AN INVESTIGATION OF POSSIBLE MECHANISMS OF TOXICITY OF THE AMINOGLYCOSIDE ANTIBIOTICS

BY

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'constant questioning is the first key to wisdom. For through doubt we are led to enquiry, and by enquiry we discern the truth.'

Peter Abelard
(1079-1142)

AN INVESTIGATION OF POSSIBLE MECHANISMS OF TOXICITY OF THE AMINOGLYCOSIDE ANTIBIOTICS

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SUMMARY

A study was made of possible mechanisms of uptake and toxicity of the aminoglycoside antibiotics, in particular gentamicin.

The uptake of gentamicin was characterised in rat kidney cortical slices and in isolated rat renal proximal tubules. In each case, the uptake of gentamicin was found to be both time and concentration dependent, showing saturable characteristics. The use of metabolic inhibitors suggested that the process was energy dependent.

Isolated rat renal tubules were used as a model system since they offer advantages over cortical slices of better oxygenation and substrate availability to the tubular cells. The preparation and characterisation of the tubules is described.

The release of the lysosomal enzymes acid phosphatase and N-acetyl-β-glucosaminidase from these tubules on incubating in the presence of gentamicin was also studied. The release was a function of a complex interaction between the gentamicin concentration and the time of incubation. The findings were interpreted as a disturbance by gentamicin of receptor-recycling by a differential effect on the two lysosomal enzyme transport pathways.

Gentamicin inhibited gluconeogenesis but did not effect protein synthesis.

The interaction of aminoglycosides with plasma membrane Na^+-K^+ ATPase as a common mechanism of drug-induced nephrotoxicity and ototoxicity is discussed.

A comparison was made of the urinary excretion of the enzymes lactate dehydrogenase, N-acetyl-β-glucosaminidase and alanine amino-peptidase with other indices of renal function in rats injected with gentamicin (40 mg/kg/day). The results confirmed that the early appearance of enzymes in urine is a sensitive indicator of kidney damage.

It is suggested that isolated rat renal tubules may be used as a relevant model for assessing toxic effects of new aminoglycosides.

KEY WORDS: Aminoglycoside antibiotics, N-acetyl-β-glucosaminidase, Nephrotoxicity, Receptor-recycling, Renal tubules.

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TO MY MOTHER

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ABBREVIATIONS

AAP	Alanine aminopeptidase
AP	Acid phosphatase
c.p.m.	counts per minute
2,4-DNP	2,4-dinitrophenol
d.p.m.	Disintegrations per minute
D.R.	Distribution ratio
KCN	Potassium cyanide
LDH	Lactate dehydrogenase
NAG	N-acetyl-β-glucosaminidase
Na^+-K^+ ATPase	Na^+ and K^+ activated adenosine triphosphatase
Pi	Inorganic phosphate
Rb^{86}	Rubidium-86
SEM	Scanning electron microscopy
s.e.m.	Standard error of mean
TCA	Trichloroacetic acid
TEM	Transmission electron microscopy

SECTION 1

INTRODUCTION

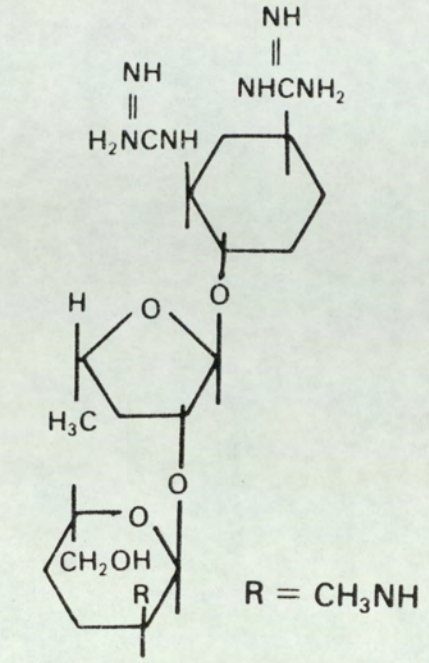
1.1 GENERAL INTRODUCTION

The aminoglycoside antibiotics are among the most effective against Mycobacterium tuberculosis and they also show marked activity against a wide variety of Gram-postive, Gram-negative and acid-fast organisms. They are often used for infections which are resistant to other less toxic drugs.

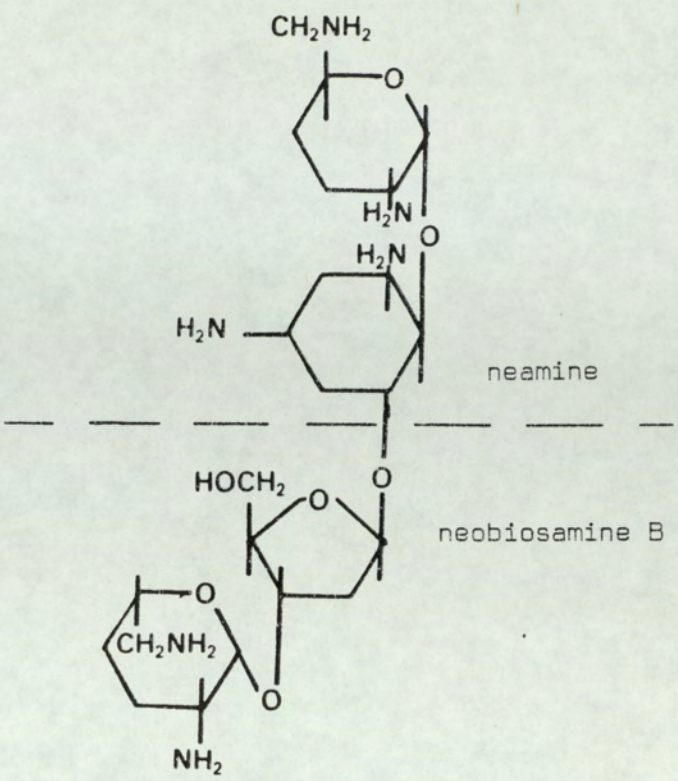
However, it has become apparent that with prolonged administration of these antibiotics, strains of aminoglycoside-resistant bacteria have emerged as a virtue of their producing inactivating enzymes. In order to combat this it has become necessary to introduce new groups on to the pre-existing structures so that they are still bacteriologically active but not sensitive to the inactivating enzymes. Thus, in addition to older products such as streptomycin, neomycin, kanamycin and gentamicin, synthetic aminoglycosides like tobramycin, amikacin and sisomicin have also appeared on the market. The structures of some of these aminoglycoside antibiotics are shown in Fig. 1.

All of these antibiotics are basic organic compounds of moderate molecular weight (mol. wt. 470-550) with pKa's between 7.5 and 8. They are highly water-soluble, may be bound to plasma proteins, not metabolised in the body and are eliminated almost exclusively in the urine in an active form. A more detailed and extensive review on the clinical use and toxicity of the individual aminoglycosides mentioned above has been reported by Harpur (1975).

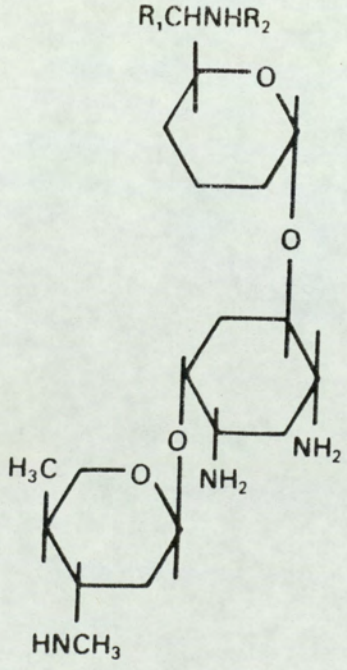
The most frequent complications arising from the long-term use of these antibiotics have been nephrotoxicity, ototoxicity and also blockade of the neuromuscular and ganglionic transmission.



STREPTOMYCIN



NEOMYCIN



Gen C_1 $R_1 = R_2 = CH_3$

C_2 $R_1 = CH_3$ $R_2 = H$

C_{1a} $R_1 = R_2 = H$

GENTAMICIN

Fig. 1. Chemical structures of some of the aminoglycoside antibiotics.

1.2 NEPHROTOXICITY

The functional unit of the kidney is the nephron (Fig. 2), which is composed of the glomerulus neck segment, proximal convoluted tubule, loop of Henle, distal tubule and the collecting duct, all of which play some role in the formation of urine.

The major route of aminoglycoside elimination is renal where 70-90% of a single dose of a drug may be recovered unchanged in twenty-four hours urine collection (Davey, Gonda, Harpur et al., 1980).

Accumulation of gentamicin by renal tissue may contribute to the reported inability to account for all of the initial dose of gentamicin in urine (Riff and Jackson, 1971), despite the belief (based on the similarity of the renal clearance of gentamicin to that of inulin) that renal elimination by glomerular filtration is the route of excretion of these aminoglycosides (Gyselynck, Forrey and Cutler, 1971). In fact, recent studies of urinary excretion of aminoglycosides have indicated complex elimination comprising glomerular filtration, tubular reabsorption and intracellular penetration at the peritubular side. The subsequent doses are apparently easily accounted for, suggesting that the renal parenchyma may rapidly become saturated with the drug. Little is known about the kinetics of this accumulation in human kidneys. It is not known whether a plateau of drug accumulation is reached, nor do we know whether the schedule of administration (e.g. continuous versus intermittant dosing)affects the kidney tissue concentrations achieved in humans. In rats, Luft, Patel, Yum et al. (1975) found that renal level of gentamicin plateaued after 5 days of a 15 day drug administration. Streptomycin, which

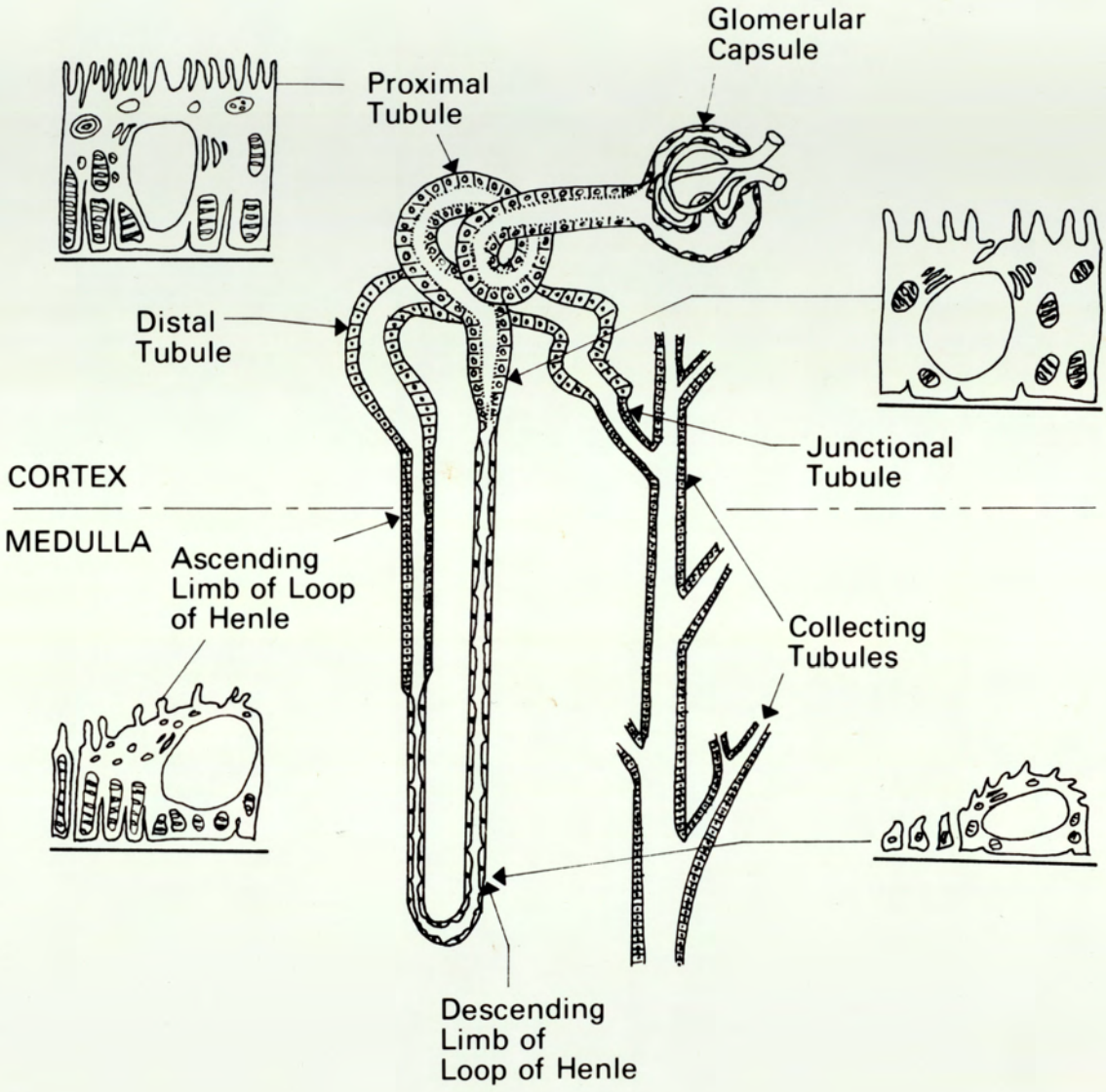


Fig. 2 **The nephron and collecting tubule diagrammatically represented**

is relatively free of nephrotoxicity, has been shown to display a much weaker affinity for renal tissue than do other aminoglycosides (Luft and Kleit, 1974).

Gentamicin has been shown to accumulate in the cortex and in particular the proximal tubular cells (Whelton and Walker, 1974). Recently, gentamicin has been found to stimulate uptake and diminish efflux of p-aminohippurate in rat renal cortical slices which was interpreted by the investigators to indicate an effect of the drug at the antiluminal membrane (Lapkin, Bowman and Kaloyanides, 1977). However, a similar effect of gentamicin was not found when accumulation of iodohippurate was studied in rabbit renal cortical slices by Dahlager and Milman (1977). They found that only the uptake was inhibited and only by very high concentrations of the drug.

Since aminoglycosides are organic bases it has been suggested that they may be eliminated by the processes which have been well demonstrated in the proximal tubule governing the elimination of organic acids or bases. However, this hypothesis does not seem very likely. Quinine, which competes with organic bases for excretion, does not alter renal concentration of gentamicin in the rat (Fabre, Rudhardt, Blanchard et al., 1977) and sulfinpyrazone which blocks the transport of organic bases, has no effect on urinary excretion of gentamicin (Neoden, Fuchs, Schroder et al., 1972). Probenecid, a competitive inhibitor of organic acid transport does not alter aminoglycoside elimination (Neoden et al., 1972).

The proximal tubular cell appears to be the primary target of the nephrotoxicity of the aminoglycosides, sparing the glomeruli. With moderate doses there is cloudy swelling of tubules and at

higher doses acute tubular necrosis has been produced in animals (Waitz, Moss and Weinstein, 1971). These effects are especially prominent in the proximal convoluted tubules (Harrison, Silverblatt and Turek, 1975) and lysosomal myeloid bodies have been noted in electron microscopical studies in rats (Kosek, Mazze and Cousins, 1974). Myelin figures in the lysosomes of renal epithelial cells were explained by them to be probably due to phagocytosed residues of damaged cellular organelles.

The prevalence of gentamicin nephrotoxicity is between 2 to 10% (Hewitt, 1974). Clinical studies of nephrotoxicity are complicated by the fact that most nephrotoxicity occurs in patients who have pre-existing renal insufficiency, other intervening events damaging the kidney or simultaneous use of aminoglycosides with other nephrotoxins, especially cephalosporins (Reiner, Bloxham and Thompson, 1978). Numerous clinical studies have failed to demonstrate conclusively whether combination of aminoglycosides plus cephalosporin increases nephrotoxicity or not. Unfortunately, animal studies have shown that cephalosporins do not augment gentamicin toxicity, and high doses of cefazolin and cephaloridine actually protected the kidney from damage (Luft, Patel, Yum et al., 1976). Dellinger, Murphy, Pinn et al. (1976) reported a decrease in tubular necrosis at each dose level of gentamicin with the addition of a cephalosporin. In the experiments of Luft et al. gentamicin still achieved high concentrations in renal cortex in the presence of cephalosporins suggesting that reduction in toxicity is not due to lower levels of gentamicin in the cortex.

Gentamicin nephrotoxicity is manifested by proteinuria and cylindruria (the presence of renal casts in the urine) with a

rising blood urea nitrogen and creatinine (Wilfert, Burke and Bloomer, 1971). In general, renal damage is usually reversible if the aminoglycoside is discontinued at the first signs of renal dysfunction (Kunin, 1966; Kovnat, Labovitz and Levison, 1973) such as rising blood urea nitrogen, serum creatinine or the presence of protein or tubular cells in the urine (Guisti, 1973). One of the earliest signs of nephrotoxicity is the finding of lysosomal hydrolase enzymes in the urine (Raab, 1972).

1.3 OTOTOXICITY

Administration of a variety of aminoglycosides has been associated with ototoxicity in humans and experimental animals (Brown and Feldman, 1978). Streptomycin, gentamicin and tobramycin tend to impair vestibular function more frequently whereas neomycin, kanamycin and amikacin primarily affect hearing. In human studies the incidence of aminoglycoside ototoxicity has been found to be between 2% and 50% (Jackson and Arcieri, 1971; Winkel, Hansen, Kaaber et al., 1978).

The ototoxic reactions of several of these antibiotics have been studied in experimental animals with physiological, histological and electron microscopical methods (Duvall and Wersall, 1964; Wersall, Bjorkroth, Flock et al., 1973; Theopold, 1977) and it has become clear that the outer hair cells are, in general, more vulnerable to aminoglycosides than the inner hair cells.

Histopathological studies pertaining to the ototoxicity of kanamycin in guinea-pigs (Hawkins and Engstrom, 1964; Kohonen, 1965) have revealed a relatively regular pattern and sequence of damage to the cochlear sensory cells by this ototoxic antibiotic. The inner row of outer hair cells (OHC) of the cochlea appeared to be the most susceptible to aminoglycoside-induced damage with the basal turn being damaged first. As the toxicity progresses other rows of OHC become involved. The damage then spreads towards the apex of the cochlea with the toxicity beginning to involve the inner hair cells, progressing from the apex to the basal coil.

Hawkins (1970) has suggested that hair cell degeneration may be secondary to action elsewhere in the cochlea. Among the

suggested primary sites are the stria vascularis, especially the mitochondria of the stria vascularis, the pericapillary elements of the outer sulcus with damage extending throughout the spiral ligament, spiral limbus and Reissner's membrane (Hawkins, 1970). The stria vascularis elements of the spiral ligament and Reissner's membrane are all involved in secretion or filtration, and a disruption of this function might easily result in marked changes in the environment of the hair cells. Such a change in hair cell environment could account for the diminution in cochlear microphonics as well as the observed morphological changes.

The aminoglycosides penetrate the inner ear via the blood circulation and then accumulate in endolymph and perilymph from which they are eliminated very slowly. Ototoxicity was explained by Stupp, Kupper, Lagler et al. (1973) to be due to high concentrations and the persistence of the antibiotics in the inner ear. The aminoglycoside antibiotics are ototoxic to varying degrees. Accordingly, Stupp et al. (1973) found that the less toxic antibiotics showed a substantially lower concentration in the inner ear. The highest concentration in the inner ear was found with neomycin which proved to be the most toxic thus indicating that drug accumulation in the perilymph is apparently related to the ototoxicity of these drugs (Brummett, Fox, Bendrick et al., 1978).

Furthermore, it is now clear that ototoxicity of aminoglycosides is potentiated by diminished renal function, presumably because of the reduced ability to excrete the drug with resultant prolonged exposure of cochlear structures to increased concentrations.

1.4 NEUROMUSCULAR BLOCKADE

Pittinger and Adamson (1972) have suggested that the streptidine or deoxystreptamine moiety of the aminoglycoside are involved in the neuromuscular blocking activity. However, other portions of the aminoglycosides must also contribute since the blocking potency of the aminoglycosides containing deoxystreptamine varies widely. The competitive hypothesis of Brazil and Franceschi (1969) which states that aminoglycosides, like magnesium compete with calcium ions and thus inhibit the presynaptic release of acetylcholine (Ach) appears to be the most consistent with the available data.

The relative presynaptic and postsynaptic effects of the aminoglycosides vary with the drug employed, the concentrations of the drug and ionised calcium, and the type of muscle preparation studied. Adams, Mathew, Teske et al. (1976) showed predominantly presynaptic effects in fast-twitch muscles and post-synaptic effects in slow-twitch muscles. A predominant presynaptic effect would occur in studies with neomycin or gentamicin, low ionised calcium, or fast-twitch muscles while postsynaptic effects would predominate in studies with streptomycin, high ionised calcium, or slow-twitch muscles. However, the presynaptic effects are probably the more important component in aminoglycoside-induced blockade in humans since the blockade is predictably reversed by calcium but not anticholinesterases. Also, the blocking potency of the aminoglycosides varies directly with their presynaptic effects (Pittinger and Adamson, 1972).

Both the presynaptic and postsynaptic components of aminoglycoside-induced blockade appear to depend upon a drug-membrane

interaction which affects the passage of positive ions through the membrane. Investigations on the effects on calcium uptake into muscles have shown that the aminoglycosides decrease total calcium uptake and increase calcium efflux.

1.5 MECHANISMS OF AMINOGLYCOSIDE TOXICITY

1.5.1 Polyphosphoinositides

The polyphosphoinositides have attracted particular attention because of their rapid turnover in excitable and secretory tissues, apparently being localised in plasma membranes (Harwood and Hawthorne, 1969). Because of their rapid turnover in kidney (Tou, Hurst, Baricos et al., 1972), inner ear (Schacht, 1976), and the brain (Kai and Hawthorne, 1969), and the strongly ionic nature of their polar head groups, it has been suggested that they may be involved in the active transport of cations (Michell, 1975).

That there is a close connection between the bioelectric events in the inner ear and the phosphoinositide turnover is shown by the parallel affects of aminoglycosides on the cochlear microphonics (CM) and on phospholipid labelling.

Nuttall, Marques and Lawrence (1977) showed that perfusion of the guinea pig cochlea with artificial perilymph containing neomycin promptly reduced the ability of this organ to generate CM in response to a sound stimulus. When radiolabelled phosphate is added to the artificial perilymph, it is incorporated into membrane phospholipids and addition of neomycin inhibits incorporation of the labelled phosphate into phosphatidylinositol diphosphate, which is one of the components of the membranes (Schacht, Lodhi and Weiner, 1977; Orsulakova, Stockhorst and Schacht, 1976). Furthermore, Orsulokova et al. (1976) have shown that chronic administration of guinea pigs with neomycin for three weeks reduces the incorporation of labelled phosphate into phosphatidylinositol diphosphate in both the organ of Corti and

the stria vascularis. However, whether this is the primary effect may be debatable since there was CM reduction and histological changes in some of the guinea pigs after two weeks and in all of them after three weeks.

Schibeci and Schacht (1977) in their studies with guinea pigs showed that neomycin profoundly altered polyphosphoinositide turnover in the kidney *in vivo* and in renal homogenates *in vitro*. Furthermore, Orsulokova *et al.* (1976) and Schacht (1976) have shown that neomycin, gentamicin and kanamycin A can inhibit binding of the cerebral tissue from guinea pigs. Neomycin has also been shown to inhibit binding of calcium to polyphosphoinositides in artificial lipid monolayers (Schacht *et al.*, 1977).

The observations on the interactions of aminoglycosides with polyphosphoinositide turnover have led Schacht (1976) to propose a detailed model of the possible interaction of these drugs with cell membranes, such as those of proximal tubule cell and in the organ of Corti. It is postulated that the basic amino groups of the antibiotic directly bind to the polyphosphoinositides in the membrane and that the binding may be further stabilised by ionic interactions between other amino groups of the neomycin molecule and anionic groups in the lipids (e.g. the diesteratic phosphate) or in neighbouring membrane components. Normally a phosphomonoesterase enzyme acts upon phosphoinositides to cleave phosphate groups, liberate bound calcium, and thereby alter permeability of the membrane to cations. Restoration of permeability would involve phosphorylation of the same groups. Aminoglycosides, which are strongly cationic, would compete with calcium for the exposed negatively charged phosphate groups.

In this respect one would expect some degree of correlation between the number of amino groups on the antibiotic and the extent of toxicity. Kunin (1970) has shown that the degree of binding is a function of the amino groups and that it correlates well with the extent of ototoxicity. The degree of binding to tissue homogenates was neomycin > kanamycin > gentamicin > streptomycin, with the number of amino groups being 6, 4, 3, and 2 respectively. The ototoxicity caused by the drugs followed the same sequence.

However, recently, Griffin, Sykes and Hawthorne (1980) have raised questions, whether the previously reported influence of neomycin on polyphosphoinositide turnover *in vivo* (Orsulokova *et al.*, 1976) is caused by a direct interaction of antibiotic and phospholipid or indirectly through an effect of neomycin on Ca^{2+} metabolism. From their studies with guinea pig synaptosomes there was no evidence of Ca^{2+} binding to membrane polyphosphoinositides, which are more likely to be located on the cytoplasmic face of the plasma membrane. The evidence suggested that neomycin does not readily enter intact synaptosomes and that the binding of neomycin to the external surface of the synaptosomal plasma membrane restricts Ca^{2+} entry through both the late Ca^{2+} and Na^{+} channels.

1.5.2 Polyphosphoinositides and Na^{+}-K^{+} ATPase

A direct link between aminoglycoside ototoxicity and Na^{+}-K^{+} ATPase inhibition has been shown by Iinuma (1967) and Kaku, Farmer and Hudson (1973). They showed that kanamycin intoxication in guinea pigs produced a reduction in ATPase activity of the stria vascularis and spiral ligament. The effect of aminoglycosides on

Na^+-K^+ ATPase activity as a possible mechanism of nephrotoxicity and ototoxicity has been investigated and discussed in section 4.2.

Furthermore, a number of studies have suggested that Na^+-K^+ ATPase activity and polyphosphoinositide turnover may be closely linked;

(i) Enzymatic characteristics of Na^+-K^+ ATPase and transport of various solutes are severely impaired in inositol-deficient mammalian cells. That this was not due to the mere disruption of the membrane was shown by the adenyl cyclase and 5' nucleotidase (both membrane bound enzymes) activities which compared favourably with normal cells (Charalampous, 1971).

(ii) Mandersloot, Roelofsen and DeGier (1978) have shown that phosphatidylinositol is the endogenous activator of the Na^+-K^+ ATPase in microsomes of rabbit kidney by systematic degradation of phospholipids associated with the enzyme by using degrading enzymes specific for a particular phospholipid.

1.5.3 Impairment of carbohydrate metabolism and energy utilisation by aminoglycosides

The hypothesis that the aminoglycosides act through impairment of carbohydrate metabolism and energy utilisation by the outer hair cells of the organ of Corti and by the kidney seems plausible. Kanamycin has been implicated in;

(i) suppression of respiratory enzyme activity in outer hair cells (Kaku, Farmer and Hudson, 1973).

(ii) inhibition of the Embden-Meyerhof pathway, but not the hexose monophosphate pathway in tissues prepared from the kidney and the organ of Corti of guinea pigs (Tachibana, Mizukoshi and Kuriyama, 1976).

(iii) impairment of ATPase activity in the organ of Corti (Kaku et al., 1973).

Furthermore, the outer hair cells of animals made diabetic with alloxan can be protected from kanamycin toxicity (Garciaqu, Glade, Norris et al., 1978) by increasing the blood glucose concentrations, possibly because glucose interferes with transport of the aminoglycosides into the cochlea. This may be possible from the observation of Owada (1962) who found that an amino sugar component of kanamycin, 3-glucosamine, was more ototoxic than the antibiotic itself.

Thus, interference with carbohydrate metabolism, inhibition of organ of Corti ATPase, and the competition between glucose and the aminoglycosides for transport into the cochlea could lead to less glucose being available for hair cell utilisation to inefficient use of the glucose that is available and to glycogen depletion. The energy requirements of the affected hair cells would then exceed readily available supplies and cell death would ensue.

1.5.4 Lysosomes

Renal dysfunction has been related to the development of ultrastructural alterations of the lysosomes in proximal tubule cells. These alterations consist of the formation of numerous "cytosegresomes", many of which contain prominent myeloid bodies (Kosek, Mazze and Cousins, 1974). Gentamicin accumulates in the renal cortex and more recently evidence for its localisation within the lysosomes of kidney cells has been presented by Just, Erdmann and Habermann (1977).

The accumulation of gentamicin in lysosomes can have profound affects on the functioning of the lysosomal system. There would

be an increase in lysosomal pH and inhibition of lysosomal functions such as breakdown of proteins. Tukens, Hoof and Tulkens (1979) reported evidence of gentamicin-induced lysosomal phospholipidosis in cultured rat fibroblasts through dysfunction of sphingomyelinase and probably other phospholipases. Eventual lysosomal labilisation would contribute towards propagation of cellular damage.

1.5.5 Protein synthesis

The bactericidal actions of the aminoglycosides are thought to be mediated by an interference with protein synthesis, due to the drug binding to a protein of the 30S ribosomal subunit (Hahn and Sarre, 1969). Since the proximal tubular cells and stria vascularis are rich in mitochondria which possess 30S ribosomal subunit, a similar mode of action has been suggested by Beard et al. (1969). Recently, several aminoglycosides have been observed to inhibit protein synthesis and elicit misreading of natural messenger RNA in extracts of eucaryotic cells, including cultured human cells (Wilhelm, Jessop and Pettitt, 1978).

1.6 AIMS OF THE PRESENT STUDY

The aim of the present study was to investigate possible mechanisms of toxicity of the aminoglycoside antibiotics, since a number of hypotheses have already been postulated by other workers (section 1.5) but none as yet have been conclusively demonstrated.

The initial stimulus for the present work was provided by the suggestion that toxic effects of gentamicin might be related to its effect on plasma membrane Na^+-K^+ ATPase and polyphosphoinositides. Histological evidence has been reported which showed depression of Na^+-K^+ ATPase activity in the ear by the aminoglycosides. This initial investigation, however, laid the foundations for a study of much wider scope.

Since the accumulation of gentamicin in the renal cortex precedes renal dysfunction, it was considered that a greater knowledge of the mechanism of aminoglycoside transport would give better insight into its primary mechanism of action. A number of mechanisms have been postulated, such as uptake by the organic base and amino acid transport system, or via endocytosis, but none have yet been substantiated.

SECTION 2

MATERIALS

2.1 DRUGS

Gentamicin sulphate (CIDOMYCIN) 1.0 mg ≡ 0.543 mg gentamicin base
Roussel Laboratories Ltd., Middlesex.

Gentamicin injections (CIDOMYCIN INJECTABLE)
2 ml ampoules ≡ 80 mg gentamicin base.
Roussel Laboratories Ltd., Middlesex.

Kanamycin sulphate.
Sigma Chemical Company.

Neomycin sulphate.
Sigma Chemical Company

Ribostamicin (RIBASTAMIN)
Laboratorios Morrith, Madrid

Dihydrostreptomycin sulphate
Sigma Chemical Company.

Streptomycin sulphate: Ref. EPB(C)11/22
specially provided by
Glaxo Laboratories Ltd., Middlesex.

2.2 RADIOCHEMICALS

All radiochemicals from 'The Radiochemical Centre', Amersham, Bucks.

|methyl-^{3}H| gentamicin-C_1-sulphate (37.8 Ci/m mol).
The position of labelling in gentamicin is shown in Fig. 3.

n-|1,2-^{3}H|hexadecane (2.17 µCi/g) reference standard for liquid scintillation counting.

|^{3}H|inulin (230 µCi/mg)

|^{14}C|carboxylic acid inulin (8.51 mCi/m mol)

DL|1-^{14}C|leucine (54 mCi/m mol)

L-|U-^{14}C|lysine monohydrochloride (342 mCi/m mol)

Rubidium-86 (1 mCi/224 µg Rb)

2.3 LIQUID SCINTILLATION COUNTING REAGENT

NE260 micellar liquid scintillation cocktail.
Nuclear Enterprises Ltd., Reading, Berks.

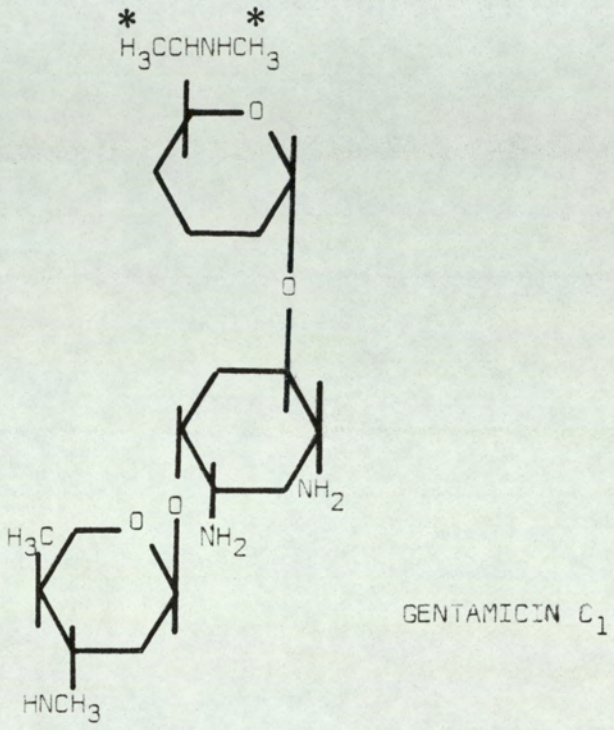


Fig. 3. The position of |methyl-^{3}H| in gentamicin C_1. The asterisk indicates the position of labelling in the radiolabelled gentamicin.

2.4 MISCELLANEOUS CHEMICALS

ATP: adenosine-5'-triphosphoric acid.
BDH Chemicals Ltd., Poole, Dorset.

L-alanine-4-nitrohydrochloride.
Merck, Darmstadt.

Collagenase Type IV.
Sigma Chemical Company.

Creatinine.
BDH Chemicals Ltd.

2,4-DNP: 2,4-dinitrophenol.
BDH Chemicals Ltd.

Glucose-6-phosphate dihydrogenase (Type XI).
Sigma Chemical Company.

Hexokinase (Type C-130).
Sigma Chemical Company.

Iodoacetic acid.
BDH Chemicals Ltd.

D-Mannose-6-phosphate (disodium salt).
Sigma Chemical Company.

NADH: β-nicotinamide adenine dinucleotide reduced form.
Sigma Chemical Company.

NADP: α-nicotinamide adenine dinucleotidephosphate.
Sigma Chemical Company.

p-Nitrophenyl-N-acetyl-β-D-glucosaminide.
Sigma Chemical Company.

Ouabain octahydrate.
Sigma Chemical Company.

Potassium cyanide.
Fisons Scientific Apparatus Ltd.

Pyruvic acid (sodium salt).
Sigma Chemical Company.

Spermine tetrahydrochloride
Sigma Chemical Company.

Triza-base: Tris(hydroxymethyl)aminomethane.
Sigma Chemical Company.

Krebs-Hensleit (1932) bicarbonate buffer.
NaCl (118 mM); KCl (5 mM); $CaCl_2$ (1.27 mM);
$MgSO_4$ (1.2 mM); KH_2PO_4 (1.2 mM) and $NaHCO_3$ (25 mM).

2.5 ANIMALS AND THEIR DIETS

All animals were supplied by Bantin and Kingman (Hull). They were held in the animal house for at least a week to acclimatise before being used for experiments.

Male Wistar rats : Diet (rat) modified 4 lb
Breeding diet.
L.A. Pilsbury Ltd.

Hartley albino guinea pigs: T.R.2. diet
L.A. Pilsbury Ltd.

SECTION 3

METHODS

3.1 EVALUATION OF A METHOD FOR DETERMINING INORGANIC PHOSPHATE IN PRESENCE OF AMINOGLYCOSIDES (see Section 4.1).

3.2 THE EFFECT OF AMINOGLYCOSIDES ON Na^+-K^+ ATPase ACTIVITY

3.2.1 Preparation of erythrocyte ghosts

Erythrocyte ghosts were prepared by hypotonic lysis according to the method of Bramley, Coleman and Finean (1971) which itself was a modification of the method of Dodge, Mitchell and Hanahan (1963).

(i) Red cells

Fresh, human heparinized blood was obtained from a Blood Transfusion Centre (West Midlands) and used within one week of the date of collection. The red cells were prepared by centrifuging the blood at 1570 g for 10 minutes and by washing twice in 4 volumes of ice cold 0.155 M $NaHCO_3$ (pH 7.6). Care was taken to remove all of the buffy layer above the red cells.

(ii) Preparation of ghosts

An outline of the procedure is given in Fig. 4. The washed cells were resuspended in the centrifuge tube with 0.155 M $NaHCO_3$ buffer to give a cell/buffer ratio of 1 : 1. This 50% cell suspension was transferred to a larger container and then diluted 1 : 4 with the same buffer and stored for 30 minutes. After centrifugation at 4000 g for 20 minutes the supernatant was removed and haemolysis performed by resuspending the sedimented cells 1 : 10 in bicarbonate buffer, 20 mOSm pH 7.6. Thirty minutes after haemolysis ghosts were sedimented at 20,000 g for 10 minutes and subsequently washed 3 times in the same 20 mOSm buffer and finally once with isotonic 0.172 M Tris-HCl (pH 7.4). The cells were resuspended in 0.172 M Tris-HCl.

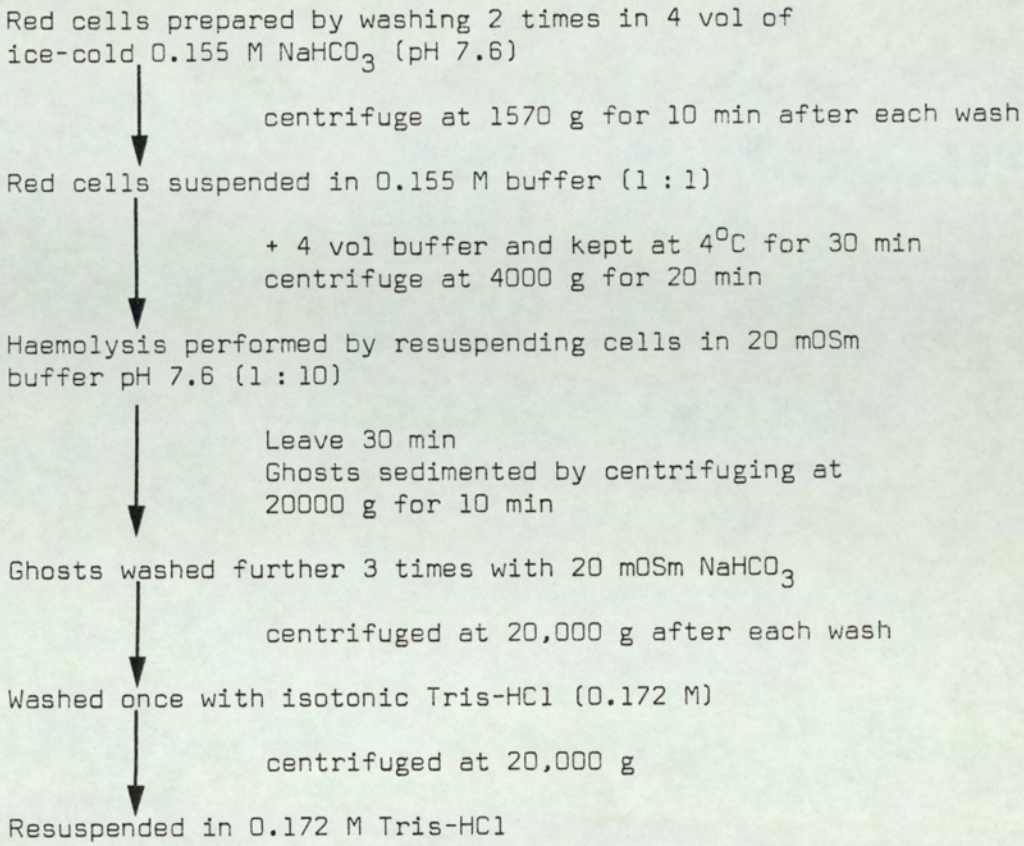


Fig. 4. Preparation of erythrocyte ghosts by hypotonic lysis.

All operations were carried out at 0 - 4°C.

(iii) Enzyme assays of Na^+-K^+ ATPase EC 3.6.1.3

Incubation time for ATPase assays was 10 minutes at 37°C with all assays performed in triplicate with appropriate blanks and controls.

The Na^+-K^+ ATPase activity was assayed by the procedure of Hokin and Hokin (1964) using the following conditions: 100 mM NaCl, 15 mM KCl, 2 mM $MgCl_2$, 2 mM ATP, 0.2 mM ouabain, 50 mM Tris-HCl pH 7.4 and 0.5 - 1.0 mg ghost protein in a final volume of 0.5 ml.

Thus, the ouabain sensitive Na^+-K^+ ATPase activity was calculated as the difference in activity with and without 0.2 mM ouabain.

The effect of aminoglycosides on Na^+-K^+ ATPase was studied by preincubating the ghosts with the drugs for 10 minutes.

At the end of the incubation period, 0.2 ml of 10% TCA was added to the reaction mixture and the inorganic phosphate released measured by the modified method of Ueda and Wada (1970)(see Section 4.1).

Protein was determined by the method of Lowry, Rosebrough, Farr et al. (1951).

3.2.2 Preparation of 'microsomal fraction' from the cortex and medulla of the guinea pig kidney

(i) Animals

Hartley albino guinea pigs (300 - 400 g) were kept in the animal house with free access to food and water.

(ii) Microsomal preparation

The procedure is outlined in Fig. 5 which was adapted from the method of Jørgensen (1974). All operations were carried out

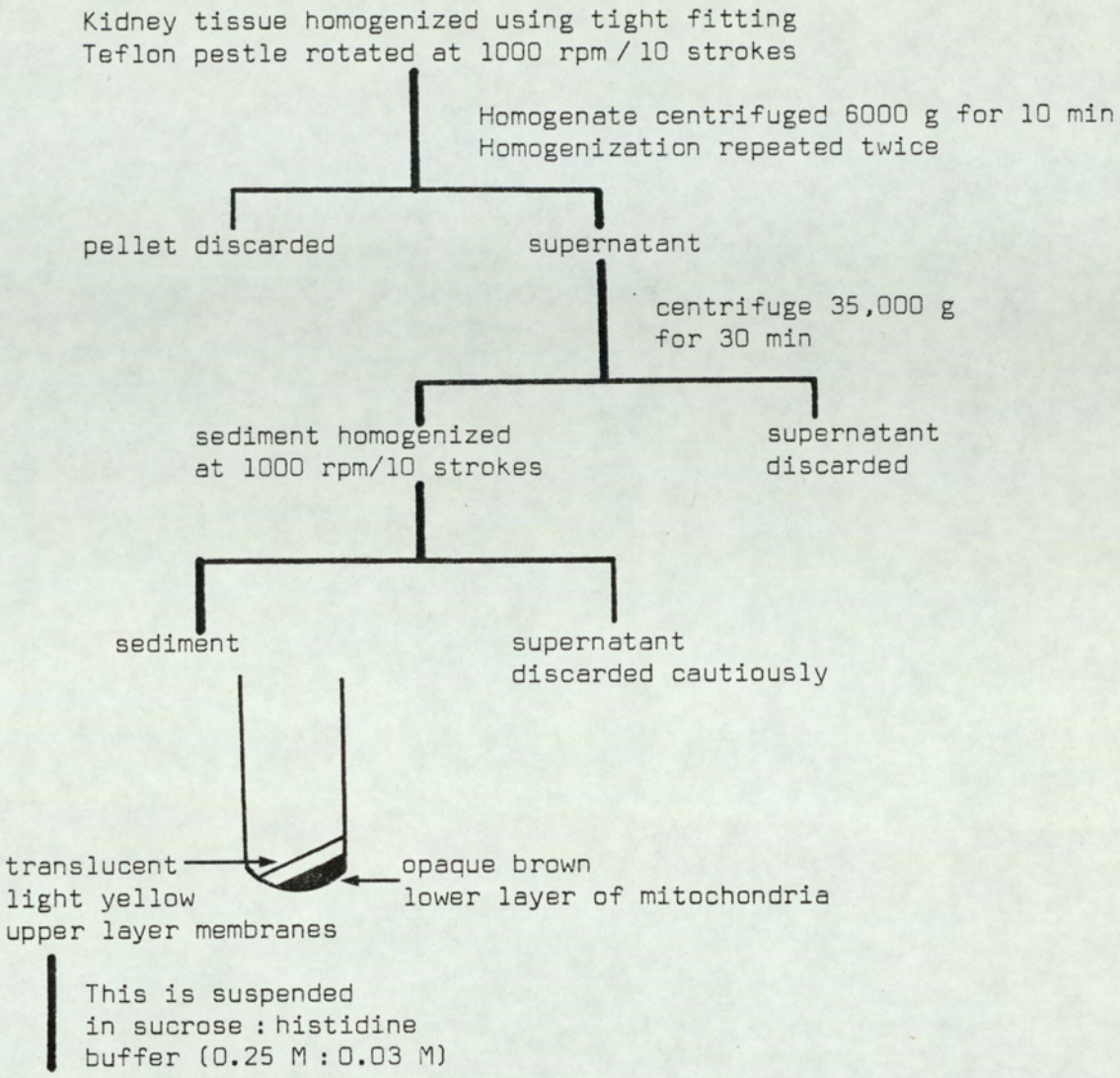


Fig. 5. Preparation of 'microsomal fraction' from the cortex and medulla region of the guinea pig kidney.

at 0 - 4°C unless otherwise specified. Guinea pigs were killed by sharp dislocation of the neck followed by exsanguination. The kidneys were removed rapidly and placed in ice-cold 0.03 M histidine, 0.25 M sucrose pH 7.2. Samples of the cortex were collected from at least 3 kidneys by cutting on the cortical side of the boundary with the outer medulla. Portions of the red outer medulla were dissected free of other tissue by cutting well inside the boundaries with the cortex and white inner medulla. The tissue from each zone was then homogenized in 10 ml sucrose-histidine solution per g tissue with 10 strokes in a glass homogenizer with a tight fitting Teflon pestle rotated at 1000 rev/min. The homogenate was centrifuged at 6000 g for 10 minutes. The sediment was resuspended by homogenisation in the original volume of sucrose-histidine solution and centrifuged again at 6000 g for 10 minutes. The combined supernatants were centrifuged at 35,000 g for 30 minutes. The sediment was resuspended in sucrose-histidine buffer and again homogenised as above. This was then centrifuged at 35,000 g for 10 minutes. The pellet, i.e. the microsomal fraction was resuspended in sucrose-histidine solution and then stored in aliquots at -20°C.

(iii) Enzyme assays of Na^+-K^+ ATPase and 5' Nucleotidase

Incubations were carried out at 37°C with all assays performed in triplicate with appropriate blanks and controls.

Na^+-K^+ ATPase EC 3.6.1.3.

ATPase activity was assayed by the procedure of Jørgensen (1974) using the following conditions: 130 mM NaCl, 20 mM KCl, 3 mM $MgCl_2$, 3 mM ATP, 1 mM ouabain, 30 mM histidine (pH 7.5) and membrane protein in a final volume of 0.5 ml. The Na^+-K^+ ATPase

activity was taken as the difference in activity with and without ouabain. The preincubation in presence of ouabain and aminoglycosides was 10 minutes.

5'Nucleotidase EC 3.1.3.5.

Enzyme activity was assayed in 100 mM Tris-HCl buffer (pH 8.5) in presence of 10 mM $MgCl_2$ and 10 mM AMP in a final volume of 0.5 ml.

The inorganic phosphate was measured by the modified method of Ueda and Wada (1970), and protein determined by Lowry et al. (1951).

3.3 IN VITRO UPTAKE OF GENTAMICIN INTO RAT KIDNEY SLICES

3.3.1 Preparation of kidney slices

Male Wistar rats (150 - 250 g) were used in all experiments. The animals were allowed free access to water and a purina rat chow diet until they were killed by stunning and decapitation. The kidneys were then quickly removed, decapsulated and placed in ice-cold Krebs-Hensleit buffer. Thin cortical slices, 0.5 mm thick and weighing approximately 30 mg were then prepared with a Steadie-Riggs microtome. The initial (polar) slice from each hemi-kidney was discarded and slices containing only cortical tissue were obtained from each hemi-kidney.

3.3.2 Incubations

Renal cortical slices were preincubated at 37^{o}C for 10 minutes in 25 ml Erlenmeyer flasks containing 4 ml of freshly gassed Krebs-Hensleit bicarbonate buffer, pH 7.4. After this equilibration period the slices were transferred to fresh incubation medium incorporating the radiolabelled compound. The flasks were continuously gassed with O_2-CO_2 (95 : 5), while being shaken at 100 cycles/minute at 37^{o}C.

In experiments performed under anaerobic conditions the incubation medium was gassed with N_2-CO_2 (95 : 5) for the 10 minute preincubation period, and the subsequent incubations performed in nitrogen atmosphere.

Incubation medium containing reduced levels of Na^+ was prepared by replacing NaCl with LiCl on an equimolar basis, whilst Na^+ free medium was prepared by further substituting 0.03 M Tris-HCl for KH_2PO_4 and $NaHCO_3$.

3.3.3 Analytical procedures

At the end of the incubation period, the slices were rinsed rapidly in cold Krebs-Hensleit buffer, blotted on filter paper and weighed on a torsion balance. The soluble radioactivity in the slice was then extracted into water by adding 0.6 ml H_2O and heating at 100°C for 15 minutes in an oil-bath. The tissue was then homogenised and centrifuged at 3,500 rpm for 10 minutes. The supernatant was removed and kept and the pellet resuspended in 0.5 ml H_2O, heated at 100°C for 5 minutes, homogenised and then centrifuged. The supernatant from the two centrifugation processes was combined and added to counting vials containing 10 ml NE260 scintillation fluor. Radioactivity in the incubation medium was also determined by counting in NE260 scintillation fluor. The vials were sealed, shaken and counted in a liquid scintillation spectrometer.

3.3.4 Total tissue H_2O

Total tissue water was determined by the difference between the tissue weight after blotting and the weight after drying at 100°C to constant weight. This value was expressed as a percentage of the wet tissue weight.

3.3.5 Extracellular space volume

In experiments of this nature a fraction of the radioactivity taken up into slices of kidney, or any other soft tissue, is due to the passive equilibration of drug between the incubation medium and the extracellular fluid. In order to quantify this phenomenon the extracellular space volume in kidney slices was measured using $|^{14}C|$ inulin. Inulin is a polysaccharide of high molecular

weight (approximately 5200) and because of its molecular size it does not penetrate cell membranes, but remains within the extracellular space. Since, at equilibrium, the concentration of inulin in the incubation medium and in the extracellular water should be the same, the amount of $|^{14}C|$ inulin taken up from the incubation medium into kidney slices at equilibrium allows quantitation of the extracellular space volume (Marlow and Sheppard, 1972).

The labelled $|^{14}C|$ inulin (0.1 μCi/ml) was incubated with cortical slices in Krebs-Hensleit bicarbonate buffer, pH 7.4, at 37°C in precisely the same fashion as that described above. At the end of the incubation period the volume of distribution of the inulin was calculated from the radioactivity recovered from the aqueous tissue supernatant (dpm), the radioactivity remaining in the medium (dpm/ml) and the wet weight of the tissue. The result was expressed as percent of total tissue wet weight.

$$\text{Extracellular space (\% wet tissue wt)} = \frac{\dfrac{\text{dpm inulin in total tissue } H_2O}{\text{dpm inulin/ml medium}}}{\text{wet tissue weight}} \times 100$$

Thus, measurement of the radioactivity of the media and water extract and estimation of total tissue H_2O (% wet tissue weight) and inulin space (% wet tissue weight) permits the distribution ratio of the compound or ratio of dpm per ml intracellular fluid to that in the media to be calculated.

$$\text{dpm in intracellular fluid/ml} = \frac{\text{dpm in tissue water} - (\text{vol. of inulin space} \times \text{dpm in media/ml})}{\text{total tissue water} - \text{inulin space}}$$

3.3.6 Scintillation counting

All radioactivity measurements were made using a Beckman LS-230 liquid scintillation spectrometer or Packard Tricarb 2660 liquid scintillation system. With the Beckman instrument, counting efficiency was monitored by internal standardisation using $|^{14}C|$ hexadecane as the internal standard whereas an external standard ratio method was used on the Packard.

3.4 PREPARATION OF ISOLATED RAT RENAL TUBULES AND THEIR CHARACTERISATION

3.4.1 Preparation of tubules

The preparation of the tubules was adapted from the method of MacDonald and Saggerson (1977). Male Wistar rats (150 - 200 g) were stunned and then killed by cervical dislocation. The kidneys were removed quickly, decapsulated and placed in ice-cold Krebs-Hensleit buffer. The medulla and papilla were excised and the pooled cortices were collected, cut into small pieces and shaken at 37°C under O_2-CO_2 (95 : 5) in a 50 ml silicone-treated Erlenmeyer flask containing 10 ml of Krebs-bicarbonate buffer (Ca^{2+} 1.27 mM) and collagenase (2 mg/ml).

After digestion for 30 minutes the tissue suspension was then gently forced through nylon mesh (No. 110) to remove undigested pieces and to aid dispersion. This was followed by centrifugation at 950 rpm for 1 minute (bench centrifuge) and the resulting pellet resuspended and washed twice by similar centrifugation with the bicarbonate buffer. Finally, this was passed through a double-layered nylon mesh to aid further dispersion and then diluted with buffer to concentration of tubules required.

3.4.2 Incubations

Incubations were commenced immediately after preparation of tubule fragments. Aliquots of stock tubule suspension was dispensed in 25 ml silicone treated Erlenmeyer flasks to give the desired tubular concentration in a final volume of 4 ml. These incubation flasks contained Krebs-Hensleit bicarbonate buffer with $CaCl_2$ (1.27 mM) and substrate pyruvate (5 mM).

The flasks were continuously gassed with O_2-CO_2 (95 : 5), whilst being shaken at approximately 70 oscillations/min at 37°C.

3.4.3 Characterisation

(i) Electron microscopy

A loose pellet of freshly isolated renal tubules were fixed by resuspending them in a 5% solution of gluteraldehyde in cacodylate buffer (0.1 M; pH 7.4). After 1.5 hours the tubules were transferred to a solution of 10% sucrose in cacodylate buffer and kept with at least two changes for 24 hours. Post-fixation was done in 1% osmium tetroxide in the same cacodylate buffer as the primary fixation.

For scanning electron microscopy (SEM), cells were dehydrated in graded alcohols followed by a stepwise transfer to amylacetate. Drying was completed using CO_2 in a critical point drier. The tubules were trapped on lint free cotton for the drying and mounting process. The tubules were sputter-coated with gold and viewed in a Cambridge S600 (SEM) instrument.

For transmission electron microscopy (TEM), prior to dehydration in graded alcohols, excess fixative was removed and the tubules resuspended in 2% agar at 46 - 47°C. This was immediately poured onto a cold glass slide and, when set, cut into 1 mm cubes. These cubes were then dehydrated and embedded in a Spurr resin. Thin sections were cut on an LKB ultramicrotome and stained with uranyl acetate and lead citrate before examining in an AEI6B (TEM) instrument.

(ii) Gluconeogenesis - glucose estimation

Glucose was measured enzymatically (Willamson and Herczeg, 1969) using hexokinase and glucose-6-phosphate dehydrogenase on

extracts prepared by deproteinisation of incubation flask contents with 0.5 ml of ice-cold 45% (w/v) $HClO_4$. These were left in ice for 20 minutes and then centrifuged to remove the protein. The supernatant was neutralised by addition of 1 M Triethanolamine HCl (0.5 ml) and concentrated K_2CO_3 (5 M) 0.45 ml.

The glucose was then measured fluorimetrically in an Aminco-Bowman Fluorimeter at an emission and excitation wavelength of 465 nm and 340 nm respectively. The Trietholamine-HCl (50 mM), pH 7.4, buffer used for assay consisted of $MgCl_2$ (10 mM) and EDTA (5 mM). During the assay the volume of the buffer in the cuvette was adjusted so that volume of buffer plus sample was 2 ml. To this 10 µl of ATP (0.1 M) and NADP (10 mg/ml) and 5 µl of glucose-6-phosphate dehydrogenase (28 EU/ml) were added. The reaction was started by addition of 5 µl of hexokinase (280 EU/ml).

Glucose calibration curve was similarly prepared by adding known amounts of glucose to the cuvette.

(iii) $|^{14}C|$ leucine incorporation into TCA insoluble material by tubules - protein synthesis

Tubules were prepared in the usual way, except that the final pellet was resuspended in Krebs-Hensleit buffer containing 20 amino acids, each at a concentration of 0.25 mM (Baxter and Turtle, 1978).

In 50 ml Erlenmeyer flasks containing a final incubation volume of 20 ml was added the radiolabelled $|^{14}C|$ leucine. At given times, 1 ml aliquots in duplicate were removed and the reaction terminated by addition of 3 ml of cold 10% TCA. The TCA precipitable extract was then filtered onto Whatman GF/C (2.5 cm) millipore filters and washed 3 times with 3 ml portions of chilled TCA. The filters were then dried under an infra-red lamp and the radioactivity counted in 10 ml of NE260 scintillation fluor.

(iv) Lysine uptake by isolated renal tubules

Tubules were incubated with continuous gassing with O_2-CO_2 (95 : 5) and shaken at 37°C in the presence of the labelled compound.

At the end of the incubation period the incubation mixture was added to tared tubes, cooled rapidly to 0°C and centrifuged at 3,500 rpm for 10 minutes. The supernatants were removed for subsequent counting and the pellet surfaces washed twice with ice-cold buffer (which was removed by suction). The tubes were weighed again to obtain the wet weight of the pellet. The cells were lysed by the addition of 1 ml of distilled H_2O and resuspended, followed by heating at 100°C for 10 minutes in an oil-bath. The lysate was then centrifuged at 3500 rpm for 10 minutes. The aqueous tissue extract thus obtained and its corresponding supernatant were separately counted in 10 ml of NE260 scintillation fluor by liquid scintillation technique.

3.4.4 Total tissue H_2O

The difference between the wet weight of the pellet and the weight after drying. The value was expressed as percentage of the wet tissue weight.

3.4.5 Extracellular space (trapped volume)

The volume of "trapped medium" in the pellet was established by the method of Burg and Orloff (1962). $|^{14}C|$ carboxy inulin was added to the tubule suspension immediately before centrifugation and then $|^{14}C|$ determined in both the medium and in the aqueous tissue extract.

Results expressed as percentage of total tissue weight.

$$\text{Extracellular space (\% wet tissue weight)} = \frac{\frac{\text{dpm in total tissue } H_2O}{\text{dpm inulin/ml medium}}}{\text{wet tissue weight}} \times 100$$

3.4.6 Volume of intracellular fluid

Difference between total tissue H_2O and the volume of "trapped medium".

3.4.7 Intracellular fluid counts

$$\text{dpm in intracellular fluid / ml} = \frac{\text{dpm in tissue water} - (\text{vol. of inulin space} \times \text{dpm in media/ml})}{\text{total tissue water} - \text{inulin space}}$$

3.5 IN VITRO UPTAKE OF GENTAMICIN INTO ISOLATED RENAL TUBULES

3.5.1 Incubations

The tubules were incubated in Krebs-Hensleit bicarbonate buffer, under conditions specified for a particular experiment in siliconised Erlenmeyer flasks with continuous gassing with O_2-CO_2 (95 : 5).

3.5.2 Analytical procedures

At the end of the incubation period 200 µl aliquots of the incubation mixture were removed and layered on a specifically prepared Beckman microcentrifuge tubes. The tubes were initially prepared by dispensing 50 µl of formic acid onto which 100 µl of silicon-corn oil mixture (10 : 3) of specific density was layered. This oil density was such that on centrifuging the tubes at 9000 g for 2 minutes (Beckman Microfuge B), the tubule would migrate through the oil and settle between the oil - formic acid interface. After centrifugation the tubes were dipped into liquid nitrogen and then cut to give two halves, one containing the pellet and the other the supernatant.

These were then placed separately into empty vials and kept for an hour in order to thaw them out. They were then counted using 10 ml of NE260 scintillation fluor.

3.5.3 Percentage "carry-over" - correction for trapped medium

This was determined after each experiment using $|^3H|$ inulin. $|^3H|$ inulin was added to the incubation mixture which was then processed as above. The counts carried over with the pellet were expressed as a percentage of the supernatant count. This percentage

was subtracted from the pellet of each experiment taking into consideration its own supernatant count.

3.5.4 Studies of the inhibition of gentamicin uptake into isolated renal tubules

In these experiments, tubules were incubated as described above, using $|^{3}H|$ gentamicin plus unlabelled gentamicin to give a final concentration of 10^{-4} M for 60 minutes in the presence of potential inhibitors. The tubules were preincubated with three concentrations of each inhibitor, 0.5 mM, 1.0 mM and 2.0 mM for 15 minutes. The inhibitors used were 2,4-dinitrophenol (2,4-DNP), potassium cyanide (KCN), iodoacetic acid and ouabain octahydrate.

3.5.5 Gentamicin binding at $4^{o}C$

Tubules were incubated at $4^{o}C$ with continuous gassing in the presence of $|^{3}H|$ gentamicin with appropriate amounts of cold gentamicin. The incubations were terminated after various times and the incubation mixtures were processed as before.

3.5.6 Scintillation counting

Radioactivity was measured by the Packard Tricarb 2660 liquid scintillation system, with quench correction using an external standard.

3.6 IN VITRO LYSOSOMAL ENZYME RELEASE FROM ISOLATED RAT RENAL TUBULES

3.6.1 Incubations

The tubules were incubated with shaking at 37°C in Krebs-Hensleit bicarbonate buffer (pH 7.4), containing 5 mM pyruvate. Each 25 ml siliconised Erlenmeyer flask contained an incubation volume of 4 ml with or without gentamicin and with continuous gassing with a mixture of O_2-CO_2 (95 : 5).

At the end of the incubation period, the incubation mixture was removed and centrifuged immediately in a bench centrifuge at 3500 rpm for 10 minutes. The supernatant was removed and assayed for the enzymes acid phosphatase (AP) and N-acetyl-β-glucosaminidase (NAG) which are thought to be primarily of lysosomal origin (Morin, Fresel, Fillastre et al., 1979).

3.6.2 Stability of AP and NAG on incubation at 37°C and in the presence of gentamicin

Tubules were frozen in liquid nitrogen and thawed 3 times in order to disrupt the cells and internal organelles and to release all the lysosomal enzymes. The extract was centrifuged (3500 rpm for 10 minutes) and the supernatant removed for stability studies.

The supernatant was incubated as above at 37°C up to 2.5 hours and then assayed for AP and NAG. Stability studies in the presence of 10^{-3} M gentamicin were similarly carried out.

3.6.3 Enzyme Assays

(i) Acid Phosphatase (AP). EC.3.1.3.2

The enzyme solution (0.1 - 0.4 ml) and sufficient 0.1 M Na-citrate buffer of pH 4.8 to give a volume of 0.9 ml was equilibrated

at 37°C. The reaction was initiated by the addition of 0.5 ml of p-nitrophenyl phosphate (4 g/litre citrate buffer) and the mixture incubated at 37°C with continuous shaking. The reaction was terminated after 30 minutes by the addition of 5 ml of 0.1 M NaOH solution and the absorption read at 410 nm.

(ii) N-acetyl-β-glucosaminidase (NAG) EC. 3.2.1.3.0

NAG was assayed similarly except that p-nitrophenyl-N-acetyl-β-glucosaminide (250 mg/litre citrate buffer) was substituted for p-nitrophenol phosphate as substrate.

Calibration curves for AP and NAG assays were prepared with known amounts of p-nitrophenol in the assay system. Tubular protein was determined by the method of Lowry et al. (1951).

3.7 GENTAMICIN INDUCED NEPHROTOXICITY IN THE RAT

3.7.1 Animals and their maintenance

Male Wistar rats weighing 150 - 250 g were housed in environmentally controlled rooms (12 hours light, 7.00 a.m. - 7.00 p.m. at 72^{o}F) and allowed free access to food and water.

After a period of acclimatisation, rats weighing 150 - 250 g were then housed singly in metabolic cages with free access to food and water. The metabolism units consisted of a stainless steel cage positioned over a glass funnel specifically designed to permit reliable separation of urine and faeces. Food (in a powder form) and water were available in containers which minimised contamination of the urine.

For at least two days prior to commencing injections and also throughout the injection period, 24 hour control measurements of body weight, food intake, water intake, urine volume, urine osmolarity, solute excretion and protein excretion were made.

3.7.2 Injections

Gentamicin sulphate, equivalent to 40 mg/kg body weight/day gentamicin base was injected subcutaneously in a dose volume of 0.1 ml/100 gm body weight. The control groups received a daily subcutaneous injection of an equivalent volume of water. The rats were injected between 9.00 a.m. - 10.00 a.m. The rats were killed 24 hours after the last injection except in studies where they were allowed to recover after termination of the injections.

3.7.3 Collection and treatment of urine for enzyme assays

Spontaneously voided urine, free of contamination by faeces or food, was collected continuously. The collection tubes, which

were changed every 24 hours immediately prior to the daily injection, were covered and surrounded by ice to prevent evaporation and denaturation of enzymes.

The urines were then centrifuged at 3000 g for 10 minutes and their volumes determined. 2 ml of the urine was placed in Visking tubing (No. 1; size 6 mm) which had previously been boiled for 30 minutes. Dialysis was carried out in a cold room (4°C) against approximately 200 volumes of distilled H_2O for 3 hours. All enzyme activities were measured on the dialysed urine.

3.7.4 Enzyme assays

(i) Alanine aminopeptidase EC. 3.4.1.2

Mondorf, Kinne, Scherberich et al. (1972)

The alanine aminopeptidase (AAP) activity was measured using L-alanine-4-nitro-anilide-hydrochloride as substrate. 0.2 ml of urine was mixed with 1.6 ml of 0.067 M phosphate buffer (pH 7.4) and equilibrated to 37°C in a water bath. The reaction was started by adding 0.2 ml of 16.6 mM substrate dissolved in distilled H_2O and absorption read at 405 nm after 15 minutes. Standard curve using various amounts of p-nitroalanine as the substrate was determined using the above assay system.

(ii) Lactate dehydrogenase EC.1.1.1.27

Leathwood and Plummer (1969)

The lactate dehydrogenase (LDH) activity was measured at 37°C by following the rate of change of absorption of $NADH_2$ at 340 nm using an automatic recording spectrophotometer. Urine (0.4 ml) was mixed with 2.4 ml of 0.067 M phosphate buffer of pH 7.4 and 0.1 ml of 3.5 mM $NADH_2$ solution. After equilibration at 37°C, the reaction was started by the addition of 0.1 ml of 32 mM sodium

pyruvate. The molar extinction coefficient of $NADH_2$ of 6.22×10^3 at 340 nm was used in enzyme activity calculations.

(iii) N-acetyl-β-glucosaminidase EC.3.2.1.30

Luft and Patel (1979)

Urine (0.4 ml) and 0.1 M Na-citrate buffer of pH 4.8 (0.5 ml) were equilibrated at 37°C. The reaction was started by the addition of 0.5 ml of p-nitrophenyl-N-acetyl-β-glucosaminide (250 mg/litre citrate buffer) and the mixture incubated at 37°C with continuous agitation. After 30 minutes the reaction was stopped by the addition of 5 ml of 0.1 M NaOH solution and the absorption read at 410 nm. Calibration curve was prepared using known amounts of p-nitrophenol in the above assay system.

3.7.5 Solute excretion

(i) Na^+ and K^+ determination

Sodium and potassium were measured by flame photometry (Corning 435 Flame Photometer) on appropriate dilutions of urine.

(ii) Ca^{2+} and Mg^{2+}

The divalent cations, Ca^{2+} and Mg^{2+} were measured by flame absorption spectrophotometry (Unicam SP90A ATOMIC ABSORPTION SPECTROPHOTOMETER).

(iii) Creatinine

Urinary creatinine was determined by the standard picrate method (Taussky, 1956). To urine (0.1 ml or less) was added 2 ml of picric acid solution followed by 0.6 ml of 1 N NaOH. The colour then developed with time and 10 minutes after the addition of NaOH, distilled water (2.3 ml) was added. They were then read immediately in a spectrometer at 520 nm.

3.7.6 Osmolarity

Urinary osmolarity was determined by freezing point depression on 0.2 ml aliquots of sample.

3.7.7 Urinary protein

Protein was determined by the method of Lowry et al. (1951) with crystalline bovine serum albumin as standard.

3.7.8 Examination of urine for abnormal constituents

All samples of urine were examined qualitatively for the presence of glucose, ketones, blood and changes in pH using Labstix (Ames Company, Stoke Poges, Slough).

3.7.9 Expression of results

(i) Units of urinary enzyme activity

All enzyme activities are given in nmoles of substrate changed per minute and expressed as activity excreted/hr/mg creatinine, i.e. mU/hr/mg creatinine.

(ii) Food and water intake, urine volumes and solute excretion were expressed per 100 g of rat.

SECTION 4

RESULTS

4.1 DEVELOPMENT OF A METHOD FOR ESTIMATING INORGANIC PHOSPHATE IN THE PRESENCE OF GENTAMICIN

4.1.1 Introduction

The interaction of aminoglycosides with plasma membrane ATPases has been speculated as one possible mechanism of drug-induced ototoxicity and nephrotoxicity (see section 1.5.1 and 1.5.2).

In order to study the activities of membrane ATPases, a convenient and dependable method is required. It is often possible to follow the ATPase reaction by determining the release of phosphate due to the hydrolysis of ATP by the ATPase enzyme. A number of spectrophotometric methods have been described for the assay of Pi in biological systems. These methods have employed molybdate to react with phosphate to form a molybdophosphate coloured-complex (Fiske and Subbarow, 1925).

The purpose of this investigation was to find a suitable method of determining phosphate in the absence and presence of aminoglycosides, in particular gentamicin.

4.1.2 Results

4.1.2.1 Calibration curve of Pi by the method of Chen, Toribara and Warner (1956)

(i) In the absence and presence of gentamicin (10^{-2} M)

Fig. 6 shows that the standard curve was linear in the absence of gentamicin. The presence of gentamicin interfered with the assay of Pi, resulting in variations which affected the reproducibility of the Pi determination.

(ii) Non-enzymatic hydrolysis of ATP

Fig. 7 shows that the drawback of this method was the high level of non-enzymatic hydrolysis of ATP. However, it was found that the hydrolysis was linear with the length of time allowed for colour development. The coloured-complex formed with 2 µg Pi was stable up to 5 hours, whereas with ATP or ATP + Pi there was a linear increase in absorbance with time.

4.1.2.2 Calibration curve of Pi by the method of Baginski and Zak (1960)

(i) In the absence and presence of gentamicin (10^{-2} M)

Fig. 8 shows that gentamicin seriously affected the determination of Pi by this method.

(ii) Precipitation of gentamicin with silicotungstic acid

The removal of gentamicin by silicotungstic acid precipitation greatly improved the assay of Pi by the method of Baginski and Zak, but it was still not very satisfactory (Fig. 9).

4.1.2.3 Calibration curve of Pi using a modification of the method of Ueda and Wada (1970)

Figs. 10 and 11 depict the calibration curves of Pi by the

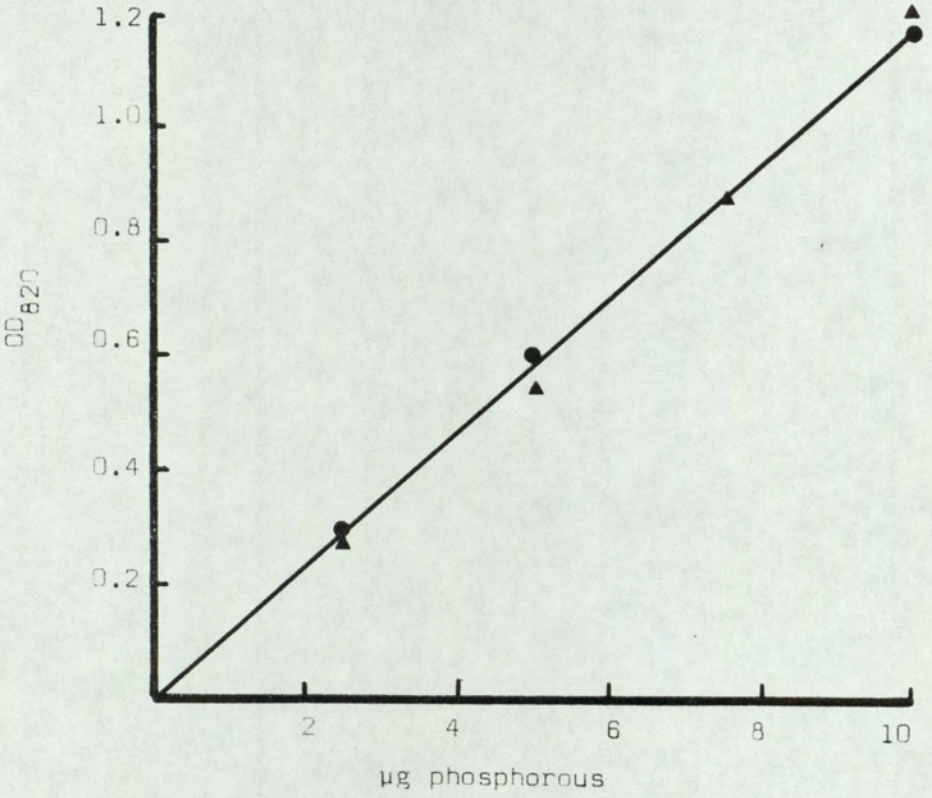


Fig. 6. Calibration curve of Pi by the method of Chen et al. in the absence (●) and presence of 10^{-2} M gentamicin (▲).

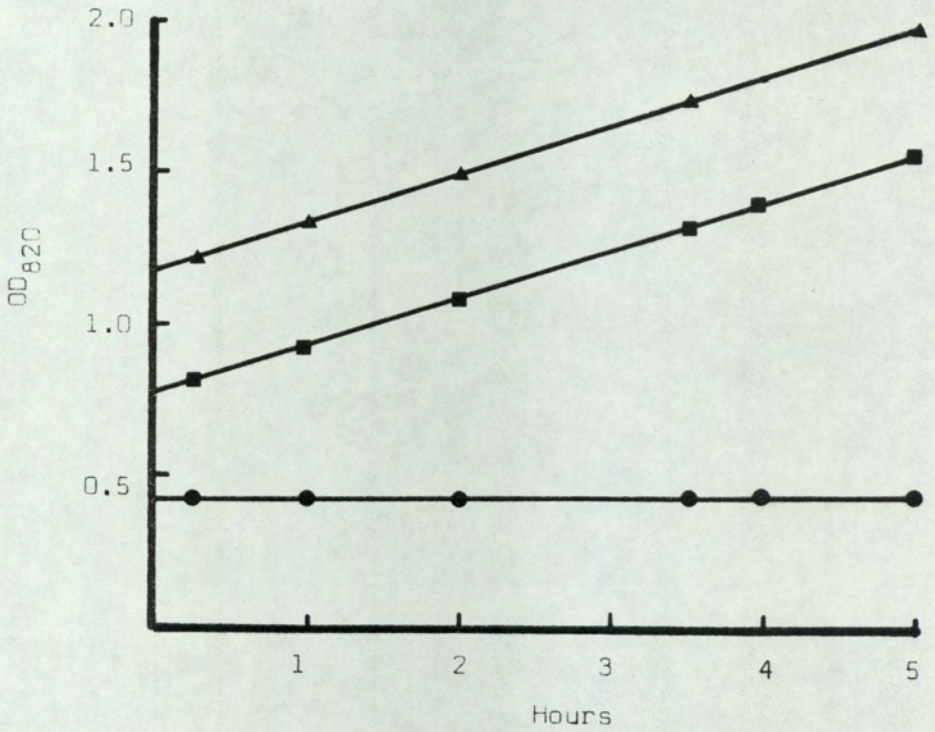


Fig. 7. Time dependent non-enzymatic hydrolysis of ATP by the method of Chen et al. 2 µg Pi (●); ATP (■); and ATP + 2 µg Pi (▲).

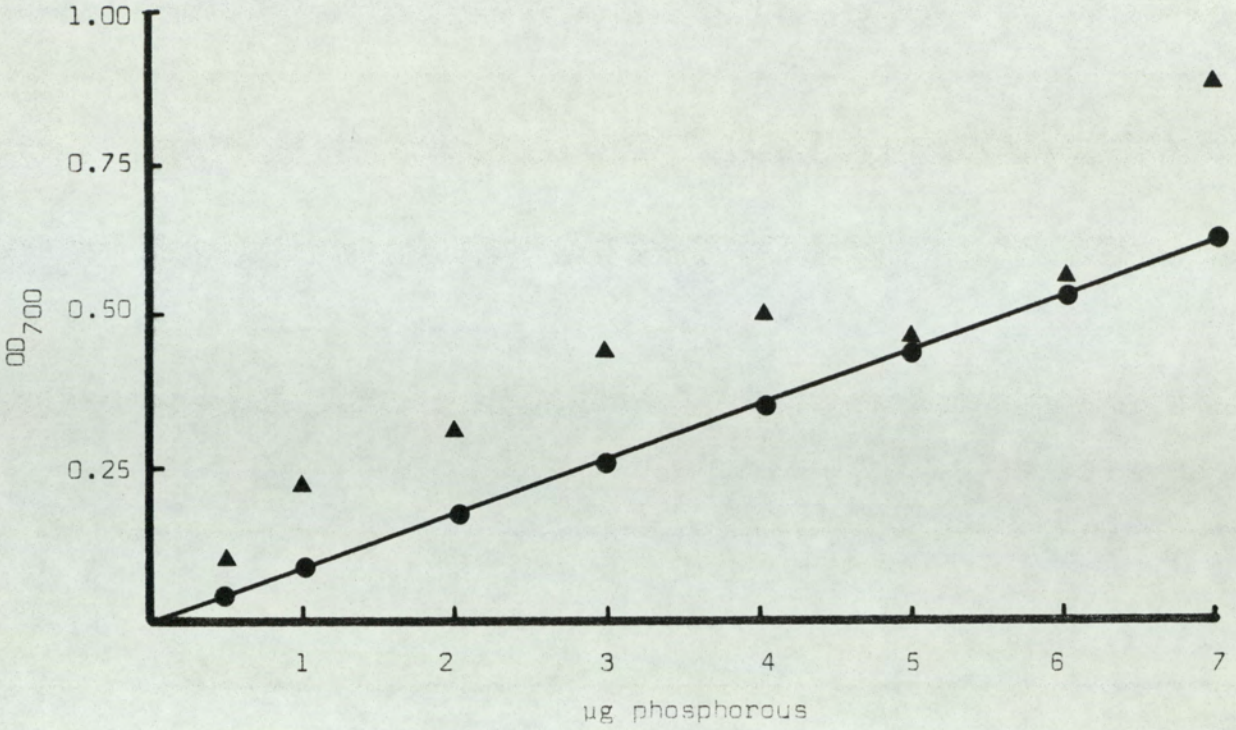


Fig. 8. Calibration curve of Pi by the method of Baginski and Zak in absence (●) and presence of 10^{-2} M gentamicin (▲).

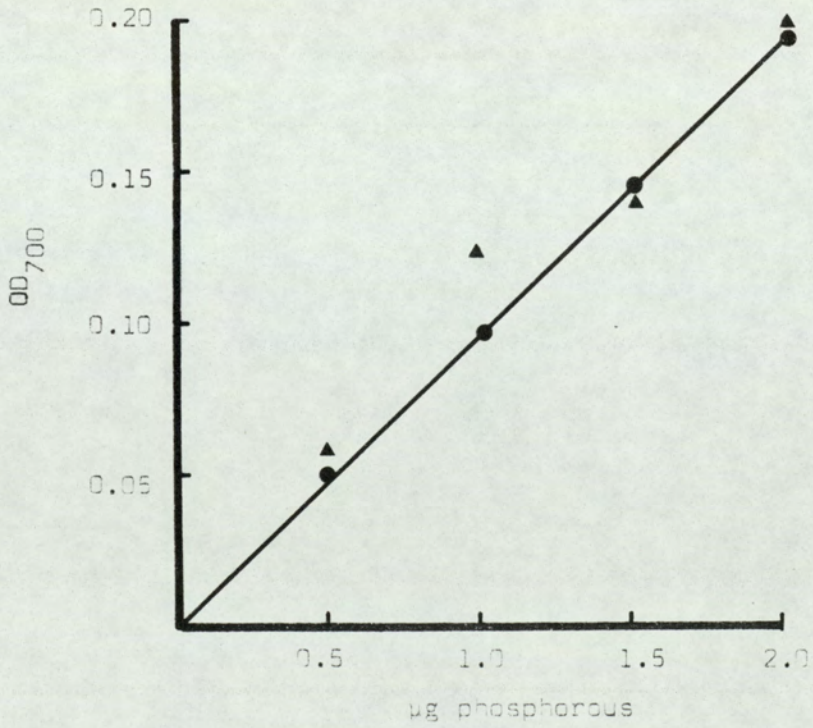


Fig. 9. Precipitation of gentamicin with silico-tungstic acid followed by a removal of an aliquot from the supernatant for the assay of Pi by the method of Baginski and Zak. Control (●); gentamicin (▲).

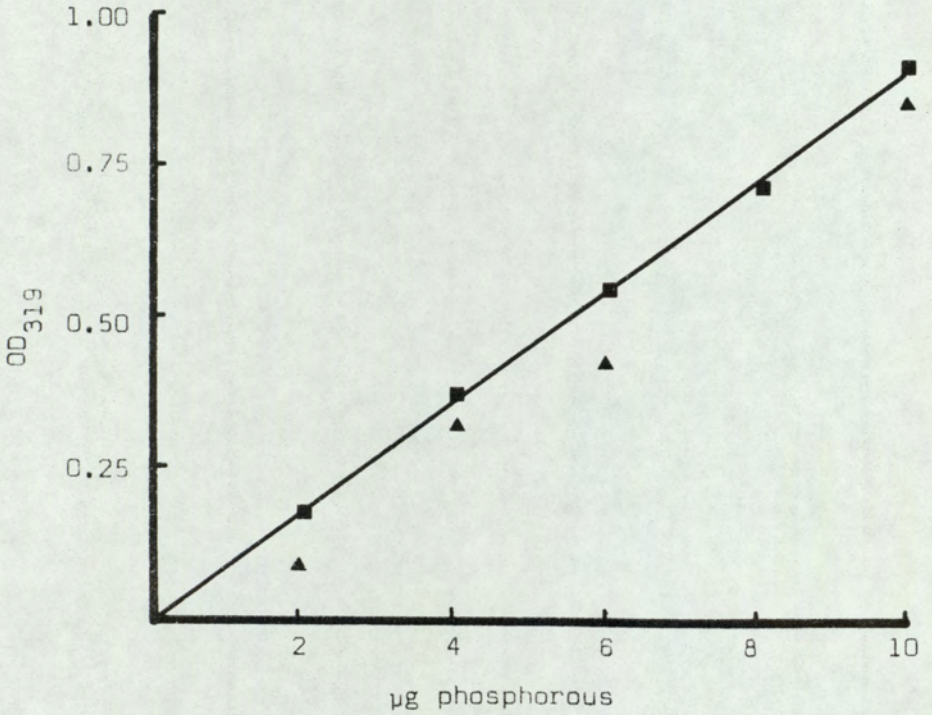


Fig. 10. Calibration curve of Pi in the absence (■) and presence of gentamicin (▲) by the modified method of Ueda and Wada, with the extraction of coloured-complex into isobutanol.

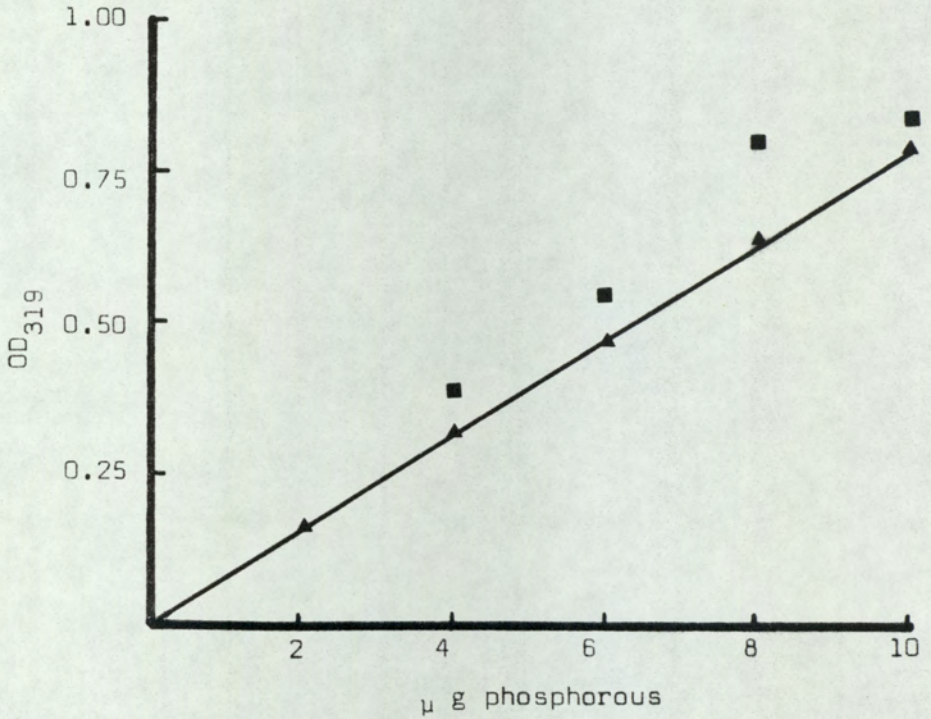


Fig. 11. Calibration curve of Pi, with the extraction of coloured complex into sec-butyl alcohol in the absence (■) and presence of methanol (▲).

modified method of Ueda and Wada. Fig. 10 shows that when the coloured-complex was extracted with isobutanol, gentamicin interfered markedly with the Pi assay. The substitution of sec-butyl alcohol for isobutanol resulted in turbidity even in the absence of gentamicin, which however could be removed by addition of methanol (Fig. 11).

Fig. 12 shows the effect of gentamicin on Pi determination by the method in which the coloured-complex was extracted into sec-butyl alcohol and the turbidity removed by addition of a few drops of methanol. The calibration curves were identical in the absence and presence of gentamicin.

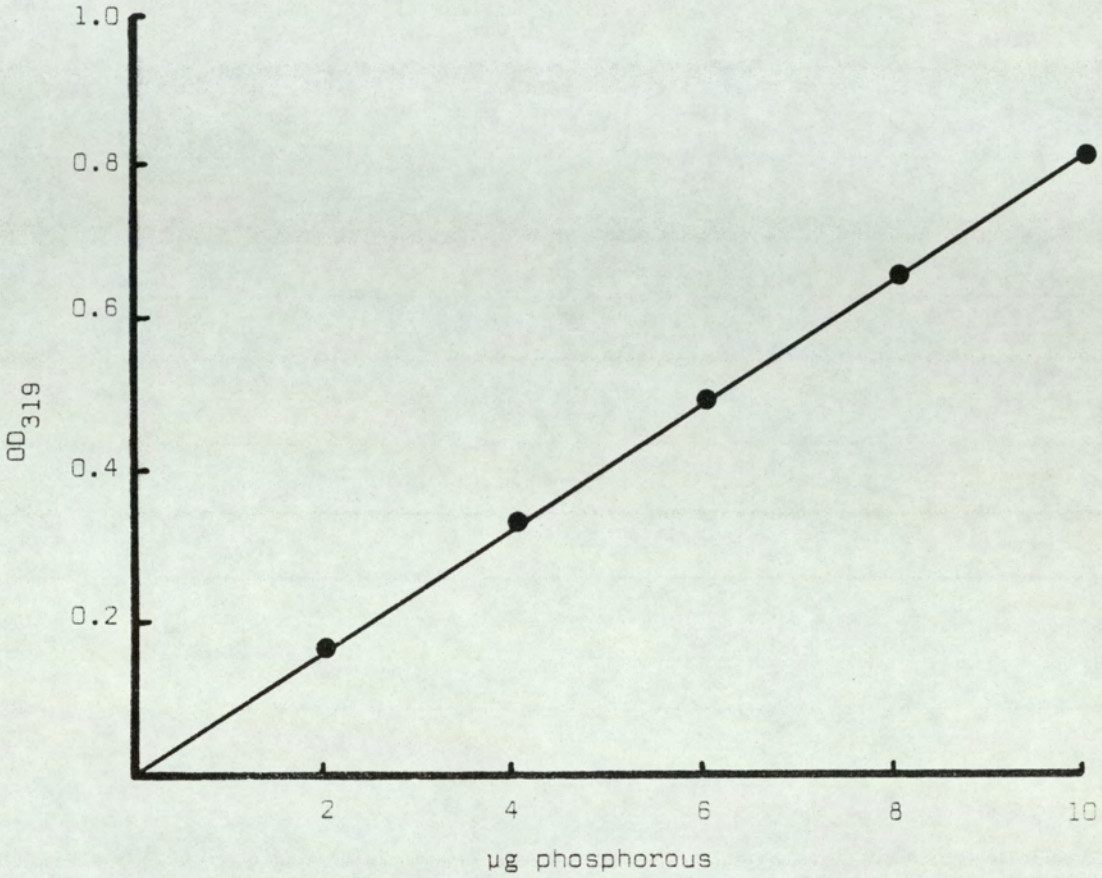


Fig. 12. Calibration curve of Pi by the modified method of Ueda and Wada, with the addition of methanol. The readings were identical in the absence and presence of gentamicin (10^{-2} M).

4.1.3 Discussion

The procedure of Chen et al. (1956) was initially used to determine Pi in the absence and presence of gentamicin. This method which employs the use of ascorbic acid for the reduction of phosphomolybdate was found to be unreliable. Gentamicin was found to interfere with the colour development and molybdate contributed to the hydrolysis of labile ATP and ADP (Figs. 6 and 7). This hydrolysis resulted in variable phosphate levels to be recorded depending on the length of time allowed for the colour development. Thus, non-enzymatic hydrolysis of ATP would give higher Pi values and so cause appreciable errors in ATPase determinations.

The method of Baginski and Zak (1960) was used next in order to remove the serious drawbacks mentioned above. This method uses a citrate/arsenite mixture as a stabilizer for the complex formed when the phosphomolybdic acid is reduced with ascorbic acid. As a result the procedure is relatively sensitive, colour stable and has the advantage of being insensitive to any newly released phosphate (i.e. Pi released by hydrolysis of ATP after stopping the enzyme reaction).

However, the calibration curve of Pi in the presence of gentamicin by this method gave unacceptable levels of variation in optical density (Fig. 8).

Roufogalis (1971), reported a method of determining Pi in the presence of interfering amines. It was a modification of the phosphomolybdate method in which silicotungstic acid (a well known alkaloid reagent) was used to precipitate a wide range of biologically active amines and non-ionic detergents. In the present study 8%

silicotungstic acid was utilised to precipitate gentamicin. The precipitate was pelleted by centrifuging at 18,000 rpm and then an aliquot of supernatants removed for Pi assay by the method of Baginski and Zak. Fig. 9 shows that this procedure was also unsatisfactory. It was also found to be very time-consuming if a number of estimations were to be made. The incomplete precipitation of gentamicin by silicotungstic acid may have been due to the very polar characteristics of gentamicin. Roufogalis found that polar amines such as l-epinephrine and amino acids did not precipitate with silicotungstic acid.

It appears that many organic bases which are sparingly soluble in water and solubilized in their protonated forms, interfere with the Pi determinations by the molybdophosphate method. However, most of these organic bases are extractable to an appreciable extent by shaking with chloroform before the addition of molybdate reagents. Ueda and Wada (1970) used this fact in their system which also included extraction of the colour complex (molybdovanadophosphate) into isobutanol. This procedure increased the sensitivity of the assay system and also minimised the non-enzymatic hydrolysis of ATP by the assay reagents.

In the present investigation, bearing in mind that gentamicin is very insoluble in chloroform and isobutanol, the principle of the above method was utilised by omitting the chloroform step and taking advantage of the extraction of the coloured-complex into isobutanol. However, even this system failed to alleviate the interference caused by gentamicin (Fig. 10). Thus, sec-butyl alcohol which is less polar but has greater basicity was substituted for isobutanol. It was found that with

this alcohol, the concentration of the molybdate had to be decreased 10-fold in order to reduce the absorbance of the blank at OD_{319}.

This method, initially did not prove to be very successful due to formation of turbidity in the alcohol during reading of the sample. A number of reagents were tried in order to remove the moisture and it was found that this could be achieved by addition of methanol (Fig. 11). Thus, this system was used to calibrate Pi curves in the absence and presence of gentamicin. The calibration curves obtained were identical as shown in Fig. 12. The method was found to be satisfactory for other aminoglycosides as well.

The methods and materials used in the determination of Pi in the presence of aminoglycosides as adapted from the method of Ueda and Wada is described below.

All reagents used were of analytical grade and all solutions were made with deionised-distilled water.

MATERIALS

(i) Ammonium molybdate solution

1 gm of ammonium molybdate tetrahydrate was dissolved in 90 ml of water and to which was added 1 ml of ammonium hydroxide (sp. gr. 0.90). The solution was made up to 100 ml with water.

(ii) Ammonium metavanadate solution

0.235 gm of ammonium metavanadate was dissolved in 40 ml of hot water and cooled under tap water. To this, 0.62 ml of concentrated nitric acid was added and then made up to 100 ml with water.

(iii) Sec-butyl-alcohol

(iv) Trichloroacetic acid (TCA) solution (10%)

10 gm of trichloroacetic acid was dissolved in water and made up to 100 ml.

(v) The colour reagent was prepared as follows

100 ml of molybdate solution was combined with 100 ml of vanadate solution and 50 ml of concentrated hydrochloric acid. This was made up to 500 ml with addition of water.

METHOD

To the incubation sample (0.5 - 1.0 ml) was added 10% TCA (0.2 ml), 2 ml colouring reagent and 4 ml of sec-butyl alcohol. This was shaken, and the two phases were allowed to separate at room temperature.

After 10 minutes, 3 ml of the final extract was removed and to which 0.2 ml of methanol was added. The yellow colour of the molybdovanadophosphate was then read at OD_{319}.

Standard curves were prepared using inorganic phosphate standards.

SUMMARY

Methods were evaluated and a modification developed for determining Pi in the absence and presence of gentamicin. The reagents and procedures for the assay were similar to those described by Ueda and Wada (1970). The techniques differed in that chloroform extraction was omitted and the molybdovanadophosphate formed was extracted with sec-butyl alcohol instead of isobutanol.

This new method is simple, rapid and the coloured complex formed is relatively stable with time and substantially free from interference by aminoglycosides.

This procedure was employed for the investigation of the effect of aminoglycosides, in particular gentamicin, on Na^+-K^+ ATPase of the erythrocyte and kidney cortical and medullary microsomal fractions (see section 4.2).

4.2 STUDIES ON THE EFFECT OF AMINOGLYCOSIDES, ESPECIALLY GENTAMICIN ON NA^+-K^+ ATPase ACTIVITY

4.2.1 Introduction

The inner ear tissue and the kidney are both involved to a very high degree in the regulation of homeostasis and ion transport.

The mammalian inner ear consists of a spirally-wound three-fold tube. The middle tube is the scala media, filled with endolymph, an extracellular fluid with a cation composition resembling an intracellular medium. The scala media is surrounded on two sides with spaces filled with perilymph, the scala vestibuli and the scala tympani. The cation composition of perilymph agrees with that of extracellular fluids. The separation between scala media and scala tympani is formed by the thick fibrous membrana basilaris upon which rests, on the endolymphatic side, the organ of Corti. The scala vestibuli and scala media are separated by Reissner's membrane, consisting of two cell layers with microvilli on the endolymphatic side. The third side of the scala media is formed by an epithelial structure, called stria vascularis, which rests on a layer of connective tissue, the spiral ligament. The cells of the stria vascularis are characterised by a large number of mitochondria, a high activity of enzymes participating in oxidative metabolism and deeply invaginated basal and lateral cell walls. These characteristics are commonly found in cells involved in active cation transport.

In fact, the distribution of Na^+-K^+ ATPase in the cochlea is well understood. Kuijpers and Bonting (1969) showed that the stria vascularis had the highest ATPase activity, with a gradient decrease of enzymatic activity from the base of the cochlea to the apex. The enzymatic activities in other cochlear structures were low,

suggesting that Na^+-K^+ ATPase in stria vascularis plays the major role in maintaining the unusual composition of the mammalian endolymph. Aminoglycosides are known to disturb endolymphatic ionic homeostasis, as shown by direct measurements of ionic concentrations in endolymph in normal and drug injected guinea pigs (Mendelsohn and Katzenberg, 1972) and also by reduction in cochlear microphonics.

There are a number of features common to both the stria vascularis and the kidney, which may contribute to the tissue specificity observed.

The kidney is the organ primarily responsible for the control of the volume and distribution of body water, solutes and electrolytes (Maffly, 1976). The cortex and the outer medulla of the kidney are characterised by the presence of different segments of the nephron.

At least part of the net reabsorption of sodium ions in the proximal tubule is thought to be the function of Na^+-K^+ ATPase localised in the basal-lateral plasma membranes. However, all of Na^+ reabsorption in the thick ascending limb of Henle, distal convoluted tubules and collecting ducts is driven by the Na^+-K^+ ATPase (Jørgensen, 1980).

The loop diuretics, ethacrynic acid and frusemide, like the aminoglycosides are toxic to the kidney and the inner ear and have been known to disrupt ionic balance in both organs. The molecular mechanism of the diuretic effect of the ethacrynic acid and frusemide is still not known. The principal site of action in the kidney seems to be the thick ascending limb of Henle, a region comparatively rich in Na^+-K^+ ATPase. Ebel (1974) has suggested that the natriuretic effect of these diuretics may well be as a result of interaction with Na^+-K^+ ATPase.

The fact that aminoglycosides accumulate in both organs and histochemical evidence suggesting depression of Na^+-K^+ ATPase activity in the ear by the aminoglycosides, led us to investigate the effect of gentamicin and other aminoglycosides on Na^+-K^+ ATPase.

4.2.2 Results

4.2.2.1 Erythrocyte membranes

(i) Na^+-K^+ ATPase activity

The erythrocytes membranes prepared by the method described in section 3.2 had a Na^+-K^+ ATPase activity of 0.09 µmoles Pi released per mg protein per hour (Table 1). This Na^+-K^+ ATPase activity represented almost 50% of the total ATPase activity of the erythrocyte membrane.

(ii) Effect of gentamicin on Na^+-K^+ ATPase

Table 2 shows that gentamicin had very little effect on the ATPase activity at a concentration of 10^{-4} M. At a gentamicin concentration of 10^{-3} M there was an inhibition of 25% with maximal inhibition of Na^+-K^+ ATPase at a concentration of 10^{-2} M.

4.2.2.2 Kidney 'microsomal fraction'

(i) ATPase activities of 'microsomal fraction' of the kidney cortex

Table 3 shows the Na^+-K^+ ATPase and Mg^{2+} ATPase activity of the microsomal fraction of the guinea pig kidney cortex. The Mg^{2+} ATPase activity was taken as the activity in the presence of 1 mM ouabain. The specific activities of Na^+-K^+ ATPase and Mg^{2+} ATPase were 0.19 and 0.17 µmoles Pi/mg protein/min respectively.

(ii) Distribution of Na^+-K^+ ATPase and other enzymes within the kidney of the guinea pig

The specific activity of the Na^+-K^+ ATPase is about 1.5 fold higher in the microsomal fraction from the outer medulla than in the preparation from the outer cortex (Table 4). The specific activity of Na^+-K^+ ATPase in whole homogenates of outer medulla

	µmoles Pi/mg protein/hr
Total ATPase activity	0.19 ± 0.01
$Na^+ - K^+$ ATPase activity	0.09 ± 0.003

TABLE 1

Total ATPase and $Na^+ - K^+$ ATPase activity of the erythrocyte membrane. ($Na^+ - K^+$) ATPase was calculated as the difference in activity with and without 0.2 mM ouabain added to the test tubes. Mean ± s.e.m. of at least three experiments performed in triplicate.

Gentamicin concentration	10^{-2} M	10^{-3} M	10^{-4} M
% inhibition of $Na^+ - K^+$ ATPase	110 ± 11 n = 8	25 ± 4 n = 8	7 ± 2 n = 3

TABLE 2

The effect of incubation with gentamicin at various concentrations on the activity of $Na^+ - K^+$ ATPase of erythrocyte membrane. Mean ± s.e.m.

ATPase activities	µmoles Pi/mg protein/min	% of total activity
Total ATPase	0.36 ± 0.03 (n = 7)	-
(Na^+ + K^+) ATPase	0.19 ± 0.02 (n = 7)	51 ± 3 (n = 7)
Mg^{2+} ATPase	0.17 ± 0.02 (n = 7)	49 ± 3 (n = 7)

TABLE 3

Na^+ - K^+ ATPase and Mg^{2+} ATPase activity of the microsomal fraction of guinea pig kidney cortex. Na^+ - K^+ ATPase was calculated as the difference in activity with and without 1 mM ouabain added to the test tubes. Mg^{2+} ATPase is the activity in the presence of 1 mM ouabain. Mean ± s.e.m.

SPECIFIC ACTIVITIES (µmol Pi/mg prot/min)	HOMOGENATE		SUPERNATANT		MICROSOMAL FRACTION	
	CORTEX	MEDULLA	CORTEX	MEDULLA	CORTEX	MEDULLA
Total ATPase activity	0.117	0.154	0.133	0.164	0.420	0.555
$Na^+ - K^+$ ATPase activity	0.023	0.037	0.026	0.026	0.182	0.284
Mg^{2+} ATPase activity	0.094	0.117	0.107	0.138	0.238	0.271
5'Nucleotidase	0.026	0.023	0.036	0.036	0.043	0.052
% $Na^+ - K^+$ ATPase	20	24	20	16	43	51

TABLE 4

Distribution of $Na^+ - K^+$ ATPase and other enzymes within the kidney of the guinea pig. Values given are means of the kidneys pooled from at least two animals.

was 0.037 µmoles Pi/mg protein per minute. This is 1.6 times that of the cortex (0.023 µmoles Pi/mg protein/minute).

The activity of the Mg^{2+} stimulated ATPase was slightly higher in outer medulla as compared to the cortex, but the difference was not so great as that for Na^{+}-K^{+} ATPase. 5'-nucleotidase, an enzyme which is thought to be localised in the plasma membrane, showed very little difference between the cortex and outer medulla. Table 4 also shows the increase in the specific activity of Na^{+}-K^{+} ATPase by almost 8 fold from whole homogenate to the microsomal fraction.

(iii) The time course of the total ATPase activity of microsomal fraction from the cortex and medulla at various concentrations of microsomal protein

The total ATPase activity was found to be linear up to 15 minutes of incubation for both the cortical and medullary microsomal fractions (Figs. 13 and 14). This linearity with time was observed for all concentrations of microsomal protein used in the range 10 - 60 µg.

(iv) The relationship between the 'microsomal' protein concentration (cortex and medulla) and the total ATPase activity after 10 minutes incubation

Fig. 15 shows that at a given concentration of microsomal protein, the ATPase activity of the medulla was greater than the cortex. Also, the ATPase activity was linear up to 50 µg microsomal protein in a final volume of 0.5 ml.

(v) Effect of preincubation of the microsomal fraction for various times on ATPase activity

The preincubation of the cortical 'microsomes' for up to 120 minutes at $37^{o}C$ did not effect its final ATPase activity,

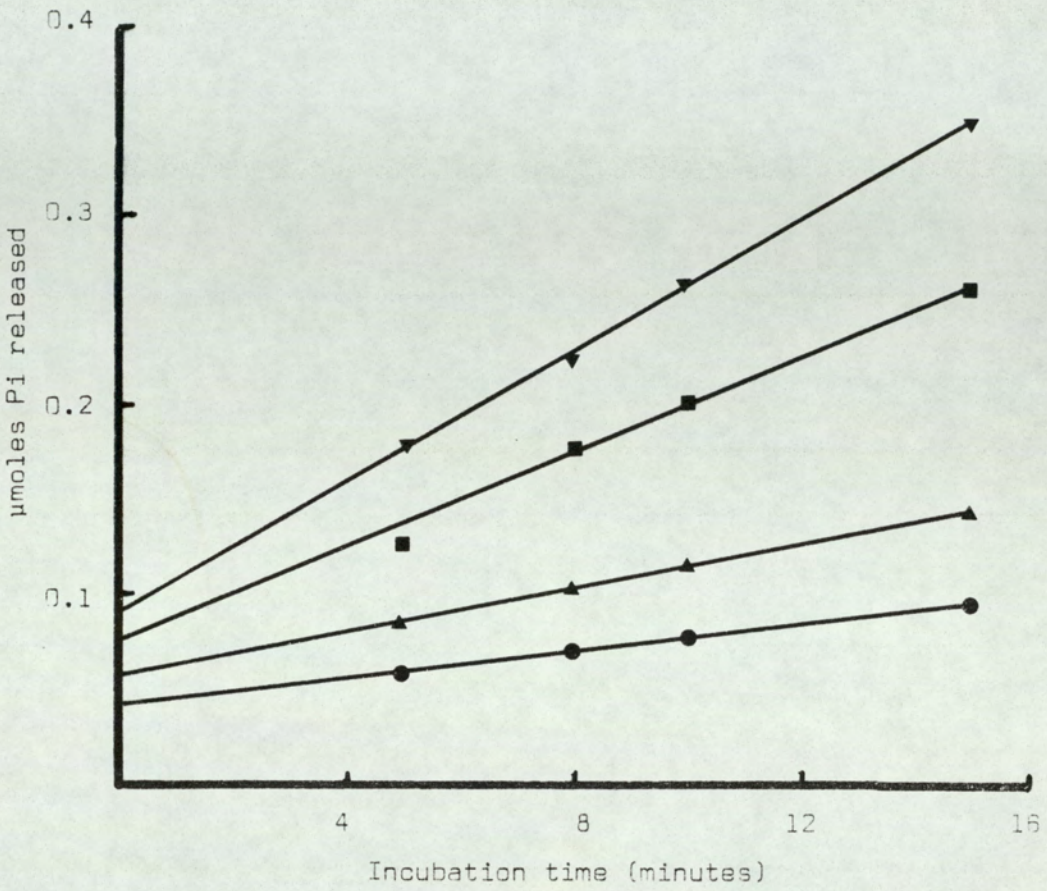


Fig. 13. The time-course of the total ATPase activity of 'Microsomal fraction' from the cortex of guinea pig kidney with increasing concentrations of microsomal protein. 10 µg (●), 20 µg (▲), 40 µg (■) and 60 µg (▼) in a final volume of 0.5 ml.

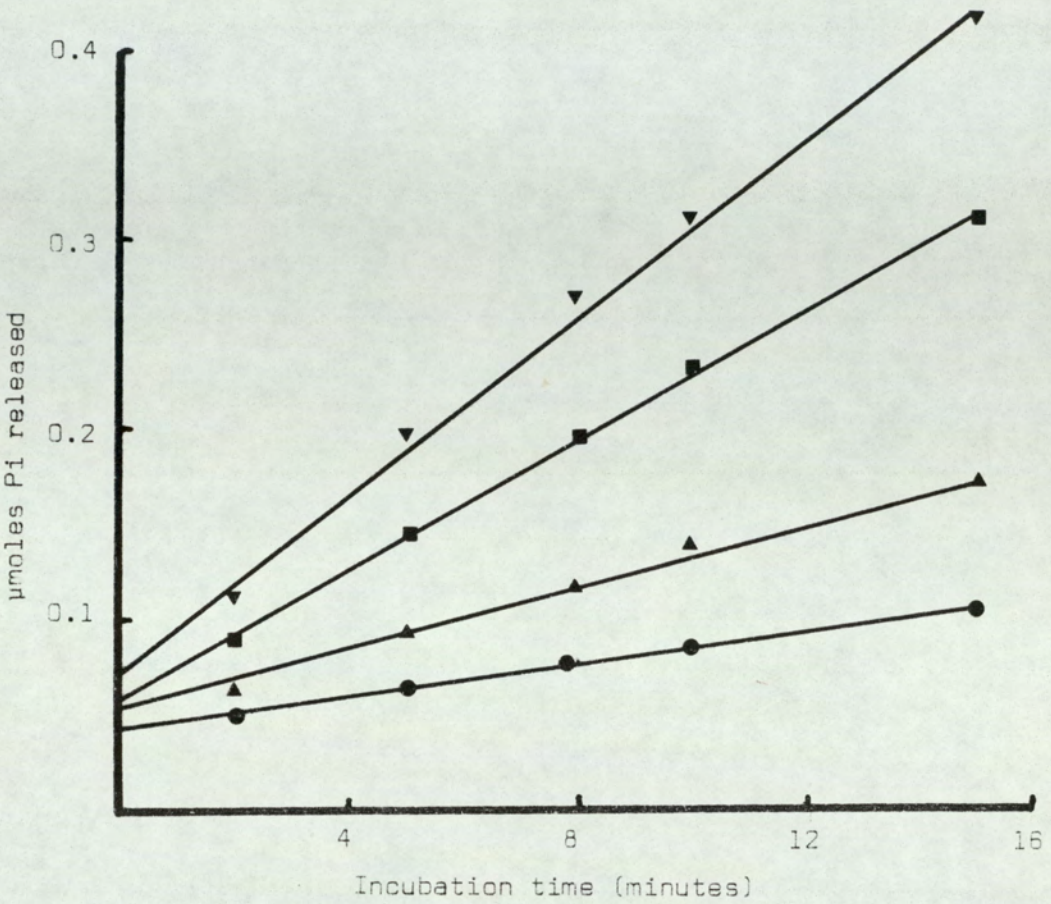


Fig. 14. The time-course of the total ATPase activity of 'microsomal fraction' from the outer medulla of guinea pig kidney with increasing concentrations of microsomal protein. 10 µg (●), 20 µg (▲), 40 µg (■) and 60 µg (▼) in a final volume of 0.5 ml.

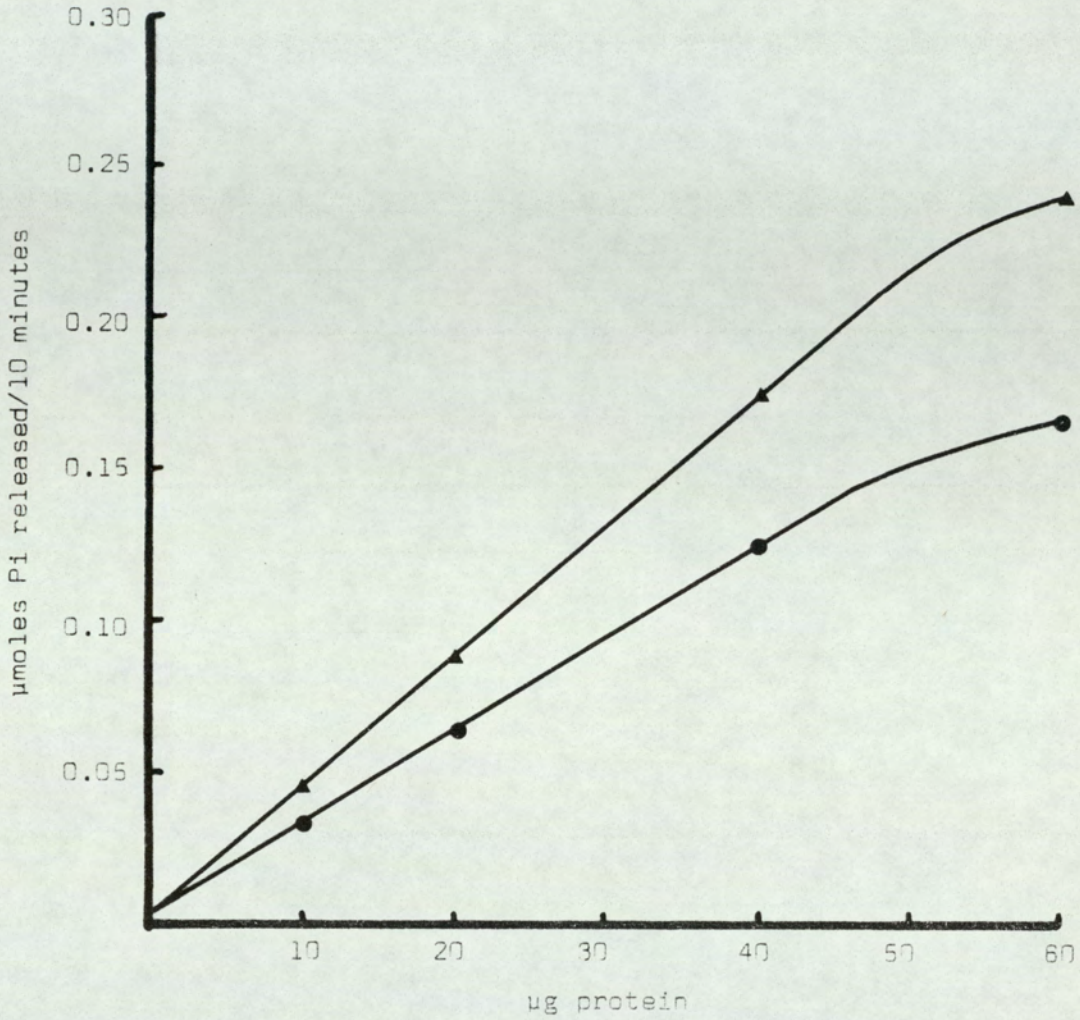


Fig. 15. The relationship between the microsomal protein concentration from the cortex (●) and medulla (▲) and the total ATPase activity after 10 minutes incubation.

which was found to be constant over the time period investigated (Fig. 16). The ATPase activity was also independent of the length of preincubation with gentamicin (10^{-2} M) and ouabain (10^{-3} M). The reduction in total ATPase activity produced by both ouabain and gentamicin was unaffected by varying the time of preincubation.

(vi) The effect of gentamicin on Na^+-K^+ ATPase of the 'microsomal fraction' of the kidney cortex

The effect of gentamicin in the concentration range 10^{-2} M - 10^{-5} M on the Na^+-K^+ ATPase appeared to show very little inhibition at low concentrations of gentamicin. At a gentamicin concentration of 10^{-2} M, Na^+-K^+ ATPase was inhibited by about 50% (Fig. 17).

(vii) The effect of aminoglycosides (10^{-2} M and 10^{-3} M) on Na^+-K^+ ATPase of the 'microsomal fraction' of the cortex and medulla

At the lower concentrations of aminoglycosides (10^{-3} M), gentamicin inhibited the cortical and medullary Na^+-K^+ ATPase by about 20%. The antibiotics, neomycin, kanamycin, ribostamicin, streptomycin and dihydrostreptomycin had no effect (Table 5). At aminoglycoside concentration of 10^{-2} M, both gentamicin and neomycin inhibited the Na^+-K^+ ATPase of the cortex and medulla to the same extent. Both antibiotics inhibited ATPase activity by about 60% with no differences between cortex and the medulla. Ribostamicin, at the same concentration, appeared to have very little effect on the Na^+-K^+ ATPase of both the cortex and medulla.

4.2.2.3 Isolated rat renal tubules

(i) Time course of Rb^{86} uptake by isolated rat renal tubules

Fig. 18 shows the time course of Rb^{86} uptake by isolated rat renal tubules at 37°C in the absence and presence of gentamicin

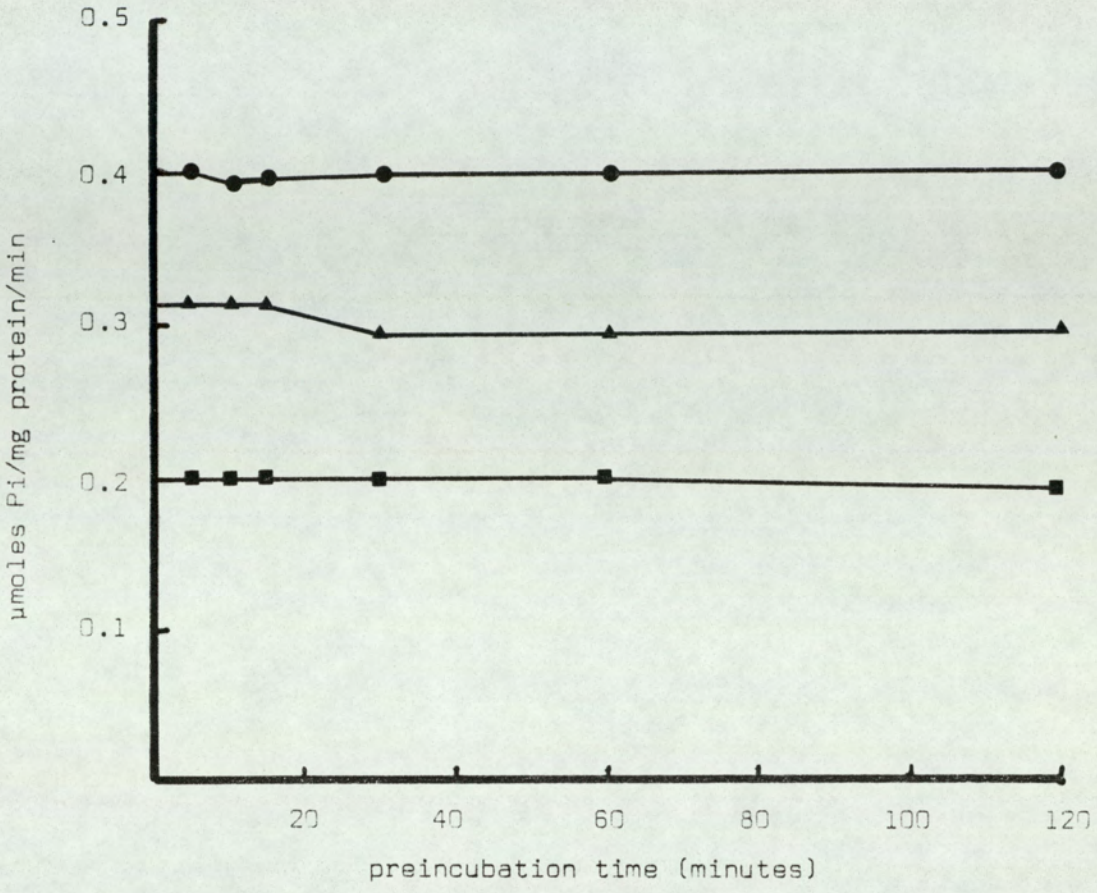


Fig. 16. The effect of preincubating the microsomal fraction from the kidney cortex for up to 120 minutes at 37°C. The preincubation was done under control conditions (●), with 10^{-2} M gentamicin (▲) and 1 mM ouabain (■), after which ATPase was assayed for 10 minutes.

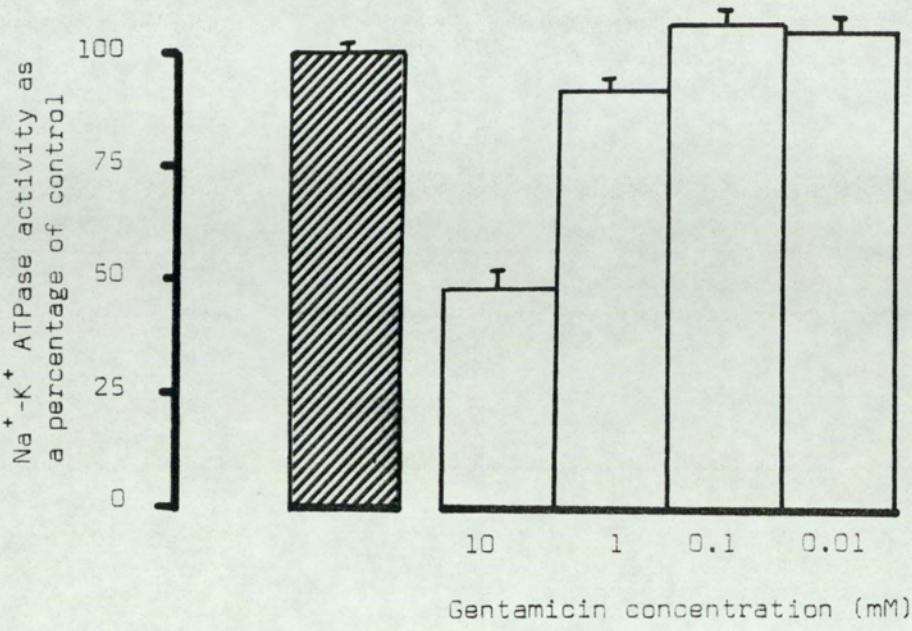


Fig. 17. The effect of incubation with gentamicin at various concentrations on the activity of Na^+-K^+ ATPase of the microsomal fraction of kidney cortex. The results shown here are expressed as a percentage of the control (closed bars - 100%) and represents the mean ± s.e.m. of 7 experiments.

AMINOGLYCOSIDE 10^{-2} M	% inhibition of $Na^+ - K^+$ ATPase CORTEX	MEDULLA
Gentamicin	59 ± 4	55 ± 1
Neomycin	59 ± 10	59 ± 8
Ribostamicin	9 ± 10	8 ± 7

AMINOGLYCOSIDE 10^{-3} M	% inhibition of $Na^+ - K^+$ ATPase CORTEX	MEDULLA
Gentamicin	18 ± 2	19 ± 2
Neomycin	11 ± 5	7 ± 2
Kanamycin	11 ± 5	4 ± 5
Ribostamicin	9 ± 5	8 ± 5
Streptomycin	7 ± 8	-4 ± 5
Dihydrostreptomycin	10 ± 4	6 ± 2

TABLE 5

The effect of incubation with various aminoglycosides at concentration of 10^{-2} M and 10^{-3} M on the activity of $Na^+ - K^+$ ATPase of the microsomal fraction of the cortex and outer medulla of the guinea pig kidney. Mean ± s.e.m. n = 3.

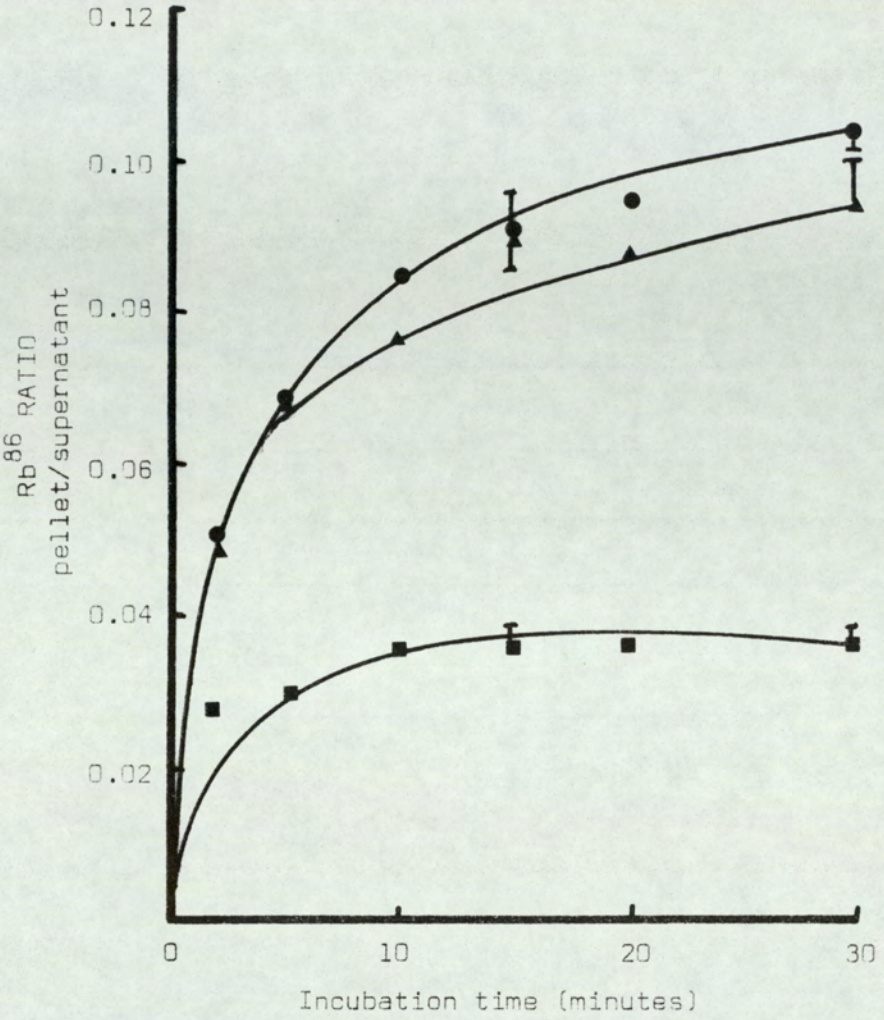


Fig. 18. The uptake of Rb^{86} by tubules in the presence of gentamicin and ouabain at 37°C. Tubules were incubated with ^{86}Rb (10 µCi/ml) in a final volume of 10 ml (●), in the presence of gentamicin at a concentration of 10^{-4} M (▲) and ouabain at 10^{-3} M (■). At the given time, 200 µl aliquots in triplicate were removed and radioactivity determined. Each point represents the mean ± s.e.m. of triplicate determinations.

(10^{-4} M) and ouabain (10^{-3} M). The uptake under control conditions was quite rapid in the first 10 minutes after which the uptake progressed more slowly. The time course in the presence of gentamicin (10^{-4} M) was very similar to the control with no appreciable difference in uptake on incubating up to 30 minutes. In the presence of ouabain (10^{-3} M), the uptake of Rb^{86} was inhibited with a plateau being reached after 10 minutes. After 10 minutes of incubation, ouabain had reduced the uptake of Rb^{86} to 35% of the control.

(ii) Effect of various concentrations of gentamicin on the Rb^{86} uptake

The uptake of Rb^{86} into tubules after incubations at 37°C for 30 minutes in the absence and presence of gentamicin in the concentration range (10^{-2} M - 10^{-5} M) is shown in Fig. 19. Gentamicin at a concentration of 10^{-2} M reduced the uptake of Rb^{86} by approximately 50% ($p < 0.001$). The uptake was not affected by lower concentrations of gentamicin. The Na^{+}-K^{+} ATPase inhibitor, ouabain (10^{-3} M) reduced the uptake of Rb^{86} to 35% of the control ($p < 0.001$).

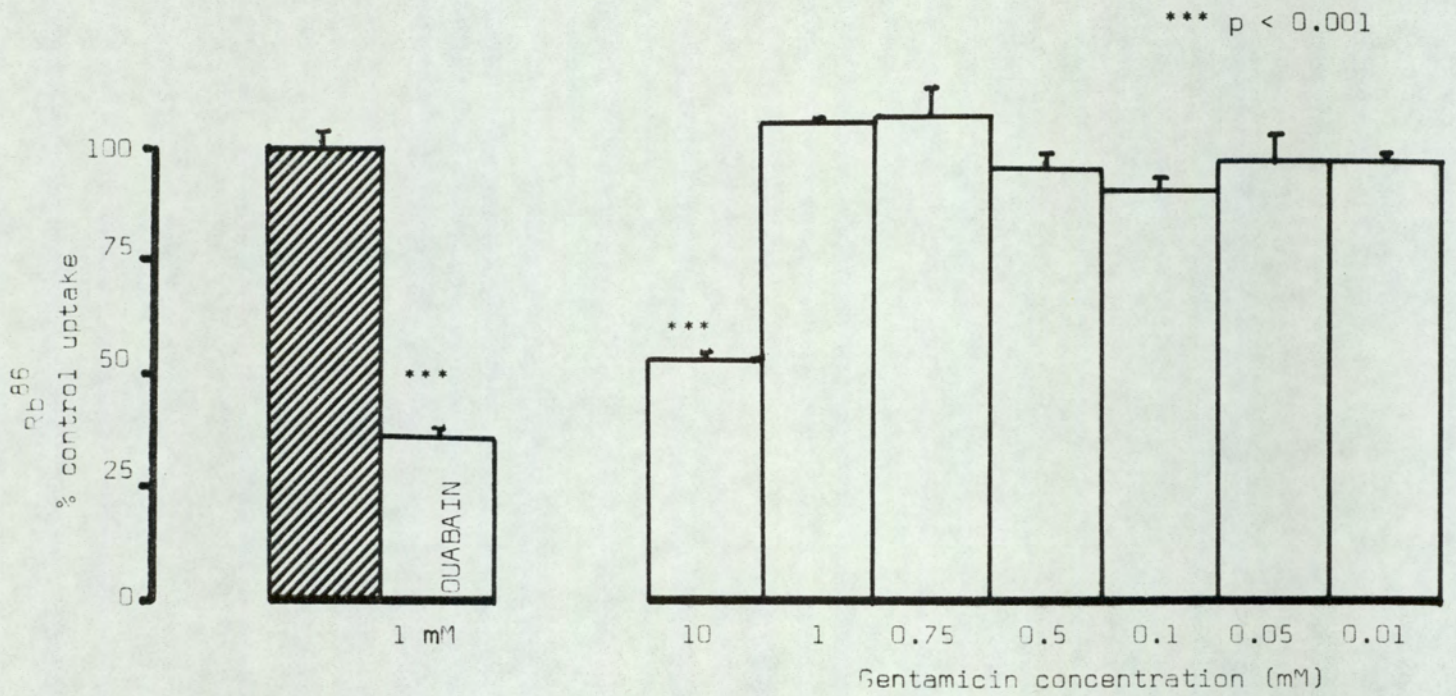


Fig. 19. The effect of various concentrations of gentamicin on the uptake of ^{86}Rb by isolated rat renal tubules after 30 min incubation at 37°C. The results shown here, are expressed as a percentage of the uptake into tubules incubated under control conditions (closed bars - 100%) and represent the mean ± s.e.m. of quadreplicate determinations.

4.2.3 Discussion

In order to study the effects of aminoglycosides on Na^+-K^+ ATPase, the human erythrocyte membrane was initially chosen. This is because of its ease of preparation and availability, apparent lack of contaminating subcellular structures and interfering enzymic activities, even though it has low ATPase activities compared with other systems (Bonting and Caravaggio, 1963). Although it is often said that this is not a typical cell since it lacks subcellular organelles, it does possess many characteristics of the normal cell type. The limiting membrane of the mammalian erythrocyte membrane catalyses reactions similar to those found in other cells, i.e. maintenance of osmotic equilibrium, transport of monovalent and divalent cations and substrate transport.

The erythrocyte membranes, prepared by the method of Bramley et al. (1971) (section 3.2), gave Na^+-K^+ ATPase activity of 0.09 µmoles Pi released per mg protein per hour. This represented 50% of the ATPase activity, the other activity was due to Mg^{2+} ATPase. There was no appreciable effect on Na^+-K^+ ATPase activity at a gentamicin concentration of 10^{-4} M but there was 25% inhibition at 10^{-3} M gentamicin. Gentamicin at a concentration of 10^{-2} M completely abolished the ouabain sensitive Na^+-K^+ ATPase activity.

Since only very high concentrations of gentamicin inhibited erythrocyte Na^+-K^+ ATPase to any appreciable extent, it was decided that kidney Na^+-K^+ ATPase may prove to be a more sensitive system.

Table 4 shows the purification of the Na^+-K^+ ATPase of the guinea pig kidney. The specific activity of the Na^+-K^+ ATPase was higher in the microsomal fraction from the outer medulla than the cortex. This is in agreement with the studies of Hendler,

Torretti and Epstein (1971) who showed that the Na^+-K^+ ATPase activity of outer medulla is higher than in the cortex. The predominant structure in the outer medulla is the thick ascending limb of Henle's loop, whereas the cortex is chiefly made up of proximal convoluted tubules (Jørgensen, 1980). Thus, the above results support the evidence that the Na^+-K^+ ATPase activity is higher in the distal portion of the nephron than in the proximal tubule. Because 5' nucleotidase activity was very similar between the medulla and the cortex, it seems improbable that a simple increase in the quantity of plasma membrane in cells of medullary region is responsible for the high activity of Na^+-K^+ ATPase. The Mg^{2+} ATPase activity was slightly higher in the outer medulla as compared to the cortex, but the difference was not so great as that for Na^+-K^+ ATPase.

The total ATPase activity of both microsomal fractions showed linearity with time and concentration of microsomal protein. The ATPase activity was linear up to 15 minutes incubation with microsomal protein concentration in the range 10 - 50 µg protein/ 0.5 ml (Figs. 13 - 15). Thus, in subsequent experiments, incubation time of 10 minutes and microsomal protein concentration of 40 - 50 µg/ 0.5 ml was chosen.

At low concentrations of the aminoglycosides (10^{-3} M), gentamicin inhibited by about 20%, with neomycin, kanamycin, ribostamicin, streptomycin and dihydrostreptomycin all showing very little inhibition. Gentamicin and neomycin at 10^{-2} M inhibited Na^+-K^+ ATPase of both microsomal fractions by about 50 - 60%. Ribostamicin (10^{-2} M) had very little effect on the Na^+-K^+ ATPase of both the medullary and cortical microsomal fraction (Table 5).

It is very often stressed that one should be aware of the dangers of extrapolating results from fragmented membranes to an intact membraneous structure.

As a consequence of this the effect of gentamicin on Na^+-K^+ ATPase of isolated rat renal tubules was also investigated. The preparation of isolated rat renal tubules by the method of MacDonald and Saggerson (1977) is described in section 3.4.

The Na^+-K^+ ATPase activity was assayed by following the uptake of Rb^{86} (a congener of K^+) by the tubules. The uptake of Rb^{86} was not affected by low concentrations of gentamicin but was inhibited by about 50% at 10^{-2} M. The extent of inhibition was very similar to that observed with the microsomal fractions suggesting a close relationship between the two model systems.

There is evidence of electrolyte imbalance occurring within the ear as a result of the effects of ototoxic drugs. Mendelsohn and Katzenberg (1972) demonstrated very marked electrolyte changes in the inner ear fluids of the guinea pig after high doses of kanamycin. A rise in endolymph sodium and a fall in potassium ion concentration was seen as early as the fifth day.

Electrophysiological studies have shown that the cochlear potentials, the cochlear microphonics (CM), indicating function of the hair cells, and the endolymphatic potential (EP), which reflects the function of the stria vascularis, are both strongly dependent on the cationic composition of endolymph and perilymph.

The guinea pig cochlear microphonics are inhibited by streptomycin but can be reversed by washing the endolymph compartment with potassium-rich Ringer's solution (Wersall and Landquist, 1968). The depression of cochlear microphonics by the Na^+-K^+ ATPase

inhibitor ouabain can also be reversed by increasing the concentration of K^+ in the fluid perfusing the perilymphatic space (Guth and Bobbin, 1971).

A more direct link between ototoxicity and Na^+-K^+ ATPase inhibition has been shown by Iinuma (1967) and Kaku, Farmer and Hudson (1973). They showed that kanamycin intoxication in guinea pigs produced a reduction in ATPase activity of the stria vascularis and spiral ligament.

The diuretics, ethacrynic acid and frusemide are also known to reduce cochlear microphonics. However, this reduction in cochlear microphonics and associated changes in the endolymphatic cation concentration may not be related to their effect on the Na^+-K^+ ATPase. Kuijpers and Wilbert (1976) and Paloheimo and Thalmann (1977) could not show an effect upon the strial Na^+-K^+ ATPase when these changes were induced by ethacrynic acid.

In the kidney, gentamicin has been reported to show an increase in excretion of K^+ in urine and that this may be an early indication of renal damage. Mitchell, Bullock and Ross (1977) have suggested that gentamicin may exert its effect on K^+ excretion as a result of molecular charge. A number of charged molecules such as basic amino acids (Giebisch, 1971) are known to promote potassium loss *in vivo*. The excretion of over 50% of filtered gentamicin results in a high concentration of charged molecules in the lumen.

Diuretics which promote K^+ loss may therefore increase the nephrotoxic effect of gentamicin, as has been observed with frusemide in studies with rats (Kahn, 1977) and humans (Noel and Levy, 1978). Adelman, Spangler, Beasom *et al.* (1979) have suggested that frusemide promotes volume depletion. This volume depletion would increase reabsorption and storage of gentamicin in the proximal tubules.

It is interesting to note that frusemide and ethacrynic acid added to the luminal perfusate at 10^{-5} M can block net NaCl transport and reduce the potential difference (Burg and Green, 1973). However, both compounds are ineffective when added to the peritubular fluid, where the Na^+-K^+ ATPase is localised.

Another very interesting observation made in this study was the lack of inhibition of Na^+-K^+ ATPase by ribostamicin compared to gentamicin and neomycin at a concentration of 10^{-2} M. The aminoglycoside ribostamicin has been reported to cause lower nephrotoxicity (Kawagishi, Nagamatsu, Nakajima et al., 1972) and ototoxicity (Harada, 1972) than gentamicin and neomycin.

Kawagishi et al. (1972), using histopathological methods found no toxic effects on the kidney when ribostamicin (200 mg/kg/day) was administered intramuscularly for 22 days. Harada (1972) observed no evidence of ototoxicity when 400 mg/kg/day of ribostamicin was administered intramuscularly for four weeks.

In conclusion, the correlation between the nephrotoxicity and ototoxicity caused by the aminoglycosides and diuretics and their ability to inhibit membrane Na^+-K^+ ATPase has not been conclusively demonstrated. Inhibition of Na^+-K^+ ATPase seems unlikely to be the primary mechanism since in the proximal tubule of the kidney, the site of drug-induced damage, the enzyme resides on the peritubular side of the cell. As for the inner ear, concern has been raised over the extent to which Na^+-K^+ ATPase of the stria vascularis is involved in the maintenance of the endolymphatic composition. Bosher (1980) has suggested an indirect role, such as maintaining the internal ionic environments of the transporting cells.

SUMMARY

The effect of aminoglycosides on Na^+-K^+ ATPase of erythrocyte membrane, cortical and medullary 'microsomal fractions' of the kidney and isolated rat renal tubules were investigated. At a concentration of 10^{-2} M there was inhibition of Na^+-K^+ ATPase by gentamicin and neomycin but not with ribostamicin. The extent of inhibition caused by gentamicin was similar in the different type of preparation used, suggesting a close relationship between the model systems.

However, from these studies it was not possible to conclude whether inhibition of Na^+-K^+ ATPase by the aminoglycosides was the primary mechanism of nephrotoxicity and ototoxicity *in vivo*.

4.3 *IN VITRO* UPTAKE OF GENTAMICIN INTO RAT KIDNEY SLICES

4.3.1 Introduction

The major route of aminoglycoside elimination is renal where 70 - 90% of a single dose of a drug may be recovered unchanged in twenty-four hour urine collection (Davey, Gonda, Harpur *et al.*, 1980). Although most of an administered dose is recovered in 24 hours, a variable proportion remains unaccounted for. There is no evidence that aminoglycosides are metabolised or excreted in bile in significant amounts.

Many authors have explained the incomplete urinary collections by suggesting binding of aminoglycosides to renal cell membranes, organelles or intracellular protein. Thus, the nephrons are exposed to high concentrations of aminoglycosides by glomerular filtration as well as through tubular secretion and reabsorption which results in nephrotoxicity (Hewitt, 1974).

The toxicity develops progressively in proportion to the dose and duration of drug therapy with a concomitant elevation of serum creatinine and blood urea nitrogen signalling deteriorating renal function (Falco, Smith and Acieri, 1969; Wilfert, Burke, Bloomer *et al.*, 1971).

Although the clearance rate of gentamicin closely parallels the rate of glomerular filtration, net reabsorption and renal cortical accumulation has been reported by Chiu, Brown, Miller *et al.* (1976), suggesting transport of gentamicin into renal tissue. Histopathological changes are seen primarily in the proximal tubular cells. In rats, within 2 days of moderate doses of gentamicin, cytosegresomes appear which are often seen to contain myeloid bodies composed of concentric dense membranes about 30Å

wide (Kosek, Mazze and Cousins, 1974). Kosek *et al*. also reported the presence of these myeloid bodies in the proximal tubular cells of gentamicin-treated patients.

This phenomenon of *in vivo* drug accumulation in the kidney is farily well documented. However, there has been relatively little research directed towards an elucidation of the mechanism underlying this phenomenon.

The present series of experiments were therefore undertaken *in vitro* in order to elucidate the mechanisms responsible for the accumulation of gentamicin in the kidney.

It was decided to use kidney slices in preference to a perfused kidney on the grounds of simpler methodology and greater ease of manipulation of experimental parameters such as incubation temperature, extracellular Na^+ concentration etc.

4.3.2 Results

4.3.2.1 Total tissue H_2O

The total tissue water content of the kidney cortical slices after drying at 100°C to constant weight was found to be 79.4 ± 0.7% of the wet weight.

4.3.2.2 Extracellular space volume

The extracellular space volume was estimated using $|^{14}C|$ inulin as an extracellular marker. As shown in Fig. 20, the extracellular space volume steadily rose from 23.0 ± 0.9% to 36.5 ± 3.3% of the tissue weight as the duration of incubation increased from 10 minutes to 90 minutes. These values for extracellular space are considerably higher than estimates made on the whole human *in vivo* (Moore, McMurrey, Parker *et al.*, 1956) but are in the same range as other reported values *in vitro* (Rosenberg, Blair and Segal, 1961). It has been suggested that the apparently large extracellular space measurement of kidney tissue studied *in vitro* is related to entrapment of medium in the tubular lamina, altered permeability of the damaged cells on the cut surface to the substrate used, and due to adherence of medium to the tussue slices (Krane and Crane, 1959).

In experiments studying the incorporation of radiolabelled gentamicin into kidney slices, all results were corrected accordingly for the amount of drug in the extracellular space after that period of incubation.

4.3.2.3 Characteristics of gentamicin uptake

(i) Time course of 10^{-4} M and 10^{-5} M gentamicin

Fig. 21 shows the results of experiments to measure the

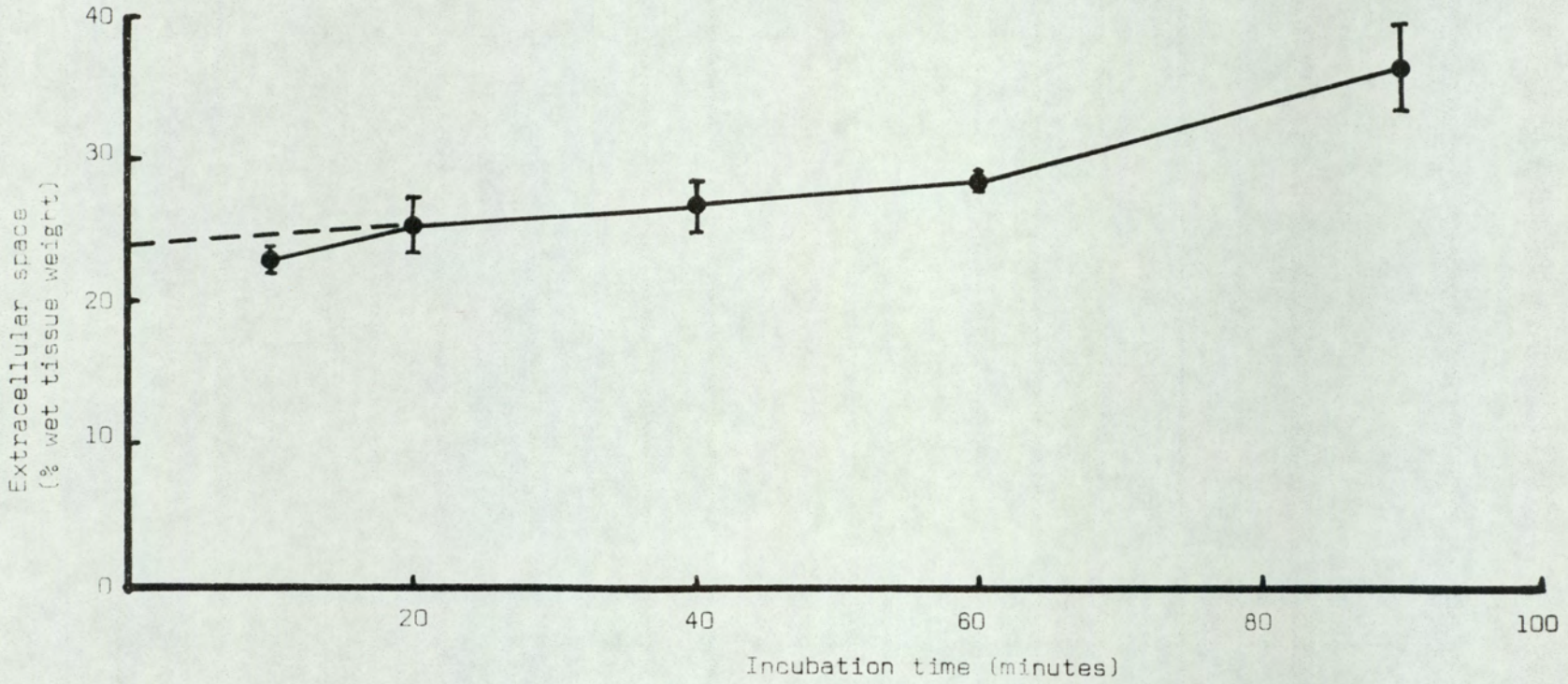


Fig. 20. Extracellular space in rat-kidney cortical slices estimated using [^{14}C] inulin as a function of time of incubation at 37^{o}C. Results are expressed as % wet tissue weight, and represent the mean $\pm$ s.e.m. of three slices.

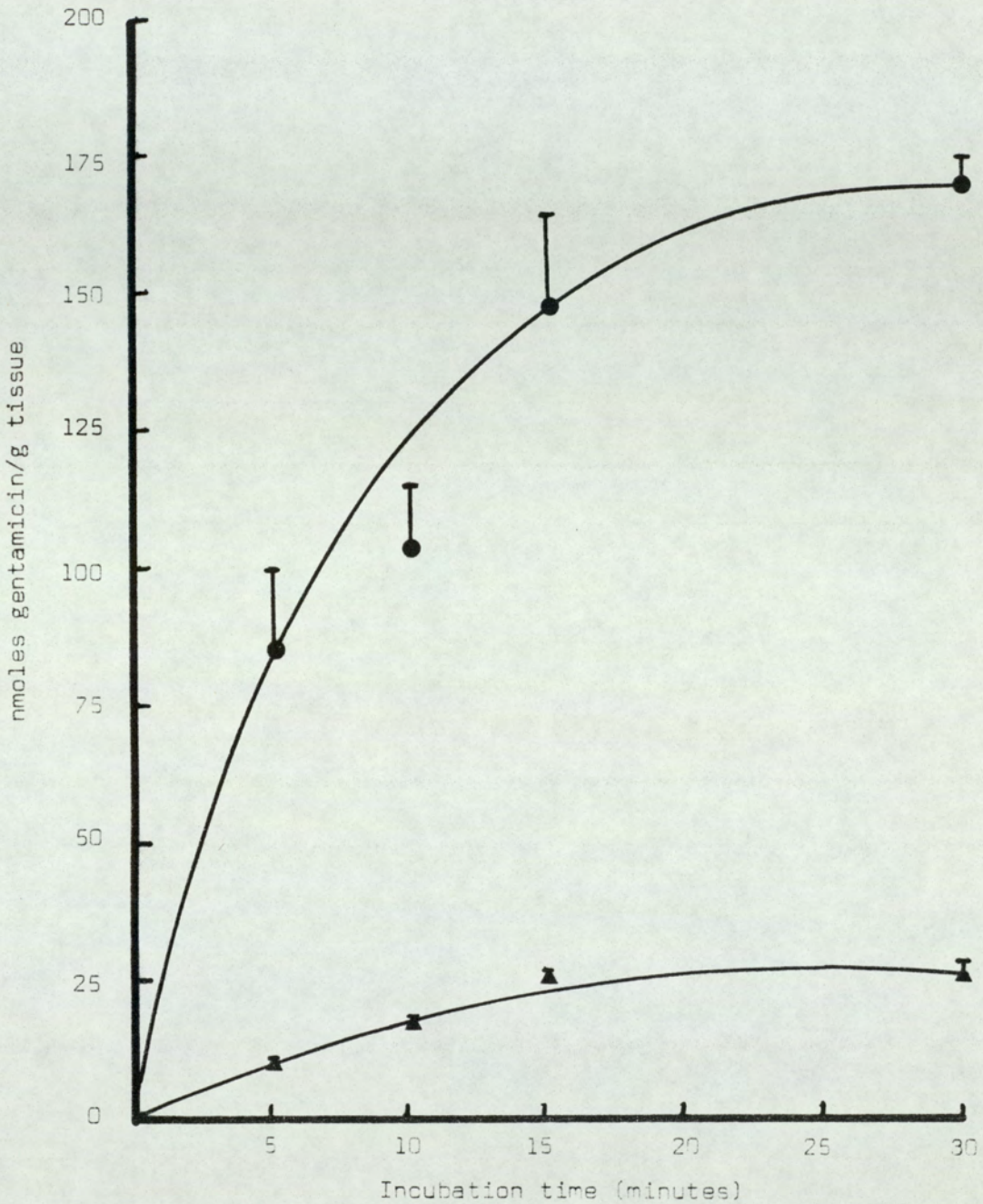


Fig. 21. Uptake of gentamicin into kidney slices as a function of time. Slices were incubated at 37°C with $|^{3}H|$ gentamicin (0.125 uCi/ml) plus appropriate amount of unlabelled gentamicin to give a final concentration of 10^{-5} M (▲) and 10^{-4} M (●). Each point represents mean ± s.e.m. of three slices

uptake of gentamicin into kidney slices at 37°C for a period of time up to 30 minutes. The uptake of gentamicin was initially quite rapid at 10^{-4} M gentamicin compared to 10^{-5} M. The uptake curve plateaued after 20 minutes indicating that an equilibration between uptake into and efflux from the tissue was reached.

(ii) Varying gentamicin concentrations

Fig. 22 shows the rate of uptake into kidney slices of gentamicin at various concentrations of the drug in the incubation medium. In these experiments uptake at 10 minutes was used as the estimate of uptake rate in order to obtain more accurate estimates of initial rates. The rate of increase of uptake clearly began to decrease as the gentamicin concentration approached 1.0 mM, indicating saturability under the conditions of these experiments.

(iii) Effect of temperature

In this experiment, and the subsequent experiments, uptake of gentamicin into kidney slices was measured at 30 minutes since a steady state was achieved by this time (Fig. 21). Fig. 23 shows the results of experiments measuring the uptake of gentamicin at three temperatures, 37°C (control), 20°C and 4°C. The rate of gentamicin uptake was highly temperature-dependent with the greatest uptake occurring at 37°C. There was a significant decrease in uptake on lowering the temperature with a resultant uptake at 20°C and 4°C of 63% and 27% respectively of the control value.

(iv) Extracellular Na^+ concentration

The effect of varying the Na^+ concentration at two concentrations of gentamicin, 10^{-4} M and 0.3×10^{-8} M is shown in Fig. 24. Lowering the medium Na^+ concentration from 143 mM

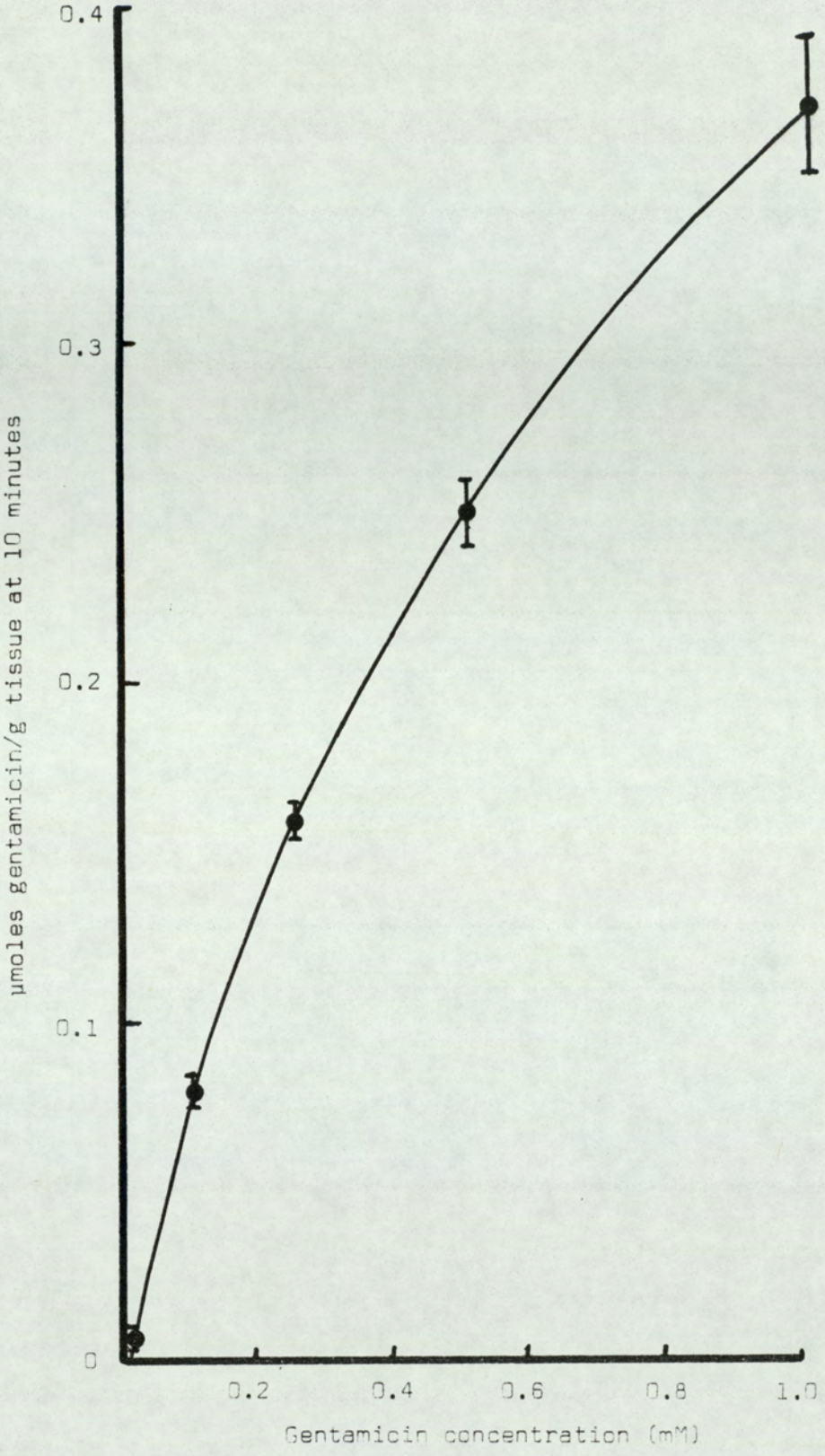


Fig. 22. Uptake of gentamicin into kidney slices after 10 minutes incubation at 37°C. Slices were incubated with [^{3}H] gentamicin (0.125 µCi/ml) plus unlabelled gentamicin to give a final concentration range from 0.01 mM to 1 mM. Each point represents mean ± s.e.m. of at least six slices.

**p < 0.01

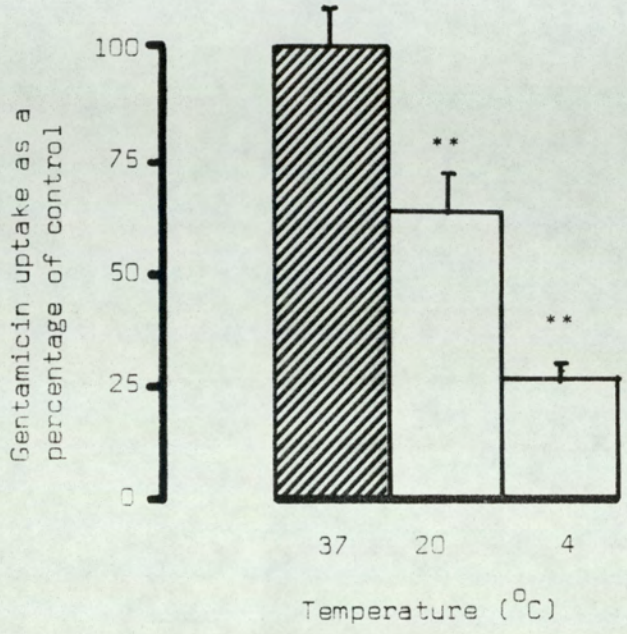


Fig. 23. The effect of temperature upon the uptake of gentamicin into kidney slices after 30 minutes incubation with [^{3}H] gentamicin (0.125 µCi/ml) plus unlabelled gentamicin to give a final concentration of 10^{-4} M. The results shown here, and in the subsequent histograms in this section, are expressed as a percentage of the uptake into slices incubated under control conditions (closed bars - 100%), and represent the mean ± s.e.m. of at least three slices.

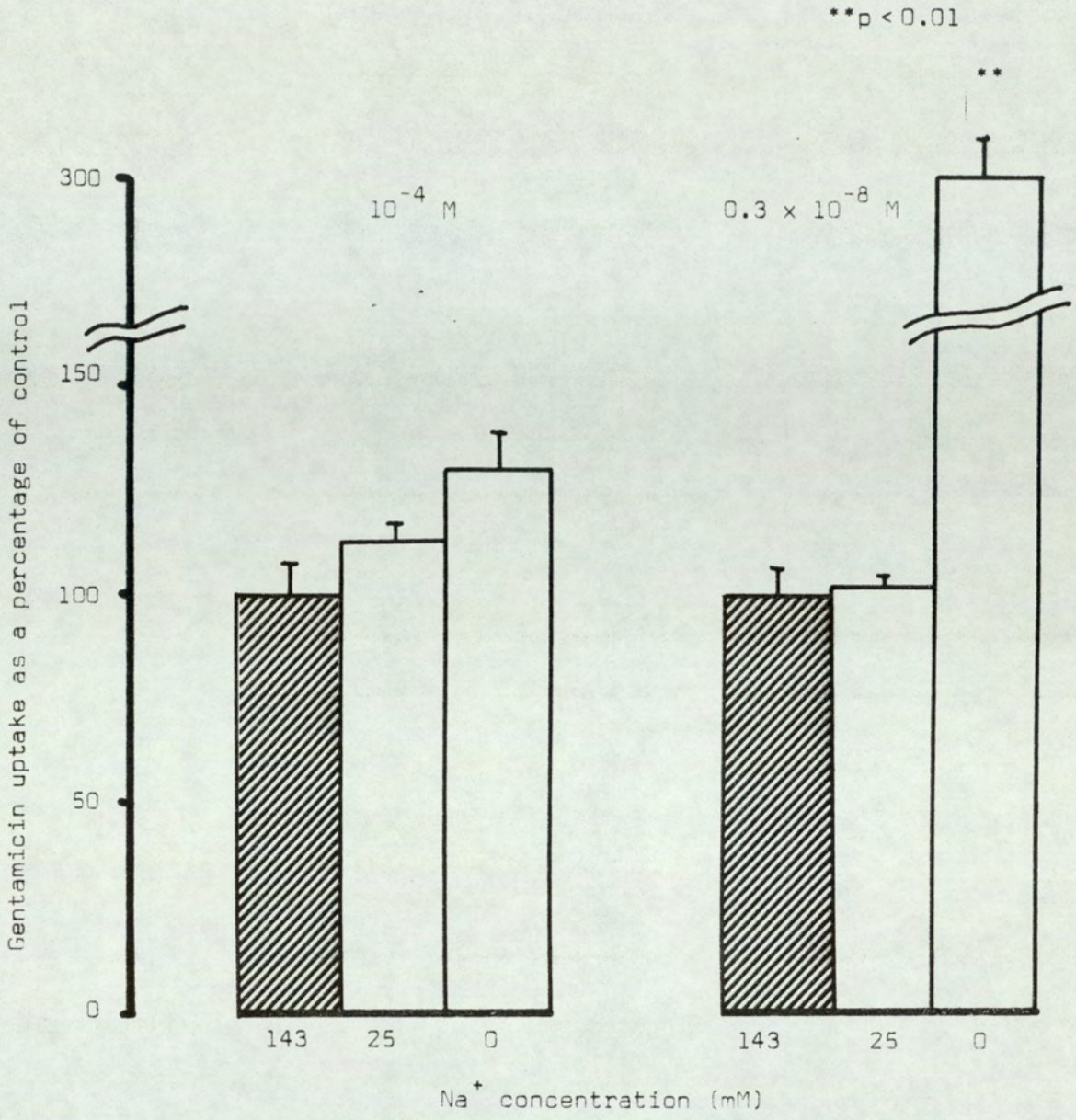


Fig. 24. The effect of extracellular Na^+ concentration upon the uptake of gentamicin into kidney slices after 30 minutes incubation at 37°C with $[^3H]$ gentamicin (0.125 uCi/ml) plus unlabelled gentamicin to give concentrations of 10^{-4} M and 0.3×10^{-8} M.

to 25 mM produced small increases (approximately 1 - 12%) in the uptake of gentamicin at both concentrations of gentamicin, but these increases were not statistically significant. However, further lowering of Na^+ concentration to virtually zero caused significant increase in uptake at 0.3×10^{-8} M gentamicin. The increase at 10^{-4} M gentamicin was not statistically significant.

(v) Extracellular Ca^{2+} concentration

An increase in Ca^{2+} concentration in the incubation medium from 1.27 mM up to 5 mM produced a reduction in the uptake at both concentrations of gentamicin (Fig. 25). These reductions were not statistically significant at 10^{-4} M gentamicin but at 0.3×10^{-8} M gentamicin an increase in Ca^{2+} concentration to 2.5 mM caused a reduction in uptake to 78% ($p < 0.05$) and at 5 mM Ca^{2+}, uptake was reduced even further to 64% ($p < 0.01$).

A decrease in Ca^{2+} concentration to zero caused an increase in uptake at both concentrations of gentamicin which was statistically significant. The greatest percentage increase was at 10^{-4} M gentamicin, 197% ($p < 0.001$) with an increase at 0.3×10^{-8} M to 136% ($p < 0.01$).

(vi) Anaerobic conditions

The uptake of gentamicin was reduced by 40 - 55% in kidney slices incubated in an atmosphere of nitrogen at 10^{-4} M and 0.3×10^{-8} M gentamicin (Fig. 26). The reduction in uptake under nitrogen compared to oxygen was statistically significant ($p < 0.01$).

(vii) Metabolic inhibitors

The incubation of kidney slices with gentamicin (10^{-4} M and 0.3×10^{-8} M), in the presence of potassium cyanide (KCN) 0.5 - 2.0 mM, resulted in reduction of gentamicin uptake compared to uptake into slices incubated under control conditions (Fig. 27).

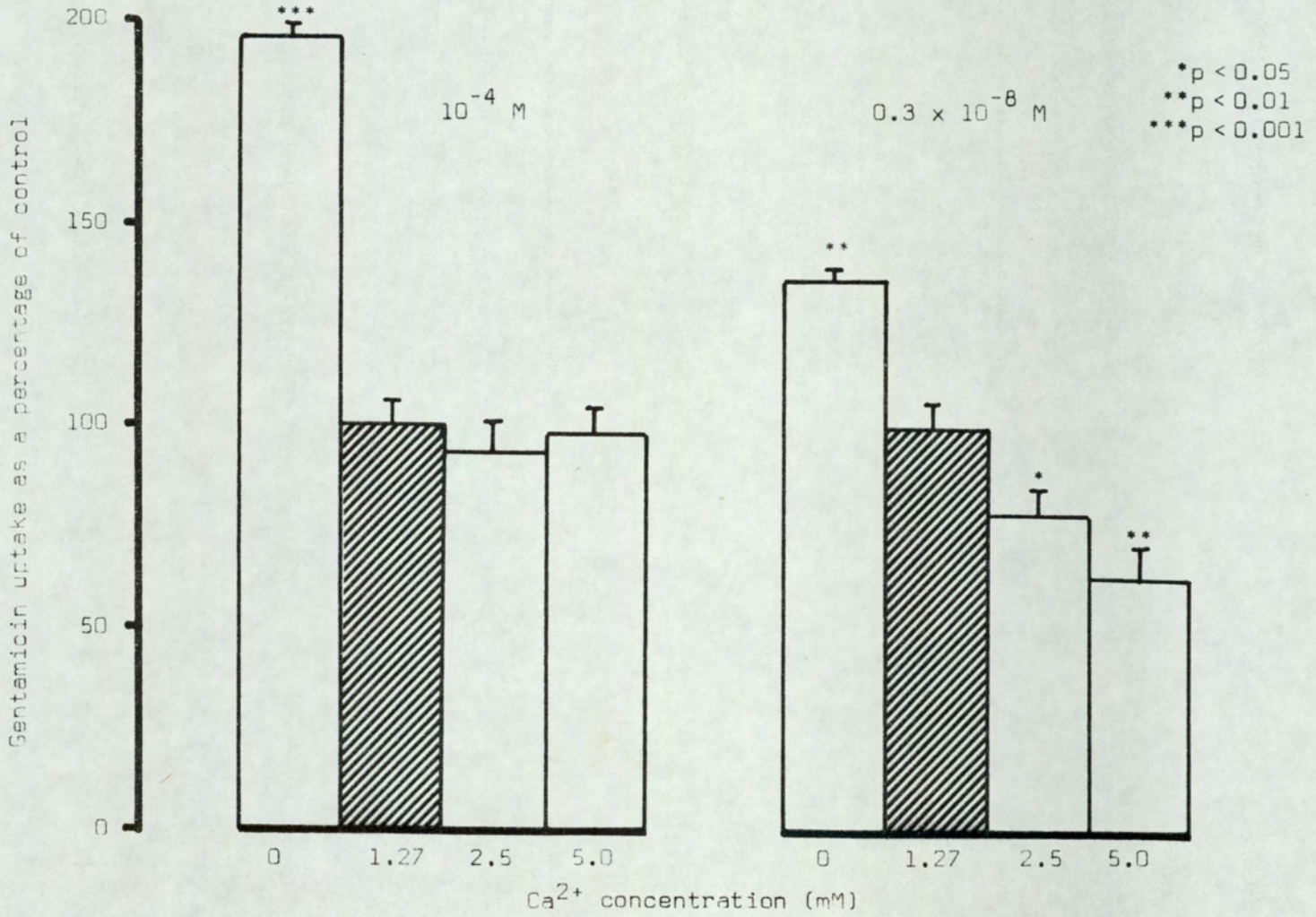


Fig. 25. The effect of extracellular Ca^{2+} concentration upon the uptake of gentamicin into kidney slices after 30 minutes incubation at 37°C with $|^{3}H|$ gentamicin (0.125 µCi/ml) plus unlabelled gentamicin to give concentrations of 10^{-4} M and 0.3×10^{-8} M.

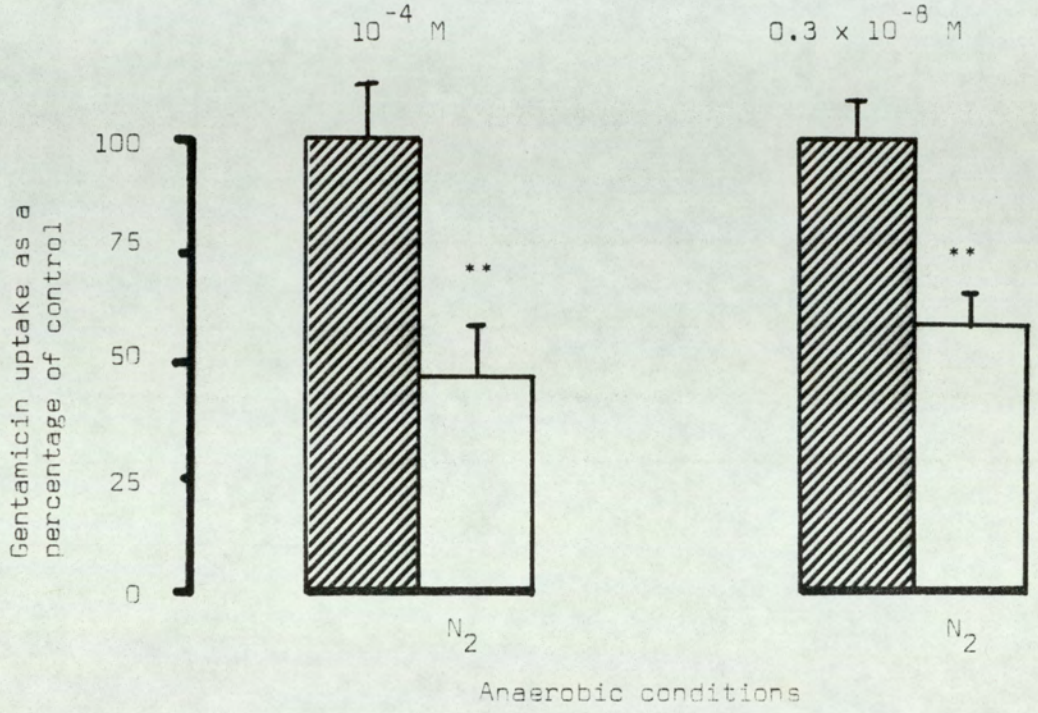


Fig. 26. The effect of anaerobic conditions on the uptake of gentamicin into kidney slices after 30 minutes incubation at 37°C with $|^3H|$ gentamicin (0.125 µCi/ml) plus unlabelled gentamicin to give concentrations of 10^{-4} M and 0.3×10^{-8} M. Closed bars represent the uptake into kidney slices incubated under control conditions, whilst the open bars represent uptake in an atmosphere of nitrogen.

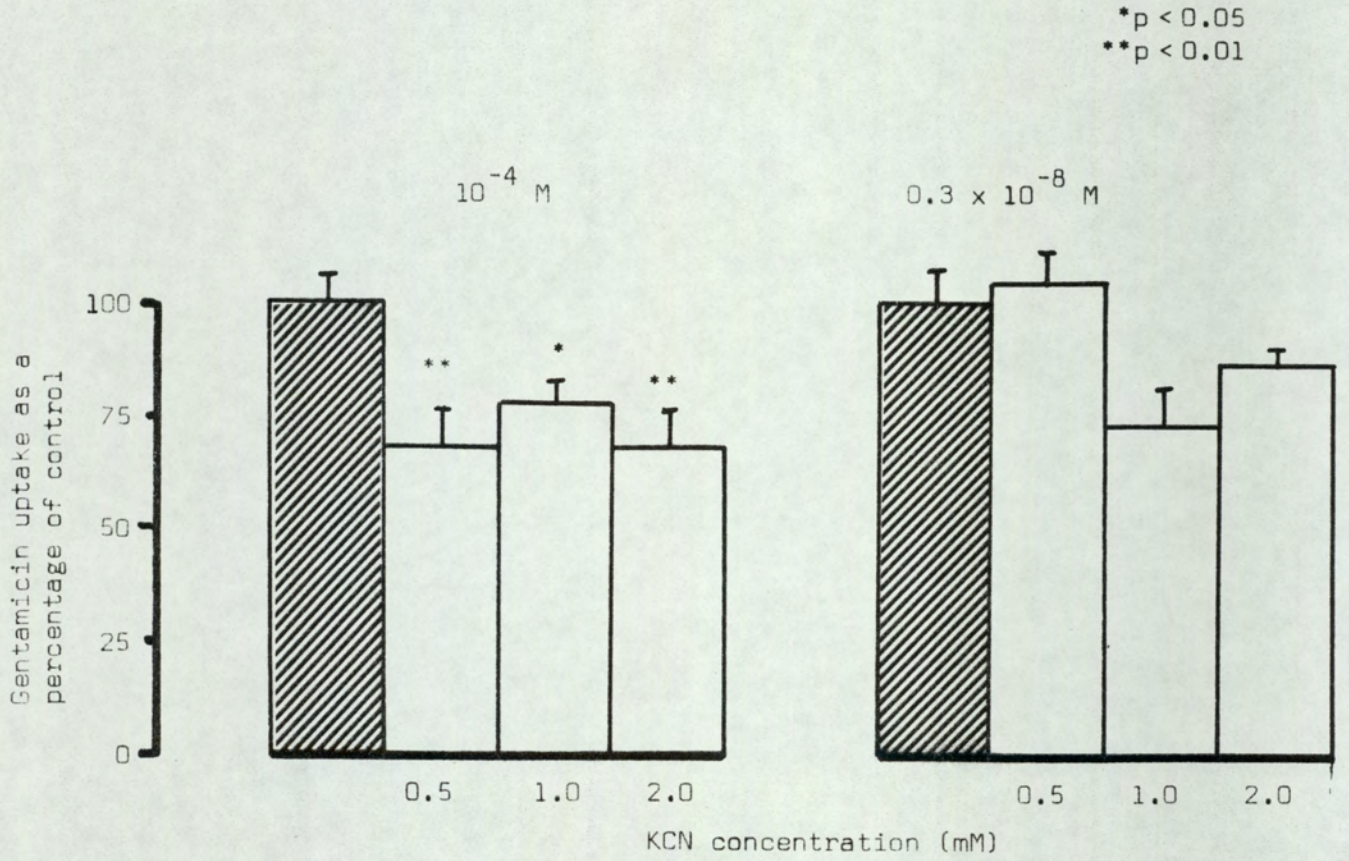


Fig. 27. The effect of potassium cyanide on the uptake of gentamicin into kidney slices after 30 minutes incubation at 37°C with $|^3H|$ gentamicin (0.125 µCi/ml) plus unlabelled gentamicin to give final concentrations of 10^{-4} M and 0.3×10^{-8} M.

This decrease was only statistically significant for the higher concentration of gentamicin in the incubation medium. The inhibitory effect was about 70% of the control value at all concentrations of KCN used (0.5 - 2.0 mM).

2,4-dinitrophenol (2,4-DNP) was also employed as a metabolic inhibitor (Fig. 28). The inhibitions observed with 2,4-DNP were of greater magnitude than those produced by KCN. In the presence of 2,4-DNP (0.5 - 2.0 mM), there was a significant concentration-dependent reduction in uptake, as compared to the uptake into slices incubated under control conditions at both concentrations of gentamicin.

At 10^{-4} M gentamicin, the uptake of gentamicin was reduced by 0.5 mM 2,4-DNP to 73% ($p < 0.05$) which was reduced even further to 56% and 45% at 2,4-DNP concentrations of 1.0 mM and 2.0 mM respectively ($p < 0.01$). At lower concentrations of gentamicin (0.3×10^{-8} M), 0.5 mM 2,4-DNP caused a decline in uptake which was not statistically significant. However, at higher concentrations of 2,4-DNP 1.0 mM and 2.0 mM, there was a significant decrease in uptake to 79% and 49% respectively ($p < 0.01$).

It appeared that 2,4-DNP was more potent in inhibiting uptake than KCN.

(viii) Effect of ouabain

The Na^+-K^+ ATPase inhibitor ouabain in the concentration range 0.5 - 2.0 mM appeared to have little or no effect on the uptake at both concentrations of gentamicin (Fig. 29).

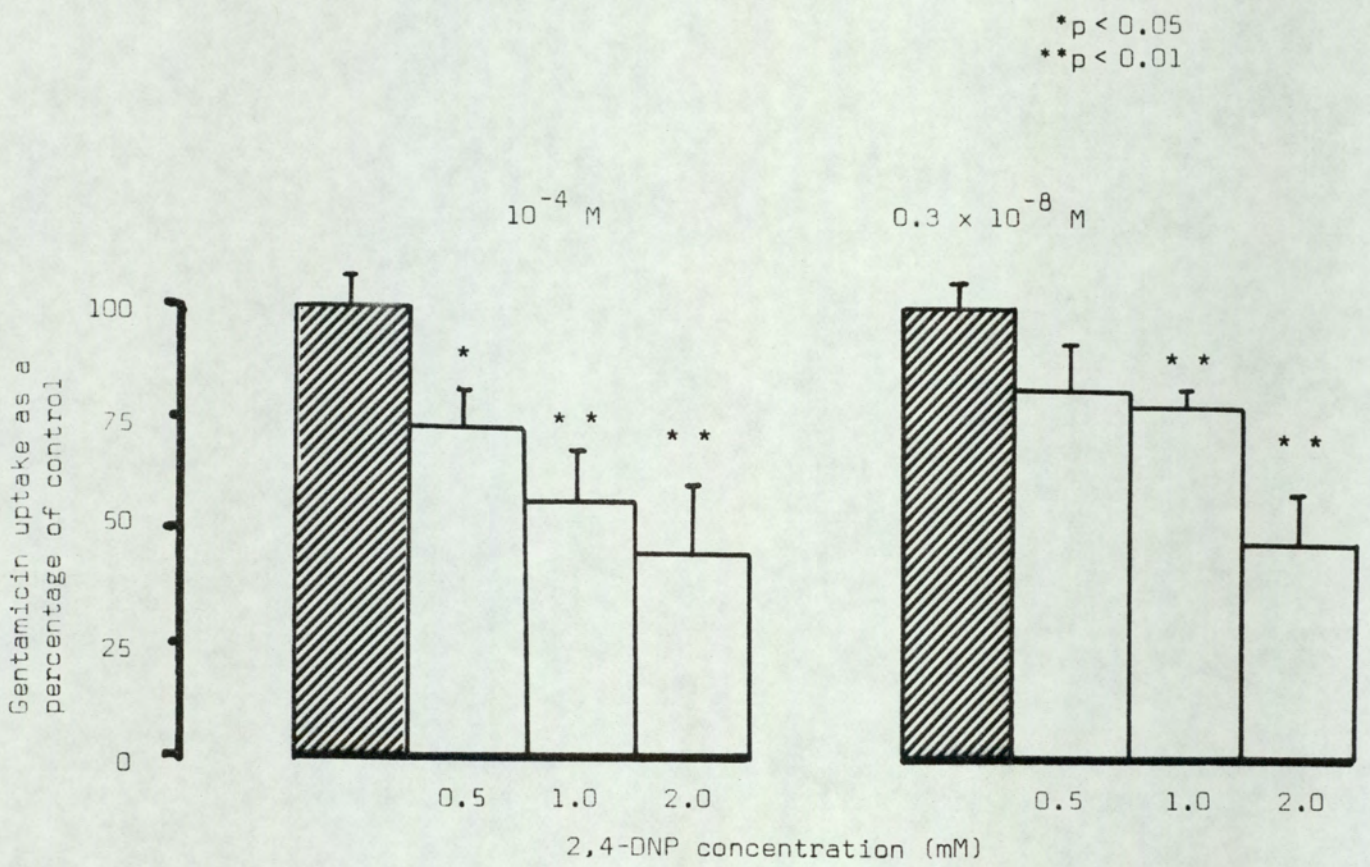


Fig. 28. The effect of 2,4-dinitrophenol upon the uptake of gentamicin into kidney slices after 30 minutes incubation at 37°C with $|^{3}H|$ gentamicin (0.125 µCi/ml) plus unlabelled gentamicin to give concentrations of 10^{-4} M and 0.3×10^{-8} M.

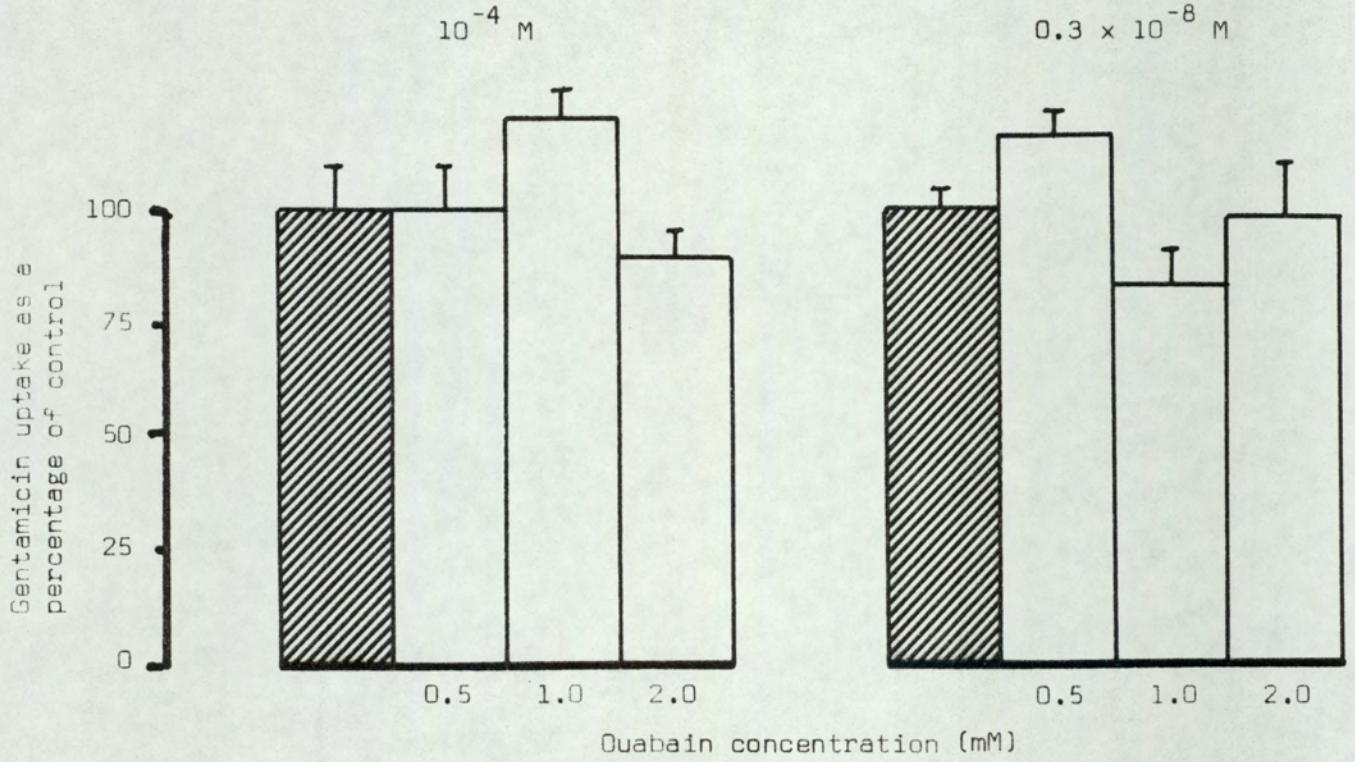


Fig. 29. The effect of ouabain on the uptake of gentamicin into kidney slices after 30 minutes incubation at 37°C with ^{3}H gentamicin (0.125 Ci/ml) plus unlabelled gentamicin to give final concentrations of 10^{-4} M and 0.3×10^{-8} M.

4.3.3 Discussion

The aminoglycoside antibiotics have been shown to accumulate *in vivo* to very high concentrations in the kidneys of animals (Gilbert, Bennett and Houghton *et al.*, 1977; Whelton and Walker, 1974) and patients (Schentag, Abrutyn and Jusko, 1976). The highest kidney aminoglycoside concentrations reported previously have been amikacin reaching concentrations of 1030 µg/gm in the cortex and 718 µg/gm in the medulla and gentamicin concentrations of 540 µg/gm in cortex and 230 µg/gm in medulla of patients (Edwards, Smith, Baughman *et al.*, 1976).

A study of renal clearance of gentamicin during treatment (Schentag and Jusko, 1977) has shown that at the beginning of treatment the clearance is less than creatinine clearance but that with repeated dosing gentamicin clearance rises to the same level. Schentag and Jusko interpreted this as evidence of gentamicin undergoing tubular reabsorption in the kidney and that this mechanism was becoming saturated with repeated dosing.

The results obtained in the present experiments with kidney slices also show a saturable uptake. The experiments studying uptake with time and varying gentamicin concentration in the medium showed the ability of the kidneys to accumulate gentamicin. This uptake of gentamicin plateaued with increasing time and gentamicin concentration. This saturability of gentamicin uptake into kidney slices has also been shown *in vitro* by Kluwe and Hook (1978).

The temperature dependence of gentamicin uptake was also studied. At a gentamicin concentration of 10^{-4} M, the kidney uptake of the drug was largely inhibited by temperature reduction to 4^{o}C (73%). The effects of varying Na^{+} and Ca^{2+} in the medium, anaerobic conditions or the presence of KCN, 2,4-DNP and ouabain

were also examined at two concentrations of gentamicin, 10^{-4} M and 0.3×10^{-8} M. The latter concentration was chosen on the basis of some interesting results obtained with lysosomal work discussed in section 4.6.

Anaerobic conditions reduced the uptake of both concentrations of gentamicin by 40 - 55%. In the presence of 2 mM concentrations of KCN or 2,4-DNP the uptake of gentamicin was inhibited by 30% and 55% respectively. However, incubation of gentamicin at both concentrations in a Na^+ and Ca^{2+} free medium resulted in a significant stimulation of gentamicin uptake.

In view of the large inhibition (73%) of uptake induced by temperature reduction, compared with the inhibition produced by anaerobic conditions and metabolic inhibitors (30 - 50%), it would appear that the accumulation of gentamicin in the tissue slices under the present experimental conditions may have some element of passive diffusion.

The extent to which the ionisation of gentamicin is temperature-sensitive will determine the relative reduction in the proportion of the unionised species and hence the apparent partition coefficient on lowering the temperature. It is possible therefore, that at lower temperatures the contribution from diffusion and non-specific hydrophobic bonding may be reduced, the magnitude of these two processes being a function of the concentration of the unionised species. This temperature sensitive uptake of gentamicin is different from the results of Kluwe and Hook (1978) who found gentamicin uptake not to be temperature dependent. However, there is agreement with incomplete inhibition of uptake under anaerobic conditions and this may in part be due to incomplete achievement of anoxia by incubation under N_2.

2,4-DNP, an inhibitor of oxidative phosphorylation, inhibited uptake at both concentrations of gentamicin whereas KCN, an inhibitor of respiratory chain had a significant effect only at the higher concentration of gentamicin. Inhibition by 2,4-DNP is similar to the findings of Hsu, Kurtz and Weller (1977), and at least in these experiments 2,4-DNP is more potent than KCN. This suggests that energy from aerobic phosphorylation may be required to transport gentamicin into the cells.

Thus, bulk of the gentamicin taken up by kidney slices in the present experiments was accumulated by a mechanism which required energy, although it is possible that a proportion of the uptake of gentamicin is accounted for by diffusion and binding.

It is possible that binding to both extracellular and intracellular sites may have contributed to the uptake observed in the present series of experiments. The binding of aminoglycosides to renal tubular brush-border have been reported by Luft, Patel and Yum et al. (1975) and Schacht (1978) has speculated interaction with the phospholipids of the membrane. It is possible that initial binding to the membrane may be the first step towards the uptake of gentamicin.

After passage into the cell, sequestration of gentamicin by intracellular binding to tissue macromolecules would effectively lower the free intracellular drug concentration and thus cause an increased concentration gradient across the cell membrane. In turn, this process would cause an enhancement of the diffusional movement of drug into the cell from the external medium, and may lead to an intracellular accumulation of drug against the 'apparent' concentration gradient. In view of the known localisation of gentamicin to lysosomes of cells (Kosek et al., 1974), it seems possible that this process of enhanced diffusion may contribute

to the kidney accumulation of gentamicin in the present *in vitro* situation. Whether this phenomenon makes a significant contribution *in vivo* is uncertain.

Diffusion of ionisable drugs across biological membranes may similarly be enhanced by the existence of pH gradients between extracellular and intracellular compartments (Christensen, 1975). However, Whelton, Carter, Bryant *et al.* (1976) and Mariel, Veyssier, Pechere *et al.* (1972) have reported renal gentamicin concentration and clearance to be unrelated to urine pH, although acidosis may increase renal damage (Hsu, Rutz, Eastling *et al.*, 1974).

Gentamicin solubility characteristics and its negligible oil-water partition coefficient precludes its dissolution or partitioning into lipoidal membranes. Its relatively large molecular size precludes transport through water filled pores or channels as has been postulated for some simple cations and anions. Therefore, it appears that its transport must involve, at least as a first step, some specific interaction with a cell membrane component.

Schacht (1978) has strongly stated a case for polyphosphoinositides as being a possible 'receptor' for aminoglycoside binding to cell membrane. He has shown an antagonistic interaction between Ca^{2+} and neomycin for the binding site on the membrane. Evidence of antagonistic interaction between the aminoglycoside antibiotics and Ca^{2+} has also been reported in experiments concerned with myocardial activity (Adams and Goodman, 1975), somatic neuromuscular transmission (Pittinger and Adamson, 1972), autonomic ganglionic transmission (Corrado and Ramos, 1958) and antibacterial activity (Ramirez-Ronda, Holmes and Sanford, 1975).

In the present study, increase of Ca^{2+} concentration in the medium resulted in a reduction of gentamicin uptake which was significant only at the lower concentration (0.3×10^{-8} M) of gentamicin used. However, reducing the Ca^{2+} levels to approximately zero resulted in a significant stimulation of gentamicin uptake at both concentrations of gentamicin.

Also the monovalent cation, Na^{+}, at zero concentration significantly stimulated gentamicin uptake at low concentrations of gentamicin. At the high concentration of gentamicin, Na^{+}-free medium increased uptake of gentamicin but was found to be not significant.

In vivo, Na^{+} deficiency has been reported to potentiate gentamicin induced renal failure by permitting higher drug levels to reach renal cortical tubules (Bennett, Harnett, Gilbert *et al.*, 1976). Adelman *et al.* (1979) have suggested that it is not the reduction in Na^{+} itself which is a highly contributing factor but the volume depletion associated with it resulting in a decline in the clearance of the drug from the kidney.

Lipsky, Cheng, Sackter *et al.* (1979), investigating gentamicin uptake by rabbit renal proximal tubule brush border membrane vesicles found inhibition of uptake when a Na^{+} electrochemical gradient of outside > inside existed. They concluded that the uptake of $|^{3}H|$ gentamicin by brush border membrane represented binding to the membrane rather than transport via a specific carrier across the membrane into an intravesicular space.

Therefore, in order to test whether gentamicin was co-transported with Na^{+} or not, the effect of Na^{+} - K^{+} ATPase inhibitor ouabain on the uptake of gentamicin into kidney slices was studied. Ouabain in the concentration range used (0.5 - 2.0 mM) had no apparent

effect on the uptake at both concentrations of gentamicin tested. This lack of inhibition by ouabain was also observed by Kluwe and Hook (1978), who used a ouabain concentration of 0.1 mM. It is well known that Na^+ - K^+ ATPase of the rat is far less sensitive to ouabain than other species, but the concentrations used in this experiment (highest 2 mM) was found sufficient to inhibit Na^+ - K^+ ATPase by 60% in isolated rat renal tubules (see section 4.2). Bakkeren, van der Beck and Bonting (1971) have reported a ouabain ID_{50} of 10^{-4} M for rat kidney Na^+ - K^+ ATPase.

If there was any connection between the transport mechanism of aminoglycosides and organic acids and bases and basic amino acids as has so often been put forward, then one might have expected an effect with ouabain. Ouabain is known to reduce *in vitro* accumulation of both organic acids (Berndt and Beechwood, 1965), organic bases (Neechay and Pardee, 1965) and basic amino acid lysine (Segal, Schwartz, Blair *et al.*, 1967).

Therefore, these findings are not consistent with the possible involvement of Na^+ dependent carrier and are somewhat paradoxical in view of the existence of a Na^+-sensitive 'uptake' at low concentrations of gentamicin.

SUMMARY

Consistent with previous *in vivo* and *in vitro* observations, the uptake of gentamicin into kidney slices was found to be both time and concentration dependent, showing saturable characteristics. Studies of the effect of temperature, anaerobic conditions and metabolic inhibitors indicated the existence of more than one mode of accumulation. The results suggested energy dependence and perhaps certain amounts of diffusion to be involved in the uptake of gentamicin by kidney slices.

Investigations with Ca^{2+} and Na^{+} suggested that binding to the membrane component may be a prerequisite for transport into the cell. However, lack of inhibition with ouabain tends to rule out Na^{+} dependent carrier mediated transport of gentamicin. The possible involvement of drug binding to the plasma membrane is considered in section 4.5

4.4 PREPARATION OF ISOLATED RAT RENAL TUBULES AND THEIR CHARACTERISATION

4.4.1 Introduction

Studies of mammalian cell function have been partially restricted by the limitations of the tissue preparations available. Whole organs, whether isolated or in situ, are difficult to manipulate and investigations with them may be limited by uncontrollable factors such as variations in the flow of perfusate to different regions of the organ. The use of thin sections of a variety of tissues have yielded significant information concerning both transport processes and tissue metabolism. However, with tissue slices most of the cells are not directly exposed to the bulk of the suspending medium and the immediate environments of many cells are affected by the rates of diffusion of substances through the extracellular space of the slice.

However, a suspension of undamaged detached cells, or small group of cells, which has none of the above disadvantages, would be a suitable preparation for the study of metabolism and other cell functions, though it should be realised that cells in isolation might behave differently from cells which are held together in an organised tissue structure.

A variety of methods have been employed to disrupt the kidney tissue in order to obtain suspensions of isolated kidney cells. These methods have included treatment with trypsin (Phillips, 1967), various chelating agents (Rappaport and Howze, 1966) as well as the purely mechanical methods of forcing kidney tissue through progressively smaller needles or homogenisation.

Despite the few successful mechanical methods of isolating apparently viable kidney cells, less drastic techniques have been developed recently. In these, the intracellular connective tissue of solid organs may be digested by enzymes such as collagenase and hyaluronidase, thus permitting gentle separation of the cells. These techniques have largely superseded the more vigorous mechanical methods of disruption.

The collagenase method of MacDonald and Saggerson (1977) was used to isolate fragmented rat renal cortical tubules and the criteria mentioned below were used to judge the viability of the tubules.

(i) cell structure as determined by phase contrast and electron microscopy.

(ii) the ability of the cells to perform integrated metabolic functions which are known to be performed by cells in the intact organ.

a) Gluconeogenesis

b) Protein synthesis

c) Basic amino acid transport

4.4.2 Results

4.4.2.1 Total tissue H_2O and volume of 'trapped fluid'

The total tissue water content of the isolated rat renal tubules was found to be 80% of the wet weight. This is in close agreement with the value reported by Foreman and Segal (1979).

The volume of 'trapped fluid' as determined by the inulin space (section 3.4.5) was 25% of the wet weight. Roth, Hwang and Segal (1976) and Foreman and Segal (1979) have quoted values in the range 15 - 25% of the wet weight.

4.4.2.2 Phase contrast microscopy

The prepared tubules when examined under phase contrast were seen to consist mainly of short fragments of tubule with some isolated cells and few clumps of cells also being present (Fig. 30). Although the cells appeared to be undamaged it was impossible to ascertain the precise degree of structural integrity.

4.4.2.3 Electron microscopy

In the present study, scanning electron microscopy (SEM) and transmission electron microscopy (TEM) were used to examine the structure of the tubules.

Fig. 31 shows the SEM of two tubules lying adjacent to each other. In one of the tubules, it appears that the lumen is patent.

The TEM of the tubular cells (Fig. 32), shows the ultra-structure to be well preserved on preparation of the tubules. The tubular epithelial cells are distinguishable by the presence of numerous microvilli on their luminal surface. The cellular organelles such as mitochondria also appear to be well preserved.

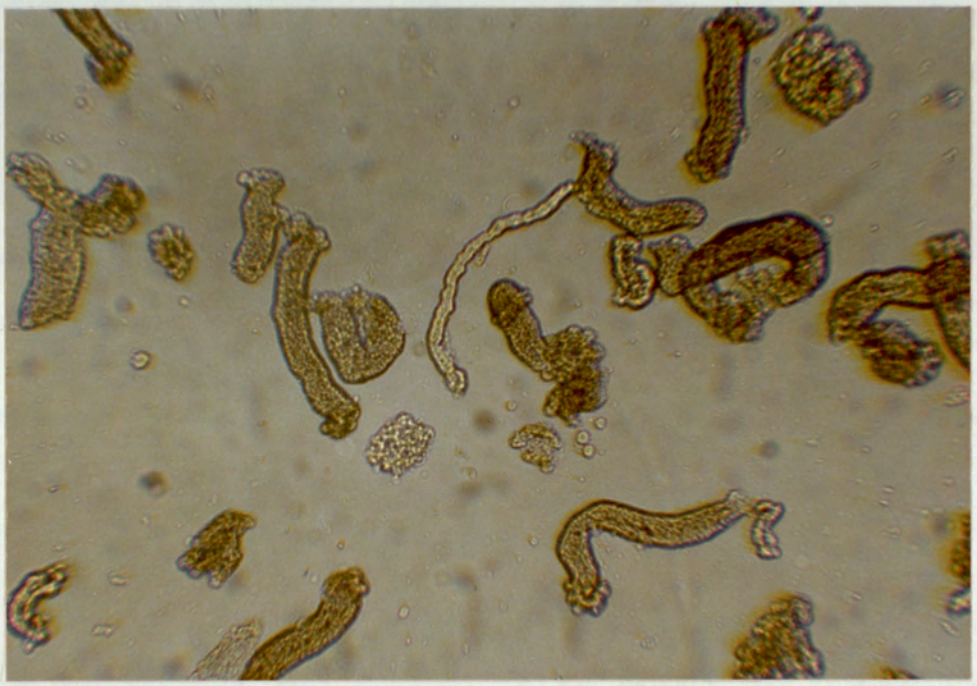


Fig. 30. Photomicrograph of freshly prepared rat kidney tubules in suspension.

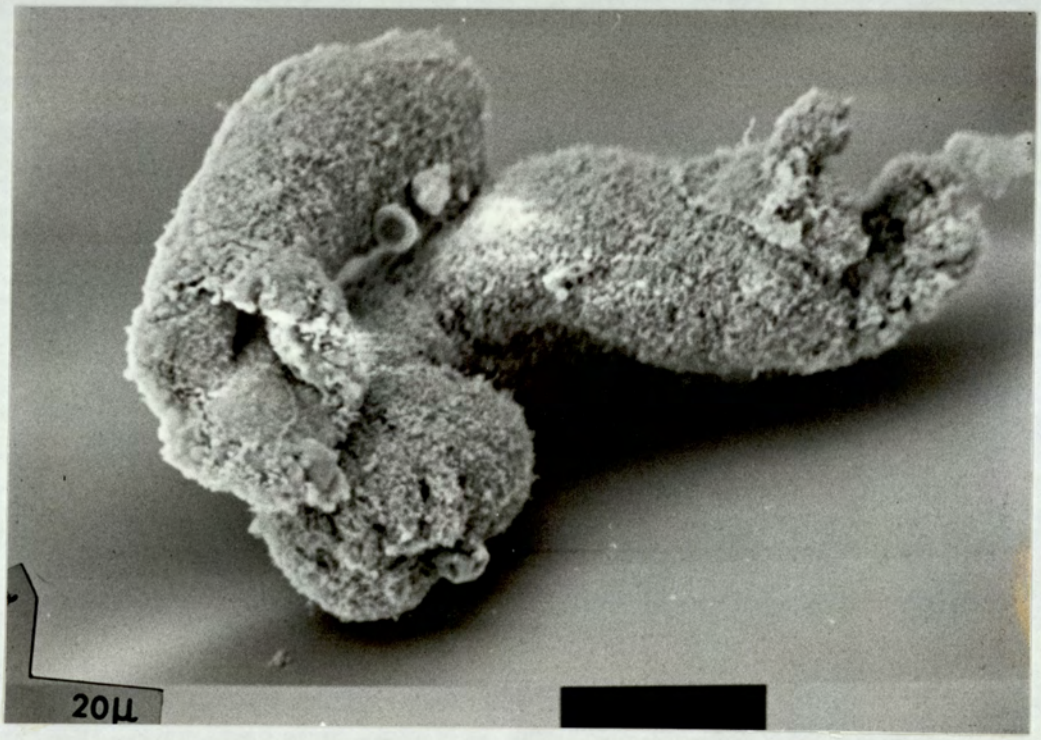


Fig. 31. Scanning electron micrograph (SEM) of isolated rat renal tubules. In one of the tubules, the lumen appears patent.

x 1250

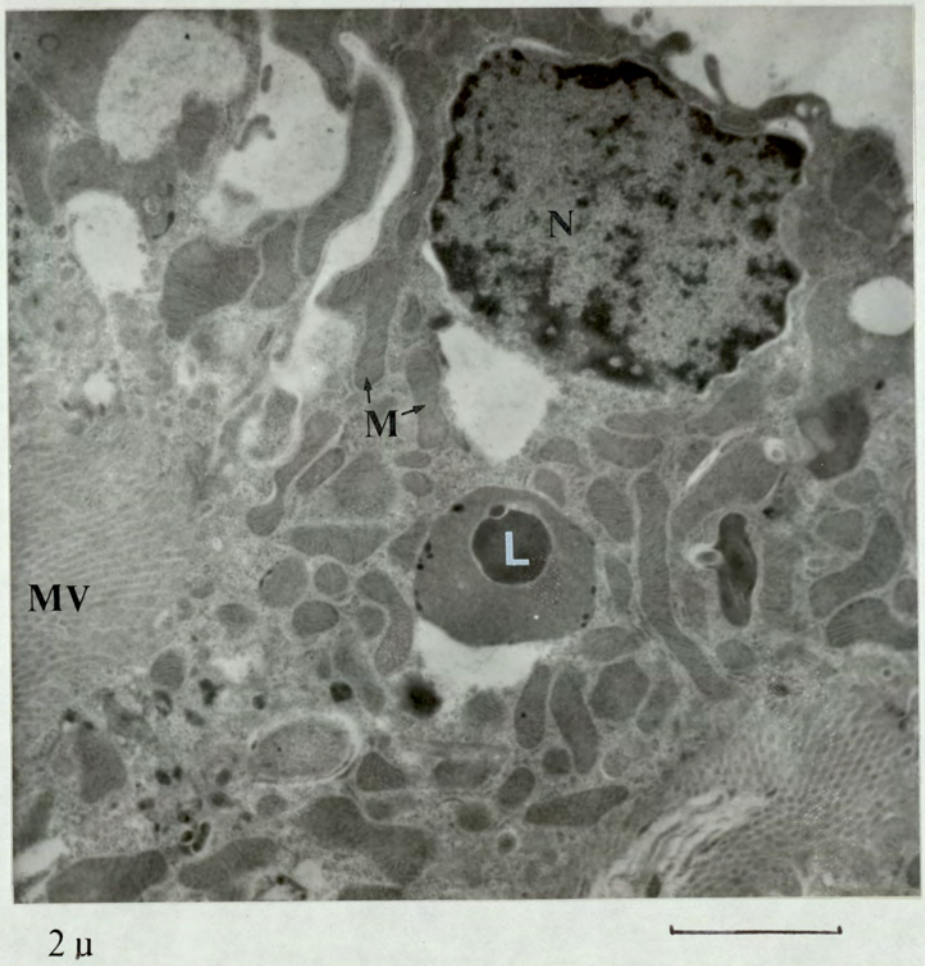


Fig. 32. Transmission electron micrograph of the tubular cells, shows the ultrastructure to be well preserved on preparation of the tubules. The epithelial cells are distinguishable by the presence of microvilli (MV), mitochondria (M), lysosomes (L) and nucleus (N).

x 11,250

4.4.2.4 Gluconeogenesis

Fig. 33 shows the time course of gluconeogenesis as measured by production of glucose by isolated rat renal tubules at 37°C. The rate of glucose formation was linear up to 60 minutes of incubation using pyruvate (5 mM) as substrate. After 1 hour, 237 nmoles of glucose per mg tubular protein had been formed.

4.4.2.5 Incorporation of $|^{14}C|$-leucine into TCA insoluble material - protein synthesis

Protein synthesis by isolated rat renal tubules was measured by looking at the incorporation of $|^{14}C|$ leucine into TCA insoluble material of tubules (Fig. 34). The ability of the tubules to perform protein synthesis was found to be linear for up to 2 hours incubation at 37°C.

4.4.2.6 Uptake of basic amino acid, $|^{14}C|$ lysine

Shown in Fig. 35 is the time course of uptake of lysine (0.065 mM) into renal tubules at 37°C, expressed as the distribution ratio (section 3.4.7). The uptake of lysine was initially rapid, reaching a steady state by about twenty minutes with a distribution ratio of about 8. A distribution ratio of greater than 1 is presumed to represent uptake in excess of simple diffusion. The results of the experiment indicates an active uptake of lysine into the tubules.

The effect of extracellular lysine concentration on the rate of lysine uptake was also studied (Fig. 36). In these experiments, uptake at 6 minutes was used as the estimate of uptake in order to obtain more accurate estimates of initial rates. The rate of increase of uptake clearly began to decrease as the lysine concentration approached 2.5 mM, showing some evidence of saturability under the conditions of these experiments.

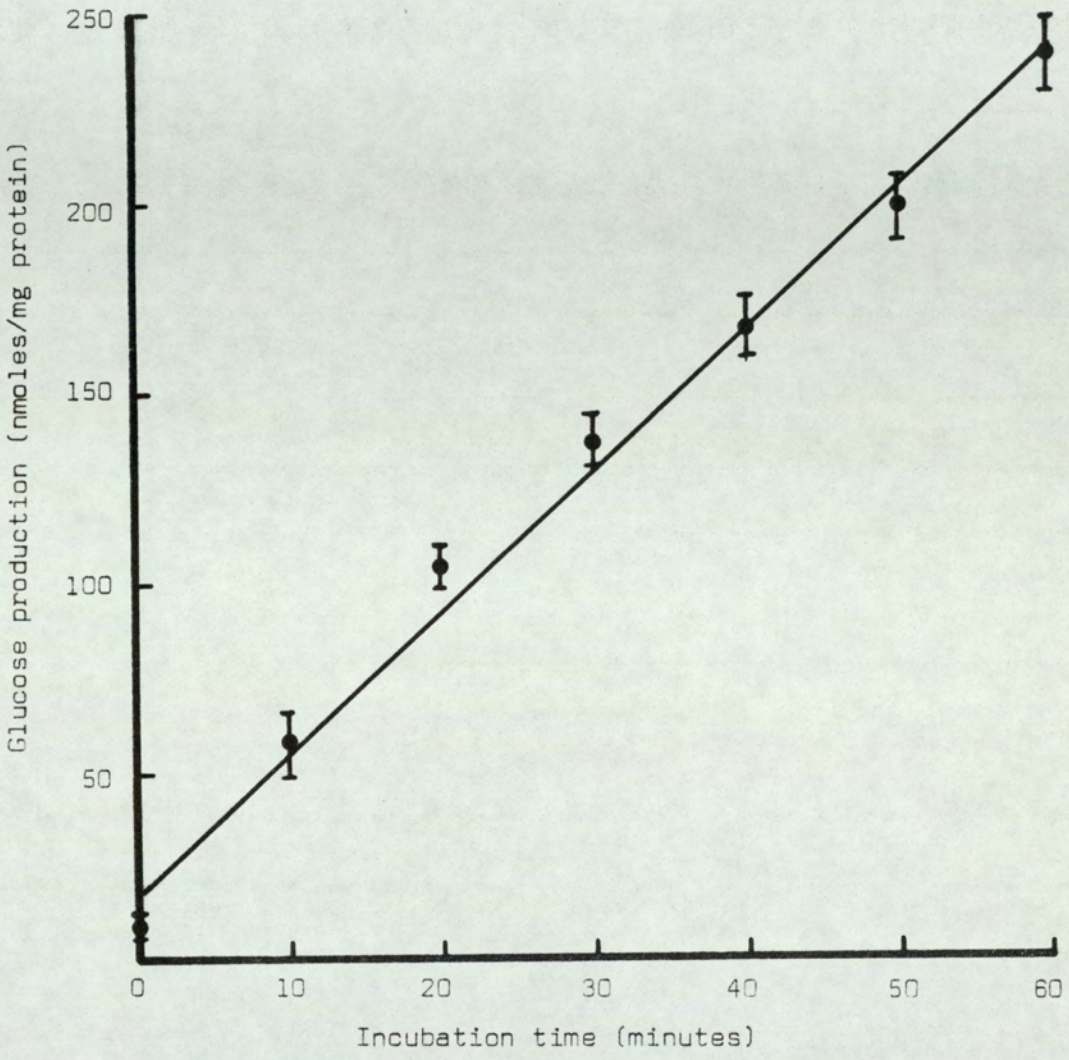


Fig. 33. The time course of gluconeogenesis in isolated rat renal tubules, incubated in Krebs-Hensleit medium (pH 7.4) in the presence of pyruvate (5mM) at 37°C. Each point represents the mean ± s.e.m. of three experiments each done in triplicate.

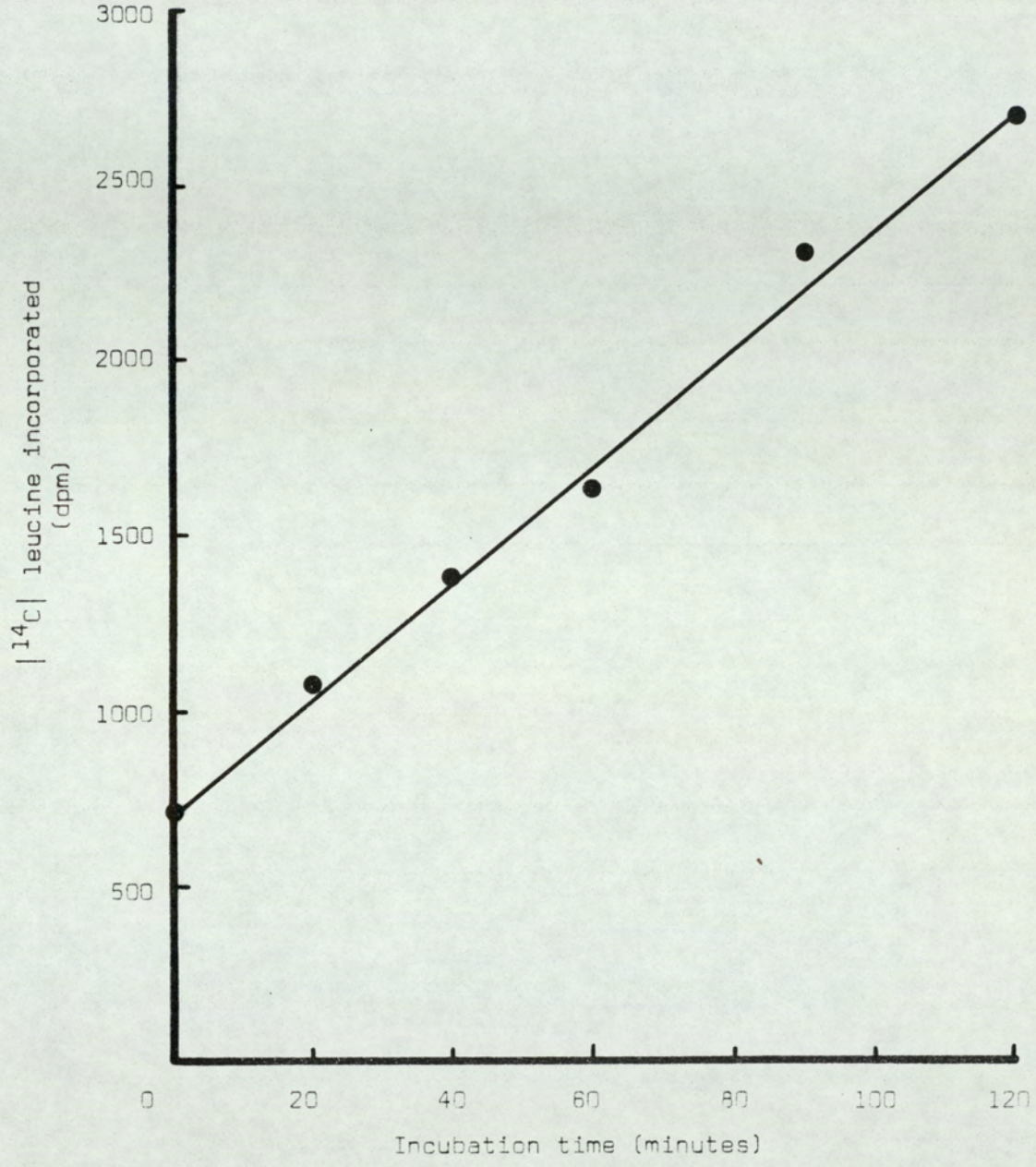


Fig. 34. Time course of incorporation of $|^{14}C|$ leucine into TCA insoluble material by isolated rat renal tubules at 37°C. Tubules were incubated with $|^{14}C|$ leucine (0.25 µCi/ml) plus unalbelled leucine to give a final concentration of 0.25 mM. Each point represents the mean of one experiment done in duplicate.

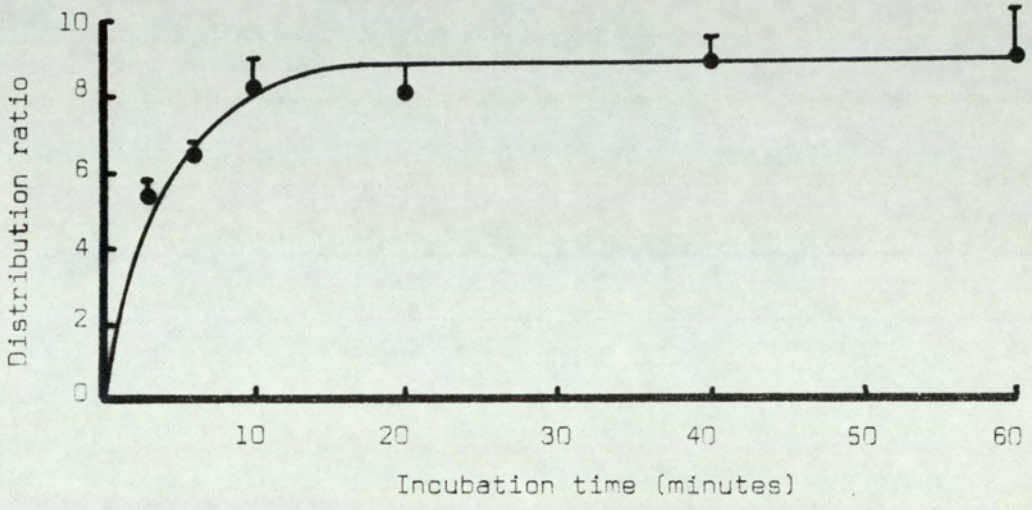


Fig. 35. Uptake of lysine into isolated rat renal tubules as a function of time. Tubules were incubated at 37°C with |^{14}C| lysine (0.1 µCi/ml) plus unalbelled lysine to give a final concentration of 0.065 mM. Each point is the mean ± s.e.m. of three experiments each done in duplicate.

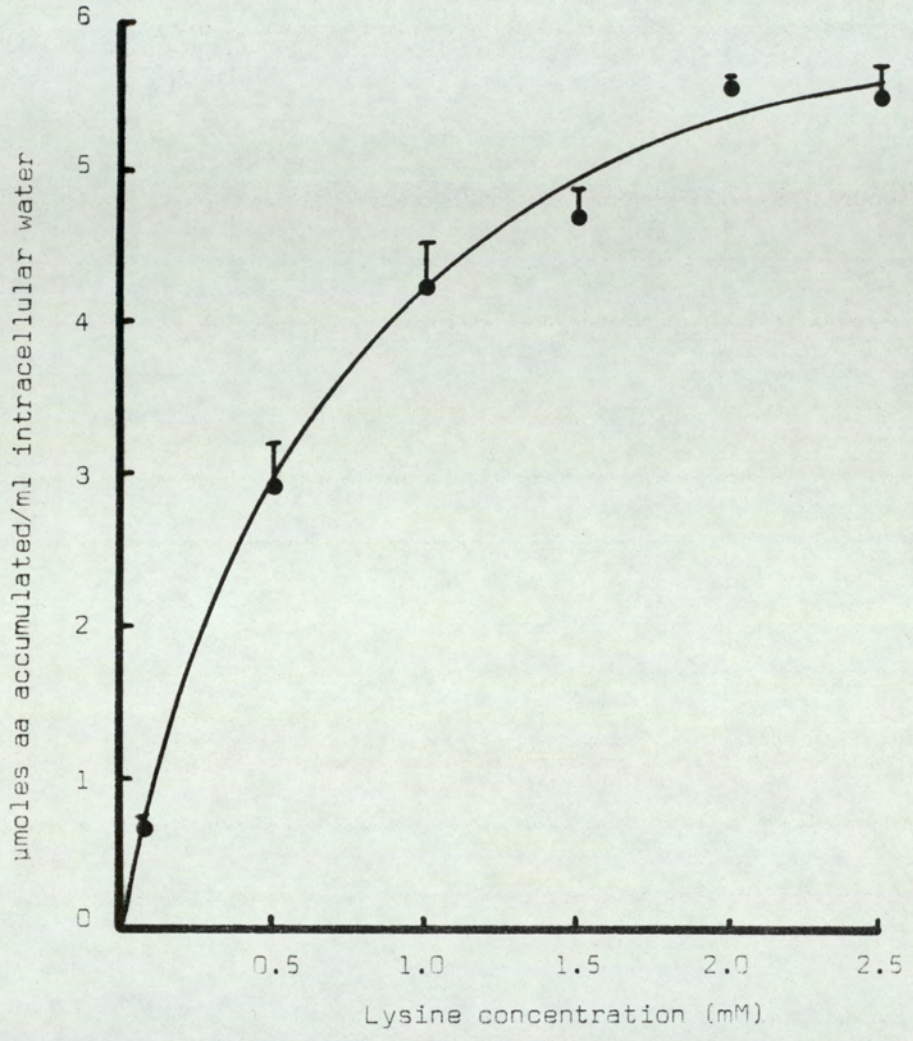


Fig. 36. Uptake of lysine into isolated rat renal tubules after 6 minutes incubation at 37°C. Tubules were incubated with [^{14}C] lysine (0.05 µCi/ml) plus unlabelled lysine to give a final concentration range from 0.065 mM to 2.5 mM. Each point represents the mean ± s.e.m. of two experiments each done in duplicate.

4.4.3 Discussion

The procedure used here for the preparations of the tubules was an adaptation of the method of MacDonald and Saggerson (1977). In the present system, defatted albumin was omitted since it has been reported by Guder, Wiesner, Stukowski *et al.* (1971) that the presence of albumin does not result in any better preparations. The presence of albumin may only complicate the interpretation of the results of gentamicin experiments since the drug is thought to bind to serum albumin *in vivo*.

The cells of the fragmented tubules appeared structurally intact when examined by phase-contrast microscopy. In experiments with kidney slices, a number of workers have expressed concern over the fact that substrate uptake in the slice model may represent peritubular rather than brush-border transport events, since it has been observed that the lumens of slices are collapsed (Foulkes, 1971 and Silverman and Turner, 1979). In the present study the SEM of the tubules showed the lumens not to be collapsed and thus the brush-border would be readily accessible to solute. Although the extent of uptake from the luminal side of the cortical slices is questionable, Roth, Holtzapple, Genel *et al.* (1979) have shown that glycine uptake by human kidney cortical slices to be completely consistent with observations made in adult rat renal brush-border membrane vesicles.

The TEM of the tubular cells showed the ultrastructure to be well preserved on preparation of the tubules by collagenase digestion.

Apart from the microscopic observations, another means of assessing the integrity of isolated cells is to study the ability

of the cells to take up or exclude certain 'vital' stains such as eosin or trypan blue. Stained cells are assumed to have membrane disruption. However, this method was not chosen in the present study because of the considerable difficulties of interpretation associated with vital staining (Black and Berenbaum, 1964). They have suggested that this test distinguishes damaged from undamaged cells with optimal efficiency only within certain limits and that these limits vary with the type of cell used, its species of origin, the nature of the suspending medium and the stain.

Therefore, instead of using this exclusion test, the time and concentration-dependent uptake of the basic amino acid lysine was used as a measure of the intactness of the outer cellular membrane.

The uptake of lysine was shown to be both saturable and active, suggesting integrity of the membrane transporting system (Figs. 35 and 36). The accumulation of amino acids in the renal cortex, against a concentration gradient, has been demonstrated both *in vivo* and *in vitro* (Segal and Thier, 1973). Numerous studies have provided evidence that the renal reabsorption of amino acids at the luminal membrane to be driven by the Na^+ gradient produced by the Na^+-K^+ ATPase located at the antiluminal side of the tubular cell (Crane, 1977).

Another criterion which has been commonly employed to judge the degree of intactness of isolated tubules is the ability of the cells to perform integrated metabolic functions which are known to be carried out by the cells of the intact organ. The synthesis of glucose from pyruvate (gluconeogenesis) and incorporation of $|^{14}C|$ leucine into proteins (protein synthesis) was used to fulfill the above criteria.

Gluconeogenic activity has been shown to be high in kidney and liver. The discovery of enzymes which catalyse specific steps in gluconeogenesis has included phosphoenolpyruvate carboxykinase which converts oxaloacetate to phosphoenolpyruvate (Utter and Kurahashi, 1954), thus providing precursors for the reversal of the 'glycolytic pathway'. This enzyme has been shown to be localised in the cytoplasm of rat kidney (Flores and Alleyne, 1971) and to be the rate limiting step in the overall glucose formation from lactate, pyruvate, glutamine and other precursors which are converted to glucose via oxaloacetate. Since the discovery of this pathway in the rat kidney, it has been shown that gluconeogenesis is limited to the cortex and in particular the proximal tubular cells (Guder and Schmidt, 1974).

The rate of gluconeogenesis by the tubules in this study was found to be linear up to 60 minutes of incubation and compared favourably with the rate obtained by Nagata and Rasmussen (1970) using pyruvate as the precursor.

This high rate of glucose formation from pyruvate by the fragmented tubules indicates a significant degree of coupling between respiration and phosphorylation. Hems, Ross, Berry et al. (1966) have emphasised that gluconeogenesis is extremely exacting in terms of ATP requirements. Moreover, when pyruvate is the substrate, the metabolic pathway requires the participation of mitochondrial and cytoplasmic enzyme systems working in unison. Therefore, gluconeogenesis might be expected to be one of the first cellular activities to be adversely affected by structural damage or by impairment of energy conservation in the cell. The observed rate of more than 200 nmoles of glucose synthesised per hour per mg of cell protein is higher than that reported for kidney

cortex slices (Krebs, Bennett, de Gasquet et al., 1963) and for isolated perfused kidney (Nishiitsutsuji-Uwo, Ross and Krebs, 1967). The ATP required for this synthesis would be provided by the coupled respiratory-chain phosphorylation. The result also suggests that the isolated fragmented tubule preparation from the rat kidney cortex consisted mainly of proximal tubular cells.

In eukaryotes, protein synthesis takes place on ribosomes attached to the endoplasmic reticulum in a highly ordered and integrated manner. The linearity of protein synthesis observed here, once again emphasises the structural intactness of the present fragmented tubule preparation.

SUMMARY

The morphology and viability of the tubules was ascertained by phase contrast and electron microscopy and also by gluconeogenesis, incorporation of $|^{14}C|$ leucine into TCA insoluble tubular material and uptake of $|^{14}C|$ lysine.

The fragmented tubule preparation exhibited a high rate of gluconeogenesis with linear rate of protein synthesis and saturable lysine uptake. These results, coupled with microscopic evidence, suggest a very high degree of cellular integrity of these tubules. A preparation such as this offers many advantages over using kidney slices and may be used to gain further insight into the mechanism of aminoglycoside nephrotoxicity.

4.5 IN VITRO UPTAKE OF GENTAMICIN INTO ISOLATED RENAL TUBULES

4.5.1 Introduction

A variety of techniques have been employed to study the uptake of aminoglycosides by renal tubular cells. Several hypotheses about the basic cellular mechanisms involved in gentamicin uptake and the resultant nephrotoxicity have been advanced. Autoradiographical evidence has indicated that the luminal side of the proximal tubular cell is the major route of access of gentamicin (Just, Erdmann and Habermann, 1977; Jerauld and Silverblatt, 1978). Just *et al.* (1977) and Silverblatt and Kuehn (1979), on the basis of autoradiographical and cell fractionation studies have suggested that aminoglycosides like some peptides, are adsorbed at the brush-border membrane followed by endocytosis and lysosomal sequestration.

Studies with cortical slices of rat kidney (section 4.3) suggested that gentamicin transport occurs by an energy dependent process because it was inhibited by metabolic inhibitors and anoxia. However, it was noted in section 4.4 that the cortical slice preparation may not be the most suitable preparation to use, since in slice preparation the innermost cells are not readily exposed to the suspending medium.

Consequently, viable isolated rat renal tubules were used to examine the following aspects of gentamicin uptake:-

(i) The kinetics of gentamicin uptake in the presence of metabolic inhibitors.

(ii) Binding of gentamicin to tubules at $4^{o}C$ and the possible nature of the 'receptor' involved in the binding process.

(iii) The effect of gentamicin on several proximal tubular functions in an attempt to localise the subcellular site of lesion.

4.5.2 Results

4.5.2.1 Time course of gentamicin uptake.

Fig. 37 shows the uptake of gentamicin into tubules at 37°C measured at intervals up to 30 minutes. At gentamicin concentrations of 10^{-4} M and 10^{-5} the uptake of gentamicin was initially rapid followed by an accumulation at a slower rate.

4.5.2.2 Efflux of gentamicin

The efflux of gentamicin from the preloaded tubules followed a biphasic pattern (Fig. 38). When preloaded tubules were reincubated in gentamicin-free medium, the initial efflux was rapid with approximately 70% of the gentamicin lost in the first 10 minutes followed by a much slower loss. The rate efflux was not affected by the presence of low or high amounts of unlabelled gentamicin (10^{-5} M and 10^{-3} M) in the medium. The preincubated tubules when resuspended in its original medium containing $|^{3}H|$ gentamicin plus unlabelled gentamicin at a final concentration of 10^{-5} M, showed continued uptake.

4.5.2.3 Effect of 2,4-DNP and N_2

Incubation of tubules with gentamicin (10^{-4} M) in the presence of 2,4-DNP or under anaerobic conditions resulted in inhibition of gentamicin uptake (Fig. 39). In the presence of 2,4-DNP (1 mM) the time course of gentamicin uptake followed that of control up to 15 minutes after which there was no further uptake with 2,4-DNP giving a resultant inhibition of 35% at 60 minutes. The effect of anaerobic conditions was very dramatic with no uptake of gentamicin observed. The negative values arose with N_2 due to subtraction of zero time uptake from uptake measured at other times.

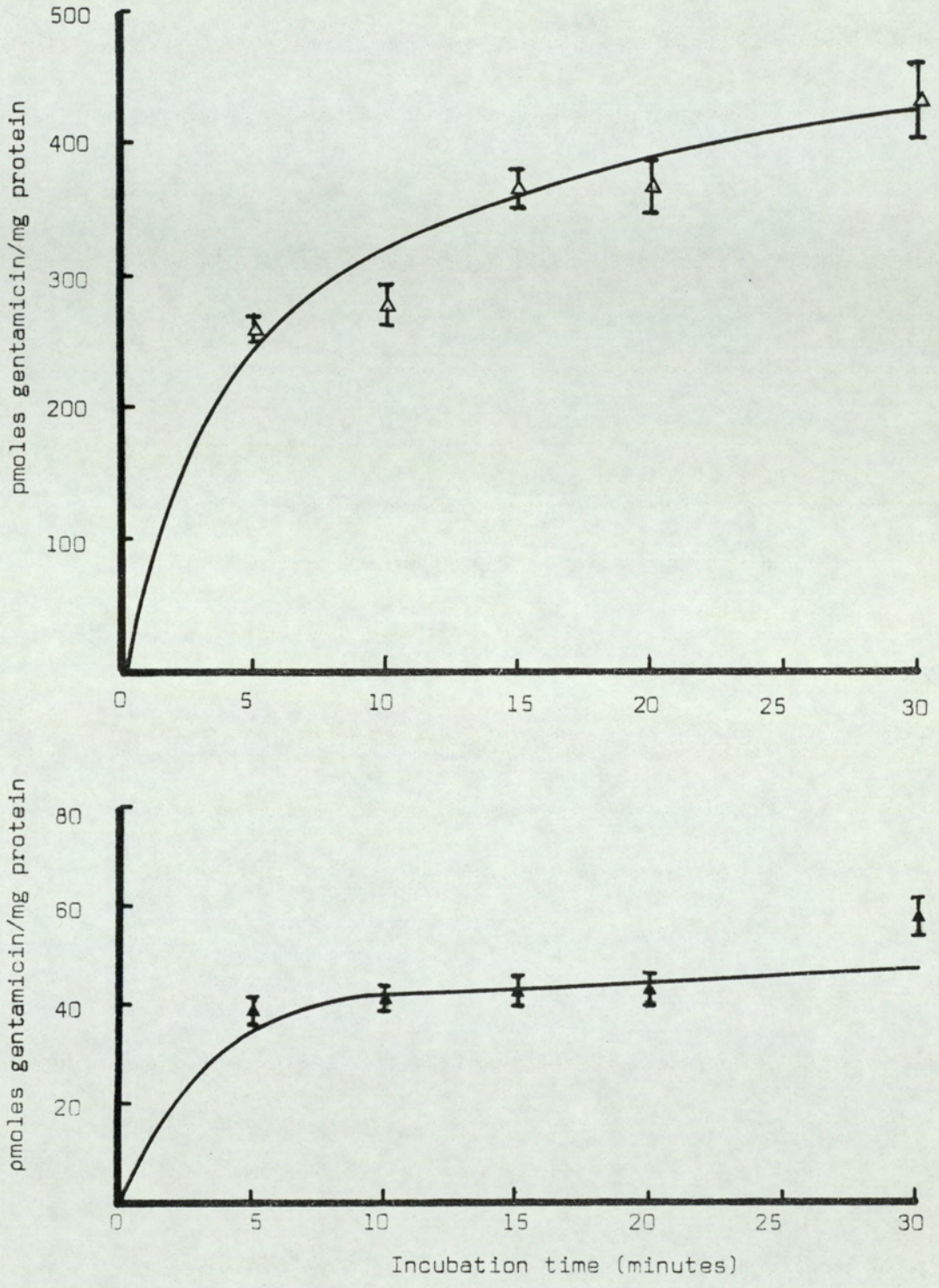


Fig. 37. Uptake of gentamicin into isolated rat renal tubules as a function of time. Tubules were incubated at 37°C with [^{3}H] gentamicin (0.5 µCi/ml) plus unlabelled gentamicin to give final concentrations of 10^{-4} M (Δ) and 10^{-5} M (▲). Each point represents the mean ± s.e.m. of experiment done in triplicate.

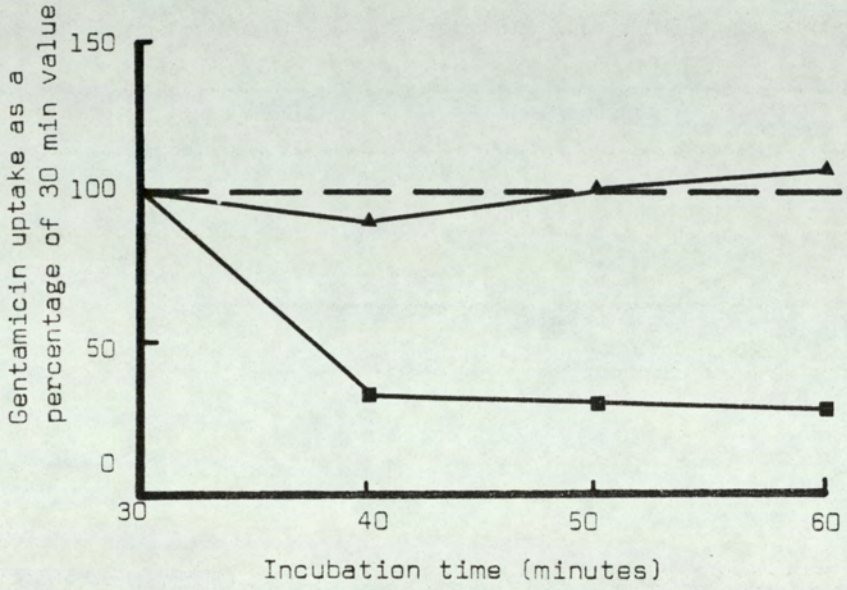


Fig. 38. The efflux of gentamicin from rat renal tubules. Tubules were incubated at 37°C for 30 minutes in medium containing $|^{3}H|$ gentamicin plus cold gentamicin (10^{-5} M). The control preparations were re-exposed to the initial medium (▲) and others resuspended in antibiotic-free medium or medium containing unlabelled gentamicin (■ ; 10^{-3} M, 10^{-5} M and 0, all the points were superimposable). Each point is the mean of experiment done in triplicate

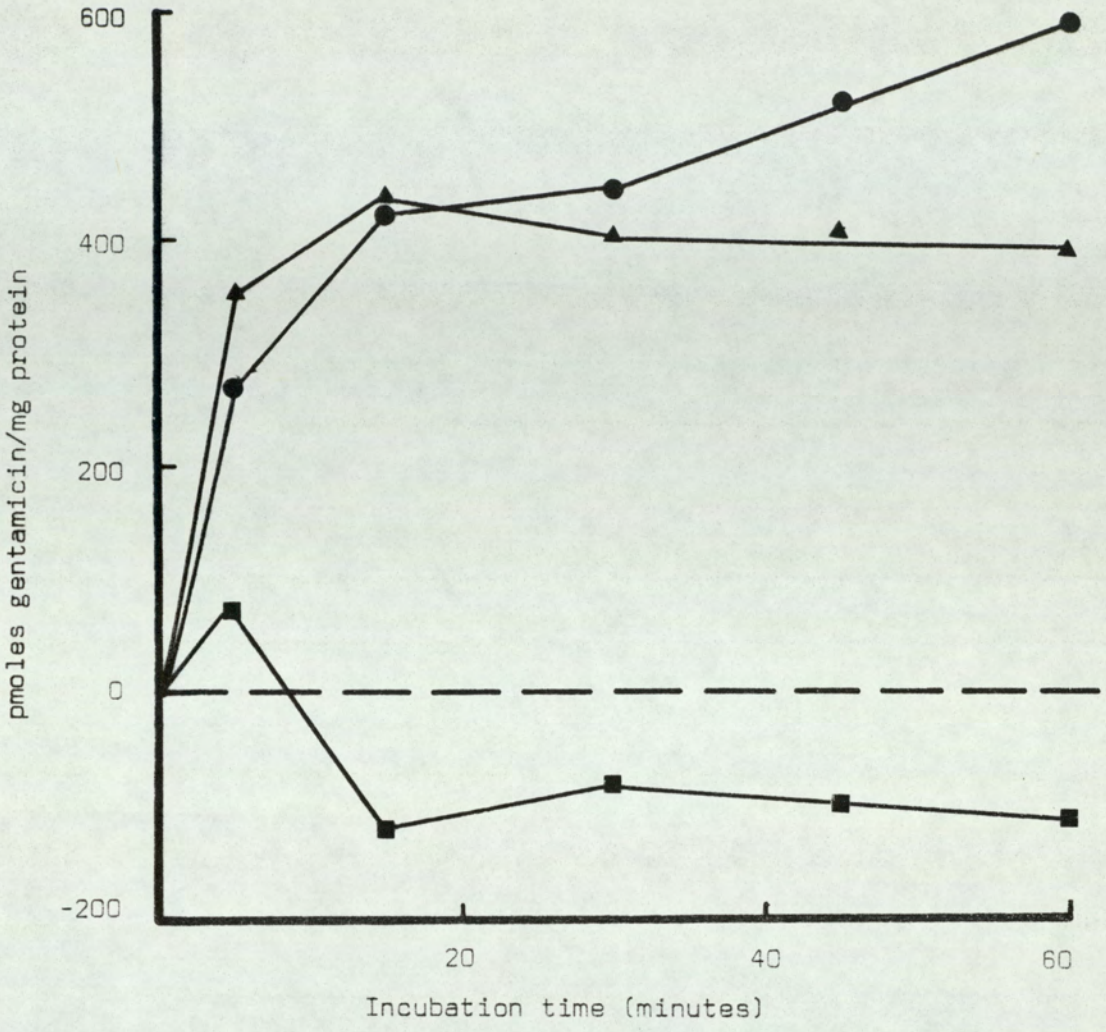


Fig. 39. Uptake of gentamicin into isolated rat renal tubules as a function time. Tubules were incubated at 37°C with $|^3H|$ gentamicin (0.5 µCi/ml) plus unlabelled gentamicin at a final concentration of 10^{-4} M. The uptake in the absence (●), in the presence of 1 mM 2,4 DNP (▲) and under N_2 (■). Each point is the mean of experiment done in triplicate

4.5.2.4 Effect of metabolic inhibitors

Isolated tubules were incubated with gentamicin (10^{-4} M) for 60 minutes at 37°C in the presence of 2,4-DNP, KCN and iodoacetic acid in the concentration range 0.5 - 2.0 mM. Compared to tubules incubated under control conditions, there was a reduction in the uptake of gentamicin to 75% and 13% at 2,4-DNP concentrations of 1.0 mM and 2.0 mM respectively (Fig. 40). KCN was far less effective than 2,4-DNP, reducing gentamicin uptake to 62% at a concentration of 2 mM (Fig. 41). Iodoacetic acid had little or no effect on gentamicin uptake in the concentration range used (Fig. 42).

4.5.2.5 Effect of ouabain

The Na^+-K^+ ATPase inhibitor, ouabain, was employed in the concentration range 0.5 - 2.0 mM. The uptake of gentamicin was reduced significantly (75% of control) at a ouabain concentration of 2.0 mM (Fig. 43).

4.5.2.6 Time dependent binding of gentamicin to tubules at 4°C

The time course of binding of gentamicin to isolated rat renal tubules at 4°C is illustrated in Fig. 44. The amount of gentamicin bound was initially very rapid occurring in the first twenty minutes followed by only a small increase on incubating further up to 90 minutes.

4.5.2.7 Varying concentrations of gentamicin binding to tubules at 4°C

Fig. 45 shows the Scatchard plot (Scatchard, 1948) of the binding of gentamicin to isolated rat renal tubules after 60 minutes incubation at 4°C. The Scatchard plot curve has an inflection

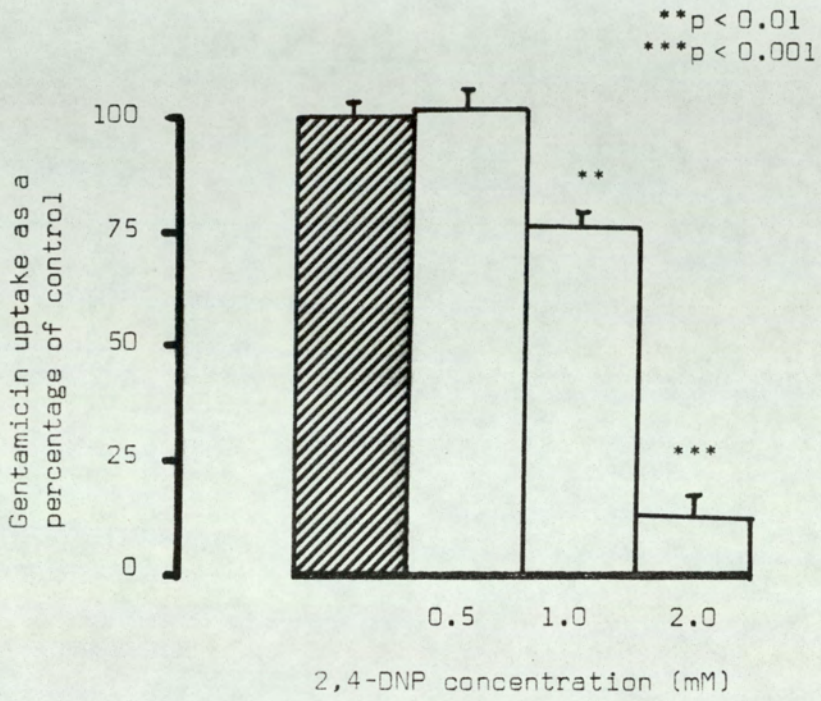


Fig. 40. The effect of 2,4-DNP on the uptake of gentamicin into isolated rat renal tubules after 60 minutes incubation at 37°C with $|^3H|$ gentamicin (0.5 µCi/ml) plus unlabelled gentamicin to give a final concentration of 10^{-4} M. The results shown here, and in the following histograms in this section, are expressed as a percentage of uptake into tubules incubated under control conditions (closed bars - 100%). Each bar represents the mean ± s.e.m. of an experiment with at least three replicates.

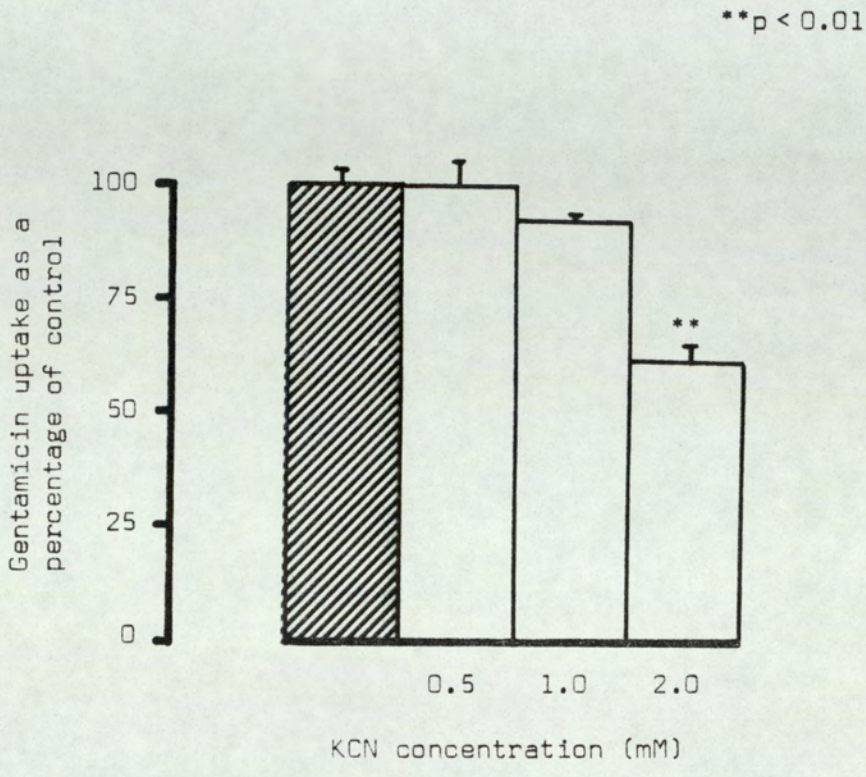


Fig. 41. The effect of KCN on the uptake of gentamicin into isolated rat renal tubules after 60 minutes incubation at 37°C with $|^{3}H|$ gentamicin (0.5 µCi/ml) plus unlabelled gentamicin to give a final concentration of 10^{-4} M.

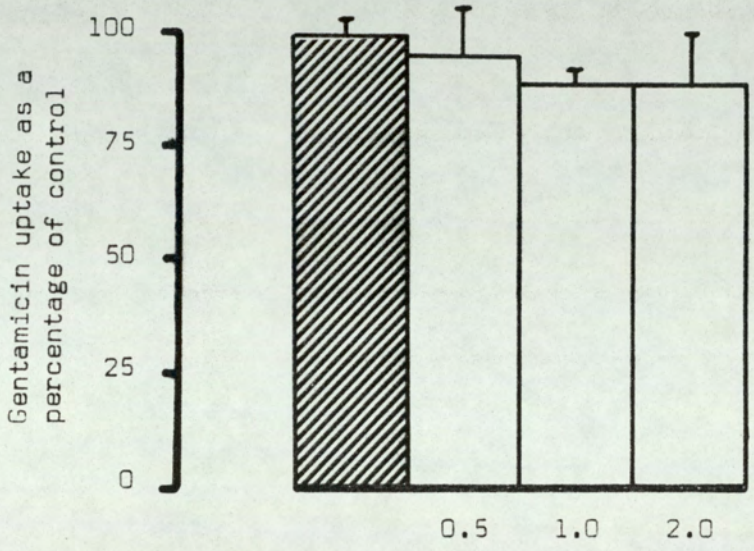


Fig. 42. The effect of iodoacetic acid on the uptake of gentamicin into isolated rat renal tubules after 60 minutes incubation at 37°C with $|^{3}H|$ gentamicin (0.5 µCi/ml) plus unlabelled gentamicin to give a final concentration of 10^{-4} M.

**p < 0.01

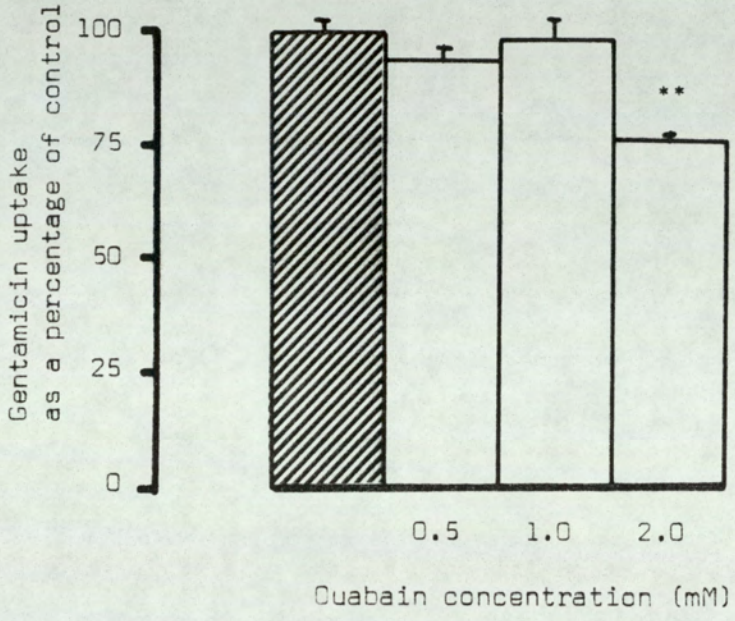


Fig. 43. The effect of ouabain on the uptake of gentamicin by isolated rat renal tubules after 60 minutes incubation at 37°C with $|^{3}H|$ gentamicin (0.5 µCi/ml) plus unlabelled gentamicin to give a final concentration of 10^{-4} M.

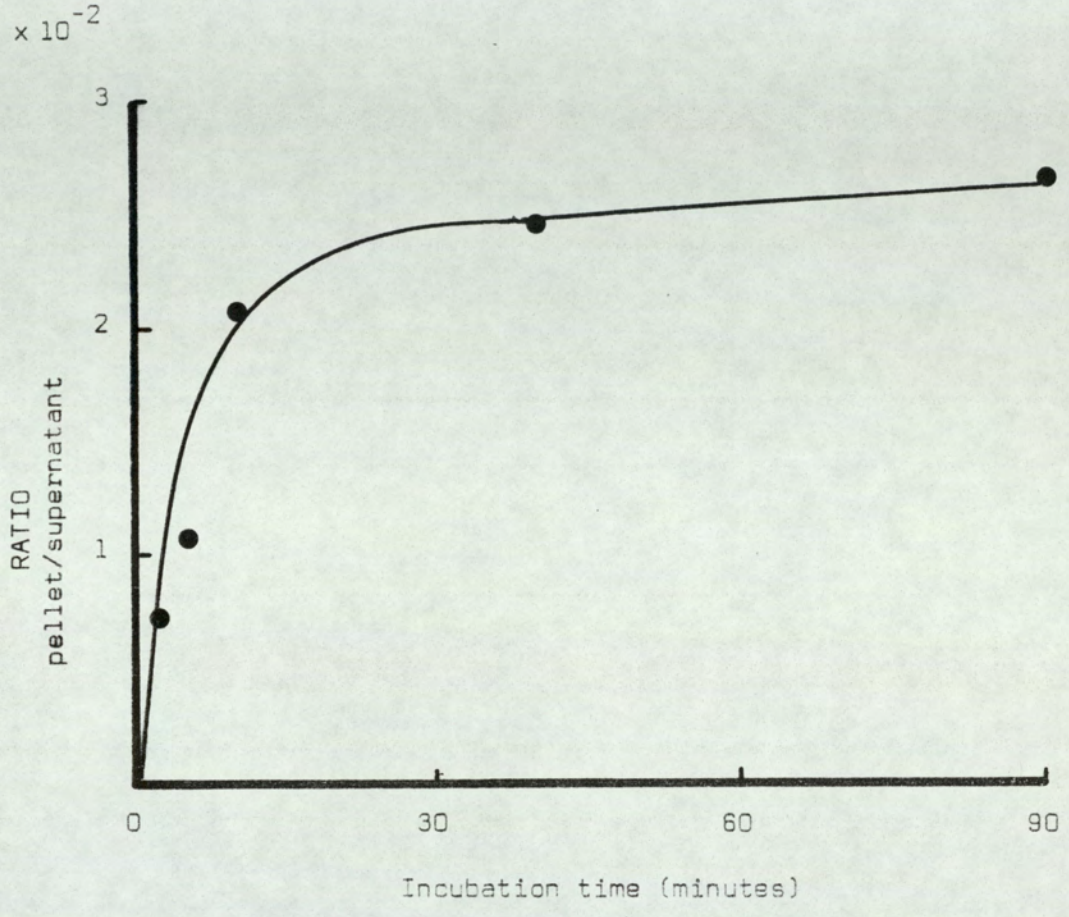


Fig. 44. Time dependence binding of $|^{3}H|$ gentamicin (1 µCi/ml) to isolated rat renal tubules at 4°C. Each point is the mean of three determinations.

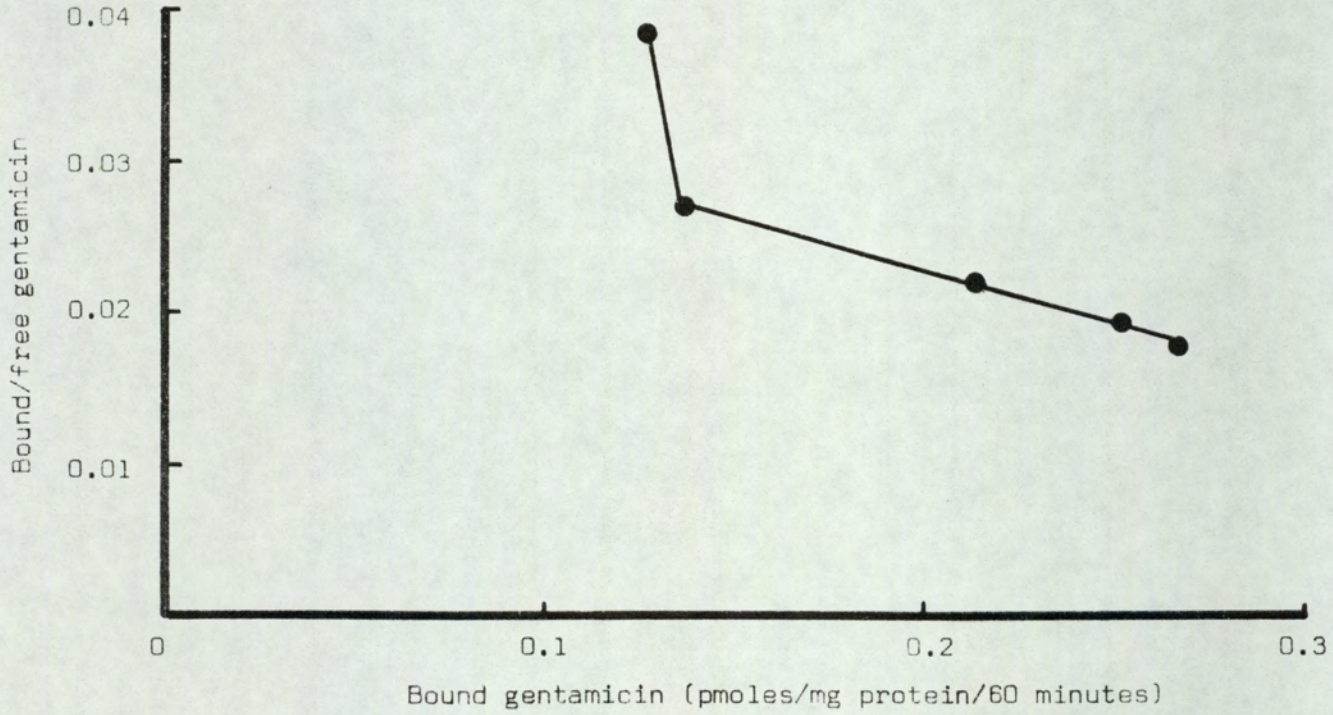


Fig. 45. Relationship between the amount of $|^3H|$ gentamicin bound to isolated rat renal tubules and the ratio of bound/free $|^3H|$ gentamicin after 60 minute incubation period at 4^oC. Each point is the mean of experiment done in triplicate.

point, suggesting that there may be more than one class of binding sites with different affinities on the proximal tubules.

4.5.2.8 Gentamicin uptake into tubules previously preincubated with trypsin

The effect of preincubating tubules with a proteolytic enzyme, trypsin (0.01%) for varying times up to 30 minutes on the subsequent uptake of gentamicin is shown in Table 6. The uptake of gentamicin compared to control was decreased by 30% and 43% on preincubating tubules with trypsin for 10 minutes and 30 minutes respectively.

4.5.2.9 Effect of spermine and mannose-6-phosphate on gentamicin (10^{-4} M) binding to tubules at $4^{o}C$

Fig. 46 shows the effect of spermine and mannose-6-phosphate in the concentration range 0.1 - 1.0 mM on gentamicin binding to tubules at $4^{o}C$ after 30 minutes incubation. Spermine and mannose-6-phosphate both inhibited gentamicin binding at all concentrations used but the reduction was only statistically significant at concentrations of 0.5 mM and 1.0 mM in both cases. At a given concentration spermine was more effective in inhibiting gentamicin binding than mannose-6-phosphate with reduction at 1.0 mM to 21% and 48% respectively compared to control. The inhibition produced in the presence of both spermine and mannose-6-phosphate (0.5 mM each) was greater than the sum of inhibition produced by each on its own.

4.5.2.10 Effect of gentamicin on lysine uptake

The time course of lysine uptake in the absence and presence of gentamicin (10^{-4} M and 10^{-3} M) at $37^{o}C$ for a period of time up to 30 minutes is shown in Fig. 47. Gentamicin had no effect on the uptake of lysine which showed a characteristic initial rapid uptake followed by a plateau.

Preincubation time with or without trypsin (minutes)	Control preincubation % gentamicin bound after incubating for 30 min	Trypsin preincubation % gentamicin bound after incubating for 30 min	Percentage reduction in amount of gentamicin bound caused by preincubation with trypsin
0	2.59	2.04	79
2.5	2.72	1.92	71
10	2.61	1.83	70
15	2.52	1.66	66
30	3.02	1.73	57

Table 6. Effect of trypsin treatment on binding of $|^3H|$ gentamicin to isolated rat renal tubules. Tubules were incubated at 37°C in a medium containing trypsin (0.01% final concentration) or medium without trypsin (control). After appropriate incubation times, aliquots were removed, centrifuged and tubules resuspended in 2 ml media containing $|^3H|$ gentamicin (0.5 µCi/ml). Incubations in the presence of gentamicin were allowed to proceed for 30 minutes and then assayed for amount of gentamicin bound.

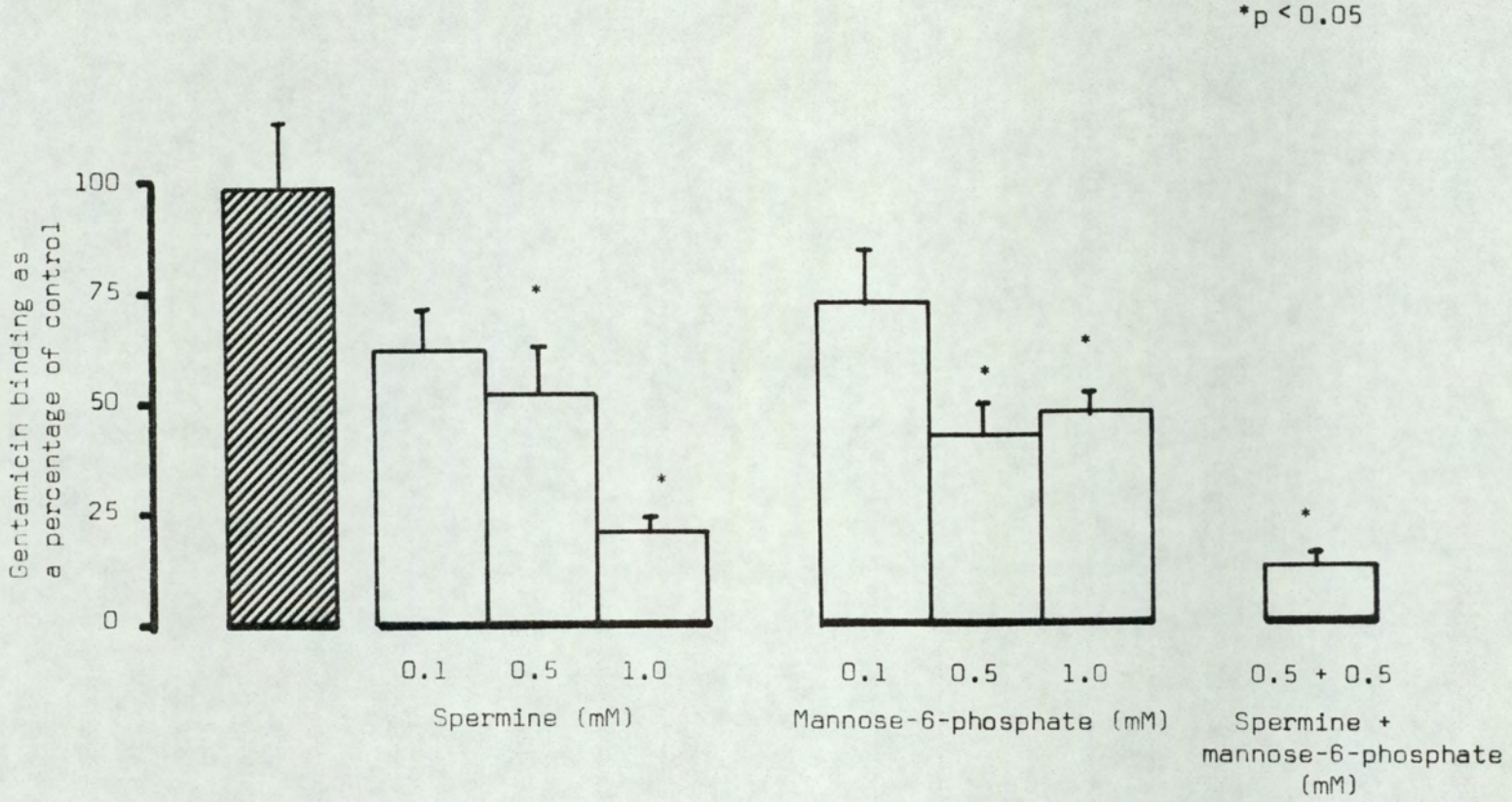


Fig. 46. Gentamicin binding to isolated renal tubules at 4°C in the absence and presence of spermine and mannose-6-phosphate. Tubules were preincubated for 15 minutes at 4°C with various concentrations of spermine and mannose-6-phosphate before addition of $|^3H|$ gentamicin (0.5 µCi/ml) plus unlabelled gentamicin at a final concentration of 10^{-4} M and incubated further at 4°C for 30 minutes. Each bar represents the mean ± s.e.m. of experiment done at least in triplicate.

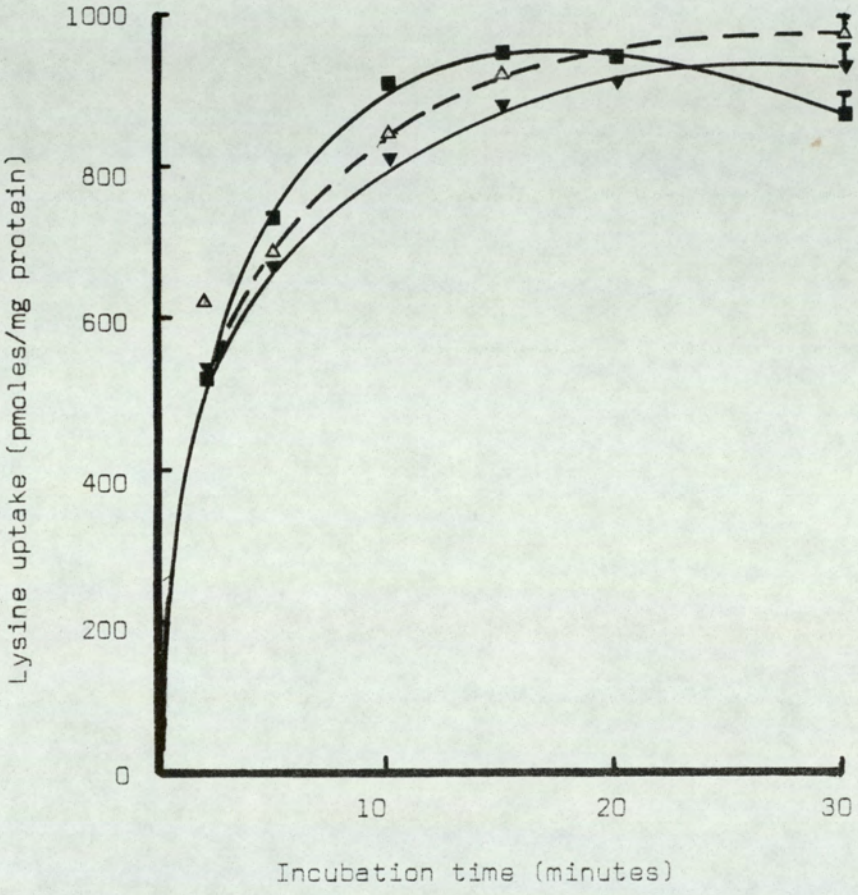


Fig. 47. The uptake of lysine in the presence of gentamicin by isolated rat renal tubules at 37°C. Tubules were incubated with [^{14}C] lysine (0.1 µCi/ml) plus unlabelled lysine at a final concentration of 0.065 mM in the absence (△) and presence of two concentrations of gentamicin 10^{-4} M (▼) and 10^{-3}M (■). Each point represents the mean ± s.e.m. of experiment done in triplicate.

4.5.2.11 Effect of gentamicin on gluconeogenesis

Fig. 48 shows the time course of gluconeogenesis by tubules at 37°C in the absence and presence of gentamicin (5×10^{-4}). Incubations of tubules under control conditions resulted in a linear rate of gluconeogenesis up to 60 minutes followed by a decline in rate on further incubation. Gentamicin affected gluconeogenesis after 30 minutes with inhibitions of approximately 24% after 60 minutes.

The effect of higher concentrations of gentamicin (5×10^{-3} M and 10^{-3} M) on gluconeogenesis was also studied (Fig. 49). The time course of gluconeogenesis shows that 5×10^{-3} M gentamicin exerted its effect very early on with plateauing of glucose production after 30 minutes of incubation. The inhibition after 60 minutes was 56% of the control incubation. Lower concentration of gentamicin (10^{-3} M) was not as effective with inhibition of gluconeogenesis at 60 minutes of 21% compared to control.

4.5.2.12 Effect of gentamicin on leucine incorporation into TCA insoluble material

The effect of gentamicin on protein synthesis as measured by incorporation of $|^{14}C|$ leucine into TCA insoluble tubular material is shown in Fig. 50. The time course of $|^{14}C|$ leucine incorporation was linear up to 2 hours of incubation and there was no statistically significant difference between the control and gentamicin (10^{-4} M) treated tubules.

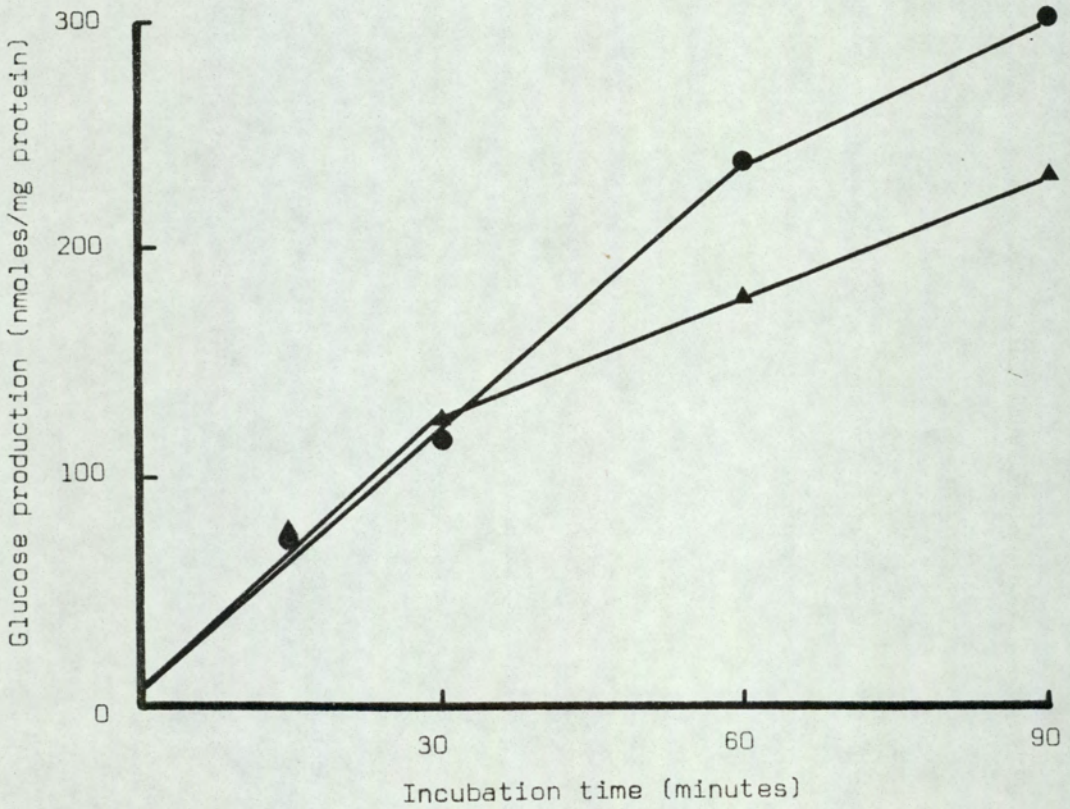


Fig. 48. The time course of gluconeogenesis as measured by production of glucose in isolated rat renal tubules in the absence (●) and presence of gentamicin at a concentration of 5×10^{-4} M (▲). Each point represents the mean of experiment done in triplicate.

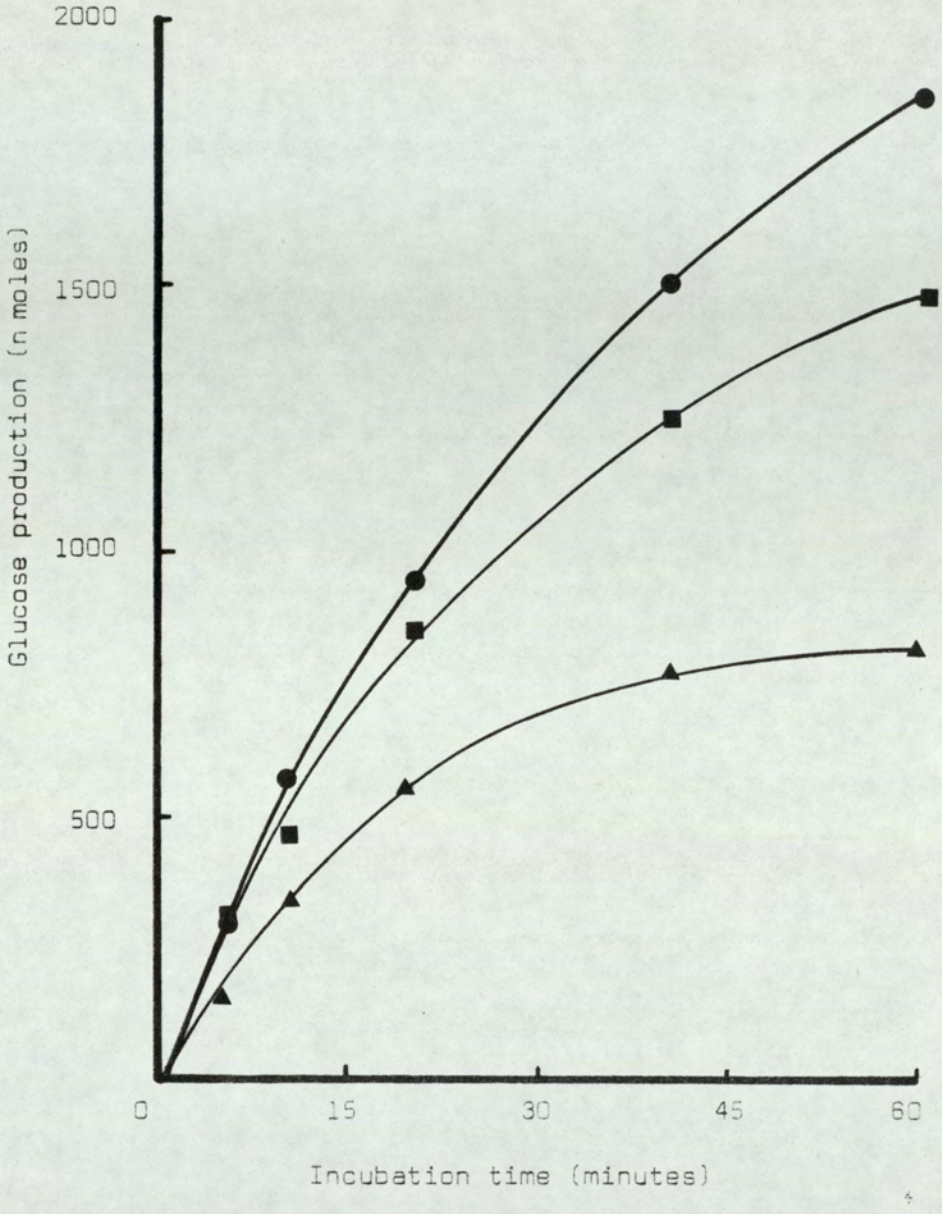


Fig. 49. The time course of gluconeogenesis in the absence and presence of various concentrations of gentamicin by isolated rat renal tubules at 37°C. The tubules were incubated in a final volume of 20 ml in the absence (●) and presence of gentamicin at concentrations of 5×10^{-3} M (▲) and 10^{-3} M (■). At given times 0.5 ml aliquots in duplicate were removed and assayed for glucose. Each point represents the mean of one experiment done in duplicate.

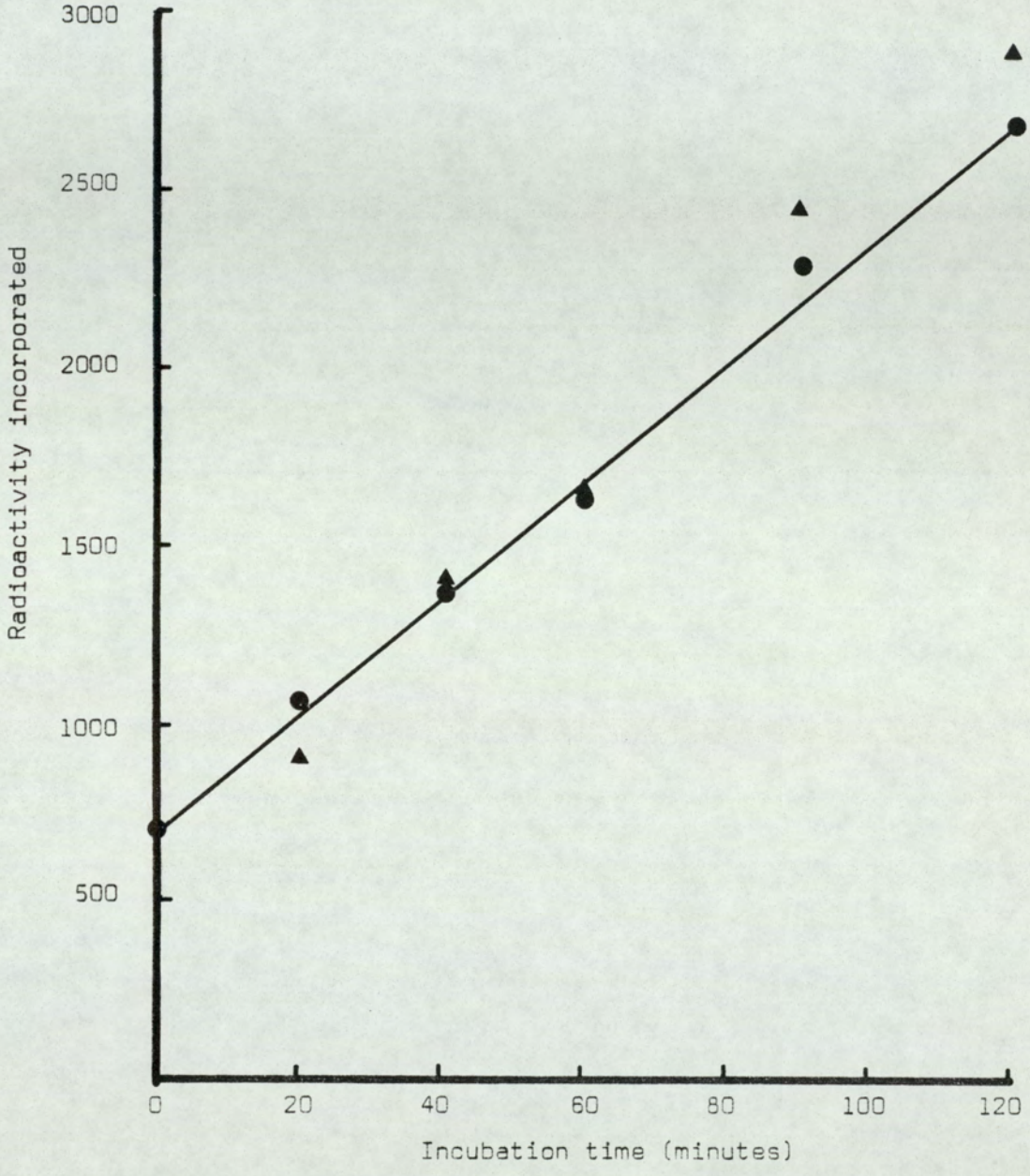


Fig. 50. The effect of 10^{-4} M gentamicin on the incorporation of $|^{14}C|$ leucine into TCA insoluble material by isolated rat renal tubules. Tubules were incubated with $|^{14}C|$ leucine (0.25 µCi/ml) plus unlabelled leucine to give a final concentration of 0.25 mM in the absence (●) and presence (▲) of 10^{-4} M gentamicin. Each point represents the mean of experiment done in duplicate.

4.5.3 Discussion

In the previous section (4.4) the preparation of isolated rat renal tubules was described. These tubules were shown to be viable in terms of morphology as seen by phase contrast and electron microscopy, ability to carry out complex metabolic functions such as gluconeogenesis and protein synthesis and also had the capacity to accumulate the basic amino acid lysine.

In the present experiments the tubule preparation showed rapid initial uptake of gentamicin followed by accumulation at a slower rate when tested at two concentrations of gentamicin (10^{-4} M and 10^{-5} M). A similar pattern of uptake has been reported by Barza, Murray and Hamburger (1980) who studied uptake of gentamicin into isolated viable renal tubules from rabbits.

The initial rapid phase of uptake of gentamicin, observed in this experiment may conceivably have been due to a non-energy mediated process such as binding of the drug to tubular cell membrane. In order to establish whether this uptake of gentamicin was to an extent energy dependent, the effect of application of various inhibitors, including anoxia, DNP, KCN, iodoacetic acid and ouabain was studied.

It can be seen from Fig. 39, which shows the effect of DNP and anoxia on the time course of gentamicin uptake that anoxia had a marked inhibitory effect on gentamicin uptake compared to DNP.

Figs. 40 - 42 show that, of the metabolic inhibitors, 2,4-DNP, KCN and iodoacetic acid used, DNP was the most effective in inhibiting gentamicin uptake after 60 minutes of incubation at 37^{o}C. At a maximal concentration of 2 mM used in these experiments the

inhibition of gentamicin uptake by 2,4-DNP, KCN and iodoacetic acid was respectively 87%, 38% and 0%.

The Na^+-K^+ ATPase inhibitor, ouabain, also produced a statistically significant reduction in gentamicin uptake (75% of control) at a concentration of 2 mM.

It appears from these data that the uptake can be best explained by postulating a metabolically active system for the transport of gentamicin. The results with DNP, KCN and ouabain suggest that mitochondrial oxidative phosphorylation as well as Na^+-K^+ ATPase may be involved. It could be argued that the effect of inhibitors seen in these experiments may have been due to result of cell damage. This seems unlikely on the basis that cell damage is generally expected to lead to leakiness and/or non-specific stickiness and an increased, rather than decreased, concentration of gentamicin (Lutz, Wyatt and Bargatze, 1978).

The gross effect of anoxia may have been due to damage of tubules as suggested by Barza et al. (1980) whose preparation showed bulging of tubular ends and uptake of trypan blue. However, this tends to contradict the above hypothesis of Lutz et al. (1978) and is difficult to interpret.

The results obtained with this tubule preparation were very similar to the kidney slice system discussed earlier in section 4.3. The one contradictory observation made was the significant effect of ouabain at 2 mM which was not seen with kidney slices.

The efflux of gentamicin from the preloaded tubules followed a biphasic pattern, when these tubules were reincubated in gentamicin-free medium. The efflux was initially rapid but this was followed by a much slower loss. The rate of efflux was not increased by the presence of high concentrations of the unlabelled gentamicin

in the medium. After incubating the tubules in fresh medium for 30 minutes, approximately 30% of the gentamicin originally taken up was still present in the tubules. Barza et al. (1980) observed a similar pattern of gentamicin efflux from rabbit tubules, although almost 60% of the gentamicin initially taken up was tightly bound. These differences may have arisen because they used longer preloading incubation periods.

The experiments so far have indicated that gentamicin is transported into the cells of the rat renal tubules and to be tightly bound to some particulate fraction of the tubule.

Silverblatt and Kuehn (1979) and Tulkens and Trouet (1978) have speculated that gentamicin is taken up by a pinocytotic mechanism. Silverblatt and Kuehn (1979) showed by autoradiography that gentamicin was transported into the proximal tubule across the luminal membrane by pinocytosis and later sequestered into lysosomes. A similar pattern of uptake was also found by Tulkens and Trouet (1978) using cultured rat fibroblasts as a model system for gentamicin uptake.

Although, usually only large macromolecules are transported by pinocytosis, there may be at least three ways in which the relatively lower molecular weight gentamicin molecule might also be transported by this mechanism.

(i) Gentamicin in solution may enter the pinocytotic vesicles in the small amount of luminal fluid which is enclosed as the pinocytic invagination separates from the surface. The amount of gentamicin accumulated by this fluid-phase uptake, as shown by Tulkens and Trouet (1974) with fibroblasts, should vary linearly with the concentration of marker in the extracellular fluid and should increase continuously with time as long as the

cell is not digesting and/or releasing the label. However, this hypothesis does not fit in too well with the observations made with macrophages. The rate of uptake of dihydrostreptomycin (Bonventre and Imhoff, 1970) by macrophages was comparable to that observed for fibroblasts although pinocytic rate is much greater and secondly no difference was observed in uptake of streptomycin between phagocytozing and non-phagocytozing macrophages.

(ii) Gentamicin may be bound to a protein undergoing pinocytosis and thus may enter the pinocytic vesicles associated with it. This seems unlikely since gentamicin binds only minimally, if at all, to serum proteins (Gordon, Regamey and Kirby, 1972). Also, in the absence of glomerular damage, the amount of protein in the lumen will be negligible.

(iii) Gentamicin binding directly to the 'receptor' site of the pinocytotic invagination. Such an interaction could be mediated by the electrostatic attraction of the positively charged gentamicin with negatively charged 'receptor'.

The evidence of very rapid initial uptake of gentamicin, suggested some form of binding process. Also, with the knowledge that formation of pinocytic vesicles and hence fluid-phase uptake is not detectable at $4^{o}C$ (Steinman, Silver and Cohn, 1974) whereas adsorption to cell surface binding sites occurs at $4^{o}C$ (Goldstein and Brown, 1974), the binding of gentamicin to tubules at $4^{o}C$ was studied. The binding of gentamicin as shown in Fig. 44 was saturable with time and a Scatchard plot of binding of gentamicin at various concentrations, showed that there may be more than one class of binding sites with different affinities on the proximal tubules.

There is evidence of saturability of gentamicin accumulation *in vivo* as well. Luft and Patel (1979) found that at high doses,

the gentamicin concentration in renal cortical tissue of rats plateaued after 5 days, whereas it took 8 days at a lower dose. This suggested a time and concentration dependent saturation of available binding sites in the renal cortex.

If the charge on gentamicin was the most important factor governing its interaction, suggesting the presence of anionic molecules, to the binding sites than the effect of cationic polyamines on gentamicin binding to tubules was of some interest. Kornguth, Bayer and Kunin (1980) showed that polyamines with three or more amino groups decreased binding of gentamicin to subcellular fractions of kidney. In fact, spermine, a naturally occurring polyamine in all mammalian tissue (Tabor and Tabor, 1964) has been shown when administered parenterally to be strongly nephrotoxic to man and experimental animals (Rosenthal, Fisher and Stohlman, 1952). In the present study, spermine in the concentration range 0.1 mM - 1.0 mM inhibited gentamicin binding to tubules at $4^{o}C$ by about 80% at 1.0 mM (Fig. 46).

Another possible site for gentamicin binding was the 'mannose/N-acetylglucosamine' receptor which has been tentatively linked to the lysosomal system and the transport of lysosomal enzymes, principally on the basis that many lysosomal enzymes have mannose or N-acetylglucosamine at their terminal ends (Stahl and Schlesinger, 1980).

The possible functions implicated for this receptor are:

(i) intracellular recognition and transport of newly synthesised lysosomal hydrolases within the endoplasmic reticulum.

(ii) within lysosomes, to retain and prevent loss of hydrolases to the extracellular compartment.

(iii) and lastly, as a membrane bound retrieval system for lysosomal enzymes which, for various physiological and/or pathological reasons, make their way into extracellular space.

Thus, it is possible for the aminoglycosides to be taken up into lysosomes by the lysosomal enzyme retrieval mechanism. Also, in view of the strong connection between gentamicin uptake and its interaction with lysosomes, the effect of mannose-6-phosphate, the terminal sugar of lysosomal enzymes, on binding of gentamicin to tubules at $4^{o}C$ was examined. Mannose-6-phosphate, in the concentration range 0.1 - 1.0 mM, inhibited binding of gentamicin to tubules by about 50% at 1 mM (Fig. 46). It has been shown using alveolar macrophages that the binding of lysosomal enzyme to the receptor is sensitive to trypsin treatment (Stahl, Rodman Miller *et al.*, 1978). In this study trypsin treatment also reduced the binding of gentamicin to tubules to 43% on preincubating tubules with trypsin for 30 minutes (Table 6).

Although spermine had a greater inhibitory effect than mannose-6-phosphate, a receptor based on the latter would be a more likely candidate for a binding site *in vivo*. Spermine based receptor appears to be very general and non-specific since the factor governing drug-receptor interaction is principally the charge on the molecule. However, in order to explain the specificity of aminoglycoside toxicity, factors other than charge must also be important, such as the glycosidic structures of aminoglycosides and mannose-6-phosphate.

An alternative hypothesis to endocytosis that has been suggested for gentamicin uptake is the entry of gentamicin into renal tubular cells by facilitated transport. The organic base

transport system and the mechanism responsible for reabsorption of amino acids are two mechanisms which have been proposed for such a role (Whelton, Carter, Craig et al., 1978).

Whelton et al. (1978) reported reduction of cortical uptake of tobramycin on intravenous infusion of amino acids into dogs. In the present experiments, gentamicin (10^{-4} M and 10^{-3} M) had no effect on the time course uptake by tubules of basic amino acid lysine (Fig. 47). This finding is similar to that of Kluwe and Hook (1978), who found no alterations of α-aminoisobutyrate transport into cortical slices of rats injected with gentamicin for 2 or 4 days with 100 mg/kg/day gentamicin.

Gentamicin, having gained entry into the proximal tubular cell may exert its effect on some of the processes occurring within the cell. The effect of gentamicin on gluconeogenesis and protein synthesis was studied, since:-

(i) the renal proximal tubules have very high gluconeogenic activity (section 4.4).

(ii) gentamicin has been shown to decrease messenger ribonucleic acid-directed protein synthesis in bacterial systems by binding with ribosomes and causing a misreading of the genetic code (Hahn and Sarre, 1969).

Gluconeogenesis by isolated tubules was reduced by gentamicin in the concentration range 10^{-4} M - 5×10^{-3} M. The inhibition after 60 minutes incubation period was 56% at gentamicin concentration of 5×10^{-3} M and approximately 20% with 10^{-3} M and 10^{-4} M gentamicin compared to control incubations (Figs. 48 and 49). Glucose formation from the exogenous substrates such as lactate and pyruvate is dependent upon the uptake of these substrates into the

mitochondria, where their interaction with the tricarboxylic acid cycle results in the formation of malate which crosses out from mitochondria to the cytoplasm where it is converted to oxaloacetate and finally to glucose.

It is conceivable that disruption of mitochondrial function by gentamicin could explain the decrease in glucose production. Alternatively, if the mitochondria were functionally intact then the rate-limiting step in gluconeogenesis catalysed by phosphoenol-pyruvate carboxykinase may be effected by gentamicin. It is localised exclusively to the proximal tubule in the rat kidney (Guder and Schmidt, 1974) and it carries out the decarboxylation and phosphorylation of oxaloacetate to phosphoenolpyruvate. Inhibition of gluconeogenesis has also been cited by Kluwe and Hook (1978), in cortical slices from preinjected rats.

Protein synthesis was unaltered in tubules incubated with gentamicin (10^{-4} M) for up to 2 hours at 37^{o}C. Roman, Krishnakantha and Cuppage (1975) also found no difference in protein synthesis in rats injected acutely with gentamicin in the dose range 0 - 160 mg/kg. Stockhorst and Schacht (1977), evaluating the mechanism of aminoglycoside ototoxicity, found that neomycin had no effect on $|^{3}H|$ leucine incorporation into protein during perilymphatic perfusion of the cochlea. Thus, the mechanism of action of gentamicin does not seem to be at the level of ribosomes as it is in sensitive bacteria (Hahn and Sarre, 1969).

SUMMARY

The uptake of radiolabelled gentamicin in separated, viable renal tubules prepared by enzymatic digestion of rat kidneys was studied. The preparation showed rapid initial uptake of gentamicin

followed by a continued slower uptake. The accumulation was significantly inhibited by anoxia, 2,4-DNP, KCN and ouabain. Binding studies at $4^{o}C$ suggested presence of possible 'receptors' with mannose-6-phosphate being a more likely candidate than spermine. Gentamicin, once inside the cell may exert its toxic effect by inhibiting gluconeogenesis. It had no effect on protein synthesis carried out by the tubules.

4.6 IN VITRO LYSOSOMAL ENZYME RELEASE FROM ISOLATED RAT RENAL TUBULES

4.6.1 Introduction

Numerous experiments have provided evidence to suggest a close association between the lysosomes of the proximal tubular cell and aminoglycoside nephrotoxicity.

Morphological studies and biochemical studies of urinary enzyme excretion in animals and man have indicated that aminoglycoside administration results in damage of the proximal tubule lysosomes. The early features of gentamicin nephrotoxicity are an increase in the number and/or volume of lysosomal structures, the formation of myeloid bodies with characteristic 'lamellae' within these structures (Kosek et al., 1974), and in the urinary sediment (Harrison, Silverblatt and Turek, 1975), and urinary excretion of lysosomal enzymes (Wellwood, Simpson, Tighe et al., 1975).

The pathological significance of these changes in the proximal tubular cell is unknown. Kosek et al. (1974) have speculated that intracellular gentamicin might enhance autophagocytic activity in these cells. The myeloid bodies would result from binding of drug to various intracellular membranes, which are subsequently sequestered in lysosomes by autophagy, where the drug-complexed membranes resist the action of lysosomal lipases or phospholipases and accumulate. Tulkens et al. (1979) reported evidence of gentamicin-induced lysosomal phospholipidosis in cultured rat fibroblasts through dysfunction of sphingomyelinase and probably other phospholipases.

Autographical evidence has suggested that gentamicin is taken up into proximal tubular cells by endocytosis and later sequestered into the lysosomes. Harrison et al. (1975) have suggested that gentamicin acted directly on lysosomes, because other renal cell

organelles were not damaged unless extremely high and prolonged doses were used. Harrison *et al*. (1975) suggested that myeloid bodies may occur through interaction of basic substances like gentamicin with acidic lipoproteins of the lysosomal membrane.

Thus, in response to an early appearance of lysosomal enzymes in urine *in vivo* (section 4.7.1) and the morphological changes discussed above, the effect of incubating gentamicin with proximal tubules on the release of lysosomal enzymes acid phosphatase (AP) and N-acetyl-β-glucosaminidase (NAG) was studied.

4.6.2 Results

4.6.2.1 Paranitrophenol calibration curve in the absence and presence of gentamicin (10^{-3} M)

Fig. 51 shows the calibration curve of p-nitrophenol in the range 0 - 30 µg in the same volume as the incubation volume. The curve was found to be linear within this range and addition of gentamicin (10^{-3} M) did not affect the absorbance of p-nitrophenol at OD_{410}.

4.6.2.2 Release of total lysosomal enzyme activity - reproducibility of freezing and thawing of tubules.

The release of enzymes by freezing and thawing of the isolated rat renal tubules was found to be very reproducible (0.748 ± 0.029 n = 12). The tubules were frozen and thawed three times in liquid nitrogen and the reproducibility ascertained by measuring the lysosomal enzyme NAG.

4.6.2.3 Stability of released AP and NAG on incubating at 37°C

(i) Time dependence

NAG was much more stable than AP on incubating at 37°C for up to 2.5 hours (Fig. 52). After 60 minutes of incubation, there was a 60% decline in the activity of AP followed by a more gradual decline on further incubation. NAG activity remained constant during the 2.5 hours incubation period at 37°C.

(ii) In the absence and presence of gentamicin (10^{-3} M)

Fig. 53 shows that the presence of gentamicin (10^{-3} M) did not significantly effect the activity of NAG throughout the 2.5 hours of incubation period. However, AP activity was increased over the control activity in the presence of gentamicin (10^{-3} M) by about

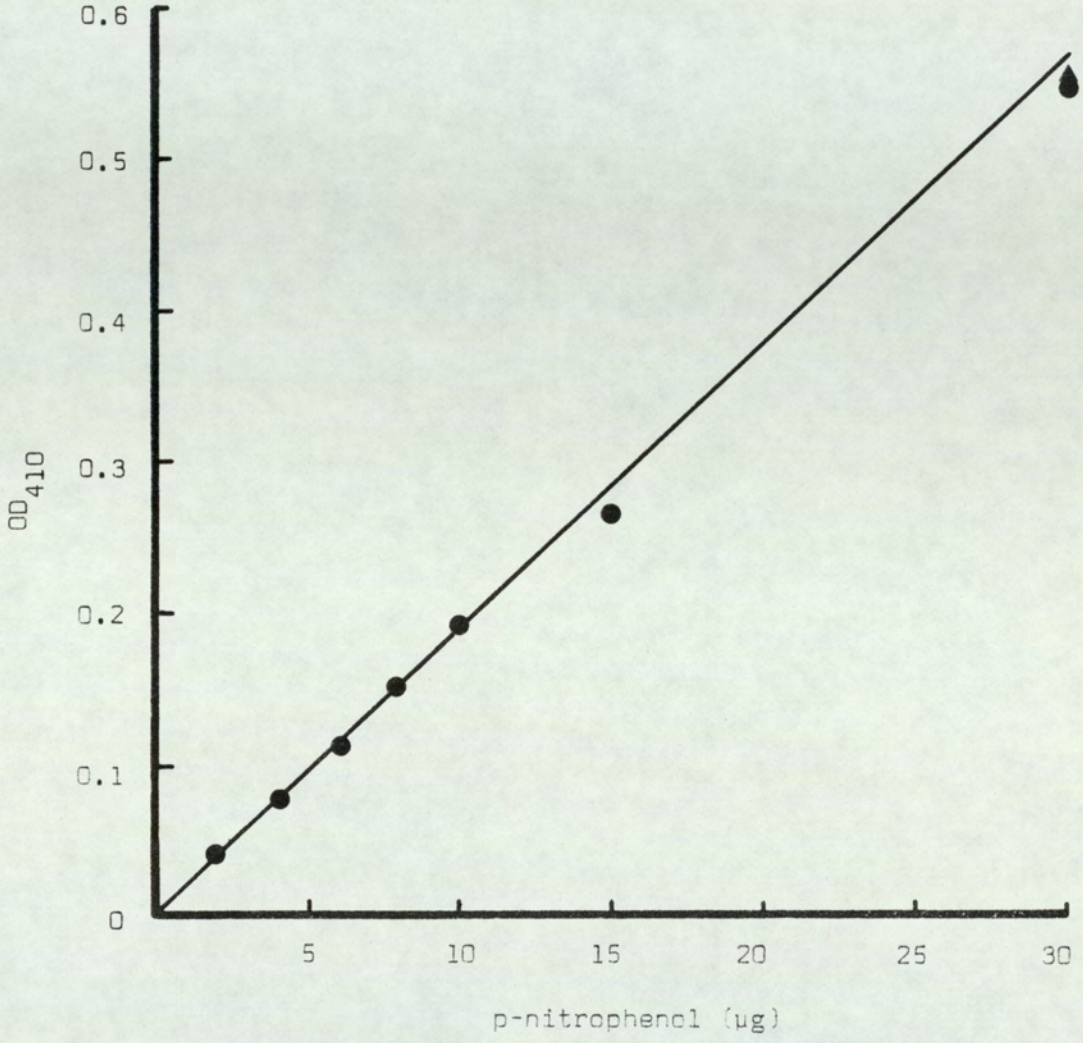


Fig. 51. Calibration curve of p-nitrophenol in the absence (●) and presence (▲) of gentamicin (10^{-3} M). The volume of this was the same as the incubation volumes. At low amounts of p-nitrophenol the points are superimposed.

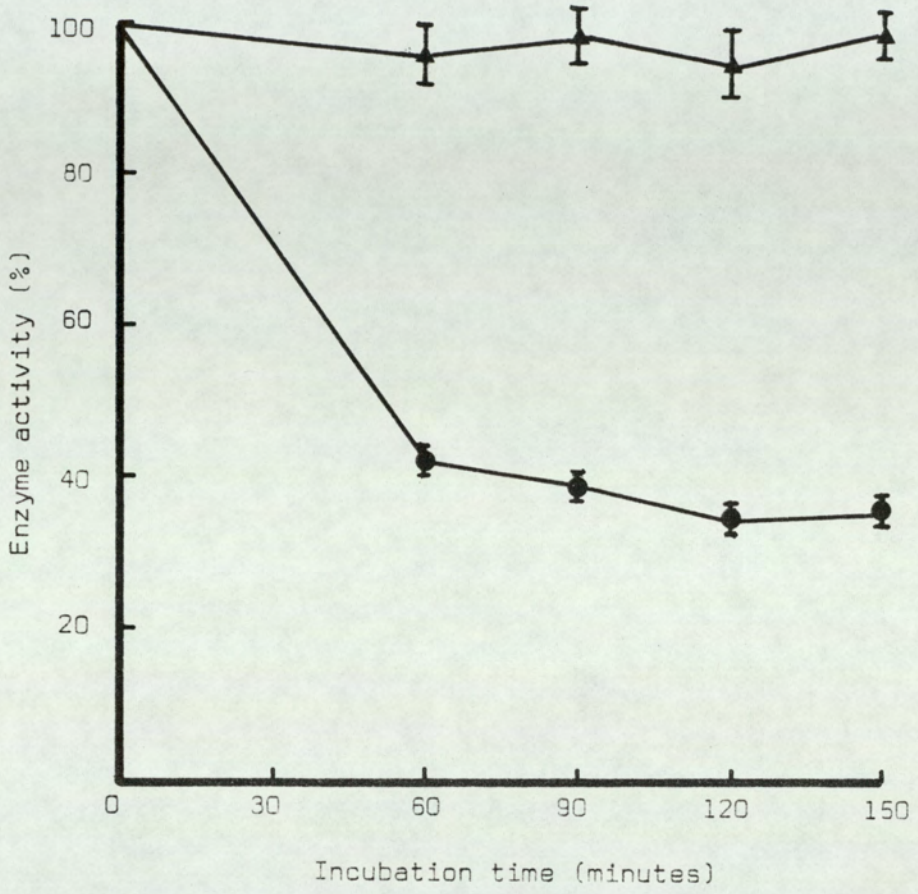


Fig. 52. Stability of free AP (●) and NAG (▲) during incubation at 37°C for up to 2.5 hours. Activities at each time are expressed as a percentage of the activity at zero time which is set to 100%. Each point is the mean ± s.e.m. of two experiments each done in triplicate.

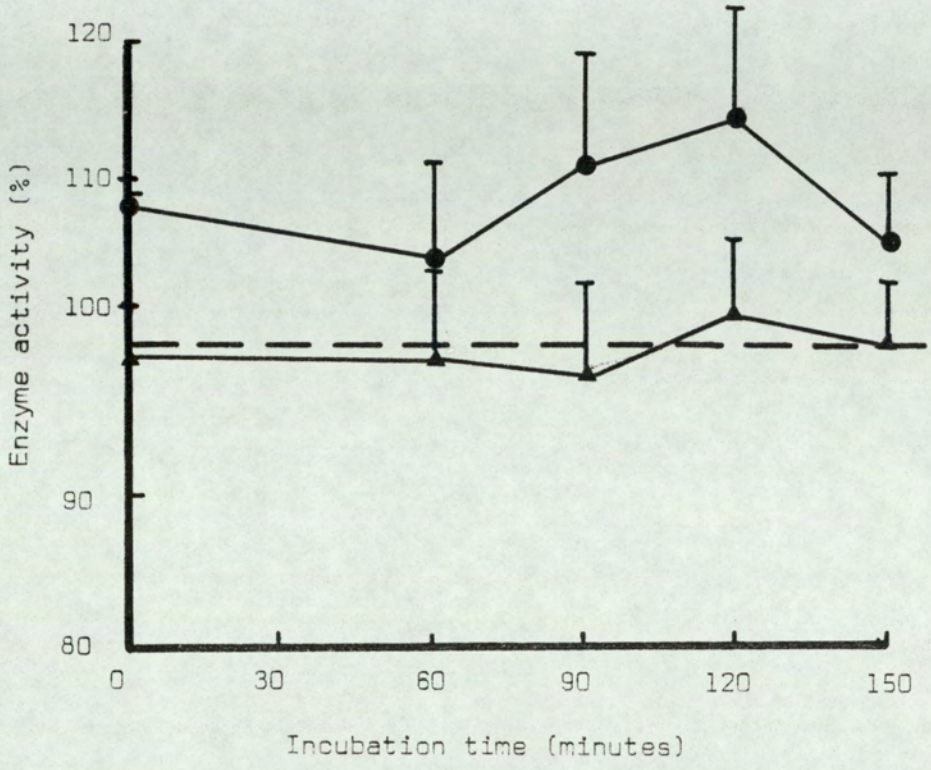


Fig. 53. Stability of free AP (●) and NAG (▲) during incubation at 37°C for up to 2.5 hours in the presence of 10^{-3} M gentamicin. Activities at each time are expressed as a percentage of the control activity at that time which is set to 100%. Each point is the mean ± s.e.m. of two experiments each done in triplicate.

15% throughout the incubation period. As a control, the effect of gentamicin on the assay was also studied using commercial acid phosphatase. No statistical significant difference was found.

4.6.2.4 Pattern of lysosomal enzyme release with gentamicin

(i) Release of AP and NAG with time

The effect of gentamicin (10^{-3} M) on the release of lysosomal enzymes from isolated rat renal tubules incubated for 2.5 hours at $37^{o}C$ is shown in Fig. 54. The release of both AP and NAG was reduced compared to the control after 60 minute incubation ($p < 0.01$). On further incubation this effect was reversed reaching control values after 120 minutes and at 150 minutes the release of NAG was elevated above the control value ($p < 0.05$). AP was also elevated above the control after 150 minutes but was not statistically significant.

(ii) Release of AP and NAG - dependence on tubular protein concentration

The influence of gentamicin (10^{-3} M) on AP and NAG release was found to be a function of the gentamicin : protein concentration ratio (Fig. 55). Whereas after 60 minutes, gentamicin caused inhibition of AP to 71% ($p < 0.01$) and NAG to 61% ($p < 0.05$) at a protein concentration of 0.2 mg/ml, there was no effect at higher protein concentrations (0.6 - 0.8 mg/ml).

(iii) Release of AP and NAG - gentamicin concentration dependence

The dependence of AP and NAG release on the concentration of gentamicin in the incubation medium was also investigated (Fig. 56). After 60 minutes incubation at very low concentrations of gentamicin (10^{-9} - 10^{-8} M) there was no difference in the release of enzymes over the control. However, at intermediate concentrations of gentamicin (10^{-7} - 10^{-5} M) the release of AP and NAG was greater

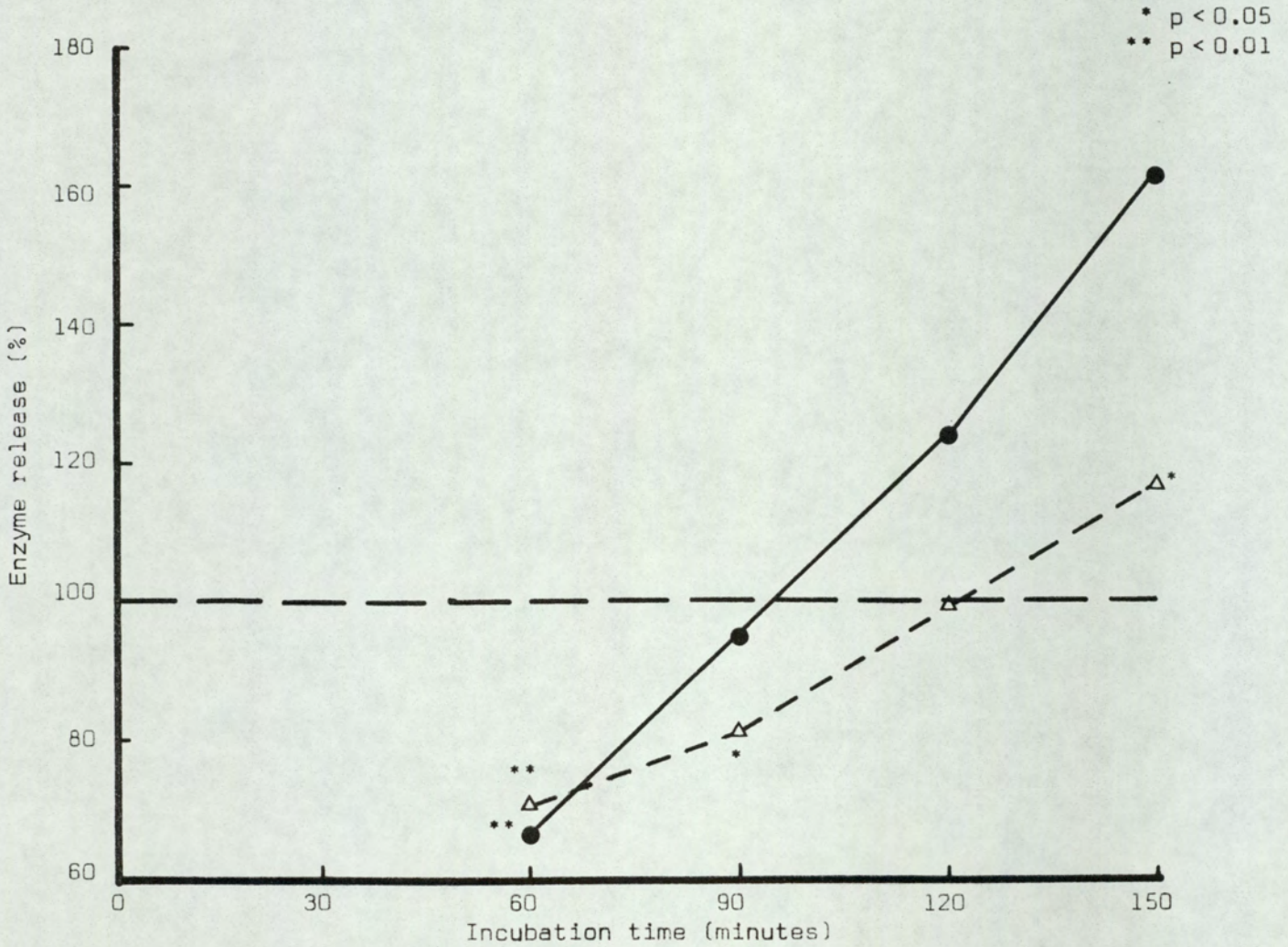


Fig. 54. Effect of gentamicin (10^{-3} M) on the release of AP (●) and NAG (△) from rat renal tubules incubated for 2.5 hours at 37°C. The release of enzymes in the presence of gentamicin is expressed as a percentage of the release in the absence of gentamicin (control set at 100%) at any given time. Each point represents the mean of three experiments, each done in triplicate.

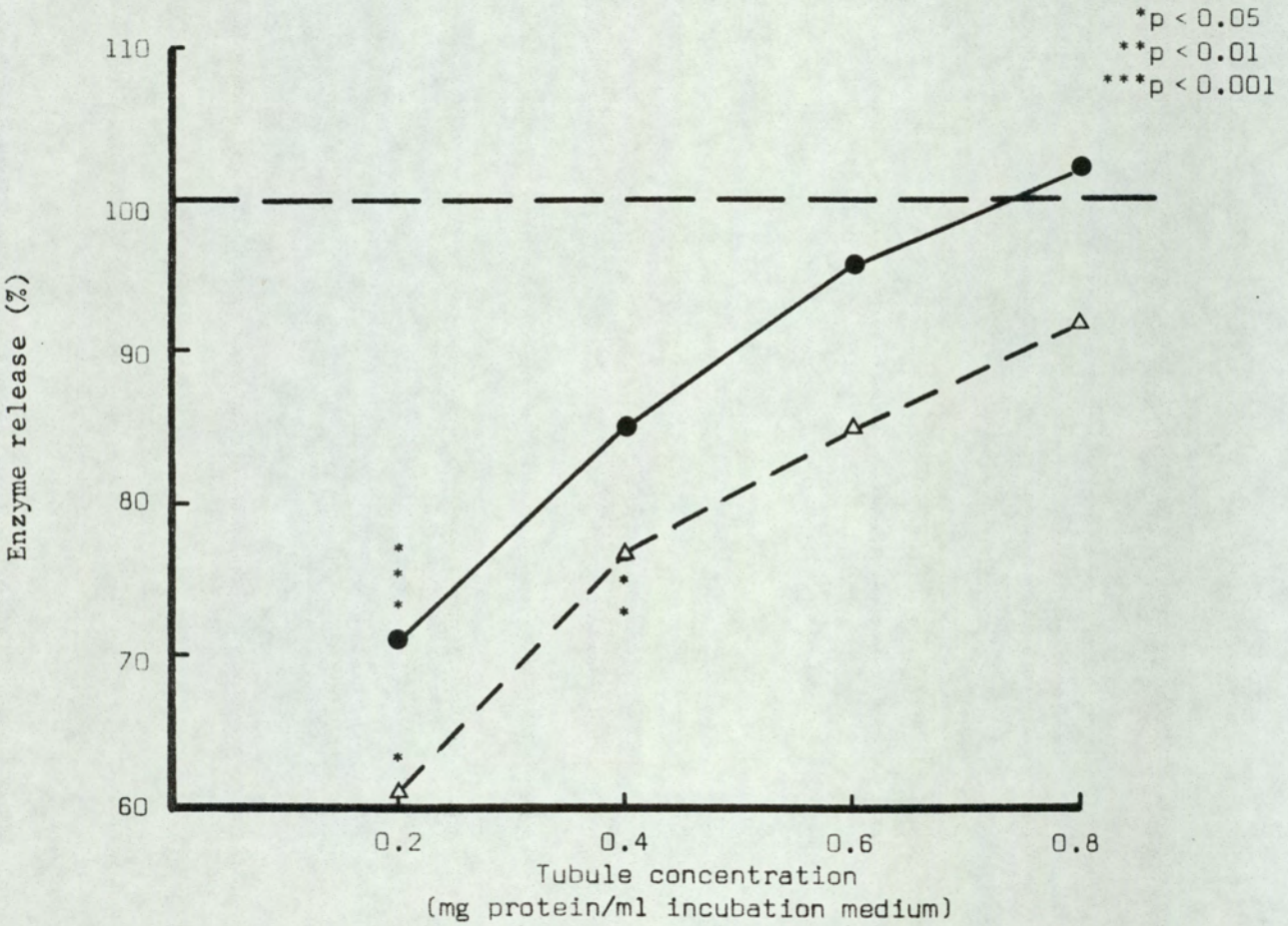


Fig. 55. Effect of gentamicin (10^{-3} M) on the release of AP (●) and NAG (Δ) from various concentrations of isolated rat renal tubules incubated for 60 minutes. Each point is the mean of three experiments, each done in triplicate.

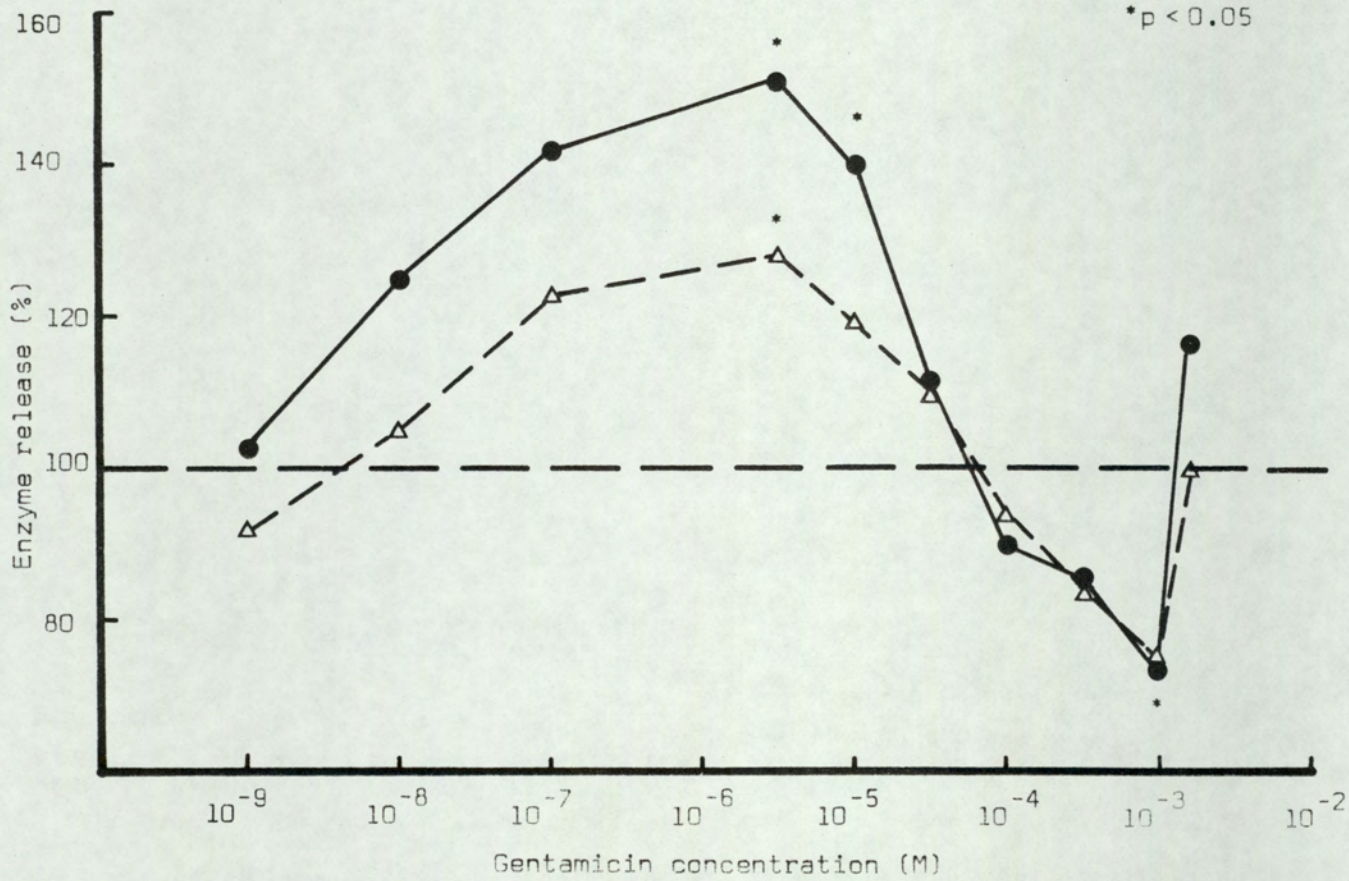


Fig. 56. Effect of gentamicin concentration on the release of AP (●) and NAG (Δ) from isolated rat renal tubules incubated for 60 minutes at 37°C. Each point is the mean of three experiments, each done in triplicate.

than the control. At 5×10^{-6} this elevation was significant ($p < 0.05$). This effect was reversed at higher concentrations of gentamicin and the release was maximally inhibited at 10^{-3} M ($p < 0.05$).

4.6.3 Discussion

The effect of gentamicin on the release of AP and NAG from isolated tubules was investigated over a 2.5 hour incubation period. During this incubation at the concentration studied (10^{-3} M) the maximal effect was observed at 60 minutes (AP = 67% and NAG = 71% of control enzyme activity). On further incubation this effect was reversed and at 150 minutes the enzyme release in the presence of the drug was AP = 162% and NAG = 117% of the control value. The dependence of AP and NAG release on the concentration of gentamicin in the incubation medium was also investigated. After 60 minutes incubation, gentamicin in the concentration range 10^{-7} M - 10^{-5} M increased the release of AP and NAG over the control. The elevation being significant at 5×10^{-6} M ($p < 0.05$). This effect was reversed at higher concentrations of gentamicin and the release was maximally inhibited at 10^{-3} M ($p < 0.05$). The influence of gentamicin on the lysosomal enzyme release was also found to be a function of the gentamicin : protein concentration ratio.

In comparing the release of AP and NAG from the tubules, the former enzyme showed the greatest variability in release and this in some way may be related to its greater instability compared to NAG. On incubating free AP and NAG at 37°C for up to 2.5 hours, NAG was much more stable than AP. After 60 minutes of incubation, there was a 60% decline in the activity of AP followed by a more gradual decline on further incubation. NAG activity remained constant during the 2.5 hours incubation period at 37°C (Fig. 52). Also, the presence of gentamicin did not significantly effect the activity of NAG throughout the 2.5 hours of incubation period. However, AP activity was increased over the control activity by

about 15% throughout the incubation period (Fig. 53). Thus, NAG being more stable, less variable and unaffected by gentamicin was chosen as the enzyme to follow.

The results of the studies of enzyme release suggest a complex interaction between gentamicin and protein concentration and the time of incubation so that different combinations can result in a decreased or increased release of enzymes compared to a control preparation.

The interpretation of these findings may be possible if the process of endocytosis and current ideas on the transport of lysosomal enzymes are considered. In section 4.5 it was suggested that the uptake of gentamicin may be via a receptor-mediated endocytosis and that the receptor may possibly be the type utilised by lysosomal enzymes.

This process of receptor-mediated endocytosis has recently become recognised as an important mechanism by which animal cells take up nutritional proteins from extracellular fluid. Biologically important molecules known to be taken up by this mechanism include plasma transport proteins (such as low density lipoprotein and transcobalamin II), certain polypeptide hormones (such as insulin and epidermal growth factors), asialoglycoproteins, and lysosomal enzymes (Goldstein, Anderson and Brown, 1979).

Noriega, Grubb, Talkad et al. (1980) have speculated from their investigations that there are two pathways for transport of lysosomal enzymes which depend on the phosphomannosyl recognition marker on acid hydrolases (mannose-6-phosphate) and its receptor. The two pathways are not alternatives but co-exist, with the second pathway being more important than the first.

The two postulated pathways are:

(i) Transport from the cell surface to lysosomes - 'secretion-recapture' hypothesis

In this first pathway, the newly synthesised enzymes are secreted into the medium, and then taken up by binding to the high affinity receptor on the cell surface. This binding which requires Ca^{2+} is followed by internalisation which requires metabolic energy. The internalisation occurs rapidly at $37^{o}C$ with the enzyme being deposited into the lysosomal compartment. The receptors are recycled back to the plasma membrane where they can participate in further uptake events.

There is evidence of exocytosis of the lysosomal enzymes from the rat kidney. Lockwood and Bosmann (1979) in their investigations demonstrated that both the isolated perfused rat kidney and the in situ organ can release approximately 20% of the total NAG activity into urine during a 24-hour period. The mechanism of the release from viable cells could consist of secretion of individual molecules or exocytosis of a common lysosomal vesicle. Some cell types are known to release the contents of the entire lysosome by fusion of the vesicle with the plasma membrane. Maunsbach (1969) using histopathological techniques has reported apparent extrusion of acid phosphatase containing vesicles at the luminal membrane of the rodent kidney.

This secretion - recapture pathway suggests several ways in which the level of intracellular and extracellular enzyme could be influenced: by an effect on the rates of secretion and internalisation, as well as in the rates of synthesis and degradation of the enzyme or receptor.

If this theory of secretion - recapture is valid then in the present study the increase in AP and NAG seen at a gentamicin concentration of 5×10^{-6} M, may have been attributed to increased exocytosis or more likely due to inhibition of internalisation by binding of gentamicin to receptor specific for lysosomal enzymes.

The inhibition of the release of AP and NAG observed at higher concentrations of gentamicin ($5 \times 10^{-4} - 10^{-3}$ M) may be related to its effect on the second pathway that has been postulated for transport of lysosomal enzymes.

(ii) Transport from the endoplasmic reticulum to lysosomes

Noriega et al. (1980) based on their observations, have suggested that most of the newly synthesised acid hydrolases are not secreted and recaptured but normally reach lysosomes without leaving the cell. The delivery of the enzymes to lysosomes depends on intracellular receptors to which bind newly synthesised enzymes bearing the phosphomannosyl recognition marker. These receptor bound hydrolases collect into specialised vesicles (primary lysosomes) that bud-off the endoplasmic reticulum or Golgi complex and deliver the hydrolases to lysosomes.

The decrease in enzyme release over the control seen at the higher concentrations of gentamicin at 60 minutes (Fig. 56) may be due to the effect of gentamicin on the transport of enzyme from the endoplasmic reticulum to the lysosomes. Interference with this pathway would result in decrease of enzymes into the lysosomes with a subsequent decrease in the release of enzymes to the medium via exocytosis.

The continued function of this intracellular pathway depends on the dissociation of enzymes from receptors in lysosomes and on

recycling of free receptors to the endoplasmic reticulum or Golgi complex. Thus, the increase in the release of enzymes seen on further incubation with 10^{-3} M gentamicin (Fig. 54) up to 2.5 hours may be due to the cisternal membrane becoming depleted of free receptors and subsequently the new synthesised hydrolases having no free receptors to bind. As a result, acid hydrolases fail to be segregated from products of the endoplasmic reticulum that are destined for export, and pass through the Golgi apparatus and are secreted.

Tulkens et al. (1979) reported uptake of gentamicin by cultured rat fibroblasts via pinocytosis followed later by sequestration into lysosomes. This resulted in a large decrease in sphingomyelinase activity which they suggested was due to gentamicin causing a net loss in the amount of sphingomyelinase within lysosomes. Either as a result of diversion of the transport of the enzyme into lysosomes or an increased degradation rate of these enzymes within lysosomes. However, Sakuragawa, N., Sakuragawa, Kuwabara et al. (1977), in their experimental model of Niemann-Pick disease in rats suggested that the reduction of sphingomyelinase activity induced by AY-9944(trans-1,4-bis(2-chloro-benzylaminomethyl)cyclohexane dihydrochloride) was due to impaired enzyme synthesis.

The effect of gentamicin (10^{-3} M) at various tubular protein concentrations can best be explained by extrapolating the ideas put forward already. At low tubular protein concentrations, each tubular cell will be exposed to high concentrations of gentamicin resulting in inhibition of lysosomal enzyme release. However, at the same gentamicin but increasing tubular protein concentration,

the effect will be reversed since the relative concentration of gentamicin that each cell is exposed to is decreased.

Chloroquine and numerous lysomotrophic amines have been shown to interfere with the maintenance of the lysosomal pH gradient and inhibit lysosomal functions (Stahl and Schlesinger, 1980). The maintenance of this transmembrane pH gradient along the endocytotic pathway may be essential for receptor retrieval. The separation of receptor from the enzyme is likely to be a pH sensitive process and thus dissipation of such a gradient may inhibit the movement of intracellular receptors. The maintenance of a distinct pH environment in lysosomes may well prove to be important to the intracellular movement and deployment of membrane receptors by cells.

Thus, the deployment of receptors explains why the uptake of gentamicin is eventually a self-limiting process, finally leading to lysosomal labilisation. The subsequent release of acid hydrolases into the cell, and eventually into the extracellular fluid are deleterious events which can contribute to propagation of cellular damage, since lysosomal enzymes include proteases which can degrade intracellular structural proteins as well as phospholipases which can disrupt cellular membrane systems.

SUMMARY

The stability studies of free AP and NAG showed that NAG was far more stable than AP on incubation at $37^{o}C$ for up to 2.5 hours in the absence and presence of gentamicin (10^{-3} M). The release of lysosomal enzymes from isolated tubules after incubation with gentamicin suggested a complex interaction between the gentamicin concentration and the time of incubation. The findings were

explained by the effect of gentamicin on receptor-recycling by a differential effect on the two lysosomal enzyme transport pathways.

The possible relationship between the pattern of enzyme release seen here in *in vitro* with that *in vivo* situation is examined in section 4.7.

4.7 GENTAMICIN INDUCED NEPHROTOXICITY IN THE RAT: COMPARISON OF FUNCTIONAL AND MORPHOLOGICAL CHANGES WITH CHANGES IN EXCRETION OF URINARY ENZYMES

4.7.1 Introduction

Aminoglycoside nephrotoxicity is manifested by proteinuria and cylindruria (presence of renal cast in the urine) with a rising blood urea nitrogen (BUN) and creatinine (Wilfert, Burke and Bloomer, 1971; Kleinknecht, Ganeval and Droz, 1973). Elevation of serum creatinine or BUN or a decrease in creatinine clearance have been used as indicators of deteriorating renal function. However, these methods are not very sensitive and largely reflect glomerular changes and are not very satisfactory if proximal tubular damage is prominent.

The renal damage is usually reversible if the aminoglycoside administration is discontinued at the first signs of renal dysfunction (Kunin, 1966; Kovnat, Labovitz and Levison, 1973). Thus, early detection of drug-induced nephrotoxicity would be desirable and potentially useful in avoiding permanent or prolonged kidney damage. Also, the reduced elimination of the drug would predispose to ototoxicity.

It has been suggested that assay of urinary enzymes may provide a convenient and sensitive means of detecting proximal tubular damage (Raab, 1972). Enzymes which are normally located in proximal tubular cells can be found in the urine of experimental animals after as few as three days of administration of aminoglycosides and several days before any changes in glomerular filtration rate (Luft, Yum, Kleit, 1976; Adelman, Counzelman, Spangler et al., 1976).

The purposes of the present investigations were to:-

(i) determine the time-course of changes incurred in order to postulate a possible primary mechanism of toxicity.

(ii) compare the excretion of urinary enzymes with indices of renal function as indicators of nephron damage induced by gentamicin in the rat.

(iii) and lastly to compare the pattern of enzyme release *in vivo* with *in vitro*. That is enzymes released in urine with those released from tubules incubated with gentamicin (section 4.6) and tubules prepared from rats injected with gentamicin (40 mg/kg/day) for 1, 3 and 5 days.

4.7.2 Results

4.7.2.1 Animals injected with gentamicin (40 mg/kg/day) for 1, 3 and 5 days

Three groups of three rats were injected daily S.C. with gentamicin (40 mg/kg/day) for either one, three or five days. A fourth group of three rats was injected with the same volume of water for five days. The rats were individually housed in metabolic cages and allowed two days to acclimatise before injections were commenced.

The increase in body weight and other measured parameters during gentamicin administration were compared to the mean ± s.e.m. obtained prior to drug injections within each group (control), using Student's t-test.

The effect of gentamicin administration on:-

(i) Body weight and food intake

Figs. 57 and 58 depict the changes in body weight and food intake of rats injected with gentamicin. Rats administered with gentamicin exhibited similar increases in body weight compared to the water-injected control group and there were no apparent changes in food intake.

(ii) Water intake and urine output

Rats injected with gentamicin for 1, 3 and 5 days showed no statistically significant changes in water intake and urine output (Figs. 59 and 60).

(iii) Osmolarity of urine

Fig. 61 shows the effect of gentamicin administration on urinary osmolarities. In general there were no changes in osmolarities observed, except for the group injected with gentamicin

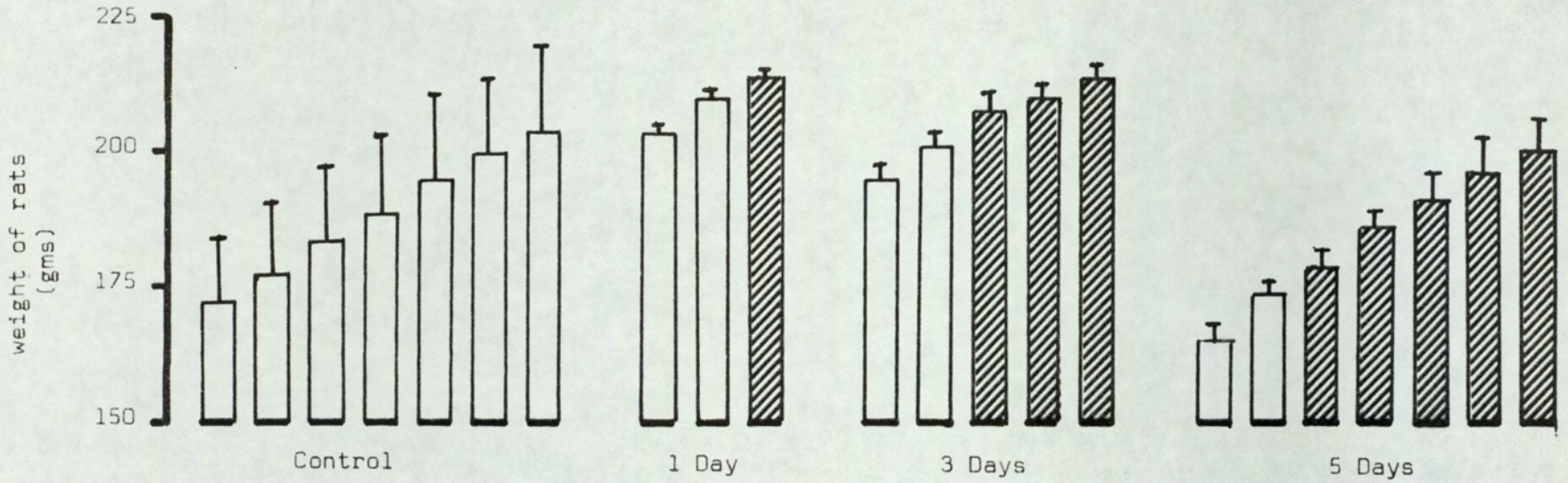


Fig. 57. Effect of saline (control) or 1, 3 and 5 days of gentamicin (40 mg/kg/day) administration on body weight. In this and subsequent histograms in this subsection the unshaded bars are control and each represents the mean ± s.e.m. of 3 rats.

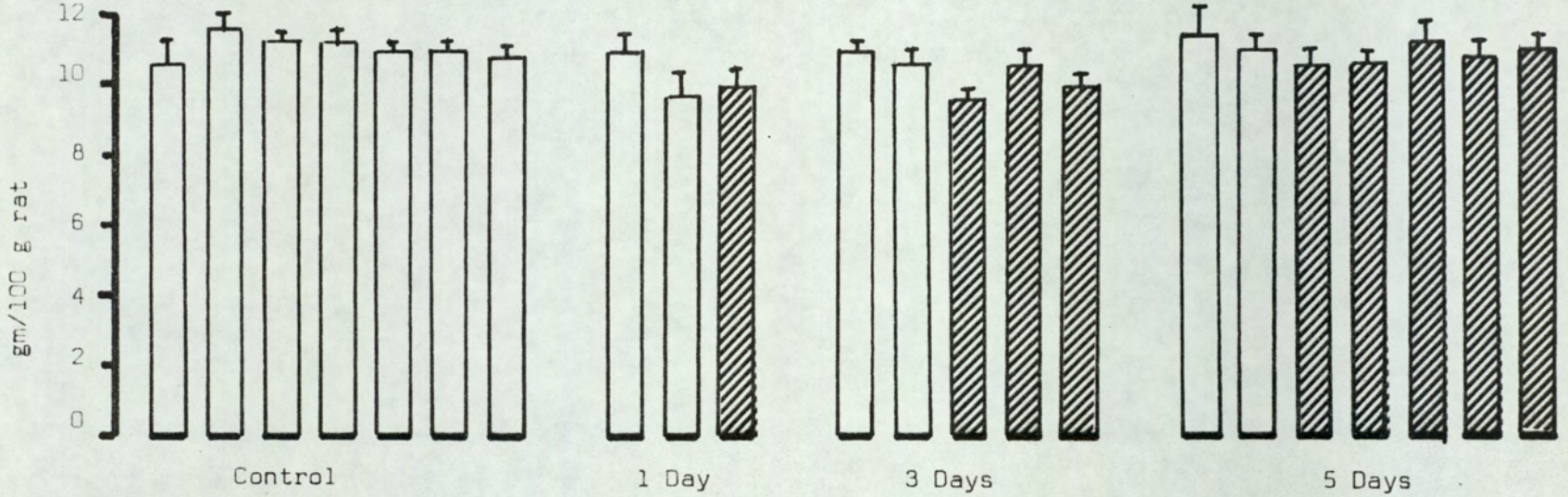


Fig. 58. Effect of saline (control) and 1, 3 and 5 days of gentamicin (40 mg/kg/day) administration on food intake.

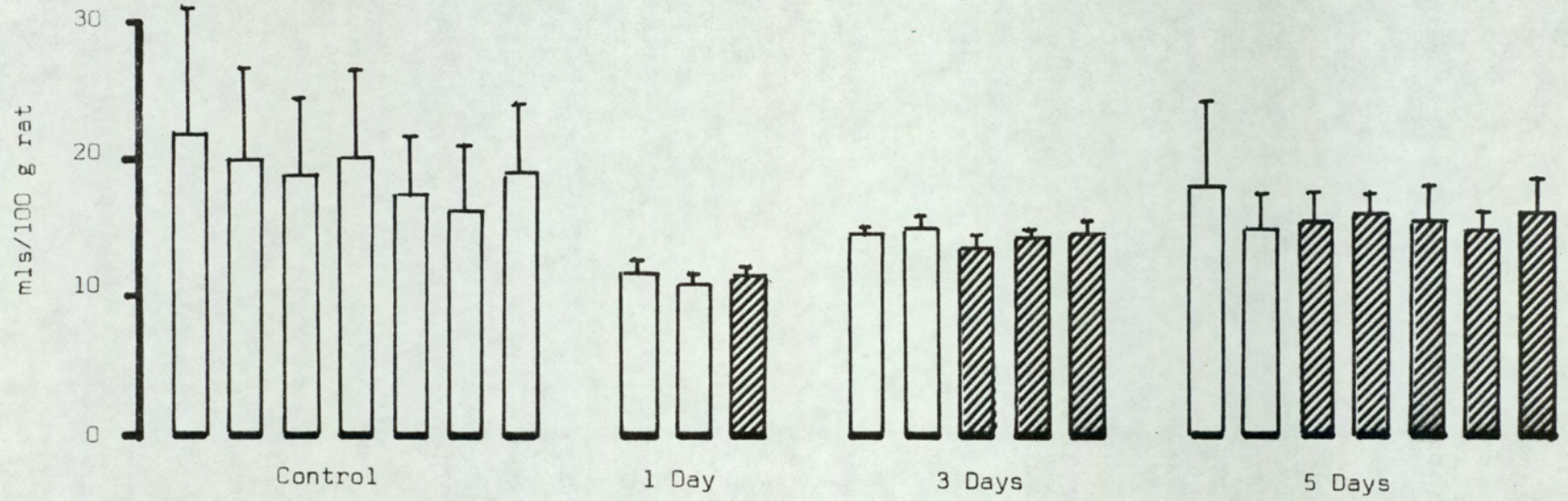


Fig. 59. Effect of saline (control) and 1, 3 and 5 days of gentamicin (40 mg/kg/day) administration on water intake.

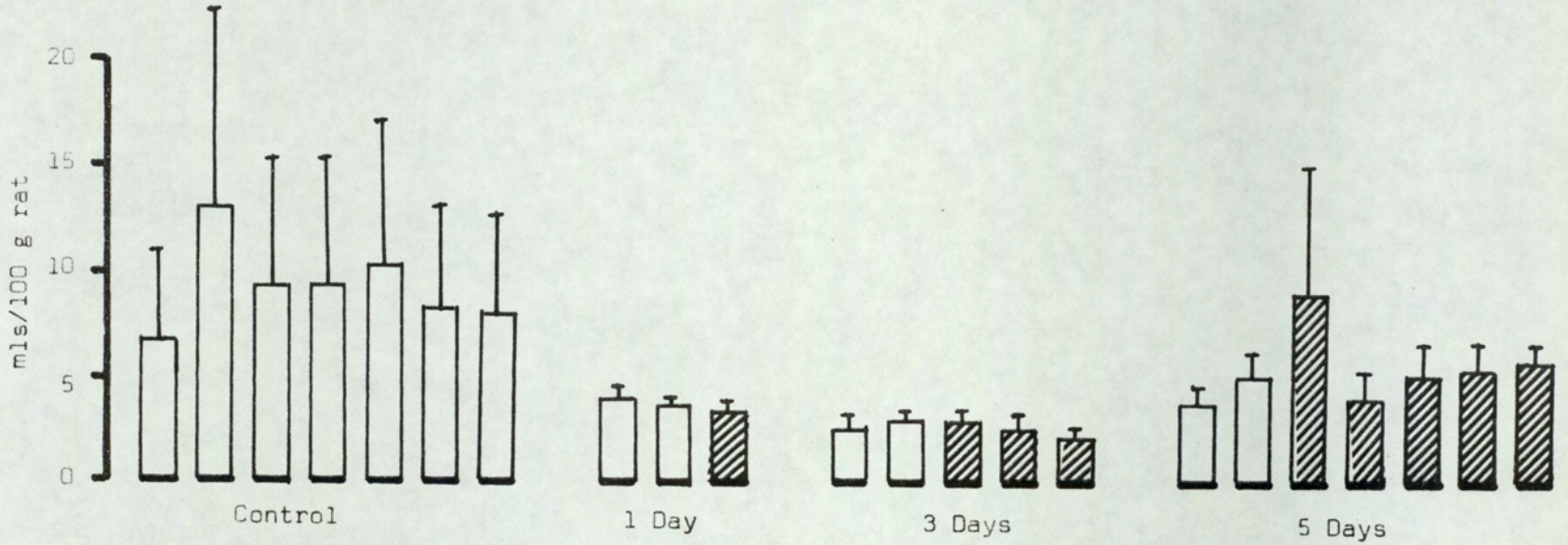


Fig. 60. Effect of saline (control) and 1, 3 and 5 days of gentamicin (40 mg/kg/day) administration on volume of urine.

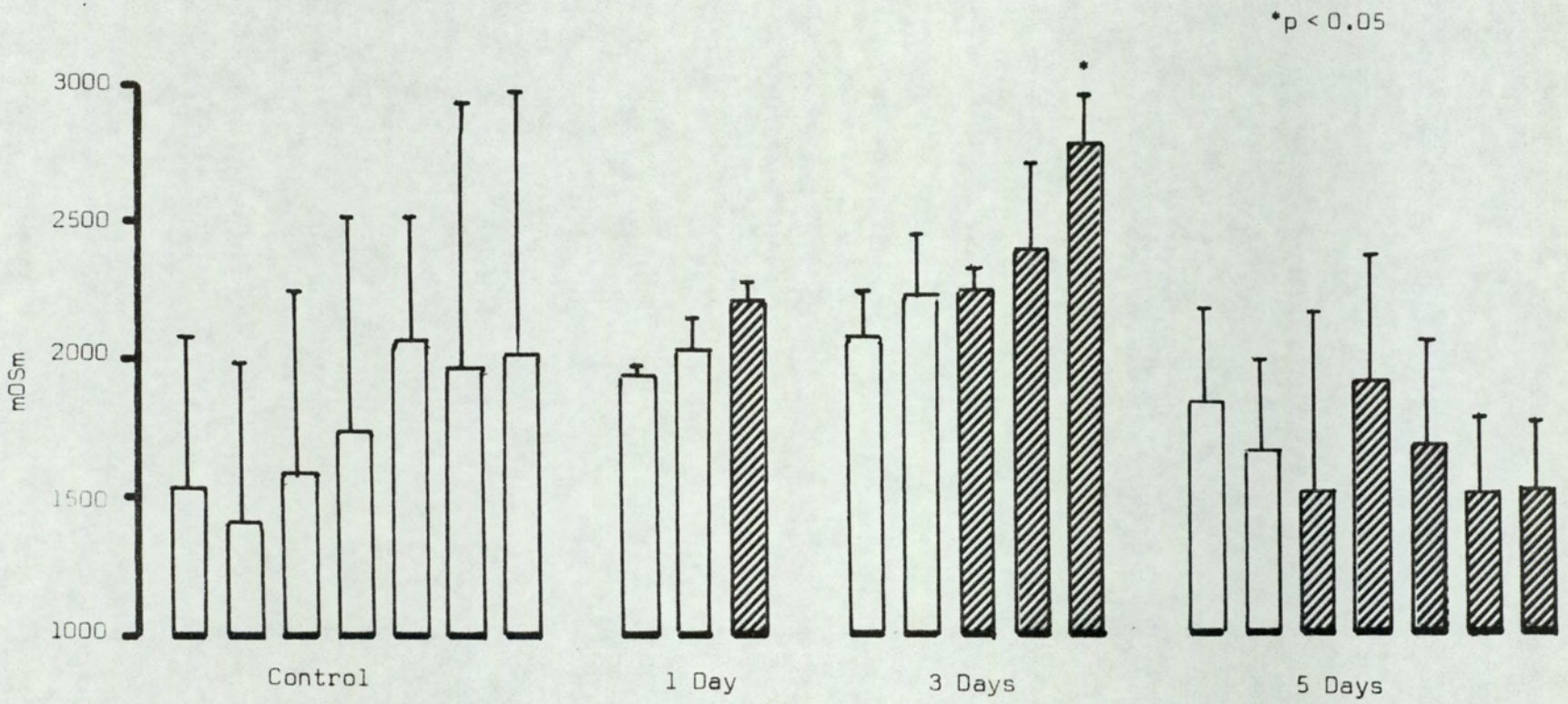


Fig. 61. Effect of saline (control) and 1, 3 and 5 days of gentamicin (40 mg/kg/day) administration on urinary osmolarities.

for 3 days. In this group there was a statistically significant rise in urinary osmolarity on the final day compared to the control ($p < 0.05$).

(iv) Excretion of protein in urine

Fig. 62 shows that there was no apparent change in protein excretion in groups injected with gentamicin for 1 and 3 days. However, in rats injected with 5 doses of gentamicin, there was significant elevation from control on the 4th and 5th day ($p < 0.05$).

(v) Na^+/K^+ ratio in urine

Na^+ and K^+ levels fluctuated from day to day and thus Na^+/K^+ ratios were used instead. Na^+/K^+ ratios were depressed initially in all groups administered with gentamicin. In the 5 day group there was a decline in Na^+/K^+ ratio after a single and second gentamicin injection, followed by a return towards the normal on further injections. Na^+/K^+ ratios were significantly reduced in 1 and 3 day groups. In both groups there was significant reduction after 1 day of injection ($p < 0.05$), with further reduction on the 2nd day ($p < 0.01$) followed by a rise on the 3rd but still reduced compared to control ($p < 0.05$) (Fig. 63).

(vi) Excretion of Ca^{2+} and Mg^{2+} in urine

Fig. 64 and 65 depict changes in Ca^{2+} and Mg^{2+} excretion respectively. In groups of rats injected with gentamicin for 1 and 5 days there was a significant increase in Ca^{2+} excretion on day 1 and 3 respectively ($p < 0.05$). In the 3 day group Ca^{2+} excretion was significantly elevated by the final day ($p < 0.01$). Highly significant changes were seen for the 5 day group on the 4th and 5th days of injections ($p < 0.001$).

There was no significant change in Mg^{2+} excretion in any of the four groups.

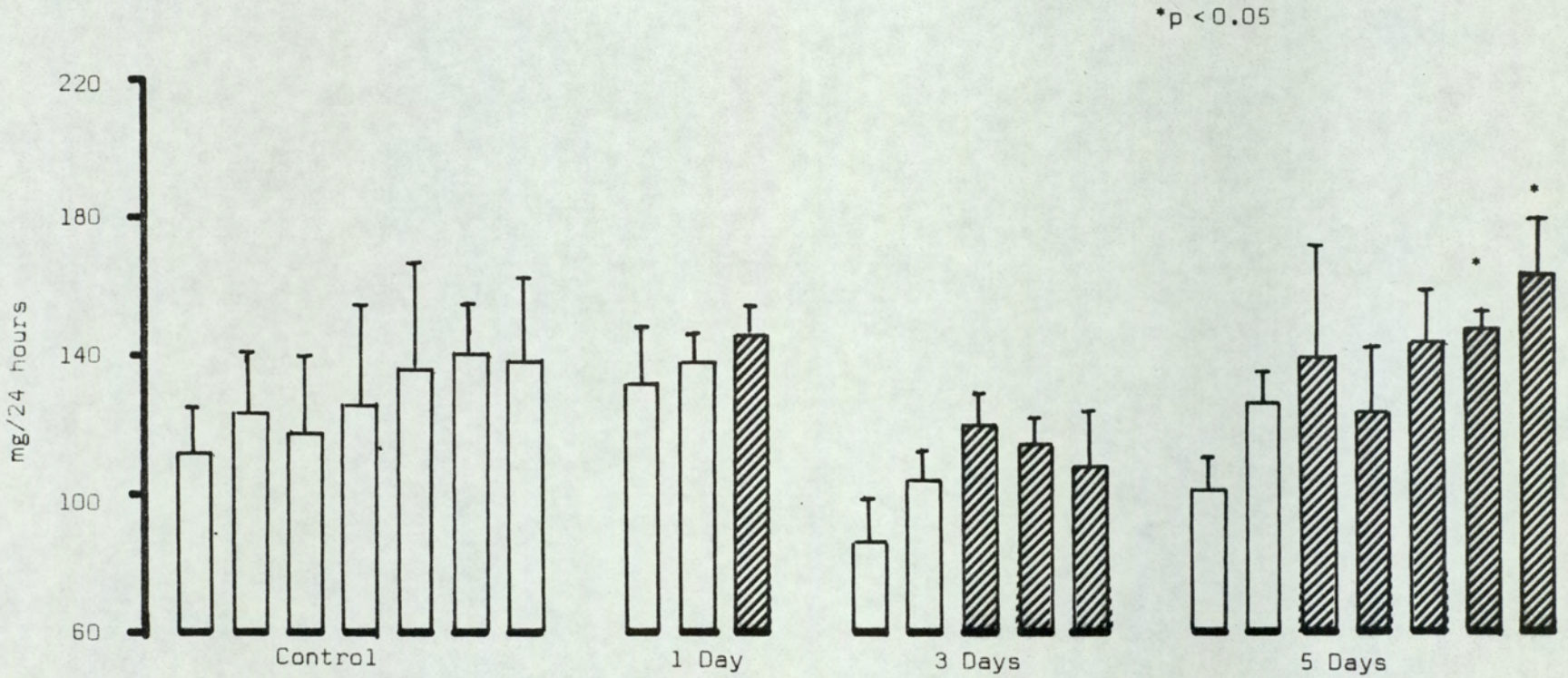


Fig. 62. Effect of saline (control) and 1, 3 and 5 days of gentamicin (40 mg/kg/day) administration on urinary protein excretion.

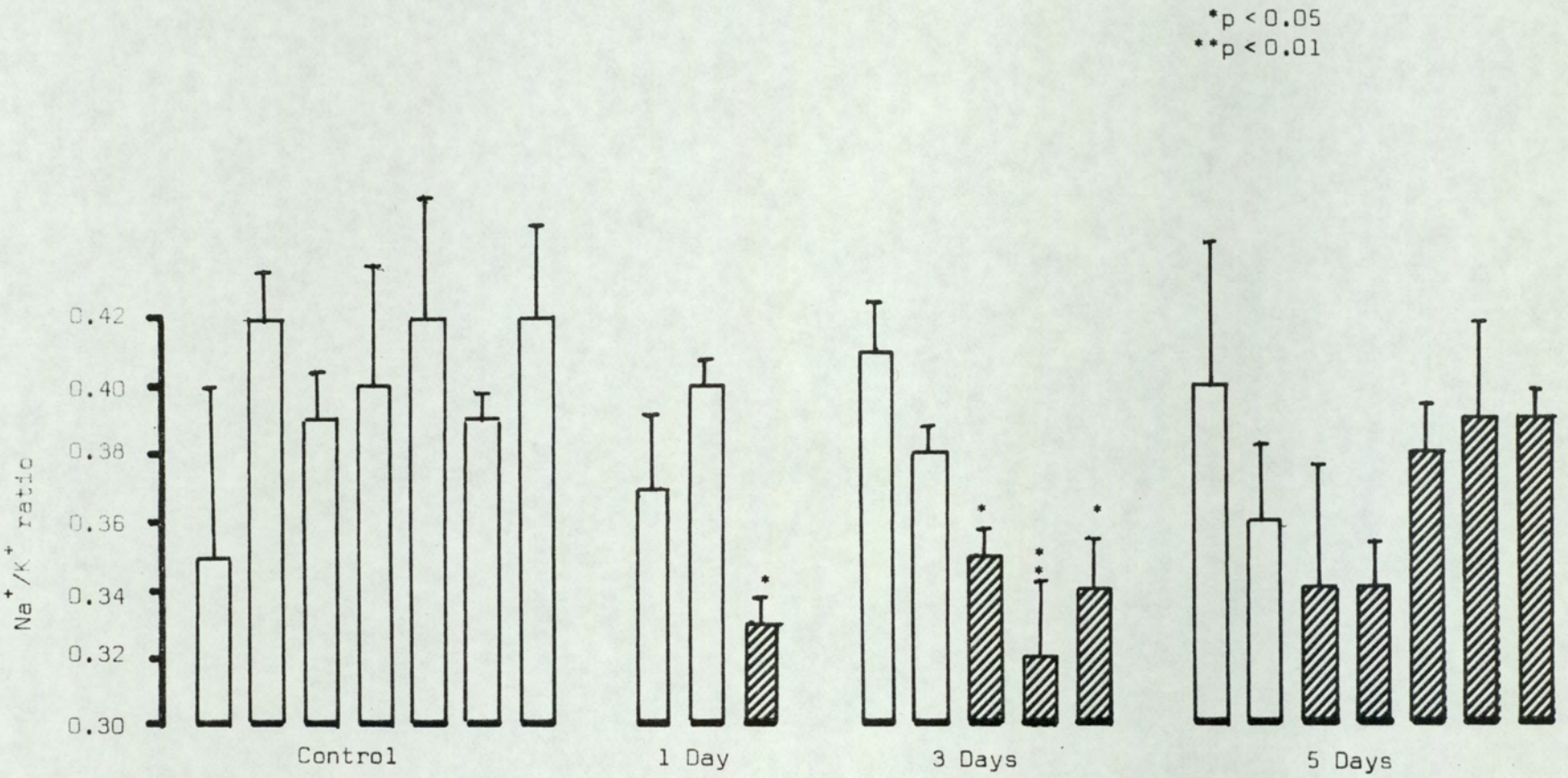


Fig. 63. Effect of saline (control) and 1, 3 and 5 days of gentamicin (40 mg/kg/day) administration on Na^+/K^+ ratios in urine.

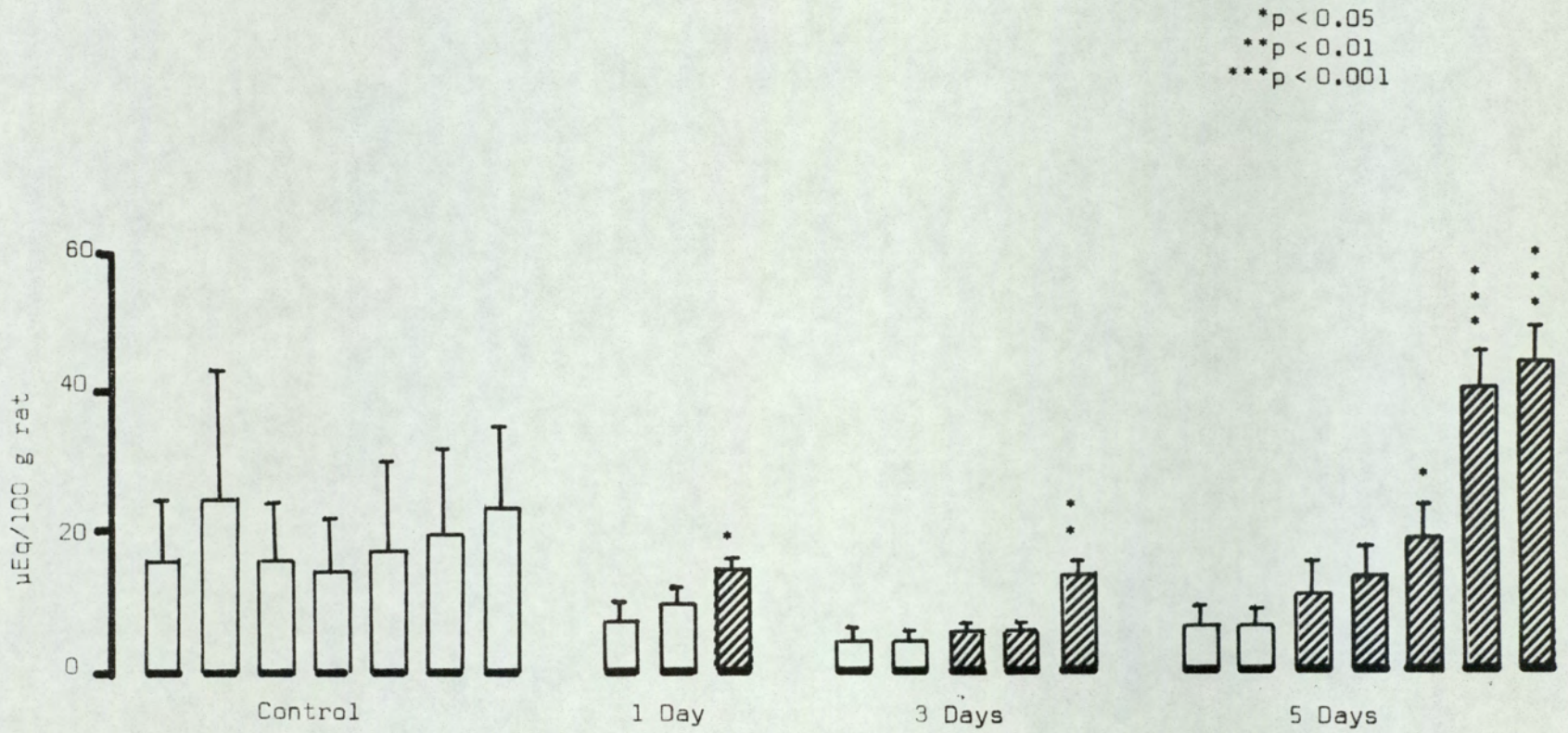


Fig. 64. Effect of saline (control) and 1, 3 and 5 days of gentamicin (40 mg/kg/day) administration on Ca^{2+} excretion in urine.

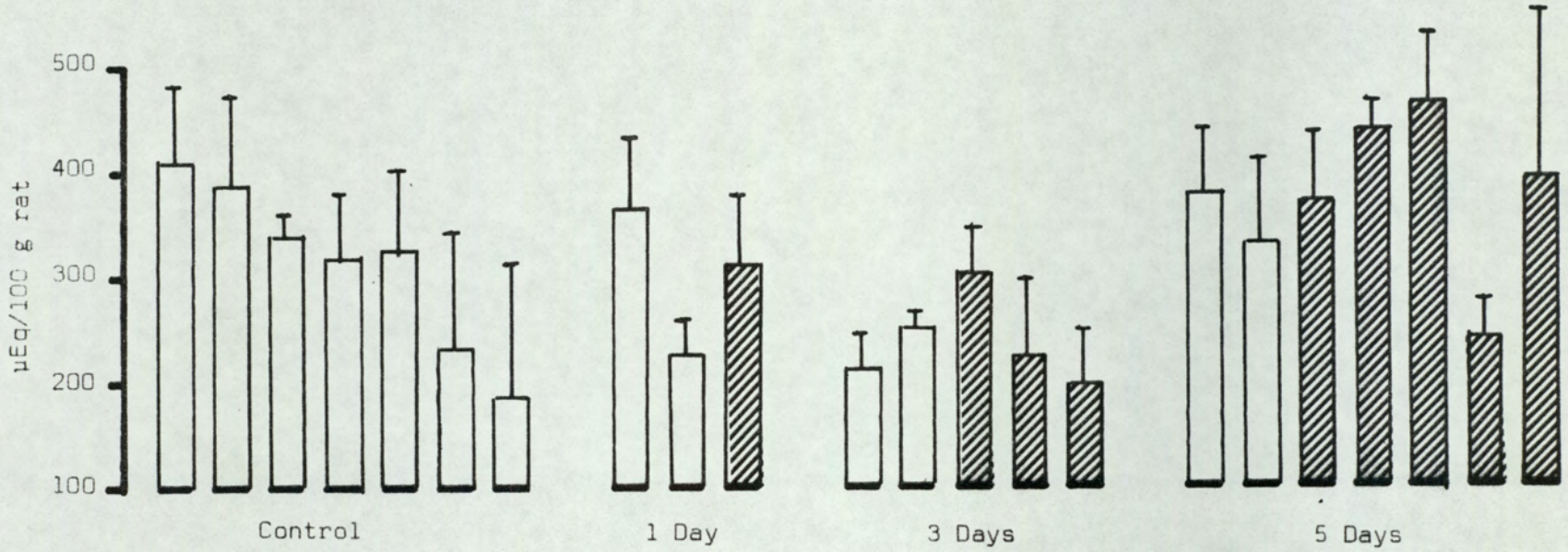


Fig. 65. Effect of saline (control) and 1, 3 and 5 days of gentamicin (40 mg/kg/day) administration on Mg^{2+} excretion in urine.

(vii) Excretion of the enzymes LDH, NAG and AAP in urine

Figs. 66, 67 and 68 show the changes in urinary excretion of LDH, NAG and AAP respectively after gentamicin administration. LDH was more significantly elevated ($p < 0.01$) than both NAG and AAP ($p < 0.05$) in the group of rats injected with gentamicin for 1 day. In the 3 day group NAG was highly elevated after two injections whereas LDH and AAP were both significantly increased after 3 days of injections ($p < 0.05$).

The activity of NAG was significantly increased on all 5 days of injection in the 5 day group. NAG was elevated on day 1 and 2 ($p < 0.01$), with further significant rise on days 3, 4 and 5 ($p < 0.001$). The LDH activity was higher on days 4 and 5 ($p < 0.01$). Gentamicin had least effect on AAP excretion. There was a significant rise in AAP after the first injection ($p < 0.05$) but there were no changes after further injections of gentamicin.

(viii) The release *in vitro* of LDH and NAG from isolated renal tubules from rats injected with gentamicin (40 mg/kg/day) for 1, 3 and 5 days

In the present study, the day of the first injection of gentamicin was staggered so that all groups of animals could be killed at the same time, 24 hours after the last injection. Isolated rat renal tubules were then prepared from the kidneys of rats and *in vitro* incubations of tubules in absence of additional gentamicin, carried out as described in section 3.6. Fig. 69 shows the release of NAG and LDH from tubules incubated up to 2 hours at 37°C. The tubules were prepared from rats which had been injected with gentamicin (40 mg/kg/day) for 1, 3 and 5 days. The release of enzyme at each incubation time was expressed as a

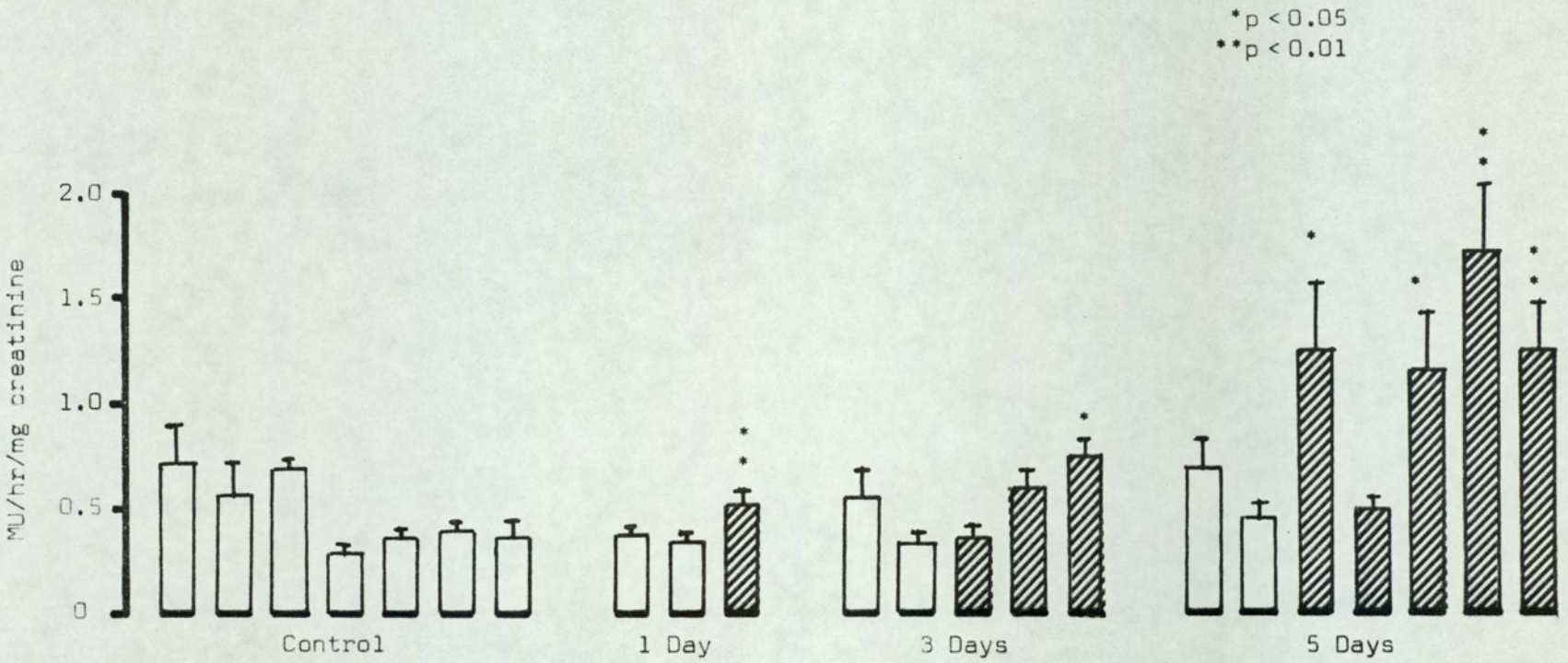


Fig. 66. Effect of saline (control) and 1, 3 and 5 days of gentamicin (40 mg/kg/day) administration on urinary LDH enzyme excretion.

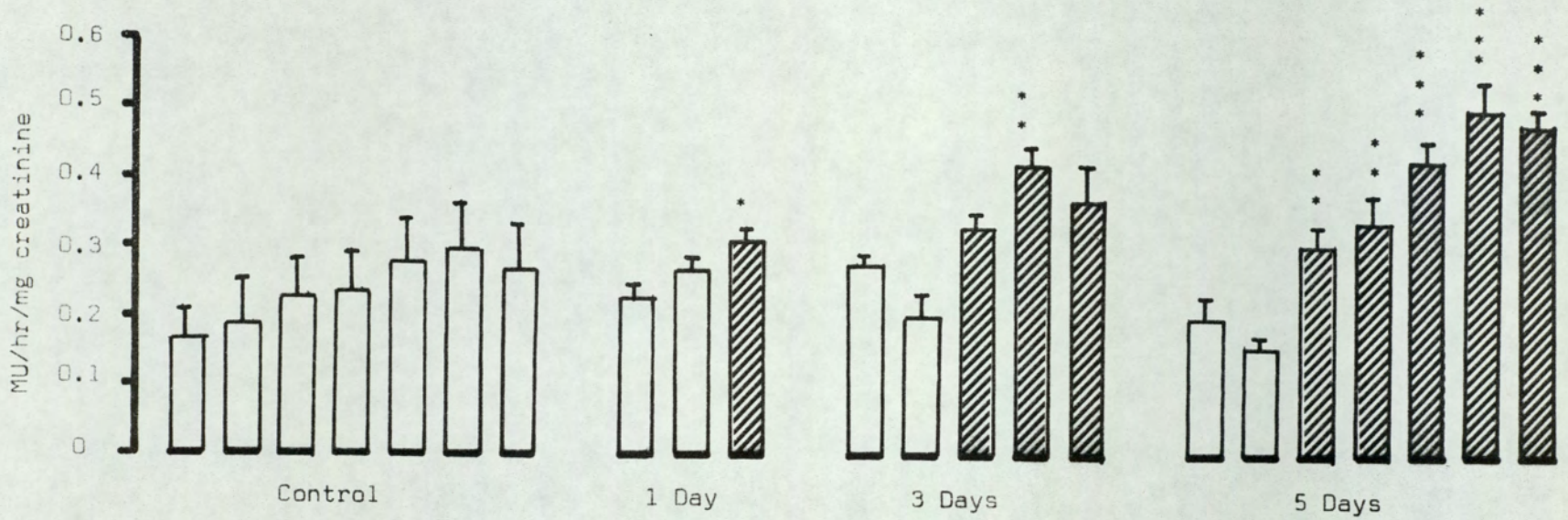


Fig. 67. Effect of saline (control) and 1, 3 and 5 days of gentamicin (40 mg/kg/day) administration on urinary NAG enzyme excretion.

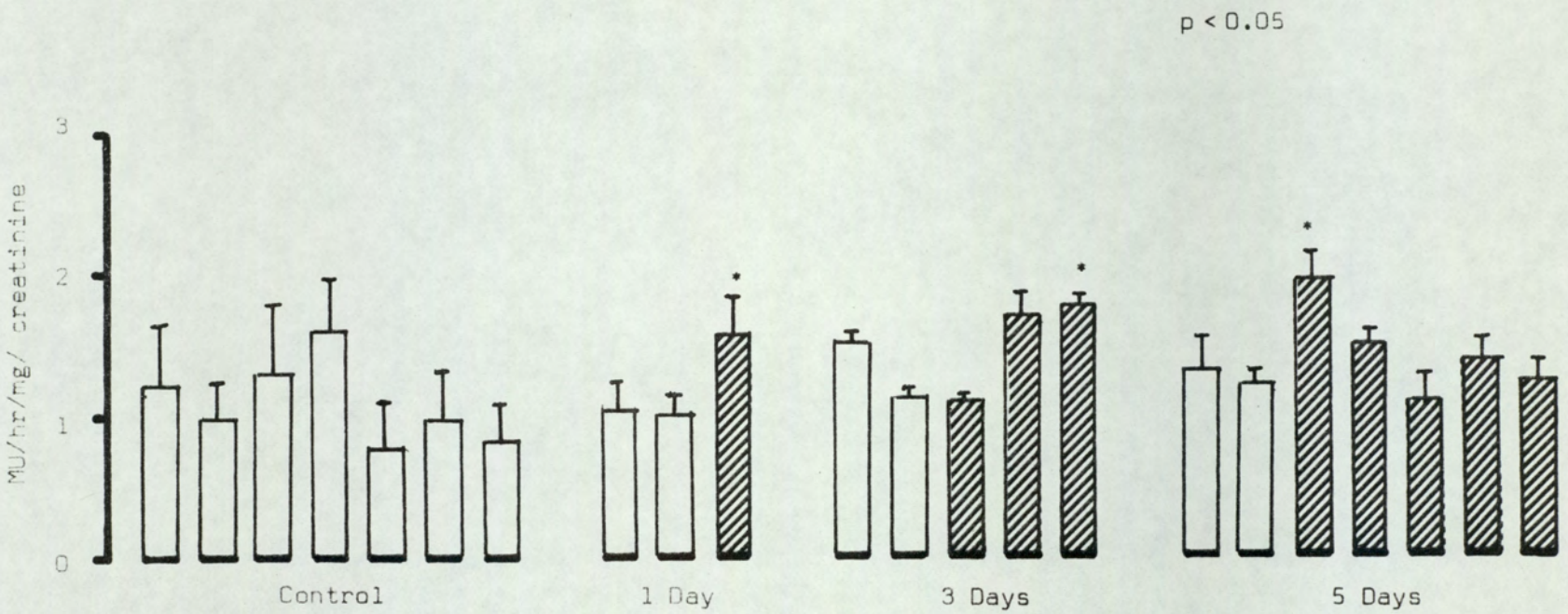


Fig. 68. Effect of saline (control) and 1, 3 and 5 days of gentamicin (40 mg/kg/day) administration on urinary AAP enzyme excretion.

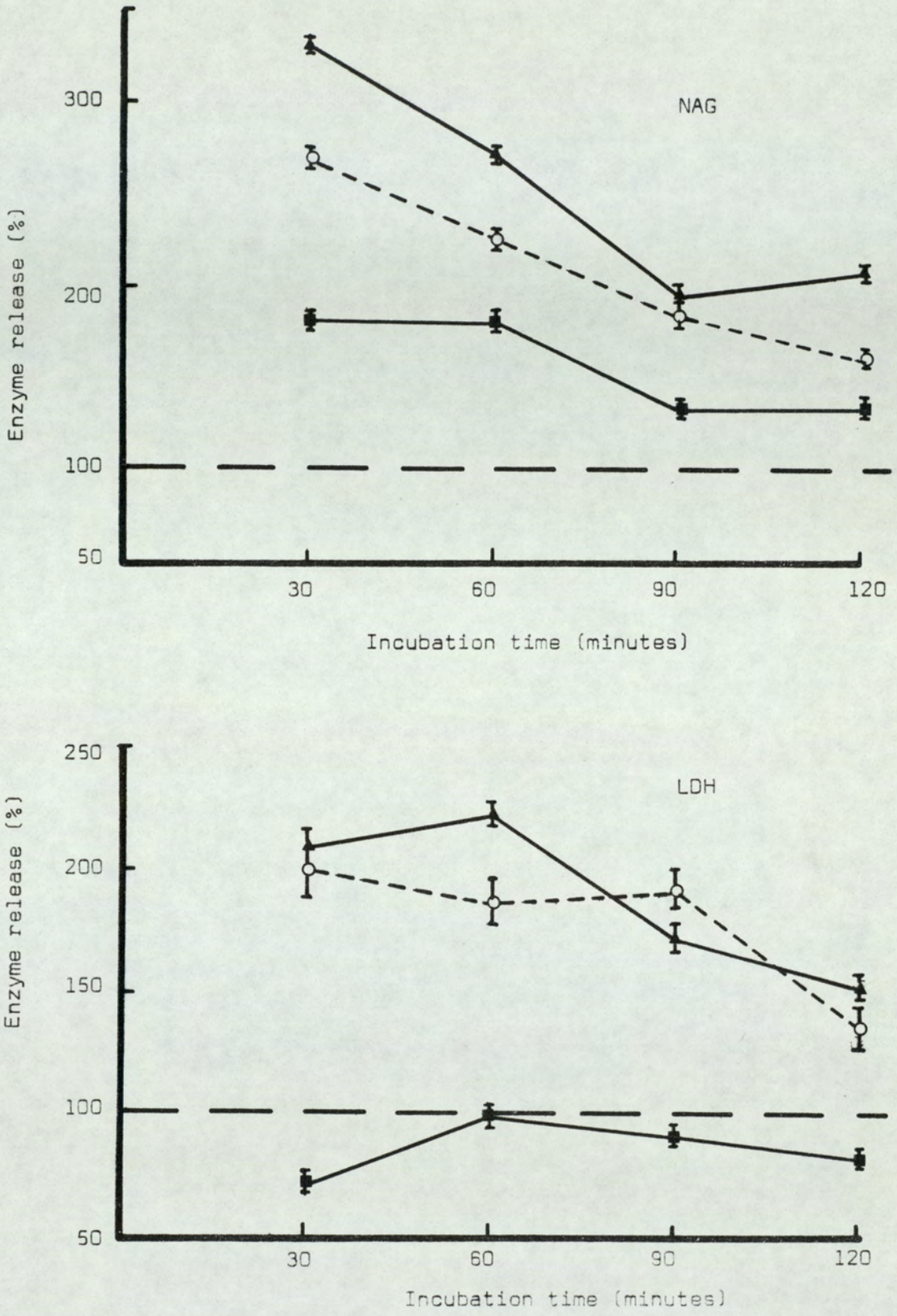


Fig. 69. The release of NAG and LDH on incubating renal tubules isolated from rats injected with gentamicin (40 mg/kg/day) for 1 (○), 3 (▲) and 5 (■) days. The release of enzymes from tubules isolated from the saline (control) group at a given incubation time was set at 100%.

percentage of the release from tubules prepared from water-injected rats (control set at 100%).

After 60 minutes incubation of the renal tubule preparation, the release of NAG was 225% of the control following a single injection, increasing to 272% for tubules prepared from rats injected for 3 days, and then returning towards the control for tubules from rats injected for 5 days (180%). In fact, at all periods of incubation the release of NAG was greatest for tubules prepared from rats injected for 3 days and least from the 5 day injected rats.

The pattern of release of LDH was similar to NAG but did not closely parallel it.

(ix) Tubular cell ultrastructure

Electron microscopy of tubular cells prepared from control (saline) rats appeared normal (Fig. 32). However, after only 1 day of gentamicin administration there were myeloid bodies seen in a few of the lysosomes (Fig. 70a). After 3 doses of gentamicin, the number of lysosomes containing myeloid bodies had increased (Fig. 70b) and mitochondrial damage was also apparent (Fig. 70c). The structural changes seen after 3 days were, it seems, absent in tubules prepared from rats given 5 doses of gentamicin (Fig. 70d).

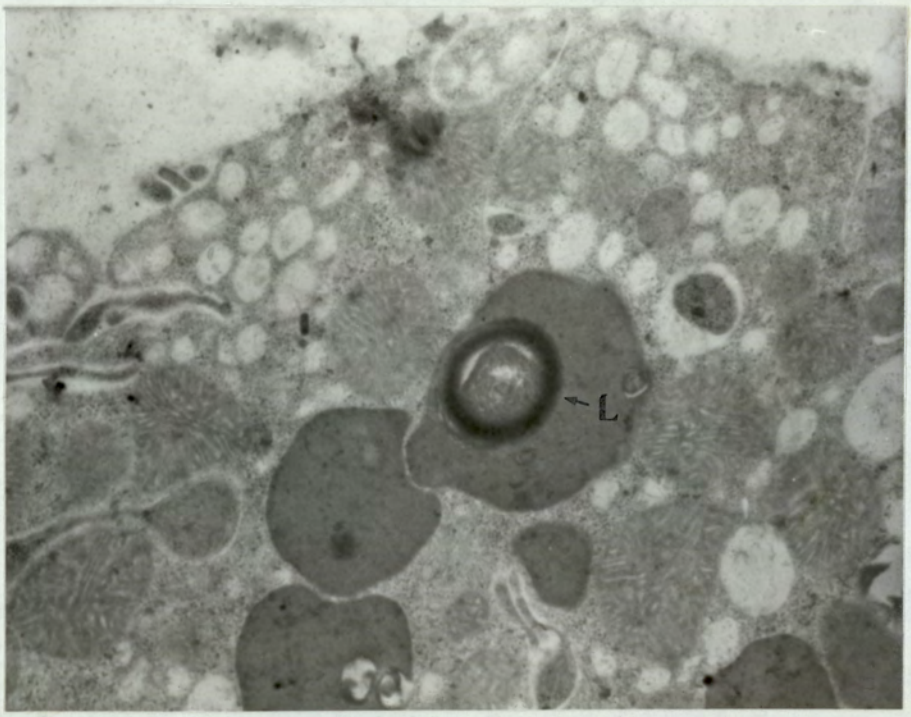


Fig. 70a. Electron micrograph showing ultrastructural appearance of tubular cell after 1 day of gentamicin (40 mg/kg/day) administration. There appears to be myeloid bodies within the lysosomes (L).

× 15,000

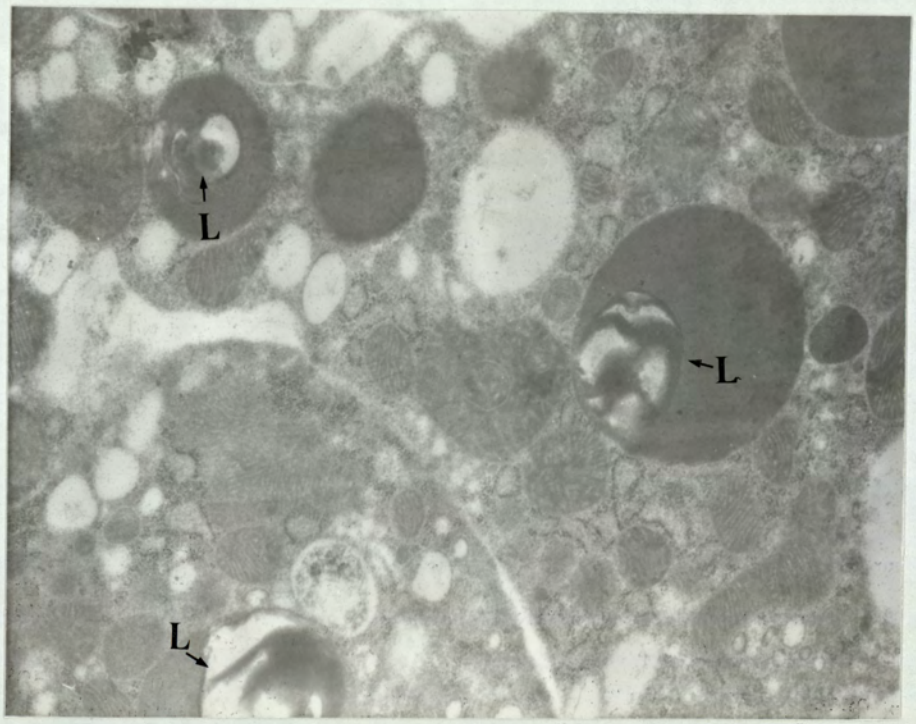


Fig. 70b. Electron micrograph showing ultrastructural appearance of tubular cell after 3 days of gentamicin (40 mg/kg/day) administration. There are numerous lysosomes (L) containing myeloid bodies.

x 15,000

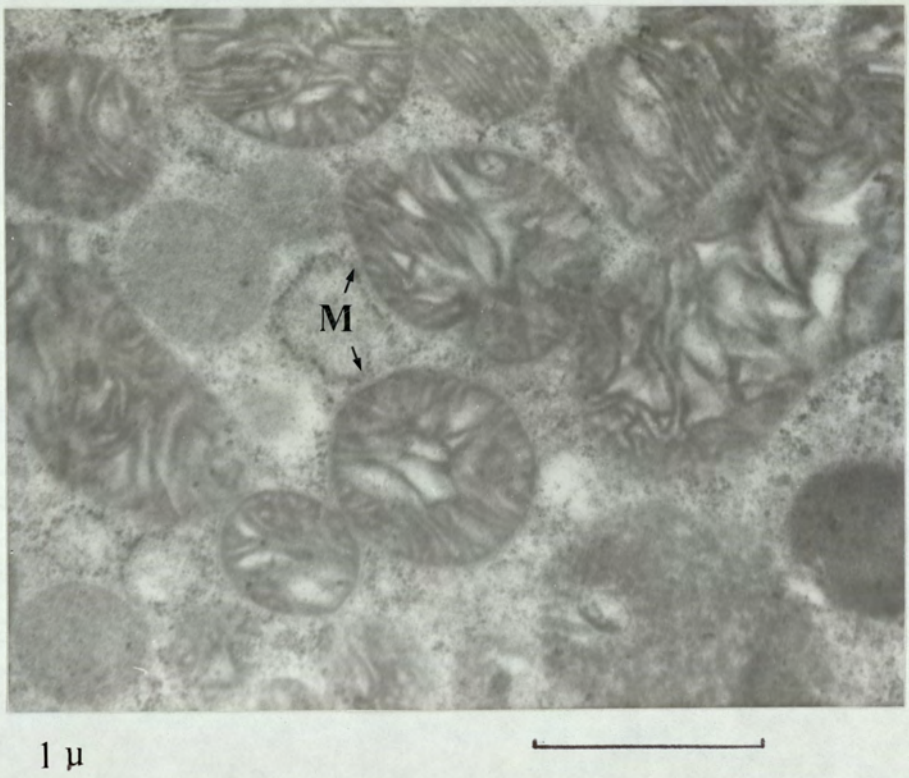


Fig. 70c. Electron micrograph showing ultrastructural appearance of tubular cell after 3 days of gentamicin (40 mg/kg/day administration. There is widespread degeneration of mitochondria (M).

x 30,000

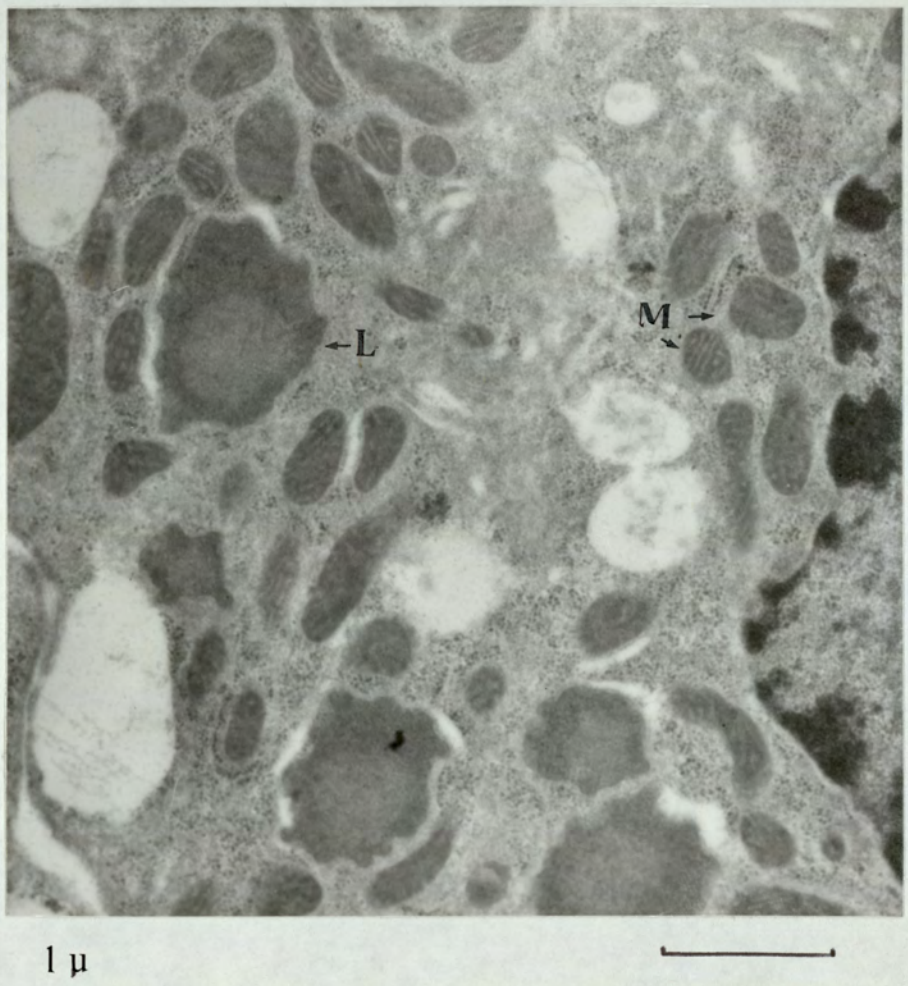


Fig. 70d. Electron micrograph showing ultrastructural appearance of tubular cell after 5 days of gentamicin (40 mg/kg/day) administration. The lysosomes (L) are devoid of myeloid bodies and the mitochondrias (M) are structurally intact.

x 22,500

4.7.2.2 Animals injected with gentamicin (40 mg/kg/day) for 7 days and then allowed to recover

Two groups of five rats each were used, with one group used as control (water-injected) and the other for gentamicin injections. The rats were individually housed in metabolic cages and allowed four days to acclimatise before injections were commenced. The various parameters of renal functions were measured, before, during and after gentamicin administration.

All the renal functional parameters measured in the gentamicin-injected group were compared to those of the control group (water-injected) on a given day by Student's t-test.

The effect of gentamicin administration on:-

(i) Body weight and food intake

Figs. 71 and 72 show that gentamicin administration had very little effect on the body weight and food intake. However, in the gentamicin group it does appear as if there is no gain in body weight from days 5 to 10, which correlates well with the sharp decline in food intake observed within this period.

(ii) Water intake and urine output

Rats injected with gentamicin showed no statistically significant difference in water intake and urine output from the control group. The water intake of the gentamicin group was higher than the control from the onset of the experiment but this daily intake was not affected by gentamicin administration. However, urine volume did appear to be elevated after 5 injections of gentamicin and remained elevated throughout the period of study (Figs. 73 and 74).

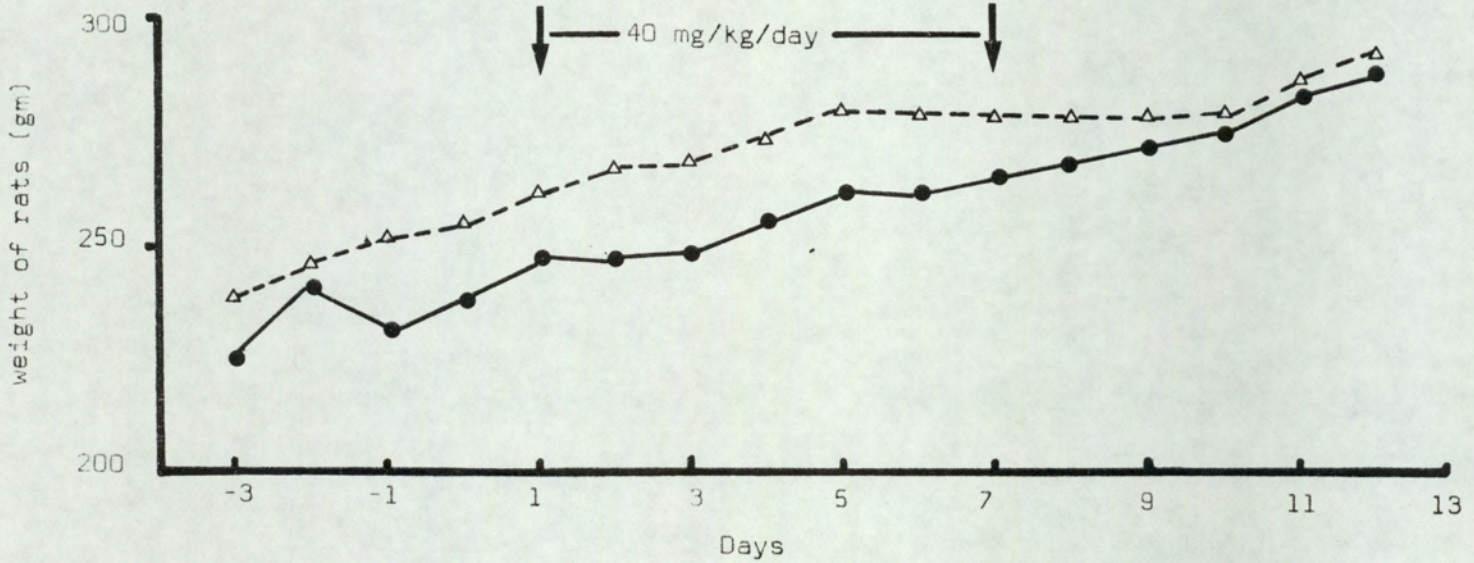


Fig. 71. Effect of 7 days of gentamicin (40 mg/kg/day) injections on body weight before, during and after drug administration. Saline control (●) and gentamicin (Δ). In this and subsequent graphs in this subsection, each point represents the mean of at least 4 rats.

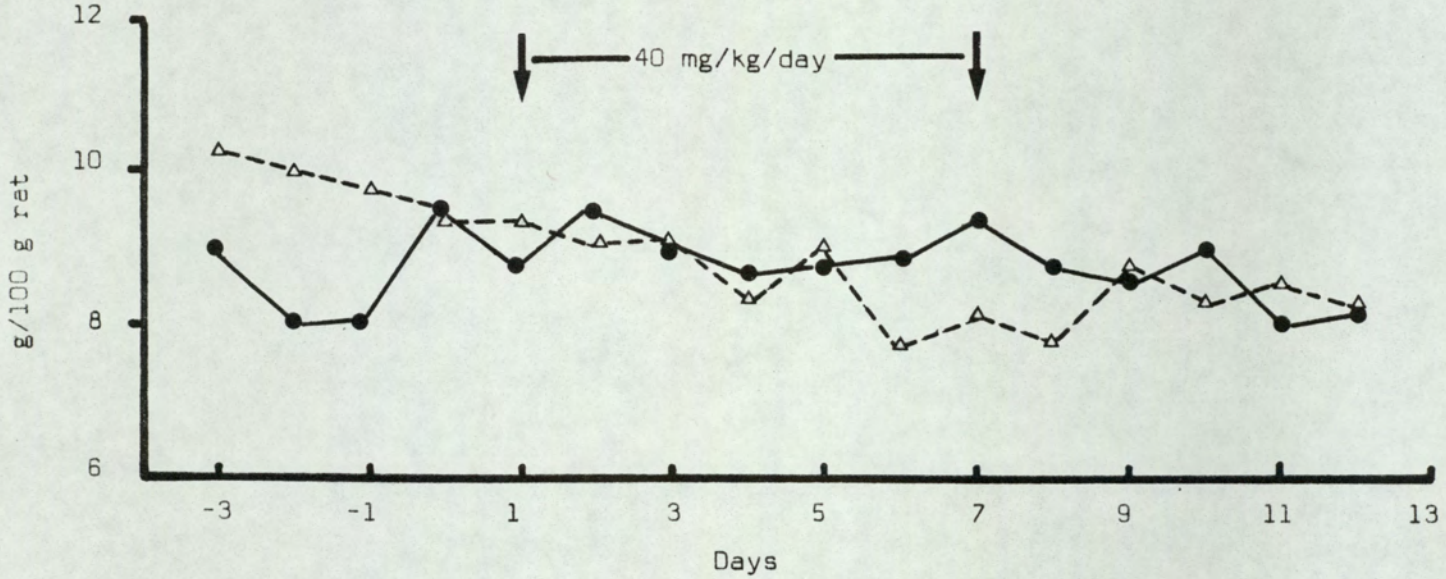


Fig. 72. Effect of 7 days of gentamicin (40 mg/kg/day) injections on food intake before, during and after drug administration. Saline control (●) and gentamicin (△).

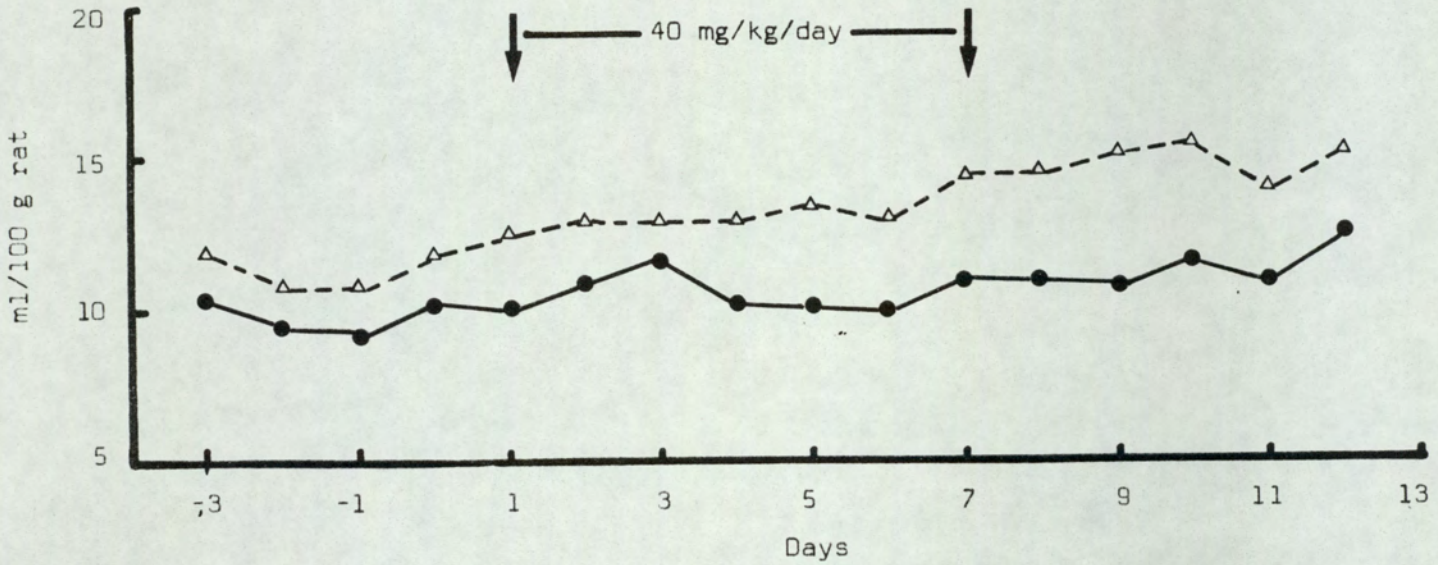


Fig. 73. Water intake before, during and after 7 days of gentamicin (40 mg/kg/day) administration. Saline control (●) and gentamicin (△).

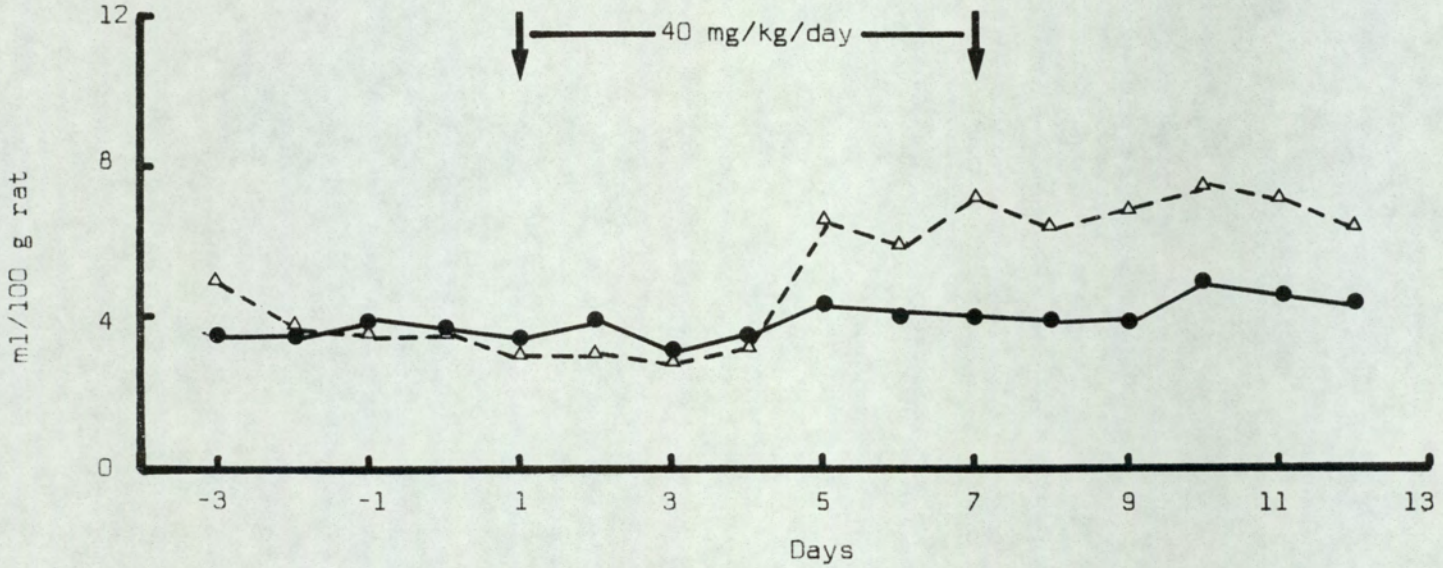


Fig. 74. Volume of urine before, during and after 7 days of gentamicin (40 mg/kg/day) administration. Saline control (●) and gentamicin (△).

(iii) Osmolarity of urine

Fig. 75 shows the effect of gentamicin administration on urinary osmolarities. Urinary osmolarity decreased progressively in the gentamicin group with a highly significant decrease by the sixth dose ($p < 0.01$). The osmolarity remained significantly reduced over the 3 days after the last injection with $p < 0.05$ on day 10. The absence of significant difference for days 11 to 13 suggests recovery of the gentamicin-injected rats towards normal.

(iv) Excretion of protein in urine

The rats receiving gentamicin displayed progressive increase in protein excretion with significant elevation after the fifth day ($p < 0.01$). This elevation remained significant during and after the last injection. There was no significant difference compared to the control group on day 13 (Fig. 76).

(v) Na^+/K^+ ratio in urine

Since the Na^+ and K^+ levels fluctuated from day to day for both groups, Na^+/K^+ ratios were used instead. Na^+/K^+ ratios were reduced significantly after the 1st and 2nd dose compared to the control group (Fig. 77). However, whether this depression is due to gentamicin or not is difficult to interpret since even before the injections were commenced the Na^+/K^+ ratio of the gentamicin group was significantly lower than the control group.

(vi) Excretion of Ca^{2+} and Mg^{2+} in urine

Figs. 78 and 79 depict the changes in Ca^{2+} and Mg^{2+} excretion respectively. The excretion of Ca^{2+} was significantly increased by day 4 ($p < 0.01$) and further increased by day 7 ($p < 0.001$). The Ca^{2+} excretion remained elevated after the last injection with $p < 0.05$ on day 12.

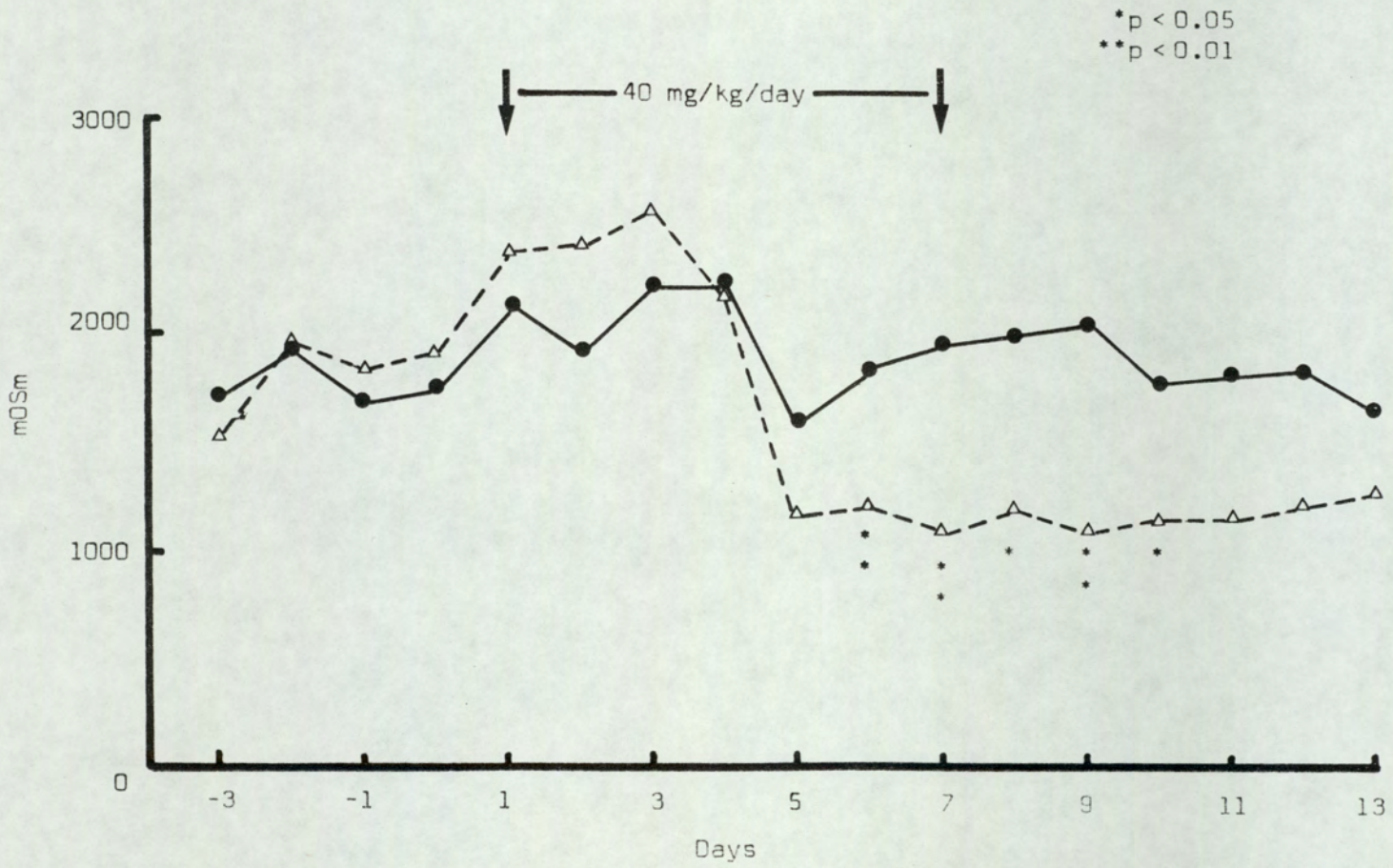


Fig. 75. Effect of 7 days of gentamicin (40 mg/kg/day) injections on urine osmolarity before, during and after drug administration. Saline control (●) and gentamicin (△). The p values relate to the significance of differences between the gentamicin-injected group and the control group on a given day.

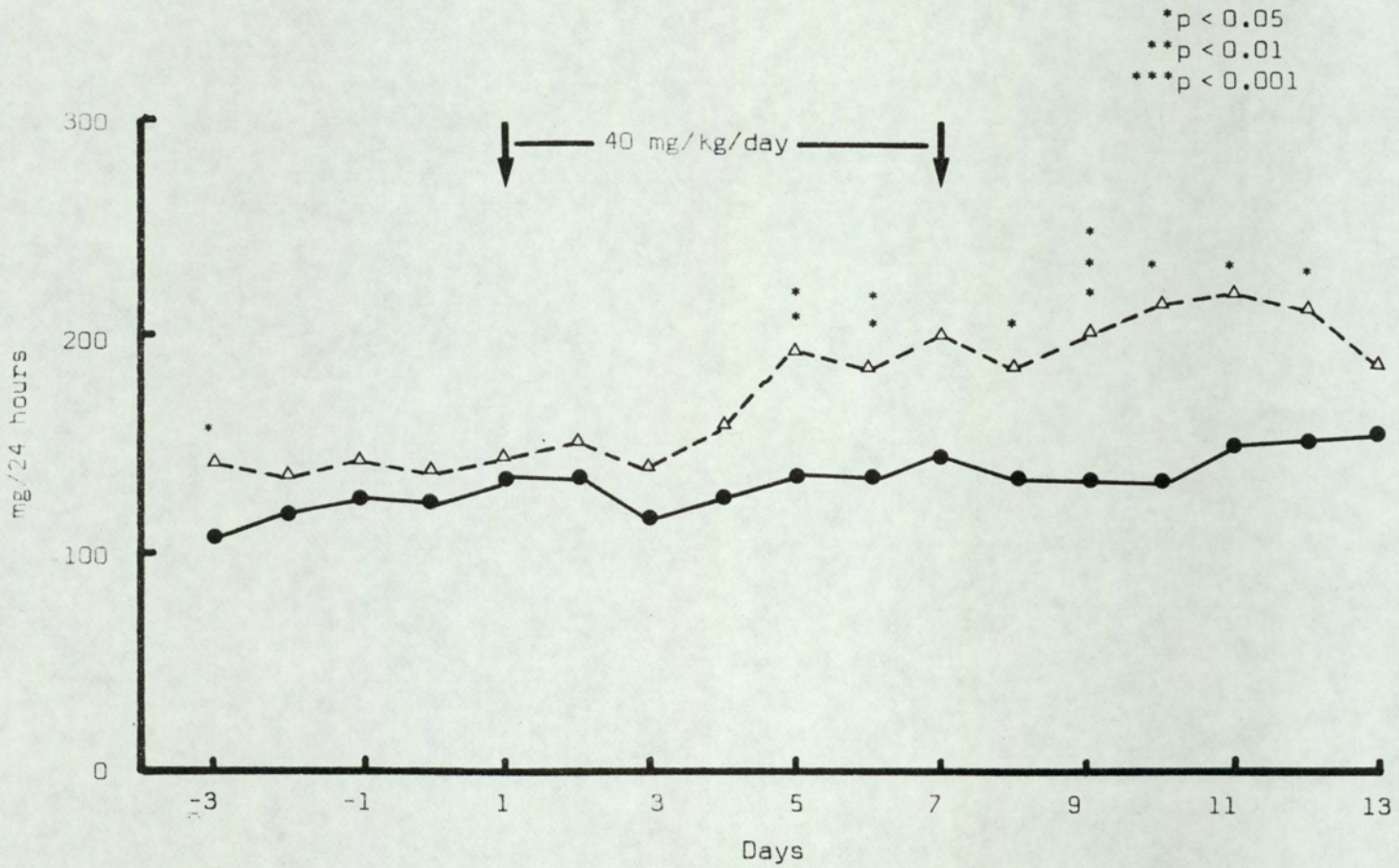


Fig. 76. Effect of 7 days of gentamicin (40 mg/kg/day) injections on urinary protein excretion before, during and after drug administration. Saline control (●) and gentamicin (Δ).

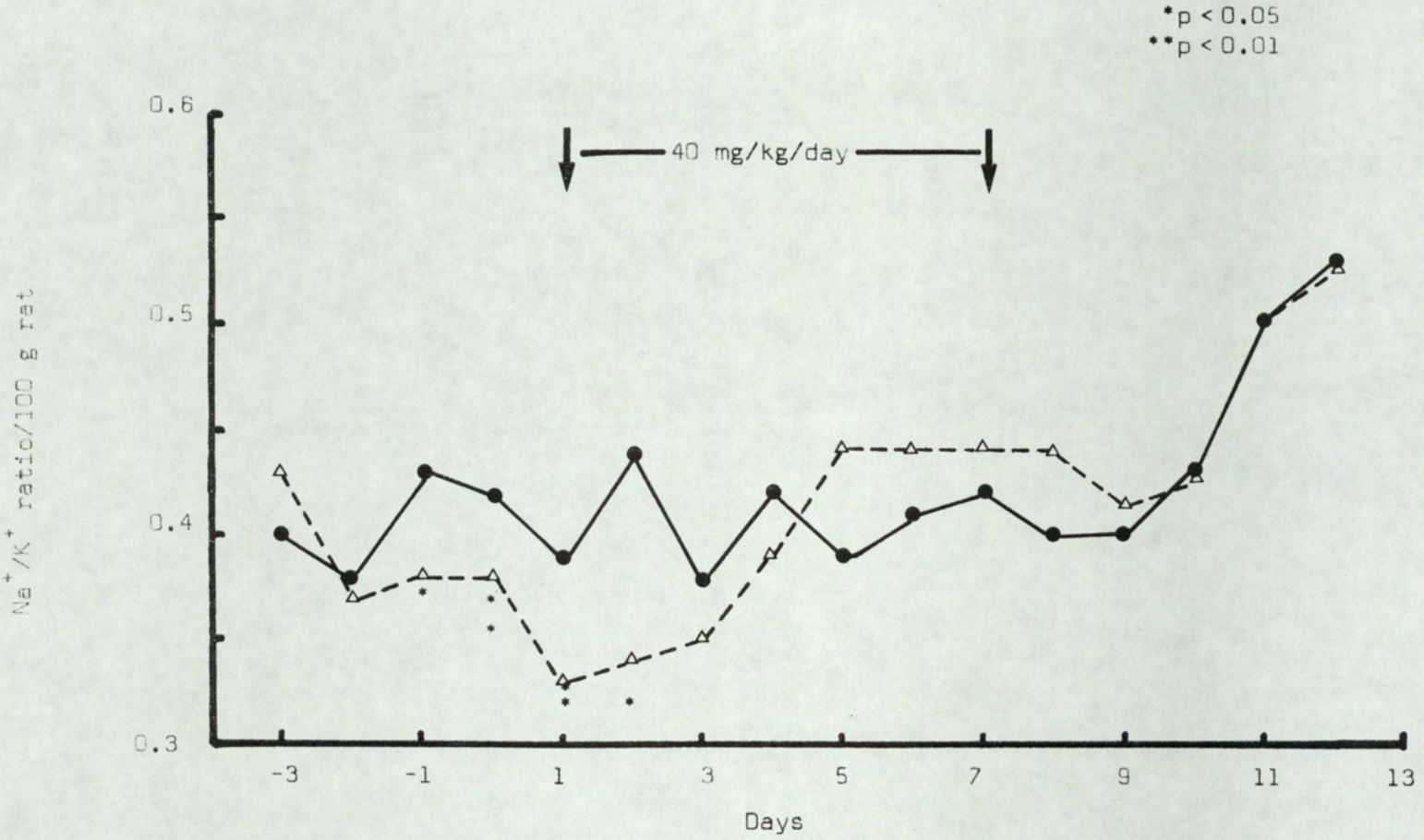


Fig. 77. Na^+/K^+ ratio, before, during and after administration of gentamicin (40 mg/kg/day) for 7 days. Saline control (●) and gentamicin (△).

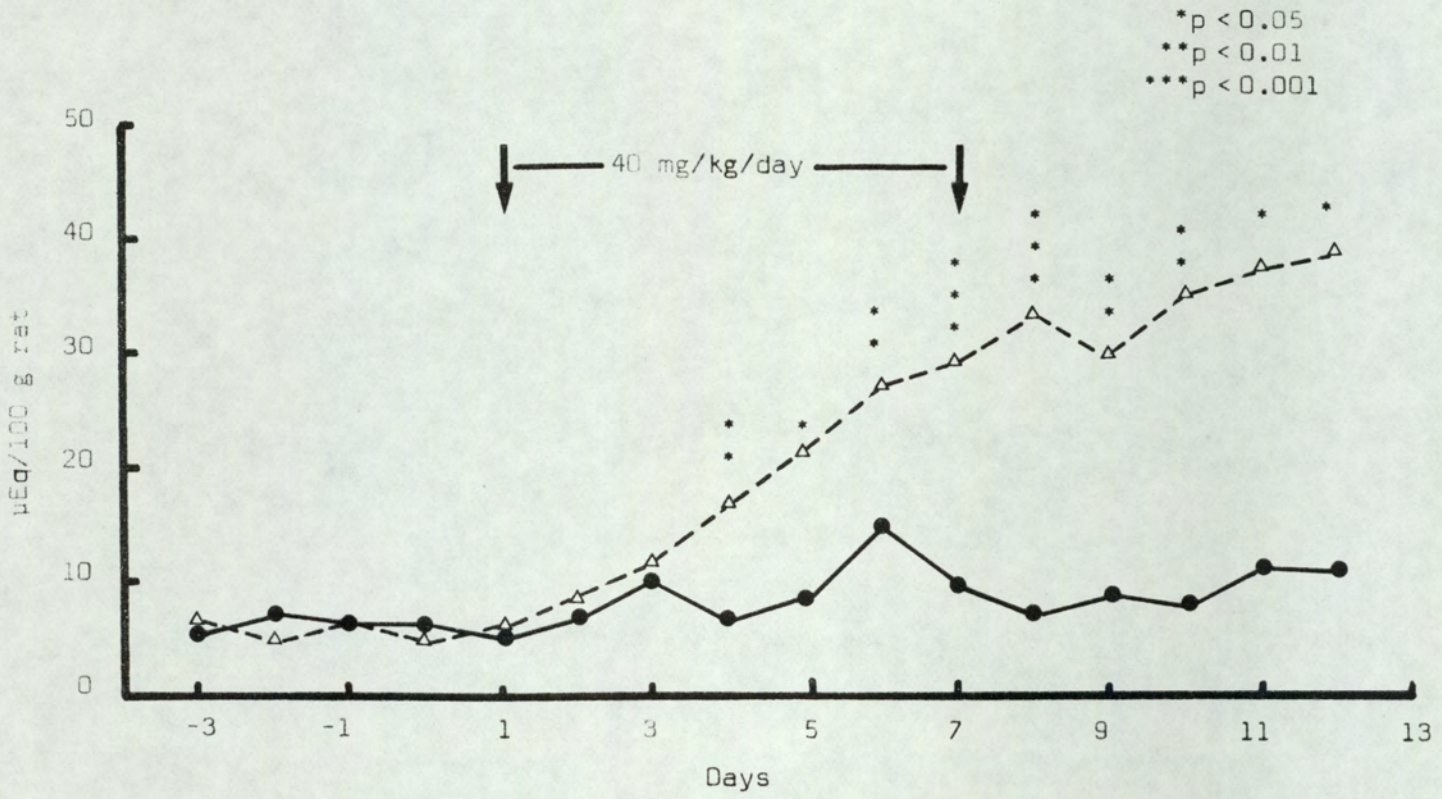


Fig. 78. Effect of 7 days of gentamicin (40 mg/kg/day) injections on urinary Ca^{2+} excretion before, during and after drug administration. Saline control (●) and gentamicin(△).

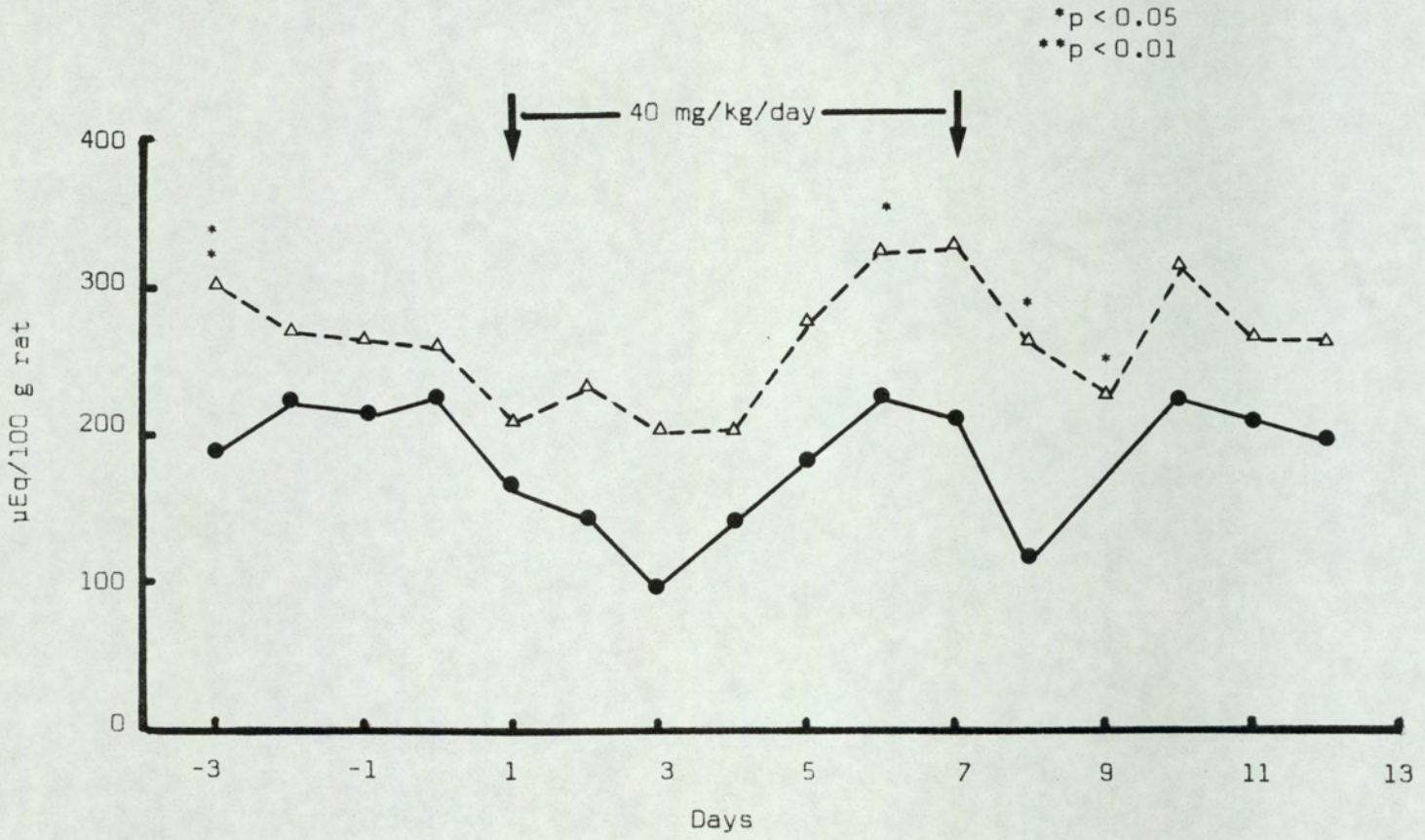


Fig. 79. Urinary Mg^{2+} excretion before, during and after gentamicin (40 mg/kg/day) administration for 7 days. Saline control (●) and gentamicin (△).

Mg^{2+} excretion was significantly elevated on days 6, 8 and 9 compared to the control group ($p < 0.05$). Whether this is a real increase is difficult to assess since there was a large fluctuation in Mg^{2+} excretion from day to day in the control group.

(vii) Excretion of the enzymes LDH, NAG and AAP in urine

Figs. 80, 81 and 82 show changes in LDH, NAG and AAP respectively, before, during and after gentamicin administration. The activity of LDH increased progressively after the administration of the first dose and enzyme excretion reached a peak by day 9, i.e. 2 days after the last injection. After day 9 there was a gradual fall in activity but it had not quite returned to the control level even after day 13. The increase over the control was very highly significant from day 1 to 13 ($p < 0.001$).

The excretion of NAG and AAP was significantly elevated after the first two doses ($p < 0.001$). The release of NAG was depressed for days 3 to 5 with highly significant elevation from day 6 and onwards ($p < 0.001$). The release of AAP was very similar to NAG since depression was observed for days 3 and 4 followed by a significant increase over the control group up to day 13 ($p < 0.001$).

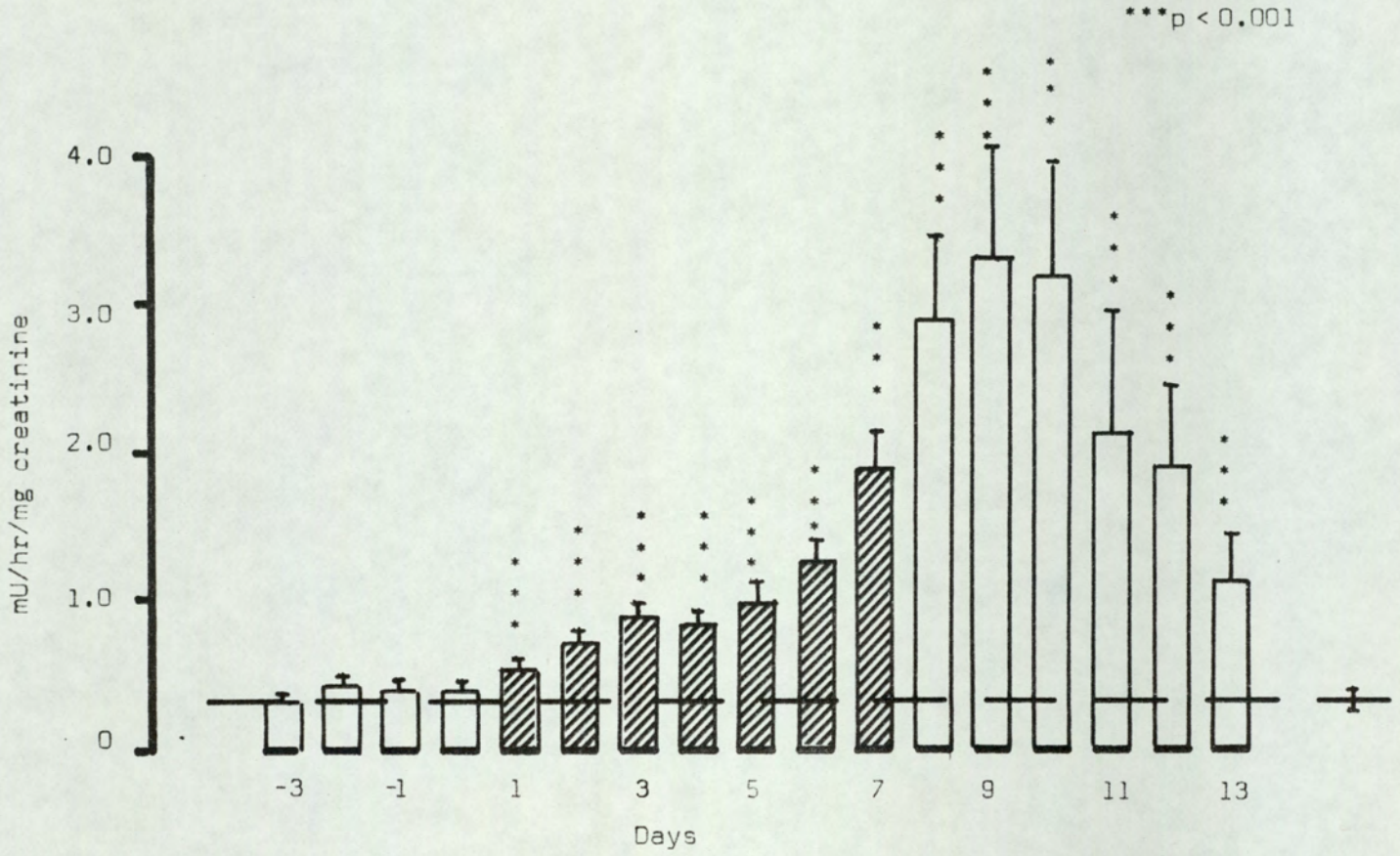


Fig. 80. LDH enzyme excretion in urine before, during and after 7 days of gentamicin (40 mg/kg/day) administration. In this and subsequent histograms in this subsection, the horizontal broken line represents mean ± s.e.m. of control group for all days. The period of gentamicin injection is shown by shaded bars and each bar is the mean ± s.e.m. of at least 4 rats.

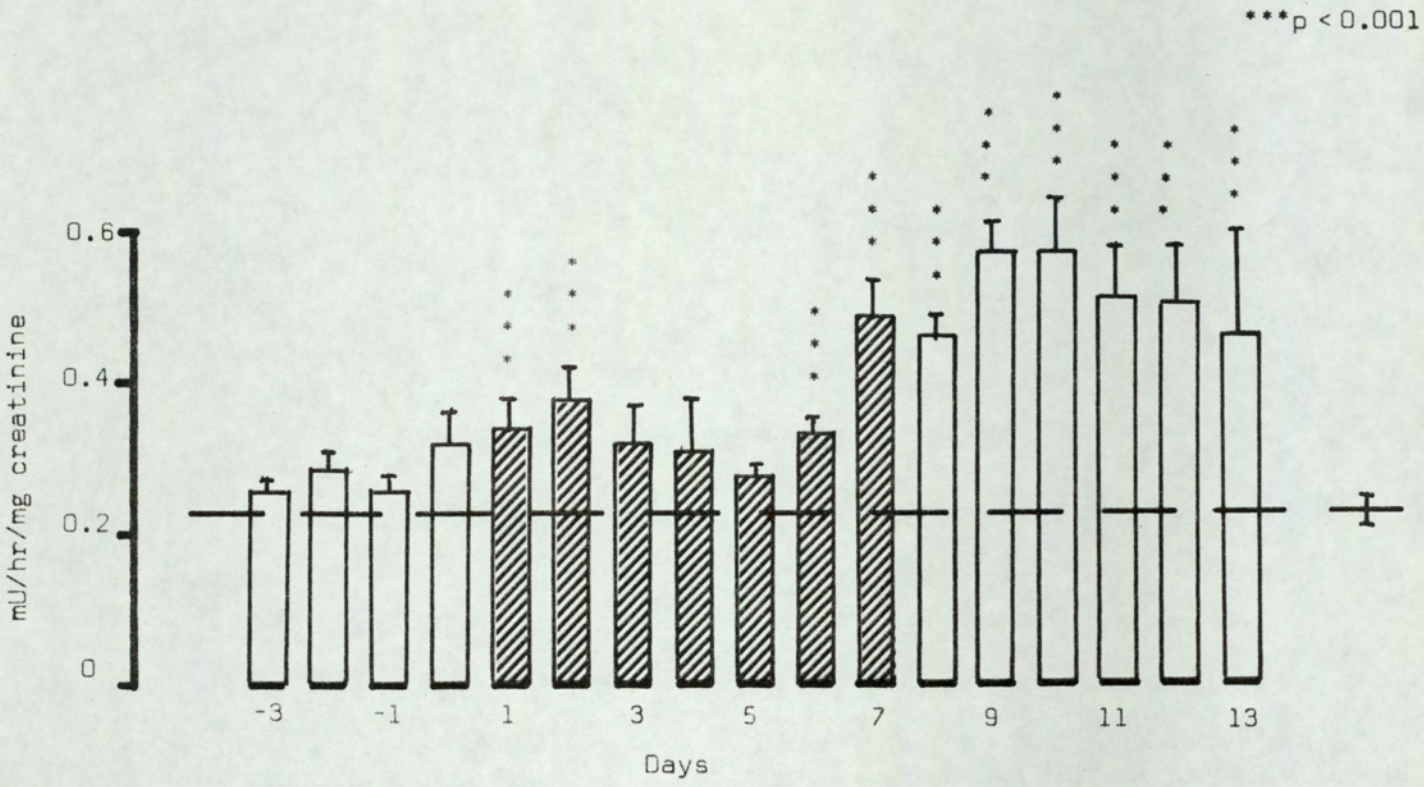


Fig. 81. Urinary NAG enzyme excretion before, during and after 7 days of gentamicin (40 mg/kg/day) administration.

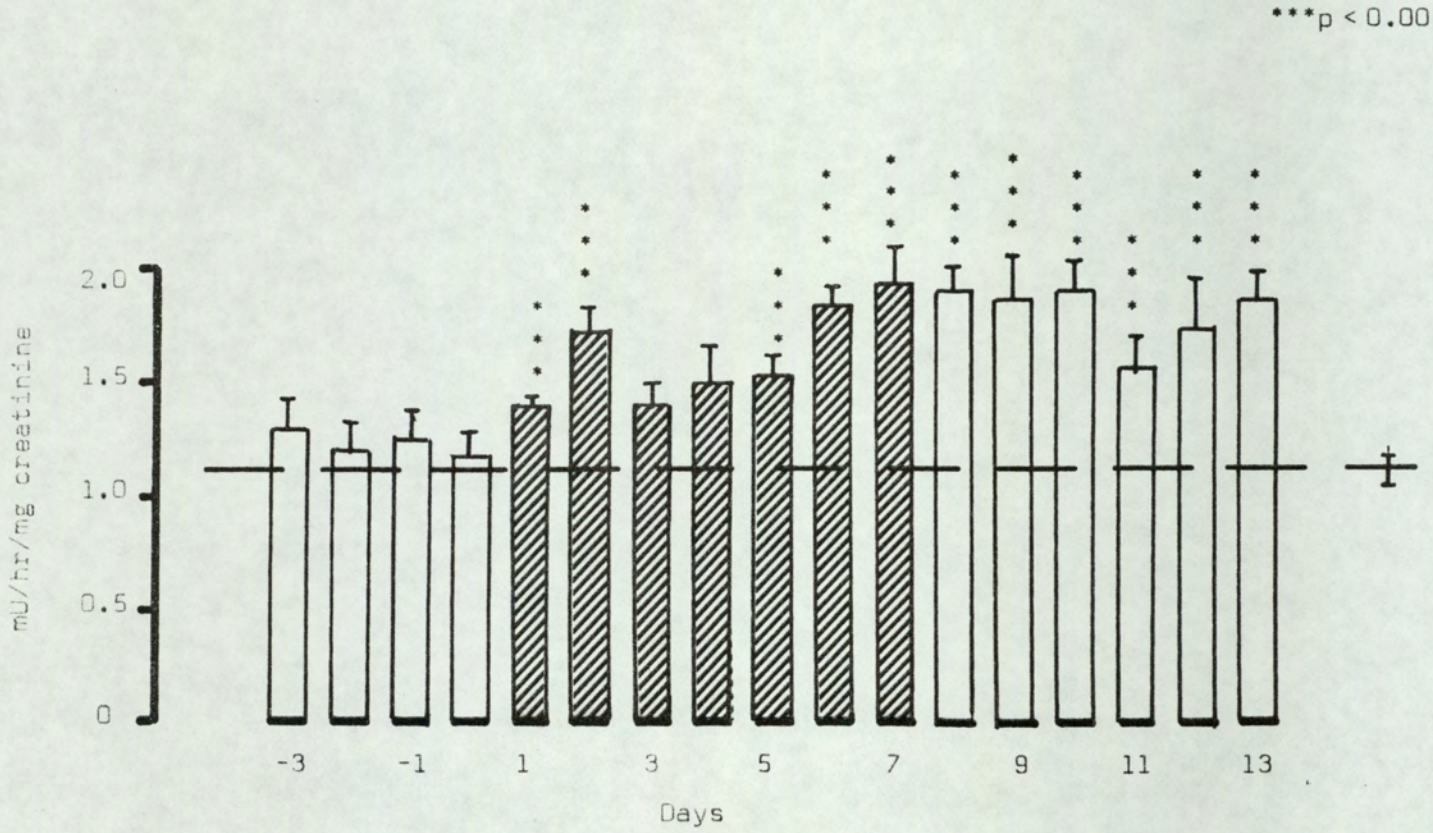


Fig. 82. Urinary AAP enzyme excretion before, during and after 7 days of gentamicin (40 mg/kg/day) administration.

4.7.3 Discussion

The excretion of enzymes in urine was compared with other parameters of renal function as indicators of nephron damage induced by gentamicin using rat as a model system, in both the short and long term studies. In addition, isolated rat renal tubules were prepared from kidneys of rats injected with gentamicin for 1, 3 and 5 days and the release of NAG and LDH determined on incubating at 37^{o}C for up to 2 hours.

It was quite clear from both the short term and long term studies that despite using the same strain of rats throughout, different groups showed varying sensitivities towards gentamicin in terms of changes in renal parameters measured. In fact, as well as group variation, individual rats within a group exhibited varying sensitivities to the drug.

There were no changes in the rate at which the rats' body weight and food intake increased on injection of gentamicin for 1, 3 and 5 days and also in the group given 7 injections. Similarly there was no significant change in water intake and urine output in 1, 3 and 5 day groups. There was however, an apparent rise in urinary volume seen by day 6 in the group administered with gentamicin for 7 days and remaining elevated even six days after the last injection.

Urine osmolarity was markedly reduced by day 6 ($p < 0.01$) and this reduction was significant up to day 10 ($p < 0.05$) (Fig. 75). This late onset of significant reduction in urine osmolarities explains why no significant changes were seen in the groups injected with gentamicin for 1, 3 and 5 days. Kosek *et al.* (1974) reported the effect of prolonged gentamicin administration (40 mg/kg/day for 28 days) in Fisher 344 strain of rats. In his studies,

animals developed oliguric renal failure with reduced urine osmolarity and urinary osmolal excretion. Body weights also changed with an average loss of 80 gm incurred during the course of the experiment.

Na^+/K^+ ratios were also determined in view of the possible effect of gentamicin on renal Na^+-K^+ ATPase as discussed in section 4.2. There did appear to be a depression of Na^+/K^+ ratio in urine at the commencement of gentamicin injection followed by a return towards the control on further injections. This apparent decrease in the ratios may have resulted from an increase in K^+ excretion although this was difficult to assess due to day to day fluctuations in the K^+ levels.

Collier, Lietman and Mitch (1979) showed potassium excretion to be slightly but significantly increased by perfusing rat kidney with gentamicin. During the 60 minutes of perfusion, gentamicin did not significantly change Na^+ reabsorption or glomerular filtration rate. The return of the Na^+/K^+ ratio towards the control may be due to a phenomenon known as 'potassium adaptation' by the rat kidney in which potassium homeostasis is maintained by a progressive increase in potassium secretion by the surviving nephrons (Fine, Yanagawa, Schultze et al., 1979).

Cuppage, Setter, Sullivan et al. (1977) reported no changes in excretion of K^+ or on the reabsorption of Na^+ in Sprague-Dawley rats given gentamicin 40 mg/kg/day for 28 days.

The divalent cations, Ca^{2+} and Mg^{2+} were also monitored since gentamicin therapy has been reported to cause hypocalcemia and hypomagnesemia (Bar, Wilson and Mazzaferri, 1975).

A large proportion of the dietary Ca^{2+} and Mg^{2+} intake is lost via the gut in the faeces (Walser, 1973) and only a small

amount of these electrolytes appear in the urine. Ca^{2+} may be actively transported from all parts of the nephron although the bulk of the filtered load is reabsorbed from the proximal tubule (Lassiter, Gottschalk and Mylle, 1963). The major site of Mg^{2+} reabsorption is the loop of Henle (LeGrimellac, Rionel and Morel, 1973).

In the present experiment Ca^{2+} excretion progressively increased during administration of gentamicin with a highly significant elevation by day 7 ($p < 0.001$). This rise over the control remained significant during the six days after the last injection. The effect of gentamicin on Mg^{2+} excretion was not as significant as Ca^{2+} excretion. The exact mechanism of gentamicin induced hypocalcemia and hypomagnesemia is not as yet understood.

It has often been suggested that assay of urinary enzymes may provide a convenient and more sensitive means of detecting kidney damage than either histological or other renal functional tests. In this study, changes in urinary excretion of LDH, NAG and AAP activities were determined. These three enzymes were chosen because of their specific locations within particular areas of the cell. LDH, an enzyme involved in glycolysis is almost completely of cytoplasmic origin whereas NAG is a lysosomal enzyme. AAP, a glycoprotein enzyme, is found in the brush border membrane (Mondorf, Breir, Hendus et al., 1978) of the kidney. Using these enzymes it was thought possible to locate the primary target of gentamicin nephrotoxicity.

All the urine samples were dialysed in order to eliminate low molecular weight inhibitors, before assaying for enzyme activity. Also, all enzyme excretion was expressed as enzyme activity per

mg creatinine in order to eliminate the effect of changes in the rate of urine flow. In this calculation, it was assumed that creatinine excretion is constant and that the urinary concentration of enzyme will depend on the rate of urine flow as well as the output of enzyme by the kidney.

In the present experiments, the groups injected with gentamicin for 1, 3 and 5 days showed considerable inter-group variation in the sensitivity to the drug and thus it was not easy to discern any specific pattern of enzyme release. However, it was observed that excretion of LDH and NAG was more significantly elevated than AAP.

The group of rats receiving gentamicin for 7 days showed a progressive increase in LDH excretion over the control, reaching a maximum two days after the last injection followed by a gradual fall in activity towards the normal. Nevertheless, LDH activity was significantly elevated all days during and after gentamicin administration ($p < 0.001$) (Fig. 80). The patterns of release of NAG and AAP were similar in that both showed an initial significant elevation ($p < 0.001$), followed by a return towards normal and then a further elevation which remained significant during the period of the experiment (Figs 81 and 82).

In this study, the maximum elevation of LDH was many times the normal, compared with the excretion of NAG and AAP. This greatly increased excretion of LDH into the urine therefore suggests an early membrane permeability change in the cells of the kidney and consequently releasing LDH into the urine.

It is interesting to note that changes in urinary enzyme excretion and other functional parameters measured in this study

tended to reach their maximum several days after gentamicin administration had been stopped. This indicates that the severity of gentamicin induced nephrotoxicity may not be fully expressed for several days and possibly imply an existence of an injury threshold. The functional expression of this threshold may be determined by the extent to which the rate of tubular cell damage is balanced by the rate of tubular cell regeneration.

This replacement of damaged tubular cell explains the reversible nature of aminoglycoside nephrotoxicity. The gradual decline in enzyme excretion seen during days 11 to 13 suggests recovery of renal function. It would have been interesting to see whether or not these rats were resistant to a second gentamicin insult. Numerous studies have been reported in which animals recovering from tubular necrosis were found to be resistant to a second nephrotoxic insult (Luft, Rankin, Sloan et al., 1978).

This would suggest that the regenerating tubular cells may be less susceptible to the toxic effect of gentamicin. It was discussed in sections 4.5 and 4.6 that gentamicin may be taken up by the proximal tubular cells by the process of endocytosis at the brush border membrane. Soberon, Bowman, Munoz et al. (1979) found in their studies that the brush border membrane of the regenerating proximal tubular epithelium to be less well developed than that of the mature cells. Thus, suggesting that these cells might accumulate gentamicin at a reduced rate and so are less likely to develop gentamicin toxicity.

The depression of urinary NAG excretion seen in the period a few days after the initial injections suggests a possible direct effect of gentamicin on the lysosomes and/or the lysosomal transporting pathway discussed in section 4.6. In order to

substantiate the hypothesis put forward in section 4.6 to explain the *in vitro* results and also the observations made here, isolated rat renal tubules were prepared from kidneys of rats injected with gentamicin (40 mg/kg/day) for 1, 3 and 5 days. These were then incubated at 37°C for up to 2 hours and the pattern of release of NAG and LDH determined.

After 60 minutes incubation of the renal tubules, the release of NAG was 225% of the tubules prepared from water-injected rats (set at 100%) following a single injection, increasing to 272% after 3 injections. The release after 5 injections (180%) was less than after 1 or 3 injections. The pattern of release of LDH was similar to NAG but did not closely parallel it.

Although tubular concentrations of gentamicin were not determined, it is reasonable to assume a progressive increase of gentamicin concentration with increasing number of injections. Sairio, Kasanen, Kangas *et al*. (1978) found a progressive increase in the concentration of free gentamicin in the rat kidney on administering gentamicin (40 mg/kg/day) for 1, 3 and 5 days. Thus, by comparing Fig. 69 with Fig. 56, it can be seen that the release of NAG from tubules prepared from 1 and 3 day injected rats is closely related to the release in the presence of gentamicin in the concentration range 10^{-9} - 10^{-8} M and 10^{-7} - 10^{-5} M respectively. The release of NAG from tubules prepared from rats injected for 5 days is related to the effect seen at very high concentrations of gentamicin (10^{-3} M).

However, it is possible that the lower level of NAG release observed for tubules from rats injected with gentamicin for 5 days represents tubular cells which are depleted of their enzyme content.

The absence of blood in all urine samples as determined by the'Labstix test' suggested that glomerular damage had not occurred to any great extent. Thus, proteinuria found in the present studies in rats receiving gentamicin (Figs. 62 and 76) was probably due to the enzymuria associated with gentamicin induced renal toxicity. Glucose, ketones and urinary pH, also appeared to be unaffected by gentamicin administration.

Electron micrographs of tubules prepared for rats injected with gentamicin (40 mg/kg/day) for 1, 3 and 5 days showed a pattern of changes related to the renal functional changes observed.

Summary

The excretion of urinary enzymes, LDH, NAG and AAP was compared with other parameters of renal function in order to determine which was the best indicator of nephron damage induced by gentamicin in a rat model. Also, by observing the chronological order of changes in renal function, it was thought possible to evaluate the primary toxic effect of gentamicin.

Proteinuria and decline in urine osmolarity which reflects polyuria did not prove to be an early indicator of nephron dysfunction due to gentamicin toxicity. Instead, the assay of the early appearance of enzymes in urine appeared to be a more sensitive means of detecting kidney damage.

The pattern of release of NAG and LDH from tubules prepared from rats injected with gentamicin for 1, 3 and 5 days correlated remarkably well with the release of enzyme from tubules incubated in the presence of gentamicin discussed in section 4.6.

SECTION 5

GENERAL DISCUSSION

General Discussion

In the present study an investigation of possible mechanisms of toxicity of the aminoglycoside antibiotics was made.

A number of hypotheses have been put forward for the basic cellular mechanism involved in aminoglycoside nephrotoxicity, but as yet none has been conclusively demonstrated.

The interaction of aminoglycosides with plasma membrane ATPases and polyphosphoinositides has been speculated as one possible mechanism of drug-induced nephrotoxicity and ototoxicity (section 4.2).

In order to study the Na^+-K^+ ATPase activity in the presence of aminoglycosides, an assay was developed which enabled inorganic phosphate to be detected in the presence of aminoglycosides. The new assay method was simple, rapid and the coloured complex formed was relatively stable with time and substantially free from interference by aminoglycosides (section 4.1).

This procedure was employed for the investigation of the effect of aminoglycosides, in particular gentamicin, on Na^+-K^+ ATPase of the erythrocyte and kidney cortical and medullary microsomal fractions. At a concentration of 10^{-2} M there was inhibition of Na^+-K^+ ATPase by gentamicin and neomycin but not with ribostamicin. The effect of gentamicin on Rb^{86} uptake by isolated rat renal tubules was also investigated. The extent of inhibition caused by gentamicin was similar in the different type of preparation used, suggesting a close relationship between the model systems. However, from these studies it seemed unlikely that inhibition of Na^+-K^+ ATPase by the aminoglycosides was the primary mechanism of nephrotoxicity and ototoxicity *in vivo* (section 4.2.).

Accumulation of gentamicin in the renal cortex precedes renal dysfunction. Thus, an analysis of gentamicin transport by rat renal cortical slices was conducted in an attempt to elucidate the mechanism by which gentamicin gains access to cortical cells, a mechanism which may be responsible for development of nephrotoxicity.

Consistent with previous *in vivo* and *in vitro* observations, the uptake of gentamicin into kidney slices was found to be both time and concentration dependent, showing saturable characteristics. Studies of the effect of temperature, anaerobic conditions and metabolic inhibitors indicated the existence of more than one mode of accumulation. The results suggested two mechanisms, one which was dependent on energy and the other involving diffusional transport in the uptake of gentamicin by kidney slices (section 4.3).

However, one of the many problems associated with using kidney slices is that the slice model may represent only the peritubular rather than brush-border transport events, since it has been observed that the lumens of slices are collapsed (Foulkes, 1971 and Silverman *et al.*, 1979).

Isolated rat renal tubules were thus prepared in view to be used as a model system for elucidating mechanisms of aminoglycoside toxicity. Isolated tubules offer advantages over cortical slices of better oxygenation and substrate availability to the tubule cells, and have lumens which are patent (Fig. 31).

The morphology and viability of the tubules was ascertained by phase contrast and electron microscopy and also by gluconeogenesis, incorporation of $|^{14}C|$ leucine into TCA insoluble tubular material and uptake of $|^{14}C|$ lysine. The fragmented tubule preparation exhibited a high rate of gluconeogenesis with a linear rate of

protein synthesis and saturable lysine uptake. These results, together with microscopic evidence, suggested a high degree of cellular integrity of the tubules (section 4.4).

This tubular preparation was used to further characterise proximal tubular aminoglycoside transport. A knowledge of the mechanism of uptake would enable protection of the kidney from aminoglycoside induced nephrotoxicity by blocking uptake either by use of competitive inhibitors or by altering the structure of the antibiotic.

The results of the study discussed in section 4.5 showed the uptake of gentamicin by the tubules to be initially rapid followed by a continued slower uptake. The accumulation was significantly inhibited by anoxia, 2,4-DNP, KCN and ouabain. The uptake was not inhibited by basic amino acid lysine, suggesting a transport of gentamicin by a mechanism other than that used by basic amino acids. It was not ascertained whether the accumulation of gentamicin occurred from the luminal side, antiluminal side or both sides of the tubular cell. Gentamicin, once inside the cell may exert its toxic effect by inhibiting gluconeogenesis. There was no effect on the protein synthesis carried out by the tubules.

Numerous experiments by other workers have provided evidence to suggest a close association between accumulation of gentamicin in lysosomes with consequent formation of myeloid bodies and aminoglycoside nephrotoxicity. Although, the participation of the lysosomes in the toxic process generated by gentamicin is readily apparent, the mechanism of its involvement has not been elucidated. Also, in response to an early appearance of lysosomal enzymes in urine _in vivo_ and the morphological changes observed, the effect

of incubating gentamicin with proximal tubules on the release of lysosomal enzymes AP and NAG were studied (section 4.6).

The release of lysosomal enzymes from isolated tubules after incubation with gentamicin suggested a complex interaction between the gentamicin concentration and the time of incubation. The release of NAG from tubules incubated in the absence of gentamicin, and prepared from kidneys of rats injected with gentamicin (40 mg/kg/day) for 1, 3 and 5 days was also studied. On comparing Fig. 69 with Fig. 56 it was shown that the release of NAG from tubules prepared from 1 and 3 day injected rats was closely related to the release from tubules incubated in the presence of gentamicin in the concentration range 10^{-9} - 10^{-8} M and 10^{-7} - 10^{-5} M respectively. The release of NAG from tubules prepared from rats injected for 5 days was related to the effect seen on incubating tubules with very high concentrations of gentamicin (10^{-3} M). The findings could possibly be explained by an effect of gentamicin on receptor-recycling by a differential effect on the two lysosomal enzyme transport pathways (section 4.6). However, it must be stressed that this is only an indirect evidence of lysosomal enzyme transport pathway implicated in the gentamicin effect.

On the basis of these findings, it may be possible to use these tubules to predict the nephrotoxic potential of a new aminoglycoside antibiotic from the pattern of enzyme release after exposure to the antibiotic. For example, an interesting comparison would be the pattern of enzyme release with gentamicin with that obtained with ribostamicin which is known to be less nephrotoxic and ototoxic than gentamicin.

Raab (1972) has suggested that in evaluation of new drugs, comparative studies using renal enzyme excretion are of highest

value to determine the relative nephrotoxic action of the new drug in comparison to a standard drug. Also, determinations of urinary enzymatic activities are easier to perform, and for the patient, are far more agreeable than most other urologic/nephrologic diagnostic tests.

The excretion of urinary enzymes, LDH, NAG and AAP were compared with other parameters of renal function in order to determine which was the best indicator of nephron damage induced by gentamicin in a rat model. The results demonstrated the ability of the rat kidney to recover from gentamicin nephrotoxicity. The recovery was evidenced by the return of the enzymatic activity in urine and defect in urine concentrating ability towards their pre-injected status. Also, in accordance with Raab (1972), it was concluded that the assay of the early appearance of enzymes in urine was a more sensitive means of detecting kidney damage (section 4.7).

The important question for the future is whether the aminoglycosides can be made less toxic and yet retain their antibacterial activity. The isolated rat renal tubules may be potentially used as a relevant model for assessing toxic effects of new aminoglycosides. They may possibly be used to resolve the problems not only of the aminoglycoside induced-nephrotoxicity but also of ototoxicity and neuromuscular blockade which possibly share a common molecular mechanism.

However, the observations made must be viewed with caution and perhaps another in vitro model system such as the isolated hepatocytes must also be used to define specificity. Also, in vitro comparisons of aminoglycoside potency may not hold true when the

agents are compared to the human situation. The use of animals such as the cat or the monkey may provide additional information, or information more comparable to the human situation.

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