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Hazard Evaluation of Chemicals That Cause Accumulation of α_{2u}-Globulin, Hyaline Droplet Nephropathy, and Tubule Neoplasia in the Kidneys of Male Rats

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This review paper examines the relationship between chemicals inducing excessive accumulation of α_{2u}-globulin (α_{2u}-g) (CIGA) in hyaline droplets in male rat kidneys and the subsequent development of nephrotoxicity and renal tubule neoplasia in the male rat. This dose-responsive hyaline droplet accumulation distinguishes CIGA carcinogens from classical renal carcinogens. CIGA carcinogens also do not appear to react with DNA and are generally negative in short-term tests for genotoxicity. CIGA or their metabolites bind specifically, but reversibly, to male rat α_{2u}-g. The resulting complex appears to be more resistant to hydrolytic degradation in the proximal tubule than native, unbound α_{2u}-g. Single cell necrosis of the tubule epithelium, with associated granular cast formation and papillary mineralization, is followed by sustained regenerative tubule cell proliferation, foci of tubule hyperplasia in the convoluted proximal tubules, and renal tubule tumors. Although structurally similar proteins have been detected in other species, including humans, renal lesions characteristic of α_{2u}-g nephropathy have not been observed. Epidemiologic investigation has not specifically examined the CIGA hypothesis for humans. Based on cancer bioassays, hormone manipulation studies, investigations in an α_{2u}-g-deficient strain of rat, and other laboratory data, an increased proliferative response caused by chemically induced cytotoxicity appears to play a role in the development of renal tubule tumors in male rats. Thus, it is reasonable to suggest that the renal effects induced in male rats by chemicals causing α_{2u}-g accumulation are unlikely to occur in humans.

Introduction

For most hazardous chemicals, adequate human data are not available, and risk analyses must rely on information from laboratory studies of rats or mice. The inference that the results of animal experiments can be applied to humans is a fundamental principle of all toxicologic research. This analysis deals with a specific case, however, where the male rat seems to respond in a manner different from other laboratory species. The possibility of a unique response in the rat among laboratory animals raises questions about the applicability of the rat data to other species, including humans. Our review evaluates the matter of human relevance and describes the types of information needed for hazard assessment of such chemicals.

A variety of organic chemicals have produced specific renal lesions in male rats in the form of a protein (hyaline) droplet nephropathy accompanied by accumulation of α_{2u}-globulin (α_{2u}-g) [reviewed in (*1,2*)]. Among the chemicals tested are paraffins (*3,4*), decalin (decahydronaphthalene) (*5,6*), petroleum-based and synthetic fuels (*7*), military aviation propellants (*8*), and 2,2,4-trimethylpentane (TMP) (*9*). As seen in Table 1, which lists a sampling of chemicals that have been tested, many are of considerable regulatory and commercial interest. For example, isophorone is a chemical intermediate of major industrial importance. Aviation and automotive fuels fit into the category, as does the natural food product, *d*-limonene found in citrus oils.

This analysis focuses on model compounds having both an adequate carcinogenesis bioassay in two species and information on α_{2u}-g or hyaline droplet accumulation in the male rat. These substances are seven chemicals, 1,4-dichlorobenzene (1,4-DCB), dimethyl methylphosphonate, hexachloroethane, isophorone, *d*-limonene, pentachloroethane, tetrachloroethylene, and a mixture, unleaded gasoline. The analysis also relies on research

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Table 1. Examples of organic chemicals that have produced renal injury in male rats characterized by hyaline droplet accumulation but not in female rats or other species.

Chemical	Species tested	Sex tested	Renal toxicity[a]	Reference
Decalin	Rats	M/F	+/–	(*10*)
	Mice	M/F	–/–	(*11*)
	Dogs	M/F	–/–	
	Guinea pigs	M/F	–/–	
Dimethyl methylphosphonate	Rats	M/F	+/–	(*12*)
	Mice	M/F	–/–	
Isophorone	Rats	M/F	+/–	(*13*)
	Mice	M/F	–/–	
JP-5 shale-derived jet fuel	Rats	M/F	+/–	(*7*)
	Mice	M/F	–/–	
	Dogs	M/F	–/–	
JP-4 jet fuel	Rats	M/F	+/–	(*7*)
	Mice	M/F	–/–	
	Dogs	M/F	–/–	
d-Limonene	Rats	M/F	+/–	(*14*)
	Mice	M/F	–/–	
	Dogs	M/F	–/–	(*15*)
Methyl isobutyl ketone	Rats	M/F	+/–	(*5*)
	Mice	M/F	–/–	(*16*)
	Dogs	M	–	
	Monkeys	M	–	
Pentachloroethane	Rats	M/F	+/–	(*17*)
	Mice	M/F	–/–	
Unleaded gasoline	Rats	M/F	+/–	(*11*)
	Mice	M/F	–/–	

[a]Positive or negative in the corresponding sex in the third column.

studies on two other model compounds, decalin and TMP, which have extensive information on α_{2u}-g nephropathy but no chronic bioassay data. More limited information on additional substances is also discussed where appropriate.

Of the selected substances tested in chronic animal bioassays, all invoked a specific type of protein droplet nephropathy in male rats but not in other sexes or species tested. It has been proposed that such renal tumors are the end product in the following sequence of functional changes in the epithelial cells of proximal tubules (*2,3,5,18,19*): *a*) Excessive accumulation of hyaline droplets in proximal tubules, representing lyosomal overload, leads to tubule cell degeneration, cell loss, and regenerative cellular proliferation. *b*) Cell debris in the form of granular casts accumulates at the "corticomedullary" junction with associated dilation of the affected tubule segment and, more distally, mineralization of tubules within the renal medulla. *c*) Single-cell necrosis accompanied by compensatory cell proliferation and exacerbation of the chronic progressive nephropathy characteristically found in aging rats occurs. *d*) Renal tubule hyperplasia and neoplasia develop subsequently.

According to this hypothesis, the increased proliferative response caused by the chemically induced cytotoxicity results in clonal expansion of spontaneously initiated renal tubule cells and increased incidence of renal tumor formation (*19–21*). This line of reasoning leads supporters of the hypothesis to conclude that the acute and chronic renal effects induced in male rats by these chemicals will be unlikely to occur in any species not producing α_{2u}-g, or a very closely related protein, in the large quantities typically seen in the male rat (*21–26*).

For clarity throughout the review, nomenclature is standardized, and abbreviations are used for frequently repeated terms. Insofar as hyaline droplet represents a morphological entity requiring only light microscopy for identification, this term will be used in preference to the synonymous protein droplet. The designation α_{2u}-globulin nephropathy is used to connote the full sequence of pathologic lesions from hyaline droplet formation to restorative hyperplasia and medullary mineralization. Toxic tubular nephropathy is a nonspecific term commonly used in rodent bioassay reports to describe various forms of nephrotoxicity induced by chemicals, including the specific lesions of α_{2u}-g nephropathy. The spontaneous age-related syndrome of rat kidney disease otherwise known in the literature as old rat nephropathy, chronic nephrosis, glomerulosclerosis, and progressive glomerulonephrosis, is standardized according to Barthold (*27*) as chronic progressive nephropathy (CPN).

In rats, the proximal tubule of the nephron is divisible morphologically into three parts. The first segment is in continuity with the parietal epithelium of Bowman's capsule surrounding the glomerular tuft. Together, the first and second segments represent the convoluted portion of the proximal tubule and are situated wholly in the cortex, the outermost zone of the rat kidney. The third segment is the straight portion of the proximal tubule (*pars recta*) comprising the outer stripe of the outer medulla but also the medullary rays arising in the cortex. The abbreviations P1, P2, and P3 are used conventionally to denote these three segments. The term renal tubule tumor describes neoplasms of the renal cortical tubule epithelium comprising collectively adenoma, adenocarcinoma, and carcinoma according to standardized nomenclature determined by the Society of Toxicologic Pathology (*28*). Except when specified, the terms adenocarcinoma and carcinoma are used interchangeably. The same neoplasms are referred to as renal cell tumors in humans, in keeping with the general literature (*29*).

Hyaline Droplets and α_{2u}-Globulin: Physiology and Biochemistry

Information on the renal processing of low molecular weight

proteins, sex and species differences in urinary proteins, and the characteritics of α_{2u}-g provides an explanatory basis for the accumulation of α_{2u}-g in hyaline droplets in the male rat following exposure to chemicals that induce α_{2u}-g accumulation (CIGA). It is pertinent, therefore, to examine the physiological and biochemical characteristics of α_{2u}-g and related proteins before exploring the possible associations between α_{2u}-g accumulation, renal toxicity, and renal tumor formation.

Filtration, Reabsorption, and Catabolism of Low Molecular Weight Proteins by the Kidney

The mammalian kidney has a major role in maintaining the plasma concentrations of circulating low molecular weight proteins at their normally low physiological levels. Thus, low molecular weight proteins are continually removed from the plasma by glomerular filtration followed by reabsorption and catabolism in the proximal tubules (*30*) or by excretion. The normal renal glomerulus freely passes proteins with a molecular weight of less than 20,000 daltons, including peptides such as insulin, lysozyme, rat growth hormone, myoglobin, and cytochrome *c* (*30*). For larger proteins such as the albumins and globulins, which have a far greater plasma concentration and much lower filtration rate than low molecular weight proteins, the kidney has no regulating role in plasma protein concentration.

Reabsorption of filtered protein occurs predominantly in the convoluted part of the proximal tubule and to a lesser extent in the *pars recta* cells. Tubular absorption of a protein is a complex process initiated by binding of the protein to the microvilli of the proximal tubule epithelium. This is followed by migration to the base of the microvilli and adsorptive endocytosis, whereby invagination of the surface membrane internalizes the protein (*31*). Although reabsorption was once considered largely nonselective, high-capacity, low-affinity transport (*30*), from recent work it now appears that the interaction between the protein and the brush border membrane is the step at which a degree of selectivity in the absorption process occurs (*31*).

Within proximal tubule cells, endocytic vesicles fuse to form endocytic vacuoles, which in turn coalesce with lysosomes derived from the Golgi apparatus, forming secondary lysosomes. The hydrolysis of proteins by protease enzymes takes place within the secondary lysosomes. The lysosomal enzymes of renal cortical tubules include two major classes of acid proteinases, cysteine proteinases (capthepsin B, H and L) and an aspartic acid proteinase, cathepsin D (*32*). Lehman-McKeeman et al. (*32*) have shown that both of these endopeptidase classes contribute to the degradation of α_{2u}-g.

Lysosomes have a large, but not unlimited, capacity to cope with increased amounts of hydrolyzable proteins, but the proteins differ in their susceptibility to hydrolysis. Protein half-lives, which are indices of their catabolism by proteases in the kidney, depend on specific molecular determinants in the protein. The primary amino acid sequence may be one important factor in determining protein half-lives (*33*). The plasma half-lives of many low molecular weight proteins are typically measured in minutes (*30*). α_{2u}-Globulin, with a half-life measured in hours (*34*), is one of the exceptions.

Whether low molecular weight proteins such as α_{2u}-g accumulate in kidney tubules depends on the balance between the rate of reabsorption by epithelium and the rate of hydrolysis in the cells. Based on the information presented below, it is believed that exposure to CIGA results in a shift of this balance in male rats.

Hyaline Droplets in Renal Tubules

The product of protein reabsorption and accumulation in renal tubule cells is visualized by light microscopy as hyaline droplets. Small protein reabsorption droplets of uniform size are a constitutive feature of normal, mature male rats and are particularly evident in the P2 segment of proximal tubules (*35–37*). Ultrastructurally, hyaline droplets are abnormallly large, dense secondary lysosomes (also called phagolysosomes), representing fusion of endocytic vacuoles with primary lysosomes. Electron microscopy shows crystalloid changes in some hyaline droplets that are not seen in the lysosomes of female rats (*38*). Crystalline formation in the normal male rat is believed to indicate the presence of a poorly catabolized protein in pure solution (*39*), presumably α_{2u}-g in the kidney lysosomes. Hyaline droplets in the proximal tubules of normal male rats contain α_{2u}-g (*5,37,40*), and their occurrence appears to parallel the variable synthesis of this protein. Thus, hyaline droplets become apparent in male rats at the time of puberty, but they decline progressively with increasing age after 18 months (*35,41*). In female rats, hyaline droplets in proximal tubules are either absent, or considerably less frequent than in males, and then they are not associated with α_{2u}-g (*35–37,42*). Hyaline droplets are substantially reduced in castrated male rats (*35*).

Abnormal accumulation of hyaline droplets in rodent kidney is seen in certain disease processes. Both male and female rats with histiocytic sarcoma show hyaline droplet accumulation in the proximal tubules, which is indistinguishable from the CIGA-induced lesion. The accumulating protein in these tumor-bearing animals has been identified as lysozyme (*43*). Similarly, in male and female mice with histiocytic tumors, abnormal accumulation of lysozyme-containing hyaline droplets sometimes occurs in proximal tubules (*43*).

In humans, the Bence-Jones proteins, a class of light-chain immunoglobulins, are produced in large amounts in multiple myeloma patients (*44*). In human cases of mononuclear cell leukemia, lysozyme is produced (*45*). The kidney injury seen with these neoplastic diseases has been described as similar to that produced by administration of decalin to male rats (*46*), including protein droplet accumulation in renal tubules (*44,47,48*). Patients with epidemic hemorrhagic fever, infused with large amounts of concentrated human serum albumin as a therapeutic procedure for shock, have also developed a comparable form of hyaline droplet accumulation (*47*).

Factors Affecting Kidney Accumulation of Low Molecular Weight Proteins

Protein accumulation in the proximal tubule can reach pathologic levels resulting in excessive hyaline droplet formation for several reasons: *a*) the rate of protein delivery to the tubule cells is abnormally high, *b*) the proteins delivered are difficult to hydrolyze, or *c*) the lysosomal hydrolysis capacity is sufficiently reduced.

The rate of protein delivery to the tubule can be abnormally

high under conditions when the capillary wall of the glomerulus fails to provide the normal filtration barrier. This happens, for example, where there is immunological, inflammatory, or toxic disease in the glomerulus, or when the permselectivity barrier is overloaded by filterable proteins (*31*).

The increased urinary excretion of low molecular weight proteins seen in diseases such as multiple myeloma in humans or histiocytic sarcoma in rats is primarily the result of an increase in plasma concentration caused by overproduction of specific small proteins (*30*). Lysozyme (histiocytic sarcoma) and light-chain immunoglobulins (multiple meyloma) are proteins also relatively resistant to hydrolysis (*30*). This suggests a combination of rate of delivery and difficulty of hydrolysis as etiologic factors in the accumulation of lysozyme in rats with histiocytic sarcoma and light-chain immunoglobulins in humans with multiple myeloma. The combination of difficult hydrolysis of the protein, as suggested by its long half-life, coupled with high rate of protein delivery to tubule cells in the sexually mature male rat, also appears to be a factor in the accumulation of α_{2u}-g in the renal tubules of male rats.

The process of protein hydrolysis can be reduced or inhibited when lysosomes are unable to maintain the low pH required for hydrolytic enzyme function. Inhibition of the metabolically driven hydrogen ion pump, by metabolic poisons or the presence of a weak base in tubule lysosomes, alters the pH and results in the accumulation of proteins (*30*). In the presence of a reduced lysosomal hydrolysis capacity, the most hydrolytically resistant proteins, such as α_{2u}-g, tend to accumulate first. In addition, testosterone is known to have a suppressive effect on the activity of some major proteolytic enzymes in the male rat kidney (*49*). Consequently, the lysosomal protease activity in male proximal tubules is lower than that of females (*49,50*), implying that the male rat could be intrinsically more prone to protein overload in the renal tubules than the female rat.

Reduction of the hydrolytic capacity of renal lysosomes and increased resistance of protein to hydrolysis can both be affected by exogenous chemicals. Although CIGA may not compromise kidney lysosomal enzyme activity per se (*32,41*), any chemically induced impediment to α_{2u}-g digestibility would be further superimposed on the factors considered above that alone can cause excessive protein accumulation in renal tubules.

The α_{2u}-Globulin Superfamily of Proteins

Alpha$_{2u}$-globulin is a member of a large superfamily of low molecular weight proteins. The complete amino acid sequence of α_{2u}-g was first deduced by Unterman et al. (*51*). With the exception of α_{2u}-g and mouse major urinary protein(s) (MUP), the sequence homology between any pair of proteins in this superfamily is small—about 20% (*52*). Statistical analysis shows, however, that the proteins are related evolutionarily (*52*).

Of the approximately 20 proteins now considered to be potential members of the superfamily (*52*), the three-dimensional structure is known for only three—retinol-binding protein, β-lactoglobulin, and insecticyanin (*53*). The central core of these three proteins is composed of eight strands with a β-barrel structure forming a hydrophobic pocket that appears to enclose the ligand (*53,54*). This structure has been described as resembling a coffee filter paper (*52*). In additon to the β-structural motif, one helical rod and several other structural elements appear to be conserved among the proteins. Protein folding patterns tend to be highly conserved in homologous proteins, even though they may diverge considerably in structure and function, suggesting that other members of the superfamily, including α_{2u}-g, possess a similar three-dimensional structure.

The only member of the protein superfamily with a clearly defined physiological function is retinol-binding protein. More circumstantial evidence suggests that the superfamily members serve as carriers of lipophilic molecules (*55*). The mode of binding in which the lipid ligand is enclosed within the β-barrel impressed Pervaiz and Brew as not unlike the protective role of the calyx to a flower (*55*). On this basis, they suggested the illustrative name, *lipocalins*, for the superfamily of proteins (*55*).

Table 2 illustrates the information available on several members of the lipocalin superfamily, which includes α_{2u}-g, retinol-binding protein, apolipoprotein D, α_1-acid glycoprotein and α_1-microglobulin, ruminant β-lactoglobulin and pyrazine-binding protein (i.e., odorant-binding protein), rat odorant-binding protein, and MUP. Some of the members of the lipocalin superfamily, such as retinol-binding protein, α_1-acid glycoprotein, and α_1-microglobulin have been identified in many species, and their properties appear to be species independent, suggesting that they share a common vital function (*52*). Others such as α_{2u}-g and MUP, seem to be species dependent.

Several functions have been suggested for α_{2u}-g. Cavaggioni et al. (*58*) speculated that α_{2u}-g may serve to transfer odorants such as ethereal lipid pheromones from male rat urine to the air for attracting females. Glandular tissue production of α_{2u}-g helps support these speculations (*65,66*). In addition, α_{2u}-g has been identified as a fatty acid-binding protein of the kidney (*61*) and may serve to transport fatty acid, an important energy source in kidney, within renal epithelial cells. Brooks (*60*) found a protein structurally related to α_{2u}-g that is synthesized and secreted by the rat epididymis under the influence of androgenic hormones. He speculated that the function of these proteins may be to carry retinoids within the lumen of the male reproductive tract.

Other members of the lipocalin superfamily, such as retinol-binding protein, apolipoprotein D, β-lactoglobulin, and α_1-acid glycoprotein, function in the transport of lipids between cells and across hydrophilic barriers (*64*). The lipids bound by the proteins differ considerably in structure and range from odorants in rat nasal epithelium to human cholesterol and retinol (vitamin A). It is not yet clear how selective these proteins are for specific ligands or whether a given protein might bind a wide spectrum of small, hydrophobic molecules. Both cases might occur because retinol-binding protein is quite specific for retinol, whereas odorant-binding proteins may have a broad specificity (*63*).

The ability of a chemical to serve as a ligand for one member of the superfamily appears to be a poor predictor of binding affinity for other members of the superfamily. Cavaggioni et al. (*59*) measured the binding affinities of a series of odorants for α_{2u}-g, MUP, and pyrazine-binding protein isolated from calf nasal mucosa. MUP bound only 1 of these chemicals, pyrazine-binding protein bound 6, and α_{2u}-g bound 12. The best ligand for α_{2u}-g was chemically unrelated to the best ligands for the other two proteins, which were also chemically unrelated.

Characteristics of α_{2u}-Globulin

α_{2u}-Globulin was first characterized in male rat urine (*67*). All

Table 2. Superfamily of lipophilic ligand-binding carrier proteins (lipocalins).[a]

Protein	Species	Tissue or body fluid	Molecular weight, daltons	No. of amino acids	Reference
α_1-Acid glycoprotein (acute phase; orosomucoid)	Mammals	Plasma	23,000 (unglycosylated) 43,000–60,000 (complex type)	184	(55,56)
Apolipoprotein D (cholesterol associated)	Mammals	Plasma	19,300	169	(57)
α_1-Microglobulin (protein HC)	Mammals	Plasma	20,619	182	(52)
Retinol-binding protein	Mammals	Liver, plasma, retina	22,868	182	(54)
Endometrial α_2-globulin	Mammals	Placenta (pregnancy associated)	25,000	NR	
β-Lactoglobulin	Ruminants, other species	Milk	18,000	162	(54)
Odorant-binding protein (pyrazine-binding protein)	Rat, cow	Nasal epithlium	18,091	172	(58,59)
α_{2u}-Globulin[b]	Rat	Male urine, preputial gland	18,709	162	(51)
Androgen-dependent secretory protein	Rat	Epididymis	18,500	184	(60)
Fatty-acid-binding protein[c]	Rat	Kidney	15,500	NR	(61)
Major urinary protein	Mouse	Urine (both sexes)	18,730	162	
Purpurin	Chick	Retina	21,924	196	
Bowman's gland protein	Frog	Olfactory epithelium	20,300	182	(62)
Insecticyanin (bilin-binding)	Tobacco hornworm, butterfly	Hemolymph	21,382 NR	189 NR	(63) (53)

NR, not reported—characterization of protein incomplete.
[a]Adapted from Pevsner et al. (64), with additional information as noted.
[b]Also occurs in other secretory glands and in liver.
[c]Described as α_{2u}-globulin by Kimura et al. (61).

isoforms of α_{2u}-g are anionic at neutral pH, although they have varying isoelectric points. The molecular weight of α_{2u}-g has been reported to be 18,000–20,000 daltons. In all rat strains tested to date, except for the NCI Black-Reiter (NBR) rat, a strain that appears to have a tissue- and gene-specific regulatory defect involving α_{2u}-g (68), the major urinary source of α_{2u}-g is the liver, where α_{2u}-g mRNA constitutes approximately 1% of the hepatic mRNA population (69,70). The hepatic isoforms of α_{2u}-g may vary throughout the lifetime (71).

Synthesis of the protein in rat liver is under multihormonal control, particularly androgen, but also glucocorticoids, thyroid hormones, insulin, and growth hormone (72,73). These hormones appear to act by regulating the steady-state level of α_{2u}-g mRNA (70). Neither α_{2u}-g nor its corresponding mRNA is detectable in the livers of sexually intact female rats (69,74,75). However, a very low background level of the mRNA has been indicated in the ovariectomized female rat (76), and ovariectomy in concert with androgen treatment induces a parallel increase in α_{2u}-g and its mRNA in female rat liver (67,74).

Although plasma and urinary α_{2u}-g derive predominantly from the liver in male rats, high levels of α_{2u}-g and its mRNA are also present in the preputial gland of both male and female rats, and neither castration nor ovariectomy significantly alters the preputial concentration of this protein and its mRNA (41). α_{2u}-Globulin mRNA has also been detected in the female mammary gland during pregnancy, and in the submaxillary, lachrymal, Meibomian, and perianal glands of rats of both sexes (66,75). The female forms of α_{2u}-g show distinct differences from male rat α_{2u}-g, suggesting that they are encoded by different genes (77).

Low levels of α_{2u}-g first become detectable in the male rat liver under the stimulus of testosterone at 35–40 days, reaching maximum adult levels by 60–80 days (71,75,78). At some stage after 5 months of age, due to the development of hepatic insensitivity to androgen during aging, hepatic synthesis of α_{2u}-g falls gradually. α_{2u}-Globulin levels are reduced by more than 90% by 22 months of age in male rats and are virtually undetectable in senescent animals (71,78,79). Renal cortical tissue content (41) and urinary excretion (78,80) of α_{2u}-g reflect the same age-related trends as synthesis in the liver.

In the mature male rat, approximately 50 mg of α_{2u}-g is filtered per day; 40% of the filtered protein is excreted in the urine and 60% undergoes reabsorption and catabolism (81,82). It is catabolized slowly relative to most other proteins in the glomerular filtrate, with a half-life in plasma, kidney cytosol, or lysosomal preparations of 5–8 hr (32,34,83). *In vitro* studies indicate that α_{2u}-g is more resistant than bovine β-lactoglobulin and lysozyme to lysosomal enzyme digestion (84). In another study comparing members of the protein superfamily, α_{2u}-g and α_1-acid glycoprotein were the most resistant to proteinase K digestion, whereas retinol-binding protein and β-lactoglobulin were 1000- to 100,000-fold more easily hydrolyzed (22). These data indicate that α_{2u}-g may be more likely to accumulate in the kidney than most other members of the superfamily if shifts in the balance between reabsorption and hydrolysis occur.

Sex and Species Comparison of Urinary Protein Content Related to the α_{2u}-Globulin Superfamily

Relative to the female rat and other species including humans, the normal, mature male rat is physiologically proteinuric. This is due to the amount of α_{2u}-g secreted in male rat urine, 1.36–8.64 mg/day/g kidney (85), which is 100–300 times greater than in female rat urine (77,86). The mouse can also be described as physiologically proteinuric because of a high urinary content of MUP (87). MUP shows the greatest similarity to α_{2u}-g in the lipocalin superfamily, sharing 90% amino acid sequence homology (88). Representing a group of proteins encoded by a multigene family, MUP is synthesized in the liver of mice of both sexes but at rates four to five times greater in males than in

females (*73,89*). Daily urinary excretion of MUP varies considerably between strains (*90*). In the B6C3F$_1$ strain, males have been shown to excrete 14.9 mg of MUP/day in the urine and females 2.1 mg/day (*91*). Adjusted for body weight, a male B6C3F$_1$ mouse therefore excretes approximately 600 mg/kg/day of MUP, some 12-fold higher than α_{2u}-g urinary excretion by the male rat. Unlike the rat, however, where 60% of filtered α_{2u}-g is reabsorbed by the kidney, MUP is not reabsorbed in the mouse and appears to be totally excreted (*82*).

Normal human urine, such as that of the female rat, contains relatively little protein, only 1% of the total concentration present in mature male rat urine (*24*). Human urinary proteins are predominantly high molecular weight species with only minor components weighing 12,000–42,000 daltons. Within this low molecular weight fraction, trace amounts of proteins represent the lipocalin superfamily, but none appear to share molecular weight identity with α_{2u}-g. The urinary excretion of retinol-binding protein, α_1-acid glycoprotein, and α_1-microglobulin has been measured at 0.0001–0.0007, 0.0006–0.002, and 0.02–0.05 mg/day/g kidney, respectively (*92–94*). Thus, the urinary excretion of α_{2u}-g in the male rat is approximately two orders of magnitude greater than the human urinary content of the three superfamily proteins combined.

Recently, a sex-dependent protein of unknown origin and function, called urine protein 1, was identified in normal human urine (*95*). The molecular features of protein 1 are similar to α_{2u}-g, as it has a molecular weight of approximately 21,000 daltons and an isoelectric point around 4.8, but its amino acids have not been fully sequenced (R. Lauwerys, personal communication). Protein 1 occurs in both sexes from an early age, but increases substantially in males after puberty, reaching up to a 50-fold difference over females during late adolescence. A 5-fold male-to-female differential persists through adulthood. Average urinary concentrations of protein 1 have been determined as 108 and 3.2 μg/L, respectively, for males and females 15–20 years old, and 24.7 and 5.8 μg/L for males and females in the 20 to 60-year age range (*95*). Such levels of protein 1 in human male urine, however, are calculated as four to five orders of magnitude lower than α_{2u}-g concentrations in the urine of male rats.

Noncovalent Binding to α_{2u}-Globulin and Its Homologues

It has been suggested that CIGA bind reversibly and noncovalently to α_{2u}-g, forming a resultant complex that is even more poorly digested in the male rat kidney than α_{2u}-g (J. A. Swenberg, personal communication).

Chemicals Complexed with α_{2u}-Globulin. In a few instances, the specific chemical entity complexed with α_{2u}-g has been identified. TMP, a branched chain aliphatic hydrocarbon present in gasoline (*96*), was the first model CIGA to be studied in this manner. When [^{14}C]TMP was administered in a single oral dose to rats, radioactivity was retained in the kidneys of males, but not of females (*97,98*). The major metabolite of TMP in the male rat kidneys was identified as 2,4,4-trimethyl-2-pentanol (TMPOH) (*98*). In a separate report, TMPOH was shown to be the only ligand for α_{2u}-g whenever TMP was administered to the male rat (*99*). TMPOH was not detected in the kidney tissue of the female rats, which excreted more conjugated TMPOH (glucuronides and sulfates) than the males (*98*). Later studies confirmed, as suspected, that the TMPOH-α_{2u}-g complex is cleared slowly from male rat kidney (J. A. Swenberg, personal communication).

For *d*-limonene, *d*-limonene-1,2-oxide has been shown to be the predominant metabolite binding to α_{2u}-g, although d-limonene also binds to some extent (*100*). For isophorone, the ligand is the parent compound (*101*). Following exposure of the male rat to 1,4-DCB, both the parent chemical and the metabolite, 2,5-dichlorophenol, bound to α_{2u}-g (*102*). About 40% of the 3,5,5-trimethylhexanoyloxybenzene sulfonate administered to male rats bound to kidney proteins, even though no protein binding was observed in the mouse or female rat kidney (*103*). Four metabolites were identified in the α_{2u}-g protein fraction, the main component being the γ-lactone of 3,5,5-trimethylhexanoic acid.

Nature of the Association. The nature of the association of CIGA with α_{2u}-g was explored initially by Lock et al. (*99*), who dosed sexually mature male Fischer 344 rats with [^{3}H]TMP, killed them 8–72 hr later, and homogenized the kidneys. Cytosol, obtained by centrifugation of the homogenate at 116,000*g*, was applied to a Sephadex G-75 column. About 26% of the cytosol radiolabel (15% of all radiolabel in the kidney) eluted in the fraction containing α_{2u}-g. Approximately 19% of the radiolabel in the cytosol was nondialyzable following overnight equilibrium dialysis against phosphate buffer. Chromatography of the dialyzed cytosol showed that the nondialyzable, radiolabeled material coeluted with the peak containing α_{2u}-g. When 0.1% sodium dodecyl sulfate (SDS), a detergent that affects the secondary and tertiary structure of proteins, was added to the dialysis buffer, there was a significant loss of binding. These results suggest a reversible binding between TMP metabolite and the protein fraction containing α_{2u}-g (*99*). The reversibility of the chemical binding with α_{2u}-g, whether parent compound or metabolite, has been confirmed with *d*-limonene(*100*), isophorone (*101*), 1,4-DCB (*102*), and 3,5,5-trimethylhexanoyloxybenzene sulfonate (*103*).

In the *d*-limonene study (*100*), the amount of radioactivity observed in the kidneys of Sprague-Dawley rats 24 hr after oral administration of [^{14}C]*d*-limonene was about 2.5 times higher in the males than in the females. Equilibrium dialysis in the presence or absence of SDS indicated that approximately 40% of the radioactive material retained in the male rat kidney was associated with proteins in a reversible manner. Gel filtration high-performance liquid chromatography (HPLC), reverse-phase HPLC, and amino acid sequencing demonstrated that this radioactive material was associated with α_{2u}-g. No *d*-limonene or *d*-limonene metabolite coeluted with female rat kidney proteins.

In the 3,5,5-trimethylhexanoyloxybenzene sulfonate study (*103*), distribution of the chemical was examined in mice and rats of both sexes. The male rat kidney contained roughly 10 times the concentration of chemical as the female rat kidney, and the concentrations in mouse kidney were even lower than those in the female rat.

Binding of CIGA to Other Macromolecules. Reversible binding generally implies a dissociable chemical–protein interaction in which the free chemical can be liberated from the protein without producing molecular damage. In contrast, in covalent binding a reactive chemical species, usually an electrophile, reacts with nucleophilic centers in target molecules comprising enzymes, other proteins, nucleic acids, or lipids. CIGA appear to dif-

fer from many known chemical toxins, nephrotoxins included, that bind covalently and irreversibly to proteins and/or DNA and through this process cause cellular injury.

A DNA binding study with F344 rats and B6C3F$_1$ mice of both sexes was performed using [1,3,5,-^{14}C]-isophorone (*104*). Twenty-four hours after the animals were administered a 500-mg dose by gavage, liver and kidneys were processed for determination of DNA binding. Neither isophorone nor its metabolites showed covalent binding to DNA. In addition, metabolically formed degradation products were not incorporated into the DNA by *de novo* synthesis of DNA from labeled fragments of the xenobiotic.

In contrast to 1,4-DCB, which is a CIGA, the closely related isomer 1,2-dichlorobenzene (1,2-DCB) does not induce hyaline droplets and appears to bind covalently to proteins in the male rat liver, plasma, and kidney (*102*). When administered orally to male rats, 1,4-DCB (and its metabolite 2,5-dichlorophenol) in the kidney cytosol eluted as a single peak in the low molecular weight fraction containing α_{2u}-g. Dialysis of the kidney cytosol with SDS led to a substantial loss of 1,4-DCB, demonstrating the reversible nature of the CIGA-protein binding. 1,2-DCB bound to low molecular weight proteins in the kidney cytosol of male rats, but it also bound to proteins in the 64,000–70,000-dalton range. Dialysis of the kidney cytosol with SDS failed to remove approximately half the 1,2-DCB, suggesting substantial covalent binding of this chemical in the male rat kidney.

Specificity of the Interaction with α_{2u}-Globulin. The capacity of CIGA to serve as ligands for other lipocalins, some of which are found in humans, has been investigated. Preliminary studies designed to determine the accumulating protein in the kidney of male rats exposed to decalin used two-dimensional gel electrophoresis of rat kidney homogenate (*5*). Although other proteins in the lipocalin superfamily are present in the male rat, decalin was associated solely with α_{2u}-g. Other preliminary studies involving the *in vitro* binding of TMPOH to lipocalins suggest that TMPOH, which binds reversibly to α_{2u}-g *in vitro*, may also bind reversibly to three other members of the superfamily, i.e., retinol-binding protein, α_1-acid glycoprotein, and β-lactoglobulin (*105*). TMPOH did not bind to β_2-microglobulin or lysozyme, low molecular weight proteins that are not members of the superfamily. *d*-Limonene-1,2-oxide also does not appear to bind to α_1-acid glycoprotein or urine protein 1 in *in vitro* studies (L. D. Lehman-McKeeman, personal communication).

Gas chromatographic analysis in experiments with liver microsomes have shown that mice are able to oxidize *d*-limonene to *cis*-*d*-limonene-1,2-oxide, as in the rat, although some quantitative and qualitative species differences were noted (*91*). However, equilibrium saturation binding studies did not demonstrate any interaction of *d*-limonene or its metabolites with MUP in male or female mice (*82, 91*). These results add further support to the specificity of the interaction between CIGA and α_{2u}-g.

When [^{3}H]retinol was administered to male rats, retinol-derived radioactivity coeluted with the protein fraction in cytosol containing α_{2u}-g. However, retinol did not produce hyaline droplet or α_{2u}-g accumulation (*106*). *In vitro* studies on the binding affinities of retinol and several CIGA for α_{2u}-g show that retinol can compete with CIGA for binding to α_{2u}-g (*107*). These studies suggest that hyaline droplet accumulation may not depend on how strongly a chemical binds to α_{2u}-g, but on whether the chemical causes a conformational change in the protein, which inhibits protein catabolism (*22*).

Binding affinities measured in *in vitro* studies generally have not correlated well with the efficacy of chemicals for causing hyaline droplet accumulation. Other factors affecting the development of hyaline droplet accumulation are the concentration of the CIGA-protein complex in the tubule lumen, the rate of breakdown of CIGA-protein complexes in the tubule cells, the death of cells resulting from abnormal accumulation of hyaline droplets, and the subsequent appearance of cell debris in the lumen of tubule cells. These factors are discussed in the following sections.

Catabolism of α_{2u}-Globulin Complexed with CIGA

Lysosomal degradation of α_{2u}-g bound to CIGA has been studied by measuring the digestion rate of the protein recovered from treated male rat kidney (*84*) or of purified urine-derived protein conjugated with CIGA *in vitro* (*91*). Charbonneau et al. (*84*) found that both a mixture of standard protease enzymes of nonrat origin or proteinase K digested α_{2u}-g from rats treated with TMP at a much slower rate than α_{2u}-g from untreated rats.

Using an *in vitro* incubation system with renal cortex lysosomes prepared from male rats, Lehman-McKeeman et al. (*91*) demonstrated that the reversible binding of *d*-limonene, 1,4-DCB, isophorone, or their metabolites impaired the degradation of α_{2u}-g by one-third. Under the experimental conditions used, this was equivalent to an extension of the apparent half-life of α_{2u}-g from 6.67 to 10 hr. The study is particularly interesting in showing that reversible binding of a CIGA to α_{2u}-g does not necessarily alter the rate of protein degradation but that this may be a function of a metabolite. Thus, d-limonene and 1,4-DCB did not impair hydrolysis of α_{2u}-g, but their respective bound metabolites, *d*-limonene-1,2-oxide and 2,5-dichlorophenol, did. With isophorone, however, it was the parent compound alone that produced the effect. This could explain why other chemicals like retinol have been shown to bind to α_{2u}-g but have not produced hyaline droplet accumulation *in vivo*.

Administration of leupeptin (an inhibitor of the lysosomal peptidase cathepsin B) to male rats caused a rapid α_{2u}-g accumulation in the kidney, indistinguishable from that induced by TMP and gasoline (*108*). These various observations provide evidence that CIGA-induced hyaline droplet accumulation may result from a reduced protein degradation rate either by making the protein harder to digest or by inhibiting enzymatic components of the proteolytic process. Studies by Charbonneau et al. (*98*) and Lehman-McKeeman et al. (*32*) favor the former by indicating that the TMP metabolite–protein complex is more resistant to hydrolysis than free α_{2u}-g. Furthermore, Murty et al. (*41*) found that unleaded gasoline was not associated with a reduction, but rather an increase, in rat kidney lysosomal proteolytic enzyme activity.

Structure–Activity Relationships for CIGA

The ability to predict those chemicals that will induce accumulation of α_{2u}-g in the male rat through structural relationships would clearly be advantageous. The fact that relatively minor metabolites such as *d*-limonene-1,2-epoxide can account

for the majority of the association with α_{2u}-g, however, restricts the present utility of structure–activity calculations as a predictive tool. Nevertheless, some associations have been observed. Lehman-McKeeman et al. (*32*) noted that retarded degradation of α_{2u}-g correlates with the presence on the active CIGA or metabolite of an oxygen function of one type or another, i.e., a hydroxyl group for TMPOH and 2,5-dichlorophenol, an epoxide for *d*-limonene-1,2-oxide, and a ketone function for isophorone.

Another recent study employed a quantitative approach to determine the structural features necessary to induce excessive hyaline droplet activity in male rats (*109*). Based on data for a number of light hydrocarbons, Bomhard et al. (*109*) surmised that an *n*-octanol/water partition coefficient above 3.5 and the presence of an isopentyl structural moiety are associated with increased hyaline droplet formation in male rats. A binding-site model for aliphatics was derived from this information. The model was then generalized to include cycloaliphatics by substituting the requirement for an isopentyl structure with a requirement for the presence of at least one tertiary carbon atom. Using this binding-site model, Bomhard et al. predicted the hyaline-droplet inducing activity of 18 previously untested hydrocarbons. These chemicals were then tested for their

Table 3. Substances that induced hyaline droplet accumulation and/or elevated levels of α_{2u}-globulin in renal proximal tubules of rats.

	Evidence for exacerbation of hyaline droplets in renal proximal tubule cells			Evidence for increased renal α_{2u}-globulin levels		
Substance/chemical	Males	Females	References	Males	Females	References
Unleaded gasoline	+	–	(*3,41,110,111*)	+	NR	(*111,112*)
2,2,4-Trimethylpentane	+	–	(*113,114*)	+	–	(*98,114,115*)
JP-4 jet fuel (mixed distillate hydrocarbons)	+	–	(*7,8*)	NR	NR	
JP-5 jet fuel (mixed distillate hydrocarbons)	+	–	(*7,8,116,117*)	NR	NR	
Diesel fuel, marine	+	–	(*8,188*)	NR	NR	
JP-10 synthetic jet fuel (exohexahydro-4,7-methanoindan)	+	NR	(*7,119*)	NR	NR	
RJ-5 synthetic jet fuel (hydrogenated dimers of norbornadiene)	+	–	(*7*)	NR	NR	
JP-7 distillate jet fuel	+	–	(*21*; Bruner, personal communication)	NR	NR	
JP-TS distillate jet fuel	+	–	(*21*; Bruner, personal communication)	NR	NR	
Stoddard solvent	+	–	(*4*)	NR	NR	
C_{10}–C_{11} and C_{10}–C_{12} isoparaffinic solvents (saturated aliphatic hydrocarbons)	+	–	(*4,120*)	+	–	(*120*)
Decalin	+	–	(*5,6,8,10,121,122*)	+	–	(*5,10,123*)
Tetralin	+	NR	(*124*)	NR	NR	
d-Limonene	+	–	(*6,14,100,125,126*)	+	–	(*100,126,127*)
Pentachlorobenzene	+	–	(*128*)	NR	NR	
1,2,4,5-Tetrachlorobenzene	+	–	(*129*)	NR	NR	
1,4-Dichlorobenzene	+	–	(*102,130,131*)	+	NR	(*102*)
Tetrachloroethylene (Perchloroethylene)	+	–	(*23,37*)	+	–	(*37*)
Pentachloroethane	+	–	(*37*)	+	–	(*37*)
Hexachloroethane	+	–	(*132*)	NR	NR	
Isophorone	+	NR	(*101*)	+	NR	(*101*)
Lindane	+	–	(*133*)	+	–	(*133*)
Dimethyl methylphosphonate	+	–	(*12*)	NR	NR	
Methyl isobutyl ketone	+	–	(*16*)	NR	NR	
Methyl isoamyl ketone	+	–	(*134*)	NR	NR	
Diisobutyl ketone	+	–	(*135*)	NR	NR	
540C (3-methylamino-1-(3-trifluoromethylphenyl)-2-pyrazoline)	+	–	(*136*)	+	NR	(*136*)
BW58C (mixture of isomeric *cis* and *trans* forms of 2-4′-*t*-butylcyclohexyl)-3-hydroxy-1-4-naphthoquinone)	+	–	(*136*)	+	NR	(*136*)
Levamisole (*levo* isomer of 2,3,5,6-tetrahydro-6-phenylimidazo-(2,1-*b*) thiazole)	+	–	(*136*)	+	NR	(*136*)
Gabapentin	+	–	(*137*)	+	–	(*137*)
3,5,5-Trimethylhexanoic acid derivatives	+	–	(*103*)	+	–	(*103*)
Tridecyl acetate	+	–	(*138*)	NR	NR	
Isopropylcyclohexane	+	NR	(*139*)	NR	NR	
1,3,6-Tricyanohexane	+	–	(*140,141*)	NR	NR	

NR, not reported.

ability to induce hyaline droplet accumulation in adult male Wistar rats. Although the binding-site model was based on the structure of the parent compound and did not allow for active metabolites, the results in the rats were described as being in good agreement with the predictions.

In an extension of the work of Lock (*99*), described earlier, Borghoff et al. (*107*) determined the apparent binding affinity to α_{2u}-g for a number of chemicals associated with α_{2u}-g nephropathy and measured their ability to compete with TMPOH. Using molecular modeling and information on the most active compounds, Borghoff et al. (*22*) concluded that the presence of an electronegative atom for hydrogen bonding is a critical factor in determining binding affinity. Lipophilicity also seemed crucial for hydrophobic interactions, but the presence of an electronegative atom was necessary for greater activity. Steric volume was also considered to play an essential role in binding activity.

α_{2u}-Globulin Nephropathy

Substances reported to induce increased formation of hyaline droplets in proximal tubule cells of male rats are listed in Table 3, along with available information on whether the accumulating protein is α_{2u}-g. The nephrotoxicity that can ensue from hyaline droplet accumulation is novel because it is associated with excessive α_{2u}-g accumulation. This α_{2u}-g accumulation is believed to initiate a sequence of events resulting in chronic proliferation of tubule epithelium, as well as an exacerbation of CPN. Because α_{2u}-g is a male rat-specific protein, nephropathy induced by accumulation of α_{2u}-g would not be expected to occur in female rats, mice of either sex, or other species.

The proposed sequence of histopathological changes is based mainly on research studies with four model substances, unleaded gasoline and TMP (*142–144*), decalin (*5,6,122,123,145*), and *d*-limonene (*123,126*). Even for these four substances, not all of the individual lesions in the proposed progression have been shown to belong to a sequence of interrelated events. Specific information pertaining to lesion nature and sequence is lacking for many of the CIGA listed in Table 3.

Much of the information useful for defining the pathologic sequelae to α_{2u}-g accumulation does not necessitate chronic exposure. In fact, the nephrotoxicity associated with accumulation of α_{2u}-g might be influenced by age because α_{2u}-g levels decline in aging male rats (*41*). Certainly, the age-related progression of CPN obscures the lesions directly related to CIGA administration, making evaluation of the chronic sequence of lesions especially difficult.

Pathologic Features of α_{2u}-Globulin Nephropathy

The first morphological manifestation of α_{2u}-g nephropathy is the rapid accumulation in proximal tubule cells of hyaline droplets (Fig. 1), which develop within 24 hr of dosing with some compounds (*126*).

The droplets stain positively with Mallory's Heidenhain stain (Fig. 2) but are negative for periodic acid-Schiff, indicating their protein composition (*5*). Mallory's Heidenhain stain is therefore more useful than conventional hematoxylin and eosin (H&E) for visualizing and quantitating the droplets. As they represent lysosome-derived entities, the droplets are strongly autofluorescent (yellow) in paraffin sections under ultraviolet illumination

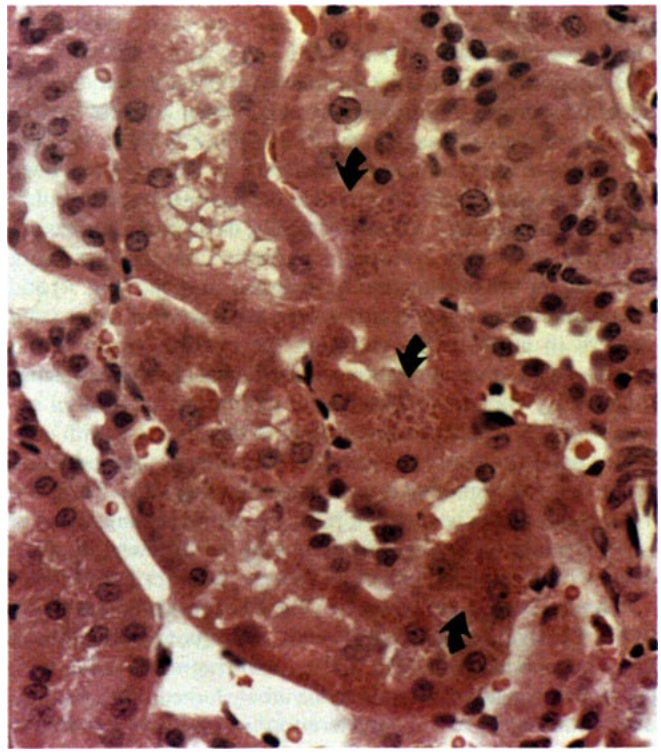

FIGURE 1. Accumulation of hyaline droplets (arrows) within convoluted proximal tubule cells following administration of *d*-limonene. H&E, 480×.

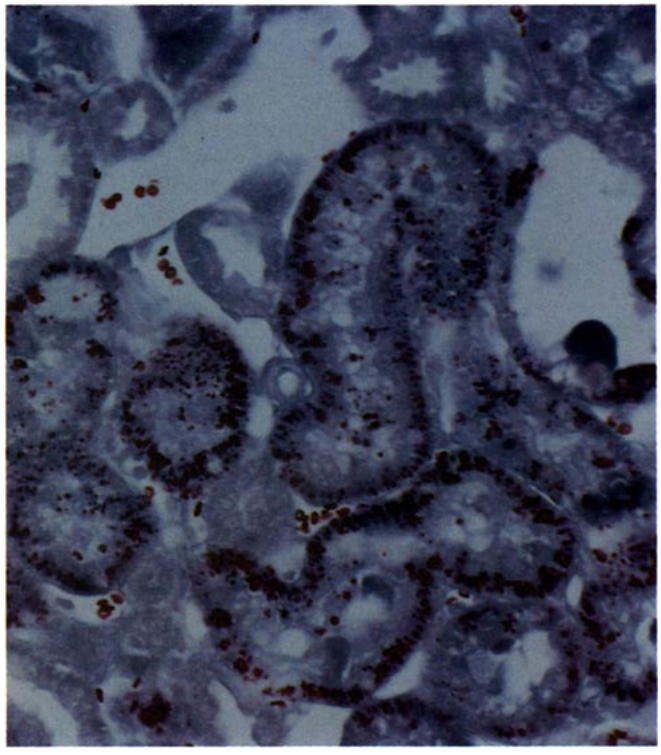

FIGURE 2. Enhanced visualization of hyaline droplet accumulation within proximal tubule cells following administration of *d*-limonene with Mallory's Heidenhain stain, 375×.

(G. C. Hard, unpublished observations). In plastic-embedded tissue, hyaline droplets can be visualized easily with Lee's methylene blue basic fuchsin (*142*).

Excessive hyaline droplet formation occurs primarily in cells of the P2 segment, but small increases in the number of hyaline droplets may also be seen in the P1 and P3 cells (*143*). By light microscopic immunohistochemistry (Fig. 3), α_{2u}-g has been clearly and specifically localized to the hyaline droplets within proximal tubules (*42*). Ultrastructurally, the hyaline droplets are enlarged secondary lysosomes partially composed of α_{2u}-g (*146*). Many are polyangular or irregular in shape (Fig. 4) and contain a condensed crystalline core suggestive of aggregated protein in pure form. Although the hyaline droplets and associated α_{2u}-g accumulation persist during chronic exposure, the severity appears to lessen with increasing duration of exposure (*144*). This apparent waning of the response with continued exposure could be related to declining α_{2u}-g production by the male rat beginning at some stage after 5 months (*71,78,79*). With continued exposure to CIGA, the initial accumulation of α_{2u}-g-containing hyaline droplets may be followed by a sequence of interrelated pathologic events, as described below.

Scattered single-cell necrosis (Fig. 5) occurs predominantly in the P2 segment cells (*143*), with subsequent exfoliation of these degenerate cells (Fig. 6) and cell fragments laden with crystalloid phagolysosomes into the tubule lumen. With decalin, a minimal degree of cell degeneration/necrosis was reported to be present in the proximal convoluted tubules after 5 days of exposure, becoming maximal at 19 days, but reverting to the minimal level after 31 days of exposure (*6*). Scattered exfoliation of droplet-affected cells was observed with up to 48 weeks of ex-

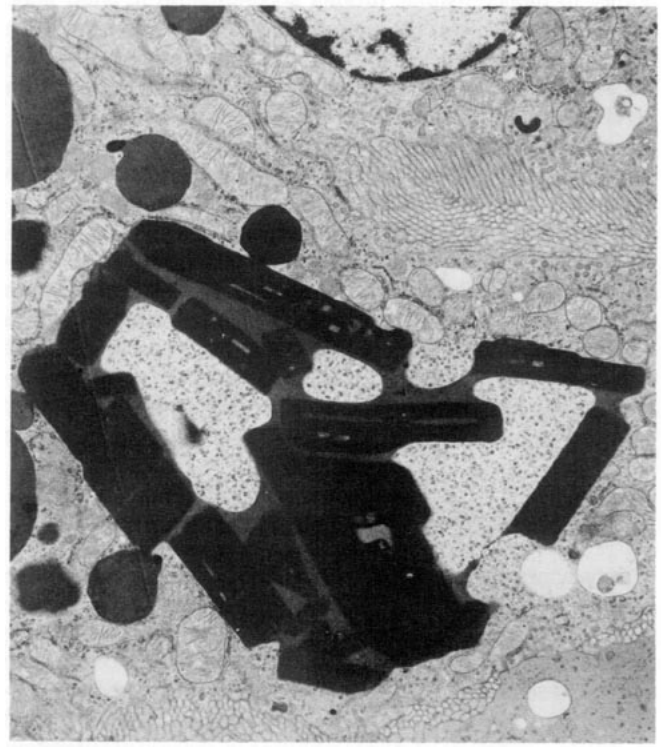

FIGURE 4. Electron micrograph of enlarged polyangular phagolysosomes induced in P2 cell by TMP, 4950×. (Photograph contributed by John Foster, ICI.)

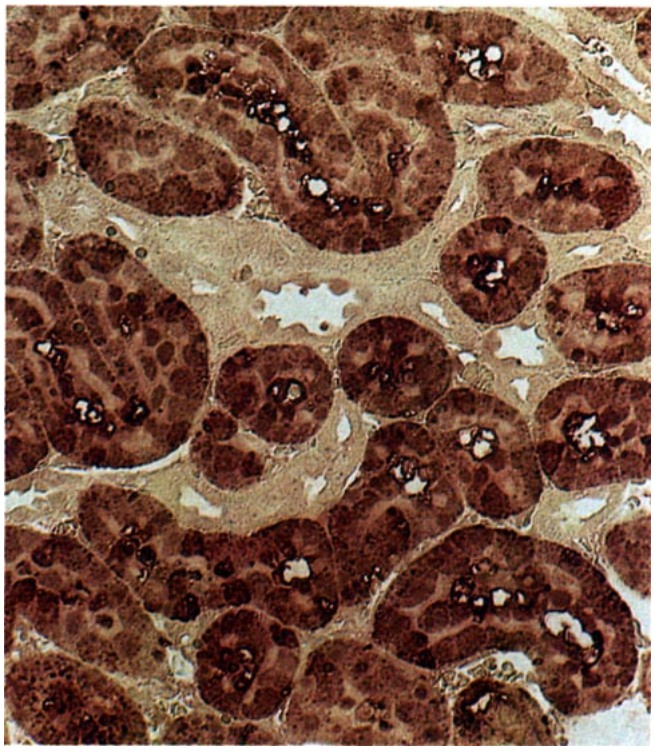

FIGURE 3. Positive immunohistochemical staining of hyaline droplets in proximal tubule cells for α_{2u}-globulin. Indirect alkaline phosphatase method with fast red as substrate, 375×.

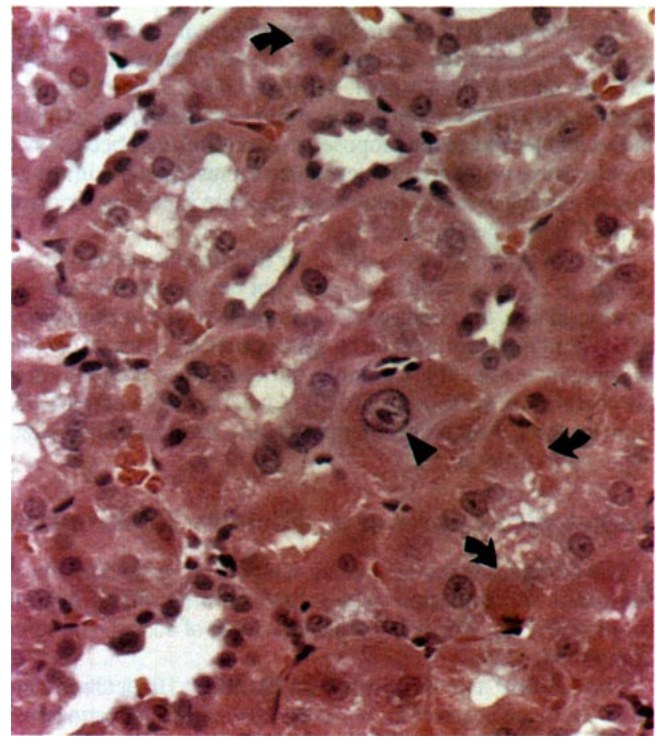

FIGURE 5. Epithelial cells undergoing degenerative change (arrows) in the lining of droplet-laden P2 tubules. A tubule near the center shows karyomegaly (arrowhead). This example was induced by 6 months of administration of *d*-limonene. H&E, 480×.

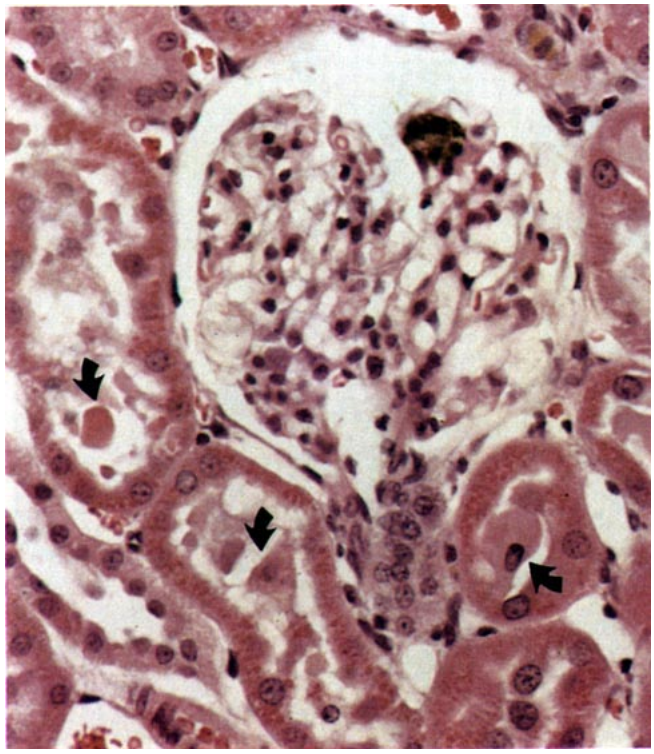

FIGURE 6. Exfoliation of degenerate epithelial cells (arrows) into the P2 tubule lumen after *d*-limonene administration. H&E, 480×.

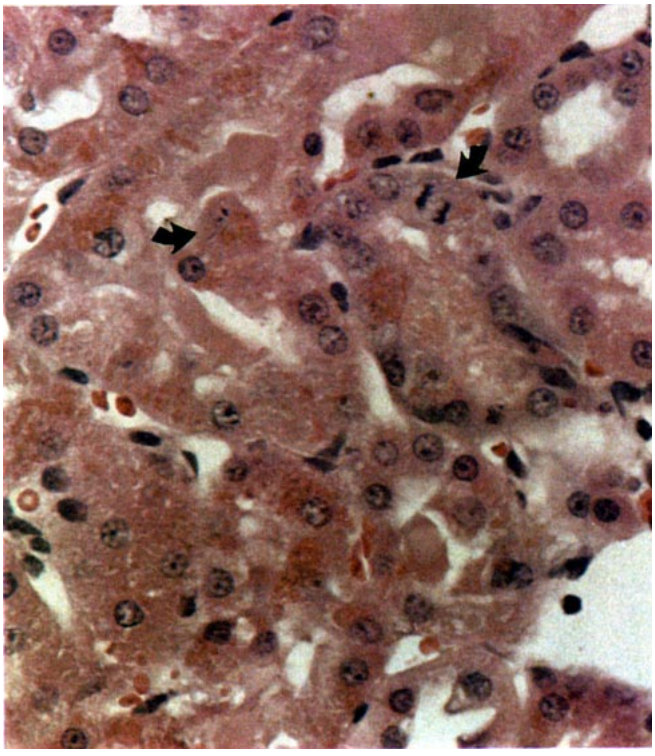

FIGURE 7. P2 tubule cells (arrows) undergoing mitosis after *d*-limonene administration. H&E, 600×.

posure to unleaded gasoline or TMP (*144*), indicating sustained single cell loss while exposure to CIGA continues.

Epithelial cell proliferation primarily involving the P2 segment occurs as a regenerative response to cell damage and loss. This can be seen as increased numbers of mitotic figures (Fig. 7) or demonstrated by labeling techniques for DNA synthesis. Increased proliferative activity has been recorded after only 3 weeks of petroleum hydrocarbon exposure (*143*), but it persisted during 48 weeks of chronic exposure (*144*).

Granular casts (Fig. 8) composed of sloughed cell debris (Fig. 9) accumulate at the junction between the P3 segment of the proximal tubule and the descending thin loop of Henle, that is, at the junction between the inner and outer stripes of outer medulla (Fig. 8), with consequent tubule dilation at this part of the nephron (*5*). This can occur as early as 2–3 weeks after initial exposure (*5,6*). As well as comprising recognizable cell debris, the granular casts stain positively for α_{2u}-g (J. R. Foster, personal communication), indicating probable derivation of the debris from cells that had accumulated this protein. Granular cast formation appears to be associated with higher doses of compound rather than with the lowest doses that can induce increased hyaline droplet accumulation. An absence of casts after treatment might therefore reflect a dose-related decrease in the severity of cell necrosis and exfoliation (*142,143*).

At chronic time points, linear mineralization develops in the renal papilla (Fig. 10), outlining affected medullary tubules, along with hyperplasia of the pelvic epithelial lining (urothelium)

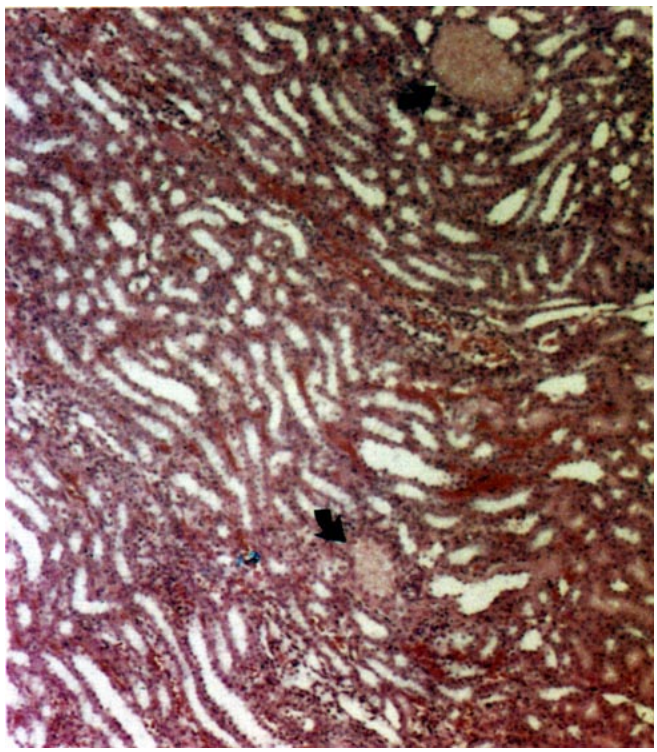

FIGURE 8. Granular cast formation within dilated tubules (arrows) at the junction of the inner and outer stripes of the outer medulla at 6 months of *d*-limonene administration. H&E, 95×.

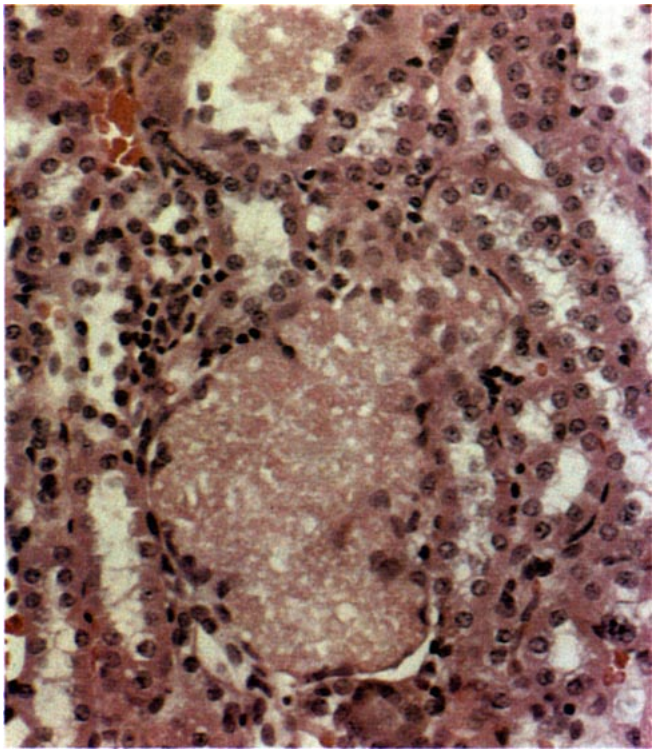

FIGURE 9. Higher power of *d*-limonene-induced corticomedullary cast showing granular nature of the contents derived from cell debris. 400×.

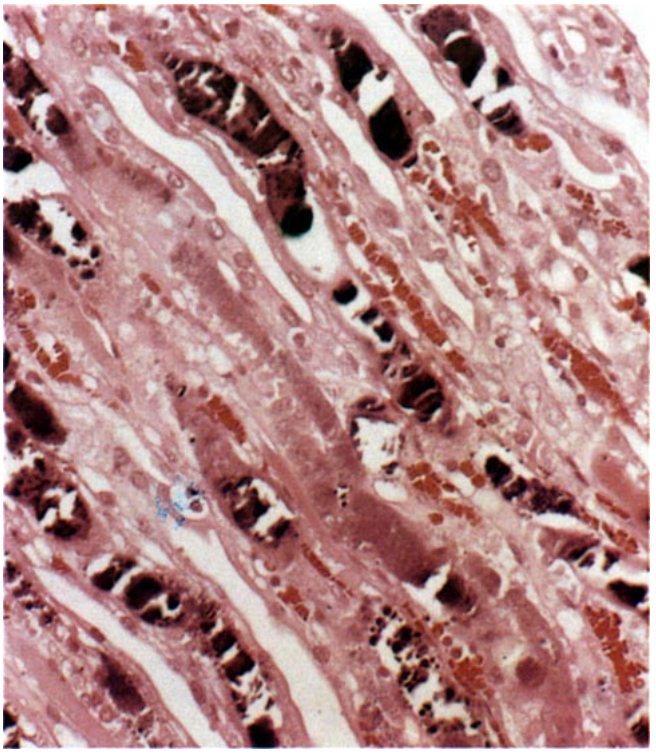

FIGURE 11. Higher power of JP-4 jet fuel-induced papillary mineralization showing involvement of Henle limbs but not collecting ducts. H&E, 375×.

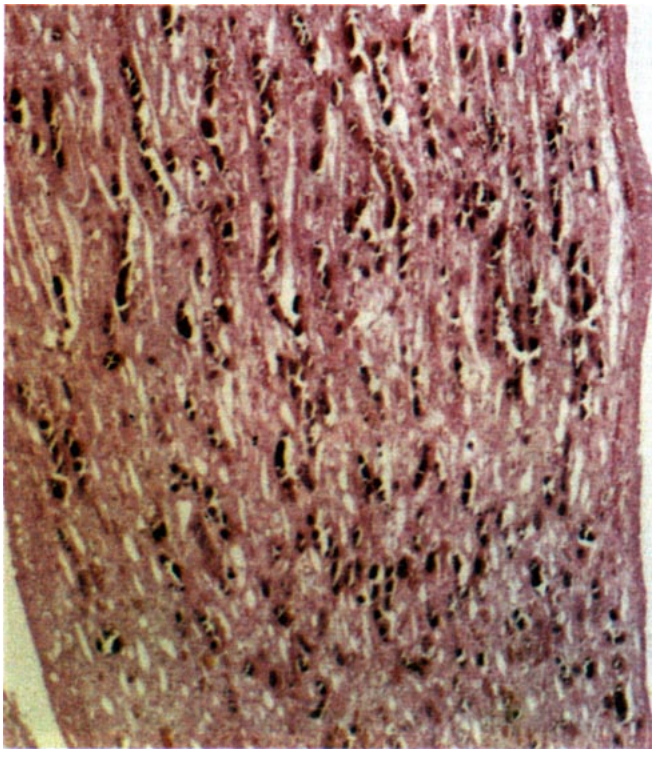

FIGURE 10. Linear mineralization of renal papilla following 1 year of JP-4 jet fuel administration. H&E, 95×.

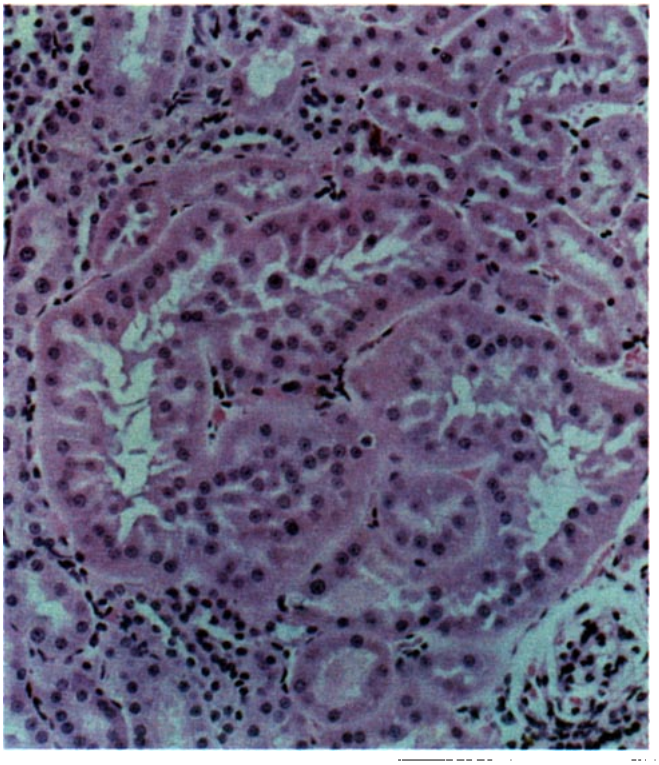

FIGURE 12. Focal hyperplasia of convoluted proximal tubule induced by *d*-limonene after approximately 1 year. H&E, 275×.

(*21*). With unleaded gasoline, this lesion was first observed at 6 months of exposure (*11*). The mineralization appears to form within the loops of Henle (Fig. 11) and has been identified as calcium hydroxyapatite (*20*). The relationship between papillary mineralization and the proximal tubule lesion remains undetermined, but the medullary lesion is presumed to represent mineralized remnants of debris from disintegrating granular casts that lodge in the prebend segments of Henle's loop (*8,21*). In turn, urothelial hyperplasia, which mainly affects the surface of the renal papilla (Fig. 12), may be a response of the renal pelvis lining to papillary mineralization (*8,147*).

Rat Urine Chemistry and CIGA

Several studies have examined renal function in rats treated with CIGA and subsequently developing α_{2u}-g nephropathy. Two days of treatment with TMP resulted in mild urinary increase in the lysosomal enzyme *N*-acetyl-β-glucosaminidase (NAG) and alkaline phosphatase, a decrease in creatinine, and mild increase in urinary cell debris. Other parameters (aspartate amino transferase [AAT], urine osmolality and volume) were not affected (*148*). A single, oral dose of TMP had no effect on renal function (*114*). In a 14-day study with decalin, of six urinary enzymes tested, only AAT, lactate dehydrogenase, and NAG were altered (increases) at days 21 and/or 28 (*149*). Similar results were obtained for levamisole except that AAT remained normal (*150*). During prolonged treatment with C_{10}-C_{11} isoparaffinic solvent for up to 8 weeks, the only urinary changes observed were mild elevation of glucose and albumin, slightly decreased concentrating power and osmolality, and epithelial cell debris in the urine. There was no alteration in urinary β_2-microglobulin content (*151*).

Taken together, these studies suggest that CIGA produce minimal changes in urinary chemistry and very little or no glomerular dysfunction or damage. The minor alterations seen in urine composition in the days following administration of CIGA suggest also that hyaline droplet accumulation is not related to increased passage of serum proteins by the glomerulus. The mild tubule toxicity of CIGA identified by clinical chemistry is a characteristic of CIGA that contrasts with the obvious urinary changes associated with nephrotoxicity induced by such classical renal toxins as mercuric chloride, hexachlorobutadiene, aminoglycosides, and papillotoxic agents (*152*).

Species Variation in the Renal Response to CIGA

The male-specific effects of CIGA have been demonstrated over a range of rat strains including Fischer 344, Sprague-Dawley, Buffalo, and Brown Norway rats (*153*). Hyaline droplet accumulation or the spectrum of lesions comprising α_{2u}-g nephropathy have not been observed in female rats or mice of either sex following treatment with CIGA (*5,19*). In addition to these studies, CIGA have been tested for toxicity in hamsters (jet fuels), guinea pigs (decalin), dogs (decalin, jet fuels, *d*-limonene and methyl isobutyl ketone), and monkeys (gasoline and methyl isobutyl ketone). No renal pathology was demonstrated in these species at CIGA doses known to cause nephropathy in male rats (*5,7,15,16,154,155*), except for one report of minor changes in dogs treated for 6 months with *d*-limonene (*156*). In this chronic study, an increased incidence of proteinaceous casts was observed in male and female beagles, but no tubule epithelium changes, tubule lumen dilation, or mineralization. However, Webb et al. (*15*) were unable to demonstrate any renal pathology in dogs after 6 months of treatment at comparable dose levels. The highest dose tested in the dogs, 1.2 mL/kg (*15,156*), is more than 10 times the doses that have caused frank nephropathy in male rats.

Knowledge concerning the renal effects of CIGA in humans is hampered by the lack of data on specific chemicals in this category, and the limitations imposed by a multiplicity of types of occupational and nonoccupational exposures. Case studies have reported a link between chronic renal disease with gasoline, solvents, and jet and diesel fuels including rare cases of acute tubular necrosis (proximal and distal tubule epithelium) following severe exposure to petroleum distillates (e.g., *157,158*). Case reports cannot be used to establish a causal relationship but may serve to initiate formal epidemiologic investigation (*159*).

Epidemiologic studies concerning non-neoplastic kidney disease and occupational exposure to hydrocarbons and solvents have been conducted only since 1975 [reviewed by Askergren (*160*), Daniell et al. (*161*), and Phillips et al. (*162*)]. A majority of these studies have indicated an association between glomerulonephritis and exposure to hydrocarbons, especially organic solvents or gasoline. Some have suggested a positive association between the presence of glomerular disease and duration and severity of occupational exposure to hydrocarbon solvents, including tetrachloroethylene, which is a CIGA in male rats (*163*). However, many of the earlier studies are considered to be methodologically limited (*159,160,162*). Their major shortcomings have been heterogeneous case definition, use of inappropriate control groups or nonblinded interviewers, and failure to consider recall bias or to adequately define hydrocarbon exposure (*162*).

More recently, Steenland et al. (*164*), investigating specific occupational exposures associated with end-stage renal disease in male workers, found elevated risks for solvents used as cleaning agents or degreasers [odds ratio (OR) 2.5; 95% confidence interval (CI) 1.56–3.95] but not for exposure to gasoline and diesel fuel (OR 0.98; 95% CI 0.49–1.06) or motor and fuel oil (OR 1.13; 95% CI 0.69–1.84). Harrington et al. (*165*) found no association (OR 1.0; 95% CI 0.16–6.3) between occupational exposure to inorganic solvents and glomerulonephritis, but the authors also concluded that the statistical power of this case-referent study was not sufficent to detect other than large risk estimates.

The glomerulonephritis reported in the positive epidemiologic studies has involved thickening of glomerular basement membranes or deposition of antibodies against glomerular basement membrane, a mild degree of albuminuria, and sometimes tubule atrophy and tubular basement membrane thickening (*162,163*).

Other indicators of renal function have also been assessed in epidemiologic studies. Levamisole, a drug used as an anthelmintic, in cancer chemotherapy, and in the treatment of rheumatoid arthritis in humans, falls into the CIGA category because it induces both hyaline droplet and α_{2u}-g accumulation in male rats (*136*). Based on an absence of elevated levels of urinary NAG in patients receiving 150 mg levamisole per day for 26 weeks, there is little evidence to indicate that this compound is nephrotoxic in humans (*166*). In addition, no positive association between urinary NAG and acute or chronic exposure was noted in a prevalence study of 180 dry-cleaning workers exposed to

tetrachloroethylene (*167*). Because urinary NAG is only slightly elevated in male rats exposed to CIGA, however, urine chemistry may not be a good biological monitor of the type of nephrotoxicity associated with CIGA.

In a study of 16 females exposed to tetrachloroethylene from their employment in dry-cleaning shops for an average of 11 years (range 1–25 years), Vyskočil et al. (*168*) found no evidence of renal damage except for an increase in lysozyme in the urine. No statistically significant increase in urinary excretion of β_2-microglobulin, lactate dehydrogenase, or glucose, which are other markers of tubular dysfunction, were noted. The authors believed these latter findings, in addition to the lack of correlation between intensity of exposure and change in biochemical parameters, supports the conclusion that renal damage is not associated with tetrachloroethylene exposure.

The evidence regarding renal injury in humans from chronic organic chemical exposure is inadequate to demonstrate whether CIGA exposure can affect the human renal tubule cell. Existing reports imply that, if the association is real, it is the glomerulus that is pathologically involved. However, this may simply reflect study designs that concentrated on clinical detection of glomerular effects. Because the injury to the rat tubule cells is relatively mild, insensitive tests, such as urine chemistry, which are generally used for evaluating humans, might be inadequate to detect changes.

Factors Affecting the Expression of α_{2u}-Globulin Nephropathy

Various conditions, including age, hormone manipulation, and genetics, have the potential for altering the expression of CIGA-induced α_{2u}-g nephropathy. Experimental studies have investigated the influence of these factors on CIGA nephrotoxicity as well as determining the effects of α_{2u}-g in female rats.

Age-Related Effects. As discussed earlier, the hepatic synthesis and urinary excretion of α_{2u}-g in the male rat are highly age-dependent, with prepubertal and aged animals showing negligible amounts of this protein (*71,79,80*). Accordingly, administration of either decalin to immature male rats (*5*) or unleaded gasoline to 26-month-old male rats (*41*) failed to produce renal cortical α_{2u}-g accumulation or an increase in hyaline droplets.

Effect of Hormone Manipulation. As α_{2u}-g synthesis is primarily under androgenic control, the effects of castration, which depresses hepatic synthesis of α_{2u}-g (*67*), were explored by Hobson et al. (*169*) using TMP. Although a significant increase in hyaline droplet formation was observed in both castrated and uncastrated male F344 rats exposed to a single oral dose of TMP, the severity of the lesion was less in the former. Thus, castration diminished but did not abolish the TMP-induced nephrotoxicity.

Estrogen is known to inhibit the hepatic synthesis of α_{2u}-g in the rat (*170*). This factor was used by Garg and co-workers (*111*) to study the influence of inhibition of new synthesis of α_{2u}-g by estradiol on recovery from CIGA-induced nephropathy. Commencing treatment on the ninth and final day of unleaded gasoline exposure, estradiol reduced renal cortical α_{2u}-g content by 25, 41, and 52% on days 3, 6, and 9 after exposure, respectively, compared to rats receiving no hormone treatment. At the same time, hyaline droplet removal appeared to be accelerated in rats treated conjointly with hormone. Hyaline droplet number and size (qualitative observations) in hormone-treated rats approached control levels at 3 days after exposure, compared with up to 9 days for complete resolution in unleaded gasoline-exposed rats not receiving estradiol.

In a subsequent study, Garg et al. (*171*) demonstrated that pretreatment of mature male rats with SC injections of estradiol for 10 days before gasoline exposure completely inhibited the renal accumulation of α_{2u}-g and hyaline droplets normally induced by gasoline.

Genetic Variants. The NBR rat has no detectable levels of hepatic α_{2u}-g mRNA in either sex and therefore is unable to synthesize α_{2u}-g in the liver, although high constitutive levels of the mRNA are present in the preputial gland (*68*). The NBR rat is capable of developing chemically induced nephropathies, but under exposure conditions that produce α_{2u}-g nephropathy in Fischer 344 rats, d-limonene, TMP, isophorone, and 1,4-DCB did not induce any detectable α_{2u}-g accumulation, hyaline droplets, or other lesions in the male NBR rat (*172*). Identical results were obtained for decalin (*153*) and lindane (*133*).

α_{2u}-Globulin Infusion in Female Rats. Ridder et al. (*153*) administered α_{2u}-g (purified from mature male rat urine) IP at hourly intervals to decalin-treated female Sprague-Dawley rats for a total of eight injections and examined kidney samples for hyaline droplets and α_{2u}-g 1 hr after the last protein injection (9 hr after decalin treatment). Although droplet formation was not evident in kidney sections stained with Mallory's Heidenhain from the α_{2u}-g-infused female rats, hyaline droplet and α_{2u}-g accumulation were clearly demonstrated in females exposed to both hydrocarbon and male urinary protein. By means of two-dimensional gel electrophoresis, the investigators showed slight, but apparent, renal cortical accumulation of α_{2u}-g in the infused females. Accumulation of the protein greatly increased in females that were both infused with α_{2u}-g and decalin-treated. These various studies indicate a direct dependence of CIGA-induced renal lesion expression on the presence of α_{2u}-g.

Chronic Progressive Nephropathy

Rats are particularly predisposed to an age-related spontaneous nephropathy, CPN, that is more severe in males than in females and that affects certain strains more than others. CPN is more common in Sprague-Dawley and Fischer 344 rats than the Wistar strain (*173*) and it is also common in the Osborne-Mendel rat (*174*). The etiology of CPN is not known, but the severity of the syndrome is influenced by a number of factors, particularly dietary manipulation affecting protein content or caloric intake (*175*).

Exacerbated CPN, involving enhanced severity and earlier onset of the disease, is generally observed after chronic administration of CIGA to male rats (*20*). It has been stated that exacerbated CPN is one component (together with hyaline droplet accumulation and granular cast formation) of a triad of lesions that specifies the nephropathic response to CIGA (*6,126*). Exacerbated CPN is usually recognized after months of continuous treatment (*20,144*) although Alden et al. (*5*) reported early signs after 2–3 weeks of decalin treatment. These authors (*5*) consider that exacerbated CPN develops as a tertiary response to nephron obstruction caused by the CIGA-induced granular casts.

Table 4. Summary of the histopathology of spontaneous chronic progressive nephropathy of aging rats.

Thickening of tubular and glomerular basement membranes
Basophilic segments of proximal convoluted tubules with sporadic mitoses indicative of tubule cell proliferation
Tubular hyaline casts of proteinaceous material originating in the more distal portion of the nephron, mainly in the medulla, and later plugging a considerable length of the tubule
Focal interstitial aggregations of mononuclear inflammatory cells within areas of affected tubules
Glomerular hyalinization and sclerosis
Interstitial fibrosis and scarring
Tubular atrophy involving segments of proximal tubule
Chronically in advanced cases, occasional hyperplastic foci in affected tubules
In some advanced cases, accumulation of protein droplets in sporadic proximal tubules

The pathologic features of CPN (listed in Table 4) include certain lesions that are also found in α_{2u}-g nephropathy as well as lesions that are distinctive. Single-cell necrosis, regenerating tubules, and focal hyperplasia of proximal tubule epithelium are common to spontaneous CPN and to α_{2u}-g nephropathy (*18*). CPN is characterized by certain lesions that are not components of α_{2u}-g nephropathy, including conspicuous thickening of tubule and glomerular basement membranes, hyaline casts consisting of homogeneous, proteinaceous material (distinct from granular casts containing cellular debris), interstitial mononuclear cell infiltration, fibrosis, tubule atrophy, and sclerotic glomeruli. Conversely, early and late stages of α_{2u}-g nephropathy exhibit a number of characteristics not associated with CPN such as hyaline droplet accumulation associated with α_{2u}-g in the P2 segment, granular casts at the junction of the inner and outer stripes of the outer medulla, and linear mineralization in the papilla (*20*). In very advanced cases of spontaneous CPN, sporadic tubules may contain excessive numbers of hyaline droplets similar in appearance to those induced by CIGA. However, these do not show immunochemical evidence of α_{2u}-g (G. C. Hard, unpublished observations). The urine and serum chemistry of advanced CPN also differs from α_{2u}-g nephropathy. Albuminuria, hypoalbuminemia, and hypocholesterolemia typify CPN, with increases in serum creatinine and urea nitrogen levels at end-stage disease (*27*).

Renal Toxicity Observed in Chronic Bioassays of Chemicals That Induced Kidney Tumors in Rats

For the purpose of the current review, bioassays were identified and the data examined on seven chemicals tested for chronic toxicity and carcinogenicity by the National Toxicology Program (NTP) or its predecessor at the National Cancer Institute (NCI). All seven produced accumulation of hyaline droplets, nephropathy, and kidney tumors in male rats. The selected chemicals were 1,4-DCB (*130*), dimethyl methylphosphonate (*12*), hexachloroethane (*132,176*), isophorone (*13*), *d*-limonene (*14*), pentachloroethane (*17*), and tetrachloroethylene (*177*). Information was also examined on unleaded gasoline, which was tested by inhalation as a totally vaporized form at International Research and Development Corporation (IRDC) for the American Petroleum Institute (*11,96,155*). Gasoline is a complex blend with CIGA properties. Although extensive acute and subchronic studies have been performed on two other chemicals, decalin and TMP, both of which cause the sequence of nephropathy in male rat kidney beginning with α_{2u}-g accumulation, carcinogenicity bioassay data are not available for these compounds.

A summary of the nonneoplastic and preneoplastic kidney effects observed in male rats after administration of the eight selected substances is presented in Table 5. Nonneoplastic and preneoplastic lesions reported in female rats and mice of both sexes are summarized in Table 6. The data in these two tables were extracted from the *NTP Technical Reports* and other relevant literature.

In male rats, at least one case of renal tubule cell hyperplasia (Fig. 13) was reported in the 2-year bioassays for the seven NTP/NCI compounds; the incidence was generally much higher and dose responsive. Although not reported in the bioassay for the eighth material, unleaded gasoline, this lesion was observed in later research studies (*178*). None of the eight bioassayed substances produced tubule cell hyperplasia in female rats, although this lesion was reported in male mice exposed to tetrachloroethylene. In male rats, renal changes described as "toxic tubular nephropathy" (encompassing degeneration of tubule epithelium, necrosis, epithelial cell regeneration, and cast formation) were seen after administration of all eight of the substances (Table 5). Some aspect of toxic tubular nephropathy

Table 5. Summary of data on non-neoplastic and preneoplastic kidney lesions in male rats associated with eight compounds that induced renal tumors in 2-year bioassays.

	Toxic nephropathy				Mineralization[a]			Hyperplasia	
Chemical	Hyaline droplets	Dose response	Increased severity	Cast formation	Present	Dose response	Karyomegaly	Present	Dose response
1,4-Dichlorobenzene	+	+*	+	+	+	+	NR	+	+
Dimethyl methylphosphonate	+	+	+	+	+	+	NR	+	+
Hexachloroethane	+	+	+	+	+	+	NR	+	+
Isophorone	+	–	+ (slight)	+	NR	NR	NR	+	+
d-Limonene	+	+	+	+	+	+	NR	+	+
Pentachloroethane	+	+	NR	+	+	+	NR	+	NR
Tetrachloroethylene	+	+	NR	+	NR	NR	+	+	+
Unleaded gasoline	+	+	+	+	+	+	+/–	+[b]	NR

NR, not reported.
[a]Localized to renal papilla.
[b]Data from research studies.

Table 6. Summary of data from 2-year bioassays on non-neoplastic and preneoplastic kidney lesions in mice and female rats exposed to eight compounds that induced renal tumors in male rats.

Chemical	Hyaline droplets	Toxic nephropathy	Cast formation	Mineralization	Karyomegaly	Hyperplasia
1,4-Dichlorobenzene	–	+ (Female rats and male mice)	–	+ (Female rats)[a]	NR	–
Dimethyl methylphosphonate	–	–	–	–	NR	–
Hexachloroethane	–	+ (Female rats and mice)	+ (Mice, hyaline)	+ (Mice, Ca deposition)	NR	–
Isophorone	–	–	–	–	NR	–
d-Limonene	–	–	–	–	NR	–
Pentachloroethane	–	–	–	–	NR	–
Tetrachloroethylene	–	NR	+ (Mice)	–	+ (Female rats and mice)[b]	+ (Male mice)[b]
Unleaded gasoline	–	–	–	–	+	–

NR, not reported.
[a]Incidence and severity much higher in male rats.
[b]Highest incidences in male rat.

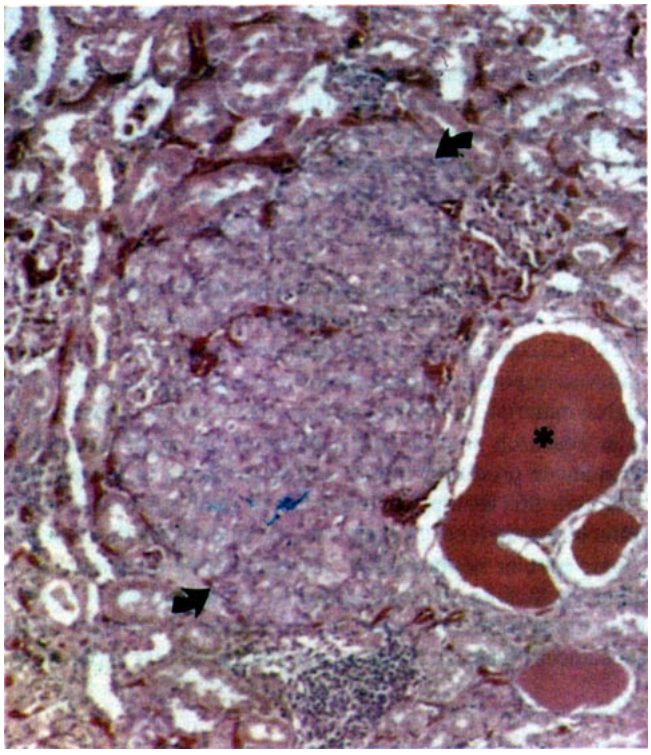

FIGURE 13. Cortically located adenoma (arrows) induced by JP-4 jet fuel after 1 year. Hyaline, proteinaceous casts (*) associated with age-related chronic progressive nephropathy are also present. H&E, 150×.

was also observed in female rats or mice administered hexachloroethane, 1,4-DCB or tetrachloroethylene (Table 6). For example, calcium deposition or mineralization was seen after administration of hexachloroethane to mice or 1,4-DCB to female rats. Cast formation was reported in mice following administration of hexachloroethane and tetrachloroethylene.

Several difficulties arise in the interpretation and utilization of the bioassay-derived data when mouse and female rat lesions are considered. The nature of casts (granular versus hyaline) is not always described, and for mineral deposits, the site (papillary versus corticomedullary) and form (linear versus globular) may not be specified. The range of lesions encompassed by the term "toxic nephropathy" is not always defined, and there is sometimes no clear distinction from CPN. Nevertheless, it appears from the data that female rats and mice do not develop as broad a spectrum of nephrotoxic lesions as those proposed to be associated with α_{2u}-g nephropathy and renal tumor formation in the male rat. Furthermore, where nephrotoxicity was reported in both male and female rats, the males had more lesions, and the female response never demonstrated the characteristics seen in the male response to CIGA. Therefore, the lesions caused by CIGA seem to be both qualitatively and quantitatively different for male rats compared to mice and female rats.

Neoplastic and Preneoplastic Lesions Observed in 2-Year Bioassays on CIGA

Incidence data for renal tubule tumors, tubule hyperplasia, and tumors at nonrenal sites from the 2-year bioassays on the eight selected substances are summarized in Table 7. For tumors occurring at sites other than the renal tubule, only statistically significant incidences are reported. For six of the eight substances, exposure was by gavage; for two, it was by inhalation. The experiments were conducted over approximately a decade, which may account for the lack of standardized terminology in describing lesions as well as differences in attention paid to recording the possible role of α_{2u}-g accumulation in the male rat kidney.

In a separate set of animal bioassays conducted for the military, male rats were exposed 6 hr/day, 5 days/week for 1 year to the synthetic hydrocarbon missile fuels RJ-5 and JP-10. At terminal sacrifice after 2 years, these animals had evidence of nephropathy characteristic of α_{2u}-g accumulation and significant increases in renal tubule tumors (9 in 65 animals exposed to RJ-5 at 150 mg/m^3; 9 in 50 animals exposed to JP-10 at 562 mg/m^3) (*7,8*). In contrast, the kidneys of female rats and female C57/BL6 mice similarly exposed to these two missile fuels were unaffected. Likewise, none of the animals, including male rats, exposed to RJ-5 continuously for 90 days and held 19 additional months before sacrifice developed renal tubule tumors.

In addition to the specific results obtained from individual

Table 7. Incidences of renal tubule preneoplasia and neoplasia in rats taken from 2-year bioassays on eight selected chemicals inducing excessive hyaline droplet accumulation.

Chemical and route	Strain	Sex	Changes	Doses, mg/kg/day			
				0	150	300	
1,4-Dichlorobenzene, gavage	Fischer 344	M	Survival, %	77	69	43	
			Hyperplasia, %	0	2	18	
			Adenomas				
			Incidence	0/50	0/50	1/50	
			Adjusted rate, %	0	0	4	
			Adenocarcinomas				
			Incidence	1/50	3/50	7/50	
			Adjusted rate, %	3	9	26	
			Combined				
			Incidence	1/50	3/50	8/50	
			Adjusted rate, %	3	9	28	
Other tumors: Hepatocellular tumors in mice							
				Doses, mg/kg/day			
				0	500	1000	
Dimethyl methylphosphonate, gavage	Fischer 344	M	Survival, %	56	34	19	
			Hyperplasia, %	0	16	18	
			Adenomas	None			
			Adenocarcinomas				
			Incidence	0/50	2/50	3/49	
			Adjusted rate, %	0	9	19	
Other tumors: Mononuclear cell leukemia; transitional cell papillomas of the renal pelvis							
				Doses, mg/kg/day			
				0	0	212	423
Hexachloroethane, gavage	Osborne-Mendel	M	Survival, %	56	65	20	18
			Hyperplasia, %	Not reported			
			Adenomas				
			Incidence	0/20	0/18	4/37	0/29
			Adjusted rate, %	0	0	11	0
			Carcinoma[a]	None			
Other tumors: Hepatocellular tumors in mice							
				Doses, mg/kg/day			
				0	10	20	
Hexachloroethane, gavage	Fischer 344	M	Survival, %	62	58	52	
			Hyperplasia, %	4	8	22	
			Adenomas				
			Incidence	1/50	2/50	4/50	
			Adjusted rate, %	3	6	15	
			Adenocarcinomas				
			Incidence	0/50	0/50	3/50	
			Adjusted rate, %	0	0	9	
			Combined				
			Incidence	1/50	2/50	7/50	
			Adjusted rate, %	3	6	24	
Other tumors: Marginal increase in pheochromocytomas in male rats							
				Doses, mg/kg/day			
				0	250	500	
Isophorone, gavage	Fischer 344	M	Survival, %	66	66	28	
			Hyperplasia, %	0	2	8	
			Adenomas				
			Incidence	0/50	0/50	2/50	
			Adjusted rate, %	0	0	8	
			Adenocarcinomas				
			Incidence	0/50	3/50	1/50	
			Adjusted rate, %	0	9	4	
			Combined				
			Incidence	0/50	3/50	3/50	
			Adjusted rate, %	0	9	12	
Other tumors: Preputial gland tumors in male rats; hepatocellular tumors, mesenchymal tumors, and malignant lymphomas in male mice							
				Doses, mg/kg/day			
				0	75	150	
d-Limonene, gavage	Fischer 344	M	Survival, %	60	68	69	
			Hyperplasia, %	0	4	7	
			Adenomas				
			Incidence	0/50	4/50	8/50	
			Adjusted rate, %	0	12	19	

Continued

Table 7, *Continued.*

Chemical and route	Strain	Sex	Changes	Doses, mg/kg/day 0	75	150
			Adenocarcinomas			
			Incidence	0/50	4/50	3/50
			Adjusted rate, %	0	12	7
			Combined			
			Incidence	0/50	8/50	11/50
			Adjusted rate, %	0	23	25
Other tumors	None in mice or rats					
				Doses, mg/kg/day		
				0	75	150
Pentachloroethane, gavage	Fischer 344	M	Survival, %	82	68	52
			Hyperplasia, %	0	0	2
			Adenomas			
			Incidence	0/50	1/49	4/50
			Adjusted rate, %	0	3	14
			Adenocarcinomas			
			Incidence	1/50	1/49	0/50
			Adjusted rate, %	2	3	0
			Combined			
			Incidence	1/50	2/49	4/50
			Adjusted rate, %	2	6	14
Other tumors	Hepatocellular tumors in mice					
				Doses, ppm		
				0	200	400
Tetrachloroethylene, inhalation	Fischer 344	M	Survival, %	48	40	24
			Hyperplasia, %	0	6	10
			Adenomas			
			Incidence	1/49	3/49	2/50
			Adjusted rate, %	4	11	11
			Adenocarcinomas			
			Incidence	0/49	0/49	2/50
			Adjusted rate, %	0	0	11
			Combined			
			Incidence	1/49	3/49	4/50
			Adjusted rate, %	4	11	22
Other tumors	Leukemia in rats; hepatocellular tumors in mice; renal tubule adenocarcinoma in one low-dose male mouse					
				Doses, ppm		
				0	67	292	2056
Unleaded gasoline, inhalation	Fischer 344	M	Survival, %	Not affected			
			Hyperplasia, %	Not reported in the bioassay			
			Adenomas				
			Incidence	0/49	1/59	2/56	1/45
			Adjusted rate, %	0	2	4	2
			Carcinomas[a]				
			Incidence	0/49	1/59	2/56	6/45
			Adjusted rate, %	0	2	4	14
			Combined				
			Incidence	0/49	1/59	5/56	7/45
			Adjusted rate, %	0	2	9	16
Other tumors	Hepatocellular tumors in female mice						

[a]Terminology used in the study, synonymous with adenocarcinoma.

bioassays, there are considerations generic to all bioassays conducted by the NTP. For example, the NTP position with regard to evaluation of rare tumors and the use of historical controls influences NTP interpretation of the evidence for carcinogenicity of CIGA (*179*). Likewise, survival rates influence the ability to analyze information from animal bioassays. These generic issues are explored before describing the results of individual studies.

Generic Considerations

Renal tubule tumors are neoplasms with a low background incidence in laboratory animals including the rat strains used in the chronic bioassays on CIGA, namely, Fischer 344 and Osborne-Mendel. The overall historical incidence of these tumors in male Fischer 344 rats is considered by the NTP to be 0.5% based on data reported on 1943 animals that served as vehicle controls in studies involving administration of chemicals via corn oil gavage (*14*). In a larger historical control database involving 2320 male and 2370 female Fischer 344 rats used as untreated controls in NTP 2-year bioassays, the incidence was 0.35% for males and 0.17% for females, suggesting a male predilection for renal tubule tumors (*180*). This is supported by spontaneous renal tubule tumor incidence rates recorded for Osborne-Mendel rats used as controls in the NCI Carcinogenesis Testing Program (*174*). In 975 males and 970 females, the incidence was 0.3 and 0%, respectively. Because of the infrequency of renal tubule tumors,

even marginal increases in their incidence in treated animals (statistically significant when compared to historical rather than concurrent controls) is regarded by the NTP as biologically significant and attributable to compound administration (*132,179*).

In the 2-year studies with the selected substances, the observed incidences of renal tumors for individual chemically dosed groups were less than 25%, and no higher than 16% for most. Because of the low background rate in both concurrent and historical controls, however, development of renal tubule tumors at these incidences was ascribed to an effect of the chemical.

The NTP bioassays provide little insight into the histogenesis of the renal tumors as they were designed and performed with the prime objective of determining the presence or absence of carcinogenic activity of the test chemical. Although an industry-sponsored study of unleaded gasoline included interim sacrifices, even this bioassay did not incorporate serial sacrifices designed to provide information on the site of origin or histogenesis of tumors.

Survival rates in high-dose male rats were poor in several of the NTP bioassays, which complicates interpretation of the CIGA data. The high mortality rate observed in some of these studies cannot be attributed to the renal tumors (181). In fact, poor survival rates usually indicated excessive toxicity. For the 1,4-DCB bioassay, survival of the high-dose males, 40% at termination, became significantly lower than that of vehicle controls after week 97 (*130*). Nearly all deaths were nonaccidental. A similar situation pertains to isophorone, where only 28% of high-dose males survived to termination (*13*).

The decreased survival rates suggest that a maximum tolerated dose (MTD) was exceeded because the early deaths could not be attributed to tumors. Administration of a chemical at dose levels exceeding an MTD may alter responses that would be seen at lower dose levels (*182*). However, exceeding an MTD at the highest dose tested, by itself, is not compelling evidence that tumors at lower dose levels are produced only when detoxification mechanisms are overwhelmed. In fact, survival of male rats in low-dose groups administered isophorone, 1,4-DCB, hexachloroethane, and tetrachloroethylene was equivalent to that of the concurrent control groups, and renal tumor incidence was elevated in these animals. Survival was excellent for all dose groups of male rats administered d-limonene or unleaded gasoline. However, it is difficult to compare tumor incidences among studies with marked differences in survival rates, especially when there is the potential for development of slow-growing tumors such as renal tubule neoplasms.

Renal Tumor Incidence

Among the eight substances, the overall unadjusted incidence rates for renal tubule tumors (adenomas and adenocarcinomas/carcinomas combined) in male rats ranged from 3 to 11% at low-dose levels and from 0 to 22% at the high dose. The highest unadjusted incidence (22%) was associated with *d*-limonene. For the remainder of the CIGA, incidences of renal tumors were 16% or less. When adjusted for intercurrent mortality, the incidence rates for combined renal tumors ranged from 0 to 28% with 1,4-DCB highest (Table 7).

For all of the eight substances, the increase in the incidences of renal tubule tumors, where adjusted for intercurrent mortality, was dose related. Because the incidence of compound-induced renal tubule tumors was low and there were confounding factors such as toxicity occurring at all dose levels in most studies, it is not possible from the NTP bioassay data to determine if there was a relationship between increasing dose and percentage of tumors classified as adenocarcinomas rather than adenomas. In its 1986 Cancer Guidelines, EPA discussed its strategy for analyzing combinations of benign and malignant tumors (*183*). In general, the Agency stated that it would consider the combination of benign and malignant tumors to be scientifically defensible if the benign tumors have the potential to progress to the associated malignancies of the same histogenic origin. The weight of evidence that a chemical is potentially carcinogenic for humans would increase when there is a dose-related increase in the proportion of tumors that are malignant. Conversely, if only benign tumors were observed, this would constitute less evidence of human cancer potential. Because the distinction between adenomas and adenocarcinomas for renal tubule tumors in rats is rather arbitrary and based mainly on size, these general principles cannot be rigidly applied.

Histogenesis of Renal Tumors

As previously indicated, NTP bioassays are designed to determine whether or not a chemical is a carcinogen. They are not designed with the intent of providing information to evaluate the developmental stages of renal neoplasia. Although renal tubule hyperplasia was reported in the male rat for seven of the eight bioassays and incidences of this lesion generally increased with increasing dose, further insight with respect to histogenesis into possible interrelationships between hyperplasia, adenomas, and carcinomas is not possible because of the low overall frequency of these lesions. The occurrence together of preneoplastic and neoplastic lesions in most studies with CIGA does provide indirect evidence of progression from tubule cell hyperplasia via adenomas to adenocarcinomas. In studies with *d*-limonene (*14*) and hexachloroethane (*132*), these lesions were stated to be part of a continuous morphologic spectrum. This accords with the generally accepted view on renal tubule tumor formation and progression (*184,185*).

Renal Tumor Latency and Progression

Renal tubule tumors produced by administration of CIGA appear to be late developing neoplasms. Times at which such tumors were first observed in bioassays of the eight model compounds usually exceeded 18 months. In general, the first renal tumor observed in each of the bioassays occurred about 5–10 weeks earlier in the high-dose than in the low-dose animals. Because renal tubule tumors are not immediately life threatening, they were usually detected in bioassays at terminal sacrifice or at death of the animal from other causes. Out of the eight bioassays, there was only one case of renal tumor metastasis, occurring in the high-dose group of hexachloroethane (*132*).

Initiation–Promotion Studies

The multistage concept of carcinogenesis, involving in its simplest form an irreversible initiation phase followed by a stage of tumor promotion (*186*), implies that chemicals may play a role

in assisting, as well as directly causing, cancer formation. There have been two research studies testing the potential of CIGA for promoting or cocarcinogenic activity in an established initiation–promotion model of renal carcinogenesis.

Using 2 weeks exposure to 170 ppm of *N*-ethyl-*N*-hydroxyethylnitrosamine (EHEN) in the drinking water as the initiating agent, the first initiation–promotion experiment of Short et al. (*178*) included both sexes of Fischer 344 rats, multiple dose levels of the test substances, short-term *versus* long-term promotion exposures, and a sequence-reversal study to discriminate any cocarcinogenic from promotional effects. The test substances were unleaded gasoline (3 inhalation concentration levels of 10, 70, and 300 ppm), and TMP (one oral dose level of 50 ppm). Treatment groups, composed of approximately 30 animals, included a control, two promotion controls, an EHEN initiation control, reverse-sequence initiation control, initiation–promotion group with a promotion phase of 24 weeks, initiation–promotion group with a promotion phase of 59 weeks, and a reverse-sequence test group where 24 weeks of exposure to unleaded gasoline or TMP preceded the 2-week period of EHEN administration. All animals were killed at 65–67 weeks after the commencement of the experiment. The results were assessed in terms of the incidence of foci of tubule hyperplasia (called atypical cell foci by the authors) and renal tubule tumors. Dose-related increases in hyperplastic foci were observed in male rats promoted with unleaded gasoline or TMP for both the short- and long-term promotion periods. A significant linear trend in the incidence of renal tubule tumors with increasing gasoline dose was also observed in male rats promoted with unleaded gasoline for 24 weeks but not for 59 weeks. The latter discrepancy reflects an experimental design weakness in the study, namely, underestimation of an optimal initiating dose of EHEN, which resulted in a low basal incidence of renal tumors. Nevertheless, the results with the single dose-level of TMP and the absence of renal tumors in any negative control group supported the observed trends with unleaded gasoline.

In the sequence-reversal study, there was no increase in renal tumors, although the incidence of hyperplastic foci was significantly elevated for both compounds. Foci of CPN were also scored in these various groups, with an increase upon CIGA exposure apparent in male rats. However, no correlation of incidence of CPN lesions with numbers of hyperplastic foci or incidence of renal tubule tumors was found.

On the basis of the results, the conclusions of the authors that unleaded gasoline and TMP have promoting activity for renal tubule tumors in the male rat, rather than acting as cocarcinogens, appear reasonable. Furthermore, there was no elevation of either hyperplastic foci or renal tumors in female rats in the study, emphasizing once again the male specificity of the renal response to CIGA.

A second initiation–promotion assay using the same EHEN model was conducted with *d*-limonene (*187*). This study specifically addressed the comparison of responses between the male Fischer 344 rat and the α_{2u}-g-deficient NBR strain. The initiating dose of EHEN was 500 ppm administered in the drinking water for 2 weeks, followed by *d*-limonene by daily gavage (5 days/week) at 150 mg/kg per day for 30 weeks. An initiation control (EHEN), promotion control (*d*-limonene), and a vehicle control were included for both strains. In the Fischer rats administered EHEN and *d*-limonene, atypical tubule cell hyperplasia and renal tubule adenomas were increased 10-fold as compared to the EHEN control group. In contrast, no tumors were observed in any of the NBR groups. Such negative results in the NBR rat strongly suggest a clear dependence on α_{2u}-g for the promoting activity of *d*-limonene.

The promotional effect of unleaded gasoline, TMP, and d-limonene may be occurring through the influence of sustained tubule cell proliferation that has been demonstrated with these same compounds (*143,187*). The extent of cell proliferation is regarded as an important factor in chemical carcinogenesis (*188–190*), and stimulation of cell turnover is one of the key mechanisms believed to operate in tumor promotion (*191*).

Evidence for Dose- and Time-Dependent Progression from Early to Late Lesions

An important aspect for examining the hypothesis that renal tumor formation is directly associated with accumulation of α_{2u}-g in the male rat kidney is a demonstration of the progression of lesions proposed to culminate in neoplasia (Table 8). For some of the steps, clear dose–response relationships have been shown. In other cases, histopathologically observable events are of a secondary nature and not in the direct progression. As the information presented below shows, data demonstrating the existence of some steps in the proposed progression are limited, restricting the confidence in judgments on the nature of the association.

Evaluation of the events leading to neoplasia is further complicated by the low incidence of renal tumors induced by the CIGA studied. Such information makes it difficult to identify possible relationships between the induced nephropathy and renal carcinogenesis.

Table 8. Summary of the histopathology and lesion progression in α_{2u}-globulin-associated nephrotoxicity and renal tubule neoplasia.

Excessive accumulation of hyaline droplets in the P2 segment of the proximal tubule region of kidney occurs after 1 or 2 days. This is reversible within 3 days to 2 weeks after exposure ceases.
Evidence of single-cell necrosis in P2 segment epithelium and exfoliation after 5 days of continuous exposure.
Accumulation of granular casts formed from the cellular debris and subsequent tubule dilation, at the junction of the P3 segment and the thin loop of Henle, following 20 to 40 days of continuous exposure. Granular casts have been observed at 3–13 weeks after commencing exposure and sometimes beyond, up to 2 years.
Increase in cell proliferation within the P2 segment following 3 weeks of continuous exposure, remaining elevated above normal at 48 weeks of exposure.
Linear mineralization of inner medullary tubules and within the renal papilla, appearing between 3 and 12 months after a 28-day exposure, and sometimes observed at the end of a 2-year study.
Hyperplasia of the renal pelvic epithelial lining observed around 1 year.
Exacerbation of the spontaneous chronic progressive nephropathy syndrome is common in aging rats.
Formation of occasional hyperplastic foci within cortical epithelium occurs at chronic time points.
Renal tubule neoplasia occurs between 18 months and 2 years.

Association between CIGA, Hyaline Droplet Formation, and α_{2u}-Globulin Accumulation

Dose-dependent relationships have been demonstrated between the administration of *d*-limonene (*101*) or gabapentin (*137*) and excessive formation of hyaline droplets, and between unleaded gasoline or TMP and α_{2u}-g accumulation (*98,112*). In the *d*-limonene study, hyaline droplets were graded on a scale of 0–12 according to size, eosinophilic intensity, and the number of tubules loaded with droplets. The droplet scores for *d*-limonene doses of 0, 0.1, 0.3, 1.0 and 3.0 mmole/kg were, control to high dose, 3, 4.5, approximately 7, 8, and 10 (*101*). The dose-response relationship with α_{2u}-g accumulation is exemplified by measurements following administration of TMP, which, given at single doses of 0.044, 0.440, and 4.00 mmole/kg, induced α_{2u}-g concentrations in rat kidney tissue at 24 hr of 10.3, 17.3, and 28.1 mg/g wet weight, respectively, as compared to a control value of 9.5 mg/g wet weight (*98*). With orally administered gasoline, the phenomenon was dose responsive only in the range of 0.04–1.00 mL/kg (*112*).

In a special NTP study, male and female F344 rats were exposed to *d*-limonene by gavage for 14 days over a 21-day period (*14*). The α_{2u}-g content, quantitated with an ELISA test in kidney homogenates, increased significantly in dosed male rats relative to vehicle controls. At 75 mg/kg, the low dose used for male rats in the 2-year bioassay, α_{2u}-g levels were approximately double those in controls. In females, increasing the dose as high as 1200 mg/kg had no measurable effect on α_{2u}-g levels in the kidney. Microscopic examination of kidney sections stained with H&E showed no visible differences between dosed male rats and vehicle controls. Sections stained with Lee's methylene blue basic fuchsin, however, showed differences in the distribution, amount, and shape of intracytoplasmic granules in the proximal tubules.

In contrast to the 21-day follow-up study, the 13 week range-finding study conducted before the *d*-limonene bioassay failed to detect an accumulation of hyaline droplets. The NTP report (*14*) acknowledged that this failure might have been related to the fact that several days passed between the time the chemical was last administered and the time the animals were killed for histological examination. Other studies have shown that renal α_{2u}-g concentrations decline rapidly, reaching preexposure levels by the third day after treatment, although hyaline droplets, being structural entities, require up to 9 days for complete resolution (*111*). This suggests that the interval between the time the chemical was last administered and the time the animals were killed for histological examination is critical to finding α_{2u}-g in hyaline droplets and probably accounts for discrepancies found among some studies.

These various observations, along with the results of α_{2u}-g localization studies and binding studies considered earlier, support a causal association between the administration of CIGA and α_{2u}-g accumulation in hyaline droplets.

Association between Hyaline Droplet Formation, Cell Necrosis, and Tubule Cell Regeneration

Hyaline droplet accumulation, single cell necrosis and cell proliferation occur predominantly in the P2 segment of the proximal tubule following CIGA administration (*143,144,178*). Although single-cell necrosis has been clearly demonstrated in association with cellular hyaline droplet accumulation (*6,143*), there are no dose–response studies quantitating the relationship between increased hyaline droplets and cell necrosis in histological sections or between cell necrosis and cell regeneration. However, Alden (*147*) has shown a correlation between the hyaline droplet response, increased mitotic index in proximal convoluted tubules, and elevation of the number of cells excreted hourly in the urine (an index of exfoliated necrotic tubule cells) using two dose levels of *d*-limonene given orally for 3 weeks.

Dose–response relationships between hyaline droplet accumulation and proximal tubule cell proliferation have been observed. Short and co-workers exposed male rats for 3 weeks to TMP (oral) or unleaded gasoline (inhalation) and then measured [^{3}H]thymidine labeling indexes (*143*). The extent and severity of hyaline droplet accumulation paralleled the extent and localization of cell proliferation in proximal tubule cells, and both parameters were increased in dose-dependent fashion. In an extended study of the same substances, Short et al. (*144*) observed 6- to 11-fold increases in labeling indexes in the P2 segment of the rat kidney after the rats received 3, 10, and 22 weeks of exposure to 300 ppm unleaded gasoline or 50 ppm TMP. These labeling indexes remained 4- to 6-fold higher than control values during week 48 of exposure.

In contrast, Viau et al. (*120*) did not observe a sustained regenerative response beyond 5.5 weeks in the kidneys of male rats exposed to an isoparaffinic solvent consisting of saturated C_{10}–C_{12} aliphatic hydrocarbons. Labeling indexes in the cortex of treated rats at 46 and 68 weeks were no different from the controls. This apparent discrepancy with the gasoline and TMP results undoubtedly reflects differences in the technique of radioactive labeling. Viau et al. (*120*) used a single injection of [^{3}H]thymidine 1 hr before sacrifice, whereas Short et al. (*144*) labeled continuously by SC osmotic minipump infusion over a 7-day period, the preferred method for cell populations with a low cell turnover, thereby increasing the amount of radiolabel incorporated into renal tissue.

In recovery studies with unleaded gasoline and TMP, Short and co-workers (*144*) showed that neither increased hyaline droplets nor cell proliferation were observable 7 days after discontinuing 3-week exposures, indicating complete recovery. However, after 10 and 22 weeks of exposure, recovery was only partial, and labeling indices remained nearly three times above controls following 10 days in a gasoline- or TMP-free environment. Thus, proximal tubule cell proliferation is a persistent phenomenon in chronic exposure to CIGA, becoming less amenable to recovery with increasing duration of exposure.

Furthermore, in promotion studies with d-limonene, cell proliferation, assessed by bromodeoxyuridine labeling via SC osmotic minipump implants, was not induced beyond background by *d*-limonene after 5 or 30 weeks of exposure in the α_{2u}-g-deficient NBR rat, compared to a 5-fold increase in the tubule cell labeling of *d*-limonene-promoted Fischer rats initiated with EHEN (*187*). This result suggests that the sustained proliferative response induced by a CIGA is dependent on the α_{2u}-g syndrome.

Thus, the sequence of events following CIGA administration involves lysosomal overload, cell necrosis, and cell replication.

All three of these occur in the same segment of the nephron in conventional strains of rats, but none occur in the NBR rat. Whereas these events are temporally correlated, it is not yet clear whether the lysosomal overload causes necrosis or whether necrosis can be associated with replication. These questions need further investigation and hypothesis development to establish mechanisms of action.

Progression to Cast Formation, Tubule Dilation, and Mineralization

Because few chronic studies incorporated serial sacrifices, it is difficult to assess the time dependence of the development and progression of the sequential lesions proposed to be associated with α_{2u}-g nephropathy. Granular cast formation was recorded exclusively in male rats for most of the CIGA evaluated in 13-week toxicity studies by the NTP and sometimes in the 2-year bioassays. In another study, Viau et al. (*120*) exposed male rats to C_{10}–C_{12} aliphatic hydrocarbons by inhalation for 5.5, 46, or 68 weeks and found granular casts at the earliest time point, but they were absent at the later time points. One explanation for these results is that certain lesions in the sequence are transitory in nature. Granular casts, for example, are presumably linked to the active hyaline droplet overload. Once α_{2u}-g levels become low because of age, after approximately 18 months, the number of new hyaline droplets being formed would presumably become minimal.

Tubule dilation is presumed to follow obstruction of the nephron by the accumulation of granular casts composed of sloughed epithelial cell debris in the tubule lumen. Male rats were administered unleaded gasoline (2 g/kg/day) for a period of 28 days and examined at five interim time points (*110*). An initial accumulation of hyaline droplets, commencing on the first day of exposure and persisting throughout, was followed at 14, 21, and 28 days by increases in epithelial cell proliferation and tubule dilation associated with lumenal accumulation of granular debris.

Linear mineralization in the renal papilla of male rats has been consistently observed in a number of 2-year bioassays with CIGA but not in their associated 13-week toxicity studies. Clear dose–response relationships were demonstrated for 1,4-DCB (*130*), JP-4 mixed distillate (*7*), and unleaded gasoline (*11*). In the 2-year unleaded gasoline study, there were interim sacrifices at 3, 6, 12, and 18 months, permitting quantitative observation on the incidence of mineralization (*11*). Although this lesion was termed "pelvic" rather than "medullary" mineralization in the original report from IRDC, it was qualified as referring to material located within tubules of the renal pelvis, thus conforming to the medullary site seen with other CIGA. Table 9 presents a summary of these data, which show a clear dose-related progression in the incidence of mineralization from 6 months up to and including the 2-year sacrifice. Parallel dose–response increases have been demonstrated for medullary mineralization and urothelial hyperplasia with JP-5 jet fuels, diesel fuel marine and decalin (*192*), supporting the notion that the pelvic hyperplasia is a urothelial response to mineralization in the papilla.

Association between CIGA and Chronic Progressive Nephropathy

Although exacerbation of spontaneous CPN by CIGA has been noted in many studies, quantitation of this response has been attempted on few occasions. Short et al. (*144*) compared the number of CPN foci per kidney section in male rats at three dose levels of unleaded gasoline exposure and two chronic time points with control specimens. For a daily dose range of 0, 10, 70, and 300 ppm unleaded gasoline, the numbers of foci observed at 22 weeks of exposure were 0.4, 0, 1.0, and 6.3, respectively, and at 48 weeks of exposure were 5.0, 4.0, 10.0, and 9.0. This study therefore supports the conclusion that there is an earlier onset of CPN, demonstrable by 5 months, and a higher incidence of disease with the middle and high doses of unleaded gasoline in male rats.

Table 9. Incidence of medullary mineralization in male rats during inhalation exposure to unleaded gasoline.

Observation time points, months	Exposure levels of unleaded gasoline vapors, ppm			
	0	67	292	2056
3	0	0	0	0
6	0	0	0	20[a]
12	0	0	20	80
18	0	0	20	80
24	0	5	63	91

[a]The incidence figures are the percentage of animals affected. Data from U.S. EPA (*11*).

In the NTP bioassay of d-limonene (*14*), treated male rats showed a spectrum of compound-related kidney lesions, including exacerbation of age-related nephropathy, mineralization in the renal medulla, hyperplasia of the epithelium lining the renal papilla, and proliferative lesions of the renal tubule cell epithelium. The severity of CPN was graded on a scale of 0–4 as "not present, minimal, mild, moderate, or marked." The mean value increased with increasing d-limonene dose from 1.5 in vehicle controls to 1.8 and 2.2 in animals dosed at 75 and 150 mg/kg, respectively.

As CPN is exacerbated by CIGA administration and CPN-affected tubules have a high cell turnover rate, it has been suggested that CPN may play a role in renal tumor production following α_{2u}-g nephropathy because enhanced regeneration is considered a risk factor for carcinogenesis (*178,193*). There is no firm evidence available to date that substantiates or disproves a link between CPN and renal tubule tumor induction. Nevertheless, in a specialized initiation–promotion study with unleaded gasoline and TMP where the authors quantified foci of CPN, some adenomas were described as arising within foci of CPN (*178*).

Evidence Concerning Progression from Nephrotoxicity to Renal Neoplasia

For the eight selected carcinogens examined in this report, there was an overall pattern indicative of dose-related increases in the incidences of toxic nephropathy, hyperplasia, and renal tubule tumors in male rats. For two CIGA, unleaded gasoline and TMP, dose-related increases in renal tubule proliferation were sustained throughout chronic administration. It is believed that the likelihood of producing a cancerous cell is increased, not only if there is a probability of a genetic transition, but also if the rate of cell replication is increased (*189,190,194*). Thus, a finding that a sustained state of cell turnover in the target cell population is a mechanistic link between α_{2u}-g nephropathy and renal neoplasia should be considered a plausible, but unproven, description of the observed results.

The hyperplastic tubules and adenomas produced by CIGA carcinogens appear to arise from the cortex, which includes the P2 segment of the proximal tubule, the main site of cellular injury in α_{2u}-g nephropathy, providing further support for their linkage. Furthermore, studies that examined cell replication rates in the renal tubule specifically noted an increase in the histologically damaged P2 segments after tetrachloroethylene (*37*), pentachloroethane (*37*), TMP (*142*), and unleaded gasoline (*143*) exposure in male rats. Under the same conditions, tubule cell replication in female rats did not differ from controls in any of these studies, nor in rats of both sexes treated with a non-CIGA, trichloroethylene.

Recent studies of the promotion potential of *d*-limonene, TMP, and gasoline also provide convincing evidence to support a linkage between α_{2u}-g nephropathy and renal tubule neoplasia. Dietrich and Swenberg (*187*) demonstrated that *d*-limonene promoted renal tubule tumors in male Fischer 344 rats, an animal that produces α_{2u}-g. In addition, there was a 5-fold increase of P2-labeling index in the Fischer 344 rats treated with *d*-limonene. In contrast, no response was recorded for proliferation, hyperplasia, or renal tubule adenomas in the NBR rat, an α_{2u}-g-deficient animal that does not develop the characteristic nephropathy. These results substantiate those of an earlier study where dose-related increases in atypical cell foci were observed in male rats promoted with unleaded gasoline or TMP for 24 or 60 weeks (*178*). In that study, there was a significant linear trend in incidence of renal tubule tumors in the male rat promoted with unleaded gasoline for 24 weeks. In contrast, none of these changes was observed in similarly treated female rats.

Finally, the nephrotoxicity seen in male rats in the selected 2-year bioassays of renal tubule carcinogens was characteristic of that proposed to result from cell damage caused by α_{2u}-g accumulation. In contrast, whenever nephrotoxicity was observed in female rats or mice of either sex, i.e., for hexachloroethane, tetrachloroethylene, and 1,4-DCB, the lesions were not characteristic of CIGA and probably were a response caused by an independent mechanism.

Genotoxicity Studies

Key evidence relevant to assessing the potential carcinogenicity of CIGA as a chemical group can be derived also from genotoxicity data. The available genotoxicity data for the eight CIGA carcinogens are summarized in Table 10. The four assays listed in the table (Salmonella [SAL], chromosome aberrations in Chinese hamster ovary cells [ABS], sister chromatid exchange in Chinese hamster ovary cells [SCE] and thymidine-kinase (*TK*) gene mutations in L5178Y cells [MLA]) are the only ones with enough common data for comparative purposes. It is not coincidental that these are the assays used by the NTP. Consequently, this analysis of genotoxicity data was limited mainly to the eight hyaline droplet inducers with bioassay data. Data from Drosophila tests conducted by the NTP (*195*) and in human lymphoblasts (*196*) are also cited in Table 10 where available.

The eight renal carcinogens selected as possible CIGA have been tested for chromosome aberrations in Chinese hamster ovary (CHO) cells (*197*) and in Salmonella (*12,198–200*). All results were negative both in the absence and presence of exogenous activation provided by S9 extracts from rat liver. Two presumed intermediate metabolites of the CIGA, *d*-limonene (the 1,2- and 8,9-epoxides) were also tested in Salmonella with and without induced S9, and no increase in revertants was observed (*201*). Several of the CIGA chemicals have tested positive, at least under some conditions, for sister chromatid exchange in CHO cells (*197*) and in the mouse lymphoma *TK* gene mutation assay (*202*). Richardson et al. (*196*) reported negative results for unleaded gasoline and its known CIGA component, TMP, in assays for TK-gene mutations and SCE in the TK6

Table 10. Summary of genotoxicity data for eight selected male rat kidney carcinogens.

Substance	Assay[a] SAL	ABS	SCE	MLA	Comments
1,4-Dichlorobenzene	–	–	–	E	MLA with S9. There was a marginal positive result in one of three experiments. Negative *in vivo* chromosome aberrations, micronuclei, and dominant lethals.
Dimethyl methylphosphonate	–	E	+	+	*MLA and SCE results positive without S9; MLA not tested with S9; SCE* negative with S9. ABS negative in two labs, with and without S9 (NTP studies), but positive in another lab [without S9 (*12*)]. Drosophila SLRL positive, but translocations negative. Dominant lethal positive in both rats and mice.
Hexachloroethane	–	–	+		*SCE reproducibly positive only with S9. No data for MLA.*
Isophorone	–	–	+	±	SCE only positive with cell-cycle delay without S9; MLA replicated positive without S9, not tested with S9 in NTP studies. CMA reported negatives for hepatocyte UDS, mouse micronuclei, and MLA (with and without S9).
d-Limonene	–	–	–	–	Clear negative in all NTP studies.
Pentachloroethane	–	–	+	+	*MLA and SCE positive without S9 (reproduced); SCE negative with S9.* Negative in rat *in vivo* kidney UDS assay.
Tetrachloroethylene	–	–	–	–	In NTP studies all clear negatives both with and without S9; also Drosophila SLRL negative. Negative in rat *in vivo* kidney UDS assay. Positive in Salmonella TA100 with GSH and kidney microsomes.
Unleaded gasoline	–	–		±	*In NTP studies both positive responses were only with S9. Other studies* confirm negative response in bacteria. In yeast, positives have been reported for mitotic recombination and both positive and negative responses for gene mutations. Negative in rat *in vivo* kidney UDS assay.

Abbreviations: SAL, Salmonella mutagenicity; ABS, chromosome aberrations in Chinese hamster ovary (CHO) cells; SCE, sister chromatid exchange in CHO cells; MLA, thymidine-kinase (*TK*) gene mutation assay in L5178Y cells; GSH glutathione; SLRL, sex-linked recessive lethal; UDS, unscheduled DNA synthesis; E, equivocal; CMA, Chemical Manufacturers Association.

[a]Positive, negative, and equivocal as defined in Haworth et al. (*198*), McGregor et al. (*202*), and Galloway et al. (*203*).

human lymphoblast cell line. A cursory appraisal of only positive and negative responses leads to the conclusions that there is significant heterogeneity and the CIGA groups are not distinguishable from non-CIGA by their genotoxic activity. Upon more detailed analysis, it becomes apparent that the majority of the positive responses of the eight carcinogens selected as hyaline droplet inducers were observed in the absence, but not in the presence, of exogenous S9 activation and at concentrations greater than 100 μg/mL.

Dimethyl methylphosphonate appears to present a unique genotoxicity profile among the eight CIGA carcinogens. Because this chemical has high water solubility and low toxicity, *in vitro* assays have employed very high concentrations, as high as 30 mg/mL. Galloway et al. (*203*) reported that at least some of the observed *in vitro* mutagenic activity seen for dimethyl methylphosphonate occurred at levels that decreased cell growth and greatly increased the osmotic strength. Similar levels of osmolality and chromosome aberrations were observed, for example, with 160 mM of potassium chloride and 30 mg/mL of dimethyl methylphosphonate. The SCE increases observed for dimethyl methylphosphonate, however, occurred at concentrations causing only slight increases in osmolality.

Of particular relevance to this report are those studies in which rodent kidney or kidney extracts were used in an assay for a genotoxic end point. Loury et al. (*204*) reported that unleaded gasoline was negative in an *in vivo/in vitro* kidney unscheduled DNA synthesis assay indicative of DNA damage and repair. Similar results were reported for pentachloroethane and tetrachloroethylene by Goldsworthy et al. (*205*). However, both studies reported significant elevation of replicative DNA synthesis in kidneys of male rats treated with these compounds.

Recently, Vamvakas et al. (*206*) reported clear dose-related positive results in Salmonella TA100 with tetrachloroethylene in the presence of glutathione and rat kidney microsomes. The glutathione conjugate *S*-(1,2,2-trichlorovinyl)glutathione was also mutagenic in the presence of kidney microsomes, and the activity was reduced in the presence of a β-lyase inhibitor. The importance of these findings in the formation of the kidney tumors of male rats exposed to tetrachloroethylene is yet unclear, but similar studies with other "nongenotoxic" kidney carcinogens seem to be in order before direct interaction with DNA can be excluded.

In summary, the preponderance of available data suggest that the CIGA group possess little, if any, genotoxic activity. However, the shortage of data on the kidney or with glutathione conjugates for these chemicals precludes closure of the question.

Comparison of CIGA with Classical Renal Carcinogens

General Features

Among the many chemicals recognized as inducers of rodent cancer, several have been used as model kidney carcinogens for studying the pathogenesis of renal tubule tumors in rats. These are dimethylnitrosamine (DMN), diethylnitrosamine (DEN), *N*-nitrosomorpholine, EHEN, lead acetate, *N*-(4′-fluoro-4-biphenylyl)acetamide (FBPA), and aflatoxin B_1 (*185*). In the mouse, certain nitrosamines, streptozotocin, and ochratoxin A are strong inducers of renal tubule tumors, whereas the classical renal carcinogen in hamsters is diethylstilbestrol (*207*).

Some classical carcinogens are effective renal tumor inducers following abbreviated dosing regimens. For example, DMN, DEN, and streptozotocin require only a single injection to produce tumors, while the EHEN regimen uses a 2-week period of oral exposure. In contrast, certain military fuels induced renal tubule tumors in male rats following lifetime observation after 1 year of intermittent exposure, but not after 90 days of continuous exposure (*8*).

Many classical renal carcinogens or their active metabolites are electrophilic species binding covalently to macromolecules and forming, in particular, DNA adducts (*147,184,207*). Such DNA reactivity is putatively the mechanistic basis of renal carcinogenesis induced by these chemicals. For example, carcinogenic nitrosamines can form various alkylation products in DNA, including O^6-alkylguanine, which is a promutagenic lesion (*208*). Accordingly, classical renal carcinogens are usually positive in short-term mutagenicity assays.

In contrast, CIGA are not known to react with DNA and are generally negative in short-term tests for genotoxicity. As described previously, CIGA binding to α_{2u}-g is reversible and not covalent in nature.

Early Nephrotoxicity

Acute toxic changes occur in the proximal tubules shortly after the administration of classical renal carcinogens. They include mild lipid droplet accumulation and scattered single-cell necrosis (*207*). Depending on the carcinogen used, this early damage can be observed in different segments of the renal tubule. For instance, with DMN it is localized to the P2 segment (*209*), and with FBPA it is localized to the P3 segment (*210,211*).

Detailed histological and/or ultrastructural observation shows that hyaline droplet accumulation is not induced by DMN (*209,212*) or DEN (G. C. Hard, unpublished observations), nor has it been described in studies using other carcinogens, such as FBPA (*210,211*), as models for renal carcinogenesis.

The lack of involvement of hyaline droplet accumulation in the early nephrotoxicity associated with classical carcinogens (definite with DMN and DEN and apparent with the others) is a major difference from the sequence of early pathologic events induced by CIGA in the male rat.

Karyomegaly

Conspicuous nuclear enlargement, indicative of increased ploidy levels without completion of mitosis (*213*), may occur in scattered proximal tubule cells during the weeks preceding development of proliferative foci induced by classical renal carcinogens. Likewise, karyomegaly in renal tubules has been reported in rats and occasionally in mice with some of the CIGA, for example, unleaded gasoline (*193*) and tetrachloroethylene (*177*).

Although karyomegaly is produced by many but perhaps not all classical renal carcinogens, there is no evidence that these cells participate in the initial formation of proliferative foci. Hence karyomegaly is not regarded as a preneoplastic lesion (*184,207,210*).

Morphologic Features of Renal Tubule Cell Carcinogenesis

Studies on the pathogenesis of renal tubule tumor formation using model carcinogens in rats agree that a continuum of chemically induced steps leads from atypical hyperplasia in tubules (also called hyperplastic tubules, tubule dysplasia, or atypical cell foci) through microscopic adenomas to macroscopic adenocarcinomas or carcinomas (*184,207*). Despite the differences in toxicity observed, the sequence of development of CIGA-induced renal tumors from tubule hyperplasia to carcinoma appears identical. These various lesions, as induced by classical carcinogens, are described below in chronological sequence.

Tubule Cell Hyperplasia. Tubule cell hyperplasia leads to the appearance of tubules with proliferating epithelium, usually multilayered, that partially or completely fills the tubular lumen. Although lumenal dilation may be pronounced (sometimes to cystic proportions), the structure of the individual tubule remains intact with a confluent basal lamina. Affected cells may be eosinophilic, basophilic, or pale-staining and often with vesicular nuclei and prominent nucleoli. Mitotic figures are variable. As a preneoplastic lesion, the hyperplastic tubule is usually associated with cellular atypia in the form of cell pleomorphism and increased nuclear-to-cytoplasmic area ratio (*184,207*). Preneoplastic tubule hyperplasia is generally considered to be distinguishable from the background tubular regeneration that is a component of spontaneous CPN (*184,214*).

Adenoma. Adenomas are small, neoplastic foci representing epithelial cell proliferation beyond the well-defined structure of individual tubules. These lesions are solid or cystic in form, and the cellular morphology and architectural appearance is similar to that of adenocarcinomas, which are described below, particularly the well-differentiated variants. Whereas adenomas and hyperplastic tubules can be differentiated on the basis of finite structure, the distinction between adenomas and adenocarcinomas/carcinomas is a more arbitrary one based mainly on size. Neoplasms in the rat kidney parenchyma less than approximately 0.5 cm tend to lack significant vascularization, hemorrhage, and degeneration, although there may be single-cell necrosis, mitosis, and cell pleomorphism (*185*).

Adenocarcinomas and Carcinomas. Renal tubule tumors comprise histological variants based on staining characteristics and architectural organization. In the rat, renal tubule tumors consist mainly of lightly basophilic, granular and/or clear cells organized in tubular, lobular, solid, or papillary patterns. Glandular differentiation as opposed to solid sheets of cells distinguishes adenocarcinomas from carcinomas, but any clear distinction between adenocarcinomas and carcinomas is often meaningless because of the admixture of both well-differentiated and poorly differentiated areas within the same tumor. Increased cellular pleomorphism tends to correlate with a decreasing degree of tubular differentiation, and anaplastic variants occur occasionally.

Cells within renal tubule tumors maintain many of the light and electron microscopic characteristics of proximal tubule epithelium, in particular, microvilli resembling brush border, basement membrane, and cytoplasmic vesicles. Brush border may occur inappropriately between adjacent cells, along any cell border, or as intracellular profiles. Adenocarcinomas/carcinomas are well vascularized and usually display areas of hemorrhage and degeneration (*18,184,185*).

Pathology reports indicate that renal tubule tumors induced by CIGA are morphologically indistinguishable from spontaneous tumors or those induced by classical carcinogens, with both granular and clear-cell types occurring. However, evidence from the bioassays suggests that the CIGA tumors may, in general, have a smaller size than other chemically induced renal tumors, probably because of the difference in potency between CIGA and classical carcinogens, which affects the latent period of tumor development.

Tumor Progression

Renal tubule tumors of the rat are slowly growing neoplasms usually requiring about 40 weeks to become clinically palpable in most experimental systems (*207*). They can grow to large dimensions, several centimeters in diameter.

Unlike their spontaneously occurring human counterparts, renal tubule tumors induced in rats by chemical carcinogens metastasize infrequently (*184*). However, effective life-span in chronic-exposure regimens may be a limiting factor. Single-dose studies with DMN, which maximize the life-span following tumor initiation, have demonstrated a link between survival period, tumor size, and incidence of metastasis in renal carcinogenesis (*215*). For example, rats that survived at least 1.5 years after dosing with DMN showed a high rate of metastasis, approximately 50%, whenever epithelial tumor dimensions exceeded 2.4 cm. These data confirm the malignant potential of renal tubule tumors induced in the rat by a classical carcinogen.

As with chronically administered classical carcinogens, metastases have rarely been reported for renal tubule tumors related to treatment by CIGA. The one case of metastasis noted with hexachloroethane indicates, however, the malignant potential of the CIGA-induced neoplasms.

Tumor Incidence

Classical renal carcinogens can induce renal tubule cancer in rats or mice in high incidence, with minimal duration of exposure, clear dose–response relationships, and with decreased latent period of development (*147,207*). Tumor frequencies are often over 50% and up to 100%, much higher than the low incidences (2–28% adjusted) recorded for CIGA. Unlike CIGA-induced renal carcinogenesis, there is usually no absolute sex specificity, with males and females both susceptible, although not always to the same degree. These differences in potency and species and sex susceptibility suggest that classical renal carcinogens and CIGA act via different mechanisms in kidney carcinogenesis.

Site of Origin of Renal Tubule Tumors

The precise location within the nephron from which experimental renal tubule tumors arise varies with the carcinogen and correlates with the site of the induced early nephrotoxicity. Thus, the P3 segment is the site of origin for FBPA-induced tumors (*210,211*), whereas DMN tumors arise from the convoluted segments of proximal tubules, probably P2 (*185*). Lead acetate- and DEN-induced tumors appear to originate in both P2 and P3 segments (*216*).

Although the specific site of origin for the renal tubule tumors

produced by CIGA is not known, the P2 region of the proximal tubule as the primary site would be consistent with existing information. Based on studies with classical carcinogens, this does not represent an unusual location.

Evidence Concerning Human Kidney Cancer

Epidemiologic studies of human renal cell cancer are reviewed for consistency with the hypothesis that CIGA-induced renal tubule cancer in male rats is an inappropriate end point for assessing human risk. Implicit in this evaluation is a presumption of male rat-to-human tumor site concordance, a supposition that is generally not made. In this special case, the hypothesized mechanism being examined depends on the accumulation of low molecular weight protein in the renal tubule, regardless of species. Hence the predicted target site for cancer in humans, as in the rats, would be the renal tubule.

Although not one of the most common neoplasms in the United States, renal cell adenocarcinoma/carcinoma is regarded as an important human cancer. This is because the disease is unpredictable, and a significant proportion of patients, approximately one third, have distant metastasis at the time of diagnosis (*217,218*). The mortality rate in these cases is high, and, overall, the survival rate for patients with renal cell cancer is 48% (*219*). In addition, the etiology of kidney cancer in humans is poorly understood.

Morphology and Histogenesis

Human renal cell tumors, which are morphologically similar to those of rodents, are classified according to cell type and cellular arrangement. Thus, two main cell forms are recognized, granular and clear, and the usual patterns of organization are tubular, solid, papillary, and cystic. Individual tumors may show an admixture of patterns and cell types. Infrequently, renal cell carcinoma presents as a sarcomatoid form composed of spindle cells (*29,217,220*).

It is generally accepted that the origin of renal cell carcinoma is the proximal tubule, based on both immunological study (*221*) and ultrastructural features (*217,220*). Electron microscopy reveals many similarities between the tumor cells and proximal tubule epithelium, including brush border elements, membrane-associated vesicles, and basilar infoldings of the plasma membrane (*222*). Ultrastructurally, the amount of intracellular lipid, particulate glycogen and organelles distinguishes clear from granular cells.

It is widely considered that human renal adenomas represent small adenocarcinomas or carcinomas as there are no microscopic, histochemical or immunologic features that discriminate them other than size, and this is not an absolute biologic parameter (*217,220,223*). Adenomas are therefore considered part of an evolutionary continuum from hyperplasia, through adenoma, to adenocarcinoma/carcinoma, as in rodents. As a general observation, there is a direct relationship between tumor size and frequency of metastasis (*223–225*).

Incidence and Mortality

Kidney cancer statistics are usually reported in a form that encompasses all types of malignant cancer affecting kidney, renal pelvis, and sometimes ureter and urethra. Renal cell cancer rarely occurs under the age of 40 years (*226,227*) and represents about 70% of all kidney tumors in adults (*219*). Kidney cancer statistics, therefore, provide an approximation only of renal cell tumor prevalence.

The number of new cases of kidney and urinary tract cancer (excluding bladder) estimated for 1991 in the United States is 25,300, with a mortality estimate of 10,600 deaths (*228*). These figures represent approximately 2% of both new cancer cases at all sites and total cancer deaths. The age-adjusted incidence rates in the United States for the period between 1975 and 1985 obtained from the NCI Surveillance, Epidemiology and End Results Program (SEER) data for renal cell cancer are 8.4 per 100,000 for males and 3.7 per 100,000 for females, with no difference among racial groups (*219*). Most studies indicate a consistent male to female ratio of 2:1 for the incidence of renal cell tumors (*219,227*).

In considering renal cell tumors specifically, the highest rates internationally have been reported from Iceland and other Scandinavian countries. Renal cell carcinoma is the fifth most common malignant tumor of males in Iceland, although it ranks only tenth in females (*229*). The lowest rates for renal cancer are recorded in Africa, Asia, and South America (*226*). Within the United States, mortality surveys indicate that the North Central region and some areas in the Northeast have the highest incidence rate for renal cell carcinoma (*230*). It has been suggested that the clustering in the North Central region may be partially explained by the predominantly German and Scandinavian origin of the area's population (*231*). Several studies have reported that the urban rates for renal cell tumor incidence are higher than for rural areas, but the correlation is considered to be weak (*232*).

In contrast to the relatively low incidence and mortality figures for malignant kidney and related tumors provided by cancer statistics data, the occurrence of renal cell adenomas at autopsy is common. The reported incidence has ranged from 15 (*29*) up to 25%, the latter for males over the age of 50 (*233*). These findings have led to speculation that a proportion of adenomas may reach a limit of growth and/or remain quiescent (*29,234*).

Over the period 1950–1985, the U.S. Cancer Statistics data indicate an increase of 82% in the incidence of kidney and renal pelvis cancer combined (*218*). For renal cell cancer alone, the increase among whites was estimated at about 30% between 1969 and 1971 and 1983 and 1985, representing an average annual percentage change in incidence of 2.0 for males and 1.8 for females (*219*). Data from Cancer Registries in Scotland between 1967 and 1979 also indicate an increase of approximately 37% in the incidence of renal cell carcinoma for males, although no overall increase in females (*223*). Despite an improvement in mortality rates since 1950 compared to incidence rates (*218*), the relative 5-year survival rates, which are close to 50%, have not altered since the early 1970s (*228*), suggesting little improvement in treatment over the past two decades. On the other hand, diagnostic detection measures have improved dramatically during this time, which may explain, at least in part, the observed increase in renal cancer incidence (*218,235*).

Renal cell carcinoma has been diagnosed with increasing frequency in patients with chronic renal failure (*232,236*). In particular, this appears to reflect an association with the develop-

ment of acquired renal cystic disease, which frequently occurs in patients on long-term hemodialysis. The incidence of renal cell carcinoma in patients with acquired cystic disease has been estimated as approximately 6% (*236*). Thus, current data suggest that a growing population of humans receiving maintenance dialysis may be at risk for developing renal cell tumors.

Environmental and Lifestyle Factors

Potential etiological associations between renal cell cancer and exogenous and endogenous environmental factors, lifestyle and occupation, have been sought in cohort and case–control studies. Of all the environmental and lifestyle factors investigated, tobacco use in the form of cigarette, cigar, or pipe smoking has been the one most consistently associated with renal cell carcinoma (*226,227,237–240*). Although a few studies have failed to identify a statistical association between smoking and renal cell cancer, it has been estimated that 30% of renal cell carcinomas in males and 24% in females may be attributable to cigarette smoking (*231*) and that there is evidence for a moderate dose response (*226,240*). One study has also linked use of chewing tobacco with renal cell carcinoma in males (*241*) and another has associated smoking with renal adenoma (*242*).

Other possible risk factors that have been reported include coffee and tea consumption, artificial sweeteners, high body mass index (maintained from 20 years of age), high dietary animal protein and fat, lower educational levels, long-term analgesic use, and diuretics (*226,227,231,237,238,241,243,244*). Of these, the evidence is least consistent for beverage consumption, artificial sweeteners, other dietary factors and socioeconomic status, and strongest for high body mass index and drug use (phenacetin and diuretics).

Occupational Factors

Although a number of epidemiologic studies have reported some association between occupation and renal cancer, clear occupational determinants have yet to be demonstrated, and it is considered that much epidemiologic research is needed to further define and quantify potential risks (*226*). Occupational exposures in North America, where at least one study has reported an association with increased kidney cancer rates, include asbestos (*245,246*), coke-oven emissions in the steel industry (*247*), printing press chemicals (*248*), laundry- and dry-cleaning agents (*249–252*), exhaust fumes in truck drivers (*239*), petroleum, tar and pitch products (*231,253–257*) and aviation and jet fuels (*258*). In these studies, information on smoking history was rarely available, so that its possible influence could not be determined.

A study of renal cancer examining the relationship with occupation as defined in the 1960 Census in Sweden, where the incidence rates are higher than in the United States, did not detect increased risk for hearth and furnace workers in the steel industry, printing workers, laundry- and dry-cleaner workers or workers in petroleum refineries and gasoline stations (*259*). Instead, the Swedish study reported an increase in incidence of renal cell cancer among health care professionals.

Renal Cancer and Hydrocarbon, Solvent or Petroleum Product Exposure

Several of the occupations listed above involve exposure to certain classes of chemicals that may fall into the CIGA category. Besides CIGA, however, non-CIGA compounds are also present, making it difficult to attribute elevations in risk with a unique exposure (e.g., CIGA). In a recent population-based case–control study, Kadamani et al. (*257*) did not observe statistically significant associations between renal cell carcinoma and high occupational exposure to hydrocarbons in males (OR 1.6; 95% CI 0.7–3.6) or in females (OR 0.8; 95% CI 0.3–2.3). The authors, however, noted a positive exposure–response relationship for those with older ages and for workers with the greatest duration of exposure.

The synthetic solvents that have been widely used in dry cleaning include one chemical shown in rodent tests to be a CIGA, namely, tetrachloroethylene, as well as Stoddard and 140F solvents, which are mixtures of hydrocarbons including straight and branched chain paraffins. Several studies analyzing proportional mortality data on laundry- and dry-cleaning workers in various states of the United States reported elevated risks for kidney cancer (*249–252*). More recent studies that were better designed, however, have not substantiated the earlier findings. No statistically significant elevations in kidney cancer risks were detected in a study of dry-cleaning workers by Blair et al. (*260*) [standardized mortality ratio (SMR) 0.5, 95% CI 0.1–1.8]. Likewise, significant elevations in kidney cancer risk in dry-cleaning workers were not observed by Lynge and Thygesen (*261*) [standardized incidence ratio (SIR) in males 1.5, 95% CI 0.6–3.3; in females 0.6, 95% CI 0.2–1.4], or Brown and Kaplan (*262*) (SMR 2.0, 95% CI 0.55–5.17). In considering occupational exposure to solvents as a general chemical category, Harrington et al. (*165*) found no relationship with renal cancer (OR 1.0, 95% CI 0.2–4.9), although the statistical power of this study, as with most of the others, was acknowledged by the authors as sufficient to identify only large risk estimates.

Siemiatycki et al. (*258*) conducted a population-based case-referent study in Montreal on cancer associations with exposure to 12 petroleum-derived liquids. These various mixtures included automotive and aviation gasolines and distillate jet fuel. Aviation gasoline differs in composition from the automotive counterpart by its high content of alkylate naphthas, constituted mainly of branched alkanes (*258*). No statistically significant risk of renal cancer was found with exposure to automotive gasoline (OR 1.2, 90% CI 0.8–1.6). Statistically significant elevations, however, were noted at the 90% confidence level with exposure to aviation gasoline (OR 2.6, 90% CI 1.2–5.8) and to jet fuel (OR 2.5, 90% CI 1.1–5.4). Six of the seven cases with exposure to aviation gasoline also had exposure to jet fuel, making it difficult to distinguish a unique exposure. In depth analyses of the two associations using logistic regression methods indicated, however, a greater role for aviation gasoline than for jet fuel.

Wong and Raabe (*263*) conducted a quantitative meta-analysis by cancer site of petroleum industry employees from the United States, Canada, United Kingdom, Europe, Australia, and Japan, critically reviewing almost 100 published and unpublished epidemiological reports. Standardized mortality ratios observed for kidney cancer in the industry as a whole were similar to those for the general population. Results from refinery studies

ranged from non-significant deficits to nonsignificant excesses. However, the possibility of an elevated kidney cancer risk was raised for one specific group within the industry. Drivers among British distribution workers (*263*) showed borderline significance for excess kidney cancer mortality. Wong and Raabe (*263*) concluded that additional data, particularly involving exposure to downstream gasoline, are needed to resolve the issue.

In a large population-based case–control study adjusted for the confounding factors of age and cigarette smoking, no overall association (OR 1.0, 95% CI 0.7–1.4) was observed between risk for renal cell cancer and employment in a range of occupations with potential for exposure to petroleum products (*264*). There was, however, a small excess risk among gasoline station attendants (OR 1.2, 95% CI 0.6–2.3), which increased with duration of employment, although individual point estimates and tests for trends were not statistically significant. A case–control study on a combined cohort of approximately 100,000 male refinery workers from five petroleum companies, sponsored by the American Petroleum Institute (*265*), suggested increases in kidney cancer risk for laborers [relative risk (RR) 1.9, 95% CI 1.0–3.9], workers in receipt, storage and movements (RR 2.5, 95% CI 0.9–6.6), and refinery unit cleaners (RR 2.3, 95% CI 0.5–9.9) when compared with a reference group of office workers, professionals, and technicians. In the cohort there were 102 kidney cancer cases among 18,323 deaths.

In evaluating unleaded gasoline, 55 relevant studies were reviewed by the U.S. EPA (*11*) to determine whether there was any epidemiologic evidence for an association between gasoline exposure and cancer risk. The evidence for drawing causal inferences between unleaded gasoline and cancer was considered inadequate under the EPA guidelines for cancer risk assessment (*183*). As Enterline and Viren (*266*) have emphasized in their review on the epidemiology of renal cancer and gasoline exposure, most of the studies have not been designed or analyzed with a specific hypothesis associating gasoline exposure and renal cancer in mind. The cohort studies of petroleum workers do not lend themselves for a comparison because they shed no light on gasoline exposure *per se*. Exposures in these studies have been varied, and the only common element is the place of work. Thus, the individuals in the cohort who had the exposure of interest, i.e., gasoline or a specific fraction, cannot be identified.

As a general conclusion from the foregoing, small risks cannot be excluded for specific job categories, but the association between human kidney cancer and exposure to petroleum distillates, if there is one, does not suggest high risks for the types of exposures that have occurred in the past.

Summary of the Evidence on the Renal Effects of CIGA

Several lines of evidence establish an association between exposure of the male rat to CIGA and nephrotoxicity and strongly support an association between this nephrotoxicity and renal tubule tumors.

Association between α_{2u}-Globulin Accumulation and Nephropathy

The information that supports an association between α_{2u}-g accumulation and male rat-specific renal toxicity following CIGA administration is summarized below.

Organic fuels, solvents, and other compounds examined in this report induced an excessive accumulation of hyaline droplets in the renal proximal tubule epithelium of male rats. In contrast, where tested, mice and female rats showed no evidence of hyaline droplet accumulation from chemical treatment. For about half the substances listed in Table 3, the accumulating protein in the hyaline droplets has been identified, and in all cases it was α_{2u}-g.

There is convincing evidence that the excessive accumulation of hyaline droplets is followed sequentially by tubule epithelial cell necrosis, granular cast formation, and other aspects of α_{2u}-g nephropathy in the male rat. Five hyaline-droplet inducers were tested in species other than the mouse or the rat, although possibly not as rigorously. Characteristic lesions were observed in the male rat kidney for these five substances, but there was no apparent nephrotoxic response in the female rat or any other species tested, which included mice (all five substances), hamsters (jet fuels), guinea pigs (decalin), dogs (jet fuels, decalin, *d*-limonene, and methyl isobutyl ketone), and monkeys (methyl isobutyl ketone and gasoline).

The increase in hyaline droplets, tubule dilation caused by granular cast formation, tubule cell proliferation, and medullary mineralization is dose dependent as shown by research studies conducted to date with four model CIGA (decalin, *d*-limonene, unleaded gasoline and TMP). In general, the chronic administration of CIGA to male rats and the ensuing nephrotoxicity enhanced the age-related renal degenerative process by exacerbating spontaneous CPN.

Specialized studies involving rats of varying age, castrated or estrogen-treated rats, the NBR strain, and α_{2u}-g-treated female rats have shown that development of the early features of α_{2u}-g nephropathy is dependent on the presence of α_{2u}-g formed in the liver.

For three of the eight CIGA carcinogens (hexachloroethane, tetrachloroethylene and 1,4-DCB), renal toxicity was observed in chronic studies of female rats or mice, but the renal toxicity appeared to be less severe or qualitatively different, not involving the same spectrum of discrete lesions associated with α_{2u}-g nephropathy.

CIGA bind reversibly to α_{2u}-g as a target molecule, and the renal accumulation of α_{2u}-g and hyaline droplet formation may be explained by chemically induced impairment of α_{2u}-g catabolism after reabsorption of the complex by the proximal tubule.

The data available on the formation of complexes between CIGA and members of the lipocalin superfamily support a conclusion that the reaction with α_{2u}-g is unique to male rats. For example, *in vivo* data showing that retinol can bind to α_{2u}-g indicate that binding alone is insufficient to demonstrate that α_{2u}-g will accumulate in hyaline droplets.

Association between α_{2u}-Globulin Nephropathy and Renal Cancer

Based on information from the rodent bioassays examined in this report and additional key data, features of renal tumors occurring subsequent to the development of nephropathy in the male rat can be identified.

The eight CIGA carcinogens produced hyperplasia, adenomas and adenocarcinomas in the renal tubule of the male rats. All eight that produced renal tumors in male rats also produced nephrotoxicity in male rats. Specifically, the nephrotoxicity that preceded renal tumor formation in male rats was characteristic of the form associated with α_{2u}-g and distinguishable from other forms of toxicity associated with nonCIGA renal toxicants.

The incidence of renal tumors produced in the male rat by the eight CIGA carcinogens was relatively low. These tumors were morphologically indistinguishable from chemically induced renal tubule neoplasia and renal tubule neoplasia that occurs rarely, but spontaneously, in male and female rats.

The renal tumors produced by the eight CIGA carcinogens occurred late, usually being found at sacrifice, metastasized rarely, and were not life threatening.

For *d*-limonene, the one CIGA examined in an initiation–promotion study comparing male rats of the NBR strain with a conventional strain, α_{2u}-g accumulation was necessary for promotion of male rat renal tubule tumors initiated by EHEN. CIGA appear to be nongenotoxic or only marginally so and may, therefore, not depend on direct genetic injury as the mechanism for tumor induction.

Information Reducing Confidence in the Conclusion That the α_{2u}-g Response is Specific to Male Rats

Although the evidence available to date supports the hypothesized association between α_{2u}-g accumulation and renal tubule tumors in the male rat, confidence in this assertion would be improved if the same results were found in an expanded database. In addition, there is a paucity of data on the lipocalin superfamily in general.

Pathologic accumulation of hyaline droplets is a reaction to excessive protein load not exclusively related to α_{2u}-g accumulation. The accumulating protein responsible for hyaline droplet formation has not been identified for about half of the substances listed in Table 3.

Data sufficient to demonstrate interdependence of the lesions in the proposed pathologic sequence from hyaline droplet accumulation to chronic toxicity exist for only a few substances. Data to define dose–response relationships for tubule cell necrosis and its association with cell proliferation are even more limited, as is dose-related information on increased cell proliferation rates over chronic exposure periods. The mechanism whereby α_{2u}-g accumulation leads to cell death has not been established.

Hexachloroethane, tetrachloroethylene, and 1,4-DCB produced renal toxicity in female rats or mice, indicating that some CIGA may have additional effects on rodent kidney not limited to the α_{2u}-g-induced sequence of lesions.

Information on a possible association between renal cell tumors and CIGA exposure in humans is inconclusive because exposures in the reviewed epidemiologic studies have been to both CIGA and non-CIGA compounds. Information on the *in vivo* binding of CIGA with other lipocalins in the α_{2u}-g superfamily of proteins suggests, but does not conclusively demonstrate, that toxicity in humans does not occur via this mechanism.

Although there are major quantitative and qualitative differences between male rats and humans in the amounts of protein excreted in urine, little is known concerning the relative quantities of low molecular weight proteins that are normally filtered by the human glomerulus and reabsorbed by the renal tubules for catabolism.

The scientific data summarized above were used to draw conclusions concerning the role of α_{2u}-g accumulation and hyaline droplet formation in producing male rat-specific nephropathy and renal tubule neoplasia and to determine the relevance of this information for assessing human risk.

Conclusions and Research Priorities

The available information on CIGA-associated renal tubule carcinogenesis in the male rat can be described by a suggested sequence of critical cellular and molecular events. According to this description, the reaction of a lipophilic compound with the low molecular weight protein α_{2u}-g appears to lead to the formation of a complex that is more resistant to lysosomal degradation than the unreacted protein. This results in a shift in balance between reabsorption and hydrolysis, leading to an abnormal accumulation of the protein in the P2 segment of the renal tubule of male rats. If exposure ceases after a short time period, recovery is complete. Continued exposure, however, results in a nephrotoxic response that is less readily reversible and a sustained increase in cell turnover, enhancing the chance that spontaneous molecular alterations occurring in DNA in the kidney may be replicated rather than repaired.

Because there are substantial data gaps, especially with regard to the expected response in humans and the critical linkages between single cell necrosis and increased cell turnover, and tubule hyperplasia and renal tubule cancer, the α_{2u}-g syndrome should be considered a satisfactory working hypothesis but not a proven mechanism of action to describe renal tubule cancer in male rats exposed to CIGA. As such, it provides an empirical description of a series of observed events in laboratory animals that could be modified or expanded upon as additional information becomes available.

Despite these limitations and the fact that α_{2u}-g accumulation also exacerbates CPN, chemically induced α_{2u}-g-associated nephropathy in the male rat can be distinguished histopathologically from other chemically induced nephrotoxicities and also from CPN. Excessive hyaline droplet formation is the earliest morphologic manifestation and an important characteristic, although a chemical can be described as a CIGA with certainty only when there is a positive identification of α_{2u}-g in the hyaline droplets. Other observable characteristics indicative of possible CIGA-induced nephrotoxicity include single cell necrosis of the tubule epithelium, granular casts at the junction of the inner and outer stripes of the outer medulla caused by sloughing of necrotic cells, mitotic figures indicative of regeneration or increased cell turnover, and often medullary mineralization.

The hepatic synthesis of the lipocalin, α_{2u}-g, is not known to occur normally in any species other than the male rat. Alpha$_{2u}$-globulin-induced nephropathy is also a distinct entity specific to the male rat among the laboratory species and genders tested to date. The characteristic nephropathy has been found only when α_{2u}-g formed in the liver is present. Thus, female rats do not

develop hyaline droplets when exposed to CIGA unless they have been administered α_{2u}-g isolated from male rat urine.

NBR rats that do not possess the mRNA for liver α_{2u}-g and castrated male rats also respond differently from conventional male rats. Of the other species, the mouse is the most thoroughly tested. Although the mouse produces large amounts of a structurally similar lipocalin, MUP, this protein is not known to bind with CIGA; it is not reabsorbed from the urine, and the mouse does not develop kidney tumors or the characteristic nephropathy seen in male rats. Limited testing in dogs, hamsters, guinea pigs, and monkeys has not shown hyaline droplet accumulation or nephropathy in these species, further suggesting that the α_{2u}-g syndrome occurs specifically in the male rat.

With regard to the potential for a chemical to produce renal tubule neoplasia in the male rat, there are common characteristics among the substances evaluated in this report. First, these compounds (and their CIGA-binding metabolites) have little or no mutagenic activity in standard batteries of tests, they are lipophiles and not electrophilic substances, and they do not appear to bind covalently to DNA. Second, the nephrotoxic response characteristic of CIGA always preceded renal tumor formation in the male rat, a finding not characteristic of classical renal carcinogens. Third, for all eight model substances examined in this report, additional sexes/species/strains were tested, and the increased incidence of renal tumors was found only in the male rat.

The manner in which the human male responds to CIGA has not been tested directly, although there are human proteins that, like α_{2u}-g, are members of the lipocalin superfamily. Human urine also contains small amounts of a sex-linked urinary protein. Epidemiologic studies have focused on glomerulonephritis or renal cancer and organic chemical exposure, in general, and not on renal tubule damage and CIGA exposure, and they do not yield results useful for testing the hypothesized mechanism in humans. Protein overload can result in formation of hyaline droplets in human kidneys, although there is no evidence that this response has occurred from lipocalin accumulation. While it is not possible to resolve the issue of how the human renal tubule responds to CIGA exposure from the available data, the uniqueness of the male rat response among the tested laboratory species and the high doses needed to produce an effect, even in the male rat, suggest that this reaction would not occur in humans, especially under typical conditions of exposure.

Several factors complicate the analysis of data on the renal effects of CIGA. Distribution studies of the compounds examined to date and information on reversible binding of CIGA to α_{2u}-g indicate that all of the substance administered does not necessarily bind to α_{2u}-g. In fact, for d-limonene the presumed CIGA is only a minor metabolite. Thus, there is always the prospect that a non-CIGA parent chemical or non-CIGA metabolites are present in the kidney along with the α_{2u}-g-bound material. The possibility that these other moieties could be toxic to the kidney, and perhaps even cause cancer, needs to be taken into account. For example, tetrachloroethylene, in addition to showing α_{2u}-g nephropathy, displays evidence of renal toxicity typical of chlorinated hydrocarbons and appears to form metabolites active through the β-lyase pathway. This example demonstrates how other mechanisms may play some role in the observed results. Although it increases the complexity of the analysis, information suggesting other mechanisms are also operative in the kidney does not preclude a determination that the α_{2u}-g sequence is involved in some manner with the renal tumor response.

At present, there is insufficient information on CIGA and their metabolites to confidently predict activity on the basis of structural analogy. Recent research on structural correlations suggests that the presence of an electronegative atom for hydrogen bonding, lipophilicity, and steric volume are important considerations. Conformational changes or other structural alterations to the protein may also be necessary because binding of the compound in the protein pocket, alone, appears to be an insufficient condition to cause reduced digestibility of the protein.

Evidence of dose responsiveness between CIGA administration and the degree of hyaline droplet or α_{2u}-g formation has been demonstrated in several studies. These findings are frequently based on subjective histopathological criteria, however, limiting their usefulness for making quantitative judgments about the relative hazard potential of different chemicals.

It is also important to recognize that for various reasons (e.g., doses administered too low, animals killed before the latency period of these slow-growing tumors is attained, number of specimens and histological sections insufficient, competing toxicity in kidney or other organs), the entire pathologic sequence culminating in renal tubule neoplasia may not be demonstrated in all cases of CIGA administration. Thus, not all CIGA would be expected to demonstrate renal tubule neoplasia in the male rat in a 2-year animal bioassay. Such a finding would not negate the applicability of the hypothesized CIGA syndrome to the evaluation of nephropathy data.

Based on the cancer bioassays and research studies of CIGA, an increased proliferative response caused by chemically induced cytotoxicity appears to play a role in the development of renal tubule tumors seen exclusively in male rats. The male rat specificity of the response to CIGA administration is emphasized by negative findings in mice and female rats. These conclusions can probably be extended to analysis of human hazard potential, especially whenever human exposure to CIGA is not excessively high for sustained periods of time, when short-term tests for genotoxicity of the compound are negative, when the nephrotoxic response and increased cell turnover characteristic of CIGA have been demonstrated in the male rat, and other species/sex combinations were tested but renal tubule tumors were observed only in male rats.

Certain other studies would fill key data gaps needed to more firmly characterize the relationship between α_{2u}-g accumulation and renal tumor formation. Epidemiologic research should be conducted, if possible, in such a manner as to explore the applicability of the α_{2u}-g hypothesis to humans, and studies of nephropathy induced by CIGA should be extended to additional laboratory species, in particular the nonhuman primate.

Studies in animals have not focused enough on identifying the specific metabolite(s) that binds reversibly to α_{2u}-g. Without such information, comparisons of structure activity and mutagenicity with α_{2u}-g accumulation could be meaningless. Furthermore, studies of the *in vitro* and *in vivo* binding of CIGA to lipocalins present in humans, such as retinol-binding protein and α_1-acid glycoprotein, and to human urine protein 1, should be extended to include compounds in addition to TMP and *d*-limonene. If binding does occur, further testing should be done

to determine if these CIGA–lipocalin complexes can accumulate in the kidney of other species, particularly the nonhuman primate, thus having the potential for involvement in nephropathy.

In terms of carcinogenic potential, key data gaps should be closed for certain model CIGA; for example, animal bioassays are needed for decalin and TMP. Future bioassays of potential CIGA should include cell proliferation studies. Additional "stop" studies would permit better characterization of the lesions from subchronic to chronic time frames, and further evaluation of CIGA and non-CIGA chemicals for carcinogenic potential in the NBR rat, which appears not to synthesize α_{2u}-g, is warranted.

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