STUDIES ON THE CLINICAL SIGNIFICANCE OF A NEW PROTEIN, URINE PROTEIN 1 (ALPHA-2 GLYCOMICROGLOBULIN).

A Thesis Submitted to the University of Surrey for the Degree of DOCTOR OF PHILOSOPHY

By

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The use of plasma proteins for the diagnosis and management of various diseases in humans has been known since the days of Hippocrates. Recently, a new protein, Urine protein 1 (UP1), has been isolated by Dakopatts Immunoglobulin, Copenhagen, from patients with renal tubular dysfunction. This study reports an investigation into the clinical significance of the protein. The purification, antibody production and semi-characterisation of the protein has also been undertaken. A sensitive, reproducible enzyme-linked immunosorbent assay has been set up and validated for the estimation of UP1.

UP1 has been shown to be a stable, negative, low molecular weight (LMW) glycoprotein, with alpha-2 electrophoretic mobility on cellulose acetate. It is suggested that the protein be called alpha-2 glycomicroglobulin (a₂GM).

a₂GM has been shown to be synthesised in the liver, and, like many other LMW proteins, cleared by the glomeruli, reabsorbed and catabolised mainly by the proximal convoluted tubules. It shows no acute phase reaction, and its serum levels are not significantly affected by pathological states such as myeloma, Hodgkin's disease, leukaemia and teratoma, unless there is associated impairment in renal function. Patients with end-stage renal failure showed the highest serum increases. Serum levels of a₂GM were not found to be significantly affected by haemodialysis.

Clinically, a₂GM has been shown to be useful in the diagnosis and assessment of the course of progression of renal disease. It has also been demonstrated that a₂GM has great potential in the identification, characterisation and monitoring of rejection episodes, and in the assessment of allograft function. Compared to other LMW plasma proteins, a₂GM indicated clearly superior sensitivity and may be useful in monitoring tubular function in cancer patients on cisplatin chemotherapy. a₂GM has also been shown to be a useful analyte in the assessment of early renal tubular involvement in diabetics.
DEDICATION

Dedicated to:

ADEIOR
DOOM
MNENA
FANEN
ACKNOWLEDGEMENT

I acknowledge the advice and motivation I received from the late Professor J Kohn, who unfortunately died a few months before the completion of this study, and Dr J Wright during the course of this study. I would also like to thank Professor V Marks for the interest he has shown in the research.

My thanks are also due to Mrs Sue Martin, and all the staff of Clinical Biochemistry, St Luke's Hospital, Guildford, who had to cope with my insistent request for samples, and through whom I learnt many of the techniques employed in this work.

I am also grateful to Drs M R Bending and J T C Kwan, S W Thames Renal Unit, St Helier Hospital, Carshalton, and Mr Koffman, Renal Unit, Guy's Hospital, London, for use of their patients; and Miss Angela Coe (Guy's Hospital), for coordinating the difficult task of sample collection for the transplant study. I am also thankful to Dr D Rowe, Top-Grade Biochemist, General Hospital, Southampton, for supplying samples from their on-going diabetes study.

I would like to thank Dr S Hampton, for her advice on ELISA, Dr C Powell, for his help during immunocytochemistry, Dr W Aherne and Mrs E Piall, for their help during antiserum production, Drs P Kwasowski and K Beysavi, for immunoaffinity column preparation, and Guildhay Antisera Ltd, for the supply of some of the antisera used in this work.

I gratefully acknowledge financial support from the Commonwealth Scholarship Commission (UK).

Lastly, but by no means the least, I place on record my appreciation for the remarkable patience and understanding my family has shown throughout the period of my absence from home. I am also grateful to God for His protection.
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CHAPTER ONE

GENERAL INTRODUCTION
1.1 INTRODUCTION

The use of proteins in biological fluids for the assessment of disease processes in humans has been known since ancient times. In the middle ages, the doctor had to observe urine for, among other things, foam (for protein). The last decades have seen the evolution of test-sticks, such as Albustix, that can indicate pathological protein levels in urine. With improvements in methodology, the list of proteins in biological fluids that can be used for diagnostic purposes, and the disease states for which they have clinical relevance, has steadily grown. Protein monitoring in biological fluids now constitutes a major diagnostic array of tests for, to mention a few, liver diseases, immune deficiency diseases, cancer, diabetes mellitus, inflammation and differential diagnosis of renal diseases. Proteins used range from those with known functions, such as immunoglobulins and albumin, to those with unestablished physiologic roles, such as alpha-1 microglobulin. Over the decades, several new proteins have been isolated, characterized and, based on their functional properties or molecular size, many have been shown to have potential or practical application in clinical practice.

Recently, a new low molecular weight (LMW) protein, Urine Protein 1 (UP1), was isolated from human urine of patients with renal tubular defects by Dakopatts Immunoglobulin, Copenhagen, Demark. As with many new proteins, nothing is known about its physiological or clinical importance. This project seeks to investigate the clinical significance of the new protein.

Because of their small size, LMW plasma proteins are rapidly cleared by the kidney. The kidney is the principle homeostatic organ in the body which, besides its endocrine functions, helps to maintain
regularity of body composition by excreting the waste products of metabolism, modifying the acidity of urine, and regulating electrolyte and water control. The functional units of the kidney, upon which this excretory and secretory burden falls, are the nephrons. Each nephron consists of a glomerulus, the ultrafiltration unit, and the tubules, through which substances are conducted and eventually appear in urine. The composition of urine, as well as plasma, reflects not only the functional disorders of the nephron but also of various systemic diseases.

With respect to protein metabolism, the kidney restricts loss into urine of high molecular weight proteins during filtration at the glomerulus, and, at the tubular level, reabsorbs and catabolises small filtrable plasma proteins. The result of these physiologically and biochemically complex set of glomerular and tubular processes is the maintainance of a fairly constant plasma and urinary protein level and composition in health. Renal and systemic disorders can alter this balance. For example, increased urinary excretion of Bence Jones proteins (light chain disease) is valuable in the diagnosis of myeloma and other lymphoproliferative disorders, whereas albuminuria may be a harbinger of renal disease.

The literature presented in this chapter reviews renal function as it relates to handling of proteins in health; and how this understanding illuminates the pathological observations in disease, and allows the formulation of strategies for the investigation of proteinuria, and hence differential diagnosis of nephropathy. Emphasis is placed on serum and urinary changes in low molecular weight protein levels as an indicator of renal function.
Nephropathy means renal damage of whatever aetiology. This damage may be functional or organic. Functional impairment may include reduction in blood flow due to haemodynamic changes, and defects in tubular function, such as concentrating capacity or glucose transport. Organic alterations may be extremely variable in site (glomeruli, vessels, tubules, interstitium), extent (patchy or diffuse), and type (degenerative, necrotic, inflammatory or allergic lesions). Renal damage may be acute or chronic, reversible or irreversible, and may be precipitated by exposure to various metabolic, chemical or immunologically-mediated insults. Nephropathies are also encountered in non-renal diseases such as diabetes, systemic lupus erythematosis, amyloidosis, cancer and hypertension. More often than not, nephropathy precipitates proteinuria. The events underlying the origin of renal damage are represented schematically in Figure 1.1 (page 5). Early detection of renal disease by a variety of insults before clinical expression of damage, preferrably at the stage of biochemical alterations, is clearly desirable.

Proteinuria is the excretion of proteins in excess of 150 mg/day, often because of renal disease. Friberg in 1950 and Kekwick in 1955 were the first to describe a distinctive type of proteinuria that occurs in cases of chronic cadmium poisoning, and Butler and Flynn (1958) described special patterns in certain congenital tubulopathies. Revillard et al (1970a) and Revillard et al (1970b) respectively reviewed and catalogued proteinuria in glomerular diseases, and renal diseases usually associated with tubular proteinuria. A term, 'NEPHROPATHO-PROTEINURIA' is introduced and taken to mean proteinuria precipitated by kidney disease. Some of the common causes of
Figure 1.1 An outline of the events underlying the origin of renal damage.

Adapted from Bonner et al (1982).
1.2.1 DIABETES AND NEPHROPATHO-PROTEINURIA

Diabetes mellitus, as presently understood, is a heterogeneous group of disorders characterized by hyperglycaemia, absolute (insulin-dependent or Type I diabetes) or relative (non-insulin dependent or Type II diabetes) insulin deficiency, and the tendency to develop certain long term complications. These complications include, but are certainly not limited to, accelerated atherosclerosis, diabetic retinopathy, diabetic nephropathy and diabetic neuropathy.

Diabetes is the single most important cause of adult end-stage renal disease. Microangiopathy involving the kidney leads to diabetic glomerulopathy. Diabetic nephropathy is the most important renal lesion that develops in all types of diabetes, and which in many cases leads to partial or eventually to complete loss of function and a state of uraemia. Diabetic nephropathy occurs less frequently in patients with type II diabetes, but when it does occur, the duration between diagnosis of diabetes and the onset of proteinuria is shorter than in patients with type I diabetes. Historically, proteinuria has been known to be associated with diabetes since the days of Richard Bright in 1827. Currently, the diagnosis of diabetic nephropathy depends upon the detection of gross proteinuria. In fact, the appearance of proteinuria in a diabetic patient, when urinary tract infection and other renal diseases have been excluded, usually indicates the presence of diabetic nephropathy, a serious complication carrying a high morbidity and mortality.
It is known that thickening of the tubular and glomerular capillary basement membranes, leakage of numerous proteins into the mesangial space, and obliteration of the mesangial space occurs within several years after diagnosis of diabetes (Steffes et al., 1985). The process of diabetic glomerulosclerosis proceeds insidiously for 10-15 years. Only after most of the mesangial space has been obliterated does proteinuria occur. (Also see section 1.4.1.) Once proteinuria is apparent, there is an exponential decrease in creatinine clearance, and end-stage renal disease usually develops within 5 years (Clements and Bell, 1985). The pathophysiologic mechanisms of diabetic nephropathy are depicted schematically in Figure 1.2 (page 8).

The glomerular barrier normally restricts passage, into urine, of proteins of molecular weight of albumin and greater (see section 1.4). However, smaller proteins and albumin do pass the barrier and despite tubular reabsorption, small quantities can still be detected in the urine of healthy subjects. In routine clinical practice albumin testing in urine is done using dipsticks such as Albustix (Ames) which changes colour when albumin concentration is of the order of 300 mg/l (Viberti and Keen, 1984).

In the study of renal disease in diabetes, very exact methods for the evaluation of kidney function are required. Measurement of excretion rate of plasma proteins is currently the method used to monitor kidney function. Measurement of excretion rate of albumin and beta-2 microglobulin (Viberti et al., 1975) has been extensively used in the last decade, and it is now widely accepted that urinary albumin excretion is a key parameter in the evaluation of early renal involvement in diabetes. Using radioimmunoassay or enzyme-linked immunosorbent assay (Mohamed et al., 1984; Watts et
Figure 1.2 The vicious circle in the kidney, with special reference to diabetes.

DISEASE

Metabolic or haemodynamic changes

DIABETIC NEPHROPATHY (and other renal diseases?)

Some damage in all glomeruli?

HYPERTENSION (now generated?)

Compensatory enlargement of remaining glomeruli

Closure of a number of glomeruli

Unknown factors

Adapted from Mogensen (1985).
al, 1986), small amounts of urinary albumin or microalbuminuria - albumin excretion rate of 30-150 ug/min (approximately 30-200 mg/day) - may be detected long before the Albustix-positive stage either basally or on provocation, as in exercise (Mogensen and Vittinghus, 1979). The amount of microalbuminuria may be reduced by careful diabetic control (Viberti et al, 1979). The measurement of albumin at low concentrations has proved a strong predictor of the development of clinical nephropathy in insulin-dependent and non-insulin dependent diabetes (Mogensen and Christensen, 1984). Albumin to creatinine ratio, too, has been found a sensitive predictor and monitor of renal involvement (Marshall and Alberti, 1986).

1.2.2 CANCER AND NEPHROPATHO-PROTEINURIA

As early as 1922 Galloway associated neoplasia (Hodgkin's disease) with nephrotic syndrome. Over the last two decades, development of several types of glomerular injury in patients with cancer has been recognized and indeed is now considered as one of the paraneoplastic syndromes (Eagen and Lewis, 1977; Gagliano et al, 1976). Several associations between glomerulopathy and neoplasia have been noted and perhaps the most prominent are the occurrence of membranous glomerulonephritis (MGN) in patients with carcinoma and the association between minimal change glomerulopathy and Hodgkin's disease (Eagen and Lewis, 1977).

It is now known that in neoplastic nephropathy, the kidney may malfunction in a variety of ways directly or indirectly as a consequence of non-renal tumours. Direct effects of tumours include metastasis, invasion or infiltration of the kidneys by tumour cells.
Indirect effects include metabolic alteration, toxic or degenerative effects, secondary infection and immunologically mediated reactions. Several reports have provided a morphologic correlate to serologic studies which show elevated levels of circulating immune complexes in most patients with cancer. The demonstration of immunoglobulin and complement in glomerular lesions and the similarity between ultrastructural features of cancer-associated MGN with idiopathic MGN suggests antibody-mediated glomerular injury, either due to circulating immune complexes or due to in situ immune complex deposition (Couser and Salant, 1980). In addition, malignancies, perhaps by affecting immune function or regulation, may render the cancer patient more susceptible to the development of immune complex nephritis due to exogenous or endogenous antigens.

In dysproteinaemias, such as occur in lymphoplasmacytic disorders, classical renal lesions due to excess immunoglobulin production are typified by the 'myeloma kidney'. In this condition, the prominent lesion is that of tubular cast nephropathy with associated tubular interstitial nephritis (Stone and Frenkel, 1975). A nonamyloidic glomerulopathy associated with direct deposition of light chains or apparent monoclonal immunoglobulins has been recognized in myeloma (Gallo et al, 1980). Glomerular lesions may also be secondary to changes consequent to severe tubulointerstitial injury. Other specific forms of glomerular injury may be seen in other dysproteinaemias such as cryoglobulinaemia and Waldenstrom's macroglobulinaemia.

In a recent review, Burkholder et al (1981) indicated a clear causal relationship between neoplastic conditions and the respective glomerular lesions reported. Cancer patients are, in addition, particularly vulnerable to renal complications because of the number
of potentially nephrotoxic drugs employed in antineoplastic therapy or in managing complications of cancer or its therapy (Luxon, 1961; Kosek et al, 1974; Madias and Harrington, 1978; Ries and Klastersky, 1986). The most common sign of glomerulopathy in patients with non-renal neoplastic disease is proteinuria or nephrotic syndrome (Burkholder et al, 1981). See also section 1.2.3

1.2.3 NEPHROTOXIC AGENT-INDUCED NEPHROPATHO-PROTEINURIA

Exposure to a variety of drugs is known to be associated with the development of renal damage in both man and experimental animals. Interstitial tubular injury is the most frequent, although glomerular lesions are also observed (Ries and Klastersky, 1986; Drukker et al, 1986). The site of renal lesion that may arise depends on the drug, and patterns of proteinuria generated may give a fair indication of the site (Whelton and Solez, 1981) and extent of damage. Various aspects of drug-induced nephropathy have been recently reviewed by Druet et al (1982), Whelton and Solez (1981), Fillastre et al (1981) and Dubach (1981).

The anatomic configuration and complexity of physiologic functions performed by the kidney render it particulary prone to the development of toxic side effects of drugs. These functional and structural relationships which confer upon the kidney a unique vulnerability to drug-induced toxicity include high blood flow and hence high rate of drug delivery; the large endothelial surface area of the renal vascular bed - which perhaps explains the high frequency of precipitation of immune complexes in the glomeruli (Fillastre et al, 1981); the secretory and reabsorptive transport of drugs by the proximal tubular cells; and the many renal
paranephral enzymic systems which can be inhibited or activated by drugs as they are excreted. In addition, the hairpin, counter-current multiplier medullary concentrating mechanism, which adds to renal efficiency, is likely to also increase the renal vulnerability to toxic agents, which will, in the deeper medulla, attain concentrations several times greater than in the other tissues of the body. This theoretical possibility is reflected structurally in the syndrome of papillary necrosis (Drukker et al, 1986).

High drug concentrations may be intraluminal, thereby exposing the epithelium to damage either when the rate of water reabsorption is higher than that of drug or when the solubility of the drug is increased at the pH of the urine. In some cases, the high concentration may be intracellular, when the drug is deposited in the tubular cells by the transport mechanism itself; or peritubular, in surrounding intertubular tissue, particularly in the medullary zone (Bonomini, 1981). Drukker et al (1986) have reviewed analgesic nephropathy, the pathologic cornerstones of which are papillary necrosis and interstitial nephritis. Nephropathy induced by cancer chemotherapy has been recently reviewed (Ries and Klastersky, 1986).

The mechanisms of drug induced nephropathy have been summarised in Table 1.1 (page 13). However, worthy of note is the genetic predisposition which may be operative in affecting the level of susceptibility to possible drug damage. In general, however, the mechanism of action of the steadily increasing heterogeneous groups of toxic agents, and the clinical pattern of intoxication, are varied, and with few exceptions the clinical picture is frequently non-specific. Thus from available evidence it appears that the same cause may give rise to different morphological and/or functional changes while, conversely, similar morphological and/or functional patterns may stem from a broad range of causes.
Table 1.1 Outline of Mechanisms of Drug nephrotoxicity

<table>
<thead>
<tr>
<th>Effect on Renal Blood Flow (RBF)</th>
<th>Drug may affect the renal vessel directly by impairing RBF and decreasing GFR as with Indomethacin (by blocking the intrarenal production of vasodilating prostaglandins) or indirectly by a combination of toxic action and dehydration.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Toxic Action in Tubular cells</td>
<td>Drug may come into intimate contact with the cells of the tubules and they may pass through them in the process of secretion or reabsorption. During this process, may affect the brush-border, various organelles, inactivate cellular enzymes and lead to functional damage or even cellular death (aminoglycosides, cephaloridin, aflatoxin, ochratoxin A, heavy metals, cadmium and carbon tetrachloride).</td>
</tr>
<tr>
<td>Immunologically Mediated Injury (Glomerular or Tubulointerstitial)</td>
<td>Drugs, as haptens, may produce immunologically mediated injury both to the glomeruli and, even more frequently, to the interstitium and start a reversible or progressive glomerular or tubulo-interstitial disease (some antibiotics, e.g. methicillin, rifampin).</td>
</tr>
<tr>
<td>Obstruction of Tubuli or Ureters</td>
<td>Drug may come out of solution during concentration of urine or change of pH of tubular fluid and block the tubuli with crystal (sulphonamides). Also the ureters can be blocked from the inside by crystals or by necrotic renal tissue (necrotic papillae) or constricted from outside by retroperitoneal periureteric fibrosis (radiation injury, methysergide).</td>
</tr>
<tr>
<td>Carcinogenic</td>
<td>Drugs or their metabolites may be carcinogenic.</td>
</tr>
</tbody>
</table>

From Brod (1982)
These nephrotic lesions, produced by drugs by whatever mechanism, elicit patterns of protein excretion that are useful in the diagnosis and follow-up of renal diseases. This is usually the basis of investigating proteinuria, especially of LMW proteins, in nephrotoxicity.

1.3 PROTEINURIA: HISTORICAL PERSPECTIVE.

The recognition that there is a connection between proteinuria and renal disease probably dates back to the time of Hippocrates (Cameron, 1970) who first noted an association of frothy urine with kidney disease. Our present understanding of the importance of proteinuria as an indicator of renal disease, however, emerged after Richard Bright observed in 1827 albumin in urine as a sign of serious kidney disease. Bright's observation, added to earlier ones by Cotunnius in 1770 and Rollo in 1798, that urine of some patients suffering from diabetes contained proteins, led Rayer in 1940 to postulate that diabetes might cause a form of Bright's disease. Pesce and First (1979) have reviewed the history of proteinuria. An outline history of proteinuria is given in Table 1.2 (page 15).

By the turn of the 19th century it had been shown that normal human urine contains soluble proteins in addition to insoluble mucous substances. Electrophoretic studies opened the way to qualitative analysis of urine and plasma (Boyce et al, 1954; McGarry et al, 1955) and suggested that part of the urinary proteins might originate from
Table 1.2 History of Proteinuria: an outline.*

<table>
<thead>
<tr>
<th>Year</th>
<th>Author(s)</th>
<th>Event</th>
</tr>
</thead>
<tbody>
<tr>
<td>1836</td>
<td>Bright¹</td>
<td>Coagulable urine and renal disease</td>
</tr>
<tr>
<td>1843</td>
<td>Lever¹</td>
<td>Proteinuria in pregnancy</td>
</tr>
<tr>
<td>1847</td>
<td>Bence Jones¹</td>
<td>Proteinuria and multiple myeloma</td>
</tr>
<tr>
<td>1878</td>
<td>Leubel¹</td>
<td>Severe exercise ('match') proteinuria</td>
</tr>
<tr>
<td>1878</td>
<td>Moxon¹</td>
<td>Postural effects on proteinuria</td>
</tr>
<tr>
<td>1941</td>
<td>Walker et al¹</td>
<td>Measurement of glomerular filtrate protein content</td>
</tr>
<tr>
<td>1951</td>
<td>Iversen &amp; Brun¹</td>
<td>Renal biopsy of patients with renal disease</td>
</tr>
<tr>
<td>1952</td>
<td>Tamm &amp; Horsfall¹</td>
<td>Isolation and characterisation of 'Tamm-Horsfall' mucoprotein</td>
</tr>
<tr>
<td>1955</td>
<td>Straus &amp; Oliver¹</td>
<td>Injected protein found in hyaline droplets in nephron tubules (on absorption)</td>
</tr>
<tr>
<td>1958</td>
<td>Butler &amp; Flynn¹</td>
<td>Proteinuria of renal tubular disease differs from glomerular disease (concept of 'tubular proteinuria')</td>
</tr>
<tr>
<td>1961</td>
<td>Farquahar &amp; Palade¹</td>
<td>Tracer protein use in electron microscopy (follow protein route through glomerulus)</td>
</tr>
<tr>
<td>1961</td>
<td>Hardwicke &amp; Soothill¹</td>
<td>Concept of glomerulus as molecular filter</td>
</tr>
<tr>
<td>1968</td>
<td>Berggard &amp; Bearn²</td>
<td>B₂-Microglobulin isolation from patients with renal tubular proteinuria</td>
</tr>
<tr>
<td>1969</td>
<td>Berggard &amp; Peterson³</td>
<td>Free immunoglobulin light chains</td>
</tr>
<tr>
<td>1969</td>
<td>Peterson &amp; Berggard⁴</td>
<td>Retinol-Binding Protein from patients with tubular proteinuria</td>
</tr>
<tr>
<td>1977</td>
<td>Ekstrom &amp; Berggard⁵</td>
<td>Alpha-1 microglobulin in normal and tubular proteinuria</td>
</tr>
</tbody>
</table>

blood. Later studies indicated that the predominant part of plasma proteins in urine originates from a process of glomerular filtration followed by tubular reabsorption.

After demonstration of proteins in urine of normal individuals, clinicians and medical scientists became primarily concerned with quantitative study of protein excretion. Values now given for protein excretion in health have varied between 24 and 133 mg/day (Berggard, 1970) depending on the method of estimation, but it is likely that values of up to 150 mg/day are two standard deviations of the mean value for total protein excretion and may therefore be accepted as 'within normal limits' (Robinson and Dennis, 1978). Citrate-buffered tetrabromophenol blue impregnated strips ('dip stick') for routine semiquantitative screening of proteinuria have been in use (Free et al, 1957), and the currently widely used Albustix (Ames Co. U.K.) begins to change colour when albumin concentration is of the order of 300 mg/day, corresponding to a total protein concentration of 0.5 g/day.

It soon became clear that pathological processes might affect the urinary excretion of individual proteins selectively and therefore estimates of total protein excretion alone may not reveal the precise physiological disturbance. It was therefore necessary to establish methods for studying patterns of proteinuria and the excretion of individual proteins rather than simply measuring the total protein excretion. During the last three decades progress in protein chemistry and immunochemical methods have enabled identification and estimation of a large number of plasma proteins in urine of healthy individuals. In addition, since the first immunoelectrophoretic study of Grant (1957), over 30 different plasma protein components have been identified in urine, and quantitative analyses are now available for over 12 plasma protein components in urine (Berggard, 1970; Mohamed et
al, 1984; Topping et al, 1986). The characterisation of various patterns of urinary protein excretion which can help in the elucidation of renal involvement has also been undertaken (Boesken et al, 1973; Mulli et al, 1974; and Hardwicke, 1975)

1.4 NORMAL HANDLING OF PROTEINS BY KIDNEYS: RELEVANCE TO PROTEINURIA.

The kidneys are a paired system of organs responsible for maintenance of the constant composition of blood and thus the optimal chemical composition of the interstitial and intracellular fluids throughout the body. This homeostatic function rests on the functional units of the kidney, the nephrons. Each nephron consists of a glomerulus, the ultrafiltration unit, with a charge and molecular-size selective sieve, through which all cleared polypeptides and other substances must pass; and the tubules, through which filtered substances are conducted and eventually appear in urine. Events that occur both at the glomerular and tubular levels, summarised below, have a direct bearing on the changes in protein levels in serum and urine in health and disease.

1.4.1 GLOMERULAR EVENTS

Diagrammatic representation of electron microscopic architecture of the glomerular capillary cell wall is given in Figure 1.3 (page 18). During transcapillary passage, plasma fluid traverses several stratified cellular and extracellular layers that make up the complex structure of the ultrafiltration unit. From inside (capillary lumen, CL) to outside (urinary space, US), it consists of endothelial fenestrae, glomerular basement membrane
Figure 1.3. Diagrammatic Representation of the electron microscopic architecture of human glomerular capillary wall.*

* Also shown is the polyanionic glomerular basement membrane (GBM). The process of transfer is from the capillary lumen (CL) to urinary space (US). Water, solutes and macromolecules must traverse three layers: the endothelial wall cytoplasm (EN), containing numerous fenestrae with diameter of 70 nm; the GBM with a mean thickness of about 320 nm, the darker area indicating the lamina densa (LD); the layer of epithelial foot processes (EP) which are separated 25 nm to 60nm from each other by slit pores, and lined by distinct membranes. The epithelial cytoplasm (EpCy) and nucleus (EpN) of a cell are also shown. LRI and LRE are lamina rara interna and lamina rara externa respectively. (Modified from Pesce and First, 1979).
(GBM), and epithelial foot processes. The integrity of each of these elements is essential for the maintenance of normal ultrafiltration.

The GBM, the barrier through which all proteins and other molecules must pass to appear in the urinary space, is a polyanionic extracellular matrix interposed between the epithelial foot processes and the attenuated endothelium. It consists of a central electron-dense layer, the lamina densa (LD), which is flanked either side by electron-lucent regions referred to as lamina rara interna (LRI) and lamina lara externa (LRE). The LRI of the GBM is readily accessible to the blood stream through the endothelial fenestrae. Most of the proteins circulating in the blood, particularly those of the molecular weight of albumin or smaller, have net negative charges; and there is an electrostatic repulsion between the protein molecules and the barrier since both are polyanionic. Molecules which are electrically balanced or neutral in the sense of electrical charge are not influenced by the polyanionic nature of this structure. However, molecules which have a net positive charge do interact with this barrier. Uncharged molecules do not readily penetrate into the LRE and accumulate at the LRI; negatively charged molecules behave in a similar manner; however, the restriction to them is greater. The few negatively charged molecules which go through the barrier appear to be absorbed at the epithelial foot processes (EP). Anionic molecules such as albumin (35 A, 3.5nm) are restricted at the LRI and are not usually visualized in the pinocytotic vesicles of the podocytes or foot processes. Molecules of neutral charge of the same size more readily penetrate the LRI and the LD into the LRE. Cationic molecules, even those of very large size, penetrate the barrier and
accumulate at the slit pore and in pinocytic vesicles. The glomerular barrier is therefore both size selective and charge selective.

Experiments (Kanwar and Rosenzweig, 1982) indicate that if electrical charge of the GBM is brought to neutrality, anionic proteins such as ferritin, albumin and insulin are adsorbed onto the GBMs by forming hydrogen bonds and, thus, clog the filter and reduce the flow of water and other solutes across the glomerular capillary. Polyanionic decrease (Wu et al, 1987) has been thought to cause partial or complete endothelial or epithelial detachment from the GBM: this may be relevant in the pathogenesis of the increased glomerular permeability that occurs in diabetes and other proteinuric states (Martinez-Hernandez and Amenta, 1983). The endothelial or epithelial detachment has also been observed in immune complex nephropathy. These changes may have a bearing on renal disease, but it is not certain whether they truly reflect charge-selective defect or account for proteinuria (Kanwar, 1984). It is however plausible that the integrity of all the layers of the glomerular capillary wall is essential for the maintainance of the size-selective as well as charge-selective properties of the ultrafiltration unit. Any disturbance that alters the 'integrated' function of the cellular and extracellular elements, regardless of how minor, can result in abnormal loss of proteins into the urinary space.

Urine is therefore essentially an ultrafiltrate of plasma. The process of ultrafiltration, by which the initial filtrate is derived from blood, as mentioned above, occurs at the level of the renal glomerulus. The glomerulus consists of an intricate network of capillaries through which the blood flows under the influence of
hydrostatic forces generated by the rhythmic contractions of the heart. The blood, driven by hydrostatic forces, traverses the glomerular capillary wall, the ultrafiltration unit, and forms the filtrate in the urinary (Bowman's) space. This ultrafiltrate is virtually devoid of blood cells and major proteins since the glomerular capillary behaves like a sieve allowing the passage of small molecules such as inulin without measurable restriction but almost completely restricting the passage of molecules of the size of albumin or larger. The transcapillary passage of plasma water and protein is regulated by renal glomerular plasma flow; the net balance between hydrostatic and oncotic pressure in the capillaries; the molecular size, charge and configuration of particulate element being filtered; the intrinsic biochemical and biophysical properties of the capillary wall; and intraglomerular haemodynamics. All these factors, in concert, maintain a delicate balance and regulate glomerular homeostasis and prevent leakage of plasma proteins into the urinary space. Conceivably any imbalance among these factors would be expected to result in an anomalous transcapillary passage of proteins resulting in proteinuria, the extent and type of which depends on the degree of the lesion. For example, damage to the glomerulus will allow proteins to pass through: minor damage will allow the passage of smaller proteins such as albumin. As the damage increases, the glomerulus will allow the passage of proteins of any size and description, including alpha-2 macroglobulins (MW 720,000).

Factors which influence the permselectivity (a term often used to indicate the selectively permeable nature of the glomerular filter) or filtration of macromolecules have been extensively reviewed (Brenner et al, 1978) and Kanwar (1984) recently reviewed
the biophysiology of glomerular filtration and proteinuria. Synthetic polymers have been used to study the sieving coefficients of glomerular membrane (Bohrer et al., 1978). [The glomerular sieving coefficient (GSC), also referred to as the fractional clearance, is the ratio of clearance of a given test molecule relative to the glomerular filtration rate (GFR) or clearance of inulin.] Unfortunately the multifactorial influence of dimension (Stoke-Einstein radius), charge and quaternary structure (asymmetry and rigidity of the molecular skeleton) is such that glomerular sieving curves obtained with artificial, charged or uncharged polymers and selected proteins cannot yet be used to estimate the glomerular sieving coefficient of any particular protein.

Several indirect methods have been used for the study of GSC of LMW proteins. Maack et al. (1979) made a description and critical appraisal of these methods. The glomerular fractional clearance of some LMW proteins in relation to size is given in Table 1.3 (page 23). From the various methods used in studying sieving properties of the glomerulus, the following general conclusions regarding the filtration of LMW proteins can be drawn from the data obtained.

Proteins smaller than 25,000 daltons (23 Å, 2.3 nm) cross the glomerular barrier, attaining concentrations in the glomerular filtrate that are usually more than 50% of their concentration in the plasma. Considering the magnitude of the GFR in mammals and the usually short half-life of most LMW proteins (Vahlquist et al., 1973), the filtration process accounts for a major proportion of the removal of circulating LMW proteins from the vascular compartment.

Apparently, molecular dimensions and molecular shape (rigidity) are the main determinants of the degree of glomerular sieving of proteins smaller than 25,000 daltons or 23 Å (2.3 nm). Below this
Table 1.3 Glomerular clearances (sieving coefficients) of LMW protein in relation to molecular size.

<table>
<thead>
<tr>
<th>Protein</th>
<th>Mol. wt. (daltons)</th>
<th>Mol. Size (Å)</th>
<th>Clearance (%GFR)</th>
<th>Source**</th>
</tr>
</thead>
<tbody>
<tr>
<td>Albumin</td>
<td>69,000</td>
<td>37</td>
<td>0.02</td>
<td>1</td>
</tr>
<tr>
<td>Amylase</td>
<td>48,000</td>
<td>29</td>
<td>3</td>
<td>1</td>
</tr>
<tr>
<td>Lambda L-chains</td>
<td>44,000</td>
<td>27</td>
<td>8</td>
<td>1</td>
</tr>
<tr>
<td>Horseradish</td>
<td>40,000</td>
<td>31.8</td>
<td>0.7 (anionic)</td>
<td>2</td>
</tr>
<tr>
<td>Peroxidase</td>
<td></td>
<td>29.8</td>
<td>6 (neutral)</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>30.0</td>
<td>34 (cationic)</td>
<td>2</td>
</tr>
<tr>
<td>Rat Growth H.</td>
<td>20,000</td>
<td>20.4</td>
<td>58-72</td>
<td>2</td>
</tr>
<tr>
<td>Myoglobin</td>
<td>16,000</td>
<td>18.8</td>
<td>75</td>
<td>2</td>
</tr>
<tr>
<td>Lysozyme</td>
<td>15,000</td>
<td>19.0</td>
<td>80</td>
<td>1</td>
</tr>
<tr>
<td>Bovine PTH***</td>
<td>9,000</td>
<td>21.4</td>
<td>66</td>
<td>2</td>
</tr>
<tr>
<td>Insulin</td>
<td>6,000</td>
<td>&lt;16.4</td>
<td>89</td>
<td>2</td>
</tr>
</tbody>
</table>

*A = 0.1 nm; ** Source: 1 = Hall and Hardwicke (1979); 2 = Maack et al (1979); ***PTH = parathyroid hormone.
size, the net charge of the protein molecule has less of an effect than observed for dextrans or proteins larger than 25 Å (2.5 nm), such as albumin or horseradish peroxidase.

The degree of sieving of a protein is not dependent on the biologic activity of the molecule itself. It is exclusively a function of the physical characteristics of the molecule (size, charge, shape) and those of the glomerular filter (GFR, permselectivity). Thus, the filtered load of a protein under normal conditions is directly related to its plasma concentration. This relationship is of fundamental importance to an understanding of the role of the kidney in the overall plasma turnover of small proteins.

The process of filtration of LMW proteins is thus obligatory in nature and homeostatic in result: obligatory in nature because it does not depend on the biological function of the protein but the physical characteristics of the molecule and those of the glomerular filter. It is homeostatic in result because filtered loads - hence renal catabolic or urinary excretion rate, or both - are directly proportional to the plasma concentration of the small protein.

1.4.2 TUBULAR EVENTS

The mechanisms for the hydrolysis and transport of LMW proteins and their catabolic products by the proximal tubule cells are given in Figure 1.4 (page 25). Filtered proteins are extensively reabsorbed by the tubular epithelium, minimal amounts appearing in the urine. The sites of accumulation within the kidney tissue has been shown by several investigators using autoradiographic and histochemical techniques to reside mainly, if not exclusively, in the proximal tubular cells (Beck and Fedynsky, 1967; Leber and
Figure 1.4. The cellular mechanisms of proximal tubular reabsorption and catabolism of proteins.*

* This schematic compares the cellular mechanisms of the proximal tubular reabsorption and catabolism of proteins or large polypeptide molecules to small, linear peptides. (a) Protein is taken up by endocytic vesicles which fuse to form phagosomes into which primary lysosomes empty their hydrolytic enzymes. Enzymic cleavage of proteins occurs in the phagolysosomes and liberated amino acids diffuse into the interstitium and return to the renal circulation. (b) Tubular handling of small, linear peptides. Hydrolysis occurs at the site of enzymes associated with the brush border of the proximal tubule. Liberated amino acids are rapidly transported across the epithelium, probably involving active pumps located at the apical cell membrane. Partially hydrolysed peptide fragments may be reabsorbed intact or undergo further intracellular cleavage prior to reabsorption. (Adopted from Carone et al, 1980).
March, 1970; Ottosen and Maunsbach, 1973). Maack (1975) showed that reabsorption of lysozyme on filtration was an active, energy dependent process with maximum capacity in excess of the usual filtered load. Major conclusions of studies on tubular uptake of proteins are summarized below.

It is generally accepted that proteins are reabsorbed by sequestration into endocytotic vesicles at the apical border of the tubular cells. This appears to be an exclusive property of the luminal cell membrane, because there is no evidence for the presence of an endocytic apparatus on the basolateral membrane of the tubular cell. Endocytotic vesicles migrate from the apical borders to the cell interior and eventually form phagosomes which fuse with lysosomes to form intracytoplasmic phago-lysosome bodies (Straus, 1964). This, as yet poorly understood process, brings into contact the reabsorbed protein with a battery of hydrolytic enzymes present in these cell organelles. After variable periods of time which may last from minutes to days, depending on the particular species of protein studied, the reabsorbed protein is no longer detectable within the secondary lysosomes or phago-lysosome. Results of intracellular localisation studies are consistent with the hypothesis that reabsorbed proteins are catabolised within the renal cells. LMW proteins may also be reabsorbed directly into the cytosol, which under normal conditions may be the main reabsorption pathway (Maack et al, 1971; Bourdeau and Carone, 1974), with reabsorption into phagolysosome bodies occuring at higher filtered load. Reabsorbed LMW proteins are catabolised by the enzymes of the proximal tubular cells to smaller peptides and amino acids, although a small proportion of reabsorbed protein may be released as intact protein but evidence for this is conflicting (Strober and Waldman,
The tubular reabsorption of proteins of the size of albumin or greater appears to be a non-selective process; the proteins are reabsorbed in proportion to their concentration in the tubular luminal fluid (Hardwicke et al, 1970). High and low molecular weight proteins may be reabsorbed by different tubular mechanisms (Maack, 1967; Harrison et al, 1968). However, there is conflicting data on the possibility of competition between individual LMW proteins for tubular reabsorption (Straus, 1962; Ravnskov, 1975). But it is not unlikely that both selective mechanisms (whether based on charge or molecular weight) and competitive tubular reabsorptive pathways may exist for some LMW proteins. It is also possible that brush border proteases can modify filtered proteins before their uptake into the renal cells (Peterson et al, 1977; Carone et al, 1982).

Though studies on the renal metabolism of small proteins indicate that LMW proteins are catabolised by enzymes of the proximal tubular cells to peptides and amino acids, other reports (Maack et al, 1971; Maack, 1975) show that intact proteins and catabolic products are released. 40% of lysozyme were released as intact protein and 60% as catabolic product, whereas more than 90% of the reabsorbed insulin was released as catabolic products. The mechanisms whereby some proteins are returned intact and others totally catabolised are not understood but may be related to differences in molecular size, susceptibility to proteolytic enzymes, or tubular transport pathway.

Contraluminal uptake of LMW proteins does occur but it is insignificant (less than 3.5%) compared to the luminal uptake (Berggard, 1970; Maack, 1975). There is also evidence that partial
hydrolysis of parathyroid hormone and insulin at the peritubular sites occurs (Katz and Rubenstein, 1973; Martin et al, 1977) but it remains to be established whether other circulating LMW proteins can also be partially hydrolysed via the peritubular route. Data obtained so far indicate that even if partial peritubular hydrolysis occurs, the major or final renal catabolism of a circulating protein takes place via the filtration-reabsorption route.

The renal clearance of a protein is, therefore, a reflection not only of the process of glomerular ultrafiltration but also that of tubular reabsorption, since all filtered molecules are candidates for reabsorption as they pass down the tubules. Under normal conditions, only minimal amounts of LMW protein are detected in urine. This is due to extensive tubular reabsorption rather than to any appreciable hindrance to the circulating LMW protein passing into the glomerular filtrate.

It must be added as a comment, however, that the physiologic significance of the process of removal of LMW proteins from the circulation by renal catabolism rather than urinary excretion, is unclear. As studies with light chains of immunoglobulins have indicated (Mogielnicki et al, 1971), the rate of renal removal from plasma is the same regardless of whether the LMW proteins are reabsorbed and catabolised or are excreted in urine. It could be argued that catabolism conserves amino acids since these are released back into circulation. But the amount of amino acids generated by renal catabolism of small proteins is probably insignificant compared to the total body pool. Another plausible speculation, devoid of clear experimental evidence, is that the catabolic products resulting from the renal hydrolysis of a protein may exert some feedback control over the production or release of
1.4.3 OVERALL RENAL PICTURE

Some aspects of renal handling of small proteins, such as filtration, reabsorption and intracellular catabolism, are common to all members of the group of LMW proteins. Other aspects are probably specific for particular proteins, for example, interaction with receptors at the peritubular side. The heterogeneity of the group, both in molecular structure and biologic function, makes it likely that some proteins may be processed by the kidney by modes not encompassed in the generalized description.

Studies of fractional turnover rate (which is the ratio of renal turnover to total plasma turnover or disappearance rate) for LMW proteins have indicated that 40-80% plasma turnover of LMW proteins is attributed to renal catabolism. Thus in the normal situation, very small amounts of plasma proteins will be excreted. Urine, however, will normally contain small quantities of proteins of renal origin, of which mucoprotein of Tamm and Horsfall is the most abundant. Based on the use of ultrafiltration and immunochemical techniques, it has been estimated that in healthy adults approximately 60% by weight of the urinary proteins consist of normal plasma proteins while the remaining fraction consists of material originating from the renal and other urogenital tissues (Poortmans and Jeanloz, 1968). The normal pattern of urinary protein composition has been stated as approximately 40% albumin, 40% tissue proteins, 15% immunoproteins and 5% other plasma proteins (Robinson and Dennis, 1978). IgM and IgD are not usually detectable (Berggard, 1970). This profile can be altered by both physiologic
and pathological factors which may affect the quantitative excretion of urinary proteins.

1.5 PATHOPHYSIOLOGY AND CLASSIFICATION OF PROTEINURIA

From the glomerular and tubular events described, it is obvious that normal kidney function with regard to metabolism of small and large proteins is characterised as follows: the small proteins have variable glomerular permeability (depending on molecular radius) and are taken up by the tubular cells where they undergo catabolism. For these proteins the kidney is the major catabolic site. In contrast, large serum proteins, exemplified by IgG, are retained by the glomerulus, do not reach the tubular catabolic sites and are not catabolised in the kidney under normal conditions. The abnormalities of urinary protein excretion seen in patients with tubular proteinuria, with nephrotic syndrome and with uraemia and nephron loss can be understood in terms of derangements of the two major functions of the kidney: the retention of proteins by the glomerulus and the catabolism of small filtrable proteins by the proximal convoluted tubules. A classification of proteinuria is given in Table 1.4 (page 32).

1.4.1 'TUBULAR' OR LOW MOLECULAR WEIGHT PROTEINURIA

Low molecular weight or 'tubular' proteinuria, as classically defined (Hall and Hardwicke, 1979), is the appearance in urine of proteins of the size smaller than albumin (MW 69,000, 37 Å, 3.7nm), in the absence of increased plasma levels. Included in this group are important peptide hormones (insulin, glucagon, parathyroid and
growth hormones), enzymes (lysozymes, RNAase), polyclonal immunoglobulin fragments, fibrin-fibrinogen degradation products and LMW proteins of unknown function (alpha1-, alpha2-, and B2-microglobulin, post-gamma globulin proteins). Circulating LMW proteins share the property of being filtered and reabsorbed by the kidney. Tubular proteinuria is usually found in patients with histological evidence of tubular and/or interstitial renal disease and/or biochemical evidence of tubular dysfunction, although it may occur occasionally as an isolated abnormality as in familial asymptomatic tubular proteinuria. Results of quantitative studies on the filtration and reabsorption of LMW proteins by several investigators have provided the basis for an understanding of the pathophysiology of LMW proteinuria (Peterson et al, 1969; Waldmann et al, 1972; Maack, 1974, 1975). The major conclusions of these studies are summarized below.

In tubular proteinuria, there is normal rate of synthesis and survival rate of LMW proteins in serum, normal glomerular permeability and normal overall metabolic rates; but vastly increased fractional proteinuric rate and correspondingly decreased fractional catabolic rate. In proteinuria due to pure tubular disease, the proximal tubules fail to take up and catabolise small proteins normally filtered by the kidney. In such cases, the normal route of LMW protein disposal, tubular catabolism, is counter-balanced by urinary excretion of intact protein, and therefore the overall rate of metabolism remains the same, and no protein accumulates in the circulation. On the basis of this pathophysiologic mechanism, patients with tubular proteinuria would have a marked increase in urinary excretion of many serum proteins that are small enough to pass through the glomerulus. This
### Table 1.4 Clinicopathologic forms of Proteinuria

<table>
<thead>
<tr>
<th>Category</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Benign proteinuria</td>
<td>Functional (fever, exercise, pregnancy)</td>
</tr>
<tr>
<td></td>
<td>Orthostatic or Postural</td>
</tr>
<tr>
<td></td>
<td>Persistent</td>
</tr>
<tr>
<td></td>
<td>Idiopathic Transient</td>
</tr>
<tr>
<td>Renal Proteinuria</td>
<td>Glomerular: Selective</td>
</tr>
<tr>
<td></td>
<td>Non-Selective</td>
</tr>
<tr>
<td></td>
<td>Haemato-proteinuria</td>
</tr>
<tr>
<td></td>
<td>Tubular</td>
</tr>
<tr>
<td></td>
<td>Mixed (both glomerular and tubular)</td>
</tr>
<tr>
<td>Pre-Renal (Overflow)</td>
<td>Destruction (myolysis)</td>
</tr>
<tr>
<td></td>
<td>Mal-production (myeloma)</td>
</tr>
<tr>
<td></td>
<td>Secretion (amylasuria in pancreatitis)</td>
</tr>
<tr>
<td>Post-Renal (Secretory)</td>
<td>Local bleeding</td>
</tr>
<tr>
<td></td>
<td>Local secretion of immunoglobulins</td>
</tr>
<tr>
<td></td>
<td>Chyluria (secondary to tumours)</td>
</tr>
<tr>
<td>Nephrogenic</td>
<td>Desquamation or destruction of kidney tissue (membrane, brush border, enzymes, mucoproteins)</td>
</tr>
</tbody>
</table>

Modified from Boesken (1979)
impairment of tubular uptake of proteins due to tubular lesion, is
typified by Fanconi syndrome, Wilson's disease, renal tubular
acidosis, congenital galactosaemia (Butler and Flynn, 1958), or
chronic cadmium poisoning (Piscator, 1966; Fleming, 1984).
Excretion of LMW proteins in these conditions is massive, up to
10,000-fold greater than in normal conditions. This is to be
contrasted with the modest increase in urinary excretion of small
proteins in lesions that affect primarily the glomerulus. In fact,
total urinary protein excretion in tubular proteinuria would not be
expected to exceed 2 g/day (Abuelo, 1983) unless there was
coexistent glomerular dysfunction. The causes of LMW proteinuria
are outlined in Table 1.5 (page 34).

Increased urinary excretion of LMW proteins may also result
from an increase in the concentration of LMW protein in the serum.
This is observed in conditions in which there is an overproduction
(or increased release to plasma) of a specific LMW protein, such as
in multiple myeloma (Bence Jones protein), monocytic and
monomyelocytic leukemias (lysozyme) (Hewell and Alexanian, 1976;
Osserman and Lawlor, 1966); myoglobinuria represents an additional
instance of this type of proteinuria. Even small increments in
plasma levels of LMW proteins may result in significant urinary
excretion, because a direct relationship between plasma
concentration and urinary excretion of a LMW protein may be found at
filtered loads below saturation of the absorption process. More
commonly, tubular damage may be accompanied by glomerular damage and
will lead to excretion of both low and high molecular weight
proteins (Defronzo et al, 1975; Grossman et al, 1974).
<table>
<thead>
<tr>
<th>Category</th>
<th>Causes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Congenital Tubulopathy</td>
<td>Faconi syndrome, oculocerebrorenal dystrophy, cystinosis, renal tubular acidosis, Bawter's syndrome, nephrogenic familial asymptomatic tubular proteinuria.</td>
</tr>
<tr>
<td>Systemic Diseases:</td>
<td></td>
</tr>
<tr>
<td>Hereditary</td>
<td>Hepatolenticular degeneration, cystinosis, glycogen storage disease, galactosaemia.</td>
</tr>
<tr>
<td>Acquired</td>
<td>Sarcoidosis, multiple myeloma, Balkan nephropathy.</td>
</tr>
<tr>
<td>Drugs</td>
<td>Drug-induced interstitial nephritis, eg methicillin, laxative abuse.</td>
</tr>
<tr>
<td>Heavy Metals &amp; Poisons</td>
<td>Cadmium, lead, arsenic, mercury, ethylene glycol, carbon tetrachloride.</td>
</tr>
<tr>
<td>Acute Renal Disease</td>
<td>Acute tubular necrosis, kidney allograft rejection.</td>
</tr>
<tr>
<td>Infective</td>
<td>Acute/chronic pyelonephritis.</td>
</tr>
<tr>
<td>Miscellaneous</td>
<td>Proteinuria of hypothermia, Nephrocalcinosis, nephrolithiasis.</td>
</tr>
</tbody>
</table>

From Hall and Hardwicke (1979)
In primary glomerular lesions, increases in glomerular permselectivity, and hence filtered loads of small proteins, is modest, because the glomerular sieving coefficient of these proteins is normally high. Pathologic changes of the glomerular membrane in primary glomerular lesions, such as occurs in the nephrotic syndrome (Waldmann et al, 1972), do not significantly affect the passage of LMW molecules which normally pass the membrane with relative ease. If tubular reabsorption is not impaired, part of the increased load of LMW protein will be reabsorbed, hence urinary excretion will be minimized. That urinary excretion does increase at all in primary glomerular nephropathies may be due to the generally low affinity of the tubular absorption process rather than to tubular absorptive maxima (Tm's) which are close to the normal filtered load. With larger proteins such as albumin, small changes in glomerular permeability will lead to large increases in filtered load. If the Tm of albumin is close to the normal filtered load, as postulated by some authors (Oken et al, 1972), the excess load will be excreted into urine. Even if the Tm of albumin is much larger than the normal filtered load, however, an increase in glomerular permeability will lead to massive albuminuria due to the low affinity of the reabsorption process. In primary tubulopathies, on the other hand, the predominantly LMW proteinuria is due to impairment of tubular uptake of the normally proportionally high filtered loads of small proteins. Albuminuria will be less prominent than in glomerular nephropathies. The term high- and low-molecular weight proteinuria, however, is relative because due to the very high plasma concentration of albumin compared to that of
small proteins, albumin may still account for a large fraction of the total protein excretion in LMW proteinuria.

In the nephrotic syndrome the glomeruli become abnormally permeable to proteins of intermediate size (albumin, IgG), although the sieving function of the glomerulus is not completely lost and large molecules such as IgM (MW 900,000) and macroglobulin continue to be retained (Joachim et al, 1964; Barth et al, 1964). In such circumstances, when over 50% of IgG metabolism is accounted for by proteinuria, over 95% of lambda L-chain is being catabolised (Bienenstock and Poortmans, 1970; Waldmann et al, 1972) presumably by uptake and breakdown by proximal tubules. It therefore appears that there is a preferential tubular uptake of small proteins compared to IgG and other intermediate size proteins.

1.5.3 MIXED PROTEINURIA: URÆMIA AND NEPHRON LOSS DISEASE

The serum concentrations of LMW proteins are markedly increased in patients with uraemia who have a greatly decreased number of functioning nephrons. This is not due to increased rate of synthesis but to prolonged serum survival which is associated with comparable reduction of fractional metabolic rate and fractional renal catabolic rate (Waldmann et al, 1972), although slight increases in production of some LMW proteins, such as parathyroid hormone (Habener et al, 1971), can not be excluded. Presumably, the reduced fractional catabolic rate of LMW proteins results from the loss of functional renal tissue and entire nephrons. Thus LMW proteins are not filtered through the glomerulus and are not exposed to their normal catabolic site, the renal tubules, and in addition are not excreted into the urine in excessive quantities. This
results, therefore, in accumulation in the circulation. The second abnormality seen in patients with uraemia is the increased proteinuric rate associated with a marked increase in proteinuric/catabolic ratio. This presumably reflects functional damage of tubules in nephrons where glomerular filtration of proteins persists, and may explain the fact that acrylamide gel analysis of urine of uraemic patients frequently shows a tubular pattern.

Reduced renal filtration would be expected to cause an elevation in serum levels of many LMW proteins normally filtered through the glomerulus. This elevation would be most marked for those small proteins that do not have extrarenal sites of metabolism and that do not have servoregulatory mechanisms that decrease the rate of synthesis in the face of an elevated serum concentration. The accumulation of LMW proteins in patients with grossly decreased numbers of functioning nephrons obviously produces widespread exposure of many physiological mechanisms to actions of the biologically active enzymes, hormones, and other proteins which are normally excreted or catabolised within the kidney. This accumulation of biologically active substances may play a role in causing some of the manifestation of the uraemic syndrome.

1.5.4 OTHER TYPES OF PROTEINURIA

Another pathological cause of increased amounts of protein in urine is a result of abnormal losses of protein not derived from the plasma filtrate, as in kidney damage which yields proteins derived from fragments of kidney tissue (Boesken, 1979). This is nephrogenic proteinuria. In individuals without primary renal
disease or multiple myeloma, transient elevations in urine proteins may occur. Fever, thyroid disorders, heart disease, intense exercise, exposure to cold, emotional stress, pregnancy, orthostatic conditions, and a number of other conditions have been reported to increase protein output (Latner, 1975; Ward, 1978). For these reasons, it may be important to determine if the proteinuria is persistent if it is to be used as an indicator of renal disease.

1.6 INVESTIGATION OF PROTEINURIA AND RENAL FUNCTION

The understanding of the various pathogenic mechanisms which precipitate increased urinary protein excretion (Hardwicke, 1975; Boesken et al, 1973), has provided the basis for the various approaches used in studying proteinuria. Of the parameters used in the investigation of proteinuria, the molecular size of the protein either in serum or excreted in urine is the most important in further analysis. Proteins have been separated on molecular sieves such as Sephadex (Hardwicke et al 1970); on sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) - a separation also based on molecular radius; or by immunochemical quantitation of proteins of known size (Berggard, 1970; Fleming, 1984; Bernard et al, 1987).

Another electrophoretic method used for analysis of proteinuria utilizes cellulose acetate membrane as support medium. Separation is based on electrostatic charge and usually gives five major fractions (albumin, alpha-1, alpha-2, beta and gamma globulin bands). Quantitation of the different fractions, after staining, can be effected densitometrically. Current practice expresses ratio of
albumin to total urinary proteins, and in normal subjects this proportion ranges from 12 - 55%. This method is of value in detecting glomerular involvement (albumin greater than 55%), but gives little information on the selectivity of proteinuria, and is of little value in tubular damage, where, though the percentage of albumin may be low, it tends to overlap with physiological values.

Immunoelectrophoresis, too, separates proteins according to their charge with subsequent identification by their interaction with antisera. Patterns are usually interpreted visually. Physiological proteinuria (concentrated to 60g/l) usually reveals albumin as the main component, together with faint amounts of gamma-globulin, although more complicated patterns may be occasionally observed. Selective proteinuria can be distinguished from non-selective proteinuria which closely resembles the control serum run simultaneously with the urine samples. Mixed patterns may remain unrecognized because high amounts of glomerular proteins hide the typical tubular features. Though sensitive, its major drawbacks are that the interpretation of the electrophoretic patterns is visual with accuracy depending on the experience of analyst; and the available antisera used contain antibodies to serum proteins and not to 'urinary proteins'.

In SDS-PAGE, SDS treatment of proteins causes them to form negatively charged protein-SDS micelles, whose migration through the gel depends on the molecular size of molecules. The patterns can be stained and read visually or quantitated densitometrically. The method is sensitive and adequate for qualitative analysis of urine proteins. Interpretation of SDS-PAGE is based on the principle that proteinuria signifies either increased permeability of the glomerular-capillary membrane or diminished tubular absorption. Glomerular disease is associated with an increased clearance of albumin and higher molecular
weight proteins (60,000 - 300,000 daltons), whereas tubular damage is associated with the predominant excretion of proteins of molecular weight less than albumin (10,000-70,000 daltons). Based on these separation patterns, proteinurias are classified as selective (60,000 - 150,000 daltons), nonselective (60,000 - 1,000,000 daltons), tubular (10,000 - 70,000 daltons) and mixed (10,000 - 1,000,000 daltons). Comparing clinical and biological data of patients suffering from renal diseases, analysis of urinary protein by this method has been proved to be a useful and non-invasive tool for diagnosis and follow-up (Boesken, 1979). SDS-PAGE analysis of urinary proteins is also useful in determining qualitatively normal or pathologic character of a quantitatively normal proteinuria, especially for early diagnosis of nephropathy in patients, such as diabetics, which are particularly prone to suffer from renal complications. It can also be used to confirm the clinical resolution or predict recurrence of renal disease, and in such situations as orthostatic, or myeloma proteinuria, or any elevation of urinary protein output of unknown aetiology (Balant and Fabre, 1979).

Another method of studying proteinuria is to measure the clearance of individual proteins of known molecular size using immunochemical methods. For assessing changes in functional intergrity of the glomerulus, the sieving coefficient of albumin, which by virtue of its molecular size and charge is just restrained by the filtration barrier in health, is commonly used. Glomerular selectivity is classically calculated from the slope of the clearances of proteins such as albumin, or IgG and alpha-2 macroglobulin in relation to transferrin clearance (Joachim et al, 1964; Boesken, 1979). This indicates the nature of glomerular damage and whether the proteinuria is selective or non-selective.
The proteinuria of tubular disease is qualitatively different from that associated with glomerular disease (Butler and Flynn, 1958). The major components are low molecular weight alpha globulins and beta globulins normally filtered at the glomerulus and reabsorbed by the proximal tubules. Tubular proteinuria may be recognized by measurement of tubular selectivity of representative low molecular weight proteins such as lysozyme (Barratt and Crawford, 1970), beta-2 microglobulin (Peterson et al, 1969), or alpha-1 microglobulin (Kusano et al, 1985). Boesken (1979) measured clearances of beta-2 microglobulin, retinol binding protein (true tubular proteins), and immunoglobulin light chains, alpha-1 antitrypsin, alpha-2 glycoprotein (a group of proteins with M.W 70,000 - 40,000 daltons) to assess tubular proteinuria. Lysozyme to creatinine ratio has been shown to be related to the proportion of filtered lysozyme escaping proximal reabsorption and increased some 100 times in Fanconi syndrome (Barrat and Crawford, 1970). Renal tubular damage also results in the release of tubular enzymes such as gamma-glutamyl transpeptidase and N-acetyl B-D-glucosaminidase whose urinary activities may also be related to creatinine. In fact there are considerable advantages in using creatinine, whose excretion is relatively constant, as a reference for concentration of urinary proteins, but reference to total protein, particularly when this is within or near normal range, may be ill-defined (Barratt, 1983). It may be preferable to measure one or two individual representative proteins rather than a whole range of cleared proteins.

Once proteinuria has been detected, its proper interpretation requires an appreciation of the physiological and pathological factors which can alter the amount and composition of urinary proteins. Consideration should, of course, be taken of the method used in
quantitating proteinuria when interpreting the results as different methods may give slightly different values for the same sample.

1.7 SUMMARY OF LITERATURE REVIEW

Nephropathy, or renal damage, may be caused by metabolic, chemical or immunologically-mediated insults, in addition to non-renal diseases such as diabetes, SLE, amyloidosis, cancer and hypertension. One of the important hallmarks of nephropathy, so recognized as far back as in the days of Hippocrates, is proteinuria - the excretion of increased protein in urine in excess of 150 mg/day. Proteinuria arises due to failure by the kidney to perform one or both of its important functions with respect to protein homeostasis: retention of high molecular weight proteins by the glomerulus, and reabsorption and catabolism of filtrable LMW plasma proteins by the proximal convoluted tubules. The former gives rise to glomerular proteinuria, which may be selective or non-selective, and the latter gives 'tubular' or low molecular weight proteinuria. Failure of both functions, as occurs in diseases characterised by loss of nephron, gives a mixed pattern of proteinuria. Proteinuria may also arise from extra-renal causes.

From available evidence, it appears that the same cause of nephropathy may give rise to different morphological and/or functional changes; conversely, similar morphological and/or functional patterns may stem from a broad range of causes. It does appear, however, that irrespective of the cause, renal involvement is commonly exemplified by proteinuria, the pattern of which depends on the site and extent of renal involvement. This is usually the basis for investigating proteinuria in the assessment of kidney function in health and disease.
As discussed, various methods and approaches have been used in studying proteinuria in the detection of early renal involvement, assessment of progression of lesion, monitoring of treatment, or prediction of recurrence of renal disease. Of the parameters used for the investigation of proteinuria, the molecular size of the proteins excreted in urine is the most important and practical. Measurement of specific proteins by immunochemical methods allows studies of their clearances or sieving coefficients, making it possible to assess changes in glomerular and tubular integrity. In addition, measurement of one or two representative 'glomerular' and/or 'tubular' proteins may be preferable to a whole range of cleared proteins in the assessment of renal function.

1.3 THE RESEARCH PROBLEM AND AIM OF THE STUDY

For many years investigators have measured levels of proteins (including enzymes) in urine and serum that might serve as markers of diseases of the kidney or individual nephron segments. Changes in the levels of non-renal proteins have been attributed to changes in glomerular permeability or to altered tubular reabsorption. Recently, attention has been focused on the role of tissue specific antigens and metabolic enzymes which may be characteristically distributed within morphologically and physiologically defined segments of the nephrons (Scherberich and Mondorf, 1979; Falkenberg et al, 1986). Quantitative or qualitative determination of such proteins in urine and changes in their excretion pattern may help to give a better insight into the localisation of kidney tissue damage. The urinary excretion profile of serum proteins or of their products resulting from catabolism in the
kidney as well as elimination of kidney specific tissue components, connected or not connected with enzymic activity, may be of further potential diagnostic value.

Measurement of low molecular weight proteins in the assessment of renal tubular function is in wide use. LMW proteins may be useful markers of renal function as they are freely filtered at the glomerulus, and reabsorbed by the lining cells of the proximal tubules. Changes in glomerular filtration or tubular reabsorption would therefore be reflected by plasma or urinary levels of LMW proteins. Lysozyme (Butler and Flynn, 1958), beta-2 microglobulin (Barratt and Crawford, 1970; Wibell et al, 1973; Ormos et al, 1984), alpha-1 microglobulin (Yu et al, 1983; Nogawa et al, 1984; Kusano et al, 1985), and retinol binding protein (Bernard et al, 1982a,b; Topping et al, 1986; Bernard et al, 1987) have been used for this purpose. Measurement of urinary albumin to assess the extent of glomerular involvement has also been undertaken (Mogensen and Christensen, 1984; Watts et al, 1986). However, the sensitivity of lysozyme as a marker may be limited as there is selectivity in protein handling with preferential reabsorption of cationic molecules such as lysozyme (Christensen et al, 1983).

Of the LMW proteins that have been employed for the assessment of renal function, $B_2$-microglobulin ($B_2M$) is by far the most widely used. But recent work (Bernard and Lauwerys, 1981; Bernard et al, 1982a; Bastable, 1983) has indicated that $B_2M$ may not be suitable for assessing renal tubular function. It is unstable, easily degraded in acid urinary pH at both room and physiological temperatures, and therefore loss of $B_2M$ can occur in the bladder. $B_2M$ levels are also elevated in plasma in non-renal diseases (Cooper and Child, 1981), and there does not appear to be a clear consensus on its clinical relevance.
in monitoring renal function and predicting rejection in kidney transplantation (Schweizer et al, 1981; Vincent et al, 1979). Another LMW protein, retinol binding protein (Peterson and Berggard, 1971; Rask et al, 1980), which is a major component in tubular proteinuria and appears in the plasma at higher concentration than B_{2}M has been suggested as an alternative marker of renal function (Scarpioni et al, 1976; Topping et al, 1986; Bernard et al, 1987). But recent work (Miller and Varghese, 1986) found retinol binding protein unreliable as a predictor of rejection in kidney transplantation. The increasing realization of the non-suitability of proteins hitherto accepted as markers of renal damage prompts the need to search for newer and more stable protein markers that may be sensitive indicators of changes in renal function.

Recently a new uncharacterised protein, Urine Protein 1 (UP1), has been reported as present in the urine of patients with tubular proteinuria (DAKOPATTS Catalogue, 1985). The origin and clinical significance of this protein has not been characterised: it might be of renal or extrarenal origin, or a breakdown product of another (renal or serum) protein. However, being a low molecular weight protein (approximate molecular weight less than 25,000 daltons) it may be potentially useful in the assessment of renal function in general and tubular integrity in particular.

This study therefore seeks to investigate the clinical significance of Urine Protein 1. The purification, characterisation and studies on the site of origin (synthesis) and route of elimination from the body would also been attempted. The clinical usefulness of UP1, if any, would be compared with other LMW proteins (a_{1}-microglobulin, retinol binding protein) already in use for the evaluation of renal function. It is hoped that this protein may be
found useful in the diagnosis and follow-up of renal diseases.

1.9 PLAN OF STUDY

The following outline plan was designed with the major aims of the study in mind:

1. Purification and characterisation of UP1:
   a). Isolation and purification of UP1 from patients with tubular lesions.
   b). Raise antiserum against purified protein in rabbits - to provide antiserum for the research and also help check purity of UP1
   c). Characterisation of purified UP1:
      - electrophoretic mobility, isoelectric point
      - lectin binding studies
      - stability studies
      - amino acid sequence
      - establish site of synthesis in the body

2. Clinical Significance Studies:
   a). Develop Enzyme-Linked Immunosorbent Assay (ELISA) method for UP1 and retinol binding protein, to be used in the clinical investigations
   b). Establish reference range for UP1 in body fluids
   c). Study, cross-sectionally, changes in serum and urine UP1 levels in renal disease
   d). Monitor urine UP1 from patients on cis-platin cancer
e). Monitor serum and urine UP1 changes pre- and post- kidney transplantation.

CHAPTER TWO

PURIFICATION AND CHARACTERISATION OF UP1

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2.1 INTRODUCTION

About 5 years ago, Dakopatts Immunoglobulin, Copenhagen, Denmark, produced an antiserum against 'tubular' proteins and proceeded to isolate a low molecular weight (LMW) protein, with a molecular weight of 20,000, not previously reported, from patients with tubular proteinuria. Because the protein was isolated in urine, it was called Urine Protein 1 (UP1).

Preliminary experiments showed that UP1 may be a new protein as it did not react with either alpha-1 microglobulin (a1M) or retinol binding protein, both LMW proteins.

In order to perform any useful investigations on the clinical usefulness of UP1 it was necessary to purify the antigen which was not commercially available. Antiserum was also produced against the purified antigen; stability of the antigen was also investigated.

2.2 PURIFICATION

2.2.1 MATERIALS AND METHODS

Material

48 hour urine samples (about 5 litres) high in UP1 was obtained from a patient on gentamicin chemotherapy at the Royal Surrey County Hospital, Guildford (Courtesy of Dr Ashraf). This urine was preserved in 0.1% azide and kept at 4°C until used. Sheep anti-human albumin and controlled pore glass beads already coated with amino groups were obtained from Guildhay, Biochemistry
Department, University of Surrey. Rabbit anti-human UP1 was supplied by Dako Ltd, High Wycombe, Bucks, UK. Glutaraldehyde and polyethylene glycol 8000 were obtained from Sigma Chemical Co., and Agarose A was supplied by Pharmacia Fine Chemicals. All reagents were of analytical grade.

**Methods**

**Concentration of Samples**

The urine sample was centrifuged to remove sediment, and concentrated about 50 times by dialysis against PEG-8000 using Visking dialysis tubing, down to a final volume of approximately 100 ml. This method was also used during the gel chromatography for concentrating fractions high in UP1. Recovery of UP1 after concentration varied between 80-90%. Later in the study, with the availability of an ultrafiltration concentrator (Amicon CH 2A, Amicon Corporation, Danvers MA 01923, USA), new samples were concentrated using the 10,000-dalton molecular weight cut-off spiral catridge, and the 30,000-dalton catridge used to exclude high molecular weight substancess. After concentration by ultrafiltration, samples were fed straight onto the immunoaffinity columns, bypassing the gel chromatographic separation step.

**Gel Chromatography on Sephadex G-75**

The concentrated urine was subjected to gel chromatography on Sephadex G-75 column (85x2.5 cm), equilibrated with 0.02M Tris-HCl buffer, pH 8.0, containing 0.5M NaCl and 0.05% azide. The eluates were assayed for protein by measurement of the absorbance at 280nm. UP1 content of the fractions was estimated by single radial
immunodiffusion on agarose gel (Mancini et al, 1965). The main part of UP1 emerged slightly later than the major protein peak (Figure 2.1, page 52). Fractions containing UP1 were pooled, as indicated in Figure 2.1 (arrowed), and concentrated by dialysis.

Immunoaffinity Chromatography:

Preparation of anti-albumin and anti-UP1 columns

2g amino-controlled pore glass beads (amino-CPG, 120-200 mesh) were washed three times in bicarbonate-saline (0.1M NaHCO₃, 0.154M NaCl), pH 8.0. This was then incubated with 40 ml bicarbonate buffer and 10 ml 25% glutaraldehyde, with gentle mixing, at room temperature for 2 hours. The amino-CPG was now activated. Unreacted glutaraldehyde was washed three times in an excess of buffer. Activated amino-CPG was then incubated with respective protein antibodies (15 mg/g CPG). This was mixed gently for 30 minutes, then left overnight at 4°C, and finally for 3 hours at room temperature with gentle mixing. The glass beads, with their respective immobilised immunoglobulins, were now poured into columns, washed extensively and stored at 4°C in bicarbonate buffer, pH 8.0, containing 0.1% azide as preservative. Rabbit anti-UP1 serum was first passed twice through anti-albumin column to remove any contaminating albumin in the anti-serum.

Affinity purification procedure

All samples were exhaustively dialysed against distilled water before application on the columns. Samples were first adsorbed onto the anti-UP1 column, then desorbed and passed through the albumin column to remove any contaminating albumin. The general steps for the process of affinity adsorption and desorption, similar to that
Figure 2.1. Gel filtration on Sephadex G-75. Urine from a patient on gentamicin was concentrated and dialysed against 0.05M Tris-HCl buffer pH 7.4 containing 0.1M NaCl and 0.5% azide. A sample of urine (5 ml containing about 1 gm total protein) was applied to the column (2.5 x 85 cm) equilibrated with the same buffer. Fractions of 5ml were collected at a flow rate of 40 ml/hour. The distribution in the effluent of UP1 was determined by single radial immunodiffusion and graph indicates squared diameter of rings (divided by 100) plotted against fraction number. Fractions containing UP1 were pooled (as indicated by arrows) and concentrated for further purification.
described by Romer and Rauterberg (1980), are summarised below:

- Adsorption of protein on the affinity column: 5-10 ml sample was added and mixed gently with the beads in column for 20 minutes.

- Washing the column between loading and elution: first with bicarbonate buffer, then with distilled water, 5 minutes each.

- Desorption and elution of protein from column: 0.1M glycine-HCl buffer, pH 2.8, added and mixed for 5 minutes before elution. The eluates were adjusted to pH 7.4 and stored at 4°C.

- Regeneration of the column with starting buffer: by washing with bicarbonate buffer for 5 minutes.

UP1 content of the affinity fractions was estimated by immunodiffusion on cellulose acetate as described by Kohn (1976). Immunofixation by floatation in anti-UP1, the use of alkaline phosphatase labelled swine anti-rabbit IgG (Dako) and subsequent visualisation using enzyme substrate were as described by Kohn et al (1983), using shorter washing periods of 1 hour. The result showed that UP1 was completely desorbed by the desorption step. Recovery of UP1 at this step was estimated to be 20-30%, with considerable amounts washed out in the washing steps.

Total protein of the final purified UP1 was determined by the Folin method of Lowry et al (1951) as modified by Hertree (1972).

Electrophoretic methods: checking purity and characterisation

The purity of the isolated UP1 was assessed using SDS-PAGE (discontinuous) as described by Hames (1981). The gel was stained using an ultrasensitive silver stain III (Merril et al, 1981); and
by gel immunoelectrophoresis as described by Kohn (1976) using polyvalent and monovalent sera.

Cellulose acetate electrophoresis (CAM) (Kohn, 1976) was used to investigate the electrophoretic mobility and lectin binding properties of UP1. Immunofixation and 'lectin fixation' were performed essentially as described by Kohn and Riches (1978).

Isoelectrofocussing technique was performed, to establish the isoelectric point of the protein, on LKB Multiphor System using commercially obtained analytical thin-layer polyacrylamide gels (5%) containing Ampholine (2.2%) and cross-linking of 3%, pH 3.5-9.5 (Code no. 1804-101, LKB-Produker AB, Bromma, Sweden). Manufacturers' instructions were religiously followed.

2.2.2 RESULTS

Purity of the isolated protein as assessed by immunoelectrophoresis is shown in Plate 2.1 (page 56). UP1 was tested against Dako polyvalent anti-urinary microglobulins; the polyvalent antiserum reacts with UP1, alpha-1 microglobulin (α1M), retinol binding protein (RBP) and B2-microglobulin (B2M). As can be seen from the plate, the polyvalent antiserum gives several precipitin lines with the starting material but only one precipitin line with purified UP1. UP1 did not react with anti-RBP.

CAM electrophoresis shows a separation of concentrated urine from a patient with mixed proteinuria, purified UP1 and normal serum. The result shows that isolated UP1 migrates in the α2-region. 'Lectin fixation' studies showed that the protein binds con A. CAM electrophoresis result is shown in Plate 2.2 (page 57). On incubating con A with UP1 antigen, the supernantant was no longer
immunofixable with anti-UP1 (Figure 2.2(b)), showing that the protein was precipitated by conc A.

SDS-PAGE showed two narrow bands barely distinguishable from one another at the molecular weight region of 10,500 daltons, showing that on SDS treatment UP1 dissociates into two almost equal units. Isoelectrofocussing showed UP1 to be a negative protein with a pi of 4.60±0.10 (see Plate 2.4, page 59).
Plate 2.1. Gel Immunoelectrophoresis of purified UP1 and starting material (SM). Abbreviations: SM = starting material, patient with on gentamicin (SM1), patient with the nephrotic syndrome (SM2); aUM = rabbit anti-urine microglobulin (a polyvalent antiserum against tubular proteins, UP1, aM, RBP and B2M); aRBP = rabbit anti-RBP; aUP1 = rabbit anti-UP1. Note that aUM gives a single precipitin line with purified material (UP1) on dilution of the antigen. Anti-RBP did not precipitate purified UP1, either neat or diluted (1/10).
Plate 2.2. Cellulose acetate electrophoresis of urine and purified UP1.

A. Normal urine (1), pathological urine (2, 3, 4); immunofixation: pathological urine (5, 6, 8); normal urine (7).

B. Lectin binding studies: pathological urine (1), purified UP1 immunofixed with anti-UP1 (2); purified UP1 pre-incubated with con A and supernatant applied, followed by anti-UP1 'fixation' (3); purified UP1 lectin-fixed with con A (4).

Note failure of anti-UP1 to fix the antigen pre-incubated with con A. Also note the alpha-2 electrophoretic mobility on both strips (A, B).
Plate 2.3. SDS-Polyacrylamide gel electrophoresis of purified UP1 using 15% gel concentration.
Purified UP1 (1, 2, 7, 8); RBP (3); Molecular weight markers (4, 10); Dako purified UP1 (5); starting materials (6, 9).
Plate 2.4. Isoelectrofocusing of purified UP1.
Isoelectric point markers (1, 4); purified UP1 (2, 3). Estimated pI of UP1 = 4.6±0.1.
2.3 ANTISERUM PRODUCTION

2.3.1 INTRODUCTION

The production of a specific antibody is one of the most important steps in the development of an immunoassay procedure, such as enzyme-linked immunosorbent assay (ELISA). After the production of purified UP1, it was therefore considered desirable to obtain antiserum for further analytical work and to be able to use it as a final biological check on the purity of the isolated antigen.

2.3.2 METHODS

Immunisation Schedule

A solution of the purified human UP1 in 0.1M PBS was emulsified with an equal volume of a non-ulcerative adjuvant. The non-ulcerative adjuvant is as effective as Freund's adjuvant in eliciting immune response but does not produce as much ulceration at the site of injection; it is routinely used by Guildhay Antisera Ltd, University of Surrey, Guildford). An adjuvant ensures that the injected immunogen is released slowly, and also facilitates phagocytosis. Two rabbits (kindly payed for by Guildhay Antisera Ltd) were injected intracutaneously on the back at 10 different spots, total antigen was 200 ug in approximately 0.5ml per rabbit. The first 'booster' injections of emulsified UP1 (100 ug/rabbit) were given subcutaneously on the third week after the initial injection. Blood was collected from the marginal ear vein on day 9, 12, and 16 following boosting. After clotting at room temperature
overnight, the blood was centrifuged at 3000 rpm for 20 minutes and the sera decanted. 0.1% azide was added as preservative and the sera stored at 4°C. Before use, immunoglobulin G was precipitated by 40% ammonium sulphate, and dialysed exhaustively against normal saline, azide was again added to a final concentration of 0.1% as preservative. Sera were assayed for specificity (using double radial immunodiffusion) and for titre (by ELISA, see Chapter 3). The highest titre was obtained between 12 to 16 days after boosting and this period was used for all post-boost animal bleeding.

The response of animals, as evaluated by the titre of the antiserum produced, was monitored after the first boost until low plateau of titre was obtained (10-12 weeks). For maximum animal response, that is production of high titre antiserum, therefore, time between successive boosts was set at about 11 weeks.

2.3.3 RESULTS

Antiserum was assessed for titre and specificity. Specificity was checked by double immunodiffusion and results are shown in Plate 2.5 (page 62). As can be seen, there was a single precipitin arc when the antiserum was tested against purified UP1. Antiserum titre was determined from the dilution curve as shown in Figure 2.2 (page 63). The titre was taken as that antiserum dilution that was needed to give 50% of the maximum absorbance. The titre of the bleeds ranged from 10k to 16k.
Plate 2.5. Double radial immunodiffusion to test antiserum produced. Antigen was applied in the centre well. Outer wells contained: non-immune rabbit serum (1); ammonium sulphate (40%) precipitated UP1 antiserum (2); neat rabbit immune serum (3, 4, 5, 6). Note absence of precipitin arc with the non-immune serum and presence of single precipitin arc for other wells.
Figure 2.2. Antiserum dilution curve for titre determination. The titre was estimated by obtaining the antiserum dilution required to give 50% maximum response (absorbance). Titre of antiserum bleeds ranged from 10 - 16k.
2.4.1 INTRODUCTION

Knowledge of the stability of a protein is essential in the appreciation of its full clinical usefulness and practicality. Absence of such knowledge may lead to questionable conclusions based on analytical results on the protein. Such was the case with B₂-microglobulin (B₂M) before its instability in pathological urine was known. In addition, stability characteristics of a protein need to be known and, if necessary, special sample collection, handling and analytical procedures may be designed to meet whatever stability problems that may exist. Therefore, to be able unequivocally to interpret data obtained in (later) clinical studies, it was decided that an investigation on the stability characteristics of UP1 was necessary.

UP1 stability was studied, in vitro, using normal and pathological acid concentration of urine, under different laboratory storage and handling conditions.

2.4.2 MATERIALS AND METHODS

Effect of pH, temperature and storage time on UP1 concentration

Early morning urine samples from healthy volunteers with UP1 concentrations ranging from 45 to 65 ug/l were pooled and divided into aliquots and the pH adjusted to 2 to 9 with HCl or NaOH (1 mol/l). Each sample was then divided into three portions, one incubated at 4°C, one at room temperature (19-22°C) and the other at
37°C for periods of 2, 4, 8, 24 and 48 hours. Incubation was stopped by diluting the sample 10 times in PBS containing Tween-20 (0.5ml/l) and gelatin (1g/l) and analysed by ELISA technique (see chapter 3).

Stability of UP1 in normal urine at room temperature, 4°C, -20°C and -40°C

The normal condition of samples in clinical laboratory usually involves refrigeration at 4°C and freezing at -20°C or -40°C. The effect of storage of normal urine, sample (pH 5.8) at these temperatures for a long period of time was investigated. All samples contained 0.1% azide as preservative.

Effect of repeated freezing and thawing on UP1 concentration

Early morning sample (pH 6.1) was divided into two portions and stored at -20 and -40°C. These were then thawed repeatedly and the UP1 concentration measured. Minimum time period between successive thaws was 48 hours.

2.4.3 RESULTS

The results of the stability of urine with varying pH is shown in Figures 2.3 to 2.5 (pages 67-69). At room temperature, there was no significant change in concentration of UP1 during periods of incubation up to 8 hours, but at 24 hours incubation the fall in UP1 concentration became significant below pH 3.0, with up to 40% loss of UP1 immunoreactivity for 48 hours at pH 2.0 (Figure 2.3). At 37°C, incubation times of 4 and 8 hours showed significance decrease in UP1 below pH 3.0. At 4°C, significant decrease in UP1 was observed at pH less than 3.5 at 24 and 48 hours incubation.
There was no significant change in UP1 concentration of normal urine (pH 5.8) after 5 months at 4°C and for over a year at -20 and -40°C, and up to one week at room temperature (19-22°C). The effect of repeated thawing on UP1 concentration are shown in Figure 2.6 (page 70). Significant change (greater than 2 standard deviations) in concentration was obtained only after 5 times of repeated thawing.
FIGURE 2.3. EFFECT OF pH AND INCUBATION TIME AT ROOM TEMPERATURE (19-22°C) ON THE STABILITY OF UP1 IN URINE
FIGURE 2.4. EFFECT OF pH AND INCUBATION TIME AT 37°C ON THE STABILITY OF UP1 IN URINE
FIGURE 2.5. EFFECT OF pH AND INCUBATION TIME AT 4°C ON UP1 STABILITY IN URINE
FIGURE 2.6. EFFECT OF REPEATED THAWING ON THE STABILITY OF UP1.
Human UP1 has been purified by combined gel and immunoaffinity chromatography and characterised. The purity of UP1, as assessed by immunoelectrophoresis and SDS-PAGE, and by the antiserum produced, is not in doubt. The molecular weight of UP1 was assessed by Dako using analytical gel chromatographic technique to be about 20,000 daltons. In this study using SDS-PAGE, UP1 showed two barely distinct bands of approximately the same molecular weight of 10,500 daltons. It appears that on SDS-treatment, UP1 dissociates into two approximately equal polypeptides or monomeric units. Molecular weight of UP1 is therefore about 21000 daltons.

On CAM electrophoresis, UP1 appeared in the alpha-2 region - migrating in a fairly homogeneously band. It is therefore electrically homogeneously charged. Isoelectrofocussing demonstrated the protein to be a negative protein, with pI of about 4.60.

Lectin fixation on CAM and lectin precipitation of UP1 shows that the protein binds to concanavalin A (con A). Concanavalin A (con A), isolated from jack bean, binds to alpha-D-glucopyranose, alpha-D-mannospyranose, alpha- and beta- fructofuranose and alpha- and beta- arabofuranose and will precipitate numerous polysaccharides containing these residues in the terminal position. Interaction with polysaccharides and glycoproteins containing these leads to formation of insoluble cross-linked complexes. Many serum glycoprotein contain mannose as a prominent part of their carbohydrate core and therefore bind con A; a mean of 48% plasma proteins have been shown to bind con A (Warren et al, 1980). UP1 is therefore a glycoprotein containing any of the carbohydrates mentioned above in a terminal position.

The stability of UP1 has been clearly demonstrated in this study -
with no significant change in concentration in pathological acid urine (pH > 4.0) at 37°C and room temperature for up to 24 hours. This obviates the need for alkalisation of urine either after collection or giving patients alkalis to ensure urine of high pH. It also indicates that UP1 concentration may not be significantly altered in the urinary bladder in overnight urine samples: therefore the measurement of urinary UP1 would be a more reliable reflection of 'true' urinary levels than say B2M which has been shown to be unstable in acid urine (Davey and Gostling, 1982; Bernard and Lauwerys, 1982a). Since no enzyme inhibitor was added to the urine samples, this could be an indication of greater resistance of UP1 to protease hydrolysis. In this respect, UP1 shows similar advantages to those suggested for RBP (Bernard and Lauwreys, 1981) and a1M (Yu et al, 1983).

In conclusion, high purity UP1 has been isolated and used to produce a monospecific antiserum. The protein has been shown to be a negative LMW glycoprotein with alpha-2 electrophoretic mobility. It is very stable in urine over a range of pH found in routine clinical practice. It exhibits remarkable stability comparable to a1M and RBP but superior to B2M. This property may be useful in clinical practice as it would obviate the need for alkalisation of urine samples without the fear of false-negative results.
CHAPTER THREE

METHODS DEVELOPMENT AND VALIDATION

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IMMUNOTURBIDIMETRIC ASSAY FOR MICROQUANTITIES OF ALBUMIN . 92
3.1 INTRODUCTION.

Urine Protein 1 (UP1) is a low molecular weight alpha-2 microglobulin first isolated in urine of patients with 'tubular' proteinuria by Dakopatts, Copenhagen (Dako Catalogue, 1985). Since no sensitive method for its estimation has been reported, it was decided to develop an Enzyme-Linked Immunosorbent Assay (ELISA) for its measurement in biological fluids in order to enable an evaluation of its clinical significance.

ELISA, like all immunoassays, combines the specificity of an antigen-antibody reaction with the sensitivity of an indicator system. It is a heterogeneous assay that employs a solid phase component which allows the separation of bound from unbound components. Since it was first described (Engvall and Perlman, 1971), ELISA has become widely used because of its simplicity and accuracy, and is now accepted as a useful method for the detection or quantitation of proteins (Brauner et al, 1983; Romer and Rauterberg, 1984). Several types of ELISA are now available (O'Beiner and Cooper, 1979). In this study, the classical 'sandwich' first antibody assay for the detection of antigen was used. This is a 'two-site' immunoassay in which the antigen is fixed by binding to excess first antibody that had been previously immobilised on a solid phase; complexes so formed are detected and quantified by their reaction with a second antibody conjugated to an enzyme. A modification of this classical sandwich ELISA that involves use of enzyme labelled anti-IgG instead of enzyme-labelled anti-antigen, has also been reported (Belanger et al, 1973). The principle of the sandwich ELISA used in this study is outlined in Figure 3.1 (page 75).

Tubular function is usually assessed by monitoring specific marker proteins in urine that are in low concentration in health. UP1, being
Figure 3.1 Principle of ELISA sandwich ('two-site') first antibody methodology.

Antibody (excess) adsorbed to plate - passive adsorption of antibody to solid phase

Wash

Test solution containing antigen added

Wash

Enzyme labelled specific antibody added

Wash

Enzyme substrate added, Colour change is proportional to amount of antigen added
a LMW protein, may be a potential candidate for this purpose. To appreciate changes in levels of UP1 in health and disease, it was necessary to also study the changes in other LMW proteins, such as RBP and alpha-1 microglobulin (a1M), which, by virtue of their molecular weight, may have a similar or comparable metabolic fate in the body. These two proteins have an established clinical significance and demonstrable stability and practicality (Bernard and Lauwerys, 1981; Bernard et al, 1987; Yu et al, 1983). At the beginning of this project there was only one sensitive method (ELISA, using alkaline phosphatase enzyme) for RBP estimation (Lucertini et al, 1984) besides Latex immunoassay (Bernard et al, 1982b). It was therefore decided to develop a sensitive ELISA for RBP utilizing peroxidase-labelled antibody because the enzyme is cheaper and may be more sensitive (Portsmann et al, 1985).

Serum and urinary a1M and serum RBP were measured using the method of Mancini et al (1965). This method was also used to measure serum and urinary levels of UP1 in some patients with chronic renal disease, and in normal urine after concentration.
3.2 ENZYME-LINKED IMMUNOSORBENT ASSAY FOR UP1

3.2.1 MATERIALS AND METHODS

Subjects - for reference range

28 healthy volunteers (13 females, 15 males) aged between 20 and 46 (mean 28) years donated a single 24 hour urine sample and a sample of blood taken between 1000 and 1100 hours from which serum was obtained. All volunteers had a normal serum creatinine (less than 125 umol/l), a negative Albustix reaction and total urinary protein of less than 150 mg/day. None had a history of previous or current renal disease. Urine was collected in 0.1% azide as preservative and aliquots were frozen at -40°C until analyzed. 20 cerebrospinal fluid samples that had no abnormality in the fluid were obtained from the Clinical Biochemistry Department, St Luke's Hospital, Guildford.

Materials

Purified rabbit immunoglobulin against human UP1 (Code A257, Lot 013A) was obtained from Dako Ltd., High Wycombe, Bucks U.K. The protein content as determined colorimetrically (Hartree, 1972) was 7.9g/l. Later in the study, an antiserum raised against immunoaffinity-purified UP1 in rabbits was used. Horseradish peroxidase labelled anti-UP1 IgG was obtained from Dako (courtesy of Jannette Sorte). o-Phenylenediamine (OPD) was obtained from Sigma Chemical Co., Poole, U.K.). All reagents were of 'Analar' grade, from BDH Chemical Ltd., Dagenham, U.K.
Disposable polyvinyl chloride microtitre flat-bottomed 96-well format (PVC) plates were obtained from Dynatech Laboratories, Billinghurst, Dorset, U.K. Only the 60 inner wells were used during the ELISA procedure, as outer wells may give greater variability in absorbance (Kricka et al, 1980).

UP1 standard was purified in the laboratory by combined gel filtration and immunoaffinity chromatography from urine samples (see Chapter 2).

**Buffered Solutions**

**Coating Buffer:** 0.1M Carbonate-bicarbonate buffer pH 9.6. Sodium carbonate (10.6g/l) was added to about 700ml of Sodium hydrogen carbonate (8.4g/l) to pH 9.6. The solution was stored at 4°C and changed every two weeks.

**Diluent/washing buffer (PBSTG):** 0.15M Phosphate buffered saline Tween 20 gelatin (sodium chloride 8.0g, potassium chloride 0.2g, disodium hydrogen phosphate 1.15g, potassium dihydrogen phosphate 0.2g, Tween 20 0.5ml, and gelatine 1.0g in 1000ml), pH 7.4. This solution was prepared daily.

**Chromogen:** Chromogen (substrate) was freshly prepared by dissolving o-phenylenediame (0.4 g/l) in a solution of citric acid (5.3g/l) and disodium hydrogen phosphate (7.1g/l) containing 40 ul of H$_2$O$_2$ (30%w/v) per 100 ml portion.

**UP1 Standard:** This was dissolved in 100ml of normal saline to give concentration of 1mg/l and stored in vials at -40°C until needed.
ELISA PROCEDURE

The general analytical protocol for the sandwich ELISA is outlined in Table 3.1 (page 80). PVC plates were pre-soaked in coating buffer for about 10 minutes before use. All incubations were in a humidity box; plates were washed three times in PBSTG using the manual Miniwash (Dynatech product).

3.2.2 RESULTS

Optimum Antiserum and Enzyme label dilutions:

PVC plate was coated with anti-UP1 at concentrations of 0, 5, 10, 15, 20 mg/l using 10 wells down the plate per antiserum concentration. 256 ug/l UP1 antigen was added to each well. Horseradish peroxidase labelled anti-UP1, diluted 2000, 4000, 8000, 16000 times in PBSTG, and PBSTG without label, were added across the plate to form a checker board. From the titration curves (Figure 3.2, page 81), working dilutions for both coating step and enzyme label were determined. Optimum coating occurred at concentration of 15 mg/l of antiserum, and although maximum absorbance occurred at enzyme label dilution of 2000, to conserve label and maintain an acceptable reaction rate, enzyme label dilution of 4000 was used. These conditions were chosen for routine assay procedure.

Linearity and Detection Limit of Assay:

Standards were diluted to concentrations of 256, 128, 64, 32, 8, 4, 2, 1, and 0.5 ug/l and applied in quadruplicates. A
Table 3.1 Summary Procedure for ELISA.

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Buffer/Solution</th>
<th>Reagent Vol. (ul/well)</th>
<th>Incubation</th>
<th>Temp (°C)</th>
<th>Wash</th>
</tr>
</thead>
<tbody>
<tr>
<td>Antibody</td>
<td>Na$_2$CO$_3$/NaHCO$_3$</td>
<td>200</td>
<td>4 hr</td>
<td>37</td>
<td>3 times</td>
</tr>
<tr>
<td>Antigen</td>
<td>PBSTG</td>
<td>200</td>
<td>3 hr</td>
<td>37</td>
<td>3 times</td>
</tr>
<tr>
<td>Enzyme label</td>
<td>PBSTG</td>
<td>200</td>
<td>Overnight</td>
<td>4</td>
<td>3 times</td>
</tr>
<tr>
<td>Substrate(OPD)</td>
<td>Substrate Buffer</td>
<td>150</td>
<td>30 min</td>
<td>37</td>
<td>None</td>
</tr>
<tr>
<td>Stop</td>
<td>2.5M H$_2$SO$_4$</td>
<td>50</td>
<td>None</td>
<td>None</td>
<td>None</td>
</tr>
</tbody>
</table>

Read at 490nm on a MicroELISA Autoplate Reader (Dynatech) linked to a microcomputer programmed to produce spline from absorbance of standards and read off concentrations of samples.
FIGURE 3.2. OPTIMISATION OF COATING ANTIBODY AND LABEL CONCENTRATIONS
representative dose response curve for the UP1 ELISA is presented in Figure 3.3 (page 83). Next, standards were run in duplicate, and 10 replicates of the zero blank (PBSTG alone) were measured in order to calculate the 2SD range for the blank. The smallest concentration of UP1 distinguishable from zero at 95% confidence was 0.75 ug/l. Linearity was 1 - 80 ug/l. For routine assay, concentrations of 1 to 128 ug/l were used. Recovery, within and between assay variation of UP1 ELISA are shown in Table 3.2 (page 84). Within assay precision was determined from values obtained for two urine and one serum samples assayed 10 times. Between assay variation was determined by measuring one serum and urine sample on 30 different occasions over a period of 5 months.

**Parallism:**

Absorbance values obtained for dilutions of test urines and serum paralleled those obtained with standard UP1, demonstrating linearity of response over the working range of the assay. In addition, the results of studies on the influence of dilution on measured values of UP1 showed no significant effect of dilution (up to 5120 times dilution) on concentration of sample. Furthermore, serum and urine samples of approximately the same concentration were diluted serially and their absorbance characteristics studied. There was no significant difference between their absorbances, indicating that serum and urine UP1 have comparable affinity for the antibody.
Figure 3.3. Dose Response Curve for UP1 ELISA.

The smallest concentration of UP1 distinguishable from zero (blank, PBSTG alone) at 95% confidence is 0.75 µg/l. Linearity of standard curve is between 1-80 µg/l.
Table 3.2. UP1 ELISA Method Validation

(a) Recoverability (mean of 10)

<table>
<thead>
<tr>
<th>UP1 (ug) added</th>
<th>UP1 (ug) measured mean±SD</th>
<th>Recovery %</th>
</tr>
</thead>
<tbody>
<tr>
<td>5.0</td>
<td>4.8±0.3</td>
<td>96.0</td>
</tr>
<tr>
<td>20.0</td>
<td>20.3±1.2</td>
<td>101.6</td>
</tr>
<tr>
<td>50.0</td>
<td>47.4±3.4</td>
<td>94.8</td>
</tr>
</tbody>
</table>

(b) Intra-assay Variation (mean of 10)

<table>
<thead>
<tr>
<th>UP1(ug/l)</th>
<th>S.D(ug/l)</th>
<th>CV%</th>
</tr>
</thead>
<tbody>
<tr>
<td>5.5</td>
<td>0.4</td>
<td>5.1</td>
</tr>
<tr>
<td>20.0</td>
<td>0.9</td>
<td>4.5</td>
</tr>
<tr>
<td>60.0</td>
<td>3.6</td>
<td>6.0</td>
</tr>
</tbody>
</table>

(c) Inter-assay Variation (mean of 30)

<table>
<thead>
<tr>
<th>UP1 (ug/l)</th>
<th>S.D (ug/l)</th>
<th>CV%</th>
</tr>
</thead>
<tbody>
<tr>
<td>45.0</td>
<td>4.4</td>
<td>9.8</td>
</tr>
<tr>
<td>422.0</td>
<td>47.0</td>
<td>11.2</td>
</tr>
</tbody>
</table>
Specificity and cross-reactivity studies - effects of serum and urine:

To evaluate the specificity and cross-reactivity of the assay, its response to increasing serum and urine concentrations in the presence and absence of UP1 was studied. Serum and urine samples from a patient with end-stage renal failure (high in LMW proteins) were used for this study. The samples were 'stripped' of UP1 by repeatedly running them through an affinity column containing porous glass beads with covalently immobilised anti-UP1 IgG. (Stripped samples gave absorbances indistinguishable from zero at 95% confidence level.) These samples were then divided into two portions, and each portion double diluted in PBSTG. To one portion of the diluted sample (serum and urine) was added UP1 to a fixed concentration. Both samples (with and without UP1) were assayed simultaneously in duplicates on the same plate. Absorbance profiles are shown in Figure 3.4 (page 87). None of the tested dilutions of samples without UP1 produced an absorbance significantly different from 2SD of the blank (PBSTG alone), showing that the capture step by anti-UP1 coated plates was specific for UP1 (with no false positives). In addition, variation in the concentration of UP1-free urine or serum produced no significant differences in the absorbance of the added standard UP1 compared to absorbance values obtained with UP1 in PBSTG alone. This may mean that other substances in serum and urine do not exhibit any significant agonist or inhibitory effects on UP1-anti-UP1 interaction.

Comparison with single radial immunodiffusion

Correlation of the ELISA with single radial immunodiffusion method is shown in Figure 3.5 (page 88). 60 (30 urine, 30 serum)
samples were measured by ELISA and their results compared to the Mancini method. The serum samples and 28 urine samples were from patients with end-stage renal disease with high values of UP1, and the other 2 urine samples from healthy individuals concentrated 30-60 times by dialysis against polyethylene glycol 8000.

Effect of type of blood sample on measured UP1 values:

Differences in UP1 values as measured in serum and plasma were investigated. 10 healthy volunteers donated 10ml of blood from which serum and plasma (heparin) were obtained. These were then assayed for UP1 on the same PVC plate. Values obtained were (mean±SEM): serum 425±35 ug/l and plasma 431±29 ug/l. There was no significant difference (P>0.001) between these values.

Reference Values:

Using this method, the reference values of UP1 are (mean±2SD): 459±186 ug/l (serum), 80±120 ug/day (urine) and 4.8±4.40 ug/l (cerebrospinal fluid). The mean urinary excretion of UP1 was higher in males than females, but the differences were not statistically significant (P>0.001).
Figure 3.4. Response of assay to UP1-free serum and urine samples. The absorbance of a fixed UP1 concentration (50 μg/l) was evaluated in different dilutions of the UP1-free serum and urine in PBSTG. Samples were stripped of UP1 by passing them several times through an anti-UP1 affinity column.
Figure 3.5 Comparison of two methods of UP1 estimation.

\[ y = 0.966x - 0.133, \ S_b = 0.93 \]
\[ r = 0.98, \ P < 0.001, \ n = 60 \]
3.3 ELISA FOR ASSAY OF URINARY RBP

3.3.1 MATERIALS AND METHODS

Rabbit anti-human RBP (code A040, lot 082) and horseradish peroxidase labelled anti-human RBP (code A040, lot 012) were obtained from Dako Ltd., High Wycombe, Bucks, UK. For standard, lyophilized protein urine concentrate (Behringwerke, FRG), reconstituted as directed by the manufacturers to give 580 mg RBP/l, was used. Other materials were the same as for UP1.

Solutions

RBP standard was prepared by diluting the reconstituted lyophilized protein standard urine concentrate in PBS to 1 mg/l. This was aliquoted into vials and stored at -40°C until needed. Other buffers and solutions were the same as for UP1.

Analytical Procedure

The general analytical procedure was the same as that outlined in Figure 3.1 (page 80). Similar procedural steps were taken for the validation and optimization of the assay of RBP as for UP1.
3.3.2 RESULTS

From optimisation studies, the optimum first and second antisera dilutions chosen for routine assay based on absorbance values and economy of antisera were 1:1000 (for first antibody) and 1:3500 (for enzyme label).

Linearity of assay was between 1 to 64 ug/l, with a detection limit of 0.85 ug/l (distinguishable from zero at 95% confidence level, 10 determinations). Assay validation results gave within and between assay coefficient of variation of 6.2% (10 determinations) and 9.5% (20 determinations) respectively, and a recovery of 95-102% (mean 98%), and a significant correlation with single radial immunodiffusion method (r=0.96, P<0.001, n=54, Figure 3.6, page 91).

Reference Value:
Urine RBP determined by the ELISA method for the healthy volunteers gave values of (mean+2SD) 130+116 ug/day.
$y = 0.93x + 0.16, \: Sb = 2.69$

$r = 0.99, \: P<0.001, \: n = 54.$

FIGURE 3G. COMPARISON OF TWO METHODS OF RBP ESTIMATION
3.4 IMMUNOTURBIDIMETRIC METHOD FOR ALBUMIN ESTIMATION

3.4.1 INTRODUCTION

In the assessment of renal function, besides looking at tubular integrity, the glomerular function is also usually assessed. The best index of glomerular permeability is the sieving coefficient of a protein which by virtue of its molecular size and charge is just restrained by filtration in health. Albumin is the obvious choice. Methods for albumin estimation already exist (Mohamed et al, 1984; Mancini et al, 1965; Spencer and Price, 1979). An immunoturbidimetric (IT) method of albumin estimation (Spencer and Price, 1979) was established on the Cobas Bio centrifugal analyzer because it is fast and can be made sensitive for the measurement of albumin.

Principle of Immunoturbidimetry

When an antigen is added to an excess amount of its specific antibody, a reaction takes place that results in the formation, in solution, of macromolecular aggregates or immunocomplexes. Both the concentration and absorbance of the complexes are proportional to the amount of antigen added. Antigen determination time can be shortened by taking advantage of the enhancing effect of polymers on antigen-antibody reaction; and the use of polymers, such as polyethylene glycol, increase the turbidity and decrease consumption of antibody. This principle was used to set up an assay on a Cobas Bio centrifugal analyzer which has a high capacity for producing analytical results with a fairly high degree of precision in
3.4.2 MATERIALS AND METHODS

Cobas Bio centrifugal analyser (Roche) was used. Sheep anti-human albumin (HP/S/608-V) was supplied by Guildhay Antisera Ltd, Biochemistry Department, University of Surrey, Guildford. Polyethelene glycol 8000 was supplied by Sigma Chemical Co., Poole, U.K. Pooled serum was prepared and used as albumin standard.

Human albumin standards were prepared in normal saline. Anti-albumin serum was diluted in a solution of normal saline (8.5g NaCl/l) containing polyethylene glycol 8000 (50g/l). Before use all solutions were filtered through Whatman No 1.

Urine samples were collected in sodium azide, centrifuged, tested for albumin with Albustix (Ames) and diluted if positive.

Procedure

In establishing the immunoturbidimetric method for albumin, the following assay variables, as suggested by Whicher and Blow (1980) for immunonephelometry and applicable to immunoturbidimetry, were optimized:

1. Position on the precipitin curve of albumin-antibody ratio range
2. Assay sensitivity and range
3. Time course of the reaction
4. Polymer enhancement
5. Economy of antiserum

Serum samples were diluted 1000 times before analysis. The following parameter settings were used in the assay:

Calculation factor = 1000
3.4.3 RESULTS

Optimum antigen-antibody ratio:

To establish optimal antigen-antibody ratio, a series of precipitin curves were produced for a wide range of antigen concentrations (0 - 100 mg/1) at different antiserum dilution (1/25, 1/50, 1/100). The curves were examined and, taking into consideration linearity of precipitin curve and economy of antiserum, an antibody dilution of 1/50, for a top albumin concentration 64 mg/1, was chosen as optimum. Equivalence point at this antibody dilution, using the parameter settings above, was about 200-250 mg antigen/1.

The time course of the antibody-antigen reaction was established using a top standard of 64mg/1 and low standard of 2mg/1. The reaction was monitored for 20 minutes and found to plateau for both standards at 5 minutes, and this was chosen as the time of incubation. Using this time, standards of concentrations 2
to 64 mg/l were run simultaneously with samples and concentration of samples read off from standard curve.

Precision and accuracy of the assay were assessed. Intra- and inter-assay coefficient of variation were 3% and 4.6% respectively, and a recovery of 97.9-99.4%. Correlation of the IT method with Rocket immunoelectrophoresis (Laurell, 1966) is shown in Figure 3.7 (page 96).

Reference Value:

The reference value obtained for albumin excretion using the IT method was 7.9±10.7 mg/day (mean±2SD).
$y = 0.95x - 1.07, \quad S_b = 9.96$
$r = 0.997, \quad P < 0.001, \quad n = 51$

**Figure 3.7.** Comparison of two methods of urinary albumin estimation.
3.5.1 MATERIALS AND METHODS

Rabbit anti-human a₁ Microglobulin (code A256, lot 013B) was obtained from Dako, Agarose A was supplied by Pharmacia Laboratories, and for standards, protein standard urine concentrate (Behringwerke, FRG), diluted to give concentrations of 15mg a₁M and 10mg RBP/l were aliquoted into vials and frozen at -40°C until needed. These were diluted to give concentration ranges of 1-15 and 1-10 mg/l for a₁M and RBP respectively.

Procedure

The method of Mancini et al (1965) was used. Agarose A (10g/l) containing PEG-8000 (40g/l) and antisem (0.9%v/v) was used. Within and between assay CV were 3.1% and 5.2% respectively.

3.5.2 RESULTS

Reference Values:
Reference range using healthy volunteers were (mean+2SD) 34.0±10.7 mg/l (serum a₁M), 3.6±3.1 mg/day (urinary a₁M), 61.2±31.1 mg/l (serum RBP).
3.6 DISCUSSION

The ELISA methods for UP1 and RBP reported in this chapter are easy to set up and rapid to perform. They make use of antisera that are already commercially available or can be easily produced. The first antibody incubation may be carried out overnight at 4°C, and second antibody added and incubated at 37°C for 2 hours, thus cutting down the assay time and allowing results to be reported the same day in routine clinical laboratory. In this case however, a slightly higher concentration of enzyme label would be needed and a higher 'noise' observed, but with no significant effect on sensitivity of the assay. Results obtained by coating plates at 37°C for 4 hours or 4°C overnight were not significantly different for both UP1 and RBP.

Parallelism and specificity studies indicate that serum and urine UP1 react with the first and second antibodies with comparable affinities and are likely to be immunologically the same antigens. Specificity studies using serum and urine samples (high in LMW proteins) which had been stripped of UP1 showed that no protein in serum or urine reacts with the antisera used for assay of UP1 or affects UP1 interaction with its specific antisera in any significant way. In addition, the assay makes no specific requirement on type of blood sample, as serum and plasma samples from the same volunteers gave no significant differences in UP1 values.

The ELISA technique has been used for the establishment of reference range for levels in urine, serum and cerebrospinal fluid. The serum and urinary levels of α1M established are in agreement with the reports of Yu et al (1983) and Kusano et al (1985), and those of RBP in accord with the work of Ormos et al (1985).

In conclusion, the evaluation of the analytical variables and
comparison with single radial immunodiffusion method provide evidence for the reliability of the UP1 and RBP ELISA procedures. The ELISA methods also have considerable advantages over single radial immunodiffusion as no preliminary concentration of urine samples is required and, for UP1, normal blood samples can be measured; there is low cost per assay when one considers the working dilutions of the coating antibody and the enzyme label, and speed, because wells in several plates can be coated simultaneously and the plates stored at 4°C without any significant effect on assay performance.
CHAPTER FOUR
PRELIMINARY INVESTIGATIONS ON UP1

IMMUNOCYTOCHEMISTRY .............................. 101

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4.1 IMMUNOCYTOCHEMISTRY

4.1.1 INTRODUCTION

In immunocytochemistry - the science of correlating antigenic cellular constituents with anatomical structure of cell - the use of labelled antibody reagents as specific probes for protein and peptide antigen allows the examination of single cells for, among other things, synthetic capability. Enzyme labels provide several advantages over fluorescent labels - they permit the use of fixed tissues embedded in paraffin which provides excellent preservation of cell morphology and eliminates the problem of antifluorescence.

A LMW protein, UP1, first isolated in urine of patients with 'tubular' proteinuria has been demonstrated in serum/plasma and cerebrospinal fluid of normal subjects (Chapter 3). Although shown as a prerenal plasma protein, its physiological role and site of synthesis has not been established. It was therefore decided to investigate and, if possible, identify the site(s) of synthesis of the protein by immunocytochemical techniques. To this end, tissues from various human organs in normal and pathological states were investigated. It is hoped that knowledge of the distribution of UP1, and probably its alteration in pathological states, may suggest the site of synthesis and a mechanism for the elimination or catabolism, and possible function for UP1.

The use of several immunoenzyme methods in cytochemistry has been reviewed (Pearse, 1980). The indirect localization of antigen using alkaline phosphatase reaction was chosen for this study because it gives less background staining compared to horseradish
peroxidase.

4.1.2 MATERIALS AND METHODS

Materials

Affinity purified donkey anti-rabbit alkaline phosphatase labelled IgG was obtained from Guildhay Antisera Ltd, Biochemistry Department, University of Surrey, Guildford.

UP1 was affinity purified and specific anti-UP1 serum raised in rabbit (see Chapter 2). Antiserum specificity was confirmed by combined agarose gel electrophoresis and double radial immunodiffusion, and by ELISA.

Naphthol AS:B1 phosphoric acid and Fast Red TR were supplied by Sigma. All other reagents were of analytical grade.

Veronal Acetate Buffer, pH 9.2: Sodium acetate (trihydrate), 0.9715g, Sodium barbitone, 1.4715g, and 0.1M HCl, 2.5ml, were dissolved and made up to 250ml with decarbonated (by boiling) distilled water.

Substrate Solution: 5mg Naphthol AS:B1 phosphoric acid (sodium salt) was suspended in 2-3 drops of dimethylformamide (BDH). 5mg Fast Red TR was dissolved in 10ml veronal acetate buffer (pH 9.2). The Fast Red solution was then added to the naphthol, mixed, and filtered. This was prepared just before use.

Tissue Specimens:

Routinely formalin-fixed and paraffin-embedded tissues were obtained from human organs (autopsy samples). The tissues were: normal kidney, liver, prostate, skin (courtesy of Mr Peter Jenkins, Pathology, Royal Surrey County Hospital, Guildford), brain stem,
artery, choroid plexus, adrenal, spleen, pancreas, lung, bladder, urethra, cervix, fallopian tube, stomach, small intestine, large intestine, skeletal muscle, smooth muscle, cardiac muscle (courtesy of Dr J P Sloane, Royal Mardsen Hospital, Sutton, Surrey), blood smears, bone marrow (normal and myeloma) (courtesy of Dr Ian Douglas, Pathology, St Luke's Hospital, Guildford).

Immunocytochemical Procedure:

The alkaline phosphatase indirect method, modified from the protocol used at the Royal Mardsen Hospital, Sutton, Surrey (courtesy of Dr J P Sloane), was employed. The modification involved blocking for non-specific binding using a non immune serum from the species in which the second antibody was raised, that is normal donkey serum. This was found to reduce background staining. An additional modification was the blueing of sections, after counter-staining with Mayer's haemalin, in tap water without use of saturated lithium carbonate. This is because saturated lithium carbonate was found to partially solubilize the coloured enzyme reaction product. Details of the modified procedure are outlined in Table 4.1 (page 104).

The dilutions and reaction times of the primary antiserum (rabbit anti-UP1) and secondary antibody (alkaline phosphatase labelled donkey anti-rabbit IgG) were determined by the result of a titration study. For convenience, the reaction of the first antibody was fixed by incubation at 4°C overnight. Primary antibody dilution was varied until there was no background staining in the positive sections, the optimal dilutions of the primary and secondary antisera for most positive sections were 1:1000 and 1:250 respectively.
Table 4.1. Protocol for indirect Alkaline phosphatase immunocytochemistry

1. Dewax, take through xylene and alcohols.

2. Incubate 5 min in 20% acetic acid to destroy any endogenous alkaline phosphatase.

3. Transfer to running tap water for 5-10 min.

4. Block with non-immune serum, 1% normal donkey serum (NDS) in PBS (pH 7.4), for 30 min at room temperature.

5. Knock off excess PBS from tissue, so that the primary antiserum is not diluted too much on the slide.

6. Incubate in a moist chamber with 150 ul per section of rabbit anti-UP1 (primary) antibody diluted in 1% NDS (in PBS) at 4°C overnight.

7. Wash (3 x 5 min) in PBS (pH 7.4) containing Tween 20

8. Wash (2 x 5 min) with PBS and wipe excess from tissue. Incubate in a moist chamber with 150 ul/slide second antibody (alkaline phosphatase conjugate) diluted in 1% NDS (in PBS), 70-80 min.

9. Repeat 7, then wash (2x10 min) in distilled water.

10. Prepare substrate solution just before use, and put 0.8 ml on each slide.

11. Incubate at room temperature for 50 min.

12. Rinse in distilled water, then wash in tap water.

13. Counterstain with Mayer's Haemalin, for 4 min.

14. Blue in running tap water

15. Mount in glycerin jelly (water based mounting medium).

16. Ring cover slip with nail varnish when dry, to stop section drying.
Control experiments were as follows: first, the primary antiserum was replaced with normal rabbit serum or was simply ommitted. Second, the primary antiserum was pre-incubated with purified UP1 (2, 5, 10, 20, ug/ml of diluted antiserum) at 4°C overnight, then centrifuged (3000 rpm, 20 min). The supernantant was then applied to the positive sections. There was no staining without primary antiserum or with normal rabbit serum. Staining was completely abolished when the antiserum was adsorbed by UP1 above 5 ug/ml of diluted antiserum (1:1000).

Stained sections were examined under a light microscope.

4.1.3 RESULTS

Results of the immunocytochemistry for the sections studied are summarised in Table 4.2 (page 106). Positive sections are shown in Plates 4.1-4.3 (pages 107-109). Only the liver, kidney and prostate glands showed specific staining. The general pattern in all sections was that of luminal staining in the blood vessels for UP1.

In the liver section, the stain appeared to be faintly localized uniformly in the cytoplasm of all hepatocytes, with more dense, probably more specific, staining occurring in specific regions within the cytoplasm. It was not possible to identify the subcellular structures that stained intensely for UP1. The kidney showed specific staining in the tubular lumen, especially in the proximal tubules; there was also staining in the luminal epithelial cells of the proermal tubules. It was not possible from the orientation of the kidney sections to identify the different segments (S1, S2, S3) of the proximal convoluted tubules. The glomeruli did not stain for UP1. In the prostate, there was
Table 4.2 Summary of Immunocytochemistry results

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Result</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal kidney</td>
<td>Positive stain in the proximal tubules.</td>
</tr>
<tr>
<td>Liver</td>
<td>Positive stain in cytoplasm of hepatocytes.</td>
</tr>
<tr>
<td>Prostate</td>
<td>UP1 antigenic stain in corpora amylacea.</td>
</tr>
<tr>
<td>Skin</td>
<td></td>
</tr>
<tr>
<td>Brain stem</td>
<td></td>
</tr>
<tr>
<td>Choroid plexus</td>
<td></td>
</tr>
<tr>
<td>Artery</td>
<td></td>
</tr>
<tr>
<td>Adrenal</td>
<td></td>
</tr>
<tr>
<td>Spleen</td>
<td></td>
</tr>
<tr>
<td>Pancreas</td>
<td></td>
</tr>
<tr>
<td>Lung</td>
<td>No specific intracellular staining.</td>
</tr>
<tr>
<td>Bladder</td>
<td>Only blood vessel lumens stained.</td>
</tr>
<tr>
<td>Urethra</td>
<td></td>
</tr>
<tr>
<td>Cervix</td>
<td></td>
</tr>
<tr>
<td>Fallopian tube</td>
<td></td>
</tr>
<tr>
<td>Stomach</td>
<td></td>
</tr>
<tr>
<td>Small intestine</td>
<td></td>
</tr>
<tr>
<td>Large intestine</td>
<td></td>
</tr>
<tr>
<td>Colon</td>
<td></td>
</tr>
<tr>
<td>Skeletal muscle</td>
<td></td>
</tr>
<tr>
<td>Smooth muscle</td>
<td></td>
</tr>
<tr>
<td>Cardiac muscle</td>
<td></td>
</tr>
<tr>
<td>Blood smear</td>
<td>No stain</td>
</tr>
<tr>
<td>Bone marrow - normal</td>
<td>No stain</td>
</tr>
<tr>
<td>- myeloma</td>
<td>No stain</td>
</tr>
</tbody>
</table>
Plate 4.1: Liver section. Upper: Section incubated with rabbit anti-UP1 (1:50), affinity purified alkaline phosphatase labelled donkey anti-rabbit IgG (1:250), then visualized using naphthol phosphate. Note the fine reaction product localized fairly uniformly in all the cytoplasm of the cells and more strongly stained specific areas (arrowed), probably the endoplasmic reticulum or/and Golgi apparatus (X630). Lower: Primary antibody IgG adsorbed with purified UP1 antigen. Note that staining is completely abolished. (X250).
Plate 4.2: Kidney Section: Upper: Section incubated with rabbit anti-UP1 (1:50), affinity purified alkaline phosphatase labelled donkey anti-rabbit IgG (1:250), then visualized using naphthol phosphate. Note that staining is confined to proximal convoluted tubular lumen and epithelial cells (arrowed) (X250). Lower: Primary antibody IgG adsorbed with purified UP1 antigen; note absence of staining (X250).
Plate 4.3: Prostate Section: Upper: Section incubated with rabbit anti-UP1 (1:50), affinity purified alkaline phosphatase labelled donkey anti-rabbit IgG (1:250), then visualized using naphthol phosphate. Note staining restricted to prostate secretions, corpora amylacea (arrowed), and not in the cells (X400). Lower: Primary antiserum IgG adsorbed with purified UP1 antigen; note that staining is completely abolished (X250).
staining of the area usually referred to as the 'prostate secretions', but no stain in the cells of the prostate glands. For all sections, staining was completely abolished in the absence of the first antibody.
4.2 ACUTE PHASE REACTION STUDIES

4.2.1 INTRODUCTION

Acute phase reaction (APR) is a fundamental response of the body to injury in which the concentration of certain plasma proteins increases. It is now recognised as a general non-specific, primitive response to most forms of infective and non-infective inflammatory processes, cellular and/or tissue necrosis and malignant neoplasia. Historically, the discovery by Tillett and Francis in 1930 of a protein in the sera of acutely ill patients, able to bind the C-polysaccharide of the cell wall of Streptococcus pneumoniae, called C-reactive protein (CRP), led to the description of APR.

Determination of 'acute phase' proteins, especially CRP, is clinically useful for screening for organic disease, for assessing the activity of an inflammatory disease such as rheumatoid arthritis, detecting intercurrent infection in SLE, in leukaemia or after surgery (secondary rise in plasma level), for detecting rejection in renal allograft recipients, as well as managing neonatal septicaemia and meningitis when collection of specimens for bacteriological investigation may be difficult, and in several other clinical settings (Pepys, 1981). The rates of CRP synthesis and secretion increase within hours of an acute injury or onset of inflammation probably under the influence of humoral mediators, such as leucocyte endogenous mediator (endogenous pyrogen) (Merriman et al, 1975) and prostaglandin PGE1 (Whicher et al, 1980); CRP and other acute phase proteins may act as mediators, participants or
inhibitors of inflammatory process.

An association of acute phase response with myocardial infarction has been known for years, and a number of acute phase proteins such as CRP, haptoglobin, anti-chymotrypsin, fibrinogen, alph-1 glycoprotein and alpha-1 antitrypsin (Agostini et al, 1970; Johansson et al, 1972) have been shown to rise in response to such infarctions. Serum CRP also excellently correlates with creatine phosphokinase (CPK-MB), and gives significantly higher values with transmural than with non-transmural infarctions.

To evaluate UP1 for APR properties, use was made of both cross-sectional studies involving patients with myocardial infarction, and longitudinal studies using patients who had undergone major surgery. For a reference acute phase protein, CRP, a protein with established acute phase reaction, was measured in all samples. It is known that CRP response is not affected by the commonly used anti-inflammatory or immunosuppressive drugs, including steroids, unless these affect activity of the underlying disease, and it covers an exceptionally broad incremental range of up to 3000-fold (Pepys, 1981). Furthermore, changes in CRP level can occur within 6-10 hours (Whicher, 1983) in response to the onset or resolution of the stimulating process. These features, coupled with the availability of fairly precise assay methods, favoured the choice of CRP as a reference acute phase protein.
4.2.2 MATERIALS AND METHODS

Patients

For cross-sectional studies, 19 serum samples from patients with myocardial infarction were obtained from the Clinical Biochemistry laboratory, St Luke's Hospital, Guildford. UP1, lactate dehydrogenase (LDH) - an enzyme that is known to be elevated in myocardial infarction, and creatinine (to assess patient's renal function) were measured on all the samples. Classification into transmural and non-transmural infarctions was not done. For the longitudinal study, 6 patients that had undergone major surgery at the Royal Surrey County Hospital, Guildford, had their serum samples monitored for UP1, CRP and creatinine prior to and following operation. Clinical details of the patients for the longitudinal study are given in Table 4.3 (page 115).

Methods

UP1 was measured by ELISA (see Chapter 3). CRP was measured by the method of Mancini et al (1965) using sheep anti-CRP (Guildhay Antisera Ltd, Biochemistry Department, University of Surrey, Guildford); CRP standard was supplied by Behring Diagnostics, La Jolla CA 92037. LDH was kindly measured by the staff of the Clinical Biochemistry laboratory, St Luke's Hospital, Guildford. Samples were frozen at -20°C and analysed simultaneously at the end of the collection.
4.2.3 RESULTS

The results of the cross-sectional, myocardial infarction study are shown in Table 4.4 (page 115). There was no significant increase in serum UP1 over and above those of normals, inspite of significantly higher levels of LDH, and markedly increased CRP.

Plots of two representative patients for the longitudinal, post-operative study are shown in Figures 4.1 and 4.2 (pages 116). No significant increases in UP1 were observed in spite of over 20-fold increases in CRP in most patients, and a secondary rise in CRP for patient A.P. due to complications (Figure 4.2).
Table 4.3. Details of patients for the longitudinal post-operative study for acute phase reaction.

<table>
<thead>
<tr>
<th>Patient</th>
<th>Sex</th>
<th>Age</th>
<th>Surgery</th>
</tr>
</thead>
<tbody>
<tr>
<td>J S</td>
<td>M</td>
<td>53</td>
<td>Total gastrectomy</td>
</tr>
<tr>
<td>J C</td>
<td>M</td>
<td>59</td>
<td>Total oesophagegastrectomy</td>
</tr>
<tr>
<td>P G</td>
<td>M</td>
<td>26</td>
<td>Pleurectomy</td>
</tr>
<tr>
<td>A P</td>
<td>M</td>
<td>39</td>
<td>Total gastrectomy</td>
</tr>
<tr>
<td>E W</td>
<td>F</td>
<td>70</td>
<td>Laparotomy / Cholecystectomy</td>
</tr>
<tr>
<td>D B</td>
<td>F</td>
<td>29</td>
<td>Cholecystectomy</td>
</tr>
<tr>
<td>K C</td>
<td>M</td>
<td>71</td>
<td>Prostatectomy</td>
</tr>
</tbody>
</table>

Table 4.4. Acute phase properties studies of UP1 in patients with myocardial infarction (cross-sectional study) (n=19).

<table>
<thead>
<tr>
<th>Analyte*</th>
<th>Serum levels (Mean ± SD)</th>
<th>Normal Values</th>
</tr>
</thead>
<tbody>
<tr>
<td>UP1 (ug/l)</td>
<td>456.3 ± 299.2</td>
<td>&lt;650</td>
</tr>
<tr>
<td>Creatinine (umol/l)</td>
<td>123 ± 23</td>
<td>&lt;125</td>
</tr>
<tr>
<td>CRP (mg/l)</td>
<td>51.7 ± 53.8</td>
<td>&lt;10</td>
</tr>
<tr>
<td>LDH (U/l)</td>
<td>1614.3 ± 889.6</td>
<td>&lt;600</td>
</tr>
</tbody>
</table>

* UP1 = Urine protein 1; CRP = C-Reactive Protein; LDH = Lactate Dehydrogenase;
J.C., Male, 39 years, had total oesophagegastrectomy.

A.P., Male, 46 years, had total gastrectomy.
4.3 SERUM UP1 LEVELS IN VARIOUS PATHOLOGICAL STATES

4.3.1 INTRODUCTION

To be able to perform any useful investigation on the clinical significance of UP1, it was thought necessary, at least as a preliminary approach, to undertake a cross-sectional study by measuring levels of UP1 in sera of patients with various pathological states, with the hope that such an investigation may give some useful clues as to the disease states that give rise to changes in levels of UP1 in serum. Levels of other LMW proteins (α₁M and RBP) were also assessed in some samples. Reference values for UP1 have already been established (Chapter 3).

4.3.2 MATERIALS AND METHODS

Serum samples were obtained from patients with myeloma, teratoma, end-stage renal failure, leukaemias and lymphomas. All samples for this study (except the functionally anephric haemodialysis samples) were obtained from St Luke's Hospital, Guildford. The anephric samples were obtained from St Helier Hospital, Cashalton, Surrey. UP1, RBP, alpha-1 microglobulin and creatinine were measured as described before.
4.3.3 RESULTS

Serum levels of UP1 in myeloma

Serum from 16 patients with myeloma were assayed for UP1, RBP and a1M. Serum creatinine was used to assess their renal function. On the basis of creatinine values, patients were grouped into two: those with serum creatinine less than 200 umol/l and those with creatinine greater than 200 umol/l. Results are summarized in Table 4.5(a) (page 120). Patients with serum creatinine greater than 200 umol/l had significantly higher UP1, RBP and a1M values, than those with creatinine less than 200 umol/l. Patients with creatinine less than 200 umol/l gave elevated values for a1M compared with healthy subjects.

Serum levels of UP1 in end-stage renal failure

10 patients (6 males, 4 females) with end-stage renal failure, functionally anephric, on maintenance haemodialysis (2-3 times per week) supplied serum samples for this study. The study was designed to give both serum levels of UP1, and also the effect of haemodialysis on UP1 levels. Thus, UP1 and creatinine were measured on both the pre and post haemodialysis samples. Results of the study are summarized in Table 4.5(b) (page 120). Patients with end-stage renal failure had 10 to 40 times increase in UP1 relative to values in health. Haemodialysis resulted in a increase in serum UP1, but the increase was not significant.

Serum UP1 levels in teratomas

36 sera from patients with various teratomas (mainly of the testis and ovary) were assayed for UP1, a1M and RBP. Results are
shown in Table 4.5(c) (page 120). There was no significant change in UP1 levels compared to normals.

Serum UP1 in leukaemias and lymphomas

12 lymphomas (6 Hodgkins, 6 non-Hodgkins) and 5 leukaemias (3 chronic amyloid, 2 acute amyloid) sera from patients aged between 29 and 85 years were assayed for UP1 and creatinine. The values are shown in Table 4.5(d) (page 120). Serum UP1 levels in these patients were all within the reference range for UP1.
### Table 4.5 Serum UP1 levels in various pathological states.*

#### (a) Myelomas (n=16)

<table>
<thead>
<tr>
<th>Analyte</th>
<th>Serum creatinine (&lt; 200 umol/1) Mean+SEM</th>
<th>Serum creat. (&gt; 200 umol/1) Mean+SEM</th>
<th>t-test</th>
</tr>
</thead>
<tbody>
<tr>
<td>UP1 ug/l</td>
<td>448±39</td>
<td>2865±1216</td>
<td>P&lt;0.001</td>
</tr>
<tr>
<td>a,M mg/l</td>
<td>55±5</td>
<td>124±21</td>
<td>P&lt;0.001</td>
</tr>
<tr>
<td>RBP mg/l</td>
<td>73±6</td>
<td>180±55</td>
<td>P&lt;0.001</td>
</tr>
<tr>
<td>Creat. umol/l</td>
<td>102±5</td>
<td>340±129</td>
<td>P&lt;0.001</td>
</tr>
</tbody>
</table>

#### (b) Functionally Anephric Patients (n=10)

<table>
<thead>
<tr>
<th>Analyte</th>
<th>Pre-HD** Mean+SEM</th>
<th>Post-HD t-test Mean+SEM</th>
<th>t-test</th>
</tr>
</thead>
<tbody>
<tr>
<td>Creatinine umol/l</td>
<td>875±64</td>
<td>463±46</td>
<td>P&lt;0.001</td>
</tr>
<tr>
<td>UP1 ug/l</td>
<td>7950±2157</td>
<td>9151±2281</td>
<td>P&gt;0.001</td>
</tr>
</tbody>
</table>

#### (c) Teratomas (n=36)

<table>
<thead>
<tr>
<th>Analyte</th>
<th>Value (Mean+SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>UP1 ug/l</td>
<td>399±126</td>
</tr>
<tr>
<td>a,M mg/l</td>
<td>57±13</td>
</tr>
<tr>
<td>RBP mg/l</td>
<td>97±19</td>
</tr>
<tr>
<td>Creatinine umol/l</td>
<td>97±10</td>
</tr>
</tbody>
</table>

#### (d) Leukaemias and Lymphomas

<table>
<thead>
<tr>
<th>Disease state</th>
<th>n</th>
<th>Creatinine (umol/l) Mean+SD</th>
<th>UP1 (ug/l) Mean+SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Leukaemias</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Chronic amyeloid</td>
<td>3</td>
<td>99±9</td>
<td>373±7</td>
</tr>
<tr>
<td>Acute amyeloid</td>
<td>2</td>
<td>159±81</td>
<td>385±14</td>
</tr>
<tr>
<td>Lymphomas</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hodgkins</td>
<td>6</td>
<td>105±25</td>
<td>353±165</td>
</tr>
<tr>
<td>Non-Hodgkins</td>
<td>6</td>
<td>104±26</td>
<td>336±126</td>
</tr>
</tbody>
</table>

* UP1 = Urine Protein 1, a,M = alpha-1 microglobulin.  
RBP = Retinol Binding Protein. ** Haemodialysis.
4.4 DISCUSSION

One of the main problems in immunocytochemistry is antibody non-specificity, arising due to structural heterogeneity of antibodies, presence of natural antibodies in normal sera, multiplicity of antibodies due to different antigenic determinants, contamination of the immunising antigens and unknown antigenic determinants in the tissue. In addition, there may be non-specific, non-immunological staining arising from the visualisation reaction due to endogenous enzyme. Due to these factors, validation of an immunocytochemical procedure must show both method specificity and antibody specificity. The first requires that no staining should occur in the absence of the primary antibody. The validation result of the method used in this study showed that there was no staining in the absence of primary antibody, indicating method specificity; there was also no staining when non-immune rabbit serum was used, demonstrating that there was no natural antibodies in the normal sera that cross reacts with UP1.

Antibody specificity requires that staining should be inhibited by adsorption of the antiserum with its specific antigen. The result obtained showed complete abolition of staining when the primary antiserum was adsorbed by purified UP1 antigen. The immunocytochemical procedure used therefore demonstrated both method and antibody specificity for UP1 antigen; results obtained may therefore be claimed to be specific for UP1.

UP1 localisation study demonstrated UP1 antigenic reaction sites in hepatocytes. The finding of UP1 reaction in the hepatocytes appears to be consistent with the established knowledge that most secretory proteins are synthesized in the liver. The location of the active sites within the cytoplasm appeared to be confined to specific areas.
It was not possible, on light microscopy, to identify the subcellular organelle(s) that were associated with this reaction. However, it looks reasonable to speculate that these areas of intense UP1 reaction could be endothelial reticulum and/or Golgi apparatus, the so called secretory pathway where most secretory proteins and glycoproteins are 'packaged' before release from the cell (Yotoko and Fahimi, 1981). Further studies are needed, on the ultrastructural level, to identify areas of intense UP1 stain within the hepatocytes.

In the kidney, UP1 antigenic reaction was confined mainly to the proximal convoluted tubules. No reactions were observed in the glomeruli. This is expected, because LMW proteins, of which UP1 is one, rapidly pass through the glomeruli with little restriction unless there is pathological glomerular clogging. The finding of UP1 in the liver and in the tubules may suggest at least one elimination pathway: that it is synthesised in the liver and cleared by the glomeruli into renal tubules. The distal convoluted tubules picked up less stain compared to the proximal tubules, probably indicating that UP1 is handled mainly by the proximal tubules; a finding that is consistent with other reports on the renal handling of other LMW plasma proteins (Stober and Waldmann, 1974).

In the prostate, UP1-like reaction was noted in the amorphous masses of the secretory product, the corpora amylacea. The staining pattern obtained was puzzlingly restricted to the secretory storage mass but not in the prostatic glandular epithelial cells. If UP1 were indeed synthesized in the prostate then intracellular staining would be obtained, as in hepatocytes. It is therefore probable that the staining of prostatic corpora amylacea, if specific for UP1 antigenic determinants, could not be explained by de novo synthesis in the prostate but by extra-prostatic delivery. In addition, if prostate
contributed to urinary appearance of UP1 either by synthesis (in its own right) and delivery (via the urethra), or merely as a post-renal route of UP1 elimination, then one would expect significantly higher urinary UP1 in males (especially in young males) than in females. Though urinary levels of UP1 are slightly higher in males, the difference is not statistically significant (Chapter 3). The reason for this difference is not clear. Further studies are needed to elucidate the role of prostate in UP1 metabolism.

Serum UP1 and other LWM proteins were measured in several pathological states and elevated UP1 found in patients with impaired renal function as assessed by serum creatinine. In all patients who showed elevated UP1, serum creatinine levels were found to be concomitantly increased either above normal or in the upper limit of reference range. This is evidenced by the myeloma patients with creatinine above 200 umol/l who had significantly increased (up to 5 times) UP1 levels. Patients with endstage renal failure and functional anephrics (about 100-200 ml urine per day) demonstrated dramatic increases in serum UP1 (up to 40 times above normal). This is consistent with existing knowledge that in impaired renal filtration such as occurs in uraemia, catabolism of LWM proteins is decreased with consequent prolongation in half-life and hence increase in serum.

Haemodialysis of patients with advanced renal disease did not have a significant effect on the levels of UP1; the slight but insignificant increase in post-haemodialysis serum may be due to haemoconcentration. This finding may be potentially useful in monitoring graft functional state in kidney transplant recipients who are still under haemodialysis, as creatinine levels may not be a true mirror of functional status at this stage.

Results of the cross sectional (using patients with myocardial
infarctions) and longitudinal (using patients who had undergone major surgery) studies indicate that UP1 does not exhibit acute phase properties; both conditions are well known to provide very powerful stimulus for the synthesis of acute phase proteins as evidenced by the many-fold increases in CRP.

**Summary of discussion**

The validity of the indirect immunocytochemical technique used in this study rests on the specificity of the anti-UP1 as well as on the method of labelling obtained. This specificity was demonstrated both by immunoelectrophoresis, by ELISA, and by the several immunocytochemical controls. On the basis of these observations, there seems little doubt that the method does in fact demonstrate the presence of UP1 in intracellular component of the hepatocytes, and proximal convoluted tubules. The demonstration of the liver as the probable synthetic site, the fact that there is UP1 staining in the proximal tubules, and the finding of increased serum UP1 in functionally anephric patients, together argue in support of the conclusion that UP1 is synthesized by the liver (that is extra-renally), cleared by the kidney and therefore in nephropathy involving impairment in glomerular filtration, serum UP1 levels are increased. Furthermore, in all the pathological states studied, serum UP1 was raised above normal only in the renal failure patients or in diseases associated with impaired renal function, as assessed by serum creatinine. The elevation of UP1, almost exclusively in patients with compromised renal function, allows a plausible speculation on the clinical significance of UP1: that it may be useful in the diagnosis and assessment of renal function in health and disease. UP1, from the preliminary studies, seems to possess an immense potential for this
purpose as it does not exhibit acute phase properties and its serum levels do not appear to be affected in any significant way by haemodialysis. Both attributes may be useful in assessment of renal function in the diagnosis and management of renal disease due to various causes.

Because of the findings in this series of studies and the potential so identified for UP1 from the results, the next four chapters in this thesis are devoted to various investigations conducted in the evaluation of the clinical usefulness of UP1 in the assessment of renal function in the diagnosis and management of renal diseases.
CHAPTER FIVE

EVALUATION OF UP1 IN

PATIENTS WITH CHRONIC RENAL DISEASES
5.1 INTRODUCTION

The ability to detect at an early stage kidney damage caused by renal or systemic disease or nephrotoxic compounds has been of great interest to clinicians and toxicologists. Renal function can be assessed by determining the clearance values of certain compounds cleared by the kidneys, or by any or all of a constellation of tests and measurements, including routine urinalysis, plasma creatinine, blood urea nitrogen, urine osmolality, and total protein. But these are insensitive, appearing abnormal only when 60-70% of the nephrons have already been compromised, and are not amenable to differential diagnosis of renal damage. Histological examination of the kidney should be more reliable and specific, but the process is long, invasive and, for routine examination, structural changes that occur at the level below the resolution of a light microscope may be missed. The ideal test for renal function should, therefore, be sensitive, specific, rapid and, above all, non-invasive.

Over the last decade, attention has been focused on the differential analysis of component proteins in urine and plasma as a more sensitive and specific index of renal damage (Peterson et al, 1969; Bienestock and Poortmans, 1970; Boesken and Noller, 1979; Ellis and Buffone, 1981; Coimbra et al, 1984; Marshall and Alberti, 1986). Low molecular weight (LMW) plasma proteins, in particular, have been used extensively for this purpose (Bernier et al, 1968; Wibell et al, 1973; Scarponi et al, 1976; Kusano et al, 1985; Bernard et al, 1987). LMW plasma proteins are readily filtered through the glomeruli and eliminated mainly by reabsorption and catabolism in the tubules, so that only minute quantities are normally excreted in urine (Bernier et al, 1968; Strober and Waldmann, 1974). In renal disease their levels
may be increased in plasma due to reduced filtration, and in urine because of impairment in reabsorption and catabolism or filtration in excess of the saturable tubular reabsorptive mechanisms. Because of the sensitivity of their changes in plasma and urinary levels in renal disease, LMW proteins have been useful in the assessment of renal function.

Human retinol binding protein (RBP) is a LMW protein (MW 21,200) isolated in plasma (Kanai et al, 1968) and urine (Peterson and Berggard, 1971). It is stable (Bernard and Lauwerys, 1981), widely distributed in the body fluids and its plasma level is elevated in patients with decreased GFR, while its urinary level is increased in patients with tubular disorders (Johansson and Ravnskov, 1972; Topping et al, 1986). Alpha-1 Microglobulin (a1M) is another LMW protein (MW 33,000) of comparable stability to RBP (Yu et al, 1983), the blood concentration of which, besides rising in renal failure (Tejler and Grubb, 1976) and decreasing in severe liver disease (Kawai and Takagi, 1980), undergoes little change in many forms of inflammatory and neoplastic disease (Takagi et al, 1980; Berggard et al, 1980). Both RBP and a1M are widely used in the assessment of renal function.

Results of studies referred to above indicate that changes in plasma and urinary concentrations of LMW proteins may reflect changes in renal function and integrity. UP1 is also a LMW protein (MW less than 25,000). It was decided to monitor urinary and plasma levels of UP1 with changes in renal function, as assessed by GFR, in order to evaluate its clinical usefulness in assessing renal function. To further evaluate the relative usefulness and sensitivity of UP1, changes in UP1 were compared with those of RBP, a1M and creatinine in the various renal diseases studied. Urinary and plasma albumin were also measured.
5.2 EXPERIMENTAL PROCEDURE

Controls and Patients

28 healthy volunteers (13 females, 15 males) aged 20-46 years donated a single 24-hour urine sample and a sample of blood taken between 1000 and 1100 hours within one day of urine collection. All normal subjects fulfilled the following criteria: a normal plasma creatinine (< 125 umol/l), a negative Albustix and total urinary protein less than 0.15 g/24hrs. None had a history of previous or current symptoms of renal disease. The study population consisted of 68 patients with various renal diseases and different renal functions: they consisted of chronic renal diseases, long-term functioning kidney transplants, and functionally anephric patients (less than 100ml urine per day) on maintenance haemodialysis attending S.W. Thames Regional Renal Unit, St Helier Hospital, Charshalton, Surrey (courtesy of Drs J Kwan and M Bending). These patients supplied 24-hour urine and a matching plasma sample. The 16 functionally anephric patients supplied only plasma samples. Details of the study population are given in Table 5.1 (page 13©). Urine was collected using 0.1% azide as preservative and aliquots were frozen at -20°C until analyzed. UP1, RBP, α1M and plasma creatinine levels were measured in both volunteers and patients. Urine total protein was also determined.

Methods

Plasma and urinary UP1 and RBP were measured by ELISA (see Chapter 3). Plasma and urinary α1M and plasma RBP were measured by single radial immunodiffusion (Mancini et al, 1965). Plasma and urinary creatinine were measured by Jaffe's (kinetic) method on Cobas Bio centrifugal analyzer by following the manufacturer's instructions. GFR
<table>
<thead>
<tr>
<th>Diagnosis</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>a) Chronic renal failure:</td>
<td></td>
</tr>
<tr>
<td>Chronic renal failure (aetiology uncertain)</td>
<td>6</td>
</tr>
<tr>
<td>Chronic glomerulonephritis</td>
<td>2</td>
</tr>
<tr>
<td>Acute renal failure due to surgical causes</td>
<td>1</td>
</tr>
<tr>
<td>Proliferative GN due to hypertension</td>
<td>3</td>
</tr>
<tr>
<td>Polycystic</td>
<td>2</td>
</tr>
<tr>
<td>Nephrotic syndrome due to minimal change GN</td>
<td>1</td>
</tr>
<tr>
<td>Neurogenic bladder</td>
<td>1</td>
</tr>
<tr>
<td>Nephrocalcinosis (due to diuretic/analgesic abuse)</td>
<td>1</td>
</tr>
<tr>
<td>Renal tuberculosis</td>
<td>1</td>
</tr>
<tr>
<td>Renal vascular disease</td>
<td>1</td>
</tr>
<tr>
<td>Chronic interstitial nephritis (chronic pyelonephritis)</td>
<td>1</td>
</tr>
<tr>
<td>Crescentic GN (rapid progressive GN)</td>
<td>4</td>
</tr>
<tr>
<td>Prune Belly syndrome</td>
<td>1</td>
</tr>
<tr>
<td>Obstructive uropathy</td>
<td>1</td>
</tr>
<tr>
<td>Haemodialysis *</td>
<td>16</td>
</tr>
<tr>
<td>b) Long-term transplants</td>
<td></td>
</tr>
<tr>
<td>Excellent function (Ccr &gt; 80ml/min)</td>
<td>3</td>
</tr>
<tr>
<td>Good function (50 &lt; Ccr &lt; 80ml/min)</td>
<td>11</td>
</tr>
<tr>
<td>Poor function (50 ml/min &lt; Ccr)</td>
<td>12</td>
</tr>
</tbody>
</table>

* functionally anephric, less than 100 ml urine/day

n = number of patients; GN = glomerulonephritis
was estimated by creatinine clearance; urine total protein was measured by a dye binding method (Bradford, 1976). Urine albumin was measured by immunoturbidimetry (see Chapter 3).

The fractional clearance of a protein (FClp), defined as the clearance of the protein (Cp) relative to creatinine clearance (Ccr), was expressed as a percentage and calculated according to the following formula:

\[
\text{FClp }\% = 100\left(\frac{C_p}{C_{cr}}\right) = 100 \left(\frac{U_p V}{S_p}\right)/\left(\frac{U_{cr} V}{S_{cr}}\right)
\]

or

\[
\text{FClp }\% = 100\left(\frac{U_p}{S_p}\right)/\left(\frac{U_{cr}}{S_{cr}}\right)
\]

where \( V \) = vol of 24-hour urine

\( U_p \) and \( S_p \) = concentrations of the protein in urine and plasma respectively

\( U_{cr} \) and \( S_{cr} \) = concentrations of creatinine in urine and plasma.

5.3 RESULTS

The levels of plasma UP1, RBP, a\text{\textsubscript{1}}M and creatinine and their respective fractional clearances for normal subjects and patients are shown in Table 5.2 (page 132); changes in fractional clearances with renal function are further shown in Figure 5.1 (page 133). The 24-hour urinary data for volunteers and patients are summarized in Table 5.3 (page 134).

**Plasma UP1 relationship with creatinine and other LMW proteins**

In order to study the relationship of plasma UP1 with other blood
Table 5.2 Plasma UP1, RBP, α1M and Creatinine levels, and the fractional clearances of the proteins in normals and patients with renal diseases. (Values given as mean ± SEM)

<table>
<thead>
<tr>
<th>Group</th>
<th>Cr</th>
<th>No.</th>
<th>Creat.</th>
<th>UP1</th>
<th>RBP</th>
<th>α1M</th>
<th>FCl UP1</th>
<th>FCl RBP</th>
<th>FCl α1M</th>
<th>FCl Alb</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>ml/min</td>
<td></td>
<td>umol/l</td>
<td>ug/l</td>
<td>mg/l</td>
<td>mg/l</td>
<td>%</td>
<td>%</td>
<td>%</td>
<td>%</td>
</tr>
<tr>
<td>A</td>
<td>Cr&gt;80</td>
<td>28</td>
<td>86±3</td>
<td>459±18</td>
<td>66.0±4.0</td>
<td>33.4±1.1</td>
<td>1.12±0.71</td>
<td>0.004±0.001</td>
<td>0.16±0.05</td>
<td>0.001±0.001</td>
</tr>
<tr>
<td></td>
<td>(112±3)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>B</td>
<td>50&lt;Cr&lt;80</td>
<td>17</td>
<td>124±6</td>
<td>711±84</td>
<td>132.3±8.0</td>
<td>77.4±6.7</td>
<td>6.73±1.82</td>
<td>0.045±0.030</td>
<td>0.38±0.08</td>
<td>0.008±0.004</td>
</tr>
<tr>
<td></td>
<td>(63±2)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C</td>
<td>30&lt;Cr&lt;50</td>
<td>4</td>
<td>184±34</td>
<td>716±213</td>
<td>117.5±33.0</td>
<td>89.3±8.4</td>
<td>2.53±0.88</td>
<td>0.121±0.080</td>
<td>0.76±0.23</td>
<td>0.003±0.002</td>
</tr>
<tr>
<td></td>
<td>(42±4)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>D</td>
<td>10&lt;Cr&lt;30</td>
<td>17</td>
<td>372±38</td>
<td>1846±331</td>
<td>200.9±20.1</td>
<td>154.5±16.3</td>
<td>15.09±2.77</td>
<td>1.074±0.227</td>
<td>3.79±1.03</td>
<td>0.103±0.034</td>
</tr>
<tr>
<td></td>
<td>(22±2)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>E</td>
<td>Cr&lt;10</td>
<td>14</td>
<td>810±63</td>
<td>3383±328</td>
<td>246.3±9.1</td>
<td>231.6±16.7</td>
<td>44.31±9.26</td>
<td>6.147±1.679</td>
<td>11.24±2.40</td>
<td>0.595±0.295</td>
</tr>
<tr>
<td></td>
<td>(6±1)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>F</td>
<td>HD</td>
<td>16</td>
<td>930±78</td>
<td>7877±1103</td>
<td>275.7±27.4</td>
<td>317.6±18.0</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>

ND = Not determined; FCl = Fractional clearance; HD = Haemodialysis; No. = Number of patients; α1M = alpha-1 microglobulin; Alb = albumin; Cr = Creat. Clearance.
Significantly different (P<0.01) from control (group A).

** Significantly different (P<0.001) from control.

Figure 5.1. Fractional clearance of UP1 in normals and patients with chronic renal diseases.
Variation is indicated as SEM.
Abbreviation of groups: A (normals) = creat. clearance, Ccr>80 ml/min (n=28); B = 50<Ccr<80 ml/min (n=17); C = 30<Ccr<50 ml/min (n=4); D = 10<Ccr<30 (n=17); E = Ccr<10 ml/min (n=14).
Group C consisted of only transplants; note the low fractional clearance relative to degree of renal insufficiency.
Table 5.3. 24-hour urine data for protein excretion in normals and patients with renal diseases.  (Values given as mean ± SEM)

<table>
<thead>
<tr>
<th>Group Cor ml/min</th>
<th>No.</th>
<th>Total protein, g (mg)</th>
<th>Albumin* (mg)</th>
<th>UP1* (mg)</th>
<th>RBP* (mg)</th>
<th>a1M* (mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>28</td>
<td>0.09±0.01 7.91±1.01</td>
<td>0.067±0.014</td>
<td>0.13±0.01</td>
<td>3.6±0.03</td>
<td></td>
</tr>
<tr>
<td>B 50&lt;Cor&lt;80</td>
<td>17</td>
<td>0.72±0.34 206.1±104.0 (26.1)</td>
<td>2.94±0.685 (43.9)</td>
<td>5.1±3.52 (25.5)</td>
<td>32.7±7.59 (4.5)</td>
<td></td>
</tr>
<tr>
<td>C 30&lt;Cor&lt;50</td>
<td>4</td>
<td>0.31±0.15 36.8±30.1 (4.7)</td>
<td>0.782±0.340 (11.7)</td>
<td>6.4±4.80 (32.0)</td>
<td>28.8±9.35 (4.0)</td>
<td></td>
</tr>
<tr>
<td>D 10&lt;Cor&lt;30</td>
<td>17</td>
<td>1.40±0.45 709.8±209.2 (89.7)</td>
<td>4.733±0.836 (70.6)</td>
<td>40.7±8.15 (203.5)</td>
<td>100.2±20.15 (13.9)</td>
<td></td>
</tr>
<tr>
<td>E Cor&lt;10</td>
<td>14</td>
<td>3.00±0.72 794.9±179.9 (100.5)</td>
<td>5.799±0.610 (86.6)</td>
<td>64.1±12.6 (320.5)</td>
<td>114.1±13.76 (15.8)</td>
<td></td>
</tr>
</tbody>
</table>

*Value in brackets is the change relative to value in health (Group A)
UP1 = Urine protein 1, RBP = Retinol binding protein, a1M = alpha-1 microglobulin, Cor = creatinine clearance.
parameters measured, plasma UP1 was correlated with creatinine, α₁M and RBP. Plasma UP1 significantly correlated with creatinine (r=0.82, P<0.001, Figure 5.2), with RBP (r=0.65, P<0.001, Figure 5.3), and with α₁M (r=0.80, P<0.001, Figure 5.4), see pages 136 to 138. The good correlation with creatinine appeared to indicate that plasma UP1 levels depend on renal function. The relationship of plasma UP1 levels with renal function, as measured by glomerular filtration rate (GFR) and estimated in this study by creatinine clearance (Ccr), was investigated. The correlation between plasma UP1 and Ccr is shown in Figure 5.5 (page 139) and that between plasma creatinine and Ccr in Figure 5.6 (page 140). Changes in plasma UP1 concentrations with changes in Ccr are similar to those of plasma creatinine, that is in inverse proportion to Ccr. Inverse plots are also shown in Figures 5.5 and 5.6 (pages 139-140). There was a positive correlation between inverse UP1 concentrations and Ccr.

To further study the association between plasma UP1 and renal function, double logarithmic plot for plasma UP1 and Ccr was made (Figure 5.7, page 141). A corresponding plot for plasma creatinine is given in Figure 5.8 (page 142). There was a significant correlation between UP1 and Ccr (r= -0.86, P<0.001) and plasma creatinine and Ccr (r=-0.96, P<0.001). A similar pattern of changes in plasma levels with creatinine and Ccr was observed for α₁M and RBP. A summary of the correlation statistics for plasma α₁M and RBP is shown in Table 5.4 (page 143).

Plasma levels and urinary excretion

In order to see whether the increased excretion of UP1 was caused by exceeding the plasma threshold for maximum tubular reabsorption, the plasma UP1 levels were correlated with urinary excretion. There was a
$y = 258 + 3.8x$

$r = 0.82, n = 80, P < 0.001$

Figure 5.2. Plot of Plasma UP1 against Creatinine.
\[ y = -56.8 + 7.78x \]
\[ r = 0.65, \, n = 80, \, P < 0.001 \]

**Figure 5.3. Plot of Plasma UP1 against Retinol Binding Protein**
y = -43.9 + 12.9 x
r = 0.80, n = 80, P < 0.001

FIGURE 5.4. PLOT OF PLASMA UP1 AGAINST ALPHA-1 MICROGLOBULIN.
FIGURE 5.5(a). PLOT OF PLASMA UP1 AGAINST CREATININE CLEARANCE.

FIGURE 5.5(b). INVERSE PLASMA UP1 AGAINST CREATININE CLEARANCE.
**FIGURE 5.6(a).** PLOT OF PLASMA CREATININE AGAINST CREATININE CLEARANCE.

**FIGURE 5.6(b).** INVERSE PLASMA CREATININE AGAINST CREATININE CLEARANCE.
\[ \log_{10} y = 3.97 - 0.64 \log_{10} x \]

\[ r = -0.66, \ n = 80, \ P < 0.001 \]

**Figure 5.7. Plasma UP1 against Creatinine Clearance (Log10 Scale).**
Log10 y = 3.45 - 0.74 Log10 x

r = -0.96, n = 80, P < 0.001

FIGURE 5.8. PLASMA CREATININE AGAINST CREAT. CLEARANCE (LOG10 SCALE).
Table 5.4. Summary of correlation statistics of plasma RBP and alpha-1 microglobulin in normals and patients with renal diseases.

<table>
<thead>
<tr>
<th>Parameter Correlated with (x)</th>
<th>Plasma Retinol Binding Protein (y)</th>
<th>Plasma Alpha-1 microglobulin (y)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n</td>
<td>r</td>
</tr>
<tr>
<td>Serum creat. (umol/l)</td>
<td>80</td>
<td>0.68</td>
</tr>
<tr>
<td>Ccr (inverse protein conc)</td>
<td>80</td>
<td>0.82</td>
</tr>
<tr>
<td>Ccr (double log scale)</td>
<td>80</td>
<td>-0.77</td>
</tr>
<tr>
<td>Urinary conc. of protein</td>
<td>80</td>
<td>0.64</td>
</tr>
</tbody>
</table>

Ccr = Creatinine clearance (ml/min/)


significant correlation ($r=0.53$, $p<0.001$) between plasma UP1 levels and its excretion in the urine. A plot of plasma levels of a substance cleared by the kidney against its urinary excretion should give an intercept on the $y$-axis (zero excretion) that corresponds to the plasma threshold concentration (that is tubular reabsorption maximum, $T_m$) above which it appears in urine. By this approach, the threshold for UP1 was calculated to be 733 µg/l with an error of 176 µg/l. With very few exceptions, all patients with plasma UP1 above 700 µg/l showed significant increases in urinary excretion of the protein, the converse was true for patients with plasma UP1 less than 700 µg/l.

One striking exception was a patient with the nephrotic syndrome due to minimal change glomerulonephritis who had increased UP1 in urine but normal plasma levels of the protein and other LMW proteins. Similar significant positive correlations were found between plasma RBP concentrations and its urinary excretion ($r=0.64$, $P<0.001$), and plasma $\alpha_1$-M and its excretion ($r=0.62$, $P<0.001$), see Table 5.4 (page 143) for summary of statistics. The $T_m$ for RBP was 109.5 mg/l with an error of estimation of 8.9 mg/l, and that of $\alpha_1$-M was 64.9 mg/l and an error of 9.5 mg/l.

**Mutual excretory correlations**

The correlations between the excretions of UP1, $\alpha_1$-M and RBP were also studied. There was a significant correlation between urinary UP1 and $\alpha_1$-M ($r=0.80$, $P<0.001$), UP1 and RBP ($r=0.50$, $P<0.001$), and between RBP and $\alpha_1$-M ($r=0.80$, $P<0.001$).

**Urinary excretions, fractional clearances and Ccr**

Fractional clearances are shown in Table 5.2 (page 132), and those of UP1 with different degrees of Ccr further illustrated in Figure 5.1.
As shown in Table 5.3 (page 134), urinary excretions of the LMW proteins and albumin increased with a decrease in Cr; however, the double logarithmic scale plots of the fractional clearance with Cr gave better correlations. The correlations (for the fractional clearances) were -0.76 (UP1), -0.88 (a1M), -0.90 (RBP) and -0.85 (albumin).

**Plasma UP1 levels in varying classes of renal function**

To evaluate sensitivity of changes in plasma UP1 with changes in renal function, plasma levels of UP1 in normal subjects (normal function), long-term transplants [with Cr>80 ml/min, 50<Cr<80 ml/min, and Cr<50 ml/min], chronic renal diseases (Cr<50ml/min), and haemodialysis patients (mean pre-haemodialysis plasma creatinine greater than 800 umol/l), were studied. (See Figure 5.9, page 146). Plasma levels of UP1 were significantly higher in patients with Cr less than 80ml/min compared with normal subjects.

### 5.4 DISCUSSION

The plasma and urinary levels and fractional clearances of LMW proteins found in this study are in agreement with values reported for RBP (Johansson and Ravnskov, 1972), and a1M (Yu et al, 1983; Kusano et al, 1985). The plasma and urinary changes in UP1, a1M and RBP with renal function are in agreement with other reports for LMW proteins (Bernier et al, 1968; Peterson et al, 1969; Scarpioni et al, 1976; Kawai and Takagi, 1982).
Figure 5.9. Plasma UP1 in healthy subjects and patients with varying degrees of renal function.

Abbreviations: Ccr = creatinine clearance ml/min; CRF = chronic renal failure; HD = haemodialysis, functionally anephric (less than 100 ml urine/day).

The horizontal bars indicate mean of plasma UP1. Note the significantly higher plasma UP1 in CRF patients compared to long-term transplants with comparable Ccr (Ccr<50).
Urinary excretion of UP1 and renal function

There was a high correlation between glomerular function and the excretion of UP1. This may have several possible explanations. Primarily, it may be an expression of tubular change parallel to changes in the glomeruli, as can be observed morphologically in progressive renal damage, regardless of aetiology. A contributing factor may be the increased plasma concentration of UP1 seen in the course of advancing uraemia, implying the creation of tubular load in excess of an eventual maximal tubular reabsorption capacity (Tm). An uraemic inhibition of proximal tubular function might also be a possibility (Preuss et al, 1966).

It was not possible to differentiate between glomerulonephritis and pyelonephritis on the basis of urinary excretion of UP1. Two long-term transplants D.S. (plasma creatinine 182 umol/l, UP1 500 ug/l) and P.V. (plasma creatinine 151 umol/l, UP1 510 ug/l) with normal Ccr for their age, had urinary UP1 excretions of over 7 and 3 mg/day respectively; but their urinary albumin was also increased, probably reflecting a mixed (tubular and glomerular) lesion. The difficulty in differentiating between glomerular and tubular lesions by urinary UP1 levels could be attributed to the fact that most of the chronic renal disease patients studied already had advanced renal lesions, so that irrespective of the original diagnosis, both tubular and glomerular function had already been compromised. It is possible that patients monitored at early stages of renal disease may show changes in UP1 more indicative of the exact anatomic region of the nephron affected. It is noteworthy, however, that with the exception of the two patients mentioned above, no patient with normal Ccr and plasma UP1 had an increased excretion of the protein, except in one patient with the nephrotic syndrome due to minimal change glomerulonephritis. The
patient had normal Ccr (97 ml/min), normal plasma UP1 (501 ug/l), but inappropriately grossly increased urinary UP1 (8.6 mg/day). Johansson and Ravskov (1972) also found increased LMW protein excretion in the nephrotic syndrome; but this result is at variance with that reported by Harrison and Blainey (1967), who noted no change in the clearance of LMW proteins in patients with glomerular disease and heavy proteinuria. Glomerular filtration of LMW proteins is usually high, approximating over 50% of plasma concentration in the ultrafiltrate, but less than 1% of the filtered load normally appears in urine. In minimal change glomerulonephritis, therefore, not more than a modest increase in UP1 excretion would be expected due to the normally efficient tubular reabsorption. The patient had no obvious signs of interstitial reaction in his kidney biopsies, there was no history of urinary tract infection and no evidence of metabolic disorders. It is tempting to suggest a competition, specific or otherwise, for the reabsorption sites between albumin, which had a markedly increased excretion, and LMW proteins. This has not been proved for UP1 or other LMW proteins. It is possible that the increased urinary excretion of LMW proteins \((a_1 \text{M, RBP})\) observed could cause increased competition with UP1 for reabsorption sites, with resultant increased excretion in urine; but there is conflicting data on the competition between individual LMW proteins for tubular reabsorption (Harrison and Barnes, 1970; Ravnskov, 1975).

Though it is statistically inadvisable, it is tempting to speculate on the possible turnover rate of UP1 based on the results for the single nephrotic patient. Obviously the maintainance of plasma UP1 at normal levels in the face of colossal losses of over 8 mg/day in urine suggests rapid synthetic and release mechanisms, and probably a short biological half-life for the protein. The biological half-life
for RBP has been reported to be 4 hours, increasing 10 to 15 times in uraemia (Valhquist et al, 1973)

It has been suggested that fractional clearance of a LMW protein is a better indicator of renal tubular damage than its 24-hour levels in urine, as this measurement attempts to quantify the protein excretion per functioning nephron (Ravnskov, 1975). The data obtained in this study (Table 5.2, Figure 5.1) show increased fractional clearance of UP1 with worsening renal function, indicating the potential use of UP1 measurement in monitoring tubular damage.

Mutual correlations between UP1, a\textsubscript{1}M and RBP

The strong correlation between UP1 and the other LMW proteins (a\textsubscript{1}M and RBP) in plasma and urine, coupled with a similarity in the pattern of change with plasma creatinine and Ccr, indicates that UP1 may be handled by the kidney in a similar way to other LMW proteins; that is, filtered by the glomerulus, reabsorbed and catabolised mainly by the proximal tubules. This seems particularly likely given the fact that its fractional clearance was found to be less than 1.0. The correlation between UP1 and RBP both in plasma and urine is not as strong as that between UP1 and a\textsubscript{1}M. This may appear suprising because both UP1 and RBP have alpha-2 electrophoretic mobility and molecular weights less than 25,000. This anomalous behaviour may in part be explained by the data reported by Peterson (1971) which showed that RBP appears in blood as a 85,000-dalton molecular weight complex with prealbumin and retinol. Only a minor part of RBP was found in the unbound form; the free form was found to increase in uraemia, with the complex remaining unchanged. Changes in glomerular filtration may not have the same effects on the plasma disappearance of RBP in the bound form compared with UP1 unless renal function becomes grossly impaired.
One of the prerequisites for sensitivity and specificity of a plasma protein in reflecting changes in filtration is that the protein should be excreted with the same molecular size in urine as it appears in blood. Plasma RBP may therefore not accurately reflect changes in glomerular filtration, emphasizing the need for caution in interpreting data for plasma RBP, especially when assessing changes in renal filtration.

**Plasma UP1 and renal function**

Bernier et al (1968) and Peterson et al (1969) showed that a linear correlation between the levels of B₂M and creatinine in plasma is present in renal insufficiency. For this reason it has been claimed that glomerular filtration is the main method of elimination of this and perhaps other LMW proteins. The dependence of the plasma levels of UP1 on GFR has been demonstrated in the present investigation, showing exponentially increasing levels of UP1 in plasma with decreasing GFR. It should be pointed out however that the regression line between log UP1 and log Ccr (Figure 5.7, page 141) is not perfectly linear and does show some scatter, especially as the Ccr becomes grossly decreased. It may be that at this low GFR, elimination by glomerular filtration may not be the exclusive route of plasma disappearance of UP1.

Changes in plasma UP1 levels with alterations in glomerular function, as shown in Figure 5.9 (page 146), presents two interesting findings. First, levels of UP1 indicate changes in glomerular filtration at an early stage, as levels are significantly raised even with only a slight reduction in Ccr. This may be useful in the early detection of changes in renal function. Second, long-term kidney transplants, irrespective of poor graft function, showed significantly lower plasma UP1 levels than chronic renal failure patients with
comparable range of Ccr and other clinical details. A comparable discrepancy between GFR and plasma levels of protein can be read from the data in the work of Peterson et al (1969), showing a higher plasma level of B\text{2}M in patients with adult Fanconi syndrome and related disorders than in patients with glomerulonephritis, despite the same degree of renal insufficiency.

These facts suggest that the glomerular elimination of LMW protein by glomerular filtration, reabsorption and catabolism in the proximal tubules, may be supplemented by a further mechanism also dependent on intact tubules, or at least intact renal tissue. This mechanism may involve an uptake in proximal tubular cells from peritubular capillaries. This type of elimination has been suggested for insulin, another LMW protein (Chamberlain and Stimmler, 1967). Thus the relatively intact renal tissue or unaffected tubules of the allograft probably allow a small but significant uptake and catabolism of UP1 by a route that does not involve filtration. Peritubular uptake, which has been shown for insulin, looks plausible for UP1 at least at high plasma levels that occur in uraemia. This explanation may be relevant in understanding the discrepancy in the general trends in Table 5.2 (Group C, page 132), since the four patients were all long-term transplants. This group showed lower values for UP1 and most of the other parameters than would be predicted from the degree of renal insufficiency.

Changes in plasma UP1 levels with GFR also show that UP1 may be potentially useful in monitoring longitudinally the progression of renal disease. Inverse plot of plasma UP1 against Ccr (Figure 5.5, page 139) shows a downward slide as renal function worsens. For long term monitoring, a plot of inverse plasma UP1 against time would be useful in monitoring treatment and course of renal disease.
The value of plasma UP1 as an estimator of GFR was also assessed. An ideal endogenous substance for the estimation of GFR should be constantly produced, should not return to the circulation after free passage across the glomerular membrane and should not have alternative route of excretion or catabolism. It is extremely difficult for any endogenously produced substance to meet all these criteria, in fact none has been found that fulfills all these conditions. The double logarithmic scale plot of plasma levels of such an ideal substance against GFR should give a gradient of -1.0 (Wibell et al, 1973). An alternative route of excretion or catabolism would cause a decrease in the value of the gradient, and inhibition of production of the substance with increasing plasma levels should affect the gradient in a similar way. From the logarithmic plots (Figures 5.5 and 5.6) the gradients are -0.64 (for UP1) and -0.74 (for creatinine). The gradient for creatinine is in agreement with earlier reports (Kusano et al, 1985). The inferior value for the gradient obtained for UP1 relative to creatinine may, at least in part, mean that the protein is handled differently by the body, probably by an increased alternative route of catabolism at high UP1 levels, but not necessarily because UP1 may be a less sensitive indicator of changes in GFR. This is evidenced by the fact that with slight reduction in GFR (50<Ccr<80), plasma levels of UP1 are elevated above normal range whereas corresponding creatinine levels are still within the normal range (<125 umol/l), see Table 5.2 (page 132) and also Figure 5.9 (page 146). UP1 may therefore be useful for the estimation of GFR when renal insufficiency has not progressed sufficiently to be detectable by plasma creatinine, and may have the added advantage of not being dependent on sex or muscle mass. Plasma UP1 may be particularly useful in GFR prediction in paediatric practice where precisely timed and complete urine collections needed for
creatinine clearance may be difficult or impracticable. B2-microglobulin has been suggested for GFR estimation (Wibell et al, 1973; van Acker et al, 1984), but it may not be very specific for this purpose as its plasma levels may change independently of glomerular filtration (Vincent et al, 1979).

Summary

In general, the LMW plasma proteins of less than 50,000 daltons are easily filtered through the renal glomerular basement membrane, and reabsorbed and degraded in the renal tubules, and eventually only minute amounts are excreted in normal urine. The proteins included in this category are B2M, RBP, a1M, lysozyme or s-nuclease. A similar metabolic pathway would also be suggested for UP1 since its fractional clearance is less than 1.0 and, as the present studies have shown, it follows patterns of changes in levels with renal function similar to those of a1M and RBP.

Changes in UP1 concentrations both in plasma and urine mirror changes in renal function, indicative of the fact that UP1 has a great potential in predicting GFR, in differential diagnosis of renal disease, in monitoring the course of progression or treatment of nephropathy, and perhaps in predicting the development of overt renal failure. Though the nature and degree of renal lesions studied makes it difficult to distinguish unequivocally glomerular from tubular lesions, the general sensitivity of changes in urinary UP1 levels and fractional clearances with even the slightest impairment in renal function is illustrative of its potential usefulness in the diagnosis and monitoring of tubular lesions. The good mutual correlations between UP1, a1M and RBP indicates that measurements of UP1 alone would suffice as a representative of changes in other LMW proteins in renal
disease.
CHAPTER SIX

STUDIES ON KIDNEY TRANSPLANT RECIPIENTS
In the management of renal transplant recipients there is considerable interest in methods for monitoring graft function, and for early identification, characterisation and monitoring of rejection episodes. In recent years many studies have investigated the use of LMW proteinuria for this purpose (Johansson and Ravnskov, 1972; Ravnskov, 1974; Woo et al, 1981). LMW proteins may be useful markers as they are freely filtered at the glomerulus, and reabsorbed by the lining cells of the proximal tubules. Changes in glomerular filtration, therefore, will alter their plasma levels and urinary excretion.

Most work on LMW proteins has involved $\beta_2$-microglobulin ($\beta_2$M) (Wibell et al, 1973; Woo et al, 1981; Van Acker et al, 1984; Ormos et al, 1985), but there has been no clear consensus on its clinical relevance in transplantation (Vincent et al, 1979; Schweizer et al, 1981). In renal transplantation, endogenous production of $\beta_2$M can change as a result of infection and increased immune activity, independently of renal function (Vincent et al, 1979). Also, recent work has cast doubt on the reliability of its measurement in urine (Bernard and Lauwerys, 1981; Davey and Gostling, 1982). Another LMW protein, RBP (Rask et al, 1980), which is a major component of tubular proteinuria and present in plasma at higher concentration than $\beta_2$M, has been advocated as an alternative marker of renal function (Scarpioni et al, 1976; Topping et al, 1986; Bernard et al, 1987). But recent work (Miller and Varghese, 1986) indicates that RBP may be an unreliable predictor of rejection. There is therefore a need to look for other LMW proteins that may be more sensitive as markers of changes in renal function during management of post-renal transplantation. Results of studies on chronic renal diseases (Chapter 5) demonstrated sensitivity
of changes in plasma and urinary UP1 levels with renal function. It was
decided, therefore, to evaluate the clinical usefulness and sensitivity
of UP1 for this purpose.

Measurement of proteins in urine, at least in the immediate
post-transplant period, is plagued with a myriad of problems.
Immediately after transplantation, especially but not exclusively of
cadaver donor kidneys, LMW proteinuria may be secondary to ischaemic
tubular damage with failure of tubular reabsorption of proteins; and
high serum levels of LMW proteins may lead to filtered load greater
than the tubular reabsorption capacity. Resultant LMW proteinuria at
this stage may not be an accurate reflection of tubular function, and
may make differentiation between acute tubular necrosis and rejection
difficult; variability in urine output and haematuria may make urinary
protein measurement at this stage meaningless. In addition, the
practical value of urine protein analyses in early detection of
rejection crises seems questionable (Ravnskov, 1974). For these
reasons, and because changes in plasma UP1 have been found to be a
sensitive mirror of renal function (Chapter 5), it was decided to
evaluate UP1 for usefulness in renal transplant monitoring and
assessment of allograft function by measuring plasma levels in kidney
transplant recipients before and following transplantation. Serum
creatinine, RBP and another LMW protein, a1-microglobulin, were also
measured.
6.2 MATERIALS AND METHODS

15 kidney transplant recipients aged between 4 and 52 years, at the Renal Unit of Guy's Hospital, London (courtesy of Mr Koffman), were used for this study. Daily plasma samples were obtained from patients before and following transplantation, for a period of at least 5 weeks. Serum samples were frozen at -20°C until analysed.

Plasma UP1 was measured by ELISA, RBP and α1M by single radial immunodiffusion method (Mancini et al, 1965). Creatinine was measured by Jaffe's (kinetic) reaction on Cobas Bio centrifugal analyser.

Rejection episodes were suspected by presence of all or any one of the following: pyrexia, graft tenderness and swelling, oliguria or sudden decrease in urine output, increase in blood pressure, and a 24-hour rise in plasma creatinine greater than 20 umol/l or 20%. Both rejection and acute tubular necrosis were confirmed by biopsy.

Patients studied are categorised as shown in Figure 6.1 (page 159), and further subdivided on the basis of graft function into four representative groups as shown in Table 6.1 (page 160).

6.3 RESULTS

Reference values for UP1, RBP, α1M and creatinine have been established in normal subjects, long-term functional transplants, and patients on maintenance haemodialysis (Chapter 5) and are shown in Table 6.2 (page 161) for comparison with transplant subjects studied.

To assess the initial function of the graft immediately following transplantation, changes in plasma levels of UP1, RBP, α1M and creatinine for patients with initial acute tubular necrosis (ATN) and
Figure 6.1 Patients studied following kidney transplantation.

Transplant
\[ n = 15 \]

Initial acute tubular necrosis
\[ n = 6 \]

Patients with no rejection
\[ n = 3 \]

Patients with rejection
\[ n = 7 \]

Initial good function
\[ n = 9 \]

Patients with no rejection
\[ n = 5 \]
Table 6.1 Representative groups of graft functions studied.

<table>
<thead>
<tr>
<th>Group</th>
<th>subgroup</th>
<th>n</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>A</td>
<td>5</td>
<td>Initial good function, no subsequent rejection</td>
</tr>
<tr>
<td>II</td>
<td></td>
<td>4</td>
<td>Initial good function, subsequent rejection</td>
</tr>
<tr>
<td>III</td>
<td>B</td>
<td>3</td>
<td>Initial ATN, but subsequent good function</td>
</tr>
<tr>
<td>IV</td>
<td></td>
<td>3</td>
<td>Initial ATN, subsequent rejection</td>
</tr>
</tbody>
</table>

n = number of patients

ATN = acute tubular necrosis
Table 6.2 Plasma UP1, RBP, \( \alpha_1 \)M and Creatinine for normal subjects, long-term functional kidney transplants, and maintenance haemodialysis patients. (Values are given as mean+SEM)*

<table>
<thead>
<tr>
<th>Group</th>
<th>No. of patients</th>
<th>UP1</th>
<th>RBP</th>
<th>( \alpha_1 )M</th>
<th>Creatinine</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal subjects</td>
<td>28</td>
<td>459±18</td>
<td>66±4</td>
<td>33±1</td>
<td>87.0±3.1</td>
</tr>
<tr>
<td>Long-term transplants</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(Ccr &gt; 50 ml/min)</td>
<td>17</td>
<td>711±84</td>
<td>132±14</td>
<td>77±7</td>
<td>124.0±6.3</td>
</tr>
<tr>
<td>Maintenance</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Haemodialysis</td>
<td>16</td>
<td>7877±1103</td>
<td>263±19</td>
<td>317±18</td>
<td>992.4±66.7</td>
</tr>
</tbody>
</table>

* Ccr = Creatinine clearance, \( \alpha_1 \)M = alpha-1 microglobulin, UP1 = Urine Protein 1, RBP = Retinol Binding Protein.
those with initial good function are compared in Table 6.3 (page 163).

Changes in levels of UP1 and the other parameters in four patients from
the representative groups (see Table 6.1) during the entire period of
the study are shown in Figures 6.2 to 6.5 (pages 164-167). Rejection
episodes are indicated by an arrow pointing downwards.

Although the numbers studied were not large enough for detailed
statistical analysis, some appraisal of the difference in pattern is
possible. In all the patients studied, there was an initial decrease
in plasma levels of LMW proteins immediately after transplantation
(Table 6.3). The decrease in UP1 was more marked and significantly
greater than RBP, a\textsubscript{1}M or creatinine; UP1 decrease was also
significantly greater in patients with good function than those with
acute tubular necrosis (ATN) (w=71.5, P=0.023 by Mann Whitney test).
The decrease in RBP was not significantly different in the two groups.

Following this decrease, there was a gradual rise in plasma LMW
proteins, except in cases where there was sustained good function
(Figure 6.2, page 164). The rise was more marked in patients with ATN.
In all cases, the plasma levels of UP1 declined with improvement in
renal function. The initial and later changes were generally more
marked for UP1 than RBP or a\textsubscript{1}M.

In the seven rejection episodes studied, increases appeared in
serum UP1 levels before clinical identification in five patients (that
is 71%), in one instance there was no significant change at all in
serum UP1, and in the last case, where mild cellular rejection was
diagnosed, UP1 showed significant rise at about the time of clinical
identification of rejection. In all the patients studied, prolonged
and sustained rise in UP1 seemed to indicate and parallel the severity
of the rejection episodes. It appeared difficult to distinguish
between ATN and mild rejection from the changes in levels of serum UP1.
Table 6.3 Plasma UP1, RBP, a1M and creatinine immediately following transplantation. (Values given as mean±SEM)

<table>
<thead>
<tr>
<th>Parameter*</th>
<th>Initial Value</th>
<th>Initial Value</th>
<th>Mean decrease</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>ATN</td>
<td>Good Function</td>
<td></td>
</tr>
<tr>
<td><strong>UP1 (ug/l)</strong></td>
<td></td>
<td></td>
<td>64</td>
</tr>
<tr>
<td>After 72hrs</td>
<td>2603±487</td>
<td>585±87</td>
<td>90**</td>
</tr>
<tr>
<td><strong>Mean decrease</strong></td>
<td></td>
<td></td>
<td><strong>10</strong></td>
</tr>
<tr>
<td><strong>RBP (mg/l)</strong></td>
<td>338±32</td>
<td>288±21</td>
<td>57</td>
</tr>
<tr>
<td>After 72hrs</td>
<td>167±23</td>
<td>111±10</td>
<td>61</td>
</tr>
</tbody>
</table>

| **a1M (mg/l)**          | 486±75        | 295±28        | 51            |
| After 72hrs             | 237±45        | 76±7          | 74**          |

| **Creatinine (umol/l)** | 1010±136      | 891±75        | 8             |
| After 72hrs             | 934±183       | 257±47        | **71**        |

* Mean decrease is expressed as percent of initial value. a1M = a1-Microglobulin, RBP = Retinol Binding protein, UP1 = Urine Protein 1.

** Initial decrease, significantly greater (W=71.5, p=0.023 by Mann Whitney test) in the group with initial good function than those with initial ATN.
Figure 6.2 Patient S.L. post kidney transplant. Upper panel: plasma creatinine, RBP, and A1M. Lower panel: plasma UP1. (Note difference in scale. Clinical history: S.L., female, 13 yrs old, had chronic renal failure (May’86) due to nephronophthisis. CAPD stared 5.9.86. First cadaver transplant Dec ‘86 (shown). Clinical course: completely uncomplicated. Horizontal line (lower panel) marks upper limit (mean±2SD) of normal serum UP1.)
FIGURE 6.3. PATIENT D.L POST TRANSPLANT. UPPER PANEL: Plasma creatinine, RBP and AIM. LOWER PANEL: Plasma UP1. (NB difference in scale). History: D.L, 6yrs. Nephrotic syndrome (Aug '85); bilateral nephrectomy Aug '86; maintained on (CAPD) until cadaver transplant Dec '86 (Shown). Clinical course: Cy A toxicity (day 5); rejection episode (arrowed, day 13); treatment started day 17; mild ATN and Cy A toxicity (day 44); persistent ATN (day 40) with oliguria. Horizontal line (lower panel) marks upper limit (mean±2SD) of plasma UP1.
FIGURE 6.4. PATIENT A.W. POST TRANSPLANT. UPPER PANEL: Plasma creatinine, RBP and A1M. LOWER PANEL: Plasma UP1. (NB difference in scale). History: A.W., 7yrs. Displastic kidneys. Bilateral nephrectomy and first transplant, Sept 86; recurrent rejection. Second transplant Dec ’86 (shown). Clinical course: maintained on HD until day 12; ATN and early pyelonephritis (day 4), graft pain (day 8), acute cellular rejection (arrowed, day 50). Horizontal line (lower panel) marks normal limit (mean±2SD) of plasma UP1.
FIGURE 6.5. PATIENT P.W POST TRANSPLANT. UPPER PANEL: Plasma creatinine, RBP and AlM. LOWER PANEL: Plasma UP1. (NB difference in scale). History: P.W., male, 45 yrs. Chronic renal failure secondary to Alport's syndrome. First transplant Sept '80, removed 1/52 due to major bleed. Second transplant Dec '86 (Shown). Clinical course: Persistent ATN (day 5, 11), Functioning transplant (day 17). Horizontal line (lower panel) marks normal limit of plasma UP1 (mean±2SD).
The plasma levels of $\alpha_1$M and RBP found in normal subjects is in agreement with previous studies (Johansson and Ravnskov, 1972; Kusano et al, 1985). Initial decrease in plasma LMW proteins followed by gradual rise is in accord with a recent report on behaviour of RBP (Miller and Varghese, 1986) and $\beta_2$M (Woo et al, 1981) in kidney transplant patients.

After transplantation, measurements of LMW proteins in plasma may be useful in the examination of two situations: the immediate post-operative period, when anuria or poor function may result from either rejection or ATN; and subsequent rejection episodes. The general initial fall in all patients appeared to be obligatory in nature, since it was independent of subsequent graft function. However the rate and extent of the decrease seemed to be related to initial and in some instances to subsequent graft function. The reason for the initial 'obligatory' fall in UP1 and other LMW proteins following transplantation is not immediately clear. It could be that the new kidneys allowed filtration and therefore increased proteinuric and renal catabolic rates of the proteins. But this was observed even in cases where there was anuria and poor perfusion. In such cases, however, the decrease was smaller than in patients with immediate perfusion. Since the decrease in LMW protein levels was significantly lower in ATN patients compared to patients without ATN, it could be argued that this is an indication of good initial tubular function possibly allowing peritubular uptake and catabolism of UP1 and the other proteins.

There is evidence for contraluminal uptake (Chamberlain and Stimmmler, 1967; Berggard, 1970) and partial hydrolysis at the
peritubular side (Katz and Rubenstein, 1973; Martin et al, 1977) for some LMW proteins. Although the contribution of this to the overall tubular (luminal and contraluminal) uptake and metabolism is reportedly small in health, it is possible that it may become a quantitatively significant contributor to overall catabolism in the face of high plasma levels of LMW proteins and anuria. This speculation becomes especially attractive in cases where the tubules of the transplanted kidneys do not have significant impairment in function, that is in the absence of ATN. This may at least in part explain the initial fall in UP1 and the other LMW proteins in all the transplant recipients, and why the fall in patients with ATN is less than those without ATN or with initial good function. That the fall is significant for UP1 and a1M but not for RBP may be due to differences in their interaction with receptors at the peritubular side.

This initial fall would, however, not be expected to be sustained unless normal function resumed following the decrease. This may be due to two reasons. First, the rapid initial fall would presumably trigger off an increase in the synthesis and/or release of UP1 as an anti-depletion mechanism. Second, if normal renal function does not resume to ensure the establishment of the normal and major filtration-reabsorption route following the initial period, the increased synthesis may be in excess of the peritubular mechanisms, and therefore an increase in serum LMW proteins may be expected to occur. This increase would, on this basis, be expected to be more rapid in cases where there is rejection or ATN or both. Since peritubular catabolism of LMW proteins is usually low (Harrison et al, 1968; Maack, 1975), one would not expect continued or sustained fall in plasma protein levels in the face of presumably increased production triggered by the initial fall, unless normal or near normal renal
function resumes. This seems to be the case in Figures 6.3, 6.4, 6.5 (pages 165 to 167), where initial fall is not maintained and is followed by a gradual rise in LMW proteins. This is to be contrasted with patient S.L (Figure 6.2, page 164), who had a perfect course with early resumption of normal function and therefore no significant rise in either LMW proteins or creatinine following the initial fall. Studies (Fermin et al, 1974) have shown that the serum concentration of microproteins, such as muraminidase, B_2^M and light immunoglobulin chains, decreased in transplants before onset of diuresis, when the glomerular filtration was zero. This observation was assumed to be due to an uptake by the proximal tubular cells of small proteins from the peritubular capillaries. A similar type of elimination, which may explain the initial fall in plasma UP1, has been suggested for insulin (Chamberlain and Stimmmer, 1967), another LMW protein. Thus, for the initial stages after transplantation a significant drop in plasma levels of UP1, before onset of diuresis, may indicate functioning tubules. Failure of this drop may suggest a diagnosis of rejection rather than ATN (which would prevent peritubular uptake) or lack of perfusion.

Most patients were on haemodialysis following transplantation and for these, serum creatinine values may not reflect accurately the state of allograft function. In most cases, rise in UP1 during rejection paralleled rise in creatinine, with UP1 levels rising 24 to 72 hours earlier than creatinine in some patients.

All the rejections studied were during the first one to seven weeks, and were acute (except in one case). Later chronic rejections, which may have different characteristics, were not studied. It is possible that the assay of UP1 may show a different pattern in these situations.
In conclusion, serum UP1 monitoring after renal transplantation has been found to be a useful and fairly sensitive procedure. It is a sensitive test for renal function and can be used for early diagnosis and treatment of rejection in many instances. In this regard, the greatest value of UP1 testing may be in the lessening of the severity of an acute rejection episode, or lessening permanent immunologic injury to the graft such as scarring and tubular atrophy, and therefore probably ensure better long-term function of the graft.
CHAPTER SEVEN

ASSESSMENT OF TUBULAR INVOLVEMENT

IN CISPLATIN CANCER CHEMOTHERAPY
Renal failure in cancer patients is a common problem. This complication is frequently multifactorial in origin. Several antineoplastic agents, for example, are potentially nephrotoxic; previous renal impairment as well as the use of other drugs, such as aminoglycosides, may further increase the risk of nephrotoxicity during chemotherapy. Cisplatin, one of the most effective antineoplastic drugs in cancer chemotherapy, is one of such agents with an established nephrotoxicity.

Ever since platinum compounds were reported to exert antitumor activity in mice and cis-diamminedichloroplatinum II (cisplatin) was found to be the most active (Rosenberg et al, 1969; Rosenberg and van Camp, 1970), cisplatin (CP) has been studied in numerous phase trials (alone and with other agents). It has proved to be one of the most potent chemotherapeutic agents, being active in cancer of the ovary, testis, bladder, head and neck, lung, endometrium, uterine cervix and many others.

The clinical use of CP, however, has been complicated by many side effects, the most important of which is its dose-related nephrotoxicity. Documented human and animal studies since 1971 have shown that CP induces dose-related (but not cumulative) microscopic changes which include epithelial cell degeneration, proximal tubular necrosis, dilation and necrosis of distal tubules, interstitial edema, and lymphocytic infiltration. Glomerular alterations are seldom observed, and any changes are usually non-specific. Although several manoeuvres such as hydration and forced diuresis, and hyperhydration with mannitol-induced saline diuresis (Al-Sarraf et al, 1982) may be used to reduce renal insufficiency and allow higher dosage
administration, the usefulness of CP is still limited.

Reliable and sensitive methods for the early detection of renal damage would therefore be useful and are clearly desirable due to the high incidence of persistent, and possibly irreversible, renal damage in patients on CP therapy. Such methods would be important in monitoring therapy and determining whether further treatment is advisable.

Several methods are currently used to detect renal dysfunction in CP therapy. Blood urea nitrogen and creatinine are commonly used but these are only raised when there is considerable damage to the kidneys, making their sensitivity questionable. Other blood substances are also relatively insensitive and ambiguous in differential diagnosis of renal disease. Urine parameters, with the advantage of being non-invasive, are ideal if they can be shown to be sufficiently sensitive.

Tubular damage is suggested by sensitive assays of tubular enzymes thought to be liberated by renal injury, such as alanine aminopeptidase and N-acetyl B-D-glucosaminidase (NAG) (Diener et al, 1981). But urinary enzymes could be contributed by serum and urino-genital tract beside renal tubules. Only the study of isoenzyme pattern, such as isoenzyme B of NAG, specific for tubular damage (Tucker et al, 1980), may increase the specificity of urinary enzymes. In addition, the presence of contaminants and endogenous inhibitors which must be removed (Pesce and First, 1979), and the fact that increase in urinary enzymes is usually only transient and not sustained in spite of continuing insult and therefore easily missed, argue against use of enzymes.

Proteins in urine appear to be more practical as an indicator of tubular lesions. The measurement in urine of low molecular weight (LMW) proteins, usually filtered by the glomeruli but reabsorbed and
catabolised by renal tubules has been shown to be a sensitive indicator of tubular function and integrity (Peterson et al, 1969; Yu et al, 1983; Bernard et al, 1987). B₂-microglobulin is one of the proteins that has been used (Cohen et al, 1981; Fleming et al, 1980). However, it is unstable and likely to be rapidly degraded at urinary pH in the bladder (Davey and Costling, 1982). Both retinol-binding protein (Bernard et al, 1982a,b) and a₁-microglobulin (Yu et al, 1983) have been shown to be useful, and suggested, for the early detection of renal dysfunction.

Results of studies on chronic renal diseases (chapter 5) indicated the potential sensitivity of UP1 in monitoring tubular lesions. In this chapter, it is sought to further investigate the clinical usefulness of measuring urinary UP1, a LMW protein presumably handled in a similar way as other LMW proteins by the tubules, as a tool in the early detection of tubular lesion in CP chemotherapy, by monitoring patients before and following therapy. Its sensitivity relative to RBP and a₁-microglobulin is also evaluated. Total urinary proteins, albumin and creatinine are also measured.

7.2 MATERIAL AND METHODS

Patients
Early morning urine samples were obtained from 8 cancer patients undergoing treatment at St Luke's Hospital, Guildford, with combination chemotherapy, consisting of CP with vinblastine and/or bleomycin or treosulphan, or CP alone, for different cancers. All patients were under hydration and diuresis. Details of the study population are summarized in Table 7.1 (page 176). Treatment regimes consisted of
Table 7.1 Details of cancer patients on cisplatin chemotherapy

<table>
<thead>
<tr>
<th>Pat. Initials</th>
<th>Course* No.</th>
<th>Sex</th>
<th>Age (yrs)</th>
<th>Diagnosis</th>
<th>Treatment**</th>
</tr>
</thead>
<tbody>
<tr>
<td>A.M</td>
<td>2 (151)</td>
<td>Male</td>
<td>35</td>
<td>Embryonal carcinoma of the testis</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>M.M</td>
<td>1 (147)</td>
<td>Male</td>
<td>28</td>
<td>Thymic carcinoid</td>
<td>5</td>
</tr>
<tr>
<td>A.H</td>
<td>1 (73)</td>
<td>Female</td>
<td>30</td>
<td>Carcinoma of the cervix</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>2 (54)</td>
<td></td>
<td></td>
<td></td>
<td>4</td>
</tr>
<tr>
<td>R.W</td>
<td>3 (177)</td>
<td>Male</td>
<td>29</td>
<td>Testicular teratoma</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>7</td>
<td></td>
<td></td>
<td></td>
<td>5</td>
</tr>
<tr>
<td>J.K</td>
<td>7 (54)</td>
<td>Female</td>
<td>29</td>
<td>Carcinoma of the ovary</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>8</td>
<td></td>
<td></td>
<td></td>
<td>2</td>
</tr>
<tr>
<td>A.G</td>
<td>3 (71)</td>
<td>Male</td>
<td>32</td>
<td>Testicular teratoma</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>4 (37)</td>
<td></td>
<td></td>
<td></td>
<td>3</td>
</tr>
<tr>
<td>S.K</td>
<td>3 (110)</td>
<td>Male</td>
<td>20</td>
<td>Testicular teratoma</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>(130)</td>
<td></td>
<td></td>
<td></td>
<td>3</td>
</tr>
<tr>
<td>P.H</td>
<td>8 (91)</td>
<td>Female</td>
<td>44</td>
<td>Cancer of the ovary</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>9 (105)</td>
<td></td>
<td></td>
<td></td>
<td>1</td>
</tr>
</tbody>
</table>

*Course No. is the particular treatment visit(s) during which the patient was monitored. Value in brackets is creatinine clearance in ml/min.

**All patients were on hydration (saline and/or mannitol) diuresis. Treatment combinations were:

1 = Cisplatin alone
2 = Cisplatin + Treosulphan
3 = Cisplatin + Bleomycin + VP16
4 = Cisplatin + Bleomycin + Vinblastine
5 = Einholm: 2L Normal Saline + Mannitol
   Cisplatin 35mg x 5 days
   1L Normal Saline
   Vinblastine 10mg x 2 days
   Bleomycin 15 mg x 3 days
monthly course of chemotherapy, each lasting 1-4 days. Urine samples were collected the morning prior to treatment and daily over a four or five day period following commencement of treatment. This was done for one or two courses of treatment. A 'course of treatment' means a single treatment visit. Blood samples were not monitored.

Samples were collected into 0.1% azide as preservative and stored at -20°C until analysed.

Methods

UP1 and RBP were measured by ELISA (see Chapter 3), a1M was measured by single radial immunodiffusion (Mancini et al, 1965), albumin was estimated by immunoturbidimetry (chapter 3), creatinine was measured by kinetic Jaffe's reaction on Cobas Bio centrifugal analyser, total urinary protein was measured by a dye binding method (Bradford, 1976). Urinary excretion of all proteins was calculated and expressed as protein per mmol creatinine, as recommended by Barratt (1983).

Urinary CP excretion was assessed by estimating urinary platinum. Total platinum in urine was measured by carbon furnace Atomic Absorption Spectrophotometry using a SP-9 Unicom Atomic Adsorption Spectrometer fitted with an autosampler. CP standards (John-Matthey Group, The Robens Institute, University of Surrey) were made in human urine diluted 1:1 with 0.1% HCl to give concentrations of 1.0, 0.5, 0.25, 0.1 ppm. Samples were diluted 1:1 with 0.1% HCl. Other details of the instrument settings are summarized below:

<table>
<thead>
<tr>
<th>Temp°C</th>
<th>Ramp</th>
<th>Hold (Sec)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Heat</td>
<td>85</td>
<td>0</td>
</tr>
<tr>
<td>Dry</td>
<td>115</td>
<td>9</td>
</tr>
<tr>
<td>Ash</td>
<td>1500</td>
<td>5</td>
</tr>
<tr>
<td>Atomise</td>
<td>2800</td>
<td>0</td>
</tr>
</tbody>
</table>
Wavelength = 265.9 nm, Slit width = 0.2 nm, D2 background correction, Purge gas = Argon, Gas flow = 11 l/min. Pyro-coated graphite tubes were used, and calibration graphs were linear with intercept at the origin.

7.3 RESULTS

Results for the patients studied are shown in Figures 7.1 to 7.8 (pages 180 to 187). Changes during treatment were related to pre-treatment protein to creatinine ratio and expressed as percentage. Because of significant differences between healthy individuals in the urinary excretion of the proteins studied it was decided, for each patient during treatment, to relate changes to pre-treatment values instead of to healthy volunteers.

The overall results and statistical analysis showed that changes in urinary excretion of UP1 and RBP were significantly correlated (P < 0.01, r = 0.60). There was a poor correlation between UP1 and α1M, and between RBP and α1M. Albumin and total urinary protein were significantly correlated (r = 0.8, P < 0.001).

Although there was a significant rise in UP1 and RBP following administration of CP in all patients studied, the rise did not correlate with CP excretion in urine. In fact, there was no significant correlation between CP and any of the proteins measured.

In four patients (JK, RW, SK and MM, Figures 7.1-7.4, pages 180-183), that is 50%, UP1 changes were significantly more sensitive, rising early and several times higher than all the other proteins. In one patient (PH, Figure 7.5, page 184), that is 12.5%, RBP was clearly
more sensitive than UP1. However, in the remaining patients (AG, AM and AH, Figures 7.6-7.8, pages 185-187), that is 37.5%, the picture was mixed, RBP and UP1 having comparable percent changes, or being more sensitive in one treatment course and less so in another for the same patient.

The pre-treatment protein to creatinine ratios did not seem to rise significantly between one course of pretreatment and the next, and most patients had their protein to creatinine levels returned to normal or pretreatment values before the fifth day. There was therefore no apparent cumulative nephrotoxic effect.

One fairly consistent observation was the late rise in albumin to creatinine levels, usually about the fourth or fifth day of treatment.

7.4 DISCUSSION

The general pattern of change in urinary excretion of proteins followed a similar pattern to that reported by other workers (Cohen et al, 1981; Litterst et al, 1986): that of a rise followed by return to normal or pretreatment values.

The interpretation of the overall data was complicated by several factors. First, some patients received different drug combinations during therapy, thus making comparison of data difficult; second, one patient had different regimes during different courses, which makes comparison between different courses for the same patient difficult; and significant patient to patient and even intra-individual variation in patterns of excretions of the proteins further compounds the difficulty in overall interpretation. Moreover, it is not unlikely that undiagnosed pre-existing renal involvement could contribute to the
Figure 7.1(a).

Figure 7.1(b).

Percent protein/creatinine ratio relative to pretreatment value for patient J.K. during the 7th [Fig 7.1(a)] and 8th [Fig 7.1(b)] course of cisplatin cancer chemotherapy.
PERCENT PROTEIN/CREATININE RATIO RELATIVE TO PRETREATMENT VALUE FOR R.W. DURING THE FIRST [FIG 7.2 (a)] AND SECOND [FIG 7.2(b)] COURSE OF CISPLATIN CANCER CHEMOTHERAPY.
PERCENT PROTEIN/CREATININE RATION RELATIVE TO PRETREATMENT VALUE FOR S.K. AS MONITORED DURING THE THIRD [FIG 7.3(a)] AND FOURTH [FIG 7.3(b)] COURSE OF CISPLATIN CANCER CHEMOTHERAPY.
FIGURE 7.4 PERCENT PROTEIN/CREATININE RATIO RELATIVE TO PRETREATMENT VALUE FOR PATIENT M.M. AS MONITORED DURING THE ONLY COURSE OF CISPLATIN
FIGURE 7.5(a).

PERCENT PROTEIN/CREATININE RATIO RELATIVE TO PRETREATMENT VALUE FOR PATIENT P.H. AS MONITORED DURING THE 8th (FIG 7.5(a)) AND 9th (FIG 7.5(b)) COURSE OF CISPLATIN CANCER CHEMOTHERAPY.
Figure 7.6(a).

PERCENT PROTEIN/CREATININE RATIO RELATIVE TO PRETREATMENT VALUE FOR PATIENT A.G. DURING THE 3rd (FIG 7.6(a)) AND 4th (FIG 7.6(b)) COURSE OF CISPLATIN CANCER CHEMOTHERAPY.
FIGURE 7.7(a).

PERCENT PROTEIN/CREATININE RATIO RELATIVE TO PRETREATMENT VALUE FOR PATIENT A.M. AS MONITORED DURING THE THIRD [FIG7.7(a)] AND FOURTH [FIG7.7(b)] COURSE OF CISPLATIN CANCER CHEMOTHERAPY

FIGURE 7.7(b).
FIGURE 7.8(a).

PERCENT PROTEIN/CREATININE RATIO RELATIVE TO PRETREATMENT VALUE FOR PATIENT A.H. AS MINITORED DURING THE 3rd [FIG 7.8(a)] AND 7th [FIG 7.8(b)] COURSE OF CISPLATIN CANCER CHEMOTHERAPY.
Despite these complications, when patients and courses of treatment are taken individually and separately, a general repetitive pattern is easily discernible. Following CP administration, excretion of UP1 was the earliest to rise and in terms of the magnitude of increase, compares only with RBP. Most patients studied had pretreatment values for UP1, RBP and $\alpha_1$M to creatinine ratio within or in the upper range of normal, probably indicating that the renal lesions that might have occurred during previous therapies were reversible with biochemical parameters returning to normal before next treatment. In addition, comparing two treatment courses for the same patient in successive months, no exaggerated or sustained rise in UP1 to creatinine ratio, over and above the preceding treatment values, was observed in the latter course. This may mean that any renal effects of in CP chemotherapy are not cumulative, a finding that is consistent with other reports (Ganzalez-Vitale et al, 1977; Madias and Harrington, 1978). The only patient (MM, Figure 7.4, page 183) whose UP1 to creatinine ratio did not appear to return to pretreatment values had compromised renal function, with mixed proteinuria, before commencement of therapy. He subsequently died.

Albumin to creatinine ratio rose during the fourth or fifth day of treatment. It is not clear whether this is an indication of a transient subclinical (primary) glomerular alterations or simply due to a partial impairment in proximal tubular reabsorption consequent on reversible tubular lesions.

There was no significant correlation between urinary excretion of UP1 (or any of the proteins studied) and urinary CP, as estimated by urinary platinum. The explanation for this is likely to be found in the mechanism of toxicity of CP and the treatment procedure used. Although
CP tissue concentrations are particularly high in the kidney after administration (Le Roy et al, 1979; Hill et al, 1975) and reported data (Patton et al, 1978) suggest an active tubular secretion of CP or its metabolites, toxicity does not depend on the overall CP concentration but on chloride concentration of the environment. Chloride ions are critical in the formation of the diversely highly toxic species of platinum (Earhart et al, 1983). Also see Figure 7.9 (page 190). Thus, intracellular reduction of CP concentration by abundant hydration, as well as tubular chloride reuptake by forced chloriuresis, might favourably influence the equilibrium between stable non-toxic CP and the toxic forms, thereby reducing tubular damage. Urinary UP1, a probable indicator of tubular lesion, would be expected to show a rise to indicate tubular damage, which is what was observed, but this rise would not be expected to correlate with CP excretion, since tissue damage is not necessarily a factor of the total CP but that of the cytotoxic species. The generation of these species was deliberately altered by therapeutic manoeuvres of hydration and forced chloriuresis. These results appear to lend credence to the work of Al-Sarraf et al (1982) which indicates that hydration chloriuresis allows higher dose administration of CP without commensurate significant increase in tubular lesion.

Histopathologic changes were not studied for obvious ethical reasons. However, in a similar study employing rats, Litterst et al (1986) found no renal tubular morphological alterations on either the first or third day after treatment. On the fifth day, a slight acute tubular necrosis was present at the corticomedullary junction; nephrosis was characterized by slight tubular dilation with flattened epithelium, cellular debris in tubular lumens, giant cells and cells with enlarged or multiple nuclei. The biochemical parameters did not
Cisplatin (CP) exerts its activity in a manner similar to alkylating agents by drug interaction with nucleophilic sites of pyrimidines in DNA. These intrastrand DNA cross-links will occur after physiologic activation of the drug, which will take place in a low chloride concentration environment. CP passage from a chloride-rich plasma milieu (103 mEq/l) to a low-chloride intracytoplasmic milieu (4 mEq/l) constitutes a passage from electrical neutrality (with a stable bis-chloro molecule, I) to aqueous activation (II, III, V), chloride ligands in cis position being replaced by water. These aquated forms are highly reactive with nucleophiles and can lose hydrogen ions to form cytotoxic hydroxyl radicals (IV, V); these latter compounds can also form cytotoxic oxygen-bridge dimers and trimers. At equilibrium, the proportion between these diversely cytotoxic complexes depends on chloride concentration and, to a lesser extent, on pH and total platinum concentration.

Adapted from Ries and Klastersky (1986)
accurately reflect histological findings which appeared after most of
the former had returned to normal in that study. This does not
invalidate the results obtained in this study since poor correlation
between biochemical and histological alterations may be expected if
early biochemical changes were as a result of structural lesions that
occurred at a level below the resolution of a light microscope, which
was used in routine histopathology. Rather, the present results
emphasise the fact that biochemical alterations, especially as
monitored by UP1 urinary excretion, are sensitive and may indicate at a
very early stage changes in tubular integrity.

In conclusion, the purpose of this study was firstly to evaluate
the usefulness of UP1 in the assessment of tubular damage, and secondly
to define the earliest protein indicator of CP nephropathy. It had
been suggested by the findings in Chapter 5 that UP1 may be sensitive
in the evaluation of tubular involvement. The present results have
indicated that UP1 has a sensitivity comparable to, and in many cases
superior to, that of RBP. Further, urinary UP1 excretion was the
earliest change detected after CP administration. This reversible
change was however not related to CP excretion as, under the
circumstances of the study, CP administration was accompanied by
pharmacologically induced chloriuresis giving rise to a reduction of
the highly toxic aqueous CP complexes in chloride-rich urine. Since no
patient developed laboratory evidence of renal toxicity, as assessed by
serum creatinine, this increased urinary UP1 may be interpreted as
reflecting subclinical renal damage. However, UP1 monitoring in urine
has been shown as the analyte which changes most or gives most abnormal
values in CP cancer therapy compared to RBP and a1M, the established,
stable indicator proteins of tubular damage, and may also be one which
is most useful in clinical decision-making in cancer treatment and
management.
CHAPTER EIGHT

ASSESSMENT OF TUBULAR INVOLVEMENT IN DIABETES MELLITUS
Renal failure secondary to diabetes mellitus has become an important health and socioeconomic issue in all nations of the world today. Epidemiologic studies have demonstrated that about 30-40% of insulin-dependent diabetic (IDDM) subjects will develop proteinuria and progressive renal failure on the average of 15-20 years after the onset of the disease (Deckert et al, 1978). Diabetic nephropathy is the major cause of renal failure in over 25% of all patients beginning therapy for end-stage renal disease. Once diabetic renal disease is well established, as evidenced by clinical proteinuria - excretion of 0.5g protein or 300mg albumin per day (Albustix-positive), attempts to modify the relentless progression of the disease have often proved essentially unsuccessful. Presumably, at this level of severity the process becomes self-perpetuating; and, although treatment of hypertension has been reported to retard the progression of the kidney disease (Mogensen, 1984), improved glycaemic control has not been shown to have obvious beneficial effects (Viberti et al, 1983). The dominant role of diabetic nephropathy as a cause of death in late diabetes therefore stresses the importance of studying the changes in renal structure and function in diabetic patients.

Over the last decade, considerable interest has emerged with the reports that have characterised a much earlier stage of diabetic nephropathy. A growing body of evidence suggests that persistent elevation of urinary albumin without clinical proteinuria, 'microalbuminuria', predicts future development of overt diabetic nephropathy (Mogensen and Christensen, 1984; Viberti and Keen, 1984). Studies on diabetic nephropathy have, in the recent past, therefore, been focused on this more promising perspective of early identification
and therapeutic intervention with a view to delaying the rate of progression to end-stage renal disease. The microalbuminuric stage is known to be preceded by a silent period of important functional and structural events, such as glomerular alterations, which occur in the kidney of diabetic patients during the 10-20 years before the onset of overt clinical proteinuria.

In normal subjects, more than 90% of the albumin filtered by the glomerulus is reabsorbed by the proximal convoluted tubules (PCTs) (Pesce and First 1979). Thus, the increase in the albuminuria in diabetic patient could possibly be the consequence of either a greater transglomerular passage, or of decrease in tubular reabsorption, or both simultaneously. Following reports, in insulin-dependent diabetes mellitus, of microalbuminuria with normal excretion of $B_2$-microglobulin ($B_2$M) (Viberti and Keen, 1984), of various glomerular alterations such as thickening and expansion of the glomerular basement membrane (Osterby, 1973) and increased renal linear extracellular membrane staining for albumin and IgG (Mauer et al, 1981), the case for glomerular origin of microalbuminuria appears to be established. On this basis, it is now argued that increased urinary $B_2$M, hence tubular alteration, is a late complication or occurs only under poor glycaemic control. However, there is a growing body of evidence which appears to indicate that LMW plasma proteins, recognised as sensitive indicators of renal tubular function, may also increase before development of overt albuminuria (Parving et al, 1976; Lopes-Virella et al, 1979; Poortmans et al, 1985; Shima et al, 1986; Rowe et al, 1987). Other studies using $B_2$M suggest that at rest, the output of the $B_2$M is higher in the diabetic patients than in the controls, which suggests decreased proximal tubular reabsorption of protein (Poortmans et al, 1985; Hermansson and Ludvigsson, 1980). Data from the above studies
undermine earlier conclusions by Mogensen (1976) and Viberti and Keen (1984) that 'tubular' proteinuria is only a late complication in diabetes, and, indeed, indicate that tubular proteinuria may also occur early in diabetic nephropathy and may warn of subclinical tubular pathology. There appears to be a fairly strong case to necessitate further study in the assessment of early tubular involvement in diabetes.

The value of urinary UP1 in the assessment of tubular involvement has been investigated and findings correlated with changes in $a_1M$ using diabetics with varied duration of the disease and renal function as assessed by albumin excretion.

8.2 MATERIALS AND METHODS

Subjects

100 patients (diabetic patients) selected for the study had albumin excretion that ranged from normal to overt albuminuria. The patients supplied untimed early morning urines. The diabetics were aged 16 to 85 (mean 51.9) years. All patients for the study had insulin-dependent (type I) diabetes. Information on the duration and arterial blood pressure was not immediately available. The control group consisted of 20 healthy volunteers aged between 20 to 50 years (11 males, 9 females; mean 29.1 years) from whom early morning urine were obtained. Urine from all subjects were stored at -20°C until analysed. Neither patients nor control showed evidence of urinary tract infection.

Assays

Red cell glycosylated haemoglobin ($HbA_{1c}$) was measured by
electroendosmosis using the Glytrac™ system (Ciba-Corning Ltd, Halsted, Essex, UK) by staff of Clinical Chemistry Dept., General Hospital, Southampton. The between-batch coefficient of variation of the method was 3.9% at a concentration of 12%. Red cell HbA1c was used as measure of metabolic control. Urine albumin was measured by radioimmunoassay with an interassay variation of 8.7% at 20 mg/l (analysed by staff of the Clinical Chemistry Dept., General Hospital, Southampton). Urine creatinine was measured by Jaffe's reaction, UP1 by ELISA, and A1M by single radial immunodiffusion (Mancini et al, 1965). Protein to creatinine ratio were used.

8.3 RESULTS

Of the 100 patients studied, 12% had clinical proteinuria (Albustix-positive reaction) with urinary albumin greater than 235 mg/l, 36% had albumin excretion greater than 30 mg/l, and 52% had less than 30 mg/l. The urine samples were analysed for UP1 and A1M, and all protein results expressed as protein/creatinine ratio. Overall, there was good correlation between albumin/creatinine (ALBCR) and UP1/creatinine (UP1CR), and ALBCR and A1M/creatinine (A1MCR) ratios at ALBCR greater than 2.5 mg/mmol. Above this ALBCR, the correlations between ALBCR and UP1CR or A1MCR were respectively 0.71 (P<0.001, n=62) and 0.88 (P<0.001, n=62). This contrasted with the relatively poor correlation between ALBCR and UP1CR (r=0.09, P<0.60, n=38), and ALBCR and A1MCR (r=0.29, P<0.40, n=38) below ALBCR 2.5 mg/mmol. Overall, there was significant correlation between UP1CR and A1MCR (r=0.85, P<0.001, n=100).

Based on the albumin and the LMW proteins (UP1, A1MCR) the
diabetic patients were classified into normal renal function (normal LMW protein/creatinine (LMWCR) ratio and ALBCR), primarily glomerular (increased ALBCR with normal LMWCR), primarily tubular (increased LMWCR with normal ALBCR), and mixed tubular and glomerular lesions (increase in both LMWCR and ALBCR). The normal cut off limits used were: ALBCR 2.5 mg/mmol, UP1CR 30 ug/mmol, and A1MCR 1.2 mg/mmol. Classifications of proteinuria were made using UP1CR and A1MCR individually and by a combined UP1CR and A1MCR (Figure 8.1, page 199). The combined classification only considered the same patients that were picked up by both proteins (that is, both UP1CR and A1MCR). There was a good agreement between the classification using combined LMWCR with either UP1CR or A1MCR alone: 91% of the same patients were picked up by both LMW proteins. Biochemical data of the patients as classified using both LMW proteins (Figure 8.1(C)) is further given in Figure 8.2 (page 200). Out of the 46 patients with increased LMW proteinuria, 6 were due to primary tubular and 40 due to a mixed glomerular and tubular lesion. Diabetic patients classified as normal did not have ALBCR and LMWCR significantly different from the control group.

All the 11 (out of 12) diabetics with overt albuminuria had mixed tubular and glomerular proteinuria.

8.4 DISCUSSION

Recently, Marshall and Alberti (1986) demonstrated the value of ALBCR of first morning urine in predicting albumin excretion rate greater than 30 ug/min (that is, microalbuminuria). This study has also used ALBCR in conjunction with LMWCR to assess renal function in diabetes. The results reported here are in agreement with recent
Figure 8.1 Classification of renal lesions based on low molecular weight (LMW) proteins and albumin excretion. (a) Using UP1/creatinine ratio (UP1CR), (b) Using alpha-1 microglobulin ratio (A1MCR), and (c) combined UP1CR and A1MCR. The classification criteria were: increased albumin/creatinine (ALBCR) ratio and LMW protein/creatinine ratio (LMWCR) - GLOMERULAR; increased LMWCR with normal ALBCR - TUBULAR; increased ALBCR and LMWCR - MIXED TUBULAR AND GLOMERULAR LESIONS; and normal ALBCR and LMWCR - NORMAL. The upper cut off limits used were: ALBCR 2.5 mg/mmol, UP1CR 30 ug/mmol, and A1MCR 1.2 mg/mmol. Note that there is 91% agreement in classification (c) with either (a) or (b). Biochemical values for the classification based on both LMW proteins (c) is given in Figure 8.2.
Figure 8.2 Graphic representation of urinary output of albumin, UP1 and alpha-1 microglobulin (A1M) and Red cell haemoglobin A1c (HbA1c) in diabetics with renal function as classified in Figure 8.1 (c) using patients that were picked up by both a1M and UP1 (91 out of 100). Abbreviations: Con. = control subjects; Norm. = diabetics with normal proteinuria; Tub. = diabetics with tubular proteinuria; Glom. = diabetics with glomerular proteinuria; Mixed = diabetics with glomerular and tubular proteinuria. Variation is indicated as SEM.

*Significantly different (P<0.001) from control.
studies by Rowe et al (1987) and Shima et al (1986) that the excretion of LMW proteins is above normal in some normoalbuminuric diabetics, with normoalbuminurics showing primary tubular lesions in 6 out of 38, but appear to conflict with data reported by Mathiesen et al (1984) and Viberti et al (1982) using B2M as an indicator protein for renal tubular function. This discrepancy may result from the greater instability of B2M (Bernard et al, 1982a, b; Davey and Gostling, 1982) relative to UP1 (see Chapter 2) or a1M (Yu et al, 1983) and the use of 24 hour urine without alkalinisation thus providing a longer period for the breakdown of B2M. In addition, increased acidity of urine during period of poor control of diabetes might be expected to increase the lability of B2M and thus conceal obvious tubular abnormality.

Studies using urinary excretion of lysozyme, another LMW protein used as a measure of tubular function, have yielded conflicting results. Farr et al (1976) found no significant increase in urinary lysozyme in Albustix-negative urines. However, in a more detailed study recently, Shima et al (1986) reported that 26% (4 out of 15) diabetics with normoalbuminuria had tubular impairment with lysozymuria and B2-microglobulinuria in inverse proportion to the rate of phenolsulphonphthalein (PSP) excretion. Lopes-Virella et al (1979) reported similar results using combined quantitative measurement of specific proteins and SDS-PAGE. Arguing for the exclusive glomerular origin of microproteinuria, Viberti and Keen (1984) attributed the results of Lopes-Virella's group to methodologic effect of SDS treatment. However, recent evidence indicates that the sensitivity of lysozyme as a marker protein for tubular function may be limited as there is selectivity in renal protein handling with preferential reabsorption, by the PCTs, of cationic molecules such as lysozyme (Christensen et al, 1983). Because of this preferential reabsorption
of lysozyme (pI>10) relative to other LMW proteins (pI<6.0) normally used for the assessment of tubular function, only considerable tubular alterations are likely to be reflected by changes in urinary lysozyme. That is, lysozyme may actually underestimate the degree of tubular problem in diabetes.

The increased LMW protein excretion in normoalbuminuric diabetics may be an indication of subclinical tubular impairment. There was a significant correlation between ALBCR and HbA1c (r=0.26, P<0.05, n=71). A weaker correlation was obtained for the LMW proteins: UP1CR (r=0.2, P<0.10, n=71), A1M (r=0.25, P<0.04, n=71). The low correlation between the degree of diabetic control and LMW excretion is probably because glomerular changes that occur during periods of poor metabolic control, such as functional microangiopathy with increased filtration pressure and/or increased porosity of the microvasculature (Parving et al, 1976), are not likely, per se, to affect urinary excretion of LMW proteins because their glomerular permeability is usually high (Maack et al, 1979). The results may also be explained by the generally fairly good metabolic control (HbA1c 9.9±2.4, range 6.7-18.8) of patients in this study. The latter explanation may also account for the low correlation between ALBCR and HbA1c.

The finding that UP1 and A1M excretion is more frequently increased in a group of diabetics with ALBCR greater than 2.5 mg/mmol makes the case for the concept of progressive renal disease, with damage advancing from the glomerulus to involve the renal tubules, very attractive. This may be relevant in explaining the better correlation between ALBCR and LMWCR at above normal ALBCR ratio (2.5 mg/mmol) compared to normal ALBCR. Since this was found in patients who had not developed overt albuminuria, it means that tubular involvement may ensue before the development of overt proteinuria.
The tubular reabsorption of albumin (and other medium MW proteins such as IgG) and LMW proteins is usually considered to occur by independent processes (Maack et al, 1979); the former probably by a non-selective process with reabsorption being proportional to filtered load (Hardwicke et al, 1970; Deen and Satvat, 1981), and the latter a more efficient, saturable, and probably energy dependent, process (Maack, 1975). Recently, Ratcliffe et al (1986) have reported that at high concentration, albumin can interfere with the reabsorption of LMW proteins. Based on this recent finding, it may be argued that the finding of LMW proteinuria in patients with already increased albumin excretion could be due to saturation of tubular reabsorption capacity in functioning nephrons where protein overload may cause the rising urinary protein excretion rather than primary tubular dysfunction. Even in such conditions, one would expect only modest increases in LMW proteinuria, as was seen in the case of glomerular proteinuria (Figure 8.2, page 200). But the colossal increase seen in patients with 'mixed lesions' is probably not due simply to a process of inhibition or saturation of the reabsorption mechanism, but true tubular involvement in the proteinuria. Earlier studies (Bienenstock and Poortmans, 1970; Waldmann et al, 1972) that reported only modest increases in lambda light chain (LMW) proteinuria in patients with the nephrotic syndrome are consistent with the above conclusion.

Diabetic kidney disease is generally accepted as a glomerulopathy, but it is not very clear whether proteinuria, recognised as clinically important as an early indicator of renal damage, may be explained purely by glomerular alterations. From available reports, an intriguing dissociation can be read when structural and morphological changes in the glomerulus are related to renal function. Thus, over 60% of patients never develop clinical renal disease despite
histological evidence of glomerulosclerosis in nearly all IDDM after only a few years of the disease (Deckert and Poulsen, 1981). It is thus becoming increasingly clear that glomerular morphological changes alone cannot be the exclusive cause of the increased urinary albumin excretion rate seen in early diabetic nephropathy. This has lead to the postulation that the primary event responsible for albuminuria is a qualitative change affecting the charge-selective properties of the glomerular barrier and that this change is independent of the basement membrane thickening, mesengial expansion and glomerular hyperfiltration (Deckert et al, 1984). In a recent review on biophysiology of glomerular proteinuria, Kanwar (1984) acknowledged the uncertainty of morphological glomerular changes seen in diabetics truly reflecting a charge-selective defect or accounting for proteinuria. It is probable that we are only beginning to understand the events that lead to proteinuria, and it is not unlikely that microalbuminuria may be influenced by tubular events. It may be interesting to study patients longitudinally and follow the progression of the diabetics with early tubular involvement.

In conclusion, this study has shown that LMW proteinuria and hence tubular alterations, may not only appear early but may occur independently of glomerular changes, as assessed by albuminuria, in diabetic patients. It is likely that this may complicate or amplify glomerular changes that are known to occur early in diabetes, may affect the course of progression in renal disease in some diabetics, and may be relevant in explaining the differences between observed morphological glomerular changes and renal function. It may be useful to follow and assess the course of progression of kidney disease in patients with early subclinical tubular alterations. The value of UP1-uria in assessing tubular lesion has again been demonstrated by the
good correlation between its levels and those of a protein with established relevance in diagnosis of renal function.
CHAPTER NINE

FINAL DISCUSSION AND CONCLUSION
9.1 HUMAN URINE PROTEIN 1

Human Urine protein 1 (UP1) has been purified from urine samples by a combination of gel and immunoaffinity chromatography and semi-characterized. It has been found to be a negative, low molecular weight (LMW) glycoprotein with electrophoresis mobility in the alpha-2 region. Based on the size and electrophoretic mobility of the glycoprotein, the name alpha-2 glycomicroglobulin (α₂GM) has been suggested for UP1. A sensitive and reproducible Enzyme-linked immunosorbent assay (ELISA) was set up and validated for the quantitation of microquantities of α₂GM (Chapter 3). This was used to study the stability characteristics of α₂GM (Chapter 2). α₂GM demonstrated remarkable stability at room, physiological, and refrigeration temperatures; in pathological acid concentration of urine the stability was comparable to RBP and α₁M (Bernard et al, 1982; Yu et al, 1983) and superior to B₂M. The stability of α₂GM in acid urine was found to be a useful property in subsequent clinical studies as it obviated the need for the alkalinisation of urine after collection, or giving subjects alkalis to ensure urine of high pH.

9.2 SITE OF PRODUCTION AND CATABOLISM

Immunocytochemical studies have established the liver as the site of synthesis of α₂GM. The cytoplasm of hepatocytes stained intensely in areas probably indicative of endoplasmic reticulum and/or golgi apparatus. Though the corpora amylacea of the prostate glands showed α₂GM reaction, it was doubtful whether this was due to a de novo synthesis within the prostate, since the glandular epithelial cells did
not stain for the protein. Therefore the prostate staining, if specific for $a_2$GM, was probably due to extra-prostatic delivery.

A study of changes in $a_2$GM and other LMW proteins (RBP and alpha-1 microglobulin) in various pathological states has been undertaken (Chapters 4-8). These experiments demonstrated remarkable mutual correlations between $a_2$GM and other LMW plasma proteins ($a_1$M and RBP) both in urine and serum. This finding, coupled with similarity in the pattern of change of $a_2$GM with serum creatinine and creatinine clearance, and the fact that the renal proximal convoluted tubules stained strongly for $a_2$GM antigenic reaction (Chapter 4), indicate that $a_2$GM is catabolised in a similar manner as other LMW proteins (Hall and Hardwicke, 1979; Maack et al, 1979; Strober and Waldann, 1974). That is, $a_2$GM is synthesised by the liver, like many glycoproteins, and, in healthy kidneys, rapidly cleared by the glomerulus, reabsorbed and catabolised mainly by the proximal tubules; under this condition only minute quantities are excreted in urine and serum levels are kept within the 'reference range' (Chapter 3). This is to be contrasted with the colossal levels of $a_2$GM in urine and serum of patients with end-stage renal failure where, though filtration is reduced giving rise to accumulation of $a_2$GM in blood, this fails to lead to a concomitant reduction in urinary $a_2$GM due to persistent glomerular filtration in nephrons where only tubular function is impaired.
9.3 DIAGNOSTIC VALUE OF $a_2$GM

From the various cross-sectional studies using patients with various diseases, $a_2$GM was identified as having potential use in the diagnosis of renal diseases. In all the patients studied, only those with increased serum creatinine, that is diseases associated with impairment in renal function, showed a significant increase in serum $a_2$GM. Serum $a_2$GM levels appeared to be reasonably specific for changes in renal function, with patients under treatment for end-stage renal disease showing up to 40 times increased $a_2$GM relative to healthy subjects. The clinical significance of UP1 was therefore assessed with specific reference to renal function.

9.3.1 OVERALL RENAL FUNCTION

Studies using patients with chronic renal diseases, long-term transplants and normal subjects (Chapter 5) have demonstrated usefulness of measuring serum and urinary $a_2$GM in the assessment of renal function. Changes in serum $a_2$GM correlated well with serum creatinine in renal insufficiency, showing exponentially increasing levels of $a_2$GM in serum with declining GFR. An indication that glomerular filtration is the main method of elimination of $a_2$GM like other LMW proteins (Bernier et al, 1968; Peterson et al, 1969). The urinary levels of $a_2$GM in renal insufficiency paralleled changes in serum, both increasing with deterioration in renal function. Increased levels of $a_2$GM may be an indication of either tubular change parallel to changes in the glomeruli as can be observed morphologically in progressive renal damage, or the increased serum concentration of urinary $a_2$GM in the course of advanced uraemia.
creating tubular load in excess of an eventual tubular reabsorption capacity, or simply due to persistent, though reduced, glomerular filtration in damaged nephrons.

The value of serum $a_2GM$ monitoring in the assessment of renal function in kidney transplant patients was evaluated. The study demonstrated the value of $a_2GM$ in assessing the overall renal function of the allograft. Changes in serum $a_2GM$ correlated with renal function, as assessed by serum creatinine. Prolonged and sustained increase in serum $a_2GM$ was useful in predicting rejection episodes, and in most cases, serum $a_2GM$ rose 48 hours before significant changes in creatinine were observed.

9.3.2 GLOMERULAR FUNCTION

Frequently, glomerular function is assessed by either creatinine clearance or, as is becoming common clinically, by serum creatinine. However, serum creatinine is known to be elevated only when considerable renal damage has already occurred, with over 50% nephron loss. Serum $a_2GM$ was assessed as a tool in the monitoring of changes in glomerular filtration (Chapter 5). Serum $a_2GM$ was elevated above normal as creatinine clearance fell below 80 (that is between 50 to 80) ml/min, at time when serum creatinine was still within the normal range. Serum $B_2M$, another LMW protein, has been suggested for this purpose, but it may not be specific for GFR changes as its levels can change independently of renal function. $a_2GM$, on the other hand is stable (Chapter 3), does not seem to be affected in any significant way by diseases that are not accompanied by impairment in renal function, nor is its serum levels affected by stimuli that elicit acute phase response such as myocardial
Infarction and trauma (Chapter 4). Serum $a_2$GM may therefore be considered as being potentially useful in the assessment of changes in glomerular filtration, especially in the creatinine 'blind region' of GFR. In addition, the linearity of change of inverse serum $a_2$GM with creatinine clearance (Chapter 5) indicates that it may have additional potential use in longitudinal assessment of progressive changes in GFR with time in chronic renal disease, and probably make it possible to predict the time course for the development of end-stage renal disease.

Another LMW protein that has been suggested for this purpose is $a_1$M (Kusano et al, 1985). But as observed in Chapter 4, serum levels of $a_1$M may be increased in patients with myeloma (also see Takagi et al, 1980; Kawai and Takagi, 1982). For this group of patients, $a_1$M may not be specific for renal injury.

9.3.3 TUBULAR FUNCTION

Several LMW plasma proteins have been investigated and proposed as indices of impaired proximal tubular function. The best known are $B_2$M, $a_1$M, lysozyme, RBP and RNAase. Currently, the ones favoured on the basis of stability and ease of measurement are $a_1$M and RBP. Several studies have shown that the excretion of these proteins in urine from patients with renal disease is generally closely related. A good correlation between $a_2$GM and $a_1$M and RBP has been demonstrated, as exemplified by the studies using patients with chronic renal diseases (Chapter 5). The potential value of urinary $a_2$GM in diagnosing or assessing tubular damage looked plausible when both urinary levels and fractional clearance of the protein, a measure that relates protein excretion to functioning
nephrons, were both increased in chronic renal disease. This potential was tested by assessing changes in patients on cisplatin cancer therapy. Cisplatin is known to be associated with tubular damage (Litterst et al, 1986; Rees and Klastersky, 1986). Urinary $a_2$GM levels were shown to change faster than $a_1$M (in all patients) and RBP (in over 50% of the patients monitored). There was, on the whole, a good correlation between $a_2$GM and the other LMW plasma proteins.

In the assessment of tubular function in diabetics, $a_2$GM gave results that were, beside being over 90% in agreement with $a_1$M, confirmed other reports (Rowe et al, 1987; Shima et al, 1986) that tubular proteinuria, hence renal tubular dysfunction, may occur early in some diabetics either in conjunction with, or independently of, glomerular alterations.

9.4 **PREDICTIVE VALUE OF $a_2$GM?**

The measurements of serum and urinary $a_2$GM gave abnormal results in patients with renal disease: therefore, $a_2$GM monitoring could become a useful test for the diagnosis of renal disease. Ideally, for every diagnostic test, it is desirable to know the degree of accuracy to which the result obtained predicts the presence or absence of the disease, that is the predictive value of the test. The predictive value of a diagnostic test for a disease is the accuracy of the test in predicting the presence or absence of the disease, and is determined by a complex interaction between the incidence of false-negative results in patients with the disease, the incidence of false-positive results in subjects without the disease, and the prevalence of the disease.
itself in the study population. It is, in essence, the percentage of all positive results that are true positives. The predictive value of \( a_2 \)GM in renal disease would therefore require knowledge of its positivity in disease (sensitivity), negativity in health (specificity) and the prevalence of renal disease in the group examined.

The possibility of establishing the predictive value of \( a_2 \)GM measurement in serum and urine in renal disease using the data obtained in this work has been explored. The results and problems of using the data for this purpose are briefly summarized below.

Out of the 29 normal subjects initially selected, all had 'normal' serum \( a_2 \)GM, but one subject had abnormal urine \( a_2 \)GM and his results eventually excluded. The subject had no clinical evidence of renal disease so the result was considered as false-positive. This gives the test a specificity of 29/29 or 100\% for serum samples and 28/29 or 96\% for urine samples.

In all the patients with chronic renal failure (excluding the long-term transplants), 41 out of 42 had above serum normal \( a_2 \)GM; one patient (out of 42) with nephrotic syndrome, had normal serum \( a_2 \)GM. All had increased urinary \( a_2 \)GM. This gives the test a sensitivity of 41/42 or 97.6\% for serum \( a_2 \)GM, and 42/42 or 100\% for urine.

This would give a predictive value of \( a_2 \)GM measurement for renal disease of 41/42 or 97.6\% (for serum) and 42/43 or 97.6\% (for urine), with a false-negative rate of 2.4\% (i.e 100-97.6) and an efficiency of 98.6\% (i.e 70/71) for both sample types. Although this is a high predictive value, it is to be viewed
against the background of the high prevalence of renal disease in the study population, with a prevalence rate of 42 out of 71 or 59%, and that most of the patients had advanced renal disease (GFR<50 ml/min). For a population with a lower prevalence of the disease, the predictive value of the test is likely to be reduced, with higher false-positives (Galen and Gambino, 1975). Since the patients had advanced renal disease, the predictive value for serum and urine samples may not accurately reflect, respectively, exclusive glomerular and tubular lesions as both functions are likely to have been compromised.

The predictive value of using \( \alpha_2 \text{GM} \) in the assessment of subclinical tubular alterations was considered. Most of the patients for this study (cancer patients on cisplatin and diabetics) did not have clinical evidence of tubular disease. Ethical difficulty of using a 'gold test', such as renal biopsy, as a reference for \( \alpha_2 \text{GM} \) results made it difficult to quantitate the predictive value of the test in the diagnosis of tubular disease. However, since urinary \( \alpha_2 \text{GM} \) paralleled closely other LMW plasma proteins (\( \alpha_1 \text{M} \) and RBP) used in the assessment of tubular function, and the fact that \( \alpha_2 \text{GM} \) was seen to rise during cisplatin treatment, it indicates that \( \alpha_2 \text{GM} \) measurement in urine may have a high predictive value.
9.5 CONCLUSIONS

From the work presented here, the following conclusions can be summarised:

1. $\alpha_2\text{GM}$ is a low molecular weight, negative glycoprotein with an alpha-2 mobility on cellulose acetate electrophoresis. It is synthesised in the liver and handled by the kidney in a similar manner to other LMW proteins such as alpha-1 microglobulin and RBP.

2. Serum and urinary $\alpha_2\text{GM}$ has been shown to correlate with renal function and it is concluded that serum and urinary $\alpha_2\text{GM}$ monitoring may be useful in predicting or assessing changes in GFR, in differential diagnosis of renal disease, in monitoring the course of progression of renal disease and perhaps in predicting the development of end-stage disease. And from the good correlation between $\alpha_2\text{GM}$ and other LMW proteins, it is suggested that measurement of $\alpha_2\text{GM}$ alone would suffice as representative of changes in pattern of other LMW proteins.

3. Serum monitoring of $\alpha_2\text{GM}$ after renal transplantation has been found to be a useful and fairly sensitive procedure. It is concluded that $\alpha_2\text{GM}$ has great potential in early characterisation and monitoring of rejection episodes, and allograft function in the management of kidney transplant recipients.
4. Urinary $a_2$GM measurement in evaluation of renal tubular function has indicated remarkable sensitivity of $a_2$GM in monitoring cancer patients on cisplatin chemotherapy. In diabetics, urinary $a_2$GM (and $a_1$M) showed that tubular alteration in function may occur early in insulin-dependent diabetics, before the development of overt albuminuria, and in a small percentage, before slight increases in albumin excretion (microalbuminuria) is observed.

9.6 FUTURE WORK

The following studies are desirable to further assess the clinical significance of UP1:

1. Further characterisation to involve amino acid analysis and sequencing of $a_2$GM. This may be useful in delineating the physiological role of the protein.

2. Studies on the site of synthesis revealed specific staining in the liver. It could not be established by light microscopy which subcellular organelles were associated with UP1 reaction. Further research is needed on the ultrastructural level to establish these subcellular structures.

3. It may be useful to assess tubular function longitudinally in patients suspected of tubular lesion from cadmium poisoning, analgesic or aminoglycoside abuse. This may reveal the extent of changes in the levels of UP1 with resolution of lesions and
thus the sensitivity of UP1 measurement in tubular disease.

4. Establish the predictive value of $a_2$GM in the diagnosis of tubular damage in particular and kidney disease in general both in patients with the disease alone and/or other diseases.


Farquhar MG (1975): The primary filtration barrier - basement membrane or epithelial slits? Kidney Int 8:197-211.


