Discussion
DISCUSSION

There are a number of methods available for the investigation of the anatomical and functional status of the kidneys. These include radiography, biopsy and the calculation of clearance values for various compounds. The measurement of the glomerular filtration rate, and the ability of the kidney to secrete an injected compound such as p-aminohippuric acid can be used to determine tubular functions.

Gross kidney damage can often be detected by determining the degree of proteinuria $p^H$, glucose, albumin and by examining the urinary sediment, but if only slight renal damage has occurred, then histological examination of the kidneys may be the only method available: This latter procedure has the disadvantage that the animal has to be killed and continuous measurement cannot be made therefore.

The extent of nephrotoxicity can be detected by measuring the activities of some enzymes excreted in urine (Wilkinson, 1968). Renal damage can be due to various diseases as well as by certain toxic compounds. Administration of nephrotoxic compounds results in the release of certain kidney enzymes into the urine (Raab, 1968). Harrison and his colleagues (1968) found elevated urinary levels of mura minidase and ribonuclease in factory workers who showed signs of multiple reabsorption defects due to cadmium poisoning.

Some enzymes act as markers for specific regions of the cells or organelles. Hence by measuring their activities in urine, it is hoped to discover the site of primary lesion and to determine the extent to which the other cellular compartments are affected.
The purpose of assaying urinary enzymes in animals is for two reasons. Firstly, they provide a sensitive non-invasive test for renal damage and with careful selection of enzymes they can be used to determine the primary site of damage in the nephron. Secondly, because the excretion of urinary enzymes is dose-related they can be used to assess the nephrotoxicity of new drugs. They also have considerable potential for the screening of industrial workers involved in hazardous processes, involving nephrotoxic materials.

In man, urinary enzymes can be used in the clinical management of patients receiving nephrotoxic drugs. The changes in the urinary profiles are easiest to interpret when patients with non-renal diseases are given nephrotoxic drugs. When nephrotoxic drugs are given to patients with renal disease (e.g., gentamicin), elevation of urinary enzymes due to the toxicity will be superimposed on those resulting from renal disease. This may cause analytical problems, but provided daily assays are carried out and the drug regimen is known, these difficulties can be overcome. (Whiting et al., 1978). This increased enzymuria reflects the influence of drugs on the proximal tubule.

It has been suggested that cationic compounds like aminoglycosides are reabsorbed into the kidney tubules by endocytosis, transferred to lysosomes and stored. Increased accumulation of aminoglycosides, leads to degradation of lysosomes, and eventually to cell death. Lipoic acid (at higher concentration) which has the ability to regenerate lost tubular cells, prevents the nephrotoxic nature of aminoglycoside by a mechanism yet to be investigated.
Pattern of proteinuria, called tubular proteinuria, consists of proteins of molecular weight below 40,000 daltons, mainly beta-2, micoroglobulin, retinol binding protein (RBP) lysozyme (LZM), immunoglobulin light chains and post-gamma globulins. The molecular weight of β2 microglobulin is 11,800 daltons. RBP is an alpha-2-microglobulin with a molecular weight of 21,000 daltons. LZM is a protein which acts as bacteriolytic enzyme with a molecular weight 14,000 daltons.

Such a reabsorption defect can be detected in various renal diseases unrelated to any antibiotic treatment e.g. congenital tubulopathies, transient proteinuria associated with hyperthermia, acute renal insufficiency and interstitial nephritis (Revillard et al., 1970). Proteinuria has also been described in heavy metal poisoning with cadmium (Peterson, 1969) or mercury (Pesce and First, 1979) and after treatment with gold (Merle et al., 1980) and in the urine of patients with renal calculus disease. Experimental models have shown that the reduction in renal mass induces an increase in glomerular filtration rate of the remnant nephrons leading to proteinuria and glomerular sclerosis (Coppo et al., 1988).

The direct effect of aminoglycoside on protein degradation may be due to an increase in intralysosomal pH and subsequent decrease in the activity of proteolytic enzymes. Treatment of rats with gentamicin decreased the lysosomal activity of cathepsin B (Morin et al., 1980), a proteolytic enzyme responsible for the lysosomal breakdown of lysozyme (Yuzuriha et al 1977). Other molecular weight proteins and peptides such as β2-microglobulin, immunoglobulin light chains and growth hormone appear to be handled like lysozyme by the kidney, whereas small linear peptides such as glucagon are degraded in the proximal tubule by enzymes localized at the brush border membrane (Peterson et al., 1982). Larger and more complex peptides and proteins such as insulin and
lysozyme undergo endocytosis and transfer to lysosomes where hydrolysis by lysosomal proteases occur (Carone et al., 1982; Christensen and Maunsbach, 1974).

Our studies also showed a marked increase in urinary proteins during gentamicin treatment and simultaneous lipoic acid treatment decreased the protein levels in the treatment group VI.

d. Creatinine

Creatinine, a commonly excreted urinary product rose significantly in gentamicin treated groups which indicates the loss of reabsorptive nature of the proximal convoluted tubules. All drugs that act on nephrons tend to increase the creatinine excretion in urine. Reports by Luft and Evan (1980), shows that administration of aminoglycoside produces glomerular and tubular alterations in the ultrastructure and function of kidney, thus leading to depression of glomerular filtration rate. Administration of 5 mg lipoic acid to gentamicin treated group, significantly reduced the excretion of creatinine, suggesting the possibility of protection of kidney tubules against the nephrotoxic effect.

e. Phosphorus

Phosphate enters the kidney by glomerular filtration and exits by tubular reabsorption and urinary excretion (Bijvoet et al., 1974). The amount of phosphorus excreted in urine naturally depends mainly on the amount of phosphorus taken in the diet. Increased phosphorus excretion in urine was obtained after administration of gentamicin. Increased levels of phosphate and uric acid excretions have been reported in conditions of foot shock stress. Administration of sodium glycollate to rats also increased the urinary excretion of phosphorus. (Murthy et al 1981; Varalakshmi et al., 1990). The
reduction in excretion of phosphorus, during administration of lipoic acid (5mg) may suggest a possible protection against tubular damage.

f. Urinary Phospholipids

Administration of gentamicin induces phospholipidosis in the rat renal cortex, with phosphatidylinositol manifesting the greatest fractional increase of the different phospholipid classes. This phospholipidosis was evident within 24 hr of a single dose of administration (Feldman et al., 1982). This increased cortical phospholipid content is reflected in the urinary excretion, leading to phospholipiduria.

It has been reported, that other classes of aminoglycosides also have the ability to induce phospholipiduria. Neomycin caused a significant increase in urinary phospholipid excretion after the first dose of drug, with further increases evident after the second and third doses. The increased urinary phospholipids are derived in part from the excretion of lysosomal myeloid bodies as well as the loss of proximal tubular cell brush border membrane (Josepovitz et al., 1986). The depression in phospholipid synthesis due to toxic injury, would magnify in urinary losses.

Studies with amikacin has shown to increase cortical phospholipidosis that would reflect in urinary excretion. (Laurent et al., 1982). It may be suggested that the increased excretion of phospholipids by aminoglycosides may be due to decreased activity of sphingomyelinase in kidney cortex. A similar pattern was seen with mercuric chloride administration (Josepovitz et al., 1985). The effect produced by lipoic acid in gentamicin treated rats was that of reduction in urinary phospholipids, excretion, which is likely to be the result of lowering of kidney cortical phospholipids as seen in glycollate treated rats (Sumathi, 1991).
II. Urinary enzymes

a. Alkaline phosphatase

Alkaline phosphatase is one of the marker enzymes assayed regularly in urine. Under normal conditions, urinary alkaline phosphatase derives from the kidneys. It serves as a marker for plasma membrane and the endoplasmic reticulum. Highest enzyme activity is found in brush border membrane and in the epithelial cells lining the convoluted tubules (Scherberich et al., 1984). Urinary alkaline phosphatase is similar to the kidney enzyme and Butterworth (1968) suggested that it is probably a subunit of kidney alkaline phosphatase. Urinary enzyme originates from the kidney enzyme and is modified after its release into the urine (Wright et al., 1972). It also possess pyrophosphatase activity. (Moss and Eaton, 1967; Felix and Fleisch, 1974).

The isoenzyme profiles of urinary alkaline phosphatase may aid in the diagnosis of renal diseases (Cornell et al., 1979; Cornell, and Hodson, 1980). In normal individuals, most of the urinary alkaline phosphatase activity is sedimentable and has similar properties to the liver enzyme (Pfleiderer et al., 1980). In contrast, in renal disease, transplant rejection or when nephrotoxic antibiotics are used, the increased urinary enzyme activity results from the presence of an intestinal like form of alkaline phosphatase. The new form of alkaline phosphatase probably arises from the plasma membrane of the proximal convoluted tubule where it is an intrinsic membrane protein (Scherberich et al., 1976).

All diseased states accompanied either by necrosis, decomposition or desquamation of renal tubular cells or by disturbances of glomerular filtration, provoke increased alkaline phosphatase activity in the urine (Gault and Steiner, 1965). Enhanced alkaline
phosphatase activity could be demonstrated in rats following toxic or shock induced kidney damage such as anaphylactic shock (Raab, 1968). Patients suffering from renal artery embolism (Donadio et al., 1986) and chronic renal diseases also showed increased activity of the enzyme in urine.

Administration of nephrotoxic drugs such as streptomycin, polymyxin-B and sulphonamides, increased alkaline phosphatase activity in urine (Jung et al., 1987). Wright and Plummer (1974) showed an maximum excretion of alkaline phosphatase activity in urine after the administration of compounds like uranyl nitrate, 4-nitrophenylarsoonic acid, 4-aminocatechol and 4-nitrocatechol.

Alkaline phosphatase excretion in urine was induced by administration of lethal doses of mercury (Sternberg et al., 1974; Stroo and Hook 1977; Braun et al., 1978), cadmium, (Bonner et al., 1980) and lithium (Emanueli et al., 1985).

Patel et al (1975) reported an increase in the activity of alkaline phosphatase, during gentamicin administration to experimental animals receiving a dosage of 30-60 mg gentamicin per kg body weight per day for 15 days. Later reports by Luft's group (1975, 1978) also confirm an increase in urinary excretion of these enzyme. The increased alkaline phosphatase activity in urine may correlated with the decrease in kidney alkaline phosphatase activity found in experimental rats. (Kacew, 1989). In the present study lipoic acid treatment was found to reduce the gentamicin induced alkaline phosphatase activity in urine by six folds.
b. Lactate dehydrogenase (LDH)

Lactate dehydrogenase (LDH) is one of the most frequently assayed enzymes in urine, and it is raised in a variety of diseases of the urinary tract. LDH is mainly found in the cytoplasm of the soluble fraction of the cell. Urinary LDH activity may be derived from serum, the kidney cells, blood cells and from prostatic secretion. In the present study, an elevation of LDH activity was noticed in gentamicin treated groups. Increased excretion of LDH, which is a cytoplasmic marker, can be considered as a biochemical marker of nephron damage.

An increase in the excretion of LDH was observed after the administration of mercuric chloride (Hofmeister et al., 1986), phenacetin and paracetamol (Metz et al., 1986), peak excretion rate of about 90 times the normal range was observed after administration of uranyl nitrate and 200 times the normal range after administration of 4-nitrophenylarsonic acid (Wright and Plummer, 1974). Assay fo LDH isoenzymes is most useful early detection for the adequate therapy for poly nephritis. The value of study of LDH isoenzymes has been demonstrated by a number of workers. (Carvajal et al., 1975; Devaskar and Montgomery, 1978). In patients with bladder infections LDH-5 was lower, than in renal infections. In the latter, LDH-4 and LDH-5 predominate. A correct diagnosis was made in 94% of patients on the basis of the iso enzyme profile in urine (Carvajal et al., 1975) and this helped to differentiate between kidney and bladder infections. Thus the determination of LDH in urine proves valuable in predicting early nephrotoxicity.

Increased excretion of LDH after administration of amikacin and gentamicin to healthy subjects was observed by Calcamugi et al (1988) which support our observation. Lipoic acid administration proved to be effective in bringing down the excretion of LDH caused by gentamicin by five folds.
c. **Inorganic pyrophosphatase**

Inorganic pyrophosphatase is a kidney enzyme and hydrolyses pyrophosphate to orthophosphate, which inhibits the enzyme action. The dialysable components in urine like oxalate and sulphate ions are found to inhibit pyrophosphatase activity at $p^H$ 3.5, while urea showed mild activation (Wakid *et al.*, 1970).

An increased excretion of urinary inorganic pyrophosphatase was observed in the present study, in gentamicin treated groups, which fell nearly to control values after the administration of lipoic acid.

d. **Gamma-glutamyl transferase: ($\gamma$GT)**

Gamma-glutamyl transpeptidase catalyses the transfer of gamma-glutamyl residues from glutathione and other gamma glutamyl peptides to various acceptors. $\gamma$-GT is a brush border enzyme, more deeply localised in the membrane. The determination of $\gamma$-GT prove useful in the diagnosis of proximal tubule damage. Elevated urinary excretion of $\gamma$-GT was observed in stone-forming rats as in patients with idiopathic calcium oxalate lithiasis (Baggio *et al.*, 1983). A moderate increase in the excretion of $\gamma$-GT was found following mercuric chloride treatment (Hofmeister, 1986). Urinary assay of $\gamma$-GT was found to be a more sensitive indicator of renal tubular damage resulting from uranyl acetate. (Nomiyama *et al.*, 1974) and sodium fluoride poisoning (Kessabi *et al.*, 1980).

Kolbe *et al.*, (1976) reported a relatively small increase in $\gamma$-GT after the administration of antibiotics. Administration of gentamicin sulphate resulted in progressive renal tubular damage, leading to increased $\gamma$-GT excretion (Gossett *et al.*, 1989). Pariat
et al (1990), observed that the excretion of γ-GT was affected by seasonal changes, during gentamicin induced nephrotoxicity.

γ-GT showed a significant increase in the urine of gentamicin treated rats when compared to controls. Lipoic acid treatment had two fold lowering effect on the enzyme level when compared to gentamicin treated animals.

e. β-Glucuronidase

β-Glucuronidase is an exoenzyme which cleaves uronic acid residue from the non-reducing end of the glycosaminoglycans. β-glucuronidase from normal mouse and human urine has been successfully purified (Pettengill and Fishman, 1962; Hygstedt and Jagenberg, 1965). β-glucuronidase is derived from the kidneys (lysosomes of tubular cells) and from the epithelial cells of the urinary tract. β-glucuronidase has been implicated in the biosynthetic path of L-ascorbic acid, since one of the products of the reaction is D-glucuronic acid, a clearly established precursor of Vitamin C. (Mora et al., 1965).

The enzyme activity was found to be increased in gentamicin treated rats significantly (Kacew, 1989) which correlate with our observation.

Increase in β-glucuronidase activity was also observed in diabetes melitus patients (Delektorskaya et al., 1990). All acute inflammatory and toxic renal lesions are accompanied by an increased urinary β-glucuronidase activity (Bank and Bailine, 1965). After administration of caffeine and furosemide (Adelman et al., 1979), there was an increased urinary β-glucuronidase activity in rats. In schistosomiasis of the bladder, an increased β-glucuronidase activity was found (Fripp, 1960). Lipoic acid treatment resulted in mild to moderate decrease in β-glucuronidase activity in gentamicin treated rats (four
fold decrease). These results may be compared to the results obtained after administration of L-tartrate in stone forming rats (Padmaja and Varalakshmi, 1989).

f. N-acetyl-beta-glucosaminidase

NAG is a hydrolase enzyme situated mainly in the lysosomal fraction of renal tubules, and with a small amount in the microsomal fraction. Its molecular weight has been found to be 1,30,000 to 1,40,000 and it is not normally filtered by the glomeruli (Prince and Dance, 1967). A good correlation has been observed between pathological changes in the renal tubules and urinary NAG activity. (Wellwood et al., 1978).

Isoenzyme profiles demonstrated that the increased NAG activity was derived from the tubular cells and not the serum. The presence of clearly distinguishable isoenzyme patterns in human serum, urine and kidney, enhances the diagnostic potential of NAG and an increase in the amount of B-form originating from the renal tubule occurs in renal disease (Ellis et al., 1975).

Urinary excretion of NAG acts as a main indicator for the tubular degeneration caused by nephrotoxic agents like antibiotics, heavy metals, analgesics, anti-inflammatory drugs, kidney transplants and in other renal and urogenital infections.

A large increase in the urinary excretion of NAG activity and its isoenzyme level, NAG-B, was observed by Cubey and Henery (1991) after gentamicin administration. Similar results were shown by Kitasato et al. (1989) and Wu et al (1990).

Colding et al (1992) observed 92% increase in urinary NAG activity in the neonates receiving continuous intravenous infusion of gentamicin.
Present study shows an approximately a ten fold increase in the excretion of urinary NAG after gentamicin treatment in rats. Administration of Lipoic acid to gentamicin treated group reverted the increased excretion of NAG towards that of control values.

Possible mechanisms of reduction of gentamicin toxicity by lipoic acid

Gentamicin, a well known aminoglycoside used in treatment of gram negative infection, is characterised as nephrotoxin, on chronic exposure. These are cationic in nature, which are filtered by the glomerulus and reabsorbed into the kidney tubular cells by endocytosis. Gentamicin is transferred to lysosomes and gets stored here. Concentration of gentamicin in lysosomes leads to rupture of lysosomes and release of hydrolases resulting in cell necrosis, and release of enzymes in urine.

Recent experiments by Loghman et al (1991) proved that membrane sulphydryl groups are essential for Na\textsuperscript{+}Pi transportation in rat kidneys. The membrane sulphydryl groups may be present on the cytoplasmic side of brush border membrane and may play an important role to maintain membrane integrity. Binding of gentamicin to membrane phosphoinositol (Sachacht, 1977), alters the membrane integrity, thus leading to loss of membrane sulphydryl groups.

DL α-Lipoic acid, a dithiol, is known to provide the source of external sulphydryl group replaces the lost membrane sulphydryl group, (Huagaard and Huagaard, 1970) due to the action of gentamicin. The supplementation of external sulphydryl group by lipoic acid might have restored the normal 3-D structure of membrane, and thereby preventing the gentamicin induced losses.
The second possibility for preventive role of lipoic acid may be due to its anionic nature in solution.

Aminoglycosides are cationic in nature. Several experiments with poly.L. aspartic acid and poly.L. glutamic acid have proved to reduce the nephrotoxicity of gentamicin. These compounds compete for the same binding sites for gentamicin, in brush border membrane, and subsequently displace them. (Kishore *et al.*, 1990).

In the present experiment, lipoic acid reduced the gentamicin induced nephrotoxicity, enzynmuria and histophtological changes. This may also be due to the anionic nature of lipoic acid in solution which might also compete for the same binding sites with gentamicin, or neutralise them in order to reduce the nephrotoxicity.