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Alpha $_{2u}$-Globulin: Association with Chemically Induced Renal Toxicity and Neoplasia in the Male Rat

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ALPHA$_{2u}$-GLOBULIN: ASSOCIATION WITH CHEMICALLY INDUCED RENAL TOXICITY AND NEOPLASIA IN THE MALE RAT

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Contents

List of Tables vi
List of Figures vii
List of Abbreviations viii
External Peer Reviewers ix
Preface xii

I. Executive Summary 1

II. Introduction 6

Part 1-Nephrotoxicity

III. Hyaline Droplets and Alpha$_{2u}$-globulin; Physiology and Biochemistry 11
- A. Filtration, Reabsorption, and Catabolism of Low-Molecular-Weight Proteins by the Kidney 11
- B. Hyaline Droplets in Renal Tubules 13
- C. Factors Affecting Kidney Accumulation of Low-Molecular-Weight Proteins 14
- D. The Alpha$_{2u}$-globulin Superfamily of Proteins 15
- E. Characteristics of Alpha$_{2u}$-globulin 19
- F. Sex and Species Comparison of Urinary Protein Content of the Lipocalin Superfamily 20
- G. Noncovalent Binding to Alpha$_{2u}$-globulin and its Homologues 21
  - 1. Chemical entities bound to alpha$_{2u}$-globulin 21
  - 2. Nature of the association 22
  - 3. Binding of CIGA to other macromolecules 23
  - 4. Specificity of the interaction of CIGA with alpha$_{2u}$-globulin 23
- H. Catabolism of Alpha$_{2u}$-globulin Complexed with CIGA 24
- I. Structure-Activity Relationships for CIGA 26

IV. Alpha$_{2u}$-globulin Nephropathy 27
- A. Pathologic Features of Alpha$_{2u}$-globulin Nephropathy 27
- B. Rat Urine Chemistry and CIGA 30
- C. Species Variation in the Renal Response to CIGA 30
- D. Factors Affecting the Expression of Alpha$_{2u}$-globulin Nephropathy 32
  - 1. Age-related effects 32
  - 2. Effect of hormone manipulation 33
  - 3. Genetic variants 33
  - 4. Alpha$_{2u}$-globulin infusion in female rats 33

Contents (cont.)

E. Chronic Progressive Nephropathy 34
F. Renal Toxicity Observed in Chronic Bioassays of Chemicals that Induced Kidney Tumors in Rats 35

Part 2-Carcinogenicity

V. Pathologic Features of Renal Carcinogenesis Induced by Classical Carcinogens 41
A. Early Nephrotoxicity 42
B. Karyomegaly 42
C. Tubule Cell Hyperplasia 42
D. Adenoma 43
E. Adenocarcinomas and Carcinomas 43
F. Tumor Progression 43
G. Site of Origin of Renal Tubule Tumors 44

VI. Neoplastic and Preneoplastic Lesions Observed in the 2-Year Bioassays 44
A. Generic Considerations 44
B. Renal Tumor Incidence 50
C. Histogenesis of Renal Tumors 50
D. Renal Tumor Latency and Progression 51
E. Induction of Other Tumor Types 51

VII. Additional Evidence Concerning the Renal Carcinogenicity of CIGA 52
A. Genetic Toxicology Studies 52
B. Initiation-Promotion Sudies 55

VIII. Comparison of CIGA with Classical Renal Carcinogens 57

IX. Evidence Concerning Human Kidney Cancer 58
A. Morphology and Histogenesis 58
B. Incidence and Mortality 59
C. Environmental and Lifestyle Factors 60
D. Occupational Factors 61
E. Renal Cancer and Hydrocarbon, Solvent, or Petroleum Product Exposure 61

X. Evidence for Dose- and Time-Dependent Progression from Early to Late Lesions 63
A. Association Between CIGA, Hyaline Droplet Formation, and $Alpha_{2u}$-globulin Accumulation 64
B. Association Between Hyaline Droplet Formation, Cell Necrosis, and Tubule Cell Regeneration 65

Contents (cont.)

C. Progression to Cast Formation, Tubule Dilation, and Mineralization 68
D. Association Between CIGA and Chronic Progressive Nephropathy 70
E. Evidence Concerning Progression from Nephrotoxicity to Renal Neoplasia 71

Part 3-Evaluation of the Hypothesis

XI. Summary of the Evidence on the Renal Effects of CIGA 73
A. Association Between $Alpha_{2u}$-globulin Accumulation and Nephrotoxicity 73
B. Association Between Nephrotoxicity and Renal Cancer 74
C. Information Reducing Confidence in the Conclusion that the $Alpha_{2u}$-globulin Response is Specific to the Male Rat 75

XII. Conclusions 76

XIII. Research Needs 78

Part 4-Science Policy

XIV. Background and Introduction 81
XV. Basis for Science Policy on Male Rat Kidney Tumors 81
A. Low-Molecular-Weight Proteins in the Rat 81
B. Progression from Chemically Induced $Alpha_{2u}$-globulin Accumulation to Nephropathy and Neoplasia 82
1. Overview 82
2. Specificity of the sequence to the male rat 83

XVI. Science Policy Statement 85
XVII. Guidance for Evaluating Chemically Induced Male Rat Renal Tubule Tumors 85
A. Renal Tubule Tumors in Male Rats and $Alpha_{2u}$-globulin Accumulation 86
B. Additional Information Useful for the Analysis 87
C. Use of the Data for Risk Assessment 88

XVIII. Nephropathy as a Toxic Endpoint 89

Appendix - Non-neoplastic Effects of Hyaline Droplet Inducers 90

References 99

List of Tables

Number | Page

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 7
2. Superfamily of lipophilic ligand-binding carrier proteins 17
3. Summary of the histopathology and lesion progression reported in $alpha_{2u}$-globulin-associated nephrotoxicity 28
4. Summary of the histopathology of spontaneous chronic progressive nephropathy of aging rats 35
5. Summary of data on non-neoplastic and Pre-Neoplastic kidney lesions in male rats associated with eight model compounds that induced renal tumors in 2-year bioassays 37
6. Summary of data from 2-year bioassays on non-neoplastic and pre-neoplastic kidney lesions in mice and female rats exposed to eight model compounds that induced renal tumors in male rats 38
7. Incidences of renal tubule preneoplasia and neoplasia in rats taken from 2-year bioassays on eight model substances 45
8. Incidences of renal tubule preneoplasia and neoplasia in rats taken from 2-year bioassays on chlorothalonil and trichloroethylene 48
9. Summary of genotoxicity data for eight selected male rat kidney carcinogens 53
10. Summary of genotoxicity data for two selected non-CIGA male rat kidney carcinogens 54
11. Incidence of medullary mineralization in male rats during inhalation exposure to unleaded gasoline 70

List of Figures

Number		Page
1.	Diagram of zonation and tubule segmentation in rat kidney	10
2.	Schematic representation of endocytic uptake of filtered proteins	12
3.	Schematic representation of the uptake and fate of $alpha_{2u}$-globulin complexed with a CIGA in hydrocarbon nephrotoxicity	25
4.	Dose-response relationship between renal hyaline droplet accumulation (D) and [^{3}H]-thymidine labeling index	66
5.	Effect of 0-2,000 ppm unleaded gasoline on continuous uptake of [^{3}H]-TdR	67
6.	Time-sequence for the development of hyaline droplets, regenerating tubule epithelium, and tubule dilation in male F344 rats	69

List of Abbreviations

AAT	aspartate aminotransferase
ABS	chromosome aberrations in CHO cells
α_{2u}-g	alpha$_{2u}$-globulin
CHO	Chinese hamster ovary
CI	confidence interval
CIGA	Chemical(s) Inducing alpha$_{2u}$-Globulin Accumulation
CPN	chronic progressive nephropathy
1,2-DCB	1,2-dichlorobenzene
1,4-DCB	1,4-dichlorobenzene
DEN	diethylnitrosamine
DMN	dimethylnitrosamine
EHEN	N-ethyl-N-hydroxyethylnitrosamine
FBPA	N-(4'-fluoro-4-biphenylyl)acetamide
H & E	hematoxylin and eosin
IRDC	International Research and Development Corporation
MLA	TK-gene mutation assay in L5178Y cells
MTD	maximum tolerated dose
MUP	mouse major urinary protein
NAG	N-acetyl-β-glucosaminidase
NBR	NCI Black-Reiter rat
NTP	National Toxicology Program
NCI	National Cancer Institute
OR	odds ratio
RR	relative risk
P1	first convoluted segment of proximal tubule
P2	second convoluted segment of proximal tubule
P3	pars recta of proximal tubule
SAL	Salmonella
SCE	sister chromatid exchange
SDS	sodium dodecyl sulfate
SEER	Surveillance, Epidemiology and End Results Program of NCI
SIR	standardized incidence ratio
SLRL	sex-linked recessive lethal
SMR	standardized mortality ratio
TK	thymidine-kinase
TMP	2,2,4-trimethylpentane
TMPOH	2,4,4-trimethylpentanol
UDS	unscheduled DNA synthesis

External Peer Reviewers

An interim draft, prepared in September 1990, was evaluated at a two-day Peer Review Workshp sponsored by the EPA Risk Assessment Forum. The meeting, held in Gaithersburg, Maryland, on November 13 and 14, 1990, was chaired by Dr. Richard Griesemer, director of the Division of Toxicology Research and Testing, National Toxicology Program (NTP). Invited participants are listed below. The Workshop proceedings will be published separately. Announcement of their availability will be through the Federal Register.

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On March 27, 1991, EPA's Environmental Health Committee of the Science Advisory Board reviewed and unanimously approved a draft report (EPA/625/3-91/019A). The FIFRA Science Advisory Panel was represented by Dr. Joe H. Grisham. The Executive Committee approved the report of the Environmental Health Committee at their July 23, 1991 meeting, although one member of the Executive Committee encouraged greater attention to the uncertainties concerning the hypothesis. In summary, the Science Advisory Board concurred with the position of the Risk Assessment Forum report.

The Technical Panel and the Risk Assessment Forum also acknowledge with appreciation the special contributions of Joseph McLaughlin, who greatly assisted in the preparation of the epidemiology section, and Jerry Blancato, Margaret M.L. Chu, Ila Cote, Richard N. Hill, and the members of the Risk Assessment Forum's Cancer Oversight Group (William Farland, Edward Ohanian, Vanessa Vu, and Jeanette Wiltse) for their thoughtful comments.

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[a] *Also reviewed a preliminary draft. Other reviewers of this draft were Carl Alden of Procter and Gamble's Miami Valley Laboratories, and Michael Lipsky of the University of Maryland Medical School.*

Preface

The U.S. Environmental Protection Agency (EPA) Risk Assessment Forum was established to promote scientific consensus on risk assessment issues and to ensure that this consensus is incorporated into appropriate risk assessment guidance. To accomplish this, the Risk Assessment Forum assembles experts from throughout the EPA in a formal process to study and report on these issues from an Agency-wide perspective.

For major risk assessment activities, the Risk Assessment Forum has established Technical Panels to conduct scientific review and analysis. Members are chosen to assure that necessary technical expertise is available. Outside experts may be invited to participate as consultants or, if appropriate, as Technical Panel members.

The use of male rat kidney tumors in risk assessment has been the subject of much recent discussion. For a certain group of chemicals, investigators have reported renal tubule tumor formation in male rats as the sequela of renal toxicity commencing with an excessive accumulation of the protein, alpha$_{2u}$-globulin (α_{2u}-g), in renal tubules. Renal tubule tumor formation with protein accumulation has not been observed in female rats or other tested species, most notably the mouse. The NCI Black Reiter rat, which does not produce α_{2u}-g, also fails to show a proliferative response in the kidney or evidence of a promotional effect when exposed to chemicals that induce protein droplet accumulation in male rats of other strains; its response has not been tested in a conventional 2-year animal bioassay. Some scientists apply the observations seen in animals to conclude that any renal tubule tumor in male rats observed in connection with α_{2u}-g accumulation is a species-specific effect inapplicable to human risk assessment. Other scientists argue that more information on humans is needed and that all male rat kidney tumors should continue to be considered as relevant to human risk as other tumors.

Because the question is relevant in assessing risk for a number of chemicals of interest to EPA, the Risk Assessment Forum established a Technical Panel to assemble and evaluate the current evidence and to develop science policy recommendations for Agency-wide use. This document is the product of that effort.

The literature review supporting this document is current as of July 1, 1991.

I. Executive Summary

This report of a Technical Panel of the U.S. Environmental Protection Agency (EPA) Risk Assessment Forum describes conditions under which the Forum advises EPA risk assessors against using information on certain renal tubule tumors or nephrotoxicity to assess human risk. Risk assessment approaches generally assume that chemicals producing tumors in laboratory animals are a potential cancer hazard to humans. For most chemicals, including many rodent kidney carcinogens, this extrapolation remains appropriate. The scientific studies reviewed by the Technical Panel indicate, however, that some other chemicals induce accumulation of alpha$_{2u}$-globulin (α_{2u}-g), a low-molecular-weight protein, in the male rat kidney. The α_{2u}-g accumulation initiates a sequence of events that appears to lead to renal tubule tumor formation. Female rats and other laboratory mammals administered the same chemicals do not accumulate low-molecular-weight protein in the kidney and they do not develop renal tubule tumors. Since humans appear to be more like other laboratory animals than like the male rat, in this special situation, the male rat is not a good model for assessing human risk.

The analysis of the scientific studies and related science policy set out in this report each stress the need for full scrutiny of a substantial set of data to determine when it is reasonable to presume that renal tumors in male rats result from a process involving α_{2u}-g accumulation and to select appropriate procedures for estimating risks to humans under such circumstances. The report also defines situations that suggest different approaches and it calls for research to clarify unanswered questions regarding the mechanisms accounting for the response in male rats.

Alpha$_{2u}$-globulin and Renal Lesions

In the male rat, the production of renal tumors by chemicals inducing alpha$_{2u}$-globulin accumulation (CIGA) is preceded by the renal lesions ascribed to α_{2u}-g-associated nephropathy. The involvement of hyaline droplet accumulation in the early nephrotoxicity associated with CIGA is a major difference from the sequence seen for classical carcinogens. The pathologic changes that precede the proliferative sequence for classical renal carcinogens also include a form of early nephroxicity, but no apparent hyaline droplet accumulation.

Investigations performed in multiple laboratories over the last decade have demonstrated a consistent association between hyaline droplets containing α_{2u}-g and production of certain lesions in the male rat kidney. These renal lesions are not found in mice, female rats, or other laboratory

species tested. The histopathological sequence in the male rat consists of the following:

- an excessive accumulation of hyaline droplets containing α_{2u}-g in renal proximal tubules;
- subsequent cytotoxicity and single-cell necrosis of the tubule epithelium;
- sustained regenerative tubule cell proliferation, providing exposure continues;
- development of intralumenal granular casts from sloughed cell debris associated with tubule dilation, and papillary mineralization;
- foci of tubule hyperplasia in the convoluted proximal tubules; and finally,
- renal tubule tumors.

Biochemical studies with model compounds show that CIGA or their metabolites bind specifically, but reversibly, to male rat α_{2u}-g. The resulting α_{2u}-g-CIGA complex appears to be more resistant to hydrolytic degradation by lysosomal enzymes than native, unbound α_{2u}-g. Inhibition of the catabolism of α_{2u}-g, a protein only slowly hydrolyzed by renal lysosomal enzymes under normal physiological conditions, provides a plausible basis for the initial stage of protein overload in the nephropathy sequence.

Comparison with Classical Carcinogens

It is instructive to compare CIGA renal carcinogens with other renal carcinogens. Several genotoxic chemicals recognized as classical inducers of rodent kidney tumors have been used to study the pathogenesis of renal tubule cancer in laboratory animals. In general, these prototypic renal carcinogens produce tumors in both males and females. Although the wide range of chemicals represented suggests multiple mechanisms of action, many of the classical renal carcinogens or their active metabolites are electrophilic species able to bind covalently to macromolecules and likely to form DNA adducts in the kidney. In contrast, CIGA renal carcinogens are not known to react with DNA and are generally negative in short-term tests for genotoxicity. CIGA renal carcinogens also interact with α_{2u}-g in a reversible and noncovalent manner.

CIGA produced minimal changes in urine chemistry and very little or no glomerular dysfunction in male rats. The mild tubule toxicity of CIGA, in contrast to the obvious urinary changes induced by renal toxins such as mercuric chloride or hexachlorobutadiene, is characteristic of CIGA and is consistent with the notion that CIGA do not bind covalently to α_{2u}-g.

Classical renal carcinogens, such as certain nitrosamines, induce renal tubule cancer in rats or mice with high incidence, minimal duration of exposure, and clear dose-response relationships. There is usually no absolute sex-specificity, although males and females may be susceptible to different degrees. In contrast, the renal tumors produced by the eight model carcinogens examined in this report tended not to be life-threatening,

occurred late in life, usually being found at terminal sacrifice, and were frequently microscopic. Even though the maximum tolerated dose was exceeded for some of the eight model carcinogens, the renal tumor incidence rate, adjusted for intercurrent mortality, was never greater than 28 percent. An increase in renal tubule tumors was not found in mice or female rats exposed to these chemicals. Initiation/promotion studies with gasoline, trimethylpentane (TMP) and d-limonene in Fischer 344 rats showed that these CIGA promoted atypical tubule cell hyperplasia and/or renal tubule tumors in males but not in females. In contrast, d-limonene did not promote these lesions in males of the NCI Black-Reiter (NBR) strain in the same initiation/promotion model. Such differences in potency and species-, strain- and sex-susceptibility suggest that CIGA renal carcinogens act via different mechanisms than classical renal carcinogens.

Renal tubule tumors produced by CIGA carcinogens also have features in common with other renal tubule tumors observed in the male rat. For renal carcinogens, in general, there is a continuum of chemically induced steps from atypical hyperplasia through microscopic adenomas to macroscopic adenocarcinomas or carcinomas. Renal tubule tumors induced by the eight model carcinogens are morphologically indistinguishable from those induced by classical carcinogens. Likewise, the sequence of development of CIGA carcinogen-induced renal tumors from tubule cell hyperplasia to carcinoma appears identical. Furthermore, none of these chemically induced tumors can be differentiated from spontaneous tumors.

Other Considerations

All eight of the model carcinogens examined in this report were also capable of producing renal tubule hyperplasia in male rats. In general, this hyperplasia became more severe with increasing dose. The occurrence of these preneoplastic lesions together with the neoplastic lesions provides indirect evidence of progression that is in accord with generally accepted views on renal tubule tumor formation.

Dose- and time-related associations between the administration of CIGA to male rats and the various histological stages have been observed. These relationships were demonstrated between CIGA administration for both hyaline droplet formation and α_{2u}-g accumulation. Although the relationships between increased hyaline droplets and cell necrosis or between cell necrosis and cell regeneration have not been quantified, a correlation between hyaline droplet response and the number of cells excreted in the urine has been observed for CIGA. Dose-response relationships between hyaline droplet accumulation and proximal tubule cell proliferation have been shown for TMP and unleaded gasoline. Clear dose-response relationships were demonstrated between linear mineralization in the renal medulla and incidence of renal tubule neoplasia in male rats in several bioassays. A recent study of d-limonene demonstrated a relationship between severity of nephropathy and renal tubule cancer in male rats.

The Technical Panel is not aware of any epidemiologic study that has been designed or conducted specifically to examine the applicability of the

CIGA hypothesis to renal cell cancer in humans. Several epidemiologic studies were reviewed for this report, but they are of limited value for this analysis because they involved exposure to complex blends, such as gasoline, or otherwise involved multiple exposures to both CIGA and non-CIGA. In addition, these studies were of limited statistical power and were not able to account for possibly confounding factors, such as smoking or obesity, which are known to influence renal cell cancer rates. In a few studies, slight increases in risk of renal cell cancer have been observed; however, the other factors described above could have easily accounted for the increased risk. These studies, therefore, are considered inadequate for purposes of exploring the relevance of the α_{2u}-g hypothesis in humans.

Low-molecular-weight proteins that probably have a three-dimensional structure similar to α_{2u}-g have been identified in mice and other species, including humans. In vitro studies have shown that the active metabolite of TMP forms complexes with some of these proteins. Other in vitro studies indicate, however, that reversible binding does not necessarily increase resistance to hydrolytic degradation, a feature apparently required for hyaline droplet formation.

Extensive studies of mice, whose urine contains large amounts of mouse major urinary proteins (MUP), have found no evidence of renal lesions similar to those associated with the α_{2u}-g syndrome. Thus, the presence of a structurally related protein, even in large quantities in the urine, does not imply that another species will respond in a manner similar to the male rat.

The form of α_{2u}-g that originates in the liver of the male rat is not detected in the female rat. Like the mouse, the female rat shows no evidence of an α_{2u}-g-like nephropathy when exposed to CIGA. In cases where nephrotoxicity was observed in mice or female rats, it was less severe or qualitatively different from that in male rats and did not involve the spectrum of discrete lesions associated with α_{2u}-g accumulation in the male rat.

Specialized studies of rats, such as those involving immature, aged and castrated male rats, males of the NCI Black Reiter (NBR) strain (which does not synthesize α_{2u}-g in the liver), and injection of male rats with estrogen and female rats with α_{2u}-g, show that development of the early features of the specific nephropathy syndrome occurs only in the presence of α_{2u}- g. Very limited information from dogs, hamsters, guinea pigs, and monkeys also supports this statement. These studies further support the hypothesis that this α_{2u}-g-related nephropathy occurs specifically in the male rat.

Summary

In summation, the reversible binding of the compound to α_{2u}-g, which results in a shift in balance between reabsorption and hydrolysis and the accumulation of α_{2u}-g in hyaline droplets in the P2 segment of the renal tubule, provides a plausible explanation for the initial steps in a sequence of events leading to the formation of renal tubule tumors in the male rat. A sustained protein overload would result in single-cell necrosis in the tubule

epithelium and increased cell regeneration, with granular cast formation and papillary mineralization as indirect consequences. The increased proliferative response caused by chemically induced cytotoxicity may be a plausible reason for the development of renal tubule tumor in male rats. Thus, renal tubule tumors produced in male rats in association with CIGA-induced α_{2u}-g nephropathy should be distinguished from other renal tubule tumors in terms of use in human risk assessment.

II. 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 paper deals with a specific case, however, where the male rat seems to respond in a different manner than other laboratory species. The possibility of a unique response in the rat among laboratory animals raises questions about the applicability of certain rat data to other species, including humans. This document provides guidance for the assessment of such information.

A variety of organic chemicals have produced specific renal lesions in male rats in the form of a hyaline droplet nephropathy accompanied by accumulation of the protein, alpha$_{2u}$-globulin (α_{2u}-g) (Health Effects Institute [HEI] 1985, 1988). Among the chemicals tested are paraffins (Halder et al., 1984; Phillips and Cockrell, 1984), decalin (decahydronaphthalene) (Alden et al., 1984; Kanerva et al., 1987a), petroleum-based and synthetic fuels (MacNaughton and Uddin, 1984), military aviation propellants (Bruner, 1984) and 2,2,4-trimethylpentane (TMP) (Halder et al., 1985). 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 animal carcinogenesis bioassay 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 methyl phosphonate, hexachloroethane, isophorone, d-limonene, pentachloroethane, and tetrachloroethylene and a mixture, unleaded gasoline. These eight substances are compared and contrasted with two related non-α_{2u}-g-inducers, chlorothalonil and trichloroethylene. The analysis also relies on research 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 24 additional substances is also discussed where appropriate.

Of the eight model substances tested in chronic animal bioassays, all invoked a specific type of protein droplet nephropathy in male rats and also produced renal tumors in male rats but not in other 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 (Universities Associated for Research and Education in Pathology [UAREP], 1983; Alden et al., 1984; Halder et al., 1984; HEI, 1988; Swenberg et al., 1989).

- Excessive accumulation of hyaline droplets in proximal tubules, representing lysosomal overload, leads to tubule cell degeneration, cell loss, and regenerative cellular proliferation.

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		Renal Toxicity	Reference
Decalin	Rats	(m/f)	+/-	Alden et al. (1985)
	Mice	(m/f)	-/-	USEPA (1987)
	Dogs	(m/f)	-/-	
	Guinea pigs	(m/f)	-/-	
Dimethyl methyl-phosphonate	Rats	(m/f)	+/-	NTP (1987b)
	Mice	(m/f)	-/-	
Isophorone	Rats	(m/f)	+/-	NTP (1986a)
	Mice	(m/f)	-/-	
JP-4 jet fuel	Rats	(m/f)	+/-	MacNaughton and Uddin (1984)
	Mice	(m/f)	-/-	
	Dogs	(m/f)	-/-	
JP-5 shale-derived jet fuel	Rats	(m/f)	+/-	MacNaughton and Uddin (1984)
	Mice	(m/f)	-/-	
	Dogs	(m/f)	-/-	
d-Limonene	Rats	(m/f)	+/-	NTP (1990)
	Mice	(m/f)	-/-	
	Dogs	(m/f)	-/-	Webb et al. (1990)
Methyl isobutyl ketone	Rats	(m/f)	+/-	Alden et al. (1984)
	Mice	(m/f)	-/-	Phillips et al. (1987)
	Dogs	(m)	-	
	Monkeys	(m)	-	
Pentachloroethane	Rats	(m/f)	+/-	NTP (1983)
	Mice	(m/f)	-/-	
Unleaded gasoline	Rats	(m/f)	+/-	USEPA (1987)
	Mice	(m/f)	-/-	

m = male

f = female

+ = positive

- = negative

- 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.
- Single-cell necrosis accompanied by compensatory cell proliferation and exacerbation of the chronic progressive nephropathy (CPN) characteristically found in aging rats occurs.
- 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 (Trump et al., 1984a; Alden, 1989; Swenberg et al., 1989). This line of reasoning leads supporters of the hypothesis to conclude that the acute and chronic renal effects induced in male rats by such 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 (Alden 1989; Borghoff et al., 1990; Green et al., 1990; Olson et al., 1990; Flamm and Lehman-McKeeman, 1991; Swenberg, 1991).

This report examines the hypothesis that the male rat is predisposed to the nephrotoxic effects induced by certain classes of chemicals, such as volatile light hydrocarbons and organohalides. It also examines data that support or contradict the concept that the renal tumors produced in male rats by these chemicals are causally related to the nephrotoxicity. Based on the Risk Assessment Forum's (RAF) conclusions regarding these data, the document describes a uniform approach for EPA to use in risk assessments dealing with this spectrum of lesions and category of chemicals.

Information for this RAF report was obtained initially from a 1988 review entitled, "Evaluation of Data Concerning the Relationships among Chemically-induced Renal Alpha$_{2u}$Globulin or Hyaline Droplet Accumulation, Nephropathy, and Renal Neoplasia" prepared for the Office of Toxic Substances by Dr. William Richards of Dynamac Corporation, Rockville, Maryland. Additional information considered in this report includes recent comprehensive reviews of the subject, comments from peer reviewers, and other original work, especially publications subsequent to the 1988 Dynamac review.

This report has four parts. Following this brief introduction, Part 1 addresses the characteristics of hyaline droplets, the protein, α_{2u}-g, and the nephropathy associated with α_{2u}-g accumulation (Sections III and IV).

Part 2 (Sections V-VIII) presents data on the carcinogenic potential of chemicals inducing alpha$_{2u}$-globulin accumulation (CIGA) in the male rat. Section V describes the preneoplastic and neoplastic lesions produced by classical renal carcinogens. Section VI considers generic factors relevant to all studies of potential renal carcinogenicity in laboratory animals and then analyzes and discusses data on the renal lesions observed in 2-year bioassays with chemicals causing the hyaline droplet nephropathy. Section VII examines additional information that assists in defining renal carcino-

gens as CIGA, in particular genotoxicity and initiation-promotion data. In Section VIII, CIGA are compared with classical renal carcinogens, while Section IX considers the human evidence for kidney cancer, its histogenesis and epidemiology. Section X examines evidence for the hypothesized dose- and time-dependent progression of lesions.

Part 3 evaluates the evidence considered in Parts 1 and 2 with regard to the hypothesis that α_{2u}-g accumulation in the kidney is an initial step in a succession of histopathologic events that may culminate in renal tubule tumor formation in male rats. This part also lists priorities for future research.

Part 4 comprises the Agency policy statement regarding approaches to risk assessment for this category of chemicals.

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 [1]. The designation, α_{2u}-g 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 (1979) as chronic progressive nephropathy (CPN). The term lipocalin is used according to the terminology of Pervaiz and Brew (1987) to describe the superfamily of low-molecular-weight proteins which appear to transport lipophilic substances.

In rats, the proximal tubule of the nephron is divisible morphologically into three parts (see Figure 1). 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

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[1] Hyaline droplets refer to spherical inclusions in the cytoplasm which are homogeneous and eosinophilic, representing overdistended phagolysosomes. They may contain various macromolecules including α_{2u}-globulin. The morphology of droplets containing different proteins may be identical and therefore immunocytochemistry is required for precise definitiion of contents.

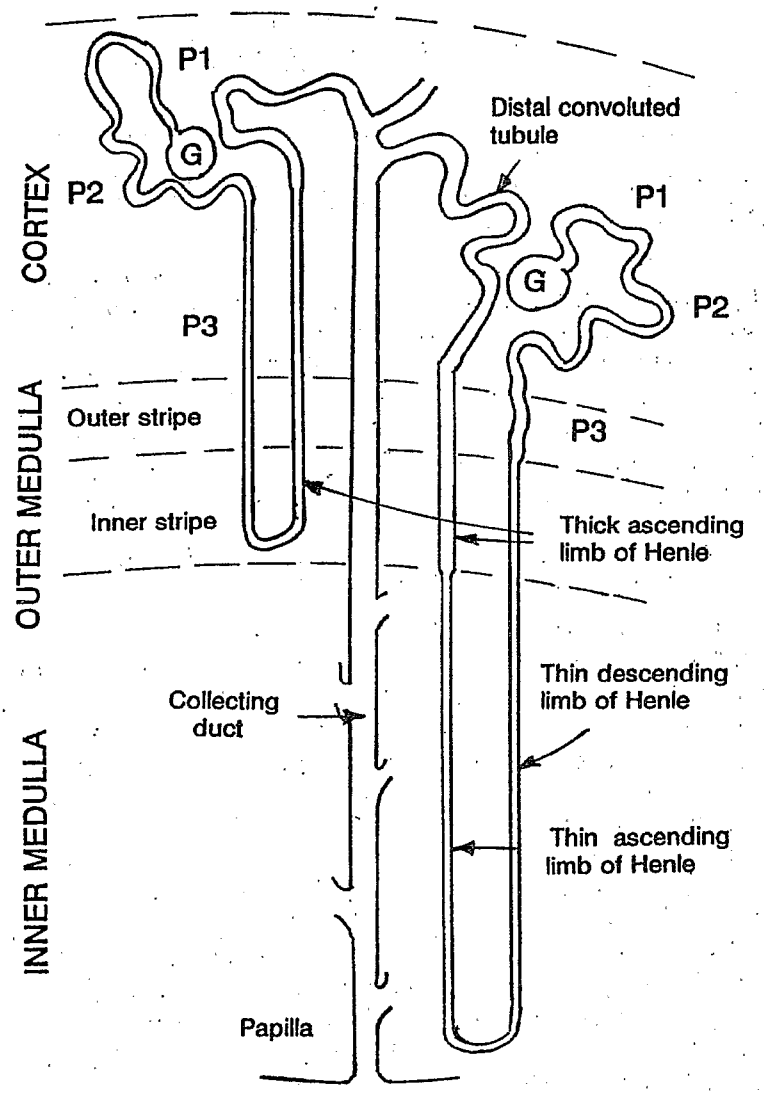

Figure 1. *Diagram of Zonation and Tubule Segmentation in Rat Kidney. G: Glomerulus; PI: First segment of proximal convoluted tubule; P2: Second segment of proximal convoluted tubule; P3: Pars recta of proximal tubule.*

Source: Adapted from Bachmann et al. (1986).

Toxicologic Pathologists (Hard et al., 1991). 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 (Bannayam and Lamm, 1980).

Part 1. Nephrotoxicity

III. Hyaline Droplets and Alpha$_{2u}$ - Globulin; Physiology and Biochemistry

Information on the renal processing of low-molecular-weight proteins, sex and species differences in urinary proteins, and the characteristics 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 CIGA. It is pertinent, therefore, to examine the physiological and biochemical characteristics of α_{2u}-g and related proteins, particularly those that occur in humans, before exploring the possible associations between α_{2u}-g accumulation, renal toxicity and renal tumor formation and their relevance to human risk assessment.

A. 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 (Maack et al., 1985) or by excretion. Figure 2 is a schematic representation of the cellular uptake and disposition of filtered proteins by the renal tubule.

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 (Maack et al., 1985). For larger proteins like 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 (Kaysen et al., 1986). While reabsorption was once considered largely nonselective, high capacity, low affinity transport (Maack et al., 1985), from recent work it now appears that interaction between the protein and the brush border membrane is the step at which a degree of selectivity in the absorption process occurs (Kaysen et al., 1986).

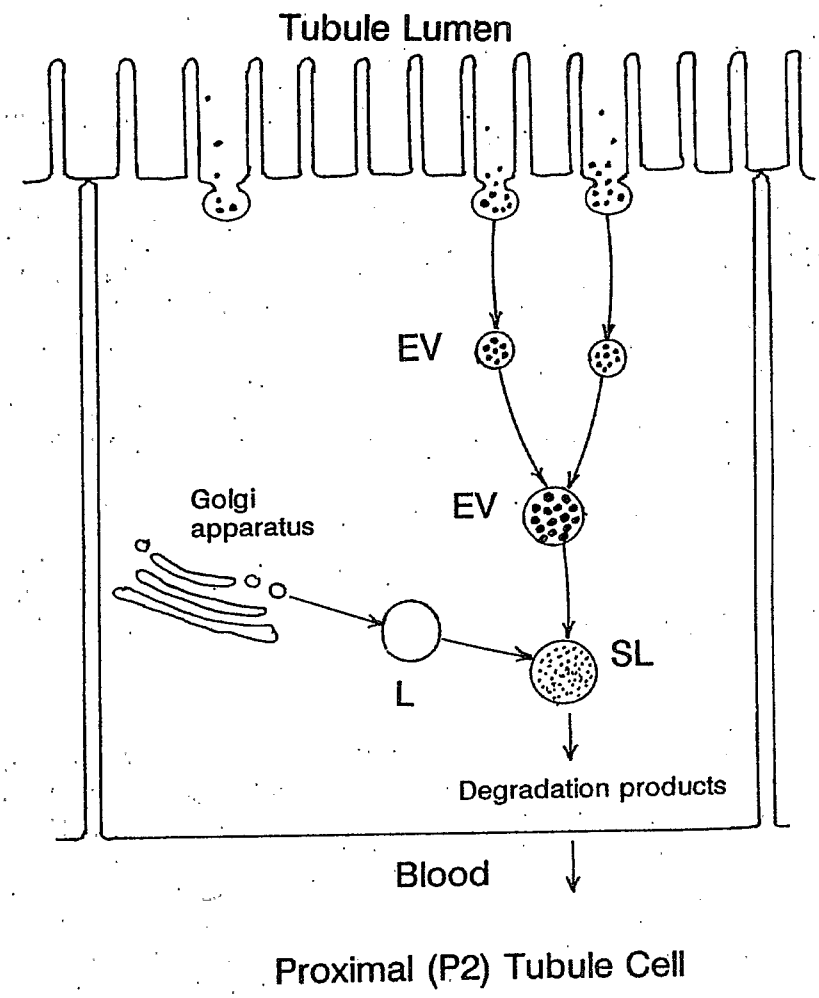

Figure 2. *Schematic representation of endocytic uptake of filtered proteins. Filtered proteins are adsorbed to endocytic sites at the lumenal membrane and segregated in endocytic vacuoles (EV). These EV migrate to the cell interior, where they fuse with lysosomes (L) to form secondary lysosomes (SL) or phagolysosomes where digestion of the protein takes place. The products of hydrolysis (amino acids) permeate the SL membrane, cross the contralumenal cell membrane, and return to the circulation.*

SOURCE: Adapted from Kaysen et al. (1986).

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, i.e., cysteine proteinases (cathepsin B, H, and L) and an aspartic acid proteinase, cathepsin D (Lehman-McKeeman et al., 1990a). Lehman-McKeeman et al. (1990a) 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 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 (Dice, 1987). The plasma half-lives of many low-molecular-weight proteins are measured typically in minutes (Maack et al., 1985). One of the exceptions is α_{2u}-g, with a half-life measured in hours (Geertzen et al., 1973).

Whether or not low-molecular-weight proteins like 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.

B. 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 being particularly evident in the P2 segment of proximal tubules (Logothetopoulos and Weinbren, 1955; Maunsbach, 1966a; Goldsworthy et al., 1988a). Ultrastructurally, hyaline droplets are abnormally large, dense, secondary lysosomes (also termed phagolysosomes), representing fusion of endocytic vacuoles with primary lysosomes. Some hyaline droplets show crystalloid changes by electron microscopy that are not observed in the lysosomes of female rats (Maunsbach, 1966b). Crystalline formation in the normal male rat is believed to indicate the presence of a poorly catabolized protein in pure solution (Pesce et al., 1980), presumably α_{2u}-g in the kidney lysosomes.

Hyaline droplets in the proximal tubules of normal male rats contain α_{2u}-g (Alden et al., 1984; Garg et al., 1987; Goldsworthy et al., 1988a), 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 (Logothetopoulos and Weinbren, 1955; Murty et al., 1988). In female rats, protein droplets in proximal tubules are either absent or considerably less frequent than in males, and they do not contain α_{2u}-g (Logothetopoulos and Weinbren, 1955; Maunsbach, 1966a; Goldsworthy et al., 1988a; Burnett et

al., 1989). Hyaline droplets are substantially reduced in castrated male rats (Logothetopoulos and Weinbren, 1955).

Because an abnormal increase in hyaline droplets has more than one etiology and can be associated with the accumulation of different proteins, it is necessary to apply special diagnostic methods such as immunohistochemical staining to make the association between chemical exposure and pathologic accumulation of α_{2u}-g.

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, indistinguishable from the CIGA-induced lesion. The accumulating protein in these tumor-bearing animals has been identified as lysozyme (Hard and Snowden, 1991). Similarly, in male and female mice with histiocytic tumors, abnormal accumulation of lysozyme-containing hyaline droplets sometimes occurs in proximal tubules (Hard and Snowden, 1991).

In humans, the Bence-Jones proteins, a class of light chain immunoglobulins, are produced in large amounts in multiple myeloma patients (Pirani et al., 1983). In human cases of mononuclear cell leukemia, lysozyme is produced (Muggia et al., 1969). The kidney injury seen with these neoplastic diseases has been described as similar to that produced by administration of decalin to male rats (Alden, 1986), including protein droplet accumulation in renal tubules (Oliver and MacDowell, 1958; Pruzanski and Platts, 1970; Pirani et al., 1983). 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 (Oliver and MacDowell, 1958).

C. Factors Affecting Kidney Accumulation of Low-Molecular-Weight Proteins

Protein accumulation in the proximal tubule can reach pathological levels resulting in excessive hyaline droplet formation for several reasons: (1) the rate of protein delivery to the tubule cells is abnormally high, (2) the proteins delivered are difficult to hydrolyze, or (3) the lysosomal hydrolyze 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 when there is immunological, inflammatory, or toxic disease in the glomerulus or when the permselectivity barrier is overloaded by filterable proteins (Kaysen et al., 1986).

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 (Maack et al., 1985). Lysozyme (histiocytic sarcoma) and light chain immunoglobulins (multiple myeloma)

are proteins that are relatively resistant to hydrolysis (Maack et al., 1985). 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 (Maack et al., 1985). In the presence of a reduced lysosomal hydrolysis capacity, the most hydrolytically resistant proteins, like α_{2u}-g, tend to accumulate first. Testosterone is known to have a suppressive effect on the activity of some major proteolytic enzymes in the male rat kidney (Kugler and Vornberger, 1986). Consequently, the lysosomal protease activity in male proximal tubules is lower than that of females (Jedrzejewski and Kugler, 1982; Kugler and Vornberger, 1986) 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 (Murty et al., 1988; Lehman-McKeeman et al., 1990a), any chemically induced impediment to α_{2u}-g digestibility caused by CIGA would be further superimposed on the causes considered previously that alone can result in excessive protein accumulation in renal tubules.

D. The Alpha$_{2u}$-globulin Superfamily of Proteins

Alpha$_{2u}$-globulin [2] 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. (1981). 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 percent (Åkerström and Lögdberg, 1990). Statistical analysis shows, however, that the proteins are related evolutionarily (Åkerström and Lögdberg, 1990).

Of the approximately 20 proteins now considered to be potential members of the superfamily (Åkerström and Lögdberg, 1990), the three-dimensional structure is known for only three, retinol-binding protein, β-lactoglobulin, and insecticyanin (Sawyer, 1987). The central core of these

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[2] The male rat urine protein, alpha$_{2u}$-globulin is named in accordance with immunoelectrophoretic nomenclature (Roy and Neuhaus, 1967). Because of its size, some authors refer to this protein as alpha$_2$- microglobulin or alpha 2μ- globulin, a term more appropriately reserved for another low-molecular-weight endometrial protein associated with pregnancy.

three proteins is composed of eight strands with a β-barrel structure forming a hydrophobic pocket that appears to enclose the ligand (Papiz et al., 1986; Sawyer, 1987). This structure has been described as resembling a coffee filter paper (Åkerström and Lögdberg, 1990). In addition to theb-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 (Pervaiz and Brew, 1987). 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. On this basis, they suggested the illustrative name, lipocalins, for the superfamily of proteins.

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, 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 (Åkerström and Lögdberg, 1990). 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. (1987) 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 (Murty et al., 1987; Mancini et al., 1989). In addition, α_{2u}-g has been identified as a fatty acid-binding protein of the kidney (Kimura et al., 1989) and may serve to transport fatty acid, an important energy source in kidney, within renal epithelial cells. Brooks (1987) 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 (Pevsner et al., 1988). 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 since

Table 2. Superfamily of Lipophilic Ligand-Binding Carrier Proteins

Protein	Species	Tissue or body fluid	Molecular weight	No. of amino acids	Reference
α_1-Acid glycoprotein (acute-phase protein) (Orosomucoid)	Mammals	Plasma	23,000 (unglycosylated) 43,000-60,000 (complex type)	184	Pervaiz and Brew (1987) Arnold and Meyerson (1990)
Apolipoprotein D (cholesterol-associated)	Mammals	Plasma	19,300	169	Drayna et al. (1986)
α_1 Microglobulin (Protein HC)	Mammals	Plasma	20,619	182	Åkerström and Lögdberg (1990)
Retinol-binding protein	Mammals	Liver, plasma, retina	22,868	182	Papiz et al. (1986)
Endometrial α_2-globulin (pregnancy-associated)	Mammals	Placenta	25,000	NR	
β-Lactoglobulin	Ruminants, other species	Milk	18,000	162	Papiz et al. (1986)
Odorant-binding protein (Pyrazine-binding protein)	Rat, cow	Nasal epithelium	18,091	172	Cavaggioni et al. (1987) Cavaggioni et al. (1990)
α_{2u}-Globulin[a]	Rat	Male urine, preputial gland	18,709	162	Unterman et al. (1981)
Androgen-dependent secretory protein	Rat	Epididymis	18,500	184	Brooks (1987)
Fatty-acid-binding protein[b]	Rat	Kidney	15,500	NR	Kimura et al. (1989)

Table 2. (cont.)

Protein	Species	Tissue or body fluid	Molecular weight	No. of amino acids	Reference
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	Lee et al. (1987)
Insecticyanin (Bilin-binding protein)	Tobacco hornworm, butterfly	Hemolymph	21,382	189	Godovac-Zimmermann (1988)
			NR	NR	Sawyer (1987)

Adapted from Pevsner et al., 1988, with additional information as noted.
NR = Not reported, characterization of protein incomplete.
a Also occurs in other secretory organs.
b Described as α_{2u}-globulin by Kimura et al., 1989.

retinol-binding protein is quite specific for retinol, whereas odorant-binding proteins may have a broad range of ligands (Godovac-Zimmermann, 1988).

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. (1990) measured the binding affinities of α_{2u}-g, MUP, and pyrazine-binding protein isolated from calf nasal mucosa for a series of odorants. MUP bound only one of these chemicals, pyrazine-binding protein bound six, and α_{2u}-g bound twelve. The best ligand for α_{2u}-g was chemically unrelated to the best ligands for the other two proteins, which were also chemically unrelated.

E. Characteristics of Alpha$_{2u}$-globulin

Alpha$_{2u}$-globulin was first characterized in male rat urine (Roy and Neuhaus, 1967). All 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 to 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 (Chatterjee et al., 1989), the major urinary source of α_{2u}-g is the liver where α_{2u}-g mRNA constitutes approximately 1 percent of the hepatic mRNA population (Sippel et al., 1976; Kurtz and Feigelson, 1977). The hepatic isoforms of α_{2u}-g may vary throughout the lifetime (Roy et al., 1983). Synthesis of the protein in rat liver is under multihormonal control, particularly androgen, but also glucocorticoids, thyroid hormones, insulin and growth hormone (Feigelson and Kurtz, 1977; Roy and Chatterjee, 1983). These hormones appear to act by regulating the steady-state level of α_{2u}-g mRNA (Kurtz and Feigelson, 1977). Neither α_{2u}-g nor its corresponding mRNA are detectable in the livers of sexually intact female rats (Sippel et al., 1975, 1976; MacInnes et al., 1986). However, a very low background level of the mRNA has been indicated in the ovariectomized female rat (Chatterjee et al., 1979), and ovariectomy in concert with androgen treatment induces a parallel increase in α_{2u}-g and its mRNA in female rat liver (Roy and Neuhaus, 1967; Sippel et al., 1975).

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 alter the preputial concentration of this protein and its mRNA (Murty et al, 1988). Alpha$_{2u}$-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 (MacInnes et al., 1986; Mancini et al., 1989). The female forms of α_{2u}-g show distinct differences from male rat α_{2u}-g suggesting that they are encoded by different genes (Vandoren et al., 1983).

Low levels of α_{2u}-g first become detectable in the male rat liver under the stimulus of testosterone at 35 to 40 days, reaching maximum adult levels by 60 to 80 days (Roy et al., 1983; Motwani et al., 1984; MacInnes et al., 1986). Due to the development of hepatic insensitivity to androgen during aging, hepatic synthesis of α_{2u}-g begins to fall gradually in male rats some time after

5 months of age. By 22 months of age, there has been a drop of over 90 percent, with α_{2u}-g being virtually undetectable in senescent animals (Roy et al., 1983; Motwani et al., 1984; Richardson et al., 1987). Renal cortical tissue content (Murty et al., 1988) and urinary excretion (Neuhaus and Flory, 1978; Motwani et al., 1984) 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 percent of the filtered protein being excreted in the urine and 60 percent undergoing reabsorption and catabolism (Neuhaus et al., 1981; Caudill et al., 1991). Alpha$_{2u}$-globulin is catabolized slowly relative to most other proteins in the glomerular filtrate with the half-life in plasma, kidney cytosol, or lysosomal preparations being 5 to 8 hours (Geertzen et al., 1973; Ekstrom, 1983; Lehman-McKeeman et al., 1990a). In vitro studies indicate that α_{2u}-g is more resistant to lysosomal enzyme digestion than bovine β-lactoglobulin and lysozyme (Charbonneau et al., 1988). 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 while retinol-binding protein and β-lactoglobulin were 1000- to 100,000-fold more easily hydrolysed (Borghoff et al., 1990). 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.

F. Sex and Species Comparison of Urinary Protein Content of the Lipocalin 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 (Neuhaus and Lerseth, 1979), which is 100 to 300 times more than observed in female rat urine (Shapiro and Sachchidananda, 1982; Vandoren et al., 1983). The mouse can also be described as physiologically proteinuric because of a high urinary content of MUP (Thung, 1962). MUP shows the greatest similarity to α_{2u}-g in the lipocalin superfamily, sharing 90 percent amino acid sequence homology (Dolan et al., 1982). 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 females (Hastie et al., 1979; Roy and Chatterjee, 1983). Daily urinary excretion of MUP varies considerably among strains (Szoka and Paigen, 1978). In the B6C3F1 strain, males have been shown to excrete 14.9 mg of MUP/day in the urine, and females, 2.1 mg/day (Lehman-McKeeman et al., 1990b). Adjusted for body weight, a male B6C3F1 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 percent of filtered α_{2u}-g is reabsorbed by the kidney, MUP is not reabsorbed in the mouse and appears to be totally excreted (Caudill et al., 1991).

In contrast, normal human urine contains relatively little protein, only 1 percent of the total concentration present in mature male rat urine (Olson et al., 1990). Human urinary proteins are predominantly high-molecular-

weight species with only minor components weighing less than 66,0(daltons. Within the low-molecular-weight fraction, trace amounts of protei represent the lipocalin superfamily, but none appear to share molecul weight identity with α_{2u}-g. The urinary excretion of retinol-binding protei α_1-acid glycoprotein and α_1-microglobulin has been measured at 0.0001 0.0007, 0.0006 to 0.002, and 0.02 to 0.05 mg/day/g kidney, respective (Berggard, 1970; Peterson and Berggard, 1971; Ekstrom and Berggai 1977). Thus, the urinary excretion of α_{2u}-g in the male rat is approximate two orders of magnitude greater than the human urinary content of the thr(superfamily proteins combined.

Recently, a sex-dependent protein of unknown origin and functio termed urine protein 1, was identified in normal human urine (Bernard et a 1989). The molecular features of protein 1 are similar to α_{2u}-g as it has molecular weight of approximately 21,000 daltons and an isoelectric poi around 4.8 although its amino acids have not been fully sequenc((personal communication, R. Lauwerys, Catholic University of Lowai Belgium, to I. Rodgers, March 25, 1991). Protein 1 occurs in both sexes fro an early age, but increases substantially in males after puberty, reaching t to a 50-fold difference over females during late adolescence. A 5-fold ma to female differential persists through adulthood. Average urinary conce trations of protein 1 have been determined as 108 and 3.2 µg/L respective for males and females aged 15 to 20 years, and 24.7 and 5.8 µg/L for mal(and females in the 20 to 60 year age range (Bernard et al., 1989). Su(levels of protein1 in human male urine, however, are calculated as four five orders of magnitude less than α_{2u}-g concentrations in the urine of ma rats.

G. Noncovalent Binding to Alpha$_{2u}$-globulin and Its Homologues

It has been suggested that CIGA bind reversibly and noncovalently α_{2u}-g, forming a resultant complex that is even more poorly digested in tl male rat kidney than α_{2u}-g (Swenberg, 1989).

1. Chemical entities bound to alpha$_{2u}$-globulin

In a few instances, the specific chemical entity complexed with α_{2u}-g h: been identified. TMP, a branched chain aliphatic hydrocarbon present gasoline 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 w: retained in the kidneys of males, but not of females (Kloss et al., 198 Charbonneau et al., 1987). The major metabolite of TMP in the male r kidneys was identified as 2,4,4-trimethyl-2-pentanol (TMPOH) (Charbonne: et al., 1987). In a separate report, TMPOH was shown to be the only ligar for α_{2u}-g whenever TMP was administered to the male rat (Lock et a 1987a). TMPOH was not detected in the kidney tissue of the female rat which excreted more conjugated TMPOH (glucuronides and sulfates) th: the males (Charbonneau et al., 1987). Later studies confirmed, as su pected, that the TMPOH-α_{2u}-g complex is cleared slowly from male r kidney (Swenberg, 1989).

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 (Lehman-McKeeman et al., 1989). For isophorone, the ligand is the parent compound (Strasser et al., 1988). 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 (Charbonneau et al., 1989). About 40 percent 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 (Lehman-McKeeman et al., 1991). Four metabolites were identified in the α_{2u}-g protein fraction, the main component being the gamma-lactone of 3,5,5-trimethyl hexanoic acid.

2. Nature of the association

The nature of the association of CIGA with α_{2u}-g was explored initially by Lock et al. (1987a) who dosed sexually mature male Fischer 344 (F344) rats with [^{3}H]-TMP, killed them 8 to 72 hours 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 percent of the cytosol radiolabel (15% of all radiolabel in the kidney) eluted in the fraction containing α_{2u}-g. Approximately 19 percent 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 percent sodium dodecyl sulfate (SDS), a detergent which affects the secondary and tertiary structure of proteins, was added to the dialysis buffer, there was a significant loss of binding. These results suggested a reversible binding between the TMP metabolite and the protein fraction containing α_{2u}-g (Lock et al., 1987a). The reversibility of chemical binding with α_{2u}-g, whether parent compound or metabolite, has been confirmed with isophorone (Strasser et al., 1988), 1,4-DCB (Charbonneau et al., 1989), d-limonene (Lehman-McKeeman et al., 1989), 3,5,5-trimethylhexanoloxybenzene sulfonate (Lehman-McKeeman et al., 1991), and lindane (Dietrich and Swenberg, 1991a).

In the d-limonene study (Lehman-McKeeman et al., 1989), the amount of radioactivity observed in the kidneys of Sprague-Dawley rats 24 hours after oral administration of [^{14}C]-d-limonene was about 2.5 times higher in males than in females. Equilibrium dialysis, in the presence or absence of SDS, indicated that approximately 40 percent 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-trimethylhexanoloxybenzene sulfonate study (Lehman-McKeeman et al., 1991), 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.

3. 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 having produced 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 differ from many known chemical toxins, nephrotoxins included, which bind covalently and irreversibly to proteins and/or DNA and through this process cause cellular injury.

A DNA binding study with F344 rats and B6C3F1 mice of both sexes was performed using [1,3,5,-^{14}C]-isophorone (Thier et al., 1990). 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, 1,2-DCB, a closely related isomer, does not induce hyaline droplets and appears to bind covalently to proteins in the male rat liver, plasma, and kidney (Charbonneau et al., 1989). 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 to 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.

4. Specificity of the interaction of CIGA with alpha$_{2u}$-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 employed two-dimensional gel electrophoresis of rat kidney homogenate (Alden et al., 1984). 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 (Borghoff et al., 1988). 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 (personal communication, L. Lehman-McKeeman, Procter and Gamble, to I. Rodgers, February 27, 1991).

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 qualitative and quantitative species differences were noted (Lehman-McKeeman, 1990b). However, equilibrium saturation binding studies did not demonstrate any interaction between d-limonene or its metabolites and MUP in male or female mice (Caudill et al., 1991; Lehman-McKeeman, 1990b). 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 accumulation of hyaline droplets or α_{2u}-g (Borghoff et al., 1989). 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 (Borghoff et al., 1991). 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 that inhibits protein catabolism (Borghoff et al., 1990).

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.

H. Catabolism of Alpha$_{2u}$-globulin Complexed With CIGA

Reduced renal lysosomal catabolism of the CIGA-α_{2u}-g complex leads to its accumulation in the cells of the proximal renal tubule, causing lysosomal protein overload and individual cell death (Swenberg et al., 1989). Figure 3 illustrates this proposed sequence of events.

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 (Charbonneau et al., 1988) or of purified urine-derived protein conjugated with CIGA in vitro (Lehman-McKeeman et al., 1990a). Charbonneau et al. (1988) found that both proteinase K or a mixture of standard protease enzymes of non-rat origin 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. (1990b) demonstrated that the reversible binding of d-limonene, 1,4-DCB, and isophorone or their metabolites impaired the degradation of α_{2u}-g by one-third. Under the experimental conditions employed, this was equivalent to an extension of the apparent half-life of α_{2u}-g from 6.67 to 10 hours. The study is particularly interesting because it shows 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-

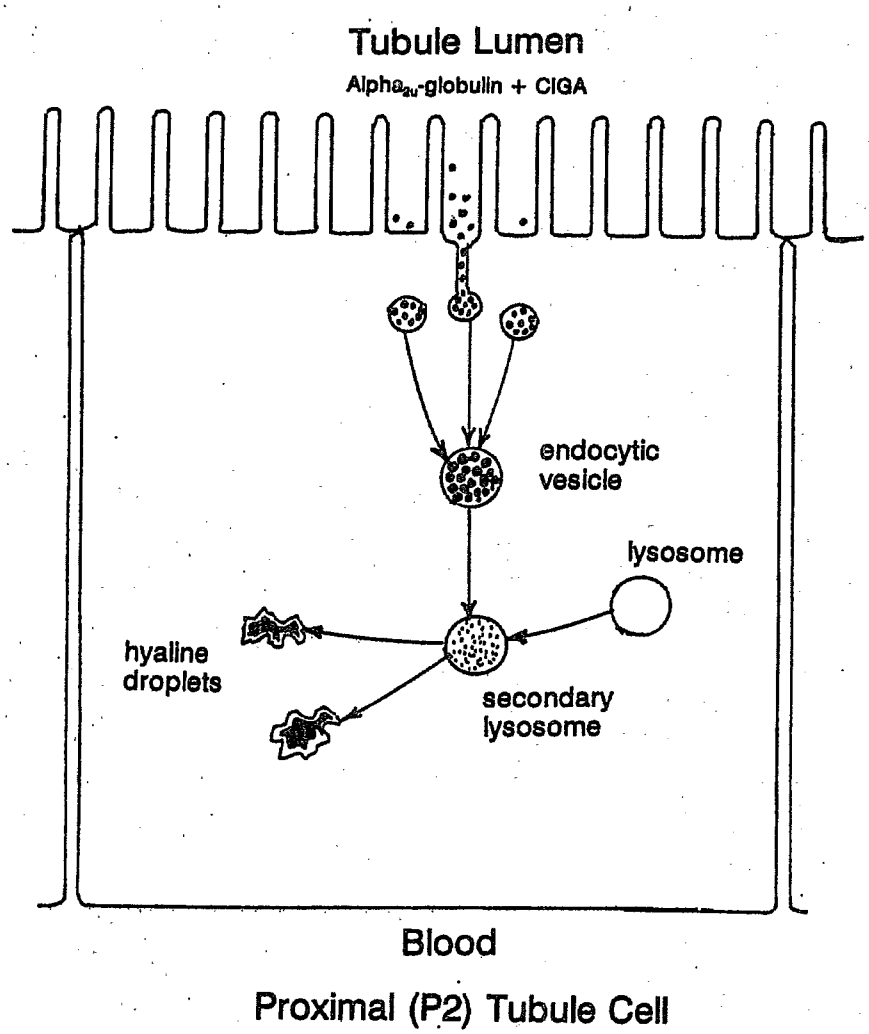

Figure 3: *Schematic representation of the uptake and fate of alpha$_{2u}$-globulin complexed with a CIGA in hydrocarbon nephrotoxicity.*

Source: *Modification of Figure 2, above.*

oxide, and 2,5-dichlorophenol, did. With isophorone, however, it was the parent compound alone which produced the effect. This apparent need to reduce protein degradation might offer an explanation to describe why chemicals, such as retinol, have been shown to bind to α_{2u}-g, without producing hyaline droplet accumulation.

Administration of leupeptin (an inhibitor of the lysosomal peptidase, cathepsin B) caused a rapid α_{2u}-g accumulation in the kidney, indistinguishable from that induced by TMP or gasoline (Olson et al., 1988). These various observations provide evidence that CIGA-induced hyaline droplet accumulation may result from a reduced protein degradation rate either by (1) making the protein harder to digest or (2) inhibiting enzymatic components of the proteolytic process. Studies by Charbonneau et al. (1988) and Lehman-McKeeman et al. (1990a) support the former by indicating that the TMP metabolite-protein complex is more resistant to hydrolysis than free α_{2u}-g. Furthermore, Murty et al. (1988) found that unleaded gasoline was not associated with a reduction, but rather an increase, in rat kidney lysosomal proteolytic enzyme activity.

I. Structure-Activity Relationships for CIGA

An ability to predict those chemicals that will induce accumulation of α_{2u}-g in the male rat through structural relationships would be clearly 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. (1990a) 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., an 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 (Bomhard et al., 1990). Based on data for a number of light hydrocarbons, Bomhard et al. 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 ability to induce hyaline droplet accumulation in adult male Wistar rats. Even though 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.

Borghoff et al. (1991) 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, these investigators 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.

The conclusions of Borghoff et al. (1991) are consistent with the notion that α_{2u}-g is capable of transporting lipophilic compounds within a binding site pocket of specific dimensions. Since binding affinity does not correlate well with hyaline droplet formation, however, the ability of this structural feature to serve as a predictive tool would appear limited.

IV. Alpha$_{2u}$-globulin Nephropathy

Substances reported to induce increased formation of hyaline droplets in proximal tubule cells of male rats are listed in Table A-1, 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 (Short et al., 1986; 1987; 1989a), decalin (Alden et al., 1984; Kanerva et al., 1987a,b,c; Stone et al., 1987), and d-limonene (Kanerva et al., 1987b; Webb et al., 1989). For even 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 hyaline-droplet inducers listed in Table A-1.

Much of the information useful for defining the pathologic sequelae to α_{2u}-g accumulation does not require chronic exposure. Accumulation of α_{2u}-g is visible within a matter of days and the response to chronic administration of CIGA might even diminish since α_{2u}-g levels decline in aging male rats (Murty et al., 1988). The nephrotoxicity associated with α_{2u}-g accumulation might also be influenced by age. 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.

A. Pathologic Features of Alpha$_{2u}$-globulin Nephropathy

Renal lesions described in scientific studies as being associated with α_{2u}-g nephropathy are listed in Table 3. The first morphological manifesta-

Table 3. *Summary of the Histopathology and Lesion Progression Reported in Alpha$_{2u}$-Globulin-Associated Nephrotoxicity*

1. 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.
2. Evidence of single-cell necrosis in the P2 segment epithelium and exfoliation after 5 days of continuous exposure.
3. 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 to 13 weeks after commencing exposure and sometimes beyond, up to 2 years.
4. Increase in cell proliferation within the P2 segment following 3 weeks of continuous exposure, remaining elevated above normal at 48 weeks of exposure.
5. Linear mineralization of tubules 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.
6. Hyperplasia of the renal pelvic epithelial lining observed around 1 year. [a]
7. Exacerbation of the spontaneous chronic progressive nephropathy syndrome common in aging rats. [a]
8. Formation of occasional hyperplastic foci within cortical epithelium at chronic time-points.

[a] Indirect consequence of progression of lesions.

tion of α_{2u}-g nephropathy is the rapid accumulation of hyaline droplets in proximal tubule cells, developing within 24 hours of dosing with some compounds (Webb et al., 1989).

The droplets stain positively with Mallory's Heidenhain stain but are negative for periodic acid Schiff, indicating their protein composition (Alden et al., 1984). 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 (unpublished observations, G.C. Hard). In plastic-embedded tissue, hyaline droplets can be visualized easily with Lee's methylene blue basic fuschin (Short et al., 1986).

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 (Short et al., 1987). By light microscopic immunohistochemistry, α_{2u}-g has been clearly and specifically localized to the hyaline droplets within proximal tubules (Burnett et al., 1989). Ultrastructurally, the hyaline droplets are enlarged secondary lysosomes partially composed of α_{2u}-g (Garg et al., 1989a). Many are polyangular or irregular in shape, containing a condensed crystalline core suggestive of aggregated protein in pure form. Although the α_{2u}-g-associated hyaline droplet accumu-

lation persists during chronic exposure, the severity appears to lessen with increasing duration of exposure (Short et al., 1989a). 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 of age (Roy et al., 1983; Motwani et al., 1984; Richardson et al., 1987).

With continued exposure, the initial accumulation of α_{2u}-g-containing hyaline droplets may be followed by a sequence of interrelated pathological events. (1) Scattered single-cell necrosis occurs predominantly in the P2 segment cells (Short et al., 1987) with subsequent exfoliation of these degenerate cells 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 (Kanerva et al., 1987a). Scattered exfoliation of droplet-affected cells was observed with up to 48 weeks of exposure to unleaded gasoline or TMP (Short et al., 1989a), indicating sustained single-cell loss while exposure to CIGA continues.

(2) 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 or demonstrated by labeling techniques for DNA-synthetic activity. Increased proliferative activity has been recorded after only three weeks of petroleum hydrocarbon exposure (Short et al., 1987) but it persisted during 48 weeks of chronic exposure (Short et al., 1989a).

(3) Granular casts composed of sloughed cell debris 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, with consequent tubule dilation at this part of the nephron (Alden et al., 1984). This can occur as early as two to three weeks after initial exposure (Alden et al., 1984; Kanerva et al., 1987a). As well as comprising recognizable cell debris, the granular casts stain positively for α_{2u}-g (personal communication, J.R. Foster, Central Toxicology Laboratory, ICI, Macclesfield, to G. Hard, November 1990) indicating probable derivation of the debris from cells which had accumulated this protein. Granular cast formation appears to be associated with higher doses of a 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 (Short et al., 1986, 1987).

(4) At chronic timepoints, linear mineralization develops in the renal papilla, outlining affected medullary tubules, along with hyperplasia of the pelvic epithelial lining (urothelium) (Alden, 1989). With unleaded gasoline, this lesion was first observed at 6 months of exposure (USEPA, 1987). The mineralization appears to form within the loops of Henle and has been identified as calcium hydroxyapatite (Trump et al., 1984a). The relationship between papillary mineralization and the proximal tubule lesion remains undetermined but the medullary lesion is presumed to represent mineral-

ized remnants of debris from disintegrating granular casts that lodge in the prebend segments of the loops of Henle (Bruner, 1984; Alden, 1989). In turn, urothelial hyperplasia, which mainly affects the surface of the renal papilla, may be a response of the renal pelvis lining to papillary mineralization (Bruner, 1984; Alden and Frith, 1991).

B. 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 aminotransferase (AAT), urine osmolality, and volume, were not affected (Fowlie et al., 1987). A single, oral dose of TMP had no effect on renal function (Stonard et al., 1986). 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 (Evans and Morgan, 1986). Similar results were obtained for levamisole except that AAT remained normal (Evans et al., 1988). During prolonged treatment with C_{10}-C_{11} isoparaffinic solvent, up to 8 weeks, the only urinary changes observed were mild elevations 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 (Phillips and Egan, 1984).

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 identified by clinical chemistry is a characteristic of CIGA, which contrasts with the obvious urinary changes associated with the nephrotoxicity induced by such classical renal toxins as mercuric chloride, hexachlorobutadiene, aminoglycosides, and papillotoxic agents (Stonard, 1987).

C. Species Variation in the Renal Response to CIGA

The male-specific effects of hyaline-droplet inducers have been demonstrated over a range of rat strains including F344, Sprague-Dawley, Buffalo, and Brown Norway rats (Ridder et al., 1990). 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 these chemicals (Alden et al., 1984; Swenberg et al., 1989). In addition to these studies, other hyaline-droplet inducers 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 doses known to cause nephropathy in male rats (Alden et al., 1984; Kuna and Ulrich, 1984; MacFarland, 1984; MacNaughton and Uddin, 1984; Phillips et al., 1987) except for one report of minor changes in dogs

treated for 6 months with d-limonene (Tsuji et al., 1975). 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 was observed. However, Webb et al. (1990) were unable to demonstrate any renal pathology in dogs after 6 months of d-limonene treatment at comparable dose levels. The highest dosage tested in the dogs, 1.2 mg/kg (Webb et al., 1990; Tsuji et al., 1975), is more than ten times the doses that have caused frank nephropathy in male rats.

Knowledge concerning 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 non-occupational 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., Barrientos et al., 1977; Crisp et al., 1979). Case reports cannot be used to establish a causal relationship but may serve to initiate formal epidemiologic investigation (Churchill et al., 1983).

Epidemiologic studies concerning non-neoplastic kidney disease and occupational exposure to hydrocarbons and solvents have been conducted only since 1975 (reviewed by Askergren, 1986; Daniell et al., 1988; Phillips et al., 1988). 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 (Kluwe et al., 1984). However, many of the earlier studies are considered to be methodologically limited (Churchill et al., 1983; Askergren, 1986; Phillips et al., 1988). 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 (Phillips et al., 1988).

More recently, Steenland et al. (1990), 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. (1989) 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 sufficient 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 (Kluwe et al., 1984; Phillips et al., 1988).

Other indicators of renal function have also been assessed in epidemiologic studies. Levamisole, a drug used as an antihelminthic, 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 (Read et al., 1988). 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 (Dieppe et al., 1978). 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 (Solet and Robins, 1991). Since 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 to 25 years), Vyskocil et al. (1990) 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 believe these latter findings, in addition to the lack of correlation between intensity of exposure and change in biochemical parameters, support 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 or not 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 which concentrated on clinical detection of glomerular effects. Since 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.

D. Factors Affecting the Expression of Alpha$_{2u}$-globulin Nephropathy

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

1. Age-related effects

As discussed earlier, the hepatic synthesis and urinary excretion of a_{2u}-g in the male rat are highly age-dependent, with prepubertal and aged animals showing negligible amounts of this protein (Neuhaus and Flory, 1978; Roy et al., 1983; Richardson et al., 1987). Accordingly, administration of either decalin to immature male rats (Alden et al., 1984) or unleaded

gasoline to aged, 26-month old, male rats (Murty et al., 1988) failed to produce renal cortical a_{2u}-g accumulation or an increase in hyaline droplets.

2. Effect of hormone manipulation

As a_{2u}-g synthesis is primarily under androgenic control, the effects of castration, which depresses hepatic synthesis of a_{2u}-g (Roy and Neuhaus, 1967), were explored by Hobson et al. (1986) 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 a_{2u}-g in the rat (Roy et al., 1975). Garg and coworkers (1988) used estradiol administration to study the influence of inhibition of new synthesis of a_{2u}-g on recovery from CIGA-induced renal tubule changes. Commencing treatment on the ninth and final day of unleaded gasoline exposure, estradiol reduced renal cortical a_{2u}-g content by 25, 41, and 52 percent on post-exposure days 3, 6, and 9, respectively, compared to rats receiving no hormone treatment. At the same time, hyaline droplet removal appeared to be accelerated in rats treated conjointly with hormones. Hyaline droplet number and size (qualitative observations) in hormone-treated rats approached control levels at 3 days post-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. (1989b) demonstrated that pretreatment of mature male rats with subcutaneous 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.

3. 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 (Chatterjee et al., 1989). The NBR rat is capable of developing chemically induced nephropathies, but under exposure conditions that produce α_{2u}-g nephropathy in F344 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 (Dietrich and Swenberg, 1991a). Identical results were obtained for decalin (Ridder et al., 1990) and lindane (Dietrich and Swenberg, 1990, 1991a).

4. Alpha$_{2u}$-globulin infusion in female rats

Ridder et al. (1990) intraperitoneally administered α_{2u}-g (purified from mature male rat urine) at hourly intervals to decalin-treated female Sprague-Dawley rats for a total of 8 injections and examined kidney samples for hyaline droplets and α_{2u}-g one hour after the last protein injection (9 hours after decalin treatment). Although droplet formation was not evident in kidney sections from the α_{2u}-g-infused female rats stained with Mallory's

Heidenhain, 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.

E. 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 F344 rats than in the Wistar strain (Gray, 1986), and it is also common in the Osborne-Mendel rat (Goodman et al., 1980). 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 (Masoro and Yu, 1989).

Exacerbated CPN, involving enhanced severity and earlier onset of the disease, is generally observed after chronic administration of CIGA to male rats (Trump et al., 1984a). 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 (Kanerva et al., 1987a; Webb et al., 1989). Exacerbated CPN is usually recognized after months of continuous treatment (Trump et al., 1984a; Short et al., 1989a) although Alden et al. (1984) reported early signs after 2 to 3 weeks with decalin. These authors (Alden et al., 1984) consider that exacerbated CPN develops as a tertiary response to nephron obstruction caused by the CIGA-induced granular casts.

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 (UAREP, 1983). 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 unlike CPN, such as hyaline droplet accumulation associated with α_{2u}-g in the P2 segment, granular casts at the "corticomedullary" junction, and linear mineralization in the papilla (Trump et al., 1984a). 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 (unpublished observations, G.C. Hard). The urine and serum chemistry of advanced CPN also

Table 4. Summary of the Histopathology of Spontaneous Chronic Progressive Nephropathy of Aging Rats

1. Thickening of tubular and glomerular basement membranes.
2. Basophilic segments of proximal tubules with sporadic mitoses indicative of tubule cell proliferation.
3. 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.
4. Focal interstitial aggregations of mononuclear inflammatory cells within areas of affected tubules.
5. Glomerular hyalinization and sclerosis.
6. Interstitial fibrosis and scarring.
7. Tubular atrophy involving segments of proximal tubules.
8. Chronically in advanced cases, occasional hyperplastic foci in affected tubules.
9. In some advanced cases, accumulation of protein droplets in sporadic proximal tubules.

differs from α_{2u}-g nephropathy. Albuminuria, hypoalbuminemia, and hypocholesterolemia typify CPN, with increases in serum creatinine and urea nitrogen levels in end-stage disease (Barthold, 1979).

F. 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 data were examined on seven chemicals tested for chronic toxicity and carcinogenicity by the National Toxicology Program (NTP) or the National Cancer Institute (NCI). All seven produced accumulation of hyaline droplets, nephropathy, and kidney tumors in male rats. These model compounds are d-limonene, dimethyl methylphosphonate, hexachloroethane, 1,4-DCB, tetrachloroethylene, pentachloroethane, and isophorone[3]. Information was also examined on unleaded gasoline (tested by inhalation as a totally vaporized form at the International Research and Development Corporation [IRDC] for the American Petroleum Institute). Gasoline is a complex blend with CIGA properties (MacFarland et al., 1984). Data on the two non-CIGA, trichloroethylene and chlorothalonil, are included for comparative purposes. Although extensive acute and subchronic studies have been performed on two other chemicals (decalin and TMP), both of which cause α_{2u}-g nephropathy in the male rat, carcinogenicity bioassay data are not available for these compounds.

[3] Several of these seven chemicals cannot be described as true "CIGA carcinogens" since the accumulating protein in the hyaline droplets has not been confirmed to be a_{2u}-g. Their use as model compounds for purposes of developing the discussion on cancer should not be construed to mean that all seven chemicals fit the Policy Statement developed in Part IV of this document.

Trichloroethylene, which was tested by the NTP, induces kidney tumors in male rats only (NTP, 1988) but does not cause an accumulation of hyaline droplets or an increase in α_{2u}-g levels (Goldsworthy et al., 1988a). There is also some evidence that trichloroethylene metabolites bind covalently to renal macromolecules (Bruckner et al., 1989). Consequently, this compound would not be considered a CIGA.

Chlorothalonil, a fungicide tested on separate occasions by industry and a government agency, induced renal tubule tumors in male and female rats and in male mice (NCI, 1978a). It also induced hyaline droplet accumulation in proximal convoluted tubules of male rats (USEPA, 1988), but these may not become apparent during the first few weeks of treatment (Killeen et al., 1990). Electron microscopic studies of male rat kidney following subchronic chlorothalonil exposure revealed angular membrane-bound lysosomes containing crystalline structures similar to those observed in α_{2u}-g nephropathy (written communication, William M. Busey and James C. Killeen, Experimental Pathology Laboratories, Inc., to Office of Pesticides Programs, 1988). However, α_{2u}-g has not been detected in the renal tubules of chlorothalonil-exposed rats (Swenberg, 1989). The progression of chlorothalonil nephrotoxicity involves initially, vacuolar degeneration of proximal tubule epithelium followed 4 weeks later by tubule cell hypertrophy, hyperplasia, and tubule dilation (Killeen et al., 1990). Therefore, this compound appears not to produce the same spectrum or sequence of lesions induced by CIGA. Furthermore, chlorothalonil has been shown to interact with cellular macromolecules including histones and thiol proteins, possibly through covalent binding of a metabolite with sulfhydryl groups (Rosanoff and Siegel, 1981). Chlorothalonil also induces overt renal dysfunction in both sexes of rats. At doses from 40 mg/kg/day, blood urea nitrogen and creatinine were increased while circulating levels of glucose and albumin were decreased (USEPA, 1988). For these various reasons, chlorothalonil is not considered a member of the CIGA class.

A summary of the non-neoplastic and preneoplastic kidney effects observed in male rats after administration of the eight substances selected as possible CIGA is presented in Table 5.

Non-neoplastic 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 (see Table A-2 in the Appendix) and other relevant literature.

In male rats, at least one case of renal tubule cell hyperplasia was reported in the 2-year bioassays for the seven renal carcinogens tested by the NTP. The incidence was generally much higher and dose responsive. Although not reported in the IRDC bioassay, this lesion was observed in later research studies of unleaded gasoline (Short et al., 1989b). 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,

Table 5. Summary of Data on Non-Neoplastic and Pre-Neoplastic Kidney Lesions in Male Rats Associated with Eight Model Compounds that Induced Renal Tumors in 2-Year Bioassays

Chemical	Toxic Nephropathy			Cast Formation	Mineralization[a]		Karyo-megaly	Hyperplasia	
	Hyaline droplets	Dose-response	Increased severity		Present	Dose-response		Present	Dose-response
1,4-Dichloro-benzene	+	+	+	+	+	+	NR	+	+
Dimethyl methylphosphonate	+	+	+	+	+	+	NR	+	+
Hexachloroethane	+	+	+	+	+	+	NR	+	+
Isophorone	+	-	+ (slight)	+	NR	NR	NR	+	+
d-Limonene	+	+	+	+	+	+	NR	+	+
Pentachloroethane	+	+	NR	+	+	+	NR	+	NR
Tetrachloro-ethylene	+	+	NR	+	NR	NR	+	+	+
Unleaded gasoline	+	+	+	+	+	+	+/-	+[b]	NR

\+ Positive
\- Negative
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 Pre-Neoplastic Kidney Lesions in Mice and Female Rats Exposed to Eight Model 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 methyl-phosphonate	-	-	-	-	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	-	-	-	-	+	-

+ Positive
- Negative
NP Not reported
Ca Calcium
a Incidence and severity much higher in male rats
b Highest incidences in male rat

epithelial cell regeneration, and cast formation) were seen following administration of all eight of the renal carcinogens (Table 6). Some aspect of toxic tubular nephropathy 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 vs. hyaline) is not always described, and for mineral deposits, the site (papillary vs. corticomedullary) and form (linear vs. 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.

Part 2. Carcinogenicity

The second major part of this document compares and contrasts information on kidney tumors induced by classical renal carcinogens with information from the NTP (or NCI) assays for renal neoplasia induced by chemicals that produced hyaline droplets and/or accumulation of α_{2u}-g. In addition, other information, such as mutagenic activity and tumor-promoting ability, which help to define a CIGA carcinogen or point to possible mechanism of action, are evaluated.

Epidemiological studies of human renal cell cancer are reviewed for consistency with the hypothesis that CIGA-induced renal cancer in male rats is an inappropriate endpoint for assessing human risk. Implicit in this evaluation is a presumption of male rat-to-human tumor site concordance, a supposition EPA generally does not make. In this special case, however, 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.

V. Pathologic Features of Renal Carcinogenesis Induced by Classical Carcinogens

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, N-ethyl-N-hydroxyethylnitrosamine (EHEN), lead acetate, N-(4-fluoro-4-biphenylyl)acetamide (FBPA), and aflatoxin B_1 (Hard, 1990). In the mouse, certain nitrosamines, streptozotocin, and ochratoxin A are strong inducers of renal tubule tumors, while the classical renal carcinogen in hamsters is diethylstilbestrol (Hard, 1987). In general, these prototypic renal carcinogens are active in both males and females.

Studies on the pathogenesis of renal tubule tumor formation using model carcinogens in rats demonstrate that a continuum of chemically induced steps leads from atypical hyperplasia in tubules (also termed hyperplastic tubules, tubule dysplasia, and atypical cell foci) through microscopic adenomas, to macroscopic adenocarcinomas or carcinomas (Hard, 1987; Lipsky and Trump, 1988).

In addition, there are invariably pathologic changes which precede the proliferative sequence of preneoplastic and neoplastic lesions including a

period of early nephrotoxicity and, often, karyomegaly. These various lesions are described below in chronological sequence.

A. 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 (Hard, 1987). 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 (Hard et al., 1984) and with FBPA, to the P3 segment (Dees et al., 1980a,b).

Detailed histological and/or ultrastructural observation shows that hyaline droplet accumulation is not induced by DMN (Hard and Butler, 1971; Hard et al., 1984) or DEN (G.C. Hard, unpublished observations); nor has it been described in studies using other carcinogens, such as FBPA (Dees et al., 1980a,b), as models for renal carcinogenesis.

More is known about DMN than other classical renal carcinogens concerning molecular interactions during the time that acute toxic changes are seen in the proximal tubules. DNA adduct formation in rat renal tissue occurs rapidly following a single administration of DMN. 0^6-Methylguanine formed in the renal cortex (Fan et al., 1989) persists at least 4 days post-injection (Nicoll et al., 1975), which is consistent with the notion that methylation of the 0^6 position of guanine in DNA is the most likely initiating event (Pegg, 1984).

B. Karyomegaly

Conspicuous nuclear enlargement, indicative of increased chromosome number without completion of mitosis (Jackson, 1974), may occur in scattered proximal tubule cells during the weeks preceding development of carcinogen-induced proliferative foci. Although karyomegaly is produced by many, but perhaps not all 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 (Dees et al., 1980a; Hard, 1987; Lipsky and Trump, 1988).

C. 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 some degree of cellular atypia (dysplasia) in the form of cell pleomorphism and increased nuclear to cytoplasmic area ratio (Hard, 1987; Lipsky and Trump, 1988). Preneoplastic tubule hyperplasia is generally considered to be distinguishable from the

background tubular regeneration that is a component of spontaneous CPN (Lipsky and Trump, 1988; NTP, 1988).

D. 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 an arbitrary one based primarily 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 (Hard, 1990).

E. 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 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 adenocarcinomas 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 (UAREP, 1983; Lipsky and Trump, 1988; Hard, 1990).

F. 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 (Hard, 1987). 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 (Lipsky and Trump, 1988). However, lifespan of the animal 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 (Hard, 1984). For example, rats that survived at least 1.5 years after dosing with DMN showed a high rate of metastasis,

approximately 50 percent, 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.

G. 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 (Dees et al., 1980a,b), while DMN tumors arise from the convoluted segments of proximal tubules, probably P2 (Hard, 1990). Lead-acetate and DEN-induced tumors appear to originate in both P2 and P3 segments (Nogueira, 1987).

VI. Neoplastic and Preneoplastic Lesions Observed in the 2-Year Bioassays

Data for all reported renal tubule tumors and tubule hyperplasia in male rats from the 2-year bioassays on the eight model substances are summarized in Table 7. For tumors occurring at sites other than the renal tubule, only statistically significant incidences are mentioned. Table 8 provides similar information for trichloroethylene and chlorothalonil. For eight of the ten substances, exposure was by the oral route; 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 and differences in attention paid to the possible role of chemically induced 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 hours/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) (Bruner, 1984; MacNaughton and Uddin, 1984). In contrast, the kidneys of female rats and female C57/BL6 mice similarly exposed to RJ-5 or JP-10 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 bioassays, there are considerations generic to all bioassays conducted by the NTP. For example, the NTP positions with regard to evaluation of rare tumors and the use of historical controls influence NTP interpretation of the evidence for carcinogenicity of CIGA (Haseman et al., 1984). Likewise, survival rates influence the ability to analyze information from animal bioassays. These generic issues are explored before describing the results of individual studies.

A. Generic Considerations

Renal tubule tumors are neoplasms with a low background incidence in laboratory animals including the rat strains used in the chronic bioassays

Table 7. Incidences of Renal Tubule Preneoplasia and Neoplasia in Rats taken from 2-year Bioassays on Eight Model Substances

Chemical	Strain	Sex	Changes	Doses (mg/kg/day) 0	150	300
1,4-Dichloro-benzene (NTP-TR-319) (NTP, 1987a)	F344	M	Survival (%)	77	69	43
			Hyperplasia (%)	0	2	18
			Adenomas			
			Incidence	0/50	0/50	1/50
Gavage			Adj. Rate (%)	0	0	4
			Adenocarcinomas			
			Incidence	1/50	3/50	7/50
			Adj. Rate (%)	3	9	26
			Combined			
			Incidence	1/50	3/50	8/50
			Adj. Rate (%)	3	9	28

Other Tumors: Hepatocellular tumors in mice.

Chemical	Strain	Sex	Changes	Doses (mg/kg/day) 0	500	1000
Dimethyl methyl-phosphonate (NTP-TR-323) (NTP, 1987b)	F344	M	Survival (%)	56	34	19
			Hyperplasia (%)	0	16	18
			Adenomas		None	
			Adenocarcinomas			
Gavage			Incidence	0/50	2/50	3/49
			Adj. Rate (%)	0	9	19

Other Tumors: Mononuclear cell leukemia; transitional cell papillomas of the renal pelvis.

Chemical	Strain	Sex	Changes	Doses (mg/kg/day) 0	0	212	423
Hexachloro-ethane (NTP-TR-68) (NCI, 1978b)	Osborne-Mendel	M	Survival (%)	56	65	20	18
			Hyperplasia (%)		Not Reported		
			Adenomas				
			Incidence	0/20	0/18	4/37	0/29
Gavage			Adj. Rate	0	0	11	0
			Carcinoma	None			

Other Tumors: Hepatocellular tumors in mice.

(cont.)

Table 7. (cont.)

Chemical	Strain	Sex	Changes	Doses (mg/kg/day) 0	10	20
Hexachloro-ethane (NTP-TR-361) (NTP, 1989)	F344	M	Survival (%)	62	58	52
			Hyperplasia (%)	4	8	22
			Adenomas			
			Incidence	1/50	2/50	4/50
			Adj. Rate (%)	3	6	15
Gavage			Adenocarcinomas			
			Incidence	0/50	0/50	3/50
			Adj. Rate (%)	0	0	9
			Combined			
			Incidence	1/50	2/50	7/50
			Adj. Rate (%)	3	6	24

Other Tumors: Marginal increase in pheochromocytomas in male rats.

Chemical	Strain	Sex	Changes	Doses (mg/kg/day) 0	250	500
Isophorone (NTP-TR-291) (NTP, 1986a)	F344	M	Survival (%)	66	66	28
			Hyperplasia (%)	0	2	8
			Adenomas			
Gavage			Incidence	0/50	0/50	2/50
			Adj. Rate (%)	0	0	8
			Adenocarcinomas			
			Incidence	0/50	3/50	1/50
			Adj. Rate (%)	0	9	4
			Combined			
			Incidence	0/50	3/50	3/50
			Adj. Rate (%)	0	9	12

Other Tumors: Preputial gland tumors in male rats; hepatocellular tumors, mesenchymal tumors, and malignant lymphomas in male mice.

Chemical	Strain	Sex	Changes	Doses (mg/kg/day) 0	75	150
d-Limonene (NTP-TR-347) (NTP, 1990)	F344	M	Survival (%)	60	68	69
			Hyperplasia (%)	0	4	7
			Adenomas			
Gavage			Incidence	0/50	4/50	8/50
			Adj. Rate (%)	0	12	19
			Adenocarcinomas			
			Incidence	0/50	4/50	3/50
			Adj. Rate (%)	0	12	7
			Combined			
			Incidence	0/50	8/50	11/50
			Adj. Rate (%)	0	23	25

Other Tumors: None in mice or rats

Table 7. (cont.)

Chemical	Strain	Sex	Changes	Doses (mg/kg/day) 0	75	150
Pentachloro-ethane (NTP-TR-232) (NTP, 1983)	F344	M	Survival (%)	82	68	52
			Hyperplasia (%)	0	0	2
			Adenomas			
			Incidence	0/50	1/49	4/50
Gavage			Adj. Rate (%)	0	3	14
			Adenocarcinomas			
			Incidence	1/50	1/49	0/50
			Adj. Rate (%)	2	3	0
			Combined			
			Incidence	1/50	2/49	4/50
			Adj. Rate (%)	2	6	14

Other Tumors: Hepatocellular tumors in mice.

Chemical	Strain	Sex	Changes	Doses (ppm) 0	200	400
Tetrachloro-ethylene (NTP-TR-311) (NTP, 1986b)	F344	M	Survival (%)	48	40	24
			Hyperplasia (%)	0	6	10
			Adenomas			
			Incidence	1/49	3/49	2/50
Inhalation			Adj. Rate (%)	4	11	11
			Adenocarcinomas			
			Incidence	0/49	0/49	2/50
			Adj. Rate (%)	0	0	11
			Combined			
			Incidence	1/49	3/49	4/50
			Adj. Rate (%)	4	11	22

Other Tumors: Leukemia in rats; hepatocellular tumors in mice.

Mixture	Strain	Sex	Changes	Doses (ppm) 0	67	292	2056
Unleaded gasoline (USEPA, 1987)	F344	M	Survival (%)	Not affected			
			Hyperplasia (%)	Not reported in the bioassay			
			Adenomas				
			Incidence	0/49	1/59	2/56	1/45
Inhalation			Adj. Rate (%)	0	2	4	2
			Carcinomas				
			Incidence	0/49	1/59	2/56	6/45
			Adj. Rate (%)	0	2	4	14
			Combined				
			Incidence	0/49	1/59	5/56	7/45
			Adj. Rate (%)	0	2	9	16

Other Tumors: Hepatocellular tumors in female mice.

Table 8. Incidences of Renal Tubule Preneoplasia and Neoplasia in Rats taken from 2-year Bioassays on Chlorothalonil and Trichloroethylene

Chemical	Strain	Sex	Changes	Doses (ppm) 0	5063	10126
Chlorotha-lonil (NTP-TR-41) (NCI, 1978a)	Osborne-Mendel	M	Survival (%)	82	40	40
			Hyperplasia (%)		none	
			Adenomas			
			Incidence	0/10	2/46	1/49
			Rate (%)	0	4	2
Diet			Carcinomas			
			Incidence	0/10	1/46	3/49
			Rate (%)	0	2	6
			Combined			
			Incidence	0/10	3/46	4/49
			Rate (%)	0	6	8
		F	Survival (%)	50	62	72
			Hyperplasia (%)		none	
			Adenomas			
			Incidence	0/10	0/48	3/50
			Rate (%)	0	0	6
			Carcinomas			
			Incidence	0/10	1/48	2/50
			Rate (%)	0	2	4
			Combined			
			Incidence	0/10	1/48	5/50
			Rate (%)	0	2	10

Other Tumors: none

Chemical	Strain	Sex	Changes	Doses (mg/kg/day) 0	0	500	1000
Trichloro-ethylene (NTP-TR-273) (NTP, 1988)	Osborne-Mendel	M	Survival (%)	42	44	34	30
			Hyperplasia (%)	0	0	10	6
			Adenomas				
			Incidence	0/50	0/50	6/50	1/50
			Adj. Rate (%)	0	0	32	6
Gavage			Carcinomas				
			Incidence	0/50	0/50	0/50	1/50
			Adj. Rate (%)	0	0	0	6
			Combined				
			Incidence	0/50	0/50	6/50	2/50
			Adj. Rate (%)	0	0	32	11

Tumors in Other Strains: 2-4% renal tubule tumors in three other strains.

Other Tumors: Malignant mesothelioma in male rats; hepatocellular tumors in male and female mice, and lymphoma in female mice.

on CIGA, namely F344 and Osborne-Mendel. The overall historical incidence of these tumors in male F344 rats is considered by the NTP to be 0.5 percent based on data reported on 1,943 animals which served as vehicle controls in studies involving administration of chemicals via corn oil gavage (NTP, 1990). In a larger historical control database, involving 2,320 male and 2,370 female F344 rats used as untreated controls in NTP two-year bioassays, the incidence was 0.35 percent for males and 0.17 percent for females suggesting a male predilection for renal tubule tumors (Solleveld et al., 1984). This is supported by spontaneous renal tubule tumor incidence rates recorded for Osborne-Mendel rats used as controls in the NCI Carcinogenesis Testing Program (Goodman et al., 1980). In 975 males and 970 females, the incidence was 0.3 percent and 0 percent, 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 (Haseman et al., 1984; NTP, 1989).

In the 2-year studies with the eight selected renal carcinogens, the observed incidences of renal tumors for individual chemically dosed groups were less than 25 percent, and no higher than 16 percent 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 data. The high mortality rate observed in some of these studies cannot be attributed to the renal tumors (Hoel et al., 1988). In fact, poor survival rates usually indicated excessive toxicity. For the 1,4-DCB bioassay, survival of the high-dose males, 40 percent at termination, became significantly lower than that of vehicle controls after week 97 (NTP, 1987a). Nearly all deaths were nonaccidental. A similar situation pertains to isophorone where only 28 percent of high dose males survived to termination (NTP, 1986a).

The decreased survival rates suggest that a maximum tolerated dose (MTD) was exceeded since the early deaths could not be attributed to tumors. Administration of a chemical at doses exceeding an MTD may alter responses that would be seen at lower dose levels (Office of Science and Technology Policy [OSTP], 1985). However, exceeding an MTD, by itself, is not compelling evidence that tumors 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 tetrachloroethane 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 neoplasms.

B. Renal Tumor Incidence

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

For all of the eight model carcinogens, and also for trichloroethylene and chlorothalonil, the increase in the incidences of renal tubule tumors, where adjusted for intercurrent mortality, was dose related. Because the incidence of 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 the 1986 Guidelines for Carcinogen Risk Assessment, EPA discussed its strategy for analyzing combinations of benign and malignant tumors (USEPA, 1986). 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. Since the distinction between adenomas and adenocarcinomas for renal tubule tumors in rats is rather arbitrary, based mainly on size, these general principles cannot be rigidly applied.

C. 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 the eight chemicals does provide indirect evidence of progression from tubule cell hyperplasia via adenomas to adenocarcinomas. In studies with d-limonene (NTP, 1990) and hexachloroethane (NTP, 1989), 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 (Lipsky and Trump, 1988; Hard, 1990).

D. 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 carcinogens usually exceeded 18 months. In general, the first renal tumor observed in each of the bioassays occurred about 5 to 10 weeks earlier in the high-dose than in 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 (NTP, 1989).

E. Induction of Other Tumor Types

Six of the eight model substances produced liver tumors in male and/or female mice but not in male or female rats. These chemicals were hexachloroethane, unleaded gasoline, isophorone, 1,4-DCB, pentachloroethane, and tetrachloroethylene. A different mechanism, independent of hyaline droplet accumulation, may be involved in the production of liver tumors by these six chemicals. Some authors suggest a mechanism involving peroxisome proliferation to account for the production of such liver tumors (Elcombe et al., 1985; Goldsworthy and Popp, 1987).

An alternative explanation for the liver tumors is that both CIGA-induced liver and kidney tumors are produced by a common mechanism (direct or indirect) not involving α_{2u}-g. Available data do not tend to support this hypothesis, although a recent inhalation toxicity study of 1,4-DCB illustrates other types of data needed before these questions can be resolved. In this study, significantly higher levels of 1,4-DCB were found in the kidneys of male rats and in the livers of female rats following exposure at 500 ppm for 24 hours (Umemura et al., 1990). Although the Umemura study may simply demonstrate reaction of 1,4-DCB with α_{2u}-g, it may also indicate metabolic differences among species and sexes that influence the effective doses delivered to the tumor sites.

Primary tumors in addition to those in the male rat renal tubule were not consistently produced in rats or mice at organ sites other than the liver following administration of the eight chemicals. The production of tumors at other sites, however, raises the possibility that other mechanisms could also be contributing to the overall kidney tumor incidence in male rats. This possibility has been suggested for tetrachloroethylene (Green et al., 1990; Dekant et al., 1989). Dekant and colleagues have proposed a mechanism involving hepatic glutathione S-conjugate formation and, ultimately,

bioactivation of renal cysteine conjugate by β-lyase in the nephrotoxic and carcinogenic response to halogenated alkenes, including tetrachloroethylene, although they also do not rule out a role for α_{2u}-g-induced nephrotoxicity. Within this context, it is noteworthy that in the tetrachloroethylene bioassay a renal tubule adenocarcinoma was observed in a single low-dose male mouse, clearly a statistically nonsignificant event, but less readily regarded as biologically irrelevant.

VII. Additional Evidence Concerning the Renal Carcinogenicity of CIGA

Key evidence relevant to providing information on carcinogenic mechanisms can be derived from short term tests, such as assays for gene mutations and DNA damage, and from studies testing the tumor-promoting effects of CIGA.

A. Genetic Toxicology Studies

The available genotoxicity data for the eight model carcinogens and for trichloroethylene and chlorothalonil are summarized in Tables 9 and 10. The four assays listed in the tables (Salmonella [SAL], chromosome aberrations in [ABS] Chinese hamster ovary cells, sister chromatid exchange [SCE] in Chinese hamster ovary cells, 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 assays are employed by NTP. Consequently, this analysis of genotoxicity data was limited, for the main part, to the 10 model substances with bioassay data. Data from Drosophila tests conducted by the NTP (Yoon et al., 1985) and in human lymphoblasts (Richardson et al., 1986) are also cited in Tables 9 and 10 where available.

The eight renal carcinogens selected as possible CIGA have been tested for chromosome aberrations in Chinese hamster ovary (CHO) cells (Galloway et al., 1987a) and in Salmonella (Haworth et al., 1983; Mortelmans et al., 1986; Ashby and Tennant, 1988; NTP, 1987b). 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 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 (Watabe et al., 1981). Several chemicals have tested positive, at least under some conditions, for SCE in CHO cells (Galloway et al., 1987a) and in the mouse lymphoma TK-gene mutation assay (McGregor et al., 1988). Four of the eight possible CIGA and both non-CIGAs were positive. Richardson et al. (1986) reported negative results for unleaded gasoline and its known CIGA component, TMP, in assays for TK-gene mutations and SCE in the TK6 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 hyaline-droplet inducing carcinogens were observed in the absence, but not

Table 9. Summary of Genotoxicity Data from Eight Selected Male Rat Kidney Carcinogens

Substance	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 methyl-phosphonate	-	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) (cited in NTP bioassay report). Drosophila SLRL positive, but translocations negative. Dominant lethal positive in both rats and mice.
Hexachloroethane	-	-	+		SCE reproducible 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[a] reported negatives for hepatocyte UDS, mouse micronuclei, and MLA (with and without S9).
d-Limonene	-	-	-	-	Clear negative in all NTP sttdies.
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 Salmonellla 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.

SAL = Salmonella; ABS = chromosome aberrations in 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.

Positive (+), negative (-), ±, and equivocal (E) as defined in Haworth et al., 1983, Galloway et al., 1987a, and McGregor et al., 1988.

[a] U.S. EPA Office of Toxic Substances submission by Chemical Manufacturers Association Document ID 40-845047, under Section 4 of the Toxic Substances Control Act.

Table 10. Summary of Genotoxicity Data for Two Selected Non-CIGA Male Rat Kidney Carcinogens

Substance	SAL	ABS	SCE	MLA	Comments
Chlorothalonil	-	+	+	+	ABS and MLA positive without S9; MLA not tested with S9. Negative in Drosophila SLRL.
Trichloroethylene	-	-	+	+	Unpublished studies negative in SAL and MLA, as well as in dominant lethal and bone marrow cytogenetic studies in mice. MLA on various catalytic fractions gave mixed results. Positive UDS in rat, mouse, and human hepatocytes. In vivo studies with gavage were positive in mouse, but not in rat liver. Kidney UDS in rats was negative (both gavage and inhalation).

SAL = Salmonella; ABS = chromosome aberrations in CHO cells; MLA = thymidine-kinase (TK)-gene mutation assay in L5178Y cells; SLRL = sex-linked recessive lethal: UDS = unscheduled DNA synthesis.
Positive (+), negative (-), ±, and equivocal (E) as defined in Haworth et al., 1983, Galloway et al., 1987a, McGregor et al., 1988.

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 model 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. (1987b) 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 are those studies in which rodent kidney or kidney extracts are combined with a genotoxic endpoint. Loury et al. (1987) 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. (1988b). However, both studies reported significant elevation of replicative DNA synthesis in kidneys of male rats treated with these compounds.

Recently, Vamvakas et al. (1989) 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 apparently 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 in the kidney or with glutathione conjugates for these chemicals precludes closure on the question.

B. Initiation-Promotion Studies

The multistage concept of carcinogenesis, involving in its simplest form, an irreversible initiation phase followed by a stage of tumor promotion (Pitot, 1982), 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 promotion or cocarcinogenic activity in an established initiation-promotion model of renal carcinogenesis.

Using 2 weeks exposure to 170 ppm of EHEN in the drinking water as the initiating agent, the first initiation-promotion experiment of Short et al. (1989b) included both sexes of F344 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, comprised of approximately 30 animals, included a control, 2 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 to 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 under-estimation of an optimal initiating dose of EHEN, which resulted in a very 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 authors' conclusions 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 (Dietrich and Swenberg, 1991b). This study specifically addressed the comparison of responses between the male F344 rat and the α_{2u}-g-deficient NBR strain. The initiating dose of EHEN was 500 ppm administered in the drinking water for two weeks, followed by d-limonene by daily gavage (5 days a week) at 150 mg/kg/day for 30 weeks. An initiation control (EHEN), promotion control (d-limonene), and a vehicle control were included for both strains. In the F344 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 which has been demonstrated with these same compounds (Short et al., 1989a; Dietrich and Swenberg, 1991b). The extent of cell proliferation is regarded as an important factor in chemical carcinogenesis (Grisham et al., 1983; Cohen and Ellwein, 1990; Cunningham et al., 1991) and stimulation of cell turnover is one of the key mechanisms believed to operate in tumor promotion (Farber, 1988).

VIII. Comparison of CIGA with Classical Renal Carcinogens

In general, classical renal carcinogens or their active metabolites are electrophilic species binding covalently to macromolecules and forming, in particular, DNA adducts (Hard, 1987; Lipsky and Trump, 1988; Alden and Frith, 1991). 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 0^6-alkylguanine which is a promutagenic lesion (Pegg, 1984). 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 (Section IIIG), CIGA binding to α_{2u}-g is reversible and not covalent in nature.

Classical renal carcinogens can induce renal tubule cancer in rats or mice in high incidences, with minimal duration of exposure, clear dose-response relationships, and with decreased latent period of development (Hard, 1987; Alden and Frith, 1991). Tumor frequencies are often over 50 percent and up to 100 percent, much higher than the low incidences (2% to 28% adjusted) recorded for CIGA. Unlike CIGA-induced renal carcinogenesis, there is usually no absolute sex specificity, with males and females both susceptible, but sometimes to varying degree. These differences in potency and species- and sex-susceptibility, suggest that classical renal carcinogens and CIGA act via different mechanisms in kidney carcinogenesis.

In addition, 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 utilizes 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 (Bruner, 1984).

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 pathological events induced by CIGA in the male rat.

Pathology reports indicate that renal tubule tumors induced by CIGA are morphologically indistinguishable from spontaneous tumors or those in-

duced by classical carcinogens, with both granular and clear cell types occurring. Likewise, despite differences in toxicity observed, the sequence of development of CIGA-induced renal tumors from tubule hyperplasia to carcinoma appears identical. However, some evidence from the bioassays suggests that the CIGA tumors may, in general, have a smaller size, probably because of the difference in potency between these chemicals and classical carcinogens, affecting the latent period of tumor development.

As with classical carcinogens, metastases have been rarely reported for renal tubule tumors related to treatment by chemicals inducing hyaline droplets and/or α_{2u}-g. The one case of metastasis noted with hexachloroethane suggests, however, that a malignant potential exists for such neoplasms.

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.

IX. Evidence Concerning Human Kidney Cancer

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 (Bennington and Beckwith, 1975; NCI, 1987). The mortality rate in these cases is high, and overall, the survival rate for patients with renal cell cancer is 48 percent (Devesa et al., 1990). In addition, the etiology of kidney cancer in humans is poorly understood.

A. 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 of cell types. Infrequently, renal cell carcinoma presents as a sarcomatoid form composed of spindle cells (Bennington and Beckwith, 1975; Bannayam and Lamm, 1980; Tannenbaum, 1983).

It is generally accepted that the origin of renal cell carcinoma is the proximal tubule, based on both immunological study (Wallace and Nairn, 1972) and ultrastructural features (Tannenbaum, 1971; Bennington and Beckwith, 1975). Electron microscopy reveals many similarities between the tumor cells and normal proximal tubule epithelium, including brush border elements, membrane-associated vesicles, and basilar infoldings of the plasma membrane (Tannenbaum, 1971). 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, histochemi-

cal, or immunologic features which discriminate them, other than size, which is not an absolute biologic parameter (Bennington and Beckwith, 1975; Ritchie and Chisholm, 1983; Tannenbaum, 1983). 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 (Bell, 1950; Hellsten et al., 1981; Ritchie and Chisholm, 1983).

B. Incidence and Mortality

Kidney cancer statistics are usually reported in a form which 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 (McLaughlin and Schuman, 1983; Asal et al., 1988a) and represents about 70 percent of all kidney tumors in adults (Devesa et al., 1990). 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 US is 25,300 with a mortality estimate of 10,600 deaths (Boring et al., 1991). These figures represent approximately 2 percent of both new cancer cases at all sites and total cancer deaths. The age-adjusted incidence rates in the US 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 (Devesa et al., 1990). Most studies indicate a consistent male to female ratio of 2:1 for the incidence of renal cell tumors (Asal et al., 1988a; Devesa et al., 1990).

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 (Thorhallson and Tulinius, 1981). The lowest rates for renal cancer are recorded in Africa, Asia, and South America (McLaughlin and Schuman, 1983). Within the US, mortality surveys indicate that the North Central region and some areas in the Northeast have the highest incidence rate for renal cell carcinoma (Pickle et al., 1987). 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 (McLaughlin et al., 1984). Several studies reported that the urban rates for renal cell tumor incidence are higher than for rural areas, but the correlation is considered to be weak (Newsom and Vugrin, 1987).

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 percent (Bannayam and Lamm, 1980) to 25 percent, the latter for males over the age of 50 (Reese and Winstanley, 1958). These findings have led to speculation that a proportion of adenomas

may reach a limit of growth and/or remain quiescent (Bannayam and Lamm, 1980; Warter, 1983).

During the period 1950 to 1985, the US Cancer Statistics data indicated an increase of 82 percent in the incidence of kidney and renal pelvis cancer combined (NCI, 1987). For renal cell cancer alone, the increase among whites, based on comparison of data from 1969 to 1971 with data from 1983 to 1985, was about 30 percent; this would be an average annual percent change in incidence of 2.0 for males and 1.8 for females (Devesa et al., 1990). Data from Cancer Registries in Scotland also indicated an increased incidence of renal cell carcinoma of approximately 37 percent for males between 1967 and 1979, although there was no corresponding increase in females (Ritchie and Chisholm, 1983). Despite an improvement in mortality rates since 1950 compared to incidence rates (NCI, 1987), the relative 5-year survival rates, which are close to 50 percent, have not altered since the early 1970's (Boring et al., 1991), 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 (Higginson et al., 1984; NCI, 1987).

Renal cell carcinoma has been diagnosed with increasing frequency in patients with chronic renal failure (Hughson et al., 1986; Newsom and Vugrin, 1987). In particular, this appears to reflect an association with the development 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 percent (Hughson et al., 1986). Thus, current data suggest that a growing population of humans receiving maintenance dialysis may be at risk for developing renal cell tumors.

C. 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 (Dayal and Kinman, 1983; McLaughlin and Schuman, 1983; Yu et al., 1986; Asal et al., 1988a; Brownson, 1988; La Vecchia et al., 1990). Although a few studies have failed to identify a statistical association between smoking and renal cell cancer, it has been estimated that 30 percent of renal cell carcinomas in males and 24 percent in females may be attributable to cigarette smoking (McLaughlin et al., 1984) and that there is evidence for a moderate dose response (McLaughlin and Schuman, 1983). One study has also linked use of chewing tobacco with renal cell carcinoma in males (Goodman et al., 1986), and another has associated smoking with renal adenoma (Bennington et al., 1968).

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 (reviewed in Dayal and Kinman, 1983; McLaughlin and Schuman, 1983; McLaughlin, 1984; McLaughlin et al., 1984; Goodman et al., 1986; Yu et al., 1986; Asal et al., 1988a; McCredie et al., 1988). Of these, the evidence is least consistent for beverage consumption, artificial sweeteners, other dietary factors, and socioeconomic status, and is strongest for high body mass index and drug use (phenacetin and diuretics).

D. Occupational Factors

Although a number of epidemiological studies have reported some association between occupation and renal cancer, clear occupational determinants have yet to be demonstrated and it is considered that much epidemiological research is needed to further define and quantify potential risks (McLaughlin and Schuman, 1983). Occupational exposures in North America, where at least one study has reported an association with increased kidney cancer rates, include asbestos (Selikoff et al., 1979; Smith et al., 1989), coke-oven emissions in the steel industry (Redmond et al., 1972), printing press chemicals (Paganini-Hill et al., 1980), laundry- and dry-cleaning agents (Blair et al., 1979; Katz and Jowett, 1981; Duh and Asal, 1984; Asal et al., 1988b), exhaust fumes in truck drivers (Brownson, 1988), petroleum, tar, and pitch products (Thomas et al., 1980; Hanis et al., 1982; Wen et al., 1983; McLaughlin et al., 1984; Savitz and Moure, 1984; Kadamani et al., 1989), and aviation and jet fuels (Siemiatycki et al., 1987). In these studies, information on smoking history was rarely available, so that its possible influence could not be determined.

A study that examined the relationship between renal cancer and occupation as defined in the 1960 Census in Sweden, where the incidence rates are higher than in the US, did not detect increased risk for hearth and furnace workers in the steel industry, printing workers, laundry and dry-cleaning workers, or workers in petroleum refineries and gasoline stations (McLaughlin et al., 1987). Instead, the Swedish study reported an increase in incidence of renal cell cancer among health care professionals.

E. 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. (1989) 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.

In a case-referent study of occupational risk indicators for renal cell adenocarcinomas, Partanen et al. (1991) examined all cases reported in

Finland in 1977 to 1978. These investigators found an elevated risk and an exposure-response relationship for gasoline exposure. Since the postulated average latency period was about 30 years, a role for lead compounds could not be ruled out.

Synthetic solvents widely used in dry-cleaning include the CIGA, tetrachloroethylene, as well as Stoddard and 140F solvents.[4] Several studies analyzing proportional mortality data on laundry- and dry-cleaning workers in various parts of the US reported elevated risks for kidney cancer (Blair et al., 1979; Katz and Jowett, 1981; Duh and Asal, 1984; Asal et al., 1988b). More recent studies that were better designed, however, have not substantiated the earlier findings. No statistically significant elevations in kidney cancer risks have been detected in the studies of dry-cleaning workers by Blair et al. (1990) (Standardized Mortality Ratio [SMR] 50; 95% CI 10-180), Lynge and Thygesen (1990) (Standardized Incidence Ratio [SIR] males, 1.5; 95% CI 0.6-3.3; females 0.6; 95% CI 0.2-1.4), or Brown and Kaplan (1987) (SMR 200; 95% CI 55-517). In considering occupational exposure to solvents as a general chemical category, Harrington et al. (1989) 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 others, was acknowledged by the authors as sufficient to identify only large risk estimates.

Siemiatycki et al. (1987) 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 (Siemiatycki et al., 1987). 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 percent 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 (1989) conducted a quantitative meta-analysis by cancer site of petroleum industry employees from the US, 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 nonsignificant deficits to nonsignificant excesses. However, the possibility of an elevated kidney cancer risk was raised for one specific group within the

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[4] Stoddard and 140F solvents are mixtures of hydrocarbons including straight and branched chain paraffins suggesting that they may also be CIGA.

industry. Drivers among British distribution workers (Rushton and Alderson, as reported by Wong and Raabe) showed borderline significance for excess kidney cancer mortality. Wong and Raabe (1989) 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 (McLaughlin et al., 1985). 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 (Poole et al., 1990), 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 USEPA (1987) 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 EPA's guidelines for cancer risk assessment (USEPA, 1986). As Enterline and Viren (1985) 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 an hypothesis specifically associating gasoline exposure and renal cancer. The cohort studies of petroleum workers do not lend themselves for a comparison since 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.

X. 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. For some of the steps, clear dose-response relationships have been shown. In other cases, histopathologically observ-

able events are of a secondary nature and not in the direct progression. As the following information shows, however, data demonstrating the existence of other steps in the proposed progression are limited, restricting 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.

A. Association Between CIGA, Hyaline Droplet Formation, and Alpha$_{2u}$-globulin Accumulation

Dose-dependent relationships have been demonstrated between the administration of d-limonene (Lehman-McKeeman et al., 1989) or gabapentin (Dominick et al., 1990) and excessive formation of hyaline droplets, and between unleaded gasoline or TMP and α_{2u}-g accumulation (Olson et al., 1987; Charbonneau et al., 1987). In the d-limonene study, hyaline droplets were graded on a scale of 0 to 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 mmol/kg were, control to high dose, 3, 4.5, ca.7, 8, and 10 (Lehman-McKeeman et al., 1989). 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 4.000 mmol/kg, induced α_{2u}-g concentrations in rat kidney tissue at 24 hours of 10.3, 17.3, and 28.1 mg/g wet weight, respectively, against a control value of 9.5 mg/g wet weight (Charbonneau et al., 1987). With orally administered gasoline, the α_{2u}-g concentrations were dose-responsive only in the range of 0.04 to 1.00 mL/kg (Olson et al., 1987).

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 (NTP, 1990). 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 employed 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 1,200 mg/kg had no measurable effect on α_{2u}-g levels in the kidney. Although microscopic examination of kidney sections stained with hematoxylin and eosin (H & E) showed no visible differences between dose and vehicle control male rats, in plastic embedded sections stained with Lee's methylene blue basic fuchsin, differences in the distribution, amount, and shape of intracytoplasmic granules in the proximal tubules were detected.

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 (1990) 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 pre-exposure levels by the

third day after treatment, although hyaline droplets, being structural entities, require up to 9 days for complete resolution (Garg et al., 1988). 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 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.

B. 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 (Short et al., 1987, 1989a,b). Although single-cell necrosis has been clearly demonstrated in association with cellular hyaline droplet accumulation (Kanerva et al., 1987a; Short et al., 1987), 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 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 (Alden and Frith, 1991).

Dose-response relationships between hyaline droplet accumulation and proximal tubule cell proliferation have been observed. Short and coworkers (1987) exposed male rats for 3 weeks to TMP (oral) or unleaded gasoline (inhalation) and then measured [^{3}H]-thymidine labeling indices. 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 (Figures 4 and 5). In an extended study of the same compounds, Short et al. (1989a) observed 6- to 11-fold increases in labeling indices 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 indices remained 4- to 6-fold higher than control values during the 48th week of exposure.

In contrast, Viau et al. (1986) did not observe a sustained regenerative response in the kidneys of male rats exposed to an isoparaffinic solvent consisting of saturated C_{10}-C_{12} aliphatic hydrocarbons beyond 5.5 weeks. Labeling indices in the cortex of treated rats at 46 and 68 weeks were no different from the controls. This apparent discrepancy between the gasoline and TMP results undoubtedly reflects differences in the technique of radioactive labeling. Viau et al. (1986) used a single injection of [^{3}H]-thymidine 1 hour prior to sacrifice, whereas, Short et al. (1989a) labeled continuously by subcutaneous osmotic minipump infusion over a 7-day period, the preferred method for cell populations with a low cell turnover, thereby increasing the amount of radioactivity incorporated into renal tissue.

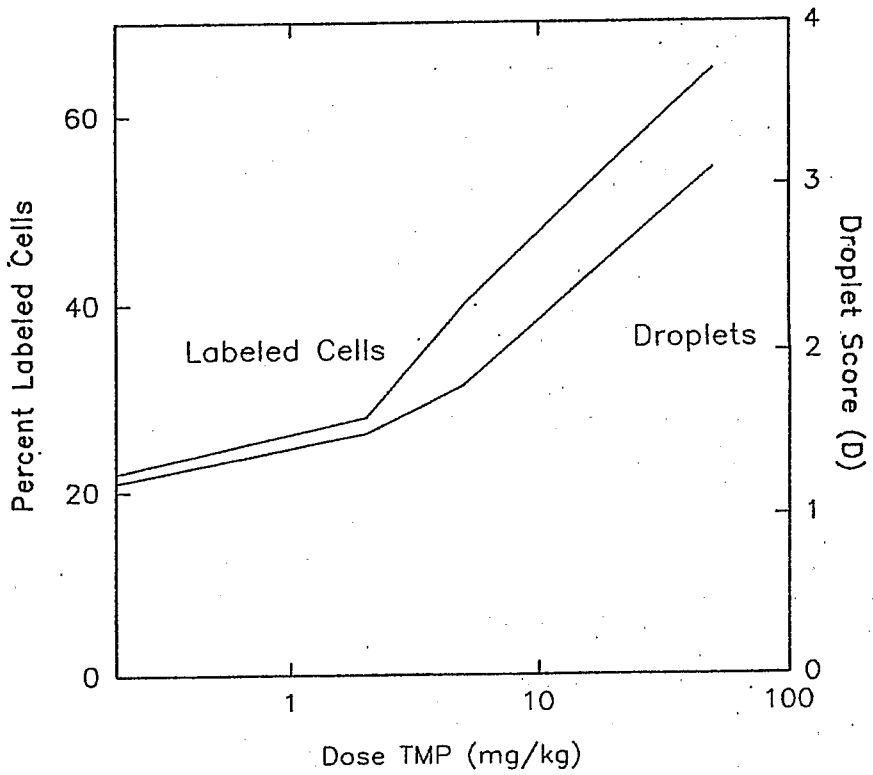

Figure 4: *Dose-response relationship between renal hyaline droplet accumulation (D) and [^{3}H]-thymidine labeling index of proximal tubule P2 cells in male F344 rats gavaged with TMP for 5 consecutive days per week for 3 weeks. Seven-day osmotic minipump implanted on twelfth day after start of dosing. Rats killed and fixed on 22nd day.*

Source: Adapted from Short et al., 1987.

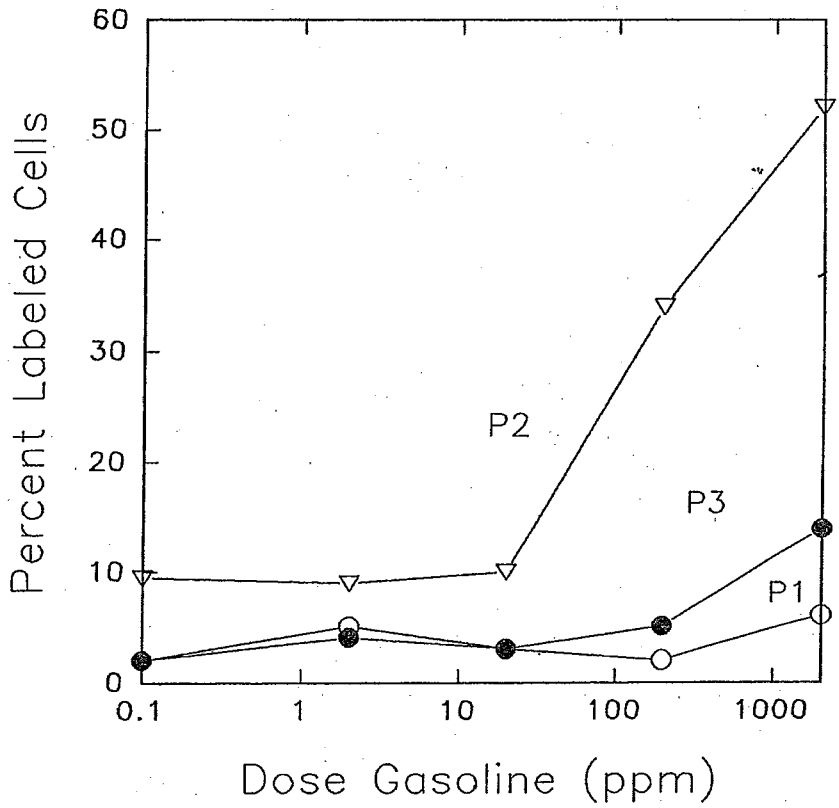

Figure 5: *Effect of 0 to 2,000 ppm unleaded gasoline on continuous uptake of [^{3}H]-TdR by P1, P2, and P3 segments of the proximal tubule epithelium. Each test point is the mean value determined from 3 rats. Dosage is presented on a log scale.*

Source: *Adapted from Short et al., 1987.*

In recovery studies with unleaded gasoline and TMP, Short and coworkers (1989a) showed that neither increased hyaline droplets nor cell proliferation were observable 7 days after discontinuing the 3-week exposures, indicating complete recovery. However, after 10 and 22 week periods of exposure, recovery was only partial, labeling indices remaining 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 subcutaneous 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 F344 rats initiated with EHEN (Dietrich and Swenberg, 1991b). 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 linked with replication. These questions need further investigation and hypothesis development in order to establish mechanisms of action.

C. Progression to Cast Formation, Tubule Dilation, and Mineralization

Since 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 selected chemicals evaluated in 13-week toxicity studies by the NTP and sometimes in the 2-year bioassays. In another study, Viau et al. (1986) 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 assumed to be 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 should become minimal. A second explanation is that subtle changes such as granular cast formation and the associated tubule dilation can be obscured by the development of CPN in later stages.

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. Figure 6 shows one example of the interrelationships

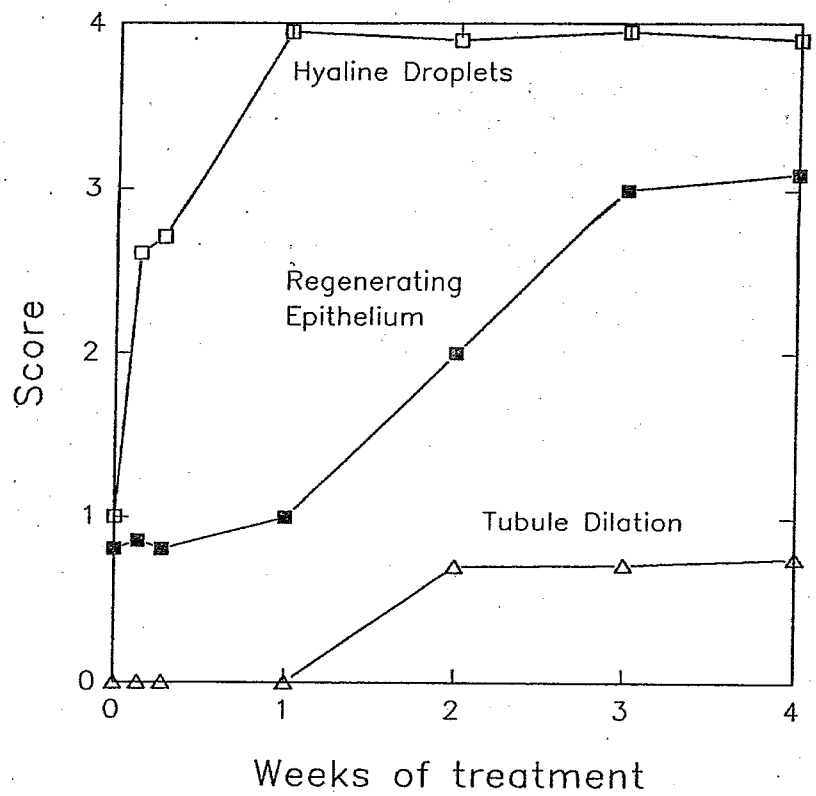

Figure 6: *Time-sequence for the development of hyaline droplets, regenerating tubule epithelium, and tubule dilation, in male F344 rats administered 2 g/kg unleaded gasoline daily by gavage for a 28-day period.*

Source: Adapted from Thomas et al., 1985.

observed between hyaline droplet formation, epithelial cell proliferation, and tubule dilation. In this study, male rats were administered unleaded gasoline (2 g/kg/day) for a period of 28 days and examined at 5 interim time-points (Thomas et al., 1985). 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 NTP and other 2-year bioassays with potential CIGA carcinogens but not in the 13-week toxicity studies. Clear dose-response relationships were demonstrated for 1,4-DCB (NTP, 1987a), JP-4 mixed distillate (MacNaughton and Uddin, 1984), and unleaded gasoline (USEPA, 1987). 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 (USEPA, 1987). 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 11 presents a summary of these data which shows 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 (Bruner 1986), supporting the notion that the pelvic hyperplasia is a urothelial response to mineralization in the papilla.

D. 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

Table 11. *Incidence of Medullary Mineralization in Male Rats During Inhalation Exposure to Unleaded Gasoline*

Observation time-points (months)	Exposure levels of unleaded gasoline vapor (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 of medullary mineralization is reported as percent of animals affected.

Source: Adapted from USEPA, 1987.

occasions. Short et al. (1989a) 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, 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 in the middle- and high-dose groups.

In the NTP bioassay of d-limonene (NTP, 1990), treated male rats showed a spectrum of compound-related kidney lesions, including exacerbation of CPN, mineralization in the renal medulla, hyperplasia of the epithelium lining the renal papilla, and proliferative lesions of the renal tubule epithelium. The severity of CPN on a scale of 0 to 4 was graded 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 (Trump et al., 1984b; Short et al., 1989b). 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 (Short et al., 1989b).

E. Evidence Concerning Progression from Nephrotoxicity to Renal Neoplasia

For the eight selected CIGA 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 (Cohen and Ellwein, 1990; Deal et al., 1989). Thus, a sustained state of cell turnover in the target cell population as a mechanistic link between α_{2u}-g nephropathy and renal neoplasia should be considered a plausible, but unproven, explanation 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 regeneration in the different segments of the male rat kidney have shown an increase in cell replication rates specifically in the histologi-

cally damaged P2 segments after administration of tetrachloroethylene or pentachloroethane (Goldsworthy et al., 1988a) or TMP or unleaded gasoline (Short et al., 1987, 1989a). 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 (Goldsworthy et al., 1988a).

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 (1991b) demonstrated that d-limonene promoted renal tubule tumors in male F344 rats, an animal that produces α_{2u}-g. In addition, there was a 5-fold increase of P2-labeling index in the F344 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 which 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 (Short et al., 1989b). 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.

Part 3 - Evaluation of the Hypothesis

XI. Summary of the Evidence on the Renal Effects of CIGA

Several lines of evidence establish an association between exposure of the male rat to chemicals that induce α_{2u}-g accumulation (CIGA) and nephrotoxicity, and strongly support an association between this nephrotoxicity and renal tubule tumors.

A. Association Between Alpha$_{2u}$-globulin Accumulation and Nephrotoxicity

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

- Although hyaline droplet accumulation per se is not necessarily diagnostic of a CIGA until proven to represent a_{2u}-g accumulation, 34 organic compounds including fuels, solvents, and other chemicals (listed in Table A-1), examined in this report, have been shown to induce 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.
- 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 a_{2u}-g nephropathy in the male rat. Five of the 34 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 5 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 model 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.
- TMPOH, the active metabolite of TMP may be able to form in vitro complexes with retinol-binding protein and α_1-acid glycoprotein, members of the lipocalin superfamily found in humans. In vivo data on retinol and α_{2u}-g, however, demonstrate that such an association does not necessarily lead to α_{2u}-g accumulation or hyaline droplet formation.

B. Association Between Nephrotoxicity 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 model carcinogens produced hyperplasia, adenomas, and adenocarcinomas in the renal tubule of the male rat.
- 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 non-CIGA renal toxicants.
- The incidence of renal tumors produced in the male rat by the eight model carcinogens was relatively low. These tumors were morphologically indistinguishable from other 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 model carcinogens occurred late, usually being found at the time of 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, therefore, may not depend on direct genetic injury as the mechanism for tumor induction.
- Trichloroethylene, a compound structurally similar to hexachloroethane and tetrachloroethylene produced renal tumors apparently by mechanisms, such as covalent binding to DNA, which do not appear applicable to the CIGA hypothesis.

C. *Information Reducing Confidence in the Conclusion that the Alpha$_{2u}$-globulin Response is Specific to the Male Rat*

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, the paucity of data on the lipocalin superfamily, in general, leaves several questions unanswered regarding the specificity of the response to the male rat.

- Pathological accumulation of hyaline droplets is a reaction to excessive protein load not exclusively related to α_{2u}-g accumulation. Although there are 34 hyaline droplet-inducing compounds identified in Table A-1 of this report, the accumulating protein responsible for hyaline droplet formation has been identified for only 17 of these compounds.
- Data sufficient to demonstrate interdependence of the lesions in the proposed pathological 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 since 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 could 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 to assessing human risk.

XII. Conclusions

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 molecular alterations in DNA occurring 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 which 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 which 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) possess 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 compounds 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. For the compounds examined to date, not all of the administered substance has been found to bind to α_{2u}-g. Thus additional CIGA/CIGA metabolites potentially exist in the kidney along with the α_{2u}-g-bound material. The possibility that these other moieties are toxic to the kidney 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. This example demonstrates how other mechanisms may play some role in the observed results.

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 since 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. However, these findings are frequently based on subjective histopathological criteria, 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 pathological 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 other 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.

XIII. Research Needs

Certain studies, suggested to fill key data gaps, are listed below. There has been no attempt to outline all the possible avenues for research on CIGA and on lipocalins, since a vast array of useful experiments could be envisioned. Instead, recommended studies would greatly improve the database on these chemicals, provide needed information to answer questions of human relevance, and set up a framework for improving the testing of chemicals that are potentially male rat renal tubule tumorigens. These research needs are listed as follows:

- Extend studies in humans, wherever possible, to determine directly the effects of hydrocarbon and solvent exposure, focusing on specific jobs with relatively pure CIGA exposure. Any human renal pathology found should be compared with

α_{2u}-g nephropathy in the male rat, and urine should be examined for the presence of cells and casts since this noninvasive technique is readily applied to humans.

- Examine human subpopulations that excrete abnormal amounts of low-molecular-weight protein in the urine to determine if they are at risk of renal disease or renal cell cancer.
- Examine additional active CIGA metabolites for binding to lipocalins, such as retinol-binding protein, α_1-acid glycoprotein, and urine protein 1. If there is binding, determine if the protein complex has a slower degradation rate.
- Thoroughly characterize any protein droplet nephrotoxicity observed as the result of administering known CIGA (e.g., d-limonene, TMP) to additional species (e.g., dog, hamster, rabbit, guinea pig, and especially nonhuman primate).
- Develop a standardized short-term protocol (e.g., in the 2-week subacute study) that will detect any abnormal accumulation of hyaline droplets in the male rat kidney before suspected CIGA are placed on chronic study. (If hyaline droplet accumulation is found, this information should be taken into account in designing the bioassay.)
- Further characterize the response of the NBR rat, which does not appear to synthesize α_{2u}-g, to CIGA and to classical renal carcinogens. These studies should verify, in a two-year chronic bioassay, that the NBR rat kidney is responsive to classical renal carcinogens already tested in other strains, and they should evaluate the suitability of this strain of rat as a test species. If the NBR rat meets these two criteria, the possibility of employing a separate test group, consisting of male NBR rats, should be considered for conventional bioassays whenever excessive hyaline droplet formation has occurred in shorter-term tests.
- Conduct serial-sacrifice studies of CIGA and non-CIGA renal carcinogens to determine if a distinctly different progression from α_{2u}-g nephropathy to tumor formation can be seen for CIGA. Studies should involve chronic exposures, examine the histogenesis of the renal tubule tumors, and include "stop" experiments and time-dependent appearance of tumor markers.
- Perform dose-response studies designed to quantitate the relationship between increased hyaline droplets and cell necrosis and between cell necrosis and cell regeneration. In addition, explore the possibility of additional steps in the progression that might further define the expression of cancer in the male rat and the cause of cell death.
- Conduct metabolism and disposition studies of CIGA in other species, compared with male rats, to determine the causative chemical for the nephropathy, and to clarify sites of biotransformation and deposition and fate of these compounds.

Additional work, not as critical as the above, but which would also assist in understanding this disease process, includes the following:

- Identify the accumulating material contained in hyaline droplets of proximal tubules for chemicals that are apparent, but unverified CIGA, and conduct 2-year bioassays for decalin and TMP.
- Perform additional in vitro assays using rodent kidney extracts to more specifically determine mutagenic potential of CIGA (or active metabolites).
- Conduct studies on the genesis of CPN and its relationship to α_{2u}-g nephropathy and examine the possible role of CPN as a cocarcinogenic factor for renal tumor induction.
- Obtain more information on the renal catabolism of α_{2u}-g and the rate and efficiency of protease-mediated hydrolysis in control and CIGA-treated rats.
- Study the binding relationships between CIGA and α_{2u}-g (e.g. affinity, concentration ranges, binding effectors) and determine the site at which binding of CIGA to α_{2u}-g occurs (e.g., liver, plasma, or urine) to investigate the hypothesis that the protein-CIGA complex is only formed at high concentrations of the chemical.
- Determine the reasons why the amount of low-molecular-weight protein in human urine is much less than it is in male rats.

Part 4. Science Policy

XIV. Background and Introduction

An increased incidence of neoplasms in laboratory animals administered test chemicals is customarily viewed by scientists as an indication of carcinogenicity in animals and as some signal that humans may be similarly affected. From this line of reasoning, EPA generally presumes that animal tumor findings indicate there may be a cancer hazard to humans, although a final judgment as to human carcinogenic potential can be made only in relation to all other relevant information. Recent studies suggest, however, that tumors produced in the tubule of the male rat kidney following the accumulation of alpha$_{2u}$-globulin (α_{2u}-g) might involve a process that occurs only in the male rat. Because of the implications to cancer risk assessment, the Risk Assessment Forum (RAF) established a Technical Panel to examine the available information on α_{2u}-g accumulation in the kidney, associated renal disease, and kidney cancer. The scientific data supporting the Technical Panel's conclusions regarding the α_{2u}-g sequence of lesions[5] are covered in depth in the preceding sections (Parts 1 through 3) of this document.

Part 4 provides guidance to EPA risk assessors regarding evaluation of male rat kidney tumors and presents RAF conclusions regarding potential human hazard and risk for a special subset of these tumors, that is, renal tubule tumors in the male rat resulting from chemically induced α_{2u}-g accumulation. Criteria for demonstrating this relationship are set forth below for use and discussion in all EPA assessments in which data on renal tubule tumors in the male rat are used to assess human risk.

XV. Basis for Science Policy on Male Rat Kidney Tumors

The information that follows highlights critical data and outlines inferential bridges used to select the most plausible explanation for the information available on male rat kidney tumors.

A. Low-Molecular-Weight Proteins in the Rat

In rat kidneys, as in those of other mammals, naturally occurring low-molecular-weight proteins are transferred from the plasma into the urine by glomerular filtration. The proteins are then partially reabsorbed from the urine into the renal tubule of the kidney where they are eventually broken down by catabolism (see section III-A). One of these low-molecular-weight proteins, α_{2u}-g produced by the liver under the stimulus of testosterone,

[5] In this report, lesion is a morphological alteration, due to disease.

reaches very high levels in the plasma and urine of young adult male rats, gradually declining with age.

Alpha$_{2u}$-globulin is regarded as a member of a large superfamily of proteins thought to be carriers of lipophilic molecules (see section III-D). Some of these proteins, e.g., retinol-binding protein and α_1-acid glycoprotein, are found in many species, including humans. Others, like α_{2u}-g, are found in specific species. The only member of the superfamily with a clearly defined physiological role is retinol-binding protein, the carrier protein for vitamin A. Although these low-molecular-weight proteins are believed to have similar three-dimensional structures, the alignment of amino acid residues between any pair of proteins in the superfamily is small, roughly 20 percent. The exception is α_{2u}-g and mouse major urinary protein(s) (MUP) which are approximately 90 percent homologous.

Alpha$_{2u}$-globulin derived from hepatic synthesis is not known to occur in the female rat or any other species, including humans. Although similar forms of α_{2u}-g are synthesized at nonhepatic sites in female rats and in the male NCI Black Reiter (NBR) rat, a strain whose males lack hepatic synthesis of α_{2u}-g, none of these other forms of α_{2u}-g nor MUP accumulates in the renal tubule following administration of the compounds discussed in Parts 1 through 3.

B. Progression from Chemically Induced Alpha$_{2u}$-globulin Accumulation to Nephropathy and Neoplasia

1. Overview

The information available provides a plausible, although probably incomplete, picture of a sequence of events occurring in the male rat kidney following chemical administration. This sequence can be portrayed on a cellular and molecular level. Initially, the test chemical appears to bind reversibly to α_{2u}-g, seemingly forming a complex more resistant to lysosomal degradation than the unreacted protein itself (see section III-H). This shifts the balance between reabsorption and catabolism and appears to result in accumulation of the protein complex in a specific area of the renal tubule, the P2 segment. Continued compound administration results in a cytotoxic response from the sustained protein overload to the renal tubule, causing single-cell necrosis of cells lining the surface of the tubule and other kidney pathology. The dead cells are replaced by cell division. As the cycle of cell death and cell replacement continues, with time tubule hyperplasia (increase in number of cells) and neoplasia may occur. It is presumed, but certainly not proven, that continued cell proliferation plays a role in the neoplastic process.

Morphologically, the sequence of events begins with an increase in the number and size of hyaline droplets[6] containing α_{2u}-g. The next

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[6] Spherical inclusions in the cytoplasm that may contain various proteins (see section III-B).

characteristic lesion, single-cell necrosis in the renal tubule, may not be seen but can be confirmed by observation of exfoliated degenerate cells in the tubule lumen[7] and granular casts.[8] Enhanced cell replication in response to cell death can be seen as increased cell division or demonstrated by labeling techniques that measure increased DNA synthesis. In chronic laboratory animal bioassays, tubule hyperplasia, linear mineralization in the renal papilla (possibly representing remnants of debris from disintegrating granular casts), and renal tubule tumors are observed.

2. Specificity of the sequence to the male rat

Consistent results from hypothesis-testing experiments conducted over the last decade in various laboratories establish the association between the accumulation of abnormal amounts of α_{2u}-g in the P2 segment of the renal tubules and a specific form of kidney disease, and they support an association between this nephropathic response and renal tubule tumors. Specifically, the male rat responds to administration of α_{2u}-g inducers with a characteristic nephropathy. The severity of this kidney disease is dose-dependent, not only with respect to the amount of compound administered, but also with respect to the concentration ofα_{2u}-g in the kidney. This alpha$_{2u}$-globulin nephropathy differs sufficiently from chronic progressive nephropathy (see section IV-E) commonly found spontaneously in aging male rats so that the two effects can be distinguished. In contrast, mice and female rats administered α_{2u}-g-inducers under the same conditions as male rats did not develop lesions characteristic of α_{2u}-g nephropathy.

Hyaline droplets in the proximal tubule of untreated male rats contain α_{2u}-g, especially in young adults. Hyaline droplets are substantially reduced in castrated male rats, further indicting the dependence of this phenomenon on male hormone levels. In female rats of any age, an observation of protein droplets is rare and α_{2u}-g is not involved.

Specialized studies involving hormone manipulation have shown that the development of the early features of α_{2u}-g nephropathy is dependent on the presence of the hepatic form of α_{2u}-g. (1) Hyaline droplet or α_{2u}-g accumulation does not occur when α_{2u}-g-inducers are administered to immature or old male rats that produce little α_{2u}-g in the liver. (2) Hyaline droplet accumulation is observed from administration of α_{2u}-g-inducers even in castrated rats, but the severity of the effect is diminished. (3) Estrogen administration to male rats reduces the severity of α_{2u}-g nephropathy. (4) Female rats administered an α_{2u}-g-inducer along with α_{2u}-g purified from male rat urine clearly showed hyaline droplet formation, α_{2u}-g accumulation in the kidney, and some nephropathy even though control female rats showed no measurable effects.

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[7] Cells inside the tubule, which show cytoplasmic deterioration, arising from the damaged P2 segment (see section IV-A).

[8] The granular casts are composed of sloughed cell debris from the dead cells. They accumulate at the junction between the P3 segment of the proximal tubule and the descending thin loop of Henle where diameter becomes narrower (see section IV-A).

The specificity of the male rat response has been tested to a limited extent in a number of other species, with no evidence of hyaline droplet nephropathy in dogs, guinea pigs, hamsters, or monkeys. Since these species (and the mouse and female rat) have proteins similar in structure to α_{2u}-g, the lack of damage to their kidney cells is consistent with the presumption that the specific α_{2u}-g produced by the liver of male rats is necessary for the expression of the renal effects.

Male rats of the NBR strain provide a unique opportunity for testing the α_{2u}-g hypothesis since this animal has no detectable levels of hepatic messenger RNA for α_{2u}-g. Under conditions of exposure that produced α_{2u}-g nephropathy in male rats of other strains, several chemicals administered to the NBR rat did not induce detectable accumulation of α_{2u}-g in the renal tubules.

Mice and female rats exposed to α_{2u}-g-inducers in chronic bioassays did not develop an increased incidence of renal tubule tumors. In contrast, male rats developed a dose-dependent neoplastic response in the kidney. Additional experimentation using a nitrosamine as the initiator of cancer and an α_{2u}-g-inducer as the promoter also support the observation that α_{2u}-g is involved in the process leading to renal tubule tumors in the male rat. In one of these studies, the promotion potential of an α_{2u}-g-inducer in NBR rats was contrasted with the response in a conventional strain, the F344 rat. Consistent with the hypothesis that α_{2u}-g is necessary to induce a response, the promoter did not enhance renal tubule tumor formation in the α_{2u}-g-deficient NBR rat, but it did promote renal tubule tumor formation in the F344 rat.

It is clear that not all renal tubule cancer in laboratory animals occurs through the hypothesized α_{2u}-g sequence. Other inducers of rodent renal tubule cancer are well known. These include, for example, certain nitrosamines in the rat and mouse and diethylstilbestrol in hamsters. In general, these prototypic renal carcinogens are active in both males and females. The acute nephrotoxic changes in the renal tubules include mild lipid droplet accumulation and scattered single-cell necrosis, but hyaline droplet accumulation and its specific associated nephropathy are not characteristic.

Based on available information, α_{2u}-g-inducers appear to have additional features that distinguish them from other rodent kidney carcinogens, such as the nitrosamines. Alpha$_{2u}$-globulin inducers appear to be nongenotoxic, or only marginally so, suggesting that the mechanism for tumor induction does not depend on direct genetic injury. So far, the incidence of renal tumors produced in the male rat by α_{2u}-g inducers has been relatively low, occurring late in life, and metastasizing rarely. In contrast, certain rodent carcinogens induce a high incidence of kidney tumors after as little as a single dose.

Distribution studies of compounds and information on chemical binding to α_{2u}-g indicate that, of the total chemical administered to the animal, only a small portion of the metabolites (possibly the parent compound) can account for all of the α_{2u}-g accumulation. Considerable amounts of the

chemical and other metabolites may also be present in the male rat kidney, not bound to α_{2u}-g. These other moieties may, at times, cause toxic effects in the kidney, possibly even cancer, that are unrelated to the accumulation of α_{2u}-g. Such information does not preclude a determination that the α_{2u}-g sequence is involved in some manner with the renal tumor response.

XVI. Science Policy Statement

Based on the analysis of the scientific literature in Parts I through III, the RAF Technical Panel reached three major conclusions. First, the sequence of events proposed to link α_{2u}-g accumulation to nephropathy and renal tubule tumors in the male rat is plausible, although not totally proven.

Second, the α_{2u}-g response following chemical administration appears to be unique to the male rat. Even though closely related proteins are present in other species, there is no evidence that these species respond to α_{2u}-g inducers in a manner similar to the male rat.

Third, the male rat kidney response to chemicals that induce α_{2u}-g accumulation is probably not relevant to humans for purposes of risk assessment.

The RAF Technical Panel's findings provide the basis for a two part EPA science policy statement regarding use of data on male rat renal tubule tumors for human risk assessment. This science policy applies to individual chemicals or chemical mixtures.

(1) Male rat renal tubule tumors arising as a result of a process involving α_{2u}-g accumulation do not contribute to the qualitative weight-of-evidence that a chemical poses a human carcinogenic hazard. Such tumors are not included in dose-response extrapolations for the estimation of human carcinogenic risk.

(2) If a chemical induces α_{2u}-g accumulation in male rats, the associated nephropathy is not used as an endpoint for determining non-carcinogenic hazard. Estimates of non-carcinogenic risk are based on other endpoints.

Even when chemically induced α_{2u}-g-related kidney tumors are present, other tumors in the male rat and any tumor in other exposed laboratory animals may be important in evaluating the carcinogenic potential of the chemical. Likewise, the role of chemically induced α_{2u}-g accumulation in the induction of renal tubule tumors in the male rat is assessed independently of evaluations made regarding tumors at other sites.

XVII. Guidance for Evaluating Chemically Induced Male Rat Renal Tubule Tumors

To determine the appropriate use of the data for EPA risk assessments, chemicals inducing renal tubule tumors in the male rat are examined in terms of three categories.

(1) The α_{2u}-g sequence of events accounts for the renal tumors.

(2) Other potential carcinogenic processes account for the renal tumors.

(3) The α_{2u}-g-associated events occur in the presence of other potential carcinogenic processes, both of which result in renal tumors.

Two questions need to be answered. The first and simplest question is whether or not the α_{2u}-g process is involved in the tumor development. The second more difficult question, given an affirmative answer to the first, is the extent to which α_{2u}-g-associated events, rather than other processes, account for the tumor increase.

A determination of the extent to which the α_{2u}-g process is involved in tumor development requires a substantial database, and not just a limited set of information confined to the male rat. For example, cancer bioassay data are needed from the mouse and the female rat to be able to demonstrate that the renal tumors are male-rat specific. Even to answer the first question affirmatively, information from toxicity studies must demonstrate whether or not the α_{2u}-g process is operative (see section XVII-A below). In the absence of this minimum information, there is no basis for judging the applicability of the α_{2u}-g process, and it would be assumed that the male rat renal tumors are relevant for risk assessment purposes. Additional data are necessary (see section XVII-B below) to answer the second question and to assign a chemical to categories 1, 2, or 3.

A. *Renal Tubule Tumors in Male Rats and Alpha$_{2u}$-globulin Accumulation*

The following information from adequately conducted studies of male rats shows that the α_{2u}-g process could be a factor in the observed renal effects; an affirmative response in each of the three categories is required. If data do not meet the criteria in any one category, the available renal tumor data should be analyzed in accordance with standard risk assessment principles.

(1) Increased number and size of hyaline droplets in renal proximal tubule cells of treated male rats

The abnormal accumulation of hyaline droplets in the P2 segment of the renal tubule is necessary to attribute the renal tubule tumors to the α_{2u}-g sequence of events. This finding helps differentiate α_{2u}-g inducers from chemicals that produce renal tubule tumors through other means.

(2) Accumulating protein in the hyaline droplets is α_{2u}-g

Hyaline droplet accumulation is a nonspecific response to protein overload in the renal tubule and may not be due to α_{2u}-g (e.g., as with chlorothalonil). Therefore, it is necessary to demonstrate that α_{2u}-g accounts for the hyaline droplet accumulation found in the male rat.

(3) Additional aspects of the pathological sequence of lesions associated with α_{2u}-g nephropathy are present.

Typical lesions include: single-cell necrosis, exfoliation of epithelial cells into the proximal tubular lumen, formation of granular casts, linear mineralization of papillary tubules, and tubule hyperplasia. If the response is mild, all of these lesions may not be observed; however, some elements consistent with the pathological sequence must be demonstrated to be present.

B. Additional Information Useful for the Analysis

If the preceding analysis (section XVII-A) indicates that the α_{2u}-g process is operative, then other information is reviewed to determine if the renal effects are solely α_{2u}-g-associated, a combination of the α_{2u}-g process and other potential carcinogenic processes, or due primarily to other processes. Many kinds of information can assist in confirming that chemically induced α_{2u}-g accumulation is involved in the renal tumor response or that other processes cannot be ruled out. Some of these findings are listed below; the information may not always be available, nor should this list be considered exhaustive.

Hypothesis-testing data: Data from specialized tests can greatly increase confidence that the α_{2u}-g sequence is involved in the renal tubule tumor response. Such information might include: modification of the nephrotoxic response through use of the NBR rat, or manipulation of sex hormones (e.g., androgens) or α_{2u}-g levels (e.g., α_{2u}-g administration to female rats). Other information might include initiation-promotion studies comparing males of the NBR strain with males of other rat strains.

Additional biochemical information: Certain in vivo and in vitro data help characterize a chemical as one that induces accumulation of α_{2u}-g. Such information might include: reversible binding of the chemical (or metabolites) to α_{2u}-g, reduction in the lysosomal degradation of the α_{2u}-g-complex, and disposition studies demonstrating sex- and species-specific retention of the test compound in the male rat kidney.

Sustained cell division in the proximal tubule of the male rat: A sustained increase in cell replication in the P2 segment of the renal tubule at doses used in the cancer bioassay and a dose-related increase in atypical hyperplasia of the renal tubule is consistent with the α_{2u}-g process, especially if other laboratory animals were tested and did not show similar responses. These endpoints are nonspecific for α_{2u}-g-inducers, however, since other renal carcinogens may also affect the P2 segment of the renal tubule.

Structure-activity relationships: Structure-activity relationships for chemicals that induce α_{2u}-g accumulation in the male rat kidney are not well defined, although there appear to be dimensional requirements to fit the protein pocket, a requirement for a degree of lipophilicity, and a need for an electronegative atom in the molecule or its active metabolite. Other structural features might suggest that a chemical belongs to a different class of suspected carcinogens.

Covalent binding to macromolecules: Some inducers of renal tubule cancer in rodents (e.g., nitrosamines) are known to bind covalently to DNA

or other macromolecules. Others do not appear to bind to DNA (e.g., isophorone) suggesting that such information may assist in distinguishing different processes leading to renal cancer.

Genotoxicity: Although renal tubule neoplasia associated with clearly genotoxic chemicals is a well known response, information to date supports a conclusion that α_{2u}-g inducers are essentially nongenotoxic and do not depend on direct genetic injury for the production of tumors. Thus, information on potential genotoxicity in a standard battery of short-term tests relevant to the evaluation of potential carcinogenicity provides a possible device for helping to distinguish between these processes.

Nephrotoxicity: Chronic progressive nephropathy (CPN) in the aging male rat can complicate the analysis of other renal lesions. However, nephrotoxicity in the male rat not attributable to either CPN or α_{2u}-g accumulation, or a nephrotoxic response in the female rat or the mouse, suggests that the possibility of other processes leading to renal cancer should be considered.

Animal bioassay data in other species-, sex-combinations: The α_{2u}-g-syndrome is specific to the male rat. Positive cancer responses in the renal tubule in female rats, mice of either sex, or any other laboratory animal imply that the α_{2u}-g syndrome alone does not account for the renal tubule tumor response in the male rats.

Confidence in determining which of the three categories applies depends on the comprehensiveness and consistency of available data. If all the data (two species, two sex combination bioassay, all elements in XVII-A, and additional information such as that described in XVII-B) are consistent with a role for chemically induced α_{2u}-g, there is a high degree of confidence that the α_{2u}-g syndrome, alone, accounts for the renal tubule tumors. In contrast, if information from adequate testing is inconsistent with the α_{2u}-g syndrome (e.g., renal tubule tumors are present in female rats or mice), other carcinogenic processes probably account for all or most of the renal tumors. Sometimes, the information will indicate that more than one carcinogenic process is occurring; in these cases, as a minimum, the criteria in support of α_{2u}-g involvement (section XVII-A) are present, but there is also evidence consistent with other mechanisms. Decisions on the applicability of the three categories can only be made on a case-by-case basis, taking all of the information into account. Whatever the finding, the risk assessor should clearly delineate and thoroughly document the basis for any decisions made.

C. Use of the Data for Risk Assessment

Once a decision on the applicability of the three categories is made, it becomes possible to determine how the response in the male rat renal tubule would apply to evaluating human hazard and to estimating human cancer risk. In general, the following guidance applies, recognizing that tumors occurring at other sites in laboratory animals administered compounds that induce α_{2u}-g accumulation in the male rat will be judged on their own merits.

Compounds producing renal tubule tumors in male rats attributable solely to chemically induced α_{2u}-g accumulation: these renal tubule tumors will not be used for human cancer hazard identification or for dose-response extrapolations.

Compounds producing renal tubule tumors that are not linked to α_{2u}-g accumulation: these renal tubule tumors are an appropriate endpoint for human hazard identification and are considered, along with other appropriate endpoints, for quantitative risk estimation.

Compounds producing some renal tubule tumors in male rats attributable to the α_{2u}-g process and some attributable to other carcinogenic processes: In general, the information needed to make a quantitative determination of the relative contribution of each process to tumor development will not be available. Thus, even though the information on the renal tubule tumors remains relevant for purposes of hazard identification, a meaningful dose-response estimate based on renal tubule tumors in the male rat is generally not possible and should not be performed. If there is enough information to determine the relative contribution of each process to the overall renal tubule cancer response in male rats, the non-α_{2u}-g-induced component may be used, as appropriate, for dose-response evaluation as well as hazard identification.

XVIII. Nephropathy as a Toxic Endpoint

If a compound induces α_{2u}-g accumulation in hyaline droplets, the associated nephropathy in male rats is not an appropriate endpoint to determine noncancer (systemic) effects potentially occurring in humans. Likewise, quantitative estimates of noncancer risk (e.g., reference doses and margin-of-exposure determinations) are based on other endpoints.

It should not be anticipated that a compound that produces nephropathy in the male rat through the sequence of events beginning with the accumulation of α_{2u}-g will always be found to induce renal tubule tumors in the male rat. The ability to detect renal tumors depends on many features that may not be present in any individual experiment, e.g., sufficient dose to induce effect without early deaths of the animals, competing toxicity from other moieties not bound to α_{2u}-g, insufficient length of exposure or followup, and incomplete histopathology. Even in the absence of renal tubule tumors in the male rat, if the sequence of lesions characteristic of the α_{2u}-g syndrome are present, the associated nephropathy in the male rat does not contribute to determinations of noncarcinogenic hazard or risk.

Appendix - Non-Neoplastic Effects of Hyaline Droplet Inducers

Table A-1. Substances that have Induced Hyaline Droplet Accumulation and/or Elevated Levels of Alpha$_{2u}$-Globulin in Renal Proximal Tubules of Rats

Substance/Chemical	Evidence for exacerbation of hyaline droplets in renal proximal tubule cells			Evidence for increased renal alpha$_{2u}$-globulin levels		
	Males	Females	References	Males	Females	References
Unleaded gasoline	+	-	Halder et al. (1984) Thomas et al. (1985) Murty et al. (1988) Garg et al. (1988)	+	NR	Olson et al. (1987) Garg et al. (1988)
2,2,4-Trimethyl-pentane	+	-	Stonard et al. (1985, 1986)	+	-	Stonard et al. (1986) Charbonneau et al. (1987) Lock et al. (1987b)
JP-4 jet fuel (mixed distillate hydrocarbons)	+	-	Bruner (1984) MacNaughton and Uddin (1984)	NR	NR	
JP-5 jet fuel (mixed distillate hydrocarbons)	+	-	Parker et al. (1981) Bruner (1984) Gaworski et al. (1984) MacNaughton and Uddin (1984)	NR	NR	
Diesel fuel, marine	+	-	Bruner (1984) Gaworski et al. (1985a)	NR	NR	
JP-10 synthetic jet fuel (exohexahydro-4,7-methanoindan)	+	NR	MacNaughton and Uddin (1984) Mattie et al. (1988)	NR	NR	

Table A-1. (cont.)

Substance/Chemical	Evidence for exacerbation of hyaline droplets in renal proximal tubule cells			Evidence for increased renal $alpha_{2u}$-globulin levels		
	Males	Females	References	Males	Females	References
RJ-5 synthetic jet fuel (hydrogenated dimers of norbornadiene)	+	-	MacNaughton and Uddin (1984)	NR	NR	
JP-7 distillate jet fuel	+	-	Bruner (1990, unpublished data) Alden (1989)	NR	NR	
JP-TS distillate jet fuel	+	-	Bruner (1990, unpublished data) Alden (1989)	NR	NR	
Stoddard solvent	+	-	Phillips and Cockrell (1984)	NR	NR	
C_{10}-C_{11} and C_{10}-C_{12} isoparaffinic solvents (saturated aliphatic hydrocarbons)	+	-	Phillips and Cockrell (1984) Viau et al. (1986)	+	-	Viau et al. (1986)
Decalin	+	-	Alden et al. (1984, 1985) Bruner (1984) Gaworski et al. (1985b) Kanerva et al. (1987a) Stone et al. (1987)	+	-	Alden et al. (1984, 1985) Kanerva et al. (1987b)
Tetralin	+	NR	Serve et al. (1988)	NR	NR	

Table A-1. (cont.)

Substance/Chemical	Evidence for exacerbation of hyaline droplets in renal proximal tubule cells			Evidence for increased renal alpha$_{2u}$-globulin levels		
	Males	Females	References	Males	Females	References
d-Limonene	+	-	Ridder et al. (1987) Kanerva et al. (1987a) NTP (1990) Lehman-McKeeman et al. (1989) Webb et al. (1989)	+	-	Ridder et al. (1988) Lehman-McKeeman et al. (1989) Webb et al. (1989)
Pentachlorobenzene	+	-	NTP (1991a)	NR	NR	
1,2,4,5-Tetrachlorobenzene	+	-	NTP (1991b)	NR	NR	
1,4-Dichlorobenzene	+	-	NTP (1987a) Bomhard et al. (1988) Charbonneau et al. (1989)	+	NR	Charbonneau et al. (1989)
Tetrachloroethylene (Perchloroethylene)	+	-	Goldsworthy et al. (1988a) Green et al. (1990)	+	-	Goldsworthy et al. (1988a)
Pentachloroethane	+	-	Goldsworthy et al. (1988a)	+	-	Goldsworthy et al. (1988a)
Hexachloroethane	+	-	NTP (1989)	NR	NR	
Isophorone	+	NR	Strasser et al. (1988)	+	NR	Strasser et al. (1988)
Lindane	+	-	Dietrich and Swenberg (1990, 1991b)	+	-	Dietrich and Swenberg (1990, 1991b)

Table A-1. (cont.)

Substance/Chemical	Evidence for exacerbation of hyaline droplets in renal proximal tubule cells			Evidence for increased renal alpha$_{2u}$-globulin levels		
	Males	Females	References	Males	Females	References
Dimethyl methyl-phosphonate	+	-	NTP (1987b)	NR	NR	
Methyl isobutyl ketone	+	-	Phillips et al. (1987)	NR	NR	
Methyl isoamyl ketone	+	-	Katz et al. (1986)	NR	NR	
Diisobutyl ketone	+	-	Dodd et al. (1987)	NR	NR	
BW54OC (3-methylamino-1-(3-trifluoromethyl-phenyl)-2-pyrazoline)	+	-	Read et al. (1988)	+	NR	Read et al. (1988)
BW58C (mixture of isomeric cis and trans forms of 2-(4'-t-butylcyclohexyl) -3-hydroxy-1-4-naphtho-quinone)	+	-	Read et al. (1988)	+	NR	Read et al. (1988)
Levamisole (levoisomer of 2,3,5,6-tetrahydro-6-phenyl-imidazo-(2,1-b) thiazole)	+	-	Read et al. (1988)	+	NR	Read et al. (1988)

Table A-1. (cont.)

Substance/Chemical	Evidence for exacerbation of hyaline droplets in renal proximal tubule cells			Evidence for increased renal $alpha_{2u}$-globulin levels		
	Males	Females	References	Males	Females	References
Gabapentin	+	-	Dominick et al. (1990)	+	-	Dominick et al. (1990)
3,5,5-Trimethyl-hexanoic acid derivatives	+	NR	Lehman-McKeeman et al. (1991)	+	NR	Lehman-McKeeman et al. (1991)
Tridecyl acetate	+	NR	Daugherty et al. (1990)	+	NR	Daugherty et al. (1990)
Isopropylcyclohexane	+	NR	Henningsen et al. (1988)	+	NR	Henningsen et al. (1988)
1,3,6-Tricyanohexane	+	-	Barnett et al. (1987) Johnson (1987)	NR	NR	

\+ Positive
\- Negative
NR = Not reported

Table A-2. Non-Neoplastic Findings Reported 2-Year Studies on Ten Selected Substances that Produce Renal Tubule Tumors in Rats

Chemical/Substance	Non-Neoplastic lesions
1,4-Dichlorobenzene species: F344 rats B6C3F1 mice route: gavage ref: NTP-TR-319 (NTP, 1987a)	Renal damage was not observed in female rats or mice in 13-week studies. Cell degeneration or necrosis of tubule epithelium was observed in male rats in a 13-week study. In 2-year studies, the severity of nephropathy was greater in male rats than female rats. Nephropathy characterized by degeneration and regeneration of renal tubule epithelium, tubule dilation with attenuation and atrophy of the epithelium, granular casts in tubules of the outer stripe of the medulla, thickening of basement membranes, and minimal accumulation of interstitial collagen. Renal tubular regeneration was noted in female mice. Nephropathy increased with dose in male mice and female rats.
Dimethyl methylphosphonate species: F344 rats B6C3F1 mice route: gavage ref: NTP-TR-323 (NTP, 1987b)	Kidneys of dosed male rats but not of dosed male mice had varying degrees of tubule cell regeneration, hyaline droplet degeneration, and cellular infiltrate (13 wks). At 2 years, the average severity of nephropathy increased and calcification was observed in the collecting tubules of the renal pelvis of male rats. The nephropathy was characterized by degeneration of the tubule epithelium, tubule dilation with attenuation and atropy of the epithelium, granular casts in the tubules of the outer stripe of the outer medulla, thickening of basement membranes, minimal to mild accumulation of interstitial collagen, and minimal to mild inflammatory cell infiltrates. The increase in severity of nephropathy was ranked, control to high dose, 1.9, 2.5, and 2.8, on scale of 1 to 4.
Hexachloroethane species: Osborne Mendel rat B6C3F1 mice route: gavage ref: NTP-TR-68 (NCI, 1978b)	Toxic tubule nephropathy was observed in all groups of treated rats and in male and female mice. The nephropathy in rats was characterized by degeneration, necrosis, and the presence of large hyperchromatic regenerating epithelial cells. Overlying the tubule lesions were chronic interstitial fibrosis and nephritis, focal pyelonephritis, tubular ectasia, cast formation, and focal glomerulosclerosis. In mice, nephropathy was characterized by degeneration of affected tubules containing hyaline casts. The kidney often showed infiltration of inflammatory cells, fibrosis, and calcium deposition. The incidences of toxic nephropathy were higher in mice than in rats.

Table A-2. (cont.)

Chemical/Substance	Non-Neoplastic lesions
Hexachloroethane (cont.) species: F344 rats route: gavage ref: NTP-TR-361 (NTP, 1989)	Nephropathy observed in nearly all males; overall average severity mild in vehicle controls and mild to moderate in dosed males. Incidence and severity of nephropathy was increased in dosed females relative to controls. Nephropathy in each sex consisted of tubule cell degeneration, regeneration and dilation, atrophy, glomerulosclerosis, interstitial fibrosis, and chronic inflammation. Linear mineralization of the renal papillae showed a dose-related increase in male rats. Hyperplasia of pelvic transitional epithelium was increased. Incidences of mineralization and pelvic epithelial hyperplasia were not increased in females.
Isophorone species: F344 rat B6C3F1 mouse route: gavage ref: NTP-TR-291 (NTP, 1896a)	Tubular cell mineralization was increased in dosed male rats but not in dosed female rats. This lesion was characterized by basophilic aggregates of minerals most often found in the medullary collecting ducts and occurred coincidentally with lesions of chronic nephropathy. The incidence of nephropathy was similar in dosed and vehicle control male rats, the severity was greater in low dose males. Hyperplasia of the renal pelvis was observed in dosed male rats but not in vehicle controls.
d-Limonene species: F344 rats B6C3F1 mice route: gavage ref: NTP-TR-347 (NTP, 1990)	Increased severity of nephropathy in male rats at 13 weeks characterized by degeneration of epithelial cells in convoluted tubules, granular casts in the outer medulla, and epithelial regeneration. No lesions in female rats. Increase in severity of nephropathy and linear deposits of minerals in the renal medulla and papilla in male rats in 2-year studies.
Pentachloroethane species: F344 rats B6C3F1 mice route: gavage ref.: NTP-TR-232 (NTP, 1983)	Chronic, diffuse inflammation, distinguishable from nephropathy seen in aging F344 rats, were found in male rats in a significant dose-related increase. Interstitial fibrosis and tubule dilation more severe than in old-age nephropathy. Mineralization of the renal papilla observed at increased incidences in dosed male rats. Some dilated tubules with giant cells and casts were observed. No indication of renal toxicity was reported for female rats or mice.

Table A-2. (cont.)

Chemical/Substance	Non-Neoplastic lesions
Tetrachloroethylene species: F344 rats B6C3F1 mice route: inhalation ref: NTP-TR-311 (NTP, 1986b)	Both male and female rats exhibited renal tubule cell karyomegaly. Karyomegaly also occurred in mice. Cast formation was noted in male and female mice. Tubule cell hyperplasia was seen in male rats and male mice.
Unleaded gasoline species: F344 rat route: inhalation ref: USEPA, 1987	Dose-related kidney lesions at 3-6 months in males included focal tubule basophilia and tubular casts at the corticomedullary junction. An interrelated increase in the incidence of renal pelvis mineralization was also reported at 12 months, 18 months, and terminal sacrifice. Progressive glomerulonephrosis was reported in one high-dose male at 12 months; the incidence was higher at 18 months but was dose-related; at the final sacrifice, nearly all male rats exhibited this lesion (MacFarland et al., 1984). Karyomegaly, i.e., very large nuclei within cells of tubule epithelium, first noted at 12 months; at 18 months, more numerous karyomegalic cells were observed in treated males, particularly in the high-dose group (UAREP, 1983).
Chlorothalonil species: Osborne-Mendel rats B6C3F1 mice route: diet ref: NTP-TR-41 (NCI, 1978a)	No non-neoplastic renal lesions were reported in the NTP bioassays.
Trichloroethylene species: ACI rats August rats Marshall rats Osborne-Mendel rats route: gavage ref: NTP-TR-273 (NTP, 1988)	Cytomegaly noted in males and females of all strains. Toxic nephropathy increased in both sexes of all strains. Calcification was produced in kidneys of ACI male and female rats.

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