INVESTIGATIONS OF THE SITE AND MECHANISMS OF DRUG-INDUCED REDUCTIONS IN THE REABSORPTION OF DIVALENT CATIONS BY THE KIDNEY

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Thesis Summary

Disturbances in electrolyte homeostasis are a frequent adverse side-effect of the administration of aminoglycoside antibiotics, such as gentamicin, and the antineoplastic agent cis-platinum. The aims of this work were to further elucidate the site(s) and mechanism(s) by which these drugs may produce disturbances in the renal reabsorption of calcium and magnesium. These investigations were undertaken using a range of *in vivo* and *in vitro* techniques and models.

Initially, a series of *in vivo* studies was conducted to delineate aspects of the acute and chronic effects of both drugs on renal electrolyte handling and to select and evaluate an appropriate animal model: subsequent investigations were focused on gentamicin. In a study of the acute and chronic effects of cis-platinum administration, there were pronounced acute changes in a variety of indices of nephrotoxic injury, including electrolyte excretion. Most effects resolved but there were chronic increases in the urinary excretion of calcium and magnesium. The renal response of three strains of rat (Fischer 344, Sprague-Dawley (SD), and Wistar) to a ranges of doses of gentamicin was also investigated. Drug administration produced substantially different responses between strains, in particular marked differences in calcium and magnesium excretion. The results suggested that the SD rat was an appropriately sensitive strain for use in further investigations. Acute infusion of gentamicin in the anaesthetised SD rat produced rapid, substantial increases in the fractional excretion of calcium and magnesium, while sodium and potassium output were unaffected, confirming previous results of similar experiments using F344 rats. Studies using lithium clearance measurements in the anaesthetised SD rat were undertaken to investigate the effects of gentamicin on proximal tubular calcium reabsorption. Lithium clearance was unaffected by acute gentamicin infusion, suggesting that the site of acute gentamicin-induced hypercalciuria may not be located in the proximal tubule.

Inhibition of Ca2+ ATPase activity was investigated as a potential mechanism by which calcium reabsorption could be affected after aminoglycoside administration. *In vitro*, both Ca2+ ATPase and Na^+/K^+ ATPase activity could be similarly inhibited by the presence of aminoglycosides, in a dose-related manner. Whilst inhibition of Na^+/K^+ ATPase could be demonstrated biochemically after *in vivo* administration of gentamicin, there were no concurrent effects on Ca2+ ATPase activity, suggesting that inhibition of Ca2+ ATPase activity is unlikely to be a primary mechanism of aminoglycoside-induced reductions of calcium reabsorption. Histochemical studies could not discern inhibition of either Na^+/K^+ ATPase or Ca2+ ATPase activity after *in vivo* administration of gentamicin.

Selection of renal cell lines for further investigative *in vitro* studies on the mechanisms of altered cation reabsorption was considered using MTT (3-(4,5,-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) and Neutral Red cytotoxicity assays. The ability of LLC-PK1 and LLC-RK1 cell lines to correctly rank a series of nephrotoxic compounds with their known nephrotoxic potency *in vivo* was studied. Using these cell lines grown on semi-permeable inserts, alterations in the paracellular transport of ^{45}Ca was investigated as a possible mechanism by which gentamicin could alter calcium reabsorption *in vivo*. Short term exposure (1 h) of LLC-RK1 cells to gentamicin, via both cell surfaces, resulted in a reduction in paracellular permeability to both transepithelial ^{3}H-mannitol and ^{45}Ca fluxes. When LLC-RK1 cells were exposed via the apical surface only, similar dose-related reductions were seen to those observed when cells were exposed to the drug from both sides. Short-term basal exposure to gentamicin appeared to contribute less to the observed reductions in ^{3}H-mannitol and ^{45}Ca fluxes. Experiments investigating transepithelial movement of ^{45}Ca and ^{3}H-mannitol on LLC-PK1 cells after acute gentamicin exposure were inconclusive. Longer exposure (48 h) to gentamicin caused an increase in the permeability of the monolayer and a consequent increase in transepithelial ^{45}Ca flux in the LLC-RK1 cell line; increases in permeability of LLC-PK1 cells to ^{45}Ca and ^{3}H-mannitol were not apparent under the same conditions.

The site and mechanism at which gentamicin, in particular, alters calcium reabsorption cannot be definitively described from these studies. However, indirect evidence from lithium clearance studies suggests that the site of the lesion is unlikely to be located in the proximal tubule. The mechanism by which gentamicin exposure alters calcium reabsorption may be by reducing paracellular permeability to calcium rather than by altering active calcium transport processes.

Keywords: Nephrotoxicity, aminoglycoside antibiotics, cis-platinum, renal wasting of electrolytes, calcium.

This thesis is dedicated to my mother,

for always believing in me.

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1. Introduction

The clinical use of aminoglycoside antibiotics and the antineoplastic agent cis-platinum frequently result in a variable degree of nephrotoxic injury. Both aminoglycosides and cis-platinum appear to exert selective effects on the renal handling of cations, particularly calcium and magnesium. The consequences of inadequate renal conservation of these ions may be manifest as lowered plasma concentrations of both calcium and magnesium, leading to potentially life-threatening clinical situations.

1.1 Aminoglycoside Nephrotoxicity

"Gentamicin, the most frequently prescribed aminoglycoside in the UK has had a prominent role in the management of hospital infection for nearly 30 years. However, toxicity due to gentamicin administration is currently one of the commonest problems for the Medical Defence Union in the field of hospital infection." (Davey, 1991). Aminoglycoside antibiotics, are widely used in the treatment of serious systemic infections caused by Gram-negative organisms but their clinical usefulness is mainly limited by their nephrotoxic potential (Appel and Neu, 1978). Unfortunately, effective therapeutic doses of aminoglycosides approach those which are also potentially toxic.

Aminoglycosides are amino sugars joined by glycosidic linkages. They are extremely polar polycations with pKa values of 8.0 or higher. The number of free amino groups is responsible for their cationic nature and hence for their ability to bind to anionic components of cellular membranes. Cell membranes are relatively impermeable to these compounds due to their polar nature and poor lipid solubility.

Aminoglycosides are excreted almost exclusively by the kidney with only 1-2% of the dose excreted in the bile. These compounds are freely filtered at the glomerulus and the clearance of gentamicin is similar to that of inulin (Glyselynck et al, 1971). Approximately 20% of the filtered load of gentamicin is accumulated by the proximal

tubular cells in an energy-dependent process (Kluwe and Hook, 1978). Initial binding of gentamicin at the cell membrane is apparently to anionic phospholipids (Sastrasinh et al, 1982; Kaloyanides and Mc Laughlin, 1983) followed by rapid internalisation of the compound by a pinocytotic mechanism with subsequent transfer to lysosomes (Silverblatt and Kuehn, 1979).

Indirect evidence which suggests aminoglycosides can gain access to proximal tubular cells via the basolateral membrane has been produced in both renal slice preparations (Bennett et al, 1982) and in the non-filtering isolated perfused kidney (Collier et al, 1979). However autoradiographic studies have shown initial binding and uptake of gentamicin occurs mainly at the apical rather than the basolateral membrane (Collier et al, 1979), and is load dependant (Pastoriza-Munoz et al, 1979). These data demonstrate that whilst gentamicin accumulation can occur at both of the plasma membrane surfaces quantitatively accumulation is greatest via the brush border membrane and is probably the major route by which aminoglycosides are taken up *in vivo*. Gentamicin binding in brush border membrane vesicles has been shown to be competitively inhibited by the presence of other aminoglycosides and by the presence of increasing concentrations of calcium (Sastrasinh et al, 1982).

As a result of binding and sequestration, aminoglycosides accumulate to levels of 5-20 times the serum concentration within the proximal tubule cells (Seale and Rennert, 1982, Pastoriza-Munoz et al, 1979). The half life of these drugs in the renal cortex is approximately 100 h, compared to a serum half life of 30 min in the rat and of 2 h in humans (Luft et al, 1976).The protracted renal half lives of these compounds results in prolonged exposure of the kidney to their harmful effects (Schentag et al, 1977).

Aminoglycosides primarily affect the functional capabilities of the proximal tubular cells but additional effects have been demonstrated on the glomeruli. Gentamicin reduces the ultrafiltration coefficient of the glomerular filtration barrier possibly by decreasing the

size and number of glomerular fenestrae (Luft et al, 1981, Cojocel and Hook, 1983). These alterations in permeability of the glomeruli appear to have little effect on the measured GFR since nearly stable GFR values have been demonstrated in the rabbit when the ultrafiltration coefficient was reduced by 50% (Savin et al, 1985). Histological changes in proximal tubule cells have been demonstrated to occur without substantial changes in more distal parts of the nephron (Kosek et al, 1974, Houghton et al, 1976). Specific toxicity in this segment is almost certainly due to the selective accumulation of gentamicin in the proximal convoluted tubule (S1 and S2 segments) in the rat (Weeden et al, 1983). Whilst substantial sequestration of gentamicin can occur in the pars recta of the rabbit (Vandewalle et al, 1981) the observed accumulation of gentamicin in the S1 and S2 segments in the rat is consistent with the toxicity shown histologically after gentamicin administration. Drug accumulation begins within 30-60 min of the first dose (Silverblatt and Kuehn, 1979) and can continue through subtle changes in cell architecture and function until eventually necrosis of the tubules is produced. At the later stages of toxicity cortical levels of the drug drop markedly since the cells lose the ability to retain and / or accumulate the compound.

Characteristically the initial ultrastructural changes seen after aminoglycoside dosing include an increase in the number and size of the lysosomal complement of the cell along with the formation of myeloid bodies consisting of partially degraded membrane phospholipid material within the secondary lysosomes (Houghton et al, 1976). Aminoglycosides inhibit the lysosomal enzymes phospholipases A and C derived from renal cortex (Hostetler and Hall, 1982, Laurent et al, 1982). Since aminoglycosides have the ability to alter the activity of phospholipases the resultant phospholipidosis which occurs after administration of the drug may be a primary cause of the toxic effects of these compounds. The aminoglycosides also appear to have a dual effect on the stability of the lysosomal membrane. At low concentrations the water permeability characteristics are altered leading to swelling of the organelle. At higher concentrations the membrane becomes further destabilised and ruptures, releasing both substantial concentrations of

the drug and potent degradative enzymes into the internal milieu (Powell and Reidenberg, 1982). This deposition of the lysosomal contents into the cell then exposes organelles such as mitochondria to direct toxic effects of these agents from which they would ordinarily be protected.

In addition to effects on lysosomal phospholipases, aminoglycosides alter the phospholipid content of the basolateral membrane soon after their administration (Kaloyanides, 1984). Derangements which the aminoglycosides induce in membrane permeability, Na/K ATPase activity, and membrane fluidity, may be produced through their actions on membrane phospholipid content and hence this may be a primary mechanism through which aminoglycosides cause toxicity.

When mitochondria are exposed to aminoglycosides specific effects at the inner mitochondrial membrane are also evident. Gentamicin competes with magnesium for membrane binding sites resulting in altered membrane permeability to sodium and potassium and perturbations in mitochondrial respiratory function (Weinberg and Humes, 1980, Weinberg et al, 1980a). Other aminoglycosides also exert similar effects on mitochondria with a relative potency which correlates with their cationic nature. Whilst the effects of aminoglycosides on mitochondrial function may not be their primary toxic action, compromised function of these organelles must undoubtedly contribute to the pathogenesis of renal failure observed after their administration. Mitochondrial swelling as a consequence of functional changes, degradation of the brush border, and eventual cytoplasmic vacuolisation have been shown to occur at the later stages of cellular disruption induced by aminoglycosides. The ultimate result of these changes is the irreversible deterioration of cellular function leading to necrotic cell death, although the precise mechanism by which this is achieved is, as yet, unresolved (Houghton et al, 1976).

With continued aminoglycoside administration regeneration of the proximal tubular cells can occur with an apparently increased resistance to the toxic effects of these compounds (Gilbert et al, 1979). Surprisingly, these areas of cells with increased resistance to aminoglycoside toxicity have been shown to coexist in tissue with substantial patches of necrosis. Newly regenerated proximal tubules cell lack the ability to transport gentamicin and therefore drug accumulation would recommence only when the cells mature sufficiently to develop transport mechanisms for the drug (Houghton et al, 1986). This asynchrony of sensitivity may reflect differences in the rate of maturation of cell types and may therefore not be a true increase in resistance of the cells to the toxic effects of aminoglycosides.

Experimentally-induced aminoglycoside nephrotoxicity consistently produces renal failure associated with a concentrating defect similar to the non-oliguric renal failure observed in humans. Additional markers of toxic injury such as various types of enzymuria (Mondorf et al, 1978, Beck et al, 1977, Patel et al, 1975), proteinuria (Soberon et al, 1978), and polyuria (Kosek et al, 1974, Cohen et al, 1975) are also measurable after aminoglycoside administration. Alterations in organic acid transport have also been shown to occur following aminoglycoside treatment (Cohen et al, 1975, Bennett et al, 1978) as have reductions in ammoniagenesis and gluconeogenesis in renal cortical tissue (Kluwe and Hook, 1978). Whilst glucose uptake in renal cortical slices obtained after *in vivo* gentamicin administration was unaffected (Kluwe and Hook, 1978), glucosuria has been observed in other studies (Ginsberg et al, 1976). The clinical criteria of renal function, elevated serum levels of blood urea nitrogen or creatinine, associated with a reduced GFR tend to be visible only during the latter stages of the toxic response to aminoglycoside dosing.

Numerous conditions modify the magnitude of aminoglycoside-induced nephrotoxicity. In the experimental situation the choice of animal species, strain, age (Flandre and Damon, 1967), gender (Bennett et al, 1982), along with the aminoglycoside (Soberon et

al, 1978), its dose, and dosing regimen (Plamp et al, 1978) can all affect the severity of the toxicity produced. Additionally extracellular volume depletion exacerbates aminoglycoside-induced nephrotoxicity (Bennett et al, 1976) presumably by increasing the concentration of the drug delivered to the tubule. Dietary magnesium or potassium depletion (Rankin et al 1981, Brinker et al, 1981) and metabolic acidosis (Elliot et al, 1980) all heighten the nephrotoxic effects of aminoglycosides by mechanisms which remain unclear.

In contrast, phosphate depletion (Eknoyman et al, 1984), and uncontrolled streptozotocin- induced diabetes mellitus (Texeira et al 1982) appear to protect the kidney from aminoglycoside-induced toxicity. Dietary supplementation of sodium has been shown to reduce the cortical content of gentamicin without altering the pathology of changes in renal function when compared to animals treated with gentamicin on a normal sodium containing diet (Bennet et al, 1976). Dietary calcium supplementation has also been shown to ameliorate gentamicin toxicity (Bennett et al, 1982, Humes et al, 1984, Quarum et al, 1984) and the mechanisms by which this may occur have been extensively studied. Since hypercalcaemia is not produced under these conditions an increase in the filtered load of calcium does not afford protection to the proximal tubular cells simply by a competitive inhibition of gentamicin uptake. Additionally, spontaneously hypertensive hypercalciuric WKY rats were not protected from the nephrotoxic action of gentamicin merely by the presence of a pre-existing hypercalciuria (Mc Carron et al, 1984). Since the effects of calcium supplementation appeared not to be a simple competitive binding interaction, attention turned to the effects of calcium supplementation on PTH levels. Anionic phospholipids, the initial binding site for aminoglycosides, show an increased rate of turnover in the presence of PTH (Farese et al, 1980). Under conditions of PTH suppression as produced with a high calcium diet, the converse may also be true producing a reduction in binding and uptake of aminoglycosides. However, tissue concentrations of gentamicin have not consistently been reduced after dietary supplementation with calcium (Humes et al, 1984, Bennett et

al, 1982, Quarum et al, 1984) which implies that the reduction in toxicity may not be due to reduced renal accumulation of the drug. Circulating levels of PTH appear to alter the sensitivity of the kidney to the effects of aminoglycosides. Parathyroidectomised rats were demonstrated to be less sensitive to gentamicin-induced nephrotoxicity than PTH-stimulated controls (Bennett et al, 1985, Elliott et al, 1987). Renal accumulation of gentamicin in parathyroidectomised rats was increased in one study (Bennett et al, 1985) but not in another (Elliott et al, 1987). Bennett et al (1985), also reported that increased accumulation of gentamicin was not accompanied by an increase in the number of binding sites in the cell membrane after PTH administration. Calcium supplementation appears to protect against aminoglycoside nephrotoxicity by altering levels of circulating PTH, but the cellular mechanisms by which this effect is achieved remain as yet undefined.

1.2 Electrolyte Disturbances arising from Aminoglycoside Administration.

Holmes et al in 1970 probably published the earliest clinical report which suggested aminoglycosides could disrupt electrolyte homeostasis. The data described the occurrence of hypokalaemic hypomagnesaemic alkalosis, in the absence of other signs of reduced renal function, in four patients undergoing treatment for pulmonary tuberculosis with a combination of drugs including gentamicin. Measurements of urinary excretion of potassium and magnesium in two patients showed inappropriately high excretions of these ions in the face of substantially reduced plasma levels. In addition to these alterations, elevations in the plasma levels of aldosterone and renin led the authors to suggest that gentamicin induced a secondary hyperaldosteronism to produce the electrolyte imbalance.

Subsequent clinical data revealed a steady flow of cases which implicated aminoglycosides in the production of electrolyte disorders, both after intramuscular injection (Rangecroft et al, 1978, Bar et al, 1975, Kelnar et al, 1978, Goodhart and Handelsman, 1985, Green et al, 1985, Wilkinson et al, 1986, Kelnar et al, 1986) and

topical application (Bamford and Jones, 1978). Symptomatic hypomagnesaemia tended to occur after repeated courses of therapy without conventional indices of nephrotoxicity necessarily being altered. However, at low total doses of gentamicin hypomagnesaemia may also be produced without significant changes in overall renal function, leading to the suggestion that hypomagnesaemia may be assymptomatic and a clinically underdiagnosed phenomenon (Zaloga et al, 1984, Davey et al, 1985). Electrolyte disturbances may not be recognised until some time after cessation of therapy (Holmes et al, 1970, Bar et al, 1975, Kelnar et al, 1978, Watson et al, 1981) and persist for many months thereafter before returning to normal plasma levels (Davey et al, 1985).

In addition to the primary hypomagnesaemia aminoglycosides also induced associated hypokalaemia and hypocalcaemia with inappropriately high excretions of these cations (Shils, 1969). Concurrently, plasma levels of PTH were found to be consistently reduced. Only one other study in a single patient (Patel and Savage, 1979) confirmed the increases in plasma aldosterone and renin found by Holmes et al (1970), after gentamicin therapy. Evidence that gentamicin produces tubular wasting of magnesium and potassium independent of effects on GFR and aldosterone levels in the rat was presented by Smith et al (1981), suggesting that in the rat experimental model gentamicin does not cause electrolyte wasting by inducing hyperaldosteronism as was suggested in the early clinical studies. Gentamicin apparently produced effects on sodium wasting which were independent of those on potassium handling. These data therefore implicated alterations in parathyroid hormone levels or action rather than renin and aldosterone as a mechanism of aminoglycoside-induced toxicity.

Hypomagnesaemia is thought to produce hypocalcaemia by impairing the release of PTH (Rude et al, 1976, Anast et al, 1976) and / or by impairing its action on the kidney (Levi et al, 1974, Estep et al, 1969). Hypokalaemia with associated potassium wasting by the kidney has also been reported to arise from the primary disorder of renal magnesium wasting (Shils, 1969, Gitelman et al, 1966), suggesting linked renal transport of the two

cations. Hypomagnesaemia induced by aminoglycosides is responsive to supplementation with magnesium salts (Davey et al, 1985, Wilkinson et al, 1986). The associated hypocalcaemia and hypokalaemia is unresponsive to supplementation with the respective salts but improves over time on supplementation with magnesium (Wilkinson et al, 1986), indicating again that the primary renal defect is associated with magnesium wasting and other electrolyte disruptions are of a secondary nature.

An increased incidence of hypomagnesaemia, of varying extent, has been reported after concurrent administration of gentamicin with cytotoxic drugs for treatment of acute non-lymphoblastic leukaemia. Davey et al (1985), showed data in which six of eleven patients became hypomagnesaemic, three of which were symptomatic. Additional secondary disorders included hypocalcaemia and hypokalaemia. These patients all demonstrated an inability to adequately conserve urinary magnesium at low plasma levels supporting the hypothesis that the maintenance of low plasma levels is due to magnesium wasting by the kidney, an effect which persisted for several months after drug administration ceased. Additional studies involving gentamicin plus cytotoxic chemotherapy confirmed the synergistic effects of these regimens and also demonstrated reduced levels of PTH, as found in the absence of the compounding effects of cytotoxic agents (Keating et al, 1977, Freeman et al, 1982). These data suggest there is a additive action of gentamicin and cytotoxic therapy on the production of hypomagnesaemia. Whether the cytotoxic agents sensitise the kidney to the toxic actions of gentamicin, or exert direct nephrotoxic effects by altering electrolyte handling, or if the disease states produce renal injury particularly sensitive to gentamicin toxicity is not known.

Experimental studies on the effects of aminoglycosides on renal electrolyte handling have yielded information which appears to vary between species. Acute effects of aminoglycosides on renal handling of calcium and magnesium excretion occur after only 30 minutes of infusion of gentamicin or tobramycin (0.56 mg/kg/min, equivalent to 100mg/kg over a 3h infusion period) (Foster et al, 1992) in the Fischer 344 rat. Another

aminoglycoside, netilmicin, was shown to produce acute increases in the fractional excretion of calcium and magnesium in the rat after one hour of administration. Fractional excretion of sodium and potassium were additionally raised after 4 hours infusion. (Pastoriza et al, 1983). In both studies increases in the excretion of these ions occurred without changes in plasma levels. In contrast, no change in the fractional excretion of either sodium or potassium were visible in the dog, (Chui et al, 1976) and the sheep model showed a decrease in the fractional excretion of sodium combined with an increase in potassium excretion and a decrease in absolute sodium excretion. These acute infusion studies clearly demonstrate a rapid and specific effect of the aminoglycosides on the renal handling of cations in the absence of overt toxicity. Evidence that these phenomena are indeed attributable to altered renal handling capabilities is shown by the evidence that gentamicin increases the fractional excretion of both calcium and magnesium, indicating a reabsorptive defect induced by gentamicin administration.

Repeated administration of aminoglycosides has also produced electrolyte disturbances but generally in conjunction with other signs of overt nephrotoxicity. Under such conditions it becomes more difficult to determine whether the effects on electrolyte handling are a specific effect of the drug or a more generalised reflection of gross tubular damage. Hypokalaemia was pronounced after gentamicin administration (30mg/kg/day) and concomitant hypocalcaemia without hypomagnesaemia was reported to occur after gentamicin dosing in the rat (Cronin et al, 1980, Chui et al, 1977). Chahwala and Harpur (1983), demonstrated a selective effect on calcium excretion after gentamicin administration (40mg/kg) for 7 days. Magnesium excretion was variable, but there were no changes in the excretion of either sodium or potassium. At therapeutic doses (5mg/kg/day for 10 days) gentamicin induced a hypomagnesaemic response in five out of six baboons tested (Finton et al, 1983). The drop in plasma magnesium levels did not prevent continued renal excretion of magnesium in four of the five hypomagnesaemic subjects. There were no indications of gross renal damage in this study, which correlates

with clinical data suggesting that assymptomatic hypomagnesaemia may be a common occurrence after aminoglycoside administration, and is a selective effect of these drugs (Finton et al, 1983, Davey et al, 1985). Harpur et al (1985), showed increases in magnesium output in the rat after gentamicin administration. Additionally a clear dose relationship between calcium excretion and gentamicin administration was observed. At the lowest dose, 10mg/kg/day, there was no evidence of toxic injury as measured by enzymuria suggesting that these alterations in renal handling of calcium, and to a lesser extent magnesium, may be a specific and direct effect of gentamicin on the kidney.

In addition to disruptions in whole body electrolyte balance alterations to the intracellular electrolyte content after gentamicin administration have also been reported. Consistent decreases in total cortical tissue potassium and magnesium have been demonstrated in both the rat (Weinberg et al, 1983) and the dog (Cronin et al, 1982). In potassium depleted animals the toxic effects of gentamicin were more marked and associated with reductions in the specific activity of Na-K ATPase. Only at stages of toxicity where either plasma creatinine levels were raised or when GFR was declining were there signs of sodium and calcium overload in cortical tissue and, specifically within mitochondria isolated from tissue containing widespread areas of necrosis (Weinberg et al, 1983). Matsuda et al (1988), studied the effects of gentamicin on the kidney using electron probe microanalysis to investigate the subcellular localisation of electrolytes. Using a dose of gentamicin which induced a decline in GFR over the study period (60mg/kg/day for 7 or 10 days) they observed increases in sodium concentrations associated with decreases in potassium and phosphorous, in non-necrotic proximal tubular cells. Necrotic cells showed a wide range of intracellular electrolyte concentrations reflecting their inability to maintain the internal environment. Interestingly, these studies also confirmed the lack of effects of gentamicin on the distal tubules where only a decrease in cell phosphorous was seen, without any apparent effects on cellular pathology. These studies appear to suggest that perturbations in intracellular electrolytes occur in the latter stages of the toxic injury with calcium and sodium overload probably representing the

progression towards cell necrosis and death. Alterations in cellular levels of magnesium and potassium similarly do not appear to be involved in the early stages of renal injury caused by aminoglycosides.

In conclusion, aminoglycosides appear to produce a complex series of effects on both intracellular and whole body electrolyte homeostasis. These effects are mediated primarily through alterations in the renal handling of calcium and magnesium, with probably secondary effects on potassium and sodium. Electrolyte disturbances have been shown to be an important clinical problem generally recognised as muscle weakness or tremulousness in the early stages of toxicity but progressing on to seizures and tetany if uncorrected. Reductions in plasma electrolyte levels may also produce marked effects on the cardiovascular system and the gastrointestinal tract, in addition to those most commonly seen on the neuromuscular system. In the absence of clinical symptoms renal wasting of important cations can occur and may represent a significant, underdiagnosed problem. Precise studies into the nature of the reabsorptive defect induced by aminoglycosides are the key to understanding the mechanisms by which aminoglycosides produce these deleterious effects and hence alleviating a significant clinical problem.

1.3 Cis-platinum Nephrotoxicity

Cis-platinum (cis-dichlorodiamminideplatinum) has proved to be one of the most effective antitumour agents available since its introduction into clinical usage about twenty years ago. It is useful against a wide variety of solid tumours, and against germ cell tumours, either as a single agent or in combination with other chemotherapeutic drugs. Its clinical use, however, is limited primarily by a dose-related nephrotoxicity although other toxicities such as ototoxicity, severe gastrointestinal disturbances and myelosuppression have also been observed (Madias and Harrington, 1978, Von Hoff et al, 1979).

In order to prevent excessive nephrotoxicity, creatinine clearance values in the normal range are required in patients beginning cis-platinum therapy. After administration cis-

platinum is rapidly deposited in the kidney and is concentrated to levels which are higher than in other organs and in plasma (Wolf and Manaka, 1976, Litterst et al, 1977). In human subjects cis-platinum appears to exhibit biphasic plasma clearance kinetics. There is an initial rapid removal from plasma of 25-49 minutes and a second slower phase, with a half life of 58-73 hours (DeConti et al, 1973). Additionally, a much slower tertiary phase of platinum clearance has also been described with platinum detectable in plasma up to three weeks after the administration of cis-platinum. This protracted final elimination phase probably represents the slow clearance of accumulated tissue deposits of platinum, particularly from the kidney.

During initial clinical trials of the drug, acute renal failure occurred at therapeutic dose levels. Renal toxicity of cis-platinum was later shown to be substantially reduced by the adoption of a dosing protocol which included vigorous hydration of the subject with either mannitol or saline (Cvitkovic et al, 1977, Hayes et al, 1977). Alternative strategies which have been employed to reduce the nephrotoxicity of this compound include the use of normal saline loading, administration of the drug in a vehicle of hypertonic (3%) saline and concomitant administration with furosemide. Experimental studies using furosemide plus cis-platinum, and the administration of cis-platinum in hypertonic saline have however yielded conflicting results as to their effectiveness in reducing nephrotoxicity (Ward et al, 1977, Lehane et al, 1979, Ozols et al, 1984, Daugaard, 1990).

The mechanism by which saline is protective when used as a vehicle for cis-platinum administration is thought to be related to the chloride ion concentration and its role in preventing the formation of more toxic entities than cis-platinum. In aqueous media cis-platinum is converted by the stepwise replacement of the chloride atoms to form the highly reactive aquated form of the compound. The aquated form of the compound may, in turn, lose hydrogen ions to form hydroxyl radicals. In the presence of increasing chloride concentrations, the parent molecule has been shown to remain stabilised in the less reactive dichloride form (Long and Repta, 1981). The consequences of this shift in

the equilibrium of the reaction toward the less toxic forms must therefore be to reduce the cytotoxicity of the compound.

Evidence that Pt is not itself the primary nephrotoxin comes from observations on the compound trans-platinum which displays minimal toxicity compared to cis-platinum. Trans-platinum has the same chemical formula as cis-platinum but exists in a different conformational state. Additional evidence that the Pt tissue concentration is less important than the form in which it exists was presented by Safirstein et al (1984); cis-platinum was metabolised intracellularly to a platinum compound which was unable to induce mutagenesis in the same manner as did cis-platinum. Depletion of glutathione has been shown to dramatically increase the toxicity of cis-platinum without substantially altering the tissue deposition of the compound. The induction of metal binding protein in another study, had no effect at low or moderate doses of cis-platinum and only reduced cis-platinum toxicity at high doses. This reduction in toxicity was also achieved without changes in tissue Pt concentrations (Litterst et al, 1985). Plasma concentrations of PTH also appear to modulate the effects of cis-platinum on renal damage. Whilst parathyroidectomy protects against cis-platinum induced nephrotoxicity, administration of exogenous PTH augments cis-platinum induced nephrotoxicity (Capasso et al, 1990).

Specific reductions in nephrotoxicity as opposed to cytotoxicity by the various methods of hydration and forced diuresis would be, however, more difficult to explain by alterations in the reactivity of the compound. In contrast to hypertonic saline which increases the chloride content of the urine, mannitol which is also protective, has been shown to reduce the chloride content of urine suggesting that either the chloride content of urine is incidental to the protective abilities of these treatments, or that they mediate their protective roles by alternative mechanisms.

Evidence that the protective effects of both these regimens is by reducing the tubular cis-platinum concentration rather than altering the chloride ion concentration comes from a variety of sources. Hydration appears to reduce cis-platinum nephrotoxicity by diluting

its intratubular concentration rather than by altering platinum content of the kidney, plasma clearance rates or total urinary excretion (Pera et al, 1979, Hayes et al, 1977, DeSimone et al, 1979). Cis-platinum pharmacokinetics are altered in patients with pre-existing renal insufficiency generally increasing the half life of the compound and hence its potential to cause toxicity. This general rule, however, does not appear to hold with the age-related decline in renal function and in cases of only a single functional kidney. Whilst both conditions generally produce a reduced 24h creatinine clearance greater renal toxicity is not observed after standard low-dose cis-platinum treatment. A suggested reason for these apparently paradoxical results is that with a lower total nephron number the ultrafiltrate passes through each tubule at a higher flow rate and hence reduces the contact time of cis-platinum to the individual nephron. This is supported by the evidence of lower urinary cis-platinum concentrations and higher 24h urine output compared to patients with two normally functioning kidneys on identical dosing schedules (Hrushesky et al, 1984). Timing of cis-platinum dosing to coincide with natural circadian rhythms of increased urine flow has also been shown to reduce nephrotoxicity, further supporting the idea that increased tubular contact time is necessary for the development of toxicity (Levi et al, 1982). Age related reductions in the renal accumulation and more rapid plasma clearance of cis-platinum contribute to the less marked nephrotoxicity shown in young animals. Alterations in the half life and hence clearance of this compound also appear to condense the time course of the observed toxicity when compared to adult rats (Appenroth et al, 1988).

Proximal tubule microinjection studies showed that platinum was not reabsorbed during its passage along the proximal tubule, but since cis-platinum recovery did not quite reach that of inulin a small amount of binding to the kidney may have occurred (Safirstein et al, 1986). Additionally, since the pars recta, the site of most Pt accumulation, is inaccessible to micropuncture, transport of cis-platinum or a metabolite may well occur in this portion of the proximal tubule. The pars recta is a major site of transport and accumulation of PAH (p-amminohippurate). On this basis, and with the evidence that Pt

is preferentially accumulated in this portion of the nephron, it has been suggested that cis-platinum or a metabolite may be transported into the cells by a similar mechanism (Dobyan et al, 1980). Evidence from isolated non-filtering perfused kidneys, where cis-platinum would have only access to the peritubular side of the cell, showed clear inhibition of both TEA (tetraethylammonium) and PAH accumulation demonstrating that cis-platinum toxicity is not entirely dependent on luminal access to the cells (Muira et al, 1987). Other studies which investigated the effect of cis-platinum on the transport of TEA and PAH *in vitro* showed that both of these independent transport systems were inhibited. The authors interpretation of this result was that the inhibition of both processes reflected a generalised toxic effect on metabolism rather than specific interactions with both transport systems (Goldstein et al, 1981). However, since the renal clearance of free platinum exceeds that of creatinine the peritubular secretion of cis-platinum or a metabolite may well occur (Jacobs et al, 1980). In addition to secretion of Pt from the peritubular side of cell it is likely that cis-platinum moves into the proximal tubule cells from the luminal membrane. Reduction of cis-platinum nephrotoxicity by inducing diuresis appears to act by reducing the intratubular concentration of cis-platinum rather than altering plasma clearance rates, total urinary excretion, or tissue distribution (Hayes et al, 1977, DeSimone et al, 1979, Pera et al, 1978). Since the luminal concentration of cis-platinum appears to be an important factor in the occurrence of nephrotoxicity it is also probable that transport of cis-platinum from the tubular lumen is important for its accumulation within the proximal tubule cells. The exact mechanism by which cis-platinum is taken up into renal cells however remains unclear. Cis-platinum is nevertheless, accumulated to high concentrations in the proximal tubule, particularly in the pars recta of the rat, by oxygen dependent processes (Safirstein et al, 1984). Intracellularly, cis-platinum is found within all cell organelles particularly the microsomes and nucleus, and also in cytosol, thus exposing the cell to the toxic effects of this compound by a variety of routes (Safirstein et al, 1980, Choie et al, 1980).

In humans cis-platinum has been shown to cause acute focal tubular necrosis mainly affecting the later parts of the nephron, the distal tubule and collecting ducts, combined with less severe histological changes in the proximal tubule (Gonzalez-Vitale et al, 1977, Dentino et al, 1978). In animals, cis-platinum consistently produces acute tubular necrosis in the proximal tubule with variable amounts of change in the latter part of the nephron, depending on the study (Ward and Fauvie, 1976, Dobyan et al, 1980, Choie et al, 1980, Safirstein et al, 1981, Lehane et al, 1975). Immediately after cis-platinum administration cells of the proximal tubule show clear signs of altered structure and concomitant changes in function. Tubular dilation, cytoplasmic vacuolisation, pyknotic nuclei, hydropic degeneration have been shown as early stages in these cells after cis-platinum dosing. The maximal injury caused by cis-platinum is however not apparent until 3-5 days after dosing. By this stage the cells of the proximal tubule, particularly those of the pars recta, have undergone further disruption and substantial areas of necrosis are apparent (Choie et al, 1980, Ward and Fauvie, 1976, Dobyan et al, 1980, Safirstein et al, 1981). Remaining cells of this region show signs of profound changes in almost all of the organelles and in overall cell morphology (Dobyan et al, 1980). Whilst regeneration may occur after cis-platinum treatment it appears to be far from complete, with cystic lesions, interstitial fibrosis, and thickened basement membranes in the kidney causing permanent renal damage, particularly after chronic treatment (Choie et al, 1980, Dobyan, 1980, Dobyan, 1981).

Characteristically, cis-platinum administration produces an immediate polyuric state, with urine of reduced osmolality with associated polydipsia (Safirstein et al, 1981, Goldstein et al, 1981). Reductions in GFR tend not to occur in the early stages of renal damage but are visible at 2-3 days after cis-platinum dosing with concomitant polyuria. The early phase of polyuria is responsive to large doses of vasopressin, although plasma levels are low, with cis-platinum interfering with its synthesis or release (Clifton et al, 1982). The polyuria in this phase may also be due to the excessive release of prostaglandins since treatment with aspirin ameliorates the polyuria (Safirstein et al,

1987). The early phase of polyuria is unaffected by changes in renal blood flow and the regional blood flow within the kidney remains the same as control animals, with normal GFR values (Daugaard et al, 1985). The later phase of polyuria is not due to the actions of prostaglandins or lack of circulating vasopressin as is the early phase. During the later phase of polyuria reductions in renal blood flow and GFR are apparent in both rat and dog experimental models. Since both arterial blood pressure and cardiac output remain the same as control animals the decreased renal blood flow must be due to an increase in the resistance of the renal vasculature (Winston and Safirstein, 1985, Daugaard et al, 1987, Abilgaard et al, 1988). The cause of the later concentrating defect is thought to be due to the reduction in papillary solute content due to diminished urea recycling (Safirstein, 1981). The polyuria is a functional manifestation of the reduction in reabsorptive capacity due to the decrease in tubule number; it appears over the same time course as the reductions in GFR. The substantial excess functional capacity of the kidney is demonstrated quite clearly by a study in the pig where despite a single dose of up to 2.5mg/kg cis-platinum there were no apparent alterations in renal function. Only when a unilateral nephrectomy was performed did the reduction in renal function (<50% of matched control) induced by cis-platinum become evident. This shows that despite marked damage whole kidney measurements of renal function may appear normal after cis-platinum dosing (Robbins et al, 1990).

1.4. Electrolyte disturbances as a consequence of cis-platinum dosing.

Clinically, cis-platinum has been reported to cause hypomagnesaemia in 50-100% of patients depending on the protocol (Shilsky and Anderson, 1979, Hayes et al, 1979, Ozols et al, 1984, Buckley et al, 1984). The incidence and extent of hypomagnesaemia appears to be dose related, with increased risk associated with short intervals between chemotherapy cycles (Buckley et al, 1984). That the defect is primarily of renal origin is demonstrated by various lines of evidence although its precise mechanism remains as yet unclear. Reductions in serum electrolyte concentrations and the associated kidney dysfunction have been reported to occur without other clinical signs of diminished renal

function (Davis et al, 1980, Pinedo et al, 1983, Reznik et al, 1991) suggesting specific effects of cis-platinum on renal handling of cations without generalised toxicity.

In the presence of substantially reduced plasma magnesium concentrations the normal response of the kidney should be to decrease the urinary fractional excretion of magnesium to almost nil, in order to conserve magnesium. Several reports clearly show that magnesium is inadequately conserved after cis-platinum dosing. Urinary magnesium excretion may simply be inappropriately high for the plasma level, or the fractional excretion may be increased over normal values from magnesium replete subjects (Schilsky et al, 1982). The problem of magnesium wasting by the kidney is exacerbated by the gastrointestinal disorders which often accompany cis-platinum dosing, leading to a reduced dietary intake of magnesium salts. Additionally, animal studies have also demonstrated alterations in the gastrointestinal absorption of magnesium during cis-platinum administration. Following the cessation of cis-platinum dosing a period of compensatory increased magnesium absorption was apparent indicating that any dysfunction of the GI. tract induced by cis-platinum is only of a temporary nature (Mavichek, 1985). In less severe cases of hypomagnesaemia the restoration of a normal dietary intake of magnesium may be sufficient to render the insult clinically silent without additional supplementation. Other commonly used manoeuvres associated with cis-platinum therapy such as saline hydration, mannitol diuresis, and the use of loop diuretics may also deplete body magnesium levels thereby increasing the likelihood of producing hypomagnesaemia (Massry and Coburn, 1973, Duarte, 1968). As with gentamicin therapy the hypomagnesaemia produced is, in the majority of cases, assymptomatic and clinically unrecognised except in the most severe cases (Shilsky, Barlock and Ozols, 1982). However, a substantial proportion of patients treated with cis-platinum may exhibit hypomagnesaemia years after the cessation of therapy (Markman, 1991). In those patients who exhibited persistent hypomagnesaemia (> 3 years after treatment) 8/9 showed inappropriately high urinary magnesium excretion (Schilsky, Barlock and Ozols, 1982). The chronic alteration in the renal handling of magnesium demonstrated in this

study suggests that cis-platinum may produce specific, permanent alterations in the renal handling of electrolytes.

In addition to the hypomagnesaemia, hypocalcaemia, hypokalaemia, hypophosphataemia, and occasionally, hyponatremia have also been reported after clinical dosing. The hypokalaemia and hypocalcaemia are unresponsive to supplementation with their respective salts but improve after supplementation with magnesium salts (Rodriguez et al, 1989). The responsiveness of these conditions to magnesium supplementation points to the hypomagnesaemia as the primary defect with other ion depletion states as secondary, related disorders. The presence of hypocalcaemia appears to be associated only with severe hypomagnesaemia (Hayes et al, 1979).

However, one clinical study has demonstrated an inappropriately raised fasting calcium excretion suggesting that renal calcium wasting may indeed occur alongside the renal magnesium wasting (Stewart, Keating and Schwertz, 1985). Alterations in the parathyroid hormone response to low serum calcium levels have been investigated as the cause of the hypocalcaemia. In children on combination therapy, including cis-platinum, those who displayed hypomagnesaemia and hypocalcaemia did not produce the expected compensatory rise in serum parathyroid hormone concentrations. Supplementation of the children with oral magnesium salts gradually resulted in a return of normal serum calcium levels. That the effect of cis-platinum on the parathyroid hormone response is via the production of a hypomagnesaemic state, and not directly on either the production and/or release of PTH, is implied by a normal increase in PTH levels on magnesium repletion (Hayes et al, 1981).

Animal studies have also shown both calcium (Mavichek et al, 1984) and magnesium wasting (Magil et al, 1986) occurs after cis-platinum dosing. Cis-platinum treated rats are able to compensate for abnormal renal losses of calcium and magnesium when fed a high calcium and magnesium diet but hypomagnesaemia became apparent when placed on a low calcium and magnesium diet. Hypocalcaemia was not evident, but dose-related

increases in the fractional excretion of both calcium and magnesium were visible in animals fed the low calcium and magnesium diet (Mavichek et al, 1984).

Several reports have shown that various transport systems may be inhibited by the action of cis-platinum, after both *in vitro* and *in vivo* exposure. However, inhibition of both Na^+/K^+ ATPase and Ca2+ ATPase was shown to require either high concentrations or prolonged incubation periods to reduce the specific activities of the enzymes (Daley-Yates and McBrien, 1982, Guarino et al, 1979, Uozumi and Litterst, 1985). After *in vivo* dosing with cis-platinum normal ATPase function was preserved until 3 days after exposure, when tissue disruption was also evident. Additionally, the highest Pt concentration achieved in the kidney after 10mg/kg dosing was substantially lower than that which was necessary to inhibit ATPases *in vitro*. These data suggest that whilst ATPases in the kidney may be inhibited by the action of cis-platinum, the concentrations and exposure required to induce this inhibition are unlikely to be achieved *in vivo*. Consequently, direct inhibition of the plasma membrane bound ATPases are unlikely to be the direct cause of reduced cation uptake along the nephron seen *in vivo*.

Disruptions of intracellular calcium homeostasis are evident from information on the concurrent effects of cis-platinum on renal endoplasmic reticulum and mitochondria. At 24 hours post cis-platinum dose, when there is no effect on BUN or serum creatinine levels, inhibition of mitochondrial uptake of both calcium and magnesium is apparent (Gemba et al, 1987). At the same time the specific activity of the calcium pump in the endoplasmic reticulum is increased in renal but not hepatic tissue (DeWitt et al, 1988). Whether the reduction in mitochondrial uptake acts as a stimulus for increased ER pump activity is not known since data at early time points for mitochondrial activity are unavailable but the consequence of the disruptions in intracellular calcium regulation combine to produce an increase in total kidney calcium concentration. The specific activity of both the plasma membrane Na^+/K^+ ATPase and Ca2+ ATPase was unaffected in these experiments. Whilst the exact nephron site of these changes in

calcium concentration is unknown, the early increases in cellular calcium levels demonstrate that intracellular homeostasis is disrupted by the action of cis-platinum.

Cis-platinum nephrotoxicity is a significant clinical problem which occurs in a substantial proportion of patients given the drug. The effects are manifest as changes in overall kidney function and also as selective effects on the renal handling of cations which may in themselves cause life threatening situations. The mechanisms by which cis-platinum affects the reabsorption of calcium and magnesium in the kidney is poorly understood and yet due to its clinical implications warrants further study.

1.3 Renal handling of cations

a. Calcium

Plasma calcium is present in three forms: bound to plasma proteins, complexed to anions, and as free ionised calcium. Only that calcium which is in the ionised form or complexed to anions is able to cross the glomerular membrane into the ultrafiltrate. Approximately 60% of the total plasma calcium is present in the ultrafiltrate (Lassiter, Gottschalk and Mylle, 1963, Harris et al, 1974, LeGrimellec et al, 1975). An estimated 98% of the calcium filtered each day is reabsorbed during its passage along the nephron; the majority (60-70%) of this reabsorption occurring in the proximal tubule (Lassiter, Gottschalk and Mylle, 1963).

The bulk transport of calcium from the ultrafiltrate in the proximal convoluted tubule is considered to be mainly passive. Calcium transport closely parallels the movement of sodium in this segment and movement of water out of the proximal tubule creates a solvent drag effect which also facilitates passive calcium movement. The ratio of tubular fluid to ultrafiltrate for calcium is 1.0 along the early part of the convoluted tubule, rising slightly toward the end of this segment (Lassiter, Gottschalk and Mylle, 1963, Harris et al, 1974, Edwards et al, 1974, Duarte and Watson, 1967, De Rouffignac et al, 1973). The increase in TF/GF for calcium suggests that calcium reabsorption lags behind water

movement achieving a small electrochemical gradient for calcium transport. Additionally the voltage across the tubule switches from lumen negative in the S1 segment to lumen positive in the S2 segment, assisting calcium reabsorption. Further evidence which suggests that the bulk of calcium reabsorption is passive along electrochemical gradients was produced in perfused rabbit convoluted S2 segments. In the absence of water movement and no electrochemical gradient no net calcium movement occurred. In fact, manipulation of the perfusate to produce a lumen-positive PD caused calcium efflux, whilst lumen-negative PD induced net calcium secretion into the tubule (Ng, Rouse and Suki, 1984). Although the bulk of calcium transport in the proximal tubule is passive and secondary to sodium flux, evidence for a small active component of calcium transport in this segment also exists. The active component of calcium transport is inhibited by reducing the sodium concentration and is abolished by the addition of ouabain, suggesting the transport of calcium is linked to the maintenance of a sodium gradient, and the presence of a Na/Ca exchange system (Ullrich et al, 1979). Additionally the presence of Ca^{2+} ATPase activity has been described in the proximal convoluted tubule although the exact contribution it makes to active calcium transport is unclear (Doucet and Katz, 1982, Gmaj et al, 1979).

In the straight (S3) segment of the proximal tubule calcium transport appears to be independent of sodium. Addition of ouabain to the perfusate, or removal of sodium does not apparently alter calcium transport. However, cooling of the preparation abolished calcium transport and suggested that the transport was active, especially since it occurred against both an electrochemical gradient, and against a lumen-negative PD (Almeida et al, 1978, Rouse et al, 1980).

Permeability to calcium is very low in the thin ascending and descending limbs of the Loop of Henle suggesting that negligible calcium transport occurs in these segments of the nephron (Rouse et al, 1980, Rocha et al, 1977). However, approximately 20% of the filtered calcium load is reabsorbed in the thick ascending limb of the Loop of Henle

(TAL). The mechanism by which this is achieved appears to vary depending on whether the thick ascending limb of the loop of Henle is located in the cortical or medullary region of the kidney. In medullary TAL calcium reabsorption appears to be mainly passive (Suki et al, 1980), whereas the cortical TAL shows evidence of active calcium reabsorption (Rocha et al, 1977, Imai et al, 1978), coupled with a substantial Ca2+/Mg2+ ATPase activity (Doucet and Katz, 1982).

Fine regulation of calcium excretion occurs in the distal nephron and the collecting duct system, where the removal of the final 10-15% of calcium remaining in the ultrafiltrate occurs. In the distal tubule calcium transport is against an electrochemical gradient and is active (Constanzo and Windhager, 1978, Shareghi and Stoner, 1978). Whilst calcium transport parallels sodium in this portion of the nephron, it is not sodium dependent and movement of the two ions can be disassociated. The entry pathway of calcium into the cells is independent of sodium (Shareghi and Stoner, 1978, Constanzo and Windhager, 1978); the extrusion at the basolateral membrane is either via Na^{+}/Ca^{2+} exchange or by the action of Ca^{2+}/Mg^{2+} ATPase, or by further stimulation of the Ca^{2+}/Mg^{2+} ATPase pump by the action of vitamin D-dependant calcium binding protein (Doucet and Katz, 1982, Borke et al, 1988).

The cortical collecting ducts reabsorb a small proportion of the filtered calcium. The reabsorption of calcium appears to occur exclusively in the early part of the segment, the "granular" portion. This portion appears to possess calcium transport characteristics similar to those found in the late distal tubule, that is calcium transport is active and the cells respond to the action of PTH by increasing calcium reabsorption (Shareghi and Stoner, 1978). In contrast, in the later part of collecting duct, the "light" portion, calcium permeability appears to be low, with any calcium transport occurring passively via a paracellular route (Shareghi and Stoner, 1978, Shareghi and Agus, 1982, Bordeau and Hellstrom-Stein, 1982).

Increases in the filtered load of calcium induced by changes in GFR tend to only cause slight rises, if any, in the excretion of calcium because both proximal and distal tubular absorption of calcium increase to compensate for the rise in the calcium concentration of the ultrafiltrate (Massry and Kleeman, 1972). In contrast, the increase in filtered load induced by hypercalcaemia causes hypercalciuria. In addition to direct effects on calcium reabsorption in the distal nephron by the suppression of PTH (Edwards et al, 1974) hypercalcaemia appears to reduce calcium reabsorption in both the proximal convoluted tubule by inhibition of the bulk reabsorption rather than the altering the active component of calcium transport (Edwards et al, 1974, Agus et al, 1977), and by inhibition of calcium reabsorption in the TAL by inhibition of calcium and magnesium extrusion at the basolateral membrane (Shareghi and Agus, 1982, Quamme, 1982).

Extracellular volume expansion increases the filtered load of calcium by increasing GFR whilst simultaneously decreasing the reabsorption of both calcium and sodium in the proximal tubule (Duarte and Watson, 1967, Dirks et al, 1984). This depression of cation reabsorption in the proximal tubule occurs independently of effects on PTH. In addition to inhibition of calcium transport in the proximal nephron suppression of calcium in the later parts of the nephron must also occur for the increase in calcium excretion to be so readily apparent. The site of this additional defect has not yet been conclusively defined, however it lies beyond the distal tubule (Agus et al, 1977).

Phosphate loading reduces calcium excretion by increasing calcium reabsorption at a site after the distal nephron (Wong et al, 1980, Lau et al, 1979, Lau et al, 1982). In contrast, phosphate depletion induces hypercalciuria (Coburn and Massry, 1970). The hypercalciuria induced by phosphate depletion appears to be only partially corrected by administration of PTH, suggesting direct actions of phosphate depletion on the tubular uptake of calcium.

Metabolic acidosis is associated with an increase in calcium excretion which is independent of changes in the filtered load of calcium or PTH (Lennon and Piering, 1970). Conversely, metabolic alkalosis causes a reduction in calcium excretion (Sutton et al, 1979).

Hypermagnesemia increases the fractional excretion of calcium by two routes; increased magnesium concentration acts in direct competition with calcium for transport in the medullary TAL reducing calcium reabsorption (Shareghi et al, 1983), and indirectly by reducing PTH secretion (Buckle et al, 1968, Slatopolsky et al , 1976).

The effect of diuretics on calcium handling in the kidney depend on the association of calcium and sodium transport at the diuretic's site of action. Loop diuretics such as furosemide, ethacrynic acid and the mercurial diuretics act on the TAL of the Loop of Henle and produce large increases in both sodium and calcium excretion (Eknoyman et al, 1970, Edwards et al, 1973, Duarte, 1968, Demartini et al, 1967). The primary cause of the effects on calcium reabsorption appear to be by the inhibition of sodium and chloride transport in the medullary TAL (Edwards et al, 1973, Seldin et al, 1966). The effects of furosemide are apparently exerted by dissipating the lumen positive PD in the medullary TAL which effectively inhibits the passive reabsorption of calcium (Bourdeau et al, 1982, Imai, 1978, Suki et al, 1980). The thiazide diuretics induce a substantial natriuresis but calcium excretion may only be slightly increased, if at all. The reason for this separation of the effects of these diuretics is that the thiazides exert their effects in the distal tubule where calcium and sodium transport are independent; their action is to inhibit sodium reabsorption whilst enhancing independent calcium reabsorption (Edwards et al, 1973, Constanzo and Windhager, 1978). When administered chronically these agents produce hypocalciuria due to enhanced proximal tubular reabsorption of calcium caused by extracellular volume depletion (Brickman et al, 1972).

Amiloride administration produces a more marked increase in sodium clearance than calcium clearance. This compound decreases the lumen-negative PD of the distal nephron portions, thereby enhancing calcium reabsorption without altering sodium transport in this segment (Constanzo and Weiner, 1976, Stoner at al, 1974). In addition, there also appears to be a direct effect of amilioride on calcium reabsorption in the distal tubule similar to that of the thiazide diuretics (Stoner et al, 1974).

Hormonal agents which alter calcium reabsorption tend to exert their effects at nephron sites distal to the Loop of Henle. Parathyroid hormone (PTH) is believed to be the primary regulator of calcium excretion and acts via the activation of adenylate cyclase. PTH appears to have a dual effect by which it reduces calcium excretion. PTH decreases GFR and thereby decreases the filtered load of calcium, in addition tubular reabsorption of calcium is increased leading to a reduction in calcium excretion (Talmage and Kraintz, 1954). PTH exerts its effects on calcium reabsorption at a variety of sites along the nephron. PTH inhibits calcium reabsorption in the proximal convoluted tubule by altering sodium and water movement, since calcium moves in parallel to sodium, calcium reabsorption is reduced also. PTH apparently directly stimulates calcium reabsorption in the cortical but not the medullary TAL via the action of cAMP (Shareghi and Agus, 1982, Bourdeau and Burg, 1980, Suki and Rouse, 1981). Enhanced reabsorption of calcium in the later parts of the nephron, the distal tubule and connecting tubule, occur without effects on sodium reabsorption suggesting a disassociation of transport of these ions at these sites (Shareghi and Stoner, 1978, Kleeman et al, 1964, Constanzo et al, 1980). Another hormone from the parathyroid gland, calcitonin, is thought to lead to an increase in calcium reabsorption by inducing hypocalcaemia and thereby decreasing the filtered load of calcium. The site along the nephron at which this increased reabsorption may occur is the medullary TAL, where calcitonin or cAMP addition stimulated calcium reabsorption has been demonstrated (Suki and Rouse, 1981) . There may however be species differences in the site of action at which calcitonin alters calcium reabsorption which remain to be resolved. The direct tubular action of vitamin D and its metabolites

also remain contentious, and vitamin D associated hypercalciuria may simply be due to its action of increasing calcium absorption from the GI. tract and thereby increasing the filtered load of calcium (Puschett et al, 1972, Brennan and Kokko, 1973).

Table 1. Summary of the principal sites of calcium reabsorption along the nephron correlated with the main sites of accumulation and injury caused by cis-platinum and aminoglycosides.

SITE	MECHANISM(S)	PROPORTION REABSORBED (%)	ACCUMULATION
Proximal tubule	Mainly passive, due to active sodium movement. May be active component too.	60	Aminoglycosides; S1 and S2 segments. Cis-platinum; PCT, but mainly pars recta.
Loop of Henle	Medullary Ca transport appears to be passive whereas cortical Ca transport in TAL appears to be active.	30	–
Distal tubule	Active; independent of Na.	10	–

b. Magnesium

At the glomerulus 70-80% of total plasma magnesium is filtered through to the ultrafiltrate (Brunette and Crochet, 1975; Le Grimellec et al, 1975). Of this, 70-80% of magnesium in the ultrafiltrate is in the ionic form with the remainder mainly complexed to anions (Walser, 1967). The proximal convoluted tubule reabsorbs approximately 25% of magnesium in the ultrafiltrate. Magnesium reabsorption in this segment follows the reabsorption of sodium and water, as does calcium, but at a lower fractional rate, leading to a ratio of tubular fluid/ultrafiltrable fluid for magnesium of 1.5-2.0 at the end of the proximal tubular segment, indicating a lower permeability of the proximal nephron to magnesium than for calcium or sodium (Wong et al, 1986, Quamme and Dirks, 1983, Quamme et al, 1978). Absolute magnesium reabsorption in this segment increases in proportion to the luminal magnesium concentration in both the rat and dog experimental model (Quamme and Dirks, 1980, Wong et al, 1983).

In vitro microperfusion studies of the straight portion of the proximal tubule have shown that magnesium is removed from the perfusate along with the removal of water, similar to the situation which occurs in the proximal convoluted tubule, with little or no back-diffusion even at elevated basolateral magnesium concentrations. However, evidence from *in vivo* studies have yielded conflicting evidence as to whether magnesium is (Brunette et al, 1975, Brunette et al, 1978) or is not (Quamme and Dirks, 1980, Levine et al, 1982) secreted into the nephron at the proximal straight tubule or the thin descending limb of the Loop of Henle. The reabsorption rate for magnesium is lower than that for sodium or calcium, as in the convoluted tubule, and is dependant on the luminal concentration of magnesium (Quamme and Smith, 1984).

Since the transport of magnesium appears to be passive along gradients generated by sodium and water in the proximal tubule, evidence of secretion of magnesium into the tubule came from studies which compared the magnesium content of the tubular fluid at the late proximal nephron and at the bend of the Loop of Henle, which suggested that

magnesium could be secreted into the thin descending limb of the loop. Evidence that magnesium secretion may occur in the early parts of the Loop of Henle comes from micropuncture studies which determined the magnesium concentration at the bend of the Loop of Henle in the desert rat (de Rouffignac et al, 1973, Jamison et al, 1979). These studies found a higher magnesium concentration at the end of the thin descending limb of the Loop of Henle than at the end of superficial proximal tubule, which suggested that addition of magnesium was occurring at some point between the two segments. Another study could only demonstrate significant addition of magnesium to the lumen in magnesium loaded rats, suggesting that the amount of backflux may depend on the concentration gradient from the interstitium to the tubule for magnesium (Brunette et al, 1975). Evidence from the microperfusion studies of the straight segment of the proximal tubule imply that back-diffusion from the interstitium is unlikely to occur at that point due to poor permeability of this segment to magnesium. Therefore the implication is that magnesium secretion may occur in the thin descending limb of the Loop of Henle although direct evidence for this hypothesis is yet to be produced.

The thick ascending limb of the Loop of Henle appears to be the major site of magnesium reabsorption along the nephron; 50-60% of the magnesium in the ultrafiltrate is reabsorbed at this site (Morel, 1969). *In vivo* loop perfusion studies carried out in the presence of normal plasma magnesium concentrations indicated increased magnesium absorption with increasing intraluminal concentrations of magnesium. There was no evidence of saturation of magnesium transport processes in the TAL up to concentrations of 5mM magnesium; approximately 80% of the filtered load at any concentration of magnesium was reabsorbed (Quamme and Dirks 1983, Quamme and Dirks, 1980). Elevation of the plasma magnesium concentration however resulted in a marked reduction in magnesium absorption. The reduction in magnesium reabsorption caused by the presence of hypermagnesemia on the basolateral side of the cells was achieved without altering the fractional reabsorption of sodium in this segment, suggesting a selective reduction in magnesium transport by hypermagnesemia in the

TAL. The transport process for magnesium in the TAL appears to be saturable at high plasma magnesium concentrations. The effect of increasing plasma magnesium concentrations was initially to stimulate the reabsorption of magnesium due to an increase in filtered load of magnesium, then, as hypermagnesemia progressed magnesium transport was depressed due to the increasing concentration of magnesium at the basal side of the cell (Massry et al, 1969, Quamme and Dirks, 1983, Shareghi and Agus, 1982). The mechanism by which magnesium is moved from the tubular lumen appears to be dependent on a transepeithelial PD generated by sodium absorption. Evidence for dependence on sodium transport is shown by the way that changes in sodium chloride transport due to increased flow rate coexist with proportional changes in magnesium absorption (Shareghi and Agus, 1982, Shareghi et al, 1983); additionally the effects of the loop diuretic furosemide, an inhibitor of active sodium transport, alter magnesium reabsorption in the TAL (Quamme, 1981).

A small proportion of the magnesium in the ultrafiltrate is reabsorbed in the distal tubule. Transport of magnesium is load-dependent in this segment but usually approaches saturation at normal magnesium loads (Quamme and Dirks, 1980). In the case of increased magnesium delivery to the distal nephron such as after furosemide administration, fractional sodium and calcium absorption increases to a greater extent than magnesium transport indicating a greater capacity for the transport of these ions than for magnesium in this segment and producing a hypermagnesuria (Quamme, 1981).

The collecting ducts of the kidney play a very limited role in the reabsorption of magnesium. Direct studies of magnesium transport in the rat along the collecting duct show that little or no net movement of magnesium occurs in this segment (Brunette et al, 1978, Shareghi and Agus, 1979, Bengele, 1981).

Both physical and hormonal factors affect the reabsorption of magnesium from the ultrafiltrate. Infusion of saline increases magnesium excretion due to a reduction in proximal tubular reabsorption of both cations (Massry et al, 1967, Brunette et al, 1969).

Whilst the some of the extra filtered load of sodium can be recouped in the more distal parts of the nephron, the capacity for magnesium reabsorption is easily overloaded leading to a greater fractional increase in magnesium excretion than sodium under these circumstances (Quamme, 1986).

Phosphate depletion results in the development of hypermagnesuria which may be severe enough to lead to hypomagnesemia, and hypercalciuria. The administration of exogenous PTH or phosphate infusions correct this defect in magnesium handling which occurs in the TAL (Kreusser et al, 1978, Coburn and Massry, 1970). The interaction of calcium and magnesium transport is clearly demonstrated by the effects of hypercalcaemia on renal magnesium handling. Hypercalcaemia increases magnesium excretion to a greater degree than calcium handling (Massry and Coburn, 1973). Conversely, hypermagnesemia results in a greater hypermagnesuria than calciuria, suggesting a linked or common pathway of transport for these ions. Alterations in PTH levels due to varying plasma levels of calcium initially confused the picture with regard to the direct effects of plasma calcium levels on magnesium reabsorption. However, microperfusion studies on thyroparathyroidectomised rats demonstrated an inhibition of magnesium and calcium absorption in the Loop of Henle with increasing plasma calcium concentrations (Quamme, 1980a). The inhibition of magnesium transport was greater than that of sodium, and occurred without concomitant changes in sodium excretion. The increase in filtered load of magnesium delivered to the distal nephron induces a small increase in magnesium reabsorption but since transport capacities for magnesium are low in this segment the excess is passed into the urine. Since elevation of luminal calcium or magnesium appears to have little effect on the reabsorption rates the interaction of the two cations appears to be at the basolateral face of the cell. Hypocalcaemia can lead to enhanced reabsorption of both magnesium and calcium (Quamme and Dirks, 1980). The enhancement of reabsorption may occur due to reduced competition for transport between calcium and magnesium at the basolateral side of the cell.

Osmotic diuretics, mannitol and urea, increase magnesium excretion in proportion to the osmotic load, by an inhibition of magnesium reabsorption in the TAL (Wong et al, 1979). The Loop diuretics, such as furosemide, have the most marked effects on magnesium excretion of all the classes of diuretics. These compounds induce a substantial magnesuria, with an associated natriuresis and calciuresis of lesser magnitude (Duarte, 1968, Eknoyan et al, 1970). The effects are mediated by an inhibition of sodium transport in the Loop of Henle and indirectly affect magnesium transport because of its sodium dependence.

PTH is probably the major hormone regulating magnesium excretion although its effects are far from clear-cut due to the marked effects of PTH on calcium handling, and its interrelationship with magnesium transport. Micropuncture studies in various animal species assessing the site of action of PTH on magnesium handling have indicated that in a sensitive species such as the golden hamster PTH administration to aparathyroid animals decreases the fractional excretion of magnesium from approximately 20% to a normal value of 5% (Harris et al, 1974). The effect of PTH was located to a region prior to the distal tubule, most probably the TAL. Infusion of cAMP mimicked the effects of PTH suggesting that PTH mediates its effects by the action of cAMP. However in other species, such as the rat, PTH showed little or no effect on magnesium reabsorption in micropuncture experiments (Quamme and Dirks, 1983). PTH also increases magnesium reabsorption when magnesium reabsorption in the Loop of Henle is inhibited by hypermagnesemia or by the action of furosemide, suggesting that PTH may also increase magnesium reabsorption at sites other than the TAL (Quamme, 1981, Quamme and Dirks, 1980). The secretion of PTH may be modulated by plasma levels of magnesium, as it is by calcium, but the parathyroid glands appear to be less sensitive to changes in plasma magnesium concentration than to plasma calcium concentration (Harris et al, 1979, Mahafee et al, 1982).

Magnesium reabsorption is also stimulated in the TAL by the concerted action of cAMP-mediated hormones glucagon, calcitonin, and ADH, in addition to PTH (Quamme ,

1980b, Elalouf et al, 1983, Bailly et al, 1984, de Rouffignac et al ,1983, Elalouf et al, 1986). The action of these hormones is a stimulation of cation reabsorption by the action of the Na/K/Cl transporter in the TAL. Changes in sodium transport by the activity of this co-transporter indirectly affect the passive reabsorption of magnesium and calcium. Further evidence suggests that PTH, calcitonin, ADH and glucagon may also stimulate reabsorption of sodium, Cl^-, calcium and magnesium in the distal tubule in addition to their activity in the Loop of Henle (Bailly et al, 1984, Bailly et al, 1985, Elalouf et al, 1983, Elalouf et al, 1984). The effect of these hormones appears to be a generalised stimulation of electrolyte transport rather than a specific effect on magnesium.

Table 2. Summary of the main sites of magnesium reabsorption along the nephron and correlation with the sites of accumulation and injury caused by aminoglycosides and cis-platinum.

SITE	MECHANISM(S)	PROPORTION REABSORBED (%)	DRUG ACCUMULATION
Proximal tubule	PCT; passive following Na movement but lower permeability to Mg than Ca	20-25	Aminoglycosides; S1 and S2 segments. Cis-platinum; PCT, but mainly pars recta.
Loop of Henle	Mg secretion may occur in the thin descending limb. Na-dependent Mg transport via production of transepithelial PD.	50-60	–
Distal tubule	Transport of Mg is load dependent but approaching saturation at normal loads. Little or no Mg transport in collecting ducts.	15	–

Chapter 2. Acute and chronic renal injury induced by cis-platinum in the rat.

2.1 Introduction

The nephrotoxicity of the antitumour agent cis-platinum has been extensively studied in both animal models and in patients with cancer. Most studies have concentrated on acute renal effects of the drug which have been thought to be reversible. Recent attention has however focused on the significant incidence of chronic hypomagnesemia in patients treated with cis-platinum. In animal models this hypomagnesemia is attributable to an impaired ability of the kidney to conserve magnesium. A reduction in more conventional indices of renal function, glomerular filtration rate (GFR) may not be apparent at the same time as the chronic defect in renal magnesium reabsorption.

The present study was initiated to study the acute and chronic effects of cis-platinum on renal function in the rat and to investigate whether altering the dose pattern reduced the toxicity of the total dose. Two dosing regimens were used in order to study whether, if the same total dose were given in 4 divided doses at two week intervals, acute and/or chronic nephrotoxicity would be attenuated compared to a single dose of the same amount. The possibility that cumulative toxicity could occur after divided doses of cis-platinum resulting in similar levels of chronic renal injury compared to a large single dose, was also considered. Of additional interest was the permanence, or otherwise, of effects of cis-platinum dosing on the renal handling of calcium and magnesium. The early phase of injury was examined in detail in order to study whether acute changes in renal function were predictive of chronic effects on the kidney.

The acute and chronic stages of renal toxicity were followed by measurements of enzymuria, calcium and magnesium outputs, urine volume and osmolality, and by occasional measurements of plasma creatinine (Pcr), plasma magnesium (PMg) and plasma calcium (PCa) concentrations.

2.2 Methods

The studies were conducted in an environmentally controlled room with a 12h light cycle (08.00-20.00), maintained at 22°C. All rats were acclimatised to this environment and to individual metabolism cages for a minimum of 7 days before any control collections were made. Spontaneously voided urine, free of contamination by food or faeces was collected over 24h periods from all drug-treated rats (n=12) and half the control rats (n=6). Urine collection tubes were covered, and surrounded by ice to prevent evaporation and denaturation of enzymes. Two urine collections were made from each group prior to the administration of the first injection. Urine collections were made on alternate days during and immediately after the dosing period, and thereafter at intervals of increasing length (maximum 2 weeks) until 4 months ± 9 days after the total dose of 6mg/kg of cis-platinum had been administered. Urine collections were scheduled to avoid the 24h immediately following an injection as it was expected that the saline load from the injection would cause an acute increase in urine volume.

Cis-platinum was administered i.p. at a dose of 6mg/kg to 24 Fischer 344 rats (weight range 145-233g) either as a single injection (CP1) or as four doses of 1.5 mg/kg at 14 day intervals (CP4). The cis-platinum was freshly dissolved in 0.9% w/v NaCl and given as a dose volume of 10 ml/kg. Control animals (n=12 for both study groups) received an equivalent volume of saline as an i.p. injection to that received by the drug groups, 24h after the drug groups were dosed with cis-platinum. The first, or only, day of injection was designated Day 0.

Cis-platinum-injected rats were allowed free access to food (Pilsbury's breeding diet, modified 41B) and tap water at all times; their daily food and water intake was measured. The experimental design was staggered so that control animals were 24h behind those animals receiving cis-platinum. Animals of the control groups could then be pair-fed with animals in the drug-treated groups. The food intake of each control rat was matched to a paired animal in the cis-platinum-injected group during periods when the

food intake of the drug treated animals was depressed, i.e. for approximately 2 weeks after the injection in the CP1 study, and on two separate occasions in the CP4 study.

Blood was obtained from all animals by cardiac puncture on Days -7, 3, 13, and 111 during the single dose study, and on Days 3, 32, 45, and 171 during the divided dose study, on each occasion at the end of a urine collection period. Animals were anaesthetised with halothane and nitrous oxide and approximately 1ml of blood was drawn into a heparinised syringe, and centrifuged to obtain plasma. Plasma calcium and magnesium concentrations were measured by atomic absorption spectroscopy, after appropriate dilution with 0.1 % w/v lanthanum chloride solution, acidified with 50mM HCl (AR). Plasma creatinine was measured by an alkaline picrate method (Taussky, 1956).

Urines were centrifuged for 10 mins at 3000 G at 4 °C to remove debris and the volume measured. The urines were checked, qualitatively, for the presence of glucose, blood, and ketone bodies, and for changes in pH using Labstix (Ames Laboratories Ltd.). Urinary creatinine was measured by the standard alkaline picrate method above, osmolality by freezing point depression using a Knauer osmometer, and protein by the method of Lowry et al (1951). The urinary creatinine excretion and the plasma creatinine concentration measured at the end of the 24h urine collection period were used to calculate approximate creatinine clearances (GFR). Enzymatic activity was measured in urine which had been dialysed against water (200 vols for 3h at 4°C) to remove low molecular weight inhibitors. Alanine aminopeptidase (AAP) was measured according to the method of Mondorf et al (1972), using l-alanine-4-nitroanilide as substrate. Aliquots (200µl) of dialysed urine were incubated in 80mM phosphate buffer at 37°C to which the substrate was added after equilibration. The absorbance which developed after 15 min was measured at 405 nm against a blank of phosphate buffer and urine. LDH activity was measured in the presence of 80 mM phosphate buffer pH 7.4 and 0.12 mM $NADH_2$. The reaction was started after equilibration to 37°C by the addition of 2.1 mM sodium pyruvate and the rate of conversion from pyruvate + NADH + H^+ to lactate + NAD^+

was followed at 340 nm. N-acetyl-ß-glucosaminide (NAG) was measured using p-nitrophenyl-n-acetyl-glucosaminide as substrate. Activities were measured as nmol of substrate converted per minute (mU) and expressed as total activity excreted per 24h. Detailed methods for each of the assays described above are included in Appendices 1-4. Urinary enzyme activities were measured until enzyme excretion returned to control values. Calcium and magnesium concentrations in urine were determined by atomic absorption spectroscopy, as above, after dilution in 0.1% $LaCl_3$ acidified with 50mM HCl.

At termination of the studies all kidneys were removed, fixed in formol saline (10% formalin in 0.9% NaCl), and processed for light microscopy. Sections (4 µM) were stained with Harris' haematoxylin and Navy Eosin, and with Periodic Acid Schiffs Reagent, and examined by a histopathologist.

Statistical comparisons were made between cis-platinum-dosed and control animals using a paired t-test. Data was taken to be significantly different at p values of 0.05 or less. Data is expressed as mean ± SEM. n = 4-12 for each data point. In figures 1-12 *** denotes $p < 0.001$, ** denotes $p < 0.01$, * denotes $p < 0.05$.

2.3 Results

Injection of a single 6 mg/kg dose of cis-platinum caused an immediate reduction in food intake and, as a consequence body weight gain was halted (Figure 1). Food intake and body weight gain gradually returned to pre-drug levels over the following two weeks. During this period there was a parallel interruption of body weight gain in the pair-fed control animals. In comparison food intake was reduced only after the second (Days 15-18), and lesserly after the fourth injections (Days 42-45) when cis-platinum was given in divided doses to the CP4 group; at all other times the food intake in the CP4 group was not reduced (Figure 2). Reduction in food intake was not as pronounced after drug treatment in the CP4 group as after the single dose of cis-platinum. However, body weight in the CP4 rats was significantly ($p<0.01$) below that of the control group

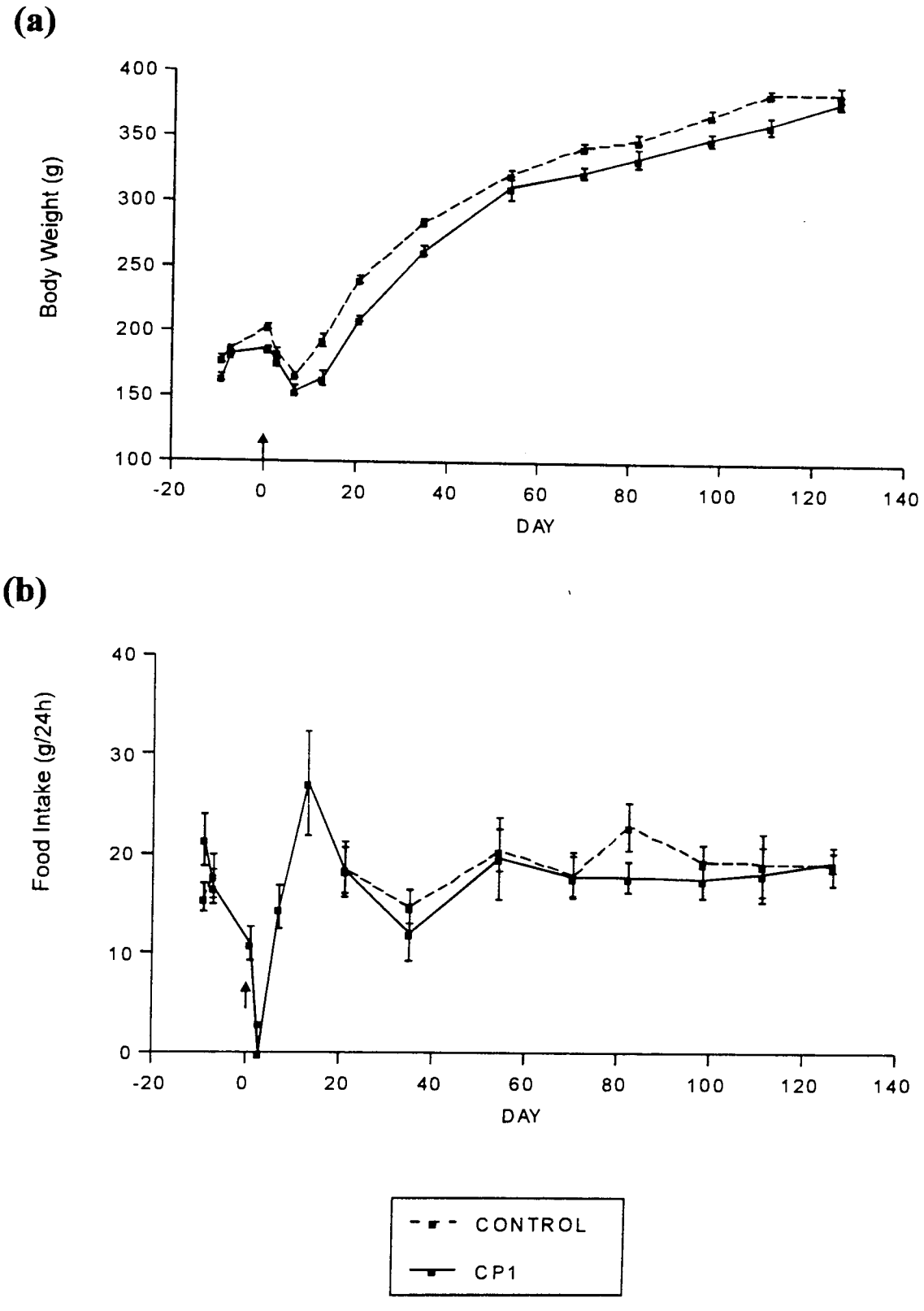


Figure 1. Effect of a single dose of 6 mg/kg cis-platinum on a) Body Weight b) Food intake. Arrows denote dosing. Data is presented as mean ± SEM. n = 3-12.

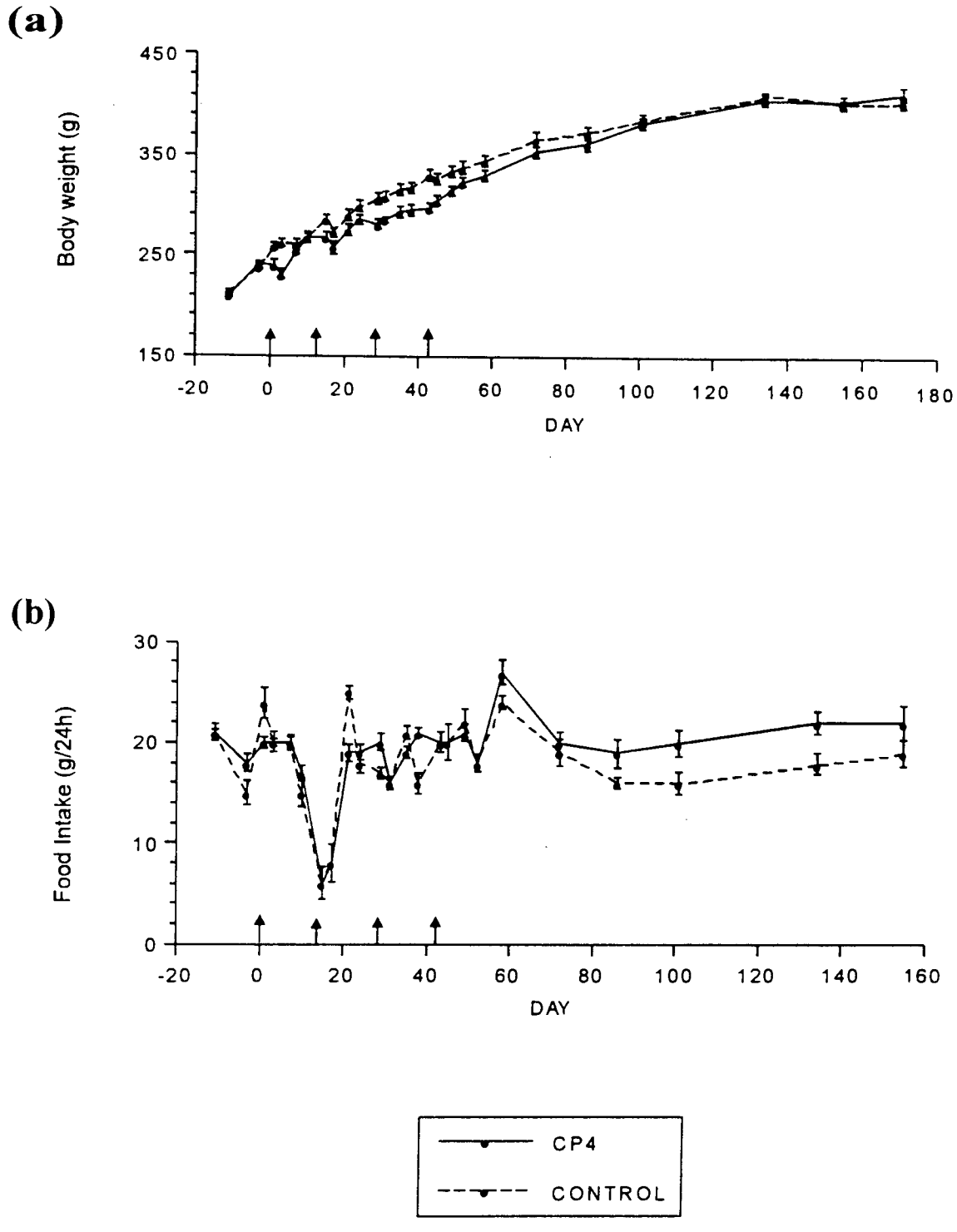


Figure 2. Effect of a divided dose of 6 mg/kg cis-platinum on a) Body Weight b) Food intake. Arrows denote dosing. Data is shown as mean ± SEM. n = 3-12.

immediately after the first injection, transiently after the second ($p<0.05$), and after third and fourth injections of cis-platinum ($p<0.01$) although the difference in group mean body weights resolved 10 days after the last injection. Possibly due to the tighter food restrictions enforced in the single cis-platinum dose study there were no significant differences in body weight in the CP1 rats compared to controls. Three days after the single 6 mg/kg injection of cis-platinum, GFR (measured as Clcr) had fallen to only 0.2 ml/min in the drug treated group compared with 0.8 ml/min for control animals (Figure 3). Although there was also a depression of GFR in the pair-fed control rats the additional reduction in GFR caused by cis-platinum administration was statistically highly significant ($p<0.001$). This fall in GFR was accompanied by a four-fold rise in Pcr to 2 μmol/l ($p<0.001$ v controls). Although by Day 13 there was evidence of some recovery Pcr remained significantly above (approximately 200% above control values), and GFR significantly below, that of controls until the last day of the study Day 111. In the CP4 study GFR was not significantly different from control values at any time during the study (Figure 4). Despite this, Pcr was persistently elevated above controls after cis-platinum administration ($p<0.001$); this increase in Pcr was still evident approximately four months after the last injection (approximately 50% above control values).

Results of the Labstix tests were positive on Day 3 in both studies. After the single injection of 6mg/kg cis-platinum there was a marked haematuria and glucosuria, and ketone bodies were present in the urine. In the CP4 study, the first injection of 1.5 mg/kg cis-platinum resulted in a marked glucosuria and in traces of blood being present in the urine. All of the tests were negative by the next urine collection (Day 7) and there were no further positive results even in response to subsequent injections of cis-platinum in the divided dose study.

No consistent drug related proteinuria was detectable in the CP1 study whereas fluctuating protein excretion during the dosing period in the CP4 study resolved into a stable proteinuria after the last injection of cis-platinum (Figures 5 and 6). The proteinuria in the CP4 group persisted for duration of the study.

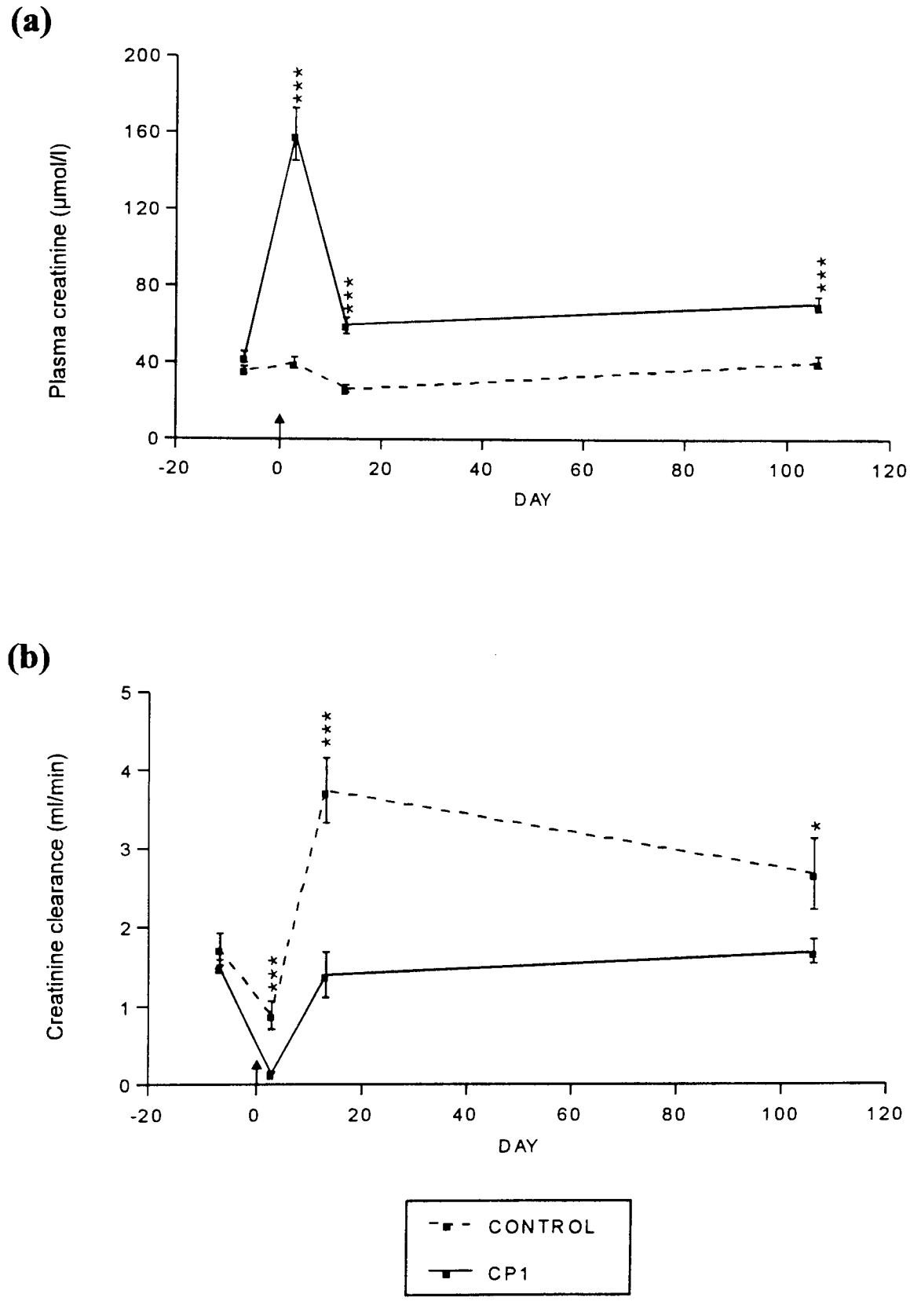


Figure 3. Effect of a single dose of 6 mg/kg cis-platinum on a) plasma creatinine b) creatinine clearance. Data are shown as mean ± SEM. n= 3 - 12.

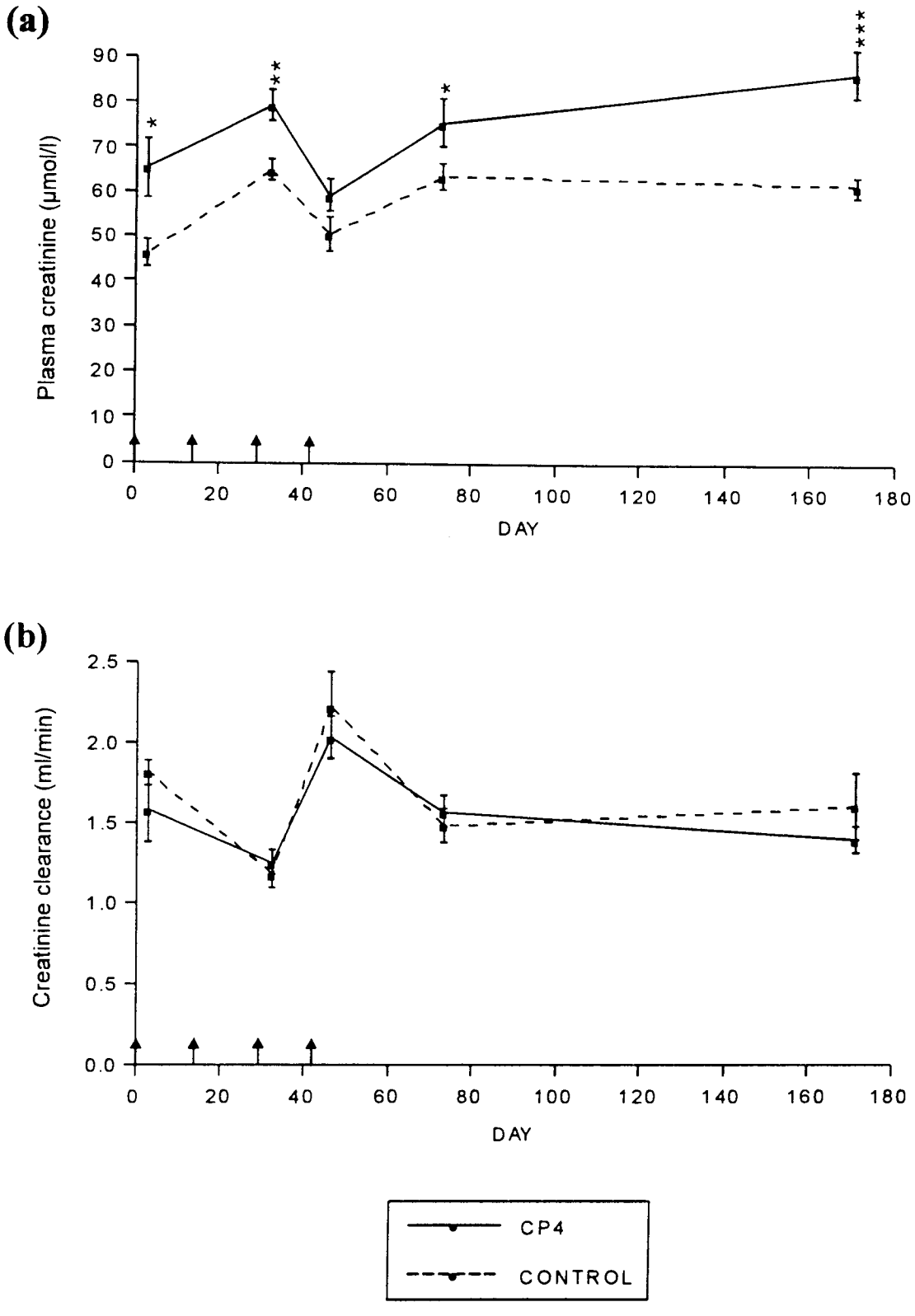


Figure 4. Effect of a divided dose of 6 mg/kg cis-platinum on (a) plasma creatinine and (b) creatinine clearance. Arrows denote dosing. Data are presented as mean ± SEM. n = 3-12.

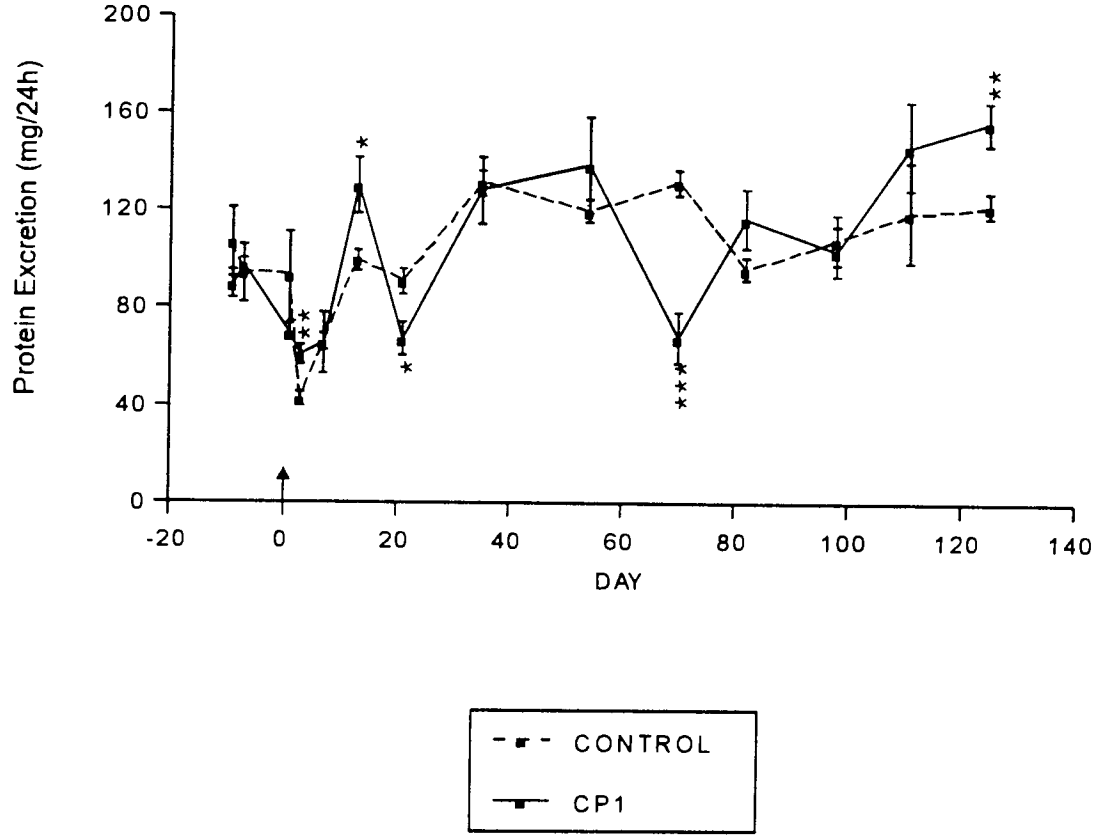


Figure 5. Effect of a single dose of 6 mg/kg cis-platinum on urinary protein excretion. Arrows denote dosing. Data shown as mean ± SEM.
n = 3-12.

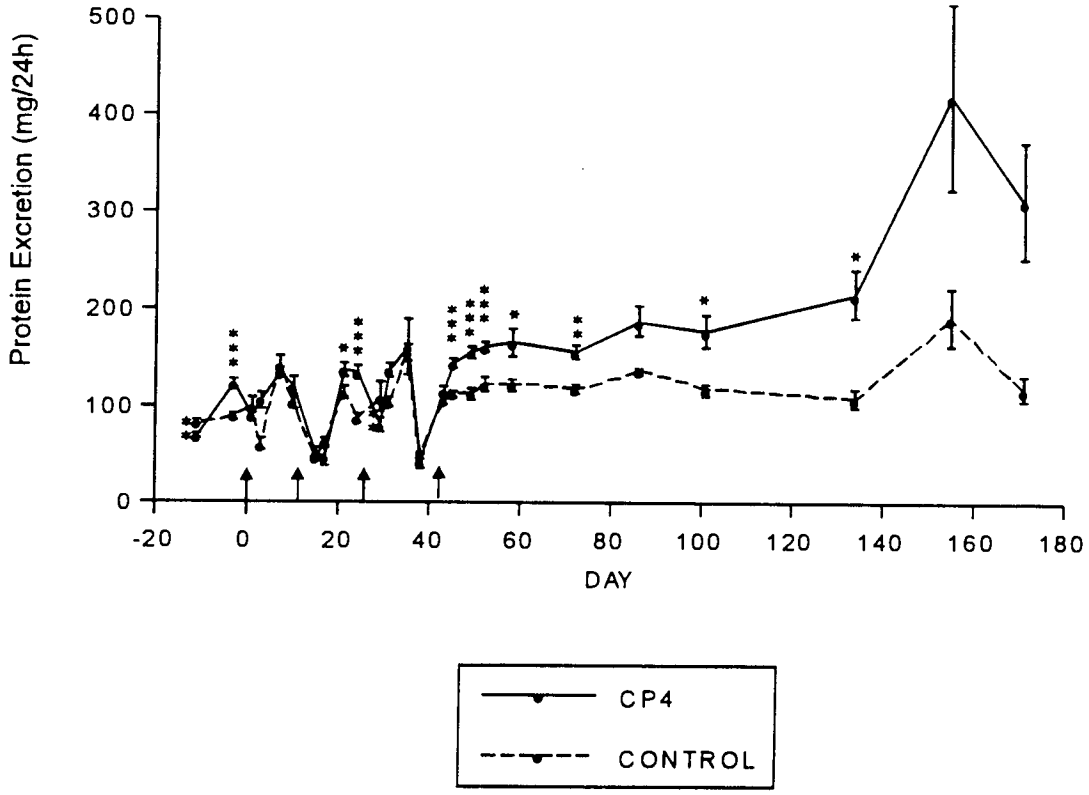


Figure 6.Effect of a divided dose of 6 mg/kg cis-platinum on protein excretion. Arrows denote dosing. Data are shown as mean ± SEM. n = 3-12.

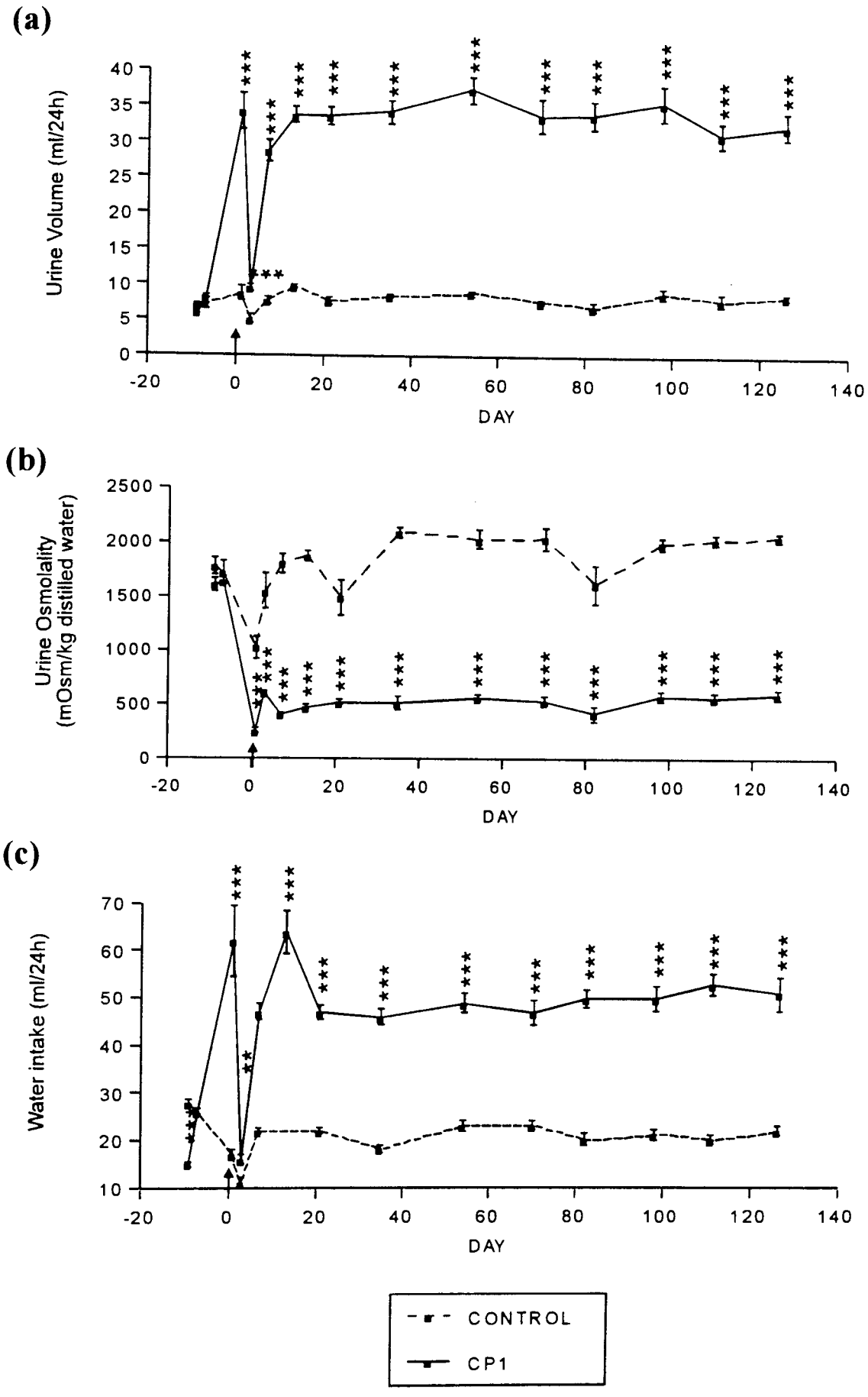


Figure 7. Effect of a single dose of 6 mg/kg cis-platinum on a) Urine volume b) Urine osmolality c) Water Intake. Arrows denote dosing. Data is shown as mean ± SEM. n = 3-12.

Following the single dose of cis-platinum the animals in the CP1 group showed an immediate in increase in urine output (Figure 7a) with an associated decrease in urine osmolality (Figure 7b) ($p<0.001$), and as a result their fluid intake was increased approximately four fold over control values (Figure7c). However, associated with the subsequent marked reduction of food intake there was an accompanying decline in fluid intake and, therefore, urine volume . This trend was also clearly visible in the pair-fed control animals. The pronounced polydipsia and polyuria returned in the cis-platinum dosed animals as food intake returned to normal; these effects then stabilised and persisted for the duration of the study ($p<0.001$ for both urine output and water intake compared to controls). In the CP4 study a much less pronounced polyuria and polydipsia was evident 3 days after the first injection but this lesion appeared to progress over the remainder of the dosing period and only stabilised several weeks after the last injection (Figure 8a, c). At this stage, urine volume was more variable between cis-platinum-treated animals in the CP4 group than in animals in the CP1 group. Whilst the mean urine volume of the CP4 group was lower than that of the CP1 group there were nevertheless marked increases in urine output in the CP4 animals compared to controls (approx. 20ml in CP4 v 35 ml in CP1 c.f. control of approx. 8 ml/24h).

The acute phase of nephrotoxic injury was monitored by measuring changes in excretion of urinary enzymes from different sites within the cell. LDH was taken to be a marker of cell membrane damage due to its location in the cytosol, NAG was used as a marker of lysosomal damage, and AAP measured to assess the extent of damage to the brush border membrane of the proximal tubule cells. Injection of a single 6 mg/kg dose of cis-platinum produced an increase in LDH excretion within 24h of administration (Figure 9a), followed 2 days later by a rise in the excretion of NAG . These effects were transient and the excretion of both enzymes in the drug-treated animals approached control values by Day 20. A small elevation in NAG excretion persisted for approximately 2 months after dosing (Figure 9c). From Day 13 onwards the cis-platinum injected animals

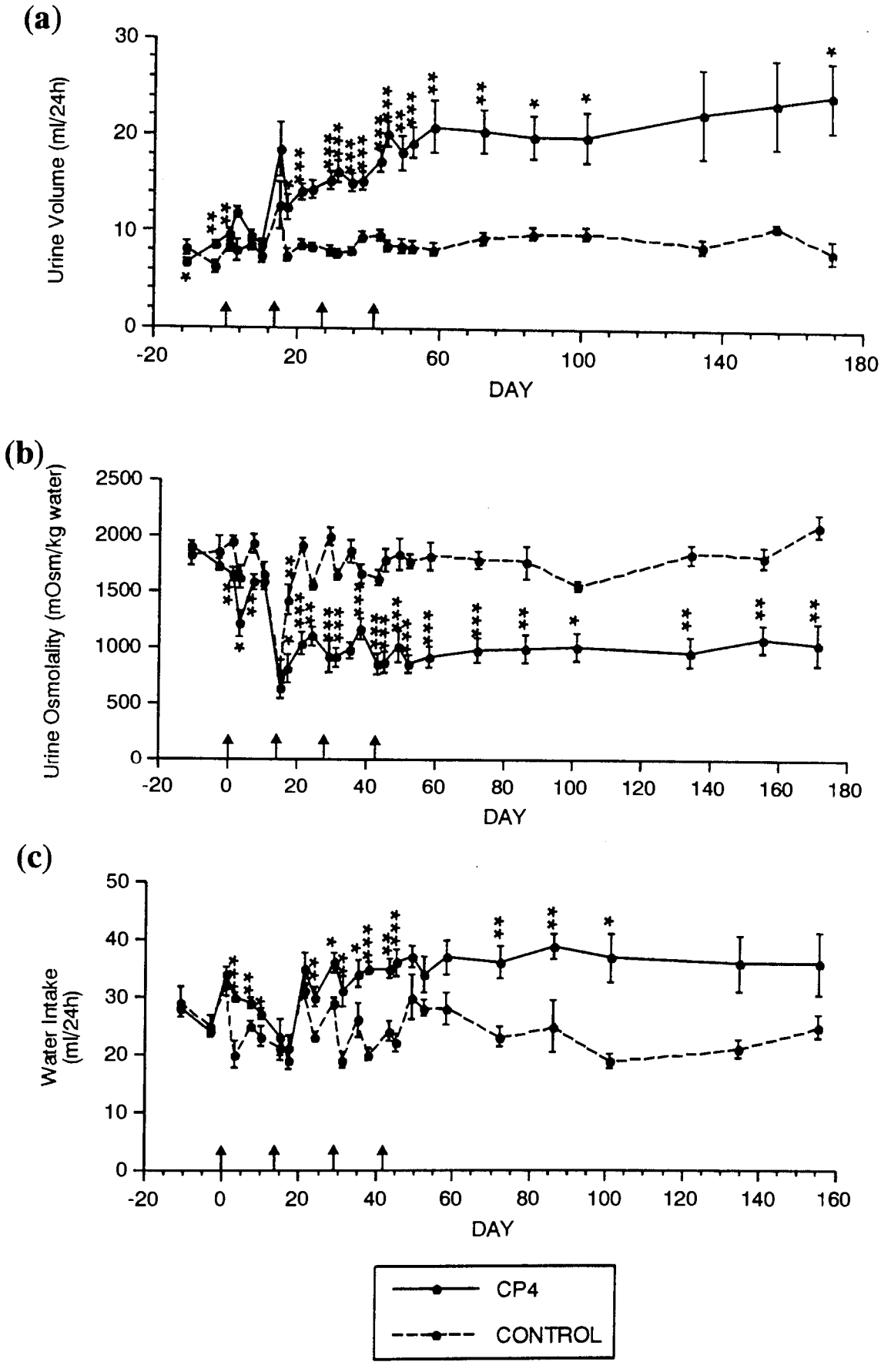


Figure 8.Effect of a divided dose of 6 mg/kg cis-platinum on a) Urine volume b) Urine osmolality c) Water Intake. Arrows denote dosing. Data are shown as mean ± SEM. n = 3-12.

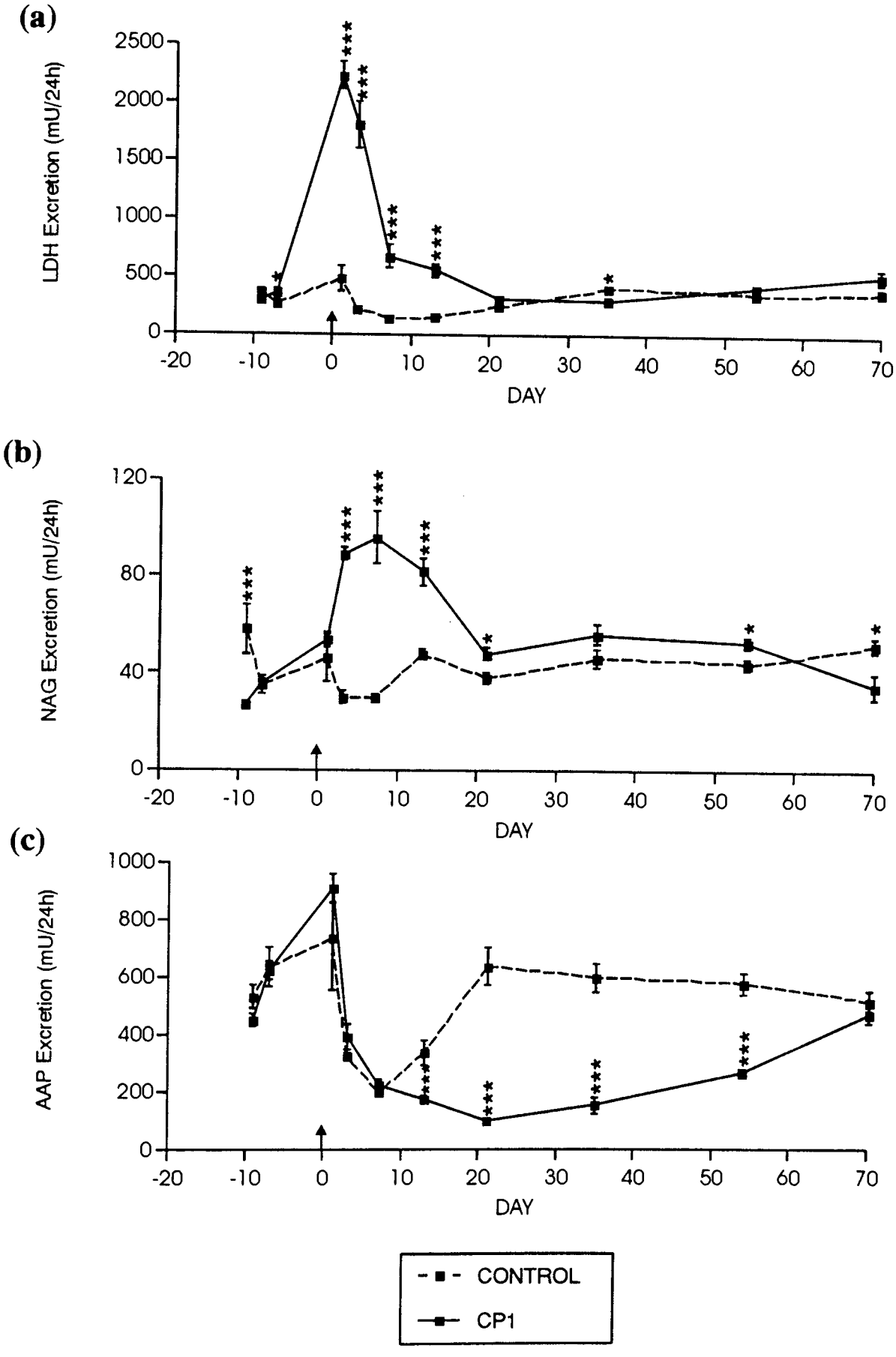


Figure 9.Effect of a single dose of 6 mg/kg cis-platinum on the urinary excretion of a) LDH b) NAG c) AAP. Arrows denote dosing. Data are shown as mean ± SEM. n = 3-12.

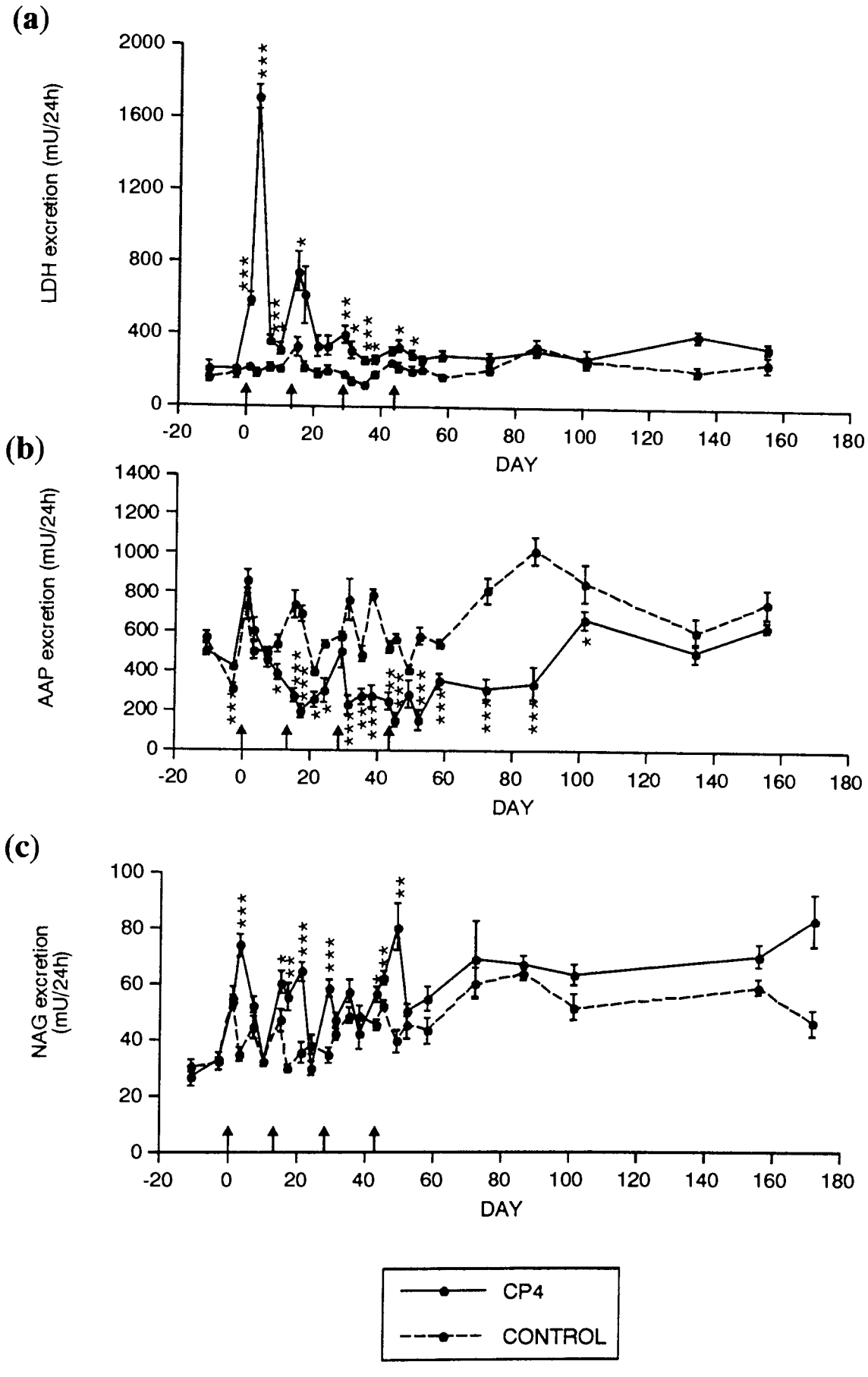


Figure 10. Effect of a divided dose of 6 mg/kg cis-platinum on the urinary excretion of a) LDH b) AAP c) NAG. Arrows denote dosing. Data are shown as mean ± SEM. n = 3-12

consistently excreted less AAP than did the control animals until the last day of measurement, Day 70 (Figure 9b). The pattern of enzymuria seen after a single dose of 6 mg/kg cis-platinum was compared to that shown by the animals given the divided doses of cis-platinum. In contrast to those animals given a single dose of cis-platinum peak LDH excretion did not occur until Day 3 after the first injection in the CP4 group (Figure 10a). The magnitude of the increase in LDH excretion, was however similar in both groups of drug treated animals (8-fold increase in CP4 animals v 11-fold in CP1 animals). Subsequent injections of cis-platinum produced significant, but much less pronounced, elevations in LDH excretion. LDH excretion decreased toward control values between injections but only returned to baseline values nine days after the final injection. A small, transient rise in AAP excretion occurred following the first cis-platinum injection in the CP4 group. Subsequent doses of cis-platinum did not produce further increases in AAP excretion. As seen in the CP1 rats, AAP excretion in the CP4 animals remained significantly below the controls during the dosing period and for a further 2 months. A gradual return of AAP excretion toward normal values was seen in both the CP1 and CP4 groups towards the end of the study period (Figure 10b). Significant increases in NAG excretion were seen following each injection in the CP4 animals. NAG excretion was however only transiently elevated following each injection and returned to control values before each subsequent injection. 10 days after the final injection, NAG excretion had returned to control values (Figure 10c).

A single 6 mg/kg dose of cis-platinum caused an immediate increase in urinary output of calcium. However output of both calcium and magnesium was transiently, but severely restricted when food intake was reduced in both the cis-platinum-treated animals and the pair-fed control rats. As food intakes returned to normal, substantial increases in Ca output were evident ($p<0.001$) but significant elevations in Mg output were not visible until three weeks after the dose of cis-platinum ($p<0.05$) (Figure11a, b). The elevated output of these cations did not produce hypocalcaemia or hypomagnesaemia, probably

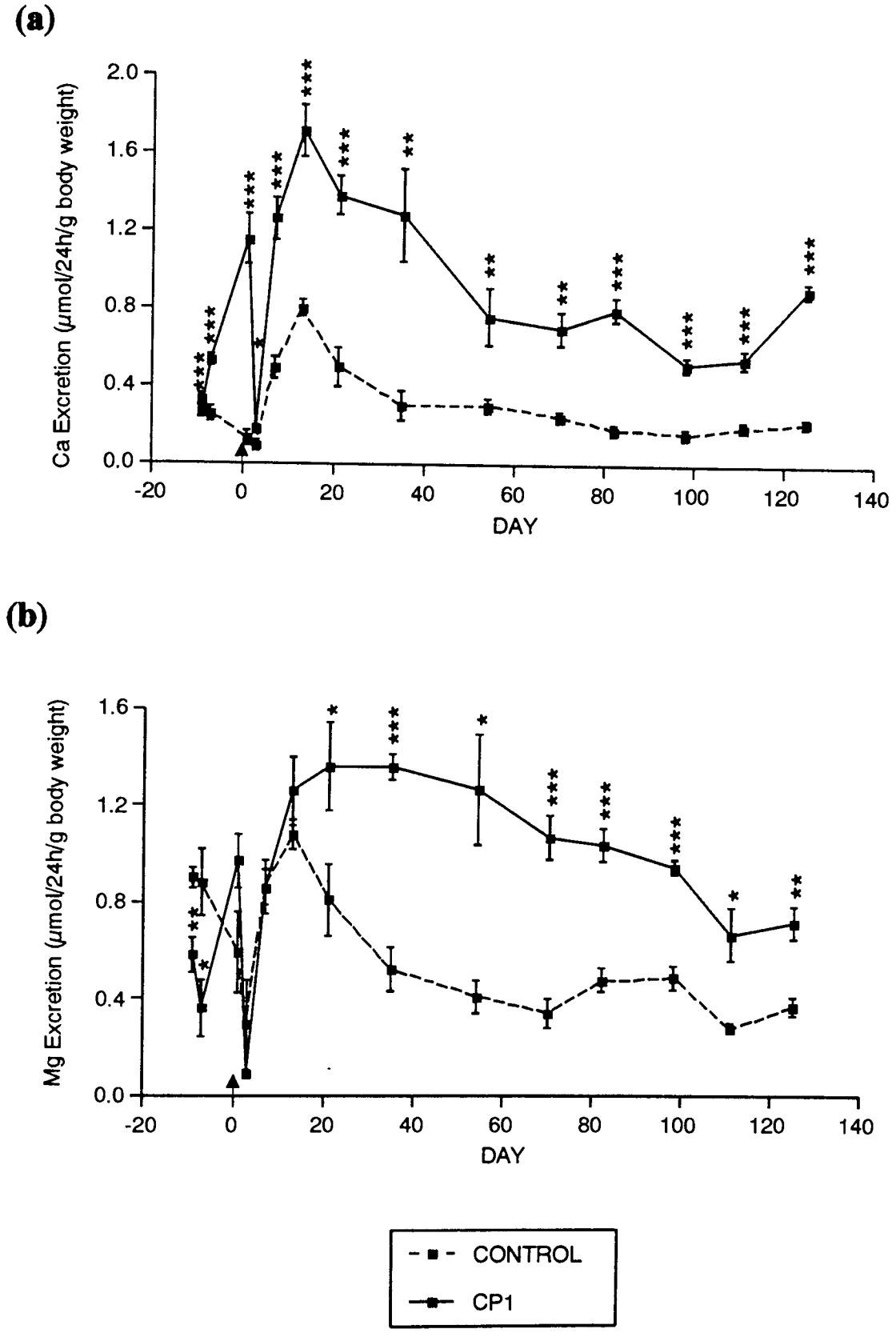


Figure 11. Effect of a single dose of 6 mg/kg cis-platinum on (a) calcium excretion and (b) magnesium excretion. Arrows denote dosing. Data are shown as mean ± SEM. n = 3-12

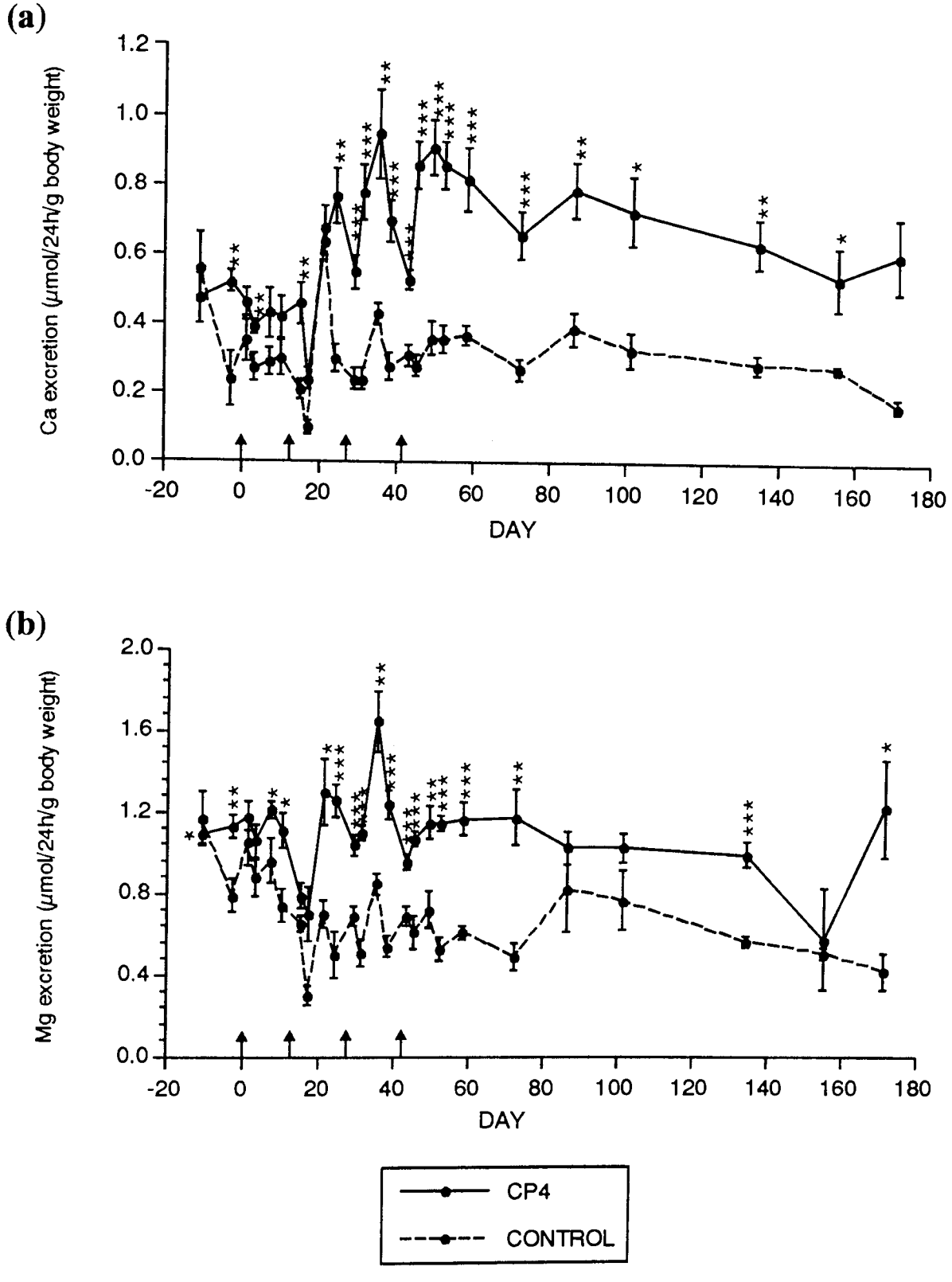


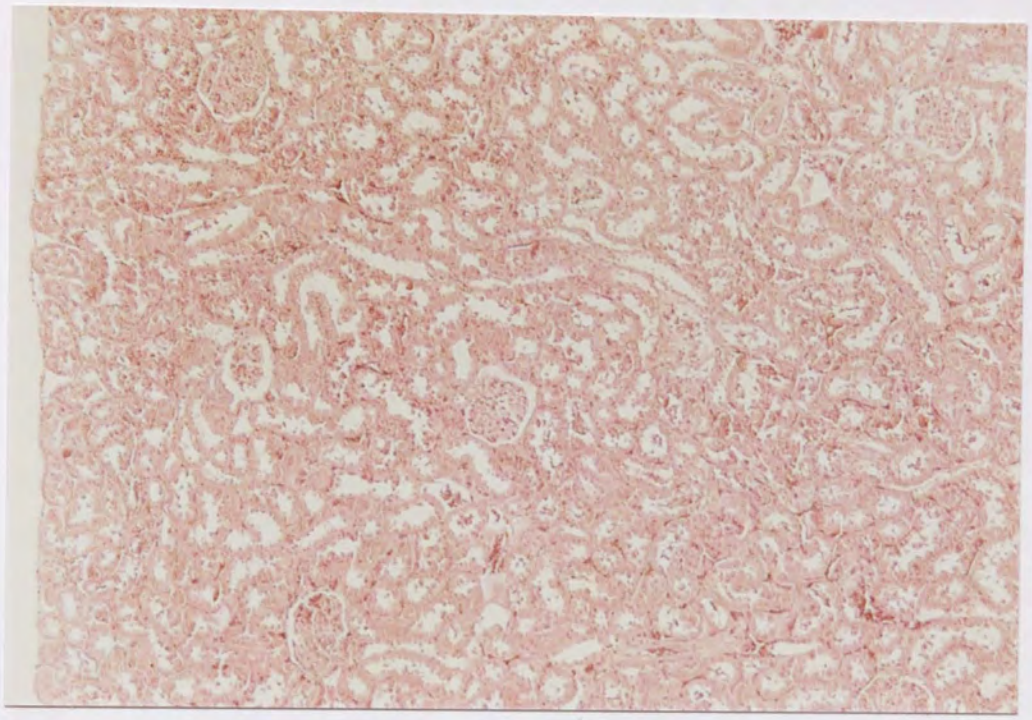
Figure 12. Effect of a divided dose of 6 mg/kg cis-platinum on (a) calcium excretion and (b) magnesium excretion. Arrows denote dosing. Data are shown as mean ± SEM. n = 3-12

due to the relatively high concentrations of these ions present in the diet supplied to the animals (Calcium 1.7%, Magnesium 0.24%). That the hypercalciuria and hypermagnesuria are attributable to decreased renal reabsorption of these ions is shown by the elevated fractional excretions of both calcium and magnesium. At the end of the study FECa in drug-treated animals was 3.4% ± 0.3 cf control animals FECa was 1.0% ± 0.1; FEMg in drug-treated animals was 20.0% ± 2.8 cf 5.1% ± 0.6 in control animals.

Calcium and magnesium excretion was also greater in the animals of the CP4 group after the first dose of cis-platinum (Figure12a, b). A decrease in calcium and magnesium output then occurred after the second dose of cis-platinum, coincident with a period of reduced food intake, but on resumption of a normal food intake hypercalciuria and hypermagnesuria were present. Further evidence that the electrolyte wasting was caused by cis-platinum administration is provided by the occurrence of peaks in output following the later doses ($p<0.001$ at the end of dosing for both calcium and magnesium) and borne out by the gradual increase in the fractional excretion of both ions. By the end of the study FECa in drug-treated animals was 3.5% ± 0.9 cf control animals 0.9% ± 0.2. FEMg in drug-treated animals was 33% ± 9 cf FEMg in control animals of 12% ± 3.3. The hypercalciuria and hypermagnesuria persisted for the duration of the study period.

The administration of cis-platinum to rats as either a single dose or as divided doses led to marked changes in the renal histopathology of the animals. Agreeing with the functional data there was variability between rats in the CP4 group in the severity of the lesions observed, but the animals in the CP1 group showed renal injury which was consistent in both nature and severity. Those animals which had the most severe histopathological damage also showed more extreme reponses of other parameters, suggesting a correlation between the biochemical and the histological findings. Animals which had received cis-platinum showed changes in morphology compatible with chronic renal injury. Dilated tubules, many of which contained hyaline casts, were prominent in both the medulla and in the cortex (Figure13b). The most striking feature for both CP1 and CP4 groups was the presence of multiple large cysts in the outer medulla. In some

a)



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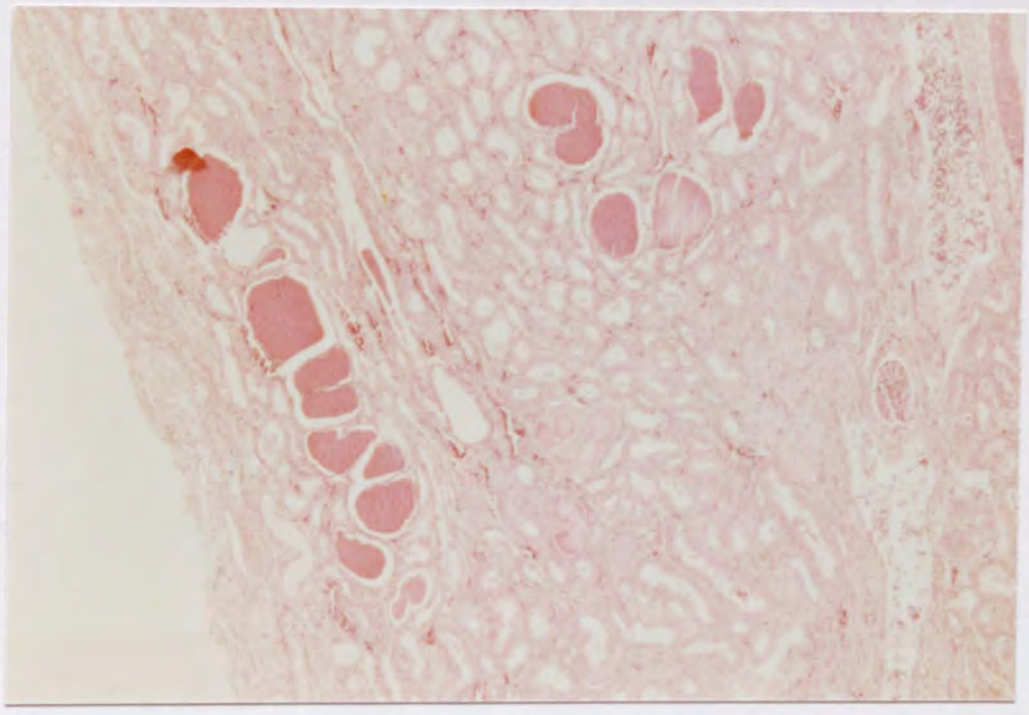
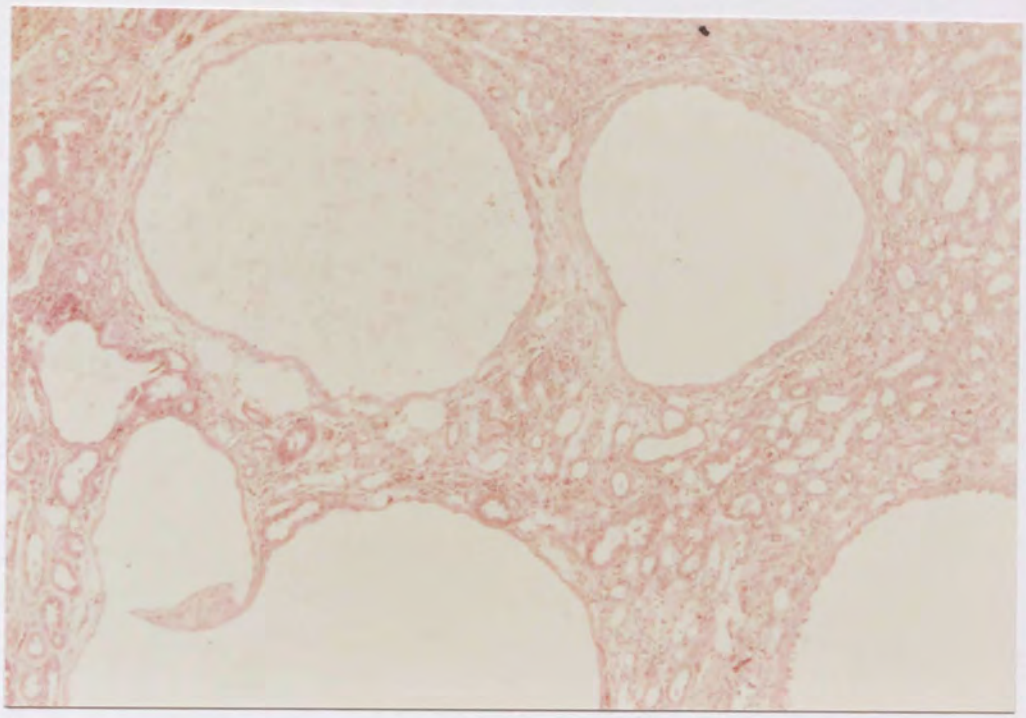


Figure 13. Histopathological injury caused by cis-platinum administration a) Control (x 90 mag) b) Hyaline casts (x 90 mag). The lesions are typical of those shown by both CP1 and CP4 groups. Micrographs are taken from CP1 animals.

a)



b)

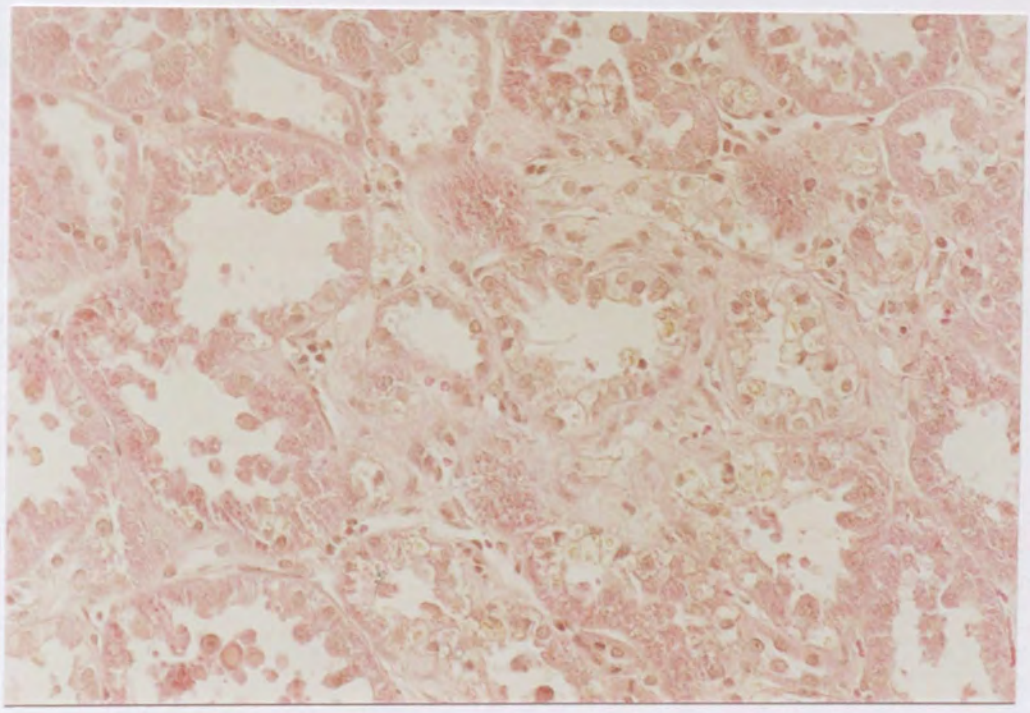
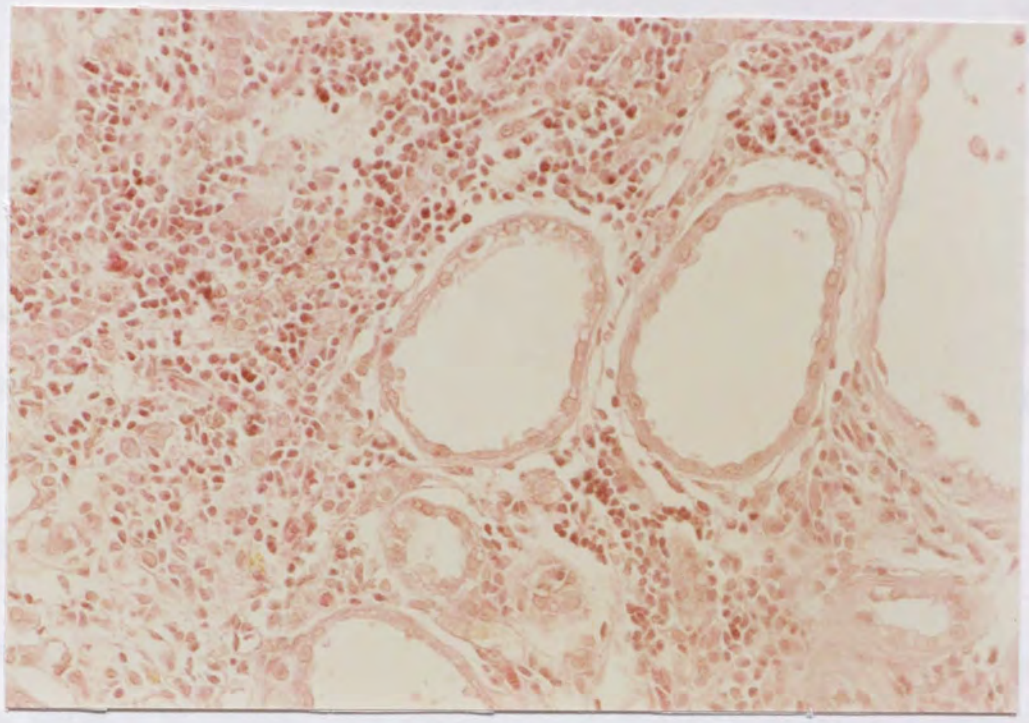


Figure 14. Histopathological injury caused by cis-platinum administration a) Cystic lesions (x 90 mag) b) Atrophic tubules (x 360). The lesions are typical of those shown by both CP1 and CP4 groups. Micrographs are taken from CP1 animals.

a)



b)

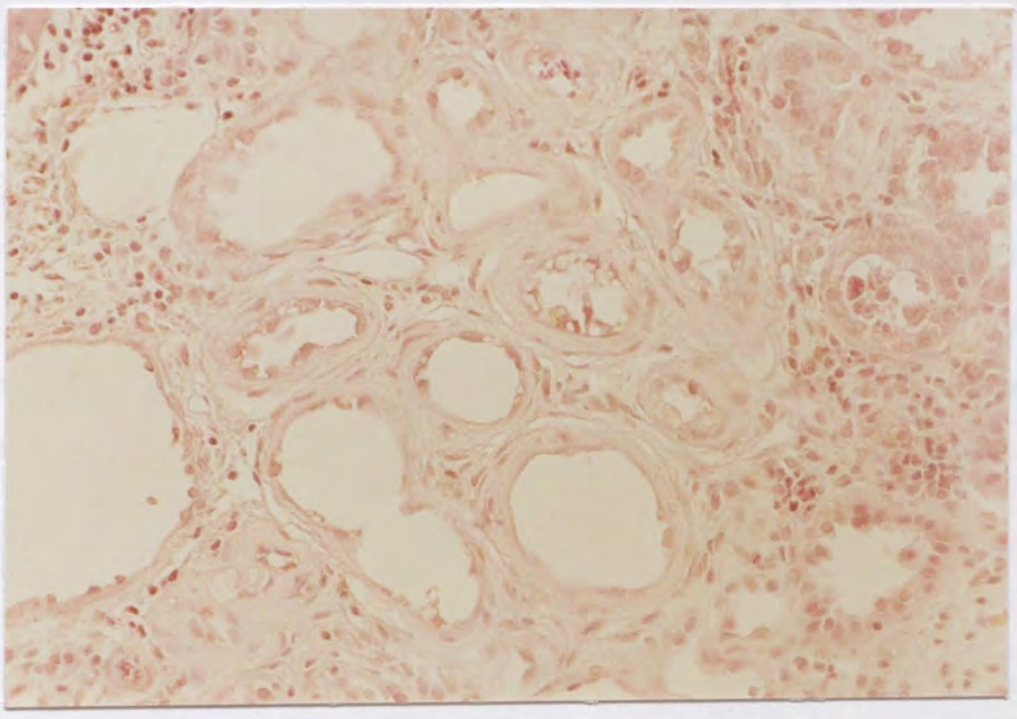


Figure 15. Histopathological injury caused by cis-platinum administration a) Lymphocytic infiltration (x 360 mag) b) Periglomerular fibrosis (x 360 mag). The lesions are typical of those shown by both CP1 and CP4 groups. Micrographs are taken from CP1 animals.

instances the cysts extended from the outer medulla into the outer cortical region of the kidney, occupying up to a third of the kidney (Figure 14a). Groups of atropic tubules were also present throughout the cortex. The atropic tubules had substantially thickened basement membranes, reduced tubular lumen space and were lined by cuboidal basophilic epithelium which was frequently vacuolated (Figure 14b). Interstitial lymphocytic infiltration was increased compared to age-matched control rats and focal interstitial fibrosis was also present (Figure 15a). Occasional glomeruli showed periglomerular fibrosis and atrophy although the majority were considered normal (Figure 15b). There were no changes in the renal pelvis after cis-platinum administration.

2.4 Discussion

Cis-platinum is routinely administered within multiple courses of drug therapy in the clinical situation. In such circumstances the kidney is exposed to repeated toxic insults at intervals only a few weeks apart. Since platinum concentrations in the kidney rapidly exceed those of plasma after only a single dose, and are cleared slowly from the kidney thereafter there may well be the expectation of long-term damage to the kidney caused by accumulated cis-platinum. The aim of the present study was to examine the extent to which cis-platinum causes permanent damage to the kidney, in particular, its effects on cation handling during, and after cessation of dosing. Both the acute phase of toxicity and the period following dosing were carefully monitored to investigate whether acute changes in renal function were predictive of the final nephrotoxicity.

The renal effects of cis-platinum were qualitatively similar for both the CP1 and CP4 animals. However whilst CP1 rats showed remarkably consistent responses, rats administered cis-platinum in 4 divided doses showed substantial variability in their responses to the drug. Some of the CP4 animals demonstrated only slight toxicity after cis-platinum treatment whereas others showed reponses approaching those observed in the single dose group. These differences in sensitivity between animals remained apparent throughout the study. This heterogeneity of toxic response has also been

observed clinically when patients are given repeated courses of cis-platinum therapy (Goren et al, 1986).

Administration of cis-platinum as a single 6 mg/kg dose caused an increase in plasma creatinine values coupled with an initial reduction in GFR. The reduction in GFR resolved by the end of the study to values not significantly lower than controls, but a significant elevation in plasma creatinine concentration was still apparent at the end of the study ($p<0.001$). Clinically, Stark and Howell (1978) reported a small, reversible, fall in GFR in patients given repeated courses of cis-platinum.

In patients hypomagnesaemia is a well recognised problem associated with cis-platinum therapy (Zumkley et al, 1982, Schilsky et al, 1982, Bell et al, 1985, Davis et al, 1980, Buckley et al, 1984). Hypermagnesuria in conjunction with symptomatic hypomagnesaemia has also been described clinically (Vozelgang et al, 1985, Schilsky et al, 1982) persisting in one study up to three weeks after cis-platinum administration (Bell et al, 1985). In the studies presented here hypermagnesuria was still apparent approximately four months after the last cis-platinum injection. Immediately following the single 6mg/kg cis-platinum injection the hypercalciuria was greater in the CP1 group than in the CP4 group after the total dose of cis-platinum was administered. However the hypercalciuria induced by both dosing regimens stabilised to similarly elevated calcium outputs over the remainder of the study period. Evidence for a primary renal reabsorption defect is shown by increases in the fractional excretion of both calcium and magnesium. The increases in fractional excretion of these ions persisted in both the CP1 and CP4 groups for the duration of the study, and at similar levels for both dosing regimens. Despite persistent hypercalciuria in both drug-injected groups the dietary content of calcium and magnesium (1.7% and 0.24% respectively) was sufficient to prevent the hypomagnesaemia and hypocalcaemia which can be induced in animal models under conditions of low dietary calcium and magnesium, following cis-platinum administration (Mavichek et al, 1984).

Polyuria and polydipsia presented early and persisted, along with reduced osmolality at a time when gross kidney function, measured by Clcr, was normal in both CP1 and CP4 groups. The magnitude of the polyuria in the CP1 group was greater, on average, than in the CP4 group overall, but some animals in the CP4 group produced urine outputs similar to those seen in the CP1 group. Safirstein et al (1981), described a polyuric state beginning only 3 days after a single 5 mg/kg dose of cis-platinum which was at that time point, unresponsive to the action of vasopressin. The chronic polyuria displayed by rats dosed with a single dose of 6 mg/kg cis-platinum has been shown to respond to the antidiuretic effect of vasopressin in the same way as control animals, but in these animals the natriuretic effect of vasopressin was reduced. The consequence of these responses was to reduce the osmolal output in cis-platinum treated animals, and therefore to reduce the maximum urine osmolality achieved after vasopressin infusion compared to control animals (Lote et al, 1987a). The disparity between these data may therefore suggest that there may either be some return of the responsiveness of the kidney to vasopressin over time or, alternatively that there may be two phases to the polyuria induced by cis-platinum, mediated by different mechanisms.

Detailed examination of the acute phase of toxicity, particularly in the CP1 rats clearly demonstrates the effects of reduced food intake on several of the parameters under study. In particular, the hypermagnesuria and calciuria were transiently interrupted when food intake was reduced. That this phenomenon is due to the reduction in food intake (and hence water intake) and not due to a direct acute effect of cis-platinum is clearly demonstrated by the same response pattern visible in the control group. Had the control group rats not had their food intake paired to the cis-platinum injected groups it would have been difficult to determine which of the observed effects were drug-related and which were merely physiological compensation mechanisms in the face of a substantial reduction in food intake.

The magnitude of the response to cis-platinum as measured by enzymuria was greater in the CP1 group than in the CP4 group. In the CP1 group LDH and NAG excretion

peaked on Day 1 and 3 respectively. There was no significant rise in AAP excretion during the acute phase of cell injury but a pronounced fall-off in AAP excretion was evident by Day 13 and which persisted to day 54. Administration of each of the 1.5 mg/kg doses of cis-platinum induced responses in the excretion of each of the enzymes studied. NAG excretion was elevated to similar levels after each injection, suggesting a repetitive, rather than a cumulative toxic effect. LDH excretion however was greatest after the first injection of cis-platinum, with smaller rises after subsequent injections. Administration of cis-platinum did not produce an initial peak in AAP excretion in the CP4 animals, but induced a persistently lower excretion of AAP with a gradual onset after the first injection. Whether this reduced AAP reflects a decrease in the amount of brush border remaining in the kidney, a decrease in brush border enzyme activity or turnover, or simply a reduction in the number of proximal tubules remaining in the kidney was not resolved in these studies.

Ward et al (1977), demonstrated that after a single dose (7.4 mg/kg) of cis-platinum regenerative lesions were clearly visible in the pars recta of the rat by Days 5-7. These regenerative changes were still apparent on the last day of their study, Day 14, the interval at which the divided doses of cis-platinum were given in this study. Regenerative changes occurring at this time may provide a means to explain the apparent reduction in toxicity of cis-platinum as measured by the decreasing effect on LDH excretion of subsequent cis-platinum doses. Newly regenerated cells after cis-platinum administration may display an increased resistance to further cis-platinum toxicity compared to those originally present, when assessed by the pattern of enzymuria.

All types of enzymuria, from both the CP1 and CP4 groups had returned to control values by the end of the study period demonstrating that monitoring urinary enzyme excretions is inappropriate in the assessment of chronic cis-platinum nephrotoxicity. A return to normal enzyme excretions after cis-platinum dosing appeared to suggest recovery of any damage caused by cis-platinum had occurred. However belying this fact was the discovery that persistent hypercalciuria and hypermagnesuria existed after

enzyme excretion levels were apparently normal, indicating serious functional impairment of the kidney still existed.

Whilst individual doses of 1.5 mg/kg of cis-platinum may cause only slight renal toxicity, evidence suggests that the effects of pre-treatment or repeated dosing with cis-platinum may lead to an increase in the renal accumulation of Pt compared to single doses of the same amount (Litterst and Schweitzer, 1984). If the determining factor for toxicity was the tissue Pt concentration one might argue that administering repeated smaller doses of cis-platinum could produce greater toxicity than a single dose of the same total amount. Repeated administration of cis-platinum (either 5 mg/kg or 3.3 mg/kg at 24h intervals) has been shown to increase the amount of Pt deposited in the kidney of the guinea pig, when compared to a single dose of the same amount (10 mg/kg) (Litterst and Schweitzer, 1984). Urinary excretion of Pt was also lower in cis-platinum pre-treated animals, suggesting that pre treatment with cis-platinum may damage the kidney severely enough to reduce the clearance of subsequent doses. A reduction in the clearance of Pt may well lead to greater tissue depositions of Pt in pre-treated animals. Effects of repetitive dosing of cis-platinum on renal accumulation of Pt were not measured at long intervals after dosing ceased. However, various lines of evidence suggest the form in which Pt exists is more important than its concentration (Safirstein et al, 1984, Litterst and Schweitzer, 1984) and evidence presented from the studies here suggest that administering cis-platinum as divided doses is certainly no more toxic than a single dose of the drug. The Pt which is accumulated within the kidney after cis-platinum dosing appears to remain very stable over time and may only cleared very slowly from the kidney (Appenroth et al, 1990). Pt levels measured after a single 6mg/kg dose of cis-platinum remained elevated over controls in the cortex and outer medulla of the rat at one month post-dose, and took a further month to return to control values (Dobyan et al, 1985). However, data from a single dog in another study showed measurable kidney Pt concentrations at 12 weeks after a single dose of 2 mg/kg cis-platinum (Lelieveld et al 1984). The variances in the apparent renal half life of Pt in kidney may be attributable to

the different species under study. The data from Dobyan et al (1985), suggest that during that later stages of the studies described herein the renal Pt concentrations should be at normal levels and any functional effects observed are a not a consequence of residual Pt in the tissue.

The histopathological lesions observed in this study were consistent with the induction of chronic renal injury after cis-platinum dosing in both CP1 and CP4 groups. The types of cellular injury (tubular dilation, atrophy, cast formation, renal cysts) seen in the cis-platinum dosed groups in this study are in accord with other published studies describing the pathogenesis of renal damage caused by cis-platinum in rats (Choie, Longnecker and Del Campo, 1981, Dobyan et al, 1981, Dentino et al, 1978) with varying dosage regimens. In humans, while damage to the proximal tubule certainly occurs, there also appears to be further damage to the distal parts of the nephron (Gonzalez-Vitale et al, 1977). The lesions appear to occur in the region of the kidney where most Pt is accumulated i.e. the corticomedullary junction and appear to progress to cystic lesions (Choie et al, 1981, Dobyan et al, 1981), as observed in the CP1 and CP4 groups of this study approximately 4 months after dosing.

These data suggest that cis-platinum alters renal function in a subtle but permanent manner which may not be measurable by conventional measurements of renal competence. At 4 months after dosing with cis-platinum there was no evidence to suggest that administration of a single bolus dose produced greater nephrotoxicity than when cis-platinum was administered as divided doses of the same total amount. At this time hypercalciuria and hypermagnesuria still existed in both CP1 and CP4 groups whilst measurements of GFR indicated no difference from controls, suggesting a disassociation between the effects on whole kidney function and the effects cis-platinum may exert on electrolyte handling in the kidney. Since cis-platinum causes extensive damage to the cortical region of the kidney it is tempting to speculate that the reduction in reabsorption of calcium and magnesium occurs, at least in part, as a direct consequence of the loss of functional proximal tubules. However this study does not provide direct evidence for the

provide direct evidence for the site at which cis-platinum impairs calcium and magnesium reabsorption in the kidney and cis-platinum could also exert its effects in the latter parts of the nephron where substantial amounts of both calcium and magnesium are also reabsorbed.

The studies presented here clearly show that the calcium and magnesium handling lesion(s) seen in human subjects can be suitably reproduced in the rat. The defects in the renal handling of calcium in particular are shown to be an immediate effect of cis-platinum dosing which persist long after more conventional measurements of renal function appear to return to normal, mimicking the clinical situation. The comparison of the responses of the animals to the different dosing regimens also highlighted differences in sensitivity to renal injury shown by animals given the same dose of cis-platinum. Since these studies were conducted using an inbred strain of rat one might expect that the responses to the drug would be more homogeneous than would occur in human subjects. This heterogeneity of response therefore highlights the problem of selecting an appropriate animal model in which to study human toxicological problems, which is the subject of the series of studies presented in the next chapter.

That cis-platinum induces a persistent renal wasting defect of calcium and magnesium is proven by this study. However, particularly in the acute stages of cellular injury, concomitant changes in whole kidney function as measured by reductions in GFR, particularly in the CP1 group, confused the situation as to whether the disruptions in electrolyte homeostasis were a consequence of wider tissue disruption rather than a selective effect of the drug. In order to study any selective effects which cis-platinum may have on the renal reabsorptive processes for calcium and magnesium it would therefore be desirable to design dosing strategies which produced increased excretion of these cations without altering other measures of kidney function. Dissociation of these acute electrolyte changes from other whole body and renal toxic effects of cis-platinum would, in all probability, require extensive and careful dose-response studies to resolve whether indeed these phenomena could be separated. To invest sufficient time and

investigations presented in subsequent chapters of this thesis became concerned with the site and mechanisms by which aminoglycoside antibiotics induce their selective, acute effects on the renal handling of calcium and magnesium.

Chapter 3. Differences between strains of rat in the renal sensitivity to the administration of gentamicin .

3.1 Introduction

Gentamicin is commonly prescribed for the treatment of gram-negative sepsis in humans but has a relatively narrow therapeutic index. Nephrotoxicity is the major problem associated with aminoglycoside therapy, and is generally manifest as proximal tubular necrosis with associated tubular dysfunction, which can deteriorate to produce acute renal failure (Bennett, 1983). In addition to causing widespread tubular disruption, aminoglycoside antibiotics have also been shown to produce alterations in the renal handling of electrolytes, particularly calcium and magnesium, which precede changes in other subtle indices of renal injury such as enzymuria (Harpur et al, 1985). Clinically, the renal wasting of calcium and magnesium may result in reduced plasma levels of these ions with potentially dangerous consequences (Zaloga et al, 1984, Davey et al, 1985).

The accurate extrapolation of data from toxicological studies using animals to human subjects requires that the species under study responds to a toxin in a similar way as do humans. The nephrotoxic effects of aminoglycosides have been clearly demonstrated in several rat strains and generate similar lesions to those observed in humans (Luft et al, 1976, Bennett, 1983). The purpose of the studies presented here was to compare the renal responses of three strains of rat commonly used in physiological and toxicological experiments to the administration of a drug with nephrotoxic potential, gentamicin. Whilst the Fischer 344 rat is thought to be similar to the human in terms of its sensitivity to drug-induced renal injury (Kosek et al, 1974), there is a lack of direct comparative information regarding inter-strain sensitivity. Of particular interest was the extent of disruption of electrolyte handling produced in the different strains of rat after gentamicin administration since no direct comparisons of this feature of aminoglycoside toxicity have been made. These studies were therefore carried out to determine which rat strain;

Fischer 344, Sprague-Dawley or Wistar, was most sensitive to gentamicin-induced renal injury.

3.2 Methods

Initially a pilot study using only one dose of gentamicin administered to the three strains of rat was undertaken. Male Fischer 344 (F344); weight range 176-195g, Sprague-Dawley (SD); weight range 169-225g, and Wistar rats; weight range 159-261g were randomly divided into gentamicin-treated and saline-treated control groups (n=6) for each strain. All animals were acclimatised to individual metabolism cages for 1 week before the study commenced. Single daily SC injections of gentamicin sulphate, at a dose of 40mg base/kg (Cidomycin, Roussel), or 0.9% saline were administered at approximately 10.00 am to each animal for 5 days. Urine samples were collected over 24 h periods into covered tubes surrounded by ice for two successive days prior to treatment, and daily over the dosing period. Measurements were made of urine volume, and osmolality by freezing point depression, creatinine by a standard picrate method, calcium and magnesium after dilution with acidified $LaCl_3$ by atomic absorption spectroscopy, N-acetyl-ß-glucosaminidase (NAG) excretion, and total protein by the method of Lowry et al, (1951). Detailed methods for these analytical measurements can be found in appendices 1-5. A pre- and post-treatment blood sample of approximately 1ml was obtained from each animal by cardiac puncture for the determination of plasma calcium, magnesium, creatinine and blood urea nitrogen (BUN) concentrations. For each animal, all measurements made after drug or saline treatments were expressed as a percentage of the mean value of both pre-treatment days i.e. % Treatment/Pre-treatment (%T/PT). Mean data (%T/PT) were obtained for each group and used to compare drug and control groups of the same strain. Comparisons were also made between the means of the absolute values obtained on the 2 days prior to dosing for the rats of each strain. Data were analysed statistically using analysis of variance. Data were taken to be significantly different from each other at P-values of 0.05 or less.

In the second part of this study a dose response of the three strains of rat to the renal effects of gentamicin was investigated. Essentially a similar protocol was followed to the pilot study, but there were some differences which are detailed below. Age-matched (6-8 weeks) male animals of F344 (weight range 100-150g), SD (150-250g) and Wistar (125-200g) strains were randomised into their treatment groups for each strain (n=6 per group). Gentamicin sulphate (Sigma) was prepared as dosing solutions of 25, 37.5, and 45 mg/gentamicin base per ml in 0.9% w/v saline, aliquoted for each day's dosing and stored at 4°C. On days 1-6, gentamicin was administered SC in a dose volume of 1ml/kg at approximately 08.00 and 16.00h giving a final total dose of 50, 75, and 90 mg gentamicin base/kg to the respective groups. Animals in the control groups were dosed with 0.9% saline. Animals were acclimatised to the study room environment and were placed in individual metabolism cages for 16h per day for one week before the study began. Prior to the start of the dosing period two consecutive control urine collections were made from each animal. Each day, after the 16.00h dosing, animals were placed into individual metabolism cages for urine collections. For the remainder of each day the rats were housed in their treatment groups in home cages. During the one week acclimatisation period, and throughout the study environmental conditions of the study room were 12h light/12h dark, at 21 ± 2 °C and 55 ± 10% relative humidity. The rats had free access to food and water whilst in home cages (approximately 08.00-16.00h each day). During the period that the animals were in individual metabolism cages animals had free access to water only, since urine collections free from food contamination could not be obtained when the pelleted diet was available to the animals during urine collection periods.

Urine volume and osmolality, creatinine, total protein, calcium and magnesium were measured in each urine sample; in addition, qualitative analysis of urine for the presence of blood and glucose using Labstix was also carried out. Desalted urine samples were used for the measurement of N-acetyl-ß-glucosaminidase (NAG), lactate dehydrogenase (LDH), alkaline phosphatase (ALP), and γ-glutamyl transpeptidase (GGT) activities.

LDH was measured as a indicator of membrane leakiness since it is located in the cytosol, GGT and ALP measured as indicators of brush-border membrane damage, and NAG as a measure of lysosomal damage.

Approximately 0.5ml of blood was collected from the tail vein of each animal into tubes containing lithium heparin on days -1, 4, and prior to termination on day 7 . Plasma samples were analysed for Ca, Mg, and creatinine. Methodological details are described in Appendix 8.

Data were analysed statistically using analysis of variance techniques. Data were taken to be significantly different from one another at P values of 0.05 or less.

At termination, approximately 24h after the last injection, both kidneys were removed, and fixed in 10% formol saline. Haematoxylin and Eosin sections were prepared from each kidney and examined by a pathologist.

3.3 Results

3.31 Pilot study results (40 mg gentamicin base/kg)

Treatment-related significant increases in NAG excretion were evident from days 1-5 in the SD strain (maximum 2-fold increase) ($P < 0.01$) and from days 2-5 in the Wistar strain (maximum 1.6-fold increase) ($P < 0.01$ by day 3). In contrast, there were no significant increases in NAG excretion caused by gentamicin administration in the F344 strain, on any day of the study (Figure 16a).

Progressive increases in calcium excretion (maximum 2-fold increase) which were significant on days 3-5 in SD rats ($P < 0.05$) and after 5 days in Wistar rats ($P < 0.05$) (Figure 16b). There was no effect of gentamicin treatment on calcium excretion on any of the study days in the F344 strain. Treatment-related increases in magnesium excretion (maximum 2-fold) were visible on days 2-4 in the SD strain ($P < 0.05$). There were no

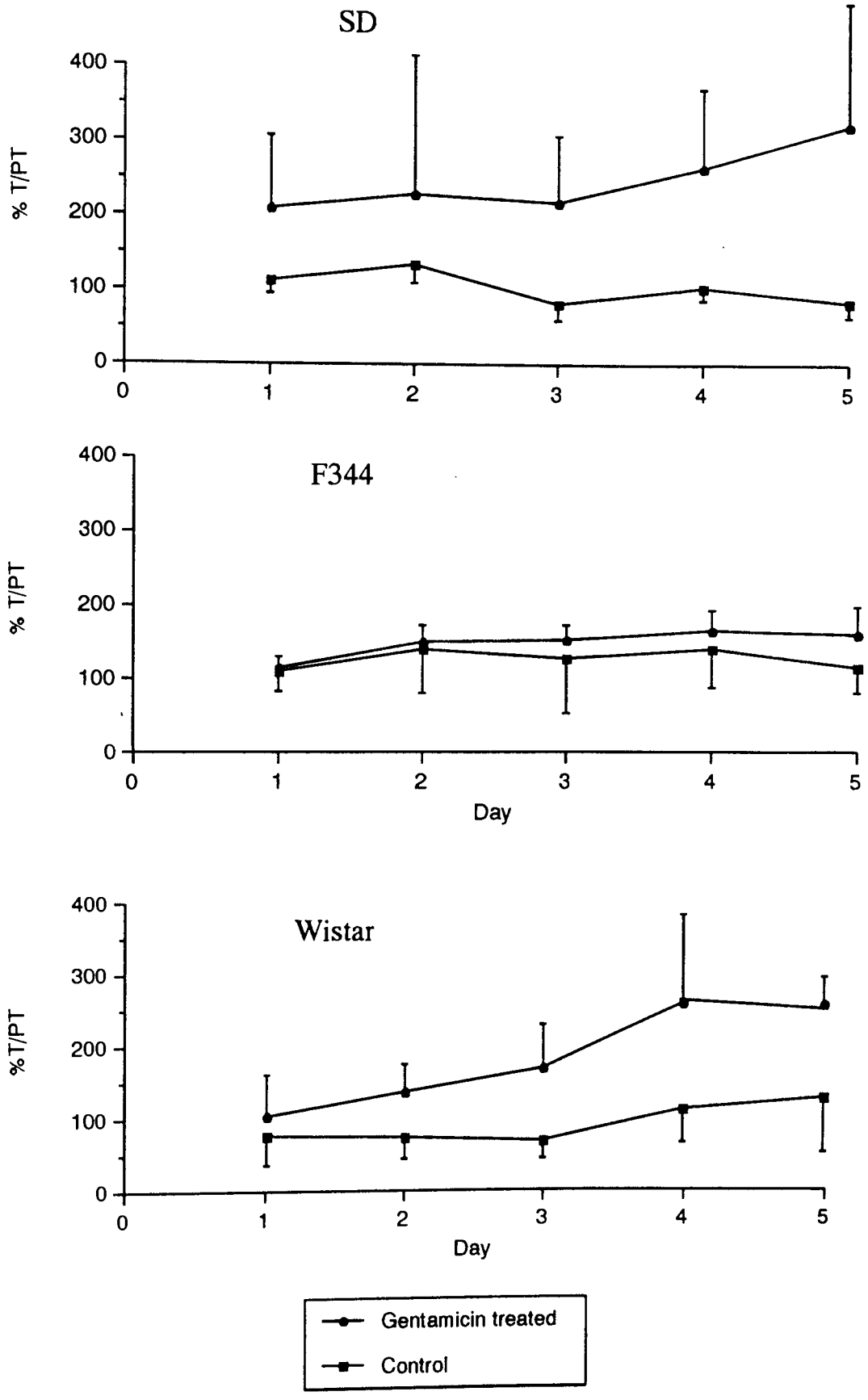


Figure 16 (a). Effect of 40 mg/kg/day gentamicin administration to F344, SD and Wistar rats on NAG excretion. Data are shown as mean ± SEM. n = 6.

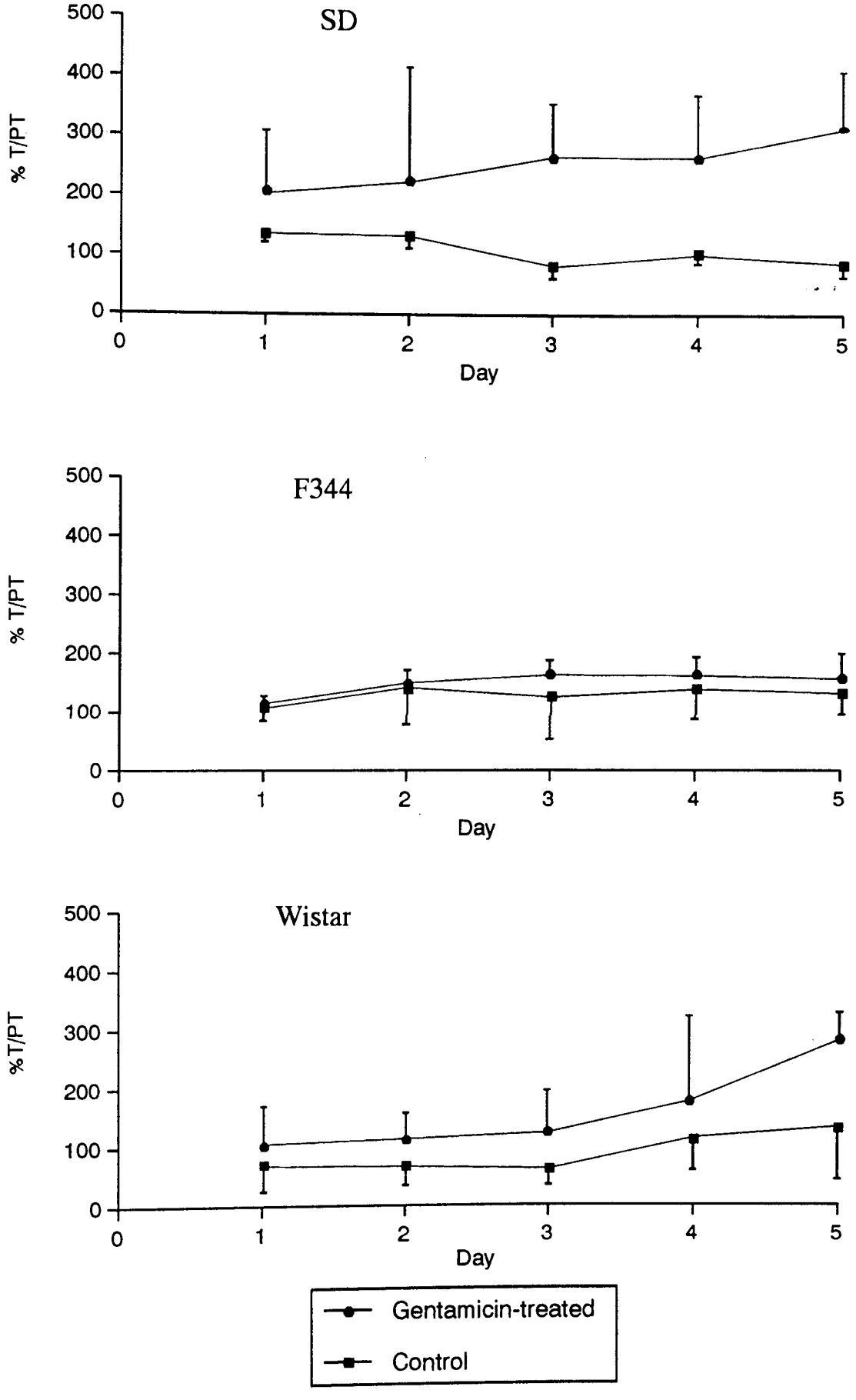


Figure 16 (b). Effect of 40 mg/kg/day gentamicin administration to F344, SD and Wistar rats on Calcium excretion. Data are shown as mean ± SEM. n = 6.

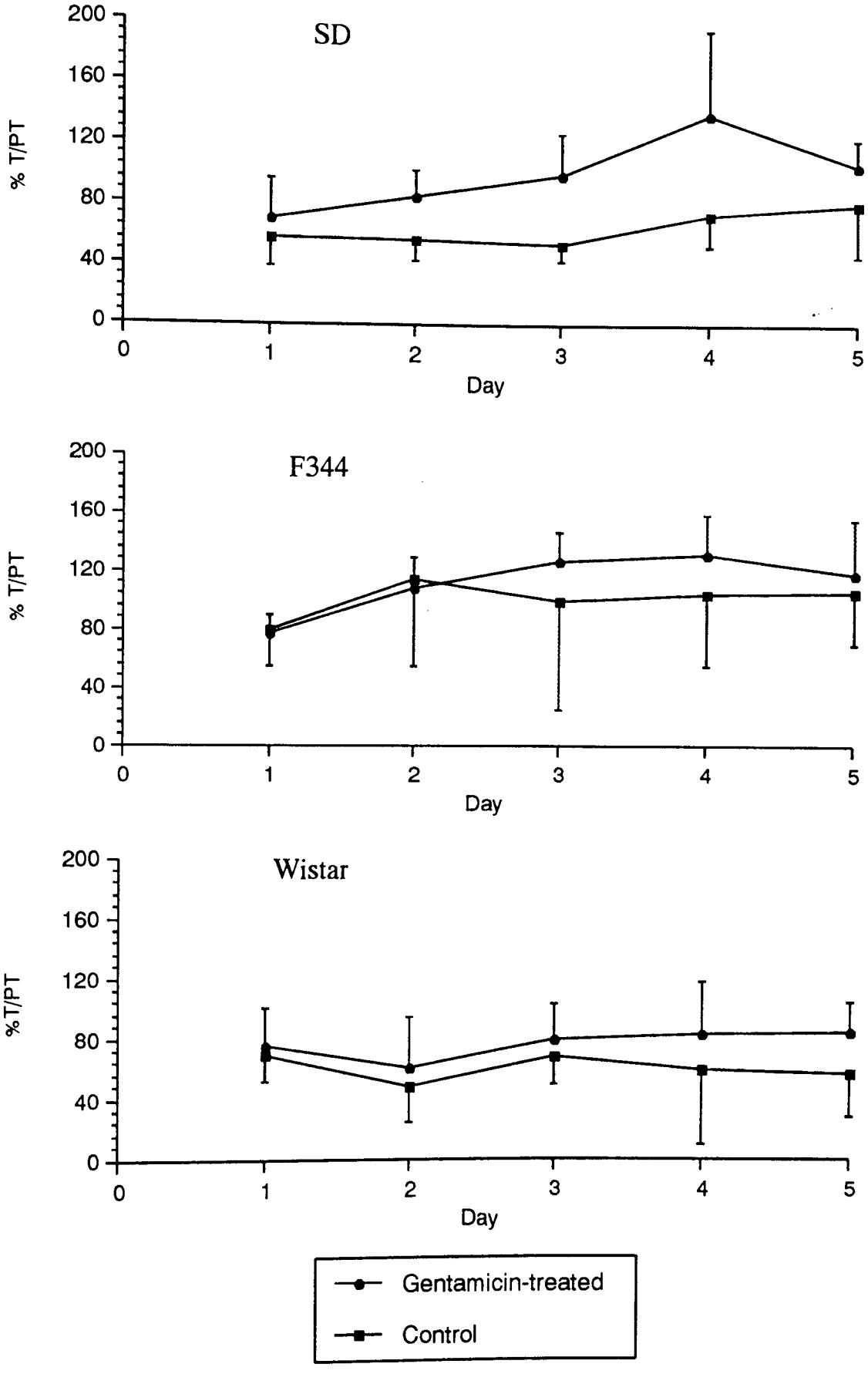


Figure 16 (c). Effect of 40 mg/kg/day gentamicin administration on magnesium excretion in F344, SD and Wistar rats. Data are shown as mean ± SEM. n = 6.

differences in magnesium excretion compared to control groups in either of the other two strains over the study period (Figure16c). There was no effect of gentamicin administration on urine volume, osmolality, or protein excretion in any of the strains of rat under study (Table 3). There were no changes in BUN, plasma creatinine, or creatinine clearance after drug administration (Table 4). Examination of the H & E sections from this study revealed no evidence of drug-related renal injury in any strain of rat.

In addition to the information gathered on the differential responses of the strains of rat to gentamicin dosing, this study also produced data which indicated substantial baseline differences, in some parameters, between the rat strains. The data are shown as mean ± SEM and, except osmolality, are 24h excretion values expressed per 100g body weight. The control excretion of NAG in the F344 strain was significantly lower at 0.9±0.6U ($P < 0.01$) than that of the Wistar (3.4±0.6U) and the SD (2.2±0.4U), which were not significantly different to each other. Urine osmolality was not significantly different between SD (1859±72 mOsm/kg H_20) and F344 groups (1895±66 mOsm/kg H_20), but the Wistar group had a significantly lower urine osmolality (1312±177 mOsm/kg H_20), ($P < 0.01$); associated with a substantially higher urine output. Urine volume was almost 2-fold greater in the strain with the highest urine output (Wistar; 7.5±0.5ml) than in the lowest (F344; 4.0±0.2ml).

There were significant differences in basal calcium excretion between all 3 strains. The rank order for calcium excretion was Wistar (62.6±9.8 µmol) > F344 (25.2±10.2 µmol) > SD (12.2±1.9 µmol). Similarly, magnesium excretion was significantly higher in the Wistar strain (210.6±33.8 µmol), ($P < 0.01$) than the other two strains which were not significantly different to each other (SD; 99.4±6.5 µmol and F344; 90.4±26.4 µmol).

Table 3. Effect of gentamicin administration on these urinary measurements compared to control animals in three rat strains. Values are taken from the last day of the pilot study (Day 5) and are expressed as mean ± S.E.M.

Rat Strain	Urine Volume (ml/24h)	Urine Osmolality (mOsm/kg H_2O)	Protein excretion (mg/24h)
Sprague-Dawley (Control)	10.5 ± 0.8	1941 ± 42	157 ± 10.6
Sprague-Dawley (40mg/kg/day gentamicin)	11.9 ± 1.1	1805 ± 78	171 ± 8.2
Fischer 344 (Control)	8.3 ± 0.4	1702 ± 95	136 ± 4.0
Fischer 344 (40mg/kg/day gentamicin)	9.1 ± 0.9	1730 ± 90	144 ± 9.6
Wistar (Control)	14.2 ± 0.9	1702 ± 46	201 ± 14
Wistar (40mg/kg/day gentamicin)	14.8 ± 1.4	1734 ± 33	217 ± 11

Table 4. Effect of gentamicin administration on plasma biochemistry compared between rat strains. Values are taken from the last day of the study, Day 5. Data are presented as mean ± S.E.M. There are no statistically significant differences between drug-treated and control groups, or between rat strains for these parameters.

Rat Strain	BUN (mg/dl)	P_{Cr} (mg/dl)	Cl_{Cr} (ml/min)
Sprague-Dawley (Control)	18.4 ± 0.5	0.57 ± 0.07	2.3 ± 0.6
Sprague-Dawley (40mg/kg/day gentamicin)	20.9 ± 0.8	0.67 ±0.08	1.3 ± 0.3
Fischer 344 (Control)	19.0 ± 0.5	0.57 ± 0.10	1.7 ± 0.5
Fischer 344 (40mg/kg/day gentamicin)	19.9 ± 1.3	0.71 ± 0.11	1.61 ± 0.6
Wistar (Control)	19.6 ± 0.6	0.54 ±0.03	2.3 ± 0.1
Wistar (40mg/kg/day gentamicin)	17.9 ± 0.5	0.56 ±0.01	2.1 ± 0.3

3.32 Dose response of the F344, SD and Wistar rat strains to gentamicin administration.

Treatment-related effects of gentamicin were apparent on urinary protein excretion, LDH, ALP and NAG excretion, and calcium excretion. In addition there were differences in baseline excretion values of creatinine, magnesium, calcium, GGT, NAG, LDH, protein, and urine volume and osmolality between the strains. Data are presented as mean ± SEM, and, except for osmolality, are expressed as excretion per hour.

Gentamicin-treated Wistar rats showed a marked dose-related progressive proteinuria which was visible in the 90 mg/kg dose group by day 2, but not statistically significant until Day 3 of dosing. SD rats also showed a dose-related proteinuria in response to gentamicin dosing which was statistically significant in the higher (50 and 75 mg/kg) dose groups by Day 4 of dosing. Gentamicin administration to F344 rats also appeared to generate small increases in urinary protein excretion in response to repeated dosing. In the F344 strain, the proteinuria did not appear to be dose-related since the magnitude of the proteinuria was similar in all dose groups; additionally the proteinuria was not statistically significant in this strain until Day 5 of dosing ($P < 0.05$) (Figure 17).

All three strains of rat showed dose- and time-related increases in LDH excretion in response to gentamicin dosing. Marked dose-related effects on LDH excretion were apparent after only two days dosing with gentamicin in the Wistar and SD strains. In contrast, LDH excretion was significantly increased only after 4 days dosing with gentamicin in the F344 strain. Wistar and SD control rats showed consistently higher baseline excretion of LDH (Wistar: 5.7±0.41 mU/h, SD: 3.3±0.61 mU/h) than the comparable F344 control animals (F344: 2.2±0.39) (Figure 18). Due to analytical problems NAG concentrations were reassessed from remaining, stored urine samples of which there were an incomplete set for all strains, due to small 16h urine volumes in some animals. The consequence of this was that there was insufficient data for statistical analysis on NAG excretion values. Therefore the description of the data for NAG

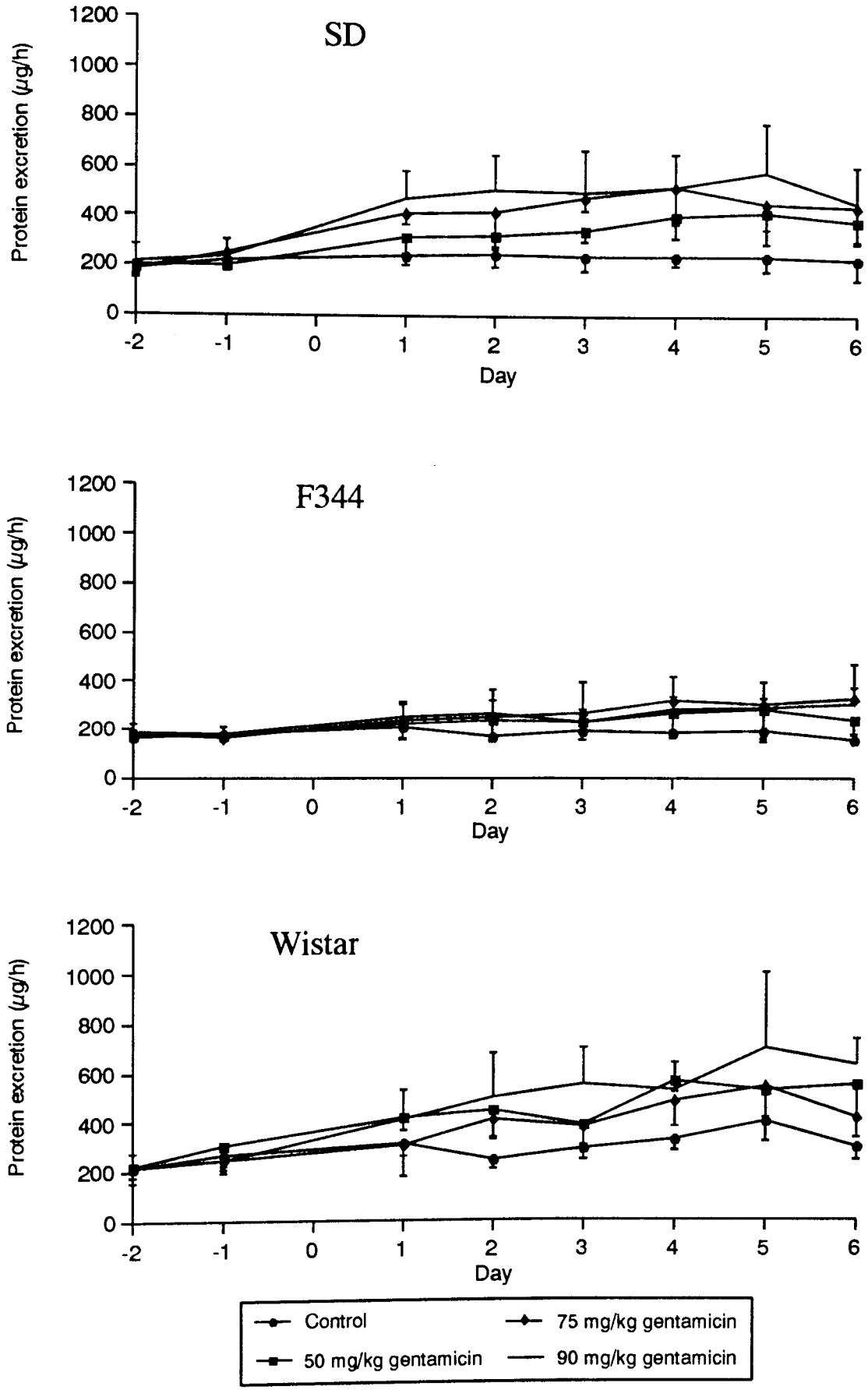


Figure 17. Effect of a range of doses of gentamicin on protein excretion in F344, SD and Wistar rats. Data are shown as mean ± SEM. n = 6.

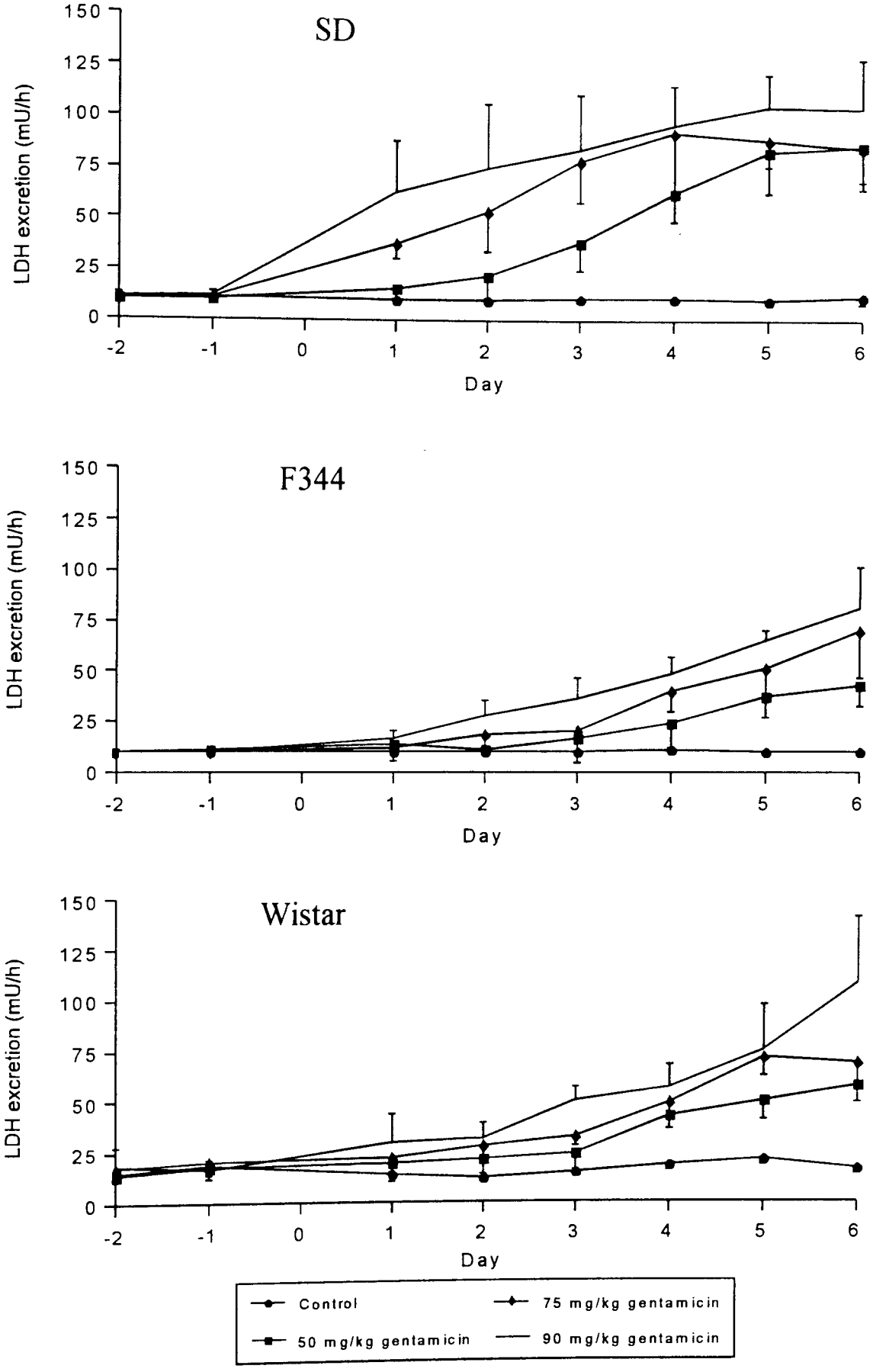


Figure 18. Effect of a range of doses of gentamicin on LDH excretion in F344, SD and Wistar rats. n = 6. Data points are mean ± SEM.

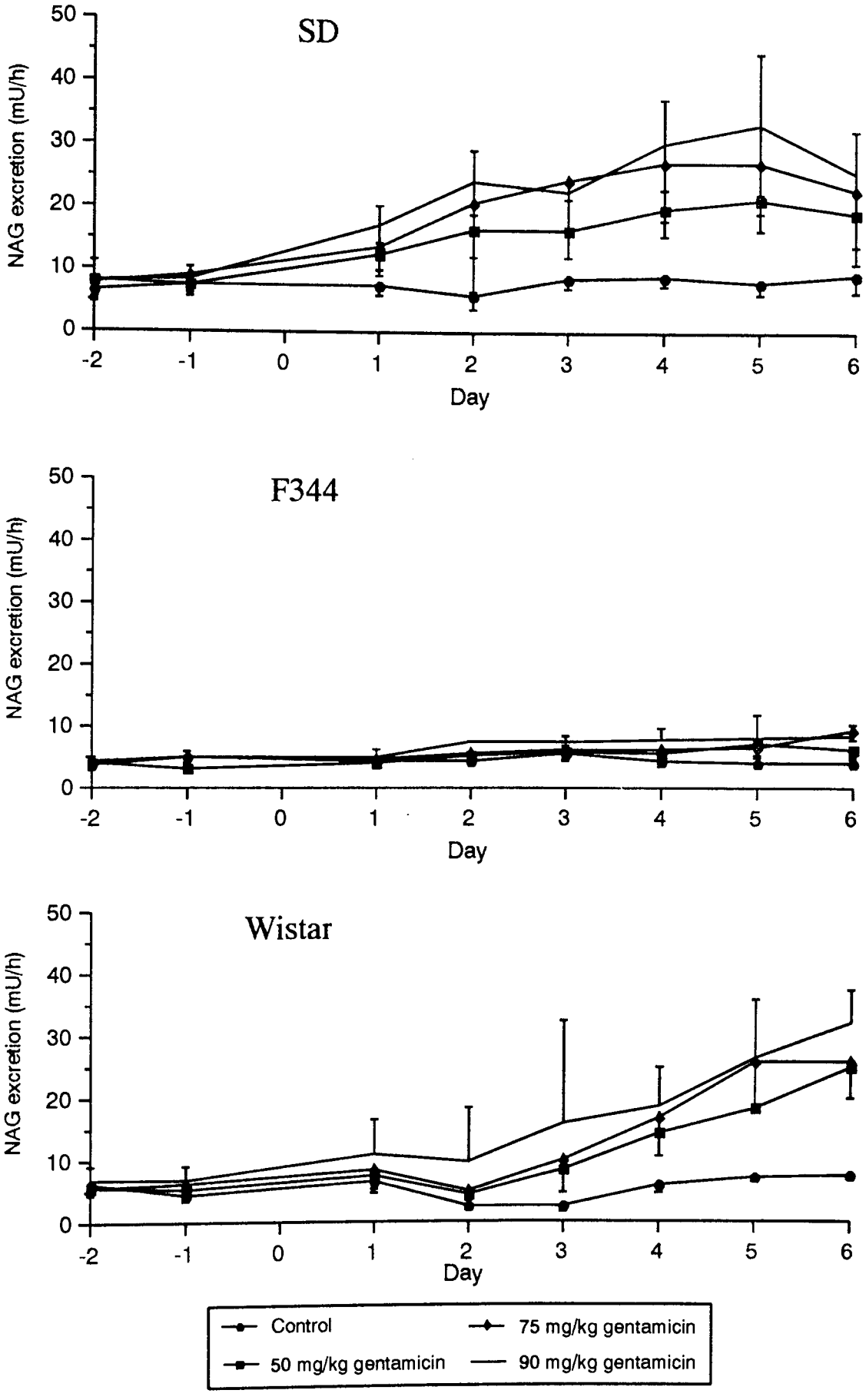


Figure 19. Effect of a range of doses of gentamicin on NAG excretion in F344, SD and Wistar rats. n = 6. Data points are mean ± SEM.

excretion is based on interpretation of plots of available data (Figure 19). SD rats showed an immediate increase in NAG excretion in all dose groups after one days administration of gentamicin. Whilst the increase in NAG excretion appeared to progress over time i.e. with repeated doses, the magnitude of the response was similar in the 75 and 90 mg/kg dose groups. Wistar rats also showed clear dose- and time-related increases in NAG excretion which were first visible in highest dose group 24h after the first dose of gentamicin. Effects of gentamicin administration on NAG excretion were also evident in F344 rats in the highest dose group after 2 days dosing, and by day 3 in the lower dose groups. Despite F344 rats showing a basal excretion of NAG excretion (0.96±0.14 mU/h) which was approximately half that of the other 2 strains (Wistar: 2.63±0.20 mU/h, SD: 2.75±0.41 mU/h), administration of gentamicin generated a dose-related increase in NAG excretion. The relative increase in NAG excretion was however less in the F344 strain than in the SD and Wistar strains. Maximum increase in NAG excretion compared to controls was 2-fold in the F344 strain, 4-fold in the SD strain, and 6-fold in the Wistar strain of rat.

Increased ALP excretion occurred in both SD and Wistar rat strains after gentamicin dosing (increases were statistically significant in SD rats by day 1 but not until day 5 in Wistars). In contrast, there was no effect of gentamicin dosing on ALP excretion in F344 rats (Figure 20). Basal excretion of ALP could be listed in the rank order Wistar (20.7±1.97 mU/h) > SD (14.98±2.23 mU/h) > F344 (8.52±0.79 mU/h) with ALP excretion in the F344 strain approximately one-third of that of the Wistar strain and half that of the SD strain. Similarly, GGT excretion was also approximately one-third to one-quarter that of the other strains in the F344 rats (F344: 55.0±7.3 mU/h, SD: 143.9±20.9 mU/h, Wistar: 219.3±11.0 mU/h). In contrast to the marked effects of gentamicin administration on the urinary excretion of NAG, LDH and ALP, there were no consistent treatment-related effects on GGT excretion in any strain.

Measurements of control urinary calcium excretion provided clear evidence of differences in baseline excretion rates between strains. While Wistar and F344 rats

excreted similar amounts of calcium in their urine under control conditions (Wistar: 1.17±0.15 μmol/h, F344: 1.70±0.50 μmol/h) SD rats excreted approximately half that of the other two strains (SD: 0.68±0.07 μmol/h). However, the amount of calcium excreted did not appear to modify the effects of gentamicin on cation excretion since progressive, significant increases in calcium excretion were apparent from day 1 after dosing in the Wistar strain ($P < 0.001$), day 5 in the SD rats ($p < 0.001$) but not at all in the F344 rats (Figure 21). There were no consistent treatment-related effects on magnesium excretion in any strain (Figure 22). Baseline magnesium excretion could be ranked in the order F344 (6.7±1.49 μmol/h) < SD (7.2±1.62 μmol/h) and Wistar (7.6±0.59 μmol/h), showing small but significant differences between the F344 and the other rat strains ($P < 0.01$).

Creatinine excretion was consistently lower in the F344 strain (0.76±0.17 μmol/h) than in the other 2 strains (SD: 1.40±0.24 μmol/h, Wistar: 1.66±0.11 μmol/h), but there was no effect of gentamicin administration on any group. There were no effects of gentamicin on urine volume or osmolality in any group. F344 rats produced lower 16h urine outputs (F344: 0.19±0.04 ml/h) ($P < 0.01$ comparing F344 to SD) than the other strains (SD: 0.29±0.06 ml/h, Wistar: 0.35±0.03 ml/h) ($P < 0.05$ comparing SD to Wistar) which may be due to the lower body weights of these inbred animals compared to their outbred, age-matched counterparts in this study.

There were no treatment-related effects on plasma calcium, magnesium, or creatinine concentrations in any strain. Plasma magnesium concentrations were found to be higher in F344 rats than both Wistar and SD rats. In addition, F344 rats showed higher plasma calcium concentrations than those of the SD strain.

The three strains of rat showed significant differences in control kidney weights, and in their response to treatment. After correction for terminal body weight, SD animals had the heaviest kidneys ($p < 0.05$); there was also an increase in kidney weight in response to drug-treatment ($P < 0.001$). Gentamicin-treated Wistar rats also showed large

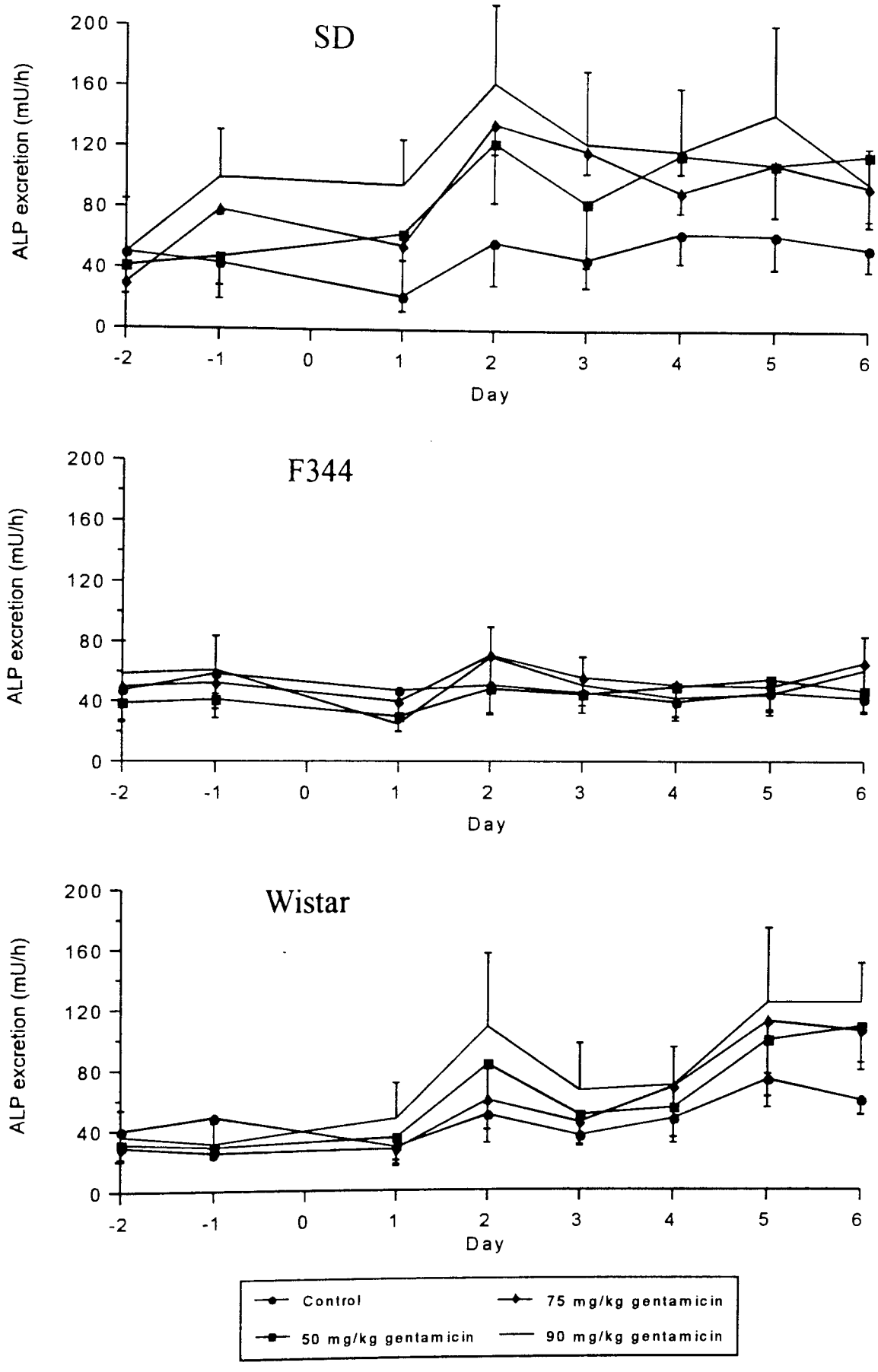


Figure 20. Effect of a range of doses of gentamicin on ALP excretion in F344, SD and Wistar rats. n = 6. Data points are mean ± SEM.

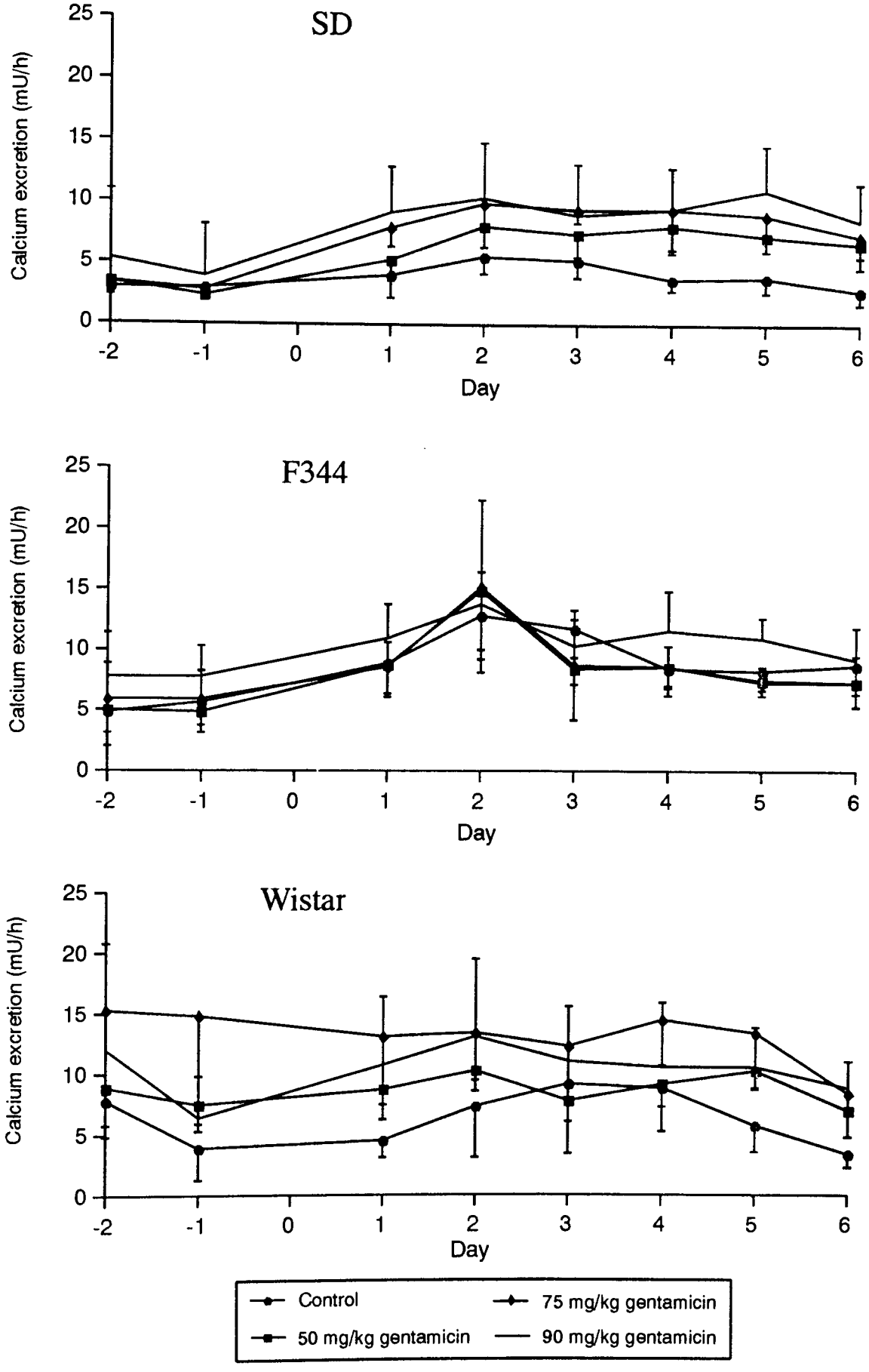


Figure 21. Effect of a range of doses of gentamicin on calcium excretion in F344, SD and Wistar rats. n = 6. Data points are mean ± SEM.

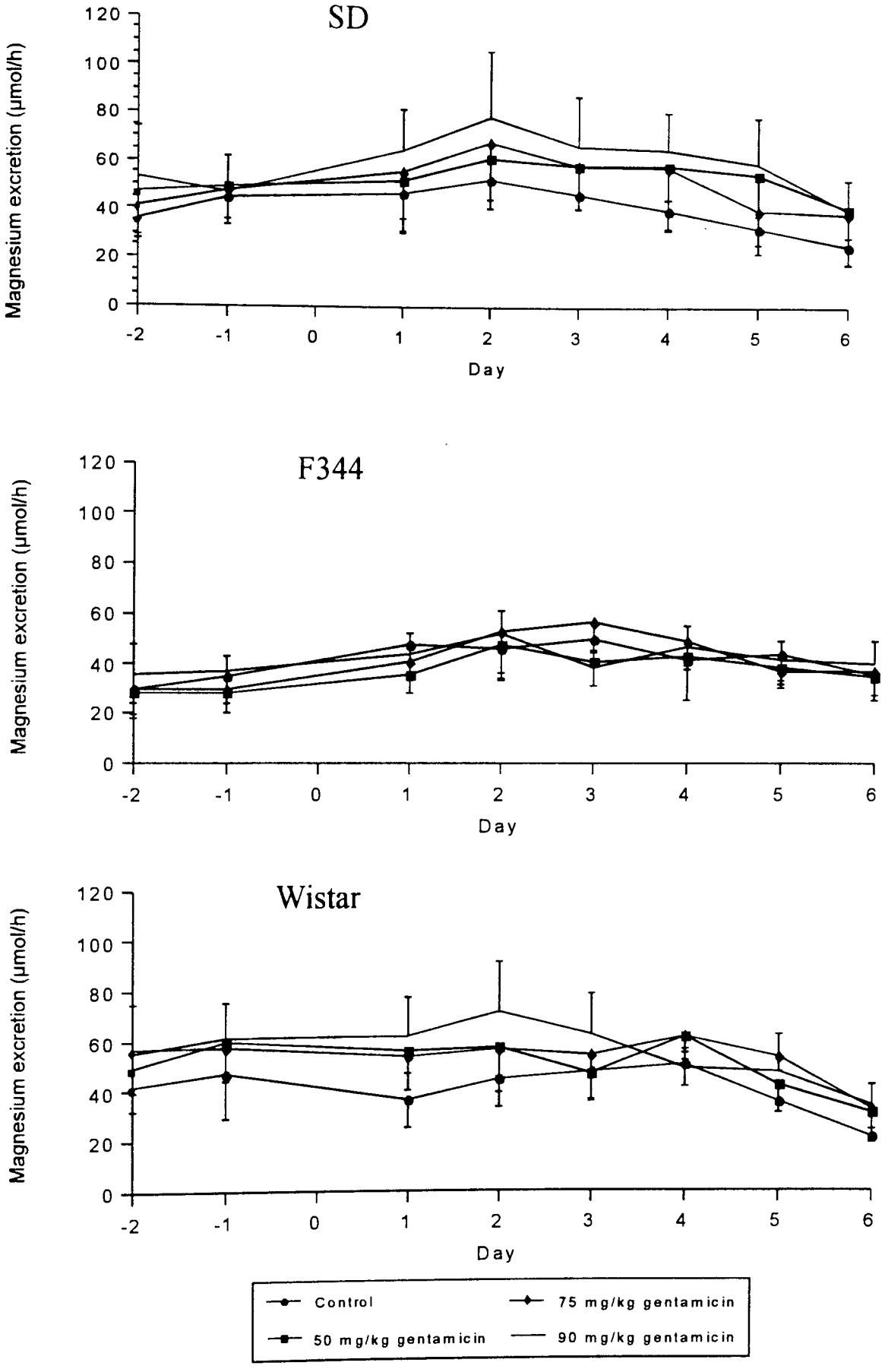


Figure 22. Effect of a range of doses of gentamicin on magnesium excretion in F344, SD and Wistar rats. n = 6 for each data point. Values are mean ± SEM.

increases in kidney weight in response to treatment ($P < 0.001$), whereas there was no effect on the kidney weight of the F344 rats after gentamicin administration. On macroscopic examination there was a clear dose-related incidence of paleness in the kidneys of F344 rats in response to gentamicin administration. In the other 2 strains only one animal from each strain (SD at 90 mg/kg dose and Wistar at 75 mg/kg) demonstrated this macroscopic alteration. In contrast to the striking macroscopic effects of gentamicin on F344 rats, microscopic examination of the tissue revealed no treatment-related lesions in these animals. There were no changes in the tissue to explain the paleness of the tissue seen macroscopically. In SD and Wistar rat strains there were only minimal signs of renal injury caused by gentamicin. In only one Wistar animal dosed with 90 mg/kg gentamicin was acute tubular necrosis visible. In other subjects in the 90 mg/kg dose group, for both Wistar and SD strains, there was evidence of single cell necrosis of the proximal tubule of a greater incidence than seen in control animals. The incidence of single cell necrosis in the 90 mg/kg dose group was 4/6 animals in the SD strain, and 2/6 animals in the Wistar strain. In the 75 mg/kg SD dose group 3/6 animals also showed the same minor alterations in response to gentamicin dosing. The lesion was of the most minor type comprising a few pyknotic nuclei in the cells of the proximal tubule. Sections from Wistar and SD rats were indistinguishable from each other on the basis of their histopathological injury.

Table 5. Data from top dose (90 mg/kg/day gentamicin) and control groups for each strain of rat in the strain difference dose response study to show lack of effect of gentamicin administration on these parameters. Drug-treated and control groups of the same strain are not significantly different.

Rat Strain	GGT Excretion (mU/h)	Creatinine Excretion (μmol/h)	Urine Volume (ml/h)	Urine Osmolality (mOsm/kg H_2O)
Sprague-Dawley (Control)	143.9 ± 20.9	1.40 ± 0.24	0.29 ± 0.06	965 ± 100
Sprague-Dawley (90mg/kg/day gentamicin)	198.8 ± 28.4	1.68 ± 0.21	0.32 ± 0.07	935 ± 107
Fischer 344 (Control)	55.0 ± 7.3	0.76 ± 0.17	0.18 ± 0.04	799 ± 56
Fischer 344 (90mg/kg/day gentamicin)	78.7 ± 11.3	1.00 ± 0.10	0.24 ± 0.05	920 ± 97
Wistar (Control)	219.3 ± 11.0	1.66 ± 0.11	0.35 ± 0.03	950 ± 31
Wistar (90mg/kg/day gentamicin)	228.5 ± 20.2	1.82 ± 0.10	0.37 ± 0.04	894 ± 58

Table 6. Data from top dose (90 mg/kg/day gentamicin) and control groups for each strain of rat in the strain difference dose response study to show lack of effect of gentamicin administration on these plasma measurements. Drug-treated and control groups of the same strain are not significantly different.

Rat Strain	P_{Ca} (mmol/l)	P_{Mg} (mmol/l)	P_{Cr} (μmol/l)
Sprague-Dawley (Control)	2.49 ± 0.02	0.84 ± 0.01	49.3 ± 2.03
Sprague-Dawley (90mg/kg/day gentamicin)	2.53 ± 0.05	0.76 ± 0.02	57.8 ± 5.25
Fischer 344 (Control)	2.50 ± 0.01	0.83 ± 0.01	42.0 ± 0.58
Fischer 344 (90mg/kg/day gentamicin)	2.52 ± 0.03	0.81 ± 0.02	48.7 ± 1.48
Wistar (Control)	2.37 ± 0.03	0.82 ± 0.04	50.0 ± 5.35
Wistar (90mg/kg/day gentamicin)	2.59 ± 0.04	0.86 ± 0.03	51.0 ± 0.86

3.4 Discussion

The initial dosing pattern of a single dose per day was selected for use in the pilot study on the basis of the growing trend for this method of administration in the clinic due to its apparently greater efficacy for bacterial killing with concomitantly reduced nephrotoxicity (Labovitz et al, 1974, Wallen et al, 1983). However, after the pilot study using a single dose of 40 mg/kg gentamicin per day yielded results which suggested that the toxicity induced at this dose was less substantial than the literature had indicated might be expected, attention turned to ways of accentuating the toxicity without altering the total dose administered. The importance of the dosing regimen and its effects on the toxicity produced after gentamicin dosing were considered with respect to route, frequency, and timing of the doses of aminoglycoside (Bennett et al, 1979, Pariat et al, 1988, Dorian et al, 1988, Cal et al, 1989). Since the administration of a divided dose appeared to increase the toxicity compared to a single dose of the same amount it was decided that the subsequent study would be conducted using divided doses of gentamicin (Bennett et al, 1979), although, in addition, the lowest dose used would be increased slightly from that used in the pilot study.

In addition to optimising the dosing interval to increase the nephrotoxic effects of the drug the timing of the dose administration was also selected to increase the potential toxicity of the compound. Several reports describe alterations in the extent of toxicity induced by aminoglycosides dependent on the time of administration after both acute and sub-chronic dosing (Pariat et al, 1986, Pariat et al, 1988). As measured by urinary excretion of GGT, NAG and ALP, maximum toxicity was apparent when doses were given at 14.00 h compared to the same dose given at 08.00, 20.00, or 02.00 h. Minimum toxicity was seen at 20.00, at the beginning of the animals activity period. Whilst there is no information on the rate at which the changeover from high to low toxicity occurs after 14.00 h, it seems reasonable to assume that since measurements taken later in the activity period of the animals (02.00 h) also indicate less toxicity than that shown after 14.00 h dosing that the sensitivity is related to the light/dark, inactivity/activity cycle and the

sensitivity to toxicity therefore changes gradually over these periods rather than abruptly. Therefore the dosing times were selected as 08.00 and 16.00h so as to maximise the toxicity of the compound, and also to aid the experimental procedure in that the animals could be placed directly into the metabolism cages for urine collections, after the 16.00 h dose was administered.

Both the 40 mg/kg pilot study and the dose ranging study produced less nephrotoxicity than reports in the literature suggested might be expected. Whilst there are no immediately apparent reasons for the differences between the studies presented here and similar experimental reports in the literature, several possibilities which cannot be readily checked are plausible. Gentamicin sulphate is produced as a mixture of isomers which have substantial differences in their nephrotoxic potential (Bennett et al, 1986). The proportions of each isomer present will therefore alter the toxicity of the mixture overall. However, details of the amounts of the various isomers within the mixture are not usually supplied with the drug and such information may also not be available from the manufacturers. Only rarely is such detail included in published reports. Since there will inevitably be some batch to batch variation, both within and between manufacturers, in the composition of the drug mixture this may account for some of the differences observed in the toxicity of the same dose of gentamicin observed by different workers.

In addition to the effects of different gentamicin isomers on nephrotoxicity modification of gentamicin-induced nephrotoxicity by dietary supplementation with calcium salts has also been reported (Bennett et al, 1982a, Quarum et al, 1984). In the pilot study presented here the animals were fed ad lib with a breeding diet containing 1.7% calcium and 0.24 % magnesium. The calcium content of this diet fed to those animals was therefore substantially higher than that found in a 'standard' rat diet and may have exerted a protective effect on the kidney against the action of gentamicin. The calcium content of the diets in which increased dietary calcium concentrations conclusively ameliorated gentamicin-induced nephrotoxicity was 4.0%, as compared with a more normal calcium concentration of approximately 0.5%. While no data is available for the range over which

increasing calcium concentration is effective in reducing gentamicin toxicity it may be that the amount of calcium present in this diet was sufficient to attenuate the nephrotoxic effects of gentamicin. In contrast, the diet supplied in the dose response study contained a more usual 0.75 % calcium but the effects of gentamicin were once again less severe than might have been predicted from the literature. Except for studies which specifically explore the effect on toxicity of altering dietary cation composition, the cation content of animal foodstuffs is not given in published reports. Hence, the influence of dietary cation content on the toxicity produced cannot be established in the majority of studies reported in the literature.

In the dose response study presented here the animals were restricted to eating only during the period 08.00 - 16.00 h while they were in home cages. Normally, rats consume the largest fraction of their daily food intake during their active period i.e. during the hours of darkness. However, when rats are only allowed access to food for limited periods they quickly adapt to these conditions and consume as much food as they require during that period, even if it is contrary to normal behaviour (personal observations). While no measurements of food intake were made in this study it is likely that this was the case. However, the effects of acute, intermittent food deprivation on gentamicin-induced nephrotoxicity have not been studied and may affect the severity of the injury produced.

At the end of the pilot study presented here in which 40 mg/kg gentamicin was administered for 5 days, there was no evidence of any histological damage caused by gentamicin in any of the three strains of rat. Additionally, in the second part of the study, where a range of doses of gentamicin was used, only minimal histopathological damage was apparent in the Wistar and SD strains, with no visible microscopic damage at all in the F344 strain even at the highest dose of 90 mg/kg. In contrast to the lack of microscopic findings, the F344 rats showed a clear dose-related paleness visible in the kidneys on macroscopic examination for which there was no microscopic correlate. Paleness of the kidney associated with gentamicin therapy has been mentioned in one

other report of gentamicin toxicity. Flandre and Damon (1967) reported paleness of the kidneys localised to the cortical region in Wistar rats which had received 200 mg/kg for 8 weeks. On microscopic examination the cortical regions of the kidney were shown to be oedematous and it was to this that the paleness visible macroscopically was attributed. In the absence of any microscopic changes in the kidneys of F344 rats, no explanation can be offered for the macroscopically observed paleness.

There are a large number of studies reporting both the histopathological effects and the alterations in functional parameters by aminoglycosides on the kidney. Aminoglycoside antibiotics consistently produce acute tubular necrosis, of varying severity, in the proximal convoluted tubule with associated renal dysfuction. Reports which describe the nephrotoxic effects of gentamicin on the F344 rat tend to indicate that low doses (40 mg/kg or less) for approximately 10 days produce substantial renal necrosis, supporting the hypothesis that the F344 rat is a sensitive model for the study of aminoglycoside induced renal injury (Clayton-Elliot et al, 1982, Kosek et al, 1974, Bennett et al, 1982a). However, for the F344 strain, as for Wistar and SD strains, other reports indicate less severe toxicity at similar doses; some by the same group of workers (Bennett et al, 1982b). There is therefore conflicting evidence for the dose required and duration of administration to produce a particular amount of renal injury, even within a single strain of rat. If the picture is widened to incorporate the influence of the strain of rat used on the ensuing nephrotoxicity the scene becomes less clear still. Damon and Flandre (1967), dosed young adult Wistar rats with gentamicin ranging from 10-200 mg/kg for 60 days with no indication of substantial renal damage at less than 100mg/kg, with only small changes in renal function, and no mortality at a dose of 200 mg/kg over the 60 days. Again, in contrast, other studies report changes in renal function of the Wistar rat produced at much lower doses (40 mg/kg) and shorter dosing periods, suggesting a similar sensitivity to the F344 rat (Chahwala and Harpur, 1983). There exists therefore a problem of direct comparability on the effects of aminoglycosides between separate studies using different strains.

In available studies where direct inter-species comparisons of the renal sensitivity to aminoglycoside nephrotoxicity between F344 and SD rats have been made, the F344 strain appeared to be more sensitive than the SD strain (Reinhard et al, 1991, Sullivan et al, 1987) or no difference between strains was observed (Cuppage et al, 1977). In these reports, nephrotoxicity was assessed by histopathologic alterations and by measurements of parameters which indicate alterations in gross kidney function, such as BUN and serum creatinine values rather than the measurements of more subtle renal injury used in the studies presented here. Sullivan et al (1987), also reported significant increases in kidney weight/body weight ratios in the F344 rats after 14 days administration of 100mg/kg gentamicin which were not seen in SD rats. These findings are in direct contrast to the findings in the study presented here where SD rats showed treatment-related increases, whereas there was no effect on F344 rats even at 90 mg/kg/day. At a similar dose to that used in the study presented here (60 mg/kg/day) Reinhard and co-workers (1991), showed that measurements of gross nephrotoxicity (BUN and S_{Cr}) were not significantly different from each other, but marked histopathological damage caused by tobramycin administration was greater in F344 rats than in SD rats. At higher doses (120 and 240 mg/kg/day) both functional and morphological parameters indicated a greater response to treatment in the F344 rat compared to the SD strain. In contrast, Cuppage et al (1977), noted only focal lesions in the proximal tubule of both SD and F344 rats after dosing with gentamicin for 28 days (dose range 3-40mg/kg). While the extent of tubular disruption was dose-dependant there were no differences in nephrotoxic injury between the two strains. Whilst data from all these studies are obtained from animals dosed for longer periods than was used in the studies presented here, the apparent reversal of sensitivities between the strains reported in two of the studies (Reinhard et al, 1991, Sullivan et al, 1987) is difficult to explain.

In addition to differences between strains in response to aminoglycoside dosing, other studies have reported differences in the extent of nephrotoxic injury arising from administration of other compounds, in different rat strains. Tarloff et al (1989), reported

differences in sensitivity between SD and F344 rats to the nephrotoxic actions of acetaminophen (APAP) and its metabolite, p-aminophenol, which were highly age-dependent. Whilst at two months of age F344 rats were susceptible to renal injury caused by APAP but SD rats were not, by three months old the two strains showed only minimal differences in sensitivity; at nine months susceptibility to renal damage from this compound was comparable between strains. Similar sensitivity between SD and F344 strains to the nephrotoxicity of $HgCl_2$ was also reported by Kroll et al (1988), who also noted a greater induction of metallothionein and greater increases in the metabolising enzyme epoxide hydrolase, in the SD strain compared to the F344. The extent of renal damage was similar in both strains. Reports which support the assertion that F344 rats are more susceptible to xenobiotic-induced renal injury than SD or Wistar strains have been produced by workers studying the effects of methoxyflurane (Mazze et al, 1973), and also the non-steroidal anti-inflammatory drugs, aspirin and phenylbutazone (Owen and Heywood, 1986). These studies showed disparate compounds produced more renal injury in F344 rats than in the other two strains.

Widely distributed out-bred stocks, such as SD or Wistar rats, may well, however, have large differences in characteristics between colonies with the same name. Genetic differences between such colonies will arise from inbreeding, stock selection, sampling errors and genetic drift, therefore stocks with the same nominal title may well be quite different when obtained from different suppliers (Festing, 1979). Inbred animals, such as the F344 strain, which are genetically uniform, should however suffer less from these problems than the outbred strains, since the original genetic pool is homogeneous. With regard to the selection of an appropriate model for toxicity studies however the outbred strains may well initially appear to be a more attractive prospect since a particular outbred colony will represent a collection of genotypes which parallels the human situation. If a sufficiently large sample is used from a particular colony then, because of the averaging effect across the different phenotypes, the mean for any particular

characteristic will tend to be nearer the mean for all animals of that species than would be the case for an inbred strain.

Whilst the data from both studies presented here lies at variance to the accepted belief in the literature that the F344 rat is more sensitive to the nephrotoxic effects of aminoglycosides than other strains of rat, the fact remains that two studies, conducted independently, produced clear and consistent evidence that the F344 rat is no more sensitive than the Wistar and SD strains, and in these studies showed substantially less renal injury than the other strains. The differences in baseline excretion values of NAG, calcium, and magnesium, were consistently lowest in the F344 rat in both studies. The significance of the effects of lower baseline excretion rates of these cations is unclear, although less aminoglycoside-induced renal injury was produced in the strain with the lowest calcium and magnesium excretion rate (F344). Neither SD or Wistar rats showed consistency in ranking between themselves for baseline excretion values of these parameters.

The agreement between the two studies presented here with respect to differences in baseline values for the parameters described above also extends to the magnitude of the renal effects induced by gentamicin dosing in the different strains. For the parameters which altered after gentamicin administration (ALP, LDH, NAG, protein excretion, calcium excretion, magnesium excretion, histopathology) effects on the F344 rats was either less severe than in the other 2 strains or not apparent at all. As with the baseline differences between strains there was no distinct ranking of sensitivity between the other strains, the magnitude and sensitivity of the response appeared to vary between the SD and Wistar rats depending on the parameter under study.

Strain-related differences alterations in gentamicin-induced changes in renal cation handling were of particular interest in these studies. Clear strain-related differences were apparent for both calcium and magnesium excretion. In the pilot study after 40mg/kg gentamicin for 5 days, only SD rats showed significant increases in magnesium excretion;

however, none of the rat strains showed significant dose-related increases in magnesium excretion at doses of up to 90 mg/kg in the second part of the study. The comparative insensitivity of renal handling of magnesium in the rat to alteration by administration of gentamicin has also been reported elsewhere (Foster et al, 1992). In comparison, marked increases in urinary excretion of calcium due to aminoglycoside dosing have been repeatedly demonstrated (Chahwala and Harpur, 1983, Harpur et al, 1985, Foster et al, 1992). In studies presented here elevated calcium excretion was clearly seen after 3 days dosing with 40 mg/kg gentamicin in the SD rat, after 5 days in the Wistar, but not at all in the F344 rat. Both Wistar and SD animals also showed treatment-related increases in calcium excretion in the dose response study, but F344 animals again showed no treatment-related response to gentamicin administration.

In conclusion, the data presented here suggests that the SD strain of rat is sensitive to the disruptions in renal cation handling which may be induced by aminoglycoside administration. Therefore, this strain may represent a more suitable model for the study of these phenomena, since it may be more sensitive to the renal wasting effects of these drugs than the other two commonly used strains, the F344, and Wistar. The SD strain was therefore chosen to study the acute effects of gentamicin dosing on renal electrolyte handling presented in the following chapters.

Chapter 4. Using renal clearance measurements to study the acute effects of gentamicin on the renal handling of electrolytes in the anaesthetised Sprague-Dawley rat.

4.1 Introduction

In previous work from this laboratory it was shown that acute infusions of aminoglycosides to F344 rats produced a rapid increase in the excretion of both calcium and magnesium without effects on GFR (Foster et al, 1992). Since the studies presented in Chapter 3 indicated that the SD rat was probably more sensitive than the F344 rat to the nephrotoxic effects of gentamicin after sub-chronic dosing, the question arose as to whether the SD rat responded in a similarly sensitive manner to acute dosing with gentamicin. Of particular interest was the altered renal handling of electrolytes induced after gentamicin administration. While striking changes in the renal handling of calcium and magnesium have been shown to occur after both acute and sub-chronic dosing with aminoglycosides, the effects on the renal handling of sodium and potassium are ambiguous, and, probably secondary to the primary renal wasting of magnesium and calcium (Foster et al, 1992, Harpur et al, 1985, Pastoriza et al, 1983, Chui et al, 1976). Therefore, studies were undertaken to discover whether SD rat strain responded in a similar manner to the F344 rat with respect to alterations in the renal handling of calcium and magnesium after acute gentamicin administration; also, to confirm that the effects of gentamicin administration were confined to alterations in calcium and magnesium handling and occurred without concurrent effects on the renal handling of sodium and potassium.

4.2 Methods

The acute effects of gentamicin on renal function were studied using renal clearance techniques in an anaesthetised rat model. Male SD rats (body weight range 226-341g) supplied by Charles River Ltd. were used. The animals were allowed free access to a diet containing 1.70% calcium, 0.24% magnesium, 0.23% sodium and 0.92% potassium, and

tap water. Animals were housed at a maximum of 6 per cage, in a room with 12h light/dark cycle (08.00-20.00), at 20-25°C for a minimum one week prior to use in experiments.

4.21 Surgical Techniques

Rats were anaesthetised with an i.p. injection of 120mg/kg body weight Inactin (sodium-5-ethyl-5(1'-methylpropyl)-2-thiobarbiturate) dissolved in 0.9% w/v saline. Animals were then placed on a thermostatically heated operating table to maintain body temperature at 37°C ± 0.5°C. A rectal thermometer was used to check body temperature.

The skin and fur were removed from the neck and the trachea exposed by blunt surgery. A tracheostomy was performed. A tapered length (about 1cm) of polyethylene tubing (P200; all tubing obtained from Portex Ltd, UK.) was tied into an opening cut into the trachea, just above the thyroid glands. The left jugular vein was cannulated using P50 polyethylene tubing connected to a 50 ml plastic syringe containing the saline infusate, via an in-line three way tap. The syringe was mounted on a continuously variable infusion pump (Harvard Apparatus Ltd., UK.) and infusion (200µl/min) was begun as soon as the cannula was securely tied into the vein. The right carotid artery was then cannulated with P50 polyethylene tubing connected to a pressure transducer filled with 0.9% saline containing 10U/ml heparin (sodium salt). The pressure transducer was connected to a previously calibrated chart recorder to measure blood pressure. The arterial cannula was in 2 parts, linked by a stainless steel tube, allowing arterial blood samples to be obtained by disconnecting the 2 sections.

The recorder was calibrated by linking the pressure sensitive unit to a sphygnomanometer and calibrating the pen deflection of the recorder to the pressure measurements on the sphygmomanometer (mm Hg). Rats which had an initial systolic blood pressure of < 100 mm Hg were not considered to be suitable experimental candidates and the experiment was terminated.

An incision was made in the lower abdomen and the abdominal wall and peritoneum pierced by electrocautery. A flared P90 catheter was inserted into a small cauterised aperture in the bladder and securely tied in place. Urine collections were made continuously once the catheter was in place.

Once all cannulations were complete the wounds in the abdomen and at the neck were closed using 6 mm Michele clips.

Saline (0.9% w/v NaCl) was infused according to the protocol set out in Figure 23. Animals were infused at 200 µl/min for 2h as a priming period to initiate the diuresis. After 2h the infusion rate was reduced to 100 µl/min and maintained at this rate for the remaining 5 h of the experiment. From the beginning of the 100µl/min infusion period the infusate also contained 1 µCi/ml ^{3}H-inulin (Amersham International Ltd.). The first 3 h of the experimental protocol (2h at 200 µl/min plus 1h at 100 µl/min infusion) served as an equilibration period so that the diuresis was stabilised at a rate approximately equal to the infusion rate; the following hour acted as a control period. Over the final 3 h of the experiment (the experimental period), the infusate was kept to the saline mixture (for control animals; n=12) or changed to a solution of gentamicin in the saline mixture (drug treated animals; n=7). Gentamicin (as Cidomycin, from Roussell) was administered at an infusion rate of 0.56mg/kg/min (equivalent to 100mg/kg over the 3h experimental period). From the beginning of the control period until the end of the experiment urine was collected into pre-weighed plastic vials which were changed at 30 min intervals. Vials containing urine were re-weighed at the end of each urine collection period and the urine volume produced was estimated by weight.

Small arterial blood samples, (approximately 300 µl in period 1 and 140 µl thereafter) were collected into microhaematocrit tubes (with heparin, sodium salt as anticoagulant) at the midpoint of each urine collection period and a larger blood sample (approx. 4ml)

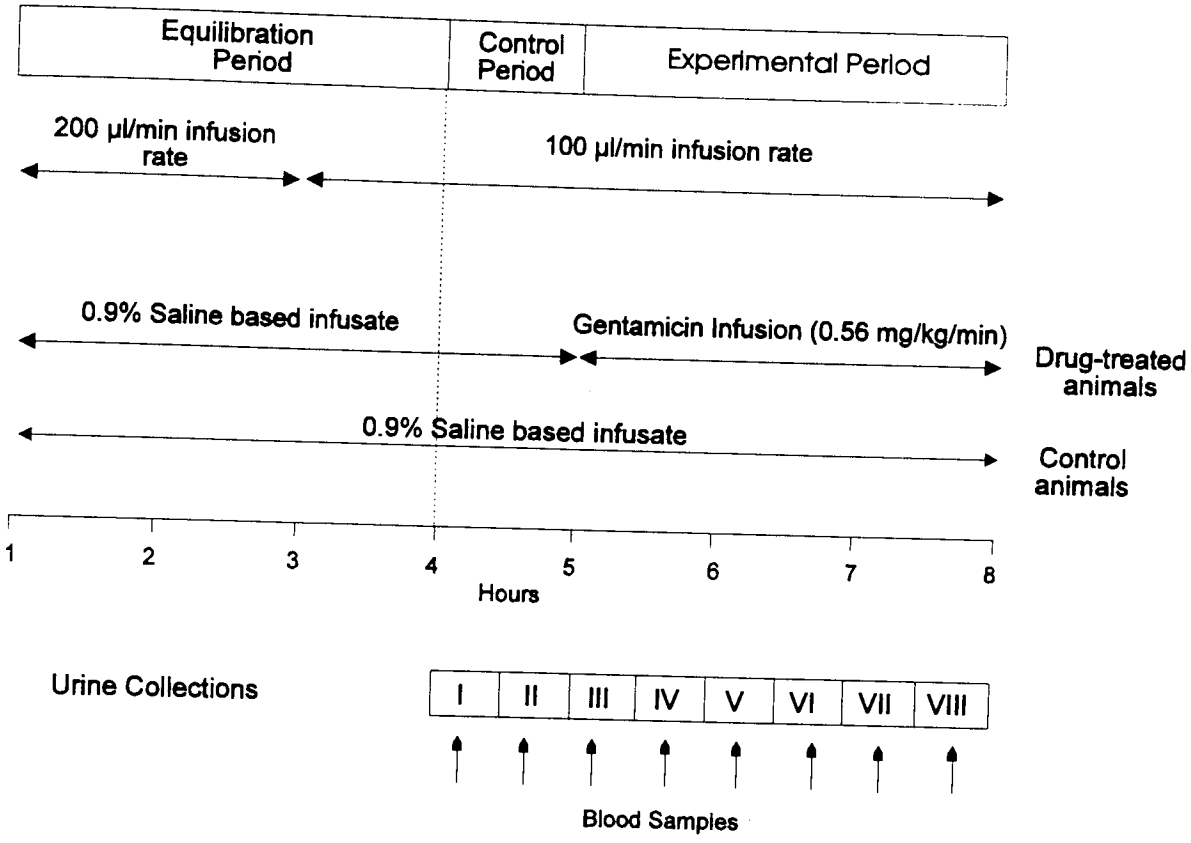


Figure 23. Experimental protocol for clearance studies in Chapter 5. Full details of the experimental procedures are provided in the chapter text.

was collected terminally into tubes containing heparin. The arterial cannula was clamped close to the tube junction and the cannula disconnected. A 1 ml syringe containing heparinised saline was connected to the tube, and the arterial cannula unclamped. The heparinised solution in the arterial cannula was withdrawn into the syringe until the contents of the cannula was simply arterial blood. Blood was then allowed to run into the required number of haematocrit tubes. The cannula was re-connected to the pressure transducer and blood remaining in the cannula was flushed back in to the carotid artery using 0.9% saline containing 10U/ml heparin. Plasma samples were obtained by sealing the haematocrit tubes with sealing compound and spinning in a microhaematocrit centrifuge for 2 min. The tubes were broken above the packed cell component and the plasma removed, in aliquots, using a positive displacement pipette.

^{3}H-inulin counts in urine and plasma samples were assessed in duplicate 10 µl aliquots by liquid scintillation counting using Optiphase MP (FSA, UK) as a scintillant. Urine and plasma samples were analysed for calcium, magnesium, sodium, and potassium by atomic absorption spectrophotometry (AAS) (See appendices 5 and 6 for methodological details). Calcium and magnesium determinations were made on plasma ultrafiltrates obtained using Amicon Centrifree Ultrafiltration cones. To obtain the ultrafiltrate approximately 0.8 ml plasma was pipetted into the ultrafiltration cones and centrifuged at 2,500 rpm at 4°C in a fixed angle rotor. Determinations of whole plasma calcium and magnesium concentrations were also made for comparative purposes. Dilutions of plasma and urine samples were made as follows for AAS measurements:

Plasma:

Ca and Mg: 1 in 101 dilution with acidified $LaCl_3$

K: 1 in 101 in distilled water.

Na: Serial dilution of the K sample 1 in 50 giving a final dilution of 1 in 5050 in distilled water.

Urine:

Ca and Mg: 1 in 101 dilution in acidified $LaCl_3$

K: 1 in 1010 dilution in distilled water.

Na: Serial dilution of the K sample 1 in 5, giving a final dilution of 1 in 5050 in distilled water.

4.3 Calculations and Definitions

I) Excretion rate

The excretion rate of a solute is the quantity of that solute excreted in the urine in unit time.

Quantity excreted = Urine concentration x Urine volume per unit time

per unit time (Q) (U) (V)

For a solute x the following therefore applies:

$$Q_X = U_X \cdot V$$

$Q_X = E_X$, the excretion rate of the solute x.

Usually :

$$E_X \text{ (mg/min)} = U_X \text{ (mg/ml)} \cdot V \text{ (ml/min)}$$

II) Clearance

The clearance of a substance is that volume of plasma completely cleared of the substance in unit time.

In more realistic terms since the quantity of solute excreted in unit time in urine, is equal to the quantity contained in a volume of plasma which equals that solutes clearance, the following equations may be constructed :

Quantity contained in

the volume of plasma = Plasma concentration X Clearance

equal to clearance

For a solute x:

$$Q_X = U_X \cdot V$$

Or :

$$Q_X = P_X \cdot C_X$$

P_X = Plasma concentration of x

C_X = Clearance of x

Hence :

$$U_X \cdot V = P_X \cdot C_X$$

Rearranging the equation therefore gives an expression for clearance :

$$C_X = (U_X \cdot V) \div P_X$$

Usually :

$$C_X \text{ (ml/min)} = [U_X \text{ (mg/ml)} \cdot V \text{ (ml/min)}] \div P_X \text{ (mg/ml)}$$

Clearance measurements of Ca and Mg were calculated using ultrafiltrable plasma values.

III) Glomerular Filtration Rate

Glomerular filtration rate (GFR) is the volume of glomerular filtrate formed per unit time and measures the flow of solvent across the glomerular membrane.

The ideal marker for the measurement of GFR is a solute which passes freely through the glomerular filter from the plasma, but is neither reabsorbed, secreted, metabolised, nor synthesised by the kidney, in addition to being excreted solely in the urine. The polysaccharide, inulin, fulfils these requirements and is therefore commonly used as a marker to measure GFR.

$$\text{Rate of inulin excretion } (U_{in} \cdot V) = \text{Rate of inulin filtration } (P_{in} \cdot GFR)$$

This may therefore be rearranged to give an equation to measure GFR :

$$GFR = (U_{in} \cdot V) \div P_{in}$$

which is the measurement of inulin clearance, therefore;

$$\text{inulin clearance} = GFR.$$

IV) Fractional excretion

The fractional excretion (FE) of a solute represents the rate of excretion of that solute, expressed as a fraction of its filtration rate at the glomerulus. Fractional excretions are generally expressed as a percentage.

The filtration rate of any freely filtered solute can be determined if concurrent measurements of GFR are made:

$$\text{Rate of filtration (FE)} = \text{Plasma concentration } (P_X) \times GFR = \underline{\text{FILTERED LOAD}}$$

As before :

$$\text{The rate of excretion of x } (E_X) = U_X \cdot V$$

Therefore :

$$\text{Fractional excretion of x } (FE_X) = (U_X \cdot V) \div [(P_X) \cdot GFR]$$

Since :

$$(U_X \cdot V) \div P_X = C_X$$

Then :

$$FE_X = C_X \div GFR$$

Thus if a solute undergoes net reabsorption during its passage along the nephron its excretion rate will be less than its rate of filtration and its FE will be less than 1. Conversely, it a solute undergoes net secretion its rate of excretion will exceed its rate of filtration and its FE will be greater than one.

4.4 Statistical Analysis

All statistical analyses were carried out using Statgraphics Version 2.0, (Lotus, UK.). Data were analysed using multivariate analysis of variance for repeated measures. Data from drug-treated and control groups of animals were compared to each other over the first two (control) collection periods, and over the remainder of the experimental period (6 collections). Data were taken to be statistically different at P values of 0.05 or less.

Data are presented graphically as mean ± SEM.

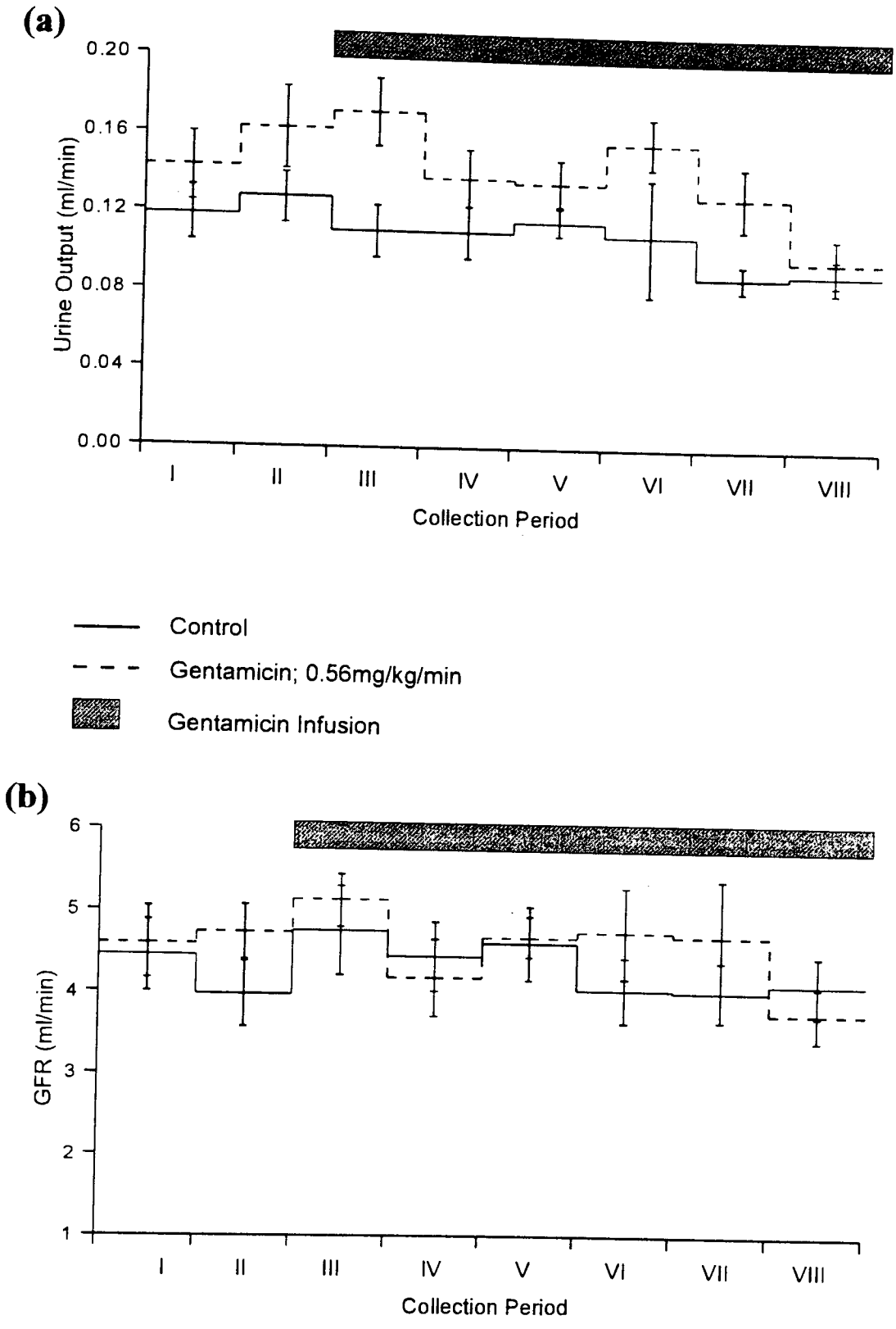


Figure 24. Urine output (a) and (b) GFR in control and gentamicin infused rats. There were no significant differences between gentamicin infused and control animals at any time during the experiment for both parameters (MANOVA).

4.5 Results

There were no differences in GFR between control and drug-treated groups either during the control period or during the period of gentamicin infusion (Figure 24b). The lack of alterations in GFR indicate that the effects which gentamicin exerts on the kidney are at a tubular, rather than at a glomerular level, after acute infusion. The urine flow rate was constant throughout the experiment in both gentamicin-infused and control rats (Figure 24a). There were no significant differences in urine output between the groups, which was similar to the infusion rate of 100 µl/min, and therefore indicates a stable diuresis in the preparations.

There were no significant differences between control and gentamicin-treated groups over the first two collection (control) periods for calcium excretion rate , and FE_{Ca}. In contrast, initiation of the infusion of 0.56 mg/kg/min gentamicin produced an immediate and striking increase (approximately 3-fold compared to control) in these parameters ($p < 0.01$) (Figure 25a, 26a). The alterations in the renal handling of calcium induced by gentamicin were fully developed in the first clearance period after the infusion of gentamicin began, and were sustained for the duration of the experiment.

There were no significant differences in the renal handling of magnesium, as measured by excretion rate or FE_{Mg}, between the gentamicin-treated and control groups, over the first two (control) collection periods. Infusion of gentamicin however produced immediate effects on magnesium handling by the kidney (Figures 25b, 26b). Significant increases (approximately 1.5-fold increase compared to controls) in magnesium excretion rate ($P < 0.01$), and FE_{Mg} ($P < 0.01$) were evident 30 minutes after the start of gentamicin infusion. However, the early increases in magnesium excretion rate produced by infusion of gentamicin appeared to decline toward control values over the experimental period, although magnesium excretion rate did remain elevated above controls for the duration of the experiment. Increases in FE_{Mg} were apparent after only

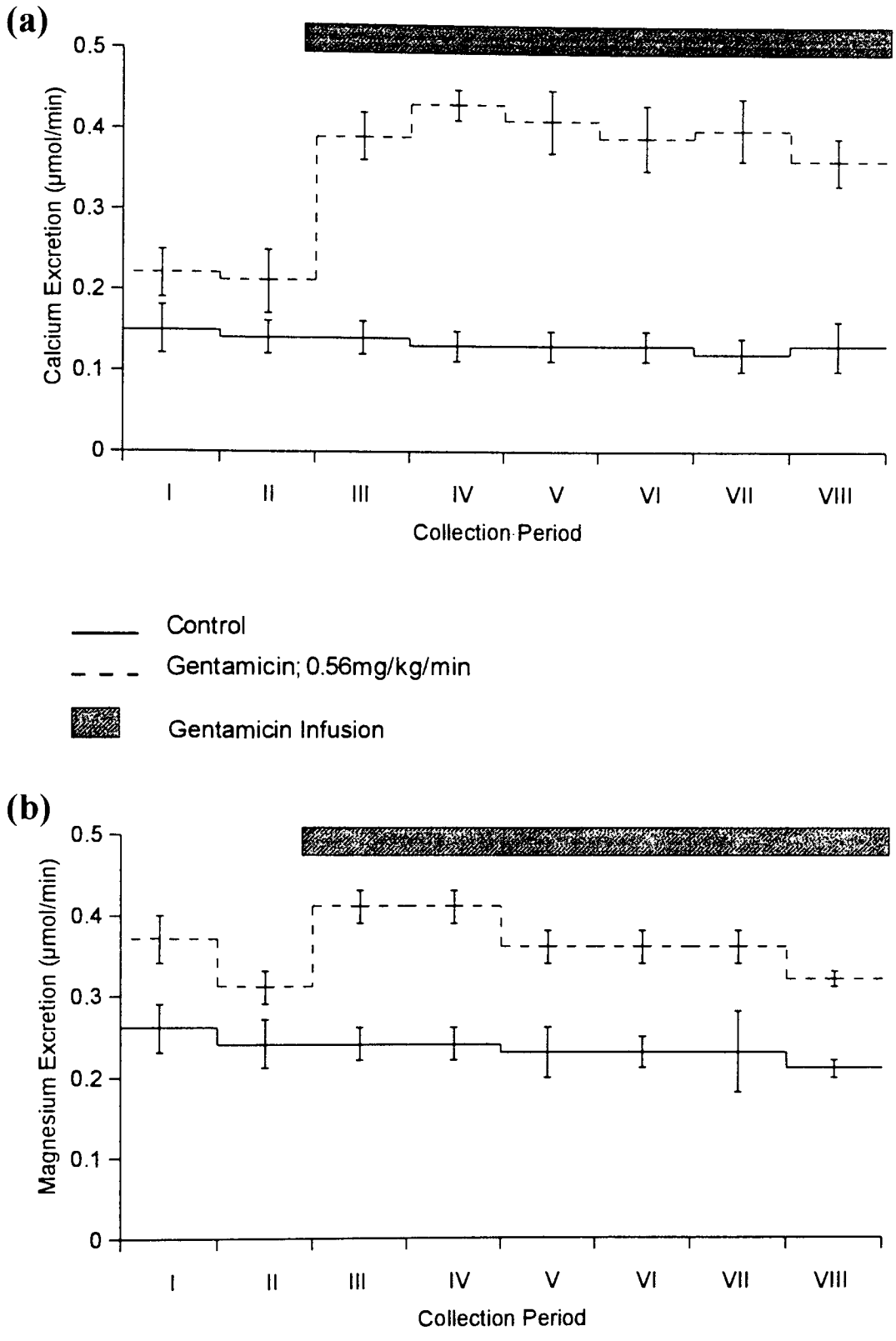


Figure 25. Calcium (a) and magnesium excretion rate (b) in control and gentamicin infused rats. There are no significant differences between groups during the control period for either parameter. Infusion of gentamicin produced significant increases in both calcium and magnesium excretion rates ($p < 0.01$) (MANOVA).

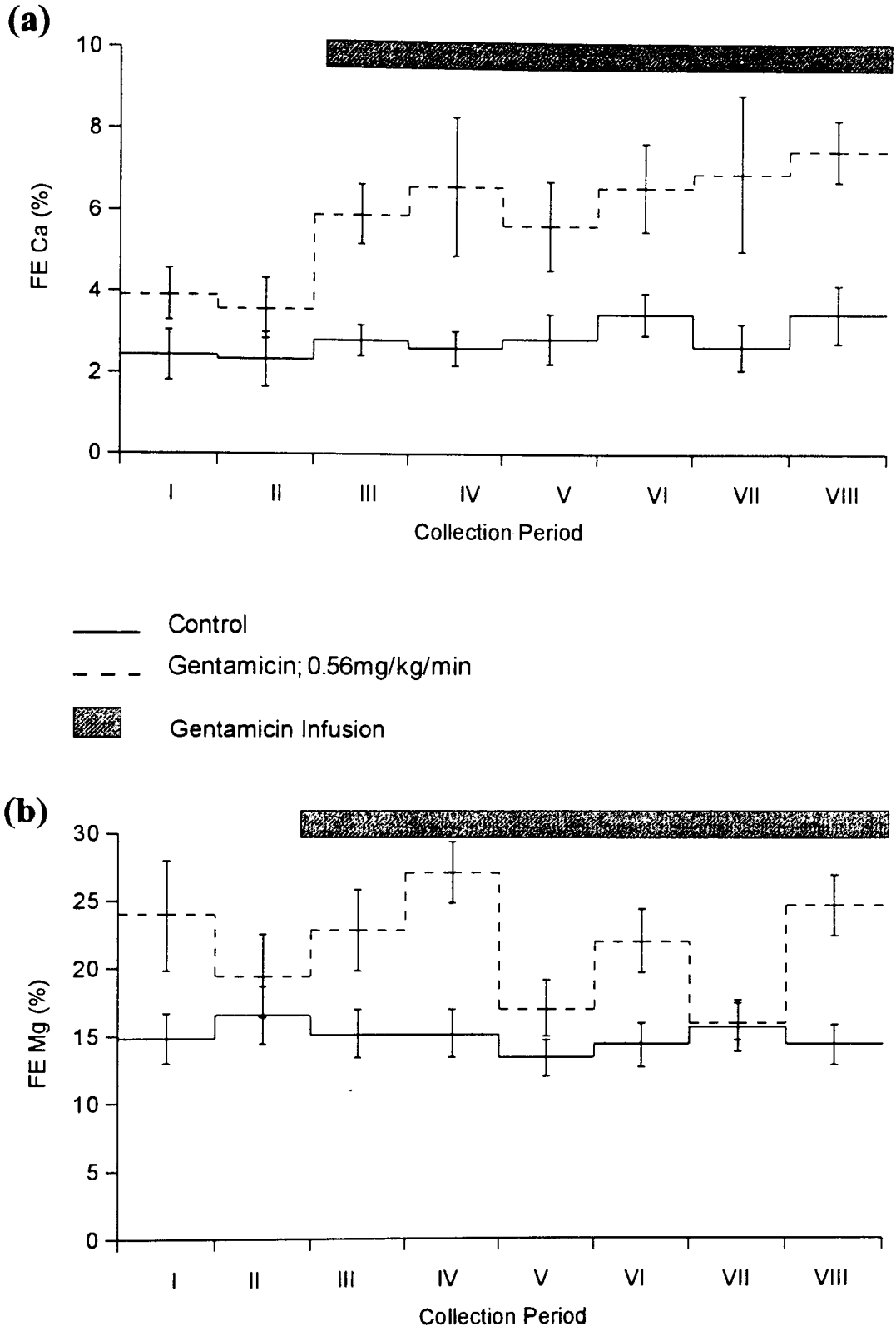


Figure 26. Fractional excretion of calcium (a) and magnesium (b). There are no significant differences between control and gentamicin infused rats over the control periods. Infusion of gentamicin produced significant differences between drug-treated and control animals for both parameters ($p < 0.01$) (MANOVA).

30 min of gentamicin infusion, but were not maximal until 1 hour after the start of infusion. The elevation of FE_{Mg} was less stable than the corresponding alterations in magnesium excretion rate over the experimental period. The magnitude of the effects of gentamicin on magnesium handling were smaller and less stable than those seen on calcium handling but nevertheless marked changes in the renal handling of magnesium certainly occurred. In contrast to the marked increases in calcium and magnesium excretion generated by the acute infusion of gentamicin there were no effects of gentamicin treatment on the renal handling of sodium.

There were no significant differences between gentamicin-treated and control groups, at any time during the control and experimental periods, in sodium excretion rate or FE_{Na}. Sodium excretion rate and FE_{Na} were essentially stable over the entire control and experimental period (Figures 27a, 28a).

There were no significant differences in FE_K or potassium excretion rate between gentamicin-infused and control rats over both the control and experimental periods. Gentamicin-infused and control animals showed similar declines in potassium excretion rate and FE_K over the study period (Figures 27b, 28b).

4.6 Discussion

Considering the response to gentamicin, neither the SD nor F344 rat strain showed alterations in GFR, urine output, or changes in the renal handling of sodium or potassium after gentamicin infusion. Infusion of gentamicin caused a rapid increase in calcium and magnesium output, fractional excretion and clearance in both strains of rat (See Table 7). In the SD and F344 rat strains the renal alterations in calcium handling were more substantial and more stable than the comparable response for magnesium. Quantitatively however, the peak response for all parameters of magnesium handling was greater in the SD rats than in F344 rats. Similar increases in C_{Ca} and E_{Ca} were shown by both rat strains after gentamicin infusion.

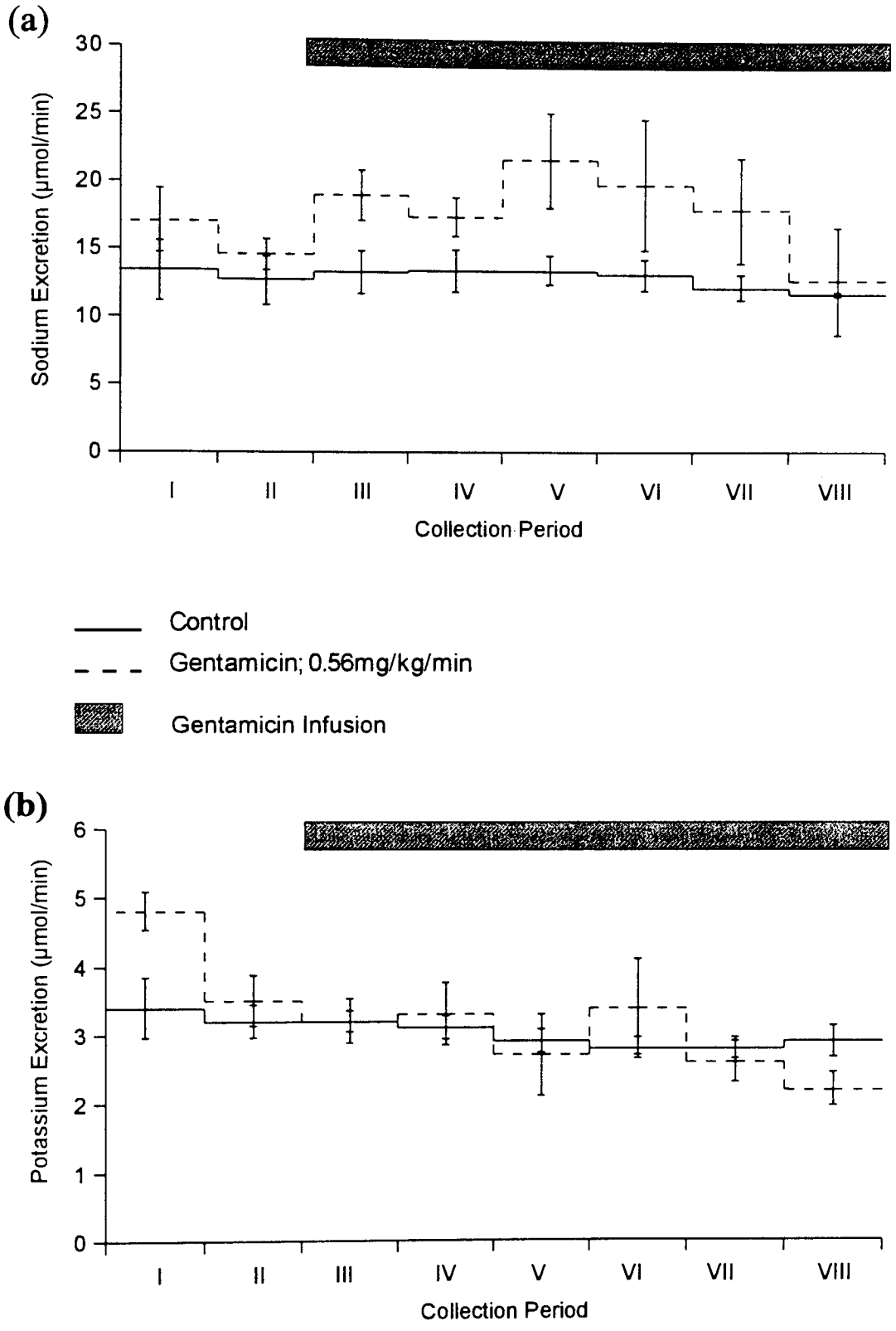


Figure 27. Excretion rate of sodium (a) and potassium (b). There are no significant differences between gentamicin-infused and control animals at any time during the experiment, for both parameters (MANOVA).

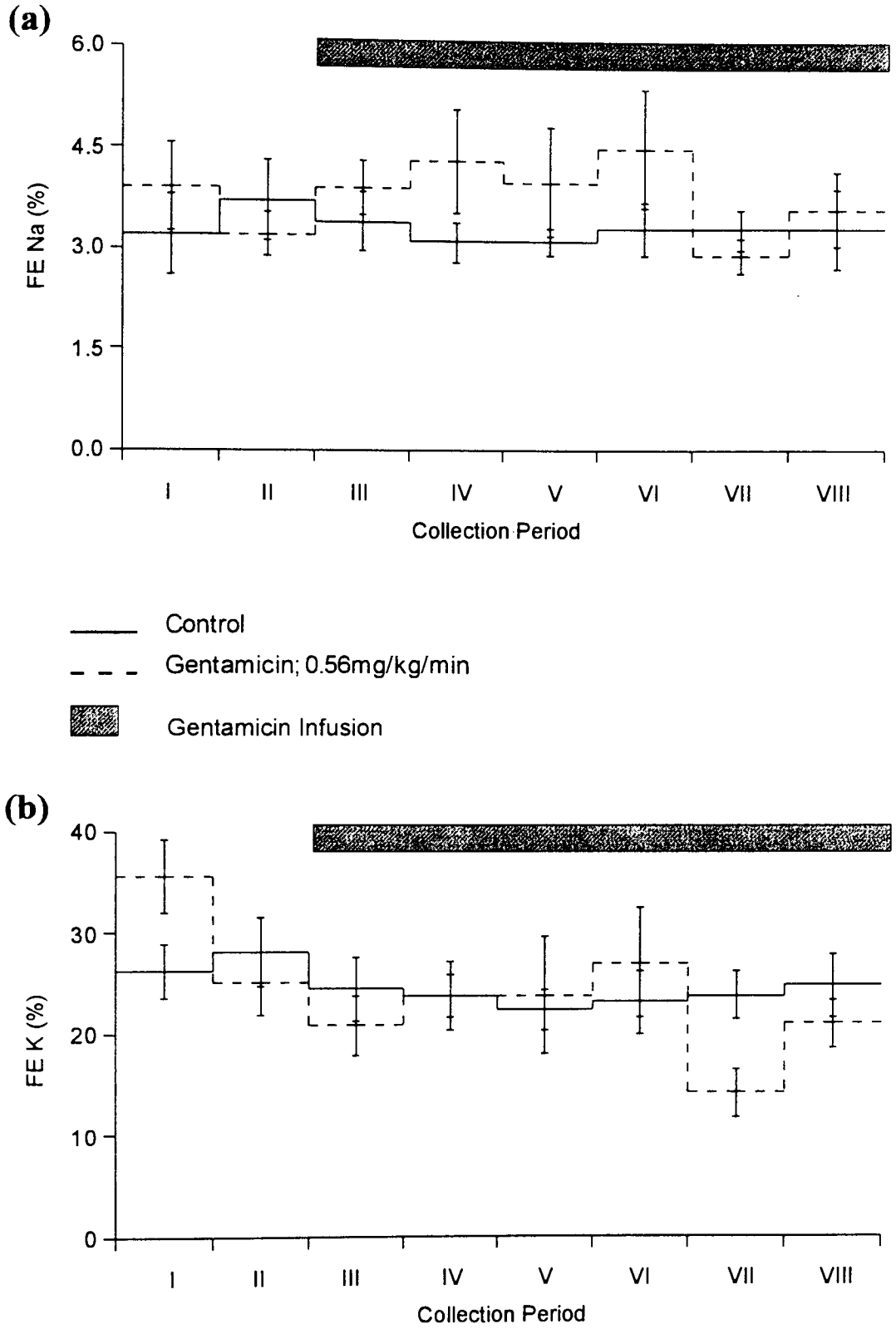


Figure 28. Fractional excretion of sodium (a) and potassium (b). There are no significant differences between gentamicin-infused and control animals at any time during the experiment, for both parameters. (MANOVA)

Table 7. Maximum increases compared to control values after infusion of 0.56 mg/kg/min gentamicin base for SD and F344 strains of rat (Data for F344 rats from Foster et al, 1992, and J.E.Foster's thesis, 1988). Clearance data is used for comparison here since GFR measurements were not made in Foster's studies, therefore measurements of fractional excretion for these ions could not be derived.

Rat Strain	C_{Mg} (% increase)	E_{Mg} (% increase)	C_{Ca} (% increase)	E_{Ca} (% increase)
Sprague-Dawley	160	160	300	300
Fischer 344	125	110-115	300	300

These data therefore confirm the work of previous experiments, described in Chapter 3, where the response to sub-chronic dosing with gentamicin was more marked in SD than F344 rats. It appears that whilst the responses of the two strains are qualitatively the same in response to acute gentamicin dosing, the alterations in the renal handling of electrolytes caused by gentamicin may be more pronounced in the SD rat strain. Other acute studies using SD rats have also shown effects of gentamicin on the renal handling of electrolytes in this strain (Garland and Harpur, 1987). Taken together, the evidence supports the use of SD rats as a suitable model in which to study the phenomenon of aminoglycoside-induced renal cation wasting.

Since there were no alterations in GFR as a response to gentamicin infusion in these acute infusion studies, and taken together with the fractional excretion data, these experiments confirm that the lesion by which gentamicin causes renal wasting of calcium and magnesium is likely to be tubular rather than glomerular in origin. In particular, increases in the fractional excretion of both calcium and magnesium indicate a reduction in the tubular reabsorption of these cations after gentamicin administration. Previous studies have reported no increases in plasma calcium or magnesium concentrations after acute administration of gentamicin in F344 rats, so alterations in the filtered load of these

cations are unlikely to be the cause of the increases in the fractional excretions seen in these experiments (Foster, 1988).

Whilst the GFR values reported in these experiments are stable in both control and drug-treated animals the absolute values of this measurement are a little higher than one would normally expect. The normal range of GFR for rats of a similar age would be approximately 2.5-3.5ml/min. GFR measurements in the experiments presented here are consistently around 4.0ml/min. However, similarly elevated values have been reported in the literature (Lote et al, 1987). The measurements of GFR serve to illustrate two important points in these experiments: i) GFR values are not significantly different between drug-treated and control animals throughout the experiment; therefore acute infusion of gentamicin does not appear to affect glomerular function. ii) GFR is stable in both groups of animals during the experiment; there is therefore no indication of a fall-off in renal function over the experimental period which could affect the renal reponses to a toxin.

Alterations in the renal reabsorption of calcium and magnesium after gentamicin dosing of the magnitude seen in these experiments could potentially occur at several sites along the nephron. However, given that both calcium and magnesium excretion are increased the most likely sites of action for changes in the reabsorption of these divalent cations are, either the site of most gentamicin accumulation, the proximal convoluted tubule or, the thick ascending limb of the Loop of Henle, the site of most magnesium, and a substantial portion of calcium reabsorption. However, transport of both ions is largely dependent, directly or indirectly, on sodium movement in these nephron segments and it is difficult to envisage how these alterations could occur without concurrent changes in sodium transport. Since there is substantial capacity in the more distal nephron segments for recouping excess delivered sodium which are independent of calcium and magnesium reabsorption (Shareghi and Stoner, 1978), compensation at these sites may be masking any concurrent effect gentamicin administration may have on sodium handling. Since there is also no apparent effect on the renal handling of potassium after gentamicin

infusion it appears that the effects of gentamicin are confined to altering the renal handling of divalent cations, rather than monovalent cations. The basis of this specific effect on divalent cation handling is unknown. The precise site(s) of the reabsorption defect caused by gentamicin administration also remains unclear at present.

This series of experiments therefore clearly demonstrates the suitability of the SD strain of rat for acute studies of the effects of gentamicin on perturbations of the renal handling of calcium and magnesium. These preliminary studies also firmly establish the correct procedures and techniques necessary to carry out further studies to identify the site of the renal lesion which causes the renal wasting of cations shown in this and other studies.

Chapter 5. Using lithium clearance techniques to determine the site of acute gentamicin-induced renal cation wasting.

5.1 Introduction

Measurement of lithium clearance has been proposed as a non-invasive method to measure sodium and water delivery from the proximal tubule of the mammalian kidney by Thomsen and his co-workers (Thomsen and Schou, 1968, Thomsen et al, 1969). Substantial bodies of evidence suggest that lithium is reabsorbed primarily in the proximal tubule, in the same proportions as sodium (Haas et al, 1986, Thomsen et al, 1981, Hayslett and Kashgarian, 1979). However, debate exists as to whether lithium is reabsorbed in later nephron segments under normal (Navar and Schafer, 1987) or modified renal sodium handling conditions (Kirchner, 1987). Since calcium, for the most part, is reabsorbed in parallel to sodium and water in the proximal tubule, measurement of lithium clearance should provide an indirect measure of proximal calcium handling. If gentamicin exerts its effects on renal calcium handling at the site at which it is accumulated (the S1 and S2 segments of the proximal tubule) one might expect that alterations in the proximal handling of sodium and hence lithium might be apparent. To fulfil the role as a marker of proximal sodium reabsorption the solute should; a) be freely filtered at the glomerulus b) not be secreted along the nephron c) have no effect on renal function and, above all, d) its reabsorption should be in proportion to sodium and water along both the pars convoluta and pars recta segments of the nephron. Lithium appears to fulfil these conditions in the majority of circumstances. In the series of experiments described below lithium clearance is used as a marker for sodium and water delivery from the proximal tubule. However, endogenous plasma concentrations of lithium are low, so for ease of analysis supplementation with a lithium salt administered either orally, or by infusion is employed. These studies were therefore conducted to investigate the possible site of altered renal calcium handling after acute gentamicin infusion, using lithium clearance measurements. The limitations of the technique and questions arising

from interpretation of the lithium clearance data will be reviewed more fully in later sections of this chapter.

5.2 Methods

Sprague-Dawley rats were prepared for clearance experiments as detailed in Chapter 4.2. Experiments were carried out according to the protocol described in Figure 29. Gentamicin was infused at 0.56mg/kg/min (equivalent to 100mg/kg over the 3h experimental period) in the drug-treated animals; control animals received 0.9% w/v saline over the same period (n=6 for each group). LiCl (AR) was infused in the saline mixture as in previous experiments so that the rats received 15 μmol Li /h over the duration of the experiment. During the 200μl/min infusion period the saline mixture contained a concentration of 1.25mM LiCl; when the infusion rate was changed to 100μl/min the infusate was replaced with a solution containing 2.5mM LiCl and ^{3}H-inulin. Urine and plasma samples were obtained as previously detailed (Section 4.2) for measurement of sodium, potassium, calcium and magnesium concentrations, and for ^{3}H-inulin counting. In this series of experiments aliquots of plasma and urine were also taken for analysis of Li concentrations by atomic absorption spectroscopy. Methodological details of this technique are described in Appendix 9.

Data were analysed using multivariate analysis of variance for repeated measures using Statgraphics Version 2.0. Data from both drug-treated and control groups of animals were compared to each other over the first two (control) collection periods, and over the remainder of the experimental period (6 collections). Data were taken to be significantly different at P values of 0.05 or less.

Data are presented graphically as mean ± SEM.

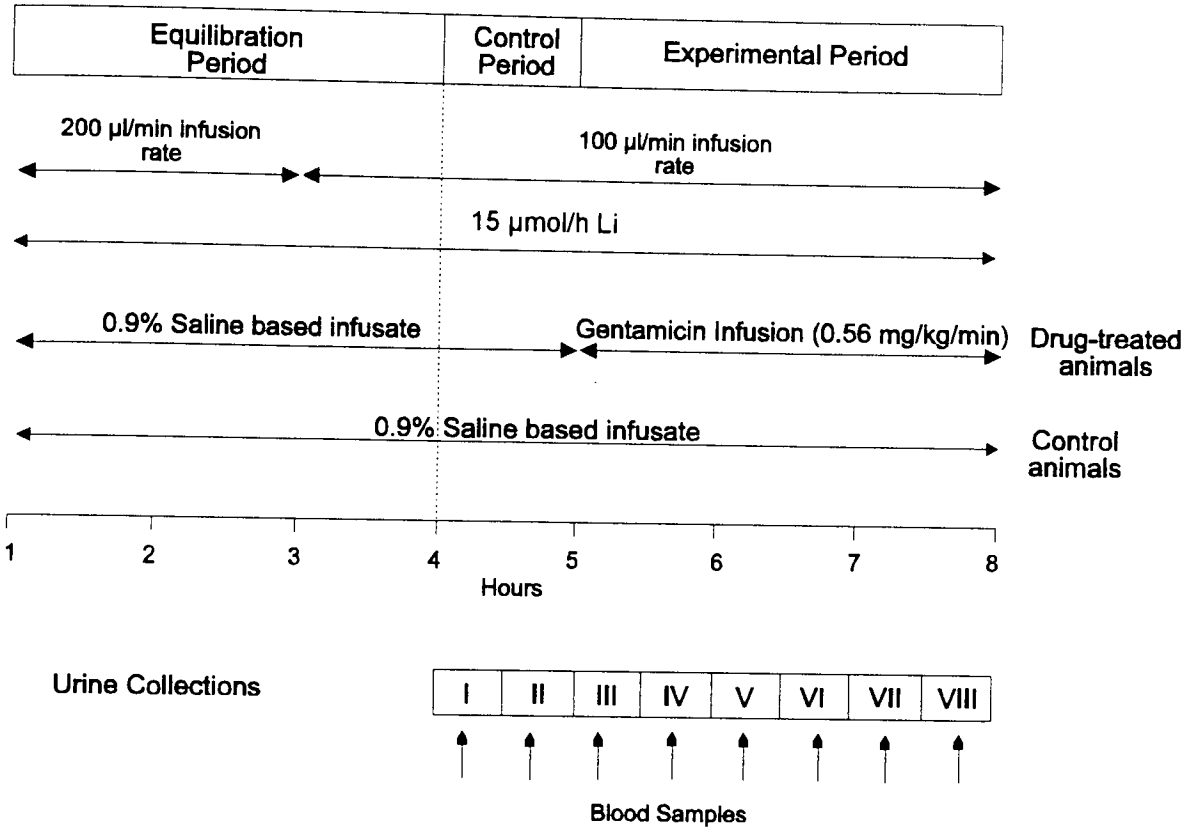


Figure 29. Experimental protocol for lithium clearance studies in Chapter 6. Full details of the experimental procedures are provided in the chapter text.

5.3 Calculations and Definitions

The proximal sodium clearance can be defined as the delivery of sodium from the pars recta to the Loop of Henle and can be described by the equation:

$$C_{Na}\ prox = (TF_{Na} \times V\ prox) \div P_{Na}$$

Where TF_{Na} = sodium concentration in tubular fluid at the terminal portion of the proximal tubule (pars recta); P_{Na} = plasma concentration of sodium.

The delivery of fluid per unit time from the pars recta to the Loop of Henle is designated Vprox.

Since TF_{Na} = PNa under normal conditions then C_{Na} prox = V prox

For C_{Li} to be a measure of C_{Na} prox then 2 criteria must be met: 1) Li must be reabsorbed in the proximal tubules to the same extent as sodium and water, and 2) Li must not be secreted or reabsorbed in the distal nephron.

When the first criterion is met:

$$TF_{Li} = P_{Li}$$

Or:

$$C_{Li} = Vprox = C_{Na}\ prox$$

When the second criterion is fulfilled TF_{Li} x V prox will equal the urinary excretion of Li :

$$C_{Li} = (U_{Li} \times V) \div P_{Li} = (TF_{Li} \times Vprox) \div P_{Li}$$

Where V = Urine flow rate.

Thus, measurement of the renal clearance of lithium (using urinary excretion and plasma concentration data) provides an indirect measurement of the proximal clearance of sodium.

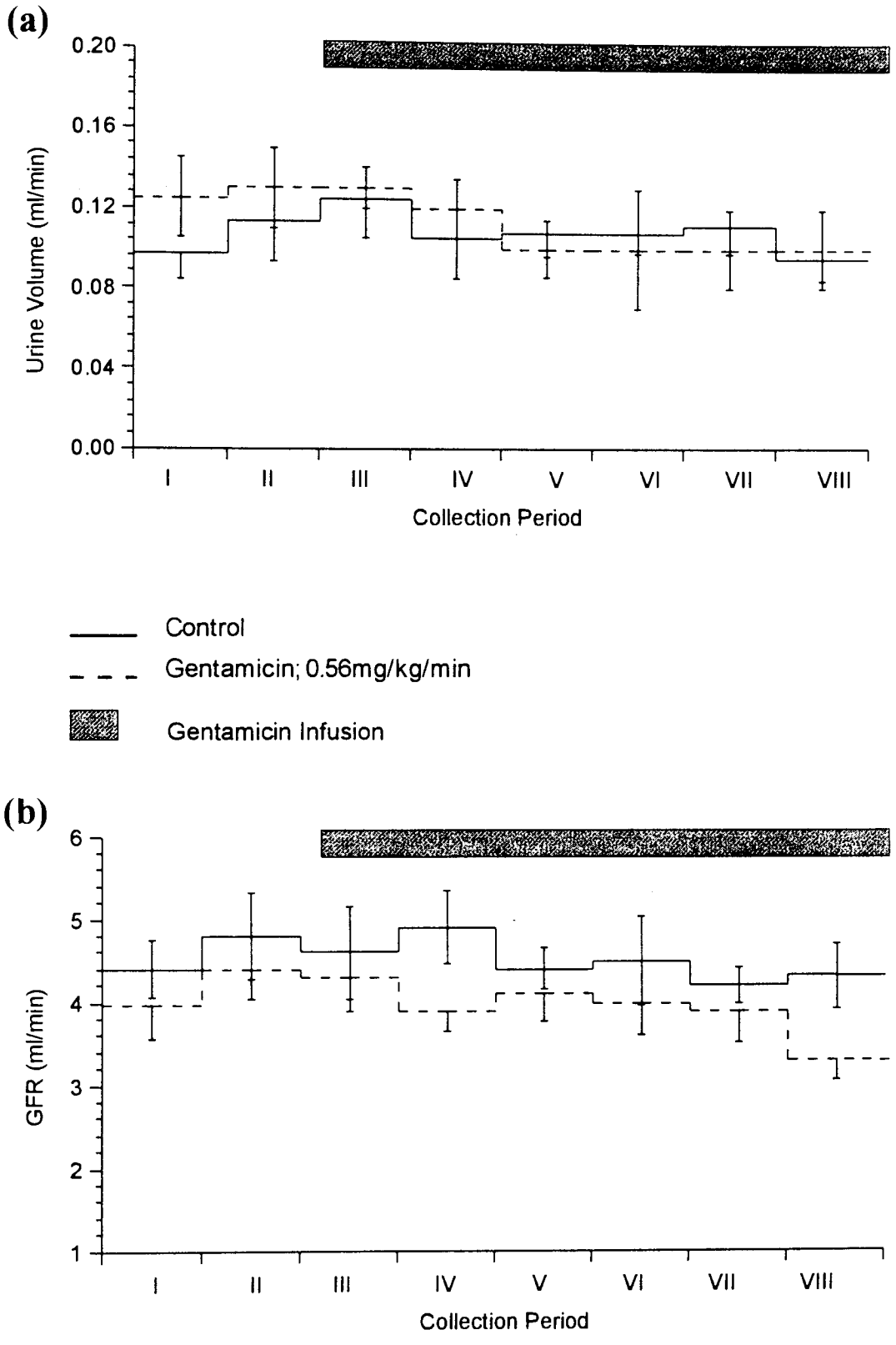


Figure 30. Urine flow rate (a) and GFR (b) in control and gentamicin-infused groups. There are no significant differences between groups during either control or experimental periods for both parameters (MANOVA).

5.4 Results

There were no differences in GFR between control and drug-treated groups either during the control period or during the period of gentamicin infusion (Figure 30 b). The urine flow rate was constant in both gentamicin-infused and control rats throughout the experiment (Figure 30 a). There were no significant differences in urine output between the groups, which was close to the infusion rate of 100 µl/min, indicating a stable diuresis in the experimental subjects.

There were no significant differences between control and gentamicin-treated groups over the first two collection periods for calcium excretion rate, FE_{Ca}, and calcium clearance. In contrast, infusion of 0.56 mg/kg/min gentamicin produced an immediate increase (approximately 2-fold) in all of these parameters ($P < 0.01$) (Figures 31a, 32a, 33a). The alterations in the renal handling of calcium induced by gentamicin were fully developed by the first collection period after gentamicin-infusion was initiated, and were sustained for the duration of the experiment.

There were no significant differences in the renal handling of magnesium, as measured by excretion rate, clearance, and FE_{Mg}, between the gentamicin-treated and control groups, over the first two (control) collection periods. Infusion of gentamicin however produced immediate effects on magnesium handling by the kidney. Significant increases (approximately two-fold) in magnesium excretion rate ($P < 0.01$), magnesium clearance ($P < 0.01$), and FE_{Mg} ($P < 0.01$) were evident 30 minutes after the start of gentamicin infusion (Figures 31b, 32b, 33b). The effects of gentamicin on magnesium handling were less stable than those seen on calcium handling but nevertheless marked changes in the renal handling of magnesium certainly occurred.

In contrast to the marked increases in calcium and magnesium excretion generated by the acute infusion of gentamicin there were no effects of gentamicin treatment on the renal handling of sodium. There were no significant differences between gentamicin-treated and control groups, at any time during the experimental procedure, on sodium excretion

rate, clearance, or FE_{Na} (Figures 34a, 35a, 36a). Sodium clearance, excretion rate and FE_{Na} were essentially steady during the entire control and experimental period.

Effects on renal potassium handling were however more ambiguous. Whilst there were no significant differences in potassium excretion rate, potassium clearance or FE_K between drug and control groups over the control collection periods, infusion of gentamicin appeared to cause alterations in various potassium measurements compared to control reponses. Against a decline in potassium excretion rate in control rats, gentamicin infusion appeared to produce significantly elevated rates of potassium excretion compared to controls ($P < 0.01$). The decline in the excretion rate of potassium shown by control animals was however exactly mirrored by those animals to which gentamicin was administered (Figure 34b). Potassium excretion rates were consistently approximately 30% higher than those of control animals throughout the experiment, which remained unaltered by the infusion of gentamicin. In contrast, infusion of gentamicin produced larger, unambiguous, increases (approximately 200%) in calcium and magnesium excretion rates above controls. A similar decline in potassium clearance was shown in both control and gentamicin-treated rats over the study period. Potassium clearance was also apparently significantly higher in gentamicin-infused animals compared to controls during the experimental period ($P < 0.01$) (Figure 35b). However, potassium clearance declined in a similar manner and extent in both the control and gentamicin-infused groups (Control: approximately 1.0 µmol/min in collection period 1 declining to 0.7 µmol/min by collection period 8. Gentamicin-infused: approximately 1.4 µmol/min in collection period 1 declining to 1.1 µmol/min by collection period 8). Mean potassium clearance values for gentamicin-infused animals were raised approximately 30 % over control values in all collection periods. Mean values for FE_K were consistently elevated over controls (approximately 40 %) throughout the entire experiment. Statistically significant increases in FE_K were evident after gentamicin infusion ($P < 0.01$) (Figure 36b). However, the differences between drug-treated and control animals occurred against a decline in FE_K which was only shown by control animals. Hence the

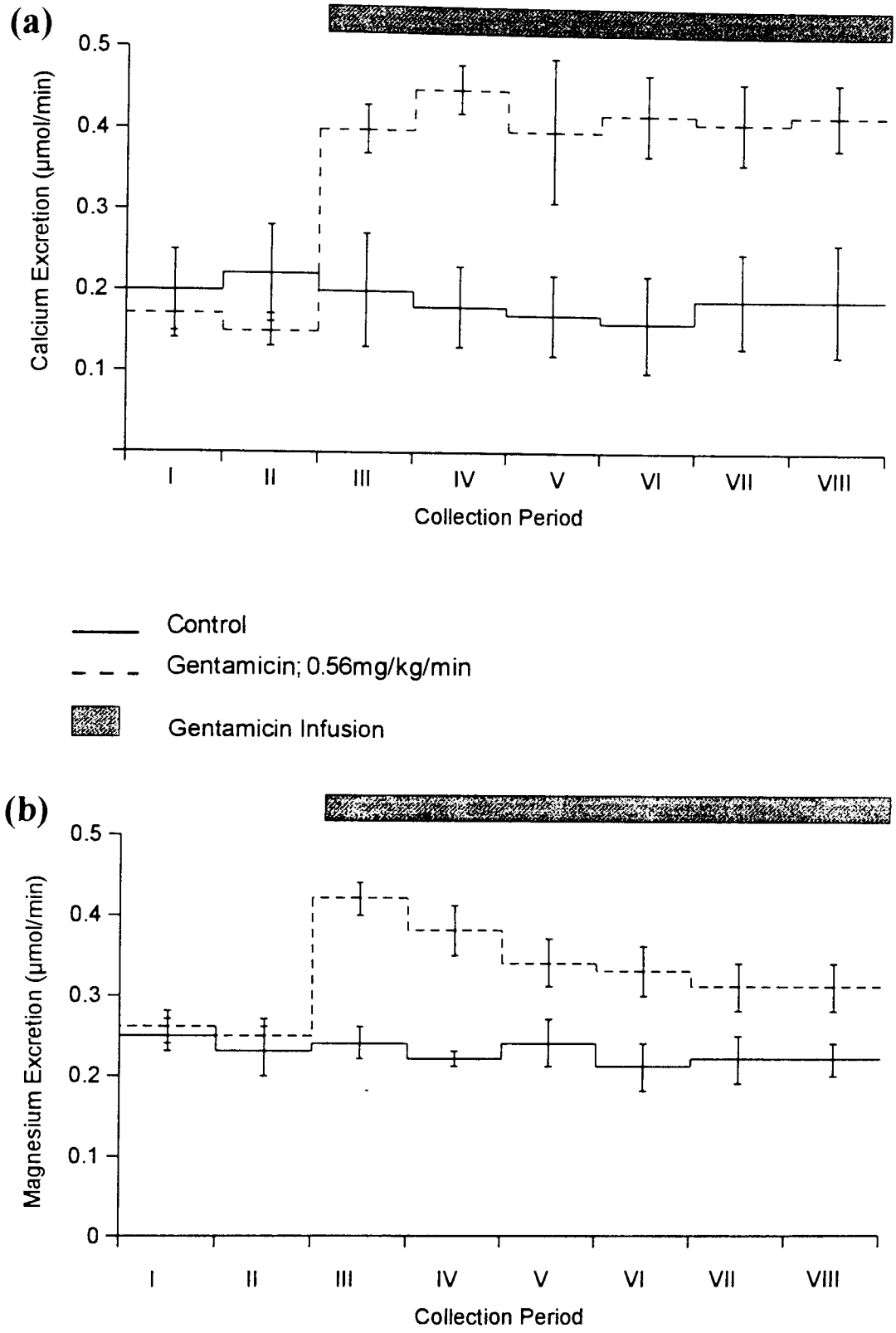


Figure 31. Calcium (a) and magnesium (b) excretion rates in control and gentamicin infused rats. There are no significant diferences during the control period for either parameter. Infusion of gentamicin produced significant increases in both calcium and magnesium excretion rates ($p < 0.01$) (MANOVA).

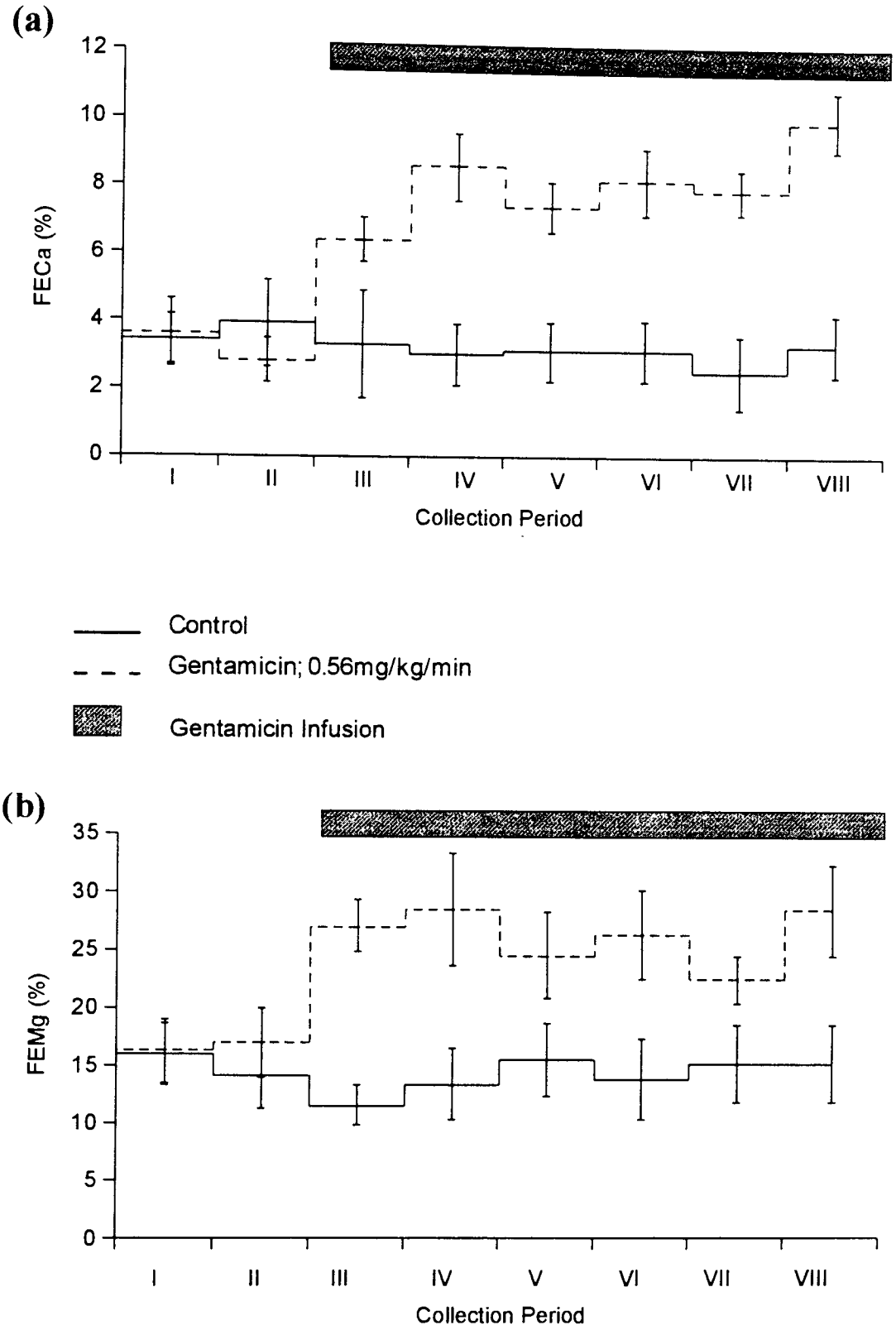


Figure 32. Fractional excretion of calcium (a) and magnesium (b) in gentamicin infused and control groups. No significant differences between gentamicin infused and control rats during control periods. Infusion of gentamicin produced significant increases in FECa and FEMg. ($P < 0.01$) (MANOVA).

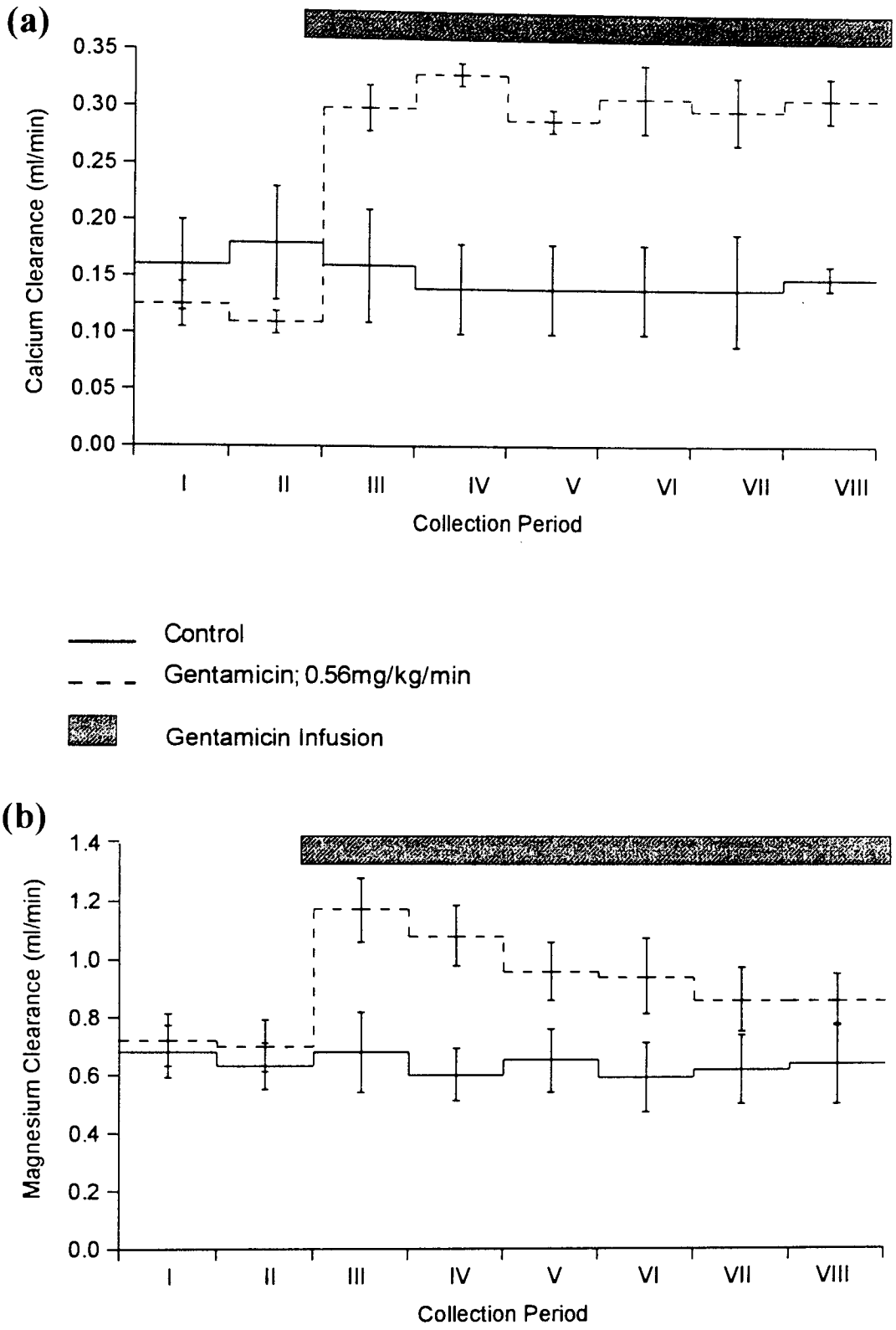


Figure 33. Clearance of calcium (a) and magnesium (b) in control and gentamicin infused rats.There were no significant differences between control and gentamicin-infused rats over the control period for either parameter. Infusion of gentamicin produced significant differences between control and drug infused groups ($P < 0.01$) (MANOVA).

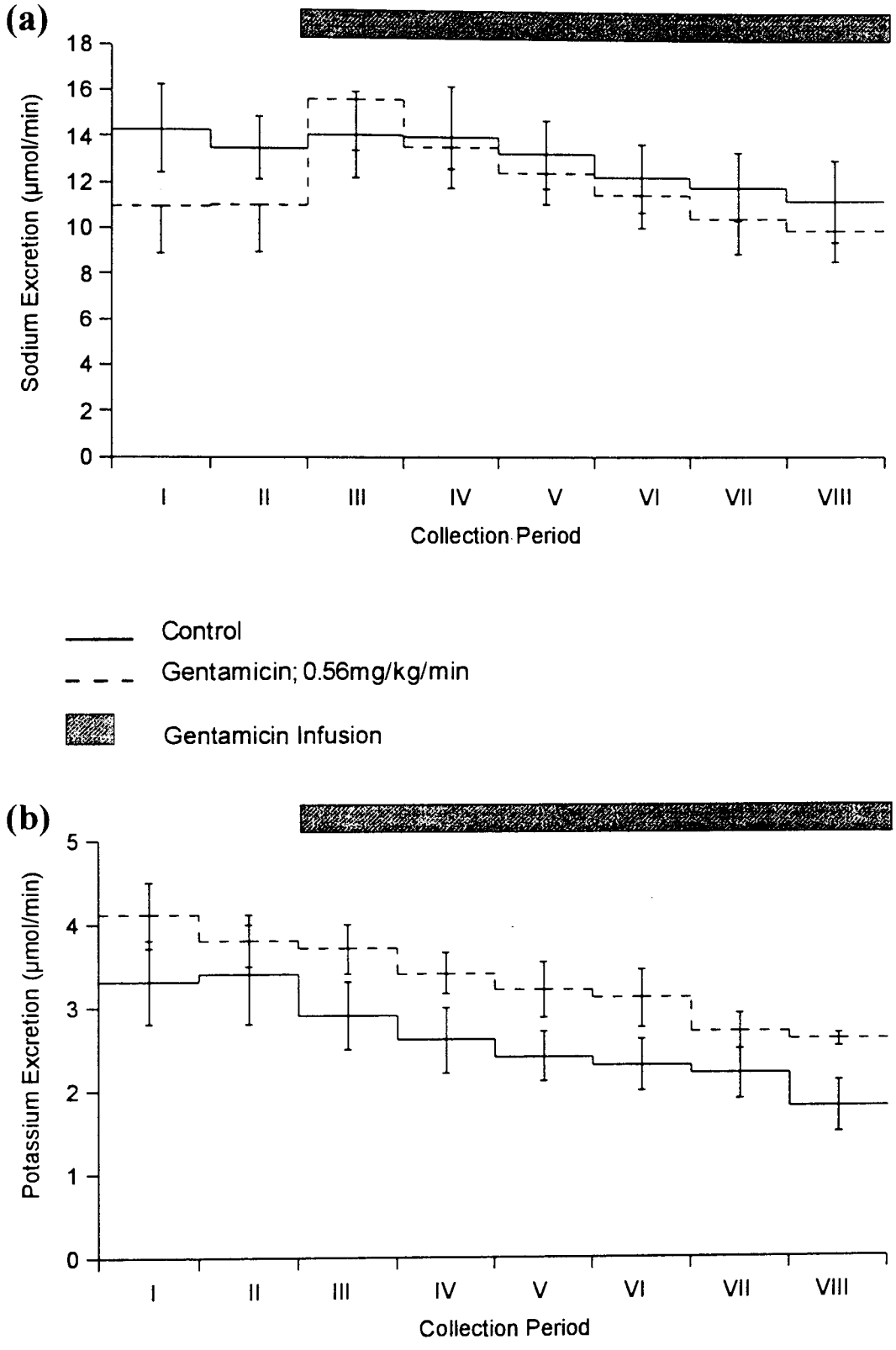


Figure 34. Sodium (a) and potassium (b) excretion rates in control and gentamicin infused rats. There are no significant differences between groups in sodium excretion rate during both the control and experimental periods. There are no significant differences between groups in potassium excretion rate during the control period. Infusion of gentamicin produced significant differences between groups in potassium excretion rate ($p < 0.01$) (MANOVA).

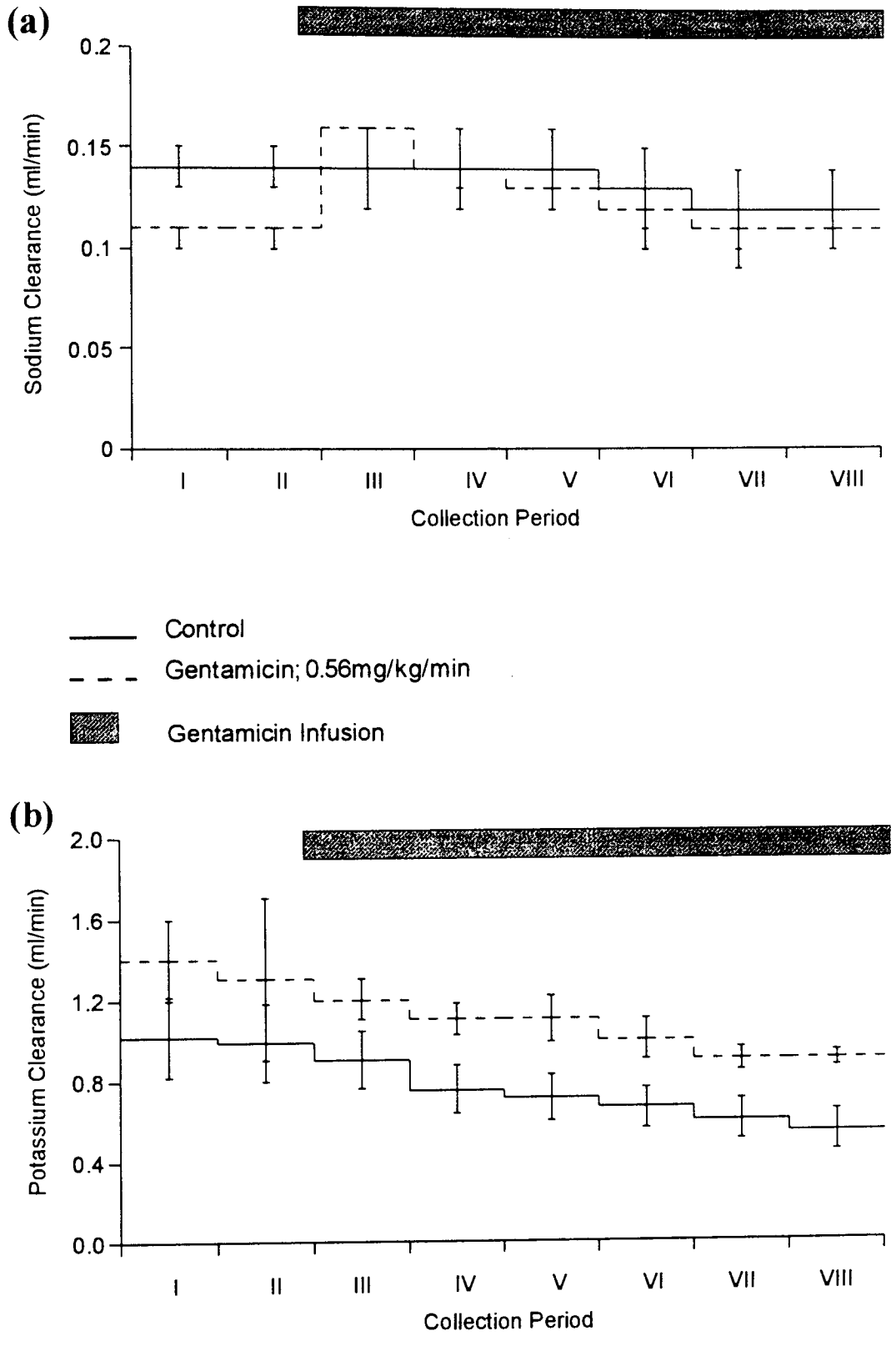


Figure 35. Sodium (a) and potassium (b) clearance in control and gentamicin infused rats. There are no significant differences between drug-treated and control groups at any time for sodium clearance. There are no signifcant diffrences between groups during the control period for potassium clearance; gentamicin infusion produced significant differences between drug-treated and control groups ($p < 0.01$) (MANOVA).

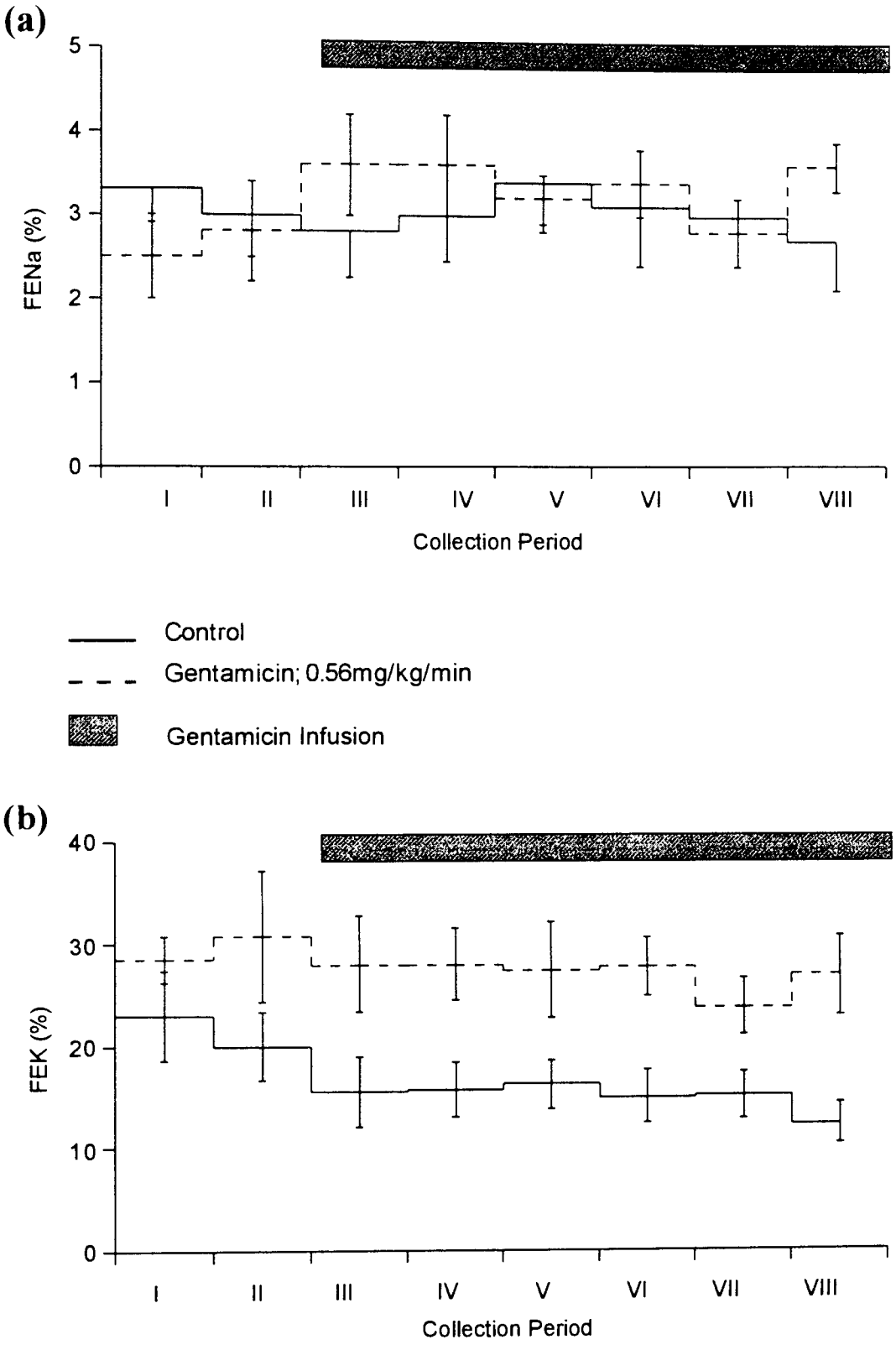


Figure 36. Fractional excretion of sodium (a) and potassium (b) in control and gentamicin infused rats. No significant differences existed between gentamicin infused and control rats during the control period for either parameter. Gentamicin infusion produced no significant alterations in FE Na, but there were significant ($P < 0.01$) differences in FE K after gentamicin infusion. (MANOVA)

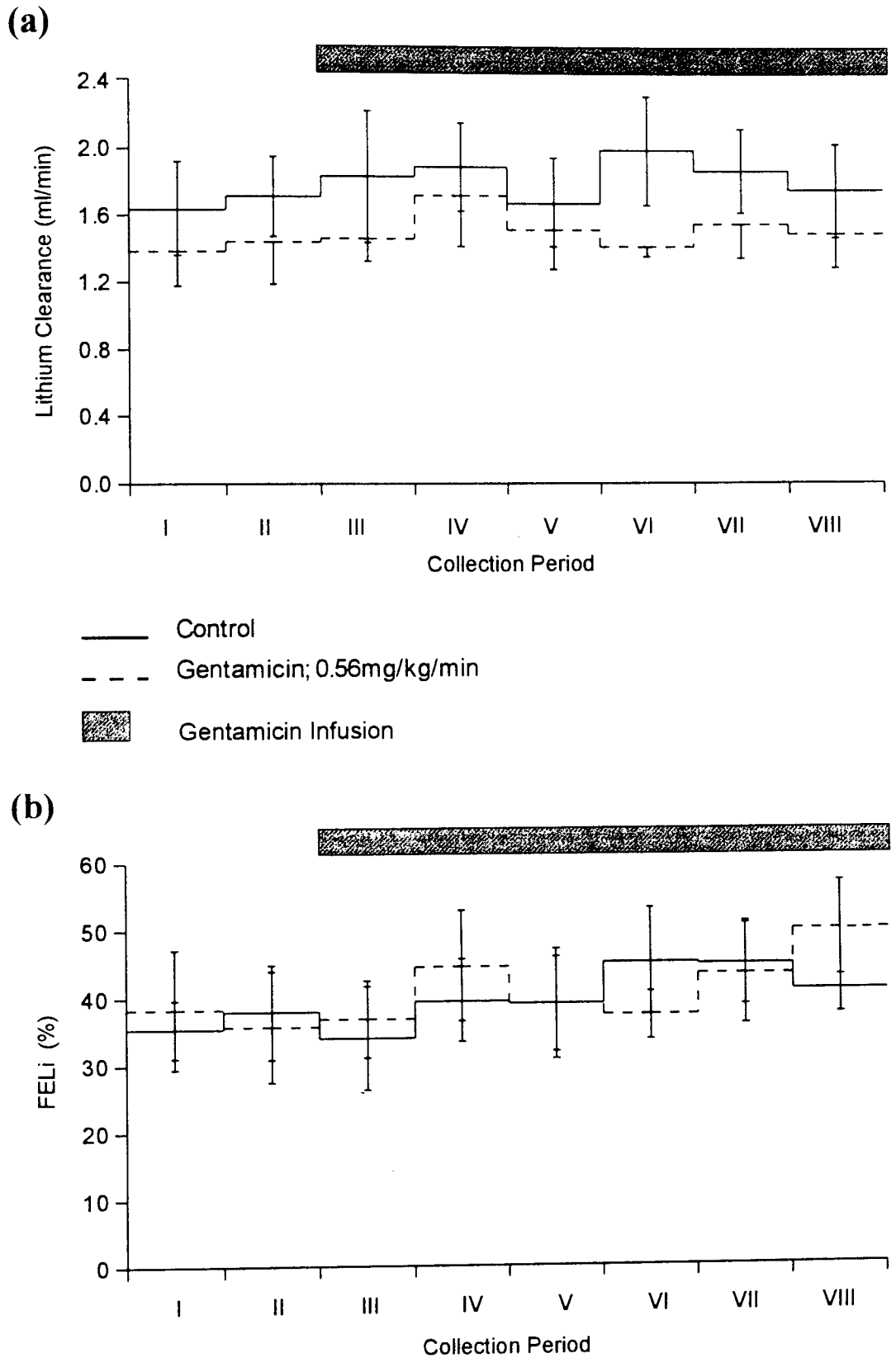


Figure 37. Lithium clearance (a) and FE Li (b) in control and gentamicin infused rats. There are no significant differences between control and gentamicin infused groups, either during the control period or after gentamicin infusion. (MANOVA)

effect of gentamicin infusion was to retard this decline in FE_K shown by control animals, rather than to produce increases of FE_K in drug-treated animals. Hence alterations in FE_K shown in these experiments, probably do not represent significant effects of gentamicin on the renal handling of potassium.

There were no significant differences over the control periods (1 and 2) for lithium clearance or FE_{Li} between the gentamicin-infused and control animals. Similarly, there were also no significant differences in lithium clearance or FE_{Li} between gentamicin-infused and control rats over the experimental period (3-8) (Figures 37a, b). Plasma lithium concentrations were 189.8 ± 23.4 μmol/l in control animals and 201.0 ± 10.7 μmol/l in gentamicin-dosed animals and were not statistically different to each other.

5.5 Discussion

Whilst the equation ;

$$C_{Li} = V \text{ prox} = C_{Na} \text{ prox}$$

is the tenet on which the C_{Li} technique is based, direct confirmatory evidence that this is the case is unavailable, since the pars recta is inaccessible to micropuncture studies. However, the structure and function of the epithelium of the pars recta resembles that of the pars convoluta. Evidence from studies in rabbits suggests while the length of the pars recta is approximately 60% of the length of the pars convoluta its reabsorptive capacity per unit length is about half that of the convoluted tubules (Hamburger et al, 1974, Hamburger et al, 1976). This would therefore suggest that the reabsorptive capacity of this segment should be about 30% of that occurring in the pars convoluta.

Experimental evidence supporting this prediction was produced in studies which examined the total lithium reabsorption between the accessible late proximal tubule and the early distal tubule (Hayslett and Kashgarian, 1979). The authors found that 32% of the total lithium reabsorption occurred between these segments, which is in excellent agreement with the predicted value. However while these findings support the hypothesis

that C_{Li} = V prox, the possibility still exists that lithium reabsorption occurs not only in the pars recta but also in the Loop of Henle. Additional studies comparing C_{Li} and direct measurements of fluid delivery from the end of the pars convoluta using micropuncture techniques, confirmed a significant degree of correlation between the 2 methods, supporting the use of C_{Li} as a measure of V prox (Thomsen et al, 1981, Shirley et al, 1983).

Whilst C_{Li} appears to provide a good estimate of V prox under normal circumstances there are situations in which lithium appears to be reabsorbed in the later parts of the nephron, particularly in the distal tubule. Several groups of experimenters have shown that rats fed a normal or raised sodium content diet C_{Li} correlates well with V prox obtained from micropuncture studies (Thomsen, 1977, Thomsen and Leyssac, 1986, Kirchner, 1987). However, reduction of dietary Na contents substantially decreased C_{Li} values, indicating reabsorption of lithium against a concentration gradient, most likely occurring in the distal tubule (Thomsen, 1977, Thomsen and Leyssac, 1986, Kirchner, 1987). Thomsen and Leyssac (1987) also investigated the effects of a low potassium diet on C_{Li} values. As in the case of a reduction in dietary sodium levels, low dietary potassium levels induced a reduction in C_{Li} values. The reduction in C_{Li} caused by low sodium and low potassium diets were additive. Rats given a diet low in both sodium and potassium showed C_{Li} values reduced to practically zero, indicating avid reabsorption of lithium in place of other monovalent cations under these circumstances.

Prevention of distal lithium reabsorption after maintenance on a low sodium diet has been demonstrated by the use of the diuretic agent amiloride (Kirchner, 1987, Thomsen and Leyssac, 1986). While C_{Li} is unaffected by amiloride administration at normal or high dietary sodium levels administration of amiloride to rats fed a low sodium diet generated increases in C_{Li} toward normal values. These data suggest that amiloride administration to animals fed low sodium diet may make C_{Li} a valid measure of V prox, and also imply that amiloride may be a suitable pharmacologic tool to investigate whether distal lithium reabsorption is present or absent in a given experimental situation.

Studies using other diuretics have also shed light on potential sites of lithium reabsorption beyond the proximal tubule and supported the hypothesis of C_{Li} as a valid estimate for V prox. Experiments using furosemide to investigate the contribution of lithium reabsorption in the Loop of Henle to C_{Li} have been subject to the flaw that while furosemide inhibits sodium reabsorption in the Loop of Henle it also alters proximal tubular output (Thomsen and Leyssac, 1987). However, a study which used a massive saline diuresis to promote maximal inhibition of proximal tubular reabsorption, produced no changes in C_{Li} or FE $_{Li}$, after furosemide dosing (Christensen et al, 1988). Whilst these data are not conclusive proof against lithium reabsorption occurring in the Loop of Henle they provide further suggestive evidence that lithium reabsorption does not usually occur beyond the proximal tubule.

Administration of diuretics which act primarily along the proximal tubule (e.g. aminophylline, acetazolamide) lead to increases in the fractional excretion of lithium (Perry et al, 1984, Petersen et al, 1974), whereas those inhibiting distal sodium reabsorption (e.g. hydrochlorothiazide, spironolactone) do not (Shirley et al, 1983, Steele et al, 1975), again supporting the belief that C_{Li} is an appropriate marker of proximal sodium handling.

Lithium reabsorption in the proximal tubule appears to occur by both paracellular, and transcellular routes probably by the action of the Na^+/H^+ exchanger in the apical membrane; the cellular extrusion route remains undefined. Lithium handling in the Loop of Henle remains uncertain. There appears to be low permeability to lithium in the thin limbs of the Loop, suggesting no net reabsorption of lithium in this segment. It remains unclear as to whether lithium can substitute for sodium on the NaCl/KCl cotransporter or the Na^+/H^+ exchanger in the thick ascending limb of the Loop of Henle, but current evidence suggests this is unlikely. Lithium reabsorption in the distal nephron appears to involve amiloride-sensitive sodium channels in the collecting tubules. However this mechanism appears to only come into action under conditions of severe dietary

restriction of sodium possibly due to channel selectivity of sodium over lithium in replete individuals (Leyssac et al, 1990).

In vitro experiments using artificial membranes have shown that lithium does not bind to plasma proteins and can pass freely across the glomerular membrane (Talso and Clarke, 1951). However endogenous plasma concentrations of lithium are low, so for ease of analysis supplementation with a lithium salt is either administered orally, or by infusion. Plasma levels of lithium above 2 mEq/l do however have significant toxic effects on renal electrolyte handling; acutely increasing sodium and potassium excretion and decreasing water reabsorption (Hecht et al, 1978).

The data presented in this chapter confirm the alterations in the renal handling of calcium and magnesium produced after acute gentamicin infusion described in the previous chapter and by Foster et al (1992). The lack of alterations in GFR indicate that the effects which gentamicin exerts on the kidney are at a tubular, rather than at a glomerular level, after acute infusion. Gentamicin induces significant increases in calcium and magnesium excretion without apparent effects on renal sodium handling. Effects of gentamicin on renal divalent cation handling were visible after only 30 min of drug infusion in these experiments. Since this equals a dose of only 5mg/kg (approximately) after the total 30 minutes of infusion it is extremely unlikely that the renal wasting of calcium and magnesium observed in these experiments represents a manifestation of gross tubular damage and is more probably a selective perturbation of the renal reabsorption of these cations induced by gentamicin. Since the major site of calcium reabsorption along the nephron, the proximal tubule, is also the predominant site of gentamicin accumulation and the only site in the nephron where histological damage is evident following the administration of gentamicin, it was initially tempting to speculate that the proximal tubule was the most likely target for the action of this compound on the renal reabsorption of calcium and, additionally, magnesium.

Information derived from the studies presented here however suggests this may not be the case. As previously described the lithium clearance technique has been used in these experiments to assess sodium handling, and indirectly calcium handling in the proximal tubule. Since the bulk transport of calcium in the proximal tubule is considered to be mainly passive, and secondary to the active reabsorption of sodium (Rouse and Suki, 1990, measurements of lithium clearance along this segment may therefore be considered as an indirect means of assessing calcium handling. As shown in Figures 37a and 35a, infusion of gentamicin produced no effects on lithium and sodium clearance over any of the experimental periods, at times when calcium and magnesium handling was substantially altered. This therefore provides indirect evidence to suggest that gentamicin does not exert its effects, on calcium handling at least, in the proximal tubule. Micropuncture data obtained in the SD rat after gentamicin infusion demonstrated no differences in recovery of ^{45}Ca in urine following injection of ^{45}Ca into the distal tubule, between control and gentamicin infused animals, indicating that decreases in calcium reabsorption induced by gentamicin are not mediated in the distal nephron (Garland et al 1992). Furthermore, microinjection studies of calcium handling in the proximal tubule in the studies of Garland et al (1992), also suggested the lack of an effect of gentamicin on ^{45}Ca handling in the segments of the proximal tubule accessible to micropuncture techniques. In the micropuncture studies of Garland et al (1992) urinary recovery of ^{45}Ca injected into the proximal tubule was greater in gentamicin-infused animals than in control animals, indicating that gentamicin exerted its effects prior to the distal tubule i.e. either the proximal tubule or the Loop of Henle. Examination of the influence of proximal micropuncture site on the extent of ^{45}Ca recovery however suggested that the effect on calcium reabsorption may not be located in the proximal tubule. Regression lines plotted through data which correlated ^{45}Ca reabsorption with the distance from the glomerulus as a site of microinjection revealed that the slopes of these lines were not significantly different between drug-treated and control animals. If the site of action of gentamicin on calcium handling is mainly proximal it would be expected that the regression lines would lie the greatest distance apart where ^{45}Ca injections were made

close to the glomerulus and converge together toward the later microinjection sites. This evidence therefore supports the hypothesis that gentamicin does not produce its effects on calcium handling by altering calcium reabsorption in the parts of the proximal tubule accessible to micropuncture. However lack of direct evidence regarding the actions of gentamicin in the late proximal tubule leaves open the question as to whether gentamicin exerts its effects solely in the straight proximal tubule rather than the convoluted segments or somewhere along the nephron between the proximal segments and the distal tubule i.e. the Loop of Henle.

If gentamicin is acting on the thick ascending limb of the Loop of Henle then this may explain the commonly observed clinical finding of hypermagnesuria with associated hypomagnesaemia (Shils, 1969, Bar et al, 1975) since the thick ascending limb is the principal site of magnesium reabsorption (Morel et al, 1969). Hypermagnesuria induced by aminoglycosides appears to be less marked in animal models than the hypercalciuria which is also produced as a consequence of aminoglycoside administration (Harpur et al, 1985, Foster et al, 1992). Nevertheless, aminoglycoside administration results in alterations in electrolyte handling in the rat (Pastoriza et al, 1983), sheep (Bennett et al, 1983) and dog (Crawford and Teske, 1978) occurring within hours of drug administration. In sub-chronic dosing studies hypercalciuria and hypermagnesuria have also been observed 24 hours after the first dose of aminoglycoside (Harpur et al, 1985, Elliot et al, 1987), occurring without concurrent increases in urinary enzyme excretion (Harpur et al, 1985).

Renal lesions caused by aminoglycoside administration tend to be confined to the proximal nephron; damage to the cells of the Loop of Henle have not been reported (Kosek et al, 1974, Houghton et al, 1976). However, since the hypermagnesiuria and hypercalciuria are evident so soon after aminoglycoside dosing it may well be that functional changes causing the renal wasting of these cations exist without corresponding alterations in cell morphology.

The significance of the lack of effect on the renal handling of sodium when concurrent changes in calcium and magnesium exist is uncertain since reabsorption of both magnesium and calcium is largely passive and dependant on active sodium transport. Limited transport of both calcium and magnesium occurs in the distal nephron; it is therefore possible that while excess sodium delivered to this segment may be recouped, the reabsorptive mechanisms for calcium and magnesium could be overloaded leading to a selective increase in excretion of both ions. Hence the data presented here confirms the effects of gentamicin on the renal reabsorption of both calcium and magnesium and suggests that the site at which this alteration occurs may be located in the Loop of Henle, rather than the proximal tubule.

In addition to sodium-dependent calcium transport, reabsorption of a small proportion of the calcium from tubular fluid may occur by the active extrusion of calcium from the cell by the activity of Ca2+ ATPase. Alterations in the activity of this enzyme therefore present an alternative mechanism by which calcium reabsorption could be inhibited. Therefore the effects of *in vitro* and *in vivo* exposure to aminoglycosides on the activity of Ca2+ ATPase were investigated and the results are presented in Chapter 6.

Chapter 6. Effect of *in vitro* and *in vivo* exposure to aminoglycosides on the activity of renal ATPases.

6.1 Introduction

The regulation of calcium excretion by the kidney is achieved through a range of processes with varying degrees of regulation and specificity. Of the total calcium filtered at the glomerulus each day in the rat only around 3% appears in the final urine, indicating a substantial reabsorptive capacity of the kidney for this cation. The majority of this reabsorption occurs along the proximal nephron, mainly as a consequence of active sodium transport, and probably via a paracellular route, although evidence for a Na^{+}/Ca^{2+} exchanger, and for Ca2+ ATPase activity in this region suggests some portion of calcium transport is transcellular. In the distal nephron segments, sodium and calcium reabsorption can be dissociated and separate transport processes for these cations exist. Both a Ca2+ ATPase and a Na^{+}/Ca^{+} exchanger have been implicated in this more distal calcium reabsorption. It is in the distal portions of the nephron that 'fine tuning' of calcium excretion can occur in response to hormonal stimulation (Rouse and Suki, 1990).

The aim of the studies reported in this chapter was to elucidate whether aminoglycosides could inhibit the activity of Ca2+ ATPase present in tissue fractions from rat kidney, as a possible mechanism by which the drugs could reduce calcium reabsorption. Previous work (Chahwala and Harpur, 1982, Williams et al, 1981) had demonstrated that aminoglycosides could inhibit Na^{+}/ K^{+} ATPase activity, after both *in vitro* and *in vivo* exposure, and showed that aminoglycosides had the potential to affect the function of this important class of plasma membrane-bound enzymes. In the experiments reported in this chapter Na^{+}/ K^{+} ATPase activity was therefore determined as a positive control. Hence both *in vivo* and *in vitro* studies were designed to investigate the possibility that a similar inhibitory effect could be exerted on Ca2+ ATPase activity after aminoglycoside exposure.

6.2 Methods

These studies employed the use of two types of kidney tissue fractions to investigate the effects of aminoglycosides on renal ATPases after both *in vivo* and *in vitro* exposure. Both Ca^{2+} ATPase and Na^{+}/ K^{+} ATPase activity was determined biochemically by estimating the amount of inorganic phosphate produced by the hydrolysis of ATP due to action of the enzymes.

6.21 Preparation of isolated tissue fractions.

Homogenate: Rats were killed by cervical dislocation, the kidneys removed and decapsulated, and placed in ice-cold 0.25M sucrose-0.03M histidine buffer, kept on ice. The tissue was weighed, coarsely chopped with scissors, and placed into the homogenising vessel with sucrose-histidine buffer at a ratio of 1g tissue: 10ml buffer. The tissue was then homogenised, over ice, at 1000 rev/min, for 10 strokes. The resulting homogenate was stored on ice prior to use, or stored as 2ml aliquots at -18 °C for later use.

Microsomal Fraction (Method of Jorgensen (1974) modified by S.B.Chahwala [1981]): A homogenate was prepared as described above and centrifuged at 6000G for 10 min, at 4 °C. The supernatant from this centrifugation step (S1) was retained on ice and the sediment resuspended in the original volume of sucrose-histidine buffer. The suspension was centrifuged at 6000G for 10 min, at 4 °C and the resulting supernatant combined with S1. After mixing, the suspension was centrifuged at 35000G for 30 min, at 4 °C and the resulting supernatant discarded. The remaining pellet was resuspended in the original volume of sucrose-histidine buffer and centrifuged at 35000G for 10 min, at 4 °C. The supernatant was discarded and the pellet resuspended in 2ml of sucrose-histidine buffer, and stored on ice prior to use. The specific activities of Ca2+ ATPase and Na^{+}/K^{+} ATPase were increased 5-10 fold in microsomal fractions compared to homogenates.

6.22 Measurement of Ca^{2+} ATPase activity

Specific activity of Ca^{2+} ATPase was measured as the amount of inorganic phosphate released from the hydrolysis of ATP per minute per mg protein. Portions equivalent to approximately 50 µg of total protein of the previously prepared tissue fractions were added to an incubation mixture containing 5mM $CaCl_2$, 5mM Tris-HCl buffer at pH 7.4 and 5mM ATP (Tris-salt), in a final volume of 1.85ml. The reaction was started by the addition of the ATP solution. The mixture was incubated at 37 °C in a shaking water bath, usually for 30 min. The reaction was stopped by the addition of 0.4 ml ice-cold 35% trichloroacetic acid. The mixture was then centrifuged at 10000G, for 10 min, at 4 °C. Aliquots of the supernatant (0.5ml) were then removed for the measurement of inorganic phosphate content by the method of S.B.Chahwala (1981), (after Ueda and Wada, 1970). Ca^{2+} ATPase activity was taken as the difference in inorganic phosphate production in the presence and absence of calcium salts in the incubation mixture. Each measurement was made in triplicate; values were within 5-10 % of each other.

Stability: In order to make the most efficient use of the available tissue a series of experiments were conducted to establish the stability of Ca^{2+} ATPase after storage at -18 °C. Microsomal fractions were prepared as previously described and assayed for Ca^{2+} ATPase activity. Samples of the microsomal fraction were aliquoted into 2 ml portions and frozen at -18 °C for storage. After varying intervals individual aliquots were rapidly thawed and the remaining activity measured. Only samples prepared from untreated or saline-injected SD rats were used for stability studies. Each sample was assayed in triplicate.

From the results presented in Table 8 below it appeared that Ca^{2+} ATPase activity present in microsomal fractions remained stable for up to 3 weeks during storage at -18 °C but longer storage resulted in a loss of activity of the enzyme. Therefore it was decided that frozen microsomal samples were suitable for use in *in vitro* studies up to 3 weeks following their isolation. These stability data are in good agreement with results

reported by Jorgensen, (1974), for the stability of Na^+/ K^+ ATPase activity after frozen storage.

Table 8. The effect of storage at -18 °C on Ca^{2+} ATPase activity in whole kidney microsomal fractions.

Storage Period (Weeks)	% of original Ca^{2+} ATPase activity (mean ± S.E.M.)	Number of independent samples
2	102 ± 20	4
3	93 ± 6	4
6	46 ± 3	2
9	53 ± 8	6

Characterisation: The specific activity of Ca^{2+} ATPase was unaffected by the presence of 1 mM ouabain (Na^+/ K^+ ATPase inhibitor) or by 1mM ruthenium red (mitochondrial Ca^{2+} ATPase inhibitor), either singly or together. Ca^{2+} ATPase activity could however be inhibited by the presence of 0.1mM $AgNO_3$ (23% inhibition of Ca2+ ATPase activity compared to controls at 0.1 mM $AgNO_3$), as reported by Thompson and Nechay (1981).

Linearity of phosphate production by Ca^{2+} ATPase and Na^+/ K^+ ATPase: The linearity of phosphate production was assessed by incubating separate samples for varying periods of time from 5 min to 1 hour. Each time point, for both enzymes, was assayed in duplicate and the reaction stopped by the addition of ice-cold TCA. The results indicate that phosphate production is linear for both enzymes for up to 1 hour (Figure 38).

6.23 Measurement of Na^+/ K^+ ATPase activity (Jorgensen , 1974)

The specific activity of Na^+/ K^+ ATPase was measured as the amount of inorganic phosphate released from the hydrolysis of ATP per minute per mg protein. The

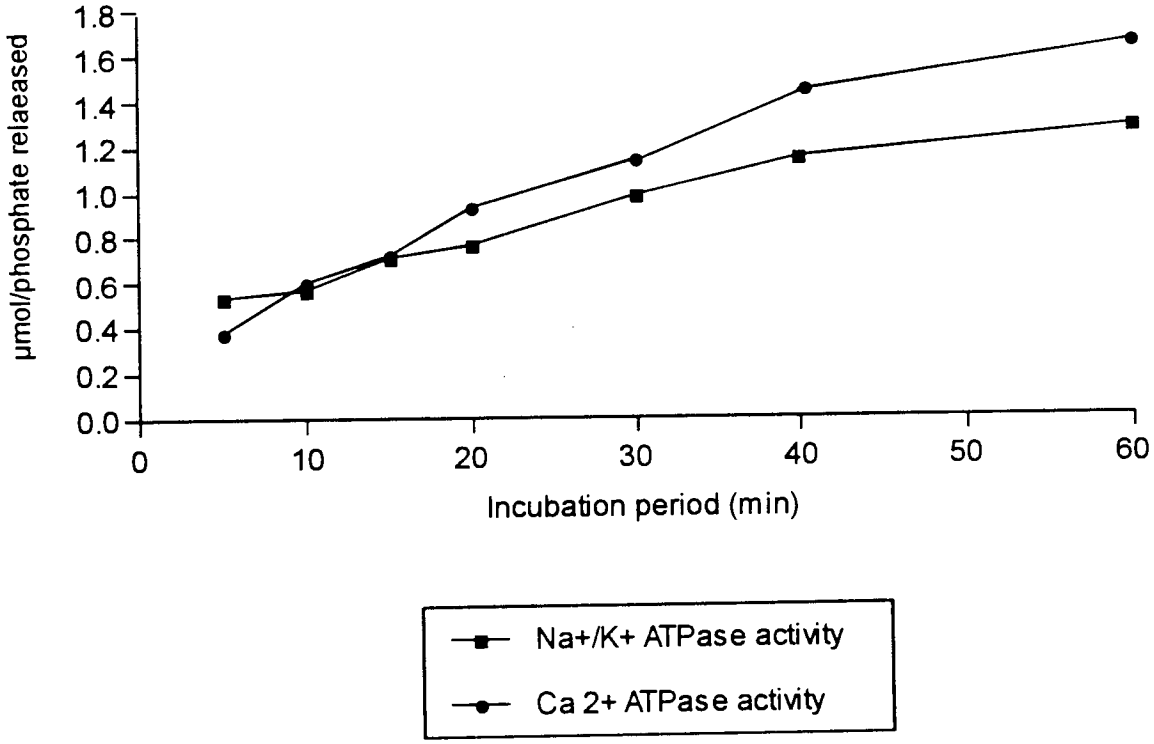


Figure 38. Linearity of inorganic phosphate production over time by Na+/K+ ATPase and Ca2+ ATPase present in kidney microsomal fractions.

incubation mixture contained 104 mM NaCl, 16mM KCl, 2.4 mM $MgCl_2$, 24 mM histidine, and 3mM ATP (magnesium salt), and in the presence or absence of 1 mM ouabain. Portions equivalent to approximately 50 µg of total protein of the previously prepared tissue fractions were added to the incubation mixture and the reaction was started by the addition of ATP. The reaction mixture was incubated for 15 min at 37 °C, in a shaking water bath. At the end of the incubation period the reaction was stopped by the addition of 0.4 ml of ice-cold 35% TCA. The mixture was then centrifuged at 10 000G, for 10 min, at 4 °C. Aliquots of the supernatant (0.5ml) were then removed for the measurement of inorganic phosphate content by the method of S.B.Chahwala (1981), (after Ueda and Wada, 1970).

6.24 Measurement of inorganic phosphate concentration.

The method quantifies the production of a molybdophosphate coloured complex.

Reagents

1. Ammonium molybdate solution: 1g ammonium molybdate tetrahydrate was dissolved in 90 ml distilled water, 1 ml of ammonium hydroxide (sp.gr. 0.90), and made up to 100ml.

2. Ammonium metavandate solution: 0.235g of ammonium metavandate was dissolved in 40 ml of hot distilled water, and allowed to cool. 0.62 ml of conc. HNO_3 was added and the solution was made up to 100 ml with distilled water.

3. Colour reagent: 100ml molybdate solution was combined with 100ml metavandate solution and 50 ml conc. HCl added. The solution was made up to 500ml and used on the day of preparation.

4.10 % w/v Trichloroacetic acid in distilled water

5. Butan-2-ol SLR.

6. Methanol SLR.

7. Standard phosphate solution: 0.4381g/l KH_2PO_4 = 1 mg/ml inorganic phosphate.

Experimental Protocol

To 0.5 ml of sample from the incubation mixture 0.2 ml 10 % TCA solution, 2ml colour reagent, and 4 ml butan-2-ol were added sequentially and vortexed thoroughly. The 2 phases were then allowed to separate, at room temperature, for 10 minutes. 3ml of the upper phase was then removed and added to another tube containing 0.2ml methanol. The mixture was then thoroughly mixed and read on a spectrophotometer at 319 nm, against a similarly prepared blank, in quartz cuvettes. A standard curve was produced using aliquots of the standard phosphate solution (range 2.5-15 µg/ml) and the method described above.

In the presence of 5mM gentamicin and 10 mM neomycin the calibration curve for inorganic phosphate showed a decrease in absorbance at 319 nm at all concentrations of phosphate used when read against blanks prepared without aminoglycoside present, and compared to absorbances of calibration curves prepared entirely in the absence of aminoglycoside. The effect of the presence of aminoglycoside was to produce a proportional decrease in absorbance for each concentration of phosphate investigated. Therefore when measured against a blank without aminoglycoside the concentration of phosphate would be underestimated. When aminoglycosides were also incorporated into the blank samples calibration curves could be superimposed with those prepared in the absence of the drugs. These experiments demonstrated the importance of preparing blanks for each sample containing the same concentration of aminoglycoside as in the test samples, so that the concentration of phosphate generated by the ATPases in the sample could be accurately determined.

6.25 Experimental Design

Experiments were conducted to investigate the effects of aminoglycoside exposure, both *in vitro* and *in vivo*, on the specific activity of Na^+/ K^+ ATPase and Ca^{2+} ATPase from renal tissue.

***In vitro* Experiments:** The effect on the specific activities of Na^+/ K^+ ATPase and Ca^{2+} ATPase of varying concentrations of gentamicin and neomycin added to the incubation mixture was assessed by measurement of phosphate production from the incubation mixtures, in the presence or absence of aminoglycoside. A final concentration of 1-5 mM gentamicin or neomycin was achieved in the incubation mixture by dilution of a stock solution of the aminoglycoside which had been adjusted to pH 7.4 using KOH. Microsomes were incubated for 30 min in the presence of the aminoglycoside and the reaction stopped by the addition of ice-cold TCA. Samples for the measurement of inorganic phosphate were taken and assayed as described in Section 6.24.

The effect of using magnesium in place of calcium as the activating ion for Ca^{2+} ATPase in the presence of inhibitory concentrations of neomycin was also investigated. A range of magnesium concentrations were added to the incubation mixture in place of $CaCl_2$ to select a concentration of $MgCl_2$ which maximally stimulated the Ca^{2+} ATPase present compared to the Ca^{2+} originally present (Figure 39).

***In vivo* Experiments:** Male SD rats were injected IP with either gentamicin sulphate at a dose of 100 mg gentamicin base/kg, or sterile distilled water, at a dose volume of 1 ml/kg. After a range of time intervals (30 min-24 hours) the animals were killed by cervical dislocation, the kidneys removed, and microsomal fractions prepared as detailed in Section 6.21. The specific activities of Ca^{2+} ATPase and Na^+/ K^+ ATPase were determined concurrently. Inhibition of renal Na^+/ K^+ ATPase by gentamicin has been documented in various preparations and was measured in these studies as a positive control (Williams et al, 1981, Cronin et al, 1982).

Additional experiments were also undertaken where SD rats were dosed with 100 mg gentamicin base/kg on Day 1 followed by a second dose on Day 2. In these experiments the animals were killed at short intervals after the second injection (30 min-4 h later). Microsomal fractions were then prepared and Ca^{2+} ATPase activities measured.(n=2 for each time point and experimental condition)

In order to verify that the effects observed on the renal ATPases were not strain-specific a number of experiments were repeated using the F344 rat strain. F344 rats were dosed with 100 mg base/kg on Day 1, followed by a second dose on Day 2. The animals were killed 4 or 24 hours after the second dose and renal ATPase activities measured. (n=2 for each time point)

6.4 Results

***In vitro* Experiments:** Incubation of microsomal fractions of renal tissue in the presence of aminoglycosides resulted in concentration-dependent inhibition of the specific activities of both Ca^{2+} ATPase and Na^{+}/ K^{+} ATPase (Figure 39). Gentamicin and neomycin both inhibited the activity of Ca2+ ATPase to the same extent at a given concentration. There appeared to be no difference in the extent of the toxic effect of these two compounds on the enzymes in these studies.

The addition of 5mM $MgCl_2$ to the incubation mixture produced the same activity of the Ca2+ ATPase as did the original activating ion, $CaCl_2$ at a concentration of 4 mM. Further increases in $MgCl_2$ concentration did not increase the specific activity of the Ca2+ ATPase present in the samples (Figure 40). Substitution of 4 mM $CaCl_2$ for 5 mM $MgCl_2$ in the incubation mixture did not afford any protection from neomycin-induced inhibition of Ca^{2+} ATPase activity. Inhibition of Ca^{2+} ATPase activity after incubation in the presence of $MgCl_2$ and 5 mM neomycin could be demonstrated in both homogenate and microsomal preparations to the same extent as seen when Ca^{2+} was

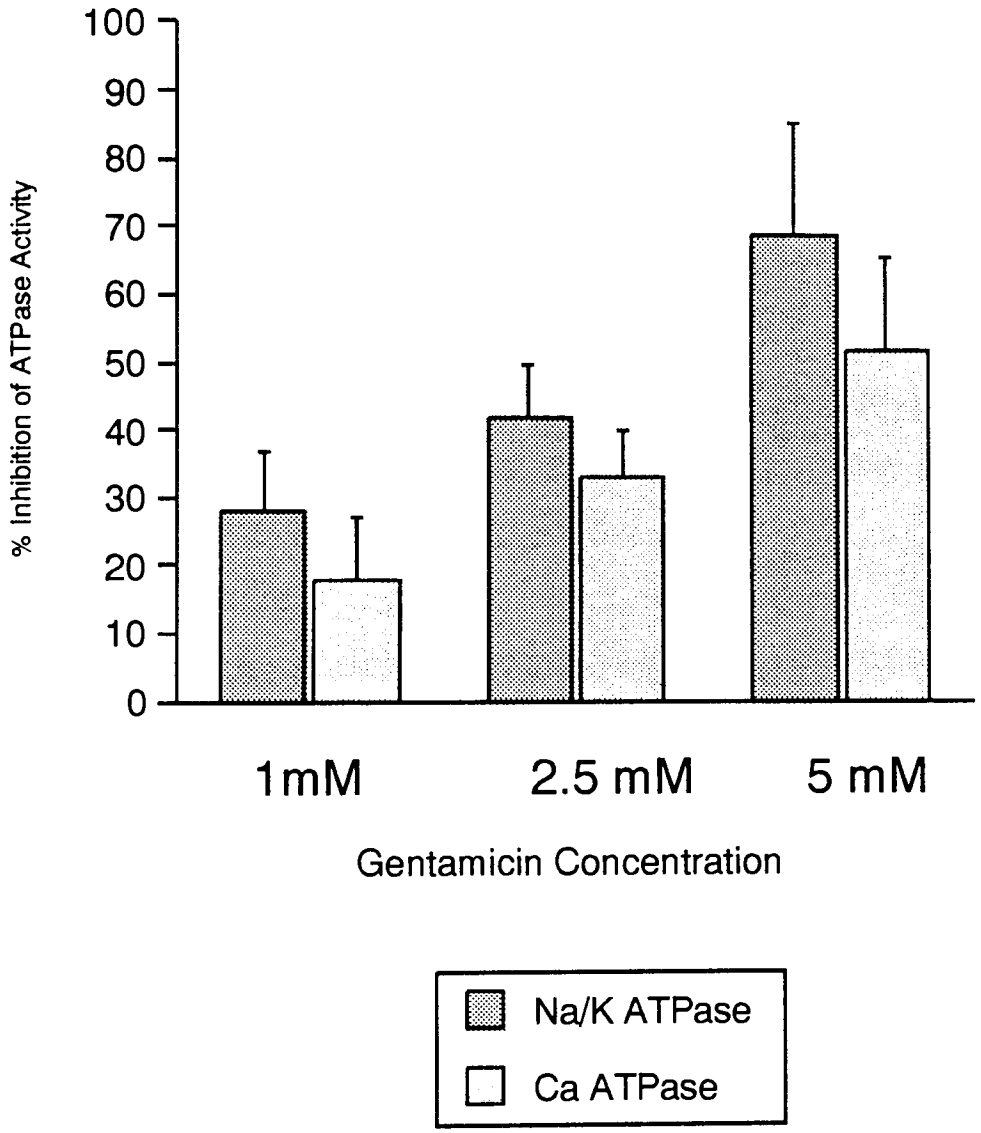


Figure 39. Effect of in vitro gentamicin exposure on Na+/K+ ATPase and Ca 2+ ATPase activity in whole kidney microsomal kidney fractions.Data are shown as mean ± SEM. n = 3.

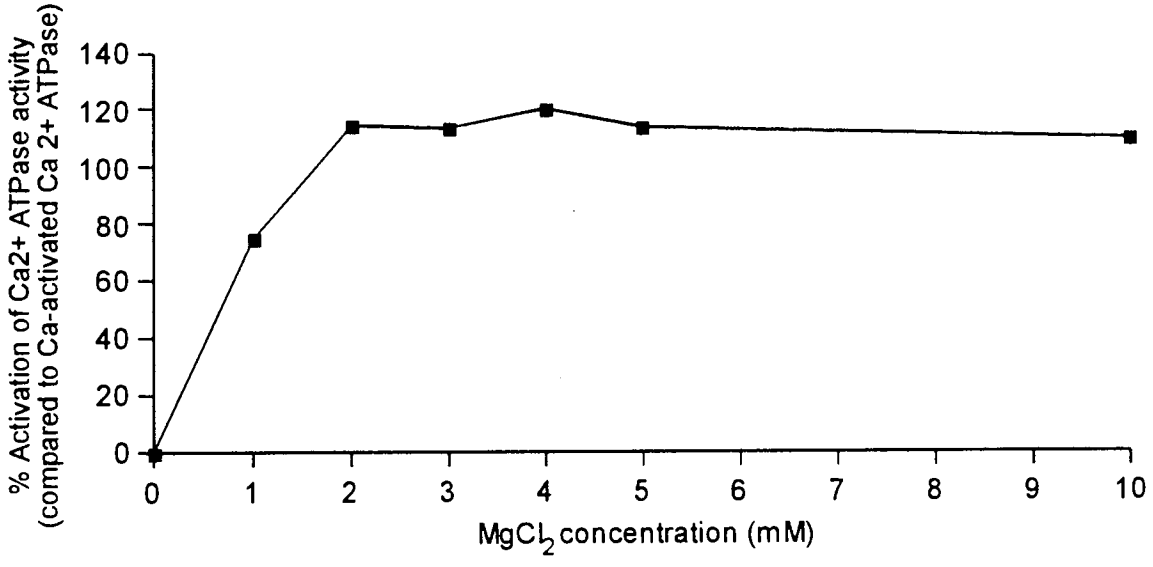


Figure 40. Effect of increasing concentrations of magnesium chloride on Ca 2+ ATPase activity.

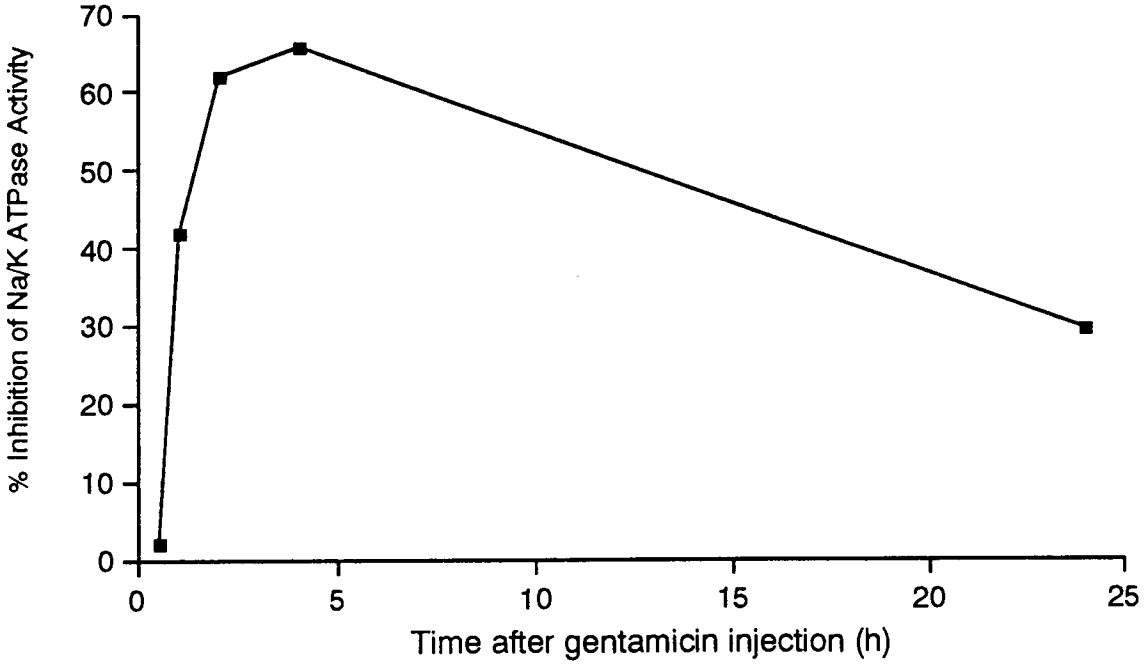


Figure 41. Effect of in vivo exposure to gentamicin on Na+/K+ ATPase activity in whole kidney microsomal fractions. n = 2 at each time point.

used as the activating ion (62 ± 6% inhibition for magnesium-activated Ca2+ ATPase compared to 64 ± 5% for calcium activated Ca^{2+} ATPase).

***In vivo* Experiments:** Inhibition of renal Na^+/ K^+ ATPase was apparent as early as 1 hour after a single injection of 100 mg gentamicin base/kg in SD rats (42% inhibition). Maximal inhibition of the enzyme appeared to be reached 4 h after the injection (67% inhibition) (Figure 41). The magnitude of gentamicin-induced inhibition of Na^+/ K^+ ATPase then declined over the following 20 hours but was still reduced compared to control animals 24 hours after the injection of gentamicin (31 % inhibition).

Despite the inhibition of Na^+/ K^+ ATPase activity following gentamicin dosing there was no concurrent effect of gentamicin dosing on Ca^{2+} ATPase activity at any time point studied, compared to controls. The mean specific activity over all treatment conditions of Ca^{2+} ATPase in control and drug treated animals was not significantly different (0.164 ± 0.02 cf. 0.168 ± 0.02 µmol inorganic phosphate produced/min/mg protein).

Experiments using SD rats given two doses of gentamicin and killed at short intervals (30 min-4h) after the second dose of gentamicin also failed to reveal any effects of gentamicin administration *in vivo* on Ca^{2+} ATPase activity. Na^+/ K^+ ATPase activity was not measured in these experiments.

Further experiments in which F344 rats were given two doses of gentamicin (100 mg base/kg) and killed 4h and 24 h after the second injection also failed to produce any effects on the specific activity of Ca^{2+} ATPase. Na^+/ K^+ ATPase activities were not measured in these experiments. There was no effect of gentamicin administration on the specific activity of Ca^{2+} ATPase in F344 rats. The specific activity of Ca2+ ATPase was not different between F344 and SD rat strains (F344 0.147 ± 0.04 compared to SD 0.146 ± 0.01 µmol inorganic phosphate produced/min/mg protein).

Table 9. Effect of injection of 100 mg/kg gentamicin base, each day for two days, on the specific activity of Ca2+ ATPase in F344 rats. Values are expressed as μmol inorganic phosphate produced/min/mg protein. n = 2 independent experiments for each experimental condition.

Sampling Time	Drug-Treated	Control
4h after last injection	0.155, 0.193	0.152, 0.145
24h after last injection	0.142, 0.187	0.118, 0.205

6.5 Discussion

The data presented in this chapter clearly demonstrate that Na^+/ K^+ ATPase and Ca2+ ATPase may both be inhibited by the action of aminoglycosides when exposed *in vitro*. The specific activities of both enzymes are inhibited by a similar amount for a given aminoglycoside concentration. The inhibition of Ca^{2+} ATPase and Na^+/ K^+ ATPase activity could however, only be achieved after exposure to millimolar concentrations of the drugs.

Marked differences between the actions of gentamicin on Na^+/ K^+ ATPase and Ca^{2+} ATPase are evident however, after *in vivo* dosing. After *in vivo* administration of gentamicin substantial inhibition of Na^+/ K^+ ATPase was already visible 1 hour after dosing. In other studies in which a similar protocol was used inhibition of Na^+/ K^+ ATPase was apparent as early as 30 minutes after a single dose of 100 mg/kg gentamicin (Williams et al, 1981). At no time point measured in these studies was Ca^{2+} ATPase inhibited after *in vivo* dosing with gentamicin. The lack of effect of gentamicin administration on Ca^{2+} ATPase activity is not specific to the SD strain but is also apparent in the F344 rat strain.

Several reports describe the importance of access to the cytoplasmic face of Na^+/ K^+ ATPase for aminoglycosides to achieve their inhibitory effects (Aramaki et al, 1986,

Fukuda et al, 1990, Williams et al, 1984, Lipsky and Lietman, 1980). If the requirement for this interaction to take place at the cytoplasmic face of the enzyme exists, it may go some way toward explaining the specific localisation of aminoglycoside toxicity, in that intracellular accumulation of the compounds is required for inhibition of Na^+/ K^+ ATPase activity. Since accumulation of aminoglycosides has been described only in relatively few locations (proximal tubular cells of the kidney, tissues of the inner ear) it appears that a correlation may exist between cellular uptake of the drug, inhibition of the Na^+/ K^+ ATPase activity and subsequent toxic injury. Evidence from the studies of Fukuda et al (1990), supports this suggestion since inhibition of Na^+/ K^+ ATPase was evident in proximal tubular segments, where gentamicin is accumulated, but not in medullary thick ascending limbs of the Loop of Henle, after *in vivo* administration of gentamicin. However similar inhibition of Na^+/ K^+ ATPase activity was produced in both of these nephron segments after *in vitro* permeabilisation and exposure to gentamicin. Since the activity of Na^+/K^+ ATPase provides the major driving force for all sodium-coupled and sodium-dependent transepithelial transport, inhibition of this crucially important enzyme may significantly disrupt cellular ionic composition and homeostasis. Indeed, it has been proposed that changes in the ionic composition of the renal cortex subsequent to inhibition of Na^+/K^+ ATPase are important factors in the production of aminoglycoside nephrotoxicity (Cronin et al, 1982).

In the *in vivo* studies presented here there was no evidence for concurrent inhibition of Ca2+ ATPase at any time after *in vivo* gentamicin exposure. In explanation of these data it is tempting to speculate that whilst the intracellular concentration of aminoglycoside is the critical event which determines the inhibitory effect of gentamicin on Na^+/ K^+ ATPase activity, it may be the extracellular concentration of the drug which is important to produce inhibition of Ca2+ ATPase activity i.e. that aminoglycosides need to interact with the extracellular face of the enzyme. Since the concentrations achieved in the blood are unlikely to approach those required to achieve inhibition of the enzyme *in vitro* this may explain the apparently selective effect of aminoglycosides on Na^+/ K^+ ATPase

activity and not Ca2+ ATPase. Whether the action of the drugs is to directly inhibit the enzyme, or whether the decrease in enzyme activity is a consequence of drug-induced alterations in the phospholipid environment of the membrane remains unclear. However, alterations in the phospholipid content of the membrane might well be expected to similarly affect both enzymes; which may therefore indicate that aminoglycosides inhibit each enzyme directly, but via different mechanisms.

However, inhibition of Na^+/ K^+ ATPase may in itself have profound effects on the renal handling of calcium since calcium transport is largely dependent on sodium flux generated electrochemical gradients. At a cellular level, the intracellular calcium concentration has been postulated as a means of feedback to regulate Na^+ transport across the cell. The presence of both Na^+/K^+ ATPase and Na/Ca exchange mechanisms within a particular cell allows interdependent regulation of transepithelial transport for both calcium and sodium. Evidence from micro-perfusion studies suggests that calcium and sodium transport are indeed closely linked. Calcium reabsorption in proximal tubules is abolished when active sodium transport is blocked by ouabain (Ullrich et al, 1976). Removal of Na^+ or addition of ouabain to the perfusate inhibited calcium transport, while increasing calcium concentrations inhibited Na^+/ K^+ ATPase activity (Gmaj et al, 1979). Ouabain induces a reduction in the rate of sodium pumping and a consequent rise in intracellular Na^+ concentration and hence a decrease in the gradient favouring sodium entry across the basolateral cell membrane. This in turn leads to reduced Na^+/Ca^+ exchange across that membrane and thereby reduced calcium extrusion out of the cell. Where Na^+/ K^+ ATPase activity is inhibited by increased intracellular calcium concentrations, the gradient across the cell for the efflux of calcium via the co-transporter is also reduced, leading to a decrease in calcium reabsorption as a consequence of a reduction in Na^+/ K^+ ATPase activity. The net effect of a reduction of Na^+/ K^+ ATPase activity may therefore be to reduce net reabsorption of both sodium and calcium across the epithelium. (Taylor and Windhager, 1979).

Another compound, cis-platinum, which can produce renal wasting of calcium and magnesium after administration also produces interesting effects on Ca^{2+} ATPase and Na^{+}/ K^{+} ATPase from renal tissue. In experimental studies which examined the effects of cis-platinum on renal ATPase activity after *in vitro* exposure, Ca2+ ATPase activity was inhibited to the same extent as was Na^{+}/ K^{+} ATPase (Daley-Yates and McBrien, 1982, Nechay and Neldon, 1984), and required almost millimolar amounts to achieve 50 % inhibition of enzyme activity. After administration of 5-10mg/kg cis-platinum *in vivo* there were no effects on total ATPase activity when measured at short intervals after dosing (1 and 6 hours post-dose). In contrast, five days after dosing with cis-platinum a dose-related inhibition of both Na^{+}/K^{+} ATPase and Ca2+ ATPase activity was apparent. This probably does not represent a specific inhibition of the enzyme by cis-platinum but is more likely to be a reflection of generalised tissue damage and disruption, and a secondary effect of the compound, since BUN concentrations were elevated in these animals. (Uozumi and Litterst, 1985). Interestingly, renal tissue concentrations of Pt were also measured in the studies of Uozumi and Litterst (1985), and found to be less than 100 μM; a concentration which produced less than 50% inhibition of the renal ATPases after 4h exposure *in vitro*. These data therefore suggest a similar conclusion to that produced from the studies presented here, in which aminoglycosides inhibited Na^{+}/ K^{+} ATPase and Ca^{2+} ATPase activity, namely that whilst renal ATPases can be inhibited *in vitro* by the action of these compounds the concentrations required to do so are unlikely to be achieved *in vivo*, and therefore may not represent a primary toxic mechanism of the drugs.

These data therefore suggest several interesting toxicological insights into the phenomenon of aminoglycoside-induced renal injury, and defects in renal electrolyte reabsorption. Firstly that two enzymes responsible for calcium transport can be inhibited by the action of aminoglycosides when exposed *in vitro*, at sufficiently high concentrations. Secondly, after exposure to the drugs *in vivo* a selective inhibitory effect on Na^{+}/ K^{+} ATPase activity was visible, which was not apparent on the activity of Ca2+

ATPase. These data suggest that inhibition of Ca2+ ATPase activity does not play a role in the reduction in calcium reabsorption which can be produced after aminoglycoside administration, but inhibition of Na^+/ K^+ ATPase activity may in itself lead to a reduction in calcium reabsorption by indirect means. In addition to possible effects of Na^+/ K^+ ATPase inhibition on renal calcium reabsorption, derangements in intracellular ion concentration as a consequence of Na^+/ K^+ ATPase inhibition may lead to other toxic manifestations of aminoglycoside-induced renal injury.

Whilst the studies presented here confirm that Na^+/ K^+ ATPase present in renal tissue can be inhibited after in vivo exposure to gentamicin, without apparent effects on Ca2+ ATPase, they do not identify the site at which this inhibition occurs. In addition, there exists the possibility that a small, possibly localised, inhibition of Ca^{2+} ATPase is occurring which is masked by using whole kidney preparations in these studies. Therefore further studies were undertaken to investigate these possibilities and are presented in Chapter 7.

Chapter 7. Application of histochemical methods to investigate the possible sites of aminoglycoside-induced inhibition of renal Na+/K+ ATPase and Ca2+ ATPase .

7.1 Introduction

In 1957 Wachstein and Meisel published the first report describing the histochemical localisation of phosphatase activity. In their method, ATP was used as a substrate for the enzyme and lead nitrate was used as a 'capture agent' for the phosphate ions liberated by the enzymatic hydrolysis of ATP. The 'captured' phosphate ions then precipitated on, or near to, the site of enzyme activity as water-soluble lead phosphate. While this original method was later found to be imperfect, further methodological development continued to improve the technique and extend its use not only for Na^+/K^+ ATPase staining but also for staining of other phosphatases such as Ca2+ ATPase. The precipitation of lead salts at the site of enzyme activity therefore became the basis of the majority of staining methods for these enzymes.

Since inhibition of Na^+/K^+ ATPase was demonstrated in homogenates and microsomal fractions prepared from whole kidney in Chapter 6, the aim of the studies presented in this chapter was to investigate the site along the nephron at which Na^+/K^+ ATPase activity was inhibited. In addition, since tissue fractions obtained from whole kidneys were used in the biochemical studies, the question arose as to whether small, localised reductions in enzyme activity of Ca2+ ATPase had been masked by studying that type of preparation. Rather than trying to produce pure populations of tubules from different regions of the kidney it was decided to evaluate methods which allowed assessment of the enzyme activity of a range of tubules simultaneously i.e. across a slice or section of the kidney. The effects of acute and sub-chronic dosing with gentamicin on the activity of renal Ca2+ ATPase and Na^+/K^+ ATPase were therefore evaluated using histochemical staining of the enzymes.

7.2 Methods

7.21 Preparation of frozen kidney sections.

Rats were killed by an overdose of barbiturate (Sagatal) and the kidneys removed. The kidneys were decapsulated and sliced, transversely, into portions approximately 3-4 mm thick. These samples were then attached onto small, labelled, squares of cork using OCT embedding medium (Raymond Lamb, UK.). The whole assembly was then placed in isopentane (SLR) cooled over liquid nitrogen, at approximately -150 °C, until thoroughly frozen (about 15-20 sec). The kidney portions were stored in individual polythene bags containing silica gel, at -70 °C until required. Frozen sections were stored for up to three weeks before use.

Prior to use the fresh-frozen or stored-frozen material was brought to approximately -20 °C in an ordinary freezer, or in the cryostat cabinet. Tissue for sectioning was attached to the cryostat chuck using OCT and cryojet (BDH). The blocks were roughly trimmed to obtain the full block face before sections were cut at 6 μM for most experimental purposes. Sections were dried onto the slides for 20 min at room temperature prior to use or prior to storage.

If sections were destined to be frozen for subsequent use, slides were wrapped back-to-back in clingfilm and stored with silica gel as desiccant at -70 °C. Slides were allowed to return to room temperature (still wrapped) for 15 min before opening out and air-drying for a further 10-15 min before use in experiments. Frozen sections were used within 3 weeks of preparation.

7.22 Method evaluation and development for staining of Na +/K+ ATPase activity in kidney sections.

Initial studies: In preliminary experiments the indirect method proposed by Firth et al (1987), for histochemical staining of Na^+/ K^+ ATPase was evaluated. In this context, 'indirect' refers to the formation of the reaction product which can be visualised. In the indirect method the initial product of the enzyme reaction is converted to insoluble lead

sulphide by a second reaction step; hence the formation of the final reaction product is 'indirect'. This method was chosen for evaluation since a comparative study by Firth et al (1987) indicated that the non-enzymatic background staining was reduced and the deposition of the reaction product was greater using this technique than using direct lead capture methods. However, several attempts to reproduce staining of the enzyme by this method failed to produce any specific staining, apparently due to the failure of the 'capture agent' ($CoCl_2$) to hold onto the liberated phosphate product. The indirect method showed that the enzyme substrate had been hydrolysed to its yellow product by the action of Na^+/ K^+ ATPase, but this product did not remain attached to the sections and came off into the staining bath. The resulting staining was pale and apparently not specific to any site in the kidney. Light fixation with Karnovsky's fixative (2.5 % glutaraldehyde, 2% formaldehyde in 0.1M phosphate buffer at pH 7.4), altering incubation temperature, or the incubation period did not improve the result. Attention therefore turned to another method of staining for renal Na^+/ K^+ ATPase.

Background to methods using p-nitrophenyl phosphate as a substrate for Na^+/ K^+ ATPase staining.

Early histochemical studies using ATP as substrate for Na^+/ K^+ ATPase activity proved to be unreliable and subject to high levels of non-specific background staining (Jacobsen and Jorgensen, 1969) leading to the commonly accepted view that there is no currently available histochemical method to determine Na^+/ K^+ ATPase activity using ATP as substrate for the enzyme (Ernst and Hootman, 1981, Firth, 1978). In 1972 (a,b), Ernst first developed a cytochemical method to localise Na^+/ K^+ ATPase using p-nitrophenyl phosphate (NPP) as a substrate in place of ATP. Since NPP is not an intrinsic substrate for Na^+/ K^+ ATPase various conditions must be satisfied to substantiate the specificity of the method for Na^+/ K^+ ATPase:

- Inhibition of the enzyme reaction by addition of ouabain to the incubation mixture.

- Demonstration of alkaline phosphatase activity, and its inhibition by an appropriate inhibitor such as levamisole since NPP can also be a substrate of alkaline phosphatase.

- Demonstration that removal of the substrate (NPP) causes a loss of precipitation of the product, to compare the sites at which non-enzymatic precipitation occurs with that produced by enzymatic hydrolysis of the substrate.

The one-step lead citrate method proposed by Mayahara et al (1980), uses lead citrate for the direct capture of the liberated phosphate ions from NPP, 25% DMSO as an activator of the enzyme, and a glycine buffer to act as a stabiliser of the incubation medium. The use of DMSO in the incubation mixture amplifies the potassium sensitive-NPPase activity and shifts the pH optimum to approximately pH 9.0 (Albers and Koval, 1972) at which the incubation is carried out, increasing the sensitivity of the method several-fold. The use of a glycine buffer in this method creates a very fine reaction product due to the chelation of lead by glycine; in addition, use of glycine in the incubation mixture also reportedly enhances Na^{+}/ K^{+} ATPase activity by chelation of heavy metal ions (Spechet and Robinson, 1973). The method of Mayahara et al (1980) describes the use of fixed material for subsequent staining purposes; use of lightly fixed material in preliminary experiments resulted in precipitation of the incubation solution, therefore unfixed material was used in all experiments described in this chapter.

Evaluation and adaptation of the method of Mayahara et al (1980)

Frozen sections 6 (μm) were incubated either individually with the incubation solution flooded onto the slide or in racks using staining troughs, depending on the number of sections processed at a particular time. The published method recommends incubation for 15 min at room temperature for staining of Na+/K+ ATPase in kidney sections. Incubations of samples at 37 °C and for varying periods of time were carried out to verify these incubation conditions (Figure 42a-d). After incubation the sections were drained, washed twice in distilled water, and developed in 1% v/v ammonium sulphide

solution for 1 minute. After developing, the sections were rinsed once in distilled water and mounted under coverslips using Aquamount (BDH). There were no differences in staining intensity between sections incubated at room temperature and at 37 °C for a given section thickness and incubation period. The intensity of staining was dependent on the thickness of the section; it was important for comparative purposes that the section was cut to a completely even thickness. Staining intensity was also clearly dependent on the incubation time; increasing the incubation period to 30 minutes or more increased the intensity of background staining to an unacceptable level. Duplicate sections were prepared for each experimental condition for all experiments.

Incubation Solution

The preparation of the incubation mixture used for these experiments is set out below, in detail, because the methods used are critical for the successful manufacture of the incubation mixture. The preparation of a usable incubation mixture is very sensitive to both the order and method of addition of some of its component parts. The reagents must be added to each other in top-to-bottom order shown. The resulting solution is a clear pale yellow solution which is stable for several hours. Additional information regarding the preparation of this solution is detailed in footnotes below.

Reagents	Volume	Final Concentration
1.0 M Glycine-KOH buffer[1] pH 9.0	2.5 ml	250 mM Glycine; 25 mM K^+
1% Lead citrate[2]	4.0 ml	4.0 mM lead citrate; 20 mM K^+
Dimethyl Sulphoxide (DMSO)[3]	2.5 ml	25% v/v
0.1 M NPP sodium salt + 10 mM $MgCl_2$[4]	1.0 ml	10 mM NPP; 10 mM $MgCl_2$
Levamisole[5]	6 mg	2.5 mM
Final Volume (pH 8.8)	10 ml	

[1]Use 5.0 M KOH to prepare the buffer

[2]Dissolve in 50 mM freshly prepared KOH with minimal dissolution. If the solution is stirred for too long or too vigorously, a lead salt (probably lead carbonate) precipitates out of solution. Once prepared this solution should be added gradually to the glycine buffer, with gentle stirring.

[3] The DMSO *must* be added *dropwise* to a slowly stirring mixture as above. Do not allow the temperature of the solution to rise more than body temerature (judge by hand) otherwise precipitation will occur.

[4]Dissolve the $MgCl_2$ in distilled water before adding the NPP. Dissolve just before addition to the main solution; add *dropwise* with gentle stirring. The magnesium salt of NPP is poorly soluble in water; hence it is easier to use the sodium-salt which dissolves readily and add additional $MgCl_2$.

[5]The levamisole should be added gradually to a more actively stirring solution until dissolved. 5mM Ouabain, if used, can be added after the levamisole in a similar manner but it is less soluble. Excessive stirring of the solution should be avoided since it may encourage precipitation to occur.

7.23 Method evaluation and development for histochemical staining of Ca2+ ATPase activity in kidney sections.

Background

A one-step method for staining Ca2+ ATPase described by Ando et al (1981) was evaluated for use with frozen kidney sections (prepared as detailed in section 7.21.).This technique uses lead citrate as the capture agent for phosphate liberated by the hydrolysis of ATP by Ca2+ ATPase activity. Since this method essentially measures the same end-point as that of the method of Mayahara (1980), for Na^+/K^+ ATPase activity, the incubation conditions need to address the problems of proving that the method demonstrates the presence of Ca2+ ATPase activity.

- The method needs to demonstrate dependence on the presence of substrate to distinguish the staining from that achieved by non-enzymatic hydrolysis of the substrate.
- The method should demonstrate that the activity is not inhibitable by classical inhibitors of other phosphatases present in the kidney such as alkaline phosphatase, or Na^+/K^+ ATPase.
- The method should show that the activity can be inhibited by Ca2+ ATPase inhibitors, or by the removal of Ca2+ from the incubation medium.

Adaptation of the method of Ando et al (1981) for the staining of Ca^{2+} ATPase activity.

The manufacture of the incubation solution proved to be only slightly less problematic than producing the incubation solution for Na^+/K^+ ATPase staining. The original method of Ando et al (1981), describes the use of a lead citrate solution produced by dissolving 1% lead citrate in 1N NaOH or 1N KOH with shaking. Despite numerous attempts to achieve this the lead precipitated out of solution and was unusable. Since the

lead solution was destined to be added to other components and made up to a larger volume (see below) it was decided to use the technique developed for Na^+/K^+ ATPase staining and use a larger volume of lower concentration lead citrate solution to make the incubation solution (20 ml of 1% lead citrate solution instead of 5ml of 4% lead citrate solution).

Stock Reagents	Volume	Final Concentration
1 % Lead citrate solution in 50 mM KOH[6]	20 ml	2mM lead citrate
500 mM glycine-KOH buffer pH 9.0	50 ml	250mM glycine
To 100 ml with distilled water		
3 mM ATP, disodium salt		3 mM ATP, disodium salt
10 mM $CaCl_2$		10 mM $CaCl_2$
10 mM Levamisole		10 mM Levamisole
5 mM Ouabain		5 mM Ouabain

The reagents should be added in the top-to-bottom order indicated in the above list, with stirring. It is also imperative that all reagents used are fresh and of analytical grade. Aged or less pure reagents will cause precipitation of the lead out of solution rendering it unusable. The solution is clear, and stable in a stoppered bottle for up to 2 hours.

The original method of Ando (1981), recommended incubation of the sections for 5-30 min at room temperature in the above solution. Initial experiments used a 30 min incubation period for method development purposes which was later reduced to 20 min when experiments showed adequate staining could be achieved after this period.

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[6] Dissolve lead citrate in 50 mM freshly prepared KOH with minimal dissolution. If the solution is stirred for too long or too vigorously, a lead salt (probably lead carbonate) precipitates out of solution. Once prepared this solution should be added gradually to the glycine buffer, with gentle stirring.

After incubation the sections were rinsed once in distilled water, developed in 1% v/v ammonium sulphide for 2 minutes, then rinsed again in distilled water and mounted under a coverslip with Aquamount (BDH).

To optimise the method for Ca2+ ATPase staining and to ensure that the technique was indeed staining the Ca2+ ATPase present in the kidney, the following experiments were carried out.

- The effect of replacing Ca 2+ with Mg 2+ in the incubation solution was examined.
- The effect of removing the activating ion (Ca 2+) from the incubation solution was tested by pre-incubation of the sections in an EDTA containing solution.
- The effects of ouabain and levamisole on the staining were observed.
- Sections were incubated at 37 °C and at room temperature to examine the effect of temperature on staining intensity.
- Sections of varying thickness were examined to assess the effect of section thickness on staining intensity and resolution.

7.24 Effects of sub-chronic dosing with gentamicin on Na^+/K^+ ATPase and Ca2+ ATPase staining.

6 μM sections were cut from kidneys of SD rats used in the dose response study detailed in Chapter 3. Briefly, the rats were dosed with either 0, 50, 75, or 90mg of gentamicin base/kg for 6 days and killed approximately 24 h after the last injection (n=6 for each group). The kidneys were removed immediately, weighed, and a centre portion of one kidney taken for cryopreservation as described previously. The sections were cut and stored at -70 °C for 2-3 weeks before staining.

Duplicate sections were cut from each kidney for staining under normal conditions; in addition another section was cut from each kidney to demonstrate inhibition of staining

by use of appropriate conditions i.e. incubation in the presence of ouabain for Na^+/K^+ ATPase staining, and cation depletion by pre-incubation with EDTA for Ca2+ ATPase staining.

For Na^+/K^+ ATPase staining the sections were incubated at room temperature for 20 minutes then developed in the normal manner as described in Section 7.22. Kidney sections which were stained for Ca2+ ATPase activity were pre-incubated in 10 mM EDTA for 30 minutes followed by two washes in deionised water prior to incubation in the staining solution for 20 minutes at room temperature. Development of the stain was carried out as described in Section 7.23.

7.25 Effects of acute administration of gentamicin on Na^+/ K^+ ATPase and Ca^{2+} ATPase staining.

In addition to staining sections from animals after sub-chronic gentamicin administration a number of sections were prepared from male SD rats after a single dose of 150 mg gentamicin base/kg. The rats were killed 4 hours after the injection of gentamicin and sections prepared for Na^+/K^+ ATPase and Ca2+ ATPase staining. Previous biochemical data had shown that maximal inhibition of Na^+/K^+ ATPase activity was apparent at 4 hours after a single injection of gentamicin (see Chapter 6).

7.26 Effect of incubation time on product intensity in sections from control and gentamicin-dosed rats.

Since the reaction for the staining of both enzymes is time-dependent an experiment was also conducted using tissue from both the control and top-dose group of the dose response study, and tissue from those animals dose acutely with gentamicin. The concern was that so much reaction product was deposited around the tubules that it would mask any differences between drug-treated and control animals which may have existed. Additional experiments were therefore conducted where incubation periods were

reduced to 2 and 5 minutes for the staining of both Na^+/K^+ ATPase and Ca2+ ATPase activity to investigate this possibility.

7.3 Results

The location of the staining visible in the sections is described by region of the kidney, rather than by individual nephron segment since it is difficult, in some instances, to be precise as to the exact segment which stained at a particular site. The following description therefore indicates the regions in which particular nephron segments are located.

When a kidney is bisected longtitudinately the cut surface shows two very distinct regions, a darker outer region, the cortex, and a lighter inner region, the medulla, which is divided by several renal pyramids extending toward the renal pelvis, each eventually forming a papilla. The renal pyramids are striated with medullary rays, which comprise the straight segments of the nephron, the collecting ducts and loops of Henle, along with blood vessels.

The glomeruli, proximal tubules and distal tubules are located in the cortex whereas the loops of Henle and the collecting ducts extend down through the medulla. The length of the loop of Henle depends on the location of that nephron's glomerulus, and two distinct populations can be identified. Nephrons with glomeruli located in the outer two-thirds of the kidney are known as cortical nephrons and have short loops of Henle which may extend only a short way into the medulla, if at all. Nephrons whose glomeruli lie in the inner third of the cortex (juxtamedullary nephrons) have long loops of Henle which extend deep into the medulla. The inner zone of the medulla therefore consists mainly of the collecting ducts, and thin limbs of the loop of Henle. Cortical and juxtamedullary nephrons may have different functional attributes.

7.31 Localisation of Na^+/K^+ ATPase staining within the rat kidney.

Light fixation of the kidney sections with Karnovsky's fixative (diluted 1 in 10; fixed for 2 min) resulted in a precipitate being deposited all over the section. Therefore it was necessary to use only unfixed sections for this staining technique. In the absence of the substrate (NPP) from the incubation mixture the whole section stained a pale, light brown colour which was not localised to any particular area, indicating only a small amount of non-enzymatic staining (figure 43a). In the presence of substrate, tubules in the cortical region of the kidney showed intense luminal staining with additional staining at the basolateral face of the cells. In contrast, tubules in the medullary region of the kidney demonstrated staining only at the basolateral side of the tubule, which was more intense than that seen in cortical tubules. There was no luminal staining of the tubules in the medullary region. Glomeruli were not stained by this technique, indicating no substantial Na^+/K^+ ATPase activity. When levamisole (2.5mM) was added to the incubation solution the luminal staining of the cortical tubules was abolished, without any effect on the intensity of staining seen at the basolateral face of the tubules in both the cortex and medullary regions of the kidney (Figure 45a-d)). The inhibition of the luminal staining in the cortical tubules by levamisole was taken to indicate that this staining represented alkaline phosphatase activity; additionally supported by its exclusive localisation in the cortical region of the kidney. The papillary region of the kidney showed only low intensity staining of the tubules. This staining was unchanged by the addition of ouabain and levamisole and may therefore represent non-specific background staining (possibly of Ca2+ ATPase activity) rather than Na^+/K^+ ATPase staining. Addition of increasing concentrations of $CaCl_2$ to the incubation solution did not however increase the intensity of the background staining. The addition of ouabain to the incubation solution resulted in a lack of specific staining in the cortical region, a massive reduction in the intensity of staining in medullary tubules (compare Figure 44c and 46b) and no discernible change in the staining intensity of the tubules of the papillary region (Figure 46c). There appeared to be some low-intensity residual staining in tubules of the medullary region in the presence of ouabain.

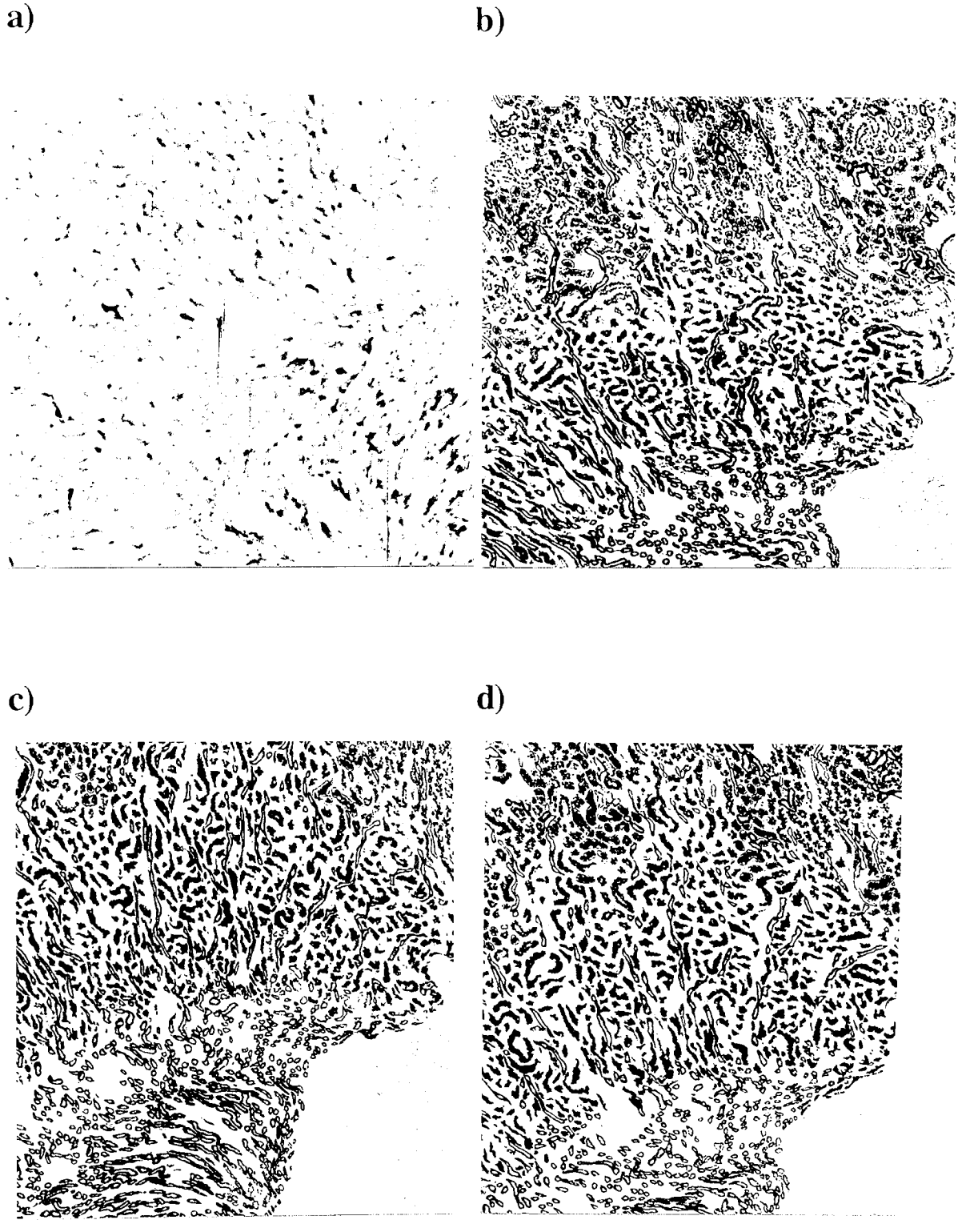
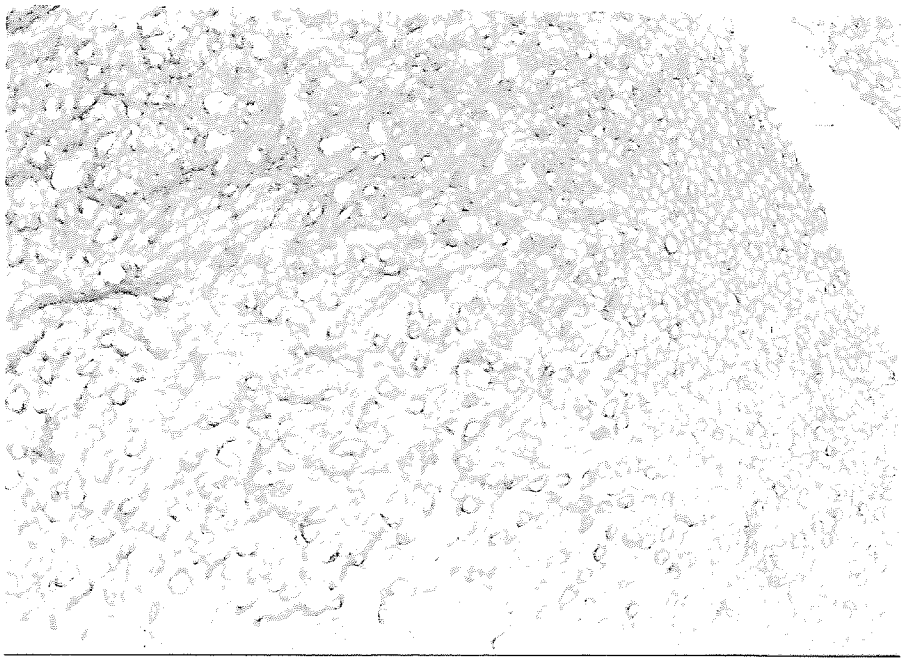


Figure 42. Effect of time on product deposition after staining for Na^+/K^+ ATPase activity **a)** 5 min incubation **b)** 15 min incubation **c)** 30 min incubation **d)** 60 min incubation. (x 30 mag)

a)



b)

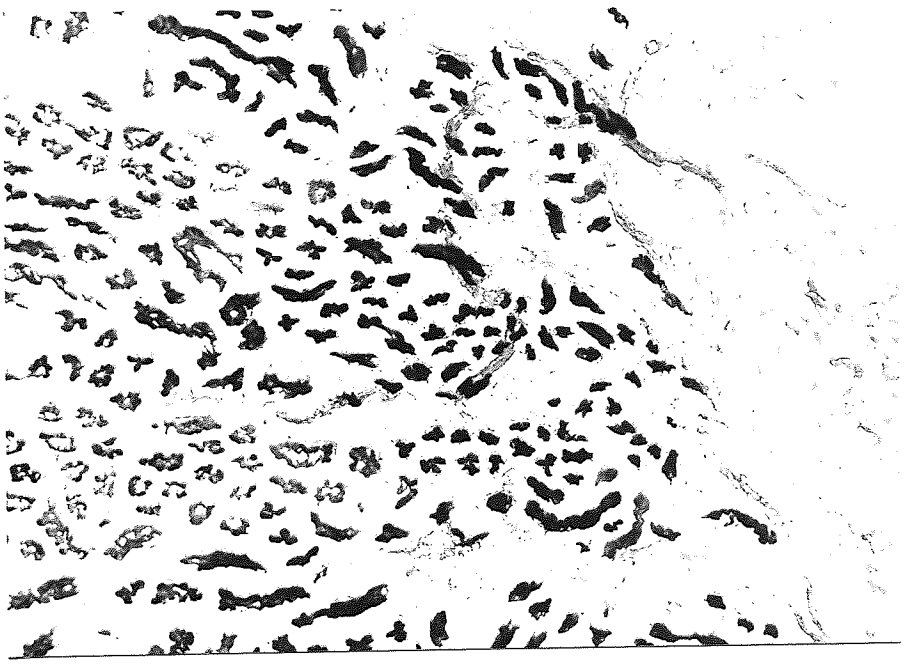
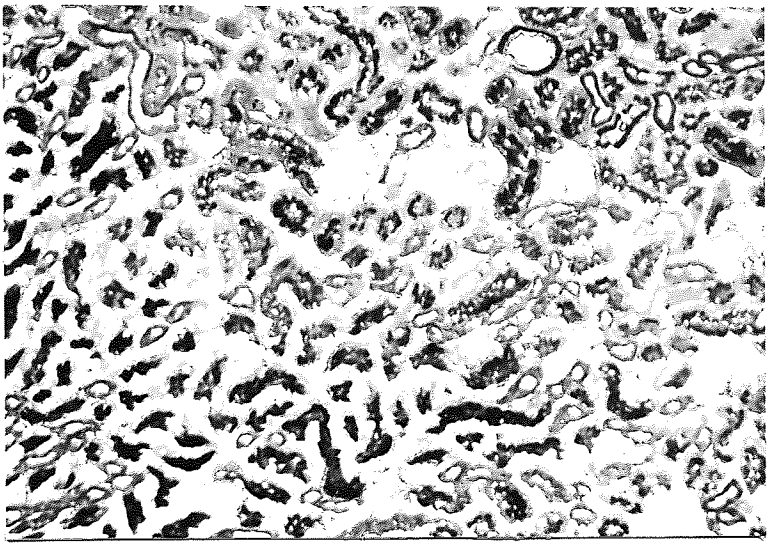
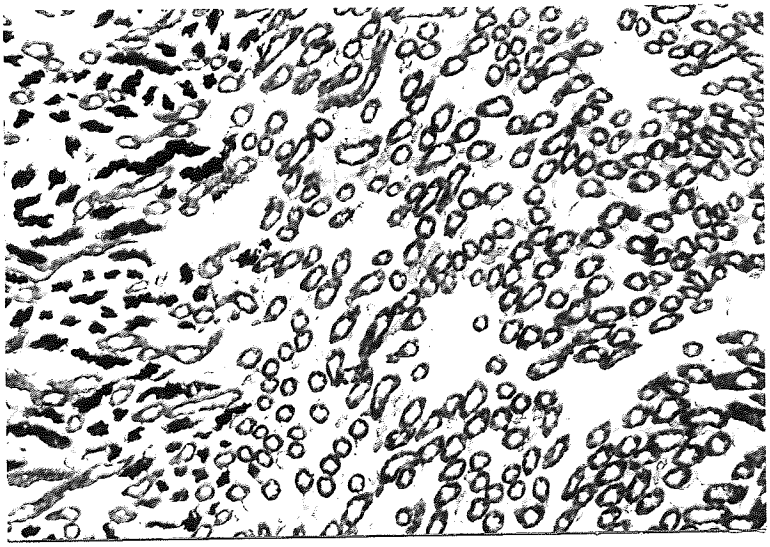


Figure 43. Histochemical localisation of Na^+/K^+ ATPase activity: a) Non-enzymatic hydrolysis in the absence of substrate b) Localisation of alkaline phosphatase activity. (x 90 mag).

a)



b)



c)

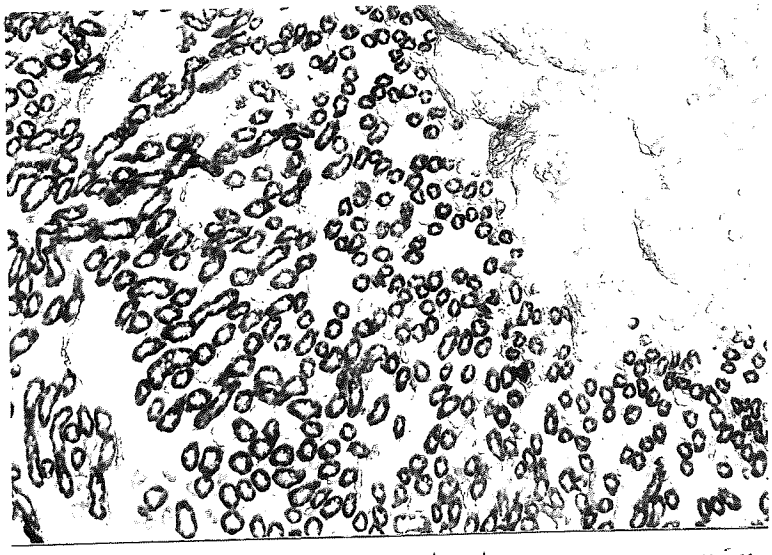


Figure 44. Histochemical localisation of Na^+/K^+ ATPase and alkaline phosphatase activity a) cortical region b) cortico-medullary junction c) medullary-papilla junction. (x 75 mag)

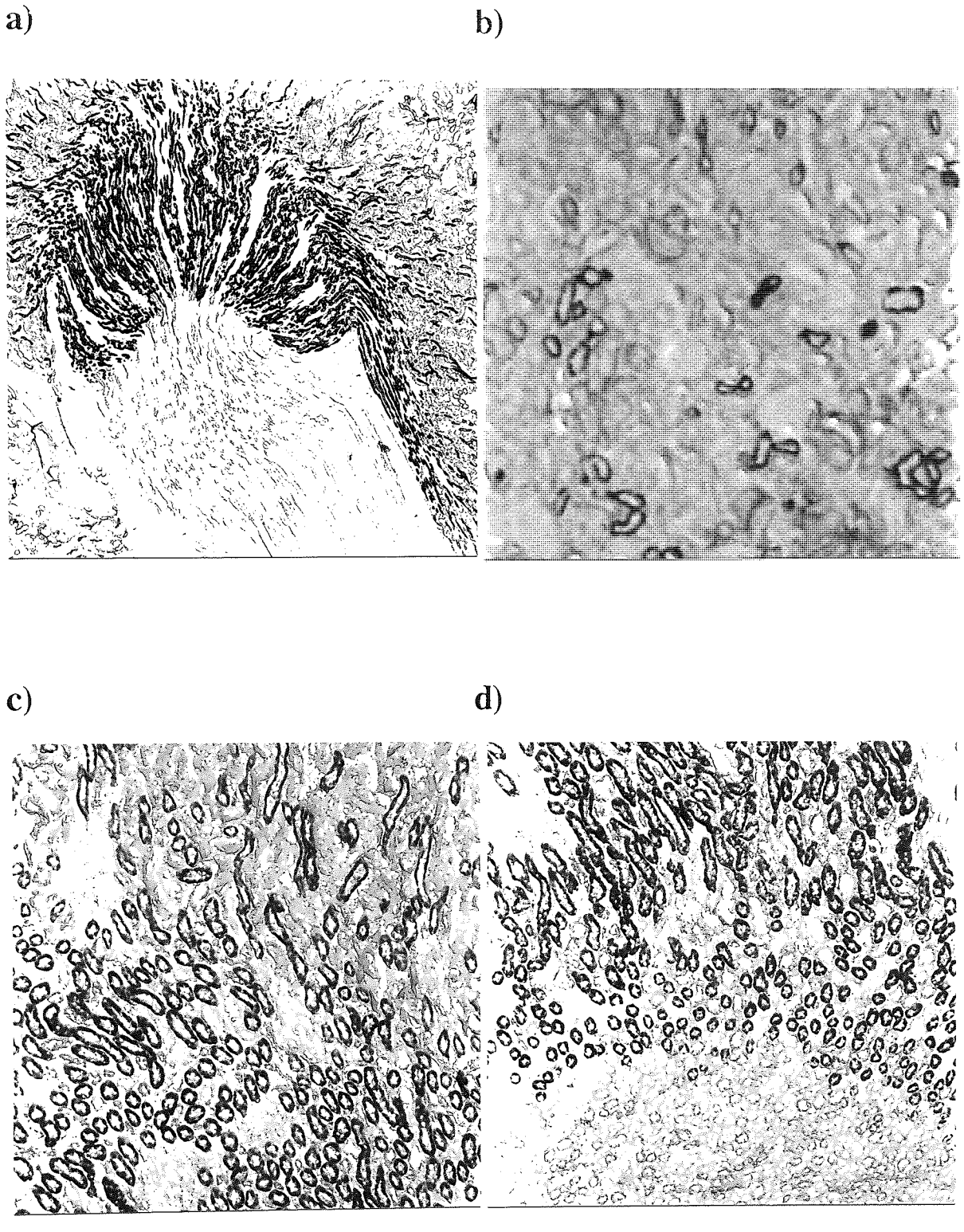


Figure 45. Localisation of Na^+/K^+ ATPase activity a) Low power overview of section (x 15 mag) b) cortex c) cortico-medullary junction d) medullary-papilla junction. (x 75 mag)

The Na^+/K^+ ATPase staining intensity was studied with respect to time of incubation period. Duplicate sections were incubated over a range of incubation periods (2-60 min) and the intensity of staining assessed. The intensity of the reaction product was clearly dependent on the incubation period. However, when the incubation period was extended to beyond 30 minutes the amount of background staining was also markedly increased, leading to a reduction in the contrast observed across the section. Therefore the optimum staining period was taken to be 20 minutes (Figure 42 a-d).

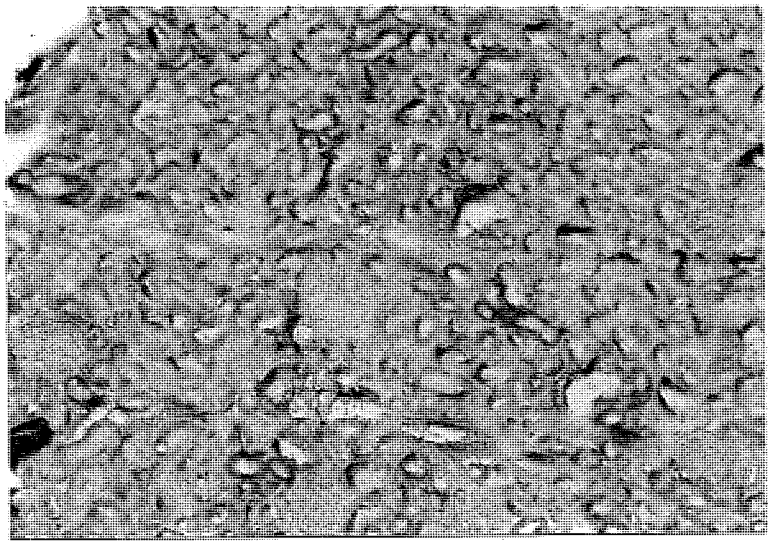
7.32 Localisation of Ca2+ ATPase staining within the rat kidney.

In the absence of levamisole from the incubation solution luminal staining was apparent in cortical tubules after staining with the method described for the localisation of Ca2+ ATPase activity. Addition of levamisole abolished the luminal staining of the tubules without affecting the intensity or distribution of the other staining products.

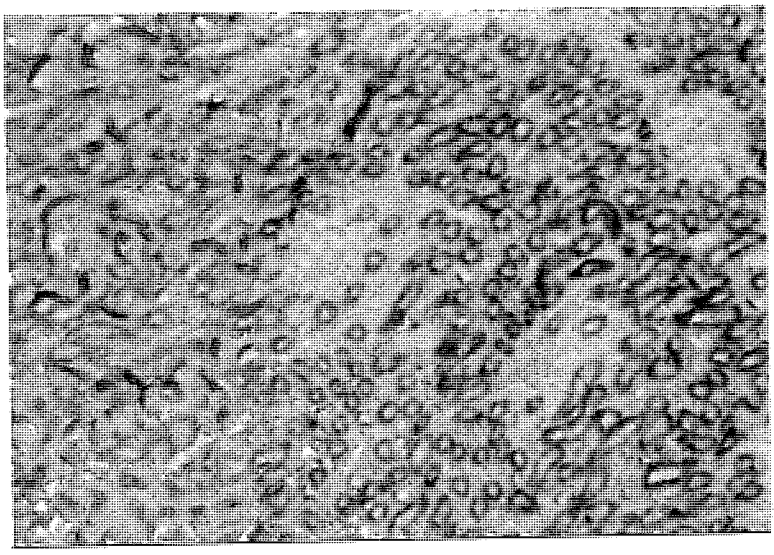
Removal of the substrate from the incubation medium resulted in very faint background coloration of the section. The coloration was evenly distributed across all regions of the kidney (Figure 47 a).

The pattern of staining for Ca2+ ATPase activity across the kidney was markedly different from that seen after staining for Na^+/K^+ ATPase activity. Ca2+ ATPase staining was most intense in the glomeruli. The basolateral edges of the cortical tubules also showed substantial amounts of reaction product, but tubules located in the medullary region of the kidney showed less Ca2+ ATPase staining than those of the cortex. Tubules in medullary rays extending into the cortex also showed less intense staining than those surrounding them. Staining in the papillary region of the kidney showed an extremely distinctive pattern of staining for Ca2+ ATPase activity. The intensity of staining for most tubules in the papilla was similar to that seen in the cortex, however there were also regular clusters of other tubules which showed more intense staining (Figure 48 a-d). The reaction product was confined to the basolateral side of the

a)



b)



c)

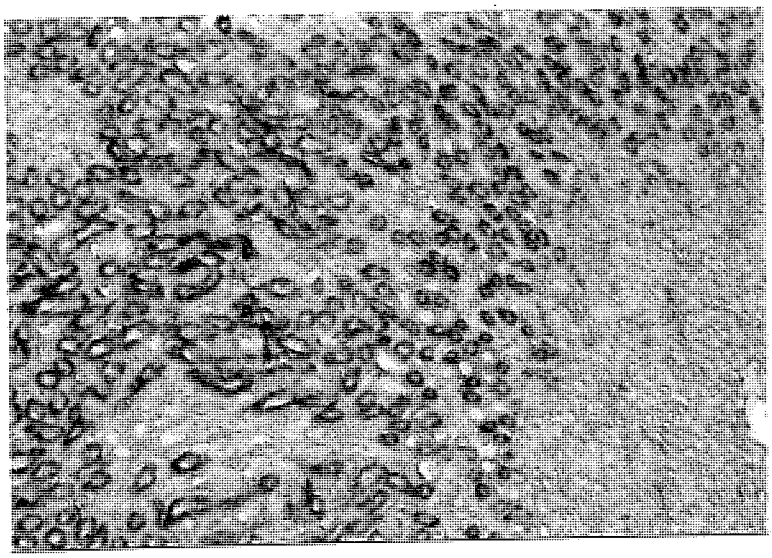


Figure 46. Inhibition of alkaline phosphatase and Na^+/K^+ ATPase activity; staining by other phosphatases a) cortex b) cortico-medullary junction c) medullary-papilla junction. (x75 mag)

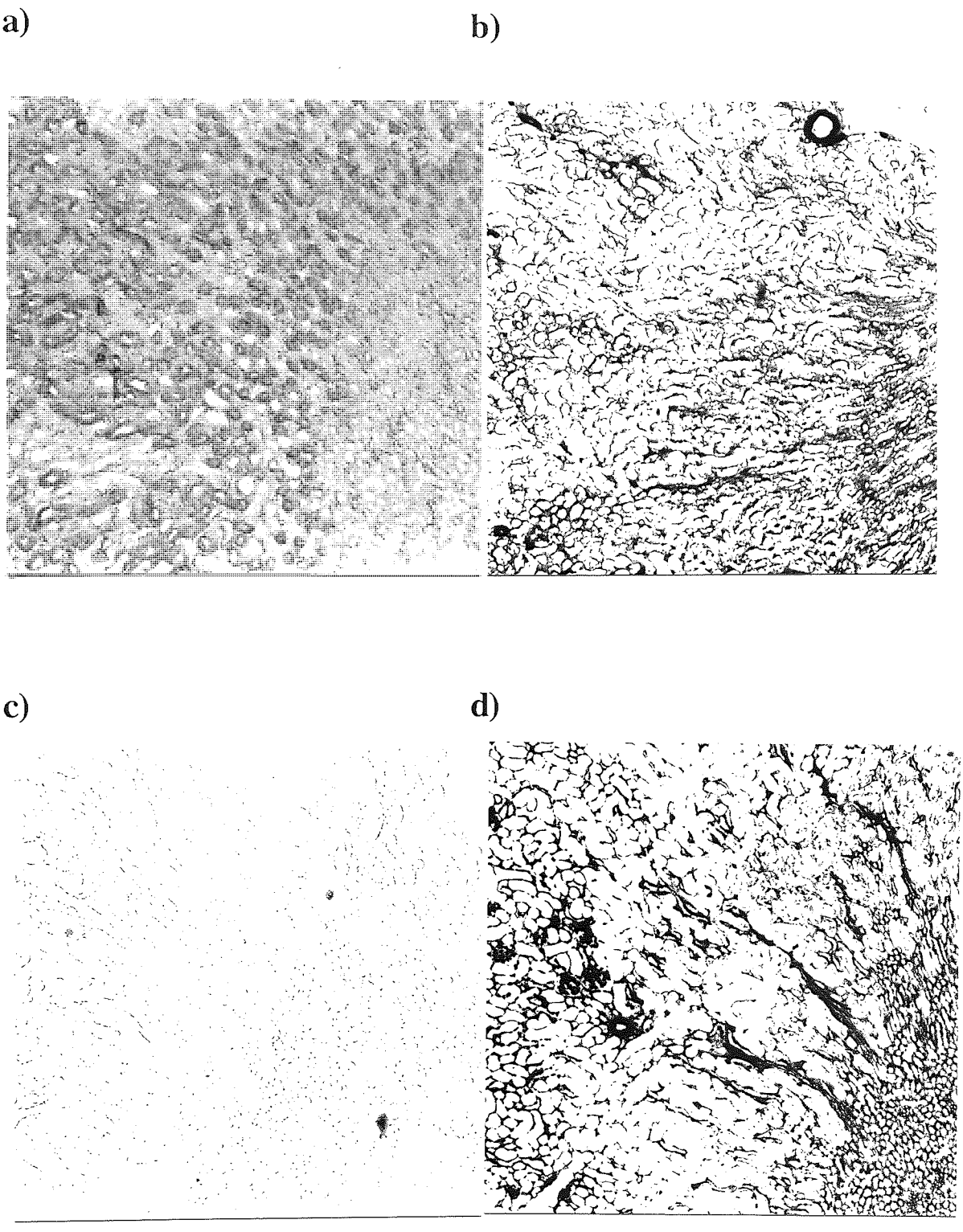


Figure 47. Histochemical localisation of Ca2+ ATPase activity; effects of various manoeuvres to demonstrate dependence on substrate and calcium. a) non-enzymatic hydrolysis (no substrate) (x 75 mag) b) no calcium in incubation solution c) EDTA pre-treatment and no calcium in the incubation solution d) No calcium depletion plus calcium in the incubation solution (Normal conditions). (x 30 mag)

tubules; there was no evidence of any Ca2+ ATPase staining on the luminal face of any tubules. There was also very intense staining of the muscular walls of the arterioles present in the kidney.

Addition of ouabain to the incubation solution did not alter the distribution or intensity of staining visible resulting from Ca2+ ATPase activity. Removal of calcium from the incubation solution resulted in a reduction of the staining intensity over the entire section, however, under these conditions Ca2+ ATPase staining was still apparent (Figure 47b). Dependence of the reaction on the presence of calcium was clearly demonstrated after depletion of endogenous calcium and magnesium in the tissue by pre-incubation in an EDTA-containing solution. Pre-incubation of the sections for 30 min in 10mM EDTA (in distilled water at pH 7.4, at 4 °C, followed by two washes in deionised water) resulted in an absence of staining when the sections were later incubated without calcium or magnesium (Figure 47c). If, however calcium (or magnesium) was added to the incubation solution then Ca2+ ATPase staining was clearly visible, although slightly less intense than sections which were not pre-treated with EDTA (Figure 47d). It appears therefore that the staining intensity is a product of the concentration (or availability) of both endogenous and exogenous calcium salts.

In experiments where the calcium concentration of the incubation was varied (0-10mM) the Ca2+ ATPase staining intensity was demonstrated to be dependent on the calcium concentration only up to 2mM $CaCl_2$; further increases in calcium concentration did not increase the intensity of the reaction product, establishing that at calcium concentrations above 2 mM the intensity of the reaction product is not limited by calcium availability. It is noteworthy however that even at higher calcium concentrations in the incubation medium those sections which were pre incubated in an EDTA-containing solution showed less intense staining than their counterparts which were not incubated with EDTA. Control sections incubated in distilled water did not show any loss of Ca2+ ATPase staining, therefore the difference in staining intensity in EDTA pre-treated sections must be due to depletion of endogenous cations. Addition of magnesium to the

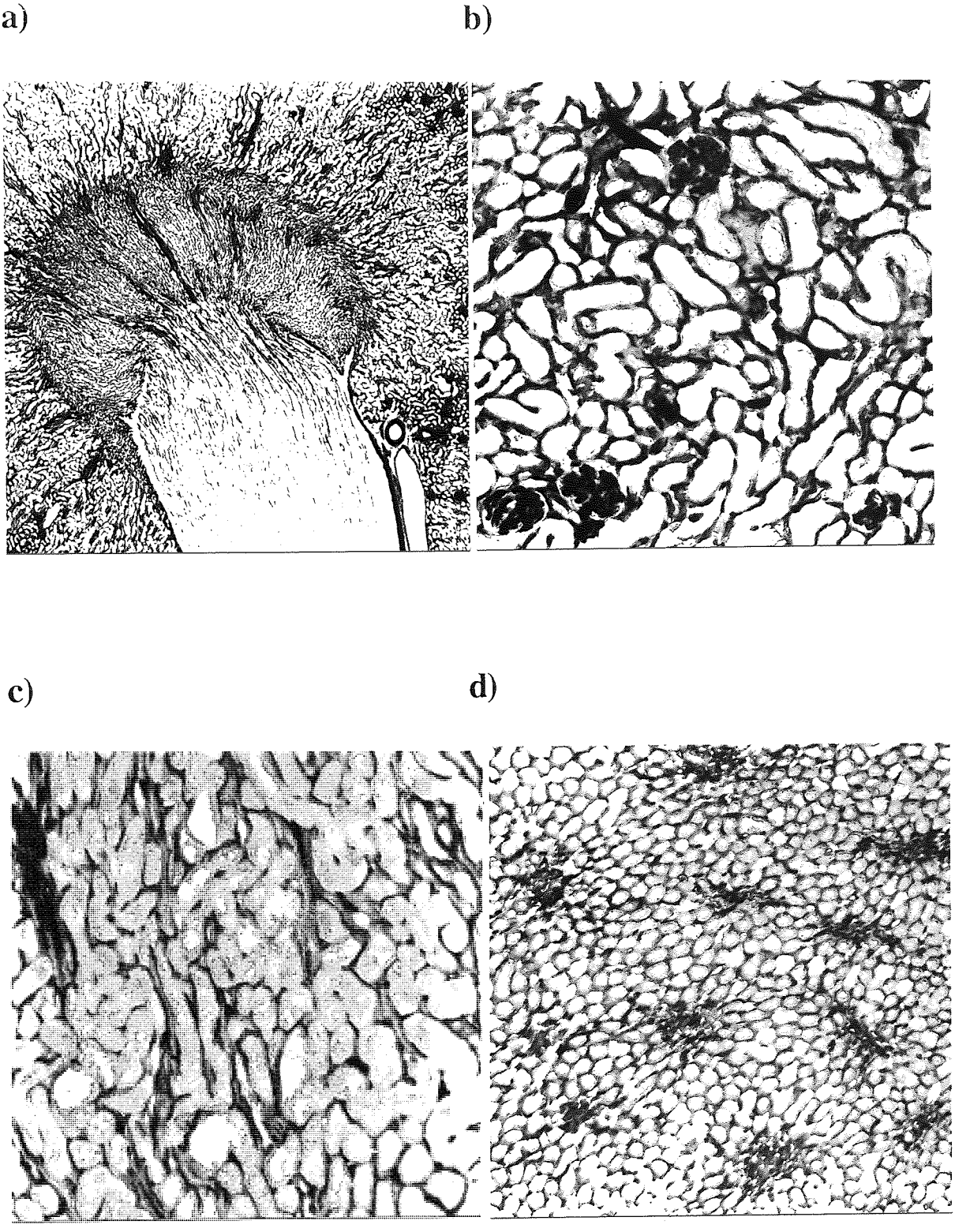
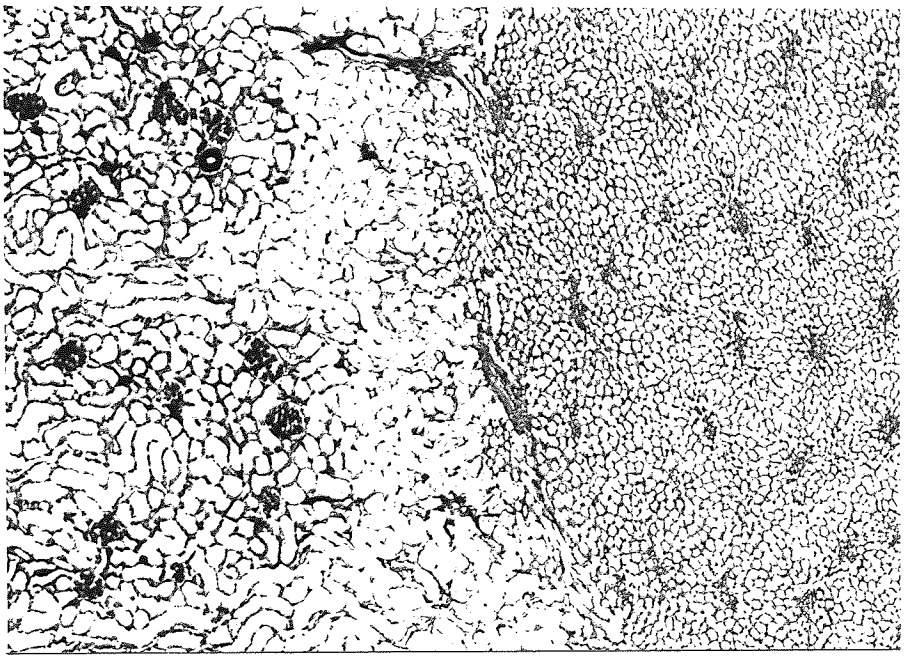


Figure 48. Ca2+ ATPase localisation a) Low power overview of section (x 30 mag) b) cortex c) cortico-medullary junction d) medullary-papilla junction. (x 75)

a)



b)

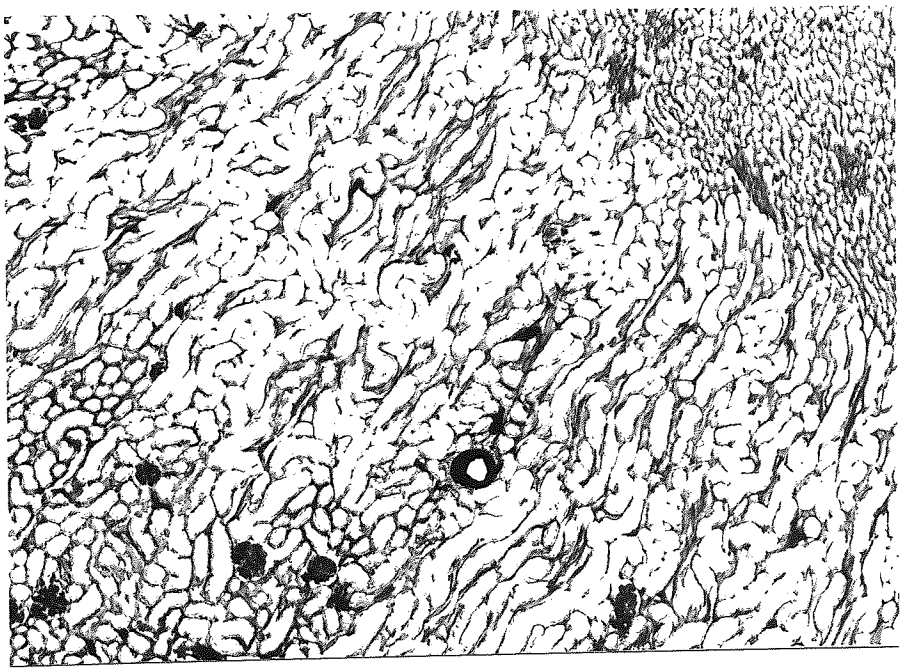


Figure 49. Dependence of staining intensity for Ca2+ ATPase activity on section thickness a) 6 µM b) 15 µM. (x 36 mag)

incubation solution in place of calcium resulted in similar levels of staining to that achieved in the presence of calcium. Attempts to inhibit Ca2+ ATPase activity by the use of $AgNO_3$ were thwarted when addition of this salt to the incubation medium resulted in precipitation.

Staining intensity was the same in sections incubated at room temperature compared to those incubated at 37 °C. Staining intensity was increased with increasing section thickness; however with increasing section thickness there was a decrease in resolution within the section (Figure 49 a,b). 6 μM sections achieved the compromise of acceptable staining intensity with good optical resolution of the section. In addition it allowed direct comparisons to be made between sections stained for Na^+/K^+ ATPase and Ca2+ ATPase activities.

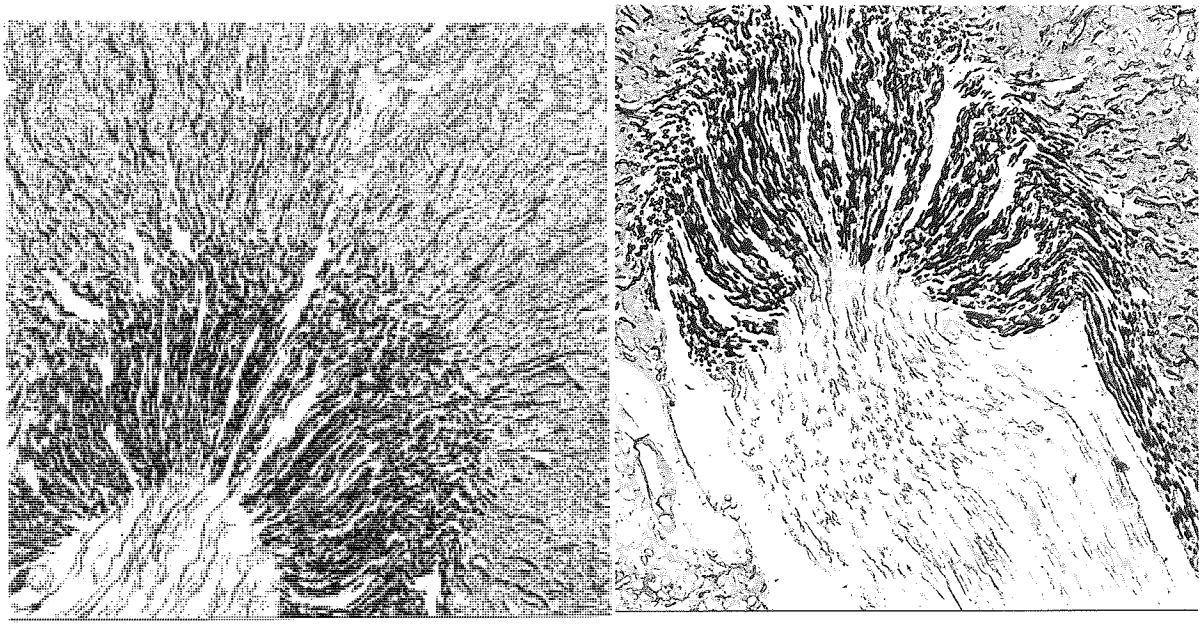
7.33 Effects of acute and sub-chronic dosing with gentamicin on the activity of renal Na^+/K^+ ATPase and Ca2+ ATPase, measured histochemically.

Sections stained for Na^+/K^+ ATPase and Ca2+ ATPase activity were examined by a pathologist with the aim of discerning whether inhibition of renal ATPase activity could be observed histochemically. There was no evidence that either Na^+/K^+ ATPase or Ca2+ ATPase activity was different between control and drug-treated animals as measured histochemically (Figure 50 a-d, 51a-d). There were no differences in the staining intensity at any particular site along the nephron for either enzyme. Neither acute nor sub-chronic dosing with gentamicin produced changes in enzyme activity which were measurable histochemically (Figure 50 a-d, 51a-d).

Experiments where the incubation period was reduced to 2 or 5 minutes (from the usual 20 minutes) also failed to demonstrate any differences in either the staining intensity or the localisation of the stain between drug-treated and control animals; this was the case for both Ca2+ ATPase and Na^+/K^+ ATPase stains.

a)

b)



c)

d)

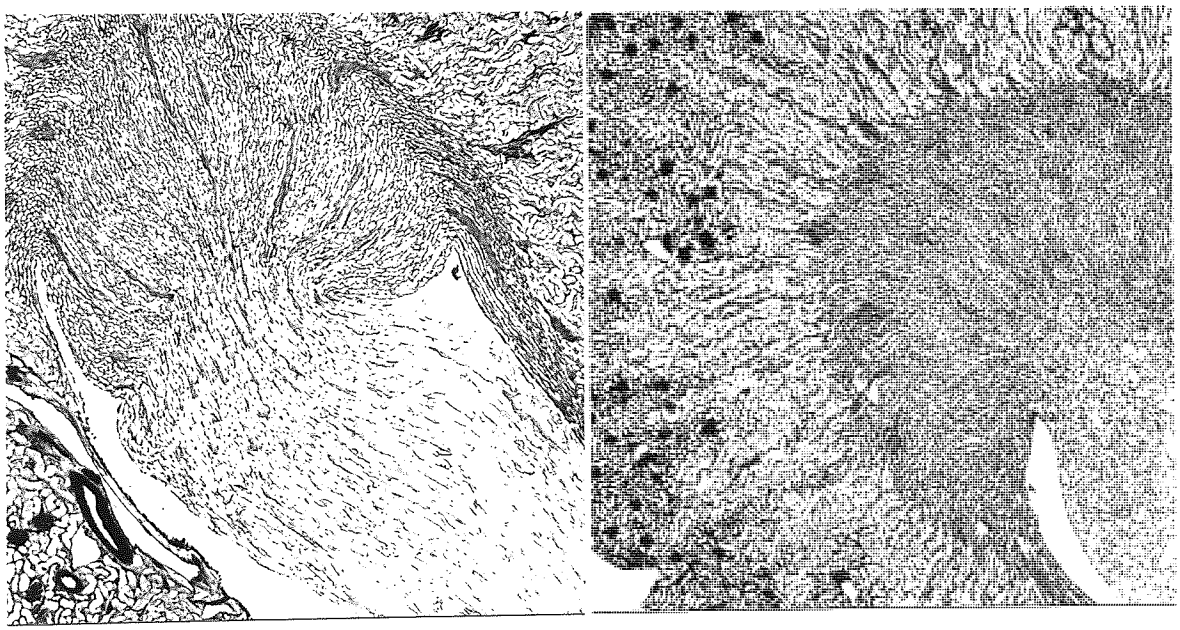
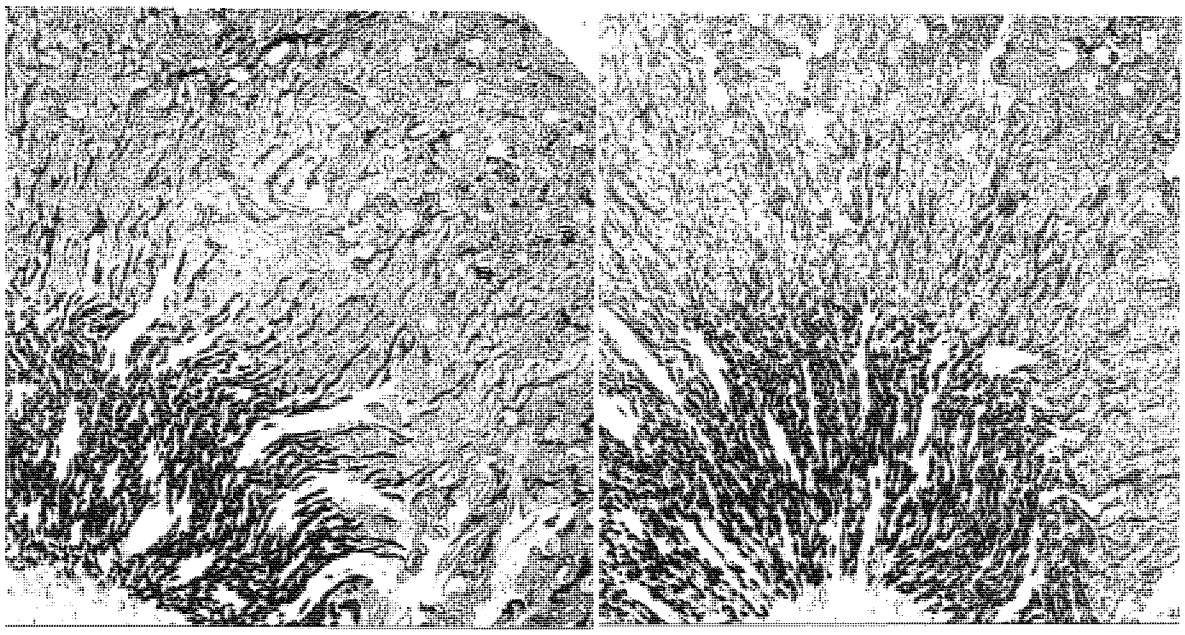


Figure 50. Effect of acute gentamicin administration on staining of Na^+/K^+ ATPase and Ca2+ ATPase activity. a) Na^+/K^+ ATPase activity; drug-treated animal b) Na^+/K^+ ATPase activity; control animal (x 24 mag) c) Ca2+ ATPase activity; drug-treated animal d) Ca2+ ATPase activity; control animal (x 15 mag)

a) b)



c) d)

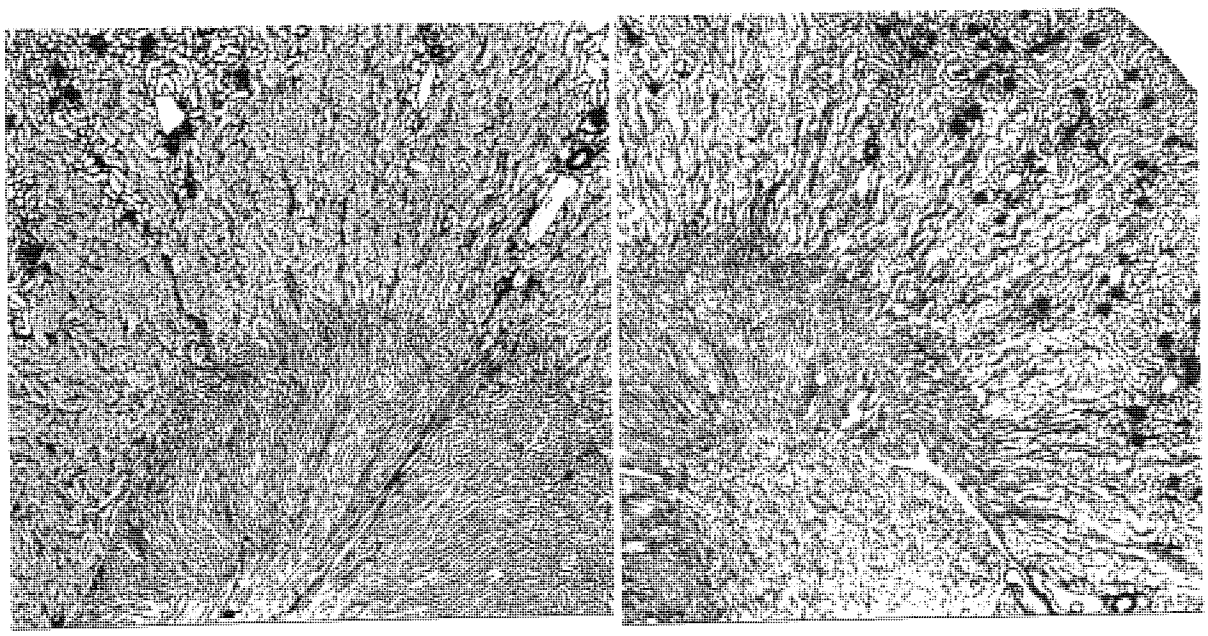


Figure 51. Effect of repeated doses of gentamicin on staining of Na^+/K^+ ATPase and Ca2+ ATPase activity a) Na^+/K^+ ATPase activity; drug-treated animal b) Na^+/K^+ ATPase activity; control animal (x 15 mag)c) Ca2+ ATPase activity; drug-treated animal (x 15 mag) d) Ca2+ ATPase activity; control animal. (x 30 mag)

7.4 Discussion

The experiments described above clearly demonstrate the specificity of the histochemical methods used for the independent staining of Ca2+ ATPase and Na^+/K^+ ATPase activity. The methodology used was optimised with respect to section thickness, incubation period and incubation temperature. In addition, specific ionic requirements of the enzymes were demonstrated, and classical inhibitors used to show that the histochemical staining could be abolished in their presence. Using these techniques to localise the site or extent of the gentamicin-induced inhibition of Na^+/K^+ ATPase were however not successful since there was no discernible difference between drug-treated and control animals, after both sub-chronic and acute dosing. Changes in enzyme-related histochemical staining could conceivably result from either direct enzyme inactivation by the drug, or as a result of more widespread tubular injury and disruption, probably at the later stages of toxic injury.

Data presented in Chapter 6, and previously published work from Williams et al (1981), clearly demonstrate inhibition of renal Na^+/K^+ ATPase four hours after a single dose of gentamicin, the time at which the animals were killed in this study. In addition, sub-chronic dosing (50 mg/kg gentamicin base for 7 days) has also been shown to inhibit Na^+/K^+ ATPase activity in proximal tubule segments, measured biochemically (Fukuda et al, 1990). Interestingly, the authors also reported that inhibition of Na^+/K^+ ATPase activity was confined to proximal tubular segments, Na^+/K^+ ATPase activity of medullary thick ascending limb segments was unaffected by sub-chronic gentamicin dosing. The studies of Fukuda et al (1992) also reported there was no effect on ouabain-insensitive ATPase activity after sub-chronic gentamicin dosing, although data was not shown. These reports therefore suggest that inhibition of Na^+/K^+ ATPase activity, at least, should be apparent at the time points selected for the histochemical studies described herein. However, biochemical measurements of ATPase activity were not made in parallel in the experiments where histochemical staining was undertaken. While

the expectation was that some degree of enzyme inhibition should have occurred this cannot be substantiated.

In conclusion, whilst this study demonstrates that staining of renal Na^+/K^+ ATPase and Ca2+ ATPase can be accomplished with relative ease it also demonstrates that precipitation staining may not be the most appropriate technique by which to demonstrate subtle changes in enzyme activity. As is the case with this type of histochemical technique the reaction product is amplified at the site of enzyme activity making quantitative assessment difficult. Other techniques using autoradiographic or immunocytochemical methodology may be more appropriate for this type of measurement.

Chapter 8. Assessment of the sensitivity of LLC-PK1 and LLC-RK1 cells to a range of nephrotoxins using cytotoxicity assays.

8.1 Introduction

Due to the substantial role the proximal tubule plays in the uptake and transport of solutes from the ultrafiltrate, this segment of the nephron is frequently the site of toxicity. Since both aminoglycoside antibiotics and cis-platinum are accumulated preferentially in the cells of the proximal tubule, the use of cultured cell lines which possess characteristics of proximal tubule cells are an attractive proposition for the study of renal injury arising from these, and other, nephrotoxic compounds. Whilst the process of de-differentiation may alter the characteristics of the cell type from those found in the original source tissue, the LLC-PK1 and LLC-RK1 continuous cell lines are thought to possess attributes of the proximal tubule cell type (Bach et al, 1985).

The LLC-RK1 cell line was originally derived from whole kidney cultures of New Zealand White rabbits (Hull et al, 1965). These cells have been shown to demonstrate polarity in culture with the apical surface possessing microvilli and the presence of tight junctions between adjacent cells (Williams et al, 1988). Additionally, these cells also show the renal marker enzyme dehydropeptidase 1 activity, and transport systems for organic cations. (Duffy and McRae, 1990). In addition, preliminary evidence for sodium-dependent glucose transport has also been reported in LLC-RK1 cells confirming that they demonstrate proximal tubular characteristics (Duffy and McRae, 1990).

The LLC-PK1 cell line was originally derived from the kidneys of a juvenile New Hampshire pig. In culture these cells display epithelial cell-like morphology, and produce domes upon attaining confluency, indicative of the ability to transport solutes across the polarised cell, typical of epithelial cells (Hull et al, 1976). LLC-PK1 cells have also been shown to possess characteristics of the proximal tubule such as sodium-dependent

glucose transport (Rabito and Ausiello, 1980, Rabito, 1981, Misfeldt and Sanders, 1981), and brush border enzyme expression (Rabito et al, 1984).

The LLC-RK1 cell line appears to retain the sensitivity to nephrotoxins demonstrated by the kidney *in vivo* (Williams et al, 1988). *In vitro* studies using LLC-RK1 cells have shown them to be more sensitive to the action of nephrotoxins than the 3T3 fibroblast cell line (Duffy and McRae, 1990), indicating some degree of tissue specificity. In a study with a series of cephalosporin antibiotics good agreement has also been shown between cytotoxicity in LLC-RK1 cells and nephrotoxicity in rabbits (Williams et al, 1988).

Although LLC-PK1 cells are more commonly studied there has been no systematic comparison of their sensitivity compared to the LLC-RK1 cell line. In this study the effects of a range of nephrotoxins with differing mechanisms of action were assessed on LLC-RK1 and LLC-PK1 cells to ascertain whether differences in sensitivity exist between the cell lines which could be exploited in toxicological studies. A particular aim of these studies was to select a single cell line which was most sensitive to the actions of aminoglycosides for subsequent development into an *in vitro* system for the study of the cellular mechanisms of disruptions in renal cation transport.

Toxicity was assessed using the neutral red uptake assay (NR) (Invitox), and the MTT assay (Mosmann, 1983), both of which provide, by different means, an estimate of the quantity of viable cells present. Neutral red is preferentially accumulated in the lysosomes of the cell by an active uptake process, whereas MTT (3-(4,5,-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) is reduced to a coloured product by the action of mitochondrial dehydrogenases in viable cells. The coloured products of these assays can be quantified spectrophotometrically thus allowing the rapid assessment of the effects of test compounds on cellular viability.

As well as examining the correlation between the two cytotoxicity indices the relationship between the *in vitro* data generated by these studies and *in vivo* data from the literature was also examined.

8.2 Methods

LLC-RK1 cells (a gift from Paul Duffy (ICI); original source Flow Laboratories) with a doubling time of approximately 32 h, were routinely cultured in medium 199 with Hanks salts and 25mM Hepes buffer, supplemented with 10 % foetal calf serum and 2mM glutamine. LLC-PK1 cells (from American Type Culture Collection), with a doubling time of approximately 16h, were routinely cultured in medium 199 with Hanks salts and 25mM Hepes supplemented with 5% foetal calf serum and 2mM glutamine. No antibiotics were included in the culture medium. Cultures were maintained in a humidified atmosphere of 95% O_2 and 5% CO_2 at 37 °C.

Both NR and MTT assays were initially optimised using LLC-RK1 cells and the experimental conditions later used with LLC-PK1 cells. Since LLC-RK1 and LLC-PK1 cells double at quite different rates the initial seeding density for each cell line was adjusted so that the cells should have achieved a visually judged 70-80% cell confluency on day 3 of the assay and so be in log growth phase when the test compound was added. At this stage the majority of cells should be at the same portion of the growth cycle and would therefore produce the most consistent response to the test compound. The effect of cell seeding number on assay linearity was investigated to ensure that the chosen seeding density produced absorbance values which lay on the linear portion of the cell number vs absorbance curve (Figure 52). The lowest concentration of reagents required to produce the coloured product was ascertained so as to maximise the absorbance attainable combined with the minimum risk of reagent toxicity (Figures 53 and 54). Additionally, the choice of solvent for the MTT assay was investigated in order to maximise final absorbance values and thereby increase the sensitivity of the assay. The absorbance spectra of reduced MTT in DMSO, acid isopropanol, and DMSO and glycine

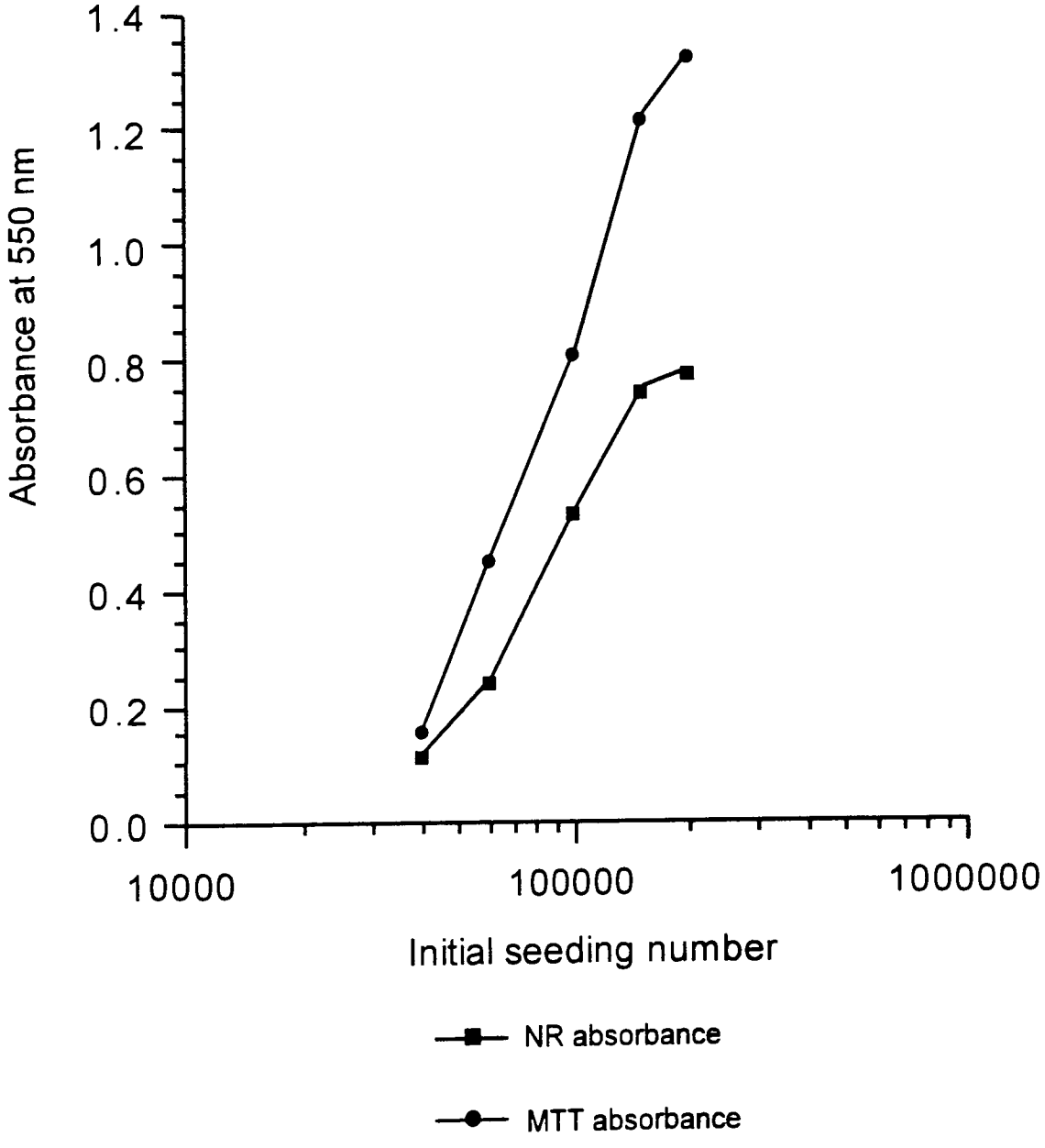


Figure 52. Effect of increasing initial cell number on absorbance values after MTT and NR assay.

buffer pH 10.5 in an 8:1 ratio (Plumb et al, 1989) were compared to locate the absorption maxima for each solvent and to contrast the relative peak heights of the MTT product in the solvent (Figure 55).

In the light of these studies (Figures 52-54) the following methodology was chosen for subsequent investigations.

LLC-RK1 cells were seeded in 200 µl aliquots at 1x 10^5 cells/ ml onto 96 well plates on day 1 of the assay. LLC-PK1 cells were seeded at 2.5 x 10^4 /ml. In all other respects LLC-RK1 cells and LLC-PK1 cells were treated identically. 24h after seeding the medium was removed and a range of doses of the test compound in 200µl medium was applied. Where necessary pH correction was made using 0.1M KOH. Each concentration was applied to a minimum of 8 replicates per experiment; plates for NR and MTT assays were prepared concurrently. 24h later (48h for gentamicin, neomycin, and cyclosporin A) the test solutions were washed off with 1x200µl PBS and the NR solution (50 µg/ml) or the MTT solution (1mg/ml) was added in 200µl aliquots. Initial dose ranging studies showed that extremely high concentrations of aminoglycoside antibiotics and Cyclosporin A were required to produce any effects on the cells. A 48h exposure period was therefore used for these compounds. After further incubation at 37 °C for 3 or 4 hours (NR and MTT assay respectively) the solution was removed and the remaining cells washed with 2 x 200µl PBS followed by gentle blotting before the addition of 200µl of final solvent, either DMSO and glycine buffer pH 10.5 (Plumb et al, 1989) for the MTT assay, or destain solution containing 1% glacial acetic acid in 50% ethanol for the Neutral Red assay. All plates were shaken until the products were evenly distributed into solution and read spectrophotometrically on a Thermomax microplate reader at 550 nm.

8.3 Calculations and Definitions

Mean absorbance values from non-drug treated cells were taken to be 100%. Absorbance values for drug exposed cells were calculated as a percentage of this value and used to determine the concentration of test compound required to produce a 50%

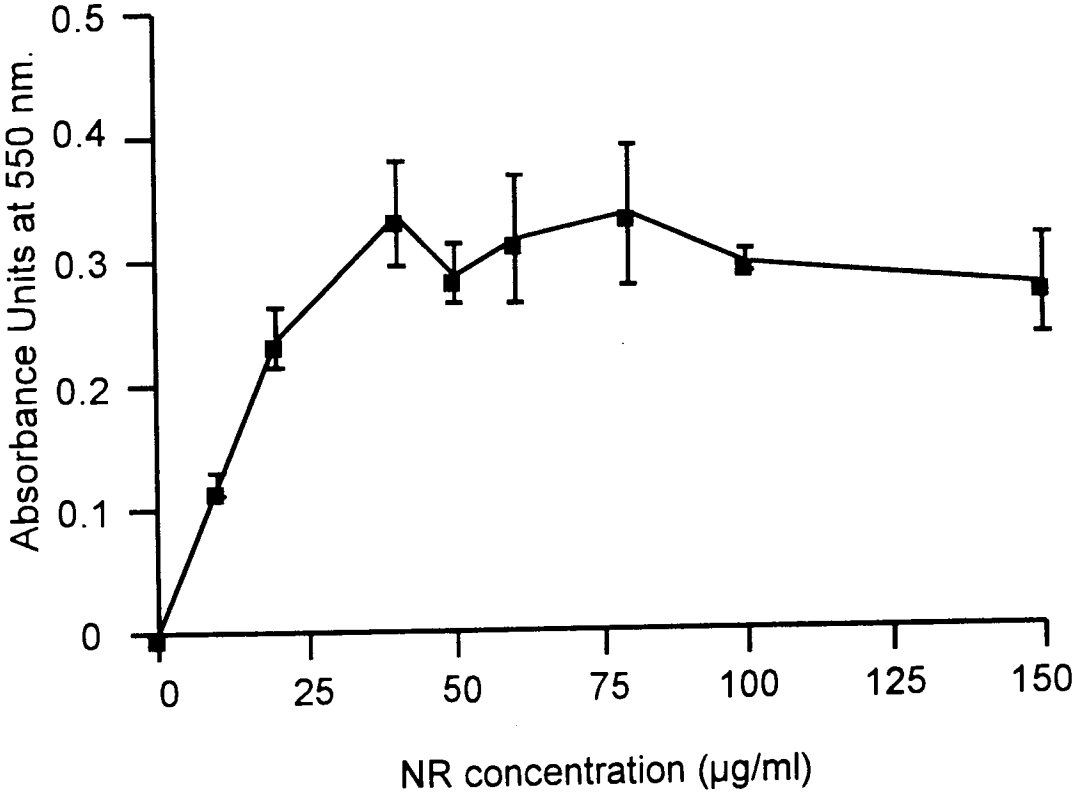


Figure 53. Effect of increasing NR concentration on absorbance values at 550nm.

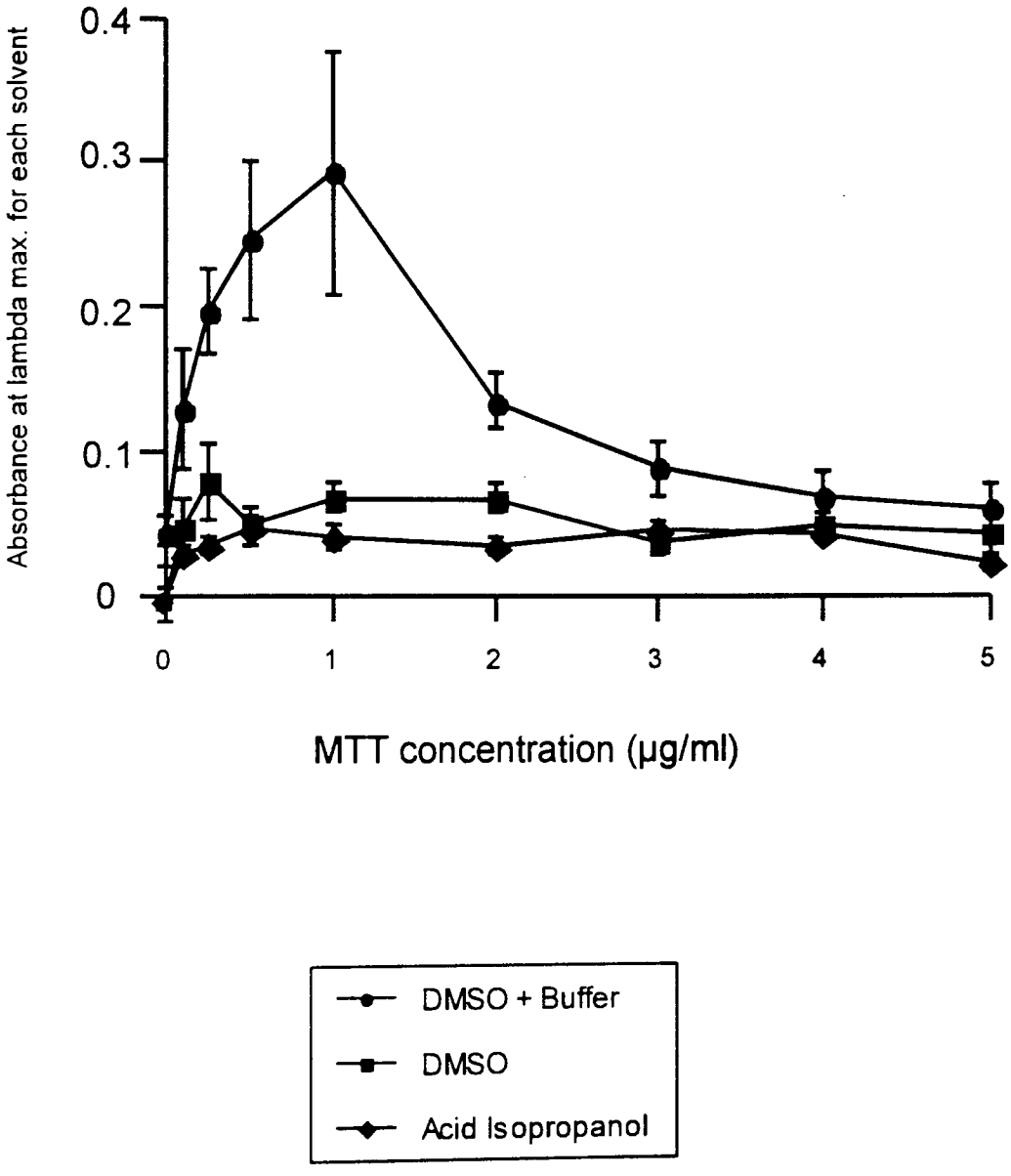


Figure 54. Effect of varying MTT concentration and solvent on absorption values at 550 nm due to MTT formazan production in LLC-RK1 cells.

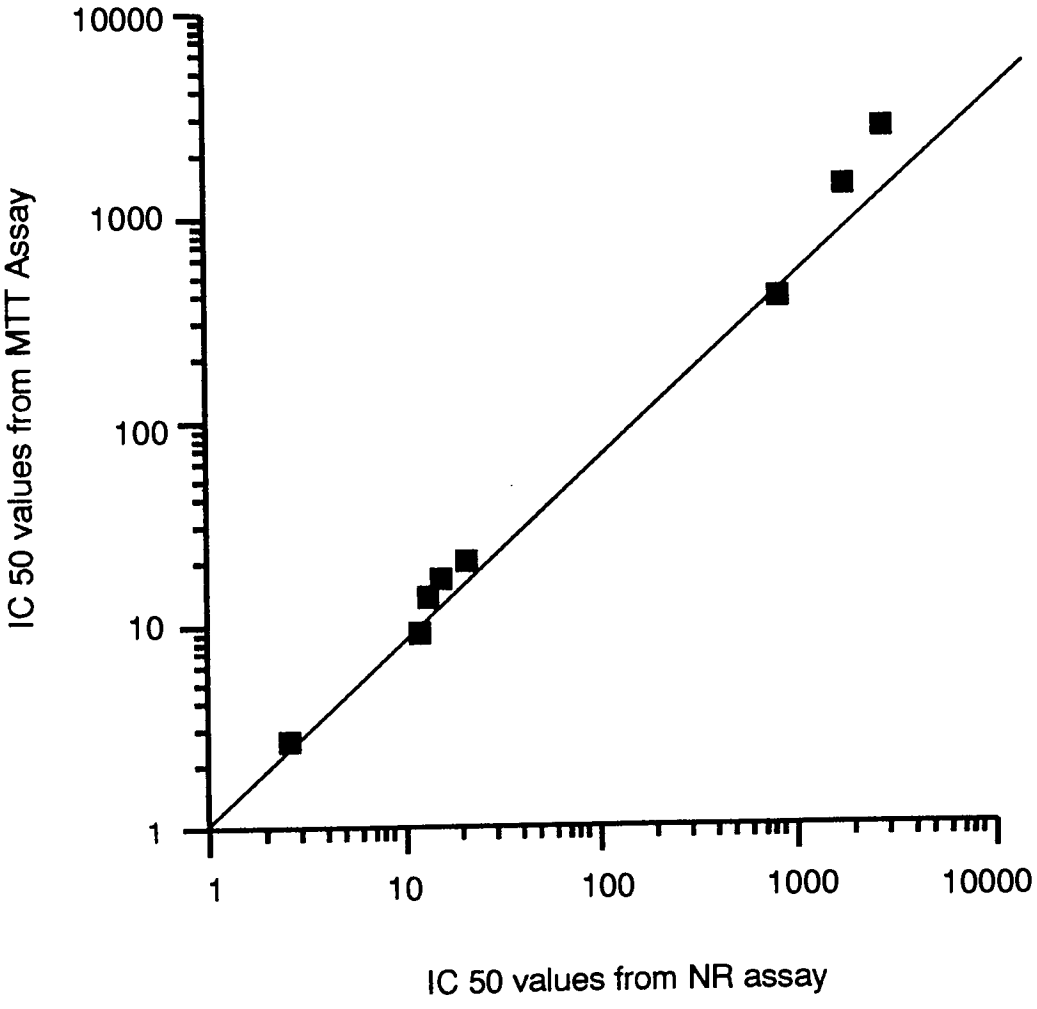


Figure 55. Correlation between IC50 values obtained from NR and MTT assays using LLC-RK1 cells.

reduction in absorbance control values (IC 50). The IC 50 values from both assays were compared to explore the correlation of the results between the assays.

8.4 Results

Both cell lines exhibited differential sensitivity to the cytotoxic action of a range of compounds known to differ widely in their nephrotoxic potential *in vivo*. Similar results were obtained with both NR and MTT cytotoxicity assays (Table 10, 11). In both cell lines NR and MTT assays produced equivalent results ($r^2 = 0.96$) under the conditions tested (Fig. 55). The use of the DMSO and glycine buffer solution at pH 10.5 produced an approximately 3-fold increase in absorbance at 550nm over the more conventionally used solvents for the MTT assay. This therefore represents a marked increase in the sensitivity of the method.

LLC-PK1 and LLC-RK1 cells were equally sensitive to the actions of acute nephrotoxins (e.g. cis-platinum, $HgCl_2$) and produced IC50 values in the micromolar range. For those nephrotoxins which require repeated administration and/or produce less severe renal damage *in vivo* (e.g. gentamicin, cyclosporin A), IC50 values in the millimolar range were obtained in both cell lines. Some of the compounds which produce nephrotoxicity *in vivo* only after repeated administration also required longer exposure periods (48 h vs 24h) to produce toxicity in this *in vitro* system. With respect to known *in vivo* nephrotoxic potency, LLC-RK1 cells correctly ranked compounds which produce nephrotoxicity after acute exposure and those which may require repeated administration to produce toxicity. In contrast, LLC-PK1 cells ranked acute nephrotoxins correctly but not those which require repeated administration and/or produce less severe renal damage *in vivo*. Structurally related compounds were correctly ranked for their nephrotoxic potential by LLC-RK1 cells but LLC-PK1 cells failed to rank the cephalosporins tested in the expected order from *in vivo* data (Table 11). Additionally, IC50 values after LLC-PK1 cells were exposed to neomycin were difficult to determine due to extreme variability of the response of the cells to this compound.

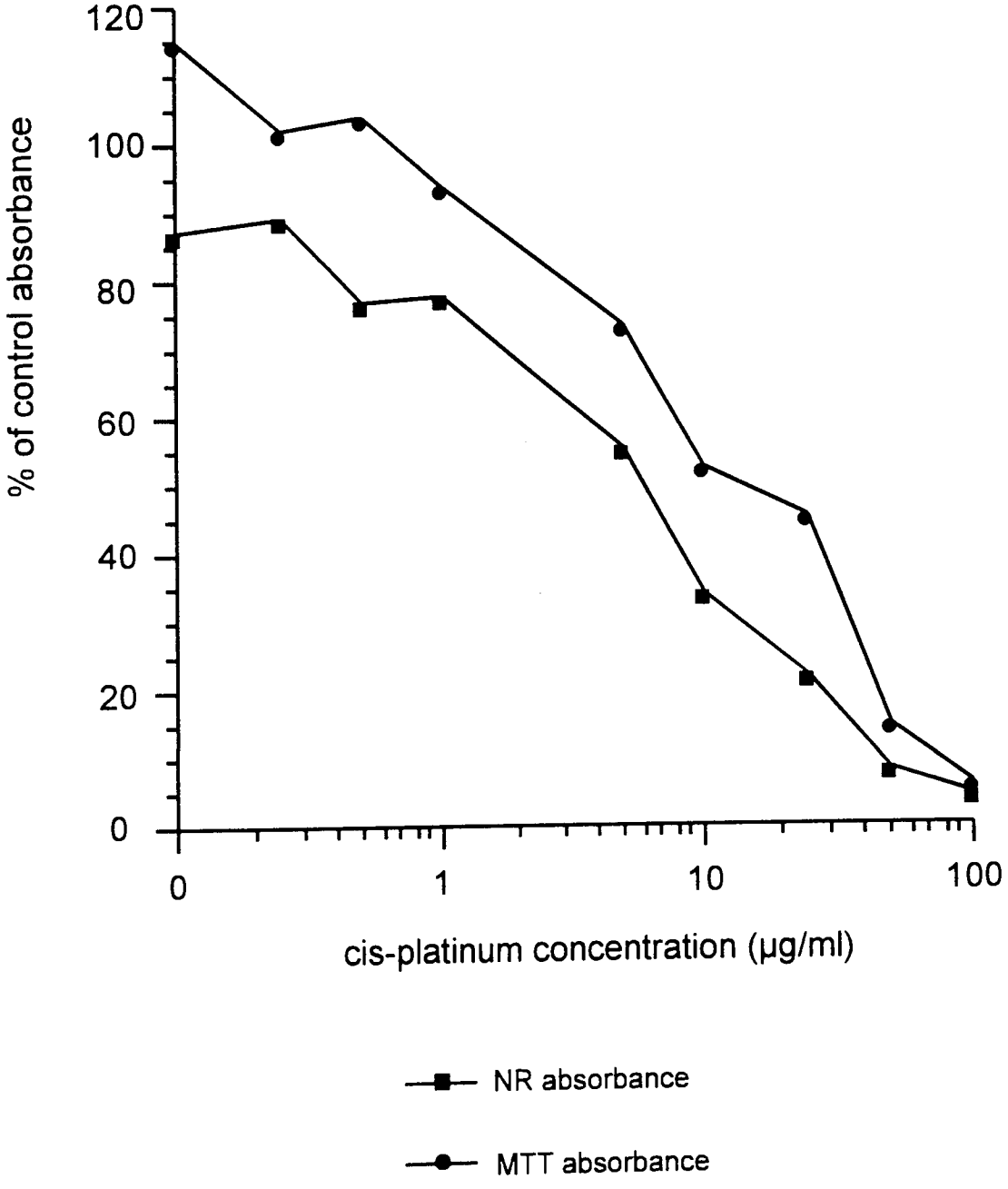


Figure 56. Effect of cis-platinum on the viability of LLC-RK1 cells as measured by NR and MTT assays. Results of typical experiments are shown. n = 8 or 16 for each data point; typical SD values were 10% or less.

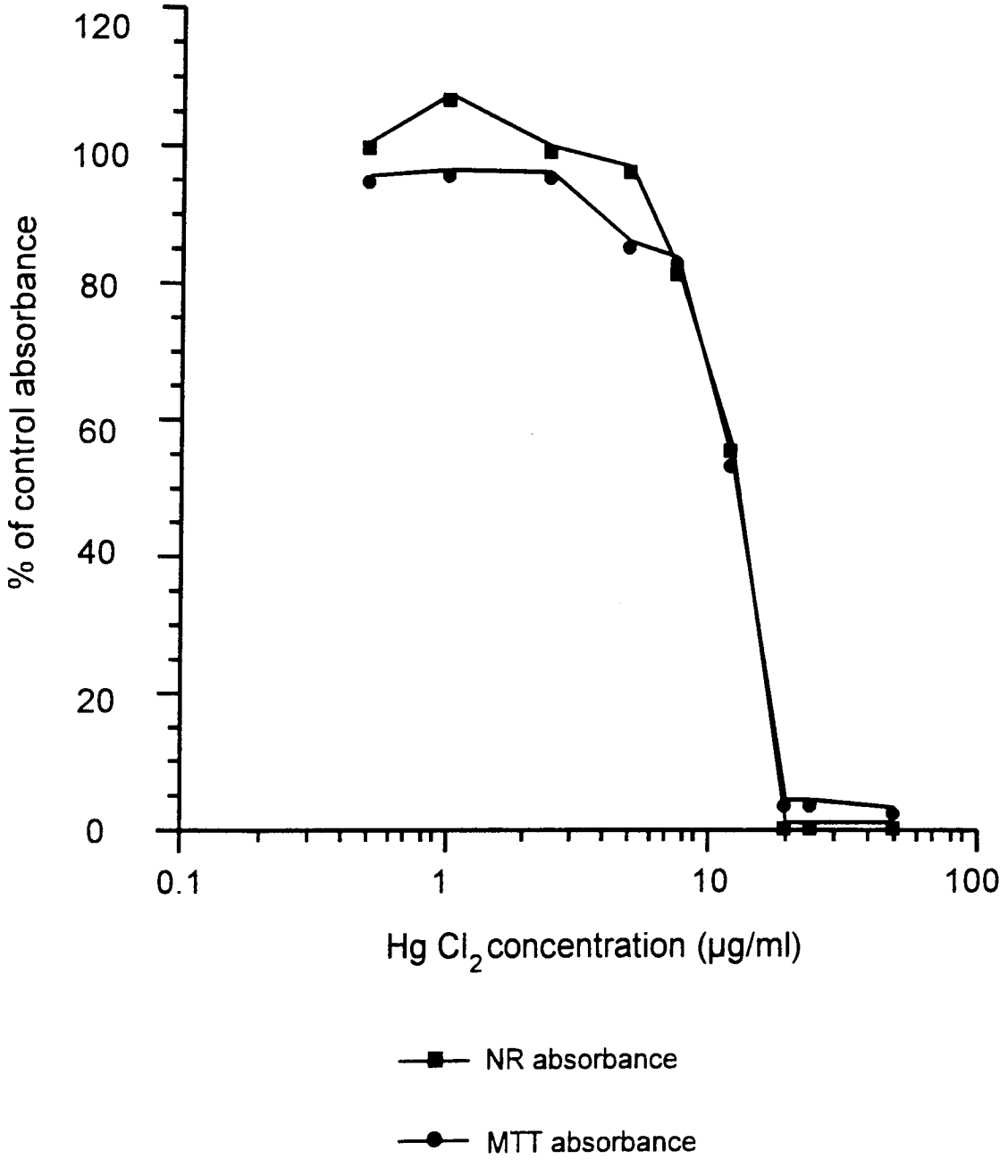


Figure 57. Effect of $HgCl_2$ on the viability of LLC-RK1 cells as measured by NR and MTT assays. Results of typical experiments are shown. N = 8 or 16 for each data point; typical SD values were 10 % or less.

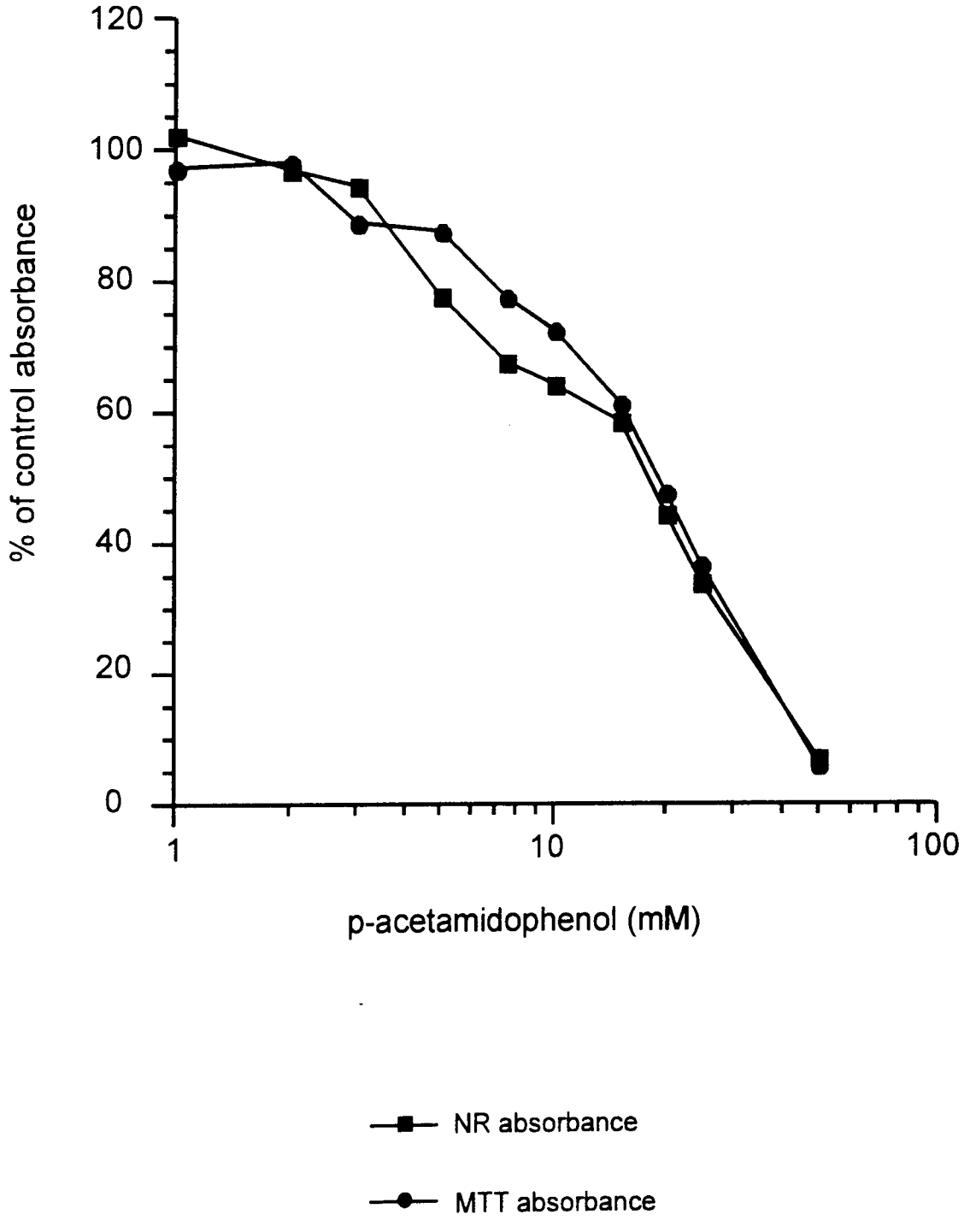


Figure 58. Effect of p-acetamidophenol on the viability of LLC- RK1 cells as measured by NR and MTT assays. Results of typical experiments are shown. n=8 or 16 for each data point; typical SD values were 10 % or less.

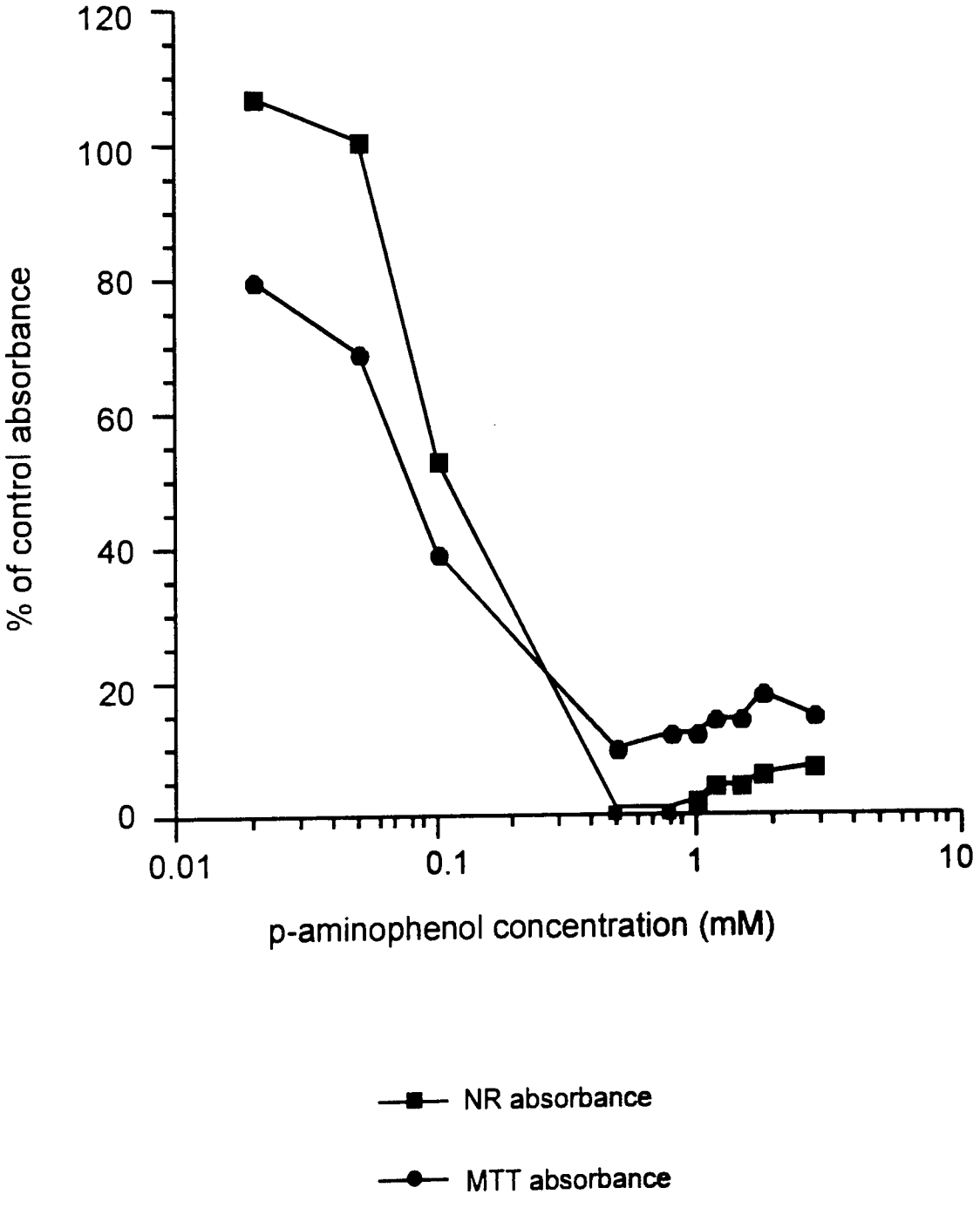


Figure 59. Effect of p-aminophenol on the viability of LLC-RK1 cells as measured by NR and MTT assays. Results of typical experiments are shown. n = 8 or 16 for each data point; typical SD values were 10 % or less.

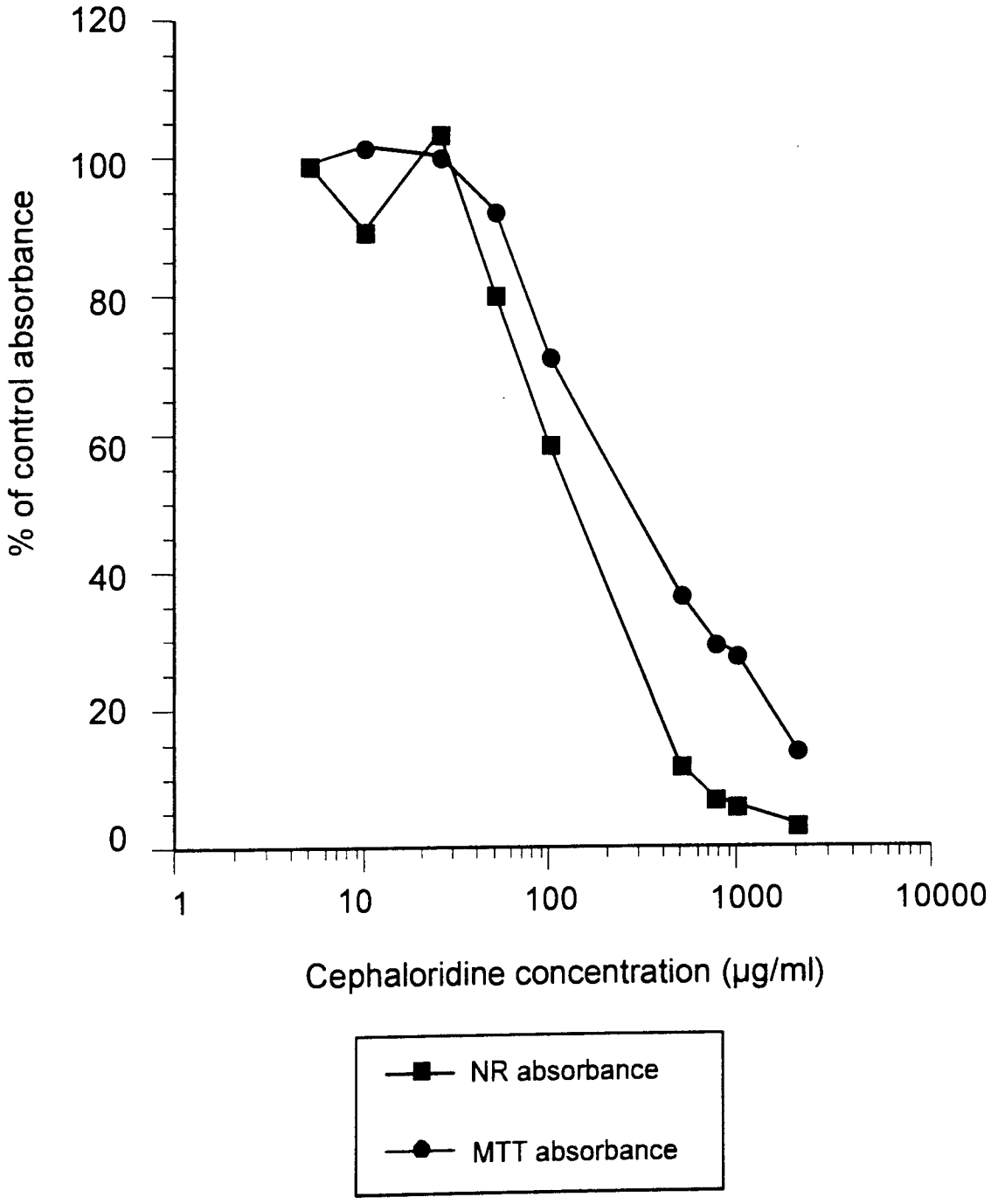


Figure 60. Effect of cephaloridine on the viability of LLC-RK1 cells as measured by NR and MTT assays. Results of typical experiments are shown. n = 8 or 16 for each data point. SD values were typically 10 % or less.

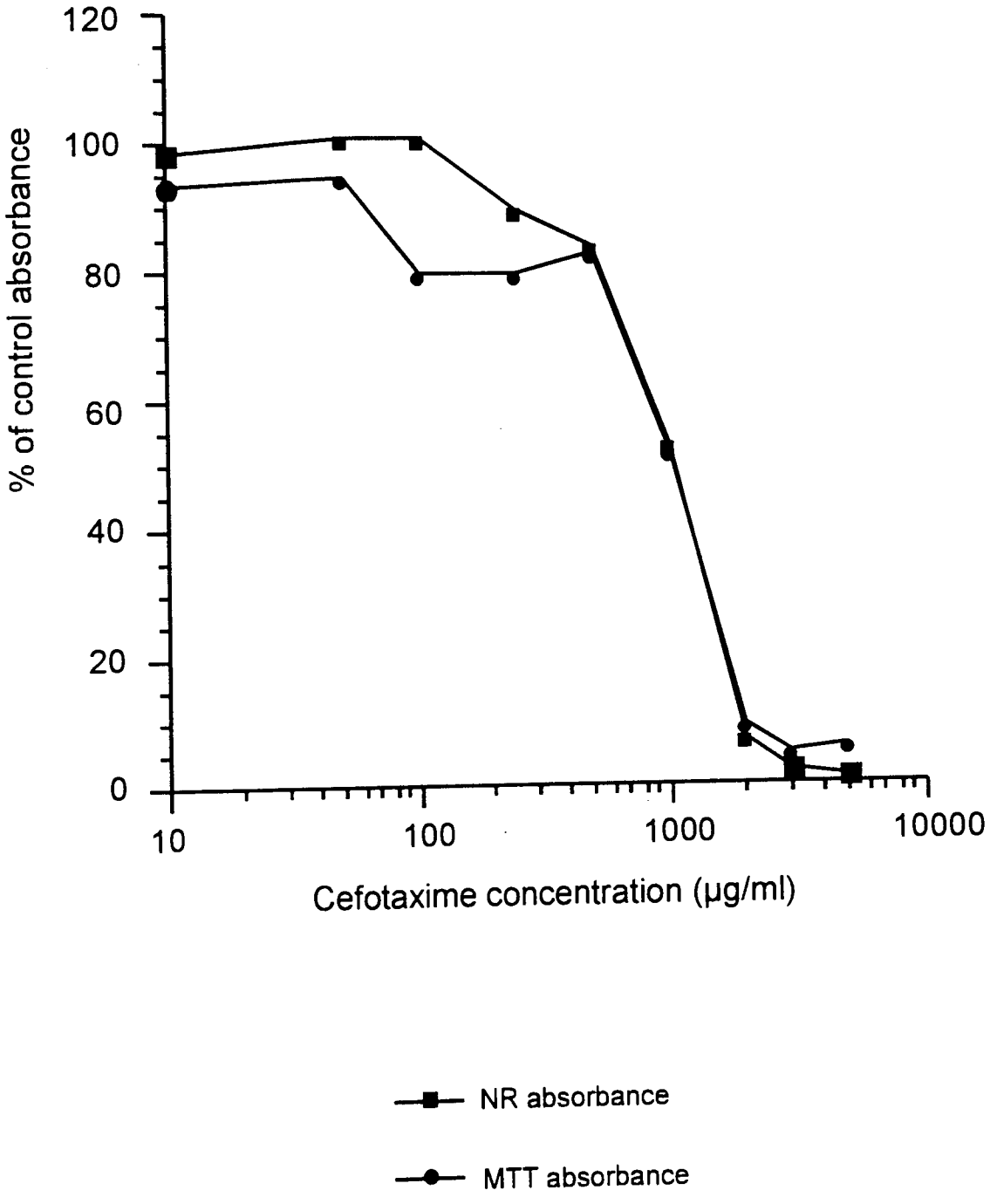


Figure 61. Effect of cefotaxime on the viability of LLC-RK1 cells as measured by NR and MTT assays. Results of typical experiments are shown. n = 8 or 16 for each data point; typical Sd values were 10 % or less.

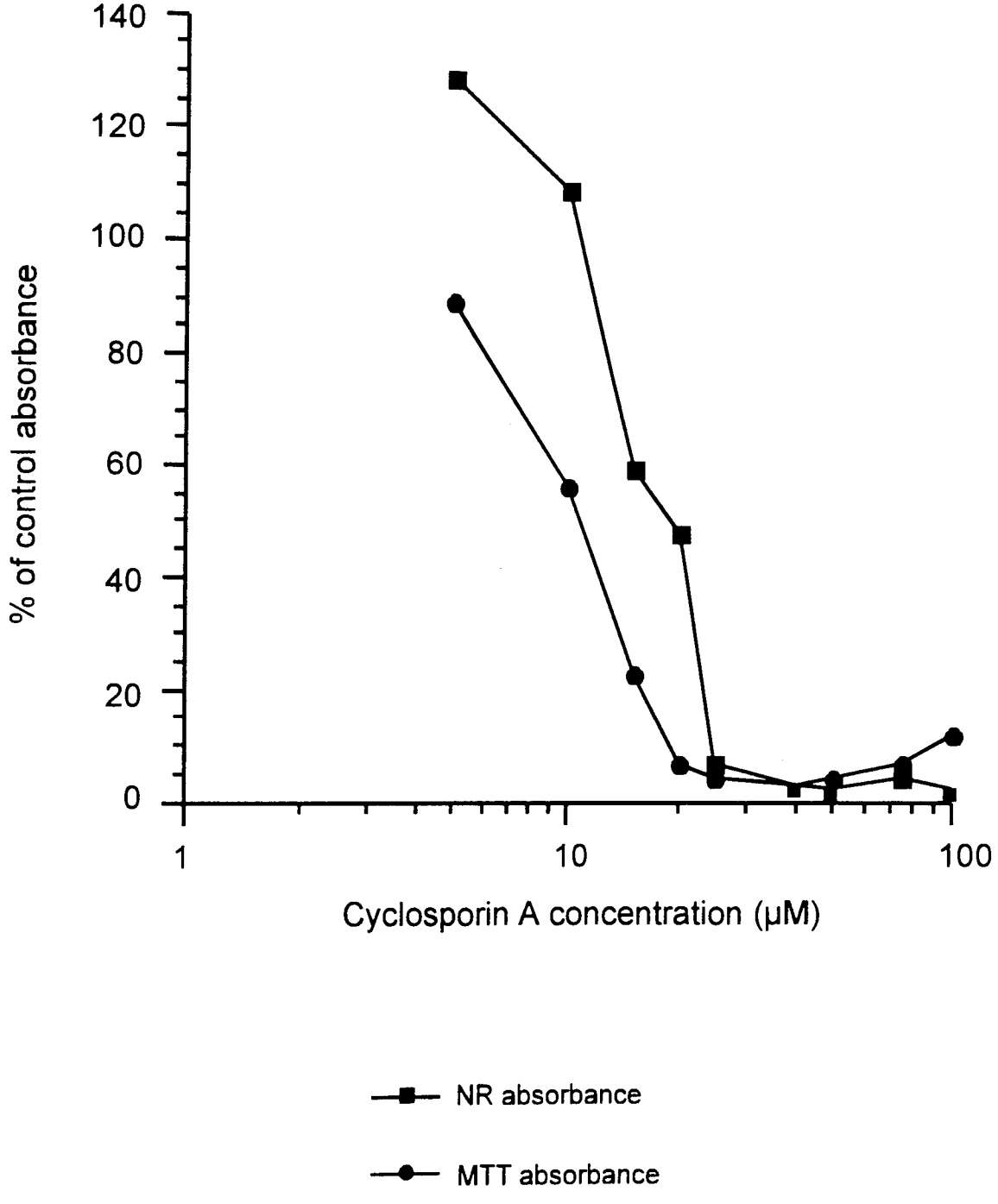


Figure 62. Effect of cyclosporin A on the viability of LLC-RK1 cells as measured by NR and MTT assays. Results of typical experiments are shown. n = 8 for each data point; SD values were typically 10 % or less.

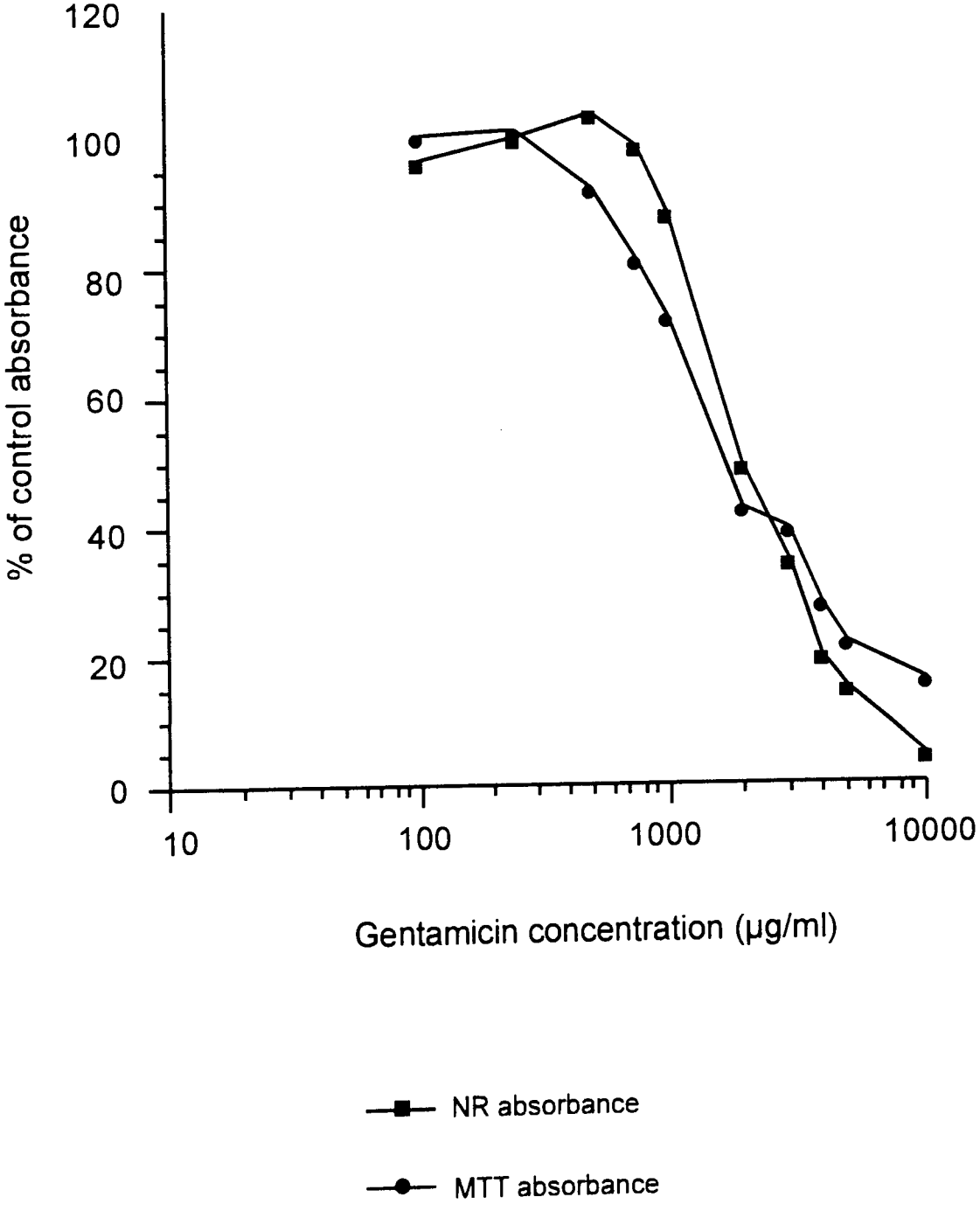


Figure 63. Effect of gentamicin on the viability of LLC-RK1 cells as measured by NR and MTT assays. Results of typical experiments are shown. n = 8 or 16 values for each data point; SD values were typically 10 % or less.

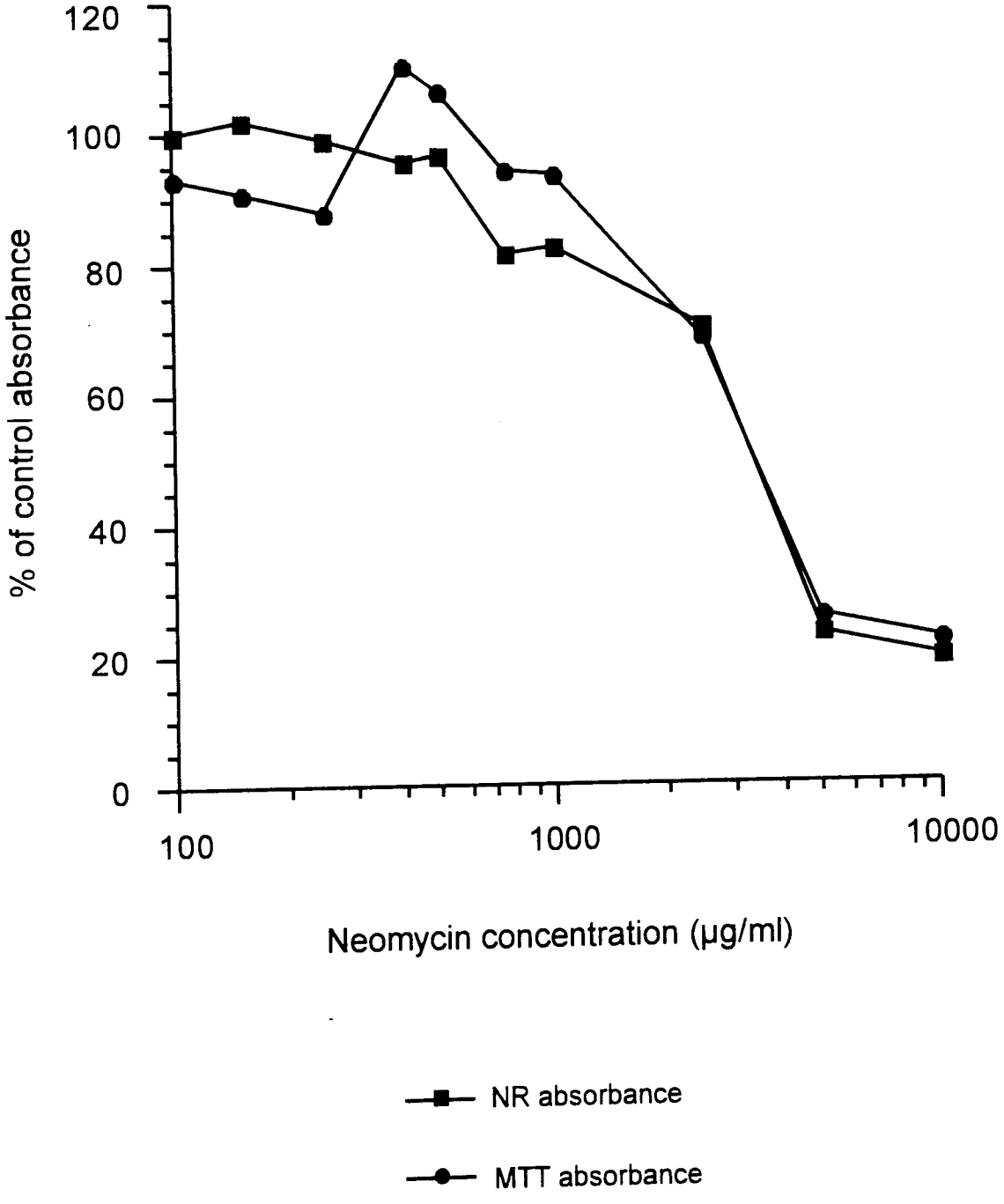


Figure 64. Effect of neomycin on the viability of LLC-RK1 cells as measured by NR and MTT assays. Results of typical experiments are shown. n = 8 or 16 for each data point; SD values were typically 10 % or less.

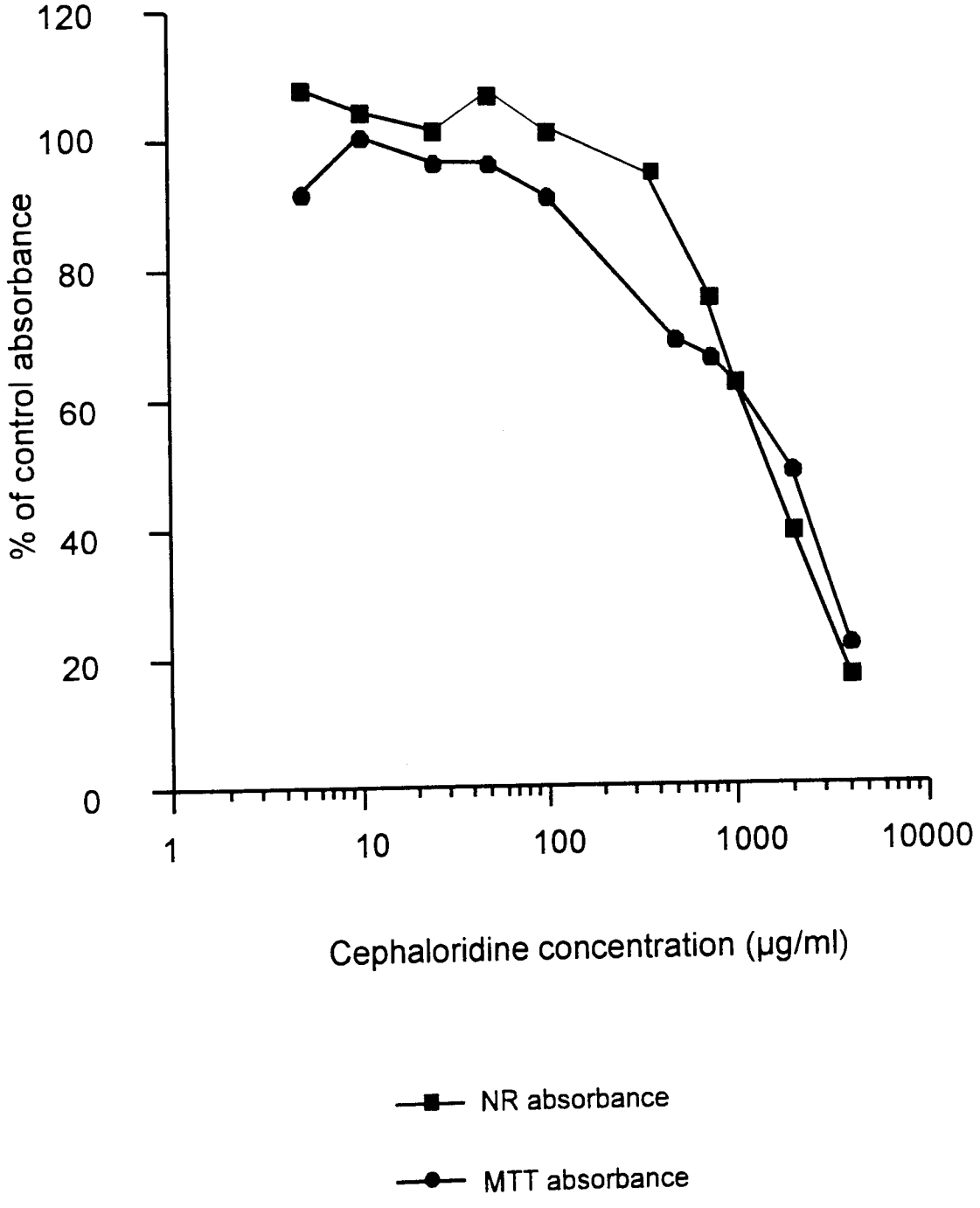


Figure 65. Effect of cephaloridine on the viability of LLC-PK1 cells as measured by NR and MTT assays. Results of typical experiments are shown. n = 8 or 16 for each data point. SD values were typically 10 % or less.

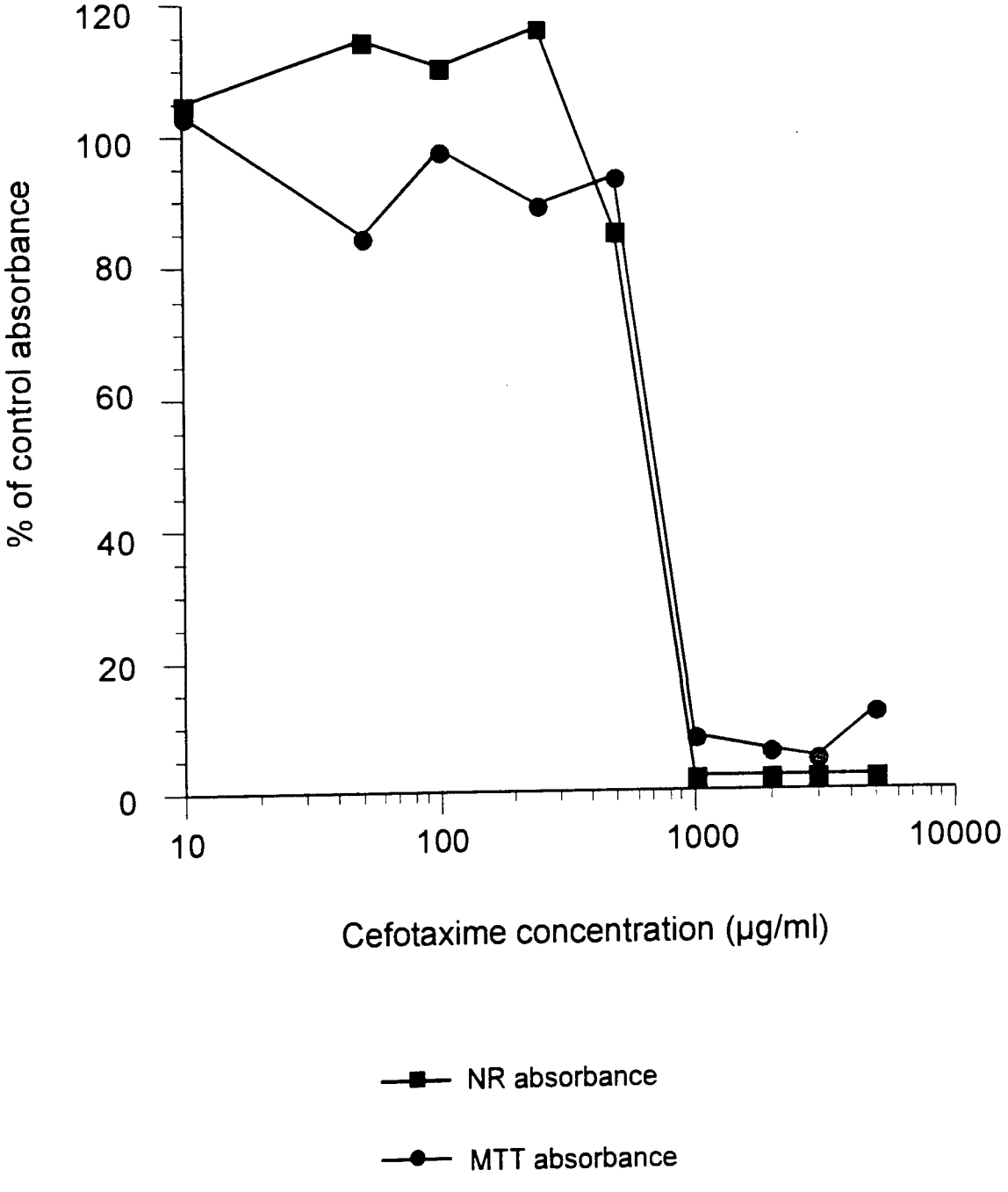


Figure 66. Effect of cefotaxime on the viability of LLC-PK1 cells as measured by NR and MTT assays. Results of typical experiments are shown. n = 8 for each data point; SD values were typically 10 % or less.

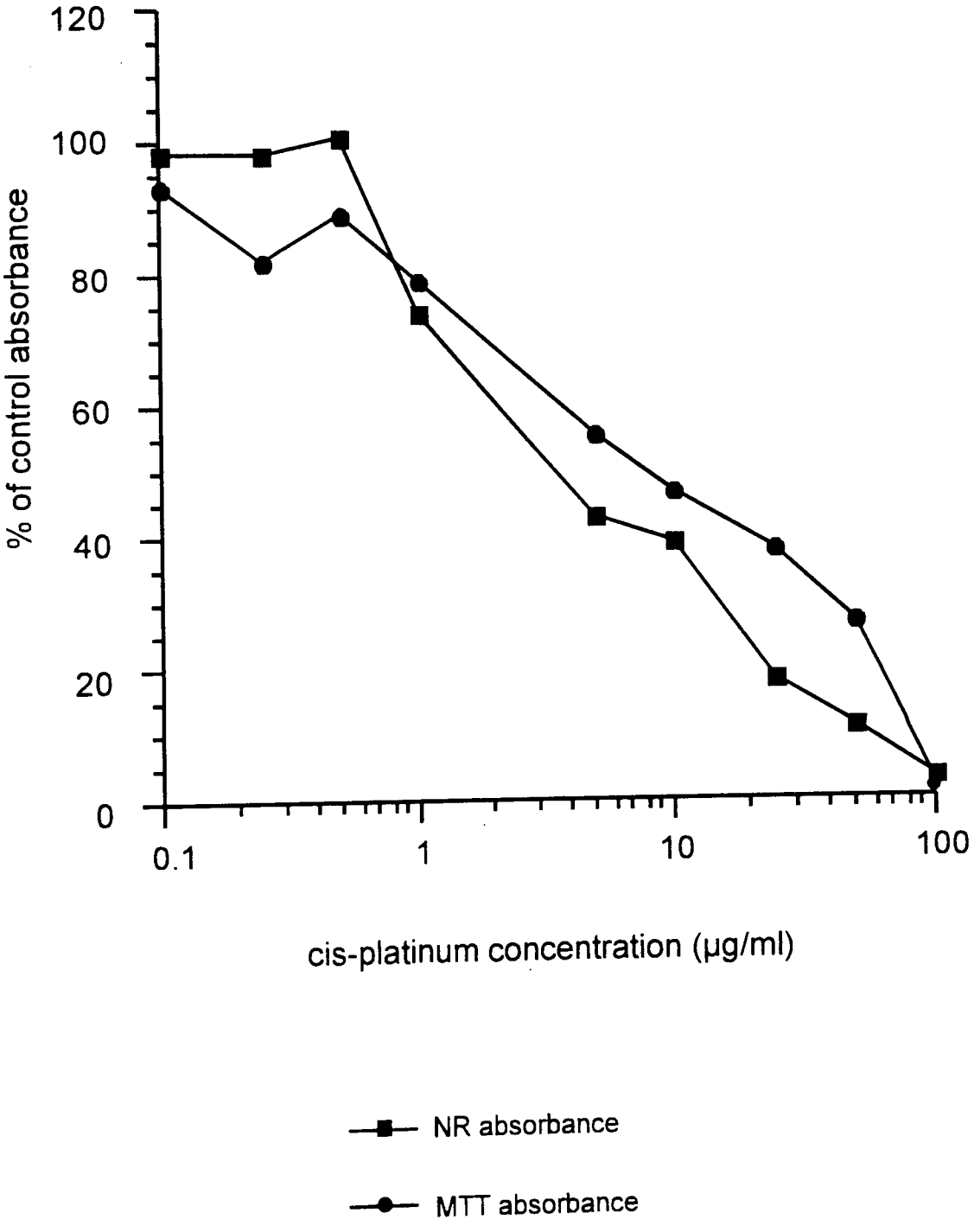


Figure 67. Effect of cis-platinum on the viability of LLC-PK1 cells as measured by NR and MTT assays. Results of typical experiments are shown. n = 8 or 16 for each data point; SD values were typically 10 % or less.

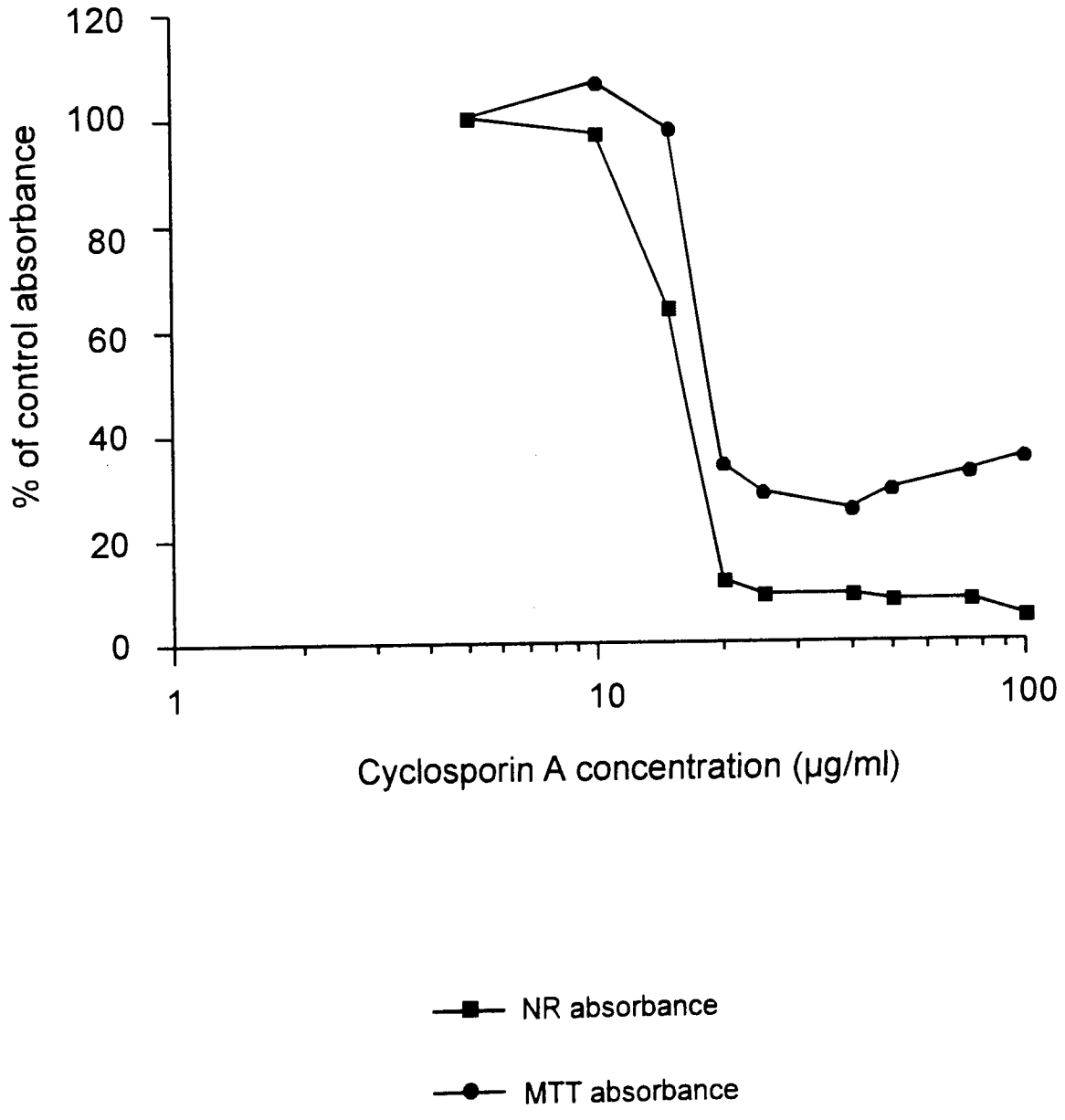


Figure 68. Effect of cyclosporin A on the viability of LLC-PK1 cells as measured by NR and MTT assays. Results of typical experiments are shown. n = 8 for each data point; SD values were typically 10 % or less.

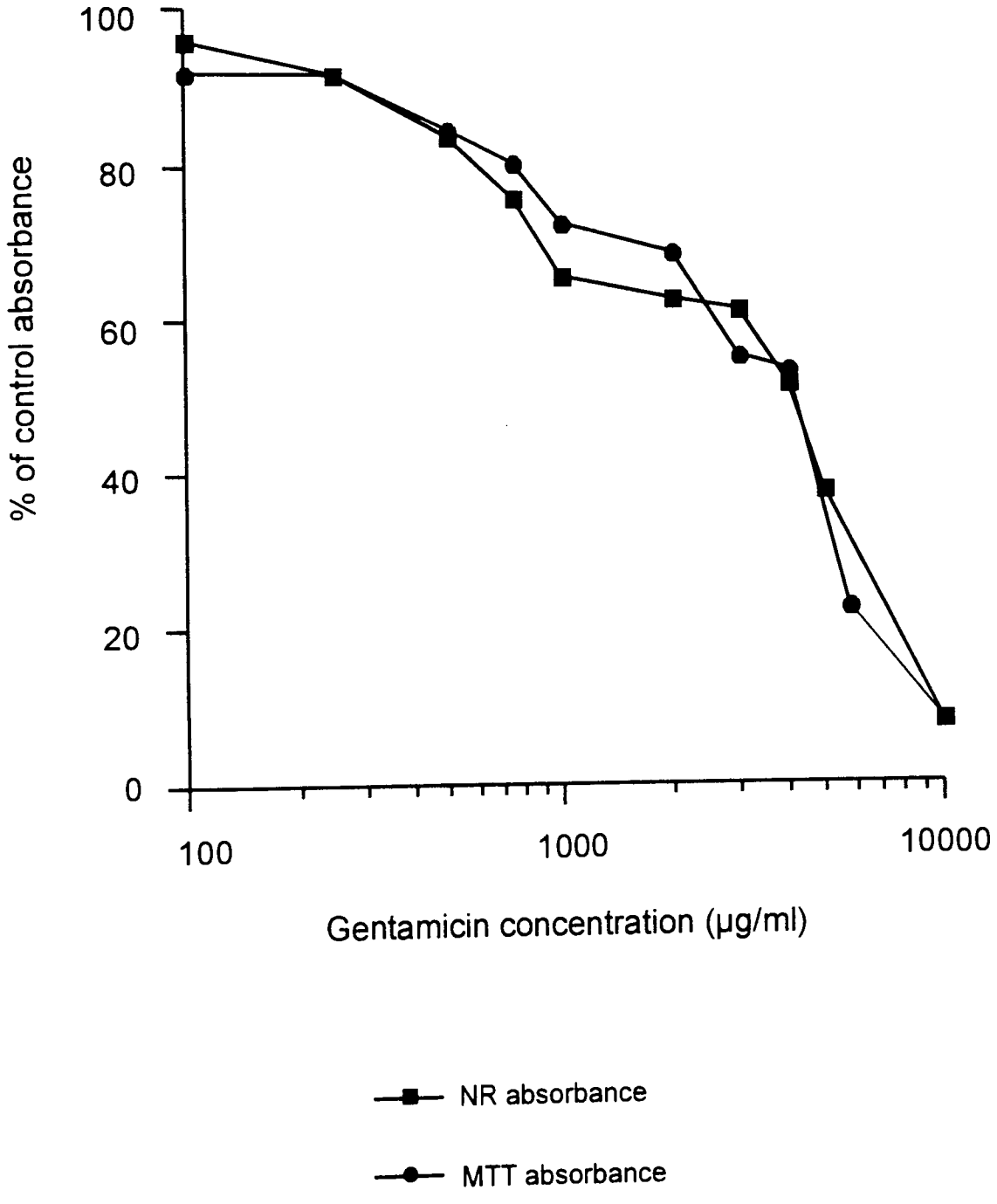


Figure 69. Effect of gentamicin on the viability of LLC-PK1 cells as measured by NR and MTT assays. Typical results are shown. n =8 for each data point; SD values were typically 10 % or less.

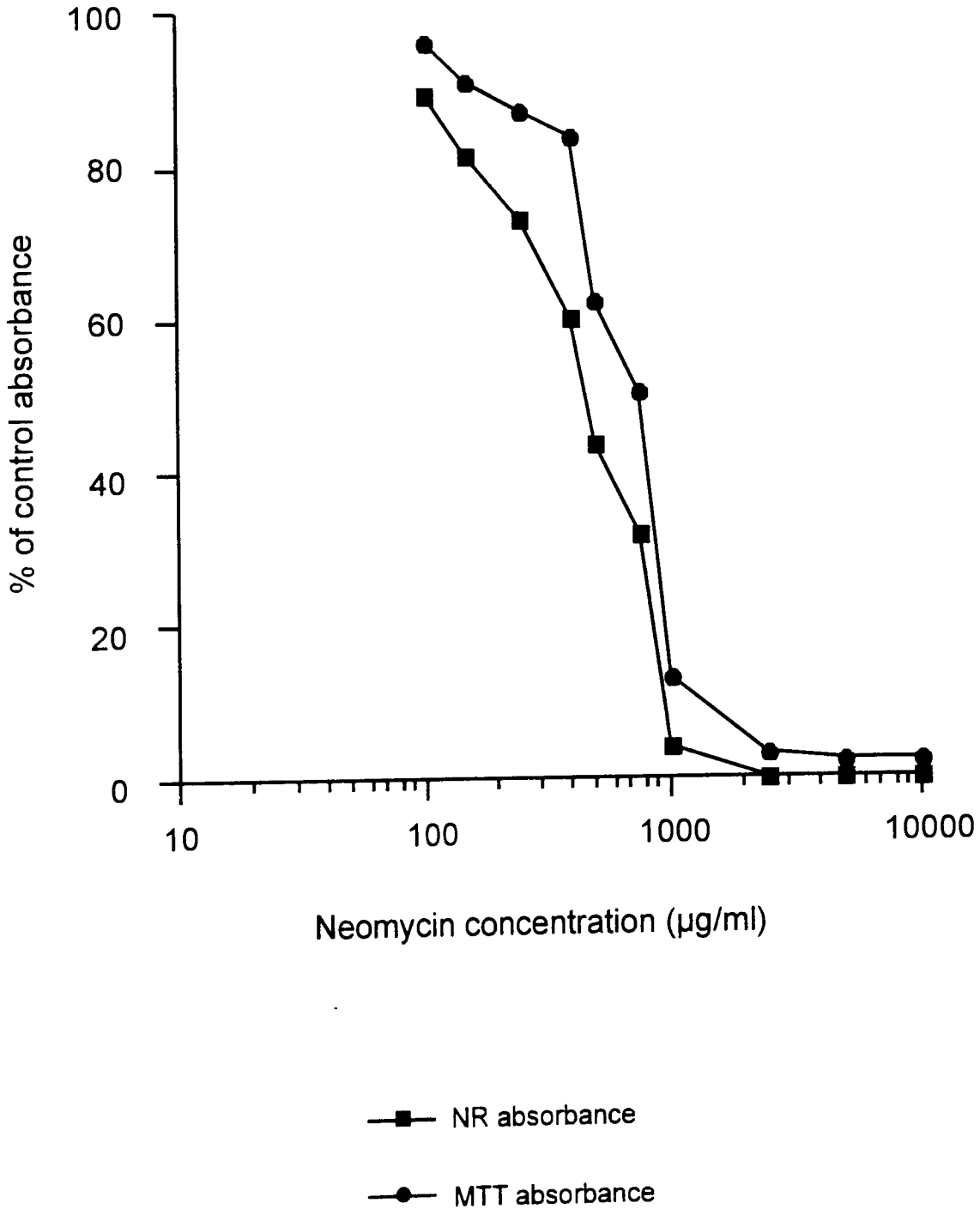


Figure 70. Effect of neomycin on the viability of LLC-PK1 cells as measured by NR and MTT assays. Typical results are shown. n = 8 for each data point; SD values were typically 10 % or less.

Table 10. IC50 values for nephrotoxins using LLC-RK1 cells, tested using NR and MTT assays. Values are the mean ±SD of 3 or 4 independent assays (each assay result being the mean of 8 or 16 replicates).

COMPOUND	EXPOSURE (h)	NR IC 50 (mM)	MTT IC 50 (mM)
cis-platinum	24	0.04 ± 0.01	0.03 ± 0.01
$HgCl_2$	24	0.05 ± 0.00	0.06 ± 0.01
p-acetamidophenol	24	18 ± 1.0	17 ± 2.3
p-aminophenol	24	0.23 ± 0.13	0.11± 0.03
Cephaloridine	24	0.80 ± 0.72	1.40 ± 1.30
Cefotaxime	24	3.40 ± 1.10	4.00 ± 1.80
Cyclosporin A	48	16 ± 4.0	19 ± 4.6
Gentamicin	48	7.60 ± 3.1	7.10 ± 2.80
Neomycin	48	>6.1*	5.40 ± 1.2

*Highest concentration tested

Table 11. IC50 values for various nephrotoxins using LLC-PK1 cells assessed using NR and MTT assays. Values are the mean +/- SD of at least 3 independent experiments.

COMPOUND	EXPOSURE (h)	NR IC50 (mM)	MTT IC50 (mM)
Cephaloridine	24	7.2 ± 2.3	4.5 ± 2.7
Cefotaxime	24	2.6 ± 1.2	2.0 ± 1.1
cis-platinum	24	0.027 ± 0.005	0.029 ± 0.017
Cyclosporin A	48	16.7 ± 3.5	18.3 ± 4.04
Gentamicin	48	10.4 ± 3.4	16.1 ± 4.7
Neomycin	48	[8.5 ± 0.9]	[11.6 ± 5.9]

Note: The results for the assays in which LLC-PK1 cells were exposed to Neomycin produced results which showed substantial interassay variation. The values in brackets above are the means of the only usable data (2/7) assays.

8.5 Discussion

The results of these studies suggest that the MTT and NR assays may be of equivalent value in assessing cytotoxicity in both the LLC-PK1 and the LLC-RK1 line. Despite the fact that the MTT and NR cytotoxicity assays measure different cellular functions, the IC50 values obtained showed a clear correlation. Williams et al (1988), also reported a good correlation between results obtained *in vitro* using the NR assay and a dye exclusion cell viability assay (although no data were shown). Additional refinement of the MTT assay by the use of DMSO and glycine buffer as the solvent for the MTT-formazan product as suggested by Plumb et al (1989) resulted in a substantial increase in the sensitivity of the assay compared to DMSO alone or the solvent originally used in the MTT assay, acid isopropanol (Mosmann, 1983). The height of the spectral peak of MTT formazan in DMSO is increased with increasing pH up to pH 10.5. Evidence from Plumb et al (1989), also pointed to the importance of optimising the MTT assay with respect to MTT concentration and cell number depending on the individual cell types under study. Their data showed that for some cell lines an excess of MTT resulted in a reduction in MTT-formazan production, as seen in this study with LLC-RK1 cells (figure 54). Cell seeding densities for both LLC-RK1 and LLC-PK1 were selected to ensure that the absorbance generated by that particular number of cells lay on the linear portion of the cell number vs absorbance curve. These studies clearly demonstrate the importance of optimising the experimental conditions of each cytotoxicity assay according to which cell-line is being used.

Studies which have contrasted the relative sensitivity of different cell preparations or cell lines to potentially nephrotoxic compounds have yielded interesting comparative information regarding the suitability of the LLC-PK1 and LLC-RK1 cell lines for toxicity studies. In a study comparing the sensitivity of a continuous cell line (LLC-PK1), freshly isolated renal tubule fragments, and primary tubule cultures, cis-platinum was shown to accumulate to a similar extent in the three cell preparations. Active uptake of radio-labelled cis-platinum was reduced by the action of metabolic inhibitors in all preparations

suggesting a qualitatively similar response for all three preparations; primary preparations of tubules however showed the most marked response to the metabolic inhibitors (Barron et al, 1989). Since the two preparations of newly isolated tubule cells might well be expected to represent the more sensitive *in vitro* model these data may suggest that the LLC-PK1 cell line is as appropriate as primary culture or freshly isolated cells for the *in vitro* study of acute nephrotoxins. However, in a study which compared the sensitivty of CHANG (human liver), SIRC (rabbit corneal) and LLC-PK1 cells to a range of aminoglycosides (Raczniak and Linseman, 1990) LLC-PK1 cells were shown to be the least sensitive of the three to gentamicin and neomycin toxicity suggesting that the toxic action of aminoglycosides may not be suitably mimicked by *in vitro* studies with LLC-PK1 cells, despite their renal origin. Additional evidence that the renal origin of LLC-PK1 cells does not confer particular selectivity or sensitivity to nephrotoxins was produced when the relative sensitivity of freshly isolated hepatocytes and LLC-PK1 cells to cisplatin was compared. *In vitro*, both cell types were shown to accumulate platinum to similar levels and required the same dose of cis-platinum to cause a 50% reduction in cell number suggesting no increase in sensitivity of LLC-PK1 cells to the toxic effects of this compound due to their renal origin. Both cell types correctly ranked the related compound carboplatin as being less toxic than cis-platinum (Poupon et al, 1990). In contrast, LLC-RK1 cells have been shown to be several times more sensitive to the toxicity of cephalosporins than the 3T3 fibroblast cell line (Duffy and McRae, 1990) suggesting that this cell line may represent a more appropriate model for the study of nephrotoxic injury than LLC-PK1 cells.

The data presented from the present study, however, suggest that whilst LLC-PK1 cells and LLC-RK1 cells appear to be equally sensitive to acute nephrotoxins, LLC-PK1 cells fail to assess the toxicity of compounds which generally require repeated administrationto produce their toxicity *in vivo* and, therefore, may be less suitable than the LLC-RK1 cell line for toxicity testing of potential nephrotoxins.

For all the compounds tested in these *in vitro* studies the IC50 value was many times higher than the clinically defined peak plasma concentration (range approximately 10-300x). Whilst it may not be entirely appropriate to directly compare these two parameters the comparative values may give some indication of the relative insensitivity of these *in vitro* tests. Obviously the *in vitro* system cannot take into account indirect changes caused by the test compounds which may increase the impact of their toxic effects on the tubular cells; for example alterations in the renal vasculature induced by cisplatinum (Daugaard et al, 1987). In addition, metabolism of the compound to its active toxic species may not occur *in vitro*, leading to an underestimate of the toxic potential of a compound.

In the studies presented in this chapter, since the effects on both types of cells were assessed by means of non-specific cytotoxicity assays, rather than by measuring alterations in specific functions of renal epithelial cells it is unlikely that these experiments will accurately model the mechanisms by which the test compounds produce their nephrotoxicity *in vivo*. Nevertheless, this *in vitro* system using the LLC-RK1 cell line produces data about the relative nephrotoxic potential of a range of compounds which supports information derived from whole animal studies. Since the difference in relative sensitivity of LLC-RK1 and LLC-PK1 cells to the toxic effects of aminoglycosides in particular appears equivocal, subsequent studies developing an *in vitro* model to study the reabsorption disorder caused by these drugs were carried out using both the LLC-PK1 and the LLC-RK1 cell lines. The results from those studies are presented in Chapter 9.

Chapter 9. Use of LLC-PK1 and LLC-RK1 cells grown on a semi-permeable insert system to investigate possible mechanisms of drug-induced alterations in cation transport.

9.1 Introduction

On attaining confluency polarised epithelial cells typically transport salt and water from the apical to the basal side of the monolayer. The presence of salts and water trapped between the basal side of the monolayer and the tissue culture plastic causes the monolayer to bulge forming a blister or dome in the monolayer. The presence of domes within a monolayer of cells is therefore indicative of cell-to-cell tight junctions and the ability of that cell line to transport solutes in a unidirectional manner. In agreement with other literature reports of this phenomenon (Hori et al, 1984, Williams et al, 1986) preliminary experiments in which confluent monolayers of LLC-PK1 and LLC-RK1 cells were exposed to gentamicin showed a dose-related reduction in the number of domes formed compared to control cultures. These early experiments therefore suggested that exposure to aminoglycosides could affect the integrity of the cell monolayer, possibly by affecting the permeability of the tight junctions between the cells. Alterations in the permeability of cell-to-cell junctions duly provoked interest as a potential mechanism by which aminoglycosides could affect calcium reabsorption, since the majority of calcium reabsorption, in the proximal tubule at least, is believed to be via a paracellular route (Rouse and Suki, 1990). In the thick ascending limb of the Loop of Henle although the majority of calcium reabsorption occurs transcellularly by active means, some paracellular reabsorption of calcium also occurs in this nephron segment (Suki et al 1980). Therefore, reductions in paracellular permeability could be a mechanism by which aminoglycosides affect calcium reabsorption both in the proximal nephron segments and in the thick ascending limb of the Loop of Henle. In the former drugs are known to accumulate and is the major site of histological damage (Kosek et al, 1974); in the latter, data from lithium clearance studies suggest gentamicin may also affect calcium reabsorption (Chapter 5).

9.2 Methods

9.21 Routine maintenance culture of cells.

LLC-RK1 cells (a gift from Paul Duffy (ICI); original source Flow Laboratories) with a doubling time of approximately 32 h, were routinely cultured in medium 199 with Hanks salts and 25mM Hepes buffer, supplemented with 10 % foetal calf serum and 2mM glutamine. LLC-PK1 cells (from American Type Culture Collection), with a doubling time of approximately 16h, were routinely cultured in medium 199 with Hanks salts and 25mM Hepes supplemented with 5% foetal calf serum and 2mM glutamine. No antibiotics were included in the culture medium. LLC-PK1 cells were split 1 : 10 twice per week; LLC-RK1 cells were divided by a split ratio of 1 : 4 twice per week. Cultures were maintained in a humidified atmosphere of 95% O_2 and 5% CO_2 at 37 °C.

9.22 Effect of gentamicin exposure on dome formation in confluent LLC-RK1 and LLC-PK1 monolayers.

Based on the report of Hori et al (1984), experiments were carried out to investigate the effect of gentamicin exposure on the ability of LLC-PK1 and LLC-RK1 cells to form the domes typical of transporting epithelial cells in culture, on the surface of a plastic culture dish. 5ml of LLC-PK1 cells at 8 x 10^4 /ml were seeded into duplicate 60 mm dishes on day 1 of the experiment. On the second day of the experiment the medium was removed and replaced with fresh medium containing 0 - 1.0 mM gentamicin. The dosing concentrations were selected to be below that of IC50 values in NR assays presented in Chapter 8. For comparative purposes a single experiment was also undertaken using a range of concentrations (0 - 0.5 mM) of cephaloridine using the same experimental conditions. Following a further 48h exposure to these conditions the cultures were harvested for quantitation of the number of domes in each culture. Medium was removed, and the monolayer washed with 1 x 10ml PBS. The cultures were fixed with 2ml of 5% gluteraldehyde in PBS for 15 min followed by staining with 2ml of Giemsa stain (BDH) for 2 minutes. The stain was aspirated off, and the cultures washed with 3 x

10 ml distilled water, before air-drying. The number of domes in each dish was counted in duplicate under an inverted microscope.

9.23 Growing cultured cells on semi-permeable inserts.

Culturing cells on inserts allows independent access to both the apical and the basolateral face of the cell. This arrangement therefore increases the usefulness of the cells by allowing study of transport processes from one compartment to the other.

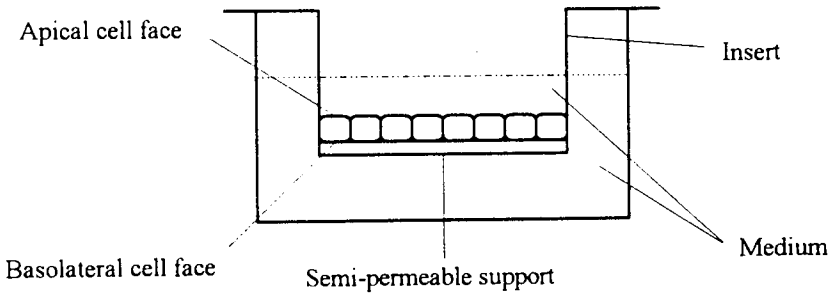


Diagram to show the arrangement of cells set up on a semi-permeable insert.

Preliminary Studies

LLC-PK1 cells were harvested using trypsin/EDTA solution in Puck's saline (Gibco, UK) into a single cell suspension from stock flasks and seeded onto Anocell and Falcon 25 mm^2 inserts at a range of cell seeding concentrations (1×10^5 - 4×10^5) in medium 199 with Hanks salts and 25mM Hepes supplemented with 5% foetal calf serum and 2mM glutamine. Inserts containing 2ml of the cell suspension were placed into the wells of a 6-well plate; each well contained 3ml of medium. Observations, using an inverted microscope, on the development of the monolayer over time showed that whilst the monolayer developed adequately over the insert there were also a variable number of peduncle-like structures attached to the monolayer (see Figure 71). These structures appeared to be multicellular and were apparent on the inserts from both manufacturers. Their presence has also been observed in LLC-PK1 cultures grown on agar-coated tissue culture plastics (Andersen et al, 1993). The presence of these protrusions appeared to

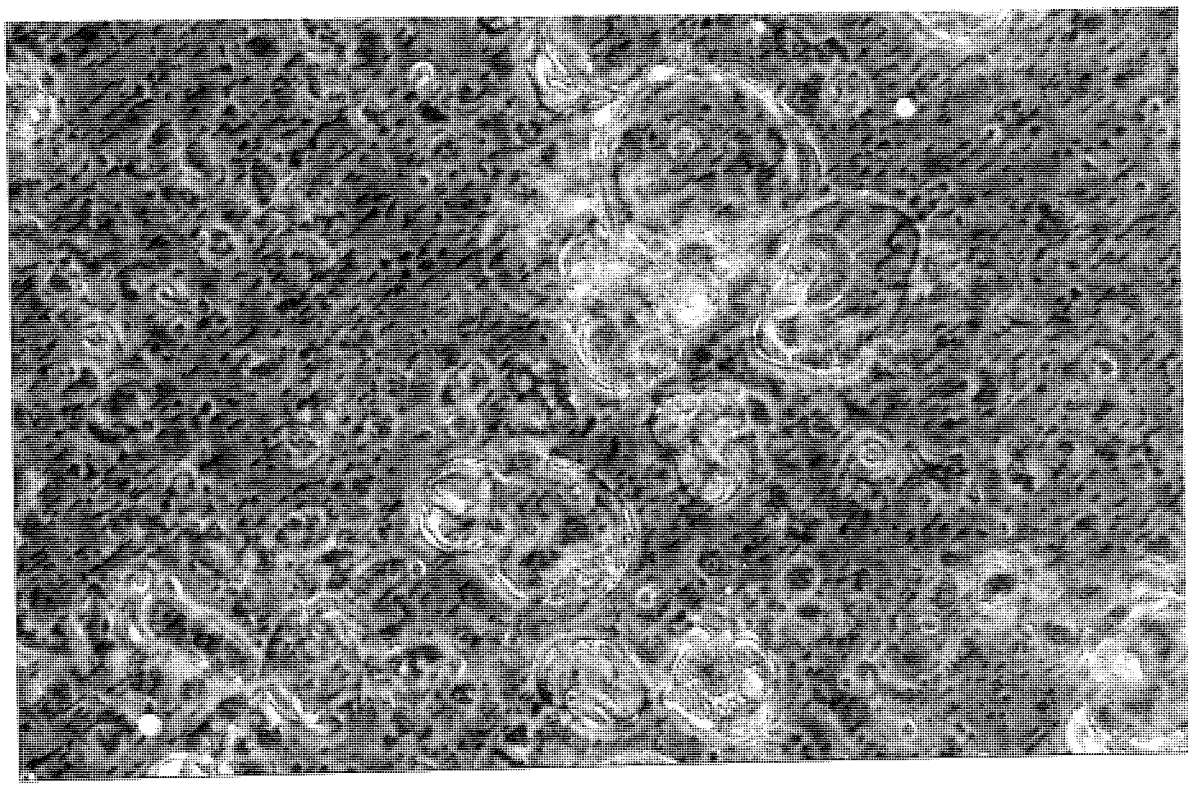


Figure 71. Light micrograph showing peduncles arising from a confluent monolayer of LLC-PK1 cells grown on Falcon semi-permeable inserts

not disrupt the integrity of the monolayer from which they arose. The peduncles were firmly attached to the monolayer; ordinary washing of the monolayer with medium or PBS did not remove them. Further preliminary studies were therefore carried out to investigate possible explanations for the presence of these peduncles and to produce strategies to remove, or minimise their number. Since the inserts available from Falcon had better optical qualities than those available from other manufacturers further investigations were carried out on these inserts only.

The following points were observed from these preliminary experiments:

- Cells harvested from stock cultures which were only 70-80 % confluent produced the same number of peduncles as those cells taken from stock cultures which were fully confluent.

- The number of peduncles on the individual inserts did not appear to be dependent on the initial seeding density. Studies in which lower seeding densities were used and observed over longer periods (up to 6 days) showed no reduction in peduncle number compared those initially seeded at a higher density, over the entire observation period.

- The introduction of a medium change step at 3 or 24 h after initial seeding did not affect number of peduncles formed, but the medium change at 3h after seeding reduced the number of cells attached as a monolayer to the insert, causing an apparent lag before the coverage over the insert equalled that of cells which did not have a medium change.

- The number of peduncles, once formed, declined over the observation period (up to 6 days). In experiments which continued over several days a complete medium change (both apical and basal compartments) was carried out on alternate days.

- The highest density of peduncles appeared to be toward the centre of the inserts.

- The possibility that the formation of peduncles was due to using a cell suspension which contained clumps of cells was investigated. By passing the LLC-PK1 cell suspension through a 19 G needle this appeared to slightly reduce the number of peduncles present on the inserts. This additional step was therefore incorporated into the standard seeding protocol.

The effect of seeding density of LLC-RK1 cells on peduncle formation on Falcon inserts was investigated. Initial seeding concentrations from 1-4 x 10^5 /ml were observed for up to 6 days after seeding. The seeding density did not appear to influence the number of peduncles produced in the LLC-RK1 cell line. In comparison to the LLC-PK1 cells, LLC-RK1 initially formed fewer peduncles when seeded onto Falcon inserts; these peduncles later became disassociated from the monolayer as confluency was attained. There were therefore fewer peduncles on the inserts seeded with LLC-RK1 cells compared to LLC-PK1 cells at the stage where the inserts would be used for transport studies (144h).

Following these preliminary studies the following protocol was used; After harvesting from a stock flask both LLC-PK1 and LLC-RK1 cells were passed through a 19 G needle prior to counting in a haemocytometer and subsequent seeding onto inserts. Both LLC-PK1 and LLC-RK1 cells were seeded onto Falcon inserts in 2ml of medium at 4 x 10^5 cells/ml.

9.24 Studies on the formation and integrity of cell monolayers on inserts

a) Growth curves for LLC-PK1 and LLC-RK1 cells grown on Falcon Inserts.

Inserts were seeded with 2ml of cell suspension of either LLC-PK1 or LLC-RK1 cells at 4 x 10^5 cells/ml. At intervals of 24h for a total of 192h one pair of inserts seeded with each cell line was washed with 2 x 3ml PBS without calcium and magnesium, followed by trypsinisation in 1 ml of trypsin/EDTA solution in Pucks's saline, for 10-15 min (as required) until all the cells were detached from the surface of the insert. The cell

suspension was aspirated from the insert and cell number was determined using a haemocytometer.

b) Rate of diffusion of ^{3}H-mannitol across Falcon inserts

Duplicate inserts were set up to investigate the time course over which ^{3}H-mannitol diffuses between the apical and basal compartments of inserts without cells. 2 ml of 0.1 µCi/ ml ^{3}H-mannitol in a carrier of 0.2 mM cold mannitol in PBS was added to the apical compartment of the inserts and duplicate 25 µl aliquots were taken from the basal compartment for counting at intervals (30 min-24h).

c) Effect of time after seeding on ^{3}H-mannitol exclusion by monolayers of LLC-PK1 and LLC-RK1 cells.

Movement of ^{3}H-mannitol from the apical to the basal compartment of the insert was measured as a means of assessing the integrity of the tight junctions of LLC-PK1 and LLC-RK1 cell monolayers. Both cell types were seeded in 2ml of medium at a density of 4 x 10^5/ml onto Falcon inserts and cultured as described in Section 9.23 for a range of periods (24-168 h). After the appropriate interval the ability of the monolayer to exclude ^{3}H-mannitol, added apically, from the basal compartment was assessed as follows. 2ml of PBS (with calcium and magnesium), supplemented with 0.2 mM mannitol and 0.1 µCi ^{3}H-mannitol was added to the apical compartment of the insert. The basal side of the insert was placed in 3 ml of the mannitol-supplemented PBS, without the radioactive mannitol. Inserts were placed in a humidified incubator at 37 °C, and duplicate 50 µl samples were removed from the basal compartment after 30 min and 1 h. The samples were mixed with 10 ml of Ultima Gold scintillant and counted on a Canberra Packard CA2200 Liquid Scintillation analyser for 10 min. Diffusion across inserts with cells present was compared to the diffusion which occurred in the absence of cells and expressed as a percentage.

d) Effect of time after seeding on the ability of LLC-PK1 and LLC-RK1 monolayers to exclude ^{3}H-inulin.

Movement of ^{3}H-inulin from the apical to the basal compartment of the insert was used as a means to assess the integrity of the tight junctions in monolayers of LLC-PK1 and LLC-RK1 cells. A solution containing 0.1 μCi ^{3}H-inulin with 5 μM cold inulin in PBS containing calcium and magnesium was prepared for use. Medium was tipped off the apical face of the inserts and both the apical and basal sides of the insert were washed with 2 x 3 ml PBS containing calcium and magnesium. Duplicate inserts for each experimental condition (each day after seeding) were then placed into the wells of a 6-well plate each containing 3 ml PBS. 2 ml of PBS containing ^{3}H-inulin was then added to the apical side of the insert. The plates were returned to a humidified incubator at 37°C and duplicate 50 μl samples were removed from the basal compartment at 30 min and 1 h intervals for scintillation counting. The samples were added to 10 ml of Ultima Gold scintillation fluid and counted for 10 min on a Canberra Packard 2200CA Liquid Scintillation analyser. Movement of ^{3}H-inulin across the monolayer was compared to movement of ^{3}H-inulin across an insert without cells, and expressed as a percentage.

9.26 Characterisation of ^{45}Ca movement across inserts.

a) Experiment to determine the rate of diffusion of ^{45}Ca as $CaCl_2$ across empty Falcon inserts.

The aim of this experiment was to determine time points at which a measurable amount of ^{45}Ca activity could be detected in the compartment opposite to which it was originally placed. Duplicate inserts were prepared to determine the rate of flux of ^{45}Ca from the apical to the basal compartment of the insert and vice versa; experiments were carried out using calcium-free PBS, and PBS containing calcium and magnesium. PBS containing 0.1 μCi/ml ^{45}Ca-$CaCl_2$ was added to either the apical compartment (2 ml) or the basal (3 ml) compartment using duplicate inserts for each experimental condition. The opposite compartment from that containing the radioactive calcium contained either

2ml 'cold' PBS (apical compartment) or 3ml PBS (basal compartment). Duplicate 25 µl samples were taken at a range of intervals (0-24h) after setting up the inserts, from the compartment opposite to which the ^{45}Ca was originally added. The experiment was carried out in a humidified incubator at 37 °C. Samples were added to 10 ml of Ultima Gold scintillation fluid and counted for 10 min on a Canberra Packard CA2200 liquid scintillation analyser. The counts obtained were corrected for volume changes over the duration of the experiment and are shown in Table 14.

b) Effect of varying calcium concentration on ^{45}Ca transport across a monolayer of cells grown on inserts.

LLC-PK1 and LLC-RK1 cells were seeded at a concentration of 2 ml cells at 4 x 10^5 per insert and grown as detailed in section 9.23. After 144 h the initial seeding medium was removed from both sides of the inserts, the inserts were washed once with 3 ml PBS without calcium and magnesium, and PBS containing varying concentrations (0.5-10 mM) of $CaCl_2$ was added; Magnesium concentration remained constant at 0.5 mM. ^{45}Ca was added to the PBS on the apical side of the inserts at an equivalent specific activity of ^{45}Ca (0.1 µCi/mM) for each $CaCl_2$ concentration. Duplicate 50 µl samples were taken from the basal compartment of each insert at 30 min and 1 h intervals to measure the amount of ^{45}Ca transport which occurred in the presence of different $CaCl_2$ concentrations. ^{45}Ca movement was compared to movement which occurred in the absence of cells on an insert and typical results are presented in section 9.33b

The effects of increasing the calcium concentration of the PBS solution on cellular viability was assessed by the ability of the cells to exclude Trypan Blue. Duplicate inserts were seeded with LLC-PK1 and LLC-RK1 cells as described earlier, and grown to 144h after seeding. Cells on the inserts were then exposed both apically and basally for 1 h to varying concentrations of $CaCl_2$, at 37 °C, in a humidified incubator. The supplemented PBS was then removed and both sides of the inserts washed with 1 x 3 ml PBS without calcium and magnesium. The monolayers were then trypsinised with 0.75 ml

Trypsin/EDTA solution for 10 or 20 min (LLC-RK1 and LLC-PK1 respectively) and 0.75 ml of 0.4 % Trypan Blue solution added. Cells were counted using a haemocytometer and the proportion of the total which excluded the dye was calculated and compared to the viability of cells which had been treated under the identical conditions but in the presence of commercially available calcium-containing PBS ($CaCl_2$ content 1.2 mM).

c) Effect of temperature on the rate of movement of ^{45}Ca across monolayers of LLC-PK1 and LLC-RK1 cells

The effect of temperature on ^{45}Ca movement across a monolayer was investigated at 4 °C, 37 °C, and 40 °C at intervals over a time course of 2 hours. ^{45}Ca in PBS (specific activity 0.1μCi/ml) and ^{3}H-mannitol was added to the apical compartment of duplicate LLC-RK1 and LLC-PK1 cells 144h after seeding, and movement of ^{45}Ca and ^{3}H-mannitol into the basal compartment measured.

d) Effect of ouabain and KCN exposure on ^{45}Ca movement

LLC-PK1 and LLC-RK1 cells at 144h after seeding were exposed, both apically and basally, to ouabain (1 and 5 mM) and, in separate experiments, to 3 mM KCN to investigate whether there was a discernible active component to the ^{45}Ca movement occurring across cells grown on inserts. In the presence of KCN or ouabain, 2 ml PBS containing calcium and magnesium, and supplemented with 0.1 μCi/ ml ^{45}Ca was added to the apical face, while the basal face of the cells were exposed to 3ml PBS only. Duplicate 50 μl samples were taken from the basal compartments after 30 min and 1 h exposure to the inhibitors and counted for ^{45}Ca activity. ^{45}Ca movement across inserts in the presence of ouabain or KCN was compared to ^{45}Ca movement in the absence of inhibitors.

9.27 Effect of drug exposure on monolayer permeability.

a) Effect of gentamicin exposure on ^{45}Ca movement across monolayers of LLC-PK1 and LLC-RK1 cells.

The aim of the experiments presented in this section was to investigate the effects of gentamicin exposure on the ^{45}Ca movement across monolayers of LLC-PK1 and LLC-RK1 cells; in addition the investigations were designed to discover whether any effects gentamicin had on ^{45}Ca movement could be attributed to alterations in the junctional permeability of the cells in the monolayer, as measured by ^{3}H-mannitol exclusion. A supplementary enquiry was whether the two cell lines responded differently to gentamicin exposure with respect to calcium movement across the monolayer.

This set of experiments was therefore broadly divided into two segments:

1. Exposure of cells to gentamicin for 24 or 48 h followed by assessment of ^{45}Ca and ^{3}H-mannitol movements across the monolayer.

LLC-PK1 and LLC-RK1 cells were cultured on inserts as previously described up to 120 h after seeding. At 120 h after seeding the medium on both sides of the insert was replaced with fresh medium containing gentamicin (1-7.5 mM) and experiments assessing transepithelial ^{45}Ca and ^{3}H-mannitol movement were carried out 24 or 48 hours later. After the addition of gentamicin to the medium the pH of the medium was adjusted where necessary using KOH. Inserts which had been cultured in the presence of gentamicin were then washed with 2 x 3 ml PBS on both sides of the inserts and 0.1 µCi/ml ^{45}Ca and 0.1 µCi/ml ^{3}H-mannitol in 2 ml of PBS was added to the apical compartment. Duplicate 50 µl samples were taken from the basal compartment for scintillation counting of both isotopes, 30 min and 1 h after addition of the solution to the inserts. Each experimental condition was prepared in duplicate for every experiment.

2. Exposure of cells to gentamicin for short periods (up to 1 h) with concurrent measurement of ^{45}Ca and ^{3}H-mannitol movements in the presence of gentamicin.

These investigations were further divided into experiments where the cells were exposed to gentamicin on both sides of the monolayer, or on the apical or basal face only.

In this series of experiments LLC-PK1 and LLC-RK1 cells were cultured on Falcon inserts to 144 h after seeding, as described previously. The medium surrounding the cells was then replaced by PBS containing calcium and magnesium plus a range of concentrations of gentamicin (1-50 mM), along with 0.1 μCi/ml ^{45}Ca and 0.1 μCi/ml ^{3}H-mannitol in 2 ml of PBS solution added to the apical compartment only. Duplicate 50 μl samples were taken from the basal compartment (which contained 3 ml PBS) for scintillation counting of both isotopes, 30 min and 1 h after addition of the solution to the inserts. Gentamicin was added to the apical compartment, the basal compartment, or both, depending on the experimental design. Each experimental condition was prepared in duplicate for every experiment.

9.28 Statistical Analysis

Data was analysed using a two-tailed Student's t-test for unpaired data, using Excel V4 (Microsoft, UK). Data was taken to be statistically different at p values of 0.05 or less.

9.3 Results

9.31 Effect of gentamicin exposure on dome formation in confluent LLC-RK1 and LLC-PK1 monolayers.

Exposure of both LLC-PK1 and LLC-RK1 cells for 48 h to gentamicin resulted in a dose-related inhibition of dome formation. A small inhibition (approximately 10 %) of dome formation was apparent after the cells were exposed to 0.1mM gentamicin; a concentration which represents only 1/70th of the IC50 value determined by NR assay (see Chapter 8). In contrast, after exposure to cephaloridine (a potent nephrotoxin which does not induce renal cation wasting) inhibition of dome formation was not apparent until the drug concentration was raised to 1/20th of the IC50 value; representing a 3.5-

fold difference between the compounds in the concentrations required to produce a similar reduction in dome formation. These preliminary results suggested that gentamicin could either alter junctional permeability, or otherwise decrease ion and water transport across the monolayer, at concentrations well below those affecting cell viability, and therefore prompted further development of an *in vitro* model in which to study this phenomenon.

Table 12. Effect of exposure to nephrotoxins on dome formation in LLC-PK1 cells grown on 60 mm Sterilin dishes. Each group of data represents a typical set of results for the compound. (Control cultures typically contained 600-700 domes per dish)

a) Gentamicin

Concentration (mM)	% Inhibition
0	0
0.01	0
0.10	12
0.50	62
1.00	88

b) Cephaloridine

Concentration (mM)	% Inhibition
0	0
0.025	2
0.05	71
0.10	98
0.5	100

9.32 Studies on the formation and integrity of cell monolayers on inserts.

a) Growth curves for LLC-PK1 and LLC-RK1 cells grown on Falcon Inserts.

Following the seeding of the Falcon inserts with 2 ml of cells at 4 x 10^5/ml for both LLC-PK1 and LLC-RK1 cell lines, a maximum cell number of approximately 1.2 x 10^6 was attained by LLC-PK1 cells 24h after seeding and after 48h for LLC-RK1 cells. The cell number then remained stable at approximately 1.2 x 10^6 over the time period studied (up to 192h) for both cell lines.

b) Rate of diffusion of ^{3}H-mannitol across Falcon inserts

At the 24h sampling point measurements of basal to apical ^{3}H-mannitol movement were similar to those shown below for apical to basal diffusion (2552 cf. 2376 cpm), indicating equilibration of ^{3}H-mannitol activity between the two compartments. Equilibration of ^{3}H-mannitol between apical and basal compartments is therefore achieved by 24 hours after its addition to one compartment only. However adequate cpm can be measured in the basal compartment, with a 50 µl sample size, after 30 min and 1 h diffusion allowing experimental sampling at these intervals.

Table 13. Diffusion of ^{3}H-mannitol from the apical to the basal compartment of empty inserts; 25 µl sample size. Values are the mean of duplicate inserts.

Sampling time (h)	Corrected cpm (measured in the basal compartment.)
0.5	339
1	548
2	1083
4	1665
6	1875
24	2552

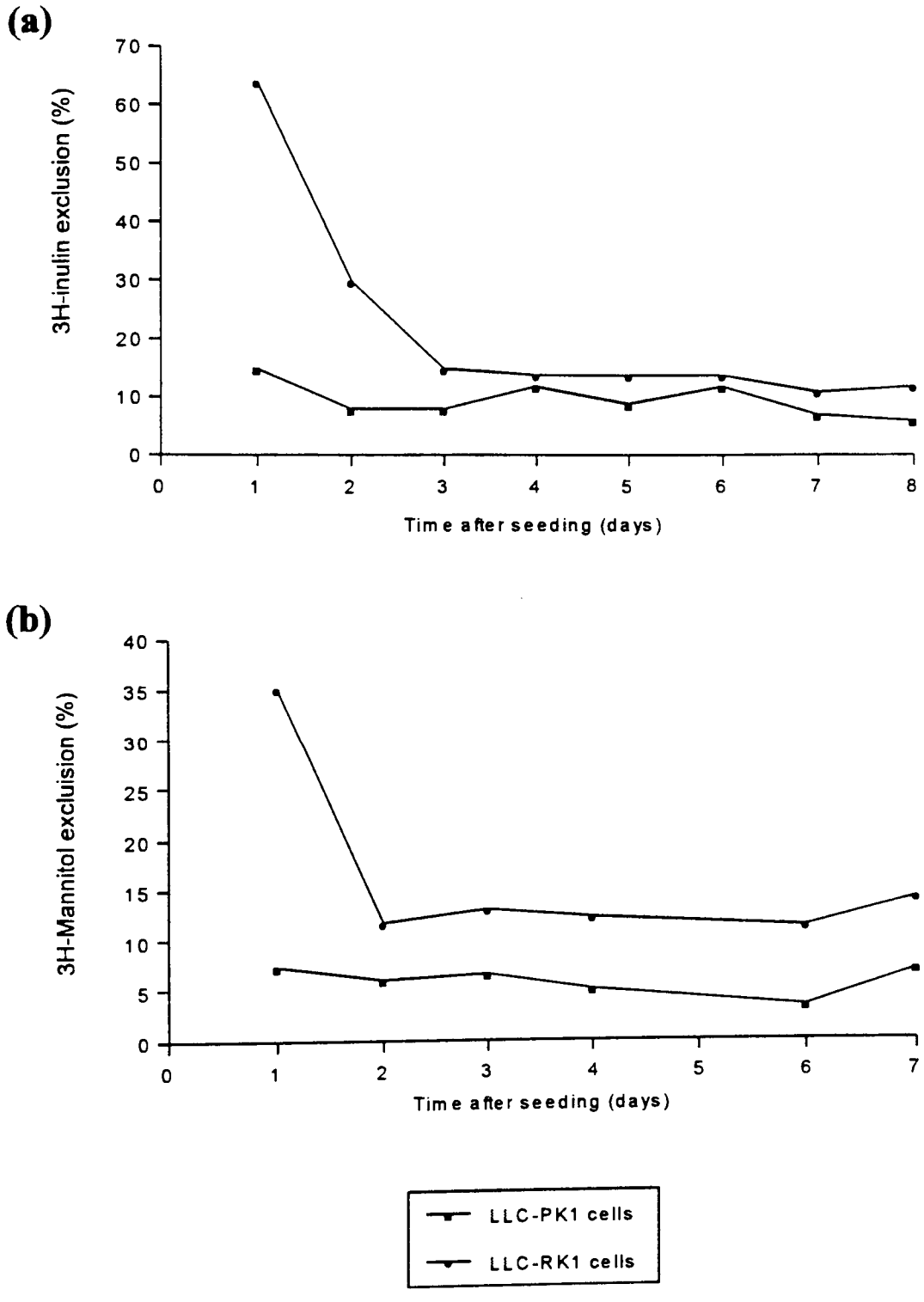


Figure 72. Effect of time after seeding on the ability of LLC- PK1 and LLC-RK1 cell monolayers to exclude a) 3H-inulin and b) Effect of time after seeding on the ability of LLC-PK1 and LLC-RK1 cell monolayers to exclude 3H-mannitol.

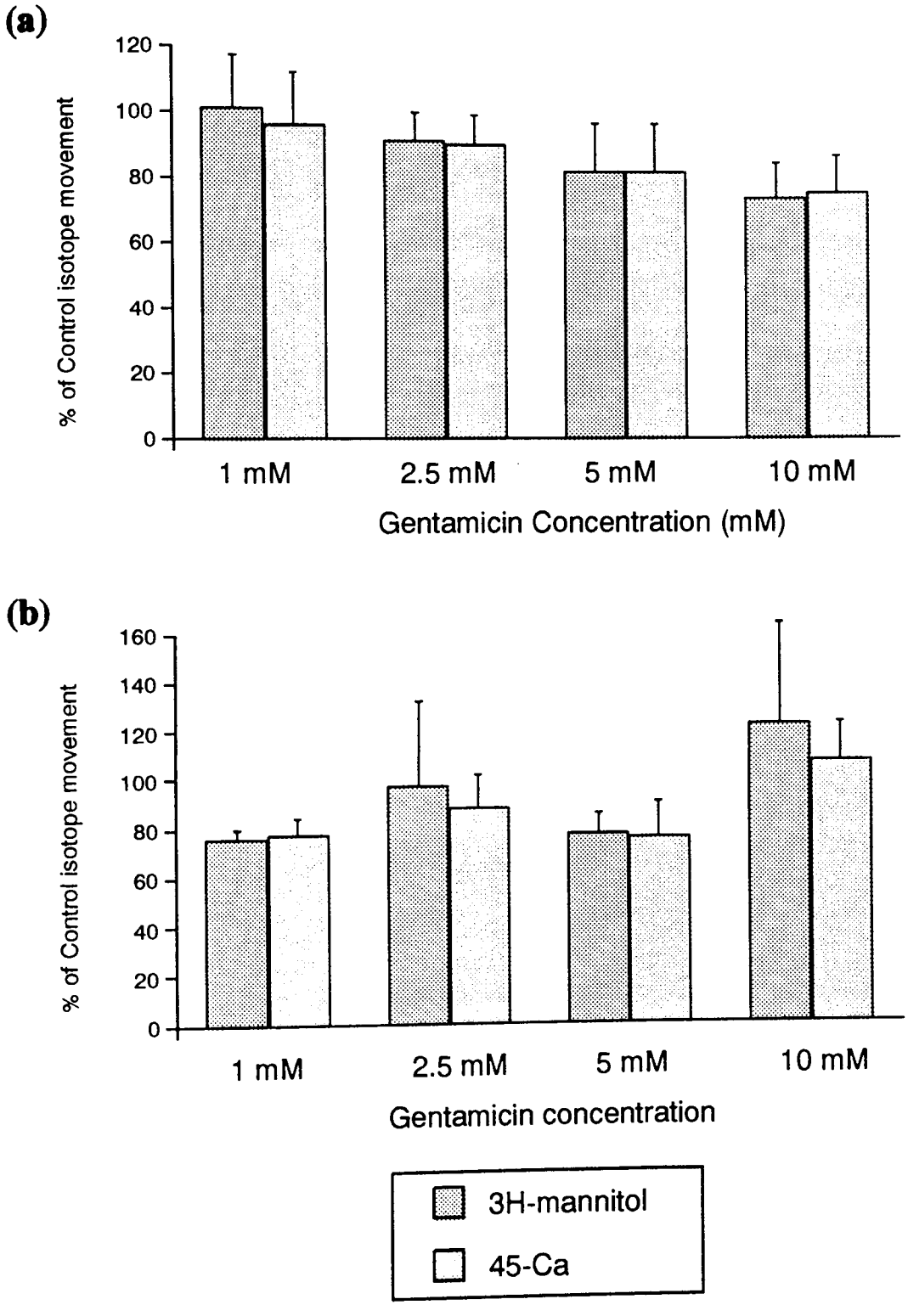


Figure 73. a) Effect of 1 hour exposure to a range of gentamicin concentrations on the movement of 3H-mannitol and 45-Ca across LLC-RK1 monolayers. b) Effect of 1 hour exposure to a range of gentamicin concentrations on the movement of 3H-mannitol and 45-Ca across LLC-PK1 monolayers. Cells were exposed via the apical surface only. Data are shown as mean ± SEM. n = 6 inserts.

^{3}H-mannitol exclusion by monolayers of LLC-PK1 and LLC-RK1 cells.

The ability of both cell lines to exclude ^{3}H-mannitol increased during the 24-48 h period after seeding; thereafter the amount of ^{3}H-mannitol excluded by each cell line remained stable over the study period (Figure 72a). The ability of LLC-PK1 cell monolayers to exclude ^{3}H-mannitol was greater (approximately double) than the ability of LLC-RK1 cells to exclude ^{3}H-mannitol. These results suggest that 144 h after seeding is a suitably stable point for the monolayer at which to carry out acute drug-exposure experiments on both cell lines.

c) Effect of time after seeding on the ability of LLC-PK1 and LLC-RK1 monolayers to exclude ^{3}H-inulin.

The ability of monolayers of LLC-PK1 and LLC-RK1 cells to exclude ^{3}H-inulin increased with time after seeding but only up to 48 h after seeding for LLC-PK1 cells and up to 72 h after seeding for LLC-RK1 cells. Thereafter ^{3}H-inulin exclusion by both cell lines remained stable for the remainder of the study period. Exclusion of ^{3}H-mannitol by both cell lines followed a similar pattern to that shown by the pattern of ^{3}H-inulin exclusion across the monolayers over time (Section 9.32b).

^{3}H-inulin exclusion was greater across the LLC-PK1 cell monolayer than across the LLC-RK1 monolayer suggesting that the cell sheet produced by LLC-RK1 cells is inherently more 'leaky' than the monolayer produced by LLC-PK1 cells (Figure 72b). These data confirm the different permeabilities of the two cell lines as seen in ^{3}H-mannitol exclusion experiments (Section 9.32b, Figure 72a). Reductions in monolayer permeability occurred in both cell lines when the cell number present was stable (Section 9.32a) suggesting further differentiation or development of the monolayer can affect monolayer permeability without changes in cell number.

9.33 Characterisation of ^{45}Ca movement across inserts.

a) Rate of diffusion of ^{45}Ca as $CaCl_2$ across empty Falcon inserts.

The rate of diffusion of ^{45}Ca across empty Falcon inserts was slightly greater when flux was measured from apical to basal compartments, at early 30 and 60 min sampling times, than when flux from basal to apical compartments was measured; however measurements of ^{45}Ca activity could be made at both sampling times. 25 µl samples contained several hundred cpm (see table 14) ^{45}Ca at 1 hour after the experiment was set up. It was therefore judged that 30 min and 1 hour sampling points would be adequate for further experiments, particularly if the sampling volume was increased from 25 to 50 µl. It was therefore decided that the radioactivity would be added to the apical compartment and samples taken from the basal compartment for subsequent experiments. The rate of diffusion of ^{45}Ca was the same whether the experiment was carried out in PBS containing calcium and magnesium, or in calcium and magnesium-free PBS.

Table 14. The rate of diffusion of ^{45}Ca across empty Falcon Inserts in PBS containing 0.9 mM calcium and 0.5 mM magnesium. Values are corrected for changes after samples were removed, and are also corrected for equivalent PBS volumes on both sides of the insert.

Sample Time	Basal to Apical Flux (cpm)	Apical to Basal Flux (cpm)
0	57	60
1 h	729	914
2 h	1279	1584
4 h	2155	2415
6 h	2784	2973
24 h	3675	3423

b) Effect of varying calcium concentrations on ^{45}Ca transport across a monolayer of cells grown on inserts.

Increasing the concentration of $CaCl_2$ above 5 mM in PBS solution in these experiments resulted in precipitation of salts out of the solutions, rendering them unusable for the assessment of ^{45}Ca transport across the monolayer, and for the assessment of viability of the cells on the inserts.

Movement of ^{45}Ca across monolayers was constant for all the concentrations of $CaCl_2$ used in these experiments. Movement of ^{45}Ca was approximately 4% of that movement which occurred in the absence of a cell monlayer (termed diffusion) for LLC-PK1 cells and approximately 15% for LLC-RK1 cells compared to diffusion, for each $CaCl_2$ concentration used.

The viability of both LLC-PK1 and LLC-RK1 cells was reduced in a concentration-dependent manner after exposure for 1 h to concentrations of $CaCl_2$ in PBS above 2 mM, compared to cells incubated in the presence of commercially available PBS (1.2 mM). The reduction in viability of the cells after exposure to increasing calcium concentrations indicated that using a concentration of 1.2 mM $CaCl_2$ (as in the standard commercially available PBS) may be optimal with regard to cell viability; this was therefore adopted for use in subsequent experiments.

Table 15. Effect of increasing $CaCl_2$ concentration on cell viability measured by Trypan Blue exclusion. Values are the mean of triplicate inserts for each condition.

Concentration $CaCl_2$ (mM)	LLC-PK1 % Viability	LLC-RK1 % Viability
Commercially Available PBS (1.2mM)	95	91
Calcium-supplemented (0.5mM)	92	90
Calcium-supplemented (2 mM)	92	83
Calcium-supplemented (4 mM)	87	75
Calcium-supplemented (5 mM)	78	70

c) Effect of temperature on the rate of movement of ^{45}Ca across monolayers of LLC-PK1 and LLC-RK1 cells

The amount of ^{45}Ca and 3H-mannitol diffusion from the apical to the basal compartment of the inserts with LLC-PK1 and LLC-RK1 cells increased slightly with increasing temperature across inserts incubated at 4-37 °C (from approximately 2% to approximately 3%); a further increase in the incubation temperature to 45 °C resulted in a dramatic increase in the permeability of the monolayer to both ^{45}Ca and 3H-mannitol (approximately 11 %). Alterations in ^{45}Ca movement were paralleled by similar changes in 3H-mannitol diffusion across the monolayer suggesting that the additional increase in temperature disrupted the integrity of the cell-to-cell junctions and increases the amount of diffusion across the monolayer. The amount of ^{45}Ca and ^{3}H-mannitol which moved from the apical to the basal compartment remained stable over the time course studied (15-120 min), at a given temperature compared to diffusion across inserts without cells. There were no differences in cell viability as assessed by Trypan Blue exclusion between the cells which had been exposed to different temperatures. The effects of increasing the ambient temperature were the same for both LLC-PK1 and LLC-RK1 cells. Since the amount of ^{45}Ca movement appeared to increase with increases in ambient temperature this may support the hypothesis that a large proportion of ^{45}Ca movement across the monolayers grown on inserts occurs by a largely passive process.

Table 16. Effect of increasing temperature on ^{45}Ca movement across monolayers of LLC-PK1 cells (144 h after seeding) after 90 min exposure to a given temperature. Data is presented as % of diffusion occurring across an insert without cells for both markers. Values are the mean of duplicate inserts for each temperature.

Temperature (°C)	^{45}Ca movement (% diffusion)	3H-mannitol movement (% diffusion)	% Viability
4	1.9	1.7	92
30	2.6	2.9	94
37	3.8	3.1	93
45	11.3	11.9	94

d) Effect of ouabain and KCN exposure on ^{45}Ca movement.

As shown in the table below, addition of ouabain to the incubation solution produced a small but discernible reduction in ^{45}Ca movement compared to controls (approximately 10 %). The reduction in ^{45}Ca movement after exposure to ouabain did not appear to be dose-related, since the addition of both 1 and 5 mM ouabain appeared to produce similar reductions in ^{45}Ca movement across the monolayers. Exposure to KCN did not however appear to alter ^{45}Ca movement across the monolayers. After observation using an inverted light microscope, cells on all inserts appeared morphologically normal even after exposure to ouabain and KCN. The responses to KCN and ouabain were similar in extent for both the LLC-RK1 and LLC-PK1 cell line.

Table 17. Effect of inhibitors on ^{45}Ca movement in LLC-RK1 cells 144h after seeding. Data is shown as mean ± SD. n = 3 for each condition.

Treatment	% of Control ^{45}Ca movement
1 mM Ouabain	86 ± 4
5 mM Ouabain	85 ± 5
3 mM KCN	94 ± 6

9.34 Effect of drug exposure on monolayer permeability

a) Effect of gentamicin exposure on ^{45}Ca movement across monolayers of LLC-PK1 and LLC-RK1 cells.

The effects of gentamicin exposure on LLC-PK1 and LLC-RK1 cells were studied with respect to junctional permeability and ^{45}Ca movement across a monolayer, over three time courses; up to 1 h exposure, after 24 h exposure, and after 48 h exposure. The assessments of ^{45}Ca and ^{3}H-mannitol movement was made in the presence of varying concentrations of gentamicin for the 1h exposure experiments. The duration of exposure

to the compound produced strikingly different effects on the permeability of the monolayers; in addition there were marked differences in the responses to gentamicin exposure between the two cell lines studied.

1 hour Gentamicin Exposure:

After apical exposure LLC-RK1 cells, but not LLC-PK1 cells demonstrated a dose-related decrease in permeability across the monolayer to both ^{3}H-mannitol and ^{45}Ca (Table 18a). The reduction in permeability to both isotopes was visible, and apparently maximal, 30 minutes after the addition of gentamicin and was sustained to 1 hour after exposure. A concentration of 1mM gentamicin and 1 hour exposure appeared to represent the dose threshold since there was no significant effect of gentamicin exposure on permeability to ^{45}Ca and ^{3}H-mannitol under these conditions. The reduction in permeability to ^{45}Ca and ^{3}H-mannitol appeared to be maximal at 25 mM gentamicin ($p < 0.001$); further increases in the concentration of gentamicin (max. dose used 50 mM) did not produce additional reductions in permeability of the monolayer (maximum reduction in ^{45}Ca and ^{3}H-mannitol flux equalled approximately 50%). The magnitude of the reduction in permeability of the monolayer was similar for both ^{45}Ca and ^{3}H-mannitol. Cellular morphology, at the light microscope level, appeared unaffected by the exposure to gentamicin.

The effects of gentamicin exposure for short periods (up to 1 hour) via the apical surface only, were less clear in LLC-PK1 cells. There appeared to be a reduction in permeability to ^{3}H-mannitol and ^{45}Ca in response to gentamicin exposure but this was smaller and less pronounced than that shown by LLC-RK1 cells (Figure 73b). Only cells exposed to 1mM gentamicin showed statistically significant differences ($p < 0.05$) compared to controls; exposure to higher concentrations of gentamicin did not produce results which reached statistically significant differences. The responses of LLC-PK1 cells to gentamicin exposure were also more variable than those shown by LLC-RK1 cells, as

previously observed when LLC-PK1 cells were used in cytotoxicity assays with aminoglycosides (see Chapter 8).

Exposure of the cell monolayers to gentamicin from the basal side only also showed differences in the responses between the cell lines. The data obtained from LLC-RK1 cells showed a reduction in permeability of the monolayers after drug exposure to the basal face of the cells (Table 18a). However, the reduction in permeability was less marked for a given concentration than was observed when the cells were dosed apically with the same concentration of gentamicin. The reductions in permeability were however statistically significant ($p < 0.05$) over dosing concentrations between 1-25 mM. The inter-experiment variation in response to basal gentamicin exposure was slightly increased in LLC-RK1 cells when they were exposed to gentamicin via the basal face only compared to the results obtained when the cells were exposed by the apical route. The trend in the data for LLC-PK1 cells showed an increase in the permeability of the monolayer at concentrations > 25 mM gentamicin after basal exposure (Table 18b). Cellular morphology at the light microscope level appeared normal.

Table 18 a) Effect of gentamicin exposure via the apical surface only for 1 hour on ^{45}Ca and ^{3}H-mannitol movement across the monolayer. Values are the mean ± SD of 3 independent experiments (6 inserts).

Gentamicin concentration (mM)	% of control ^{45}Ca movement	% of control ^{3}H-mannitol movement
LLC-RK1 (1 mM)	83 ± 3	80 ± 4
LLC-RK1 (10 mM)	74 ± 11	70 ± 14
LLC-RK1 (25 mM)	57 ± 2	49 ± 2
LLC-RK1 (50 mM)	58 ± 5	43 ± 5
LLC-PK1 (1mM)	74 ± 15	66 ± 14
LLC-PK1 (10 mM)	86 ± 14	80 ± 12
LLC-PK1 (25 mM)	81 ± 33	69 ± 28
LLC-PK1 (50 mM)	95 ± 26	79 ± 26

b) Effect of gentamicin exposure via the basal surface only, for 1 hour, on ^{45}Ca and ^{3}H-mannitol movement across the monolayer. Values are the mean ± SD of 3 independent experiments (6 inserts).

Gentamicin concentration (mM)	% of control ^{45}Ca movement	% of control ^{3}H-mannitol movement
LLC-RK1 (1 mM)	82 ± 0.6	82 ± 2
LLC-RK1 (10 mM)	79 ± 14	77 ± 18
LLC-RK1 (25 mM)	79 ± 4	73 ± 2
LLC-RK1 (50 mM)	93 ± 2	90 ± 6
LLC-PK1 (1mM)	103 ± 9	102 ± 18
LLC-PK1 (10 mM)	90 ± 6	89 ± 11
LLC-PK1 (25 mM)	110 ± 28	134 ± 56
LLC-PK1 (50 mM)	126 ± 26	149 ± 18

24 h Gentamicin exposure:

After exposure of LLC-RK1 cells to a range of concentrations of gentamicin for 24 h there were apparent increases of monolayer permeability to ^{45}Ca and ^{3}H-mannitol at concentrations from 1-7.5 mM. The increases in permeability of the monolayer to ^{45}Ca did not appear to be dose-related since they were similar across the range of concentrations tested and were not significantly different from controls at any dose used. There were no significant differences in permeability of LLC-PK1 cell monolayers to ^{45}Ca and ^{3}H-mannitol at any concentration used. Cellular morphology at the light microscope level appeared unaffected by the exposure to gentamicin. In NR and MTT cytotoxicity assays IC 50 values for 48h gentamicin exposure were approximately 7.5 mM for LLC-RK1 cells and 10-16 mM for LLC-PK1 cells.

Table 19. Effect of 24 h exposure to gentamicin on the permeability of LLC-PK1 and LLC-RK1 monolayers to ^{45}Ca and ^{3}H-mannitol. Cells were exposed to gentamicin via both the apical and the basal surface. Results are expressed as mean ± SD and are results from 3 independent experiments.

Gentamicin Concentration (mM)	% of control ^{45}Ca movement	% of control ^{3}H-mannitol movement
LLC-RK1 (1.0 mM)	136 ± 24	173 ± 29
LLC-RK1 (2.5 mM)	138 ± 32	153 ± 33
LLC-RK1 (5.0 mM)	121 ± 12	119 ± 13
LLC-RK1 (7.5 mM)	135 ± 20	127 ± 15
LLC-PK1 (1.0 mM)	90 ± 11	89 ± 11
LLC-PK1 (2.5 mM)	99 ± 19	91 ± 8
LLC-PK1 (5.0 mM)	102 ± 26	87 ± 10
LLC-PK1 (7.5 mM)	137 ± 22	107 ± 10

48 h gentamicin exposure:

After 48h exposure to gentamicin there was a dose-related increase in permeability to ^{3}H-mannitol and ^{45}Ca across monolayers of LLC-RK1 cells which reached statistical significance at 5 and 7.5 mM ($p < 0.05$). The changes in ^{3}H-mannitol permeability were closely paralleled by alterations in ^{45}Ca movement across the monolayer although the magnitude of increases in the latter appeared to be greater. In LLC-PK1 cells exposed to gentamicin for 48 h there appeared to be small increases in permeability of the monolayer to ^{3}H-mannitol and ^{45}Ca at concentrations of 2.5-7.5 mM, although these did not reach statistical significance. The effects of gentamicin exposure on the permeability of LLC-PK1 cell monolayers were more variable than those shown by LLC-RK1 cells under the same experimental conditions.

Table 20. Effect of 48 h exposure to gentamicin on the permeability of LLC-PK1 and LLC-RK1 monolayers to ^{45}Ca and ^{3}H-mannitol. Cells were exposed to gentamicin via both the apical and basal surfaces. Results are expressed as mean ± SD and are results from 3 independent experiments.

Gentamicin Concentration (mM)	% of control ^{45}Ca movement	% of control ^{3}H-mannitol movement
LLC-RK1 (1.0 mM)	101 ± 5	97 ± 17
LLC-RK1 (2.5mM)	118 ± 11	102 ± 22
LLC-RK1 (5.0 mM)	133 ± 22	113 ± 20
LLC-RK1 (7.5 mM)	148 ± 11	119 ± 5
LLC-PK1 (1.0mM)	88 ± 11	105 ± 40
LLC-PK1 (2.5 mM)	95 ± 10	123 ± 23
LLC-PK1 (5.0 mM)	98 ± 14	122 ± 6
LLC-PK1 (7.5 mM)	106 ± 22	134 ± 51

9.4 Discussion

When epithelial cells are grown in culture on inserts their orientation of apical face upwards and basolateral face downwards may be envisaged to represent an *in vitro* model of a nephron segment, with the apical face of the cells representing the luminal side of the nephron. Therefore, movement of solutes from the apical compartment to the basal compartment may represent a model of reabsorption from the tubular lumen to the interstitium. Having made this assertion the following provisos must apply when considering this system as a suitable model for the study of solute reabsorption occurring *in vivo*. Whichever cell line is chosen as a model for the reabsorptive process it must be borne in mind that some transport mechanisms for a given solute may not be present in a particular cell type or, under the culture conditions used, may not be expressed to the same extent, as in cells of that type *in vivo* (Toutain and Morin, 1992). LLC-PK1 cells have been shown to possess Ca^{2+} ATPase activity and the presence of a putative Na^{+}/Ca^{2+} exchange system has been demonstrated (Parys et al, 1986, de Smedt et al, 1988). The specific capabilities of LLC-RK1 cells to transport calcium have not yet been reported. The LLC-RK1 cell line has been far less extensively characterised than the LLC-PK1 cell line, consequently whilst the implication exists that cell lines with a similar origin may have the same properties, proof of this is not yet available for LLC-RK1 cells.

Various reports in the literature describe the development of transport functions such as Na+ dependent hexose, and ouabain-sensitive K+ and Na+ transport in LLC-PK1 cells after the cell number has stabilised allowing further differentiation of the cellular phenotype (Larsson at al, 1988, Gilberti et al, 1991, Amsler and Cook, 1982). In addition to the improved differentiation of cells after reaching quiescence, growing cells on inserts has also been reported to improve differentiation of the cells compared to those grown on tissue culture plastics, possibly by improving nutrient supply to the basal side of the cell (Pitt and Gabriels, 1986, Handler et al, 1984). Therefore the cells used in the studies presented in this chapter might well be expected to possess a well-differentiated phenotype, suitable for the study of calcium transport processes.

Initially, measurement of transepithelial flux of ^{3}H-inulin was tested as a marker of the permeability of the monolayers of LLC-PK1 and LLC-RK1 cells in the studies presented here. However further reading suggested that there was some controversy over whether tight junctions between cells were truly impermeable to this substance and suggesting ^{3}H-mannitol as a more appropriate marker (Ma et al, 1991). Subsequent studies presented here using ^{3}H-mannitol as a marker for paracellular permeability showed very similar results to ^{3}H-inulin permeability data, for both LLC-PK1 and LLC-RK1 cell lines; both ^{3}H-mannitol and ^{3}H-inulin exclusion experiments indicated that LLC-PK1 and LLC-RK1 monolayers were quite different with respect to their development of tight junctions and hence monolayer permeability (section 9.32b, c). This preliminary data therefore indicated that ^{3}H-mannitol and ^{3}H-inulin may be equally suitable for use in these cell lines as monitors of monolayer permeability.

In early experiments where the extracellular calcium concentration was varied (the specific activity of ^{45}Ca present was kept constant across the concentration range), the proportion of ^{45}Ca movement from the apical to basal compartment remained constant over the range of calcium concentrations used for both LLC-PK1 and LLC-RK1 cells (see Section 9.26). Although an EGTA containing-buffer was not used (so that the true available calcium concentration could not be calculated) the interpretation of the data showing a constant proportion of the total ^{45}Ca present moving from the apical to the basal compartment suggests that the majority of calcium movement across these monolayers occurs by a non-saturable process, involving diffusion. This substantial amount of calcium transport occurring by a passive means is in good accord with data on the mechanisms of calcium reabsorption in the proximal nephron *in vivo* (Rouse and Suki, 1990). This mechanism of passive movement of calcium, by a paracellular route, may also occur in the thick ascending limb of the Loop of Henle (in particular, thick ascending limbs in the medullary region of the kidney) (Suki et al, 1980). Data presented in this chapter showed that the presence of ouabain in the incubation medium resulted in a small reduction (approximately 10%) of ^{45}Ca movement from apical to basal

compartments, suggesting a small fraction of sodium-dependent calcium transport was occurring in both LLC-PK1 and LLC-RK1 cells. However, the amount of ^{45}Ca movement achieved by this means was relatively small and close to the methodological limits of detection, so that the hypothesis that gentamicin could also inhibit ^{45}Ca transport by this (probably transcellular) route was not further pursued in these early experiments.

In the series of experiments where LLC-PK1 and LLC-RK1 cells were acutely (1h) exposed to gentamicin via the apical surface (see Section 9.34), a situation which could represent the presence of drug in the tubular lumen, LLC-RK1 cells showed a clear reduction in transepithelial permeability to both ^{45}Ca and ^{3}H-mannitol in the presence of increasing concentrations of gentamicin. This reduction in permeability was clearly dose-related and apparently saturable at the highest concentration used (50 mM). Whilst this data offers intriguing possibilities as a mechanism by which gentamicin could reduce calcium reabsorption *in vivo*, it must be borne in mind that the reductions in transepeithelial flux of both ^{3}H-mannitol and ^{45}Ca occurred only at high concentrations of gentamicin which may not be achieved in the tubular lumen *in vivo*.

Interestingly, the reduction in both ^{45}Ca and ^{3}H-mannitol movement after 1 hour gentamicin exposure was more marked when the LLC-RK1 cells were exposed apically than when the cells were exposed from the basal side only. The magnitude of the inhibition suggests that the majority of the reduction in ^{45}Ca movement is due to a reduction in the permeability of the tight junctions; a supposition which is confirmed by the close parallelism of the effects of gentamicin on ^{3}H-mannitol movement. Whether any of the reduction in ^{45}Ca movement is due to inhibition of active transport mechanisms for calcium was not investigated in this series of experiments. As far as I am aware a reduction in ^{45}Ca movement across an epithelial monolayer in response to gentamicin exposure has not previously been reported.

Exposure of LLC-PK1 and LLC-RK1 cells to gentamicin for 24h produced quite different results to those seen after acute exposure to gentamicin. There were no significant changes in permeability in response to 24h gentamicin exposure in either cell line.

After 48 h exposure of LLC-RK1 cells to gentamicin there appeared to be limited disruption of the monolayer which resulted in a consequent increase in permeability to both ^{45}Ca and ^{3}H-mannitol. The effects of protracted gentamicin exposure (48h) on LLC-PK1 cells were less marked than those shown by LLC-RK1 cells. There were no significant differences in permeability to ^{45}Ca and ^{3}H-mannitol between control and gentamicin exposed LLC-PK1 cells. However exposure to concentrations of 5 and 7.5 mM for 48 h to gentamicin produced statistically significant increases in permeability in LLC-RK1 cells ($p < 0.05$). McGlynn et al (1992), have reported reductions in transepithelial resistance of LLC-PK1 cells, with a consequent increase in membrane permeability after protracted (up to 48 h) gentamicin exposure. These disruptions in permeability of the monolayer observed *in vitro* may correlate with the damage to the integrity of the tubular structure observed *in vivo* after aminoglycoside exposure.

Since gentamicin is a polycationic molecule it is tempting to speculate that the reduction in permeability across the monolayer caused by the compound is a simple direct competitive effect between two positively charged molecules for permeation at the tight junctions. The effects of varying calcium concentration in an EGTA containing buffer, or other strongly cationic molecules, with a constant gentamicin concentration in this insert system may indicate whether this is the case or whether the interaction between gentamicin and the tight junctions is a more specific one. Competitive inhibition of gentamicin binding by increasing concentrations of calcium and by other aminoglycosides has been demonstrated in brush border membrane vesicles (Sastrasinh et al, 1982). If there is a more specific change induced by the presence of gentamicin, perhaps in the phospholipid composition of the membrane, the mechanism by which gentamicin alters calcium reabsorption may still be by reducing the paracellular permeability to this ion by

an indirect means, rather than by altering transcellular transport. In the studies presented here the inhibition of both ^{45}Ca and ^{3}H-mannitol movement was maximal after only 30 minutes of exposure to gentamicin, and was sustained at that level of inhibition for the remainder of the experiment (up to 1 hour). In clearance studies presented in Chapters 4 and 5, and by Foster at al (1992), the effects of gentamicin infusion on calcium reabsorption were visible after only 30 minutes of infusion and were sustained for the duration of the experiments (up to 4 hours). The acute effects of gentamicin on the renal clearance of calcium have also been shown to be rapidly reversible once the drug administration is halted, suggesting a 'washing out' of the drug and therefore a cessation of the drug interaction (Foster, 1988).

Studying the same phenomena in two separate cell lines has yielded additional comparative information about the functions of these cells, and yet also highlights the difficulties of interpretation of *in vitro* data. Whilst using LLC-RK1 cells produced clear data showing inhibition of ^{45}Ca movement in the presence of gentamicin, LLC-PK1 cells studied under the same conditions produced more equivocal results. The fundamental question which arises from such differences must therefore be 'Which cell line actually represents the true *in vivo* situation ?' and is a necessarily recurring one in the extrapolation of *in vitro* data to the *in vivo* situation.

In conclusion, this *in vitro* study has shown two quite distinct responses in membrane permeability to gentamicin exposure. In the early acute phase (up to one hour) of gentamicin exposure there may be a decrease in cellular permeability to calcium, possibility due to a reduction in the permeability of the paracellular junctions between the cells. After more protracted exposure to gentamicin (48h) the permeability of the monolayer is disrupted, resulting in an increase in permeability to ^{3}H-mannitol and ^{45}Ca. Therefore, the possibility that alterations in paracellular transport of ^{45}Ca may be a mechanism by which electrolyte wasting from the kidney could occur, is an intriguing one and deserves further study. An *in vitro* cell culture model grown on a semi-

permeable insert system offers an attractive proposition for the continuation of the studies presented in this chapter.

Chapter 10. General Discussion

The data presented in the preceding chapters provide further insights into the toxicological phenomenon of aminoglycoside-induced nephrotoxicity, and in particular into the impairment of renal reabsorption processes for calcium and magnesium. The increased urinary loss of calcium and magnesium observed after aminoglycoside dosing may well contribute to the hypomagnesaemia and hypocalcaemia associated with clinical aminoglycoside therapy.

The effects of aminoglycosides on the impaired conservation of calcium and magnesium may exhibit two phases. In the early phase, shortly after initial dosing, or in acute studies, aminoglycosides appear to perturb normal electrolyte handling by the kidney resulting in specific decreases in the renal reabsorption of calcium and magnesium without concurrent effects on the renal handling of the other major electrolytes, sodium and potassium. These changes in the renal handling of calcium and magnesium have been clearly demonstrated to arise as a reduction in the tubular reabsorption of calcium and magnesium rather than as a consequence of changes in glomerular function, or from altered filtered load delivered to the nephron (Chapter 4, and Foster et al, 1992). In acute studies where the drug is administered by infusion, these derangements in electrolyte handling have been shown to be rapid in onset (30 min after the commencement of drug infusion; after as low a dose as approximately 5mg/kg gentamicin base), and are sustained for the duration of the infusion (up to 4 h). In other studies where aminoglycosides were administered on a daily basis, low doses of gentamicin have also produced increased urinary excretion of calcium before any enzymuria was apparent (Harpur et al, 1985), supporting the hypothesis that any effects on cation handling precede the substantial renal injury produced in the later phase of nephrotoxicity and are a selective effect of these compounds.

Functionally, the proximal tubule is extremely important for the reabsorption of solutes from the ultrafiltrate. This segment is the major site of calcium reabsorption along the

nephron; additionally a substantial minority of magnesium reabsorption also occurs during its transit along the proximal tubule. The proximal tubule is, in addition, the predominant site of drug accumulation for both cis-platinum and the aminoglycoside antibiotics. Therefore, the hypothesis that alterations in the renal reabsorption of calcium and magnesium due to drug toxicity could also occur in this segment was perhaps a logical extension of these data. However, the magnitude of the increases in fractional excretion of both calcium and magnesium after acute gentamicin dosing (from approximately 2% to 7% for FECa and from 15% to 25% for FEMg) did not exclude later nephron segments as potential sites of drug action, since approximately 30 % of total calcium reaborption occurs along the Loop of Henle and a further 10 % in the distal tubule; in the case of magnesium reabsorption approximately 50-60% of the total filtered load of magnesium is reabsorbed in the Loop of Henle, and an additional 15 % in the distal nephron segments. Important, albeit indirect evidence which indicated that the site of the calcium reabsorption defect was unlikely to be located in the proximal tubule was shown in the lithium clearance studies presented in Chapter 5. Lithium clearance measurements were used as a marker of proximal tubular sodium reabsorption, and indirectly proximal tubular calcium reabsorption, since calcium reabsorption in this segment is passive and largely dependent on active sodium transport. Acute infusion of gentamicin did not alter either lithium or sodium clearances; in contrast, concurrent measurements of calcium and magnesium clearances showed marked increases. Micropuncture data from Garland et al, 1992 showed that calcium lesion was not located in the distal nephron. Taken together, these data suggested that the site of the renal reabsorptive defect for calcium may be located in the Loop of Henle. The link between the acute effects on renal calcium handling, possibly located in the Loop of Henle, and the subsequent histological damage observed in the cells of the proximal tubule remains unclear in the pathogenesis of nephrotoxic injury caused by aminoglycosides.

In the later phases of nephrotoxic injury caused by both aminoglycoside antibiotics and cis-platinum the increased excretion of calcium and magnesium which can be produced is

probably a reflection of more widespread damage and disruption within the kidney rather than selective effects of these compounds, and as such is a reflection of gross tubular damage and malfunction. At this later stage, there may well be other indications of substantial renal injury such as reduced GFR in both experimental animals and human subjects treated with these drugs.

The effects of gentamicin on renal electrolyte handling have been suggested as a contributor to the pathogenesis of subsequent renal injury at a cellular level. Effects on intracellular calcium concentrations and perturbations of whole body electrolyte balance may indeed be a common pathway to cellular injury and cell death (Schanne et al, 1979). There are however complex interrelationships between transport of calcium and other ions, particularly sodium, which may be significant with respect to this cellular injury. Reductions in the specific activity of Na^+/K^+ ATPase could potentially affect both intracellular elctrolyte homeostasis, and may also provide an indirect means by which renal calcium reabsorption could be reduced. Both Ca2+ ATPase and Na^+/K^+ ATPase activity have been shown to be similarly inhibited after *in vitro* exposure to aminoglycosides (Chapter 6) In addition, inhibition of Na^+/K^+ ATPase activity has been reported after both acute and chronic dosing with aminoglycosides *in vivo*, but transcellular calcium movement as a result of Ca2+ ATPase activity is apparently unaffected by the actions of these drugs since the specific activity of this enzyme remains at control levels after *in vivo* drug administration (Chapter 6). The result of modifications in the activity of key enzymes may have profound effects on the intracellular ionic balance, consequently affecting subcellular organelles which may be the ultimate site of impairment of calcium and magnesium transport. In addition, inhibition of Na^+/K^+ ATPase activity alone may also have important consequences for reducing calcium reabsorption due to the interdependence of their cellular transport mechanisms.

However, since the majority of calcium reabsorption is believed to occur paracellularly, rather than by active transcellular processes, particularly in the proximal tubule, alterations in the permeability of the tight junctions between the apical surface of these

highly polarised cells also provoked interest as a potential site through which reabsorption could be altered. Pursuing this hypothesis by the use of *in vitro* models provided indirect evidence suggesting gentamicin could indeed alter the leakiness of the intercellular tight junctions (Chapter 9). In agreement with *in vivo* data there appeared to be two types of injury which aminoglycosides could also produce in *in vitro* model systems. After acute exposure (1 h), and in the presence of gentamicin, a clear dose-related reduction in transepithelial flux of ^{45}Ca could be observed across monolayers of LLC-RK1 cells. However, the inhibition of ^{45}Ca flux across the cell monolayer could only be observed at substantial gentamicin concentrations (10 mM or greater). After more protracted exposure (48 h) the effect of gentamicin exposure was to increase the permeability of the LLC-RK1 monolayer to ^{45}Ca. Permeability to ^{3}H-mannitol was also increased in these experiments suggesting a disruption of cell-to-cell contact caused by gentamicin. These preliminary results therefore suggest an intriguing mechanism by which aminoglycosides could acutely reduce calcium reabsorption from the tubular fluid *in vivo*. i.e by reducing the paracellular permeability of tubule cells to this important cation. It appears therefore that in addition to producing disruptions in intracellular electrolyte homeostasis by altering Na^+/K^+ ATPase activity, aminoglycosides may also have the capacity to disrupt paracellular electrolyte flux. The data presented in this thesis therefore suggest that the cellular mechanisms by which aminoglycosides could alter renal cation reabsorption may be by inhibiting Na^+/K^+ ATPase activity, or by altering paracellular permeabilty to calcium, and, as a consequence of either, or both, of these actions alter whole body calcium and magnesium balance.

Whilst the use of both animal and *in vitro* models to study toxicological phenomena originating in humans is attractive, extrapolation of the results from such studies may be compounded by difficulties. As shown for 3 different rat strains and for two continuous cell lines in this thesis (Chapter 3, and Chapters 8 and 9) the choice of different experimental subjects may lead to quite disparate results, and therefore conclusions, from the same experimental protocol. The question which then must arise is 'Which is the most

accurate representation of the original toxicological problem in the human?'. However, since any original data must be considered in the light of a considerable body of other evidence, both animal studies and *in vitro* model systems continue to supply further insights into a variety of toxicological problems. Judiciously interpreted, data from such experiments can considerably enhance our knowledge and understanding of significant clinical problems, ultimately minimising or eliminating the toxic side-effects which may arise from therapeutic drug administration.

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Appendices

1. Alanine Aminopeptidase (AAP) Assay (Mondorf et al, 1972)

Reagents

1a. 0.1 M $Na_2 HPO_4$ in distilled water

1b. 0.1 M KH_2PO_4 in distilled water

Mix solutions 1a and 1b in an approximate ratio of 9:1 to give a buffer solution of pH 7.4

2. Substrate: l-alanine p-nitroanilide hydrochloride; 5mg/ml in distilled water.

Method

1. For each sample to be assayed the following measurements need to be made. Assays are carried out in triplicate. Since the reaction product is yellow measurements of the absorbance due to the intrinsic urine colour need to be made for each sample and subtracted from the absorbance produced in the presence of substrate (tube 5 vs. 6). The reaction is measured against a blank containing buffer and substrate but without urine present (tubes 1-3 vs. 4).

TUBE	*PO_4 BUFFER*	*URINE SAMPLE*	*Mix*	*SUBSTRATE*	*DIST. H_2O*	*Mix*	*FINAL VOL*
1	1.6 ml	0.2 ml	*mix*	0.2 ml	-	*mix*	2.0 ml
2	1.6 ml	0.2 ml	*mix*	0.2 ml	-	*mix*	2.0 ml
3	1.6 ml	0.2 ml	*mix*	0.2 ml	-	*mix*	2.0 ml
4	1.6 ml	-	*mix*	0.2 ml	0.2 ml*	*mix*	2.0 ml
5	1.6 ml	0.2 ml	*mix*	-	0.2 ml	*mix*	2.0 ml
6	2.0 ml	0.2 ml	*mix*	-	-	*mix*	2.0 ml

*Equivalent to volume of sample used in tubes 1-3

2. Equilibrate assays to 37 °C before addition of substrate. After addition of substrate, mix and incubate for up to 15 min at 37 °C.

3. After reaction period read samples 1-3 immediately, since the reaction is not halted, against a blank of tube 4 at 405 nm.

4. Read tube 5 against tube 6 to compensate for the absorbance due to the urine colour. Subtract this value from the absorbance reading obtained for the complete reaction (from tubes 1-3).

Calculation of activity

$$\frac{\text{ABS @ 405nm x 202.5 x Factor to give 1ml of urine x Dialysis Factor (if appropriate)}}{\text{Time (mins)}}$$

= Units AAP activity/min.

2. Lactate dehydrogenase (LDH) Assay (Leathwood and Plummer, 1969).

Reagents

3.5 mM $NADH_2$ in distilled water.

32mM Sodium pyruvate (pyruvic acid, sodium salt) in distilled water.

Phosphate buffer: 0.1M KH_2PO_4 and 0.1M $Na_2HPO_4 . 2H_2O$. Mix in an approximate ratio of 1:9 to give pH 7.4.

Method

1. Add 2.4 ml phosphate buffer, 0.1 ml $NADH_2$ solution, and 0.4 ml urine sample into a cuvette and allow to equilibrate to 37°C.

2. The blank (reference) contains the same quantities of buffer and $NADH_2$ solutions as the sample to be assayed, plus an additional 0.1 ml of buffer to replace the substrate in

the assay mixture. After equilibration add 0.1 ml of the pyruvate solution to the sample cuvette, mix rapidly, and follow the reaction at 405 nm.

3. Measure the rate of change of absorbance over time and use this to calculate the LDH activity in the sample.

Calculation

Slope/min x 50.24 = mU LDH activity/min.

3. N-acetyl-ß-glucosaminidase (NAG) Assay (Luft and Patel, 1979)

Reagents

0.1M Na-citrate buffer pH 4.8

25 mg/100 ml p-nitrophenyl-N-acetyl-ß-glucosaminide (substrate) in Na-citrate buffer:

0.1M NaOH solution.

Method

1. 0.4 ml of urine and 0.5 ml of Na-citrate buffer were mixed together and equilibrated in a water bath to 37 °C. Samples were assayed in duplicate.

2. The reaction was started by the addition of 0.5 ml of substrate and the entire mixture was incubated, with shaking, for 30min.

3. After 30 min the reaction was stopped by the addition of 5 ml NaOH solution and the absorption measured against a blank comprising buffer, urine, and NaOH solution at 410 nm.

Calculation

$$\frac{\text{Absorbance @ 410nm x 370.5 x Factor to give 1 ml sample volume x Dialysis factor}}{\text{Incubation period (30 min)}}$$

= mU NAG activity/min

4. Creatinine Assay (Taussky, 1956)

Reagents

1M NaOH solution

Saturated aqueous picric acid

Standard solution of 1 mg/ml creatinine.

Method

1. 2 ml of picric acid solution was added to duplicate 0.1 ml urine samples, and mixed. The reaction was started by the addition of 0.6 ml NaOH solution followed by further mixing.

2. The reaction mixture was left at room temperature for 10 min, after which time 2.3 ml of distilled water was added.

3. The absorption of the samples was measured immediately at 520 nm against a blank containing picric acid, NaOH solution and distilled water.

4. A calibration curve using known amounts (range 25-100 µg) of creatinine standard was prepared in order to estimate creatinine concentrations present in the samples.

5. Measurement of calcium and magnesium concentrations in plasma and urine samples by atomic absorption spectroscopy (Fell and Peaston, 1973).

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6. Measurement of sodium and potassium concentrations in urine and plasma by atomic absorption spectroscopy.

Determinations of total sodium and potassium concentrations were made in samples of urine and plasma diluted in distilled water as detailed in Chapters 3 and 4. The diluted samples were measured on a Perkin Elmer 560 atomic absorption spectrophotometer using standard calibration solutions containing both sodium and potassium. After optimising the instrument with respect to lamp set up, burner height and emission wavelength (589.0 nm for sodium and 766.5 nm for potassium) the instrument was set up with an oxygen/acetylene flame. Stock solutions of 1000 ppm NaCl and 1000 ppm KCl were obtained from Sigma and diluted to the required concentration in distilled water. The instrument set-up was optimised using a combined standard containing 1ppm Na and 2 ppm K until a stable maximal absorbance was obtained. the instrument was then zeroed against distilled water and the signal rechecked for stability. A one point calibration of 1 ppm was used for Na determinations and 2 ppm for K determinations. The sensitivity limits were 0.015 ppm for Na determinations and 0.04 ppm for K determinations. Samples were assayed in batches of 10 samples after which the concentrations of the standards were re-assessed. If the variation of the standard solutions had altered by a maximum of 5% then the instrument was checked for problems and recalibrated and the samples re-assayed. If the concentration of the element in the diluted sample proved to be greater than the linear range of the instrument then the sample was further diluted to bring it within the working range of the instrument and re-measured.

7. Urine osmolality measurements.

For all studies presented in this thesis urine osmolality was measured using the principle of freezing point depression. The osmotic concentration of a sample is measured relative to the freezing point achieved by pure H_20 and is a direct measurement of solute concentration. The osmometers were calibrated using pure H_20 and a 300mOsm/kg H_20 standard.

8. Analytical methods used for the study investigating the response of three strains of rat to the nephrotoxic effects of a range of doses of gentamicin (Chapter 3).

Quantitative measurements of plasma creatinine, calcium and magnesium and measurements of the urinary concentrations of LDH, NAG, GGT, ALP, calcium, magnesium, creatinine and total protein in the study reported in Chapter 3 were assayed using a COBAS FARA II automatic analyser. The COBAS FARA II is a centrifugal analyser which may be programmed to carry out a number of selected tests, using differing methodologies, on an individual urine or plasma sample. In addition, dilution of samples is achieved within the programmed method so that manual dilution inaccuracies are removed.

Determination of magnesium in urine and plasma: Magnesium ions in the sample form a stable complex with Calmagite in alkaline solution which is quantified at 520 nm. Interference from Ca ions in the sample is removed by the addition of EGTA in the reagent mixture. The kit used for this method on the Cobas Fara is supplied by Bio Merieux Cat. No. 6 141 1.

Determination of calcium in urine and plasma: Urine and serum plasma calcium concentrations were measured using an Arsenazo III method on the Cobas Fara Centrifugal analyser. Arsenazo III binds specifically to calcium forming a stable coloured complex. Addition of an EDTA reagent removes non-specific interferences. The EDTA

reagent removes calcium from the calcium-Arsenazo complex and allows for an accurate blank measurement. The difference in absorbance of the calcium-Arsenazo III complex and that of the EDTA treated complex is due to calcium. The kit used for this technique was obtained from Roche Diagnostics Cat. No. 44033.

Determination of creatinine in urine and plasma: Urine and serum plasma creatinine concentrations were measured using a modified method of Jaffe on the Cobas Fara centrifugal analyser. Creatinine in the sample reacts with alkaline picrate to form a red-orange complex, quantitated at 520nm. The kit used for this technique was obtained from Sigma Diagnostics Cat. No. 557-A/557-M.

Determination of total protein in urine: Urinary total protein is measured using the Coomassie Blue method. In this method the shift in absorbance which occurs when protein binds to Coomassie Blue G250 in acidic solution, from 465nm to 595nm, is measured. The kit used for this technique was obtained from Pierce Chemical Company Ltd. Cat. No. 23200.

Enzyme activities: In this study urine samples were passed through Pharmacia Nap5 Gel filtration columns (Cat. No. 17-0853-02) and eluted with 0.9% saline to remove enzyme inhibitors prior to analysis on the Cobas Fara.

Determination of γ-Glutamyl transpeptidase (GGT) in urine: Urine GGT is quantified using a kinetic colorimetric method on the Cobas Fara centrifugal analyser. GGT catalyses the transfer of a gamma-glutamyl group from gammaglutamyl-3-carboxy-4-nitroanilide to glycylglycine with the formation of gammaglutamylglyclglycine and aminonitrobenzoate. The resultant increase in absorbance is measured at 405 nm. The kit used for this technique was obtained from Boehringer Mannheim GmbH Diagnostica Cat. No. 1 582 08.

Determination of n-acetyl-β-glucosaminidase (NAG) in urine : Urinary NAG concentrations were measured using a colorimetric method on the Cobas Fara centrifugal

analyser. NAG hydrolyses 3,3'-dichlorophenol sulphophthalein-n-acetyl-β–D-glucosamidine, sodium salt, releasing chlorophenol red which is quantitated at 575 nm. The kit used for this technique was obtained from Boehringer Mannheim GmbH Diagnostica Cat. No. 1 087 657.

Determination of Alkaline Phosphatase activity (ALP) in urine: Urinary ALP concentrations were measured using a kinetic colorimetric method using the Cobas Fara centrifugal analyser. ALP catalyses the hydrolysis of p-nitrophenyl phosphate to yield p-nitrophenol, which is quantified at 405 nm. The kit used for this technique was obtained from Boehringer Mannheim GmbH Diagnostica Cat. No. 782 874/782 858.

Determination of Lactate Dehydrogenase activity (LDH) in urine: Urinary LDH activity was measured using a kinetic UV method using the Cobas Fara centrifugal analyser. LDH catalyses the reversible conversion from pyruvate to lactate, with the corresponding conversion of NADH to NAD^+ measured at 340 nm. The kit used for this technique was obtained from Boehringer Mannheim GmbH Diagnostica Cat. No. 158 186.

9. Measurement of lithium in plasma and urine samples by atomic absorption spectroscopy. (Chapter 5)

The lithium concentration in plasma and urine samples were determined by flameless atomic absorption spectroscopy, on a Perkin-Elmer Zeeman 30/30 atomic absorption spectrophotometer. Duplicate 10 µl plasma samples were diluted 1 in 200 with 0.2 % triton-X containing 0.1M HNO_3. Urine was diluted using the same diluent so that the lithium concentration lay within the linear range of the instrument (100-600 absorbance units); usually 1 in 1000. Calibration solutions were prepared from stock solutions containing 146.3mM LiCl (Spectrosol, BDH). Using the triton-X/HNO_3 diluent as a blank, the instrument was calibrated with a one-point calibration using a standard solution containing 0.2mM Li, 140mM NaCl, and 5mM KCl for plasma determinations.

For lithium determinations on urine samples the calibration solution contained 0.4 or 2 mM Li; the instrument was calibrated with a one-point calibration.

Publication List

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