EPHROTOXICITY IN THE EXPERIMENTAL AND CLINICAL SITUATION

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Nephrotoxicity in the experimental and clinical situation

Part 2

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PART 1

PREFACE

There are many aspects of renal function and malfunction that we still do not understand. Homeostasis is central to renal function, but its maintenance also serves to mask the earliest features of malfunction. Thus renal dysfunction is buffered and cannot be identified until degeneration has reached a level at which homeostasis is severely compromised. Because of this, diagnosis of the vast majority of nephropathies are often so late as to preclude therapeutic intervention. More importantly, it has been impossible to establish the aetiology of many nephropathies.

The kidney is known to be a frequent target for toxicity, because of its size in relation to the many functions it must perform. All to often in the past there has been a failure to adequately perceive this in the early development of new therapeutic agents, their clinical trials and subsequent drug usage. Industrial and environmental chemicals have also been implicated in several nephropathies, but the causal link with exposure to the offending chemical may not have been immediately established.

These volumes cover the different methods that are used to assess renal function in health and disease. The biology of many model nephropathies that are directly relevant to the clinical situation (especially those where a mechanistic understanding is helping to define the primary lesion and its secondary consequences) and a broader appreciation of the different types of clinical nephrotoxicity and factors that may affect their diagnosis and progression. The objective of NEPHROTOXICITY IN THE EXPERIMENTAL AND CLINICAL SITUATION is to use a multidisciplinary scientific approach as the foundation to better understand nephrotoxicity. The experimental systems will serve to provide a basis for improved screening for potentially nephrotoxic drugs and chemicals, the development of less nephrotoxic drugs, they will improve the approach to the prevention of nephrotoxicity and also provide a rational basis for the more successful clinical management of all nephropathies.

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15
LIGHT HYDROCARBON NEPHROPATHY AND RENAL CARCINOMA*

M.M. LIPSKY AND B.F. TRUMP

I. INTRODUCTION

Recent studies have demonstrated that acute exposure of male rats to volatile hydrocarbons induces a renal toxicity. Chronic exposure of male rats to a few of the volatile hydrocarbons resulted in an increased incidence of renal adenomas and carcinomas. These effects have not been observed in female rats or any other species of experimental animals exposed to these volatile hydrocarbons.

Volatile (or light) hydrocarbons range in boiling point from 60 °C to 220 °C and can include single, pure compounds, such as 1,1,2,2-trimethyl pentane, or complex mixtures of compounds such as gasoline[1]. All of the different hydrocarbons which have been shown to induce this male rat renal toxicity also produce a very similar spectrum of pathological alterations after acute exposure. Only a few complex hydrocarbons have, however, been examined in chronic, bioassay experiments.

In this chapter the pathological changes which occur in male rat kidneys and the pathogenesis of the changes after acute and chronic exposure to light hydrocarbons will be described. Where possible these changes will be related to the potential mechanisms of hydrocarbon toxicity.

II. EFFECTS OF ACUTE EXPOSURE OF MALE RATS TO VOLATILE HYDROCARBONS

Exposure of male rats, by inhalation or gavage, to volatile hydrocarbons produces an acute nephropathy which has four

*Contribution No. 2329 from the Pathobiology Laboratory.

characteristic pathological hallmarks[2-6]. These are: accumulation of hyaline droplets in proximal tubule epithelial cells; focal degeneration and necrosis of epithelial cells in the proximal tubule; the appearance of areas of focal tubule cell regeneration; and dilated tubule lumina at the junction of the proximal tubule and the descending thin limb.

While these primary lesions have been described, detailed evaluation of the histogenesis and localization to a particular tubular segment has not been performed.

A. Hyaline droplets

The most prominent component of the hydrocarbon-induced nephropathy in male rats is accumulation of hyaline droplets in cells of the proximal tubule. Hyaline droplet accumulation has been produced by exposure by gavage and inhalation exposure to a variety of petroleum-derived hydrocarbon compounds and mixtures (Table 1) including unleaded gasoline[2-5], decalin[6], 2,2,4-trimethylpentane[7], solvents[3,4,8,9], and synthetic hydrocarbon fuels[10,11]. These droplets were characterized by positive staining using Gram's stain, Mallory-Heidenhien and Toluidine blue. The droplets were diffuse and located in focal aggregates of proximal tubule profiles in the kidney cortex[2,3,6]. Hyaline droplets varied in size, some being as large as the nucleus. The appearance of hyaline droplet accumulation can be rapid, occurring within 1-2 days in rats treated with decalin by gavage[6]. The accumulation of hyaline droplets is readily reversible after cessation of exposure[6,12,13]. The ultrastructural appearance of these lesions has been evaluated[4,6,12]. These studies showed characteristic, round accumulations consistent with phagolysosomes and large, angular profiles, often containing crystalline inclusions. Both the round and angular cytoplasmic bodies displayed a positive reaction for lysosomal acid phosphatase[4,12]. Studies utilizing gel electrophoresis and the Western blotting technique demonstrated that a major protein component of the decalin and trimethylpentane-induced hyaline droplets was α_2-μ-globulin, a low molecular weight protein, normally produced by the male rat[6,7,12]. α_2-μ-Globulin is a major male rat urinary protein that readily passes through the glomerulus and is resorbed by proximal tubule epithelial cells in mature rats[6,14]. The mechanism by which volatile hydrocarbons induce hyaline droplet accumulation is unknown. Alden et al.[12] proposed several possibilities for decalin-induced hyaline droplet accumulation. Preliminary evidence from that laboratory (Alden, personal communication) indicates that a possible mechanism involves the production of α_2-μ-globulin/decalin or decalin metabolite adduct. It is this hydrocarbon-protein adduct which is believed to accumulate in the lysosomes of the male rat proximal tubule epithelial cells.

B. Hydrocarbon-induced renal tubular degeneration/regeneration

Subsequent to the accumulation of hyaline droplets, male rats treated with volatile hydrocarbons develop focal areas of proximal

tubular cell degeneration and necrosis and areas of regeneration. Occasional cell necrosis was observed, although this has not been considered a prominent component of hydrocarbon nephropathy[2,4,6]. Additional cellular changes included swollen mitochondria and loss of brush border[4,6]. More prominent than cellular necrosis was the observations of focal areas of basophilic, flat epithelial cells lining some proximal tubules[2-6]. These have generally been considered as areas of tubular cell regeneration, as distinct from basophilic, atrophic tubules seen as part of the ageing rat nephropathy[15]. While the hyaline droplet accumulation can occur within 24 hours after an oral exposure to volatile hydrocarbons, tubular cell regeneration is not observed until 7-14 days after dosing[12,16].

C. Hydrocarbon-induced granular casts

The other major morphological component of the volatile hydrocarbon nephropathy is the formation of granular casts. The casts were located at the junction of the inner and outer stripes of the outer medulla[5,10,11]. Although not precisely localized, they appear to occur in the descending portion of the thin limb of Henle. The casts are eosinophilic and composed of cellular debris which are presumed to be the remnants of necrotic cells denuded from more proximal tubular segments[11,15]. The casts are observed 10-14 days after exposure, appear to be dose- and time-dependent and would seem to represent the accumulation of progressive focal necroses over time[6,11,12,15].

The hydrocarbon-induced nephropathy has only been demonstrated to occur in mature male rats. Studies with immature male rats, female rats and both sexes of dogs, guinea pigs, mice and male primates have been consistently negative with respect to the hydrocarbon nephropathy[4-6,17,18]. This trend, with respect to the spectrum of lesions comprising the nephropathy, parallels the occurrence in hyaline droplet accumulation between species. Hyaline droplets can be observed in proximal tubule epithelial cells of mature (>40 day old) male rats, and many of these contain α_2-μ-globulin[7]. Hyaline droplet accumulation occurs rapidly after exposure (24-48 hours) and appears to be completely reversible after cessation of exposure[6,7]. The persistence and extended accumulation of the hyaline droplets leads to tubular cell necrosis. The induction of hyaline droplets and the other lesions associated with the hydrocarbon nephropathy has not been correlated with alterations in renal function[4,7]. Administration of 2,2,4-trimethylpentane (2 ml/kg), by gavage, produced no significant alteration in common urinalysis parameters, i.e. glucose and protein or in two urinary enzymes, alkaline phosphatase and N-acetyl-β-glucosaminidase[7]. Inhalation exposure of male rats to C_{10}-C_{11} isoparaffin for 8 weeks produced minimal changes in renal function. Urinary glucose and protein levels were elevated to double the control levels in isoparaffin-treated rats[4,16]. These changes were not seen in rats treated for 4 weeks or less.

III. RELATIONSHIP BETWEEN HYDROCARBON STRUCTURE AND INDUCTION OF THE NEPHROPATHY

Re-evaluation of studies performed in the 1970's demonstrated the induction of hydrocarbon nephropathy in male rats exposed to a group of petroleum-derived industrial solvents[4,13]. Not all of the solvents, however, induced the nephropathy in male rats[4]. Halder et al.[3,19] investigated the ability of selected naphtha refinery streams (petroleum-derived) to induce the nephropathy. Differences were noted between different naphthas with respect to nephropathy induction. Differences were also noted in the nephrotoxic potency between napthas which were able to induce the nephropathy[3,19].

Table 1. Hydrocarbons shown to induce the nephropathy in male rats

Compound		References
A.	Solvents	
	1. 60 Solvent	4,8
	2. 70 Solvent	4,9
	3. C_{10}-C_{11} Isoparaffin	4,15
	4. High naphthenic solvent	4
	5. VM & P naphtha	4
	6. Stoddard solvent	4
	7. Napthenic aromatic solvent	4
B.	Mixed distillate high energic fuels	
	1. RJ-5	10
	2. JP-4	10
	3. JP-5	10,11
	4. JP-10	10,11
C.	Petroleum-naphtha stream	
	1. Light alkylate naphtha	3,19
	2. Thermal cracked naphtha	3,19
	3. Polymerization naphtha	3,19
	4. Light catalytic cracked naphtha	3,19
	5. Light catalytic reformed naphtha	3,19
	6. Heavy catalytic naphtha	3,19
D.	Unleaded gasoline	2,3,19
E.	Pure hydrocarbons	
	1. 2-Methylpentane	19
	2. 2,3-Dimethylbutane	19
	3. 2,2,5-Trimethylheptane	19
	4. 2-Methylhexane	19
	5. 2,3-Dimethylpentane	19
	6. 2,2,4-Trimethylpentane	7,19
	7. Decalin	6,12,19

These studies demonstrated the diversity of nephrotoxic potential of complex hydrocarbon mixtures and pointed out the need to identify the nephrotoxic components of the mixtures. Table 1 lists the hydrocarbons shown to induce the nephropathy in male rats. The studies of Halder et al.[3,19] indicate that the alkane (paraffin) components of petroleum-derived hydrocarbon mixtures were primarily responsible for induction of the nephropathy. Cycloalkane components were also shown to be capable of inducing the nephropathy[3,6,12]. In contrast, alkanes and aromatic constituents of hydrocarbon mixtures did not induce the hydrocarbon nephropathy. Halder et al.[3,19] also studied the relationship between alkane structure and nephrotoxicity. In general, their data indicates the degree of branching of alkanes correlated with nephrotoxicity, and the more branching of the alkane backbone, the greater was the nephrotoxic potential of the compound. In reviewing the earlier data by Halder et al.[3] on the ability of naptha refinery streams to induce the nephropathy, the relationship between alkane components and nephrotoxicity induction is maintained. The light alkylate naphtha was the most nephrotoxic mixture and it was composed of almost all alkanes with 79% known or suspected nephrotoxic alkanes.

IV. EFFECTS OF SUBCHRONIC AND CHRONIC EXPOSURE OF MALE RATS TO VOLATILE HYDROCARBONS

The effects of more chronic exposure to some volatile hydrocarbons in male rats have also been studied. The hydrocarbon mixtures included unleaded gasoline and petroleum or shale oil-derived and synthetic fuels used by the military, i.e. RJ-5, JP-10, JP-4, and JP-5[3,5,10,11]. The subchronic exposure of male rats to these four fuels of military interest produced a spectrum of morphological changes in the kidney similar to the hydrocarbon nephropathy induced by acute exposure[10,11]. This included accumulation of hyaline droplets, granular casts and focal areas of tubule regeneration. The hydrocarbon nephropathy was also present in male rats exposed by inhalation to a generic, unleaded gasoline for 3 and 6 months[5].

Chronic exposure to volatile hydrocarbons produced toxic hyperplastic and neoplastic alterations in the kidney of male rats. The complete picture of toxic kidney lesions induced by chronic light hydrocarbon exposure is difficult to describe. This is in large part due to the confounding effects of the ageing rat nephropathy[16,18,20]. While the acute and subchronic studies show a clear difference between lesions comprising the hydrocarbon nephropathy and lesions comprising the ageing rat nephropathy, this distinction becomes blurred in the chronic studies. In common with the acute changes of the hydrocarbon nephropathy are accumulation of hyaline droplets in the proximal tubule epithelial cells and the presence of granular casts. However, the extent of tubular cell degeneration, necrosis and their sequelae attributable to hydrocarbon exposure is impossible to assess. The kidneys of male rats exposed to hydrocarbons also contained large areas of basophilic tubules, varying amount of basal lamina thickening,

fibrosis, inflammation and glomerular sclerosis[5,10,11,16]. While many of these lesions are components of the ageing rat nephropathy, the extent of changes was far greater in treated than in control animals. It appears that light hydrocarbon exposure exacerbates the ageing rat nephropathy, however, the mechanism is not clear[16,18].

One lesion which is unique to chronic light hydrocarbon exposure is the mineralized deposits accumulated in the renal pelvis[5,10,11,16]. These precipitates have been characterized as calcium hydroxyapatite[16,20]. The mineralized deposits appear to be located in the loops of Henle, and most probably represent the cellular debris which accumulated from chronic, proximal tubular cell necrosis and subsequent phospholipid-vesicle-induced calcification[16,21].

V. PRENEOPLASTIC AND NEOPLASTIC RENAL LESIONS INDUCED BY LIGHT HYDROCARBON EXPOSURE

Only a few complex hydrocarbon mixtures have been studied with respect to their neoplastic potential. They include a generic unleaded gasoline[5,22] and two fuels of military interest[10,11] designated RJ-5 and JP-10. All of these hydrocarbon mixtures induced primary renal neoplasia in male rats exposed for extended periods of time (i.e. >1 year).

The studies of RJ-5 and JP-10 used 1 year inhalation exposures followed by an additional post-exposure observation or 12 months observation until the terminal sacrifice[10,11]. Both RJ-5 and JP-10 induced renal adenomas and renal adenocarcinomas, primarily in the high dose groups of male rats. These neoplasms were identical in morphological appearance to neoplasms induced in male rats by a number of different chemical carcinogens[16,18].

The studies of unleaded gasoline were conducted as a 2-year inhalation bioassay, with an industrial mode of exposure, i.e. inhalation for 6 hours a day, 5 days a week for 24-26 months[5]. Animals were sacrificed for histological evaluation after 3, 6, 12, 18 and 24-26 months of exposure to three different doses of whole vapours of unleaded gasoline. As early as 12 months after exposure, focal karyomegalic cells were noted in some of the high-dose animals[16,22]. These cells were located in the proximal tubules and appeared as enlarged cells with greatly enlarged hyperchromatic nuclei containing prominent nucleoli. These karyomegalic cells were not seen in the control rat kidneys. The development of karyomegalic cells has often been associated with exposure of experimental animals to renal chemical carcinogens[23,24]. The precise relationship between karyomegaly and carcinogenesis in the kidney is not known. It has been shown, however, that there is no quantitative relationship between karyomegaly and the later development of renal preneoplasia and neoplasia[25]. Karyomegaly of the type described above is not a component of the ageing rat nephropathy and in carcinogen-treated animals is usually noted prior to neoplasia development.

At the 18 month and terminal sacrifice period, renal tubular cell hyperplasia was noted[16,22]. These lesions appeared to be lo-

cated in the cortex and in the outer stripe of the outer medulla including the medullary rays of the pars recta. The tubular cell hyperplasias were similar in morphological appearance to hyperplasias induced by a large number of renal carcinogens of diverse chemical classes[16]. The hyperplasias were composed of basophilic cells which were generally smaller than normal proximal tubule epithelial cells. The cells contained hyperchromatic nuclei and often grew as papillary projections into the tubule lumens. It is not possible to determine from which segment of the proximal tubule the hyperplasias or tumours arose. It is clear, however, that these lesions are different from focal areas of basophilic tubules common to the ageing rat nephropathy[18,20]. Although it is not currently possible to determine, mechanistically, the relationship between the lesions induced by acute hydrocarbon exposure and the hyperplasias, recent evidence suggests a link may exist. Using continuous tritiated thymidine infusion via an osmotic mini-pump, Short et al.[26] reported extensive labelling of proximal tubule epithelial cells, primarily in the S_2 segment, after subchronic exposure to unleaded gasoline vapour. While this type of study does not demonstrate the genesis or determine the aetiology of distinct hyperplastic lesions, it highlights the ability of hydrocarbon exposure to produce the physiological conditions necessary to induce tubular cell division. What is unclear is the relationship between cell division, tubular cell necrosis and the sustained division present in renal preneoplastic lesions commonly referred to as tubular cell hyperplasia. At present, we do not know where the lesions occur.

Three renal adenomas and nine adenocarcinomas were observed in the unleaded gasoline bioassay primarily at the terminal sacrifice period[5,22]. One adenoma occurred at the 18 month sacrifice period. These neoplastic lesions appear to be identical in morphological appearance to neoplasms induced by a multitude of renal carcinogens and identical to neoplasms induced by the other hydrocarbon fuels. The adenomas were solid and papillary in architectural organization being composed of basophilic cells with large hyperchromatic nuclei[16,22]. Some of these adenomas appeared to arise in dilated tubular profiles and were generally localized in the cortex. Due to the design of the study and the extensive renal damage occurring in rats at the terminal sacrifice period, the precise tubular localization of the renal adenomas was not possible. The renal adenocarcinomas varied from well to poorly differentiated, with one being classified as a mixed adenocarcinoma-sarcoma[22]. There were no metastatic lesions observed in the animals containing the renal neoplasms. As with the adenomas, the precise tubular localization, i.e. cells of origin, could not be determined. Based, however, upon their rather characteristic morphological appearance, the origin of the hydrocarbon-induced neoplasms is probably identical to the origin of renal neoplasia induced by other chemical carcinogens, i.e. from proximal tubule epithelial cells[16,23].

VI. MECHANISMS AND RELATIONSHIPS BETWEEN TOXIC AND NEOPLASTIC RENAL LESIONS INDUCED BY LIGHT HYDROCARBON EXPOSURE

The primary components of the light hydrocarbon nephropathy are the accumulation of hyaline droplets and focal, tubule epithelial cell necrosis. These two changes ultimately lead to the granular casts, tubular regeneration and probably to papillary mineralization. Although there is a temporal relationship between hyaline droplet accumulation and other renal changes, this does not necessarily imply a cause and effect relationship. Hyaline droplets consist of accumulated protein within the lysosomal compartment that generally stains with anionic dyes such as Mallory-Heidenhie. In the hydrocarbon-induced lesions the droplets appear to consist primarily of α_2-μ-globulin[6,7]. This small protein is produced in the liver of male rats and is usually absent in females and other species. In this regard, therefore, the limitation of hydrocarbon nephropathy to the male rat corresponds to the presence and accumulation of α_2-μ-globulin. This association, however, does not mean that α_2-μ-globulin, itself initiates cell injury, necrosis or is involved in the neoplastic process. As previously discussed, one hypothesis is that an adduct of α_2-μ-globulin-decalin (or decalin metabolite) may be implicated in the genesis of decalin-induced nephropathy[12]. Studies of the metabolism of decalin in the kidney indicate that male Fischer 344 rats produced cis- and trans-2-decalone, while the female rat kidney extracts did not contain these metabolites[27]. This suggests that a ketone (decalin metabolite) could be related to renal toxicity of decalin and/or may interact with α_2-μ-globulin. A similar finding, i.e. the presence of a ketone metabolite, was observed in male but not in female rats exposed to the military fuel JP-10[28]. The specific chemical structure and nature of such an adduct is not known, although the binding between the hydrocarbon and the protein is not covalent. The α_2-μ-globulin may be able to strongly link to hydrocarbons by some as yet undefined mechanism and act to transport the hydrocarbon into the proximal tubule cells. Recent evidence from our laboratory using an in vitro, renal tubular epithelial cell culture system, indicates that α_2-μ-globulin by itself is toxic at a concentration of 3.0 mg/ml (M.M. Lipsky, unpublished observation). Hydrocarbon exposure does not increase α_2-μ-globulin synthesis in the liver and does not affect glomerular structure or function[5,6,14]. Its accumulation in proximal tubule cells must be due to some alteration in tubular function. A likely site of alteration is the lysosome, where a decrease in catabolic activity could lead to α_2-μ-globulin accumulation. On an acute basis, however, any reduction in catabolism must be transient since cessation of hydrocarbon exposure results in rapid resolution of the hyaline droplets with a return to normal morphological appearance. Therefore, the specific mechanism by which light hydrocarbons induce a nephropathy and the role of α_2-μ-globulin remains unclear.

Chronic exposure of male rats to light hydrocarbons results in renal neoplasia. This is accompanied by extensive renal damage, including primary toxic lesions and exacerbation of lesions comprising the ageing rat nephropathy. The mechanism of hydrocarbon-induced neoplasia is not understood. In general, the light

hydrocarbon compounds are weakly to non-mutagenic, as determined by a variety of in vitro and in vivo assays[16]. The light hydrocarbons have not been assayed to assess their pure, promoting capabilities in the kidney. The limitation of acute and chronic toxicity and carcinogenicity of light hydrocarbons to male rats indicates that the mechanisms may be related, and are linked to physiological factors unique to the male rat. As mentioned previously this could relate to the male rat protein, α_2-μ-globulin and its interactions with hydrocarbons or their metabolites and with proximal tubule epithelial cells.

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16

METALLOTHIONEIN AND ITS INVOLVEMENT IN HEAVY METAL INDUCED NEPHROPATHY

K. CAIN

I. INTRODUCTION AND HISTORICAL PERSPECTIVE

The interest in metallothionein and its involvement in heavy metal-induced nephropathy is a direct result of studies on the toxic metal, cadmium. This metal, although not abundant, is widely distributed throughout the biosphere[1] and contamination of the food chain cannot be avoided. Although absorption of the metal in man and animals via the gastrointestinal tract is low[2], small quantities of the cation are absorbed and retained principally in the liver and kidney. The biological half-life of the metal is long, for example in rats and man it is 200-300 days and 17-30 years respectively[2]. Thus, animal species even in relatively uncontaminated environments will accumulate significant body burdens. Early studies (1950-1960) in Sweden and Britain[3] showed that in industrial workers, long-term exposure to cadmium caused nephrotoxicity. At the same time, evidence from Japan[4] strongly implicated cadmium as a causative factor in the Itai-Itai disease which was endemic in the Toyama prefecture. Consequently there was considerable interest in determining the mechanisms behind cadmium nephrotoxicity and research was stimulated by two key findings. Firstly, Margoshes and Vallee[5] in 1957 demonstrated that the equine kidney cortex, which is particularly rich in cadmium, contains a soluble metalloprotein with a high affinity for the cation. Kagi and Vallee[6] showed that the protein contained 2-9% Cd^{2+}, 0.6% Zn^{2+} and 4% sulphur (as cysteine) and proposed it should be known as metallothionein. Later Piscator[7] extended these studies, demonstrating that rabbits chronically dosed with cadmium also accumulated the metal and metallothionein in their kidneys. Piscator[7] suggested that the protein was synthesized in response to the metal as a "detoxifying mechanism", and it was only when the latter was exceeded that the toxic effects were produced. This important conclusion offered an explanation for the

long-term toxicity of cadmium, and much research has been carried out to validate this hypothesis. Other metals, particularly zinc and copper, are also potent inducers of metallothionein synthesis[8]. It has been suggested that the detoxification role of metallothionein[8] in heavy metal toxicity is probably nothing more than a fortuitous interaction with the normal homeostatic mechanisms for zinc and copper. Regardless of this conclusion however, it is clear that metallothionein has a protective role in heavy metal nephropathy. In contrast, there has been considerable concern that extrarenal cadmium-metallothionein (Cd-Mt), particularly from the liver, may have a primary role in the production of renal toxicity. Research into this problem stems from the original proposal by Piscator[7] that Cd-Mt is synthesized in the liver and then carried in the blood to the kidney; a concept supported by the fact that the parenterally administered metallothionein is a potent nephrotoxin[9,10]. This chapter reviews the mechanisms involved in this process, its relevance to the long term toxicity of cadmium and the possibility that circulating and urinary metallothionein levels can be used as an index of cadium exposure and nephropathy.

II. CHEMISTRY OF METALLOTHIONEIN

IIA. Introduction

The structure and physical properties of metallothionein play an important role in determining its biochemical effects within the cell. Metallothionein is an unusual protein and a great deal of research has been carried out on the protein chemistry and physical properties of the metalloprotein (see reviews 12-15), but only the more important and well-established findings are outlined here.

IIB. Isolation and purification of metallothionein

Metallothionein is a small (MW 6000-7000), soluble protein which has been identified in a variety of organs and species (Table 1). The concentration of metallothionein in a particular organ depends on a variety of factors, including age, sex and the extent, if any, of heavy metal exposure. A large number of methods for isolating and purifying metallothionein have been published (see Webb[13] for review). All involve homogenizing the tissue and separating the cytosol by centrifugation. The metallothionein fraction is then isolated from the cytosol by gel filtration, usually using Sephadex G-75 (Figure 1). As metallothioneins have no known enzymic activity and poor absorbance[6] at 280 nm, the only reliable method of determining the metallothionein peak which elutes at a V_e/V_o of 1.8 to 2.0 is to assay the metal content of the eluant by atomic absorption spectroscopy. In the case of Cd-Mt, use can be made of the proteins high 254 nm absorbance, which, as shown by Kagi and Vallee[6], is due to the presence of Cd-S mercaptide bonds. It is still necessary, however, to determine the metal content of the protein. Indeed, the lack of a suitable protein assay for thionein[13]

has resulted in the almost universal practice of using the metal content as a means of quantitation of metallothionein.

Table 1 Occurrence of metallothionein in mammalian tissues[a]

Species	Tissue	Metallothionein criteria[b]	References
Man	Kidney	+++	16-19
	Liver	++++	17-21
	Heart	+	18
	Testis	+	18
	Embryonic fibroblasts	+	22
	Skin epithelial cells	+	23
Horse	Kidney	++++	5,6,11,24,25
	Liver	++++	24,25
	Intestine		26
Rat	Kidney	+++	17,27-30
	Liver	++++	17,27,29-34
	Intestine	+++	35-37
	Testis	+	38
Mouse	Kidney	+	39
	Liver	++++	39,40,41

a This table is not meant to be comprehensive and references 13-15 list other species and tissues in which metallothioneins have been isolated.

b + Metallothionein identified by behaviour on gel filtration and high metal content with low 280 nm absorbance. ++ As above - plus separation into isoforms and evidence of high SH content. +++ As above - plus amino acid composition characteristics of metallothionein. ++++ As above - plus evidence of amino-acid sequence which is compatible with previously described sequences. This is the most unambiguous definition and is equivalent to that proposed by Nordberg and Kojima[42].

Gel filtration offers a very convenient means of separating metallothionein from the cell cytosol. However, metallothionein exists in at least two isomorphic forms, usually designated as metallothionein 1 (Mt1) and metallothionein 2 (Mt2). These are easily separated by anion-exchange chromatography as they have different negative charges[20,28] and typical separations from rat liver and rat kidney are shown in Figure 2. The concentrations of Mt1 and Mt2 in the cell are not constant, and vary under different conditions (see later). Isometallothioneins, as isolated from anion-exchange columns, exhibit a high degree of purity, and further purification is not normally necessary; although in some cases, such as amino-acid sequencing studies, further purification steps may be necessary[40].

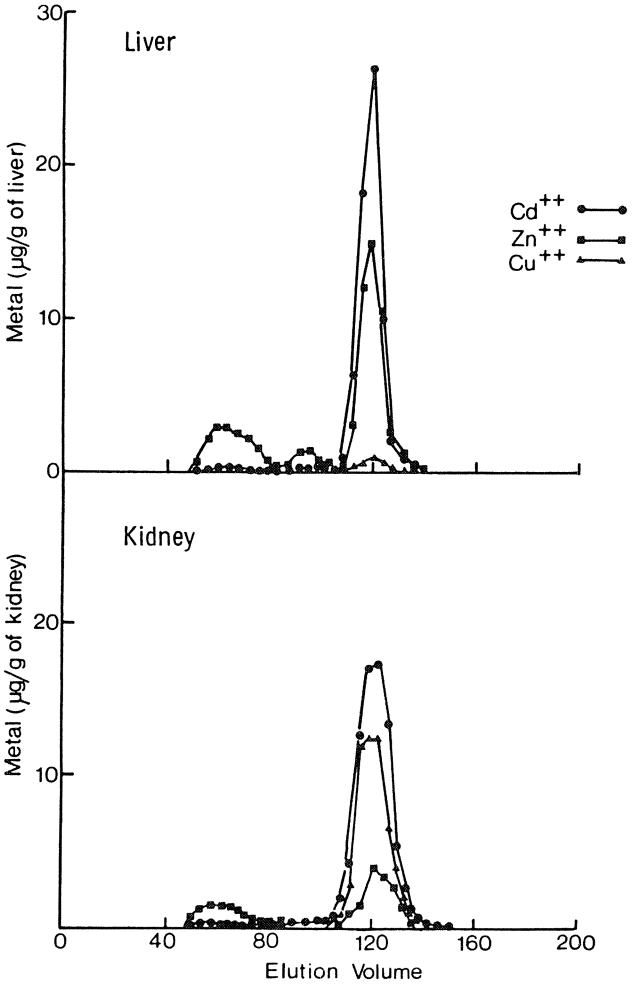

Figure 1 Sephadex G-75 gel filtration elution profiles for liver and kidney cytosol fractions obtained from animals dosed six times on alternate days with Cd^{2+} (1 mg/kg b.w., s.c.). Cytosols were isolated as described by Cain and Holt[29] and the columns were eluted with 10 mmol/l Tris-HCl, pH 8.0 at a flow rate of 7-10 ml/h and fraction volume of 3 ml. All fractions were analysed for Cd^{2+}, Zn^{2+} and Cu^{2+}. The metallothionein peak elutes at approximately 100-140 ml, i.e. V_e/V_o = 1.8-2.0. (Previously unpublished results, K. Cain and D.E. Holt.)

IIc Primary structure

The amino-acid composition of some mammalian thioneins is shown in Table 2. Paradoxically, although the kidney is the primary organ showing toxicity after chronic exposure of cadmium and other heavy metals, it is the liver thioneins which have been studied in most detail. Only the equine kidney thioneins, for example, have been sequenced, whereas hepatic thioneins from horse, human, mouse, rat and rabbit have been fully or partially elucidated[15].

From Table 2 it is clear that thioneins have a very high cysteine content, which in the liver ranges from 18 to 21 residues/mol out of a total amino-acid content of approximately 61. After cysteine the most abundant amino-acids are serine, alanine, lysine and aspartate. Aromatic amino-acids and histidine are not usually detected (see Webb[13]) and there is a remarkable homology between the amino-acid composition of hepatic thioneins.

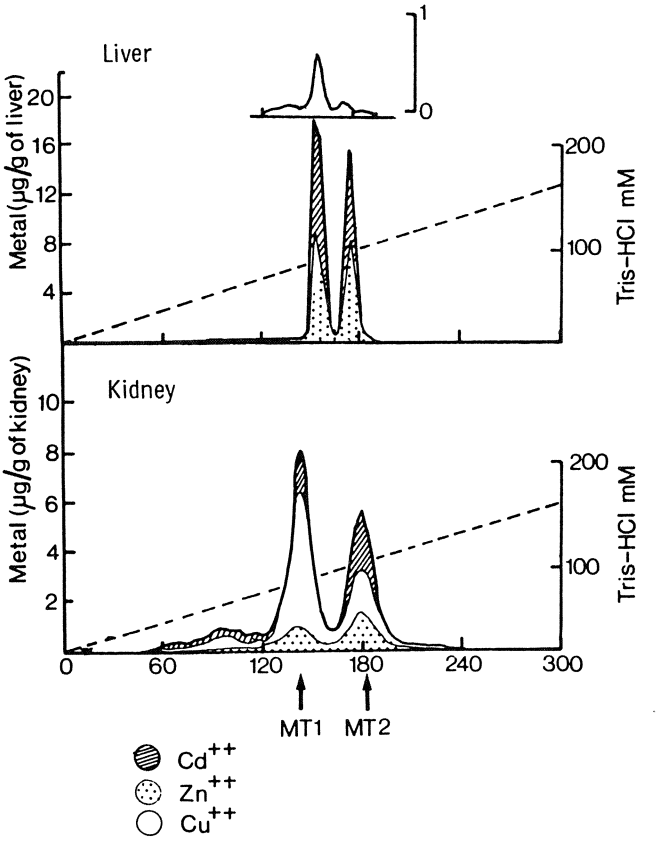

Figure 2 Anion-exchange chromatography of liver and kidney Cd-Mt. Metallothionein (100 µg thionein bound Cd^{2+}) was purified as described in Fig 1 and applied to a DEAE D52 cellulose column. The charge separable forms (Mt1 and Mt2) were eluted with a 10-200 mmol/l Tris-HCl, pH 8.0 gradient (400 ml total volume) at a flow rate of 30 ml/h. Fractions (3 ml) were collected and analysed for Cd^{2+}, Zn^{2+} and Cu^{2+}. The copper content of the liver metallothionein is shown inset with a different scale. (Data redrawn from Cain and Holt[29].)

Assessment of the differences between the renal and liver proteins is difficult due to the relative lack of data. However, there is some evidence to show that the equine renal and liver thioneins are very similar. Indeed the amino-acid sequencing data[25] (Figure 3) demonstrate that although equine Mt1a and Mt1b have different sequences there are no inter-organ variations. On the

other hand, the rat renal and hepatic thioneins appear to be quite different, with the kidney proteins having a lower cysteine and higher serine content. These differences may only be a reflection of the difficulties experienced in purifying rat kidney thioneins[29,30] which usually contain copper as the secondary cation. Copper-containing thioneins are notoriously difficult to purify as the proteins are susceptible to oxidation and polymerization[42-44]. Bremner et al.[30], for example, found that the rat renal copper, zinc thioneins have a relatively low cysteine content (12 residues/mol) even when isolated under anaerobic conditions. Significantly, not all cadmium induced renal thioneins contain copper. Suzuki[45], for example, showed that rat and guinea-pig renal thioneins contained copper, with little zinc, whereas the mouse, rabbit and hamster all have zinc as the major secondary cation. Furthermore, Cd/Zn renal metallothioneins (i.e. equine[25] and rabbit[46]) have amino-acid compositions very similar to the liver proteins. Thus the unusual amino-acid complement of the rat kidney thioneins may be unique to this species.

Table 2 Amino acid composition of hepatic and renal thioneins

	Hepatic metallothionein								Renal metallothionein			
Amino acid[a] (residues/mol of thionein)	Human[20]		Horse[25b]		Rat[43]		Mouse[40]		Horse[25]		Rat[28]	
	Mt1	Mt2	Mt1a	Mt1b	Mt1	Mt2	Mt1	Mt2	Mt1a	Mt1b	Mt1	Mt2
Total	61	62	57	57	59	60	61	61	61	62	61	60
Cysteine	18	21	18	18	21	21	20	20	20	21	17	15
Serine	8	8	7	7	8	7	9	10	7	7	10	9
Glycine 5	5	6	5	6	4	5	4	7	6	6	5	
Aspartic ($-NH_2$)	3	4	3	3	4	4	4	4	3	3	4	5
Threonine	2	2	3	1	3	2	5	1	3	1	4	3
Glutamic ($-NH_2$)	3	2	2	3	2	4	1	3	3	3	2	4
Proline	2	2	3	2	2	2	2	2	3	2	1	2
Alanine	6	7	5	7	3	5	5	6	5	7	5	5
Valine	2	1	1	3	2	1	2	1	1	3	2	2
Methionine	1	1	1	1	1	1	1	1	1	1	1	1
Leucine	1	0	0	0	0	0	0	0	0	0	1	1
Isoleucine	1	1	0	0	0	1	0	1	0	0	1	1
Arginine	0	0	2	1	0	0	0	0	2	1	0	0
Lysine	8	8	6	6	7	8	7	8	6	7	7	7
Metal complement	Zn/Cd/Cu		Zn/Cd/Cu		Cd/Zn		Cd/Zn		Cd/Zn/Cu		Cd/Zn	

a Residues/mol thionein values are expressed as whole numbers. In the case of the rat liver the values have been calculated from the original residue percentage data using 61 as the total amino acid value.

b Mt1a and Mt1b are the original nomenclature given by Kojima et al. and differ from other mammalian isothioneins (i.e. Mt1 and Mt2) in that anion-exchange chromatography at pH 10 is required for separation.

The amino-acid sequences (Figure 3) show there is considerable homology between species, and that most mammalian thioneins have 20 cysteines which are aligned in a specific cys-x-cys tripeptide arrangement, where x is another amino-acid (often serine). There are seven tripeptide sequences in the molecule and they form the primary chelation sites for the seven Cd^{2+} or Zn^{2+} binding sites usually found in this protein[26]. The amino-acid sequences show that the isometallothioneins (Figure 3) have small but distinct differences, the significance of which are as yet unknown, although it is probable that these amino-acid changes will alter the binding affinities for heavy metals and/or change the negative charge of the isoproteins.

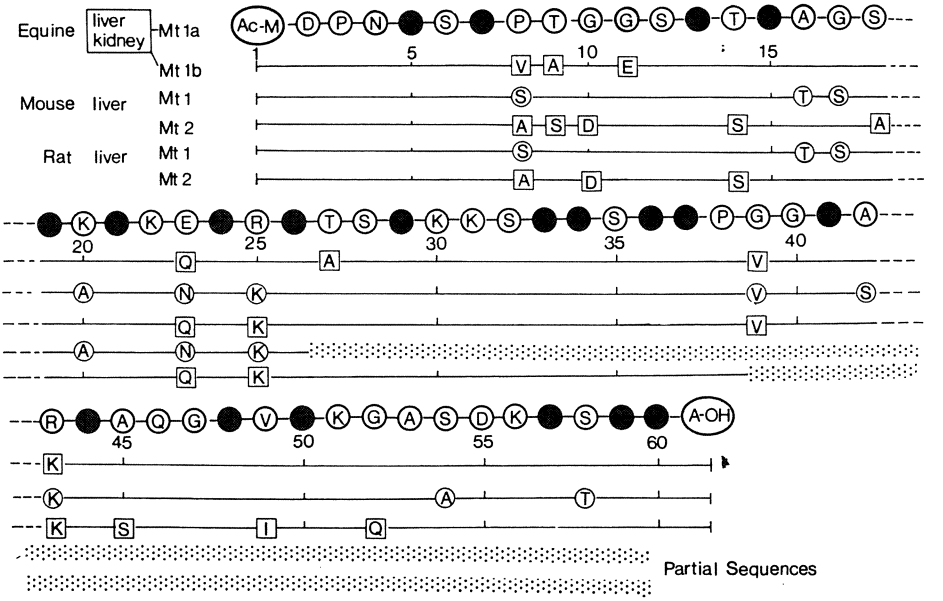

Figure 3 Amino-acid sequences of some mammalian metallothioneins. Filled in circles are cysteine amino-acids, all other amino-acids are designated according to the conventional one letter symbols. (Data from Kagi et al.[15].)

IID. Secondary structure and metal binding properties of thionein

Thioneins containing cadmium, zinc, copper, mercury, silver, gold and bismuth have been isolated from mammalian liver and/or kidney (see Ref. 42 for survey). In addition lead[47], and more recently platinum[48], have been reported to bind to metallothionein in vitro. However, detailed studies of metal binding to thionein have been confined mainly to cadmium, zinc and copper (see Refs. 13-15, 49-51). Early studies by Kagi and Vallee[6,11] showed that all the cysteinyl residues are deprotonated and that there is a 3:1 stoichiometry between mercapto and metal atoms (i.e. Cd^{2+} or Zn^{2+}). These experiments also showed that all the zinc could be displaced at around pH 4.5, whereas a pH of 3 was required to

remove the cadmium. On the basis of these experiments, it was calculated that the binding affinities for Cd^{2+} and Zn^{2+} at pH 7.0 are 10^{-15}M and 10^{-11}M respectively[42]. The affinities of other metals for thionein have not been accurately calculated. However, from metal and $[H^+]$ concentration competition experiments it is possible to draw some reasonable conclusions. The removal of Cu^{2+} and Hg^{2+} require pH values of less than 2[16,52]. Silver will displace copper, cadmium and zinc but not mercury, reacting with a 1:1 stoichiometry with the cysteinyl residues. Thus, the order of metal binding for thionein is $Hg^{2+}>Ag^{2+}>Cu^{2+}>Cd^{2+}>Zn^{2+}$. The relative affinities of other metals such as platinum, gold and bismuth are as yet unknown.

A variety of spectrophotometric techniques, including ultraviolet (UV)[15], circular dichroism (CD)[52], X-ray photoelectron spectrometry (XPS) and ^{113}Cd nuclear magnetic resonance (NMR)[53] have been used to investigate the metal binding sites of metallothionein. These studies have shown that Cd^{2+}, Zn^{2+} and Hg^{2+} are tetrahedrally coordinated. Hepatic $^{113}Cd/Zn$-metallothioneins induced by injecting animals with $^{113}Cd^{2+}$, contain zinc as a secondary cation and yield NMR spectra[54], which have several well dispersed resonances. These spectra can be simplified[51] by fully saturating $^{113}Cd/Zn$-Mt in vitro with ^{113}Cd and rationalized in terms of seven separate resonances which are produced by the seven ^{113}Cd atoms. The metals are arranged in two polynuclear clusters (Figure 4). Cluster A contains four metal atoms and 11 cysteines, and cluster B, three metal atoms and nine cysteines. The binding of metals to the two clusters is a very ordered (cooperative) process and there is a marked specificity and independence between the two clusters. Thus Cd^{2+} preferentially binds to cluster A[55], which under the right conditions can be isolated intact in a 31 residue, polypeptide (i.e. the α domain or fragment, Figure 4), obtained by subtilisin cleavage at Lys 30[43]. Zn^{2+}, on the other hand, has a greater affinity for the B cluster[51].

The binding of copper to thionein is a complex phenomenon which is not fully understood. The protein is particularly susceptible to oxidation and polymerization, giving rise to spurious metal and amino acid analyses[44,57]. Isolation of rat liver copper thioneins under anaerobic conditions yields metallothioneins with 9-11 Cu atoms and 18 cysteines/molecule[57]. This agrees with the generally accepted view[14] that the copper/cysteine ratio is close to 2. In anaerobically prepared samples the copper is ESR silent[52,57,58], strongly suggesting that the cation is either Cu^+ (diamagnetic) or Cu^{2+} which is antiferromagnetically coupled[52]. Oxidation of the copper atom yields an ESR signal[52] which is consistent with the concept that Cu^+ is converted to Cu^{2+}. This oxidation reaction, which occurs very readily in mammalian thioneins, is accompanied by disulphide bond formation between adjacent cysteines leading to aggregation[58] and a reduced affinity of thionein for the cation[59]. This can lead to a loss of metal and apparent discrepancies in the cysteine content of copper thioneins[44,58,59]. The binding of Cu^+ to thionein is apparently (like Cd^{2+} and Zn^{2+}) a cooperative process in which the B cluster is filled preferentially[60]. The exact coordination of Cu^+ in thionein is not known, although extended X-ray absorption fine structure

(EXAFS) spectra[14] suggest that Cu^{+} is trigonally liganded in contrast to the tetrahedrally coordinated Cd^{2+} or Zn^{2+} ions.

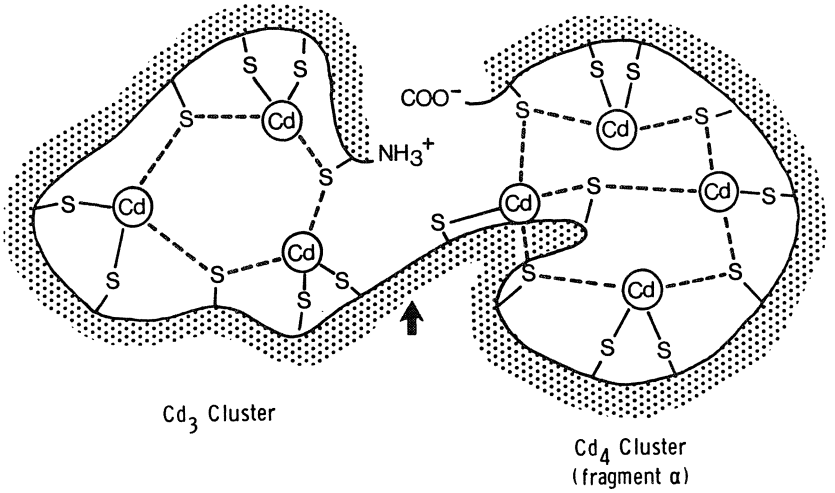

Figure 4 Metal cluster structure of metallothionein. (Redrawn from Winge et al.[50].)

It is clear from the above discussion that the in vitro binding of Cd^{2+}, Zn^{2+}, Cu^{2+} and other heavy metals to hepatic thionein is a very ordered process. The occurrence of two structured domains with different specificities for Cd^{2+}, Zn^{2+} and Cu^{2+} suggest that they may each have a specific function in heavy metal homeostasis. However, the metal compositions of metallothioneins isolated from the liver and kidneys of heavy metal exposed animals vary according to the length of exposure, age, sex and species. In this respect, factors other than the intrinsic metal binding properties of thionein may have a greater influence on the metal composition of metallothionein in vivo. It is perhaps significant that native rat liver $^{113}Cd/Zn$ isometallothioneins have quite different NMR spectra and exhibit 14-22 major resonances[54] (compared with the seven resonances in ^{113}Cd substituted metallothionein[51]). These clearly show that although Cd^{2+} ions are tetrahedrally coordinated, there are more than seven different binding environments. This may reflect some heterogeneity in the isoforms, or alternatively the binding of metals to thionein in vivo may be a more random event than the ordered cluster filling process seen in vitro. It should also be remembered that virtually all of the structural studies have been carried out on the liver proteins and there is little or no information on the kidney metallothioneins. Although the affinity of thionein for heavy metals is very high, other proteins in the cell may have a greater affinity for some metals than thionein. This is illustrated by reconstitution studies which have shown that zinc-thionein can reactivate zinc depleted enzymes such as carbonic anhydrase[61]. In essence the concentration of thionein bound metal in a particular cell is a complex relationship depending on the metal's affinity for thionein and other cellular components. This relationship varies with the individual metals (see

later) and is also influenced by the concentration of thionein in the cell. The latter is determined by the relative rates of thionein synthesis and degradation and this is discussed in the next section.

III. SYNTHESIS AND DEGRADATION OF METALLOTHIONEINS INDUCED BY:

IIIA. Cadmium

The endogenous levels of thionein in the liver and kidneys of adult animals are very low[22,29,31] and a parenteral injection of Cd^{2+} stimulates (induces) Cd-Mt synthesis in both these organs[13]. In this dosing regime the liver accumulates most of the cadmium[62] and within 1 h the hepatic uptake of the cation is complete. The majority of the Cd^{2+} is initially bound to high molecular weight (HMW) proteins and after a lag phase of 2-3 h thionein synthesis is initiated and the Cd^{2+} is gradually transferred to the newly formed protein. The synthesis and hence incorporation of radiolabelled amino-acids and Cd^{2+} into Cd-Mt is blocked by cycloheximide (a translational inhibitor)[27] and actinomycin D (a transcriptional inhibitor)[63]. This strongly suggested that thionein synthesis was controlled at the transcriptional stage in protein synthesis. Confirmation of this came from studies on Zn-Mt synthesis. Webb[27] and Bremner et al.[64] had shown that Zn^{2+} also induced thionein synthesis, and Cousins and his co-workers[65] demonstrated that the primary event was the increased production of a metallothionein specific poly (A^+) mRNA. The increase in metallothionein mRNA synthesis after a Cd^{2+} or Zn^{2+} injection is a short lived event that is maximal at 4-5 h and then slowly declines[65,66] to normal levels by 16-20 h. However, the initial induction seems to leave the cell in a primed state as Cempel and Webb[62] found that a second injection of Cd^{2+}, 24 h later, stimulates thionein synthesis without a lag phase. Furthermore, Squibb et al.[67] demonstrated that a second injection of Zn^{2+} 20 h after the first dose produced a five-fold greater stimulation of hepatic thionein synthesis, and multiple injections of Cd^{2+} lead to much higher levels of mRNA than after a single injection[68].

Less is known about the induction mechanism in the kidney. Originally Piscator[7] suggested that Cd-Mt was only synthesized in the liver and transported to the kidney. It is now recognized[13] that Cd-Mt is synthesized in the kidney as well as the liver. Initially it was reported[69] that the synthesis of the renal protein was not affected by actinomycin D. However, studies by Durnam and Palmiter[70], using recombinant DNA technology, have shown that renal thionein synthesis, like its counterpart in the liver, is regulated at the transcriptional stage in protein synthesis. In the mouse liver and kidney, Mt1-mRNA levels are increased after Cd^{2+} injection by a process which involves an increased rate of transcription[70].

There is continual turnover of the thionein moiety which has a half-life for Cd-Mt of 3-6 days[29,71-73] and it is likely that Cd^{2+} is released by degradation and continually stimulates mRNA levels to maintain the synthesis of thionein. The latter sequesters the

free toxic metal and overall the Cd-Mt content of the cell remains constant. In contrast, Zn-Mt synthesis after parenteral Zn^{2+} injection reaches a maximum at 18 h, thereafter the Zn-Mt as measured by the Zn^{2+} and ^{35}S-cysteine incorporation decreases[65,67] with a half-life of 18-20 h. Clearly, Zn^{2+} is not reincorporated into newly synthesized thionein and is removed from the liver. Thus the long biological half-life of Cd^{2+} in the liver and kidney is not due to the presence of metallothionein, but rather to the liver and kidneys' inability to excrete the cation.

The renal and hepatic proteins, although induced by Cd^{2+}, always contain other metals. In the rat, for example, the hepatic protein is a Cd:Zn-Mt whereas renal thionein contains Cu[29]. The ratio of thionein bound Cd to Zn or Cu varies with exposure. Thus with an increasing Cd^{2+} concentration there is a corresponding increase in the Cd:secondary cation ratio. Intriguingly, the turnover time of the thionein moiety also increases as the Cd:Zn or Cd:Cu ratio increases[29]. Thus the Cd^{2+} content of the thionein does not alter although the protein is turning over, but there is some confusion as to the fate of the secondary cation during this process. Chen et al.[71] reported that, after a single subcutaneous Cd^{2+} injection, hepatic thionein-bound Cd^{2+} reached a maximum at 4 days and remained constant for a further 7 days. In contrast the Zn^{2+} content also reached a maximum, but thereafter decayed at a rate similar to the turnover time of the protein. This suggests that Zn^{2+} is removed from the thionein as the protein is degraded, and is not taken up again by newly synthesized thionein. However, it is likely that this only occurs shortly after Cd^{2+} administration when a steady state between synthesis and degradation has not been achieved. In support of this idea, Riddlington et al.[75] injected animals with Cd^{2+} and then asssayed the hepatic Cd:Zn-thionein ratio at 1, 2, 3, 4, 5 and 6 months after injection, and found that the metal ratio remained constant. Thus in the Cd^{2+}-exposed animal, Zn^{2+} which is released from the degraded thionein is either not removed from the cell (in contrast to Zn-Mt) and hence reincorporated into new Cd:Zn-Mt or, alternatively, the presence of the latter elevates and maintains the Zn^{2+} concentration of the cell.

Current evidence favours the role of Cd:Zn-Mt maintaining cellular Zn[21,39,76,77], because Cd^{2+} uptake precedes that of Zn^{2+}, which only begins to increase in response to Cd-Mt synthesis. The time course experiments of Sugawara[39], for example, show that in the liver, after a Cd^{2+} injection, the synthesis of thionein, which binds the inducing metal and any "free zinc", is stimulated. The effect of "trapping" the zinc causes an influx of the cation from the serum, which in turn leads to more metallothionein synthesis but with a lower Cd:Zn ratio. The exact mechanisms underlying the increased liver zinc content are not understood. Winge et al.[76] maintain that excretion of zinc into the bile is blocked, probably by the "trapping" of free zinc by thionein. The increase in liver zinc can then be explained by the normal uptake of the cation from the serum. This, however, does not explain the transient decrease in zinc serum levels reported by Suzuki and Yamamura[77] after Cd^{2+} injection. This result implies that Zn^{2+} uptake after Cd^{2+} injection is stimulated or, alternatively, that the reverse transfer of

the cation to the serum is blocked, presumably in an analogous fashion, to the inhibition of excretion into the bile. Both the above mechanisms require the Zn^{2+} to be "trapped" in Cd-Mt, and this is not readily explained by the available physical chemistry data, which (as discussed above) show that Cd^{2+} has a greater affinity for thionein than Zn^{2+}. It is therefore difficult to envisage how the latter would actually displace the former cation. The problem is even more puzzling with the rat renal metallothionein which in the rat has Cu^{2+} as its secondary cation[29]. A possible explanation is that newly synthesized Cd-Mt has unoccupied binding sites which can trap Zn^{2+} or Cu^{2+}. This hypothesis is supported by the α and β domain metal binding studies (see previous section), which have suggested that the α domain is filled with Cd^{2+} in a cooperative fashion before the β domain is occupied. As discussed by Hunt et al.[51], the binding affinity of Cd>Zn>Cu for cluster A (α domain) and the reverse order applies for cluster B (β domain). However, one would expect that the Cd:Zn and Cd:Cu ratios to be close to 4:3 and 4:6[59] respectively, irrespective of the Cd^{2+} content of the tissue. Native ^{113}Cd:Zn-Mt (Cd:Zn ratio ≈ 2) exhibits an NMR spectrum which, although containing 15 separate resonances, can be interpretated as being due to a non-homogeneous but limited population of mixed-metal thioneins[78]. The Cd^{2+} and Zn^{2+} are not randomly distributed among the binding sites, but are preferentially bound to the 4 and 3 metal clusters respectively. Significantly, Nettesheim et al.[78] found that the NMR spectrum cannot be reproduced by replacing Zn with ^{113}Cd in vitro. However, mixtures of Cd_7-Mt and Zn_7-Mt undergo a hitherto unknown metal-exchange reaction to give a ^{113}Cd:Zn-Mt with the same NMR spectrum as the native metallothionein. These observations suggest that in vivo Cd_7-Mt and Zn_7-Mt are synthesized and then undergo metal dismutation reactions to give the observed Cd:Zn ratio. Nettesheim et al.[78] further suggest that the homeostatic role of Zn-Mt can be carried out only when Zn is bound to specific sites in the protein (i.e. the B cluster). Thus, when there is excess Cd-Mt in the cell, cadmium exchanges with the Zn in the B cluster. The cell's response is to synthesize more Zn-Mt until the metabolically active Zn binding sites are regenerated. It is suggested that this is optimally achieved when the Cd:Zn ratio is approximately 2. This is an attractive hypothesis, and may explain the results of Sugawara[39]. However in continual exposure experiments, which are more analogous to the chronic effects of cadmium, there is clear evidence that thionein Cd:Zn and Cd:Cu ratios are not approximately 2. For example, Sato and Nagai[79] gave repeated subcutaneous injections of 0.3 mg Cd^{2+}/kg b.w. to rats 6 days a week for 4 months. The liver Cd^{2+} concentration plateaued at about 380 μg/g after 12 weeks of dosing. In contrast, the Zn^{2+} content reached a maximum of 82 μg/g after 4 weeks and then slowly declined to 52 μg/g by the end of the experiment. The Cd:Zn ratio of thionein increased linearly up to the 9th week, after which there was a further non-linear increase in the ratio. In the kidney of the rat the Cu^{2+} content also increases and then plateaus, and this is accompanied by an increasing metallothionein Cd:Cu ratio (K. Cain and M. Webb, unpublished results). Autopsy material from horses and humans have shown that the kidney thionein con-

tains Zn^{2+} as the secondary metal and that the Cd:Zn ratio increases with the increasing renal Cd^{2+} concentration[80]. Elinder and Nordberg[80] estimate that the zinc content of thionein will not increase once the Cd^{2+} concentration in the human and equine renal cortex exceed 157 μg/g and 135-180 μg/g respectively. The uptake of zinc or copper into the liver or kidney in response to Cd-Mt synthesis is thus saturable and it is difficult to see how this can be related purely to the different binding affinities of the metals for clusters A and B. It is also important to reiterate that the binding properties of the clusters are based on in vitro studies.

The Zn^{2+} or Cu^{2+} content of Cd-Mt also presents another question in that thionein degradation presumably releases Cd^{2+} and Zn^{2+} or Cd^{2+} and Cu^{2+}, which may all be contributing to the induction of new metallothionein-mRNA. In this respect it is generally assumed that all the metals induce the same mRNAs and therefore thioneins are identical except for metal content. This is a difficult theory to prove as a variety of metals, stress and glucocorticoid hormones can all induce thionein synthesis[8]. Furthermore, in the case of the heavy metals it is difficult to distinguish between a direct induction effect and an indirect effect in which the toxic metal displaces intracellular Zn^{2+} or Cu^{2+}, the increased concentration of which induces metallothionein synthesis. It is important to understand the mechanisms that control the rate and extent of cellular thionein synthesis as this is the major factor in determining what proportion of the tissue Cd^{2+} content is rendered harmless by thionein binding (see later). Conventional techniques have given little insight into these problems, and it is only in recent years, with the advent of recombinant DNA technology, that significant progress has been made. Figure 5 shows a proposed model[81] for the induction of thionein synthesis in which the apothionein acts as a repressor on the promotor region of the gene. Induction takes place when a metal with a high affinity for thionein binds to the protein forming the metallothionein which is inactive as a repressor. The transcription rate of the metallothionein gene increases and mRNA and thionein synthesis is stimulated. Ultimately the level of thionein is sufficient to bind all of the inducing metal and the concentration of the apothionein rises and switches off the gene. Presumably in the long-term situation, where there is continual thionein turnover, the release of the metal by degradation would be sufficient to maintain the elevated level of mRNA synthesis. This scheme is supported by the fact that there is a marked initial uptake of the cation into the nucleus, the content of which then decreases at a rate proportional to the synthesis of metallothionein[82]. Immunohistochemical localization of intracellular metallothionein also supports this conclusion[34,83,84]. Metallothionein (or apothionein) is mainly localized in the cytoplasm of the hepatocyte, renal collecting duct and distal tubular epithelium in control animals. However, in Cd^{2+} injected rats thionein is located in both the nucleus and cytoplasm of the hepatocytes and proximal convoluted tubular epithelium. Thus, in Cd-Mt loaded tissues a large proportion of the protein is located in the nucleus and similar observations have been reported for Zn-Mt in the neonatal and newborn liver[84]. In this situation there are high levels of Zn^{2+} and Zn-Mt[8] and according to Panemangalore et

al.[84] the latter is predominantly located in the nucleus until 9 days post partum. At 11 days there are equal levels of thionein in the cytoplasm and at day 14 the metalloprotein is mainly found in the cytoplasm. Several aspects of the apothionein-repressor model need further investigation. For example, it is unclear as to how the apothionein can be transported from the cytoplasmic ribosomes to the nucleus without picking up "free Zn^{2+} or Cu^{2+}". In addition, subcellular fractionation studies[85] show that in the Cd^{2+}-exposed animal less than 5% of the Cd^{2+} is bound to the nuclei. Furthermore, in the study of Panemangalore et al.[84], isolated nuclei did not contain metallothionein, which is in complete contrast to the immunohistochemical staining results. A possible explanation for this discrepancy is that the cell homogenization and sub-cellular fractionation techniques lead to a rapid redistribution of the metallothionein to the cytosol.

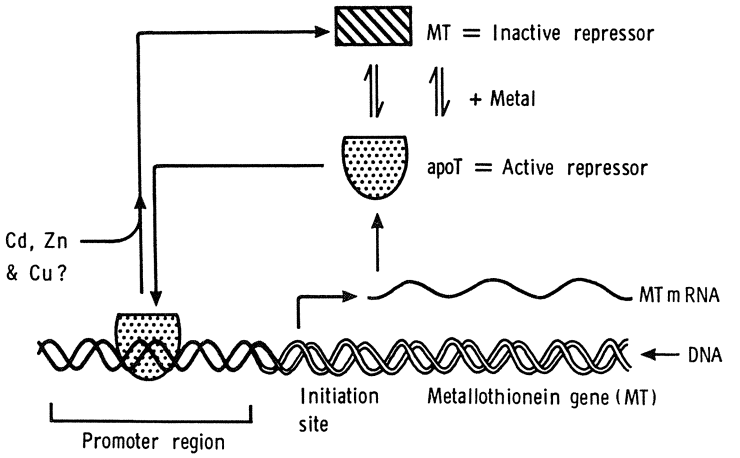

Figure 5 Proposed model for regulation of thionein synthesis. (Adapted from Karin and Richards[81].)

Our understanding of the mechanisms which control thionein synthesis is further complicated by the recognition that there are multiple metallothionein genes. In man, for example, there are two separate classes of gene which encode for the isometallothioneins[81]. Of the several Mt2 genes only one (Mt2a) is functional. The Mt1 class of genes are arranged in tandem and at least one (Mt1a) and possibly more of these genes are functional. Karin and Richards[81] have shown that although the Mt2a gene is induced to the same extent by Cd^{2+}, Zn^{2+}, Cu^{2+} and dexamethasone, the Mt1a gene is only fully induced by Cd^{2+}. This demonstrates that the two genes, and hence the synthesis of the isometallothioneins, are regulated differently, perhaps as a consequence of having separate functions in the cell. The latter concept is not new, and is supported by the fact that the two isoproteins have different rates of degradation[29] and that in the regenerating liver[86] Zn-Mt2 synthesis and accumulation is two-fold greater than Zn-Mt1. However, what is novel is the possibility that Mt1 is synthesized as a protective mechanism

against Cd^{2+} toxicity and Mt2 is involved in the homeostatic control[81] of Zn^{2+} and Cu^{2+}. Based on this hypothesis the ratio of Mt1 to Mt2 should reflect different metabolic demands. In the equine kidney, which apparently has four functional Mt1 genes[81], the predominant isometallothionein can be correctly predicted to be Cd-Mt1[24]. In the neonatal and regenerating[86] rat liver, Zn-Mt2 seems to be the predominant form and this isoform may be involved in cell proliferation[8]. However, in the Cd^{2+}-exposed rat the Mt1:Mt2 ratios are around 1.3 to 1.5 (see Refs. 29, 71, 74). So, although there is a greater concentration of Cd-Mt1, it is not markedly in excess of Mt2. This discrepancy may be due to the absence of multiple Mt1 genes in the rat, or because although induction may be metal specific, it is unlikely that the isometallothioneins themselves only bind specific metals. This can be illustrated by partially hepatectomizing the livers of Cd^{2+}-pretreated rats. Regeneration leads to increased Zn-Mt2 synthesis and concomitant re-distribution of Cd^{2+} (Figure 6). It is perhaps significant that Suzuki and Yamamura[77] have shown that, after the injection of Cd^{2+} and Zn^{2+}, the isometallothionein ratios in the liver and kidney change markedly with time. Clearly the synthesis of Mt1 and Mt2 are regulated by complex mechanisms in which the induction susceptibility of the isometallothionein gene is just one factor. Further research needs to focus on the differing isometallothionein ratios and their role in the toxic effects of cadmium.

IIIB. Mercury

The role of thionein in the toxicity of Hg^{2+} was suggested by Pulido et al.[16], who showed that thionein, isolated from human renal tissues (following the use of mercurial diuretics), contained levels of Hg^{2+} which were equivalent to the Cd^{2+} content on a μg/g basis. Competitive binding studies (see section IID) also showed that Hg^{2+} had greater affinity for thionein than Cd^{2+}. The inductive effect of Hg^{2+} on thionein synthesis in the liver and kidney is, however, quite different from Cd^{2+}. This is probably more a reflection of the organ distribution of Hg^{2+} than a specific difference in the mechanism by which the cation induces thionein synthesis. For example, Rothstein and Hayes[88] showed that Hg^{2+} is always deposited in greater concentrations in the kidney than in the liver. Furthermore, although the liver initially accumulates a significant content of Hg^{2+}, this is very rapidly cleared (less than 2% of the dose within 6 days is located in the liver). In contrast the kidney content continues to rise and by the fifteenth day 90% of the body burden is in the kidney. Thus the excretion of mercury from the kidney is very slow, whereas in other tissues (particularly the liver) elimination is rapid. It is therefore not surprising that Cherian and Clarkson[89] showed that after mercury vapour (Hg^0) exposure, the kidney deposition of Hg^{2+} (derived by oxidation) is very much greater than the deposition in the liver. Furthermore, 60% of the Hg^{2+} content of the kidney was located in the supernatant, 70% of which was bound to a heat stable, small

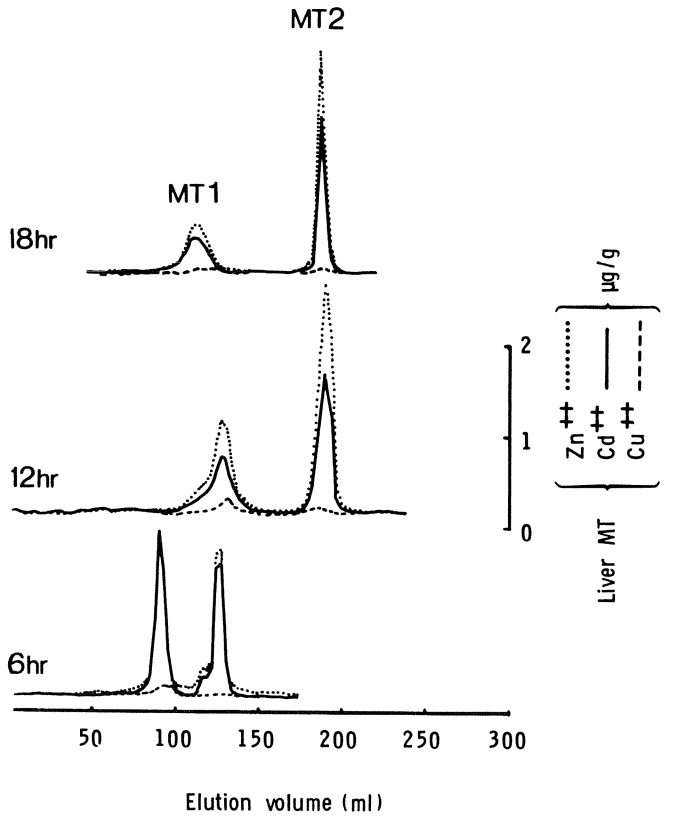

Figure 6 Anion-exchange elution profiles of cadmium isometallothioneins isolated from the regenerating liver. Rats were pretreated with 3 x 1 mg Cd^{2+}/kg b.w., s.c. doses on alternate days and left for 1 month before partial hepatectomy. (Previously unpublished results, K. Cain and B.L. Griffiths.)

molecular weight protein, which separated into two forms on isoelectro-focusing, characterizing it as a metallothionein. Hg-Mt was not detected in the liver, but Winge et al.[90] have reported hepatic Hg-Mt in rats injected subcutaneously with $^{203}HgCl_2$. The metalloprotein was shown to have two isoforms on anion-exchange chromatography and to contain Zn^{2+} as a secondary cation. The amino acid analysis of Mt2 induced by Hg^{2+} was very similar to that induced by Cd^{2+} and Zn^{2+}. Bryan and Hayes[91] have also reported the presence of hepatic Hg-Mt in the mouse after the administration of $HgCl_2$ in the drinking water. Thus there seems to be little doubt that Hg^{2+}, Cd^{2+} and Zn^{2+} induce the same thioneins. However, in both of these studies[90,91] the levels of Hg^{2+} and Hg-Mt in the liver were low, and the kidney content was not analysed. In this respect Piotrowski et al.[92] have shown that in rats given repeated doses of mercuric chloride (0.5 mg Hg^{2+}/kg on alternate days) the hepatic content of thionein bound ^{203}Hg is the same as that seen after a single dose[93]. Furthermore, using an in vitro ^{203}Hg binding assay, these workers were able to show that the hepatic thionein content of control and mercury exposed

rats were virtually the same. In contrast, the kidney content of Hg^{2+} and Hg-Mt increased with the time of exposure, eventually plateauing after 10 doses. It is therefore likely that the Hg-Mt which has been identified in the liver is nothing more than the binding of trace amounts of Hg^{2+} to endogenous zinc thionein (see also below).

The induction of renal metallothionein biosynthesis by Hg^{2+} is probably identical to that produced by Cd^{2+}. Shaikh and Smith[94] have shown renal Hg-Mt synthesis is blocked by cycloheximide and actinomycin D. More recently, Durnam et al.[70] have shown that Hg^{2+} is a potent inducer of mouse Mt1-mRNA synthesis in both the liver and kidney. In the liver, after the injection of 5 mg Hg^{2+}/kg, mRNA levels rose slowly, reaching a maximum at 24 h. In comparison, the Mt1-mRNA levels after Cd^{2+} (5 mg/kg) reached a slightly higher maximum at 6 h post injection. These results are surprising, because the Hg^{2+} content of the liver must be much less than that of Cd^{2+} and furthermore on a g ion basis there would be even less Hg^{2+}. This implies that Hg^{2+} is a far better inducer of Mt1-mRNA than Cd^{2+}. However, in the same experiment the thionein content was estimated by ^{35}S-cysteine incorporation, and on this basis there was an approximately 4-fold greater concentration of Cd-Mt than Hg-Mt. This would suggest that the Mt-mRNA induced by Hg^{2+} is not being efficiently translated. If this is the case, it must be confined to the liver, as in the kidney both Cd^{2+} and Hg^{2+} are almost equipotent in inducing mRNA synthesis and the amount of thionein synthesized[70]. Clearly, further work is required and it is unfortunate that Durnam et al.[70] did not equate the Mt1-mRNA levels with the actual concentrations of the cation in the two tissues. This type of comparison would provide very useful information on the relative potency of the various metals as thionein inducers.

In any event, it is clear that Hg^{2+} can induce Mt1-mRNA synthesis in the rat kidney, and this is an important finding as there is some confusion about the identity of the renal Hg^{2+} binding protein. This confusion stems from the behaviour of these proteins on anion-exchange chromatography[95] and gel electrophoresis[96]. In the rat, Hg-Mt has been identified by gel filtration on Sephadex G-75 and contains copper as a secondary cation[92-96]. This partially purified protein is separated into at least four Hg/Cu containing proteins on gel electrophoresis[95]. Using anion-exchange chromatography Webb et al.[95] isolated at least five Hg/Cu proteins. Peak B, the major Hg^{2+}-containing protein, seems to be analogous to a small diffuse peak which is often seen eluting in front of Mt1 after Cd^{2+} induction (see Figure 2, and Refs. 96, 97 for comparison). This peak seems to be derived from Mt1 and may be caused by the Cu^{2+} content of the protein, making the latter more susceptible to oxidation and chemical degradation. In this respect it is possible[96,97] that Hg/Cu thioneins are more unstable, and even under anaerobic conditions there may be considerable chemical alteration and charge differences which show up on anion-exchange chromatography. It is therefore difficult to assess what percentage of the total kidney Hg^{2+} is bound to thionein, and the involvement of the latter in the nephrotoxic effects of mercury. In addition very little is known

about the turnover of Hg-Mt. On the basis of ^{35}S-cysteine incorporation and disappearance from Hg-Mt, Durnam et al.[70] have suggested that the rate of degradation of Hg-Mt in the kidney is similar to that of Cd-Mt. It is not known, however, if the Hg^{2+} which is released by degradation is capable of further stimulating Mt synthesis. The early studies of Rothstein and Hayes[88] clearly show that Hg^{2+} is gradually cleared from the kidney, and it is likely Hg-Mt must also decrease. This result has been confirmed by Webb et al.[95], who also showed that the total and thionein bound Hg^{2+} content of the rat kidney decrease in parallel.

IIIC. By other heavy metals and the involvement with copper

Bismuth, gold, platinum, lead and silver are all known to bind to thionein (see section IID) in vitro. However, the evidence that they can induce thionein synthesis is not so clear. In the case of silver, Winge et al.[90] have shown that this metal induces a metallothionein in the liver in which Zn^{2+} is the predominant cation. In the kidney, however, silver apparently does not induce thionein synthesis[96] and for the purposes of this review need not be discussed further. Lead is a potent nephrotoxin and binds to thionein in vitro by displacement of zinc[47]. However, recent studies[98] have shown that in vivo soluble lead is bound mainly to a 63,000 M_r component with only a small proportion bound to a 11,500 M_r protein. The latter is not increased by Cd^{2+} pretreatment and Pb^{2+} does not appear to be an inducer of metallothionein synthesis. There is therefore no evidence that thionein has any biological role to play in Pb^{2+} nephrotoxicity.

Cisplatin (cis-dichlorodiamine platinum) is a potent antitumour drug with nephrotoxic side effects which are dose dependent and cumulative[99]. The possibility that thionein synthesis could ameliorate the toxic symptoms of cisplatin was suggested by Bakka et al.[100]. These workers demonstrated that Cd^{2+} resistant cell lines were also resistant to cisplatin. The Cd^{2+} and cisplatin resistance was attributed to increased intracellular concentrations of metallothionein which bound the toxic metals. However, there was no evidence that cisplatin induced metallothionein synthesis in the cell lines. Zelazowski et al.[48] subsequently showed that cis and transplatin bind to metallothionein both in vivo and in vitro by Zn^{2+} replacement. However, both the cis and trans isomers failed to induce metallothionein synthesis in either the liver or kidney. Cis and transplatin probably bind to thionein by interacting with -SH groups of the protein in an analogous manner to other alkylating compounds (and heavy metals) which react with thiols. In this respect platinum binding to thionein is fortuitous and is of limited significance in the nephrotoxic effects of the cation, unless the pre-existing levels of thionein in the kidney are high.

The use of bismuth-containing salts in the treatment of peptic ulcers, and after colostomy and ileostomy can have nephrotoxic side effects[101]. Piotrowski et al.[96] have shown that bismuth accumulates predominantly in the rat kidney (approximately 55% of the body burden) where it is bound to a metallothionein-like protein (designated renal metal binding protein, RMBP). The syn-

thesis of the latter is apparently inducible, as Szymanska et al.[102] showed that after one injection bismuth is bound to the HMW proteins of the cytosol, whereas after multiple injections the majority of the metal is bound to the RMBP fraction. Bismuth, however, does not seem to be a very good inducer of this protein in the liver[96], presumably because of its poor uptake into the liver (compare with Hg^{2+}). Although the identification of the RMBP as a metallothionein has not been unequivocally proven, there is sound evidence to justify the statement that bismuth induces metallothionein. Firstly, Zelazowski et al.[103] have shown that on polyacrylamide gel electrophoresis the Bi-RMBP separates into three separate proteins all of which contain bismuth. In the same study Cd-Mt and Hg-Mt also separated into three components. Secondly, antisera raised against the Bi-RMBP cross-reacts with the renal Hg- and Cd-Mt. In addition, the bismuth-binding proteins also contain (like Hg- and Cd-Mt) copper as a secondary cation, the renal concentration of which increases after bismuth administration[104]. There is thus reasonable evidence that bismuth administration stimulates metallothionein synthesis in the kidney. However, there is no absolute evidence that bismuth is itself an inducer of thionein synthesis. In this respect it is significant that the effects of bismuth on thionein synthesis have been studied only in the rat. The latter is unusual in that copper is the secondary cation bound to thionein. Furthermore other studies, particularly on gold (see below), suggest that metal binding to thionein is a consequence of disturbances in the metabolism of copper and it is the latter which is the true inducer of thionein. Such a system may also be operating for bismuth.

The use of gold salts in the treatment of rheumatoid arthritis is well established, although there are undesirable nephrotoxic side effects[104]. Gold, whether given as $NaAuCl_4$[105,106] or sodium aurothiomalate[107,108] accumulates predominantly in the subcellular organelles and cytoplasm of the kidney. In the rat the cytoplasm accounts for approximately 20% of the total tissue burden of the metal and only 30% of this soluble gold is bound to low molecular weight binding proteins[105,107]. The concentration of the latter, however, increases with repeated administration and the synthesis of the protein is blocked totally by cycloheximide and partially by actinomycin D respectively[105]. The Au-binding low molecular weight proteins can be separated into at least three, probably four, isoforms[105] on anion-exchange chromatography, and although the evidence is not unequivocal there are good reasons[107] to believe that they are all Au-containing metallothioneins. The complex elution patterns of these proteins on anion-exchange chromatography are almost certainly due to the fact that Au treatment leads to an increase in renal copper content in both the tissue and metallothionein fraction. Indeed, the thioneins contain more copper than gold and by analogy to Hg/Cu-Mt such proteins are inherently unstable and difficult to purify (see Section IIIB).

The involvement of copper in the induction of Au-Mt, however, is even more complex. Studies on different species[105,107] have shown that the rat and guinea-pig accumulate Au/Cu-Mt, whereas hamster, mouse and rabbit do not[109]. In the latter three species there is no increase in renal copper and furthermore Cd-Mt

obtained from these animals contains Zn^{2+} as a secondary cation[45]. It is therefore likely that the induction of Au/Cu-Mt is a result of the increase in copper in the kidneys of the rat and guinea-pig and it is improbable that Au is the primary inducing metal.

The degradation of Au-Mt has not been studied in detail. Sharma and McQueen[107] showed that the level of Au in the HMW and metallothionein fractions of the cytosol slowly decrease with time after a single injection (s.c.) of aurothiomalate. Mogilnicka and Webb[110] demonstrated that in female-rats after Au(I) or Au(III) injection the gold content of the kidney decreases exponentially with half-lives of 23.9 and 32 days respectively. On the other hand the half-lives of the metallothionein bound gold are slightly shorter at 29.6 and 23.9 days respectively. Thus the thionein bound gold content is very closely correlated with the total kidney content. Clearly, in this situation (unlike Cd^{2+}) Au released by thionein degradation is not re-bound by newly synthesized thionein. However, the Cu content of the Au-Mt does not decrease in parallel with the loss of gold. Indeed, after Au(III) administration, not only does the thionein bound Cu remain high, but after 60 days it begins to increase. Thus the thionein and Au content of the kidney appear to be independent of one another, and are only linked indirectly by the effects on copper metabolism.

IV. THIONEIN SYNTHESIS AS A PROTECTIVE MECHANISM AGAINST THE NEPHROTOXICITY INDUCED BY HEAVY METALS

It can be seen from the previous sections that the high affinity for metals and induceability of thionein make it an ideal defence mechanism against heavy metals. However, it is important to stress that thionein is an intracellular protein and any potential protective function must be restricted to cations which have entered the cell. This is an important proviso as many metals can exert toxic effects extracellularly by interaction with the cell membrane[111]. Thus, although thionein synthesis may be sufficient to provide an effective defence against the intracellular toxicity, this may not be enough to save the cell from death, due to membrane damage. It is also clear from the previous discussion that thionein synthesis can provide an effective defence only against Hg^{2+} and Cd^{2+} (and in certain situations Zn^{2+} and Cu^{2+}).

IVA. Cadmium

Cd^{2+} is a very potent in vitro inhibitor of enzymes and biochemical pathways[112,113]. Thionein has a very high affinity for Cd^{2+} and as yet there is no evidence that "intact Cd-Mt" is an inhibitor of enzymes or biochemical pathways. Indeed, in metal donation experiments Cd/Zn-Mt is equally as good as Zn-Mt at reactivating zinc depleted apo-carbonic anhydrase[114] and there is no transfer of Cd^{2+} to the enzyme. It is therefore a reasonable assumption that thionein bound Cd^{2+} is a non-toxic form of the heavy metal. It follows also that in vivo the toxic species is "free Cd^{2+}" or non-

thionein bound cadmium. In Figure 7 a model scheme to explain the toxicity of Cd^{2+} is shown, and it is obvious that the concentration of "free Cd^{2+}" is determined mainly by the level of thionein in the cell. In this section the effect of the latter on Cd^{2+} toxicity is discussed.

IVA1. The involvement of thionein in acute cadmium nephrotoxicity

After a parenteral injection of Cd^{2+}, most of the metal is accumulated in the parenchymal cells of the liver[62,115] and acute liver damage results if the dose is high enough. Thus, after acute injections of Cd^{2+}, significant renal concentrations of the cation are only obtained at dose levels which are not only hepatotoxic but also damaging to other organs[116]. It follows that acute injections of Cd^{2+} do not provide an ideal experimental model for evaluating the protective role of metallothionein in nephrotoxicity.

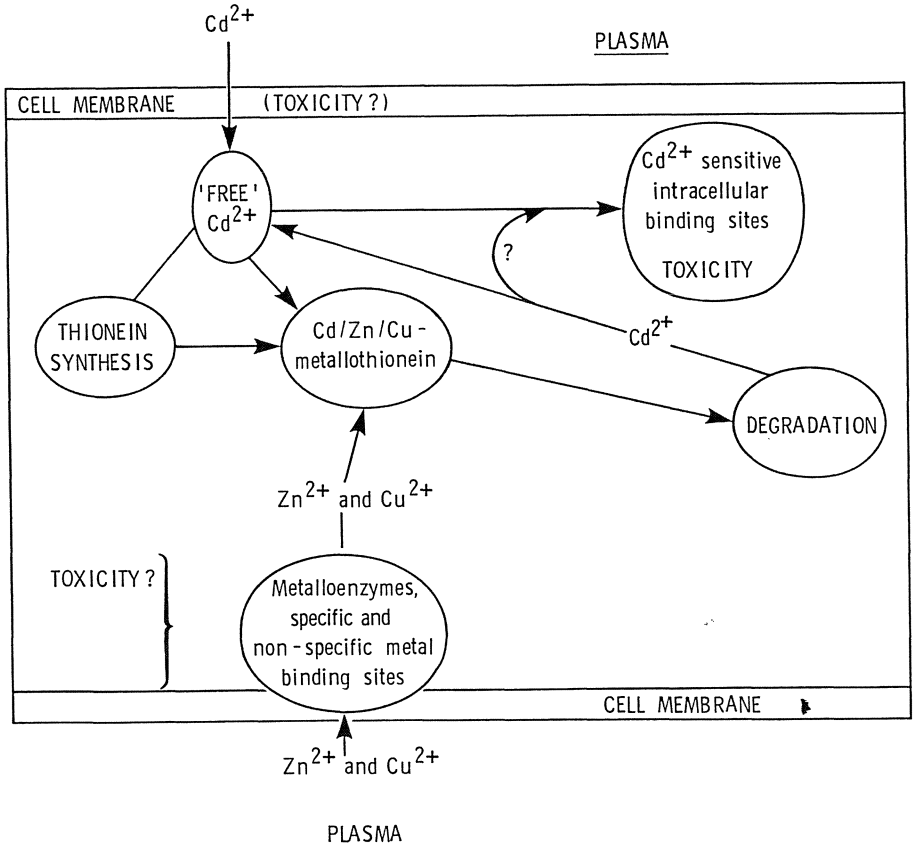

Figure 7 Model scheme to explain Cd^{2+} toxicity in liver and kidney.

However, the organ distribution of Cd^{2+} after acute injection can be markedly altered by the simultaneous administration of the metal and a suitable chelating agent (e.g. EDTA, 2,3-dimercaptopropanol, penicillamine, dimercaptosuccinic acid and cysteine). The effects of the latter on renal and hepatic uptake of cadmium are complex[117-124], varying with the relative doses of metal and chelating agent.

Table 3 Effect of L-cysteine on the kidney and liver uptake of cadmium 1 after parenteral injection (from Murakami and Webb[121])

Time (h) after injection	Cd^{2+} concentration (μg/g wet weight)			
	Liver		Kidney	
	Cd^{2+} alone	Cd^{2+} plus L-cysteine	Cd^{2+} alone	Cd^{2+} plus L-cysteine
1.0	4.0	1.5	0.7	21.3
2.0	5.2	2.5	0.7	19.5
4.0	7.2	3.3	0.9	18.0
24.0	10.7	5.3	1.7	16.2

Rats were given either Cd^{2+} (1.12 mg/kg b.w. s.c.) alone or together with L-cysteine (5 mmol/kg b.w. i.p.)

The appropriate combination of metal and complexing agent can change the organ and the cellular distribution[125] of Cd^{2+}. As shown in Table 3, Cd^{2+}, when given simultaneously with a 500-fold molar excess of L-cysteine, is preferentially taken up by the kidney. In this study[121], the renal cadmium concentration was maximal (21-22 μg Cd^{2+}/g) at 4 h, and this was accompanied by extensive and progressive renal tubular damage. The latter was largely restricted to the straight proximal tubules (S_3) located in the outer stripe of the outer zone of the medulla. The damage to the cells which was observed at 1 and 4 h, by electron and light microscopy, included cytoplasmic vesiculation, irregularly shaped nuclei and swollen mitochondria. At 24 h the lesions had progressed to produce cellular necrosis and karyorrhexis. Autoradiography with $^{109}Cd^{2+}$ confirmed that the highest concentrations of the metal were correlated with the histological lesions. Appreciable thionein synthesis was also detected between 2 and 7 h, but was insufficient to prevent the development of the toxic syndrome. Thus kidney damage can be caused by much lower concentrations of Cd^{2+} than are needed to produce the chronic effects. In chronic Cd^{2+} toxicity and after Cd-Mt administration, the damage is localized in the S_1 and S_2 regions of the proximal tubules[126,127]. In this respect the Cd^{2+} + cysteine injection regime

does not provide an exact model of the effects of chronic Cd^{2+} exposure, although it is likely that the underlying biochemical events are similar. Irrespective of this, it is clear that in the kidney of the unexposed animal, metallothionein synthesis cannot provide a complete defence against sudden influxes of Cd^{2+}. This is perhaps a not unexpected finding, because as discussed previously (see section IIIA) there is a "lag phase" before thionein synthesis is initiated, and during this time the renal cadmium uptake is nearly complete[121].

The protective effect of thionein must therefore be confined to those situations in which thionein synthesis is already preinduced. This would then provide the cell with two possible lines of defence. The initial protection would be provided by the preexisting thionein scavenging incoming cadmium, and the second defence would come from the ability of the cell to produce more thionein without a "lag phase" between synthesis and cellular uptake of the cation.

These possibilities have been investigated by a number of workers[128-131] who have studied the protective effect of pretreatment with Cd^{2+} or Zn^{2+} against a subsequent challenge dose of cadmium. Suzuki and Yoshikawa[129], for example, demonstrated that in Cd pretreated rats the liver accumulates more of the cation which binds to the Cd/Zn-Mt by zinc displacement. They postulated that as a result the liver effectively immobilizes a greater percentage of the injected cadmium and thereby decreases the content and toxicity of the cation in other organs. Studies by Probst et al.[130] showed that in mice a range of Cd^{2+} pretreatment doses produced dose-dependent increases in the hepatic Cd-Mt content and proportional decreases in acute cadmium toxicity. These suggested that a threshold concentration of hepatic metallothionein must be exceeded in order to provide adequate protection against acute doses of cadmium. This and the studies described above support the concept that elevated levels of thionein in the liver (and perhaps the kidney) can provide an effective defence against normally toxic doses of cadmium. However other findings cast some doubt on this conclusion. Webb and Verschoyle[132], for example, demonstrated that the protective effect varies according to the time elapsed between pretreatment and challenge doses, and appears to be independent of the thionein content. However, a greater percentage of the lethal cadmium dose is accumulated in the liver of the Cd-pretreated animals (see Suzuki and Yoshikawa[129]) than in control animals. In contrast, the uptake of Cd^{2+} into the heart, pancreas, spleen and kidneys is unaffected, although in the latter a greater proportion of the tissue metal content is bound to the metallothionein fraction of the cytosol. Thus, the increased hepatic uptake of cadmium in pretreated animals has no effect on the uptake of the metal into other organs. Indeed it is unlikely that the concentration of thionein in the cell has any great influence on the uptake or retention of cadmium. This can be illustrated in the neonatal rat, the liver of which has a very high thionein concentration[133-135]. However, the uptake of Cd^{2+} is not related to the concentration of pre-existing thionein[134,135]. Similarly, in the kidney of the 7-day-old and 28-day-old rat the thionein concentrations are the same, and yet the initial renal concentration of

Cd^{2+} in the neonate after a parenteral injection is half that of the adult[134]. Furthermore, in spite of the high hepatic thionein concentration, the newborn rat is more susceptible than the adult to the lethal effects of a cadmium injection. In contrast, immature rats are more resistant to the testicular toxicity of cadmium than are adult animals[136]. This is in spite of the fact that the testis of the newborn rat accumulates a much higher concentration of cadmium than the adult. Furthermore, the thionein content of the testis of the immature and adult rat are the same, and thus the susceptibility of this organ to an acute dose of cadmium is not affected by the thionein content. In conclusion, the protective effect of high levels of endogenous metallothionein in the various organs of the body is of minimal significance against acute toxic (and therefore high) doses of cadmium.

IVA2. Role of thionein in chronic cadmium toxicity

Renal damage is not a normal response to a single acute dose of cadmium as the metal accumulates predominantly in the liver while kidney uptake is small. In the chronically exposed animal, however, the liver/kidney distribution can be quite different. This is illustrated in Table 4, and it is accepted that in both man and animals which are exposed to cadmium in the diet it is the kidney which ultimately accumulates the highest concentration of the cation. The consensus is that this accumulation is not accompanied by progressive renal damage, and an apparently critical concentration has to be exceeded to initiate toxicity. The concept of the critical concentration has been reviewed recently by Friberg[3], who stresses that the idea of a discrete critical level of 200 μg Cd^{2+}/g cortex for man and animals, at which concentration renal damage begins, is too simplistic as it does not take into account individual biological variation. Nevertheless, most evidence supports the concept that the kidney can accumulate large concentrations of cadmium without damage, and then at a certain concentration (between 100 and 200 μg Cd^{2+}/g cortex) nephropathy is initiated. As discussed previously, the toxic species is almost certainly non-thionein-bound cadmium ("free cadmium") and according to Nomiyama and Nomiyama[137] it is the critical concentration of the latter which is important.

The build up of toxic levels of free cadmium can be explained by either one or both of the following:

1. In the Cd^{2+}-exposed animal a small percentage of the Cd^{2+} in the kidney (or liver) is always "non-thionein-bound", and if the renal concentration is high enough then the absolute concentration of "free Cd^{2+}" will be in excess of the toxic threshold.
2. The second hypothesis is more complex and suggests that thionein synthesis is saturable. Below the "critical level" thionein production can cope with both the incoming cadmium and also the metal which is released by degradation. At or around the "critical level" thionein synthesis is saturated and "free Cd^{2+}" levels increase and produce toxicity.

An alternative hypothesis[138] suggests that thionein degradation can exceed synthesis, thus releasing high concentrations of non-thionein-bound cadmium, but there is no direct evidence that thionein synthesis is saturable at or around the critical level. As discussed by Webb[13], acute injection studies show that both the liver and kidney have a large capacity for thionein synthesis. In these situations the "free Cd^{2+}" concentration must be much greater than that produced in chronic feeding experiments where the only source of the metal is from the diet and metallothionein degradation. There is also no evidence that thionein degradation can exceed synthesis. Indeed the turnover studies discussed in section IIIA demonstrate that it is the metal content of the thionein which determines the rate of degradation. Thionein degradation studies in older animals (K. Cain, unpublished results) also indicate that the protein's turnover is not significantly affected by old age. All in all there is no conclusive evidence that there are alterations in thionein synthesis or degradation at the critical level which produce toxic increases in the "non-thionein-bound cadmium" levels.

Table 4 Organ distribution of Cd^{2+} after various types of dosing (Cd^{2+} μg/g of tissue)

		Chronic dosing by			
Acute intravenous injection[a]		Subcutaneous injection[b]	Feeding[c]	Drinking water[d] 10ppm	100ppm
Kidney	4.9	160	78	12.2	100
Liver	15.7	350	23	4.2	78

a 1.6 mg Cd^{2+}/kg b.w. into male rats[62] and liver and kidney cadmium levels determined 2-6 h later.

b 0.5 mg Cd^{2+} b.w. 6 days a week for 10 weeks[126].

c 100 ppm Cd^{2+} in the diet for 35 weeks[79].

d Rats given cadmium in drinking water as indicated, and values refer to concentrations after 37 weeks[126].

It is more likely that the concentration of "free cadmium" is dependent only on the total Cd^{2+} content of the kidney. However, the evidence for this conclusion is equivocal, and much of the uncertainty is directly related to experimental problems in achieving the "critical level" and measuring the "non-thionein-bound cadmium". Ideally, the "critical level" should be achieved with animals fed on cadmium supplemented diets, as this is more analogous to human exposure. In this experimental model Cd^{2+} absorption is low, and the effects on other organs can be considered to be minimal as it is the kidney which accumulates the highest concentration of the metal. However, with this dosing regime it

takes a long time to attain the critical concentration. The alternative is to repeatedly inject small quantities of Cd^{2+} over a period of time. This experimental approach was pioneered as early as 1960 by Bonnell et al.[139] who showed that repeated i.p. dosing of cadmium (0.75 mg Cd^{2+}/kg b.w., thrice weekly) led to very high levels of the metal in the liver and kidney. In both organs the Cd^{2+} concentrations were maximal after 3-4 months, at approximately 350 μg/g and 250 μg/g respectively. Histologically the renal damage was not observed until the 4th month. Thereafter, as dosing continued the number and severity of the renal lesions increased. However, during this time the kidney and liver cadmium concentrations declined slightly, demonstrating that in both organs the cadmium uptake was saturable. Thus, repetitive dosing apparently can be used to reproduce the chronic nephrotoxic effects of cadmium, and a number of workers have used similar experimental regimes to study the role of "non-thionein-bound cadmium" in the toxic process.

For example, Nomiyama and Nomiyama[137] gave subcutaneous injections of cadmium (0.5 mg Cd^{2+}/kg b.w.) to rabbits 6 days a week. At intervals, total thionein and "non-thionein-bound cadmium" concentrations in the liver and kidney were determined and compared with renal function disorders as measured by changes in the urinary metabolites. Between 0 and 4 weeks the renal cortex concentration rose rapidly to 240 μg Cd^{2+}/g and then more slowly to reach a maximum of 425 μg Cd^{2+}/g at the end of 14 weeks. In contrast, "the non-thionein-bound" metal attained a maximum of 75 μg Cd^{2+}/g by the 4th week and thereafter remained constant until the 14th week, before decreasing rapidly. The maximum "non-thionein-bound cadmium" concentration corresponded with the onset of renal disorders as determined by proteinuria, aminoaciduria, low molecular weight proteinuria and increased excretion of alkaline phosphatase. The subcellular distribution of the "non-thionein-bound-cadmium" was not determined, although gel filtration chromatography showed that at 3-4 weeks the cytosolic cadmium was not only bound to thionein but also to HMW proteins and low molecular weight (LMW) components. Similar results have been reported by Suzuki[140] and Sato and Nagai[141]. The latter workers found that in the liver and kidney there was a linear relationship between metallothionein bound and total Cd^{2+} content. However, binding of the cation to other cytosolic fractions (i.e. HMW and LMW components) was not observed until the tissue levels of cadmium had reached approximately 100 μg Cd^{2+}/g. These findings suggest that the distribution pattern of cadmium in the cytosol changes when the kidney content is high.

In this respect the chemical form of cadmium in the other subcellular fractions may be important. Most studies have isolated the various subcellular fractions by conventional centrifugation techniques, and a typical example is shown in Table 5, which demonstrates that 80% or more of the tissue cadmium is located in the cytosol. The accuracy of subcellular distribution studies is influenced by the homogenization and centrifugation methods. These are usually never 100% successful and some unbroken cells will pellet with the nuclei (cell debris) pellet. In the case of the centrifugation steps, further errors can be introduced as "cytosol"

is trapped in the pellets. The extent of these errors may be considerable, as Colluci et al.[142] have shown that the latter

Table 5 Subcellular distribution of cadmium in kidney and liver 1 taken from rats fed for two years on 50 ppb $^{109}CdCl_2$ in the drinking water and 11 ppb Cd^{2+} in the diet

Subcellular fraction	Intracellular distribution, percentage of total homogenate	
	Liver	Kidney
Nuclei[a]	5.0 ± 1.45	3.9 ± 1.33
Mitochondria[b]	7.5 ± 0.6	7.0 ± 0.71
Lysosomes[c]	3.3 ± 0.7	3.0 ± 1.4
Microsomes[d]	2.6 ± 0.3	2.8 ± 0.4
Cytosol[e]	81.6 ± 1.7	88.3 ± 4.3
Metal content (ng Cd)	63.5 ± 6.7	217.0 ± 22.2

Data taken from Sabbioni et al.[85], organs homogenized in 0.25 mmol/l sucrose, 10 mmol/l HEPES, pH 7.4.

a 700 g x 10 min fraction.
b 900 g x 15 min fraction.
c 2,5000 g x 25 min fraction.
d 10,5000 g x 110 min fraction.
e 105,000 g supernatant.

fractions are contaminated with the cellular supernatant and the amount of soluble Cd^{2+} is thus underestimated. This is a significant observation, as most studies have demonstrated that about 90% of the soluble (i.e. 105,000 **g** supernatant) cadmium is recovered in the metallothionein fraction (see Refs. 39, 78). Therefore if in the kidneys of chronic Cd^{2+}-exposed animals all of the metal is soluble, then the cystolic "non-thionein-bound cadmium" mentioned earlier may be the true toxic species. However, this is as yet unproven, as the quantitation of the "free Cd^{2+}" is very difficult. In Nomiyama and Nomiyama's experiments[137], "non-thionein-bound cadmium" in the cytosol was measured by a cadmium/saturation/competition method. This method was evaluated by Onsaka and Cherian[143], and involves saturation of all the thionein metal binding sites with excess cadmium. Any "free" and "non-thionein-bound cadmium" is removed by treatment with haemoglobin. Heat precipitation and centrifugation are then needed to remove the Cd-haemoglobin complexes, and the supernatant is used to assay the Cd-Mt content. This method is quick and its

sensitivity can be improved[144] by using $^{109}Cd^{2+}$. However, the method has only been used on cytosol preparations[137,143,144] and the possible inaccuracies introduced by incomplete homogenization and trapping of cytosol in pellets remains. In this respect, Waku[145] gave rats drinking water containing 250 ppm Cd for 12 months, and fractionation of the livers and kidneys showed that 90% of the tissue Cd^{2+} was present in the cytosol, 3-5% and 5-7% in the mitochondrial and microsomal fractions respectively. The Cd^{2+} content of the particulate fractions was not removed by repeated washing. However, treatment of the mitochondrial and microsomal fractions with 1% deoxycholate solubilized 98% of the particulate Cd^{2+}, the major proportion (89-94%) of which was characterized as metallothionein by Sephadex G-75 chromatography and high 250 nm absorbance. The remaining Cd^{2+} was bound to the HMW proteins eluting in the void volume of the column which is consistent with similar studies on the cadmium content of mitochondria and lysosomes, as reported by Sato and Nagai[141]. Waku[145] has attempted to evaluate the toxicological significance of these Cd-HMW complexes by preparing the latter in vitro. This complex was prepared in vitro by incubating Cd^{2+} with liver microsomes and 1% deoxycholate, and isolating the Cd-HMW proteins by Sephadex G-75 chromatography. The isolated Cd-protein complexes were then tested for their inhibitory potency against alcohol dehydrogenase (an -SH containing enzyme). Increasing the Cd^{2+}:protein ratio of the complexes increased the inhibitory potency of the complex and enzymic activity was inhibited 75% at a Cd^{2+} concentration of 7.16×10^{-5}M. Waku[145] calculates that if the kidney contained 250 μg Cd^{2+}/g this would correspond to 2×10^{-3}M Cd^{2+}, and if only 1% of this were bound as Cd-HMW complexes this would be equivalent to 2×10^{-5}M Cd^{2+} and therefore potentially toxic. This is an interesting speculation; however if it were true it should be possible to detect enzyme inhibition in key organelles, such as the mitochondria. The bioenergetic pathways of the latter are particularly sensitive to micromolar concentrations of Cd^{2+} (see Webb[146] for review). However, as shown in Table 6, there is no demonstrable inhibition of mitochondrial phosphorylation even though the mitochondria contain 2 ng ion Cd^{2+}/mg of protein. The latter, if it were "free Cd^{2+}" would cause marked inhibition of succinate and NADH linked ATP synthesis. As shown in Table 6 it does not; and the only noticeable difference is that in vitro the mitochondria from Cd^{2+} treated animals are more resistant to endogenous Cd^{2+} inhibition (Figure 8), probably because the metal is bound to the endogenous mitochondrial metallothionein. The lack of mitochondrial inhibition is to be expected because, according to Waku[145], less than 10% of the mitochondrial Cd^{2+} would be "non-thionein bound", which reinforces the idea that the cellular concentration of "non-thionein-bound cadmium" is never very high. This conclusion is supported by the findings of Minkel et al.[147], who maintain that the Cd^{2+} found in the HMW proteins and LMW fractions is an artefact of the preparation procedures. Under aerobic conditions, metals are displaced due to metallothionein oxidation and binding of thionein to HMW proteins and particulate fractions of the cell. The addition of 5 mM mercaptoethanol to the homogenization and chromotographic buffers prevents thionein

oxidation and the formation of inter-protein disulphide bonds. As a result, a greater proportion of the tissue Cd^{2+} is bound to metallothionein and Minkel et al.[147] estimate that over 95% of the tissue Cd^{2+} is thionein-bound. The sensitivity of Cd-thionein oxidation

Table 6 The effect of 100 ppm Cd^{2+} in the diet on in vitro rat kidney mitochondrial function

Time on diet (wks)	Kidney Cd^{2+} content		Oxidative phosphorylation (nmol ATP/mg protein/min)			
			Succinate		Pyruvate/malate	
	Whole tissue (μg/g net weight)	Mitochondria (ng ion/mg protein)	Control	100 ppm Cd^{2+}	Control	100 ppm Cd^{2+}
25	87.8 ± 9.7	1.5	381 ± 33 (0%)*	346 ± 11 (19%)	143 ± 24 (0%)	143 ± 5 (100%)
50	139 ± 9.4	2.0	318 ± 37 (0%)*	298 ± 52 (28%)	131 ± 30 (0%)	156 ± 13 (100%)
75	165.9 ± 7.0	1.7	296 ± 24 (0%)*	319 ± 22 (22%)	91 ± 8 (0%)	149 ± 14 (100%)

Rats were fed on a diet containing 100 ppm Cd^{2+}, and at the indicated times, animals were killed and mitochondria from the kidneys isolated. Oxidative phosphorylation was measured with the indicated substrates by measuring ATP synthesis with a glucose-hexokinase trap system. Cd^{2+} contents in the whole kidney and mitochondria were measured with flame and flameless atomic absorption spectroscopy respectively. (Unpublished results, K. Cain and M. Webb.)

* The percentage figures refer to predicted inhibition, that is based on in vitro studies (see Figure 12) and the concentration of μgCd^{2+}/mg protein.

may be related to the copper content which is increased when the isolation is carried out in 5 mM mercaptoethanol. The concentration of the latter is similar to the thiol concentration of the liver[147] and suggests that the metallothioneins of the cell are normally in a reduced environment. These conclusions are supported by Mehra and Bremner[148] who used mercaptoethanol to solubilize Cu-thionein from the particulate fraction of pig livers. All these studies demonstrate that "non-thionein-bound cadmium" represents at most 5% of the total, which is equivalent to 10 μg Cd^{2+}/g at the critical level (assuming an average figure of 200 μg Cd^{2+}/g). This value is much lower than the 30-75 μg Cd^{2+}/g figure for "non-thionein-bound cadmium" estimated by Nomiyama and Nomiyama[137], although it is in the same range of concentrations[149] produced after the injection of Cd^{2+} plus cysteine[121] and Cd-Mt. However, in the latter

examples the Cd^{2+} reaches the kidney as a bolus and therefore "local concentrations" (e.g. at the cell membrane of the tubular epithelia) must be much higher. It is therefore likely that "other factors" in addition to the concentration of "non-thionein-bound cadmium" are involved in the production of chronic renal toxicity.

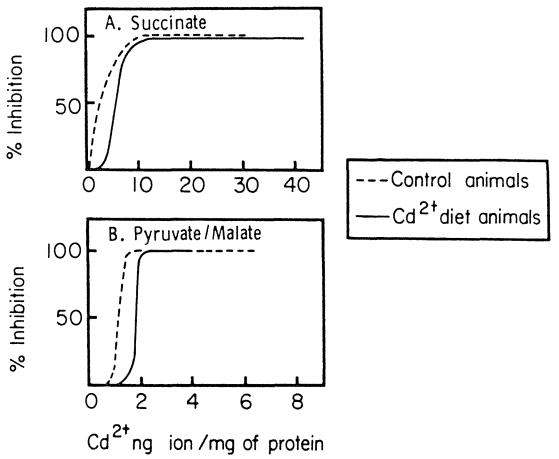

Figure 8 In vitro Cd^{2+} inhibition of ATP synthesis in kidney mitochondria isolated from rats fed on control and 100 ppm Cd^{2+} diets for 75 weeks. ATP synthesis was measured as described in Table 6. Mitochondria were pre-incubated with the various Cd^{2+} concentrations for 10 min before substrates were added. (Previously unpublished results, K. Cain and M. Webb.)

In this respect, Kawai et al.[126] maintain that the type and extent of renal damage are markedly influenced by the daily dose of cadmium used to produce chronic nephropathy. They compared and contrasted the renal histology produced by a range of different dosing regimes which they classified as described in Table 7. Two types of lesion could be identified: an acute lesion characterized by hydropic changes and acidophilic necrosis of the proximal convoluted tubules, and a chronic lesion which was characterized by tubular degeneration and interstitial oedema. The severity of the acute lesion roughly corresponded to the size of the daily dose, rather than the cadmium content of the kidney. The latter, and also the Cd^{2+} content of the liver, however, appeared to be very important in "triggering" the onset of the final renal lesions. Thus this scenario envisages that the liver and kidney have a finite loading capacity, and once this is exceeded the level of blood cadmium is much higher and renal toxicity, possibly through acute vascular damage, is produced. With lower daily doses the incidence of acute lesions is reduced, but even at 0.5 mg Cd^{2+}/kg (subchronic) there is an intermittant incidence of acute lesions. Therefore this dosing regime induces both chronic and acute lesions, each of which contribute to the development of nephropathy. This experiment also demonstrated that the liver is damaged as well as the kidney and Dudley et al.[150] (using the

same dosing regime) maintain that the hepatic injury precedes kidney damage. In this study the liver damage peaked at 10-12 weeks, and coincided with a sharp increase in plasma metallothionein levels, presumably as a result of that released from damaged hepatocytes. Injected Cd-Mt[149] is a potent nephrotoxin, and the sudden increase in plasma levels may also contribute to the onset of the renal lesion. Other toxic metabolites may also be released from the liver and would contribute to the overall nephrotoxicity. It is therefore possible that the apparent increases in "non-thionein-bound cadmium" observed at or around the "critical level" by some workers[137,141] are as a result of nephrotoxicity rather than the cause of renal damage.

Table 7 Classification of renal pathology in relation to dose and duration of dosing (modified from Kawai et al.[125])

				Type of lesion	
Classification	Daily dose (mg/kg)	Latency period[a]	Morphology in latency period	Moderate	Chronic
Acute latency	3-6 s.c.	Several days	Widespread acute type lesions	Severe	None
Subacute	1-2 s.c.	2-10 weeks	Some acute type lesions	Moderate	None
Subchronic	0.5 s.c.	2-4 months	Occasional acute type lesions	Slight	Yes
Chronic	Oral	4 months or more	None	None	Yes

a Time between beginning dosing and development of necrotic lesions.

The importance of liver damage to the development of the kidney lesions may be highly significant, as in feeding experiments where the Cd^{2+} concentration in the blood is low there is no evidence of liver damage[125,151] and the acute type renal lesions described by Kawai et al.[126] are not observed. The latter workers found that in chronic feeding experiments the renal lesions appeared as scattered foci in the deep cortex which, with increasing time of exposure, became more numerous and extended into the outer zone of the cortex. Significantly, some lesions were observed at rat kidney cadmium concentrations as low as 44.4 μg/g (i.e. well below the 200 μg/g critical level), although the scattered nature of the lesions suggests that the cadmium concentrations in the renal tubules are not uniform. Kawai et al.[126] conclude that the

development of the chronic lesion may not be solely related to the cadmium concentration in the kidney and that the duration of exposure is important. In a later study Kajikawa et al.[151] also examined the histological changes in the kidneys of rats fed 200 μg/g Cd^{2+} in the drinking water. They concluded that the lesions were relatively subtle and confined to the proximal convoluted tubules. The extent of the lesions varied from tubule to tubule and even individual cells in the same tubule were not affected to the same degree. The earliest lesions were observed at 16 weeks when the kidney contained approximately 80 μg Cd/g. The extent of the damage increased with time and renal cadmium content. The latter reached a maximum at 40 weeks of 200 μg/g (cortex) and thereafter declined to 140 μg/g at 91 weeks. During this time renal damage was greatest; however, marked spontaneous nephropathy was also observed in the control animals, and this seemed to be exacerbated in the cadmium fed rats. In support of this, Lauwerys et al.[152] have shown that rats given 200 ppm Cd in the drinking water for 11 months develop proteinuria which is largely glomerular in origin. In this respect it resembles the proteinuria which results from the chronic progressive nephrosis seen in older animals. The cadmium treatment seems to amplify this normal ageing process, which is not reversible.

In conclusion, renal lesions produced by chronic Cd^{2+} feeding experiments are usually less marked than are seen after repetitive Cd^{2+} injections. The onset of the lesions may occur at fairly low Cd^{2+} concentrations (see also Aughey et al.[153]) which are well below the critical concentration. In this situation it is improbable that thionein synthesis is insufficient to accommodate all of the Cd^{2+}. It is therefore more likely that the real cause of the renal damage is extracellular Cd^{2+}, i.e. in the blood, which could damage tubular, glomerular and vascular membranes before entry into the kidney cell. This damage would probably be slight (as cadmium concentrations in the blood are low) and presumably in the young animal such damage would be compensated for by the normal repair mechanisms. However, with increasing age these lesions would be synergistic with the spontaneous nephropathy, producing marked renal damage.

IVB. Mercury

Although there is evidence that thionein synthesis is an important protective mechanism against Cd^{2+} toxicity, the evidence for a similar role in mercury nephrotoxicity is not so clear cut. It is important to stress that it is only the Hg^{2+} ion which binds strongly to thionein, and the protein therefore has no significant role in the detoxification of CH_3Hg^+, except to scavenge the divalent cations which are released by biotransformation in the body[154]. As the latter is a slow process, thionein synthesis must be of minimal significance as a protective mechanism against methylmercury. Thionein scavenging of Hg^{2+}, released from organomercurials used as diuretics, is perhaps more important, because these compounds are rapidly metabolized to Hg^{2+}, which is probably the active agent[155]. This explains why Pulido et al.[16] originally found that

human metallothionein contained mercury.

Mercuric chloride is a potent nephrotoxin and acute injections, as discussed by Magos[155], produce both acute renal failure and proximal tubular damage. The former is mediated through the release of renin, and the latter is a consequence of the localized accumulation of Hg^{2+} in the proximal tubules. The acute renal failure is mediated in part through effects on the renal baroreceptors, and is unlikely to be affected by intracellular thionein synthesis. The proximal tubular damage is similar to that induced by cadmium.

In the non-thionein induced animal it is unlikely that thionein synthesis can be effective against acute high doses of Hg^{2+}. The reasons for this conclusion are identical to that discussed above for Cd^{2+}. Thus, high concentrations of Hg^{2+} probably cause renal damage by a combination of extracellular (e.g. tubular membrane effects[111]) and intracellular (e.g. mitochondrial damage[156]) effects. Thionein cannot protect against the former, and the onset of the latter is rapid and complete before the initiation of the metalloprotein's synthesis.

Pretreatment with Hg^{2+} or Cd^{2+} protects against subsequent doses of mercury[157] and the most logical explanation is that the former treatment induces thionein, which is the protective agent. However, Webb and Magos[158] found that after a toxic injection of Hg^{2+} only a small percentage (8%) of the renal mercury was thionein bound. Cd^{2+} pretreatment increased the kidney mercury content by 60% and the thionein bound content 6-fold. However, the non-thionein bound Hg^{2+} also increased and, as the latter would expect to be the toxic species, it is apparent that the protective mechanism is not mediated through the increased concentrations of thionein. This conclusion is borne out by findings that other nephrotoxic agents[155], which do not induce thionein synthesis, also protect against mercury. After such pretreatments the proximal tubular epithelia regenerate. These cells are initially without a fully differentiated brush border and are resistant to the effects of mercury[155]. As the early effects of Hg^{2+} are on the brush border membranes of the proximal tubule, it seems that the protective effect is related to the insensitivity of the regenerating cells to Hg^{2+}. This may explain why the newborn rat kidney is relatively resistant until 3 weeks old when the organ acquires a fully differentiated brush border membrane[155]. In any event it seems certain that neither endogenous nor pre-induced thionein can offer any protection against acute Hg^{2+} toxicity.

The possible protective effect of thionein against chronic low-dose exposure to Hg^{2+} is more problematic as there is very little information on this topic. However, Bogden et al.[159] showed that male rats given 20 ppm $HgCl_2$ in the drinking water for 7 weeks accumulated 152 μg Hg^{2+}/g kidney. Although the kidney weight increased by 20%, there were no specific histological lesions or signs of tubular regeneration. As in acute-dose experiments[157] the kidneys are severely damaged at tissue concentrations as low as 15-16 μg Hg^{2+}/g, and it is possible that the kidneys are protected by some mechanism other than tubular regeneration. In this respect, although Bogden et al.[159] did not measure the metallothionein levels they did record the fact that copper levels in

the kidney increased 5-fold, which would be expected if the Hg/Cu-Mt levels were elevated. This conclusion is supported by the results of Piotrowski et al.[92], who subcutaneously injected female rats every other day with 0.5 mg Hg^{2+}/kg for 14 weeks. In these animals 72-83% of the Hg^{2+} kidney burden was located in the metallothionein fraction, and this suggests that thionein has a protective role in chronic mercury exposure. The extent of this protection must depend on the distribution of Hg^{2+} between thionein and other ligands in the cell. The affinity of Hg^{2+} for the latter is, however, very high and according to Webb[109] all forms of Hg^{2+} bound to cellular components are in equilibrium. As a result there is always a significant proportion of the total Hg^{2+} which is non-thionein bound. The toxicological significance of this form of Hg^{2+} is difficult to evaluate. On the basis of the cadmium protection experiments described above it is clear that not all non-thionein bound Hg^{2+} is necessarily toxic. It is possible that Hg^{2+} toxicity results from specific interactions with sites whose affinities for the metal are not necessarily the highest in the cell. If this is the case, then the finding that HMW proteins of the cytosol apparently have a higher affinity for Hg^{2+} than thionein[95] may be irrelevant. Thus, the only requirement for a protective effect is that thionein has a higher affinity for Hg^{2+} than binding sites which are toxicologically significant.

V. ROLE OF HEPATIC METALLOTHIONEIN IN CADMIUM NEPHROTOXICITY

Thionein synthesis provides a potentially effective means of protecting the kidney against Cd^{2+} and to a lesser extent Hg^{2+} toxicity. With inorganic mercury there is a gradual clearance of the metal from the kidney[95] after exposure ceases, and as the latter contains most of the body burden the danger of toxicity decreases with time. The protective mechanism is not ideal with Cd^{2+}, which accumulates in both the kidney and liver, where in man the half-lives are 17-33 years and 7 years respectively[160]. The faster rate of elimination of the metal in the liver has been demonstrated in animals and is the result of a slow transfer of the cation from the liver to the kidney[7,119,161]. Piscator[7] originally suggested that thionein was synthesized solely in the liver and then transported to the kidney. Although the synthesis theory has been disproved (see section IIIA), there is considerable evidence that Cd-Mt is transported from the liver to the kidney via the blood.

In this section the nephrotoxic effects of Cd-Mt and the evidence that metallothionein is the carrier protein are discussed. The use of plasma or urinary levels of metallothionein as a diagnostic test for Cd^{2+} exposure is also reviewed. In addition, the effect of hepatic damage on the kidney Cd^{2+} content is assessed.

VA. The renotoxicity of cadmium metallothionein

Studies in the rat[10] and in the mouse[9] showed that Cd-Mt was a

very potent nephrotoxin. The LD_{50} for parenterally injected Cd-Mt in the rat has been estimated to be 0.28 mg Cd-Mt/kg[10] which is much less than the LD_{50} value for cadmium chloride (2.24 mg Cd^{2+}/kg)[10]. In the mouse also, Nordberg et al.[9] reported that the thionein bound Cd^{2+} was more toxic than the ionic form, and concluded that death was as a result of renal failure. Two theories have been advanced to explain the nephrotoxicity. The predominant theory originally suggested by Webb and Etienne[10] and supported later by Squibb et al.[162], and Cain and Holt[149], proposes that Cd-Mt is reabsorbed in the proximal tubules by endocytosis, degraded in the lysosomes, releasing Cd^{2+} which is subsequently toxic (Figure 9). The alternative theory put forward by Cherian and co-workers[12,163] also proposes that Cd-Mt is reabsorbed by endocytosis, and suggests that toxicity is produced by the intact Cd-Mt, which causes cell membrane rupture and damage to the mitochondria.

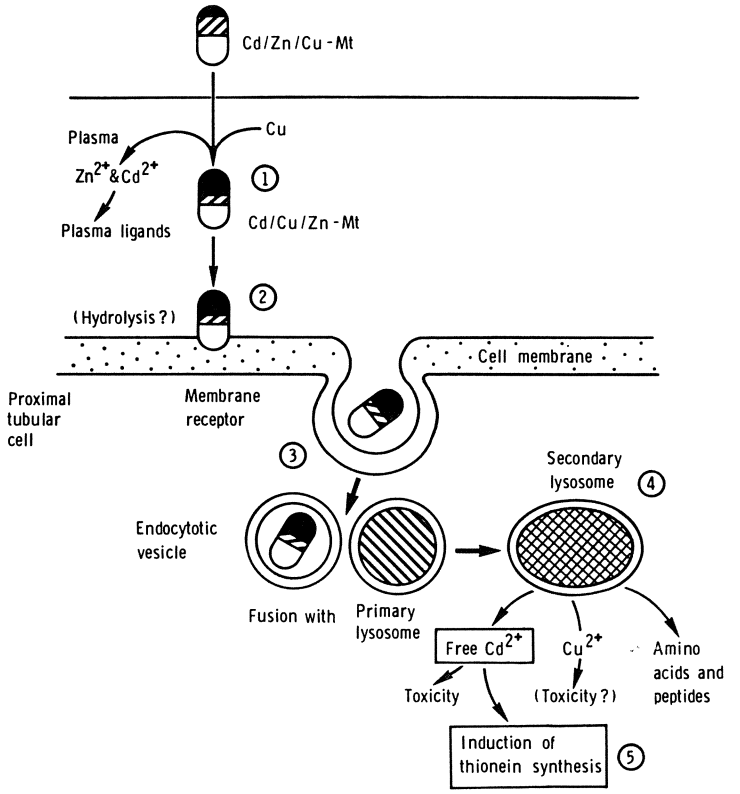

Figure 9 Cd-Mt nephropathy. The scheme shows the principal biochemical events leading to renotoxicity after a parenteral Cd-Mt injection.

As shown in Figure 9, the injected metallothionein first undergoes a metal exchange reaction (Step 1) in the plasma before reaching the kidneys. The evidence for this conclusion comes from

studies on the rat. Tanaka et al.[164], for example, noted that after an injection of ^{109}Cd/Zn-Mt a significant proportion of the dose was excreted in the urine. However, the urinary ^{109}Cd-Mt contained much less zinc than the injected metallothionein. Similarly, Bremner et al.[165] showed that after Cu/Zn-Mt or Zn-Mt injection, Cu in the plasma exchanged with thionein bound zinc. This cation-exchange reaction explains why Suzuki and Yamamura[166], and Cain and Holt[149], found marked changes in the renal Cu content after Cd/Zn-Mt injection. In the latter study the animals were injected with [^{3}H]Cd/Zn-Mt which had been labelled in vivo with [^{3}H]cysteine. The time-course of Cu accumulation paralleled the uptake of Cd. Furthermore the DPM:μg Cd^{2+} ratio of the kidneys at 10 min (the earliest time measured) was almost double that of the injected metallothionein. Thus, Cd^{2+} as well as Zn^{2+} is displaced by the copper in the blood.

The displacement of thionein-bound Cd^{2+} and Zn^{2+} by copper must be a rapid process, because the protein is cleared very rapidly from the blood[149,162,165] after an i.v. injection. As shown from the example in Table 8,and in other studies[10,163], this rapid clearance from the blood can be explained by reabsorption of the metallothionein in the kidneys. The hepatic uptake of Cd^{2+} after a metallothionein injection is very small, and in view of the cation-exchange reactions discussed above, it is likely that some of the liver uptake of cadmium results from the displaced cation (i.e. Step 1 in Figure 9) rather than the transport of thionein-bound Cd^{2+} into the hepatocyte. In support of this conclusion, Bremner et al.[165] studied the organ distribution of [^{35}S]Cd-Mt after parenteral injection using a metallothionein preparation in which the protein was labelled with ^{35}S-cysteine. The results showed that 30 min after injection only 0.6% of the radioactivity was located in the liver, compared with the 20% which was taken up by the kidney. Thus, there is negligible reabsorption of metallothionein in the liver.

Table 8 Organ distribution of metallothionein 1 h after an 1 intravenous injection of Cd-Mt

Tissue	Radioactivity (cpm/g tissue)
Liver	246
Kidney	32,156
Spleen	368
Testis	136
Lung	233
Small intestine	132
Heart	147
Blood	217

The data are taken from Tanaka et al.[164] and rats were given 0.23 mg Cd-Mt/kg b.w. and the organ contents determined by assaying the radioactivity.

The renal accumulation of Cd-Mt in the kidney is a very rapid process and irrespective of the dose is virtually complete within 1 h of administration (Figure 10). It can also be seen from the figure that Cd-Mt uptake is a dose-dependent saturable process, which, in the rat at least, is maximal at a dose level of approx. 0.8 mg Cd-Mt/kg b.w. The kinetics of reabsorption of Cd-Mt in the kidney have been studied in rabbits with the use of bolus and constant infusion techniques[167-170]. Foulkes[167,168] demonstrated that Cd-Mt was freely filtered by the glomerulus and suppression of glomerular filtration abolished the reabsorption of metallothionein. In the same studies it was shown that there was no peritubular uptake of the protein, and thus renal reabsorption of Cd-Mt takes place entirely from the glomerular filtrate. The constant infusion experiments[167] also showed that there were two reabsorptive mechanisms; one of which was saturated at relatively low blood concentrations and another which was not saturated at the highest concentrations used in these studies.

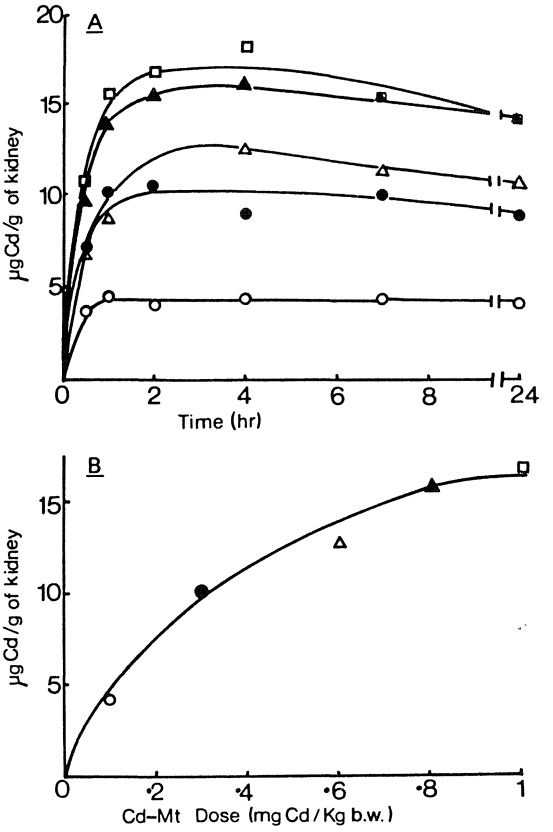

Figure 10 Time course and dose-dependent renal uptake of Cd-Mt. Rats (100 g) were given intravenous doses of Cd-Mt and at the indicated times (A) killed and the kidney removed and assayed for Cd^{2+}. Part B shows the maximum uptake plotted as a function of dose. The doses (in mg Cd^{2+}/kg b.w.) were 0.1 (○), 0.3 (●), 0.6 (△), 0.8 (▲) and 1 (□). (Previously unpublished results, K. Cain and D.E. Holt)

The latter uptake mechanism was also shown to be fully saturable when bolus injections of 1 mg Cd-Mt were given[169], and this explains the urinary excretion of metallothionein after large parenteral doses of the protein.

The significance of two transport mechanisms for Cd-Mt is not understood. However, it appears on the basis of in vitro binding studies[171] with isolated renal brush-border membranes (BBM), that for both uptake processes the initial and possibly rate-limiting step is the interaction of metallothionein with specific receptors on the luminal cell membrane (Step 2 in Figure 9). Two classes of receptor have been identified with differing affinities for Cd-Mt. The high affinity receptors (K_a = 2.2 x $10^7 M^{-1}$) have a concentration of 0.13 μg Cd-Mt/mg BBM protein, whereas the low affinity receptors (K_a = 6.5 x $10^5 M^{-1}$) are more abundant with a concentration of 1.07 μg Cd-Mt/mg BBM protein. Binding of Cd-Mt to the low affinity site is antagonized by myoglobin, and in this respect parallels the apparent competition between these proteins for renal reabsorption in vivo[168,169]. Furthermore, Cd-Mt reabsorption is decreased[168,169] in animals chronically treated with Cd^{2+} and BBM isolated from such animals bind 36% less Cd-Mt. Thus the uptake of Cd-Mt in the kidneys is correlated with its binding to the luminal cell membrane.

Reabsorption of proteins by endocytosis involves binding of the protein to the plasma membrane, prior to its invagination (see for reviews, Maunsbach[172] and Besterman and Low[173]) to form endocytotic vesicles. Although the formation of the latter has been demonstrated conclusively for other proteins[172] and endocytotic vesicles have been observed after Cd-Mt administration[162,163], there was, until recently, no unequivocal evidence that the same mechanism was responsible for the uptake of the metalloprotein. However, Murakami et al.[174] have shown by autoradiography that ^{125}I-labelled thionein can be detected within 30 min in endocytotic vacuoles and heterolysosomes in the apical and middle regions of the cells of the proximal convoluted tubules. There is thus no doubt that Cd-Mt enters the cells internalized in endocytotic vesicles (Step 3, Figure 9).

It is the latter process which Cherian and Goyer[12] consider to be toxic, through membrane rupture. If this is the case then Cd-Mt must be a very unusual protein, because many other proteins are reabsorbed in the same manner without apparent damage. Indeed there is good evidence that any membrane which is internalized during endocytosis is rapidly recycled without loss of cell surface area, volume or integrity[173]. In addition, Zn-Mt which is reabsorbed by the same mechanism[165] is not nephrotoxic[10]. Furthermore, assuming that the saturable uptake of metallothionein is a function of protein concentration rather than the metal content, it should be possible to get more or less Cd^{2+} in the kidneys by injecting Cd/Zn-Mts with differing Cd:Zn ratios. The toxic effects of the latter, when given to rats at constant dosage (i.e. on a protein basis), produce nephrotoxicity, the severity of which increases in proportion to the Cd^{2+} content of the injected protein[175]. The simplest explanation for the latter finding is that the toxic species is "free Cd^{2+}" which is liberated by lysosomal degradation as shown in Step 4 (Figure 9). The latter process has

been shown to be true for other proteins which are reabsorbed by endocytosis[172,176]. However, subcellular studies on kidney homogenates isolated from animals injected with ^{109}Cd-Mt have shown that 1 h after injection less than 7% of the radioactivity is associated with the "lysosomal fraction"[177]. At 4 h the cytosol contained 52% of the kidney radioactivity, 90% of which was associated with the metallothionein fractions as determined by Sephadex G-75 gel filtration. Whilst accepting that the thionein moiety is degraded and resynthesized, Cherian[177] concludes that nearly all (90%) of the metal (Cd) is bound to thionein and the role of the lysosomal system in the renal toxicity of Cd-Mt is minimal. This conclusion assumes that the ^{109}Cd (i.e. 48% of the total at 4 h) located in the various other cell fractions (i.e. nuclei, mitochondria, lysosomes and microsomes) has the same distribution as the "cytosol" (i.e. 90% is thionein-bound). In complete variance with these results, Squibb et al.[162] carried out similar subcellular experiments after ^{109}Cd-Mt injections. Although these workers used different centrifugation procedures to Cherian[177], they found that at 30 min, 39% of the kidney radioactivity was located in three particulate fractions which were correctly designated as mixtures of mitochondria and lysosomes. At 3 h the same fractions contained only 6.2% of the ^{109}Cd. The cytosolic fraction also showed marked changes containing 26% and 62% of the radioactivity at 0.5 and 3 h respectively. This rapid redistribution of the cadmium from the particulate fractions to the cytosol is entirely consistent with, but does not prove, lysosomal degradation.

However, studies with ^{3}H-, ^{35}S- and ^{14}C-labelled metallothioneins provide more convincing evidence of degradation. Thus Cherian and Shaikh[177] and Bremner et al.[165] have shown that the thionein moiety of injected Cd-Mt is rapidly catabolized. In a detailed time-course study, Cain and Holt[149] showed that all of the ^{3}H-labelled thionein is degraded by 4 h and, as shown in Figure 11 this is accompanied by the production of ^{3}H-labelled, lower molecular weight fragments, probably peptides and amino-acids. This degradation was accompanied by redistribution of Cd^{2+} to HMW proteins (Figure 11). Subcellular fractionation revealed that there was no major accumulation in the mitochondrial/lysosome fraction. However, in the first 2 h post-injection the nuclei/cell debris fraction contained nearly 70% of the cadmium and 80% of the total tritium. This subcellular fraction contains not only nuclei, but also a mixture of endocytotic vesicles, primary and secondary lysosomes (see Cain and Holt[149]), the lysis of which liberates disproportionately less Cd^{2+} than ^{3}H into the cytosol. This is not unexpected, as non-thionein-bound Cd^{2+} is unlikely to distribute in the cell in the same way as the negatively charged intact metallothionein. From these data it was calculated that a maximum of 11-12 μg Cd^{2+}/g (i.e. 70% of the total renal Cd^{2+} burden) is non-thionein bound in the first 4 h post-injection, and is thus more than enough to explain the toxic effects (compare also with the effects of Cd + cysteine[121]). This contrasts with Cherian's assertion that after Cd-Mt injection most of the Cd^{2+} is always bound to thionein. However, it must be remembered that rat kidney contains an endogenous Zn/Cu-Mt[149] which is capable of binding Cd^{2+} and that endogenous thionein synthesis is initiated between 2 and 4 h

(Step 5, Figure 9). The amount of cadmium binding to this endogenous thionein will only be a small percentage of the total when the renal Cd-Mt uptake is maximal, and complete within 2 h (i.e. as is the case after a large intravenous dose[169]). However, if the renal Cd-Mt uptake is low, then the amount of Cd^{2+} which can be bound by the endogenous thionein will be a significant proportion of the total kidney burden, particularly at later times (i.e. 4 h onward) when new thionein is being synthesized. This is likely to be the case in Cherian's experiments where a sub-lethal dose of metallothionein (0.3 mg Cd-Mt/kg b.w.) was given intraperitoneally. The latter route of administration produces a much slower rate of renal Cd-Mt uptake[163,176] and it is therefore not surprising that at 4 h a significant percentage of the cytosolic Cd^{2+} is bound to metallothionein.

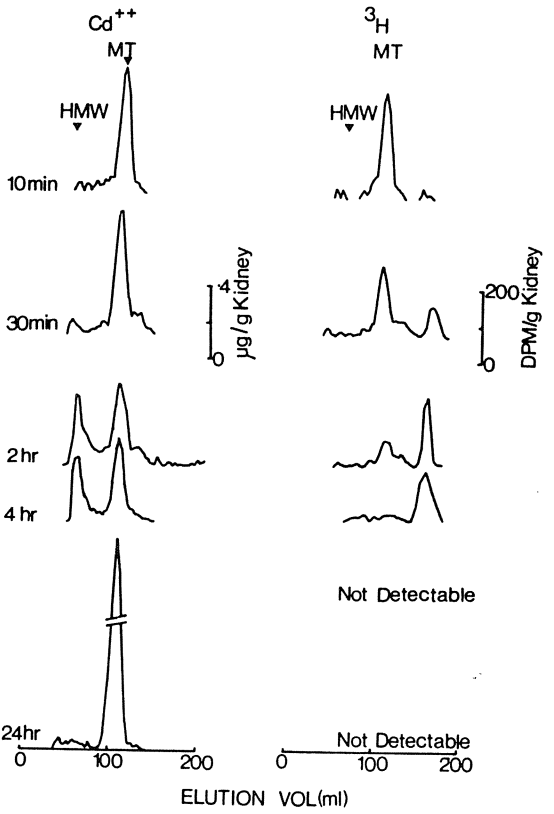

Figure 11 Sephadex G-75 chromatography of cytosol isolated at various times from the kidneys of rats injected with 0.8 mg [^{3}H] Cd/Zn-Mt/kg. The elution positions of metallothionein (MT) and high molecular weight proteins (HMW) are indicated in the figure. The Cd^{2+} (µg/g kidney) and ^{3}H (DPM/g kidney) are given and are shown as scale bars. (Adapted from Cain and Holt[149].)

Thus it is the rapid degradation, or more accurately the

rapid liberation of "free Cd^{2+}", which exceeds the binding capacity of the endogenous thionein that causes toxicity. In this respect there is evidence that Cd^{2+} is liberated even before the protein is totally degraded. Since autoradiographic studies with ^{109}Cd-Mt have shown that even at early times (e.g. 10 min) after injection, cadmium is uniformly distributed throughout the cell with no obvious accumulation in endocytotic residues or lysosomes. This is in contrast with similar studies[174] with ^{125}I-labelled metallothionein which have shown that the thionein moiety is definitely concentrated in endocytotic vesicles and heterolysosomes. Obviously then, the Cd^{2+} must be removed before the protein is fully degraded. This may be as a consequence of the acid pH of the lysosome which can displace thionein bound metals (see section IID). Alternatively, partial digestion may lead to unfolding of the tertiary structure with the release of bound cations. Some degradation may also take place at the luminal surface of the proximal tubule cell membrane which contains amino peptidases capable of hydrolysing small linear peptides like angiotensin II[178].

The initiation of thionein synthesis between 2 and 4 h post-injection[149,177] is also indicative of the release of "free Cd^{2+}", as it is the latter, and not the thionein-bound cation, which induces the synthesis of metallothionein (see section IIIA). By 24 h virtually all of the "cytosolic Cd^{2+}" is thionein-bound and in the rat, at least, the new metallothionein contains Zn^{2+} as the secondary cation[149,166,175]. This is in contrast to the Cd/Cu-Mt which is normally produced after $CdCl_2$ administration[29].

The morphological effects of Cd-Mt on the kidney have been investigated at both the light- and electron-microscopical level. These studies have shown that the damage is largely concentrated in the S_1 and S_2 regions of the proximal convoluted tubules and the other portions of the nephron (i.e. glomerulus, straight proximal tubules, distal tubule and collecting duct) are essentially unchanged[126,179]. Apical vesiculation is the earliest sign of damage which occurs within 1 h of administration[126] and by 4 h is extensive in some of those cells surrounding the larger arteries in the cortex. At early times (0-8 h), the ultrastructure of the brush border membranes appear undamaged and in vitro, brush border vesicles are unimpaired in their ability to transport glucose[180]. The lesions progress with time and at 24 h the initially affected cells exhibit extreme necrosis and most of the proximal convoluted epithelia are hydropically or vacuolarly degenerated[126]. These changes are maximal at 48 h and are associated with an increase in kidney weight (due to the oedema) which reaches a maximum at 4 days[149]. The necrotic changes are maximal at 48 h, thereafter regeneration begins and by day 7 most of the cell debris has been removed. At this time the morphology of the regenerating or regenerated proximal tubular epithelia is similar to that of the normal kidney[126].

The morphological changes suggest that the toxic lesions are initiated in the first few hours after Cd-Mt injection. Studies on functional and biochemical alterations are also consistent with this view. Thus 8 h after a 0.6 mg Cd-Mt/kg b.w. injection, there is a 2.5-fold increase in urine volume, a 2-fold increase in urine sodium and a 35-fold increase in protein excretion[180]. Lysosomal

cathepsin D and acid phosphatase activity are also inhibited at early times[181]. Fowler and Squibb[181] believe that in the first 8 h after Cd-Mt administration the protein is rapidly degraded and that high levels of non-thionein bound Cd^{2+} inhibits primary lysosome formation and the fusion of the latter with pinocytotic vesicles. As a result the reabsorption of low molecular weight proteins is inhibited and protein excretion increases. This may in part explain the abundant apical vesiculation which has been observed in electron microscopy studies[126,162,163,180,181]. An analogous cellular vesiculation has been observed with sodium maleate[176], which induces a pronounced proteinuria and decreased protein degradation in proximal tubular lysosomes. The latter may be explained by decreased endocytosis and impaired fusion of endocytotic vesicles and lysosomes. It is perhaps also significant that one of the earliest morphological signs after a Cd^{2+} plus L-cysteine injection is apical vesiculation[121] and in the liver at least Cd^{2+} is known to inhibit heterolysosome formation[182]. Thus the endocytotic reabsorption of protein in vivo may be particularly susceptible to the action of Cd^{2+}, and this may explain the proteinuria which is a characteristic feature of chronic cadmium exposure.

The location of the site of damage (i.e. in the S_1 and S_2 regions of the proximal tubule) after Cd-Mt administration and chronic cadmium exposure are similar[126], thus the former treatment provides a convenient experimental model for studying Cd^{2+} nephrotoxicity. However, the effect of Cd-Mt must be regarded as an acute type of toxicity in response to a relatively large bolus injection of Cd^{2+}. In the chronic situation the levels of Cd-Mt in the blood are much lower, and the levels of metallothionein in the kidney are much higher. The latter provide a large reservoir of potential Cd^{2+} binding sites, which, according to the "lysosomal theory", would protect the kidney against Cd^{2+} liberated by hydrolysis. In this respect Webb and Etienne[10] showed that pretreatment of rats with Zn-Mt or ionic Cd^{2+} protected the animals against the toxic effects of a subsequent dose of Cd-Mt.

Furthermore, in rats dosed repeatedly with Cd-Mt (0.2 mg Cd-Mt/kg b.w., every 3-4 days) the first dose, although nephrotoxic, seems to protect the kidney against further tubular damage by subsequent doses[183]. In addition when very low amounts of Cd-Mt are infused from subcutaneously implanted mini-pumps, there is a selective renal accumulation of Cd^{2+}, and this is achieved without kidney damage[170]. Cd-Mt is thus unlikely to have a primary role in the renotoxicity of animals or humans chronically exposed to Cd^{2+}.

VB. Plasma and urinary metallothionein levels as a measure of cadmium nephrotoxicity

Until recently the role of metallothionein as an hepatorenal carrier protein has been assumed rather than proven. This assumption is largely based on the evidence that there is a liver to kidney transfer of cadmium, and that metallothionein is selectively accumulated by the kidney (see above). However, direct evidence for such a

transport role has been difficult to establish. The reasons for this are that even large bolus injections of Cd-Mt are cleared very rapidly from the blood, and in the chronic situation where the levels are very low it is difficult to accurately measure the Cd^{2+} content let alone the metallothionein concentration. Nordberg et al.[184] attempted to overcome these problems by injecting mice with high doses of $^{109}CdCl_2$ (0.5 mg Cd/kg b.w.) 5 days a week for 6 months. At the end of this period 60% of the Cd^{2+} in the blood was associated with a low molecular weight whose behaviour on Sephadex G-75 was compatible with that of metallothionein. Shaikh and Hirayama[185] used a similar experimental regime with rats and detected a metallothionein-like protein in the plasma and urine at 4 and 10 weeks respectively. Thereafter, increasing levels of thionein-bound ^{109}Cd were found in both urine and plasma. However, in both these experiments[184,185] the dosing regime was severe, and it is almost certain that the appearance of metallothionein in the plasma and urine was as a result of liver and kidney damage respectively. Under these conditions the concentrations of the protein in the plasma and urine will be relatively high. In absolute terms, however, the concentration of thionein bound Cd^{2+} is less than 10 ng/ml of plasma[185]. This level of Cd^{2+} cannot be accurately assayed with atomic absorption techniques, and it is therefore impossible to assess with conventional techniques whether or not metallothionein is normally found in plasma of either humans or animals chronically exposed to cadmium.

However, in the past few years sensitive radioimmunoassays (RIA) for metallothionein have been developed. The basic characteristics of three of the most sensitive assays are shown in Table 9. In all cases the RIAs are based on the principle that unlabelled antigens will compete with the labelled antigen (tracer) for the antibody binding sites. In practice the antibody and tracer concentrations are fixed and the concentration of unlabelled antigens varied. The tracer is usually prepared by iodination of "pure" metallothionein using the chloramine-T method, and it is possible to detect low amounts (pg) of metallothionein. The production of antiserum with high antibody titre requires some manipulation as the native monomeric protein is a poor immunogen[189], and it is necessary to polymerize or conjugate the thionein with itself or other proteins to produce a good antibody response (Table 9). Once the antibody is fully reacted with the tracer and unlabelled antigens it is necessary to separate the latter complex from the unbound (free) tracer with either a second antibody[186,188] or ammonium sulphate precipitation step. Usually a standard curve is prepared and, as shown in Table 9, RIAs are sensitive, and can be highly specific for the isoforms of metallothionein[188].

The advantages of this increased sensitivity over conventional methods were demonstrated in an early study by Vander Mallie and Garvey[190]. Thus, with rats dosed intraperitoneally (600 μg Cd^{2+}/rat/week) 5 days a week, increases in the serum metallothionein levels could be detected within 2 weeks of commencing dosing. This was earlier than in another study[185], which used the same injection protocol but relied on conventional techniques for metallothionein analysis. In a more detailed time-course experiment Garvey and Chang[192] reported that resting serum levels were

1-2 ng Mt/ml, which increased to 13 ng/ml 36 h after an injection of 0.8 mg Cd/kg b.w. Thereafter the serum levels dropped to 8 ng/ml, at which concentration they remained static for at least 13 days. Subsequent injections of Cd^{2+} at days 3 and 6 produced only small transient changes. Injections of Zn^{2+} (5 mg Zn^{2+}/kg b.w.) at first produced little effect on serum levels. However, the second injection at day 3 elicited an approximate 3-4-fold increase in the serum levels. The latter then returned to nearly normal by day 6. A third injection of zinc at this time then produced a similar

Table 9 Comparison of metallothionein radioimmunoassay (RIA)

Antigen	Antibody	Labelled antigen	Precipitation method	Working range	Cross-reactivity	Comments	Reference
Rat liver Cd-Mt1 and Cd-Mt2 polymerised with glutaraldehyde	Rabbit anti-rat liver Cd-Mt 1 and 2	[^{125}I] Cd-Mt1	Goat anti-rabbit serum	200-10,000 pg of protein 1:20,000 dil.	Both Cd isoforms from equine, human and rat liver; Chinese hamster cell and also Zn-Mt2 and apoprotein from rat liver	Reacts with most Mt, relatively high antibody titre	186
Rat liver Cd-Mt2 conjugated to albumin with glutaraldehyde	Rabbit anti-rat liver Cd-Mt2	[^{125}I] Cd-Mt2	Ammonium sulphate	1.2-36 ng of protein at 1:2500 dil.	Rat liver Cd-Mt1 and Cd-Mt2; human renal Cd-Mt2	Low antibody titre	187
Rat liver Cd-Mt1 conjugated with rabbit IgG	Sheep anti-rat liver Cd-Mt1	[^{125}]I Zn-Mt1	Donkey anti-(sheep IgG) antiserum	100-11,000 pg of protein at 1:35,000 dil	Cd-Mt1, Zn-Mt1 and Cu-Mt1 from rat liver	Specific for Mt1 with very little cross-reaction with Mt2	188

response to that produced by the second injection. Garvey and Chang[192] maintain that these experiments show that metallothionein appears in the plasma before overt renal damage and that the protein can act as a transport protein for Zn^{2+} or Cd^{2+}. However,

it should be realized that liver damage can also elevate the thionein concentration in plasma[193] and some liver damage would be expected after intraperitoneal injections of Cd^{2+} or Zn^{2+}. Also it is significant that Cd^{2+} elicits a much greater and prolonged increase in serum metallothionein levels than does Zn^{2+}, which produces a transient increase. The serum responses mirror what would happen to metallothionein levels in the liver and suggest that the latter are directly responsible for the former. Further evidence for the latter conclusion comes from studies by Tohyama and Shaikh[187] who analyzed the metallothionein levels in the plasma and urine of rats given subcutaneous injections of 0.56 mg Cd^{2+}/kg b.w./day, 5 days a week for 15 weeks. In these animals the thionein concentrations in the plasma increased progressively during the first 6 weeks and then gradually plateaued. The plasma metallothionein accumulation curve thus approximated the time course of Cd^{2+} uptake in the liver. The appearance of metallothionein in the urine was much lower at first and then rose steeply at about 8 weeks, which coincided with maximum levels of Cd^{2+} in the kidney of around 200 μg/g (i.e. the critical concentration). The latter suggests that the determining factor for increased urinary metallothionein concentrations is the onset of renal damage which liberates intracellular Cd-Mt. This conclusion is supported from the kinetics of Cd-Mt absorption (see section VA) and Foulkes[195] maintains that the efficiency of Cd-Mt reabsorption is so great that under chronic exposure conditions very little of the protein will be directly excreted into the urine. Immunohistochemical studies[84] have also shown that in rats repeatedly injected with $CdCl_2$, metallothionein was localized intracellularly at 2 and 4 weeks. However after 6 and 8 weeks of dosing, intraluminal staining was observed, and this coincided with proximal tubular damage as shown by cytoplasmic vacuolation. It is also relevant that in epidemiological studies carried out on women with Itai-Itai disease[194], it was found that the urinary metallothionein concentration was significantly correlated with increased excretion of total protein, glucose, β_2-microglobulin, retinol-binding protein, α-amino nitrogen and proline. These workers concluded that the elevation of excretion of metallothionein is an index of renal dysfunction caused by chronic exposure to Cd^{2+}. A similar conclusion was reached by Falck et al.[196], who demonstrated that in men exposed occupationally, metallothionein excretion was linearly related to β_2-microglobulin excretion. The simplest explanation for the latter is that Cd-Mt is released from damaged renal epithelia and is excreted in the urine. However, there is some evidence that this conclusion may be an over-simplification. Thus Chang et al.[197] found that Mt concentrations in the urine were similar in cadmium exposed workers, irrespective of renal damage. In another study Nordberg et al.[198] demonstrated that in Cd^{2+} exposed workers with urinary β_2-microglobulin concentrations >500 μg/l, the median metallothionein concentration in the urine was statistically significantly higher than that in workers with β_2-microglobulin levels <500 μg/l. However, they also found, in individual cases, a considerable increase in urinary β_2-microglobulin without any increase in urinary metallothionein.

Another puzzling aspect is that if the urinary excretion of

Cd-Mt is due to renal cell damage, then it would be expected that Cd^{2+} excretion would also be related to renal damage. The evidence for this is, however, contradictory. Tohyama et al.[199], in an earlier study, found people living in a Cd^{2+}-polluted area had similar levels of urinary Cd^{2+}, irrespective of renal damage. However, in a later paper, Tohyama et al.[194] found that urinary Cd^{2+} and Cu^{2+} concentrations were significantly correlated with the metallothionein concentration. The increased excretion of Cu^{2+} in the urine suggests that the excreted protein is a Cd/Cu-Mt. The human renal Cd-Mt usually contains Zn^{2+} as a secondary cation[16], and this result implies that the increased urinary excretion of metallothionein is due to a defect in the reabsorption of the protein from the blood as this would be expected to contain Cu^{2+} (see section VA). It is also significant that, in rats[200], cadmium exposure increases the urinary excretion of Cu-Mt in the absence of cadmium-induced renal damage. These results reinforce the idea that some of the metallothionein excreted in the urine is non-reabsorbed Cd/Cu-Mt, and conflicts with the view of Foulkes[195] that virtually all the filtered metallothionein is reabsorbed in the chronic situation. However, it should be remembered that Foulkes observed that Cd-Mt reabsorption was depressed by 30% in Cd^{2+}-exposed animals when the renal cortical concentrations of the cation exceeded the critical level. More recently, Holt et al.[183] showed that after repeated doses of Cd/Zn-Mt, the renal uptake of Cd (and presumably metallothionein) in rats does not remain a constant proportion of the dose, and either ceases or is reduced to a very low level when the renal Cd^{2+} concentration exceeds 140 μg/g. This is a significant finding, as it suggests that the increased excretion of Cd-Mt in the urine is due to decreased reabsorption of the protein. This, in part, perhaps explains why the kidney Cd^{2+} levels plateau after chronic administration of the cation (see section IVA2).

In conclusion it seems that metallothionein in the urine is a combination of decreased reabsorption and/or renal tubular damage releasing intracellular thionein. In any event there is little justification that the measurement of urinary metallothionein is the best index of Cd-exposure or Cd-induced renal damage. Indeed, the latter is probably better assessed by measuring the excretion of β_2-microglobulin or other low molecular weight proteins (see also Lauwerys et al.[152]).

The studies on plasma metallothionein concentrations in both experimental animals[187,188] and in humans[189,196,199] clearly show that metallothionein is found in the blood, and there can be no doubt that Cd is transported from the liver to the kidney in this form. However, the relationship between plasma and hepatic metallothionein is still not completely understood. As discussed above, in experimental animals after the injection of Cd^{2+} and Zn^{2+} the plasma concentration of metallothionein is proportionally related to that of the liver. This implies that there is some form of concentration dependent transport or secretion from the hepatocyte and raises the question, "How is metallothionein released from the liver?" It is unlikely that the release is analogous to the mechanisms normally associated with secretory proteins, as these are synthesized on ribosomes bound to the endoplasmic reticulum

and released via the golgi apparatus[201]. Two other possibilities exist;

1. The protein is released from dead cells and thus is a consequence of normal cell turnover.
2. The protein is released by an exocytotic or transport mechanism.

With regard to the first idea there is good evidence that this can happen. For example, when the hepatotoxin, aflatoxin, is given to Cd^{2+}-pretreated animals there is a marked and rapid redistribution of Cd^{2+} to the kidney[202]. Later studies by Tanaka et al.[193] and Tanaka[203] have shown that other hepatotoxins (i.e. CCl_4, galactosamine and ethionine) also cause a similar redistribution of Cd^{2+}. This is correlated with increased blood and urine levels of metallothionein, and is concomitant with an elevation of liver marker enzymes (e.g. lactate dehydrogenase) in the plasma. Thus in a situation where there is enhanced liver cell death there is a much greater release of metallothionein. As pointed out previously[202], this has clinical implications in that individuals who have been exposed to Cd^{2+} without any obvious symptoms may at a later stage suffer hepatic injury (e.g. alcohol-induced cirrhosis) which accelerates the transfer of hepatic Cd^{2+} to the kidney and eventually the critical concentration may be reached. Clearly, hepatic cell turnover and cell death must be a contributory factor in determining the metallothionein concentration in the blood.

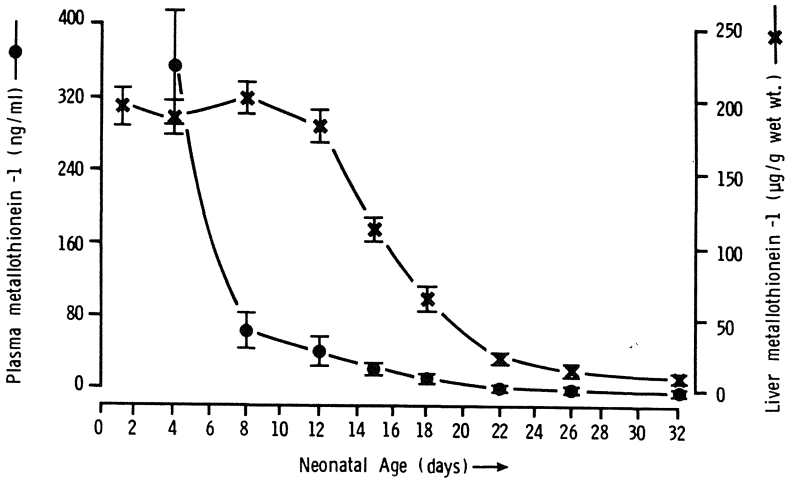

Figure 12 Concentrations of metallothionein-1 determined by RIA in the liver and plasma of neonatal rats. Drawn from the data (Table 1) of Mehra and Bremner[205].

However, there is also evidence that metallothionein can be released from the liver without cell death. Thus, when the liver from Cd^{2+}-fed rats are perfused, Cd-Mt (as measured by RIA and

Cd^{2+} measurements) is released into the perfusate[204]. The concentration of metallothionein in the perfusate eventually plateaued at about 1.1 μg/ml of perfusate. This release was apparently not due to cell damage, as this was monitored by lactate dehydrogenase release and light and electron microscopy of the liver after perfusion. Further evidence that liver damage is not a prerequisite for metallothionein release comes from studies on neonatal rats[205]. These animals have very high levels of hepatic Zn-Mt[8], and Mehra and Bremner[205] have shown by RIA that 4-day-old male and female rats have Mt1 plasma concentrations of 350 and 750 ng/ml respectively. Thereafter there was a steady decline to the adult levels of 3.5 ng/ml at 32 days. However, as shown in Figure 12, the liver levels do not parallel the plasma concentrations, and it is only at day 12 that the hepatic Mt1 concentration begins to drop. The plasma concentrations in the newborn animal are thus extraordinarily high, and it is clear that the equilibrium between the plasma and liver thionein levels is not a simple concentration-dependent phenomenon.

In conclusion, the plasma content of metallothionein will be predominantly determined by the liver content. However, release from the latter will depend on both liver damage and some as yet uncharacterized transport mechanisms. The complex interactions between these two complementary methods of release make the relationship between liver and plasma concentrations very hard to predict. It is therefore extremely doubtful that plasma levels of metallothionein can be used to accurately assess liver concentrations and hence the extent of Cd^{2+} exposure. The latter is probably better assessed by in vivo neutron capture τ-ray analysis to directly analyze the liver and kidney content[206].

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17
DETECTION OF NEPHROTOXICITY OF FOREIGN CHEMICALS WITH THE USE OF IN VITRO AND IN VIVO TECHNIQUES

S. KACEW

INTRODUCTION

The kidney is of paramount importance in the excretion of many potentially toxic drugs and chemicals. Investigations into the possible renal toxicity of such agents helps identify those substances that may be hazardous to health, and also provides information about normal renal function. This chapter will examine the methods used to evaluate chemical-induced nephrotoxicity, in normal experimental animals (and in some cases where some degree of renal failure has been experimentally produced); study the properties and mechanisms involved in organic anion and organic cation transport; and compare the sensitivity of various function tests in assessing nephrotoxicity.

One of the methods used to study the secretory mechanism for organic anions and cations is based on an in vitro renal cortical slice technique developed by Cross and Taggart[1]. p-Aminohippurate (PAH) is commonly used as the prototype for organic anions, while tetraethylammonium (TEA) and N-methyl-nicotinamide (NMN) represent the two most commonly used organic cations. In this procedure, renal cortical slices are prepared from excised kidneys after drug treatment of animals for varying lengths of time. The tissue slices are incubated for 90 min under 100% O_2 in an isotonic medium containing PAH and [^{14}C]-NMN or [^{14}C]-TEA. After incubation, the PAH content of the slices and medium is analysed colorimetrically, while the organic base slice and medium content is determined by scintillation counting, and the active uptake of each of these compounds by kidney slices is reported as the slice to medium (S:M) ratio.

A simplified illustration of the general approach in such studies is shown in Figure 1. In addition to measurement of PAH and NMN transport, a number of other measurements of renal function have been utilized. Glucose synthesis by renal cortical slices

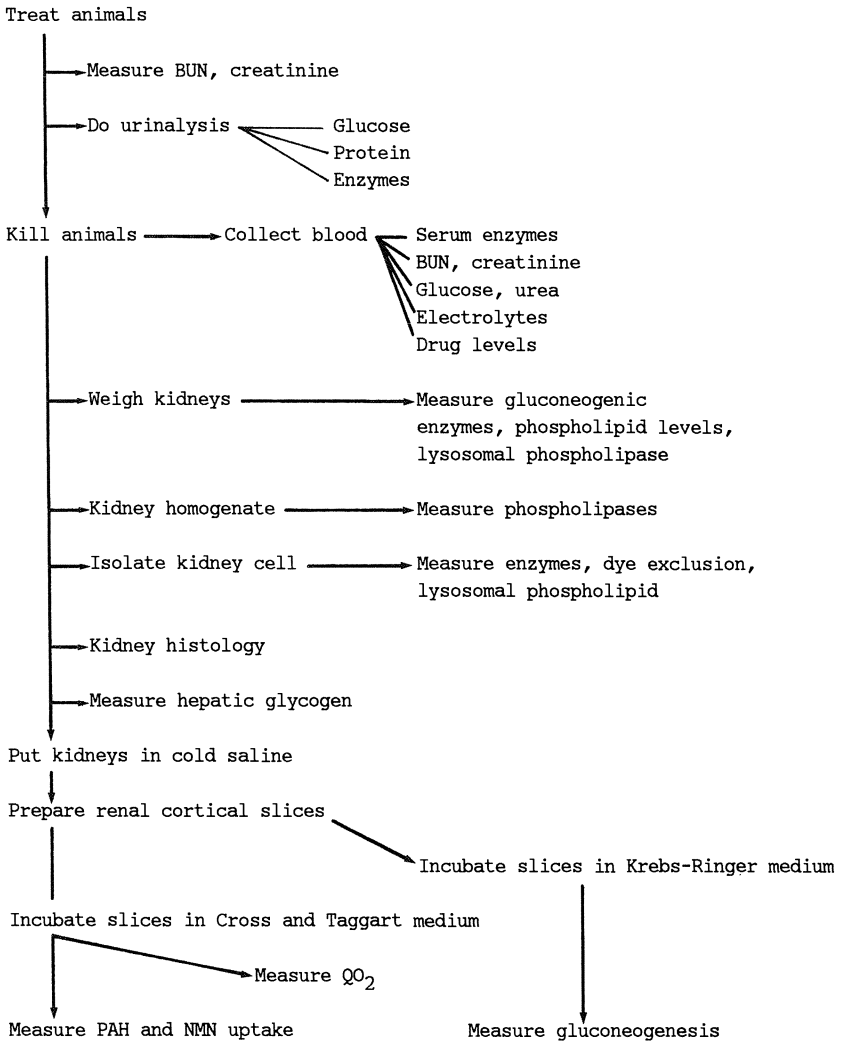

Figure 1 Design of experimental protocols to test renal function.

was determined after incubation under 95% O_2-5% CO_2 for 60 min, with results expressed as μmol glucose/g wet weight of tissue per hour, while oxygen consumption was measured by conventional manometric techniques[1]. Urine was collected during drug treatment for measurement of volume, osmolality, protein, glucose or enzyme content. Blood samples were obtained for determination of blood urea nitrogen (BUN), glucose, creatinine or enzyme levels. The liver was excised for the measurement of hepatic glycogen, while

gluconeogenic enzyme activities were measured in kidneys. Recent studies have revealed that alterations in renal phospholipids are an early manifestation of nephrotoxicity. Hence kidneys were excised for the determination of phospholipid content both biochemically and morphologically.

The kidney slice technique offers several advantages for in vitro studies of renal function and metabolism. Most of the cells are intact; the preparation of experimental samples is straightforward, rapid and samples are easy to handle, and the incubation medium can be manipulated to study effects of substrate or ion changes, etc. Slices from several animals may be pooled, or alternatively slices from a single animal may be divided for use in different flasks. Thus one can compare various parameters directly while minimizing tissue variability; and physiological factors such as tubular obstruction, dehydration or blood pressure changes are eliminated. The reliability of the kidney slice technique decreases in chronic drug-induced renal dysfunction; and thus in vivo function tests are a necessary complement. Ideally, both in vitro and in vivo measurements should be undertaken to establish the nephrotoxic potential of drugs. Furthermore, the assessment of the nephrotoxic potential of chemicals must also include factors such as disease state, age, sex and diet.

A. STUDIES WITH HEAVY METALS

1. Potassium dichromate toxicity

Cellular damage, primarily to renal proximal tubules, occurs following administration of potassium dichromate[2]. Hirsch[3] and Kacew and Hirsch[4] found that 24 h after a single s.c. injection of 5 mg/kg the PAH S/M ratio was significantly increased, without an associated change in NMN transport. Increasing the dose of potassium dichromate to 40 mg/kg enhanced NMN uptake in renal cortical slices, while PAH accumulation was significantly decreased. Significant increases in the kidney:body weight ratio were also observed at the higher dose. The nephrotoxicity following 40 mg/kg potassium dichromate was thus evidenced as a significant reduction in the PAH S/M ratio accompanied by increased tissue water content. Forty-eight hours after 40 mg/kg renal necrosis was extensive and both PAH and NMN transport were significantly decreased[5]. Thus although potassium dichromate produces renal structural damage, histological changes are not directly associated with the effect on organic base transport.

The effect of potassium dichromate on renal anion and cation transport was also investigated in vitro (Table 1). PAH accumulation by renal cortical slices was increased at 10^{-6} mol/l potassium dichromate but decreased at 10^{-3} mol/l, while QO_2 values progressively declined with increasing concentrations of potassium dichromate. NMN accumulation increased as the concentration of potassium dichromate increased up to 10^{-4} M, but at 10^{-3} M potassium dichromate all of the parameters measured, including NMN accumulation, were reduced[3]. Thus, these in vitro observations with PAH and NMN parallel the results obtained after treating the rats

with potassium dichromate in vivo.

Table 1 Summary of effects of incubation of control renal slices with heavy metals on organic ion transport

Metal	Concentration	PAH accumulation	NMN or TEA accumulation	Ref.
Potassium dichromate	10^{-6} mol/l	↑	↑	4,5
	10^{-4} mol/l	↓	↑	4,5
	10^{-3} mol/l	↓	↓	4,5
Organic mercury	10^{-4} mol/l	↓	↓	17,19
Cisplatin	500 µg/ml	↓	↓	77

↑ = stimulation ↓ = inhibition

The runout or efflux of [^{14}C]NMN or [^{14}C]PAH was studied in renal cortical slices preloaded with NMN or PAH for 90 min[6]. The slices were then transferred through a series of 12 beakers containing incubation medium, but no NMN or PAH, and the data were expressed as dpm of NMN or PAH remaining in the slices as a function of the runout beaker number (time). The efflux of NMN from slices from treated animals was significantly less than that from control animals. The initial concentration of NMN was higher in slices from treated rats, and the rate of efflux significantly less; whereas PAH efflux was similar in slices from control rats and rats injected with 5 mg/kg potassium dichromate, and significantly greater in slices obtained from rats given 40 mg/kg potassium dichromate[6]. This is consistent with the observation that 40 mg/kg potassium dichromate produced substantial inhibition of renal slice PAH content.

The cortex to serum (C/S) ratio of [^{14}C]NMN was higher in rats which had been given 40 mg/kg potassium dichromate 24 h earlier, while urinary NMN excretion was substantially less than controls[7]. Stimulation of the NMN C/S ratio in vivo and the decreased efflux of NMN in vitro indicate that an accumulation following potassium dichromate treatment is related to enhanced organic base retention in the renal cortical cells[7]. Whether this retention is due to specific binding of NMN by organic base receptors, or merely to binding of NMN to denatured protein, has not been established. Potassium dichromate increased BUN levels and inhibited glucose synthesis[3] by cortical slices 24 h after injection of 25 or 40 mg/kg. It should be emphasized that the tissue slice method is an effective tool for measuring the early response to

acute injury. The use of urinary protein and glucose may be more sensitive metabolic indicators of subsequent renal dysfunction. Baines[8] demonstrated that between 7 and 10 days after a single s.c. injection of potassium dichromate (10 mg/kg), urinary glucose and protein levels increased in the rat. Thus, it is evident that the in vivo experiments corroborate the in vitro observations with kidney slices.

2. Uranyl nitrate toxicity

Histopathological damage in the proximal convoluted tubules is a well-established consequence of uranyl nitrate[9,10]. In vivo uranyl nitrate was found to decrease inulin clearance (glomerular filtration rate) and PAH clearance[9,11,12]. Blantz[12] demonstrated that the uranyl nitrate-induced decrease in glomerular filtration rate could be attributed to a reduction in total glomerular permeability. It is of interest that this decreased glomerular permeability occurs prior to the onset of morphological alterations. This suggests that measurement of renal function is a sensitive early indicator of uranyl nitrate toxicity.

In an effort to establish the nephrotoxic action of various agents by means of function tests in vitro, Hirsch[10] found that treatment of adult male rats with uranyl nitrate produced a specific decrease in cortical NMN uptake, while PAH accumulation remained unchanged. Forty-eight hours after a single i.p. injection of 6 mg/kg uranyl nitrate the body weight was not significantly altered, but the kidney/body weight ratio increased, representing a drug-induced elevation in renal weight. Additional experiments demonstrated a general effect of uranyl nitrate on organic cation transport, since both TEA and NMN accumulation were enhanced in cortical slices 24 h after uranyl nitrate treatment (Table 2). Doses of uranyl nitrate as low as 0.5 and 1.0 mg/kg specifically stimulated NMN uptake by renal cortical slices, without affecting PAH transport 24 and 48 h after dosing. The degree of structural damage elicited by 0.5 mg/kg uranyl nitrate was mild proximal tubular cell hydropic change, with isolated areas of cellular necrosis, which was not directly related to the degree of stimulation of NMN transport[4,5]. When 6 mg/kg uranyl nitrate was administered, extensive cellular necrosis was evident, although the degree of NMN stimulation produced at these two doses was comparable.

Although both uranyl nitrate and potassium dichromate enhanced cortical NMN accumulation, the stimulation of this proximal tubular transport system appears to be a non-specific response to cellular injury. Administration of mercuric chloride (2.5 or 5 mg/kg) produced renal damage that was both time- and dose-related as measured by histological and metabolic techniques[3]. PAH and NMN S/M ratios were not stimulated following mercuric chloride, suggesting that this nephrotoxin may act at different sites in tubular cells as compared with uranyl nitrate or potassium dichromate.

Table 2 Summary of alterations in organic ion transport in renal slices obtained from metal-treated animals

Metal	Dose (mg/kg)	PAH accumulation	NMN or TEA accumulation	Ref.
Potassium dichromate	5	↑	--	3,4
	25	↓	↑	4,7
	40	↓	↓	5
Uranyl nitrate	1	--	↑	10
	6 at 24 h	--	↑	10
	6 at 48 h	--	↓	10
Organic mercury	1 for 20 days	--	↓	23
	0.5 to 2 for 6 days	↓	N.D.	30
Inorganic mercury	1 at 8 or 12 h	↑	N.D.	29
	1 at 16 or 24 h	↓	N.D.	29
	1 for 20 days	↓	↑	23
Lead	4% in diet	--	--	44
Cadmium	1	↓	N.D.	62
	5	↓	N.D.	78
Cisplatin	5 (normal diet)	↓	↓	77
	5 (restricted diet)	--	--	77

↑ = stimulation; ↓ = inhibition; -- = no change; N.D. = not done

The age related changes in tubular transport, distribution of renal blood flow and the perfusion of superficial glomeruli[13,14] appear to play a role in the responsiveness of kidney to nephrotoxic agents. Pelayo et al.[15] found that 24 h after administration of a 10 mg/kg dose of uranyl nitrate, the glomerular filtration rate was not markedly altered in 1-2-week-old dogs, but was significantly suppressed in 3-5-week-old animals. In addition, morphological alterations in proximal tubules were most evident in the older animals. At present the effect of uranyl nitrate on PAH and NMN uptake of renal cortical slices from immature animals remains to be determined. However, in vivo data suggest the developing kidney is less sensitive to the nephrotoxic action of uranyl nitrate, and that further studies are necessary to elucidate the basis for this protective mechanism.

3. Mercury toxicity

The organic acid mercurial diuretics meralluride and mercaptomerin and the non-organic mercurial chlormerodrin inhibit PAH[16] and NMN[17] transport with approximately the same potency (50% inhibition near 5 x 10^{-4} mol/l) and decrease glucose transport[16]. These findings using cortical slices obtained from mercurial-treated dogs and rats suggested that mercurial compounds exert a general inhibitory action on anion transport.

Since the S/M ratio of PAH and NMN is measured in a steady state, it was considered possible that the effect of the mercurials was on the retention of organic ions, rather than directly on the transport processes. Ross and Farah[18] demonstrated that the rate of PAH accumulation during very early incubation times can be used to measure the rate of transport (since at these early time periods the intracellularly accumulated PAH is not of sufficient concentration to alter the net flux). Both the organic acid mercurial mercaptomerin and non-acidic chlormerodrin decreased the rate of PAH entry into the rat renal cortical slices[17]. PAH and NMN uptake was also inhibited by about 50% by mercuric chloride and methyl mercury at 10^{-4} mol/l[19]. These slice studies thus demonstrated that the inhibitory effect of organic mercurial compounds on PAH transport is non-specific, and probably reflects a general decrease of metabolic activity produced by these compounds.

The environmental pollutant methyl mercury is a well-established nephro- and neuro-toxin. Ultrastructural evidence of nephropathy includes swelling of proximal tubule epithelial cells in guinea pigs[20], degeneration of proximal tubule associated with basophilic cytoplasm, and disappearance of brush border in weanling rats[21]. In adult rats occasional exfoliation, and an increase in cellular cytoplasm, and mitochondrial damage has been observed in the pars recta[22]. Using renal cortical slices taken from rats administered methyl mercury (1 mg/kg daily) for 20 days, Stroo and Hook[23] demonstrated a significant decrease in NMN accumulation, while the uptake of PAH remained unchanged. A similar decrease in the ability of kidney slices to concentrate NMN and PAH was noted by Hirsch[19] in animals exposed to methyl mercury. The observed mitochondrial alteration associated with lowered S/M ratios was suggested by Stroo and Hook[23] to reflect mitochondrial dysfunction, a possible early functional sequela of methyl mercury poisoning.

Methyl mercury-induced nephrotoxicity has also been demonstrated in vivo. Daily i.p. injections of methyl mercury for 45 days produced a decrease in hepatic glycogen associated with a rise in blood glucose[24]. The observed hyperglycaemia was accompanied by glucosuria and an elevation in the kidney gluconeogenic enzymes. There is some evidence to suggest that methyl mercury produces an initial decrease in gluconeogenesis, followed by stimulation. Whereas Stroo and Hook[23] noted that gluconeogenesis was not altered in renal cortical slices from 20-day methyl mercury-treated rats, Chang et al.[25] reported that methyl mercury injection for 4 weeks decreased renal glucose-6-phosphatase. Verschuuren et al.[26] found that 12 weeks exposure to 25 ppm

methyl mercury resulted in glucosuria, hepatic glycogenolysis and increased glucose-6-phosphatase activity. Methyl mercury also produces a proteinuria[27] and increases the concentration of blood urea[24,26]. In contrast to the actions of cadmium, urinary enzyme excretion was not markedly altered by methyl mercury[23]. However, histochemical examination of several enzymes in methyl mercury-treated animals indicated that this metal is nephrotoxic to the proximal convoluted tubule[25]. This indicates that renal enzyme histochemistry may be used as an adjunct to aid in the establishment of methyl mercury intoxication.

One agent used to produce an experimental model of acute renal failure is inorganic mercury, which produces selective necrosis to proximal tubules, calcification and vacuolization of the cytoplasm, as well as mitochondrial swelling[28]. Intravenous injection of 1 mg/kg per rat of mercuric chloride increased PAH uptake into renal cortical slices after 12 h; however, there was a marked decrease in PAH accumulation by 16 h[29]. The observed decrease in PAH uptake by cortical slices corresponded with the appearance of renal necrosis. Phillips et al.[30] also demonstrated that mercuric chloride reduced the uptake of PAH by kidney cortical slices, and that the observed fall occurred prior to any morphological evidence of damage. In addition to a decrease in PAH uptake, Stroo and Hook[23] found that mercuric chloride-treated renal cortical slices displayed an increased ability to accumulate NMN.

Ellis et al.[31] reported that PAH clearance and T_m were significantly decreased only in the late stages of mercuric chloride intoxication in dogs, suggesting that other functional tests are necessary for the in vivo determination of inorganic mercury poisoning in this species. Under the experimental conditions employed, the assay of urinary alkaline and acid phosphatases proved the most sensitive in the detection of mercuric chloride nephrotoxicity. A similar increase in urinary enzyme excretion was found by Stroo and Hook[23] in the rat. An elevation in serum urea nitrogen and proteinuria also resulted from inorganic mercury intoxication[32]. Abnormal protein metabolism, as evidenced by a marked reduction in renal succinic dehydrogenase, ATPase, and alkaline phosphatase, was suggested by Chang et al.[25] to be an index of kidney dysfunction. In contrast, Ellis et al.[31] found that mercuric chloride generally failed to alter the activities of certain serum enzymes. Based on these results it would appear that estimation of urinary rather than serum enzymes is a more reliable index of mercury intoxication.

Finally, one of the indications of mercuric chloride-induced renal damage is the presence of glucosuria[33]. It is conceivable that the urinary source of glucose may be derived from gluconeogenesis. However, Chang et al.[25] found that mercuric chloride decreased the activity of both renal and hepatic glucose-6-phosphatase. A similar decrease in glucose-6-phosphatase activity was reported by Phillips et al.[30] in mercuric chloride-treated kidneys. In addition to glucose-6-phosphatase, mercuric chloride reduced the activities of pyruvate carboxylase, phosphoenolpyruvate carboxykinase and fructose-1,6-diphosphatase. Indeed, Phillips et al.[30] found that PAH uptake was a more sensitive index of mercuric chloride-induced nephrotoxicity compared to altered

gluconeogenic capacity. At present the source of urinary glucose observed in inorganic mercury poisoning is not known, but hepatic glycogenolysis or decreased proximal tubule glucose reabsorption may play a contributory role.

Searches for agents which ameliorate mercuric chloride-induced acute renal failure have used morphologic evaluation and renal function tests such as glomerular filtration rate (GFR) and BUN[34]. Dobyan and Bulger[34] demonstrated that pretreatment of rats with chlorpromazine prevented ultrastructural changes, and the mercury-induced decrease in GFR and increase in BUN. In contrast, Klonne and Johnson[35] found the amelioration of mercuric chloride-induced nephrotoxicity by dithiothreitol in rats was independent of GFR. These findings suggested that the use of GFR as an index of restored renal function may not be adequate. Since dithiothreitol restores the sulphydryl status of kidney enzymes[35], the ability to elevate sulphydryl levels may serve as an indicator that a compound is useful in providing protection against mercuric chloride. Indeed, Fukino et al.[36] reported that zinc pretreatment prevented mercuric chloride-induced kidney damage by increasing glutathione. However, depletion of glutathione by diethyl maleate also afforded some protection against mercuric chloride-induced renal dysfunction[37,38]. Clearly the mechanisms involved in amelioration of mercuric chloride-induced acute renal failure by various agents require further examination.

The role of age in mercuric chloride-induced nephrotoxicity is controversial. Some evidence indicates that the stage of kidney development may be a primary factor in the responsiveness, but the data are equivocal. Wold et al.[39] demonstrated that mercuric chloride was equi-nephrotoxic in newborn adult rabbits using tubular necrosis and an increase in BUN as the criteria. Bidani et al.[40], however, found that mercuric chloride produced a greater mortality rate and elevated BUN in 2-week-old rats as compared to more mature 4- and 8-week old animals, indicating that a young rat was more sensitive to the nephrotoxic action of this metal. By contrast, in a study using numerous indices of renal function, Daston et al.[32,33] demonstrated that neonatal rats appeared relatively insensitive to mercuric chloride-induced kidney damage, and that nephrotoxicity was enhanced in frequency and severity with increasing age.

4. Lead toxicity

The toxic manifestations of chronic plumbism include the formation of intranuclear bodies, interstitial nephritis and contracted kidney tumours[41-43], which have been studied by a variety of methods[44-60]. In order to correlate functional defects with lead-induced histological changes, Hirsch[44] placed lead acetate (2 or 4%) in the diet of dams so that newborn rats were exposed from birth via the milk. Immediately after weaning, these lead-exposed newborns received the metal in their diet for up to a period of 40 weeks. Some vacuolar degeneration occurred in the S_3 segment of the proximal tubule after 30 days on 2% lead acetate, and in addition cellular necrosis occurred when 4% lead acetate was used. The most

pronounced effects were observed when rats were maintained on diets containing 2% lead acetate for the first 10 weeks of life; in this group lead inclusion bodies, cellular necrosis and sloughing of tubular cells were observed. These minimal histological changes were associated with functional abnormalities using kidney slices. While lead exposure did not markedly alter the renal transport of organic anion (PAH) or organic cation (TEA), there was a significant decrease in the capacity of kidney slices to synthesize glucose and metabolize pyruvate. While the tissue slice method has proved to be an effective tool in the measurement of acutely induced renal tubular necrosis, this experimental technique is somewhat limited in the study of chronic toxicity, a situation encountered in plumbism. It has been established that a sensitive in vivo metabolic index of kidney function involves the measurement of glucose in urine. Singhal et al.[54] demonstrated that daily i.p. injection of 0.2 or 1.0 mg/kg lead for 45 days resulted in an increased excretion of urinary glucose. The observed urinary glucosuria was accompanied by an increase in the concentration of blood glucose. A similar increase in urinary glucose excretion associated with morphological evidence of renal damage was reported by Goyer[45], Chisolm[46] and Zook[47].

Although a slight increase in blood glucose was found in sheep exposed to lead for 27 weeks[48] hyperglycaemia was a marked feature in humans with plumbism[49]. In order to determine whether hepatic glycogenolysis contributed to the observed blood glucosuria, Singhal et al.[54] measured the effects of prolonged lead treatment on liver glycogen levels. Daily administration of lead for 45 days decreased the concentration of hepatic glycogen, suggesting that the liver may be a source for the urinary glucose. These data are supported by the findings of Cornell and Filkins[49] and Rippe and Berry[50], who reported that lead decreased hepatic glycogen levels.

Children are generally more susceptible than adults to poisoning by lead. In order to simulate human lead poisoning with an animal model, Stevenson et al.[51] administered 2% lead as a solution in the drinking water of rat dams for 21 days. After weaning, the metal-exposed pups were maintained for 35 days on drinking water containing either 20, 40 or 80 ppm lead. As in the case of adult rats[54] all three concentrations of lead decreased hepatic glycogen levels and elevated blood glucose in a dose-dependent manner (Table 3). The 20 ppm dose of lead significantly enhanced the activities of renal phosphoenolpyruvate carboxykinase and pyruvate carboxylase. When the concentration of lead was increased to 40 or 80 ppm a resultant elevation in the quartet of kidney gluconeogenic enzymes occurred. The lead-induced increase in gluconeogenic enzyme activity was related to the dose with maximal changes produced by the highest metal concentration used. In contrast to these findings, in vitro measurements using rat kidney slices[44] or isolated hepatocytes[49] demonstrated that lead inhibited the process of gluconeogenesis. Since differences in experimental protocol yield variations in data obtained, it would appear that measurement of glucosuria is not a sensitive measure of lead-associated nephropathy.

Table 3 Influence of cadmium or lead on various parameters associated with renal function in rats

Metabolic parameter		Control[a]	Cadmium	Control[a]	Lead
Blood glucose (mg/100 ml)		81±2	150±2[b]	72±4	179±5[b]
Serum urea (mg/100 ml)		26±1	34±1[b]	18.6±1.5	24.1±1.5[b]
Liver glycogen (g/100 g)		2.2±0.1	0.9±0.1[b]	3.6±0.6	0.8±0.2[b]
Kidney cortex gluconeogenic enzymes (μmol/h/mg protein)	Pyruvate carboxylase	74±3	160±7[b]	94±3	213±20[b]
	Phosphoenol-pyruvate carboxykinase	25±2	34±1[b]	21.5±1.4	58.7±8.6[b]
	Fructose-1,6-diphosphatase	7.2±0.5	11.9±0.3[b]	5.2±0.5	13.5±2.1[b]
	Glucose-6-phosphatase	10±1	13±1[b]	7.5±1.2	13.5±1.6[b]

[a] Means S.E.M. represents 5 animals in each group. Cadmium (1 mg/kg/day) was administered i.p. for 45 days while controls received physiological saline. Lead (0.2%) was present in maternal drinking water for the first 21 days and for the subsequent 35 days 80 ppm metal was present in the infant water supply. Corresponding controls had free access to physiological saline in drinking water.

[b] Statistically significant difference when compared with corresponding control values ($p<0.05$).

Renal dysfunction produced by lead has been determined with the use of other metabolic parameters. Inhibition of Na^+,K^+-ATPase was demonstrated in human and rat kidney[52,53]. Chronic administration of lead was found to elevate the concentration of BUN[44,54]. The increased urea levels in blood are associated with enhanced excretion of phosphate, uric acid and amino acids in urine[45,46]. In particular, the measurement of the urinary amino acid, δ-aminolaevulinic acid, is considered a specific indicator of lead poisoning[55]. In addition to enhanced excretion of certain proteins, Choie and Richter[56,57] demonstrated that lead treatment initiated the synthesis of RNA, protein and DNA in renal tissue. Similarly, Cihak and Seifertova[58] noted an augmentation in the incorporation of thymidine into renal DNA in mice given an intracardiac injection of lead acetate. Stevenson et al.[59] also found that a single i.p. injection of lead to adult rats increased the incorporation of thymidine into renal DNA, and this observed elevation was preceded by a rise in the incorporation of orotic acid into RNA. In order to assess whether lead exerts any influence on growth processes in tissues of developing rats, 1-day-old pups were administered 50 μg of lead daily via gastric intubation for 3 weeks, and thereafter received 80 ppm metal in their drinking water for 35 days. This treatment produced no appreciable differences between corresponding controls and metal-administered animals with respect to the ability to gain weight, and thus the observed changes could be attributed to lead and not to dietary or nursing habits. Treatment of rat pups from birth with lead revealed that kidney retained the metal during the entire exposure period with the highest amount noted after 2 weeks[60]. Although metal administration failed to alter thymidine incorporation into renal DNA after 2 weeks, a reduction was noted after 4 weeks. This was sequentially followed by elevation in the incorporation of thymidine into kidney DNA in animals treated for 8 weeks. In summary, it would appear that lead may not produce renal damage in all cases, and hence a diversity of metabolic correlates are needed to establish the presence of nephrotoxicity.

5. Cadmium toxicity

A variety of experimental techniques have been utilized to establish the nephrotoxic actions of cadmium. Cadmium produces degenerative changes in kidney tubules and glomeruli of rats, rabbits and humans[61]. Renal cortical slices obtained from rabbits given a single intravenous injection of cadmium showed markedly inhibited PAH uptake[62]. Gieske and Foulkes[62] substantiated these in vitro results with the in vivo findings that cadmium decreased the clearance and extraction of PAH in rabbits. Indeed, it was suggested that this inhibition of PAH transport might reflect a direct cytotoxic action of cadmium on cortical tissue rather than via intrarenal blood flow distribution. A similar cadmium-induced decrease in the ability of kidney tubules to secrete PAH was found following acute cadmium treatment of dogs[63] and subacute cadmium administration to rabbits[64]. However, chronic cadmium administration to rabbits for 24 weeks failed to significantly alter PAH

transport[65], indicating that the duration of exposure may play a critical role in the use of PAH transport as a measure of renal function.

Cadmium produces proteinuria and glucosuria. Nomiyama et al.[65] found that the earliest signs of cadmium intoxication in rabbits were amino-aciduria and enzymuria, subsequently followed by proteinuria and glucosuria. In addition to these urinary indices of nephrotoxicity, cadmium elevated blood glucose and decreased hepatic glycogen (Table 3); biochemical parameters that contribute to glucosuria[66,67]. Using several renal function tests, Nomiyama et al.[65] concluded that the presence of urinary acid phosphatase, alkaline phosphatase, glutamic pyruvic transaminase and glutamic-oxaloacetic transaminase, was the method of choice for detection of early cadmium intoxication in chronically exposed rabbits. In humans, however, Nogawa et al.[68] found that a cadmium exposed individual excreted elevated amounts of glucose and protein in urine; with the most sensitive and consistent finding being an increase in β_2-microglobulin.

It is of interest that renal dysfunction as evidenced by glycosuria and proteinuria was more severe in older rabbits administered cadmium[69], a phenomenon previously shown for mercuric chloride[32,33] and uranyl nitrate[15]. Additional studies using an in vitro approach are necessary to clarify observations found in developing animals exposed to cadmium.

6. Nickel toxicity

Although nickel accumulates to the greatest extent in kidney[70], relatively few studies have focussed on the potential consequences. Histopathologically, nickel-produced nephropathy as evidenced by multifocal proximal tubule degeneration with necrosis[71]. In vitro parameters of renal dysfunction remain to be examined, but nickel was reported to produce proteinuria, glycosuria and hyperglycemia[71-73]. Waalkes et al.[71] demonstrated that zinc pretreatment reduced the severity of nickel-induced nephropathy as assessed by ultrastructural damage and decreased hyperglycaemia compared to rats given only nickel. It is of interest that zinc has also proved effective in protection against cadmium-induced nephrotoxicity[74].

7. Platinum toxicity

The nephrotoxic actions of the platinum-containing antineoplastic agent cisplatin have been well-documented. Histologically, the renal lesions induced by cisplatin have been characterized by acute proximal tubular necrosis[75,76]. Incubation of kidney slices from control rats with cisplatin resulted in a marked decrease in PAH and TEA accumulation[77]. When renal slices were obtained from male Wistar rats administered 5 mg/kg cisplatin, a decrease in PAH accumulation was noted[78]. Goldstein et al.[77] reported that PAH and TEA accumulation in kidney slices of male Fischer-344 rats administered 5 mg/kg cisplatin was reduced; however, this functional

difference disappeared when compared to pair-fed animals. These data suggested that food intake played a role in the observed in vivo effects of cisplatin on renal cortical slice function. Since diet was not controlled in the study, reported by Cojocel et al.[78] the decreased kidney function might be due to lower food intake. In comparison between in vivo and in vitro renal slice techniques, the reduction in PAH and TEA accumulation is far more pronounced in the in vitro model, probably due to a higher concentration of cisplatin in direct contact with the organic ion transport systems[77].

Cisplatin-induced renal dysfunction has also been demonstrated by means of several metabolic indices. Cisplatin decreased glomerular filtration rate, and increased creatinine, BUN, glucose and glucagon in serum[77,79,80]. It is of interest that the cisplatin-induced hyperglycaemia was not associated with enhanced renal or hepatic gluconeogenesis[80], which is in contrast to cadmium[66] or mercury[24]. In an attempt to establish more sensitive tests for the early detection of cisplatin-induced nephrotoxicity, evidence has been accumulated to show that urine analysis provides more consistent results than in vitro transport studies[77]. Various investigators demonstrated that cisplatin produced glucosuria and proteinuria, particularly increased excretion of the low molecular weight protein β_2-microglobulin in the absence of serum changes[77,81]. Further, urinary magnesium wasting was attributed to result in the observed hypomagnesemia[82,83]. While it is clear that cisplatin-induced nephrotoxicity can be demonstrated with a whole host of in vitro and in vivo parameters, the mechanism(s) that cause kidney dysfunction remain to be elucidated.

8. Lithium toxicity

Although it does not belong to the category of heavy metals, lithium exerts a toxic effect on the kidney comparable to mercuric chloride and may lead to acute renal failure[84]. Chronic lithium administration was found to produce focal atrophy and fibrosis in human kidney[85]. In rats, lithium treatment resulted in renal tubule cell damage characterized by mitochondrial swelling, dilatation of rough endoplasmic reticulum and swelling of apical cytoplasm[86]. Schou[87] demonstrated that one of the metabolic indices of lithium-induced nephrotoxicity was an elevation in BUN. In addition to increased BUN levels, Thomsen and Olesen[84] found that lithium significantly decreased the tubular maximum for PAH and clearance for inulin. In common with lead[58,59], lithium was found to augment the incorporation of thymidine into DNA[88,89]. In a recent study, Kling et al.[89] were unable to produce interstitial nephritis and tubular atrophy in lithium administered rats. As there was a major difference in the type of diet employed between Kling et al.[89] and Hestbech et al.[85], it was suggested that lithium-induced nephropathy was derived from the synergistic interaction between dietary factors and perhaps metals. This may also explain why renal failure is not a consistent finding in patients on lithium therapy[88,90]. At present, data using the in

vitro kidney slice technique to support the nephrotoxicity of lithium are lacking.

B. ANTIBIOTIC TOXICITY

The in vitro slice technique has also been used to evaluate the nephrotoxicity of gentamicin, following the clinical reports of toxicity[91-93]. This aminoglycoside antibiotic is closely related to neomycin, kanamycin and streptomycin, and they are all ototoxic. Vestibular damage has been reported, following gentamicin particularly in patients with impaired renal function[94]. Next to vestibular damage, nephrotoxicity is the most important side effect of gentamicin. After parenteral administration, elimination of gentamicin is primarily via the kidneys by glomerular filtration, with 80 to 90% of the injected dose being recovered in the active, unchanged form in the urine[95]. In patients with renal failure, prolonged elevation of plasma gentamicin levels is observed due to its decreased clearance[96]. The early signs of nephrotoxicity in patients are elevated BUN and plasma creatinine levels. However, recent evidence suggests that the increase in serum creatinine is secondary to the aminoglycoside-induced damage to renal tubules, and that other more stringent indices of kidney damage need to be determined in humans[97]. Urinary β_2-microglobulin is very sensitive to gentamicin-induced renal injury, and may therefore provide early warning signs of impending creatinine rise[98]. In critically ill patients, Schentag[97] found that while aminoglycoside therapy eventually elevated serum creatinine, the measurement of urinary casts and β_2-microglobulin was a more sensitive early indicator of impending serum changes.

Hirsch[99], using the in vitro slice technique to investigate the possible nephrotoxicity of gentamicin, made the following observations. At the lowest dose used (15 mg/kg), no significant changes were observed in the parameters measured after 3 weeks of treatment. In addition, Cojocel et al.[78] reported that 15 and 30 mg/kg gentamicin for 5 days did not markedly alter PAH uptake by rat renal cortical slices. These findings are in agreement with those of Forrey et al.[100] who reported that a dose as low as 2 mg/kg gentamicin given every 8 h for 3 days failed to affect human renal function as evidenced by no marked changes in creatinine and PAH clearance, nor in urinary enzyme excretion. However, by increasing the dose to 40 mg/kg, Hirsch[99] found some indications of renal toxicity. Both glucose synthesis and O_2 consumption were inhibited, and the kidney:body weight ratio was increased. NMN transport, glucose synthesis and changes in kidney weight appeared to be most sensitive to gentamicin treatment., these parameters were also significantly different from those in controls after 1 or 2 weeks of treatment at 70 mg/kg gentamicin. PAH accumulation and BUN levels were not changed[99]. Williams et al.[101] reported no significant alteration in renal cortical slice transport of PAH and NMN; 1.5 h after a 100 mg/kg dose of gentamicin, however, the activity of Na^+,K^+-ATPase was decreased. When the duration of treatment was increased to 4 days, Kluwe and Hook[102] demonstrated that 100 mg/kg gentamicin decreased the up-

take of NMN by renal cortical slices, while PAH transport remained unchanged. In contrast, Cohen et al.[103] reported that gentamicin increased PAH uptake by kidney cortical slices without changing NMN transport. Kluwe and Hook[102] also showed that gentamicin reduced gluconeogenesis and ammoniagenesis, and that this was accompanied by an increased excretion of glucose and B-galactosidase in urine. Kluwe and Hook[102] concluded that using the renal cortical slice technique, transport of organic ions, resting ammoniagenesis and glucosuria appeared to be the most sensitive indicators of gentamicin-induced nephrotoxicity. The finding that inhibition of Na^+,K^+-ATPase occurred prior to alterations in organic ion transport suggested that the transport system may not be involved in the pathogenesis of drug-induced dysfunction, but serve merely as a consequence of the disorder. The use of basolateral kidney membranes for the study of organic ion transport has an advantage over tissue slice techniques in that initial rates can be determined, and steady-state values quantified. Using this technology, Williams et al.[104] found that gentamicin inhibited the uptake of NMN in the basolateral membrane preparation without a marked effect on PAH transport. In common with tissue slices, gentamicin reduced the activities of membrane Na^+,K^+-ATPase and adenylate cyclase and calcium content. Although the precise role of these parameters in the pathogenesis of gentamicin nephrotoxicity remains to be elucidated, the measurement of organic cation transport is a more sensitive index of drug-induced kidney dysfunction.

In the search for parameters of in vitro toxicity, investigators have taken a step beyond a plasma membrane preparation to isolated rabbit kidney cells. The release of lactic dehydrogenase by kidney cells is proposed to reflect cell lysis[105] while uptake of the dye, neutral red, is an index of cellular viability[106]. Incubation of rabbit kidney cells with gentamicin resulted in inhibition of dye uptake, this parameter being indicative of renal damage. In addition, Viano et al.[105] noted a significant increase in lactic dehydrogenase activity in the incubation medium. Consequently it was proposed that measurement of lactic dehydrogenase in kidney cell media might serve as an important pre-screening parameter of potential antibiotic toxicity.

Exacerbation of gentamicin nephrotoxicity by methoxyflurane has been reported by Barr et al.[107]. Hirsch[99] has also shown that gentamicin toxicity was increased in rats suffering from glycerol-induced renal failure. Gentamicin alone (70 mg/kg i.m. for 8 days) produced changes in kidney weight and glucose synthesis, while glycerol (1 ml/100g of 50% glycerol in saline s.c., on days 1 and 8) produced no changes. Administration of both compounds, however, caused increased BUN levels and kidney weight, reduced PAH and NMN transport by kidney slices, and inhibited glucose synthesis. Measurement of gentamicin in serum confirmed the in vitro slice observations, in that the gentamicin serum concentrations were significantly higher when both gentamicin and glycerol were administered[99]. Thus, the in vitro method accurately demonstrated the changes in toxicity and reflected the changes in gentamicin serum concentration. Although the in vitro slice techniques remain to be performed, gentamicin-induced nephrotoxicity can be modified in different ways.

Table 4 Factors influencing aminoglycoside-induced nephrotoxicity

Enhanced toxicity	Ref.	Amelioration of toxicity	Ref.
Methoxyflurane	107	Calcium-supplemented diet	109
Glycerol	99	Streptozotocin-induced diabetes	110
Low potassium diet	108	Modification in chemical structure to hydroxy-gentamicin	139
Advancing age	129-132		
Male (more susceptible)	111,112		

Gentamicin produced a more prominent renal tubular necrosis and organ dysfunction in dogs placed on a low potassium diet compared to control or potassium diet-supplemented dogs[108]. It is of interest that gentamicin levels were highest in renal cortex of the low potassium diet-administered dogs. In contrast, administration of gentamicin to rats given a calcium-supplemented diet resulted in amelioration of antibiotic-induced acute renal failure as measured by BUN and mitochondrial function[109]. Surprisingly, calcium supplementation did not lower kidney gentamicin content suggesting that calcium does not interfere with antibiotic uptake but may exert an effect at a subcellular level. Recently, Teixeira et al.[110] noted that in streptozotocin-induced diabetes in rats, treatment with gentamicin failed to produce kidney dysfunction. The ability of the diabetic state to confer protection against antibiotic-induced renal damage was associated with reduced kidney gentamicin content. While the role of the hormone, insulin, in the accumulation of gentamicin in kidney cortex and subsequent tissue damage needs further investigation, the influence of sex hormones has been examined. Bennett et al.[111] demonstrated that in female rats gentamicin did not markedly alter PAH uptake by kidney cortical slices. However, gentamicin produced a transient stimulation of PAH uptake in tissue slices followed by a marked decrease in male rats[111,112]. Although NMN transport was decreased in kidney slices of both sexes, the male transport system was more severely altered and was associated with higher gentamicin levels in renal tissue. Clearly, these studies demonstrate that the ability of agents to modify gentamicin transport into the renal cortex ultimately plays a role in antibiotic-induced alterations in organ function (Table 4).

Cohen et al.[103] studied the effect of gentamicin on renal function in Sprague-Dawley rats, in an attempt to identify "the early functional correlates of gentamicin toxicity". BUN and serum creatinine levels were somewhat elevated after 4 days of gentamicin treatment at 100 mg/kg, while the urinary concentrating capacity

was reduced. Gentamicin nephrotoxicity has been reported[113] to be associated with structural changes in the proximal tubules. In particular, Kluwe and Hook[102] provided evidence to suggest that gentamicin produces a mitochondrial lesion in proximal tubules. Cohen et al.[103] found that gentamicin-treated rats secreted PAH at a higher rate than control animals. The results observed in their in vivo experiments were then confirmed by in vitro studies, in which renal cortical slices from rats treated with gentamicin (100 mg/kg/day, i.m.) accumulated PAH to a greater degree than slices from control animals. The PAH S/M ratio was elevated to the greatest extent 4 days after treatment, with incubation times varying from 60 to 180 min. An increase in PAH accumulation was also reported in renal cortical slices obtained from gentamicin-treated male rats after 7 days; however, this was followed by a decrease after 10 days[111,112]. Similarly, the uptake of NMN in kidney slices was impaired 10 days after gentamicin. The possibility thus exists that gentamicin affects organic anion transport prior to organic cation accumulation.

The production of nephrotoxicity implies the development of impaired renal function, usually accompanied by histological changes. The relationship of enhanced PAH transport to the subsequent appearance of renal failure and proximal tubular necrosis is not clear. Indeed, Bennett et al.[111] found female rats were far less susceptible to gentamicin-induced changes in PAH uptake than males, yet histologically renal damage was identical. This same dilemma between function and histopathology is applicable to the enhancement of NMN accumulation and ultrastructural changes produced by uranyl nitrate or potassium dichromate[3,10].

The kidney slice technique was also used to demonstrate differences in sensitivity to gentamicin in different strains of rats. The Fischer-344 strain of rat has been reported to be more sensitive to a number of drugs, including gentamicin[113]. In one series of experiments Fischer-344 rats were treated with 10 mg/kg gentamicin i.m. twice daily for 15 days[5]. BUN levels were not changed, but kidney weight was increased, and NMN uptake was significantly reduced, suggesting that some nephrotoxicity had been produced using this dosage schedule. PAH accumulation by renal cortical slices from gentamicin-treated rats was significantly enhanced when compared with controls, supporting the observations of Cohen et al.[103]. In another set of experiments the treatment duration for gentamicin was 4 days (twice daily at 10 mg/kg). The results suggested that substantial nephrotoxicity was not produced since kidney weight, BUN levels and NMN transport were not significantly different from control values. However, the PAH S/M ratio was still significantly enhanced after gentamicin treatment. Since the earlier experiments had demonstrated that gentamicin produced only moderate nephrotoxicity when given at 10 mg/kg twice daily for 2 weeks, the same dose was administered three times daily for 2 weeks[4]. Nephrotoxicity was now evident, with kidney weight, BUN and serum creatinine levels all being significantly increased. As expected, the NMN S/M ratio was reduced. The PAH response was different from that seen earlier, since the PAH S/M ratio was now significantly lower than that in controls. Thus, with this regime in Fischer-344 rats gentamicin-induced sub-

stantial nephrotoxicity, and both NMN and PAH S/M ratios served as useful indicators of the degree of nephrotoxicity. Histological observations support the slice studies on PAH and NMN uptake. When gentamicin was given at 10 mg/kg twice daily for 2 weeks, minimal structural damage was produced; however when the dose was increased to 10 mg/kg three times daily for 2 weeks, obvious toxicity was produced[3,4]. The above data suggest that gentamicin, like potassium dichromate at low doses, causes a stimulation of PAH transport, while at larger doses, which cause cellular damage, PAH transport is reduced.

In recent years numerous investigators have concentrated on elucidating the metabolic steps underlying gentamicin-induced renal dysfunction. Gentamicin was found to be transported into renal proximal tubule cells and sequestered in lysosomes[114], a process that resulted in the formation and accumulation of lysosomal myeloid bodies[114,115]. The gentamicin-induced morphological change was accompanied by a significant increase in renal phospholipid content[116-118]. Further, Cojocel et al.[119] proposed that the lysosomal accumulation of gentamicin was responsible for the observed decrease in endocytic reabsorption of proteins from renal tubular fluid resulting in enhanced protein concentration in this tissue. With the use of an isolated liver lysosomal preparation, incubation with gentamicin resulted in an inhibition of phospholipases A and C[120]. These data provided evidence that the gentamicin-induced elevation in renal phospholipid and subsequent impairment in protein reabsorption may be due to inhibition of lysosomal phospholipases[121]. Indeed, treatment with gentamicin was found to inhibit phospholipase A in rat and human kidney[122,123]. Similarly, Lipsky and Leitman[124] demonstrated that gentamicin inhibited phospholipase C activity in rat kidney. The toxic effects exerted by gentamicin on phospholipid accumulation appear to be selective towards the kidney. Kornguth and Kunin[125] reported that gentamicin accumulates predominantly within renal cortex. While gentamicin produced a phospholipidosis in rat kidney, no marked change was apparent in hepatic or pulmonary phospholipid levels[116,126]. However, in vitro incubation of human amniotic tissue with gentamicin was found to stimulate phospholipase C without an associated alteration in phospholipase A[127]. It would appear that in the pregnant state, gentamicin exerts an effect directly on fetal membranes as opposed to an indirect action through the maternal kidney.

The susceptibility to gentamicin-inflicted nephrotoxicity is dependent on age. Rajchgot et al.[128] demonstrated that gentamicin increased the excretion of urinary N-acetyl-β-glucosaminidase, a lysosomal enzyme marker in premature infants. In newborn rats gentamicin was found to produce a marked renal phospholipidosis[126], an early manifestation of nephrotoxicity[116]. However, in a comparative study between young and old rats, McMartin and Engel[129] demonstrated that gentamicin induced a two-fold increase in the number of tubules damaged in older animals. While proteinuria occurred regardless of age in gentamicin-treated rats, microscopic evidence indicated that young animals were more resistant to aminoglycoside nephrotoxicity. Additional support for the view that aminoglycoside nephrotoxicity increases with age has

been reported by several investigators[130,131]. In a recent study with newborn infants, Tessin et al.[132] found that gentamicin failed to alter urinary β_2-microglobulin and serum urea, parameters of renal function that are commonly increased in the adult, confirming that the newborn are less susceptible to antibiotic-induced kidney damage.

Cinoxacin is a synthetic antibiotic with a spectrum similar to that of nalidixic acid, a urinary antiseptic[133]. Incubation of rat renal cortical slices in vitro with cinoxacin reduced PAH uptake, but did not change ATP content, oxygen consumption or N-acetyl-β-glucosaminidase release[134]. The uptake of PAH was also inhibited while ATP levels remained similar to control in kidney slices obtained from rats administered cinoxacin[135]. Thus, alterations in PAH transport in renal cortical slices appear to be the most sensitive index of cinoxacin-produced kidney dysfunction in rats[134]. At present the basis for species-specific renal impairment induced by cinoxacin is not known.

Cephaloridine exerts a nephrotoxic action in man and animals that is characterized by necrosis of proximal tubules[136]. In adult rabbits cephaloridine decreased the accumulation of PAH and TEA, and inhibited gluconeogenesis in cortical slices[137,138]. The observed change in renal ion transport was accompanied by a rise in BUN. Recently, Cojocel et al.[78] demonstrated that the cephaloridine-induced decrease in PAH accumulation was associated with reduced lysozyme degradation in rat kidney slices as well as ultrastructural alterations. As these changes occurred simultaneously, and inhibition of protein degradation did not precede morphological or transport system parameters[78], lysozyme changes are believed to reflect a non-specific index of cellular dysfunction. Unlike other antibiotics, cephaloridine is unique in its cationic character. It is of interest that inhibitors of cation transport, such as cyanine and mepiperphenidol, which by themselves are not nephrotoxic, significantly potentiated the kidney dysfunction produced by cephaloridine[137]. In particular, pretreatment with cyanine or mepiperphenidol markedly decreased cephaloridine-induced renal cortical slice PAH accumulation and gluconeogenesis as well as elevation in BUN. Wold et al.[137] thus proposed that inhibition of the cationic transport system required for removal of cephaloridine from the renal cortex may be responsible for the observed toxicity.

Age is also a factor in cephaloridine-induced nephrotoxicity. Fleming and Jaffe[140] reported that immature rabbits were not susceptible to the nephrotoxic action of cephaloridine. Wold et al.[39] demonstrated that cephaloridine produced renal tubular necrosis and elevation in BUN and creatinine in adult and 30-day-old rabbits, and confirmed that neither renal morphology nor serum metabolic parameters were altered in 5-day-old animals.

At present, few data are available on the nephrotoxic actions of the antimicrobial agents tricloran and chlorhexidine. Chow et al.[141] demonstrated that incubation of renal cortical slices with tricloran or chlorhexidine resulted in a significant decrease in the accumulation of PAH and NMN. The observation that kidney slices obtained from tricloran- or chlorhexidine-treated rats exhibited a decreased capacity to accumulate PAH and NMN provides supportive

evidence for nephrotoxic action attributed to these antimicrobial agents. Whereas chlorhexidine elevated BUN, tricloran failed to markedly affect this parameter. Thus Chow et al.[141] concluded that chlorhexidine- or tricloran-induced inhibition of PAH and NMN may be considered a more sensitive index of kidney function when compared with BUN, since the latter may only be rendered abnormal after considerable tissue injury.

Finally, administration of racemomycin-D was found to accumulate and produce a delayed toxicity in rat kidney tubules[142]. With the use of renal cortical slices, incubation with racemomycin resulted in a decrease in PAH accumulation accompanied by no marked change in ATP content. However, when racemomycin-D was incubated with renal microsomes, the activity of Na^+,K^+-ATPase was significantly decreased. Although Inamori et al.[142] could not account for the lack of change in kidney slice ATP after racemomycin treatment, data show that in vitro methodology may be employed to establish the development of antibiotic nephrotoxicity.

Table 5 Summary of amphiphilic drugs producing renal phospholipidosis

Compound	Pharmacological class	Ref.
Gentamicin	Antibiotic	113,121
Citalopram	Antidepressant	145
Diethylamino-ethoxyhexestrol	Coronary vasodilator	146
Chloroquine	Antimalarial	147,148
Iprindole	Antidepressant	149
Imipramine	Antidepressant	149
Chlorcyclizine	Antihistaminic	150
Tilorone	Interferon inducer	151
Chlorphentermine	Anorectic	126,149,152,153

C. AMPHIPHILIC DRUG TOXICITY

Gentamicin belongs chemically to a class of drugs known to induce a phospholipidosis and an accumulation of myeloid bodies in kidney[143,144] and this could account for the observed nephrotoxicity[120]. Morphological evidence of phospholipidosis as indicated by an accumulation of foam cells in renal tissue was observed in response to various amphiphilic drugs with differing pharmacological actions (Table 5). In contrast to the findings of Lullmann-Rauch[149], Sgaragli et al.[154] reported that there were no apparent morphological changes in kidneys of rats treated with the antidepressant, chlorimipramine. As expected, total and individual renal phospholipid class levels remained insignificantly different from control. Although the antidepressant-induced phospholipidotic

response in kidney may be controversial[149,154], treatment with chlorphentermine increased total renal phospholipid content as well as individual phospholipid class levels[126,153]. Chlorphentermine failed to significantly alter morphology or phospholipid content in rat liver as well as in kidney and liver of chick embryos. In addition, Lullmann et al.[155] demonstrated that during chronic daily [^{3}H]chlorphentermine treatment, accumulation of this anorectic occurred in rat kidney but not in liver. These observations suggested that the chlorphentermine-induced phospholipidosis was both species- and tissue-dependent[152]. It is of interest that the morphological and metabolic lesions produced by chlorphentermine were associated with impaired renal function, as evidenced by a rise in BUN and decreased creatinine clearance[156]. With the use of renal cortical slices obtained from rats administered chlorphentermine, Christensen et al.[157] demonstrated a decreased ability of these slices to degrade the protein, isozyme, supporting the view that the anorectic impairs kidney function. Based on studies in which cultured kidney cells were incubated with amphiphilic drugs, Hostetler[144] and Hostetler and Richman[158] have proposed that these compounds prevent intralysosomal phospholipid degradation. The consequent phospholipidosis may then be responsible for the observed renal dysfunction.

CONCLUSIONS

In conclusion, the kidney slice technique has been extremely useful in assessing renal toxicity, produced by a number of different compounds. In many cases it is desirable to do concomitant histological studies, measure serum enzymes, creatinine or BUN levels, or various in vivo physiological procedures as additional sources of data for toxicological evaluation. There are, of course, a number of disadvantages inherent in the slice technique as an in vitro approach, although the advantages of this technique have been stressed. The in vitro approach obviously cannot completely replace in vivo methods, but it can be used in many cases to assess renal toxicity, or in some situations to verify or clarify results obtained in vivo.

In this chapter the use of the in vitro kidney slice technique as a measure of nephrotoxicity is compared with in vivo methods. The kidney slice technique can be used to determine the effect of prior drug treatment of laboratory animals on renal organic anion (PAH) or organic cation (NMN) transport, on glucose synthesis, and on oxygen consumption by renal cortical slices. The nephrotoxic agents uranyl nitrate and potassium dichromate exert inhibitory effects on renal function, although both agents enhance organic cation transport at low doses, and potassium dichromate enhanced organic anion transport at low doses. Enhanced phospholipid accumulation and PAH transport has been found to be a sensitive indicator of gentamicin-induced nephrotoxicity, while inhibition of other parameters has been reported. The tissue slice method is less effective in evaluating chronic nephrotoxicity such as that produced by lead. The inhibitory effect of mercurial diuretics has been shown to be due to the general decrease of me-

tabolic activity by mercury. In the case of amphiphilic drug-induced nephrotoxicity where the renal slice methodology remains to be performed, the observed damage is associated with enhanced intralysosomal accumulation of phospholipid. Differential effects of compounds on in vitro organic anion and cation transport provide information about the transport of these compounds as well as about their nephrotoxicity. Although it is desirable to perform other in vivo or in vitro renal function tests, the renal cortical technique has proved to be extremely useful in toxicological evaluation.

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18

PROTEINS, ENZYMES AND CELLS IN URINE AS INDICATORS OF THE SITE OF RENAL DAMAGE

M.D. STONARD

I. INTRODUCTION

A comparison of the composition of fluid entering and leaving the kidney shows that many of the normal plasma constituents are handled individually - some are eliminated, some are conserved. For example, we have to account for the near absence of glucose and protein and for the appearance of high concentrations of creatinine and urea. To fulfil its role in maintaining a relatively constant internal environment the kidney is able to perform many complex and diverse functions simultaneously. These major functions include filtration, reabsorption, secretion and excretion. Changes in any of these functions will be reflected by a change in the composition of the urine. It is widely accepted that a variety of chemical substances can alter renal function, and the ability to produce a mild and reversible change is important from a pharmacological viewpoint, e.g. diuresis. However, in some instances irreversible effects may occur following incidental exposure to toxic substances. There are several reasons why the kidney may be particularly susceptible to injury. Firstly, renal blood flow is approximately 25% of cardiac output and as such delivers large quantities of blood and contents to the kidney. Secondly, the anatomical and functional properties of the kidney ensure that ample and often unwanted exposure to chemicals occurs by virtue of the blood supply to peritubular membranes and by virtue of tubular fluid at the brush-border membranes in the proximal tubule. Thirdly, the ability to concentrate the glomerular fluid may mean an increase in concentration of 100-1000 fold of certain chemical substances at critical regions of the nephron, e.g. the proximal tubule.

Most of the current simple screening tests of renal function used to assess toxicity in animal studies and in clinical practice, have been in existence for many years, e.g. blood urea, dipstick test in urine for presence of protein. Although these tests may lack sensitivity or specificity or both, they remain useful in the

initial evaluation of renal toxicity. Whilst these screening tests may, along with renal histology, provide evidence or otherwise of nephrotoxicity, they often fail to indicate the site of damage within the kidney. Having established that a substance is nephrotoxic by the traditional screening tests, secondary and more selective renal techniques are often required to determine the nature, time-course and site of the lesion. Some of the secondary techniques which are non-invasive, and which provide further insight into site-specific lesions or particular functions performed by the kidney, will now be discussed.

Although urine is the major body fluid for the non-invasive investigation of injury to the kidney it must be recognized that organs other than the kidney can affect the biochemical and cellular composition of urine, e.g. bladder. Before considering the measurement of specific substances in urine it is important to appreciate that many factors can affect the composition of urine. The precautions that need to be taken depend upon which measurements are to be made, the origin of the urine sample, whether animal or human, and the way in which it is collected. For instance, in experimental animal studies, dietary and faecal contamination may provide chemical substances which interfere with a particular analysis. Cooling and/or addition of a preservative, e.g. azide, may need to be added to the urine sample during a timed collection period in order to eliminate the risk of bacterial contamination and subsequent alkalinization of the urine. In human studies it may be necessary to request an individual to refrain from excessive volume of alcohol intake prior to providing a urine sample, or to request a midstream sample for certain measurements.

II. PROTEINURIA

The appearance of abnormal amounts of protein in the urine is often the first manifestation of renal disease. The simplest detection method for urinary protein involves the dipstick method, in which a test strip impregnated with tetrabromophenol blue at pH 3 changes from a yellow colour to various shades of green in the presence of protein. If a colour change is detected by the dipstick method, then a quantitative protein estimation may be performed to confirm the original semi-quantitative finding. Most of the available quantitative methods for estimating protein in urine have been discussed by Pesce[1]. The most accurate quantitative methods are those based on the biuret or Folin-Lowry reactions. Acid precipitation methods involving the use of trichloroacetic, sulphosalicylic or phosphotungstic acid often form an initial step in protein estimation although such methods are not always quantitative. Dye-binding assays similar in principle to the dipstick test are also widely used. No one method can be singled out as the technique of choice since all suffer from at least one of the following disadvantages - lack of sensitivity (biuret), endogenous chemical interferences (Folin-Lowry), and/or they measure predominantly albumin (dye-binding).

A. Protein separation - practical considerations

Historically, the presence of protein in urine has often been referred to as "albuminuria". This is an anachronism which arose because of the inability to discriminate different proteins, and a preoccupation with renal glomerular disease. However, with the use of paper electrophoresis a significant finding[2] focused attention away from albuminuria and on to a group of proteins of low molecular weight (LMW) which were excreted in the urine from patients with Fanconi syndrome. It was suggested that the proteinuria acquired its distinctive pattern from impaired tubular reabsorption of certain normal proteins passing through the glomeruli. With the advent of chromatographic methods, involving Sephadex, this finding was substantiated[3]. Although these early electrophoretic and chromatographic methods showed that urinary protein could be differentiated into fractions, these approaches were unable to discriminate the 40 or more proteins thought to be present.

Several immunological studies have established the presence of many plasma proteins in normal urine. Renal clearance studies with plasma proteins have shown that the normal glomerulus is relatively impermeable to molecules with a molecular weight (MW) >40,000. Molecules of lesser size pass through the glomerulus more readily but their appearance in urine is governed by subsequent tubular reabsorption. It is now known that several factors other than molecular size, e.g. charge, shape, are determinants of glomerular permeability[4], but a classification of proteinuria based upon the MW pattern seemed an obvious goal. To a large extent this target has been achieved by the development and application of sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE). When a detergent such as SDS reacts with a protein, a negatively charged protein-SDS micelle is formed with a constant charge to mass ratio. When proteins are electrophoresed in the presence of SDS, a linear relationship is found between the log MW and the distance of migration on the gel[5]. Whilst this linear relationship between log MW and migration distance is obeyed by most proteins, anomalous behaviour may be observed by carbohydrate-rich glycoproteins which in the presence of SDS show a reduced mobility and hence a higher apparent MW[6].

The major advantage of the SDS-PAGE technique is that large numbers of urine samples can be compared in a single run with high resolution of individual protein bands. As with most methods, there are limitations to its convenience and certain pitfalls which should be avoided. The main disadvantage of the method is that urine samples usually need to be concentrated to a fixed protein concentration prior to electrophoresis. The methods available for concentrating proteins in urine have already been reviewed[1]. They include filtration through polymer membrane (Minicon), ultrafiltration through polymer membranes (Amicon Diaflo), lyophilization and osmotic concentration against a polymer (carbowax). Again, no single method embraces all the necessary criteria of speed, convenience, and concentration of several samples simultaneously without significant loss of some material. There are several ways in which protein may be lost and these

have been evaluated for the type B-15 "Minicon" concentrators[7]. It was concluded that although some loss of protein occurred by adsorption onto the membrane, and by mechanical loss, the concentrator was reproducible and convenient to use with large numbers of urine samples. However, some loss of protein of MW <15,000 is to be expected from this device[8].

Many SDS-PAGE methods incorporate β-mercaptoethanol to reduce disulphide bridges to sulphydryl groups; however, this should be avoided with urine samples since the reduction of disulphide bridges in immunoglobulins will produce heavy and light chains and remove the effectiveness of the technique for discriminating proteinurias of different renal origins.

B. Protein separation methods - assessment of renal disease

The technique of SDS-PAGE has found extensive use in the clinical investigation of renal disease. Chronic renal diseases are rarely confined to one region of the kidney, but it has been found that primary tubular disorders, e.g. pyelonephritis, can be discriminated from glomerulonephritis by the ratio of macroproteins to microproteins[9]. When the classification of proteinuria is compared with the clinical investigation it has been found that the former permits a good forecast of the site of the renal lesion[10]. This has been confirmed by other investigations in which a close correlation was found between the classification of proteinuria and the clinical and renal biopsy data[11].

Quantitative estimation of stained protein bands in separating media such as polyacrylamide is less reliable than specific immunochemical methods and the densitometric results obtained should be interpreted cautiously. Most protein staining methods usually involve Coomassie blue or Amido black, although more recently silver staining methods have been introduced. The relationship between the staining intensity and the amount of protein present is largely dependent upon the extent to which these dyes are taken up and bound by the different proteins. It is possible, however, to make the method more quantitative where a protein has been positively identified and purified. If a known amount of this pure protein is run concurrently with mixtures containing the protein, then the staining intensity which corresponds to a known amount of this protein may be determined by densitometry[12].

Isoelectric focusing (IEF) is an alternative electrophoretic technique for the separation of urinary proteins in normal health and disease[13]. This technique is based on the migration of proteins to their respective isoelectric point, which is the pH at which the net charge on the protein is zero. The main advantages of the technique are not only speed and high resolution but also that no preconcentration of urine samples is usually needed. However, pretreatment of samples is needed to remove salts which may interfere with the pH gradient. This technique, like SDS-PAGE, has been used to differentiate glomerular and tubular proteinurias in overt renal disease and in individuals exposed to nephrotoxic substances in the workplace, e.g. cadmium[12].

Although IEF has not found wide application as a technique

in its own right, when combined with SDS-PAGE in a two-dimensional separation system it offers enormous potential for the detection of subtle changes in urinary protein patterns indicative of renal disease[14] and the detection of novel kidney-specific proteins[15].

Although electrophoretic techniques have been widely used to classify different types of proteinuria, the recent introduction of high-performance gel- and ion-exchange-chromatography techniques may with further improvements offer certain advantages, e.g. speed of analysis, over electrophoretic techniques[16,17]. From the results of clinical investigations the classification of the proteinuria based upon gel permeation chromatography revealed a good prediction of the site of the renal lesion. Furthermore, good agreement was observed between the urinary protein patterns obtained by gel permeation chromatography and by SDS-PAGE[16]. However, at the present time SDS-PAGE appears to offer the best screening method in renal disease[18].

C. Specific proteins as indicators of renal disease

1. Albumin

Several investigators have shown that the normal glomerulus passes only traces of albumin (MW = 68,000) amounting to <0.1% of glomerular filtration rate in man.

The maximal tubular reabsorptive capacity for albumin is fairly low, and is almost saturated under normal conditions. Since the filtration coefficient is small, a relatively large increase in albumin excretion may occur with only a slight change in glomerular permeability. The urinary excretion of albumin in species such as dog and man is very low, unlike the rat[19]. Because albumin clearance is of diagnostic importance in the detection of minimal glomerular disease, specific radioimmunoassay methods have been developed to measure accurately the concentration of albumin in urine[20,21]. The kidney appears to play little or no role in the catabolism of high MW proteins such as albumin and immunoglobulins[22].

Proteins of MW <40,000 are more readily filtered by the glomerulus, and subsequently undergo extensive tubular reabsorption and degradation. The kidney appears to be involved in the reabsorption and catabolism of several LMW proteins[23-25]. Much of the insight into the renal handling of LMW proteins has been provided by the proteins lysozyme and β_2-microglobulin. Studies with purified lysozyme have shown that the reabsorption process occurs by endocytosis in the proximal tubule[26] and is a saturable process[27].

2. β_2-Microglobulin

β_2-Microglobulin (a LMW protein of 11,800 daltons) was purified from the urine of patients with tubular dysfunction, where it occurs in relatively large amounts[28]. Because the concentration of

β_2-microglobulin is low in normal human urine, a radioimmunoassay (RIA) technique was developed to measure these low concentrations[29,30]. A solid phase RIA is commercially available as a kit (Pharmacia, Uppsala, Sweden). An alternative and less expensive assay for β_2-microglobulin has been developed which uses latex particles coated with antibody and quantitation by particle counting or light scattering[31].

β_2-Microglobulin has been shown to be eliminated from the body almost exclusively by the kidneys, predominantly by glomerular filtration. After filtration the protein is almost totally reabsorbed by the renal proximal tubules[32] and catabolized by the kidney[33,34].

A close inverse relationship has been found between plasma β_2-microglobulin and inulin clearance[35].

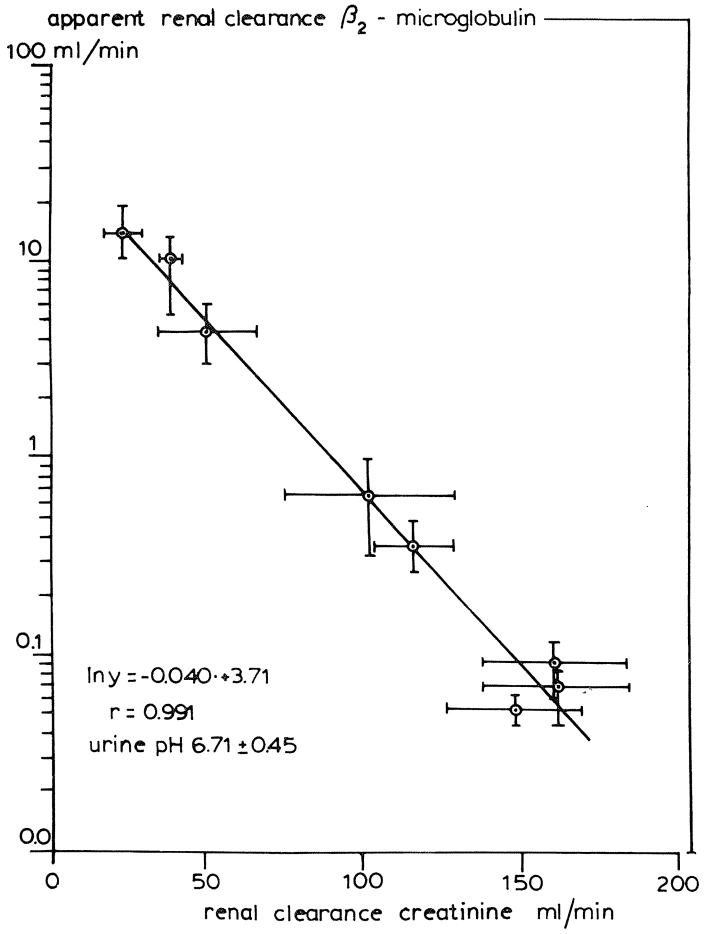

Figure 1 Average apparent renal clearance of β-microglobulin (ml/min) plotted against the corresponding average renal clearance of creatinine (values ± SD). (Reproduced from ref. 37).

The measurement of plasma β_2-microglobulin appears to be at least as useful as creatinine for estimation of GFR from a single serum determination[36]. The relationships between the renal clearances of creatinine and β_2-microglobulin are shown in Figure 1. However, as β_2-microglobulin undergoes tubular reabsorption and catabolism in the kidney, the clearance should be considered as an apparent renal clearance[37]. The measurements of β_2-microglobulin in serum and urine as indices of renal integrity have found wide application in both clinical and occupational practice. β_2-microglobulin measurements have been used to distinguish between upper and lower urinary tract infections[38], as an early marker of aminoglycoside nephrotoxicity in patients before alterations in the conventional tests of renal function were observed[39] and in several other clinical situations[40]. The excretion of both albumin and β_2-microglobulin has been explored in several studies of renal disease. Patients with glomerular disease exhibited a marked elevation of albumin excretion with an essentially unchanged β_2-microglobulin excretion; in contrast, β_2-microglobulin excretion was increased with normal or modest increases in albumin excretion in tubular disorders[41]. The urinary excretion of albumin and β_2-microglobulin have been compared in a population where Balkan nephropathy is endemic. This disease is manifested as a chronic nephritis due to a primary interstitial disease but is of unknown aetiology. An early sign of the disease is the occurrence of a tubular proteinuria. It has been shown that an increased excretion of urinary β_2-microglobulin may provide an early indication of the onset of the disease[42] and that the calculation of the albumin/β_2-microglobulin ratio may provide additional information where an elevated β_2-microglobulin is found[43].

In the field of occupational health the measurement of β_2-microglobulin has been most prominent in studies of workers exposed to toxic metals, e.g. cadmium which is known to produce renal proximal tubular damage. Although a tubular type proteinuria with an increased excretion of β_2-microglobulin has been observed especially in workers with a long history of exposure to cadmium there is also evidence from measurements with other specific proteins (albumin, transferrin, orosomucoid) that a glomerular proteinuria may also be present[44]. The finding of a mixed-type proteinuria in some workers exposed to cadmium has been confirmed experimentally in the rat[45]. In a cooperative study between Japan and Sweden of Itai-Itai disease, one manifestation of which is tubular proteinuria and in which cadmium exposure has been implicated, it was concluded that the radioimmunoassay of β_2-microglobulin in urine is a sensitive indicator of cadmium-induced proteinuria[46]. With the recent development of an immunoassay for metallothionein, a cadmium-binding protein found in the kidneys, the measurement of this protein in urine is being evaluated as an index of renal dysfunction[47].

A possible association between exposure to solvents, mainly hydrocarbons, and glomerular disease has been suggested by several groups on the basis of epidemiological surveys[48-52]. The excretion of albumin and β_2-microglobulin has been measured in urine samples from groups of workers exposed to styrene, toluene and toluene/xylenes and compared to a reference group. The ex-

posed population, and especially the styrene group, excreted significantly greater amounts of albumin than the reference group, but there were no differences in β_2-microglobulin excretion between the exposed and reference populations[53].

3. Other LMW proteins

One of the major disadvantages of the use of β_2-microglobulin as an index of renal tubular function is its instability at slightly acidic pH. Several publications have emphasized that it is essential to raise the pH of the urine immediately after collection in order to stabilize the protein. Even with this precaution, some protein may have already been degraded in the bladder depending on the time elapsing before voiding. In an effort to overcome this drawback, several authors have proposed alternative LMW proteins as indices of renal tubular function. In particular, retinol-binding protein (RBP) has been proposed, mainly on account of its stability down to pH 4.5 and the development of a more sensitive assay[54,55]. Retinol-binding protein exists in the plasma free with a MW of 21,400 or bound to prealbumin as a high molecular weight complex[56,57]. RBP clearance appears indirectly correlated with creatinine clearance as a measure of GFR; as the GFR is reduced progressively, then RBP clearance approaches creatinine clearance. Its renal handling appears similar to other LMW proteins with an increased excretion, relative to albumin, in renal tubular disease[58]. In a comparison between β_2-microglobulin and retinol-binding protein, both proteins were shown to exhibit similar sensitivity and specificity as indices of tubular proteinuria[54]. Although competition between LMW proteins for common reabsorptive sites appears to exist[22], there may be differences since gentamicin blocks the renal uptake of β_2-microglobulin but not retinol-binding protein[59].

Another possible alternative protein as an indicator of renal tubular function is α_1-microglobulin. This protein is a glycoprotein with a MW of 31,500[60,61]. In a recent study the use of α_1-microglobulin as an index of renal tubular function has been compared with that of β_2-microglobulin and retinol-binding protein. Although the urinary concentrations of β_2-microglobulin and retinol-binding protein were highly correlated in acute tubular disorders, the correlations between these two proteins and α_1-microglobulin were far less strong[62]. Although LMW proteinuria, characterized by an increased excretion of several LMW proteins, can now be considered as a reasonable predictor of renal tubular disorders, the raised excretion of a single LMW protein may not necessarily reflect renal tubular disease but a disease process occurring in another organ[63]. Several examples can be cited to support this view. These include the well-established presence of light chains derived from immunoglobulins in Bence Jones proteinuria associated with myeloma, and myoglobinuria which may be due to disorders of muscle metabolism or muscle injury. Recent developments with the separation of human urine proteins by two-dimensional electrophoresis have revealed the presence of proteins in the urine associated with various muscular diseases[64] and with prostatic

cancer[65].

4. α_{2U}-Globulin in the rat

Although albumin is the major protein found in the urine of humans in normal health, it is not the major protein in all species. The rat, which is widely used in the toxicological assessment of new substances, shows several qualitative and quantitative differences in protein excretion from man[66]. In this respect the detection of protein in rat urine with a dipstick test is not wholly appropriate. Male rats excrete more protein than females, and the amount of protein excreted increases with age. The major portion of protein excreted in the male rat consists of LMW protein and not albumin immediately post puberty, but with increasing age the protein excretion changes from a predominantly LMW to a high MW pattern because of the onset of spontaneous renal disease[67,68]. For obvious reasons the rat is not the most appropriate species for nephrotoxic studies, since any renal effects due to treatment with a test substance may be superimposed on a high background of spontaneous renal disease. The magnitude and relative proportions of LMW proteins to albumin are also strain-dependent[69]. The protein composition of male rat urine, and the possible origin of different proteins have been studied immunologically[70]. Approximately 50% of the proteins found in normal rat urine appear to have immunological identity with serum[71]. The other proteins of non-serum origin appear to arise from kidney and other accessory organs of the urogenital tract[72].

Although several sex-related proteins may account for the quantitative difference in protein excretion between male and female rats, one protein in particular, α_{2U}-globulin, has attracted most interest[73]. This protein of MW 20,000 is known to be synthesized by the liver but not the kidney[71], and its synthesis is subject to multi-hormone control including androgens[74]. α_{2U}-Globulin, as expected for a LMW protein, undergoes extensive reabsorption by the kidney but in contrast to other LMW proteins is excreted in large amounts[75]. Although this protein was first characterized some 20 years ago, its physiological function remains unknown. Several recent studies have shown α_{2U}-globulin to be a family of proteins exhibiting microheterogeneity in both MW and charge[76,77], with evidence for multigene encoding[78].

The urinary excretion of albumin and α_{2U}-globulin have been compared in experimental models of nephrotoxicity. Rats with glomerular damage induced by puromycin amino-nucleoside exhibited a massive loss of albumin without affecting α_{2U}-globulin excretion. In contrast, sodium maleate-induced tubular damage was characterized by an increased loss of both albumin and α_{2U}-globulin excretion[79].

Whilst the functional significance of α_{2U}-globulin remains to be established, recent studies suggest that an association may exist between α_{2U}-globulin re-absorbed by the kidney and the presence of renal hyaline (protein) droplets[80,81]. Although the presence of these droplets in the kidney has been known for many years[82,83] the detection of these droplets in male but not female

rats appears to correlate with the more pronounced proteinuria which can be largely accounted for by α_{2U}-globulin[84]. Furthermore, these droplets are absent in pre-pubertal rats which are unable to synthesize α_{2U}-globulin in the liver. Support for the association is provided by substances which stimulate the further production of hyaline droplets; α_{2U}-globulin has been shown to be present in hyaline droplets induced in rats by decalin treatment[80]. In addition, recent studies in the author's laboratory with another compound, 2,2,4-trimethylpentane, which also stimulates droplet formation, have shown firstly that staining due to α_{2U}-globulin by an immunoperoxidase technique is localized within the hyaline droplets, and secondly that as the number of droplets increases there appears to be a corresponding increase in the concentration of renal α_{2U}-globulin[81]. Further studies are needed to understand the relationship between the hepatic synthesis and the renal tubular handling of this protein.

The presence of a sex-related protein in urine is not unique to the rat since a similar phenomenon is seen also in the mouse. Although a LMW protein in mouse urine appears to be synthesized in the liver, and to undergo reabsorption and excretion by the kidney, it is qualitatively different from the rat protein, being prealbumin in nature[85-87].

III. ENZYMURIA

The presence of enzymes in urine has been recognized for many years, with several enzymes showing a basal activity which reflects normal cell turnover with exfoliation of intact or damaged cells into the urine. Although the kidney appears to be the major source of urinary enzymes[88,89], other tissues of the urogenital tract, e.g. bladder and prostate, may contribute to the enzyme composition of urine. Enzymes in urine may also arise from blood plasma but these are likely to be restricted to those of LMW which can readily permeate the glomerulus in normal health, and most if not all of these will be reabsorbed by the renal tubules.

The detection of abnormally high activities of enzymes in urine has been interpreted as evidence of renal cellular damage, especially when a reciprocal decrease in the tissue enzyme has been demonstrated[90]. Several reviews of the diagnostic use of enzymes in urine as indicators of renal damage have been published[91-94]. Although the potential of urinary enzymes to detect renal damage has been used for diagnostic purposes in the experimental situation, and for diagnostic and prognostic purposes in the clinical situation, it is clear from all the reviews that relatively few enzymes appear to be of diagnostic relevance. Those enzymes which have been most thoroughly investigated include those which are found in high concentration in the kidney[95], and can be assayed conveniently. Several of these are localized to the proximal renal tubule, a region which is particularly vulnerable to several agents (Table 1).

Table 1 Localization of renal tubular enzymes in the rat

Enzyme	EC No.	Localization	Ref.
Alanine aminopeptidase (AAP)	EC 3.4.11.1 (cytosol)		
	EC 3.4.11.2 (microsomes)	PST > PCT	99
Alkaline phosphatase (ALP)	EC 3.1.3.1	PCT > PST > distal tubule	96
β-Galactosidase (GAL)	EC 3.2.1.23	PST > PCT > distal tubule	98
Gamma-glutamyl transferase (GGT)	EC 2.3.2.2	PST > PCT > distal tubule	97
Lactate dehydrogenase (LDH)	EC 1.1.1.27	Distal tubule > proximal tubule	96
β-N-Acetyl glucosaminidase (NAG)	EC 3.2.1.30	PCT > PST = distal tubule	98

PST = proximal straight tubule; PCT = proximal convoluted tubule

A. Urinary enzymes - practical considerations

The assay methods for those urinary enzymes which are most commonly used are shown in Table 2; they can be classified under five broad headings. Most assays involve a spectrophotometric method with fluorimetry as an alternative method for several lysosomal enzymes and phosphatases.

Table 2 Measurement of enzyme activities in urine

Principle of the method	Enzymes	Ref.
1. Formation of 4-nitroaniline	AAP, GGT	100-102,105
2. Formation of 4-nitrophenol	NAG, GAL, ALP	104,108
3. NADH-linked oxidation	LDH	106
4. Formation of 4-methyl-umbelliferone*	NAG, GAL, ALP	103,107
5. Formation of 2-methoxy-4-[2'nitro-vinyl] phenol	NAG, GAL	109

* Fluorimetric.

In several early studies upon urinary enzymes a non-linear relationship was found between enzyme activity and urine volume, which suggested the presence of enzyme inhibitors[110]; these endogenous inhibitors appear to be of LMW and need to be removed prior to enzyme assay. These inhibitors may be removed either by dialysis against water or by gel filtration[111,112]. The latter method is considered to remove inhibitors more completely than dialysis. For enzymes such as alkaline phosphatase (ALP) and lactate dehydrogenase (LDH), the need to remove these inhibitors is well established; however, it remains controversial whether this is needed for all urinary enzymes, e.g. GGT. If doubt exists, then the need to pretreat urine samples to remove interfering substances should be assessed in pilot studies. This pretreatment may be circumvented with the fluorimetric assay involving 4-methylumbelliferone, in which any endogenous inhibitor can be appreciably diluted in the assay system because of the greater sensitivity of the method[113].

Because of the hostile medium in which urinary enzymes exist, some may be less stable than others. Although instability may arise for several reasons, e.g. the presence of proteases, the most likely source of instability appears to be the pH of the urine. Most urinary enzymes appear unstable as the pH is reduced to 5 [114,115]. However, NAG appears particularly robust, not only being stable over the pH range 5-8 but also stable to storage at +4°C or freezing[116]. Most of the urinary enzymes are not stable to freezing and the collection of urine samples, removal of inhibitors and enzyme assays should be performed on the same day if erroneous

results are to be avoided.

In animal studies a source of contamination of urine which is often overlooked is from faeces. Although most metabolic cages used for the collection of urine and faeces separate the two materials reasonably efficiently, certain enzymes, e.g. ALP and glutamate dehydrogenases have a much higher activity in faeces than in urine.

The kidney is a major route for the elimination of drugs and foreign chemicals, and chemical interference with enzyme activity is always a possibility. This can be tested by the in vitro incubation of the enzyme with the chemical, at concentrations expected to be found in vivo. However, this does not eliminate the possibility that enzyme activity may be affected by a metabolite, the structure of which may or may not be known. Interference with urinary enzyme activity has been reported with methylmercury[117].

B. Urinary enzymes - assessment of renal damage

The main applications of urinary enzymes have been

1. the potential to detect impending renal transplant rejection;
2. the detection of drug-induced nephrotoxicity;
3. the detection of renal disease of unknown aetiology;
4. in experimental studies to assess the nephrotoxicity of new or existing substances;
5. the assessment of renal integrity in individuals occupationally exposed to potential or actual nephrotoxic agents.

1. Clinical studies

The urinary enzyme which has undergone the most exhaustive investigation for advance warning of renal transplant rejection is NAG. Following injury to the human kidney, immediate rises in urinary NAG activity were found which were roughly proportional to the degree of damage[118]. Using an automated fluorimetric method it has been possible to show that an abnormal elevation of NAG in renal transplant patients provided an early warning of rejection[119]; furthermore, studies in patients with acute and chronic renal diseases showed that NAG could be used as a sensitive indicator of renal disease[120]. In patients with hypertension, NAG can be used cheaply and conveniently to screen out those individuals who develop renal disease[121]. The enzyme NAG has also been characterized into different isoenzymic forms A (normally found in urine), B and I and the individual forms have been used to refine the diagnostic interpretation of renal disease[122,123].

Single and/or repeated doses of aspirin have been shown to cause an increase in the urinary excretion of LDH[124] and NAG in man[125], and LDH in rats[126]. The latter group have adopted a systematic approach to the detection of kidney damage by measuring a combination of urinary enzymes derived from different subcellular fractions in the kidney. By using this approach it has been possible to determine which organelles within the cell are affected,

and the sequence of cell injury. In contrast to the effect of aspirin upon LDH without affecting ALP, acid phosphatase and glutamate dehydrogenase activities, all enzyme activities were elevated after phenacetin, indicating more generalized cellular injury[126].

The measurement of urinary enzymes has been used in the early detection of renal injury induced by aminoglycosides and/or cephalosporins. At high dosage (50-100 mg/kg), the aminoglycoside gentamicin causes renal tubular necrosis with electron microscopy revealing an increase in the number and size of tubular lysosomes. Lysosomal enzymuria has been shown to be an early manifestation of gentamicin nephrotoxicity in several studies in both humans and rats[127-129]. An increased excretion of the brush-border enzymes AAP[130] and GGT[131] has also been observed in patients or volunteers receiving aminoglycosides. At low doses (5 mg/kg per day) renal tubular degenerative changes similar to those seen in humans have been observed in rats with raised activities of urinary NAG, GAL and ALP[129]. The enzymes NAG and AAP, in combination with the LMW protein, β_2-microglobulin, have been shown to be of predictive value in nephrotoxicity caused by gentamicin[132].

The concern relating to nephrotoxicity of the cephalosporins is in the main confined to cephaloridine[133] and to cephalothin at exaggerated doses of the drug. At a high dose of 2 g/kg, cephaloridine produced a raised excretion of ALP and LDH in rats[134]. At a much lower dose of cephaloridine (0.25 g/kg), an increased excretion of ALP, leucine aminopeptidase and LDH in rat urine was still present[135]. After acute cephaloridine treatment of rats the enzymuria has been correlated with the proximal tubular injury[136]. The combination of an aminoglycoside with a cephalosporin has been reported to increase the incidence and severity of nephrotoxicity in man[137] but not in rats[138,139]. However, a study of AAP excretion in human volunteers receiving aminoglycoside or cephalosporin, or both, suggested that the tubular nephrotoxicity was mainly related to the aminoglycosides[130].

The detection of upper urinary tract infection has been examined by the measurement of the urinary excretion of various markers of renal tubular involvement. Although the isoenzyme, LDH_5, was the most valuable parameter for the early diagnosis of this condition, the overall diagnostic accuracy was reinforced by the inclusion of either NAG, lysozyme (muramidase) or β_2-microglobulin[140].

A tubular proteinuria of unknown aetiology is a key feature of endemic Balkan nephropathy. The proteinuria is accompanied by an elevated excretion of ribonuclease[141], which may reflect some lysosomal damage in this disease.

2. Experimental studies

The role of enzymes in urine as early markers of renal injury in the toxicological evaluation of novel substances has been reviewed previously[142]. Since the evaluation of urinary glutamate-oxaloacetate transaminase as a renal test for use in toxicity studies in rats[143], many experimental studies with several established

nephrotoxic agents have used urinary enzymes, not only to indicate renal injury but also to localize the site of injury. In some of these experimental studies the relationship between dose of nephrotoxin, urinary enzyme excretion and renal histopathology has been examined. On the basis of studies with mercuric chloride in the rat, it was concluded that enzyme excretion and enzyme histochemistry were of a similar sensitivity as indices of renal damage in the 24 h period following a single dose of the toxic agent, and were more sensitive than renal histology[144]. A change in the urinary excretion pattern of enzymes has been shown to closely parallel the ultrastructural changes which occur in renal tubular cells of the rat following a single dose of the nephrotoxin[90]; however, all measurements were made at a single dose in this study. Finally, increases in the urinary excretion of LDH were well correlated with the dose of the nephrotoxin, as well as with the extent of the renal damage shown by histopathology and enzyme histochemistry[145]. Experimental models of renal tubular damage have been produced in the rat by 4-nitrophenyl-arsonic acid[134,146], uranyl nitrate[90,117,134], and in the rabbit[147], mercuric chloride[117,144,145,148-150], and in the dog[151], dichromate in the rat[117], hexachlorobutadiene in the rat[152], carbon tetrachloride in the rat[117,148] and distal tubular injury in the rat produced by folic acid[150]. Those enzymes located on the brush-border of the proximal renal tubule appear to be earlier indicators of renal damage than other renal tests at the lowest dose levels of nephrotoxins used[142]. However, in certain experimental studies urinary enzymes appear less sensitive than other renal parameters[117,148].

In a dose-response study with hexachlorobutadiene, which produces selective damage to the pars recta in the rat, the urinary enzymes ALP and NAG were shown to be less sensitive than urinary protein and of approximately the same sensitivity as plasma urea for the detection of renal damage (Table 3).

Selectivity of urinary enzymes can be used to distinguish papillary damage from proximal tubular injury. NAG is one of the few enzymes with a relatively high concentration in the renal papilla. Studies with the papillotoxic agents, ethyleneimine in the rat[154] and dog[155] and with 2-bromoethylamine in the rat (Figure 2), show that the production of a voluminous dilute urine is accompanied by an early rise in NAG excretion and precedes the excretion of brush-border enzymes indicative of secondary tubular involvement.

The usefulness of urinary enzymes as aids to the diagnosis of renal glomerular damage appears still uncertain. Although much information about the protein composition of the glomerular basement membrane has been accrued[156], little is known about the enzyme composition of this structure[157]. The excretion of enzymes has been evaluated in renal glomerular damage in the rat induced by anti-rat kidney antibodies[146], puromycin aminonucleoside[152,158,159] and by bovine serum albumin[150]. Whilst a marked proteinuria, characterized by high MW proteins, is the key feature of glomerular damage, enzymuria can be used to monitor the secondary tubular involvement which usually accompanies damage to the glomerulus.

These experimental studies with site-specific nephrotoxins

Table 3 The effect of hexachlorobutadiene on urine volume, the excretion of urinary protein, N-acetyl-β-D-glucosaminidase, alkaline phosphatase and plasma urea concentration in rats 24 h after a single dose

Treatment	Urine flow rate (ml/h)	Urinary protein (μg/h)	Alkaline phosphatase excretion (units/h)[a]	N-acetyl-β-D-glucosaminidase excretion (units/h)[a]	Plasma urea (mmol/l)
Control	0.26 ±0.02 (33)	164 ± 16 (34)	176 ± 23 (19)	125 ± 7 (30)	5.8 ±0.2 (38)
HCBD					
300 mg/kg	0.83[b]±0.09 (5)	1442[b]± 65 (3)		1378[b]±607 (4)	24.7[b]±1.4 (18)
200 mg/kg	0.39[b]±0.03 (5)	1291[b]±135 (6)	1145[b]±185 (6)	623[b]± 70 (6)	18.7[b]±1.8 (10)
100 mg/kg	0.36[b]±0.03 (11)	1075[b]±140 (10)	470[b]± 93 (6)	255[b]± 20 (11)	9.7[b]±0.8 (11)
50 mg/kg	0.26 ±0.01 (5)	296[b]± 20 (4)	190 ± 5 (3)	157 ± 15 (3)	5.7 ±0.3 (11)
20 mg/kg	0.29 ±0.02 (6)	316[b]± 10 (6)	142 ± 22 (5)	142 ± 22 (5)	5.8 ±0.2 (5)

[a] Enzyme excretion is expressed as total units excreted during the 24 h collection period divided by 24. A unit is the amount of enzyme catalysing the formation of 1 nmol 4-methylumbelliferone/h.

[b] Significantly different from control, $p < 0.05$. (Modified from Lock and Ishmael[153].)

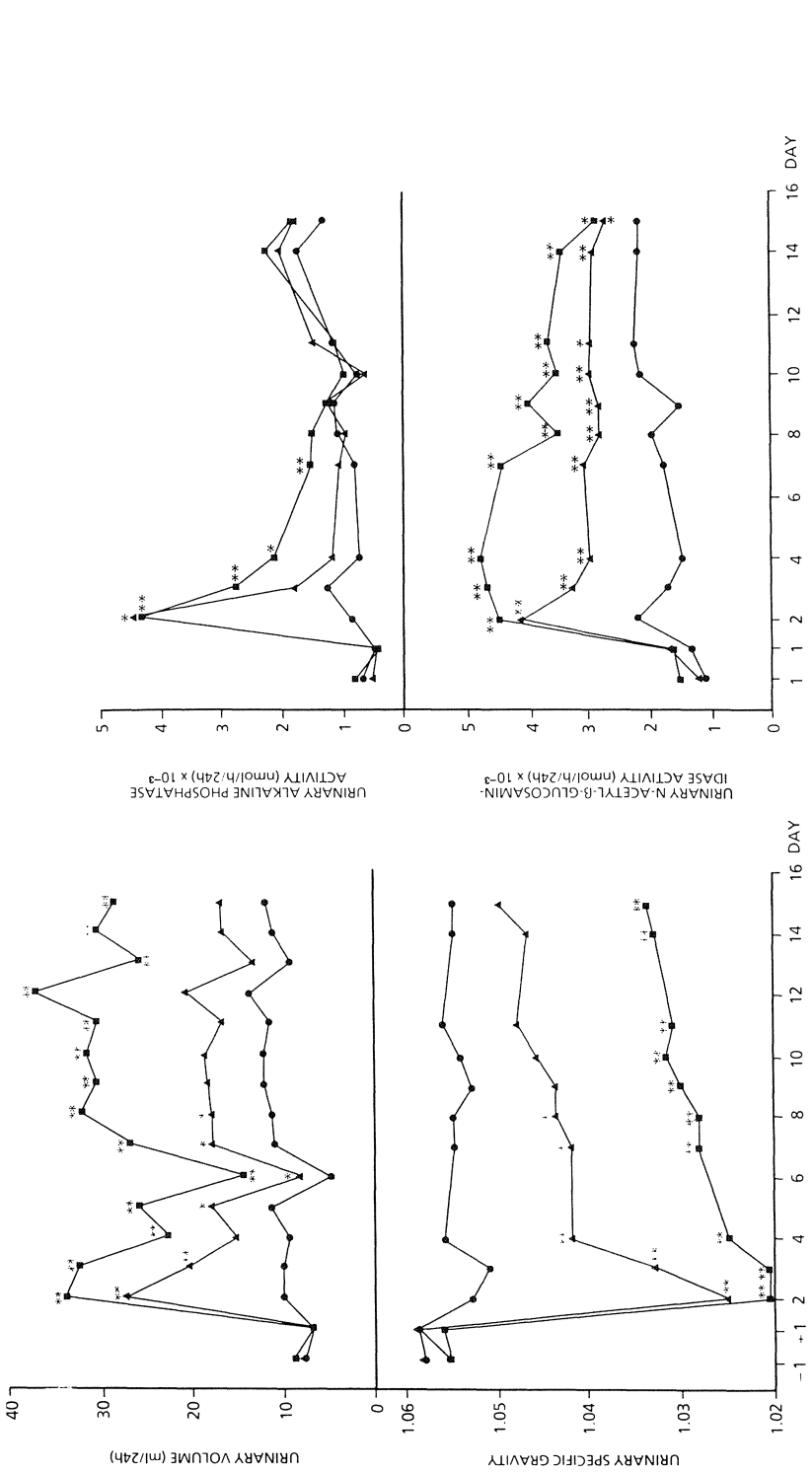

Figure 2 The effect of 2-bromoethylamine (BEA) on urine volume, specific gravity and the excretion of N-acetyl-β-glucosaminidase and alkaline phosphatase in rats after a single dose. Each point is the mean of eight animals from the control group (●), from the 50 mg/kg BEA group (▲) and from the 200 mg/kg BEA group (■). Animals were dosed intraperitoneally on day +1. Statistically significantly different from the control group mean at the 5% (*) and 1% (**) levels. (Stonard, M.D., unpublished observations.)

illustrate the fact that each segment of the nephron contains a characteristic group of enzymes which are released into the lumen when damage to one particular region of the nephron occurs. However, even the brush-border enzymes are not evenly distributed (Table 1); whilst GGT and AAP have the highest activities in the pars recta, the activity of ALP is highest in the pars convoluta of rat kidney[160]. Studies on the brush-border membranes from human kidney cortex also demonstrate differences in the subcellular distribution of these three enzymes. Protease treatment of these membranes caused almost total release of AAP, partial release of GGT and virtually no release of ALP, the latter only being released by harsher treatment with detergent[161]. These studies indicate that AAP and a proportion of GGT are surface enzymes of the brush-border whilst the remaining GGT and all of the ALP are integral constituents of the membranes.

It has been suggested that enzymuria may be more sensitive in chronic toxicity studies where renal compensatory mechanisms may mask alterations in other renal functions[117]. However, this remains to be established as such studies have yet to be carried out. Under such circumstances, for a modest increase in enzyme excretion to be detected, the analytical and animal variation will need to be exceedingly small. Where there is a lack of information about a novel substance to be tested, traditional renal function tests should be carried out in the first instance to establish nephrotoxicity and then be augmented by a battery of enzymes, selected on the basis of regional distribution, to localize the site of the lesion. Studies with isolated nephron segments may help to identify further enzymes which may be of diagnostic significance[160].

3. Occupational studies

Enzyme excretion, in conjunction with urinary protein determinations, have been used to evaluate the effects of exposure to potential or actual nephrotoxins in the workplace. Several toxic metals, e.g. cadmium, lead and mercury, are known to produce deleterious effects upon the kidney which are dependent upon the intensity and duration of exposure. A raised excretion of the lysosomal enzyme, GAL, but not other enzymes, has been observed in workers with long-term exposure to cadmium. This observation, which appears suggestive of tubular damage, has been reinforced by the findings of an elevated excretion of β_2-microglobulin and of tubular proteinuria after urinary protein electrophoresis[162]. On the basis of both plasma and urine assays for GAL it was concluded that GAL originated from the kidney and not from plasma, which could only account for a minute fraction of urinary GAL, even making allowance for slight glomerular dysfunction which is observed in some workers exposed to cadmium.

Occupational exposure to mercury vapour may lead in some individuals to slight glomerular and tubular changes as reflected by a raised excretion of albumin and GAL respectively[163]. In a more recent study of mercury vapour exposure a small increase in the prevalence of higher activities of the urinary enzymes GGT and

NAG was observed, again suggestive of tubular effects[164].

The excretion of NAG has been evaluated as a sensitive indicator of renal injury in workers exposed to lead, mercury or organic solvents. Although none of the individuals had clinically evident renal disease and all exhibited normal urinalysis, slight increases in NAG excretion were found in both metal exposures and in two out of three of the solvent exposures[165]. In a cross-sectional renal function study of exposure to various individual solvents and solvent mixtures, slight elevations in urinary lysozyme and β-glucuronidase, indicative of tubular change, were observed in some but not all exposure groups[166]. Several points of caution are necessary when interpreting the results of enzyme excretion obtained in the workplace. Enzyme excretion studies measure activity and not the concentration of the enzyme; thus a change in activity need not necessarily reflect an alteration in the amount of enzyme released into the lumen. Inter- and intra-individual variability may be considerable. There are several factors unrelated to occupation which may confound the interpretation of an enzymuria, e.g. medications, diurnal rhythm etc. Because urinary enzymes appear in most instances to be sensitive markers of early renal dysfunction, they should be carried out in conjunction with traditional, but less sensitive, parameters of renal integrity. Finally, the increases in urinary enzyme excretion seen in most occupational studies are slight and almost certainly do not pose an immediate health hazard to the individual, although a continuous monitoring programme may be required in some instances.

IV. CELLURIA

A. Urinary cells - practical considerations

The quantitative evaluation of cell excretion in urine has been used both clinically and experimentally to investigate renal injury. After centrifugation of urine samples to obtain the sediment, several cell types are normally present in the sediment. These may include leucocytes, erythrocytes, renal tubular cells, squamous epithelial cells with or without casts, crystals, bacteria, etc. In order to distinguish between the different cell types, special staining techniques are necessary[167,168]. After appropriate staining of the sediment it is resuspended and the cell numbers determined in a counting chamber. The practice of reporting the number of cells counted "per high-power field", rather than in a counting chamber, should be avoided[169]. This is especially important when defining normal values. In humans, renal cell excretion is claimed to be very constant, whilst leucocyte excretion is highly variable in centrifuged samples[170]. However, centrifugation of urine samples prior to counting has been criticized as it leads to an unpredictable loss of cells[171]. Irrespective of the need to centrifuge or not, renal cell excretion is dependent on urinary flow rate and results should be presented as a rate of excretion.

B. Urinary cells - detection of renal tubular injury

1. Clinical studies

An increased urinary excretion of renal tubular cells has been reported in man following administration of several drugs. Repeated administration of aspirin caused an increased cell excretion which was maximal on days 2-3 but which was not sustained throughout the entire treatment period[172]. When cell excretion was monitored both before and after aspirin treatment, an increased excretion of renal tubular cells and erythrocytes but not leucocytes or protein was observed[173]; a similar change was seen after caffeine but not after paracetamol. Increased excretion of renal tubular cells was observed after volunteers had ingested a single dose of aspirin[124] and after administration of the diuretic, frusemide, to volunteers[174]. None of these drugs which cause a celluria is regarded as a frank nephrotoxin, and the mechanism by which the increased cell excretion occurs is unclear.

2. Experimental studies

Cell excretion studies in experimental animal models of nephrotoxicity are relatively few, and have mostly involved mercuric chloride. The production of renal proximal tubular cell necrosis of different degrees of severity in the rat by a single dose of mercuric chloride was characterized by an increased excretion of tubular cells, with the rise being more rapid in the more severe lesions. However, the peak cell excretion was poorly correlated with the severity of the renal lesions[175]. Mercuric chloride has been shown to induce a tolerance phenomenon, similar to that seen with aspirin in man, upon repeated administration to rats. Cell excretion rose rapidly to a peak after an initial delay and then declined more slowly, despite the daily administration of the nephrotoxin. Furthermore, a recovery period was necessary before a further cell excretion could be elicited by mercuric chloride, and which was of lesser magnitude[176].

In an attempt to positively identify cells in urine which originate from the renal tubules, a histochemical assay for succinic dehydrogenase (SDH), an enzyme with a high concentration in proximal tubular cells, was developed[177]. After treatment of rats with several nephrotoxins, cell excretion increased and the majority of cells were SDH positive. As the proportion of positive to negative staining cells appeared to vary with time after neomycin treatment, it was suggested that the histochemical approach might be used to indicate the severity of the lesion. However, the change in the proportion which stain positively may reflect differences in the numbers of necrotic and viable cells which are released, since the former cells may be devoid of activity.

In a recent chronic dosing study with an isoparaffinic solvent in rats, a large excretion of cells was observed in urine after 4 and 8 weeks of treatment, with a return to normal after a 4-week recovery period. These changes in cell excretion were accompanied by a very small increase in glucose and protein output, a small

reduction in urine concentrating ability and minimal tubular histopathology[178]. The authors concluded that renal cell excretion is a sensitive method for detecting proximal tubular damage but is not suitable for evaluating the degree of damage.

The rate at which renal tubular cells are excreted into urine is to be regarded as a sensitive and reliable indicator of acute damage to a specific region of the nephron, the proximal tubule[169]. Its specificity for this region needs to be established conclusively. On the basis of the relatively few studies which have investigated cell excretion, there is a generally poor correlation between the degree of renal tubular damage and the rate of tubular cell excretion.

V. CONCLUSIONS

1. The separation of urinary proteins according to molecular weight by electrophoretic methods can be used to classify proteinuria patterns and to provide a reliable indication of the site of renal injury.
2. Glomerular filtration and tubular reabsorption are the two major factors which affect the renal handling of proteins. Measurement of specific proteins in urine (e.g. albumin and β_2-microglobulin or retinol-binding protein) can be used as sensitive indices of functional changes to the glomerulus and/or the proximal tubule.
3. A direct relationship appears to exist between the presence in male rat kidney of a LMW protein, α_{2U}-globulin, and the appearance of hyaline (protein) droplets in renal tubular cells. Although this protein undergoes extensive reabsorption by the kidney, it is also excreted in large amounts. The physiological function of α_{2U}-globulin is unknown.
4. The kidney is the major source of urinary enzymes but relatively few of these enzymes are of diagnostic significance. Several precautions need to be taken prior to the assay of enzyme activity.
5. Urinary enzymes are sensitive indicators of renal damage and can be used clinically to detect renal disease, to give advance warning of an impending renal transplant rejection and to monitor the nephrotoxic effect of anitibotics and other therapeutic substances.
6. The selective measurement of enzyme activities in urine can be used in experimental situations to detect the site of the renal lesion after traditional renal toxicity tests have established the presence of acute renal injury.
7. Several urinary enzymes and specific proteins can be used to screen for renal damage/dysfunction in individuals occupationally exposed to potential or actual nephrotoxic substances.
8. Renal cell excretion in urine is a sensitive and reliable indicator of acute damage to the proximal tubule. The rate of cell excretion is not a good predictor of the extent of renal tubular injury.

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THE MEASUREMENT OF KIDNEY-DERIVED IMMUNOLOGICALLY REACTIVE MATERIAL IN URINE AND PLASMA FOR STUDYING RENAL INTEGRITY

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I. INTRODUCTION

It is the purpose of this chapter to describe the need for sensitive, site-specific immunoassays for identifying renal damage. These tests should complement rather than replace the established assays, both in clinical practice and in assessing the information derived from animal and in vitro studies. In common with all other chemical pathology assays, the interpretation of these data demands a thorough understanding of the pathophysiology of the condition and the methodological factors which can influence results. The advantages and disadvantages of these different methods will be discussed. The chapter deals with the problems of identifying renal-derived material, recent advances in assay development, and outlines the need for future development, in particular non-invasive tests for the detection of acute interstitial nephritis.

II. ASSESSMENT OF RENAL DAMAGE - CURRENT STATUS

The most serious consequence of kidney disease is loss of glomerular excretory function. Retention of waste products results in renal failure and death. Not surprisingly, screening tests of renal functional integrity have in the past concentrated on tests for glomerular damage such as proteinuria or reduced glomerular excretory function, as manifest by increased levels of plasma urea or creatinine. There are, however, serious limitations to this approach. Primary glomerular damage is for the most part due to immunologically mediated disease, diabetic glomerulosclerosis or, much less commonly, amyloid disease. Glomerular damage is a late secondary consequence of tubulointerstitial disease induced by drugs or industrial toxins. The glomerulus has little metabolic ac-

tivity and not surprisingly is less affected directly by toxins or drugs, save where there is immunologically mediated damage (e.g. penicillamine or gold therapy). It follows that, in screening for drug-induced renal damage, tests of glomerular function are insensitive indicators, save where severe tubulointerstitial disease has already developed. The use of plasma urea or creatinine as screening procedures is also unsatisfactory as the normal reference range is wide on account of variations in diet, muscle mass, tissue catabolic rates and non-specific assay interference. Thus small elderly individuals on a low-protein diet and with small muscle mass may have a considerable elevation of their levels of urea or creatinine due to glomerular disease, but these levels still fall within the normal range. Conversely a large muscular male eating a high-protein diet may have values above the normal range, yet have normal glomerular function. In general it is accepted that plasma urea and creatinine levels may not rise above the normal range until some 60% of glomerular excretory function has been lost.

The solution to the problems inherent in interpreting levels of plasma urea and creatinine lies in direct measurement of glomerular filtration rate, most often by creatinine clearance. This, however, is cumbersome in that it requires timed urine collections - usually over 24 h to eliminate defects in bladder emptying - and may be inaccurate due to errors in timing or missed collections. Radiolabelled chromium EDTA clearances using sequential blood sampling eliminates problems with urine collection, but is not generally available and is more expensive. Proteinuria, usually identified by commercial "dip stix" testing, certainly suggests glomerular disease, but is commonly absent in those patients with tubulointerstitial disease caused by nephrotoxins and is therefore a poor monitor for renal damage in trials on drug safety. Finally there is evidence that, even with primary glomerular disease, the associated abnormality of tubular function may be of greater prognostic value than the degree of glomerular dysfunction. For all these reasons it is more logical and more desirable to develop and employ tests which screen for tubular dysfunction, particularly in monitoring for possible chemically associated kidney damage.

Metabolically active tubular cells are particularly susceptible to toxins due to the large renal blood flow (25% of cardiac output) and because high tubular concentrations of non-reabsorbable toxins can develop in the normal process of urine concentration. Dysfunction due to nephrotoxicity should therefore be manifested at an early stage - long before progressive tubulointerstitial disease due to toxicity affects glomerular function.

Tests of renal tubular functional integrity are available. Glycosuria, phosphaturia, amino aciduria or low molecular weight proteinuria assess proximal tubular function, and urine concentrating ability and acidification measure distal tubular function. All of these tests are cumbersome, difficult to interpret, or are of diagnostic value only through excluding other possible causes. Due to the large reserve capacity of the kidney, demonstrable functional abnormality may be late in appearance in contrast with sensitive tests of renal cell damage. The assays of urinary enzymes do reflect cell damage, but enzymatic measurements have many disad-

vantages over the use of immunoreactivity (vide infra).

Tissue-derived proteins are normally present at a steady-state level in serum and urine as a consequence of cell turnover, thus altered levels due to cell damage are readily detected. The measurement of such proteins is widely used to detect damage to the heart, liver and pancreas, but the kidney remains an exception which is routinely monitored on the basis of biochemical function. This chapter reviews our current knowledge of how kidney-derived material may be used to screen for nephrotoxicity, and discusses the relative merits of using assay methods which measure immunoreactivity.

III. TOXICITY TESTING

Extensive drug toxicity trials are carried out in laboratory animals of various species with the aim of assessing risk in humans. Although invaluable, such studies have many shortcomings:

1. There is considerable variation in toxicity between laboratory animal species, and especially between animals and man, due to differences in pharmacokinetics, pharmacodynamics and organ susceptibility.

2. Detection of toxicity is more dependent on histological examination of tissues rather than sensitive, non-invasive biochemical measures. Detection of morphological changes is, however, susceptible to sampling error and early, subtle changes are not always easily seen.

3. Chronic studies in laboratory animals are of doubtful value for assessing long-term toxicity in man.

4. Renal dysfunction has not invariably been observed in the experimental animal, but a host of drugs cause toxicity when used clinically, as illustrated most recently with Cyclosporin A[1].

Pre-clinical drug trials are usually carried out in young healthy people and conducted over short periods of time, despite the fact that the drugs are often for chronic use in patients who may have age- or disease-related diminished renal functional reserve. The elderly have a higher rate of adverse reactions to drugs[2], which may result from non-compliance[3], age- and/or disease-related changes in pharmacokinetics and pharmacodynamics, and interaction with other prescribed drugs[4]. Given these observations, short-term trials in young healthy volunteers may demand more sensitive tests for nephrotoxicity to assess the potentially greater risk in patients.

Sensitive tests of tubular function are also needed to compare the relative toxicity of different drugs with the same therapeutic spectrum and as an early indicator of renal damage. This is especially important when chronic administration is envisaged. The therapeutic effects of many drugs far outweigh their

nephrotoxic potential, particularly if the loss of renal function is minor and reversible. Provided that the results are interpreted correctly, such tests need not be "over sensitive".

IV. INTERPRETATION OF RESULTS

A. Pathophysiological factors

With the introduction of any new assay, a problem which is often not thoroughly addressed is that of reference ranges. Physiological factors which can affect "normal values" include age (e.g. menopause, puberty), sex, race, posture, exercise, stress, diet, biological rhythms (e.g. diurnal, circadian and menstrual cycle variations) and pregnancy. In addition, in the sick patient the underlying disease may have secondary effects on renal function. In such cases evidence of drug-induced nephrotoxicity requires study of untreated matched controls or sequential study of individual patients before, during and after drug treatment. It was reported recently that transient proteinuria (that took up to 10 days to resolve) was a result of postural change, exposure to cold, emotional stress, adrenalin administration, abdominal operations, congestive heart failure and fever, all in the absence of renal disease[5]. This example emphasizes how important it is to assess changes in renal function that are so often automatically attributed to simultaneous drug administration.

Problems may arise due to non-pharmacological side-effects of drugs which may be interpreted as evidence of toxicity. Gentamicin, a known nephrotoxin, can, in common with other compounds which contain free amino groups, cause low molecular weight proteinuria due to saturation of reabsorption sites in the proximal tubule[6]. Separating such pharmacological effects from actual toxicity requires a thorough understanding of renal pathophysiology. When a change in serum or urine levels of proteins of unknown function is observed, it is always necessary to consider the possibility of a pharmacological rather than a toxic effect. Monitoring the rapidity with which such changes return to baseline after drug withdrawal can be a useful guide. Thus a return paralleling the known half-life of the drug would suggest a pharmacological effect, whereas a prolonged abnormality with more gradual return may suggest a toxic effect.

The most commonly measured kidney-derived analytes used in the detection of nephrotoxicity have been urinary enzymes. The methods employed have not usually been specific for the kidney isoenzyme but, as the enzymatic proteins are generally of relatively high molecular weight, interpretation of results has relied on the integrity of the glomerular barrier to exclude urinary enzymes from extrarenal sources. The specificity of these methods is thus questionable in the presence of proteinuria due to a glomerular leak. For example with N-acetyl-β-D-glucosaminidase (EC 3.2.1.30) (NAG), serum levels are high relative to the normal tubular derived urine output[7]. Given even a small glomerular leak, a marked increase in urine output due to glomerular filtration can occur in the presence of normal tubular function.

B. Methodological factors

Immunoassays facilitate the specific and sensitive measurement of a range of compounds previously present in quantities too small to assay, or not possessing easily measurable biological activity. Such assays have greatly increased the potential for measuring tissue-specific proteins. As yet there has been little impact on the routine measurement of enzymes because comparatively simple, cheap and easily automated (for high sample throughput) assays are already established. The measurement of urine enzyme activity is therefore still the mainstay for kidney-derived analytes. A scarcity of pure enzyme preparations for producing antisera is now less of a problem with the advent of mass-produced monoclonal antibodies.

Measurement of enzyme activity is still important in situations where increased or decreased activity has clinical relevance - for example renin in hypertension, blood clotting enzymes in blood disorders, etc. However, when enzyme levels are measured purely as markers of tissue injury, measurement of biological activity is not necessary, and has several disadvantages. Thus the activity of an enzyme depends on the functional integrity of its active site which, in turn, is dependent on the preservation of tertiary structure and therefore conformation. In the process of active tissue destruction, or during passage through the urinary tract, enzymes may be degraded and lose their activity. They still, however, retain immunoreactive sites and these fragments can be measured immunologically despite loss of biological activity. Haemolysis and lipaemia in sera, and the presence of chromophores and fluorophores in serum and urine, can affect the end-point detection and therefore enzyme assays require the use of sample blanks. Immunoreactivity is not affected by these factors. Enzyme activity is also dependent on temperature, substrate, protein matrix, pH, ionic strength and the presence of inhibitors or activators in the sample, the latter two in particular being difficult to investigate and yet potentially highly variable between samples[8]. Inactivation of an inhibitor would result in an apparent increase in enzyme activity, whereas simultaneous release of an inhibitor in tissue damage would mask an increase in enzyme activity. In many cases these problems have led to a pre-assay requirement to dialyse urine samples to remove low molecular weight interference, a process which in itself may result in loss of enzyme and is not suitable for large numbers. Although antigen-antibody binding is affected by pH, ionic strength and the protein matrix, these factors are readily investigated over the range of physiological and pathological variability, and reaction conditions can be optimized to exclude their interference. The presence of drugs and their metabolites is also more likely to interfere with enzyme activity than with immunoreactivity.

Total enzyme activity is affected by the relative proportions of isoenzymes, which often vary in disease and possess different reactivities under the same assay conditions. Isoenzyme specificity requires electrophoretic separation, which is not suitable for routine use, or immunoinhibition, which is not widely available for many enzymes. In immunoassays, antisera may be selected to be

more or less specific for any one (or more) of the isoenzymes.

The specificity of the antisera for a single analyte is of utmost importance in unlabelled immunoassays (i.e. nephelometric, turbidimetric, radial immunodiffusion, electroimmunoassay), whereas in immunoassays employing a labelled antigen the presence of antibodies to other immunoreactive proteins will not interfere provided the labelled antigen is free from such proteins and that they do not cross-react with the analyte. With the advent of monoclonal antibodies a greater number of immunoassays are being developed for tissue-specific proteins. However, it should be remembered that, due to the very nature of their monoclonality, and therefore specificity for a single epitope, such antibodies are more likely to cross-react with different proteins than are polyclonal antiserum. Rigorous investigation of their specificity is therefore required. In order to develop antigen-specific immunoassays it is often necessary to combine several monoclonal antibodies, and so minimize the likelihood of non-specific interference.

C. Expression of results

Non-reabsorbable substances are excreted in the urine at a rate independent of urine flow, and therefore their concentration varies inversely with urine volume. There may also be diurnal variations in excretion rate. Timed urine samples, typically over 24 h, are the classical solution to both these problems. This immediately introduces one of the greatest sources of error - that of obtaining a complete, correctly timed sample. The excretion rate of creatinine is relatively independent of urine flow rate[9] and is more or less constant throughout the 24 h. Provided the excretion rate of a compound is constant then a simple ratio to creatinine may be calculated on a random, untimed sample. If there is a diurnal variation in excretion rate then random samples may still be used, provided that they are always obtained at the same time each day. It is worth noting that creatinine output is higher in males than in females due to greater muscle mass. If a compound is excreted at the same rate in both sexes then factoring to creatinine will give slightly different reference ranges, being higher in females than in males. By taking the serum creatinine into account the variation in output due to muscle mass will be abolished, and excretion is then expressed relative to glomerular filtration rate, reflecting the number of functioning nephrons. Although the latter will vary with kidney size, so too should any kidney-derived compound. Compensation for body surface area is therefore not necessary.

In order to relate the clearance of a filtered substance (X) to creatinine clearance, the ratio is expressed in the following formula:

$$\frac{[X]u.V}{[X]p} \cdot \frac{[Cr]p}{[Cr]u.V}$$

where [X]u and [Cr]u denote the urine concentrations of X and creatinine respectively, and [X]p and [Cr]p denote their plasma

concentrations. The urine flow rate, V, is constant for both analytes and can therefore be eliminated from the calculation. The clearance ratio, a dimensionless quantity, may thus be obtained from a random urine and simultaneous plasma sample for estimation of both X and creatinine. In the case of a non-filtered analyte, [X]p is omitted from the calculation and a clearance ratio cannot be calculated. The amount excreted ([X]u.V) is then expressed per ml of creatinine clearance.

This calculation is also useful when comparing results from patients with varying degrees of glomerular impairment. With increasing loss of functioning nephrons the output of creatinine will remain constant due to the increased filtered load per nephron resulting from an increased serum concentration. However the output of kidney proteins per functioning nephron may be increased or decreased depending on whether there is active damage, on the balance between cell necrosis and regeneration and on the amount which may be released from any cell stores. Simply expressing output per unit time or factoring to urine creatinine without any estimate of the amount of functioning renal tissue may give misleading results.

V. APPLICATION OF IMMUNOLOGICAL METHODS

The measurement of tissue-derived materials as markers of tissue integrity should aim to satisfy several criteria. Ideally they should be specific for the tissue of interest and for the type and site of the lesion within the tissue, and not affected by disorders in other tissues. Levels should reflect the extent of damage and whether or not it is progressive. Sensitive, specific and precise assays should be available and other factors which may affect levels should be known. It will become apparent that although the number of assays for renal integrity are growing rapidly, most still fail to satisfy these criteria.

A. Demonstration of kidney-specific protein

The urine contains proteins derived from serum, kidney tissue and the urogenital tract[10-13]. Early studies of urine protein of non-serum origin depended on raising antisera to either kidney homogenates or urine concentrates, and absorbing these with serum proteins. By inference, the remaining antibodies were specific for proteins of the urinary tract. Immunoelectrophoresis separated at least six urine proteins and three specific renal proteins[14,15]. These figures are undoubtedly an underestimate because: (1) a relatively insensitive end point detection was used; (2) those antigens present in low concentrations would not elicit an immune response; and (3) any membrane-bound antigen may not have been solubilized prior to immunization. Although these studies provided useful qualitative evidence of the excretion of renal proteins, such methods are not capable of either quantifying or identifying the specific antigens involved. Nonetheless, Antoine[16] was able to demonstrate increased excretion of tissue antigens in urine of

patients with a variety of tubular disorders. Although these antigens were not kidney-specific, their absence from serum suggested a renal origin. Using similar techniques, greater amounts of kidney antigens were shown to be excreted in rats suffering from a wide range of experimentally induced nephropathies[17].

B. Immunoassays for uncharacterized renal antigens

In a series of papers, Mondorf and colleagues[18-24] described the isolation of brush-border membranes of the human proximal tubule and the production of antisera to this particulate fraction. Following absorption with human plasma proteins to increase kidney specificity, the antiserum was found to cross-react with alkaline phosphatase, alanine aminopeptidase and gamma glutamyl transferase[18,19]. An increased excretion of these brush-border antigens was demonstrated qualitatively on immunoelectrophoresis in the urine of patients with acute tubular necrosis and during transplant rejection, in acute and chronic glomerulonephritis, and following administration of nephrotoxic drugs and radio-constrast media[18,20-22]. This group also developed a quantitative radioimmunoassay by using the same antiserum to isolate, from the urine of a patient with kidney disease, a heterogeneous fraction of high molecular weight brush-border antigens[23] which was then ^{131}I-labelled. A correlation was found between antigen excretion and kidney damage during transplant rejection episodes and following ingestion of nephrotoxic drugs[23,24]. However, both qualitative and quantitative methods exhibited a 1% cross-reactivity with plasma proteins causing non-specific interference in the presence of significant proteinuria. In the rat, antisera to brush-border membranes of the proximal tubule also stain many non-renal epithelia, e.g. jejunal mucosa, bile canaliculi, pancreatic acinar cells, which have a common function as absorbing and/or secreting surfaces[25]. In a series of experiments, Zager and colleagues[26-29] also isolated and labelled a proximal renal tubular epithelial antigen. An abnormal urinary excretion was found in some 50% of patients with chronic tubulointerstitial disease and 79% with acute tubular necrosis, but not in those with glomerulopathies. In rats, following bilateral ureteral obstruction, increased excretion was found in proportion to the length of time of obstruction and the degree of brush-border effacement seen histologically[29]. An increased excretion of "renal antigen" of undefined specificity has also been described in children with active renal infection or other renal disease compared with children with normal renal function[30]. While demonstrating the potential of this approach for the detection of kidney damage, the simultaneous detection of a number of antigens and the ill-defined renal specificity does not allow precise identification of the site of renal dysfunction.

With the advent of monoclonal antibody technology, attempts are now being made to define the specificity of antisera to crude kidney extracts for sites within the nephron. Numerous monoclonal antibodies were raised to human kidney cortex plasma membrane fractions and their specificity evaluated by indirect immunofluorescence on kidney sections[31]. Most reacted with tubular antigens al-

though antibodies specific for glomeruli, blood vessels and the interstitial spaces were found. Some antibodies reacted with all tubular cells while others were more specific for different parts of the nephron. The specificity also varied according to the type of membrane, and whether intracellular antigens were stained either diffusely of focally. Such monoclonal antibodies have been used in the development of sandwich enzyme-linked immunosorbent assays for measurement of the antigens in urine[32,33]. The results were expressed as arbitrary units per 24 h, as no pure antigen was available for calibration. The lack of pure antigen also prevented full validation of the assays through parallelism and recovery experiments. Preliminary studies have shown differences in excretion of proximal and distal tubule antigens from healthy individuals and those with renal disease[24]. Whether these monoclonal antibodies react only with renal antigens has not yet been established. It may be that their specificity for detecting renal damage relies on the maintenance of glomerular impermeability.

Mutti and co-workers[34] have used an enzyme-linked immunosorbent assay employing a monoclonal antibody recognizing an antigen of molecular weight 50,000 (BB50) which is located on human proximal tubule brush-borders and also reacts with a component on the endothelial membrane of the peritubular capillaries, and compared its urinary excretion with that of retinol-binding protein and albumin. In chromate workers there was a significant increase in BB50 and retinol-binding protein but not albumin. In cis-platinum treated patients the excretion of all three was increased, presumably reflecting greater nephrotoxicity of this compound. The increased excretion of albumin was within the range which could be expected if due solely to diminished tubular reabsorption. It is unlikely that monitoring the excretion of antigens from this part of the nephron will reveal damage any different from that reflected by the assay of proximal tubular enzymes, although the move away from enzyme activity to immunoactivity is to be welcomed. This type of approach will be of most interest with the advent of markers for other parts of the nephron.

The production of monoclonal antibodies to both human and animal renal tissue is increasing rapidly[35-38], although most have not yet been employed in immunoassays. In most cases these antibodies have yet to be thoroughly screened for renal specificity. By using them on affinity columns to purify the antigens from kidney tissue, antibodies could be raised to other immunodeterminants on the antigen, and thereby increase the specificity. Monoclonal antibodies of defined specificity are not only of use as markers of kidney injury. They can also be used in renal cell culture work to identify cell types[35], and in studying the relationship between the kidney and those cells of the immune system which share common antigenic determinants and may be of significance in immunologically mediated renal injuries[36].

C. Immunoassays for characterized renal antigens

1. Ligandin

Ligandin is a cytosolic protein of molecular weight 46,000, found in the liver, kidney and small intestinal mucosal cells[39,40]. It is involved in organic anion binding and is also the major glutathione-S-transferase. In the kidney it has been found in the proximal tubule in the rat and rabbit[40], and in humans it also occurs in the thick ascending limb of the loop of Henle[41]. It comprises about 2% of the soluble protein in renal homogenates. Comparable results can be obtained by either immunological or enzymatic assay, although the latter is less sensitive[40]. Ligandin is generally undetectable in the serum of both normal individuals and patients with fulminant hepatic failure[42]. Therefore, although it is not renal-specific, and is of sufficiently low molecular weight to be filtered by the glomeruli, it is unlikely that significant amounts of ligandin in urine could originate from non-renal sources. Normal urine output[40,42] in humans is <120 μg per 24 h. It is not increased in chronic renal disease of various aetiologies, including glomerulonephritis, pyelonephritis and polycystic disease, nor in acute glomerulonephritis or hepatorenal syndrome. A variety of toxic and acute ischaemic tubular injuries in humans and rats[40,42] resulted in increased ligandin excretion which tended to mirror the increased levels of serum creatinine. Administration of iodinated contrast agents, which can cause acute renal failure, resulted in transiently increased excretion of ligandin in some patients with no change in serum creatinine[40], suggesting it may be of use in detecting subclinical tubular injury. In subclinical cadmium nephrotoxicity in the rat (of insufficient severity to raise serum creatinine), the increased excretion of ligandin paralleled that of lactate dehydrogenase, a more widely studied cytosolic enzyme, indicating that the two are of similar sensitivities[43]. The data suggest that increased ligandin excretion reflects ongoing active tubular damage.

2. Tamm-Horsfall glycoprotein

Tamm-Horsfall glycoprotein (THG) is a kidney-specific protein located in the thick ascending limb of the loop of Henle and distal convoluted tubule of human, rat and hamster kidneys[44-46] and also found in normal human[47,48] and rat[49] serum and urine. It is the only well-characterized distal nephron protein for which immunoassays are available, and is therefore the only marker for damage at this site. THG has a molecular weight of 75,000 but can aggregate in urine to polymers of several million molecular weight which, unless dissociated before assay, can cause aberrant results[48]. In humans, normal serum levels range from 100 to 500 μg/l[47], and daily urine excretion[50-55] from approximately 22 to 66 mg per 24 h.

The factors which control THG synthesis and secretion in health are unknown, but its specific location suggests that it may have a role in salt- and water-handling by the kidney. In chronic

renal disease serum and urine levels are generally decreased in relation to the diminished glomerular filtration rate[50,53,55,56]. None is detectable in the serum from anephric patients, but following successful kidney transplantation both urine and serum levels return to the normal reference range[57-59]. Conversely, unilateral nephrectomy, as in kidney donors, results in diminished serum levels, although not directly reflecting the loss of functioning renal mass[57]. In acute renal failure, and in transplant rejection, transiently elevated levels of serum THG are often found, but are not diagnostically useful[57,59]. The excretion of urine THG falls dramatically during transplant rejection[58], in contrast to the elevation of urine NAG[60]. This suggests that large amounts of THG are not stored in tubular cells to be released on cell necrosis.

The urine excretion of THG is more easily assessed by relating it to creatinine output or, better, to the rate of glomerular filtration[55]. In chronic renal disease of primarily tubular origin, such as Fanconi syndrome, cadmium nephropathy and Balkan nephropathy, an increased amount of THG is found per functioning nephron, although daily excretion rates are reduced in proportion to the diminished glomerular filtration rate[50,61,62]. In chronic renal disease of primarily glomerular origin a lower excretion of THG was found in patients with histologically observed tubular atrophy compared with those with well-preserved tubules[55]. The excretion of THG may therefore be a useful non-invasive marker of the initial site of renal lesions.

It is only recently that THG has been measured as a marker of nephrotoxicity. The concentration of serum THG in renal transplant recipients receiving cyclosporin A was significantly lower than in those on conventional immunosuppression with an equivalent level of serum creatinine[63]. In patients with primary biliary cirrhosis being treated with cyclosporin A, both serum and urine levels of THG were significantly lower than in a control group who had not received cyclosporin A, although other markers of tubular dysfunction (urine β_2-microglobulin, retinol-binding protein, albumin and NAG) were not significantly different between the two groups[64].

In a group of healthy volunteers taking non-steroidal anti-inflammatory drugs (piroxicam or naproxen), serum THG was significantly decreased, although neither urine THG nor other tubular markers were affected[65]. Whether these effects on THG reflect actual toxicity or are secondary to the pharmacological actions of the drugs, remains to be elucidated, and illustrates the problem of interpreting concentrations of analytes when the physiological control mechanisms are unknown.

3. Basement membrane antigens

Many of the components of renal basement membranes have been identified[66] although they are not renal-specific. Normal human urine contains small amounts of glycoproteins immunoreactive with antisera to basement membrane[67-69]. Low molecular weight fragments of type IV collagen and fibronectin have been identified[70]. Traces have also been found in normal human serum, and in lesser

amounts following bilateral nephrectomy, suggesting only a partial contribution from the kidney[68]. In rabbits with experimental acute nephrotoxic glomerulonephritis an increased urinary excretion of two basement membrane antigens normally present in urine, in addition to two other basement membrane antigens, was found, but only when polymorphonuclear leucocytes (PMNL) were involved in the glomerular lesion[67]. This was probably due to the presence of enzymes in PMNL which have been shown to hydrolyse glomerular basement membrane in vitro. The basement membrane antigens were not detectable in serum from these rabbits, suggesting their origin was renal and most likely glomerular.

With the more recent development of radioimmunoassays for the 7-S collagen domain of type IV collagen, and for the P_1 and P_2 fragments of laminin, there is increasing evidence that their levels in serum reflect disturbed basement membrane metabolism[71-73]. Such disturbances may also be reflected in the increased urine excretion of acid mucopolysaccharides[74] derived from connective tissues. Patients with renal disease have not yet been studied, but by simultaneously measuring the serum and urine levels of such compounds it may be possible to determine whether their origin is renal or not.

4. Prostanoids

Prostanoids are a family of chemically related compounds comprising prostaglandins (PG) and thromboxanes (Tx). The most abundant prostanoids synthesized in the healthy kidney are PGI_2 and PGE_2, with small amounts of TxA_2, $PGF_{2\alpha}$ and PGD_2. Their precise role in renal function has yet to be elucidated, but they can act directly on the nephron to stimulate water and electrolyte reabsorption, alter the distribution of blood flow within the kidney and affect the activity of renal sympathetic nerves. They can also interact with local hormones such as angiotensin II, bradykinin and vasopressin[75] in the kidney.

Prostanoids can be measured by bioassay, gas chromatography and radioimmunoassay. Due to the chemical similarity of these compounds their assay by immunological methods demands rigorous attention to the problem of cross-reactivity. It is apparent, retrospectively, that not all assays have been as specific as claimed. Measurements in serum are difficult to interpret as the kidney is only one of many sources of prostanoids. In the urine, PGE_2 and $PGF_{2\alpha}$ are believed to be solely of renal origin in females and pre-pubertal males[76]. In adult men, commonly high urine prostaglandin levels are probably the result of contamination with seminal fluid prostagladins[77].

In the context of nephrotoxicity the prostanoids have been of major interest as possible mediators of the renal dysfunction caused by non-steroidal anti-inflammatory drugs[78-84]. These drugs reversibly inhibit cyclo-oxygenase, a key enzyme in the synthesis of prostaglandins. Renal failure is most likely to occur in patients with already compromised renal perfusion who rely on increased prostaglandin synthesis to maintain intra-renal blood flow[80]. In these circumstances, inhibition of prostaglandin synthesis results

in acute renal failure, which is reversible on withdrawal of the non-steroidal anti-inflammatory drug. Renal failure may also occur due to interstitial nephritis[85], but it is unclear if this is prostaglandin-dependent. Although the frequency of clinically adverse renal effects is uncommon, the widespread use of these drugs has resulted in a huge population at risk[86]. They are one of the most common (perhaps second only to aminoglycosides) causes of drug-induced acute renal failure[80]. Because of the difficulties in measuring and interpreting prostaglandin levels, most renal effects of non-steroidal anti-inflammatory drugs on prostaglandin activity have been studied by monitoring end-organ responses compared with the responses to the administration of known prostaglandins.

Assay of PGE_2 has shown that its urinary excretion relative to functioning renal mass is increased in progressive chronic renal disease, presumably in an attempt to improve the decreased blood flow caused by the disease[87]. Dimished excretion was found following ingestion of a variety of non-steroidal anti-inflammatory drugs[79,88-90]. As more specific assays are developed, the measurement of prostanoids will probably become more commonplace. Nonetheless, it is likely that they will remain of most interest in understanding the pathogenesis of nephrotoxicity rather than as markers of renal integrity.

D. Future developments

Although enzyme activity along the nephron has been well studied[91,92] there is still a relative paucity of comparable information on the localization of immunoreactive material which could be used as markers for specific cell types. Increasing numbers of renal antigens are being identified immunologically[93-99] and may prove to be suitable markers for different parts of the nephron once assays are available.

The nature of the binding proteins involved in calcium reabsorption in the distal nephron[100-103], which may interact with cyclic nucleotides, is still being elucidated. These proteins may prove to be useful markers, as some are thought to have tissue specificity[104]. The measurement of cyclic nucleotides per se has been of great value in studying hormonal effects, e.g. the action of parathyroid hormone on the kidney[104]. Such studies may also help elucidate the role of calcium as a mediator of renal tubule cell injury[105,106] due to nephrocalcinosis, ischaemia and nephrotoxins. There is also a growing appreciation of the interaction of cyclic nucleotides with prostaglandins[107,108]. However, the assay of cyclic nucleotides is likely to remain of most interest in understanding the pathogenesis of renal dysfunction rather than as a marker of renal integrity.

From the clinical point of view, immunoassays for kidney-derived material have yet to make a contribution to the specific diagnosis of acute interstitial nephritis (AIN), probably the most common consequence of drug-induced nephrotoxicity[109]. Many drugs and occupational toxins cause AIN[109-111], which is characterized histologically by infiltration with plasma cells, lymphocytes, mononuclear cells and PMNL[109,112,113]. Diagnostic tests for detect-

ing subclinical AIN, and for the differential diagnosis in patients presenting with acute renal failure, would represent a major advance. With the identification of a growing number of leucocyte-specific proteins and other inflammatory mediators it may be possible to measure their urine output as a non-invasive means of assessing renal infiltration.

VI. CONCLUSIONS

The number of immunoassays for renal antigens, specific for different functional segments of the nephron, has increased rapidly over the past few years, especially with the advent of monoclonal antibodies. The chemical characterization, physiological function and controlling factors of most of these markers remains to be elucidated. Because of this, caution is advised when monitoring drug toxicity in ascribing changes in their levels as simply due to toxic effects. Using a range of such non-invasive assays should allow the localization of the early lesions in both toxic and other forms of renal disease in the human, and decrease the present reliance on animal studies.

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EXTRAPOLATION OF ANIMAL DATA TO MAN: THE CONCORDANCE BETWEEN TOXICITY SCREENING AND CLINICAL CONSEQUENCE

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I. INTRODUCTION

Exposure of humans to nephrotoxins can occur through ingestion, inhalation or contact. While the offending agent is often a prescribed drug or illicit compound, the two other categories of nephrotoxin exposure which are gaining clinical prominence are chemicals in the workplace and environmental contaminants. This chapter focuses on the problems of extrapolating experimental data to humans.

There are at least four reasons for conducting animal studies. Foremost is the desire to provide information regarding both safety and efficacy. The second purpose is to explore the mechanism of injury. An understanding of the mechanisms of cell injury allow rationale modifications of the drugs' structure to eliminate or minimize the adverse effects. In addition, the information obtained while searching for the mechanism of drug-induced damage often has relevance to natural occurring renal diseases. Furthermore, by understanding the mechanisms of drug-induced renal injury, techniques for protecting the kidney can be tested which may reduce toxicity without impairing clinical effectiveness.

With the increasing sophistication of drugs, the potential for drug-drug interaction is increasing. Obviously, testing these interactions is another application for animal models. However, in order for studies on models to be clinically meaningful, the drugs' pharmacokinetics/pharmacodynamics in the test animal must resemble those in humans.

The fourth use of animal models is in evaluation techniques for modifying toxicity. This may be the outgrowth of studies directed at mechanisms of injury, but, it is also possible to conduct such studies without knowing the mechanism(s) of renal injury. There are two factors which can be defined as influencing

drug toxicity - those that are intrinsic to the drug and those that are unique to the organism being treated. Once the general mechanism of drug actions is defined one can usually speculate on the cause of its toxicity. In addition, there are several characteristics of the patient in whom nephrotoxicity has a greater likelihood of occurring. Elderly patients and individuals who are fluid volume-depleted are particularly susceptible. For drugs which are excreted by the kidney, a permanently reduced renal function and liver disease may represent a risk factor. Equally important is the role of enzyme inducers in altering the renal response to drugs. An example is the effect of polyhalogenated chemicals which do not produce any direct renal cell injury, but induce microsomal enzymes and may lead to the increased production of reactive metabolites from otherwise innocuous agents[1].

As outlined in Table 1, there are three phases to animal research in nephrotoxicity. Phase 1 compares how closely the characteristics of the renal injury in animals relates to the clinical counterpart using both structural and functional parameters.

Table 1 Animal model

Phase 1:	Characterize (describe) the drug-induced injury pattern in the animal model. How does it compare with clinical counterpart?
Phase 2:	Mechanism of drug-induced organ/cell damage to test hypothesis.
Phase 3:	Factors which modify drug-induced nephrotoxicity. What risk factors are associated with enhanced toxicity? What maneuvers can be used to reduce potential for toxicity?

If there is not an exact replica, the animal model may still be close enough to allow meaningful data collection and progression to phases 2 and 3. If nephrotoxic damage is the result of a combination of circumstances involving the toxicant and the recipient, it may be impossible to reproduce these changes in the laboratory setting. The failure to mimic pre-existing disease, or the absence of age-related changes in the animal, can be a serious handicap to a reproducible model that parallels a clinical nephrotoxicity. In many animal models, toxicants must be administered in amounts substantially exceeding those recorded in their clinical counterpart in order to reproduce the characteristic lesion. Excess dosage may also induce other organ dysfunction which will compound the variables acting to reduce renal injury. For example, high doses of aminoglycoside antibiotic yield significant neurotoxicity in rats which can cause premature death, independent of renal damage[2]. However, once an animal model is a suitable reproduction of the clinical state, one can progress on to phases 2 and 3 to search for the mechanistic basis of an abnormality. Furthermore, a stepwise progression from in vivo observation through isolated organ and individual nephron measurement to single cell preparation is

required in order to characterize the injury pattern. However, it must be recognized that phases 2 or 3 can proceed in parallel and are not interdependent, but rather complementary. From various clinical studies have emerged certain well defined risk factors increasing nephrotoxic reactions (Table 2).

Table 2 Clinical risk factors for nephrotoxicity

Volume depletion (? diuretics)
Old age
Hypotension/renal ischemia
Pre-existing renal disease
Liver disease
Sex of patient
Co-morbid illness
Concomitant nephrotoxic drugs
Bacteraemia
Acidosis
Hypokalaemia

Table 3 Factors protective against drug-induced acute renal failure

Ion loading: Na^+, K^+, Ca^{2+}
Volume expansion
Inhibiting renin/angiotensin
Concomitant drugs
Thyroxine
Young age
Dosing modifications

Other factors include recent dosage with the same class of drug, abnormal liver metabolism, enzyme induction, drug-drug interactions, and sex. Furthermore, a similar tabulation of protective factors, especially in the case of experimentally-induced renal failure, have been identified (Table 3). Thus, it is possible to provide broad suggestions regarding modulations of clinical nephrotoxicity through the expedient of these generic modifying factors.

Animal models have both advantages and disadvantages in exploring nephrotoxicity. A major advantage is the ability to control variables. Unlike humans with their multiple genetic and environmental variability, in-breed strains of animals are much more predictable and thus more consistent in providing toxicity data. A well-characterized animal model also allows the testing of those manipulations designed to modify or eliminate the nephrotoxicity. The major disadvantage of using animal models as a predictor of human nephrotoxicity is the inability to mimic the clinical condition under which administration of the drug usually occurs. For example, in exploring antibiotic nephrotoxicity, infected animals are rarely used so that the clinical indication for prescribing the drug, along with the altered physiology which infection induces are absent. A final disadvantage of using animals is that there are examples of human nephropathies (e.g. radiocontrast-induced nephrotoxicity) which cannot be conveniently reproduced in animals.

Table 4 Relative merits of animal models

Advantages
Variables can be controlled.
Pre-screening possible resulting in a quick answer.
Allows direct drug-drug comparison.
Disease mechanisms can be explored at cellular level.
Preventative intraventions can be tested.
Disadvantages
Often does not mimic clinical conditions.
Excessive dosages are often required.
Endpoints are often extreme.
Excessive sensitivity due to in-breeding may lead to elimination of valuable drugs in early testing.

In order for the animal model to provide interpretable data, there must be a detailed description of the characteristics of the clinical presentation of the drug-induced nephrotoxicity. If one is going to develop strategies for preventing or modifying the toxic response in patient, then the degree to which the clinical condition can be duplicated in animals is a fundamental consideration. Two examples of drugs in which animal models have been used to provide understanding of a clinical nephrotoxicity will be reviewed.

The clinical presentation of drug-induced nephrotoxicity may either be acute or chronic and involve the renal circulation or the parenchymal cells. Distinguishing between vascular injury and cell injury often requires substantial research. However, when the injury has been fully defined, it allows rational decisions to be made on preventive measures and potential co-existing risks.

Table 4 summarizes the advantages and disadvantages of using animal models in the evaluation of drug-induced nephrotoxicity. However, even after a suitable animal model is developed, the problem of translating experimental results to clinically useful information may be difficult. One of the principle problems which confound this transfer is the lack of a sensitive, easily obtained measure of renal tubular function and/or reserve in humans. Such a statement seems paradoxical when so much experimental information is known about both the cellular, subcellular and integrated function of the kidney, and yet the most often used parameter to estimate renal functions in man is serum creatinine, an indirect assessment at best of glomerular filtration rate (GFR). The lack of a reliable and reproducible estimate of tubular function and/or reserve is a problem because most toxic injuries to the kidney involve cellular damage. However, when nephrologists consider renal function or measuring "renal reserve" they are referring to GFR.

Table 5 Source of urinary enzymes

Lysosomal	Brush border	Low molecular weight
N-Acetyl-β-glucosaminidase(NAG)	Alanine aminopeptidase(AAP)	β_2-Microglobulin
α-Glucosidase	Alkaline phosphatase	Malate dehydrogenase
β-Glucoronidase	α-Glutamyltransferase	Lysozyme
β-Galactosides		Ligandin
α-L-Fucosidase		Amylase
		Light chain, λ, κ

This discrepancy arises because, inexact as it may be, the measurement of GFR and its interpretation have gained near

uniform acceptance. Furthermore, while the measurement of endogenous creatinine clearance requires a timed urine collection and a simultaneous blood sample, it does not require the administration of exogenous indicators nor the technical expertise that inulin clearance or the radioisotopic techniques demand. Furthermore, a simple serum creatinine can often suffice as an estimate of GFR[3]. Obviously, if changes in GFR reflected damage to the nephron then this test would be a convenient monitor of early acute or chronic toxic effects. However, this is not the case. While Bohle et al.[4] have shown a correlation between a decrease in renal cell volume and reduced GFR for patients with chronic progressive interstitial nephritis, other investigators report a wide disparity between these two parameters[5,6]. The dissatisfaction with GFR as a sensitive predictory of toxic renal injury has prompted the use of other techniques. In particular, quantitative urinary enzyme excretion patterns have been proposed as a practical method. Timed urine samples are, however, required, and there are a wide variety of enzymes from which to select[7]. The choice of enzyme, depends on the cellular component of the nephron in which damage is suspected, and can be tailored (Table 5) to confirm this[8]. However, significant increases in urinary enzyme excretion are often considered "too sensitive" since they do not correlate with other parameters of renal dysfunction (usually some estimate of GFR)[9].

II. MECHANISM OF RENAL INJURY

Traditional, experimental acute renal failure (ARF) has been divided into an initial or initiating event and a maintenance phase[10]. Initiating events can be mediated either through an effect on vessels causing altered haemodynamics, or a direct cellular injury and cell death.

The haemodynamic effect resulting from blood vessel changes often represent an exaggeration of normal circulatory control. For example, infusion of the endogenous occurring pressor epinephrine will raise renal vascular resistance (RVR) and diminish renal blood flow (RBF). It will also activate the production and release of the intrinsic vasodilator substance, prostacyclin (PGI_2). When nonsteroidal anti-inflammatory drugs are given simultaneous with epinephrine, RBF is more severely curtailed, thus exaggerating the ischaemia. Clearly, prolonged and persistent reductions in RBF are associated with a time dependent tubular cell injury in the ischaemic model of ARF that culminates in the maintenance phase[11].

The maintenance phase of ARF is characterized by renal structural changes that can be verified by microscopic abnormalities and dysfunction. The abnormalities which have been identified are:

(1) nephron lumen obstruction with cellular debris arising from membrane bleb formation[12].

(2) luminal fluid back-leak across denuded basement membrane[13], and

(3) altered glomerular capillary permeability (K_f)[14]. Some combinations of these abnormalities may persist and lead to subacute or chronic renal failure[15].

Most clinically important nephrotoxic drugs are considered to cause structural and functional abnormality by acting directly on cellular components to cause necrosis. A notable exception may be cyclosporin in which a vascular mediation has been proposed[16,17]. In addition, many of the industrial toxins and environmental intoxicants may share both vascular and cellular mechanisms.

Before mechanisms can be proposed to account for renal cell injury the possible sites of nephron involvement are identified, based upon structural and functional changes in either man or animals. Agents damaging the proximal tubule may induce a Fanconi-like condition with glucosuria, aminoaciduria and proximal tubular acidosis secondary to bicarbonate wasting and magnesium wasting. In addition, proximal tubular enzymuria may occur along with β_2-microglobulinuria and renal phosphate wasting. Recently, availability of site specific urinary antigens for both proximal or distal tubules, have been reported[18]. It must be emphasized that interpretation of such observations must be cautious when used to design animal experiments.

Other classifications of nephrotoxic damage are based on structural location and the suspected subcellular site of injury (Table 6). If this can be identified, then the potential mechanisms of injury can be narrowed and appropriate experiments designed.

Table 6 Tubular cell injury

Intracellular site	Mediator cell injury
Plasma membrane	Permeability/osmotic rupture
Lysosome	Enzyme activation/peroxidation
Mitochondria	Permeability/energy disruption
Endoplasmic reticulum	Drug metabolism/metabolite
Cell sap	Ca^{++} maldistribution
Nucleus	Histone binding/chromosome damage
Vascular Cell Injury	
Endothelial fenestri	Altered K_f

Many drugs (Table 7) cause renal reactions, with frequencies that range from common to rare[19]. The toxicological information for many drugs of contemporary interest, i.e. cyclosporin, non-steroidal anti-inflammatory drugs, cisplatin, is evolving so rapidly that the availability of confirmatory experimental data is either lacking or conflicting. At present, data on environmental nephrotoxicants is often less well defined, even for well studied heavy metals such as lead[20].

Table 7 Drug-induced renal disease (offending drugs)

ANTIMICROBIAL AGENTS:	**AMINOGLYCOSIDES, PENICILLINS, TETRACYCLINE, AMPHOTERICIN B,** ACYCLOVIR, SULFONAMIDES, CEPHALOSPORINS, POLYMIXIN/COLISTIN, RIFAMPIN
ANALGESICS:	**COMBINATION ANALGESICS (PHENACITIN, ASA, ETC.),** ACETYLSALICYLIC ACID, PARACETAMOL (ACETAMINOPHEN)
ANTIHYPERTENSIVES:	**DIURETICS,** CAPTOPRIL, HYDRALAZINE
ANTI-INFLAMMATORY:	**NSAI, GOLD SALTS, PENICILLAMINE,** ALLOPURINOL, URICOSURICS
ANTINEOPLASTICS:	**CISPLATIN, CYCLOPHOSPHAMIDE, STREPTOZOTOCIN,** MITOMYCIN C, NITROSOUREAS, ADRIAMYCIN, METHOTREXATE, MITRAMYCIN, VINBLASTINE
ANAESTHETICS:	**METHOXYFLURANE,** ENFLURANE, HALOTHANE
MISCELLANEOUS:	**CONTRAST MEDIA, LITHIUM, HYPOGLYCEMICS,** PHENYTOIN, PHENINDIONE, TRIDIONE

Key: **COMMON**, UNUSUAL, RARE

III. AMINOGLYCOSIDE NEPHROTOXICITY

Shortly after gentamicin became widely used, there were clinical reports that the drug had a significant incidence of nephrotoxicity[21]. However, due to its unique bacterial spectrum, clinical efficacy and lack of a suitable non-toxic substitute, it was still widely used.

Aminoglycosides are not metabolised by man and after saturation of tissue stores, undergo quantitative excretion by the kidney[22,23]. In addition to renal accumulation, significant concentrations can be found in the endolymph and cartilage. Gastrointestinal absorption is minimal so that clinical administration requires a parenteral route.

Gentamicin has been the most exhaustively studied member of the aminoglycoside antibiotic group. While differences do exist with some of the more recently introduced aminoglycosides, as a class,

the renal handling of these compounds are similar. Because of minimal protein binding[24] renal handling of aminoglycosides is principly by glomerlular filtration. An initial interaction between the aminoglycoside and the vascular endothelial cells of the glomerular capillary may account for a portion of the drug-induced nephrotoxicity. The explanation invokes the fact that aminoglycoside antibiotics carry substantial cationic charges with injury occurring because of a charge interaction with fixed membrane anionic sites distributed along the endothelial surface of the glomerular capillary wall[14]. Conflicting data exists with regard to tubular secretions of aminoglycosides[25], although studies in isolated perfused kidney[26] and in vitro renal cortical slices confirm peritubular uptake[27], an observation compatible with a component of tubular secretions, substantial luminal uptake of gentamicin occurs along both the convoluted and straight portion of the proximal tubule[28]. Furthermore, this proximal tubular luminal uptake is judged to be concentration dependent, based on radioisotopic studies. Vanderville et al.[29] reported a progressively decreasing tubular cell aminoglycoside concentration downstream from Bowman's capsule. The cellular uptake of drug is also energy dependent, and inhibited by anoxia and 2,4-dinitrophenol[27] using isolated renal cortical slices. Experiments using isolated rabbit tubules, have shown that gentamicin uptake occurs in all three segments of the proximal tubular[30]. Once cellular uptake occurs, tissue half-life is prolonged exceeding that of plasma by up to 100-fold[31].

Aminoglycoside uptake by proximal tubular cells is not influenced by glucose[32], N-methylnicotinamide (NMN)[33], p-aminohipputate (PAH)[32,33] or probenecid[32], indicating that neither the sugar nor the organic ion transport systems contribute to intracellular accumulation. Using radioautographic techniques, aminoglycoside binding to the apical plasma membrane has been shown[34], with engulfment into apical vesicles occurring within 10 minutes[28]. The apically derived vesicles fuse with lysosomes and form cytosegresomes within 60 minutes. The possibility of the existence of unbound aminoglycoside within the cytosol has been suggested from the observations of Weeden[35].

The kinetics of renal cortical cell uptake have been studied both in vitro and in vivo. Several studies[31,33,36,37] have characterized the exponential nature of gentamicin uptake by human kidneys. While the total amount of aminoglycoside does not predict nephrotoxicity with certainty, it is considered to be a factor which enhances risk[38]. However, there is a growing appreciation that the initial kinetics of cellular uptake of the drug may be closely related to the eventual manifestation of cellular toxicity. Recent observations from Giuliano et al.[39], suggest that gentamicin/amikacin may follow Michaelis-Menton kinetics while tobramycin kinetics are linear. This difference may explain the reduced toxicity of tobramycin in animals.

To determine the applicability of animal models of aminoglycoside nephrotoxicity to the clinical situation in patients, a comparison of each is required. Aminoglycoside antibiotics are the most common cause of drug-induced acute renal failure for hospitalized patients[40]. Therefore, understanding and preventing this nephrotoxicity is important since aminoglycoside antibiotics provide

a significant treatment modality in serious gram-negative infections.

The clinical manisfestation of aminoglycoside nephrotoxicity usually takes the form of non-oliguric acute renal failure. Less frequently, oliguric acute renal failure may develop, or one of a variety of tubular syndromes, i.e. nephrogenic diabetes insipidus, Fanconi syndrome, renal magnesium or potassium wasting[25]. It is not clear if repeated insults might culminate in chronic interstitial nephritis. A drug-induced concentrated defect, characterized by polyuria and secondarily increased thirst, precedes the detectable rises in blood urea nitrogen (BUN) and serum creatinine, which begins after 5 to 7 days of parenteral aminoglycoside therapy. Mild proteinuria and granular casts have been identified in the urine[9] but are diagnostically non-specific. The most sensitive index of aminoglycoside administration is the early appearance of an increased quantity of either β_2-microglobulin or proximal tubular brush-border enzymes, i.e. N-acetyl-β-glucosaminidase (NAG), alanine aminopeptidase (AAP), etc. (Table 5) in the urine at between 2 and 4 days of treatment. While enzymuria is a highly sensitive indicator, the specificity with regard to identifying clinical-significant aminoglycoside nephrotoxicity has been questioned[41]. Obviously, advocates of urinary enzyme monitoring can criticize the clinical criteria for confirming aminoglycoside nephrotoxicity[46] - as failing to utilize appropriate markers of tubular toxicity. More recently, Mandal et al.[43] have proposed that quantitative increases in urinary myeloid bodies correlate with significant deterioration of renal functions. Further evaluation of the urinary myeloid bodies will be needed to assign its diagnostic significance. Parallel studies of altered renal histopathology in human aminoglycoside nephrotoxicity are not as numerous as the measurement of GFR. However, patchy tubular necrosis has been confirmed in patients experiencing acute aminoglycoside induced nephrotoxicity. In addition, electron microscopic evaluations have demonstrated the presence of autophagocytosis and prominent cytosegregosomes in selected proximal tubular cells patients[44]. It should be noted that these "myeloid bodies" may also be present following the ingestion of non-nephrotoxic drugs, i.e. quinine[45] and may therefore represent a histological marker of aminoglycoside administration rather than an intermediate step in the cascade of cell injury. Various risk factors for aminoglycoside nephrotoxic reactions have been identified by analysis of variance using double blind crossover clinical trials[46] and these are summarized in Table 2. Clinical aminoglycoside nephro-ototoxicity occurs principally as a non-oliguric acute renal failure in 2 to 20% of patients being treated for serious gram negative infections. The fall in GFR, measured as creatinine clearance (C_{Cr}), usually occurs between day 5 and 10 of parenteral treatment, although longer latency periods have also been reported[47]. Polyuria, mild proteinuria with granular cast, proximal tubular brush-border enzymuria, urinary β_2-microglobuluria, and urinary myeloid bodies excretion precede or coincide with the fall in GFR. Certain patient characteristics such as old age and liver disease are associated with an increased risk of nephrotoxic response, while infants and small children tolerate larger doses of the drug without adverse renal effects.

This pattern of renal dysfunction can be reproduced in

animals given aminoglycoside. Most of the animal studies have been conducted using gentamicin as the prototype chemical structure. Other aminoglycosides may share the potential for renal injury, but they do manifest lesser degrees of damage on a dose/response basis.

Using Fischer 344 rats and a standard dosing protocol[38,48,49] a predictable, dose-dependent and progressive form of aminoglycoside nephrotoxicity has been induced in animals. The rat has become the most frequently reported laboratory animal for evaluating aminoglycoside-induced nephrotoxicity. Characteristically, a dose-dependent non-oliguric acute renal failure develops over a 7 to 14 day period followed by near normal recovery despite continued administration of drug[15]. A persistent defect is urinary concentrating capacity which is evident during prolonged administration[50]. Glucosuria and proteinuria and the urinary elimination of potassium, calcium and magnesium[51-53] occur during the second week of aminoglycoside dosing. Complementary changes in tubular function parallel the increased urinary electrolyte excretion and increased fractional excretion of both Na^+ and K^+ have been reported by Luft et al.[49]. According to Kluwe and Hook[27] reduced tubular reabsorption of glucose accounts for the glucosuria. Cronin et al.[54] have pursued the adverse consequences of the kaliuretic action of aminoglycoside in the dog. While interference with proximal tubular reabsorption of filtered protein has been confirmed by Cojocel et al.[55]. Measurement of organic ion transport sequentially throughout the period of experimental ARF result in a progressive decline in NMN uptake, while that of PAH is biphasic, initially increased, then decreased[33]. Concomitant reduction in renal blood flow has been documented by Appel et al.[56]; however, outer renal cortical blood flow was retained, a finding characteristic of experimental non-oliguric renal failure. They did note a disparity in that while RBF fell by 67%, simultaneous reductions in GFR varied from 15-50%. Urinary enzymes such as NAG, leucine aminopeptidase (LAP), β_2-microglobulin, all increase when aminoglycoside dosing starts.

Structural correlates of the functional changes detailed above have been well documented. Proximal tubular necrosis of a patchy, dose-dependent nature has been reported[34,38,44,49,57,58]. Cytosegrosomes with prominent myeloid bodies develop within the first 48 hours even at the very low dose of 1 mg/kg/day[59]. Such findings are limited to electron microscopic evaluation, but it is rare to confirm changes by light microscope at doses less than 10 mg/kg/day. The typical light microscopic lesion is a focal, patchy proximal tubular necrosis which peaks at 10 days of aminoglycoside treatment and has co-existing evidence of cellular regeneration. Prominent swollen mitochondria and dilated endoplasmic reticulum precede frank cell necrosis[58]. Using histochemical techniques, Heinert et al.[59] identified an antecedent change in cellular protein composition of proximal tubular prior to aminoglycoside-induced cell necrosis. Laurent et al.[60], using ^3H-thymidine, detected significant cellular regeneration at aminoglycoside dosages which failed to induce light microscopic evidence of cell necrosis.

How good is the concordance between the experimental lesion in animals and that reported for humans? Schentag and Plaut[61]

evaluated 201 patients given 267 courses of aminoglycoside antibiotics. Using the criteria of Smith et al.[62], 17% of their patients developed clinical nephrotoxicity. Most cases where non-oliguric acute renal failure in which serum creatinine and/or BUN increased between the 7th and 10th day of therapy. This rise was preceded by an increased excretion of β_2-microglobulin on day 2 or 3, followed in succession by an increase in urinary NAG and increased urinary casts in the sediment. Using a computer-based pharmacokinetic model, they concluded that those patients with nephrotoxicity had significantly greater tissue accumulations of gentamicin during the first 24 hours of dosing, despite the fact that comparable amounts were given. Kaye et al.[63] found that gentamicin induced a mild, transient defect in proximal tubular reabsorption of low molecular weight proteins, i.e. β_2-microglobulin, amylase and light chain, but without overt nephrotoxicity. Abnormal enzymuria in both human volunteers[64] and patients receiving aminoglycoside[41,65,66] have all demonstrated this to be a measurement of much greater sensitivity than the usual clinical parameters of serum creatinine or some derivative. The interpretation of enzymuria continues to be controversial with respect to predicting significant nephrotoxicity, but clearly resembles the findings in animals. Also, histological damages adjudged by light and electron microscope parallel those reported in animal experiments[57,58] and also include the presence of myeloid bodies in the urinary sediment from treated patients[67].

Table 8 Features of gentamicin nephrotoxicity

Man	Rat
Renal failure is non-oliguric.	Polyuria and a decreased U_{osm} precede fall in GRF.
Renal cortical concentrations of gentamicin exceed serum and medullary levels 5 to 10 fold.	Renal cortical tissue has 20 times more gentamicin than serum and four times more than renal medulla.
Proximal tubular necrosis is the predominant lesion. EM shows "myeloid bodies" in most patients irrespective of clinical toxicity.	Ultrastructural changes occur early and independent of development of proximal tubular necrosis.
Recovery of glomerular filtration rate is usual without dialysis.	Glomerular filtration rate improves and proximal tubular regeneration occurs despite continued gentamicin administration.

The similarity between aminoglycoside nephrotoxicity in humans and laboratory animals is summarized in Table 8. A remarkable similarity is that reported by Trollfors[68], who identified six patients with nephrotoxicity that cleared despite continuous dosing. This finding paralleled earlier studies on animals[15].

Having established that the animal model reproduces clinical aminoglycoside nephrotoxicity, investigators have pursued techniques to reduce or eliminate the risk of nephrotoxicity. Several risk factors (Table 2) have emerged from clinical trials with aminoglycoside therapy and these have guided subsequent animal experiments. The adverse effect of volume depletion in aminoglycoside toxicity was confirmed in both rats[69,70] and dogs[71]. Furthermore, in the dog the interaction of aminoglycoside with diuretics was separated from the diuretic-induced volume depletions. Age-related sensitivity has been confirmed as young animals are resistant and old ones susceptible[72]. The resistance of female animals to aminoglycoside-induced injury[73,74] was confirmed. However, the role that sex hormones play is still disputed, and a similar resistence in female patients to the antibiotic remains to be substantiated. The route and frequence of aminoglycoside administration have been extensively evaluated. In animals, the magnitude of the nephrotoxic response increased with daily dosing and also with frequency of dosage[75,76]. Thus, experimental animals given identical total daily doses in three, rather than one, injection have earlier and more profound renal failure. While data in humans is less convincing, Powell et al.[76] did conclude that intermittent dosage was preferable to continuous infusion. Another area of potential risk of aminoglycoside-induced nephrotoxicity involves electrolyte interactions. Cronin et al.[77] have provided clear evidence that potassium depletion enhances aminoglycoside nephrotoxicity in dogs. Conversely, Bennett and co-workers[78] have reported significant protection from experimental aminoglycoside nephrotoxicity in rats given a sustained calcium load, a finding confirmed by Hume and Weinberg[79]. Whether magnesium depletion and/or loading might modify the aminoglycoside nephrotoxicity must await further studies. While the additive nephro-toxicity of concomitantly administered cephalosporins and diuretics was reported in the early clinical literature, reproducing this interaction in animal models proved difficult[80]. Since both the rate and extent of tissue accumulation of aminoglycoside were considered to be important determinants of experimental toxicity[81], it followed that attempts to selective block proximal tubular uptake could be clinically important. Our group examined compounds that would provide competitive inhibition of aminoglycoside uptake by proximal tubular cells. Polyamines are naturally occurring metabolites which are filtered and reabsorbed by proximal tubule. While they are capable of displacing tissue-bound aminoglycoside in vitro, they provided to be too toxic for in vivo use. The second approach was also based on competitive binding and the difference in toxicity between recently introduced aminoglycosides, i.e. amikacin and netilmicin[82]. However, this approach also failed to prevent the manifestation of acute nephrotoxicity[83]. The interesting observation[84] that diabetic animals are protected from the toxic effects of various aminoglycosides, has been confirmed[85,86], but Bergeron et al.[87]

were unable to demonstrate any decreased incidence of netilmicin-induced nephrotoxicity in diabetic patients.

As noted above, diuretic-induced volume depletion has been implicated as potentiating aminoglycoside-induced nephrotoxicity in the dog[71], but not humans. Smith and Lietman[88] analyzed three prospective, controlled, randomized trials in which patients were given aminoglycosides with or without concomitant furosemide. Patients receiving the combination had a 20% incidence of nephrotoxicity which did not differ from 17.1% in the non-diuretic treated group; an additional increase in ototoxicity could be identified. Lawson et al.[89] did, however, report that furosemide reduced renal gentamicin clearance, with a resulting transient rise in serum gentamicin levels. While acute changes in GFR cause measurable, parallel changes in gentamicin clearance, chronic changes have a less dramatic effect. In a study of the effect of aging on the pharmacokinetics of gentamicin Bauer and Blouin[90] were unable to establish any age related effect in either gentamicin clearance, volume of distribution or half-life. A significant difference between aminoglycoside nephrotoxicity in humans and rats involves the occurrence of cephalosporine-aminoglycoside synergistic toxicity. While several well documented cases and control trial have revealed synergism in humans[91], repeated attempts to induce a similar state in rats have failed[92] and some degree of protection has been found. Protection has also been noted when ticarcillin and an aminoglycoside is given to rats[93]. In concluding that cephalothin with aminoglycoside had an additive nephrotoxicity, both Wade et al.[94] and Klastersky and co-workers[95] compared the combination to one of aminoglycoside plus semisynthetic penicillin. Both methicillin and ticarcillin, had a protective effect rather than an additive effect of cephalothin to aminoglycoside. One important difference between aminoglycoside nephrotoxicity in experimental animals and in man is the co-existing infection. To more faithfully reproduce the clinical setting, our group undertook experiments in infected rats given 2 different aminoglycoside dosage regimens, i.e. every 4 h or every day - same total daily dose of 60 mg/kg/day[96]. The result supports our previous findings that the every 4 h dosage was significantly more nephrotoxic than the every day dosage. However, both dosing schedules were equally effective with respect to antibacterial action.

The ultimate usefulness of animal experiments is derived from their ability to predict nephrotoxic potential of aminoglycosides in a clinical setting. From experiments in a variety of laboratories[25-27,31-34,38,47,54,60,96] the nephrotoxicity for a series of aminoglycoside (most to least) is as follows: neomycin > gentamicin > sisomicin = amikacin = kanamycin > tobramycin > netilmicin > streptomycin[2]. Of the 23 comparative clinical toxicity studies which have been reported[47], the majority have failed to show a significant difference between two or more aminoglycoside in man. However, there are six studies in which significance was achieved, and five of these studies gave consistent clinical toxicity data with the predictions from animal experiments.

IV. LITHIUM-ASSOCIATED NEPHROPATHY

A. Animal data

Over the last ten years, lithium has gained widespread acceptance in the long-term treatment of recurrent mania and manic-depressive illness. However, the use of lithium to treat gout and rheumatism was already established and the toxic effects were well recognized[97]. In addition to nausea, vomiting, diarrhoea, anorexia, muscle weakness and tremors, polyuria and occasional oliguria were part of the presentation of acute lithium intoxication.

From a wide variety of animal experiments it is known that the absorption, distribution and elimination of lithium resembled that of sodium[98]. Absorption of lithium from the intestinal tract probably shares the same pathways as sodium. Analysis of the plasma disappearance of lithium is compatible with a two-compartment model, with a rapid distribution and equilibration within the extra-cellular space, followed by a slower elimination phase which parallels GFR. Once filtered, lithium undergoes reabsorption in both the proximal tubule[99] and thin limb of Henle[100]. A 3-fold cortico-medullary lithium gradient has been reported in rats that developed vasopressin-resistant polyuria after seven days of dietary loading[101]. The absence of distal tubular reabsorption of lithium is the basis for the experimental use of fractional lithium clearance as a measurement of proximal tubular rejection fraction[102]. By sharing at least a part of the renal sodium regulation pathway, it is not surprising that the most critical determinates of renal lithium excretion are those factors which alter proximal tubular salt and water reabsorption[103]. Excess lithium reabsorption occurs in association with low salt diet, cardiac failure, diuretic use and volume depletion[104]. Conversely, volume expansion coupled with loop-diuretic administration will enhance renal elimination of lithium[105]. Lithium has been reported to induce major changes in both electrolyte and acid-base composition of the urine. While the latter effect on acid secretion is probably lithium specific[106], Myers et al.[98] showed virtually identical changes in urinary electrolyte composition in response to acute sodium loads compared to acute lithium loads. These authors concluded that the similarity was most likely explained by "volume-induced" depression of proximal tubular function. The contention that lithium-induced kaliuresis is linked to the vasopressin-resistant concentrating defect[107] is not supported by experimental observations using micropuncture techniques[108]. The mechanism responsible for the distal renal tubular acidosis reported in man[109] has been evaluated by Arruda et al.[110] using the isolated turtle bladder. From these studies the authors were able to exclude a direct lithium inhibition of the H^+ pump. Based on these findings, they concluded that the reduction in H^+ secretion induced by lithium was an indirect effect which involved inhibiting active Na^+ transport, which resulted in a less favourable electrical diffusion gradient for intracellular H^+ secretion.

The most intense experimental effort regarding lithium's renal effect has centred on defining the mechanism of the concentrating defect. While the rat has been the laboratory animal most

frequently investigated, the dog and the pig, and also the isolated amphibian urinary bladders have all contributed to our understanding. Lithium, given either in the diet[101,111] or intraperitoneally[112] result in a polyuria that is resistant to exogenous vasopressin after only seven days. While many authors have classified the lithium-induced concentrating defect as mimicking nephrogenic diabetes insipidus, experimental confirmation is confusing. For example, using the amphibian urinary bladder Harris and Jenner[111] found serosally applied lithium inhibited anti-diuretic hormone (ADH)-mediated water flow, Singer et al.[113] reported that mucosally applied lithium was effective in inhibiting ADH-mediated hypoosmotic water flow and Bentley and Wasserman[114] were unable to demonstrate any lithium inhibition. A consistent finding was an interference with active Na^+ transport. Similar confusion results from studies on the effect of lithium on the adenyl cyclase-c-AMP system, the second messenger for ADH intracellular action. In some experiments lithium depressed ADH-stimulated adenyl cyclase[115] while in others lithium was without effect[112,117].

Micropuncture studies by Hecht et al.[108] and Carney et al.[118] have provided substantial insight regarding the mechanism of lithium-induced urinary concentrating defect in the rat. Based upon tubular fluid/plasma (TF/P) inulin values from proximal and distal tubular puncture sites Hecht et al.[108] concluded that lithium induced polyuria in the rat was associated with depressed fluid reabsorption from proximal convoluted tubule, but not the pars recta or the loop of Henle. These observations were refined and extended by Carney et al.[118] who concluded, based upon the increased percentage of water reabsorbed by the distal tubule and collecting duct in rats with lithium-induced polyuria, that the action of endogenous ADH on distal and collecting duct cells was not impaired. They explained the absence of exogenous ADH's effect on a new state of equilibrium induced by lithium. In addition to depressing proximal tubular fluid reabsorption, they also found impaired reabsorption from the loop of Henle. The latter observation is consistent with reports that lithium interfered with thick ascending limb chloride transport[119] and with a reduced medullary solute gradient[120], although no change in medullary solute composition could be measured[101]. Thus, once lithium-induced polyuria stabilizes the increased delivery of proximal tubular fluid into the thick ascending limb of Henle combined with the restricted Cl^- transport and reduced medullary solute gradient act in concert to prevent maximum endogenous ADH action on urine concentrating capacity. Since the collecting duct is under maximum ADH stimulation, no additional effect is registered with exogenous ADH and thus it is concluded that end organ unresponsiveness exists and the condition is labelled nephrogenic diabetes insipidus.

Good[121] and Radomski et al.[122] provided early descriptions of histolopathological changes in kidneys from rats with lithium toxicity. The main pathologic features were localized to the distal tubule and collecting duct and consist of nuclear and cellular polymorphisms, nuclear hyperchromasia, focal tubular luminal dilatation, with occasional tubular cell atrophy[123-125]. Despite relatively long-term treatment in rats, no evidence of pure interstitial fibrosis has been verified. Furthermore, attempts to correlate

structure changes with the functional defect and attempts to potentiate the lithium-induced structure changes by combining them with other neuroleptic agents have failed[126]. Monitoring histochemical changes, Jacobsen et al.[127] detected early changes in structural proteins of renal tubular cells which preceded microscopic evidence of pathology. In addition, ^{3}H-thymidine autoradiographic studies confirmed increased DNA synthesis in collecting ducts of lithium-treated rats, and subsequent cellular proliferation being detected by conventional light microscopy. These changes were maximal at the junction of the outer and inner medullary junctions. The extent of lithium-induced structural damage is dose dependent[128], but is modified in residual renal tissue following partial surgical ablation[129]. More recently, Kling et al.[130] have provided a detailed histological analysis of sequential changes in distal tubule and collecting duct of rats given lithium for up to 18 weeks. Proliferation of collecting duct cells dominates the late pathologic findings with no interstitial inflammation or fibrosis being identified. Similar changes were not evident in either pair fed Brattleboro rats, nor glucose-treated controls despite similar degree and duration of polyuria. These authors suggest that the proliferating cells of the collecting duct may be more susceptible to otherwise trivial insults which lead to the occasional chronic tubulo interstitial nephropathy reported in man. Finally, the histological changes seem to be reversible once lithium is withdrawn; although more severe lesions required longer[125].

Thus a consistent, reversible polyuria can be induced in rats at serum concentration well within the human therapeutic range. While this lesion was originally classified as a functional nephrogenic diabetes insipidus, recent micropuncture studies question lithium inhibition of end organ responsiveness to ADH as the sole mechanism. Dose dependent structural changes are limited to the distal tubule/collecting duct region and prolonged treatment causes significant proliferation of collecting duct cells.

B. Human data

Cade[131] first identified the therapeutic benefits of lithium for patients with affective disorders. However, the potential nephrotoxicity of lithium was appreciated since the turn of the century[97,121]. Through the mid-1970's such toxicity was not considered a significant risk based on the paucity of adverse reactions reported[132]. Where nephropathy was documented, it was limited to patients who had experienced repeated episodes of acute lithium intoxication[133-135]. However, the safety of chronic lithium treatment has been questioned[123].

Polyuria and secondary polydipsia regularly follow the prescription of lithium salts. The frequency of polyuria varies from 12 to 50%[101,136] and is most prominent during initiation of treatment. This functional lesion is ADH-resistant and has the characteristics of nephrogenic diabetes insipidus. Its occurrence does not correlate precisely with lithium plasma levels, can be reproduced in animals, and is usually reversible once the lithium is discontinued. In addition to a proposed effect of lithium on ADH-activated adenyl

cyclase in the renal collecting duct cells[115,116], Martinez-Maldonado[119] has reported evidence which suggests a central pituitary effect which modifies ADH release to appropriate stimuli.

The report of chronic interstitial nephritis in 14 patients without prior acute lithium toxicity or prominent symptoms of diabetes insipidus was the first clear implication of irreversible renal damage[123]. Conformations of chronic interstitial fibrosis occurring in select patients given lithium followed[137,138]. However, questions which remained included:

(1) is acute intoxication a mandatory pre-condition?

(2) do other neuroleptics contribute to this renal lesion?

(3) is the lesion correlated with dose, total amount or blood level[136]?

(4) does age, concomitant drugs, and/or co-existing diseases explain these findings?

Clearly, the concern raised by Hestbeck et al.[123] stimulated a variety of clinical and experimental studies. Clinical investigations included longitudinal studies of both glomerular and tubular functions in patients given long-term lithium. Some studies utilized serial renal biopsies while others combined both structure and functional observations. Hullin and associates[139] using non-lithium treated patients with affective disorders as controls were unable to identify any significantly increased incidence of renal dysfunctions in lithium treated patients. Two things stand out in their results. The first was the lack of excess blood lithium levels; many were below the accepted minimum, i.e. <0.5 mEq/L. The second was the unexpected reduction in renal functions in the non-lithium treated patients as compared to published norms. This observation led to the speculation that there was a concordance between affective disease (and/or other neuroleptic drugs) and mild renal dysfunctions. Relative polyuria had been reported in affective patients receiving neither lithium nor neuroleptic agents[140]. Building on the question, does the renal impairment of lithium treatment include other aspects of nephron function, a series of studies have examined GFR and proximal tubular injury, using quantitative enzymuria, conducted both as cross-sectional and longitudinal studies. Grof et al.[141] failed to detect changes in creatinine clearance associated with lithium treatment, but did note that so-called "non-responders" to lithium therapy had larger 24-hour urine volumes and more impressive impairment of maximum concentrating capacity. Wallin et al.[142] evaluated 278 patients on long-term lithium. They noted that one out of every two patients could not concentrate their urine above 600 mOsm/kg, and that this defect was positively correlated with both the durations of lithium treatment and character of tablet administered, but was independent of the other neuroleptic drugs that were being taken. Urinary β_2-microglobulin was not increased, but 17% of the group had a significant reduction in GFR when compared to age-corrected controls. The reduction in GFR paralleled the fall in concentrating

capacity. A similar association between long-term lithium treatment, i.e. 10 years, and a downward trend in GFR was reported by Tyrer et al.[143], although Bendz and associates[144] failed to detect a significant reduction in GFR despite over 50% of patients having an impaired concentrating capacity. Waller et al.[145] also failed to substantiate a direct correlation between accumulative lithium dosage and decreased GFR. Nor were they able to show consistent urinary NAG or β_2-microglobulin changes. Once again, maximum urinary concentrating capacity was inversely correlated with the durations of lithium treatment but, in addition, they reported microalbuminuria in 40% of patients that was independent of duration of treatment. Jensen and Richer[146] conducted a prospective study of change in GFR in 13 consecutive patients who had never received lithium. Over 16.5 months, a small but significant fall in mean GFR was reported. However, when correlated to body surface, this significance was traced to the increase in weight associated with lithium treatment.

In parallel with studies of renal functions in patients receiving long-term lithium, investigators[123] sought to confirm the renal pathology. Burrows and associates[137] reported chronic tubulointerstitial nephritis in the biopsies of three patients receiving long-term lithium treatment. Kincaid-Smith and co-workers[147] used three patient groups, those with affective disorders receiving lithium, similar patients not treated with lithium and healthy kidney donors. Pathological changes were confined to the patients with affective disorders, but lithium treatment versus none could not be separated by blind assessment. These results questioned the renal histopathologic changes as being lithium induced. An expanded study of renal structure-function relationships in patients receiving long-term lithium therapy confirmed and expanded the conclusion of the Australian group[148] that no evidence of lithium specific tubulointerstitial disease or reductions in GFR could be substantiated.

To explore the mechanism of renal function abnormality, Hansen et al.[149] evaluated enzymuria as a marker of tubular injury. In 15 patients with significant lithium-induced concentrating defect, 13 of whom had chronic tubulo interstitial disease confirmed by biopsy, only one patient showed significant increase in β_2-microglobulin. This led Hansen to conclude that damage was restricted to the distal nephron. Batlle et al.[106] using measurements of urine to blood PCO_2 in addition to concentrating capacity, reported a mild distal tubular acidification defect in patients with short-term lithium treatment, a defect that can preceed the loss of maximum concentrating capacity. More recently, this group[84] has reported that amiloride could reverse lithium-induced concentrating defect by counteracting the ions inhibitor effect at the collecting duct. Penney and co-workers[151] reported increased amounts of arguinine vasopressin in the morning urine of lithium treated patients as compared to controls and were able to correlate these increases with plasma lithium values.

While the plasma lithium level has been shown to correlate with the concentrating defect, the dosage frequency have also been shown to modify expected renal dysfunctions. Plenge et al.[152] compared the effect on renal function of a single total daily dose of lithium versus the same total dose divided 2 or 3 times per day.

Functional and structural changes were more evident in patients receiving divided dosage. A similar finding was reported by Schou et al.[153]. Recently it has been reported that substantially less interstitial fibrosis occurs in biopsies taken from patients given single daily doses of lithium[154,155].

Thus, it is recognized that lithium induces a polyuria in both man and animals which has the functional characteristics of a nephrogenic diabetes insipidus. This functional concentrating defect is reversible in most instances although in man it has been reported to remain for 6 to 12 months after ceasing lithium therapy. Polyuria is neither life threatening nor does it predict a future decline in GFR. The aetiology of the chronic interstitial nephritis reported in patients with affective disorders remains unresolved. There is compelling evidence that this lesion is not directly lithium related although the question is unresolved as to whether other psychotropic agents may be involved. It is the consensus that the lithium-induced polyuria is related to the frequency of dosage rather than the total daily dose and recommend once a day dosing for patients who can be maintained at the lower blood levels of lithium, i.e. 0.4-0.7 mEq/L. The role of the lithium salt prescribed as a feature of nephrotoxic potential remains a question. Lithium therapy is a very effective treatment for patients with affective disturbances, especially if the dosage can be adjusted to minimize the inconvenience of polyuria. The serial monitoring of lithium levels to assure they fall within accepted therapeutic ranges is extremely important. It is critical to avoid salt depletion or other conditions which induce extracellular contractions since these will enhance both sodium and lithium reabsorption and therefore disturb the steady state. In elderly patients where a normal serum creatinine does not reflect a normal GFR, adjustments of lithium intake by monitoring serum levels is an effective means of preventing chronic toxicity.

A disturbing aspect of lithium's effect on the kidney involves the controversial "chronic" lithium nephropathy. The pathologic lesion is one of chronic interstitial nephritis that is restricted to patients on long-term lithium treatment. The frequent episodes of acute lithium intoxication reported in such patient studies have led to a definite question as to whether or not such histological changes are the result of acute overdose rather than chronic therapeutic administration of lithium. Furthermore, the contention that long-term lithium administration was causally related to chronic interstitial nephritis has been questioned because of similar renal pathology in biopsies from non-lithium treated patients with affective disorders. The renal histological appearance of chronic interstitial nephritis was distinct from a group of kidney donor controls. This unique renal pathology in patients with affective disorders was subsequently substantiated in another group of non-lithium treated patients. However, the possibility of other neuroleptic agents being involved has not been excluded. The chronic interstitial lesion described is distinct from the nuclear filamentous changes of distal tubular cell described by Kincaid-Smith[147].

In summary, animal models of drug-induced nephrotoxicity can be helpful in both explaining mechanism(s) of cellular injury

and evaluating how to modify renal insult. However, in order to be interpreted, the model must faithfully reproduce the nephropathy which occurs in humans. An ongoing dilemma surrounds the excessive dosages which many animal experiments require to insure a distinct endpoint.

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21

ANTIBIOTICS: THE EXPERIMENTAL AND CLINICAL SITUATION

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I. INTRODUCTION

This chapter will review information gained from animal experiments to help prevent and manage antibiotic nephrotoxicity in humans. The comparison is based on the effects of aminoglycosides on animals, normal human volunteers and patients being treated for infection. These data have been used to highlight the problem of studying nephrotoxicity in patients with severe infections and highlight the need for research into more realistic animal models.

II. THE MANIFESTIONS OF AMINOGLYCOSIDE NEPHROTOXICITY IN ANIMALS AND HUMANS

The functional consequences of aminoglycoside nephrotoxicity in animals have been reviewed[1-4] and can be summarized as follows:

(a) manifestations of tubular injury:
    enzymuria;
    impairment of proximal tubular reabsorption;
    impairment of acid-base homeostasis;
(b) impairment of glomerular filtration.

Enzymuria and failure to reabsorb low molecular weight proteins have both been demonstrated in normal human volunteers[5,6] and in patients[7-10], after a single dose of gentamicin. This had led to the hypothesis that gentamicin is competing for proximal tubular reabsorption of low molecular weight proteins[5]. Both phenomena have been used as markers of aminoglycoside nephrotoxicity in humans.

Although glucosuria is not a major manifestation of the renal dysfunction caused by aminoglycosides in humans[3], there have been occasional reports of a Fanconi type syndrome - which in-

cludes glucosuria - in humans treated with gentamicin[11,12]. There may be species differences in the occurrence of this symptom of proximal tubular dysfunction. It has occurred in rabbits[13] given gentamicin, 4-8 mg/kg twice daily, doses which are only slightly greater than those used clinically. On the other hand, a much higher dose of gentamicin failed to produce glucosuria in rats[14]. By contrast, in the rat[15] and the dog[16] glucosuria appeared as a late manifestation of gentamicin-induced tubular injury, occurring only when glomerular function had been impaired. Gentamicin appeared to have a greater effect on urinary glucose excretion in rats which were rendered acidotic[17]. Some workers have failed to find any impairment of the tubular capacity for reabsorption of glucose in the rat[18] or renal cortical slice accumulation of glucose analogues[19]. Thus it is clear that both in animals and humans alteration of renal handling of glucose is neither an early nor a prominent sign of aminoglycoside nephrotoxicity.

In humans receiving aminoglycosides, electrolyte disturbances, such as those occurring in the Fanconi type syndrome are rare, but recent reports suggest that the frequency, particularly of hypomagnesaemia[20,21], has been considerably underestimated. Earlier reports used hypokalaemia and hypocalcaemia to identify patients with aminoglycoside-induced tubular malfunction[22], but Zaloga[20] and Davey[21] demonstrated that these abnormalities occurred only in patients with severe hypomagnesaemia. This implies that hypomagnesaemia is the primary event, and that failure to measure serum magnesium resulted in the underestimation of the incidence of electrolyte disturbances.

Experimental studies in animals have demonstrated effects of aminoglycosides on electrolyte homeostasis, but the results are variable and seem to be species dependent. The most consistent finding in animal studies is an increased fractional excretion of potassium, after administration of high doses of aminoglycosides to the rat[15] and the dog[16] and in acute single-dose studies in the rat[23,24] and the sheep[25]. In contrast, fractional excretion of sodium (FENa) has been shown to be increased[24] or unchanged[23] in acute studies in the anaesthetized rat but decreased in the conscious sheep[25]. Appel[3] found that in rats treated chronically with gentamicin, FENa increased in parallel with an increase in urine flow and a decline in glomerular filtration rate (GFR).

Recently it has been shown that renal handling of other divalent cations is also altered by aminoglycoside administration. Hypercalciuria was a prominent finding in one study when gentamicin, 40 mg/kg daily, was given to rats for 7 days[14]. Changes in magnesium excretion were difficult to interpret because of fluctuations in control animals. Sodium and potassium excretion were unaffected. Further work[26,27] has shown that altered handling of calcium and magnesium is an immediate response of the rat kidney to gentamicin administration. In one of these studies[26] there was a dose-related increase in excretion of calcium which was significant after just one dose. Magnesium excretion was also increased, but without a clear relationship to dose, and it developed later than calcium changes. Gentamicin, at a dose of 10 mg/kg, increased the excretion of calcium and magnesium, but there were no changes in urinary enzymes, volume or osmolality. This sug-

gested that electrolyte homeostasis might be disrupted prior to the occurrence of significant cell injury. In this study the elevated excretion of calcium and magnesium returned to normal, at all doses, within a week of stopping the drug. However, if the drug was given at high doses or for prolonged periods the increased urinary excretion of calcium and magnesium persisted long after the drug administration was stopped[28].

Although altered renal handling of electrolytes by aminoglycoside administration to animals has been demonstrated frequently, aminoglycoside-induced alterations in plasma electrolytes have been observed only occasionally. This is presumably a consequence of the excess of electrolytes in the standard diets for laboratory animals and the absence of other factors predisposing to electrolyte imbalance. Cronin et al.[16] observed that hypokalaemia developed in dogs towards the end of a 10 day course of gentamicin, 30 mg/kg daily. Hypocalcaemia occurred only 7 days after the drug administration was stopped and at a time when GFR was reduced. Plasma magnesium levels did not change at any time. The authors speculated that the hypocalcaemia might have developed as a consequence of resistance to the action of parathyroid hormone, known to develop in acute renal failure. Finton et al.[29] induced hypomagnesaemia in baboons by 10 day administration of gentamicin, 5 mg/kg daily. Dosage was adjusted to keep the gentamicin levels in the therapeutic range. Serum magnesium levels fell (in four of six animals to the hypomagnesaemic range) in the absence of any change in serum creatinine, calcium or phosphate. Urinary concentrations of magnesium were judged to be inappropriately high for the level of serum magnesium.

Thus, these animal studies suggest that there are two facets to aminoglycoside-induced changes in renal handling of electrolytes:

1. Effects which occur as an immediate response to the drug administration. These include an increased urinary excretion of K^+, Ca^{2+} and Mg^{2+}, and a decreased renal content[30,31] of K^+ and Mg^{2+}. These changes appear to precede all others and may be a cause of renal cellular injury as well as representing a consequence of disordered whole-body electrolyte homeostasis. This suggestion is supported by the observations that both potassium deficiency[32] and magnesium deficiency[33] (caused by a potassium- or magnesium-deficient diet) have been associated with augmented nephrotoxicity caused by gentamicin in dogs and rats. Conversely, a calcium supplemented diet delayed the development of nephrotoxicity and reduced its severity[34].

2. Long-term effects which occur at times when there are other indicators of renal damage and which may persist after recovery of conventional indices of nephrotoxic damage. This renal wasting of electrolytes may result from proximal cell injury or be an independent event but, in either case, it may proceed insidiously when renal function seems normal.

Table 1 Cases of hypomagnesaemia associated with aminoglycoside use

(a) Cases with no cytotoxic therapy

Reference	Number of cases	Drug	Duration of treatment	Other predisposing factors
Holmes et al.[40]	4	gentamicin	5-11 months	
Bar et al.[37]	1	gentamicin	8 days	
Bamford and Jones[38]	3	topical neomycin	16-30 days	colistin, polymyxin B
Kelnar et al.[41]	1	gentamicin	4 months	
Patel and Savage[35]	1	gentamicin	3 courses of 10, 13 and 16 days	
Davey et al.7	1	gentamicin	8 days	alcoholism
Zaloga et al.[20]	21	gentamicin, tobramycin or amikacin	9.9 ± 1.2 days	dietary magnesium deficiency
Goodhart and Handelsman[42]	1	gentamicin	28 days	
Davies and Murray[43]	1	gentamicin	9 days	amphotericin B
Wilkinson et al.[44]	1	gentamicin	50 days	

(b) Cases with cytotoxic therapy

Reference	Number of cases	Drug	Duration of treatment	Malignancy	Other potentially nephrotoxic drugs
Bar et al.[37]	1	gentamicin	3 courses of 7-14 days	ANLL[a]	doxorubicin
Keating et al.[22]	17	gentamicin, tobramycin or amikacin	5-58 days	14 ANLL 3 "solid tumour"	14 doxorubicin, full list not given
Freedman et al.[45]	9	gentamicin	not stated	6 ANLL 3 ALL[b]	doxorubicin or daunorubicin
Davey et al.[21]	6	gentamicin	2-4 courses of 7-14 days	ANLL	doxorubicin

[a] Acute non-lymphoblastic leukaemia

[b] Acute lymphoblastic leukaemia

Despite a number of animal studies the precise aetiology of hypomagnesaemia in humans remains unclear. Studies in patients have given rise to several possible explanations. Early reports focused on coincident hypoparathyroidism[22,35] but this can occur as a secondary manifestation of hypomagnesaemia even when the latter is induced by pure dietary deficiency[36]. Patients who have hypomagnesaemia during aminoglycoside therapy excrete inappropriate amounts of magnesium in the urine[21,37,38] but it is important to emphasize that these amounts would be considered normal but for the presence of hypomagnesaemia. Excessive urinary magnesium excretion has not been shown to initiate the problem, and it may be that aminoglycoside therapy superimposes impaired magnesium reabsorption upon a pre-existing state of magnesium depletion[20]. A significant decrease in serum magnesium levels was noted after 4 days of gentamicin therapy in nine patients who had no other cause for a decline in serum magnesium[39]. However, the majority of symptomatic cases of hypomagnesaemia have received prolonged courses of aminoglycoside therapy or have other predisposing causes for hypomagnesaemia (Table 1a). Patients with leukaemia seem to be particularly at risk[21,22,45], possibly because of underlying renal injury from the disease process[46] or from interaction with cytotoxic drugs[21]. These patients also tend to receive prolonged or repeated courses of aminoglycosides (Table 1b). In addition they are likely to have dietary deficiency of magnesium[45]. The influences of all of these other factors would in part explain the difficulty in demonstrating similar problems in animals. However hypomagnesaemia is potentially lethal[47], and its aetiology in the context of nephrotoxicity requires further study, both in relation to the aminoglycosides and other drugs such as amphotericin B [43] and cyclosporin[48].

Non-oliguric renal failure is characteristic of patients in whom aminoglycosides are strongly implicated as an aetiological factor[49], and is an almost universal feature of aminoglycoside nephrotoxicity in animals[2]. Alkalosis has been demonstrated in humans receiving topical neomycin and long-term gentamicin, but in both cases it was associated with abnormalities such as hypokalaemia and hypochloraemia, which may also have contributed[38,40].

Impaired glomerular filtration, as manifested by an increase in serum creatinine, is the standard marker of nephrotoxicity in clinical trials[50]. It is generally assumed to follow tubular injury but in animals gentamicin has direct functional and histological effects on the glomerulus[3]. Studies with chromium labelled EDTA, a much more sensitive measure of glomerular filtration than serum creatinine, have demonstrated that impaired glomerular function occurs early in aminoglycoside therapy of patients and this may also reflect direct action on the glomerulus[51].

In summary the manifestations of aminoglycoside toxicity in animals and humans are broadly similar. The most obvious difference is the existence of hypomagnesaemia in patients and this is an important area for future study. A more realistic animal model might include animals with pre-existing magnesium depletion and animals that have been treated with cytotoxic agents, notably the anthracyclines[21].

III. THE INFLUENCE OF SEPSIS UPON RENAL FUNCTION, AND ITS POTENTIAL INTERACTION WITH ANTIBIOTIC INDUCED NEPHROTOXICITY

Septic shock is associated with multiple organ failure including acute renal failure (ARF). This syndrome can follow infection with Gram-negative or Gram-positive organisms. In both cases it is due principally to the effect of bacterial cell wall components, probably peptidoglycan in Gram-positive organisms and lipopolysaccharide in Gram-negative organisms[52,53]. The Gram-negative endotoxin is by far the most potent of these agents[52] and, as the aminoglycosides and cephalosporins are primarily used to treat Gram-negative infection, we will focus on the role of Gram-negative endotoxin in the aetiology of ARF.

A discussion of the pathophysiology of Gram-negative septic shock has been reviewed[52-56]. In addition Wardle[57] has reviewed the experimental evidence concerning the mechanisms by which endotoxin may cause renal failure. The actual role of sepsis in the aetiology of renal failure remains controversial. For example Galpin et al.[58] described 43 consecutive cases of renal failure of which they stated that aminoglycosides alone were responsible for 10 cases, sepsis plus aminoglycosides for four cases and sepsis plus cephalothin for one case. In contrast Wardle[59] found measurable blood levels of circulating endotoxin and associated abnormalities of platelet function in 12 of 16 consecutive cases of renal failure and concluded that endotoxin mediated damage "appears to account for most cases of ARF in man".

In fact most cases of ARF are probably multifactoral[60]. Rasmussen and Ibels[61] conducted a multivariate analysis of the causes of, and risk factors for, ARF and concluded that "excessive aminoglycoside exposure" was a significant risk factor and that "sepsis" was not. However this study does not resolve the issue because they used hypotension as the dependent variable and examined the additional role of each of the other potential risk factors. As hypotension is integral to the pathophysiology of endotoxic shock[55] it is perhaps not surprising that sepsis did not emerge as an additional independent variable. Moreover sepsis was diagnosed on clinical grounds which may have resulted in the inclusion of patients who did not have circulating endotoxin and the exclusion of patients who did. From the clinical description of Wardle's cases[59] it seems unlikely that all of the patients with circulating endotoxin would have been classified as having sepsis by the criteria of Rasmussen and Ibels[61].

It is theoretically possible that therapy of Gram-negative infection with any antibiotic may result in the liberation of large amounts of endotoxin following the death of the bacteria and the disintegration of their cell walls. The origin of this idea is generally attributed to Galpin[62] who commented on the sudden deaths which followed the initiation of chloramphenicol therapy in some patients with typhoid fever. The idea has received support from recent studies in an experimental rabbit model of Escherichia coli septicaemia[63,64]. Chloramphenicol inhibited the growth of bacteria but had no effect on endotoxin levels, whereas gentamicin and moxalactam killed bacteria and resulted in substantial increases

in the ratio of endotoxin to viable bacteria. Moreover this effect was significantly greater with moxalactam, a drug which kills bacteria by disrupting the cell wall, than with gentamicin, an inhibitor of protein synthesis.

It is important to put this information into perspective. In a study of 612 patients with Gram-negative septicaemia the mortality in patients with organisms sensitive to the initial antibiotic therapy was one half that of patients in whom appropriate antibiotic therapy was delayed; other therapeutic measures were similar in the two groups[65]. Moreover two randomized trials reported that tobramycin therapy was associated with a greater incidence of renal malfunction than moxalactam or cefotaxime[66,67]. Moxalactam and cefotaxime kill bacteria by disrupting the cell wall and would be expected, therefore, to cause greater release of endotoxin than tobramycin. This clinical evidence suggests that the beneficial effects of prompt, appropriate antibiotic therapy outweigh harmful effects of increased endotoxin release and that the increased release of endotoxin by antibiotics acting on the bacterial cell wall does not offset the inherent nephrotoxicity of tobramycin.

Evidence has emerged from a recent study that the nephrotoxicity of gentamicin in rats is increased by the prior induction of an experimental pyelonephritis[68]. The explanation for this increase in the toxicity of gentamicin may have been increased intrarenal accumulation of the drug in infected kidneys, which had been demonstrated in an earlier study[69]. Increased accumulation of antibiotic in pyelonephritic kidneys is not an invariable finding as the concentration of ampicillin was found to be lower in pyelonephritic kidneys than normal kidneys[70].

The possibility that both the increased accumulation of drug in the renal cortex and the increased nephrotoxicity in pyelonephritis might be attributable to the presence of bacterial endotoxin during antibiotic therapy has been tested in several recent studies. Tune and Hsu[71] found that the acute nephrotoxicity in rabbits of neomycin - and of the cephalosporins, cephaloglycin and cephaloridine - was augmented by prior administration of endotoxin from Escherichia coli. It was thought that the effect of endotoxin might be to increase the concentration of the antibiotics in the renal cortex but a subcellular interaction could not be discounted; at least for the cephalosporin-endotoxin synergy. Other studies[72,73] suggest that increased renal uptake of aminoglycosides in the presence of endotoxin may be the mechanism by which their toxicity could be increased. Bergeron and Bergeron[72] found that endotoxin from Escherichia coli increased the accumulation of gentamicin, netilmicin, tobramycin and amikacin, but not cephalothin, in the rat kidney. The endotoxin-induced changes in the renal accumulation of aminoglycosides appeared to occur in the absence of significant changes in cardiovascular function.

Halkin et al.[74] found altered pharmacokinetics of gentamicin in rabbits in which pyrexia was induced by administration of Escherichia coli endotoxin and they attributed the changes in gentamicin pharmacokinetics to the raised temperature. However, it has been claimed that fever did not contribute to the increased renal accumulation of aminoglycosides caused by endotoxin in the rat[72]; these animals were anaesthetized, which may have modu-

lated any disruption of temperature regulation caused by the endotoxin.

Table 2 Risk factors for aminoglycoside nephrotoxicity in patients

Greater risk associated with	
	Increasing age
	Female sex
	Higher initial peak level
	Higher initial creatinine clearance
	Liver disease
	Shock
Reduced risk associated with	
	Pre-existing abnormal renal function
Factors without influence on risk	
	Initial trough level
	Total dose or duration of treatment
	Serum bicarbonate
	Bacteraemia
	Diabetes
	Concomitant frusemide or cephalothin

Source: Moore et al.[79]

It is important to note that these studies suggest that bacterial endotoxin can affect the renal distribution of aminoglycosides and possibly increase the renal injury they cause without precipitating shock. Ambinder et al.[75] found no evidence for any interaction between tobramycin and shock in their effect on renal function in patients. Data were analyzed from a trial of cefotaxime v. nafcillin plus tobramycin for treatment of suspected Gram-negative infection. Both therapy with tobramycin and the occurrence of shock were independently associated with a decline in renal function but there was no evidence of any interaction between these two risk factors.

Clearly the interaction of antibiotic therapy and endotoxin is an important area for future research both in animals and in patients. The discovery of the J5 antiserum against endotoxin and the demonstration that it works both prophylactically and therapeutically was a major advance in the clinical management of Gram-negative septicaemia[76,77]. This antiserum may also prove to be a powerful tool for unravelling the interactions between antibiotic therapy, endotoxin release and renal failure in animal models.

IV. THE INFLUENCE OF UNDERLYING DISEASE AND OTHER DRUGS UPON AMINOGLYCOSIDE NEPHROTOXICITY

The majority of patients who receive aminoglycosides have underlying diseases such as hypertension and diabetes mellitus or undergo major surgical procedures or receive other drugs such as diuretics, anaesthetic and cytotoxic agents all of which may independently affect renal function or influence the effects of aminoglycosides. There are reports in which unexpectedly high incidences of renal failure have been attributed to the interaction of one or more of these with aminoglycoside therapy, for example the use of frozen blood and neomycin wound irrigation[78]. However, there is only one large body of information which has been subjected to multivariate analysis[79]. Several of the risk factors identified in this study (Table 2) have also been documented in animal studies. However there has been no animal study of the influence of liver disease on nephrotoxicity, nor of the possible protective effect of pre-existing renal impairment provided that drug levels are carefully controlled. Some risk factors in animal studies, such as frusemide therapy and acidosis, were not risk factors in the patient population. The authors are careful to point out the limitations of their study. In a clinical study such as this every patient has multiple potential synergistic and antagonistic factors and only the most potent will emerge as independently significant. The problems inherent in such clinical trials are discussed in detail below.

The greatest contrast between the results of human and animal studies in this area concerns the interaction of cephalosporins with aminoglycosides. In animals cephalothin reduces renal uptake of aminoglycosides and protects against nephrotoxicity, whereas in humans there is either no effect or increased nephrotoxicity. Luft[80] concluded that "a reconciliation of all of the reported findings cannot be made". However a recent study[81] has gone some way towards achieving a reconciliation by showing that cephalothin was protective against gentamicin toxicity in normal rats but in rats that were acidotic, dehydrated or had unilateral nephrectomy the combination was unpredictable, appearing to be less toxic than gentamicin in some animals but causing lethal renal failure in others. If this study is confirmed then it has important implications for human studies. It may explain why cephalothin and gentamicin have produced synergistic toxicity in some studies because patients with severe sepsis are frequently acidotic or dehydrated. This study also raises the possibility that examining multiple risk factors in animal models might resolve some of the other conflicts between findings in animals and in patients.

V. COMPARATIVE STUDIES OF ANTIBIOTIC NEPHROTOXICITY IN ANIMALS AND HUMANS

In rats the rank order of aminoglycoside nephrotoxicity is as follows[2]:

gentamicin > amikacin > tobramycin > netilmicin

In addition there is some evidence that the aminoglycosides have different sites of toxicity in rats: gentamicin affects both glomerular and tubular function but netilmicin and tobramycin impair only glomerular or tubular function respectively.

There have been three recent reviews of all of the published comparative studies in patients[50,82,83]. These reviews adopt two very different approaches: one is to amalgamate all of the information, in which case there is no difference between the drugs[82,83]; the other is to review the methodology employed in the trials and consider only those that had a reasonable chance of detecting a real difference, in which case tobramycin may be less toxic than gentamicin[50]. Subsequently two additional studies have been published in which there was no significant difference in the nephrotoxicity of netilmicin and tobramycin[84] or of gentamicin, netilmicin and tobramycin[85].

Only one large comparative study in patients has used indices of proximal tubular function and glomerular function: tobramycin caused less nephrotoxicity than gentamicin by both criteria[86].

Consideration of the above might lead to the conclusion that there is very little relation between the relative toxicities of these drugs in rats and in patients. A middle ground is provided by the studies of AAP excretion by Mondorf's group[87], in which human volunteers were treated with aminoglycosides for 3 days. In this model both netilmicin and tobramycin were less toxic than amikacin or gentamicin, results which are similar to those obtained in the rat. These results were given some clinical credibility by the finding of Davey et al.[7] that AAP excretion in the first 3 days of gentamicin therapy did identify patients who later developed clinically detectable abnormalities in renal function.

The reason for the conflict between the results of animal or human volunteer studies and the results of clinical trials almost certainly rests in the multiple other factors alluded to in section IV. The most convincing evidence for this hypothesis comes from the work of the Johns Hopkins group. The study of risk factors[79] used data collected in two earlier studies[88,89]. In the original studies patients were excluded if they had identifiable causes of renal malfunction prior to the start of aminoglycoside therapy, whereas all of the patients were included in the analysis of risk factors. The conclusions of the individual studies were that the combination of cephalothin with gentamicin is more toxic than the combination of methicillin with gentamicin[88], and that gentamicin is more toxic than tobramycin[89]. When all the information was pooled neither of these findings proved to be significant[79].

These two approaches typify what Feinstein[90] has called the "fastidious" and the "pragmatic" approach to clinical trials. Both are perfectly valid but they address totally different questions. Thus the first two studies[88,89] addressed a question of the form "Is drug A inherently less toxic than drug B?" and it was therefore reasonable to exclude all other potential causes of renal failure or factors which might affect the toxicity of either drug. The third study[79] addressed the pragmatic question "If drug B is sub-

stituted for drug A in all patients will the incidence of renal malfunction be reduced?" The answer in this case was no, because the inherently lower toxicity of tobramycin was obscured either by other causes of renal failure or by uneven distribution of factors which increase aminoglycoside toxicity.

The same group has used yet a third approach, namely cost effectiveness analysis, on the data from the trial which demonstrated that tobramycin was less toxic than gentamicin[89]. In this case the question is "Can the benefits of reduced nephrotoxicity be quantified and do they offset the extra cost of tobramycin?" In this case the answer was that gentamicin appeared to be more cost effective than tobramycin, despite the statistically significant lower toxicity of tobramycin in these patients[91].

In summary tobramycin and netilmicin are less nephrotoxic than gentamicin in rats and in a normal human volunteer model. Clinical trials have demonstrated that the reduced toxicity of tobramycin can only be demonstrated in patients who have no other factors predisposing them to renal malfunction, and that in this group the benefits of tobramycin do not offset its extra cost.

CONCLUSIONS

Superficially, it may seem that there are major differences between the results of studies of antibiotic nephrotoxicity in animals and the results of clinical trials. However, we believe that the apparent conflict is largely attributable to the complex interactions of disease states with treatment. There is clearly a need for experimental studies to take this into consideration. We have also drawn attention to the need for further studies into the aetiology of aminoglycoside-induced hypomagnesaemia, which is potentially much more harmful to patients than transient reduction in glomerular filtration. Finally, it is essential to understand that clinical trials may not be designed to show a biological difference in the nephrotoxic potential of two drugs, but rather whether such a difference will be apparent in clinical practice amidst all the other causes of renal damage. Even when such a difference is demonstrated in clinical trials it will be necessary to demonstrate that reduction in nephrotoxicity results in tangible benefits, either to the patient or, by saving on additional treatment, to the health service. Experimental scientists must appreciate these considerations in order to resolve apparent conflict between the results of clinical trials and experimental studies.

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22
MECHANISMS OF METAL-INDUCED NEPHROTOXICITY

B.A. FOWLER, P. MISTRY AND P.L. GOERING

I. INTRODUCTION

The kidney is frequently a target organ for metal toxicity since it concentrates many of these elements during excretion and possesses a large number of metabolic processes which are highly sensitive to metal-induced perturbation. Metals such as lead (Pb), mercury (Hg), cadmium (Cd), chromate (Cr), uranium (U), and the metalloid arsenic (As) are all known to be concentrated by the kidney and to produce a spectrum of organelle/biochemical injuries to the nephron by a number of mechanisms. A number of factors such as dietary status, concomitant exposure to several trace elements, presence of high-affinity metal-binding proteins, or other intracellular depots for metal sequestration and cell type are all known to play major roles in determining both the nature and extent of metal- or metalloid-induced nephrotoxicity.

The mammalian kidney is composed of over 15 different cell types, but only a few are commonly affected by toxic processes. Cells of the renal vasculature and proximal tubule cells (PTC) are most frequently involved in metal-induced nephrotoxicity while other types are spared. The present overview of metal nephrotoxicity will not consider vascular lesions (i.e. interstitial fibrosis) since these cell types are not technically a component of the kidney, but will instead focus on mechanisms of metal toxicity to renal PTC.

In addition to cellular specificity, metal-induced nephrotoxicity to PTC also appears to frequently exhibit both organelle and biochemical selectivity since PTC are highly organized cells (Figure 1) which contain specialized metabolic systems that perform a large number of functions at a high metabolic rate. In part, as a result of this intracellular specialization, most metals/metalloids appear to produce rather selective subcellular responses. For example, some metals (depending upon dose and chemical form) appear to primarily exert toxicity to PTC via the cell membrane and

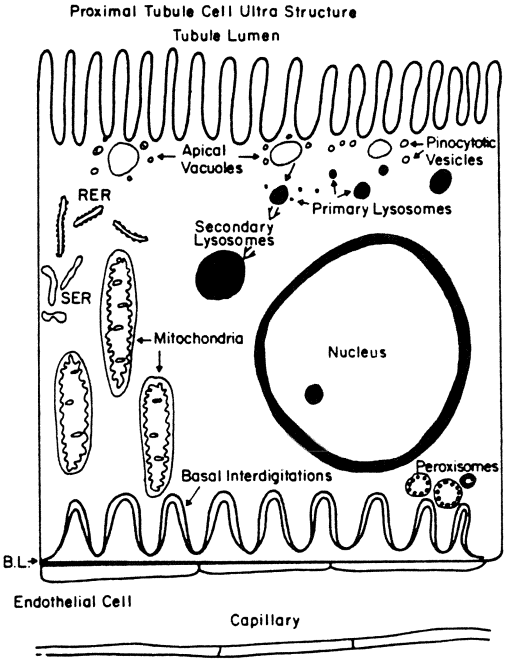

Figure 1 Diagram of kidney proximal tubule cell showing organelles and brush border.

associated transport processes, while others appear to produce deleterious effects by acting on the mitochondrial, lysosomal or nuclear endoplasmic reticulum systems or a combination of systems. In recent years it has also become appreciated that the intracellular distribution and activity/toxicity of a given metal is highly dependent upon high affinity metal-binding proteins or other "sinks" which appear to regulate metal toxicity within the PTC. The following discussion will focus on the relationships between intracellular binding and organelle system/biochemical effects, and will provide some insights into our current understanding of metal-induced nephrotoxicity.

II. TRANSPORT OF METALS INTO RENAL PROXIMAL TUBULE CELLS

Relatively little is known about the precise biochemical mechanisms of metal uptake by PTC, but metal-specific brush-border membrane transport systems have **not** been demonstrable for the metals[1-6]. Uptake of metals at the luminal side of PTC occurs via endocytosis following either binding of the metal to the anionic surface coat of the brush border membrane[5] or via normal endocytosis of metal-protein complexes such as Cd, Zn-metallothionein[7]. This endocytotic event is followed by intracellular release of the metal

from the membrane or metal-protein complex via lysosomal proteolysis at acid pH (Figure 2). In addition to this general

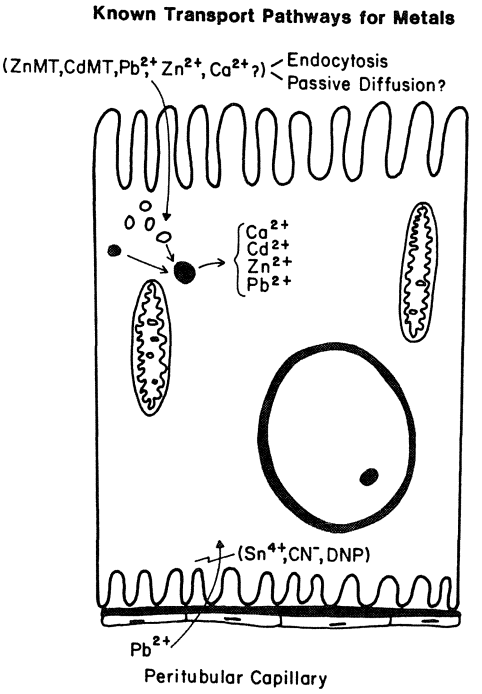

Figure 2 Diagram showing endocytosis of metal cations and subsequent intracellular release from lysosomes at low pH.

mechanism, passive diffusion and basolateral transport of metals such as lead has also been suggested[3-4]. A number of studies[3-5] have also demonstrated an interaction between renal uptake of a metal, metabolic inhibitors, other competing metal cations, and chelators. Following uptake into PTC, the intracellular distribution of a given metal is markedly influenced by its intracellular binding patterns and the presence of high-affinity sinks such as metallothionein or intranuclear inclusion bodies (see below).

III. SPECIFIC METALS/METALLOIDS

A. Arsenic

In discussing the nephrotoxicity of arsenic it is important to examine not only the chemical form/oxidation state of the chemical species involved in the exposure, but also the apparent metabolic pathway of arsenic in vivo.

1. In vivo metabolism

Early studies by Ginsburg and Lotspeich[8] and by Ginsburg[9] examined the renal uptake of arsenate (As^{5+}) and reported the apparent partial reduction of As^{5+} to As^{3+} in the kidney. Recent studies (see ref. 10 for review) have confirmed the reduction of As^{5+} to As^{3+} in vivo and further demonstrated the formation of methyl arsonic and dimethyl arsinic acid species in the liver. These species of arsenic are the major chemical forms excreted in the urine by man and other species[10-12] following exposure to inorganic arsenic. The importance of recognizing the in vivo metabolic conversions of inorganic arsenic rests with a better understanding of the relationship between intracellular speciation and mechanisms of toxicity.

Of the major toxic trace elements, arsenic and its related compounds are perhaps the least studied under both clinical and experimental conditions. The effects of this element on the kidney of humans or animals appear to primarily involve the proximal tubule portion of the nephron and appear to exert effects specifically on mitochondrial structure and function[21].

2. Clinical observations

There are relatively few clinical reports of inorganic arsenic-induced nephrotoxicity. Frejaville et al.[13] reported four cases of arsenical intoxication with extensive PTC damage primarily involving swollen mitochondria and cellular necrosis. Arsine gas, which is a potent haemolytic agent, also produces acute nephrotoxicity in exposed persons, apparently secondary to blockage of the tubule lumens[14-17]. PTC lesions were characterized by cloudy swelling and necrosis with concomitant albuminuria, azotaemia, altered serum electrolyte levels, and decreased organic ion transport. Recovery from acute arsine poisoning is slow, with renal functional impairment continuing for many months[14-18].

3. Mechanisms of arsenical nephrotoxicity

Ultrastructural and biochemical studies by Brown et al.[19] demonstrated mitochondrial swelling and reductions in mitochondrial respiratory function for NAD-linked substrates (e.g. pyruvate/malate) in rats chronically exposed to arsenate (As^{5+}) in drinking water. Similar results with respect to selective inhibition of NAD-linked substrate respiration have also been reported for kidney slices incubated in vitro with arsenite (As^{3+}) or arsine gas[20]. The specific effects of arsenicals on mitochondrial NAD-linked substrate respiration, and hence cellular energy production in PTC, is believed to result from trivalent arsenical complexing with vicinal thiol groups of the lipoic acid cofactor necessary for oxidation of these substrates (see ref. 21 for review).

B. Cadmium

1. Clinical observations

Clinical effects of chronic cadmium exposure on the kidney are primarily characterized by toxicity to PTC with development of a low molecular weight tubular proteinuria, glucosuria and calcuria among workers occupationally exposed to this element and Japanese ingesting cadmium-contaminated rice[22,23,29]. Development of calcuria and proteinuria in cadmium workers has been associated with development of osteomalacia which is similar to that observed in Itai-Itai patients in Japan[30]. Specific proteins which have been identified in the urine following cadmium exposure include β_2-microglobulin, retinol-binding protein, and RNAase[22]. Glycosuria, decreased creatinine clearance, elevated serum creatinine, and renal calculi have also been attributed to occupational exposure to cadmium[141]. A major aspect of cadmium nephrotoxicity in humans is the slow accumulation of this element in the renal cortex over decades as a result of its transport in blood bound to metallothionein. The onset of clinical renal disorders associated with cadmium occurs at renal cadmium concentrations on the order of 200 μg Cd/kg kidney, which is regarded as the "critical concentration" for this element in the kidney[22]. Interestingly, the onset of renal tubular toxicity is associated with release of cadmium into the urine and a concomitant reduction in renal cadmium burden[22].

2. Mechanisms of cadmium nephrotoxicity

Numerous experimental studies using a variety of animal species[24-28] have reported patterns of cadmium-induced nephrotoxicity virtually identical to those described in humans; however, the underlying mechanism of toxicity was difficult to elucidate due to the slow accumulation of cadmium in the kidney, and the insidious nature of the developing lesion within the PTC. More recently the central role played by the low molecular metal-binding protein metallothionein in the toxic process has been appreciated[31,32].

A more complete discussion of metallothionein and its known roles in the toxicity of several metals is given below (see Figure 2). Cadmium absorbed from the gastrointestinal tract or lungs is initially transported to the liver, where it induces the synthesis of metallothionein which has a molecular weight of about 6800 in mammals. Following continual exposure to cadmium, liver injury ensues with subsequent leakage of cadmium-thionein from hepatocytes into the circulation[28]. The metal-protein complex is transported to the kidney and readily filtered by the glomerulus with subsequent reabsorption by the PTC in a manner similar to other low molecular weight proteins[1,27,33]. The reabsorbed metal-protein complex is rapidly degraded by the renal PTC lysosome system with release of Cd^{2+} ion[27,34,35] which induces the synthesis of renal metallothionein. This process continues until the capacity of the PTC to synthesize metallothionein is exceeded and Cd^{2+} is available to

react with sensitive biological target systems. Alternatively, under chronic exposure conditions where the overall dimensions of the cadmium and metallothionein pools are quite large, the absolute amount of non-metallothionein-bound cadmium may exceed a threshold, and toxicity will ensue. The PTC lysosome system appears to be highly susceptible[27,36] to perturbation by Cd^{2+}. Prior to the binding of Cd^{2+} to nascent renal metallothionein, a major portion of the intracellular Cd^{2+} is bound to various high molecular weight ligands. The most critical ligands are those which appear to play a role in the normal biogenesis of the PTC lysosome system, which involves the fusion of primary lysosomes with pinocytotic vesicles to yield mature secondary lysosomes. Combined ultrastructural morphometric/biochemical studies[27] have shown that Cd^{2+} selectively disrupts this process leading to loss of lysosomal proteolytic enzyme activity and development of tubular proteinuria patterns with attendant calcuria[37,38] similar to those observed[30] in workers chronically exposed to Cd^{2+}. The effects produced in experimental studies may be reversed by prior induction of renal metallothionein synthesis with Zn, enlarging this Cd-binding compartment[27] and reducing the absolute amount of non-metallothionein-bound cadmium. While the exact molecular lesion underlying this compromise of the renal PTC lysosome system is not presently known, it is possible that prior to induction of metallothionein, Cd^{2+} ions are activating cellular calmodulin[39]. Activation of calmodulin, the major Ca^{2+}-binding receptor in eukaryotic cells, results in a cascade of events leading to disruption of the cytoskeleton. Since the cytoskeleton in PTC is believed to play a role in the fusion of primary lysosomes with pinocytotic vesicles[40], disruption of this fusion process would result in the inability of PTC to reabsorb proteins; hence tubular proteinuria would ensue.

The major points to be derived from these studies concern the central roles played by metallothionein in regulating both the transport of Cd^{2+} to the kidney and the availability of Cd^{2+} within the PTC. Disruption of PTC lysosomal biogenesis is a central component of Cd^{2+} nephrotoxicity. Development of tubular proteinuria secondary to decreased lysosomal catabolism of reabsorbed proteins is a major clinical manifestation of Cd^{2+} nephrotoxicity. At present, calcuria observed in this model appears to result from Ca^{2+} binding to excreted proteins rather than a defect in normal renal tubule membrane transport of Ca^{2+} ion[37].

C. Chromium

1. Clinical observations

Acute exposure to chromium, usually as the hexavalent chromate ion (Cr^{6+}), has been reported to produce acute necrosis of the proximal convoluted tubule in humans[41-43]. Percutaneous biopsies performed on a patient with chromium-induced PTC necrosis have also demonstrated large membranous myeloid bodies within these cells, which were suggested to be lysosomal in character[42]. Functional impairment of the kidney associated with this event involves subsequent oliguric renal failure with a prognosis which depends

on the severity of the lesion and efficacy of clinical intervention[42,43].

2. Experimental studies

Numerous experimental studies involving acute administration of chromate (Cr^{6+}) to rodents have confirmed and extended observations on chromium-induced nephrotoxicity in humans. A number of these studies have demonstrated that the first two proximal tubule segments (S_1 and S_2) are the primary cellular targets for Cr^{6+} toxicity[41,44-49]. Time-course ultrastructural studies demonstrated that the earliest ultrastructural lesions involved disruption of the brush border membranes in these cells[44]. This structural change has been associated with loss of PTC reabsorptive capacity as measured by glucosuria, tubular proteinuria and enzymuria[41,44]. In addition, this sequence of events has been accompanied by a reduction in the specific activity of several brush border marker enzymes in the kidney[48] and decreases in para-aminohippurate transport[45,47,49] progressing ultimately to renal failure as measured by increased BUN and serum creatinine[46]. Prolonged exposure to Cr^{6+} via repeated subcutaneous injections demonstrated ultrastructural and biochemical lesions similar to those reported for acute administration studies[41].

Intraperitoneal injection of Cr^{6+} has also been reported to produce DNA interstrand cross-linking, strand breaks and DNA-protein cross-linking in kidney nuclei[50]. At 40 h after treatment the DNA cross-links had been repaired, but DNA-protein cross-links remained. The authors suggested that the kidney was more sensitive to these effects than the liver in which repair of the lesions was rapid[50].

D. Gold

1. Clinical observations

Nephrotoxicity from gold salts has been extensively described in patients with rheumatoid arthritis treated with these compounds[51-53]. Renal lesions from administration of these agents may involve both acute tubular necrosis[51] and immune complex glomerulonephritis[52-54,137,139]. The resultant functional defects characteristically include haematuria, nephrotic syndrome, proteinuria, and acute renal failure[53,54]. A study by Merle et al.[51] compared urinary excretion of leucine aminopeptidase, N-acetylglucosaminidase and β_2-microglobulins in the urine of patients receiving aurothioglucose or gold thiomalate. Excretion of leucine aminopeptidase and N-acetylglucosaminidase was elevated in 55-70% of the patients relative to untreated controls, while excretion of β_2-microglobulin was increased in 30% of the female patients but in none of the males. The mechanism for this sex difference in excretion of β_2-microglolubin was not evident.

2. Experimental studies

Experimental studies by Mogilnicka and Piotrowski[55] demonstrated the extensive binding of gold in the kidneys of rats to a low molecular weight protein with an estimated mass of 10,000 to 12,000 daltons. Subsequent studies[56,57] confirmed these findings and demonstrated that this protein peak could be further fractionated by DEAE ion exchange chromatography into four distinct gold-containing peaks[56]. Further studies demonstrated that gold will bind to induced renal metallothionein suggesting that the renal gold-binding protein could represent endogenous renal metallothionein[58]. Direct evidence concerning the specific chemical characterization of these molecules is still lacking, and further studies are needed to elucidate whether they are, in fact, metallothioneins.

E. Lead

Lead is the most abundant of all the nephrotoxic elements, and there have been both extensive clinical studies of lead-induced nephropathy and corroborative studies in experimental animal models which have yielded extensive insight into both the mechanisms of cellular toxicity and factors regulating these processes.

1. Clinical observations

Morphologically, lead-induced nephropathy is characterized by development of pathognomonic, acid-fast, lead intranuclear inclusion bodies, karyomegaly and cytomegaly of the renal PTC and interstitial fibrosis. At the ultrastructural level these lead inclusion bodies exhibit an electron-dense core with a fibrillar outer margin[59]. Clinically, persons with severe chronic, lead-induced nephropathy may present with azotaemia and a host of functional tubular defects including aminoaciduria, proteinuria, glucosuria, and phosphaturia[59-64]. In addition, several recent case reports, including an exhaustive mortality study of lead smelter workers, have suggested an increased incidence of renal cancer among lead-exposed workers with extensive lead nephrotoxicity[65-67].

2. Experimental studies

Chronic exposure of experimental animals to lead salts via enteral or parenteral routes produces a spectrum of renal effects identical to those observed in humans[59]. In recent years a number of studies have examined some of the possible mechanisms of lead-induced tubular injury. The major findings of these studies, and the emerging relationships which exist between the intracellular binding of lead in PTC and toxicity, are described below.

a. Renal lead transport. Lead uptake by the kidney has been studied in vivo and in vitro using both renal slices and isolated brush border membrane vesicles. Vander et al.[2] performed renal clearance studies in dogs 2 h after a single intravenous dose of lead acetate and found that 44% of the plasma lead was ultrafiltrable, with kidney reabsorption values of 89-94% for the ultrafiltrable fraction. Subsequent stop-flow studies by Victery et al.[6] in dogs given a single intravenous dose of lead acetate showed both proximal and distal tubular reabsorption sites for lead. Distal reabsorption was not linked to sodium, chloride, or calcium transport pathways. These authors also examined the influence of acid-base status on renal accumulation, and excretion of lead in dogs intravenously infused with lead acetate or in rats given access to drinking water containing 500 ppm lead for 2-3 months[68]. Data from these studies showed that alkalosis increased lead accumulation in tubule cells via both luminal and basolateral membranes, with a resultant increase in both renal tissue concentration and urinary excretion of lead. Alkalosis increased lead excretion in rats previously given access to lead via drinking water.

In vitro studies using rabbit kidney slices showed a steady-state uptake of lead, and that lead could enter the slices as a free ion[4]. Tissue slice uptake was reduced by a number of metabolic inhibitors, suggesting the possibility of an active transport mechanism for lead. Uptake of lead was markedly reduced by tin (Sn^{4+}) which did not alter either lead efflux or para-aminohippurate accumulation. This finding raises the possibility that lead and tin compete for a common carrier. Other studies showed that co-transport of lead into rabbit kidney slices in the presence of organic anions such as cysteine, citrate, glutathione, histidine, or serum ultrafiltrate was relatively small compared with uptake due to ionic lead[3]. Victery et al.[5], using isolated brush border membrane vesicles, demonstrated extensive lead binding to the vesicle surface and an apparent absence of a direct transport mechanism. Thus lead may enter the proximal tubule cells via either endocytosis of the membrane or passive diffusion.

b. Intracellular binding patterns. After transport into renal PTC, lead is presumably released at low pH from secondary lysosomes, and initially binds primarily to several high-affinity cytosolic lead-binding proteins[69-72]. These proteins are capable of facilitating nuclear translocation of lead[70] prior to initiation of lead intranuclear inclusion body formation, which involves de novo synthesis of a unique protein[73-75]. The formation of these inclusions results in a marked shift in the distribution of lead from various organelle compartments to the nucleus[76]. The lead inclusions have also been shown to contain the highest intracellular concentrations of lead following chronic exposure[73-77]. The importance of lead intranuclear inclusion bodies in mediating injury to renal proximal tubule cells cannot be understated both with respect to direct lead toxicity to sensitive subcellular systems[76,78-80] and lead-induced changes in renal PTC gene product expression[81]. The effects of lead on specific intracellular processes within the kidney are described below.

c. Mitochondrial respiratory function and energy-linked structural transformations. Mitochondria are extremely sensitive to perturbation by lead. Lead apparently disrupts normal structure/function relationships between mitochondrial membranes and a number of integrated membrane-dependent biochemical processes[59,77,82].

Combined ultrastructural and biochemical studies conducted on PCTs following chronic in vivo lead administration have demonstrated various degrees of in situ mitochondrial swelling associated with decreased respiratory control ratios and the ability to undergo energy-linked structural transformations[59,77,83,84]. A short-term lead injection regimen which induced the formation of both nuclear and cytoplasmic inclusions (but not in situ mitochondrial swelling), decreased respiratory control ratios of renal cortical mitochondria compared to untreated controls. It did not, however, change the energy-linked membrane binding of ethidium bromide[76]. These data suggest that loss of respiratory control is the most sensitive parameter for assessing lead effects on mitochondrial respiratory function, and that high-amplitude mitochondrial swelling and loss of energy-linked membrane configurational changes only occur at higher membrane concentrations of lead[59].

d. Mitochondrial haem biosynthetic pathway enzymes. A number of mitochondrial enzymes which are part of the haem biosynthetic pathway are known to be highly sensitive to alterations by lead. The net effects of lead exposure on δ-aminolaevulinic acid synthetase (ALAS), which is the first and rate-limiting enzyme in haem biosynthesis, represent the sum of two opposing actions. Renal ALAS is sensitive to direct inhibition by lead[77]; however, synthesis of the enzyme is regulated by feedback inhibition such that inhibitory effects of lead on other enzymes in the pathway result in increased ALAS synthesis. The combination of these two perturbations has resulted in mixed findings with respect to lead effects on renal ALAS activity including decreases[77], increases[85], or no change[86].

The other mitochondrial enzyme involved in haem biosynthesis which is sensitive to lead inhibition is ferrochelatase, the terminal enzyme in the pathway. Several studies have demonstrated inhibition of ferrochelatase activity in kidney following chronic in vivo lead exposure[77,80]. The enzyme has been shown to require both the transmembrane movement of and concomitant reduction of Fe^{3+} to Fe^{2+} for activity[87]. Thus the deleterious effects of lead on mitochondrial inner membrane structure and function noted above may play a major role in the observed inhibition of this enzyme. An important aspect of this hypothesis is the prospect that the observed lead-induced effects on ferrochelatase (like those for ALAS) may represent changes in normal mitochondrial structure/function relationships.

e. Cytosolic haem biosynthetic pathway enzymes. The second step in the haem biosynthetic pathway is catalyzed by the zinc-dependent enzyme, δ-aminolaevulinic acid dehydratase (ALAD). This enzyme is highly sensitive to lead inhibition (IC_{50} of approximately 10^{-7}mol/l) in most tissues except kidney and brain due

to the presence of endogenous metallothionein and cytosolic Pb, Zn-binding proteins in these organs[69,72-88]. These proteins protect the enzyme from lead inhibition via both sequestration of lead into a relatively inert complex and donation of zinc from the protein to ALAD, with subsequent activation of the enzyme.

f. Consequences of decreased renal haem biosynthesis. Several studies have demonstrated the apparent effects of chronic lead administration on renal haemoprotein function. Rhyne and Goyer[89] demonstrated quantitative decreases in the terminal electron transport chain cytochromes aa_3 (cytochrome oxidase) suggesting decreased haem availability for these essential haemoproteins in renal mitochondria. Functional evidence of an overall decrease in the activity of mitochondrial respiratory function, consistent with a general depletion of haem from the electron transport chain cytochromes, was reported by Fowler et al.[77]. These workers demonstrated decreases in both NAD-linked and succinate-supported mitochondrial respiration rates with concomitant marked decreases in ALAS and ferrochelatase activities following chronic lead exposure. For shorter exposures, lead preferentially inhibits respiration supported by NAD-linked substrates, but spares that supported by succinate. A general decrease in the flow of electrons through the mitochondrial electron transport chain due to a decrease in cytochrome content, a consequence of decreased renal haem biosynthesis, provides a reasonable explanation for inhibition of both substrate types.

F. Mercury

1. Metabolism

The toxicology of mercury is complex due to the fact that multiple chemical forms of the metal exist, each with different physical, chemical, and toxicological properties[90]. Elemental mercury is not soluble in water and is poorly absorbed in the gut. This form is, however, highly volatile and lipid soluble, and therefore is the form of greatest concern following occupational inhalation exposure. Mercurous (Hg^{1+}) compounds are typically insoluble, non-volatile, and regarded as relatively non-toxic, although there is good evidence that their inclusion in teething powders has been associated with the infant syndrome acrodynia or pink disease[91]. Mercuric (Hg^{2+}) salts are water soluble and potentially nephrotoxic, whereas organic compounds such as methyl mercury are lipid soluble, volatile, and highly neurotoxic in inverse proportion to the size of the organic moiety. Although the toxic manifestations of organic mercury on the nervous system are the most well-known aspects of mercurialism, toxic effects produced by these forms of mercury in the kidney also deserve attention. Particular attention will be focused on the discussion of methyl mercury, which is the most toxic form of mercury, and is also the species of greatest environmental concern.

The highest concentration of mercury following exposure to inorganic mercury is found in the kidney, particularly in the distal

parts of the proximal tubules[92-96]. Although very little mercury penetrates the brain, the amount that does enter this tissue turns over at a very slow rate. Within the kidney, mercury is bound primarily to proteins, including the low molecular weight metal binding protein metallothionein. Kidney lysosomes also accumulate mercury[97-99].

After administration of methyl mercury the metal is more evenly distributed between organs compared to distribution following inorganic mercury administration. High levels are found in the kidney, liver, spleen, pancreas, and red blood cells. Brain mercury concentrations are low compared to kidney and liver, but are much higher than those seen after inorganic mercury exposure[100].

2. Clinical observations

Acute exposure of humans to mercuric salts usually arises from attempted suicides or accidental ingestion. The target organ in such cases is the kidney, and the acute lethal dose is estimated at 0.5-1.0 g for an adult[90].

The pattern of damage resulting from chronic mercury exposure in humans varies greatly according to the chemical form of the mercurial and the duration of exposure. Metallic mercury poisoning following exposure to mercury vapour results in the classic syndrome of mercurialism, which includes central nervous system effects.

Studies of the pathology of human inorganic mercury poisoning are sparse and are restricted to descriptions of kidney damage resulting from ingestion of mercuric salts[101]. By comparison, methyl mercury toxicity has received much more comprehensive study. Hunter and Russell[102] described the pathology from a mild case, and Takeuchi et al.[103-104] subsequently reported pathology from fatal cases of Minamata disease. In all these cases extensive central nervous system damage occurred. In addition to neurotoxic effects, porphyrinuria was also reported in these patients, clearly indicating toxicity to other organ systems including the kidney[105].

Attempts at chelation therapy in humans with various types of mercury poisoning have met with mixed success. In cases of attempted suicide or large acute accidental doses of mercuric salts, prompt use of BAL (British Anti-Lewisite) (2,3-dimercaptopropanol) is effective in preventing kidney damage. Following chronic inorganic mercury exposure, however, BAL has little or no benefit, and EDTA (ethylenediamine tetra-acetic acid) actually increases toxicity[106]. BAL is also usually ineffective in decreasing the toxicity of organic mercury compounds, although the overall effect is dependent on the type of organic mercury compound[106,107]. The penicillamines, however, are somewhat effective against organic mercurials if administered early in the clinical course[108]. The relative ineffectiveness of these chelators is presumably due, in part, to the binding of Hg^{2+} to metallothionein in the kidney and other organs as described below.

3. Experimental studies

Tolerance to mercuric salts by the kidney has been noted in other studies[109]. Thus, although a single intravenous injection of 1 mg/kg $HgCl_2$ causes acute renal failure resulting in extensive degenerative changes in the pars recta of kidney proximal tubules[110], chronic oral exposure to 50 ppm $HgCl_2$ for 22 weeks[111] causes only local damage to proximal tubules. Although the liver is not generally considered a target organ in inorganic mercury toxicity, chronic exposure to mercuric chloride may also lead to liver damage in rats, as evidenced by periportal lipid accumulation and foci of hepatocyte necrosis[105].

Inorganic mercury is known to selectively damage the pars recta of the proximal tubule[53,112,113]. Gritzka and Trump[112] and Ganote et al.[110] demonstrated that loss of the PTC brush border was among the earliest structural lesions to occur following acute administration of mercuric chloride. Concomitant increases in tissue water and calcium in the inner cortex were also observed, while changes in organic cation transport and renal slice oxygen consumption occurred later in the process of cell injury[110]. Ultrastructural damage to the PTC brush border was correlated with increases in the urinary excretion of brush border marker enzymes, e.g. alkaline phosphatase, with no change in excretion of basolateral membrane markers[114]. Thus inorganic mercury may induce cell injury by a direct interaction with the brush border membranes; however, the mechanisms underlying this effect are unknown.

Little attention has been focused on the toxicity of methyl mercury to non-neuronal tissues, although the kidney is also an important target organ. Fowler[115,116] demonstrated a more severe ultrastructural damage to the pars recta of the renal PTC than the convoluted portions following chronic exposure to low concentrations of methyl mercury in the diet. These changes included increased amounts of smooth endoplasmic reticulum (SER), apical blebbing of SER-containing casts, degenerating mitochondria, and cell necrosis. The kidneys of female rats were more sensitive to these effects than males. Pars recta tubules of female rats exposed to methyl mercury were dilated and contained spherical, cytoplasmic masses described as cytosomes which were characterized ultrastructurally by the presence of a SER bundle and an occasional microbody[115,116]. These cytosomes were postulated to serve as a mechanism for sequestering mercury. The inhibition of microsomal enzymes (see above) in pars recta cells could render SER aggregates non-functional, and thus provides a mechanism for their extrusion in cytoplasmic masses. This may account for the proteinuria observed in persons occupationally exposed to organic mercury compounds[121,122].

The higher sensitivity of female rats to methyl mercury nephrotoxicity may reflect sex-based differences in the enzymatic capacity to convert methyl mercury to inorganic mercury[115,116]. Organic mercury compounds are known to be converted to inorganic mercury in the kidney[117-119]. Swollen mitochondria, proliferation of SER, and cellular necrosis characterize acute inorganic mercury poisoning[112,113]; however, the same lesions are ob-

served following methyl mercury exposure[115,116]. The inorganic mercury formed in vivo following methyl mercury exposure may react with microsomal enzymes as a non-competitive inhibitor. The mechanism of the inhibition involves the interaction of mercury with sulphydryl groups present at the active site of many enzymes[120].

The renal mitochondrion is also a primary subcellular target following chronic oral methyl mercury exposure. Methyl mercury exposure preferentially inhibits respiration of NAD-linked substrates such as pyruvate/malate and α-ketoglutarate with no effect on succinate-supported respiration[123]. Renal haem biosynthesis is also a key cellular pathway affected by methyl mercury exposure. Woods and Fowler[124] demonstrated inhibition of renal mitochondrial ferrochelatase with concomitant urinary excretion of the haem precursors, coproporphyrin and uroporphyrin.

An interesting aspect of mercury toxicity relates to interactions with other metals or organic compounds which may alter the toxicity of mercury. Vitamin E, at least in large amounts, appears to provide some protection against methyl mercury, but no mechanism is known[125]. Substances which induce microsomal enzymes, such as phenobarbital and dieldrin, a chlorinated hydrocarbon pesticide, reduce methyl mercury toxicity by increasing renal excretion of mercury or stimulating demethylation of methyl mercury by the microsomal mixed function oxygenase system[115,126].

The best known interaction between another element and mercury involves selenium, which is itself toxic. Although the basis of the interaction between selenium and methyl mercury is not known, some information regarding the interaction with inorganic mercury is now available[111]. Simultaneous administration of mercuric chloride and sodium selenate protects against the weight loss produced by either substance alone and prevents the pathological lesions produced by mercury in the liver and kidney. This protection is associated with the appearance of intracellular electron dense inclusion bodies in the nuclei of kidney PTC which appear to play a role in decreasing the nephrotoxic effects of mercury. In addition, electron dense material is found in the extracellular space in the liver, often associated with collagen fibres. X-ray microanalysis has revealed that this material contains both mercury and selenium. No similar effect has been reported for either substance alone, and it is generally accepted that the reduction of toxicity is brought about by the direct association of these two elements in vivo.

Chromium, administered as potassium dichromate, has also been reported to enhance the effect of mercuric chloride on renal transport of the organic ions, para-aminohippurate, and tetraethylammonium[49]. The effects were produced at doses of potassium dichromate which alone elicited no response. The interaction was described as a potentiation response, and illustrates the need to study the interaction of metals and other compounds under multiple-exposure conditions.

A number of studies have demonstrated that Hg^{2+} binds tightly to metallothionein (MT) in vivo[127,129] while methyl mercury does not[128]. The importance of these studies rests with understanding the relatively long retention time for mercury in the kidney, and the relative ineffectiveness of chelating agents in remov-

ing this element from the body. Interestingly, administration of selenium[130] reduces binding to this protein, possibly as the result of formation of the mercury-selenium nuclear inclusions described above.

Mercury has been shown to induce an immunologically mediated glomerulonephitis in both humans and experimental animals[137-140]. The mechanisms of chemically induced, immunologically mediated glomerulonephritis are poorly understood, and different mechanisms may operate depending upon species and strain. The mechanisms involved include interaction of free circulating antibodies with structural glomerular membrane-bound antigens, deposition of circulating immune complexes onto the glomerular membrane, and/or cell-mediated (T cell) immune responses. The autoimmune disease induced by mercury in a Brown-Norway rat model is characterized by the presence of circulating anti-glomerular basement membrane antibodies which are found deposited along the glomerular capillary wall[138,140]. Deposition of these antibodies is associated with a nephrotic syndrome including proteinuria. The most widely accepted mechanism responsible for the disease induced in this model involves polyclonal activation of B cells. This activation results from a modification of T-helper cells which are capable of stimulating normal T-helper cells and B cells. These effects are maximal after 3 weeks following injection of mercuric chloride at a dose of 1 mg/kg body weight three times per week, and can be detected at doses as low as 0.1 mg/kg. The glomerulonephritis can be induced by a number of mercury species, including mercuric chloride, methyl mercury, and many mercurial pharmaceutical compounds.

In general, specific mechanisms of mercury nephrotoxicity are not well understood. Mercury can be shown to inhibit a large number of enzyme-mediated biochemical reactions in vitro, due generally to its high affinity for active sulphydryl groups[131]; however, it has been difficult to identify the protein most sensitive to mercury in vivo[132]. Generally, mercury toxicity occurs in the organs in which appreciable amounts of the metal accumulate, and varies with the chemical form. Methyl mercury and mercury vapour are lipid soluble and can therefore easily penetrate the blood-brain barrier, while inorganic forms of mercury accumulate primarily in the kidney.

G. Uranium

1. Clinical observations

The nephrotoxic effects of uranium are also focused on the PTC. The kidney is the major excretory route for uranium and also highly concentrates the metal. Early studies demonstrated that exposure to this element produced necrosis with subsequent oliguric renal failure in humans and leads to proteinuria and increased excretion of catalase[133]. The determination of urinary catalase combined with a test for proteinuria has been suggested to be a good indicator of exposure to uranium[133].

2. Experimental studies

Animals treated with uranium salts develop necrosis of the terminal segments of the PTC. Hyaline casts and cellular necrosis are the primary morphological features of uranium-induced tubular necrosis. In addition to the tubular changes, damage to glomeruli has been noted, characterized by glomerular capillary basement membrane changes as well as hyaline droplets and cytoplasmic vacuoles in epithelial cells[134]. The corresponding functional changes are characterized by proteinuria, aminoaciduria, glucosuria, and reduced para-aminohippurate clearance. Studies by Hirsch[135] reported stimulation of N-methyl nicotinamide and tetraethylammonium transport by renal cortical slices from rats treated in vivo with uranyl nitrate with no change in the uptake of para-aminohippurate. More recent studies have examined increased renal excretion of lactate dehydrogenase, alkaline phosphatase, and leucine aminopeptidase following injection of uranyl nitrate as a function of age[136]. These studies demonstrated that urinary excretion of these enzymes was a reliable indicator of nephrotoxicity in adult and aged rats. In 15- to 20-day old rats, only urinary excretion of alkaline phosphatase proved to be a reliable indicator of uranium-induced nephrotoxicity[136].

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23
NATURALLY OCCURRING ENVIRONMENTAL CONTAMINANTS

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Although any number of chemical substances can be correctly identified as "naturally occurring", the material in this chapter will focus only on some of the substances associated with the plant kingdom. It is quite clear that from very early in man's existence there have been encounters with undesirable effects of certain harmful plants, mushrooms, etc. For the most part, in the so-called advanced countries, poisoning by consumption of plants has been considerably reduced. Nonetheless, there still exists concern about the adverse effects of plants not only on man, but also on various domestic animals. This may be particularly troublesome in some agricultural areas. It would appear that the most widespread outbreaks of poisonings due to plants have come from the consumption of foods containing toxic chemicals. For example, the outbreak of ergotism in the Middle Ages was caused by the use of grain contaminated with the fungus, Claviceps purpurea. The chemical substances produced by this fungus, as is true with most plants, represented so-called secondary products of metabolism, i.e. these substances are not essential to the survival of the plant. The exact role of these secondary products in the plant economy is highly debated, but the poisonous substances may serve as survival devices, at least to some extent.

A very wide diversity of chemical substances with adverse effects in mammalian systems exist in plants. Not only is the chemical variety interesting, but also so are the various biochemical pathways by which these compounds are generated. Consistent with the wide diversity of chemical substances, there is an equally wide diversity of effects on biological systems. Well defined actions have been identified on the nervous system, the respiratory system, the cardiovascular system, the liver, the gastrointestinal tract, skeletal muscles, and the kidneys. The comments in this chapter will be directed only at those substances with documented effects on the kidney. If there should be overlap between the actions of a given substance on the kidney and other organs, par-

ticularly the liver, that material will be alluded to. Three sections will be presented: toxins in higher plants, mushroom toxins, and toxins produced by filamentous fungi, i.e. the mycotoxins. For those with a general interest in plant toxins, several excellent reviews and books are available. The following may be examined both for factual material and for references to the literature: Tampion[1] and Rechcigl[2].

HIGHER PLANT TOXINS

A. Organonitriles

Various cruciferous plants (Table 1) commonly cultivated as vegetables, condiments, etc. contain various glucosinolates which are capable of hydrolysis to a variety of organonitriles, thiocyanates, and isothiocyanates. Nitrile formation appears more likely during autolysis of the crushed, wet, natural plant material[3,4]. The major organonitriles formed are three, S-1-cyano-2-hydroxy-3-butene and two diastereoisomers of two S-1-cyano-2-hydroxy-3,4-epithiobutanes (CHEB)[5]. Nishie and Daxenbichler[6] investigated the toxicity of these organonitriles and various other substances found in Cruciferae. No functional studies were undertaken. However, the morphological studies suggested damage to

Table 1 Examples of some higher plant toxins

Chemical types	Plants	Chemical compounds
Organonitriles	Cruciferous plants	S-1-cyano-2-hydroxy-3,4-epithiobutanes
Pyrrolizidines	Widely distributed, e.g. Senecio, Crotalaria, Echium, etc.	Monocrotaline, jacoline

the kidneys. Specific nephron segments were not identified, nor were cell types identified, but it was reported that kidney tubular cells were swollen and the nephrons dilated. In addition, some areas appeared to show severe vacuolation and necrosis. These studies were done with acute, subcutaneous administration of various doses of CHEB rather than with the butene nitrile.

Gould et al.[7] undertook a 90 day feeding study with CHEB (44% threo and 56% erythro) at three dose levels in the diet. The highest dose in the diet yielded a daily intake of approximately 22 mg/kg, a dose significantly below that administered subcutaneously in the acute experiments of Nishie and Daxenbichler[6]. Relatively modest effects on renal function were observed. Indeed, blood urea nitrogen (BUN) was depressed rather than increased after the 90 day feeding at the highest dose. Histological changes in the

kidney were described as a "nephrocytomegaly" wherein both nuclear cytoplasmic enlargement of proximal tubular cells throughout the cortex and outer medullary stripe were observed. The karyomegaly was the most striking effect and the authors remind us of a number of other substances which produce karyomegaly prior to the production of tumours. For example, alkali-treated soya protein and lysinoalanine[8] produce a marked renal tubular karyomegalic response, but unlike some other substances have not been reported to be carcinogenic.

Subsequently, Gould et al.[9] have undertaken both functional and structural studies after acute administration of CHEB. Oral doses of from 50 to 100 mg/kg were administered. Depending somewhat on dose, CHEB produced a high output renal failure with an increase in plasma creatinine and BUN and decreased glomerular filtration rate 48 to 72 hours after administration of the toxin. Urinary N-acetyl-β-D-glucosaminidase (NAG) excretion was elevated at the lower dose over 48 to 72 hours after administration of the toxin, but was decreased at the 100 mg/kg dose. Light microscopic studies indicated a preferential involvement of the straight part of the proximal tubule, although in some experiments the convoluted tubule was also involved. The lesions were necrotic in nature at their severest, although lesser effects were also observed. In general, the morphological changes correlated well with the functional changes.

No studies were undertaken specifically to examine mechanisms of nephrotoxicity, but Gould et al.[9] offered some interesting speculations. Davis[10] and Willhite and Smith[11] have demonstrated that organic nitriles can undergo biotransformation which will result in the release of cyanide. Further, Ahmed and Faroqui[12] were able to demonstrate increased concentrations of cyanide in liver, kidney and brain of animals which received various organonitriles. These workers did not say, however, whether or not there were any renal lesions associated with the increased cyanide content. Nonetheless, although renal lesions are not generally associated with classical cyanide intoxication, the local production of cyanide within a specific tissue might yield different results. Certainly nitriles other than CHEB have been associated with reports of renal lesions[13,14], and it is possible these effects resulted from local cyanide production.

B. Pyrrolizidine alkaloids

The pyrrolizidine alkaloids represent a group of esters of amino alcohols derived from the heterocyclic pyrrolizidine nucleus (Table 1). These substances appear throughout the plant kingdom and are represented in the genera Senecio, Crotalaria, Echium, Heliotropium, etc. Monocrotaline (Crotalaria spectabilis), senecionine and seneciphylline (Senecio alkaloids) have been studied most commonly.

The pyrrolizidine alkaloids primarily affect three organs: liver, lung, and kidney, and which organ is attacked is apparently species-dependent[15,16]. Severe hepatic necrosis has been reported[17]. With the lungs the bronchiolar epithelium as well as

alveolar cells are effected after feeding animals with seeds containing pyrrolizidine alkaloids; in addition, various vascular disorders are noted in the lungs. Indeed, these vascular effects are also noted in the liver and the kidney, and may also underlie much of the toxicity in these organs. In all likelihood the vascular damage resulting in a venous occlusion may be effects of the alkaloids or their metabolites on the capillary endothelia. It is noteworthy, for example, that regardless of the organ similar vascular disruption is observed.

Considerable species variation appears to occur with respect to pyrrolizidine alkaloid-induced renal damage[18-20]. Both the mouse[17] and the pig[21] seem to be sensitive to the effects of pyrrolizidine alkaloids, while the rat[22] appears to be relatively insensitive. Although not discussed by the authors[22], it may be that the difference in sensitivity reflects a difference in the architecture and function of the different kidneys. Megalocytosis has been reported with significant nuclear as well as cytoplasmic enlargements in the epithelial cells of the nephron. Primarily these changes are in the proximal tubule, but also occur in glomerular cells. The latter effects are not surprising, given the degenerative changes noted in the capillaries of other organs. The cytoplasmic swelling in the glomeruli may lead to occlusion of those capillaries, possibly because of dense deposits of proteinaceous material. With more severe damage, endothelial cells are observed to become detached from the basement membrane and significant haemorrhaging may occur. Hence, as with other organs, the primary effect noted in the kidney appears to relate to the vasculature, although effects on proximal tubular epithelia have also been reported. It is unclear which of these events may be primarily responsible for the overt renal damage, i.e. the so-called "nephrosis".

Regardless of the details of the toxicities observed, regardless of the organ system involved, and regardless of the species, it would appear that all of the toxicities produced by the pyrrolizidine alkaloids are mediated through the same mechanisms, although it must be admitted that these mechanisms are poorly understood. Pyrrolizidine alkaloids are metabolized to pyrrole derivatives as well as to N-oxide compounds. Although several workers have suggested[18,22,23], that it is the pyrrole derivatives that are the toxic compounds, others have noted that the allylic ester function is a good alkylating agent[23-25]. Indeed, these two suggestions may indicate the same mechanism of action since the pyrrole derivatives are potential alkylating agents themselves and as such can produce damage to intracellular as well as membrane structures. Actions of this sort would lead to mitochondrial dysfunction as well as other metabolic abnormalities. Chesney and Allen[26] examined the relative resistance of the guinea pig to these alkaloids. The studies were done with monocrotaline and it was found that the pyrrole derivatives were formed much more readily in the rat than in the guinea pig, while the N-oxide compounds were produced equally in both species. Since administration of the monocrotaline pyrroles to the guinea pigs did produce toxicity, these data suggested that the absence of the synthetic pathway for the pyrroles, or at least its activity at a reduced level, was

responsible for the relatively greater resistance of the guinea pig to these substances than observed in the rat. Not all examples of resistance relate to metabolism[27]. The rabbit is relatively resistant, apparently, because of poor gastrointestinal absorption of the alkaloids. This observation was proven by the administration of the purified alkaloids intravenously with the development of a relatively predictable toxicity. Furthermore, it has been observed that the rabbit liver can readily generate pyrroles, suggesting that where the alkaloids are absorbed, toxicity would ensue. Finally, studies from Buhler's laboratory[28] have demonstrated that butylated hydroxyanisole (BHA) can protect against the toxicity of monocrotaline. Indeed, BHA reduced the degree of pyrrole generation by the liver. Further, BHA appeared to increase liver sulphydryl concentrations. Hence, Buhler's group concluded that the protective effects of BHA stemmed not only from the reduction of available pyrroles, but also by an increase in sulphydryl content of the liver. Whether or not any of these metabolic processes occur in the kidney for pyrrolizidine alkaloids is uncertain. To date, experiments have not been undertaken to determine whether or not pyrroles are formed locally or whether or not alkylation occurs in the kidney.

Buckmaster and colleagues[29] have undertaken, at least indirectly, studies to examine some of the problems given above. For example, these workers reason that cysteine might be expected to react with electrophilic metabolites and thereby prevent their attack on cellular components. In these experiments animals were fed crude plant extracts and treated with either cysteine or methionine. Cysteine afforded better protection against the pyrrolizidine-containing plant materials and tended to confirm the working hypothesis.

In general, pyrrolizidine alkaloid toxicity is thought of as a problem for grazing farm animals. Dickinson et al.[30] found that at least one of the five major alkaloids in ragwort, jacoline, was contained in cow's milk. Hence the possibility exists for transfer of this alkaloid to the human. In addition, since bees collect pollen from ragwort as well as other plants, and the alkaloids are present in pollen, it is not surprising that Deinzer et al.[31] found some pyrrolizidine alkaloids in honey. Again, there is at least the potential for human intoxication with these alkaloids, although it must be admitted that quantitative considerations may preclude the development of serious human toxicities.

The above two examples of toxic substances in plants which may have effects on renal function in man and his animals, might well be taken only as a caution. The wide variety of secondary metabolites of plants with important effects in biological systems would suggest that there are other chemical substances in plants with effects on renal function and structure. This is an area which has not been investigated adequately.

MUSHROOM TOXINS

Several important reviews on mushroom poisons have been written in recent years. These do not focus specifically on toxins which

act on the kidney, but rather survey the whole field of poisonous mushrooms. The interested reader is referred to Lampe[32,33], Lincoff and Mitchell[34], Rumack and Salzman[35], Bertelli et al.[36] and Faulstich et al.[37]. This flurry of publishing activity on the general subject of fungal poisonings reflects an increasing interest and concern about this problem. For example, there is now evidence of potential health hazards for agricultural workers involved with the commercial harvesting of mushrooms. Further, there seems to be an ever increasing desire for new mushroom tastes, not only in Europe but also in North America, and this is contributing to our heightened awareness of the potential medical problems involved. Indeed, mycetism or mycetismus (mushroom poisoning) all too frequently results from individuals collecting and eating wild mushrooms without the proper background for being able to identify those that are safe and those that are not.

In general it is possible to divide virtually all systemic mushroom poisonings into two main groups depending upon the latency involved between consumption of the mushrooms and the appearance of symptoms. There are those mushrooms which will produce their toxicity within a matter of 2 or 3 hours after ingestion. Some mushrooms only cause toxicity after ingestion with significant quantities of alcohol. These mushrooms usually involve primarily a gastrointestinal response such as gastroenteric irritation, nausea, vomiting, diarrhoea, etc. Short-latency mushroom poisoning, in addition, will frequently display other signs of parasympathetic hyperactivity, such as sweating. Delirium and hallucinations may be present after consumption of psilocybin-containing mushrooms. Some species of Coprinus or Boletus have been reported to produce a disulphiram-like reaction in individuals who consume alcohol at the time they are eating the mushrooms.

Those mushrooms that produce intoxications with a delayed onset of usually 6 to 8 or even more hours are frequently associated with very serious poisoning often with a grave prognosis.

Table 2 Types of mushroom toxins with effects on renal function

Amanita phalloides, Galerina sp.,	α-amanitin	Liver and kidney damage
Cortinarius orellanus, C. speciosissimus, C. gentilis	orellanine	Paraquat-like action
Hypholoma fasciculare	?	?

It is these mushrooms that produce renal damage (Table 2). Delayed-onset toxicity is observed after ingestion of Amanita phalloides and a variety of related species and subspecies. Usually after several hours (5 to 10) the subject will be struck with severe gastroenteritis with vomiting, severe abdominal pain, watery diarrhoea, etc., which will pass fairly promptly. Because fluid loss may be quite severe during this gastrointestinal phase of the

toxicity, the potential exists for dire consequences to the cardiovascular system. After the initial illness the patient will appear nearly normal for several days, after which the severe, often life-threatening toxicity develops. This latter toxicity is first observed as jaundice and other signs of hepatic cellular necrosis.

There are two classes of orally active toxins in A. phalloides, both of which are cyclic polypeptides. The phallotoxins are cyclic heptapeptides and possess a relatively low toxicity for mammals. The amatoxins are apparently responsible for all of the observed clinical symptoms of poisoning with these mushrooms. These compounds are cyclic octapeptides. Although as many as seven or eight octapeptides may exist in varying species, α-amanitin would appear to be the most commonly occurring substance and one with extremely high toxicity.

Clearly hepatic damage is the primary concern with these substances, but occasionally renal failure has also been reported[38]. Electron microscopic examination of biopsy samples has suggested that both proximal and distal tubular cells may be affected, although a classically defined necrosis was not present; glomerular damage was not seen. Both kidney and liver cells appeared to demonstrate a vesicular-like damage to the cell nucleus. The nuclear damage can appear quite rapidly, i.e. in a matter of a few hours after administration of amatoxins to experimental animals[39]. Very early Stirpe and Fiume[40] suggested an action of α-amanitin on nucleic acids, specifically, they found the toxin to inhibit the activity of RNA polymerase. Recent studies[41-45] have demonstrated beyond any doubt that RNA polymerase II is inhibited by amatoxins thereby disrupting protein synthesis in the cell. Most of these studies have been conducted with hepatic tissue, however, and whether or not similar effects are seen in the kidney is less clear, but highly likely.

Species variability appears to exist with respect to α-amanitin and renal damage. Fiume et al.[46] demonstrated the sensitivity of the mouse to this toxin, with damage restricted to the proximal convoluted tubule. Subsequently, the rat was found to be relatively insensitive[47]. This lack of toxicity in the rat was suggested to be due to the inability of this species to transport this substance into or out of the tubular fluid, so that sufficient concentrations within the proximal tubular cells were never achieved. Renal damage could be produced in the rat if the toxin was first bound to albumin, presumably because the albumin would be taken into the cells by pinocytosis. Bonetti et al.[48] demonstrated that hepatocytes could accumulate more toxin when the toxin was bound to albumin than when it was free.

The genus Cortinarius contains several species of mushroom capable of producing renal failure several days after ingestion of the mushroom. Toxicities have been reported in several countries, and these have been summarized by Lampe[33] and Schumacher and Hoiland.[49]. The species involved appear to be C. orellanus, C. speciosissimus and C. gentilis. The latter two species have been studies in the experimental laboratory and at least one of them involved in a human poisoning[50-54]. Necrotic lesions have been observed in both male and female rats 2 to 4 days after administration of a single dose of Cortinarius extract. In female rats the

most dramatic damage seemed to be in the inner cortex. Post-mortem examination of human tissue showed tubular necrosis as well as fatty degeneration of the liver, although morphological sites were not specified. At low doses the course of the human toxicity usually involves an onset of relatively high urine output and consumption of large volumes of liquid. Ultimately this progresses to renal failure thought to be responsible for deaths in humans. Large doses of toxin will result in oliguria or anuria early in the course. Non-fatal poisonings have a very long course of recovery.

The toxin, orellanine, has been suggested to be the primary agent in the production of toxicity, and its chemical structure is under study[55,56]. Details of the chemistry are debated, but agreement exists on the most important points. The toxin is heat stable. Its mechanisms of nephrotoxicity are yet to be defined unequivocally, however, Schumacher and Hoiland[49] have raised some interesting observations. The proposed structure for orellanine is similar to that of paraquat and diquat, both of which can produce severe nephrotoxicity[53-59]. These cationic bipyridyls can be reduced to free radicals and re-oxidized by molecular oxygen to superoxide radicals[60]. Either directly, or through formation of singlet oxygen, lipid free radicals may be generated resulting in membrane damage[61]. It is likely that orellanine can undergo similar redox reactions to form free radicals and this may underlie Cortinarius toxicity. Details of these and other studies are reviewed by Schumacher and Hoiland[49].

MYCOTOXIN-INDUCED NEPHROPATHY

Man's involvement with mycotoxicoses is not new. Abundant data exist even in the ancient literature to demonstrate the effects of various secondary fungal metabolites on various organ systems in man and other animals. The magnitude and severity of many of these intoxications were great and have continued to the present day. The relatively recent involvement of the aflatoxins in human cancers and the possible involvement of one or more mycotoxins in human renal disease (see below) are examples of the potential importance of these substances in human health.

Because the topic of mycotoxin-induced diseases in humans and other animals is of considerable importance, much has been written on various aspects of these problems in recent years. Accordingly, no attempt will be made here to give an exhaustive review of the literature of mycotoxin effects in general or of the mycotoxin-induced nephropathies. Instead, the interested reader is referred to any of several recent articles such as Berndt[62,63], Hall[64], Hayes[65], Wilson[66], Uraguchi and Yamazaki[67], and Rodricks et al.[68]. In addition, the multi-volume work edited by Wyllie and Morehouse[69] contains much useful information not only about biological effects of the toxins, but also about their chemistry. Further, all of these reviews give many references to the original literature.

The material to be presented below will focus on two fungal toxins with well-documented effects on renal function and structure, the possible role of fungal toxins in one human renal dis-

ease, endemic Balkan nephropathy, and a review of other fungal toxins which have less well documented actions on the kidney (Table 3). Because of the interest in the effects of fungal toxins on farm animals, many important studies of potential nephrotoxicities have been done in those species. However, various studies also have demonstrated the utility of laboratory model for the study of mycotoxin-induced nephropathy[70-77]. Despite the difficulties involved in species to species comparisons, data from the laboratory model and from various "field" studies[78-81] agree remarkably well.

Table 3 Mycotoxins with actions on the kidney

Active compound	Fungus
Citrinin	Penicillium citrinium, P. veridicatum
Ochratoxin A	Aspergillus ochraceus, P. veridicatum
Aflatoxin B	Aspergillus flavus
Rubrutoxin B	Penicillium rubrum
Oxalate	Penicillium veridicatum
unknown	Rhizopus nigricans

A. Citrinin

Citrinin, produced by several aspergilli and penicillia, was first studied as a possible antibiotic, and is now of concern because of contamination of foodstuffs. Citrinin often occurs in conjunction with ochratoxin A, another mycotoxin capable of altering renal function[82].

In the laboratory rat citrinin administered intraperitoneally has been demonstrated to disrupt both renal function and structure. Acute tubular necrosis of the earliest proximal tubular segment occurs with peak effects being seen at 48 to 72 hours after administration of a single dose[76]. Animals that survive the initial insult will regenerate nephron cells, and by 5 to 7 days after the single dose of toxin will exhibit nearly normal renal function and structure. Distal tubular damage was not observed morphologically, nor was there glomerular damage. These observations may be of some interest in that Carleton and colleagues[83,84] suggested the occurrence of distal tubular lesions in the mouse and the dog. Except for the possibility of species differences, explanations for these differences have not been forthcoming.

As with the morphological studies, a sustained disruption of renal function occurs after a single dose of citrinin. The disruption of function followed the same time course as the alterations in structure. Depending on dose, most rats exhibited a "high-output" renal failure. Accompanying the excretion of a large volume of very dilute urine there was an increase in urinary glucose excretion and a marked proteinuria. The blood urea

nitrogen (BUN) was elevated in a dose- and time-dependent manner. This overall pattern of response agreed well with that reported in the literature for porcine nephropathy[78,80] and with a similar disease reported in poultry[81]. Larger doses of citrinin administered to the rat produced anuric renal failure and death.

Although the first evidence of onset of nephrotoxicity in the unanaesthetized rat occurred several hours to 1 day after administration of the citrinin, when the fungal toxin was administered acutely to the anaesthetized rat alterations in renal function were observed within 2 hours[77]. These effects involved not only alterations in electrolyte excretion, but also a decrease in glomerular filtration and excretion of p-aminohippurate (PAH). Although the effects on PAH excretion might be interpreted as a change in renal blood flow, in vitro studies would indicate that citrinin can directly effect the transport of PAH by renal tubular cells[77]. These data suggest that the decrease in PAH clearance observed in the intact animal may reflect decreased transport of PAH rather than an alteration in renal blood flow. Citrinin also affects the transport of tetraethylammonium (TEA), an organic cation.

Taken together, these data would suggest that the primary action of citrinin is to disrupt renal tubular function rather than glomerular function. Glomerular filtration falls, but it would appear that the primary insult is to the nephron itself not the glomerulus.

Pharmacokinetic studies[73,74] indicated that citrinin was excreted relatively rapidly in the urine when renal function was unimpaired. Indeed, as much as 80% or more of administered label (^{14}C) appeared in the urine by 48 hours after the dose was given. Most of the citrinin in the urine was unmetabolized compound. However, a significant 10-20% of the administered substance was metabolized in the liver[75] (also Mehendale and Berndt, unpublished observations). Studies with the isolated perfused kidney failed to demonstrate the generation of any metabolites by that organ (Berndt, unpublished observations). Although the chemical nature of the metabolites has not been identified, neither incubation with glucuronidase nor sulphatase had any effect on the metabolites. Because the metabolites are more polar than citrinin itself, they could be mercapturic acid derivatives resulting from glutathione conjugation. Alternatively, they could be oxidation products - for example, alcohols of citrinin.

The mechanisms underlying the nephrotoxicity produced by citrinin are as of yet poorly defined. Citrinin does deplete renal glutathione, and "covalent binding" of citrinin or a citrinin metabolite by renal tissue has been demonstrated[62,63,75]. These data would suggest that the formation of a metabolite is very important in the production of toxicity, at least based on the usual scenario reported for other substances such as acetaminophen. On the other hand, the usual pretreatment routines expected to alter metabolism (e.g. phenobarbital, 3-methylcholanthrene, etc.) fail to produce the expected results. Hence, although there is some evidence of metabolism of citrinin that may have an important impact on nephrotoxicity, a final answer to the question is still lacking.

This story is complicated by the fact that there are other

data generated to understand mechanisms which appear relevant, but may suggest a different mechanism. For example, Berndt et al.[85] demonstrated that the fungal toxins citrinin or ochratoxin A could disturb tissue calcium balance. Enhanced renal slice calcium accumulation or accumulation of calcium by the intact kidney occurred earlier than any other observed disruption of renal physiology or biochemistry. Further, these effects were seen at doses known to produce a nephrotoxic response in the intact animal. Of course, given the rapidity of metabolic processes one might argue that these effects were produced by a metabolite, not the parent citrinin, and that they represent only one step, albeit an early one, in the generation of the acute tubular necrosis.

The question of whether or not a metabolite or the parent compound is involved in the production of toxicity is an important issue, and one which has been addressed at least in part. Berndt and Hayes[86] demonstrated that probenecid, an inhibitor of organic anion transport, could reduce the toxicity generated by citrinin. Since probenecid is not known to have any other effect on the kidney, and since citrinin is an organic anion transported by the renal organic anion transport system[87], the probenecid data are at least consistent with an effect to prevent the entrance of citrinin into renal tubular cells. Since the isolated perfused kidney data (see above) did not demonstrate metabolism of citrinin by the kidney, the probenecid experiments are at least consistent with citrinin being the proximate toxin. Of course, the possibility exists that citrinin metabolism in the liver, which is known to occur, could generate an organic anion metabolite, the transport of which is blocked by citrinin. That issue has not been resolved.

Hence, although there appear to be several pieces of evidence to suggest that the parent compound is the toxic species in the rat, the data are not unequivocal. Whatever the mechanism of toxicity, it is clear that citrinin can produce an acute tubular necrosis and either a high output or anuric renal failure. Pharmacokinetic studies do not suggest a cumulative toxicity with this substance, but the acute events cannot be overlooked.

B. Ochratoxin A

Ochratoxin A has been isolated from cultures of A. ochraceus. Since its identification, it has been suggested to be involved in various nephrotoxicity episodes. Krogh et al.[78], Krogh[79], and Golinski et al.[80] have supported the role of this toxin in the production of porcine nephropathy.

Even in the laboratory model there can be no doubt that ochratoxin A has effects on renal function[88]. The nature of the response, however, was clearly different from that observed with citrinin. No single dose of ochratoxin A could be found that would produce a renal dysfunction. When doses large enough to produce effects on urine flow, etc. were used, the animals invariably became overtly ill and many died. Only after the administration of small doses on a daily basis was it possible to see an alteration in renal function that was not accompanied by other, gross toxicities. In those animals, after several days of treatment, the urine volume

increased and its osmolality fell. In addition, urinary excretion of glucose and protein increased. This response was complicated by the fact that the animals treated with ochratoxin A lost large amounts of body weight, although appropriate control experiments indicated that the loss in body weight was not responsible for the alterations in renal function.

As with citrinin, ochratoxin A altered specific renal transport processes. The animals which had been pretreated with low doses of ochratoxin A over several days showed a reduction in the renal transport of organic anions and organic cations similar to the effects seen with citrinin. The addition of ochratoxin A to fresh renal cortex slices also resulted in inhibition of organic ion transport. These data suggest a direct effect of this nephrotoxin on renal tubular events, perhaps similar to those produced by citrinin.

Pharmacokinetic studies with ochratoxin A comparable to those with citrinin have not been performed. However, the toxicological data would suggest that this toxin has a cumulative toxicity which is not seen with citrinin. Such an effect presents an even greater challenge in terms of the potential human toxicity since small doses of ochratoxin A consumed on a daily basis might result in renal dysfunction at some later time. Perhaps such an observation would be consistent with the role of ochratoxin A in the production of porcine nephropathy or the similar human disease, endemic Balkan nephropathy.

Few, if any, studies have been undertaken to examine the mechanisms by which ochratoxin A might produce its effects on the kidney. Creppy et al.[89] demonstrated ochratoxin A to be a competitive inhibitor with phenylalanine in the phenylalanyl-tRNA synthetase catalysed reaction in various cells. Effects on hepatomous cells in culture paralleled inhibition of protein synthesis. Whether or not these effects might be translated into alterations in renal function is undecided. Further, little information is available concerning the possible role of xenobiotic metabolism in the ochratoxin effects.

C. Fungal toxins and endemic Balkan nephropathy

No attempt will be made here to define all of the pathological or pathophysiological parameters that define endemic Balkan nephropathy. All of this material has been presented by Hall[64] and by Hall and his colleagues in various earlier publications.

The effects of ochratoxin A in many experimental animals are very similar to those reported in the human. There is little doubt that ochratoxin A has a role in porcine nephropathy, and since the outcome in humans is similar it is a likely candidate for a causative agent in endemic Balkan nephropathy. Ochratoxin A has been identified in foodstuffs from some of the villages where the disease has occurred[90]. In addition, ochratoxin A has been isolated from the serum of some patients with endemic Balkan nephropathy[91]. Studies in humans would suggest that the primary lesion of endemic Balkan nephropathy is in the proximal tubule[92]. Further, virtually all of the actions of ochratoxin A in the experimental

situation are exerted on the proximal tubule[75,88,93]. This observation includes not only transport functions and reabsorptive functions, but also metabolic functions. Taken together, these data would suggest that ochratoxin A is a prime candidate as an aetiological factor in the production of endemic Balkan nephropathy.

However, since ochratoxin A and citrinin are known to occur together in many situations it may be important to examine further the possible role of citrinin, along with ochratoxin A, as an aetiological agent. Furthermore, the possibility of an interaction between the two fungal toxins cannot be overlooked. Such an interaction might result in potentiation of the action of one or the other toxin.

OTHER MYCOTOXINS

The Sassoon Hospital syndrome[94] has been suggested to be related to a fungal toxin. The disease entity and the possible causative agent are less well defined than for endemic Balkan nephropathy. Nonetheless, reports indicate that the Sassoon Hospital syndrome may be due to the consumption of mould-contaminated foodstuffs. Unfortunately, little or no experimental work to address this problem has been undertaken.

Aflatoxin B_1 has been reported to produce renal damage in dogs[95,96], pigs[96-99], rats[100-102] and horses[103]. Overall, the renal damage produced is somewhat less than with other toxins, but a 50% reduction in GFR was reported in the rat[100]. Further, the hepatic effects of aflatoxin are so much more dramatic that the renal effects appear less important.

Rubratoxin B also has effects in the dog[99], BUN was elevated and morphological evidence suggested renal tubular damage. The nature of the renal damage was not well defined, but cortical necrosis seemed to be prominent.

Although relatively few mycotoxins have been identified as primary nephrotoxins, that may simply reflect the extent to which this phenomenon has been studied. Furthermore, the possible interaction of two nephrotoxins, to produce a greater effect on the kidney than observed with either one alone, is a distinct possibility, and a preliminary suggestion to this effect has been reported by Hayes and Williams[99] for rubratoxin B and aflatoxin B_1. Mechanisms which might underlie this sort of interactive effect were not defined.

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24
RADIOGRAPHIC CONTRAST MEDIA

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INTRODUCTION

Contrast media are used in diagnostic radiology to enhance the X-ray attenuation between a body structure of interest and the surrounding tissue. A detail becomes perceptible on a roentgenogram only when its contrast exceeds a minimum value in relation to the background. Small areas of interest must have higher contrast than the background[1]. The contrast effect depends on concentration of the contrast media within the body. A high contrast media concentration difference thus gives rise to more morphological details in the radiographs (Figure 1).

Contrast media can be divided into negative contrast media such as air and gas which attenuate X-rays less than the body tissues, and positive contrast materials which attenuate X-rays more than the body tissues. The positive contrast media all contain either iodine (atomic number 53) or barium (atomic number 56) and can be divided into water-insoluble and water-soluble contrast media.

For gastrointestinal investigations barium sulphate ($BaSO_4$) is the most commonly used contrast medium. This is a suspension of barium sulphate particles in water. Many different additives, such as carboxymethylcellulose, gelatin, polystyrene, etc., have been used in barium sulphate contrast media[2]. These additives are used: to improve patient acceptance; to improve suspension properties, such as viscosity and coating ability; to reduce flocculation, mixing and bubble formation; and as preservatives. The barium sulphate used in commercial suspensions has a particle size in the range 0.1-3.0 μm. There is therefore no absorption of barium sulphate from the gastrointestinal tract into blood and thus the barium sulphate never reaches the kidneys. Toxic effects of barium sulphate contrast media on the kidneys has not been reported.

Water-soluble contrast media are all iodine-containing substances, and are based on the triiodinated benzene ring structure (Figure 2A-D). They are excreted either mainly through the kidneys (urographic agents) or through the liver via bile secretion

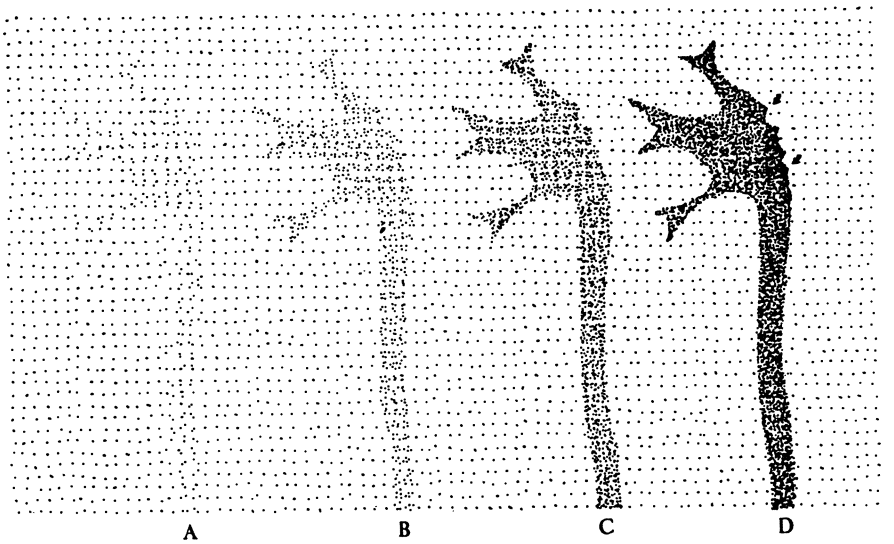


Figure 1 The importance of the contrast medium concentration in the renal pelvis and ureter. In (A), (B), (C) and (D) the number of black points is respectively 2, 4, 8 and 16 times higher in the renal pelvis than in the background. In (A) the size of the ureter cannot be estimated. In (D) the highest contrast medium concentration, an irregular surface (a tumour?) in the renal pelvis can be discovered.

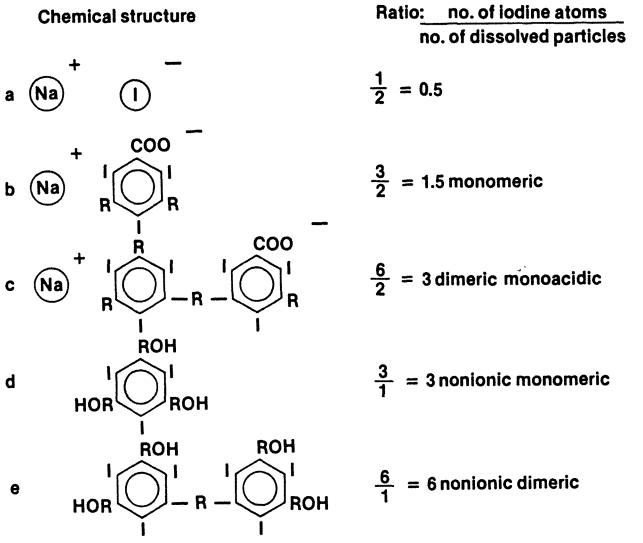


Figure 2A Basic structure of present (I-V) and future contrast media. Exemplification of different ratio contrast media in Figures 2B-2C.

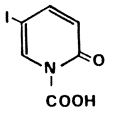
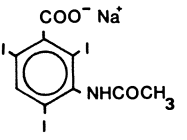
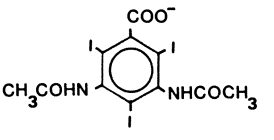


Figure 2B Uroselectan. A ratio 0.5 medium.

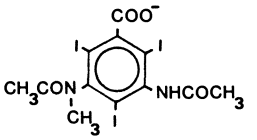


Acetrizoate

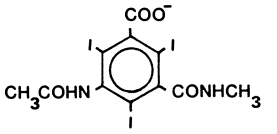
ratio 1,5



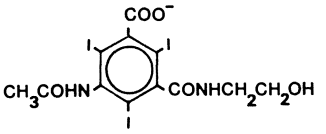
Diatrizoate



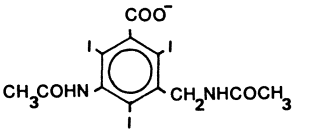
Metrizoate



Iothalamate

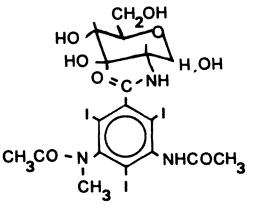


Ioxithalamate

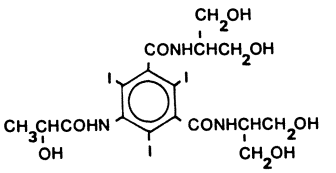


Iodamide

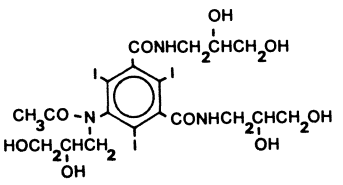
Figure 2C Ratio 1.5 urographic contrast media currently used contrast media.



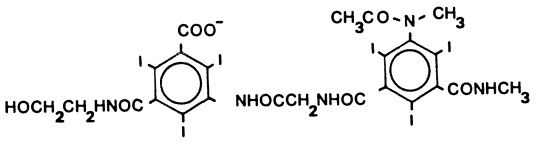
Metrizamide



Iopamidol



Iohexol



Ioxaglate

Figure 2D Ratio 3.0 urographic contrast media developed for future use.

(cholegraphic agents). The character of the benzene side chains of the molecule determines the pharmacokinetic and pharmacodynamic behaviour of the contrast drugs. Continuous research since the introduction of iodine into radiological practice in 1918[3] has been directed towards two main targets: an increase in iodine concentration within the body (increased X-ray attenuation) and a reduction of the toxicity.

The water-soluble contrast media excreted mainly by the kidneys are either used for radiological diagnosis of the vascular system (angiography) or for diagnosis of the kidneys(urography).

Urography was developed by Osborne et al.[4], who used sodium iodine for obtaining roentgenograms of the kidneys. Urography as known today was introduced into clinical practice in the 1930s by Swick et al.[5] and Binz[6]. The contrast medium used at that time was the sodium salt of 5-iodo-2-pyridone-N-acetic acid with the commercial name Uroselectan (Figure 2B). It contained one iodine atom per molecule and dissociated in solution into one cation and one anion, the ratio between the number of iodine atoms and number of particles in solution being 0.5 (Figure 2A). The contrast media used in the 1940s had a ratio of 1.0. In 1950 acetrizoate was introduced, and it was the first contrast medium to have a ratio of 1.5 between the number of iodine atoms and particles in solution (Figure 2C). Acetrizoate (Urokon^{R}) caused many adverse reactions during its clinical use and facilitated the development of the present ionic contrast media, diatrizoate, metrizoate, iothalamate, iodamide, ioxithalamate, with a ratio of 1.5 (Figure 2C). These media are all in common clinical use today. They are marketed either as pure methylglucamine salts or as mixtures of sodium salts and methylglucamine salts (Table 1). The sodium salts in general have insufficient water solubility to form highly concentrated solutions. Methylglucamine salts have a higher solubility, but also have the disadvantage of a higher viscosity than the sodium salts. Viscosity is an important parameter when rapid injection is wanted during angiography.

The ionic contrast media all have an osmolality of more than 1500 mOsm/kg water at iodine concentrations exceeding 280 mg I/ml. All the clinically used formulations are highly hypertonic in relation to human serum. It is the hypertonicity which is responsible for many of the physiological-toxicological effects noted after contrast medium administration. Immediately after injection into the vascular system the hypertonicity of the ionic contrast media results in withdrawal of water from blood cells and surrounding tissues (Figure 3), damage to the vascular endothelium[7-9] and blood-brain barrier[10,11]. It also causes the pains associated with angiography[12,13]. Secondary to the increase in plasma water and changes in membranes, increases in pulmonary arterial pressure[14], hypervolaemia[15], and vasodilation[16,17] have been reported.

As a consequence of these adverse reactions related to the high osmolality of the contrast media, research has focused on the development of new media with lower hypertonicity.

Metrizamide was the first non-ionic contrast medium introduced. It has a ratio of 3 (Figure 2D) for the number of iodine atoms per particle in solution, and a considerably reduced osmolality, of 430 mOsm/kg H_2O at an iodine concentration of 280 mg/ml.

Other non-ionic contrast media have been developed and two of these (iopamidol and iohexol) have been in clinical use since 1977 and 1979 respectively. Ioxaglate (Figure 2D) represents another approach to reducing the osmolality by forming an ionic dimer with six iodine atoms per two particles in solution, giving a ratio of 3. The osmolality of all these media is approximately the same, about 500 mOsm/kg H_2O at 280 mg I/ml, and all show a general reduction in osmotic side-effects.

Table 1 Product names of the presently used urographic contrast media

Non-proprietary name	Product name	Ratio
Diatrizoate - Na and/or meglumine	Renografin, Renovist, Hypaque, Urografin, Angiografin, Urovision, Pielografin, Urotrast, Radioselectan, Triombrin, Visotrast	
Iothalamate - Na and/or meglumine	Conray, Angio-Conray, Meglumine, Contrix, Angio-Contrix, Cardio-Conray	1.5
Metrizoate - Na and/or meglumine	Isopaque, Ronpacon, Triosil	
Iodamide - Na and/or meglumine	Uromiro, Uromiron, Iodamide, Iodoradiopaque, Iodomiron	
Ioxithalamate - Na and/or meglumine	Telebrix	
Metrizamide	Amipaque	
Iopamidol	Iopamiro, Solutrast	
Iohexol	Omnipaque	3.0
Ioxaglate - Na and meglumine	Hexabrix	

Recently developed non-ionic dimers, with a ratio of 6, are essentially isotonic at 280 mg I/ml. These non-ionic dimers fit the ideal osmolality requirements for the radiologist, by offering clinically useful contrast at concentrations which are isotonic to blood. However, the low osmolality of the experimental non-ionic dimers causes a very low osmotic diuresis, and as a result these dimers attain a very high concentration in the lumen of the renal tubules. It is therefore of importance to examine carefully the nephrotoxicity of these dimers. Contrast medium-related renal failure in patients has been reviewed by Mudge and co-workers[18,19], Harkonen and Kjellstrand[20], and Berkseth and Kjellstrand[21], but

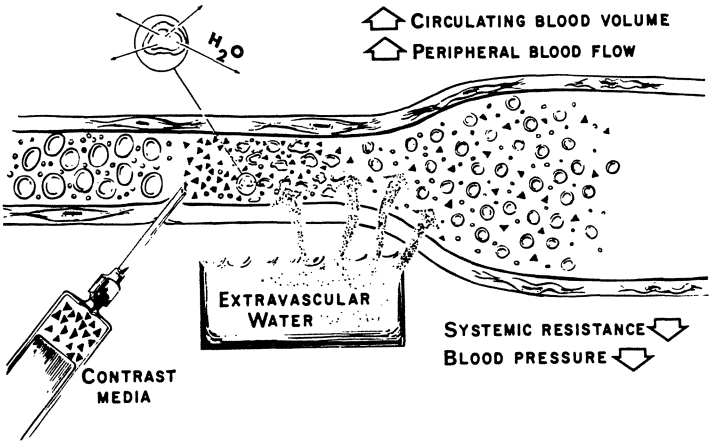


Figure 3 The immediate effects of contrast medium injections.

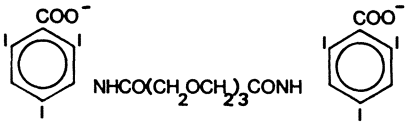
the new low-osmolality contrast media (non-ionic monomers and dimers) have not been considered in these reports, which summarized clinical experience with the ionic ratio 1.5 media.

The general conclusion of these reviews was that the pathogenesis of contrast medium-induced nephropathy is uncertain. Many different mechanisms have been suggested (Table 3) and some of these will be discussed in this chapter.

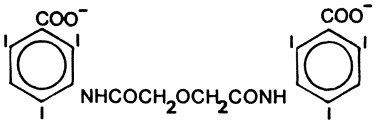
NEPHROTOXICITY

Biliary contrast media: clinical evidence

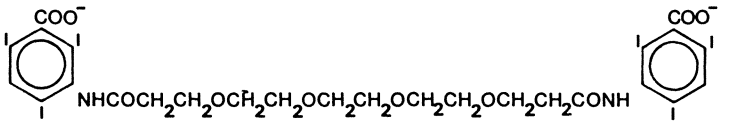
The biliary contrast agents are the intravenous media iotroxamate, ioglycamate, iodoxamate, and the oral media iopanoic acid and iocetamic acid (Figure 4A, 4B). They are either ratio 1.5 or 2.0 contrast media. They are more lipophilic than the urographic agents and, as one might expect, the toxicity of these agents is higher than that of the urographic contrast media. Despite the relatively low clinical doses of intravenous biliary contrast media (100 mg I/kg), severe complications are expected at a frequency of 1 in 450 and a mortality of 1 in 6500. The corresponding figures for urography are 1 in 20,000 to 1 in 40,000[22]. The excretion of all the biliary contrast media takes place mainly via active transport mechanisms in the liver. Renal excretion, and consequently renal toxicity problems, may occur when conditions are present which favour renal excretion - i.e. liver diseases or high blood concentrations of biliary contrast media. The use of biliary contrast media has been reduced during the past decade due to other new diagnostic modalities such as ultrasound and computerized tomography (CT). The number of reported cases of renal failure after exposure to these agents is therefore rather limited[19], and does not allow any general description of the mechanisms involved.



Iotroxamate
(Biliscopin)

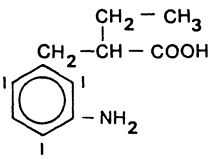


Ioglycamate
(Bilivistan)

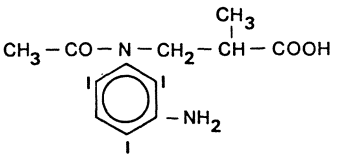


Iodoxamate
(Cholevue)

Figure 4A Intravenous cholangiographic media.



Iopanoic acid
(Telepaque)



Iocetamic acid
(Cholebrin)

Figure 4B Oral cholecystographic media.

Urographic contrast media: clinical evidence

Urographic contrast media are widely used, and more than 10 million urographies were performed in 1986. The frequency of reported contrast media-induced renal failure varies from about zero up to 90% of the patients included in an individual report. There is an extreme variation in incidence between different reports. This is partly related to the design of the investigations,

i.e. inclusion and exclusion criteria, prospective or retrospective study, diagnostic criteria for contrast media-induced acute renal failure, time of renal function assessment after the contrast media dosage.

The total number of reported contrast medium-induced acute renal failures in 1983 was 101[23-27]. This number, and the numbers given from the previous years (Figure 5), only reflect a minor proportion of the actual number of patients having their kidney function affected by the contrast medium. One reason for this difficulty is the problem of separating the effect of the contrast medium from effects of the disease process itself. Also, renal function tests applied in general clinical practice (serum urea and creatinine) are slow-reacting indicators of nephrotoxicity.

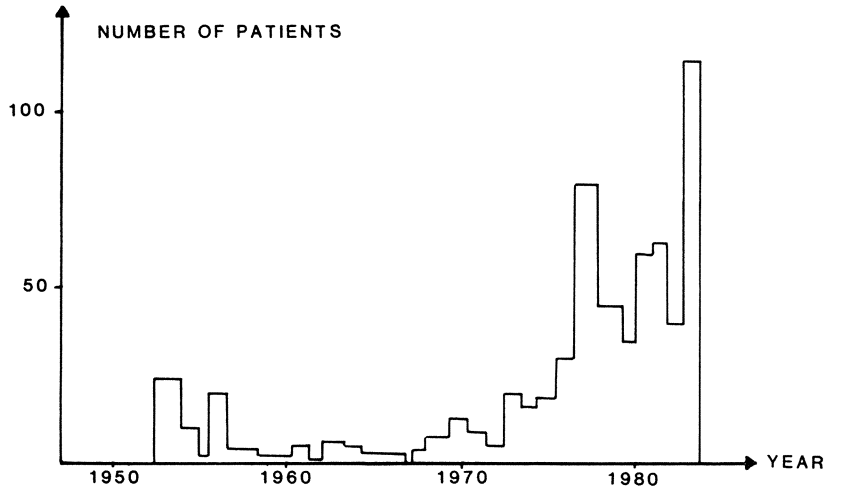


Figure 5 Number of yearly reported cases with contrast medium-induced renal failure. Data in the period to 1980 from Mudge et al.[19].

Recognition of how common acute renal failure is after administration of contrast media and other pharmaceuticals is related to diagnozing the acute renal failure. Diagnosis of transient renal failure is easily missed because many patients have no oliguria or other symptoms indicating the likelihood of acute renal failure; the only sign of acute renal failure may be a transient increase in creatinine or urea in serum.

Contrast media-induced renal failure is characterized by a reduction in the glomerular filtration rate (GFR) and may be accompanied by oliguria or anuria. In most cases the renal impairment is transient, with a peak serum creatinine concentration within the third or fourth day followed by a slow return to precontrast clearance levels 7-12 days after injection[28,29]. Severe cases of contrast media-induced acute renal failure or exacerbated chronic renal failure leading to chronic haemodialysis and death have been reported[30,31]. The most severe cases occurred generally in high-risk patients. A definition of a high-risk patient has been

Table 2 Risk factors reported to be connected with contrast medium-induced acute renal failure[32]

Diabetes mellitus[8,11,14,17]
Pre-existing renal insufficiency[8,68,69]
Advanced age[8,15]
Multiple myeloma[52,53]
Transplanted kidneys[32,54]
Dehydration[54-56]
Hypertension[8,51]
Dosage of contrast medium[8,50,57]
Short interval between contrast medium procedures[15,58]

suggested by Törnquist[32] (Table 2), who summarized the factors which contribute to contrast media-related acute renal failure. The main factors are:

(a) Diabetes mellitus

Studies involving diabetic patients have indicated that the risk of contrast medium nephropathy is directly correlated to the degree of renal impairment in connection with diabetes. This means that diabetic patients with normal functioning kidneys (serum creatinine <130 μmol/l), are not expected to be at higher risk for renal failure[33-35] than a normal patient group. Diabetic patients with moderate renal impairment (serum creatinine 130-400 μmol/l) appear to be at significantly greater risk than those with normal renal function or with similar levels of renal insufficiency from causes other than diabetes.

Harkonen and Kjellstrand[36] found that up to 90% of patients with juvenile-onset diabetes mellitus may experience a further deterioration of renal function after the contrast media administration. They also found that more than half of the patients injected with contrast media needed permanent haemodialysis. Patients with diabetes and renal failure are therefore at higher risk than patients with the same degree of renal failure without diabetes.

(b) Advanced age (over 60 years)

This may be related to the fact that there is a progressive loss of renal mass, renal blood flow and a decrease in perfusion per gram of renal tissue with increasing diseases[37] (diabetes, renal insufficiency, coronary heart disease, hypertension). The importance of the age factor alone is difficult to assess. Slasky and Lenkey[30] proposed that elderly patients should be considered in the same category as borderline renal insufficiency patients because of their expected 30% decrease in GFR.

(c) Reduced kidney function

The importance of pre-existing renal insufficiency as a predisposing factor are shown by the results of Shafi et al.[38], who in a group of 40 well-hydrated chronic renal failure patients observed a contrast medium-induced nephrotoxic response in 70% of the patients who had a 25% increase in serum creatinine or a decrease in creatinine clearance after the contrast medium examination. Van Zee et al.[29] reported that the incidence of renal damage after urography was directly related to the precontrast creatinine in serum. Pre-existing renal insufficiency should therefore be treated as one of the most significant risk factors for the development of contrast nephropathy.

In addition to the preinjection condition of the patient the choice of contrast medium will affect the degree of damage to the kidney. It has been found[39] that the new ratio 3 contrast medium iohexol did not affect the serum creatinine in 34 diabetic patients, while a similar group of patients injected with the ionic ratio 1.5 medium metrizoate showed an increased serum creatinine level the day after the urographic examination.

Differences in renal effects between non-ionic ratio 3 media and ionic ratio 1.5 media have also been found in animal studies. So far, however, it has not been possible to completely clarify the basic pathways leading to the measured differences in renal toxicity.

POSSIBLE MECHANISM OF CONTRAST MEDIA NEPHROPATHY

Urographic contrast media are highly hydrophilic compounds with insignificant protein binding[40-42] which distribute throughout the extracellular space with a distribution volume of 0.20-0.25 l/kg body weight. They are mainly excreted by glomerular filtration[43-45] and 24 hours after an i.v. injec tion more than 90% of the contrast media is found in the urine. Contrast media may affect kidney function by a number of different mechanisms (Figure 6, Table 3). About 25% of the cardiac output is directed towards the kidneys. Consequently the pathophysiology of the acute renal failure has been thought to be initiated by a decrease in renal blood flow resulting in renal cell ischaemia. Contrary to other vascular beds, the renal vasculature responds to intra-arterial contrast medium exposure by both vasodilation and vasoconstriction. Contrast media cause an initial increase followed by a prolonged reduction in blood flow after renal arteriography (Figure 7). These changes are less pronounced with the low osmolar ratio 3 contrast media than with the ionic ratio 1.5 media[46]. Hypertonic solutions of control substances (i.e. saline, dextrose, urea) elicit haemodynamic responses qualitatively similar to contrast media, whereas isotonic solutions do not produce significant haemodynamic changes. It is therefore reasonable to assume that the hyperosmolality of the contrast media is of importance for haemodynamic response.

The renin-angiotensin system and the prostaglandins were naturally thought[47-49] to be involved in the generation of the renal vasoconstriction. Workman et al.[50] measured aortic and renal

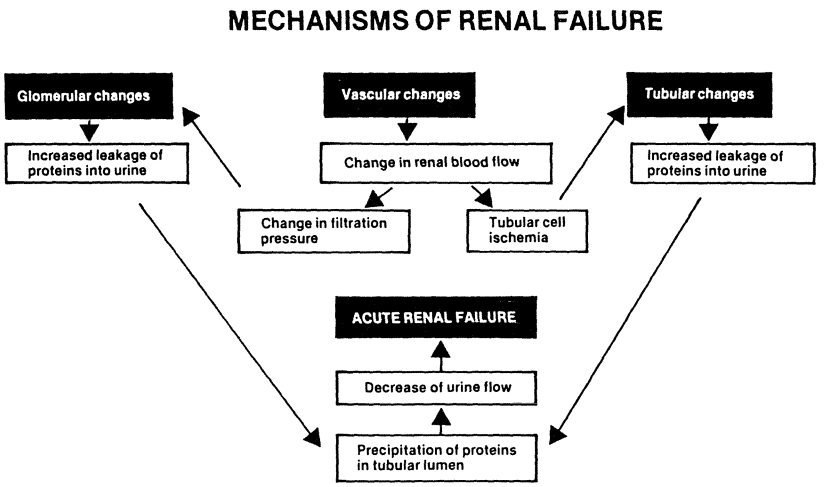


Figure 6 Suggested pathways for contrast medium-induced renal failure.

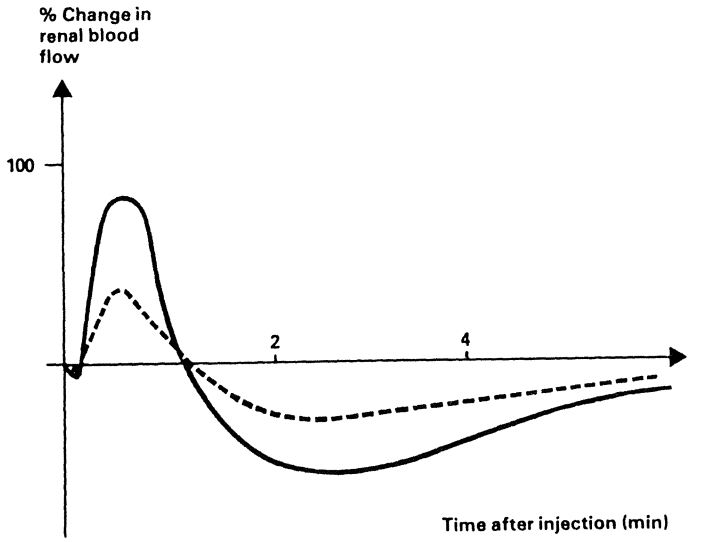


Figure 7 Change in renal blood flow during nephroangiography with the equivalent dose of: ——— ratio 1.5 contrast medium and – – – ratio 3.0 contrast medium.

venous levels of angiotensin II, renin, 6-keto-prostaglandin-$F_{1\alpha}$, the stable metabolite of prostacyclin, after intra-aortic injection of 50 ml (2.5-3.3 ml/kg) of meglumine iothalamate in dogs. Besides the biphasic change in renal blood flow they observed a parallel biphasic increase and decrease in creatinine clearance, but no changes from the baseline of normal angiotensin and renin concentrations. The direct involvement of the renin-angiotensin system was therefore questioned. However, a decrease in 6-keto-prostaglandin-$F_{1\alpha}$ was observed, and this may be related to a rela-

tive deficiency of prostacyclin which normally opposes the action of the renin-angiotensin system. Contrast media injections also affect the release of prostaglandins from the lung[51] and although further studies are required it is likely that prostaglandin is involved in the haemodynamic response of the kidney to contrast media.

Table 3 Various mechanisms that may be involved in the pathogenesis of contrast medium-induced acute renal failure. A number of observed effects of contrast media that are compatible with the hypothetical mechanisms are given

Vascular Changes
reduction in renal blood flow[8,48]
increased blood viscosity[28]
erythrocyte alterations[28]
endothelial damage[78]
platelet aggregation[79-81]
thrombus formation[63,64]
increased intratubular pressure with capillary compression[27,82]
shift in oxyhaemoglobin dissociation curve[77]
Tubular Changes
gel formation of Tamm-Horsfall mucoproteins[86,87]
increased excretion of uric acid[88]
increased excretion of oxalate[89]
increased intratubular pressure[27,82]
enzymuria[26,90]
acute tubular necrosis[15,69]
osmotic nephrosis[91]
increased mitotic rate[92]
reduced PAH clearance[93]
depression of sodium transport[94]
Glomerular Changes
albuminuria (glomerular leakage)[48]
reduced glomerular filtration rate[82-85]
proliferative glomerulonephritis[95]
antibody formation[96]

Immediately after intra-arterial or intravenous injection of both ionic and non-ionic contrast media, the size of the kidney changes[52]. The maximum increase in size is reached within the first 5 minutes after injection. Depending on dose, the kidneys may remain enlarged for hours. The size increase is related mainly to the osmotic diuresis. The increased intrarenal pressure which follows the volume increase within the relatively non-elastic kidney capsule may thus be another cause to the decrease in renal blood flow. The non-ionic contrast media do not cause as much osmotic diuresis as the ratio 1.5 media[53], and the changes of the kidney size are less; consequently a smaller effect on renal blood flow has

been observed. This led to the suggestion that the increased intrarenal pressure was the common pathway in contrast media-induced renal blood flow alterations and the acute renal failure[32,54].

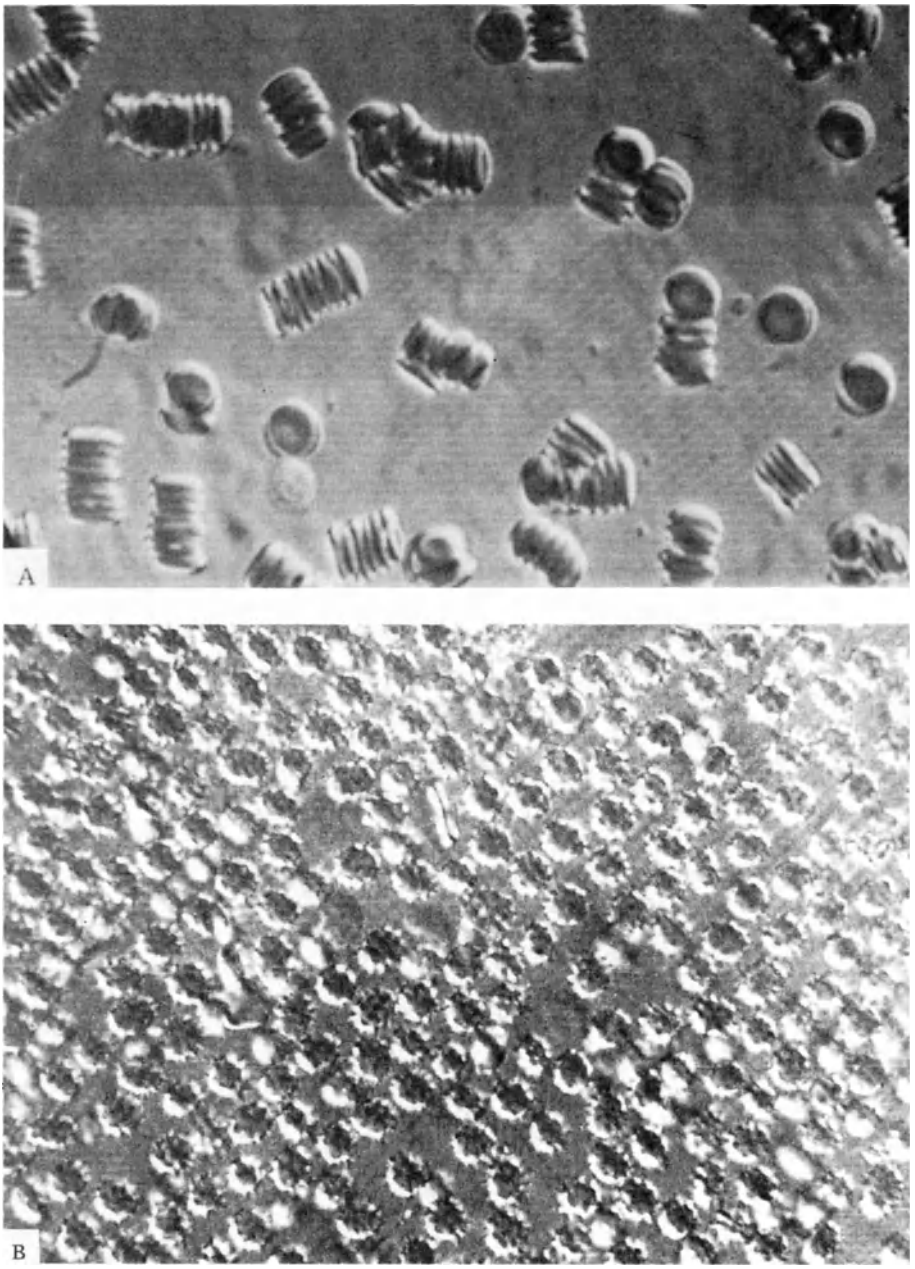


Figure 8A Normal red blood cells. **Figure 8B** Echinocyte deformation of red blood cells after exposure to contrast media.

Blood flow alterations could, however, also be related to the effects of contrast media on erythrocytes. Aspelin et al.[55] studied the in vitro effects of contrast media on red blood cell morphology, aggregation and deformability. All contrast media, to varying degrees, induced rigidification of red blood cells. Aspelin[55] observed two separate effects on red cells: osmotic shrinkage (desiccocytes) which was mainly due to the hyperosmolality of the contrast media (Figure 8) and formation of echinocytes (crenated cells) in isotonic solution due to chemical toxicity. Only the desiccocytes showed a significantly reduced deformability. Therefore the osmotic effects were considered to have more clinical relevance. A reduction of flow at the capillary sites of the kidneys must be expected due to the reduced deformability of erythrocytes when they are passing the narrow capillaries. The concentration of contrast medium in the renal arteries influences the dynamic behaviour of the kidneys. Therefore, as mentioned above, there may be different mechanisms leading to renal failure when comparing intravenous urography and selective renal arteriography. During selective renal angiography extremely high concentrations of contrast medium are obtained in the vascular bed of the kidney. It is therefore not surprising that during renal arteriography an irregular, non-homogeneous nephrogram (Figure 9) has been observed. This has been seen in both clinical and experimental studies[56,57] using the ionic ratio 1.5 contrast media like diatrizoate and metrizoate. Törnquist[32] measured a marked reduction of renal blood flow in dog kidneys during the renal angiography and simultaneously noted a "patchy contrast medium retention". It could be shown that the patchy contrast medium retention in the renal parenchyma correlated with injection of large single doses of the contrast medium, but not to the total dose when injected as several smaller doses. Large single doses expose the endothelium for a longer time to the contrast medium leading to surface changes which provoke platelet accumulation. Microscopically demonstrable transient platelet aggregates and thrombi in glomerular capillary tufts and in afferent arterioles have been reported[58]. The reduction in renal blood flow could be caused not only by obstruction of the vessel lumen by the platelets, but also by release of vasoactive substances such as serotonin from the platelets. Endothelial damage may be another factor in the pathogenesis of contrast medium nephropathy. This factor may be of special importance in diabetic patients who already have advanced degenerative changes of the microvascular wall[70,75,76]. Non-ionic and low-osmolality contrast media cause less endothelial damage in experimental models[7,8] than high-osmolality ionic contrast media. They produce a smaller effect on blood cells, less osmotic diuresis and influence renal blood flow less. Their nephrotoxic potential, at least on the vascular side of the kidney, can therefore be considered lower than those of conventional ionic media (ratio 1.5 media).

In some special selected cases factors other than contrast agents can influence the renal blood flow during the radiological procedure. Practically all commercial contrast media vials, rinsing solutions, and angiographic equipment are contaminated with foreign bodies[59] (Figure 10). Due to the large volumes administered during radiological procedures such foreign body contamination

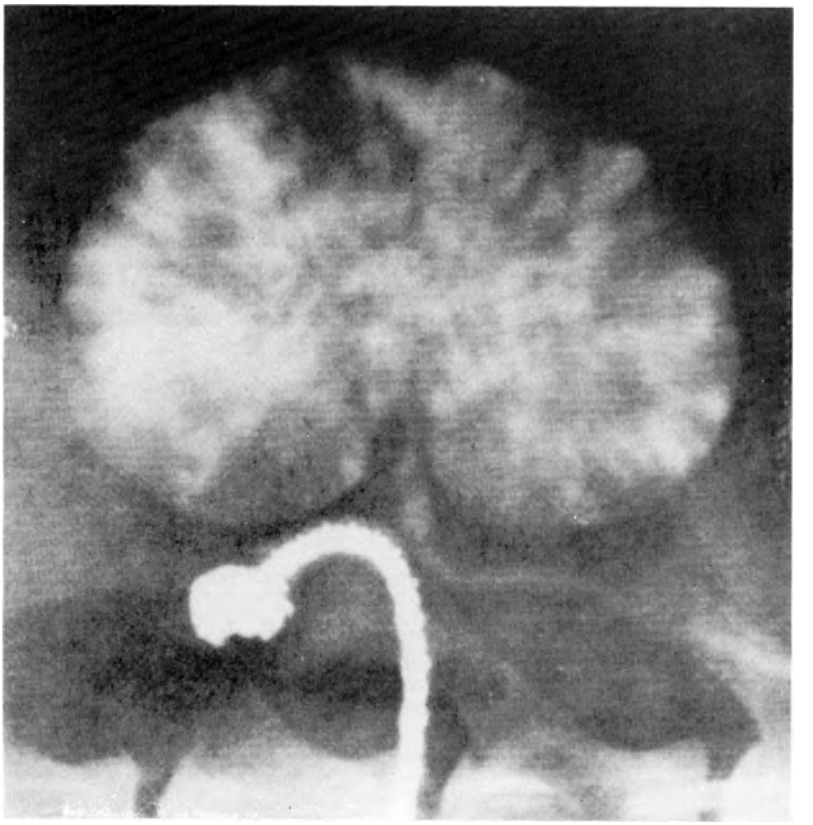


Figure 9 "Patchy contrast medium retention" within the kidney after nephroangiography in a dog.

might be of greater significance to the vascular bed, and for contrast media toxicology than other pharmaceuticals administered[60].

GLOMERULAR CHANGES

The occurrence of abnormal amounts of proteins in urine and their molecular weight distribution is used as an indicator of renal disease. Nephroangiography and urography increase glomerular permeability, thus causing leakage of proteins into the urine[61]. Albuminuria can be so severe that the urinary concentration of albumin increases above 100 g/g creatinine. After selective renal arteriography with the ionic ratio 1.5 media in humans, values as high as 330 g albumin/g creatinine have been found[62]. This suggests that intratubular obstruction due to precipitation of excessive amounts of proteins could be one possible cause of acute renal failure.

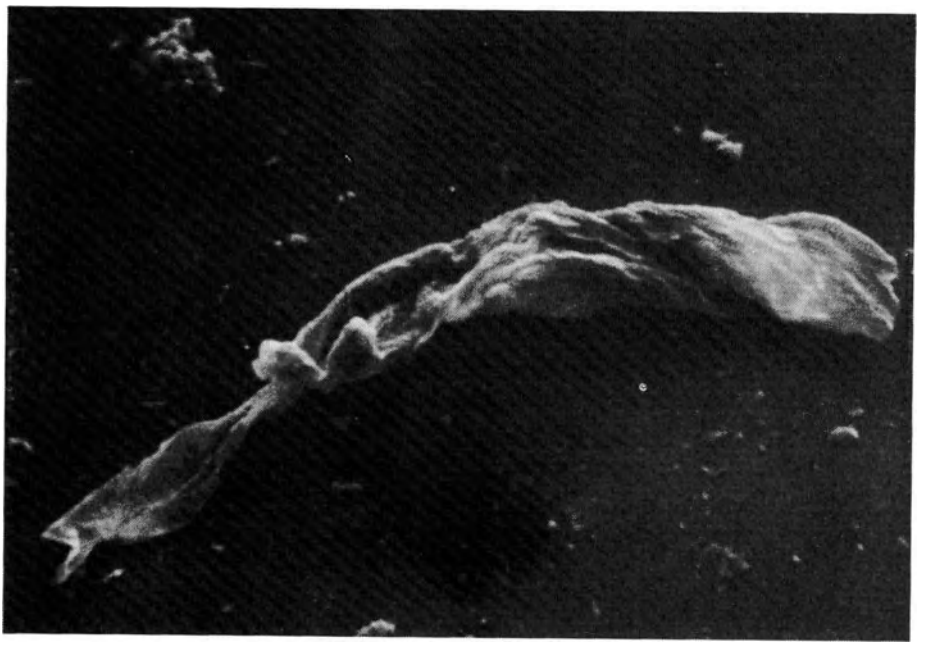


Figure 10 Scanning EM (x1000) of a foreign body contamination found in a urographic contrast medium solution. The particle consists of organic material. Longest dimension approximately 105 μm. Courtesy of O. Winding.

Several studies[63] have been performed in order to clarify what happens to the glomerular membranes. Use of light microscopy did not reveal any morphological changes, and even electron microscopy was unable to show changes in the glomerular barrier from dogs showing a severe proteinuria. Present theories point to the possibility that the contrast media induce changes in electric charge of the barrier. The osmolality of contrast medium seems not to be of importance for the quantitative leakage of albumin. The non-ionic ratio 3 contrast agent metrizamide caused the same degree of proteinuria as diatrizoate and metrizoate in dogs[64], rats[65] and humans[66]. The non-ionic compounds iohexol, iopamidol, iopromide and iopentol, and the monoacidic dimer ioxaglate, caused far less albuminuria than the above mentioned media[66,67]. The maximum postangiographic proteinuria is always observed in the first minutes after exposure, and generally returns to normal after a period of 2-24 hours (see also Table 3).

The renal failure found after urography is probably not induced by the proteinuria as intravenous injection of contrast media would not be expected to expose the glomerular endothelium to high concentrations of contrast media. A dose twice that used in selective nephroangiography given intravenously was found to give an albuminuria of 1/300 of that after selective artery injection.

This supports the notion that different causative mechanisms of renal failure may exist following urography or nephroangiography.

TUBULAR CHANGES

Injection of radiographic contrast media is known to cause a vacuolization of the proximal tubules in the renal cortex. The lesion is characterized by the appearance of small vacuoles along the tubular basal membrane, which may grow and eventually fill the cytoplasm. The nuclei and the basal membrane of the cells are not affected, and necrosis was not reported in studies using clinically relevant doses. The degree of vacuolization may vary depending on the species, the time course of development of the lesion, and the health status of the kidney. Further, the histopathological appearance may be further influenced by different types of fixation[97].

A dose-response relationship has not been clearly established. Moreau et al.[97] reported vacuolization in human biopsies taken at different time intervals after urography or renal arteriography. The extent of vacuolization was the same after 0.5 g I/kg or 0.8 g I/kg. The lesion, called osmotic nephrosis, can be observed after injection of iodine equivalent doses of highly hypertonic (ionic) ratio 1.5 contrast media, slightly hypertonic (non-ionic or monoacidic dimeric) ratio 3.0 contrast media and isotonic (non-ionic dimeric) ratio 6 contrast media. Hypertonic solutions of sugar and mannitol, and certain types of dextran blood substitutes, cause a similar lesion. The osmolality thus only plays a partial role, whereas the chemotoxicity related to the physicochemical properties of the contrast media may be of greater importance. The pathogenesis of the lesion is unclear, and its relation to functional alterations of the kidney has not been established. In experimental studies of tubular vacuolization there are, in general, no clinical chemically measurable signs of reduced kidney function such as an increase in serum urea or creatinine. From human studies[97,98] a direct relationship between tubular vacuolization and development of renal failure could not be drawn.

Vacuolizations of renal cells are signs of acute injury caused by contrast media. The appearance of enzymes in the urine, normally found intracellularly or attached to the epithelial layers of the nephron, is another sign, and can be used to depict the lesion and its location within the nephron. Rabbits given large doses of iothalamate or metrizamide showed increased excretion of enzymes normally localized in tubular cells and on the brush border of cells of the proximal tubules[99]. After renal arteriography the urinary excretion of the enzymes lactate dehydrogenase, glutamic oxalacetic transaminase, catalase[90] and glutathione[100] were increased. The occurrence of enzymes in the urine was time dependent, with peaks during the first hour after dosing the contrast medium. Both vacuolization and enzymuria may represent the transient damage to tubular cells which occurs after contrast agent exposure.

Although there have been only a few reports on contrast media-related tubular distribution and precipitation of Tamm-Horsfall glycoprotein (THG)[86,87] its relevance for contrast media-

induced renal failure has attracted considerable attention[18,71,72]. THG is a glycoprotein, normally present in, and secreted by, the ascending thick limbs of Henle's loop and the distal convoluted tubules[101,102]. It has a molecular weight of about 75,000 and can aggregate to form higher molecular weight molecules. THG is the major constituent of PAS-positive hyaline casts. Dawnay et al.[103] reported the effects of sodium iothalamate on THG aggregation and excretion in 19 patients before and after a routine urography. They used a specific THG radioimmunoassay and concluded that intratubular precipitation of THG is unlikely to play any role in the pathogenesis of renal failure after urography. This was based on the observation that the urinary concentration of THG was not increased. Further the urinary osmolality decreases due to the diuretic effect of the contrast medium, a factor which should favour the solubility of THG. It has also been impossible to show precipitation of any contrast medium-THG complexes with either ionic or non-ionic urographic contrast media in vitro[104], and in an experimental study in rodents using non-ionic contrast agents no evidence was found for intratubular obstruction due to THG. One may assume that the THG is involved in the contrast medium-induced renal failure in a few selected cases, but it is far from being the general pathogenic pathway of the contrast medium-induced renal failure.

SUMMARY ON VASCULAR, GLOMERULAR AND TUBULAR CHANGES

Injection of all types of contrast media can affect the vascular bed, the glomerular membrane and the tubular cells. A general pathogenic pathway has not been established; most likely because there is no general pathway, but a contribution from several damaging factors which lead to the contrast medium-induced reduction in renal function.

PREVENTION AND DETECTION OF CONTRAST MEDIUM-MEDIATED RENAL FAILURE

To draw a concise relationship between administration of contrast medium and the possible development of acute renal failure is not possible. The primary effects of contrast medium toxicity which could lead to renal failure may be different in individual patients. This makes the detection of early general renal deterioration an important factor in the prevention of acute renal failure.

There are a number of methods for the determination of GFR using the endogenous marker creatinine or the clearance of radiolabelled GFR markers such as inulin, Cr-EDTA or Tc-DTPA. However, the contrast medium itself may be used to detect the glomerular filtration capacity of the kidney by measuring the blood iodine concentration after a radiological investigation[105]. The blood disappearance curve of contrast media gives information about the early changes in terms of hours after the administration of the contrast medium, whereas the serum creatinine or urea concentrations are indicators of the metabolic accumulation due to kidney

dysfunction in terms of days after contrast medium injection. Using the "single injection technique" for the determination of contrast medium clearance it is possible to find acute renal failure at an early stage, and possibly avoid those severe complications that develop later.

During reconstructive surgery of obstructions or aneurysms of aorta and iliac arteries, the renal arteries may be occluded for periods that do not, per se, seriously damage renal function. However, these operations are preceded by angiography; they are, furthermore, often performed in patients with a reduced renal function, as a result of which acute renal failure may be precipitated[18]. These cases of acute renal failure might have been avoided if an examination of the contrast medium clearance had been included in the preoperative angiography. The contrast clearance which is a measure of GFR might have revealed a deterioration in renal function from the angiography and, if possible, the reconstructive surgery could have been delayed. The intraoperative renal artery occlusion might then be undertaken without causing acute renal failure because it was made at a period when contrast medium concentrations and contrast medium effects in the kidneys were lower. The damaging effects on kidneys from contrast media and from renal artery obstruction appear to potentiate each other[38]. In renal transplantation the donor kidney may in several clinical situations be subjected to both these damaging factors. Nephrectomy is often performed early after angiography while contrast medium remains in the kidneys. Then a period of renal vascular obstruction for several hours may follow while the kidneys, still filled with contrast media, await transplantation. Donor kidneys from cadavers subjected to angiography shortly before their removal show a poorer function, and a shorter period of survival after transplantation when compared to cadaver kidneys not subjected to angiography[73,77].

The precise screening of renal function after contrast medium exposure is especially important in premature infants where high doses of contrast media are often given up to 2-3 g I/kg body weight. The combination of high doses of contrast media and a not fully developed renal function may yield severe or even fatal outcome.

After one injection of an intravascular contrast medium it is possible to make the roentgen diagnostic examination and then some hours later (by blood sampling) measure contrast medium clearance (glomerular filtration rate). Thus both radiological information (renal anatomy) and a post-radiologic information on renal function is obtained; data that could sometimes reveal an early acute renal failure.

In summary the ratio 3 contrast media introduced into the clinic during the past decade all seem to have a smaller effect on the vascular bed, glomerular membranes and tubular cells than the ionic ratio 1.5 media, and as a consequence the number of contrast medium-induced renal failures may be reduced. An additional way of decreasing the effects of contrast medium-induced renal damage may be to secure early detection of the evolving renal function impairment by measuring the contrast medium clearance within the first few hours after injection.

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25
IMMUNOLOGICALLY MEDIATED NEPHRITIS INDUCED BY TOXINS AND DRUGS

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I. INTRODUCTION

Immunologically mediated nephropathies are usually of unknown origin. Many drugs and toxins have, however, been recognized as potential aetiological factors, and the number of chemical agents implicated in this lesion is still growing.

It is often difficult to identify new drugs causing immunologically mediated nephritis because only few individuals are affected. Thus careful clinical observations are of major importance. Single case reports by different groups may be the only way to implicate new chemicals. It is important to recognize the causative agent as soon as possible, since its withdrawal or termination of exposure usually leads to remission. The basic mechanisms of drug- or toxin-induced nephropathies are still very poorly understood. The development of experimental models has, however, shed some light on the mechanisms of drug-induced autoimmunity. Aspects of drug-induced glomerulonephritis have recently been reviewed[1-3].

This review will consider the possible mechanisms of drug-induced autoimmunity, and then the mechanisms of immunologically mediated nephritis. The various drugs or toxins associated with immunologically mediated glomerulonephritis or interstitial nephritis in humans and in experimental conditions, and the pathogenetic mechanisms, will be reviewed.

II. MECHANISMS OF DRUG- OR TOXIN-INDUCED AUTOIMMUNITY

These mechanisms are still poorly understood. We will first consider the various immune reactions which may occur after drug exposure, and then the possible ways by which a drug or a toxin may trigger such mechanisms. We will then discuss the symptoms

that tend to indicate that immunologically mechanisms are at play in a patient exposed to a drug.

A. Immune reactions induced by a drug or a toxin

There are four main types of hypersensitivity reactions, and drugs and toxins may induce at least one of these. However, several of the other reactions may also be observed to be associated with drug hypersensitivity, and it is not easy to assess the role of each in renal injury.

Immediate hypersensitivity (type I) is observed when the first contact with an antigen, or hapten bound to proteins, has elicited the production of IgE antibodies. These antibodies bind (through their Fc fragments) to basophils and mast cells. After reintroduction of the immunogen, the latter binds to the cytophilic IgE antibodies. Binding of the antigen to the specific IgE present at the cell surface results in the liberation of histamine, serotonin, slow-reacting substance of anaphylaxis and eosinophilic chemotactic factor. These mediators induce contraction of smooth muscle and produce the classical manifestations of anaphylaxis. There is evidence that drugs or toxins may induce the formation of IgE antibodies in the clinical and experimental conditions[4,5]. The finding of IgE-containing plasma cells or eosinophils[6] in the interstitium of some cases of acute interstitial nephritis also supports a role for immediate hypersensitivity in immunologically mediated drug-induced nephritis. However, the role of this type of hypersensitivity in the pathogenesis of drug-induced nephritis has not been clearly demonstrated.

Type II or cytotoxic hypersensitivity is characterized by the destruction of cells after binding of specific antibodies. Destruction of the target cell can occur either as a consequence of lysis due to complement activation or elimination by the reticuloendothelial system. Anti-tubular basement membrane (TBM) or anti-glomerular basement membrane (GBM) antibodies are classical examples of antibodies which are bound to basement membrane antigens rather than to the cell itself[7]. Heymann's type nephritis, and related glomerulonephritis, also represent this type of immunonephropathy, since circulating antibodies have been found to bind the membrane of visceral epithelial cells[7] and subsequently give rise to activation of the complement cascade. Anti-TBM or anti-GBM antibodies have been observed following drug or toxin exposure.

Immune complex type hypersensitivity (type III) is characterized by the deposition of circulating immune complexes, as seen in the classical model of serum sickness, or by formation of immune complexes, in situ in the Arthus phenomenon. Acute and chronic serum sickness were first considered to be due to the deposition of circulating immune complexes in the mesangial area and/or in a subepithelial position[7,9]. Recent experiments suggest that the successive deposition of antigen and antibody resulting in the in situ formation of immune complexes[10] is more likely. There are several examples of similar glomerular lesions (so-called membranous GN) following exposure to drugs and toxins in humans

or experimental conditions.

Cell-mediated type IV hypersensitivity is mediated by specific T lymphocytes and by lymphokines released by T cells. There are now several examples that suggest cell-mediated hypersensitivity is the basis of experimentally induced nephritis. There is also some evidence that such mechanisms could be involved in several forms of drug-induced acute interstitial nephritis.

B. Mechanisms of drug and toxin induction of hypersensivity reactions

These mechanisms are still poorly understood. A drug or a toxin may induce an immune reaction in several ways[11]. The toxic agent may act as a hapten after binding to serum or tissue protein. In this situation an immune response is mounted against the hapten bound to the self antigen. Such an example is provided by methicillin-induced acute interstitial nephritis. A metabolite of methicillin (dimethoxiphenylpenicilloyl) binds to the TBM and circulating anti-hapten antibodies then deposit on the hapten fixed to renal structures[12]. Alternatively the hapten may be bound to the structural antigen that then modifies the carrier determinant (autoantigen)[13]. This modified structure is subsequently recognized by the immune system as foreign, which results in the production of antibodies against both the modified and the native form of the structural antigen. Anti-TBM antibodies have been found after methicillin and diphenylhydantoin therapy. Anti-nuclear antibodies produced after treatment with hydralazine or procainamide have also been considered to be a consequence of an interaction of drugs with DNA[14]. Drugs or toxins may also deregulate the immune system. This hypothesis is tempting since, in many situations, several autoantibodies have been observed following drug exposure. Though it is possible that a toxic agent modifies different autoantigens leading to the production of several autoantibodies, it is also logical to consider that so many different autoantibodies are produced as a consequence of an effect of the toxic agent on the immune system. Methyldopa[15] and procainamide[16] have been shown respectively to inhibit suppressor cells or activate helper T cells. Drugs and toxins could also theoretically deregulate the immune system if, as suggested by Gleichmann et al.[17] and Eisenberg and Cohen[18], they are able to modify Ia determinants. Such modified cells would then be stimulated by T helper cells and would produce a variety of autoantibodies. This model has been devised from experiments performed in mice with autoimmunity appearing as a consequence of chronic graft-versus-host reaction[17], a situation where T helper cells stimulate allo-Ia positive cells. However it remains to be shown that drugs can modify Ia determinants.

C. Features suggesting the involvement of autoimmunity in the presence of drug-induced nephritis

Clinical findings are of great value when present. (1) Renal in-

volvement is only observed in a small percentage of individuals. (2) The occurrence of the nephritis does not depend either on the amount of drug taken or on the duration of treatment. (3) The adverse effect which usually disappears after withdrawal of chemical exposure returns after rechallenge. (4) Other manifestations are occasionally associated (rash, fever, arthralgias, lymphadenopathy, eosinophilia and eosinophiluria) and eventually other autoimmune disorders can be observed such as autoimmune haemolytic anaemia and leucopenia.

Laboratory findings may also help by demonstrating some kind of hypersensitivity. An increase in total serum IgE level, the presence of specific IgE antibodies, positive basophilic degranulation test or positive skin tests will suggest type I hypersensitivity. Specific IgG or IgM antibodies may be recognized by a haemagglutination assay provided the drug binds to erythrocytes. Other autoantibodies, for example towards leucocytes, may be detected, but the presence of circulating immune complexes is probably of little help if the antigen is not characterized. Other assays (positive patch test, lymphocytic blastic transformation after introduction of the antigen and inhibition of macrophage migration) would suggest the involvement of cell-mediated immunity.

Renal biopsy is by far the most contributive procedure. Renal histology must include electron microscopy and/or immunofluorescence analysis. The use of monoclonal antibodies and of anti-IgE antiserum are of great interest to characterize infiltrating cells and to dissect more accurately the mechanism involved. That renal biopsy is essential to diagnosis is further demonstrated since indisputable cases of immunologically mediated nephritis are often observed following drug exposure without any extrarenal symptom or laboratory abnormality favouring hypersensitivity. This is the case for gold- or D-penicillamine-induced membranous GN. In contrast, in the presence of obvious extrarenal immunoallergic manifestations and acute renal failure, renal biopsy may exhibit acute tubular necrosis without any evidence of immunologically mediated process. This has been described, for example, during captopril therapy[19].

III. MECHANISMS OF IMMUNOLOGICALLY MEDIATED NEPHRITIS

A. Immunologically mediated glomerulonephritis

Immunologically mediated glomerulonephritis can be due to the deposition of free circulating antibodies interacting with a structural glomerular antigen or with endogenous "planted" antigens. In both situations, immune complexes are formed "in situ". Alternatively, glomerular injury may be the consequence of the deposition of circulating immune complexes. Recent experiments suggest that cellular immunity could also play a major role (Table 1).

1. Antibodies to structural glomerular antigens

The antigenic composition of the glomerulus is highly complex and is currently being actively studied[20]. The GBM and the mesangium

Table 1 Mechanisms of immunologically mediated nephritis

Humoral immunity	Antibodies to structural antigens	Basement membrane (glomerular or tubular). Non-basement membrane (visceral epithelial cell, Tamm-Horsfall protein).
	Antibodies to exogenous-planted antigens	Lectins, cationic or cationized molecules, DNA. Immune material (anti-isotype, anti-idiotype).
	Circulating immune complexes	(Role of charge, size, antibody affinity etc.)
Cell mediated immunity	Participation in antibody-mediated glomerulonephritis. Major role in immunologically mediated tubulo-interstitial nephritis.	

consist of collagen (types IV and V), and of glycoproteins such as laminin, fibronectin, entactin and proteoglycans such as heparan sulphate and chondroitin sulphate. Moreover, antigens expressed by glomerular epithelial cells are being characterized and appear to be of major importance in several immunologically mediated GN.

a. Anti-GBM mediated nephritis. Anti-GBM nephritis was first described in experimental animals at the beginning of the century. Heterologous anti-GBM antibodies, when passively administered, are immediately fixed to the GBM as assessed by immunofluorescence. Several days later, in response to the introduction of the heterologous antibodies, autologous antibodies are formed, resulting in a second phase (autologous phase) of glomerular injury. Although this model has been widely described, several uncertainties persist. Firstly, the exact nature of the antigen(s) responsible for glomerular injury (defined by proteinuria and renal insufficiency) remains controversial. Secondly the mechanisms leading to glomerular injury are not adequately understood. Polymorphonuclear leucocytes are attracted as a consequence of complement activation and are responsible for initial proteinuria. Schreiner et al.[21], using an accelerated model of anti-GBM nephritis, have pointed out the role of macrophages. Recent experiments have shown that T lymphocytes can be detected very early in the glomerulus, and suggest that they may be at least as important as anti-GBM antibodies[22]. Several models of anti-GBM antibody-mediated GN have been described. The most relevant to human pathology is probably the autoimmune disease induced in sheep by immunization with homologous antigens[23], resulting in a severe anti-GBM GN.

Human anti-GBM GN (Goodpasture's syndrome when associated with lung haemorrhage) is a rare but severe disease charac-

terized by the deposition along the GBM of IgG in a typical linear continuous pattern resulting in a necrotic glomerulonephritis. Anti-GBM antibodies can be eluted from the diseased kidneys and are found in the circulation.

b. Antibodies to non-basement membrane structural antigens. Heteroantibodies to rat proximal tubule brush border antigen designated FX1A when injected intravenously in rats result in the appearance of subepithelial deposits (so called membranous GN). Granular IgG deposits (considered until recently as the hallmark of immune complex mediated GN) are seen along the glomerular capillary wall[7]. It has now been clearly demonstrated that this glomerular disease is due to the binding of free circulating antibodies to a glycoprotein (designated g.p. 330) expressed on glomerular epithelial cells as well as on proximal tubule brush border[24].

Autologous immune complex GN (active Heymann's nephritis) results[25] from immunization with homologous FX1A. This GN, which was considered until recently to be due to the deposition of circulating immune complexes[26], is probably also due to the binding of free circulating antibodies[27]. Related antigens are probably responsible for other membranous GN occurring spontaneously in rabbits or induced in mice and rats.

It is quite likely, but not yet demonstrated, that similar antibodies are responsible for at least some cases of human membranous GN, but the antigens involved are unknown in most instances.

2. Antibodies to exogenous planted antigens

Circulating antibodies may also react with molecules which have been trapped for immune or non-immune reasons in glomerular structures.

Concanavalin A binds to sugars of the GBM[28], cationic or cationized molecules such as lysozyme, IgG, bovine serum albumin or ferritin bind to anionic sites of glomerular structures[29]. Aggregated IgGs injected intravenously are also transiently entrapped in mesangial areas[30]. DNA has been shown to bind in vitro to GBM collagen[31]. The subsequent induction or passive injection of antibodies to these non renal "planted" antigens will result in the formation of in situ immune complexes which may induce glomerular injury. The role of non-renal planted antigens in the pathogenesis of human GN is still speculative; however it has recently been demonstrated that cationic antigens derived from micro-organisms such as streptococci are involved in the pathogenesis of post-streptococcal GN[32]. Similarly immune material deposited in glomerular structures for any reason may give rise to anti-isotypic or anti-idiotypic antibodies, which may accelerate the progression of the disease[33].

3. Deposition of circulating immune complexes

From initial studies by Dixon et al.[7] and Germuth[9] it has been

widely accepted that soluble circulating immune complexes may be deposited in glomerular structures (in subepithelial, subendothelial or mesangial locations) and in vessels. These conclusions were mainly derived from studies in the acute and chronic models of serum sickness induced by bovine serum albumin.

The characteristics of immune complexes able to induce an "immune complex type disease" have been the subject of numerous studies. The charge of antigens and/or of antibodies constituting the complexes are of major importance, since only passively administered cationic complexes can localize in a subepithelial position[34].

Circulating immune complexes were considered responsible for most human GN characterized by granular IgG deposits, but from the experimental studies reviewed above it now appears that antibodies to renal antigens or to "planted" antigens give rise to similar immunomorphological patterns. It is therefore difficult to deduce the mechanisms from the immunofluorescent patterns.

4. Role of cell mediated immunity

Humoral mechanisms are considered to play a crucial role in the pathogenesis of most experimental or human GN. T lymphocytes are also essential, since they are required to obtain an antibody response to most antigens, and monocytes are effector cells, for example, in acute serum sickness[35]. However the monocytic influx is usually considered to derive from antibody deposition.

It is difficult to answer the central question whether cell mediated immunity participates directly in the development of glomerular diseases. Humoral immunity, resulting in easily detectable antibodies, may represent only the tip of the iceberg. Recent experiments suggest, for example, that T lymphocytes are important in anti-GBM-mediated glomerulonephritis[22,36]. Morever, Bolton et al.[37] have shown that bursectomized chickens immunized with GBM develop severe GN in the absence of a humoral antibody response.

There is also good indirect evidence (see below) that cell mediated immunity is involved in the pathogenesis of idiopathic nephrotic syndrome with minimal glomerular changes in humans[38]. Thus drugs that induce nephrotic syndrome with minimal glomerular changes will be considered as immunologically mediated in this review.

B. Immunologically mediated tubulo-interstitial nephritis

The mechanisms involved will not be extensively described since they are similar to those mentioned above. Antibodies to basement membrane antigens[6] and to non-basement membrane antigens, such as Tamm-Horsfall protein[39], have been shown to cause several forms of experimental autoimmune nephritis. Circulating immune complexes have also been considered to be responsible for extraglomerular deposition, for example, in chronic serum sickness[40].

Tubulo-interstitial diseases are considered separately because

the role of cellular mediated immunity has been demonstrated more clearly. The introduction of an exogenous antigen into the kidney cortex of a sensitized animal results in a local delayed hypersensitivity reaction[41]. More relevant to interstitial nephritis, T lymphocytes participate in cytotoxic reactions seen in guinea pigs immunized with heterologous tubular basement membrane[42]. The T cell infiltration of the interstitium also plays a role in the progression of the disease in Brown-Norway (BN) rats immunized with homologous tubular basement membrane. It has also been possible to transfer the disease to syngeneic recipients with lymph node cells from immunized donors[43] in this model.

IV. DRUG AND TOXIN INDUCED GLOMERULONEPHRITIS

In this chapter we will review the numerous drugs known to be associated with the occurrence of immunologically mediated GN. The GN observed is usually a so-called immune complex type GN, most commonly a membranous GN. Though a causal relationship is firmly established, the mechanisms involved, the nature of the antigen(s) and the specificity of the antibodies are still unknown. These drugs known to induce the nephrotic syndrome, with minimal glomerular changes, will also be covered, since there are data suggesting that this glomerular damage is immunologically mediated (Tables 2 and 3).

Table 2 Main immunologically mediated glomerular lesions induced by drugs, and drugs most frequently associated

Membranous glomerulonephritis*	Nephrotic syndrome with minimal glomerular changes*
Gold salts	Lithium
Mercurials	NSAID
D-penicillamine	Rifampicin
Other drugs with sulphydryl group	

* The glomerular lesion mentioned is the most frequently observed associated with the corresponding drugs. However most drugs may be associated with the other glomerular lesion.

NSAID: Non-steroidal anti-inflammatory drugs.

A. Glomerulonephritis induced by heavy metals

1. Gold salts are used to treat patients with rheumatoid arthritis, but cause proteinuria and the nephrotic syndrome in 6-17.2% and 2.6-5.3% (respectively) of the patients. Other side-effects of gold therapy such as rash, leucopenia or thrombocytopenia are less frequent. Most of the 122 rheumatoid patients treated with gold

salts who developed proteinuria had a membranous GN (89.5%) and 9.6% had minimal glomerular changes. Renal insufficiency was, however, quite rare, and the proteinuria subsided within 4-18 months. Gold therapy has always been stopped when proteinuria appears; reintroduction has very rarely been attempted.

Table 3 Drugs or toxins that have been found associated with immunologically mediated glomerulonephritis

Well documented
Heavy metals (gold, mercury)
D-penicillamine
Other drugs with a sulphydryl group (thiopronine, 5-thiopyridoxine, pyrithioxine, methimazole, captopril)
Lithium salts
Non-steroidal anti-inflammatory agents
Rifampicin
Anticonvulsant drugs (diones, hydantoin, ethosuximide, mephenytoin)
Drugs responsible for lupus-like syndrome (hydralazine)
Heroin-associated glomerulonephritis
Likely
Drugs responsible for lupus-like syndrome (procainamide), toxin-induced connective tissue disease (silica exposure, toxic-oil syndrome)
Hydrocarbon exposure
Interferon
Not well documented or few cases published
Ampicillin
Probenecid
Phenindione
Carbutamide, tolbutamide, chlorpropamide
Potassium chlorate
Quinidine
Dapsone
PUVA
Levamisole
Nifedipine
Human adjuvant disease

Immunomorphological studies of gold-induced membranous GN gave results quite similar to those obtained in idiopathic membranous GN. Granular IgG deposits (often focal) are observed along the glomerular capillary walls, together with subepithelial electron-dense deposits[44]. Recent studies suggest however that the terminal complement complex (C5 b-9 complex or membrane attack complex) is absent, while it is always found in idiopathic membranous GN[45]. The significance of this finding is unclear, but suggests a different pathogenic mechanism.

Most authors agree that there is a causal relationship

between the occurrence of membranous GN and gold treatment, since such glomerular lesions have been rarely reported among rheumatoid arthritis patients who did not receive gold salts, D-penicillamine or related drugs. The occurrence of membranous GN is not correlated with the cumulative dose of gold, with the duration of treatment, or with the gold salts used[3]. Proteinuria does, however, seem to occur much less after treatment with the gold salt auranofin given orally[46]. Proteinuria is only observed in some patients and is not dose-related, which suggests susceptibility may be genetically determined. This is supported by the fact that patients with HLA-B8 or DRW_3 antigens are at higher risk[47]. The pathogenesis of gold-induced membranous GN is unclear and will be discussed later.

2. Mercury-induced membranous GN has been described in patients treated with organomercurial diuretics, using ammoniated mercury topically or using mercurous chloride-containing laxatives[1-3]. Such situations are expected to be encountered much less frequently since mercury-containing drugs are rarely used nowadays. Antiseptics, laxatives or vaginal contraceptives containing mercury, are however, still in use in some countries.

Interestingly the nephrotic syndrome has also been observed in women using mercury-containing skin lightening creams[3] and after environmental or occupational exposure to mercurials. Eighteen cases have been published and renal biopsies exhibited either a membranous GN or minimal glomerular changes[3]. It is possible that unknown mercury exposure might account for some cases of apparently idiopathic membranous GN. Either minimal glomerular changes, membranous GN or other forms of GN with Ig deposits were observed. It is also worthy of note that a few patients had glomerular linear IgG deposits, suggestive of the presence of anti-GBM antibodies. Similar glomerular lesions could be induced in rabbits after application of the incriminated creams.

The pathogenesis of mercury induced GN in humans is also unknown, but experimental studies, described in detail below, shed some light on the possible mechanisms.

3. Silver exposure may also induce the nephrotic syndrome in humans, but there is good evidence that silver-induced proteinuria is a consequence of silver deposition within the lamina densa and is not immunologically mediated[1-3].

B. Glomerulonephritis induced by drugs with sulphydryl groups

Several drugs containing a sulphydryl group are able to induce immune type GN. Among them, D-penicillamine is responsible for the greatest number of cases.

D-penicillamine is used in the treatment of rheumatoid arthritis, Wilson's disease, cystinuria, chronic active hepatitis and systemic sclerosis. Proteinuria has been reported to occur in all these situations but more often in rheumatoid arthritis patients who account for the largest number of patients treated with this drug.

Proteinuria is usually the only abnormality encountered. It

has been observed in 7-20% of rheumatoid arthritis patients treated with D-penicillamine. In recent reviews[1,2], 190 cases were collected. The nephrotic syndrome was present in 49.6%. The characteristics of proteinuria or of the nephrotic syndrome are similar to those mentioned for gold salts. Renal insufficiency is rare, as is hypertension. The occurrence of proteinuria is neither correlated with the cumulative dose of the drug nor with duration of treatment, suggesting individual susceptibility; Emery et al.[48] showed the role of HLA-DR_3 and sulphoxidation status. Renal biopsies performed in 147 of these patients exhibited a membranous GN quite similar to that induced by gold salts in 85.2% of the cases and more rarely (10.4%) a mesangioproliferative GN. Minimal glomerular changes and focal necrotizing GN have been observed, each in three patients and, more recently, a rapidly progressive GN in one[49]. The prognosis is good in most cases and proteinuria progressively disappears. Interestingly, since D-penicillamine is given in severe diseases such as Wilson's disease, and because drug-induced GN usually has a good prognosis, therapy has occasionally not been interrupted. It has been reported that proteinuria may disappear despite continued treatment. Similarly, reintroduction of the drug was not always associated with recurrence of proteinuria.

Glomerulonephritis as a part of a lupus-like syndrome[1-3] or associated with lung haemorrhage (Goodpasture's syndrome)[1-3] has been observed in six and nine patients respectively. In the latter situation, diffuse extracapillary necrotizing proliferative glomerulonephritis is usually observed. The prognosis is poor though favourable outcome has been observed after plasma exchange associated with immunosuppressive therapy. It is of note that while a linear pattern of deposition of IgG along the glomerular capillary wall has been occasionally observed, circulating anti-GBM antibodies were not detected.

Other drugs with a sulphydryl group have been associated with an immune type GN. Proteinuria occurs in 1% of hypertensive patients treated with captopril, and a membranous GN has been found in 18 of the 19 patients who were biopsied. Prognosis is usually good on withdrawing the drug. Proteinuria (with or without the nephrotic syndrome) has also been reported in patients treated with thiopronine, pyrithioxine or 5-thiopyridoxine, where renal biopsies exhibited a membranous GN or minimal glomerular changes[1-3]. The nephrotic syndrome has also been observed in three patients treated with methimazole for Grave's disease.

C. Drugs inducing lupus-like syndromes

Several drugs are known to induce lupus-like syndromes. Renal involvement is usually absent, but there are several exceptions. D-penicillamine can induce a lupus-like syndrome associated with renal involvement. Renal manifestations seem to be very rare after procainamide treatment, and only six renal biopsies have been reported[3]. Different glomerular lesions were reported: normal glomeruli by light microscopy with Ig deposits, focal GN, focal thickening of capillary walls with mesangial proliferation and

granular Ig deposits, membranous GN, and crescentic GN. A glomerulosclerosis with segmental mesangial proliferation, IgM and C3 deposits was observed associated with subendothelial electron-dense deposits in one patient who presented with the nephrotic syndrome[50].

Renal involvement is less rare in patients treated with hydralazine. We collected 24 cases, 16 of whom underwent renal biopsy[1-3,51-54]. Mild renal abnormalities (proteinuria, haematuria) were observed in seven of them. Of the other 17 patients renal insufficiency developed in 14. Renal biopsy in most instances exhibited a focal and segmental GN with crescents and necrotizing GN.

Anti convulsant drugs such as the oxazolidinedione derivatives, hydantoin derivatives or ethosuximide, are also able to induce lupus-like syndrome. Renal involvement has also been described. However, in most cases renal involvement is not associated with systemic manisfestations of the lupus-like syndrome. The nephrotic syndrome has been occasionally observed, usually in children treated with dione derivatives. Various renal lesions have been reported, including membranous GN, minimal glomerular changes and, in one patient, a peculiar glomerular lesion defined by an infiltration of capillary loops with eosinophils[55]. The prognosis was favourable in all cases after withdrawal of the drug.

Renal abnormalities have also been reported in the presence or absence of lupus-like syndrome in a few patients treated with mephenytoin, diphenylhydantoin or in children receiving both hydantoin and oxyzalidinedione derivatives, and in one patient treated with ethosuximide[1-3].

D. Connective tissue diseases associated with occupational or accidental exposure, or cosmetic surgery

1. Occupational exposure to silica may lead to renal insufficiency. Interstitial fibrosis and glomerulosclerosis were initially considered as the consequence of a direct nephrotoxic effect of silica. However it has been also shown that silica exposure may be responsible for the induction of connective tissue disorders such as scleroderma, systemic lupus erythematosus, and rheumatoid arthritis[56]. Several reports have mentioned the occurrence of glomerular disease associated with a lupus-like syndrome[57,58]. A rapidly progressive nephropathy has been reported in four patients[59], all of whom had a proliferative GN with occasional crescents, interstitial lymphocytic and plasma cell infiltrates, and immune reactants and/or electron-dense deposits in glomeruli. A proliferative GN has also been reported in another patient[60].

2. Renal involvement seems to be rare in the toxic-oil syndrome recently described in Spain. This syndrome is usually responsible for a scleroderma-like syndrome. However, circulating anti-GBM antibodies have been reported in few patients, but no renal biopsies were taken. Moreover, a GN with proteinuria and renal failure has been reported in four patients[61]. Renal biopsies exhibited a proliferative endocapillary (two cases) or extracapillary

(one case) GN or membranoproliferative GN (one case).

3. Another intriguing disease, often called "human adjuvant disease", has been reported following cosmetic surgery. Systemic sclerosis, rheumatoid arthritis, polymyositis or the lupus-like syndrome have been described following injections of silicone or paraffin. Proteinuria has been reported in five out of 46 women affected with this syndrome[62], but there are no data from renal biopsies. One woman who underwent breast augmentation and later developed systemic lupus erythematosus with the nephrotic syndrome and a proliferative GN has recently been observed (unpublished observation).

4. Various autoimmune abnormalities have been described in workers exposed to vinyl chloride, including scleroderma-like syndromes mainly in patients with the DR3 HLA antigen. Although Ig deposits have been found in the skin, muscle or lung in a few patients, the presence of proteinuria or renal involvement is not documented[63].

E. Lithium induced nephrotic syndrome

Lithium salts are widely used in the treatment of some psychiatric disorders and can affect renal function in several ways. Although only 10 patients have been reported[1-3], a causal relationship has been well established since three patients who had recovered from a first episode of the nephrotic syndrome relapsed when rechallenged with lithium sulphate or lithium carbonate. Serum lithium levels were in the therapeutic range and the delay between the institution of the treatment and the occurrence of the nephrotic syndrome varied from 6 weeks to 2 years. Heavy proteinuria with nephrotic syndrome was present in all cases, associated with renal insufficiency severe enough to require haemodialysis in two patients. The nephrotic syndrome and proteinuria rapidly disappeared and renal function returned to normal shortly after withdrawal of the drug. Even in patients who relapsed after rechallenge, the nephrotic syndrome disappeared after the drug was stopped.

Renal biopsies exhibited minimal glomerular changes with negative immunofluorescent findings and/or without electron-dense deposits in most patients. Diffuse granular IgG deposits suggestive of membranous GN were reported in only one patient[3]. In another patient who developed acute renal failure a small collection of lymphocytes in the interstitium suggested that acute renal failure could be due to an allergic interstitial nephritis, as is the case for fenoprofen nephropathy. However, these histological descriptions do not allow any firm conclusions.

F. Other drugs

Rare cases of GN have been reported in association with other drugs[1-3], but a causal relationship is difficult to establish in so

few cases. Compounds incriminated are: probenecid, phenindione, carbutamide, tolbutamide, chlorpropamide, potassium chlorate, quinidine, dapsone, PUVA and levamisole[1-3], and more recently nifedipine[64].

G. Hydrocarbon and agrochemical exposure

A causal association between hydrocarbon exposure and development of GN has been suggested from case reports and from case control studies. Immunofluorescent studies were performed in 11 of 12 cases of proliferative necrotizing GN[65-70]. All exhibited linear IgG deposits, and circulating anti-GBM antibodies were found in eight cases[66,69,70]. Glomerulonephritis was associated with lung haemorrhage in eight of these 11 patients[65,67-70]. The prognosis was usually bad, but a favourable outcome has been reported after cessation of exposure and immunosuppressive therapy[67]. Six cases of membranous GN have also been reported in patients exposed to hydrocarbon[71-73]. There is therefore a strong suspicion from these case reports of a causal association between hydrocarbon exposure and anti-GBM-mediated GN or membranous GN. Five case control studies have reported a greater exposure to hydrocarbon among patients with GN when compared to control groups, but such a relationship was not observed by Van Der Laan[74]. In a recent report, Churchill[75] has critically reviewed these case control studies and pointed out methodological deficiencies which put the conclusions into question.

Experimental studies have, however, shown that chronically exposed rats develop symptoms of Goodpasture's syndrome or the nephrotic syndrome, but unfortunately immunofluorescent studies were not performed[65].

Polla et al.[76] reported one case of anti-GBM-mediated GN after exposure to herbicides and insecticides.

H. Heroin-associated glomerulonephritis

Drug addiction is responsible for a number of cases of glomerular disease. In a recent review comprising 167 cases, most were observed in Black American males. Renal biopsies were performed in 124 patients and variety of glomerular lesions such as focal glomerulosclerosis, focal proliferative GN, diffuse glomerulosclerosis, type I and II membranoproliferative GN, membranous GN, and pure proliferative GN[3] were observed.

The pathogenesis of these glomerular lesions is unclear, but it is quite possible that either the drug or the vehicle used have a direct toxic effect. It is also likely that, at least in some cases, the introduction of antigenic adulterant material, together with the heroin, initiates an immune reaction responsible for the glomerular disease. It is also possible that either the drug or unrelated antigenic material induces a dysregulation of the immune system.

V. DRUG- AND TOXIN-INDUCED EXPERIMENTAL GLOMERULONEPHRITIS

In order to better understand the mechanism(s) of action of drugs in inducing immunologically mediated nephritis, several experimental models have been devised (Table 4). All of the available models concern GN, and at present no experimental model of immunologically mediated interstitial nephritis has been reported after drug exposure.

Table 4 Experimental immunologically mediated glomerulonephritis induced by drugs and toxins

Drug and toxin	Species	Glomerular lesion	References
Mercury	Rat	Anti-GBM GN	76
		Membranous GN	77,102,103
	Rabbit	Anti-GBM GN	105
		Membranous GN	
	Mouse	Immune complex GN	106
Gold salts	Rat	Membranous GN	108
D-penicillamine	Rat	Membranous GN	109
		Anti-GBM GN	110
Hydralazine	Mouse	Immune complex GN	111
Alfalfa sprout (L-canavanine?)	Monkey	Immune complex GN	112
Hydrocarbon	Rat	Goodpasture's syndrome*	65
CCl_4	Rat	IgA nephropathy	113

* Immunofluorescence studies not performed.
CCl_4 induces cirrhosis of the liver.

B. Mercury-induced glomerulonephritis

1. Mercury-induced autoimmune disease in the Brown-Norway rat

a. Description of the disease. The GN observed following injections of $HgCl_2$ (thrice weekly at a dose of 100 μg/100 g body weight) is species- and strain-dependent. One week after the first injection a host of autoantibodies can be detected, including anti-GBM antibodies and anti-SS DNA antibodies[77-79] in the Brown-Norway (BN) rat. A striking polyclonal IgE[5] and IgG increase is also observed. These autoimmune abnormalities reach a peak by day 15 and progressively disappear even when $HgCl_2$ injections are continued[79]. Circulating anti-GBM antibodies are readily found deposited in a typical linear and smooth pattern along the glomerular capillary wall and in other organs[77,80]. They can be eluted from the diseased kidneys and have been characterized as

anti-GBM antibodies. The circulating antibodies are directed towards collagenase-digested GBM, laminin and fibronectin, but the antibodies responsible for the pathogenic effect have not been identified[79]. Indeed the deposition of anti-GBM antibodies is associated with the occurrence of heavy proteinuria with the nephrotic syndrome. There is no significant renal insufficiency, and light and electron microscopy at peak illness showed a mild influx of monocytes without extracapillary proliferation. Rats may die at this time, probably as a consequence of intravascular coagulation due to antibody deposition[81]. The mechanism of proteinuria is not clearly understood, but has been shown to be complement-independent[82].

When rats survive this phase of the disease, glomerular linear IgG deposits progressively diminish and granular IgG deposits are observed superimposed along the glomerular capillary wall and in a subepithelial position as shown by electron microscopy[77,83]. Granular IgG deposits are also found in the mesangium and in the vascular walls in the kidneys and in several other organs[80]. Transient circulating immune complexes are probably responsible[79]. The antigen and the antibodies constituting such complexes are unknown at present.

It is important to note that the autoimmune disease observed in the BN strain is not dose-dependent since very low doses of $HgCl_2$ (10 μg instead of 100 μg) also induce autoimmunity[77]. Also important is the fact that several mercurials were tested (mercurous chloride, methylmercury and various pharmaceutical agents containing mercury), and all were found to induce various degrees of autoimmune abnormalities. Finally, the route of administration of the compound (parenteral injections, oral route, digestive route, topically applied) does not influence the manifestations observed[84,85]. This is probably of interest since mercurials are found in the environment, and may therefore be responsible for cases of apparently idiopathic GN in man.

b. Genetic control of susceptibility. The BN rat strain is the only one among the 22 tested to develop such autoimmune abnormalities. Interestingly the four strains with the RT-1 l haplotype are completely resistant (the RT-1 complex is the equivalent of the HLA complex in man). The susceptibility of Lewis (LEW) rats has been more extensively studied. Doses which induce acute tubular necrosis are unable to induce autoimmune phenomena. In order to more precisely identify the genetic control of susceptibility in that model, the behaviour of segregants between the resistant LEW strain and the susceptible BN strain was assessed. BN rats have the RT-1 n haplotype. The haplotype was determined in all the segregants (F_1 and F_2 hybrids and backcrosses) and the occurrence of linear or granular IgG deposits in response to $HgCl_2$ was studied in all of these animals. The total serum IgE level was also sequentially determined. We were thus able to demonstrate that susceptibility is inherited as an autosomal dominant trait and depends upon three or four genes, one of which is RT-1 linked[86-88]. This is of interest since, as mentioned above, MHC-linked genes are also involved in gold-induced membranous GN in humans.

The resistance of LEW rats is not due to the absence of the corresponding basement membrane antigen, since kidney acid eluate

from BN rats cross-reacts with GBM from LEW rats as assessed by indirect immunofluorescence[86]. Moreover when a LEW rat kidney was transplanted into binephrectomized F_1 hybrids (later injected with $HgCl_2$), both linear and granular glomerular IgG deposits were observed[89]. The mechanism of the resistance in LEW rats has not been elucidated, but these animals do become susceptible when lethally irradiated and rendered chimaeric by reconstitution with bone marrow cells from susceptible (LEW x BN) F_1 hybrids[90].

c. Mechanisms of induction of mercury-induced autoimmune disease in BN rats. In vivo findings in BN rats injected with $HgCl_2$ strongly suggest that some kind of polyclonal activation of B cells occurs. Indeed, these rats develop spleen and lymph node enlargement from day 8, and we have shown (unpublished observations) that the number of both B and T cells significantly increases in these organs. Moreover, as mentioned above, BN rats develop a polyclonal increase in IgG and IgE serum levels, and several autoantibodies are produced such as anti-GBM and anti-SS DNA antibodies. Using the plaque-forming cell assay we also observed that the number of anti-TNP and anti-sheep red blood cells plaque-forming cells produced by the spleen was increased when compared with controls[78]. These in vivo observations were confirmed by in vitro experiments. Indeed normal BN rat spleen cells exposed in vitro to non-cytotoxic doses of $HgCl_2$ also produced anti-TNP and anti-sheep red blood cell antibodies as shown by the plaque-forming cell assay. T cells and/or macrophages were found to be required for this polyclonal activation to occur[78].

This was confirmed more recently by using another approach. When spleen cells from BN rats were fused with the non-secreting IR 983 F rat myeloma cell line, clones were obtained which produced antibodies with various specificities including anti-GBM and antinuclear antibodies[91]. A number of clones produced IgE without detectable antibody specificity in most cases. The significance and the role of the IgE produced in that model is obscure, but confirms that the polyclonal activation observed is highly T-dependent. Moreover this suggests that $HgCl_2$ acts by dysregulating the normal immune response. The possible role of $HgCl_2$ modifying lymphocyte functions in BN rats was studied using the popliteal lymph node assay and mixed lymphocyte cultures. Unfractionated spleen cells and T cells from BN rats treated with $HgCl_2$ induce a proliferation in the draining popliteal lymph node when injected in the footpad of naive syngeneic recipients. Both T helper cells and B cells were also found to proliferate[92]. The findings were confirmed in mixed lymphocyte culture. Irradiated T helper cells from $HgCl_2$-treated BN rats or irradiated normal T helper cells exposed in vitro to $HgCl_2$ induced normal BN rat spleen cells to proliferate. Both normal T helper cells and normal Ia positive cells were required among responder cells. This response was specific since T suppressor/cytotoxic cells or B cells from rats injected with $HgCl_2$ were unable to induce the proliferation[93].

In conclusion, we have shown that $HgCl_2$ acts, at least in part, by inducing polyclonal activation. Evidence has been obtained that this polyclonal activation occurs as a consequence of a modification of T helper cells which are able to stimulate normal

T helper cells and B cells. Several hypotheses are currently under investigation to explain this abnormal cooperation. It is possible that T helper cells exposed to $HgCl_2$ release non-specific factors. It is also possible that modified T helper cells induce the generation of autoreactive T helper cells recognizing Ia positive cells[13].

These findings do not confirm the hypothesis put forward by Gleichmann et al.[17] from chronic graft-versus-host experiments. These workers showed that autoimmune manifestations in that model are a consequence of a reaction of T helper cells with allo-Ia positive cells[94]. They suggested that Ia determinants modified by the virus or drugs could function as allo-Ia and stimulate autologous T helper cells[17]. No evidence for a role of modified Ia determinants could be found in the studies outlined above[93].

d. Spontaneous regulation of mercury-induced autoimmune disease. Another interesting feature of the disease induced in BN rats is the spontaneous regulation that occurs after 3 weeks even when $HgCl_2$ injections are continued[79]. All the autoimmune manifestations subside progressively so that no abnormalities can be detected by the end of the second month in most of the animals, except for the persistence of granular IgG deposits in kidney structures. Bowman et al.[95] observed that the disease cannot be induced again in those animals that have recovered, and that BN rats can be rendered tolerant if they are first injected with low $HgCl_2$ doses. This suggests that suppressor cells could be responsible for the spontaneous regulation observed. Indeed spleen cells from animals that have recovered are able to transfer resistance when injected into naive syngeneic recipients. Other experiments also suggest that these cells have the suppressor/cytotoxic phenotype since spleen cells depleted in OX-8+ cells (suppressor/cytotoxic) are unable to transfer the resistance[95].

The role of auto-anti-idiotypic antibodies has also been investigated. Indirect evidence suggesting that such antibodies are present was reported by Chalopin and Lockwood[96]. Using an anti-GBM plaque forming cell assay, they showed that some rats had an antigen augmentable plaque-forming cell response, and that this response could be inhibited by the serum of these rats. This phenomenon is similar to that described by Goidl et al.[97]. However Chalopin and Lockwood[96] could not directly demonstrate the presence of auto-anti-idiotypic antibodies. It therefore seems clear that spontaneous regulation is mediated by suppressor/cytotoxic cells, but a role for auto-anti-idiotypic antibodies, although likely, remains to be proven.

e. Immunomodulation. Mercury-induced autoimmune GN in the BN rat is a potentially useful model to test the effect of various immunomodulating agents. Cyclophosphamide given at a daily dosage of 10 mg/kg completely prevents the appearance of anti-GBM antibodies and the total IgG increase. The effect of the drug, when given after polyclonal activation has started, has not been evaluated. It is of interest to note that Pusey et al.[98] observed that a single dose of the drug given the day before the first $HgCl_2$ injection completely prevented the appearance of autoimmune disorders. In contrast methylprednisolone given daily at a dose of 1.5 mg/kg has no effect (unpublished).

Prostaglandins, and especially PGE_1, have been shown to attenuate autoimmune manifestations in B/W mice, probably due to its immunosuppressive properties. PGE_1[99] given twice daily at a dose of 12 μg/kg, significantly reduced all the autoimmune abnormalities in the mercury model compare to control animals[99]. More recently the effect of cyclosporin A at daily doses of 10, 7 or 5 mg/kg completely prevented or considerably attenuated the severity of the disease[100]. This drug was also found to be effective when given at a time when autoimmune disorders have already appeared.

Vendeville and Druet[101], using a new technique for plasma exchange in the rat, found a temporarly decrease in the amount of autoantibodies and the total IgE level. This model has the potential to evaluate the effect of various immunosuppressive regimens.

2. Mercury-induced glomerulonephritis in other strains of rats

We have previously described the disease observed in BN rats and mentioned that rats with the RT-1 l haplotype are resistant to the induction of the autoimmune disease. Several other rat strains have been tested using the same experimental protocol[102] (Table 5).

Table 5 Glomerular lesions in various rat strains after injection of $HgCl_2$ (refs. 76,77,102)

Strain	RT-1	Glomerular lesion
BN	n	Anti-GBM GN IC-type nephritis*
PVG/c, AVG	c	IC-type nephritis
AVN, DA	a	IC-type nephritis
BDV	d	IC-type nephritis
BUF	b	IC-type nephritis
OKA	k	IC-type nephritis
AS_2	f	IC-type nephritis
LEW, F. 344	l	None
AS, BS	l	None
WAG, LOU	u	None
WF	u	IC-type nephritis

* IC - Immune complex

About 30% of outbred Wistar rats develop a membranous GN, but the antigen responsible has not been identified. Mesangial IgG deposits can also be induced in outbred Wistar rats. Several strains with the RT-1 u haplotype were then tested. Most of them (Wistar AG, LOU) were found to be resistant, but a membranous GN was only found in a small percentage of inbred Wistar-Furth rats injected with 0.4 mg of $HgCl_2$. This suggests that, in Wistar

rats, the susceptibility to the induction of membranous GN does not depend only on MHC-linked genes, but that environmental factors are most important. Among the other strains of rats tested[102] several developed only an immune complex-type GN characterized by granular IgG deposits in various locations (subepithelial, mesangial or in vascular walls). This was the case for PVG/c and AUG rats (RT-1 c), for DA and AVN rats (RT-1 a), for BDV rats (RT-1 d) and for OKA rats (RT-1 k). Antinuclear antibodies were found by indirect immunofluorescence and anti-SS DNA by the Farr assay in several of these strains. Anti-SS DNA antibodies could be eluted from the kidneys of PVG/c rats, suggesting that these antibodies may be of pathogenic significance in that strain[102]. The GN induced in PVG/c rats has been studied by Weening et al.[103], who found antinuclear antibodies. Moreover, Weening et al.[104] obtained evidence that the occurrence of the disease was associated with an inhibition of suppressor cell function, because adult thymectomy worsened the disease. It therefore seems that the mechanisms responsible for the induction of autoimmune GN are different depending on the strain tested. An enhancement of T helper function appears to be at play in BN rats, while a decrease in T suppressor function would be responsible for the occurrence of the autoimmune GN in PVG/c rats.

3. Mercury-induced autoimmune GN in other species

Roman-Franco et al.[105] reported that $HgCl_2$ induces a disease in outbred rabbits quite similar to that seen in BN rats. Rabbits develop anti-GBM antibodies and circulating immune complexes. Linear IgG deposits along the glomerular capillary wall (and other organs) are followed by granular IgG deposits in a subepithelial position in glomeruli and in the vessel walls of several other organs. Interestingly using $^{203}HgCl_2$ and autoradiography, mercury was deposited in the proximal tubular cells, but not within the immune deposits[105].

Several authors have studied the effect of $HgCl_2$ in mice[106]. Most authors have found that a mesangial glomerulopathy could be induced in BALB/c mice and Swiss mice. Granular IgG deposits along the glomerular capillary wall have also been reported in BALB/c mice. No anti-GBM antibodies have been observed.

B. Other experimental autoimmune GN induced by drugs and toxins

Several other experimental models have been described, but usually the mechanisms responsible have not been sought. It has been reported that diones induce the nephrotic syndrome in Wistar rats[107]. Nagi et al.[108] showed that a membranous GN occurs as a consequence of gold thiomalate administration in Wistar rats. It was suggested, but not demonstrated, that antibodies against the brush-border tubular antigen were responsible. Wistar rats have also been shown to develop a membranous GN after prolonged feeding of D-penicillamine[109], but there is no mechanismic data. More

recently, Donker et al.[110] observed that BN rats fed D-pencillamine develop a disease quite similar to that described in that strain after injections of $HgCl_2$. Indeed linear IgG deposits were observed along the glomerular capillary wall and several rats died as a consequence of intravascular coagulation. These authors have moreover shown that LEW rats and Sprague-Dawley rats are resistant. There are therefore striking similarities between disease observed in BN rats receiving $HgCl_2$ or D-penicillamine, which suggests similar mechanisms of induction. In contrast, captopril does not induce any autoimmune manifestations in BN rats.

Several reports have been published concerning experimental induction of lupus-like syndromes. Ten Veen and Feltkamp-Vroom[111] demonstrated that hydralazine may give rise to anti-nuclear antibodies and also to mesangial IgG deposits. Ajax mice appeared to be more affected than BALB/cj mice. Lympho-proliferation and polyclonal Ig increases were also reported. Recently we tested the effect of hydralazine in BN and LEW rats. We could not find antinuclear antibodies or glomerular IgG deposits in either strain, or significant alterations in the total serum IgE level.

Another lupus-like syndrome with renal involvement has been induced in monkeys fed alfalfa sprouts[112], and a role for L-canavanine, a non-protein amino acid present in alfalfa sprouts, was proposed. Rats exposed to hydrocarbon have been reported to develop a Goodpasture's syndrome[65]; however immunofluorescence studies were not performed. It has also been known for a long time that puromycin, and more recently adriamycin, are responsible for the appearance of the nephrotic syndrome in rats, but there is no evidence that these glomerular diseases are immunologically mediated.

Finally CC_{14} induces cirrhosis of the liver in rats associated with mesangial IgA deposits[113]. This model is similar to the changes observed in humans with cirrhosis of the liver.

VI. DRUG INDUCED ACUTE INTERSTITIAL NEPHRITIS

A. General considerations

Drug-mediated allergic interstitial nephritis is a well-known side-effect of drug therapy. Since its first description with sulphonamides in the 1940s, more than 40 different drugs have been associated with immunological interstitial nephritides. This aetiology represents 0.8%[114] to 8%[115] of all causes of acute renal failure.

Fever, skin rash, arthralgia, and macroscopic haematuria suggest an allergic drug reaction, but these symptoms are absent in 60%-90% of patients[4,115,116]. Diuresis is preserved in 30-40% of cases[4,116]. Thus this diagnosis may be missed, especially if the drug has a potential direct nephrotoxic effect, or if it was introduced to treat an illness with possible kidney involvement. The onset of acute renal failure is not dose-dependent. The clinical signs develop usually within 15 days (2-44 days) after initiation of therapy, and this delay may be shorter after rechallenge with the same drug[117].

Microscopic or macroscopic haematuria, and mild proteinuria may occur but do not aid in diagnosis. Nephrotic range proteinuria is common with acute interstitial nephritis induced by non-steroidal anti-inflammatory drugs[118] and in some cases of treatment with penicillin[4], ampicillin[119], and thiazides[4]. Eosinophiluria, when sought specifically, would be present in more than half of the patients[115,116]. It seems to be the most reliable diagnostic index. Blood eosinophilia is observed in 60-100% of cases[117] and serum hyper-IgE in about 50%[4,115,117]. Although in many circumstances there is strong evidence for a major role of cellular immunity, anti-drug antibodies using complement fixation by antibody-coated erythrocytes, leucocytes or platelets has been demonstrated with several drugs such as amoxicillin[117], rifampicin[117], glafenine[119], floctafenine[117], and iothalamate[120]. Acute intravascular haemolysis may also be associated[119]. Circulating anti-tubular basement membrane antibodies have been detected after methicillin[13,121,122], cefalothin[123], and diphenylhydantoin[124] treatment. Except for rechallenge with the putative offending drug, the validity of the different tests proposed to incriminate certain drugs is questionable. Several tests such as allergen-specific IgE detection[125,126], allergen-induced histamine release from leucocytes[126], lymphocyte transformation test[124,127], macrophage inhibition assay[128], degranulation basophil test[129], and skin tests have been used[117].

Kidney histological examination shows a diffuse or focal interstitial infiltration with lymphocytes, plasmocytes and most often eosinophils. Interstitial oedema and varying degrees of tubular damage are usually associated, whereas glomeruli and vessels are almost normal[4,115,117]. In the absence of eosinophils, lesions lack specificity. Granulomas with epithelial and giant cells are observed in 32% of the patients and the association of granulomas and acute mononuclear interstitial nephritis is almost specific to drug induced interstitial nephritis[117,119]. Indeed, in a series of 10 AIN with epithelioid granulomas, nine were drug-induced[119]. This association has been described with penicillin[130], methicillin[122,131], oxacillin[132], thiazides[133], thiazide-triamterene[117,134], co-trimoxazole[117], phenindione[135], glafenine[117], floctafenine[117], noramidopyrine[117], paracetamol[117], diflunisal[136], and bethanidine[136]. Immunofluorescent studies using anti-heavy chain and anti-complement antisera are usually negative, but linear tubular basement membrane staining with IgG, IgM or factor B has been observed after methicillin[12,121,137-139], ampicillin[139], diphenylhydantoin[124,127,140] and allopurinol[141] use. Characterization of the infiltrating cells using monoclonal antibodies has been undertaken in some cases. In certain cases sclerosing transformation of the interstitial infiltrate may lead to permanent renal failure[12,117,122,130] and thus steroid treatment may be justified in severe cases.

Interstitial infiltration is the sole indication of drug-induced immunological injury, and renal biopsy is necessary for diagnosis. Even in the presence of clinical and/or laboratory findings typical of allergy, acute renal failure may be due to lesions other than acute interstitial nephritis, and renal biopsy is also warranted[19]. Finally, drug-induced acute interstitial nephritis occurs often in

complex clinical situations in which it is not always possible to precisely delineate the exact mechanisms of the observed lesions. Bacterial or viral infection, direct drug toxicity, shock, and haemolysis may all play a role.

B. Drugs responsible for acute interstitial nephritis (Table 6)

Allergic interstitial nephritis has been described for more than 40 different drugs, principally penicillin and its analogues, rifampicin, sulphonamides, cimetidine and non-steroidal anti-inflammatory drugs.

1. Antibiotics

About 150 patients with methicillin nephritis have been reported (see full references in ref. 117). The disease begins 1-60 days (average 15 days) after initiation of therapy with clinical picture of

Table 6 Drug immunologically mediated acute interstitial nephritis without the nephrotic syndrome*

1. Antibacterial therapy
   (a) Penicillin derivatives
   Methicillin
   Ampicillin
   Oxacillin
   Carbenicillin
   Penicillin G
   Nafcillin
   Amoxcillin
   (b) Cephalosporin
   Cephalothin
   Cephalexin
   Cephradin
   Cephoxitin
   (c) Others
   Rifampicin
   Piromic acid
   Erythromycin
   Minocycline
   Vancomycin
   Salicylazosulfapyridine
   Sulphonamides
2. Diuretics
   Chlorothiazide
   Cyclothiazide
   Hydrochlorothiazide
   Tienilic acid
   Chlorthalidone
   Triamterene
   Furosemide
3. Analgesics
   Glafenine
   Floctafenine
   Anthrafenine
   Aminopyrine
   Noramidopyrine
   Clometacine
   Phenazone
   Sulphinpyrazone
   Paracetamol
4. Others
   Phenindione
   Cimetidine
   Allopurinol
   Diphenylhydantoin
   Carbamazepine
   Clofibrate

The most frequently incriminated drugs are underlined.
* For NSAID see Table 7. See references in the text.

fever (87% of patients), proteinuria (94%), gross haematuria (85%), leucocyturia (93%) and usually eosinophiluria but skin rash is present in only 24% of cases. Renal failure is noted in 61% of patients older than 16 years, 30% of whom are oliguric and need dialysis. Recovery usually occurs within a few days of the discontinuation of therapy and 90% of the patients are well within 1 year.

Table 7 NSAID responsible for acute interstitial nephritis

With the nephrotic syndrome			Without the nephrotic syndrome		
Drug	No. of cases	References	Drug	No. of cases	References
Fenoprofen	28	in 3	Mefenamic acid	21	36,184
Zomepirac	6	201,206,207	Zomepirac	2	187,188
Piroxicam	3	211,213	Benoxaprofen	1	181
Fenclofenac	2	203,204	Diflusinal	1	182
Ibuprofen	2	200,202	Ketoprofen	1	184
Phenylbutazone	2	210	Niflumic acid	1	117
Tolmetin	2	208	Phenylbutazone	1	185
Aclofenac	1	205	Tolmetin	1	186
Indomethacin	1	209			
Naproxen	1	195			

The remainder manifest continued azotaemia for at least a year[12,13,116,122,142]. There is a possible cross sensitization with other penicillin derivatives or cephalosporins[130,143]. The mechanism of the renal lesions is hypothetical. In some patients linear tubular deposits of IgG and factor B, together with the dimethoxyphenyl penicilloyl hapten (DPO) (an antigenic determinant of a methicillin metabolite) are found on the TBM[12,13,121], and in one case along the GBM[12]. Circulating anti-TBM antibodies have been detected only rarely[13,121,122,131]. DPO is secreted by tubular cells and cannot be eluted, suggesting its fixation rather than simple deposition[13]. Border[13] has suggested that DPO combined with TBM induces the formation of anti-TBM antibodies, but it is also possible that antibodies are directed towards DPO which is bound to the tubular basement membrane[12].

Penicillin G is rarely involved. Ampicillin was responsible for at least seven cases[12,115,117,129,144] and amoxicillin, oxacillin, carbenicillin, and nafcillin for isolated ones (see ref. 117). Clinical symptoms are usually mild, but haemorhagic cystitis and ureteritis have been described in association with acute renal failure in one case[145].

Rifampicin is responsible for about 60 cases of acute interstitial nephritis[117,146,147]. The large majority of cases occurred in patients receiving interrupted or discontinuous therapy. High fever with chills, lumbar pains, dark urine and myalgia are invariably present; rash and eosinophilia rare. Renal failure with or without oliguria supervenes. Haemolysis, thrombopenia and hepatic cytolysis are sometimes associated. When sought, anti-rifampicin antibodies detected by an antiglobulin test are usually found. Renal biopsy shows an acute monocytic interstitial nephritis without eosinophils. Renal abnormalities may persist despite rifampicin withdrawal (see references in ref. 147). At least four cases of acute renal failure have been described during a first course of continuous rifampicin therapy[148,149]. In one case renal failure was insidious in onset and histological lesions were typical of drug-induced interstitial nephritis[149]. Patients receiving discontinuous treatment have a higher anti-rifampicin antibody titre than those on continuous therapy, and seem to be at higher risk for acute renal failure. The specific antibodies may persist several months after cessation of therapy. When rifampicin is to be reintroduced after several weeks or months, a search for specific antibodies may be judicious[117].

Co-trimoxazole, the most commonly used coformulation of sulphonamide in recent years, has been limited to at least 14 well documented cases of AIN[115,117,139,150-152] with a disseminated rash or a Stevens-Johnson-like syndrome. Renal failure is a predisposing factor, mainly when dosage has not been adapted to renal function[151].

Other antimicrobial drugs described in association with acute interstitial nephritis are: minocycline[153], vancomycin[154], piromic acid[155,156], erythromycin[157], and salicylazosulfapyridine[158].

2. Cimetidine

At least 13 documented cases of acute interstitial nephritis have been described after cimetidine therapy[115,159-168]. In most of them there is a clear clinical and biological picture of allergy with, in one case[159], a positive lymphocyte transformation test and macrophage inhibition assay.

3. Diuretics

In spite of the wide use of diuretics only a small number of patients have been described with diuretic-related acute interstitial nephritis. In the majority of the cases thiazides were implicated (chlorothiazide[169], hydrochlorothiazide[170], cyclothiazide[117]), but triamterene is often associated, and certain observations suggest a

potentiating role for this drug[117,133,171]. In one case[171], T cell subsets have been determined using monoclonal antibodies. The vast majority of T cells were T4+, whereas only a minority expressed the T8+ phenotype. These latter cells were found predominantly in close proximity to the tubules. Interstitial granulomas have been found in several cases and may have been induced by T cells[117,133,171]. Pre-existing chronic GN also be a predisposing factor[169,170]; chlorthalidone[4], triamterene[172] and tienilic acid[117,173] are also responsible for isolated cases. No biopsy-proven allergic interstitial nephritis has been described with the widely used furosemide, but some case records suggest acute interstitial nephritis following this drug.

4. Analgesic and non-steroidal anti-inflammatory drugs

a. Glafenine and derivatives. Glafenine-induced acute renal failure is a common problem. In the majority of cases renal failure seems to be due to a direct toxic effect without evidence for an immune mechanism. In some cases acute haemolysis (often mediated by immune mechanisms) is associated with renal failure. The basophil degranulating test or lymphoblastic transformation test may be positive, and anti-glafenine antibodies can be detected by the anti-globulin test[117]. At least three cases of glafenine-induced acute renal failure have been described with a typical histological pattern of allergic interstitial nephritis, but without haemolysis[117]. Glafenine derivatives anthrafenine and floctafenine can induce acute renal failure by the same mechanisms[117].

b. Other analgesics. Other analgesics responsible for isolated cases of immune interstitial nephritis include phenazone[174], noramidopyrine and aminopyrine[117,175], sulphinpyrazone[115,176], and paracetamol[117].

c. Clometacine. Clometacine is an indomethacin analgesic derivative without anti-inflammatory effects, widely used in western Europe. Three cases of AIN and cholestatic jaundice have been associated with its use[177-179]. In one case the degranulation basophil test was positive in the presence of clometacine[178]. In another, the study of the interstitial infiltrate with monoclonal antibodies showed that 75% of the total lymphocytes were T cells with an OK T8/OK T4 ratio of 0.5. Furthermore, preincubation of the patient's peripheral blood lymphocytes with clometacine resulted in an increased sensitivity to interleukin II, and in a positive syngeneic mixed lymphocyte culture[179].

d. Non-steroidal anti-inflammatory drugs. Non-steroidal anti-inflammatory drugs (NSAID) can induce allergic interstitial nephritis. Most of the reported cases have been associated with the nephrotic syndrome, and many cases of acute renal failure in association with NSAID have been described since the first reports which implicated phenylbutazone[118], but are poorly or incompletely documented without histological data. In some reports the role of other drugs administered with NSAID cannot be excluded, and it is not possible to distinguish between vasomotor nephropathy and acute interstitial nephritis. In only some, proteinuria or total serum protein are documented. In a recent review, Abraham and

Keane[180] mentioned six cases of acute interstitial nephritis without the nephrotic syndrome, but no more than 10 well-documented cases are in the recent literature with eight different drugs (see Table 7 for drugs and references). It is of interest to note that in two patients glomeruli displayed 15%[186] and 30%[187] foot process effacement. Thus there may be some overlap between NSAID-induced acute interstitial nephritis with and without nephrotic syndrome.

5. Other drugs

Other drugs are responsible for isolated cases of acute interstitial nephritis, such as allopurinol[141,189-191], carbamazepine[192], clofibrate[193], diphenylhydantoin[124,127], and phenindione which is no longer widely used.

In conclusion, given the large number of drugs available one marvels at the small number of recognized drug-induced immunological interstitial kidney injuries. It is likely that new products will be implicated but the lag period is unpredictible. For example cimetidine-induced interstitial nephritis was described a short time after its introduction, whereas the first case of chlorpropamide-induced nephritis[194] was described many years after widespread use of the drug. It is also necessary to point out that extrarenal autoimmune disorders may affect patients simultaneously. The association between AIN and immune haemolysis is well-documented with glafenine[119], as is the association with myositis after cimetidine therapy[159] and with hepatitis after clometacine[178-180] and allopurinol[191]. Even if drugs are able to induce both acute interstitial nephritis and systemic vasculitis (allopurinol, thiazides, sulphonamides, penicillin G), the two are rarely seen in the same patient. It is unlikely that the same drug is responsible for causing both entities in these few case reports.

C. Drug-induced immunological interstitial nephritis and glomerulopathy

The association of acute interstitial nephritis and the nephrotic syndrome has been attributed to several drugs but principally to non-steroidal anti-inflammatory drugs (NSAID). It must be stressed that fenoprofen, which belongs to the propionic acid family was the culprit in more than half of the 50 reported cases. The first two cases of acute interstitial nephritis with the nephrotic syndrome to fenoprofen were reported by Brezin et al. in 1979[195]. About 30 similar cases have been published since[3], but according to Lorch et al.[196] about 100 cases were compiled by the manufacturer in a single year (1980). An accurate description of what has been called "fenoprofen nephropathy" can be made from an analysis of the published cases (see references in ref. 3). Female (male/female ratio = 4/16) and elderly patients (mean age 64 years, range 50-84) are particularly at risk. Mean duration of exposure to fenoprofen was 7.9 months (range: 1-36 months). Daily dosage of fenoprofen were always in the therapeutic range. Except for the rare case of

eosinophilia there are no extrarenal symptoms of hypersensitivity. Renal failure, always present, may be severe enough to necessitate dialysis. Immunological tests: complement components, antinuclear and anti-DNA antibodies, cryoglobulins, rheumatoid factor and circulating immune complexes are normal or negative. Renal function returned to normal and proteinuria disappeared in the vast majority of patients upon withdrawal of the drug. However, in some patients renal failure persisted until steroid therapy was instituted, and Stachura et al.[197] recently expressed the opinion that patients with severe uraemia should be treated with steroids. A few patients retained some degree of renal failure and/or proteinuria for several months.

Renal histological findings were reported in 26 cases. Glomeruli were normal by light microscopy and the interstitium was oedematous and infiltrated with large numbers of mononuclear cells associated in 40% of cases to eosinophils. By immunofluorescence, there was no staining with anti-IgA, IgG, IgM, C3 antisera, although a granular pattern for C3 and IgG was found in TBM in occasional cases[195,198]. Ultrastructurally, extensive foot process fusion was noted in all the cases studied, and a few electron-dense deposits were seen on the epithelial side of the GBM[50]. In one case histological abnormalities had almost completely disappeared at a second biopsy performed 2 months later[197]. Studies of the lymphocyte subpopulations within the inflammatory interstitial infiltrates were performed in six patients[197,200,201]. T cells constituted about 80% and B cells about 20% of the total lymphocyte population. Cytotoxic/suppressor T cells predominated over helper/inducer T cells. The great majority of B cells were IgE bearing cells[197].

Non-steroidal anti-inflammatory agents other than fenoprofen can induce an acute interstitial nephritis with minimal glomerular change albeit less frequently. Although most cases have been published as short reports, it seems that clinical and histological data in these cases are not appreciably different from what is observed in "fenoprofen nephropathy". However the female predominance of "fenoprofen nephropathy" is not observed here. Of the 20 cases reported to implicate nine different drugs zomepirac was incriminated in six cases and piroxicam in three (see Table 7 for cases and references). In addition to NSAID-induced acute interstitial nephritis (with and without nephrotic syndrome) it must be stressed that minimal glomerular change without acute interstitial nephritis and renal failure has been observed in at least two cases, one with sulindac[214] and one with tolmetin[215]. In contrast with the aforementionned studies induced by NSAID, Bender et al.[216] found no histological differences between cases of acute interstitial nephritis and minimal glomerular changes (three with fenoprofen, one with ibuprofen, one with zomepirac, one with tolmetin) and three that were not drug-induced. The percentage of B cells and the OK T4/OK T8 ratio were not different in the two groups. These authors questioned the existence of a distinct "fenoprofen nephropathy", and mention the presence of giant collecting duct cells observed exclusively in NSAID-induced renal failure with minimal glomerular changes. Acute interstitial nephritis with minimal glomerular changes has also been reported in in-

dividual cases of therapy with ampicillin[119], rifampicin[217] and recombinant leucocyte A interferon[218], where the histological findings were similar to those typically found in "fenoprofen nephropathy".

Acute renal failure has been attributed to rifampicin in six other patients, where interstitial and glomerular lesions coexisted in three patients[219-221], whereas glomerular lesions were predominant in the others[222-224]. In one patient[224] renal biopsy disclosed a severe immune extracapillary GN with minimal inflammatory interstitial infiltrate. In this case acute renal failure supervened during a first continuous daily antituberculous therapy including rifampicin.

Extracapillary immune complex-type glomerulonephritis associated with acute interstitial nephritis has also been reported after chlorpropamide therapy[194]. A second renal biopsy performed after discontinuation of the drug showed disappearance of extracapillary proliferation. It is worthwhile to point out two cases of a reversible overt nephropathy with Henoch-Schönlein purpura due to piroxicam[225], and one case of an anaphylactoid reaction with focal cortical necrosis due to zomepirac[226]. A few cases of allergic interstitial nephritis with severe necrotizing glomerulitis and/or vasculitis have been described after penicillin[227], sulphonamide, thiazide and allopurinol therapy[130]. In at least some of these cases the relationship between renal involvement and drug exposure is questionable because of the underlying disease (septicaemia, endocarditis, drug abuse) and/or the absence of satisfactory immunomorphological studies. Two cases of spontaneously resolving acute interstitial nephritis with necrotizing GN have been observed ; one with cutaneous vasculitis, the other with articular and digestive tract involvement after co-trimoxazole and indomethacin therapy respectively (unpublished observations).

VII. PATHOGENESIS OF IMMUNOLOGICALLY MEDIATED NEPHRITIS IN HUMANS

a. Immune complex nephritis

These nephritides are the consequence either of deposition of circulating antibodies or of in situ immune complex formation. The numerous experimental models that have been described are probably the most convincing evidence that drug exposure may induce such immunologically mediated GN. However the subtle mechanisms at play are usually unknown, and there is little evidence that drugs or toxins may induce renal damage by modifying self antigens or by acting as haptens. Probably the only example is that of methicillin-induced antibodies (either anti-TBM or directed towards a metabolite of methicillin). It is usually considered that gold- or mercury-induced membranous GN could be due to the induction of antibodies similar to those observed in Heymann's nephritis, but such antibodies have never been demonstrated. It has also been claimed that gold or mercury could act as haptens, but neither gold nor mercury could be detected within the immune material deposited. Although D-penicillamine interferes with collagen metabo-

lism this has not been shown to result in auto-antibodies synthesis.

In contrast, several experiments suggest that many drugs which induce immunologically mediated nephritis have an immunomodulatory effect. Gold, D-penicillamine and mercury may also interfere with the immune response. Besides the immunosuppressive effect of both gold and D-penicillamine, these agents may have stimulatory effect. D-penicillamine has been shown, for example, to act as a polyclonal activator in mice, and mercury clearly interferes with the immune response in BN rats. More recent experiments suggest that this drug could induce a graft-versus-host like disease in mice. We have already mentioned the immunomodulatory effect of mercury in the rat. Charpentier et al.[228] also showed, in a patient with mercury-induced membranous GN, that mercury was able to modify the allogeneic response.

Whatever the precise mechanisms of action of the drug, it is clear that the effect observed depends on genetic factors:

1. Drug induced lupus-like syndrome; gold- and D-penicillamine-induced membranous GN are more frequently observed in patients with the DR4[229] and DR3[47,48] antigen respectively. The role of class II antigens is also confirmed from experimental studies.
2. Antinuclear antibodies are more frequently encountered in slow acetylators[230], and it is noteworthy that the acetylated form of procainamide does not induce antinuclear antibodies[231].

In conclusion, it is most likely that the mechanisms are highly complex and that one drug may act in different ways. It is of interest to mention in that respect that recent experiments show that drugs such as hydralazine interact in vitro with the fourth component of complement and may therefore modify the clearance of immune complexes[232].

b. Drugs inducing the nephrotic syndrome with minimal glomerular changes

It is well known that drugs such as adriamycin or puromycin induce the nephrotic syndrome with minimal glomerular changes in the rat. This glomerular lesion is dose-dependent, has been observed in all the strains tested and is considered to be the consequence of a direct toxic effect of the drug.

There is good evidence that, in humans, drug-induced nephrotic syndrome associated with minimal glomerular changes may be of immune origin:

1. Only a few of the patients exposed develop the glomerulopathy which is against a toxic effect.
2. Several drugs such as D-penicillamine, gold salts or mercury compounds may induce either a membranous GN or the nephrotic syndrome with minimal glomerular changes. This suggests that, depending on still unknown factors, either

humoral immunity or cellular immunity are involved.

3. Similarly, drug-induced nephrotic syndrome with minimal glomerular changes is often associated with immuno allergic acute interstitial nephritis characterized by interstitial infiltration with T lymphocytes. It is therefore tempting to speculate that activated T cells could play a role in the appearance of the nephrotic syndrome.
4. The steroids and cyclophosphamide have been reported to have a beneficial effect on these drug-induced nephrotic syndromes.
5. Finally there are several arguments suggesting that the idiopathic nephrotic syndrome is immunologically mediated. Shaloub[38] first suggested that lipoid nephritis could be a consequence of T cell dysfunction. Others have then suggested that lymphokines released by activated T cells could increase vascular permeability[233]. It is therefore tempting to speculate that both lipoid nephrosis and the drug-induced nephrotic syndrome with minimal glomerular changes have similar pathogenesis. Unfortunately there is no experimental model of drug-induced nephrotic syndrome with minimal glomerular changes of immune origin.

c. Immunoallergic acute interstitial nephritis

Although there is no experimental model of drug-induced immunoallergic acute interstitial nephritis available, there are data supporting a role for the involvement of immune reaction. Both humoral and cellular immunity are probably at play. As is the case in experimental models of immunologically mediated nephritis, it is often difficult to delineate their respective roles.

As already mentioned there is good evidence that anti-TBM antibodies are involved in at least some cases of methicillin- or diphenylhydantoin-induced immunological nephritis. Lymphocytes have, however, been found in similar situations, and it is difficult to know the respective roles of antibodies and cell infiltration. Indeed, in the majority of acute interstitial nephritis no immune reactants are found deposited and the most striking feature is the presence of cells infiltrating the interstitium with occasional granulomatous reactions, with mononuclear cells and occasional eosinophils or plasma cells. The phenotype of mononuclear cells has been studied in a few cases of acute interstitial nephritis due to non-steroidal anti-inflammatory agents[198], cimetidine[160] or thiazide[173]. T cells were found to constitute the majority of cells comprising a majority of T4 (helper/inducer) cells and a minority of T8 (suppressor/cytotoxic) cells. Interstingly, in one study T8 cells were found in close proximity of tubules, and in another one, T8 cells were found to be mostly cytotoxic cells, but a similar pattern was observed in three patients with interstitial nephritis and proteinuria without drug exposure[216]. The reason for this influx of T cells is unclear. The most tempting hypothesis is that T cells are activated as a consequence of drug exposure. It is also possible that T cell influx is secondary to a direct drug-induced toxic effect.

CONCLUSION

Numerous questions remain to be answered. It is often very difficult to assess the relationship between drug or toxin exposure and the occurrence of nephritis. It is also difficult to make sure that drug-induced nephritides are of immune origin. The availability of reliable assays would be most helpful is that respect. Another important question for the clinician is to know whether the withdrawal of a suspected drug is always necessary. Drug-induced autoimmunity in the BN rat is spontaneously autoregulated and gold- or D-penicillamine-induced GN in man may have a non-remissive course and withdrawal of the drugs may be unjustified. Once a drug has been recognized as a culprit it becomes important to develop non-deleterious new drugs with a similar beneficial effect. Auranofin, or an acetylated form of procainamide, or hydralazine, are good examples. Another interesting way to prevent the appearance of drug-induced immune reactions would be to characterize those patients at risk (e.g. slow acetylators, or patients with DR antigens associated with increased susceptibility). Finally, the mechanisms of drug-induced autoimmunity are still unclear and constitute an exciting research area, for which the development of experimental models is important.

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26

CLINICAL AND EXPERIMENTAL NEPHROTOXICITY OF CANCER CHEMOTHERAPEUTIC AGENTS

C.L. LITTERST AND R.B. WEISS

INTRODUCTION

Cancer chemotherapeutic agents classically affect populations of rapidly dividing cells, in both malignant and normal tissue. Hence the most commonly encountered toxic effects are in the bone marrow, gastrointestinal tract, gonadal tissue, and hair follicles. Several drugs in this group also affect other tissues, such as the kidney. This chapter examines those agents which have been implicated in nephrotoxicity in the clinical setting and presents the clinical manifestations and pathogenesis, as well as the effect of drug interactions and clinical methods of blocking the renal toxicity. Four agents (cisplatin, doxorubicin, daunorubicin, and mitomycin C) are examined in more detail to consider the information that has been generated in animal studies that relate to the total picture of nephrotoxicity, as well as the molecular pathogenesis of the renal lesion. Other agents which are known to produce clinical renal toxicity are not covered in detail because: (1) the effect is purely physical and not specific for renal cells (methotrexate; see clinical discussion), (2) the major lesion is in the urinary bladder, rather than the kidney (also the mechanism of action is relatively well understood for cyclophosphamide and ifosfamide), (3) little in-depth experimental work has been conducted in animals, or (4) the drugs represent only a minor clinical problem (nitrosoureas).

I. CLINICAL STUDIES

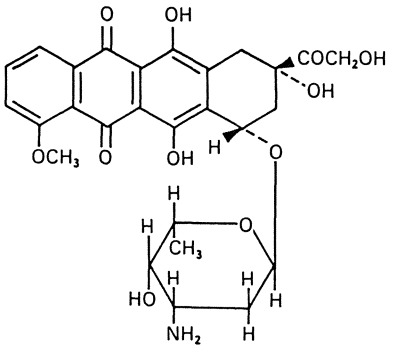
Myelosuppression is the major dose-limiting effect of most cancer chemotherapeutic agents. For a few drugs (e.g. cisplatin and streptozocin) renal toxicity is dose-limiting, while bone marrow suppression is much less of a problem. Some agents (e.g. the nitrosoureas) will produce appreciable renal toxicity only when the

drug is used over a long period. Other drugs (e.g. dacarbazine and L-asparaginase) produce azotaemia that rarely becomes a clinical problem. By contrast, other anticancer agents (e.g. vincristine and vinblastine) have never been known to cause any renal dysfunction. The drugs reviewed in this chapter include those agents commercially available in the United States and Europe, and those agents undergoing investigative studies that already have a well-defined role in cancer treatment. Table 1 lists the drugs under discussion according to their risk of causing nephrotoxicity.

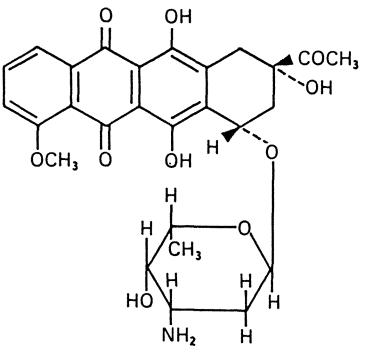
Table 1 Cancer chemotherapeutic agents and their risk of nephrotoxicity

High risk of immediate nephrotoxicity	
Cisplatin	
High-dose methotrexate	
High-dose mithramycin	
Streptozocin	
High risk of nephrotoxicity from long-term use	
Lomustine	
Mitomycin	
Semustine	
Low or moderate risk of nephrotoxicity	
5-Azacytidine	
Intravenous high-dose 6-thioguanine	
Low-dose methotrexate	
Pentostatin	
Azotaemia without risk of nephrotoxicity	
Dacarbazine	
L-Asparaginase	
Very low risk of nephrotoxicity	
Carmustine	
Cyclophosphamide	
Not known to have nephrotoxicity	
Amsacrine	Hexamethylmelamine
Bleomycin	Hydroxyurea
Busulfan	Mechlorethamine
Chlorambucil	Low-dose melphalan
Cytarabine	6-Mercaptopurine
Dactinomycin	Mitotane
Daunorubicin	Procarbazine
Doxorubicin	Teniposide
Etoposide	Oral 6-thioguanine
5-Fluorouracil	Thiotepa

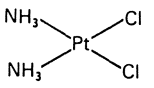
In assessing a patient with a malignancy who develops renal dysfunction while on treatment, one must always keep in mind that the cancer itself may be involved in the aetiology of the renal problem[1]. The cancer chemotherapeutic agents are not always necessarily the cause.



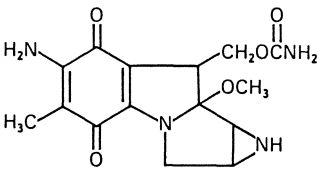
DOXORUBICIN (Adr)



DAUNORUBICIN (Dauno)



cis-PLATINUM (cis Pt)



MITOMYCIN C (Mit C)

Figure 1 Chemical structures of selected antineoplastic drugs.

A. Cisplatin

Cisplatin (cis diammine dichloroplatinum II) is an inorganic complex of platinum with two ammonia groups and two chloride ligards (Figure 1). Early studies in experimental animals with this agent indicated that it was toxic to the proximal convoluted tubules[2], and it is not surprising that cisplatin caused renal dysfunction in patients treated in the initial clinical trials[3,4]. Renal dysfunction was more prevalent as drug doses were increased, and the development of irreversible renal failure necessitated stopping cisplatin administration in some patients.

It is now well established that cisplatin can produce both acute and chronic renal dysfunction, sometimes leading to irreversible renal failure[5,6]. Repeated cisplatin doses of 50 mg/m^2 or greater, administered either as a single dose or over several days, will eventually result in renal dysfunction in most patients, unless measures are taken to minimize this toxicity. If the renal dysfunction is not severe it generally reverses when the drug is discontinued. However, most often, subclinical damage remains in the form of a decreased glomerular filtration rate (GFR)[6].

Clinical manifestations

A rise in blood urea nitrogen (BUN) and/or serum creatinine is the initial indication of cisplatin-induced renal dysfunction. Creatinine clearance is usually also reduced, and this can be decreased even without significant changes in the serum creatinine or BUN. This fact necessitates a baseline determination of the creatinine clearance before initiating cisplatin therapy and periodic repeat testing, especially if the serum creatinine rises above the normal range. Serum urea and creatinine concentrations may not rise appreciably until considerable renal function is lost. Most patients receiving repetitive cisplatin doses develop a reduction in creatinine clearance over a period of time. This may stabilize at a moderate level of dysfunction which is insufficient to warrant stopping cisplatin[6]. There is little correlation between the total amount of cisplatin administered and decremental changes that occur in creatinine clearance.

Proteinuria is generally not a feature of cisplatin-induced nephrotoxicity because cisplatin is primarily a tubular toxin and little, if any, glomerular injury occurs. If severe nephrotoxicity occurs, the urinary sediment may contain a few red and white blood cells. Renal tubular cells and granular casts may be present, but these usually occur only when severe renal dysfunction has occurred[6].

A single dose of cisplatin can cause large urinary losses of calcium, amino acids, and magnesium[7]. Magnesium loss is very common and is usually asymptomatic, but it can be severe enough to cause personality changes, muscle cramps, tremor, and twitching[8,9]. Once cisplatin is discontinued, serum magnesium levels usually return to normal, but persistent hypomagnesaemia up to 3 years later has been reported[10]. Hypomagnesaemia appears to increase in frequency as repetitive cisplatin doses are administered[11]. The serum creatinine is usually normal when hypomagnesaemia is present.

Hypocalcaemia can also occur as part of the magnesium wastage phenomenon. The combination of hypomagnesaemia and hypocalcaemia may result in tetany or other manifestations of major central nervous system dysfunction such as emotional lability, ataxia, and cranial nerve palsies[12,13]. These more severe neuromuscular abnormalities probably occur as a result of the additive effect of the two electrolyte imbalances. The hypocalcaemia may be due to reduced parathormone release or diminished end-organ responsivness to parathormone mediated by the hypomagnesaemia.

Cisplatin has also been recognized to induce severe renal sodium wasting leading to neuromuscular irritability and possibly seizures[14,15]. Cisplatin causes high urinary sodium excretion by inhibiting sodium resorption in the loop of Henle.

The mechanism of the ion wasting is not fully understood, but it is assumed to be related to inhibition of important active transport systems in the tubules. Cisplatin has been shown to inhibit PAH transport in the flounder[16]. This is a non-specific effect, however, and does not explain the defective magnesium conservation.

Subclinical defects in tubular function induced by cisplatin have been demonstrated in studies of urinary microprotein excretion. Measurement of urinary β_2-microglobulin and β-glucuronidase indicate that a single cisplatin dose causes an increased excretion of these low molecular weight proteins[17,18] even in the absence of changes in the serum creatinine, and despite the use of those techniques that protect against nephrotoxicity. Cisplatin affects tubular function to at least a slight degree, despite active efforts to prevent it.

Age relationships

Renal function diminishes with advancing age - a factor that could exacerbate cisplatin nephrotoxicity. This issue has been addressed in a study that compared serial creatinine clearances in young and old patients receiving the same cisplatin dose and forced diuresis[19]. There was no age-related difference in the degree of deterioration of renal function induced by cisplatin. Therefore cisplatin dose does not need to be modified based on the patient's age. This study also demonstrated that dose modifications are unnecessary if the patient has only one kidney[19].

When cisplatin is used in young children, reliance on serial determinations of serum creatinine and creatinine clearance may underestimate the degree of renal dysfunction produced by cisplatin. It is difficult to obtain accurate timed urine collections in paediatric patients; and serial measurement of GFR has been recommended as a more reliable indicator of cisplatin-induced renal dysfunction[20].

Cisplatin interaction with other drugs

Aminoglycoside antibiotics and amphotericin are both nephrotoxins, and may be necessary to treat infections in patients who have received cisplatin. Additive nephrotoxicity of cisplatin and these antimicrobial agents has been reported by many observers[21,22]. Subclinical cisplatin-induced renal dysfunction may be present, even months after cisplatin administration, and when these other nephrotoxins are given, acute renal failure occurs. These antimicrobial agents must therefore be used with great caution in anyone previously treated with cisplatin. An interaction between cisplatin and antihypertensive agents has been reported to accentuate renal toxicity[23]. Such agents should also be used with caution in the patient receiving cisplatin.

An interaction between cisplatin and bleomycin can enhance the pulmonary toxicity of bleomycin[24,25]. Cisplatin-induced renal dysfunction decreases urinary excretion of bleomycin, the predominant route of its elimination. Prolonged bleomycin excretion results in greater exposure of the lung to the drug which can cause severe, and even fatal, pulmonary toxicity[26].

Histology

The physiological site of cisplatin nephrotoxicity (the tubule) is also the site of histological abnormalities. Focal epithelial necrosis occurs in the distal convoluted tubules and collecting ducts[21]. Tubular lumina contain eosinophilic material, and hyaline and granular casts are seen in the collecting ducts, but the glomeruli are usually unaffected.

Nephrotoxicity protection

The initial clinical studies with cisplatin[3] indicated that it had useful antitumour activity, but the frequent irreversible renal toxicity somewhat discouraged interest in this new agent. In the mid-1970s investigators at the Memorial Sloan-Kettering Hospital devised means to minimize and even totally circumvent nephrotoxicity. After first proving the concept was valid in experimental animals[27], these investigators initiated a clinical trial using hydration and diuresis to reduce nephrotoxicity[28]. The forced diuresis prevented cisplatin-induced nephrotoxicity in most patients, even when high and repetitive doses were used. This hydration technique opened the door to widespread, safe use of cisplatin, and allowed the drug to be successfully utilized as a curative treatment for some cancers and an effective palliation for others.

Since this initial work the hydration techniques have become widely standardized. The usual practice is to begin hydration about 12 hours before, and continue it for 24-48 hours after, administering cisplatin. There are many different methods employed to cause a forced diuresis. Most involve use of high volumes of saline or half-normal saline (approximately 3000 ml/24 h). Sodium chloride solutions are preferentially used because the high chloride concentration prevents hydrolysis and inactivation of the administered cisplatin. Diuretics such as furosemide and mannitol have been used as additional means of forcing diuresis. A randomized trial comparing furosemide versus mannitol, each with saline, demonstrated no difference in efficacy of nephrotoxicity protection[29]. Many investigators have used saline alone, without diuretics, and have achieved good protection from nephrotoxicity[30]. Thus there is no evidence to indicate that furosemide and/or mannitol are necessary to augment the protective effect of saline hydration.

Magnesium sulphate can be administered with cisplatin to prevent hypomagnesaemia, especially when high cisplatin doses are used. The addition of 1 to 3 g of magnesium sulphate to the hydration fluids is safe, simple, and effective in preventing symptomatic hypomagnesaemia[31].

Why does hydration protect the kidney? Experiments conducted by Pera et al.[32] show that the afforded protection is not the result of increased cisplatin excretion, more rapid plasma clearance, or decreased levels of cisplatin in the kidneys. It appears to be due to a lower concentration of cisplatin and a shorter duration of drug exposure in the renal tubules.

Prolonged hydration with large intravenous fluid volumes is

inconvenient and may require the patient to be hospitalized to receive each cisplatin course. Also, patients with cardiac dysfunction may tolerate the hydration poorly. Other methods of nephrotoxicity protection have thus been evaluated to circumvent these problems.

One approach was to reduce the cisplatin dose to a level ($\leq$ 40 mg/m^2) below that usually associated with nephrotoxicity and administer it weekly without hydration[33]. Unfortunately, dose-limiting nephrotoxicity still occurred in one-half of the patients studied.

Another approach has been to use sodium thiosulphate for nephrotoxicity prevention. This substance has been tested in experimental animals, based on the theory that cisplatin tubular toxicity is mediated through platinum binding to sulfhydryl groups on tubular transport enzymes[34]. Thus, if an effective platinum chelator were administered before irreversible tubular damage occurred, renal toxicity might be decreased. Clinical trials of cisplatin administered intraperitoneally with intravenous thiosulphate as the protective agent have demonstrated that nephrotoxicity is markedly reduced, while antitumour efficacy is maintained[35]. Thiosulphate cannot be used when cisplatin is given intravenously, because of probable reduction in antitumour effect as has been demonstrated in animal studies[34].

A method has been devised to allow administration of very high doses of cisplatin without nephrotoxicity. Animal studies suggested that cisplatin-induced nephrotoxicity could be reduced if a concentrated sodium chloride solution were administered concomitantly[36]. Clinical studies later showed that cisplatin doses of 200 mg/m^2 could be safely used if 3% NaCl was co-administered[37]. Combination chemotherapy using this cisplatin dose was highly efficacious in patients with testicular cancer and high tumour burdens[37].

An interesting, and relatively simple, method of reducing cisplatin nephrotoxicity is to administer the drug at a chronotoxicological advantageous time of the day. Hrushesky and colleagues[38,39] have shown in experimental animals and in clinical studies that nephrotoxicity varies relative to when the drug is administered in the circadian rhythm. The clinical studies indicated that the optimal time for drug administration is the evening[39]. Further studies showed that when cisplatin was administered at or near the time of peak urinary excretion of potassium, there was negligible nephrotoxicity.

Heavy metals such as platinum are well known to be nephrotoxic, but the presence of the platinum atom is not the only factor in the renal effect. The configuration of the platinum derivative is also important. Only the cis isomer of the platinum complex is nephrotoxic; the trans isomer is not. Moreover, structure modification of the ligands of the cisplatin complex can greatly alter the frequency and degree of nephrotoxicity. These facts have spurred investigators to synthesize and evaluate over 1000 analogues of cisplatin, seeking agents with enhanced antitumour efficacy and/or reduced nephrotoxicity[16]. Some of these agents have reached phase I clinical trials but proved to be no less nephrotoxic than cisplatin. An example is the analogue named TNO-6. This analogue

offered no advantage in therapeutic index to cisplatin and probably will not be studied further[40]. Two other analogues, carboplatin (already marketed in some countries) and iproplatin, have shown more promise. Carboplatin appears to have equivalent or greater antitumour efficacy to cisplatin but little, if any, nephrotoxicity[41]. However, it has greater haematological toxicity, especially to platelets. Iproplatin, which has not been studied as extensively as carboplatin, also has less nephrotoxicity and more haematological toxicity than cisplatin[42]. The development of cisplatin analogues is an ongoing process, and any promising compound will be brought to clinical trial. Those analogues that provide an advantage in therapeutic index (especially relative to nephrotoxicity) will be developed further and marketed.

B. Streptozocin

Streptozocin is a naturally occurring nitrosourea-glucosamine that is useful for the treatment of advanced islet cell carcinomas and carcinoid tumours. In the first phase I trial reported with this drug, all 18 treated patients developed renal dysfunction, and two of them became anuric[43]. Schein et al.[44] treated 106 patients and noted renal abnormalities in 28%; this was the most common form of toxicity. Nephrotoxicity contributed to the death of four patients. Moertel and co-workers[45] saw evidence of nephrotoxicity in two-thirds of 38 patients treated with streptozocin. It also occurred in two-thirds of 52 patients in another series, and five of these patients died of renal failure[46]. Renal toxicity occurs frequently with streptozocin and is the dose-limiting side effect. The incidence rises with prolonged drug administration so that eventually most patients display nephrotoxicity.

The major excretion pathway of streptozocin is the urine[44]. Streptozocin injures the glomerulus and tubule, based on histological abnormalities that have been observed in both sites[46]. However, the mechanism for nephrotoxicity is not known.

Clinical manifestations

The clinical features of streptozocin nephrotoxicity involve abnormalities of both glomerular and tubular function. The earliest sign of renal dysfunction is hypophosphataemia, and this can occur after only a single dose[43]. Glycosuria, acetonuria, hyperchloraemia, and aminoaciduria are also seen, indicating renal tubular acidosis[44]. Proteinuria occurs in half of the patients, making this the most frequent finding. It can appear in some patients after only a few doses. Protein excretion is usually mild, and only rarely does it approach the levels associated with nephrotic syndrome. Elevations in BUN and serum creatinine are later findings, and sometimes do not occur despite the presence of renal abnormalities of other types[44]. If the indications of renal toxicity are only mild, and the drug is stopped, they will return to normal. However, with continued treatment irreversible, and sometimes fatal, nephrotoxicity can occur.

Histology

The most prominent features histologically are tubular (mainly proximal) changes[47]. Extensive tubular atrophy, interstitial inflammatory infiltrates and glomerular lesions in the form of cellular tufting are seen.

Nephrotoxicity protection

Streptozocin is equally nephrotoxic to cisplatin, but it has not been accorded the same degree of scientific investigation to minimize this adverse effect. Streptozocin is an effective drug for only two rare cancers. Thus, it is only used occasionally, and investigation into means of decreasing nephrotoxicity has not been of a high priority.

C. Mitomycin

Preclinical studies with mitomycin showed that acute tubular necrosis occurred in monkeys given a single intravenous dose[48], and thus clinical nephrotoxicity was to be expected. Liu and colleagues[49] described mitomycin-induced nephrotoxicity in humans in 1971. By a decade later mitomycin was recognized to cause two major forms of nephrotoxicity. One is a slow, progressive deterioration in renal function, and the other is an acute renal failure associated with features of the haemolytic uraemic syndrome[50-52]. The overall incidence of nephrotoxicity is 8-10% with an approximately equal frequency of the two forms of renal injury. Most of the cases of haemolytic uraemic syndrome are fatal, although early recognition and/or vigorous treatment have sometimes reversed the process[53-55].

Clinical manifestations

A rising serum creatinine or BUN is usually the initial manifestation of the slowly progressive form of nephrotoxicity. Proteinuria, hypertension, and occasionally haematuria are observed.

The haemolytic uraemic syndrome is usually characterized by an abrupt onset. Erythrocyte fragmentation, microscopic haematuria, proteinuria, azotaemia, maculopapular rash, hypertension, anaemia, and thrombocytopenia are seen. The renal impairment frequently progresses to renal failure that requires dialysis. Neurological symptoms may occur. Some patients also have pulmonary oedema and severe hypoxaemia as part of the syndrome[56]. Decreased serum fibrinogen levels and increased fibrin split products can be seen.

The progressive renal impairment without the haemolytic process usually begins 6-12 months after mitomycin treatment is initiated[50-52]. It appears to have a relationship to cumulative dose, but some patients develop renal dysfunction after only several doses.

The haemolytic syndrome also usually appears after some 6 months of therapy, but it can also occur after only one mitomycin dose[51-56]. In some patients the haemolysis and renal failure occur months after discontinuation of the mitomycin. It is thus an unpredictable condition and can develop at any time a patient is receiving the drug or after its discontinuation.

Histology

Light microscopy shows focal glomerular abnormalities in most cases. Lesions noted are mesangiolysis and cellular atypia[50,56,57]. Nuclear abnormalities are also prominent. The haemolytic uraemic syndrome produces capillary thrombi and luminal narrowing. The glomerular basement membranes are thickened and disrupted. Fibrin deposition and arteriolar endothelial proliferation are also seen. The tubules are less apt to be affected, but cellular atypia can occur[56]. Staining for immunoglobulins and components of complement usually demonstrates no abnormalities. Fibrin stains show conspicuous glomerular capillary thrombi[56].

Pathogenesis

Most of an administered dose of mitomycin is metabolized by the liver and urinary excretion is limited[58]. The mechanism of mitomycin nephrotoxicity is therefore probably not related to urinary elimination. There have been many proposed explanations for the haemolytic uraemic syndrome[51]. Immune complex formation and the presence of cancer are probably not mediators of the syndrome. The toxicity (both the slowly progressive form and the haemolytic uraemic syndrome) may be mediated by a direct mitomycin effect on glomerular capillaries. Experimental work in rats supports this theory[59]. The endothelial damage leads to platelet deposition and activation of the coagulation system, and fibrin deposition occurs in the renal microvasculature.

Nephrotoxicity protection

The haemolytic uraemic syndrome has a high mortality. When the accompanying anaemia is treated with transfusions, the renal failure frequently worsens. Various methods of treatment using steroids and antiplatelet drugs have been generally ineffective[51]. Plasmaphaeresis has had variable success. Plasma perfusion over filters containing staphylococcal protein A moderated the renal failure in some patients[54].

Treatment frequently fails to reverse the renal failure, but at present there is no known method of preventing mitomycin nephrotoxicity. Therefore one must be aware of the risk of nephrotoxicity and monitor the renal function of any patient on this drug, especially after 6 months or more.

D. Nitrosoureas

Streptozocin is a nitrosourea, but its high nephrotoxic potential warrants a separate section. Renal toxicity was also noted in the preclinical toxicological studies of carmustine (BCNU), lomustine (CCNU), and semustine (methyl CCNU)[60]. Lomustine in particular caused fatal chronic interstitial nephritis in monkeys.

In the earliest clinical trial of carmustine, 10% of the patients had unexplained elevations of BUN, but there were no instances of severe renal problems[61]. Numerous subsequent reports of trials with these drugs specifically stated that no instances of significant renal toxicity had occurred. Thus renal problems were believed not to be of major importance with any of these drugs. In the past decade, however, semustine and lomustine have been recognized to produce nephrotoxicity when large cumulative doses are administered[62,63].

The incidence of nitrosourea-induced nephrotoxicity is somewhat difficult to determine because it occurs only after unusually long treatment. Only four cases related to lomustine and 29 cases related to semustine have been collected[62,63]. All instances of nephrotoxicity appeared after cumulative drug doses of 1400 mg/m^2 or more. The number of patients treated with total doses this high is unknown, so no reliable incidence figure can be derived, but it may be as great as 25-35% for patients receiving high cumulative doses[62].

Clinical manifestations

Clinical manifestations are usually limited to the insidious development of an elevated BUN and serum creatinine. Urinary sediment abnormalities are rare and usually minimal. Renal size measured by serial renal scans can progressively decrease, especially in patients who have received at least 1500 mg/m^2 of semustine[64]. Kidney size can sometimes continue to decrease even after semustine is stopped[64].

Histology

For those patients who progress to renal failure the histological features are similar whether the dysfunction is caused by semustine or lomustine. There is marked glomerular sclerosis, interstitial fibrosis, and extensive tubular atrophy[62,63]. Glomerular immunofluorescence is not seen.

Pathogenesis

The mechanism of renal toxicity induced by these three nitrosoureas is no better defined than that caused by streptozocin. All of the drugs are rapidly metabolized, and parent drug cannot be detected in the plasma for more than 20 minutes after administration[60]. Metabolites are excreted in the urine for up to 72

hours after drug administration. Urinary excretion accounts for about 60% of the elimination of these drug products. No parent compound is excreted in the urine, so nephrotoxicity is most likely due to one of the metabolites. There is no explanation for the delayed onset of nephrotoxicity, which in some cases occurs many months after the drug has been discontinued.

Nephrotoxicity protection

Presently, there is no known method of preventing nitrosourea nephrotoxicity other than limiting the cumulative dose that a patient receives. Because amounts greater than 1400 mg/m^2 of semustine seem to be associated with a high risk of nephrotoxicity, this should be the dose limit. The same is probably true for lomustine. Carmustine has a lower nephrotoxic potential. Use of dose limits for pulmonary toxicity from this drug (about 1200 mg/m^2) will probably prevent the onset of serious renal problems.

E. Methotrexate

When methotrexate is administered in conventional oral or intravenous doses, nephrotoxicity is only an occasional problem, but there are rare reports of patients receiving long-term methotrexate treatment (for psoriasis) who died of renal failure[65,66]. Condit and colleagues[67] studied 13 patients with cancer on conventional doses of methotrexate, and assessed its adverse renal effects. They found frequent evidence of subclinical renal dysfunction. Inulin and p-aminohippuric acid clearances were decreased while the BUN stayed normal or increased only slightly. Three patients died of tubular necrosis and renal failure[67]. Even though it is an uncommon problem, patients can die of nephrotoxicity from low dose methotrexate. Because of the subclinical damage methotrexate can induce, a patient with compromised renal function probably should not receive this drug.

Very high doses of methotrexate plus citrovorum factor rescue are widely used for treatment of certain cancers. Nephrotoxicity can occur with serious consequences in this situation. Renal toxicity develops most often when hydration is inadequate for complete renal clearance of the drug. Delayed drug clearance may result in severe stomatitis and myelosuppression.

Clinical manifestations

There are no specific clinical findings. The BUN and/or serum creatinine are elevated, and oliguria or anuria can occur with high-dose drug administration. Electrolyte disturbances are also present.

Methotrexate interaction with other drugs

Acute renal failure secondary to methotrexate treatment has occurred when the patient has been taking indomethicin[68]. It is postulated that there is some subclinical nephrotoxic effect of indomethicin that inhibits rapid and complete elimination of large methotrexate doses. A similar interaction between methotrexate and ketoprofen, causing fatal acute renal failure, has been observed[69]. The non-steroidal anti-inflammatory drugs appear to hinder methotrexate excretion, and a patient should not take any drugs of this class while receiving treatment with methotrexate.

Pathogenesis

Methotrexate is excreted rapidly in the urine whether it is administered orally or parenterally. Henderson and co-workers[70] found that 54-88% of conventional doses are excreted in the urine during the first 24 hours after administration. When Pratt et al.[71] administered very high intravenous doses over 6 hours, the urinary excretion varied from 35% to 53% during the infusion. Ninety per cent of the parent compound had been excreted by 30 hours. At these very high doses a metabolite of methotrexate (7-hydroxymethotrexate) is also excreted in the urine[72]. Methotrexate is cleared both by glomerular filtration and tubular secretion.

The mechanism of methotrexate nephrotoxicity is not fully understood. High-dose methotrexate administration causes a subclinical, transient decrease in GFR without accompanying clinical toxicity[73]. This action may be mediated by either direct tubular toxicity or methotrexate precipitation in the tubular lumen. At physiological pH the drug is fully ionized, but when a saturated solution of methotrexate is lowered to a pH of 5.7 a dense precipitate forms. During urinary excretion, drug precipitation could occur as the urine is concentrated and acidified in the tubules, thus causing luminal obstruction. This process could be reversible. However, when overt nephrotoxicity occurs the GFR decrease is more long-lasting, and there can be cumulative toxicity[73]. These facts suggest that methotrexate directly damages tubular epithelium.

Nephrotoxicity protection

Whatever contribution tubular precipitation of methotrexate makes to this drug's nephrotoxicity, it can be alleviated by the simple process of forced diuresis and alkalinization of the urine. This corrects the problem of high tubular drug concentration and acid urine that facilitates drug precipitation when high drug doses are given. Patients are hydrated intravenously and sodium bicarbonate is liberally administered. This treatment has successfully reduced the problem of nephrotoxicity induced by high-dose methotrexate. As a further precaution, if the patient has a serum methotrexate concentration of greater than 1×10^{-7} mol/l more than 24 hours after a drug infusion, hydration and administration of citrovorum

factor are extended until the drug concentration reaches this non-toxic level. If severe nephrotoxicity develops despite these manoeuvres, then thymidine administration may be necessary in addition to the citrovorum factor[73].

F. Anthracyclines

Daunorubicin

Daunorubicin is a nephrotoxin in various experimental animals[74,75]. It will produce both chronic glomerulonephritis with persistent nephrotic syndrome and renal tumours. In fact, daunorubicin is such a consistent glomerular toxin and inducer of nephrotic syndrome that it has been used to create an experimental model for the study of this renal disease[74]. Despite this clear-cut animal nephrotoxicity, daunorubicin does not cause renal dysfunction in man.

Doxorubicin

Doxorubicin differs structurally from daunorubicin only by the addition of a hydroxyl group, and also produces nephrotoxicity in experimental animals[76,77]. The glomerulus is affected primarily, but vacuolization of the tubular epithelium and interstitial fibrosis have also been seen[76]. In rabbits the renal pathology is dose-dependent and has an onset in parallel with doxorubicin cardiomyopathy. Again, despite this well-demonstrated animal toxicity, doxorubicin has no demonstrable nephrotoxicity in man.

G. 5-Azacytidine

This pyrimidine causes renal tubular abnormalities in experimental animals, and its elimination pathway in man is the urine[78]. Despite these facts, 5-azacytidine is not clearly a nephrotoxin when it is administered by itself. When it is part of a combination anti-leukaemia regimen it does appear to have some nephrotoxic effect. Peterson et al.[79] noted a high rate of urine and blood abnormalities indicative of renal tubular dysfunction in patients being treated with drug combinations that included 5-azacytidine. One or more manifestations of polyuria, glycosuria, sodium wasting, aminoacidura, hypophosphataemia and renal tubular acidosis occurred in 88% of the treatment courses given to 22 patients. Although all patients were treated with other cytotoxic agents, the fact that these investigators did not observe such toxicity using similar treatment without 5-azacytidine suggests that this agent was a major contributor to the renal dysfunction.

H. Mithramycin

In the past mithramycin was used for treatment of testicular car-

cinoma in a dose of 50 μg/mg. Azotaemia occurred in 40% of 54 patients given this drug[80]. Six eventually died of renal insufficiency. It was recommended that the drug be stopped if any rise in serum creatinine occurs, because renal injury is frequently permanent.

Use of mithramycin for testicular carcinoma has now been supplanted by more effective drugs. However, it is widely used as a means of treating hypercalcaemia secondary to malignant disease, where the dose is 25 μg/kg. Serial measurements of renal function during mithramycin infusions at this lower dose showed no significant alterations in one study[81]. A single case of severe nephrotoxicity related to this lower mithramycin dose has been reported[82]. However, this patient had obstructive uropathy and had previously been treated with a large cumulative dose of amphotericin. Thus, if no underlying renal dysfunction is present, it appears that this lower dose of mithramycin will be generally free of nephrotoxic effects.

I. 6-Thioguanine

Oral 6-thioguanine, in the doses usually employed in the treatment of acute leukaemia, has not been associated with nephrotoxicity. However, when high doses are administered intravenously, renal toxicity has been observed[83,84]. This was manifested by mild to moderate azotaemia, and in most cases it was reversible within 2 weeks. Marked azotaemia only occurred at doses of 800 mg/m^2 or more. Whether this dose schedule has meaningful antitumour activity is presently unknown. Thus, nephrotoxicity of this drug may not be a clinical problem.

J. Dacarbazine

Dacarbazine is rapidly excreted in the urine both as intact drug and as the major metabolite, 5-aminoimidazole-4-carboxamide[85,86]. Excretion is via glomerular filtration and tubular secretion. No clinical studies have displayed any significant dacarbazine renal toxicity. However, some investigators[87] treating patients with advanced cancer have reported a few instances of mild to moderate azotaemia, but it is difficult to ascertain whether this was due to the drug or the severity of the illness.

K. Hydroxyurea

Minor alterations of creatinine clearance and renal tubular function have been reported from this drug[88,89]. However, these are reversible and no severe nephrotoxicity has been known to occur even with long-term treatment of benign disease.

L. L-Asparaginase

This drug causes azotaemia of a mild to moderate degree in about 50% of patients receiving it[90,91]. However, it does not cause concomitant alterations of the serum creatinine, urinary sediment, or creatinine clearance. The azotaemia is prerenal and caused by decreased protein synthesis and liver dysfunction that results in high serum ammonia levels. There has been no definite nephrotoxicity from this drug.

M. Cyclophosphamide and ifosfamide

Cyclophosphamide and its metabolites are excreted in the urine in high concentrations[92]. It can cause tubular necrosis in experimental animals, but the main urinary problem with cyclophosphamide is the well known chemical cystitis, caused by one of the metabolites, acrolein[93]. Despite these facts, no clinical nephrotoxicity occurs, even when carefully assessed. For example, De Fronzo and associates[94] could detect no changes in a battery of renal function tests in 17 patients treated with high doses of cyclophosphamide.

Although there are no detectable alterations of renal function tests, some subtle changes in tubular physiology do occur. Bode and associates[95] studied the mechanism of water retention that results from cyclophosphamide administration. They determined that cyclophosphamide directly affected the tubules, causing increased water reabsorption and sodium loss. This water retention is usually self-limited, lasts only a day or two, and is not a major clinical problem.

The cyclophosphamide analogue, ifosfamide, has a greater propensity to produce a haemorrhagic cystitis. This cystitis is so common that mercaptoethane sulphonic acid (MESNA), which covalently binds with acrolein, must be administered with ifosfamide to minimize this toxicity. The water retention and hyponatraemia, commonly seen with cyclophosphamide, have not been reported with ifosfamide. Tubular dysfunction does occur, however, in the form of renal tubular acidosis, Fanconi syndrome, and nephrogenic diabetes insipidus[96]. Patients with compromised renal function are at an increased risk for ifosfamide-induced renal toxicity, but MESNA not only protects the bladder epithelium, it also helps prevent glomerular and tubular damage[97]. If renal abnormalities develop, forced diuresis and MESNA will usually reverse the renal dysfunction.

N. Other alkylating agents

Melphalan is partially excreted in the urine, but only one case of nephrotoxicity from this drug has been recorded[98]. Acute renal failure occurred after administration of a very high dose (240 mg/m^2) and rescue with autologous marrow transplantation.

O. Pentostatin (deoxycoformycin)

This agent is being studied in the treatment of leukaemias and lymphomas. Acute renal failure has been observed, especially when high doses are administered[99]. Renal toxicity occurs most often in those patients who have some underlying compromise in renal function. Published information on the features of nephrotoxicity from this agent is limited at present.

P. Epipodophyllotoxins

One case of acute renal failure secondary to an immune-related acute haemoylsis induced by teniposide has been reported[100]. This case represents renal dysfunction secondary to another form of drug reaction. Neither etoposide nor teniposide appears to have any direct nephrotoxicity.

Q. Combination chemotherapy

Certain combination regimens of chemotherapeutic agents have caused renal toxicity. In these cases it is not possible to implicate one particular drug as the nephrotoxin.

Various forms of vascular lesions such as Raynaud's phenomenon have been caused by the regimen of bleomycin, vinblastine, and cisplatin[101]. A similar sort of vascular damage can occur in the renal arterioles and glomerular capillaries resulting in hypertension and acute renal failure[102]. The drug most suspect as the aetiology of this vascular injury is bleomycin, but it may be the drugs acting in concert that initiates the damage. The hypomagnesaemia induced by cisplatin may also play a role[102].

Another unusual form of renal toxicity from combination regimens is induction of the Schwartz-Bartter syndrome. Two patients with metabolic alkalosis, urinary wasting of potassium, increased serum renin, and normal blood pressure have been reported in relation to combination chemotherapy[102,103]. Both patients received cyclophosphamide as part of the combination regimen. Since this drug affects tubular function subclinically, it would be the one drug most suspect as contributing to the renal dysfunction.

II. EXPERIMENTAL NEPHROTOXICITY

A. Cisplatin

The renal toxicity of cisplatin appears in all animals that have been examined, including mice, rats, dogs, monkeys, fish, and chickens[2,105-112]. Cisplatin has generated a large amount of animal work in an attempt to define the renal lesion and to study its mechanism of action. As explained below, this plethora of work has contributed discouragingly little to elucidate the mechanism of cisplatin nephrotoxicity. The toxicity is dose-related, and acute

lethality demonstrates a steep dose-response curve[2,107]. The main renal toxicity is an acute renal failure that presents similarly in all species. There does, however, appear to be an age-related change in susceptiblity. Newborn rats develop anuria with decreased GFR[113]. There were no histological changes in 5-day-old rats, even though the azotaemia was greater than in adults[114], but 10-15-day-old rats appear to be relatively resistant to the nephrotoxic effect of low, but otherwise toxic, doses. The reason for these differences is not completely understood. In addition there is accumulating evidence that there may be a small, but obvious, strain difference in sensitivity to the renal toxicity of cisplatin between Fisher 344 and Sprague Dawley rats (Litterst, unpublished data), although no differences were noted between F344 and Wistar rats[107]. There also appears to be a difference between male and female Sprague Dawley rats, with the female exhibiting a greater sensitivity, while at the same time demonstrating a slower renal clearance of cisplatin[115]. An apparent difference in sensitivity between male and female monkeys has been suggested[2].

Following parenteral administration of cisplatin a biphasic diuresis with hypo-osmolar urine and a striking azotaemia develops. Daily determinations of plasma and urinary parameters of nephrotoxicity show a striking increase in urine volume with a concomitant decrease in urine osmolality beginning 24 h after a nephrotoxic dose of cisplatin. BUN, however, is rarely if ever reported to be increased until 3 days after toxic, but non-lethal doses, although elevations can be detected within 48 h after high, lethal doses. Increases in BUN are consistently 5-8 times above controls at the peak of the azotaemia. BUN elevations peak on days 4-6 and then gradually decline through the next 10 days, when normal values are achieved. Plasma creatinine concentration is also elevated and increases in a parallel manner to BUN. Other serum parameters of renal toxicity have not been studied, except for uric acid, which like BUN and creatinine increases qualitatively, but does not show the very large quantitative changes[107,112].

Urinary parameters of nephrotoxicity have been widely studied, however. There is a significant early diuresis first observed 24 or 48 h following cisplatin administration[111,116-120], although not observed by some investigators[121,122]. Accompanying the increased urine volume is a decrease in urine osmolality[111,118,121]. Although urine volume returns to normal within 1 or 2 days, urinary osmolality remains reduced for as long as 15 months after a single injection of cisplatin[123]. Urinary concentration of protein rises dramatically 4-7 days after a single injection and remains elevated for an additional 7-10 days. Urinary glucose is also increased dramatically[108,111,124,125]. These changes in glucose concentration are most likely a function of the severe tubular injury which has reduced the kidneys' capacity to reabsorb glucose. However this contrasts with recent studies showing that the reabsorption of high molecular weight polymers was unaffected during the first 6 days after cisplatin treatment of rats[126]. This finding suggests that the tubular damage is not a total breakdown of tubular structure and function, but is restricted to the reabsorption of low molecular weight compounds. The source of the urinary protein is not yet established with certainty, although the

damaged tubular cells are a likely source. This is suggested by the near-universal reporting of a lack of an early glomerular lesion produced by cisplatin[112,120,121,127,128]. In addition, numerous enzymes of the proximal tubule are found in elevated quantities in the urine[2,119,124,125], with a 5-fold increase in urinary alkaline phosphatase, N-acetyl-β-glucuronidase (NAG), and gamma-glutamyl transpeptidase (GGT) reported[124]. Enzymuria appears to be a sensitive measure of cisplatin renal toxicity, and many enzymes show substantial elevations at cisplatin doses which were previously thought to be below the threshold of effect[119,123-125]. The presence of high concentrations of GGT and NAG in urine suggests that the primary damage occurs in the proximal tubule, and this suspicion is borne out by histological studies. A single toxic dose of cisplatin produces proximal tubular degeneration, with necrosis and sloughing of cells and debris into tubular lumen. The lesion begins as early as 72 h in the outer stripe of the outer medulla and initially presents as cellular swelling and degeneration. Necrosis is evident by the end of the first week. Tubules are lined with flattened epithelial cells, but the basement membrane remains intact. By 10-11 days after dosing signs of regeneration are evident, and these persist and become more numerous by the beginning of week 4. Cystic tubules have been reported by several authors[107,126] and shown to persist for as long as 15 months after treatment[123]. The production of these cysts is so characteristic that cisplatin has been used to produce an in vitro model of polycystic kidneys using organ cultures of fetal kidney tissue[129]. The renal lesion is most severe at the cortico medullary junction, and rarely invades either into the medulla or into the outer cortex. Glomerular lesions (either to the tuft or the interstitium) are rare during the first few weeks after a single dose of cisplatin, although glomerular sclerosis has been reported in animals surviving 15 months after treatment[123].

Grossly, kidneys gain weight, so that kidney/body weight is elevated. Kidneys appear swollen and have a grainy, yellowish surface. These changes are more obvious at higher doses and at longer times after treatment. Histologically, the earliest change reported is cytoplasmic vacuolation and tubular swelling that occur on the first day after injection[112]. The peak histopathological incidence of necrosis occurs at the end of the first week. The proximal tubular lesion is striking in its specificity for the pars recta or P_3 portion of the tubule, with no lesion evident in the P_1 or upper part of the P_2 and only occasional mild involvement[128] of the lower part of the P_2. Although the lesion occurs almost exclusively in the proximal tubules, occasional reports of distal tubular damage do occur[112]. Most authors report indications of regeneration beginning 7-10 days after treatment, but there is a single report of no evidence of regeneration[112]. Ultrastructural changes include cytoplasmic vacuoles, mitochondrial swelling, aggregation of the endoplasmic reticulum membranes, and nucleolar changes. Brush borders remain intact, in spite of flattened epithelial cells and sloughing of cells into the tubular lumens. Focal loss of basement membrane has been observed, however. Extensive regeneration of the epithelial cells lining the pars recta of the proximal tubule is seen, even though necrotic cells are still

evident.

Only rarely have investigators followed the renal lesions longer than 4 weeks post-treatment. Dobyan et al., however, investigated the long-term histopathologicalal changes that occurred in the kidney for up to 15 months following injection of a low dose of cisplatin to rats[123,128,130,131]. Their main finding was that lesions at later times are basically a continuation of what is reported at earlier times. Widely dilated tubules and evidence of regeneration were widely observed, and restricted to the pars recta. Interstitial fibrosis was striking and a large number of variably sized cysts were observed[130]. Although cysts had been reported previously by others, Dobyan et al. first described them in detail and speculated on their genesis and long-term relevance[131]. Remaining tubules at the cortico medullary junction showed a loss of brush borders and an increase in the number of intracellular inclusions. By 6 months the lesions were detectable in S_1 and S_2, as well as in the S_3 portion of the proximal tubule. Inflammatory foci were also evident for the first time, as were multiple areas of hyperplasia in at least two kinds of cell types. At 15 months there was isolated evidence of damage to glomeruli, mainly with sclerosis of tufts and increases in the interstitial area. The number of cysts appeared to be reduced at this time, however.

Although not as widely studied as the acute lesion, the chronic toxicity of small doses of cisplatin has also been studied[112]. Rats were treated with a dose or 1 or 2 mg/kg twice weekly for 12 weeks. Animals dosed at 1 mg/kg lived for the entire treatment regimen. BUN, creatinine and kidney weight were all significantly elevated and the kidney tissue contained high concentrations of platinum (2% of the administered dose). The major histological lesions were tubular dilation and focal necrosis. Enlarged tubules were lined with flattened epithelial cells and the lumens were filled with protein casts. Interstitial and periglomerular fibrosis were observed, with thickening of the basement membrane. Electron microscopic evalution revealed mainly mitochondrial swelling, and reduction in total amount of cell organelles. No evidence of regeneration was observed.

An interesting insight into the manner in which animals may handle multiple administrations of cisplatin is provided in a study of multiple injections of guinea pigs with several small doses of cisplatin[132]. Injection of several small doses proved to be more toxic than a single injection of the same total dose. In this study the amount of platinum distributed into kidney and the amount excreted into urine was less from the last injection than from the first injection. The authors used radioactive cisplatin as the final treatment, and thus were able to distinguish between the way in which initial and final injections of cisplatin were handled and distributed.

The major functional change in cisplatin-induced renal failure is a decrease in GFR. However, as is the case with other indicators of renal toxicity (BUN, creatinine, proteinuria), GFR changes are not apparent until 48 h after cisplatin injection and do not become severe until days 3-6. The decrease in GFR is maintained for at least 30 days after treatment[120]. The decrease in GFR has been studied extensively in a series of elegant experi-

ments by Saferstein and co-workers[120,133,134], who found that single-nephron GFR is reduced, as well as whole-animal GFR. They found further that inulin permeability of the tubule and ateriolar pressure were not altered, but that renal plasma flow and stop-flow pressure were reduced in micropuncture studies of proximal tubules. When plasma was infused, both GFR and single-nephron GFR were increased, but not to control levels. Taken together these findings suggest that the defect is one of increased vascular resistance, but the mechanism is not known with certainty. Other investigators have also concluded that the decreased GFR is caused by an increased vascular resistance[121].

Studies to eliminate the possibility of angiotensin II involvement as the mechanism for the decreased GFR have used captopril, an inhibitor of angiotensin II formation, and verapamil, which blocks the microvascular effects of angiotensin II. Neither drug was able to restore GFR or to block its decrease following cisplatin administration[134]. These results agree with conclusions reached from studies in patients where cisplatin-induced reductions in GFR were also unaffected by pretreatment with verapamil or captopril[135,136].

Even though some authors report cisplatin-induced renal failure as being non-oliguric[121,122], polyuria is observed by most investigators after cisplatin administration. The polyuria is often extensive, with 24 h urine volumes in some animals[116] being greater than 50 ml. The diuresis is biphasic[117,118,134]. The initial increase in urine volume is accompanied by decreased osmolality (but normal GFR) and is responsive to vasopressin[134]. Cisplatin administration has been shown to produce decreased vasopressin concentration in blood[134], suggesting an effect on vasopressin synthesis or release from the pituitary. The potential involvement of prostaglandins is suggested by data which show that aspirin pretreatment can block the hypo-osmolality by inhibiting the production of PGE_2 synthesis from arachadonic acid[134].

The second phase of polyuria, however, is distinctly different from the first, and is accompanied by a falling GFR, which is unresponsive to vasopressin, and not affected by aspirin[134]. This phase of polyuria is not caused by increased rates of solute or fluid flow, nor an inability to maintain a transepithelial concentration gradient. The only abnormality detected by micropuncture studies is that urea is reabsorbed from, rather than secreted into, the tubular fluid in the loop of Henle[120]. Thus the effect is an increased fluid reabsorption in the collecting duct or the distal tubule. Again, the mechanism underlying the change in urea flux or fluid absorption is unknown.

There is an apparent discrepancy in the literature regarding the significance of back diffusion of solute in the damaged nephron. Some authors report a substantial back-diffusion of [^{3}H]inulin, with a 26% decrease in recovery from the dosed kidney, a 14% increased recovery in the opposite kidney, and a decrease in total recovery of 40% relative to the initial values[121]. These data were interpreted as being consistent with a back-diffusion of glomerular filtrate through the severely damaged pars recta epithelium. Others, however, found no back-leakage of inulin across the tubular epithelium[120,133]. One possible explanation for

this difference was that the former study[121] was conducted following the administration of a larger dose of cisplatin, and thus may have assessed a more severely damaged nephron than was the case for those studies that did not find inulin leakage[120,133]. Both authors, however, were consistent in concluding that tubular obstruction was not the cause of the decreased GFR, and that GFR decreased to a greater extent in single nephrons than in the whole animal.

The pathogenesis of cisplatin nephrotoxicity is not well understood. The drug is rapidly and extensively bound to plasma proteins so that within a few hours after administration more than 90% of the circulating platinum is protein bound. However, within that first few hours, 50-70% of the administered dose is also excreted in the urine and only trace quantities are excreted thereafter. Excretion by any other route has not been demonstrated, although the presence of platinum in the bile has been reported[137-139]. Thus the free drug is rapidly and extensively excreted in urine and it is assumed that this high exposure of renal tubule cells is in part responsible for the renal toxicity. Hydration is the single most effective way to limit clinical renal toxicity, and the mechanism for this has been suggested to be a dilution of the platinum concentration in tubular fluid[16]. However, platinum does not appear to be actively reabsorbed from the tubular fluid into renal cells (it may be secreted) and renal cell exposure has been shown to be via the peritubular border[109,134].

The exact mechanism of the handling of cisplatin by the kidney is still not certain. While some authors have studied anaesthetized animals given a steady-state infusion of cisplatin[139,140], others have used a single bolus injection of cisplatin to conscious animals[141]. In vitro methods include the study of transport in renal slices[142], or use micro vesicles[143]. Interpretation is further confused because some investigators study the effect of cisplatin on transport of marker chemicals such as tetraethylammonium, N-methylnicotinamide, or para-aminohippurate, while others report on the effect of the marker chemical on platinum transport. Despite the variables in experimental design, there is some agreement in the area of platinum renal clearance. While some authors have shown the clearance of platinum to be equal to (or only slightly above) that of inulin[139,140], i.e. equal to the GFR, others[141] have reported platinum clearance to be greater than 3. One study determined free rather than total platinum[140], and most studies use steady state infusions of cisplatin[139,140] rather than a single injection of cisplatin[141]. The consensus appears to be that only small increases, if any, above GFR are ever observed for cisplatin. This is in contrast to the only clinical study, where an increase in cisplatin clearance in patients was interpreted to mean secretion[142]. No evidence exists for the reabsorption of cisplatin. Several investigators have concluded that cisplatin is transported via a cationic transport mechanism, but the data are conflicting. Cisplatin inhibited organic cation transport, but had no effect on anion transport in membrane vesicles[143]. Cisplatin does, however, inhibit both the cation and anion transport systems in mouse kidney slices[144], and in the chicken studied by the Sperber technique[109]. The transport of cisplatin was not affected by N-

methylnicotinamine, a chemical transported by the cation transport mechanism, but probenecid, an anion, caused a 70% increase in platinum clearance. This suggests that the transport of cisplatin was by the anion transport mechanism[140]. Cisplatin affected both the cation and anion transport systems in the Sperber chicken preparation[109], but inhibitors of the cation system blocked the cisplatin uptake. In addition, the organic base transport sytem was shown to be involved in cisplatin transport, because inhibited tetraethylammonium cisplatin and transport of other organic bases into rat kidney slices[145].

Cisplatin transport into the renal cell is of questionable significance to nephrotoxocity. The consensus is that exposure of the renal cell to cisplatin is via the peritubular, and not luminal, side of the cell[109,134]. It has been suggested that the unbound platinum in the renal cell is biologically inactive[134]. This was concluded because platinum in an ultrafiltrate of plasma and urine was mutagenic, but platinum in cell cytosol ultrafiltrate was not. This implies either that the platinum concentration in renal cells may not be important for cisplatin-induced renal toxicity, or that the kinetics of intracellular platinum are more complex than previously assumed. Thus cisplatin may be converted in the cell to a toxic metabolite which interacts with critical sites. A second metabolic step may then occur which detoxifies the unbound platinum to form the biologically inactive species. This hypothesis also suggests that there may be a finite number of binding sites within the cell, so that there are only a limited number of reactive platinum molecules which can be bound before the detoxification step occurs. Finally, the kinetics of cisplatin binding to protein may be different from the kinetics of its metabolism.

The variability of the renal response to cisplatin administration deserves comment. In two separate experiments it has been reported that 4/16 and 3/13 rats injected intraperitoneally with cisplatin failed to develop renal failure[121]. Whereas an incidence of 5-10% could be attributable to injection into the colon or other intraperitoneal organ, a consistent incidence of 20-25% in two separate experiments is unlikely to be injection error. This is substantiated by a recent study that showed 25% of rats did not develop renal failure following intravenous (i.v.) injection of cisplatin[118], where blood levels of platinum confirmed the success of the injections. In addition, histological evaluation of kidneys from cisplatin treated rats often report areas of moderate to severe pathology which are adjacent to regions with normal architecture[127], and this heterogeneity of response of individual nephrons to the effect of cisplatin has been discussed[121]. Furthermore, the exceptionally large diuresis of some rats, but not others, supports this variable response. Taken together these data suggest a population of resistant animals that could be used to study mechanism of cisplatin nephrotoxicity.

The role played by renal sulfhydryl-containing compounds has been studied in an attempt to explain the mechanism of cisplatin renal toxicity, but the data generated are not consistent or enlightening. A minor decrease (7-14%) in total and non-protein bound sulfhydryls was reported beginning 24 hours after cisplatin treatment, along with the absence of an interaction between

cisplatin and sulfhydryl-containing amino acids in vitro[146]. Others, however, have shown the opposite effect with increases of 34% and 69%, respectively, in reduced and total glutathione (GSH) 1 day after cisplatin[147]. A decrease in GSH reductase throughout a 12 day time course and a striking increase in total GSH 1-2 weeks after dosing was also reported[147]. These authors presented data that showed 30% of the cytosolic platinum co-eluted with authentic GSH in a thin layer chromatographic assay, suggesting that a platinum-glutathione complex may be formed[147]. Leyland-Jones et al. found higher values of GSH at 20 minutes[148] that were consistent with the previously reported increase in total glutathione at 24 hours[147]. The return of GSH levels to normal by 24 hours[148] may be related to the lower dose of cisplatin used. Renal gamma-glutamyl transferase activity was reported to be unchanged[148]. Thus changes in GSH levels produced by cisplatin are minimal and their involvement with cisplatin renal toxicity is not clear. However, when endogenous levels of GSH are altered by drug pretreatments before cisplatin administration dramatic changes in cisplatin toxicity occur. Decreases in endogenous levels of GSH were produced by pretreatment with diethylmaleate or buthioninesulphoximine[149] and increases in endogenous GSH levels by pretreatment with reduced glutathione[150], and it was found that an increased toxicity correlated with a decreased level of blood and kidney glutathione. These studies also investigated changes in the subcellular distribution of platinum following the various pretreatments but found no correlation between increasing toxicity and changes in platinum distribution. GSH pretreatment, however, led to an overall increase in platinum concentration in kidney cytosol and a decrease in microsomal levels beginning 2 minutes after treatment and lasting through 24 hours. Mitochondrial platinum showed varying changes. The significance of these changes is as yet unknown. Thus glutathione may play a role in the renal toxicity of cisplatin, but perhaps only as a non-specific binding molecule.

The possible role of sulfhydryl interactions in cisplatin toxicity has been further pursued in studies of the role of metallothionein (MT) as a possible site of cisplatin interaction. MT is a low molecular weight (c.a. 12,000) protein whose amino acid composition is 30% cysteine, thus providing substantial sites for potential interaction if cisplatin were to react with sulfhydryl groups. MT has been shown to actively bind heavy metals such as mercury, cadmium, zinc and copper, and to have its synthesis induced by these metals. Bakka et al[151]. first suggested the involvement of MT in the toxicity of cisplatin by demonstrating that the in vitro cytotoxicity of cisplatin was correlated with the MT content of MT-deficient and MT-normal cells. In vivo work has shown that cisplatin does interact with an MT-like compound, the exact identity of which is disputed[149]. It has been shown that toxicity of cisplatin correlates with platinum binding to an elution fraction corresponding to MT. Although platinum did bind to this fraction, pretreatement with cisplatin did not increase the binding[149]. These authors did not isolate the fraction, nor did they fully characterize the binding protein. Other authors have studied this molecule but disagree on the identity of the protein. Thus one report using an

antibody to MT claimed platinum binds to a protein precipitable with the anti-MT antibody[152], while another claimed there are small differences between authentic MT and the platinum-binding protein[153]. Suffice it to say that platinum from cisplatin binds to a low molecular weight protein with a high concentration of sulfhydryl groups, which may be MT. The issue has been further clouded by data on the interaction of cisplatin with chemicals known to induce the synthesis of MT[154]. Some inducers alter toxicity but others have no effect, and these data are inconclusive with regard to the role of MT in modulating cisplatin nephrotoxicity.

Another potential site of cisplatin toxicity is the renal ATPase system. Guarino et al. first suggested this mechanism, based on the in vitro inhibition of ATPase after incubation with very high cisplatin concentration[16]. Others have since confirmed this in vitro finding[155-157], but have shown no effect on renal ATPase activity in vivo[157]. One reason for this lack of detectable in vivo effect on ATPase is that very high concentrations were required in order to produce decreased activity in vitro. Thus, concentrations of 0.7 to 5 mmol/l have been reported as inhibitory in vitro[16,134,155-157]. These high concentrations are unphysiological and not achievable in vivo[157], where the drug is continually being removed from the circulation by excretion and protein binding.

Other specific compounds or sites through which cisplatin might exert its renal toxic action have not been explored in detail, but it is known[145] that cisplatin is actively taken up into renal cells by an oxygen dependent process, so it is likely that the P_3 segment of the proximal tubule contains some highly specific molecule to which cisplatin binds - perhaps some sulfyhydryl containing molecule whose conformation is changed by cisplatin.

B. Anthracyclines

The anthracyclines are a class of antitumour antibiotics which are produced by Streptomyces sp. fungi. The drugs are chemically composed of a tetracyclic nucleus to which are attached various substituents. The two anthracyclines to be discussed here are similar in that they both contain an amino sugar (daunosamine). They differ, however, only in that one contains an hydroxyl substitution on the 14-carbon atom and the other does not (Figure 1). The former is adriamycin (doxorubicin; Adr) and the latter is daunomycin (donorubicin; Dauno). These drugs are somewhat unique vis-à-vis renal toxicity because the sensitivity of the kidney to these agents appears to be species specific, with humans being relatively resistant to the effects and rodents, particularly rats and rabbits, particularly sensitive.

Adr and Dauno have both been shown to be model compounds for producing a nephrotic syndrome in rats and rabbits after single or multiple doses. The nephrosis is characterized by a dramatic proteinuria accompanied by a hypoalbuminaemia and peripheral fluid accumulation and followed by severe hyperlipaemia. BUN and creatinine either are not elevated or are only minimally increased. A single injection of Adr or Dauno to rats is the stand-

ard regimen for producing the nephrotic syndrome but chronic, multiple-dose regimens have also been used in both rats and rabbits.

The syndrome is characterized by a striking decrease in plasma albumin accompanied by a corresponding increase in urinary albumin. These changes in albumin status are delayed, however, and do not occur for several weeks[158]. In one study the changes were evident by 10 days after treatment[159], but not by day 5. Values do not return to normal until 30 days after treatment[159]. Following the onset of albumin changes, plasma lipids such as cholesterol, and high- and low-density lipoproteins, are all elevated beginning 2-3 weeks after treatment. They are still elevated 70 days after treatment and this increase is assumed to represent an irreversible change[160]. Although plasma lipid levels are dramatically altered, tissue lipid composition is relatively unchanged, with only mild changes in triglycerides, phospholipids and cholesterol observed in liver, kidney, and small intestine[160].

The nephrotoxicity of Dauno was first reported in 1970[75]. In spite of the striking structural similarity between Dauno and Adr, no renal toxicity was reported for Adr in early animal toxicity testing[161]. Extensive chronic toxicity data, however, suggested no histologically detectable renal damage in dogs, but rabbits injected with a very high dose of Adr showed mild increases in BUN, mild decreases in serum protein, and severe tubular nephrosis 90 days after treatment[162]. Although the nephrosis was first demonstrated with Dauno, both drugs produce the same syndrome and most subsequent studies have been undertaken with Adr. The syndrome is commonly induced in the rat with a single i.v. dose of Adr, although multiple injections of smaller doses have also been used successfully. Early proteinuria and hypoproteinaemia are first detected 4-5 days later, and the syndrome reaches its maximum intensity between 14 and 21 days after injection. Although some authors report histological signs of regeneration in the damaged kidney at 4 weeks after treatment[124], striking proteinuria and hypoproteinaemia are still evident as long as 70 days after treatment and chronic histopathological lesions (fibrosis and sclerosis) are present a full year later.

It has commonly been assumed that the rat and rabbit were the only animal species sensitive to the anthracycline nephrotic syndrome. This assumption has been strengthened by the fact that renal toxicity from anthracyclines is rare in humans. However the lack of a good, routinely used non-invasive marker for chronic renal failure, or the use of inappropriate test protocols for detection of this syndrome, may simply have precluded detection of this lesion in other species. Thus Gralla et al. looked at single and multiple injections of Adr in dogs and monkeys and failed to detect any renal toxicity[163]. However, the dogs were injected with a single high dose that resulted in death within 5 days and the dogs given multiple doses died within 6 days. There was no indication that plasma or urinary protein or lipid levels were investigated in this study. The earliest changes in plasma protein and lipid values occur in rodents and rabbits 8 days after treatment, and histopathological changes do not occur during the first week after injection of doses that lead to death in 4-7 days[77,158]. The dog was

also found to be refractory to nephrotoxicity in another study[162] where there were no altered serum parameters of renal function, or histopathological changes in the kidney at 30, 60 or 90 days after a chronic treatment regimen of Adr. Monkeys injected daily for a week had no renal pathology, even though most of them survived for longer periods. This strengthens the argument that primates, including the human, are not sensitive to the renal toxicity of Adr. Interestingly, a subchronic study of Adr in rabbits where the drug was administered every other day for 3 weeks (total dose 10 mg/kg) revealed no change in several parameters used to monitor renal toxicity (BUN, glucose, total protein and albumin). Histological results in that same study, however, showed dilated tubules and other renal lesions. Pigs have also been shown to exhibit the renal syndrome following multiple i.v. dosing with Adr[164]. This study did not, however, report clinical chemistry data, and gross examination of kidneys was negative. Microscopically dilated proximal tubules with abnormal cell nuclei were observed, and a chronic nephrosis with early signs of interstitial fibrosis was observed in some animals. Mice have been widely used in acute lethality studies, but only rarely have blood chemistry determinations or histological evaluations been conducted. Shurig et al. reported no BUN changes 4 days after a single intraperitonal (i.p.) injection of 0.5, 0.75 or 1.0 times the LD_{50} dose of Adr[165]. In another study mice were injected i.v. on a 4- or 5-day schedule and a lipid accumulation in proximal tubule cells, with casts and hyaline droplets in tubules, was reported 8 days after the end of treatment[166]. In addition, the mouse was found to be non-responsive to the tumourigenic effects of anthracyclines[167]. Apparently, therefore, the mouse does not demonstrate the same type of renal sensitivity seen in rats and rabbits. Thus it appears as if the dog, monkey and human may be refractory to the nephrotoxicity of the anthracyclines but rats, rabbits and pigs are sensitive. It would be interesting to know if there were any differences in drug distribution among the sensitive and resistant species that might account for the differences in renal responsiveness.

Most investigators examine blood protein and lipid values at weekly intervals. Bertani et al., however, looked at earlier times following Adr administration and reported urinary protein to be normal on day 3 but elevated at day 5 with a peak value 100-fold normal at 4 weeks after treatment[168]. The values then began to decline but were still 30-fold above normal 10 weeks after treatment. Serum protein in this study was normal up to day 5 and then decreased from day 7 up to 4 weeks, after which it returned to normal. An interesting distinction was shown, however, between serum protein and serum albumin levels. The latter decreased on day 7, but was normal until week 4. Beginning on week 4 serum albumin was reduced throughout the remaining 10 weeks of the study. Calandra et al.[159] also investigated plasma albumin after Adr and reported no change at day 5 and decrease between day 10 and 30, with a nadir between days 15 and 25. Urinary albumin also was normal until day 10 when it dramatically increased, reaching a maximum value between day 20 and the end of their study (day 30). Litterst and Copley similarly showed normal urinary protein values on day 4 after a single i.v. dose of Adr, but a significant

increase on day 7[124]. Values peaked at day 12 and then declined, although values had not returned to control by day 23. These authors also investigated urinary glucose and found normal values throughout a 23-day period. This is an interesting finding in light of the major leakage of high molecular weight compounds through the GBM, and suggests that the reabsorptive capacity of the proximal tubules was not significantly impaired by Adr treatment, at least during the first 3 weeks after treatment. O'Donnell et al. similarly investigated both urinary protein and albumin in their study 4-5 weeks after treatment, and reported a qualitative similarity, with both compounds elevated but with a dramatic quantitative difference, with protein increased 15-fold, but albumin increased 200-fold above control values[169]. No explaination was given to account for the failure of the increased albumin values to affect the total protein values to a greater degree. These authors further reported normal plasma protein values 4-5 weeks after treatment. Grond et al. have studied urinary protein for 12 weeks and found no return to normal by that time, after a 100-fold increase after 14 days[170]. Other long term studies showed a 33% decrease in serum albumin for as long as 7 to 9 weeks after treatment[171]. The minor discrepancies in the first date of elevation or return to normal probably reflects differences in dose, animal species and analytical methodology used to study protein values.

Bizzi[160] has performed a comprehensive study of lipid changes following a single i.v. injection of Adr, and found plasma triglycerides decreased by 50% on days 1 and 3 after treatment, with no further change throughout the following 67 days. In this dose response study they also found only a high dose of Adr causing increased cholesterol and phospholipids on days 21, 30, and 70. On day 30 the chylomicron fraction, high- and low-density lipoproteins were all increased. They found no change in serum or diaphragmatic lipoprotein lipase activity, but there was a doubling of activity in heart and an 87% reduction of activity in fat. No values were reported for kidney. At day 21 there was a decrease in phospholipid content in kidney, which contrasts to other data which showed an increase in lipid deposition in the kidney after a chronic treatment schedule. Grond[170] also showed an increase in cholesterol and triglycerides at week 12, and Calandra[159] showed cholesterol and triglycerides to be elevated between day 15 and 30, with a maximum between days 20 and 25. Kunimoto et al.[172] found that chronic treatment of rats with very low doses of Adr led to increases in cholesterol, triglycerides and phospholipids beginning after 4 weeks, in an 8-week treatment regimen.

Increases in BUN or plasma creatinine, which are normally used as markers for renal toxicity, are unreliable in anthracycline-induced nephrosis. Some authors report no change in these parameters, and even when changes are reported they are minimal and not indicative of a serious renal failure[173,174]. Even in uninephrectomized animals which received Adr, Giroux et al.[175] reported only minimal BUN elevations.

The first detectable lesion appears 2-6 hours after i.v. injection of rats with an LD_{50} dose of Adr[176]. Only glomerular epithelial cells are affected, and they exhibit disruption of "nucleolonemal" strands, with a separation of granular and fibrillar

components. This leads to enlargement of the nucleolus by 24 hours after treatment. These lesions are characteristic of drugs which interact with DNA and may not be specific for Adr or the anthracyclines in general.

Sternberg has looked for histological changes characteristic of the nephrotic syndrome following a single very high dose of Dauno to rats, and found no changes by light or electron microscopy during the first 2 days after treatment[75]. By day 4 there were occasional glomeruli with dilated capillary loops. Ultrastructural changes were more impressive, however, and showed visceral epithelial cells with microvilli and vacuoles, lipid inclusion bodies and a focal loss of foot processes. In addition, at this early time there appeared cross-striated fibrils that had the appearance of contractile elements and may replace the foot processes. By day 7 dilated glomeruli are common, and mitochondrial damage and generalized loss of foot processes apparent. It is not until 4 weeks after treatment, however, that there is a thickening of Bowman's capsule, including a thickening of the GBM and the first appearance of collagen in some damaged glomeruli. A full year after drug treatment the kidney is characterized by a lobular sclerosing glomerulonephritis with fibrotic changes in the loops and a sclerotic capsule. There are persistent GBM changes and occasional loss of foot processes, with intact foot processes demonstrating an irregular shape. Light microscopic evidence of renal cell damage, however, has been reported not to be present 7 days after an acute, lethal i.v. dose of Adr[77]. This is consistent with other results where proximal tubular degeneration in the inner cortex first appeared 8 days after a single dose of Adr[124]. By day 11 tubules were lined with flattened epithelial cells that extended in rays out into the cortex from the cortico medullary junction. The nephrosis was severe by day 16 but scattered foci of regeneration became more apparent by day 23. The appearance of signs of regeneration has also been reported by other authors, although the lesion progresses to a fibrotic involvement 4-6 months after treatment[171].

Bertani et al. have also shown no change in glomeruli, interstitium or tubules 3 hours after a single injection of a standard dose of Adr to rats[168]. By 28 hours however, there were occasional focal losses of foot processes and by day 13 vacuolation of epithelial cells, atrophy of tubules with extensive filling with protein and cellular casts, and extensive loss of foot processes, but with no apparent change in GBM, which is similar to that observed by others[75].

A sequence of similar lesions has been observed following thrice-weekly dosing of rabbits with Adr[76]. This extensive study showed the lesion beginning in glomeruli and tubules at the medullary level, and showed that superficial cortical tubules were unaffected. Early changes included vacuolization of glomeruli which led to a compression of the glomerular tuft and a hyperplasia of epithelial cells. There was fibrosis of the stroma and the advanced disease was characterized by glomerular sclerosis with numerous microthrombi. No vascular changes were seen and there was no inflammatory infiltrate[76]. There were extensive retractions of foot processes and a thickening of Bowman's capsule, with a thickened

and irregular GBM. Tubular dilatation was present, with atrophy of epithelial cells. Microvilli develop as an early response to Adr, and their origin appears to be dilation of the endoplasmic reticulum that then project into the urinary space. The loss of these microvilli correlates with the flattening of the epithelial cells. A study of 17 weekly i.v. injections of Adr in rabbits also showed large vacuolated podocytes in the glomerulus[177]. Epithelial cells showed an interstitial infiltrate with mild fibrosis. The severity was related to cumulative dose of Adr. Following chronic Adr administration to the rabbit[178] and pig[164] cytomegally with chromatin aggregates was also seen in the central portion of the epithelial cell. Fijardo et al., however, reported only rare nuclei that were enlarged or misshapen[76]. The renal lesion presents in surviving animals a long time after anthracycline treatment has been reported to be either fibrosis[75,76,164,179,180] or sclerosis[75,169,170,175]. Sclerosis occurs at a very low frequency, in terms of affected animals and the incidence of sclerotic lesions in any individual animal. Of particular interest is the lack of correlation between severe proteinuria and glomerular capillary wall damage, and the appearance of sclerosis[170]. The former changes are usually considered to lead to the development of sclerosis. This is not the case, however, in rats given a single i.v. dose of Adr, where extensive proteinuria was present for 4-5 months, but the incidence of sclerotic glomeruli was very low[170]. Similarly, sclerosis is usually associated with lipid deposits in glomerular cells. Although protein and lipid deposits are prominent components of the anthracycline-induced nephrotic syndrome, these deposits are usually not associated with prominent sclerosis. In spite of chronic nephrosis, several authors report focal areas of regeneration in kidneys of Adr-treated animals. In one study the regenerative sites appeared on day 16 after a single i.p. injection of Adr and were commonly observed[124] by day 23. Regenerative changes were also reported following a chronic multiple dose treatment schedule in rats when evaluated at the end of the treatment sequence[171]. These regenerative changes were reported only in tubular epithelium, with no reports of glomerular renewal. The apparent ability to partially recover from the nephrosis is also obvious from the report of Sternberg, who found the loss of foot processes was diminished at long times after a single injection of a very high dose of Dauno to rats, even though some of the remaining foot processes were irregular[75]. This same study noted that animals killed 1 year after treatment were in a debilitated condition, and thus recovery in a clinical or functional sense is probably relative, and pertains only to focal areas of damaged tubules.

The cellular specificity of the anthracycline-induced nephrotic syndrome is of interest. Morphological changes are reported only in epithelial cells of the glomerulus. Even after chronic treatment with very high doses tubular epithelial cells were unaffected and changes in tubular function have not been reported. The manner in which a glomerular epithelial cell lesion could lead to fusion and disappearance of foot processes, and ultimately to disruption of the GBM, can be relatively easily envisioned and the subsequent proteinuria easily explained.

The lesion, then, is a slowly developing vacuolation of both

glomeruli and tubules that leads to a thickening of the GBM and atrophy of tubular cells. The lesion begins in the cortico medullary junction and extends outward into the cortex. It is likely that the glomerular lesion produces a disruption of protein retention, with the subequent proteinuria that characterizes the syndrome. Recovery can probably occur to some extent, but fibrosis is extensive and morphological changes are still apparent a year after treatment.

The pathogenesis of the Adr-induced nephrotic syndrome is at the present time unknown, but several authors have speculated on possible causes. The proteinuria is not fully understood, but obviously reflects an increased permeability of the GBM to large molecular weight substances. In elegant studies of the relationship of thromboxane (Tx) synthesis and proteinuria in Adr-induced nephrosis, Remuzzi et al. have concluded that the proteinuria is caused by an increased permeablity of the GBM produced by an increased Tx synthesis[181]. These authors first correlated the extent and duration of proteinuria with the degree and duration of Tx elevation. Using an inhibitor of Tx synthesis, the authors then showed that inhibition of Tx synthesis correlated with a less severe and less prolonged proteinuria. The manner in which Adr may stimulate synthesis of Tx is not yet understood. Careful control of their studies led to the conclusion that the effect was not due to a change in GFR. Further, although there is an apparent effect of Adr on glomerular capillaries, the change in Tx synthesis is not related to the vascular effects of Adr, because isolated glomeruli responded to Adr exposure with a decrease in Tx synthesis. These authors[181] also eliminated as causative, changes in availability or synthesis of Tx precursor pools and activation of the renin-angiotensin system. Finally, the increased production of Tx was not caused by the hypoalbuminaemia-induced stimulation of platelet Tx synthesis, or to an increase in the number of circulating cells such as leukocytes or platelets.

On the other hand, Bertani et al. have postulated a different mechanism underlying the increased permeability of the GBM and consequent extensive proteinuria[168]. These authors studied the presence of sialic acid-associated polyanions on the surface of epithelial cells using histochemical staining. They observed a decreased staining intensity by 3 hours after treatment with Adr, a marked decrease by 28 hours, and total absence of polyanions on days 13 and 18 after treatment. The disappearance of these sialic acid proteins clearly preceded the onset of both proteinuria and ultrastructural changes detectable by electron microscopy. A cause-effect relation is therefore more likely because the changes preceded the proteinuria, rather than merely correlating with it, as was the case with the increased Tx synthesis discussed above. Again, however, the direct involvement of Adr was not demonstrated.

A more extensive study[182] included assessing glomerular permselectivity changes in rats up to 55 days after an i.v. injection of Adr. A decrease in surface polyanion concentration was also found in this study, and appeared to be due to a decrease in cell surface glycoproteins. However, these authors found no change in the amount or the distribution of anionic sites in GBM, using

polyethyleneimine staining and electron microscopy, nor did they find the expected changes in absorption of positively charged molecules. Thus they concluded that the charge barrier of the GBM was unaltered by Adr treatment and that the proteinuria was a simple consequence of the defective glomerular capillary wall which allowed leakage of proteins.

Similarly, Kubosawa et al.[183], using a different method, found that anionic sites in the GBM and on podocytes were no different in Adr-induced proteinuria compared to controls until a far more advanced stage of glomerular damage had developed, and concluded that the loss of polyanion staining that did occur was a consequence of the advanced proteinuria and GBM leakage, rather than a cause of it.

Ismail et al.[179] correlated morphological changes with extensive Adr-specific fluorescence throughout the renal sections, suggesting that the lesions were caused by the active presence of the drug throughout the time that the lesions were developing. Other authors have similarly shown that Adr is present in very high concentrations in the kidney following a chronic treatment regimen[184] and in renal interstitial fluid after intra-arterial infusion to dogs[185]. Hjelle et al.[186] have shown that both Adr and Dauno undergo intracellular metabolism in rabbit proximal tubular cells in vitro, suggesting the possibility that these drugs may also be metabolically activated in renal cells in vivo.

The presence of drug at the site of toxicity, active metabolism of anthracyclines in tubule cells, and an evident dose response curve strongly suggest that the lesion is related directly to presence of drug in the affected renal cells. This, then, forms the basis for an explanation for the striking species sensitivity of the rat and rabbit. If toxicity is dependent on tissue binding, then the less susceptible species may have fewer appropriate binding sites on target proteins. Similarly, if binding is dependent on metabolic transformation the less susceptible species may have a deficiency of activating enzymes or different detoxication or protection mechanims.

Several authors have studied immunofluorescence in renal sections from Adr-treated animals and found these sections negative for IgG, IgM, C3, complement, fibrinogen or albumin deposition, strongly suggesting the lack of an immune-mediated mechanism for toxicity[168-170,175]. Bristow et al.[187] have postulated that the toxic mechanism may be an Adr-induced release of vasoactive substances, which would lead to a prerenal or early renal vasoconstriction and hence ischaemia. These authors used various chemicals to block release or binding of both histamine and catecholamines. Total histaminic blockade given during Adr administration to rabbits weekly for 19 weeks produced a significant decrease in renal damage, and blocked the Adr-induced hypoalbuminaemia, cholesterolaemia, and proteinuria. Total alpha- and beta-adrenergic blockade did not reduce renal toxicity. When adrenergic and histaminic blockade were both utilized, however, a near complete protection greater than merely the histamine blockade was obtained. This latter effect suggests that both histamine and catecholamines may be involved in the pathogenesis, but the mechanism by which the catecholamines act is probably secondary

to the histaminic effect. The authors argue persuasively that Adr metabolism and tissue kinetics in vivo are not altered by the pretreatments and thus they conclude a toxic effect of vasoactive amines is mediating the Adr renal toxicity. The exact mechanism by which either or both of these chemical classes acts remains to be fully defined. An encouraging note on this study was that antitumour efficacy was not altered by inhibition of vasoactive amine function, thus presenting the possibility that a chemical blockade of serious toxicity might be discovered without affecting clinical effectiveness. The involvement of catecholamines in the mechanism of action of anthracyclines is also indicated by the very early observation that Dauno produced a rapid increase in circulating levels of epinepherine[188].

The argument for an ischaemic mechanism is given further credence by the knowledge that glomerular ischaemia produces abnormal tubular cells which may be characteristic of glomerular nephritis. Although there are obvious differences in the distribution of such abnormal cells in anthracycline-induced nephrosis, abnormal tubular cells are a prominent component of the latter stages of the anthracycline nephrosis. This argument is further supported by the changes observed in capillary walls from affected glomeruli. It is reasonable to expect this vascular change to result in ischaemia due merely to altered vascular transport.

The effect of Adr on renal clearance also supports an ischemic mechanism. Weening and Rennke found a minor decrease in GFR (20%) accompanied by a similar decrease in renal plasma flow[182]. A similar decrease in clearance of endogenous creatinine has also been observed[173] and a decrease in single-nephron GFR was seen, accompanied by a decreased ultrafiltration fraction[169,189]. Furthermore, Tvete et al.[190] recorded a substantial drop in renal blood flow during the first 2-4 minutes of an Adr infusion, and concluded that Adr had a direct vasoconstricting effect on renal vessels and that there may be an extrarenal origin of the systemic increase in vascular resistance. Remuzzi et al., however, concluded from their studies that there was a direct toxic effect on the glomerular capillaries[181].

An interesting speculation on the mechanism of Dauno-induced nephrosis is reported by Gartner[191], who postulated that the widely recognized ability of anthracyclines to inhibit nucleic acid synthesis leads to a generalized disruption in protein synthesis resulting in the defective GBM, possibly even the de novo synthesis of a defective GBM[191]. A similar mechanism was proposed by Kronenberg et al.[192], who attributed sclerosis to a progressive folding of the basement membrane caused by excessive GBM synthesis. The data to support such a suggestion, however, are merely the coiling and folding of the GBM reported by these authors[192] and others[75].

Not only is the kidney the site of histopathological and functional lesions, but it is also the site of uncommon renal tumours following Dauno administration. One of the histological lesions mentioned by several authors is the presence of giant cells with abnormal nuclei[164,176,177]. These cells are frequently precursors of drug-induced cancer. Sternberg et al.[193] investigated the carcinogenic properties of a single i.v. injection of Dauno and found a

total of 27 renal tumours in 16 of 33 experimental animals and no renal tumours in controls. In addition, there was a clear dose-response relationship evident, with 10/14 high dose rats having tumours and 6/19 low dose rats having tumours. The doses used bracketed those most widely administered to induce the nephrotic syndrome. There were two distinct histological types of tumours. Clear-cell adenocarcinomas were found in two animals treated with the low dose of Dauno and killed 1 year later. These tumours were similar to the clear-cell carcinomas found in human kidney. The most prevalent type of tumour was a tubular adenoma that occurred in five additional animals. In addition there were tumours of mammary gland, lung, liver and several other organs. All rats with renal tumours had severe glomerulonephritis. Dauno has also been found to be strongly carcinogenic following i.v. injection to mice[194]. In contrast to the strong carcinogenicity of Dauno, Adr had only questionable carcinogenic activity when tested by the same route. Whereas Dauno induced mainly adenocarcinomas, Adr induced mainly fibroadenomas. In addition, a study of the carcinogenicity of Adr following p.o. or i.p. administration to mice was negative, although s.c. administration produced a low incidence (25%) of fibrosarcomas at the site of injection[167]. The IARC subsequently classified Dauno as a carcinogen in animals[195], but felt the data base was too limited to similarly classify Adr[196].

C. Mitomycin C

Mitomycin C (MitC) is one of several chemically related antitumour antibiotics (Figure 1) and was isolated from cultures of Streptomyces caespitosis in the late 1950s. Although the dose limiting clinical toxicity is myelosuppression, several large clinical studies have suggested that renal complications frequently have been implicated in the cause of death, and up to 10% of patients experience renal toxicity. Because of the complexity of the multi-drug treatment regimens in which MitC has been used, it has been difficult to positively identify MitC as the causative agent in the clinical toxicity. In-depth laboratory animal studies have not been conducted until recently.

Very early toxicity screening studies with multiple injection of low doses of MitC in dogs showed normal BUN, suggesting no compromise in renal function, although the renal cortex was reported to be haemorrhagic[48]. Furthermore, there was no change in volume or specific gravity of daily urine collections throughout the course of the treatments, again suggesting no change in functional integrity of the kidney. Animals receiving a lethal dose of MitC and sacrificed on days 10 or 20 of a multiple treatment regimen showed a haemorrhagic renal cortex, but no further histological description was given. No renal toxicity was reported in rats receiving lethal single or multiple injections of MitC[48].

Monkeys, in contrast, showed striking renal toxicity[48]. Animals were moribund 5 and 8 days after a single i.v. injection, and necropsy showed haemorrhage and marked pallor of renal cortex. Histologically, the kidneys had a necrotizing nephrosis with recent haemorrhages. The proximal convoluted tubules were

necrotic, many nuclei were absent, but those that remained were irregular and enlarged, there was a loss of cellular boundaries, and protein casts were present in tubular lumens.

Other renal lesions have also been produced in experimental animals by MitC. Daily injections of non-toxic doses for 6-8 weeks produced hydronephrosis in several strains of mice at an incidence of at least 56%. This hydronephrosis was not caused by impaired peristalsis of the ureter[197].

The only extensive study into the pathogenesis of MitC induced renal toxicity, however, utilized perfusions of MitC for short times directly into the rat kidney in situ[59]. Control animals were perfused similarly with vehicle and the short period of ischaemia shown to have no adverse effect on renal structure or function during the following month after treatment. Gross appearance of the MitC perfused kidney showed reddening of the cortex with foci of haemorrhage, as observed previously in monkeys. Very low doses also produced cortical pallor.

Light microscopic evaluation 24 hours after perfusion showed lesions characteristic of acute haemolytic uraemia[59]. Focal and diffuse glomerular congestion and thrombosis were present, and some glomeruli demonstrated endothelial swelling, loss of nuclei, and decreased integrity of the capillary lumens. Epithelial cells, on the other hand, were normal. Many arterioles showed fibrinoid necrosis with or without thrombosis. The interstitium was edematous, with capillary haemorrhage and polymorphonuclear cell infiltrate. In the tubules there was loss of brush border, cytoplasmic vacuolation and various nuclear abnormalites.

At the end of the first week there was glomerular thrombosis with cellular infiltrate. Tubular damage was more extensive and severe than at 24 hours, and in regenerating tubules nuclei were abnormal. Interlobular arteries showed fibrinoid necrosis, and there was interstitial oedema, fibrosis and cellular infiltrates. One month after perfusion, areas of normal cortex were adjacent to abnormal areas, which showed glomerular sclerosis, thickened capillary walls, and increased mesangial matrix. Arteries and arterioles were normal, but there was severe tubular atrophy with interstitial cell infiltrates.

Although electron microscopic examination was conducted 1 and 3 hours after perfusion, the first changes were not detected until 6 hours, when loss of endothelial fenestrations and lifting of endothelium from the basement membrane. Epithelial cells and arterioles were normal. By 24 hours after perfusion, glomerular changes were widespread, with endothelial loss in GBM and loss of fenestrations and cell swelling. Visceral epithelial cells showed loss of foot processes. Tubules were dilated and lumens contained debris and there was some endothelial swelling of arterioles. One week after perfusion glomerular capillary lumens were reduced due to edema and hypercellularity. There was extensive destruction of foot processes and severe tubular damage, with some necrosis. After 1 month, glomerular capillaries were collapsed, epithelial cells were denuded of foot processes and extensive tubular damage was still present.

In summary, the histological picture of MitC-induced renal damage produced by a short perfusion of kidneys with MitC was

very similar to that described in human autopsy specimens as haemolytic uraemic syndrome. One author has concluded that MitC produces a pathological effect on endothelial cells which results in stimulation of microvascular thrombosis, which in turn leads to the haemolytic uraemic syndrome and ultimately to renal failure[59].

This appears to be a valid hypothesis that is well-supported by experimental evidence. At the present time systemic administration of MitC to rats has not been shown to produce the haemolytic uraemic syndrome, but studies in this area are currently in progress[59]. That MitC should produce nephrotoxicity may not be surprising in light of high concentrations of drug in urine of MitC treated animals and the fact that highest tissue concentrations of drug are found in kidney of MitC treated guinea pigs 5 minutes after i.v. injection[58].

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27

RADIATION-RELATED RENAL DAMAGE

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1. HISTORICAL INTRODUCTION

In 1896, a year after the discovery of X-rays by Roentgen[1], several reports appeared on the effects of X-rays on deep seated tissues[2-4]. The initial report of signs appearing after the exposure of abdominal organs to roentgen rays was by Walsh[5], and the deleterious effects of radiation on the kidney were first reported by Baermann and Linser[6]. Rabbit kidneys were exposed to X-rays for 1 hour, after which a transitory albuminuria was noted, but no identifiable histological changes were seen in the renal epithelium. Histological evidence of renal damage due to radiation was initially described by Buschke and Schmidt[7] and Schulz and Hoffman[8]. These authors reported glomerular and tubular atrophy and increased interstitial tissue after the initial albuminuria. Although no clinical data were available at that time, Edsall[9] inferred from these experimental findings that patients who either had some impairment of renal function, or whose kidneys were "under special strains" should be excluded from, or followed closely after, X-ray exposure. Subsequently, marked nitrogen retention was reported in both animals and patients following exposure of the renal area to roentgen rays[10].

Domagk[11] presented the first accurate description of radiation-induced injury to the human kidney. He described a 9 year old girl who had received abdominal irradiation for tubercular mesenteric lymph nodes. Four months after treatment the patient exhibited oliguria and albuminuria, death occurring 2 months later. At post mortem the kidneys were found to be small. Histological studies showed glomerular hyalinization or thickening of the Bowman's capsule, tubular atrophy or necrosis and hyaline material in the arterial walls. Experimental studies confirmed this pattern of radiation-induced renal damage[12].

The early experimental studies on radiation nephritis, which have been reviewed extensively[13,14], were carried out before

precise physical measurements of radiation dose and the distribution of that dose were possible. It was not until the classic study of Kunkler et al.[15] that the clinical radiation tolerance of the kidney was adequately defined. Kunkler and co-workers studied a number of patients who had received bilateral renal irradiation during the treatment of testicular tumours with 250 kV X-rays; they established that a total dose of 23 Gy*, given over a 5 week period, produced a high risk of renal damage. Subsequently Luxton[16] did a further follow up on 54 of these patients. On the basis of this study radiation nephritis was classified into five categories: acute radiation nephritis, chronic radiation nephritis, proteinuria, benign hypertension and malignant hypertension.

Acute radiation nephritis (ARN) usually presents after a latent period of 6-12 months in adults, but may develop earlier in children[17]. The clinical picture is one of oedema, dypsnoea, headaches, moderate hypertension and anaemia. The symptoms and physiological abnormalities are usually moderate, but may develop into severe progressive hypertension or malignant hypertension. In those patients that survive the acute phase of nephritis (ARN) chronic radiation nephritis (CRN) may develop. However, CRN may occur after a latent period in patients with no previous history of ARN. The prognosis of patients in this group is better. The clinical symptoms of CRN are proteinuria, hypertension and a reduction in renal function, which may result in chronic anaemia.

Proteinuria which is either intermittent or permanent appears approximately 11 years after radiotherapy; although renal function is normal, these patients appear to have an impaired renal reserve and exhibit temporary renal failure after stress.

Benign hypertension may develop within 2-5 years after radiotherapy. The condition may persist for many years, with patients exhibiting the usual complications resulting from hypertension. They often die of congestive heart failure.

Late malignant hypertension may be associated with ARN, developing from 18 months to 11 years after irradiation. However, it can also develop after the irradiation of one kidney and can be relieved by the removal of the irradiated kidney.

2. RADIOBIOLOGICAL CONSIDERATIONS

Following irradiation with doses in the range responsible for the development of progressive functional and morphological changes in the kidney, primary damage is most likely to result from the loss of the reproductive integrity of the cells. This is sometimes termed reproductive cell death and, as the name implies, cell death is contiguous with the process of cell division, although not necessarily at the first division after irradiation[18]. While the reproductive integrity of cells may be impaired by single doses of a few Gray (Gy), disruption of metabolism within the cell, which may result in interphase death, requires doses of approximately 100 Gy.

* Gray, unit of absorbed radiation dose; 1 Gy = 100 rad (official unit of dose prior to 1985).

Lymphocytes are a notable exception to this rule; very low doses of 0.05-0.25 Gy will result in the interphase death of some of these cells[19]. Death of the cells occurs within hours of irradiation.

Classically estimates of reproductive cell death have been obtained from mammalian cells grown in culture[20]. In effect the converse of cell death is determined by counting the number of colonies produced by reproductively viable cells relative to the number of irradiated cells deposited on a culture plate. In addition to these 'in vitro' techniques 'in vivo' systems have been developed and are well established for such tissues as skin and gut epithelium[21,22]. An 'in vivo' assay has recently been developed for the kidney[23] which, it is claimed, measures the reproductive survival of kidney tubular cells. However, it is not clear whether the tubules counted in this assay, 62-68 weeks after irradiation, have truly regenerated from a surviving cell or cells, or if they simply represent tubules that persist after irradiation, the other tubular epithelial cells having been lost as a result of primary or secondary damage.

The relationship between radiation dose and the loss of the reproductive capacity of cells, which is thought to be mediated by damage to nuclear DNA, is complex and dependent on a number of factors. For sparsely ionizing radiations (low linear energy transfer [LET]) which includes X- and γ-rays, the primary damage to DNA is well separated and the cellular response curve has an initial shoulder region, after which the level of cell survival falls approximately exponentially with dose. Many model equations have been proposed to fit this dose response relationship[24]. In terms of the classical multi-target model the initial shoulder represents the ability of cells to repair some radiation damage, referred to as 'sublethal damage'. This concept is important in the subsequent understanding of the effects of dose fractionation. The slope of the terminal part of the curve is said to be truly exponential, and its slope is frequently used as a measure of the radiosensitivity of cells. This is usually referred to in terms of the D_0 or D_{37} dose, the amount of radiation that will reduce the surviving proportion to 37%, when exponential kinetics are reached. Using the 'in vivo' assay mentioned above for mouse kidney a D_0 dose of 1.25 Gy was obtained[23]. This is within the accepted range for mammalian cells. The overall equation for cell survival based on the multi-target model is:

$$\text{Survival (for dose D)} = 1 - [1 - e^{-(D/D_0)}]^n$$

where 'n' is the number obtained by a back extrapolation of the exponential part of the curve to the survival axis.

An alternative model, which has received much attention in recent years[25,26], assumes that the reproductive survival of cells can be fitted by a linear quadratic equation, where the survival (S) for a dose (D) is such that:

$$\log_e S = - [\alpha D + \beta D^2]$$

In such a model the initial part of the curve (α) would be linear while the terminal portion (β) would be continuously bending. This

model, with a continuously bending as opposed to an exponential terminal portion to the curve, is consistent with 'in vivo' experimental data[27]. The radiation survival studies for human kidney cells in culture have also resulted in a continuously bending curve[28]. In reality it is unlikely that any one single model equation adequately fits cell survival data.

A schematic cell survival curve following single doses of X- or γ-rays is illustrated in Figure 1. Using such a curve the effect of fractionated as opposed to single doses of radiation can be explained. The repair of sublethal damage between fractions results in a considerable dose sparing as a consequence of fractionation. The time scale for the recovery of sublethal damage indicates that the maximum effect is reached between 4 and 12 hours[20]. Shorter time intervals may result in incomplete repair and a reduction in the overall effect of dose sparing produced by fractionation. The effectiveness of fractionation may also be increased by cell proliferation between fractions.

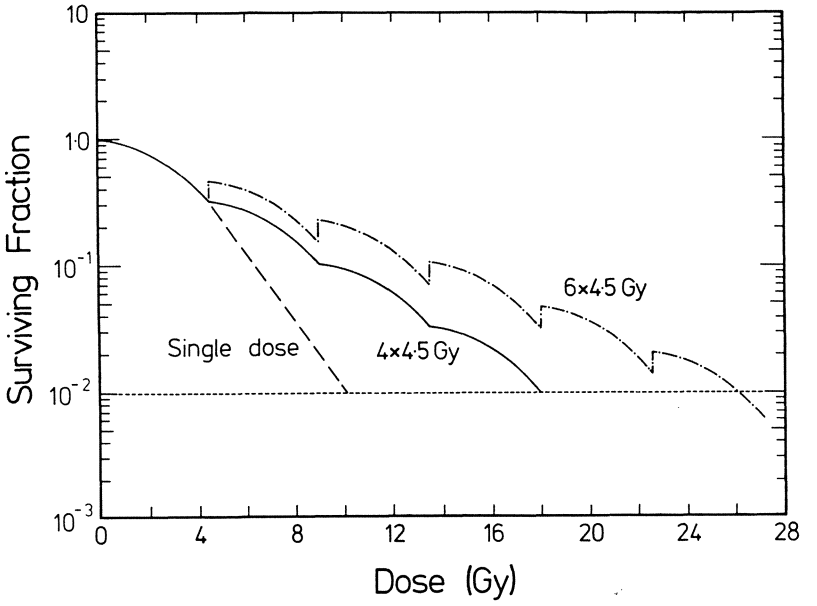

Figure 1 Schematic cell survival curves where the log of the percentage reproductive cell survival is plotted against radiation dose. With single doses the survival falls exponentially with dose after an initial shoulder (— — —). In the case of fractionated irradiation the first part of the survival curve is repeated at every dose, resulting in an increase in the total dose required to produce the same effect (—). An increase in the number of reproductively viable cells between doses (—·—) will produce a more pronounced dose sparing.

In Figure 1 one cell doubling has been assumed in 50% of the surviving population between each fraction. Thus nearly two addi-

tional fractions of 4.5 Gy (total dose 27 Gy) would be required to produce the same effect as four fractions of 4.5 Gy (total dose 18 Gy) with no repopulation. In normal tissues repopulation will only be significant when the damage is recognized. Thus it is more likely to be of importance in influencing the response of a tissue to fractionated irradiation in rapidly as opposed to slowly proliferating cell systems[29,30].

For X- and γ-rays the oxygen status of cells also influences the shape of the response curve, the curve for hypoxic cells being less steep (Figure 2). The dose-modification factor has a maximum value of three for equivalent levels of survival for oxic and anoxic cells. In radiobiological terms full oxygenation occurs at an oxygen tension of 30 mmHg; this compares with the range 20-40 mmHg for pooled venous blood. The relatively high oxygen utilization by some areas of the kidney may result in some degree of radiobiological hypoxia in situations of reduced blood flow.

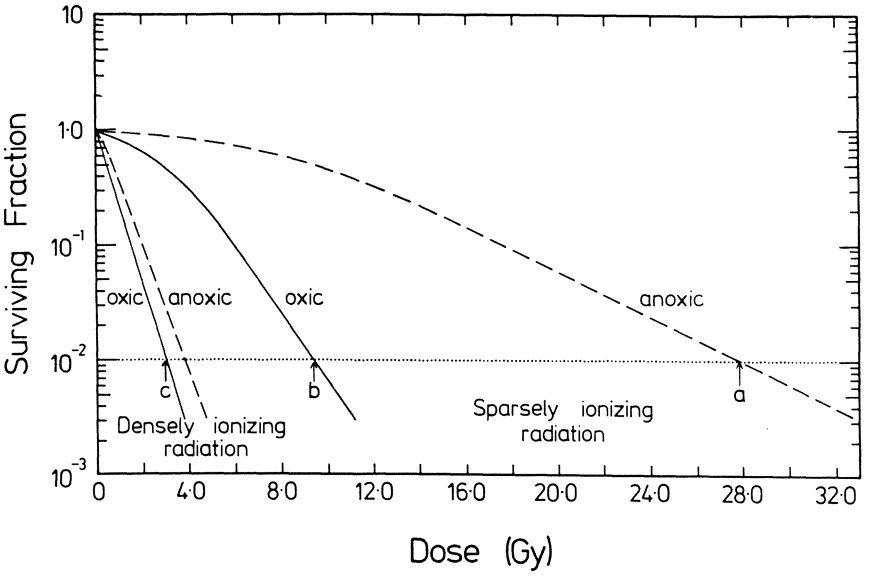

Figure 2 Schematic reproductive cell survival curves. For sparsely ionizing radiations (low LET) the dose modification resultant on irradiating anoxic compared with oxic cells has a maximum value of 3 (a/b). The RBE for the densely ionizing radiation (high LET) is obtained by comparing the ratio of doses required to produce the same biological effect (b/c). In these examples the dose levels that result in the survival of 1 cell in 100 are compared.

For densely ionizing radiations (high LET), such as fast neutrons or α-particles, the ionizations resultant on interaction are close together. In these circumstances, where a large amount of damage is accumulated in a small volume, little repair is possible

and the cell survival curve has little or no shoulder region (Figure 2). For high LET radiations, dose fractionation results in little or no dose sparing[31]. The protection afforded to cells by hypoxia is also lost. The lack of repair with high LET radiation means that they are more effective than X- or γ-rays. The ratio of doses to produce the same level of effect is a measure of the 'relative biological effectiveness' (RBE). Because of the nature of the initial part of the cell survival curve for the two types of radiation the RBE tends to increase with decreasing level of effect[32] (Figure 3). In terms of fractionated irradiation treatments this translates into an increasing RBE value with decreasing dose per fraction. If the cell survival curve for X- or γ-rays is of the form that has an initial linear component then there will be a maximum upper RBE value. Below this dose per fraction, further fractionation will not increase the maximum tolerated dose[33].

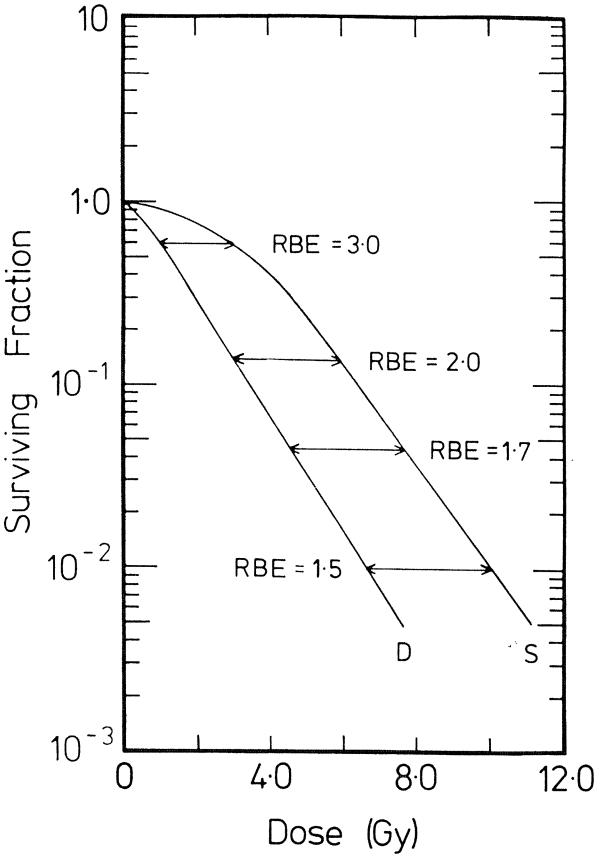

Figure 3 Schematic cell survival curves produced following irradiation with both a low LET and high LET radiation. Shows how the RBE value would increase with decreasing level of effect.

3. RADIATION TOLERANCE OF THE KIDNEY

The tolerance or maximum radiation dose that may be given to the kidney without the development of life-threatening complications is influenced both by radiobiological and physiological factors. In the clinical study of the effects of radiation on the kidney these factors often conflict with each other and a meaningful interpretation of the observations is frequently difficult. Thus for the independent evaluation of the importance of any single factor much emphasis has to be placed on the results from animal studies.

(a) Radiobiological factors

In clinical terms the kidney is seen as a radiosensitive organ and on the basis of the evaluation of clinical data a maximum dose of 20 Gy of a low LET radiation is considered the upper limit of tolerance[15]. This would appear to have been adopted somewhat arbitrarily, since this value is used for a broad spectrum of dose fractionation regimes.

The influence of fraction number and hence fraction size and overall treatment time, using low LET radiation, has been investigated extensively in recent years in both small[34,35] and large animals[36]. The experimental systems used by the various authors include single kidney irradiation[36], irradiation of both kidneys[34,35] and the treatment of a single hypertrophied kidney after unilateral nephrectomy (UN)[37,38]. In order to establish the total doses that result in an equivalent level of effect for the different dose fractionation regimes, damage to the kidneys has been assessed using a number of different physiological assay techniques, including renography[36], renal blood flow[39], urine output and [^{51}Cr]EDTA excretion[35]. Other authors have used either an histological grading system[38] or animal survival to determine iso-effect doses[37,40]. Despite these differences in experimental technique there is some degree of agreement in the influence of dose fractionation on the radiation tolerance of the kidney.

When fractionated doses of radiation were given within a period of 21 days, the total dose required to produce a given effect increased as the fraction number was increased. When the log of the total iso-effect dose was plotted against the log of the number of fractions (Figure 4), the slopes of the iso-effect curves were found to decrease as the total number of fractions increased (fraction size decreased). This would be expected if the dose response relationship for damage was fitted by a linear quadratic expression, where less repair would be seen between fractions as the dose per fraction was reduced and approached the initial linear portion of the dose effect curve. Between 2 and 15 fractions the slope of this iso-effect line varied between 0.45 and 0.21, the slope being steeper at the lower end of the fraction range due to the relatively low tolerance doses for total doses given as two fractions (Figure 4). Data for 2-15 fractions obtained by Jordan et al.[38] would not appear to be in agreement with these more general findings. These authors obtained an iso-effect plot with a steeper slope of 0.49. However, these studies differed in one important

respect: the doses were given as daily fractions, i.e. 2 fractions/24 hours, 15 fractions/19 days, and thus both time and fraction number were changing.

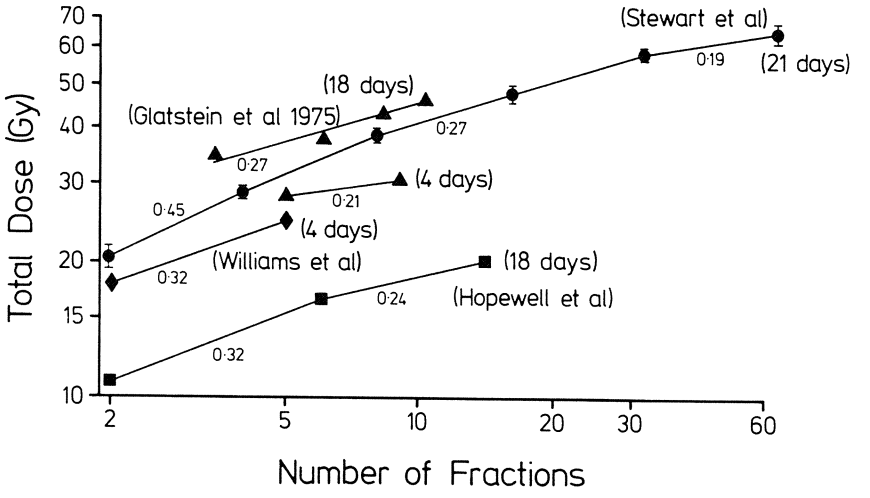

Figure 4 Log-log iso-effect plots for renal irradiation damage. Shows the change in iso-effect dose with fraction number for treatments given in 53 weeks (■ 46,47; ♦ 35; ● 50; ▲ 39).

In a few studies fractionated doses of radiation have been given over 35-40 days (Figure 5). In these studies the slope of the iso-effect plots was much steeper, 0.43-0.49, and did not change as a function of fraction number, even when total doses were given in 60 fractions. Sixty fractions were delivered as 2 fractions/day with a 6 hour interval between fractions.

It is these changes in the iso-effect dose with fraction number (dose per fraction) after X-rays that mainly influence the RBE value for fractionated neutron irradiation[31]. For large single doses of neutrons (≈10 Gy) the RBE was 1.7[41]. This increases rapidly with decreasing neutron dose per fraction reaching a value of 3-8 at ≈1 Gy per fraction[31,42]. One additional factor is that the absolute RBE value varies as a function of neutron energy[43].

A comparison of the data obtained for X-rays for both short and longer overall treatment times suggests an increase in the iso-effect dose with time for a given number of fractions, particularly when treatment times are increased from 18 to 40 days (Figure 6). However, the effect of overall time seems to be very variable with few, if any, general patterns emerging. In studies with mice, involving irradiation with 16 dose fractions, the slope of the iso-effect curve varied between 0.04 and 0.2 depending on the assay system employed[44]. Results based on urine output tended to

produce a larger time factor than those based on [^{51}Cr]EDTA excretion. The same was true in some earlier studies with two fractions[45]. For two fractions the time relationship appears to be greater in the pig kidney[46]; the same is true if the 14 fraction pig[46] and 16 fraction mouse[44] data are compared. However, when 5 (mouse)[45] and 6 (pig)[46] fractions are compared the reverse is found to be true. In other studies involving two fractions[37], no recovery was seen between 20 and 40 days. This is of interest, since in this model a single hypertrophied kidney was irradiated.

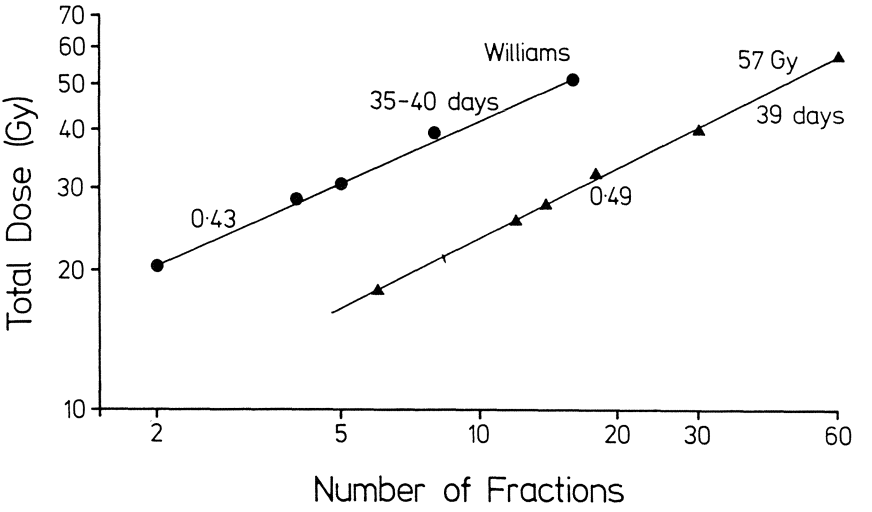

Figure 5 Log-log iso-effect plots for renal irradiation damage. Shows the change in iso-effect dose with fraction number for treatments given in 35-40 days (● 35; ▲ 46, plus some previously unpublished data).

It has been suggested that the repair seen between 3 and 6 weeks could be due to stimulated repopulation[47]. Thus in a UN model[37] where the remaining hypertrophied kidney has already expressed its proliferative potential, no additional recovery would be possible. Early radiation-induced changes in the kidney which might initiate renal growth have been reported (see Sections 4 and 5). However, the case for stimulated repopulation remains unproven in the kidney where renal growth is not always associated with extensive cell proliferation[48]. Alternatively, radiation induced changes in the kidney during radiation treatment, such as reduced blood flow and oedema[34] within the confines of the renal capsule may produce relative renal ischaemia and hence some degree of radiobiological hypoxia, rendering the kidney less sensitive to subsequent doses of radiation. Other authors favour a slow repair process[44], previously only reported in the lung[49].

The appearance of a significant time related effect for 9 frac-

tions given over 4 or 11 days[39] would seem to be at variance with the two fraction results obtained for pig and mouse. It may be that with 9 fractions/4 days the minimum inter-fraction interval of 6 hours was insufficient to allow for the complete repair of sublethal injury, resulting in a fall in the total iso-effect dose. When 60 fractions were given over 39 days to the pig kidney, or 64 fractions over 21 days in the mouse with a minimum 5-6-hour gap between fractions, then the results were consistent with the complete repair of sublethal injury. However, the fraction size used in these studies of 3.5 and >1 Gy respectively may be associated with different rates of repair of sublethal injury[51].

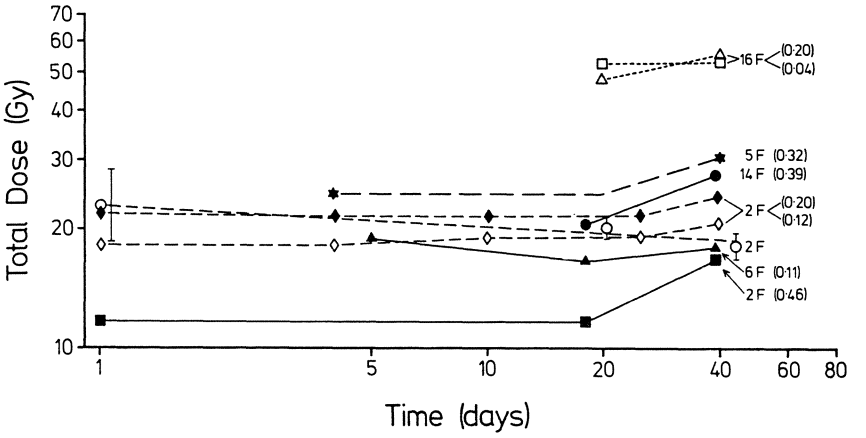

Figure 6 Log-log iso-effect plots for renal irradiation damage. Shows the change in iso-effect dose with variation in treatment time. The slopes of the lines between 3 and 6 weeks are indicated (△——□ 44; ▲——■——● 46,47; ◇——◆——✱ 45; ○——○ 37).

Thus the relationship between fraction number and overall treatment time and their influence on the kidney are extremely complex. Some of these changes can be explained in terms of well established radiobiological factors. However, these are unlikely to provide a complete explanation and thus in order to provide a fuller understanding physiological factors also need to be considered.

(b) Physiological factors

In addition to the above radiobiological factors there are physiological parameters which influence the functional status of an irradiated tissue and may lead to changes in overall radiation tolerance. This may be of particular importance when considering the renal response to irradiation in which there is a paired organ system. It is likely that the proportion of renal tissue irradiated

will have a marked effect on the subsequent 'apparent' renal tolerance. It has been shown that if one kidney is irradiated with the other 'in situ' then the irradiated kidney becomes reduced in size while the unirradiated kidney hypertrophies. However, if the contralateral kidney is removed prior to irradiation of the remaining kidney then that kidney does not undergo subsequent shrinkage[13,52,53], but remains at its pre-treatment size. The hypertrophic stimulus produced by unilateral nephrectomy and the contraction resulting from irradiation appear to be balanced. Redd[14] concluded that the degree of recovery of the kidney from a pathological process was determined, to a large extent, by the presence of the other kidney. This means that if another kidney were present to maintain life, the damaged kidney would show less tendency to recover than if it was being called upon to maintain life by itself. Thus in a unilaterally irradiated animal the kidney may be more radiosensitive than would be apparent from studies in which both kidneys were irradiated. There is some experimental evidence to substantiate this view. Van der Kogel[54] reported that the renal tolerance dose of rats in which both kidneys were irradiated was 1.3-1.6 times greater when compared with that of a single kidney both before and after contralateral nephrectomy. In addition, recent studies in pigs in which both kidneys were irradiated with 8.8-12.6 Gy have shown that individual kidney function, expressed in terms of effective renal plasma flow, was 2-2.5 times greater than that predicted from the results of unilateral renal irradiation studies[55].

There is little clinical evidence for an increased radiosensitivity in patients in whom a single kidney was irradiated. A study of 13 patients with non-Hodgkin's lymphoma, in which all or half of the left kidney received between 25.5 and 49.5 Gy, over 5-6 weeks, revealed no clinically demonstrable acute radiation nephropathy[56]. However, seven patients showed functional and/or morphological changes in the left kidney. The lack of demonstrable clinical radiation nephritis is likely to reflect the compensatory response of the unirradiated kidney, preventing any overall reduction in total renal function. The lack of clinical nephropathy does not necessarily preclude a reduction in functional renal status.

A number of experimental investigators have adopted the procedure of unilaterally nephrectomizing animals prior to irradiation[57-59], allowing the remaining kidney to exhibit compensatory growth before irradiation. The basis for such experimental systems is that it allows the total remaining renal tissue to be irradiated. The subsequent renal damage can be detected more easily. This is particularly relevant in small animal studies, where it is technically difficult to measure function in individual kidneys. On the other hand, total renal function can be assayed readily and such procedures can be adopted following UN. Such studies can be criticized for not having control data for comparative studies. In addition there remains the question as to the normality of the radiation response of a single hypertrophied kidney.

There is some evidence to suggest that age may exert some influence on renal tolerance. Whole body exposure to sublethal doses of ionizing radiation during the neonatal period results in the formation of abnormal renal glomeruli in the dog[60,61]. Gagnon

et al.[62] reported that in mice aged 5, 8 or 11 weeks, whose kidneys were exposed to continuous low dose rate β irradiation over a 16 hour period, the functional changes observed were inversely correlated with age. In a clinical report[17] radiation nephritis was described in three children after a latent period of only 3-5 weeks. It should be noted that these children underwent unilateral nephrectomy before radiotherapy and the remaining kidney may well have been under physiological stress. Thus the question of an age-related effect remains controversial, since other authors have reported the absence of an age effect[63,64].

In the clinical situation, as suggested above, the overall picture is often complicated by the presence of other factors which may influence renal tolerence to radiation. An important factor is the use of chemotherapeutic drugs in combination with radiotherapy. Arneil et al.[65] described two children who, subsequent to UN for nephroblastoma, received 15 and 20 Gy to the remaining kidney. This was preceded by chemotherapy with actinomycin D in association with vincristine. ARN developed 12 and 16 weeks after therapy. It was proposed that this was due to increased renal radiosensitivity as a consequence of the previous chemotherapy. This proposal was later supported by the observations of Moskovitz and Donaldson[66], who described two patients whose remaining kidney failed to hypertrophy after renal irradiation and chemotherapy comprising actinomycin D, vincristine and adriamycin. The complexity of factors influencing renal tolerance to radiation in the patient is compounded by the finding of a variability in susceptibility of the individual to renal irradiation[16,67]. Thus the likely renal tolerance must be carefully evaluated with regard to the particular clinical situation pertaining to that patient prior to therapy.

4. EFFECTS OF RADIATION ON RENAL FUNCTION

(a) Experimental studies

The early investigations into the effects of radiation on renal function have been reviewed extensively elsewhere[13,14] and will not be discussed here. The results from more recent studies in a variety of animal systems are summarized in Table 1. The direct comparison of results is difficult as a number of treatment schedules have been used by the different authors. These include UN followed by irradiation, irradiation of both kidneys and unilateral irradiation. Moreover, both single and fractionated doses of radiation have been used. Despite this, several conclusions can be drawn from these data.

Renal irradiation results in a decline in both glomerular filtration rate (GFR) and effective renal plasma flow (ERPF) within a few weeks[68-73]. The decline appears to be both dose dependent and progressive in nature. In studies where the acute changes in renal function have been assessed an initial hyperaemic response has been reported[42,68,73-75]. There is then a progressive decline in both GFR and ERPF. These changes in renal haemodynamics have been detected both by using classical clearance techniques

and more recently with radioisotopic methods. Renography has proved particularly valuable in the early detection of radiation nephropathy. In the dog the effects of single doses of 5, 10 or 20 Gy of X-rays to the left kidney were studied using [^{131}I]hippuran renography[72]. Changes were seen from the fourth to the sixth week in all animals treated with doses >5 Gy, despite the fact that they all had normal intravenous pyelograms (IVPs). The usefulness of renography has been confirmed by several authors[36,76]. The evidence for a radiation induced reduction in renal haemodynamics comes mainly from studies with large animals, since the techniques available for measuring GFR and ERPF are more easily applied to these species. However, similar results are available from small animal studies. Glatstein[77] assessed RBF using ^{86}Rb extraction in mice in which both kidneys had been irradiated with single doses of 11-19 Gy of X-rays. One day after irradiation RBF was increased; this was followed by a significant dose-dependent, decline in RBF in all dose groups after 2-3 months. Significant reductions in ERPF following renal irradiation have also been observed in rats[78]. Thus there is general agreement the functional changes resulting from renal irradiation reported in a wide variety of experimental animals exposed to a range of dose schedules, i.e. an initial hyperaemic response followed by a decline in both GFR and ERPF (particularly marked) within a few weeks of irradiation.

Table 1 Effects of kidney irradiation on renal haemodynamics

Species		Date	Radiation conditions dose (Gy)	Renal blood or plasma flow	Glomerular filtration rate	Reference
Dog	unilaterally nephrectomized	1953	20.1 fractionated	Normal	Normal	Mendelsohn and Caceres[68]
			27.5	↑ @ 7 days; ↓ @ 9-11 wks	↑ @ 7 days; ↓ @ 9-11 wks	
			36.8	↑ @ 7 days; ↓ @ 7-8 wks	↑ @ 9-11 wks ↓ @ 9-11 wks	
Rat	exteriorized kidneys	1957	25.0 single	↑ @ 7 days; normal @ 28 days	↑ @ 7 days; normal @ 28 days	Smith and Boss[69]
			30.0	↑ @ 7 days; normal @ 28 days	↑ @ 7 days; normal @ 28 days	
			40.0	↑ @ 7 days; ↓ @ 28 days	↑ @ 7 days ↓ @ 28 days	
Dog	unilaterally nephrectomized	1959	18.0 fractionated	↓ in 3 days	Normal	Klapproth et al.[70]
			36.0	↓ in 3 days	Normal	
			11.5 to 1/3 of kidney	Normal	Normal	

Table 1 Continued

Species		Date	Radiation conditions dose (Gy)	Renal blood or plasma flow	Glomerular filtration rate	Reference
Dog	both kidneys	1964	19.0 fractionated	Slight reduction @ 10 wks	Slight reduction @ 10 wks	Concannon et al.[71]
			25.0	↓ @ 10 wks	↓ @ 10 wks	
			31.0	↓ @ 10 wks	↓ @ 10 wks	
Dog	explanted kidneys unilateral irradiation	1967	5.0 single	↓ @ 5-7 months	↓ @ 5-7 months	Gup et al.[72]
			10.0	↓ @ 5-7 months	↓ @ 5-7 months	
			10.0 fractionated	↓ @ 5-7 months	↓ @ 5-7 months	
			20.0	↓ @ 5-7 months	↓ @ 5-7 months	
Mouse	both kidneys	1973	11.0 single	↑ @ 1 day;	--	Glatstein[39]
				↓ @ 2-3 months		
				↑ @ 1 day;	--	
				↓ @ 2-3 months		
				↑ @ 1 day;	--	
				↓ @ 2-3 months		
Rat	unilateral irradiation	1976	10.0 single	Normal	--	Chauser et al.[78]
			20.0	↓ @ 18 wks	--	
			30.0	↓ @ 12 wks		
Monkey	unilaterally nephroctomized	1978	9.6 fractionated	↑ @ 5 days;		Raulston et al.[42]
				↓ @ ≈50 days	--	
				↑ @ 5 days;		
				↓ @ ≈50 days	--	
				↑ @ 5 days;		
				↓ @ ≈50 days	--	
				↑ @ 5 days;		
				↓ @ ≈50 days	--	
Monkey	unilaterally irradiated	1982	25.0 fractionated	--	↑ @ 1-4 wks;	Anderson et al.[75]
					↓ @ 1-18 months	
Pig	unilaterally irradiated	1985	7.0 single	Normal	↑ @ 2 wks;	Robbins et al.[73]
					normal @ 4-24 wks	
			8.8	Normal @ 2 wks;	↑ @ 2 wks;	
				↓ @ 8-12 wks	normal @ 8-12 wks	
			10.7	Normal @ 2 wks;	↑ @ 2 wks;	
				↓ @ 6-8 wks	normal @ 6-8 wks	
			12.6	Normal @ 2 wks;	↑ @ 2 wks;	
				↓ @ 6-8 wks	normal @ 6-8 wks	

(b) Clinical studies

Support for a similar pattern of changes in the clinical situation is limited due to the practical limitations of a prolonged follow-up of patients. However, there have been studies where the acute effects of radiation on renal function have been investigated. Avioli et al.[79] specifically addressed the problem of acute changes in 10 patients who had histologically confirmed intra-abdominal and/or retroperitoneal malignancies with no evidence of renal disease. Standard clinical assays, such as blood urea nitrogen levels (BUN) and IVPs indicated no apparent changes in renal function following radiotherapy. However, a reduction in both GFR and RBF was detected both during and after completion of radiotherapy. The majority of patients showed evidence of relative renal ischaemia by the end of therapy. Changes in renal function were undetected by standard techniques in two patients examined 1 year after treatment, where both GFR and ERPF had fallen to 38 and 43% of control values respectively. Both these patients were clinically well. Avioli et al.[79] concluded that subtle subclinical alterations in glomerular and tubular function occurred during the latent period before the clinical expression of radiation nephritis, and that these were not reflected by corresponding changes in the routine, relatively insensitive, indices of renal function. Other studies have confirmed the poor diagnostic value of routine laboratory renal function tests to reflect the degree and nature of renal damage following irradiation[80].

Assays such as BUN and serum creatinine levels are poor indicators of acute radiation changes as they do not identify small decrements in renal function. This is not surprising, since more than 50% of the functioning nephrons need to be damaged before there is any elevation of serum creatinine levels. BUN levels are unreliable since urea metabolism can be influenced by extrarenal factors such as protein intake, the catabolic state of the individual and hepatic function[81]. Reliance on serum creatinine as the sole indicator of GFR may be misleading, particularly in patients with advanced malignant disease. In a steady state situation the serum creatinine concentration is the result of the constant production of this substance by skeletal muscle and its excretion by glomerular filtration. Malignant disease may result in a rapid and significant reduction in the body's muscle mass, which in itself lowers the serum creatinine concentration independently of glomerular filtration. Therefore it is imperative to provide highly accurate measurements of renal function and to follow the progression of renal damage in these patients as a possible guide for subsequent therapy. Scintillation imaging and renography appear to provide such practical and non-invasive methods[82].

The value of the renogram in detecting early changes in patients receiving radiotherapy to the pelvis and abdomen was assessed by Quinn et al.[82]. Serial renography demonstrated significant dynamic alterations in renal function that were undetected using IVPs. Similar results indicating the sensitivity of radioisotopic techniques have been reported[56,83,84]. Therefore it seems reasonable to conclude that the latent period before the clinical presentation of ARN reflects a gradual loss of function

which culminates in the appearance of gross clinical signs[85,86], these initial changes being undetected by standard renal function tests.

The question remains, can these modifications in renal functional parameters following irradiation be correlated with histological changes? This might help in ascertaining the particular cell type(s) at risk. Although histological changes have been reported in the kidney following irradiation there has been some disagreement between authors as to the nature of the changes produced. In many studies, particularly of clinical cases, damaged kidneys are seldom examined before the pathological process is well advanced. Therefore it is difficult to determine the sequence of the observed structural changes, or equally to define the primary as opposed to the secondary alterations. While some authors believe that the primary lesion is confined to the fine interstitial vasculature, and that tubules and glomeruli are only destroyed as a consequence of vascular damage[87,88], others state that the tubules, and in particular the proximal tubules, are markedly sensitive to radiation and that tubular degeneration and necrosis results in interstitial fibrosis, subsequently leading to renal sclerosis[89,90].

5. HISTOLOGICAL CHANGES

(a) Experimental investigations

Schulz and Hoffman[8] first described histological changes in the kidney: following the irradiation of rabbits they noted atrophy of glomeruli and tubules with a concomitant increase in interstitial tissue. The early studies have been reviewed extensively elsewhere[13,14], and will not be discussed here.

Sequential histological changes in rats in which one kidney was irradiated with a single large dose of 48 or 96 Gy were described by Madrazo et al.[91,92]. They concluded that radiation induced injury was mainly the result of damage both to parenchymal cells and the intercellular substance. There was a loss of contact between the cells and their basement membrane, with a subsequent overproduction of intercellular material leading to interstitial fibrosis. Arterial and arteriolar changes appeared 3-4 months after radiation, when renal atrophy was already evident. In a subsequent study using more radiotherapeutic doses (15 and 20 Gy) these authors reported similar changes[93]. Glomeruli showed progressive degeneration of epithelial, endothelial and mesangial cells with the eventual complete collapse of the glomerular capillaries. The latter was thought to be due to alterations in the basement membrane and/or loss of support due to endothelial cell detachment. The tubular changes, involving both the proximal and distal convolutions, consisted of degeneration and necrosis of tubular cells with the detachment of cells from the basement membrane. The basement membrane exhibited characteristic layering and thickening, with the accumulation of intact cells and cell debris between the layers. This was taken as being indicative of severe, progressive, tubular cell atrophy and repair. Interstitial capillary changes were mild and, combined with the apparent ab-

sence of vascular damage at the initial stages of injury, indicated that radiation injury was mainly the result of direct damage to the parenchymal cells. Vascular injury was a later complicating factor. Overall these studies indicated that the radiation induced changes were the same over the dose range 15-96 Gy, increase in the dose merely accelerated the process. Similar progressive and dose-dependent changes at the glomerular and tubular level have been reported in the rabbit[94].

Glomerular changes were reported by Glastein et al.[34] in a sequential morphological study of radiation-induced murine renal disease. The most striking alteration, seen from 3 months onwards, was in the glomeruli. This consisted of the progressive replacement of capillary walls and lumina by acidophilic, periodic acid-Schiff positive material. Tubular atrophy and stromal fibrosis was also evident, but was not seen before 4 months; although progressive, tubular atrophy and stromal fibrosis were less severe than the glomerular lesion. In contrast Philips and Ross[57], using UN mice, reported the relative sparing of glomeruli following fractionated radiation doses of 10-15 Gy. The major changes occurred in the tubules, and were associated with damage to tubular capillaries with subsequent atrophy and fibrosis. These changes were restricted to the cortical region. Other investigators have reported radiation damage to renal tubules[59,95].

The early progression of radiation damage has been studied in adult beagles[96]. Biopsy specimens taken 3-5 weeks after irradiation showed early parenchymal degeneration with focal regeneration. Vascular changes consisted of mildly damaged and thickened vessel walls with occluded capillaries. By 7-9 weeks there was extensive proximal tubular epithelial degeneration and interstitial fibrosis; focal tubular regeneration was clearly evident. Biopsies taken at 11-13 weeks revealed evidence of extensive repopulation except in a few wedge-shaped areas which showed marked parenchymal depletion and fibrosis. The data inferred that acute radiation induced vascular damage, such as endothelial cell separation and swelling, could initiate radiation nephropathy.

(b) Clinical investigation

Histological observations from clinical cases of radiation nephritis have been reviewed previously[13,14]. The first reported microscopic observations[11] were of glomeruli largely obliterated by connective tissue, thickening of Bowman's capsule and compressed glomerular tufts. Tubules appeared dilated, atrophic or necrotic, and the intima of large vessels showed evidence of connective tissue thickening. Zuelzer et al.[17] described different stages of glomerular and tubular damage in children who died 3-7 months after radiotherapy. The changes described ranged from complete scarring and glomerular obliteration to recent degenerative changes and acute necrosis, indicative of recurrent renal injury. The characteristic glomerular lesions consisted of prominent endothelial cell damage, basement membrane thickening, obstruction of capillary loops and focal necrosis. In contrast, Davey et al.[97] stated that the primary lesion was tubular, and suggested that this portion of

the nephron, particularly the proximal and distal convoluted tubules, was more sensitive to radiation than the glomerulus. However, later studies confirmed the presence of definite glomerular changes in man[98,99].

The electron microscopic evaluation of radiation induced histological changes produced evidence for glomerular hypocellularity, with degeneration of both endothelial and epithelial cells with the occlusion of capillary lumens[100]. Proximal tubular epithelium exhibited nuclear pleomorphism, hyperchromasia and focal proliferation. Mostofi and Berdjis[101] observed primarily marked interstitial fibrosis with variable vascular changes and relatively well-preserved glomeruli in patients evaluated many years after treatment. However, patients evaluated 6-12 months after therapy often had marked glomerular changes with variable interstitial findings. In biopsies from two patients[102] who had presented with renal insufficiency 10 months after abdominal irradiation for ovarian carcinoma, there were marked glomerular changes; 2 months later these changes had progressed to include interstitial fibrosis and vascular damage. During this interval the patients were only moderately hypertensive. This indicates the difficulties involved in classifying radiation nephritis either as a primary interstitial or glomerular process. Ultrastructural glomerular changes included the marked deposition of electron-lucent material between the basement membrane and the endothelial cells. These were similar to the radiation induced glomerular abnormalities described in the reports of experimental studies[34,93]. These observations have subsequently been confirmed in histological material from other patients[103,104].

Mostofi[105] grouped the pathological changes found following renal irradiation into various categories. The most frequently observed category was termed sclerosing nephrosis; this consisted of early tubular atrophy followed by more severe tubular atrophy, interstitial fibrosis and glomerular hyalinization. Vascular narrowing was evident but was minor in comparison with the extent of tubular atrophy. In the extreme form there is considerable tubular loss and atrophy, glomerular hyalinization and vascular necrosis, leading to a 'shrinking kidney'[98]. This corresponds to the clinical condition known as CRN[16]. A second category, nephroglomerulosis, manifests as a mild degree of sclerosing nephrosis, consisting largely of glomerular changes; this corresponds to ARN[16]. Mostofi[105] suggested that the term radiation nephritis was unsatisfactory, as it implied an inflammatory process. In reality it was a progressive degenerative lesion. The term radiation nephropathy appears to be more generally acceptable.

White[86] used the term nephroglomeruloendotheliosis to describe the earliest renal lesions. The lesions involved alterations in the microvasculature with swelling and disturbance of intercellular connections between renal capillaries and arteriolar tissue. These progress into areas of micro infarction leading to necrosis. The above studies highlight the need to use suitably sensitive histological techniques in order to detect the initial lesion induced by radiation. Ultrastructural studies have revealed changes which then progress, only becoming detectable with light microscopy at a later stage when it is difficult to determine the true development of the lesion. It seems likely that there is a continuous spectrum of early

and intermediate glomerular and tubular changes which lead, after an apparent latent period, to clinical radiation nephritis[16]. The gradual progression of the anatomical lesions resulting from radiation are further complicated by the development of hypertension.

6. RADIATION-INDUCED RENAL HYPERTENSION

Hypertension has frequently been shown to be associated with irradiation injury. Although the mechanism responsible has not been fully elucidated its renal origins have been clearly demonstrated in clinical cases where the removal of a single damaged kidney resulted in hypertensive patients becoming normotensive[80,106,107]. In a case described by Dean and Abels[80] a woman who received 46 Gy, over 25 days, to the upper quadrant of the left kidney, presented 7 years later with hypertension and evidence of impaired function in the left kidney. After removal of the left kidney the patient recovered and became normotensive.

The development of hypertension following the irradiation of both kidneys has also been clearly demonstrated[17,108]. The results of studies in rats[67] led to the conclusion that renal irradiation produced two distinct and independent biological effects, namely interstitial fibrosis and hypertension. These could develop together or independently. Moreover, hypertension could be induced by renal irradiation in the apparent absence of the structural changes normally associated with radiation sclerosis. When these structural changes were found to occur later their origin was uncertain. They closely resemble lesions found in human essential malignant hypertension and could be secondary to the high blood pressure and not the result of primary radiation damage. However, the fact that renal hypertension could develop in the apparent absence of anatomical lesions might reflect the use of inappropriate histological techniques rather than a true absence of anatomical lesions.

The pathogenesis of radiation-induced hypertension, as stated previously, is unknown. Vascular necrosis was found in arterioles and in the interlobular arteries of the kidneys in patients presenting with radiation induced hypertension, while they have only been seen in hypertensive non-irradiated patients when the hypertension was considerably more marked. Asscher et al.[109] demonstrated that radiation and hypertension could have a synergistic action on blood vessels. They suggested that irradiation increased arterial wall susceptibility to hypertensive damage by causing an excessive myogenic response to changes in intravascular pressure. Excessive vasoconstriction might result, leading to necrosis of the arterial wall. This increased vasoconstriction might also lead to ischaemia, causing hypertension due to vascular occlusion. The importance of ischaemia in the development of essential hypertension was originally proposed by Ellis[110], who suggested that the mechanism of hypertension was renal ischaemia. He added that this ischaemia was likely to be of functional origin, due to an 'extrarenal factor', rather than to renal arteriosclerosis. It has been suggested that the increased blood pressure results from an increased secretion of renin by the irradiated kidney(s)[111,112], the stimulus for the enhanced secretion being a renal ischaemia as

a result of subtle damage to intrarenal arterioles[113]. Structural as opposed to functional occlusive changes have been reported in irradiated renal vessels[88].

In an investigation of unilateral radiation nephritis in rats[114] it has been shown that irradiated kidneys exhibited microangiographic alterations; these consisted of a reduction in cortical vasculature relative to medullary vasculature, apparently due to glomerular lesions induced by radiation. This change in intrarenal blood flow distribution resulted in a relative cortical ischaemia. It has been established experimentally[115,116] that renal ischaemia may give rise to arterial hypertension, and that a reduced cortical blood flow is responsible for the development of hypertension in ischaemia[117].

This may also be the case in man, and might provide a mechanism whereby radiation-induced injury to the kidney can result in hypertension. Radiation induced vascular alterations in the rabbit kidney[118] were very similar to those found earlier in rats[113], i.e. in the early stages following irradiation there appears to be a shunting of the contrast medium through the medullary portion of the kidney at the expense of the peripheral glomeruli. This could not be easily identified in histological sections taken at the same time. This suggests a possible cause and effect relationship between these radiation-induced functional changes and hypertension.

7. PATHOGENESIS OF RADIATION DAMAGE

The results of animal studies into the pathogenesis of radiation-induced nephropathy have led to two opposing schools of thought concerning the primary site of radiation damage, namely vascular or parenchymal. Proponents of the former consider that the initial lesion is vascular, and that tubules and glomeruli are only lost as a consequence. Conversely it has been suggested that the late effects after irradiation, namely parenchymal or stromal depletion, are a primary effect and are not secondary to ischaemia.

Casarett[119] reported arterionephrosclerosis following ?-particle irradiation from polonium, intravenously injected into rats. Serial sacrifice of these animals revealed a slow progressive nephrosclerosis. Initial changes within 2 months included arteriolar constriction resulting in a relative ischaemia[120]. Progressive obstruction of arterioles continued, in association with atrophy, fibrosis, hyalinization of glomeruli and degeneration and atrophy of the tubules. The reduction in calibre of renal arterioles was also reported to result in multiple sites of ischaemia, and thus localized atrophy. It was stressed that vascular change preceded parenchymal damage. The occlusion of the lumens of arterioles appears to be due to endothelial cell swelling[121], which may or may not progress to necrosis. If the endothelium remains viable then proliferation occurs, leading to further occlusive changes, resulting in a further compromising of the renal blood supply. Radiation-induced occlusive changes in glomerular arterioles and capillaries have been reported both in experimental[34,88,96] and clinical[17,100,102] studies. Such occlusive changes have been

proposed on a theoretical basis[88]. In addition, reduced cortical perfusion has been demonstrated angiographically following experimental[118] and radiotherapeutic[122] treatments. It has been well documented that such occlusive changes and the subsequent renal ischaemia, can cause specific tubular cell loss[34,86]. Indeed, the particular loss of proximal tubules following radiation injury can be explained, in part, in terms of their particular susceptibility to ischaemic injury[123]. The occlusion of arterioles and the resulting ischaemia after irradiation, are changes which are not restricted to the kidney, suggesting a general pattern for radiation-induced vascular damage[124].

The precise mechanism by which endothelial swelling occurs is unknown. There is some evidence to suggest that it is a direct effect of radiation[125,126], perhaps being an indication of the reproductive death of endothelial cells. However, endothelial swelling has been reported during epinephrine-induced vasoconstriction[127], suggesting that radiation may act indirectly via vasoconstrictive agents. Keane et al.[102] speculated that radiation induced endothelial cell damage resulted in local activation of the coagulation system with subsequent thrombosis in the renal microvasculature.

There appears to be a considerable amount of evidence to implicate the primary role of the vasculature in radiation induced nephropathy, whereby a reduction in cortical perfusion is induced. In view of the similarity between the haemodynamic and angiographic patterns of radiation nephropathy and those seen in acute renal failure caused either by a number of nephrotoxic agents, shock, trauma or haemolysis, it is tempting to implicate a local intrarenal vasomotor mechanism as a common pathway.

Withers et al.[23] have proposed that radiation nephritis results from parenchymal cell depletion and state that there is no evidence that vascular injury plays a role in the aetiology. Radiobiologically the kidney has been regarded as a late responding system, since the kidney does not exhibit acute radiation injury due to the low proliferative activity and slow turnover rate of normal renal parenchymal cells[128]. Since radiation damage is only likely to become manifested as cells attempt mitosis, then a long latent period would be expected, indeed there appears in clinical acute radiation nephritis to be a latent period of some 6-12 months[15]. This argument has been extended by Williams and Denekamp[45], who state that stimulated repopulation, which has been shown to be important in the sparing of acutely responding normal tissues to fractionated irradiation, seems an unlikely explanation for the time-related recovery found for fractionated irradiation in late-responding tissues, such as the kidney (see section 3). They have expressed the view that the observed increment in dose sparing to fractionated irradiation occurs before radiation damage has been expressed functionally and hence a stimulus for such proliferation does not exist. Thus the parenchymal argument consists of two basic assumptions: (1) tubular cell loss occurs without any apparent vascular change, and (2) radiation induced injury causes late effects since renal parenchyma exhibits a slow cell turnover, with little evidence for an early expression of functional damage.

The argument that there is no evidence for vascular injury causing late effects in the kidney ignores considerable evidence to the contrary. Furthermore the assay method of Withers et al.[23] fails to distinguish whether damage to tubular cells is direct or indirect, in effect reflecting damage to the vasculature. With respect to the question of the time for expression of renal damage, it has been shown that if suitably sensitive tests are employed then structural and functional changes can be seen in the kidney a short time after irradiation. That these changes are associated with increased proliferation due to cell loss has also been demonstrated[96,129]. Thus where sensitive tests have been used, early radiation induced changes have been detected. The importance of the assay systems used has recently been illustrated by Jordan et al.[130] in an investigation of radiation-induced injury in the mouse kidney. They noted that functional changes in the rodent kidney were relatively poor indicators for evaluating radiation damage due to technical difficulties, possible compensatory changes and repair mechanisms. Histological endpoints appeared to have considerable potential for predicting late tissue damage. It is interesting to note that in studies with a large animal, the monkey, functional changes were the best indicators[75]. These examples illustrate the need to use suitable biological parameters and the application of these to the appropriate system.

Instead of the vascular and tubular mechanisms of radiation injury being regarded as mutually exclusive it seems more reasonable to consider that both play a major role in the development of radiation nephropathy. Vascular changes causing a relative cortical ischaemia may lead to proximal tubular cell loss, in addition to tubular cell loss due to the direct action of radiation on the cells; the two events do not preclude each other.

8. FUTURE DEVELOPMENTS

In the clinical situation where the kidney is included in the treatment volume, there is likely to be a move to investigate, in more detail, the effects of hyperfractionation regimes, i.e. many small dose fractions. Observations based on recent fractionation studies[26] indicate that tolerance doses for late effects increase more steeply with the decline in radiation dose per fraction than do early effects. If late-responding tissues determine the tolerance dose, then each dose per fraction should not be greater than the dose at which the survival curve for the target cells responsible for the late effects bends down. It is at such doses that optimum sparing of the late-responding tissue, of which the kidney is believed to be one, may be found. If tumours are early-responding tissues then some therapeutic gain will be obtained. Initial experimental findings tend to support this concept[31].

Investigations into the pathogenesis of chronic radiation injury will, it is hoped, detect early and possibly reversible changes that might anticipate chronic irreversible alterations. One can speculate that if steps could be taken to alleviate the initial ischaemia then the resultant renal damage might be reduced. There is some evidence, albeit indirect, from clinical studies to support

such a proposal. Surgical correction of RBF has been shown to restore renal function in previously non-functioning kidneys[131-133]. Renal biopsies have shown that such revascularization has resulted in an apparent regeneration of initially atrophic tubules to produce a kidney with a normal appearance[132]. A similar restoration of renal function has been demonstrated in unilaterally irradiated pigs[134]. Removal of the unirradiated contralateral kidney 6 months after radiation resulted in a rapid and pronounced increase in renal size and function (GFR and ERPF) in the previously non-functioning irradiated kidney.

There are possible parallels between these clinical observations in vascular occlusive disease and the effect of UN on unilaterally irradiated kidneys in the pig. In both cases there was, initially, a small essentially non-functioning kidney. Following intervention vascular supply was improved, resulting in an increase in renal size and functional status. Since the reduction in RBF following irradiation may, at least in part, be of functional origin it is possible that the use of vasoactive compounds may increase RBF and specifically cortical nephron flow[135,136]. This may modify the initial effects of radiation and subsequently reduce the deleterious effect of radiation on renal function.

In patients treated to full renal tolerance with X-rays it is likely that a high proportion of patients will incur some degree of kidney damage which will not produce clinical symptoms. In these patients the kidneys may be under some degree of physiological stress and further nephrotoxic insult might lead to marked loss of function. More precise information is needed concerning the upper limit of radiation which can be administered to patients concomitantly receiving cytotoxic agents, particularly in view of the increased use of treatment regimes involving radiotherapy combined with chemotherapy. The use of chemotherapeutic agents such as cisplatin, which is known to be nephrotoxic[137], further highlights the need to accurately monitor subtle changes in renal function; even if an irradiated kidney shows no evidence of clinical nephritis there may be an impairment of that kidney's recovery potential. More information is also required concerning the importance of renal functional reserve. Bosch et al.[138] have shown that in patients with a reduced number of nephrons the functional reserve may be diminished or absent. Such a patient would be particularly susceptible to further radiotherapy and/or chemotherapy. In all cases there is an urgent need to accurately monitor the renal status of the patient. Whether or not the treatment of early, possibly reversible, alterations in renal function may improve the patient's prognosis remains to be elucidated.

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28
EPIDEMIOLOGY IN THE ASSESSMENT OF NEPHROTOXICITY

D.P. SANDLER

I. BACKGROUND

Numerous environmental and occupational exposures have been identified as potential human nephrotoxins. Animal and experimental studies as well as clinical case reports all suggest that the kidney is at risk for damage due to acute and chronic exposure to metals, solvents, and a wide variety of drugs. Acute renal failure following many toxic exposures has been well documented, and in many of these instances case reports by clinicians or industrial hygienists have been sufficient to establish the existence of disease risk.

Links between environmental exposures and chronic renal disease, however, are more difficult to identify. Associations between renal disease and exposures which occurred long in the past are often difficult to establish. Prior exposures are difficult to document, and long-term follow-up of exposed groups is costly and difficult to carry out. Few well-designed epidemiological studies have examined the prevalence, risk factors or natural history of occupationally or environmentally induced chronic renal disease.

Environmental epidemiologists have traditionally focused on mortality from all causes or from cancer among occupationally exposed groups, or have conducted retrospective studies of easily identified or counted groups of patients, such as patients identified from cancer registries. Few of the simple methods available to epidemiologists are easily applied to the study of chronic renal disease, and this perhaps accounts for the comparative lack of studies.

Accumulated evidence from available epidemiological and clinical studies suggests that nephrotoxic exposures contribute substantially to morbidity and mortality from chronic renal disease, but evaluation of the evidence is made difficult by problems inherent to the study of chronic renal disease. Such problems include the lack of uniform criteria for diagnosis and classification of

renal disease patients, the often insidious onset of renal disease, the potentially long latency between exposure and disease onset, the high frequency of other chronic conditions in renal disease patients which may or may not be aetiologically significant, the possible interaction between underlying conditions which affect renal function and nephrotoxic exposures, the possibility that the aetiology is multifactorial, and the fact that chronic renal disease comprises a heterogeneous group of conditions with apparently similar manifestations. These problems are not unique to the study of chronic renal disease, but they have nonetheless contributed to a general lack of clinical awareness that a large proportion of chronic renal disease may be due to environmental exposures. This lack of awareness, together with a focus on palliation rather than prevention of kidney damage, continues to make epidemiological evaluation of environmentally induced renal disease difficult.

II. EPIDEMIOLOGICAL APPROACHES

Epidemiology is essentially observational. However, with careful attention to the limitations and advantages of different methodologies, much can be learned from evaluation of the clinical and epidemiological literature. Observational research studies that have been used to evaluate some potential nephrotoxins include case reports and case collections, ecological and cross-sectional studies, retrospective or "case-control" studies, and prospective or "cohort" follow-up studies. These approaches and their limitations will be described briefly below as a basis for later evaluation of the literature on selected potential nephrotoxins, including lead, cadmium, solvents, and analgesic drugs.

A. Case reports and case collections

Numerous case reports and case collections describe patients with renal disease and some recent or prior exposure to a potential nephrotoxin, such as lead, solvents or specific medications. These reports are an important starting place for epidemiological research, but they offer no indication of the magnitude of the association, if any, between the exposure and renal disease. Case reports are biased towards positive associations. There would be little interest in a report of five patients with renal dysfunction in whom lead exposure was not documented, for example. Such reports are also biased towards unusual exposures and towards exposures which immediately precede disease onset. Case reports offer no information about the population from which the cases arise. Without any indication of the frequency of the same exposure in the general population, no inferences regarding disease risk can be made.

B. Ecological and cross-sectional studies

Ecological studies have been useful in identifying potential links

between environmental exposures and renal disease. In such studies, areas with high disease incidence are compared with areas of low disease incidence to identify potentially important exposure differences. Alternatively, disease incidence or prevalence in areas with known high and low exposures are compared. Studies have compared areas of high and low analgesic consumption and have compared areas with high and low cadmium exposure to evaluate morbidity or mortality from chronic renal disease. Unfortunately, associations identified in this way may be misleading. Although mortality from chronic renal disease may be higher in a community with known cadmium pollution than in a cadmium-free community, for example, it is not clear that individuals who are most exposed to cadmium are those who are dying from renal disease. Some other exposure that is also more common in the cadmium-polluted area which is also more common among individuals who develop renal disease, such as use of analgesic drugs, may produce a spurious association between cadmium pollution and renal disease. This is known as an "ecological fallacy".

A related approach has been to compare renal morbidity and mortality rates in a particular community before and after a change in exposure to a potential nephrotoxin. For example, studies have compared deaths from renal disease before and after an "epidemic" of lead poisoning or before and after a ban on the sale of phenacetin-containing analgesic mixtures. As with all ecological studies, inferences based on community-wide data are not necessarily true of individuals, and some other factors may be responsible for any apparent changes in disease rates. Time trend studies are also subject to biases introduced by changes over time in diagnostic expertise or rigour. Simple awareness that an exposure may produce a specific renal abnormality may result in increased reporting of such abnormalities, for example, without any real change in the prevalence or incidence of the condition. Such awareness bias could also account for differences between communities in any one time period. Thus, geographical differences in the reporting of renal disease associated with analgesic abuse could be a function of differences in awareness of the potential problem.

Cross-sectional studies, which simultaneously measure renal disease or dysfunction and exposures, have also been used to evaluate potential nephrotoxins. For example, workers potentially exposed to lead have been screened for evidence of lead exposure and early renal dysfunction. Cross-sectional studies may avoid the "ecological fallacy" that potentially plagues studies at the population level, but do not provide strong support for any causal relationship. Thus, while individuals who consume large quantities of analgesic drugs may be found to have renal dysfunction, it is not always possible to establish that the exposure preceded the renal damage.

C. Cohort studies

Potential nephrotoxins have been evaluated through long-term follow-up of occupationally exposed cohorts. Generally these studies have involved retrospectively identifying workers employed

in the past in a particular industry, and using vital statistics and occupational records to trace the cohort forward to the time of the study. Mortality from all causes is the traditional endpoint for such studies, although morbidity is occasionally evaluated. Other types of cohorts, such as a cohort of women known to consume analgesic drugs, have also been studied. Advantages of cohort studies include unbiased ascertainment of exposure status (that is, exposure status is determined before disease onset and is thus independent of disease status), and the ability to evaluate risks from exposures that are unusual in the population at large.

Cohort studies, however, often require large numbers of workers and/or a very long-term follow-up to identify potential risks. Mortality rates attributed to chronic renal disease are relatively low, and associations may be missed because too few workers are studied. The latency between exposure and fatal (or even clinically evident) renal disease may be quite long, requiring follow-up intervals as long as 30 or more years. Many of the industries involving exposure to potential nephrotoxins are small, and identifying large enough groups of workers is difficult.

Nephrotoxic exposures may result in disabling, but not necessarily fatal chronic renal disease, and studies of mortality only may be misleading. Furthermore, cohort studies often suffer from low rates of follow-up, and workers who develop chronic disease may be those least likely to be traced.

Many occupational cohort studies rely only on data available from existing records, and it is not often possible to obtain information on other potentially important exposures that may be associated with renal disease risk. Exposed workers may be more likely to consume analgesic drugs, to smoke cigarettes or drink alcoholic beverages, or to have had other jobs with hazardous exposures. If these additional exposures also produce renal disease, a spurious association between the occupational exposure of interest and disease will result. Information on such potential confounding factors is rarely available through existing occupational records.

Information is also rarely available on other medical conditions which might relate to renal disease risk. While persons with hypertension and diabetes are at increased risk of developing renal dysfunction, not all persons with these conditions are affected. It is possible that individuals with underlying compromised renal function are most at risk from occupational exposure to particular nephrotoxins, or that only those with secondary exposures are at risk. Baseline differences between exposed cohorts and comparison groups in the prevalence of conditions such as hypertension could also account for any observed differences in renal disease risk. Thus small observed differences in renal disease risk between cohorts known to consume analgesics and those who do not consume analgesics could be explained by differences in the prevalence of hypertension, which may or may not be a function of other hazardous exposures.

Finally, many cohort studies do not provide information on actual level of exposure to the supposed nephrotoxins. Good exposure monitoring data are not usually available on a wide scale. Exposure in retrospective studies is usually inferred from job

titles, or simply from exposure in a particular industry. Little information is usually available on other exposures in the same industry, or on exposures that may have occurred during prior or subsequent jobs.

In some instances exposure monitoring data are available for small subsets of workers who have been followed in epidemiological studies. Because the sample sizes are small, these studies are not often able to evaluate mortality from renal disease, or even to evaluate clinically significant renal dysfunction. Recent studies have measured markers of early renal dysfunction in groups of workers with documented exposures. These studies provide important information on the existence of potential risks. However, the long-term implications of some of these very early changes have yet to be established.

D. Case-control studies

Epidemiological case-control studies are quite useful for establishing risk factors for relatively rare diseases such as end-stage renal disease. In these studies, individuals with disease and appropriate comparison subjects are studied for evidence of prior exposure to specific substances. Such studies often rely on questionnaire data to ascertain exposure histories, but may also take advantage of existing records or even biochemical tests to confirm past exposures. Case-control studies, unlike cohort studies, offer the opportunity to simultaneously evaluate risk associated with several exposures and to evaluate potential interactions.

Patients with end-stage renal disease and other hospital patients have been compared to evaluate risk associated with analgesic consumption, and a few groups of patients with glomerular disease have been compared with other patients to evaluate risk associated with solvents. Case-control studies "nested" in cohort studies have also been used to evaluate risk from cadmium or lead exposure when exposure levels were difficult to document for large groups of workers. There have been, however, relatively few studies of renal disease involving this approach. Reasons for this may include difficulties in:

1. identifying renal patients other than those on dialysis;
2. classifying patients with renal disease; and
3. identifying patients with renal disease who do not have other associated illnesses which might also be related to toxic exposures.

These problems have important implications for the study of chronic renal disease and will be discussed later in this chapter.

Case-control studies allow for the identification of large numbers of patients in relatively short periods of time, although for uncommon exposures it may not be possible to identify enough cases from the population at large to adequately evaluate risk. Case-control studies are also often limited by the quality of retrospective exposure data that can be collected, by difficulties establishing that exposures preceded disease and that exposure (or

the lack of exposure) is not a consequence of disease, and by difficulties in generalizing from select groups of patients and controls to the population at large.

The inferences that can be drawn from case-control studies are often limited by how cases are identified. If only selected groups of patients with renal disease progress to end-stage disease, or are eligible for dialysis services, findings from studies of dialysis patients may not be generalizable. If patients with early renal disease are not likely to be diagnosed unless they have other conditions which bring them to medical attention, results from studies of patients identified through medical records also may not be generalizable. The way in which patients are diagnosed and identified for study will also influence whether or not correct inferences can be made from study findings. In case-control studies, care must be paid to insure that the diagnosis of renal disease is completely independent of exposure information. For example, it is essential that a history of analgesic abuse or lead exposure not be included in the criteria for making a diagnosis of interstitial nephritis. Otherwise no estimate of risk given such exposures can be made.

"Population-based" studies are often carried out so that findings may be generalized, and so that the amount of disease in the population attributable to particular exposures can be determined. This approach is not readily available for renal disease studies because the population with renal disease is not easily recognized. Dialysis and transplant registries, unlike cancer registries, are not likely to account for all patients with renal disease or dysfunction.

Determining the appropriate comparison group in case-control studies is also quite difficult. Textbooks describing the advantages and disadvantages of different types of comparison groups, including other hospitalized patients and randomly selected individuals from the population, are available, and this material will not be duplicated here. However, the problem of appropriate comparison groups is particularly relevant to evaluating studies of some particular nephrotoxins. For example, in a study of risk associated with use of analgesic drugs it may be difficult to identify hospitalized comparison subjects whose diseases are not related in some way to use of analgesics (either the disease is medicated with analgesics or analgesics may be proscribed). In another example, in a study evaluating occupational exposure to solvents in patients with acute renal disease, use of hospital controls with chronic conditions might overestimate any risk associated with occupational exposure because persons with chronic diseases may be less likely to be employed. Alternatively, studies comparing healthy subjects with patients who have chronic renal disease and who are no longer employed, could underestimate risk if attention is paid only to recent occupational exposures.

III. PROBLEMS IN THE STUDY OF CHRONIC RENAL DISEASE

A. The scope of the problem

The contribution made by environmental and occupational exposures

to chronic renal disease needs to be understood in the context of the morbidity and mortality attributed to chronic renal disease. Vital statistics and other existing data, unfortunately, do not adequately indicate the magnitude of the renal disease problem. Renal disease may be under-reported in vital statistics data on underlying cause of death. An estimate of the incidence of end-stage disease can be obtained from dialysis and transplant registry data, but this is not an adequate source for ascertainment of time trends or geographical differences in disease incidence. Recent changes in the number of patients receiving dialysis reflect expanding criteria for eligibility rather than a true increase in incidence. Incidence data for renal disease that is not end-stage are not available, yet the bulk of renal disease attributable to nephrotoxins may fall in this category. Mortality statistics also may not be useful for evaluating time trends or geographical differences because of changes over time in diagnostic practice, death certificate coding systems, and renal classification schemes.

1. Prevalence and incidence of chronic renal disease

The reported death rate from chronic renal failure that is not considered secondary to other causes such as hypertension or diabetes is low, comparable to that of leukaemia, for example. In 1970 the crude United States death rate from chronic renal failure coded to "chronic and unqualified nephritis and nephrosis" (ICDA 582-584) plus deaths coded to "chronic infections of the kidney" (ICDA 590.1) was 7.7 per 100,000 population[1,2]. Death rates for males and females were similar, but the death rate for non-whites was twice that for whites (13.7 versus 6.9). Deaths from chronic renal failure increase with age for all race and sex groups from less than 1 per 100,000 under age 20, to 15.7 at ages 60-64, 35.1 at ages 70-74, and 88.4 at ages 80-84.

Reported incidence rates for end-stage renal disease are similar in magnitude to mortality rates. The incidence of end-stage disease requiring dialysis in several countries has been estimated to be between 3.8 and 7 per 100,000 population per year[3,4]. The incidence of treated end-stage renal disease in the United States has recently been estimated to be between 4 and 6 per 100,000 population per year overall, but between 10 and 12.5 per 100,000 for blacks[5]. Another more recent study[6], which began with serum creatinine laboratory values to identify cases rather than with physician diagnoses, estimated the incidence of end-stage disease in Finland to be 11.9 per 100,000. In that study the incidence of moderate renal failure was reported to be as high as 31.7 per 100,000.

While mortality and incidence may be low, morbidity from chronic renal disease is significant and costs for maintenance dialysis are high[7-9]. The prevalence of treated end-stage disease is reportedly twice the incidence, although these figures are also influenced by availability of dialysis facilities[10,11]. Accurate estimates of the prevalence of chronic renal disease are as elusive as estimates of incidence.

There is little basis for determining what level of renal dys-

function should be considered clinically significant. Often in medical practice no steps are taken to evaluate or reverse elevated serum creatinine values that are below 2.0 mg/dl. At least half of normal function may be lost, however, before the concentration of BUN or creatinine in plasma rises above clinically detectable abnormal ranges[12]. Approximately 3% of the US population has serum creatinine values greater than 1.5 mg/dl, but it is not clear what proportion of patients with renal dysfunction will ultimately require dialysis.

Renal disease may be asymptomatic or produce non-specific symptoms in early stages. This also helps make it difficult to estimate the prevalence or incidence of renal disease that does not require dialysis. Because of its insidious onset and non-specific symptoms, early disease does not always come to medical attention. Patients are often diagnosed incidentally during treatment of other conditions. Not all patients with early renal disease will be identifiable through hospital or even outpatient medical records, and those that are identified in this way may represent an unusual subgroup of patients. Routine monitoring of clinical or laboratory studies, on the other hand, could overestimate renal disease incidence, as many acute conditions can produce temporary elevations in routinely monitored renal function parameters.

2. Prevalence and incidence of toxic nephropathies

The proportion of renal disease that is related to toxic exposures is even more difficult to determine. However, there are large numbers of individuals with occupational or environmental exposure to compounds that have been shown to be nephrotoxic[13]. Despite substantial evidence that numerous exposures can cause kidney damage, toxic exposures have been systematically ignored in clinical practice. This is probably due to several factors. First, by the time most patients come to medical attention there is little therapeutic value in identifying the causes of renal dysfunction. Second, chronic exposure to nephrotoxins may lead to insidious or subtle tubular injury which later is apparent as an interstitial nephropathy that is indistinguishable from interstitial disease from any cause[14]. In end-stage disease there may be no distinguishing pathological features at all. Toxic exposures may also result in glomerular injury which is no different from that in immune complex disease or other conditions with glomerular manifestations. Studies of early dysfunction in workers exposed to some metals such as cadmium or mercury have demonstrated changes related to glomerular as well as tubular damage[15,16]. Furthermore, long-term decreases in glomerular filtration resulting from toxic interstitial nephropathies can produce changes such as proteinuria which may lead to a diagnosis of primary glomerular disease[17].

Patients with toxic nephropathies may have other conditions which also mislead the clinician. For example, as many as one third of patients with possible analgesic associated nephropathy have frequent urinary tract infections which could lead to a mistaken diagnosis of pyelonephritis[18]. Other toxic exposures, such as lead, also give rise to kidney damage that can give the appearance of

chronic pyelonephritis[19]. For many years it was quite common for patients with non-glomerular renal disease to be diagnosed as having chronic pyelonephritis[20]. This may have been especially common among women who are prone to frequent urinary tract infections. However, more recent evidence suggests that very few of these individuals actually have renal damage due to bacterial infection of the kidney. Those who do are the very small percentage of patients with structural abnormalities leading to reflux[21]. The tendency to label patients in this way may also have contributed to the lack of attention paid to obtaining detailed histories of toxic exposures.

Patients with lead nephropathy may be mistakenly diagnosed as having nephrosclerosis[19]. Although there is substantial evidence that chronic lead exposure can lead to hypertension, not all studies agree[22]. It is not clear whether renal disease from lead exposure is mediated through hypertension, or whether both diseases are independent outcomes of exposure. Nonetheless, hypertension, which is quite common, especially among black males in the United States, may be a coincidental finding in patients with toxic nephropathy which prevents the initiating exposure from being detected.

Data collected by dialysis and transplant registries are potentially useful for determining the proportion of end-stage disease that is due to environmental exposures. However, the clinical limitations described above make it difficult to draw conclusions from these data. A recent profile of treated end-stage renal disease patients in the United States shows that only a small fraction of patients are diagnosed as having toxic nephropathies[5]. Most patients are classified histologically, rather than aetiologically. Almost 38% of patients are listed simply as having glomerulonephritis or interstitial nephritis. A further 17% are classified as having hypertensive nephrosclerosis which, although presumed to be primary, may only be a coincidental finding in these patients. Only 26% of treated patients have some condition such as diabetes, obstruction, hereditary or congenital disease, or other systemic diseases which accounts for their renal dysfunction. Even diabetic nephropathy is problematic in that recent reports suggest that not all patients with renal dysfunction and diabetes can be shown to have diabetic glomerulonephrosclerosis on biopsy[23]. Thus as many as three quarters of end-stage renal disease patients may have renal disease of toxic aetiology.

Data concerning end-stage renal disease patients in different countries are similar and often indicate circular reasoning leading to diagnosis or labelling of patients[3,4,6,11,24-28]. There is, however, considerable geographical variation in the proportion of patients described as having interstitial as opposed to glomerular disease, which may reflect differences in toxic exposures or in clinical practice. Race and sex differences in the distributions of purported causes of renal disease may also reflect differences in aetiological agents or in clinical perceptions leading to evaluation and diagnosis.

B. Additional limitations of existing data

1. Vital statistics data

Vital statistics data are potentially useful for monitoring trends in renal disease, but specific deficiencies in the coding of chronic renal disease limit their usefulness. In addition, many epidemiological studies of occupational exposures and chronic renal disease rely solely on vital statistics data to identify potential nephrotoxins. The quality of death certificate data, therefore, is important to consider in evaluating these studies. Mortality data which rely on the coded underlying cause of death may seriously undercount the number of persons who die with chronic renal failure. Modan found that only 56% of patients in Israel who died in end-stage renal failure had a death certificate coded to renal disease codes or to uraemia[29,30]. Some additional patients had deaths coded to related causes such as diabetic nephropathy for a total of 66% of patients identified by underlying cause of death. If all causes listed on the death certificate were considered, which is rarely done in mortality follow-up studies of exposed workers, up to 84% of patients with known end-stage renal disease can be detected through death certificates. It is likely that even fewer of patients who are not on dialysis would be identified through death certificate data.

2. Coding of renal disease deaths

There are a number of different International Classification of Diseases (ICD) codes under which renal disease may be classified, and it is not always clear how these are assigned. Changes in coding practices and rules over the years make it difficult to monitor trends or to compare results between studies conducted in different time periods. Currently, renal disease deaths may be coded to "nephritis and nephrosis", "hypertensive heart and renal disease", "diabetic renal disease", or "infections of the kidney" among other possibilities. Within many of these categories, deaths may be specified as due to acute, chronic, or unspecified disease. Given the clinical difficulties described above, it is not clear that deaths attributed to infections of the kidney or to hypertensive renal disease, for example, can be ignored in studies of occupational exposures. Most occupational studies have focused on deaths coded non-specifically as nephritis and nephrosis, or chronic and unspecified nephritis and nephrosis. In earlier years most toxic nephropathies would probably be labelled in this way. However, with the introduction of the 8th revision of the International Classification of Diseases in 1968[1], deaths mistakenly attributed to pyelonephritis could be coded to infections of the kidney. As many as 50% of chronic renal disease deaths are coded in this way, yet few occupational studies have reported on risks for renal deaths coded to chronic infections of the kidney. Furthermore, many deaths labelled as hypertensive or diabetic renal disease might be associated with toxic exposures. Such associations could be missed if these causes are not also evaluated.

C. Ascertainment and classification of renal disease patients

There are no uniformly agreed upon standards for identifying and classifying individuals with renal disease. Because of the insidious onset and wide range in severity, the population with renal disease is difficult to characterize. This becomes more of a problem for epidemiological studies if risk factors vary with disease severity or disease course. Such variation is likely because chronic renal disease appears to be a heterogeneous group of disorders with common superficial appearance. Differences in findings between studies may result from inclusion of different populations of renal disease patients. Observations from studies of clinically recognized disease, for example, may not be generalizable to all patients with renal dysfunction.

It is essential that epidemiological studies clearly define the patient population that has been included and categorize these patients according to disease subtype. Unfortunately it may be quite difficult to do so. Once a patient requires dialysis it is often no longer possible to make a specific pathological diagnosis. Even for newly diagnosed patients there are no uniformly agreed upon standards for basic renal disease evaluation and classification. Because biopsy results do not usually affect prognosis, biopsies are not often done - at least in the United States. Patient evaluation and diagnosis may therefore depend more upon clinical judgement and biases than on standardized pathological review.

Unlike some acute conditions which allow precise case-definition, there are many possible choices for defining chronic renal disease, and study findings may be affected by the particular definition chosen. Cases may be defined on the basis of renal function, clinical diagnosis, morphology, specific combinations of clinical and laboratory findings, or by the need for maintenance dialysis. Cases may then be identified through dialysis units or registries, hospital records, or population screening.

Measures of renal function such as serum creatinine, BUN or creatinine clearance are continuous variables, and artificial cut-points must be used to distinguish diseased from non-diseased. Because measures of renal function are influenced by factors such as age, race, sex, weight, and concurrent disease, choice of cut-points for epidemiological studies requires information on population norms. The choice of cut-points will also affect how controls should be selected. A very low cut-point makes it difficult to use population controls unless it is feasible to screen controls to rule out modest changes in renal function. If a control group included substantial numbers of individuals with undetected renal dysfunction, comparison of cases and controls would underestimate any disease risk associated with specific exposures. On the other hand, with very high cut-points, although controls are not likely to be "misclassified cases", only the most unusual cases will be identified, limiting study generalizability.

Not all patients with chronic renal disease receive medical attention. This is especially true for asymptomatic patients who do not progress to overt kidney failure, or for patients with rapid case-fatality. In a study designed to evaluate dialysis needs, McCormick identified 218 patients with BUN greater than

100 mg/dl, of whom only a fraction were seen for genitourinary symptoms[11]. Most were seen for non-specific symptoms or identified during routine examinations or evaluations for other conditions, suggesting that, in the earlier stages at least, receiving a diagnosis may be random for a large proportion of the case population.

Even when patients come to medical attention, not all will be labelled as having renal disease. This limits the usefulness of medical records for case identification. In a study recently completed in North Carolina, one third of hospital patients identified because of serum creatinine values greater than 1.5 mg/dl who also had evidence of chronic renal disease were not discharged with a diagnosis suggesting chronic renal dysfunction[31]. The circumstances under which renal dysfunction is observed may have an effect on whether a diagnosis is made. For example, a serum creatinine of 2.0 mg/dl in an otherwise healthy individual would be more likely to be evaluated than the same level of dysfunction in an individual with other problems requiring medical attention. Such selective factors may have implications for evaluating findings from studies based on cases identified from routinely collected medical records. Other selective factors, such as age, race, or history of a particular exposure, may influence not only whether an evaluation is carried out, but may also decide the type and extent of evaluation that is undertaken and the particular diagnostic label that is applied.

Chronic renal disease includes multiple conditions which all result in loss of renal function. These different disorders may each be characterized by their underlying pathology, pathogenesis, clinical appearance, or aetiology. Particular risk factors for chronic renal disease may be more apparent in studies of well-characterized discrete subgroups of renal disease patients. Because existing medical records are likely to be quite variable and subject to potential clinical biases, biopsy records might be a more appropriate source for identifying patients for epidemiological study. Unfortunately, in the United States at least, few patients are biopsied. While limiting studies to patients who have had a biopsy may make risk factor identification easier, other problems limit the generalizability of findings from such studies. Patients who have a biopsy represent a highly selected group, and some of the selective factors leading to biopsy may also relate to the probability of having particular environmental exposures.

Dialysis registries offer a ready source of identifiable patients for epidemiological study, but they too include a highly selected and limited subgroup of renal disease patients, limiting the generalizability of findings from studies of dialysis patients. Dialysis patients may also have altered their behaviour or exposures as a result of long-standing chronic disease, which makes it difficult to identify some aetiological agents.

Groups of dialysis patients and patients identified earlier often differ in the distribution of underlying pathology or renal diagnoses. Patients with end-stage disease are more often classified as having glomerulonephritis, whereas patients with less severe dysfunction are more likely to be described as having interstitial disease[3,4,11,24,28,32,33]. This may be because the severity of dysfunction varies with pathology, the pathological picture changes

as dysfunction progresses, or only selected patients obtain dialysis. Nonetheless, there are important implications of these differences for epidemiological studies. For example, reports linking renal disease to analgesic abuse generally involve patients with interstitial disease[32,33], whereas reports concerning solvent exposure involve patients with glomerular disease[34,35]. Findings regarding particular exposures may vary between studies if the mixture of patients also varies. Therefore, careful attention needs to be paid to the adequacy of case classification in individual studies when evaluating findings from different epidemiological studies.

IV. EPIDEMIOLOGICAL STUDIES OF SELECTED NEPHROTOXINS

Keeping in mind the advantages and disadvantages of different epidemiological study designs, as well as problems specific to the study of chronic renal disease, it is possible to review the clinical and epidemiological literature to identify exposures which are likely to increase renal disease risk in the population. These exposures include heavy metals such as lead and cadmium, medications such as phenacetin-containing analgesic mixtures, and chemical solvents. While there are no definitive studies linking any of these exposures with risk of chronic renal disease, the bulk of evidence suggests such risk exists. Although many of the individual studies have limitations because of design flaws, if they all reach similar conclusions, then the effects might be real. Furthermore, many of the inadequacies present in the literature result in potential underestimation of the magnitude of any effect of toxic exposures on renal disease risk, rather than overstating the problem.

A. Lead

Since the late nineteenth century, chronic lead poisoning has been a well-known cause of granular contracted kidneys[36]. Legislation introduced in the early 1900s set limits for lead exposure and presumably reduced the prevalence of chronic lead poisoning and its renal consequences. While there is no doubt that acute lead poisoning can induce proximal tubular dysfunction, the consequences of chronic low-level lead exposure are less clear.

Tubular injury in lead poisoning is associated with intranuclear inclusion bodies and cytoplasmic damage. These effects are reversible following brief exposures but are progressive in association with severe, persistent exposures. Lilis et al.[37] have shown that the recovery process is slowed with successive exposures, suggesting that some residual damage remains from each episode, although renal function may appear within the range of normal. A small but undocumented proportion of individuals with renal failure from acute lead poisoning may go on to develop chronic renal failure.

The chronic failure associated with lead exposure is characterized by an indolent, progressive, renal insufficiency. The renal cortices are characteristically atrophied, with loss of glomeruli and

interstitial fibrosis. Such insufficiency may develop even without continued lead exposure, and it may result from exposure to other potentially nephrotoxic agents whose effects are superimposed upon residual lead damage. Conversely, it may be that individuals who develop chronic renal insufficiency following lead exposure are those with underlying renal changes from other exposures or structural defects.

The small contracted kidneys seen following lead exposure are indistinguishable from interstitial nephritis from other causes. In addition, patients with renal disease from lead may also develop gout or hypertension, making it difficult to recognize lead exposure as the cause of disease. Thus nephropathy from chronic or low-level lead exposure may be underreported in the clinical literature.

The epidemiological literature on chronic lead exposure deals primarily with occupational exposures and exposure to lead paint during childhood. Other sources of chronic lead exposure have been less well studied. These include the home brewing of alcohol in stills made from lead pipes or automobile radiators, recycling batteries in cottage industries, or burning discarded battery casings for home heating[38-40]. These exposures have been reported in association with impaired renal function in patients with documented lead intoxication and even in the absence of lead poisoning symptoms[36,38,39]. For example, a strong correlation between lead levels in water and elevated serum urea levels was seen in a survey of households with lead plumbing[41].

Much of the evidence suggesting that childhood lead exposure is related to chronic renal failure comes from Australia. As early as the 1890s, reference was made to the frequency of chronic nephritis in Queensland[42]. In the early 1900s, reports focused on the frequent occurrence of lead poisoning in Queensland children[43,44]. Death rates from chronic nephritis in 1920 to 1940 were higher in Queensland than elsewhere in Australia[43,45]. A series of studies linked this excess to childhood lead poisoning from ingestion of lead paint dust from veranda railings.

In 1929, Nye[43] collected 80 cases with chronic renal failure, in which 14 had clinically documented childhood lead poisoning. Nye interviewed a third of the remaining cases without documented lead poisoning and found that almost all were likely to have ingested lead paint dust. No comparison subjects were interviewed.

In a follow-up study of 401 Queensland individuals diagnosed with lead poisoning between 1915 and 1935, Henderson[46] found that almost half of the 352 individuals located had died before age 40, a death rate almost five times that expected from vital statistics data. Of the deaths, 108 were attributed to chronic nephritis or other renovascular causes. Additional renal failure cases were identified through interviews with surviving lead poisoning victims. No adjustments were made for social class or other differences between those with lead poisoning and the population at large that might have explained the mortality differences.

None of the Queensland reports included appropriate control groups to determine the incidence of chronic renal failure in a similar non-exposed group, or the frequency of lead paint dust exposure in a similar but healthy population. Other reports sug-

gest a high frequency of analgesic nephropathy in Queensland[47], although this may not be a factor for so young a group with renal disease. A characteristic picture of progressive renal failure in the Queensland patients, which included years of generalized ill-health and malaise, has been described[43]. This allows for the possibility that large quantities of analgesics could have been consumed. While studies have documented bone absorption of lead in some of the patients, a causal link has not been proven, and other factors acting alone or in concert must be considered.

In 1963, Tepper[48] followed a cohort of individuals treated in Boston for childhood lead poisoning that had been diagnosed between 1924 and 1941. He identified 165 individuals with evidence of lead absorption and intoxication who survived the acute episode. There were ten deaths among the 139 subjects traced, but only one attributed to chronic renal failure. This represents a much lower risk than that reported for the Queensland group, but is greater than would be expected for so rare an occurrence among young people.

The lead exposure in the United States series was different from that in Australia, which might account for the differing level of apparent renal disease risk. Other factors, such as the incomplete follow-up of the Boston cases, might also explain the lower level of risk reported for this group.

Renal dysfunction in the absence of overt renal failure has frequently been reported for workers exposed to lead. Wedeen[49] identified eight workers with suspected excessive lead exposures and found renal function abnormalities in four - one with asymptomatic failure and three with preclinical dysfunction. Only two of these patients had been hospitalized for lead poisoning symptoms suggesting renal effects of lower level exposure. In another series, Wedeen et al.[50] evaluated renal function in 57 of 113 asymptomatic workers who excreted detectable amounts of lead in urine. Focal interstitial nephritis was found for six patients in whom a biopsy was performed. Abnormal glomerular filtration rate was documented for 21 workers. Other reports have focused only on renal dysfunction among workers with known lead poisoning. For example, Lilis et al.[37] studied 102 patients hospitalized for lead poisoning over a 10-year period, and found evidence of renal dysfunction in 17. In contrast, Buchet et al.[51] reported no renal dysfunction among 25 workers who had been exposed to very low lead levels for an average of 20 years.

Most reports have employed clinical measures of renal dysfunction. However, recent occupational studies have utilized assays to detect very early renal damage in exposed workers. For example, Meyer et al.[52] found increased urinary excretion of N-acetylglucosaminidase in 29 workers exposed to lead in whom renal function was apparently normal.

It appears that continuous exposure to lead, even at presumably non-toxic levels, can produce renal damage. While very low-level exposures have not been well documented as causing overt failure, the presence of reduced renal function in exposed workers leaves open the possibility that failure could develop after very long intervals. This is supported by studies in rats in which the long-term administration of lead produced renal damage typical

of that seen in acute lead poisoning[53].

While lead exposure alone might produce renal insufficiency, a limited number of reports suggest that effects could be greater when multiple exposures are involved. Buchet et al.[54] studied renal function in workers exposed simultaneously to lead and cadmium, and found that effects were greater in the group with combined exposures than in the group with either exposure alone, although the abnormalities seen were more characteristic of cadmium than of lead exposure. Studies in animals also support the possibility of enhanced effects of lead exposure in the presence of underlying compromised renal function[55].

Chronic lead nephropathy was commonly recognized in the early 1900s in European lead workers[36]. Before 1930, when occupational standards were implemented, numerous cases of occupational lead nephropathy were reported[56]. Since that time only a few reports suggest a risk for symptomatic renal failure due to occupational exposures. Lane[57] conducted a mortality follow-up study of battery workers exposed prior to the implementation of standards, and found nine chronic renal failure deaths among approximately 150 workers exposed for 18 to 32 years. A more recent follow-up of men from the same factories who were exposed primarily after 1927 uncovered four additional renal failure deaths[58]. Only one death would have been expected based on population death rates. In a larger follow-up study, Cooper and Gaffey[59] found a slight excess of deaths from hypertensive disease, chronic nephritis, and other renal sclerosis among 7032 battery and smelter workers who were employed between 1946 and 1970. Information on actual lead exposure was available for only a small proportion of the cohort.

McMichael and Johnson[60] calculated proportionate mortality ratios for 140 deceased smelter workers with lead poisoning diagnosed between 1928 and 1959. Compared with 695 deceased smelter workers without lead poisoning, lead-poisoned workers experienced an increased proportion of deaths from chronic renal disease and stroke. In addition, a larger proportion of deceased workers without lead poisoning died from chronic nephritis than did other Australians, suggesting a possible effect from low-level lead exposure.

Most recently, Selevan et al.[61] reported follow-up of a cohort of almost 2000 lead smelter workers from Idaho employed between 1940 and 1965. Overall mortality, and mortality from hypertensive disease, were similar to that of other United States males. However, there were almost twice as many deaths as expected from chronic and unspecified nephritis. There was also a dose-response relationship between the inferred level and duration of exposure and nephrosis, despite the small numbers of workers studied. Additional evidence of renal disease risk from low-level exposure is provided by the fact that the incidence and prevalence of end-stage renal disease is also increased in the Idaho community in which the smelter is located[62].

In these studies the magnitude of risk associated with lead exposure may have been underestimated, since only underlying causes of death were evaluated. Other causes of death - such as hypertensive renal disease, diabetic renal disease, or infections of the kidney - were also not uniformly examined, and these

categories might include deaths related to lead exposure and more properly classified elsewhere. Even in the larger cohorts, statistical power to detect excess renal disease mortality is low. Few of the studies are able to evaluate risk for specific renal disorders and most combine mortality from acute and chronic disease, which might be misleading. Furthermore, in many occupational cohorts, workers are exposed to more than one metal as well as other potential nephrotoxins, making it difficult to attribute risk to lead exposure. In addition, the most exposed workers are often lost to follow-up or have had previous jobs with exposures to other potential nephrotoxins, which further complicates the interpretation of study findings. Workers who develop chronic disability may also be among those least likely to be traced.

In most of the mortality follow-up studies exposure data are inadequate to determine actual exposure levels. High and low exposure is inferred from job titles and from duration of employment and date of hire. Often "high" level exposure is assumed only if lead poisoning symptoms occur. Entire plants or work stations are often considered to be "exposed" in the absence of actual exposure measurements. This practice may lead to underestimation of risk from lead exposure if large numbers of workers with little or no actual exposure are considered to be exposed. Furthermore, effects of duration of exposure and latency are extremely difficult to sort out. In smaller cross-sectional studies of exposed workers, blood lead levels provide a more accurate measure of exposure and allow for evaluation of less direct exposure measures.

B. Cadmium

There has been little evidence of symptomatic chronic renal failure in large numbers of cadmium-exposed individuals. However, cadmium's affinity for the kidney, its long biological half-life and the well documented proteinuria in cadmium-exposed workers suggest that cadmium may play a role in the development of chronic renal failure.

Occupational exposure to cadmium is common with approximately 1.5 million United States workers potentially exposed. Cadmium is used in metal plating, in the production of pigments, in nickel-cadmium batteries, in stabilizers for plastics and in other industries. In addition to occupational sources, cigarette smoking and diet are potentially important sources of cadmium exposure[63-65].

Acute exposure to cadmium can lead to swelling and necrosis in the proximal tubules and ultimately to acute renal failure. Long-term low-level cadmium exposure may lead to renal dysfunction characterized by low grade proteinuria with excretion of low molecular weight proteins. Chronic renal failure is associated with low tissue cadmium levels, presumably due to fibrosis of tubular cells[66]. While the renal damage caused by cadmium is generally described as tubular[16], high weight molecular proteinuria has also been reported, suggesting a glomerular component as well[67,68]. Glomerular changes may result from long-standing tubular dysfunction, rather than as a direct result of cadmium exposure[69].

Cadmium exposure has been associated with non-malignant lung disease, respiratory cancer, prostate cancer, hypertension and increased renal stone formation[69-72], although reports are conflicting[73]. Apparent renal dysfunction following cadmium exposure could possibly result from hypertension or stone disease. Few epidemiological studies, however, have taken this into consideration. A possible interaction between cigarette smoking and cadmium body burden and effects on renal function has also been suggested, with increased urinary cadmium levels seen among exposed workers who smoke[74]. Occupational cohort studies and other epidemiological studies generally have also not taken cigarette smoking into account.

Renal dysfunction and possibly overt renal disease have been reported for residents of cadmium polluted areas. Lauwerys et al.[75] compared cadmium body burdens and renal function in long-term female residents of a cadmium-polluted area in Belgium with that of residents from a similarly industrial but non-exposed community. Cadmium pollution was attributed to non-ferrous smelters that had operated for many years. Both cadmium body burden and the frequency of renal dysfunction were higher among residents of the cadmium-polluted area. In a later report, Lauwerys and De Wals[76] noted increased mortality from renal and urinary tract disease in this same cadmium-polluted area. The proportion of deaths due to nephritis and nephrosis was greater than the proportion elsewhere in Belgium. This observation held for males and females, and applied mostly to individuals older than age 60. Others have, however, raised the possibility that the observed excess renal disease could also be explained by analgesic abuse which was reported to be common in the area[77].

Nogawa et al.[16] compared renal function in inhabitants of Japanese communities with differing levels of cadmium exposure. All 96 adults over age 40 from two exposed communities were compared with 42 adults over age 50 from a non-polluted area. A higher prevalence of low molecular weight proteinuria, glucosuria, and aminoaciduria was seen in the polluted areas. Nogawa et al. also reported elevated serum creatinine, lower creatine clearance and differences in percentage renal phosphorus reabsorption. Results were similar, although not statistically significant, in a larger multistage screening programme[78].

Older women who were long-term residents of three German communities with differing amounts of cadmium pollution were also evaluated for evidence of renal dysfunction[79]. Urine and blood cadmium levels as well as serum creatinine levels were higher for women residents of an industrial area with known cadmium pollution, although other measures of renal dysfunction, including urinary excretion of low molecular weight proteins, did not differ between communities. Participation rates for this study were extremely low and community differences which might have accounted for the apparent lack of renal function differences were not taken into consideration. For example, residents of the control community were more often smokers or diabetics, and were more likely to report a history of prior renal disease.

In a 40-year follow-up study of residents of Shipham, Somerset potentially exposed to high soil cadmium levels, a slight

excess mortality from nephritis and nephrosis was seen[80]. There were twice as many genitourinary disease deaths among males from Shipham as expected. No excess was seen for a nearby control village. Hospital admissions data for Shipham residents were also evaluated over a 4-year period[81]. No increased morbidity was noted for Shipham residents as compared with regional hospital admission rates. However, the number of admissions studied was small, and only the first diagnosis listed on a multiple problem list was evaluated. Thus chronic or benign renal conditions which are likely to appear as secondary problems on individual admission records are likely to be under reported. In a third study of Shipham residents, urinary measures of cadmium exposure and renal dysfunction were compared with results of similar studies in residents from a nearby community[82]. Only very slight differences attributable to cadmium were seen.

There is a growing body of evidence concerning renal dysfunction and disease in workers exposed to cadmium. As with studies of workers with lead exposure, actual exposure data for large numbers of workers are lacking. Many studies utilize limited air sampling data along with job titles and descriptions to classify workers into high and low exposure categories, or simply study entire cohorts of workers without regard to whether or not exposure actually occurred. Such classification schemes also do not take into account possible use of protective equipment or other factors which would modify actual exposure levels. More detailed exposure evaluations may be available for small groups of workers in whom body burden and renal function are simultaneously evaluated. While there is generally good correlation between measures of exposure and body burden, long-term exposure data are often unavailable.

Numerous reports describe renal dysfunction in cadmium workers, even among those exposed below current permissible exposure levels[51,67,68,83-85]. Lauwerys et al.[68] compared renal function in several groups of cadmium workers with that of control groups matched on age, sex, weight, height, smoking habits and socioeconomic status. The 31 female workers studied were exposed for an average of 4 years. Although urinary cadmium levels differed between exposed females and controls, there were no significant differences in renal function measures. Differences were seen among male workers, and proteinuria was evident in almost 70% of those exposed more than 20 years. Bernard et al.[67], also reported mixed proteinuria in 15 of 18 workers exposed to cadmium for an average of 28 years. Matched non-exposed workers from the same factories were also studied. While both groups had higher mean urinary cadmium levels than the general population, only the group with an exposed job category had significant proteinuria and aminoaciduria. In another report on renal function in 42 cadmium workers and 77 controls, both tubular and glomerular type proteinuria were seen, mainly in workers with more than 25 years of exposure[83].

Falck et al.[85] evaluated renal function in 33 volunteers exposed to cadmium at a plant producing refrigeration compressors, and non-exposed controls. Exposure data dating back to 1961 were available. Workers were exposed to cadmium at or below the cur-

rently enforceable permissible exposure level. Controls turned out to be significantly younger than exposed workers. Even so, there were no differences in renal function measures between the exposed workers and the control group. However, among the exposed workers a dose-response relationship was seen between exposure level renal dysfunction.

Recent studies have focused on possible mechanisms and natural history of renal damage from cadmium exposure. Elinder et al.[69] studied 60 workers exposed to cadmium-containing solder between 1955 and 1978 and a non-exposed industrial control group. Findings suggested that glomerular changes such as albuminuria might be secondary to tubular damage. Gompertz et al.[86] suggested that long-term exposures were necessary to produce both significant kidney tissue burden and renal dysfunction. Proteinuria and enzymuria were strongly correlated with cadmium body burden and duration of exposure. Gompertz et al.'s work also underscores the need for follow-up studies of the natural history of kidney damage following chronic cadmium exposure. The long-term significance of urinary excretion of enzymes or even low molecular weight proteins has not been well established. However, Kazantzis[87] documented significant clinical disease in a very small group of cadmium-exposed workers who had been followed because of tubular proteinuria found in a preliminary screening study.

The mortality of cadmium-exposed workers has been evaluated in several studies. Kjellstrom et al.[88] studied 269 cadmium-exposed Swedish workers with five or more years of work experience in a cadmium-nickel battery factory. Between 1949 and 1975, 43 deaths occurred. Overall mortality was less than expected based on population rates. This is a common result in occupational follow-up studies, and is known as the healthy worker effect. Mortality among workers exposed before 1947 when cadmium levels were greatest was, however, greater than expected. For the entire cohort, while there were fewer than expected cancer and cardiovascular diseases, mortality from renal and respiratory disease was increased. In an expanded report, mortality between 1951 and 1983 among 522 battery workers was examined[89]. Three deaths attributed to nephritis and nephrosis were observed when only one such death was expected. Only underlying cause of death as coded on the death certificate was included. However, examination of death certificates indicated that a further death should have been attributed to renal disease.

Other studies offer only modest support for an effect of cadmium exposure on renal disease mortality. A carefully conducted well-designed mortality study involving 19 plants in England in which cadmium was used found no increase in renal disease mortality among exposed workers[71]. The study included 6995 males born before 1940 and employed for at least 1 year on or near a cadmium process between 1942 and 1970. Vital status was ascertained and underlying cause of death, as well as other causes, were examined. Exposure levels were determined from job descriptions and industrial hygiene data. Workers came from five main industries: primary cadmium production, copper-cadmium alloys, silver-cadmium alloys, pigments and oxides, and stabilizers. Most were exposed only at very low levels. Fewer than expected deaths

from nephritis and nephrosis and from other genitourinary causes were seen. Among the very small group considered to be highly exposed, one nephritis death was observed when none would have been expected based on population death rates. Negative findings were not affected by inclusion of causes other than the underlying cause of death.

Sorahan et al.[90] reported on mortality among a further 3025 cadmium-exposed workers from plants in England. Nickel-cadmium battery workers who began work between 1923 and 1975, and had at least 1 month of work experience, were followed to ascertain vital status and underlying cause of death. Almost twice as many as expected deaths from nephritis and nephrosis were observed. After statistical adjustment for potential modifying factors, including sex, year of hire, age, and duration of employment, renal disease risk was not significantly elevated. The study also included 39 workers known to have had low molecular weight proteinuria in 1968. One nephritis death was observed for this group when 0.25 was expected statistically. The authors believed that this death was related to phenacetin abuse rather than to cadmium exposure, but long-term renal dysfunction could have preceded the intake of analgesics.

Mortality from renal disease was assessed for the above two cohorts combined and a nested case-control study was conducted in order to permit more detailed exposure assessment[91]. Twice as many as expected deaths from nephritis and nephrosis were observed for workers who had "high" cadmium exposure. However, only four deaths were observed, and this finding was not statistically significant. The case-control study included 23 deaths from nephritis and nephrosis, and a further 11 cases where nephritis and nephrosis was mentioned elsewhere on the death certificate. Deaths attributed to chronic pyelonephritis, hypertensive renal disease, or other possible renal categories were not included. A 2-3-fold risk of borderline statistical significance was reported for workers having "high" or "moderate" exposure to cadmium at some time.

Most recently, Thun et al.[92] reported on mortality among employees of a United States cadmium recovery plant. All hourly workers and foremen with at least 6 months experience in a production area between 1940 and 1969 were followed through 1978. Exposure estimates were determined based on length of employment and categories of jobs within the plant. No excess renal disease risk was observed among the 602 white males studied when underlying cause of death was evaluated. Only one death was attributed to renal disease. However, when other causes listed on the death certificate were included, four additional renal disease deaths were seen. Unfortunately, population data for multiple causes of death were not available for comparison.

Differences between countries in reported renal disease risk from cadmium exposure could be due to differences in death certificate coding practices or to under-reporting of known renal disease deaths. Differences in exposure levels, exposure assessment, and quality of follow-up records also might affect results. Furthermore, the United States and Swedish cohorts were relatively small, and the statistical uncertainty of resulting risk estimates

was large.

C. Solvents

Acute tubular necrosis is most often reported following acute exposure to organic solvents[93,94]. Reports have also linked chronic solvent exposure and glomerulonephritis[34,93,95]. Potentially nephrotoxic compounds include aromatic hydrocarbons (toluene, xylol), halogenated hydrocarbons (carbon tetrachloride, chloroform, trichloroethylene), glycols, and aliphatic-aromatic hydrocarbons (gasoline, turpentine)[93]. Exposures can occur occupationally, during household activities, in hobbies, or through such activities as glue sniffing.

Solvent exposure has been linked with different forms of glomerular disease in several case reports[93,95]. For example, Beirne and Brennan[95] reported that six of eight patients with glomerular disease (Goodpasture's syndrome or antiglomerular basement membrane disease) had extensive industrial solvent exposure, and Ehrenreich et al.[96] reported four cases with epimembranous glomerulonephritis following solvent exposure.

Small case-control studies provide stronger support for an association between solvent exposure and chronic glomerulonephritis, although methodological weaknesses in most of these studies have been cited[97]. Zimmerman et al.[34] interviewed 63 patients with end-stage renal disease and hospital controls who did not have renal disease, and reported that patients with known or suspected glomerulonephritis had greater occupational exposure to hydrocarbon solvents than either patients with other forms of renal disease or controls. However, a larger proportion of patients with glomerular disease than comparison subjects were males, increasing the likelihood of employment in solvent-related industries.

Finn et al.[35] surveyed 87 patients with end-stage disease and found that patients with glomerulonephritis were twice as likely to have had solvent exposure as renal patients with non-glomerular disease. However, 49 of the 52 non-glomerular comparison patients had systemic diseases such as diabetes, lupus, or polycystic kidney disease. Patients with these conditions may have limited opportunities for employment in industries with solvent exposure. Thus poor choice of controls could have created a spurious association between glomerular disease and solvents. In addition, the study does not allow for inferences regarding solvent exposure and other non-systemic renal diseases. Lagrue[98] also reported more solvent exposure among 108 patients with glomerulonephritis than among 56 hospital controls with hypertension or renal stones but without renal disease.

Ravenskov[99] compared 50 biopsy-diagnosed glomerulonephritis patients with two age- and sex-matched control groups (one with non-glomerular renal disease and one with acute appendicitis). Patients with glomerulonephritis were more likely to have had occupational exposure to organic solvents, and a dose-response relationship with degree of exposure was suggested. In an earlier publication, Ravenskov[100] also reported an association between solvent exposure and post-streptococcal glomerulonephritis in a study

comparing patients with post-streptococcal glomerulonephritis with patients with streptococcal infection who did not develop renal complications. Subsequent reports by Ravenskov and others[101,102] support findings from the case-control study[99].

In contrast, in a small study, Van der Laam[103] failed to find a significant association between mixed hydrocarbon solvent exposure and glomerulonephritis. Inclusion of patients with glomerular disease due to other systemic conditions, and the possible inclusion of controls with diseases influenced by solvent exposure, may have minimised the likelihood of observing an effect of solvent exposure in this study. A similar study reported by Franchini et al.[104] also did not demonstrate a statistically significant risk of glomerulonephritis from solvent exposure, although the reported relative risk of 1.7 was of borderline significance. Furthermore, after restricting analysis to patients with membranous glomerulonephritis, a significant relative risk of 4.1 was observed.

Most recently, Bell et al.[105] reported findings from a case-control study of 50 patients with biopsy proven non-systemic proliferative glomerulonephritis and 100 age-, sex-, and social class-matched controls with acute non-renal hospital admissions. Solvent exposure was significantly more common among the renal disease patients than among the controls.

In these studies determination of solvent exposure was based on interviews with patients or chart reviews to ascertain employment histories and non-occupational sources of solvent exposure. Several studies used an exposure scale developed by Ravenskov et al.[99] in which various intensity-of-exposure scores were assigned to different industries, occupations, and activities. None of these studies verified actual exposure levels. Reported findings relate to general solvent exposure rather than to any individual chemical solvent or class of chemicals.

While individual studies have been criticized for problems in exposure assessment and other aspects of study design, such as small sample sizes, potential recall bias, poor control selection, and unblinded interviews, weaknesses vary between studies[97,106]. Taken together, these studies suggest that a true association between solvent exposure and glomerular disease may exist. While there has been some apparent controversy over the reports by Ravenskov, disagreements may be political rather than scientific[107,108].

Studies have primarily addressed the risk of chronic glomerular disease from long-term solvent exposure, although most of the involved solvents are acutely toxic to the renal tubules[106]. Zimmerman et al.[34] have suggested that direct chemically induced tubular injury could lead to the formation of autoantibodies and deposition of an immune complex in the glomeruli. If so, glomerular changes following solvent exposure may be secondary to tubular injury. This is supported by a study in which urinary findings suggestive of tubular damage were seen[109]. The case-control studies of chronic glomerulonephritis do not rule out a possible association between chronic solvent exposure and non-glomerular renal disease, although several did report greater solvent exposure among patients with glomerulonephritis than among patients with other forms of renal disease. Sex and age differences between subjects,

however, may have affected these results.

In addition to the report by Franchini et al.[109], which suggested tubular changes following solvent exposure, studies have found early indications of glomerular damage, including proteinuria. Van Ganse et al.[110] reported increased proteinuria among patients with non-renal solvent-related diseases. Askergren[111] demonstrated increased albuminuria in subjects exposed to organic solvents, but found no evidence of tubular damage. Meyer et al.[52] reported increased urinary excretion of enzymes in two groups of solvent-exposed workers without clinical proteinuria compared with unexposed subjects. Differences in other renal function parameters were not presented. On the other hand, neither glomerular nor tubular type changes were found in a study reported by Krusell et al.[112] of 43 solvent-exposed printers and 43 non-exposed controls. Differences in exposure level and duration, and in chemical mixtures, might account for these discrepancies.

While no cohort studies have been carried out specifically to evaluate renal disease risk in solvent-exposed workers, several studies designed to evaluate cancer mortality are relevant[113-115]. These offer weak support for an effect of solvent exposure on renal disease mortality. Most studies of solvent-exposed workers, however, fail to include information on deaths due to diseases of the genitourinary system[116-118]. It is not clear if the lack of mention indicates that no deaths were observed, or if categories were excluded because they were not felt to be relevant. Even studies where genitourinary deaths are reported cannot be used to evaluate risk of specific forms of renal disease. Most often, specific renal causes are not separately reported, and the number of observed deaths is too small for meaningful statistical analysis. In addition, these studies rely on underlying cause of death, and renal disease is often under-reported on death certificates, presenting problems for interpretation. In addition, deaths are generally grouped by major body system, which does not allow for examination of renal deaths that might be coded to non-renal sites, such as hypertensive heart disease or diabetic renal disease.

D. Analgesics

There have been numerous case reports of nephropathy associated with analgesic abuse. Reports have also described frequent analgesic consumption among collections of cases with interstitial nephritis[33,119]. The analgesic most often cited is phenacetin, although phenacetin-containing mixtures, aspirin alone, and other analgesics have been reported[120-124]. The pathological lesion most often found in association with analgesic abuse is papillary necrosis. Cases without papillary necrosis have also been reported[122,125]. Papillary necrosis also occurs in the absence of demonstrated analgesic exposure[120].

Several clinical reports suggest an improved prognosis for patients who discontinue use of analgesics compared with those who continue use[126-130]. In many of these reports only a small proportion of original cases are successfully followed. More important, however, is the lack of adjustment for potential differences in dis-

ease severity and concurrent risk factors which might affect both prognosis and compliance with medication restrictions.

Clinically, patients labelled as having analgesic-associated renal disease tend to appear older than their age, they smoke, and often have mild hypertension and frequent urinary tract infections, all of which might increase renal disease risk. In the absence of biopsy and good analgesic use history it is not generally possible to distinguish analgesic-associated nephritis from interstitial nephritis from other causes. The small scarred kidneys seen radiographically are indistinguishable from those seen in other conditions. This may introduce the possibility of diagnostic bias and result in wide variation in recognition and reporting of analgesic-associated disease. Even when a biopsy is performed, papillary necrosis may not be apparent.

In many reports, history of analgesic use is a major component of the diagnosis of analgesic-associated interstitial disease[32,33]. This may, however, be misleading for a number of reasons. Analgesic histories are difficult to obtain and specific clinical interests may affect the rigour with which an analgesic history is sought. For example, such a history may not be sought when other conditions or risk factors such as proteinuria and haematuria or diabetes are present. Conversely, recognition of analgesic abuse in a renal disease patient may limit the extent to which other factors are evaluated. Thus it becomes difficult to determine the true prevalence and incidence of analgesic-associated renal disease using existing studies and records. Population based assessment using standardized diagnostic criteria might eliminate some of the apparently wide geographical variation in the prevalence of analgesic-associated disease.

Because analgesic use histories are most likely to be obtained for patients in whom other more obvious reasons can be ruled out, histories are not generally obtained for patients with glomerular disease marked by clinically evident haematuria and proteinuria. As a result, the notion that analgesics specifically cause tubulointerstitial damage has gained widespread belief. Few reports provide information that can be used to assess the possibility of an association between analgesics and other forms of renal disease. At least one report suggests that some patients with analgesic-associated interstitial nephritis also have glomerulonephritis that either preceded or occurred simultaneous with the interstitial changes[131]. Lack of attention to the possibility that some glomerular disease might be due to analgesic use may result in an underestimation of the magnitude of the problem of analgesic-associated kidney damage.

There is wide geographical variation in the prevalence of analgesic-associated nephropathy. Analgesic-associated nephropathy is commonly reported in Switzerland, Sweden, and Australia[120,122,132,133]. The geographical differences in the prevalence of analgesic-associated renal disease also suggest that there may be geographical differences in patterns of use of analgesics, frequency with which the diagnosis is made (due to differences in diagnostic acuity, quality of drug histories obtained, or interest in making the diagnosis), factors which might effect analgesic toxicity, or frequency of other renal disease risk factors

in persons who consume analgesics.

Ecological studies indicate that areas of high analgesic consumption and high analgesic-associated renal disease generally coincide[120,122,125,132,133]. It has also been shown that time trends for consumption of phenacetin-containing preparations and time trends in interstitial nephritis morbidity and mortality correspond[134,135]. A number of reports have suggested a decline in the prevalence of analgesic-associated renal disease following restrictions placed on the sale of analgesics containing phenacetin in Canada and Scandinavia[134,136-139]. It is difficult to evaluate whether or not these are true time trends or reflect less intensive screening for analgesic-associated renal disease after withdrawal of the "culprit" (phenacetin-containing) medications.

In the United States and Britain, analgesic consumption has only recently been recognized as a potential factor in renal disease. The prevalence of regular analgesic use in the United States and Canada has been reported to be between 6 and 10%[140,141]. The frequency of analgesic abuse is as common as it is in countries where analgesic-associated nephropathy is reported to be a problem[142,143], although patterns of use and specific analgesics taken may differ.

A significant proportion of renal disease reportedly occurs in association with analgesic consumption even in some areas of the United States. Murray and Goldberg[32] reported that 20% of interstitial nephritis patients abused analgesics. Because interstitial nephritis accounted for 30% of the chronic renal failure patients included in the series, at least 7% of chronic renal failure was reported related to analgesic consumption. Where analgesic use is more common, such as in Australia, as much as 20% of end-stage renal disease has been attributed to analgesic use[47,144]. In a report from North Carolina, analgesics were said to account for 10% of end-stage renal disease[33]. Analgesic consumption may be more common in the southeastern USA, but the frequency of analgesic consumption in a comparable disease-free group was not examined in this and in other case series.

Assigning a causal role to analgesic consumption is more difficult than establishing an association. The definition of "abuse" varies with each report, a dose response relationship has not been established, and the responsible analgesic has not been determined. In many reports pyelonephritis is also present in from 50 to 100% of patients with analgesic-associated nephropathy[120]. It is unclear whether consumption precedes infection or if preclinical renal disease produces symptoms which motivate analgesic consumption.

Studies that have utilized appropriate comparison groups have produced conflicting results. Two cross-sectional studies have not demonstrated a strong association between analgesic consumption and renal disease. The Boston Collaborative Drug Surveillance Program screened 6407 consecutive hospital admissions for history of analgesic abuse and evidence of renal dysfunction[141]; 7% were found to be daily analgesic users, among whom the frequency of renal disease was 4.5%. Renal disease was present in 3.8% of persons who did not admit to regular analgesic use. Measures of renal function did not differ with analgesic consumption. Only 1% of the population used analgesics containing phenacetin, which would ex-

plain the lack of demonstrated association with renal disease if phenacetin were truly the responsible agent.

Waters et al.[143] surveyed approximately 3000 women aged 20-64 living in a defined area of Wales. Renal function and renal symptoms in individuals who took four or more analgesic tablets a day were compared with function in a 10% random sample of the remaining women. Only 1.9% of the population were analgesic users. Women in the analgesic group reported significantly more urinary tract symptoms than did other women. There were, however, no differences in plasma urea or creatinine, although these measures did show a steeper rise with age in the analgesic group.

Additional cross-sectional studies of the general population have also shown no association between analgesic consumption and renal dysfunction[145]. Similar studies conducted in factory workers in Sweden[139], and in rheumatology patients from New Zealand and Australia[146,147], have, however, found an increase in some types of renal disease among users of phenacetin-containing analgesics.

Sorenson[148] compared renal function, bacteriological studies, renal biopsies and radiological evaluations of analgesic users and non-users. He found no apparent connection between the magnitude of analgesic consumption and the incidence of papillary necrosis, but did find that analgesic consumers had a higher incidence of papillary necrosis than did non-users. Larsen and Moller[149], on the other hand, found not only that three times as many phenacetin users as controls had reduced renal function, but that both dose and duration of consumption correlated with renal disease.

Lawson and Maclean[150] compared autopsies and medical records of 60 patients with rheumatoid arthritis and 120 age- and sex-matched controls. Renal changes were found in 73% of patients but only 25% of controls. Papillary necrosis was seen in 21% of patients and the frequency and type of renal disease appeared related to the specific analgesic (phenacetin) and the quantity consumed. However, no information on drug use in the controls was given, and renal disease may be more common in arthritis patients for reasons unrelated to analgesic consumption. In another autopsy study, Burry et al.[151] found that papillary degeneration was seen in 73% of individuals known to have consumed large amounts of phenacetin. Death from papillary necrosis with pyelonephritis was seen in 37% of the abusers.

Several other studies of autopsy patients have also found increased prevalence of renal disease among users of phenacetin-containing analgesics as compared with non-users[152-154]. In these studies no attempt was made to control for potential confounding factors, and the time course of analgesic use and renal damage cannot be established. Furthermore, autopsy series are generally highly selected. Factors associated with selection for study might also relate to either analgesic use or renal disease or both, making interpretation of findings difficult.

Only a small number of studies employed traditional epidemiological designs. A study by Dubach and colleagues[142,155,156] was carried out in several stages. In the first stage over 7000 women aged 30-49 employed in various industries in Switzerland

were screened for urinary evidence of phenacetin ingestion[142]. Phenacetin use was found in 1376 (18.8%) women. Of these, 672 were also positive a second time and were designated the "exposed" group. A matched comparison group was selected from among those with no urinary evidence of phenacetin consumption. The exposed and non-exposed groups were similar in age, parity, and marital status, but the exposed were more often machine workers (80 vs. 69%) than non-exposed and thus may have been more likely to have had other nephrotoxic exposures. There were no statistically significant differences between the groups in prevalence of haematuria, bacteriuria, or elevated serum creatinine. There were, however, differences in prevalence of proteinuria and history of past kidney disease. No dose-response relationship was found for level of phenacetin metabolites in urine and renal abnormalities. The cross-sectional nature of this first stage limited interpretation of any observed differences.

Follow-up of this group was conducted yearly between 1969 and 1972, and again in 1975 to determine the incidence of renal dysfunction among those initially free of disease[155]. Overall mortality was higher in the exposed group, but only two deaths were attributed to renal failure. General mortality differences might relate to reasons for taking analgesics (underlying medical conditions) rather than to effects of phenacetin. Small differences were seen in some measures of renal function such as renal concentrating ability, serum creatinine and bacteriuria, but these might also be explained by other factors such as occupational exposures. One potential limitation of the study is that urinary and questionnaire measurement of phenacetin were not in good agreement. Approximately 75% of the "exposed" group and 24% of the controls admitted on questionnaire to use of phenacetin. This substantial level of potential misclassification might affect study findings. The study is also limited in that only phenacetin was measured. Exposure to other analgesics with similar or different effects might vary between groups.

Additional follow-up was conducted in 1978[156]. Again, differences in some measures of renal dysfunction were reported, and there was evidence of a dose-response relationship for phenacetin consumption and several of these measures. Mortality among phenacetin users was also significantly greater than that among non-users. This was true for genitourinary diseases as well as for cancer, cardiovascular disease, and total mortality. The excess deaths from non-renal cancers and cardiovascular disease suggest there may have been important baseline differences between users and non-users of phenacetin. Potential differences, such as differences in the prevalence of hypertension might influence both analgesic use and renal dysfunction, and might explain some of the findings relating phenacetin and renal dysfunction.

Two case-control studies have been conducted[157,158]. Findings in the two studies are quite different, but could be explained by the inclusion of different case groups and by differing prevalence of analgesic consumption.

McCredie et al.[157] studied patients with papillary necrosis and several groups of controls. These included patients with renal diseases which were judged unrelated to analgesics, women who

were friends or relatives of the female cases, and women who attended a health screening clinic. Questionnaire data on analgesic use were obtained. A strong association between use of phenacetin and papillary necrosis was seen, with a reported relative risk of 17.7 among users and a dose-response relationship between cumulative phenacetin dose and disease. No increased risk was associated with other analgesics after adjusting for phenacetin use. The authors did not determine if analgesic consumption began before or after the onset of renal disease, and they did not control for other conditions that might predispose to both renal disease and analgesic use. It is unlikely, however, that so large a relative risk could be entirely "explained" away by such oversights.

In the second study, patients with end-stage renal disease undergoing dialysis and age-, race-, and sex-matched hospital controls were interviewed regarding analgesic exposure prior to dialysis (or hospital admission for controls)[158]. Relative risks were close to one (no risk) for the use of any single ingredient analgesics. The relative risk for the use of combination analgesics was also close to one, but the risk associated with heavy or long-term use of combination analgesics was 2-fold (borderline statistical significance). Relative risks associated with some specific analgesics were also elevated, but no consistent dose-response relationships were seen, perhaps due to small numbers of users.

This study, unlike that of McCredie et al.[157], included patients with a variety of renal diseases. If analgesic abuse is only associated with a particular subset of renal disease, such as interstitial nephritis, an association might be obscured if the majority of patients had other diagnoses. The authors were not able to classify many of the patients included in their study by primary renal diagnosis. Furthermore, analgesics might lead to forms of renal disease that are less severe or less rapidly progressive, which would be under-represented in a dialysis series.

Controls in the study by Murray et al.[158] included patients hospitalized for conditions associated with analgesic use. This might also explain the apparent lack of strong association between analgesic use and renal disease, as might the failure to adjust for differences between patients and controls in prevalence of conditions such as hypertension or other factors that might relate to renal disease risk. Further limitations of this study include the low prevalence of analgesic abuse in either cases or controls, and the resulting low power to detect small differences in risk, and the inability to determine if the presence of renal disease prior to dialysis affected analgesic use patterns.

V. COMMENT

The clinical and epidemiological literature concerning potential nephrotoxins is large and diverse. Reports indicate that nephrotoxic exposures clearly play an important role in renal disease incidence. However, a large proportion of the literature consists of case reports, case series, and ecological studies which fall short of providing strong evidence of particular associations. Even studies that utilized more appropriate study designs generally have

methodological weaknesses which limit the usefulness of individual studies. The greatest weakness in the literature, however, is the lack of standardized criteria for ascertainment, diagnosis, and classification of renal disease and dysfunction. Until such criteria are established, renal disease will continue to be difficult to study. Interpretation and evaluation of the existing literature will continue to be problematic, and the magnitude of the problem of renal disease due to nephrotoxins will continue to be under-appreciated.

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