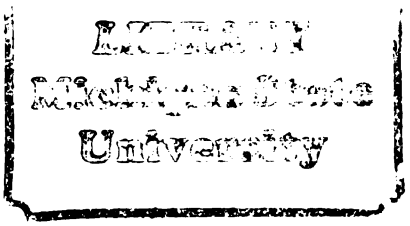

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BIOCHEMICAL MECHANISMS OF CEPHALORIDINE-INDUCED NEPHROTOXICITY

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CHAO-HEN KUO

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Major professor

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BIOCHEMICAL MECHANISMS OF CEPHALORIDINE-INDUCED NEPHROTOXICITY

By

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ABSTRACT

Biochemical Mechanisms of Cephaloridine-Induced Nephrotoxicity

By

Chao-Hen Kuo

The purpose of this investigation was to determine the mechanism(s) responsible for cephaloridine-induced nephrotoxicity. Phenobarbital treatment induced mixed-function oxidases in rabbit renal cortex and also potentiated cephaloridine nephrotoxicity. On the other hand, treatment with piperonyl butoxide reduced cephaloridine nephrotoxicity in both rabbits and rats. A higher renal cortical concentration of cephaloridine was detected in phenobarbital-treated rabbits. Similarly, treatment with piperonyl butoxide decreased renal cortical accumulation of cephaloridine. Therefore, the potentiating and protective effects of phenobarbital and piperonyl butoxide, respectively, on cephaloridine nephrotoxicity might be due to increased and decreased renal cortical accumulation of the parent drug.

Cephaloridine was found to be most nephrotoxic to rabbits, intermediate in toxicity to rats, and least toxic to mice. The relative susceptibility of these three species to GSH depletion in the renal cortex shortly after administration of cephaloridine paralleled species difference in nephrotoxicity of cephaloridine. In addition, pretreatment of animals with diethyl maleate potentiated cephaloridine

nephrotoxicity. Furthermore, cephaloridine markedly increased GSSG [coupled with the decreased GSH] in rat and rabbit renal cortex. These changes between GSH and GSSG could be the result of increased lipid peroxidation. Formation of conjugated diene in renal cortex was increased shortly following administration of cephaloridine. Removal of selenium and/or vitamin E from the diet potentiated cephaloridine nephrotoxicity suggesting that lipid peroxidation may be involved with cephaloridine nephrotoxicity. Alternatively, the high ratio of GSSG to GSH concentrations could be due to decreased NADPH concentrations in renal cortex. Low NADPH concentrations have been shown to inhibit several essential cellular functions. In addition, high concentration of GSSG alone will block many biochemical processes vital to the functions of renal cortical cells. Thus, cephaloridine-induced nephrotoxicity may be explained by one or a combination of the following three closely related potential changes, increased lipid peroxidation, increased intracellular GSSG and decreased NADPH.

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INTRODUCTION

In 1945 Brotzu isolated a fungus, Cephalosporium acremonium, from the sea near a sewage outlet in Sardinia. Crude extract from cultures of this fungus was found to cure typhoid fever and Brucella infection. A culture of this microorganism was sent to Oxford, where seven antibiotic substances were isolated (Florey, 1955). Three of these were cephalosporin N, a new type of penicillin which is active against gram-negative and gram-positive microorganisms; cephalosporin P, which has a steroid structure and is active only against gram-positive microorganisms; and cephalosporin C which is less potent than cephalosporin N but possesses the same range of antimicrobial effectiveness. The nucleus of cephalosporin C is 7-aminocephalosporanic acid and with the addition of side chains, it became possible to produce semisynthetic cephalosporins with antibacterial activity much greater than that of the parent substance.

Cephaloridine (Figure 1), 7-[(2-thienyl)acetamido]-3-(1-pyridylmethyl)-3-cephem-4-carboxylic acid betaine, is one of the semisynthetic derivatives of cephalosporin C (Loder et al., 1961). This antibiotic has a broad spectrum of activity against many gram-positive and gram-negative microorganisms (Murdoch et al., 1964; Turck et al., 1967). It has no action against fungi, protozoa, or helminths and has only low

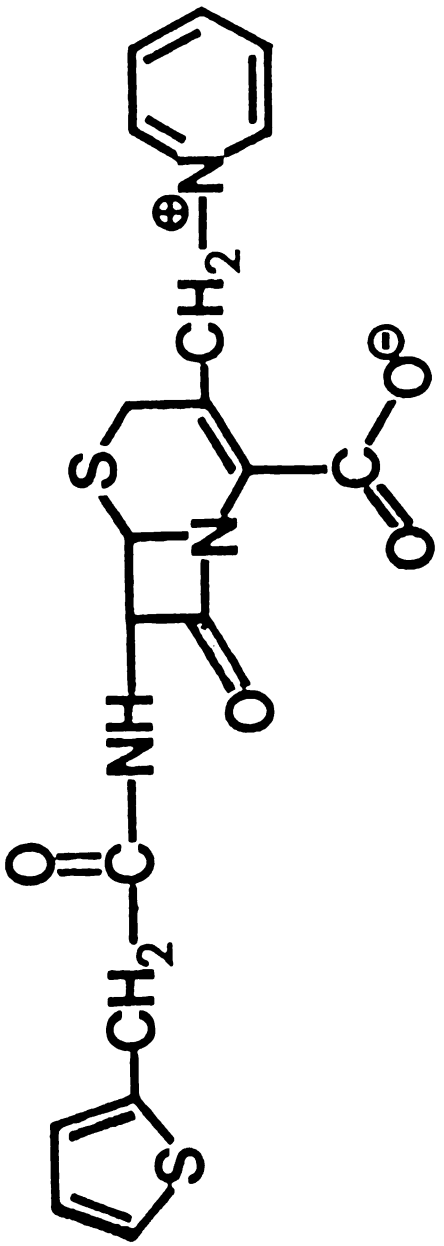


Figure 1. The structure of cephaloridine.

activity against Mycobacterium tuberculosis. The antibiotic is highly bactericidal, particularly against gram-negative organisms.

Pharmacokinetics of Cephaloridine

Absorption and Distribution. Cephaloridine is poorly and irregularly absorbed from the gastrointestinal tract (Kislak et al., 1966). Serum concentration of the drug in humans after an oral dose of 1 g in the fasting state never achieved a level of 0.5 μg/ml at any time within 2 hours. In contrast, high serum concentrations (10-25 μg/ml) were achieved within 15 minutes when the drug was given parenterally. Welles et al. (1966) reported that the serum half-life of cephaloridine was 0.5 hr in the dog after a single intravenous injection. A similar serum half-life was also observed when the drug was administered intramuscularly, indicating a rapid absorption from the injection site. A relatively longer serum half-life was observed in humans. Kirby et al. (1971) reported a serum half-life of 1.12 hr in healthy volunteers. This value is very close to the 1.5 hr serum half-life in patients with normal kidney function (Kabins and Cohen, 1966). The serum half-life of cephaloridine is prolonged to 20-23 hr in patients with renal impairment (Kunin and Atuk, 1966; Kirby et al., 1971). Unlike cephalothin, another cephalosporin, cephaloridine is less protein-bound, about 20% compared to 65% for cephalothin (Kirby et al., 1971). Cephaloridine is distributed in a variety of tissues and also is found in the fetus and milk (Welles et al., 1966). Kidney tissue has the highest concentration of the drug; other tissues contain only one-tenth or less of the

concentration in the kidney (Welles et al., 1966). Most of the drug in the kidney is found in the cortical region (Tune et al., 1974).

Excretion. Only a small percentage of cephaloridine is excreted through the bile (Mandell, 1973). Biliary concentrations of the drug were found to be dose-unrelated and also to be lower than serum concentrations (Nishida et al., 1976). Elimination of cephaloridine occurs mainly through the kidney. In dogs, approximately 92% of intravenously administered drug was recovered in the urine within 6 hours (Welles et al., 1966). More than 80% of the drug was excreted in the urine within 24 hours in humans (Kirby et al., 1971). Child and Dodds (1966) first investigated the mechanism of urinary excretion of cephaloridine in various species of animals. Because of low protein binding, most cephaloridine appeared to be excreted by glomerular filtration (Child and Dodds, 1966). In addition, using the method of Sperber (1948), Child and Dodds found that excretion of cephaloridine by chickens was higher on the infused side and these authors suggested that tubular secretion of cephaloridine occurred in hen kidneys. However, this may not be true for other species. Renal clearances of cephaloridine in anesthetized rabbits, cats, dogs and monkeys were 1 to 5 ml/kg/min, very closed to the value found in humans, 3 ml/kg/min (Kirby et al., 1971). The ratios of cephaloridine to creatinine clearance in these animals ranged from 0.30 to 1.20, indicating that there was not secretion but a small reabsorption of cephaloridine. A saturable tubular reabsorption in humans was also suggested (Arvidson et al., 1979). Although renal clearance studies did not provide evidence of renal tubular secretion of cephaloridine, it is possible that bidirectional tubular transport of

the drug may occur. In order to investigate this possiblity, Child and Dodds (1967) used stop-flow techniques (Malvin et al., 1958) to re-examine nephron transport of cephaloridine in dogs and rabbits. The results indicated that cephaloridine was neither secreted nor reabsorbed by the renal tubules. Furthermore, probenecid, an inhibitor of organic acid transport, was shown to block the active secretion of cephaloridine in the hen kidneys (Child and Dodds, 1966), but this inhibitor had no effect on the stop flow pattern of cephaloridine in dogs and rabbits (Child and Dodds, 1967) nor the renal clearance of cephaloridine in dogs (Welles et al., 1966). These results led Child and Dodds to conclude that cephaloridine might not be handled by an organic acid transport system, at least in dogs and rabbits. However, several lines of evidence argued against this suggestion. Child and Dodds (1967) found that prior administration of probenecid and other inhibitors of organic acid transport prevented the renal lesion caused by cephaloridine (cephalorodine-induced nephrotoxicity will be described in detail later) in mice. This protecting effect was also demonstrated in rabbits (Tune, 1972). Furthermore, cephalosporin drugs are structurally related to the penicillins (Loder et al., 1961), which have been shown to be actively secreted by renal organic acid transport (Beyer, 1950). In addition, cephalothin was found to be transported by an organic acid transport system (Lee et al., 1963). All this information strongly suggested a possible connection between cephaloridine and organic acid transport. Interestingly, all the investigations on the renal handling of cephaloridine were directed solely towards cephaloridine secretion and

completely neglected the possibility that cephaloridine might be concentrated in the renal proximal tubular cells in the absence of secretion. This possibility was confirmed a few years later by Tune and his coworkers (Tune, 1972; Tune and Fernholt, 1973). Tune (1972) studied renal uptake of cephaloridine in rabbits by giving single subcutaneous injections of the drug and measuring its concentration in serum, renal cortex and medulla 30 minutes later. Cortex/serum ratios of cephaloridine were approximately 8 times higher than those of inulin, suggesting that renal cortical cephaloridine concentration was significantly greater than that which could be accounted for by glomerular filtration alone. Prior administration of probenecid or benzylpenicillin markedly reduced cortex/serum ratios of cephaloridine. Furthermore, Tune and Fernholt (1973) were able to show that the uptake of cephaloridine by renal cortical slices was oxygen dependent and inhibited by dinitrophenol, indicating that the transport of cephaloridine into renal cortical tissue was an active process. Thus, although there is no significant cephaloridine secretion into the urine, there is active cortical cephaloridine uptake by organic acid transport. In order to understand why cephaloridine is accumulated in renal cortex, Tune and his coworkers (1974) conducted a series of elegant experiments. These investigators ligated the ureter to interrupt the movement of PAH from proximal tubular fluid into the urine subsequently retarding PAH movement from cell water into luminal fluid. Six minutes of ureteral ligation resulted in a doubling of renal cortical PAH concentration but had no effect on renal cortical cephaloridine concentration, indicating

cephaloridine movement from cell water to luminal fluid was much slower than PAH movement. Furthermore, abrupt inhibition of transport by an intravenous bolus of probenecid resulted in a rapid loss of PAH from proximal tubular cells. However, intravenous administration of probenecid only resulted in a slow decrease of the renal cortical concentration of cephaloridine. These results and those from other investigators led Tune (1975) to conclude that cephaloridine, like PAH, was actively transported into the proximal tubular cells at the peritubular cell membrane, but unlike PAH, cephaloridine did not move readily across the luminal cell membrane into the tubular fluid. It can be concluded from these studies that although there is no net secretion of cephaloridine, cephaloridine does depend on active transport for uptake into cortical tubular cells and this transport can be blocked by organic acid transport inhibitors. The role of organic acid transport in renal cortical accumulation of cephaloridine was further confirmed by Wold and Turnipseed (1978) who investigated the accumulation of cephaloridine in the renal cortex of rabbits of various ages. The cortical concentration of cephaloridine rose from newborn to adult levels by approximately one month of age. This pattern correlated well with the development of organic acid transport studied with the classical substrate PAH (Hirsch and Hook, 1970). Furthermore, pretreatment of immature rabbits with procaine penicillin G has been shown to stimulate the development of organic acid transport (Hirsch and Hook, 1970). Similarly, penicillin G treatment stimulated the ability of immature rabbit renal cortical cells to accumulate cephaloridine (Wold and Turnipseed, 1978).

Even though it is known that the egress of cephaloridine from renal cortical cells is much slower than PAH, the process for cephaloridine efflux was not studied until recently. Wold and Turnipseed (1980) found that pretreatment of rabbits with cyanine, an inhibitor of organic base transport, led to retention of cephaloridine in the renal cortex. In addition, the efflux of cephaloridine from preloaded renal cortical slices was significantly slowed by the presence of cyanine in the efflux media. These results indicated that cephaloridine depended on, at least in part, a base transport step for exit from proximal tubular cells.

Toxicity of Cephaloridine in Humans and Laboratory Animals

Cephaloridine and other cephalosporins share several toxicities, which are mainly allergic reactions. However, the main adverse effect of cephaloridine is nephrotoxicity. Many studies have documented serious and even fatal nephrotoxicity from cephaloridine in humans with previously normal kidneys who received doses greater than 6 grams daily (Kaplan et al., 1968; Hinman and Wolinsky, 1967). Although treatment with doses of 4 g or less daily in patients with normal renal function does not result in detectable renal impairment (Fair, 1972), the risk of nephrotoxicity by cephaloridine may be enhanced by other agents such as aminoglycosides and diuretics (Lawson et al., 1970; Kleinknecht et al., 1974). In addition, patients who have illnesses that directly or indirectly affect renal function may develop nephrotoxicity with doses of 4 g cephaloridine daily or less (Mandell, 1973).

Nephrotoxicity of cephaloridine has been demonstrated in many species of laboratory animals (Atkinson et al., 1966), especially monkeys and rabbits (Perkins et al., 1968). Cephaloridine-treated

rabbits showed dose-dependent alteration in renal function. Those given 50-100 mg/kg per day had no significant change in creatinine clearance and no glycosuria or proteinuria, whereas a dosage of 200 mg/kg per day caused marked changes in renal function. All animals receiving this dose developed proteinuria and some animals had concomitant glycosuria. Rabbits given 500 mg/kg of cephaloridine per day, developed glycosuria and severe proteinuria (Perkins et al., 1968). Alterations in renal function were compatible with histopathological findings. Silverblatt et al. (1970) reported that rabbits receiving 200 mg/kg developed alteration in the proximal tubular brush border at one hour and frank necrosis was evident at 16 hours. Early changes seen with the electron microscope were loss of microvilli and the disappearance of structures associated with endocytosis. Later, disorganization of lateral interdigitations of cell membranes and mitochondrial swelling were observed. Necrosis did not result from 50 mg/kg cephaloridine treatment. Proximal tubular necrosis developed in some rabbits receiving 100 mg/kg of cephaloridine. In addition, uptake of horseradish peroxidase was blocked in damaged tubules one hour after administration of cephaloridine, indicating interference with endocytosis. These data demonstrated that in rabbits, cephaloridine produced a dose-dependent lesion of the proximal tubules that resulted in early disruption of structure and function of the cell membrane. Perkins et al. (1968) also reported a dose-related nephrotoxicity in Rhesus monkeys. Dosage of 100 mg/kg per day resulted in little change in renal function. Monkeys given 200 mg/kg per day had a marked drop in PAH clearance and a concomitant marked rise in BUN and serum creatinine. Proximal tubular alteration

and necrosis were observed in these animals (Perkins et al., 1968). A similar finding was also reported by Atkinson et al. (1966). In addition to rabbits and monkeys, renal tubular necrosis was seen in rats and mice treated with higher dosage of cephaloridine (Welles et al., 1966; Atkinson et al., 1966).

It has been shown that when the kidney is affected by the action of certain toxic compounds, enzymes are usually released from the damaged cells and pass into the blood, urine and other extracellular fluids. Measurements of such enzyme activities and their characterization may provide a useful index of kidney damage. For instance, lactate dehydrogenase, alkaline phosphatase, muramidase, acid phosphatase and β-glucosidase have been shown to be elevated in the urine following damage to the kidney in animals and man. Ngaha and Plummer (1977) monitored the change in the rat urinary lactate dehydrogenase, alkaline phosphatase and muramidase activities following cephaloridine administration; urinary lactate dehydrogenase and muramidase were elevated within 12 hours following subcutaneous administration of 2 g/kg cephaloridine and reached a peak at 60 hours. A concomitant decrease in kidney lactate dehydrogenase and alkaline phosphatase was also determined. Serum enzymes were also studied to determine the possible sources of urinary enzymes in cephaloridine-induced nephron damage. The results indicated that serum enzyme levels were not much affected. Raab and Moerth (1976) also reported an increased excretion of alkaline phosphatase, leucine aminopeptidase and lactate dehydrogenase after the administration of cephaloridine to rats at a dose of 250 mg/kg. In addition, Sack (1976) observed increased urinary enzyme excretion as well as an increased rate

of excretion of renal epithelial cells after the administration of cephaloridine at a dose of 500 mg/kg per day to Wistar rats. In contrast, cephaloridine appeared to have different effects on lysosomes. Following 2 g/kg, lysosomal enzyme acid phosphatase in the urine was suppressed (Wright et al., 1974). This decrease was shown to be due to the stabilization of lysosomal membranes by cephaloridine (Fry et al., 1975; Ngaha et al., 1979). Later, cephaloridine was shown to inhibit a lysosomal membrane-bound phospholipase 2, the enzyme which digests phospholipids of lysosomal membrane. Such an inhibition may explain the cephaloridine-induced stabilization of rat kidney lysosomes (Fry and Plummer, 1979). More recently, the toxicity of cephaloridine in rabbits has been characterized by other techniques, and the antibiotic has been shown to produce changes in transport and metabolism of the kidney as measured by the ability of renal cortical slices to accumulate the anion PAH or the cation TEA and to perform gluconeogenesis (Wold et al., 1979).

Renal Cortical Concentrations and Toxicity

Several lines of evidence have suggested a close correlation between cephaloridine nephrotoxicity and renal cortical concentration of cephaloridine. The early report of the nephrotoxicity of cephaloridine by Welles et al. (1966) showed an unusually high concentration in the renal cortex, although they did not suggest that this unique high concentration caused renal injury. As described previously, cephaloridine nephrotoxicity in rabbits and mice was prevented by probenecid (Child

and Dodds, 1966; Tune, 1972). This protection appeared to be associated with decreased cephaloridine concentration in renal cortex (Tune et al., 1977a). Tune et al. (1977a) examined the effect of various doses of probenecid on the nephrotoxicity and renal cortical concentration of cephaloridine in rabbits. Probenecid at doses of 20 to 60 mg/kg produced a dose-related decrease in the severity of cephaloridine-induced renal cortical necrosis. Interestingly, probenecid also caused a dose-related decrease in renal cortical concentration of cephaloridine. A similar effect of probenecid on cephaloridine concentration and nephrotoxicity was demonstrated in guinea pigs (Tune et al., 1977a).

Additional evidence for this causal relationship between renal cortical concentration and nephrotoxicity of cephaloridine came from the studies by Fleming and Jaffe (1967) and Wold et al. (1977a; 1978). Fleming and Jaffe (1967) first reported that newborn rabbits were less susceptible to cephaloridine nephrotoxicity. This observation was extended by Wold et al. (1977a), who investigated the susceptibility of rabbits of various ages to cephaloridine nephrotoxicity. Cephaloridine administered at doses of 200 or 400 mg/kg produced severe renal cortical tubular necrosis and elevations of BUN and creatinine in rabbits 30 days of age and in adult rabbits. However, doses as high as 800 mg/kg produced no evidence of cortical tubular necrosis or alteration in renal function in rabbits 5 days of age. The relative resistance of the newborn rabbit kidney to cephaloridine nephrotoxicity did not appear to be a generalized property of the immature kidney since mercuric chloride, a nephrotoxicant which also produced cortical tubular necrosis, has

been shown to be equally toxic in the immature and adult rabbits (Wold et al., 1977a). The development of susceptibility to cephaloridine nephrotoxicity paralleled development of the ability of renal cortical cells to accumulate cephaloridine (Wold et al., 1978). Further evidence for the link between renal cortical concentration and nephrotoxicity of cephaloridine was obtained from experiments in which rabbits were pretreated with PAH or penicillin G. Pretreatment with either PAH or penicillin markedly enhanced the susceptibility of animals of 15 to 18 days of age to cephaloridine nephrotoxicity (Wold et al., 1977a) and similarly enhanced the uptake of cephaloridine in renal cortex (Wold et al., 1978).

Cephaloridine nephrotoxicity varied among the species. Atkinson et al. (1966) calculated the ND_{50}, which they defined as the nephrotoxic dose for each species producing histologically evaluable changes at 48 hr in 50% of the animals treated. ND_{50} was lowest in the rabbit (90-140 mg/kg) and increased in other species as follows: monkey, 300 mg/kg; guinea pig, 400-700 mg/kg; rat, 1000-1400 mg/kg; and mouse, 600-3100 mg/kg. The dog and the cat were not affected by 1000 mg/kg. On the basis of this information, Tune (1975) measured renal cortical concentrations of cephaloridine in rabbits, guinea pigs and rats following s.c. administration of 100 mg/kg of cephaloridine. Cortical concentration was highest in the rabbit and lowest in the rat, which was consistent with the severity of cephaloridine nephrotoxicity in these species studied.

The nephrotoxicity of cephaloridine exhibited a threshold in rabbits and guinea pigs (Tune et al., 1977a). In both species, cortical

concentrations of cephaloridine above 2000 μg/g at 3 hours following cephaloridine administration produced significant tubular necrosis, while concentrations remaining below 1000 μg/g caused little or no cell damage. This finding was extended by the studies of Wold (1981). He found that renal cortical function (e.g. organic acid and base transport and gluconeogenesis) began to decline when cortical concentrations of cephaloridine were approximately 1200 μg/g. When cortical concentrations were above 1500 μg/g, marked nephrotoxicity evidenced by BUN elevation was observed.

Although all cephalosporins possess the carboxyl functional group of the beta-lactam ring necessary for interaction with renal organic anion transport, cephaloridine is unique in that it possesses a cationic functional group in the quaternary nitrogen of the pyridinium substitution on the beta-lactam ring. Because of this cationic property of cephaloridine, Wold et al. (1979; 1980) suggested that cation transport, like anion transport, might also play a role in determination of renal cortical concentrations of cephaloridine. These investigators found that pretreatment of rabbits with the cation transport inhibitors cyanine 863 or mepiperphenidol (Peters et al., 1955; Rennick et al., 1956; Kandel and Peters, 1957), markedly enhanced the nephrotoxicity of cephaloridine, but had no effect on the nephrotoxicity of cefazolin, a cephalosporin which has no cation functional group. Furthermore, administration of cyanine at intervals of up to 3 hr after administration of cephaloridine was also shown to enhance cephaloridine nephrotoxicity, suggesting that the inhibition of cation transport might

result in a decreased efflux of cephaloridine out of cells of the renal cortex. Examination of the effect of cyanine pretreatment on cortical concentrations of cephaloridine as well as evaluation of the effect of cyanine on the efflux of cephaloridine *in vitro* clearly indicated that although peak concentrations of cephaloridine obtained *in vivo* were unaltered the renal cortical concentrations were prolonged and efflux *in vitro* was slowed by inhibition of cation transport (Wold and Turnipseed, 1980).

Biochemical Mechanisms of Cephaloridine Nephrotoxicity

Since cephaloridine was recognized as being capable of producing renal cortical damage, several biochemical mechanisms have been proposed to explain this toxic effect. Boyd *et al.* (1971; 1973) first extensively investigated the mechanism of cephaloridine nephrotoxicity. Cephaloridine solution was shown to contain polymeric macromolecules. Using Sephadex column chromatography and ultrafiltration, Boyd *et al.* (1971) obtained 4 fractions from commercial cephaloridine preparations. The first was found in only minute amounts and had a molecular weight (MW) greater than 5000; this fraction was inconstant and had the characteristics of a polypeptide-polymer complex. The next 3 fractions were constantly present from batch to batch. The first of these, polymer I ($5000 \geq MW \geq 3500$) occurred as 45% of starting material. The second, polymer II ($3500 \geq MW \geq 1000$) formed 26% and the final fraction ($1000 \geq MW$) was present as 29% of starting material. Because of inconstant presence and minute amounts, these investigators did not study the first fraction. Boyd and coworkers (1973) studied the remaining fractions and attempted to determine which fraction(s) was responsible for cephaloridine nephrotoxicity. Different degrees of nephrotoxicity were

observed with these fractions. Renal tubular damage was found to be least with the high molecular weight polymer (polymer I), greater with the medium-sized polymer (polymer II) and greatest with the fraction with molecular weight less than 1000 (polymer III). The polymer III fraction contained monomers and dimers of cephaloridine and probably degradation products. Since pyridine may be released from cephaloridine and retained in the polymer III fraction, these investigators also studied the nephrotoxicity of pyridine. Pyridine failed to cause any significant renal tubular cell damage at equimolar doses. In addition, reconstitution by mixing these three fractions (polymer I, II and III) only led to about half of the renal tubular damage caused by the starting material, indicating that the untested polypeptide-polymer complex fraction coud play some part in causing nephrotoxicity. However, these investigators (Boyd et al., 1973) did not favor this possibility, because not all batches of commercial cephaloridine used in their studies contained this fraction, whereas all batches appeared to be equally nephrotoxic. Although none of the fractions was solely responsible for cephaloridine nephrotoxicity, the severity of nephrotoxicity was decreased with an increase in the degree of polymerization. The greatest tubular damage resided with the fraction (polymer III) which had a strong tendency to polymerize; less damage occurred with polymer II which was probably halfway through the polymerizing process and least damage occurred with polymer I which was the most stable fraction, being nearest to the end of the polymerizing process. These possibilities led Boyd et al. (1973) to suggest that the process of polymerization might be responsible for the tubular damage; this process

was more likely to occur in concentrated solutions such as those in the lumen of the proximal tubule. Such an effect might also occur in the cytoplasm of tubular cells. These investigators did not exclude the possibility of precipitation of cephaloridine from concentrated or supersaturated solutions. Precipitated antibiotic might complex with protein to form haematoxyphilic granular deposits.

The second possible biochemical mechanism of cephaloridine nephrotoxicity was proposed by Tune et al. (1979), who suggested that cephaloridine first interfered with renal cortical mitochondrial function which then led to cell damage and death. In order to test their hypothesis, these investigators examined the effect of cephaloridine on respiration by mitochondria in intact cells (renal cortical tubules) and isolated renal cortical mitochondria (Tune et al., 1979; Tune and Fravert, 1980). In the isolated mitochondria studies, the effect of cephaloridine on respiration was examined under two conditions: (a) in vitro exposure: respiration studied before and after exposure of isolated mitochondria to cephaloridine; (b) in vivo exposure: respiration studied in mitochondria isolated from animals that received cephaloridine prior to sacrifice. In these studies, respiration by renal cortical mitochondria was studied with each of three substrate groups including glutatmate plus maleate, succinate, and tetraethylphenylenediamine plus ascorbate. The respiratory control ratio (RCR), which is the ratio of the higher rate of oxygen consumption in the presence of ADP (state 3) to the lower rate after the consumption of ADP (state 4), was used as a functional measure of the integrity of mitochondria and their ability to generate ATP. In in vivo studies, renal cortical

mitochondria were isolated from rabbits which received a toxic dose of cephaloridine (200 mg/kg) subcutaneously 2 hr before sacrifice. In vivo exposure to cephaloridine resulted in a significant reduction of mitochondrial respiratory rates. In in vitro studies, measurements of mitochondrial respiration were made before and after addition of 2000 μg/ml of cephaloridine, which was equal to the concentration found in renal cortex in which significant tubular necrosis was observed. This concentration reduced state 3 rates and RCR's with the natural substrates and respiration with tetramethylphenylenediamine plus ascorbate. The effect of cephaloridine on respiration was further examined in studies of isolated renal cortical tubules under three conditions: (a) in vitro short-term exposure (5 to 10 min) to 2000 μg/ml of cephaloridine; (b) in vitro longer term exposure (2 hr) to the same concentration; and (c) in vivo exposure by the subcutaneous injection of 400 mg/kg of cephaloridine 2 hours before the animals were sacrificed. Respiration rate was significantly reduced under the last two conditions (65 and 53% of control, respectively) but was not altered in the in vitro short-term exposure.

One possible reason for this delayed expression of cephaloridine-induced cytotoxicity (inhibition of respiration by isolated renal tubules) is the requirement for biotransformation of cephaloridine to a toxic metabolite. Such a requirement was suggested by McMurtry and Mitchell (1977) and Mitchell et al. (1977). These investigators reported that two inhibitors of cytochrome P450 monooxygenase activities, cobaltous chloride and piperonyl butoxide, either completely blocked or

markedly reduced the renal proximal tubular necrosis produced by cephaloridine in rats and mice (McMurtry and Mitchell, 1977). Furthermore, the lack of effects of these inhibitors on the direct nephrotoxicants, mercuric chloride and sodium fluoride, provided additional support for their hypothesis. They failed to increase cephaloridine nephrotoxicity by pretreatment of animals (rats and mice) with phenobarbital (McMurtry and Mitchell, 1977). However, phenobarbital has been shown to induce drug-metabolizing enzymes in the liver but not in the kidney in rats (Lake et al., 1973). The lack of effect of phenobarbital on renal drug-metabolizing enzyme activities as well as the lack of potentiation of cephaloridine toxicity by phenobarbital led these investigators (Mitchell et al., 1977) to conclude that metabolic activation of the antibiotic presumably occurred in situ in the kidney. Furthermore, these investigators reported that pretreatment of rats and mice with cysteine decreased cephaloridine renal injury and produced a shift in the zone of necrosis from the outer to the inner cortex. Even though the nature of the toxic cephaloridine metabolite was not demonstrated, on the basis of their findings, Mitchell et al. (1977) suggested that it was an electrophilic reactant since nucleophilic sulfhydryl compounds decreased its toxicity. Furthermore, cephaloridine contains a 5-membered thiophene ring, and many thiophenes produced renal or hepatic necrosis in animals after metabolic activation (McMurtry and Mitchell, 1977), suggesting that a thiophene epoxide metabolite of cephaloridine might be generated.

Renal Drug-Metabolizing Enzymes

Although the precise biochemical mechanism responsible for cephaloridine nephrotoxicity is not clear, understanding of renal drug-metabolizing enzyme systems may help to extend knowledge of cephaloridine toxicity. The kidney is one of the most sensitive organs in the body to the harmful effects of toxic chemicals. This high susceptibility to toxicants is the result of several factors. The two kidneys together receive approximately 25% of cardiac output, even though they comprise less than 1% of total body mass. High renal blood flow can rapidly deliver toxic chemicals to the kidneys. Because a large amount of plasma water is filtered and reabsorbed, and a variety of materials (e.g., cephaloridine) are actively transported into the tubular cells, many toxic chemicals can be concentrated in the kidney and have a greater opportunity to injure kidney cells than most other cells in the body. However, high concentrations of chemicals (e.g., penicillin G) do not necessarily produce cell damage. Under many conditions, chemicals (e.g., chloroform, acetaminophen and bromobenzene) have to be metabolically activated prior to causing cell injury. Since cephaloridine has been suggested to be bioactivated *in situ* in the kidney, it becomes important to understand the characteristics of renal drug-metabolizing enzymes (DMEs).

Many DME activities including cytochrome P450 monooxygenase, glucuronyltransferase (Fowler *et al.*, 1977), glutathione S-transferase (Fine *et al.*, 1978), epoxide hydrolase (Oesch, 1972) and others (Zenser *et al.*, 1978; Orrenius *et al.*, 1973) are measurable in renal tissues.

However, the distribution of DMEs in the kidney appears to be heterogeneous. Zenser et al. (1978) found high activities of laureate hydroxylase and aminopyrine demethylase in kidney cortex but little or no activity in medulla. Fine et al. (1978) measured glutathione S-transferase activity in isolated segments of the rabbit nephron using 1-chloro-2,4-dinitrobenzene as substrate. Enzyme activity was confined to the proximal tubules and was not detectable in the loop of Henle and collecting tubules. Tetrachlorodibenzo-p-dioxin (TCDD) has been administered orally to rats and produced marked proliferation of smooth endoplasmic reticulum in the renal proximal tubular cells of the straight segments. These changes were associated with pronounced induction of the microsomal enzymes glucuronyl transferase and benzo(a)pyrene hydroxylase (Fowler et al., 1977). Renal dissection studies disclosed that the activities of these enzymes in TCDD-treated animals were not uniformly distributed within the kidney. Enzyme activities were higher in the outer stripe of the medulla and cortex and lower in the medulla (Fowler et al., 1977). Therefore, in general, DME, especially mixed-function oxidases, are concentrated in the cells of the renal cortex.

In general, renal enzyme activities appear to be lower than activities of hepatic enzymes (Orrenius et al., 1973). Based on a comparative study of drug metabolism by hepatic and extrahepatic tissue from five different species, Litterst et al. (1975) reported that kidney activities were usually 15-40% of those found in liver. However, substrate specificity may be different for hepatic and extrahepatic enzymes. The renal MFO system has higher laurate hydroxylase activity

compared with the hepatic system in adult rabbits, suggesting that the kidney enzyme may metabolize fatty acid more rapidly (Zenser et al., 1978). In addition to substrate specificity, kidney tissues in several species of animals responded to barbiturate-type inducer (e.g., phenobarbital and DDT) differently than did hepatic tissues. Phenobarbital increased hepatic but not renal microsomal enzyme activities in mice (Kluwe et al., 1978) and rats (Uehleke and Greim, 1968; Feuer et al., 1971). However, the lack of effect of phenobarbital on renal enzymes is not a uniform phenomenon. Phenobarbital was reported to induce MFO activities, cytochrome P450 and cytochrome b5 content in rabbit kidneys (Uehleke and Greim, 1968). Rabbits have been reported to be more susceptible to cephaloridine nephrotoxicity than rats (Atkinson et al., 1966). This species difference in susceptibility may be due to qualitative or quantitative differences in the ability of kidney cells to metabolize cephaloridine to reactive intermediates. As described previously, rabbit renal MFO activity can be induced by phenobarbital but rat and mouse MFO activities are not altered by phenobarbital treatment. This differential response suggests that the rabbit renal MFO system is somehow different from the rat and mouse MFO system. If this assumption is correct, then the rabbit kidney could have higher capacity to activate cephaloridine than rat and mouse kidney and therefore produce greater damage in the rabbit kidney.

Role of Glutathione in Cellular Protection Against Toxic Chemicals

Glutathione conjugation has been considered a detoxification pathway for many toxic chemicals. Several metabolite-mediated toxic chemicals such as chloroform (Brown et al., 1974; Docks and Kirshna, 1976),

acetaminophen (McMurtry et al., 1978) and vinyl chloride (Hefner et al., 1975) produce liver and/or kidney necrosis associated with depletion of glutathione (GSH). Cell injury or death results only under conditions in which this sulfhydryl compound is depleted (Mitchell et al., 1976). As described previously, Mitchell et al. (1977) reported that pretreatment of rats and mice with cysteine decreased cephaloridine renal injury. Since cysteine has been shown to be a precursor for glutathione synthesis (Meister and Tate, 1976; Boyland and Chasseaud, 1967; Reed and Orrenius, 1977), this finding suggests that cephaloridine or its metabolite(s) may react with glutathione. Glutathione (GSH) is a physiologically important tripeptide with the sequence L-γ-glutamyl-L-cystinylglycine. This ubiquitous molecule is the most abundant cellular peptide and accounts for about 90 percent of intracellular non-protein thiols. The structure of this tripeptide is unusual in that the amino-terminal peptide bond utilizes the γ-carboxyl moiety of glutamate. This unusual arrangement makes GSH resistant to the hydrolytic action of many proteases and aminopeptidases. The cellular concentration of GSH at any given time is the result of a dynamic system and reflects the balance of degradation and biosynthesis processes. GSH is synthesized from its constituent amino acids (glutamate, cysteine and glycine) in two separate reactions, each requiring one molecule of ATP (Snoke and Bloch, 1952; Snoke et al., 1953). The first step involves formation of a γ-glutamyl linkage between L-glutamate and L-cysteine, catalyzed by γ-glutamyl cysteine synthetase (Meister, 1974). This reaction is relatively specific for L-glutamate but less specific for L-cysteine. In the second step, L-glycine is added to γ-glutamyl cysteine to form GSH,

the reaction being catalyzed by glutathione synthetase (Meister, 1974). Regulation of GSH biosynthesis can be through feedback inhibition; GSH at physiological concentrations was reported to inhibit the formation of γ-glutamylcysteine (Richman and Meister, 1975). Similarly, ADP was shown to inhibit glutathione synthetase (Snoke et al., 1953; Yanari et al., 1953) and γ-glutamylcysteine synthetase (Mandeles and Block, 1955). In addition, the rate of GSH biosynthesis is limited by the availability of substrates (Tateishi et al., 1974). The breakdown of glutathione is catalyzed by γ-glutamyl transpeptidase, a membrane-bound enzyme (Meister and Tate, 1976), which may be the only enzyme that is capable of hydrolyzing the γ-glutamyl-peptide bond since the usual peptidases appear to be unable to hydrolyze this linkage. The reaction catalyzed by γ-glutamyltranspeptidase involves transfer of the γ-glutamyl moiety of glutathione to an amino acid acceptor to yield a γ-glutamyl peptide and cysteinylglycine. Cysteinylglycine is then split to cysteine and glycine by a peptidase (Semenaz, 1957; Hughey et al., 1978).

The most widely known biological role of GSH is to form GSH conjugates with foreign compounds or their metabolites. These conjugation reactions occur non-enzymatically and are also catalyzed by glutathione S-transferases (Chasseaud, 1973). The glutathione S-conjugates are then converted to mercapturic acid through three sequential reactions. The first reaction is to remove the γ-glutamyl moiety from the GSH conjugates, which is catalyzed by γ-glutamyl transpeptidase (Meister and Tate, 1976). The resulting S-conjugates of cysteinylglycine are then converted to the cysteine conjugates by removing glycine, the reaction

being catalyzed by a number of aminopeptidases and dipeptidases (Hughey et al., 1978). The cysteine conjugates are subsequently acetylated by N-acetyltransferase to mercapturic acids (Green and Elce, 1975), which are nontoxic and quickly excreted. Conjugation with GSH not only facilitates the excretion of toxic chemicals, but also serves to intercept highly reactive compounds before they can covalently bind to tissue macromolecules leading to cell damage or death. The role of GSH in detoxification of reactive metabolites has been studied with many compounds such as acetaminophen and bromobenzene. Evidence from the studies by Zampaglione et al. (1973) and Jollow et al. (1974) demonstrated in vivo direct correlation between hepatic GSH concentrations and the severity of hepatic necrosis, the degree of covalent binding of bromobenzene to liver macromolecules and the urinary excretion of bromophenyl mercapturic acid. In addition, GSH has also been shown to exhibit a protective effect against acetaminophen-induced necrosis in the liver (Mitchell et al., 1973) and kidney (McMurtry et al., 1978).

In addition to the mercapturic acid detoxication pathway, via glutathione peroxidase glutathione can prevent cellular damage by blocking intracellular oxidative processes such as lipid peroxidation. Glutathione peroxidase, an enzyme found in most mammalian tissues, specifically uses GSH as a hydrogen donor to reduce chemically reactive hydroperoxides (e.g., H_2O_2 and lipid hydroperoxides) to H_2O or chemically stable alcohols and thereby protect the integrity of the cells. Meanwhile two molecules of GSH are oxidized to GSSG (Cohen and Hochstein, 1963; Wendel, 1980) ($ROOH + 2GSH \rightarrow ROH + H_2O + GSSG$). The

resulting GSSG is then reduced back to GSH by glutathione reductase with NADPH, which is generated from the hexose monophosphate pathway (Beutler, 1974) (GSSG + NADPH + H^+ → 2GSH + NADP). Several lines of evidence demonstrated the role of glutathione peroxidase in preventing tissue lipid peroxidation. In in vitro studies, glutathione peroxidase was shown to prevent lipid peroxidation and functional impairment of isolated mitochondria (Flohe and Zimmermann, 1970). Furthermore, a genetically-determined deficiency of glutathione peroxidase in human erythrocytes has been correlated to drug-induced hemolysis and chronic hemolytic anemia (Necheles et al., 1970; Steinberg and Necheles, 1971) when exposed to prooxidative drugs. In addition, selenium was shown to be an essential cofactor for glutathione peroxidase (Rotruck et al., 1973; Flohe et al., 1973; Oh et al., 1974) and animals fed with selenium-deficient diet had a decreased glutathione peroxidase activity (Rotruck et al., 1973). The selenium-deficient animals were shown to be unable to prevent hydrogen peroxide-induced erythrocyte hemolysis (Rotruck et al., 1973) and became more susceptible to the toxicity of paraquat (Bus et al., 1975), which was shown to act through generation of superoxide with subsequent initiation of lipid peroxidation (Bus et al., 1974; 1976).

Lipid Peroxidation and Its Role in Cellular Toxicity

Lipid peroxidation has been considered as a basic deteriorative reaction in the toxicity of a variety of xenobiotics (Plaa and Witschi, 1976). The peroxidative process is the reaction of oxidative deterioration of polyunsaturated lipids. Peroxidation of lipids involves the

reaction of oxygen with polyunsaturated lipids to form free radical intermediates and lipid hydroperoxides, which then promote free radical chain oxidation (Tappel, 1973). Lipid peroxidation is damaging because of the subsequent reactions of free radicals, mainly peroxy radicals. Several endogenous oxidation reactions and oxidation of xenobiotics can convert oxygen to superoxide and hydroxyl radical. These various forms of oxygen are then capable of either directly or indirectly initiating lipid peroxidation (Tien et al., 1981; Svingen et al., 1978; Tyler, 1975; Fong et al., 1973; Kellogg and Fridovich, 1975, 1977). Generation of superoxide occurs in many endogenous biological reactions such as autooxidations of reduced flavins, hydroquinones and catecholamines and from the aerobic actions of enzymes such as xanthine oxidase, aldehyde oxidase and flavin dehydrogenases (McCay and Poyer, 1976; Fridovich, 1975). In addition, the toxicity of several chemicals such as adriamycin (Goodman and Hochstein, 1977; Myers et al., 1977), alloxan (Heikkila and Cohen, 1975), dialuric acid (Cohen and Heikkila, 1974), 6-hydroxydopamine (Heikkila and Cohen, 1973) and paraquat (Bus et al., 1975) have been shown to be associated with superoxide formation.

Plasma membranes and membranes of subcellular organelles are major sites of lipid peroxidative damage. Membranes are complex mixtures of lipids and proteins. Mitochondrial and microsomal membranes contain relatively large amounts of polyunsaturated fatty acids in their phospholipids (Rouser et al., 1968). Furthermore, some of the most powerful catalysts involved in lipid peroxidation, such as coordinated iron and hemoproteins, are in close molecular proximity to these polyunsaturated

lipids (Tappel, 1973). Because of these particular characteristics, mitochondrial and microsomal membranes are highly susceptible to lipid peroxidative damage. Lipid peroxidative damage of mitochondrial membranes has been demonstrated to correlate with swelling and lysis of the mitochondria and impaired mitochondrial respiration (Hofsten et al., 1962; Vladimirov et al., 1980). Lipid peroxidation also affects microsomal membranes, decreasing microsomal drug metabolism and cytochrome P450 concentration (Plaa and Witschi, 1976). In addition, lipid peroxidation damages lysosomal membranes (Fong et al., 1973; Wills and Wilkinson, 1966), with resultant intracellular release of hydrolytic enzymes. Proteins and enzymes in aqueous solution are also susceptible to lipid peroxidative damage (Tappel, 1973). Thus, a great number of cellular functions are altered as a result of lipid peroxidation (Vladimirov et al., 1980).

Since lipid peroxidation is an exceedingly damaging biological process and the peroxidative process is very likely constantly occurring in the cells, in order to survive cells possess several protective mechanisms. Superoxide dismutase has been shown to scavenge the superoxide radicals, which will prevent the initiation of lipid peroxidation (Hassan and Fridovich, 1980). Several endogenous antioxidants such as glutathione and vitamin E are known to prevent the propagation of lipid peroxidation (Tappel, 1978). The roles of glutathione, glutathione peroxidase and selenium against lipid peroxidation have been described previously. Vitamin E is an important nutritional antioxidant and a free radical scavenger (Wasserman and Taylor, 1972; Tappel and Green, 1972; Urano and Matsui, 1976), which is able to interrupt the free

radical chain reactions of lipid peroxidation and prevent peroxidative damage (Tappel, 1978). Tappel and Zalkin (1959) and Dillard and Tappel (1971) have shown that liver mitochondria and microsomes isolated from rats fed vitamin E-deficient diets had greater peroxidation rates than rats fed basal diets supplemented with vitamin E. Expiration of pentane and ethane deriving from lipid peroxidation (Dumelin and Tappel, 1977), has been used as an *in vivo* index of lipid peroxidation (Riely *et al.*, 1974; Dillard *et al.*, 1977). Dillard *et al.* (1978) demonstrated that dietary vitamin E decreased pentane expiration in rats. Hafeman and Hoekstra (1977) also reported ethane expiration in carbon tetrachloride-treated rats to be diminished by dietary vitamin E. Vitamin E deficiency was reported to increase the susceptibility of animals to the toxicity of oxygen (Mino, 1973), ozone (Goldstein *et al.*, 1970) and paraquat (Bus *et al.*, 1975), which were shown to be associated with lipid peroxidative damage. Tudhope and Hopkins (1975) also reported an increased susceptibility of erythrocytes to lipid peroxidation on exposure to hydrogen peroxide vapor when erythrocytes were obtained from patients with low plasma vitamin E. Thus, by altering vitamin E (antioxidant) and selenium (a cofactor of glutathione peroxidase) concentrations in the diet, it has become possible to evaluate indirectly lipid peroxidation *in vivo*.

One of the common features between paraquat (Figure 2) and cephaloridine (Figure 1) is the pyridinium ring. Paraquat contains two pyridinium rings and cephaloridine has one pyridinium substitute on the β-lactam ring. Pyridinyl free radicals have been found to exist in a

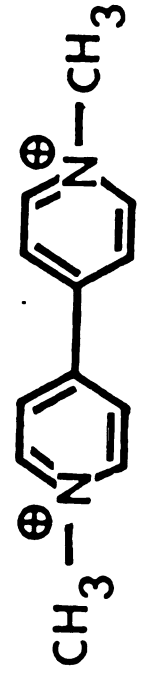


Figure 2. The structure of paraquat.

variety of pyridinium-containing compounds (Kosower, 1976). Studies by Mees (1960), Gage (1968), Farrington (1973), Davies and Davies (1974) and Bus et al. (1975, 1976) demonstrated that paraquat can be reduced to the paraquat pyridinyl free radical by a single electron reduction reaction catalyzed by cytochrome P450 reductase and NADPH (Pederson and Aust, 1973; Pederson et al., 1973); paraquat free radical is then non-enzymatically converted back to paraquat and meanwhile superoxide radicals are generated from oxygen (Figure 3). Superoxide radicals can subsequently initiate reactions with unsaturated lipids associated with cell membranes to form lipid hydroperoxides. The pyridinium ring of cephaloridine may be metabolized through this reduction-oxidation cycle and produce superoxide radicals (Figure 4).

Objectives

The purposes of this investigation were three-fold: (a) to determine the role of biotransformation in cephaloridine-induced nephrotoxicity; (b) to evaluate the role of glutathione conjugation against cephaloridine toxicity; and (c) to estimate the role of lipid peroxidation in cephaloridine nephrotoxicity.

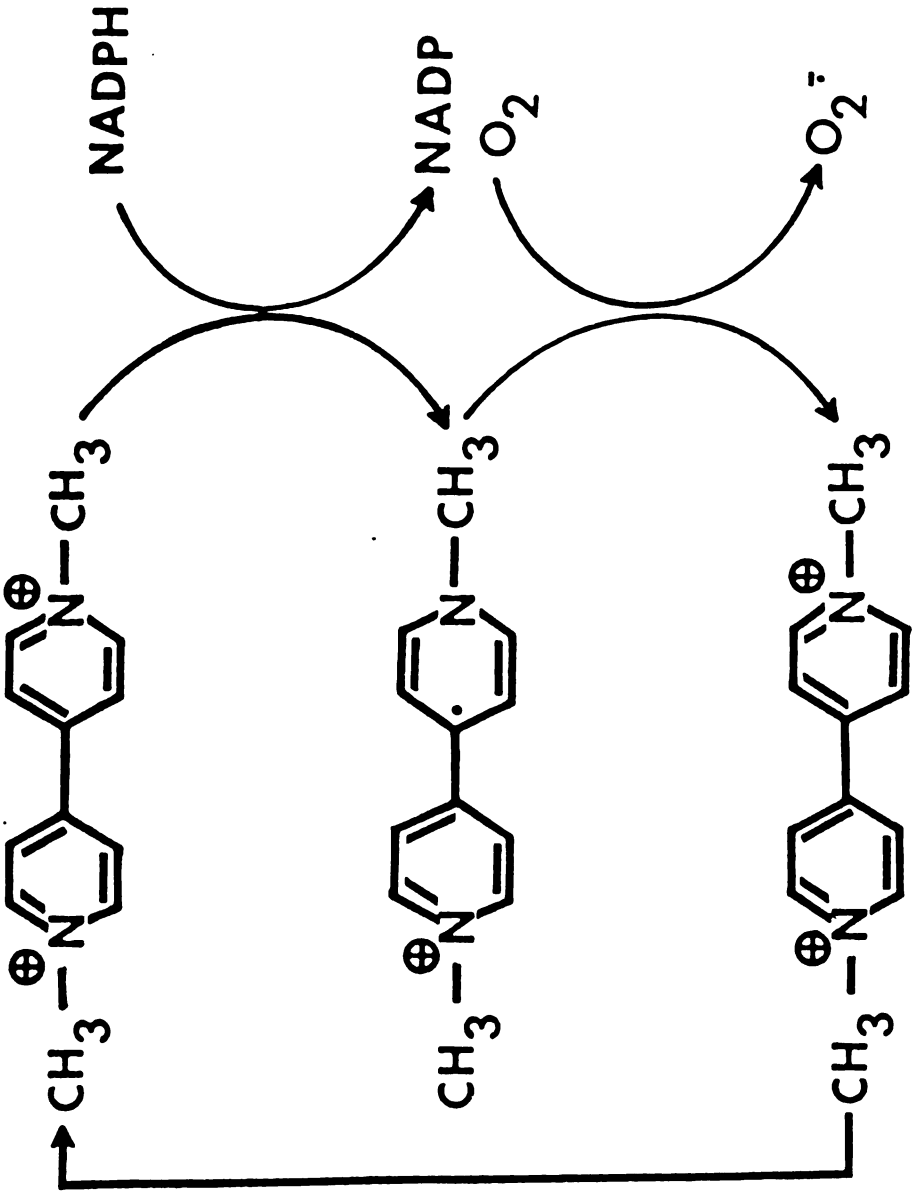


Figure 3. The cyclic reduction-oxidation of paraquat and concomitant formation of superoxide.

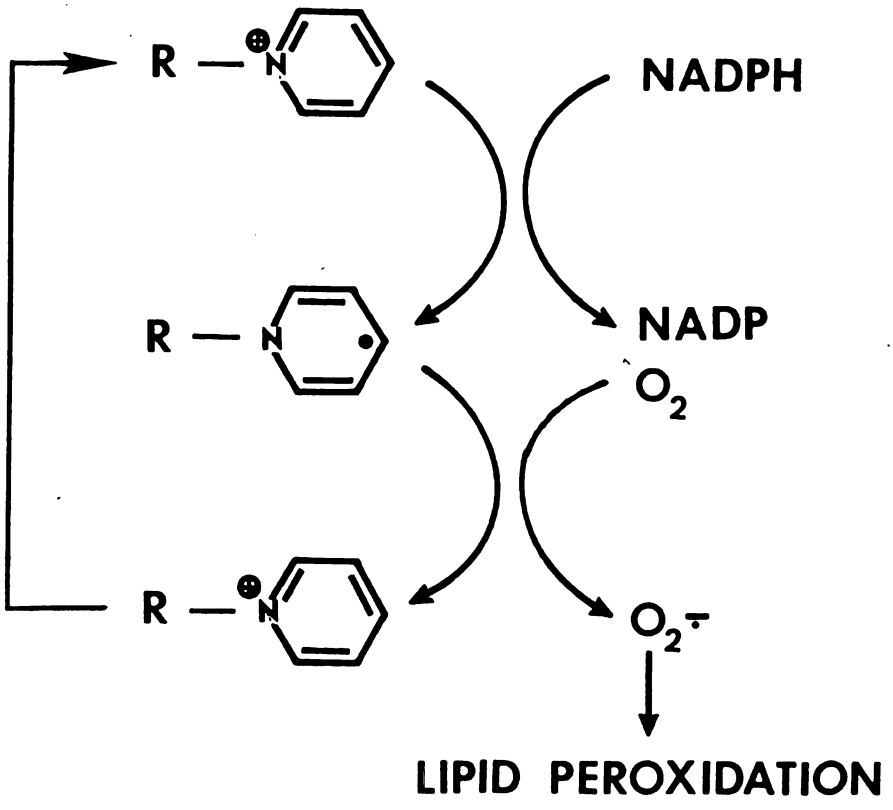


Figure 4. Proposed cyclic reduction and oxidation of cephaloridine.

METHODS

Animals

Male Fischer 344 rats (200-250 g) and female New Zealand White rabbits (1.5-3.0 kg) were purchased from Harlan Industries, Inc. (Indianapolis, IN) and a local breeder, respectively. In a few experiments, male Sprague-Dawley (SD) rats (200-250 g) and male ICR mice (25-35 g) obtained from Spartan Farms (Haslett, MI), were also used. Animals were maintained under standardized conditions of light (7 a.m.-7 p.m.) and temperature (25±2°C) and allowed free access to food (Wayne Lab-Blox, Allied Mills, Inc., Chicago, IL; Lab Rabbit Chow No. 5321, Ralston Purina Co., St. Louis, MO) and water until use.

In the dietary study, 5-6 week old male Fischer 344 rats (56-85 g) were purhcased from Harlan Industries, Inc. and housed in wire mesh hanging cages, maintained in a room with a 12 hr light-12 hr dark cycle and allowed free access to food and water.

Induction of Renal Microsomal Monoxygenases and Glutathione S-Transferases by Phenobarbital

Rats and rabbits were given a single i.p. injection of phenobarbital (80 and 60 mg/kg, respectively) once daily for four days. Animals were killed by cervical dislocation 24 hr after the last dose. Livers and kidneys were quickly excised and placed in ice-cold 1.15% KCl.

After being weighed, renal medulla and papilla were carefully dissected out and the remaining cortical portion was minced in 20 mM Tris-HCl buffer (pH 7.4) containing 1.15% KCl, rinsed 3 times and homogenized in 3 volumes of the same solution using a Potter-Elvehjem homogenizer with a Teflon pestle followed by centrifugation at 10,000 x g for 20 minutes. The resulting supernatant was then centrifuged at 100,000 x g for 60 minutes. The pellet was resuspended in 66 mM Tris-HCl buffer, pH 7.4 and recentrifuged at 100,000 x g for 60 minutes. The pellet was resuspended in 66 mM Tris-HCl buffer, pH 7.4 containing 0.25 M sucrose and 5.4 mM EDTA to a final concentration of 10-20 mg protein/ml.

The activities of benzphetamine-N-demethylase (Prough and Ziegler, 1977), and ethoxycoumarin-O-deethylase (Ullrich and Weber, 1972) were then determined in the final microsomal suspension. Cytochrome P450 concentrations were determined according to the method of Omura and Sato (1964). Protein was quantified by the method of Lowry (1951).

In the second series of experiments, animals were treated similarly and after cervical dislocation, portions of liver and renal cortex were minced and homogenized in 3 volumes of ice-cold 66 mM Tris-HCl buffer, pH 7.4. The homogenates were centrifuged at 10,000 x g for 20 minutes. The resulting supernatants were further centrifuged at 100,000 x g for 60 min. Arylhydrocarbon hydroxylase activity was measured in the 10,000 x g supernatant by the method of Nebert and Gelboin (1968) as modified by Oesch (1976) with quinine sulfate as the standard. Glutathione S-transferase activity was measured in the 100,000 x g supernatant using 1-chloro-2,4-dinitrobenzene as the substrate (Habig *et al.*, 1974).

Effects of Phenobarbital Pretreatment on Cephaloridine Toxicity

Rabbits and rats received 60 and 80 mg/kg of phenobarbital in saline, i.p., respectively, once daily for 4 days. The control group received saline alone. Twenty-four hours after the last injection of phenobarbital or saline, rabbits and rats were dosed with a single administration of cephaloridine (Eli Lilly and Company, Indianapolis, IN). Rabbits were killed 48 hr later. Because in the preliminary experiments, some rats died between 24 and 48 hr after administration of cephaloridine, all rats were killed 24 hr following cephaloridine administration. Nephrotoxicity was evaluated by determining changes in blood urea nitrogen (BUN) and p-aminohippurate (PAH) and tetraethylammonium (TEA) accumulation in renal cortical slices. After animals were killed, blood samples were collected for determination of BUN and serum glutamic pyruvic transaminase activity (SGPT). Livers and kidneys were removed immediately and weighed. Renal cortical slices were prepared for determination of PAH and TEA accumulation.

Effects of Piperonyl Butoxide Pretreatment on Cephaloridine Toxicity

Rats received a single i.p. injection of piperonyl butoxide (1000 mg/kg) 30 min prior to a single i.p. administration of cephaloridine. The control animals received corn oil prior to cephaloridine administration. All animals were killed 24 hr after cephaloridine administration. BUN and renal cortical accumulation of PAH and TEA were determined. In the second series of experiments, rabbits received a single i.p. injection of piperonyl butoxide (135 or 750 mg/kg) 30 min prior to a single s.c. administration of cephaloridine (150 mg/kg) and

were killed 48 hours later. Histopathological alterations in renal cortical tissues were determined.

Effects of Phenobarbital Pretreatment on Renal Cortical Uptake and Runout of Cephaloridine

In the first series of experiments, animals were pretreated with phenobarbital or saline as described previously, and killed one, two or three hours after a single administration of cephaloridine (150 or 1000 mg/kg). After animals were killed, blood was collected to prepare serum samples, tissues were removed immediately and portions of the tissues were homogenized in five volumes of distilled water using a Potter-Elvehjem homogenizer. Concentrations of cephaloridine in sera and tissue homogenates were then determined.

In the second series of experiments, rabbits were treated with phenobarbital or saline as described previously. Twenty-four hours after the last phenobarbital injections, animals were killed and renal cortical slices were prepared. Cortical slices were then incubated in 4.0 ml of phosphate-buffered medium containing 275 μg/ml of cephaloridine at 25°C under 100% O_2 for 45 and 90 minutes. Following incubation, slices were removed, blotted, weighed and homogenized in one ml of water and mixed with one ml of CH_3CN. In addition, one ml of medium was mixed with one ml of CH_3CN. Concentrations of cephaloridine in these mixtures were then determined.

Cephaloridine runout was determined by the method described by Farah et al. (1963) with a few modifications. Kidney slices were preloaded with cephaloridine by incubating tissue in 4.0 ml of medium

which contained 250 μg/ml of cephaloridine under 100% O_2, at 25°C for 90 min. Slices were then removed, rinsed and transferred at 1 min intervals through a series of beakers containing 4.0 ml of cephaloridine-free medium. At 0, 5, 10, 15, 20 and 25 min, slices were removed, blotted, weighed, homogenized in 1 ml of H_2O and mixed with 1.0 ml of CH_3CN. Cephaloridine concentration in the homogenates was then determined.

Effects of Piperonyl Butoxide Pretreatment on Renal Cortical Accumulation of Cephaloridine

In the first series of experiments, rabbits received a single i.p. injection of piperonyl butoxide 30 min prior to administration of cephaloridine (150 mg/kg) and were killed 15 or 60 min after cephaloridine administration. Serum and renal cortical concentrations of cephaloridine were determined spectrophotometrically.

In the second series of experiments, rabbits received a single i.p. injection of piperonyl butoxide (750 mg/kg) or 1% Tween 80 (vehicle) and were killed 45 min later. Cortical slices were prepared and incubated in 4.0 ml of phosphate-buffered medium containing 125 μg/ml of cephaloridine at 25°C under 100% O_2 for 30, 60 and 90 minutes. Following incubation, slices and medium were treated as described previously and cephaloridine concentrations in the slices and medium were then determined.

Effects of Phenobarbital Pretreatment on Renal Cortical Accumulation of Inulin and p-Aminohippurate In Vivo and Cortical Slice Accumulation of p-Aminohippurate and Tetraethylammonium In Vitro

Rabbits were pretreated with phenobarbital or saline as described previously. Twenty-four hours after the last phenobarbital injection,

animals were infused with a solution containing PAH and inulin according to the method of Tune and Fernholt (1973). Rabbits were given a priming dose of PAH, 17.5 mg/kg body weight, and 2.0 ml of 10% inulin solution through an ear vein. A solution containing 10% inulin and 20 mg/ml of PAH was then infused, i.v., at a rate of 0.11 ml/min. After one hr of infusion the animals were killed by cervical dislocation. Blood was collected and allowed to clot for one hr at room temperature and serum was prepared by centrifugation at 2,000 x g for 10 min. A 0.25 ml aliquot of serum was mixed with 0.6 ml 10% TCA, brought to a final volume of 2.0 ml with distilled water and centrifuged at 2,000 x g for 10 min and the precipitate discarded. Portions of renal cortex and liver were homogenized with 20 volumes of 3% TCA and centrifuged at 2,000 x g for 10 min. PAH concentrations in the resulting supernatants from tissue and serum samples were then determined by the method of Smith et al. (1945) and inulin concentrations in the tissue supernatants were also measured following the method of Schreiner (1950). In addition, a 0.1 ml aliquot of serum was mixed with 0.2 ml of 0.75 N NaOH, 0.2 ml of $ZnSO_4$ in H_2SO_4 (100 g of $ZnSO_4 \cdot 7H_2O$ and 40 ml of 6N H_2SO_4 diluted to 1000 ml with distilled water) and 1.5 ml distilled water and centrifuged at 2,000 x g for 20 min. Inulin concentrations in the supernatants were then determined by the method of Walser et al. (1955).

In the second series of experiments, rabbits were pretreated with phenobarbital or saline four days. Twenty-four hours after the last phenobarbital or saline injection, animals were given a bolus ear vein injection of PAH (40 mg/kg) and inulin (200 mg/kg) in saline and killed

one hour later. Serum and tissue PAH and inulin concentrations were determined as described previously.

In the third series of experiments, rabbits were treated with phenobarbital or saline as described previously. Twenty-four hours after the last phenobarbital injections, animals were killed and renal cortical slices were prepared. Accumulation of PAH and TEA in renal cortical slices were then measured.

Effects of Piperonyl Butoxide Pretreatment on Renal Cortical Accumulation of p-Aminohippurate, Tetraethylammonium and Inulin

In the first series of experiments, rabbits received a single i.p. injection of piperonyl butoxide (750 mg/kg) 30 or 90 min prior to infusion with a solution containing PAH and inulin according to the method of Tune and Fernholt (1973) as described previously.

In the second series of experiments, animals received piperonyl butoxide (750 mg/kg) in 1% Tween 80 (vehicle). Control animals received the vehicle alone. Forty-five minutes later animals were killed and uptake of PAH and TEA by renal cortical slices were determined.

Depletion of Renal Glutathione Concentration and Nephrotoxicity of Cephaloridine in Rabbits, Rats and Mice

The purpose of the first series of experiments was to determine cephaloridine toxicity in rabbits, rats and mice forty-eight hours following cephaloridine administration. Animals were administered cephaloridine i.p. or s.c., at doses ranging from 150 to 2000 mg/kg in saline. Control animals were given saline only. All animals were killed 48 hours later by cervical dislocation followed by decapitation.

BUN and SGPT activity in the serum were measured. Livers and kidneys were removed immediately and weighed. PAH and TEA accumulation by renal cortical slices were determined.

The second series of experiments was designed to quantify glutathione (GSH) depletion in kidneys and livers one to four hr following a single administration of cephaloridine. In order to avoid diurnal changes in tissue GSH concentration (Jaeger et al., 1973; Hassing et al., 1979), animals were dosed between 7 a.m. and 9 a.m. and killed before 1 p.m. GSH and water content in the livers and kidneys were determined. In addition, PAH and TEA accumulation and gluconeogenesis in renal cortical slices were measured.

In the third series of experiments, animals were treated with diethyl maleate (0.4 ml/kg, i.p.) in corn oil, 30 min prior to administration of cephaloridine. Animals were killed 24 or 48 hours later and PAH and TEA accumulation in renal cortical slices were determined. Blood urea nitrogen was also measured.

Dose-Dependent Effect of Cephaloridine, Cephalothin and Gentamicin on Tissue GSH Concentration

Experiments were designed to quantify GSH depletion in tissues shortly following a single administration of cephaloridine, cephalothin (Eli Lilly and Company, Indianapolis, IN) or gentamicin (Schering Corporation, Kenilworth, NJ). Animals were dosed between 7 a.m. and 9 a.m. and killed before 10 a.m. One hour after a single i.p. injection of cephaloridine (0-2000 mg/kg) or cephalothin (0-2000 mg/kg), animals were killed, kidneys and livers immediately removed and tissue GSH was

measured. In addition, some animals received a single i.p. injection of gentamicin (0-1000 mg/kg). Because some animals receiving the highest dose of gentamicin died within 45 min, all animals were killed at 45 min instead of 60 min.

Effect of Cephaloridine on Tissue Reduced and Oxidized Glutathione (GSH and GSSG) and Conjugated Dienes

In the first series of experiments, rabbits and rats received a single administration of cephaloridine or saline and were killed one hour later. Concentrations of reduced and oxidized glutathione in the liver and renal cortex were determined immediately.

In the second series of experiments, rats and rabbits received a single administration of cephaloridine and were killed at various times. Portions of liver and renal cortex were used to prepare microsomes as described previously and concentrations of conjugated diene in the microsomes were then determined.

Effect of Vitamin E and/or Selenium Deficiency on Cephaloridine Toxicity in Rats

Five to six weeks old male Fischer 344 rats (56-85 g) were housed in wire mesh hanging cages. Diets (Teklad Test Diets, Madison WI) and water were supplied ad libitum. The tolura yeast-basal diet (Tables 1 and 2) was deficient in vitamin E and selenium (Se). Supplemental levels of 121 U/kg vitamin E and 0.2 ppm Se were used. Animals were divided into four groups. Group 1 was fed the basal diet with no additional vitamin E or Se, group 2 received vitamin E-supplemented diet, group 3 received 0.2 ppm Se-supplemented diet and group 4 received

TABLE 1

Composition of Basal Diet

Ingredient	Composition (g/kg)
Torula Yeast	300.0
DL-Methionine	3.0
Sucrose	593.98
Lard, Tocopherol-Stripped	50.0
Mineral Mix, Hubbell-Mendel-Wakeman	50.0
Manganese Sulfate	0.154
p-Aminobenzoic Acid	0.11
Biotin	0.0004
Vitamin B_{12} (0.1% trituration in mannitol)	0.03
Calcium Pantothenate	0.04
Choline Dihydrogen Citrate	2.44
Folic Acid	0.002
Menandione	0.05
Niacin	0.05
Pyridoxine HCl	0.015
Riboflavin	0.01
Thiamin HCl	0.01
Vitamin A Palmitate, in Corn Oil (200,000 U/g)	0.099
Vitamin D_2, in Corn Oil (400,000 U/g)	0.0055

TABLE 2

Mineral Mix, Hubbell-Mendel-Wakeman

Ingredient	Composition (g/kg)
Calcium Carbonate ($CaCO_3$)	543.0
Magnesium Carbonate ($MgCO_3$)	25.0
Magnesium Sulfate ($MgSO_4$)	16.0
Sodium Chloride (NaCl)	69.0
Potassium Chloride (KCl)	112.0
Potassium Phosphate, Monobasic (KH_2PO_4)	212.0
Ferric Pyrophosphate	20.5
Potassium Iodide (KI)	0.08
Manganese Sulfate ($MnSO_4 \cdot H_2O$)	0.35
Sodium Fluoride (NaF)	1.0
Aluminum Potassium Sulfate ($AlK(SO_4)_2 \cdot 12H_2O$)	0.17
Cupric Sulfate ($CuSO_4$)	0.90

both supplements. Forty-two days after being fed the experimental diets, animals received a single i.p. injection of cephaloridine (0, 500 or 1000 mg/kg) in saline and were killed 24 hr later. BUN and SGPT activity in the serum were measured. Kidneys and livers were removed and weighed. Gluconeogenesis by renal cortical slices were determined. Histopathological alterations in the kidneys and livers were also examined.

Analytical Methods

Determination of PAH and TEA Accumulation and Gluconeogenesis in Renal Cortical Slices. After animals were killed, the kidneys were removed and placed in ice-cold saline until use. Thin renal cortical slices were prepared and incubated in 4.0 ml of phosphate-buffered medium (Cross and Taggart, 1950) which contained 7.4×10^{-5}M PAH and 1.0×10^{-5}M [^{14}C]TEA (specific activity, 2.0 Ci/mole, New England Nuclear, Boston, MA). Incubations were carried out in a Dubnoff metabolic shaker at 25°C under a gas phase of 100% O_2 for 90 min. After incubation, the slices were removed, weighed and homogenized in 10 ml of 3% trichloroacetic acid. Two ml of incubation medium was treated similarly. After centrifugation, the supernatant was assayed for PAH and [^{14}C]TEA concentrations. PAH was determined by the method of Smith et al. (1945). To quantify [^{14}C]TEA, one ml of slice or medium supernatant was added to 10 ml of ACS counting cocktail (Amersham, Arlington Heights, IL) and radioactivity was determined. The accumulation of PAH and TEA in renal cortical slices was expressed as a slice-to-medium (S/M) concentration

ratio, where S represents mg of PAH or TEA per gram of tissue and M represents mg of PAH or TEA per ml of medium.

Gluconeogenesis by renal cortical slices in vitro was measured according to the method of Roobol and Alleyne (1974) with a few modifications. Approximately 100 mg of tissue slices were incubated in 5.0 ml of Krebs-Ringer bicarbonate medium containing 10 mM pyruvate. The incubation flasks with the media were gassed with 95% O_2/5% CO_2, capped tightly, and incubated for 60 min at 37°C in a Dubnoff metabolic shaker. The tissues were then removed, blotted and weighed. After removal of the tissue, the incubation medium was centrifuged at 2,000 x g for 10 min, and glucose concentration in the supernatant was determined using reagents obtained from Sigma Chemical Co. (Sigma Technical Bulletin No. 510, St. Louis, MO).

Determination of Blood Urea Nitrogen (BUN) and Serum Glutamic Pyruvic Transaminase (SGPT) Activity. The blood samples were allowed to clot at room temperature and centrifuged at 600 x g for 10 min and BUN and SGPT activity in the sera were then determined. BUN was measured spectrophotometrically (Fawcett and Scott, 1960; Chaney and Marbach, 1962) with Sigma reagents (Sigma Technical Bulletin No. 640, Sigma Chemical Co., St. Louis, MO). SGPT activity was assayed (Reitman and Frankel, 1957) with Sigma reagents (Sigma Technical Bulletin No. 505) and the activity was expressed as Sigma-Frankel (SF) units/ml. One SF unit of SGPT activity will form 4.82×10^4 μmoles glutamate/min in phosphate buffer, pH 7.5 at 25°C.

Determination of Tissue Water Content and Reduced Glutathione (GSH) and Oxidized Glutathione (GSSG) Concentration. Water content was determined as weight loss after drying to constant weight at 85°C. Reduced glutathione (GSH), more precisely non-protein sulfhydryl content, was measured according to the method of Ellman (1959) with a few modifications. Kidney or liver portions were homogenized in 20 volumes of ice-cold 6% trichloroacetic acid (TCA) and centrifuged at 10,000 x g for 20 min. After an adequate dilution with ice-cold 6% TCA, 0.5 ml of the diluted supernatant was added to 2 ml of 0.3 M Na_2HPO_4 solution. 0.5 ml of 0.04% 5,5'-dithio-bis-(2-nitrobenzoic acid) in 10% sodium citrate was then added and immediately after mixing the absorbance at 412 nm was measured.

In the second series of experiments, both reduced and oxidized glutathione (GSH and GSSG), more precisely both reduced and oxidized non-protein sulfhydryl, were determined according to the method of Ellman (1959) modified by Van Doorn et al. (1978). After animals were killed, portions of liver and renal cortex (0.4-0.6 g) were homogenized in 20 volumes of ice-cold 0.15 M KCl containing 30 mM EDTA. For the determination of GSH, 2 ml of homogenates were deproteinized by the addition of 3 ml of a solution containing 0.3 g/ml NaCl, 0.0167 g/ml metaphosphoric acid and 0.002 g/ml EDTA. After centrifugation at 10,000 x g for 20 min at 4°C, 0.5 ml of the diluted supernatant was added to 2 ml of 0.3 M Na_2HPO_4 solution. 0.5 ml of 0.04% 5,5'-dithio-bis-(2-nitrobenzoic acid) in 10% sodium citrate was then added and the absorbance at 412 nm was measured immediately after mixing. Total glutathione (GSH and GSSG) was assayed as follows: One ml of

deproteinized supernatant was reduced with 1 ml of 5% $NaBH_4$ and then incubated at 45°C for 60 min. The mixture was neutralized with 0.5 ml of 2.7 N HCl and SH groups were assayed as described previously.

Measurement of Conjugated Diene in Microsomes. Conjugated diene concentrations of microsomal lipids were determined by the method of Recknagel and Ghoshal (1966) modified by Sell and Reynolds (1969). One to two grams of liver or renal cortex were homogenized in 3 volumes of ice-cold 0.3 M sucrose, 0.003 M EDTA solution and centrifuged at 10,000 x g for 20 min. The resulting supernatants were further centrifuged at 100,000 x g for 60 min. The pellets were weighed and an adequate volume of 0.3 M sucrose, 0.003 M EDTA solution was added to make a final concentration of 300 mg microsome/ml. 0.5 ml of microsomal suspension was extracted with 9.5 ml of chloroform:methanol (2:1) solution. The mixture was shaken for 15 min and filtered. The filtrate was separated into two phases by the addition of 2.0 ml of water and the upper phase was discarded. 0.2 ml of methanol was added to the lower phase and the absorbance was determined between 210 and 300 nm using freshly prepared lower phase as blank (25 ml water to 95 ml 2:1 chloroform:methanol). Peak absorption for diene conjugation products of lipid peroxidation was at 240 nm. Absorbance at 240 nm, expressed as OD units per gram microsomes, was used as an indicator of the extent of lipid peroxidation.

Determination of Serum and Tissue Cephaloridine Concentration. After animals were killed, blood was collected to prepare serum samples. Tissue were removed immediately, and portions of the tissues were homogenized in five volumes of distilled water using a Potter-Elvehjem homogenizer. A 0.3 ml aliquot of the homogenate was mixed with

0.7 ml of acetonitrile (CH_3CN). Meanwhile a 0.3 ml aliquot of serum was also mixed with 0.7 ml of CH_3CN. The mixtures were centrifuged at 2,000 x g for 10 min and precipitates discarded. The supernatants were removed and filtered through a 0.45 μ regenerated cellulose membrane (Anspec Inc., Ann Arbor, MI) and 10-50 μl aliquots were injected onto an HPLC column. HPLC analyses were performed on a Model 440 chromatograph (Waters Associates, Inc., Milford, MA), equipped with a model U6K injector (Waters Associates, Inc.) at ambient temperature. A Radial-PAK C_{18} reverse phase column (8 mm ID x 10 cm) was used. The mobile phase consisted of 60% CH_3CN/40% H_2O at a flow rate of 2.5 ml/min. Pressure was 1,000-1,500 p.s.i. Effluent absorbance was monitored at 254 nm and recorded at 0.02-0.1 absorbance units full scale. Peak heights or areas were determined and converted to concentrations of cephaloridine by interpolation from a standard curve.

In some experiments, cephaloridine concentrations in serum and tissue were determined according to the method of Tune (1972). After animals were killed, approximately 0.1 g renal cortex was homogenized in 5 ml of distilled water. Serum or tissue suspensions were diluted with distilled water to achieve cephaloridine concentrations of 5 to 10 μg/ml. 500 μl of each solution were then added to 2 ml of 8.75% trichloroacetic acid to precipitate protein. After centrifugation, 500 μl of the supernatant were added to 500 μl of 2 N HCl and the mixture was heated in a stoppered tube at 98°C for one hour to release pyridine by acid hydrolysis. The mixture was then allowed to cool to room temperature. One drop of 0.1% methyl red in 50% aqueous ethanol was added. The mixture was titrated to a yellow color (pH 6.2) with 2 N sodium

hydroxide and left at room temperature for three hours. To each tube were added 1 ml of a freshly prepared solution of 1% aniline in a 2.5% sodium phosphate buffer (pH 6.0) and 500 µl of fresh cyanogen bromide (1 ml of bromine in 50 ml of distilled water, decolorized by dropwise addition of 10% potassium cyanide). Finally, water was added to adjust the total volume to 5 ml. Fluorsecence was read six minutes after the last addition with excitation at 475 nm and emission at 515 nm.

Determination of Cephaloridine Concentrations in Renal Cortical Slices and Incubation Media. Following incubation, cortical slices were removed, blotted, weighed and homogenized in one ml of water and mixed with one ml of CH_3CN. The homogenates were then centrifuged and filtered. In addition, one ml of medium was mixed with one ml of CH_3CN and treated similarly. A 10-50 µl aliquot of filtrate was injected onto the HPLC column. HPLC analyses were conducted in a similar manner as described previously with a few modifications. A 5 mm ID x 10 cm Radial-PAK C_{18} column was used. The mobile phase consisted of 30% CH_3CN/70% H_2O/5 mM PIC B8 (Waters Associates, Inc.). A flow rate of 2.5 ml/min was maintained, with a pressure of 1,000-1,500 p.s.i.

Determination of Potential GSH Conjugates of Cephaloridine or Its Metabolite(s). The purpose of the first series of experiments was to determine the possibility of nonenzymatically catalyzed reactions between GSH and cephaloridine. A final volume of 100 µl incubation mixture containing 50 mM Tris-HCl (pH 7.2-9.0) or 50 mM sodium phosphate buffer (pH 5.7-7.5), 120 µg/µl cephaloridine and 10 mM [^{35}S]GSH (specific activity 6.9 Ci/mole, New England Nuclear, Boston, MA) was incubated

at 25° or 37°C for 60 min. The control blanks only contained 50 mM buffer and 10 mM [^{35}S]GSH. After incubation, samples were then analyzed by thin layer and high pressure liquid chromatography.

In the second series of experiments, possible enzymatically catalyzed GSH conjugating reactions were investigated. A portion of non-treated rabbit or rat hepatic or cortical tissues was homogenized in 3 volumes of 66 mM Tris-HCL buffer, pH 7.4 or 50 mM sodium phosphate buffer, pH 7.0 and then centrifuged at 10,000 x g for 20 min. The resulting supernatant was then used as an enzyme source. In some experiments, 10,000 x g supernatant was further centrifuged at 100,000 x g for 60 min. The resulting 100,000 x g pellet was resuspended in either 66 mM Tris-HCl or 50 mM sodium phosphate buffer to a final concentration of 10-20 mg/ml (microsomal fraction). A final volume of 250 µl incubation mixture containing 10-50 µl enzymes (10,000 x g supernatant, 100,000 x g supernatant or microsomal fraction), 25 µg/µl cephaloridine, 5 mM [^{35}S]GSH (specific activity, 6.9 Ci/mole), 3 mM $MgCl_2$ and 1 mM NADPH was incubated at 37°C for 30 min. The control blank(s) contained the same ingredients but the enzyme sources. The denatured enzymes (boiled at 100°C for 5 min) were used in the control blanks. After incubation, the samples were analyzed by TLC and HPLC.

For TLC analysis, an aliquot (50-150 µl) of incubation mixture was spotted on an activated silica gel GF plate (Analtech, Inc., Newark, Delaware), dried under air flow, and then developed with a solvent system consisting of n-butanol:acetic acid:water (2:1:1, by volume). After being developed, every 1-cm wide band was scrapped into a

scintillation vial containing 10 ml Aquasol-2 solution (New England Nuclear, Boston, MA) and 4 ml water. The samples were mixed well and radioactivities were determined.

For HPLC analysis, after incubation, the incubation mixture was deproteinized by the addition of an equal volume of CH_3CN and centrifugation. The supernatant was filtered and an aliquot of filtrate (10-100 μ) was then injected onto a Radial Pak C_{18} reverse phase column (8 mm ID x 10 cm). The mobile phase consisted of CH_3CN and water at a flow rate of 2.0 ml/min and a linear gradient from 0.5 to 60% CH_3CN was used. Effluent absorbance was monitored at 254 nm and every 1.0 ml of effluent was collected in a scintillation vial containing 10 ml ACS and the radioactivity was determined.

Histology

After animals were killed, portions of kidney tissues were quickly fixed in 10% neutral buffered formalin (3.7% formaldehyde in 75 mM sodium phosphate buffer, pH 7.0). After fixation, tissue samples were embedded in paraffin. Sectionss of kidney were then stained with hematoxylin and eosin (H and E), and evaluated for glomerular, tubular and papillary abnormalities. Degrees of renal cortical necrosis were quantified as follows: mild, less than 10% of proximal tubules necrotic; moderate, 10 to less than 25% of tubules necrotic; severe, 25% or more of the tubules necrotic.

Statistics

The data were analyzed by Student's t-test or analysis of variance, completely randomized design, and treatment means were compared with the least-significant difference test (Steel and Torrie, 1960). The 0.05 level of probability was used as the criterion of significance.

RESULTS

Induction of Renal Microsomal Monooxygenases and Glutathione S-Transferase Activities

Treatment with phenobarbital for four consecutive days had no effect on rat renal cytochrome P450 concentration, ethoxycoumarin-O-deethylase or arylhydrocarbon hydroxylase activity. In contrast, phenobarbital treatment increased rabbit renal cytochrome P450 content (385% of control), ethoxycoumarin-O-deethylase (1400%), benzphetamine-N-demethylase (422%) and arylhydrocarbon hydroxylase (440%) activities (Table 3).

In control animals, both renal and hepatic glutathione S-transferase activities were higher in rabbits than in rats (Table 4); rabbit renal enzyme activity was approximately 6-fold higher than rat. Treatment with phenobarbital significantly increased renal enzyme activity in rabbits but not in rats (Table 4). In contrast, the same treatment increased rat hepatic enzyme activity but had little effect on rabbit hepatic enzyme activity.

Inhibition of Renal Glutathione S-Transferase Activities by Cephaloridine In Vitro

Cephaloridine inhibited renal glutathione S-transferase activities in a dose-dependent manner. For example, at concentrations of 1.8 and

TABLE 3

Effect of Phenobarbital Treatment on Renal Microsomal Monooxygenases in Rats and Rabbits[a]

Enzymes	Rabbit		Rat	
	Control	Phenobarbital	Control	Phenobarbital
Cytochrome P450[b]	0.112±0.025	0.432±0.020[f]	0.066±0.002	0.058±0.004
Ethoxycoumarin O-Deethylase[c]	96.9±36.0	1359.3±65.1[f]	17.8±2.0	16.4±1.2
Benzphetamine N-Demethylase[d]	236.0±6.0	997.0±1.8[f]	---	---
Arylhydrocarbon Hydroxylase[e]	4.43±1.38	23.74±11.90[f]	5.12±0.60	4.82±1.23

[a]Rabbits and rats received a single ip injection of 60 and 80 mg/kg of phenobarbital, respectively, once daily for four days and were killed 24 hr after last injection. Control animals received saline alone. Values are means ± SE of four or more animals.

[b]nmoles/mg microsomal protein.

[c]pmoles 7-hydroxycoumarin formed/mg protein/min.

[d]nmoles formaldehyde formed/mg protein/min.

[e]Relative fluorescent units/mg protein/min.

[f]Significantly different from controls ($p<0.05$).

TABLE 4

Effect of Phenobarbital Treatment on Renal and Hepatic Glutathione S-Transferase Activity in Rats and Rabbits[a]

Animal	Treatment	Glutathione S-Transferase Activity	
		Kidney	Liver
Rabbit	Control	2.62±0.25	5.08±0.44
	Phenobarbital	3.36±0.19[b]	4.56±0.45
Rat	Control	0.42±0.04	1.95±0.20
	Phenobarbital	0.49±0.05	3.30±0.23[b]

[a]Rabbits and rats received a single ip injection of 60 and 80 mg/kg of phenobarbital, respectively, once daily for four days and were killed 24 hr after last injection. Control animals received saline alone. Glutathione S-transferase activity was measured using 1-chloro-2,4-dinitrobenzene as substrate and expressed as μmoles per mg protein per minute. Values are means ± SE of three animals.

[b]Significantly different from control ($p<0.05$).

54 mM, cephaloridine reduced transferase activities by 13 and 62%, respectively (Table 5).

Effect of Phenobarbital on Cephaloridine Nephrotoxicity

In rats, a single administration of cephaloridine caused a dose-dependent increase in blood urea nitrogen (Figure 5). Similarly, cephaloridine treatment depressed renal cortical accumulation of PAH and TEA. Pretreatment of rats with phenobarbital did not alter these effects (Figure 5).

Forty-eight hours following a single injection of cephaloridine to rabbits, BUN was increased while PAH S/M and TEA S/M ratios were reduced in a dose-related manner (Table 6). Phenobarbital pretreatment alone did not have any effect on these functions but did potentiate cephaloridine toxicity. For example, 300 mg/kg of cephaloridine increased BUN by 510%, and decreased PAH and TEA accumulation by 84 and 73%, respectively, in control animals. After phenobarbital treatment the same dosage of cephaloridine increased BUN by 726%, and decreased PAH and TEA accumulation by 92 and 94% (Table 6).

Effect of Piperonyl Butoxide Pretreatment on Cephaloridine Nephrotoxicity

A single injection of piperonyl butoxide (1000 mg/kg) alone had no significant effect on kidney-to-body weight ratio or BUN in rats (Table 7); but slightly inhibited PAH and TEA S/M. Pretreatment with piperonyl butoxide markedly protected rats against cephaloridine nephrotoxicity (Table 7). For example, in the non-pretreated animals, cephaloridine at a dose of 2000 mg/kg, increased BUN by 227%, and decreased PAH and TEA

TABLE 5

In Vitro Effect of Cephaloridine on Rat Renal Cortical Glutathione S-Transferase Activity[a]

Cephaloridine (mM)	Glutathione S-Transferase Activity (μmoles/mg protein/min)
0 (control)	0.232±0.012
1.8	0.201±0.007[b]
9.0	0.137±0.004[b]
18	0.124±0.004[b]
36	0.096±0.009[b]
54	0.088±0.013[b]

[a]Cephaloridine was added into the incubation media. Values are means ± SE of three animals.

[b]Significantly different from control ($p<0.05$).

Figure 5. Effect of phenobarbital pretreatment on blood urea nitrogen and renal cortical slice accumulation of PAH and TEA in rats treated with cephaloridine. Animals were treated with 80 mg/kg of phenobarbital or saline once daily for four days and then received a single administration of cephaloridine (0, 500, 750, 1000 or 1500 mg/kg) and were killed 24 hr later. Each value represents mean ± SE of six or more animals. (*) Significantly different from the control animals receiving an equivalent dose of cephaloridine ($p<0.05$).

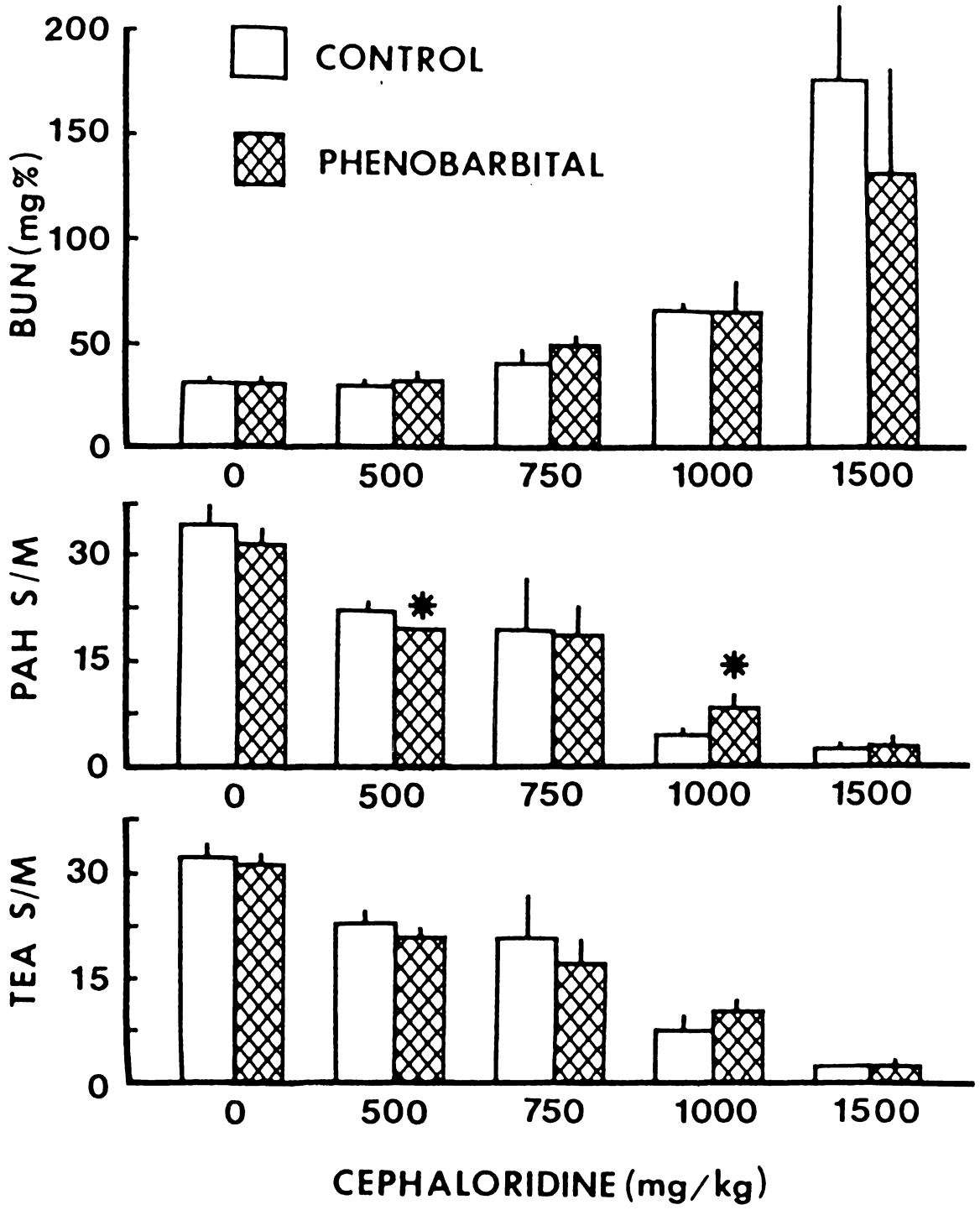

CONTROL
PHENOBARBITAL
BUN (mg%)
200
150
100
50
0
0
500
750
1000
1500
PAH S/M
30
15
0
*
*
TEA S/M
CEPHALORIDINE (mg/kg)

Figure 5

TABLE 6

Effct of Phenobarbital Pretreatment on Cephaloridine Toxicity in Rabbits[a]

Pretreatment	Cephaloridine (mg/kg)	SGPT (SF units/ml)	BUN (mg %)	PAH S/M	TEA S/M
Saline	0	17.3±2.6	15.2± 0.5	15.22±1.44	18.55±1.18
Phenobarbital	0	30.3±3.4[b]	17.7± 1.3	19.59±3.57	18.26±2.03
Saline	150	28.4±2.9	32.6± 8.3	8.84±1.16[b]	13.76±3.21[b]
Phenobarbital	150	34.3±5.3	59.5±17.8	3.37±0.62[b]	6.03±1.08[b]
Saline	300	35.9±5.5[b]	93.2± 6.4[b]	2.39±0.19[b]	5.09±0.97[b]
Phenobarbital	300	63.8±6.6[b]	146.6±19.4[b]	1.61±0.28[b]	1.18±0.22[b]

[a]Animals received a single ip injection of phenobarbital (60 mg/kg) or saline once daily for four days and 24 hr after last injection received a single administration of cephaloridine. All animals were killed 48 hr following cephaloridine injection. Values are means ± SE of four or more animals.

[b]Significantly different from the saline group receiving an equivalent dose of cephaloridine ($p<0.05$).

TABLE 7

Effect of Piperonyl Butoxide Pretreatment on Cephaloridine Nephrotoxicity in Sprague-Dawley Rats[a]

Pretreatment	Cephaloridine (mg/kg)	$\frac{\text{Kidney Wt}}{\text{Body Wt}}$ (g/kg)	BUN (mg %)	PAH S/M	TEA S/M
Corn Oil	0	8.12±0.25	24.2± 0.8	25.2±2.0[b]	24.7±2.0[b]
Piperonyl Butoxide	0	7.73±0.22	24.7± 0.8	21.9±0.9[b]	19.5±1.1[b]
Corn Oil	1000	10.14±0.28[b]	45.9± 5.0[b]	8.5±2.2[b]	9.8±2.6[b]
Piperonyl Butoxide	1000	7.85±0.29[b]	21.3± 2.0[b]	22.3±2.1[b]	14.0±1.1[b]
Corn Oil	2000	11.58±0.25[b]	79.1±11.9[b]	4.1±0.4[b]	6.5±0.7[b]
Piperonyl Butoxide	2000	9.95±0.54[b]	55.3± 5.5[b]	11.3±2.2[b]	11.2±1.5[b]

[a]Piperonyl butoxide (1000 mg/kg) was given 30 min prior to cephaloridine administration. The control groups received corn oil. All animals were killed 24 hr following cephaloridine administration. Values are means ± SE of four or more animals.

[b]Significantly different from the corn oil group receiving an equivalent dose of cephaloridine ($p<0.05$).

S/M by 84 and 74%, respectively. However, the same dosage of cephaloridine after piperonyl butoxide increased BUN by 124% and decreased PAH and TEA by approximately 56 and 55% (Table 7).

A similar protective effect of piperonyl butoxide was also found in rabbits (Table 8). A single administration of cephaloridine (150 mg/kg) produced proximal tubular necrosis in 75% of nonpretreated animals. However, the same dose of cephaloridine only produced tubular necrosis in 25% of piperonyl butoxide pretreated (750 mg/kg) group.

Effect of Sex Difference on Cephaloridine Toxicity

Cephaloridine treatment produced a dose-related nephrotoxicity in male and female rats (Table 9). However, there was no significant difference between the sexes.

Effect of Phenobarbital on Renal Cortical Accumulation of Cephaloridine In Vivo

Cephaloridine was accumulated in renal cortex to concentrations much greater than in serum. In control rabbits, one hour after a single administration of 150 mg/kg of cephaloridine, the cortex-to-serum (C/S) ratio was 26.2±2.7 (Table 10). This ratio declined to 19.4±2.9 by two hours but reached 36.1±7.0 by three hours. Pretreatment of rabbits with phenobarbital increased cephaloridine C/S ratios at two and three hours to 40.9±2.3 and 61.9±9.6, respectively. The increase in cephaloridine C/S ratios in rabbits produced by phenobarbital treatment resulted from a higher concentration of the drug in renal cortex and was not due to a decrease in serum cephaloridine concentrations (Table 10).

TABLE 8

Effect of Piperonyl Butoxide Pretreatment on Cephaloridine Nephrotoxicity in Rabbits[a]

Pretreatment	No Necrosis	Proximal Tubular Necrosis		
		Mild	Moderate	Severe
None (control)	2	1	4	1
Piperonyl Butoxide				
135 mg/kg	1	3	0	0
750 mg/kg	6	1	1	0

[a]Cephaloridine (150 mg/kg sc) was administered 30 min following piperonyl butoxide injection. Animals were killed 48 hr after cephaloridine.

TABLE 9

Cephaloridine Toxicity in Male and Female Rats[a]

Cephaloridine (mg/kg)	Sex	SGPT (SF units/ml)	BUN (mg %)	PAH S/M	TEA S/M
0	Male	36.4± 1.9	36.5± 1.2	30.73±1.33	25.51±2.53
0	Female	29.5± 1.1[b]	35.0± 2.3	29.46±2.21	28.21±2.79
500	Male	32.9± 2.1	41.9± 4.7	23.23±1.30	22.72±1.18
500	Female	33.2± 1.7	36.4± 1.3	24.06±1.00	24.75±1.24
1000	Male	34.6± 2.3	73.2± 6.9	6.85±1.71	8.36±2.09
1000	Female	38.7± 2.1	95.8± 5.5[b]	7.78±0.64	12.11±1.87
2000	Male	81.3±21.8	268.2±87.0	2.37±0.18	1.17±0.14
2000	Female	64.5± 4.5	293.0±54.5	2.50±0.24	0.91±0.55

[a]Animals received a single i.p. injection of cephaloridine or saline and were killed 48 hours later. Values are means ± S.E. of four or more animals.

[b]Significantly different from male rats receiving an equivalent dose of cephaloridine ($p<0.05$).

TABLE 10

Effect of Phenobarbital Pretreatment on Renal Cortical Accumulation of Cephaloridine in Rabbits[a]

Pretreatment	Time (hr)	Cephaloridine		
		Cortex (μg/g)	Serum (μg/ml)	Cortex/Serum
Control	1	10246±1257	397±44	26.2±2.7
Phenobarbital	1	8832±1197	279±30	32.8±4.8
Control	2	4862± 729	259±29	19.4±2.9
Phenobarbital	2	8890± 649[b]	223±17	40.9±4.3[b]
Control	3	653± 81	20.4±2.9	36.1±7.0
Phenobarbital	3	1176± 132[b]	20.7±3.2	61.9±9.6[b]

[a]Animals received phenobarbital (60 mg/kg, ip) or saline (control) once daily for four days and then a single injection of 150 mg/kg cephaloridine 24 hr after last administration of phenobarbital. Animals were killed one, two or three hr after cephaloridine administration. Values are means ± SE of four or more animals.

[b]Significantly different from the control group killed at the same time following cephaloridine administration ($p<0.05$).

Unlike in rabbits, treatment of rats with phenobarbital did not significantly increase cephaloridine C/S ratios (Table 11). In fact at three hours, the serum cephaloridine concentration was less in phenobarbital pretreated animals than in controls (Table 11).

Effect of Phenobarbital Pretreatment on Cephaloridine Uptake and Runout in Rabbit Renal Cortical Slices

Pretreatment of rabbits with phenobarbital increased uptake of cephaloridine by renal cortical slices *in vitro* (Table 12). However, the same treatment did not appear to alter the slope of the efflux curve (Figure 6).

Effect of Piperonyl Butoxide Pretreatment on Renal Cortical Accumulation of Cephaloridine In Vivo and In Vitro

Pretreatment of rabbits with piperonyl butoxide (750 mg/kg) significantly decreased renal cortical accumulation of cephaloridine (Table 13). For example, in the controls, a concentration of 2.68 and 4.00 mg/g of cephaloridine was accumulated in renal cortex, 15 and 60 min, respectively, following a single administration of 150 mg/kg cephaloridine. However, in piperonyl butoxide pretreated animals, significantly less cephaloridine (1.10 and 2.23 mg/g) was accumulated. Meanwhile, piperonyl butoxide pretreatment increased serum concentration of cephaloridine (Table 13). Furthermore, pretreatment of rabbits with piperonyl butoxide markedly inhibited uptake of cephaloridine by renal cortical slices *in vitro* (Table 14).

TABLE 11

Effect of Phenobarbital Pretreatment on Renal Cortical Accumulation of Cephaloridine in Rats[a]

Pretreatment	Time (hr)	Cephaloridine		
		Cortex (µg/g)	Serum (µg/ml)	Cortex/Serum
Control	1	2580±301	328±63	8.34±1.80
Phenobarbital	1	1881±405	307±77	6.94±0.88
Control	2	2585±458	294±53	8.93±2.39
Phenobarbital	2	3096±569	343±57	10.08±1.87
Control	3	2386±311	363±28[b]	6.50±0.69
Phenobarbital	3	1548±238	182±23[b]	8.50±0.92

[a]Animals received phenobarbital (80 mg/kg, ip) or saline (control) once daily for four days and then a single injection of cephaloridine (1000 mg/kg) and were killed one, two or three hr later. Values are means ± SE of four or more animals.

[b]Significantly different from the control group killed at the same time following cephaloridine administration ($p<0.05$).

TABLE 12

Effect of Phenobarbital Pretreatment on Cephaloridine Accumulation in Rabbit Renal Cortical Slices[a]

Pretreatment	Time (min)	Cephaloridine		
		Slice (µg/g)	Medium (µg/ml)	Slice/Medium
Control	45	332±20	265± 7	1.26±0.10
Phenobarbital	45	706±38[b]	265±11	2.68±0.23[b]
Control	90	590±56	255± 3	2.32±0.23
Phenobarbital	90	1353±43[b]	222±12	6.10±0.21[b]

[a]Rabbits received a single ip injection of phenobarbital (60 mg/kg) or saline (control) once daily for four days and were killed 24 hr after last injection. Renal cortical slices were incubated in 4.0 ml of phosphate-buffered medium containing 275 µg/ml of cephaloridine at 25°C under 100% O_2 for 45 and 90 min. Cephaloridine in slices and media were determined and expressed as µg cephaloridine per gram of tissue and per ml of medium, respectively. Values are means ± SE of three animals.

[b]Significantly different from control ($p<0.05$).

Figure 6. Effect of phenobarbital pretreatment on cephaloridine runout in rabbit renal cortical slices. Animals received 60 mg/kg of phenobarbital or saline (control) once daily for four days and were killed 24 hr following the last injection. Renal cortical slices were then preloaded with cephaloridine and runout was conducted at 25°C under air. Each point and vertical bar represents mean ± SE of three animals.

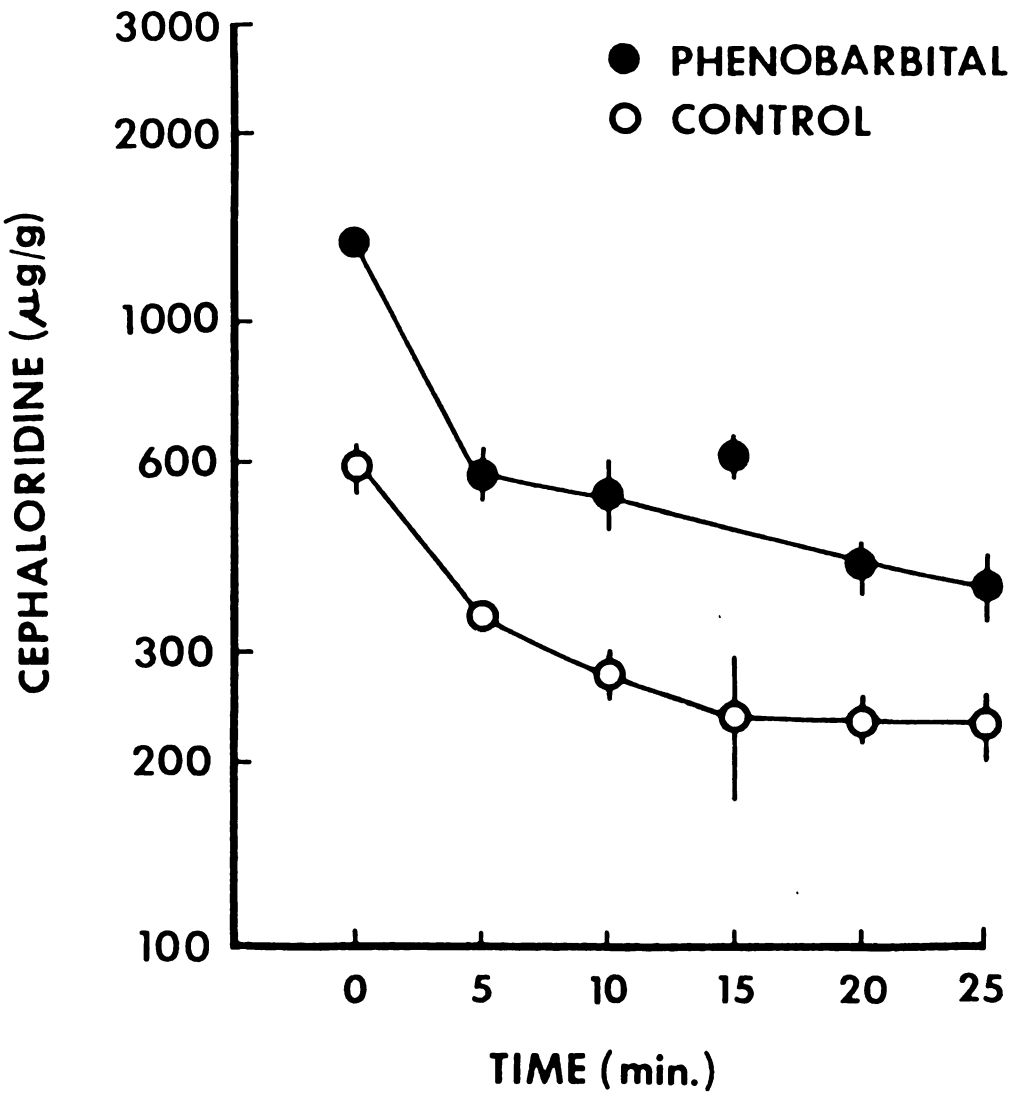

3000
2000
1000
600
300
200
100
CEPHALORIDINE (µg/g)
PHENOBARBITAL
CONTROL
0
5
10
15
20
25
TIME (min.)

Figure 6

TABLE 13

Effect of Piperonyl Butoxide Pretreatment on Cephaloridine Accumulation by Rabbit Renal Cortex[a]

Pretreatment	Serum Cephaloridine (μg/ml)	Cortical Cephaloridine (μg/g)	Cortex/Serum
1/4 hour			
Control	482±31	2684±176	5.8±0.7
Piperonyl Butoxide	717±53[b]	1104±321[b]	1.7±0.6[b]
1 hour			
Control	168±18	4000±471	26.1±5.1
Piperonyl Butoxide	292±62[b]	2231±452[b]	9.7±2.9[b]

[a]Animals received piperonyl butoxide (750 mg/kg, ip) 30 min prior to a single sc injection of cephaloridine (150 mg/kg). The control groups received piperonyl butoxide vehicle (1% Tween 80) prior to cephaloridine administration. Animals were killed 15 or 60 min after cephaloridine. Values are means ± SE of four or more animals.

[b]Significantly different from control ($p<0.05$).

TABLE 14

Effect of Piperonyl Butoxide Pretreatment on Cephaloridine Uptake in Rabbit Renal Cortical Slices[a]

Incubation Time (min)	Slice Cephaloridine (μg/g)		Medium Cephaloridine (μg/ml)		Slice/Medium	
	Control	Treated	Control	Treated	Control	Treated
30	153.1±1.9	74.8± 3.3[b]	92.9±4.1	119.6±7.3[b]	1.66±0.08	0.63±0.03[b]
60	196.2±8.9	92.2± 6.9[b]	94.7±4.0	105.4±1.9[b]	2.08±0.13	0.88±0.07[b]
90	314.5±7.4	135.3±20.1[b]	73.9±8.9	98.3±4.7[b]	4.42±0.48	1.38±0.21[b]

[a]Rabbits received a single administration of piperonyl butoxide (750 mg/kg) or 1% Tween 80 (vehicle) and were killed 45 min later. Values are means ± SE of four animals.

[b]Significantly different from control ($p<0.05$).

Effect of Phenobarbital Pretreatment on Rabbit Renal Cortical Accumulation of PAH and Inulin In Vivo and PAH and TEA In Vitro

In the first series of experiments, animals (controls and phenobarbital treated) were continuously infused with a solution containing PAH and inulin for one hour (Table 15). Phenobarbital treatment did not significantly alter renal cortical concentration of PAH or inulin. In the second series of experiments, animals were given a single bolus administration of PAH and inulin and killed one hour later. Phenobarbital treatment significantly decreased PAH cortex-to-serum concentration ratios. Cortical PAH concentration was also reduced in phenobarbital pretreated animals (Table 15). Phenobarbital treatment had no effect on PAH or TEA accumulation by rabbit renal cortical slices (Table 16).

Effect of Piperonyl Butoxide on Rabbit Renal Cortical Accumulation of PAH and Inulin In Vivo and PAH and TEA In Vitro

Pretreatment of rabbits with piperonyl butoxide did not significantly alter accumulation of PAH and inulin in renal cortex, when PAH and inulin were administered i.v. (Tables 17 and 18). Cortical slices from rabbits pretreated with piperonyl butoxide for 90 min did not accumulate less PAH or TEA than the slices from the controls (Table 19). The cortical slices from the rabbits pretreated with piperonyl butoxide for 45 min accumulated somewhat less PAH and TEA than the controls (Table 20); however, this inhibition was not persistant and much less profound than the inhibition of cephaloridine accumulation (Table 14).

TABLE 15

Effect of Phenobarbital Pretreatment on Renal Cortical Accumulation of PAH and Inulin in Rabbits

Treatment	PAH			Inulin		
	Cortex (μg/g)	Serum (μg/ml)	Cortex/Serum	Cortex (μg/g)	Serum (μg/ml)	Cortex/Serum
Control	274±28	44.0±2.5	6.18±0.42	1645±110	895± 95	1.70±0.07
Phenobarbital[a]	220±39	32.3±5.4	7.93±0.97	1412±139	872±120	1.71±0.14
Control	66.5±11.4	13.8±1.6	4.73±0.32	903± 75	445± 19	2.03±0.13
Phenobarbital[b]	38.8± 3.7	11.2±0.5	3.48±0.39[c]	708± 50	415± 23	1.72±0.16

[a]Animals received phenobarbital (60 mg/kg, ip) or saline (control) once daily for four days and then a bolus ear vein injection of PAH (17.5 mg/kg) and inulin (2 ml, 10%) followed by a continuous infusion of 20 mg/kg PAH and 10% inulin at a flow of 0.11 ml/min for one hour. Values are means ± SE of four or more animals.

[b]Animals received phenobarbital (60 mg/kg, ip) or saline (control) once daily for four days and then a bolus ear vein injection of 40 mg/kg PAH and 200 mg/kg inulin. Animals were killed one hour later. Values are means ± SE of four animals.

[c]Significantly different from controls ($p<0.05$).

TABLE 16

Effect of Phenobarbital Pretreatment on Accumulation of p-Aminohippurate and Tetraethylammonium in Rabbit Renal Cortical Slices[a]

Pretreatment	Incubation Time (min)	PAH S/M	TEA S/M
Control	45	11.64±1.28	12.94±1.40
Phenobarbital	45	15.18±0.94	15.34±0.95
Control	90	18.08±1.32	17.84±1.78
Phenobarbital	90	20.66±1.92	19.38±2.72

[a]Rabbits received a single ip injection of phenobarbital (60 mg/kg) or saline (control) once daily for four days and were killed 24 hr after the last injection. Renal cortical slices were incubated in 4.0 ml of phosphate-buffered medium containing 7.4×10^{-4}M PAH and 1.0×10^{-5}M [^{14}C]TEA at 25°C under 100% O_2 for 45 and 90 min. Values are means ± SE of three animals.

TABLE 17

Effect of Piperonyl Butoxide Pretreatment (30 min) on PAH and Inulin Accumulation in Rabbit Renal Cortex[a]

Pretreatment	Serum PAH (μg/ml)	Cortical PAH (μg/g)	Cortex-to-Serum Ratio
1% Tween 80	42.1±15.1	377.6±130.0	8.77±1.32
Piperonyl Butoxide	66.0±12.7	518.9±124.0	7.88±0.77
Pretreatment	**Serum Inulin (μg/ml)**	**Cortical Inulin (μg/g)**	**Cortex-to-Serum Ratio**
1% Tween 80	1367±508	2471±744	1.98±0.17
Piperonyl Butoxide	1532±332	2572±490	1.72±0.10

[a]Animals received piperonyl butoxide (750 mg/kg) 30 min prior to a single i.v. injection of 17.5 mg/kg of PAH and 2 ml of 10% inulin, followed by a one-hour infusion with 10% inulin and 20 mg/ml of PAH at an infusion rate of 0.11 ml/min. Control animals received 1% Tween 80 prior to infusion with inulin and PAH. Each value represents mean ± SE of four or more animals.

TABLE 18

Effect of Piperonyl Butoxide Pretreatment (90 min) on PAH and Inulin Accumulation in Rabbit Renal Cortex[a]

Pretreatment	Serum PAH (μg/ml)	Cortical PAH (μg/g)	Cortex-to-Serum Ratio
None[b]	36.5± 5.3	190.4± 14.5	5.76±1.14
1% Tween 80	48.8± 2.5	247.6± 48.4	5.51±0.92
Piperonyl Butoxide	44.6± 5.6	242.5± 38.8	6.92±1.05

Pretreatment	Serum Inulin (μg/ml)	Cortical Inulin (μg/g)	Cortex-to-Serum Ratio
None[b]	1767±293	1716±158	1.03±0.11
1% Tween 80	2096±228	2700±248	1.46±0.19
Piperonyl Butoxide	1876±174	2223±231	1.28±0.29

[a]Animals received piperonyl butoxide (750 mg/kg) 90 min prior to a single i.v. injection of 17.5 mg/kg of PAH and 2 ml of 10% inulin, followed by a one-hour infusion with 10% inulin and 20 mg/ml of PAH at an infusion rate of 0.11 ml/min. Control animals received 1% Tween 80 prior to infusion with inulin and PAH. Each value represents mean ± SE of four or more animals.

[b]Animals did not receive piperonyl butoxide or 1% Tween 80 before PAH and inulin infusion.

TABLE 19

Effect of Piperonyl Butoxide Pretreatment (90 min) on p-Aminohippurate and Tetraethylammonium Accumulation in Rabbit Renal Cortical Slices[a]

Incubation Time (min)	PAH S/M[b]		TEA S/M[b]	
	Control	Treated	Control	Treated
15	4.21±0.34	3.51±0.32	4.58±0.34	3.94±0.41
30	6.54±0.44	6.42±0.73	6.88±0.31	6.07±0.37
60	10.25±0.56	9.84±0.49	10.64±0.58	10.04±0.54
90	11.96±0.77	12.76±1.05	13.31±0.67	12.10±1.17

[a]Female New Zealand rabbits received a single i.p. injection of 750 mg/kg of piperonyl butoxide in 1% Tween 80. Control animals received 1% Tween 80 (vehicle) alone. Animals were killed 90 min later and renal cortical slices were prepared and incubated in a phosphate-buffered medium containing 7.4×10^{-5}M PAH and 1.0×10^{-5}M TEA under 100% O_2 for 15, 30, 60 and 90 min.

[b]Accumulation of PAH or TEA in renal cortical slices is expressed as the concentration of PAH or TEA in slices to the concentration in the medium ratio. Each value represents mean ± SE of 7 or more animals.

TABLE 20

Effect of Piperonyl Butoxide Pretreatment (45 min) on p-Aminohippurate and Tetraethylammonium Accumulation in Rabbit Renal Cortical Slices[a]

Incubation Time (min)	PAH S/M		TEA S/M	
	Control	Treated	Control	Treated
15	4.63±0.50	3.50±0.46	4.76±0.64	3.83±0.45
30	6.91±0.38	4.71±0.65[b]	6.84±0.29	5.15±0.49[b]
60	12.32±1.58	9.48±0.65	11.97±1.40	9.01±0.57
90	17.34±2.41	13.10±0.56	16.16±1.69	11.94±0.84[b]

[a]Female New Zealand rabbits received a single i.p. injection of 750 mg/kg of piperonyl butoxide in 1% Tween 80. Control animals received 1% Tween 80 (vehicle) alone. Animals were killed 45 min later. Each value represents mean ± SE of four animals.

[b]Significantly different from control animals ($p<0.05$).

Kidney and Liver Toxicity in Rabbits, Rats and Mice Forty-Eight Hours After A Single Dose of Cephaloridine

Treatment of rabbits with 150 mg/kg cephaloridine did not alter the kidney-to-body weight ratios, but a higher dose (300 mg/kg) significantly increased the ratio by 17% (Table 21). Similarly, kidney-to-body weight ratios increased by approximately 7, 12 and 34% in rats following the administration of 500, 1000 and 2000 mg/kg cephaloridine, respectively (Table 22). In contrast, cephaloridine treatment had very little effect on liver-to-body weight ratios (Tables 21 and 22). Even though there was no change in the liver-to-body weight ratio, cephaloridine slightly, but significantly, elevated SGPT activity in rabbits (Table 21) and rats (Table 22). However, the lower doses (500 and 1000 mg/kg) appeared to have no influence on SGPT activity in rats.

In rabbits and rats, cephaloridine treatment increased BUN in a dose-related manner (Tables 21 and 22). Cephaloridine, at a dose of 150 and 300 mg/kg, elevated rabbit BUN by approximately 110 and 510%, respectively (Table 21). In rats, 500 mg/kg cephaloridine did not significantly alter BUN, but the higher doses, i.e., 1000 and 2000 mg/kg, increased BUN about 2- and 6-fold (Table 22). In contrast, the same doses of cephaloridine had very little influence on mouse blood urea nitrogen (Table 23).

Renal PAH and TEA transport were impaired following cephaloridine treatment. This treatment appeared to have a greater effect on PAH transport, especially with the lower doses. For example, treatment with 150 mg/kg cephaloridine reduced PAH accumulation by about 40%, but had little effect on TEA transport in rabbits (Table 21). Similarly,

TABLE 21

Cephaloridine Toxicity in Rabbits[a]

Parameter	Cephaloridine (mg/kg)		
	0 (Control)	150	300
Kidney Wt/Body Wt (g/kg)	6.82±0.27	6.18±0.26	7.99±0.39[b]
Liver Wt/Body Wt (g/kg)	32.27±0.71	33.67±2.18	32.98±2.04
BUN (mg %)	15.24±0.51	32.55±8.86[b]	93.19±6.41[b]
SGPT (SF units/ml)	17.25±2.57	28.41±2.88[b]	35.92±5.48[b]
PAH S/M	15.22±1.44	8.84±1.16[b]	1.61±0.28[b]
TEA S/M	18.55±1.18	13.76±3.21	5.09±0.97[b]

[a]Animals received a single sc administration of cephaloridine and were killed 48 hr later. Values are means ± SE of five or more animals.

[b]Significantly different from control ($p<0.05$).

TABLE 22

Cephaloridine Toxicity in Rats[a]

Parameter	Cephaloridine (mg/kg)			
	0 (Control)	500	1000	2000
Kidney Wt/Body Wt (g/kg)	9.35±0.22	10.03±0.19[b]	10.48±0.28[b]	12.51± 0.60[b]
Liver Wt/Body Wt (g/kg)	39.15±1.26	42.54±1.25	38.40±0.80	41.61± 1.65
BUN (mg %)	36.5 ±1.2	41.9 ±4.7	73.2 ±6.9[b]	268.2 ±87.0[b]
SGPT (SF units/ml)	36.35±1.94	32.91±2.12	34.62±2.27	81.31±21.75[b]
PAH S/M	30.73±1.33	23.23±1.30[b]	6.85±1.71[b]	2.37± 0.18[b]
TEA S/M	25.51±2.53	22.72±1.18	8.36±2.09[b]	1.17± 0.14[b]

[a]Animals received a single ip injection of cephaloridine and were killed 48 hours later. Values are means ± SE of four or more animals.

[b]Significantly different from control ($p<0.05$).

TABLE 23

Cephaloridine Toxicity in Mice[a]

Parameter	Cephaloridine (mg/kg)			
	0 (Control)	1000	1500	2000
Kidney Wt/Body Wt (g/kg)	13.93±0.31	15.29±0.50	14.52±0.47	14.24±0.57
Liver Wt/Body Wt (g/kg)	50.16±1.55	46.80±2.27	50.14±1.11	48.60±1.32
BUN (mg %)	34.54±1.68	34.49±3.72	37.88±3.52	31.08±1.81
SGPT (SF units/ml)	18.80±2.03	24.80±1.66	17.40±1.47	14.60±2.69
PAH S/M	11.87±0.45	12.68±0.57	11.31±0.23	11.83±0.71
TEA S/M	30.04±0.80	29.48±0.78	28.08±1.26	26.40±0.34[b]

[a]Animals received a single ip injection of cephaloridine and were killed 48 hours later. Values are means ± SE of four or more animals.

[b]Significantly different from control ($p<0.05$).

cephaloridine at a dose of 500 mg/kg, significantly reduced PAH accumulation, but did not alter TEA transport in rats (Table 22). However, cephaloridine had little effect on mouse renal PAH or TEA transport except the highest dose (2000 mg/kg), which significantly impaired TEA transport (Table 23).

Effect of Cephaloridine on Kidney and Liver Reduced Glutathione (GSH) and Water Content in Rabbits, Rats and Mice

A single administration of cephaloridine (300 mg/kg, s.c.) to rabbits rapidly decreased the concentration of renal cortical GSH such that it reached 60% of control by one hour (Figure 7) and then returned to the control value by four hours. In contrast, no significant change in the liver GSH occurred (Figure 7). In addition, cephaloridine treatment did not alter renal medullary GSH. Cephaloridine (2000 mg/kg, i.p.) also markedly decreased rat renal cortical GSH (Figure 8). In contrast to rabbits and rats, mouse renal cortical GSH was not decreased after cephaloridine (Table 24A). Paradoxically, GSH was significantly elevated three hours after the administration of the drug in mice.

The reduction in tissue GSH content did not appear to be due to a change of tissue water content (Table 25). Cephaloridine (300 mg/kg, s.c.) did not change rabbit liver water content, but slightly increased renal cortical water content (Table 25). Similarly, cephaloridine (2000 mg/kg, i.p.) altered rat liver and cortex water content slightly (Table 25) and actually decreased liver water content.

Cephaloridine depleted renal cortical GSH in a dose-related manner (Figures 9 and 10). A single administration of 250 mg/kg cephaloridine

Figure 7. Time course of rabbit tissue GSH following a single sc administration of cephaloridine. Rabbits received 300 mg/kg of cephaloridine and were killed at the required time. Each point and vertical bar represents mean ± SE of four or more animals. (*) Significantly different from the animals killed at zero hour (controls) after cephaloridine administration ($p<0.05$). Hepatic and renal cortical concentrations of GSH in the control animals were 12.99±2.40 and 3.90±0.47 μmoles/g tissue, respectively.

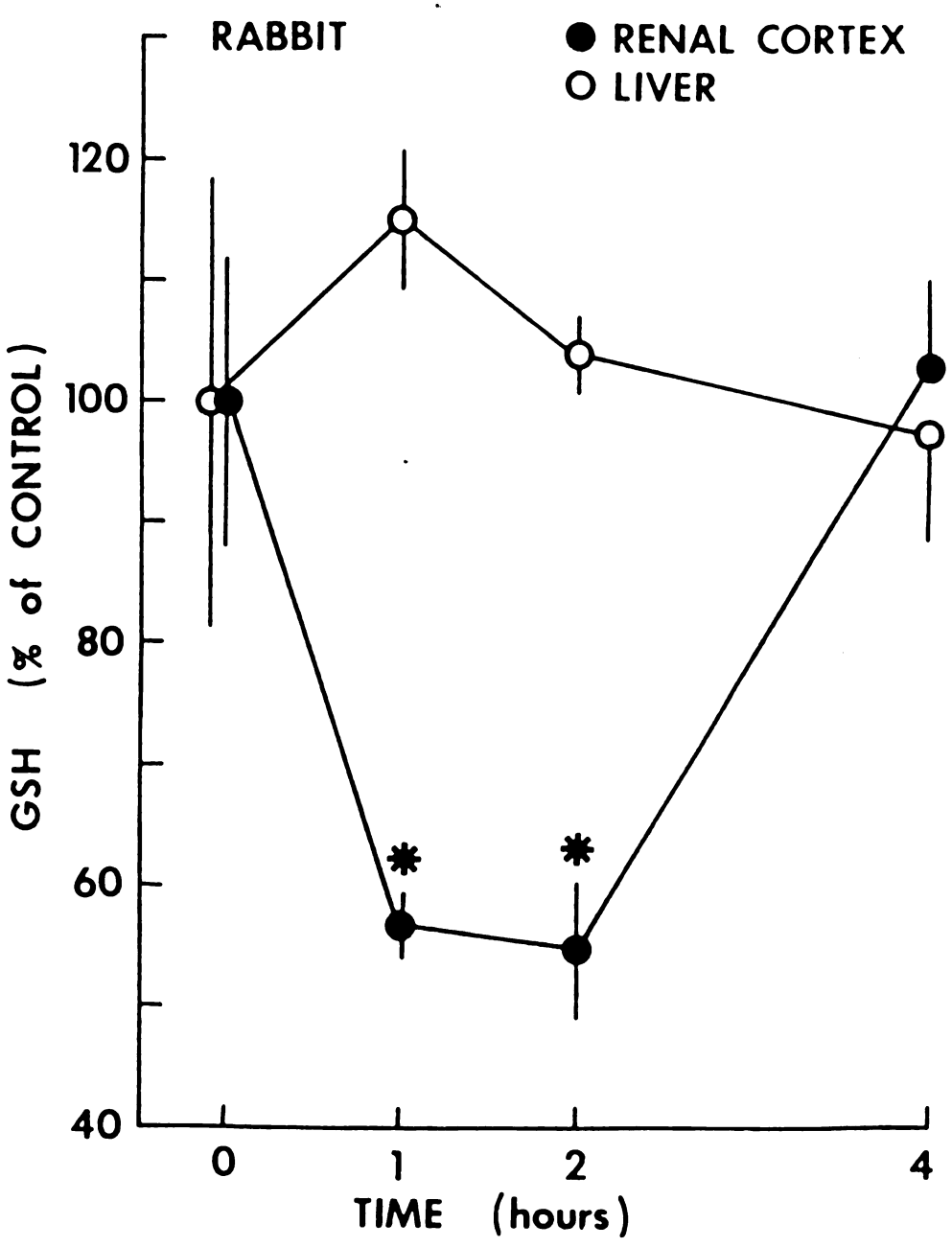

RABBIT
● RENAL CORTEX
O LIVER
120
100
80
60
40
GSH (% of CONTROL)
*
*
0
1
2
4
TIME (hours)

Figure 7

Figure 8. Time course of rat tissue GSH following a single ip administration of cephaloridine. Animals were dosed 2000 mg/kg of cephaloridine and killed at the required time. Each point and vertical bar represents mean ± SE of four or more animals. (*) Significantly different from the animals killed at zero hour (controls) after cephaloridine ($p<0.05$). Renal cortical concentration of GSH in the control animals was 2.57±0.08 μmoles/g tissue.

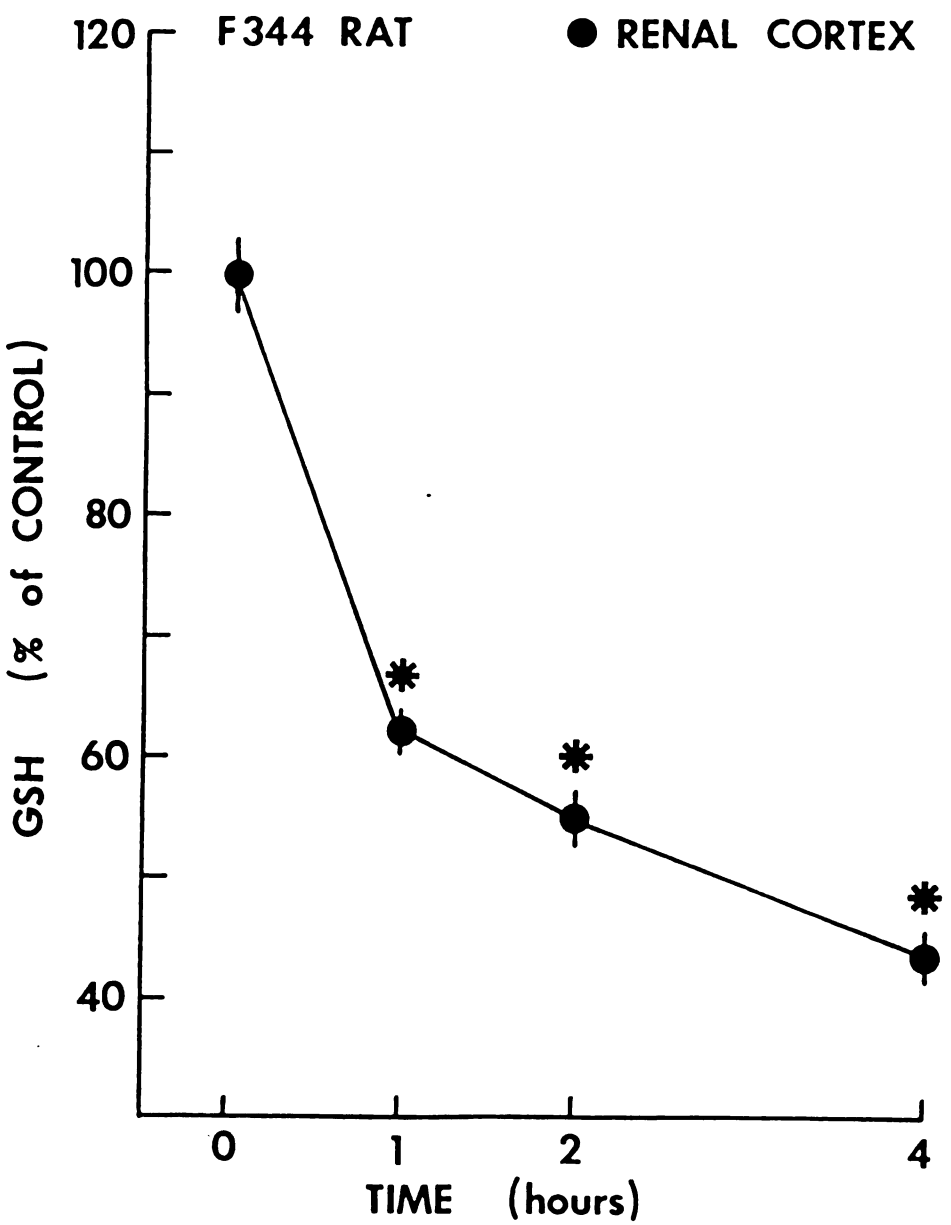

F344 RAT
RENAL CORTEX
120
100
80
60
40
GSH (% of CONTROL)
0
1
2
4
TIME (hours)

Figure 8

TABLE 24

Effect of Cephaloridine Treatment on Mouse Renal Cortical GSH Concentration

A		B	
Time[a] (hrs)	GSH (μmole/g)	Cephaloridine[b] (mg/kg)	GSH (μmole/g)
0 (control)	4.72±0.65	0 (control)	4.57±0.41
3/4	4.41±0.15	1000	5.67±0.19[c]
1	5.17±0.26	2000	4.61±0.53
2	5.91±0.37	3000	5.11±0.12
3	6.71±0.44[c]		

[a]Animals were dosed a single ip injection of cephaloridine (2000 mg/kg) and killed 0, 3/4, 1, 2 and 3 hrs later. Values are means ± SE of four animals.

[b]Animals received a single ip administration of cephaloridine or saline (control) and were killed one hour later. Values are means ± SE of three or more animals.

[c]Significantly different from controls ($p<0.05$).

TABLE 25

Water Content in Rabbit and Rat Renal Cortex and Liver Following Cephaloridine Administration[a]

Time (hrs)	Rabbits		Rat	
	Cortex	Liver	Cortex	Liver
0 (control)	100	100	100	100
1	100.5±0.8.	101.8±2.3	99.4±0.3	98.1±0.5[b]
2	102.9±0.7[b]	99.1±0.3	103.3±0.2[b]	98.3±0.3[b]
4	103.1±0.9[b]	98.0±0.2[b]	104.6±0.8[b]	98.3±0.5[b]

[a]Animals received a single administration of 300 mg/kg (rabbits) or 2000 mg/kg (rats) of cephaloridine and were killed 0, 1, 2 and 4 hours later. Data are expressed as percentage of controls. Each value represents mean ± SE of four or more animals.

[b]Significantly different from control ($p<0.05$).

decreased rabbit cortical GSH by about 45% and 750 mg/kg reduced GSH by 70% by one hour (Figure 9). In rats, 1000 and 2000 mg/kg cephaloridine depleted cortical GSH by about 20 and 30%, respectively (Figure 10). In contrast, cephaloridine had no effect on rabbit liver GSH nor rat medulla GSH (Figures 9 and 10). In addition, at the highest dose (2000 mg/kg) cephaloridine reduced rat liver GSH by about 17%, even though the lower doses (500 and 1000 mg/kg) did not alter GSH. Cephaloridine did not deplete mouse cortical GSH, even when a dose as high as 3000 mg/kg was used (Table 24B).

Effect of Diethyl Maleate Pretreatment on Cephaloridine Nephrotoxicity

Diethyl maleate depleted renal cortical GSH in a dose-dependent manner (Table 26). Treatment of rats and rabbits with diethyl maleate (0.4 ml/kg) alone had no effect on BUN, PAH or TEA accumulation (Tables 27-29). However, this treatment significantly potentiated cephaloridine nephrotoxicity in Fischer 344 rats. For example, a single dose of 750 mg/kg cephaloridine slightly increased BUN (128% of control) in non-pretreated Fischer 344 rats, but the same dose of cephaloridine markedly elevated BUN (720% of control) in diethyl maleate pretreated animals (Table 28). Similarly, 1000 and 2000 mg/kg of cephaloridine increased BUN by about 90 and 200% in corn oil-pretreated SD rats, respectively; however, the same doses of cephaloridine elevated BUN by 315 and 510% in diethyl maleate-pretreated S-D rats (Table 29). Furthermore, administration of 1000 and 2000 mg/kg of cephaloridine decreased PAH S/M by approximately 60 and 85% in corn oil-pretreated S-D rats. But the same doses reduced PAH S/M by 77 and 87% in diethyl maleate-pretreated S-D rats (Table 29).

Figure 9. Dose-dependent depletion of rabbit tissue GSH following cephaloridine administration. Rabbits were injected with a single dose of cephaloridine and killed one hour later. Each point and vertical bar represents mean ± SE of four or more animals. (*) Significantly different from the animals receiving no cephaloridine ($p<0.05$). Hepatic and renal cortical concentrations of GSH in the animals receiving no cephaloridine (controls) were 12.29±0.71 and 3.19±0.27 μmoles/g tissue, respectively.

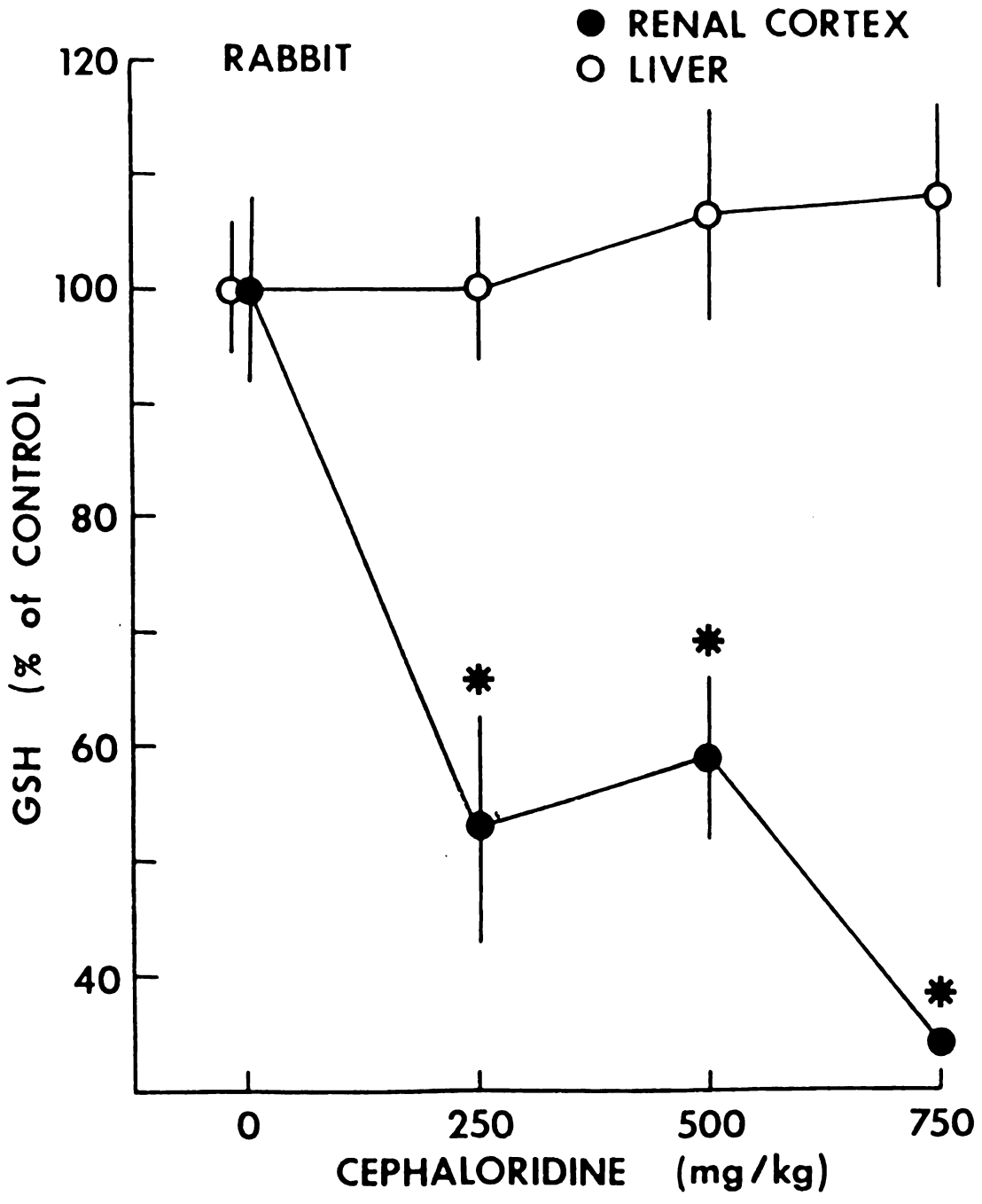

RENAL CORTEX
LIVER
RABBIT
120
100
80
60
40
GSH (% of CONTROL)
0
250
500
750
CEPHALORIDINE (mg/kg)

Figure 9

Figure 10. Dose-dependent depletion of rat tissue GSH following cephaloridine administration. Animals were injected with a single dose of cephaloridine and killed one hour later. Each point and vertical bar represents mean ± SE of four or more animals. (*) Significantly different from the animals receiving no cephaloridine ($p<0.05$). Renal cortical and medullary concentrations of GSH in the animals receiving no cephaloridine (controls) were 4.23±0.09 and 2.69±0.10 μmoles/g tissue, respectively.

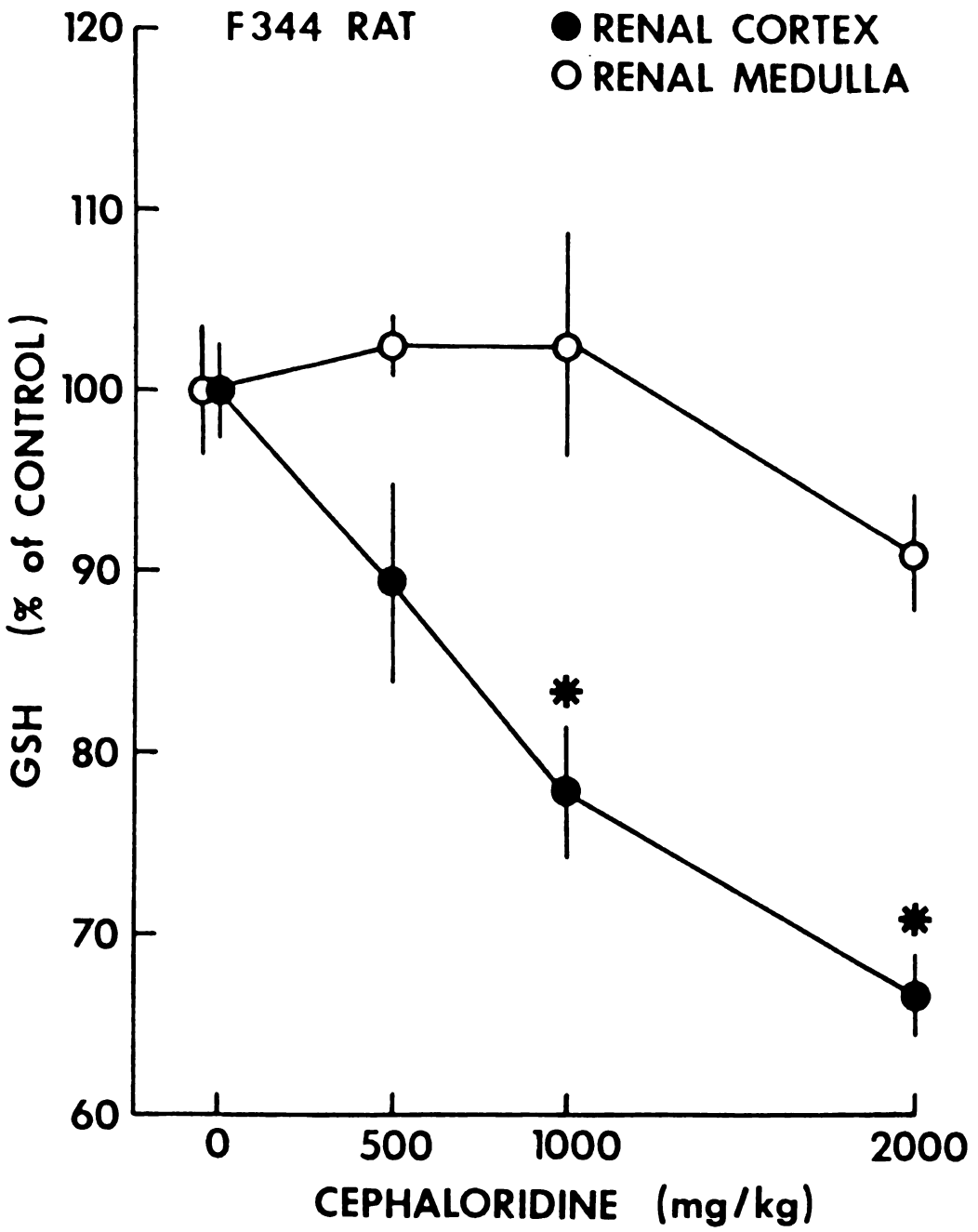

F344 RAT
RENAL CORTEX
RENAL MEDULLA
120
110
100
90
80
70
60
GSH (% of CONTROL)
0
500
1000
2000
CEPHALORIDINE (mg/kg)
*
*

Figure 10

TABLE 26

Effect of Diethyl Maleate Treatment on Rat Renal Cortical GSH Content[a]

Diethyl Maleate (ml/kg)	Renal Cortical GSH (μmoles/g)
0 (control)	3.20±0.09
0.1	2.51±0.13[b]
0.2	1.58±0.05[b]
0.4	1.22±0.06[b]

[a]Animals received a single ip injection of diethyl maleate or corn oil (control) and were killed one hour later. Values represent means ± SE of three animals.

[b]Significantly different from control ($p<0.05$).

TABLE 27

Effect of Diethyl Maleate Pretreatment on Cephaloridine Nephrotoxicity in Rabbits[a]

Pretreatment	Cephaloridine (mg/kg)	BUN (mg %)	PAH S/M	TEA S/M
Corn Oil	0	19.02± 1.08	14.55±1.16	16.19±1.03
Diethyl Maleate	0	22.58± 2.43	13.63±2.43	14.25±0.79
Corn Oil	100	24.18± 3.52	12.48±2.66	17.08±2.22
Diethyl Maleate	100	29.96± 3.83	11.91±2.92	13.43±1.50
Corn Oil	300	66.10±10.67	3.04±0.49	6.55±1.13
Diethyl Maleate	300	163.48±26.81[c]	1.88±0.32	3.79±0.82[c]

[a]Animals received a single injection of diethyl maleate (0.4 ml/kg) or corn oil 30 min prior to the administration of cephaloridine, and were killed 48 hours later. Values are means ± SE of four or more animals.

[b]50% of animals died between 24 and 48 hours after cephaloridine administration.

[c]Significantly different from the group receiving an equivalent dose of cephaloridine without diethyl maleate pretreatment ($p<0.05$).

TABLE 28

Effect of Diethyl Maleate Pretreatment[a] on Cephaloridine Nephrotoxicity in Rats

Pretreatment	Cephaloridine (mg/kg)	BUN (mg %)	PAH S/M	TEA S/M
Corn Oil	0	27.5±0.7	38.6±5.0	32.4±3.4
Diethyl Maleate	0	24.2±1.4	39.0±2.2	34.9±2.6
Corn Oil	750	35.2±1.7	11.5±0.6[b]	11.6±1.0[b]
Diethyl Maleate	750	173.4±43.7[b]	6.0±1.5[b]	7.3±1.8[b]

[a]Male Fischer 344 rats received a single ip injection of diethyl maleate (0.4 ml/kg) or corn oil 30 min prior to the administration of cephaloridine (750 mg/kg) or saline, and were killed 24 hours later. Values are means ± SE of four or more animals.

[b]Significantly different from the group receiving an equivalent dose of cephaloridine without diethyl maleate pretreatment ($p<0.05$).

TABLE 29

Effect of Diethyl Maleate Pretreatment on Cephaloridine Nephrotoxicity in Sprague-Dawley Rats[a]

Pretreatment	Cephaloridine (mg/kg)	BUN (mg %)	PAH S/M	TEA S/M
Corn Oil	0	24.23± 0.84	27.89±1.15	24.72±1.97
Diethyl Maleate	0	25.66± 2.35	25.03±1.71	21.60±1.06
Corn Oil	1000	45.92± 4.97	11.15±1.75	13.24±1.70
Diethyl Maleate	1000	106.48±19.19[c]	5.77±2.02[c]	8.36±2.38
Corn Oil	2000[b]	73.27±11.67	4.08±0.27	8.52±2.08
Diethyl Maleate	2000[b]	157.40± 7.10[c]	3.14±0.20	3.92±0.74[c]

[a]Animals received a single ip injection of cephaloridine 30 min after administration of diethyl maleate (0.4 ml/kg) or corn oil and were killed 48 hours later. Values represent means ± SE of four or more animals.

[b]57% of animals died within 48 hours after administration of cephaloridine. Values represent means ± SE of three surviving animals.

[c]Significantly different from the group receiving an equivalent dose of cephaloridine but without diethyl maleate pretreatment ($p<0.05$).

Effect of Cephaloridine, Cephalothin and Gentamicin on Tissue GSH in SD Rats

When cephaloridine, at doses of 1000 and 2000 mg/kg, were administered, renal medullary GSH was not affected, but renal cortical GSH concentration was significantly depleted (Figure 11). A single injection of a high dose of gentamicin (1000 mg/kg) caused death within 45 minutes, therefore all animals treated with gentamicin were killed 45 min after drug administration. In contrast to cephaloridine, administration of gentamicin did not deplete renal cortical GSH. Similarly, gentamicin had no effect on renal medullary GSH (Table 30). In addition, renal cortical and hepatic GSH were not depleted by cephalothin (Figure 12). Instead, higher doses of cephalothin increased renal cortical GSH.

Formation of Glutathione Conjugate(s) of Cephaloridine or Its Metabolite(s) In Vitro

No glutathione conjugate(s) was detected through either nonenzymatically or enzymatically catalyzed reactions in vitro.

Effect of Cephaloridine on Renal Cortical and Hepatic GSH and GSSG in Rabbits and Rats

In rats, cephaloridine decreased renal cortical GSH concentration by approximately 30% and increased GSSG concentration by 1300% one hour after drug administration (Table 31). In contrast, cephaloridine treatment had no significant effect on either GSH or GSSG concentration in rat liver. Similarly, cephaloridine (500 mg/kg) treatment depleted GSH (45%), and increased GSSG (2360%) in rabbit renal cortex, but had little effect on hepatic GSH or GSSG concentration (Table 32).

Figure 11. Dose-dependent depletion of Sprague-Dawley rat tissue GSH following cephaloridine administration. Animals were injected with a single dose of cephaloridine and killed one hour later. Each point and vertical bar represents mean ± SE of five animals. (*) Significantly different from the animals receiving no cephaloridine ($p<0.05$). Renal cortical and medullary concentrations of GSH in the animals receiving no cephaloridine (controls) were 3.92±0.19 and 2.34±0.22 μmoles/g tissue, respectively.

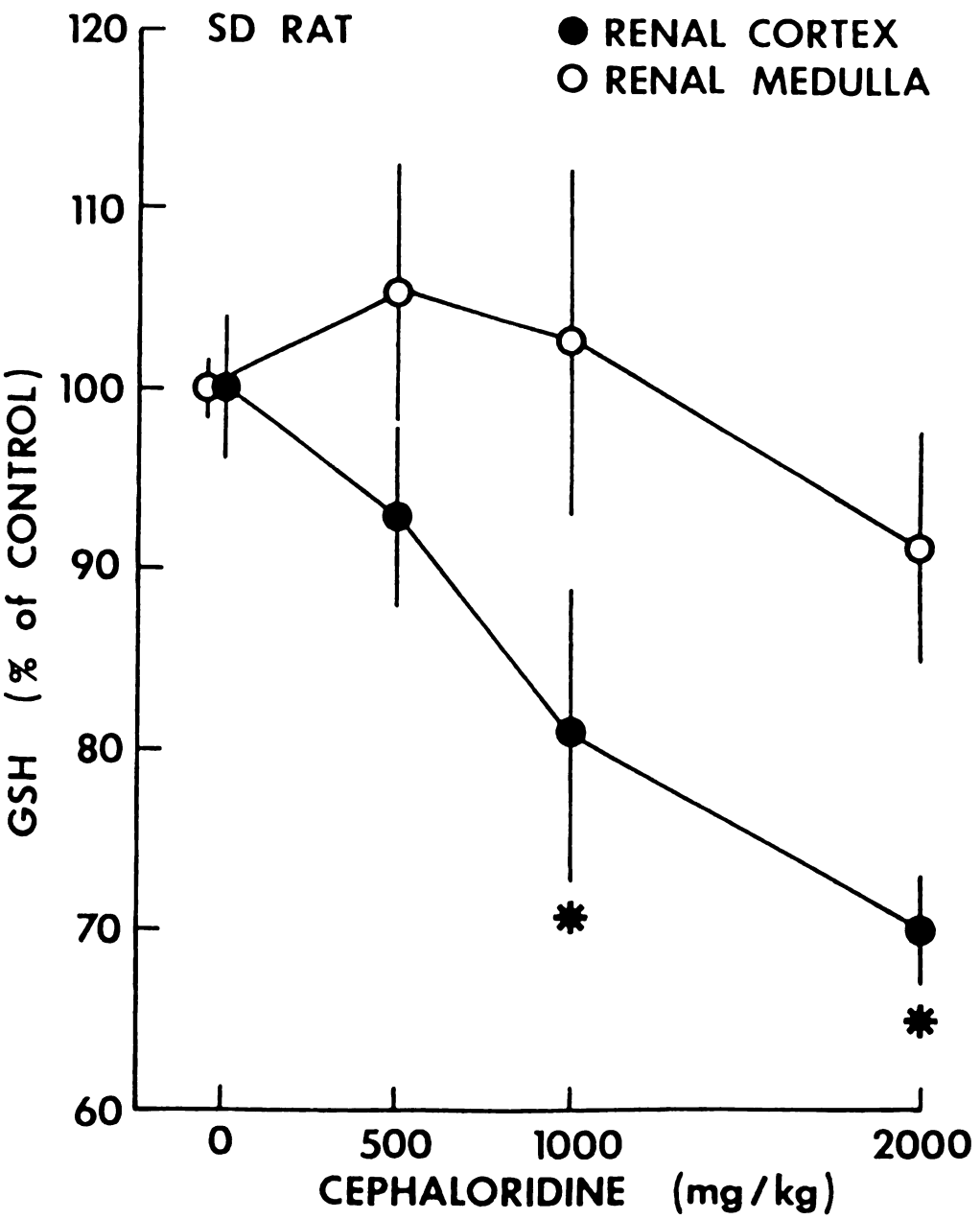

SD RAT
● RENAL CORTEX
○ RENAL MEDULLA
120
110
100
90
80
70
60
GSH (% of CONTROL)
0
500
1000
2000
CEPHALORIDINE (mg/kg)
*
*

Figure 11

TABLE 30

Effect of Gentamicin Treatment on Sprague-Dawley Rat Tissue GSH Concentrations[a]

Gentamicin (mg/kg)	GSH (μmoles/g)		
	Renal Cortex	Renal Medulla	Liver
0	3.82±0.14	2.09±0.11	7.44±0.21
250	3.66±0.04	2.14±0.18	7.45±0.27
500	3.50±0.17	2.27±0.07	7.90±0.19
1000[b]	4.06	2.10	7.44

[a]Animals received a single administration of gentamicin or saline and were killed 45 minutes later. Values are means ± SE of four or more animals.

[b]75% of animals died within 45 minutes.

Figure 12. Dose-dependent depletion of Sprague-Dawley rat tissue GSH following cephalothin administration. Animals received a single ip injection of cephalothin or saline and were killed one hour later. Renal cortical and hepatic GSH concentrations in control animals were 3.59±0.13 and 7.22±0.40 μmoles/g tissue, respectively. Values represent means ± SE of three or more animals. (*) Significantly different from control ($p<0.05$).

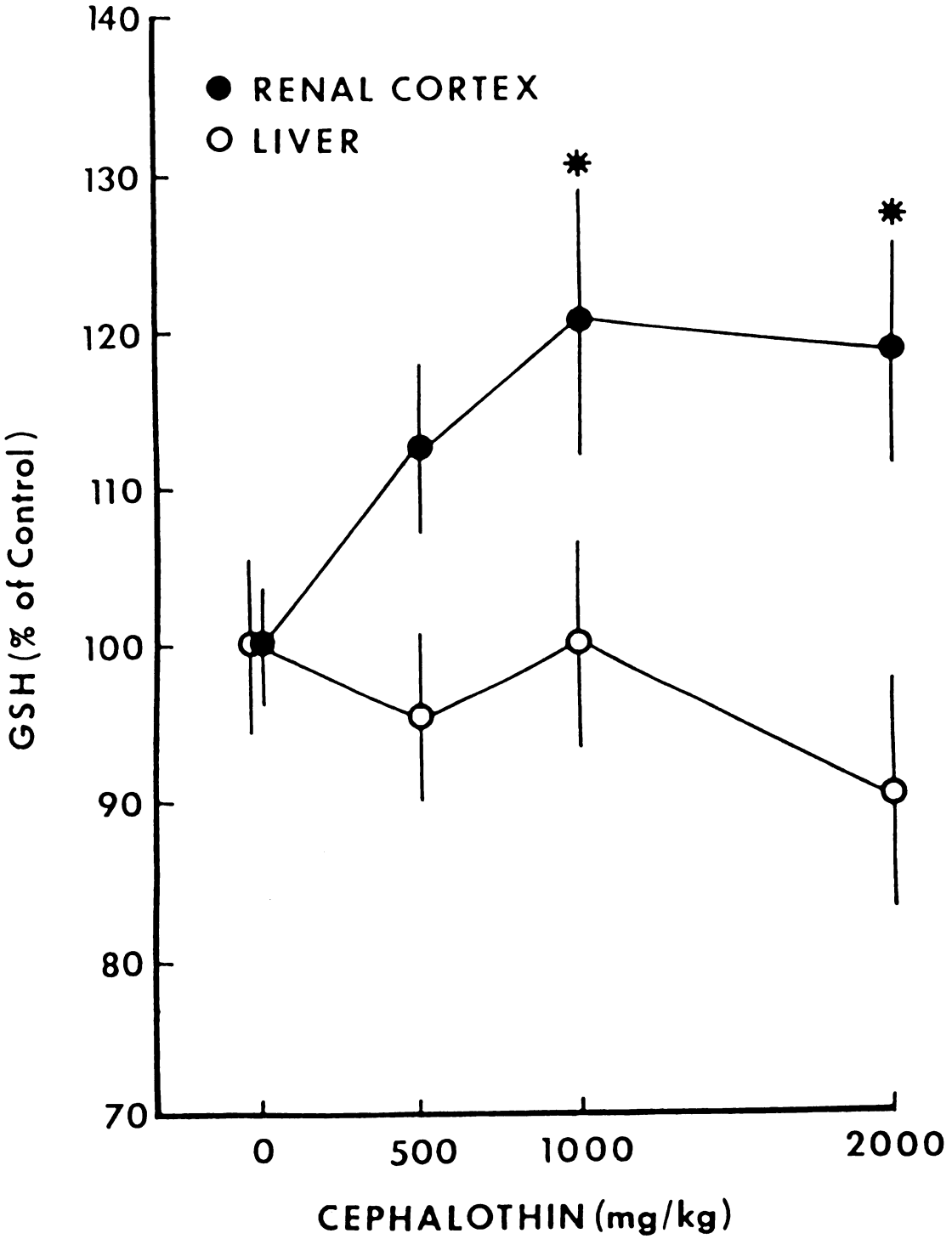

140
130
120
110
100
90
80
70
● RENAL CORTEX
○ LIVER
*
*
GSH (% of Control)
0
500
1000
2000
CEPHALOTHIN (mg/kg)

Figure 12

TABLE 31

Effect of Cephaloridine Treatment on Reduced and Oxidized Glutathione Concentrations in Rat Kidneys and Livers

Tissue	Glutathione (μmoles/g)	
	Control	Cephaloridine[a]
Renal Cortex		
GSH	4.71±0.13	3.40±0.06[c]
GSH + GSSG[b]	5.14±0.15	9.47±0.25[c]
Liver		
GSH	6.55±0.13	6.29±0.13
GSH + GSSG	7.65±0.21	8.01±0.13

[a]Rats received a single ip injection of cephaloridine (2000 mg/kg) or saline (control) and were killed one hour later. Values are means ± SE of four animals.

[b]Concentration of GSH and GSSG expressed as total GSH concentration.

[c]Significantly different from control ($p<0.05$).

TABLE 32

Effect of Cephaloridine Treatment on Reduced and Oxidized Glutathione Concentrations in Rabbit Kidneys and Livers

Tissue	Glutathione (μmoles/g)	
	Control	Cephaloridine[a]
Renal Cortex		
GSH	4.44±0.11	2.44±0.23[c]
GSH + GSSG[b]	4.82±0.09	11.41±0.51[c]
Liver		
GSH	12.62±0.81	12.64±0.44
GSH + GSSG	13.13±0.84	13.60±0.33

[a]Rabbits received a single sc injection of cephaloridine (500 mg/kg) or saline (control) and were killed one hour later. Values are means ± SE of four animals.

[b]Concentration of GSH and GSSG was expressed as total GSH concentration.

[c]Significantly different from control ($p<0.05$).

Effect of Cephaloridine on Renal Cortical and Hepatic Conjugated Dienes

Concentration of conjugated dienes in rat renal cortical microsomes increased shortly following cephaloridine administration and maintained at a level higher than control for at least 10 hours (Table 33). In contrast, the same treatment had no effect on concentration of conjugated diene in rat liver microsomes. In addition, cephaloridine increased conjugated diene concentration in rabbit renal cortical microsomes (Figure 13).

Effect of Vitamin E and/or Selenium Deficiency on Cephaloridine Toxicity in Rats

In the dietary study, diet alone had no significant effect on body, liver or kidney weight (Figure 14). Neither vitamin E nor selenium deficiency altered BUN or renal cortical gluconeogenesis (Figures 15 and 16). In addition, there was no significant histopathological change in the kidneys of the animals fed the deficient diets (Figures 17A-D).

Cephaloridine, at a low dose (500 mg/kg), had little effect on BUN in the control and deficient-diet groups (Figure 15). In contrast, the same dose of cephaloridine increased renal cortical gluconeogenesis in the control group; but it did not increase gluconeogenesis in the deficient-diet groups (Figure 16). Some histological differences after cephaloridine were observed among the control and deficient-diet groups (Figures 18A-D). No changes related to cephaloridine treatment were found in the kidneys of the control group (Figure 18A). However, cephaloridine (500 mg/kg) produced slight to moderate vacuolation of the proximal tubular cells in the vitamin E and selenium deficient animals

TABLE 33

Formation of Conjugated Dienes in Rat Renal Cortex and Liver Following Cephaloridine Administration[a]

Time (hr)	Conjugated Diene (OD_{240}/g microsome)	
	Renal Cortex	Liver
0 (control)	17.00±1.12	11.65±1.18
1	20.88±1.67	12.36±0.67
2	26.70±2.05[b]	---
3	25.38±2.86[b]	12.06±0.53
4	24.21±1.96[b]	---
6	23.64±2.03[b]	13.01±0.93
12	26.14±2.45[b]	13.06±0.51

[a]Rats received a single ip injection of cephaloridine (2000 mg/kg) and were killed at various times. Values are means ± SE of three or more animals.

[b]Significantly different from control ($p<0.05$).

Figure 13. Effect of cephaloridine on rabbit renal cortical conjugated dienes. Animals received a single sc injection of cephaloridine (500 mg/kg) and were killed two or four hours later. Values represent means ± SE of four animals. (*) Significantly different from control ($p<0.05$).

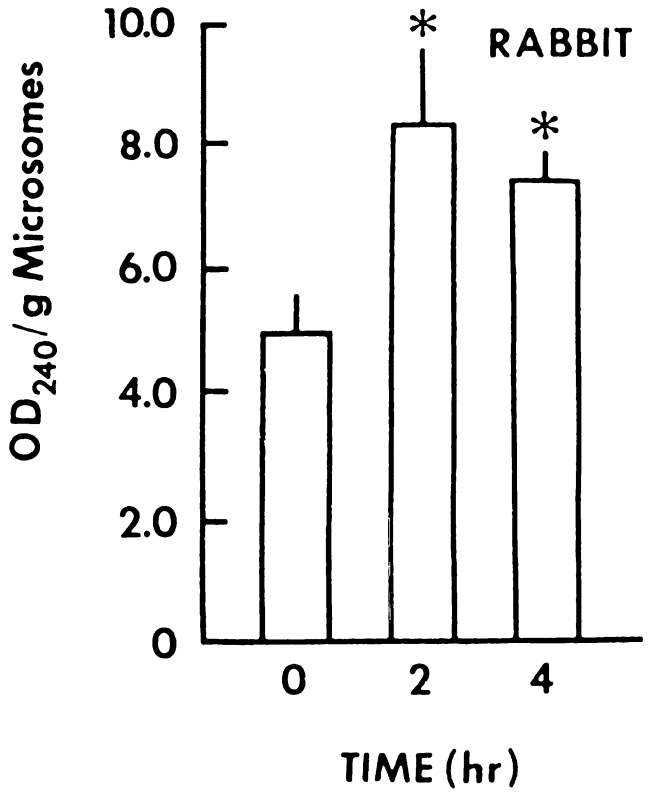


Figure 13

Figure 14. Effects of vitamine E and/or selenium deficiency on cephaloridine toxicity in rats. Animals were divided into four groups: Group 1 (+Se+E) were fed the basal diet with both Se (0.2 ppm) and vitamin E (121 U/kg) supplements, Group 2 (-E) received only vitamin E supplement, Group 3 (-Se) received only Se supplement and Group 4 (-Se-E) were fed the basal diet with no additional vitamin E or Se. Forty-two days later, animals received a single ip injection of cephaloridine (0, 500 or 1000 mg/kg) in saline and were killed 24 hr later. Values represent means ± SE of seven or more animals. (*) Significantly different from Group 1 receiving an equivalent dose of cephaloridine ($p<0.05$). (†) Significantly different from Group 1 receiving saline only ($p<0.05$).

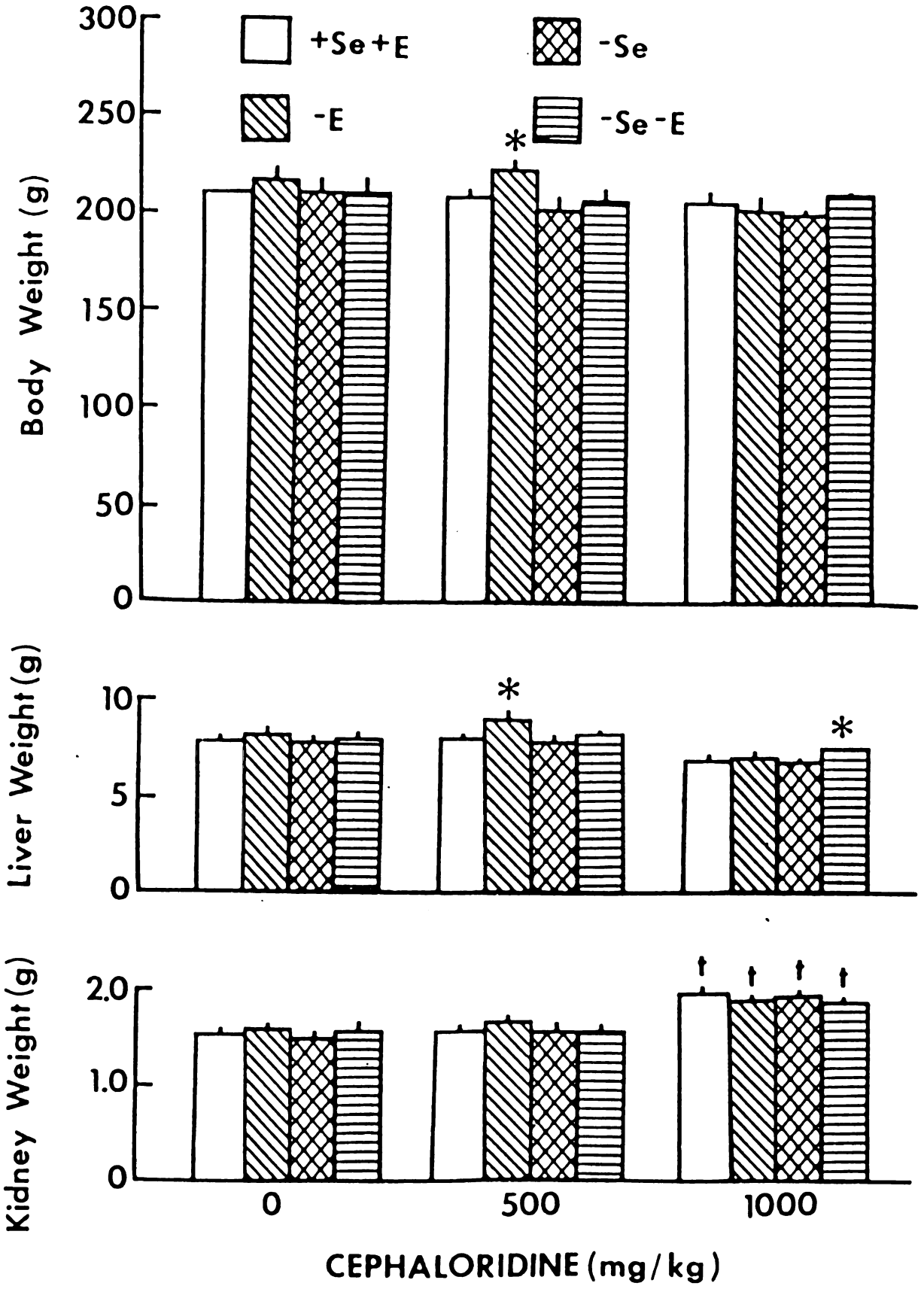

300
250
200
150
100
50
0
+Se+E
-Se
-E
-Se-E
*
Body Weight (g)
10
5
0
*
*
Liver Weight (g)
2.0
1.0
0
†
†
†
†
Kidney Weight (g)
0
500
1000
CEPHALORIDINE (mg/kg)

Figure 14

Figure 15. Effects of vitamin E and/or selenium deficiency on blood urea nitrogen in rats after cephaloridine. Animals were treated the same as described in Figure 14. Values are means ± SE of seven or more animals. (*) Significantly different from Group 1 receiving an equivalent dose of cephaloridine ($p<0.05$). (+) Significantly different from Group 1 receiving saline only ($p<0.05$).

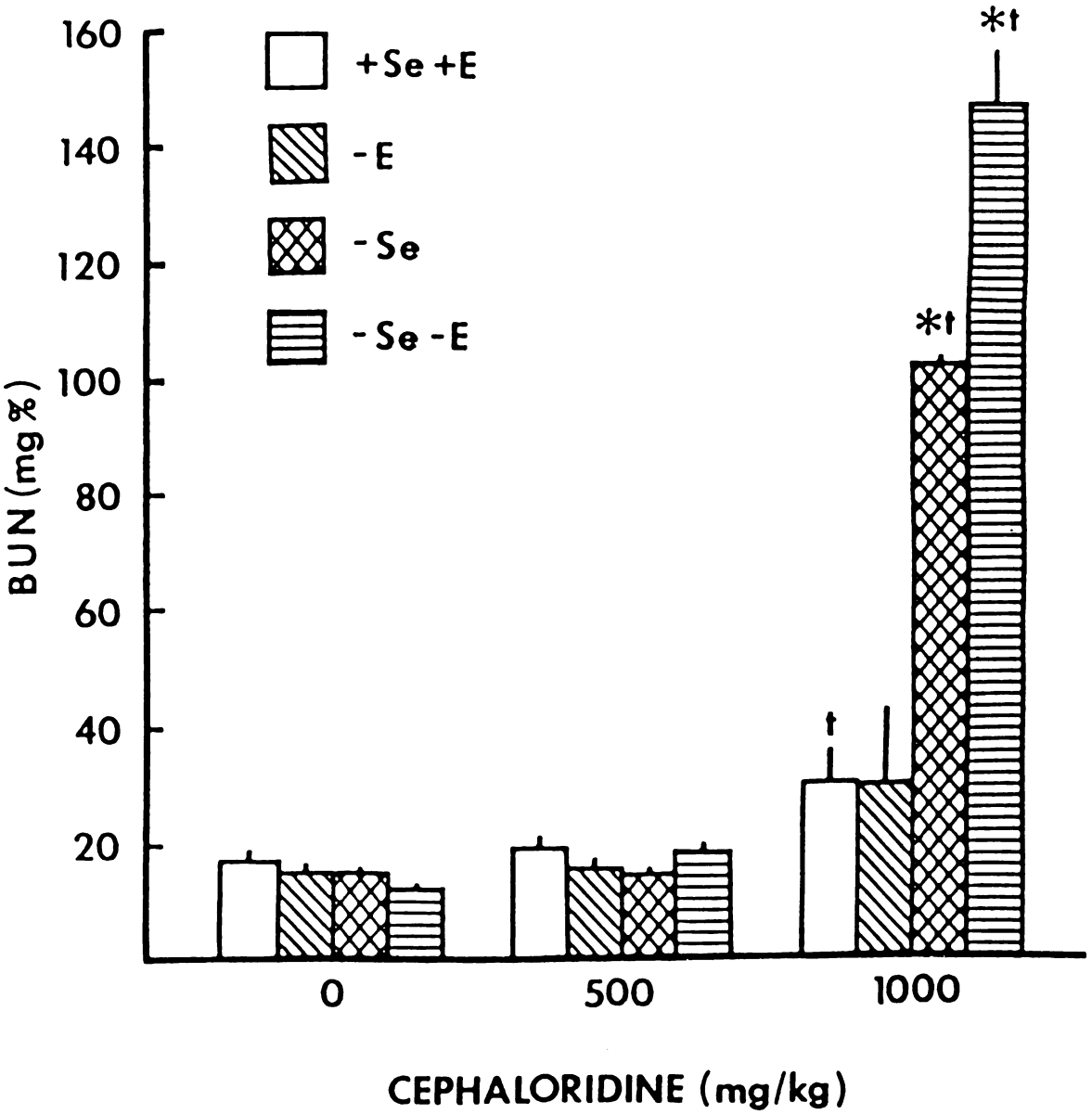

160
140
120
100
80
60
40
20
BUN (mg%)
+Se +E
-E
-Se
-Se -E
*t
*t
t
0
500
1000
CEPHALORIDINE (mg/kg)

Figure 15

Figure 16. Effects of vitamin E and/or selenium deficiency on gluconeogenesis by renal cortical slices in rats after cephaloridine. Animals were treated the same as described in Figure 14. Values are means ± SE of four or more animals. (*) Significantly different from Group 1 receiving an equivalent dose of cephaloridine ($p<0.05$). (†) Significantly different from Group 1 receiving saline only ($p<0.05$).

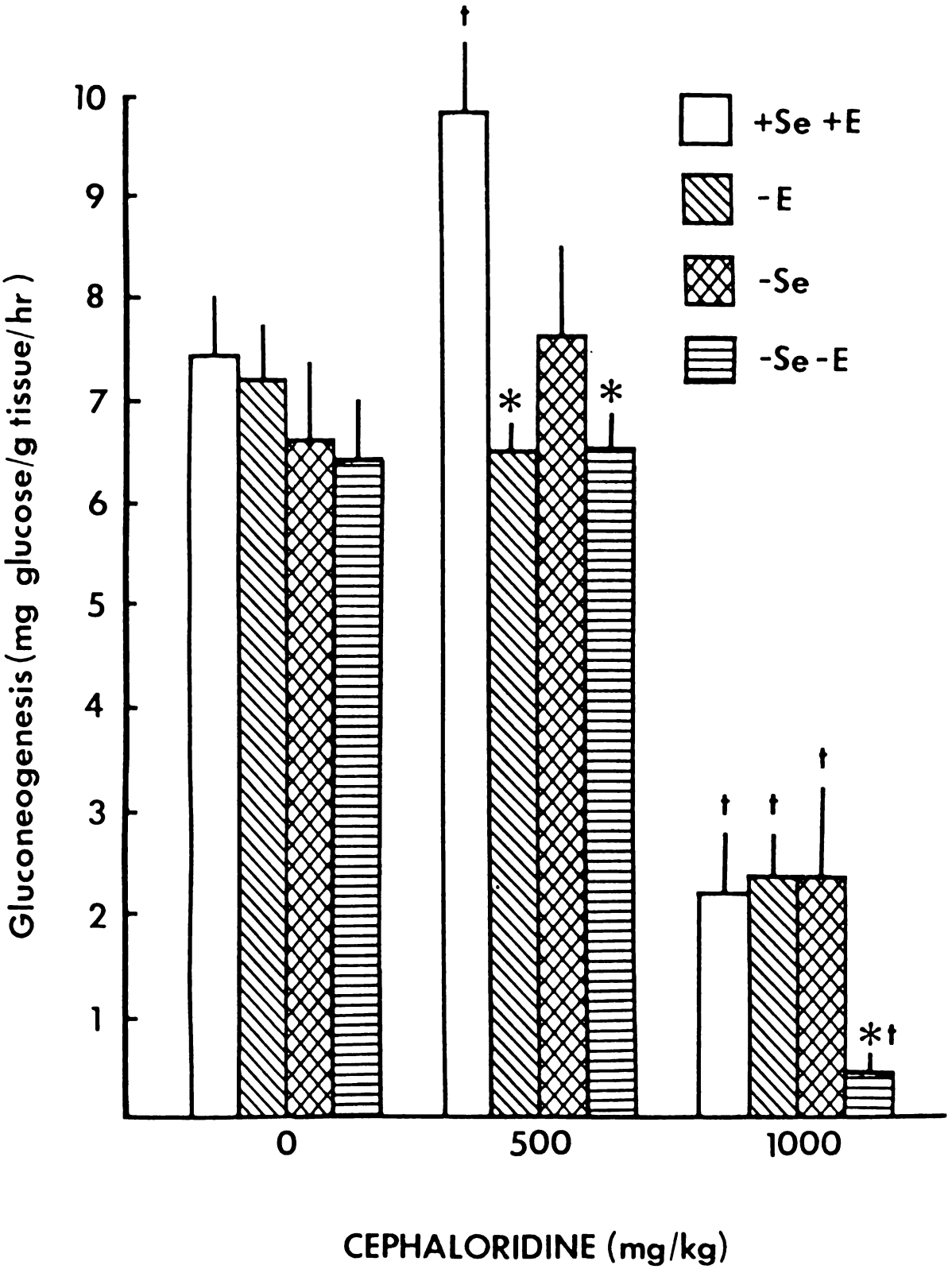

Gluconeogenesis (mg glucose/g tissue/hr)
10
9
8
7
6
5
4
3
2
1
+Se +E
-E
-Se
-Se-E
0
500
1000
CEPHALORIDINE (mg/kg)

Figure 16

Figure 17. Effects of vitamin E and/or selenium deficiency alone on rat renal histologic structure. Animals were fed the diets as described in Figure 14 for 42 days and then received a single injection of saline and were killed 24 hours later. No abnormality was observed between control (17A, +Se+E) and deficient groups (17B, -E; 17C, -Se; 17D, -Se-E). Sections were stained with H and E (x100).

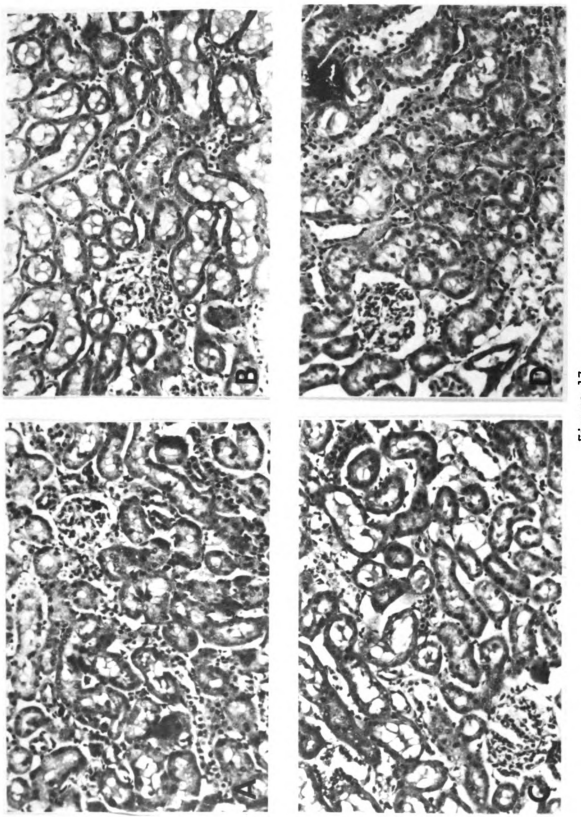


Figure 17

Figure 18. Effects of vitamin E and/or selenium deficiency on rat renal histologic structure after 500 mg/kg of cephaloridine. Animals were fed the diets as described in Figure 14 for 42 days and then received a single injection of cephaloridine (500 mg/kg) and were killed 24 hours later. No abnormality was observed in the control diet group (18A, +Se+E). In vitamin E deficient (18B) and selenium deficient (18C) groups, occasional vacuolation was seen in the proximal tubular cells. In both the vitamin E and selenium deficient group (18D), vacuolation and degeneration in the proximal tubular cells were prominent and occasional necrosis was observed. Sections were stained with H and E (x100).

Figure 18

(Figures 18B and C). The same dose of cephaloridine produced more severe vacuolation accompanied with occasional coagulative necrosis in the group fed the diet deficient in both vitamin E and selenium (Figure 18D).

A higher dose of cephaloridine (1000 mg/kg) significantly increased kidney weight in both control and deficient groups (Figure 14). It also elevated BUN in the control group; however, the same dose of cephaloridine markedly increased BUN in the selenium-deficient and both deficient groups (Figure 15). Similarly, 1000 mg/kg of cephaloridine significantly decreased renal cortical gluconeongenesis; the greatest effect was found in the group deficient in both vitamin E and selenium (Figure 16). In the histopathological evaluation, coagulative necrosis of the proximal tubular cells was a common sign in all four groups; but there were some differences in the distribution and severity of the lesions among the groups. In the control diet group, the high dose of cephaloridine produced necrotic lesions in the outer cortex; proximal tubular cell vacuolation was frequent but the necrotic process was not prominent (Figure 19A). However, the same dose of cephaloridine also caused some necrosis in the inner cortex along with swelling and hydropic degenerated tubular cells in both vitamin E and selenium deficient groups; necrotic areas of the proximal tubules was dispersed in the cortex (Figures 19B and C). Eosinophilic cylinders were occasionally observed in the pars recta of the proximal tubules of the outer medulla in the vitamin E deficient group. The most destructive change was found in the group deficient in both vitamin E and selenium after the high dose of

Figure 19. Effects of vitamin E and/or selenium deficiency on rat renal histologic structure after 1000 mg/kg of cephaloridine. Animals were fed the diets as described in Figure 14 for 42 days and received a single injection of cephaloridine (1000 mg/kg) and were killed 24 hours later. In the control group (19A, +Se+E), vacuolation of proximal tubular cells was profound in the outer cortex but necrotic process was slight. In vitamin E deficient (19B) and selenium deficient (19C) groups, moderate necrotic areas of the proximal tubule cells were dispersed in the cortex. In both the vitamin E and selenium deficient group (19D), large portion of cortex was changed to necrotic tissue along with frequent occlusion of tubular lumens with proteinous cylinders. Sections were stained with H and E (x100).

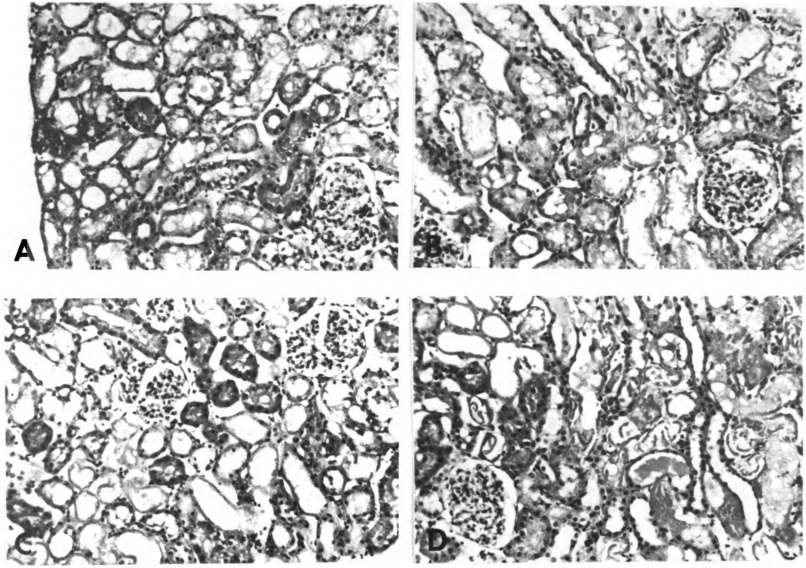
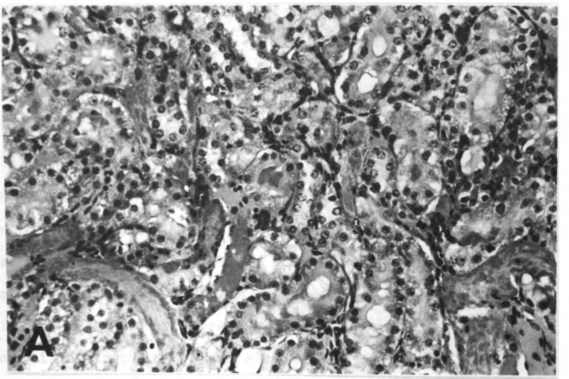


Figure 19

cephaloridine. A large portion of cortex was replaced by necrotic tissue and tubular lumens were filled with amorphous cellular debris or serous fluid with occasional deposits of mineral substance (Figure 19D). Eosinophilic cylinders were found in Henle's loop, distal tubules and pars recta of the proximal tubules in the outer medulla (Figures 20A and B).

Figure 20. Effects of deficiency in both vitamin E and selenium on rat renal histological structure after 1000 mg/kg of cephaloridine. Animals were treated the same as described in Figure 18. The tubular cells in the outer strip of outer medulla showed moderate to severe swelling with variable degree of vacuolation. In addition, necrosis was observed (20A). In the inner strip of outer medulla, proteinous cylinders occluded the lumens of the distal tubules (20B). Sections were stained with H and E (x100).



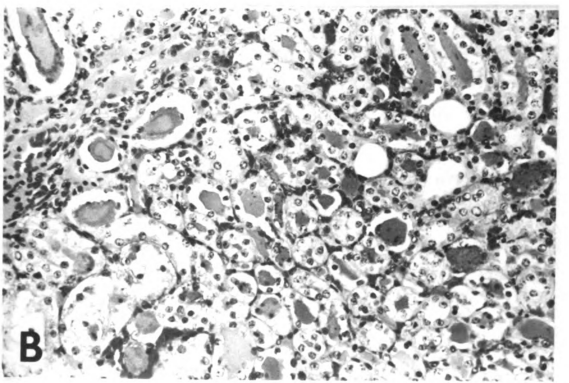


Figure 20

DISCUSSION

Drug-metabolizing enzyme inhibitors such as piperonyl butoxide, SKF-525A and cobaltous chloride, as well as inducers such as phenobarbital, 3-methylcholanthrene and β-naphthoflavone have been widely used to study xenobiotic metabolism and toxicity (Jollow et al., 1977; Gram, 1980). Using these inducers and inhibitors, investigators obtained valuable information regarding whether metabolic activation of xenobiotics is necessary to produce tissue injury. In the present study, pretreatment of rats with piperonyl butoxide signicantly decreased animal susceptibility to cephaloridine nephrotoxicity (Table 7). A similar result was also observed in the rabbit on the basis of histopathological findings (Table 8). The protective effect of piperonyl butoxide on cephaloridine nephrotoxicity has been demonstrated previously in the mouse and rat (McMurtry and Mitchell, 1977). Pretreatment of rats and mice with piperonyl butoxide decreased the incidence and severity of the renal proximal tubular necrosis caused by cephaloridine. Furthermore, McMurtry and Mitchell (1977) reported a protective effect by cobaltous chloride. The results from these inhibitor studies suggested that cephaloridine might have to be metabolically activated in order to cause renal damage. However, this type of result

can not adequately predict the site where biotransformation takes place. In general, the liver will be the first choice because of its great metabolic capacity. It has been difficult to identify extrahepatic sites of biotransformation *in vivo*. In order to overcome this problem, phenobarbital has been used as a tool, since this inducer has different effects on hepatic and extrahepatic drug-metabolizing enzyme systems in some species (Uehleke and Greim, 1968; Feuer *et al.*, 1971).

In the present study, pretreatment of rabbits with phenobarbital (60 mg/kg daily for four days) significantly increased susceptibility to cephaloridine nephrotoxicity (Table 6). In contrast, a similar treatment (80 mg/kg daily for four days) had very little effect on rats (Figure 5). These species-specific effects are consistent with the inducibility of renal mixed-function oxidases by phenobarbital. Evidence obtained from the present study (Table 3) and others (Uehleke and Greim, 1968; Feuer *et al.*, 1971) have shown that treatment with phenobarbital increases rabbit renal microsomal cytochrome P450 and cytochrome b5 concentrations and mixed-function oxidase (e.g., ethoxycoumarin-O-deethylase, benzphetamine-N-demethylase, arylhydrocarbon hydroxylase, etc.) activites, but does not alter P450 or b5 concentrations or enzyme activities in the rat kidney. The lack of effect of phenobarbital on renal toxicity of cephaloridine was also demonstrated in mice (McMurtry and Mitchell, 1977). Interestingly, phenobarbital also showed very little effect on mouse renal mixed-function oxidase activity (Kluwe *et al.*, 1978). Thus, potentiation of cephaloridine-induced nephrotoxicity seems closely associated with the inducibility of renal mixed-function oxidases by phenobarbital, but not with the inducibility

of hepatic mixed-function oxidases since phenobarbital induces hepatic mixed-function oxidases in all three species (Uehleke and Greim, 1968; Feuer et al., 1971; Kluwe et al., 1978). This correlation indicates that the bioactivation of cephaloridine may take place in situ in the kidney.

However, phenobarbital treatment has been shown to increase renal blood flow (Ohnhaus and Siegel, 1974) and urinary excretion of xenobiotics (Ohnhaus, 1972). Treatment with phenobarbital may also alter the pharmacokinetics of cephaloridine and thus vary renal cortical accumulation of the antibiotic, which could lead to a change in the drug toxicity. In the present study, phenobarbital treatment altered pharmacokinetics of cephaloridine in rabbit kidneys, resulting in significant retention of cephaloridine in rabbit renal cortex (Table 10). In contrast, phenobarbital treatment did not significantly alter pharmacokinetics of cephaloridine in rat kidneys (Table 11). Higher renal cortical concentration of cephaloridine in phenobarbital-treated rabbits was not the consequence of a higher serum concentration of cephaloridine (Table 10), but rather was an intrarenal event. It could have been due to a decreased efflux or an increased uptake of cephaloridine or a combination of the two processes. Cephaloridine is known to be actively transported into the proximal tubular cells via an organic anion transport system (Tune and Fernholt, 1973). Concomitant administration of an organic anion such as PAH or probenecid with cephaloridine inhibits renal cortical accumulation of cephaloridine and the drug toxicity (Tune et al., 1977a,b). In addition, the organic anion transport system is incompletely developed in newborn rabbits (New et al., 1959) and because

of this delayed development, the newborn kidneys accumulate less cephaloridine and the nephrotoxicity of cephaloridine is minor in newborn (Wold et al., 1977a; Wold and Turnipseed, 1978). Furthermore, multiple administration of penicillin (a substrate for the renal organic anion transport) stimulates the development of organic anion transport (Hirsch and Hook, 1969) which increases the ability of the kidney cells to accumulate cephaloridine and increases nephrotoxicity (Wold and Turnipseed, 1978). These studies strongly suggest that cephaloridine and PAH are handled by the kidney in a similar manner. However, in the present study, treatment of rabbits with phenobarbital did not increase uptake of PAH in renal cortex either in vivo or in vitro (Tables 15 and 16) even though the same treatment increased renal cortical uptake of cephaloridine (Table 12). Thus, phenobarbital treatment appeared to have different effects on PAH and cephaloridine transport.

Unlike PAH, cephaloridine is a zwitterion, having a positive charge on the quarternary nitrogen of the pyridinium substitute on the beta-lactam ring in addition to possessing an anionic carboxyl group. Thus, the renal cation transport system may also influence the movement of cephaloridine into or out of the proximal tubular cells. Wold and Turnipseed (1980) found that pretreatment of rabbits with cyanine (an organic cation inhibitor) delayed the disappearance of cephaloridine from renal cortex in vivo and this inhibitor also decreased the efflux of cephaloridine from preloaded renal cortical slices in vitro. These results suggest that the exit of cephaloridine from cortical cells is dependent on a cation transport system. Since phenobarbital treatment induces mixed-function oxidase activities in rabbit kidneys, like

tetrachlorodibenzo-p-dioxin (Fowler et al., 1977), the induction may be associated with membrane alteration. This could modify the organic cation transport system and therefore alter the exit of cephaloridine from renal cortical cells. However, phenobarbital treatment had very little effect on renal cortical accumulation of organic cations (e.g., TEA, Table 16). Furthermore, treatment with phenobarbital did not appear to decrease efflux of cephaloridine from renal cortical slices (Figure 6).

Piperonyl butoxide is known to inhibit microsomal mixed-function oxidase systems (Anders, 1968; Jaffe et al., 1968; Casida, 1970), but little information is available regarding its effect on renal transport. The data from the present study demonstrated that systemic administration of piperonyl butoxide did not affect renal cortical concentration of PAH (Tables 17 and 18); however, the same treatment markedly reduced renal cortical accumulation of cephaloridine (Table 13). Furthermore, renal cortical slices from piperonyl butoxide treated animals accumulated much less cephaloridine compared to those from control animals (Table 14). The cortical slices from the same piperonyl butoxide treatment accumulated almost equal or slightly less PAH than those from the controls (Tables 19 and 20). These in vivo and in vitro results indicated that the effects of piperonyl butoxide on cephaloridine and PAH transport are not the same. In addition, the in vitro data (Tables 14, 19 and 20) suggested significant differences between cephaloridine and TEA transport.

Organic anion (PAH) and cation (TEA) transport are two independent systems existing in the renal proximal tubular cells (Rennick, 1972). These transport processes can be divided into three steps. The first step is transport from the blood into the proximal tubular cells across the peritubular membrane. The second step is intracellular retention and the third step is transport out of the cells across the luminal membrane. The difference between renal cortical accumulation of PAH and cephaloridine has been reported to be in the third step. Tune and his associates (1974; 1975) suggested that cephaloridine, unlike PAH, was not readily transported across the luminal cell membrane and was therefore retained in renal tubular cells. This concept was further studied and extended by Wold et al. (1979; 1980), who reported that efflux of cephaloridine from cortical tubular cells was markedly inhibited by organic cation inhibitors (e.g., cyanine and mepiperphenidol). On the basis of these results and studies from other investigators, they proposed that cephaloridine transport depended on organic anion transport for entry into the cortical tubular cells from blood, but, because of the cationic nature of the quaternary nitrogen of the pyridinium side chain, required a cation transport step for exit from cortical tubular cells. They further suggested that organic cation transport appeared to be only moderately efficient for the exit of cephaloridine, which therefore allowed for accumulation and maintenance of high concentrations of cephaloridine in the renal cortex.

Although a high concentration of cephaloridine can result from inefficient cation transport, alternatively it may be due to

intracellular binding. Binding of organic anions to intracellular organelles or cytoplasmic proteins has often been considered as an integral component of the organic anion transport system. Arias and his colleagues (Kirsch et al., 1975; Arias et al., 1976) suggested that the cytoplasmic protein, ligandin (GSH S-transferase B), served as a transport carrier of organic anions (e.g., PAH and penicillin) in the kidney. Several lines of evidence supported this hypothesis. The GSH S-transferase fraction isolated from homogenates of renal cortex bound various organic anions including PAH and penicillin (Kirsch et al., 1975). Probenecid administration inhibited the binding of penicillin to renal ligandin following in vivo injection (Kirsch et al., 1975). Pretreatment of animals with enzyme inducers (e.g., TCDD) increased the concentration of renal GSH S-transferases as well as urinary excretion, plasma disappearance and renal binding of organic anions (Kirsch et al., 1975; Arias et al., 1976). However, some observations by Pegg and Hook (1977) did not support this hypothesis. Renal GSH S-transferase activities did not parallel the development of PAH transport in neonates. The apparent lack of correlation between PAH transport capacity and GSH S-transferase activity was also observed in several instances including inducer treatment, chronic ammonium chloride acidosis, unilateral nephrectomy, etc. Nevertheless, intracellular binding may be an important compound of net transport of some organic anions.

As described previously, the results from the present investigation demonstrated that phenobarbital had different effects on PAH and cephaloridine transport. Recently, cephalothin (another cephalosporin

antibiotic) was reported to bind to GSH S-transferase (ligandin) and to inhibit transferase activities (Ketley et al., 1975). Like cephalothin, cephaloridine also inhibited GSH S-transferase activity (Table 5) and this antibiotic may bind to transferases and therefore move slowly out of the kidney cells. Two lines of evidence from the present study support this suggestion. Treatment with phenobarbital increased rabbit but not rat renal GSH S-transferase activity (Table 4). Kirsch et al. (1975) also reported that pretreatment of rats with phenobarbital did not increase renal ligandin concentration but doubled hepatic ligandin concentration. This increase in rabbit renal transferase may be responsible for the higher concentration of cephaloridine accumulated in phenobarbital-treated rabbit kidney. Furthermore, in the untreated animals, rabbit renal cortical GSH S-transferase activity was much higher than rat renal enzyme activity (Table 4). These differences are consistent with the ability of rabbit and rat kidney cells to accumulate cephaloridine. Rabbit renal cortex has been shown to accumulate more cephaloridine than rat renal cortex (Tune, 1975). Thus, these results suggest that GSH S-transferases (ligandins) may be major binding sites for cephaloridine in renal cortical cells.

Since both phenobarbital and piperonyl butoxide are modulators of cytochrome P-450 systems and also produce significant alterations in renal cortical accumulation of cephaloridine, it is possible that cytochrome P-450 molecules are the intracellular binding sites of cephaloridine. Several lines of evidence support this hypothesis. Pretreatment of rabbits with phenobarbital increased kidney cytochrome P-450

concentration (Uehleke and Greim, 1968; Table 3) and also enhanced renal cortical accumulation of cephaloridine (Tables 10 and 12). In contrast, phenobarbital pretreatment had no effect on rat kidney cytochrome P-450 concentration (Uehleke and Greim, 1968; Feuer et al., 1971) or renal cortical accumulation of cephaloridine (Table 11). In rat kidney, there is no sex-related differences in cytochrome P-450 concentrations (Litterst et al., 1977) and similarly, no sex-related difference in cephaloridine nephrotoxicity was observed (Table 9). Piperonyl butoxide has been shown to bind to cytochrome P-450 (Matthews et al., 1970; Philpot and Hodgson, 1971; Philpot and Hodgson, 1972) and this interaction may prevent cephaloridine from binding to cytochrome P-450. In the present studies, piperonyl butoxide pretreatment decreased cephaloridine accumulation in the renal cortex (Tables 13 and 14).

The hypothesis proposed by Wold et al. (1980) to explain a high concentration of cephaloridine in renal cortex (described previously) cannot rationalize the effect of phenobarbital and piperonyl butoxide on cephaloridine accumulation, since both phenobarbital and piperonyl butoxide had little effect on PAH or TEA transport. Based on the present results and the information from others, a schematic expression of renal transport of cephaloridine may be suggested (Figure 21). Cephaloridine molecules appear to be actively transported into the proximal tubular cells from the blood via an organic anion transport system which can be influenced by probenecid, penicillin, etc. After cephaloridine molecules enter the cells the antibiotic may share some binding sites with organic acids. In addition, cephaloridine may bind

Figure 21. Schematic diagram of the proposed transport of cephaloridine in renal proximal tubular cells. ⊕ Pretreatment with penicillin increases uptake of cephaloridine from the blood into the tubular cells. Phenobarbital pretreatment increases concentrations of cytochrome P450 and/or GSH transferases, which may bind more cephaloridine. ⊖:Piperonyl butoxide decreases the binding of cephaloridine to cytochrome P450 and/or GSH transferases. Cyanine decreases efflux of cephaloridine from the tubular cells to the lumen. Probenecid decreases uptake of cephaloridine from the blood into the tubular cells.

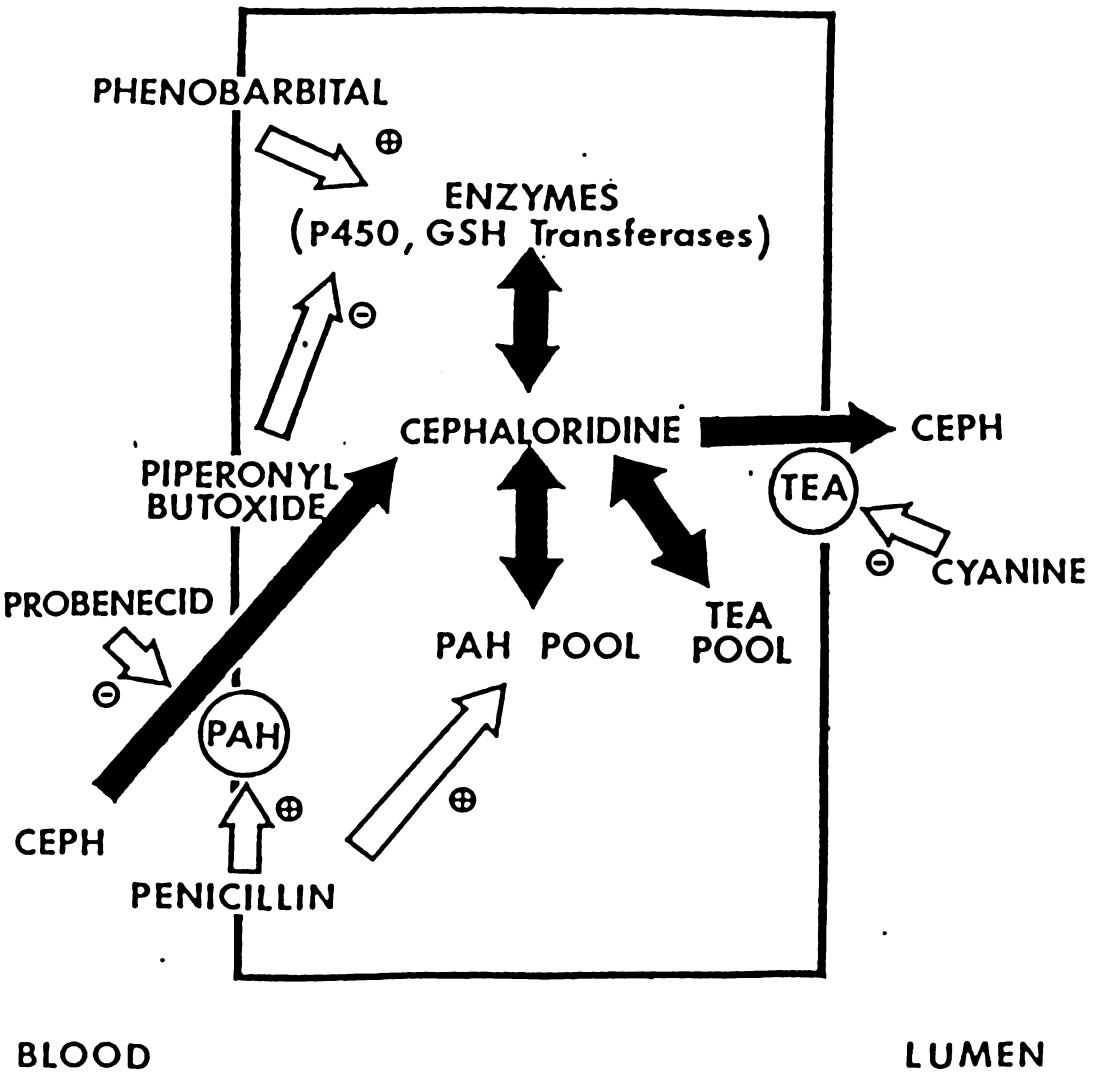

PHENOBARBITAL
ENZYMES
(P450, GSH Transferases)
CEPHALORIDINE
CEPH
PIPERONYL
BUTOXIDE
TEA
CYANINE
PROBENECID
PAH POOL
TEA
POOL
PAH
CEPH
PENICILLIN
BLOOD
LUMEN

Figure 21

to other proteins such as cytochrome P-450 and GSH S-transferase which are probably incapable of reacting with other organic anions. Because of the zwitterion nature, cephaloridine may also react with cation binding proteins (Rennick, 1981). All these possible binding reactions may retain cephaloridine in the cortical tubular cells.

Even though the effects of phenobarbital and piperonyl butoxide on cephaloridine-induced nephrotoxicity may, as described previously, result from alterations in renal cortical accumulation of cephaloridine, these effects still can be, at least in part, due to changes in the metabolic activation of cephaloridine. If this process, indeed, occurs in the kidney, the activated metabolites (presumably epoxides) of cephaloridine are very likely to react with intracellular GSH. GSH is present in all types of living cells and is able to conjugate a variety of chemically active compounds including reactive intermediates formed during the metabolism of certain xenobiotics (Mitchell et al., 1976; Boyland and Chasseaud, 1969). These glutathione conjugates usually are nontoxic and readily excreted. When glutathione content is sufficiently depleted and glutathione conjugation becomes less efficient, some reactive metabolites will be able to react with vital cellular macromolecules and cause cell injury and death. Many toxic chemicals such as chloroform (Docks and Krishna, 1976), adriamycin (Olson et al., 1980), acetaminophen (McMurtry et al., 1978; Hassing et al., 1979), bromobenzene (Jollow et al., 1974) and vinyl chloride (Hefner et al., 1975) have been reported to produce tissue damage associated with depletion of GSH. When intracellular GSH is available, most reactive intermediates

are conjugated with GSH. Although reactive intermediates may be detoxified by other pathways (Bucker et al., 1979), glutathione conjugation appears to be the most important one. Tissue damage (e.g., necrosis) and covalent binding of cellular macromolecules occur only when GSH is depleted. Because of this endogenous protective process, a dose threshold exists for many chemical (e.g., acetaminophen and bromobenzene) induced tissue injury (Mitchell et al., 1973; Jollow et al., 1973, 1974). Interestingly, a dose threshold for cephaloridine nephrotoxicity also was observed (Tune et al., 1977a; Wold, 1981). In further support of this view, Mitchell et al. (1977) reported that pretreatment of animals with cysteine decreased cephaloridine renal injury in rats and mice. Since cysteine is a precursor of glutathione, administration of cysteine will increase the availability of intracellular GSH (Leaf and Neuberger, 1947; Boyland and Chasseaud, 1967; Meister and Tate, 1976; Reed and Orrenius, 1977). Cysteine protection also has been demonstrated in many circumstances of tissue injury produced by potential toxic chemicals such as acetaminophen and bromobenzene (Mitchell et al., 1973; Potter et al., 1974; Jollow et al., 1974) and these protective effects have been shown to be associated with increased glutathione conjugation.

Two possible pathways for GSH conjugation of cephaloridine may exist (Figure 22). First, as suggested by Mitchell et al. (1977), cephaloridine may be activated to an epoxide intermediate and this intermediate can then react with GSH. In addition, the positive charge at the quaternary nitrogen of the pyridinium ring can shift to the para

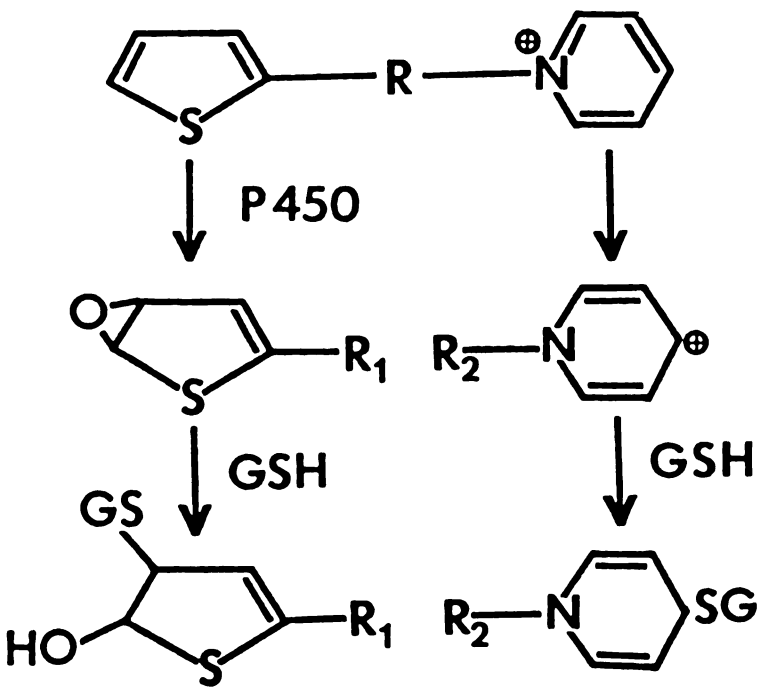


Figure 22. Possible pathways for the formation of glutathione conjugate(s) of cephaloridine or its metabolite(s).

position of carbon atom through π-bond resonance and this new positively charged center, with less steric hindrance, may react with GSH (Figure 22). Both potential GSH conjugates could be less toxic and rapidly excreted. A positive correlation has been shown between GSH depletion and tissue injury produced by many chemicals (Jollow et al., 1974; McMurtry et al., 1978). A greater degree of glutathione depletion is associated with a greater magnitude of tissue injury. If, indeed, glutathione conjugation is an important protective pathway against cephaloridine nephrotoxicity, this type of correlation should be observed. In the present investigation, the results from 48-hr toxicity studies demonstrated that cephaloridine is more nephrotoxic to rabbits than to rats, and is least toxic to mice (Tables 21-23). These species-different sensitivities to cephaloridine toxicity are consistent with the previous histopathological findings (Atkinson et al., 1966; Welles et al., 1966). Taking advantage of these species-related sensitivities, we have investigated changes of tissue GSH in these three species following cephaloridine administration (Figures 7-10, Table 24). Cephaloridine produced a dose-related depletion of GSH in rabbit and rat (Figures 9 and 10) but not mouse renal cortical GSH (Table 24). An apparent reduction in tissue GSH content can be due to a change of tissue water content (e.g., edema). However, the data from the present study (Table 25) indicated small changes (less than 5% of controls) in water content which could not be responsible for the depletion of tissue GSH. This depletion was greatest in rabbits, intermediate in rats, and least in mice, a pattern which is consistent with the species

susceptibility to cephaloridine nephrotoxicity. In addition, cephaloridine had very little effect on GSH concentration of liver (Figure 9) or renal medulla (Figure 10). Previous reports have demonstrated cephaloridine caused renal cortical injury, especially proximal tubular necrosis, but had little effect on the medulla (Silverblatt et al., 1970; Atkinson et al., 1966) and the liver (Barza, 1978). All these findings suggested that glutathione depletion in renal cortex is closely associated with cephaloridine nephrotoxicity. This suggestion was further supported by diethyl maleate studies (Tables 27-29). Diethyl maleate is known to react with GSH and will deplete hepatic GSH shortly following administration (Boyland and Chasseaud, 1970). This compound has been used to potentiate xenobiotic toxicity by depleting tissue GSH (Jollow et al., 1974; Mitchell et al., 1973; Harris and Anders, 1980), if tissue toxicity produced by xenobiotics can be prevented by GSH conjugation. In the present study, treatment with diethyl maleate also markedly depleted renal cortical GSH (Table 26) and pretreatment of rabbits and rats with diethyl maleate significantly enhanced cephaloridine nephrotoxicity. The present results and the cysteine studies by Mitchell et al. (1977) strongly suggests a protective role of glutathione against cephaloridine nephrotoxicity. In order to determine further whether an epoxide of thiophene is involved with cephaloridine toxicity, we have used another cephalosporin, cephalothin, which also possesses a thiophene ring but has no pyridinium ring. In contrast to cephaloridine, renal cortical GSH was not reduced following cephalothin administration (Figure 12). Furthermore, using [^{35}S]labelled glutathione, in the presence of cephaloridine and required enzymes, we could

not detect any glutathione conjugate of cephaloridine. One possibility, although unlikely, is that the analytic methods used still are not sensitive enough to detect the potential metabolites. However, most likely, no glutathione conjugate of cephaloridine can be generated in the kidney. In support of this view, Stewart and Holt (1964) analyzed urine samples from cephaloridine treated patients and did not detect any metabolites. Furthermore, using high pressure liquid chromatography, Wold and Turnipseed (1977b) also did not identify any metabolites.

Alternatively, depletion of GSH in renal cortex by cephaloridine can be due to a decrease in glutathione synthesis. The synthesis of glutathione involves two sequential enzymatic steps in which ATP is required (Meister, 1976). As described previously (Introduction section), Tune et al. (1979) reported impaired respiration in renal cortical mitochondria isolated from rabbits that had received a toxic dose (200 mg/kg) of cephaloridine. Decreased mitochondrial respiration will reduce the synthesis of ATP and lower its intracellular concentration such that GSH synthesis can be limited, resulting in a decrease in renal cortical GSH. This possibility has been investigated by using another nephrotoxic antibiotic, gentamicin (Barza and Miao, 1977). Gentamicin, like cephaloridine, also is accumulated in the cells of the renal cortex (Luft and Kleit, 1974) and high renal cortical concentrations of this aminoglycoside antibiotic have been shown to inhibit renal cortical mitochondrial respiration (Simmons et al., 1980). However, gentamicin, unlike cephaloridine, had no effect on renal cortical GSH concentration, even when an acutely lethal dose (1000 mg/kg) was used (Table 30).

Thus, decreased ATP synthesis resulted from impaired mitochondrial respiration cannot be the cause of renal cortical GSH depletion produced by cephaloridine.

Intracellular GSH concentration results from a dynamic balance between GSH degradation and synthesis, and also is determined through a redox status between GSH and GSSG. A decrease in renal cortical GSH after cephaloridine may be due to an increased oxidation of GSH. The results from the present studies in rabbits and rats clearly demonstrated a decreased cortical GSH with a concomitant increased GSSG shortly following cephaloridine administration (Tables 31 and 32). This alteration in GSH/GSSG ratio appeared to be selective, since the same treatments did not have any significant effect on hepatic GSH and GSSG levels (Tables 31 and 32). Furthermore, total renal cortical glutathione (GSH and GSSG) concentrations in cephaloridine-treated animals also increased, indicating some de novo synthesized GSH had been oxidized to GSSG. Thus, a shift from GSH to GSSG appeared to be a reasonable explanation for GSH depletion after cephaloridine treatment. The next question is how this can happen. An increase in GSSG concentration can be due to an increased oxidation of GSH, a decreased reduction of GSSG or a combination of both. GSH may be oxidized through thiol-disulfide exchange reactions, but mainly is oxidized by activated oxygen, radicals or peroxides (Flohe and Gunzler, 1976). Thus, increased formation of these reactive species in the cells will convert more GSH to GSSG. The question now is "can cephaloridine increase the formation of these reactive species?" As described previously

(Introduction section), cephaloridine possesses a pyridinium ring, the specific structure also existing in paraquat. Several lines of evidence (Farrington et al., 1973; Bus et al., 1974, 1975; Trush et al., 1981) indicated that paraquat undergoes a single electron reduction to form the reduced free radical with NADPH serving as a source of electrons for the reduction. Reduced paraquat radical is rapidly reoxidized by molecular oxygen with formation of oxidized paraquat and superoxide radical. Superoxide radicals may then react with iron, which can attack unsaturated lipids of cell membranes to produce lipid hydroperoxides. Lipid hydroperoxides spontaneously decompose to lipid free radicals, initiating the chain reaction process of lipid peroxidation. Since all these components required for the reduction-oxidation reaction and lipid peroxidation have been shown to be present in the kidney (Monserrat et al., 1969; Grinna and Barber, 1973; Yonaha et al., 1980; Yonaha and Ohbayashi, 1980), the pyridinium ring of cephaloridine may undergo the similar reduction-oxidation cycle to generate superoxide radicals and then initiate lipid peroxidation (Figure 23). If, indeed, this proposed cascade for cephaloridine metabolism occurs in renal proximal tubular cells, several studies can be conducted to evaluate this hypothesis. A variety of methods have been developed to measure lipid peroxidation (Buege and Aust, 1978; Csallany and Ayaz, 1976; Recknagel and Ghoshal, 1966; Riely et al., 1974; Dillard et al., 1977), however most of them cannot accurately determine in vivo lipid peroxidation at a specific tissue. Measurement of conjugated dienes (Recknagel and Ghosal, 1966;

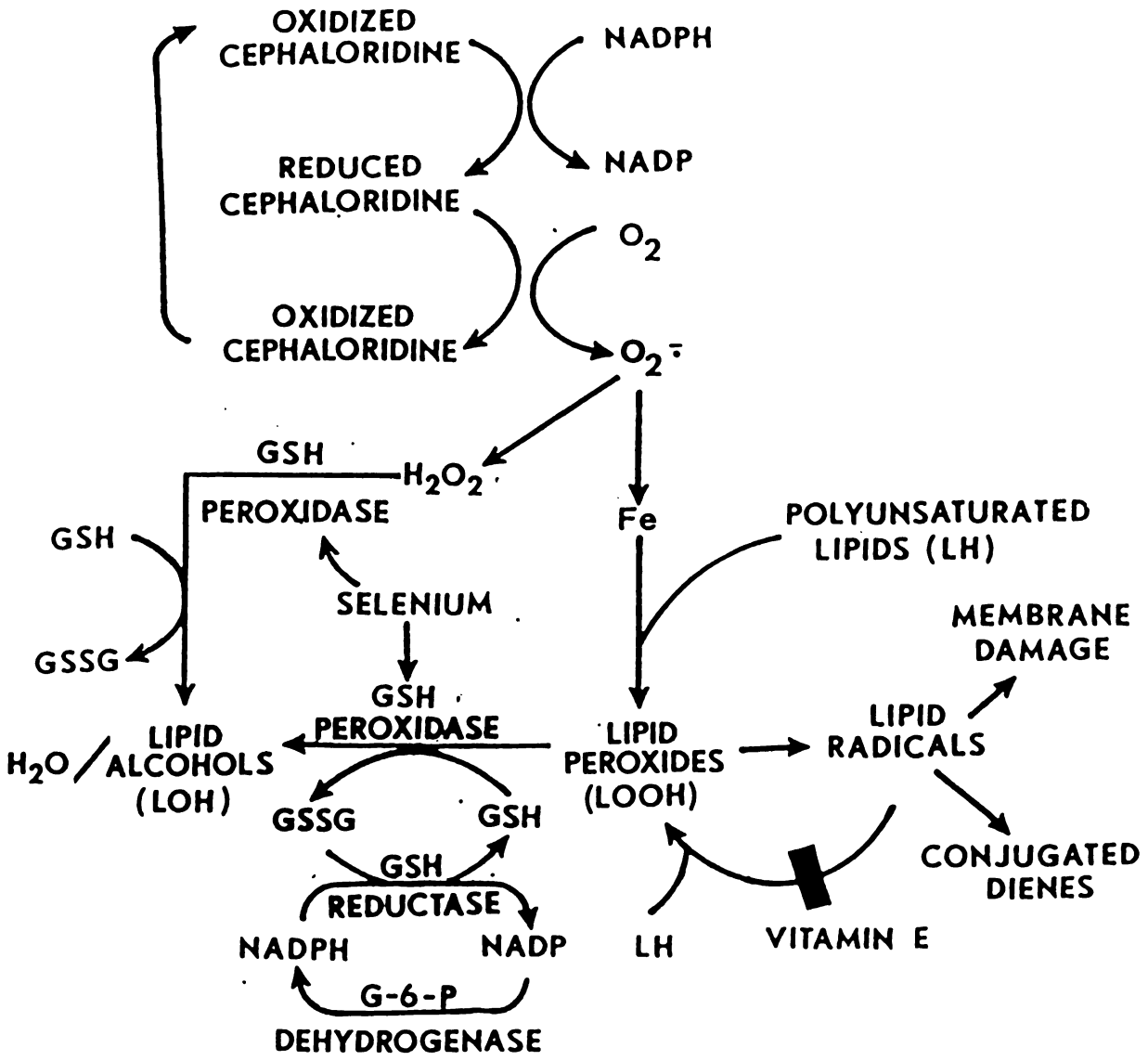


Figure 23. Proposed mechanism for cephaloridine nephrotoxicity involving lipid peroxidation.

Sell and Reynolds, 1969) appears to be a relatively suitable method for this purpose. The results from the present study demonstrated that cephaloridine increased conjugated diene concentration in renal cortex (Table 33 and Figure 13). This increase appeared to be very selective, because the same treatment did not alter hepatic conjugated diene levels (Table 33). Furthermore, increased conjugated dienes occurred shortly after cephaloridine administration (Table 33 and Figure 13), indicating that lipid peroxidation may be a cause of renal cortical cell injury rather than a result of cell damage. This hypothesis was further supported by the results from the dietary study. Removal of vitamin E and selenium from the diet appeared to potentiate cephaloridine nephrotoxicity (Figures 15-20). Vitamin E is known as a free radical scavenger (Myers et al., 1977; Goodman and Hochstein, 1977; Thayer, 1977; Urano and Matsuo, 1976) and presumably acts in the membrane by interrupting the free radical chain reactions of lipid peroxidation (Tappel, 1980). Increased vitamin E concentrations in tissues has been shown to prevent or reduce toxicity of several lipid peroxidation-inducing agents such as carbon tetrachloride (Hafemann and Hoekstra, 1977), adriamycin (Myer et al. 1976), ozone (Fletcher and Tappel, 1973), ethanol (Litov et al., 1978) and methyl ethyl ketone peroxide (Litov et al., 1981). Conversely, vitamin E deficiency increased toxicity of oxygen (Mino, 1973) and paraquat (Bus et al., 1975) which are shown to be caused by lipid peroxidation. On the other hand, selenium has been shown to be an essential constituent of GSH peroxidase (Rotruck et al., 1973; Flohe et al., 1973; Oh et al., 1974), which is the key enzyme to reduce radical-producing lipid hydroperoxides to stable lipid alcohols (Christopherson,

1969; Wendel, 1980) or to remove hydrogen peroxide formed in the cells (McCay et al., 1976; Hoekstra, 1975). Therefore, GSH peroxidase has been considered a detoxifying enzyme for hydrogen peroxide and organic hydroperoxides. Removal of selenium from the diet significantly reduced GSH peroxidase activities in many tissues including kidney (Hoekstra, 1975) and increased the toxicity of several compounds which were shown to act through lipid peroxidation (Bus et al., 1975; Wandel and Feuerstein, 1981). Similarly, in the present study, animals fed a diet deficient in vitamin E and selenium appeared to reduce the ability of converting hydroperoxide and/or hydrogen peroxide to nontoxic metabolites and therefore became more susceptible to cephaloridine toxicity.

Although lipid peroxidation may be an important mechanism responsible for cephaloridine-induced nephrotoxicity, the possible damage due to a dramatic change in renal cortical GSH-GSSG ratios should be considered. A significant number of destructive effects have been reported to be due to alterations of intracellular GSH-GSSG status. Kosower et al. (1969) and Harris et al. (1971) have studied a thiol-oxidizing agent, diamide, and reported that this compound penetrated the red blood cells and nucleated mammalian cells and stoichiometrically and rapidly oxidized intracellular GSH to GSSG. Addition of diamide to the cells depressed protein synthesis and this inhibitory effect was reversed after the cells regenerated GSH (Harris et al., 1971). This observation was further studied by Zehavi-Willner et al. (1971), who found that diamide inhibited both translation and initiation steps of protein synthesis. However, initiation was more sensitive to GSH-GSSG alteration than translation. Processes involved in translation recovered

after a short lag following regeneration of 40-60% of the original GSH; however initiation processes recovered after regeneration of 70-80% of the original GSH (Zehavi-Willner et al., 1971). Later, using a cell-free preparation (rabbit reticulocyte lysate), Kosower et al. (1972) was able to show a direct inhibitory effect of GSSG on protein synthesis. Addition of a small amount of GSSG to the lysate preparation caused a conversion of polysomes to monosomes accompanied with a loss of associated amino acids, and markedly inhibited the initiation of protein synthesis (Kosower et al., 1972). Thus, protein synthesis appeared to be closely linked to the intracellular GSH-GSSG status and to be regulated by GSSG concentrations in the cells. Cephaloridine markedly increased intracellular GSSG in renal cortex (Tables 31 and 32) and this change may inhibit protein synthesis.

It has been shown that glutathione in the reduced (GSH) or in the oxidized (GSSG) form can enter the disulfide-sulfhydryl exchange reactions with disulfides (Prot·SS·Prot) or sulfhydryl (Prot·SH) groups of proteins resulting in the formation of mixed disulfides (GSS·Prot) (Modig, 1969; Harrap et al., 1973; Issac and Binkley, 1977; Mannervik and Axelsson, 1975). Through disulfide-sulfhydryl exchange reactions, a variety of enzymes have been shown to be inhibited by GSSG. Purified glycogen synthetase was inactivated by GSSG and this inactive form could be reactived by the addition of GSH. Inactivation of glycogen synthetase was the result of the formation of mixed disulfides between GSSG and the sulfhydryl group(s) of glycogen synthetase (Ernest and Kim, 1973). Similarly, pyruvic kinase was shown to be converted by GSSG to a

thermolabile and less active form which had a lower affinity for the substrate phosphoenol pyruvate and the oxidized enzyme was reduced by GSH (Van Berkel et al., 1973). In support of this view, patients with deficiency of glutathione reductase were also deficient in pyruvic kinase (Schroter, 1970; Nowicki et al., 1972). Glutathione reductase is the key enzyme in the reduction of GSSG to GSH and a deficiency of this enzyme will increase intracellular GSSG concentration, which then inhibits pyruvic kinase activity. GSSG also was reported to inhibit human erythrocyte acid phosphatase activity subsequent to formation of mixed disulfide bonds in the enzyme proteins (Bottini and Modiano, 1964). Consistent with this concept, a lower level of acid phosphatase activity was found in erythrocytes from patients with glucose-6-phosphate dehydrogenase deficiency (Oski et al., 1963). Glucose-6-phosphate dehydrogenase is the important enzyme in the formation of NADPH from NADP coupled with the dehydrogenation of glucose-6-phosphate, and NADPH is required for the reduction of GSSG. Deficiency of glucose-6-phosphate dehydrogenase in erythrocytes will reduce NADPH concentrations and subsequently increase GSSG concentration, which can then inhibit acid phosphatase activity.

Alteration of intracellular GSH/GSSG status has been reported to have a variety of effects on kidney functions. Treatment with diamide specifically inhibited several important enzyme activities, such as protein kinase, Na^+,K^+-dependent ATPase and glucose-6-phosphatase; inhibition of these enzymes is reversible upon addition of GSH (Pillion et al., 1977a; Leibach et al., 1978). In addition, treatment with

diamide inhibited several renal cortical functions such as gluconeogenesis (Pillion et al., 1977b), amino acid transport (Hewitt et al., 1974; Chesney et al., 1979) and sugar transport (Pillion et al., 1975, 1976). Inhibition of these transport systems has been related to intracellular GSH-GSSG status.

Thus, on the basis of this information, it seems reasonable to suggest that cephaloridine may also alter functions of proximal tubular cells through an alteration in GSH-GSSG status. Although increased lipid peroxidation will increase formation of GSSG, high concentrations of intracellular GSSG do not necessarily follow because a great percentage of GSSG can be reduced by glutathione reductase with the necessary reducing equivalent, NADPH. While GSSG is reduced back to GSH, concomitantly NADPH is converted to NADP. The regeneration of NADPH from NADP is mainly catalyzed by glucose-6-phosphate dehydrogenase coupled with oxidation of glucose-6-phosphate. Rose and coworkers (1976) reported that the hexose monophosphate pathway was stimulated in lung tissue, even when animals were exposed to paraquat for only a very short period. Furthermore, addition of paraquat into the incubation medium increased the hexose monophosphate pathway in lung slices taken from nontreated animals (Rose et al., 1976). This increase was related to paraquat concentration in the lung slices. The stimulation of the activity of the hexose monophosphate pathway indicates a requirement for NADPH. Recently, Witschi et al. (1977) and Smith et al. (1979) showed that the ratio of NADPH to NADP was reduced in lungs taken from paraquat-treated rats. Furthermore, paraquat treatment actually decreased

absolute NADPH concentration of the lung (Smith et al., 1979). Although paraquat stimulates the hexose monophosphate pathway, it markedly reduced pulmonary net NADPH concentration, suggesting that the rate of NADPH oxidation by cyclic reduction and oxidation of paraquat is greater than the rate of NADPH generation by the hexose monophosphate pathway. Following paraquat treatment, the intracellular NADPH concentration may fall below that required to sustain vital physiological processes. In support of this view, Smith et al. (1979) have shown that some important NADPH-dependent processes such as fatty acid synthesis in lung was inhibited following paraquat poisoning. A decreased intracellular NADPH concentration also can affect the GSH/GSSG ratio. Because NADPH is an important reducing equivalent to convert GSSG to GSH, a reaction catalyzed by glutathione reductase, a marked decrease in NADPH will attenuate the rate of reduction of GSSG. Although in the present study renal cortical NADPH and NADP concentrations were not measured, the high ratio of GSSG to GSH (Tables 31 and 32) could be the result of a reduced NADPH/NADP ratio. Through the cyclic reduction and oxidation reactions, cephaloridine, like paraquat, may oxidize NADPH and eventually deplete renal cortical NADPH concentration. GSSG was accumulated in renal cortex after cephaloridine (Tables 31 and 32), suggesting that NADPH might be markedly depleted. This marked depletion of NADPH very likely will interfere with normal function of proximal tubular cells.

A decrease in the concentration of NADPH may not only debilitate vital physiological processes but render the cells more susceptible to lipid peroxidative injury. As described previously, decreased NADPH will deplete GSH concentration. Depletion of tissue GSH has been shown

to result in an increase in lipid peroxidation (Wendel et al., 1979; Younes and Siegers, 1980, 1981). This increase is possibly due to the lack of glutathione as a scavenger against free radicals and/or hydroperoxides. Anundi et al. (1979) showed that glutathione depletion alone could evoke cell damage and they related this effect to an increase in lipid peroxidation. But an enhanced lipid peroxidation was seen only after 80% or more of normal GSH level was depleted (Younes and Siegers, 1981). In the present study, cephaloridine treatment did not deplete renal cortical GSH to that magnitude. However, renal cortex contains various types of cells in addition to proximal tubular cells, where cephaloridine is selectively accumulated, therefore the actual GSH depletion in the proximal tubular cells should be much greater. Thus, lipid peroxidation in proximal tubular cells may take place after GSH was depleted by cephaloridine treatment.

CONCLUSION

In conclusion, even though cephaloridine is excreted mainly through glomerular filtration, a significant amount of the drug is actively transported into renal proximal tubular cells via an organic anion transport system. In the proximal tubular cells the drug presumably shares common "binding sites" with organic acids and bases. In addition, cephaloridine may bind to other proteins such as cytochrome P450 and glutathione S-transferase. Unlike the entry of the drug into the cells, cephaloridine appears to be transported out of the proximal tubular cells through an organic cation transport system (Figure 21). Because of these unique transport and intracellular binding systems, high concentrations of cephaloridine are developed selectively in renal proximal tubular cells. Through cyclic redox reaction, the high concentration of cephaloridine will consume large amounts of NADPH and generate abnormally high concentrations of intracellular lipid hydroperoxides. A marked depletion of NADPH and increased lipid hydroperoxides will result in a high intracellular GSSG/GSH ratio (Figure 23). High lipid peroxidation rate, high intracellular GSSG/GSH ratio, low intracellular NADPH or a combination of these changes appeared to be responsible for cephaloridine-induced renal proximal tubular injury (Figure 24).

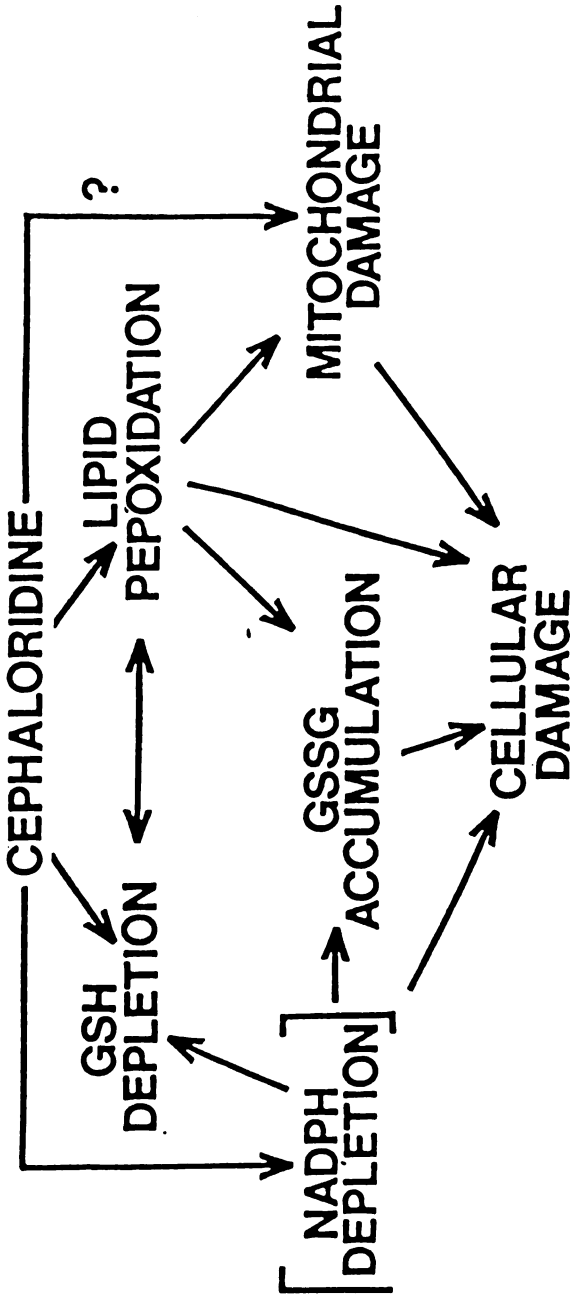


Figure 24. Schematic diagram of the proposed mechanisms for cephaloridine-induced nephrotoxicity.

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