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REPORT OF THE EPA PEER REVIEW WORKSHOP ON $ALPHA_{2u}$-GLOBULIN: ASSOCIATION WITH RENAL TOXICITY AND NEOPLASIA IN THE MALE RAT

RISK ASSESSMENT FORUM
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SECTION ONE

OVERVIEW AND ISSUES PAPER

OVERVIEW

This workshop report highlights issues and conclusions from a U.S. Environmental Protection Agency (EPA) workshop on the question of using certain rat kidney tumors for human risk assessment. The workshop was convened to acquire expert opinion on a draft EPA report entitled "Alpha$_{2u}$-Globulin: Association with Chemically Induced Renal Toxicity and Neoplasia in the Male Rat" (55 Federal Register 46994, 8 November 1990). The final Risk Assessment Forum (Forum) report, which is based in part on information described in this workshop report, is scheduled for publication in the fall of 1991. EPA is making the final Forum report and the workshop report available to the public through notices in the Federal Register.

Background

The report on alpha$_{2u}$-globulin (α_{2u}-g) and renal effects in the male rat and the workshop were organized by the EPA's Risk Assessment Forum. The Forum, which is composed of Agency scientists selected for their expertise in various areas of risk assessment, studies controversial risk assessment issues in order to promote scientific consensus within EPA. For major projects, the Forum formally assembles a technical panel of EPA scientists to provide an Agency-wide perspective on the issues. Before recommendations of the technical panel are incorporated into EPA's risk assessment approach, Forum reports are subject to rigorous internal and external peer review.

The laboratory rat has been, and continues to be, a reliable source of information on potential risk to humans from exposure to environmental carcinogens. However, the use of data on certain male rat kidney tumors to predict human risk has been a topic of controversy for several years. Specifically, recent studies show that administration of certain chemicals to the

male rat results in the accumulation of the low-molecular-weight protein, α_{2u}-g, in the P2 segment of the renal tubule. The protein build-up is followed by kidney disease and an increased incidence of kidney tumors. Because the male rat response to α_{2u}-g inducers is unlike that of other laboratory species tested with these chemicals, questions have been raised about the use of such data to project risk to other species, including humans. Clearly, any suggestion that a finding of cancer in laboratory animals is irrelevant to human hazard is controversial, warranting evaluation by the Forum.

A draft EPA report ("workshop draft") examining the hypothesis that α_{2u}-g accumulation can lead to male rat-specific nephropathy and renal tubule tumor formation was prepared by a Risk Assessment Forum technical panel chaired by Dr. Karl Baetcke of the Office of Pesticide Programs (Appendix A). This workshop draft examined current studies on the linkage between α_{2u}-g accumulation and male rat-specific nephropathy, giving special emphasis to hypothesis-testing studies (e.g., estrogen administration to male rats, castration, α_{2u}-g administration to female rats, and use of animals not producing α_{2u}-g). Based on this review of recent studies, the workshop draft concluded that the nephropathy observed in male rats following administration of α_{2u}-g inducers is unlikely to occur in other species.

The remaining question, whether the kidney cancer seen in male rats administered α_{2u}-g inducers is a consequence of α_{2u}-g nephropathy or whether these tumors arise through some other process, has important implications for cancer risk assessment. Based primarily on the specificity of the response, the workshop draft concluded that a well-defined progression of lesions in the male rat, beginning with chemically induced α_{2u}-g accumulation, is a plausible explanation for the observed renal tubule tumors.

The Peer Review Workshop

To provide peer review of the workshop draft, EPA's Risk Assessment Forum sponsored a two-day workshop, "Alpha$_{2u}$-Globulin: Association with Renal Toxicity and Neoplasia in the Male Rat." This workshop was held in Gaithersburg, Maryland, on November 13 to 14, 1990. Sixteen scientists involved in research activity directly related to the male rat kidney tumor issue

and seven risk assessment experts were invited to discuss the workshop draft and related concerns, which are outlined below in an issues paper that follows this overview.

In general, the peer reviewers endorsed EPA's analysis of the male rat kidney tumor issue as presented in the workshop draft. Although questions remain regarding the exact mechanism by which chemically induced α_{2u}-g accumulation leads to renal tumors, participants reached provisional decisions regarding use of these tumor data for risk characterization, pending the availability of additional information from research studies. On this basis, the peer reviewers concluded that, under the conditions enumerated in EPA's analysis, α_{2u}-g-associated male rat renal tubule tumors are probably not relevant for human risk assessment.

Workshop participants formed several different workgroups and each workgroup had useful suggestions, now incorporated in the final report, for improving the workshop draft (see Section Five, Workgroup Reports). For example, the nephropathy workgroup's concern about the potential reactivity of other members of the α_{2u}-g protein superfamily caused the technical panel to greatly expand this section in the final EPA report. Also, based on comments of the cancer workgroup and new information showing that d-limonene induced renal tubule tumors did not form in male rats of an α_{2u}-g-deficient strain, the technical panel concluded that such renal cancer responses are probably linked to α_{2u}-g accumulation. Elsewhere, recommendations of the criteria workgroup played a key role in the development of the science policy statement for the final Forum report, laying a state-of-science foundation for distinguishing renal carcinogens associated with α_{2u}-g accumulation from other renal carcinogens. Finally, the science policy statement in the final Forum report is founded on recommendations of the risk characterization workgroup.

Workshop participants did not fully resolve several important points raised at the meeting. One of these was the use of male rat kidney tumor data for risk characterization when tumors are also present at other sites in the male or female rat, or in other species. In addition, specific guidance for evaluating mutagenicity data was not developed even though participants agreed that rat renal tumors would be irrelevant for human risk assessment only if the chemical is judged as having little or no genotoxic activity.

In this same vein, workshop participants pointed out a number of important research gaps, some of which are illustrated below.

- The level of α_{2u}-g in a cell leading to its death is undefined.
- Whether or not chemicals that induce α_{2u}-g accumulation are promoting spontaneous lesions of the kidney is unknown.
- Although lysosomal fragility is speculated as the mechanistic link between hyaline droplet accumulation and cell death, other explanations, including intrinsic toxicity of α_{2u}-g and α_{2u}-g as a vehicle for concentrating the chemical at the site of action, have also been proposed.

For these reasons, it is premature to consider the α_{2u}-g hypothesis and the biological processes described in the literature and in the Forum report as a true mechanism of carcinogenic action for induction of male rat renal tumors.

Following this overview and the issues paper (Section One), the workshop report presents the chairperson's summary report (Section Two). Section Three presents the workshop agenda, and Section Four contains introductory comments by the chairperson of each workgroup. Section Five contains the reports from each of the four workgroups, and presentations by other workshop participants are included in Section Six. The appendices to the workshop report include a list of technical panel members (Appendix A), workshop participants (Appendix B), and workshop observers (Appendix C).

The workshop was organized by Dr. Imogene Sevin Rodgers, Dr. Karl Baetcke, Ms. Letitia Tahan, and Drs. Marion Copley, Julie Du, Robert McGaughy, and William Pepelko of EPA and Ms. Leslie Beyer of ERG. Dr. Rodgers and Ms. Beyer assembled this workshop report.

The revised EPA report, "Alpha$_{2u}$-Globulin: Association with Chemically Induced Renal Toxicity and Neoplasia in the Male Rat" (EPA/625/3-91/019F) is available to the public.

ISSUES PAPER

PEER REVIEW WORKSHOP

DRAFT EPA RISK ASSESSMENT FORUM REPORT "ALPHA$_{2u}$-GLOBULIN—ASSOCIATION WITH RENAL TOXICITY AND NEOPLASIA IN THE MALE RAT"

The purpose of this workshop is twofold: (1) to develop information and opinions on the scientific analyses presented in the draft report, "Alpha$_{2u}$-Globulin—Association with Renal Toxicity and Neoplasia in the Male Rat," and (2) to obtain comments on use of this information to characterize human risk.

ISSUES FOR DISCUSSION AT PEER REVIEW WORKSHOP

Each peer review panel should examine the conclusions, suppositions, and limitations stated below for their consistency with available data and applicable scientific principles.

PANEL 1

Alpha$_{2u}$-Globulin Biochemistry and Nephropathy

The workshop draft concludes that the acute and chronic renal effects induced in male rats by chemicals that induce alpha$_{2u}$-globulin accumulation (CIGA) are unlikely to occur in any species not producing alpha$_{2u}$-globulin (α_{2u}-g) or a closely related protein in the large quantities typically seen in the male rat.

Conclusions and Suppositions Used in Reaching This Position

- There is no evidence that the nephropathy induced by α_{2u}-g accumulation occurs in female rats or other species, including humans.

- Accumulation of α_{2u}-g in hyaline droplets would always result in nephrotoxicity given sufficient dose and length of exposure to a CIGA.

- Male rats of strains commonly employed in toxicology testing—e.g., Sprague Dawley, Osborne Mendel, Fischer 344—would be expected to respond similarly to chemicals that induce α_{2u}-g accumulation.

- Because concentrations of protein homologues in human urine are well below those found for α_{2u}-g in male rats, it is highly unlikely that enough protein could accumulate in the human kidney following exposure to CIGA to result in hyaline droplet formation (i.e., the threshold for response would not be crossed).

Data Limitations

- Analysis of the available data is hampered by inconsistent use of terminology, e.g., "casts," "toxic nephropathy."

- It would be useful to know if any CIGA can bind to human homologues of α_{2u}-g and if such a complex is formed, its catabolism rate relative to the rat.

- There is little information on whether chemical-protein complexes of homologous protein are more easily digested than chemical-α_{2u}-g complexes.

- There is no discernable structure-activity relationship that clearly defines a chemical that induces α_{2u}-g accumulation.

- Data on other species regarding α_{2u}-g accumulation and hyaline droplet formation are extremely limited.

PANEL 2

Alpha$_{2u}$-Globulin Accumulation and Renal Neoplasia

The workshop draft concludes that the progression of lesions described in the α_{2u}-g hypothesis provides a plausible explanation for the renal tubule cancer observed in the male rats exposed to CIGA, although this explanation may not be exclusive.

Conclusions and Suppositions Used in Reaching This Position

- The critical step for examining the hypothesis linking α_{2u}-g accumulation with neoplasia is the presumption that neoplasia in the male rat kidney occurs because of an increase in replicative rate.
- It is not possible to conclusively demonstrate that increased replicative rate is the cause for renal neoplasia in the male rat exposed to CIGA, but available evidence does not refute this.
- No CIGA has been shown to induce renal neoplasia in female rats or mice of either sex.
- If a CIGA is a mutagen or reacts covalently with DNA, then other pathways that could result in renal neoplasia are plausible.
- Most CIGA that produced renal neoplasia in male rats also produced cancer in the liver of mice suggesting that neoplasia may be induced by more than one mechanism of action.
- Chemicals that are not CIGA, e.g., trichloroethylene and chloroform, also produce renal tumors in male rats at a significantly higher rate than in female rats, raising the question that CIGA might also induce tumors by non-CIGA mechanisms.

Data Limitations

- There is no discernable structure-activity relationship that clearly defines this group of renal carcinogens.
- Epidemiologic studies do not answer questions of human relevance regarding the association of CIGA to kidney tumors.
- Differences in species responses could be related to different patterns of metabolite formation and the binding of these metabolites or their parent compounds to α_{2u}-g or its homologues; this possibility needs to be explored further.
- Data actually showing progression from nephropathy to an increase in replicative rate to hyperplasia followed by neoplasia are extremely limited.
- Investigators have not routinely looked for α_{2u}-g accumulation or hyaline droplet formation in male rats in classical renal carcinogenesis testing (in some cases, e.g., chlorothalonil, at least one of these phenomena have been seen).
- Standard techniques for a 2-year chronic bioassay will not provide information adequate to identify a CIGA.

PANEL 3

Criteria for Identifying Renal Carcinogens as CIGA

The workshop draft concludes that data on renal neoplasia in the male rat exposed to CIGA require special screening in assessing risk to humans exposed to these chemicals. To accomplish this objective, the risk assessor must be able to distinguish whether renal tumors in male rats are CIGA-induced. The technical panel seeks the advice of the workshop participants on this issue. The information provided below reflects the technical panel's present thinking.

- A renal carcinogen is probably a CIGA when there is: (1) evidence of hyaline droplet formation, (2) the presence of α_{2u}-g in the hyaline droplets, (3) granular cast formation at the corticomedullary junction and/or linear mineralization, (4) increased cell replication in the renal tubules, and (5) a progression of lesions.

- It cannot be determined that the renal tumors seen in male rats are α_{2u}-g-associated without confirmation that they do not occur in female rats.

- Additional information useful for testing the applicability of the hypothesis would include data on species other than the rat.

- Evidence of mutagenic activity precludes the determination that renal tubule tumors in male rats are exclusively related to the chemical's properties as a CIGA.

Information from Previous Sections and Assumptions

- The first morphological manifestation of α_{2u}-g nephropathy is the accumulation of hyaline droplets in proximal tubule cells, developing within 24 hours of exposure; severity decreases with increasing duration of exposure beyond about 3 weeks.

- Alpha$_{2u}$-g can be clearly and specifically localized to the hyaline droplets within the proximal tubules.

- Granular casts formed from the cellular debris accumulate at the junction of the P3 segment and the thin loop of Henle within 20 to 40 days of continuous exposure.

- Linear mineralization of inner medullary tubules and within the renal papilla is a common occurrence, but it is not a necessary finding to identify a CIGA.

- No CIGA tested to date has produced renal tumors in mice or female rats.

- Standard animal bioassay techniques do not provide the information needed to demonstrate that a chemical is a member of the CIGA class.

PANEL 4

Characterization of Risk

The workshop draft does not propose a uniform policy for dealing with the spectrum of lesions associated with α_{2u}-g accumulation. Based on comments received at the workshop, the technical panel intends to develop such an approach. The technical panel can identify three separate situations that need to be addressed: (1) when the only tumor observed in laboratory animals is in the male rat kidney and the chemical is clearly a CIGA, (2) when the renal tumors cannot be related to the spectrum of lesions, and (3) when the information is suggestive but not definitive. The outline below reflects the present thinking of the technical panel.

- If a chemical induces α_{2u}-g accumulation in hyaline droplets, nephropathy in male rats should not be used as the endpoint for determining a NOAEL for non-cancer effects.

- Extreme caution should be used when basing a NOAEL on other toxic endpoints in the male rat since the animal may have compromised ability to handle the CIGA because of the kidney effects.

- If a chemical is not mutagenic, there is α_{2u}-g accumulation in hyaline droplets, and the only neoplasia observed is in the renal tubules of male rats, this is extremely limited evidence for a carcinogenic risk in humans.

- If a chemical is not mutagenic and α_{2u}-g accumulation in hyaline droplets and renal neoplasia occur in the male rat, but there are other tumors in the rat or tumors in other species, human relevance must be determined on a case-by-case basis viewing all of the data together. Dose-response relationships should not be based on kidney tumors in male rats, however.

- If a chemical is a mutagen as defined by EPA's Guidelines and causes cancer at any site, EPA's default assumptions for cancer risk assessment should apply.

Information from Previous Sections and Assumptions

- A chemical can be designated as a CIGA in the absence of evidence of carcinogenic activity.

- A chemical could have properties of a CIGA and still be a mutagen or cause cancer at other sites in the male rat.

- The hypothesis that renal neoplasia in the male rat is related to α_{2u}-g accumulation is incompatible with default presumptions regarding dose-response models in the 1986 Cancer Guidelines.

SECTION TWO

CHAIRPERSON'S SUMMARY OF THE WORKSHOP

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On November 13 and 14, 1990, EPA convened a workshop for review and comment on a draft report prepared by the EPA Risk Assessment Forum, "Alpha$_{2u}$-Globulin: Association with Renal Toxicity and Neoplasia in the Male Rat." See Section Three for the agenda and Appendices A, B, and C for the list of workshop participants.

The peer review workshop format consisted of (a) plenary presentations and discussions to ensure common understanding of the scientific basis for the contents of the draft report and clarification of the issues of scientific and Agency concern; (b) meetings of four subject-specific workgroups on nephropathy, renal cancer, criteria for categorization, and risk characterization; and (c) followup plenary sessions to review and comment on workgroup reports and to arrive at conclusions and recommendations. The workshop participants were aided in their evaluations by presentations by Dr. McLaughlin on epidemiologic aspects of renal cancers, by Dr. Trump on the pathology of human renal disorders, and by Dr. Swenberg on his recent studies with resistant NBR rats and on modeling of molecular binding sites.

In addition to this summary report (Section Two), the reports from the four workgroups are included in Section Five.

In general, the workshop participants found the draft report to be an excellent, balanced review of the available, published information on the subject and found little of substance to criticize. One exception is the section on epidemiologic studies, which participants considered to be too brief and incomplete. The reviewers recommended that this topic be revised by the authors, perhaps with outside help, to include a missing reference and to expand the text so that the reader has a clear idea of what is known about renal cancers in humans, especially incidence, risk factors, and tumor biology. A section on non-neoplastic renal disorders in humans, focused on those lesions

characterized by lysozymal overload, would also be desirable. The reviewers are aware that the bulk of the information available on α_{2u}-globulin and related substances comes from experimental data, but the available knowledge from human studies is necessary for judgments about the relevance of animal studies to humans.

The workshop participants reached the following conclusions:

- The chemically induced lesions in the P2 segment of the male rat kidney that are associated with lysozymal accumulation of α_{2u}-globulin (α_{2u}-g) constitute a discrete and distinctive pathologic entity.

- Renal lesions characterized by lysozymal overload occur in humans and in other animal species but in general are associated with macroglobulins rather than microglobulins.

- Substances similar in molecular structure to an α_{2u}-g (varying degrees of amino acid sequence homology) occur in mice, cattle, humans, and other species but do not produce nephropathy, perhaps because their concentrations in plasma are much lower or because the male rat kidney cells handle such substances differently, or both.

- Because the characteristic nephropathy associated with α_{2u}-g appears (from the available information) to occur only in male rats of certain strains, this response need not be considered in assessing potential non-neoplastic health risks to humans.

- Those chemical substances studied thus far that produce α_{2u}-g nephropathy also produce renal tubule adenomas and carcinomas in male rats.

- The tumors produced in rats are morphologically similar to other chemically related tubule cell tumors in rats but tend to occur at lower incidences and are generally microscopic in size, slow growing, and not life-threatening.

- The pathogenetic sequence of development of the renal tumors has not been proved but can be assumed to be related to the preceding nephropathy.

- Inclusion of substances in the category of α_{2u}-g-related nephropathy and renal carcinogenicity require (a) that the substance be non-mutagenic, (b) that α_{2u}-g be demonstrated immunochemically or biochemically in the renal tubule cells of male rats, (c) that induced tumors be found nowhere in male rats other than the kidneys, and (d) that no tumors be induced anywhere in female rats or in mice of either sex in adequately performed studies.

- Renal tumors in male rats that are produced by exposure to substances that produce α_{2u}-g nephropathy and fit the criteria for inclusion in this category need not be considered in assessing potential neoplastic health risks to humans.

- Molecular modeling studies that show promise for predicting which chemical structures might produce α_{2u}-g nephropathy should also be useful for developing methods for future predictions about the potential of other members of the superfamily of chemical substances to have adverse biological effects.

- It is not yet known whether the sex and animal strain specificity of the α_{2u}-g is related to the quantities of α_{2u}-g produced and filtered through the kidneys or to an as yet uncertain and unique pathologic mechanism for responding to what appears to be lysozymal overload.

- Some uncertainty remains about whether there might be sensitive human subpopulations and the molecular identity, kinetics, and biology of similar proteins in humans. The animal database is relatively small and the Agency is advised to continue to assess future research results as they are obtained.

Based on the information on hand, it was the consensus opinion of the workshop participants that non-mutagenic animal carcinogens that produce only male rat kidney tumors through an α_{2u}-g mediated mechanism are probably not carcinogenic to humans. Further details can be found in the individual workgroup reports (see Section Five).

SECTION THREE

WORKSHOP AGENDA

MONDAY, NOVEMBER 12

7:30-9:00 p.m.	Early Registration/Check-in

TUESDAY, NOVEMBER 13

7:30-8:30 a.m.	Conference Registration
8:30-8:45 a.m.	Introductory Remarks ..... Dr. Griesemer
8:45-9:30 a.m.	$Alpha_{2u}$-Globulin and Nephropathy Discussion ..... Dr. Olson
9:30-10:15 a.m.	$Alpha_{2u}$-Globulin and Kidney Cancer Discussion ..... Dr. Ashby
10:30-11:15 a.m.	Criteria for Identifying CIGAs Discussion ..... Dr. Hard
11:15 a.m.-NOON	Characterizing Risk for CIGAs Discussion ..... Dr. Page
1:00-6:00 p.m.	Subject-Specific Workgroups: Discussion and Drafting of Reports

WEDNESDAY, NOVEMBER 14

8:30-9:00 a.m.	Nephropathy Workgroup Presentation
9:00-9:30 a.m.	Cancer Workgroup Presentation
9:30-10:00 a.m.	Criteria Workgroup Presentation
10:00-10:30 a.m.	Risk Characterization Workgroup Presentation
10:45 a.m.-NOON	Comments from Observers
1:00-3:30 p.m.	Discussion by Panelists

SECTION FOUR

INTRODUCTORY COMMENTS FOR PEER REVIEW WORKSHOP ON ALPHA$_{2u}$-GLOBULIN ASSOCIATION WITH RENAL TOXICITY AND NEOPLASIA IN THE MALE RAT

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EVALUATION OF THE ASSOCIATION BETWEEN ALPHA$_{2u}$-GLOBULIN AND CHEMICALLY INDUCED NEPHROPATHY IN THE MALE RAT

This session of the peer review workshop focuses on the nature and quality of data linking, and limiting, chemically induced hyaline droplet nephropathy (HDN) of male rats to expression of the urinary protein α_{2u}-globulin (α_{2u}-g). Workshop participants should refer to pp. 1-4 of the Executive Summary of the draft report "Alpha$_{2u}$-Globulin: Association with Renal Toxicity and Neoplasia in the Male Rat" for a synopsis of the information linking HDN to α_{2u}-g. Full-length treatment of the association of renal pathology in male rats with derangement of the normal physiologic processes of α_{2u}-g synthesis, catabolism, and excretion may be found on pp. 7-67 of the draft report. Rather than attempt to recapitulate the detailed information assembled in the workshop draft report, the comments provided here are intended to focus critical attention on the strength of the hypothesis that expression of α_{2u}-g by male rats is integral to susceptibility of these animals to HDN. A principal purpose of such an analysis is to determine whether further hypotheses linking chronic, chemically induced HDN to renal carcinogenesis by an epigenetic mechanism are well founded. During this session of the workshop and the associated workgroup breakout, it is expected that discussion will evolve from the issues paper contained in the premeeting materials. Commentary elicited in the first session of the workshop will be used as background material in subsequent sessions and will form an integral part of the hazard evaluation phase of human risk assessment for chemicals inducing α_{2u}-g accumulation and HDN in male rats.

A first topic for consideration, since HDN is properly understood only in the context of the unique physico-chemical nature of α_{2u}-g, is a summary of the properties of this protein. The draft

document provides this information and describes the proteins, some identified in humans, related to α_{2u}-g (pp. 23-25). In addition, the complete amino acid sequence of α_{2u}-g has been deduced (Unterman et al., 1981), and this information, along with sequence homology to other members of the lipocalin (proteins that bind lipophilic ligands) superfamily (Pervaiz and Brew, 1987), has been used to suggest the tertiary structure of the protein (Miller et al., 1989; Borghoff et al., 1990). Although a relatively small molecule (162 amino acids), α_{2u}-g is envisioned to form a hydrophobic binding pocket within an eight-stranded β barrel configuration similar to human retinol-binding protein and bovine lactoglobulin. The interior of the folded protein is largely composed of hydrophobic amino acid residues. Such a structure is consistent with the non-covalent binding of lipophilic exogenous chemicals as surrogates for the uncharacterized natural ligand(s) of α_{2u}-g.

Secondly, while an association with α_{2u}-g has been shown for only a few selected chemicals, a much larger group of economically important hydrocarbons have been shown to cause HDN limited to the male rat. Appendix I of the draft document provides a comprehensive listing of hyaline droplet-type nephrotoxins and points out specific chemicals for which an association with renal accumulation of α_{2u}-g has been made. Experimental evidence substantiates the conclusion that selected hydrocarbons or hydrocarbon metabolites (e.g., d-limonene-1,2-oxide and 2,4,4-trimethyl-2-pentanol) bind α_{2u}-g in vivo (reviewed on pp. 29-34 of the draft document). While these data further the hypothesis that HDN occurs only in male rats expressing high levels of α_{2u}-g, such data are lacking for the majority of HDN-type nephrotoxins. One hope for efficiently accumulating data on hydrocarbon-α_{2u}-g binding for a wide variety of male rat nephrotoxins is in vitro binding studies with α_{2u}-g. Such studies are possible (Borghoff et al., 1991) and provide a mechanism to identify and characterize hydrocarbon-protein interactions. A chief conclusion of these studies, however, is that calculated binding affinities for α_{2u}-g-hydrocarbon interaction are quite variable and do not correlate well with the efficacy of chemicals for causing HDN. Thus, factors other than binding affinity define the action of a particular chemical as an inducer of HDN. (It should be pointed out that to date no chemical has been identified that induces HDN in the absence of interaction with α_{2u}-g.) Other gender-specific features such as bio-distributional (pharmacokinetic) phenomena, which control the target tissue toxicant dose, probably also contribute to the development of HDN. Further, as pointed out recently (Borghoff et al., 1991), certain factors such as lipophilicity, the presence of oxygen (as an electronegative atom), the steric volume of a chemical, and the possibility of hydrogen bonding between protein and chemical within the

hydrophobic region of the protein may confer α_{2u}-g-binding activity and control the affinity of such binding. However, at this time, with the exception of empirically identified structure-activity relationships for limited numbers of chemicals (notably the branched chain alkanes), a priori prediction of HDN-inducing potential for untested chemicals is not generally possible.

One result of α_{2u}-g binding of HDN-type hydrocarbons (or metabolites) is speculated to be increased resistance to proteolytic digestion within secondary lysosomes of the renal proximal tubule epithelium. As explained on pp. 34-37 of the workshop draft, a variety of data exist to support not only the relative resistance of native α_{2u}-g to catabolism but also a decreased rate of catabolism of α_{2u}-g-hydrocarbon complexes. Evidence from a number of investigators suggests that male rats are proteinuric not only because of the large amount of α_{2u}-g produced but also because of low proteolytic enzyme activity in male rat kidney. Apparently, testosterone has a suppressive effect on the synthesis of several major renal proteolytic enzymes (cathepsins) in the male rat (Jedrzejewski and Kugler, 1982; Kugler and Vornberger, 1986); these cathepsins appear to play a major role in α_{2u}-g degradation (Murty et al., 1988; Olson et al., 1988; Lehman-McKeeman et al., 1990). Furthermore, the physiologic response to protein overload, i.e., transient induction of lysosomal cathepsins, observed in other tissues such as involuting mammary gland and exercised muscle apparently does not occur in male rat kidney while hydrocarbon is present (Olson et al., unpublished). Thus, it is probable that the renal tubular transport system for resorbing proteins as well as the mechanism for degrading resorbed proteins must operate at maximal levels to cope with the urinary ultrafiltrate load of α_{2u}-g and other proteins in male rats. Therefore, small decreases in the efficiency of protein degradation caused by hydrocarbons in male rats may be magnified by the constant requirement to resorb large amounts of protein. Overloading of renal tubular epithelial cells is apparently also further exacerbated by the resistance of α_{2u}-g and α_{2u}-g-protein complexes to proteolytic digestion.

A key argument limiting the risk of HDN only to animals expressing α_{2u}-g is the expectation that significant levels of hydrocarbon-protein binding occur only with α_{2u}-g. Although data suggest that certain hydrocarbons that are bound by α_{2u}-g in vivo also form complexes with other members of the lipocalin family in vitro (Borghoff et al., 1988), there is no evidence at this time to support the conclusion that such binding occurs in vivo in humans. Even if such information should become available, it is important to keep in mind that binding of hydrocarbons to low molecular weight

serum proteins is not tantamount to induction of HDN since female rats that lack α_{2u}-g, but express many lipocalin-type proteins (which presumably bind hydrocarbons), do not develop HDN. Also, mice express MUP, a protein sharing the highest known degree of sequence homology with α_{2u}-g, but are not susceptible to HDN when exposed to hydrocarbons known to induce HDN in male rats. Furthermore, as reviewed on pp. 27-29 of the workshop draft, human urine is much different in the range of protein molecular weights and charges than urine of male rats; male rat urine is relatively very protein-rich, specifically due to the high concentration of α_{2u}-g (Olson et al., 1990). Although there are trace quantities of lipocalin proteins in human urine (e.g., α_1-acid glycoprotein), normal human urine contains relatively little protein and much of this protein is of high molecular weight, suggesting an origin in the distal urinary tract rather than in serum. Thus, the male rat seems uniquely predisposed to renal protein accumulation for reasons in addition to the actions of α_{2u}-g in binding hydrocarbons.

Having gained an appreciation of the chemical nature of α_{2u}-g and the unique renal physiology of male rat kidney and its role in processing of proteins resorbed from the urinary ultrafiltrate, we now turn to a consideration of the sequence of events interposed between toxicant exposure and the appearance of HDN in male rats. As outlined on p. 38 of the workshop draft, experimental evidence points to a defined sequence of events at the cellular and molecular level resulting in the characteristic histopathology of HDN in male rats. Alden et al. (1984) originally proposed the following developmental sequence for HDN induced by decalin:

> Xenobiotic administered and metabolized → Renal accumulation of hyaline droplets → Tubule cell necrosis occurs proportional to toxicant dose and quantity of α_{2u}-g accumulated → Sloughing of necrotic cells and formation of granular casts at the junction of inner and outer bands of outer zone of renal medulla → Exacerbation of spontaneous glomerulonephrosis (chronic progressive nephrosis).

As presented initially, the appearance of increased numbers of hyaline droplets in the kidney of toxicant-treated male rats is predicated upon decreased renal catabolism of α_{2u}-g. Hyaline droplets, which are a constitutive feature of the proximal tubule epithelium of male rats, contain α_{2u}-g (Olson et al., 1987). By either light (Olson et al., 1987; Short et al., 1989) or electron microscopic (Garg et al., 1987) immunohistochemistry, a marked increase in lysosomal α_{2u}-g content

accompanies hyaline droplet accumulation. The accumulation of both α_{2u}-g and hyaline droplets is reversible upon cessation of toxicant exposure (Garg et al., 1988). While these conclusions appear sound for commonly used rat strains, as pointed out in the issues paper, little or no evidence exists in non-rat species to support or refute a role for proteins homologous to α_{2u}-g in the sequential renal pathology detailed above. From the limited multi-species testing done, however, no lesion analogous to HDN is known in hydrocarbon-treated animals other than the male rat.

Rapid accumulation of hyaline droplets and α_{2u}-g following initiation of chemical exposure of male rats precedes development of necrosis of proximal tubule epithelial cells. Necrosis is confined to individual cells of the proximal tubule (principally segments P1 and P2) and does not progress to confluent necrosis, an observation differentiating HDN from lesions induced by other types of chemical tubulo-toxins. One of the major unresolved questions pertaining to the sequence of pathology in HDN is, "How is hyaline droplet accumulation related to necrosis?" Lysosomal instability (fragility) has been speculated to contribute to necrosis, but other hypotheses such as intrinsic toxicity of α_{2u}-g or the action of α_{2u}-g as a renal "bioconcentrator" for hydrocarbons may also pertain.

As pointed out on pp. 40-42 of the workshop draft, exposure of male rats to HDN-type nephrotoxins is associated with minimal degree of alteration in renal function. The most pronounced change in urinary composition parameters consistently observed with these toxicants is a large increase in cellular casts or debris in the urine. Such findings are consistent with the limited necrosis observed and suggest that renal toxicity screening panels based on urine chemistry may be ineffective in identifying HDN-type nephrotoxins. Lack of pronounced pathognomonic alterations in urine chemistry or composition hamper efforts to identify effects of HDN-type nephrotoxins in species in which histopathologic study at multiple time points is ethically or economically infeasible.

Chronic administration of HDN toxicants to male rats is accompanied by granular cast formation and dilation of portions of the nephron distal to the proximal convoluted tubule, mineralization of medullary tubule segments, and hyperplastic changes in the urothelium of the renal pelvis (Alden et al., 1984). However, the principal sequela of tubule cell necrosis relevant to the carcinogenic effects of HDN-type nephrotoxins appears to be induction of cell replication in

the proximal convoluted tubule (Short et al., 1989). Increased rates of cell replication correlate with the species-, gender-, and organ-specificity of carcinogenesis by several HDN toxicants (e.g., unleaded gasoline and 2,2,4-trimethylpentane (TMP)). Furthermore, an increased rate of cell replication is observable as long as hydrocarbon exposure continues, rather than being a transient, early event (Short et al., 1989). In the case of the male rat kidney, increased cell replication associated with HDN-type toxicant exposure is not seen in the absence of α_{2u}-g accumulation. Likewise, female rats do not experience renal tubule cell proliferation when treated with chemicals that induce HDN in male rats. However, the experimentation upon which these conclusions and assumptions are based is limited to work with only a few toxicants (e.g., TMP and unleaded gasoline) and renal cell proliferative responses in species other than the rat have not been explored. Interestingly enough, it has been reported that the P3 segment of the male rat proximal tubule shows an increase in the rate of cell proliferation associated with gasoline or TMP administration (Short et al., 1989). This tubule segment is not involved in hyaline droplet accumulation. Thus, the possibility remains that, at least in the male rat, HDN-type toxicants may exert mitogenic effects independent of cell regeneration following hyaline droplet accumulation. Important studies of the rate of renal cell proliferation in male rats lacking α_{2u}-g expression either due to a genetic anomaly (i.e., the NBR strain of rat mentioned on p. 46 of the draft report) or hormonal manipulation might be used to substantiate the necessity of α_{2u}-g accumulation as a triggering event for cell replication.

In conclusion, the weight of the available evidence supports strongly the notion that α_{2u}-g expression is intimately involved in determining susceptibility to HDN. Thus, because this protein is synthesized at significant levels only by male rats, there appears to be no risk of HDN in species other than rat. Full acceptance of these conclusions for the diverse group of HDN-inducing chemicals is hampered by the lack of extensive studies of the pathogenesis of HDN induced by all but a few nephrotoxicants. However, the general hypothesis that male rats only are susceptible to HDN appears sound.

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INTRODUCTORY COMMENTS FOR PEER REVIEW WORKSHOP ON ALPHA$_{2u}$-GLOBULIN: ASSOCIATION WITH RENAL TOXICITY AND NEOPLASIA IN THE MALE RAT*

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EVALUATION OF THE ASSOCIATION BETWEEN ALPHA$_{2u}$-GLOBULIN AND NEOPLASIA IN THE MALE RAT

The first set of slides summarizes the gross picture, and the second set applies specifically to the kidney. Alpha$_{2u}$-globulin renal carcinogens are actually a very small part of the very large, worldwide picture of chemical carcinogenicity. I want to start by showing the total picture, because I think it will help us in this afternoon's discussions.

First of all, let's remember that this is the third such meeting that EPA has organized. The previous two, the Williamsburg meeting on short-term tests and the Virginia Beach meeting on carcinogen risk assessment, provided the seeds of this meeting. At the Virginia Beach meeting, the central issue was whether or not there are different mechanisms of chemically induced cancer in rodents and whether we can have differential extrapolation of these different carcinogens to humans. At that time (2 years ago), trimethylpentane and limonene were already in the possibly-not-relevant-to-man classification, and of course, a vast amount of data have come in since then. The driving force is, hopefully, to separate the extrapolation of chemicals such as dibromochloropropane from limonene.

The second slide reminds us that although knowledge of carcinogenic mechanisms is dramatically growing at the moment, in fact the data have been available for a long time. The third slide summarizes data that are 10 or 12 years old. The reason I show it is that for two well

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*This is a summary of remarks based on a taped presentation. It has been reviewed and approved by the speaker.

established carcinogens, dimethyl benzanthracene (DMBA) and 2-acetaminofluorene (2-AAF), you can modulate the incidence of cancer by interrupting the biology in rats, either by feeding them other chemicals or, in the case of DMBA, performing ovariectomies. This finding indicates that toxicity, hormonal control, and mitogenesis are of critical importance in the expression of carcinogenicity, even for genotoxic reference compounds like 2-AAF. Additionally, chemicals causing secondary effects such as toxicity, hormonal imbalances, or mitogenesis may, in the correct tissues of the correct species of animals, appear to be carcinogens because of spontaneous or naturally present DNA mutations in the tissues.

From this larger picture of carcinogenicity, we move on to a slide that summarizes what most researchers are doing in the area of chemically induced cancer. Ten years ago, most people were solely concerned with DNA in the nucleus, as most of the classical human carcinogens were mutagenic. This is the area in which, 15 years ago, we began conservative, low-dose extrapolation. Because of the failure to correlate genotoxicity with rodent carcinogenicity, we have all been forced to look elsewhere—in the nucleus, outside of the nucleus, and into the tissue where nongenotoxics are producing changes in networks that may modulate tumor incidence. I think we're actually in the middle of this area today. I don't think there is any primary interaction with genomes for most of the CIGAs we are discussing. Again, the interest in this separation is that with nongenotoxins there is a much higher chance that there will be a threshold-dependent effect, although a threshold cannot be assumed and needs to be clearly established.

The next slide is an alerting one, because when you're not really considering the chemical interacting with DNA, you lose DNA reactivity as a very important thread of continuity from bacteria right through to humans, and the door is wide open for false correlations. Some of the data that Jim Swenberg has published indicate that limonene and trimethylpentane are classic promoting agents, rather than initiating carcinogens. So it is legitimate to talk of limonene as a tumor-promoting agent and as a carcinogen; terminology is important.

I will present two examples of associations that we assume we wouldn't make. We know that trimethylthiourea (TMTU) produces goiter and that it is positive in the L51 assay. We would not attempt to associate the L51 response with goiter, because we know that the mechanisms for the two responses are different. Likewise, aniline produces cyanosis and you can contrive an Ames

positive response for this chemical, but I don't think we would automatically relate the Ames positive response with the cyanosis. So there are some areas where we know there's not a correlation between two phenomena. We don't know everything in advance about a new mechanism of carcinogenic action, and although it is very tempting to correlate everything that happens, parallel events may not be causative events. This is a general caution that we should bear in mind.

The next point I would like to make is that the problem of nongenotoxic carcinogenicity is growing, and CIGAs are a very small part of it. Ray Tennant and I developed a chart while looking at the whole database (301 chemicals) of the National Toxicology Program's (NTP's) rat/mouse bioassay program. The chemicals have been classified according to whether they are structurally alerting or not. We used very simple rules: if they had nitro groups or alkylating species, for example, we considered them structurally alerting; if not, we considered them structurally negative. Splitting the database by positive and negative structure-activity results in a dramatic picture. In going from two-species carcinogens, of which 58 were structurally alerting, to noncarcinogens, 33 of which were structurally alerting, we found that the Salmonella assay was firing overtime. The Salmonella assay indicated that 94 percent of the carcinogens were mutagenic and 67 percent of the noncarcinogens were mutagenic. Mutagenicity, therefore, is very prevalent in structurally alerting chemicals, both carcinogens and noncarcinogens. The actual resolution between carcinogenicity and noncarcinogenicity is quite small.

There is another large group of nonalerting chemicals, such as trimethylpentane (TMP) and limonene, which run the gamut from two-species carcinogens to noncarcinogens, with single carcinogens and equivocal chemicals in between. For these chemicals, the Salmonella assay has essentially nothing to say, since no more than 4 percent of any of these groups are mutagenic. This is a large number of chemicals, and this is the area in nongenotoxic carcinogenicity that people are investigating at the moment.

To emphasize that the kidney is a very small part of a much bigger problem, I have a slide of the nonreactive chlorinated compounds in the NTP database. Although they are nonalerting and Salmonella negative, many of them are, nevertheless, mouse liver carcinogens. So there are much bigger problems related to mitogenesis and other secondary mechanisms, which we should bear in

mind, because we want to come to conclusions that are consistent with the bigger pattern. We can't adopt a very hard position on the importance of mitogenesis to cancer if it doesn't fit in with other areas of current research.

The idea that cancer is a black and white phenomenon is confusing. In fact, chemical carcinogenicity is a great spectrum from potent, multisite carcinogens to very weak carcinogens, to noncarcinogens. We are involved in this boundary area between strong and weak effects. Here are, for example, the NTP data on percent of tumor-bearing animals for isophorone.

These are generic ideas. Now let's view some slides on the issues we will hopefully be looking at this afternoon and the traps we can try to avoid. This is a very important area we're looking at, and we must not come to premature or simplistic conclusions.

First of all, we need to consider male rat kidney specificity in terms of its importance and uniqueness. We have to be careful because we're still working with a relatively small number of bioassay results. I went through the NTP database, looked at the lung carcinogens, and arranged them provocatively (see Table 1). They all happen to be structurally alerting with one exception—selenium sulfide—and they are all Salmonella mutagens. Lung tumors occur in male and female rats, and in male and female mice. Only one chemical, tetranitromethane, affects all four test groups (i.e., male rat, female rat, male mouse, and female mouse). You have to be careful, because microgroups, such as a group of six chemicals that are associated with lung cancer only in female mice, can be identified where there is apparent specificity. Nevertheless, in this particular example, the identification of this microgroup would not be interpreted as indicative of a new mechanism; it is probably the result of differences in pharmacokinetics or relative toxicity.

Table 2 summarizes all of the kidney carcinogens in rats and mice in the NTP database. There are only 22, the majority are not structurally alerting, and most are nonmutagenic. They do not fit our historical perceptions of carcinogenicity. A little over half of the compounds are only carcinogenic in the male rat, but there are also some that are only active in the female rat, and there are others that are active in all four test groups. Some of these chemicals are also associated with tumors at other sites, as shown.

Table 1 Test Groups Affected by Lung Tumours

Chemical Name	Structural Alert	Salmonella Assay	Rats		Mice	
			Male	Female	Male	Female
5-Nitro-*o*-anisidine	▲	▲	▲	▲		
3,3'-Dimethylbenzidine	▲	▲	▲	▲		
p-Nitrosodiphenylamine	▲	▲	▲			
Bromoethane	▲	▲	▲			
Dimethyl hydrogen phosphite	▲	▲	▲			
1,2-Epoxybutane	▲	▲	▲			
H.C. Blue 1	▲	▲		▲		
2,4,5-Trimethylaniline	▲	▲		▲		
1,2-Dibromoethane	▲	▲		▲	▲	▲
1,2-Dichloroethane	▲	▲			▲	▲
Dichloromethane	▲	▲			▲	▲
Tris (2,3-dibromopropyl) phosphate	▲	▲			▲	▲
Bis (2-chloro-1-methylethyl) ether	▲	▲			▲	▲
Sulfallate	▲	▲			▲	
Glycidol	▲	▲			▲	
1,3-Dichloropropene	▲	▲				▲
4,4'-Methylenedianiline	▲	▲				▲
1,5-Naphthalenediamine	▲	▲				▲
Selenium sulfide	○	▲				▲
Trifuralin	▲	▲				▲
4-Vinyl-1-cychohexene diepoxide	▲	▲				▲
Tetranitromethane	▲	▲	▲	▲	▲	▲

○ = No effect

▲ = Lung tumour

Table 2 Groups With Renal Tumour

Chemical Name	Structural Alert	Salmonella Assay	Rats		Mice	
			Male	Female	Male	Female
Chlorinated paraffins (C-12)	○	○	▲ ●	●	●	●
Isophorone	○	○	▲			
d-Limonene	○	○	▲			
α-Methylbenzyl alcohol	○	○	▲			
Cinnamyl anthranilate	○	○	▲ ●		●	●
1,4-Dichlorobenzene	○	○	▲		●	●
Hexachloroethane	○	○	▲		●	●
Hydroquinone	○	○	▲	●		●
Monuron	○	○	▲ ●			
Dimethyl methylphosphonate	▲	○	▲			
2-Amino-4-nitrophenol	▲	▲	▲			
Nitrofurantoin	▲	▲	▲			●
Chlorthalonil	○	○	▲	▲		
Nitrilotriacetic acid	○	○	▲ ●	▲ ●	▲	▲
Phenylbutazone	○	○		▲	●	
Benzofuran	○	○		▲	●	●
Tris (2-chloroethyl) phosphate	▲	○	▲	▲		
C.I. Acid Orange 3	▲	▲		▲		
Tris (2,3-dibromopropyl) phosphate	▲	▲	▲	▲	▲ ●	▲
1-Amino-2-methylanthraquinone	▲	▲	▲ ●	●		●
o -Anisidine	▲	▲	▲ ●	●	●	●
Bromodichloromethane	▲	▲	▲ ●	▲ ●	▲	●

● = Tumour at sites other than kidney

○ = No effect

▲ = Renal tumour

One of the complicating issues that we'll face this afternoon is that most of these chemicals are associated with cancer in other organs in addition to the rat kidney. The technical panel's report addresses this issue very well, stating that we either have to assume that there are several parallel, independent mechanisms of nongenotoxic cancer, or we have to assume we know nothing about that whole area. This is well stated, because there is a small possibility that we are following correlations. There are very few chemicals (e.g., the isophorones and limonenes) where the only cancer observed is in the male rat kidney. So that's a general alert about not putting too much weight on small groups. Incidently, there appear to be some cases, such as nitrofurantoin, that are very clearly rat-kidney specific. It may be possible that mutagenic chemicals can still elicit the secondary mechanism. I think we should have our minds open to that.

I suggest that we address the issue of genotoxicity very simply and assume that CIGAs are pure, nongenotoxic agents, although in reality most of them probably are not. Limonene seems to be the most likely to remain a nongenotoxin with the passage of time. Trimethylpentane will probably never affect any genetic toxicity system, but unfortunately, we have no data on it in the report, which is a shame, because it should become the gold standard. I suggest we don't waste time on tetrachloroethylene. Let's concentrate on the pure nongenotoxins like limonene.

Here is the proposed mechanism.

Chemical dose → possible metabolism → insoluble complex with α_{2u}-globulin

↓

compensatory hyperplasia ← cell necrosis ← precipitated protein complex

↓

tumors

This afternoon we will, I hope, decide which of these steps are critical, which we have data on, and which we can link to the next step. The whole question of the formation of this complex either in vivo or in vitro, and its hydrolytic degradation is a big area for discussion; the other big area is compensatory hyperplasia, which links cell necrosis to tumors. There is actually a vast chasm between hyperplasia and tumors. A lot of data indicate that hyperplasia is absolutely not a

carcinogenic effect; an equal amount indicate that it is. These are the two major areas where we'll be looking at cancer-critical mechanisms, and we need to be cautious in doing so.

I'd like to say a few words on hyperplasia because Bruce Ames and Lois Gold have thrust it into the common mind recently through their article in *Science*. In case you haven't seen it, I'll just summarize what was written in the article entitled, "Too Many Rodent Carcinogens." The suggestion is that the administration of chemicals at maximum tolerated dose increases cell division, which in turn increases rates of mutagenesis, and thus carcinogenesis. That's pretty slick, but it's also pretty untrue. The paper goes on to say, "... then *any* chemical that increases mitogenesis is a likely rodent carcinogen." Well, that's just wrong. Then somewhere else it says "... but mitogenesis was not measured even though it can be high without histologically observed lesions" (this is in reference to 2-AAF in the mouse), and finally, "... agents causing mitogenesis are proper carcinogens and are important in human cancer." This article underscores the fact that we should be very careful about what we mean when we discuss hyperplasia.

Here is a slide that really brings this home. This is one of Jim Swenberg's reports (CIIT, 1990), which is not fully published yet. He and his coworkers conducted an inhalation study with dimethylamine using Fischer 344 rats and B6 mice of both sexes. They found nasal irritation and hyperplasia in all four test groups, but no cancer. So the event of hyperplasia is not pivotal. What matters is the underlying mechanism of the hyperplastic response, its magnitude and duration, and the particular tissue affected (see Butterworth, 1990. Mutat. Res. 239: 117-132).

My last slide shows the product of unclear thinking in order to focus clear thinking. This is a summary of work from Loury[a], who was testing unleaded gasoline (UG) and trimethylpentane, a nephrotoxic compound of UG, in both sexes of Fischer rats and B6 mice. Male rat kidney cancer was observed for UG. In addition, UG was associated with liver cancer in the female mouse. For both substances, administration in vivo resulted in UDS-positive results for UG in hepatocytes isolated from mice, but negative results for trimethylpentane. Positive results for UDS in vivo are actually quite rare, so that's significant. It's probable, the paper suggests and other people agree,

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[a]Loury, D.J., Smith-Oliver, T., Strong, S., Jirtle, R., Michalopoulos, G., and Butterworth, B.E. 1986. Toxicol. Appl. Path. 85:11-23.

that these data are the result of impurities in the unleaded gasoline. Nevertheless, these data may influence the interpretation of some of the gasoline data.

My last point concerns interpreting hyperplasia in terms of cancer. In the case of Loury's study, the authors report that only female mice had liver cancer when exposed to unleaded gasoline. On the other hand, the S-phase results were negative for female mice treated with UG, but positive in male mice. These data argue against a correlation between hyperplasia and cancer. Except that it's not particularly good toxicology, because the cancer data came from a two-year inhalation study at relatively low doses, and the S-phase data came from one-shot, high-dose, gavage studies. Mixing together observations from grossly acute and long-term studies should be done carefully. I don't think this study ends the debate. I'd like to see an inhalation study and see the S-phase results before I dismiss the correlation. These are the considerations we must bear in mind because long-term and short-term toxicity are not always the same.

This afternoon, we will be trying to find out how much of the mechanism is hard and believable—which steps exist, which we can really believe in, and which steps link other steps. The issue of dose response is quite important. In the report, we have clear evidence that these male rat kidney effects are being produced below the maximum tolerated dose. Some of the markers, however, are not dose-related. We will try to approach the questions of thresholds, and, hopefully, we will list experiments that need to be done.

INTRODUCTORY COMMENTS FOR PEER REVIEW WORKSHOP ON ALPHA$_{2u}$-GLOBULIN ASSOCIATION WITH RENAL TOXICITY AND NEOPLASIA IN THE MALE RAT

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CRITERIA FOR IDENTIFYING CIGAS

Discussion on establishing criteria for the categorization of renal carcinogens as CIGAs can be addressed under some of the headings listed for hazard identification in the EPA Risk Assessment Guidelines of 1986.

1. Short-term tests. As a group, chemicals inducing α_{2u}-globulin (α_{2u}-g) show little or no genotoxic activity, although more multitest data may be needed for comparisons across the range of substances. Where tested, CIGA have proved negative in assays for Salmonella mutations, chromosome aberrations, and micronucleus formation, and in tests with human cells.

Some CIGA have proved positive for sister chromatid exchange and in the mouse lymphoma gene mutation assay. However, with the exception of dimethyl methylphosphonate, such positive responses have been observed only in the absence of exogenous S9 activation and at relatively high concentrations. For practical purposes, might clear evidence of mutagenic activity in a range of conventional tests including the Salmonella mutation and chromosome aberration assays preclude categorizing a renal carcinogen as a CIGA? It may also be useful to consider the relevance of the replicative DNA synthesis assay in rat kidney cells to this issue.

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Toxicity of CIGA

CIGA induce a sequence of renal lesions, some of which, individually, are not commonly encountered in other forms of chemically induced nephrotoxicity. Excessive formation of hyaline droplets in the P2 segment of proximal tubules is a special type of lesion, but is also seen in both sexes of rat and mouse in association with histiocytic sarcoma. The demonstration of α_{2u}-g in chemically induced hyaline droplets represents an essential criterion and discriminates these from tumor-associated droplets, which stain positively for lysozyme and not for α_{2u}-g in immunohistochemical techniques.

Granular cast formation at the corticomedullary junction and linear mineralization in tubules of the renal papilla also constitute unusual lesions. CIGA-induced papillary mineralization involving Henle limbs, in particular, is a discrete lesion distinct from the usual form of cortico-medullary mineralization or pelvic mineralization.

The specific sequence of progression of these lesions, implying an interdependence between specific lesions, may be a useful discriminator. In this context, there should be some consideration given to the demonstration of increased cell replication in renal tubules as this currently provides the putative mechanistic link from α_{2u}-g nephropathy to renal cell tumor induction. Perhaps information on the presence or absence of renal pathology induced in other strains or species by a test chemical would support the CIGA categorization.

Carcinogenicity Bioassay Data

Among the laboratory species tested, CIGA have induced renal tumors in male rats only. Long-term bioassay data must, therefore, be acquired at least in the mouse and the rat to demonstrate this characteristic. However, on its own, the carcinogenicity study is not sufficient to classify a test chemical as a CIGA, but acts as a pointer to the need for establishing additional criteria related to the nephropathic sequence.

The nature of the renal tumors and their apparent progression from hyperplasia through adenoma to carcinoma with a potential for metastasis (the latter exemplified by hexachloroethane) are not distinguishable from the lesions produced by classical renal carcinogens. On the other hand, renal tumor incidence appears to be consistently low in studies with CIGA in contrast to the higher frequency of tumors induced by genotoxic renal carcinogens. In addition to hyperplasia, the presence of linear mineralization in papillary tubules, which tends to be observed during the long-term assay, might also serve as an additional pointer at this stage of testing. Panel discussion should address the significance of chemicals that are known to induce the special sequence of nephrotoxicity without apparent renal tumor formation.

In the broader context, the panel needs to consider the classification of chemicals that induce both the special form of nephropathy culminating in renal tumor formation, and the occurrence of dose-related increased incidence of tumors at other sites. The panel should also consider whether there might be a distinction in criteria-setting for the induction of α_{2u}-g nephropathy on the one hand, and renal neoplasia on the other.

Metabolic/Mechanistic Factors

Binding, essentially of a reversible nature, of CIGA metabolites or, in some cases, of only the parent compound itself (e.g., isophorone) is considered to be critical to the development of protein overload. Recent data suggest that factors other than binding are involved in the abnormal renal accumulation of α_{2u}-g and that the important effect may be whether the bound xenobiotic prolongs the rate of protein degradation in the kidney cells. Consequently, the panel should consider the question of what information is needed on metabolites and comparative metabolism between species, the nature of binding, demonstration of impairment of α_{2u}-g hydrolysis, and the possibility of covalent binding to macromolecules. In particular, the distinction between a specific chemical's nephropathic effect involving reversible binding to α_{2u}-g and the concomitant covalent binding by metabolites to DNA in other organs and/or the kidney needs discussion and clarification.

Structure-Activity Relationships

Substances that have been shown to induce renal accumulation of α_{2u}-g and/or hyaline droplet nephropathy represent a seemingly diverse range of chemical structures. However, research is proceeding in this area of structure-activity evaluation. Recent studies from several laboratories have already provided some information for consideration. Lipophilicity, hydrogen bonding, and steric volume appear to play a role in binding activity while the capacity for an α_{2u}-g xenobiotic to prolong renal lysosomal digestion of α_{2u}-g correlates with the presence of an oxygen function in a limited number of chemicals tested. The panel should consider whether structure-activity relationships can assist in categorizing renal carcinogens as CIGA.

Finally, in addressing the issue of criteria-setting from these various aspects, the panel should determine whether CIGA categorization for chemicals is feasible, and if so, how a classification scheme could be constructed.

INTRODUCTORY COMMENTS FOR PEER REVIEW WORKSHOP ON ALPHA$_{2u}$-GLOBULIN: ASSOCIATION WITH RENAL TOXICITY AND NEOPLASIA IN THE MALE RAT

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CHARACTERIZING RISK FOR CIGAS

EPA, in preparing the draft report, intentionally refrained from proposing a Science Policy position (Part IV). A science policy in regard to the relevance and use of data pertaining to CIGAs will depend on the conclusions reached by the other three workgroups, especially workgroup 3, which has the responsibility for developing the criteria for identifying CIGAs. Nevertheless, certain approaches can be suggested based on the draft report, fully aware that the approaches may be inappropriate if the draft report and its conclusions are modified.

Conceptually, several scenarios can be envisioned based on differing conclusions that might be arrived at from those of the draft report. The key conclusions that pertain to CIGAs considered most likely to drive the thought processes of those performing the risk assessment are listed below.

1) Chemically induced α_{2u}-globulin (α_{2u}-g) nephropathy in the male rat can be distinguished histopathologically from chronic progressive nephropathy, and designating a chemical as a CIGA requires positive identification of α_{2u}-g in the hyaline droplets.

2) Alpha$_{2u}$-globulin nephropathy appears to be a distinct entity specific to the male rat among the tested laboratory species and genders.

3) Epidemiology studies are inconclusive although it appears that the human male is probably unlike the rat in nephrotoxic response to CIGAs, and even if the human

male is more like the rat, there would be quantitative differences in response, with the risk of nephropathy reduced in the human from that observed in male rats.

4) CIGAs evaluated so far have not been shown to possess mutagenic activity and do not appear to bind covalently to DNA.

5) A nephrotoxic response of CIGAs has always preceded renal tumor formation in the male rat.

6) The mechanism for neoplasia may be promotional in nature.

7) Renal tumors have only been observed in male rats and not in any other species tested with CIGAs.

8) The conclusion that increased proliferative response caused by chemically induced cytotoxicity appears to play a role in the development of renal tumors in male rats is most applicable when short-term tests for genotoxicity are negative, when nephrotoxic responses characteristic of CIGA are also observed, and when renal tumors are observed only in male rats and not in other species/sex combinations.

The following three scenarios can be envisioned based, respectively, on acceptance of all of the draft conclusions, only some, or none of the conclusions:

1) <u>The conclusions are accepted, neoplasia is considered the end stage of the nephrotoxicity progression, and the chemical meets the criteria that indicate that the nephrotoxicity and renal neoplasia are unique responses for the male rat and the data are irrelevant for human risk considerations</u>.

Under this scenario, the renal toxicity and neoplasia are discarded and the data used for risk assessment will consist of other organ effects (or effects on the kidney not typical of the CIGA response). Procedures to use for the risk assessment are those currently used by EPA.

2) The link between nephrotoxicity and renal neoplasia is not established although the nephrotoxicity is considered to be specific to the male rat, the mechanism for renal neoplasia is unknown, and the nephrotoxicity is considered to enhance the response in the male rat.

Under this scenario, the data pertaining to nephrotoxicity in male rats are discarded and the data pertaining to renal neoplasia are utilized in the risk assessment. Procedures to use for the risk assessment will then involve weight-of-evidence of the data and selection of the appropriate quantitative method(s) to use.

In the absence of sufficient relevant data to adjust for the enhancement caused by nephrotoxicity, the default multistage model will be employed along with other mathematical models to determine the best fit. If relevant data are available, then other models that take into consideration those data may be employed, e.g., physiologically based pharmacokinetic model (PB-PK) or Moolgavkar model. It is also possible that the enhanced replicative rate caused by the nephrotoxicity may contribute to an independent mechanism of action leading to a response observable in male rats. Conceivably, the use of the safety factor approach may also be considered if the evidence strongly suggests a threshold for the neoplastic response.

3) The link between nephrotoxicity and renal neoplasia is established and neither nephrotoxicity nor renal neoplasia are considered specific for the male rat.

Under this scenario, the data pertaining to nephrotoxicity in male rats as well as the tumor data are acceptable for risk assessment. Procedures to use for the risk assessment will then involve standard methodologies employed by the EPA, i.e., weight-of-evidence evaluation with selection of the appropriate quantitative methods.

The default multistage model will be employed along with other mathematical models to determine the best fit in the absence of mechanistic data. If data pertaining to mechanism and pharmacokinetics are available, then other models that take into consideration those data may be employed.

Other scenarios may also be appropriate depending upon the conclusions reached by the other panels. For example, it may be decided that the nephrotoxicity data are acceptable only for acute/subchronic toxicity evaluations but not for chronic/carcinogenicity analyses. In that case, another scenario might be chosen for the risk assessment science policy.

SECTION FIVE

WORKGROUP REPORTS

NEPHROPATHY WORKGROUP

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$Alpha_{2u}$-Globulin Biochemistry and Nephropathy

Review of the workshop draft report "$Alpha_{2u}$-Globulin: Association with Renal Toxicity and Neoplasia in the Male Rat" by the nephropathy workgroup focused on identification of data supporting the principal conclusion that expression of α_{2u}-globulin (α_{2u}-g) is integral to susceptibility to hyaline droplet nephropathy caused by CIGA. To facilitate review, the workgroup's discussion revolved around the key points raised in the section on α_{2u}-g biochemistry and nephropathy in the issues paper. The outcome of our review consists of restatement of some of the conclusions of the issues paper with modifications made to reflect the consensus of the workgroup.

The workshop draft concludes, and the workgroup concurs, that the acute and chronic renal pathological effects induced in male rats by chemicals causing α_{2u}-g accumulation (CIGA) are unlikely to occur in any species not producing α_{2u}-g or proteins with a structurally similar binding domain to α_{2u}-g, in the large quantities typically seen in the male rat.

Conclusions and Suppositions Used in Reaching This Position

There is no evidence that the nephropathy induced by α_{2u}-g accumulation occurs in female rats or either gender of other species examined to date, including humans.

Accumulation of α_{2u}-g in hyaline droplets will always result in nephrotoxicity given sufficient dose and length of exposure to a CIGA.

Male rats of strains commonly employed in toxicology testing—e.g., Sprague Dawley, Osborne Mendel, Fischer 344—would be expected to respond in a qualitatively similar manner to chemicals that induce α_{2u}-g accumulation.

Concentrations of protein homologues of α_{2u}-g in human urine are well below those found for α_{2u}-g in urine of male rats.

There are no known differences in structure and function of the proximal convoluted tubule epithelium between human male and female. However, numerous biochemical differences of the renal proximal tubules of male and female rats have been identified. Among these are key differences that render the male rat more susceptible to nephropathy induced by CIGA.

Based upon these considerations, it is unlikely that human renal toxicity or tumors could occur by the α_{2u}-g mechanism hypothesized to operate in the male rat. Certain elements of the conclusions could be made more forcefully except for the recognition of limitations in the available data. These limitations are catalogued below. Implicit in the statement of these limitations are recommendations for carefully planned further experimentation.

Data Limitations

It would be useful to know if any CIGA can bind to human homologues of α_{2u}-g, and if such a complex is formed, its catabolism rate relative to the male rat and its toxicity to human proximal tubule epithelium.

Experimentation on other species examining possible renal protein accumulation and hyaline droplet formation by CIGA (defined in male rats) is extremely limited.

The site of tumor origin within the kidney of CIGA-treated male rats is unknown for most CIGA that have been identified as nephrocarcinogens by bioassay. Early time points for serial sacrifice in future bioassays of suspect CIGA are desirable.

There is no known mechanistic link between α_{2u}-g and hyaline droplet accumulation and the induction of necrosis in proximal convoluted tubules of CIGA-treated male rats.

There is uncertainty regarding the triggering stimulus for increased rates of cell replication following administration of CIGA to male rats.

CANCER WORKGROUP

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This report is written from the viewpoint of one wishing to predict a new, male rat-specific renal carcinogen operating by the α_{2u}-globulin (α_{2u}-g) mechanism. It is assumed that the further along in the carcinogenicity sequence one proceeds, the stronger will be the presumption of carcinogenicity.

1. Chemical structure. The majority of CIGAs are chemicals that are not expected to be electrophilic, and, therefore, they separate out from classical genotoxic carcinogens. However, essentially unpublished data indicate that a separate structure-activity relationship (SAR) exists for CIGA. The prospective use of such an SAR model will be hampered by the need to predict metabolism, both to active ligands and to detoxification products. The fact that limonene epoxide, the active ligand derived from limonene, is a minor metabolite illustrates this potential problem. Also, different types of binding to α_{2u}-g exist, as for the dichlorobenzenes. Clear SARs should, however, develop with time based on current disclosures. Isolation of ligand-α_{2u}-g complexes from dosed animals is the preferred way to study these SARs. Lipophilicity, hydrogen bonding, and shape correlations may evolve, but they must also evolve for predicted metabolites if they are to be useful in screening.

2. Genotoxic status of test agent. In order to have credibility for a nongenotoxic mechanism of tumor induction, it is necessary to evaluate the test agent for genotoxic potential. This must include the conduct of an appropriate Salmonella assay, rodent bone marrow cytogenetic assay, and if possible, a rat kidney DNA repair assay. The conduct of ancillary in vitro assays, such as the mouse lymphoma or SCE assays, is debatable. Most of the current CIGAs are active in these last two assays at high-dose levels in the absence of S9 mix. It is hard to accept that this defines them as being DNA-reactive. The quality of test protocols is often the critical variable here. However, a believable and adequate level of negative genotoxicity data should exist for a candidate CIGA carcinogen. In the short-term, concentration on clear nongenotoxins such as limonene will aid mechanistic studies.

3. Alpha$_{2u}$-globulin binding studies. Based on point (1) it should be possible to conduct in vitro binding studies, coupled to measurements of the critical variable of reduced protein breakdown in the presence of proteinases. Metabolite identification complicates the extension of such studies into a screening test, and this is not recommended in any case.

4. Hyaline droplet accumulation in vivo. Although points (1) and (3) may prove useful in mechanistic studies, the chemical induction of hyaline droplet accumulation is best achieved and studied in rodents. The minimum protocol for such studies depends on the detection techniques employed. Thus, using immunochemical detection of α_{2u}-g, it should be possible to detect all CIGA within a 14-day dosing protocol. In such a limited assay, use of young adult male rats that are clearly producing α_{2u}-g is mandatory. Use of less definitive hyaline droplet detection systems (i.e., hematoxylin and eosin [H & E] or methylene blue) would reduce the sensitivity of the test and is not recommended. Fourteen days would also fit in with the detection of peroxisomes in the liver. The potential problem of hyaline droplets that do not contain α_{2u}-g, as alleged for chlorothalonil, should be born in mind in any screening program. Dose levels in all such acute studies should, where possible, be limited by the chronic maximum tolerated dose (MTD) (i.e., expected bioassay dose level). This avoids the generation of potentially misleading acute high-dose data (e.g., the 1.2 g/kg data for limonene whose chronic MTD is 150 mg/kg). Observations should be made within 1 day of cessation of dosing.

5. Morphology of α_{2u}-g-accumulated droplets (here referred to as hyaline droplets). Data exist indicating that the morphology and the presence of crystalloid patterns in hyaline droplets can help distinguish CIGA-induced from control droplets. This can be a useful parameter for interpreting weak accumulations. The accumulation of α_{2u}-g appears to be common across proximal tubule epithelial cells, the extent of accumulation being measured within cells. There are no data indicating the critical level that will lead on to cell necrosis, but such can be expected in the future. Anecdotal accounts of two chemicals that induce α_{2u}-g accumulation but that are noncarcinogenic were ignored pending data to review the validity of the claim.

6. Single cell necrosis. The next step in the sequence is considered to be single cell necrosis leading to compensatory cell proliferation. Although a critical part of the carcinogenicity sequence, single cell necrosis is not a sensitive parameter as assessed by standard H & E methods. Specifically, the absence of such effects cannot be used to conclude termination of the proposed carcinogenic sequence at this point. The necrosis, when determined, occurs primarily, although not exclusively in the P2 region, the presumed site of tumor origin. Again, the level of α_{2u}-g in a cell that triggers cell death has not been determined.

The mechanism of cell death has also not been defined. The assumption of lysosomal overload leading to mechanical cell death is usual in male rats (unusual in humans); however, it may be that α_{2u}-g acts as a method of accumulating the CIGA in the P2 cells. Subsequent breakdown of the CIGA-α_{2u}-g complex could release the CIGA which itself may kill the cell. This latter concept was considered the less likely as it is hard to accept that all CIGA will be appropriately toxic to renal cells (the implicit assumption).

7. Exfoliation/cast formation/mineralization. These three phenomena were regarded as inevitable but irrelevant effects that occur beyond the P2 segment. Thus, although they may provide useful adjunct data, especially in chronic studies, they are not regarded as measurable events on the cancer critical pathway and are not always present. Nonetheless, the *linear* foci of mineralization in the renal papilla seems to be CIGA-specific.

8. Induced cell proliferation. This is both measurable and critical to the process of carcinogenesis. As with single cell necrosis, observation of mitotic figures (using H & E) is not the

most sensitive of techniques. Rather, determination of semi-conservative DNA synthesis using ^{3}HTdR or bromodeoxyuridine (BrDU) is recommended. Antibody recognition of BrDU is, in fact, the most used and best method. The current best practice is the use of 3-7 day minipumps. Specifically, the use of single intraperitoneal (ip) injections of the bases is not recommended for reasons of reduced sensitivity.

In the rodent liver, the assumption that the incidence of cells in S-phase gives a direct measure of the incidence of cells undergoing mitosis is questionable. This is because liver cells can either binucleate or stay in a higher ploidy state without undergoing cytokinesis. These effects are apparently not encountered in the rodent kidney. Further, programmed apoptosis complicates estimation of cell proliferation in the liver, but apparently not in the kidney. Thus, the careful determination of S-phase activity in the kidney appears to provide a reliable measure of mitogenesis in the kidney.

It is, nonetheless, highly important to realize that the balance between mitogenesis and cell death may be such that increases in cellularity do not occur, i.e., formal hyperplasia may not be indicated by measurements of S-phase activity. It is therefore possible that some chemicals may proceed as far as cell proliferation, yet not proceed further to the induction of hyperplastic foci (the low-dose level in the UG bioassay may fit these criteria).

The 1988 review of toxicity/hyperplasia in NTP studies by Hoel et al. was not definitive in the above respects and should therefore not be related directly to the present debate.

9. Atypical tubules/atypical hyperplasia. These modified pathological terms were devised by Swenberg, and provide the next cancer-critical observation that can be made. Observations are primarily in the P2 section of the tubule, consistent with perceptions of tumor aetiology. These lesions do not regress, or regress less rapidly than acutely induced hyaline droplets. The morphology and description of these lesions have been formalized and should be generally adopted. As with preneoplastic foci in the rodent liver, the incidence of atypical hyperplasia far exceeds the eventual incidence of tumors, so additional selective processes must ensue. The continuum through to adenomas and adenocarcinomas remains to be defined, but will be primarily dependent on lesion size. Little can be gained at this stage from the observation by Bannasch that at least five distinct

cell types are involved in preneoplastic lesions found in the P2 segment. However, the fact that basophilic cells are faster growing may explain why CIGA tumors are mainly basophilic. Clear cell tumors occur, but are apparently rare for CIGA. No data currently exist regarding cell-type, quantitation of atypical tubules, hyperplasia, and tumors.

10. Tumor morphology. CIGA-induced tumors are essentially indistinguishable from either spontaneous or genotoxin-induced tumors; they are solid, basophilic, and generally nonmetastatic. They are also slow growing. The data are therefore consistent with a promotional mechanism, as opposed to an initiatory mechanism of CIGA-induced tumors.

11. Promotional studies. If CIGAs promote spontaneous lesions, as opposed to initiating new lesions, this should impact low-dose risk assessment. The evaluation for carcinogenicity to the male rat kidney of standard renal-promoting agents (e.g., sodium barbitol), would be worth doing. (Barbitol used to be only a mouse-liver-promoting agent until lifetime studies were conducted when it was found to be capable of producing tumor incidence increases without initiation by diethyl nitrosamine [DEN].) Oncogene studies, such as those conducted by Reynolds et al. in the mouse liver, may also contribute to the confirmation of promotion versus initiation. Another issue, whether the male rat kidney contains rare, existing mutant cells or whether the process of cell division itself induces new mutations, is worthy of future study.

Summary. We found the proposed sequence of events leading to CIGA-induced tumors to be credible and capable of providing key events capable of use as predictive markers.

Recommended Studies

1. Conduct of standard NTP tumor bioassays in male rat of some classical renal-promoting agents (e.g., sodium barbitol). This will establish that cytotoxic-induced cell proliferation can promote renal cancer in F344 rats. Decalin is also worthy of study for male rat renal carcinogenicity.
2. Definition of genotoxic status of decalin and trimethylpentane.

3. Use of interim kills in future bioassays of CIGA to evaluate early toxicities that may be swamped by CPN at sacrifice.

EPA Draft Conclusions

1. Agree.
2. We would remove this. The presumption of a link is justified, but not absolutely established.
3. Agree. New Swenberg data enhances further.
4. Agree.
5. Not too useful. We accept the parallel induction of tumors in different tissues by different mechanisms.
6. We would drop this rather confusing conclusion.

EPA Data Limitations

1. This is wrong.
2. For others to answer.
3. We agree. We would make it species/strains. We endorse strongly the need for metabolic studies. We endorse the need for human protein homologue studies.
4. Some appropriate and firm data do exist. This is too strong and negative.
5. Badly written (e.g., "or"). But we encourage interim kills for this purpose.
6. Agree, add interim kills with appropriate measurements.

CRITERIA WORKGROUP

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The workgroup determined that the evidence concerning categorization of CIGA falls into four categories: essential, supportive, preclusive, and unrelated.

A. Essential criteria. All of the following evidence is considered by the workgroup to be essential for categorizing a renal carcinogen as a CIGA. No single criterion provides sufficient evidence to place a renal carcinogen in the CIGA class.

1. Abnormal increase in the number and size of hyaline droplets in the renal proximal convoluted tubule cells of male rats in which the accumulating protein is demonstrated by appropriate technique to be α_{2u}-globulin (α_{2u}-g), is pathognomonic of a CIGA.

2. Although a chemical can be assigned to the CIGA class in the absence of renal tumors, when a tumorigenic effect in the kidney is observed, it occurs in the male rat only and not in female rats or other species and must involve induction of renal tubule cell tumors. The tumors observed with CIGA generally occur in relatively low incidence, are often microscopic, and are usually observed at study termination (2 years).

3. Based on a weight-of-evidence approach involving a range of accepted short-term tests, CIGA should be nongenotoxic or of limited genotoxicity only.

4. Under appropriate experimental conditions, some aspects of the pathological sequence representing α_{2u}-g nephropathy should be demonstrated. These lesions may include single (tubule) cell necrosis, exfoliation of epithelial cells into the proximal tubular lumen, formation of granular casts with associated tubule dilation at the junction of the inner and outer stripes of the outer medulla, and linear mineralization of papillary tubules.

5. When renal tubule cell cancer is observed in a 2-year carcinogenicity bioassay, increased cell replication at the doses used in the bioassay should be demonstrated at an appropriate time-point in the proximal convoluted tubule because it represents a possible mechanistic link between α_{2u}-g, nephropathy and neoplasia.

B. Supportive evidence. The following evidence is considered as supporting (but not essential) for the categorization of a chemical/renal carcinogen into the CIGA class, because it provides additional information explaining the mechanistic basis of action.

1. The demonstration of reversible binding of the chemical (or metabolites) to α_{2u}-g adds to the weight of evidence by showing the interaction between the xenobiotic and the protein. The identification of the moiety responsible for this binding, although useful for establishing structure-activity relationships, is not essential to classify a CIGA.

2. Demonstration of a reduction in the lysosomal degradation of the α_{2u}-g complex establishes the effect of the chemical on the catabolism of the protein.

3. Disposition studies will demonstrate a sex- and species-specific retention of the test chemical, resulting from the interaction with α_{2u}-g in the male rat kidney.

4. Demonstration of cell replication data should be considered as additional supportive evidence when renal tumors are not induced in the carcinogenicity bioassay and the characteristic nephrotoxicity is the endpoint.

5. The observation in a 2-year bioassay of a dose-related increase in atypical hyperplasia in male rat kidney that is not evident in female rats or mice of either sex may be supportive of the long-term effects of a CIGA in the absence of renal tumors.

6. Data on structure-activity relationships are not adequate for chemical classification, but are supportive.

C. Preclusive evidence. The following aspects constitute evidence that precludes placing a renal carcinogen in the CIGA category.

1. Renal carcinogens that are consistently genotoxic in a battery of short-term tests should not be considered as CIGA.

2. Demonstration of renal tumors in female rats and/or mice or other species precludes the classification of a chemical as a CIGA.

3. The finding of an extremely high incidence of tumors in the male rat kidney in the carcinogenicity study, renal tumor multiplicity, and/or clear reduction of tumor latency (in a standard 2-year bioassay) suggests that alternative mechanisms of renal carcinogenesis may also be operative.

D. Unrelated (nonspecific) evidence. Certain nonspecific events can occur with both CIGAs and other compounds. These should not influence the decision concerning CIGA categorization.

1. Spontaneous, age-related chronic progressive nephropathy (CPN) may be exacerbated in rats dosed with CIGA. This is a nonspecific effect also associated with other compounds and/or physiological/nutritional conditions.

2. Presence of a qualitatively different form of nephrotoxicity in female rats, or mice of either sex, should not prevent classification of a compound as a CIGA.

3. An increased occurrence of neoplasms at organ sites other than the male rat kidney should not confound CIGA classification.

RISK CHARACTERIZATION WORKGROUP

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The draft report reviewed at the peer review workshop did not contain suggested approaches and policies regarding risk characterization of CIGAs. Rather, the risk characterization (RC) workgroup was assigned the responsibility to suggest policies, taking into consideration the technical panel's conclusions regarding CIGA nephrotoxicity and the α_{2u}-globulin (α_{2u}-g) hypothesis for CIGA renal neoplasia. In developing this policy section, the RC workgroup was to keep in mind existing science policy of the Agency. In pursuing its charge, the RC group considered both qualitative and quantitative aspects to risk characterization. The workgroup found that a simple flowchart was useful in discussing various options and considerations. This flowchart was provided on an informal basis to the technical panel for its information, not as a strict recommended operational procedure. Further discussion and clarification would be necessary before it could serve as a firm recommendation, thus the flowchart is not included in this report.

In formulating recommendations for the technical panel, the RC workgroup, in addition to its review of the draft report, considered the viewpoints expressed by the other workgroups in the plenary panel discussions. There are still some uncertainties preventing complete acceptance of the male rat specificity and α_{2u}-g hypothesis for CIGA renal neoplasia. These concerns are indicated

below and are followed by specific recommendations that should be considered by the technical panel in the further development of the Agency policy regarding the risk characterization of CIGAs.

Concerns

1. Concerns expressed by the workgroup centered primarily on the epidemiology data. It was felt that the data were not clear and may need more detailed analysis in order to be able to elaborate as to the power and significance of the studies. This is especially true, since several of the epidemiology studies are suggestive as currently described in the report (raising a flag of concern).

2. There are a number of chemicals for which the kidney is a target in both humans and animals. Indirect diagnostic tools that are currently used in humans may not be as definitive as direct examination used in animals. Future research should address issues of comparison between humans and animals, including:

   - Binding of CIGA chemicals to human proteins.
   - Characterization, including level and variability (i.e., age, disease state, genetic factors) of α_{2u}-g analogues.
   - Pharmacokinetics of α_{2u}-g analogues.

3. The workgroup expressed concern that the terms chemical and agent do not include mixtures. Therefore, if not already done, a statement in the document should say that these terms include mixtures.

Recommendations (Issues)

1. If a chemical induces α_{2u}-g accumulation in hyaline droplets, the associated nephropathy in male rats may not be an appropriate endpoint and therefore should not be used for determining a no-observed-adverse-effect level (NOAEL) for noncancer effects.

2. Caution should be used when basing a NOAEL on other toxic endpoints whose occurrence may be related to the kidney toxicity in the male rat, since the animal may have compromised ability to handle the CIGA because of the kidney effects.

3. If a chemical has been adequately tested and judged not to be mutagenic (or not to be of mutagenic concern for carcinogenicity), there is α_{2u}-g accumulation in hyaline droplets, and the only neoplasia observed is in the renal tubules of male rats (assuming adequate testing for carcinogenic potential including the female rat and other species), this is probably not (one person preferred to say *marginal*) evidence for a carcinogenic risk in humans. A quantitative risk assessment for cancer should not be performed using these data.

4. If a chemical has been adequately tested and judged not to be mutagenic (or not to be of mutagenic concern for carcinogenicity), there is α_{2u}-g accumulation in hyaline droplets, and renal neoplasia in male rats (assuming adequate testing for carcinogenic potential including the female rat and other species), but there are other tumors in the rat or tumors in other species, human relevance must be determined on a case-by-case basis considering all of the data together. A quantitative risk assessment for cancer should not be performed using the kidney tumor data in male rats, however.

5. If a chemical that causes α_{2u}-g accumulation has been judged to be mutagenic (or to be of mutagenic concern for carcinogenicity), and causes cancer at any site including the kidney, EPA's current approach for cancer risk assessment should apply.

SECTION SIX

PRESENTATIONS BY OTHER WORKSHOP PARTICIPANTS[a]

RECENT DATA FROM AN INITIATION-PROMOTION EXPERIMENT WITH 8-WEEK-OLD NBR and F344 RATS

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Primarily through the hard efforts of Dan Dietrich, a postdoctoral associate in my lab, we have completed the initiation-promotion study outlined in Figure 1 in time for this meeting. The objectives of this study were to show that d-limonene promotes renal tumors only in the presence of α_{2u}-globulin (α_{2u}-g) and that increased cell proliferation is the link between α_{2u}-g-induced nephropathy and the observed neoplasia. D-limonene was chosen because it is the gold standard for inducing α_{2u}-g nephropathy in that it is nongenotoxic and only causes male rat kidney tumors. The dose of d-limonene was the same as that used in the original 2-year carcinogenicity bioassay by the NTP: 150 mg/kg/day in corn oil by oral gavage, 5-days per week. The controls were treated with corn oil. Prior to d-limonene treatment, half of the animals of each strain were initiated with 500 ppm N-ethyl-N-hydroxyethylnitrosamine (EHEN) in drinking water for 2 weeks. The other half (initiation controls) received distilled water. We used two strains of male rats: the Fischer 344 (F344), which was used by the NTP, and the NCI Black-Reiter (NBR). The NBR rat is the only rat strain that does not synthesize the androgen-dependent hepatic form of α_{2u}-g. The Xs in Figure 1 at 7 and 32 weeks denote the cell proliferation groups. The numbers in parentheses are the numbers of animals in each of these groups. At the Xs, we implanted BrdU-filled 7-day osmotic mini-pumps to characterize cell proliferation.

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[a]This section includes a summary of three presentations given on November 13 and November 14. Because EPA did not ask the speakers to prepare formal papers, the following texts are based on tape recordings of each speaker's presentation and have been edited for clarity. Each speaker has reviewed and approved the material presented here.

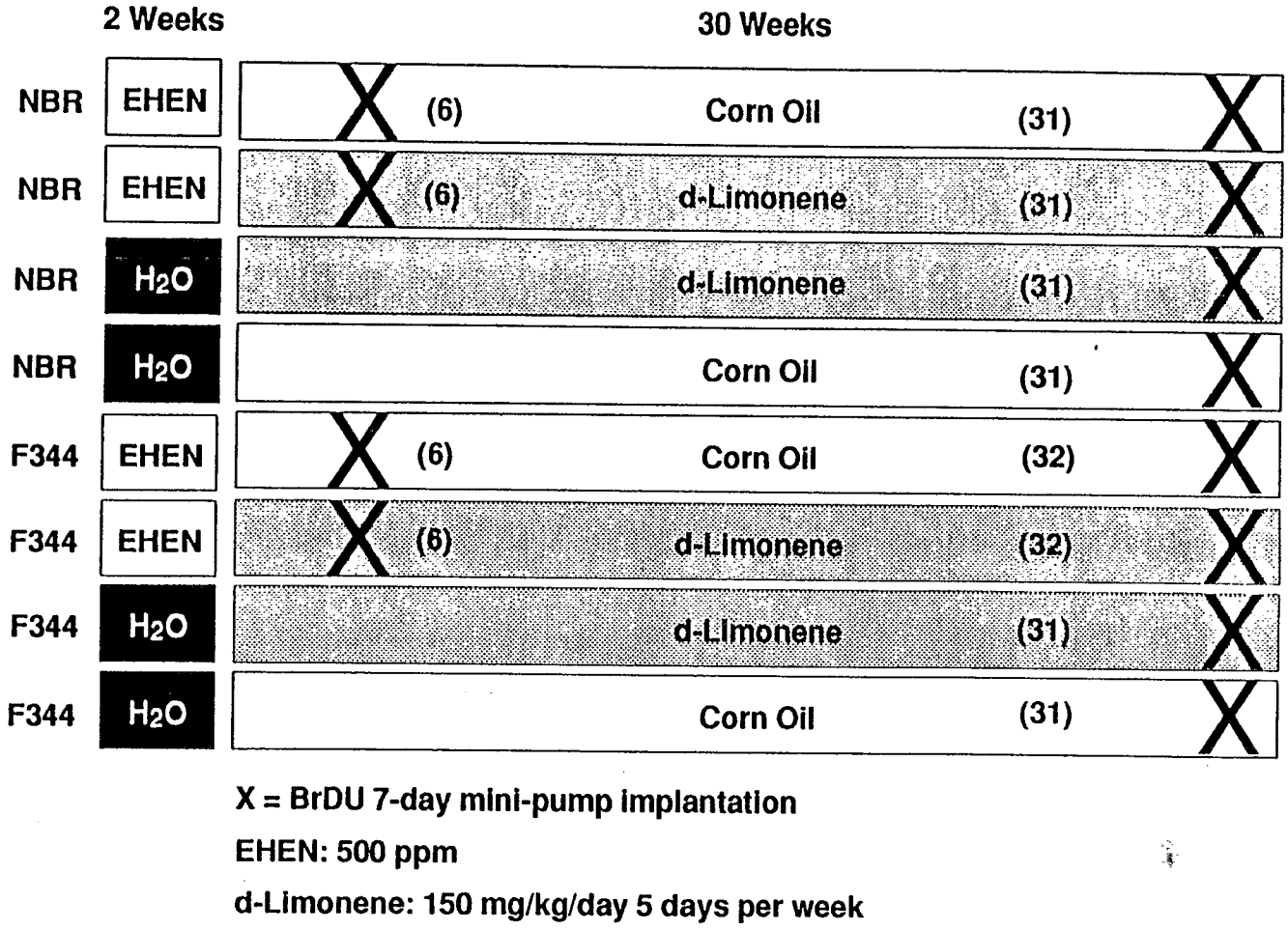

Figure 1. Initiation-Promotion Experiment with 8 Week-Old Male NBR and F344 Rats.

If we look at the water consumption and calculate from this the EHEN uptake per gram of body weight (Figure 2), you can see that both the NBR and Fischer rats received approximately the same dose of the initiating agent, EHEN.

After 7 weeks, the cell proliferation labeling index in control animals, treated for 2 weeks with EHEN and 5 weeks with corn oil, was approximately 5 percent for both strains (Table 3). However, treating male F344 rats with EHEN for 2 weeks and d-limonene for 5 weeks yielded roughly a fivefold increase in cell proliferation. The d-limonene treated NBR rats, on the other hand, which do not synthesize α_{2u}-g, did not have an increase in cell proliferation (their rates were identical to those of controls). This demonstrates that the protein-chemical interaction is responsible for the increased rates of cell proliferation.

After a total of 32 weeks, i.e., after 30 weeks of promotion, we observed sustained increases in cell proliferation in d-limonene treated F344 rats (Table 3). The F344 control rats, whether initiated with EHEN or not, again had a labeling index of about 5 percent. F344 rats treated with d-limonene, whether initiated with EHEN or not, had a fivefold increase in cell proliferation. On the other hand, NBR rats, whether initiated with EHEN or not, or promoted with d-limonene or not, had cell proliferation comparable to that of controls. This demonstrates that the protein, α_{2u}-g, is causal for the increased cell proliferation and provides the link between nephrotoxicity and cell proliferation.

We then looked at the tumors at the end of the experiment (32 weeks). We observed one adenoma in the F344 EHEN-initiated group and nine in the F344 EHEN-initiated d-limonene-promoted group (Table 4). All tumors were located in the renal cortex. None of the other groups—the noninitiated control group, the d-limonene treated F344 group, or any of the NBR groups—had tumors.

One of the questions that had been asked this morning was: "Is there linkage between preneoplastic events and carcinogenesis?" We classified two types of preneoplastic lesions: atypical tubules and atypical hyperplasia. Of these preneoplastic lesions, atypical tubules are least committed to tumor formation. Atypical hyperplasias were found predominantly in the P2 segment of the renal proximal tubule of all groups and rats strains (Table 5). However, treatment of F344 rats with EHEN and d-limonene gave rise to a tenfold increase in the total number of atypical hyperplasias. We also saw a statistically significant increase of this lesion in F344 rats treated with

Figure 2 Water and EHEN Consumption During 2 Week Initiation With EHEN

H_2O

Initiated NBR	22 ml/rat/day
Non-initiated NBR	32 ml/rat/day
Initiated F344	16 ml/rat/day
Non-initiated F344	23 ml/rat/day

EHEN

Initiated NBR	55.3 mg/kg/day
Initiated F344	58.0 mg/kg/day

TABLE 3

AVERAGE LABELING INDEX (LI) IN P2 CELLS MEASURED 7 AND 32 WEEKS AFTER THE BEGINNING OF THE STUDY

		Labeling Index (%)[a]			
Exposure Groups	**Strain**	**7 Weeks**	**n**	**32 Weeks**	**n**
EHEN-Corn Oil	NBR	5.46+/-0.51	6	5.22+/-0.39	7
EHEN-d-Limonene	NBR	5.44+/-0.44	6	5.60+/-0.45	7
H_2O-d-Limonene	NBR	n.m.[b]	-	4.66+/-0.20	7
H_2O-Corn Oil	NBR	n.m.[b]	-	4.48+/-0.32	7
EHEN-Corn Oil	F344	5.95+/-0.27	6	5.18+/-0.39	6
EHEN-d-Limonene	F344	26.19+/-1.76[c]	6	20.55+/-1.29[c]	7
H_2O-d-Limonene	F344	n.m.[b]	-	24.15+/-1.49[c]	6
H_2O-Corn Oil	F344	n.m.[b]	-	4.63+/-0.26	6

[a]Values are means +/- standard error of mean; n depicts the number of animals.

[b]n.m. = not measured.

[c]Significantly higher than F344 EHEN-corn oil or water-corn oil group, Student's t-test ($p<0.001$).

TABLE 4

INCIDENCE, TOTAL NUMBER, AND NUMBER OF RENAL ADENOMAS (RA) PER RAT IN NBR AND F344 RATS

Treatment	Strain	Incidence	%	Total RA	RA/rat
EHEN-Corn Oil	NBR	0/31	0	0	0.00
EHEN-d-Limonene	NBR	0/30	0	0	0.00
H_2O-d-Limonene	NBR	0/31	0	0	0.00
H_2O-Corn Oil	NBR	0/30	0	0	0.00
EHEN-Corn Oil	F344	1/30	3	1	0.03
EHEN-d-Limonene	F344	9/31[a]	29	11[b]	0.35
H_2O-d-Limonene	F344	0/31	0	0	0.00
H_2O-Corn Oil	F344	0/31	0	0	0.00

[a]Significantly higher than the corresponding F344 EHEN-corn oil group, Fisher's exact test ($p<0.05$).

[b]Significantly higher than the corresponding F344 EHEN-corn oil group, Student's t-test ($p<0.05$).

TABLE 5

INCIDENCE, TOTAL NUMBER, AND NUMBER OF ATYPICAL HYPERPLASIAS (AH) PER RAT IN NBR AND F344 RATS

Treatment	Strain	Incidence	%	Total RA	AH/rat
EHEN-Corn Oil	NBR	6/31	19	6	0.2
EHEN-d-Limonene	NBR	5/30	17	6	0.2
H_2O-d-Limonene	NBR	2/31	6	2	0.1
H_2O-Corn Oil	NBR	3/30	10	4	0.1
EHEN-Corn Oil	F344	20/30[a]	67	37[d]	1.2
EHEN-d-Limonene	F344	31/31[b]	100	480[e]	15.5
H_2O-d-Limonene	F344	10/31[c]	32	13[f]	0.4
H_2O-Corn Oil	F344	0/31	0	0	0.0

[a]Significantly higher than F344 water-d-limonene or water-corn oil group, Fisher's exact test ($p<0.01$).

[b]Significantly higher than F344 EHEN-corn oil, water-d-limonene, or water-corn oil group, Fisher's exact test ($p<0.001$).

[c]Significantly higher than F344 water-corn oil group, Fisher's exact test ($p<0.01$).

[d]Significantly higher than F344 water-d-limonene or water-corn oil group, Student's t-test ($p<0.001$).

[e]Significantly higher than F344 EHEN-corn oil, water-d-limonene, or water-corn oil group, Student's t-test ($p<0.001$).

[f]Significantly higher than F344 water-corn oil group, Student's t-test ($p<0.001$).

d-limonene only, compared to control animals. In the NBR rats, d-limonene treatment did not affect the incidence of atypical hyperplasia.

Atypical tubules were located primarily in the P2 segment of the renal proximal tubule, and were observed in all groups and strains in this experiment. Again, promotion of EHEN-initiated rats with d-limonene resulted in a fivefold increase in total number of atypical tubules in male F344 rats, but not in NBR rats (Table 6). A highly significant difference ($p<0.001$) can also be seen between these early lesions in F344 rats treated with d-limonene alone and the control F344 rats. For NBRs, there is an increase only in conjunction with EHEN treatment, with d-limonene treatment having no effect. I think these data are extremely important. I should point out that the genesis of this study came from a prompt at the Virginia Beach meeting by Kim Hooper, who wanted to know whether or not the protein is really causal. I believe this work makes it quite clear that the protein is causal and that there is linkage between cell proliferation and neoplasia in these animals.

In addition, we've collated some new data on human protein 1 with data on some animal proteins (Figure 3). If you look at the amounts of these proteins that are present in humans and rats, and determine the amount of protein that is going through the glomerulus on a normalized basis (i.e., per gram of kidney), you'll find that male rats have much higher amounts. In male rats, for example, the level of α_{2u}-g excreted in the urine is four orders of magnitude greater than the highest level of protein 1 excreted by human males of any age group. $Alpha_{2u}$-g is an androgen-dependent, male-rat-specific protein. Although female rats also synthesize and excrete α_{2u}-g, they do not synthesize the androgen-dependent hepatic form of α_{2u}-g. Females synthesize α_{2u}-g primarily in the salivary glands and excrete it at concentrations 1/100th or less than what is found in the urine of male rats. Even though female rats excrete α_{2u}-g, they do not develop α_{2u}-g nephropathy, display increased rates of cell proliferation, or develop treatment-related renal tumors. I think this is fairly strong evidence for a threshold-type activity. In humans, the protein levels are much lower than those observed in female rats. The final important point that I would like to make, which hasn't been made earlier today, is that a number of these proteins that are present in humans are also present in female F344 and male NBR rats, and despite the presence of these proteins these rats do not develop protein-associated nephropathy or cancer.

Figure 3 Comparative Urinary Excretion of alpha2u-Globulin Superfamily Proteins

Protein		Source	Serum (mg/L)	Excreted in Urine [a] (mg/day)	(mg/day/g kidney)
Retinol binding protein		Human	36-58	0.04-0.22	0.0001-0.0007
Protein HC		Human	14	-	-
Apolipoprotein D		Human	62	-	-
alpha1-Acidglycoprotein		Human	500-1500	0.18-0.67	0.0006-0.002
Protein 1	male (05-10y)	Human	-	0.006	0.00002
	male (10-15y)	Human	-	0.035	0.0001
	male (15-20y)	Human	-	0.162	0.0005
	male (20-60y)	Human	-	0.037	0.0001
alpha2u-Globulin		male Rat	23-37	3-19	0.45-8.64
		female Rat	0.6	0.028	0.0125

a: average daily urine excretion rate: 20 ml/kg/day (Humans); 55 ml/kg/day (Rats)
average body weight: 70 kg (Humans); 0.25 kg (Rats)
average kidney weight: 300 g (Humans); 2.2 g (Rats)

TABLE 6

INCIDENCE, TOTAL NUMBER, AND NUMBER OF ATYPICAL TUBULES (AT) PER RAT IN NBR AND F344 RATS

Treatment	Strain	Incidence	%	Total AT	AT/rat
EHEN-Corn Oil	NBR	27/31	87	287[b]	9.3
EHEN-d-Limonene	NBR	27/30	90	241[b]	8.0
H_2O-d-Limonene	NBR	26/31	84	143	4.6
H_2O-Corn Oil	NBR	27/30	90	140	4.6
EHEN-Corn Oil	F344	30/30[a]	100	469[c]	15.1
EHEN-d-Limonene	F344	31/31[a]	100	2019[d]	65.1
H_2O-d-Limonene	F344	30/31[a]	97	330[c]	10.7
H_2O-Corn Oil	F344	21/31	68	103	3.3

[a]Significantly higher than F344 water-corn oil group, Fisher's exact test ($p<0.01$).

[b]Significantly higher than NBR water-d-limonene or water-corn oil group, Student's t-test ($p<0.01$).

[c]Significantly higher than F344 water-d-limonene or water-corn oil group, Student's t-test ($p<0.01$).

[d]Significantly higher than F344 EHEN-corn oil, water-d-limonene, or water-corn oil group, Student's t-test ($p<0.001$).

[e]Significantly higher than F344 water-corn oil group, Student's t-test ($p<0.001$).

THE EPIDEMIOLOGY OF RENAL CELL CANCER

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Renal cell cancer accounts for about 70 percent of the total renal cancers (ICD 189) found in adults (not the 85 to 90 percent that is suggested in the review). Of the remaining kidney tumors, 15 percent are from the renal pelvis, 8 percent are from the ureter, 4 percent are from the urethra, and 3 percent are others. There are two different types of kidney tumors in adults: adenocarcinomas (i.e., renal cell cancers)—our present topic—and transitional cell tumors (i.e., renal pelvis, ureter, and urethra cancers). The epidemiology of these two types of tumors is different. Transitional cell tumors of the ureter and renal pelvis are similar to bladder cancer in their epidemiology and risk factors.

Since about 1970, renal cell cancer has risen about 2 percent a year among most races and sex groups in the United States. (This rate of increase is a little less than that suggested in the review, because the rate of increase of renal pelvis and ureter cancer is closer to 3 or 4 percent per year). Among American white males, the incidence rate of renal cell cancer is 8.4 per 100,000 not the 10 or 11 suggested in the review. The rate is very similar for blacks and whites. For black males, the rate is 8.6 per 100,000. Female rates are about half those of males. For white females, the rate is 3.7; for black females, the rate is about 3.8. (The data on incidence rates that I have been presenting have not been published. Published SEER data, which is the main source for incidence data in the U.S., do not distinguish between renal cell and renal pelvis and ureter cancers.) Rates are similar among Hispanics and Asians in the U.S.; their rates are very low. Internationally, Scandinavian countries have the highest rates. In Iceland and Sweden, the rates among males were a little over 9 per 100,000, which are not much higher than those in the U.S. Areas of the world with low rates include China, Japan, and Central and South America.

The relative survivorship for renal cell cancer is about 50 percent at 5 years. In fact, if it is diagnosed reasonably early and the kidney is removed, the patient is usually considered cured.

By contrast, the 5-year relative survivorship of transitional cell tumors of the renal pelvis and ureter is about 70 percent, which is very similar to the survivorship for bladder cancer.

There are two established risk factors for renal cell cancer: cigarette smoking and obesity. Cigarette smoking has been consistently identified as a risk factor in most case-control and cohort studies. Cohort studies are problematic, since 20 to 30 percent of the tumors are likely to be transitional cell tumors, not renal cell tumors. Mortality data are, therefore, somewhat misleading because they include transitional cell tumors (i.e., renal pelvis and ureter tumors), which are more strongly related to cigarette smoking.

In most of the renal cell cancer case-control studies a weak-to-moderate association with cigarette smoking was found. For people who have never smoked cigarettes, the risk of developing renal cell cancer ranges from about 1.2 to 2; for heavy smokers, the risks range from slightly over 2 to almost 3. A dose-response relationship has been demonstrated in the larger and better conducted case-control studies. In the largest case-control study published to date, a significant decline in risk associated with the number of years that an individual had stopped smoking was observed. This is persuasive evidence that there is probably a causal association between renal cell cancer and cigarette smoking.

Obesity, or high relative weight, which is usually measured by a body mass index (w/h^2), has been associated with renal cell cancers more consistently than any other risk factor. Regardless of their scope or degree of precision, all known studies of this topic have concluded that increased relative weight brings increased risk. When a body mass index distribution is derived and divided into quartiles, the risk in the upper quartile is about 3. In the top 10 percent, that risk can go up to five- or sixfold. The risk is higher in women than in men; all studies have found the increased risk in women, and some studies have found it in men.

Based on animal studies in which Syrian golden hamsters developed renal tumors when administered estrogens, it is thought that obesity in humans results in higher levels of endogenous hormones that may increase kidney cancer risk. In studies of humans, no association between renal

cell cancer and estrogens (i.e., birth control pills or post-menopausal estrogens) or reproductive history has been found. This is another example of an association found in animals but not in humans.

Another major area of risk is medication usage, especially use of analgesics. Phenacetin-containing analgesics, which are no longer available in the United States or in most other countries, are associated with a very high risk for renal pelvis tumors. Starting in the early 1980s, a number of case-control studies reported associations between the use of phenacetin-containing analgesics and adenocarcinoma of the kidney. (More recently, these drugs have been associated with bladder cancer.) Heavy phenacetin use is a moderate risk factor for renal cell cancer and a very strong risk factor for transitional cell tumors of the lower urinary tract; the observed risks for renal cell cancer are approximately two- to threefold.

An added concern is that renal cysts and acquired cystic disease increase the risk of renal cell cancer. This risk, however, may be overstated since some patients on renal dialysis were also analgesics abusers, and that is frequently overlooked in their history.

Aspirin- and acetaminophen-containing analgesics do not appear to be associated with renal cancer, although a recent cohort study published this year suggested that regular use of aspirin increases the risk of renal cell cancer. This study, which was of retired people in the Los Angeles area, was based on only six cases of renal cell cancer. (Renal cancer is not a very common cancer, so cohort studies rarely have more than a handful of cases.) In addition, this study did not adjust for cigarette smoking, relative weight, or other potential confounders such as diuretics usage, which is common among the elderly and has been associated in other epidemiologic studies with a high relative risk of renal cell cancer.

Two published epidemiologic studies reported very high risk associated with the use of diuretics. I personally reviewed unpublished studies showing the same association, so I think there is a real association. Whether it is a causal relationship or not remains to be seen, but diuretics are a risk factor of approximately five- or sixfold. The issue is somewhat complicated because diuretics are used to treat hypertension, so it is important to determine when the hypertension started and why it developed. A further complication is that hypertension can accompany kidney cancer

because as the tumor grows, it displaces tissue in the parenchyma, inducing hypertension. It is important to determine in each case when and for what purpose the use of diuretics began.

A study conducted in Minnesota concluded that, of the people who had used diuretics (most of whom were women), those who were not hypertensive had the highest risk of renal cancer—about a sixfold increased risk. Since these women were not diagnosed with high blood pressure, they were obviously using diuretics for other reasons. Unfortunately, the study did not inquire into the reason for use.

Animal studies on the two major diuretics used in the United States and in other parts of the western world—hydrochlorothiazide and furosemide—have been conducted. The results are suggestive of an excess of renal tumors and liver cancers in mice and rats.

Occupation also has been studied as a potential risk factor. Unlike bladder cancer, renal cell cancer is not considered an occupationally induced tumor. Although there are a number of citations of occupational studies in your review, these studies are not equal; some are better than others.

An association between occupational exposure to asbestos and renal cancer was consistently found in two or three reasonably well-done cohort studies and in one case-control study. Autopsy studies have found asbestos fibers in the kidney.

Another consistent occupational association in the literature has been among laundry and dry cleaning workers. Proportional mortality studies have shown an increased risk of renal cancer among workers in this industry. However, these were proportional mortality studies or PMR studies; a PMR study is not a very good study design, as it is prone to a number of biases. This year, my colleague Aaron Blair and his coworkers have published the largest study of laundry and dry cleaning workers to date. It was a straightforward cohort study in which they calculated Standard Mortality Ratios (SMRs). So, the study design was better, and it was a bigger study, but no association was found. (The SMR was 0.5.)

Associations between kidney cancer and other occupations are frequently mentioned. An association between coke oven workers in the steel industry and kidney cancer has been found in only one study, but it is often included in reviews. Other studies have not been able to replicate that association. Cadmium and lead are two additional agents thought to have the kidney as a target organ. However, epidemiologic studies have reported no excess of renal cancer among workers exposed to cadmium or to lead, even though inorganic lead is associated with kidney cancer in animals.

More than 20 cohort studies of petroleum or oil refinery workers have been conducted. Overall, an excess of kidney cancer has not been observed. The International Agency for Research on Cancer (IARC) has concluded that the data are insufficient to establish a causal link; I would agree. Your review mentions Wong and Raabe's review. They conducted a meta analysis of 147 renal cancer deaths (which is a large number of renal cancer deaths) aggregated from a number of cohort studies, and found an SMR of 0.98. So there's not much evidence that supports an excess renal cancer among petroleum or refinery workers.

Gasoline exposure has also been investigated as a risk factor in kidney cancer. I think it was MacFarland who published in the early 1980s a paper presenting the results of a 2-year study of rats and mice exposed to unleaded gasoline. After that, gasoline came under suspicion and several studies were conducted. As far as I know, there are only two positive studies, one of which is included in your review.

In 1980, we conducted a very large population-based case-control study in Minnesota in which we obtained a complete occupational history. We asked industrial hygienists to review the occupational codes and estimate potential exposures to various petroleum products. When we analyzed the data, we found no association with any type of petroleum product. But when we looked at the occupational code for gasoline service station attendants, we found a suggestive upward trend in risk with duration of employment. The results were as follows:

Service Station Attendants

Work History	95% Confidence Limits	Odds Ratio
Never worked as a service station attendant	—	1.0
Ever worked as a service station attendant	0.6 - 2.3	1.2
Worked 1 to 2 years	0.3 - 2.4	0.9
Worked 3 to 5 years	0.3 - 4.5	1.3
Worked more than 5 years	0.4 - 6.5	1.7

None of these individual odds ratios was statistically significant as a point estimate. The trend was not statistically significant, either. But we did find that duration of employment increased risk. The lower bound for the highest odds ratio is 0.4, because it is based on 6 cases and 5 controls; it is difficult to make very much of this 70 percent excess risk because of this lower bound. Unfortunately for epidemiologists, being a gasoline station attendant is rarely a career position, so we found very few people who were employed as gasoline station attendants for more than a few years. These results were adjusted for cigarette smoking and weight. Exposure to gasoline in this study was assured, since these were gasoline station attendants. However, the results were based on small numbers; there were only 20 cases and 23 controls who ever worked as service station attendants. So it is weak evidence at best.

The other study is of British workers who distributed petroleum products. In the 1982 published report no excess risk for kidney cancer was observed; the SMR was 1.2 based on over 15 cases of death from renal cancer. Since then, someone analyzed a subset of this population, and, although it has never been published, Wong and Raabe had access to it in their review. As far as I'm aware, a subset of gasoline truck drivers had a suggestive increase in risk that was not statistically significant. However, what you see reported in your review is that for a subset of the

subset, men 55 to 65 years of age, a statistically significant SMR of 1.89 was observed. Unfortunately, I don't know much about this particular analysis, as it has never been published.

There is one further suggestive study—that of Siemiatycki. His hypothesis-generating study, which was conducted in Canada on aviation gasoline, inferred an increased risk for renal cell cancer. He looked at 12 different exposures and 20 different cancers, and he found a couple of provocative things. One problem with the study is that Siemiatycki used cancer controls.

Let me say a few things about the rest of the studies. In terms of increased risk associated with gasoline, other U.S. case-control studies have reported no association. There have been at least three nested case-control studies of petroleum workers. To conduct these studies, epidemiologists identify a cohort of petroleum workers, determine all the people who died of kidney cancer, carry out a nested case-control study, and compare the cases who had kidney cancer versus other people in the cohort. All of them were negative for gasoline exposure.

Finally, there's the Poole study. Charles Poole, Rothman, and others completed a study for the American Petroleum Institute. I have never seen the paper, and the first time I heard of it was in reading the review. It is presented as a case-control study in which the cases were derived from a large number of combined cohorts. I don't know if this is really a nested case-control study within a group of cohorts. Since I'm not sure exactly how it was done, I'm uncomfortable commenting on it. I prefer not to comment on the results because, as I recall, they used a 90 percent confidence limit, which exaggerates the potential for statistical significance. Furthermore, I'm not sure what the authors' conclusions were.

There is a new generation of case-control studies of renal cell cancer now being conducted in the United States, Sweden, Denmark, Germany, Australia, and China. These studies will specifically look at the issue of gasoline as an exposure and service station attendants as an occupation. Since these studies use basically the same protocol and questionnaire, their results can be pooled, so there will be thousands of renal cell cancer cases to compare to population-based controls. The IARC has concluded in one of their volumes on carcinogenesis that the evidence concerning gasoline exposure in humans is inadequate. The new case-control studies should help fill this data gap.

Let me just say one further thing. One of the advantages of animal studies (although after sitting through yesterday's meeting, I'm not sure how many advantages there are anymore) is randomization; the animals are randomly assigned the exposure. When you randomize between two groups, exposed and unexposed, known and unknown biases should be randomly distributed between the two groups, and they should be more or less balanced. In epidemiologic studies we can't assign exposures; it is usually unethical. So, we normally do not have the luxury of randomization, and therefore cannot randomize known and unknown bias. Thus, the potential for bias, confounding, and chance have to be very critically evaluated in each study.

However, as the report correctly concludes, one cannot exclude a weak risk of, say, 1.2 to maybe 1.4, or even 1.5. That is, we can't exclude a 15 to 40 percent excess risk. In epidemiology, it is difficult to exclude a weak association with any confidence, because the lower the risk ratio and the closer it gets to 1, the more important bias, confounding, and chance become. If the risk ratio is consistently found to be in the range of 3 to 5, it is hard to explain it away on the basis of bias, uncontrolled confounding, or chance association. But risks of 1.2 to 1.3 or 1.4 could be explained away by uncontrolled confounding or bias in the study design. The strength of the association is a very important issue.

In my opinion, the case-control studies of this cancer site have more or less consistently identified weak-to-moderate associations with cigarette smoking and phenacetin usage, and a moderate-to-strong association with obesity. But an association with gasoline exposure has not been convincingly demonstrated.

THE RELATIONSHIP OF HYALINE DROPLETS AND RENAL CELL CANCER IN HUMANS

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I will present the current state of knowledge regarding the normal kidney, hyaline droplets, and renal cell cancer. As mentioned in the report, the normal proximal tubule of the human has the same sort of segmentation as that of the rat. In the rat and related rodents, the demarcation of nephron segments is very clear. The kidney zones can even be seen grossly and identified by the locations of P1, P2, and P3. This is not the case in the human or similar primates where the P3 segment, in particular, is not as long or as well developed. In humans, no known established metabolic differences between males and females are known to exist. In contrast, male and female rats have lysosomal differences, as well as many morphological and biochemical differences in the endoplasmic reticulum, the peroxisomes, and in various oxidative enzymes of intermediary metabolism; the male and the female rat kidney proximal tubules are really very different.

Hyaline droplets of similar morphology to those seen in rats are very common in human renal disease. They almost always occur with either infusion or release of a filterable protein into the plasma. Hyaline droplets in humans are associated with glomerular disease where the glomerular capillary wall is leaking. Examples of this include glomerulonephritis and, most prominently, the chronic nephrotic syndrome in children and adults where over a relatively long period of time, a continual leak of plasma proteins, such as albumin and lipoproteins, leads to a tremendous overload of the proximal tubule phagolysosomes. This overload resembles what you see in rats exposed to CIGAs. In humans, however, the proteins have a higher molecular weight and are normally nonfilterable. No association has ever been suggested between the accumulation of such proteins and renal cell carcinoma. But, of course, individuals with this overload of protein are medically treated, removing the hyaline droplets and excess proteins. In addition, the type of karyomegaly that's so characteristic in the P2 and P3 segments of rats exposed to carcinogens is very unusual or does not occur in human proximal tubules.

In humans, the other commonly observed cause of nephropathy is intravascular hemolysis, which arises either from disease or from a blood transfusion reaction. In persons with this condition, hemoglobin (sometimes myoglobin) is filtered, forming hyaline droplets containing hemoglobin and/or myoglobin.

In autopsy studies and in studies we have done on the kidneys of individuals with surgical resection for renal cell carcinoma, we have found a relatively high incidence of small adenomas (10 to 20 percent, depending on the number of sections and the depth of investigation). Areas of atypia and hyperplasia also have been seen. The classification of these tumors has been a dilemma for years in surgical pathology, which developed the size rules: if it is less than 3 centimeters it is an adenoma; if it is greater it is a carcinoma. In fact, cytologically, these small tumors can be indistinguishable from big ones, and tumors less than 2 centimeters in size have metastasized in humans. I believe that these small tumors often represent carcinomas in situ; they're just growing very slowly. The same phenomenon occurs in the rat. If you give rats a high dose of a mutagenic carcinogen that produces high tumor incidence, such as nitrosamines or fluorobiphenylylacetamide (which we have studied), there will be hundreds or thousands of hyperplasias, adenomas, carcinomas in situ, etc., representing all stages of the progression. I think that, in all probability, these are just very slow growing lesions that do not cause a problem over a typical rodent lifetime.

I took part in a study of renal cell carcinomas conducted in Oklahoma for the American Petroleum Institute.[a] The pathology slides were divided into two subsets: dry cleaning workers who were still using carbon tetrachloride, and a group of inpatients with no history of such exposure. All of the slides were blinded, and I and two other pathologists looked at them. We set up hypotheses based on the previous findings in the rat. It was a very extensive study of the preneoplastic lesions and other toxic lesions, including all the things we've talked about in the rat. When it was completed, no differences could be found between the two groups. In other words, there was no difference in any kind of preneoplastic or other lesion in the workers with a history of exposure.

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[a]Pitha, J.V., Hemstreet, G.P., III, Asal, N.R., Petrone, R.L., Trump, B.F., and Silva, J.V. (1987). Occupational hydrocarbon exposure and renal histopathology. Toxicol. Ind. Health. 3, 491-506.

APPENDIX A

LIST OF TECHNICAL PANEL MEMBERS

U.S. Environmental Protection Agency

PEER REVIEW WORKSHOP
ALPHA-2u-GLOBULIN: ASSOCIATION WITH RENAL TOXICITY AND NEOPLASIA IN THE MALE RAT

Gaithersburg Marriott
Gaithersburg, MD

November 13-14, 1990

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APPENDIX B

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PEER REVIEW WORKSHOP
ALPHA-2u-GLOBULIN: ASSOCIATION WITH RENAL TOXICITY AND NEOPLASIA IN THE MALE RAT

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