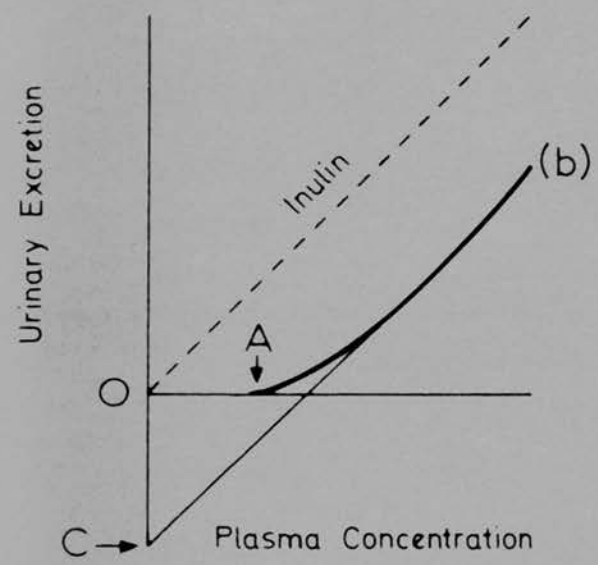
Sellars et al calculated that the daily reabsorption of protein in the proximal tubule of the rat came to 120 mg. They pointed out that since the total protein content of rat plasma was about 360 mg, the rat apparently reabsorbed one third of its plasma protein daily.

Figure 1

The effect of varying plasma concentrations on the urinary excretion of crystalloids handled in different ways by the renal tubules.



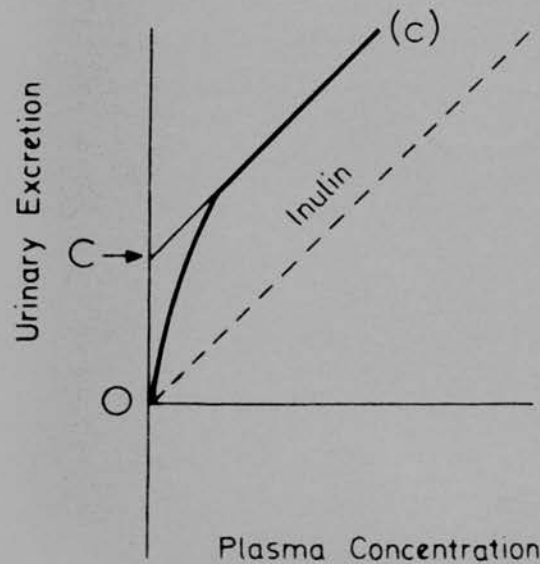
(a) A crystalloid (e.g. inulin) which is freely filtered and which is neither reabsorbed nor secreted by the tubules.



(b) A crystalloid (e.g. glucose) which is freely filtered and which is reabsorbed but not secreted by the tubules.

A - renal threshold.

C - (negative intercept with Y axis)
= tubular reabsorptive capacity.



(c) A crystalloid (e.g. para-amino hippuric acid) which is freely filtered and which is secreted but not reabsorbed by the tubules.

C - (positive intercept with Y axis)
= tubular secretory capacity.

d. Clearance Studies, Stop Flow Analysis, and Renal Threshold Determinations with Respect to Protein

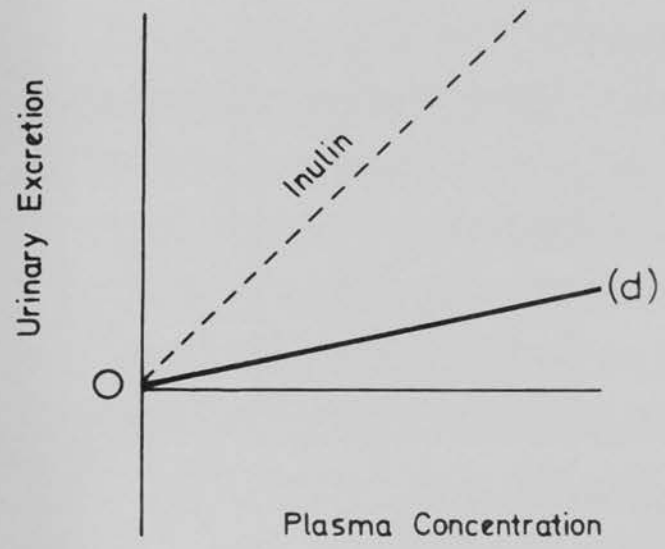
Clearance techniques (Smith, 1956) have shed light on the renal handling of many substances. If a substance is cleared from the plasma at a rate equal to that of inulin over a wide range of plasma concentration, it is filtered at the glomerulus but is neither secreted nor reabsorbed by the tubules.

Provided the substance is of low molecular weight and is not protein-bound, the finding of a clearance less than that of inulin indicates tubular reabsorption, while a clearance greater than that of inulin indicates tubular secretion. The amount of a substance filtered at the glomerulus is proportional to the plasma concentration. At high plasma levels, tubular mechanisms become saturated and the line relating excretion per unit time to plasma concentration becomes parallel to the similarly constructed line for inulin. For a substance subject to tubular reabsorption, the line is below that obtained for inulin, and makes a negative intercept with the "y" axis at a point which gives a measure of the tubular reabsorptive maximum for the substance concerned. For a substance subject to tubular secretion, the line relating excretion per unit time to plasma concentration is above the line obtained for inulin, and makes a positive intercept with the "Y" axis which is a measure of the tubular secretory maximum (figure 1).

Where macromolecules are concerned, the demonstration of a clearance which is less than that of inulin can be explained in terms of limited glomerular permeability, tubular reabsorption,

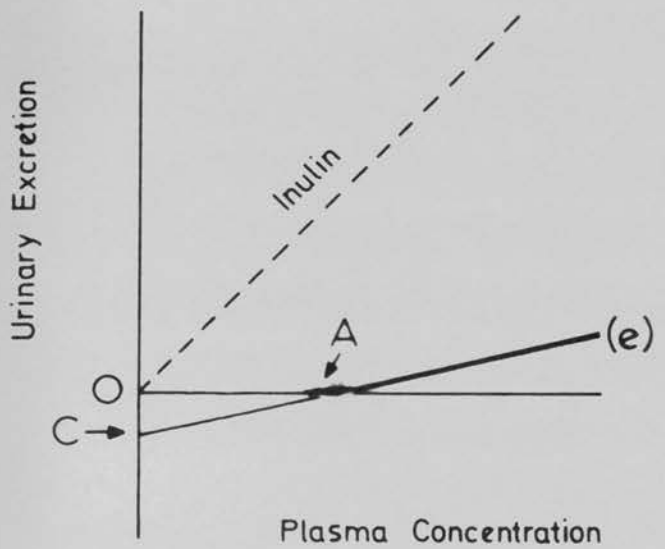
Figure 2

The effect of varying plasma concentrations on the urinary excretion of colloids handled in different ways by the renal tubules.



(d) A colloid (e.g. dextran) which has restricted glomerular filtration and which is neither reabsorbed nor secreted by the tubules.

The slope of the line relating excretion to plasma concentration is a measure of glomerular pemeability to substance (d); it is considerably less than that obtained for inulin.



(e) A colloid (e.g. albumin) which has restricted glomerular filtration and which is reabsorbed but not secreted by the tubules.

A - renal threshold

C - (negative intercept with Y axis)
= tubular reabsorptive capacity.

protein-binding effects, or a combination of all these factors.

If the concentration of the macromolecule in plasma is increased, a point will be reached where the relationship between plasma concentration and urinary excretion rate becomes linear. The slope of the line is flatter than the slope with respect to inulin; this slope in fact is a measure of glomerular permeability. Provided there is no protein binding, the negative intercept of the line with the "Y" axis again gives a measure of tubular reabsorption (figure 2).

Monk and Yuille (1940) determined simultaneous creatinine and haemoglobin clearances in the dog. They found that at plasma levels of under 100 mg%, no haemoglobin appeared in the urine. At levels of between 100 mg% and 250 mg% the renal clearance rose with the plasma concentration. At a plasma concentration of 250 mg% or more, the haemoglobin clearance became constant and amounted to 3% of the creatinine clearance. Monk and Yuille calculated the tubular maximum reabsorption rate for haemoglobin to be 2 mg per minute, in dogs with a glomerular filtration rate of (on average) 66 ml/min.

Yuille and Clark (1941) studied the renal handling of myoglobin in the dog. Myoglobin contains one atom of iron per molecule and has a molecular weight of 17,500, as compared with haemoglobin which has four atoms of iron per molecule and a molecular weight of 68,000. The renal threshold for myoglobin was found to be 17 mg%. The renal clearance of myoglobin increased with increasing plasma concentrations to become constant at 60% of the creatinine clearance when the plasma myoglobin

level was over 50 mg%. Yuille and Clark interpreted the existence of a renal threshold for myoglobin as being evidence of tubular reabsorption of this substance.

Lichtz, Havill, and Whipple (1932) demonstrated that repeated haemoglobin injections over a period of days led to a marked lowering of the renal threshold for haemoglobin. They considered that the injections had led to saturation of the reabsorptive mechanisms for haemoglobin, and that these mechanisms, once saturated, remained blocked for an appreciable time.

Yuille and his colleagues tested this hypothesis (Yuille, Steinman et al, 1941). They studied two groups of dogs, one of which had been pretreated by a number of haemoglobin injections, and the other which had not. They assessed tubular reabsorption of haemoglobin by clearance techniques, as in their earlier studies, and also measured the radioactivity accumulating in tubular cells following the injection of haemoglobin labelled with radioactive iron.

In the group of dogs which had been pretreated with haemoglobin injections, clearance studies indicated reabsorption of 20% of the injected haemoglobin. Measurement of the radioactivity accumulating in the tubular cells of these animals gave a recovery of between 19 and 23% of the injected dose - a figure in good agreement with the clearance studies.

In the dogs which had not been pretreated by haemoglobin injection, the situation was very different. Clearance studies suggested that 45% of the injected dose had been reabsorbed by the tubules. Only 17-19% of the injected dose of radioactivity, however, could be recovered from the tubular cells. Yuille et al

could not explain these puzzling findings at the time.

Some years later, Allison and Reeves (1957) and Vanderveiken et al (1957) showed that haemoglobin is normally present in the circulation bound to a carrier protein, haptoglobin. The haemoglobin-haptoglobin complex is of high molecular weight and passes the glomerular filter in trace amounts only. Since the haemoglobin-haptoglobin complex is rapidly removed from the circulation by the reticulo-endothelial system, repeated injections of haemoglobin lead to haptoglobin depletion.

For haemoglobin to appear in the urine, the binding capacity of haptoglobin must be saturated as well as the tubular re-absorptive capacity.

The conflict between the results of the clearance studies and the radioactive iron recovery from the tubules in Yuille's study (Yuille, Steinman et al, 1941) can be explained on the following basis. In the dogs which had not been pretreated by haemoglobin injection, only a proportion of the plasma haemoglobin was available for glomerular filtration. The rest was bound to haptoglobin. The clearance studies gave an erroneously high estimate of tubular reabsorption, and therefore failed to agree with the radioactive iron recoveries. In the dogs which had been pretreated with haemoglobin injection, haptoglobin depletion had occurred. The bulk of the haemoglobin present in the plasma was therefore filterable, and the figures obtained for tubular reabsorption from the clearance studies were therefore correct, or nearly so. In this instance, therefore, the clearance studies gave results which were in agreement with the figures obtained

from estimating tubular recovery of radioactive iron.

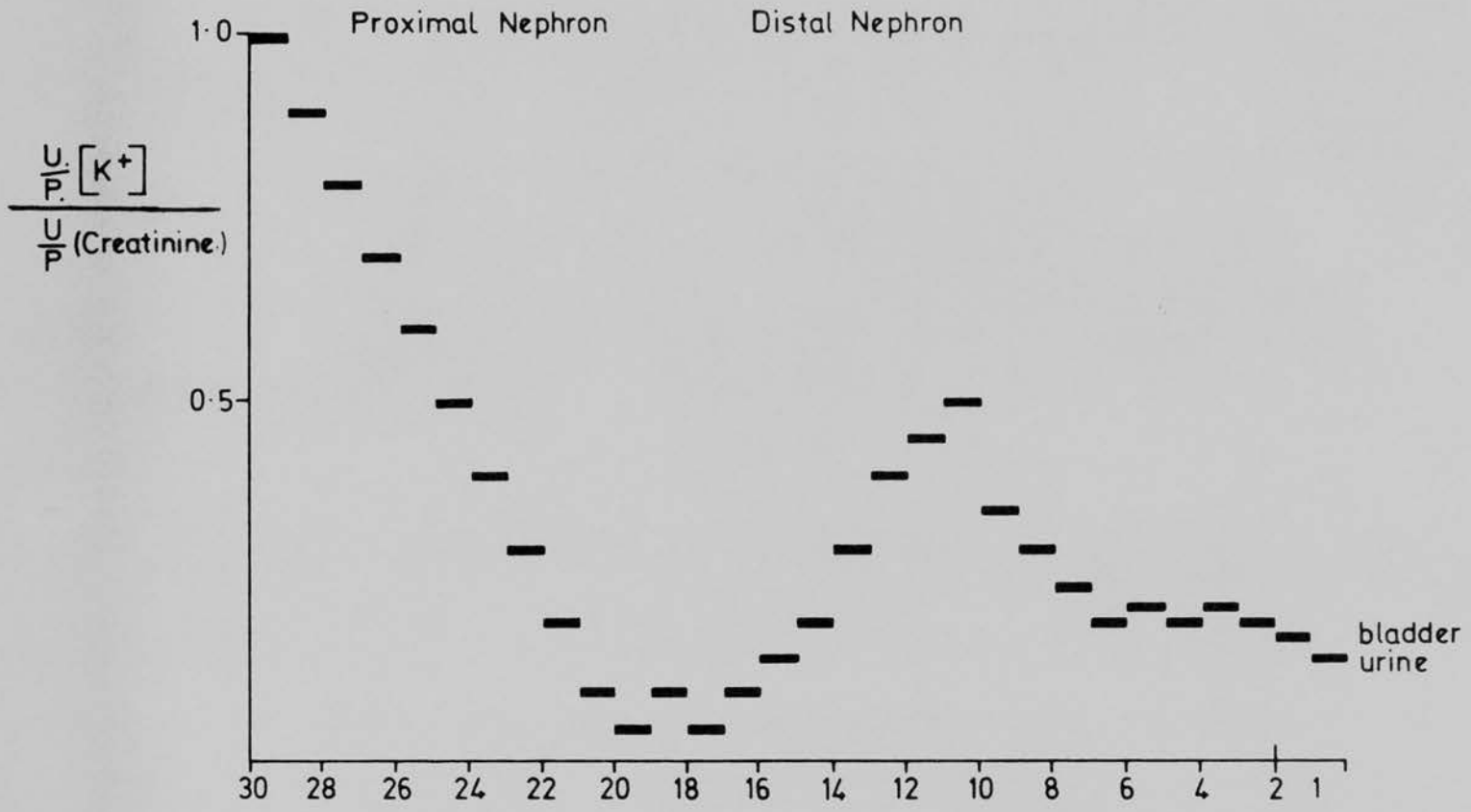
In the light of what is now known of the role of haptoglobin in haemoglobin transport, the careful work of Monk, Yuille and their colleagues requires re-evaluation. Their observation that at high haemoglobin levels the clearance of haemoglobin is about 3% of that of creatinine remains valid, since haptoglobin binding is saturated at high plasma haemoglobin levels. The values which they calculated for tubular reabsorption of haemoglobin, however, are obviously erroneous.

Lathem (1959) studied the renal clearance of haemoglobin in adult humans. 14G to 18G of haemoglobin (derived from haemolysis of the subject's own red cells) was infused intravenously over a four hour period, following a priming dose. Free haemoglobin and bound haemoglobin were assayed separately on the plasma samples, and the clearances calculated were for free haemoglobin. Inulin clearances were determined simultaneously.

It was found that haemoglobin was bound to haptoglobin until its plasma lavel exceeded (on average) 130 mg%. No convincing threshold for free haemoglobin was demonstrated, since haemoglobin appeared in the urine almost as soon as free haemoglobin appeared in the blood. From the slope of the line relating haemoglobin excretion to plasma haemoglobin concentration, the glomerular permeability to haemoglobin appeared to be $5.0 \pm 1.0\%$ of the permeability to inulin in the ten subjects studied. From extrapolation of this line, it appeared that the renal threshold for free haemoglobin ranged from 0 to 54 mg,

Figure 3.

Illustrating the technique of stop-flow analysis.



The earliest tubes collected are at the right of the diagram.

Tubes 30 to 20 represent proximal nephron.

Tubes 16 to 7 represent distal nephron.

Tubes 6 to 1 represent dead space.

There is a fall in the urine to plasma ratio of potassium (corrected for reabsorption of water) in tubes 30 to 20, indicating proximal potassium reabsorption.

There is a rise in the urine to plasma ratios of potassium (corrected for water reabsorption) in tubes 16 to 7, indicating distal potassium secretion.

Adapted from Berliner (1960).

with a mean of 25 mg. The maximum tubular reabsorptive capacity for haemoglobin was 1.3 ± 0.9 mg per minute per 1.73 sq. m. surface area. Lathem pointed out that when the error of the method was taken into account the tubular reabsorption which he had demonstrated did not vary significantly from zero.

Lathem et al (1960) then turned to the technique of stop-flow analysis - a technique introduced by Malvin, Sullivan and Wilde (1958). The basis of the method is as follows. Ureteric obstruction is applied acutely to produce a static column of fluid within the renal tubules. This allows each tubular segment to perform its functions practically to completion. Following release of the obstruction, under conditions of rapid urine flow, successive aliquots of ureteric urine are collected. Early aliquots reflect function in the collecting ducts. Subsequent samples reflect the activity successively of the distal tubule, the loop of Henle, and the proximal tubule. To correct for reabsorption of water, the measured concentrations of the substance under test in the aliquots obtained are adjusted by a factor derived from the urine to plasma concentration ratio of creatinine. Figure 3 illustrates the use of this technique in determining the approximate sites of tubular reabsorption and secretion of potassium.

In stop-flow experiments on dogs, Latham et al (1960) showed that a marked fall occurred in the urine to plasma ratio of free haemoglobin in samples which appeared to be derived from the proximal tubule, after correcting for water reabsorption. This fall could only be explained in terms of reabsorption of

haemoglobin by the cells of the proximal tubule. It is of interest that the histochemical studies of Oliver (1950), Smetana (1942), and Rather (1948) indicate that the proximal tubule is where protein reabsorption occurs. A second but smaller fall in the urine to plasma haemoglobin ratio (again corrected for reabsorption of water) was noted in samples obtained from the distal tubule.

In summary, Lathem and his colleagues demonstrated definite tubular reabsorption of haemoglobin in the dog, using stop-flow analysis, but showed by clearance techniques that the amount reabsorbed by the human kidneys was small, being in the region of 1 mg per minute. It should be noted that in neither series of experiments was there any evidence of tubular secretion of haemoglobin.

Free haemoglobin is, of course, not a normal constituent of plasma. Clearance studies and threshold determinations involving albumin are probably of more direct physiological relevance than similar studies involving haemoglobin.

As has been stated in an earlier section, comparison of the protein content of the glomerular filtrate with that of the urine suggests that some protein passes the glomerular filter, tubular reabsorption of this protein being virtually complete. This concept of the renal handling of serum proteins is supported by the results obtained from protein infusion studies by a number of workers in the 1940s.

Thorn, Armstrong, and Davenport (1946) used salt-poor albumin in the treatment of five patients who had hepatic cirrhosis complicated by hypoproteinaemia and ascites. After the intravenous

infusion of between 150G and 300G of albumin at a rate of 50G per day, proteinuria of over 1.0G daily occurred in all five patients. In every case, protein disappeared from the urine within a few days of stopping the infusions.

Terry et al (1948) conducted a number of experiments in normal dogs in order to determine if nitrogen balance could be maintained with intravenous plasma infusions as the only source of protein. Proteinuria was produced in all the animals. In each case it disappeared within a few days of discontinuing the plasma infusions. The threshold for proteinuria varied from 9.6G to 10.4G protein per 100 ml plasma. Repeat experiments on the same dogs showed that for an individual animal the renal threshold for protein was remarkably constant.

Waterhouse, Bassett, and Holler (1949) gave salt-poor albumin by intravenous infusion to four patients who were protein depleted as the result of malnutrition. After the infusion of between 200G and 300G of albumin at a rate of 60G per day all four patients developed proteinuria. In three patients the protein excretion ranged from 3.0G to 7.0G per day. In the case of the fourth patient - a man with trace proteinuria prior to the start of the experiment - protein excretion rose to 30G per day, following albumin infusion. Proteinuria occurred in each case following a rise in serum albumin concentration of about 2.0G % and an increase in plasma volume of about 25%.

These experiments demonstrated the existence of a renal threshold for albumin in dogs and humans with normal renal function.

Quantitative clearance studies were not carried out by any

of the groups whose work is referred to above.

Malmendier and Lambert (1955) carried out albumin infusion experiments on dogs using clearance techniques. These indicated that the permeability of the glomerulus to albumin was about 0.6% of its permeability to creatinine. They conducted similar experiments on normal humans (Malmendier, Gregoire and Lambert, 1957) which suggested glomerular permeability to albumin was about 0.3% of the permeability to creatinine. The tubular reabsorptive maximum for protein appeared to be less than 55 mg per minute. The albumin threshold for normal man was found by these workers to be about 7.0G per 100 ml. As albumin infusion causes plasma expansion, Malmendier, Gregoire and Lambert were unable to raise the serum albumin levels sufficiently to investigate the relationship between the serum albumin concentration and the urine albumin excretion over a wide range. For this reason, the figures obtained for tubular reabsorption and glomerular permeability with respect to albumin in normal subjects were estimates rather than precisely determined values.

As subjects with the nephrotic syndrome are proteinuric at relatively low serum albumin concentrations, it is possible to investigate protein excretion in these individuals over a much wider range of serum protein concentrations. Chinard et al (1954a and b) carried out albumin clearance studies on patients with the nephrotic syndrome, before and after intravenous albumin infusion. They held that proteinuria in such patients might either result from decreased tubular reabsorption of protein in nephrons with normal glomerular permeability, or from increased

glomerular permeability leading to saturation of tubular reabsorptive mechanisms. They considered it unlikely that tubular secretion was an important factor in the proteinuria of the nephrotic syndrome, and they cited work by Gitlin and Janeway (1952) to the effect that dysproteinaemia was not a factor in the proteinuria of the nephrotic syndrome. (Gitlin and Janeway had established immunochemical identity between the proteins of nephrotic urine and the proteins of both normal and nephrotic sera).

Chinard et al (1954a) calculated the minimum possible concentration of albumin in the glomerular filtrate on the basis of the following formula, previously used by Bing (1936) and by Berglund, Scriver and Medes (1935):-

$$\text{Min } G_{Alb} = U_{Alb} V / GFR$$

Min G_{Alb} is the minimum possible concentration of albumin in the glomerular filtrate. It is the value obtained by assuming tubular reabsorption of protein to be zero.

U_{Alb} is the urine albumin concentration in mg%.

V is the urine volume in unit time.

GFR is the glomerular filtration rate, derived in Chinard's experiments from the creatinine clearance.

Min G_{Alb} was estimated on 34 occasions in 6 patients. The mean value found was 69 mg%, with a range of from 11 mg% to 193 mg% and a standard deviation of ±48 mg%.

Chinard's patients were hypoproteinaemic. He pointed out that the albumin concentration of the glomerular filtrate depended on the serum albumin concentration as well as on glomerular

permeability, and (on the basis of simple proportionality) corrected his filtrate albumin concentrations to correspond to a serum albumin concentration of 4.0G%. In his series Min G_{Alb} after applying this correction had a mean value of 183 mg%, with a range of from 55 mg% to 305 mg% and an SD of 154 mg%.

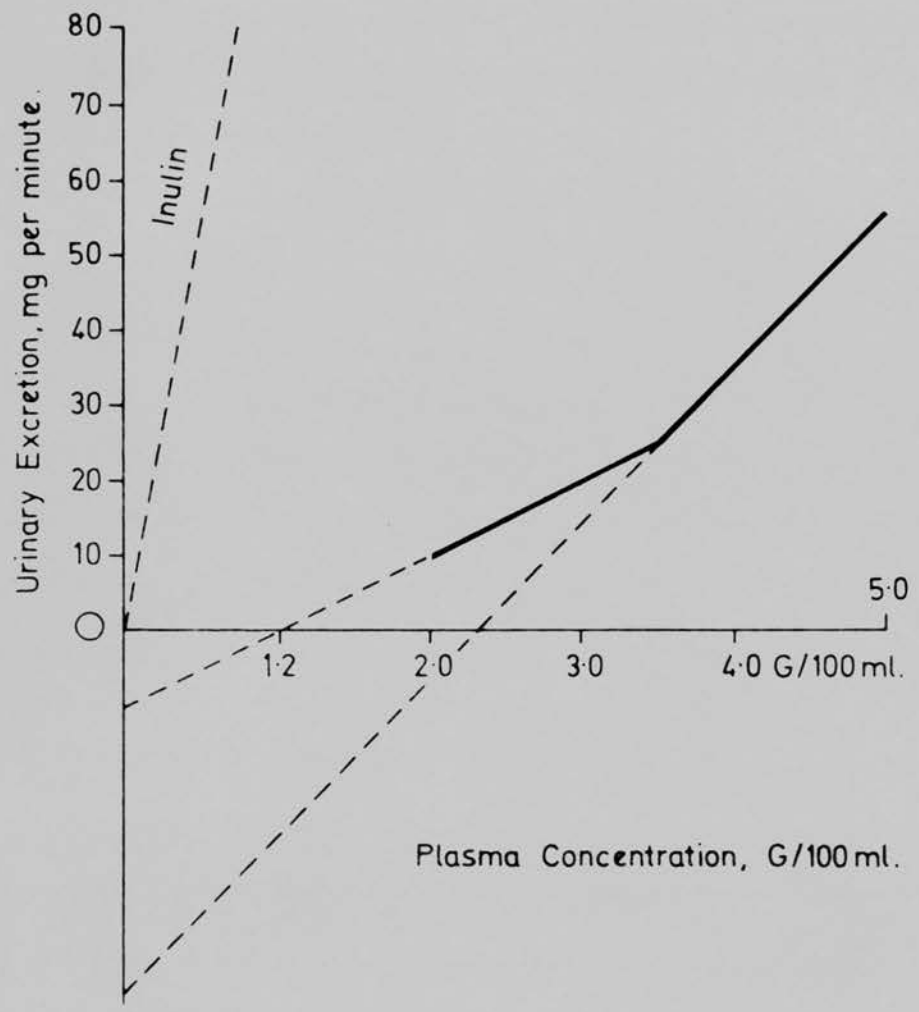
As has been stated in an earlier section, micropuncture and other methods of assessing the protein content of the glomerular filtrate suggest that in normal kidneys this is of the order of 10 to 30 mg%. Chinard's results - especially after taking into account the lowered serum albumin levels - indicate that patients with the nephrotic syndrome have albumin concentrations in the glomerular filtrate which are substantially in excess of these normal values. This suggests that the proteinuria of the nephrotic syndrome results from increased glomerular permeability to protein.

Chinard et al found that the relationship between albumin excretion and the serum albumin concentration was linear over the earlier part of the curve, but at higher levels of serum albumin concentration a break appeared in the line, which suddenly became steeper. This break in the line was thought to be due to an abrupt increase in glomerular permeability to protein, and was thought by Chinard to be the result of plasma expansion

Gregoire, Malmendier and Lambert (1958) carried out similar experiments on 18 patients with proteinuria. By plotting the albumin excretion against the serum albumin concentration before and after the intravenous infusion of albumin they were able to calculate the tubular reabsorption of albumin and also the clearance of albumin relative to that of creatinine. The values

Figure 4.

The effect of plasma expansion on glomerular permeability to albumin.



Relationship of urinary excretion of albumin to plasma concentration during albumin infusion.
The "break in the line" at a certain point in the infusion is attributed to plasma expansion causing altered glomerular permeability.

Adapted from Gregoire, Malmendier and Lambert (1958).

obtained for tubular reabsorption of albumin ranged from 2.2 mg per minute to 20.5 mg per minute, with a mean of 10.5 and a standard deviation of 7.1. The clearance of albumin relative to that of creatinine ranged from 0.57% to 3.93% with a mean value of 1.7% and a standard deviation of ± 1.0%.

They infused rather more albumin in their experiments than the amount used by Chinard et al. At serum albumin levels which varied from 2.4G to 4.4G in different patients, they noted a break in the line relating albumin excretion to plasma albumin concentration (figure 4). They pointed out that this could theoretically be accounted for by the existence of a second nephron population with a very great tubular reabsorptive capacity for protein contributing albumin to the urine only at high plasma albumin levels. They felt that explanation to be an unlikely one, however. They ascribed the break in the line to an abrupt increase in glomerular permeability to protein occurring as the result of plasma expansion with consequent stretching of the glomerular pores. This explanation was similar to that used by Chinard to account for the same phenomenon.

Albumin infusion experiments in proteinuric patients were also carried out by Hardwicke and Squire (1955). Using an electrophoretic technique, they determined simultaneous clearances with respect to albumin, creatinine and four globulin fractions.

Following infusion, there was invariably a rise in the albumin clearance. Hardwicke and Squire pointed out that this observation was strong evidence for the existence of a quantitatively important tubular reabsorptive mechanism for albumin, since in

the absence of such a mechanism the clearance would remain constant, although albumin excretion rates would rise with rising plasma levels of albumin.

In four patients, the albumin permeability relative to creatinine ranged from 1.1% to 2.3%, with a mean value of 1.4%. The calculated values of tubular reabsorption of protein ranged from 14 mg per minute to 32 mg per minute, with a mean value of 21 mg per minute.

An unexpected finding was that the clearance of each of the globulin fractions rose proportionately with the albumin clearance following albumin infusion - despite a transient fall in plasma globulin levels consequent on plasma expansion. This led Hardwicke and Squire to suggest that the tubular reabsorptive process for protein was a nonspecific one - all serum proteins sharing a common pathway. With all the protein moieties in the glomerular filtrate competing for this single pathway the amount of any individual protein reabsorbed would be proportional to its concentration in the glomerular filtrate. Such a mechanism would lead to the concentrations of proteins relative to each other in the urine being the same as their concentrations relative to each other in the glomerular filtrate - although the absolute amounts of protein reaching the final urine would obviously be influenced considerably by the amount reabsorbed in the tubules.

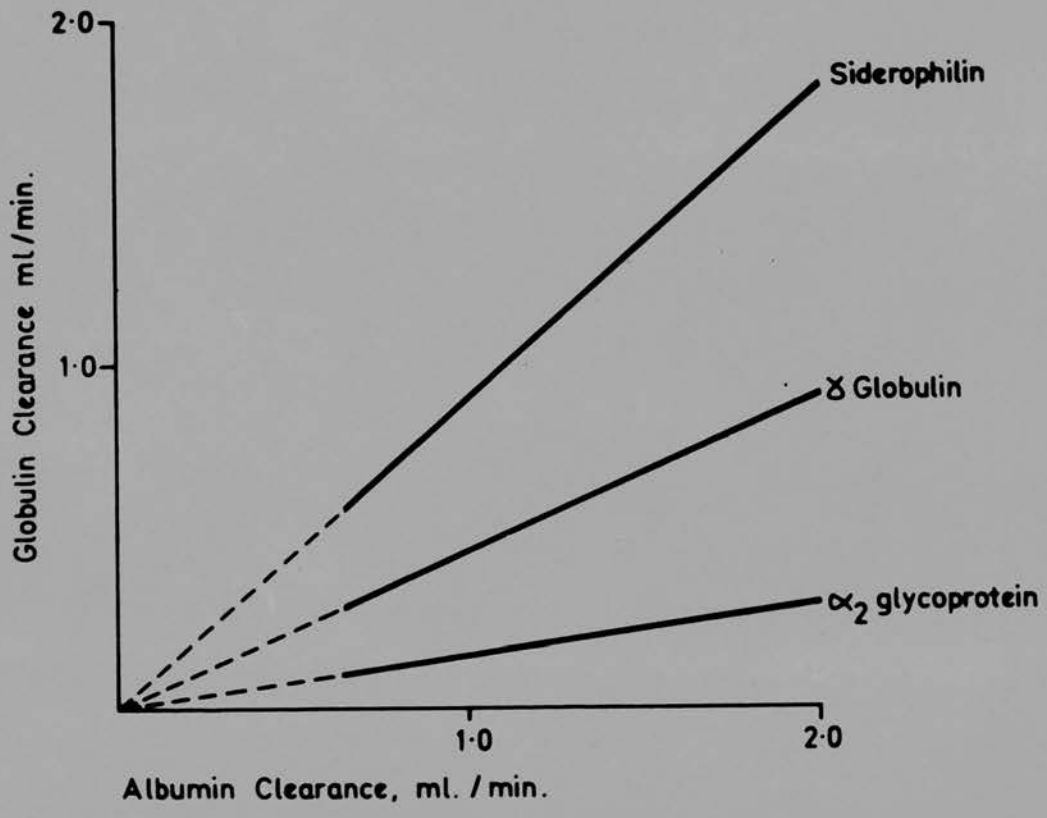
In the course of Hardwicke and Squire's experiments, plasma expansion occurred - as evinced by a fall in packed cell volume and in serum globulin concentrations. This plasma expansion

could have led to stretching of the pores of the glomerulus, and had this happened, a rise in clearance values of all proteins would have followed. The globulin clearances measured by Hardwicke and Squire (1955) were proportional to the simultaneously determined albumin clearances, and the lines relating globulin clearances to albumin clearances were straight ones, passing through the origin. Hardwicke and Squire pointed out that such a relationship would be expected if the various proteins involved shared a common reabsorptive pathway, but that an increase in pore size would have given a curvilinear relationship and lines which did not pass through the origin.

This argument would appear, on the fact of it, to be a strong one. Plasma beta globulin, however, contains at least two major components of widely differing molecular weight. The larger of these components is a lipoprotein, present in large amounts in nephrotic serum, but almost undetectable in nephrotic urine. Since urine beta globulin is almost entirely made up of the small molecular weight component, the beta clearance, as measured electrophoretically, has little real validity. This was admitted by Hardwicke and Squire. Since similar discrepancies between the composition of the serum and urinary fractions of other globulins exist, the globulin clearance figures obtained by Hardwicke and Squire in 1955 can only have been approximate. The Birmingham group were, of course, fully aware of the limitations of the eletrophoretic method as a means of measuring globulin clearance, and Hardwicke and Soothill (1961) repeated the work reported in 1955, using an immunological method of assay for the various

Figure 5.

The relationship between albumin clearance and the clearances of three other proteins following albumin infusion.



globulin fractions. Within the limits of experimental error, straight lines passing through the origin were obtained when the clearances of a number of globulins were plotted against the simultaneously determined albumin clearances (figure 5).

This concept of a shared reabsorptive pathway for protein is supported by the observation that albumin infusion does not alter glomerular permeability to protein (Marchena and Becker, 1968) or to dextran (Petrie et al, 1970). An analagous competitive process is involved in the reabsorption of certain amino-acids (Pitts, 1944). In certain disorders of the renal tubules, however, evidence for this type of reabsorptive mechanism appears to be lacking.

In the Fanconi syndrome, glomerular permeability appears to be normal, but there are multiple defects in tubular function. A significant degree of proteinuria exists. The proteins excreted are predominantly of low molecular weight. They are of glomerular rather than tubular origin, their presence in urine being due to failure of tubular reabsorption. (Flynn and Platt, 1968; petersen et al, 1969). If the reabsorptive pathway for these small molecular weight proteins was the same as that for albumin and other proteins of over 60,000 molecular weight, escape of the small proteins into the urine would be accompanied by significant excretion of larger protein molecules. The excretion of albumin and larger molecules in tubular proteinuria is, however, negligible. (Flynn and Platt, 1968; Petersen et al, 1969; Harrison, personal communication, 1970).

Petersen (1969) has suggested that the reabsorptive pathway

for proteins such as beta-2 microglobulin, with molecular weights of 40,000 and under is different from that for proteins such as albumin with molecular weights of over 60,000.

(e) Electron Microscope Studies Relating to the Renal Handling of Protein

The introduction of renal biopsy by Perez (1950) made it possible to evaluate renal morphology during life, and hence it has become possible to correlate structure and function in a variety of renal diseases. It has also become possible to classify renal disease into a number of histological categories, and although several disease entities are probably contained in each category, when one compares (for example) a series of patients with minimal lesion glomerulonephritis in one renal centre with that from another centre, one is comparing like with like to a greater extent than was possible with any previously employed system of classification.

The use of electron microscopy in the evaluation of renal biopsy specimens has revealed a great deal about the cellular and subcellular basis of renal function (Hall, 1957; Pease, 1955; Rhodin, 1955 and 1967; Bencosme and Bergman, 1962). These morphological findings will be discussed briefly in so far as they have a bearing on the subject of proteinuria.

The glomerular filter consists of three layers. The capilaries are lined by endothelial cells. The layer of endothelial cells is breached by a series of fenestrations which vary in diameter from 200 to 900 A. These fenestrations are closed by a thin, single-layed membrane. External to the endothelial cells is the glomerular basement membrane. The structure of this membrane has been the subject of some controversy. Mueller (1958) and Movat and Steiner (1961) distinguished a central

dense layer lying between two layers of lesser density. More recent observations (Rhodin, 1967) suggest that these layers were perhaps an artefact caused by earlier methods of fixation, and that the basement membrane, apart from having a fine fibrillary ultrastructure, is relatively homogeneous. Spiro (1959) found apertures in the basement membrane which he regarded as the sites of filtration of macromolecules. Other workers (Movat, 1960; Farquhar and Palade, 1961) failed to confirm the existence of such pores, and it is now thought that such pores (like the previously noted trilaminar structure of the basement membrane) are a fixation artefact.

External to the basement membrane is the layer of epithelial cells. Each cell possesses numerous extensions which branch to form delicate pedicels, or foot processes. These are attached to the glomerular basement membrane. Between the pedicels are small spaces which are termed slit-pores. These have a width of between 70 and 100 A. Like the endotholial fenestrations, the epithelial slit pores are closed by a thin membrane.

The glomerulus affords a considerable barrier to the passage of albumin, which has a glomerular clearance in health of about 0.6% of the inulin clearance (Chinard, Lauson and Eder, 1952; Malmendier et al, 1957). Since albumin has a molecular radius of about 36A, it is unlikely that either the 200 Å endothelial fenestrations or the 100 Å epithelial slit pores would hinder its filtration to this extent. The main barrier to the filtration of macromolecules would appear to be the glomerular basement membrane. With the exception of Spiro (1959) electron

microscopists have been unable to demonstrate pores in the basement membrane, and it may be that macromolecules cross this structure by a process of gel diffusion. For the purpose of this thesis, however, the glomerulus will be regarded as a porous seive. The results of permeability studies fit both the diffusion concept and the filtration concept equally well (Hardwicke and Soothill, 1961) and the discussion of variations in "functional pore size" involves less in the way of verbal gymnastics than a discussion of varying restrictions on diffusion.

Studies on particles such as ferritin (identifiable on electron microscopy because of their high density) show that the passage of such macromolecules through the endothelial cells is slight, and access to the basement membrane by such particles is mainly at the endothelial fenestrations (Farquhar, 1960; Farquhar and Palade, 1960; Farquhar, Wissig and Palade, 1961; Farquhar and Palade, 1962). At these fenestrations, aggregation of ferritin particles occurs on the capillary aspect of the basement membrane, while the basement membrane on its epithelial aspect contains fewer particles. This suggests that a restriction to the passage of these macromolecules is exerted by the basement membrane. Much of the ferritin which succeeds in traversing the basement membrane is taken up by the epithelial foot processes of Bowman's capsule, but some particles reach the capsular space - most being taken up in turn by cells of the proximal tubule.

There is a considerable accumulation of large particles such as colloidal gold, ferritin, or thorotrast in the subendothelial space following injection. Naturally occurring macromolecules (not

readily demonstrable on electron microscopy) may show a similar accumulation. Such an accumulation, if persistent, might well interfere with filtration (Farquhar and Palade, 1962; Latta et al, 1960). These workers showed, however, that macromolecules do not persist in the subendothelial space for a prolonged period, but are - by a poorly understood process - transported to the mesangial cells of the glomerular capillaries.

Electron microscopy studies have also shed light on the mechanisms involved in the tubular reabsorption of protein. The cells of the proximal convolution have numerous long and slender brush border prolongations. The effect of this type of ultra-structure is to bring a thin layer of filtrate into intimate apposition with the tubule cell (Miller, 1960) - a situation favouring efficient tubular reabsorption. While the smaller molecules of the filtrate appear to pass readily through the cell membrane, larger molecules, such as proteins, are taken up by tiny openings in the cell surface. These openings are invaginations of the cell membrane. The invaginations can become distended to form vacuoles (Richter, 1958; De Duve, 1959; Rhodin, 1967). Distended vacuoles break off their connection with the surface of the tubular cell and move into more deeply situated parts of the cytoplasm. Acid hydrolic enzymes enter the vacuoles (at this stage these are sometimes called lysosomes) and the protein, reabsorbed by a process akin to phagocytosis, is digested (Miller and Palade, 1964). The lysosome system of reabsorption appears to have a limited capacity (Rhodin, 1967) and protein entering the cells in excess of this capacity appears to coat mitochondria

(Oliver et al, 1954a and b). Fusion of several protein-mitochondrial complexes often occurs, and large accretions then occur in the cell. These accretions are visible on light microscopy, and would appear to represent the protein reabsorption droplets discussed in section 1c. This scheme of protein reabsorption (Rhodin, 1967) - which is still the subject of some controversy (Oliver et al, 1954 a and b; Miller and Palade, 1964) - would appear to explain why, although protein reabsorption occurs in the proximal tubules continuously, droplets are only observed occasionally in normal kidneys. If the lysosome method of reabsorption is the method which operates when only small concentrations of protein are present in the tubular fluid, since lysosomes can not be detected on light microscopy, droplets would only be expected to appear when the lysosome system was saturated by the appearance in the glomerular filtrate of unusually large amounts of protein.

Although the stop-flow studies of Lathem et al (1960) raise the possibility of a secondary site of haemoglobin reabsorption in the distal nephron, there is no evidence, either on light microscopy or electron microscopy, that protein reabsorption occurs at any sites other than Bowman's capsule and the proximal tubule (Rhodin, 1967).

2. The Concept of "Protein Selectivity" and its Relationship to the Functional Integrity of the Glomerular Filter

In mammals - and in most other animals also - tubular fluid is produced by filtration at the glomerulus. Provided that the changes produced by tubular reabsorption do not distort the size distribution of the macromolecules present in the filtrate to an excessive degree, the study of this size distribution in urine can be expected to yield information concerning the functional pore size of the glomerular filter. Strauss (1916) noted that the albumin to globulin ratio in the urine of nephrotic patients differed markedly from the ratio present in the serum of these patients. As a result of this, he stated that it was necessary to postulate that protein excretion was "a selective process carried out by the glomerulus". Although Bayliss and Tookey-Kerridge (1933) did not use the term selectivity, they showed that whether or not a given protein was filtered by the glomerulus depended on its size; in other words they demonstrated that the glomerulus was a selective filter.

All filters are, by definition, selective - in that they will hold back particles of over a certain size while allowing smaller particles to pass into the filtrate. In relation to proteinuria, the glomerulus is said to be highly selective if it allows small protein molecules to pass through to the filtrate, but holds back all but trace amounts of larger protein molecules. Where the cut off is less sharp, and relatively large amounts of globulins are excreted in addition to albumin, the glomerulus is said to be poorly selective.

In major proteinuria (over 1 g/day), almost all the protein appearing in the urine is of glomerular origin. The tubular reabsorption of protein appears to be a non specific process, resulting in the concentrations of proteins relative to each other in the glomerular filtrate being the same as those obtaining in the urine. Since the size distribution of the proteins of the urine represents the size distribution of the proteins in the glomerular filtrate (Hardwicke and Squire 1955; Hardwicke and Soothill, 1961), it is to be expected that the protein excretion pattern in major proteinuria might yield information concerning the functional integrity of the glomerulus.

A simple if inaccurate way of assessing this size distribution is to measure the urinary albumin to globulin ratio. Hoffman (1882) did this using a gravimetric method following fractional precipitation. He did not report his findings in detail, but stated that a low albumin/globulin ratio in the urine was indicative of a severe destructive process in the kidney, while a high albumin/globulin ratio indicated a more benign lesion. Hiller, McIntosh, and van Slyke (1927) measured the albumin/globulin ratio in 29 cases with major proteinuria. In four cases the ratio was above 10. One of these cases made a spontaneous and complete recovery, while the remaining three showed little deterioration over a follow up period of just under two years. In the 16 cases where the A/G ratio was below 5, 13 died within 18 months, while the remaining three showed marked deterioration. Similar results were reported by Blackman et al (1941).

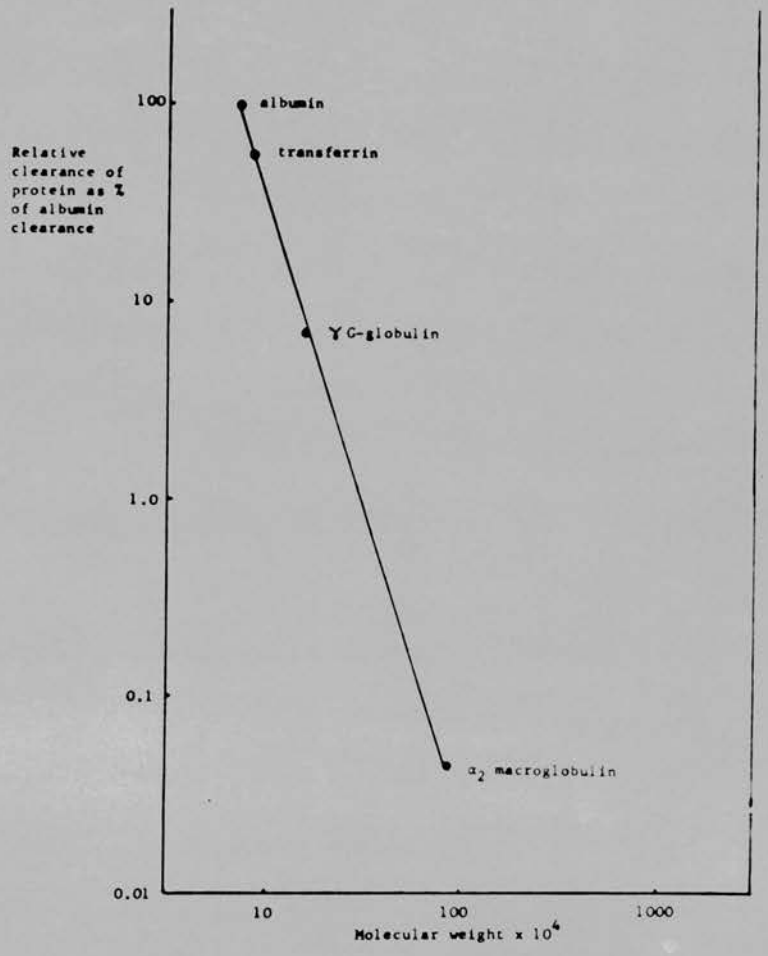
Other workers, however, (Strauss, 1916; Movat et al, 1962) failed to find any consistent relationship between the protein excretion pattern and the severity of the disease process. The excretion of a protein depends not only on its molecular size in relation to the functional pore size of the glomerulus, but also on its plasma concentration. Since proteinuria leads to substantial changes in serum protein concentrations, it is important to assess the relative clearances of the urinary proteins and not simply their relative concentrations. The urine albumin/globulin ratio can obviously be affected considerably by a marked reduction in the serum albumin concentration, and this fact (as well as the rather imprecise methods of protein fractionation available before the second world war) may well account for the conflicting results obtained by the early workers in the field of urine protein excretion patterns.

Wolvius and Verchure (1957) carried out a study on the protein excretion pattern in a number of types of proteinuria. When they simply expressed the albumin excretion as a percentage of the total protein excretion, no consistent pattern emerged. When they divided the globulin clearance by the albumin clearance, however, using the formula $\frac{U\ \text{globulin}}{P\ \text{globulin}} \times \frac{P\ \text{albumin}}{U\ \text{albumin}}$, they found that this ratio lay between 0.1 and 0.2 in all their cases of nephrotic syndrome but was considerably higher in their other groups of patients.

Hardwicke and Squire (1955) assessed the clearances of a number of fractions of plasma by an electrophoretic method and suggested that the assessment of the renal clearances of a variety

Figure 6

Showing the linear relationship which is obtained when relative clearance is plotted against molecular weight on a double logarithmic scale.



of proteins of varying molecular weights would provide a parameter of renal function of diagnostic and prognostic significance. This Birmingham group gave substance to their prediction in 1960, when Blainey, Brewer, Hardwicke, and Soothill published a study of patients with the nephrotic syndrome where clinical features were correlated with the results of protein clearance studies using an immunodiffusion method (Soothill, 1962) to determine the clearances of individual proteins and with renal biopsy appearances. When the urine to plasma ratios of the various protein concentrations were plotted against the molecular weights of these proteins on a logarithmic scale, a straight line was obtained. (See figure 6). In minimal lesion glomerulonephritis, the slope of the line was steep, indicating that the clearance of high molecular weight protein was very substantially less than that of albumin. The slope of the line obtaining in membranous glomerulonephritis was much flatter, indicating a less sharp cut-off in the filtration of high molecular weight protein. In proliferative glomerulonephritis, the slope of the line relating the logarithm of protein clearance to the logarithm of the molecular weight of the protein concerned occupied an intermediate position. In minimal lesion glomerulonephritis, therefore, it appeared that the proteinuria was highly selective and in membranous glomerulonephritis the proteinuria appeared unselective.

Since the publication by Blainey et al (1960) of this important paper, the idea that protein selectivity determinations are capable of yielding information of considerable diagnostic and prognostic value concerning patients with proteinuria has steadily

gained ground. Many workers (e.g. Joachim et al, 1964; Robson, 1967; Vere and Walduck, 1966; and Hitzig et al, 1965) have reported results in overall agreement with those of Blainey et al (1960). These findings and those of others in the same field will be discussed in detail in a later section of this thesis.

3. Glomerular Permeability to Macromolecules other than Protein

From the above account of the renal handling of protein, it will be seen that protein clearance studies can give considerable information regarding glomerular permeability - provided that the amount of protein excreted in the urine is substantial. In situations where protein is present in the urine in trace amounts only, or where the glomerular origin of the protein is in doubt, protein clearance studies are difficult to interpret.

The use of macromolecules other than protein to study glomerular permeability is obviously of interest. Shaffer et al (1948) showed in the dog that low molecular weight fractions of polyethlene glycol had a renal clearance equal to that of inulin, and that the clearance of higher molecular weight fractions was substantially less. Brewer (1951) reported similar findings with regard to dextran. Campbell et al (1953) found that the clearance of low molecular weight fractions of polyvinylpyrrolidone was equal to the glomerular filtration rate, while the clearance of larger molecules was considerably less.

These workers suggested that the low clearances observed with the high molecular weight fractions of various polymers was due to restriction of glomerular filtration on the basis of molecular size.

Of these macromolecules, dextran is the one which has been studied most intensively. It is a polysaccharide synthesised by Leuconostoc mesenteroides and a number of related bacteria when growth occurs in a medium containing saccharose. It is a glucose polymer, the main chains of which occur as the result of 1:6 linkages, with side chains arising from 1:4 linkages. The molecular weight of the

parent substance is about 2 x 10^6, but incomplete acid hydrolysis can be used to obtain fractions in almost any desired molecular weight range.

Ingelman and Halling (1949), Grotte et al (1951) and Friberg et al (1951) showed that following injection of various dextran fractions, the quantity of dextran appearing in the urine bore an inverse relationship to the average molecular weight of the preparation injected.

Brewer (1951) carried out clearance studies using dextran in the rabbit. He found that for fractions of average molecular weight 7,000 or less the dextran clearance was equal to the creatinine clearance. Dextran molecules with an average molecular weight of 25,000 had a renal clearance which was 19% that of creatinine, while for a fraction with an average molecular weight of 35,000, the renal clearance was 6% of the creatinine clearance. Giebisch, Lauson and Pitts (1954) carried out renal clearance studies on the dog which gave substantially similar results. After correcting for the renal elimination of dextran, they assessed the volume of distribution of dextran. This showed a continuing increase with time, and from this they deduced that the metabolic breakdown of dextran in the body gave a plasma clearance of between 0.5 and 1.0 ml per minute.

Wallenius (1954) published a monograph which gave the results of his extensive and meticulous studies of the renal handling of dextran, in man and in animals. He prepared dextran standards of narrow molecular weight range by alcohol precipitation and determined the precise molecular weights of these standards

by viscosity, ultracentrifugation, and diffusion measurements. He devised a method of turbidimetric titration of urine and plasma samples following alcohol precipitation, using his narrow fractions for calibration purposes. This gave an estimate of the molecular size distribution of the dextran present in his samples. The total amount of dextran present was assessed colorimetrically with anthrone following acid hydrolysis.

Serial determinations of dextran clearance were performed following a single injection of a dextran fraction with an average molecular weight of 20,000. The clearance values obtained fell with time. Since the smaller molecules of a polydispersate substance are excreted more rapidly than the larger molecules, the average molecular weight of the substance remaining in plasma rises with the passage of time, and the clearance of the polydispersate substance falls.

The clearance of low molecular weight dextran (M.W. 4,000 average) was measured in the dog, and compared with the exogenous creatinine clearance. For dextran molecules of this size, there was substantial agreement between the renal clearance and that of creatinine, over a wide range of plasma concentration. The dextran clearance was between 92% and 109% of the creatinine clearance. These results indicate that no appreciable re-absorption of dextran occurs in the renal tubules.

Several workers have demonstrated dextran within the tubular cells following intravenous injection. [Zettergren (1962); Maunsbach et al (1962); James and Asworth (1961).] Wallenius admitted that passive diffusion of dextran into cells almost

certainly occurs. He pointed out, however, that the presence of small amounts of dextran in the tubular cells occurred only in the presence of much larger amounts of dextran in the tubular lumen. There was no demonstrable renal threshold for dextran. The "reabsorption" of low molecular weight dextran was therefore passive, occurring along a concentration gradient, and the quantities involved were so small as to be undetectable by clearance studies. As far as the larger molecular weight fractions of dextran are concerned, their ability to penetrate the tubular cell by a passive process would be much more restricted than that of dextran molecular weight 4,000. Wallenius concluded, therefore, that the low renal clearances observed for high molecular weight dextran fractions were a function of restricted passage through the glomerulus rather than of tubular reabsorption.

Clearance studies using a range of dextran sizes were carried out on dogs. Dextran of molecular weight 8,500 was found to have a clearance which was 91% that of creatinine, while dextran of molecular weight 28,500 had a clearance which was 11% that of creatinine. The close inverse relationship obtaining between molecular size and renal clearance suggested that the glomerulus was behaving like a porous seive with selective properties as regards dextran. Wallenius pointed out, however, that these results, like the results discussed above for protein, could be equally well explained in terms of diffusion through a gel - a process where molecular size has an equally sharp limiting effect on rate of transfer.

Wallenius also studied the excretion of dextrans in the molecular size range of serum protein. The amounts of these larger dextran molecules which appeared in the urine were too small to allow formal clearance determinations by his technique to be meaningful. He therefore assessed the size of the largest dextran molecules appearing in the urine in detectable amounts following dextran injection. In rabbits, guinea pigs, and dogs, the largest dextran molecules found in the urine ranged from 37,000 to 47,000 in molecular weight. The rats which he used were normally proteinuric, and dextran of molecular weight 63,000 could be detected in their urine. In ten normal human subjects, dextrans of up to molecular weight 48,000 were excreted. In sixteen patients with proteinuria the upper size limit of the dextran detected in the urine ranged widely, lying between 49,000 and 104,000 molecular weight.

It should be noted that the above figures are for the upper limits of molecular size <u>detected</u> in the urine, not for the upper limit <u>present</u>. With increasing molecular size, the clearance of dextran in the urine approaches zero asymptotically. The upper limit of size detected in the urine is therefore a function, inter alia, of the specificity and sensitivity of the methods used. This reservation does not affect the validity of Wallenius's conclusion that dextran of high molecular weight enters the urine more readily in the presence of proteinuria than in its absence. Impaired selectivity of the glomerular basement membrane with respect to protein appears to be associated with impaired selectivity with respect to dextran.

The studies of Brewer (1951) and of Wallenius (1954) were concerned mainly with dextran molecules substantially smaller than the protein molecules which appear in the urine in renal disease. The amounts of dextran of molecular weight 40,000 and above which are excreted in the urine are too small to allow of accurate quantitation by the methods they used.

In the Edinburgh renal unit over the past five years our aim has been to quantitate the permeability of the glomerulus to macromolecules, as the amount of information which can be gained from purely qualitative studies is limited. We wished to compare renal selectivity to protein with renal selectivity to other substances of similar molecular size. It was therefore necessary to utilise a method which would allow fractionation and estimation of dextran with considerable accuracy at the upper end of the molecular weight scale. The alcohol fractionation method of Wallenius was too insensitive for use in this context. We therefore used the technique of gel filtration to obtain separation by molecular size of our blood and urine samples. This separation technique was also found to be applicable to protein and polyvinylpyrrolidone selectivity determinations. It will be discussed fully in the section of this thesis which is devoted to methodology.

4. Nature and Aim of the Studies Described in this Thesis

At this point, it is appropriate to outline the aspects of the renal handling of macromolecules which are discussed in the later sections of this thesis.

i) Although the non specific nature of protein reabsorption in the tubules results, by and large, in the urine protein excretion pattern being a reflection of the pattern obtaining in the glomerular filtrate, the existence of tubular reabsorption complicates the interpretation of protein clearance studies. Clearance studies were therefore carried out using dextran and polyvinyl pyrrolidone (PVP) (substances not demonstrably subject to tubular reabsorption), and the results obtained were compared with those derived from protein clearance studies.

ii) The method used routinely for protein clearance determinations was immunodiffusion; that used for dextran and PVP studies was gel filtration. These methods differ considerably in their theoretical basis. In order to determine the validity of comparing dextran or PVP results obtained by one method with protein results obtained by another, protein selectivity determinations using immunodiffusion were compared with protein selectivity determinations using gel filtration, and the degree of correlation between the two methods was assessed.

iii) The renal tract contributes trace amounts of protein to the urine, and where the total amount of protein present in the urine is small, these trace amounts become a significant proportion of the total protein excreted. One cannot therefore assume that selectivity values for protein obtained from normal subjects or

from subjects with proteinuria of less than 1.0G per day reflect glomerular permeability with any degree of accuracy. In this situation, and where the glomerular origin of protein is unclear it was felt that clearance studies on macromolecules not subject to tubular reabsorption would give a better indication of glomerular permeability than clearance studies using protein. Dextran and PVP clearances were carried out on normal subjects, on subjects with postural proteinuria, and in patients with acute tubular necrosis. The results were compared with those obtained from protein clearance studies.

iv) Individuals with normal renal function and without proteinuria can be rendered proteinuric for a time by massive intravenous infusions of plasma or albumin. Whether this effect arises solely from saturation of tubular reabsorption, or whether "pore stretching" arising from plasma expansion plays a part is not as yet clear. Haemophilic patients, infused with large amounts of plasma to control bleeding, occasionally become proteinuric. Since protein is absent from the urine before infusion and disappears some 48 hours after infusion it was felt that the results of protein selectivity determinations in such patients would give a close approximation to the selectivity pattern for protein which exists in the normal kidney, but which is obscured by almost complete reabsorption of the filtered protein. Protein and dextran clearance studies were therefore carried out in a number of these patients in the presence of proteinuria. To assess the importance of any "pore stretching" effect, dextran clearance studies were also carried out when proteinuria was absent.

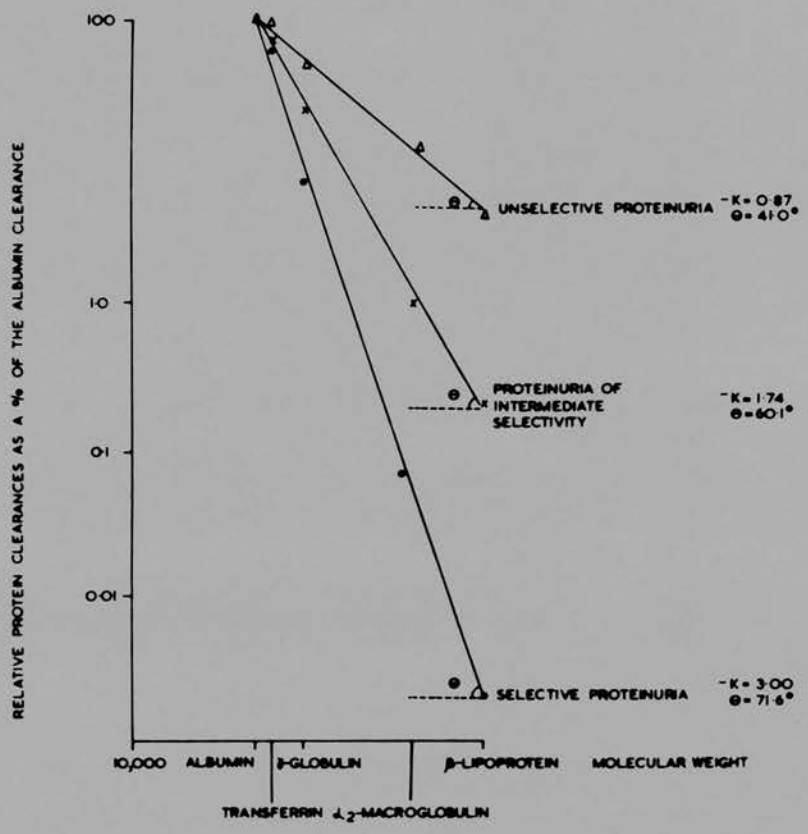
v) Protein selectivity determinations provide information concerning glomerular permeability, and thus add an additional parameter to the assessment of a patient with proteinuria. It is obviously important to attempt to determine the usefulness of this parameter in clinical terms. Towards this end, the results of protein selectivity determinations in 207 patients have been assessed in terms of the histological diagnosis on renal biopsy. The relationship between protein selectivity and a number of other parameters of renal function (such as creatinine clearance and total protein excretion) has been examined. The diagnostic value of selectivity determinations has been evaluated, and their prognostic value - both in terms of rate of deterioration of renal function and responsiveness to steroid therapy - has been assessed.

SECTION II

METHODS

Figure 7

The relationship between log clearance and log molecular weight observed in patients with selective, unselective, and intermediately selective proteinuria.



1. Assessment of Protein Clearances by Immunodiffusion

a. Principle of the Method

Urine to plasma concentration ratios of a number of proteins were determined by gel diffusion against specific antisera, using Ouchterlonie plates. The method was that of Gell (1957) as modified by Soothill (1962) and by MacLean and Robson (1966). Prior to the immunodiffusion process, the urine was concentrated by osmotic ultrafiltration against polyethylene glycol. The individual urine to plasma concentrations ratios were expressed as a percentage of the urine to plasma ratio of a reference protein (albumin or transferrin).

The relationship between the relative clearance values thus obtained and the molecular weights of the proteins concerned was found to be inverse and linear when both variables were expressed in logarithmic units (figure 7). The slope of the line relating $\log_{10}$ relative clearance to $\log_{10}$ molecular weight was taken as the index of selectivity. (-K)

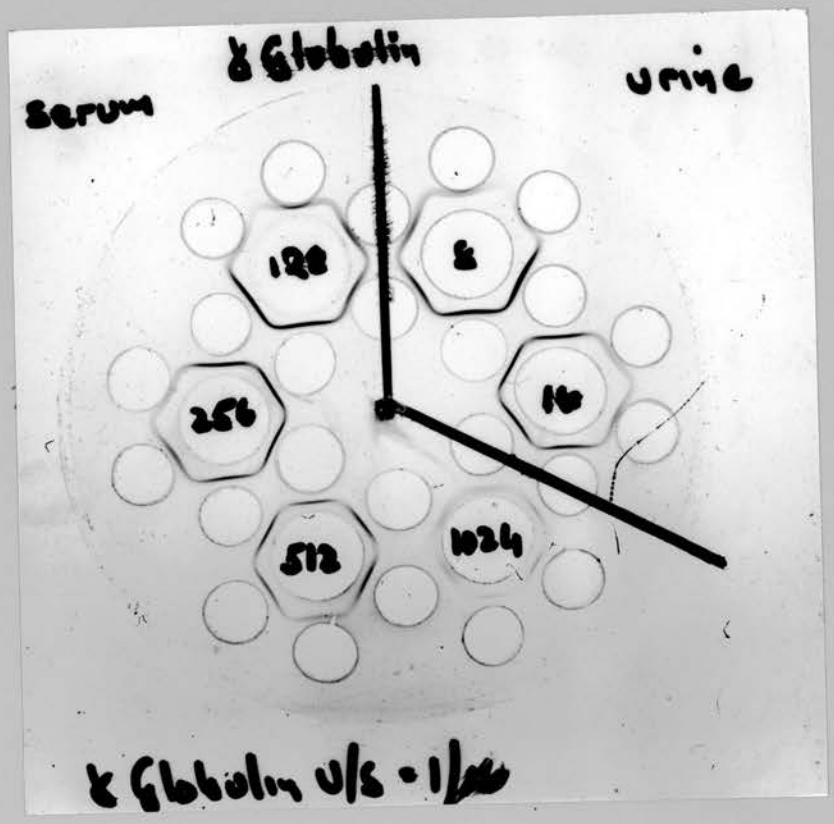
b. Details of the Method

(i) Concentration of the Urine

An aliquot of urine was filtered, transferred to Visking dialysis tubing, and then dialysed overnight against cold running tap-water. The dialysis sac was then placed in a solution of poly-ethylene glycol (Carbowax 20 M) and concentrated to a volume of 1 to 2 ml. This method of concentration is essentially that described by Berggard and Rissinger (1961). Some counterdialysis of polyethelene glycol occurs, but this does not interfere with

Figure 8

The determination of urine to plasma concentration ratios of specific proteins by the technique of immuno-diffusion.



The protein being assayed on the plate illustrated is gamma globulin (IGG). The hexagon representing a 1/16 dilution of urine corresponds to that representing a 1/256 dilution of serum. The urine to serum ratio of IGG is therefore 16/256, or 1/16.

immunological reactions (Howe, Groome and Carter, 1964; MacLean, 1966). The dialysis membrane is not absolutely impermeable to albumin (Grant, 1957). Recovery experiments carried out by Dr. MacLean, however, showed that in our experiments the loss of albumin during urine concentration was between 1 and 2% (MacLean, 1966).

(ii) Clearance Determinations of Individual Proteins

Agar plates were prepared containing phosphate saline buffer, 0.2M, pH 7.0. On each plate, six large holes (for antigen) each surrounded by six smaller holes (for antiserum) were cut, using a special template. Four dilutions of serum and two of urine concentrate were placed in the antigen holes, while serial dilutions of an antiserum specific for the protein under assay were placed in the surrounding antibody holes (see figure 8). A separate plate was used for each protein assayed. The plates were left at room temperature for 24 to 48 hours to allow diffusion. Precipitin lines formed where the proportions of antigen and antibody were optimal. When the antigen concentration was high relative to the antibody concentration, the precipitin line was further from the antigen containing well than when the antigen concentration was low. Since each antigen-containing well was surrounded by six wells containing antiserum, the precipitin lines formed a hexagonal pattern. For estimations carried out on a single plate using predetermined dilutions of the same batch of antiserum, the size and shape of each hexagon depended solely on the amount of antigen present in the central well. By matching the position and intensity of the lines forming the urine hexagons

TABLE 1

The clearance of IgG globulin can be expressed as

$$\frac{Ug.V}{Pg.T}$$

The clearance of albumin can be expressed as

$$\frac{Ua.V}{Pa.T}$$

where

V = volume (millilitres)

T = time (minutes)

Ug and Ua = urine concentration of IgG and albumin respectively (mg per 100 ml.)

and Pg and Pa = plasma concentration of IgG and albumin respectively (mg per 100 ml.)

If the IgG clearance is expressed as a percentage of the albumin clearance, we arrive at

$$\left(\frac{Ug.V}{Pg.T} \times \frac{Pa.T}{Ua.V} \times 100\right)$$

V and T cancel out, and the expression simplifies to

$$\frac{Ug}{Pg} \times \frac{Pa}{Ua} \times 100$$

with those derived from the plasma dilutions, the urine to plasma concentration ratio for the protein being assayed could be determined. (See figure 8). For clearance studies urine to plasma concentration ratios are as satisfactory as absolute figures. It was not necessary therefore to include a standard of known composition in the assay.

The antisera used were manufactured by Behringwerke, A.G. (Marburg-Lahn, Germany) and supplied by Hoescht Pharmaceuticals. Their specificity was checked by immunoelectrophoresis and found to be satisfactory (MacLean, 1966).

Between 1962 and 1966, urine to plasma ratios of five proteins were determined for estimations of protein selectivity - albumin, transferrin, IGG gamma globulin, alpha 2 macroglobulin, and beta lipoprotein. Between 1966 and 1969, however, the urine to plasma ratios of two proteins only were determined routinely - transferrin and alpha 2 macroglobulin.

In conventional clearance studies, accurate timing and completeness of the urine collection are of vital importance. If the clearance of the various proteins are expressed as percentages of the clearances of a reference protein, however, the terms for volume and time disappear from the equation (see opposite, table 1). The use of a method of calculation which compensates automatically for collection errors is obviously useful for studies which, as often as not, are carried out on an out-patient basis.

The relationship between relative clearance and molecular weight was found to be linear on a double logarithmic plot (figure 7). For the five protein method, the slope of the line

TABLE 2

Calculation of protein selectivities by immunodiffusion.

Protein	Molecular Weight (M.W.)	Log_{10} M.W.	Clearance (% of Albumin)	Log_{10} of 10 x % Clearance
		x		y
Albumin	69,000	4.84	100	3.00
Transferrin	90,000	4.95	88	2.94
gamma(I_gG) globulin	150,000	5.18	22	1.34
alpha Macroglobulin	840,000	5.92	1.4	1.15
Beta Lipoprotein	2,500,000	6.40	0.11	0.04

K, the slope of the line relating Log clearance and Log M.W. is determined by the method of least squares.

$$K = \frac{E.xy - \frac{E.xE.y}{n}}{E.x^2 - \frac{(E.x)^2}{n}}$$

$$= \frac{48.26 - 51.68}{150.76 - 148.94} = \frac{-3.42}{1.82}$$

$$= -1.88$$

Thus $(-K) = 1.88$

relating $\log_{10}$ clearance (as a percentage of albumin clearance) to $\log_{10}$ molecular weight was determined from the five points obtained by the method of least squares. For the two protein method, the transferrin clearance was taken as 100% and the alpha 2 macroglobulin clearance was expressed as a percentage of this. Here the slope of the line relating log clearance to log molecular weight was determined from two points only. This slope was taken as the index of selectivity. (-K)

To clarify the arithmetic involved in the calculation, a worked example is shown in table 2.

c. Discussion of Method: Estimation of the Error of the Method

Studies of the reproducibility of the method for the estimation of the urine to plasma ratios of the individual proteins have been carried out by Dr. Pamela MacLean and are discussed fully in her Ph.D. Thesis. (MacLean, 1966). Using ten urine and ten serum aliquots of the same specimens, the following coefficients of variation (CV) were found.

Albumin urine to plasma ratio - 16%

Transferrin urine to plasma ratio - 8%

Gamma (IG) globulin to plasma ratio - 11%

Alpha 2 macroglobulin urine to plasma ratio - 7%

Beta lipoprotein U to P ratio - 11%

When the coefficient of variation was determined for the 5 protein index of selectivity it was found to be only 2%, although in a second series of duplicate determinations, a CV of 4% was obtained. This surprisingly high reproducibility was partly due to the "averaging out" effect of using five points in constructing the

line, and also to the fact that the logarithms of the urine to plasma ratios were employed in the determination of the slope of the line, rather than the ratios themselves.

The change from the five-protein method of selectivity determination to a method employing clearance determinations on two proteins only requires explanation. Cameron and Blandford (1966) compared the results of selectivity determinations based on transferrin and IGG globulin only with determinations based on clearance studies involving six proteins. They showed that the results of the simplified method correlated well with those obtained using six proteins.

In terms of time and expense, a method employing two proteins has obvious advantages over one employing five or six. It was felt in Edinburgh, however, that Cameron and Blandford had perhaps not chosen the two best proteins. An analysis of our results showed that a better correlation with the results of our five protein method was obtained when transferrin and alpha 2 macroglobulin were used for selectivity determinations than when transferrin and IGC globulin were the proteins assayed (MacLean and Robson, 1967).

The computation of the slope of a line from the position of two points one it is obviously a potentially inaccurate procedure. If the two points chosen represent only a small portion of the line, the inaccuracy will tend to be greater than when the two points chosen are more widely separated on the X axis. Transferrin has a molecular weight of 90,000 (Koechlin, 1952) and I 9G globulin has a molecular weight of 150,000 (Isliker, 1957). Alpha 2

Figure 9a

Comparison of selectivity results obtained by the standard five protein immunodiffusion method with results obtained by a two protein method employing transferrin and gamma (IGG) globulin.

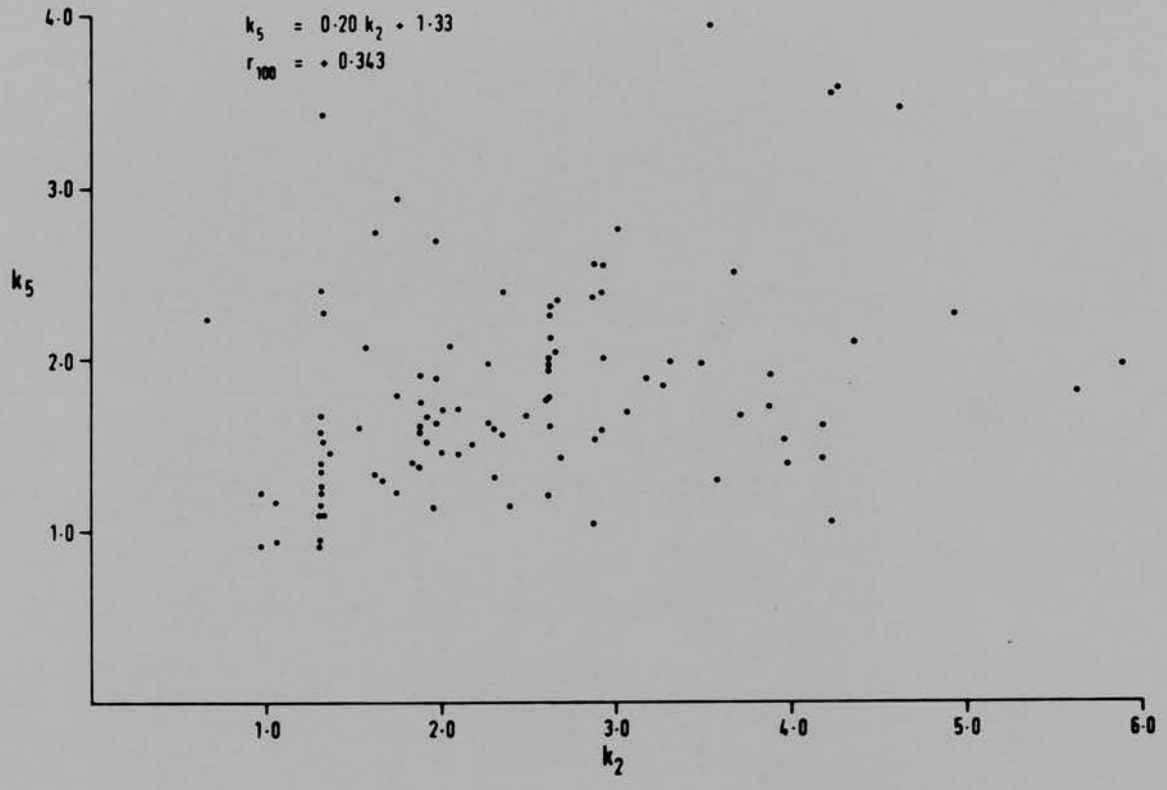
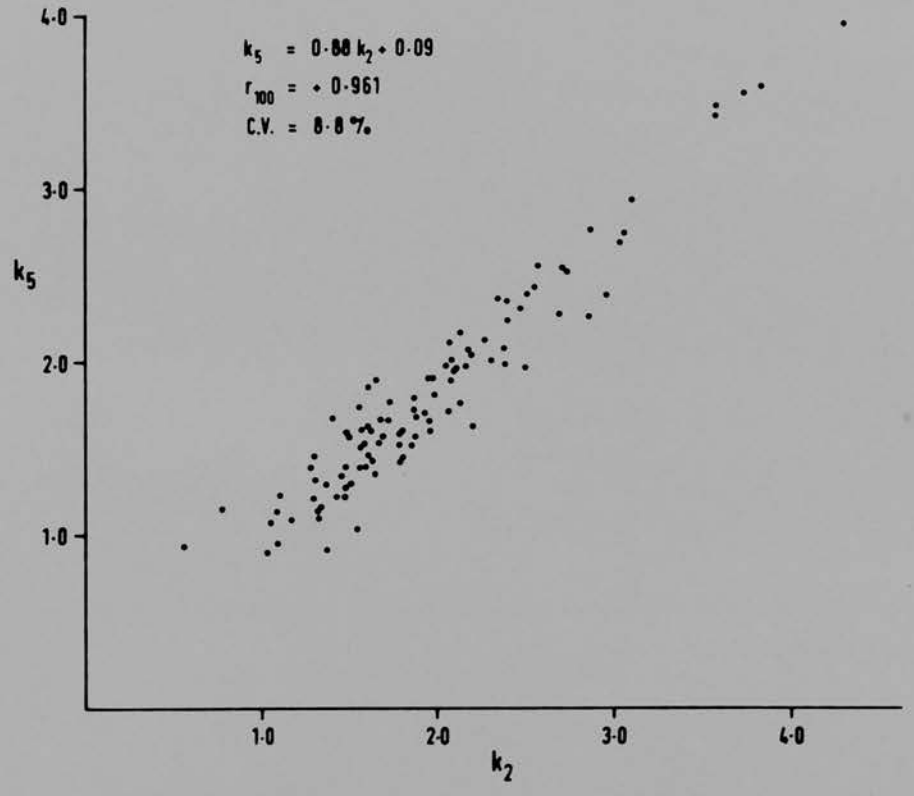


Figure 9b.

Comparison of selectivity results obtained by the standard five protein immunodiffusion method with results obtained by a two protein method employing transferrin and alpha 2 macroglobulin.



macroglobulin has a molecular weight of 840,000 (Schultze, 1957). Using alpha 2 macroglobulin as the second protein instead of IGG globulin means that two proteins of widely dissimilar molecular weights are being employed instead of two proteins of relatively similar molecular weight. Because of the wider separation of the two points on the X axis, the transferrin - alpha 2 macroglobulin system of selectivity determination might be expected to prove more accurate than the transferrin - IGG globulin system. This expectation was realised in practice. For 100 selectivity determinations, the transferrin - IGG globulin system gave a correlation coefficient of 0.343 when compared with the five protein method, while the transferrin - alpha 2 macroglobulin system gave a correlation coefficient of 0.961. (See figure 9. MacLean and Robson, 1967).

Where the two protein transferrin / alpha 2 macroglobulin method was employed, selectivity determinations were found to have a coefficient of variation of 9.2%, as compared with 2.0% to 4.0% using the five protein method.

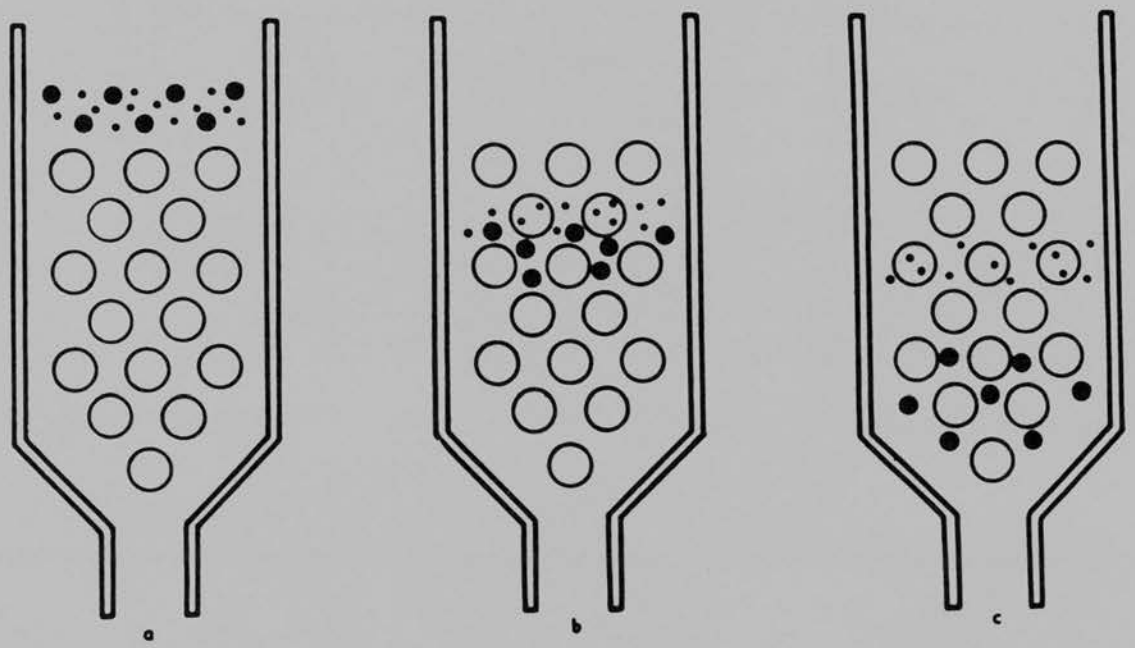
Had albumin been used in place of transferrin in the two protein method, a third plate to determine the concentration factor for the urine would have been required, as a urine concentrated sufficiently to give alpha 2 determinations often contains an amount of albumin in excess of the optimum for this method of immunoassay. The change to the transferrin /α_2 macroglobulin two protein method, therefore, involved a change in the reference protein as well as a loss in accuracy. This change in reference protein altered the position of the line relating log clearance to log molecular weight. It did not however alter the

slope of the line - and it is the slope of the line which is the index of selectivity.

Although the results of the five protein method and those of the two protein method do not have equal accuracy, there is no systematic difference between the results obtained by the two methods (MacLean and Robson, 1967).

Figure 10

Separation of molecules on the basis of molecular size by gel filtration.



The large particles are excluded from the interstices of the Sephadex molecules, and are eluted in a volume of fluid equal to Vo, the external liquid phase. The small particles are able to enter the internal liquid phase of the gel, and in consequence are eluted later.

2. The use of Gel Filtration in Determining Relative Renal Clearances of Macromolecules

a. Principle of the Method

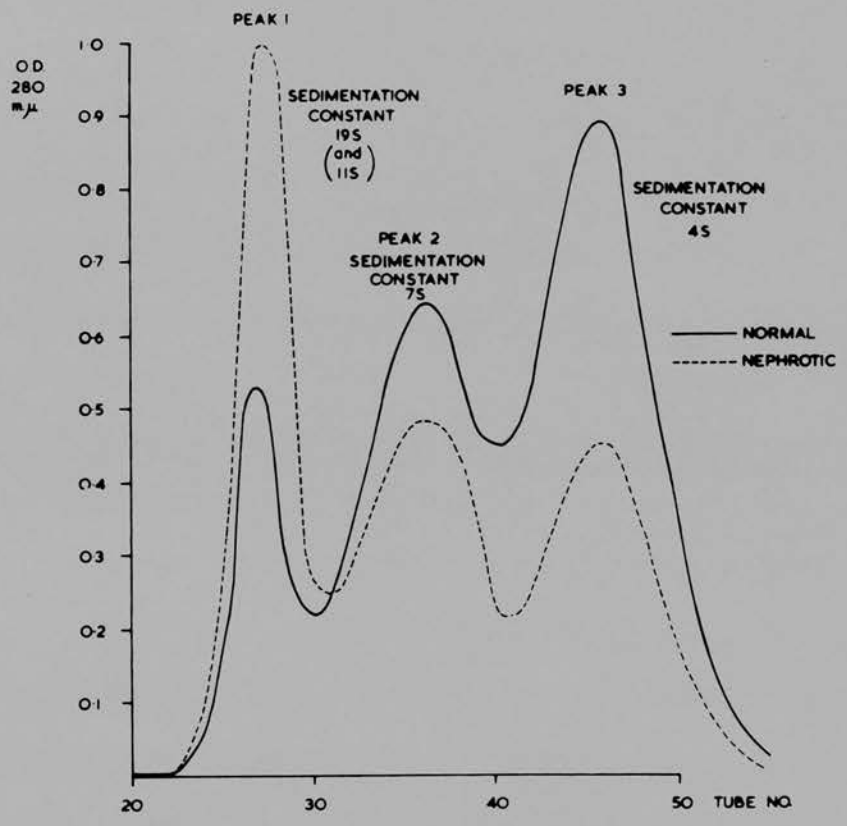
This technique has a number of names, the most descriptive of which is possibly "molecular seive chromatography". It is based on the fact that columns packed with any one of a number of substances (e.g. agar, starch, or cross-linked dextran) will fractionate the component parts of a mixture of macromolecules on the basis of molecular size. This fractionation is reproducible, and given appropriate calibration of the system, the "effective molecular radius" of a substance can be calculated from the position of its elution peak (Porath, 1967, Ackers, 1964). Provided that the substances under test and those used in calibration are of comparable molecular shape and possess comparable partial specific volumes, gel filtration techniques can also be used to estimate molecular weights (Ackers, 1964, Andrews, 1965, Andrews, 1967).

The media most often used for gel filtration are the cross-linked dextrans manufactured by Pharmacia and marketed under the generic title of "Sephadex". The liquid phase of such a gel is in two compartments, one compartment being within the interstices of the dextran molecules, and the other being outwith the molecules. We can denote the volume of the entire liquid phase with the symbol Vt, and use Vi and Vo to denote respectively the volumes of the compartments within and without the dextran molecules. Vt is equal to Vi plus Vo.

Vo, the external liquid phase, can be measured by determining the volume of elution of a substance of very high molecular weight

Figure 11

The elution patterns of normal and nephrotic sera on G 200 Sephadex.



(e.g. for G 200 Sephadex, a substance of molecular weight over 10^6). When such a substance is passed through a gel filtration column, it is excluded from the interstices of the gel because of its molecular size. As it is confined to the external liquid phase, its volume of elution gives a measure of Vo.

Vt, the volume of the entire liquid phase, can be measured by determining the elution volume of a very small molecule (e.g. for G200 Sephadex, a substance with a molecular weight of under 500). Such a substance enters the entire liquid phase of the system freely, and its elution peak measures the volume of this phase.

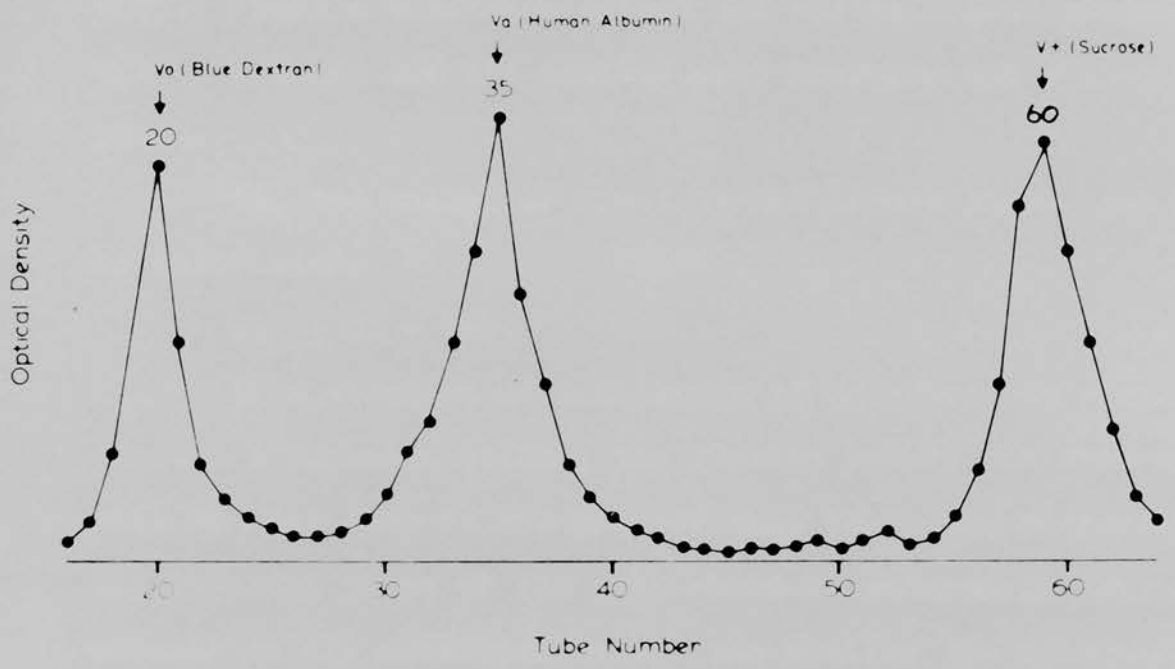
Vi, the volume of the internal liquid phase, can readily be calculated.

(Vi = Vt - Vo).

If a solution of a molecule of intermediate size be passed through a Sephadex column, the elution volume for this molecule will be less than Vt and greater than Vo. The proportion of Vi, the internal phase of the gel, which is entered by this molecule depends on its molecular size; the smaller the molecule, the more completely it will penetrate the internal liquid phase, and the later will be its elution peak. The proportion of the interna luqiid phase penetrated by such a molecule can be regarded as being its "distribution coefficient" for the system concerned, and can be represented by the term Kd. Kd is constant for a given molecule fractionated on a given column under a given set of conditions. If the elution volume of substance x be represented as Ve (x) then Kd (x) equals $\frac{(Ve(x) - Vo)}{Vi} = \frac{(Ve(x) - Vo)}{(Vt - Vo)}$ (see figure 12, facing p.63).

Figure 12

Calibration run on G 200 Sephadex, illustrating the determination of the Kd value for albumin, M.W. 68,000, molecular radius 37A.



Vo = 20 tubes; Va = 35 tubes; Vt = 60 tubes

Kd albumin = (Va-Vo)/(Vt-Vo) = 15/40 = 0.375.

With suitable calibration, once this term Kd (x) has been determined, the effective molecular radius of x can be determined, or the molecular weight of x estimated.

It should be noted that these considerations only hold good where there is no adsorption of the substance x under test on to the column.

Figure 11 shows the elution patterns of normal and nephrotic sera.

Figure 12 shows a calibration run illustrating the determination of the Kd value for albumin.

b. Details of the Method

(i) Preparation and Operation of the Gel Filtration Column

The techniques used were broadly those outlined by Flodin (1957). As macromolecules within the molecular weight range of the serum proteins were of greatest interest, the gel used was G200 Sephadex (Pharmacia, Uppsala). This substance fractionates effectively over the molecular weight range 5,000 to 800,000 with respect to globular proteins, and over the range 1,000 to 200,000 for polysaccharides.

The dry Sephadex gel was mixed with about 1,000 times its weight of water, and left to soak for at least 24 hours. The supernate was discarded, and the residual gel mixed again with an excess of distilled water. This process was repeated some twenty times to remove the finer Sephadex particles, which (if allowed to remain) tend to pack tightly during column operation and impede flow. The gel was washed with the eluant buffer (0.1M tris/HCl: 0.1M NaCl, pH 8.0), and then left for at least 24 hours in a further excess volume of buffer for equilibration.

The details of actually packing the column are fully described by Flodin (1957). Care had to be taken that the columns were mounted vertically and that packing occurred evenly.

A number of different columns were used over a period of some five years. These varied in length from 60 to 100 cm, and in diameter from 2 cm. to 4 cm. The operating pressure was, as a rule, between 5 and 15 cm. of buffer and the flow rates employed were in the range 5 ml. to 15 ml. per hour. Constant volume

fractions of between 2 ml. and 5 ml. were obtained using an LKB fraction collector with a syphon attachment. A Marriott bottle was used as the eluant reservoir.

(ii) Preparation of Serum and Urine Samples for Gel Filtration Protein samples

The urine samples were concentrated as for immunodiffusion studies, then dialysed against 0.58M NaCl. This final step was introduced because gel filtration (in contrast to immunodiffusion) does not differentiate between proteins of renal tract origin and those of serum origin. The most important renal tract protein present in urine, from the quantitative viewpoint, is the muco-protein of Tamm and Horsfall. (Tamm and Horsfall, 1952; Grant, 1957; Maxfield, 1961). This protein is precipitated in 0.58M NaCl.

Dextran samples

Samples used for dextran selectivity studies did not as a rule require to be concentrated; in the few instances where this step was required, the method used for immunodiffusion was employed. As the method used in estimating dextran in fact measures total carbohydrate (Dubois et al 1956), the urine and the serum were both deproteinised with trichloracetic acid (final concentration, 5% TCA) immediately before application to the column. This step removed the glycoproteins. When exposure to 5% TCA is brief and at room temperature, it does not lead to any detectable hydrolysis of dextran (see MacLean, 1966, Ph.D. Thesis).

Polyvinyl-pyrrolidone samples

In a few instances the urine required concentration by the method described under immunodiffusion. Otherwise, unmodified urine was used. There is a possibility (see appendix) that slight protein binding of PVP may occur; for this reason deproteinisation of serum and urine samples prior to column application might have been desirable. Unfortunately, all methods of deproteinisation tried resulted in co-precipitation of PVP. Since the binding of PVP to protein is only just detectable it is unlikely that the use of protein-containing solutions introduced an appreciable error.

(iii) Estimation of Protein, Dextran and PVP concentrations in eluate fractions from G 200 column

Protein Estimations

This was, as a rule, by measurement of ultraviolet extinction at 280 mμ , (Warburg and Christian, 1941). The protein concentration in the eluate ranged from 0 mg.% to 200 mg.%, and over this range Beer's Law is followed. This method depends on the aromatic amino acid content of the protein being estimated. It is not absolutely specific for protein, but almost all substances which interfere are of small molecular weight and are separated from the protein by the process of gel filtration.

In some pilot experiments, the method of Lowry was used, as adapted for the autoanalyzer (Mandl, 1961). This is a modified Folin Ciocalteau method, and is particularly useful where high lipid concentrations give turbidity in the tubes corresponding

to the high molecular weight protein range. It is also of use where very small aliquots of eluate are collected. By collecting column fractions with a minimum volume of 2 ml., and by obtaining fasting blood specimens in situations where lipaemia was a problem, we were able to avoid having to use the Lowry method as a routine. Selectivity results obtained by the two above methods of protein estimation were in substantial agreement (MacLean, Ph.D. Thesis, 1966).

Estimation of Dextran Content of Column Eluate Fractions

Dextran was estimated by a modification of the method of Dubois et al (1956). In this method, dextran is hydrolysed to glucose by the action of concentrated sulphuric acid, and the amount of carbohydrate present is estimated by the colour reaction which occurs with phenol. The reagents for this method are stable; for this reason we found it preferable to the anthrone method (Semple, 1957).

A 1 ml. aliquot of column eluate was placed in a boiling tube. 2 ml. of a 5% w/v solution of phenol was added, using an automatic pipette. To this mixture, 5 ml. of concentrated sulphuric acid was added, again using an automatic pipette. As the intensity of the final colour depends on the speed of addition of the acid and on the speed and thoroughness with which mixing is achieved, the plunger of the automatic pipette containing acid was depressed in a constant manner, and the boiling tubes were agitated by hand after the addition in a manner which was as constant as possible. When the tubes had cooled, the extinction due to the brown colour was read at 490 m in a Unicam SP 600

spectrophotometer, against a blank consisting of the phenol and sulphuric acid reagents and the buffer used in elution from the column. The calibration curve, prepared from dilutions of Rheomacrodex (Pharmacia) was linear over the range 0 to 10 mg% of dextran, and departed only slightly from linearity between 10 mg% and 20 mg%. The coefficient of variation for dextran estimations on single samples was 1.5%. Once the tubes had cooled, the colour was stable for about five days, but at ten to twelve days a detectable change in optical density became apparent.

As protein-containing solutions from which dextran was absent gave detectable readings with the Dubois method, samples were deproteinised before being added to the column. Glucose present in blood or urine did not interfere, being separated from the much larger dextran molecules by the gel filtration process.

Scrupulously clean glassware was essential to accurate dextran estimations. The use of sulphuric acid of less than A.R. grade gave a variety of colours and inaccurate results. Careless packing of the column, resulting in the passage of Sephadex in to the eluate, gave extremely high and variable readings for the column blank.

Estimation of I 125 Labelled Polyvinyl-Pyrrolidone

Aliquots of column eluate in glass tubes of standard size were placed in a well scintillation counter. The results obtained were expressed in terms of counts per standard time interval, with adjustment of the figures to make the time interval the same for serum and urine runs from a given patient. Where

Figure 13a

Illustrating the use of gel filtration in the determination of protein selectivity values.

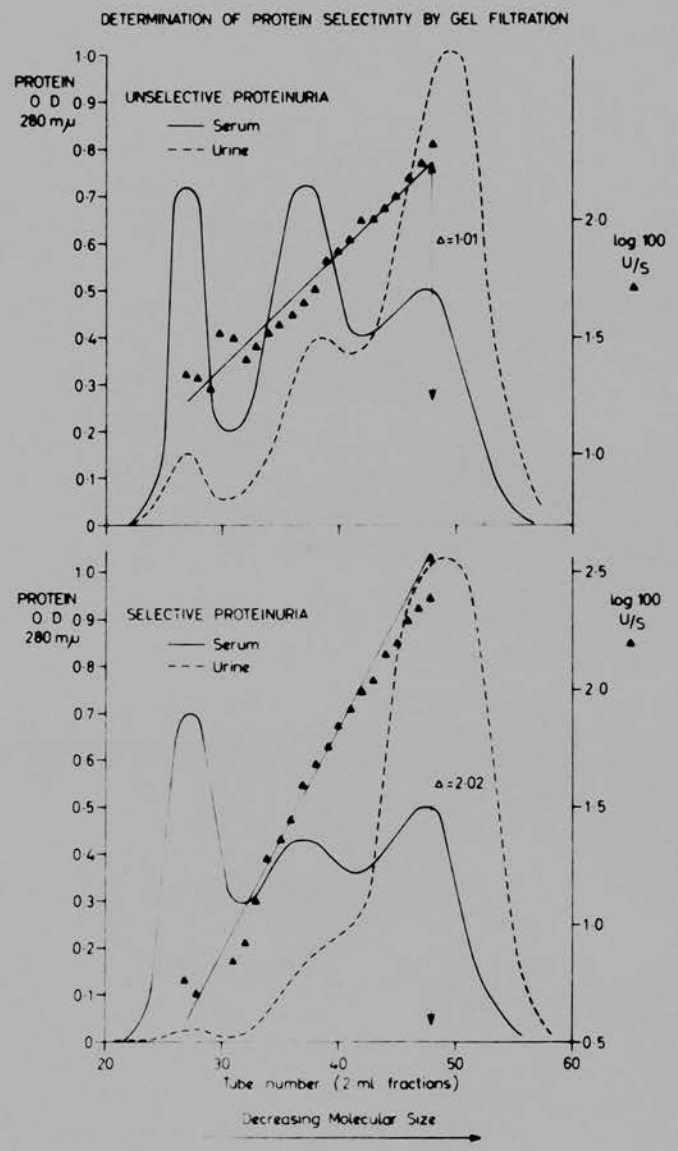
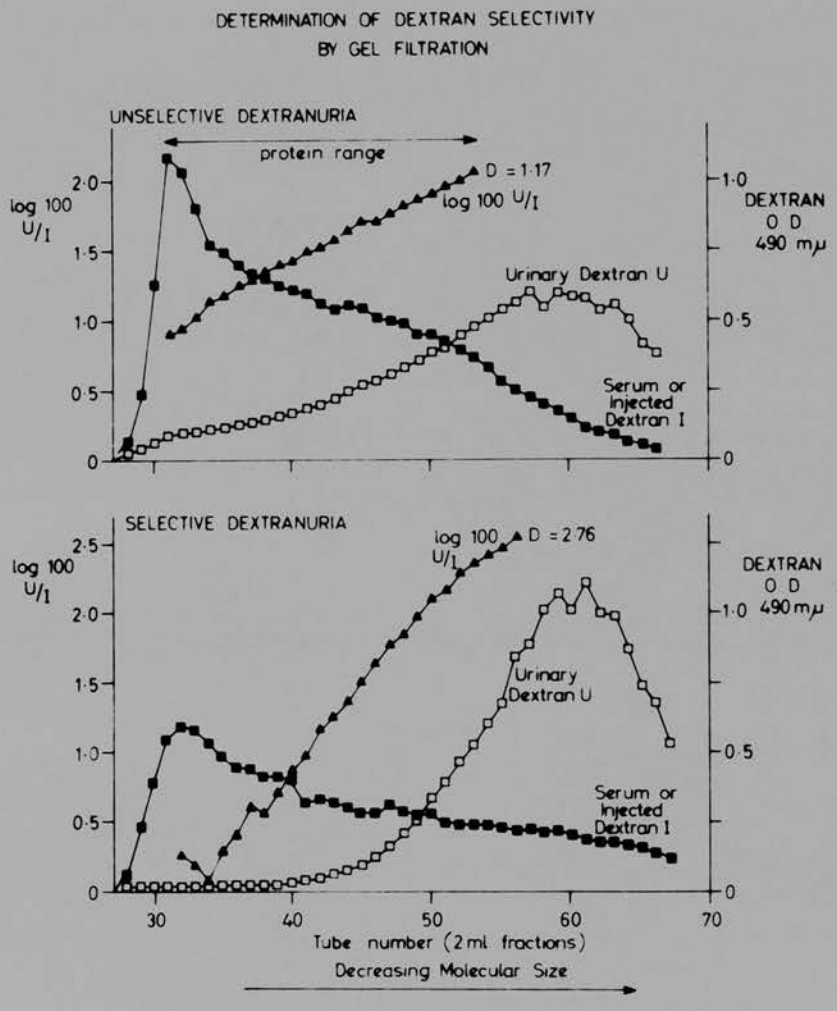


Figure 13b

Illustrating the use of gel filtration in the determination of dextran selectivity values.



a period of several days elapsed between urine and plasma counting, a correction was made for the decay in radioactivity of I 125.

(iv) Selectivity Determinations Using Gel Filtration

This was by the method of Hardwicke (1965). A standard solution was applied to the column, and constant volume fractions were obtained using a fraction collector with a syphon attachment. The standard generally employed consisted of "blue dextran" (Pharmacia), M.W. over 10^6, purified human serum albumin (supplied by the Edinburgh blood transfusion service), and sucrose. The dextran peak gave a measure of Vo, and the sucrose peak gave a measure of Vt. The albumin peak gave a Kd value for a substance of known molecular radius, from which column calibration could be carried out.

An aliquot of urine, treated as in section (ii) above, was then passed through the column, followed by the corresponding serum. A further calibration solution was then passed through the column in order to check the stability of the column characteristics. The concentration of the solute under test was then estimated for each tube from the serum and urine runs, and the urine to plasma ratio calculated for each tube of eluate. Log_{10} of each urine to plasma ratio was taken, and plotted against tube number. Elution volume is of course directly proportional to the tube number, and Andrews (1967) has shown that elution volume varies inversely as log molecular weight, for globular proteins. The plot of $\log_{10} \frac{100U}{P}$ against tube number (as far as protein is concerned), is therefore equivalent to a plot of $\log_{10}$

TABLE 3

Calculation of protein selectivities by gel filtration.

Tube Number	Urine (U) (optical density)	Plasma (P) (optical density)	$\frac{100U}{P}$	$\text{Log}_{10} \frac{100U}{P}$
x				y
1	60	660	9.1	0.96
2	52	330	15.8	1.20
3	53	260	20.4	1.31
4	71	277	25.6	1.41
5	121	336	36.0	1.56
6	175	347	50.4	1.70
7	271	337	80.4	1.91
8	360	322	112	2.05
9	551	350	157	2.20
10	860	410	210	2.32

= n (number of tubes) x m (the slope of the line relating log $\frac{100U}{P}$ and tube number) over the range Va - Vo.

$$= \left\{ \frac{\left(E.xy - \frac{E.xE.y}{n}\right)}{\left(E.x^2 - \frac{(E.x)^2}{n}\right)} \right\} n$$

$$= \frac{(103.7 - 91.4)}{(385 - 302.5)} \times 10$$

$$= \frac{(12.3)}{(82.5)} \times 10$$

$$= 1.49.$$

molecular weight, but (since the number of tubes depends on the size of the column and the volume of the fractions collected) has no fixed units. The slope of the line obtained was therefore multiplied by the number of tubes between the origin (Vo) and the albumin peak. The index of selectivity obtained in this way was assigned the symbol △ , where protein was under assay, D when dextran was being estimated, and P when the macromolecule under test was PVP. (See figures 13a and b).

Hardwicke has suggested (personal communication) that the gel filtration selectivity is more fundamentally expressed in terms of the change in $\log_{10} U/P$ per unit change in Kd value. Since the mean Kd value for albumin found in our calibration runs was 0.38, the multiplication of the values for delta or D found in this thesis by a factor of 2.63 provides a reasonable approximation to selectivity values in terms of the units suggested by Hardwicke.

In the PVP studies, where a molecular weight range wider than that of the serum proteins was studied, it was felt (for reasons discussed below) that the use of "tube number" for the abscissa was not adequate for all purposes. The column was calibrated in terms of effective molecular radius (Ackers, 1964), and $\log_{10}$ 100 U/P was plotted against molecular radius (<u>not</u> log radius). For purposes of comparison, " ? PVP", equivalent to ? and to D, was also calculated for the PVP clearances, and in some instances a probit scale was used for the ordinate.

c. Gel Filtration Determination of Selectivity

Discussion: Error of the Method

The methods used for the estimation of protein, dextran, and polyvinyl-pyrrolidone were accurate in comparison with the errors involved in the process of gel filtration. Duplicate analyses (10 pairs of samples in each case) gave a coefficient of variation of 1.9% for protein, 1.5% for dextran, and 2.2% for PVP.

For selectivity determinations, solute concentrations have to be assigned to their appropriate elution volume (tube number), and serum and urine runs have to be correctly aligned with respect to each other. The column has to give good separation. Where the peaks are unduly broad an excessive amount of low molecular weight solute appears in the tubes corresponding to high molecular weight values, and an erroneously low selectivity result is obtained. The syphon used for collection of eluate aliquots must deliver consistent volumes; a dirty syphon will deliver widely varying fraction volumes and distort the column elution pattern. Even when the syphon is scrupulously clean, varying protein concentrations, by altering the viscosity of the eluate, will result in a slight variation of the fraction volumes obtained from the syphon.

Frequent calibration runs were employed to check column characteristics. A column was not used for gel filtration until the positions of the calibration peaks were constant, plus or minus one tube.

Estimations on ten separate aliquots of serum and ten

separate aliquots of urine taken from a single sample of urine and plasma gave a CV for the method of 7% for protein selectivities by gel diffusion; twelve duplicate determinations of dextran selectivity gave a coefficient of variation of 9.1%. (MacLean, 1966). These studies probably overestimate the reproducibility of the method as they were carried out on a single column with "excellent" rather than "good" separation properties. From results of parallel experiments carried out by Dr. MacLean and myself using different columns of more variable separation qualities, it would appear that the error of the method lies in the range 10% to 15%.

This is a significant error, but it must be remembered that a selective proteinuria gives a gel filtration selectivity of about 2.5, while an unselective proteinuria gives a value of about 1.0. The error is not large in comparison with the differences being examined.

TABLE 4

Pilot Experiments Involving Dextran

Dextran infused - 22 to 33G of 2 to 1 "Dextran" - "Rheomacrodex" mixture in 350 to 500 ml. of normal saline.

Subject	Blood samples used in calculation of D	Duration of urine collection period	D (dextran index of selectivity)	Notes
1	A(start) and B(end) of first collection period	3hrs.	2.91	
	B(start) and C(end) of second collection period	40 minutes	2.81	
2	A(start) and B(end) of first collection period	40 minutes	1.82	
	B(start) and C(end) of second collection period	50 minutes	2.13	
	C(start) and D(end) of third collection period	60 minutes	2.11	
3	A(start) and B(end) of first collection period	20 minutes	1.75	(-K) before dextran infusion = 2.48 (-K) after dextran infusion = 2.61
	B(start) and C(end) of second collection period	30 minutes	1.84	
	C(start) and D(end) of third collection period	20 minutes	2.0	

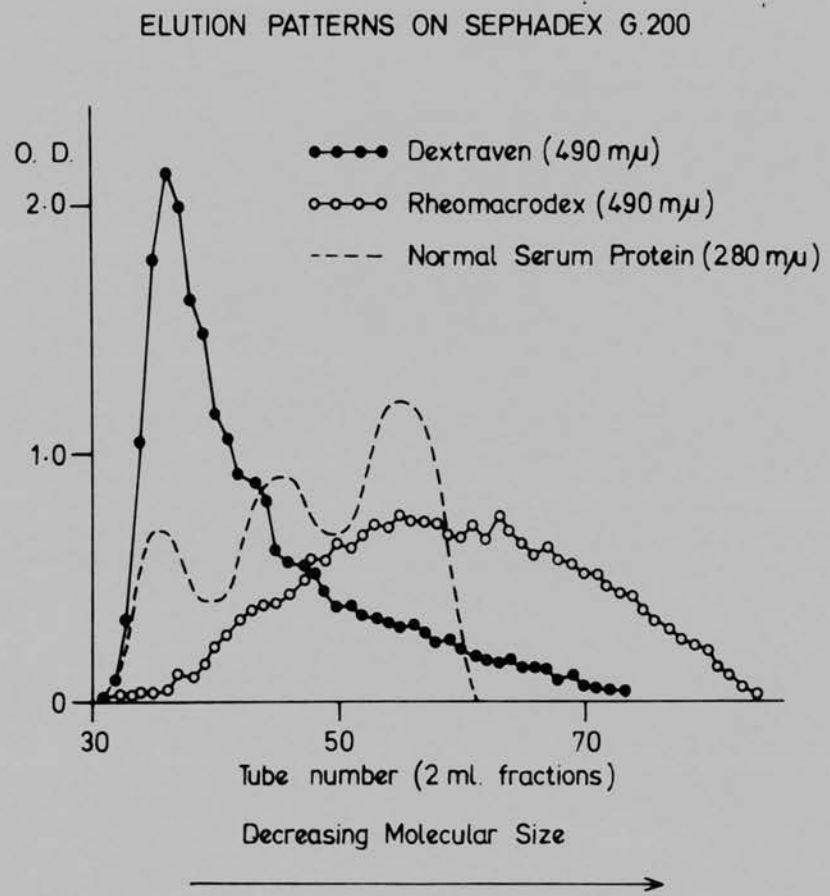
...contd.

TABLE 4 contd.

Subject	Blood samples used in calculation of D	Duration of urine collection period	D (dextran index of selectivity)	Notes
4	A(start) and B(end) of first collection period	45 minutes	1.12	(-K) before dextran infusion = 1.42
	B(start) and C(end) of second collection period	45 minutes	1.36	(-K) after dextran infusion = 1.34
5	A(start) and B(end) of first collection period	40 minutes	2.42	
	B(start) and C(end) of second collection period	40 minutes	2.30	
6	A(start) and B(end) of first collection period	30 minutes	1.95	(-K) before dextran infusion = 1.74
	B(start) and C(end) of second collection period	30 minutes	2.10	(-K) after dextran infusion = 1.80

Figure 14

The elution patterns of two commercially available dextran preparations on G 200 Sephadex.



3. Injection and Collection Techniques used for Clearance Studies involving Dextran and I^{125} labelled Polyvinylpyrrolidone (PVP)

a. Dextran

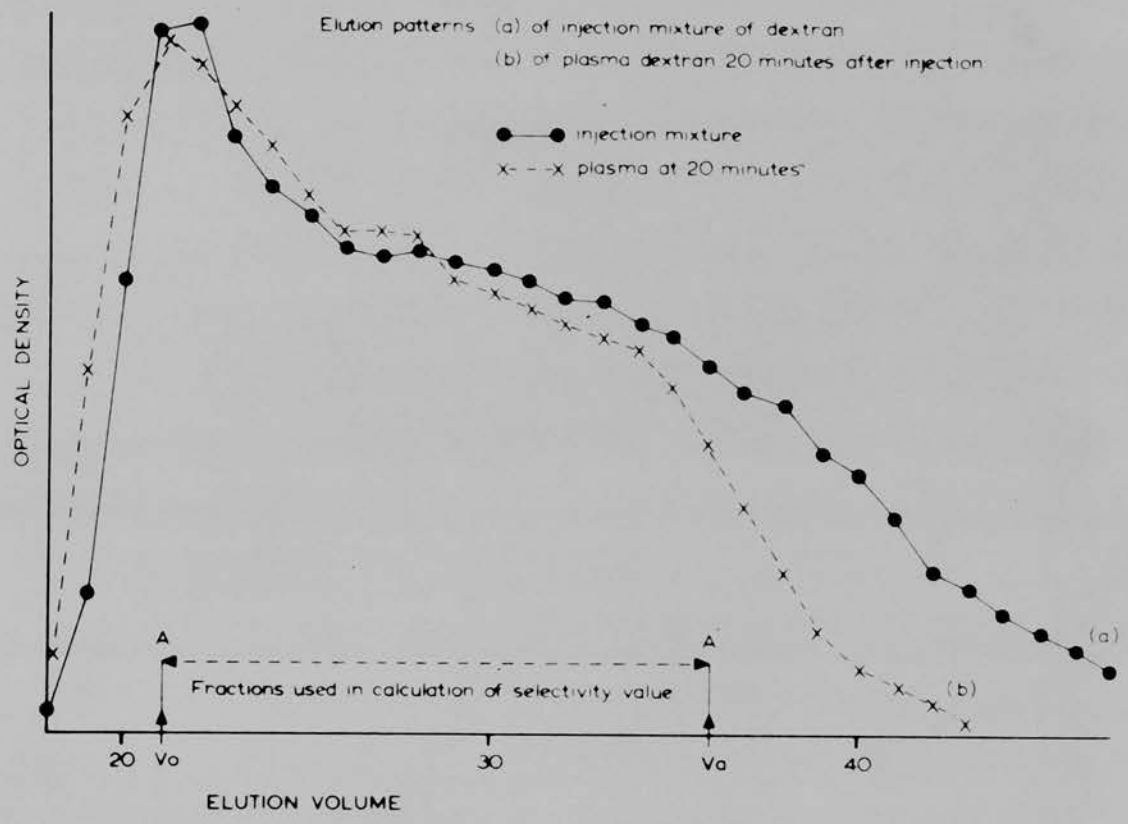
The elution patterns on G200 Sephadex of two commercially available preparations of dextran for injection are shown in fig. 14. In order to obtain a range of dextrans comparable in molecular size with the serum proteins a mixture containing two parts of the high molecular weight preparation (Dextraven, Benger Laboratories) and one part of the low molecular weight preparation (Rheomacrodex, Pharmacia) was employed. The elution pattern of this mixture is shown in fig. 15.

Six pilot studies were carried out using 350 to 500 ml of this mixture, containing 22 to 33G of dextran in normal saline. Timed urine specimens were obtained by catheterisation and blood specimens for dextran determination were obtained at the beginning and end of each collection period. The results of these pilot studies are shown in tables 4 and 5.

The measurement of the renal clearance of macromolecules of varying size raises a number of practical and theoretical problems. The standard technique for the measurement of inulin clearances is to give a loading dose of inulin followed by a continuous infusion, carrying out determinations once a constant plasma level has been reached. This principle cannot be applied to dextran selectivity determinations, however. The different molecular size moieties are cleared at different rates, and an

Figure 15

The rapid elimination of low molecular weight dextran from the circulation.



The very slight alteration at 20 minutes in the molecular size distribution over the range (Va-Vo) allows the injection mixture to be used in place of a plasma sample in the calculation of the dextran selectivity.

infusion rapid enough to maintain a constant level of small molecular weight fractions leads to a continuing rise in the concentration of the larger dextran molecules.

A single injection technique for dextran clearance determinations is therefore preferable. Following a single injection the rate of disappearance of the smaller molecules is more rapid than that for the larger molecules. This is illustrated in fig. 15. Fortunately the rate of clearance of dextrans in the molecular size range of the serum proteins (i.e. between Vo and Va on G 200 Sephadex elution) is reasonably slow, and for this elution range the change in molecular size distribution over a period of 20 minutes is relatively slight. The relative unimportance of this change is illustrated by table 5, where the effect of using inappropriate sera in dextran selectivity determination is shown. Were really small dextran fractions to be included in the selectivity calculations the situation would be different; fig. 15 illustrates the rapid loss from the plasma of dextrans eluting later than Va.

For studies which frequently involved outpatients it was thought desirable to avoid bladder catheterisation if at all possible. There is evidence to suggest that plasma expansion can alter glomerular permeability (Chinard et al, 1954; Gregoire et al, 1958). Protein selectivity determinations carried out before and after dextran infusion did not show any particular trend, but in spite of this it was felt advisable to avoid significant changes in plasma volume. The following technique was therefore adopted as a routine in dextran selectivity

TABLE 5

Pilot Experiments Involving Dextran

Effect on dextran selectivity of

(a) using appropriate sera in calculation (i.e. $\frac{A(initial) + B(fi}{2}$

(b) using initial serum (A) only

(c) using final serum (B) only

(d) using injection mixture (I) in place of serum

D (using $\frac{A+B}{2}$)	D using A only	% error	D using B only	% error	D using I	% error	duration of urine collection	
1.82	1.77	-2.7	1.87	+2.7	1.77	-2.7	40 min.	Injec
1.75	1.77	+1.1	1.71	-2.3	1.76	+0.6	20 min.	less 30'
2.46	-	-	-	-	2.50	+1.6	25 min.	from midpo
2.58	2.55	-1.3	2.63	+1.9	2.51	-3.0	30 min.	of colle
2.36	-	-	-	-	2.28	-3.4	50 min.	perio
1.12	1.10	-1.8	1.14	+1.8	0.97	-13.0	45 min.*	Injec
2.91	2.89	-0.7	2.97	+2.1	-	-	3 hrs.	more 30' f
2.40	2.06	-14%	2.80	+171.	1.95	-18.5	3 hrs.	midpo colle
								perio

* duration of infusion prior to start of collection was 30'; "correct" blood for clearance period was therefore 52½' after start of infusion.

determinations.

The patient was given 300 to 500 ml of water orally to ensure a good urine flow. Twenty minutes later he emptied his bladder, and immediately after this he was given an intravenous injection of 50 ml of a 2:1 mixture of "Dextraven" and "Rheomacrodex" in 0.9% saline. (Several dozen aliquots of such a mixture were made up and then resterilised in the pharmacy department; this ensured that the injections given throughout the study were comparable.)

The patient was instructed to empty his bladder 25 minutes after the dextran injection, and this urine was retained for selectivity determination. If the patient had not succeeded in emptying his bladder 35 minutes after the injection, the experiment was repeated on another occasion. Where there was persistent failure to obtain a urine specimen within the stated period, the attempt to measure dextran selectivities was as a rule abandoned. In a handful of cases where it was thought that the results would be of particular interest, catheterisation was resorted to after a full discussion of the situation with the patient.

The blood dextran concentrations after the injection of 50 ml of injection mixture were too low to be estimated reliably after column fractionation. A small aliquot of the injection mixture was therefore retained after each experiment and used in place of serum in the selectivity determination.

The above technique would obviously be quite inadequate for the estimation of absolute clearances. In many patients the

volume of the collection must have been rendered inaccurate by incomplete bladder emptying, and with short collection periods the error due to renal tract dead space would be quite appreciable. Selectivity determinations, however, involve the measurement of relative clearances. One is interested in the relationship between change in clearance and change in molecular size. Since collection errors affect all size fractions of dextran equally, they do not affect the accuracy of selectivity determinations.

The most important thing to ensure is that the injection mixture used in place of a blood sample has a molecular size distribution representative of that existing in the blood during the period when the dextran present in the urine was filtered at the glomeruli. Dead space urine formed prior to dextran injection contains no dextran. It therefore dilutes the dextran in the urine produced after injection, but does not alter the molecular size distribution of the dextran molecules. Dead space time will alter the effective length of the collection period, as will delay in bladder emptying. A patient with a dead space time of 12 minutes emptying his bladder 25 minutes after dextran injection would produce a urine specimen containing dextran filtered between 0 and 13 minutes after injection; in this patient the blood specimen appropriate for selectivity studies would be one obtained 6½ minutes after injection. A patient with a dead space time of 2 minutes emptying his bladder 35 minutes after injection would produce a urine specimen containing dextran filtered between 0 and 33 minutes after injection. The appropriate blood specimen would be one obtained 16½ minutes following injection.

Figure 15 shows that over the range Vo to Va, used for the calculation of dextran selectivities, the elution pattern for dextran in blood taken 20 minutes after injection is very close to that of the injection mixture. Table 5 shows that if the collection period follows immediately after the dextran injection and is 40 minutes or less in duration the error introduced by using injection mixture in place of blood is of the order of 3%.

b. Clearance techniques using I 125 labelled polyvinyl pyrrolidone

Following an intravenous dose of 50 microcuries of I 125 labelled PVP, there was no difficulty in measuring the activity in the serum elution fractions. There was therefore no need to use the injection mixture in place of blood in the PVP studies.

To have used the simplified technique described above for dextran in the PVP studies would have been inappropriate on two counts. Firstly, clearance studies with PVP were extended into the low molecular weight range, where renal clearance was rapid. Secondly, even in the high molecular weight range, the injection mixture would not have reflected the serum elution pattern with accuracy. Tothill (1965) has shown that labelled PVP is taken up rapidly by the liver. This uptake is particularly avid for high molecular weight fractions.

Orthodox clearance techniques were therefore used in the PVP studies. The bladder was catheterised to ensure complete emptying, and blood was taken five minutes before the midpoint of a 30 minute collection period. I 125 uptake by the thyroid was blocked by potassium iodide administration.

SECTION III

RESULTS

1. Comparison of gel filtration and immunodiffusion in the assessment of the selectivity of protein excretion

a. Introduction

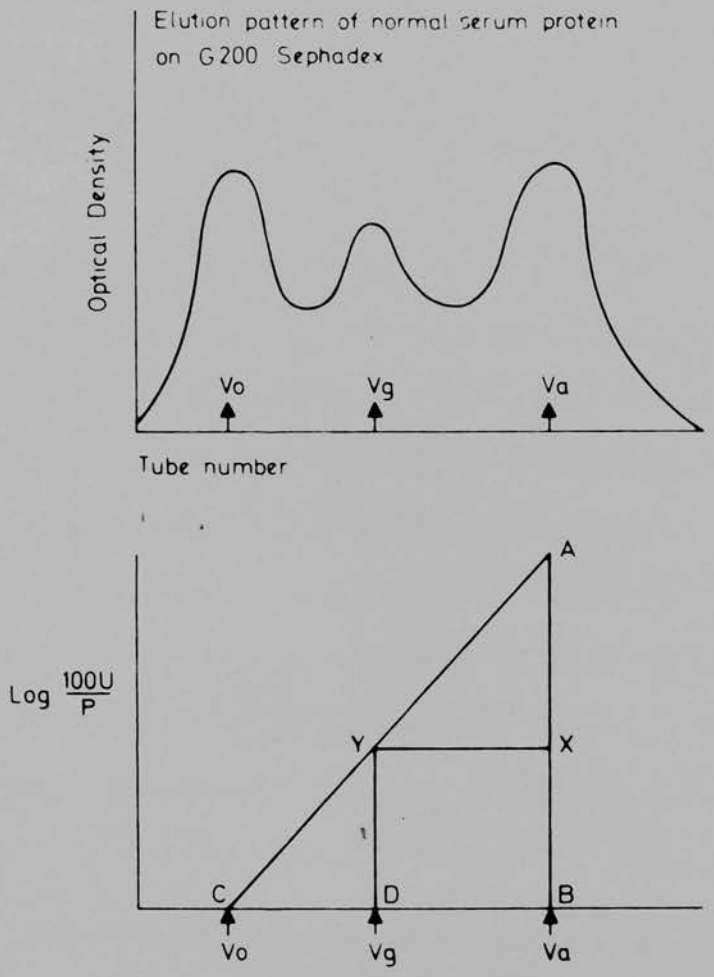
The established technique for the determination of protein selectivity is immunodiffusion. As this method utilises antisera specific to human protein, it is not applicable to non-protein macromolecules such as dextran. The technique of gel filtration is the one of choice for dextran selectivities.

As it was proposed to compare protein and dextran selectivities in order to assess (inter alia) the effects of tubular reabsorption of protein on clearance studies, it was necessary to compare the results obtained by gel filtration with those given by immunodiffusion, using protein as the macromolecule under assay.

b. Theoretical relationship between gel filtration results and immunodiffusion results

In the technique of gel filtration, each tube of eluate from the column contains a mixture of proteins of varying molecular weights. The peak elution volume of each protein, however, is a function of log. molecular weight, and in the calculation of selectivity it is assumed that each tube contains a pure solution of a protein with its elution peak at the point concerned. Hardwicke (1965a) has shown that this assumption is not as unjustified as might appear at first sight; the errors introduced by the assumption largely cancel each other out. Since this is the case, the expected relationship between $\triangle$ (the selectivity as determined by gel filtration) and -K (the selectivity determined by immunodiffusion) can be calculated in the following way:

Figure 16



AB represents Δ (d log c for the range Vo to Va, represented by CB)

AX represents

d log c for the range Vg to Va, represented by DB. DB = XY.

By the method of similar triangles $\frac{AX}{AB} = \frac{XY}{BC}$

But XY = BD and $\frac{BD}{BC} = 0.46$

Thus $\frac{AX}{AB} = 0.46$, or $AX = 0.46\ AB$

Thus d log c for the range (Va-Vg) = 0.46 Δ

(d log c for a given range of log MW is the change in $\log_{10} \frac{100U}{P}$ for that range).

$\triangle$ is the change in $\log_{10}$ in the clearance of protein between Vo, the first protein peak, and Va, the third (albumin) elution peak. (Fig. 16). Representing "change in $\log_{10}$ clearance" as d log. c

$\triangle$ = d log. c for the range (Va - Vo).

The resolution of the column at the point Vo is poor, and the molecular weights of the proteins having an elution peak at this point are indeterminate. The second and third protein peaks, however (Vg and Va) lie in the region of optimal column resolution. Vg is the elution peak for IgG (MW 150,000) and Va is the elution peak for albumin (MW 69,000). Andrews (1965) has shown that for proteins elution volume is proportional to log MW; $\log_{10}$ of 150,000 is 5.176 and $\log_{10}$ of 69,000 is 4.839. Thus over the elution range (Va-Vg) there is a change in $\log_{10}$ MW of 0.362. The molecular weight range over which $\triangle$ is calculated can be found by determining what proportion (Va - Vg) is of the total elution range (Va - Vo).

The ratio (Va - Vg)/(Va - Vo) was determined experimentally on 20 separate occasions on three different columns. The mean value for this ratio was 0.46, S.E.M. $\pm$ 0.01.

Since log. M.W. is proportional to elution volume

$$\frac{\text{The change in Log. MW over the range (Va - Vg)}}{\text{The change in log. MW over the range (Va - Vo)}} = \frac{(Va - Vg)}{(Va - Vo)} = 0.46$$

Since the relationship between log clearance and log MW is linear, it can be shown (see fig. 16 opposite)

$$\frac{\text{d. Log c for the range (Va - Vg)}}{\text{d. log c for the range (Va - Vo)}} = 0.46$$

TABLE 6

VALUES OF SELECTIVITY ESTIMATED BY GEL FILTRATION (Δ) AND BY IMMUNODIFFUSION ($-K$) AND MEAN VALUES OF Δ AND $-K$ FOR EACH PATIENT

The number of estimations is given in brackets. The mean protein excretion is also given.

Patient	Δ	$-K$	*Mean* Δ	*Mean* $-K$	*Mean urinary protein g/24h*
1	2.51	2.95	2.51 (1)	3.20 (8)	1.6
2	2.29	2.46	2.24 (4)	2.68 (19)	2.8
	2.27	2.60			
	1.97	3.29			
3	2.11	2.92	2.11 (4)	2.68 (9)	15.4
4	2.08	2.58	2.08 (2)	2.54 (4)	4.0
5	1.95	2.86	2.01 (6)	3.03 (5)	24.0
	2.13	3.41			
6	1.95	2.27	1.95 (1)	2.43 (15)	12.1
7	1.85	3.03	1.80 (3)	2.67 (10)	1.3
	1.66	2.81			
	1.88	2.59			
8	1.80	2.37	1.80 (1)	2.50 (8)	11.3
9	1.92	1.88	1.74 (6)	1.96 (7)	19.6
	1.84	2.22			
	1.43	1.82			
10	1.71	1.94	1.71 (1)	1.84 (3)	1.1
11	1.64	2.24	1.64 (1)	2.21 (15)	4.0
12	1.64	1.55	1.64 (1)	1.74 (6)	7.4
13	1.50	1.69	1.50 (1)	2.02 (4)	10.0
14	1.11	1.69	1.29 (3)	1.62 (8)	10.3
	1.39	1.67			
15	1.29	2.38	1.29 (1)	2.15 (3)	5.5
16	1.28	1.56	1.28 (1)	1.45 (7)	13.2
17	1.22	1.16	1.22 (1)	1.47 (10)	8.8
18	1.20	1.55	1.20 (2)	1.67 (9)	6.1
19	1.20	2.30	1.20 (5)	2.11 (12)	16.0
	1.19	1.87			
20	1.16	1.79	1.16 (1)	1.77 (3)	2.2
21	1.33	1.47	1.14 (6)	1.49 (12)	6.9
	0.87	1.34			
22	0.98	1.47	1.12 (6)	1.70 (3)	3.1
	1.26	1.58			
23	1.10	1.82	1.10 (1)	1.67 (5)	3.6
24	1.07	1.93	1.06 (2)	1.74 (8)	1.6
	1.04	1.81			
25	1.02	1.94	1.02 (1)	1.69 (2)	5.0
26	1.02	1.56	1.02 (1)	1.75 (9)	14.1
27	1.01	1.67	1.01 (1)	1.74 (10)	7.0
28	0.95	1.48	0.98 (1)	1.30 (3)	2.3
29	0.89	1.29	0.89 (1)	1.28 (2)	9.1
30	0.89	1.17	0.89 (1)	1.55 (6)	6.6
31	0.86	1.36	0.82 (5)	1.38 (8)	10.8
	0.81	1.53			
32	0.73	0.88	0.69 (3)	1.04 (4)	3.6
	0.64	1.02			
33	0.40	0.82	0.50 (2)	0.58 (2)	3.4
	0.61	0.34			
34	1.70	2.17			0.4
	1.50	3.15			
10	1.29	3.40			0.6
	1.20	3.40			
35	1.10	2.68			0.8
36	0.90	2.43			0.8
	0.64	2.98			
37	0.78	2.15			0.5
38	0.64	2.98			0.5
39	0.54	1.60			0.5
40	0.47	1.01			0.6

Taken from MacLean and Petrie (1966).

Since d. log. c. for the range (Va - Vo) = $\triangle$

d. log c for the range (Va - Vg) = 0.46 $\triangle$ (Equation 1)

Now (-K) is d. log. c for a change in $\log_{10}$ MW of 1.0.

The change in $\log_{10}$ MW for the range (Va - Vg) is 0.362.

Thus d. log. c for the range (Va - Vg) = 0.362 (-K) (Equation 2).

Combining equations 1 and 2

0.362 (-K) = 0.46 $\triangle$

Thus $\triangle$ = 0.73 (-K) or (-K) = 1.37 $\triangle$

c. Results

Selectivity results obtained by gel filtration were compared with those using immunodiffusion on 59 paired specimens of blood and urine from 40 patients with proteinuria. The methods used have been discussed in section II of this thesis. The results are shown in Table 6. This table also shows the mean values for (-K) and $\triangle$ for each patient, and the mean 24-hour protein excretion for each patient over the period of the study.

The mean value of $\triangle$ was 1.4, and the mean value of (-k) was 1.9. On inspection of these figures, there appeared to be a reasonably close correlation between $\triangle$ and (-k) for the patients excreting over 1.0 G of protein in 24 hours, but in patients 34 to 40 inclusive (all excreting less than 1.0 G of protein daily) the values of $\triangle$ relative to (-K) were much lower than in the series as a whole. In these patients, the measurement of protein in the column eluates was difficult; column chromatography results in the dilution of samples and in some cases the limits of the sensitivity of the method were approached. Furthermore, the small amount of protein in the urine which is not contributed by

Figure 17a

Comparison of protein selectivity values obtained by gel filtration with values determined by immunodiffusion.
Individual values from 33 patients excreting 1.0G or more of protein in 24 hours.

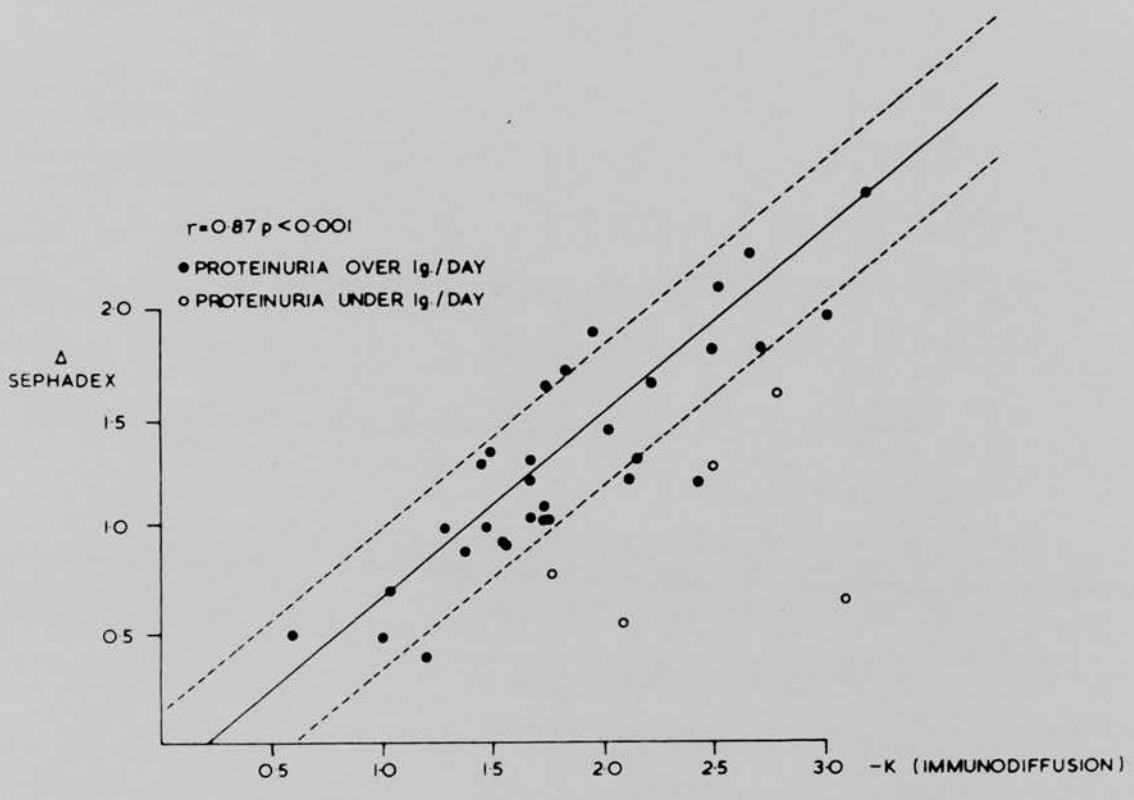
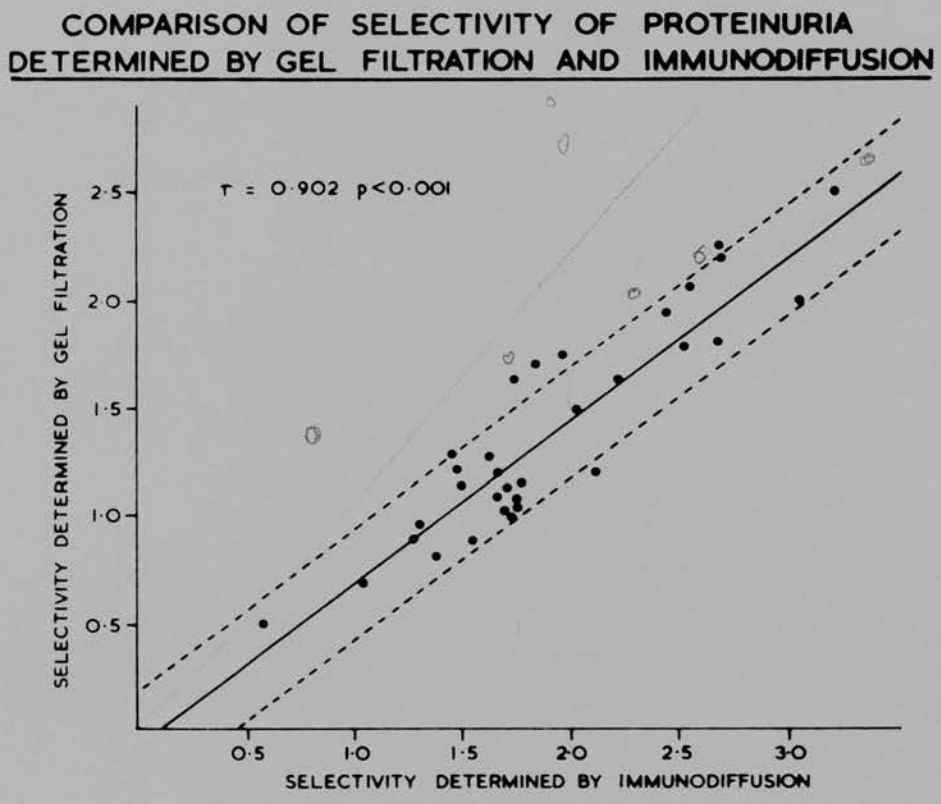


Figure 17b

Comparison of protein selectivity values obtained by gel filtration with those obtained by immunodiffusion. Mean values from 33 patients excreting 1.0G or more of protein in 24 hours.



glomerular filtration is quantitatively more important in cases of trace proteinuria than when massive glomerular proteinuria is present. For these reasons it was decided to analyse the results in patients excreting over 1.0 G daily separately from those obtained in patients excreting less than 1.0 G per day.

Figure 17a shows a plot of gel filtration values of protein selectivity (values) against immunodiffusion values of protein selectivity (-K values). 48 values from 33 patients with proteinuria over 1.0 G daily are shown. There is a certain degree of scatter, as is to be expected when the experimental error for each method approaches 10%. There is, however, a strong positive correlation (r_{48} = +0.857, p 0.001), and the best straight line for the relationship between and (-K) is = 0.65 (-K) + 0.13. The standard error of the estimate of is ± 0.26, or 18.7%.

In figure 17b the mean value of for each of the 33 patients excreting over 1.0 G of protein daily is plotted against the mean value of (-K) in each patient. There is again a strong positive correlation (r_{33} = 0.902; p 0.001).

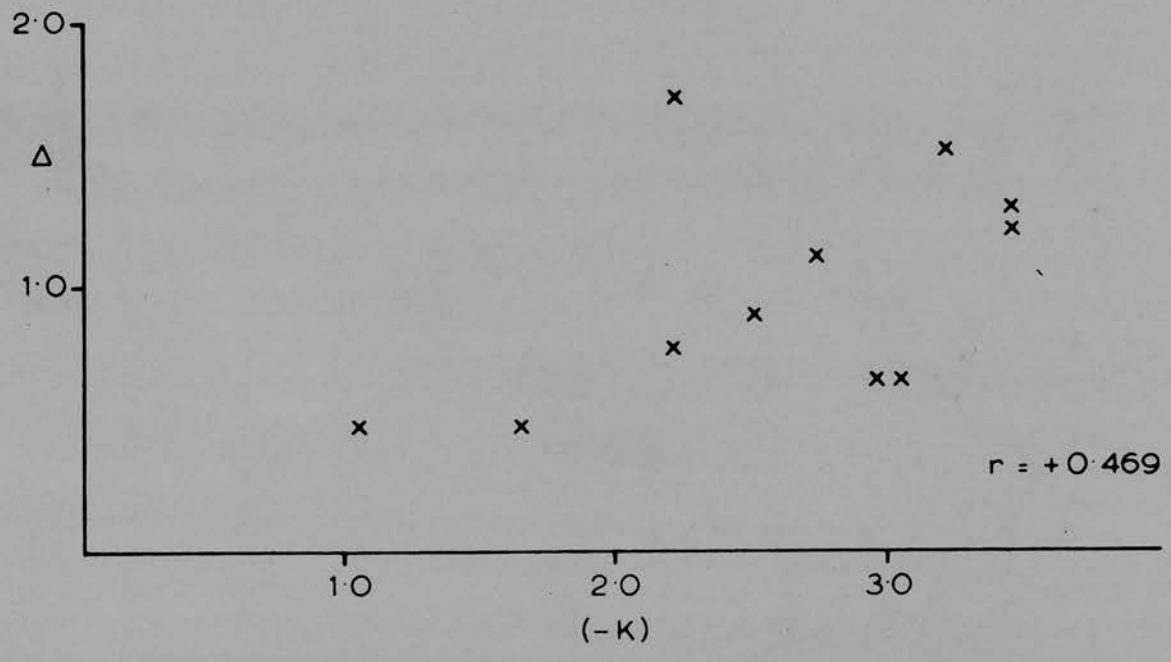
The best straight line for the relationship between d and (-K) in this instance is = 0.76 (-K) - 0.08,
and the standard error of the estimate of is ± 0.21.

and (-K) were also determined for the 21 patients with glomerulonephritis discussed in Section III 2a of this thesis. (See table 7 and figure 19). For these patients the relationship between and (-K) was found to be = 0.69(-K) + 0.07. The standard error of the estimate of is ± 0.17.

The expected relationship between and (-K) is = 0.73(-K).

Figure 18.

Comparison of protein selectivity values obtained by gel filtration with those obtained by immunodiffusion in patients excreting less than 1.0G of protein in 24 hours.



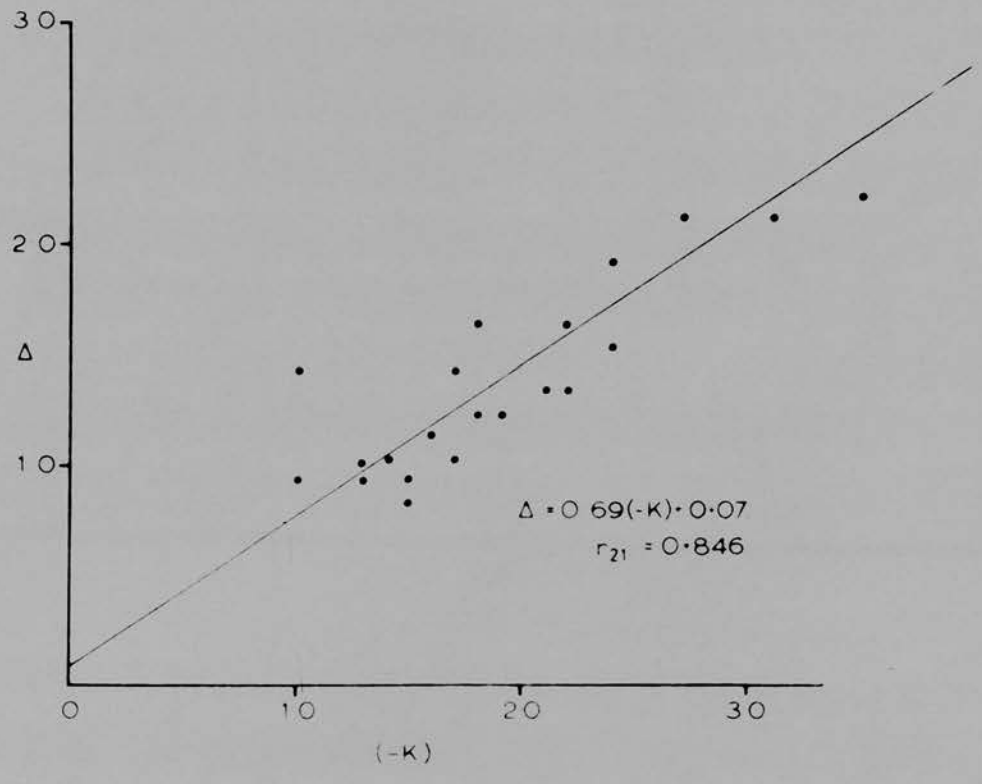
This is very similar (within the limits of experimental error) to the three observed relationships described above. The expected line passes through the origin, and the three observed lines pass reasonably near to the origin. If the simplifying assumption is made that the line relating $\triangle$ to (-K) does in fact pass through the origin, the calculation of the ratio $\triangle/(-K)$ becomes meaningful. For the mean values of $\triangle$ and (-K) in the 33 patients with a protein excretion of over 1.0G in 24 hours, the ratio of $\triangle/(-K)$ was 0.725 ± 0.017. This is extremely close to the expected ratio of $\triangle/(-K)$ of 0.73.

For the patients excreting less than 1.0G of protein in 24 hours, the situation was different. The relationship between $\triangle$ and (-K) for these patients is shown in figure 18; the line relating $\triangle$ to (-K) has a slope of 0.25 only. For the 11 estimations carried out on these 7 patients, the ratio $\triangle/(-k)$ was 0.40 ± 0.05. This differs significantly from the ratio of 0.725 ± 0.017 found in the 33 cases with over 1.0G of proteinuria daily. ($t = 7.57$, $p < 0.001$).

This indicates that either gel filtration, or immunodiffusion, or both, give unreliable results in proteinuria of under 1.0G daily. Examination of fig. 18 shows that it is the gel filtration results which are uniformly low in the patients with minor proteinuria; the range of immunodiffusion results does not differ appreciably from the range seen in the series as a whole. Both methods are reliable in the presence of massive proteinuria. Gel filtration (which does not distinguish proteins of serum origin from those of tubular origin) is unreliable at protein excretion

Figure 19

Comparison of protein selectivity values obtained by gel filtration with those obtained by immunodiffusion. These patients are discussed in detail in section III 2a; they comprise a separate series from the patients depicted in figure 17.



rates of under 1.0G daily, while immunodiffusion continues to give reasonably reliable results down to about 0.5G per day of protein excretion.

Figure 20

Comparison of dextran selectivity values in patients with glomerulonephritis with immunodiffusion values of protein selectivity. Showing the whole series of 21 patients.

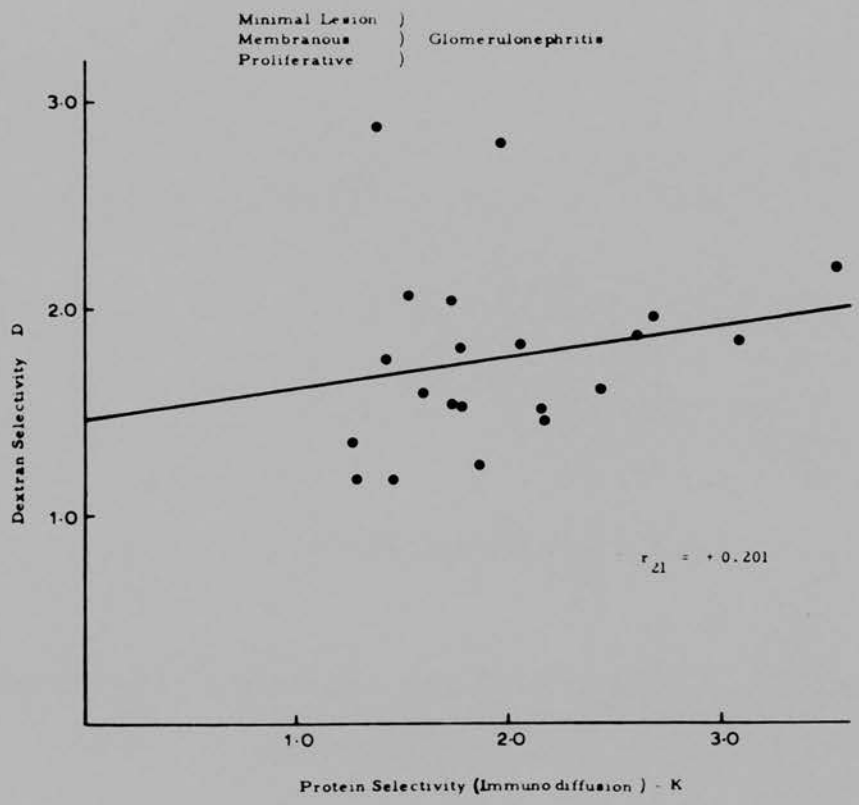


Figure 21

Comparison of dextran selectivity values in patients with glomerulonephritis with gel filtration values of protein selectivity. Showing the whole series of 21 patients.

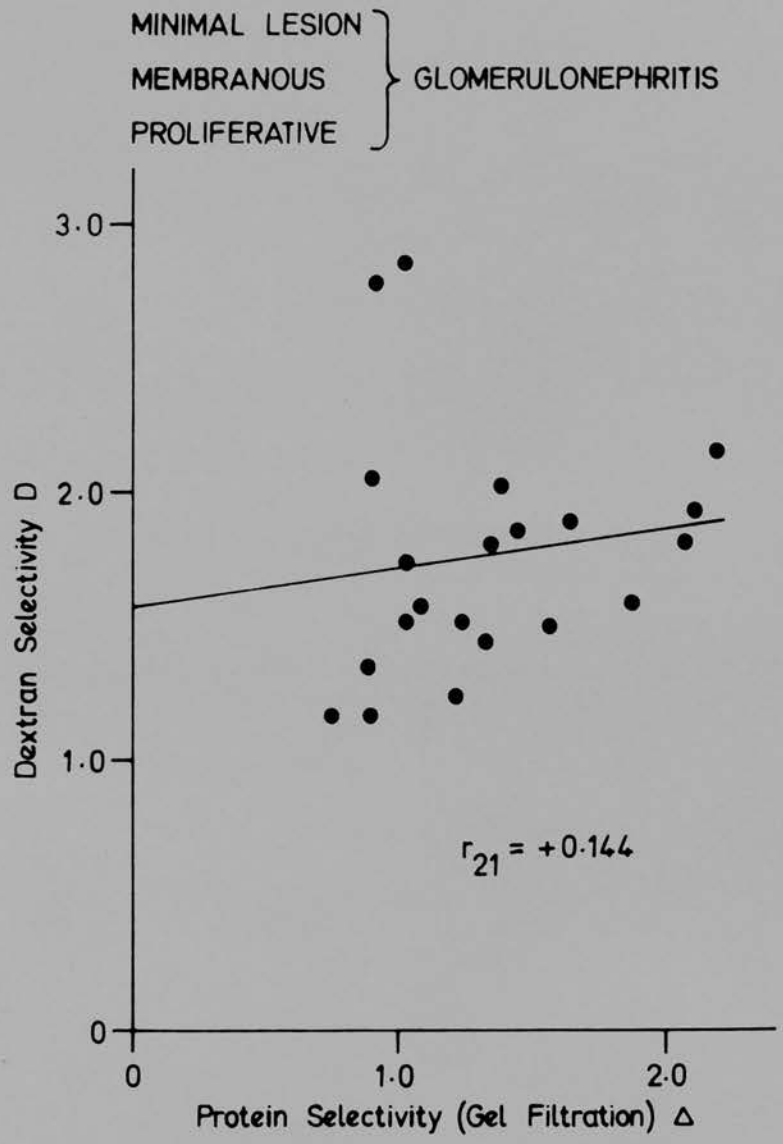


TABLE 7

Dextran and protein selectivity values in patients with glomerulonephritis.

Subject	Diagnosis	Protein Excretion (G/24hr)	Creatinine Clearance (ml/min)	D	(-K)	△
1	Minimal	4.8	122	2.18	3.53	2.20
2	Lesion	14.9	97	1.95	2.68	2.11
3	Glomerulo-	10.0	32	1.86	2.60	1.44
4	nephritis	4.8	110	1.83	3.08	2.07
5		6.6	38	1.53	1.73	1.02
6	Membranous	9.1	101	1.52	1.77	1.23
7	Glomerulo-	0.7	102	1.51	2.15	1.56
8	nephritis	1.7	12	1.35	1.26	0.88
9		8.1	73	1.24	1.86	1.20
10		9.0	7	1.17	1.45	0.75
11		1.5	105	2.87	1.38	1.01
12	Proliferative	0.9	90	2.78	1.96	0.91
13	Glomerulo-	4.4	9	2.05	1.53	0.89
14	nephritis	4.5	77	2.03	1.73	1.38
15		6.0	128	1.90	1.77	1.64
16		6.8	168	1.81	2.05	1.34
17		11.7	66	1.75	1.42	1.02
18		13.4	33	1.60	2.42	1.88
19		6.8	99	1.59	1.61	1.07
20		7.1	27	1.45	2.16	1.32
21		8.4	53	1.27	1.28	0.89

Reported in Petrie, MacLean and Robson (1970).

2. Comparison of Dextran Selectivities and Protein Selectivities in Renal Disease, in Normal Subjects, and in Induced Proteinuria

a. Patients with Glomerulonephritis

21 patients with glomerulonephritis and proteinuria of over 1.0G per day were studied. The diagnosis in each case was established by renal biopsy. Four patients had minimal lesion glomerulonephritis. Six had membranous glomerulonephritis. The remaining eleven had proliferative glomerulonephritis.

Table 7 shows the diagnosis, the 24-hour protein excretion, and the creatinine clearance in each patient, along with dextran selectivity values, determined by gel filtration and protein selectivity values determined both by immunodiffusion (-K) and by gel filtration .

Figure 20 shows the relationship between D (the dextran index of selectivity) and (-K), the index of protein selectivity by the technique of immunodiffusion for the entire series of 21 patients. By the method of least squares, the best straight line for the relationship between D and (-K) is $D = +0.15\ (-K) + 1.47$ The correlation coefficient for D and (-K) for the series as a whole was not statistically significant, r_{21} being +0.201.

Figure 21 shows the relationship between D and $\triangle$, the index of protein selectivity determined by gel filtration. The best straight line for the relationship was found to be

$$D = 0.15\triangle + 1.57.$$

Again, the correlation coefficient was not statistically significant,

Figure 22

The relationship between dextran selectivity and immunodiffusion values of protein selectivity in 11 patients with proliferative glomerulonephritis.

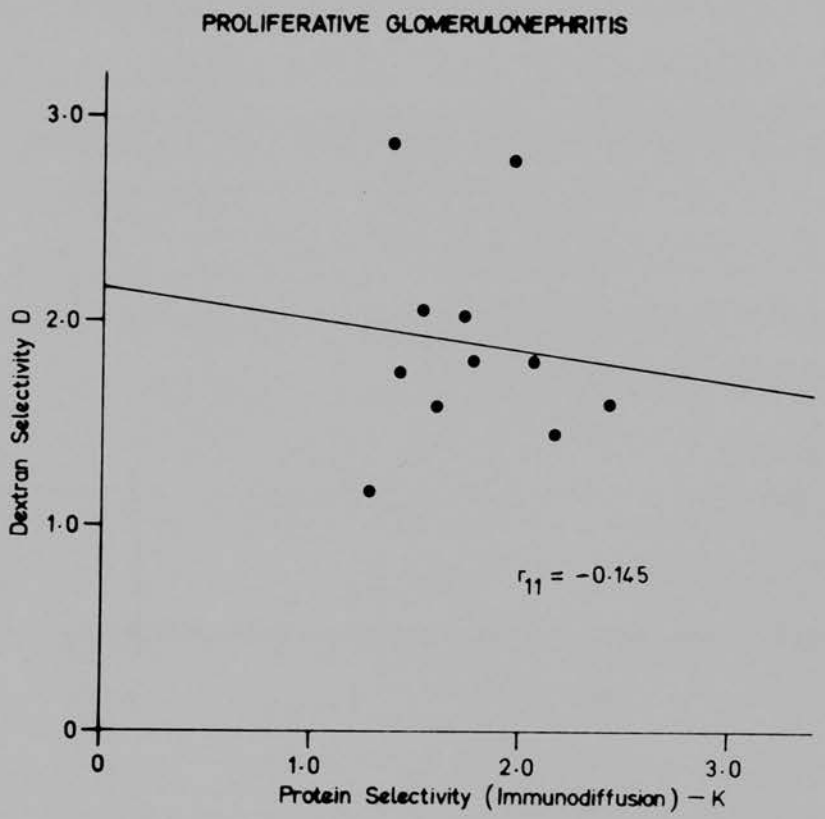
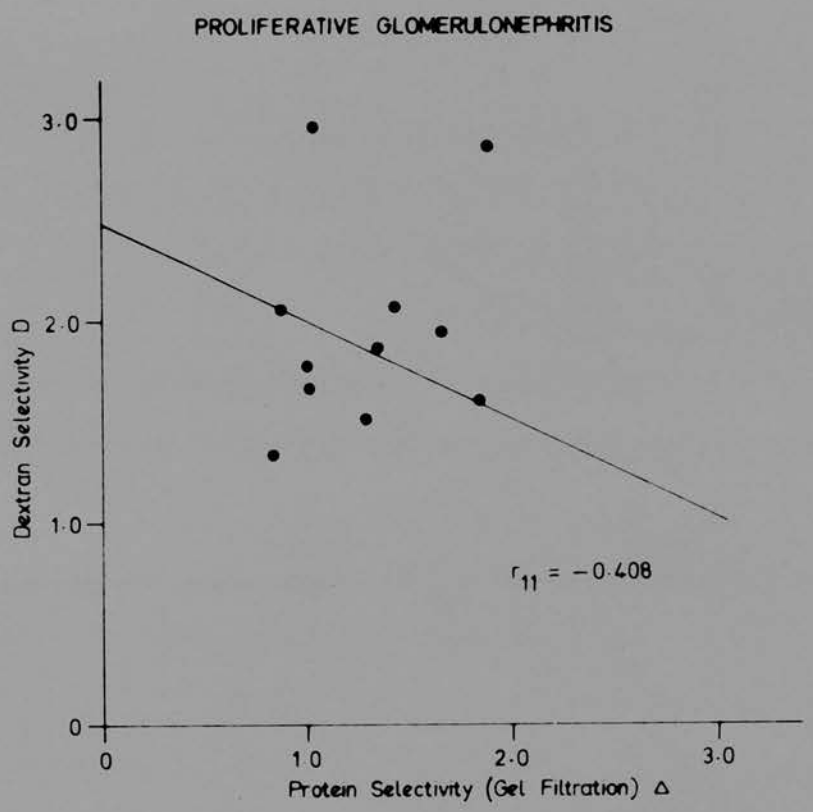


Figure 23

The relationship between dextran selectivity and gel filtration values of protein selectivity in 11 patients with proliferative glomerulonephritis.



r_{21} being + 0.144.

If dextran and protein are handled in the same way by the kidneys and on Sephadex gel filtration, on the basis of the relationships shown in section 1 of the results, the relationship to be expected between D and (-K) would be

$$D = 0.73\ (-k)$$

while that between D and △ would be

$$D = \triangle$$

Some divergence from these theoretical relationships is to be expected, in view of the physical differences between the two molecular species, but it would be surprising if the poor correlation found could be explained on this basis alone. The mean value of D for the series of 21 patients was 1.77, and this was considerably higher than the mean value of 1.32 found for △. The difference in means was significant ($t = 3.23$, P less than 0.0025). On inspection of the results in table 7, the discrepancy between D and △ appears to be greatest in those patients with proliferative glomerulonephritis. In figure 22, D is plotted against (-K) for the 11 patients with proliferative glomerulonephritis. Figure 23 shows the relationship between D and △ in these same cases.

From figure 22, the best straight line relating D and (-k) in the proliferative group was

$$D - 0.45\ (-K) + 2.16.$$

r_{11} was -0.145 (not statistically significant). From figure 23, the best straight line relating D and △ in the proliferative group was

Figure 24

The relationship between dextran selectivity and immunodiffusion values of protein selectivity in 10 patients with membranous or minimal lesion glomerulonephritis.

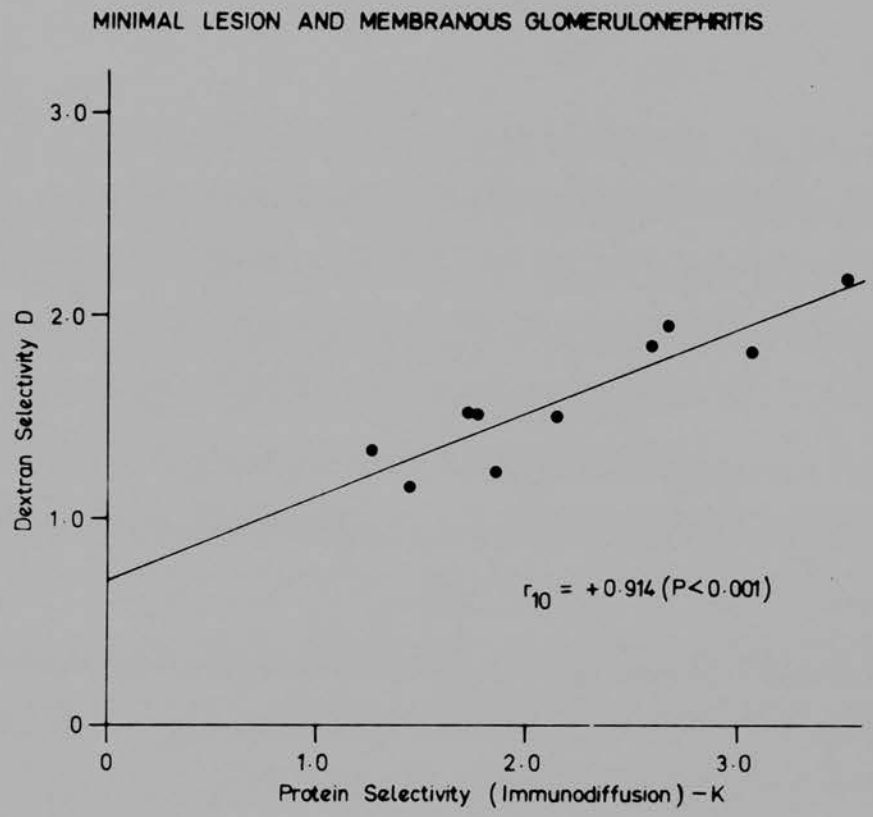
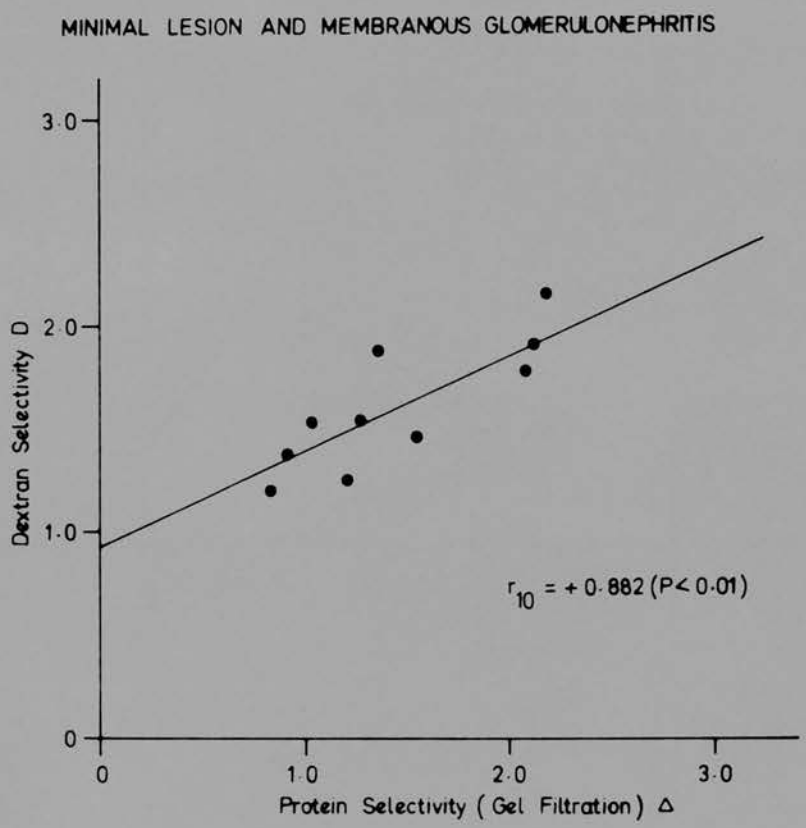


Figure 25.

The relationship between dextran selectivity and gel filtration values of protein selectivity in 10 patients with membranous or minimal lesion glomerulonephritis.



$$D = -0.41\triangle + 2.40$$

r_{11} was -0.408 (again, not significant statistically).

It appeared that the poor correlation between dextran and protein selectivities observed in the series as a whole might be due very largely to the total lack of correlation shown in the 11 patients with proliferative glomerulonephritis.

The relationship between dextran selectivity and protein selectivity was therefore assessed in the remaining 10 patients, four of whom had minimal lesion glomerulonephritis and six of whom had membranous glomerulonephritis.

Figure 24 shows the relationship between D and (-K) in these 10 cases. The best straight line gave the formula

$D = 0.41\ (-K) + 0.71$. $r_{10} = 0.914$; P being less than 0.001.

Figure 25 shows the relationship between D and $\triangle$ in the 10 cases with minimal lesion or membranous glomerulonephritis.

$D = 0.55\triangle + 0.82$.

r_{10} was +0.882, P being less than 0.001.

In view of the physical differences which exist between dextran and protein molecules, and the differences in the renal mechanisms involved in their excretion, precise numerical correlation between protein and dextran selectivities is not to be expected. Nevertheless, in the patients with minimal lesion and membranous glomerulonephritis, a highly selective pattern of protein excretion was associated with a highly selective pattern of dextran excretion, and unselective proteinuria was associated with unselective dextranuria.

In the patients with proliferative glomerulonephritis, this

was not the case. The correlation coefficient between the protein and the dextran indices of selectivity did not differ significantly from zero. The dextran selectivity values were consistently much higher than the protein selectivity values in the proliferative patients.

This difference between the situation in proliferative glomerulonephritis on the one hand, and that in minimal lesion and membranous glomerulonephritis on the other is deserving of comment. Possible reasons for the observed difference are discussed in section IV of this thesis.

TABLE 8

Protein selectivity in normal subjects.

Subject	(-K)	U/P ratio of albumin
1	0.90	1/5,120
2	1.28	1/10,750
3	0.59	1/2650
4	1.18	1/8750
5	1.56	1/8830
6	0.96	1/8250
7	1.28	1/3200
8	1.54	1/10,900
9	0.78	1/6160
10	1.29	1/1600

These subjects have been reported in MacLean and Robson (1966).

The mean value for (-K) is 1.14 ± 0.11

TABLE 9

Dextran selectivity values in normal subjects.

Subject	Dextran Selectivity (D)	DX1.37
1	2.38	3.26
2	2.50	3.43
3	2.76	3.78
4	2.98	4.08
5	3.19	4.37
6	3.65	5.00
7	3.58	4.90
8	3.12	4.27
9	2.81	3.85

Multiplication of values of D by 1.37 makes the units used in measuring dextran selectivities (D) comparable with those used in table 9 for protein selectivities (-K).

Subjects 1 - 6 reported in MacLean, Petrie and Robson (1970).

The mean value of (Dx1.37) = 4.1 ± 0.21

2. Comparison of Protein Selectivity Values with Dextran Selectivity Values in Normal Subjects

Protein selectivity values were estimated in 10 normal subjects by the immunodiffusion technique. The results are shown in table 8. The range of selectivity values obtained was from 0.09 to 1.56 with a mean value for (-K) of 1.14.

Dextran selectivity values were estimated in 9 normal subjects by the gel filtration technique. The results are shown in table 9. The range of selectivities obtained was from 2.38 to 3.65, with a mean value for D of 2.95. On the assumption that D (the dextran index of selectivity) is numerically comparable to $\triangle$ (the protein index of selectivity as determined by gel filtration), since $\triangle = 0.73$ (-K), the above dextran selectivity values can be converted to (-K) units by multiplying by a factor of 1.37. Thus, in terms of (-K) units, dextran selectivities in normal subjects ranged from 3.26 to 5.00 with a mean value of 4.10. Because of the differences in physical properties between dextran and protein molecules, it cannot be assumed that these (-K) equivalents are precise. Whether or not a conversion factor is used, however, it is apparent that normal subjects have a highly selective pattern of dextran excretion and a very unselective pattern of protein excretion. The implications of these findings will be discussed in Section IV of this thesis.

TABLE 10

Protein and dextran selectivity values in postural proteinuria.

Patient	Alb. U/P ratio	Protein Selectivity (-K)	Dextran Selectivity D	Dx1.37	Posture
1	1/26 1/640	1.06 1.19	- -	- -	Erect Supine
2	1/112 1/5250	1.38 0.95	- -	- -	Erect Supine
3	1/112 1/768	0.75 1.48	- -	- -	Erect Supine
4	1/160 1/640	1.26 1.27	- -	- -	Erect Supine
5	1/16 1/2130	1.01 0.73	- -	- -	Erect Supine
6	1/180 1/1280	1.05 -	2.38 2.45	3.26 3.36	Erect Supine
7	1/330 -	1.41 -	3.47 -	4.75 -	Erect Supine
8	1/370 -	1.22 -	3.26 -	4.47 -	Erect Supine
9	1/220 -	1.18 -	3.22 -	4.41 -	Erect Supine
10	1/120 1/2240	1.30 1.28	2.77 2.60	3.79 3.56	Erect Supine

Multiplication of D values by 1.37 makes them comparable with (-K) values.

In erect posture, mean of (-K) = 1.16; mean of (Dx1.37) = 4.14

Patients 1 - 5 reported in Ruckley, McDonald, MacLean, and Robson (1966).

Patients 6 - 9 reported in MacLean, Petrie and Robson (1970).

Comparison of Protein Selectivity Values with Dextran Selectivity Values in Patients with Orthostatic (Postural) Proteinuria

Ten subjects with orthostatic proteinuria were studied. In all subjects proteinuria was present in the lordotic postiion, and absent (on the basis of albustix testing) in the supine position. In 9 of the 10 subjects renal biopsy was carried out, confirming the absence of glomerulonephritis and the presence of changes on light and electron microscopy characteristic of postural proteinuria (Ruckley et al, 1966). The results are shown in table 10.

Protein selectivity values in the presence of significant proteinuria ranged from 0.75 to 1.41, the mean value being 1.16.

Protein selectivity value in the absence of significant proteinuria (i.e. albustix -ve, albumin S to U ratio 640 to 5250) ranged from 0.73 to 1.48, with a mean value for (-K) of 1.12.

Dextran selectivity values in the presence of significant proteinuria ranged from 2.38 to 3.47, with a mean value of D of 3.02. Applying the conversion factor of 1.37, the range of these dextran selectivities in terms of (-K) units was from 3.26 to 4.75, with a mean value of 4.14.

Only two estimations of dextran selectivity were made in the absence of significant proteinuria. The values of D obtained were 2.45 (subject 6) and 2.60 (subject 10). These values were not noticeably different from the value obtained in these subjects when proteinuria was present.

In summary, patients with postural proteinuria were found

to have a highly selective pattern of dextran excretion and a very unselective pattern of protein excretion. The amount of protein in the urine changed markedly with the posture of the patient, but there was no appreciable change in selectivity with posture. The implications of these findings will be discussed in Section IV of this thesis.

TABLE 11

Protein and dextran selectivity values in patients with acute ischaemic renal failure.

Patient	Albumin U/P ratio	Protein Selectivity (-K)	Dextran Selectivity (D)	Dx1.37
1	1/196	0.66	-	-
2	1/128	1.10	-	-
3	1/112	1.13	-	-
4	1/216	0.80	-	-
5	-	0.40	-	-
6	1/84	1.08	-	-
7	1/96	0.77	1.99	2.73
8	-	-	2.02	2.77
9	-	-	2.10	2.88
10	1/256	0.93	2.59	3.55
11	1/448	0.25	2.65	3.63
12	1/224	1.40	3.28	4.49

Mean value for (-K) = 0.85

Mean value of D = 2.44

Mean value of (Dx1.37) = 3.34

Patients 1 to 6 reported in MacLean and Robson (1966).

Patients 7 to 12 reported in MacLean, Petrie and Robson (1970).

Comparison of Protein Selectivity Values with Dextran Selectivity Values in Patients with Acute Ischaemic Renal Failure (Acute Tubular Necrosis)

12 patients were studied, the diagnosis of acute tubular necrosis being confirmed by renal biopsy in 9 instances. Selectivities were determined early in the diuretic phase in each case, at a time when significant proteinuria was present. In 6 cases protein selectivities only were determined, in 2 cases dextran selectivities only were assessed, and in the remaining 4 cases determinations were carried out both with protein and with dextran. The results are shown in table 11.

The values for protein selectivity by the technique of immunodiffusion ranged from 0.25 to 1.40, the mean value of (-K) being 0.85.

The values for dextran selectivity by the technique of gel filtration ranged from 1.99 to 3.28, the mean value of D being 2.44. Using the factor 1.37 (see section III, 1) to convert D values to their (-K) equivalents, the range of dextran selectivities in terms of (-K) was from 2.73 to 4.49 with a mean value equivalent to 3.34 (-K).

The protein selectivity values in acute renal failure were thus found to be consistently low. Dextran selectivity values, on the other hand, were consistently high, though the mean dextran selectivity in acute renal failure of 2.44 (in terms of D) was somewhat lower than the mean dextran selectivity in normal subjects (D = 2.91) and the mean value in postural proteinuria (D = 3.02). The implications of these findings will be discussed in Section IV

e. Glomerular permeability during proteinuria induced by plasma infusion.

(i) Introduction

The normal glomerulus is highly selective in its permeability to dextran (MacLean, Petrie, and Robson, 1970) and also in its permeability to polyvinyl pyrrolidone (Hulme and Hardwicke, 1966). The excretion pattern of the small amount of protein excreted by normal subjects is, however, very unselective (Rowe and Soothill, 1961; MacLean and Robson 1966; Poortmans, 1968).

It has been suggested that protein selectivity values only reflect glomerular permeability to protein when the quantity of protein derived from glomerular filtration is enough to swamp the quantity of protein reaching the urine from non-glomerular sources. In normal subjects under normal conditions, a glomerular proteinuria of this degree does not occur.

Proteinuria can however be induced by protein infusion. This has been demonstrated in the dog (Brull, 1934; Terry et al, 1948), in the rat (Post, 1960; Lipmann, 1948; Lannigan and McQueen 1962), and in man (Waterhouse et al, 1948). This proteinuria has been ascribed to saturation of tubular reabsorption in the presence of normal glomerular permeability (Terry et al, 1948). If this is in fact the mechanism involved, selectivity studies on induced proteinuria would be expected to give an indication of the permeability of the normal glomerulus to protein. The infusion of large amounts of biological material is not devoid of risk, and there was some hesitation about giving plasma or albumin in large repeated

Table 12

Protein solutions given to patients with induced proteinuria

Patient	Diagnosis	Platelet-poor Plasma		Anti-haemophilic Fraction		Total protein infused		Infusion period * (days)
		ml.	G.	ml.	G.	ml.	G.	
1	Haemophilia	2400	132	600	18	3000	150	3
2*	Christmas Disease	7400	407	-	-	7440	407	9
3	Haemophilia	8400	462	2000	60	10400	522	8
4	Haemophilia	7200	396	2400	72	9600	468	8

*"Infusion period" is the time taken for persistent proteinuria to develop.

*Patient 2 also received 40 ml of prothrombin complex.

doses to normal volunteers. Patients with bleeding tendencies, however, receive sizeable infusions of protein-containing solutions as part of their haemostatic therapy. Thanks to the co-operation of Dr. Howard Davies and his colleagues in the Royal Infirmary Department of Haematology, it was possible for us to study these patients before, during, and after the protein infusions which they received as part of their routine therapy. These patients were not, of course, normal subjects, but all those selected for study had normal kidneys - as far as could be judged from clearance studies, electrolyte estimations, and urine examination.

Over a period of nine months, admission specimens of urine were tested for protein on about 30 haemophilic patients. No patient had significant proteinuria on admission (apart from 2 with frank haematuria) and in no patient was proteinuria induced by less than 2.5 litres of plasma given at a rate of 800ml daily.

Of the 10 patients who received more than 2.5 litres of plasma at the rate specified above, 6 developed proteinuria of over 1.0G per day. Because of a variety of clinical circumstances, studies on 2 of these 6 patients were fragmentary, but in the remaining 4, dextran and protein selectivity estimations were carried out before, and during induced proteinuria and also after significant proteinuria had disappeared. Three of these four patients had haemophilia and the fourth had Christmas disease. All received fresh frozen platelet-poor plasma (prepared by the Blood Products Unit, Edinburgh). The three haemophilics also received purified antihaemophilic fraction (Cohn fraction 1) and the patient with Christmas disease was given prothrombin complex (concentrate of

Table 13

Selectivity Results in Induced Proteinuria

Patient	Creatinine Clearance ml/min	Serum Protein (Prior to proteinuria) Total	Albumin	Serum Protein (During proteinuria) Total	Albumin	Urine Protein (Prior to infus-ion) mg/2hhr	Urine Protein (During protein G/2hhr	(-K) During Protein-uria	D During Protein-uria	D (No Protein-uria)
1	150	7.7	4.2	8.3	4.8	19	12.6	3.13	3.15	2.89
2	76	7.3	3.9	8.8	5.0	7	4.2	2.49	2.47	2.44
3	129	6.9	3.8	9.2	4.5	20	4.5	2.66	2.48	2.99
4	105	7.8	3.8	9.4	4.4	23	11.8	2.61	2.56	3.03

(-K) is the protein index of selectivity.

D is the dextran index of selectivity.

factors II, VII, IX, and X). The protein concentrations in each solution and the volume of each solution used are shown in table 12.

The methods used in assessing protein and dextran selectivities have already been described.

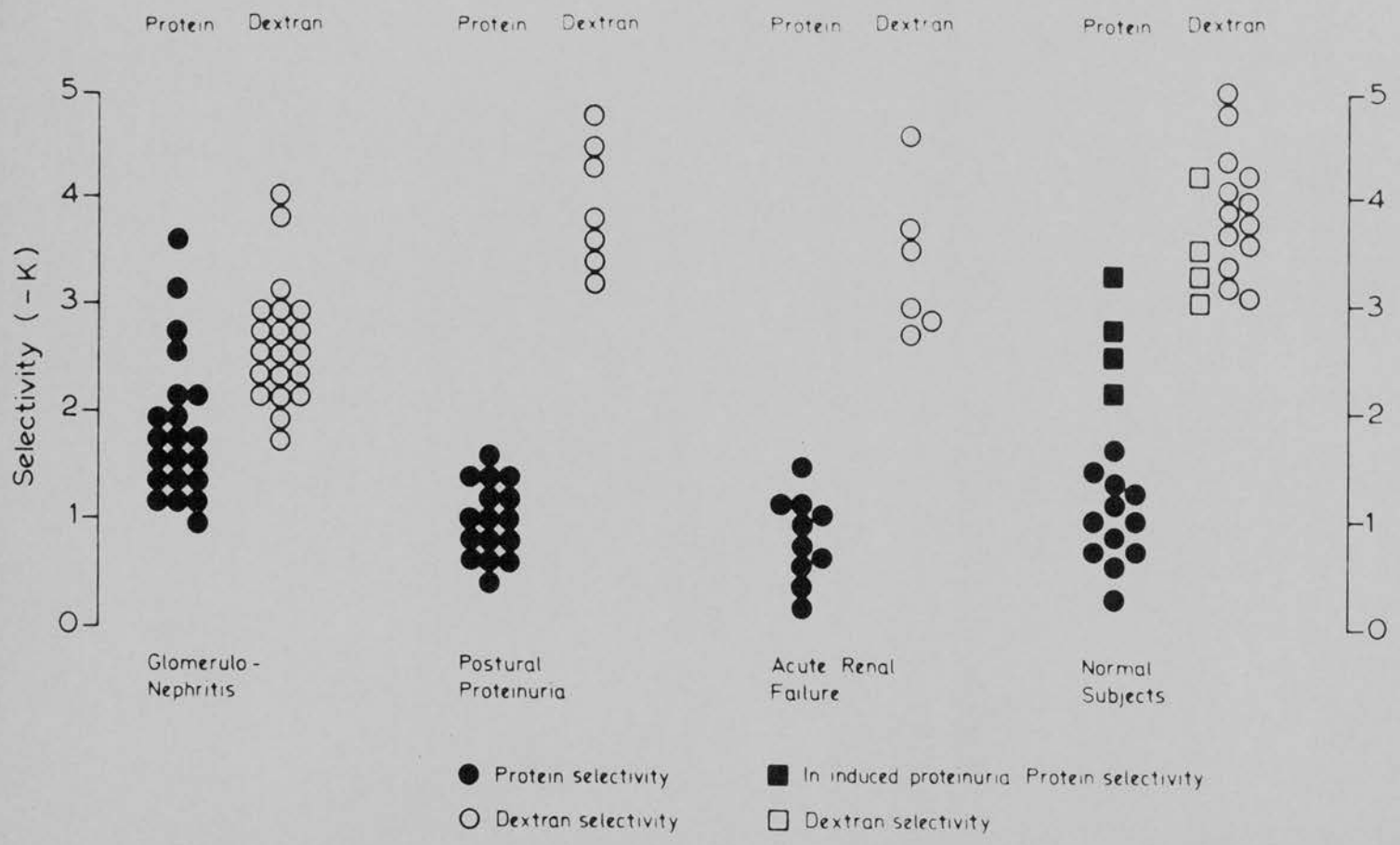
(ii) Results

The four patients studied all had less than 30 mg of protein per 100 ml in their urine and/or a urine to serum albumin concentration ratio of less than 1 in 1600 both before the protein infusion and five days after the cessation of therapy. The values for creatinine clearance ranged from 76 to 150 ml per minute. Urine microscopy (performed at least twice on each patient) failed to show red cell or granular casts on any occasion. A few hyaline casts were observed in the presence of induced proteinuria. Plasma protein concentrations prior to infusion ranged from 6.9 to 7.8 per 100 ml with albumin concentrations of between 3.8 and 4.2G/100 ml. Measurements during induced proteinuria gave total plasma protein concentrations ranging from 8.3 to 9.4 G per 100 ml. with albumin levels ranging from 4.4 to 5.0 G per 100 ml.

The range of protein selectivity values obtained during induced proteinuria was from 2.49 to 3.13. (See table 13). These values indicate a highly selective pattern of protein excretion, comparable to that found in minimal lesion glomerulonephritis. Dextran selectivity values were also high, ranging from 2.47 to 3.15. Converting these D values into their (-K) equivalents, we arrive at a range of dextran selectivities in the

Figure 26

Protein and dextran selectivity values in glomerulonephritis, acute tubular necrosis, postural proteinuria, and in normal subjects.



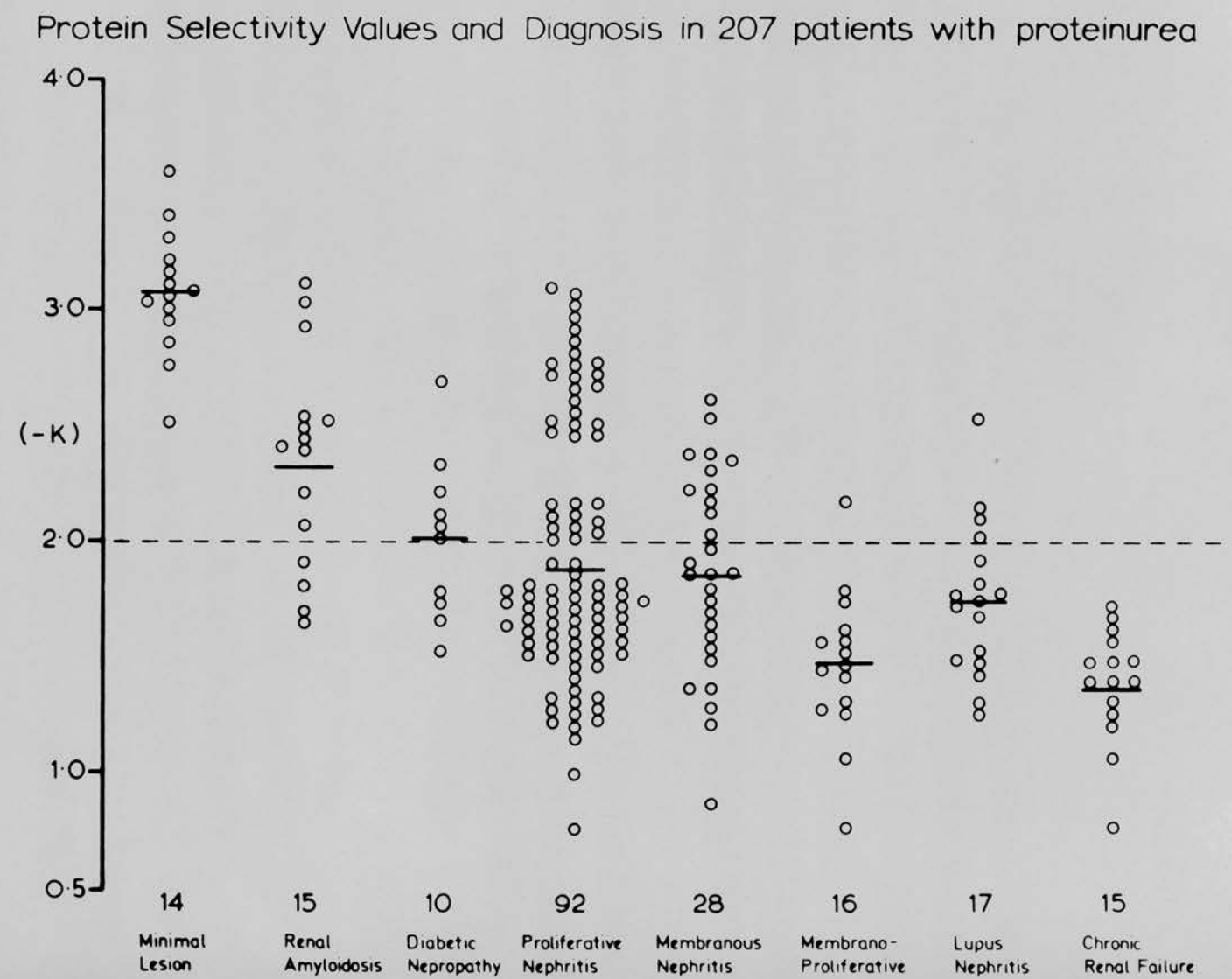
presence of induced proteinuria ranging from 3.38 to 4.32 (-K) units. The values obtained in the absence of induced proteinuria were essentially similar, the D values ranging from 2.44 to 3.03 and their (-K) equivalents from 3.34 to 4.15 (-K) units.

Thus when proteinuria is induced in subjects with normal kidneys by plasma infusion, selectivity values for both dextran and protein are high, and the dextran selectivity does not change appreciably from that noted in the absence of proteinuria. The possible importance of this observation will be discussed in section IV of this thesis.

In this section, protein and dextran selectivity values have been compared in a variety of situations. Figure 26 summarises these comparisons. Dextran and protein results are both expressed in terms of (-K).

In all situations there is a tendency for dextran values to be higher than protein values. In glomerulonephritis and in induced proteinuria, however, dextran and protein selectivity results are comparable. In normal subjects under normal conditions, in postural proteinuria, and in acute renal failure, dextran selectivity results are very much higher than protein selectivity results, in every case.

Figure 27


Protein Selectivity Values and Diagnosis in 207 patients with proteinurea
4·0
3·0
(-K)
2·0
1·0
0·5
14
15
10
92
28
16
17
15
Minimal Lesion
Renal Amyloidosis
Diabetic Nepropathy
Proliferative Nephritis
Membranous Nephritis
Membrano-Proliferative
Lupus Nephritis
Chronic Renal Failure

3. Protein Selectivity Values in Patients with Major Proteinuria

During the period 1964-1970, 221 patients came under the care of the Medical Renal Unit in Edinburgh who fulfilled the following criteria.

1. They had major proteinuria of over 1.0G in 24 hours, and proteinuria of this order persisted for four weeks or longer following their referral to the unit.
2. They were followed up for a minimum period of three months.
3. Data concerning creatinine clearance, 24 hour protein excretion, serum proteins and protein selectivity were obtained.

This group consists, therefore, of patients with persistent major proteinuria. Patients with pyelonephritis or tubular necrosis do not figure among the 221 cases, as these patients either did not excrete as much as 1.0G of protein daily, or else did so very transiently. Patients with classical and rapidly resolving acute nephritis are also excluded because of the transient and variable nature of their protein excretion. None of the patients with postural proteinuria excreted as much as 1.0G of protein per 24 hours except on one or two isolated occasions.

Of the 221 patients in this group, 195 underwent renal biopsy. The pathological diagnosis was made in each case by Dr. Mary MacDonald of the University Department of Pathology; it was based on both light and electron microscopy findings. (MacDonald and Ruckley, 1966; Robson, 1967). Average values for protein selectivity, 24 hour protein excretion, creatinine clearance, and serum albumin are shown in table 14.

The 221 patients were classified histologically as follows:-

Table 14

Protein Selectivity values in Patients with Major Proteinuria.

	-K (mean)	GER (mean) ml./min	Serum Albumin G/100ml. (mean)	Protein Excretion G/24hr. (mean)	Number of Patients.
Proliferative glomerulonephritis	1.86	73	2.5	4.7	92
Membranous glomerulonephritis	1.90	88	2.2	7.7	28
Mixed membranous and Proliferative	1.52	88	2.5	5.9	16
Minimal lesion glomerulonephritis	3.08	84	1.6	6.6	14
Renal lupus erythematosus	1.74	74	2.5	5.7	17
Renal amyloidosis	2.33	60	1.9	6.8	15
Diabetic nephropathy	2.10	65	-	3.2	10
No biopsy (contracted Kidneys)	1.32	8.2	3.2	2.8	15
Other undiagnosed patients	-	-	-	-	14
				Total	211

Proliferative glomerulonephritis	- 92
Membranous glomerulonephritis	- 28
Mixed membranous and proliferative	- 16
Minimal lesion glomerulonephritis	- 14
Renal Lupus erythematosus	- 17
Renal amyloidosis	- 15
Diabetic nephropathy	- 10
Miscellaneous or unclassified	- 8
No biopsy	- 21

a. Patients with Proliferative Glomerulonephritis

This large and heterogenous group was comprised of several disease entities. In 20 cases, a streptococcal aetiology appeared clear-cut, though only a minority of these 20 cases had a classical Ellis type 1 history. In 8 cases there was clinical evidence of Henoch-Schönlein disease. 3 patients had unequivocal Goodpasture's syndrome, and 2 had Alport's syndrome. The remaining 59 cases presented as the nephrotic syndrome, as symptomless proteinuria, or as cases of impaired renal function of insidious onset.

Some of the patients fell clearly into the histological subgroups of proliferative glomerulonephritis described by Cameron (1971) but in the majority of cases sub-classification by these criteria would have proved somewhat arbitrary and possibly misleading. An additional 36 cases shown on biopsy to have proliferative change were excluded from the series because of the minor or transient nature of their proteinuria. In over half of these excluded cases a streptococcal aetiology was either established or strongly suspected.

The 92 patients with proliferative glomerulonephritis included in the study had a mean protein excretion of 4.7G per day.

The mean creatinine clearance value for the group was 73 ml per minute, and the mean serum albumin level was 2.5G per 100 ml. The average duration of follow up was 2.8 years.

26 of the patients with proliferative glomerulonephritis developed renal failure leading to either death or dialysis. 5 died of causes not immediately related to renal disease - 3 from bronchogenic carcinoma, one from pulmonary embolism, and one from myocardial infarction.

The mean selectivity value for this large and diverse group was 1.86; S.D. $\pm$ 0.54.

b. Patients with Membranous Glomerulonephritis

There were 28 patients in this group. All the patients with the histological appearances of membranous glomerulonephritis seen during the period 1964 to 1970 had major proteinuria which persisted for months or years; no patient had to be excluded on the ground of minor or transient proteinuria.

The mean daily protein excretion in the 28 cases was 7.7G. The mean creatinine clearance value was 88 ml per minute, and the mean serum albumin concentration was 2.2G per 100 ml. The average period of follow up for the patients in this group was 3.4 years.

4 patients with membranous glomerulonephritis developed renal failure during the period of follow-up; 2 died and 2 were started on long term haemodialysis. 2 further patients died of causes not immediately connected with renal disease - one from bronchogenic carcinoma and one from carcinoma of the colon.

The mean selectivity value with respect to protein for these 28 patients was 1.90; S.D. $\pm$ 0.08.

c. Patients with Mixed Membranous and Proliferative Glomerulonephritis

There were 16 patients in this group. All patients with histological changes indicative of mixed membranous and proliferative glomerulonephritis on renal biopsy had persistent proteinuria of over 1.0G in 24 hours; no case had to be excluded on grounds of transient or trace proteinuria.

The mean daily protein excretion in this group was 5.9G. The mean value for creatinine clearance was 88 ml per minute and the mean serum albumin concentration was 2.5G per 100 ml. The average period of follow-up for the patients in this group was 3.8 years.

During the period of follow-up, 5 patients developed renal failure. 3 of these died; the remaining 2 were started on long term haemodialysis. No patient in this group died of causes unconnected with renal disease.

The mean selectivity value for protein in the group of 16 patients with mixed membranous and proliferative glomerulonephritis was 1.52; S.D. $\pm$ 0.09.

d. Patients with Minimal Lesion Glomerulonephritis

14 patients had changes indicative of minimal lesion glomerulonephritis on renal biopsy, and in each of these cases the diagnosis was confirmed by electron microscopy. 8 patients initially considered on light microscopy to have minimal lesion glomerulonephritis were found on electron microscopy to have proliferative changes; these cases have been included in section 3a. 3 patients with light and electron microscopy evidence of minimal lesion glomerulonephritis were excluded from this series because a steroid-induced remission had occurred before satisfactory selectivity values had been obtained.

11 of the 14 cases with minimal lesion glomerulonephritis were adults. The remaining 3 were 5, 6 and 12 years old respectively at the time of their first presentation to the renal unit.

The mean 24 hour protein excretion in this group of patients was 6.6G. The mean creatinine clearance value was 84 ml per minute and the mean serum albumin concentration was 1.6G per 100 ml.

The average period of follow-up for the 14 cases with minimal lesion glomerulonephritis was 5.0 years. 2 patients died during the period of follow-up. Both had excellent renal function at the time of death. One patient died of myocardial infarction while on steroid therapy; the second, a girl of 15 with idiopathic epilepsy, was accidentally drowned - probably as the result of an epileptic convulsion.

The mean selectivity value for protein in the 14 patients with minimal lesion glomerulonephritis was 3.08; S.D. $\pm$ 0.11.

e. Patients with Renal Amyloidosis

15 patients had renal biopsy appearances indicating amyloidosis. In 5 the amyloid infiltration appeared to be secondary to reheumatoid arthritis, and in 2 cases it appeared to be due to chronic pulmonary suppuration. In 1 patient renal amyloidosis occurred in conjunction with dental sepsis; in this patient proteinuria disappeared following extraction of the affected teeth. Amyloidosis was secondary to multiple myeloma in 1 patient, and in one further case the presence of para-proteinaemia suggested a similar aetiology, though abnormal plasma cells could not be demonstrated. In 6 patients there was no apparent underlying aetiology to account for the amyloidosis.

2 patients with renal biopsy appearances indicative of amyloidosis had to be excluded from this series because of inadequate follow-up and incomplete data.

The mean 24 hour protein excretion in the patients with amyloid disease was 6.8G. The mean creatinine clearance was 60 ml per minute and the mean serum albumin concentration was 1.9G per 100 ml. The average period of follow-up for the 15 patients with renal amyloid was 2.0 years. 7 of the patients died during the period of follow-up. One case, with good renal function, died of a gastro-intestinal haemorrhage complicating steroid therapy. The remaining 6 died of renal failure.

The mean protein selectivity value for the 15 patients with renal amyloidosis was 2.33; S.D. $\pm$ 0.16.

f. Patients with Renal Lupus Erythematosus

In 17 patients the renal biopsy appearances were indicative of renal lupus erythematosus. One case, with similar appearances, was excluded from the series because of the minor and transient nature of his proteinuria. Two further cases were excluded because of inadequate data.

In 10 of the patients in this series the histological diagnosis was supported by the following additional evidence of lupus.

1. A strongly positive test for antinuclear factor obtained on 2 or more occasions, and/or a positive L.E. cell preparation obtained on 2 or more occasions.
2. Two or more of the following:-

a rash compatible with S.L.E.
a haemolytic anaemia responding to steroid therapy.
involvement of the uveal tract.
a white cell count of 4,000 per cubic millimetre or under.
arthropathy or arthritis.
pleurisy, not explicable in terms of infection.
pericarditis, not explicable in terms of uraemia.
pyrexia unexplained by infection and responsive to steroid therapy.

These 10 patients were regarded as having definite renal lupus.

In 7 of the 17 cases the pathological diagnosis was less strongly supported by laboratory and clinical evidence. These patients were regarded as having probable renal lupus erythematosus.

The mean 24 hour protein excretion in the 10 cases with definite renal lupus was 4.6G per 100 ml. The mean creatinine

clearance was 75 ml per minute and the mean plasma albumin concentration was 2.6G per 100 ml. The average period of follow-up was 3.1 years. Two of the 10 cases with definite lupus died during the period of follow-up, death in both instances being due to renal failure.

The mean protein selectivity value for the patients in this sub-group was 1.80.

In the 7 cases classified as having probable renal lupus, the mean 24 hour excretion of protein was 7.2G. The mean value for creatinine clearance was 72 ml/min and for serum albumin it was 2.4G per 100 ml. The average period of follow-up was 2.2 years. During this period of follow-up 2 patients died, both of renal failure. The mean value for protein selectivity in this sub-group was 1.66.

Combining the results for all 17 cases with histological appearances suggesting lupus, the mean 24 hour protein excretion was 5.7G. The mean creatinine clearance value was 74 ml per minute. The average serum albumin concentration was 2.5G per 100 ml. The average period of follow-up for the 17 patients was 2.7 years and four patients died during the follow-up period, all of renal failure.

The mean value for protein selectivity in the 17 patients with definite or probable renal lupus was 1.74; S.D. $\pm$ 0.12.

g. Patients with Diabetic Nephropathy

The 10 patients with diabetic nephropathy represented a small fraction of the several hundred diabetic patients with proteinuria who attended the diabetic department of Edinburgh Royal Infirmary in the period 1964-70. Since the priority with these patients was the management of their overall diabetic state, they were followed up at the diabetic department rather than at the renal clinic. Prolonged follow-up information on this group of patients was therefore not available.

The mean protein selectivity in the 10 patients with diabetic nephropathy was 2.01. Twenty-four hour protein excretion averaged 3.2 and the mean creatinine clearance for the group was 65 ml/minute.

h. Miscellaneous other patients

Of the 221 patients discussed in this section, 29 could not be assigned to a precise diagnostic category. In 8 cases the biopsy findings were equivocal. In the remaining 21, no biopsy tissue was obtained.

15 of the 21 patients in whom biopsy was not undertaken had radiological evidence of very small kidneys, and it was because of the size of the target organs that biopsy was not attempted. These 15 cases all had creatinine clearances of under 15 ml per minute at the time of their presentation to the renal unit. In spite of the absence of a histological diagnosis, it was possible to group them together as having contracted kidneys, major proteinuria, and moderate to severe renal failure.

The mean protein selectivity for this group of 15 patients was 1.32. The 24 hour protein excretion averaged 2.8G and the creatinine clearance averaged 8.2 ml per minute. The mean serum albumin level was 3.2G per 100 ml. The mean period of follow-up for this group of patients was 1.7 years, and 10 of the 15 either died or were started on dialysis during the period of follow-up.

The remaining 14 patients had nothing in common except for the absence of a diagnosis; their results have therefore not been analysed in detail.

4. The relationship between protein selectivity values and prognosis in patients with major proteinuria.

It is generally agreed that in patients with major proteinuria there is a correlation between selectivity and the severity of the underlying renal lesion. (Hardwicke and Squire, 1955; Joachim et al, 1964; Cameron and White, 1965; Robson, 1967.) Since a highly selective proteinuria tends to be associated with relatively slight pathological changes, it might be expected that a high selectivity index would be associated with a favourable prognosis and that a low index of selectivity would be prognostically unfavourable.

From the previous section, however, it will be seen that patients with renal amyloidosis tend to have selective proteinuria, and that 6 out of 15 patients with this condition died of renal failure during a period of follow-up which averaged less than 2 years. A selective proteinuria is therefore not always associated with a prolonged survival. This observation prompted an examination of the relationship between selectivity and survival.

Of the 221 patients with major proteinuria discussed in the preceding section, 197 were included in this study on prognosis. The 14 patients discussed in section 3h who could not be classified were excluded, as were the 10 diabetic patients discussed in section 3g. The diabetic cases were excluded because they were not followed up at the renal clinic, and information concerning their progress was incomplete.

The relationship between selectivity and prognosis was assessed in two ways.

(a) The status of each of 146 patients was recorded three years after the first attendance of that patient at the renal clinic. The patients were divided into four groups on the basis of the selectivity of their protein excretion, and the survival rate at three years was assessed for each group.

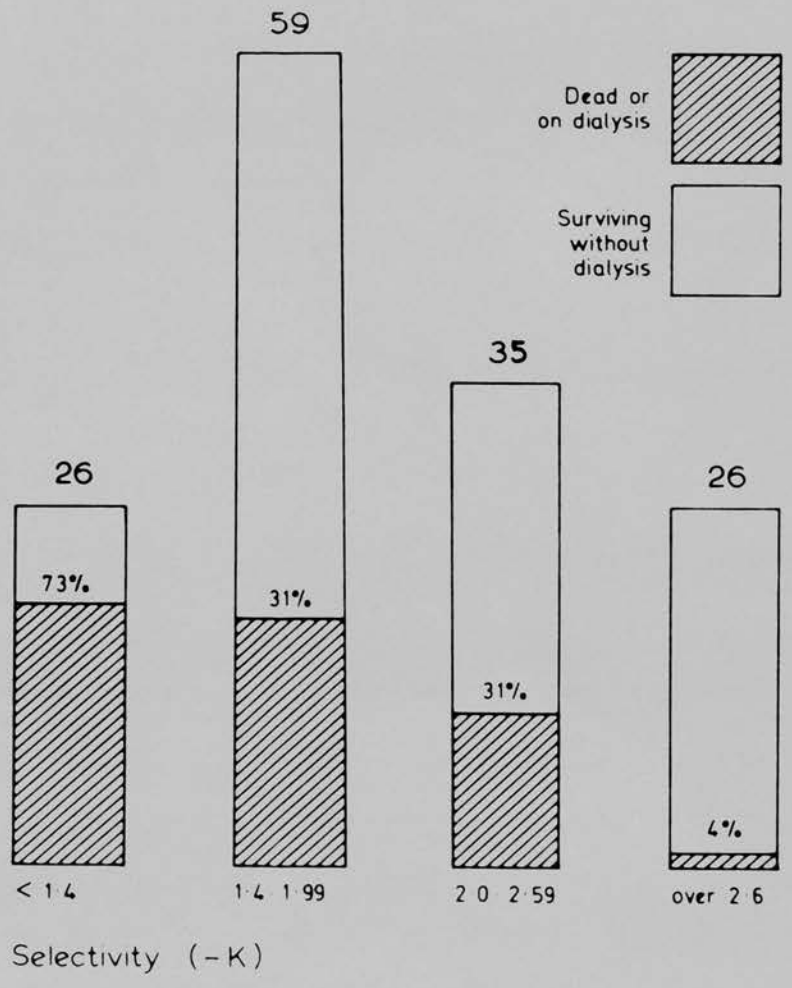
(b) 183 patients were divided into two groups, the first comprising 116 cases with unselective proteinuria, and the second 67 cases with selective proteinuria. Survival in each group was assessed by an actuarial method. (Barnes, 1965 a and b).

In every case "survival" was taken as meaning survival with independent renal function. Failure of survival was taken as either the death of the patient, or the development of chronic renal failure necessitating long term dialysis or renal transplantation (i.e. death of the kidneys).

Patients developing end stage renal failure were regarded as having sustained "renal death" on the date of their first dialysis. Dialysis preceded transplantation in all cases receiving a kidney graft.

Figure 28

Survival with independent renal function related to selectivity in 146 patients with proteinuria of over 1.0G per day.



a. Three year survival with independent renal function in patients with varying indices of protein selectivity.

Of the 197 patients considered in the study on selectivity and prognosis, 179 were seen for the first time on or before December 31st, 1967, and could therefore have been followed for three years by 31st December 1970, when the study was concluded. Of these 192 patients, 49 were either dead or dependent on an artificial or transplanted kidney within three years of their first attendance. 97 patients were known to have survived three years with independent renal function. 33 patients, comprising 18% of those who could in theory have been followed for three years, had been lost to follow-up.

The 149 patients who were followed for 3 years or until dialysis was needed or death ensued were divided into four groups on the basis of their selectivity index.

The first group consisted of patients with very unselective proteinuria. It comprised 26 patients in whom the value for (-K) was less than 1.4. 19 patients in this group (73%) were either dead or on dialysis by the end of three years.

The second group comprised patients with moderately unselective proteinuria, i.e. a (-K) value of 1.40 to 1.99. 59 patients were in this category, 18 of the 59 (31%) were either dead or on dialysis at the end of three years.

The third group consisted of patients with moderately selective proteinuria, i.e. a (-K) value of 2.00 to 2.59. 35 patients had selectivity indices in this range. 11 of these 35 patients (31%) were either dead or on dialysis by the end of three years.

Table 15.

Diagnosis	Selectivity under 1.4			Selectivity 1.4 to 1.99			Selectivity 2.0 to 2.59			Selectivity 2.6 and over			Total in all 4 groups		
	Total in group	Dead or on dialysis	Survival 3 years	Total in group	Dead or on dialysis	Survival 3 years	Total in group	Dead or on dialysis	Survival 3 years	Total in group	Dead or on dialysis	Survival 3 years	At start	Surviving 3 years	% Surviving
Proliferative Glomerulonephritis	10	7*	3	30	10	20	18	7*	11	10	0	10	68	44	65%
Membranous Glomerulonephritis	3	2	1	9	1	8	5	0	5	1	0	1	18	15	83%
Mixed M and P Glomerulonephritis	5	2	3	6	1	5	1	0	1	0	0	0	12	9	75%
Minimal Lesion Glomerulonephritis	0	0	0	0	0	0	0	0	0	14	1*	13	14	13	93%
Renal Lupus	2	2	0	6	2	4	5	1	4	0	0	0	13	8	62%
Renal Amyloid	0	0	0	3	0	3	6	3	3*	1	0	1	10	7	70%
No biopsy (Contracted Kidneys)	6	6	0	5	4	1	0	0	0	0	0	0	11	1	9%
Total in each group	26	19	7	59	18	41	35	11	24	26	1	25	146	87	

*Includes 1 non renal death.

Three year survival with independant renal function in patients with major proteinuria.

The fourth group was made up of patients with highly selective proteinuria, i.e. a (-K) value of over 2.6. Of the 26 patients in this group, all but 1 (4%) survived for three years with independent renal function. The single death in this group was an elderly patient with minimal lesion glomerulonephritis whose demise from myocardial infarction occurred in the presence of good renal function.

The relationship between selectivity and status at three years is shown in figure 28. Table 15, in addition to showing the fate of the patients in each group, also shows the diagnoses of the patients concerned, and the survival in relation to selectivity for each diagnostic category. Since any hospital series is a selected one in which mild cases are under-represented, table 15 cannot be taken as reflecting the prognosis of (say) the general run of patients with proliferative glomerulonephritis. Many patients with mild acute nephritis were not referred to the renal unit. Of the patients who were referred, about 30 were excluded from this study because rapid resolution of proteinuria prevented adequate selectivity studies from being undertaken.

Although the series was a selected one (see p.97 for the precise selection criteria) protein selectivity was not one of the factors involved in the selection. As the number of patients involved in the study was large, the observed relationship between selectivity and prognosis is almost certainly a real one. Summarising these results,

a) in very unselective proteinuria (-K less than 1.4) the prognosis appears to be bad, with 73% of patients dying or developing end-

stage renal failure within 3 years.

b) in very selective proteinuria (-K over 2.6), the prognosis is good, the 3 year survival being in excess of 95%.

c) The two intermediately selective groups did not differ appreciably from each other in terms of prognosis. In each group, the number of cases dying or developing end-stage renal failure was 31%. This survival rate was significantly better than in the patients with very unselective proteinuria, and significantly worse than in the patients with highly selective proteinuria.

Table 16. Prognosis Related to Protein Selectivity.

(a) Diagnostic Categories of Patients included in Actuarial Calculation.

(i) Unselective Proteinuria: (-K) 1.90 and under.

Diagnosis	Dead or on dialysis						Survivors, followed for							Total in Group
	in 1yr	1-2yr	2-3yr	3-4yr	4-5yr	Total	1yr	1-2yr	2-3yr	4-5yr	4-5yr	5yr	Total	
Proliferative	10	6	2	3	1	22	1	8	12	1	3	10	35	57
Membranous	0	1	1	0	0	2	0	1	3	3	0	5	12	14
Mixed	3	1	0	0	1	5	1	2	0	0	1	5	9	14
Minimal	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Amyloid	1	0	0	1	0	2	1	0	0	0	0	1	2	4
Lupus	0	0	3	0	0	3	3	1	2	2	0	1	9	12
Contracted Kidneys	4	4	2	0	0	10	3	1	0	1	0	0	5	15
Total	18	12	8	4	2	44	9	13	17	7	4	22	72	116

(ii) Selective Proteinuria: (-K) 2.10 and over.

Diagnosis	Dead or on dialysis						Survivors, followed for							Total in Group
	in 1yr	1-2yr	2-3yr	3-4yr	4-5yr	Total	1yr	1-2yr	2-3yr	3-4yr	4-5yr	5yr	Total	
Proliferative	2	1	3	0	1	7	0	6	4	4	0	9	23	30
Membranous	0	0	0	0	0	0	2	1	5	1	0	1	10	10
Mixed	0	0	0	0	0	0	1	0	0	0	0	0	1	1
Minimal	1	0	0	1	0	2	0	0	1	1	3	7	12	14
Amyloid	2	0	0	1	0	3	1	3	1	1	0	1	7	10
Lupus	1	0	0	0	0	1	0	0	0	0	0	2	2	3
Contracted Kidneys	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Total	6	1	3	2	1	13	4	10	11	7	3	20	54	67

b. <u>The relationship between protein selectivity and projected survival with independent renal function in patients with major proteinuria.</u>

The actuarial method used in this part of the survival study is that used by Barnes (1965a and b) in the compilation of the statistics of the International Transplant Register. In section 4a only those patients who had been followed for three years or until the termination of independent renal function could be included in the calculation. Using the actuarial method, patients lost to follow-up, or seen for the first time during the past three years, could be included in the study.

As the actuarial method is unreliable where small groups of patients are concerned, cases were divided into two groups on the basis of selectivity, instead of into four, as in the preceding study. (-K) values for the unselective group were 1.9 and below; there were 116 patients in this group. The selective group, (patients with (-K) values of 2.1 and above), was made up of 67 patients. Table 16 shows the diagnostic categories and the outcome of the patients in these two groups. 14 patients had selectivity values in the range 1.9 to 2.1; as these patients did not fall into either group they were excluded.

The method of calculating projected survival is as follows.
Let n be the number of patients surviving at the start of time period x1.
Let b be the number of patients dying or requiring dialysis during the period x1.
And let c be the number of patients lost to follow-up during the period x1.

Table 17.

Projected survival* in patients with major proteinuria.

1. Unselective proteinuria: -K under 1.9.

Period	n (Patients surviving at start of period)	b (Patients dying or started dialysis during period)	c (Lost to follow-up during period)	q $\left(\frac{b}{n-c/2}\right)$	p Survival for period (1 - q)	f.s. Percent surviving at end of period	Error ø %
First year	116	18	9	0.161	0.847	84.7	±3.4
Second year	89	12	13	0.145	0.855	72.4	±4.3
Third year	64	8	17	0.144	0.856	62.0	±4.8
Fourth year	39	4	7	0.072	0.928	57.5	±5.2
Fifth year	28	2	4	0.077	0.961	55.3	±5.8
2. Selective proteinuria: -K over 2.1.							
First year	67	6	4	0.092	0.908	90.8	±3.5
Second year	57	1	10	0.019	0.981	89.1	±3.8
Third year	46	3	11	0.074	0.926	82.5	±5.0
Fourth year	32	1	7	0.034	0.966	79.7	±5.4
Fifth year	24	1	3	0.047	0.953	75.9	±6.2

*Survival means survival with independant renal function.

ø Percentage error = F.S. x $\sqrt{\Sigma\left(\frac{q}{n-b}\right)}$ (Barnes 1965a)

Then q, the proportion of patients dying or requiring long term dialysis during the period x1, is

$$\frac{b}{n - c/2}$$

The survival for the period x1 can be represented by p; p is equal to (1-q).

The survival for the second period of follow-up (x2) can be similarly calculated.

The percentage surviving at the end of period x2 (F.S.) is $p_{x1} \times p_{x2} \times 100$

The error of the actuarial method is given by Barnes (1965a) as being equal to

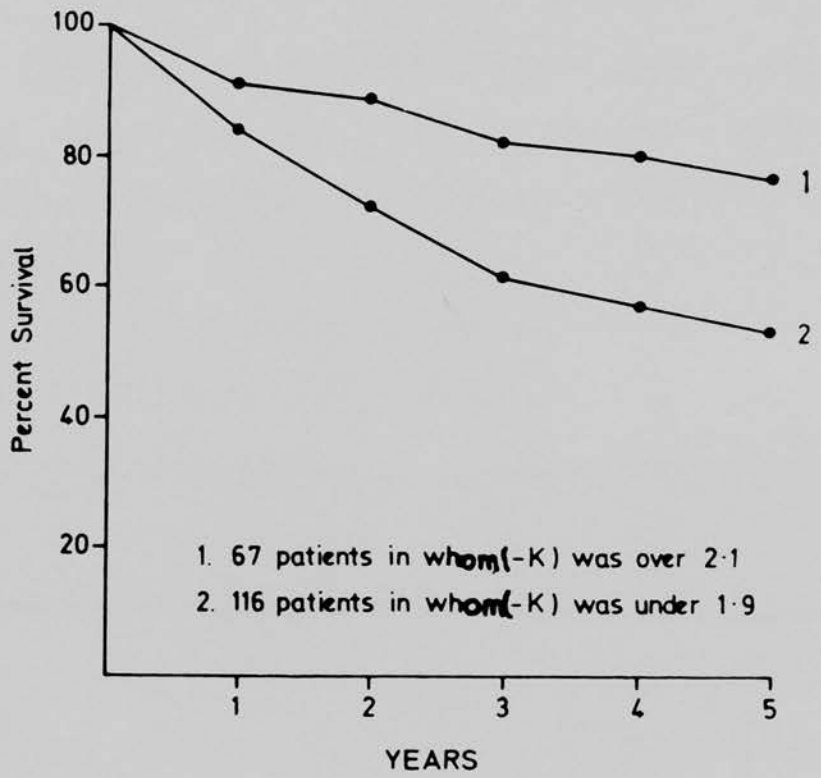
$$\text{F.S.} \times \sqrt{\Sigma \frac{q}{(n - b)}}$$ (Greenwood 1926)

Table 17 shows the calculation of the survival quotients in the two groups for each of the five years of follow-up considered. The number of patients followed for more than five years was 22 in the first (unselective) group and 20 in the second (selective) group. These numbers were too small to allow calculation of survival quotients for the sixth and subsequent years.

As shown in table 17 and in figure 29, survival with independent renal function was consistently better for the patients with (-K) values of over 2.1 than for patients with selectivity values of under 1.9. The difference was greater than could be accounted for by the fact that all the patients with minimal lesion glomerulonephritis were in the selective group. The difference in survival for the two groups was 6.1% at the end of the first year; this was

Figure 29

Projected survival with independent renal function in 183 patients with proteinuria of over 1.0G per day.

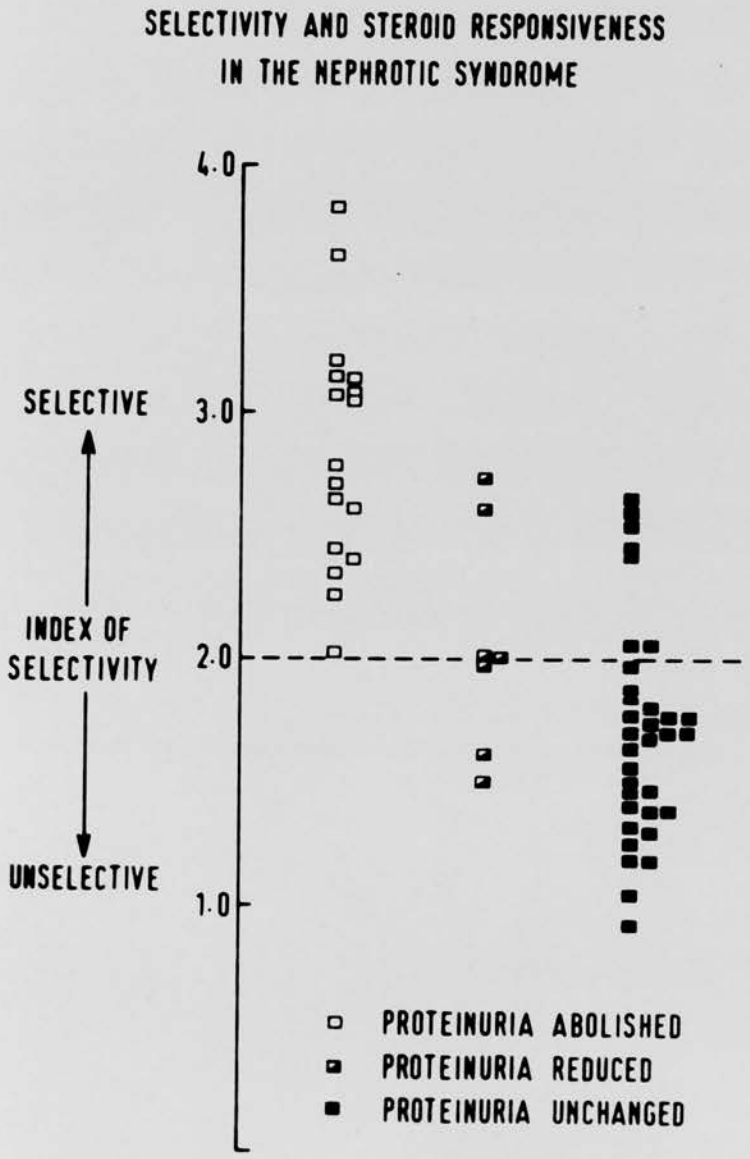


not significant in view of the combined error of 6.9%. For the second year (difference 16.8%; combined error 8.1%) through to the fifth year (difference 20.1%; combined error 12.0%) survival with independent renal function was significantly better for the selective group than for the unselective group.

Of the 13 patients with selective proteinuria who died or required dialysis, 5 died of non-renal causes in the presence of good renal function. One of these died of bronchogenic carcinoma, two from myocardial infarction, 1 from accidental drowning, and one from gastric haemorrhage complicating steroid therapy.

Of the 44 patients with unselective proteinuria who died or required dialysis, only 2 did not develop end-stage renal failure. Both these patients died of bronchogenic carcinoma.

Figure 30


SELECTIVITY AND STEROID RESPONSIVENESS
IN THE NEPHROTIC SYNDROME
4·0
3·0
2·0
1·0
SELECTIVE
INDEX OF
SELECTIVITY
UNSELECTIVE
PROTEINURIA ABOLISHED
PROTEINURIA REDUCED
PROTEINURIA UNCHANGED

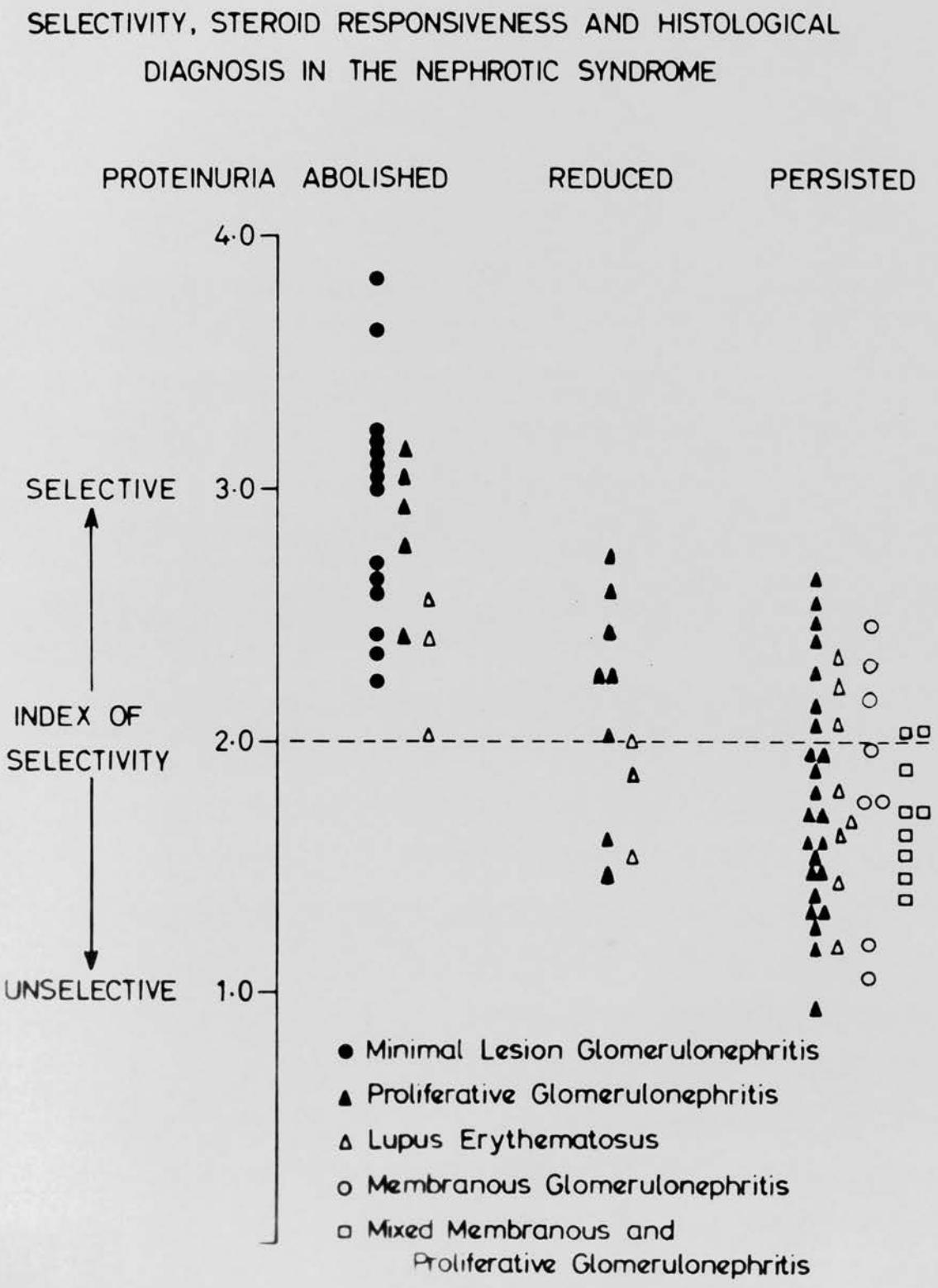
5. Protein selectivity and responsiveness to steroid therapy

Patients with a high protein index of selectivity tend, on the whole, to respond more favourably to steroid therapy than patients with unselective proteinuria (Joachim et al, 1964; Cameron, 1968). In this section, this relationship between selectivity and steroid responsiveness is examined with a view to determining whether, in the individual case, protein selectivity is an important factor in the decision as to whether or not to use steroids. Since the M.R.C. trial on the nephrotic syndrome (Black et al 1970) has shown that the overall mortality in adults treated with steroids is greater than that in matched controls, steroid therapy should obviously not be used except where there is a strong possibility of a favourable response.

Our preliminary findings in 1966 (Robson, Petrie and MacLean, unpublished) are shown in figure 30. Of 58 patients (predominantly adults) who were treated with steroids for the nephrotic syndrome, proteinuria disappeared in 17 and was reduced to 20% or less of its initial quantity in a further 7. In 34 patients, there was no significant response to steroid therapy. All of the 17 patients in whom proteinuria was abolished had protein selectivity values of over 2.0; these 17 cases comprised 61% of the total number of patients with selective proteinuria who were treated with steroids. Out of 30 cases with protein selectivity values of less than 2.0, proteinuria was not abolished in a single instance.

As a result of these findings, we came to the conclusion that an unselective pattern of protein excretion was a strong contra-indication to steroid therapy, except possibly in patients with

Figure 31


SELECTIVITY, STEROID RESPONSIVENESS AND HISTOLOGICAL DIAGNOSIS IN THE NEPHROTIC SYNDROME
PROTEINURIA
ABOLISHED
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1·0
SELECTIVE
INDEX OF SELECTIVITY
UNSELECTIVE
● Minimal Lesion Glomerulonephritis
▲ Proliferative Glomerulonephritis
△ Lupus Erythematosus
○ Membranous Glomerulonephritis
□ Mixed Membranous and Proliferative Glomerulonephritis

systemic lupus erythematosus, where there is some evidence (Pollak et al, 1964) that the use of this form of therapy may delay the progression of the disease.

In view of a favourable response rate of over 50%, we felt that a selective proteinuria might constitute at least a relative indication for steroid therapy, except of course in those cases where the degree of proteinuria was so slight that there was no clear need for any therapy at all.

There is, however, a strong body of opinion which holds that steroids are only of proven value in patients with minimal lesion glomerulonephritis (Sharpstone et al, 1969b, White et al, 1970, Cameron, 1971), though these authors would probably agree that this form of therapy should not be withheld in patients with renal lupus. A substantial proportion of the patients with selective proteinuria who responded to steroids in our initial series had minimal lesion glomerulonephritis, and in this condition the proteinuria is invariably selective. It was obviously necessary to reassess our results to discover if the favourable response to steroids shown in the patients with selective proteinuria could be accounted for entirely by the responsiveness of the patients with minimal lesions.

Figure 31 shows the 1966 series brought up to date by the addition of a further 28 cases. In addition to selectivity and responsiveness to therapy, the diagram shows the histological diagnosis in each case. Of 82 patients with the nephrotic syndrome treated with steroids, 44 had selective proteinuria (-K over 2.0). 22 of these 44 patients lost their proteinuria completely following

steroid therapy; a response rate of 50%. In none of the 38 patients in whom selectivity was low (-K under 2.0) did proteinuria disappear completely after steroids.

Relating steroid responsiveness to histological diagnosis, all 14 patients with minimal lesion glomerulonephritis sustained a complete remission following steroid therapy. None of the 8 patients with membranous glomerulonephritis responded to steroids, and the same total lack of responsiveness was shown by the 9 patients with mixed membranous and proliferative changes.

There were 18 cases of proliferative glomerulonephritis with protein selectivities of 2.0 or over. Abolition of proteinuria occurred in 5 of these patients following steroids and reduction in proteinuria to 20% or less of the initial value occurred in a further 6. In only 7 of these selective cases (39%) was there complete unresponsiveness to steroids. Of the 19 cases of proliferative glomerulonephritis having (-K) values of under 2.0, proteinuria was not abolished in a single case, and was reduced to 20% or less of the initial value in only 2. Seventeen patients (90%) with proliferative glomerulonephritis and unselective proteinuria were totally unresponsive to steroids.

Of 7 cases of renal lupus with selective proteinuria, 3 showed a complete response to steroids, and 1 showed a partial response. Three of the 7 selective cases (43%) were totally unresponsive. Of 7 cases of lupus with unselective proteinuria, in no instance was proteinuria abolished completely by steroids. Two patients showed a partial response, with proteinuria falling to 20% or less of the initial value. Five of the 7 unselective cases (71%)

showed no response to steroids.

As a result of these figures, one can concur with Cameron (1971) to the extent of admitting that only in minimal lesion glomerulonephritis can resolution of proteinuria be confidently expected following steroid therapy. There would appear to be little or no place for steroid therapy in the nephrotic syndrome with membranous or with membrano-proliferative histology. Cameron suggests that steroids are never indicated in proliferative glomerulonephritis. Our results however would appear to suggest that at least some patients with this histological picture benefit from steroids, the benefit being largely confined to the cases with selective proteinuria.

The disappearance of proteinuria following steroid therapy does not of course prove that the remission was caused by steroids. The incidence of spontaneous remission in the nephrotic syndrome can be as high as 36% in children over a one year period (Arneil, 1961) and of the order of 15 to 20% in adults (Nesson et al, 1963; Ross and Smith, 1963). In proliferative glomerulonephritis - particularly where the onset is 'nephritic' rather than 'nephrotic' (see De Wardener, 1967) remission within a few weeks of onset is very common. It was, however, our practice to withhold steroids for at least four weeks in all cases, and to avoid them altogether in patients fitting the clinical classification of Ellis Type I nephritis. Steroids were only given to patients with major proteinuria and oedema; they were withheld if clinical, biochemical, or histological criteria suggested the likelihood of a reasonably

Table 18 Patients with proliferative glomerulonephritis.

Proteinuria at 8 weeks expressed as a per centage of initial proteinuria.

Unselective patients (-K less than 2.0) Untreated controls.

181%
169%
133%
125%
125%
123%
119%
118%
89%
88%
78%
75%
67%
42%
26%
20%
11%

Mean at 8 weeks = 93.5% ± 12.1

Unselective patients (-K less than 2.0) Treated with steroids.

183%
130%
118%
104%
94%
89%
88%
88%
79%
73%
68%
40%
37%
24%

Mean at 8 weeks = 86.79% ± 10.9

Selective patients (-K over 2.0) Untreated controls.

176%
150%
128%
109%
101%
98%
92%
71%
59%

Mean at 8 weeks = 109.3% ± 12.3

Selective patients (-K over 2.0) Treated with steroids.

146%
89%
86%
75%
67%
55%
47%
47%
31%
26%
(- 0 at 12 weeks)
18%
16%
11%
(- 0 at 10 weeks)
8%
(- 0 at 12 weeks)
3%
0%

Mean at 8 weeks = 45.4% ± 9.9

prompt spontaneous recovery.

Judgements as to which patients are likely to recover spontaneously and promptly are, however, notoriously difficult. The only way of eliminating spontaneous recovery as a factor in the apparent responsiveness to steroids of some patients with proliferative histology is to relate the progress of treated cases to the progress of an untreated control group, taking a fixed interval of time as that over which responsiveness is assessed.

Protein excretion 8 weeks after the start of steroid therapy in 30 patients with proliferative histology was therefore compared with protein excretion eight weeks after initial assessment in 26 untreated controls. The date of the second protein selectivity determination was taken as the "onset of no treatment" in the control cases.

Table 14 shows the protein excretion at 8 weeks, expressed as a percentage of protein excretion immediately prior to therapy (or, in the control cases, immediately prior to the second protein selectivity estimation).

There were 17 unselective control patients, and at the end of 8 weeks, their protein excretion was, on average, 93.5% ± 12.1% of the initial value.

There were 9 selective control patients, and at the end of 8 weeks their proteinuria was, on average 109.3% ± 12.3% of the initial value.

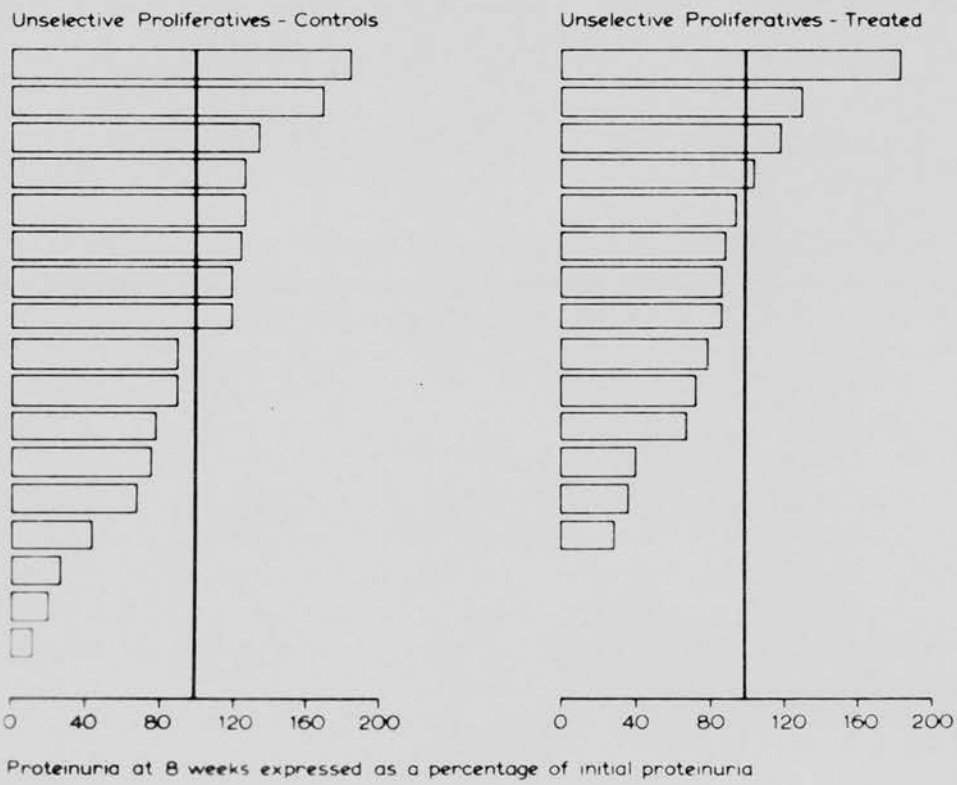
In 14 patients with unselective proteinuria treated with steroids, proteinuria at the end of 8 weeks averaged 86.8% ± 10.9%

Figure 32a

Responsiveness to steroid therapy of patients with proliferative glomerulonephritis.

Each horizontal bar represents one patient. The length of the bar represents proteinuria at 8 weeks expressed as a percentage of initial proteinuria.

Unselective proteinuria, (-K) less than 2.0.



of the initial value. This represents some reduction in comparison with the unselective controls (93.5% ± 12.1%) but the difference was not significant ($t = 0.403$, p over 0.7).

In 16 treated patients with selective proteinuria, protein excretion at 8 weeks averaged 45.4% ± 9.9% of the initial value. Compared with 109.3% ± 12.3% in the selective controls, this represents a significant reduction in proteinuria ($t = 3.958$, p less than 0.001). The responsiveness of the selective proliferatives was also greater than that of the unselective proliferatives ($t = 2.813$, p less than 0.01).

While the 24 hour protein excretion is perhaps the simplest basis on which to judge steroid responsiveness, this excretion is dependent on the glomerular filtration rate and the serum albumin concentration as well as on glomerular permeability to protein. A reduction in protein excretion may arise as a result of a fall in the glomerular filtration rate, and a rise in the serum albumin concentration (other things being equal) will lead to an increase in the amount of albumin excreted. (Hardwicke and Squire, 1955). It is therefore helpful to adjust the protein excretion figures to take into account the creatinine clearance and the serum albumin figures.

In every patient, therefore, initial protein excretion and protein excretion after 8 weeks was adjusted according to the following formula.

$$\text{Adjusted excretion} = \frac{\text{Actual excretion} \times 100 \times 4.0}{\text{Creatinine clearance} \times \text{Serum albumin (g.\%)}}$$

(Since the bulk of the excreted protein is albumin, relating

Figure 32b

Responsiveness to steroid therapy of patients with proliferative glomerulonephritis.
Each horizontal bar represents one patient. The length of the bar represents proteinuria at 8 weeks expressed as a percentage of initial proteinuria.
Selective proteinuria, -K over 2.0.

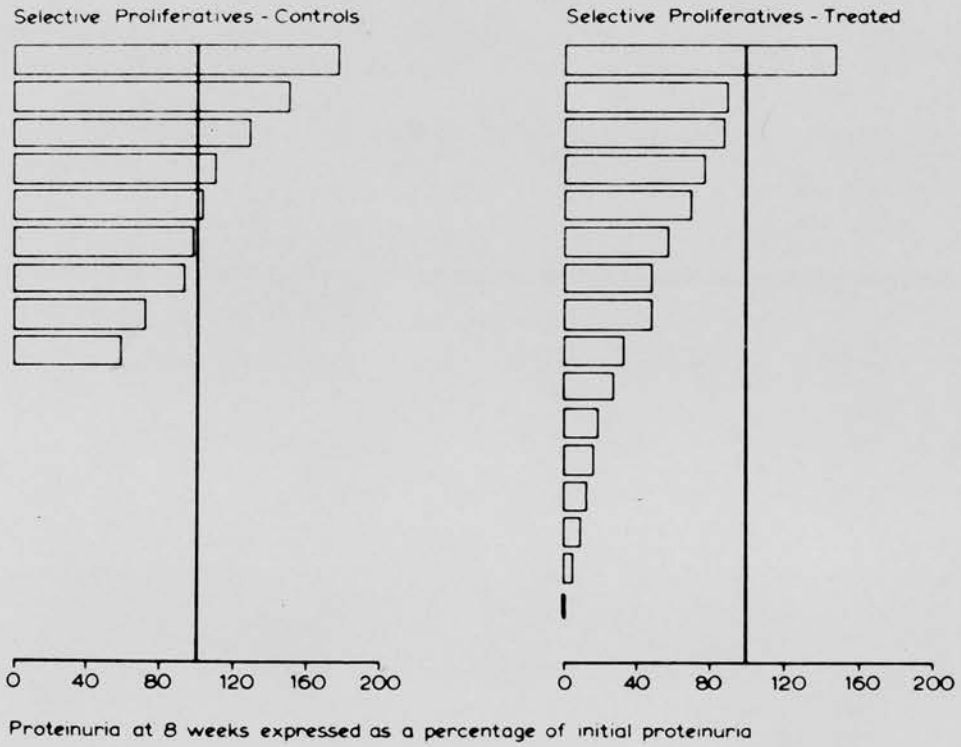


Table 19 Patients with proliferative glomerulonephritis. Proteinuria adjusted to a creatinine clearance of 100 ml/minute and a serum albumin concentration of 4.0 G per 100 ml. Adjusted protein excretion at 8 weeks expressed as a percentage of adjusted initial protein excretion.

Unselective patients (-K less than 2.0) Untreated controls	Unselective patients (-K less than 2.0) Treated with steroids
221%	34%
163%	184%
161%	113%
205%	102%
73%	79%
96%	46%
187%	43%
229%	100%
121%	48%
157%	49%
55%	71%
42%	39%
63%	50%
38%	18%
11%	
15%	
3%	
Mean at 8 weeks = 108.2% ± 18.8	Mean at 8 weeks = 69.7% ± 11.59

Selective patients (-K over 2.0) Untreated controls	Selective patients (-K over 2.0) Treated with steroids
107%	101%
115%	64%
221%	11%
59%	34%
88%	18%
101%	24%
108%	85%
60%	32%
82%	15%
	12%
	15%
	3%
	2%
	2%
	0.2%
	0.0%
Mean at 8 weeks = 104.6% ± 16.0	Mean at 8 weeks = 26.3% ± 7.7

excretion to serum albumin concentration is more accurate than relating it to serum total protein concentration). In each case the adjusted protein excretion after 8 weeks was expressed as a percentage of the initial adjusted protein excretion.

In the 17 control patients with unselective proteinuria, the mean adjusted protein value at 8 weeks was 108.2% ± 18.8% of the initial adjusted protein excretion.

In the 9 control patients with selective proteinuria, the mean adjusted protein excretion at 8 weeks was 104.6% ± 16.0% of the initial value. The mean adjusted protein excretion in the 14 treated patients with unselective proteinuria at 8 weeks was 69.7% ± 11.6% of the initial value. In comparison with the 108.2% ± 18.8% in the unselective controls, this might suggest some benefit from steroids in the treated unselective proliferatives. The value for t, however, was 1.656, giving a p value of over 0.10 (not significant).

In the 16 treated patients with selective proteinuria, the mean adjusted value for protein excretion as a percentage of the initial adjusted value was 26.3% ± 7.7%. Compared with the figure for control patients with selective proteinuria (104.6 ± 16.0), the difference was highly significant (t = 4.98; p less than 0.001). The adjusted values for protein excretion in treated selective proliferatives was also significantly different from that in unselective treated proliferatives (25.3% ± 7.7% as against 69.7% ± 11.6%; t = 3.193; p less than 0.01).

These calculations indicate that patients with proliferative glomerulonephritis and selective proteinuria show a significant

reduction in proteinuria after 8 weeks treatment with steroids.

This response cannot be compared with that seen in minimal lesion glomerulonephritis, however. In 14 cases with minimal lesion, 12 had lost their proteinuria completely at 8 weeks after steroid therapy. The remaining two cases lost their proteinuria 10 and 12 weeks respectively after the onset of therapy.

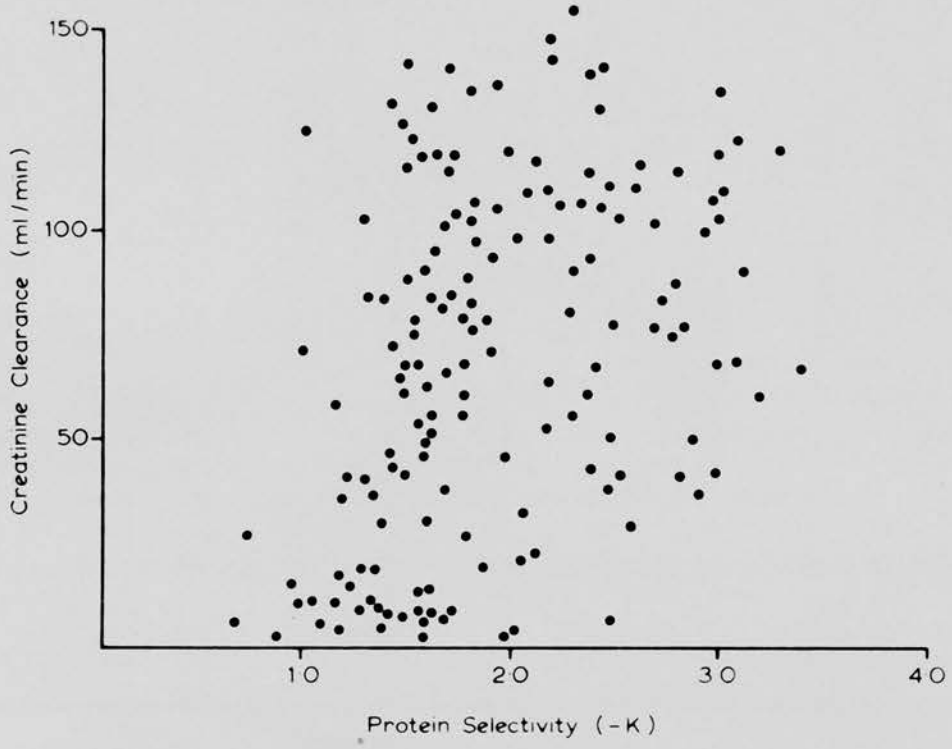
In only 1 of the 14 selective proliferatives was protein excretion abolished inside 8 weeks. 1 further case had lost her proteinuria at 10 weeks and a further 2 cases were free of urinary protein 3 months after the start of therapy.

The situation with regard to proliferative glomerulonephritis, therefore, is that a partial response to steroid therapy is likely, with reduction in proteinuria in those patients with selective proteinuria. Abolition of proteinuria is to be expected in only about 30% of cases, and this abolition will tend to take about 3 months to occur. The response of proliferative cases with unselective proteinuria is unimpressive.

A selective proteinuria in patients with proliferative histology can therefore be regarded as a relative indication for steroid therapy. In most cases the response is only partial and, if the nephrotic state can be adequately controlled by diet and diuretics, it is probably not worth risking the side-effects of steroids. All the patients who eventually responded completely to steroids showed a substantial reduction in protein excretion at 8 weeks.

Figure 33

The relationship between protein selectivity and creatinine clearance in 160 patients with proteinuria of over 1.0G per 24 hours.



6. The relationship between protein selectivity and creatinine clearance.

In figure 33 creatinine clearance (ml per minute) has been plotted against protein selectivity (-K) in 160 patients with proteinuria of over 1.0G per 24 hours. The best straight line for the plot was found to be

$$y = 25.2x + 22.9$$

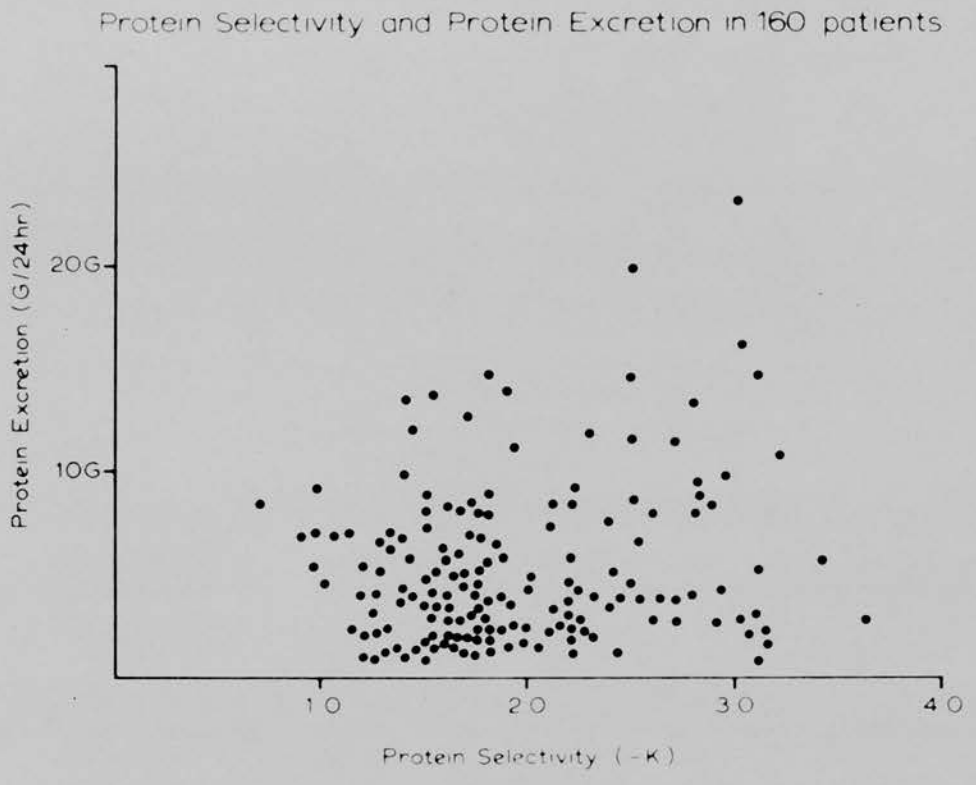
where y is the creatinine clearance and x is the protein selectivity. In view of the extremely wide scatter of the points, this line has very little meaning. A positive correlation between protein selectivity and creatinine clearance did exist, however; r was +0.361 and p was less than 0.01.

Despite this significant correlation for the series as a whole, from figure 33 it will be seen that many patients with (-K) values of less than 1.6 had creatinine clearances of over 80 ml/minute, while a substantial number of patients with selectivity values of over 2.0 had creatinine clearances of less than 30 ml per minute.

Protein selectivity does not, therefore, reflect overall renal function in the individual case with any useful degree of precision.

Figure 34

The relationship between protein selectivity and 24 hour protein excretion in 160 patients with proteinuria of over 1.0G per day.



7. The relationship between protein selectivity and 24 hour protein excretion.

In figure 34 protein excretion (G per 24 hours) has been plotted against protein selectivity (-K) in 160 patients with proteinuria of over 1.0G per day. The best straight line for the plot was found to be

$$y = 1.18\,x + 3.28$$

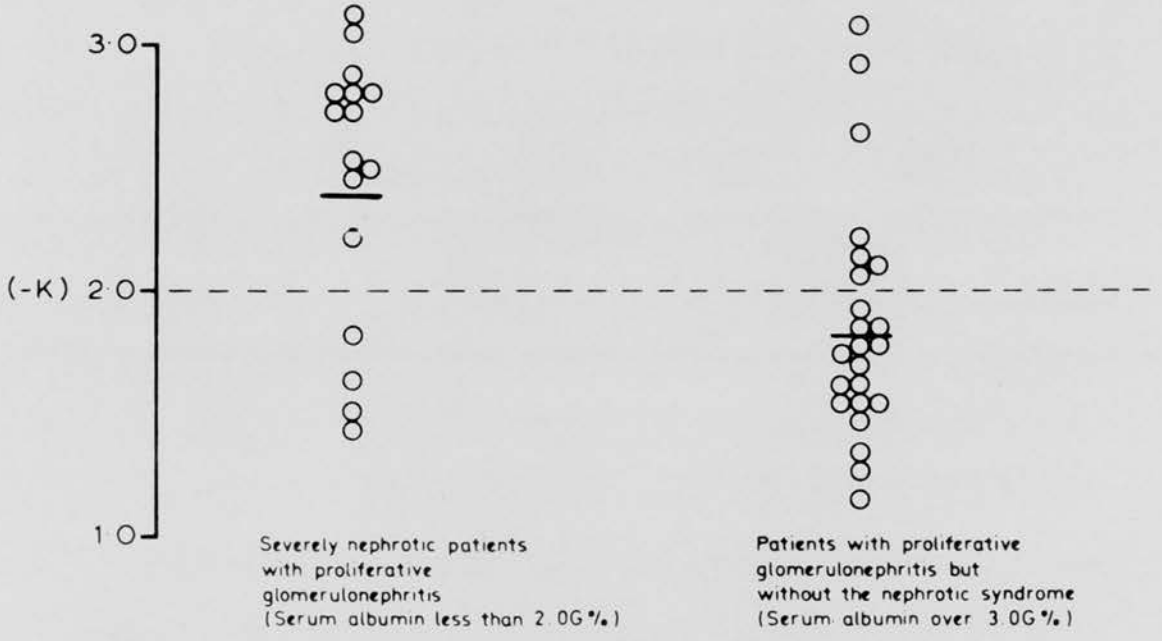
where y is the protein excretion and x the protein selectivity. In view of the wide scatter of the points, the calculated line has very little meaning. A positive correlation between selectivity and protein excretion was found; r was +0.179 and p was just less than 0.05.

Since a low value for selectivity indicates a relatively large glomerular pore size, a negative correlation between selectivity and protein excretion might have been expected. The total amount of protein excreted, however, depends on the total area of the basement membrane which is permeable to protein and not simply on the average size of the pores. Selectivity determinations give some indication of pore size (Hardwicke and Soothill, 1961) but they give no indication regarding pore number.

Protein excretion is dependent on the glomerular filtration rate and the plasma protein concentration, as well as on the permeability of the glomerular filter to macromolecules. It is therefore reasonable to "adjust" the observed protein excretion to take these variables into account. Since albumin is the most important urinary protein in quantitative terms, the following formula gives a reasonable approximation to what might be expected

Figure 35

Comparison of protein selectivity values in patients with proliferative glomerulonephritis and an unequivocal nephrotic syndrome, and patients with similar histology but without the nephrotic syndrome ("nephritic proliferatives").



on the basis of a creatinine clearance of 100 ml per minute and a serum albumin concentration of 4.0G per 100 ml.

$$\text{Adjusted protein excretion} = \frac{\text{Observed protein excretion} \times 4 \times 100}{\text{Serum albumin (G per 100 ml)} \times \text{Creatinine clearance (ml per min.)}}$$

When the 24 hour protein excretion was adjusted according to the above formula for the 160 patients, a negative correlation between selectivity and protein excretion was found; r was -0.267 and p was less than 0.05. This negative correlation may well be an artefact caused by the method used in calculating adjusted protein excretion, however. Creatinine clearance appears in the bottom line of the equation used to determine "adjusted excretion", and a positive correlation between creatinine clearance and protein selectivity has already been shown in the preceding section.

In proliferative glomerulonephritis there appears to be a genuine tendency for patients with massive proteinuria to have a moderately selective mode of protein excretion. Figure 35 shows the selectivity values in 16 nephrotic patients with serum albumin concentrations of less than 2.0G per 100 ml along with selectivity values in 23 "nephritic" patients in all of whom the albumin concentration in serum was over 3.0 per 100 ml. The mean protein selectivity for the nephrotic group was 2.41. The mean selectivity for the "nephritic" group was 1.82. This difference in means was significant; t was 3.870 and p was less than 0.001.

8. Studies involving I 125-labelled polyvinyl-pyrrolidone (PVP)

a. Introduction

I 125 polyvinyl pyrrolidone is a radioactively labelled polydisperse macromolecule. The material generally used has an average molecular weight of 38,000; it can be obtained in a form suitable for intravenous injection from the Radiochemical Centre, Amersham, Bucks. Its use in the study of glomerular permeability to macromolecules has already been reported by Hulme and Hardwicke (1966), Hardwicke et al (1968, 1970) and Lambert et al (1970).

The methods used for measuring PVP clearances by gel filtration have already been described. The sensitivity and flexibility of the radiometric assay enabled clearance studies with PVP to be conducted over a much wider molecular size range than was possible with the methods employed for assaying protein and dextran.

Although the principle of the PVP method was fundamentally simple, progress with PVP studies was delayed for a time by a number of methodological problems. Most of these problems were related to binding of radioactive material.

In some of our early pilot experiments, "Blue Dextran" (M.W. 2×10^6, Pharmacia) was used as an internal standard for urine and plasma fractionation runs in order to determine the position of Vo* with greater accuracy. The radioactive label,

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*Note: Vo is the origin of the Sephadex fractionation run. See pp. 61 to 63 of this thesis.

however, became partially bound to the dextran marker, giving erroneously high counts in the earlier tubes of the fractionation run. It became obvious that Blue Dextran was unsuitable for use as a marker in this context.

A second binding problem occurred in relation to Sephadex itself. It was found that after the application of larger than usual amounts of radioactive material to the column, the background radioactivity of subsequent elution runs often rose to an unacceptable level. This phenomenon was due to adsorption of radioactive material on the column, with subsequent gradual elution. Blue Dextran proved useful in this context, as the passage of about 5mg of this material through a "hot" column stripped it of much of its radioactivity and restored the column blanks to acceptable levels.

These experiences raised the question of binding effects between protein and I 125 labelled PVP. Such binding effects were in fact demonstrated. There was a slight but definite shift in the elution pattern of PVP-protein mixtures in comparison with the pattern on column chromatography of pure I 125 PVP. This took the form of an increase in radioactivity in the region of the three protein peaks, with a corresponding decrease in the region of the intervening troughs. The effect was more noticeable following the injection of PVP into a patient than when the labelled material was simply added to plasma in the laboratory.

It was not possible to eliminate this possible source of error by deproteinising the samples prior to chromatography. Following protein precipitation, either with tungstate or trichloracetic acid, less than 20% of the radioactivity originally

present remained in the supernate.

Protein-binding of I 125 labelled PVP was, however, detectable rather than obvious. Since the amount of protein in the early tubes of a fractionation run of unconcentrated urine from normal subjects or from patients with selective proteinuria is small, it was thought that binding would not cause appreciable errors in such patients. In one patient who had heavy proteinuria and an unselective mode of excretion, however, there was an unexpected rise in PVP clearance with increasing molecular size. The rise was confined to the first four tubes of the fractionation run. It was felt that protein-binding might have been the factor responsible for this particular anomaly.

Our investigation of these various binding phenomena proved somewhat time-consuming. The progress of actual clearance studies was delayed, and at the time of writing satisfactory PVP clearance data had been obtained in only 14 subjects - 8 patients with glomerulonephritis and 6 normal individuals. Even with this small series it was possible to confirm many of the observations reported by Hulme and Hardwicke.

One advantage of using labelled PVP in clearance experiments is that observations can be made over a wider range than is possible with either protein or dextran. The inclusion in clearance studies by the gel filtration technique of macromolecules which elute later than Va* does, however, cause certain difficulties when it comes to selecting the method of choice for expressing the

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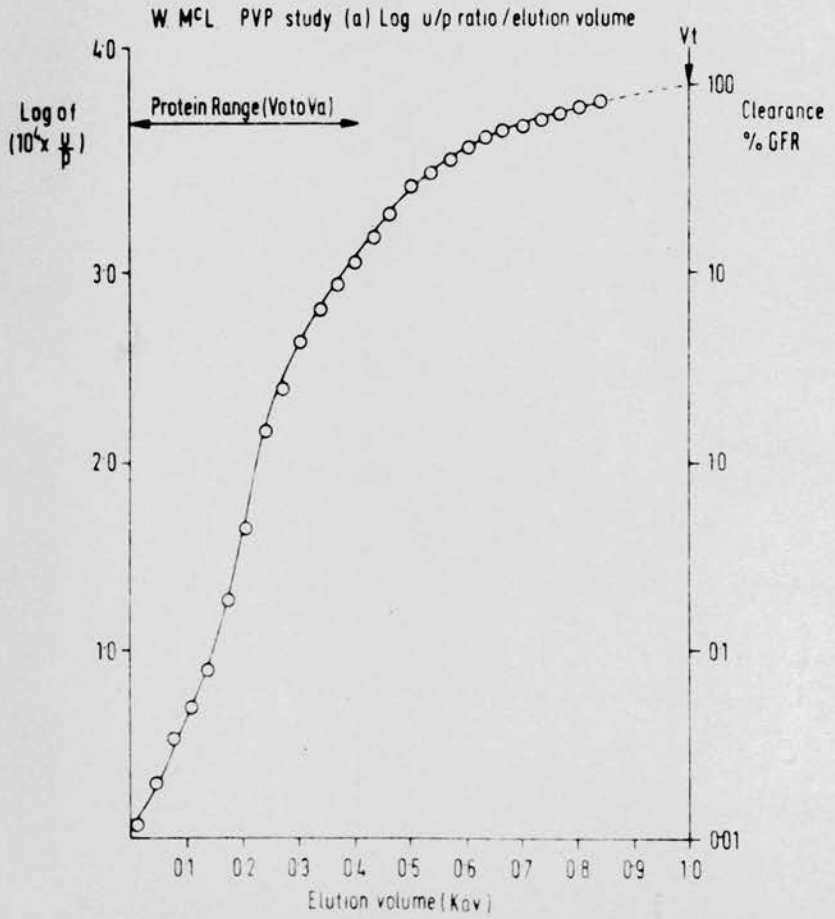
*Note: Va is the elution peak of human serum ablumin. This term is defined, along with Vo, Ve and Vt on pp.61 to 63 of this thesis.

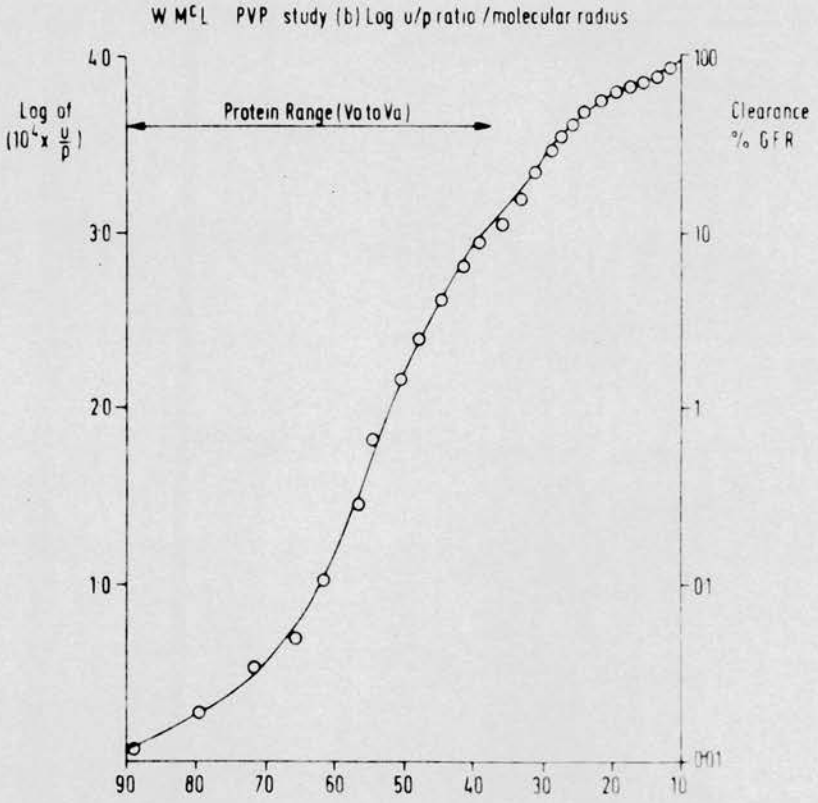
Figure 36

PVP indices of selectivity

a) Log clearance related to elution volume (Kav).



b) Log clearance related to molecular radius.



results. This point is illustrated in figure 36, which shows the data from a single PVP clearance experiment treated in four different ways.

In figure 36a, log clearance has been plotted against elution volume. In order to eliminate variables such as column size and column volume, elution volume has been expressed in terms of Kav. Kav (Laurent and Killander, 1964) is synonymous with Kd (Ackers, 1964). As explained on p.62 of this thesis,

$$Kd = Kav = \frac{(Ve - Vo)}{(Vt - Vo)}$$

The relationship between log clearance and Kav is linear over the range Vo to Va. In the range between Va and Vt, however, the line becomes a curve with a continually changing slope.

In figure 36b, log clearance is plotted against effective molecular radius. Effective molecular radius can be calculated from elution position on G 200 Sephadex, following appropriate calibration of the column, using the formulae and tables given by Ackers (1964).

From figure 36b, it can be seen that the relationship between log clearance and molecular radius takes the form of a sigmoid curve.

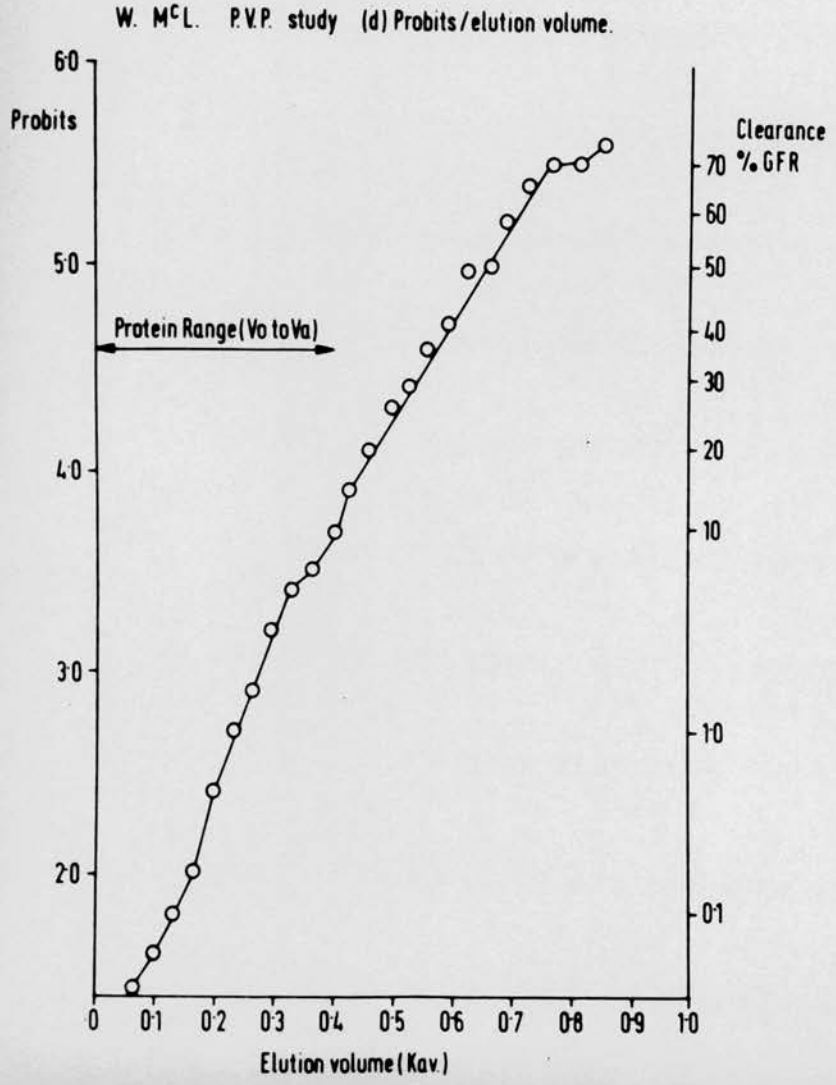
Hulme and Hardwicke (1966) and Hardwicke et al (1968) expressed PVP clearances as percentages of the glomerular filtration rate and subjected these percentage values to probit transformation (Gaddum, 1945). The probit units obtained were then plotted against elution volume or molecular radius.

Figure 36d shows PVP clearances expressed in terms of probit units and plotted against molecular radius; 36c shows

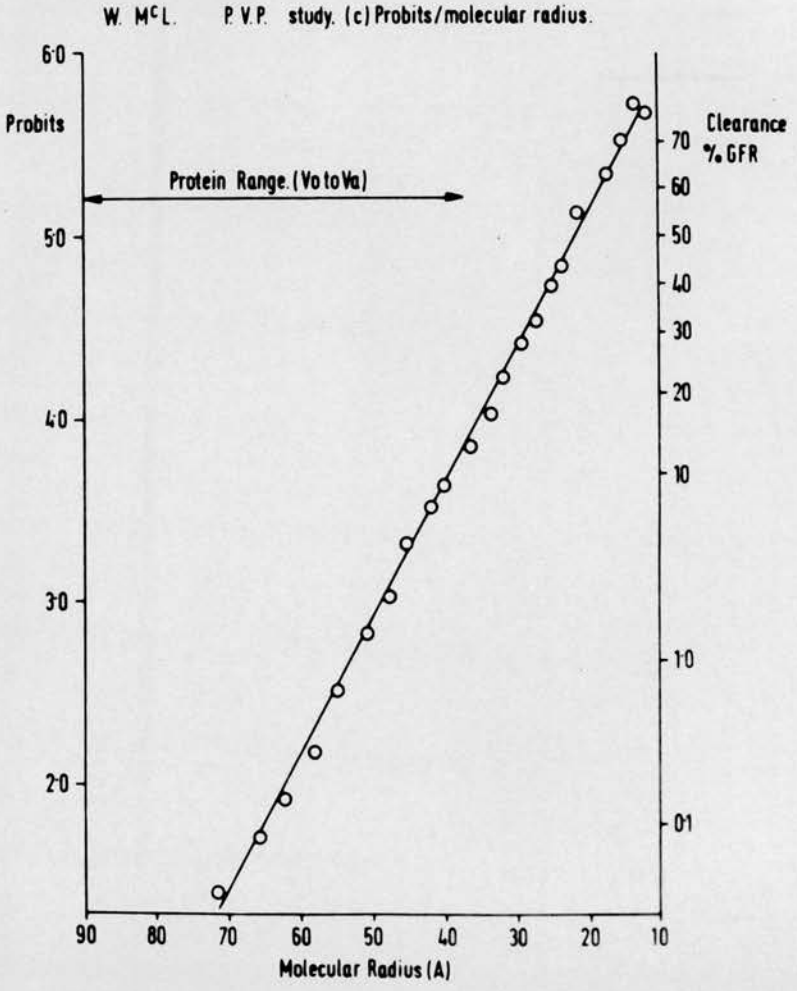
Figure 36 (cont.)

PVP indices of selectivity

c) PVP clearance as a % of GFR plotted on a probit scale against elution volume (Kav.)



d) PVP clearance as a % of GFR plotted on a probit scale against molecular radius.



probit units plotted against elution volume. In both of these figures the use of probit units gives a relationship which is linear over a wide molecular size range.

For clearances calculated over the elution range Vo to Va, it would seem appropriate to plot log clearance against elution volume.

For clearances calculated over a wider molecular size range it would seem more appropriate to use probit units. We have in fact plotted clearances as a percentage of GFR in probit units against molecular radius.

Table 20.

PVP Selectivity Values in Normal Subjects

Patient	Δ PVP	"S"*	(-K)ø
1	3.60	9.00	4.99
2	3.55	8.87	4.86
3	2.76	6.90	3.78
4	2.84	7.10	3.89
5	3.18	7.95	4.36
6	2.70	6.75	3.70
Mean value	3.10 ± 0.40	7.75 ± 1.0	4.26 ± 0.56

* "S" in the change in log clearance per unit change in Kav.

$$S = 2.5 \times \Delta \text{ PVP}$$

ø (-K) is the index of selectivity for protein as determined by immunodiffusion.

$$-K = 1.37 \times \Delta \text{ PVP}$$

b. PVP clearance studies in normal subjects

Clearance studies using I 125-labelled polyvinyl pyrrolidone were carried out in 6 normal subjects, using the technique of gel filtration. On Sephadex G 200 fractionation, the clearance rises as molecular size decreases, and in the fractions eluting just before Vt a constant value for clearance is reached. This constant value is termed the "limiting clearance"; it is a measure of the glomerular filtration rate. (Hulme and Hardwicke, 1966). The clearances of the various PVP fractions eluting earlier can be expressed as percentages of this limiting clearance.

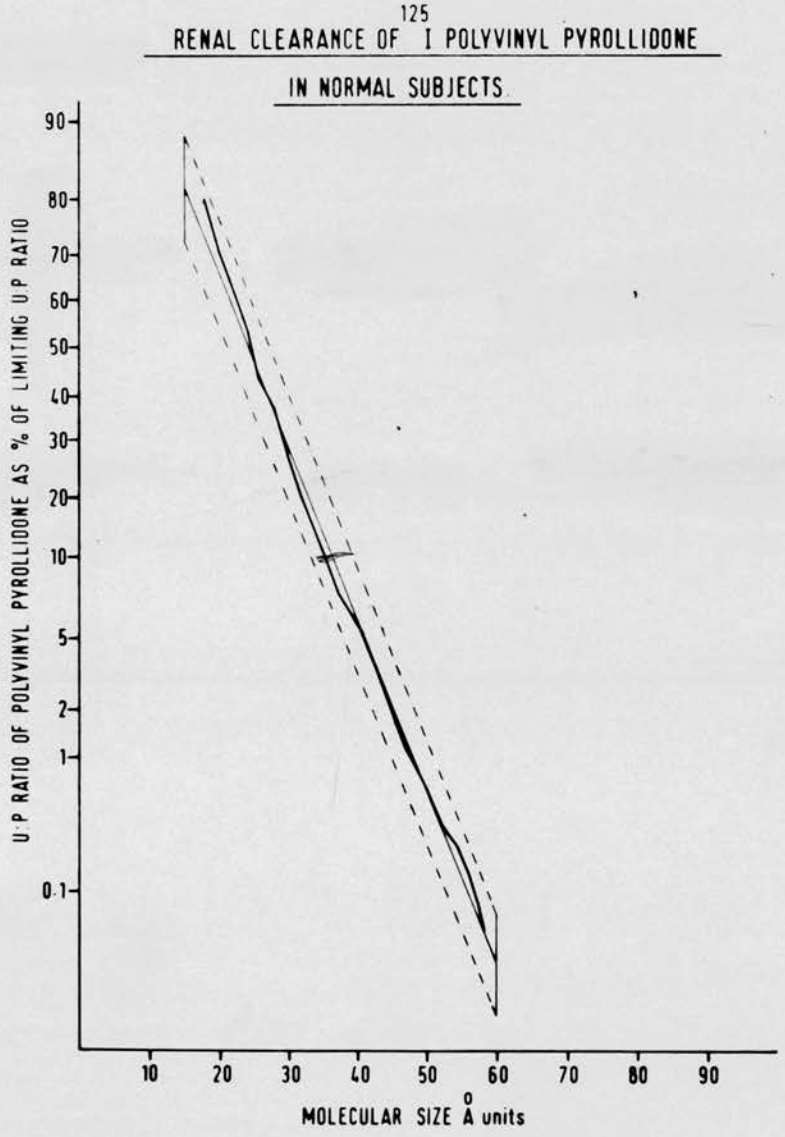
For studies over the protein elution range Vo to Va, the index of selectivity " $\triangle$ PVP" was calculated. This index, like $\triangle$ (the index of protein selectivity as determined by gel filtration) and D (the gel filtration index of dextran selectivity) represents the total change in log clearance over the range Vo to Va. The method used in its calculation is shown in table 3, facing p.70.

Table 20 shows the values for $\triangle$ PVP obtained in the six normal subjects. This table also shows PVP selectivity in terms of -K, the index of selectivity determined by immunodiffusion, and in terms of "S", which is the symbol we have used to denote Hardwicke's index of gel filtration selectivity. (Hardwicke et al 1968).

Values for $\triangle$ PVP in normal subjects ranged from 2.70 to 3.60, with a mean value of 3.10. These values were in good general agreement with the similarly calculated indices of dextran selectivity in normal subjects, which had a mean value of 2.95 (see p.88).

It would appear that PVP, like dextran, is filtered by the

Figure 37


125
RENAL CLEARANCE OF I POLYVINYL PYROLLIDONE
IN NORMAL SUBJECTS
U·P RATIO OF POLYVINYL PYROLLIDONE AS % OF LIMITING U·P RATIO
90
80
70
60
50
40
30
20
10
5
2
1
0·1
10
20
30
40
50
60
70
80
90
MOLECULAR SIZE Å units

normal glomerulus in a highly selective manner.

For clearance studies incorporating the results found with the low molecular weight fractions eluting later than Va, results are better expressed in terms of probit units than in terms of log clearance. Figure 37 summarises the findings in the 6 normal subjects over a wide elution range, the probit values of the various PVP clearances as percentages of GFR being plotted against molecular radius.

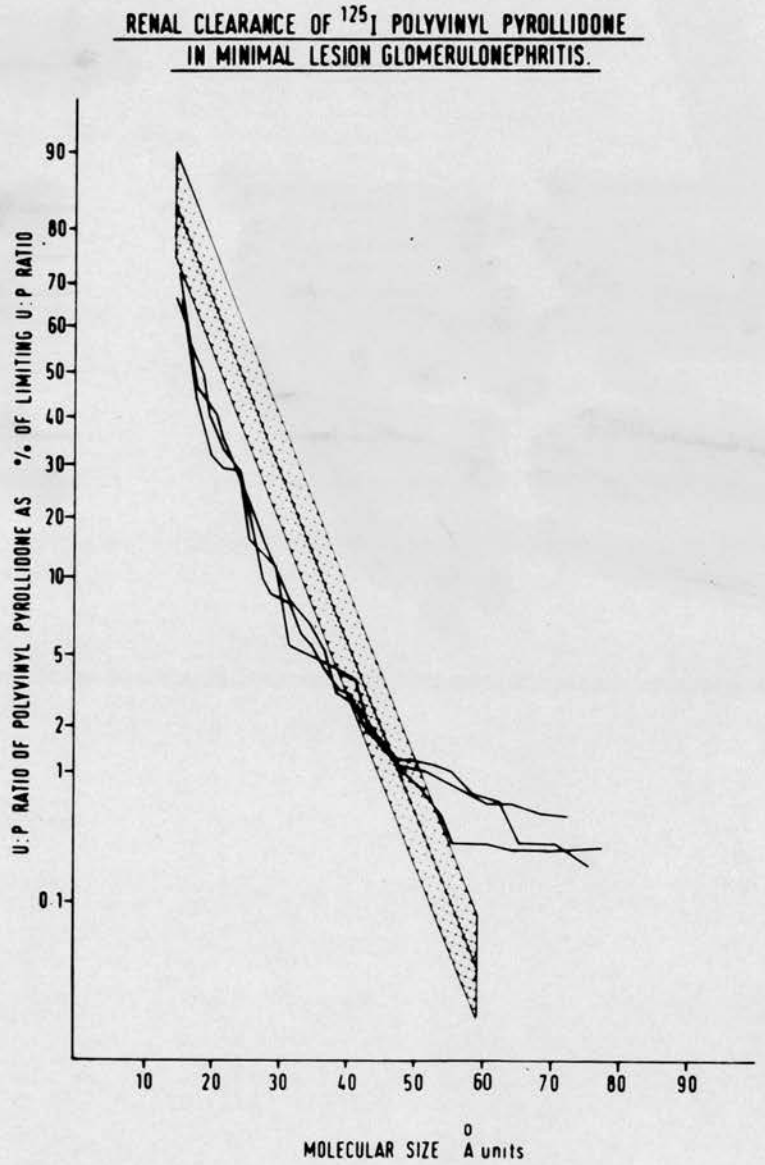
Hulme and Hardwicke (1966) showed a linear relationship between PVP clearances expressed in probit units and molecular radius, in normal subjects. Our results confirm this observation. The linear relationship suggests that the pores of the healthy glomerular basement membrane are normally distributed with respect to pore size.

It is interesting to note from figure 37 that the clearance of PVP molecules with an effective molecular radius of 36A is approximately 15% of the glomerular filtration rate. Hardwicke et al (1968) obtained even higher values for PVP clearances at this molecular size. The molecular radius of human serum albumin is 36A. Even in severely nephrotic patients, the glomerular clearance of albumin rarely reaches 10% of GFR; the highest value recorded for renal clearance by Gregoire et al (1958) was 4% of GFR. Albumin loading experiments on normal individuals (Malmendier et al, 1957; Lambert et al, 1970) have shown that the glomerular clearance of albumin for the normal kidney is about 0.3% of the creatinine clearance. The permeability of the glomerulus to PVP would appear to be much greater than its permeability to protein

molecules of equivalent size; the differences between PVP and albumin clearances are too great to be explained in terms of the relatively small tubular reabsorptive capacity which exists for protein. Lambert et al (1970), using formulae suggested by Pappenheimer (1953) and by Landis and Pappenheimer (1962) have calculated that the mean pore radius of the glomerulus is 37.7A with respect to albumin and 62.8A with respect to PVP.

This disparity presumably reflects differences between the molecular species involved. Albumin, being a charged molecule, may well be subjected to more in the way of steric hindrance at the site of filtration than PVP, which is uncharged. Albumin molecules are roughly spherical, while PVP molecules have an elongated structure. This difference in molecular shape may also be a factor in the differing behaviour of protein and PVP at the glomerular filter.

Figure 38


RENAL CLEARANCE OF ^{125}I POLYVINYL PYROLLIDONE
IN MINIMAL LESION GLOMERULONEPHRITIS.
U:P RATIO OF POLYVINYL PYROLLIDONE AS % OF LIMITING U:P RATIO
90
80
70
60
50
40
30
20
10
5
2
1
0.1
10
20
30
40
50
60
70
80
90
MOLECULAR SIZE Å units

c. PVP clearance studies in patients with glomerulonephritis

Clearance studies using I 125-labelled polyvinyl-pyrrolidone were carried out in 8 patients with glomerulonephritis. Three of the patients had minimal lesion glomerulonephritis, two had proliferative glomerulonephritis, and three had membranous glomerulonephritis. In each case, proteinuria at the time of the PVP clearance studies was over 1.0G per 24 hours.

Figure 38 shows the results in the patients with minimal lesion glomerulonephritis. PVP clearance as a percentage of GFR has been plotted on a probit scale against molecular radius. Figures 39 and 40 show the results in the patients with proliferative and membranous glomerulonephritis respectively, expressed in a similar fashion. In all three diagrams the results obtained from the six normal subjects have also been shown for purposes of comparison.

In the patients with minimal lesion glomerulonephritis (fig. 38) the line relating clearance in terms of probit units to molecular radius is parallel to the line obtained in normal subjects over the range 15 to 45A. The line for each patient, however, is slightly to the left of the normal range, suggesting that in minimal lesion glomerulonephritis the clearances of small molecular weight fractions of PVP may be slightly reduced. For PVP molecules with a radius of 50A, the clearance is the same as in normal subjects. For molecules with a larger radius than 50A, the clearances are greater than in normal subjects.

The two patients with proliferative glomerulonephritis showed somewhat different patterns of PVP excretion. In the first

Figure 39

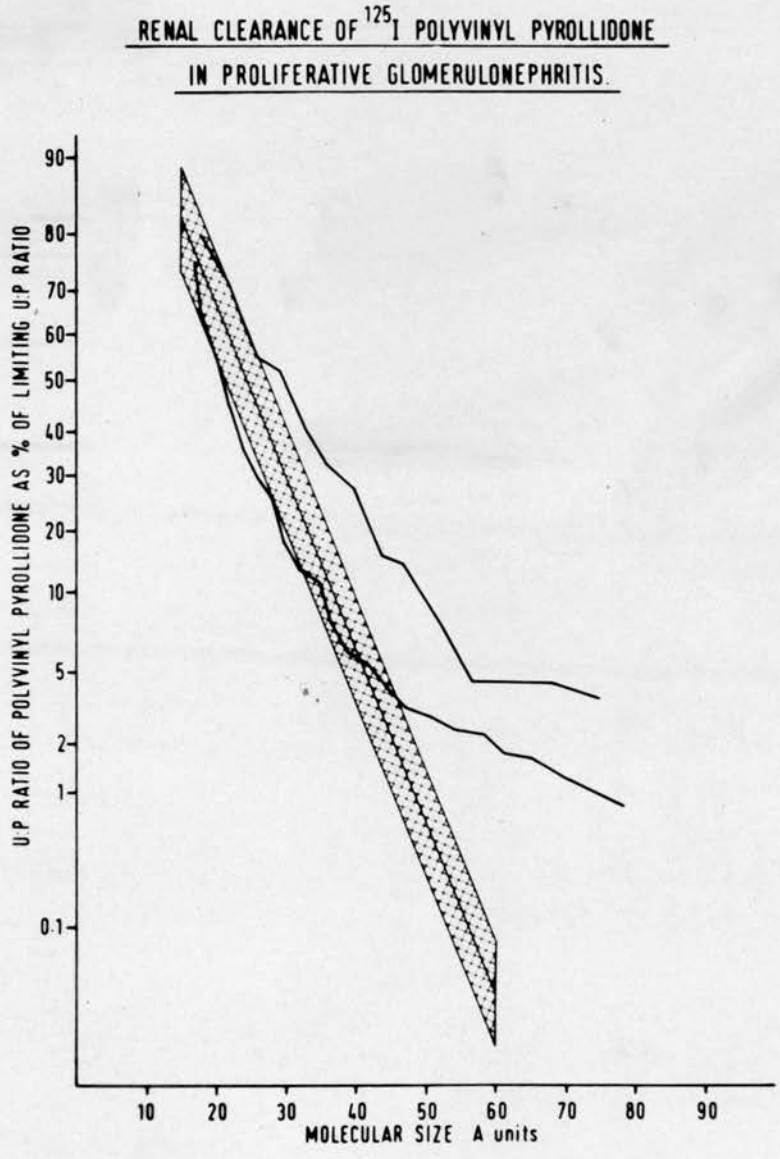

RENAL CLEARANCE OF ^{125}I POLYVINYL PYROLLIDONE
IN PROLIFERATIVE GLOMERULONEPHRITIS.
U:P RATIO OF POLYVINYL PYROLLIDONE AS % OF LIMITING U:P RATIO
90
80
70
60
50
40
30
20
10
5
2
1
0.1
10
20
30
40
50
60
70
80
90
MOLECULAR SIZE A units

Figure 40

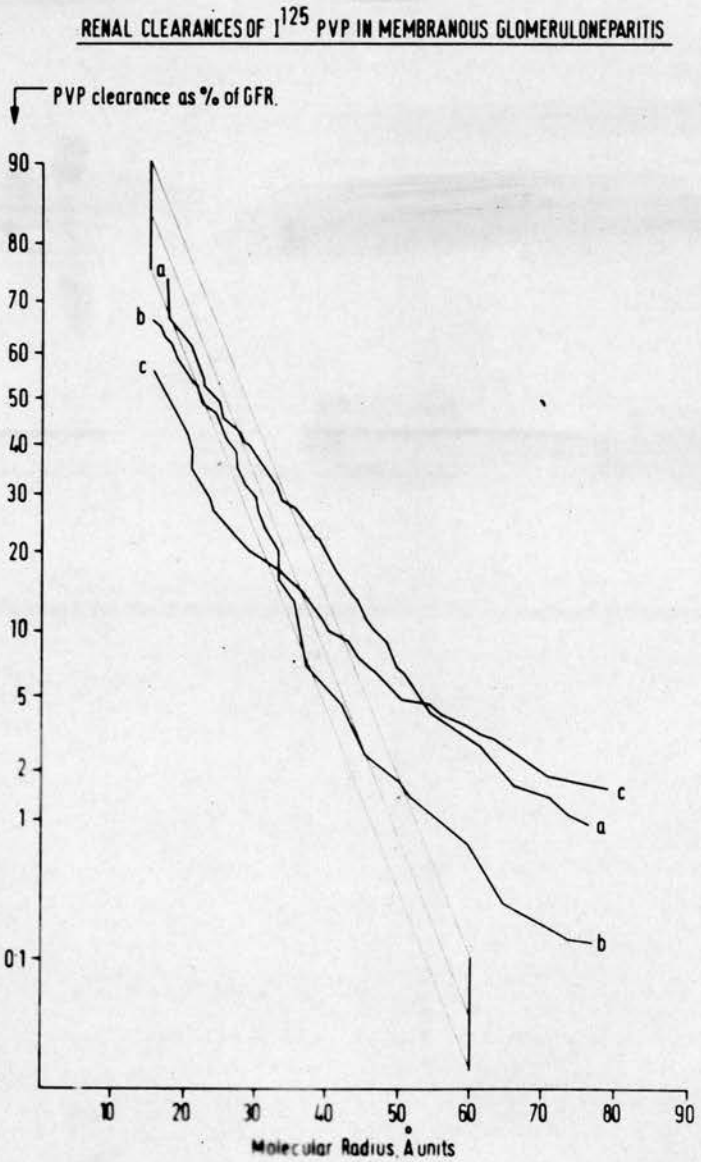
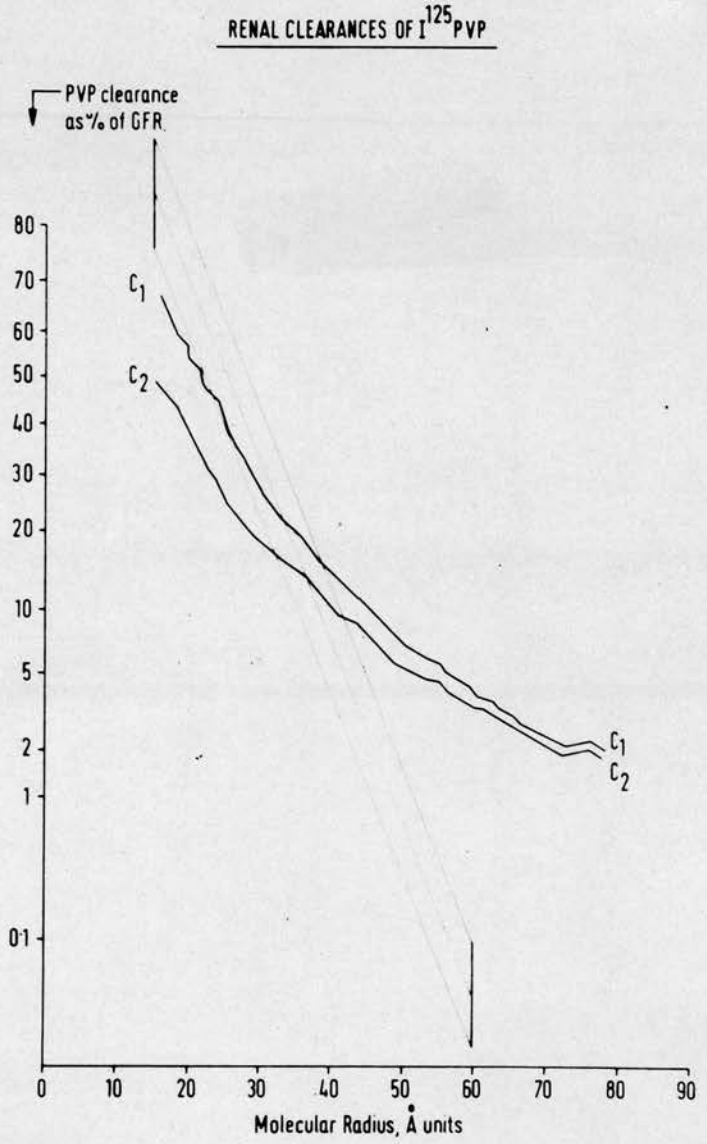

RENAL CLEARANCES OF I125 PVP IN MEMBRANOUS GLOMERULONEPARITIS
PVP clearance as % of GFR.
90
80
70
60
50
40
30
20
10
5
2
1
0·1
a
b
c
c
a
b
10
20
30
40
50
60
70
80
90
Molecular Radius, Å units

Figure 41

Patient c, figure 40



In c_1, the limiting clearance of PVP has been taken as 100% GFR.

In c_2, the creatinine clearance has been taken as 100% GFR.

In the case of the patient represented in figure 40 by line c, it was difficult to be certain whether the "cross-over" phenomenon was a genuine finding or a methodological artefact. Provided that both creatinine and the limiting clearance of PVP give a measure of the glomerular filtration rate, it should be possible to use either as the 100% reference point for the plot of clearance (in probit units) against molecular radius. In figure 40, line c was in fact calculated taking the urine to plasma ratio of creatinine as 100%. In this particular experiment, however, the creatinine clearance was 30% higher than the limiting PVP clearance.

In figure 41, line c in figure 40 has been reproduced as line "c_2". Line "c_1" shows the effect of plotting the same data from the same patient taking the limiting clearance of PVP as 100%. In line "c_1" the cross-over effect is negligible.

Figure 41 illustrates an important difference between scales using probit units and scales employing logarithmic units. Had the clearances been expressed in logarithmic units, lines c_1 and c_2 would have differed slightly in position but not at all in slope. Because probit units have been employed, however, a 30% variation in the value taken to represent 100% GFR has produced a very substantial difference between the lines - particularly at the left hand, low molecular weight end of the plot. Thus when a probit scale is employed, precise measurement of GFR is critical. Using a logarithmic scale, it makes little difference whether inulin, creatinine, or limiting OVP clearance is used to measure GFR.

The interpretation of these results in patients with

glomerulonephritis will be discussed in section 4 of this thesis. In view of the small number of patients in each diagnostic category it has not been possible to evaluate the diagnostic usefulness of PVP clearance studies. The similarity between the pattern obtained in one patient with membranous glomerulonephritis (line b, figure 40) and the patterns noted in minimal lesion glomerulonephritis (figure 38) suggests that PVP clearances may be less valuable in the diagnosis of the latter condition than protein clearance studies. This impression is supported by Hulme's findings in renal transplant cases. (Hulme, 1970). It would appear that, at present, clearance studies using PVP are more of value in research than in the clinical situation.

Table 21.

Protein selectivity values before therapy and during the reduction in protein excretion induced by steroids

Patient	Initial Protein Excretion (G/24 hr)	Protein Excretion on Steroids (G/24 hr)	Percentage change in Protein Excretion	Initial Selectivity (-K)	Selectivity on Steroids (-K)	Percentage change in Selectivity
1	4.7	0.9	-81%	3.74	3.92	+4.8%
2	17.5	2.4	-86%	2.82	2.75	-2.4%
3	4.9	0.8	-84%	2.60	3.22	+ 24%
4	3.7	0.5	-86%	4.17	3.65	- 12%
5	7.6	1.8	-76%	2.70	2.82	+3.7%
6 (1966)	17.6	0.4	-98%	3.10	3.40	+9.7%
6 (1968)	9.4	0.7	-93%	3.64	3.16	- 13%
Mean Value	9.3	1.1		3.25	3.27	

All patients had minimal lesion glomerulonephritis.

9. The effect of steroid therapy on protein selectivity in patients responsive to steroids.

In patients with minimal lesion glomerulonephritis, steroid therapy leads to abolition of proteinuria in the majority of cases. The mechanism of action of steroids in this situation is not known. Vere and Walduck (1966) suggested that if steroids acted to reduce glomerular permeability by reducing functional pore size, this effect should produce a measurable change in protein selectivity values. As protein selectivities cannot be measured with accuracy in situations of trace proteinuria (Maclean and Petrie, 1966) it was necessary to carry out protein clearance studies before therapy and after a substantial but incomplete reduction in protein excretion had occurred as a result of therapy. In over half the patients with minimal lesion glomerulonephritis studied, proteinuria had been abolished before the follow up selectivity determinations were attempted. We were, however, able to measure protein clearances before and during the response to steroids on seven occasions in six patients with minimal change lesions.

Table 21 shows protein selectivity values before steroid therapy and after a substantial steroid-induced reduction in proteinuria had occurred. Protein excretion fell by between 76% and 98%, the mean protein excretion before treatment being 9.3G and during treatment 1.1G. In four instances the protein selectivity rose during steroid therapy and on three occasions protein selectivity fell. The mean value for (-K) before therapy was 3.25, and the mean value following a substantial reduction in proteinuria was 3.27. In other words, there was no change in selectivity which

could not be accounted for by a combination of experimental error and normal biological variation.

In their investigation of this problem, Vere and Walduck (1966) studied six patients in all - four by the somewhat approximate technique of electrophoretic protein fractionation, and two by the gel filtration method of Hardwicke (1965). By neither techniques could these authors show any significant change in what they termed the "selective permeability" of the glomerulus.

As they pointed out, however, the substantial reduction in protein excretion points to a very definite steroid induced reduction in overall glomerular permeability to protein. Had this reduction been due to a steroid-induced dimination in the pore size of the postulated abnormal glomerular pores, protein selectivity values would have increased during the decrease in protein excretion. This did not happen. The reduction in total protein excretion, however, indicates that the area of the membrane permeable to protein underwent a reduction. Since this reduction in area did not result from a reduction in pore size, it must have been the result of a reduction in pore number.

From this brief study, therefore, it can be concluded that steroids act by reducing the number of abnormally enlarged pores in minimal lesion glomerulonephritis rather than by reducing their size. On the basis of similar results, Vere and Walduck (1966) draw similar conclusions.

SECTION IV

DISCUSSION

1. Indices used to express the results of clearance studies on macromolecules

Discussions on the topic of macromolecular clearance studies are complicated by semantics and arithmetic.

The expression "selective proteinuria" has rightly been criticised. Protein molecules are not endowed with a capacity for selection. The adjective applies to the manner in which protein molecules are handled at the glomerular filter and not to the proteinuria itself.

In the search for a more acceptable mode of expression, "glomerular permeability" has been used as a synonym for selectivity. This term is not strictly applicable to differential protein clearance determinations, however, and to use it in this sense is to substitute a scientific error for a grammatical one. Glomerular permeability is related to the absolute glomerular clearance of a macromolecule, or to its sieving coefficient (Pappenheimer, 1953), or to its glomerular clearance expressed as a percentage of the glomerular filtration rate. It is a function of the area of membrane available for the filtration of the macromolecule concerned.

When the clearance of one macromolecule is expressed as a percentage of the clearance of another, overall glomerular permeability is not measured. The index obtained reflects the size of the (presumed) abnormal pores in the basement membrane, but gives no indication of their number.

Provided that it is clearly understood that "selective proteinuria" is a shorthand way of saying "selective filtration of protein", there is no need to abandon a terminology which has

become ingrained over a decade.

The grammar of selectivity may irritate the purist, but it is unlikely to cause serious confusion. The arithmetic of selectivity is far more baffling. The problem arises because when a measurement is made it is necessary to attach a number to the result.

When Blainey and his colleagues plotted log clearance against log molecular weight in 1960, it was enough for them to say that the regression line obtained in patients with minimal lesion glomerulonephritis was steeper than that obtained in membranous glomerulonephritis. Subsequent workers, however, wanted to know how much steeper; they needed numbers in place of "steep" and "not so steep".

Hitzig et al (1965) were content with qualitative descriptions, but in other centres the search began for numbers. It has been said that if ten Frenchmen were placed on a desert island, some months later ten political parties would emerge. Nephrologists also tend to be rugged individualists, and while the ratio of indices of selectivity to workers in the field does not quite approach 1 to 1, there is a plethora of coefficients, slopes, angles and ratios which cannot but be confusing to the average clinician. Edinburgh, alas, has contributed as much to the proliferation of selectivity indices as any other centre.

Before we can discuss the findings described in this thesis in relation to those of other workers, it is necessary to define the arithmetic which has been employed from time to time in the attempt to give numerical expression to the concept of selectivity.

a. Indices used in immunodiffusion studies

Blainey et al (1960) plotted log clearance against log molecular weight. An inverse linear relationship was found. Steep regression lines were termed "selective"; less steep lines were termed "unselective".

Joachim et al (1964) also plotted log clearance against log molecular weight. They used θ, the (acute) angle made by the regression line with the X axis as their index of selectivity. θ was in fact derived from the calculated slope of the regression line; this slope being the tangent of the angle θ.

In Edinburgh, we also expressed the results of our immunodiffusion studies in terms of the relationship between log clearance and log molecular weight. We did not use the angle θ as our index of selectivity, however, but its tangent. Tan θ is a direct measure of the slope (or steepness) of the regression line. The angle θ also reflects the steepness of the line, but in a non linear fashion. Since log clearance is inversely proportional to log molecular weight, the regression line has a negative slope. To avoid working with negative numbers, we designated our index of selectivity as -K.

The advantage of this mode of expression is that -K can be precisely defined in readily understandable terms. It is the change in $\log_{10}$ clearance per unit change in $\log_{10}$ molecular weight. Since the molecular weight of alpha 2 macroglobulin is approximately 10 times that of transferrin and the log of 10 is 1, a -K value of 1.0 indicates that the transferrin clearance is 10 times the alpha 2

clearance. A -K value of 2.0 indicates that the transferrin clearance is 100 times the alpha 2 globulin clearance.

The method of Soothill (1962) is very suitable for the determination of differential protein clearances in specialised research laboratories. It is, however, very demanding in terms of time and expertise. If selectivity determinations are to be used on a routine basis for the investigation of patients with proteinuria, a simpler technique is required.

In 1966, Cameron and Blandford produced a much needed simplification in methodology by introducing single radial immunodiffusion in place of Soothill's double diffusion technique. In this innovation they were to be followed, with speed and with enthusiasm, by Soothill himself (Soothill and Hendrickse, 1967).

Instead of basing their results on the clearances of five or six proteins, Cameron and Blandford (1966) restricted their clearance determinations to transferrin and IgG. These two proteins can readily be estimated by the radial immunodiffusion technique, but some criticisms can be made of selectivity studies employing them.

The relationship between log clearance and log molecular weight does not always give a completely linear regression. The plot is often "dog-leg" in appearance, the deviation from linearity being due in most instances to the anomalous behaviour of IgG (Soothill and Hendrickse, 1967). The behaviour of IgG on G 200 Sephadex is aberrent (Andrewss, 1965). Rowe and Soothill (1961 a and b) have shown that small molecular weight fragments of IgG giving the immunological reactions of the parent molecule

can often be detected in urine.

Both Θ and -K reflect the inverse relationship between log molecular weight and log clearance which, it is believed, is a function of the effective size of the glomerular pores. Since IgG is sometimes excreted in an idiosyncratic manner, the slope of log clearance against log molecular weight based on transferrin and IgG alone does not always agree with that found when the plot is based on the results obtained with five proteins. (MacLean and Robson, 1967). Even where IgG is not cleared aberrantly, since the molecular weights of transferrin and IgG differ relatively slightly, a relatively small error in determining the clearance of one of the two proteins leads to a greater error in slope than would be the case if there was a greater difference between the molecular weights of the proteins selected.

Cameron and Blandford were, of course, aware of these difficulties. Since results based on the clearances of IgG and transferrin do not always reflect the true slope of the regression of log clearance on log molecular weight, they expressed their results not in terms of Θ or -K, but as the simple ratio $\frac{\text{IgG clearance}}{\text{Transferrin clearance}}$. Yet another index of selectivity had been born!

The single diffusion technique is to be preferred to the double diffusion technique in terms of simplicity, accuracy, and speed (Soothill and Rowe, 1967). The use of alpha 2 macroglobulin is to be preferred to the use of IgG for clearance studies involving two proteins (MacLean and Robson, 1967). The best "simplified" technique for selectivity determinations might be one estimating

transferrin and alpha 2 macroglobulin by the single diffusion method. As single diffusion is less sensitive than the double diffusion technique, the urine would have to be concentrated (often by a factor of 100 or more) prior to assay of alpha 2 macroglobulin. The addition of this step would make the technique considerably less simple than that of Cameron and Blandford. Whether the general introduction of alpha 2 macroglobulin in place of IgG would be worthwhile or not is a matter of opinion. The author of this thesis feels that the undoubted benefits gained by Cameron and Blandford in terms of simplicity are outweighed by the price paid in terms of loss of accuracy and interpretability. An opposite view has been expressed in the excellent book edited by Manuel et al (1970); if the opinion stated was the unanimous one of Hardwicke, Cameron, Harrison, Hulme and Soothill (the authors of the chapter concerned), we in Edinburgh are both outnumbered and outgunned!

In summary, the indices used for the expression of selectivity results obtained from immunodiffusion studies are Θ (Joachim et al, 1964), -K (MacLean and Robson, 1966) and the ratio, IgG clearance/transferrin clearance. (Cameron and Blandford, 1966).

-K is the slope of the line relating log clearance to log molecular weight.

Θ is the angle made by this line at its intersection with the X axis;

-K is the tangent of the angle Θ

The ratio IgG clearance/transferrin clearance can be expressed in terms of -K on the basis of the following equation -

$$-K = \frac{\left(2 - \log \frac{(10^2 \times \text{IgG clearance})}{(\text{transferrin clearance})}\right)}{(\log \text{MW IgG} - \log \text{MW transferrin})}$$

This equation assumes that the clearance of IgG falls on the linear regression line derived from clearance studies involving five proteins. Where the renal clearance of IgG is anomalous, there is no way of relating the ratio of IgG clearance/transferrin clearance to the other indices of selectivity.

b. Selectivity indices employed in gel filtration studies

Hardwicke (1965a) introduced the use of G 200 Sephadex gel filtration in the assessment of protein selectivity. Since elution volume is inversely proportional to log molecular weight for Sephadex fractionation of spherical proteins (Andrews, 1965) the plot of log clearance against elution volume (or tube number) is analogous to the plot of log clearance against log molecular weight introduced by Blainey et al (1960). The relationship is linear over the elution range Vo to Va.

Hardwicke (1965a) plotted log clearance against tube number over the range Vo to Va and in this paper he used the change in log clearance per eluted tube as his index of selectivity. To make this index independent of column size and fraction volume he adjusted the number of tubes between Vo and Va to 15 in each case. If we take m to represent the change in log clearance per tube and n to represent the number of tubes eluting between Vo and Va, Hardwicke's index was

$$\frac{m \times n}{15}$$

We repeated Hardwicke's experiments (MacLean and Petrie, 1966) but were disinclined to incorporate the number 15 into our index of selectivity; this number is of course an arbitrary one. We expressed our gel filtration index of protein selectivity by the symbol $\triangle$; it represented the total change in log clearance over the range Vo to Va. Taking m as the change in log clearance per tube and n as the number of tubes eluting between Vo and Va,

$$\triangle = m \times n.$$

We used an identical method of calculation for our indices of selectivity with respect to dextran and PVP and used the symbols D and $\triangle$ PVP respectively for these indices.

Vere and Walduck (1966) also used gel filtration to determine what they termed "the selective permeability of the glomerulus with respect to protein". They calculated their protein clearances over the range Vo to Va but expressed their results in terms of the change in log clearance per unit change in Kav.

Now Kav is (Ve-Vo)/(Vt-Vo); its value is unity when Ve is equal to Vt. Thus the selectivity index used by Vere and Walduck was the change in log clearance for the total elution range Vt, calculated from results over the range Vo to Va only. For the purposes of this discussion, we have designated Vere and Walduck's slope by the symbol "S". S is the change in log clearance per unit change in Kav.

Hardwicke, like ourselves, wanted to get rid of the mysterious number 15 which appeared in his initial paper on gel filtration (Hardwicke, 1965a). In order to do this, he adopted a unit very similar to that employed by Vere and Walduck; this unit was in fact the change in log clearance per unit change in (1-Kav) (Hardwicke et al, 1968). Since a unit change in Kav is also a unit change in (1-Kav), the index used by Hardwicke et al (1968) did not differ numerically from that employed by Vere and Walduck (1966), and we have used the symbol "S" to refer to this index also.

Since $\triangle$ is the change in log clearance over the elution

Table 22. Equivalent values for Δ, θ, S and $\frac{CIgG}{C\ transferrin}$ at intervals of 0.2 in (-K).

-K	θ	Δ	S	$\frac{CIgG}{C\ transferrin}$	-K	θ	Δ	S	$\frac{CIgG}{C\ transferrin}$
0.2	12	0.14	0.36	0.89	3.2	72.5	2.33	5.84	0.19
0.4	22	0.29	0.73	0.81	3.4	73.5	2.48	6.20	0.17
0.6	31	0.44	1.09	0.72	3.6	74.5	2.63	6.57	0.15
0.8	39	0.58	1.46	0.65	3.8	75.5	2.77	6.93	0.14
1.0	45	0.73	1.82	0.59	4.0	76	2.92	7.30	0.12
1.2	50	0.88	2.19	0.54	4.2	76.5	3.07	7.66	0.11
1.4	55	1.02	2.55	0.48	4.4	77.5	3.21	8.03	0.10
1.6	58	1.17	2.92	0.44	4.6	78	3.36	8.39	0.09
1.8	61	1.31	3.28	0.39	4.8	78.5	3.59	8.76	0.08
2.0	64	1.46	3.65	0.35	5.0	79	3.65	9.12	0.07
2.2	65.5	1.60	4.01	0.32	5.2	79.2	3.80	9.49	0.07
2.4	67.5	1.75	4.38	0.29	5.4	79.5	3.94	9.85	0.06
2.6	69	1.90	4.74	0.26	5.6	79.9	4.09	10.21	0.06
2.8	70.5	2.04	5.11	0.23	5.8	80.2	4.23	10.58	0.05
3.0	72	2.19	5.47	0.21	6.0	80.6	4.38	10.95	0.05

-K is the slope of the regression line relating log clearance and log molecular weight. (MacLean and Robson, 1966)

θ is the angle at which this line cuts the X axis. (Joachim et al, 1964)

Δ is the change in log 100 U/P over the range Vo to Va. (Maclean and Petrie, 1966)

"S" is the symbol used in this thesis to denote the change in log 100 U/P per unit change in Kav (Vere and Walduck, 1966) or per unit change in (1-Kav) (Hardwicke et al, 1968)

$\frac{CIgG}{C\ transferrin}$ is the ratio of IgG clearance to transferrin clearance. (Cameron and Blandford, 1966)

range (Va-Vo) and S is the change in log clearance over the range (Vt-Vo)

$$\frac{\Delta}{S} = \frac{(Va - Vo)}{(Vt - Vo)}$$

The expression (Va-Vo)/(Vt-Vo) is in fact the Kav value for albumin. This has a value on G 200 Sephadex of approximately 0.40.

Thus $\Delta = 0.4\ S$

and $S = 2.5\Delta$

The relationship between selectivity values determined by gel filtration and selectivity values obtained by immunodiffusion is not immediately apparent. This relationship has, however, been investigated empirically and considered theoretically in section 3 of this thesis. In this section the following equations were derived.

$$\Delta = 1.37\ (-K)$$

$$(-K) = 0.73\ \Delta$$

Since it has been shown in the previous paragraph that $S = 0.4\Delta$

$$S = 1.82\ (-K)$$

and $(-K) = 0.55\ S.$

Table 22 shows equivalent values of Δ, θ, S and the ratio IgG clearance/transferrin clearance at intervals of 0.2 in (-K). This table allows the results of different workers to be compared even when these results are expressed in different units. It is to be hoped that those involved in the field of differential protein clearance studies will at some time reach agreement over a single, mutually acceptable index of selectivity. Although results in one laboratory will in all probability differ from results in another because of differences in methodology, without a common

mode of expressing results it is not even possible to make comparisons.

The differences between probit units and logarithmic units are so fundamental that no attempt has been made to include indices of selectivity based on probit transformation in the above discussion.

2. Macromolecular clearance studies related to the mechanisms involved in proteinuria.

a. General considerations

In section 3.1 of this thesis, protein selectivity values obtained by the gel filtration technique have been compared with protein selectivity values obtained by immunodiffusion. For patients excreting over 1.0G of protein per 24 hours, the two methods give results which are in substantial agreement. In terms of methodology, it is therefore permissible to compare the results of gel filtration studies on dextran and polyvinyl pyrrolidone with the results of immunodiffusion studies on protein.

The handling of dextran and PVP by the kidneys, however, differs from the handling of protein in two important respects. Firstly, neither of the exogenous macromolecules is reabsorbed by the tubules to any appreciable extent (Wallenius, 1954; Hulme and Hardwicke, 1966). Secondly, clearance values for PVP molecules of a given molecular radius are much higher than the clearance values for protein molecules of equivalent size. (Hardwicke et al, 1968; Lambert, 1970; this thesis, section 3.8). Dextran is similar to PVP in this respect (Hardwicke et al, 1968).

The fact that dextran and PVP are filtered more readily than protein molecules with the same effective molecular radius does not affect the selectivity index directly. The position of the line relating log clearance to elution volume is altered, but the slope of the line is not, and it is this slope which is the selectivity index. The fact that protein is subject to tubular

reabsorption, and the fact that it penetrates the filtration barrier less readily than the exogenous macromolecules, will however lead in certain situations to the population of glomerular pores assessed in protein clearance studies being different from the population assessed in dextran and PVP studies.

This important concept requires careful explanation. The mean pore radius of the normal kidney with respect to PVP is about 60A (Lambert et al, 1970). The sieving coefficient for PVP molecules with a molecular radius of 36A is about 0.5* (Hardwicke et al, 1968). Thus even in patients with heavy proteinuria, where a substantial proportion of the filtration area is occupied by abnormally large pores, much of the PVP of radius 36A and larger will have been filtered through normal pores. PVP selectivity results will therefore reflect the sieving characteristics of both the normal population and the abnormal population of glomerular pores. Similar considerations apply to dextran clearance studies.

The mean pore radius of the normal kidney with respect to albumin (molecular radius 36A) is about 38A. The sieving coefficient for albumin is approximately 0.003 (Lambert et al, 1970). Since the glomerular clearance of albumin through normal pores is only 0.3% of GFR, in a situation where the renal clearance of albumin is 1% of GFR, at least 70% of the protein in the urine must have been filtered at abnormal pores, and 30% at most of the protein will have been filtered at normal pores. Once tubular reabsorption is taken into account, however, it will be seen that the contribution of the normal pores to the total protein excretion is even less than the proportion suggested above.

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*i.e. The clearance of PVP of 36A radius is about 50% of GFR.

The sieving coefficient of 0.003 has been calculated on the basis of albumin loading experiments in normal subjects where, as a result of raising the plasma albumin level, tubular reabsorption was saturated. It represents the glomerular clearance of albumin; the renal clearance of albumin in normal subjects under normal conditions is of course negligible. Proteinuric patients are not protein loaded. If in such patients the renal lesions are patchily distributed, and some glomeruli escape damage, these undamaged glomeruli will contribute no protein to the urine. If the number of glomeruli remaining normal is at all substantial, the renal clearance of albumin by way of normal pores will be very much less than the normal glomerular clearance of 0.3% GFR. In this situation, if the albumin clearance is 1% of GFR, considerably more than 70% of the protein in the urine will have been derived from abnormal pores.

In summary, then, since dextran and PVP are not reabsorbed and since they penetrate the normal filtration barrier with relative ease, dextran and PVP clearance studies will reflect the permeability of normal as well as abnormal pores in normal as well as abnormal glomeruli. Since protein is subject to tubular reabsorption, and since it penetrates the normal filtration barrier with difficulty, clearance studies involving endogenous protein will reflect the permeability of abnormal glomeruli only, and of abnormal pores in these glomeruli predominantly.

The above account is based on the view that a substantial amount of protein is filtered at the glomerulus in health and that virtually all of this protein is reabsorbed by a pathway which, as

far as albumin and proteins larger than albumin are concerned, is non-selective.

This view has recently been challenged. Studies on the Fanconi syndrome and allied disorders have shown that low molecular weight proteins such as beta 2 microglobulin can have a renal clearance of about 75% of GFR in the presence of an albumin excretion of a few hundred milligrams of albumin only. (Flynn and Platt, 1968; Harrison and Blainey, 1967; Petersen et al 1969).

If the reabsorption of albumin in the Fanconi syndrome is impaired to the same extent as that of beta 2 microglobulin, the absence of heavy proteinuria in this syndrome would suggest that the albumin content of the glomerular filtrate is negligible, being of the order of 0.3 mg% (Flynn and Platt, 1968).

There is, however, no reason to believe that albumin shares a reabsorptive pathway with molecules of the size of beta 2 microglobulin. The experiments of Hardwicke and Squire (1955) demonstrated a shared reabsorptive pathway for albumin and certain molecules larger than albumin. Lambert (1933) has shown that small protein molecules are reabsorbed at a more proximal site than large protein molecules; in the Fanconi syndrome it is the proximal part of the proximal tubule which is most severely disorganised. (Clay, Darmady and Hawkins, 1953).

It was Cushny (1926) who postulated that the capsular fluid was a true ultrafiltrate of plasma, being entirely protein free. Over the past half-century, however, numerous workers have established by numerous techniques that filtration of protein occurs at the glomerulus and that reabsorption of protein occurs in the

tubules. This work on the basic mechanisms involved in the handling of protein by the kidneys has been reviewed at some length in the introduction to this thesis; Cushny's theory has been undermined by many separate lines of enquiry. The results of micropuncture experiments, stop-flow experiments, clearance determinations, histochemical observations, and electron microscopy studies outlined on pp.1-40 of this thesis need not be repeated here. These findings cannot be discounted solely on the basis of indirect evidence derived from the study of a single pathological situation.

b. Protein and dextran selectivity values in normal subjects

The results of protein selectivity determinations in 10 normal subjects are shown in table 8, facing p.88. The values obtained ranged from 0.59 to 1.56, and the mean value for (-K) was 1.14 ± 0.11.

Dextran selectivity values in 9 normal subjects are shown in table 9, also facing p.88. When these results were expressed in terms of (-K) units, they ranged from 3.28 to 5.00, and the mean value was 4.10 ± 0.21

PVP selectivity values in six normal subjects are shown in table 20, facing p.132. The mean value for PVP selectivity, again in terms of (-K) units, was 4.25 ± 0.21.

Thus the excretion pattern for protein in normal subjects was found to be very unselective, while the excretion pattern for dextran and PVP proved to be very selective.

Our finding of an unselective pattern of protein excretion in normal subjects has been confirmed by other workers. (Hardwicke, personal communication; Rowe and Soothill, 1961a; Poortmans, 1968). In 1968, however, Braun and Merrill reported protein selectivity values in normal subjects which were substantially higher than those obtained by ourselves. Expressing their results in terms of the angle θ, they found the mean value for protein selectivity in normal subjects to be 65°. This is equivalent to a (-K) value of 2.14. Even this relatively high figure probably does not reflect the true permeability to protein of the normal glomerulus. It indicates an excretion pattern which is considerably less selective than that found in minimal lesion glomerulonephritis

where the mean value for (-K) in our series was 3.08. It is, however, much higher than our mean value in normal subjects of 1.14. Braun and Merrill used polyelectrolyte membranes in the concentration of their urine samples (Blatt et al, 1965) and it is likely that this difference in methodology was responsible for the difference between their results and ours.

Cameron and White (1965) noted that protein selectivity values in children with the nephrotic syndrome were not normally distributed, but were instead skewed upwards. They speculated that the skew was in the direction of the true selectivity to protein of the normal glomerulus, and suggested that if this were so, the selectivity of the normal glomerulus to protein would be about 75°, in terms of the angle θ. This speculative figure of Cameron's corresponds to a (-K) value of 3.7, which is reasonably close to our mean values for protein and dextran selectivity - (4.10 and 4.25 respectively, in terms of (-K)).

Because of the virtually complete tubular reabsorption of the protein filtered by the normal kidney, the pattern of protein excretion in normal subjects cannot be taken as representing the pattern of protein filtration. Only a proportion of the 50 mg of protein excreted each day in health is of serum origin. (Berggard, 1970; Bourillon, 1970; Grant, 1970). Although the assay methods used in protein selectivity determinations on normal subjects were specific to proteins of serum origin, low molecular weight fragments giving the immunological reactions of their parent proteins are to be found in urine, and in normal urine, such fragments make up a significant proportion of the total protein excreted. (Rowe and Soothill, 1961a). Serum protein added to the urine distal to the

glomerular filter (e.g. by transudation across bladder epithelium) may represent a significant proportion of the total amount of protein excreted by normal subjects, even if the amount of protein excreted in this way is as little as 10 mg daily. Since a relatively large proportion of the protein in normal urine is not of glomerular origin, it is not to be expected that protein selectivity studies on normal subjects under normal conditions will reflect the filtration pattern of the glomeruli with accuracy.

An alternative approach is to determine the selectivity pattern which obtains when normal kidneys are rendered proteinuric by plasma infusion. In this situation, tubular reabsorption of protein becomes saturated, and it is where there is saturation of the reabsorptive mechanisms for protein that one can expect the protein excretion pattern to correspond with the pattern of protein filtration.

The induction of proteinuria by the parental administration of homologous protein has been described in the dog (Terry et al, 1948), in the rat (Post, 1960) and in man (Waterhouse et al, 1948). The results outlined on pages 93 to 96, however, represent the only selectivity determinations which have so far been recorded in proteinuria induced in this way.

The four patients with bleeding disorders described in section 3.2 of this thesis had normal renal function and were free of proteinuria prior to plasma infusion. The prompt disappearance of the proteinuria following cessation of infusion, along with the dose-related nature of the proteinuric response, argued against immunological mechanisms being involved in the genesis of the

proteinuria. It is considered that the most important mechanism involved was an increase in protein filtration at the glomeruli consequent on the raising of serum protein levels by plasma infusion.

The protein and dextran selectivity values obtained in these patients are shown in table 13, facing p.95. Values for protein selectivity ranged from 2.49 to 3.13, the mean value of (-K) being 2.72. Dextran selectivity values, expressed in terms of (-K) units, averaged 3.66 when measured during the period of induced proteinuria. (See also figure 26, reproduced opposite.)

Thus when proteinuria is induced in patients with normal kidneys, the protein excretion pattern and the dextran excretion pattern are both highly selective.

Gregoire et al (1958) and Malmendier et al (1967) showed in the course of albumin infusion experiments in patients with proteinuria that plasma expansion led to an increased glomerular permeability to albumin. Their findings are discussed on p.30 and illustrated in figure 4. The four patients in our series developed proteinuria following very considerable plasma expansion, and it is possible that this expansion might have modified glomerular permeability by a pore-stretching effect.

Table 13 shows dextran selectivity values determined both in the presence and in the absence of proteinuria. There was no apparent difference between the two sets of dextran selectivity results, and consequently we obtained no direct evidence of a pore stretching effect.

A slight increase in glomerular permeability resulting from

plasma expansion could not be excluded, however. The dextran selectivity values obtained were at the lower end of the normal range. The values obtained for protein selectivity were comparable to those found in minimal lesion glomerulonephritis. Since it is generally accepted that proteinuria in the nephrotic syndrome is due to increased glomerular permeability to protein (Chinard et al, 1954) it might have been expected that the true selectivity of the normal glomerulus with respect to protein would be rather higher than the selectivity values found in patients with minimal change lesions. It is therefore conceivable that a slight degree of pore-stretching was induced by plasma expansion in the patients with induced proteinuria, and it may be that the true selectivity of the normal glomerulus in terms of the protein filtration pattern is slightly higher than the values for protein selectivity obtained in these patients.

There is no evidence to suggest that "pore-stretching" occurs with albumin infusions of the order of 25G to 40G. Marchena and Becker (1968) showed a sharp rise in protein excretion following the infusion of 25G of albumin in proteinuric patients, but this increased excretion was not accompanied by any detectable alteration in protein selectivity.

Following 40G albumin infusions in a number of our own nephrotic patients, we obtained similar results. The patients in whom proteinuria was induced, however, were in a somewhat different situation, as each received at least 150G of protein intravenously.

c. Comparison of dextran and protein selectivity values in patients with glomerulonephritis.

The results of clearance studies involving endogenous protein and exogenous dextran in 21 patients with glomerulonephritis are summarised in table 7, facing p.84. In figure 26, facing p.96 protein selectivity values and dextran selectivity values are both expressed in terms of (-K) units, for purposes of comparison. The following three points emerge.

1. In glomerulonephritis, protein selectivity values and dextran selectivity values (when expressed in the same units) are of the same order of magnitude. From figure 26 it will be seen that although the dextran values tend to be somewhat higher than the protein values, there is considerable overlap.
2. The individual values for dextran selectivity in the patients with minimal lesion glomerulonephritis and membranous glomerulonephritis correlate well with the corresponding values for protein selectivity. (See figures 22 and 23, facing p.86).
3. The individual values for dextran selectivity in patients with proliferative glomerulonephritis correlate poorly with the corresponding values for protein selectivity. (See figures 24 and 25, facing p.87).

The most striking feature of these results is the contrast between the poor correlation between values obtained for dextran and protein selectivity in patients with proliferative glomerulonephritis on the one hand, and the high degree of correlation between such values in patients with minimal lesion or membranous glomerulonephritis on the other.

It has already been pointed out that indices of selectivity are affected by the functional state of the nephrons which contribute the macromolecule concerned to the urine, and of these nephrons only. The protein in the filtrate from a normal glomerulus is almost completely reabsorbed. It is only when the glomerulus is functionally abnormal that it leaks enough protein to saturate reabsorption. Thus it is only abnormal glomeruli which contribute protein to the urine. Since dextran is not reabsorbed, dextran selectivity values will reflect the filtration characteristics of all functioning glomeruli, while protein selectivity values will reflect the characteristics of abnormal glomeruli only.

If the renal lesion is uniform and all the glomeruli are similarly affected, then dextran and protein selectivity values will be in substantial agreement. If the glomerular lesion is patchy, however, with some glomeruli more affected than others, their permeability will also vary. Glomeruli leaking protein to an extent which is less than the tubular reabsorptive maximum, however, will contribute no protein to the urine and the filtration characteristics of these glomeruli will not be reflected in the protein selectivity value. Protein selectivity values will disproportionately represent the permeability of those of the more severely affected glomeruli which remain functional. Since the selectivity of the normal glomerulus is high, protein selectivity values will be lower than dextran selectivity values when the renal lesion is not uniform.

As table 7 shows, this was found to be the case in proliferative

glomerulonephritis, where the values for D (the dextran index of selectivity) were found, with only one exception, to be greater than the corresponding values for Δ (the index of protein selectivity by the gel filtration method). In proliferative glomerulonephritis, the severity of the glomerular lesions is characteristically patchy.

In minimal lesion glomerulonephritis and in membranous glomerulonephritis, however, the renal lesions are more uniform. In these two conditions a better correlation between protein and dextran selectivity was found. Even here there was a slight tendency for dextran selectivity values to be higher than the corresponding protein selectivity values. Such a tendency is to be expected even when both macromolecules are being excreted by the same nephrons. It will be remembered that dextran molecules pass through normal pores rather more readily than protein molecules. Thus if an abnormal, proteinuric nephron contains both normal and abnormal pores, the contribution of the normal pores to the dextranuria will be significant, and the dextran selectivity index will in part reflect the permeability of such pores. Since the glomerular clearance of albumin through normal pores is of the order of 0.3% of GFR, however, most of the protein excreted by an abnormal glomerulus will have been filtered at abnormal pores.

A very heavy proteinuria implies that a large proportion of the total filtration area is taken up by abnormal pores. In this situation, regardless of the histological diagnosis, it is likely first of all that the vast majority of functioning nephrons will be contributing protein as well as dextran to the urine, and

secondly that a major proportion of the dextran in the urine eluting over the Vo to Va range will have been filtered at abnormal pores. Thus where the proteinuria is massive, dextran selectivity values and protein selectivity values will tend to reflect the function of the same pores in the same glomeruli, and will thus tend to be in agreement.

This concept receives support from the fact that even in patients with proliferative glomerulonephritis dextran and protein selectivity values were in reasonable agreement in those patients who excreted over 6.0G of protein per 24 hours, but were widely divergent in patients excreting 4.5G or less.

d. Protein and dextran selectivity values in postural proteinuria

Table 10, facing p.89, shows the values obtained for protein and dextran selectivity determinations in patients with postural proteinuria. Protein selectivity values were uniformly low, both in the presence and in the absence of significant proteinuria. Dextran values for selectivity, on the other hand, were uniformly high. (See figure 26, facing p.96).

Postural proteinuria has been defined by Robinson (1970) as "a laboratory syndrome whose diagnosis requires the absence of qualitative proteinuria during recumbency and its presence during quiet upright ambulation or standing." The mechanisms which underlie it are in doubt.

Bull (1948) found that proteinuria could be induced in erect lordosis in about 70% of adolescent males. This proteinuria could be abolished in many instances by a tight abdominal binder. He suggested that in the erect posture in thin subjects the liver might rotate to compress the inferior vena cava, with proteinuria arising from a rise in renal venous pressure. Lagerlof et al (1951) found that the pressure in the inferior vena cava actually fell in the erect posture, however.

Parallels were then drawn with renin-induced proteinuria, (Addis et al 1949) and it was felt that renal haemodynamic changes induced by posture might be the cause; in patients with postural proteinuria such changes might differ from the changes found in healthy subjects. King and Baldwin (1954) showed, however, that the effects of posture on renal haemodynamics were identical in subjects with postural proteinuria and in subjects without this

condition. Castenfors (1967) produced evidence which suggested that changes in the volume and distribution of renal blood flow were responsible for exercise proteinuria, but Rowe and Soothill (1961) had shown significant differences in the pattern of protein excretion between this condition and postural proteinuria. Robinson (1970) conceded that the haemodynamic changes induced by the erect posture (i.e. a fall in renal plasma flow, a less marked fall in glomerular filtration rate, and a rise in filtration fraction) did not differ in patients with orthostatic proteinuria from the changes seen in normal subjects. He suggested that these effects might cause proteinuria in certain individuals by increasing the permeability of glomeruli which were already slightly more permeable than normal.

This view is perhaps more widely accepted than any other, but Wolman (1945) has drawn attention to the absence of casts in orthostatic proteinuria as being evidence against a glomerular origin of the protein.

Quinke (1912) suggested that the renal lymphatics were the source of the urinary protein in this condition. He based his theory on anatomical observations of the close relationship between the renal lymphatics and the lower urinary passages. Lowgren (1955) demonstrated reflux of contrast media from the renal pelvis and calyces into renal lymphatics on retrograde and intravenous pyelography. He suggested that posture might alter both the pressure and the direction of flow in the renal lymphatics, giving rise to extravasation of lymph into the urine in the erect position in certain subjects.

This view has been attacked. In the course of experiments with labelled protein, Slater et al (1960) found close agreement between the specific activity of urinary albumin and that of serum albumin at the presumed time of urine formation. These workers held that this finding was against a lymphatic origin of postural proteinuria; because of the blood-lymph barrier they expected the specific activity of the albumin in renal lymph to differ from that of plasma. The observations of Slater et al, however, invalidate Lowgren's hypothesis only if certain assumptions are made concerning the mechanisms of production of renal lymph.

Microscopic examination of biopsy specimens in postural proteinuria have not elucidated the mechanism of protein excretion. Light microscopy shows no abnormality (Robinson et al, 1961); Ruckley et al, 1966). The changes on electron microscopy are "both subtle and focal" (Robinson, 1970). There are foci of foot-process fusion. This fusion is more marked than that seen in normal subjects, but less marked than the changes seen in minimal lesion glomerulonephritis. There is a patchy, irregular ballooning of epithelial cytoplasm. The glomerular basement membrane is normal in thickness and appearance (Ruckley et al, 1966).

The similarities between postural proteinuria and minimal lesion glomerulonephritis in terms of the electron microscope appearances might suggest that postural proteinuria is glomerular in origin. In minimal lesion glomerulonephritis, however, the proteinuria is selective; in postural proteinuria, it is very unselective. The pattern of dextran excretion suggests that the overall permeability of the glomeruli is normal; in terms of (-K)

units dextran selectivity in postural proteinuria (mean 4.14) was identical to dextran selectivity in normal subjects (mean 4.10). If the proteinuria is glomerular in origin, since the overall permeability of the glomeruli is normal, filtration must be through a small number of relatively large pores. In this situation, one might expect to see patchily distributed areas of relatively severe glomerular damage; no such changes have as yet been described in postural proteinuria.

Comparison of the protein pattern of lymph with that of plasma (Schultze and Hereman, 1966) suggests that lymph is formed by a relatively unselective process. Our results would be consistent with the suggestion made by Quinke (1912) and Lowgren (1955) of a post glomerular mechanism derived from the renal lymphatics.

e) Protein and dextran selectivity values in acute ischaemic renal failure.

Table 1, facing p.91, shows the results of protein and dextran clearance studies in 12 patients with acute renal failure. Protein selectivity values were uniformly low, (-K) having an average value of 0.85. Dextran selectivity values were considerably higher. In terms of (-K) units the mean dextran selectivity value in acute renal failure was 3.34; this value is appreciably lower than the mean value of 4.10 found in normal subjects. (See figure 26, facing p.96).

Acute renal failure following haemorrhage, depletion of body fluids, severe infection, or trauma is initiated by renal ischaemia. The factors which perpetuate severe impairment of renal function for days or weeks after the correction of the precipitating cause, however, are not clearly understood.

Bull et al (1950) concluded from clearance studies that renal plasma flow remained depressed throughout oliguria. In situations where the extraction of PAH from renal arterial blood is abnormally low, however, it cannot be assumed that PAH clearance is a measure of renal plasma flow.

Meroney and Rubini (1959) suggested that obstruction of the renal tubules by debris within them was responsible for the impaired flow of urine. This theory remains unproven.

Oliver, McDowell and Tracy (1951) suggested that the glomerular filtrate might be reabsorbed virtually in toto by leakage back through the damaged tubular epithelium. This hypothesis has received little recent support. Hardaway (1966) suggested that intravascular coagulation might be an important factor

both in the genesis and in the persistence of acute renal failure. Clarkson et al (1970) provided convincing evidence in support of this concept in the course of a detailed study of electron microscope appearances and fibrin degregation products.

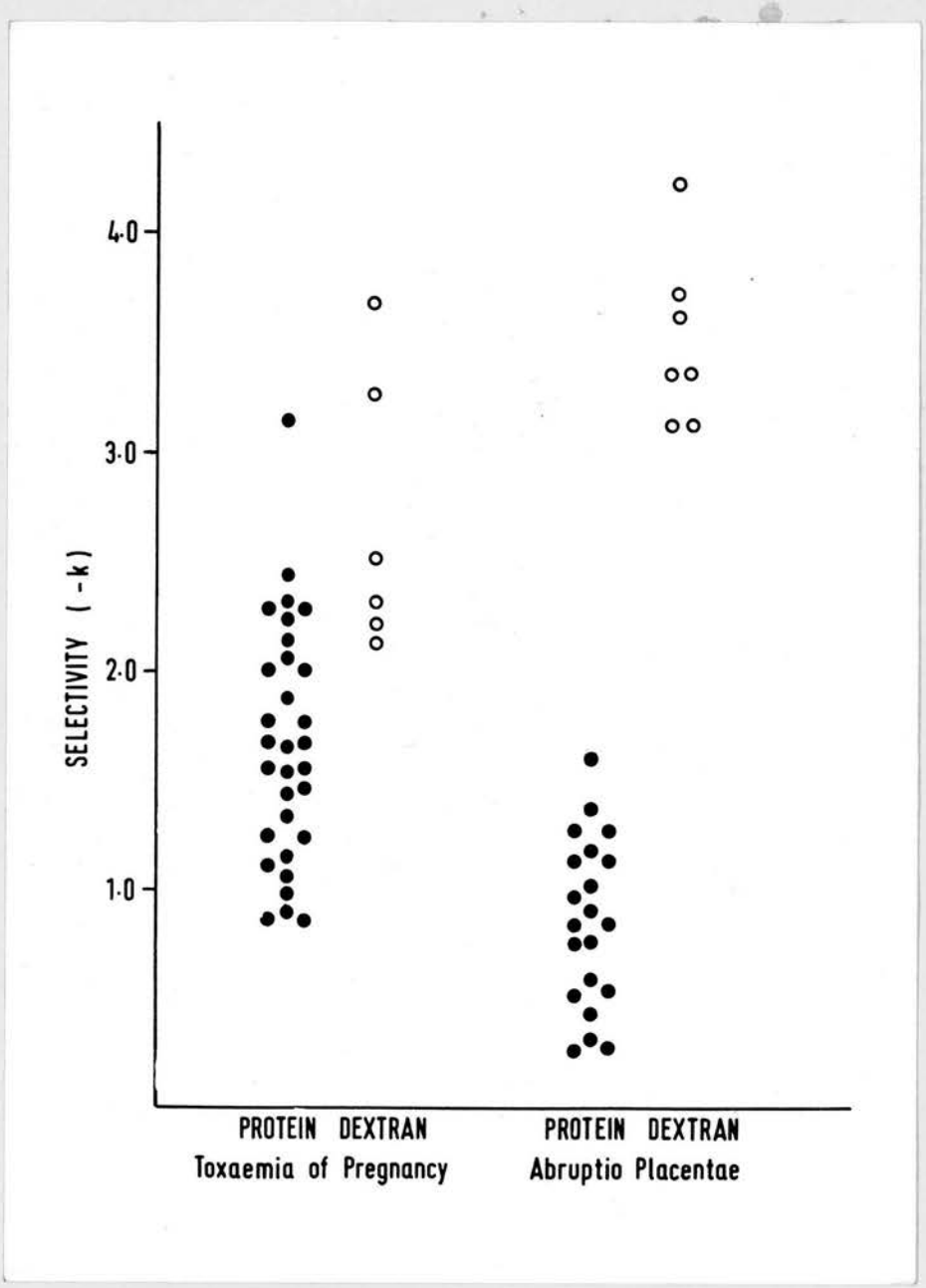
Protein excretion in acute ischaemic renal failure is of two kinds. There is a "tubular proteinuria" similar to that seen in the Fanconi syndrome, characterised by the presence in the urine of low molecular weight proteins giving bands in the alpha 2 and beta 2 regions on paper electrophoresid. (Butler and Flynn, 1958; Butler and Flynn, 1961). Proteinuria of this kind has been attributed to failure of the tubular reabsorptive mechanisms for low molecular weight proteins. (Harrison and Blainey, 1967). In addition, there is excretion of high molecular protein of serum origin by a non-selective pathway. (Revillard et al, 1970).

Our clearance studies in patients with acute renal failure were confined to high molecular weight proteins and to high molecular weight dextrans; we obtained no information about the low molecular weight protein excretion described by Butler and Flynn. We found a selective dextranuria, and the total amount of protein excreted was never massive. There was thus no evidence of a generalised increase in the permeability of the glomeruli to macromolecules in acute renal failure.

The protein excretion pattern, however, was very unselective. It is possible that this unselective pattern of protein excretion was due to filtration at the site of severe, patchy, glomerular abnormalities. An alternative explanation, which receives support

Figure 42

Protein and dextran selectivity values in proteinuria complicating pregnancy.



from the extreme lack of selectivity of the proteinuria and from the histological observation of areas of tubular rupture, is that the proteinuria derived in whole or in part from leakage of plasma through damaged tubular epithelium. If this were indeed the route of entry of protein to the urine, a small amount of dextran would enter the urine by the same route, but the bulk of the filtered dextran would be derived from filtration at glomeruli of normal or near normal permeability. This would be consistent with the observation that dextran selectivity values in acute renal failure are slightly lower than dextran selectivity values in normal subjects.

Since tubular reabsorption of low molecular weight protein is impaired in acute renal failure (Butler and Flynn, 1961) it would be tempting to suggest that the excretion of large protein molecules in this condition was also due to impaired tubular reabsorption in the presence of normal glomerular permeability. There is, however, no evidence that high molecular weight proteins share a reabsorptive pathway with low molecular weight proteins (Lambert et al, 1970) and it has been shown that the saturation of tubular reabsorption by protein-loading gives rise to a highly selective proteinuria. (Petrie et al, 1970; this thesis, pp.93-96.)

It has recently been shown (Smart et al, in preparation) that protein selectivity values are low in abruptio placentae, but that dextran selectivity values are high in this condition. In uncomplicated toxaemia of pregnancy, on the other hand, protein and dextran selectivity values are in the intermediate range and

correlate well. The glomerular lesions are remarkably similar in toxaemia and in patients with abruptio but without preceding toxaemia (Thomson and MacDonald, in preparation). Abruptio placenta is an obstetrical catastrophe which frequently gives rise to acute renal failure, and it may be that in this condition part or all of the protein in the urine has found its way there via a leak through damaged tubular epithelium. In toxaemia, where the protein and dextran selectivity values are similar, it is likely that the proteinuria is of glomerular origin.

In summary, it is considered the unselective pattern of protein excretion which occurs in acute renal failure is indicative either of unselective filtration of protein through a relatively small number of large abnormal glomerular pores, or of a leak of plasma through severely damaged renal tubular epithelium.

3. Comparison of our clinical findings with those of other workers

In this thesis the results of protein selectivity determinations on 207 patients have been presented in Table 14, facing p.97, and fig. 27, facing p.98. The mean value of -K for this series was 1.92. In the group of 14 patients with minimal lesion glomerulonephritis, the average value for -K was 3.08.

Joachim et al (1964) reported the results of selectivity determinations in 48 patients. Their series, like ours, consisted mainly of adults. The mean value for θ for their series was 58.6°; this represents an average value for -K of 1.64. The histological criteria used by Joachim et al differed from those used by ourselves, and it was not possible to determine which of their patients had minimal change lesions. 33 patients were treated with steroids, and in four instances proteinuria was abolished. All 4 of the steroid-responsive cases had selectivity values of over 67.5° in terms of θ, or 2.4 in terms of -K.

Hitzig et al (1965) carried out differential protein clearances in 49 patients. They expressed their results in qualitative terms; it was therefore not possible to compare their findings with our own.

Cameron and White (1965) assessed protein selectivity in 28 children with the nephrotic syndrome. In terms of θ, the mean selectivity value calculated from their data was 69.1°, corresponding to a -K value of 2.62. This mean value was considerably higher than that found by us (1.92) and that found by Joachim et al (1.64). The selectivity values in Cameron and White's series were not normally distributed, but were skewed upwards to a mode of 74°

in terms of θ , or 3.49 in terms of -K. 12 of the 28 children studied by Cameron and White had minimal lesion glomerulonephritis. The mean selectivity for these patients was 73° in terms of θ , or 3.27 in terms of -K.

Selectivity values in children tend to be higher than in adults. This fact is only partially accounted for by the high proportion of patients with minimal lesion glomerulonephritis in paediatric populations. In 15 children with proliferative glomerulonephritis studied by Cameron and White, the mean protein selectivity was 67.2° in terms of θ , or 2.38 in terms of -K. Most of our 92 patients with proliferative glomerulonephritis were adults; the mean value for -K in this condition was found by us to be 1.86. This indicates that proteinuria is more selective in children with proliferative diseases than in adults with similar histology.

White et al (1966) studied 18 patients with steroid resistant proliferative disease. The mean selectivity value in this series was 1.71 in terms of -K.

Cameron and Blandford (1966) studied transferrin and I g G clearances in 134 patients, and expressed the results in terms of the IgG/transferrin clearance ratio. In 35 children, steroid therapy led to complete abolition of proteinuria. 34 of these steroid-responsive children had I g G/transferrin clearance ratios of 0.22 or less. In 40 patients with minimal lesion glomerulonephritis, the ratio was 0.22 or less in 38, the proteinuria being unselective in only 2 cases. For reasons which have been stated earlier in this discussion, the I g G/transferrin clearance ratio

cannot be expressed precisely in terms of -K. From table 22, however, it will be seen that a ratio of 0.22 or less corresponds to a -K value of 2.9 or more.

Cameron (1968) and Sharpstone et al (1969) expressed their selectivity results in terms of the I g G/transferrin clearance ratio, while Soothill and Hendrickse (1967) used the I g G/albumin clearance ratio. The results in these papers cannot therefore be compared with ours directly, but the following points are worth noting.

Soothill and Hendrickse showed that Nigerian children, unlike British children, tend to have unselective proteinuria and to be unresponsive to steroid therapy. The steroid-sensitive minority, however, have selective proteinuria.

Cameron (1968) gave selectivity results for 400 patients, both adults and children. A diagnosis of minimal lesion glomerulonephritis was made in 116. In 108 of these patients (93%) the proteinuria was selective. 4 of the 8 patients who were diagnosed as having minimal lesions but who had unselective proteinuria were treated with steroids, but only in 1 of these was proteinuria abolished. Histology in this series was by light microscopy only, and it is possible that some of the "unselective minimals" may have been misdiagnosed. 233 of Cameron's patients were treated with steroids. 168 had selective proteinuria, and protein excretion was abolished in 99 of these cases (69%). 65 treated patients had unselective proteinuria, and in only 2 of these (3%) was proteinuria abolished. Only 7 patients with proliferative glomerulonephritis lost their proteinuria following steroids. 6 of

these responsive proliferatives had selective proteinuria.

Sharpstone et al (1969) treated 20 patients with proliferative histology with steroids and azathiaprine. In only 2 cases was there abolition of proteinuria. Both these patients had very selective proteinuria.

The above findings, in conjunction with our own, give some indication of the usefulness of selectivity determinations to the clinician. An unselective proteinuria virtually excludes the diagnosis of minimal lesion glomerulonephritis, and is a strong contra-indication to steroid therapy, since a favourable response is not to be expected. There is also a highly significant relationship between prognosis and selectivity. The prognosis in patients with very unselective proteinuria is poor, while that in patients with very selective proteinuria is good (see fig. 28, facing p.111).

Differential protein clearance studies have not revolutionised the practice of nephrology and have not led to a major breakthrough in our understanding of the aetiology of the nephrotic syndrome. Although the contribution of such studies has been undramatic, it has nevertheless been real. Steroids are dangerous drugs. Patients with unselective proteinuria do not respond to steroids. If this single message is appreciated by those concerned in the treatment of nephrotic patients, the saving in terms of morbidity will be substantial, and in terms of mortality, worthwhile.

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A COMPARISON OF GEL FILTRATION AND IMMUNODIFFUSION IN THE DETERMINATION OF SELECTIVITY OF PROTEINURIA

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SUMMARY

Gel filtration on Sephadex G 200 has been used to estimate the selectivity of proteinuria. The method has been found simple and reproducible. Selectivity values by gel filtration have been compared with selectivity values by immunodiffusion on 40 patients. Indices of selectivity correlate extremely well except in cases of proteinuria of under 1 g/day.

INTRODUCTION

In recent years protein excretion patterns have been studied by immunochemical techniques, estimating the relative clearances of individual serum proteins of known molecular weight by the use of specific antisera[1-4]. When the logarithms of these relative clearances are plotted against the logarithms of the molecular weights of the proteins concerned a linear relationship is found. The slope of the line obtained is the index of the "selectivity" of the proteinuria. If the urine contains a relatively small amount of high molecular weight protein and a relatively large amount of low molecular weight protein, the slope of the line is steep and the proteinuria is said to be selective. Conversely in unselective proteinuria the slope obtained is flatter because of the relatively larger amount of high molecular weight protein present in the urine.

Since the tubular reabsorption of filtered protein is a non-specific process[5], the relative concentrations of the proteins in the urine remain the same as their relative concentrations in the glomerular filtrate, when marked proteinuria of glomerular origin is present. Selectivity determinations therefore give information about the permeability of the glomerular basement membrane. It has been suggested that in a number of renal diseases there is an association between selectivity and the type and degree of the glomerular lesion, and that this may have a bearing on the response to treatment with corticosteroids[1,2]. Selectivity determinations, therefore, may have important clinical implications.

Immunochemical determination of selectivity has been carried out in this unit for the past three years, and the technique has been found satisfactory. The antisera

are, however, expensive. Also, it is possible that degraded but antigenically active protein fragments of lower molecular weight than their parent molecules might give rise to erroneous clearance values. Hardwicke[6] described the estimation of selectivity using Sephadex G 200 to fractionate the proteins of serum and of urine. Sephadex separates proteins according to the logarithms of their molecular weights[7]. Since the amount of a given protein appearing in the urine depends on its plasma concentration as well as on the permeability of the glomerulus, selectivity must be assessed on the basis of urine to plasma ratios and not on the basis of urinary composition alone. By comparing the protein elution patterns of serum and urine, relative renal clearances of proteins over a range of molecular weights can be assessed, and selectivity can be calculated.

This paper compares the results obtained by gel filtration with those obtained by immunodiffusion in 40 patients with proteinuria.

MATERIALS AND METHODS

1. *Immunodiffusion*

A modification of the method of Soothill, described elsewhere[4], was used. Selectivity ($-K$) is expressed by the slope of the line relating relative protein clearance to molecular weight on a double log plot.

2. *Gel filtration*

The method used was an adaptation of that of Hardwicke[6]. Two columns were employed, one 100 × 2 cm, and the other 60 × 2 cm. The preparation of the gel for these columns and the method of operating them was as outlined in the monograph by Flodin[8]. The eluant was 0.1 *M* tris buffer in 0.1 *M* saline, pH 8.0. The columns were packed and operated at a pressure head of 10–25 cm of buffer, giving flow rates of 5–15 ml per h. Constant volume aliquots of eluant were obtained using syphons of 3 ml capacity for the larger column and 2 ml for the smaller one, in conjunction with a fraction collector.

An aliquot (0.5 to 2 ml) of serum containing 30 to 90 mg of protein and subsequently a similar volume of the corresponding urine, concentrated to contain approximately the same amount of protein, were added to the column. In order to remove all non-protein low molecular weight substances which might absorb at 280 mμ, the column was washed with at least one elution volume of buffer between samples and the effluent was then checked at 280 mμ. Concentration of the urine was achieved by the use of polyethylene glycol (carbowax 20 M). The urine was first dialysed overnight against running tap water, and then concentrated by dialysis against polyethylene glycol. The concentrate was dialysed against distilled water for 24 h, brought to 0.58 *M* by addition of 3.5 *M* NaCl, dialysed against 0.58 *M* NaCl and then centrifuged before being applied to the column. This adjustment of tonicity precipitates Tamm–Horsfall protein, a high molecular weight urinary tract protein[9] comprising about 70% of the total urinary tract proteins.

The dialysis and centrifugation subsequent to concentration are important as some polyethylene glycol is known to cross the dialysis membrane[10]. Counter-dialysed polyethylene glycol is eluted over the protein range on Sephadex G 200; moreover, it interferes with the methods used for protein estimation in this study

since it absorbs at 280 mμ and causes precipitation in the Folin–Ciocalteau reaction. From model experiments which were carried out the amount of polyethylene glycol remaining in solution after concentration, dialysis, and centrifugation is very small and is unlikely to introduce significant errors. However, in order to assess any effect of counterdialysed polyethylene glycol on the accuracy of the results, some of the urines were also concentrated by ultrafiltration using an LKB ultrafilter; otherwise the urine was treated as described above.

The protein concentrations of the effluent fractions from the column were determined either by reading the optical density at 280 mμ or by a modified Folin–Ciocalteau method using an AutoAnalyzer[11].

3. Calculation of selectivity

The protein concentrations of the serum eluates and of the corresponding urine eluates were plotted against tube number. The urine to serum ratio for each tube was calculated and (for ease of subsequent calculation) multiplied by 100. The logarithm of

$$\frac{100 \times \text{urine protein concentration (U)}}{\text{serum protein concentration (S)}}$$

was plotted against tube number. Since protein separation on Sephadex is proportional to the logarithm of the molecular weight, the resulting graph showed relative protein clearance against molecular weight on a double log plot. The relationship was linear over the range of effective protein separation on Sephadex G 200.

Using the method of least squares the slope of the line relating $\log_{10} \frac{100\ U}{S}$ against tube number was calculated, using the points lying between the first and third serum protein peaks, as Sephadex G 200 is known to resolve well within this area[12]. In order to obtain an index of selectivity which was independent of the column size and the volume of the effluent fractions, the total change in $\log_{10} \frac{100\ U}{S}$ between the first and third serum peaks was taken. This index (Δ) was calculated by multiplying the slope of the line by the number of tubes between the apex of the first serum peak and the apex of the third serum peak. The indices of selectivity by gel filtration and immunodiffusion are therefore directly comparable, since both are based on the slope of a line relating relative renal clearance of protein to molecular weight on a double log plot.

RESULTS

Typical serum and urine separations and the relationship between relative clearance of protein ($\log_{10} \frac{100U}{S}$) and tube number are shown in Figs. 1 and 2. Fig. 1 shows the pattern obtained from a selective proteinuria. The bulk of the urine protein is eluted in the third peak and the slope of the line relating $\log_{10} \frac{100U}{S}$ to tube number is very steep. The index of selectivity (Δ) was 2.02. Fig. 2 shows the pattern obtained from an unselective proteinuria and in this case there is rela-

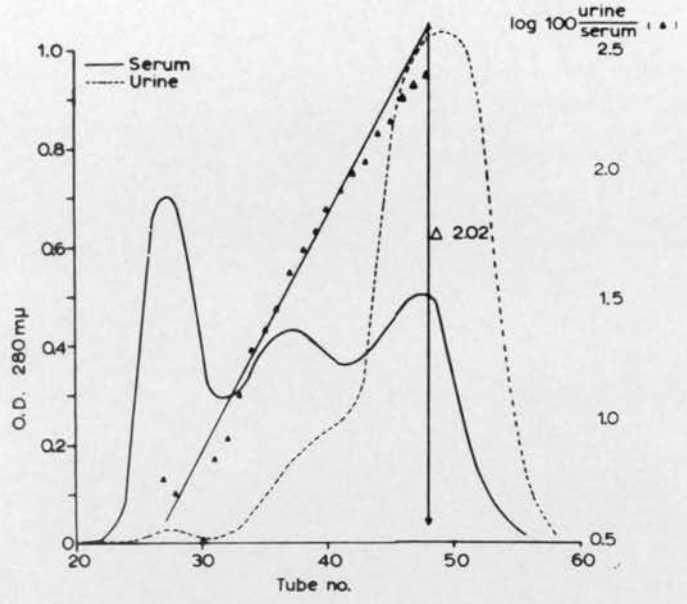

Fig. 1. Serum and urine elution patterns from a Sephadex G 200 column. The plot of $\log_{10} \frac{100 \text{ urine}}{\text{serum}}$ against tube number is also shown. The proteinuria is selective and the index of selectivity, Δ (total change in $\log_{10} \frac{100 \text{ urine}}{\text{serum}}$ between the first and third serum peaks) is high.

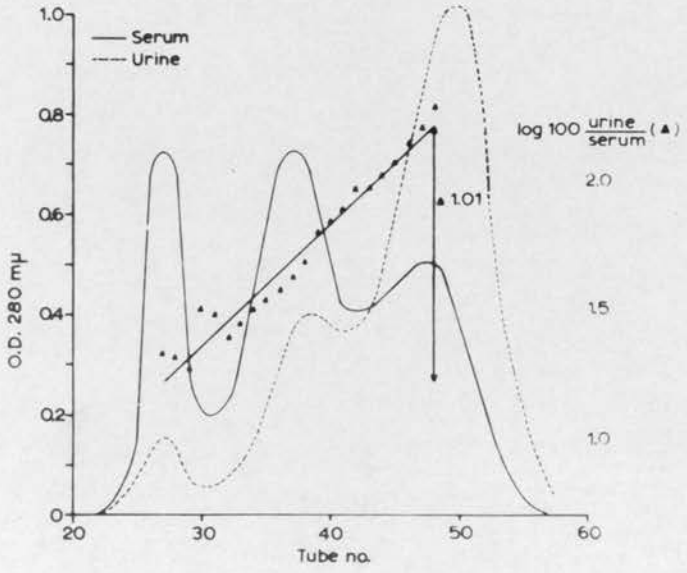

Fig. 2. Serum and urine elution patterns from a Sephadex G 200 column. The plot of $\log_{10} \frac{100 \text{ urine}}{\text{serum}}$ against the tube number is also shown. The proteinuria is unselective and the index of selectivity, Δ (total change in $\log_{10} \frac{100 \text{ urine}}{\text{serum}}$ between the first and third serum peaks) is low

tively more urine protein eluted in the first two peaks. The slope of the line relating $\log_{10} \frac{100\ U}{S}$ to tube number is much flatter and the index of selectivity was 1.01.

Possible sources of error and the accuracy of the method were investigated in the following ways.

(a) *Recovery of protein.* In five experiments recovery of added protein ranged from 101 to 105% with a mean of 102.6%.

(b) *Linear relationship between renal clearance of protein and tube number.* The plot of $\log_{10} \frac{100\ U}{S}$ against tube number consistently had highly significant correlation coefficients of between + 0.92 and + 0.99 with a mean from 19 plots of +0.974. Coefficients of variation for the points about this line averaged 7% with a range of 2% to 12%. In cases with proteinuria under 1 g/day the correlation coefficients tended to be lower, and in 5 plots ranged from + 0.77 to + 0.99 with a mean of + 0.910.

(c) *Reproducibility of the index of selectivity.* Five determinations of selectivity (Δ) were estimated on one serum and the corresponding urine. Five aliquots of serum and five aliquots of urine were passed through the column and these five pairs of separations were used in the calculations of Δ. Values ranged from 1.15 to 1.30, mean = 1.22, S.D. ± 0.07. The coefficient of variation was 5.8%.

(d) *Measurement of effluent proteins.* Selectivities calculated on the basis of measurement of protein at 280 mμ were compared with those calculated on the basis of measurement of protein by the modified Folin–Ciocalteau reaction. The results are shown in Table I. Measurement of the effluent protein by the Folin method tended to give consistently higher values of selectivity than those obtained when the effluent protein was estimated at 280 mμ. The mean overall difference, however, was 5.3%, an amount comparable to the reproducibility of the method.

(e) *Method of concentration of protein.* Selectivities estimated when the urine protein is concentrated by polyethylene glycol and by ultrafiltration are compared

TABLE I

COMPARISON OF VALUES OF SELECTIVITY (Δ)

Obtained when the urine is concentrated by polyethylene glycol (PEG) and ultrafiltration (UF) and when the effluent proteins are estimated by reading the optical density at 280 mμ or by a modified Folin–Ciocalteau reaction.

Patient	*PEG*			*UF*			*Mean PEG*	*Mean UF*	*% Difference (PEG–UF)*
	280 mμ	*Folin*	*% Difference 280 mμ–Folin*	*280 mμ*	*Folin*	*% Difference (280 mμ–Folin)*			
10	1.22	1.35	+ 10.7						
14	1.05	1.16	+ 10.5						
4	2.00	2.16	+ 8.0						
21	1.34	1.29	− 3.7						
19				0.99	1.15	+ 16.2			
31	0.80	0.86	+ 7.5	0.77	0.81	+ 5.2	0.83	0.79	− 4.8
21	1.05	1.16	+ 10.5	0.95	0.97	+ 2.1	1.10	0.96	− 12.7
9	1.34	1.35	+ 0.7	1.47	1.53	+ 4.1	1.35	1.50	+ 11.1
3	1.92	2.16	+ 12.5	2.07	2.27	+ 9.7	2.04	2.17	+ 6.4
5	2.08	2.04	− 1.9	2.31	2.07	− 10.4	2.06	2.19	+ 6.3
Mean	1.42	1.50	+ 6.1	1.43	1.47	+ 4.5	1.48	1.52	+ 1.3

in Table I. Although the use of polyethylene glycol to concentrate the urine protein could theoretically introduce small errors into the effluent urine protein values, this was not found to be significant. The mean difference in selectivity between the two concentration methods was only 1.3%.

(f) *Matching of serum and urine effluent protein patterns.* The elution pattern from a Sephadex G 200 column was found to change with time. There was, however, practically no change between successive runs. Since a urine concentrate was always run immediately after the corresponding serum, changes in column characteristics did not contribute appreciably to errors in the estimation of Δ. As a rule the tubes of serum eluate could be aligned with the corresponding tubes of urine eluate on the basis of tube number. Correct alignment was indicated by the fact that the first urine protein peak coincided with the first serum protein peak. Occasionally the first serum and urine peaks were found to be separated by one, or at the most, two tubes. The urine tubes were then renumbered to make the first urine peak coincide with the first serum peak. With patients who had very selective proteinuria, this adjustment could not easily be made as the amount of urinary protein in the early tubes was small and a definite first peak was not always detected. Because of this occasional difficulty in matching the effluent patterns, it seemed important to estimate the errors involved when selectivity was calculated from deliberately misplaced plots. Table II gives values of Δ over a range of selectivity calculated from plots with the serum and urine first peaks matching, and with the urine plot shifted by one tube and by two tubes. An alignment error of $\pm$ one tube varies the calculated Δ by a mean of 6%, which is of the order of magnitude of the overall error of the method; an error of two tubes varies it by a mean of 10%.

TABLE II

VALUES OF SELECTIVITY (Δ)

Calculated from elution patterns in which the 1st peak of serum and urine are at the same tube number compared with values calculated when the urine plot is shifted 1 or 2 tubes to the left. This shift tends to make the third peaks of serum and urine coincide. Δ is changed by similar amounts but to lower values when the urine plot is shifted to the right.

Peak I matching	*1 Tube moved*	*% Error*	*2 Tubes moved*	*% Error*
0.68	0.72	6	0.75	10
0.86	0.92	7	0.95	10
1.01	1.14	13	1.21	20
1.26	1.34	6	1.40	11
1.52	1.56	2.6	1.57	3.3
1.71	1.77	3.5	1.85	8.2
1.92	1.98	3.1	2.05	6.7

It should be noted that lining up serum and urine patterns on the basis of the third protein peak is not a sound procedure. Because of the relatively high clearance of small molecular weight protein, urine contains a relatively large proportion of proteins with a molecular weight less than that of albumin (60000) whereas these small molecular weight proteins constitute a very small proportion of the serum protein. The composition and average molecular weight of the proteins eluted in the third urine peak is therefore different from those of the third serum peak, and the urine peak is usually eluted two or three tubes after the serum peak.

TABLE III

VALUES OF SELECTIVITY ESTIMATED BY GEL FILTRATION (Δ) AND BY IMMUNODIFFUSION ($-K$) AND MEAN VALUES OF Δ AND $-K$ FOR EACH PATIENT

The number of estimations is given in brackets. The mean protein excretion is also given.

Patient	Δ	$-K$	*Mean* Δ	*Mean* $-K$	*Mean urinary protein g/24h*
1	2.51	2.95	2.51 (1)	3.20 (8)	1.6
2	2.29	2.46	2.24 (4)	2.68 (19)	2.8
	2.27	2.60			
	1.97	3.29			
3	2.11	2.92	2.11 (4)	2.68 (9)	15.4
4	2.08	2.58	2.08 (2)	2.54 (4)	4.0
5	1.95	2.86	2.01 (6)	3.03 (5)	24.0
	2.13	3.41			
6	1.95	2.27	1.95 (1)	2.43 (15)	12.1
7	1.85	3.03	1.80 (3)	2.67 (10)	1.3
	1.66	2.81			
	1.88	2.59			
8	1.80	2.37	1.80 (1)	2.50 (8)	11.3
9	1.92	1.88	1.74 (6)	1.96 (7)	19.6
	1.84	2.22			
	1.43	1.82			
10	1.71	1.94	1.71 (1)	1.84 (3)	1.1
11	1.64	2.24	1.64 (1)	2.21 (15)	4.0
12	1.64	1.55	1.64 (1)	1.74 (6)	7.4
13	1.50	1.69	1.50 (1)	2.02 (4)	10.0
14	1.11	1.69	1.29 (3)	1.62 (8)	10.3
	1.39	1.67			
15	1.29	2.38	1.29 (1)	2.15 (3)	5.5
16	1.28	1.56	1.28 (1)	1.45 (7)	13.2
17	1.22	1.16	1.22 (1)	1.47 (10)	8.8
18	1.20	1.55	1.20 (2)	1.67 (9)	6.1
19	1.20	2.39	1.20 (5)	2.11 (12)	16.0
	1.19	1.87			
20	1.16	1.79	1.16 (1)	1.77 (3)	2.2
21	1.33	1.47	1.14 (6)	1.49 (12)	6.9
	0.87	1.34			
22	0.98	1.47	1.12 (6)	1.70 (3)	3.1
	1.26	1.58			
23	1.10	1.82	1.10 (1)	1.67 (5)	3.6
24	1.07	1.93	1.06 (2)	1.74 (8)	1.6
	1.04	1.81			
25	1.02	1.94	1.02 (1)	1.69 (2)	5.0
26	1.02	1.56	1.02 (1)	1.75 (9)	14.1
27	1.01	1.67	1.01 (1)	1.74 (10)	7.0
28	0.95	1.48	0.98 (1)	1.30 (3)	2.3
29	0.89	1.29	0.89 (1)	1.28 (2)	9.1
30	0.89	1.17	0.89 (1)	1.55 (6)	6.6
31	0.86	1.36	0.82 (5)	1.38 (8)	10.8
	0.81	1.53			
32	0.73	0.88	0.69 (3)	1.04 (4)	3.6
	0.64	1.02			
33	0.40	0.82	0.50 (2)	0.58 (2)	3.4
	0.61	0.34			
34	1.70	2.17			0.4
	1.50	3.15			
10	1.29	3.40			0.6
	1.20	3.40			
35	1.10	2.68			0.8
36	0.90	2.43			0.8
	0.64	2.98			
37	0.78	2.15			0.5
38	0.64	2.98			0.5
39	0.54	1.60			0.5
40	0.47	1.01			0.6

(g) *Lipaemic serum.* Nephrotic sera tend to be lipaemic and occasionally the elution of large amounts of lipid into the early tubes of serum eluate gave a spuriously high first protein peak at 280 mμ which was due to turbidity. This difficulty could often be alleviated by obtaining fasting blood. When the serum eluate was turbid, however, reading the protein by the Folin Ciocalteau method rather than at 280 mμ gave more accurate values for the protein concentrations.

Correlation of selectivity by gel filtration and immunodiffusion

A total of 78 values of selectivity on 57 specimens were estimated on 40 patients with various forms of renal disease by both gel filtration and immunodiffusion. Table III shows values of Δ and $-K$ for each specimen, and also gives the mean value of Δ and mean value of $-K$ for each patient. Mean values for Δ and $-K$ were 1.4 and 1.9 respectively, with ranges for Δ of 2.51–0.47 and for $-K$ of 3.40–0.58. Fig. 3 shows the correlation between the two methods of measurement of selectivity for patients with proteinuria of over 1 g/day. Fig. 3a is plotted from the individual values of selectivity and Fig. 3b from the mean values of selectivity. The relationships between Δ and $-K$ for Fig. 3a and 3b are $\Delta = 0.65(-K) + 0.13$. and $\Delta = 0.76(-K) - 0.08$ respectively. The correlation coefficient, r, was + 0.857 ($p = < 0.001$) and the standard error of estimate of $\Delta = 0.26$ or 18.7% for Fig. 3a. For Fig. 3b the correlation coefficient was + 0.902 ($p = < 0.001$) and the standard error of estimate of $\Delta = 0.21$ or 14.7%. Correlation in each case is highly significant, although the scatter in Fig. 3a is slightly greater than in Fig. 3b.

Patients with less than 1 g/day of urinary protein have been analysed separately and form a quite different statistical population. The correlation is shown in Fig. 4. Values of Δ when compared to values of $-K$ are very much lower than for patients with proteinuria above 1 g/day, and the correlation between Δ and $-K$ is not significant statistically ($r = + 0.469$).

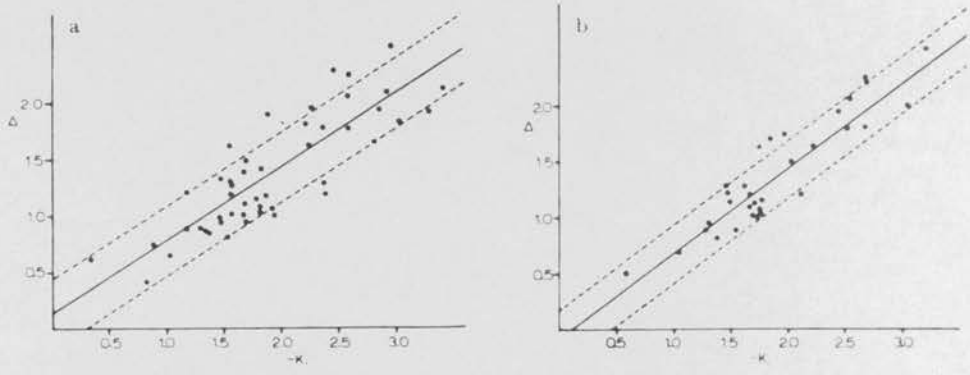

Fig. 3. Correlation of selectivity estimated by gel filtration (Δ) and immunodiffusion ($-K$) for patients with over 1 g/day of urinary protein.
(a) Correlation of individual values of selectivity. Δ and $-K$ are related by $\Delta = 0.65(-K) + 0.13$, correlation coefficient $r = + 0.857$ $p = < 0.001$ and the standard error of estimate of $\Delta = 0.26$ or 18.7%.
(b) Correlation of mean values of selectivity for each patient. Δ and $-K$ are related by $\Delta = 0.76(-K) - 0.08$, correlation coefficient $r = + 0.902$ $p = < 0.001$ and the standard error of estimate of $\Delta = 0.21$ or 14.7%.

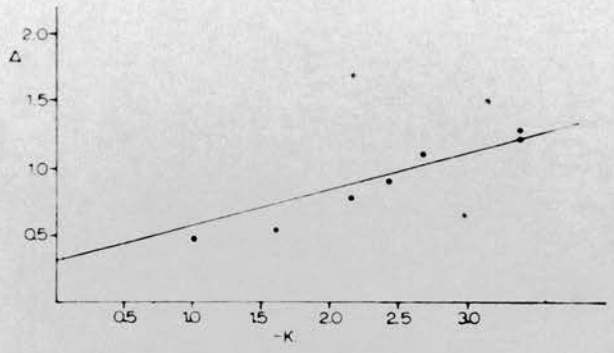

Fig. 4. Correlation of selectivity estimated by gel filtration (A) and immunodiffusion ($-K$) for patients with less than 1 g/day of urinary protein. The correlation coefficient r is not significant statistically ($r = +0.469$), and A is lower relative to $-K$ than for patients excreting over 1 g/day of protein.

DISCUSSION

Gel filtration of serum and urine on Sephadex G 200 has proved to be a valuable method for assessing the selectivity of proteinuria. The method is simpler than immunodiffusion and less expensive in terms of materials required. It is, however, more time-consuming and slightly less accurate. The technique and the errors involved have been investigated in this study.

Gel filtration and immunodiffusion are methods which differ considerably in principle. Both have inherent limitations. Selectivity by immunodiffusion is estimated from the relative clearances of five individual serum proteins. Although only proteins derived from the serum are measured, the method is unable to distinguish between protein fragments with antigenic activity and the parent protein molecules. Gel filtration on the other hand measures relative clearances of mixtures of proteins of known gradation in molecular size, but no distinction can be drawn between proteins of serum origin and proteins from the urinary tract. Hardwicke[6] described the use of gel filtration to assess renal permeability to protein in 10 patients. His results are not directly comparable with ours as he used the slope of the line relating $\log_{10} \frac{100\ U}{S}$ to tube number as his index of selectivity. Multiplication of the slopes by the average number of tubes used in his calculations gives comparable values for A ranging from 0.7–2.4. An attempt was made in his paper to correlate the relative clearances of individual proteins obtained by immunodiffusion with their relative clearances obtained by gel filtration. Hardwicke did not, however, indicate how individual protein clearances were determined using Sephadex G 200; it would seem that for the purposes of the calculations he assumed that each individual protein eluted at a single tube. The basis for his choice of tube number is not clear from his paper. Since any protein elutes from Sephadex over a number of tubes, and since any tube contains a mixture of proteins[12], the validity of gel filtration as a method for determining the clearance of an individual protein is dubious.

In this paper a comparison is made of overall selectivities by gel filtration and immunodiffusion in 40 patients. Although the units of A and $-K$ are not the

same, they are both based on the linear relationship between relative renal clearance of protein and molecular weight on a double log plot. The index of selectivity by immunodiffusion ($-K$) is the change in renal clearance of protein per unit change in molecular weight, whereas the index of selectivity by gel filtration (A) is the total change in renal clearance of protein over a fixed range of molecular weight. It is therefore valid to compare A and $-K$.

Hardwicke's series did not contain any patients excreting less than 250 mg % of protein. By concentrating the urine it has proved possible to determine selectivities on patients with urinary protein concentrations of down to 30 mg %. Gel filtration, however, is of limited value in the estimation of selectivity where the urine protein is under 1 g/day. This is most likely to be due to the presence of a relatively large contribution of urinary tract protein to the total urinary protein. Selectivity measurement by immunodiffusion is more reliable in cases of minor proteinuria, since this method only estimates protein derived from the serum.

Selectivity values measured by gel filtration and immunodiffusion correlate extremely well in patients excreting over 1 g protein/24 h, and the correlation is improved when mean values of both A and $-K$ from two or more determinations are taken. These results suggest that gel filtration constitutes a satisfactory alternative method for the determination of selectivity in patients with moderate or severe proteinuria.

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GLOMERULAR PERMEABILITY TO SERUM PROTEINS AND HIGH MOLECULAR WEIGHT DEXTRANS IN GLOMERULONEPHRITIS

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SUMMARY

1. Renal permeability to dextran of a molecular weight range approximating to that of the serum proteins has been studied in twenty-one patients with glomerulonephritis and significant proteinuria. Results are expressed in terms of dextran selectivity indices, which relate the clearance of dextran to its molecular weight.

2. The indices of dextran selectivity found were compared with similarly determined indices of protein selectivity.

3. The dextran and protein selectivity indices are found to correlate well in membranous glomerulonephritis and in minimal lesion glomerulonephritis ($r_{10} = + 0{\cdot}914$; $P<0{\cdot}001$) but the correlation is poor in proliferative glomerulonephritis ($r_{11} = -0{\cdot}145$).

4. It is concluded that the difference found in correlation with respect to protein and dextran selectivities between minimal lesion and membranous glomerulonephritis on the one hand, and proliferative glomerulonephritis on the other, reflects differences in the homogeneity of the renal lesion.

The assessment of glomerular permeability to serum proteins has become increasingly important in recent years in the evaluation of patients with the nephrotic syndrome (Blainey *et al.*, 1960; Joachim *et al.*, 1964; Cameron & White, 1965; Robson, 1967). When the glomerulus is permeable to proteins of predominantly low molecular weight (i.e. in selective proteinuria) minimal lesion glomerulonephritis is frequently present and the response to treatment with steroids is usually good. An unselective proteinuria, on the other hand, in which serum proteins of large molecular weight are found to a greater extent in the urine, tends to exclude the diagnosis of minimal lesion glomerulonephritis, and is almost always associated with a poor response to steroids (Cameron & White, 1965; MacLean & Robson, 1967).

It is generally believed that protein filtered by the glomerulus is reabsorbed by the renal tubules and appears in the urine when the tubular reabsorptive capacity is exceeded (Rather,

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1952). Hardwicke & Squire (1955) showed that when the albumin clearance is raised by the infusion of albumin in patients with the nephrotic syndrome, the clearance of other proteins rises also. On this basis they suggested that the reabsorption of protein is non-specific. It has since been assumed that in major degrees of proteinuria the concentrations of the urinary proteins relative to each other are the same as their relative concentrations in the glomerular filtrate. It is possible, however, that tubular reabsorption may modify the pattern of proteins which have been filtered by the glomerulus, in which case protein clearance studies would not accurately reflect glomerular permeability. Furthermore, protein could theoretically be added to post-glomerular fluid and obscure the glomerular pattern. Thus, in some respects, protein is not theoretically the ideal macromolecule for the assessment of glomerular permeability.

Brewer (1951) and Wallenius (1954) showed that the renal clearance of dextrans of molecular weight 4000–10 000 equals that of inulin. The clearances of dextrans of higher molecular weight are considerably less, and this is likely to be due to restricted glomerular permeability. Dextrans appear to be neither reabsorbed nor secreted by the tubules (Brewer, 1951; Wallenius, 1954) and are thus theoretically ideal substances for use in the study of glomerular permeability.

Brewer (1951) and Wallenius (1954) and Arturson & Wallenius (1964a, b) have used dextrans to assess the permeability of the normal glomerulus and have defined the relationship between renal clearance and molecular size over the molecular weight range 16 000–60 000. In this study glomerular permeability to dextran in subjects with three forms of glomerulonephritis has been investigated using dextrans of a rather higher range of molecular weight. The results have been compared with those obtained from similar studies using serum and urinary proteins as the macromolecule.

MATERIALS AND METHODS

Total urinary protein was measured by the biuret method of Hiller, Grief & Beckman (1948). AutoAnalyzer (Technicon) was used to estimate creatinine in serum and urine (Stevens *et al.*, 1962).

Protein selectivities

Protein selectivities were determined by immunodiffusion technique of Soothill (1952) as modified by MacLean & Robson (1966) and by gel filtration as described by MacLean & Petrie (1966). In our hands the estimation of selectivity using immunodiffusion has a coefficient of variation (CV) of 4%, and using gel filtration it has a CV of 7%. The indices of selectivity calculated from both methods are derived from the slope of the plot of the logarithm of relative renal clearance of protein against the logarithm of molecular weight, and when the methods are compared the results correlate well. The gel filtration selectivity index (Δ) is related to the immunodiffusion selectivity index ($-K$) by the formula $\Delta = 0{\cdot}76\,(-K) - 0{\cdot}08$ (MacLean & Petrie, 1966).

Dextran selectivities

Dextran selectivities were estimated by a method which, in principle, was the same as that used in determining protein selectivities by gel filtration. Two parts 'Dextraven' (Benger Laboratories) or 'Intradex' (Glaxo Laboratories) to one part 'Rheomacrodex' (Pharmacia) or 'Intraflodex' (Glaxo Laboratories) provided a dextran mixture with a molecular weight

range covering that of the serum proteins as determined by gel filtration (Fig. 1). In four pilot studies 22–33 g of dextran in 350–500 ml of saline were infused intravenously. Carefully timed urine specimens, collected by catheterization, were obtained, and blood specimens were taken at the beginning and the end of each collection period.

Since these studies showed that the disappearance from the plasma of dextrans of the molecular weight range under study was slow, the technique was simplified in further experiments by giving a single injection of 4–11 g dextran. Urine was then collected over a 20–30-min period without catheterization, and a sample of the injected dextran mixture was used in place of blood specimens to determine selectivity. This simplified procedure allowed a smaller dose of dextran to be given and so minimized any error in selectivity determination which might have been caused by plasma expansion. Since relative dextran clearances, rather than absolute clearances, were being calculated an accurately timed collection period was not essential.

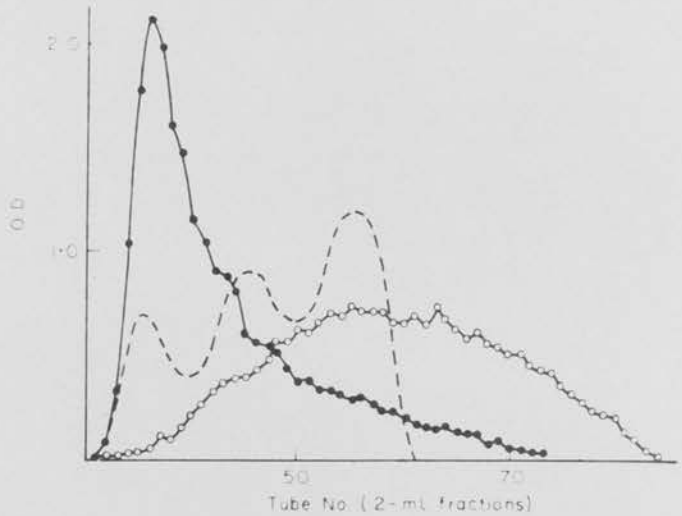


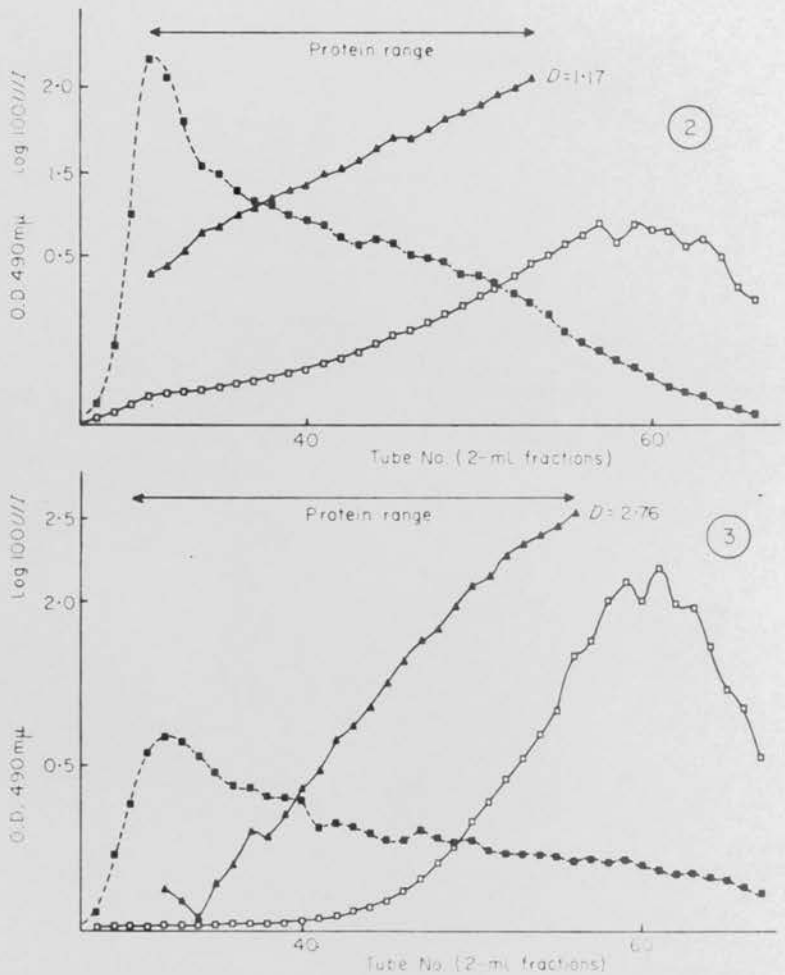
FIG. 1. Elution patterns of dextran and proteins on Sephadex G-200. Optical density (directly proportional to concentration) is plotted against tube number (inversely proportional to the logarithm of molecular size). 'Dextraven', ●; 'Rheomacrodex', ○; normal serum protein, - - - -.

Samples containing 5–10 mg of dextran were applied to Sephadex G-200 columns of 2 × 100 cm and 2 × 60 cm. These were run at pressure heads of 5–15 cm using 0·1 M-Tris buffer, pH 8·0, in 0·1 M-saline, as the eluting fluid and samples of 2 or 3 ml were collected. After every second or third dextran run a normal serum was fractionated on the column in order to check the protein separation, since calculations of dextran selectivity were made over the molecular weight range of serum protein.

Dextran in the column effluents was estimated by the method of Dubois *et al.* (1956). This method estimates total carbohydrate but small carbohydrate molecules (e.g. glucose) were eliminated by gel filtration, and protein-bound carbohydrate was removed by the use of trichloracetic acid. Hydrolysis of dextran by trichloracetic acid was not found to occur. A calibration curve for the determination of dextran was linear over the range 0–20 mg/100 ml

and the CV for the dextran estimations was 1·5%. When necessary, concentration of the sample of urine was carried out prior to gel filtration by dialysis against polyethylene glycol (Howe, Groom & Carter, 1964).

It was found that several factors could produce spuriously high values for dextran in the



FIGS. 2 and 3. The elution patterns for the injection mixture (I) and for the urinary dextrans in patients Nos. 10 and 12, respectively (Table 2). The plot of $\log_{10} 100\ U/I$ against tube number is also shown. ■, Injection mixture (I); , urine dextran (U); ▲, $\log_{10} 100\ U/I$. In Fig. 2, unselective dextranuria is shown. D (the dextran index of selectivity) is 1·17. Fig. 3 illustrates selective dextranuria. D is 2·76.

column effluent. A faint trace of Sephadex washing from the column into the collecting tubes gave large errors. Bacterial contamination of the buffer, tubes which were not scrupulously clean and tubes which had been washed in acetone also gave high readings. Care was taken to eliminate these sources of error, and a constant check was kept on the column blank and

buffer blank. In order to eliminate interference from small molecular weight sugars, the column was washed with at least two elution volumes of buffer between each run.

The ratios of urine–serum or of urine–injection mixture were calculated for dextran for each elution tube between the first and third protein peaks.

The logarithm of

$$\frac{100 \times \text{Urine dextran concentration}}{\text{Serum or injection mixture dextran concentration}}$$

($\log_{10} 100U/S$ or I), was plotted against tube number, and a linear relationship was obtained. The dextran index of selectivity (D) was taken as the total change in $\log_{10} 100U/S$ or I between the first and third protein peaks. This index, D, is arithmetically directly comparable to the protein selectivity index Δ (MacLean & Petrie, 1966). Figs. 2 and 3 show typical serum and urine elution patterns, the linear relationship between $\log_{10} 100U/S$ or I and tube number, and values of D, in selective and unselective dextranuria.

TABLE 1. The use of injection mixture and of various sera in the determination of dextran selectivity

D (using $(A+B)/2$)	D (using A only)	% error	D (using B only)	% error	D (using I injection mixture)	% error
2·91	2·89	−0·7	2·97	+2·1	–	–
1·82	1·77	−2·7	1·87	+2·7	1·77	−2·7
1·75	1·77	+1·1	1·71	−2·3	–	–
1·12	1·10	−1·8	1·14	+1·8	0·97	−13·0*
2·46	–	–	–	–	2·50	+1·6
2·58	2·55	−1·3	2·63	+1·9	2·51	−3·0
2·36	–	–	–	–	2·28	−3·4

D is the dextran selectivity; A is the serum taken at the beginning of the urine collection period; B is the serum taken at the end of the collection period; I is 'injection mixture'.

* Error partly explained by slowness of dextran infusion.

Reproducibility and limitations of the method

A series of twelve duplicate determinations of dextran selectivity (D) gave a CV of 9·1%. Since the variation was greater than that obtained for the comparable protein index (Δ), most of the dextran selectivities were determined in duplicate and the average value taken.

Correct tube alignment is essential for accurate determinations of selectivity by gel filtration (MacLean & Petrie, 1966). Despite the precaution taken in these experiments of frequent protein 'marker' runs, it is possible that an error of alignment of one or two tubes may have occurred in some of the experiments. The magnitude of this possible error was assessed by calculating the effect of deliberate tube misalignment. It was found that a shift to the right of the urine plot of two tubes introduced an error of bias in selectivity of +8·7%, while a shift of two tubes to the left introduced one of −4·0%.

A satisfactory linear relationship was found for the log–log plots of relative dextran clearance against molecular weight over the protein molecular weight range. In ten experiments the correlation coefficients of this relationship were found to be highly significant, ranging from +0·954 to +0·996, with a mean of +0·983.

The pilot studies enabled a comparison to be made of values of selectivity which were calculated from a serum taken at the beginning and the end of the collection period and on the basis of the mixture of dextrans injected. Results are shown in Table 1. The errors introduced are not appreciable and in particular the injection mixture had no significant effect on the index of selectivity when used in place of the serum.

There is no change in molecular composition of dextran after 5 years when stored at 4° (Maycock & Rickets, 1961). However, a dextran splitting enzyme has been found in animal tissues, including the kidney (Rosenfeld & LuKomskaya, 1957). The stability of the dextran in urine was, therefore, tested. A significant loss of dextran was found after 8 weeks storage at 4 . This was not noted at 4 weeks storage at this temperature. Because of the uncertain stability of dextran in the urine, the dextran selectivity was always determined within 2 weeks of collecting the specimens.

RESULTS

Indices of dextran selectivity were determined on twenty-one patients with significant proteinuria suffering from several different types of renal disease.

Variation in dextran selectivity

Results of the four pilot experiments are shown in Table 3. Values for dextran selectivity showed a tendency to rise after the infusion, but the mean change in the four subjects, over all the collection periods, was +8·2%, a value within the reproducibility of the method. No significant difference in protein selectivity was detected.

Values of dextran and protein indices of selectivity

Values of dextran and protein selectivity are shown in Table 2, which also gives the total urinary protein, creatinine clearance, and diagnosis based on renal biopsy. Values of dextran selectivity (D) ranged from 2·87 to 1·17, mean 1·77, while those of protein selectivity determined by gel filtration (Δ) were generally lower ranging from 2·20 to 0·75, with a mean of 1·32. This difference is statistically significant ($t = 3{\cdot}23$, $P < 0{\cdot}0025$). Values of protein selectivity determined by immunodiffusion ($-K$) which are not directly comparable arithmetically with those of Δ and D ranged from 3·53 to 1·28, with a mean of 1·97. Using the relationship $\Delta = 0{\cdot}76(-K) - 0{\cdot}08$, this mean value for immunodiffusion remains significantly different from D.

Correlation of dextran and protein indices of selectivity

Dextran and protein selectivity values for the twenty-one patients studied are compared in Figs. 4 and 5. The overall correlations between D and $-K$ and D and Δ are poor with correlation coefficients $r_{21} = +0{\cdot}201$ and +0·144, respectively. However, when patients with proliferative glomerulonephritis are excluded from the series, a high degree of correlation between dextran and protein values of selectivity is found (Figs. 6 and 7). The correlation

TABLE 2

Subject	Diagnosis	Protein excretion (g/24 hr)	Creatinine clearance (ml/min)	D	$-K$	Δ
1	Minimal lesion glomerulonephritis	4·8	122	2·18	3·53	2·20
2		14·9	97	1·95	2·68	2·11
3		10·0	32	1·86	2·60	1·44
4		4·8	110	1·83	3·08	2·07
5	Membranous glomerulonephritis	6·6	38	1·53	1·73	1·02
6		9·1	101	1·52	1·77	1·23
7		0·7	102	1·51	2·15	1·56
8		1·7	12	1·35	1·26	0·88
9		8·1	73	1·24	1·86	1·20
10		9·0	7	1·17	1·45	0·75
11	Proliferative glomerulonephritis	1·5	105	2·87	1·38	1·01
12		0·9	90	2·78	1·96	0·91
13		4·4	9	2·05	1·53	0·89
14		4·5	77	2·03	1·73	1·38
15		6·0	128	1·90	1·77	1·64
16		6·8	168	1·81	2·05	1·34
17		11·7	66	1·75	1·42	1·02
18		13·4	33	1·60	2·42	1·88
19		6·8	99	1·59	1·61	1·07
20		7·1	27	1·45	2·16	1·32
21		8·4	53	1·27	1·28	0·89

D is the dextran selectivity, determined by gel filtration; Δ is the protein selectivity, determined by gel filtration; $-K$ is the protein selectivity, determined by immunodiffusion.

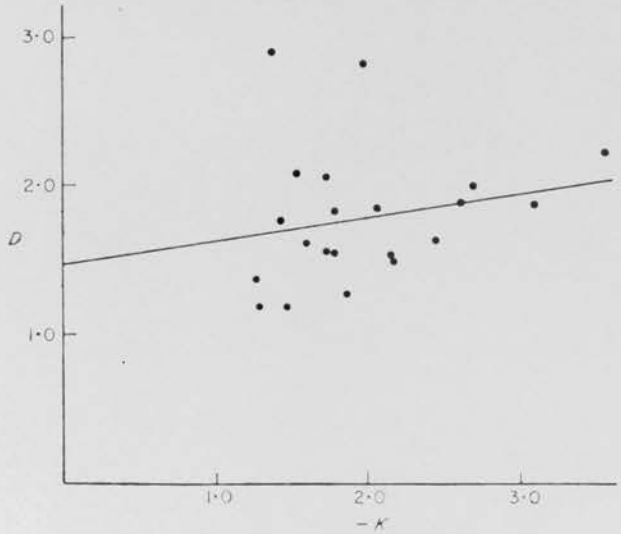


FIG. 4

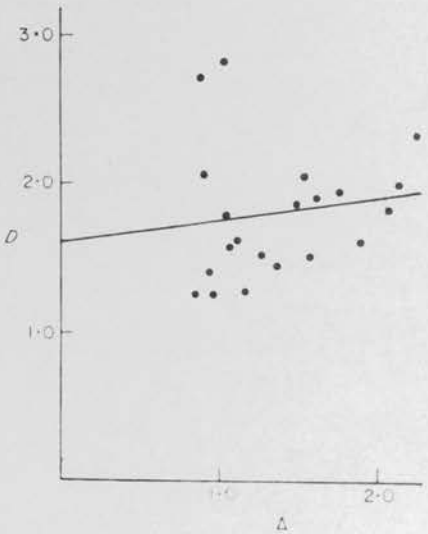


FIG. 5

FIGS. 4 and 5. Dextran selectivity plotted against protein selectivity. All the patients studied are included. In Fig. 4, the protein selectivity is determined by immunodiffusion, and is expressed by the symbol $(-K)$. Dextran selectivity $(D) = 0{\cdot}15(-K) + 1{\cdot}47$; $r_{21} = +0{\cdot}201$. In Fig. 5, the protein selectivity is determined by gel filtration, and is expressed by the symbol (Δ). $D = 0{\cdot}15\Delta + 1{\cdot}57$; $r_{21} = +0{\cdot}144$.

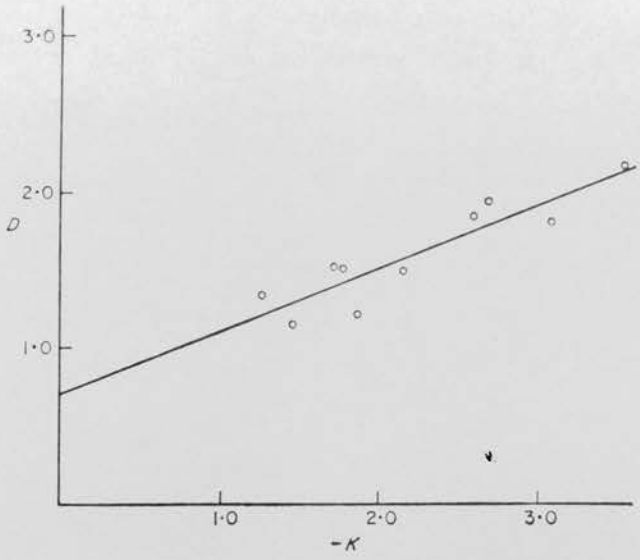


FIG. 6

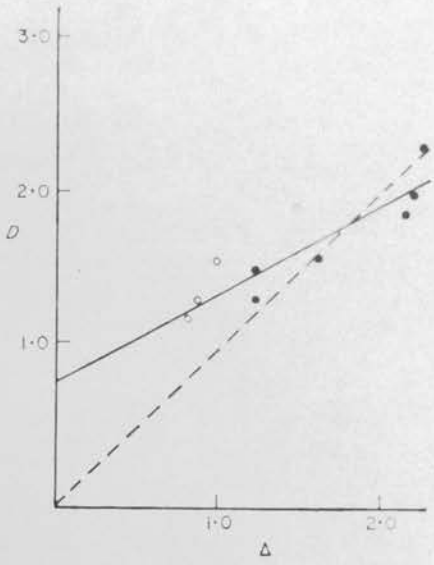


FIG. 7

FIGS. 6 and 7. Dextran selectivity (D) plotted against protein selectivity. Patients with proliferative glomerulonephritis are excluded, and the relationship is for patients with minimal lesion glomerulonephritis and membranous glomerulonephritis. In Fig. 6, protein selectivity ($-K$) is determined by immunodiffusion. $D = 0{\cdot}41(-K)+0{\cdot}71$; $r_{10} = +0{\cdot}914$; $P < 0{\cdot}001$. In Fig. 7, protein selectivity (Δ) is determined by gel filtration. ●, Patients with creatinine clearances over 70 ml/min; ○, patients with creatinine clearances less than 70 ml/min; - - - -, $D = \Delta$; ———, $D = 0{\cdot}55\Delta + 0{\cdot}82$; $r_{10} = +0{\cdot}882$; $P < 0{\cdot}001$.

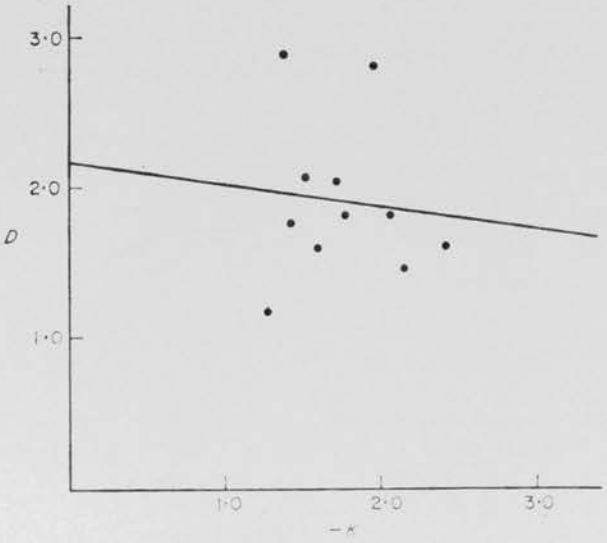


FIG. 8

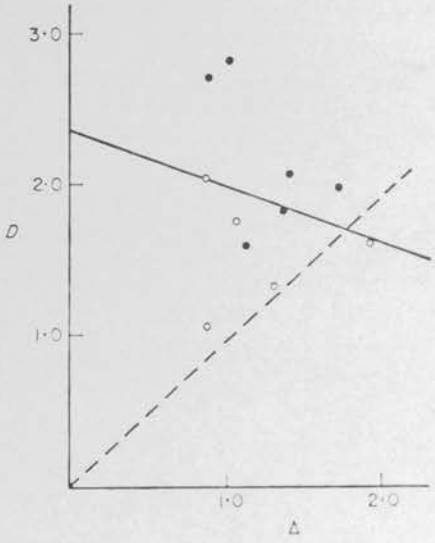


FIG. 9

FIGS. 8 and 9. Dextran selectivity (D) plotted against protein selectivity. Only patients with proliferative glomerulonephritis are included. In Fig. 8, protein selectivity ($-K$) is determined by immunodiffusion. $D = -0{\cdot}45(-K) + 2{\cdot}16$; $r_{11} = -0{\cdot}145$. In Fig. 9, protein selectivity (Δ) is determined by gel filtration. ●, Patients with creatinine clearances over 70 ml/min; ○, patients with creatinine clearances less than 70 ml/min; - - - -, $D = \Delta$; ———, $D = -0{\cdot}41\Delta + 2{\cdot}40$; $r_{11} = -0{\cdot}408$.

coefficients within this group of patients with minimal lesion glomerulonephritis or membranous glomerulonephritis were $r_{10} = +0{\cdot}914$, $P<0{\cdot}001$, and $r_{10} = +0{\cdot}882$, $P<0{\cdot}001$ for D and $-K$, and D and Δ, respectively. Dextran and protein indices were related by the equations:

$$D = 0{\cdot}41(-K)+0{\cdot}71 \quad (1)$$

$$D = 0{\cdot}55\Delta+0{\cdot}82 \quad (2)$$

TABLE 3. Pilot studies using i.v. infusions of 22–33 g of dextran

Subject	Samples of blood used in calculation of	Duration of urine collection period†	Δ	Notes
1	*A* (start) and *B* (end) of first collection period	3 hr	2·91	
	B only	5 min	2·61	
	B (start) and *C* (end) of third collection period	40 min	2·81	
2	*A* (start) and *B* (end) of first collection period	40 min	1·82	
	B (start) and *C* (end) of second collection period	50 min	2·13	
	C (start) and *D* (end) of third collection period	60 min	2·11	
3	*A* (start) and *B* (end) of first collection period	20 min	1·75	$-K$ protein before dextran infusion = 2·48
	B (start) and *C* (end) of second collection period	30 min	1·84	$-K$ protein after dextran infusion = 2·61
	C (start) and *D* (end) of third collection period	20 min	2·0	
4	*A* (start) and *B* (end) of first collection period	45 min	1·12	$-K$ protein before dextran infusion = 1·42
	B (start) and *C* (end) of second collection period	45 min	1·36	$-K$ protein after dextran infusion = 1·34
	C (start) and *D* (end) of third collection period	16½ hr*	1·30	

* This length of collection period only possible since patient had GFR of less than 10 ml/min.
† Urine collections in each patient obtained successively.

Figs. 8 and 9 show the correlation between dextran and protein indices in proliferative glomerulonephritis. The correlation coefficients were not significant with $r_{11} = -0{\cdot}145$ and $r_{11} = -0{\cdot}408$ for D and $-K$, and D and Δ, respectively.

DISCUSSION

The most striking feature of these experiments is the contrast between the poor correlation of values obtained for dextran and protein selectivity in patients with proliferative glomerulonephritis on the one hand, and the high degree of correlation in patients with minimal lesion glomerulonephritis or membranous glomerulonephritis on the other.

Indices of selectivity are affected only by the nephrons which contribute macromolecules to the urine. Since dextran is not reabsorbed by the renal tubules, the dextran in the urine represents the contribution from all the functioning glomeruli. Proteins, on the other hand, are reabsorbed by the tubules and the protein in the filtrate from a normal glomerulus is almost entirely reabsorbed (Dirks, Clapp & Berliner, 1964). Only the protein from functionally abnormal glomeruli is sufficient to saturate the tubular reabsorptive mechanism. Thus protein in the urine represents the contribution from abnormal glomeruli only. It follows that dextran selectivity values will reflect the function of all the glomeruli, while those of protein will reflect only the function of the abnormal glomeruli.

If the renal lesion is uniform and all the glomeruli are similarly affected then theoretically values for protein and dextran selectivity should agree. If the glomerular lesion is patchy, however, with some glomeruli more affected than others, it is likely that their permeability will also vary. In this event dextran selectivity will reflect the permeability of all functioning glomeruli and an overall picture of glomerular permeability will be obtained. As far as the proteins are concerned, tubular reabsorption will magnify the influence on selectivity of the glomeruli contributing the greater amount of protein and will eliminate the contribution of the glomeruli filtering protein below the tubular reabsorptive maximum. Thus, values of protein selectivity will disproportionately represent the permeability of the more abnormal glomeruli. Since the selectivity of the normal glomerulus is high (Wallenius, 1954; Arturson & Wallenius, 1964b; Hulme & Hardwicke, 1966) values of protein selectivity will therefore tend to be lower than those of dextran selectivity when the renal lesion is not uniform. As Fig. 9 shows, this was found to be the case in patients with proliferative glomerulonephritis, where the values obtained for D were found (with one exception) to lie above the line $D = \Delta$. In this condition the severity of the glomerular lesion is typically patchy.

In minimal lesion and membranous glomerulonephritis, however, the renal lesion is more homogenous and a much better correlation between protein and dextran selectivity values was found. The correlation appears even better when the four patients with low creatinine clearances in this group are excluded. These patients (open circles, Fig. 7) all have values for D lying above the line $D = \Delta$. For the remaining six patients the values obtained fit the line $D = \Delta$ fairly well, but in order to establish this statistically the number of patients with minimal and membranous glomerulonephritis would need to be increased.

Hulme & Hardwicke (1966) have studied glomerular permeability to labelled polyvinyl pyrollidone (PVP). The molecular weight range was lower than that of the serum proteins but it is of interest that in six out of seven cases their PVP selectivity was higher than the protein selectivity.

While it appears likely that our results are best explained in terms of heterogeneity of the nephron population, there are several other theoretical possibilities which need discussion:

(1) After injection, dextran is lost from the circulation, the smallest molecule leaving most

rapidly (Arthurson & Wallenius, 1964a). The pilot studies, however, showed this to be a relatively slow process for large molecules having an insignificant effect on the present study.

(2) Dextran *in vivo* has been reported to undergo chemical changes (Cargill & Bruner, 1951). However, the work of Terry *et al.* (1953) and Gray (1953) has indicated that this occurs only after dextran has been taken up by the tissues. Over the short time intervals of the present experiments this effect is unlikely to be significant.

(3) Dextran itself might affect renal function. Tubular changes following dextran infusion have been described (Zettergren, 1962; Maunsbach, Madden & Latta, 1962), but there are no significant glomerular changes (James & Ashworth, 1961). Rises in glomerular filtration rate have been reported by Klutsch, Heidland & Kammerer (1965), and it has been suggested that glomerular permeability is increased (James, Gordeilo & Metcalf, 1956; Malmendier, de Koster & Lambert, 1960). Such changes, however, are only detectable after considerable plasma expansion. The constancy of protein selectivity before and during the test period (Table 3) argues against substantial changes in glomerular permeability occurring in these experiments.

(4) Dextran and protein may behave differently on gel filtration. Sephadex G-200 has been widely used to determine molecular weights of proteins and, in general, results are in good agreement with data obtained by ultracentrifugation (Ackers, 1964; Squire, 1964; Andrews, 1965). Although dextran is separated on a molecular weight basis by gel filtration (Granath & Flodin, 1962) there may be a difference between the behaviour of dextran molecules and protein molecules on Sephadex G-200. Carbohydrate-containing proteins and elongated molecules do not conform precisely to the relationship of elution volume and molecular weight (Andrews, 1965), such molecules as a rule being eluted earlier than expected. In addition to its carbohydrate composition dextran has a high axial ratio. However, since there is no break in the linearity of the plot of log U/S against tube number over the whole molecular weight range studied for both dextran and protein, this particular problem of a possible small difference in the absolute molecular weight ranges studied should not materially affect the results.

Even if some of the above theoretical considerations are relevant, it is difficult to see how they would provide an explanation for the negligible correlation between dextran and protein selectivities in one group of diseases, and the highly significant correlation in two other groups. The difference between the results for proliferative glomerulonephritis and the results for minimal and membranous glomerulonephritis is therefore more likely to be due to characteristics of the kidneys of the two groups.

ACKNOWLEDGMENT

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GLOMERULAR PERMEABILITY TO HIGH MOLECULAR WEIGHT DEXTRANS IN ACUTE ISCHAEMIC RENAL FAILURE AND POSTURAL PROTEINURIA

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SUMMARY

1. Renal permeability to dextran of a molecular weight range approximating to that of the plasma proteins has been studied in six patients with acute ischaemic renal failure, four patients with postural proteinuria and six healthy subjects.

2. Results are expressed in terms of dextran selectivity indices which relate the clearance of dextran to its molecular weight. Indices of dextran selectivity were found to be high in acute ischaemic renal failure, postural proteinuria and in normal subjects. Comparable indices of plasma protein selectivity in these groups were low.

3. It is suggested that in postural proteinuria and acute ischaemic renal failure the proteinuria is not glomerular in origin, and that in these conditions macromolecules are filtered quite normally and urinary protein arises from a post glomerular source characterized by a lack of selectivity.

The renal clearance of macromolecules has been studied by several workers in order to assess glomerular permeability in health and disease. Plasma proteins have been widely used for this purpose, results being expressed as indices of selectivity (Blainey *et al.*, 1960; Joachim *et al.*, 1964; Robson, 1968). Proteins are not ideal macromolecules with which to study glomerular permeability since they are reabsorbed by the renal tubules. This is particularly relevant in the investigation of normal glomerular function and in minor degrees of proteinuria, when almost all the protein filtered by the glomeruli is reabsorbed and only small amounts escape into the urine (Dirks, Clapp & Berliner, 1964).

Dextran is a more suitable macromolecule for this purpose, since virtually all the dextran filtered at the glomerulus appears in the urine and no demonstrable amount is reabsorbed by the tubules (Brewer, 1951; Wallenius, 1954). Normal glomerular permeability to dextran has been studied by Arturson & Wallenius (1964a, b) and the relationship between renal clearance and molecular weight over the range 16 000–60 000 has been defined. Petrie, MacLean & Robson (1968) also studied the glomerular permeability to higher molecular weight

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dextran in patients with different forms of glomerulonephritis and compared the results with protein studies carried out simultaneously. In proliferative glomerulonephritis, dextran selectivity studies gave results in marked disagreement with those of protein; on the other hand, in minimal lesion and membranous glomerulonephritis the selectivity results using dextran and protein were substantially the same. These findings are attributed to differences in the degree of uniformity of the glomerular lesions in the two groups of patients.

The protein clearance patterns in patients with acute ischaemic renal failure and in postural proteinuria are unselective (MacLean & Robson, 1966). The lack of selectivity is surprising in view of the almost normal glomerular appearance reported by most authors (Dalgaard & Pederson, 1961; Ruckley *et al.*, 1966). In view of this, glomerular permeability to dextran and plasma proteins of comparable molecular size has been compared in patients with acute renal failure and postural proteinuria. The glomerular permeability to dextran was also studied in healthy subjects.

METHODS

Total urinary protein was measured by the biuret method of Hiller, Grief & Beckman (1948) and by a modification of the microbiuret method of Itzhaki & Gill (1964). An AutoAnalyzer (Technicon) was used to estimate creatinine in serum and urine (Stevens *et al.*, 1962).

Protein selectivities were determined by the immunodiffusion technique of Soothill (1962) as modified by MacLean & Robson (1967). The index of protein selectivity by this method is denoted by $-k$.

Dextran selectivities were determined as described by Petrie *et al.* (1968). The method is similar in principle to that used to determine protein selectivity by gel filtration (MacLean & Petrie, 1966), but the coefficient of variation for the dextran method is higher (9%) and most of the estimations were therefore performed in duplicate and the average taken. The index of dextran selectivity (D) represents the change in renal clearance of dextran, calculated over a fixed range of molecular size, and is arithmetically and methodologically comparable to the index of protein selectivity (Δ) when determined by gel filtration. Since Δ is related to $-k$ by the equation $\Delta = 0{\cdot}76\,(-k) - 0{\cdot}08$ (MacLean & Petrie, 1966) then the indices of D and $-k$ should be compared according to the equation $D = 0{\cdot}76\,(-k) - 0{\cdot}08$. High values for indices of selectivity indicate that the glomerulus is filtering macromolecules in a selective fashion and allowing very few large molecules to escape into the urine. Conversely low values for indices of selectivity indicate that the glomerulus is allowing a higher proportion of large molecules to escape into the urine.

The diagnosis of acute ischaemic renal failure and of postural proteinuria was based on appropriate clinical findings and light and ultrastructural appearances of renal biopsies (Ruckley *et al.*, 1966). Patients with postural proteinuria were shown to have proteinuria which was intermittent and related to posture.

RESULTS

Table 1 shows indices of dextran (D) and protein ($-k$) selectivity in six patients with acute ischaemic renal failure, at the beginning of the diuretic phase, in four patients with postural proteinuria, and in six healthy subjects. Fig. 1 shows these results and, for comparison, dextran

and protein indices of selectivity in minimal lesion and membranous glomerulonephritis (Petrie *et al.*, 1968). Table 1 also includes values for total 24 hr urine protein, creatinine clearance, and serum:urine albumin concentration ratio. Values for D were high, ranging from 1·99 to 3·28 (mean 2·41) for patients with acute ischaemic renal failure, from 2·38 to 3·47 (mean 3·08) for patients with postural proteinuria and from 2·38 to 3·65 (mean 2·91) for normal subjects. In contrast, the values for protein selectivity in patients with acute ischaemic renal failure were low, ranging from 0·25 to 1·40, mean 0·84. These patients all had low creatinine clearances, and the total protein excretion was over 200 mg/24 hr. The albumin serum:urine concentration

TABLE 1

Subject	D	$-k$	Alb S:U	Creatinine clearance (ml/min)	Total urine protein (mg/24 hr)
Acute ischaemic renal failure					
1	1·99	0·77	96	8	600
2	2·02	—	—	41	—
3	2·10	—	—	22	—
4	2·59	0·93	256	13	600
5	2·65	0·25	448	10	800
6	3·28	1·40	224	4	200
Postural proteinuria					
7	2·38	1·05	180	62	600
8	3·47	1·41	330	—	—
9	3·26	1·22	370	134	900
10	3·22	1·18	220	95	500
Normal subjects					
11	2·38	—	—	93	14
12	2·50	—	—	40	25
13	2·76	—	—	37*	87
14	2·98	—	—	104	21
15	3·19	—	—	100	45
16	3·65	—	—	92	15

D = index of dextran selectivity; $-k$ = index of protein selectivity by immunodiffusion; Alb S:U = albumin serum:urine concentration ratio. Values for $-k$ in the four patients with acute ischaemic renal failure are similar to those previously obtained (MacLean & Robson, 1966).

* Subject 13 had one kidney.

ratio was also abnormal, the upper limit in normal subjects being 1600 (MacLean & Robson, 1966). The values for protein selectivity in postural proteinuria were also low, ranging from 1·05 to 1·41 (mean 1·22) and the serum:urine albumin concentration ratio was abnormal.

DISCUSSION

The results show that the glomerular permeability to dextran in acute ischaemic renal failure, postural proteinuria and normal subjects is highly selective. The normal glomerulus has

previously been suggested to be highly selective (Hulme & Hardwicke, 1966), in spite of the unselective pattern of protein excretion (Rowe & Soothill, 1961; MacLean & Robson, 1966). Similarly this study shows that in acute ischaemic renal failure and postural proteinuria a highly selective dextranuria accompanies an unselective proteinuria.

The methods used to determine protein and dextran selectivity are different in principle.

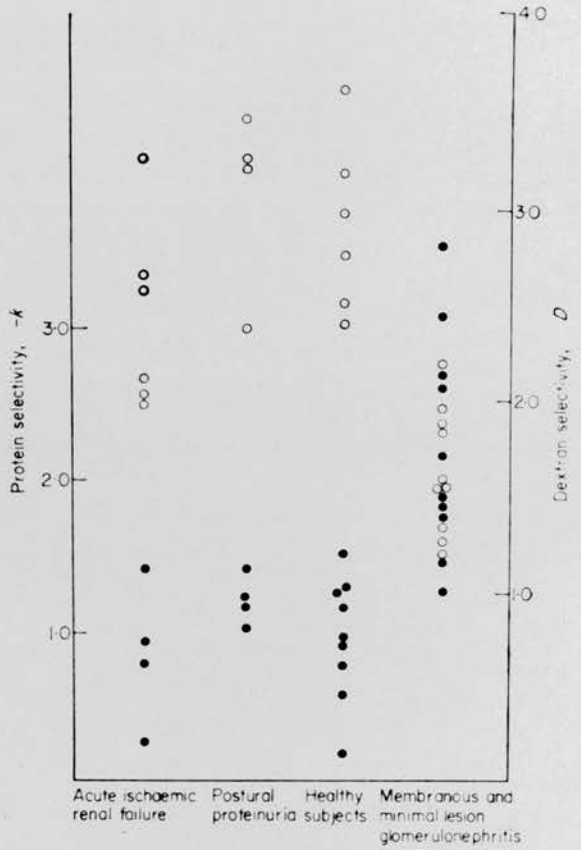

FIG. 1. Dextran (○) and protein (●) selectivity, denoted by $-k$ (left ordinate) and D (right ordinate) respectively. The scales for $-k$ and D differ according to the relationship $D = 0{\cdot}76(-k) - 0{\cdot}08$. In acute ischaemic renal failure and postural proteinuria (two left-hand columns) there is wide disparity between the dextran and protein values for selectivity. By contrast in minimal lesion and membranous glomerulo-nephritis (extreme right-hand column) values for dextran and protein selectivity are comparable (Petrie *et al.*, 1968). Values obtained in healthy subjects for dextran (from Table 1) and for protein (from MacLean & Robson, 1966) are also shown.

In proteinuria greater than 1 g/day, results of gel filtration and immunodiffusion correlate well (MacLean & Petrie, 1966). In proteinuria of less than 1 g/day the gel filtration method is a doubtful measure of glomerular selectivity because urinary tract protein is present in the urine in a relatively greater amount. However, the immunodiffusion method appears to be reliable at lower protein concentrations of 0·2–1 g/day. In postural proteinuria

and acute ischaemic renal failure values of selectivity are constant in the face of wide variations in protein excretion (Ruckley *et al.*, 1966; MacLean & Robson, 1966). Although it is theoretically possible that degraded protein in the urine will react antigenically and influence the value of selectivity, studies of urinary proteins show in general that the molecular weights are similar to those of the serum proteins (Gitlin & Janeway, 1952; Neale, 1955; Hardwicke & St. Cyr, 1961). Although small molecular weight immunoglobulins can be detected in the urine there is evidence that they are derived by plasma clearance rather than by degradation (Fagelman, McGhee & Chaplin, 1966).

Protein and dextran differ considerably in molecular configuration. Dextran has the more expanded structure, and the effective molecular radius for dextran of molecular weight 70 000 is substantially larger than that for a globular protein of the same molecular weight (Laurent & Granath, 1967; Anderson & Stoddart, 1967). This fact results in dextrans being eluted earlier from a gel filtration column than proteins of the same molecular weight, and, compared to protein, leads to a difference between the lines relating clearance to molecular size. This shift makes only a small difference in the range of molecular size used for calculating selectivity and does not influence the slope of the line. Since indices of selectivity are a function of this slope, and do not depend on precise measurements of molecular weights, comparisons between dextran and protein selectivities are valid.

In discussing the mechanism of dextran and protein excretion in glomerulonephritis, Petrie *et al.* (1968) concluded that the differences in dextran and protein selectivity in proliferative glomerulonephritis resulted from different tubular treatment of these macromolecules by different nephrons. Since dextran is not reabsorbed by the renal tubules the urinary dextran reflects the behaviour of every functioning nephron, whereas estimates of selectivity based on protein can reflect only the behaviour of glomeruli which are associated with tubules where the reabsorptive capacity is saturated. It follows that when the glomerular lesions are uniform throughout all nephrons, protein and dextran selectivities would be expected to agree as, e.g., in minimal lesion and membranous glomerulonephritis (Fig. 1), whereas when the glomerular lesion is patchy, values for dextran selectivity will be higher than those of protein, assuming of course that the normal glomerulus is highly selective.

However, in acute ischaemic renal failure this mechanism is unlikely to account for the association of a highly selective dextranuria and a highly unselective proteinuria. In this condition, there is no histological evidence of a patchy glomerular lesion and the glomeruli appear uniformly almost normal. The high dextran selectivity, which is similar to that found in normal subjects, is in accord with the virtually normal glomerular appearances. The source of the urinary proteins in this condition is uncertain, but it has been suggested that fragmented tubules may allow the passage of plasma protein into the tubular lumen from the peritubular fluid (MacLean & Robson, 1966). The finding that the range of dextran selectivity in acute ischaemic renal failure is slightly lower than in normal subjects is compatible with the view that the glomerular dextran of high selectivity may be admixed with dextran from a more unselective route, which could be the source of the urinary protein.

In postural proteinuria the dextran values are also highly selective and have a range similar to that of healthy subjects, indicating that the glomeruli are functioning normally. The unselective protein pathway appears to make no significant contribution to the dextran selectivity, possibly because in these patients there is a good renal function and the amount of glomerular dextran is large in relation to the small amount of dextran from any other source. Thus al-

though the origin of the urinary protein is obscure it is most unlikely to be glomerular. Renal haemodynamic changes have been postulated as a cause of postural proteinuria (Bull, 1948; King & Baldwin, 1954; Robinson *et al.*, 1963). Because of the renal portal system, back pressure from the renal veins could conceivably result in a tubular leak of protein. However, there is no histological or electron microscopic evidence of rupture of the tubular basement membrane in postural proteinuria. Lowgren (1955) suggested a post-glomerular mechanism derived from the renal lymphatic system and, although there is little information in support of this, studies comparing lymph and serum protein patterns suggest that lymph is produced by a relatively unselective process (Schultze & Heremans, 1966). Although our results do not resolve the question of the origin of the protein in postural proteinuria, the normal glomerular permeability, indicated by the high dextran selectivity, coupled with the low protein selectivity provides indirect support for a renal lymphatic origin.

While the source of urinary proteins in normal subjects is uncertain, the dextran results confirm that protein selectivity at normal levels of protein excretion does not reflect glomerular permeability and that the normal glomerulus is highly selective.

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GLOMERULAR PERMEABILITY DURING PROTEINURIA INDUCED BY PLASMA INFUSION

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SUMMARY

1. Glomerular permeability to protein and dextran was studied in four patients with bleeding disorders who had normal renal function and in whom proteinuria occurred during the large infusions of plasma needed for treatment of the primary disorder.

2. Results are expressed in terms of selectivity indices which relate clearance to molecular weight.

3. Dextran selectivity was high both in the presence and absence of proteinuria and protein selectivity during proteinuria was also high.

4. Proteinuria induced by plasma infusion is likely to be glomerular in origin and the results indicate that the normal glomerulus is highly selective to plasma protein as well as to dextran.

In recent years, clearances of plasma proteins have been used in the assessment of patients with proteinuria, and the results have been interpreted in terms of glomerular permeability. This function is usually expressed in terms of the degree of selectivity of the glomerulus in permitting the passage of macromolecules of different sizes (Blainey, Brewer, Hardwicke & Soothill, 1960; Joachim, Cameron, Schwartz & Becker, 1964; Cameron & White, 1965; Hardwicke, 1965; Hitzig, Auricchio & Benninger, 1965; Ruckley, MacDonald, MacLean & Robson, 1966; Robson, 1968). In selective proteinuria the glomerulus restricts the passage of larger protein molecules to a very marked degree, and the clearance of albumin is about 1000 times that of α_2 macroglobulin. In unselective proteinuria the passage of large molecules is much less restricted and the clearance of albumin is about five to 100 times that of α_2 macroglobulin.

The normal glomerulus is highly selective for dextran and polyvinyl pyrrolidone (Hulme & Hardwicke, 1966; MacLean, Petrie & Robson, 1970), but the excretion pattern of the small amount of protein which appears in normal urine is highly unselective (Rowe & Soothill, 1961; MacLean & Robson, 1966; Poortmans, 1968). It has been suggested that this disparity may be

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due to the fact that a high proportion of normal urinary protein arises from the renal tract (Grant, 1957) and differs immunologically from serum protein, and/or that a relatively high proportion of the plasma protein in normal urine is derived from tubular or more distal sources rather than from glomerular filtration.

In health, filtered protein is largely reabsorbed by the renal tubules (Rather, 1952; Dirks, Clapp & Berliner, 1964) and only very small amounts escape into the urine. In glomerulonephritis on the other hand, it is believed that the tubular reabsorptive capacity for protein is saturated and that the bulk of the urinary protein is derived from glomerular filtration (Hardwicke & Squire, 1955). Only under such circumstances can studies of protein clearance be expected to be a valid reflection of glomerular permeability to protein and these conditions clearly do not prevail in health.

Proteinuria, however, can be induced in normal dogs (Brull, 1934; Terry, Hawkins, Church & Whipple, 1948; Vernier, 1961) and in human subjects not previously showing proteinuria (Waterhouse, Bassett & Holler, 1949) by the intravenous infusion of large amounts of albumin or plasma. This urinary protein is almost certainly of glomerular origin, the protein infusion leading to an increase in the protein content of the glomerular filtrate which is sufficient to saturate tubular reabsorption. Although electron microscopy studies in the dog indicate that this type of proteinuria is associated with loss of structure of the epithelial foot processes of Bowman's capsule, its disappearance following cessation of protein infusion suggests that the renal damage is of minor degree (Vernier, 1961).

Patients with bleeding disorders frequently require large infusions of plasma or plasma products to achieve haemostasis, and a proportion of such patients develop transient, heavy proteinuria with this treatment. The estimation of protein clearances in these circumstances thus provides the opportunity to estimate glomerular permeability to protein in what are believed to be normal human kidneys. In the present study, proteinuria was induced by plasma infusion in four patients with bleeding disorders, but with no evidence of renal disease, and protein selectivity was estimated. Since plasma expansion *per se* may cause haemodynamic changes resulting in altered glomerular permeability (Chinard, Lauson, Eder, Greif & Hiller, 1954; Malmendier, de Koster & Lambert, 1960), glomerular permeability to dextran was also measured both in the absence of proteinuria and after it had been induced.

METHODS

The patients studied suffered from haemophilia or Christmas disease and were admitted to hospital for treatment of haemorrhagic complications or for haemostatic therapy prior to elective surgery. Haemostatic therapy was controlled by the staff of the Department of Haematology and no alterations in therapy were made for the purpose of this study. Of ten patients infused with more than 2·5 l of plasma at a rate of over 800 ml/day, six developed proteinuria of over 1·0 g/day. Four of these patients have been studied in detail. In addition to fresh frozen platelet-poor plasma (prepared by the Blood Products Unit, Edinburgh), four received purified antihaemophilic fraction (Cohn fraction I) and case 3 received Prothrombin Complex (concentrate of factors II, VII, IX, X). The protein concentrations and volumes of the fresh frozen plasma and protein fractions are shown in Table 1.

Protein selectivities and urine to serum ratios of albumin were determined by a modification

TABLE 1. Details of protein containing solutions given to patients prior to onset of persistent proteinuria. The infusion period is the time taken for proteinuria to develop.

Patient	Diagnosis	Platelet-poor plasma		Antihaemophilic fraction		Prothrombin complex	Total protein infused		Infusion period
		(ml)	(g)	(ml)	(g)	(ml)	(ml)	(g)	(days)
1	Haemophilia	2400	132	600	18	—	3000	150	3
2	Christmas disease	7400	407	—	—	40	7440	407	9
3	Haemophilia	8400	462	2000	60	—	10400	522	8
4	Haemophilia	7200	396	2400	72	—	9600	468	8

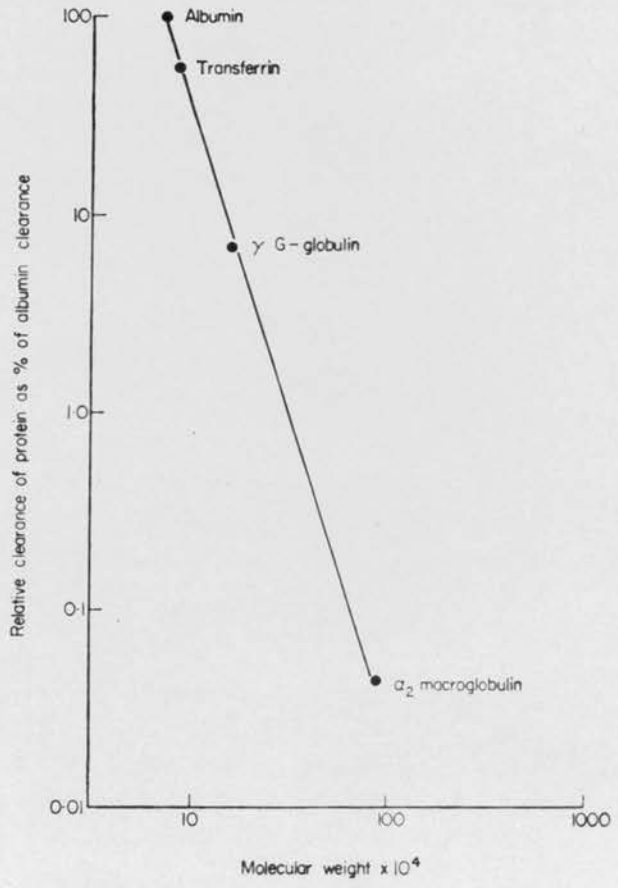

FIG. 1. Calculation of $-k$, the index of selectivity for protein in patient 1. The renal clearance, expressed as a percentage of the clearance of albumin, is plotted against molecular weight for each of four proteins, using a double logarithmic scale. $-k$ is the slope of the regression line obtained, using the method of least squares. In this case the proteinuria is selective and $-k$ is 3·13.

of the immunodiffusion technique of Soothill (MacLean & Robson, 1966; MacLean & Robson, 1967). The index of selectivity obtained is denoted by the symbol $-k$. (Fig. 1).

Dextran selectivities were determined by a method which involves the separation of serum and urinary macromolecules by Sephadex G 200 on the basis of molecular size (Petrie, MacLean & Robson, 1968). The dextran selectivity index is denoted by the symbol D.

Creatinine in the serum and urine, total serum protein and serum albumin were estimated employing the AutoAnalyzer (Technicon Methodology, Files N11b and N19b, Northam & Widdowson, 1967). Total urinary protein was measured by the biuret method (Hiller, Grief & Beckman, 1948), and by a modification of a microbiuret method (Itzhaki & Gill, 1964).

RESULTS

The four patients studied (Table 2) all had less than 30 mg/100 ml of protein in their urine, or a urine to serum albumin ratio of less than 1:1600 both before the protein infusions, and 5 days after the cessation of treatment. The values for creatinine clearance ranged from 76 to 150 ml/min. Plasma protein levels prior to the protein infusions ranged from 6·9 to 7·8 g/100 ml, with albumin concentrations of between 3·8 and 4·2 g/100 ml. The serum protein concentration in these patients at the time of proteinuria ranged from 8·3 to 9·4 g/100 ml with albumin levels ranging from 4·4 to 5·0 g/100 ml.

Protein selectivities measured during the period of proteinuria ranged from $-k$ values of 2·49 to 3·13. These values indicate a highly selective pattern of protein excretion, comparable for example to that seen in glomerulonephritis with minimal lesions (Robson, 1968). The technique for determining dextran selectivities differs somewhat from the immunological technique used in assessing protein selectivities, but gel filtration selectivity values have been shown to be related to $-k$ values for protein selectivity by the formula $D = 0{\cdot}76\,(-k) - 0{\cdot}08$ (MacLean & Petrie, 1966). D values measured during proteinuria ranged from 2·47 to 3·15 and are therefore comparable to the simultaneously measured $-k$ values for protein. These D values show the highly selective excretion pattern which is characteristic of the normal kidney (MacLean *et al.*, 1970). Essentially similar values for dextran selectivity were obtained in the absence of proteinuria. These ranged from 2·44 to 3·03.

DISCUSSION

The proteinuria induced by plasma infusion is highly selective and simultaneously measured dextran clearances show a similar pattern. There are three possible explanations of the results.

Firstly, the glomeruli may be structurally normal, but the increase in plasma volume produced by protein infusion leads to stretching of 'glomerular pores'. In this event the glomerular clearance of protein would be slightly raised and this, along with the increased serum protein concentration, would result in an increased amount of protein being delivered to the tubules, with consequent saturation of tubular protein reabsorption. This mechanism has been shown to occur in patients with the nephrotic syndrome following albumin infusion (Chinard *et al.*, 1954; Malmendier *et al.*, 1960). However, since no alteration in dextran selectivity could be demonstrated following plasma infusion in our patients 'pore stretching' due to plasma expansion appears unlikely.

Secondly, the glomeruli may be structurally and functionally abnormal. The abnormality, if

TABLE 2. $-k$ = index of protein selectivity, D = index of dextran selectivity

Patient	Creatinine clearance (ml/min)	Serum protein (no proteinuria) (g/100 ml)		Serum protein (during proteinuria) (g/100 ml)		Urine protein (no proteinuria) (mg/24 h)	Urine protein (during proteinuria) (g/14 h)	Albumin serum:urine ratio (no proteinuria)	$-k$	D (during proteinuria)	D (no proteinuria)
		Total	Albumin	Total	Albumin						
1	150	7·7	4·2	8·3	4·8	19	12·6	2560	3·13	3·15	2·89
2	76	7·3	3·9	8·8	5·0	7	4·2	5430	2·49	2·47	2·44
3	129	6·9	3·8	9·2	4·5	20	4 5	7920	2·66	2·48	2·99
4	105	7·8	3·8	9·4	4·4	23	11·8		2·61	2·56	3·03

it exists, coincides in time with the plasma infusion and is therefore presumably caused by it. All the patients studied had had repeated previous infusions of plasma, and may well have possessed antibodies to various components of the infused material. It is known that antigen-antibody complexes can be deposited in the basement membrane with resultant glomerular damage and proteinuria (Dixon, 1968; Cochrane, 1968). While the present results do not exclude glomerular damage of this type, this mechanism is unlikely. In the first place the proteinuria is highly selective. Secondly, it occurs only after the serum protein concentration has been raised significantly. It also disappears promptly following cessation of protein infusion when the plasma protein concentration falls towards normal.

Finally, the glomeruli may be structurally and functionally normal, the proteinuria being due entirely to saturation of tubular reabsorption. This saturation can be attributed to more protein than usual being filtered through normal glomeruli as a result of the raised concentration of plasma proteins. In this event the selectivity is a valid reflection of glomerular permeability.

The haemostatic disorder in these patients naturally prevented renal biopsies being undertaken during or after the proteinuria induced by the protein infusion. In view of the values for dextran selectivity however and the transient nature of the proteinuria it is believed that glomerular function and structure in these patients is not significantly abnormal. The results therefore indicate that the normal glomerulus filters plasma protein in a highly selective manner.

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