THE EFFECT OF GENTAMICIN THERAPY ON URINARY EXCRETION OF RETINOL-BINDING PROTEIN, LYSOZYME AND ELECTROLYTES

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by

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SUMMARY

The urinary excretions of the low molecular weight proteins, retinol binding protein (RBP) and lysozyme, were investigated in 28 patients treated with gentamicin (an aminoglycoside antibiotic) at East Birmingham Hospital. A control group of 21 surgical patients, not receiving aminoglycoside antibiotics, was also studied. The aim of the study was to investigate these two proteins as urinary indicators of drug-induced proximal tubular damage, such as that caused by the aminoglycoside antibiotics. The urinary excretions and plasma levels of calcium and magnesium were also studied in these patients to investigate any changes in electrolyte homeostasis due to aminoglycoside therapy.

Lysozyme was measured by an established turbidometric assay. An enzyme-linked immunosorbent assay for determination of RBP was specially developed for this study. Calcium and magnesium were determined by atomic absorption spectrophotometry.

RBP and lysozyme both proved too sensitive and non-specific to use in clinical practice as urinary markers of drug-induced proximal tubular damage. It was not possible to identify those patients who later developed nephrotoxicity, as excretion of both proteins was raised in most patients treated with gentamicin, whether or not plasma creatinine was significantly raised. Urinary excretions of both these proteins were also raised, after surgery, in patients who did not receive aminoglycosides and showed no significant rise in Pcr. The highest excretions of RBP and lysozyme were shown by gentamicin-treated surgical patients, possibly due to a combination of surgery, infection and gentamicin therapy. Patients with cystic fibrosis who were treated with gentamicin had normal or only slightly raised excretions of both proteins despite longer courses of therapy.

Hypomagnesaemia and hypocalcaemia, which were very mild, occurred in only a few patients and no evidence was found for an effect of gentamicin on electrolyte excretion. The absence of significant effects on electrolyte homeostasis may be attributable to the relatively short courses of gentamicin therapy, whereas previously published reports of hypomagnesaemic hypocalcaemia involved long-term, high-dose gentamicin therapy.

Key words:Gentamicin-nephrotoxicity, retinol binding protein, lysozyme, calcium, magnesium.

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I would especially like to thank my project superviser Dr E S Harpur (MRC Mechanisms of Drug Toxicity Group, Department of Pharmaceutical Sciences, Aston University), for his constant support, advice and encouragement.

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Collaborative Work in the Studies

A student at Aston University, Miss Catherine Burn, carried out the stability study of retinol-binding protein. All other studies were carried out by myself. Use has also been made of data from the Clinical Chemistry Laboratory at East Birmingham Hospital. The presentation and analysis of the results set out in this thesis is entirely my own.

Ethical Approval of the Study

A protocol of the study was approved by the Ethical Committee at East Birmingham Hospital, prior to commencement of the study. The decision to start gentamicin therapy was made by the doctor responsible for the patient and was in no way influenced by this study. Before asking for their consent to take part in the study, all patients in treated and control groups were informed that daily urine collections were required; it was also explained that the information obtained was for research purposes alone and would not affect their treatment.

List of Abbreviations Used

AAP	Alanine Aminopeptidase
B_2M	Beta-2-Microglobulin
BSA	Bovine Serum Albumin
Ca	Calcium
CBC	Carbonate Bicarbonate Buffer
CF	Cystic Fibrosis
CI	Chest Infection
Cl_{cr}	Creatinine Clearance
EBH	East Birmingham Hospital
ELISA	Enzyme-Linked Immunosorbent Assay
F	Female
GFR	Glomerular Filtration Rate
HSE	Health and Safety Executive, Edgware Road, London
LDH	Lactate Dehydrogenase
Lysozyme	Muramidase
M	Male
Mg	Magnesium
NAG	N-Acetyl-beta-D-Glucosaminidase
op	Operation
PBS	Phosphate Buffered Saline
P_{cr}	Plasma Creatinine
PSP	Protein Standard Plasma (Behring)
RBP	Retinol-Binding Protein
S_{cr}	Serum Creatinine
SD	Standard Deviation
SDS/PAGE	Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis

TBS	Tris Buffered Saline
TEMED	NNN' Tetramethylethylenediamine
UPCR	Urine to Plasma Creatinine Ratio
UTI	Urinary Tract Infection
WI	Wound Infection.

INTRODUCTION

The aminoglycoside antibiotics are potent bactericidal agents which are most frequently used for the treatment of serious gram-negative infections. One of the major limitations to the use of these antibiotics is, however, their ability to cause nephrotoxicity. The reported incidence of aminoglycoside-induced nephrotoxicity in clinical studies depends on the methods and criteria used for assessing nephrotoxicity and varies from 4 - 24%[1].

The aminoglycosides are highly polar cations consisting of one or more amino-sugars joined by a glycosidic linkage. The number of amino groups per molecule is responsible for the degree of cationic character and also the ability to interact with anionic components in biological membrances[2]. Different aminoglycosides show differing potential to cause nephrotoxicity, which appears to be related to the number of amino groups per molecule and cationic structure[2].

Renal Handling of Aminoglycosides

The kidney is the main route of elimination of aminoglycosides from the body. Aminoglycosides are not metabolised and only 1 - 2% of the administered dose is excreted in the bile. After a single injection, 60 - 80% of the drug is recovered unchanged over a subsequent 24 hour urine collection. Therefore, with repeated dosing, the daily urinary recovery approaches 100% of the injected dose[3]. After discontinuation of aminoglycoside therapy, gentamicin may be detected in the urine for up to 4 weeks[4], as it is gradually released from the tissue stores.

Aminoglycosides are first filtered by the glomerulus, then have been shown by clearance studies to be reabsorbed into the cells of

the proximal tubule. No reabsorption of aminoglycoside has been detected in the distal tubule[5].

Aminoglycosides appear to be taken up across the luminal and basolateral membranes of the proximal tubular cells[6], by an energy-dependent mechanism[7]. It is thought that aminoglycosides undergo an uptake process involving binding to anionic sites on the brush border of the luminal membrane, then endocytic uptake and transfer to lysosomes[8]. The active transport into the proximal tubular cells does not appear to be dependent on the mechanisms by which organic acids and bases, or glucose, are reabsorbed[9].

The aminoglycosides have been shown to compete for binding sites on the brush border[10], with filtered cationic proteins[11], polypeptides, polyamines, polylysine, amino acids and also other aminoglycosides[12]. This binding appears to be both competitive and saturable[12].

Aminoglycoside Nephrotoxicity

Most of the work to determine the histopathology and mechanisms of aminoglycoside nephrotoxicity has been carried out in the animal model. Although the threshold for aminoglycoside nephrotoxicity is different in different animals and humans, once the threshold has been exceeded all animal models show similar functional and histopathological lesions. In the rat model aminoglycosides have been shown to damage both the proximal tubule and the glomerulus[13,14,15]. Damage may be reversible or irreversible and is dependent on such factors as dose, duration of therapy[16], age[17], and sex[18]. Concomitant therapy with other potentially nephrotoxic drugs, sodium depletion[19], metabolic acidosis[20], and pre-existing renal disease are pre-disposing factors in aminoglycoside nephrotoxicity.

1. Histology.

A: Proximal tubular damage

Aminoglycosides induce proximal tubular cell necrosis. The first identifiable lesion to be seen by electron microscopy, in rat proximal tubular cells, is an increase in the number and size of secondary lysosomes (also known as cytosegresomes) which contain myeloid bodies[5]. Myeloid bodies consist of densely packed membranes, and have been detected in proximal tubular cells within 48 hours of gentamicin administration[21]. Later, other changes such as a decrease in the number and height of microvilli in the brush border membrane[22], swelling of mitochondria, cytoplasmic vacuolisation and swelling of the endoplasmic reticulum can be seen[5]. These changes can progress to frank tubular necrosis. However, even after severe damage with cell necrosis, tubular cells can regenerate, whether or not aminoglycoside treatment is continued[23,24].

B: Glomerular damage

A reduction in both the number and size of glomerular endothelial fenestrae has been shown to occur with aminoglycosides in an order corresponding to the nephrotoxic potential of particular drugs[14,15]. This may be responsible, in part, for the decrease in glomerular filtration rate (GFR) caused by aminoglycosides. It is possible that this effect may be due to polycationic aminoglycosides neutralising negatively charged sites around endothelial fenestrae, leading to a reduction in diameter and eventual collapse of the fenestrae[2].

2. Pathways of Aminoglycoside Nephrotoxicity.

Little is known about how aminoglycosides cause damage to the glomerulus. It is possible that they may have a direct toxic action on the glomerulus, or that the damage may be secondary to tubular injury.

As aminoglycosides exert their bactericidal action by binding to ribosomal units causing inhibition of protein synthesis, it is possible that accumulation of aminoglycosides within proximal tubular cells may inhibit protein synthesis, leading to cell injury.

One theory of the pathway of aminoglycoside nephrotoxicity is that it may be due to the inhibition of Na^+-K^+ATPase, within the proximal tubular cell, by aminoglycosides. However, an investigation showed that aminoglycosides only demonstrated a marked inhibition (50-100%) of Na^+-K^+ATPase at very high concentrations of the drug[25]. Therefore, it was suggested that this effect on Na^+-K^+ ATPase was unlikely to be the primary mechanism of nephrotoxicity.

Another proposed theory of aminoglycoside nephrotoxicity is that it may be due to the effects of aminoglycosides on mitochondrial function. It has been found that gentamicin causes decreased ammonia and glucose production in rat proximal tubular cells, which suggests a mitochondrial lesion.[26]. Other investigations found that, *in vitro*, gentamicin is a competitive inhibitor of mitochondrial Ca^{2+} uptake in a dose-dependent matter[27]. Therefore, it appears that aminoglycosides alter mitochondrial function, but it is not yet known whether this is a contributory factor in aminoglycoside-induced nephrotoxicity.

The most widely accepted theory of how aminoglycosides damage the proximal tubular cells is due to their effect on lysosomal degradation processes. It has been found *in vitro*, that lysosomal

accumulation of aminoglycosides inhibits the activity of phospholipases, the enzymes which break down phospholipids within the lysosome[28]. In vivo gentamicin has been shown to cause an increase in phospholipid concentration in the renal cortex and an increase in the urinary excretion of phospholipids[29]. Therefore, it appears that the aminoglycoside-induced phospholipidosis leads to an accumulation of undegraded cellular membranes within lysosomes, leading to an increase in lysosomal size and in the formation of myeloid bodies[8]. There is also a reduction in the degradation of low molecular weight proteins, normally filtered by the glomerulus then taken up and catabolised in the proximal tubular cells. This reduced degradation of low molecular weight proteins may be due, in part, to an inhibitory effect of aminoglycosides on protein binding to the brush border membrane. Lysosomal accumulation of aminoglycosides leads to an increase in lysosomal size and the lability of the lysosomal membrane. Eventually, the lysosome may disrupt leading to liberation of lytic enzymes and subsequent cell necrosis[2]. Although there is much evidence for the effect of aminoglycosides on lysosomal function, whether this effect is the primary mechanism of aminoglycoside nephrotoxicity has yet to be determined.

3. Functional Manifestations of Aminoglycoside Nephrotoxicity.

Aminoglycoside-induced nephrotoxicity is manifested, at first, as polyuria, decreased urinary osmolarity, enzymuria and low molecular weight proteinuria. Later signs are raised serum creatinine and urea, caused by a decrease in the GFR[5]. These functional changes have been found in both human subjects and the animal model.

The enzymuria is caused by an increase in the excretion of both brush border[30,31] and lysosomal enzymes[32,33] of the proximal tubular

cells. Lysosomal enzymuria may occur due to the exocytosis of lysosomes causing release of enzymes into the proximal tubular lumen, or from actual proximal tubular cell necrosis. The increased urinary excretion of brush border enzymes such as γ-glutamyl transferase and alanine aminopeptidase, is probably due to accelerated turnover of the brush border membrane.

The low molecular weight proteinuria occurring early in aminoglycoside nephrotoxicity is mild and of tubular origin. Proteins with a molecular weight of less than 30,000 are usually filtered by the glomerulus, reabsorbed by the proximal tubular cells, then broken down within the lysosomes, the resulting amino-acids being returned to the circulation[34]. Therefore only trace amounts of these proteins are normally found in the urine. However, if the cells of the proximal tubule are damaged, or their normal function disrupted, then increased amounts of these proteins appear in the urine[35].

Polyuria and a decrease in urine osmolarity have been found to be early manifestations of aminoglycoside nephrotoxicity in the rat model[21,33,36,37]. The urine concentrating defect occurs before the GFR is measurably reduced. This defect is not accompanied by an increased urinary solute excretion or by a fall in tubular reabsorption of solute-free water, and administration of exogenous vasopressin does not correct it[36]. This polyuria has been proposed to be due to a decreased number of functioning nephrons accompanied by a compensatory increase in filtration rate and a mild solute diuresis per residual nephron[36]. Other investigators have found evidence suggesting that aminoglycosides interfere with the action of vasopressin on the distal nephron[38].

A decrease in the GFR is a relatively late manifestation of aminoglycoside nephrotoxicity, being preceded by enzymuria,

proteinuria and decreased urine concentrating capacity. The decrease in GFR may be due to a direct action on the glomerular epithelium[15], or secondary to proximal tubular cell damage. The decrease in GFR leads directly to increased serum concentrations of urea and creatinine.

4. Potential Clinical Indicators of Aminoglycoside Nephrotoxicity.

A: Serum creatinine/Creatinine clearance.

Presently the most widely accepted method for the assessment of drug-induced nephrotoxicity is the measurement of the serum creatinine or creatinine clearance. Creatinine clearance can only be determined by collecting 24-hour timed urine samples, which are notoriously difficult to obtain accurately. Therefore, often the serum creatinine value is used to estimate creatinine clearance, using an equation such as this one developed by Cockroft and Gault[39]:

$$Clcr = \frac{(140\text{-age})\ (wt.kg)}{72 \times Scr\ (mg\%)}$$

(15% less in females)

Clcr = creatinine clearance (ml/min)
Wt.kg = weight in kg.
Scr = serum creatinine

As previously stated, however, raised serum creatinine is one of the later signs of nephrotoxicity and occurs only when the GFR is markedly impaired. As in aminoglycoside-induced nephrotoxicity, extensive tubular damage may precede any change in GFR, serum creatinine is not really an appropriate marker. Raised serum creatinine is also not specific to proximal tubular damage, as it will increase in any conditions decreasing the GFR.

In normal or impaired renal function, serum creatinine concentration is determined by rate of creatinine production, endogenous creatinine clearance, and during changing renal function,

by the apparent volume of distribution for creatinine. These determinants of serum creatinine concentrations are affected by age, sex and body weight[40].

Therefore, a more sensitive indicator of aminoglycoside nephrotoxicity, which is more specific to proximal tubular cell damage, and which shows tubular damage before any appreciable decrease in GFR, would be very useful.

B: Urinary Enzymes.

As enzymuria is one of the early manifestations of nephrotoxicity, many urinary enzymes have been investigated as potential markers of aminoglycoside nephrotoxicity. The evidence as to the usefulness of various urinary enzymes as markers of nephrotoxicity is often contradictory.

In one study, the urinary excretion of the enzymes β-D-galactosidase, α-L-fucosidase and N-acetyl-β-D-glucosaminidase (NAG) were found to rise in nearly all aminoglycoside-treated patients, in the absence of other clinical signs of nephrotoxicity[41]. In another study, some aminoglycoside patients who did not show any elevation in serum creatinine, showed raised urinary levels of NAG, β-D-galactosidase and β-glucuronidase[42]. The urinary excretion of the brush border enzyme alanine aminopeptidase (AAP) has been found by some workers to be too non-specific to use in clinical practice[43,44].

However, some studies have found the measurement of some urinary enzymes to be useful. For example, the lysosomal enzyme NAG has been found to be predictive of nephrotoxicity in patients receiving aminoglycosides, in several studies[44,45]. The isoenzymes of NAG have also been found to be of use in distinguishing the site of renal injury[46,47].

Therefore, the evidence as to the usefulness of various urinary enzymes in the detection of aminoglycoside-induced nephrotoxicity is not at all conclusive. In a recent review on urinary enzymes, it was summarised that the assay of NAG and AAP, together with perhaps lactate dehydrogenase (LDH), may provide useful information on the renal toxicity of antibiotics and the degree of renal damage caused by their administration[48].

C: Urinary Excretion of Low Molecular Weight Proteins

As a low molecular weight proteinuria is an early event in aminoglycoside nephrotoxicity, the measurement of certain low molecular weight proteins have been investigated as potential markers of nephrotoxicity.

Aminoglycosides have been shown to effect several processes in the renal handling of the cationic low molecular weight proteins. A study in rats showed that gentamicin caused impairment of glomerular filtration, tubular reabsorption and lysosomal catabolism of the low molecular weight protein, lysozyme, due to interactions between the two cations[10]. Therefore, an increased excretion of protein in the urine may be due to competition with aminoglycoside for uptake, protein not being catabolised due to the effect of aminoglycosides on lysosomal catabolism or frank cell necrosis so that proteins cannot be reabsorbed.

The low molecular weight protein, B_2-microglobulin (B_2M) has been extensively studied as a possible indicator of aminoglycoside nephrotoxicity. However, recent studies suggest that B_2M is unstable in urine with a pH of 5.5 - 6.0 or less[49,50]. Also as B_2M is cationic, aminoglycosides have been shown to inhibit its proximal tubular reabsorption[51], therefore it tends to be excreted in large amounts in most patients receiving aminoglycosides.

AIMS AND OBJECTIVES OF THE STUDY

For this study it was decided to investigate the urinary excretion of two low molecular weight proteins, muramidase and retinol-binding protein (RBP), with the objective of evaluating these two proteins as potential markers of aminoglycoside nephrotoxicity. It was also hoped that studying the excretion of these two proteins may increase our knowledge of the mechanisms by which aminoglycoside nephrotoxicity is caused.

Muramidase, which is more commonly referred to as lysozyme, is a cationic protein, having a pI of 10.5 - 11.0[52]. The tubular reabsorption of lysozyme appears to be competitively inhibited by the cationic aminoglycosides[10]. Therefore, an increased urinary excretion of lysozyme may be due to competition with aminoglycosides for anionic binding and subsequent uptake into proximal tubular cells, the inhibition of the lysosomal degradation by aminoglycosides, tubular necrosis, or a combination of these effects.

RBP is an anionic protein, having a pI of 4.4 - 4.8[51], which would not be expected to have its tubular uptake appreciably inhibited by aminoglycosides, as it should not compete for anionic binding sites on the brush border membrane. Therefore, urinary excretion of RBP would be expected to rise appreciably only when tubular necrosis had occurred, so that RBP could not be reabsorbed, or due to inhibition of the lysosomal catabolism of the protein by aminoglycosides.

Therefore it was thought of interest to investigate and compare the urinary excretion of these two low molecular weight proteins in aminoglycoside-treated patients. To evaluate their potential as markers of nephrotoxicity, the excretion of these proteins needed to be compared with serum creatinine data (as an absolute indicator of renal damage).

As aminoglycosides are often given post-surgically, it was decided to study the urinary excretion of these proteins in a control group of surgical patients not receiving aminoglycosides, to investigate any effects which surgery may have on the excretion of these proteins.

It was also thought of interest of study the plasma levels and urinary excretion of calcium and magnesium in these patients, due to recent reports of aminoglycoside-induced hypomagnesaemia with hypocalcaemia in patients.

Lysozyme

Muramidase, a bacteriolytic enzyme usually referred to as lysozyme, has a molecular weight of 15,000-16,000[52]. Lysozyme in the blood originates in the leuocytes and is probably concerned with defence against bacterial infection[53]. Lysozyme has already been studied, in patients, as a marker of proximal tubular damage due to aminoglycosides or other drugs[41,53-61]. Several of these studies concluded that urinary lysozyme measurement may be a useful indicator of proximal tubular cell damage[54,56-61]. One study, however, found that increased urinary excretion of lysozyme occured with hypokalaemia, post-operative collapse, electrolyte depletion, severe extrarenal infection, acute pyelonephritis, nephrotic syndrome and uncomplicated surgical procedures, therefore was of limited value in the clinical diagnosis of proximal tubular damage[53]. Other studies found that lysozyme excretion increased in all patients treated with aminoglycosides, whether there were other clinical signs of nephrotoxicity or not[41,55].

Therefore, the evidence gathered so far does not confirm whether measurement of urinary lysozyme is a useful predicter of aminoglycoside nephrotoxicity.

To establish that increased urinary excretion of lysozyme is due to impaired renal function, it is necessary to establish that serum lysozyme does not exceed the renal threshold, as this would cause an overflow of lysozyme into the urine. Therefore, both serum and urinary levels of lysozyme need to be measured. The normal serum levels of lysozyme are 5.1 - 14.0 mg/L[56], the normal urinary levels are 0 - 2 mg/L[58], and the renal threshold is around 45 mg/L[53]. Serum levels above the renal threshold commonly occur in leukaemic patients[62].

Retinol-binding protein

Retinol binding protein has a molecular weight of about 21,000[63,64]. It is the specific carrier protein for retinol (Vitamin A alcohol) in the plasma. It is synthesised by the liver and transports retinol from the liver to its target tissues[63].

In the plasma, RBP circulates as a 1:1 molar complex with thyroxine-binding prealbumin (molecular weight 55,000)[63,64]. The affinity between the two proteins is specific and the affinity constant is high[65].

In addition to the prealbumin-bound RBP, free RBP is present in small amounts in normal plasma[66]. In normal circumstances, about 95% of RBP is bound to prealbumin in plasma, only about 5% remaining uncomplexed[67,68]. Of this free RBP, 80-90% is apo RBP, i.e. not attached to retinol, whereas the rest is attached to retinol (holo RBP)[66]. In patients with impaired glomerular filtration, the concentration of free RBP is greatly increased [66-68].

When retinol has been delivered to the tissues by the complexed RBP, RBP is believed to lose some[70], if not all[71], of its affinity for prealbumin. This loss of affinity for prealbumin may result from

a conformational change in RBP when retinol is lost[70]. The resulting free RBP is filtered by the glomeruli, then taken up and catabolised in the proximal tubular cells[67]. Therefore, very little RBP, about 0.11 mg per 24 hr volume[72], appears in the urine under normal circumstances.

Both apo and holo uncomplexed RBP have been found in the urine and both appear to be of the same type as the free apo and holo RBP found in plasma[73]. The bulk of this urinary RBP lacks retinol, only about 20% of urinary RBP is holo[73]. For prealbumin-bound RBP to be found in the urine, significant deterioration of the glomerular filtration barrier would need to take place to allow high molecular weight proteins to pass through.

The total pool of RBP in the body is about 410 mg[68], slightly more than half of which is present in the plasma compartment (220 mg)[67] and the rest in the interstitial compartment. As uncomplexed RBP has a fractional filtration of 0.4, about 240 mg of RBP should be filtered each day by the glomeruli and subsequently metabolised in the proximal tubular cells[68]. In patients with tubular proteinuria, excretion of up to 150 mg RBP per 24-hour volume has been reported[72].

No reference has been found to the renal threshold of RBP, but the normal serum range is 30-60 mg/L[74]. The levels of RBP in normal urine range from 0-0.56 mg/L[75].

Very little work has yet been attempted to determine whether RBP would be a useful indicator of aminoglycoside-induced nephrotoxicity. One study, however, investigated the relationship between RBP excretion and glomerular and tubular nephropathies and found that RBP excretion increased only in patients with tubular nephropathies[76].

RBP has been shown to be stable in urine down to a pH of 4.5[50], and as it is anionic, it should prove to be a more reliable and specific index of proximal tubular damage than B_2-m[51].

Electrolyte disturbances in aminoglycoside therapy.

There have been several reports of patients developing hypomagnesaemia, often accompanied by hypolcalcaemia with hypokalaemia, thought to be due to aminoglycoside therapy[77-83]. The mechanism of the magnesium depletion is not clear, except that the hypomagnesaemia appears to be due to the renal wasting of magnesium[78,79,82,83]. The hypocalcaemia and hypokalaemia appear to occur secondary to the hypomagnesaemia.

The mechanism underlying the development of hypocalcaemia due to hypomagnesaemia is not clear, but it is thought to include decreased parathyroid hormone synthesis[84,85] and/or enhanced net movement of calcium from extracellular fluid to bone[84].

The conclusion of one study[81], where patients on long term gentamicin therapy developed hypomagnesaemia and hypokalaemia, was that these symptoms were due to gentamicin-induced hyperaldosteronism. This theory was supported by another study[79], where elevated levels of plasma renin and aldosterone were found in a patient thought to be suffering from gentamicin-induced magnesium depletion. However, other workers found normal levels of plasma aldosterone in their hypomagnesaemic patient[80].

Most of these studies involved long courses of high dose aminoglycoside therapy in single patients[77-81,83]. In one study, however, short courses of the normal therapeutic doses of gentamicin, tobramycin or amikacin produced hypomagnesaemia in more than one third of the 55 patients studies[82]. However, it was found in the study that it was the patients who had an initial serum magnesium at the lower end of the normal range that developed hypomagnesaemia during therapy. Therefore, it was suggested that it was the patients who were having total parenteral nutrition, eating poorly or not

receiving supplemental magnesium who were most at risk of developing this side-effect[82]. Work carried out in rats shows that a magnesium deficiency accentuates the nephrotoxic effect of the aminoglyoside[86].

Because of these reports of aminoglycoside-induced magnesium depletion and also due to a study undertaken in our laboratory[87], which showed gentamicin-induced hypercalcuria in the rat, it was decided to study the serum levels and renal excretion of calcium and magnesium in patients receiving aminoglycoside therapy.

MATERIALS AND METHODS

Patients Studied and Methods of Sample Collection

Patients from East Birmingham Hospital (EBH), who received gentamicin therapy between 1/1/85 and 1/3/86 were entered into the study. For a patient to be entered into the study, a 2 h timed urine collection (9 a.m. to 11 a.m.) had to be taken within 48 h of the first dose of gentamicin being given.

A 2 h urine collection was taken every day that the patient was receiving gentamicin and for three to four days after completing the therapy, if possible. Patients were asked to empty their bladders at 9 a.m., then all urine passed between 9 a.m. and 11 a.m. was collected. Patients from whom less than four urine samples were collected, were excluded from the study.

Surgical patients, not receiving aminoglycosides, were included in the study as a control group. One 2 h urine collection was taken prior to surgery, either on the day before surgery or on the morning of surgery. Then a daily 2 h urine collection was taken for at least two days post-surgery. Control patients from whom less then three urine samples were obtained, were excluded from the study.

Urine samples were taken to Aston University for analysis. After centrifugation to remove solid matter from the urine, each urine sample was divided into aliquots for the measurement of RBP, lysozyme and creatinine. These aliquots were frozen until analysis. Samples were diluted in 0.1% acidified lanthanum chloride for the measurement of calcium (Ca) and magnesium (Mg), and stored at 4°C.

Plasma samples were routinely collected from each patient, at intervals of a few days, for analysis by the clinical chemistry

department at EBH. These samples were obtained from the clinical chemistry department after their analyses had been carried out. Occasional plasma samples were routinely taken from patients receiving aminoglycosides at EBH, for the determination of aminoglycoside plasma levels, by the antibiotic assay laboratory. These plasma samples were also obtained for this study. Although no detailed analysis of aminoglycoside plasma levels was attempted in this study, these data were examined in patients where excretion of RBP and lysozyme proved to be very high.

Aliquots of the plasma samples were frozen until analysis for RBP and lysozyme. Further aliquots were diluted in 0.1% acidified lanthanum chloride and stored at 4°C until analysis for plasma Mg. Plasma Ca, creatinine and albumin were routinely measured by clinical chemistry, therefore their results were obtained for this study.

Study Patients

Twenty eight patients who received gentamicin therapy were entered into the study. This group of patients consisted of 19 male and 9 female patients. The age range of this group was from 19 to 80 years, mean age 50 years. Two patients with cystic fibrosis who each received two separate courses of gentamicin therapy were included, each course being treated as a separate patient. Gentamicin was given at normal therapeutic doses to each study patient and the duration of therapy varied from 2 to 17 days; the mean duration was 6 days. Patient details and indications for gentamicin therapy are shown in Table 1, and a summary of the indications for gentamicin therapy is given in Table 2.

Control patients

Twenty one surgical patients, not receiving aminoglycoside therapy, were entered into the study as a control group. Details of

these patients are given in Table 3. The control group consisted of 14 male and 7 female patients aged from 39 to 79 years, mean age 63 years. Details of pre-operative and post-operative antibiotics and potentially nephrotoxic drugs given to control patients, are also shown in Table 3.

The form used for the recording of patient details for the study is shown in Appendix 1.

Table 1 Details of Gentamicin-treated Patients

Patient Course No.	Age	Sex	Duration of Therapy	Illness or Operation	Indication for Gentamicin Therapy
2	36	F	14	Scleroderma	Chest Infection
3	67	M	4	Abdominal surgery	Prophylactic post-op
4	62	M	6	Abdominal surgery	Pyrexial post-op
5	48	M	7	Gallstone removal	Prophylactic pre-op
6	22	F	4	Persistant cystitis	Urinary tract infection
8	74	F	5	Abdominal surgery	Prophylactic post-op
9*	25	M	3	Cystic Fibrosis	Chest infection
10	49	M	4	Abdominal surgery	Pyrexial post-op
11	41	F	4	Chest surgery	Wound infection later post-op
12	75	M	7	Abdominal surgery	Chest infection post-op
13**	19	M	10	Cystic fibrosis	Chest infection
14	50	F	17	Colectomy	Pyrexial pre-op and post-op
15	80	M	2	Abdominal surgery	Prophylactic post-op
16	74	M	6	Abdominal surgery	Pyrexial post-op
17	64	M	3	Abdominal surgery	Prophylactic post-op
18	25	M	2	Cystic fibrosis	Chest infection
19	77	F	6	Septicaemia (cause unknown)	Septicaemia
20**	19	M	10	Cystic fibrosis	Chest infection
21	68	F	2	Bronchiechsis and asthma	Chest infection
22*	25	M	11	Cystic fibrosis	Chest infection
23	20	F	14	Cystic fibrosis	Chest infection
25	62	F	10	Diabetic amputation	Pyrexial post-op

Table 1 cont.

26	76	M	5	Cancer prostate	Urinary tract infection
28	69	M	6	Urinary tract infection	Urinary tract infection
29	70	M	6	Kidney operation	Urinary tract infection
30	29	M	4	Kidney operation	Chest infection later post-op
42	47	M	4	Appendectomy	Wound infection later post-op
43	32	M	4	Abdominal surgery	Wound infection later post-op

* 9 and 22 represent two different courses of gentamicin therapy in the same patient

** 13 and 20 represent two different courses of gentamicin therapy in the same patient

Table 2 Summary of Indications for Gentamicin Therapy

Indication for Gentamicin Therapy		No of Patient courses
1) Associated with surgery		
i) Prophylactic prior to surgery		1
ii) Prophylactic immediately post surgery		4
iii) Pyrexia (therapy started within three days after surgery)		7
2) Not associated with surgery		
i) Chest infection	a) Cystic fibrosis	*6
	b) Others	3
ii) Wound infection		3
iii) Urinary tract infection		3
iv) Septicaemia (cause unknown)		1
		28

*There were 6 patient courses of gentamicin in cystic fibrosis patients, but only 4 patients.

Table 3 Details of Control Patients

Patient no.	Age	Sex	Operation	Antibiotics and potentially nephrotoxic drugs Before Operation (daily)	Pre-op Medication (one dose)	Post-operatively (daily)
32	63	M	Femoral artery bypass	-	Cefuroxime Metronidazole	Cefuroxime Metronidazole
33	45	M	Cholecystectomy + liver biopsy	-	Cefuroxime	-
34	50	F	Vagotomy	-	Cefuroxime	-
36	63	F	Removal adenocarcinoma	-	Cefuroxime Metronidazole	Cefuroxime Metronidazole
37	75	M	Repair aortic aneurysm	-	Cefuroxime Metronidazole	Cefuroxime Metronidazole
38	62	M	Right aorto-iliac decron graft	-	Cefuroxime Ampicillin	Cefuroxime Flucloxacillin
40	69	M	Repair aortic aneurysm	-	? Metronidazole	- Metronidazole
41	72	F	Cholecystectomy	Frusemide	Cefuroxime Frusemide	Cefuroxime Frusemide
44	67	M	Partial thyroidectomy	-	?	Ampicillin Benzylpenicillin

Table 3 cont.

Patient no.	Age	Sex	Operation	Antibiotics and potentially nephrotoxic drugs Before Operation (daily)	Pre op Medication (one dose)	Post-Operatively (daily)
45	58	F	Hernia operation	Frusemide	Cefuroxime	Frusemide
46	66	F	Carotid surgery	-	-	-
47	39	F	Femoral artery	-	-	Ampicillin
48	57	M	Hernia operation	-	-	Cephalexin
49	56	F	Carotid surgery	-	-	-
50	62	M	Femoral artery	-	Cefuroxime	Cefuroxime
51	53	M	Arterial surgery	-	Cefuroxime	-
52	75	M	E.U.A. and colostomy	Metronidazole	Cefuroxime Metronidazole	Cefuroxime Metronidazole
53	66	M	Repair aortic aneurysm	-	Ampicillin Flucloxacillin	-
54	78	M	T.U.R. (investigative kidney operation)	Cephalexin	Cefuroxime	Cefuroxime
55	65	M	Repeat T.U.R.	Cephalexin	-	Cephalexin
56	79	M	Litholapaxy (crushing renal stones)	Amoxycillin	Frusemide Cefuroxime	-

Materials

1) ELISA Assay

A: RBP standard preparation

Protein standard plasma for NOR Partigen (Behring Diagnostics, Hoechst U.K. Ltd., Hounslow, Middlesex), was used as the standard preparation for the ELISA assay. This was reported to contain 90 mg/l of RBP.

B: Antibody to RBP

(i) Rabbit antiserum to human RBP (Behring Diagnostics), was used during the development of the assay procedure. The concentration of this antibody preparation was reported as 120 mg/l.

(ii) Rabbit immunoglobulins to human RBP (Dako Ltd., High Wycombe, Bucks) were used once the assay had been developed. The concentration of this antibody preparation was quoted as 150 mg/l.

C: Antibody to RBP/enzyme conjugate

Horse-radish peroxidase conjugated rabbit immunoglobulins to human RBP (Dako Ltd.) were used as the enzyme-conjugate preparation.

D: Colour substrate

The colour substrate used for the assay was ortho-phenylene diamine (Sigma Chemical Company Ltd., Poole, Dorset). This produced an orange colour when broken down by the enzyme of the conjugate mentioned above.

E: Microtiter plates

96-well, flat-bottomed, irradiated immulon plates, type 129B (Dynatech Laboratories Ltd, Billinghurst, Sussex), were used for the assay.

F: Plate-reader

A Titertek Multiskan (Flow Laboratories, Rickmansworth, Herts) was used to read the absorbance of the developed plate.

G: Buffers

All chemicals used were of analytical grade.

i) Antibody coating buffer

Carbonate-bicarbonate buffer (CBC),

pH 9.6 : Na_2CO_3 1.59 g) made up to 1 litre with

$NaHCO_3$ 2.93 g) distilled water

This buffer could be stored at 4°C for up to three weeks.

ii) 'Blocking' buffer

Phosphate buffered saline with

Bovine serum albumin (PBS/BSA), pH 7.4:

NaCl	8.0 g)	
KH_2PO_4	0.2 g)	made up to 1 litre
$Na_2HPO_4,_{12}H_2O$	2.9 g)	with distilled
KCl	0.2 g)	water
BSA	1.0 g)	

This buffer was made freshly before each assay.

iii) Washing buffer

Phosphate buffered saline with Tween (PBS/T), pH 7.4 : Formula same as above except 0.5 ml Tween 20 was added instead of BSA

This buffer was made freshly before each assay.

iv) Substrate buffer

Phosphate citrate buffer, pH 5 :

Na_2HPO_4 0.730 g) made up to 100 ml

Citric acid 0.467 g) with distilled water

To this buffer 40 µl of 30% hydrogen peroxide and 40 mg of orthophenylene diamine were added to produce the substrate solution. This buffer made freshly made directly before use.

2) Lysozyme Assay

A: Lysozyme standard preparation

Chicken egg white lysozyme, grade 1 (Sigma Chemical Company Ltd) was used as the standard for the assay.

B: Substrate preparation

Micrococcus lysodeikticus dried cells (Sigma Chemical Company Ltd.) were used as the enzyme substrate.

C: Phosphate buffer

The buffer used for the experiment was a sodium phosphate buffer 67 mM, pH 6.2.

D: Spectrophotometer

A Cecil 5095 High Performance Scanning Spectrophotometer was used for the experiment.

3) To check the specificity of the antibody and the antibody enzyme conjugate preparations used in the ELISA assay.

A: 15% Polyacrylamide gel and Stacking gel

	15% Gel	Stacking Gel
Stock solution 1	18.5 ml	-
Stock solution 2	-	5.0 ml
Sodium dodecyl sulphate (10% w/v)	1.5 ml	0.3 ml
Tris buffer 1.5 M (pH 8.8)	18.5 ml	-
Tris buffer 0.5 M (pH 6.6)	-	7.5 ml
Distilled water	20.0 ml	16.0 ml
NNN' Tetramethylethylenediamine (TEMED)	0.14 ml	0.08 ml
Ammonium Persulphate (AMPS)	0.20 ml	0.10 ml

Stock 1 = 44% w/v acrylamide and 0.8% N,N'-methylene-bis acrylamide (BIS)

Stock 2 = 30% w/v acrylamide and 0.8% w/v BIS

B: Sample buffer pH 6.8

Sample buffer pH 6.8	
Tris 0.5 M pH 6.8	5.0 ml
SDS 10% w/v	10.0 ml
Mercaptoethanol	0.5 ml
Glycerol	5.0 ml
Distilled water	10.0 ml
Bromophenol blue solution	0.1 ml

C: Electrode buffer pH 8.3

Electrode buffer pH 8.3

Tris	6.0 g
Glycine	28.8 g
SDS 10% w/v	20.0 ml
Distilled water to 2 litres	

4) Determination of Calcium and Magnesium

A: Atomic absorption spectrophotometer

Perkin Elmer 560 Atomic Absorption Photometer

B: 0.1% acidified lanthanum chloride

0.1% aqueous solution $LaCl_3$ containing 50 mM HCl.

5) Determination of Creatinine in Urine

A: Creatinine standard

Creatinine (BDH Chemicals Ltd., Poole, England).

B: Spectrophotometer

Cecil 5095 High Performance Scanning Spectrophotometer.

6: Determination of creatinine in plasma and urine for reabsorption study

Kit for determination of creatinine in serum and urine (Sigma Chemicals).

Experimental Methods

1) Lysozyme Assay

Lysozyme content in serum and urine may be measured by several different methods, most of which are based on the clearing phenomenon of the sensitive organism Micrococcus lysodeikticus. These methods, however, differ with respect to buffer composition, pH and ionic strength, concentration of micrococcus substrate, spectrophotometer wavelength, and temperature and time of incubation. In this study, a turbidometric method originally described by Boasson[88], and later modified and evaluated by Houser[89], was used.

A) Assay procedure

Lysozyme standards of 0.25 - 2.5 mg/l were freshly prepared, at room temperature, in the phosphate buffer. A suspension of M.lysodeikticus, 200 mg/l in phosphate buffer, was also freshly prepared. Urine samples were tested undiluted at first, whereas plasma samples were routinely diluted 1 in 10.

The assay was performed in triplicate. 250 µl aliquots of standard, sample, or buffer alone were added to each test-tube containing 2.5 ml of micrococcus substrate. The additions were performed at 15 second intervals. The resulting solutions were incubated in a gently shaking water bath for 30 minutes at 25 C.

After incubation, the absorbance of the solutions were read at 15 second intervals against buffer alone, in a 1 cm lightpath glass cuvette, using a double-beam spectrophotometer (see Materials) at a wavelength of 570 nm. The absorbance reading had been adjusted so that phosphate buffer, without substrate, read zero.

The mean absorbance of each standard was subtracted from the mean absorbance of the buffer/substrate solution (i.e. blank

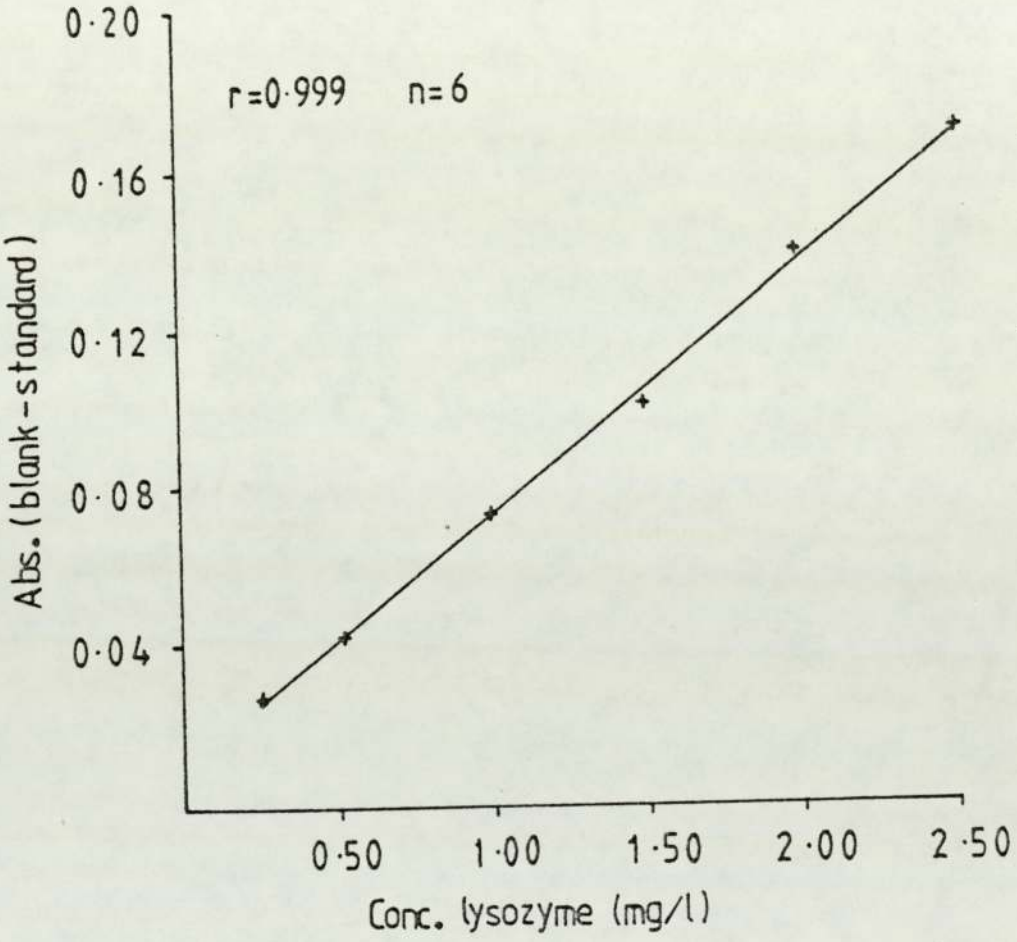


FIGURE 1: A typical calibration curve for the lysozyme assay

solution, no lysozyme present). A standard curve of absorbance (Abs. buffer/substrate - Abs. standard) against concentration of egg white lysozyme (mg/L) was generated by linear regression. The lysozyme concentration of samples was determined from the calibration curve. A typical calibration curve for the lysozyme assay is shown in Figure 1.

As human lysozyme is more active (about 3 times) than chicken egg white lysozyme, results were reported as mg. of egg white lysozyme per litre of plasma or urine.

The range of linearity of this assay method extended up to a concentration of 2.5 mg/l and was sensitive down to 250 μg/l of egg white lysozyme. As the calibration line of the assay intercepted the y axis and did not pass through the origin, it was not possible to measure lower concentrations of lysozyme using this method.

B) Stability of lysozyme in urine

As urine samples for this study were stored for up to one week in the fridge or up to four weeks frozen, before being assayed for lysozyme, it was decided to determine the stability of lysozyme in urine.

Twelve freshly collected spot urines, from EBH patients receiving a range of antibiotics were used for this study. The samples were centrifuged to precipitate out any solid matter, then divided into four aliquots each. No preservatives were added to any of the samples. Two aliquots were stored at -20°C in the freezer, one aliquot was stored at 4°C in the fridge and one aliquot was immediately assayed for lysozyme.

After one week, the aliquot of each sample which had been stored at 4°C and one of the aliquots which had been stored at -20°C, were

assayed for lysozyme. After four weeks, the remaining aliquots, stored at -20°C, were assayed for lysozyme.

2) **Retinol Binding Protein Assay**

A) Principle of the assay

RBP was assayed by a double antibody sandwich enzyme-linked immunosorbent assay (ELISA) technique, developed specifically for this study. The principle of such an assay is as follows (see Figure 2):

i) Antibody to the antigen to be measured is first adsorbed to a microtiter plate. The plate is then washed to remove excess antibody.

ii) The sample containing the antigen to be measured is then added and binds to the antibody. The plate is then washed again.

iii) A second antibody to the antigen to be measured is then added, which binds to the antigen. This second antibody is already bonded to an enzyme, forming an enzyme-antibody conjugate. The plate is washed to removed excess conjugate.

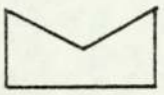
iv) A substrate for the enzyme is then added, which, when broken down by the enzyme, produces a colour. The amount of colour is proportional to the amount of antigen present in the sample.

B) The assay procedure

The ELISA assay developed for this study measured RBP in plasma and urine, within the range of 1.5 - 45 μg/l. The assay procedure was as follows:

1) Microtiter plates were coated overnight, at 4°C, with 100 μl per well of a 1 in 100 (1.5 mg/l) solution of antibody to RBP (Dako) in CBC buffer.

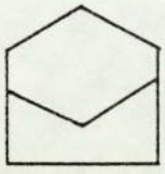
1)



Antibody attached to plate

wash

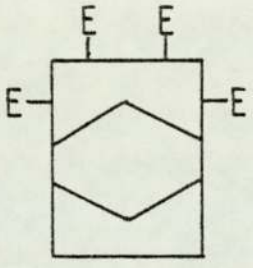
2)



Antigen attached to antibody

wash

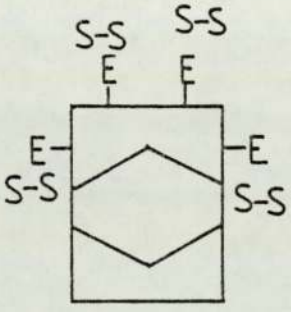
3)



Second antibody (enzyme -linked) attached

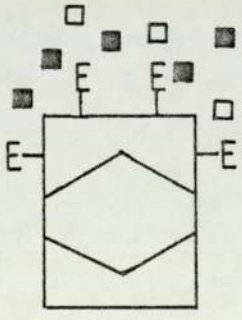
wash

4)



Colour substrate added

5)



Colour produced ≡ amount antigen

FIGURE 2: The Double-Antibody Sandwich ELISA

2) Plates were then washed three times with PBS/BSA buffer. Washing involved filling each well with 200 µl of washing solution, leaving to stand for a few moments, then shaking the solution out of the wells. The third washing was left on the plate for 1 h, at room temperature, to 'block' the sites on the plate unoccupied by antibody, i.e. to prevent non-specific binding. The plate was then shaken empty.

3) Protein standard plasma (PSP), which was used as the reference standard, and samples of urine or plasma were diluted in PBS/T buffer. PSP was diluted from 1 in 2000 (45 µg/l RBP), in doubling dilutions down to a 1 in 64000 dilution (1.5 µg/l RBP). Duplicates of each dilution were applied to the first two columns of microtiter plate, 100µl per well. To the rest of the plate, in columns, duplicates of doubling dilutions of urine or plasma samples were applied, 100 µl per well. Urine samples from gentamicin-treated patients were diluted from 1 in 40 down to 1 in 5120, urine samples from control patients were diluted from undiluted down to 1 in 128. Plasma samples were diluted from 1 in 2000 down to 1 in 64000. Control wells, containing only PBS/T, were included on each plate. The plates were then left for 2 h at room temperature, before washing three times with PBS/T.

4) 100 µl per well of conjugate, diluted 1 in 300 (0.01 mg/l) in PBS/T was then added to each well. The plates were then left for 2 h at room temperature, before washing five times with PBS/T.

5) The freshly prepared colour substrate solution was then applied, 100 µl per well. The plates were then wrapped in foil and left in a dark place (the colour substrate is light sensitive) for 1 h, at room temperature.

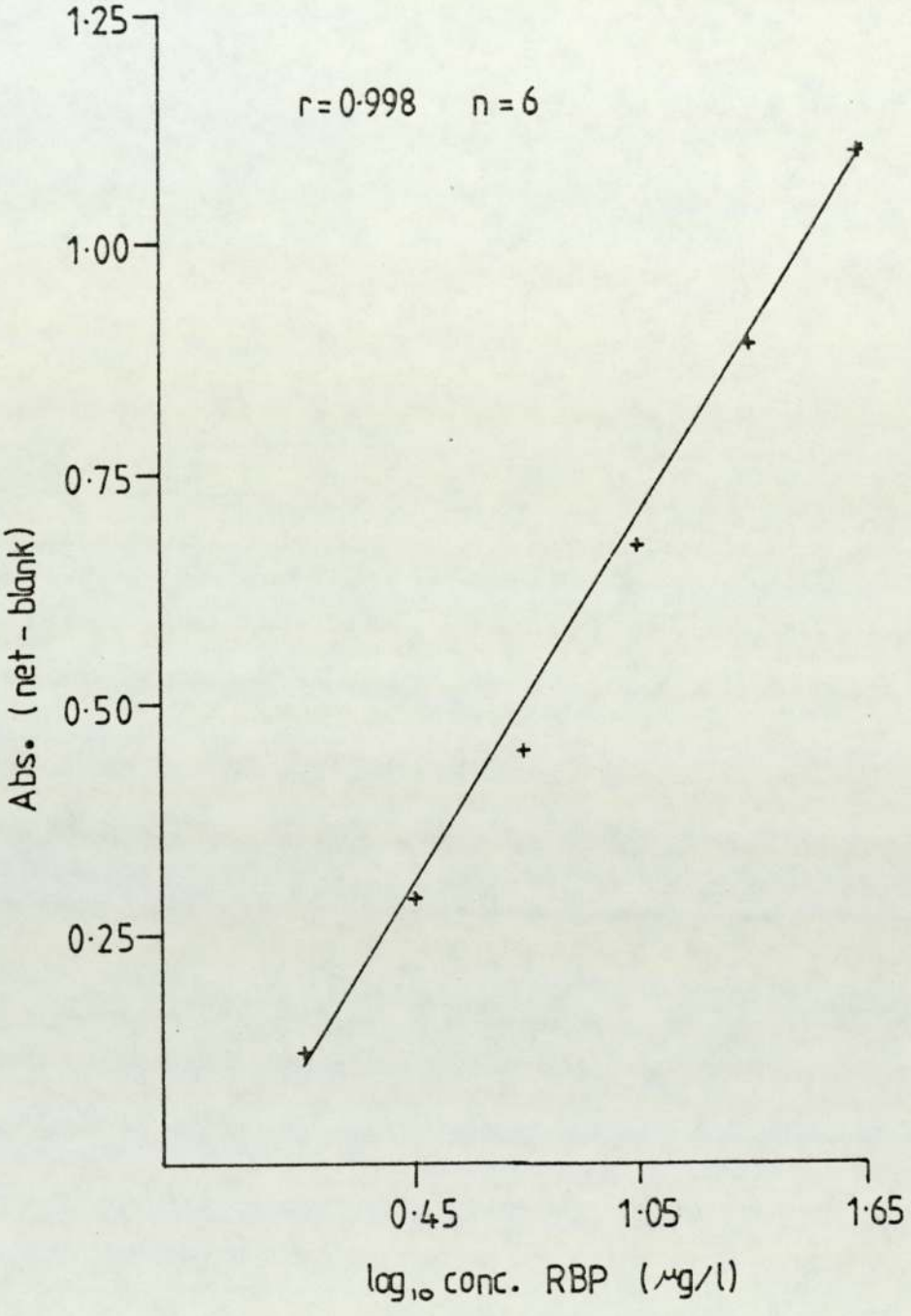


FIGURE 3 : A typical calibration curve for the ELISA assay of RBP

6) 100 µl per well of 2.5 M H_2SO_4 was applied to stop the colour reaction, then the absorbance of each well was read at 492 nm, using the plate reader.

Calculation of assay results

The amount of RBP present in each sample was calculated using a calibration curve of the absorbance of different concentrations of PSP standard (mean of the duplicates), plotted against the $\log_{10}$ of the concentration of RBP(µg/l) in each dilution. Linear regression analysis was used to calculate the calibration line. Figure 3 shows a typical calibration curve for the ELISA assay of RBP.

From the absorbance of each dilution of sample, the amount of RBP present in each dilution was read from the calibration curve. This value was then multiplied by the dilution factor to give the amount of RBP present in the sample. A mean of the values obtained from all the dilutions of each sample, whose absorbance fell within the calibration range, was then taken as the actual amount of RBP present in the sample.

C) Development of the assay

The ELISA method was developed from a basic procedure for double antibody sandwich ELISA, described by Voller[90]. Two ELISA methods for the measurement of RBP have previously been described[74,91], but both of these methods have involved the synthesis of an antibody to RBP/enzyme conjugate. As a commercially synthetised antibody to RBP/enzyme conjugate had recently become available, it was decided to develop an ELISA assay using this conjugate.

Experiments were carried out to determine the optimum concentrations of antibody, plasma standard and conjugate to use for the assay. The Behring antibody to RBP was used at first, and gave a very poor absorbance response. It was therefore decided to try using the Dako antibody to RBP. An ELISA assay was carried out to compare the two antibody preparations:

One microtiter plated was coated overnight in a 1 in 100 (1.2 mg/l) solution of the Behring antibody in CBC buffer. Another microtiter plate was covered overnight in a 1 in 100 (1.5 mg/l) solution of Dako antibody. The ELISA was carried out by the usual method. The resulting calibration curves for the two plates are shown in Figure 4. Using the Dako antibody seemed to give a far better absorbance response than using the Behring antibody. The developed plate, where the Dako antibody had been used, showed a three-fold difference between a highly positive and a negative sample. The plate where the Behring antibody had been used, however, showed only a 1.5-fold difference. Therefore, it was decided to change to the Dako antibody for all subsequent assays.

An intraassay coefficient of variation was determined for the ELISA assay, by measuring the absorbance of a number of samples of four different dilutions of plasma standard.

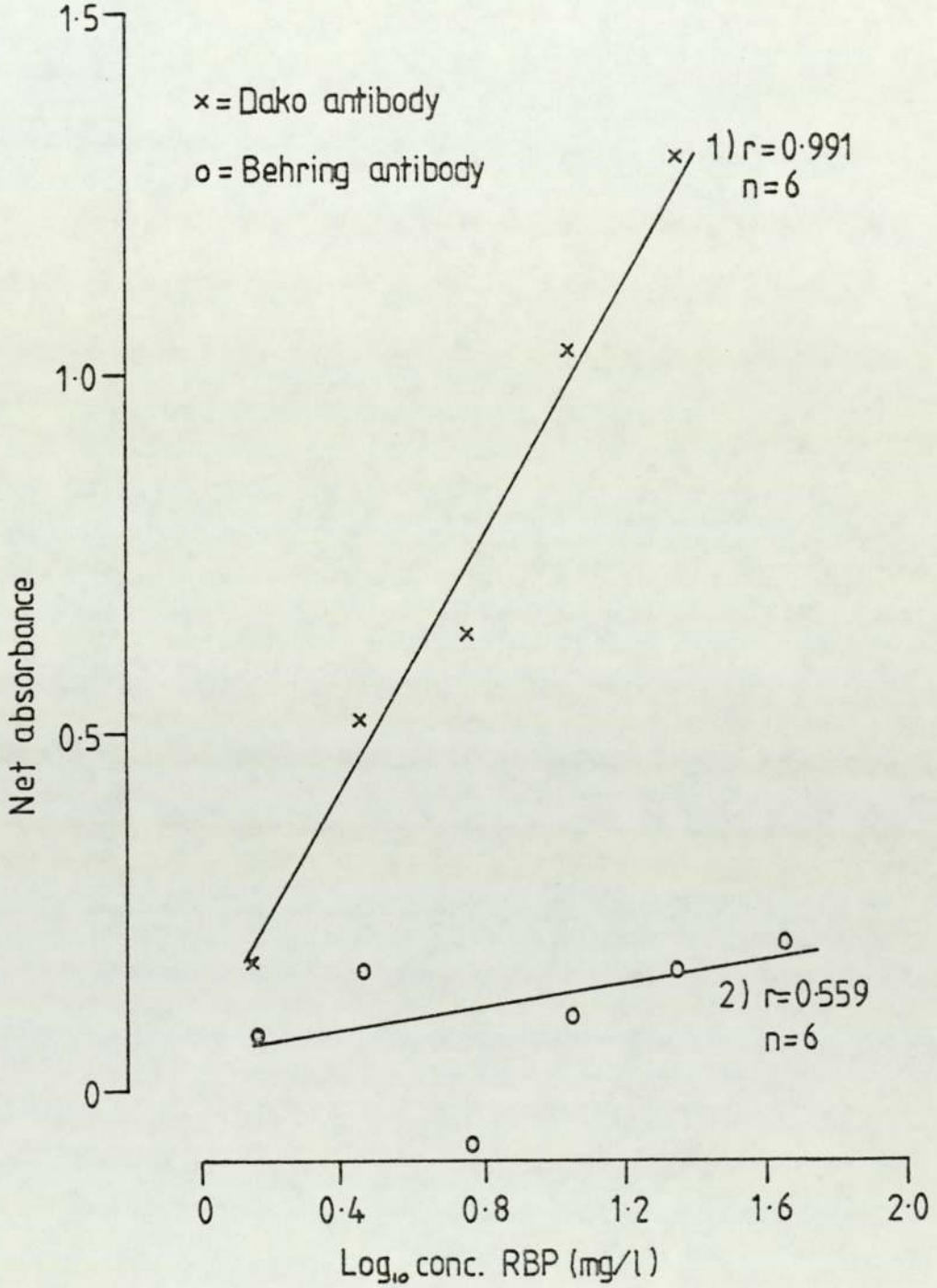


FIGURE 4: A graph to compare the calibration curves produced when 1) Dako and 2) Behring antibody to RBP were used in the ELISA.

Table 4 The intraassay coefficient of variation of the ELISA assay

Dilution of plasma standard	Absorbance (mean ± S.D.)	No. of samples	Coefficient of variation (%)
1 in 2000	1.337 ± 0.020	7	1.5
1 in 8000	0.870 ± 0.028	8	3.2
1 in 16000	0.758 ± 0.025	8	3.2
1 in 32000	0.533 ± 0.024	8	4.4

Therefore the mean intraassay coefficient of variation was 3%.

An interassay coefficient of variation was determined for the ELISA method, by measuring the concentration of RBP in four different dilutions of plasma standard in five ELISA assays (carried out on different days). The results are summarised in Table 5.

Table 5 The interassay coefficient of variation of the ELISA assay.

Dilutions of plasma standard	Concentration of RBP determined (mean ± S.D.)	No. of assays	Coefficient of variation (%)
1 in 4000	19 ± 2.6	5	14
1 in 8000	10 ± 1.8	5	18
1 in 16000	5.0 ± 0.58	5	12
1 in 32000	2.9 ± 0.36	5	12

Therefore the mean interassay coefficient of variation was 14%.

D) To check the specificity of the antibody and the antibody enzyme conjugate preparations used in the ELISA assay.

To check the specificity of the antibody to RBP (Dako) and the antibody/enzyme conjugate (Dako), electrophoresis using a Sodium Dodecyl Sulphate Polyacrylamide Gel was carried out, by the method described by Lugtenberg et al[92]. This method (SDS/PAGE) separates proteins according to their molecular weight, so by separating out the RBP in urine samples and plasma standard, it was possible to see whether the antibody and conjugate bind exclusively to RBP. The experiment was carried out by the following method;

Method

A 15% Polyacrylamide gel and a stacking gel were made up according to the formula described in materials.

Solutions were mixed together, polymerisation being started by the addition of TEMED. The 15% gel, when set between 2 glass plates, was topped by the stacking gel.

A standard solution with proteins of known molecular weight (band 3 standard) was used so that RBP (M.Wt. = 21,000) could be identified from the separated proteins. The standard, two urine samples, one known to be high in RBP and one known to be low in RBP, a solution of the protein standard plasma (Behring), and a plasma sample were to be run on the gel. The protein standard plasma and the plasma sample were diluted 1 in 100 in tris buffered saline, then the urines, the band 3 standard, the diluted protein standard plasma, and the plasma sample were mixed in a 1 : 1 ratio with the sample buffer (see Materials)

The solutions were then boiled for 10 minutes to denature the proteins. Samples were run in three sections, with 40 μl of each sample and standard being loaded onto each section.

The electrophoresis was performed at room temperature at a constant current of 40 mA, using the electrode buffer described in Materials.

The samples were allowed to run on the gel for about 4 h, then all three sections of the gel were blotted onto nitrocellulose overnight at 50 volts, by the method described by Towbin et al[93]. Then one section was stained immediately with amido black solution to show up the protein bands of known molecular weight in the band 3 standard. The other two sections were soaked in Tris buffered saline containing 0.5ml TWEEN 20 per litre (TBS/T) for an hour at 37 C, to block off any sites on the nitrocellulose not occupied by protein in order to prevent non-specific binding of the conjugate or antibody. The nitrocellulose sections were then rinsed in TBS (without TWEEN). One section was then soaked in a 1 in 100 (1.5 mg/l) solution of antibody to RBP/enzyme conjugate (Dako) for 1 h. The antibody soaked section was then soaked in protein A peroxidase for 1 h. Protein A binds to the fc region of antibodies. As previous SDS-Page experiments had shown a poor visual reaction, it was thought that using Protein A peroxidase on the antibody blot would amplify the colour reaction. The blots were visualised using 4-chloronapthol (2.5 mg) and 10μl hydrogen peroxide in 100 ml tris buffer.

E) Investigation of the relationship between plasma standard and purified RBP standard for the ELISA assay.

Another group of workers (Dr. M. Topping, Health and Safety Executive, Edgware Road, London) had also developed an ELISA method for the estimation of RBP. It was decided to compare the results obtained using the two methods.

Ten urine samples were assayed by our ELISA method, then sent to the Health and Safety Executive (HSE) for analysis by their ELISA method. HSE assayed a further 10 urine samples by their method, then sent these to our laboratories for measurement by our method.

The correlation between the London results and our results is shown in Figure 5. Results were too low to be measured in three samples, and one sample was lost, therefore only the results from 16 samples could be compared. The correlation between the two sets of results is 0.914, which is highly significant, $p < 0.001$. The slope of the regression line is, however, significantly different from a slope of 1, $p < 0.05$. There appeared to be a positive bias in our results compared to the HSE results. The HSE group thought that this positive bias was probably due to the fact that we were using a plasma preparation as the standard for our ELISA assay. They had found that using a plasma preparation as standard led to an overestimation of the amount of RBP in urine, compared to the results obtained using RBP purified from human urine as the standard preparation. They had also found that adding prealbumin to this purified RBP standard led to a reduction in the gradient of the calibration curve. From their results, it appeared that RBP bound to prealbumin, as in plasma, seemed to cause a reduction in the gradient of the calibration curve of the RBP assay, as compared to the calibration curve produced using purified RBP as standard. This would lead to an overestimation of the amount of RBP in urine samples.

Therefore it was decided to investigate the relationship between plasma standard and purified RBP standard in the ELISA assay. Some RBP, purified from the urine of patients with tubular proteinuria, was obtained (courtesy of Dr. A. Bernard, Unite de Toxicologie

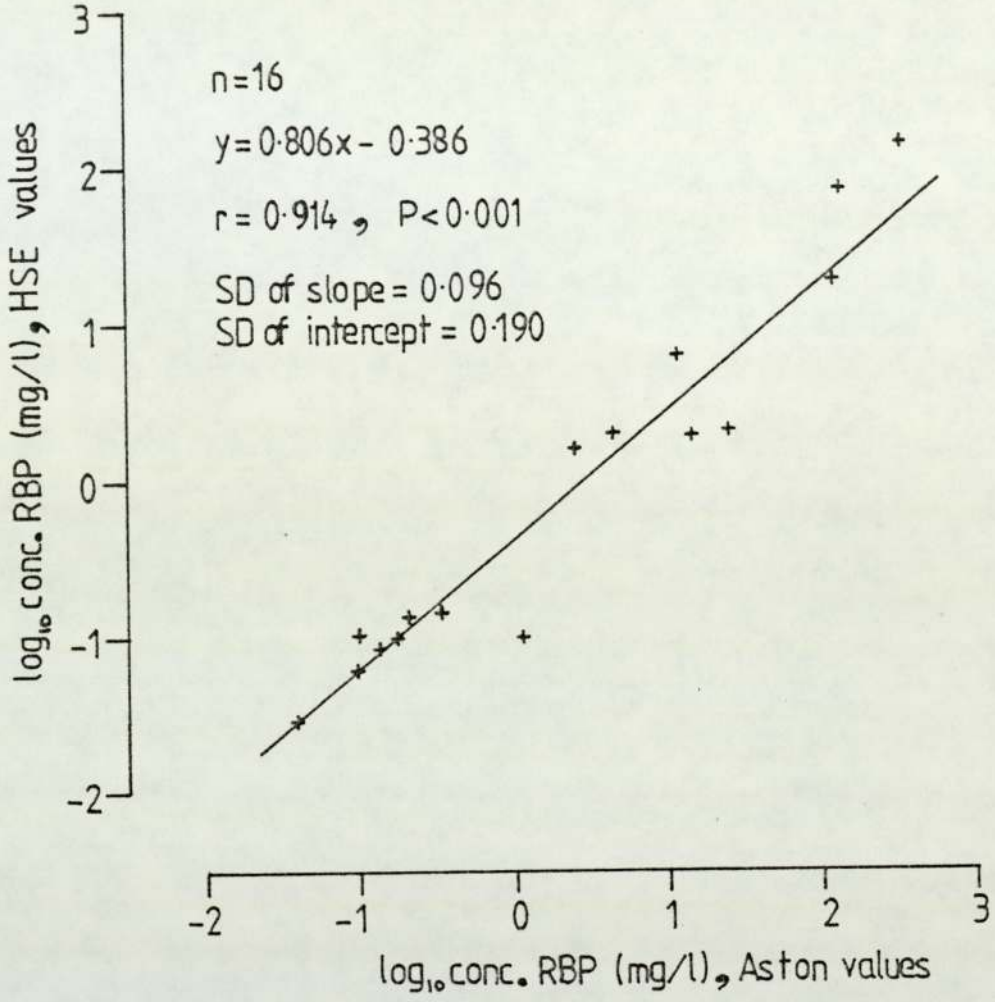


FIGURE 5: Correlation of the results obtained when the RBP concentrations of 16 urine samples were measured using:

1) Our ELISA method, with protein standard plasma (Behring) as standard
2) The London HSE ELISA method

Industrielle et medicine du travail, Brussels). The concentration of this purified RBP was quoted as 864 mg/l. An ELISA assay was carried out by the following method:

Plates were coated overnight with antibody and the next morning the PBS/BSA 'blocking' stage was carried out as usual. Protein standard plasma (PSP) was diluted, as usual, in doubling dilutions from 45 μg/l RBP down to 1.5 μg/l RBP. These diluted standards were applied, in duplicate, to the plate in the usual manner. Purified RBP was diluted from 43 μg/l RBP down to 2.7 μg/l RBP. Control wells to which PBS/T was added instead of sample or standard, were included on each plate. The remainder of the assay was carried out as usual.

Calculation of results

The mean absorbance of the duplicates of each dilution of PSP was determined. The mean blank absorbance determined was then subtracted from each mean absorbance value. The mean absorbance values were then plotted against $\log_{10}$ of the concentration of RBP in each dilution of PSP. The calibration line was calculated from these points using linear regression analysis. This whole process was repeated for the purified standard. Therefore, it was possible to compare the calibration lines produced using PSP and purified RBP standard. This assay was repeated four times.

Results

Although the two calibration lines appeared close in each assay, the gradient of the purified RBP line was always slightly greater than that of the PSP line. The significance of the difference between the means of the two sets of slopes (purified standard and PSP) determined in the five experiments was calculated using a paired t-test. Each of pair of data was the slopes of the two calibration lines determined using the same plate. A significant difference was found between the two sets of slopes, $p < 0.05$.

Figure 6 shows the general relationship found between the PSP and purified RBP calibration lines, using lines determined from the mean slopes and intercepts from the five experiments. The lines are close, crossing towards the lower end of the calibration curve. As RBP concentration of each urine sample was taken as the mean of the values for RBP concentration calculated from the absorbance of each dilution of sample which fell within the calibration range, using PSP as standard would lead to a general overestimation of the amount of RBP present.

As all urine samples previously tested for RBP had been measured using PSP as standard, it was decided to correct these values to read as if purified RBP had been used as standard. A simple method for achieving this was to take the true RBP content of samples as the value calculated from the dilutions of samples whose absorbance (blank subtracted) fell below 0.5. At this level, using PSP as standard would lead to, at most, approximately a 15% overestimation of the amount of RBP in urine. An overestimation as high as 15% would be very unlikely to occur using this correction method; however, as this could only happen if only one dilution of sample was available, whose absorbance was exactly 0.5. Therefore this method of correction provides an estimate of the true RBP content with an approximate theoretical maximum error of 15%.

The RBP concentrations of urine samples corrected by this method differed from the uncorrected RBP values by a mean of -15% (range -33% - +33%), showing that using PSP as standard had caused a general overestimation of the amount of RBP present in urine.

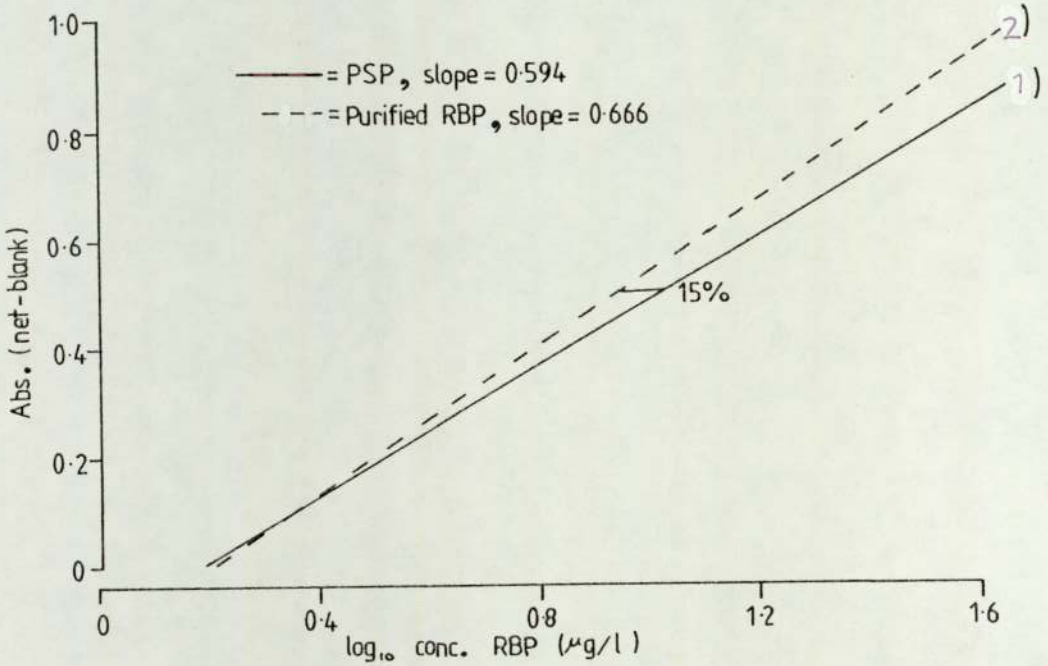


FIGURE 6: The general relationship between the calibration lines produced using:

1) Protein standard plasma (Behring)

2) Purified RBP (from human urine)

as standard in the ELISA assay

F) Comparison of the modified ELISA method, using purified RBP as standard, with the method in use at London Health and Safety Executive.

Due to the results obtained in the previous experiments, it was decided to check the results obtained using the modified ELISA method (using purified RBP as standard), with the method in use at HSE. Therefore, 10 urine samples were assayed by each laboratory, then sent to the other laboratory for analysis, as before.

Figure 7 shows the correlation between the results obtained by HSE and the results obtained by Aston. The results show an excellent correlation, $r = 0.945$, which is highly significant, $p < 0.001$. The slope of the line is not significantly different from a slope of 1.

Therefore, it was decided to use the purified RBP as standard for all further ELISA assays of RBP in urine, but to use protein standard plasma for assays of RBP in plasma. This modified ELISA method measured RBP in urine and plasma, within the range of 3-43 µg/l.

G) Stability of RBP in urine

Twenty three spot urine samples collected from patients at EBH who were receiving a range of antibiotics were used for this study. The samples were centrifuged to precipitate out any solid matter, then divided into three aliquots. No preservatives were added to any of the samples. One aliquot was assayed immediately for RBP. One aliquot was stored at 4°C for one week, then assayed for RBP. One aliquot was stored at -20°C for four weeks, then assayed for RBP. All assays were carried out by the modified ELISA technique i.e. using purified RBP as standard. Urine samples were routinely diluted 1 in 40 before testing. The assays were carried out by a project student,

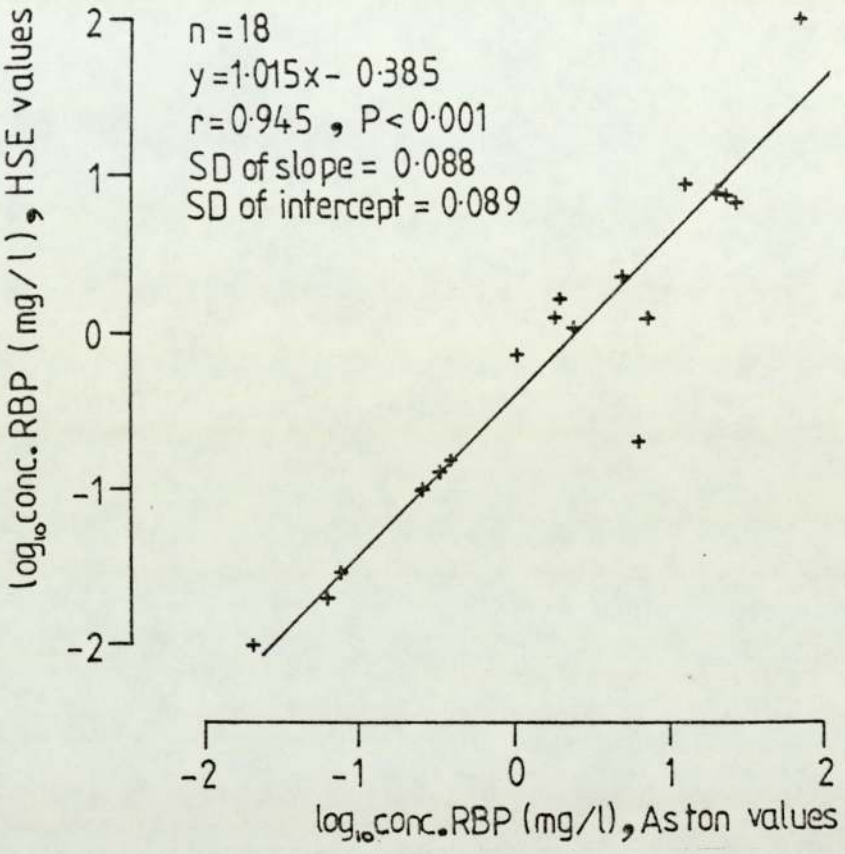


FIGURE 7: Correlation of the results obtained when the RBP concentrations of 18 urine samples were measured using:

1) Our ELISA method, using RBP purified from human urine as standard
2) The London HSE ELISA method

Miss Catherine Burn. The student calculated an interassay coefficient of variation for her ELISA assays of approximately 30%.

3) Experiment to determine the percentage reabsorption of RBP and lysozyme by the kidney

It was decided to determine the percentage reabsorption of both RBP and lysozyme by the kidney proximal tubular cells, in healthy human volunteers. This was thought of interest as if one of the proteins has a higher percentage reabsorption than the other, then the magnitude of the rise in excretion due to a certain decrease in proximal tubular function would be greater than that of the protein with the lower percentage reabsorption. Therefore, differing percentage reabsorptions may explain differing magnitude of rises in excretion of these two proteins in the event of proximal tubular damage.

Method

A 2 h timed urine collection from 9 a.m. to 11 a.m., was obtained from four healthy volunteers (one female and three male). Mid-way through the urine collection (at 10 a.m.) a blood sample was taken from each volunteer.

After centrifugation of the blood to separate out the plasma and centrifugation of the urine to precipitate out solid matter, creatinine, lysozyme and RBP concentations were determined in the plasma and urine samples. Plasma and urinary creatinine were determined using a kit for determination of creatinine in serum and urine (Sigma Chemicals).

From the creatinine results, a creatinine clearance was determined for each volunteer, as a check of normal renal function. From the lysozyme and RBP results, the percentage reabsorption of RBP and lysozyme were calculated for each volunteer.

4) Determination of Calcium and Magnesium

A) Determination of calcium and magnesium in urine

For the determination of the amount of calcium in urine samples, the samples were diluted 1 in 100 in 0.1% acidifed lanthanum chloride. For the determination of magnesium, urine samples were diluted 1 in 500 in 0.1% acidified lanthanum chloride. Measurements were carried out using an atomic absorption spectrophotometer, and were calibrated against a standard solution containing known amounts of calcium and magnesium.

B) Determination of magnesium in plasma

To determine the amount of magnesium in plasma, samples were diluted 1 in 50 in 0.1% acidified lanthanum chloride. Magnesium concentration was determined as described above.

C) Determination of calcium in plasma

Values for plasma calcium were obtained from the clinical chemistry department at EBH. This measured plasma calcium was corrected to take account of the binding of calcium to albumin in hypoalbuminaemic states, using the following formula:

$$\text{Corrected calcium} = \text{Plasma calcium} + (32 - A) \times 0.025$$

where A = plasma albumin

Plasma albumin results were also obtained from clinical chemistry.

5) Determination of creatinine in urine

For the determination of the amount of creatinine in urine, the following method was used:

Method

A standard solution containing 100 mg% of creatinine was made up in distilled water. Five test tubes were set up as follows:

Tube	Amount 100 mg% creatinine	Amount distilled water
1	0 µl	100 µl
2	25 µl	75 µl
3	50 µl	50 µl
4	75 µl	25 µl
5	100 µl	0 µl

Further tubes contained 50 µl of the urine samples to be measured and 50 µl of distilled water.

2 ml of saturated picric acid was added to each tube. Then 600 µl of 1 M sodium hydroxide was added to each tube at 30 second intervals. The tubes were then incubated for 10 min before adding 2.3 ml of water per tube at 30 second intervals.

Each tube was then read against tube no. 1 at 520 nm using a scanning spectrophotometer. A calibration curve of absorbance against amount of creatinine present was then generated by linear regression analysis. The amount of creatinine present in each sample was determined from the curve.

RESULTS

Experimental Results and Discussion

1. Stability of lysozyme in urine

i) Results

Table 6 shows the change and percentage change in lysozyme activity of the 12 urine samples tested, after one week at 4°C and after one week and four weeks at -20°C. The percentage change in lysozyme concentration varied greatly between samples. The mean percentage change in lysozyme activity after one week at 4°C was an increase of 9% although percentage change varied between -24% and +75%. The mean percentage change in lysozyme activity after one week at 20°C was 0%, although the percentage change varied between -46% and +110%. After four weeks at -20°C, the mean percentage change in activity was a decrease of 25%, although the percentage change varied between -68% and +60%.

Figure 8 shows the correlations between initial concentration of lysozyme and concentrations of lysozyme under the three storage conditions. The correlation between the two sets of results was very good in each case. From 8a) and 8b) it appears acceptable to store samples for one week at either 4°C or 20°C before assay. From Figure 8c) it can be seen that there is a positive bias towards the x axis i.e. lysozyme activity decreases significantly on storage for four weeks at -20°C.

Table 6 The change and percentage change in lysozyme activity of urine samples stored for one week at 4°C, and one and four weeks at -20°C.

Sample no.	Initial lysozyme conc. (mg/l)	Lysozyme conc. after 1 week, 4°C (mg/l)	% change in lysozyme conc.	Lysozyme conc. after 1 week -20°C	% change in lysozyme conc.	Lysozyme conc. after 4 weeks -20°C	% change in lyzozyme conc.
1	0.90	0.99	+10	1.0	+11	0.93	+ 3
2	4.5	5.0	+11	4.6	+ 2	3.4	-24
3	26	23	-12	23	-12	21	-19
4	1.1	1.5	+36	1.2	+ 9	1.1	0
5	26	24	- 8	22	-15	21	-19
6	0.40	0.38	- 5	/	/	0.13	-68
7	1.8	2.9	+61	2.5	+38	0.27	-85
8	1.2	1.2	0	0.9	-25	1.4	+17
9	2.0	3.4	+75	4.2	+110	3.2	+60
10	5.1	4.3	-16	3.6	-29	2.2	-57
11	1.3	1.1	-15	0.76	-42	0.78	-40
12	1.7	1.3	-24	0.91	-46	1.4	-18

/ = sample lost

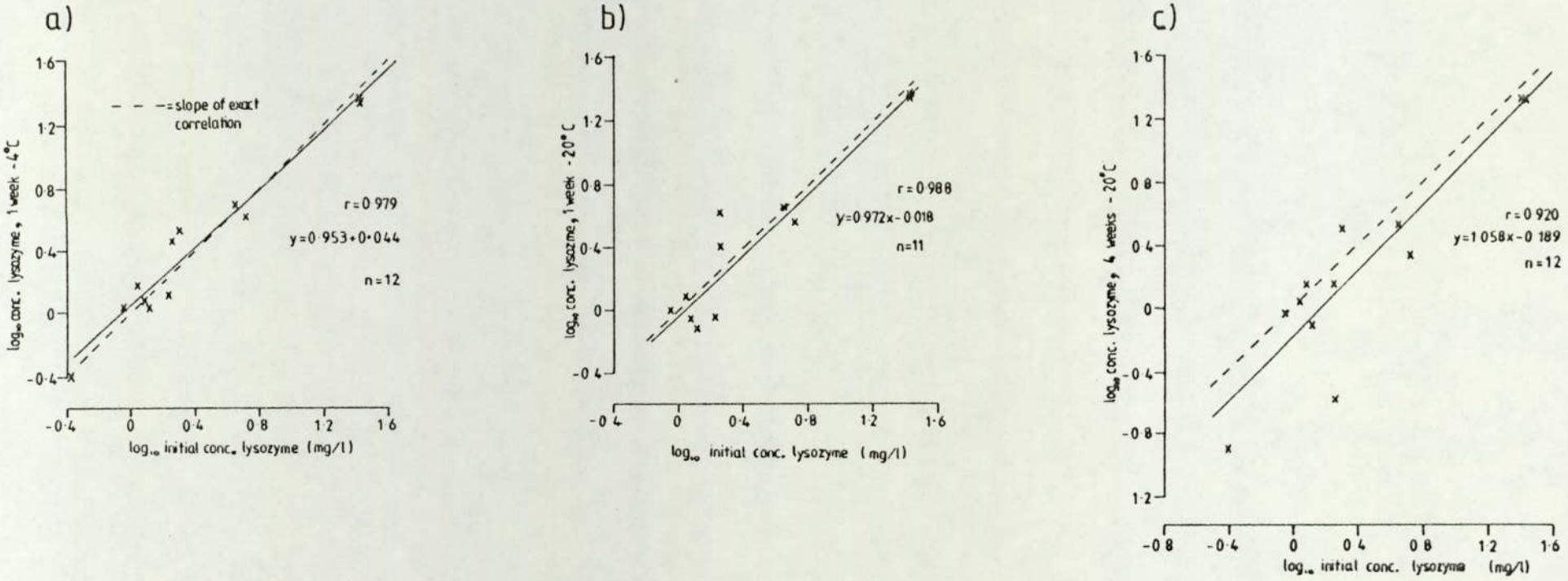


FIGURE 8: Log_{10} initial lysozyme concentration plotted against log_{10} lysozyme concentration after:

a) one week at 4°C
b) one week at -20°C
c) four weeks at -20°C

ii) Discussion

The large percentage increases in lysozyme activity which occurred in some of the urine samples may have been due to the inaccuracy of the assay at low dilutions of urine. The samples which contained a high concentration of lysozyme and therefore were well diluted before assay, showed a decrease in activity on storage, as expected. The samples which showed an increase in lysozyme activity on storage, were tested either undiluted or diluted 1 in 2. This increase in lysozyme activity in some urines may have been due to the presence of inhibitors, the activities of which decrease on storage. At higher dilutions of urine, the effect of these inhibitors would be minimised. Another reason for the possible inaccuracy of the lysozyme assay at low dilutions is that the assay does not take account of the colour of the urine, and the colour may change on storage. At higher dilutions the colour of the urine would make a negligible contribution.

As it was only significantly raised urinary lysozyme levels which were important for this study, possible inaccuracy of the assay at low dilutions of urine was not of great importance.

It does appear that some urine samples showed a large decrease in lysozyme activity when stored for four weeks at -20°C. Therefore, it is advisable to store samples for just one week at 4°C or -20°C, if great accuracy of results is required. For this study, however, only large absolute rises in excretion of lysozyme were important.

2. To check the specificity of the antibody and antibody-conjugate preparations used in the ELISA assay.

i) Results

Figure 9 shows a copy of the nitrocellulose blots produced in this experiment. A band of approximately 21,000 Da appeared in the track of the urine sample known to be high in RBP. This protein band appeared in the amido-black stained blot and in the antibody and conjugate treated blots. No band appeared in the region of 21,000 Da in the track of the urine sample known to be low in RBP, or in the tracks of the plasma sample or plasma standard. Faint bands did appear, however, in the region of high molecular weight proteins (such as albumin and globulins) in the antibody and conjugate-treated blots. Only one band appeared for the plasma standard and plasma sample in the conjugate-treated blots but two bands appeared for both the plasma standard and plasma sample in the antibody-treated blot.

ii) Discussion

The protein band which appeared in the track of the urine known to be high in RBP, in the region corresponding to a molecular weight of approximately 21,000 Da, was presumed to be unbound RBP. There was probably not enough unbound RBP present in the plasma sample or plasma standard to produce a colour reaction, as only about 5% of RBP is unbound in plasma[67,68]. The bands in the region of high molecular weight proteins, in the tracks of the plasma sample and plasma standard were probably due to the binding of the conjugate and the antibody to prealbumin-bound RBP. However, as there were two bands present in the antibody-treated blot, it is possible that the antibody binds non-specifically to other high molecular weight proteins. These two bands may, however, represent apo- and holo-prealbumin bound RBP.

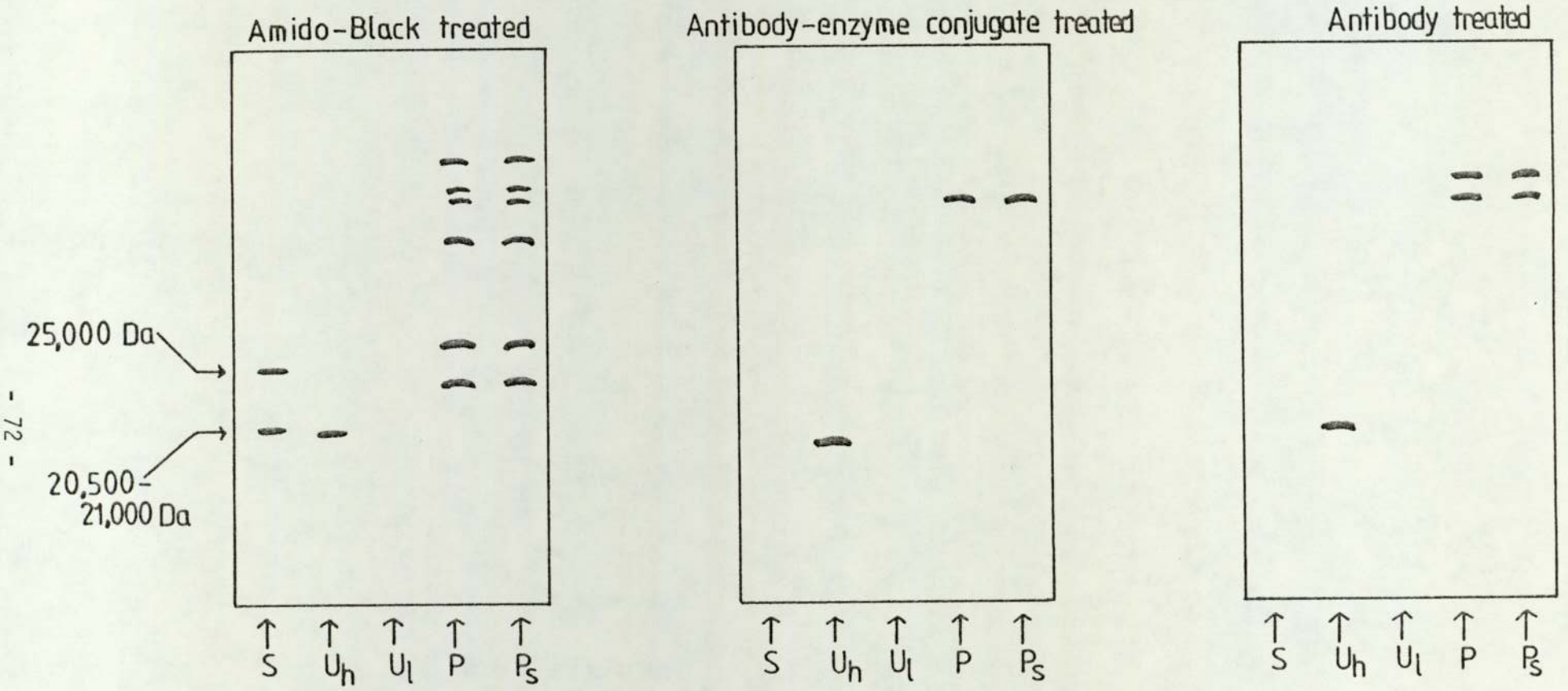


FIGURE 9: A copy of the nitrocellulose blots produced by the SDS-Page electrophoresis to test the specificity to RBP of the antibody and antibody-enzyme conjugate prepations used in the ELISA assay.
S='band 3' standard; U_h=urine, high in RBP; U_l=urine, low in RBP; P=plasma sample; P_s=protein standard plasma

Therefore, it seems probable that antibody to RBP (Dako) and antibody to RBP/enzyme conjugate (Dako) bind exclusively to RBP, whether free (as in urine) or pre-albumin bound, as in plasma. Therefore, both the antibody and conjugate preparations are suitable to use for the ELISA assay of RBP in plasma and urine.

3) The ELISA method

Discussion

As using a plasma preparation as standard for the ELISA assay produced a calibration curve which had a lower gradient than the calibration curve produced using RBP purified from urine as the standard preparation, this would suggest a difference in the activity of RBP in plasma compared to RBP in urine, in the ELISA assay. It seems that RBP in plasma, which is mostly pre-albumin bound, may show less affinity for the antibody to RBP than the free form of RBP which occurs in urine.

It has been reported, however, that the pre-albumin/RBP complex dissociates in the presence of antibody to RBP, therefore pre-albumin binding should have no effect on the antigenicity of RBP[94]. Also it has been reported that RBP in plasma and urine are identical[73], and that whether RBP is of the holo or apo form (with or without retinol) and whether free or in complex with pre-albumin, it exhibits identical reactivity with antibody to RBP[95,96]. However, the results of the present study, suggesting some difference in antigenicity between bound and free RBP, are supported by the London HSE findings that adding pre-albumin to purified RBP led to a reduction in gradient of the calibration curve of the ELISA assay compared to using purified RBP alone.

Therefore, it was concluded that purified RBP should be used as the standard for the ELISA assay of RBP in urine, whereas a plasma standard is probably more suitable for the assay of RBP in plasma. The ELISA method for determination of RBP in urine, therfore, has a range of sensitivity of 3-43 μg/l and the ELISA method for determination of RBP in plasma has a range of 1.5-45 μg/l.

There was always a moderately high background absorbance in the ELISA assay. The control wells, which were treated in the usual manner, except that they contained no antigen (i.e. no plasma, urine or standard was added), always showed a significant amount of background absorbance. However, if the control wells were not coated with antibody, or if no antibody/enzyme conjugate was added, hardly any background absorbance was present. Therefore, it is possible that this background activity may be due to antibody to RBP reacting to some extent with the antibody/enzyme conjugate. Interference in the ELISA assay may, however, be due to a complicated interaction of the solid phase, antigen, antibody, enzyme label and substances in test fluids[97]. By decreasing the background activity a better range of activity between positive and negative samples would be achieved, which would increase the accuracy of the results. It was attempted without success to reduce the background absorbance by increasing the concentration of TWEEN 20 in the washing buffer.

In each ELISA assay there were several spurious results. Often wells at the edge of the microtiter plate showed a very high absorbance. This phenomenon of an 'edge effect', where antibody or antigen attaches unevenly to the microtiter plates, has been previously reported[98]. Any erroneously high results at the edge of the plate were ignored.

Lysozyme has also been reported to interfere in the ELISA assay, as it strongly associates with proteins which have low isolectric points. As immunoglobulins have an isoelectric point of about 5, lysozyme may form a bridge between the first antibody and enzyme-labelled antibody[99]. It may be possible to reduce this effect by masking the lysozyme using Cu^{2+} ions or ovalbumin. No consideration of the effect of lysozyme on the ELISA method was taken into account in this study.

The ELISA method for determination of RBP in serum and urine developed for this study, has advantages over such existing methods as radial immunodiffusion[100] or radioimmunoassay[96], Radio-immunoassay requires a γ-radiation source and special facilities for radioactive counting and disposal. Radial immunodiffusion requires 2-3 days incubation and has an assay range of 5-82 mg/l. (Behring LC Partigen Radial Immunodiffusion plates). Therefore, although concentrations may be measured by this technique, most urine samples need concentrating prior to the assay, to bring them within the assayable range.

The ELISA method described in this thesis has advantages over the two previously reported ELISA methods for RBP[74,91] because all the immunological ingredients are commercially available. The assay is easy to perform, requiring no specialised equipment although the assay is facilitated by the use of an ELISA plate reader. The assay takes about 7h to perform, excluding the overnight coating with antibody, and material costs are approximately £1 per sample. The main advantage of this method is that it can be used for determination of RBP in both plasma and urine.

4) Stability of RBP in urine

i) Results

Table 7 shows the change and percentage change in the RBP concentration of the urine samples tested, after one week at 4°C and four weeks at -20°C. After one week, three urine samples showed concentrations of RBP which were below the sensitivity of the assay, in this < 0.12 mg/l, as all urine samples were diluted 1 in 40 and the sensitivity of the assay using undiluted urine was 3 µg/l. After four weeks, six urine samples had concentrations of RBP which were below the sensitivity of the assay, and another six samples were not assayed, therefore only 11 results are shown.

The percentage change in RBP concentration, after storage, varied greatly between samples. After one week at 4°C, most samples showed a decrease in RBP activity, although five samples actually showed an increase in RBP activity. Three of these samples, 3,8 and 11, showed large percentage increases in activity, although the actual change in activity was quite small for samples 8 and 11. The RBP concentration after one week at 4°C for sample 3 was based on just one dilution which fell within the calibration range, and therefore may not have been a very accurate result. The RBP concentration after one week at 4°C, for patient 8, was determined from a poor correlation curve, and therefore may also have been unreliable.

After four weeks at -20°C, all samples but one (15) showed a decrease in RBP activity.

Figure 10 shows the correlations between initial concentration of RBP and concentration of RBP under both storage conditions. There is a strong correlation between the two sets of variables in each case. There is, however, a bias towards the x axis under both storage conditions indicating that, generally, there is a decrease in

Table 7 The change and percentage change in RBP concentration of urine samples stored for one week at 4°C and four weeks at -20°C.

Sample No.	Initial RBP conc. (mg/l)	RBP conc. after one week 4°C (mg/l)	% change in RBP conc.	RBP conc after 4 weeks -20°C (mg/l)	% change in RBP conc.
1	1.2	0.53	-56	0.63	-48
2	0.71	0.33	-54	ND	-
3	20	42	+110	/	-
4	0.51	0.39	-24	0.12	-76
5	0.75	ND	-	0.56	-25
6	0.54	0.73	+35	ND	-
7	0.37	0.16	-57	ND	-
8	0.14	1.7	+1114	ND	-
9	0.68	ND	-	0.22	-68
10	69	53	-23	33	-52
11	0.13	0.57	+338	ND	-
12	1.3	0.71	-45	0.18	-86
13	0.14	ND	-	ND	-

Table 7 continued/

14	0.34	0.15	-55	ND	-
15	3.4	1.5	-56	4.5	+32
16	0.83	0.27	-67	0.49	-41
17	1.7	1.1	-35	0.78	-54
18	1.9	0.83	-56	0.86	-55
19	0.18	ND	-	/	-
20	2.0	3.1	+55	/	-
21	97	45	-54	/	-
22	1.9	0.9	-53	/	-
23	1.8	1.4	-22	/	-

ND = below the sensitivity of the assay.

/ = sample lost.

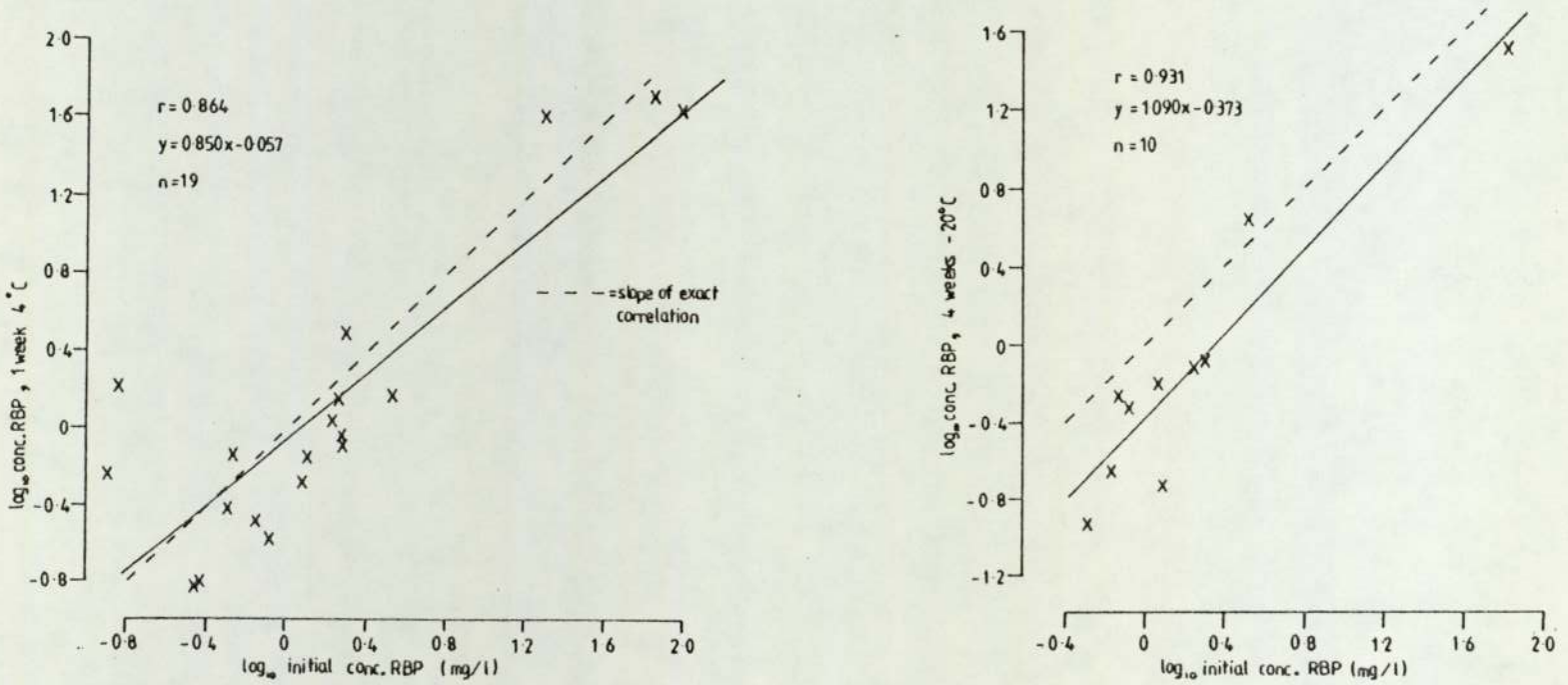


FIGURE 10: Log_{10} initial RBP concentration plotted against log_{10} RBP concentration after:

a) one week at -4°C

b) four weeks at -20°C

RBP activity under both storage conditions. This decrease is more pronounced after four weeks storage at -20°C than after one weeks storage at 4°C.

ii) Discussion

Although in this stability study, there appeared to be large fluctuations in RBP activity after storage, when the large interassay coefficient of correlation found by Miss Burn for her assay is taken into account, these fluctuations appear to be acceptable.

Although there is a general decrease in RBP activity after storage, this decrease would only be important in this study if values which were raised above normal decreased to below normal on storage. This happened in samples 12, 17, 18 and 22, but in each case the initial RBP concentrations in these samples were just above normal limits. One sample, 8, showed a normal level initially, which became raised after storage, but this was probably due to experimental error. Therefore, these minor fluctuations in RBP concentration after storage would not significantly affect the results for a study such as this, where only major changes in RBP concentration are important.

5) Experiment to determine the percentage reabsorption of RBP and lysozyme by the kidney.

i) Results

a) Creatinine Clearance

Creatinine clearance was determined for each person, using the following equation:

$$Cl_{cr}\ (ml/min) = \frac{U_{cr} \times V}{P_{cr}}$$

Cl_{cr} = Creatinine clearance

U_{cr} = Urinary creatinine

V = volume of urine/min (total vol. ÷ 120)

P_{cr} = Plasma creatinine

Table 8 Determination of creatinine clearance in four healthy volunteers.

Volunteer	Urine total volume (ml)	Urinary creatinine (mg/dl)	Plasma creatinine (mg/dl)	Creatinine Clearance (ml/min)
1	80	168	0.892	126
2	119	133	0.931	142
3	275	124	1.138	250
4	83	154	0.724	147

Table 8 shows the determination of creatinine clearance in the four volunteers. Normally creatinine clearance is 85-125 ml/min in males and 75-115 ml/min in females (clinical chemistry reference values, EBH). Therefore these values were slightly above normal, showing that the volunteers did not have renal impairment.

b) Percentage reabsorption of lysozyme

As the urinary concentrations of lysozyme determined for each volunteer were all below the sensitivity of the assay, i.e. below 250 µg/l, it was decided to determine the minimum percentage that could be reabsorbed. Therefore, the urinary concentration of lysozyme was set at 250 µg/l for each volunteer.

Percentage lysozyme reabsorbed was determined by the following equation:

$$\% \text{ lysozyme reabsorbed} = \frac{P_L U_{cr} - U_L P_{cr} \times 100}{P_L U_{cr}}$$

P_L = Plasma lysozyme (mg/l)

U_L = Urinary lysozyme (mg/l)

Table 9 Determination of minimum percentage reabsorption of lysozyme in four healthy volunteers

Volunteer	Plasma lysozyme (mg/l)	Urinary lysozyme (mg/l)	Minimum % lysozyme reabsorbed
1	9.8	0.25	99.99
2	4.4	0.25	99.96
3	5.5	0.25	99.96
4	4.0	0.25	99.97

Table 9 shows the minimum percentage of lysozyme reabsorbed in four healthy volunteers.

c) Percentage reabsorption of RBP

The percentage reabsorption of RBP was determined in the volunteers using the following equation:

$$\% \text{ RBP reabsorbed} = \frac{P_{RBP}U_{cr} - U_{RBP}P_{cr} \times 100}{P_{RBP}U_{cr}}$$

P_{RBP} = Plasma RBP (mg/l)

U_{RBP} = Urinary RBP (mg/l)

Table 10 Determination of percentage reabsorption of RBP in four healthy volunteers.

Volunteer	Plasma RBP (mg/l)	Urinary RBP (mg/l)	% RBP reabsorbed
1	37	0.09	100.00
2	25	0.25	100.00
3	45	0.07	100.00
4	70	0.09	100.00

Table 10 shows the percentage reabsorption of RBP in the volunteers.

ii) Discussion

From the creatinine clearance results, it appeared that none of the volunteers showed impaired renal function. The percentage reabsorption values for RBP are 100% and for lysozyme are very close to 100% in each case. These results show that it is unlikely that differences in percentage reabsorption between the two proteins could account for any large differences in the magnitude of the rise in excretion between the two proteins, due to proximal tubular damage.

Clinical Results

Introduction

Although it was intended to take daily urine collections for all study and control patients, inevitably some urine collections were forgotten or mislaid. Because of problems in identifying gentamicin-treated patients, the first urine collection was often not taken until the third day of therapy. Also, when patients had completed their course of gentamicin therapy, often they were sent home immediately, therefore no samples were available after therapy in many cases. Hence, although a day of peak excretion of proteins and electrolytes has been quoted, this is only the peak excretion which is apparent from the available data.

When describing events occurring in gentamicin-treated patients, day 1 refers to the first day of therapy and day-1 refers to the day before therapy commenced. Therefore, positive numbers denote days after the commencement of therapy and minus numbers denote days before commencement of therapy.

When describing events occurring in control patients, minus numbers relate to number of days before surgery, day 0 refers to day of surgery and positive numbers relate to number of days after surgery.

1) Plasma Creatinine

Table 11 shows plasma creatinine (P_{cr}) data for gentamicin-treated patients. Normal values for P_{cr} are 50-120 μmol/l (Clinical Chemistry reference values, EBH). A significant rise in P_{cr} was defined by the criteria of Smith et al[101] as a rise of >44.2 μmol/l

Table 11 Plasma Creatinine Data for Gentamicin-Treated Patients

Patient Course	Duration of therapy	a) Lowest P_{cr} μmol/l (day)	b) Peak P_{cr} μmol/l (day)	Significant rise in P_{cr}
2	14	57 (2)	99 (7)	-
3	5	160 (7)	208 (5)	-
4	6	77 (9)	117 (2)	-
5	7	75 (10)	90 (7)	-
6**	4	104 (2)	104 (2)	-
8	5	69 (4)	134 (2)	+
9	3	61 (4)	70 (2)	-
10	4	67 (3)	87 (1)	-
11*	4	-	-	-
12	7	107 (5)	149 (8)	-
13	10	81 (2)	99 (9)	-
14	17	44 (15)	67 (18)	-
15	2	151 (2)	379 (7)	+
16	6	87 (7)	133 (4)	+
17	3	65 (7)	74 (4)	-
18	2	46 (3)	59 (1)	-
19	6	121 (2)	169 (8)	+
20	10	75 (3)	93 (1,10)	-
21	2	67 (3)	67 (3)	-
22	11	55 (3)	110 (1)	+
23	14	37 (4)	90 (11)	+
25	10	69 (14)	96 (7)	-
26	5	123 (2)	123 (2)	-
28	6	93 (2)	93 (2)	-

+ = Significant rise in P_{cr}

Table 11 cont.

Patient Course	Duration of therapy	a) lowest P_{cr} μmol/l (day)	b) Peak P_{cr} μmol/l (day)	Significant rise in P_{cr}
29**	6	87 (2)	87 (2)	-
30*	4	-	-	-
42	4	69 (2)	69 (2)	-
43	4	82 (3)	102 (2)	-

a) The lowest available P_{cr}, and the day on which this occurred.

b) The apparent peak of P_{cr} and the day on which this occurred.

* No p_{cr} data was available for patients 11 and 30

** This was the only available P_{cr}

if the initial peak was <265 μmol/l. In no patient was the initial P_{cr} >265 μmol/l. If P_{cr} fell by >44.2 μmol/l from the peak level and no pre-treatment levels were available, this was also considered to be significant, as this suggested that the P_{cr} was returning to pre-treatment levels and the peak level represented a rise of >44.2 μmol/l above the patient's normal level.

Therefore, patients 15, 19 and 23 showed a significant rise in P_{cr}. Patients 8, 16 and 22 may also have shown a significant rise in P_{cr}, as levels fell by >44.2 μmol/l during the period of study, and no pre-treatment levels were available. To determine whether these rises in P_{cr} were due to a renal cause, a urine to plasma creatinine ratio (UPCR) was calculated for each of these patients. UPCR of <10 is diagnostic of a renal cause, UPCR of 10-40 favours a renal cause, and UPCR of >40 is diagnostic of a pre-renal cause. UPCR ratios calculated for patients 8, 15, 19, 22 and 23 were between 10 and 40, therefore it is probable that the rise in P_{cr} had a renal cause in these patients. In patient 16, UPCR was >40 so there was probably a pre-renal cause for the rise in P_{cr}.

Patient 8 showed a moderately elevated P_{cr} of 134 μmol/l on day 2 of a 5 day course of gentamicin therapy, which decreased to 69 μmol/l on day 4. As this raised P_{cr} occurred early in therapy, it was probably not attributable to gentamicin nephrotoxicity.

Patient 15 received only a 2 day course of gentamicin therapy, therefore the rise in P_{cr} in this patient was probably not caused by gentamicin therapy alone. The P_{cr} continued to rise in this patient, who died of renal failure seven days after the last day of gentamicin therapy.

Patient 19 already had impaired renal function, when commenced on gentamicin therapy. Prior to the 6 day course of gentamicin

therapy, this patient had a high P_{cr}, which decreased to normal levels early in therapy, then increased by >44.2 μmol/l later in therapy. Therefore, the rise in P_{cr} in this patient was probably not completely due to gentamicin therapy.

Patient course 22 was the second course of gentamicin therapy in a cystic fibrosis patient. P_{cr} was within normal limits, at 110 μmol/l on day 1 of an 11 day course of therapy, but had dropped by >44.2 μmol/l on day 3. It is unlikely that gentamicin nephrotoxicity caused the initial rise in P_{cr}, as P_{cr} decreased after day 1 of therapy.

Patient 23 was a cystic fibrosis patient who received a 14 day course of gentamicin therapy. No pre-treatment values were available in this patient, but P_{cr} rose by 53 μmol/l between day 4 and day 11 of therapy. No other causes of renal impairment could be identified in this patient, therefore it is possible that P_{cr} rose due to gentamicin nephrotoxicity.

Therefore, although patients 8, 15, 19, 22 and 23 showed a significant rise in P_{cr} which was probably due to a renal cause, gentamicin nephrotoxicity was probably only a contributory cause in patients 15, 19 and 23.

Table 12 shows pre-surgical and post-surgical peak P_{cr} data in control patients. P_{cr} rose after surgery in 11 of the 14 patients in whom both pre- and post-surgical data were available. Only one patient, patient 37, showed a signficant rise in P_{cr}. UPCR showed that this rise in P_{cr} had a renal cause. This patient had chronic renal failure and developed on acute chronic episode after surgery. No potentially nephrotoxic drugs were given to this patient (see Table 3).

Table 12 Plasma Creatinine Data for Control Patients

Patient	Pre-surgery P_{cr} μmol/l (day)	Post-surgery peak P_{cr} μmol/l (day)
32	-	85(4)
33	50 (-1)	-
34	-	76 (1)
36	83 (-2)	91 (1)
37	170 (-2)	296 (5)
38*	-	-
40	66 (-2)	103 (4)
41	148 (-1)	-
44	111 (-1)	116 (1)
45	77 (-1)	103 (1)
46	73 (-1)	87 (1)
47	77 (-3)	103 (1)
48	121 (-1)	-
49	58 (-1)	86 (1)
50	112 (-1)	124 (1)
51	91 (-1)	80 (5)
52	113 (-2)	121 (1)
53	93 (-1)	-
54	88 (-1)	71 (6)
55	116 (-1)	121 (4)
56	59 (-3)	59 (3)

* No P_{cr} data was available for patient 38

2) Plasma and Urinary RBP and Lysozyme Results

A: Plasma lysozyme levels

The normal range for the concentration of lysozyme in plasma has been reported as 5.1 - 14 mg/l[56], in normal healthy subjects. In all but one of the gentamicin-treated patients, patient 19, plasma lysozyme levels were well below the normal renal threshold of 45 mg/l[53]. Patient 19 showed plasma lysozyme levels in the region of 100 mg/l.

The mean level (± S.D.) of lysozyme in plasma, determined from all plasma samples assayed for lysozyme in gentamicin-treated patients, was 16 ± 6 mg/l (n = 92). This was slightly greater than the published normal range for healthy subjects.

Plasma lysozyme levels were found to be well below the renal threshold in all control patients, both before and after surgery. The mean pre-surgery plasma lysozyme concentration (± S.D.) was 16 ± 5 mg/l (n = 14). The mean post-surgery plasma lysozyme concentration (± S.D.) was 15 ± 6 mg/l (n = 16).

B: Plasma RBP levels

The normal range for the concentration of RBP in plasma has been reported as 30-60 mg/l[74], in normal healthy subjects. Gentamicin-treated patients 2, 13, 14, 15, 17, 21 and 25 all showed plasma levels of RBP >60 mg/l, but <80 mg/l. The normal renal threshold for RBP is not known, but is unlikely to be below 80 mg/l, as the normal range is 30-60 mg/l. Patient 28, however, showed a plasma RBP of 110 mg/l which may have exceeded the renal threshold. The mean plasma concentration (± S.D.) of RBP, determined from all plasma samples assayed for RBP in gentamicin-treated patients was 37 ± 22 mg/l (n = 82).

In control patients, plasma RBP levels were raised (above 60 mg/l) in patients 33, 34 and 37. Patients 34 and 37 showed RBP levels of <80 mg/l, but patient 33 showed a plasma RBP of 98 mg/l, which may have exceeded the renal threshold. The mean plasma concentration (± S.D.) of RBP prior to surgery was 25 ± 26 (n = 13). The mean (± S.D.) of RBP post-surgery plasma RBP was 27 ± 25 (n = 15).

C: Urinary excretion of RBP in gentamicin-treated patients

Although a normal urinary excretion of RBP of <0.18 mg/g creatinine (mean +2 S.D.) has been published[91,102], this value was determined for normal healthy patients. Therefore, a normal urinary excretion of RBP in hospital patients was calculated from the pre-surgery urinary excretion values for control patients (see Table 20). The value for control patient 52 was excluded from this calculation as RBP excretion was significantly higher in this patient than for the rest of the control group. The mean excretion (± S.D.) for the remaining control patients was found to be 0.33 ± 0.35 mg/g creatinine. Therefore, an upper limit for the normal urinary excretion of RBP in hospital patients was determined as the mean + 2 S.D. = 1.0 mg/g creatinine.

Table 13 shows the peak urinary excretion of RBP and lysozyme in gentamicin-treated patients. The peak urinary excretion of RBP was above the upper normal limit of 1.0 mg/g creatinine in 22 of the 28 patients. The mean value (± S.D.) for the peak urinary excretion of RBP in gentamicin-treated patients was 56 ± 110 mg/g creatinine (range 0.13 - 530 mg/g creatinine).

The 22 patients with raised levels of RBP included five of the six patients who had shown a significant rise in P_{Cr}; 8, 15, 16, 19

Table 13 Peak Urinary Excretion of Lysozyme and RBP in Gentamicin-Treated Patients

Patient Course	Duration of therapy (days)	Peak urinary excretion RBP (mg/g creatinine)	Day of peak	Peak urinary excretion lysozyme (mg/g creatinine)	Day of peak
2	14	28	14	0.7	7,13
3	5	21	≤3	31	≥8
4	6	7.4	≤3	0.9	≤3
5	7	6.5	≥10	ND	-
6	4	0.31	≤3	ND	-
8	5	14	≤3	5.6	≤3
9	3	0.31	≤3	ND	-
10	4	0.83	5	2.4	3
11	4	19	≤3	1.3	≤3
12	7	260	5	340	≥8
13	10	0.50	4	ND	-
14	17	530	17	540	12
15	2	130	≤2	30	3
16	6	150	≤3	290	5
17	3	38	≤2	24	4
18	2	0.13	≤2	ND	-
19	6	32	6	2000*	≥10
20	10	2.0	7	50	8
21	2	12	4	3.4	≥7
22	11	1.0	7	ND	-
23	14	2.0	8	1.6	8
25	10	82	≥14	220	8
26	5	21	≤2	44	6

Table 13 continued/

Patient Course	Duration of therapy (days)	Peak urinary excretion RBP (mg/g creatinine)	Day of Peak	Peak urinary excretion lysozyme (mg/g creatinine)	Day of Peak
28	6	5.2	5	9.9	1
29	6	100	⩽3	21	4
30	4	42	2	1.0	3
42	4	35	3	2.9	2
43	4	18	⩽3	ND	-

*Plasma lysozyme above renal threshold in this patient.

ND = Not detectable.

⩽ = Peak appeared to occur on this day but may have occurred beforehand as this was the first available sample.

⩾ = Peak may have occurred later than this, as this was the last available sample.

and 23. Patient 22, who had shown a significant rise in P_{cr} showed a normal excretion of RBP. However, urinary excretion of RBP was also raised in many of the patients who had shown no significant rise in P_{cr}. Patients 15 and 16 showed a peak excretion of >100 mg/g creatinine, but patients 8, 19 and 23 showed a peak excretion which was below the mean peak RBP excretion for gentamicin-treated patients, the peak for patient 23 being just above normal levels. In 17 of the gentamicin-treated patients, therefore, urinary excretion of RBP was raised above normal in the absence of a significant rise in P_{cr}.

Table 14 The distribution of peak urinary excretions of RBP among gentamicin-treated patients

Peak urinary excretion of RBP	No. of patients
⩽1.0	6
1.1 - 9	5
10 - 24	6
25 - 99	6
⩾100	5

Table 14 shows the number of patients falling within various ranges of peak excretion of RBP. Five of the patients (12, 14, 15, 16, 29) showed a peak excretion ⩾100 mg/g creatinine. Patient 14 had shown a peak plasma RBP of 70 mg/l and patient 15 had shown a peak plasma RBP of 65 mg/l. Although these values were above the normal plasma levels of 30-60 mg/l, it is unlikely that they were above the renal threshold for RBP, especially as patient 28 had shown a plasma RBP of 110 mg/l and had a peak excretion of only 5.2 mg/g creatinine. Patients 12, 16 and 29 had all shown plasma RBP levels below 60 mg/l. Therefore it is very unlikely that these large peak excretions of RBP were a consequence of plasma levels exceeding the renal threshold.

Table 15 The relationship between the duration of gentamicin therapy and the peak urinary excretion of RBP

Duration of therapy (days)	No. of patients	Mean peak excretion RBP ± S.D. (mg/g creatinine)
2 - 5	14	25 ± 33
6 - 10	10	65 ± 88
> 10	4	140 ± 260

Table 15 relates the duration of therapy to the peak excretion of RBP. It appears that the peak of urinary excretion of RBP is greater in patients receiving longer courses of gentamicin therapy. This relationship is quite difficult to assess in so few patients, as the variance about the mean peak excretion for each group is so great.

Table 16. The relationship between the day of peak urinary excretion of RBP and the duration of gentamicin therapy.

Day of peak urinary excretion of RBP	No. of patients	Mean duration of therapy ± S.D.
1 - 3	15	4 ± 1
4 - 7	8	7 ± 3
8 - 17	5	12 ± 4

Table 16 relates day of peak excretion to the duration of therapy. It appears that the peak in excretion occurs later in patients receiving longer courses of gentamicin therapy. Therefore, patients receiving longer courses of gentamicin therapy show a larger and

later peak excretion of RBP than those patients receiving shorter courses of therapy.

D: Urinary excretion of lysozyme in gentamicin-treated patients

It was not possible to determine normal levels for lysozyme excretion in hospital patients using pre-surgery levels in control patients, as most control patients showed pre-surgery levels below the sensitivity of the lysozyme assay (see Table 20). Therefore, a published normal excretion of $\leq$0.58 mg/g creatinine[89] was used, although this value was determined in normal healthy subjects.

The urinary excretion of lysozyme was raised above normal levels in 21 of the 28 gentamicin-treated patients (see Table 13). Patient 19 showed such a high lysozyme excretion because plasma lysozyme levels exceeded the renal threshold.

It was not possible to determine the mean peak excretion of lysozyme in gentamicin-treated patients, as many patients showed lysozyme levels which were below the sensitivity of the lysozyme assay. The range of peak excretions of lysozyme was <0.25 - 540 mg/g creatinine.

The 21 patients who showed raised excretion of lysozyme included five of the six patients who had shown a significant rise in P_{Cr}; 8, 15, 16, 19 and 23. Patients 16 and 19 showed a peak excretion of >100 mg/g creatinine, but in patient 19 this was due to plasma lysozyme levels exceeding the renal threshold. Patients 8, 15 and 23 all showed a peak excretion of lysozyme which was well below 50 mg/g creatinine, the peak excretion in patient 23 being just above normal levels. Therefore, in 16 of the 28 gentamicin-treated patients, urinary excretion of lysozyme was raised above normal in the absence of a significant rise in P_{Cr}.

Table 17 The distribution of peak urinary excretions of lysozyme amongst gentamicin-treated patients

Peak urinary excretion lysozyme (mg/g creatinine)	No. of patients
⩽0.58	7
0.59 - 9	10
10 - 24	2
25 - 99	4
⩾100	5

Table 17 shows the number of patients who showed various peak excretions of lysozyme. Five patients showed a peak excretion of ⩾100 mg/g creatinine; 12, 14, 16, 19 and 25. Plasma levels of lysozyme in patients 12, 14, 16 and 25 were well below the normal renal threshold for lysozyme.

Table 18. The relationship between the duration of gentamicin therapy and the peak urinary excretion of lysozyme

Duration of therapy (days)	No. of patients	No. of patients showing peak lysozyme excretion <50 mg/g creatinine	⩾50 mg/g creatinine
2 - 5	14	14	0
6 - 10	*9	6	3
>10	4	3	1

*Patient 19 excluded.

Table 18 relates the duration of therapy to the peak urinary excretion of lysozyme. None of the patients who received short (2-5 day) courses of gentamicin therapy showed a peak excretion of lysozyme of ≥50 mg/g creatinine. Patients receiving longer courses of therapy tended to show higher peak excretions of lysozyme. However, there was not a direct relationship between duration of therapy and size of peak, as only one of the four patients who received more than ten days of therapy showed a peak excretion of ≥50 mg/g creatinine.

Table 19 The relationship between the day of peak urinary excretion of lysozyme and the duration of gentamicin therapy in those patients in whom lysozyme levels were detectable.

Day of peak urinary excretion of lysozyme	No. of patients	Mean duration of therapy ± S.D. (days)
1 - 3	8	4 ± 1
* 4 - 7	6	6 ± 4
* 8 - 17	8	10 ± 4

* Patient 2 is included in both these categories as this patient showed two peaks of identical size, one on day 7 and one on day 13 of therapy.

Table 19 relates the day of peak excretion to the duration of therapy. The peak urinary excretion of lysozyme thus appeared to occur later in the longer courses of therapy. As for RBP, in general

patients on longer courses of gentamicin therapy showed larger and later peaks in lysozyme excretion than patients on shorter courses.

Also of interest is that only eight patients showed a peak lysozyme excretion on days 1 - 3 of therapy, compared with 15 patients who showed a peak of RBP.

E: Comparison of RBP and lysozyme excretion in gentamicin-treated patients

Lysozyme and RBP excretion seemed to follow a similar pattern of excretion in most patients. The graphs of patients 3, 17 (see Figure 11), 12 and 25 (see Figure 12) illustrate this point. In 17 patients the peak excretion of RBP was greater than that of lysozyme, in 10 patients the peak excretion of lysozyme was greater, and in one patient it was not known which was greater. In 11 patients the peak of RBP excretion occurred before that of lysozyme excretion, in six patients the peak in lysozyme excretion occurred first and in four patients the peaks occurred on the same day. In seven patients it was unknown which occurred first as lysozyme levels were undetectable.

Patients on shorter courses of gentamicin therapy tended to show just one peak in excretion of both RBP and lysozyme (see Figure 13 - patient 16), whereas patients on longer courses of therapy tended to show more than one peak (see Figure 12 - patient 25).

To examine the results in more detail, the gentamicin-treated patients were divided into the three subgroups (see Table 2); patients who began gentamicin therapy within three days of surgery (surgical patients); patients with cystic fibrosis who were treated for chest infection; and patients who fit into neither of these two groups.

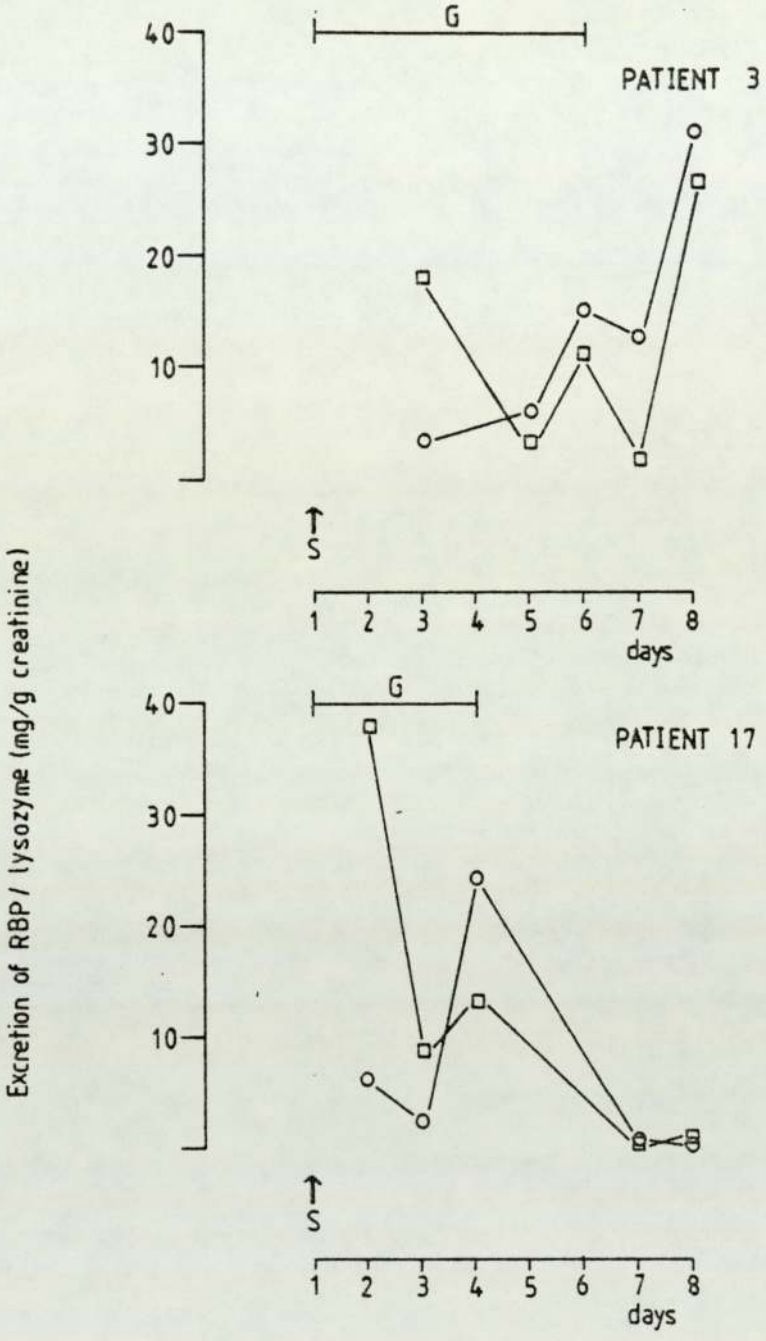


FIGURE 11: Urinary excretion of lysozyme and RBP by patients 3 and 17. S = surgery, G = gentamicin therapy. o = lysozyme, □ = RBP

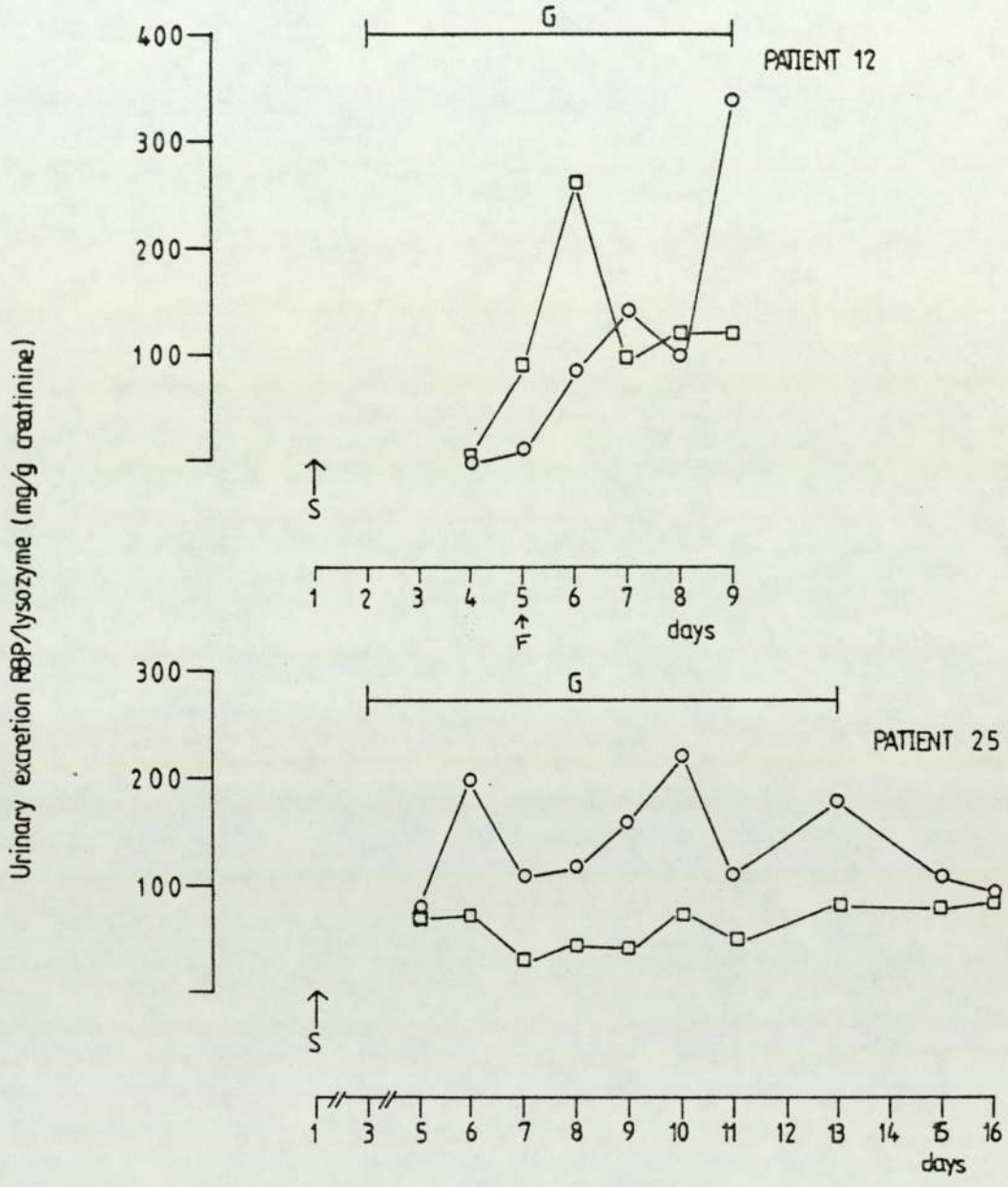


FIGURE 12: Urinary excretion of lysozyme and RBP by patients 12 and 25. S=surgery, G=gentamicin therapy, F=frusemide. o=lysozyme, □=RBP.

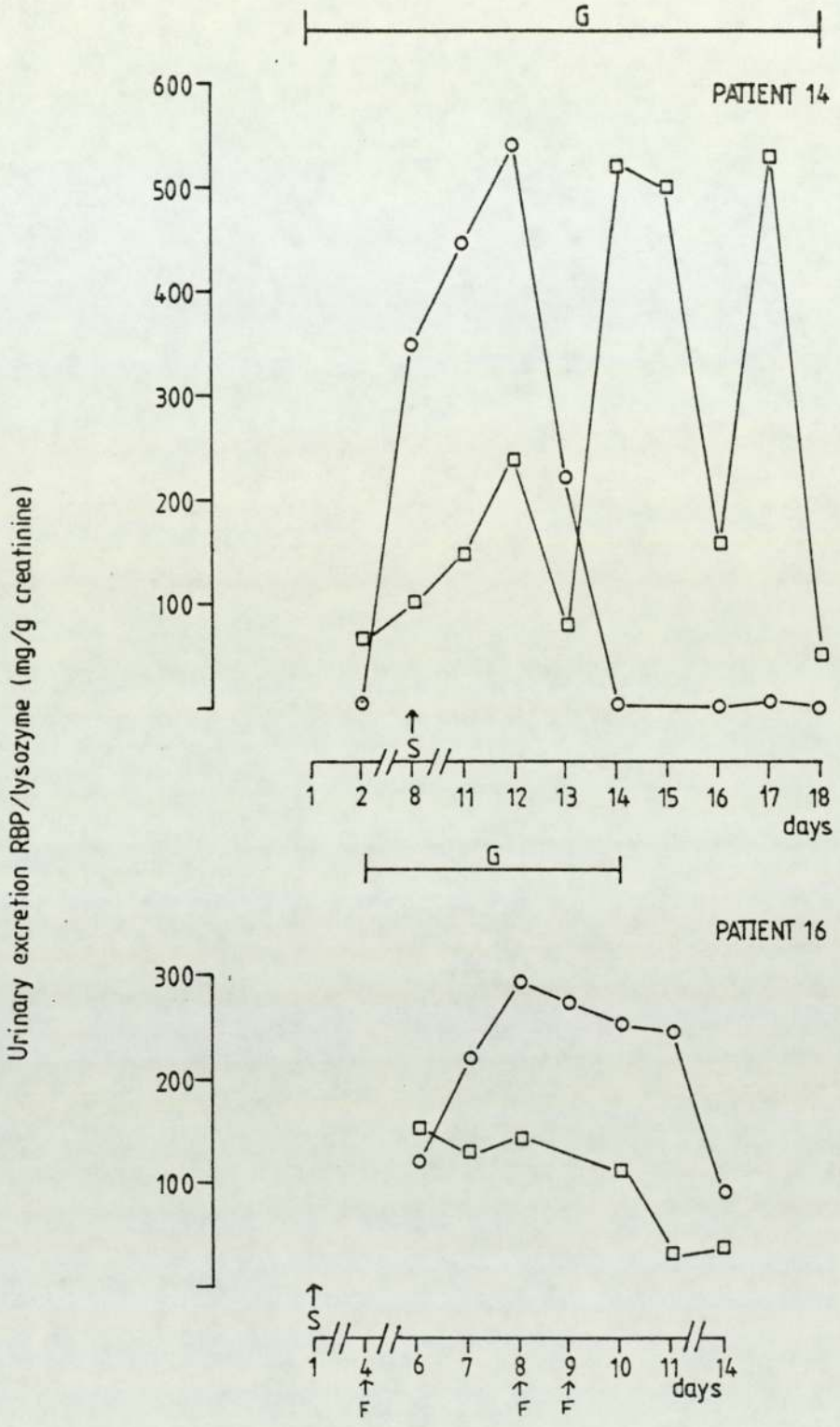


FIGURE 13: Urinary excretion of lysozyme and RBP by patients 14 and 16. S = surgery, G = gentamicin therapy, F = frusemide. o = lysozyme, □ = RBP.

Figures 14 and 15 represent the peak urinary excretions of RBP and lysozyme respectively, in the various subgroups of gentamicin-treated patients, and the post-surgery peak excretions of RBP and lysozyme in the control group.

F: Urinary excretion of RBP and lysozyme in surgical control patients

Table 20 shows the pre-surgery and post-surgery peak excretion data for RBP and lysozyme in the surgical patients used as a control group for the study. Appendix II shows graphs of RBP and lysozyme excretion in surgical control patients. From Figure 14 it can be seen that the range of peak excretions of RBP for the control group show a great deal of overlap with the range of peak excretions for the gentamicin-treated patients.

Pre-surgery peak excretion of RBP was within normal limits in 19 of 21 control patients, and just above normal limits in one patient. Post-surgery peak excretion was, however, above normal limits in 14 patients. The excretion of RBP actually increased by a factor of 10 or more post-surgery, as compared to pre-surgery levels, in 10 patients. Figure 16a shows pre-surgery RBP excretion, compared to excretion of RBP one day after surgery. Figure 16b shows pre-surgery RBP excretion compared to peak post-surgery RBP excretion. These diagrams show that levels of RBP rose in all control patients after surgery, and that this rise often occurred immediately after surgery.

The mean excretion (± S.D.) of RBP prior to surgery was 0.60 ± 1.3 mg/g creatinine (range 0.05 - 6.0). The mean excretion (± S.D.) of RBP post-surgery was 19 ± 27 mg/g creatinine (range 0.14 - 110). Two patients showed a post-surgery peak excretion of > 50 mg/g creatinine; patient 37, who showed a peak excretion of 110 mg/g

Table 20 Pre-surgery Excretion and Post-surgery Peak Excretion of RBP and Lysozyme in Control Patients

Patient	a) Days after surgery studied	b) Pre-surgery RBP (mg/g creatinine)	Post-surgery RBP (mg/g creatinine)	c) Day of peak	b)Pre-surgery lysozyme (mg/g creatinine)	Post-surgery lysozyme (mg/g creatinine)	c)Day of peak
32	3	0.05	1.1	2	ND	ND	-
33	4	0.08	0.24	⩾4	ND	ND	-
34	4	0.05	0.34	2	ND	ND	-
36	4	0.19	29	3	ND	ND	-
37	4	0.16	110	⩾4	ND	130	1
38	4	1.1	15	1	ND	110	3
40	2	0.82	39	⩾2	ND	2.0	⩾2
41	3	1.0	9.7	⩾3	ND	0.14	1
44	4	0.46	36	1	ND	0.51	2
45	4	0.10	12	2	ND	ND	-
46	2	0.07	0.26	1	ND	ND	-
47	4	0.12	0.39	1	ND	ND	-
48	4	0.06	0.14	2	ND	ND	-

Table 20 continued/

Patient	a)Days after surgery studied	b)Pre-surgery RBP (mg/g creatinine)	Post-surgery RBP (mg)g creatinine)	c)Day of peak	b)Pre-surgery lysozyme (mg/g creatinine)	Post-surgery lysozyme (mg/g creatinine)	c)Day of peak
49	3	0.08	0.85	1	ND	ND	-
50	5	0.12	8.9	3	ND	0.67	1
51	6	0.28	27	1	ND	1.0	1
52	4	6.0	56	2	2.65	19	2
53	7	0.17	40	3	ND	2.1	2
54	7	0.44	3.6	1	ND	0.77	2
55	4	0.26	0.33	⩾4	ND	0.43	1
56	6	0.95	4.4	3	40	5.8	⩾6

a) = No. of days after surgery that urine samples were taken.

b) = Pre-surgery levels were taken on the day before surgery or the morning of the day of surgery.

c) = Day of peak = day of peak in excretion after surgery.

ND = Not detectable.

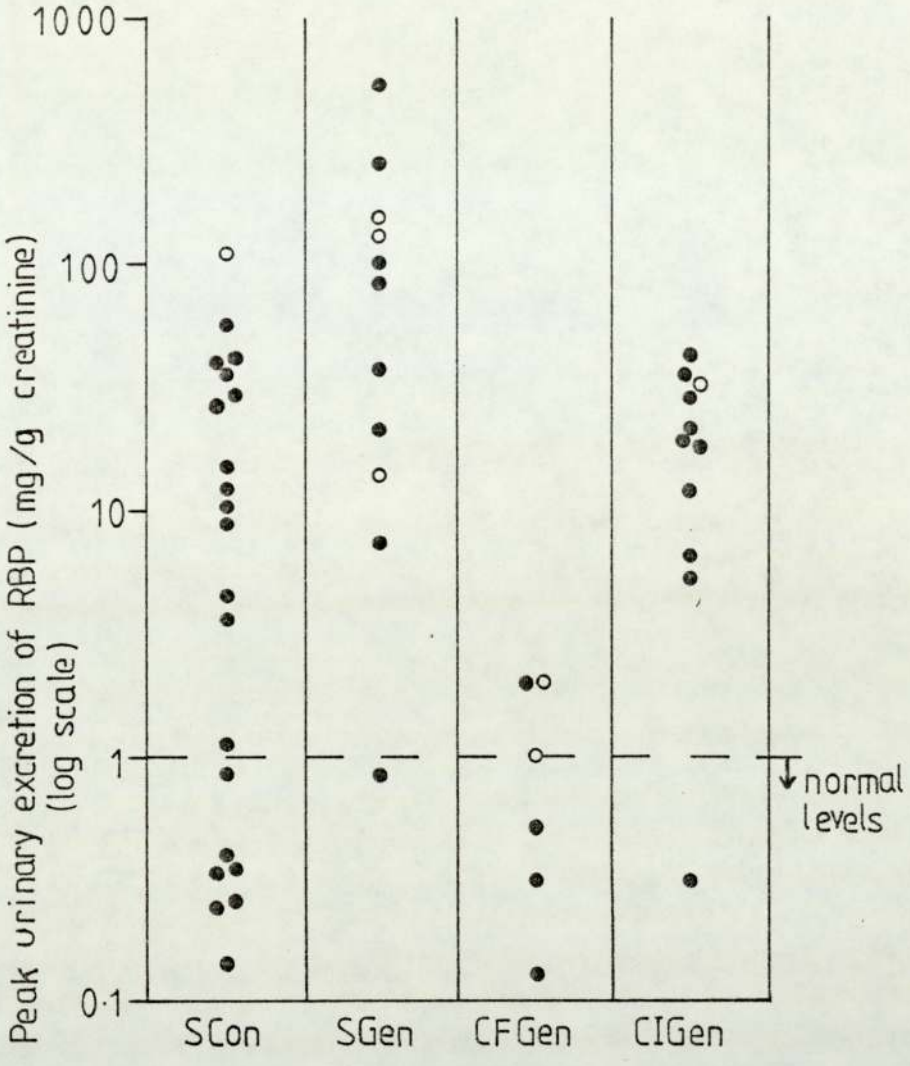


FIGURE 14: Peak urinary excretions of RBP in the three sub-groups of gentamicin-treated patients and post-surgical peak excretions of RBP in control patients. SCon = surgical control patients; SGen = gentamicin-treated surgical patients; CFGen = gentamicin-treated cystic fibrosis patients; CIGen = gentamicin-treated patients with chest infection etc.; o = patients with a significent rise in P_{cr}

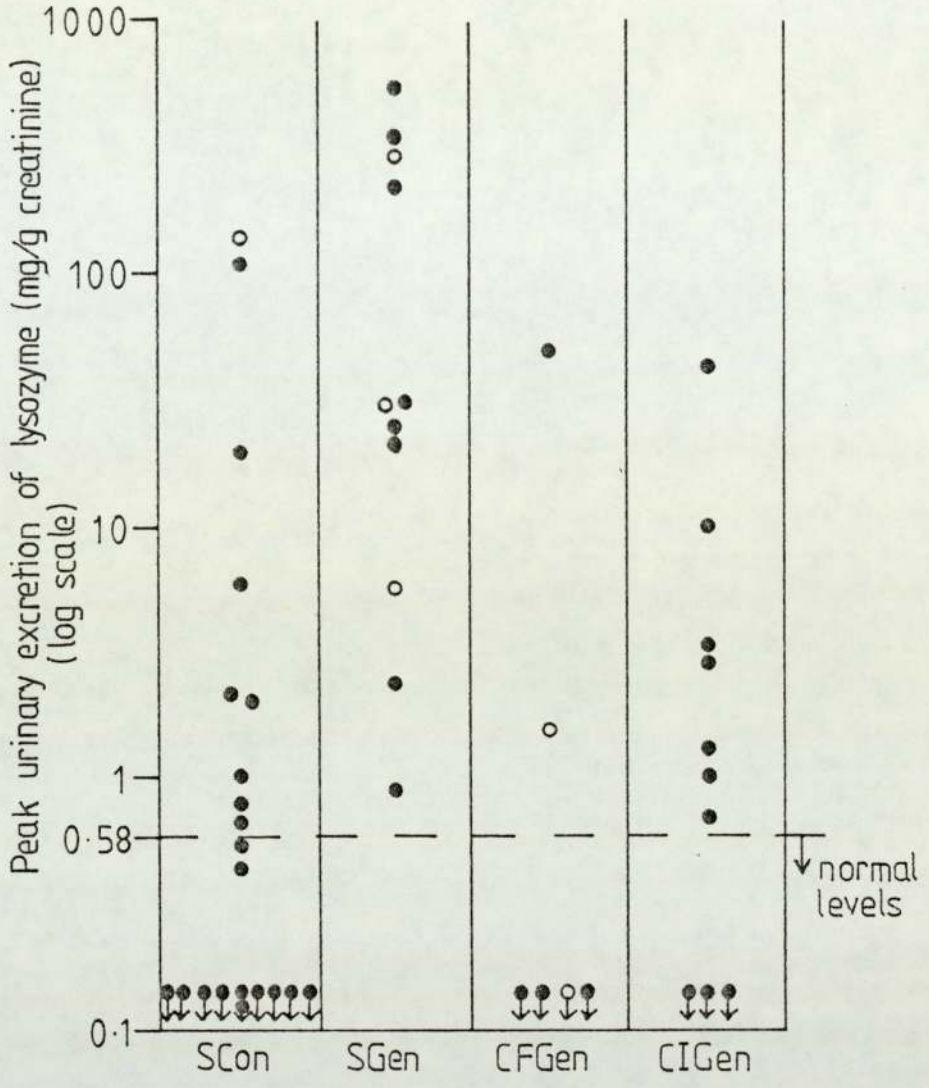


FIGURE 15: Peak urinary excretions of lysozyme in the three sub-groups of gentamicin-treated patients and post-surgical peak excretions of lysozyme in control patients. SCon = surgical control patients; SGen = gentamicin-treated surgical patients; CFGen = gentamicin-treated cystic fibrosis patients; CIGen = gentamicin-treated patients with chest infection etc.; o = patients with a significent rise in P_{cr}. ⚲ = below the sensitivity of the lysozyme assay.

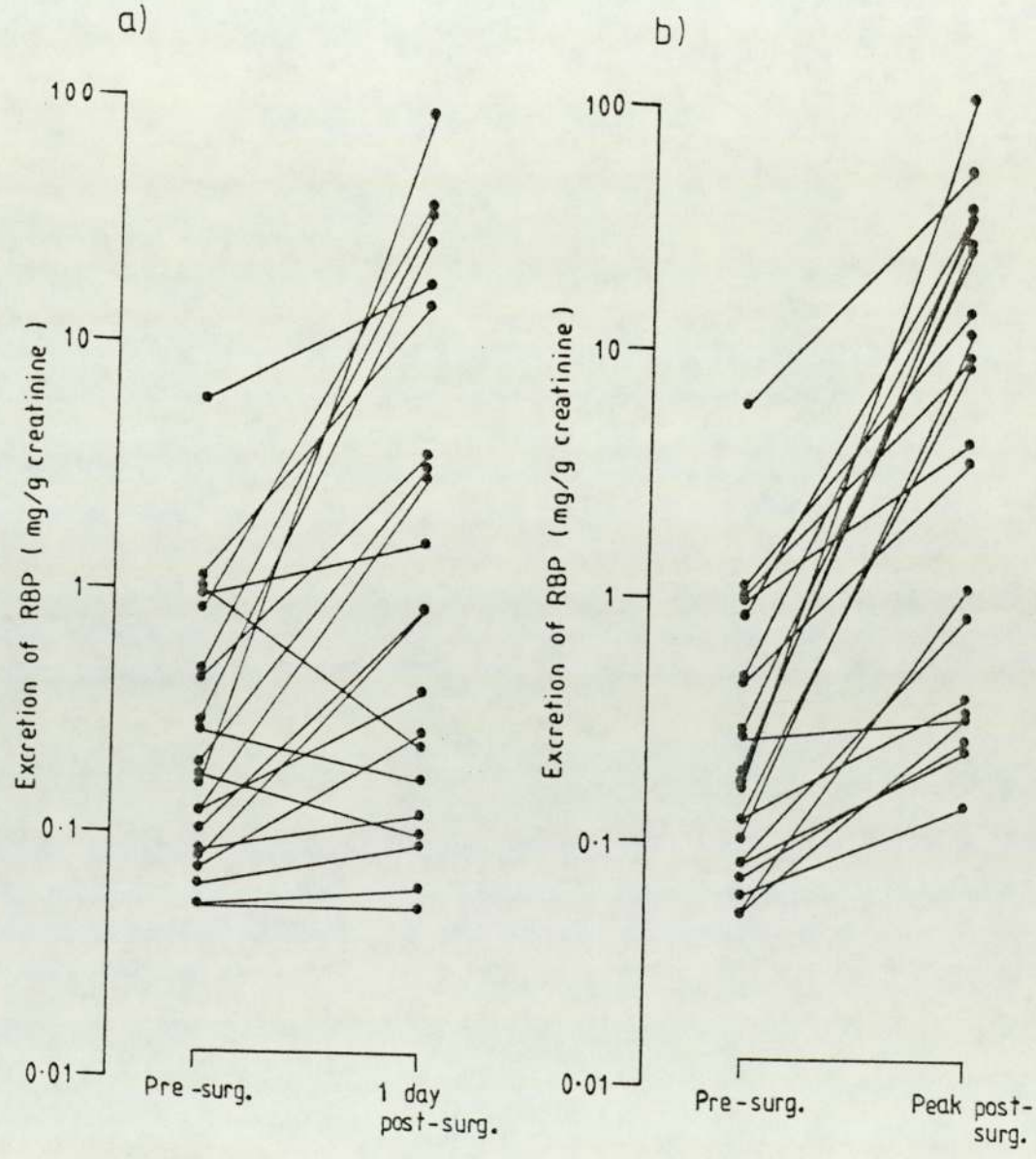


FIGURE 16: The pre-surgery excretion of RBP compared with:

a) excretion one day post-surgery

b) peak excretion post-surgery

in surgical control patients.

creatinine and patient 52, who showed a peak excretion of 56 mg/g creatinine. The peak excretion of RBP occurred on the first or second day after surgery in 12 of the 21 patients.

The pre-surgery urinary excretion of lysozyme was raised above normal values in only two patients, 52 and 56. In patient 56, pre-surgery excretion of lysozyme was 40 mg/g creatinine. After surgery, the peak excretion of lysozyme was raised above normal in nine of the 21 control patients.

Table 21 The distribution of the pre-surgery excretions and the post-surgery peak excretions of lysozyme in control patients

Pre-surgery excretion of lysozyme (mg/g creatinine)	No. of patients	Post-surgery peak excretion of lysozyme (mg/g creatinine)	No. of patients
⩽0.58	19	⩽0.58	12
0.59 - 9	1	0.59 - 9	6
10 - 49	1	10 - 49	1
⩾50	0	⩾50	2

Table 21 shows the distribution of various pre-surgery lysozyme excretion values and post-surgery peak lysozyme excretion values among control patients. The two patients who showed a post-surgery peak excretion of ⩾50 mg/g creatinine were patients 37 and 38. Patient 37 showed a peak excretion of 130 mg/g creatinine and patient 38 showed a peak excretion of 110 mg/g creatinine.

From Figure 15 , it can be seen that the range of peak excretions of lysozyme in the control group shows a great deal of overlap with the range of peak excretions of lysozyme in the gentamicin-treated groups.

In nine of the 12 patients in whom urinary lysozyme levels were detectable, the peak in excretion occurred on day 1 or 2 after surgery.

In several of the control patients, post-surgery lysozyme excretion was within normal levels or only slightly raised, whereas RBP excretion was greatly raised, compared to pre-surgery levels. The graph of RBP and lysozyme excretion in patient 50 (see Figure 17) demonstrates this large post-surgical rise in RBP excretion, while lysozyme excretion remains low. In patients 37 and 38, however, lysozyme excretion was raised above RBP excretion, although excretion of both proteins was greatly raised above normal (see Figure 18).

Patient 52 was the only patient in whom both lysozyme and RBP were raised above normal levels before surgery (see Figure 17).

The three patients in whom one or both proteins showed a post-surgery peak excretion of $\geqslant$50 mg/g creatinine were patients 37, 38 and 52.

Patient 37 showed a peak excretion of RBP of 110 mg/g creatinine and a peak excretion of lysozyme of 130 mg/g creatinine (see Figure 18). Excretion of both proteins was raised after surgery to over 500 times the pre-surgery excretion. Patient 37 had heart surgery with a post-operative antibiotic cover of cefuroxime and metronidazole (see Table 3). This patient was the only control patient to show a significant rise in P_{cr} after surgery (see Table 12). UPCR in this patient showed that this rise in P_{cr} was attributable to a renal cause. However, this was not due to drug therapy as this patient received no potentially nephrotoxic drugs.

Patient 38 showed a post-surgery excretion of lysozyme of 15 mg/g creatinine, and a post-surgery peak excretion of lysozyme of 110 mg/g creatinine (see Figure 18). This patient had also received heart surgery and was treated post-operatively with ampicillin and

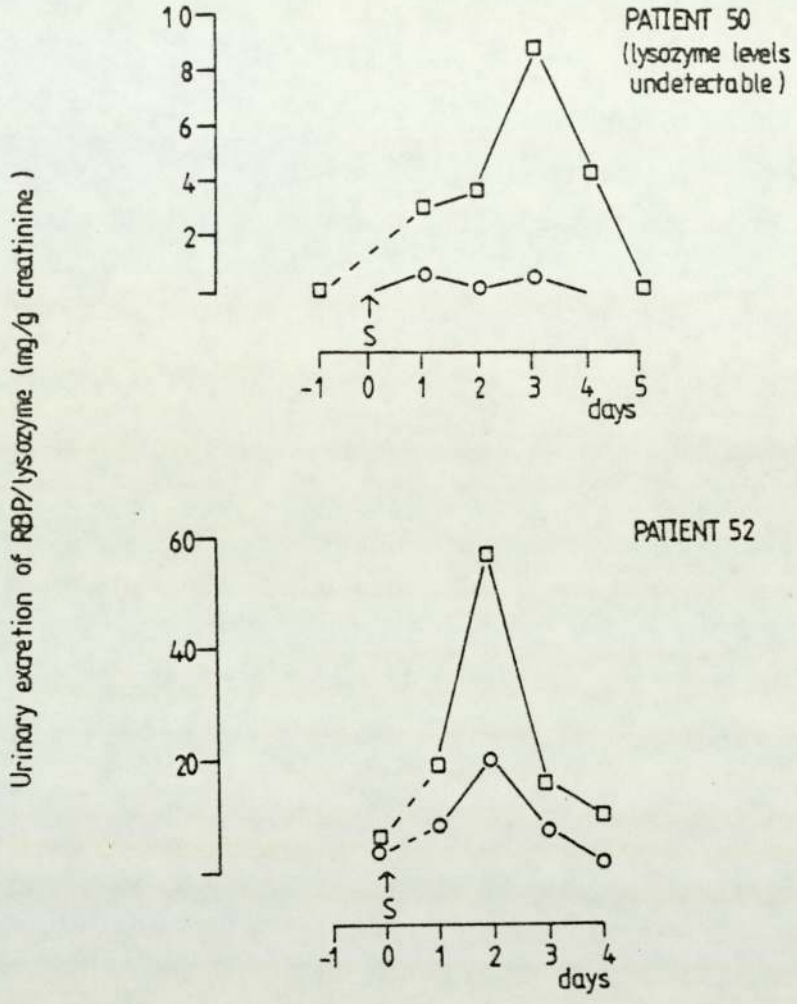


FIGURE 17: Urinary excretion of lysozyme and RBP by patients 50 and 52. S = surgery. o = lysozyme, □ = RBP.

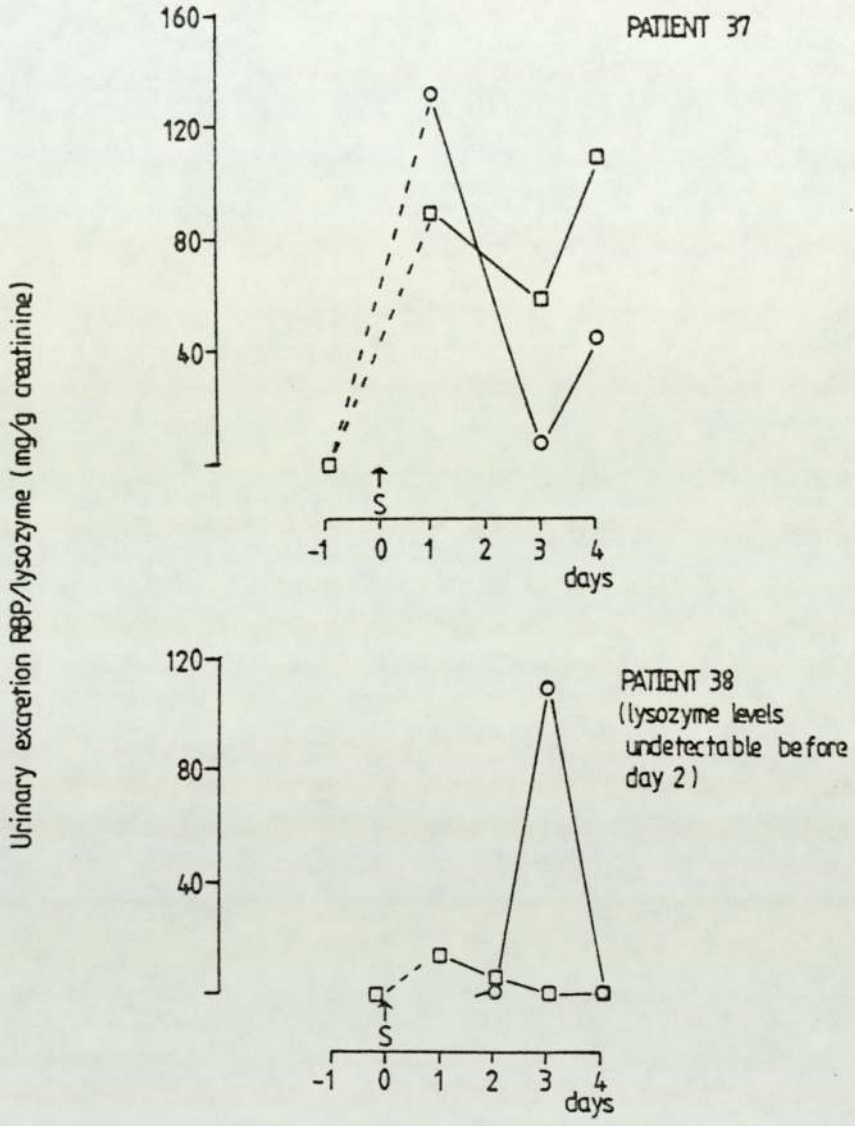


FIGURE 18: Urinary excretion of RBP and lysozyme by patients 37 and 38. S = surgery. o = lysozyme, □ = RBP.

flucloxacillin. Therefore any renal impairment was not due to nephrotoxic drugs. Unfortunately, no P_{cr} data was available for this patient.

Patient 52 (see Figure 17) showed raised levels of lysozyme and RBP before surgery, which rose further after surgery to give a peak of RBP excretion of 56 mg/g creatinine, and a peak of lysozyme excretion of 19 mg/g creatinine. Patient 52 had major abdominal surgery and was given a post-operative antibiotic therapy of cefuroxime and metronidazole. P_{cr} rose only slightly after surgery in this patient.

Therefore, both lysozyme and RBP rose above normal limits after surgery in many control patients, even though these patients were receiving no aminoglycosides or other potentially nephrotoxic drugs.

G: RBP and Lysozyme excretion in the different subgroups of gentamicin-treated patients

It can be seen from Figure 14 that urinary excretion of RBP is raised over 50 mg/g creatinine in only six of the gentamicin-treated patients, and all of these patients were in the surgical group. All cystic fibrosis patients showed peak excretions of RBP, which were within or only slightly above normal levels. The other patients, i.e. those treated with gentamicin for chest infection (C.I.), wound infection (W.I.), urinary tract infection (U.T.I.) etc. had peak excretions of RBP which varied from normal levels to almost 50 mg/g creatinine. There was a great deal of overlap between the peak excretions of RBP in the three subgroups of gentamicin-treated patients.

From Figure 15 it can be seen that, excluding patient 19, lysozyme was only raised over 50mg/g creatinine in four gentamicin-treated patients, all of whom were surgical patients. Cystic

fibrosis patients showed low peak excretions of lysozyme, within or slightly above normal levels, apart from one patient who showed a peak excretion of around 50 mg/g creatinine. The other subgroup of gentamicin-treated patients showed peak lysozyme excretions which ranged from near normal levels to almost 50 mg/g creatinine. Again there was a great deal of overlap between the peak excretions of lysozyme in the three subgroups of gentamicin-treated patients.

i) RBP and lysozyme excretion in patients treated with gentamicin post-surgically (within three days of surgery)

Table 22 shows the peak urinary excretion data of RBP and lysozyme for the 11 patients who received gentamicin post-surgically. Appendix III shows graphs of lysozyme and RBP excretion in these patients. All but one of these patients, patient 10, showed a greater than normal excretion of RBP. The mean peak excretion (± S.D.) of RBP in this group was 121 ± 157 mg/g creatinine (range 0.83 - 530 mg/g creatinine). This mean value is more than double that found for the group of gentamicin treated patients as a whole. RBP excretion was ⩾100mg/g creatinine in five patients; patients 12, 14, 15, 16 and 29.

The peak excretion of RBP occurred on day ⩽3 in seven of the 11 patients, all of whom had <7 days therapy. Of the remaining four patients, three had gentamicin therapy for ⩾7 days. As the peak excretion of RBP occurred in the first sample obtained after surgery in seven patients, it seems probable that the peak in excretion was associated with the surgery. The graphs of RBP excretion in patients 4, 8, 15, 16, 17 and 19 (see Appendix III) demonstrate this initial peak early in therapy, then excretion gradually decreasing towards normal as therapy continues.

In longer courses of therapy, the peak seemed to occur later,

Table 22 Peak urinary excretion data for RBP and Lysozyme in patients receiving gentamicin post-surgically (within 3 days of surgery).

Patient	a) Day of surgery	Duration of therapy	Peak excretion RBP (mg/g creatinine)	Day of peak	Peak excretion lysozyme (mg/g creatinine)	Day of peak
3	1	5	21	≤3	31	8
4	1	6	7.4	≤3	0.9	≤3
8	1	5	14	≤3	5.6	≤3
10	1	4	0.83	5	2.4	3
12	-1	7	260	5	340	8
14	8	17	530	17	540	12
15	1	2	130	≤2	30	3
16	-3	6	150	≤3	290	5
17	1	3	38	≤2	24	4
25	-2	10	82	≥14	220	8
29	1	6	100	≤3	21	4

a) Day of surgery. Minus numbers denote days before gentamicin therapy.

Day 1 = First day of gentamicin therapy

and therefore was probably associated with the gentamicin therapy rather than the surgery or a combination of the two. Graphs of RBP excretion in patients 12, 14 and 25 show this peak excretion later in therapy (see Figures 12 and 13). Patient 12, who had a seven day course of gentamicin therapy, showed one peak in RBP excretion on day 5 of therapy (five days after surgery). Patient 14, who had surgery on day 8 of a 17 day course of gentamicin therapy, showed three peaks in RBP excretion after surgery, on days 12, 14 and 17 of therapy (4, 6 and 9 days after surgery). Patient 25, who had a 10 day course of gentamicin therapy, showed four small peaks of excretion on days 4, 8, 11 and 14 of therapy (5, 9, 12 and 15 days after surgery).

Lysozyme excretion was raised above normal in all 11 patients treated with gentamicin post-surgically. The mean peak excretion (± S.D.) of lysozyme for this group was 137 ± 184 mg/g creatinine (range 0.90 - 540 mg/g creatinine).

Lysozyme excretion was $\geqslant$100 mg/g creatinine in four patients; 12, 14, 16 and 25. The peak excretion of lysozyme occurred on day $\leqslant$3 in only four patients, on day 4-7 in three patients and on day $\geqslant$8 in four patients. In the four patients who showed a peak excretion in the first three days of therapy, the peak in excretion may have been associated with surgery. However, in the other seven patients who showed a peak later in therapy, the peak was more likely to be associated with gentamicin therapy or a combination of surgery and gentamicin therapy. The graph of lysozyme excretion in patient 8 (see Appendix III) demonstrates this peak in lysozyme excretion soon after surgery.

The graphs of patients 12, 14 and 25 (see Figures 12 and 13 show a peak excretion later in therapy. The graphs of patients 12 and 14 show only one peak in lysozyme excretion. In patient 12 the peak occurred on day 9 (9 days after surgery), in patient 14 the peak

occurred on day 12 of therapy (4 days after surgery). The graph of patient 25 shows three clearly defined peaks of excretion on days 4, 8 and 11 (5, 9 and 12 days after surgery).

a) Possible factors involved in patients where both RBP and lysozyme excretion were > 100 mg/g creatinine

In patients 12, 14 and 16 excretion of both RBP and lysozyme were ⩾100 mg/g creatinine.

Patient 12 was a 75 year old man who had major abdominal surgery, one day before the commencement of gentamicin therapy (for chest infection). This patient received intravenous frusemide on day 4 of gentamicin therapy. Frusemide has been reported to enhance the nephrotoxicity of aminoglycosides in dogs[103]. As can be seen in Figure 12, both RBP and lysozyme excretion started to rise steeply after day 4 of gentamicin therapy. Only one gentamicin plasma level was taken in this patient on day 3 of therapy. The pre-dose (trough) plasma concentration was 1.3 mg/l and the post-dose (peak) plasma concentration was 4.4 mg/l. These levels are well below the recommended maximum peak level of 12 mg/l and trough level of 2 mg/l (reference values, antibiotic assay laboratory, EBH). P_{cr} rose slightly above normal in this patient, being 107 μmol/l on day 4 of gentamicin therapy, 113 μmol/l on day 6 and 149 μmol/l on day 8. This patient died on day 9. It was possible, therefore, that this patient had developed nephrotoxicity as P_{cr} values were rising up to the time of death. This patient was receiving intravenous feeding and was critically ill, bedridden and immobile. Therefore the large increase in excretion of RBP and lysozyme may have been due to aminoglycoside nephrotoxicity (possibly enhanced by Frusemide), surgical trauma, infection or critical illness.

Patient 14 was a 50 year old woman who had one operation a week

before commencement of gentamicin therapy (for pyrexia), then major abdominal surgery on day 8 of the 17 day course of gentamicin. Gentamicin plasma levels were taken on days 2, 4 and 10 of therapy; all trough levels were <1 mg/l, peak levels were 3.4, 6.4 and 8.5 mg/l respectively. All peak and trough levels were well within the normal range. After this second operation, the patient was immobile, bedridden and receiving intravenous feeding. P_{cr} remained low (around 60 μmol/l) throughout therapy. From Figure 13 it can be seen that excretion of both RBP and lysozyme were very high after surgery (on day 8 of gentamicin therapy). Lysozyme excretion showed a very large peak on day 12 of therapy (4 days after surgery). There were three large peaks in RBP excretion on days 12, 14 and 17 of therapy (4, 6 and 9 days after surgery), the largest peak being on day 17. It is not known why this patient should exhibit such very large peak excretions of RBP and lysozyme, unless due to surgical trauma, infection, critical illness or gentamicin therapy.

Patient 16, who was a 75 year old man, had major abdominal surgery three days before commencement of a six day course of gentamicin therapy (for pyrexia). Gentamicin plasma levels, taken on day 3 of therapy, showed a trough of <1 mg/l and a peak of 4.9 mg/l. These levels were well within normal range. This patient received single doses of intravenous frusemide on days 1, 5 and 6 of gentamicin therapy. It can be seen from Figure 13 that lysozyme and RBP excretion were both high by day 3 of gentamicin therapy. Lysozyme excretion continues to rise to a peak on day 5 of therapy, whereas RBP excretion gradually decreases towards normal. This patient showed significant rise in P_{cr} during therapy, but UPCR showed a pre-renal cause for this. Therefore the only explanation for the large amounts of RBP and lysozyme excreted in the urine is sub-clinical nephrotoxicity, major surgical trauma, infection or

critical illness.

b) Excretion of RBP and lysozyme in gentamicin-treated surgical patients compared with control surgical patients

From Figure 14 it can be seen that the post-surgery peak RBP excretion values for the control group overlap to a large extent those in the gentamicin-treated surgical group. However, there are some higher peak RBP excretions in the gentamicin-treated group, and the control group shows many more normal peak RBP excretions. As most control patients were only studied for 3 - 4 days after surgery, peak RBP excretion appeared to be on day ≤ 3 after surgery, whereas in the gentamicin-treated surgical group some peaks in RBP excretion occurred later than this. Therefore it was decided to compare post-surgical peak excretion of RBP in control patients with the first available post-surgical RBP excretion value in gentamicin-treated patients (see figure 19). The range of first available post-surgical RBP excretions is very similar to the range of post-surgery peak excretions in the control group, although the control group still showed more peak excretions within normal levels than the gentamicin-treated group.

Figure 20 shows post-surgical peak excretion of lysozyme in control patients, compared with the first available post-surgery lysozyme excretion value in gentamicin-treated patients. Again, the range of the post-surgery peak excretions in the control group is very similar to the range of first available post-surgical lysozyme excretions in the gentamicin-treated group. Therefore, it seems highly possible that surgery was the major causative factor in the large excretion of both RBP and lysozyme in both control and gentamicin-treated surgical patients. Therefore, any peaks in excretion of RBP and lyozyme occurring in the first few days after

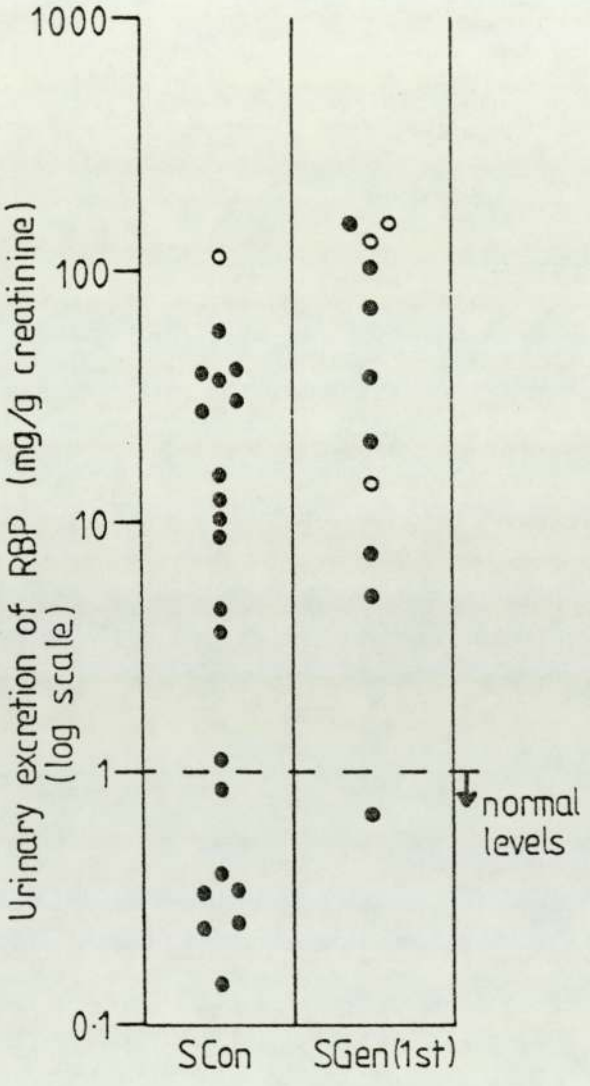


FIGURE 19: Post-surgical peak excretion of RBP in surgical control patients (SCon) and first available post-surgical excretion of RBP in gentamicin-treated surgical patients (SGen(1st)). o = patients with a significent rise in P_{cr}

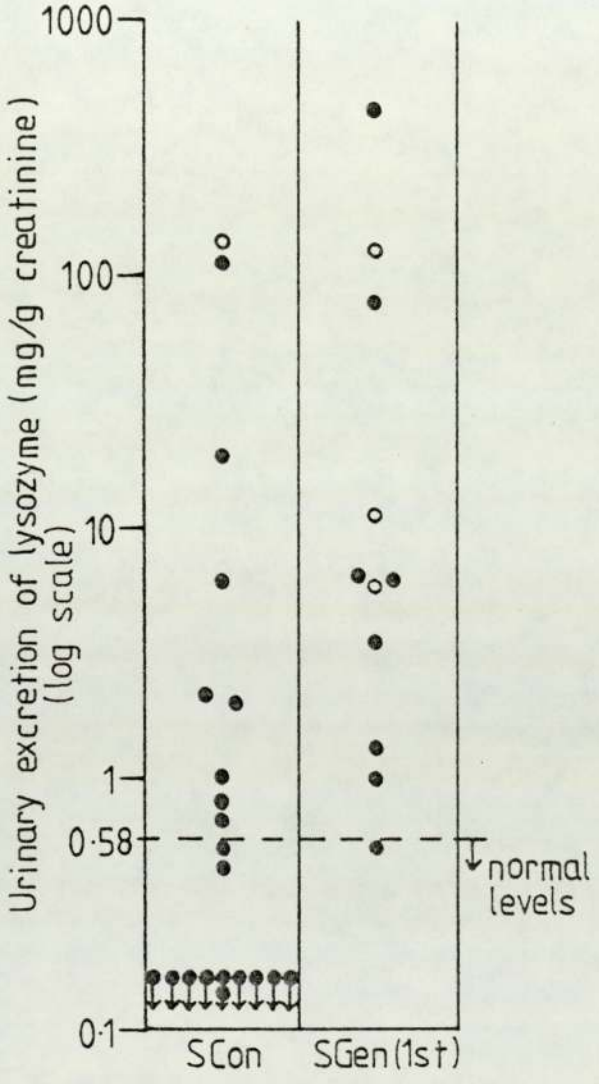


FIGURE 20: Post-surgical peak excretion of lysozyme in surgical control patients (SCon) and first available post-surgical excretion of lysozyme in gentamicin-treated surgical patients (SGen(1st)). o=patients with a significent rise in P_{cr}; ↕ = below the sensitivity of the lysozyme assay

surgery, in gentamicin-treated patients, were probably due to surgery alone, or a combination of surgery and gentamicin therapy.

ii) Excretion of RBP and lysozyme in cystic fibrosis patients receiving gentamicin for chest infection

As shown in Figure 14, cystic fibrosis patients showed near normal excretion of RBP in all cases. Table 23 shows the peak urinary excretion data for RBP and lysozyme in the six patient courses of gentamicin (four patients). Appendix IV shows the graphs of RBP and lysozyme excretion in these patients. The mean peak excretion (± S.D.) of RBP in this group was 0.99 ± 0.83 mg/g creatinine (range 0.13 - 2.0 mg/g creatinine), which was much lower than the mean excretion for the group of gentamicin-treated patients as a whole. RBP excretion was raised slightly above normal in two of the courses, and within normal limits in the other four courses.

Patient course 22, which followed course 9 in the same patient by four months, produced a higher peak RBP excretion than course 9. This may well have been due to the first course of gentamicin therapy being of only three days duration, and the second course being of 10 days duration.

Table 23 Peak urinary excretion data for RBP and lysozyme in cystic fibrosis patients treated with gentamicin

Patient course	Duration of therapy	Peak excretion RBP (mg/g creatinine)	Day of Peak	Peak excretion Lysozyme (mg/g creatinine)	Day of Peak
9*	3	0.31	$\leqslant$ 3	ND	-
22*	11	1.0	7	ND	-
13**	10	0.50	4	ND	-
20**	10	2.0	7	50	8
18	2	0.13	$\leqslant$ 2	ND	-
23	14	2.0	8	1.6	8

* Patient courses 9 and 22 are two separate courses of gentamicin therapy in the same patient.

** Patient courses 13 and 20 are two separate courses of gentamicin therapy in the same patient.

N.D. = Not detectable.

Patient course 20, which followed course 13 in this patient by one month, produced a higher peak excretion of RBP even though both courses were of 10 days duration.

Lysozyme excretion was below normal limits and too low to be measured in patients 9, 13, 18 and 22. Patient 23 showed only slightly raised excretion of lysozyme. Patient 20, however, showed a large peak in lysozyme excretion on day 7 of therapy. It is not known why this sudden peak occurred.

All these cystic fibrosis patients were routinely receiving frequent courses of gentamicin therapy.

iii) RBP and lysozyme excretion in patients treated with gentamicin for chest infection, wound infection, urinary tract infection, etc.

As shown in Figures 14 and 15, peak RBP excretion values ranged between normal values and nearly 50 mg/g creatinine in this group, whereas peak lysozyme excretion values were around normal levels except for two patients. Table 24 shows the peak urinary excretion data of RBP and lysozyme in the 11 patients in this group. The graphs of RBP and lysozyme excretion in this group are shown in Appendix V.

The mean peak excretion (± S.D.) of RBP in this group was 20 ± 13 mg/g creatinine (range 0.31 - 42 mg/g creatinine) which was much lower than the mean for the whole of the gentamicin-treated group. Only one patient in this group, patient 6 (U.T.I.) had a peak excretion of RBP within normal limits.

Lysozyme excretion was greater than normal in eight patients. Patient 19 showed a peak urinary excretion of 2000 mg/g creatinine, but this was due to plasma lysozyme levels exceeding the renal threshold.

The range of peak urinary excretions of lysozyme in this group of patients, excluding patient 19, was 0.25 - 44 mg/g creatinine.

In the patients with C.I., 2, 21 and 30, RBP excretion was greatly raised above lysozyme excretion. Peak RBP excretion in these patients ranged from 12 - 42 mg/g creatinine, and peak lysozyme excretion ranged from 0.7 - 3.4 mg/g creatinine. In the three patients with U.T.I., 6, 26 and 28, lysozyme excretion rose above RBP excretion in patients 26 and 28, whereas both RBP and lysozyme excretion were normal in patient 6 (see Appendix V). Peak RBP excretion in these patients ranged from 0.31 - 21 mg/g creatinine and

Table 24 Peak urinary excretion data for RBP and lysozyme in patients receiving gentamicin for chest infection (not cystic fibrosis patients), urinary tract infection, wound infection, pre-operatively or for septicaemia (unknown cause).

Patient	Duration of therapy	Illness	Peak excretion RBP (mg/g creatinine)	Day of Peak	Peak excretion Lysozyme (mg/g creatinine)	Day of peak
2	14	C.I.	28	14	0.7	7,13
21	2	C.I.	12	4	3.4	⩾7
30	4	C.I.	42	2	1.0	3
6	4	U.T.I.	0.31	⩽3	N.D.	-
26	5	U.T.I.	21	⩽2	44	6
28	6	U.T.I.	5.2	5	9.9	1
11	4	W.I.	19	⩽3	1.3	⩽3
42	4	W.I.	35	3	2.9	2
43	4	W.I.	18	⩽3	N.D.	-
5	7	Pre-op	6.5	⩾10	N.D.	-
19	6	Septicaemia	32	6	*2000	⩾10

C.I. = Chest Infection

U.T.I. = Urinary Tract Infection

W.I. = Infected wound from recent surgery (more than 3 days before gentamicin therapy started).

*Patient 19 had a plasma level of lysozyme far above the renal threshold.

lysozyme excretion ranged from <0.25 - 44 mg/g creatinine. In all three patients with W.I., 11, 42 and 43, peak excretion of RBP occurred on day ≤3 of therapy, and was very much greater than lysozyme excretion (see Appendix V). Peak RBP excretion in this group ranged from 18 - 35 mg/g creatinine and peak lysozyme excretion ranged from <0.25 - 29 mg/g creatinine.

In patient 5, who was treated pre-operatively with gentamicin, lysozyme levels were undetectable , whereas RBP excretion rose to 6.5 mg/g creatinine (see Appendix V). In patient 19, who was treated with gentamicin for septicaemia of unknown cause, peak lysozyme excretion was 2000 mg/g creatinine due to plasma lysozyme levels exceeding the renal threshold. RBP excretion rose to a peak of 32 mg/g creatinine in this patient. The high plasma lysozyme in patient 19 was thought to be due to the septicaemia or to the fact that this patient already had mild renal failure, before gentamicin therapy. However, if the high plasma levels of lysozyme were due to renal impairment leading to accumulation of lysozyme in the plasma, then RBP levels would also be expected to be high. As RBP plasma levels were within normal levels in this patient, it is thought that the plasma lysozyme concentration was probably not due to renal failure.

3) Plasma and Urinary Calcium and Magnesium Results

A: Plasma calcium in gentamicin-treated patients.

Table 25 shows the plasma and urinary calcium data for the 28 gentamicin-treated patients. Five patients (13,15,16,18 and 23) developed hypocalcaemia i.e. plasma calcium of <2.10 mmol/l (Clinical Chemistry reference values, EBH), during or immediately after gentamicin therapy. None of these five patients showed a large depression in plasma calcium, the lowest value being 1.97 mmol/l for patient 18. The hypocalcaemia developed towards or just after the end of gentamicin therapy in each case. The development of hypocalcaemia did not seem to be related to duration of therapy, as therapy ranged from two to 14 days in the hypocalcaemic group. Patients 13,18 and 23, who developed hypocalcaemia, were cystic fibrosis patients. Patients 15 and 16 were receiving gentamicin post-surgically. Patients 15 and 23 had both shown a significant rise in P_{cr} during therapy (which may have been due to gentamicin nephrotoxicity).

Twenty-two patients did not appear to develop hypocalcaemia during therapy (no plasma calcium data was available for patient 11). The lowest plasma calcium value occurred during the first four days of gentamicin therapy in 21 of the 28 patients. Plasma calcium data for patients 2,14,20,22 and 25 show that the lowest plasma calcium value did not tend to occur later in the longer courses of therapy.

Plasma calcium levels did not fluctuate greatly in any patient. Many patients showed a small transient decrease in plasma calcium in the first few days of gentamicin therapy, which tended to recover whether therapy was continued or not. The graph of plasma

Table 25a) Plasma and Urinary Calcium data for Patients with Hypocalcaemia (< 2.10 mmol/l)

Patient	Duration of therapy (days)	Lowest plasma Ca (mmol/l)	a) Day of lowest plasma Ca	Peak urinary excretion Ca (mmol/g creatinine)	a) Day of peak
13	10	2.01	9	4.6	5
15	2	2.04	2	1.8	5
16	6	2.00	7	2.3	5
18	2	1.97	3	14	≥7
23	14	2.00	11	39	8

a) Where day 1 = First day of gentamicin therapy.

Table 25b) Plasma and Urinary Calcium data for Patients with Plasma Calcium Levels > 2.10 mmol/l

Patient Course	Duration of therapy (days)	Lowest plasma Ca (mmol/l)	a) Day of lowest plasma Ca	Peak urinary excretion Ca (mmol/g creatinine)	a) Day of peak
2	14	2.60	7	14	≥17
3	5	2.33	7	0.94	≤ 3
4	6	2.17	7	35	8
5	7	2.45	7	18	7
6	4	*2.34	2	13	≥ 8
8	5	2.33	4	6.2	8
9	3	2.20	2	1.9	4
10	4	2.28	1	2.6	≥10
11	4	-	1	2.9	≥8
12	7	2.15	2	1.5	7
14	17	2.10	1,3	22	14
17	3	2.18	4	2.8	7

Table 25b continued/

19	6	2.15	4	2.8	4
20	10	2.29	3	8.4	8
21	2	*2.51	3	550	4
22	11	2.17	1,8	25	7
25	10	2.24	1	28	4
26	5	*2.35	2	9.6	6
28	6	2.64	2	5.2	1
29	6	2.18	3	4.1	8
30	4	2.31	2	12	≥ 6
42	4	2.18	2	15	3
43	4	2.18	2	12	7

* = only one plasma calcium value was available

No plasma calcium data was available for patient 11

a) = where day 1 is first day of gentamicin therapy

calcium in patient 17 (see Figure 21), shows this decrease in plasma calcium, which recovers when gentamicin therapy is stopped. The graph of plasma calcium for patient 23 (see Figure 21), who received a longer course of therapy, shows plasma calcium decreasing early in therapy, recovering to normal levels, then decreasing and recovering again later in therapy.

In patient 15, who developed hypocalcaemia, the lowest plasma calcium value was the first plasma sample obtained after the beginning of gentamicin therapy (on day 2). Therefore, this patient may have been hypocalcaemic before therapy. In patient 16, plasma calcium values gradually decreased throughout therapy, hypocalcaemia developing on the last day of the six day course of gentamicin therapy. In patient 13, plasma calcium levels rose on day 7 of gentamicin therapy, then dropped sharply so that hypocalcaemia occurred on day 9 of the 10 day therapy. In patient 18, hypocalcaemia developed one day after the end of a two day course of therapy.

B: Plasma magnesium in gentamicin-treated patients.

Table 26 shows the plasma and urinary magnesium data for the gentamicin-treated patients (no plasma magnesium was available for patient 11). Hypomagnesaemia, i.e. a plasma magnesium of < 0.70 mmol/l (Clinical Chemistry reference values, EBH), developed in two of the patients; 8 and 18. Hypomagnesaemia was mild in each case, being just below the normal limits for plasma magnesium.

Patient 8 was a patient receiving gentamicin post-surgically who developed hypomagnesaemia one day after the end of a 5 day course of therapy. This patient showed a significant rise in P_{Cr} during therapy (not thought to be due to gentamicin nephrotoxicity). Patient 18 was cystic fibrosis patient who developed hypomagnesaemia

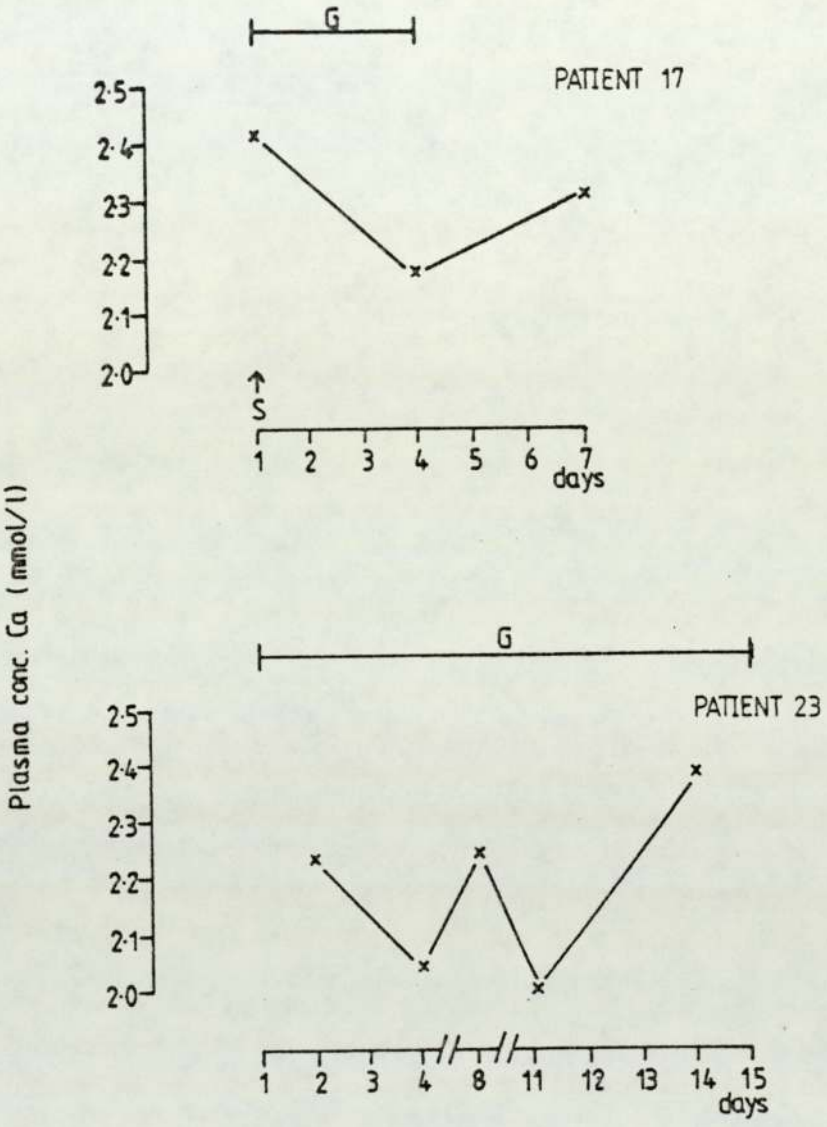


FIGURE 21: Plasma calcium concentration in patients 17 and 23. S = surgery, G = gentamicin therapy.

Table 26a) Plasma and Urinary Magnesium data for Patients with Hypomagnesaemia (< 0.70 mmol/l)

Patient Course	Duration of therapy (days)	Lowest plasma Mg (mmol/l)	a) Day of lowest plasma Mg	Peak urinary excretion Mg (mmol/g creatinine)	a) Day of peak
8	5	0.65	6	2.9	5
18	2	0.66	3	7.8	≥7

a) Where day 1 = first day of gentamicin therapy.

Table 26b) Plasma and Urinary Magnesium data for Patients with Plasma Magnesium Levels > 0.70 mmol/l.

Patient Course	Duration of therapy (days)	Lowest plasma Mg (mmol/l)	a) Day of lowest plasma Mg	Peak urinary excretion Mg (mmol/g creatinine)	a) Day of peak
2	14	0.76	2	20	13
3	5	0.75	7	3.8	⩽ 3
4	6	0.80	7	16	⩾10
5	7	0.82	10	7.0	7
6	4	1.02*	2	7.9	⩾ 8
9	3	0.91	2	9.1	6
10	4	0.83	3	2.8	7
11	4	-	-	4.4	4
12	7	0.83	3	11	5
13	10	1.03	2	7	3
14	17	0.82	1	28	14
15	2	0.98	2	6.1	5
16	6	0.91	1	29	7
17	3	0.71	7	4.8	7
19	6	0.82	8	5.4	5

Table 26b) continued

20	10	0.90	10	3.8	8
21	2	1.04*	3	108	4
22	11	0.87	1	37	7
23	14	0.87	11	24	8
25	10	0.70	1	20	4
26	5	1.14	2	7.1	5
28	6	0.75	2	4.5	3
29	6	0.72	2	5.0	4
30	4	1.15*	2	5.1	2
42	4	1.00	2	9.6	3
43	4	0.87	5	4.9	$\leq$ 3

a) = where day 1 is first day of gentamicin therapy

* Only one plasma magnesium value was available

No plasma magnesium data was available for patient 11

one day after the end of a two day course of gentamicin therapy. This patient also developed hypocalcaemia.

Twenty-six patients did not appear to develop hypomagnesaemia during therapy. The lowest plasma magnesium occurred on day 1-4 of gentamicin therapy in 18 of the 28 gentamicin-treated patients.

Plasma magnesium levels fluctuated by a small amount in most patients throughout therapy, but showed no definite trends. As patients 8 and 18 both received only short courses of gentamicin therapy, and patients 2,14,20,22 and 25 received long courses of therapy and did not develop hypomagnesaemia, it appears that hypomagnesaemia is not related to duration of therapy in these patients.

C: Urinary excretion of calcium in gentamicin-treated patients.

Patient 23, who developed hypocalcaemia, showed a very raised peak excretion of calcium. However, the peak urinary excretion of calcium did not appear to be greater in the remainder of those patients with hypocalcaemia (see Table 25) than in these patients with normal plasma calcium, as would be expected if hypocalcaemia was due to the renal wasting of calcium. The mean peak excretion (± SD) of calcium for the hypocalcaemic patients was 12 ± 15 mmol/g creatinine, and the mean for the remainder of the group was 11 ± 9 mmol/g creatinine. The mean peak excretion for the whole of the gentamicin-treated group was 11 ± 11 mmol/g creatinine. The results for patient 21 were excluded from this calculation, as this patient showed an erroneously high excretion of calcium, due to a very low concentration of creatinine in the urine. The actual urinary concentration of calcium (mmol/l) for patient 21 was similar to the values shown by the rest of the group. Patient 21 showed a low plasma creatinine and very low creatinine clearance (< 10 ml/min).

The peak excretion of calcium occurred on day 4-7 of gentamicin

therapy in 15 of the patients. The peak often occurred after the gentamicin therapy had finished. In three of the patients who developed hypocalcaemia, the peak in excretion preceded the lowest plasma calcium. In two patients hypocalcaemia preceded the peak in calcium excretion.

From the pre-surgery urinary excretion data for control patients shown in Table 27, normal values were established for the urinary excretion of calcium in hospital patients. The mean pre-surgery excretion (± SD) of calcium for control patients was 6 ± 4 mmol/g creatinine. Therefore, an upper normal limit for urinary excretion of calcium in hospital patients was established as the mean + 2SD = 14 mmol/g creatinine. Therefore, urinary excretion was above normal in only one of the gentamicin-treated patients with hypocalcaemia; 23, although excretion was at the upper limit of normal in patient 18. Of the patients who did not develop hypocalcaemia, urinary excretion of calcium was above normal in seven patients; 4,5,14,21,22,25 and 42.

Figure 22 shows the peak calcium excretion values in the three sub-groups of gentamicin-treated patients (excluding patient 21) and the peak post-surgery calcium excretion values for control patients. There is a great deal of overlap in the ranges of peak excretions in the control and gentamicin-treated groups. The highest peak excretions were for the gentamicin-treated surgical patients and the cystic fibrosis patients, whereas results for the chest infection, etc. group were mostly within normal levels. Urinary excretion of calcium was above normal in only one of the control patients, but in eight of the gentamicin-treated patients; three post-surgical patients, two cystic fibrosis patients and three patients from the chest infection etc. group (including patient 21).

The highest peak excretion of calcium (excluding patient 21) was

Table 27 Plasma and Urinary Calcium data for Surgical Control Patients.

Patient	a) Days after surgery urine samples taken.	b) Pre-surgery plasma Ca, mmol/l (day)	b)Post-surgery plasma Ca, mmol/l (day)	Pre-surgery urinary excretion mmol/g creatinine	c)Post-surgery peak urinary excretion of Ca mmol/g creatinine (day)
32	3	-	1.92 (4)	7.8	10 (2)
33	4	2.16 (-1)	-	10	6.1 (≥4)
34	4	-	2.12 (1)	13	8.1 (1)
36	4	2.29 (-2)	2.06 (1)	11	7.6 (3)
37	4	2.30 (-2)	1.79 (1),2.20(5)	4.1	3.6 (≥4)
38	4	-	-	4.5	11 (≥4)
40	2	2.28 (-2)	2.07 (4)	4.7	0.45 (1)
41	3	2.30 (-1)	-	3.3	7.4 (2)
44	4	2.10 (-1)	2.04 (1)	7.8	5.3 (≥4)
45	4	2.20 (-1)	2.13 (1)	5.7	11 (≥4)
46	2	2.56 (-1)	2.23 (1)	4.7	7.3 (≥2)

Table 27 continued/

47	4	2.18 (-1)	2.05(1),1.78(4)	6.1	5.1	(1)
48	4	2.47 (-1)	-	1.9	2.5	(≥4)
49	3	2.13 (-3)	2.31 (1)	8.2	6.0	(≥3)
50	5	2.33 (-1)	2.18 (1)	5.7	4.3	(4)
51	6	2.28 (-1)	2.27 (5)	6.7	1.3	(≥6)
52	4	2.25 (-2)	2.05 (1)	6.9	3.9	(2)
53	7	2.27 (-1)	2.33 (1)	2.2	2.4	(6)
54	7	2.35 (-1)	2.51 (6)	12	6.2	(3)
55	4	2.26 (-1)	2.28 (4)	2.1	1.9	(≥4)
56	6	2.24 (-3)	2.27 (3)	2.8	18	(3)

a) No. of days after surgery that urine samples were taken.

b) Minus numbers denote days prior to surgery, day 0 = day of surgery, positive numbers denote days after surgery.

c) The peak excretion of calcium post-surgery is quoted with the day after surgery when this peak occurred.

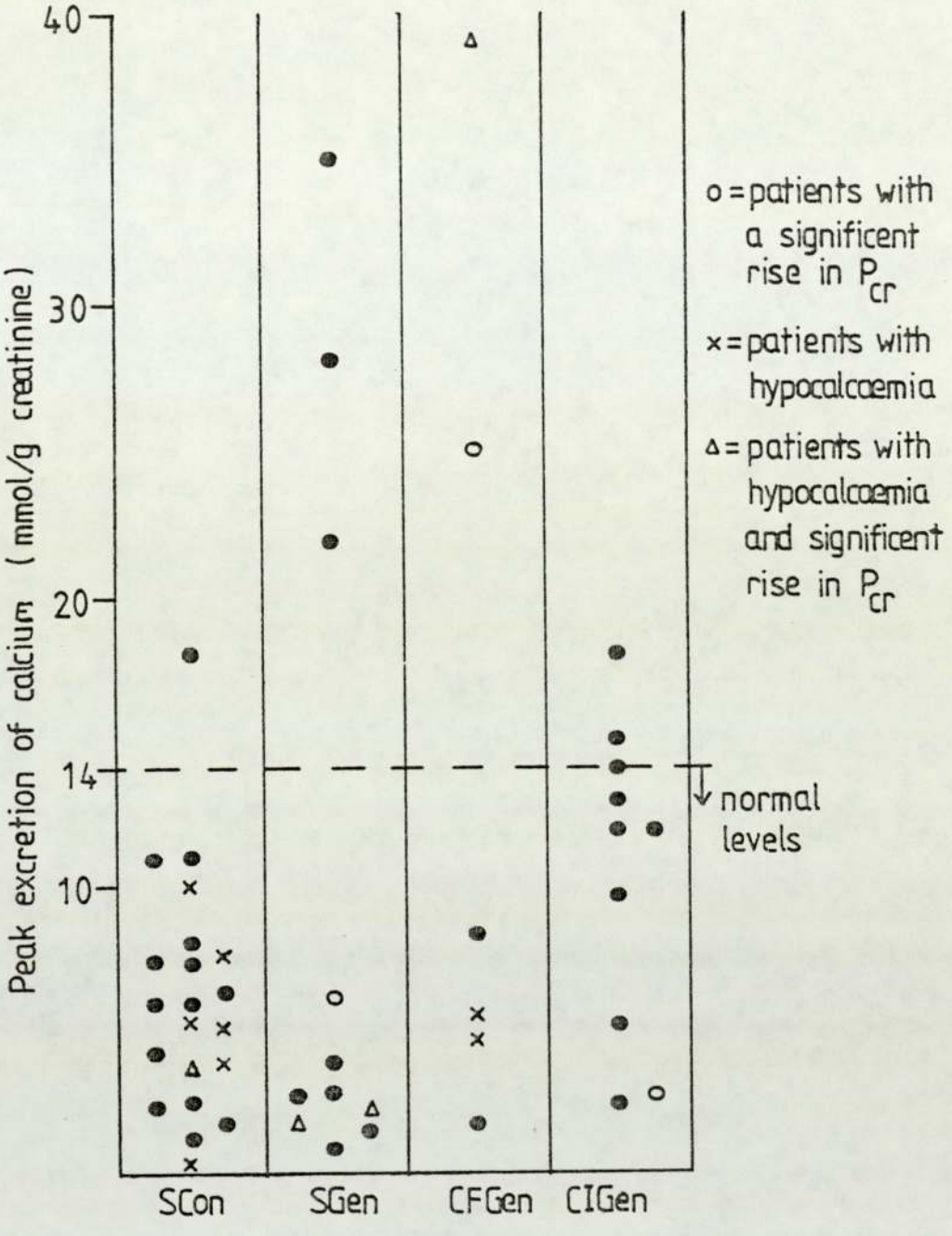


FIGURE 22: Peak urinary excretions of calcium in the three sub-groups of gentamicin-treated patients and post-surgical peak excretions of calcium in control patients. SCon = surgical control patients; SGen = gentamicin-treated surgical patients; CFGen = gentamicin-treated cystic fibrosis patients; CIGen = gentamicin-treated patients with chest infection etc.

(patient 21 excluded)

exhibited by patient 23, a cystic fibrosis patient who had shown a significant rise in P_{cr} (which may have been due to gentamicin nephrotoxicity) and developed hypocalcaemia. Patient 22, who had shown a significant rise in P_{cr} (not thought to be due to nephrotoxicity) also showed a raised peak calcium excretion. All other patients with hypocalcaemia, a significant rise in P_{cr}, or both, had normal levels of calcium excretion.

D: Urinary excretion of magnesium in gentamicin-treated patients.

The peak urinary excretion of magnesium did not seem to be greater in those gentamicin-patients who developed hypomagnesaemia, as would be expected if the hypomagnesaemia was due to renal wasting of magnesium. The peak urinary excretion of magnesium for the two patients with hypomagnesaemia was <10 mmol/g creatinine, whereas the mean peak excretion (±SD) for the rest of the group was 11 ± 9 mmol/g creatinine. The mean peak excretion of magnesium for the group of gentamicin-treated patients as a whole was 11 ± 9 mmol/g creatinine. These calculations excluded the results of patient 21 for the reasons stated under calcium excretion. The peak excretion occurred on day 4 - 7 of gentamicin therapy in 16 patients. The peak excretion of magnesium occurred three days after the last dose of gentamicin in patient 18 and on the last day of gentamicin therapy in patient 8. The peak in magnesium excretion preceded the lowest plasma magnesium in patient 8, and followed the lowest plasma magnesium in patient 18.

From the pre-surgery urinary excretion data for control patients, shown in Table 28, normal values for the urinary excretion of magnesium in hospital patients were calculated. The mean pre-surgery urinary excretion (± SD) of magnesium in control patients was 5 ± 3 mmol/g creatinine. Therefore, an upper normal limit of

Table 28: Plasma and Urinary Magnesium Data for Surgical Control Patients.

Patient	a) Days after surgery urine samples taken	b) Pre-surgery Plasma Mg. mmol/l(day)	b) Post-surgery plasma Mg. mmol/l(day)	Pre-surgery urinary excretion Mg (mmol/g creatinine)	c) Post-surgery peak urinary excretion mmol/g creatinine (day)
32		-	1.43 (4)	6.2	6.8 (≥3)
33	4	0.98 (-1)	-	7.1	3.9 (≥4)
34	4	-	0.98 (1)	5.4	5.4 (3)
36	4	-	-	9.5	7.7 (3)
37	4	1.05 (-2)	0.84(1), 1.14(5)	3.9	5.4 (≥4)
38	4	-	-	2.6	9.7 (3)
40	2	1.01 (-2)	-	3.7	0.46 (1)
41	3	1.20 (-1)	-	3.5	0.76 (≥3)
44	4	1.14 (-1)	0.92 (1)	4.9	4.8 (≥4)
45	4	1.26 (-1)	1.07 (1)	3.3	8.4 (≥4)
46	2	0.99 (-1)	0.84 (1)	2.5	2.6 (≥2)
47	4	0.95 (-1)	0.97(1),1.17(4)	2.4	1.7 (2)
48	4	0.98 (-1)	-	2.7	6.8 (≥4)
49	3	1.17 (-3)	1.18 (1)	3.2	5.0 (1)

Table 28 continued.

50	5	-	0.91 (1)	4.3	4.3 (1)
51	6	0.99 (-1)	0.94 (5)	2.5	1.4 (≥6)
52	4	1.34 (-2)	1.21 (1)	15	30 (2)
53	7	-	1.15 (1)	2.9	5.9 (1)
54	7	-	-	0.83	6.8 (2)
55	4	1.13 (-1)	0.88 (4)	5.1	4.9 (≥4)
56	6	1.08 (-3)	1.02 (3)	0.79	34 (3)

a) = No. of days after surgery that urine samples were taken.

b) = Minus numbers denote days prior to surgery, day 0 = day of surgery, positive numbers denote days after surgery.

c) = The peak excretion of magnesium post-suirgery is quoted with the day after surgery when this peak occurred.

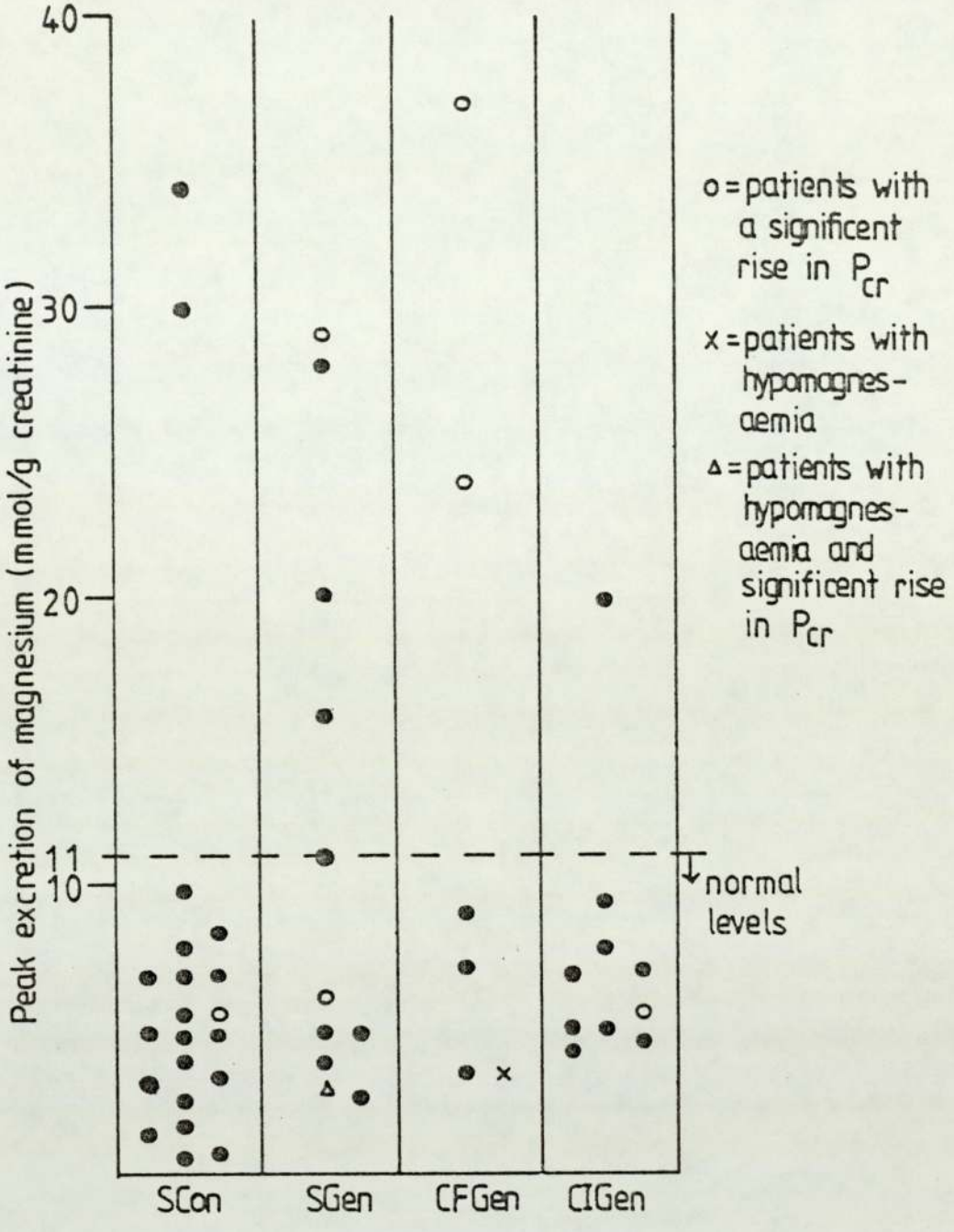


FIGURE 23: Peak urinary excretions of magnesium in the three sub-groups of gentamicin-treated patients and post-surgical peak excretions of magnesium in control patients. SCon=surgical control patients; SGen=gentamicin-treated surgical patients; CFGen=gentamicin-treated cystic fibrosis patients; CIGen=gentamicin-treated patients with chest infection etc.
(patient 21 excluded)

magnesium excretion was established as mean + 2SD = 11 mmol/g creatinine. Urinary magnesium excretion was not raised above normal in either of the two patients with hypomagnesaemia. Of the patients who did not develop hypomagnesaemia, urinary excretion of magnesium was above normal in eight patients, 2,4, 14,16,21,22,23 and 25. Therefore both calcium and magnesium excretion were raised in patients 4,14,21,22,23 and 25.

Figure 23 shows the peak excretion values for magnesium in the three subgroups of gentamicin-treated patients (excluding patient 21), and the post-surgical peak excretion of magnesium in the control group. Again there is a lot of overlap between the range of peak excretions in the four groups. The highest peak excretion values are again for the surgical and cystic fibrosis patients. The control patients all showed normal excretions of magnesium, except for one patient. The eight gentamicin-treated patients who showed raised excretion of magnesium included four surgical patients, two cystic fibrosis patients, and two chest infection etc. patients.

The highest peak excretion of magnesium (excluding patient 21) was exhibited by patient 22, a cystic fibrosis patient who showed a significant rise in P_{cr}, but did not develop hypomagnesaemia. Patients 16 and 23, who had shown a significant rise in P_{cr}, but did not develop hypomagnesaemia, also showed a raised peak excretion of magnesium. None of the other patients who had shown a significant rise in P_{cr}, or had developed hypomagnesaemia, showed a raised peak excretion of magnesium.

E: Comparison of calcium and magnesium excretion in gentamicin-treated patients.

In most gentamicin-treated patients, calcium and magnesium excretion seemed to follow a very similar course, with magnesium

excretion being higher in 50% of patients, and calcium excretion being higher in the other 50%. In some patients magnesium excretion was greatly raised above calcium excretion, see graph of patients 15 and 16, (figure 25). The graphs of calcium and magnesium excretion in patients 13 and 25 (see Figure 24) show the excretion of calcium and magnesium following a similar pattern.

The peak in magnesium excretion occurred before the peak in calcium excretion in 11 of the 28 patients, the peaks occurred on the same day in 11 patients and the peak in calcium excretion occurred first in six patients. Patients on shorter courses of therapy tended to show just one peak in excretion of calcium and magnesium, for example, patients 15 and 16 (see Figure 25). Patients on longer courses of therapy, however, tended to show two or more peaks, for example, patient 25 (see Figure 24).

F: Plasma calcium and magnesium data and urinary excretion of calcium and magnesium in surgical control patients

Table 27 shows the plasma and urinary calcium data for the surgical control patients. Prior to surgery, none of the patients in whom plasma samples were available, showed hypocalcaemia. Post-surgery, however, seven of the 17 patients in whom plasma samples were available had developed some degree of hypocalcaemia. This hypocalcaemia occurred immediately after surgery in most cases. Hypocalcaemia was very mild in each case, the lowest plasma calcium being 1.78 mmol/l for patient 47. In another four patients plasma calcium decreased after surgery, but did not fall below normal levels.

In two patients, more than one post-surgery plasma sample was available. In patient 37, plasma calcium decreased from 2.30 mmol/l

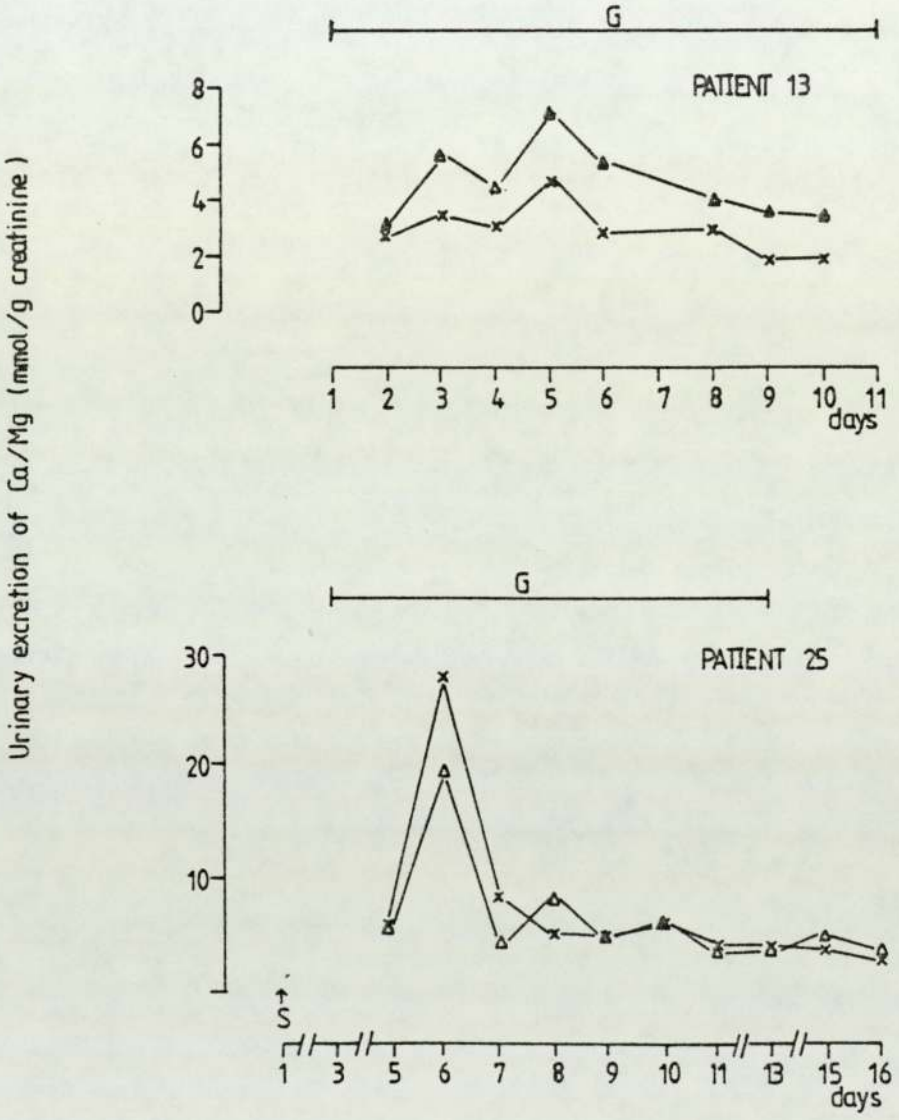


FIGURE 24: Urinary excretion of calcium and magnesium by patients 13 and 25. S=surgery, G=gentamicin therapy. ×=calcium, △=magnesium.

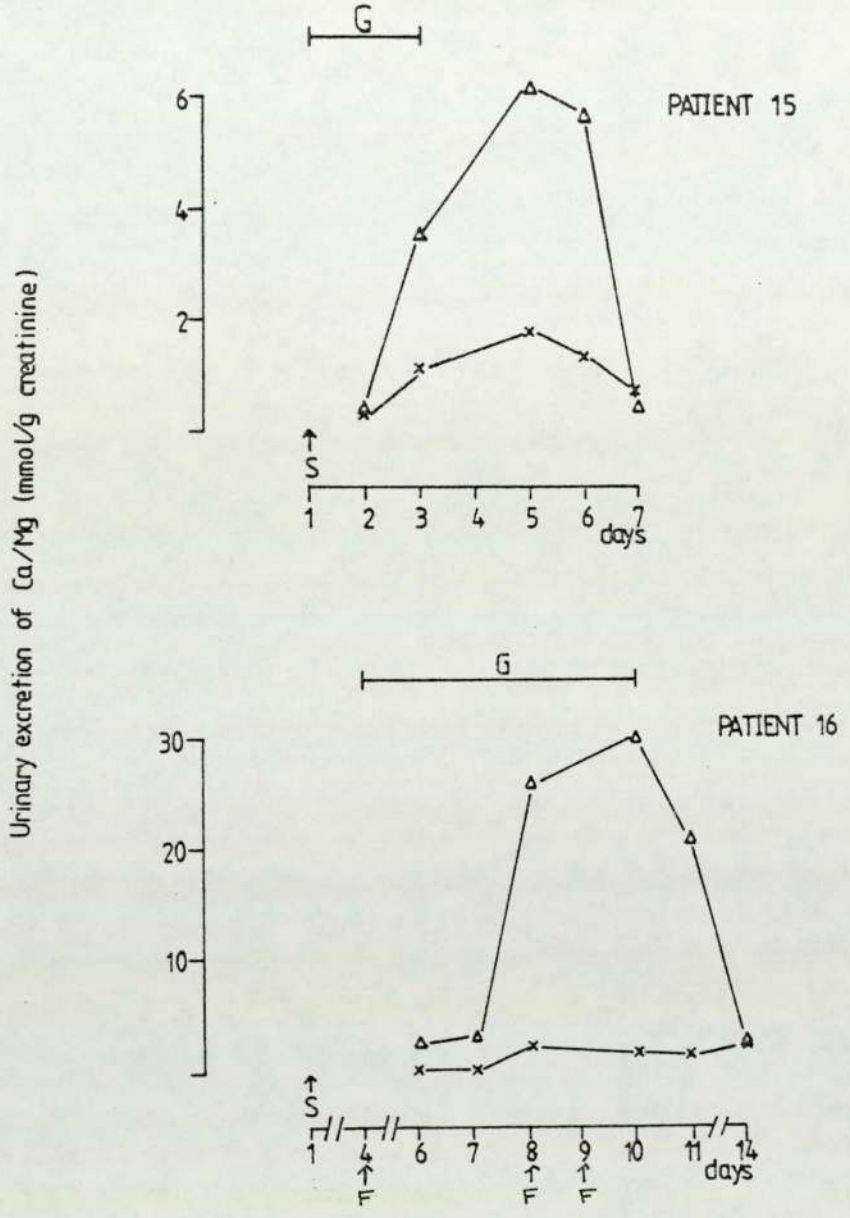


FIGURE 25: Urinary excretion of calcium and magnesium by patients 15 and 16. S= surgery, G=gentamicin therapy, F= frusemide. × = calcium , △ = magnesium.

two days before surgery, to 1.79 mmol/l one day after surgery, and then recovered to 2.20 mmol/l on day 5 after surgery. In patient 47, plasma calcium decreased from 2.18 mmol/l on the day before surgery, to 2.05 mmol/l one day after surgery then decreased further to 1.78 mmol/l four days after surgery.

Graphs of urinary excretion of calcium and magnesium in all control patients are shown in Appendix VI. Urinary excretion of calcium was within normal limits in all control patients prior to surgery. Post-surgery, peak urinary excretion of calcium was raised above normal in only one patient, 56, who showed a peak urinary excretion of 18 mmol/g creatinine on day 3 after surgery. This patient did not, however, develop hypocalcaemia.

Six of the seven patients who developed hypocalcaemia after surgery (36,37,40,44,47 and 52) did not show a rise in urinary calcium excretion after surgery. Therefore, it would appear that the hypocalcaemia which had developed after surgery was not caused by an increase in calcium excretion.

It can be seen from Figure 22 that post-surgical peak excretions of calcium are clustered below 10mmol/g creatinine in most control patients, whereas peak excretions for the gentamicin-treated groups cover a wider range of values. The mean post-surgical peak excretion (±SD) of calcium in the control group was 6 ± 4 mmol/g creatinine which was lower than that for the gentamicin-treated group of 11 ± 11 mmol/g creatinine.

Table 28 shows the plasma and urinary magnesium data for the control patients. Prior to surgery, none of the patients in whom plasma samples were available, showed hypomagnesaemia. Post-surgery, none of the patients showed hypomagnesaemia, but plasma magnesium decreased after surgery in eight of the 10 patients in whom both pre- and post-surgery samples were available.

Pre-surgery excretion of magnesium was above normal in just one patient, 52. Post-surgery peak excretion of magnesium was above normal in patients 52 and 56, who showed peaks of 30 and 34 mmol/g creatinine, on days 2 and 3 after surgery respectively.

Four of the eight patients in whom plasma magnesium decreased after surgery, showed an increase in urinary excretion of magnesium, but only two patients (52 and 56) showed a great increase in excretion. Therefore, it is possible, in these cases, that the decrease in plasma magnesium may have been due to renal wasting of magnesium.

From Figure 23 it can be seen that the post-surgical peak excretions of magnesium are all clustered below 10 mmol/g creatinine, except for two patients. The mean post-surgical peak excretion (± SD) for the control group was 7 ± 9 mmol/g creatinine, which is less than that for the gentamicin-treated group of 11 ± 9.

The graphs of calcium and magnesium excretion for the control group show that, in most cases, the excretion of the two electrolytes followed a similar pattern. The excretion of both calcium and magnesium seemed to decrease immediately after surgery in most patients, then increased towards or above pre-surgery excretion levels. Graphs of calcium and magnesium excretion in patients 36 and 44 (see Figure 26) demonstrate this point.

G: Calcium and magnesium excretion in the various subgroups of gentamicin-treated patients

i) Calcium and magnesium excretion in patients treated post-surgically with gentamicin (within three days of surgery).

Table 29 shows the peak urinary excretion data for calcium and magnesium in patients receiving gentamicin post-surgically. Appendix

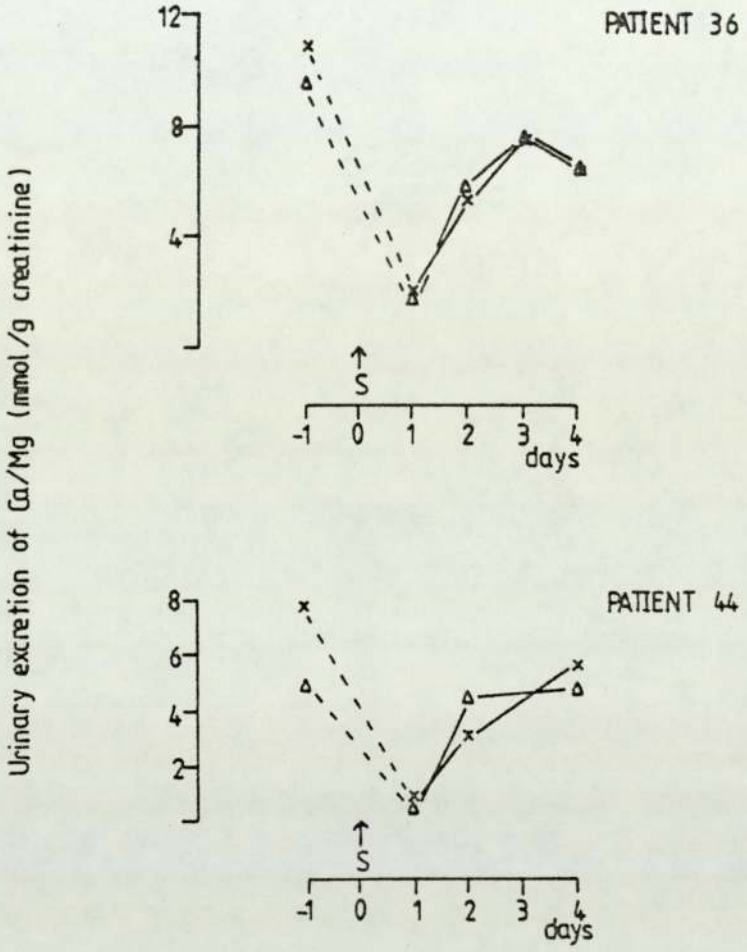


FIGURE 26: Urinary excretion of calcium and magnesium by patients 36 and 44. S = surgery. × = calcium, △ = magnesium.

Table 29. Peak Urinary Excretion data for Calcium and Magnesium in Patients Receiving Gentamicin Post-surgically (within 3 days of surgery).

Patient	a) Days of surgery	Duration of therapy	Peak excretion Ca (mmol/g creatinine)	Day of peak	Peak Excretion Mg(mmol/g creatinine)	Day of peak
3	1	5	0.94	⩽ 3	3.8	⩽ 3
4	1	6	35	8	16	⩾10
8	1	5	6.2	8	2.9	5
10	1	4	2.9	⩾10	2.8	7
12	-1	7	1.5	7	11	5
14	8	17	22	14	28	14
15	1	2	1.8	5	6.1	5
16	-3	6	2.3	5	29	7
17	1	3	2.8	7	4.8	7
25	-2	10	28	4	20	4
29	1	6	4.1	8	5.0	4

a) Day of surgery. Minus numbers denote days before gentamicin therapy. Day 1 = First day of gentamicin therapy.

VII shows the graphs of calcium and magnesium excretion for this group of patients.

Calcium excretion was above normal levels in three of the 11 patients; 4,14 and 25. Patients 14 and 25 both received courses of gentamicin therapy of $\geq$ 7 days durations. Patient 4 received a 6 day course of gentamicin therapy. From Figure 22 it can be seen that calcium excretion was < 10 mmol/g creatinine for the rest of the group.

The mean peak excretion (±SD) of calcium in the gentamicin treated surgical group was 10 ± 12 (range 0.94 - 35 mmol/g creatinine). This result was similar to the mean peak excretion of calcium for the whole group of gentamicin-treated patients of 11 ± 11 mmol/g creatinine.

The peak in calcium excretion occurred on day 4-7 of gentamicin therapy in six patients and day $\geq$8 in four patients. Therefore, the peak in calcium excretion did not usually occur in the first few days of therapy and thus did not appear to be directly associated with surgery.

Magnesium excretion was greater than normal in four patients; 4,14,16 and 25. Patient 16 received a 6 day course of gentamicin therapy. The mean peak excretion (± SD) of magnesium in this group was 12 ± 10 mmol/g creatinine (range 2.8 - 29 mmol/g creatinine), which was very similar to the mean for the whole group of gentamicin-treated patients, of 11 ± 9 mmol/g creatinine.

The peak excretion of magnesium occurred on day 4-7 in eight patients, and on day $\geq$8 in two patients. Therefore, it appeared that the peak in magnesium excretion was not directly associated with surgery.

Patients 4,14 and 25, therefore, showed a raised excretion of both calcium and magnesium during gentamicin therapy. Patient 14

(see Figure 27) showed multiple peaks in both calcium and magnesium excretion after surgery, the largest peak occurring on day 14 of gentamicin therapy (6 days after surgery). The graph of patient 25 (see Figure 24) shows a large peak in both calcium and magnesium excretion on day 4 of therapy (5 days after surgery) followed by multiple smaller peaks. The graph of patient 4 (see Figure 27) shows the peaks in calcium and magnesium excretion occurring after the end of a 6 day course of gentamicin therapy, 7 and 8 days after surgery respectively.

ii) Calcium and magnesium excretion in cystic fibrosis patients treated with gentamicin for chest infection.

Table 30 shows the peak urinary excretion data for calcium and magnesium in the four cystic fibrosis patients (six patient courses) treated with gentamicin. Appendix VIII shows the graphs of calcium and magnesium excretion in this group.

Two patients courses, 22 and 23, showed a raised peak excretion of calcium. Both of these courses of gentamicin therapy were of > 10 days duration. The mean peak calcium excretion (+SD) in this group was 15 ± 14 mmol/g creatinine (range 1.9-39 mmol/g creatinine). This was slightly higher than the mean peak excretion for the whole group of gentamicin-treated patients. The peak in calcium excretion occurred on day 4-7 in four of the six patient courses.

Magnesium excretion was also raised above normal in patients 22 and 23. The mean peak excretion (± SD) of magnesium in this subgroup was 15 ± 13 mmol/g creatinine, which was slightly higher than that for the gentamicin-treated group as a whole. The peak in magnesium excretion was on day 4-7 in three of the patient courses.

The graphs of calcium and magnesium excretion during patient courses 9 and 22, which were consecutive courses of gentamicin

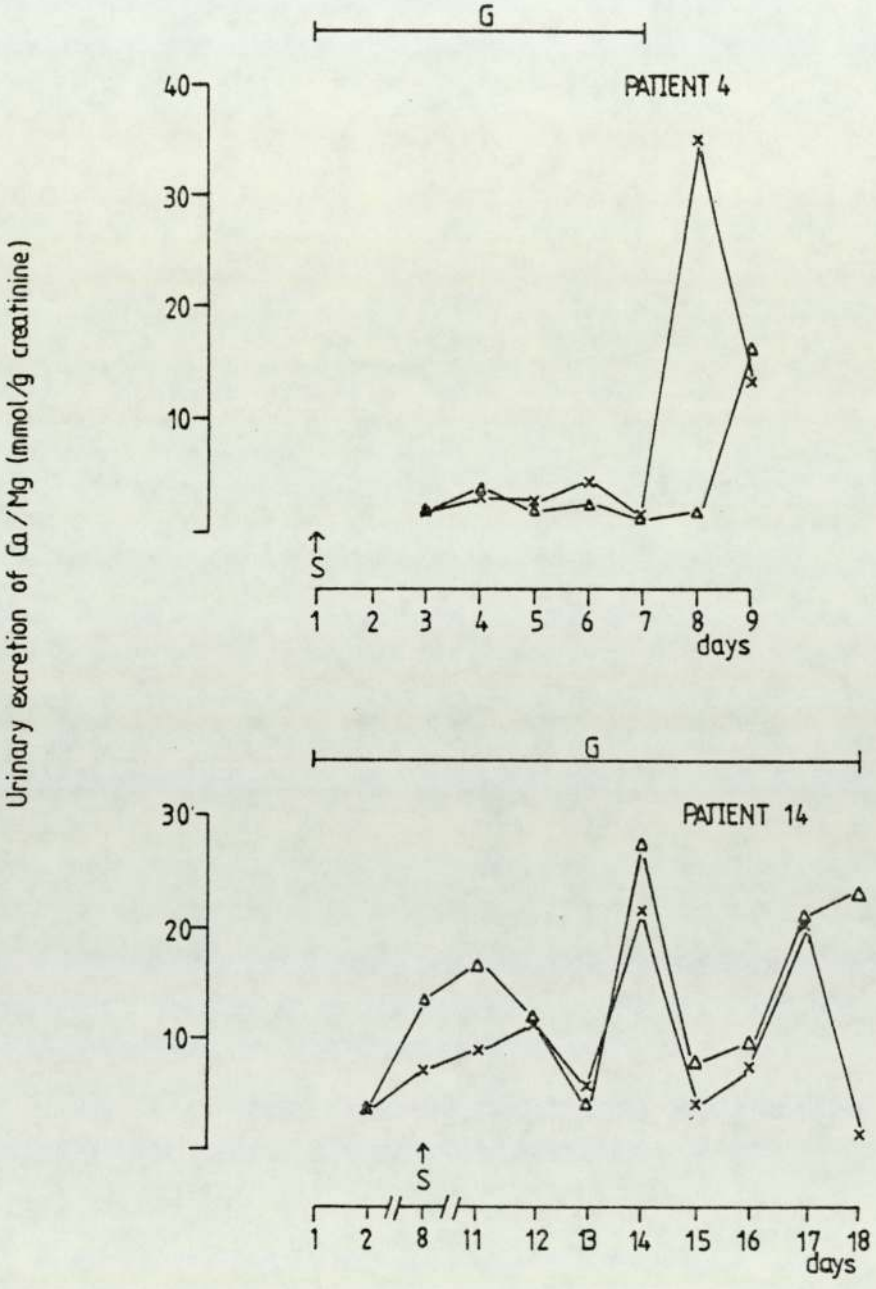


FIGURE 27: Urinary excretion of calcium and magnesium by patients 4 and 14. S = surgery, G = gentamicin therapy. × = calcium, △ = magnesium.

Table 30. Peak Urinary Excretion data for Calcium and Magnesium in Cystic Fibrosis Patients Treated with Gentamicin.

Patient courses	Duration of therapy (days)	Peak excretion Ca.(mmol/g creatinine)	Day of peak	Peak excretion Mg (mmol/g creatinine	Day of peak
* 9	3	1.9	4	9.1	6
*22	11	25	7	37	6
**13	10	4.6	5	7.1	3
**20	10	8.4	8	3.8	8
18	2	14	≥7	7.8	≥7
23	14	39	8	24	8

* Patient courses 9 and 22 are two separate courses of gentamicin therapy in the same patient.

** Patient courses. 13 and 20 are two are two separate courses of gentamicin therapy in the same patient.

therapy in the same patient (separated by four months), are shown in Figure 28. The graph of course 9 shows that both calcium and magnesium excretion remained within normal limits in this patient. The graph of course 22 shows a large peak in both calcium and magnesium excretion early in therapy, then a larger peak of 37 mmol/g creatinine of magnesium and 25 mmol/g creatinine of calcium on day 7 of therapy. Therefore, the excretion of both calcium and magnesium was greater in the second course of therapy. The first course of therapy, however, was only of three days duration, whereas the second course was of 10 days duration. The graph of calcium and magnesium excretion for patient 23 (see Figure 28) shows calcium and magnesium excretion rising to peaks of 39 mmol/g creatinine and 24 mmol/g creatinine, respectively, on day 8 of the 14 day course of gentamicin therapy.

iii) Calcium and magnesium excretion in patients treated with gentamicin for chest infection, wound infection, urinary tract infection, etc.

Table 31 shows peak urinary excretion data for calcium and magnesium in patients treated with gentamicin for chest infection, wound infection, urinary tract infection etc. Appendix IX shows the graphs of calcium and magnesium excretion in these patients.

Excluding patient 21, for reasons mentioned under 'urinary excretion of calcium in gentamicin-treated patients', urinary excretion of calcium was greater than normal in two patients, 5 and 42. Patient 5 received gentamicin pre-operatively for seven days, finishing therapy a few days before an operation. Patient 42 received four days of gentamicin therapy for a wound infection.

The mean peak excretion (± SD) of calcium in this group (excluding patient 21) was 10 ± 5 mmol/g creatinine. This was only

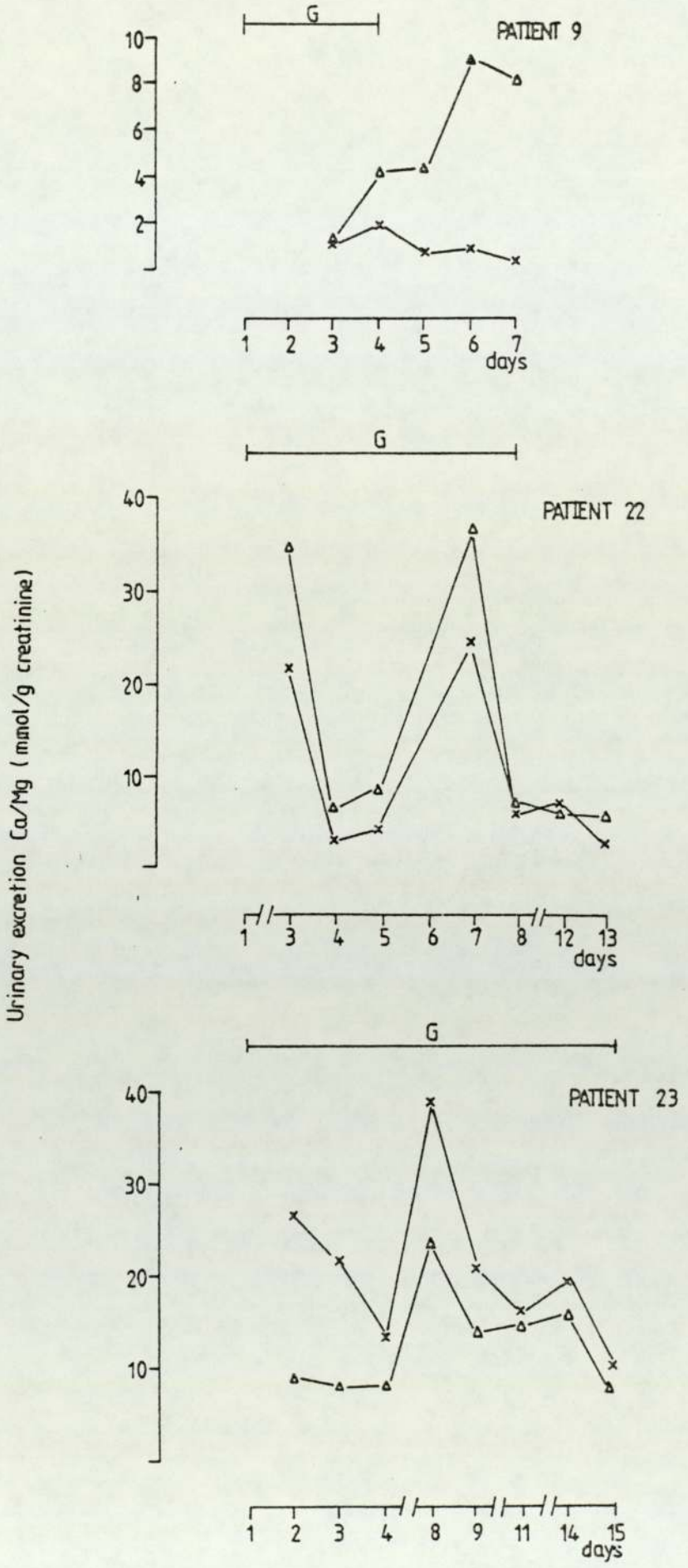


FIGURE 28: Urinary excretion of calcium and magnesium by patients 9, 22 and 23. G=gentamicin therapy. ×=calcium, △=magnesium

Table 31. Peak Urinary Excretion data for Calcium and Magnesium in Patients Receiving Gentamicin for Chest Infection (not cystic fibrosis patients), Urinary tract infection, Wound infection, Pre-operatively or for Septicaemia (unknown cause).

Patient	Duration of of therapy	Illness	Peak excretion of Ca (mmol/g creatinine)	Day of peak	Peak excretion of Mg (mmol/g creatinine)	Day of peak
2	14	C.I.	14	$\geq$17	20	13
21	2	C.I.	550	4	108	4
30	4	C.I.	12	$\geq$ 6	5.1	2
6	4	U.T.I.	13	$\geq$ 8	7.9	$\geq$ 8
26	5	U.T.I.	9.6	$\geq$ 6	7.1	5
28	6	U.T.I.	5.2	1	4.5	3
11	4	W.I.	2.6	$\geq$ 8	4.4	4
42	4	W.I.	15	3	9.6	3
43	4	W.I.	12	7	4.9	$\leq$ 3
5	7	Pre-operatively	18	7	7.0	7
19	6	Septicaemia	2.8	4	5.4	5

C.I. = Chest infection

U.T.I. = Urinary tract infection

W.I. = Infected wound from recent surgery (more than 3 days before gentamicin therapy commenced).

slightly lower than the mean peak excretion of calcium for the whole of the gentamicin-treated group. The peak in calcium excretion occurred on day $\leq$ 3 of gentamicin therapy in four patients, on day 4-7 in three patients and on day $\geq$8 in four patients. Therefore, there seemed no common pattern as to when the peak occurred.

Excluding patient 21, urinary excretion of magnesium was greater than normal in only one patient: 2. This patient had a chest infection and received 14 days of gentamicin therapy.

The mean peak magnesium excretion (± SD) for this group was 8 ± 5 mmol/g creatinine, which was lower than that for the group as a whole. The peak in magnesium excretion occurred on day 1-4 of gentamicin therapy in nine of the 11 patients. Patient 2 showed multiple peaks in calcium and magnesium excretion, the highest peak in magnesium excretion occurring on day 13 of the therapy and the highest peak in calcium excretion occurring on day $\geq$ 17. The peak in magnesium excretion was above normal limits. The other two patients with chest infection, 21 and 30, showed calcium and magnesium excretion within normal limits.

All three patients with U.T.I. (6,26 and 28) showed excretion of calcium and magnesium within normal limits.

Patients 11 and 43, who were treated with gentamicin for wound infection, showed excretion of calcium and magnesium within normal limits. Patient 42 showed a normal excretion of magnesium but calcium excretion rose to a peak of 15 mmol/g creatinine on day 3 of a 4 day course of gentamicin therapy.

Magnesium excretion for patient 5 was within normal limits, but calcium excretion rose to a peak of 18 mmol/g creatinine on the last day of a 7 day course of gentamicin therapy. Patient 19 shoed normal excretions of calcium and magnesium.

DISCUSSION

Discussion of the RBP and Lysozyme Results

The urinary excretions of both RBP and lysozyme were raised in most gentamicin-treated patients, whether or not there was a significant rise in P_{cr}. In some patients who showed no increase in P_{cr}, excretion of one or both proteins was raised to over one hundred times the normal values. Therefore, monitoring the urinary excretion of RBP and lysozyme failed to identify those patients who developed increases in P_{cr} during gentamicin therapy. There were large inter individual variations in excretion of both proteins, patients with similar conditions and durations of gentamicin therapy showing entirely different patterns and ranges of excretion of these proteins.

There was some evidence that the size and timing of the peaks in RBP and lysozyme excretion were related to the duration of gentamicin therapy. There appeared to be a relationship between duration of therapy and peak of excretion of these proteins, patients receiving longer courses of therapy tending to show longer and later peaks in excretion. Patients receiving longer courses of therapy also tended to show multiple peaks in excretion of both RBP and lysozyme. As timing of peaks was quite variable for both proteins, occasional daily urine collections which were missed may have led to an underestimation of the peak excretion of these proteins in some patients. The results of a previous study had shown that missing one days urine collection could lead to a 34% underestimation of the peak excretion of lysozyme[104].

The excretion of RBP was raised to a greater extent than the excretion of lysozyme in many gentamicin-treated patients. This observation was unexpected, as the tubular reabsorption of the cationic lysozyme has been shown to be competitively inhibited by the

cationic gentamicin[10]. As RBP is anionic (pI 4.4-4.8)[51], it should not have its tubular uptake appreciably inhibited by gentamicin, as it should not compete with gentamicin for anionic uptake sites on the brush border membrane. In a study carried out in rats, it was found that urinary excretion of low molecular weight proteins during gentamicin therapy was dependent on the electrical charge of the proteins. The cationic protein B_2-m was excreted to a much larger extent than the anionic RBP, during gentamicin administration[51]. As the excretion of RBP and lysozyme differed in magnitude in many patients in the present study, it is possible that the excretions of these two proteins are mediated by different processes. The relative excretions of these two proteins did not depend on filtered load, as the filtered load of RBP was almost always greater than that of lysozyme, even when the urinary excretion of lysozyme was much greater than that of RBP.

RBP excretion rose above normal values after surgery in most control patients, although only one of these patients (37) had shown a significant rise in P_{cr}. Only patient 37, however, showed a post-surgery peak excretion of RBP of 100 mg/g creatinine. Lysozyme excretion rose above normal levels after surgery in less than half the control patients, only two patients showing a peak excretion of >100 mg/g creatinine; patient 38 in whom P_{cr} values were not known and patient 37. The peak in excretion of both RBP and lysozyme tended to occur in the first few days after surgery. Lysozyme excretion has been reported to increase in many conditions including post-operative collapse, uncomplicated surgical procedures and severe extrarenal infection[53]. The excretion of B_2-m has also been found to rise due to surgery or trauma, the increase in excretion only lasting for one to three days after surgery and thought to be due to decreased tubular reabsorption or increased glomerular filtration of

B_2-m[105]. Plasma RBP has been found to decrease after surgery[106] and during infections[107]. This decrease in plasma RBP may be due to decreased synthesis of RBP or increased urinary excretion.

Some control patients and most gentamicin-treated patients had some form of infection. 'Febrile proteinuria' ie proteinuria occurring in generalised sepsis, has been previously reported[108-110] and may be glomerular and/or tubular in origin. Tubular proteinuria has been reported to be of short duration, disappearing once the fever subsides, whereas glomerular proteinuria may persist for one or two weeks[108]. It is thought that this tubular proteinuria may be due to a transient impairment of tubular reabsorptive capacity[108]. One study found that urinary lysosyme excretion was raised in nine of 39 patients with an infectious disease, whereas none of the control patients (who did not have an infectious disease) showed a rise in excretion[109]. Another study found an increased urinary excretion of B_2-m in generalised sepsis and also found another, smaller, increase in excretion of B_2-m in non-septic patients who were critically ill[111]. To further investigate this phenomenon, an animal model of generalised sepsis was developed in the sheep. Electron microscopy of the kidney showed a normal glomerulus, but that 30-50% of the proximal tubular cells were abnormal. Abnormalities included cell swelling, loss of brush border, protrusion of cytoplasm into the lumen, endocytosis and swollen mitochondria[111]. It is not known how non-renal infections may cause this damage to proximal tubular cells and subsequent low molecular weight proteinuria. It has been suggested that the non-renal infections may induce transient immunological or haemodynamic disorders of the kidneys[110]. It is also possible that the infective agents themselves directly cause damage to the proximal tubular cells.

The highest urinary excretions of RBP and lysozyme occurred in

the gentamicin-treated surgical group of patients. The peak in excretion of both proteins usually occurred soon after surgery, suggesting that the rise in excretion was associated with surgery. Three patients showed peak excretions of both proteins which were >100 mg/g creatinine. All three of these patients had serious infections, had had major surgery and were critically ill. Only one of these patients, however, had shown a significant increase in P_{cr}. Therefore, it is possible that the surgery, illness and infection all contributed to the proximal tubular damage or dysfunction which was further potentiated by the gentamicin therapy. Gentamicin itself has been found to cause some degree of proximal tubular damage in virtually all patients receiving therapeutic doses[112].

Gentamicin treated patients with cystic fibrosis showed very low excretions of RBP and lysosyme, compared to other gentamicin-treated patients, even though two cystic fibrosis patients had shown a significant increase in P_{cr} (which in one case was thought to be attributable to gentamicin nephrotoxicity). Excretions of both proteins were normal or only slightly raised in all but one patient (patient 20 showed a large rise in lysosyme excretion), despite relatively long courses of gentamicin therapy. It is not known why these patients did not develop a greater degree of tubular proteinuria, unless due to some difference in the renal handling of these proteins by patients with cystic fibrosis. It is also possible that these patients were somehow resistant to the nephrotoxic action of gentamicin, as all patients with cystic fibrosis were receiving regular courses of aminoglycosides. Plasma RBP has also been reported to be low in cystic fibrosis patients[113,114]. The cystic fibrosis patients in the current study also showed relatively low plasma levels of RBP compared to other patients. Therefore it is possible that urinary excretion of RBP is decreased in these patients due to low plasma levels.

Discussion of the Calcium and Magnesium Results.

Hypomagnesaemia developed in just two of the 28 gentamicin-treated patients (one surgical patient and one patient with cystic fibrosis) and was very mild in each case. Hypomagnesaemia did not appear to be related to duration of gentamicin therapy or to the renal excretion of magnesium, as both patients who developed hypomagnesaemia had short courses of therapy and showed low urinary excretions of magnesium. The surgical patient who developed hypomagnesaemia had shown a significant rise in P_{cr}, but if hypomagnesaemia had been due to gentamicin nephrotoxicity, renal wasting of magnesium would have been expected[78-82,115-116]. Therefore, hypomagnesaemia in this patient was probably due to reduced dietary intake of magnesium around the time of surgery or a metabolic response to surgical trauma. It was not known why hypomagnesaemia should develop in the patient with cystic fibrosis, unless due to poor nutritional status commonly associated with cystic fibrosis. It has been reported that poor diet prior to gentamicin therapy can predispose patients to hypomagnesaemia[82]. Hypomagnesaemia has been previously reported in aminoglycoside-treated patients with cystic fibrosis and was thought to be due to such predisposing factors as recurrent courses of aminoglycoside therapy, increasing renal sodium load and food manabsorption (occurring in most cystic fibrosis patients)[117].

Mild hypocalcaemia developed in five of the gentamicin-treated patients (three surgical patients and two patients with cystic fibrosis), only one of whom had hypomagnesaemia. Whereas in previous studies hypocalcaemia had been thought to occur secondary to gentamicin-produced hypomagnesaemia[78-82,115,116], in this study hypocalcaemia occurred in the absence of hypomagnesaemia. Hypo-

calcaemia did not appear to be related to duration of gentamicin therapy or urinary excretion of calcium in most cases. One of the cystic fibrosis patients (patient 23) had shown a very high excretion of calcium and another (patient 18) had shown an excretion that was just below the upper limit of normal, therefore it is possible that hypocalcaemia was due to renal wasting of calcium in these patients. Patient 23 had also shown a significant increase in P_{cr}, which may have been due to gentamicin nephrotoxicity, therefore it is possible that renal wasting of calcium was due to gentamicin nephrotoxicity in this patient. Poor nutritional status may be a factor leading to hypocalcaemia in the patients with cystic fibrosis. In the gentamicin-treated surgical patients, hypocalcaemia may be related to poor dietary intake of calcium at around the time of surgery or associated with metabolic changes due to surgery.

The occurrence of hypomagnesaemia in this study was much less than reported in one study, where hypomagnesaemia developed in more than one third of patients treated with normal therapeutic doses of aminoglycosides[82]. However, most reports of hypomagnesaemic hypocalcaemia associated with aminoglycosides have involved patients receiving chronic, high-dose or repeated treatment[78-81,115,116]. As none of the patients in this study received gentamicin-therapy for longer than 17 days (most courses being less than 7 days duration) and no patient received high-dose therapy, perhaps hypomagnesaemic hypocalcaemia should not be expected in these patients.

None of the surgical control patients developed hypomagnesaemia prior to surgery. After surgery, although no patients had developed clinical hypomagnesaemia, plasma magnesium levels had decreased in most patients. This decrease was again probably due to decreased intake of magnesium caused by the relative starvation for two or more

days around the time of surgery. One study reported a lowered plasma magnesium in 56% of patients after surgery, thought to be due to dietary restrictions, which usually corrected itself by the 2nd or 3rd post-operative day[118].

After surgery, only two surgical control patients showed an excretion of magnesium which was raised above normal. Many patients do, however, show an increase in magnesium excretion after surgery. In most surgical control patients, excretion of magnesium decreased immediately after surgery, then rose towards or above pre-surgery levels. This pattern of excretion is similar to that reported in a previous study of magnesium excretion, where urinary excretion of magnesium decreased on the day of operation, then rose to a maximum on the first or second post-operative day before steadily declining[118]. In another study, it was reported that magnesium excretion decreased on the day of operation, returned to normal excretion on the first day post-operatively, then was raised from the second to the fifth or sixth post-operative day[119]. This increase in the urinary excretion of magnesium post-surgery was unexpected during a period of deprivation of dietary magnesium and was therefore thought to be associated with the surgical trauma.

None of the surgical control patients showed hypocalcaemia prior to surgery, whereas seven patients had developed mild hypocalcaemia after surgery and plasma calcium had decreased in most patients. This decrease may have been due to poor dietary intake of calcium at the time of surgery or metabolic changes associated with surgical trauma. Urinary calcium excretion in surgical control patients followed a similar pattern to magnesium excretion, in most patients decreasing immediately after surgery then rising up to or above pre-surgical levels. Urinary excretion of calcium was not raised above normal

levels in any control patients prior to surgery and in only one patient post-surgery.

In most gentamicin-treated patients, urinary excretion of calcium and magnesium followed a similar course. Urinary excretion of both electrolytes appeared to be greater in most cases than for the surgical control patients. Most patients showed a peak in excretion of both calcium and magnesium on day 4-7 of gentamicin therapy. A previous study in which patients had received normal therapeutic doses had shown a small transient rise in urinary excretion of calcium and magnesium between days 3 and 7 of gentamicin therapy[120]. However, most patients in the current study showed little evidence of the large rise in calcium excretion reported to occur in rats treated with aminoglycosides[87]. It has been postulated that the increase in magnesium excretion in aminoglycoside-treated patients is due to aminoglycosides disturbing the reabsorption of magnesium in the renal tubules[82].

The largest excretions of calcium and magnesium were exhibited by patients in the gentamicin-treated surgical and cystic fibrosis groups. The two cystic fibrosis patients (22 and 23) who showed raised excretion of magnesium and calcium also showed a significant increase in P_{cr}, but this rise in P_{cr} was only thought to be due to gentamicin nephrotoxicity in patient 23, therefore it is possible that gentamicin nephrotoxicity may have caused the renal wasting of calcium and magnesium in this patient. It is not known why else cystic fibrosis patients should show such high excretions of these electrolytes, unless due to the disease state. A previous study had, however, reported no differences in the renal handling of calcium between normal children and children suffering from cystic fibrosis[121].

Four gentamicin-treated surgical patients had shown raised excretions of magnesium (three of whom had also shown raised excretions of calcium). The peak excretion of both electrolytes occurred on day 4-7 of gentamicin therapy in most surgical patients, which was similar to the pattern of excretion in surgical control patients. As the mean peak excretion for the gentamicin-treated surgical group was higher than than for the control surgical group, it appears that gentamicin accentuates the increase in excretion normally seen in patients after surgery.

There are many other factors which should be considered when examining calcium and magnesium excretion in patients. Variables such as age, sex, dietary intake of calcium and magnesium and exercise should be taken into account, as all these factors may influence excretion of calcium and magnesium[122]. The diet of each patient is different and patients get varying amounts of exercise. While urinary calcium has been found to be reduced by exercise[123], it has been found to markedly increase with continuous supine beadrest[124]. As many of the patients studied were bed-bound for most of the time, this may have increased their calcium excretion. Many patients also received frusemide, a loop diuretic which is known to increase the urinary excretion of these electrolytes[125,126]. Therefore, for the results of this type of study to be really meaningful, diet, exercise and drug therapy should be controlled in these patients. Plasma levels of parathyroid hormone also should be measured, as this can affect the homeostasis of calcium and magnesium[127-129].

CONCLUSIONS

Conclusions of the RBP/lysosyme study.

There are several complicating factors involved when trying to evaluate potential urinary markers of drug-induced proximal tubular damage. Firstly, the relationship between proximal tubular damage and clinically apparent nephrotoxicity (as determined by an increase in P_{cr}) has not yet been fully established. Secondly, the loss of these potential markers in the urine may be due to other caused than drug-induced proximal tubular damage. From the results of this study, it appears that neither lysosyme nor RBP are entirely suitable to use as urinary markers of aminoglycoside-induced nephrotoxicity in clinical practice, as their appearance in urine may be due to a variety of factors such as surgery, trauma, infection, critical illness or merely being treated with aminoglycosides. As one or more of these factors are always present in patients treated with aminoglycoside antibiotics, these low molecular weight proteins would not prove to be reliable indicators of aminoglycoside nephrotoxicity.

Conclusions of the Calcium/Magnesium Study.

Hypomagnesaemic hypocalcaemia did not appear to be a feature of gentamicin treatment in the patients involved in this study. Hypocalcaemia developed in more patients than did hypomagnesaemia and only one patient developed both conditions. Hypocalcaemia may have been caused by the renal wasting of calcium in some cases, but did not appear to be associated with duration of gentamicin therapy or increased plasma creatinine. Hypomagnesaemia did not seem to be associated with renal wasting of magnesium, the duration of gentamicin therapy or increased plasma creatinine.

Hypomagnesaemia did not occur after surgery in control patients, but a decrease in plasma magnesium commonly occurred. Hypocalcaemia developed in some control patients after surgery and a decrease in plasma calcium frequently occurred. Therefore, when investigating plasma calcium and magnesium levels in gentamicin-treated patients who have undergone surgery, the decrease in the plasma levels of these electrolytes associated with surgery should be taken into account.

Urinary excretions of calcium and magnesium were usually higher in gentamicin-treated patients than control patients. Calcium and magnesium excretion followed a similar pattern of excretion in gentamicin-treated patients, rising to a peak on day 4-7 of therapy. However, when evaluating urinary excretion of these electrolytes in gentamicin-treated patients who have undergone surgery, the increase in the urinary excretion of these electrolytes following surgery should be taken into consideration.

It is difficult to draw any definite conclusions as to the effect of gentamicin therapy on the homeostasis of calcium and magnesium due to the many complicating factors influencing these parameters.

APPENDIX

PATIENT NAME/STUDY NO.	HOSPITAL NO.	WARD
DATE	PATIENT NO.	FIRM/CONSULTANT
AGE	SEX	BODY WT (KG)

DOSE OF AMINO-GLYCOSIDE	FREQUENCY	DATE	DAYS OF COURSE

DIAGNOSIS/HISTORY(PREV. AMINOGLYCOSIDE THERAPY)

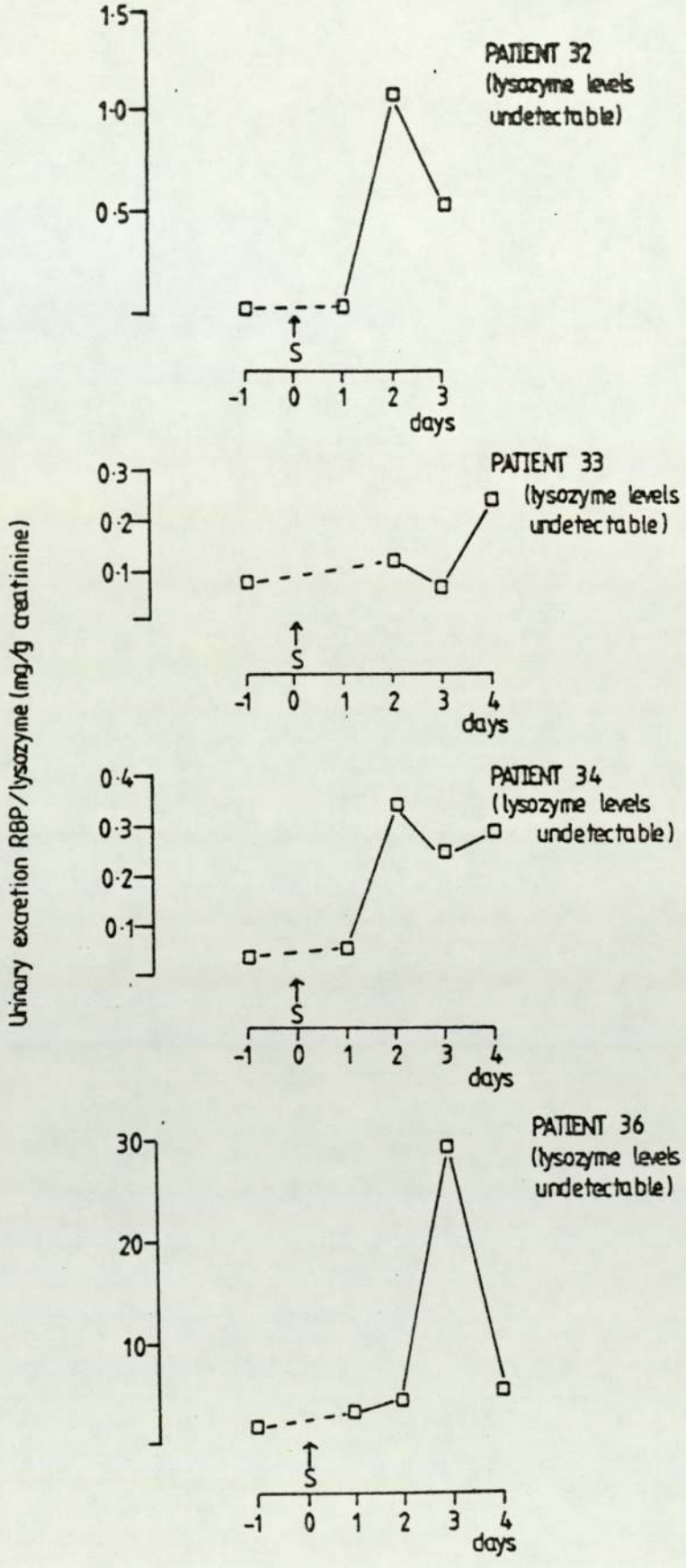
OTHER DRUG THERAPY

CREATININE CLEARANCE (μmol/L)

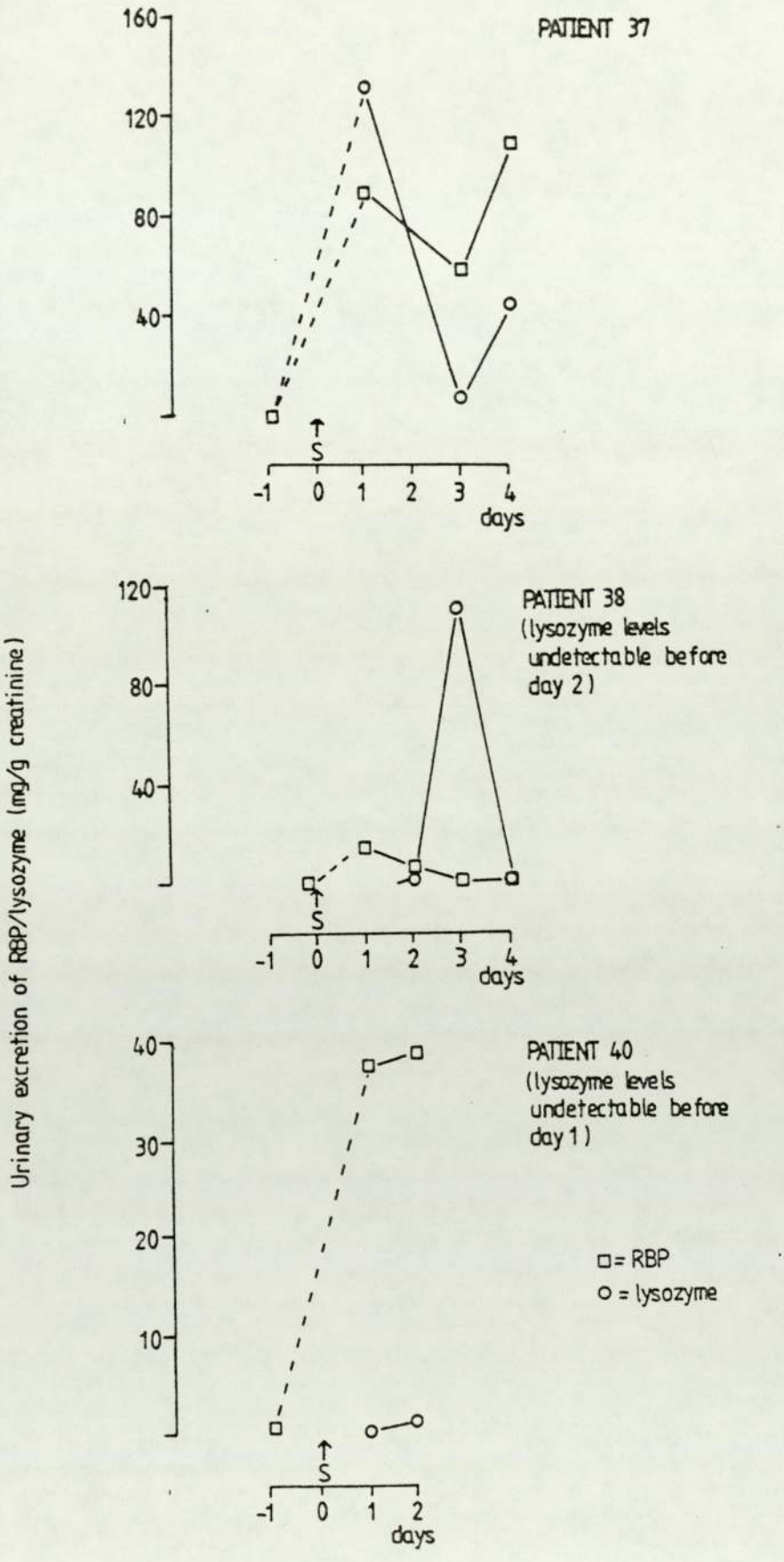
AMINOGLYCOSIDE PLASMA LEVELS (μg/ml)

OTHER DETAILS

Appendix I: The form used to record patient details for the study



Appendix II(AII): Urinary excretion of lysozyme and RBP in surgical control patients S=surgery, G=gentamicin therapy
□=RBP, o=lysozyme, F=frusemide



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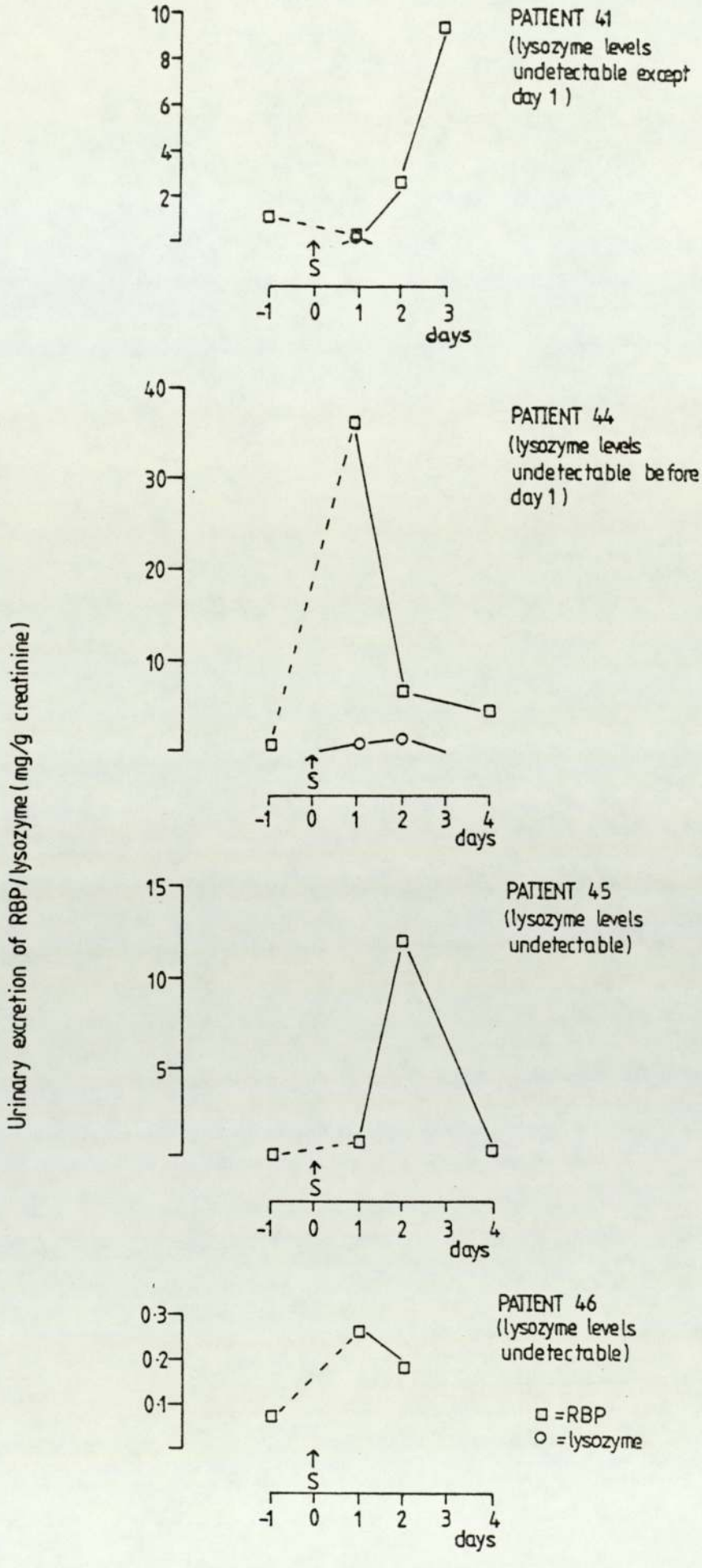

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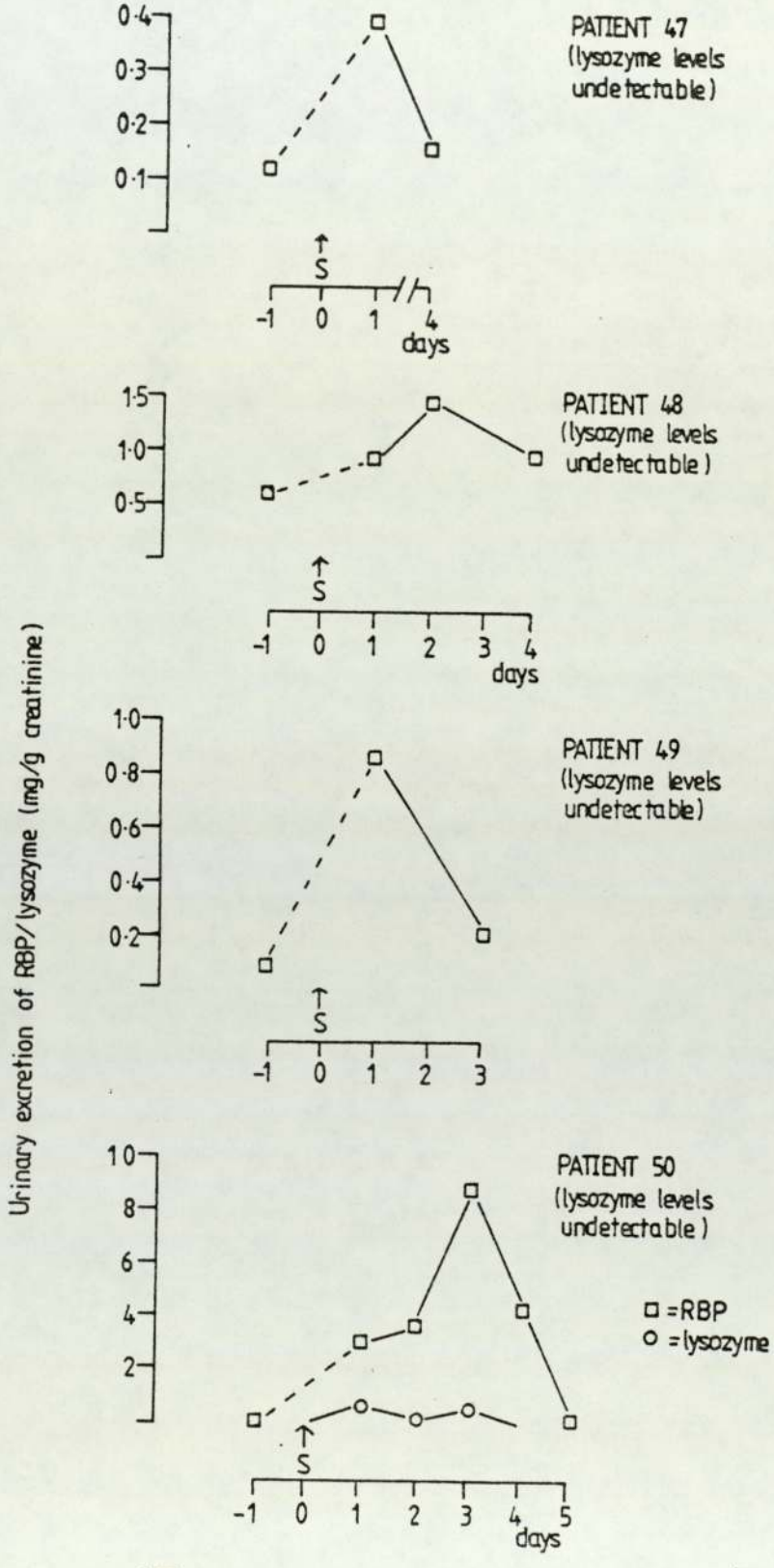

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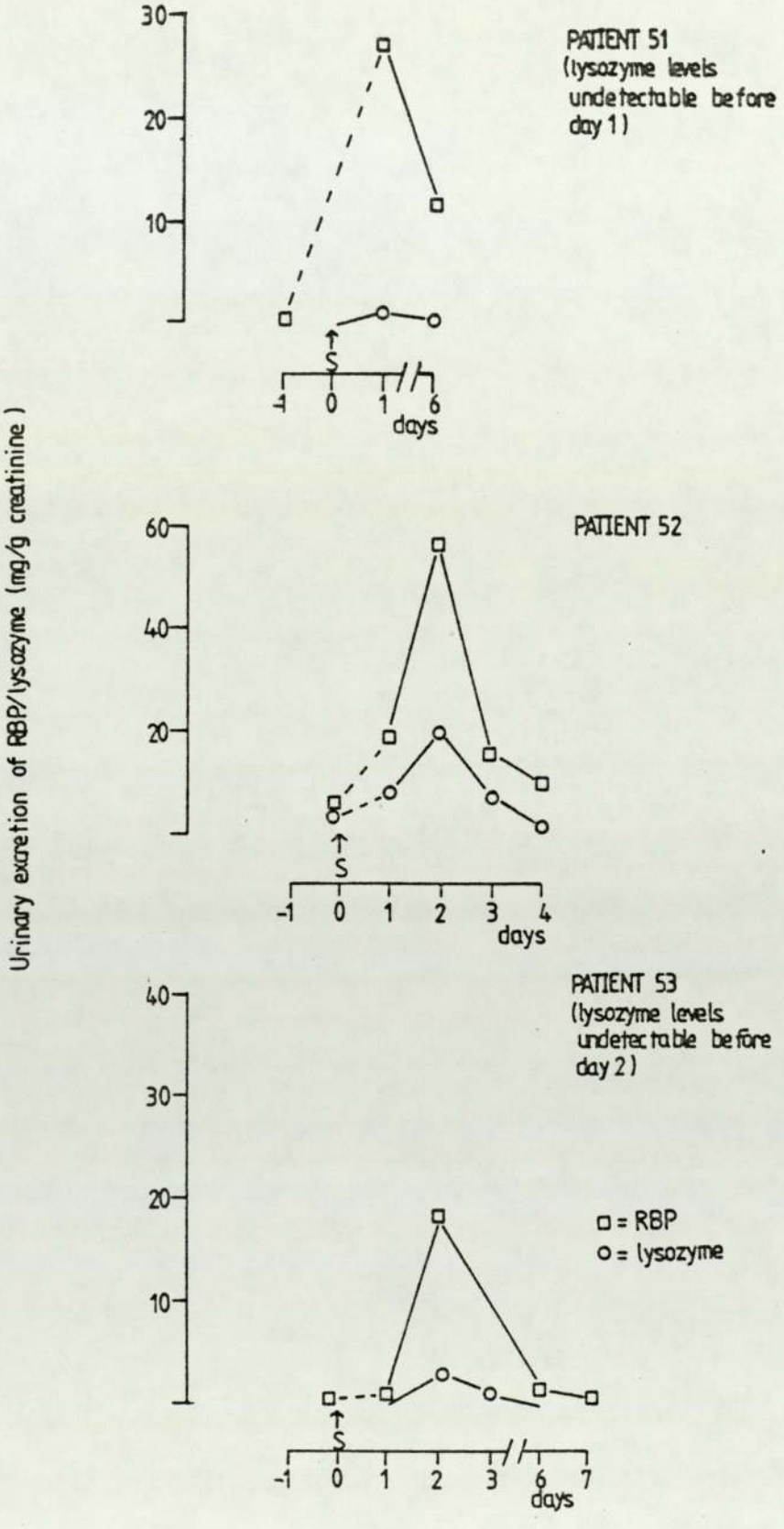

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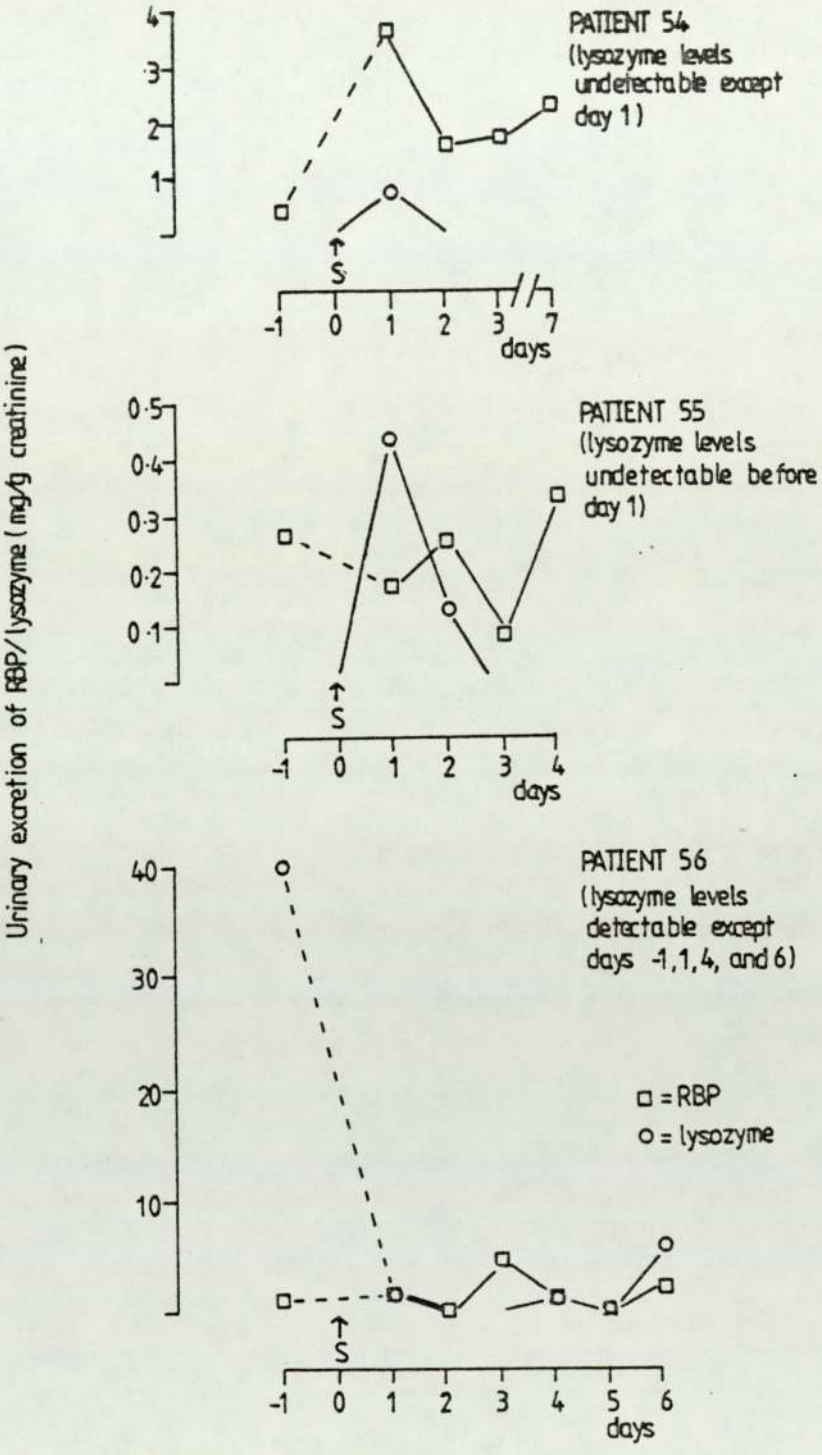

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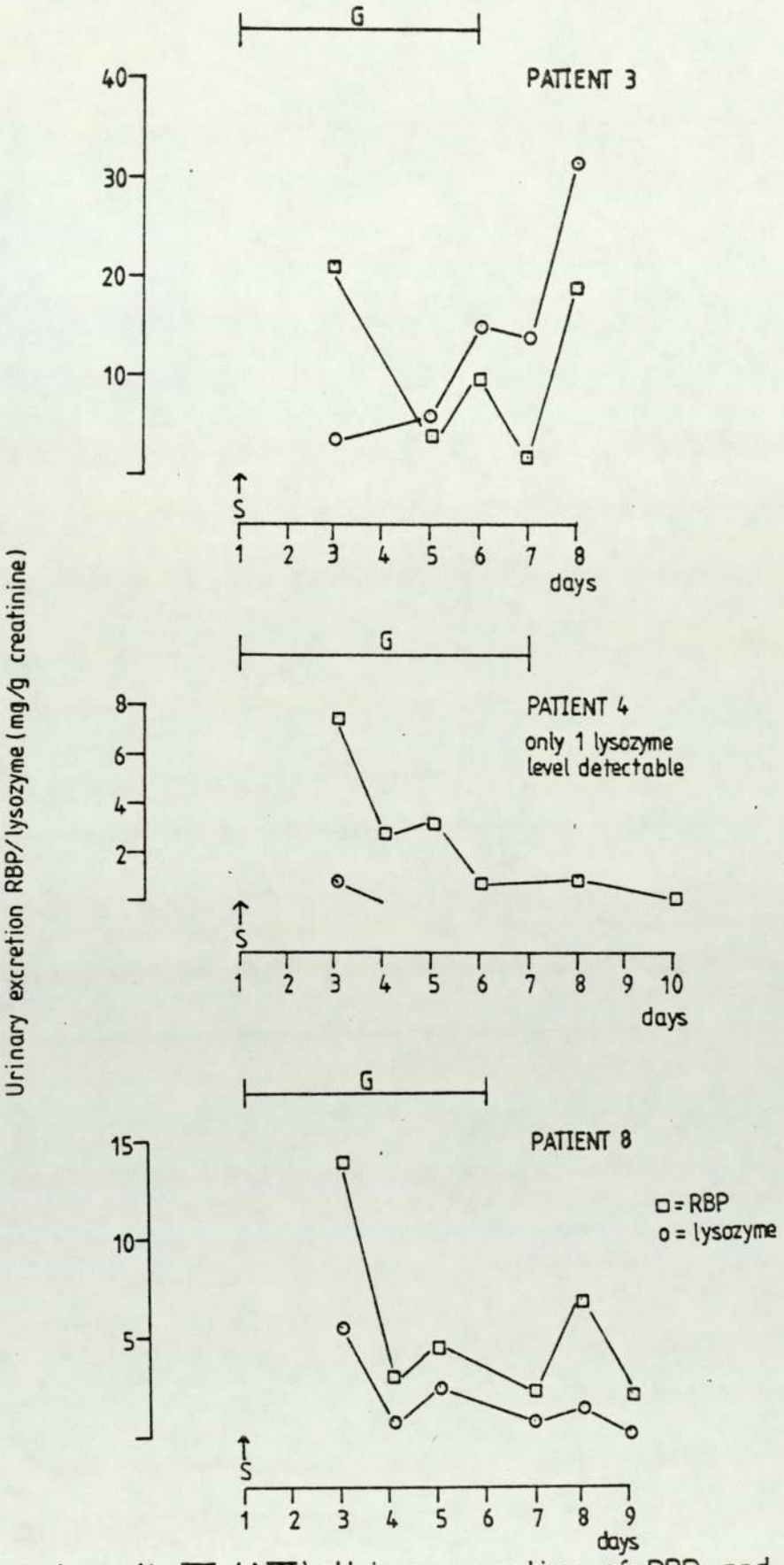
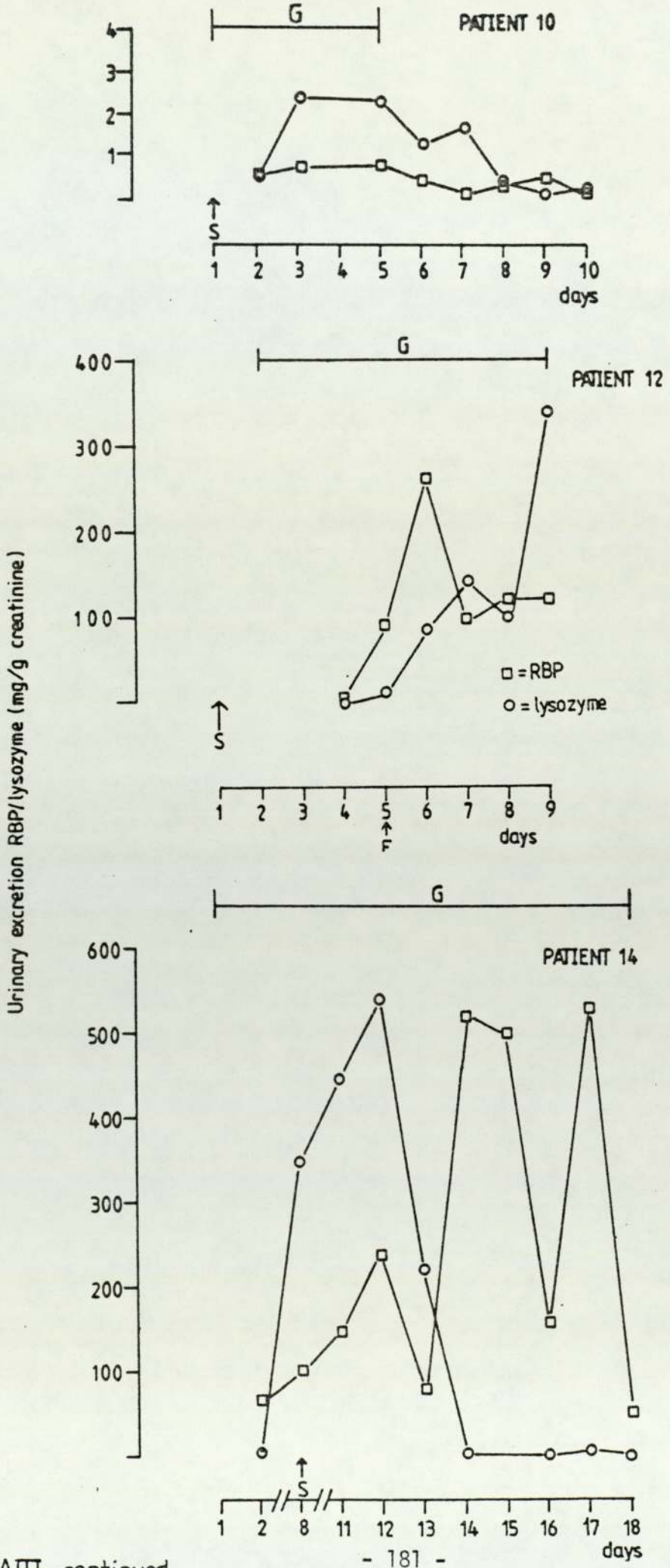


Appendix III (AIII): Urinary excretion of RBP and lysozyme in gentamicin-treated surgical patients



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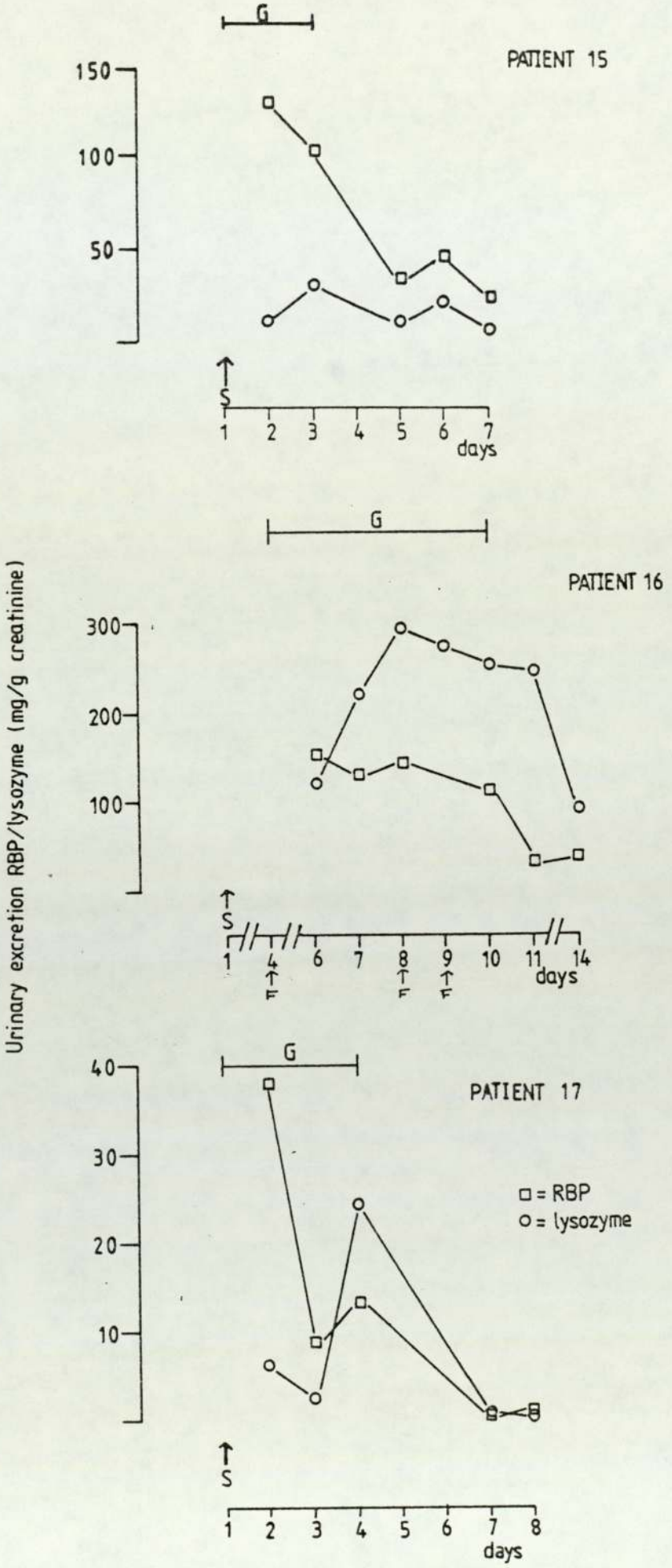

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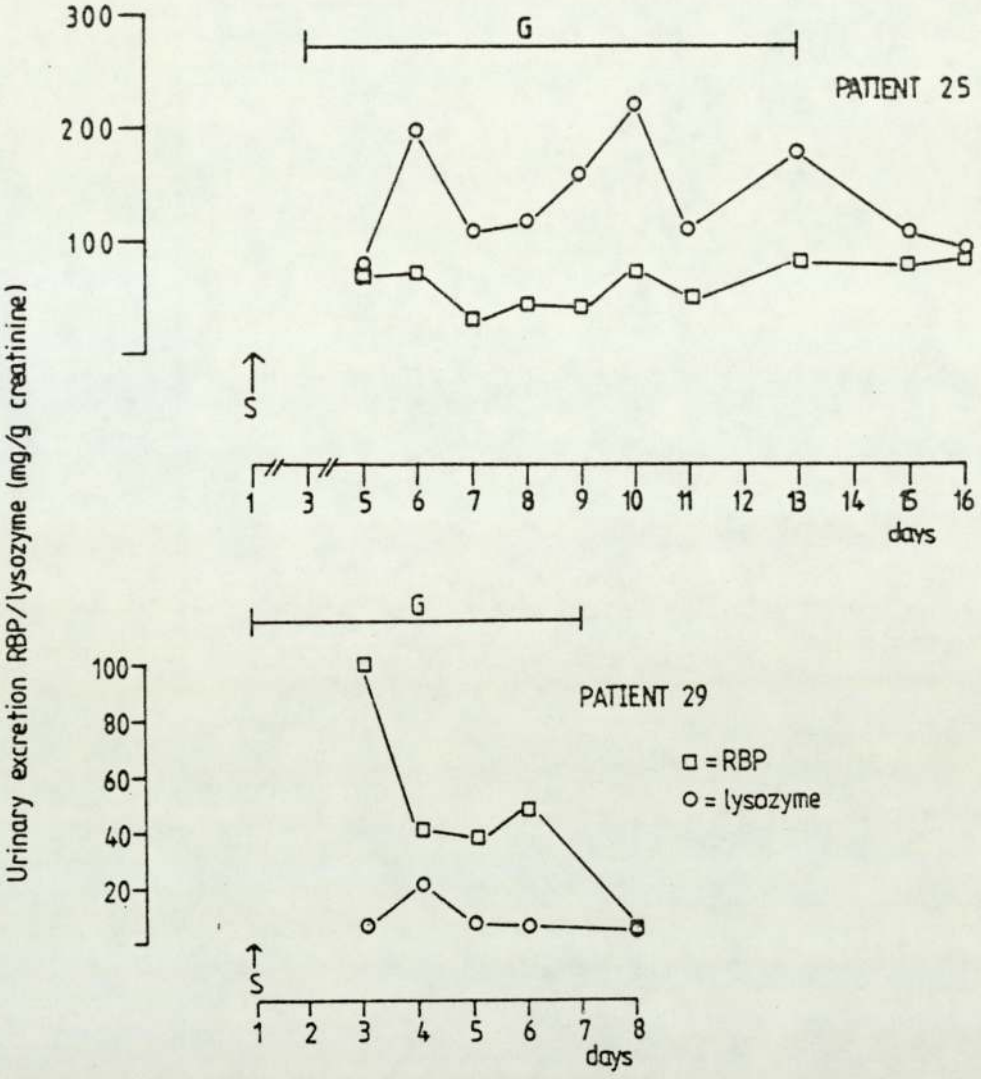

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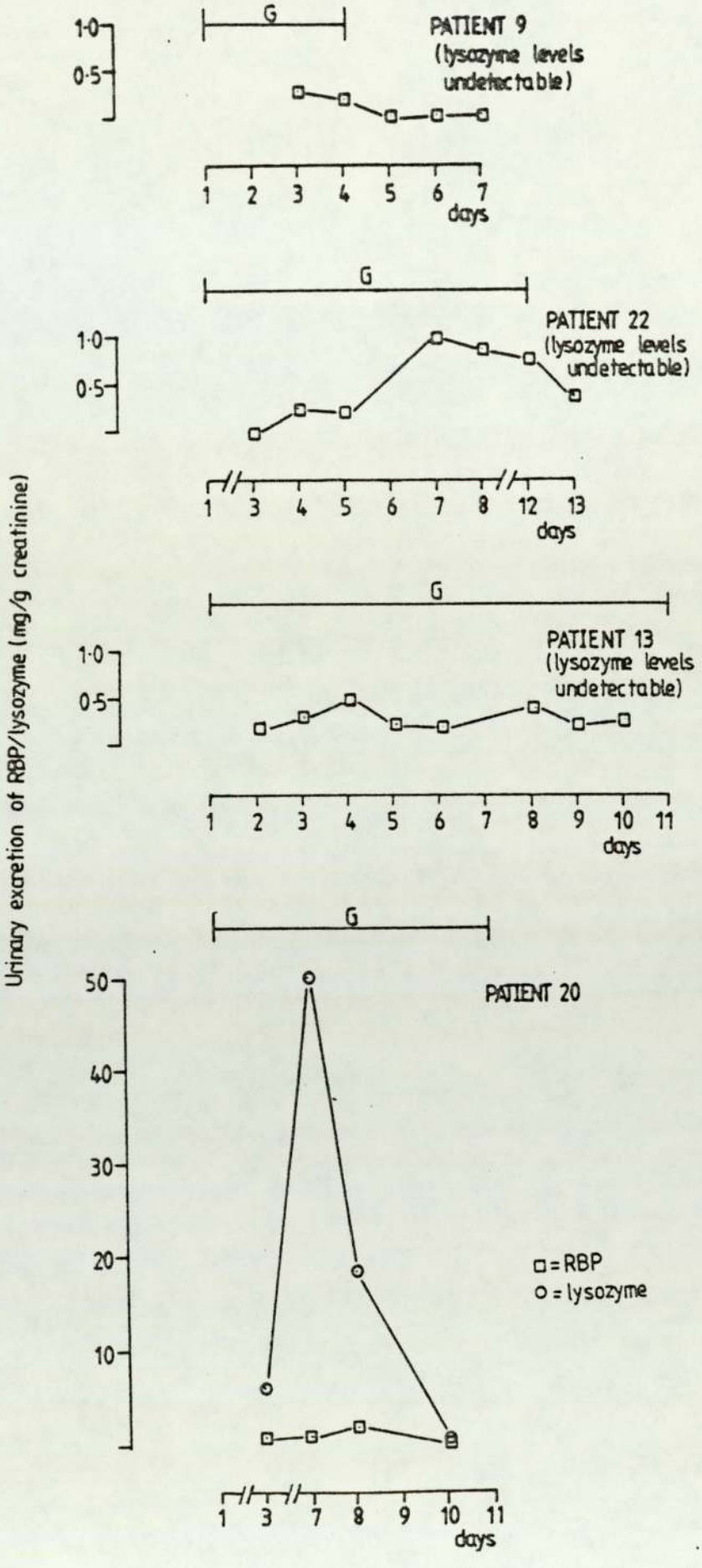
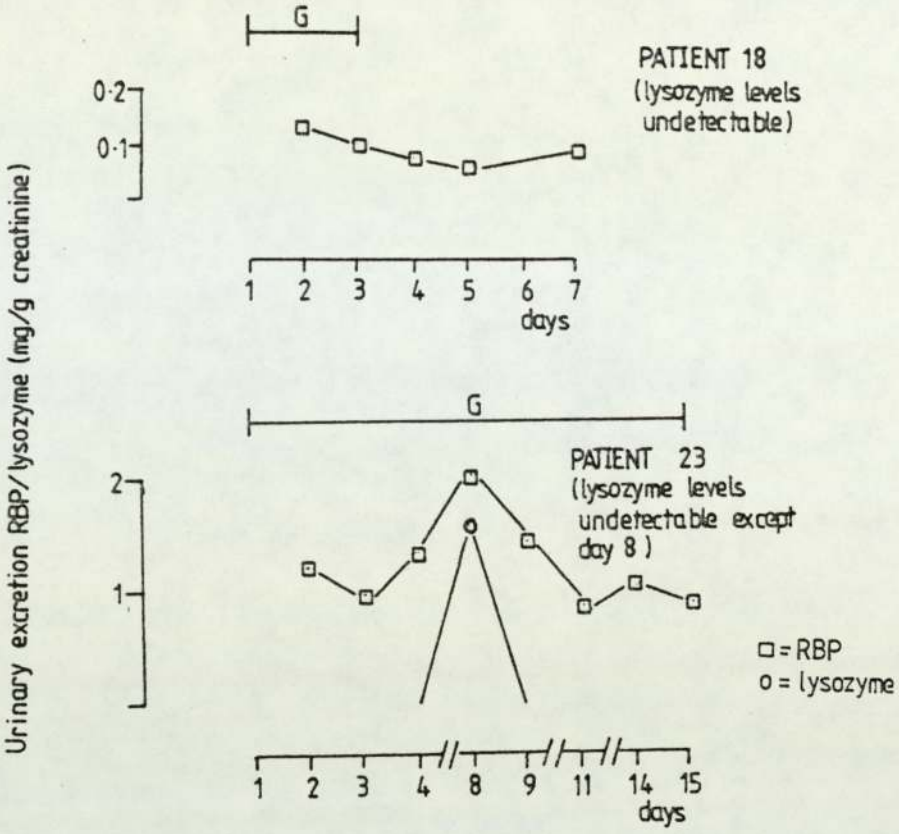


Appendix IV: Urinary excretion of RBP and lysozyme in patients with cystic fibrosis.
(AIV)



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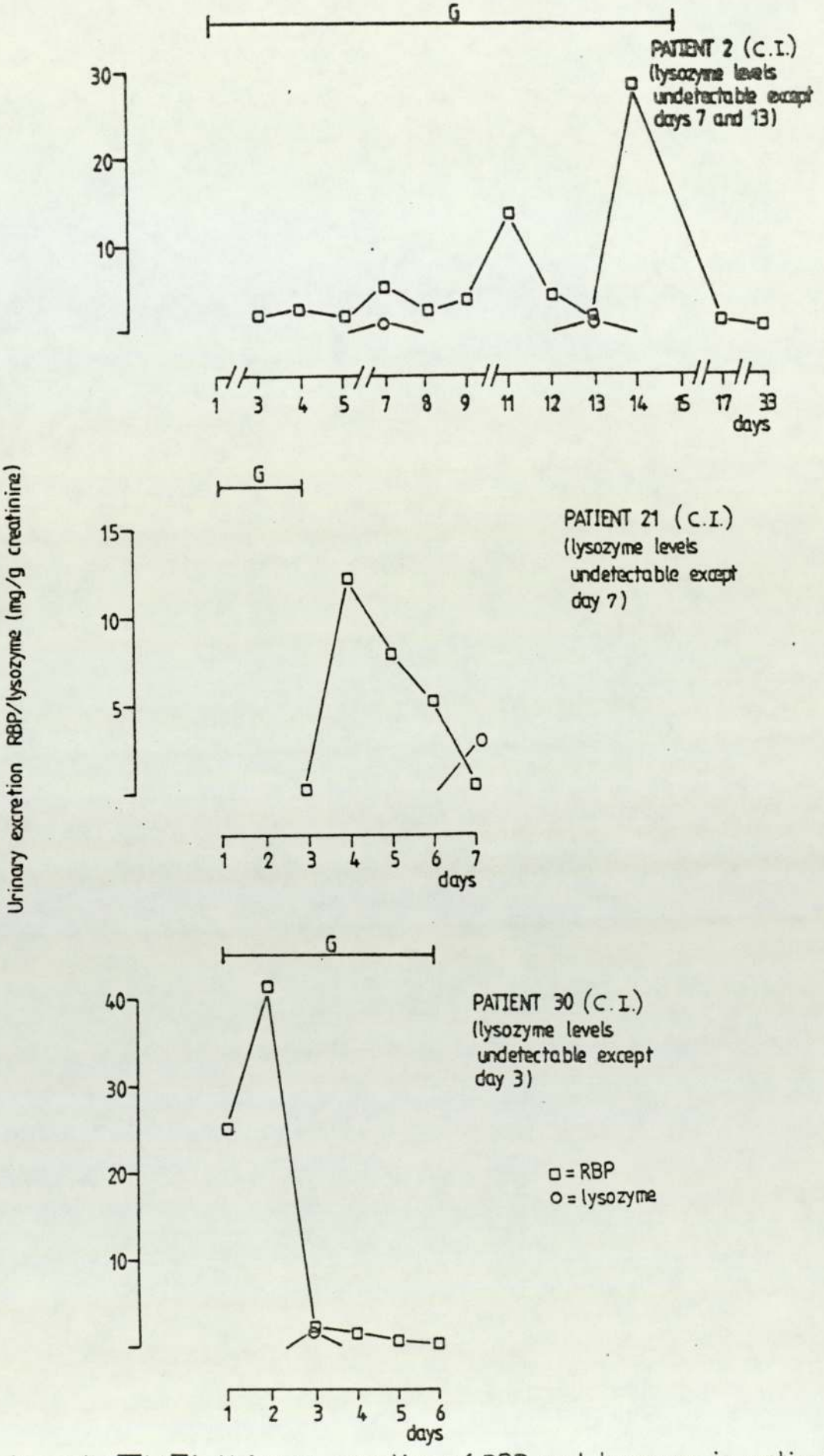
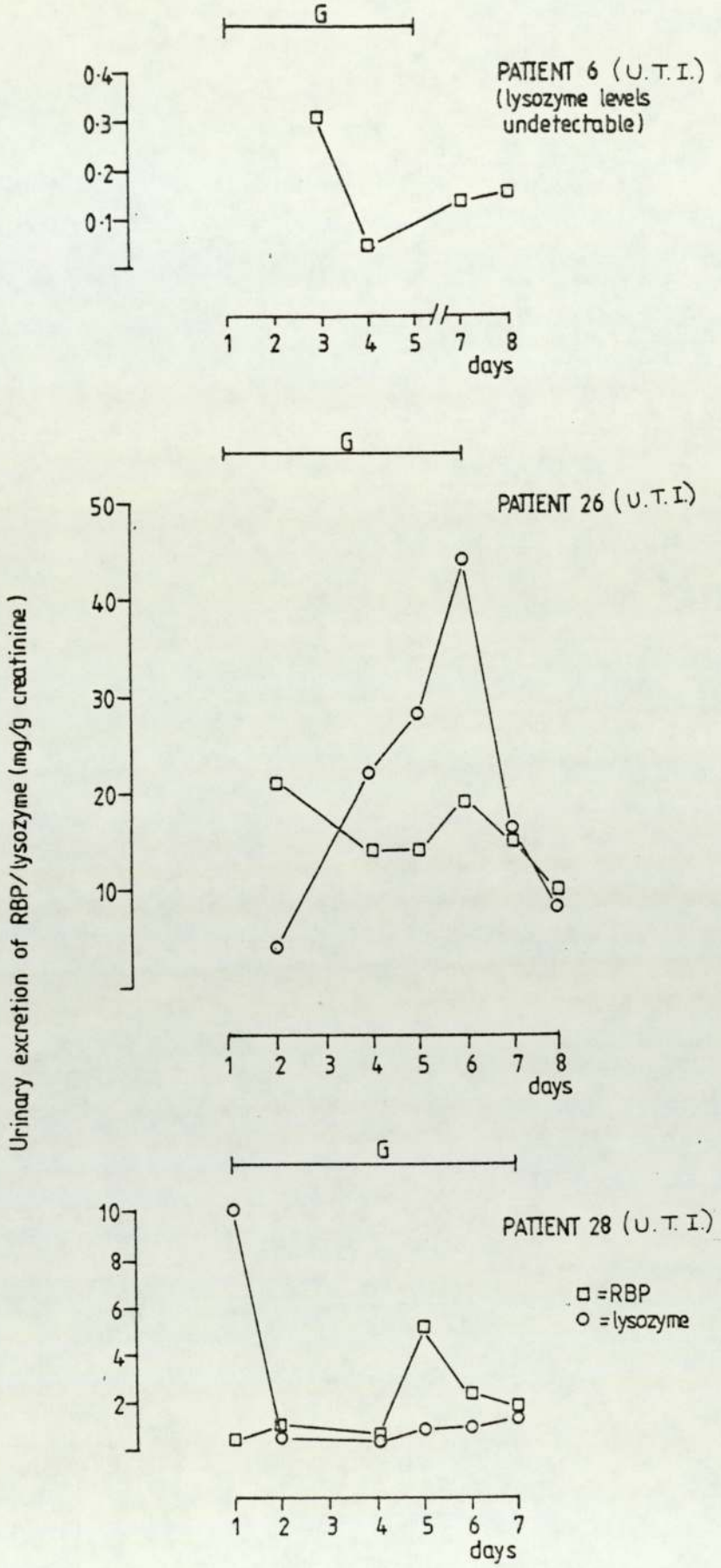


Appendix V(AV): Urinary excretion of RBP and lysozyme in patients with CI, UTI, WI etc.



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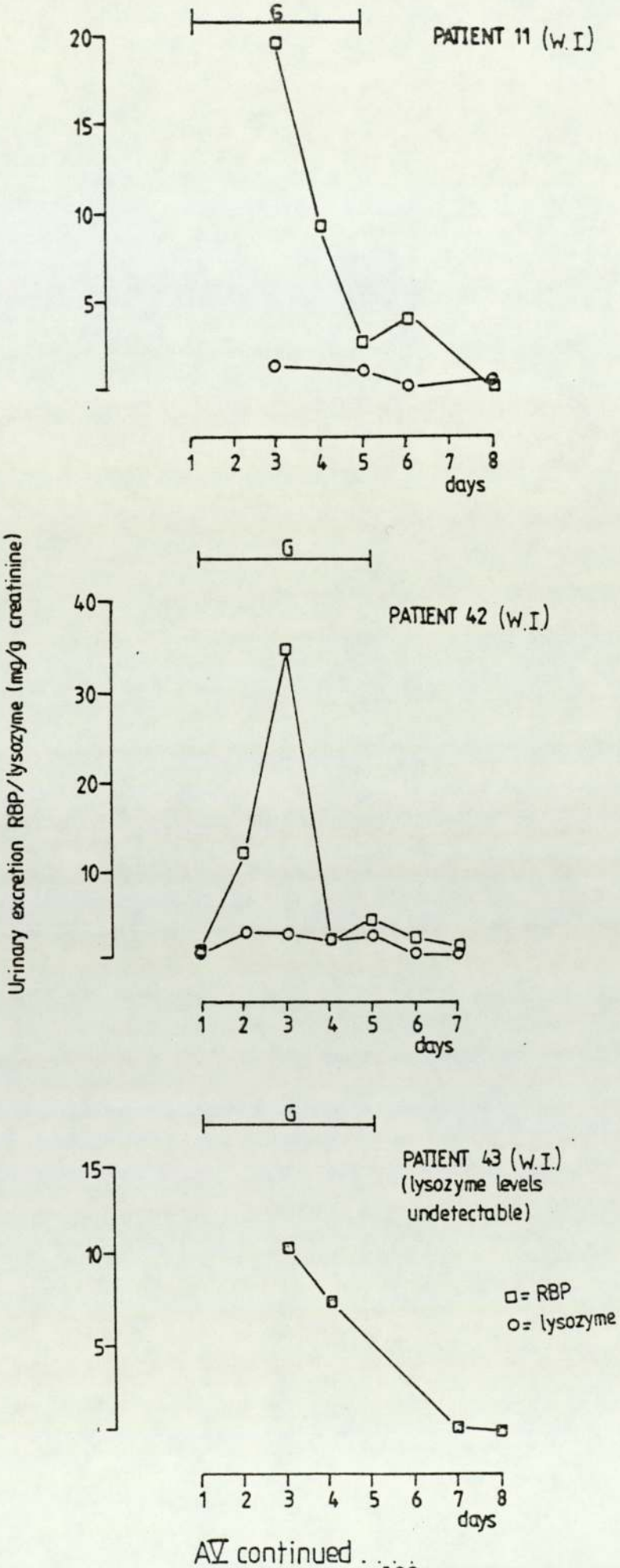

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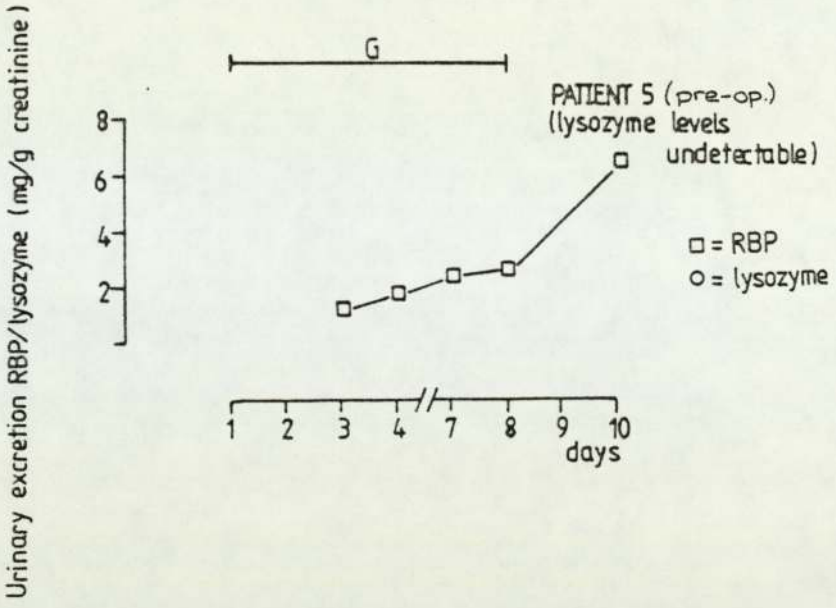

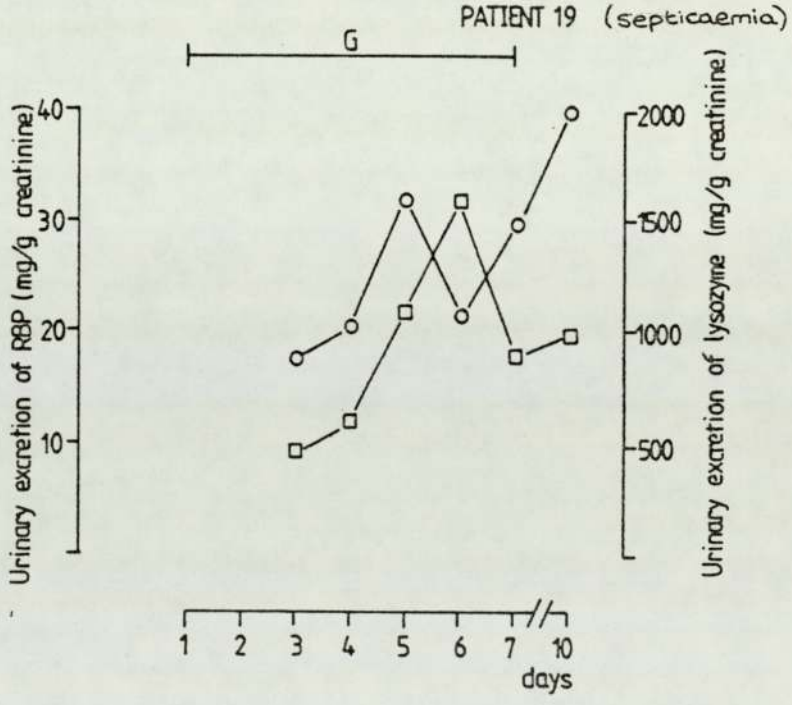

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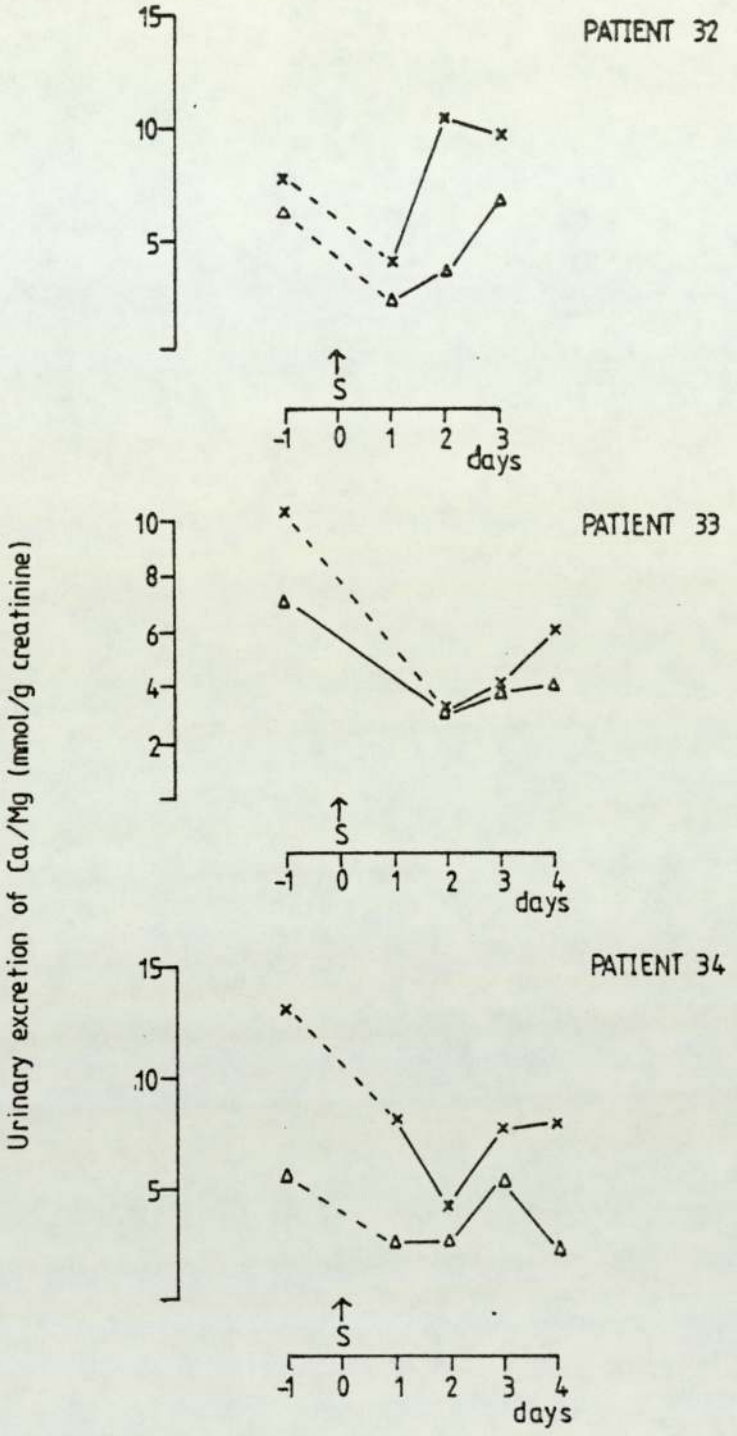
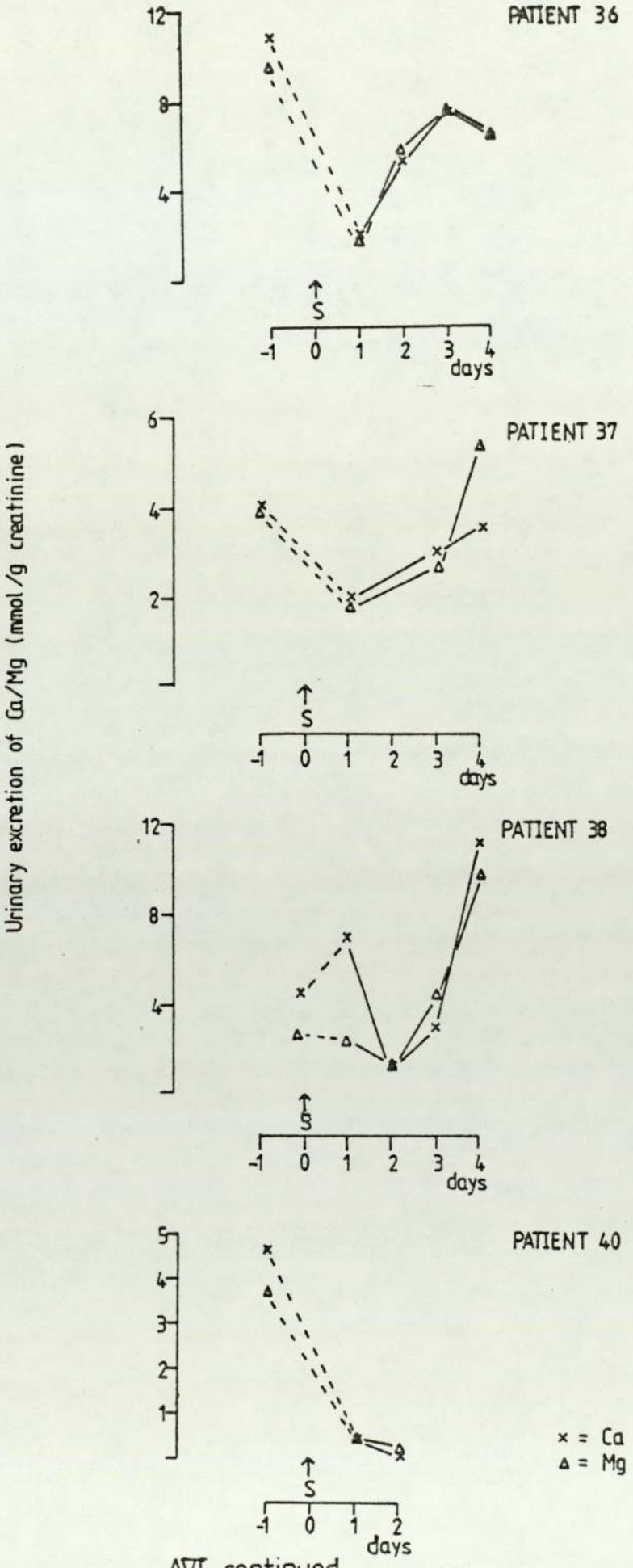


Appendix VI (AVI) : Urinary excretion of calcium and magnesium by surgical control patients. ×=Ca, △=Mg



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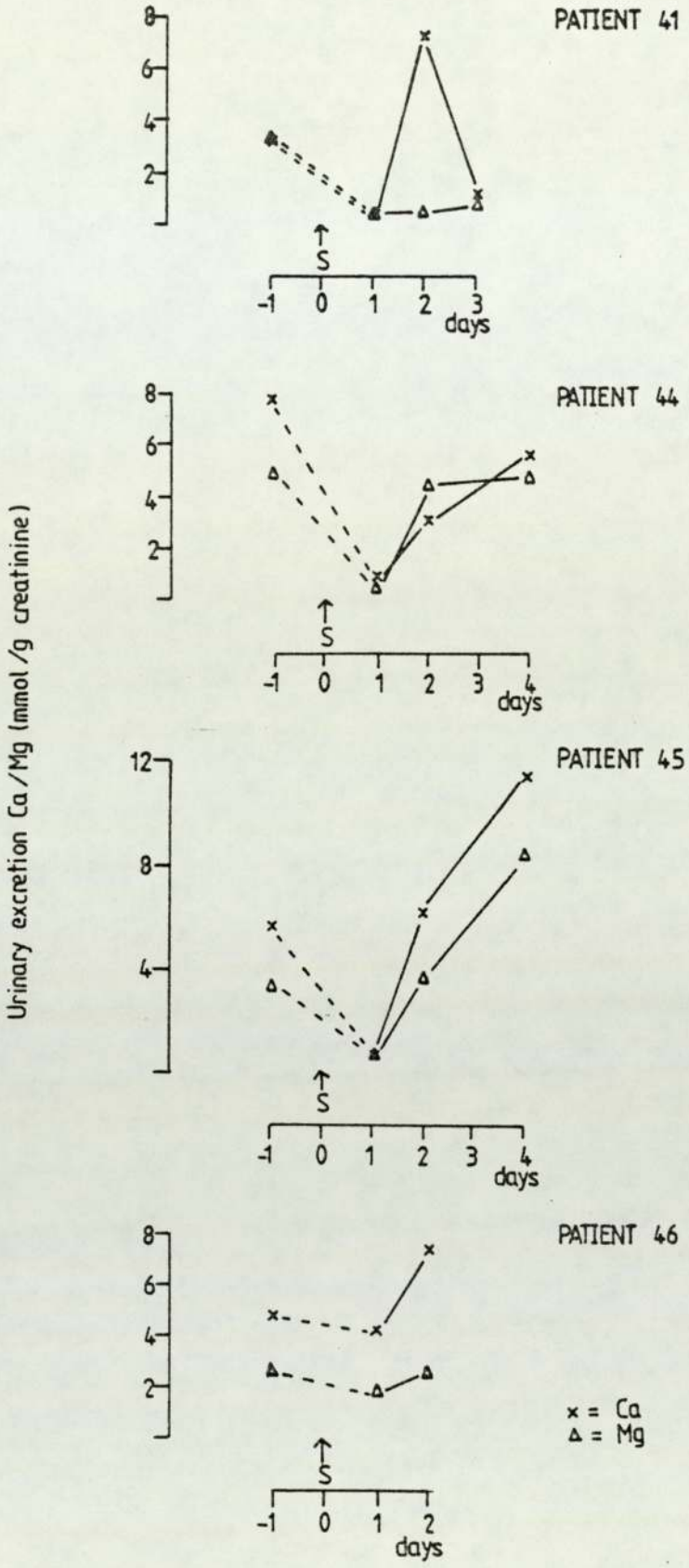

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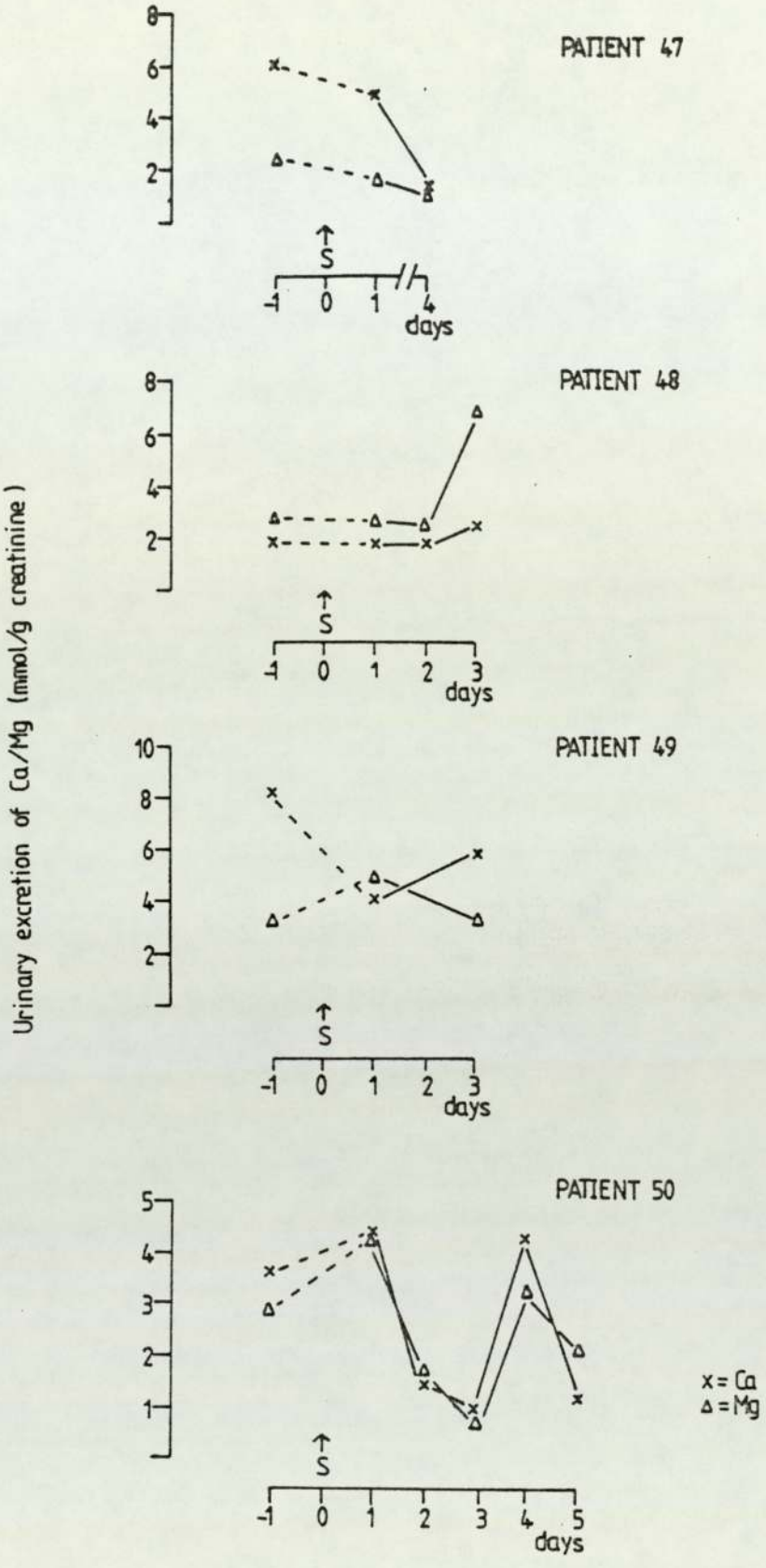

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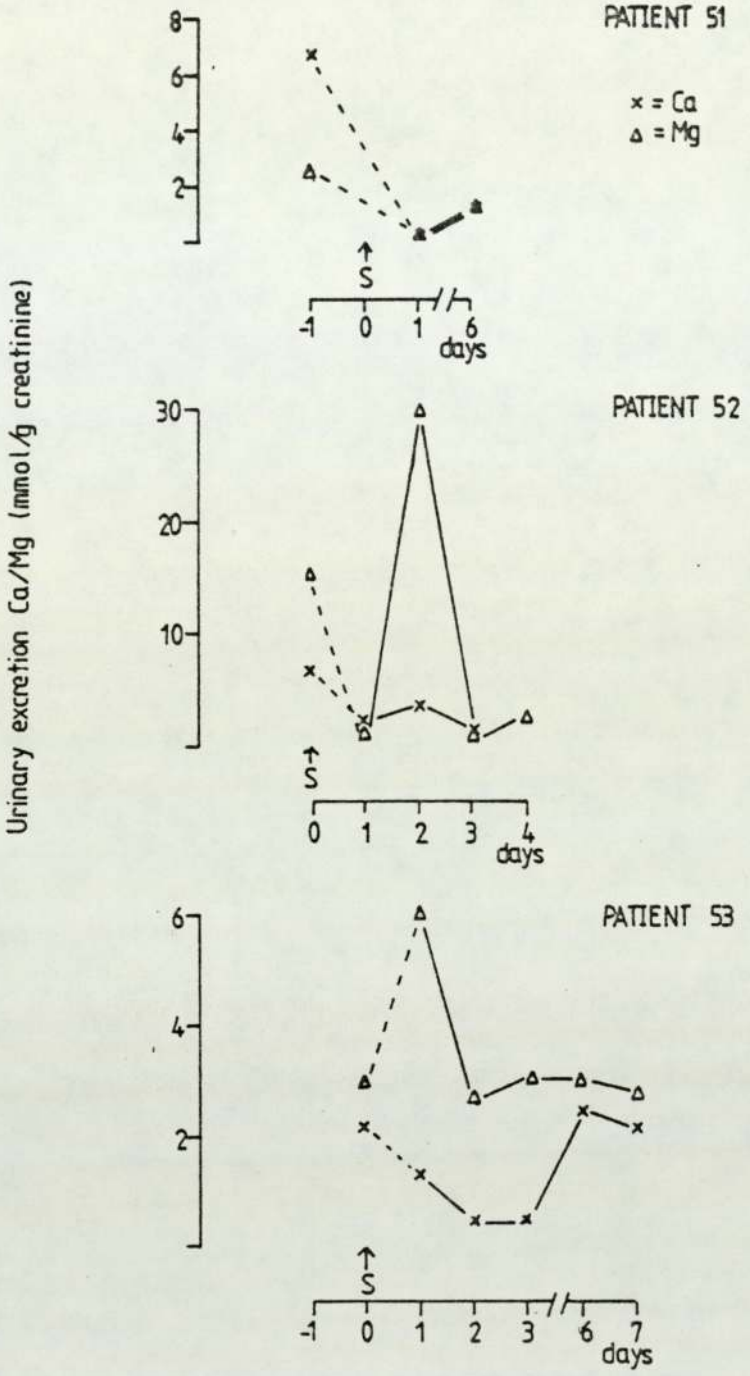

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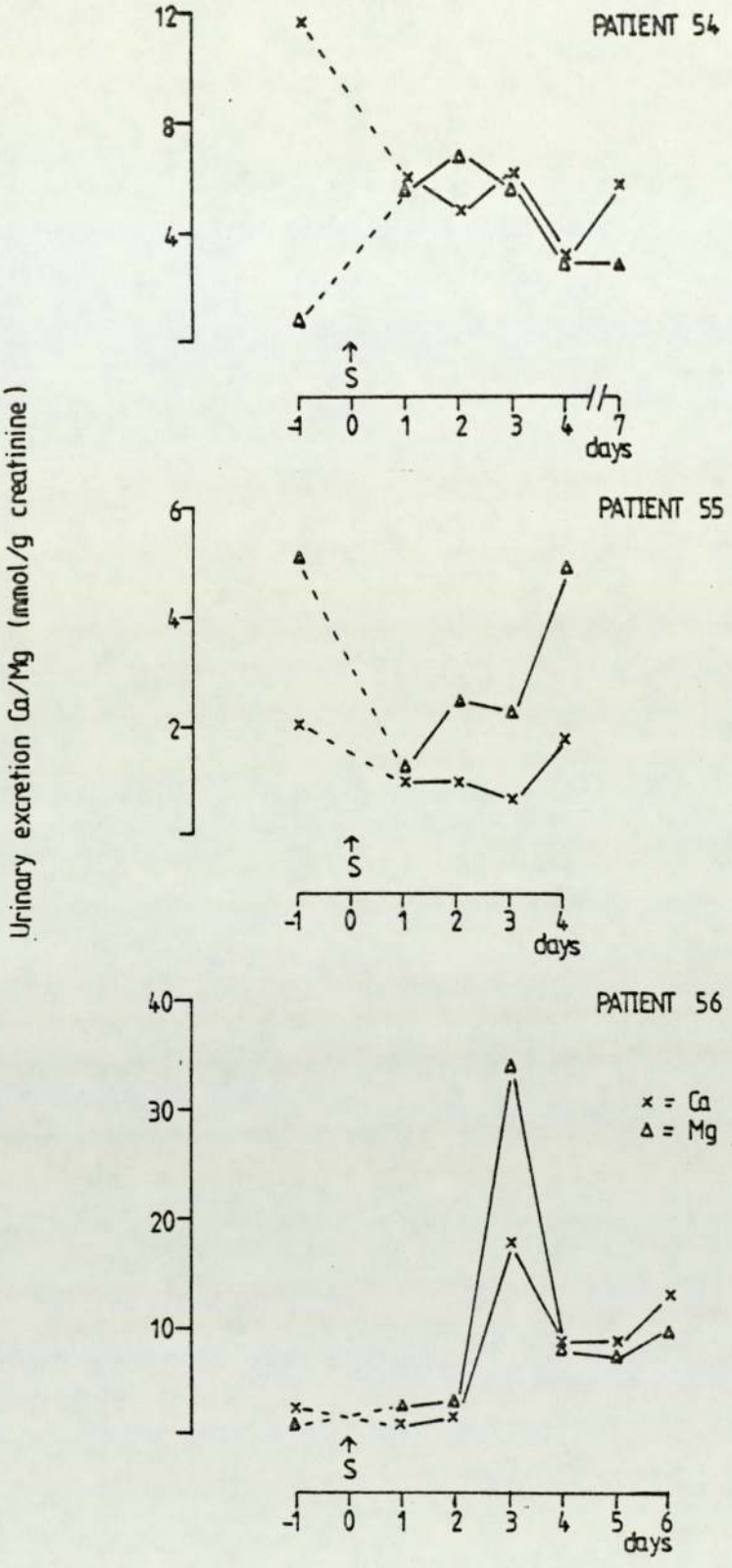

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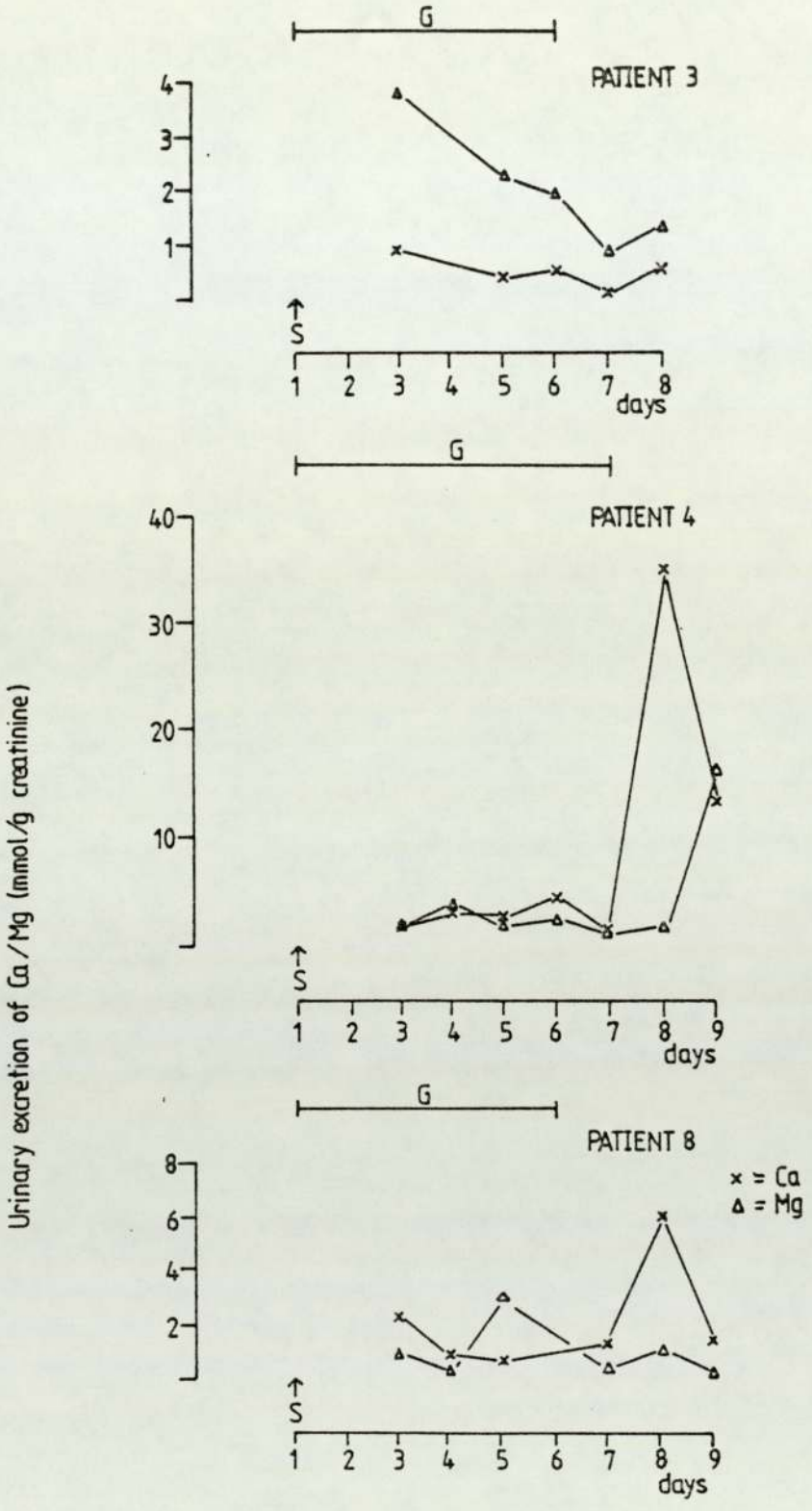
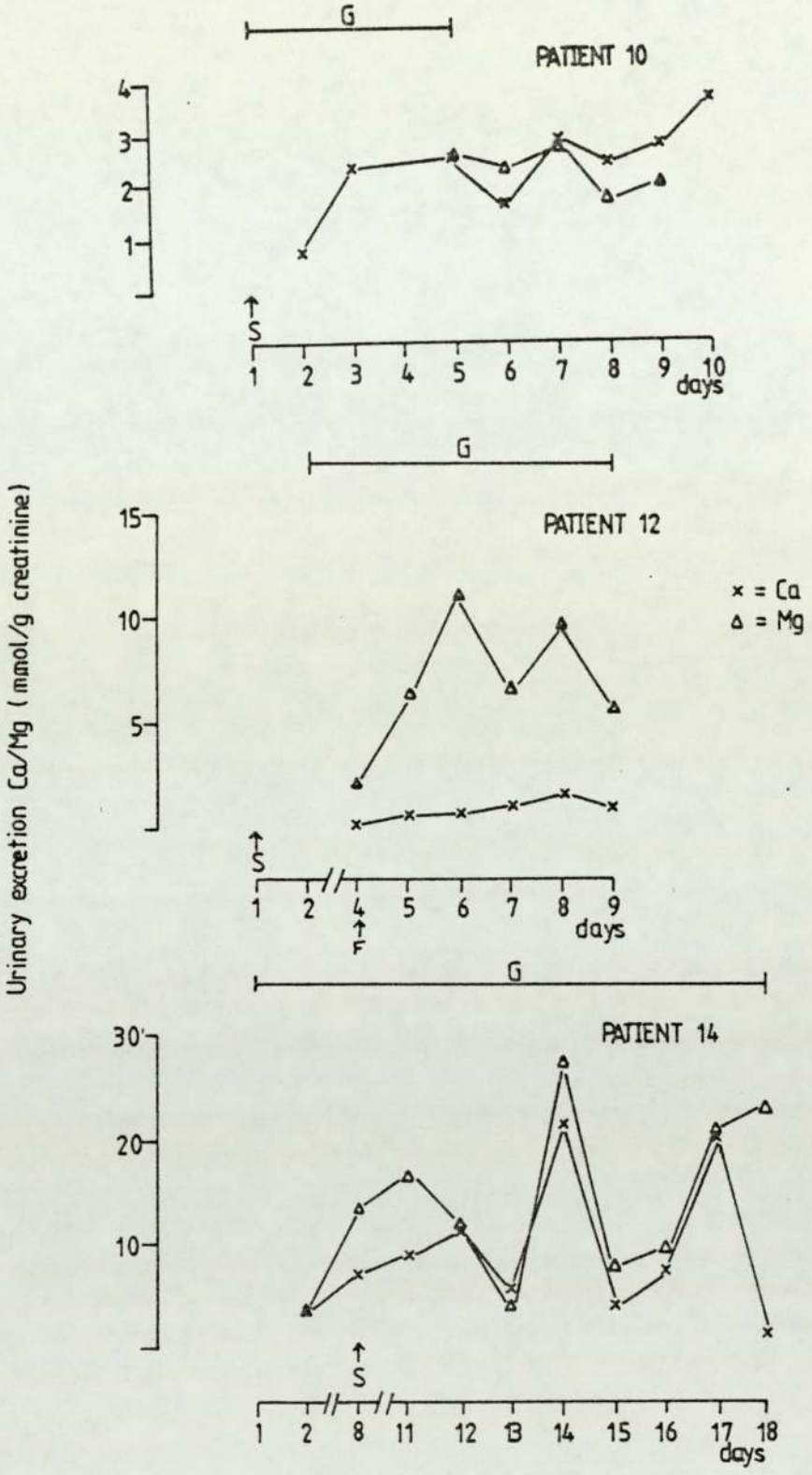


Appendix VII (AVII): Urinary excretion of calcium and magnesium in gentamicin-treated surgical patients.



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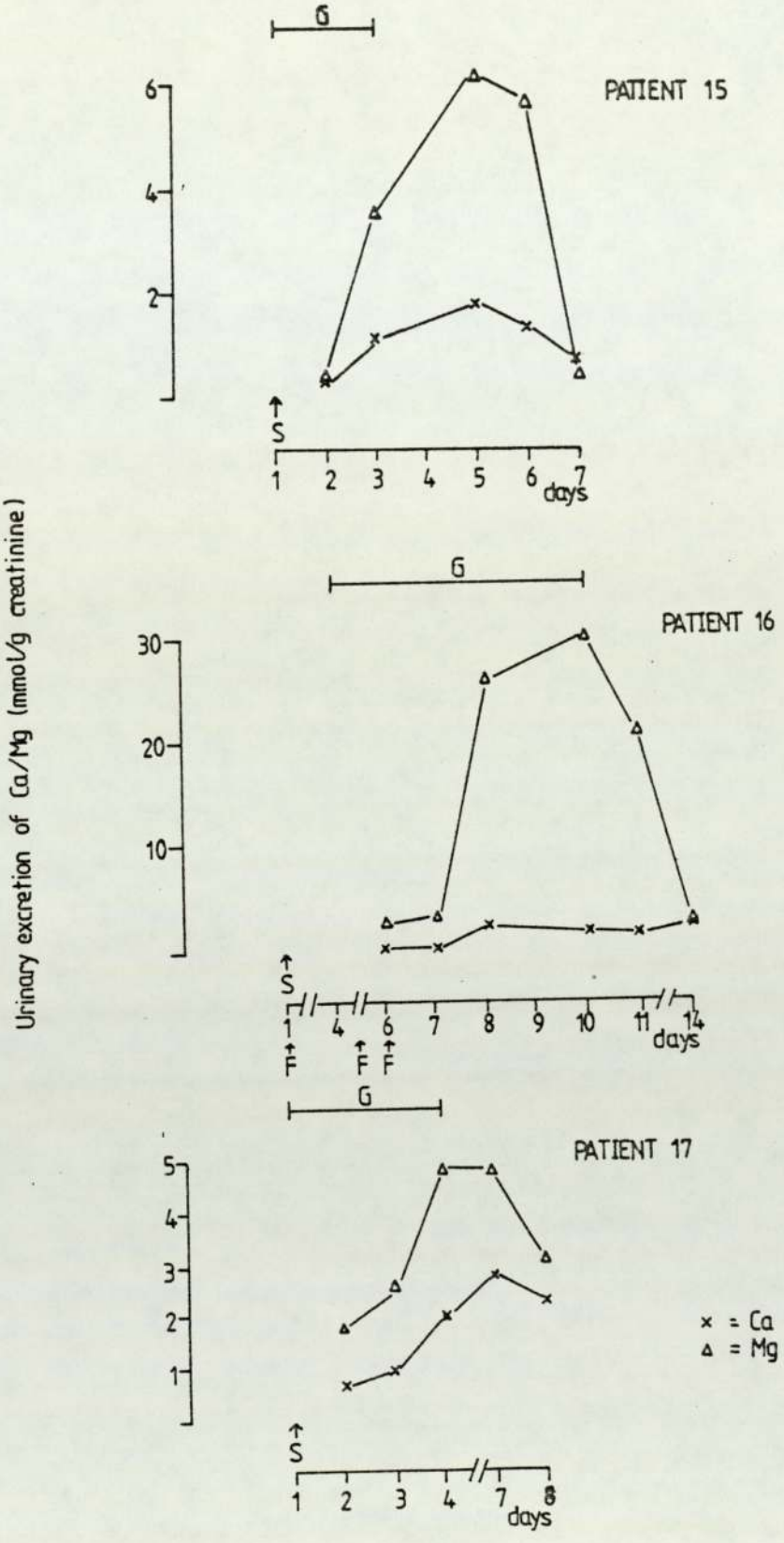

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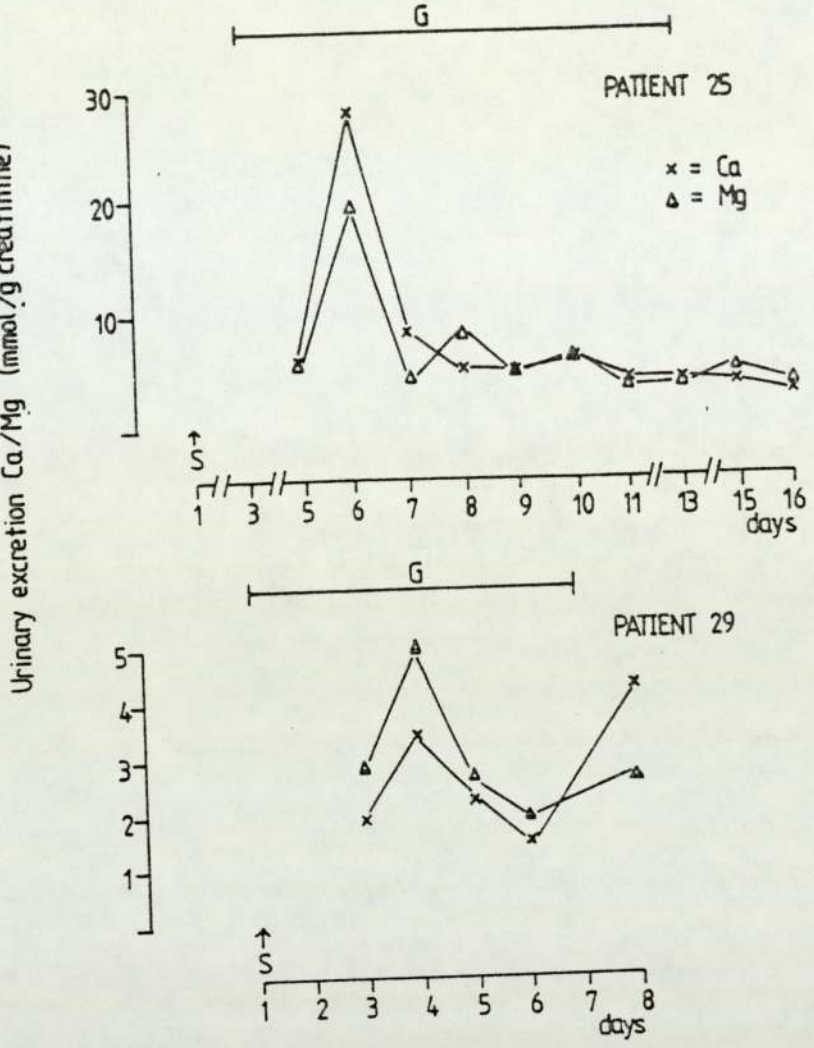

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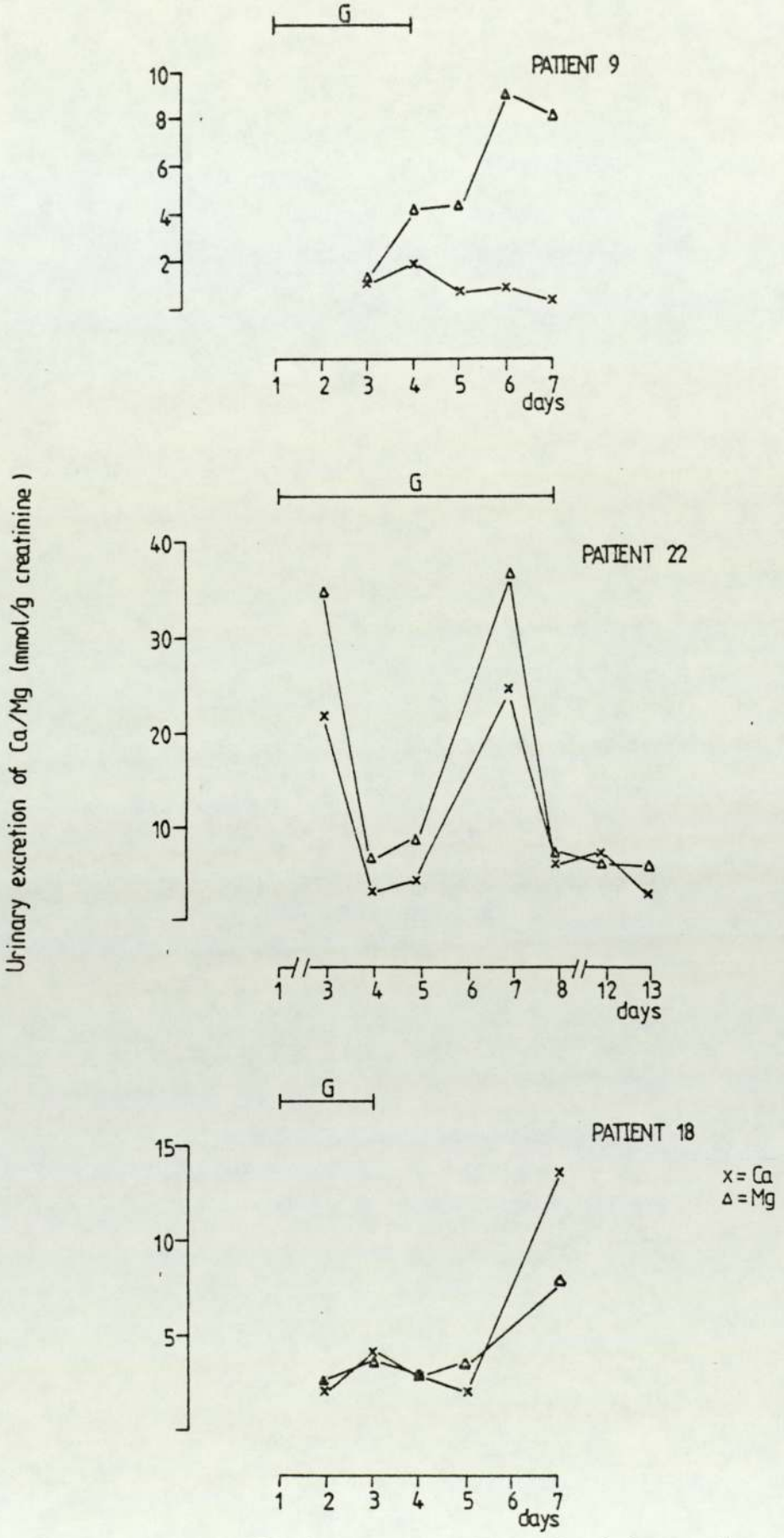
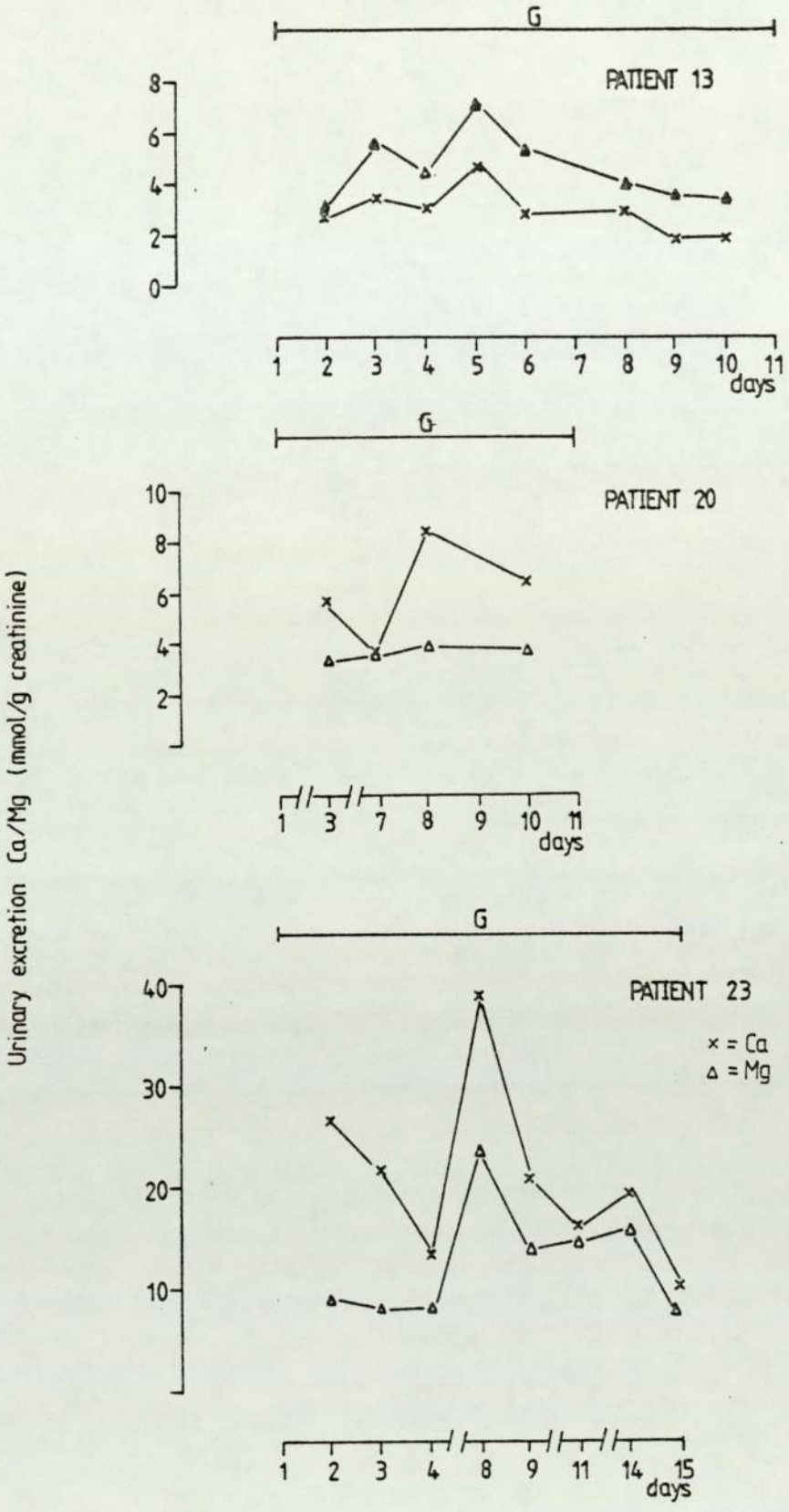


Appendix VIII (AVIII): Urinary excretion of calcium and magnesium by patients with cystic fibrosis.



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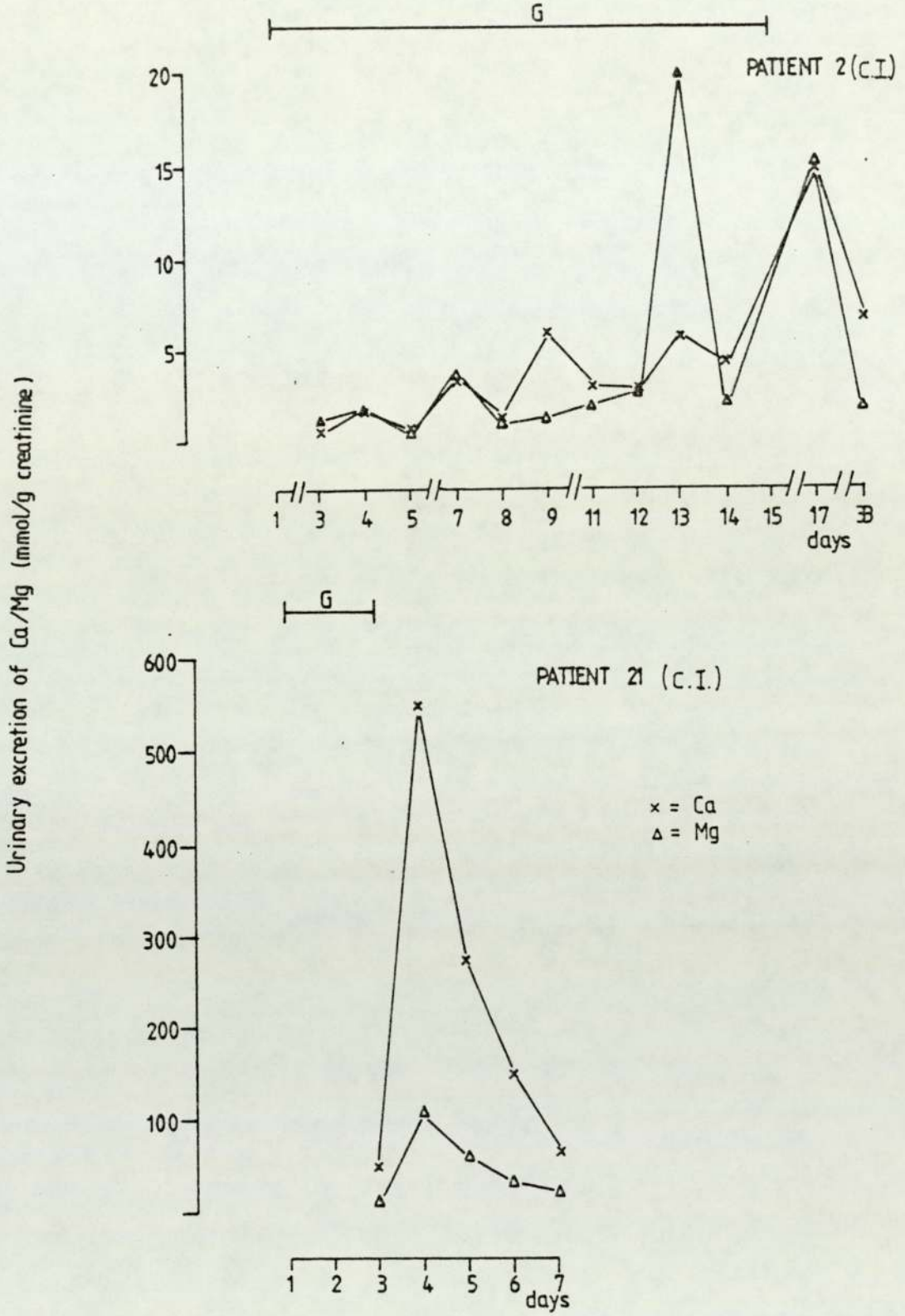
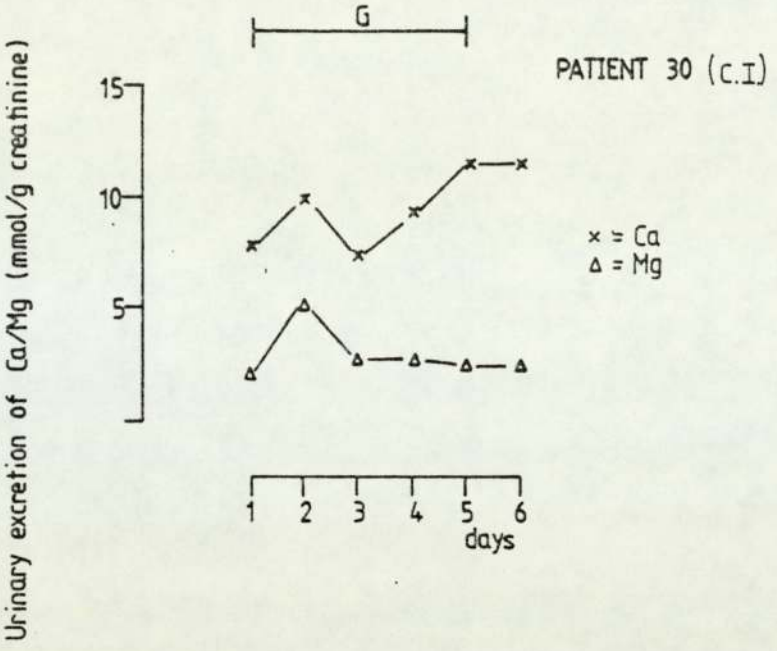


Appendix IX (AIX): Urinary excretion of calcium and magnesium by patients with CI, UTI, WI etc.



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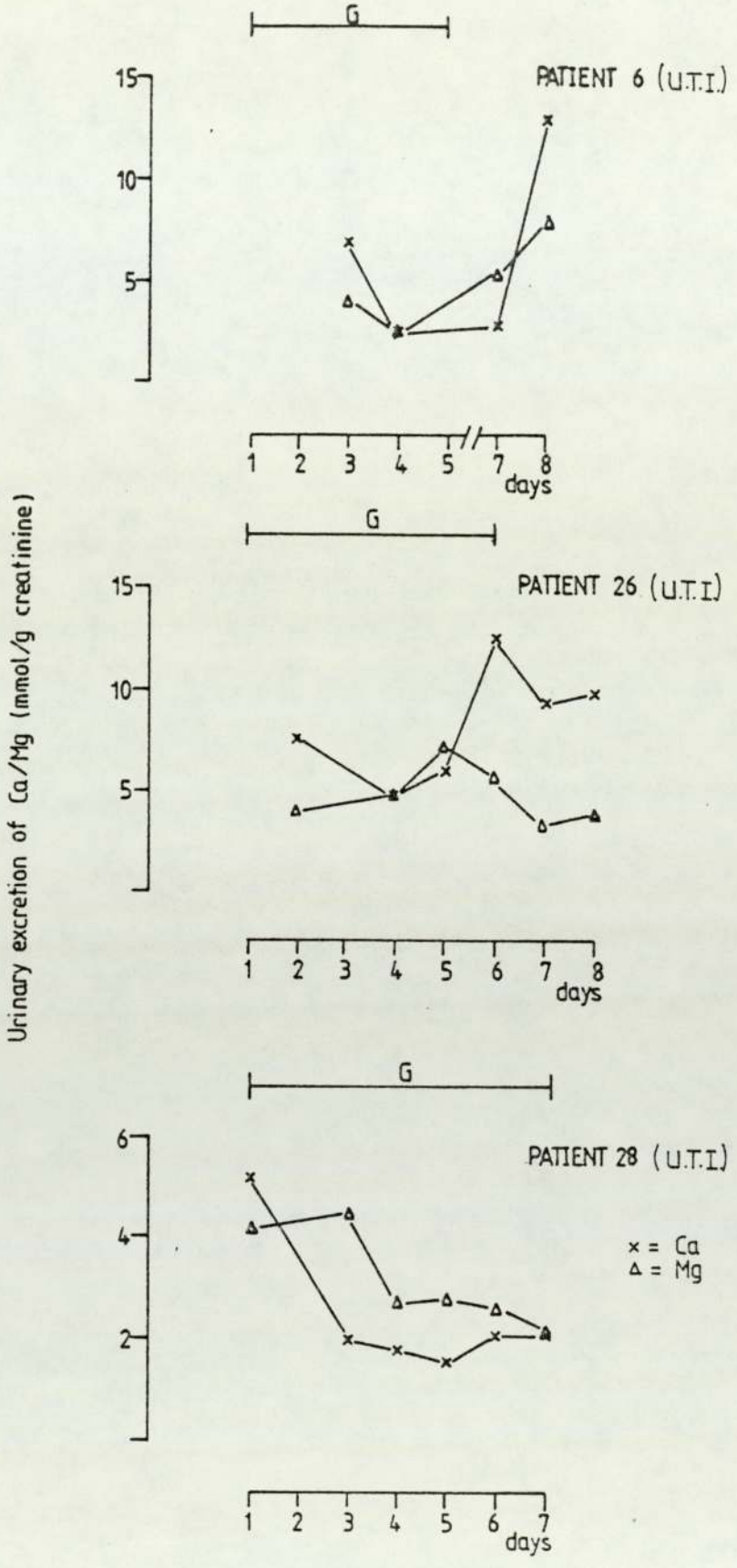

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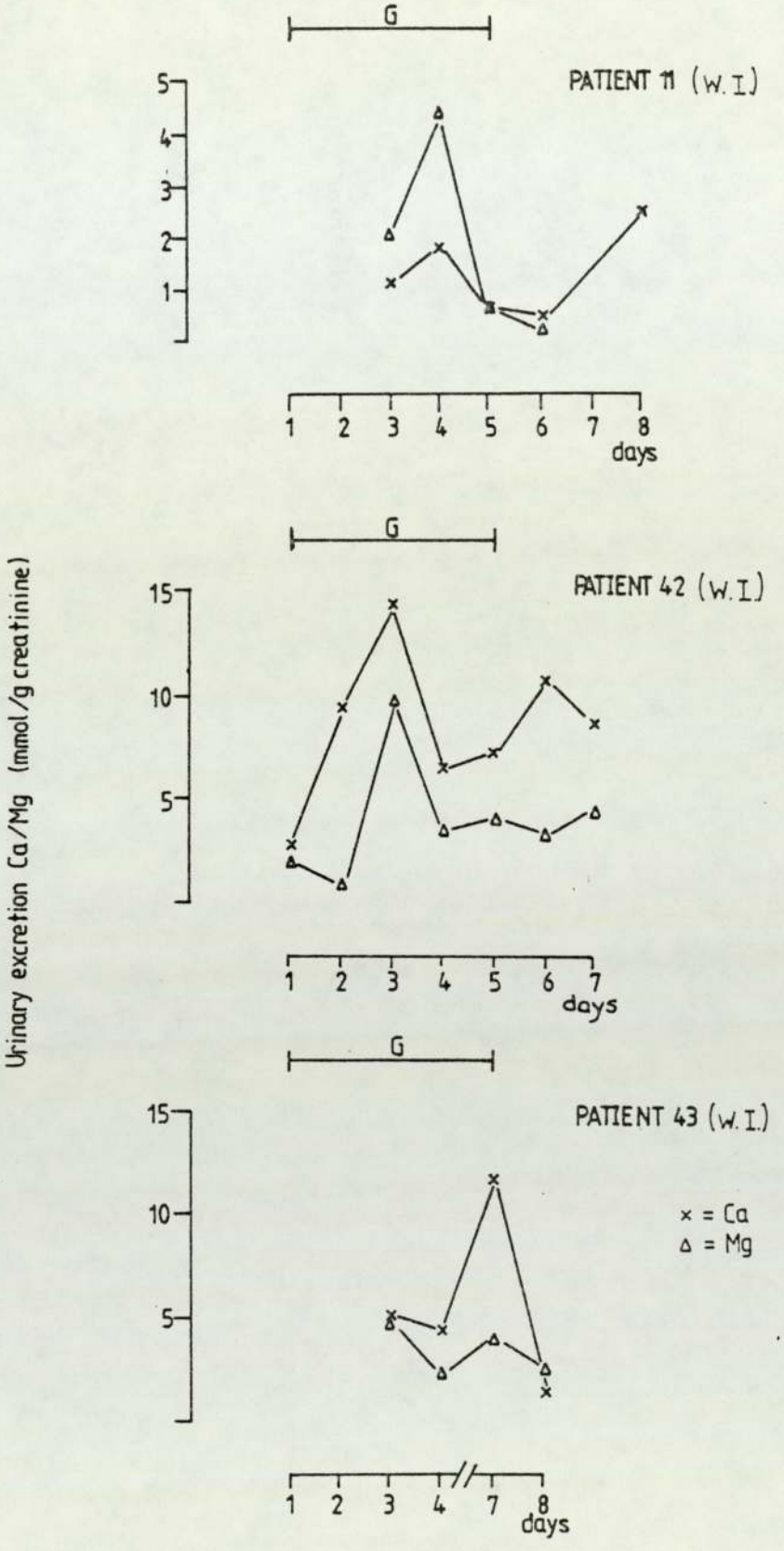

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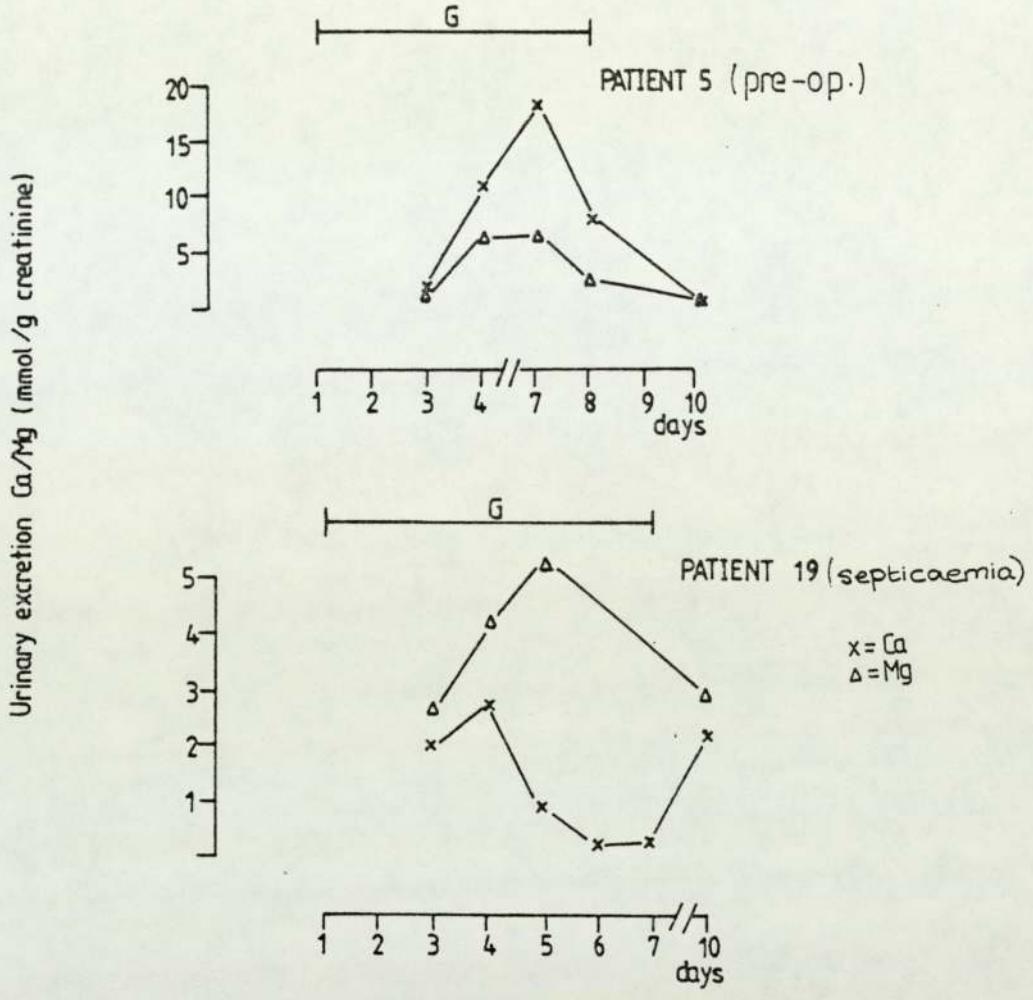

AIX continued . . . .

REFERENCES

1. Kahlmeter,G. and Dahlager,J.I. 1984. Aminoglycoside Toxicity: A Review of Clinical Studies Published between 1975 and 1982. J. Antimicrob. Chemother. 13: Suppl. A:9-22.

2. Cojocel, C. and Hook, J.B. 1983. Aminoglycoside Nephrotoxicity. Trends Pharmac. Sci. 4: 174-179.

3. Appel, G.B. and Neu, H.C. 1978. Gentamicin in 1978. Ann. Intern. Med. 89: 528-538.

4. Dahlager, J.I. 1980. The Effect of Netilmicin and Other Aminoglycosides on Renal Function: A Survey of the Literature on the Nephrotoxicity of Netilmicin. Scand. J. Infect. Dis. Suppl. 23: 96-102.

5. Kaloyanides, G.J. and Pastoriza-Munoz, E. 1980. Aminoglycoside Nephrotoxicity. Kidney Int. 18: 571-582.

6. Pastoriza-Munoz, E., Bowman, R.L. and Kaloyanides, G.J. 1979. Renal Tubular Transport of Gentamicin in the Rat. Kidney Int. 16: 440-450.

7. Kluwe, W.M., Hook, J.B. 1978. Analysis of Gentamicin Uptake by Rat Renal Cortical Slices. Toxicol. Appl. Pharmacol. 45: 163-175.

8. Silverblatt, F.J. and Kuehn, C. 1979. Autoradiography of Gentamicin Uptake by the Rat Proximal Tubule Cell. Kidney Int. 15: 335-345.

9. Trestman, I., Parsons, J., Santoro, J., Goodhart, G. and Kaye, D. 1978. Pharmacology and Efficiency of Netilmicin. Antimicrob. Agents Chemother. 13: 832-836.

10. Kuo, C.H. and Hook, J.B. 1979. Specificity of Gentamicin Accumulation by the Rat Renal Cortex. Life Sci. 25: 873-878.

11. Cojocel, C. and Hook, J.B. 1983. Effects of Acute Exposures to Gentamicin on the Renal Handling of Proteins. Toxicology 28: 347-356.

12. Just, M. and Habermann, E. 1977. The Renal Handling of Polybasic Drugs: 2. In Vitro Studies with Brush Border and Lysosomal Preparations. Naunyn-Schmiedeberg's Arch. Pharmacol. 300: 67-76.

13. Baylis, C., Rennke, H.R. and Brenner, B.M. 1977. Mechanisms of the Defect in Glomerular Ultra Filtration Associated with Gentamicin Administration. Kidney Int. 12: 344-353.

14. Cojocel, C., Dociu, N., Ceacmacudis, E. and Baumann, K. 1984. Nephrotoxic Effects of Aminoglycoside Treatment on Renal Tubular Reabsorption and Accumulation. Nephron 37: 113-119.

15. Luft, F.C., Aronoff, G.R., Evan, A.P. and Connors, B.A. 1981. The Effect of Anminoglycosides on Glomerular Epithelium: A Comparative Study. Res. Commun. Chem. Pathol. Pharmacol. 34: 89-95.

16. Fabre, J., Fillastre, J.P., Morin, J.P. and Rudhardt, M. 1978. Nephrotoxicity of Gentamicin: Action on Subcellular Organelles and Pharmacokinetics in the Kidney. Cont. Nephrol. 10: 53-62.

17. McMartin, D.N. and Engel, S.G. 1982. Effect of Aging on Gentamicin Nephrotoxicity and Pharmacokinetics in Rats. Res. Commun. Chem. Pathol. Pharmacol. 38 (2): 193-207.

18. Bennett, W.M. Parker, R.A., Elliott, W.M., Gilbert, D.N. and Houghton, D.C. 1982. Sex-related Differences in the Susceptibility of Rats to Gentamicin Nephrotoxicity. J. Infect. Dis. 145: 370-373.

19. Bennett, W.M., Hartnett, M.N., Gilbert, D., Houghton, D. and Porter, G.A. 1976. Effect of Sodium Intake on Gentamicin Nephrotoxicity in the Rat. Proc. Soc. Exp. Biol. Med. 151: 736-738.

20. Hsu, C.H., Kurtz, T.W., Easterling, R.E. and Weller, J.M. 1974. Potentiation of Gentamicin Nephrotoxicity by Metabolic Acidosis. Proc. Soc. Exp. Biol. Med. 146: 894-897.

21. Kosek, J.C., Mazze, R.I. and Cousins, M.J. 1974. Nephrotoxicity of Gentamicin. Lab. Invest. 30: 48-57.

22. Morin, J.P., Olier, B., Viotte, G. and Fillastre, J.P. 1982. Nephrotoxicite des Aminoglycosides et Toxicite Lysosomiale: Approche Biochimique et Morphometrique. In: (Ed) Fillastre J.P. Nephrotoxicite-Ototoxicite. Publications de l'Universite de Rouen: p. 113-129.

23. Luft, F.C., Rankin, L.I., Sloan, R.S. and Yum, M.N. 1978. Recovery from Aminoglycoside Nephrotoxicity with Continued Drug Administration. Antimicrob. Agents. Chemother. 14 (3): 284-287.

24. Houghton, D.C., Hartnett, M., Campbell-Boswell, M., Porter, G. and Bennett, W. 1976. A Light and Electron Microscopic Analysis of Gentamicin Nephrotoxicity in Rats. Am. J. Pathol. 82: 589-612.

25. Chahwala, S.B. and Harpur, E.S. 1982. An Investigation of Effects of Aminoglycoside Antibiotics on Na^+-K^+ ATPase as a Possible Mechanism of Toxicity. Res. Commun. Chem. Pathol. Pharmacol. 35 (1): 63-78.

26. Kluwe, W.M. and Hook, J.B. 1978. Functional Nephrotoxicity of Gentamicin in the Rat. Toxicol. Appl. Pharmacol. 45: 163-175.

27. Sastrasinh, M., Weinberg, J.M. and Humes, H.D. 1982. The Effect of Gentamicin on Calcium Uptake by Renal Mitochondria. Life Sci. 30: 2309-2315.

28. Carlier, M.B., Laurent, G. and Tulkens, P. 1984. In Vitro Inhibition of Lysosomal Phospholipases by Aminoglycoside Antibiotics: A Comparative Study. Arch. Toxicol. Suppl. 7: 282-285.

29. Kaloyanides, G.J. and Feldman, S. 1982. Gentamicin Induces a Phospholipidosis in the Rat. In: (Ed)Fillastre, J.P. Nephrotoxicite-Ototoxicite. Publications de l'Universite de Rouen: pp. 131-145.

30. Beck, P.R., Thompson, R.B. and Chaudhuri, A.K.R. 1977. Aminoglycoside Antibiotics and Renal Function: Changes in Urinary γ-Glutamyl Transferase Excretion. J. Clin. Pathol. 30: 432-437.

31. Mondorf, A.W., Hendus, J., Brier, J. Scherberich, J.E., Schoeppe, W. 1978. Tubular Toxicity Induced by Aminoglycosides in Human Kidneys. In: (Ed) Fillastre, J.P. Nephrotoxicity. Masson Publ. U.S.A. Inc., New York: pp. 168-174.

32. Wellwood, J.M., Lovell, D., Thompson, A.E. and Tigne, J.R. 1976. Renal Damage Caused by Gentamicin: A Study of the Effects on Renal Morphology and Urinary Enzyme Excretion. J. Pathol. 118: 171-182.

33. Luft, F.C., Patel, V., Yum, M.N., Patel, B. and Kleit, S.A. 1975. Experimental Aminoglycoside Nephrotoxicity. J. Lab. Clin. Med. 86: 213-220.

34. Maack, T., Johnson, V., Kau, S.T., Figueiredo, J. and Sigulem, D. 1979. Renal Filtration, Transport and Metabolism of Low Molecular Weight Proteins: A Review. Kidney Int. 16: 251-270.

35. Cojocel, C., Dociu, N., Maita, K., Sleight, S.D. and Hook, J.B. 1983. Effects of Aminoglycosides on Glomerular Permeability, Tubular Reabsorption and Intracellular Catabolism of the Cationic Low-Molecular-Weight Protein Lysosyme. Toxicol. Appl. Pharmacol. 68: 96-109.

36. Cohen, L., Lapkin, R. and Kaloyanides, G.J. 1975. Effect of Gentamicin on Renal Function in the Rat. J. Pharmacol. Exp. Ther. 193 264-273.

37. Bowman, R.L., Silverblatt, F.J. Kaloyanides, G.J. 1977. Comparison of Nephrotoxicity of Netilmicin and Gentamicin in Rats. Antimicrob. Agents Chemother. 13: 474-478.

38. Porter, G., Elliott, W.C., Bennett, W. and McClung, M. 1982. Further Evaluation of the Polyuria Associated with Experimental Aminoglycoside Nephrotoxicity. In: (Ed) Fillastre, J.P. Nephrotoxicite-Ototoxicite. Publications de l'Universite de Rouen: pp 96-108.

39. Cockcroft, D.W., Gault, M.H. 1976. Prediction of Creatinine Clearance from Serum Creatinine. Nephron. 16: 31-41.

40. Bjornsson, T.D. 1979. Use of Serum Creatinine Concentrations to Determine Renal Function. Clin. Pharmacokin. 4: 200-222.

41. Reed, M.D., Vermeulen, M.W., Stern, R.C., Cheng, P.W., Powell, S.H., Boat, T.F. 1981. Are Measurements of Urine Enzymes Useful during Aminoglycoside Therapy? Pediatr. Res. 15: 1234-1239.

42. Adelman, R.D., Halsted, C.C., Jordan, G.W. and Russo, J. 1981. Use of Urinary Enzyme Activities in the Early Detection of Aminoglycoside Nephrotoxicity: A Study in Children and Adults Receiving Gentamicin or Netilmicin. Proc. West. Pharmacol. Soc. 24: 261-264.

43. Davey, P.G., Geddes, A.M. and Cowley, D.M. 1983. Study of Alanine Aminopeptidase Excretion as a Test of Gentamicin Neprotoxicity. J. Antimicrob. Chemother. 11: 455-465.

44. Gibey, R., Dupond, J.L., Alber, D., Leconte des Floris, R. and Henry, J.C. 1981. Predictive Value of Urinary N-acetyl-beta-D-glucosaminidase (NAG), Alanine-aminopeptidase (AAP) and Beta-2-microglobulin (B_2M) in Evaluating Nephrotoxicity of Gentamicin. Clin. Chim. Acta. 116: 25-34.

45. Sethi, K. and Diamond, L.H. 1981. Aminoglycoside Nephrotoxicity and its Predictability. Nephron. 27: 265-270.

46. Halman, J., Price, R.G. and Fowler, J.S.L. 1984. Urinary Enzymes and Isoenzymes of N-acetyl-B-D-Glucosaminidase in the Assessment of Nephrotoxicity. In: (Eds) Werner, M. and Goldberg, D.M. Selected Topics in Clinical Enzymology. Vol. 2. Publ. Walter de Gruyter, Berlin, New York: pp 435-452.

47. Gibey, R., Dupond, J.L. and Henry, J.C. 1984. Urinary N-acetyl-beta-D-glucosaminidase (NAG) Isoenzyme Profiles: A Tool for Evaluating Nephrotoxicity of Aminoglycosides and Cephalosporins. Clin. Chim. Acta. 137: 1-11.

48. Price, R.G. 1982. Review Paper: Urinary Enzymes, Nephrotoxicity and Renal Disease. Toxicology. 23: 99-134.

49. Davey, P.G. and Gosling, P. 1982. B_2-Microglobulin Instability in Pathological Urine. Clin. Chem. 28 (6): 1330-1333.

50. Bernard, A.M., Moreau, D. and Lauwerys, R. 1982. Comparison of Retinol-binding Protein and B_2-microglobulin Determination in Urine for the Early Detection of Tubular Proteinuria. Clin. Chim. Acta. 126: 1-7.

51. Viau, C., Bernard, A., Ouled, A., Lauwerys, S.R. and Tulkens, P. 1984. Polycationic Gentamicin Inhibits the Tubular Reabsorption of Plasma Proteins According to their Electrical Charge. In: (Eds) Bach, P.H. and Lock, E.A. Renal Heterogeneity and Target Cell Toxicity. Proceedings of the Second International Symposium on Nephrotoxicity. Publ. J. Wiley, Chichester: pp 353-356.

52. Clyne, D.H., Kant, K.S., Pesce, A.J. and Pollak, V.E. 1979. Nephrotoxicity of Low Molecular Weight Serum Proteins: Physico-chemical Interactions between Myoglobin, Hemoglobin, Bence-Jones Proteins and Tamm-Horsfall Proteins. In: (Eds) Dubach., U.C. and Schmidt, U. Current Probs. in Clin. Biochem. 9: Diagnostic Significance of Enzymes and Proteins in Urine. Publ. Huber, Vienna: pp 299-308.

53. Harrison, J.F., Parker, R.W. and DeSilva, K.L. 1973. Lysozymuria and Acute Disorders of Renal Function. M. Clin. Path. 26: 278-284.

54. Nicot, G., Merle, L., Charmes, J.P., Valette, J.P. and Lachatre, G. 1980. Letter: Lysozymuria and Gentamicin Nephrotoxicity. New Eng. J. Med. 303: 48-49.

55. Valette, J.P., Nicot, G., Charmes, J.P., Merle, L. and Benevant, D. 1979. Low Molecular Weight Proteins as Urinary Markers of Aminoglycoside Nephrotoxicity in Man. Proc. Eur. Dial. Transplant. Assoc. 16: 597-602.

56. Harrison, J.F., Lunt, G.S., Scott, P. and Blainey, J.D. 1968. Urinary Lysozyme, Ribonuclease and Low Molecular Weight Protein in Renal Disease. Lancet. 1: 371-375.

57. Hayslett, J.P., Perillie, P.E. and Finch, S.C. 1968. Urinary Muramidase and Renal Disease: Correlation with Renal Histology and Implication for the Mechanism of Enzymuria. New Eng. J. Med. 279: 506-512.

58. Prockop, D.J. and Davidson, W.D. 1964. A Study of Urinary and Serum Lysozyme in Patients with Renal Disease. New Eng. J. Med. 270: 269-274.

59. Barratt, T.M. and Crawford, R. 1970. Lysozyme Excretion as a Measure of Renal Tubular Dysfunction in Children. Clin. Sci. 39: 457-465.

60. Favre, H., Wauters, J.P. and Mach, R.S. 1971. Clinical Diagnostic Value of Lysozymuria. Adv. Nephrology. 1: 195-205.

61. Evan, A.P. and Dail, W.G. 1974. The Effects of Sodium Chromate on the Proximal Tubules of the Rat Kidney. Lab. Invest. 30: 704-715.

62. Perillie, P.E., Kaplan, S.S., Lefkowitz., Rogaway, W. and Finch, S.C. 1968. Studies of Muramidase in Leukaemia. J.A.M.A. 203: 317-326.

63. Kanai, M., Raz, A., Goodman, D.S. 1968. Vitamin A Transport in Human Plasma. J. Clin. Invest. 47: 2025-2044.

64. Peterson, P.A. 1971. Characteristics of a Vitamin A Transporting Protein Complex Occurring in Human Serum. J. Biol. Chem. 246 (1): 34-43.

65. Peterson, P.A. and Rask, L. 1971. Studies on the Fluorescence of the Human Vitamin A-transporting Plasma Protein Complex and its Individual Components. J. Biol. Chem. 246: 7544-7550.

66. Peterson, P.A. 1971. Demonstration in Serum of Two Physiological Forms of the Human Retinol Binding Protein. Eur. J. Clin. Invest. 1: 437-444.

67. Vahlquist, A., Peterson, P.A. and Wibell, L. 1973. Metabolism of the Vitamin A-transporting Protein Complex. 1: Turnover Studies in Normal Persons and in Patients with Chronic Renal Failure. Eur. J. Clin. Invest. 3: 352-362.

68. Fex, G., Albertsson, P.A. and Hansson B. 1979. Interaction between Prealbumin and Retinol Binding Protein Studied by Affinity Chromatography, Gel Filtration and Two-Phase Partition. Eur. J. Biochem. 99: 353-360.

69. Smith, F.S. and Goodman, De W.S. 1971. The Effects of Diseases of the Liver, Thyroid and Kidneys on the Transport of Vitamin A in Human Plasma. J. Clin. Invest. 50: 2426-2436.

70. Raz, A., Shiratori, T. and Goodman, De W.S. 1970. Studies on the Protein-Protein and the Protein-Ligand Interactions Involved in Retinol Transport in Plasma. J. Biol. Chem. 245: 1903-1912.

71. Heller, J. and Horwitz, J. 1973. Conformational Changes Following Interaction between Retinol Isomers and Human RBP and between Human RBP and Prealbumin. J. Biol. Chem. 248: 6308-6316.

72. Peterson, P.A. and Berggard, I. 1971. Isolation and Properties of a Human Retinol Transporting Protein. J. Biol. Chem. 246: 25-33.

73. Fex, G. and Hansson, B. 1979. Retinol-Binding Protein from Human Urine and its Interaction with Retinol and Prealbumin. Eur. J. Biochem. 94, 307-313.

74. Bosin, E. and Monji, N. 1983. Quantitation of Serum Retinol-Binding Protein by an Enzyme-Linked Immunosorbent Assay. Anal. Biochem. 133: 283-287.

75. Parvianen, M.T. and Ylitalo, P. 1983. Immunonephelometric determination of Retinol-Binding Protein in Serum and Urine. Clin. Chem. 29 (5): 853-856.

76. Scarpioni, L., Dall'Aglio, P.P., Poisetti, P.G. and Buzio, C. 1976. Retinol Binding Protein in Serum and Urine of Glomerular and Tubular Nephropathies. Clin. Chim. Acta. 68: 107-113.

77. Davey, P., Gozzard, D., Goodall, M. and Leyland, M.J. 1985. Hypomagnesaemia: An Underdiagnosed Interaction between Gentamicin and Cytotoxic Chemotherapy for Acute Non-lymphoblastic Leukaemia. J. Antimicrob. Chemother. 15: 623-628.

78. Kelnar, C.J.H., Taor, W.S., Reynolds, D.J., Smith, D.R., Slavin, B.M. and Brook, C.G.D. 1978. Hypomagnesaemic Hypocalcaemia with Hypokalaemia caused by Treatment with High Dose Gentamicin. Arch. Dis. Child. 53: 817-820.

79. Patel, R. and Savage, A. 1979. Symptomatic Hypomagnesaemia Associated with Gentamicin Therapy. Nephron 23: 50-52.

80. Bar, R.S., Wilson, H.E. and Mazzaferri, M.D. 1975. Hypomagnesaemic Hypocalcaemia Secondary to Renal Magnesium Wasting: A Possible Consequence of High-Dose Gentamicin Therapy. Ann. Intern. Med. 82: 646-649.

81. Holmes, A.M., Hesling, C.M. and Wilson, T.M. 1970. Drug-Induced Secondary Hyperaldosteronism in Patients with Pulmonary Tuberculosis. Q.J. Med. 39: 299-315.

82. Zaloga, G.P., Chernow, B., Pock, A., Wood, B., Zaritsky, A. and Zucker, A. 1984. Hypomagnesaemia is a Common Complication of Aminoglycoside Therapy. Surg. Gyn. Obst. 158: 561-565.

83. Goodhart, G.L., Handelsman, S. 1985. Gentamicin and Hypokalaemia (Letter). Ann. Intern. Med. 103: 645-646.

84. Juan, D. 1979. Hypocalcaemia: Differential Diagnosis and Mechanisms. Arch. Intern. Med. 139: 1166-1171.

85. Suh, S.M., Tashjian, A.J., Matsuo, N., Parkinson, D.J. and Frazer, D. 1973. Pathogenesis of Hypocalcaemia in Primary Hypomagnesaemia: Normal End Organ Responsiveness to Parathyroid Hormone, Impaired Parathyroid Gland Function. J. Clin. Invest. 52: 153-160.

86. Rankin, L.I., Krous, H., Whong, R. and Fryer, A.W. 1981. The Effects of Magnesium Deficiency on Gentamicin Nehprotoxicity. Clin. Res. 29 (4): 745A.

87. Chahwala, S.B. and Harpur, E.S. 1983. Gentamicin-induced Hypercalciuria in the Rat. Acta pharmacol. et toxicol. 53: 358-362.

88. Boasson, E.H. 1937. On the Bacteriolysis by Lysosyme. J. Immunol. 34 (4): 281-293.

89. Houser, M.T. 1983. Improved Turbidometric Assay for Lysozyme in Urine. Clin. Chem. 29 (8): 1488-1493.

90. Voller, A., Bidwell, D.E. and Bartlett, A. 1979. The Enzyme-Linked Immunosorbent Assay: A Guide with Abstracts of Microplate Applications. Publ. Dynatech Laboratories Ltd., Billingshurst, Sussex.

91. Lucertini, S., Valcavi, P., Mutti, A. and Franchini, I. 1984. Enzyme-linked Immunosorbent Assay of Retinol-Binding Protein in Serum and Urine. Clin. Chem. 30 (1): 149-151.

92. Lugtenberg, B., Meijers, J., Peters, R., van der Hoek, P. and van Alphen. 1975. Electrophoretic Resolution of the Major Outer Membrane Protein of Escherichia Coli K12 into Four Bands. FEBS Lett. 58: 254-258.

93. Towbin, H., Staehelin, T. and Gordon, J. 1979. Electrophoretic Transfer of Proteins from Polyacrylamide Gels to Nitrocellulose Sheets. Proc. Natl. Acad. Sci. U.S.A. 76: 4350-4354.

94. Peterson, P.A. 1971. Studies on the Interaction between Prealbumin, Retinol-Binding Protein and Vitamin A. J. Biol. Chem. 246 (1): 44-49.

95. Vahlquist, A. and Peterson, P.A. 1973. Combination of Specific Antibodies with the Human Vitamin A-transporting Complex. J. Biol. Chem. 248 (11): 4040-4046.

96. Smith, F.R., Raz, A. and Goodman, De W.S. 1971. Radioimmunoassay of Human Plasma Retinol-Binding Protein. J. Clin. Invest. 49: 1754-1761.

97. Ishikawa, E. Enzyme-Linked Immunoassay Techniques. In: Techniques in Metabolic Research B217. Publ. Elesvier/North-Holland Scientific Publishers Ltd.: pp 1-18.

98. Chessum, B.S. and Denmark, J.R. 1978. Inconstant ELISA (Letter) Lancet, i: 161.

99. Essink, A.W.G., Arkesteijn, G.J.M.W. and Notermans, S. 1985. Interference of Lysosyme in the Sandwich Enzyme-Linked Immunosorbent Assay. (ELISA). J. Immunolog. Methods, 80: 91-96.

100. Mancini, G., Carbonara, A.O. and Heremans, S.J.F. 1965. Immunochemical Quantitation of Antigens by Single Radial Immunodiffusion. Immunochem. 2: 235-254.

101. Smith, C.R., Lipsky, J.J., Laskin, O.L., Hellman, D.B., Mellits, E.D., Longstreth, J. and Lietman, P.S. 1982. Double-Blind Comparison of the Nephrotoxicity and Auditory Toxicity of Gentamicin and Tobramycin. New Eng. J. Med. 302: 1106-1109.

102. Bernard, A.M., Moreau, D. and Lauwerys, R.R. 1982. Latex Immunoassay of Retinol-Binding Protein. Clin. Chem. 28 (5): 1167-1171.

103. Adelman, R.D., Spangler, W.L., Beasom, F., Ishizaki, G. and Conzelman, G.M. 1981. Frusemide Enhancement of Netilmicin Nephrotoxicity in Dogs. J. Antimicrob. Chemother. 7: 431-440.

104. Davey, P.G., Cowley, D.M., Geddes, A.M. and Terry, J. 1984. Clinical Evaluatin of B_2-Microglobulin, Muramidase and Alanine Aminopeptidase as Markers of Gentamicin Nephrotoxicity. Contr. Nephrol. 42: 100-106.

105. Wide, L. and Thoren, L. 1972. Increased Urinary Clearance for Albumin, B_2-Microglobulin, Insulin and Luteinising Hormone Following Surgical or Accidental Trauma. Scand. J. Clin. Lab. Invest. 30: 275-279.

106. Gofferje, H. and Maintz, E. 1978. Das Verhalten von Pralbumin, Retinol-bindendem Protein, Transferrin und Haptoglobin in der Postoperativen und Posttraumischen Phase. Infusionstherapie 5: 268-272.

107. Arroyave, G. and Calcano, M. 1979. Descenso de los Niveles Sericosde Retinol y su Proteina de Enlace (RBP) Durante los Infecciones. Arch. Latinoamericanos. Nutr. 29 (2): 233-260.

108. Hemmingsen, L. and Skaarup, P. 1977. Urinary Excretion of Ten Plasma Proteins in Patients with Febrile Diseases. Acta. Med. Scand. 201: 359-364.

109. Jensen, H. and Henriksen, K. 1974. Proteinuria in Non-Renal Infectious Diseases. Acta. Med. Scand. 196: 75-82.

110. Ehrich, J.H.H., Foellmer, H.G., Krull, F., Withycombe, C., Horstmann, R.D., Dietrich. M. 1981. Proteinuria in Non-Renal Infectious Diseases. Contr. Nephrol. 24: 122-133.

111. Linton, A.L., Richmond, J.M., Walker, J.F. and Sibbald, W.J. 1984. Proteinuria and Renal Pathology in Generalised Sepsis. Contr. Nephrol. 42: 118-122.

112. Trollfors, B., Alestig, K., Krantz, I. and Norrby, R. 1980. Quantitative Nephrotoxicity of Gentamicin in Non-Toxic Doses. J. Infect. Dis. 141: 306-309.

113. Smith, F.R., Underwood, B.A., Denning, C.R. Varma, A. and Goodman De W.S. 1972. Depressed Plasma Retinol-Binding Protein Levels in Cystic Fibrosis. J. Lab. Clin. Med. 80: 423.

114. Navarro, J. and Desquilbet, N. 1981. Depressed Plasma Vitamin A and Retinol-Binding Protein in Cystic Fibrosis, Correlations with Zinc Deficiency (Letter). Am. J. Clin. Nutr. 34 (7): 1439-1440.

115. Wilkinson, R., Lucas, G.L., Heath, D.A., Franklin, I.M. Boughton, B.J. 1986. Hypomagnesaemic Tetany Associated with Prolonged Treatment with Aminoglycosides. Br. Med. J. 292: 818-819.

116. Keating, M.J., Sethi, M.R., Bodey, G.P. and Samaan, N.A. 1977. Hypocalcaemia with Hypoparathyroidism and Renal Tubular Dysfunction Associated with Aminoglycoside Therapy. Cancer. 39: 1410-1414.

117. Green, C.G., Doershuk, C.F. and Stern, R.C. 1985. Symptomatic Hypomagnesemia in Cystic Fibrosis. J. Pediatr. 107: 425-428.

118. Heaton, F.W. 1964. Magnesium Excretion in Surgical Patients. Clin. Chim. Acta. 9: 327-333.

119. Walker, W.F., Fleming, L.W. and Stewart, W.L. 1968. Urinary Magnesium Excretion in Surgical Patients. Br. J. Surg. 55: 466-469.

120. Mountokalakis, T., Tzouvelekis, E. and Mayopoulou-Synvoulidou, D. 1985. Effects of Gentamicin in Renal Magnesium Excretion. In: (Eds) Halpern and Durlach. Magnesium Deficiency: First European Congress on Magnesium, Lisbon 1983: pp 172-174.

121. Gruskin, A.B., DiLiberti, J.H. and Baluarte, H.J. 1973. Calcium Excretion in Cystic Fibrosis. 82: 344-345.

122. Robertson, W.G. 1976. Urinary Excretion. In: (Ed) Robertson, W.G. Calcium, Phosphate and Magnesium Metabolism. Publ. Churchill Livingstone: pp113-161.

123. Heaton, F.W. and Hodgkinson, A. 1963. External Factors Affecting Diurnal Variation in Electrolyte Excretion with Particular Reference to Calcium and Magnesium. Clin. Chim. Acta. 8: 246-254.

124. Moore-Ede, M.C. Faulkner, M.H. and Tredre, B.E. 1972. An Intrinsic Rhythm of Urinary Calcium Excretion and the Specific Effect of Bedrest on the Excretary Pattern. Clin. Sci. 42: 433-445.

125. Stier, C.T. and Itskovitz, H.D. 1986. Renal Calcium Metabolism and Diuretics. Ann. Rev. Pharmacol. Toxicol. 26: 101-116.

126. Rude, R.K. and Singer, M.D. 1981. Magnesium Deficiency and Excess. Ann. Rev. Med. 32: 245-259.

127. Goldberg, M., Agus, Z.S. and Goldfarb, S. 1976. Renal Handling of Calcium and Phosphate. In (Ed) Thurau, K. International Review of Physiology. Kidney and Urinary Tract Physiology 2, Volume 2. University Park Press, Baltimore: pp. 211-247.

128. Sutton, R.A.L. 1970. Plasma Magnesium Concentration in Primary Hyperparathyroidism. Br. Med. J. 1: 529-533.

129. King, R.G. 1970. Stanbury, S.W. 1970. Magnesium Metabolism in Primary Hyperparathyroidism. Clin. Sci. 25: 475-485.