

# **Species Differences in Thyroid, Kidney and Urinary Bladder Carcinogenesis**

## International Agency For Research On Cancer

The International Agency for Research on Cancer (IARC) was established in 1965 by the World Health Assembly, as an independently financed organization within the framework of the World Health Organization. The headquarters of the Agency are in Lyon, France.

The Agency conducts a programme of research concentrating particularly on the epidemiology of cancer and the study of potential carcinogens in the human environment. Its field studies are supplemented by biological and chemical research carried out in the Agency's laboratories in Lyon and, through collaborative research agreements, in national research institutions in many countries. The Agency also conducts a programme for the education and training of personnel for cancer research.

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## Cover

Depiction of the ligand-binding cavities in  $\alpha_{2u}$ -globulin (blue cloud) and mouse urinary protein (MUP) (red cloud) as determined by X-ray crystallography. For  $\alpha_{2u}$ -globulin, the shape of the binding cavity is essentially spherical, resulting from the orientation of two phenylalanine residues which close off the back of the cavity. The two phenylalanine residues in  $\alpha_{2u}$ -globulin are replaced by leucine and alanine, respectively in MUP, resulting in a binding cavity that is elongated and flattened (red cloud) relative to the binding pocket in  $\alpha_{2u}$ -globulin. Although the binding cavities of the two proteins are similar in volume, the actual overlap in shape (the highlighted, pink region) is very small. The spherical binding cavity in  $\alpha_{2u}$ -globulin is far less restrictive, thereby allowing for a broad spectrum of chemicals to bind to the protein.

# **Species Differences in Thyroid, Kidney and Urinary Bladder Carcinogenesis**

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# Foreword

In the absence of adequate human cancer data, it is biologically plausible and prudent to regard agents for which there is sufficient evidence of carcinogenicity in experimental animals as if they presented a carcinogenic risk to humans. However, the possibility that an agent causes cancer in animals through a mechanism that does not operate in humans must be taken into account. During recent years, evidence has developed that certain neoplasms commonly seen in bioassays for carcinogenicity in rodents can develop through such species-specific mechanisms. These include urinary bladder carcinomas associated with urolithiasis and with certain urinary precipitates; renal cortical neoplasms arising specifically in male rats in association with alpha-2 urinary ( $\alpha_{2u}$ ) globulin nephropathy, and thyroid follicular-cell tumours associated with imbalances in thyroid stimulating hormone levels. All of these conditions involve persistent hyperplasia in specific cell types from which neoplasms arise.

This publication originates from a Workshop held in Lyon during 3-7 November 1997, which was attended by invited experts from different countries, to consider how rodent tumours of urinary bladder, renal cortex, and thyroid gland should be treated within the *IARC Monographs on the Evaluation of Carcinogenic Risks to Humans*. The main product of the workshop was the Consensus Report, which is published in the first part of this volume, and was agreed by all participants. Each participant also prepared and presented an authored paper, which includes the views of the authors and also reflects the outcome of specialty group discussions held during the meeting.

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**P. Kleihues**  
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# Species Differences in Thyroid, Kidney and Urinary Bladder Carcinogenesis

Lyon, 3-7 November 1997

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# Consensus Report

## Introduction

The IARC *Monographs* programme is an international consensus approach to the identification of chemicals and other agents that may present carcinogenic hazards to humans. The *Monographs* assess the strength of the published scientific evidence for such identifications, which are based primarily on epidemiological studies of cancer in humans and bioassays for carcinogenicity in mice and rats. Information that may be relevant to the mechanisms by which the putative carcinogen acts is also considered in making an overall evaluation of the strength of the total evidence for carcinogenicity to humans.

Following a meeting on mechanisms of carcinogenesis<sup>1</sup> held in 1992, a series of IARC publications have dealt with specific topics on generic mechanisms of carcinogenesis that are relevant to overall evaluations of carcinogenic hazards of certain groups of chemicals to humans. These include reports on *Peroxisome Proliferation and its Role in Carcinogenesis* in 1995<sup>2</sup> and *Mechanisms of Fibre Carcinogenesis* in 1996<sup>3</sup>.

In the IARC *Monographs* programme, an agent with *sufficient evidence* of carcinogenicity in experimental animals and *inadequate evidence* of carcinogenicity in humans will ordinarily be placed in the category *Group 2B – the agent (or mixture) is possibly carcinogenic to humans*. When there is strong evidence that carcinogenesis in experimental animals is mediated by mechanisms that do operate in humans, the agent may be upgraded to *Group 2A – probably carcinogenic to humans*. However, the classification scheme allows for down-grading into *Group 3 – the agent (or mixture) is not classifiable as to its carcinogenicity to humans*

if there is strong, consistent evidence that the mechanism of carcinogenicity in experimental animals does not operate in humans or is not predictive of carcinogenic risk to humans.

Numerous agents that are carcinogenic to the thyroid follicular epithelium, renal cortical epithelium or urinary bladder urothelium in experimental animals, humans or both have been evaluated in the IARC *Monographs* (see Appendix 1). A recurring theme is that both clearly genotoxic and some apparently non-genotoxic agents cause tumours in each of these tissues in experimental rodents. A second recurring theme is that, in bioassays for carcinogenicity in rodents, tumours in these tissues are often accompanied by tumours at other sites.

As a general rule, tumour morphology in rodents is similar for tumours at a given site, irrespective of the nature of the inducing agent. Thus, agents that may be acting by fundamentally different carcinogenic mechanisms may not be distinguishable by histopathology alone. However, in some cases, carcinogenic activity has been detected only in the thyroid follicular epithelium in rodents in association with a defined hormonal mechanism, in the male rat renal cortex in the presence of  $\alpha_{2u}$ -globulin<sup>4</sup>-associated nephropathy or in the urinary bladder in rodents in the presence of urinary precipitates or calculi. The predictive value of tumours arising in any of these circumstances may be different from that of histologically similar neoplasms that arise in the same organs in rats or mice when these mechanisms or processes do not operate.

This IARC workshop was held in Lyon on 3–7 November 1997 to examine the scientific basis for possible species differences in mechanisms by which thyroid follicular-cell tumours in mice and

<sup>1</sup>Vainio, H., Magee, P.N., McGregor, D.B. & McMichael, A.J., eds (1992) *Mechanisms of Carcinogenesis in Risk Identification* (IARC Scientific Publications No. 116), Lyon, IARC

<sup>2</sup>IARC (1995) *Peroxisome Proliferation and its Role in Carcinogenesis. Views and Expert Opinions of an IARC Working Group* (IARC Technical Report No. 24), Lyon, IARC

<sup>3</sup>Kane, A.B., Boffetta, P., Saracci, R. & Wilbourn, J.D., eds (1996) *Mechanisms of Fibre Carcinogenesis* (IARC Scientific Publications No. 140), Lyon, IARC

<sup>4</sup>The protein is a urinary globulin and is properly designated  $\alpha_{2u}$ -globulin (Roy, A.K. & Neuhaus, D.W. *Proc. Soc. Exp. Biol. Med.*, **121**, 894–899, 1966). It is not a micro( $\mu$ )-globulin, as the term  $\alpha_{2u}$ -globulin would suggest and this latter designation which has sometimes been used for the urinary globulin is to be avoided.

rats, renal tubule-cell tumours in male rats and urinary bladder tumours in rats may be produced. The workshop also addressed the predictive value of these tumours for the identification of carcinogenic hazards to humans when they occur alone and when they occur along with tumours in other organs. The workshop did not formally evaluate the carcinogenic hazard posed by any agent; such evaluations are undertaken in the context of the *IARC Monographs* programme.

In the following sections, etiological risk factors in humans and experimental animals as well as the hypotheses underlying the proposed species-specific mechanisms for each of the above-mentioned tumour types are summarized. The applicability of the underlying mechanisms to humans is also discussed, and current gaps in knowledge are highlighted. Finally, for each tumour type, recommendations are presented on how the mechanistic data could be used in the overall evaluation of carcinogenicity to humans.

The Consensus Report uses the background scientific reviews that were prepared before the workshop by individual participants. These individual authored papers formed the basis for the discussions that were held and are included in the second part of this volume.

## **Follicular-cell neoplasms of the thyroid**

### ***Human thyroid follicular-cell tumours***

#### ***Incidence, etiology and pathology of thyroid tumours in humans***

Thyroid cancer represents about 0.5% of cancers recorded among men and about 2% of those recorded among women. In most countries, incidence rates are below 2 per 100 000 men and 4 per 100 000 women. Mortality rates are 5–10-fold lower than incidence rates, especially among women. There is evidence that in the last three decades mortality has been slowly decreasing, while the recorded incidence has been increasing in most developed countries (see Franceschi & Dal Maso, this volume).

Ionizing radiation is the only known human thyroid carcinogen; a history of adenoma and goitre are established risk factors for thyroid carcinoma. The evidence for other factors, including iodine deficiency and iodine excess, hormonal and reproductive factors, is less consistent. Wide

variations exist in the clinical behaviour of thyroid cancer, depending upon the histological type.

Benign thyroid tumours derived from follicular cells are relatively common in humans, while carcinomas are uncommon. Tumours derived from C cells (medullary carcinoma) are not included in this discussion. Carcinomas are subdivided into differentiated tumours, which retain both structural and functional follicular cell differentiation, and the undifferentiated tumours, which lack these features. Undifferentiated carcinomas are rare, rapidly progressive tumours. Differentiated carcinomas form up to 90% of clinically detected thyroid carcinomas and can be divided into two main groups: papillary carcinoma and follicular carcinoma. This classification was originally based on the dominant architectural pattern of the tumour, but it is now applied to describe tumours that are characterized by differing cytology, encapsulation, distribution of metastases and molecular biology; architectural pattern is of less importance in classification. Small papillary carcinomas, formerly called occult, are relatively frequent, but of little clinical significance. It seems likely that no more than a small proportion of microcarcinomas progress to clinically significant tumours. In the past, these microcarcinomas were usually found incidentally in surgical specimens or at autopsy. They are now increasingly found using ultrasound or other techniques.

The differing histological types occur at different frequencies in different countries, with Iceland and Hawaii showing the highest incidence of thyroid carcinomas, 80% of which are papillary carcinomas. These countries have a high dietary iodine intake, but it is not clear whether this plays a role in the high thyroid cancer incidence. Follicular carcinomas are proportionally more common in countries with low iodine intake, and the ratio of papillary to follicular carcinoma has changed in several countries after iodination of the diet. It is not certain, however, that the increasing incidence of papillary carcinoma is causally related to an increase in dietary iodine, as there has been a general trend for papillary carcinomas to increase for several decades. Incidence figures are influenced by several factors, including changing diagnostic techniques. In patients with congenital defects in thyroid hormone synthesis, with consequently elevated levels of thyroid-stimulating

hormone (TSH), follicular adenomas are common and cancer, when it occurs, is almost always follicular in type. It therefore seems likely that high circulating levels of TSH in humans are associated with an increased incidence of tumours of the follicular type.

The role of other hormonal factors (see Franceschi & Dal Maso, this volume) has been suspected on the basis of the high female-to-male incidence ratio and the rapid rise in the incidence of thyroid cancer (largely papillary carcinoma) in females at puberty. Ten published case-control studies from the USA, Europe and Asia have studied the influence of reproductive and menstrual factors and use of exogenous female hormones on the incidence of thyroid cancer. A moderately increased risk was seen in parous women and in those who underwent an early menopause or who used oral contraceptives. The level of risk diminishes with time after these events or cessation of oral contraceptive use. Diagnostic ascertainment bias may play a role, since the majority of thyroid carcinomas in young women are detected as a consequence of medical examination for other reasons (e.g., oral contraceptive use, premenopausal complaints).

#### *Mechanistic considerations in humans*

Papillary carcinoma appears to arise directly from the follicular cell without any precursor lesion; it is the major type of thyroid cancer induced by radiation exposure and there is no evidence that it is causally linked to elevated TSH. Rearrangements of tyrosine kinase receptor genes (*ret* or *trk*) occur in a proportion of cases of papillary cancer and *ret* rearrangements have been identified in papillary microcarcinomas (see Thomas & Williams, this volume).

Follicular carcinoma appears to arise by progression from follicular adenoma. *Ras* mutations have been found in a proportion of follicular cancers, whereas tyrosine kinase receptor gene rearrangements have not been detected. The relationship between incidence of follicular carcinoma and iodine intake and the association of follicular but not papillary carcinoma with dys-hormonogenesis suggest that prolonged TSH elevation may be associated with an increased risk of follicular carcinoma in humans. This effect does not appear to be large. The effect of TSH is mimicked in patients with Graves' disease with circulating autoantibodies

that bind to the TSH receptor; these patients are often treated with drugs such as methimazole, which may also cause a TSH increase. There is no evidence of a substantial increase in frequency of thyroid cancer in these patients, suggesting that short-term elevation of TSH in adults is not relevant to the incidence of human thyroid carcinoma.

In humans, ionizing radiation induces mostly papillary carcinomas. Children are much more sensitive to the thyroid carcinogenic effects of ionizing radiation than are adults.

#### **Thyroid follicular-cell tumours in rodents**

##### *Incidence, etiology and pathology*

Spontaneous thyroid tumours derived from the follicular cell occur in 1–3% of laboratory rats and mice (adenomas and carcinomas combined in a variety of strains of rats aged two or more years). In general, male rats show a higher incidence of follicular-cell tumours than female rats (see Thomas & Williams, this volume).

In rodents neoplasms derived from the thyroid follicular cell are not subdivided on the basis of pathological and molecular biological criteria into papillary and follicular types as in human patients. Thyroid tumours in rodents are usually follicular in architecture and do not possess the morphological or cytological features that are considered to be diagnostic for papillary thyroid carcinoma in humans. Most thyroid neoplasms in rodents that develop spontaneously or following exposure to xenobiotic chemicals are well demarcated lesions composed of variably sized colloid-containing follicles lined by hyperchromatic follicular cells. There often is evidence of progression of focal hyperplastic lesions to follicular cell adenomas and occasionally carcinomas. In contrast, the thyroid tumours that develop in mice transgenic for the human papillary carcinoma oncogene (*ret/PTC1*) do show morphological features seen in human papillary carcinomas (papillary infoldings, frequent nuclear grooves and intranuclear cytoplasmic inclusions). In comparison to the human tumours, little is known about the molecular biology of rodent thyroid tumours.

The thyroid follicular cell is one of the more common target sites for tumorigenesis in long-term toxicological studies in rats. Both genotoxic and non-genotoxic agents have been shown to induce thyroid follicular-cell tumours. Non-genotoxic agents can be divided into those which have

effects directly on the thyroid (blocking uptake of iodine into the follicular cell, e.g., perchlorate; inhibiting thyroid peroxidase, e.g., thioureas, etc.; or inhibiting hormone release, e.g. lithium) and those which have effects on thyroid hormone catabolism and excretion (e.g., agents such as lupidine which increase uptake into the hepatic cell or those such as phenobarbital which increase thyroid hormone loss from the liver through enzyme induction). The only known common pathway through which these agents act is the pituitary-thyroid feedback mechanism involving TSH. Among the genotoxic agents that cause thyroid tumours, some (e.g., certain dianilines) may also affect thyroid homeostasis.

A group of agents that induce thyroid follicular tumours by a non-genotoxic mechanism are also associated with the induction of liver tumours, although not necessarily in the same species (see McClain & Rice, this volume).

Radiation (exposure to either external radiation or to isotopes of iodine) has been shown to induce thyroid tumours in both rats and mice. At high doses, the mechanism of radiation thyroid carcinogenesis may involve not only genotoxic activity but also an effect through interference with thyroid hormone homeostasis leading to an increase in plasma TSH. Experimental evidence from rats shows a bell-shaped rather than a linear dose-response curve at high radiation levels, with the dose which gives the peak tumorigenic response in the rat thyroid lying within the range which demonstrably interferes with thyroid function (see Thomas & Williams, this volume).

#### *Mechanistic considerations in rodents*

The role of TSH-induced growth in the development of thyroid follicular tumours in rodents may be related to the increased chance of mutation that accompanies increased mitotic activity or to the increased chance of expansion of clones of cells bearing pre-existing mutations. The evidence that in rodents thyroid hormone imbalance alone leads to tumour formation is based on studies on the effect of iodine deficiency, partial thyroidectomy and transplantation of TSH-secreting tumours. Each of these regimes induced thyroid tumours in rodents without the use of any other agent. The frequency of tumour induction is higher if administration of a mutagen is followed

by goitrogen-induced sustained high TSH than if either the mutagen or the goitrogen is given separately. The importance of post-mutagenic growth is shown by the abolition of mutagen-induced carcinogenesis if all TSH-induced growth is suppressed. Additional evidence for the carcinogenic role of TSH-induced growth due to agents interfering with thyroid hormone homeostasis has been provided by experiments showing that return of the TSH level to normal using thyroid hormone abolishes the tumour-promoting effect of phenobarbital on the thyroid (see McClain & Rice, this volume).

#### *Species differences relevant to thyroid carcinogenesis*

##### *Species differences in thyroid biochemistry and physiology*

There are several species differences in thyroid physiology. Thyroxine-binding globulin (TBG) is the predominant plasma protein in humans and non-human primates that binds and transports thyroid hormone in the blood. This protein has binding affinities 3 and 5 orders of magnitude greater than the other two thyroxine-binding proteins, albumin and pre-albumin, respectively. The lack of TBG in the adult rat is an important difference.

Major differences are also present in the half-life of thyroxine, 12 h in the rat versus 5-9 days in humans, and in the serum level of TSH which is 25 or more times higher in the rodent than in humans. The rat also exhibits enhanced thyroid hormone elimination with less efficient entero-hepatic recirculation than humans. The histology of the resting rodent thyroid is similar to that of the stimulated human gland, with small follicles lined by tall follicular cells. Thus, both the physiological parameters and the histological appearance indicate that the rodent thyroid gland is more active and operates at a higher level with respect to thyroid hormone turnover as compared to the human gland.

##### *Species differences in goitrogenic effects*

In general, the goitrogenic effects of chemicals do not operate via a species-specific mechanism. An exception is the goitrogenic effect of sulfonamides.

Some species, including rodents, are sensitive to sulfonamides, whereas no goitrogenic effects are observed at high dosages in monkeys or at

therapeutic doses in humans. The biochemical basis for this species difference has been shown to be a marked species difference in the inhibition of thyroid gland peroxidase *in vitro* between rats and primates. The monkey is also less sensitive to the inhibition of thyroid peroxidase and the goitrogenic effects of propylthiouracil; however, this difference was not as great as with the sulfonamides.

#### *Species differences in thyroid carcinogenesis*

The weight of the evidence suggests that rodents are more sensitive than human subjects to thyroid tumour induction due to hormonal imbalances that cause elevated TSH levels.

#### *Gaps in our knowledge*

At the population level, it would be useful to obtain incidence and mortality data from areas of the world which have never been assessed but which are likely to have extreme variations with respect to iodine intake and other exposures (e.g., low-level radiation) potentially associated with thyroid cancer risk. Concurrent surveys on distribution by histological type should also be planned. The role of dietary habits, low-level radiation and cancer family history in different types of thyroid cancer should also be studied further by means of analytical epidemiology techniques.

Molecular studies of genes known to be involved in human thyroid tumours (e.g., *ret*, *ras*, *trk*, *p53*) should be expanded to link genetic changes to environmental factors where the latter are known (e.g. radiation). Other genes involved in thyroid carcinogenesis need to be identified, including germ-line mutations involved in familial thyroid tumours.

Similar molecular biological studies need to be carried out in animal thyroid tumours, both spontaneous and induced, so that any alterations can be linked to morphological changes and to the inducing agents used.

### **Renal-cell neoplasms of the kidney**

#### ***Incidence, etiology and pathology of renal carcinomas in humans***

Approximately three-fourths of human renal-cell carcinomas are conventional (clear-cell) carcinomas, the majority of which are associated with somatic alterations on chromosome 3 including

the von Hippel-Lindau (*VHL*) gene. Other major renal-cell carcinoma subtypes are associated with distinctive cytogenetic features not involving the *VHL* gene. The most common of these are the papillary (chromophil) carcinomas associated with polysomies of chromosomes 7, 17 and other chromosomes and with deletion of the Y chromosome in tumours of males. Chromophobe carcinomas are another cytogenetically and morphologically distinct subgroup, characterized by hypodiploidy with loss of multiple chromosomes. It is important for future epidemiological and biological studies to consider these and other recently characterized tumour subgroups as potentially distinct entities involving different pathogenic mechanisms (see Beckwith, this volume).

The incidence rates of renal-cell carcinoma are highest in northern Europe, Australia and North America (6–8 per 100 000 in men, 3–4 per 100 000 in women), intermediate in southern Europe and Japan and low in the rest of Asia, Africa and South America. Incidence rates have been increasing in most populations, although the rate of increase seems to be slowing down in some countries and levelling off in a few others. The incidence in men is 2–3 times that in women (see Møller, this volume).

Two risk factors (cigarette smoking and obesity) have consistently been found to increase the risk of renal-cell carcinoma and together account for approximately 40% of cases in high-risk countries. Cigarette smoking increases the risk approximately two-fold and a dose-response effect as well as an effect of cessation have been demonstrated. Other tobacco products have not been associated with renal-cell carcinoma risk to the same extent.

Obesity (defined as a high body-mass index) increases risk in women and, to some extent, also in men. The increase in risk may be as high as 3–4-fold in very obese women. It is not known whether the diet, peripheral oestrogen synthesis or some other factors are responsible for the association.

Human exposure to gasoline and *d*-limonene is particularly interesting in the light of these agents' ability to induce  $\alpha_2$ -globulin nephropathy and renal tumours in male rats. Although some case-control studies have found an approximately 50% increase in risk among individuals exposed to gasoline after adjustment for other risk factors,



other studies gave negative results and cohort studies of refinery workers and gasoline station attendants have yielded inconsistent findings. Furthermore, no studies have looked at leaded and unleaded gasoline separately. No epidemiological study has focused on *d*-limonene, but studies of diet and renal-cell carcinoma have not suggested an association with intake of fruit juice or citrus fruit which contain *d*-limonene.

The use of phenacetin increases the risk of urothelial cancer and, to a lesser extent, that of renal-cell carcinoma, but this drug is no longer available in most western countries. Other analgesics (e.g., aspirin and paracetamol) do not seem to influence the risk of renal-cell carcinoma.

Some studies have shown a moderate increase in risk of renal-cell carcinoma among users of diuretics or other antihypertensive drugs. It is unknown whether it is the medical condition, the drug itself or shared risk factors for renal-cell carcinoma and the medical condition that are responsible.

A possible role of diet and several occupational and environmental exposures (e.g., asbestos, cadmium, lead, pesticides, dry-cleaning processes, trichloroethylene, fungal toxins) in inducing human renal-cell carcinoma remains unresolved.

#### ***Mechanisms of renal-cell carcinogenicity in rodents***

In the rodent kidney, several mechanisms of chemically induced carcinogenesis have been identified based on reasonably robust data-sets from the published literature. These mechanisms can be categorized as follows:

- A. *Direct DNA reactivity.* Some model genotoxic carcinogens (or their metabolites), particularly certain N-nitroso compounds, are known to interact directly with DNA of renal tubule cells, causing genomic alterations.
- B. *Indirect DNA damage mediated by oxidative stress.* At least two compounds (potassium bromate and ferric nitrilotriacetate) have been shown to generate reactive oxygen species in rodent kidneys, which in turn have the potential to cause genomic alterations.

C. *Sustained stimulation of cell proliferation.* A number of non-genotoxic chemicals appear to lead to the development of renal cell tumours through a process involving prolonged renal tubule cell injury coupled with regenerative cell proliferation. This mechanistic pathway can be further subcategorized as involving either

(i) a regenerative response to chemically induced cytotoxicity (chloroform has been suggested as an example of a chemical that directly affects proximal tubule cells causing cytotoxicity, cell death and compensatory cell proliferation); or

(ii) a regenerative response not dependent on direct chemical cytotoxicity but on cytotoxicity resulting from impairment of a physiological process; this is the proposed mechanism for  $\alpha_{2u}$ -globulin nephropathy and the associated renal carcinogenesis.

Kidney tumours induced by chemicals in category C tend to occur in low incidence (usually less than 30%), even at high doses, with a long latency, and may exhibit sex-dependent differences and in some cases, species-specificity. This is in contrast to chemicals representing mechanistic categories A and B that can induce high (up to 100%) incidences of renal tumours which may have relatively short latent periods, and are not typified by sex-specificity.

#### ***$\alpha_{2u}$ -Globulin-associated nephropathy as a mechanism of renal tubule cell carcinogenesis in male rats***

$\alpha_{2u}$ -Globulin nephropathy is a syndrome that occurs exclusively in male rat kidney. A diverse group of non-genotoxic chemicals have been shown to cause acute renal changes manifested as the accumulation of this urinary globulin in phagolysosomes of renal proximal tubule cells. The toxicity appears to be caused by the accumulation of a single, major male rat-specific protein,  $\alpha_{2u}$ -globulin (see Swenberg & Lehman-McKeeman, this volume). The  $\alpha_{2u}$ -globulin accumulating in the male kidney is synthesized in the liver, where its expression represents about 1%

of total hepatic mRNA. Hepatic synthesis of  $\alpha_{2u}$ -globulin is regulated by complex hormonal interactions, particularly androgens, whereas in female rats, oestrogens repress hepatic synthesis. Lysosomal accumulation of  $\alpha_{2u}$ -globulin leads to death of individual renal cells and compensatory cell proliferation which may result in atypical tubule hyperplasia and ultimately renal tubule tumours. Several agents that induce  $\alpha_{2u}$ -globulin nephropathy have been shown to promote both spontaneously and chemically initiated tubule epithelial cells to preneoplastic and neoplastic lesions in male rat kidney. Furthermore, a quantitative relationship between sustained renal-cell proliferation and the promotion of preneoplastic and/or neoplastic lesions has been established, providing support for the conclusion that sustained renal cell proliferation is causally related to the development of renal tumours in male rats.

The contribution of  $\alpha_{2u}$ -globulin to the species-specificity of the nephropathy has been shown in several ways. First, it has been determined that the NBR strain of rats that does not synthesize the hepatic form of  $\alpha_{2u}$ -globulin, does not develop  $\alpha_{2u}$ -globulin nephropathy and is not susceptible to renal tumour promotion by these agents. Additionally, it has been shown that, although mice are resistant to renal toxicity following exposure to agents that induce  $\alpha_{2u}$ -globulin nephropathy, transgenic mice engineered to synthesize  $\alpha_{2u}$ -globulin developed the nephropathy. Mechanistic studies have demonstrated that the requisite step in the development of the syndrome is the ability of a chemical (or metabolite(s)) to bind reversibly, and specifically, to  $\alpha_{2u}$ -globulin. Binding of chemicals to  $\alpha_{2u}$ -globulin appears to alter the lysosomal degradation of the protein, leading to its accumulation in phagolysosomes. Furthermore, a comprehensive survey of structurally-related proteins along with experimental analyses has provided evidence that, although other species, including humans, synthesize proteins that are similar to  $\alpha_{2u}$ -globulin, differences in ligand-binding properties, physiological function and renal handling of these homologues preclude their involvement in this protein droplet nephropathy. For example, although mice synthesize mouse urinary protein (MUP), a protein that shares approximately 90% amino acid sequence identity with  $\alpha_{2u}$ -globulin and which is considered the murine

homologue of  $\alpha_{2u}$ -globulin, this protein does not contribute to a similar syndrome in mice.

An alternative hypothesis to the accumulation and impaired degradation of  $\alpha_{2u}$ -globulin (see Melnick & Kohn, this volume) proposes that  $\alpha_{2u}$ -globulin in male rats may serve to transport and concentrate the ligand within proximal tubule cells. Release of the ligand or subsequent metabolism of the ligand may produce cytotoxicity. However, one compound, 2,4,4-trimethyl-2-pentanol, has been tested and not found to be cytotoxic in primary cultures of renal tubule fragments. Although there are no data supporting this alternative hypothesis, it can be concluded that a process involving  $\alpha_{2u}$ -globulin as a vector for chemically induced injury would remain exclusive to the male rat.

There are differences in opinion as to whether the hypothesis linking  $\alpha_{2u}$ -globulin nephropathy to the induction of kidney tumours is consistent with the available data on chemicals or mixtures proposed to act through this mechanism. Such differences have been attributed to a lack of compliance with the criteria that must be fulfilled in order to state that the mechanism by which chemicals induce renal tubule cell tumours is exclusively through an accumulation of the rat male-specific  $\alpha_{2u}$ -globulin. The Working Group therefore formulated the following criteria (all of which must be met) for concluding that an agent causes kidney tumours through an  $\alpha_{2u}$ -globulin-associated response (Table 1).

Numerous chemicals have been shown to induce hyaline droplets in the kidneys of male rats. However, for many of these there is a lack of sufficient data and/or adherence to previous developed criteria, that characterize a chemical causing kidney tumours solely through an  $\alpha_{2u}$ -globulin-associated response. Swenberg and Lehman-McKeeman (see this volume) have judged that only seven of 40 chemicals and mixtures which induce hyaline droplets in male rat kidney have fully met the criteria in Table 1 for an  $\alpha_{2u}$ -globulin-associated response. All of the remaining chemicals need additional data in order to determine whether they meet these criteria. Some chemicals which induce hyaline droplets in the kidney in male rats are excluded by these criteria (see Swenberg & Lehman-McKeeman, this volume).

**Table 1. Criteria for an agent causing kidney tumours through an  $\alpha_{2u}$ -globulin-associated response in male rats**

- Lack of genotoxic activity (agent and/or metabolite) based on an overall evaluation of in-vitro and in-vivo data
- Male rat specificity for nephropathy and renal tumorigenicity
- Induction of the characteristic sequence of histopathological changes in shorter-term studies, of which protein droplet accumulation is obligatory
- Identification of the protein accumulating in tubule cells as  $\alpha_{2u}$ -globulin
- Reversible binding of the chemical or metabolite to  $\alpha_{2u}$ -globulin
- Induction of sustained increased cell proliferation in the renal cortex
- Similarities in dose-response relationship of the tumour outcome with the histopathological end-points (protein droplets,  $\alpha_{2u}$ -globulin accumulation, cell proliferation).

### ***Do similar mechanisms occur in humans?***

A requisite step in the development of  $\alpha_{2u}$ -globulin nephropathy is the binding of a chemical (or metabolite) to  $\alpha_{2u}$ -globulin.  $\alpha_{2u}$ -Globulin is a member of a superfamily of proteins that bind and transport a variety of ligands. Many of these proteins are synthesized in mammalian species, including humans. Therefore, the question of a similar mechanism occurring in humans can be addressed by determining whether these structurally homologous proteins can function in humans in a manner analogous to  $\alpha_{2u}$ -globulin. This question can be answered both qualitatively and quantitatively.

The protein content of human urine is very different from that of rat urine, as humans excrete very little protein (about 1% of the concentration in male rats). Human urinary protein is also predominantly a species of high molecular weight, and there is no protein in human plasma or urine identical to  $\alpha_{2u}$ -globulin.

With respect to the  $\alpha_{2u}$ -globulin superfamily, it has been shown that MUP, the protein most similar to  $\alpha_{2u}$ -globulin, does not contribute to a similar syndrome in mice, and the lack of a response in female rats, which synthesize many other proteins of this superfamily, demonstrates that these proteins are unlikely to contribute to the renal toxicity.

Saturable binding of 2,4,4-trimethyl-2-pentanol and *d*-limonene-1,2-oxide to  $\alpha_{2u}$ -globulin can be shown *in vitro*, but these chemicals do not bind to other members of the  $\alpha_{2u}$ -globulin superfamily. Furthermore, it has been shown that 2,4,4-trimethyl-2-pentanol does not bind specifically to

any low molecular weight protein isolated from male human kidney, indicating that there are no proteins constitutively present in human kidney that are similar to  $\alpha_{2u}$ -globulin. The X-ray-derived crystal structure of  $\alpha_{2u}$ -globulin further supports the unique binding property of this protein. Other superfamily proteins are characterized by flattened, elongated binding pockets, whereas the ligand-binding site in  $\alpha_{2u}$ -globulin is distinguished by its spherical, non-restrictive shape.

From a quantitative perspective, adult male rat kidneys reabsorb about 35 mg of  $\alpha_{2u}$ -globulin per day. Female rats synthesize less than 1% of the amount of  $\alpha_{2u}$ -globulin reabsorbed by male rats, but no  $\alpha_{2u}$ -globulin is detected in the female rat kidney and female rats do not develop the nephropathy. The most abundant  $\alpha_{2u}$ -globulin superfamily protein in human kidney and plasma is  $\alpha_1$ -acid glycoprotein, and this protein does not bind to agents that induce  $\alpha_{2u}$ -globulin nephropathy in rats.

Taken together, there is no evidence that a mechanism similar to  $\alpha_{2u}$ -globulin nephropathy occurs in humans.

### ***Gaps in knowledge***

For many chemicals that induce hyaline droplets in male rat kidney, there is insufficient information to evaluate whether this response is associated with an  $\alpha_{2u}$ -globulin-related mechanism. The most important data gaps are therefore those required to establish whether such chemicals fulfil the criteria for an  $\alpha_{2u}$ -globulin-associated response.

Because *d*-limonene is currently being evaluated as a cancer chemopreventive agent, it should be possible to establish in humans whether this

agent produces nephrotoxicity at the high doses used in those clinical trials.

There is also a need for further epidemiological studies of gasoline exposure, preferably with quantitative exposure assessment and sufficient data on other risk factors to allow adjustment for potential confounding factors.

There is a need for further evaluation of quantitative relationships between the various intermediate steps in order to improve prediction of the carcinogenic response of chemicals operating through the  $\alpha_{2u}$ -globulin-associated mechanism.

Information on effects of chemicals on lysosomal function relative to their abilities to cause  $\alpha_{2u}$ -globulin accumulation is needed.

Kinetic models linking  $\alpha_{2u}$ -globulin accumulation and ligand dosimetry after multiple dosing are needed.

Clarification of whether an endogenous ligand exists for  $\alpha_{2u}$ -globulin would help in understanding the physiological function of this protein and might further delineate the mechanisms underlying  $\alpha_{2u}$ -globulin-associated pathology in the kidneys of male rats. Mechanisms of cell death should be investigated.

A hypothesis has been put forward that  $\alpha_{2u}$ -globulin serves to concentrate nephrotoxic ligands and that the renal pathology is related to a release of such bound ligands. Additional experiments designed to examine this hypothesis are warranted.

## Urinary bladder neoplasms

### *Incidence, etiology and pathology of urinary bladder tumours in humans*

Incidence and mortality rates for bladder cancer tend to be higher in developed countries than in developing countries. Between 1973 and 1994, bladder cancer incidence in the United States showed moderate increases in both sexes and various races, whereas mortality tended to decline, particularly in men. In the early 1990's, most age-adjusted mortality rates for men within Europe fell within the range of 5–8 per 100 000. Rates for women were between 1 and 3 per 100 000 in most countries and showed no appreciable changes over time. In most of western Europe, mortality has declined in generations born since 1940.

Transitional-cell carcinoma of the bladder accounts for about 95% of bladder cancer, although squamous-cell carcinomas are more common in certain regions, such as the Middle East (see La Vecchia & Airoldi, this volume).

Cigarette smoking is recognized as the main cause of bladder carcinoma and accounts for about 50% of cases in most developed countries. A high risk of bladder carcinoma has been observed in workers exposed to some aromatic amines. Based on these and other occupational risks (e.g., among leather workers, truck drivers and aluminium production workers), it has been estimated that 5–10% of bladder carcinomas in industrialized countries were due to exposures of occupational origin.

A number of other factors have been evaluated as possible human bladder carcinogens. Several studies have investigated the association between coffee consumption and bladder cancer risk. Although excess risks have been reported in some studies, the lack of dose- or duration-risk relationships leaves this issue open to discussion. There is also no convincing evidence of any appreciable risk of bladder cancer from personal use of hair dyes.

Two cancer chemotherapeutic drugs, cyclophosphamide and chlornaphazine, and treatment with ionizing radiation have been shown to cause bladder cancer in humans. Heavy consumption of phenacetin-containing analgesics was strongly associated with lower urinary tract carcinomas including bladder carcinomas in several studies, but paracetamol-containing analgesics have not been shown to have such effects.

Infectious agents and other diseases of the urinary tract, which may cause chronic inflammation, have a major influence on bladder cancer risks in northern Africa and the Middle East and other areas where *Schistosoma haematobium* infestation is endemic. In these regions, there is a consistent relationship between carcinomas (especially squamous-cell carcinomas) of the bladder and urinary schistosomiasis.

The role of urinary tract infections other than those associated with schistosomiasis on bladder carcinogenesis is more difficult to assess. Most findings, however, are consistent with a 2–3-fold elevated risk. The association appears to be stronger for squamous-cell carcinomas and may be stronger in women.

With reference to urinary tract stones, four case-control studies published between the 1960s and the 1980s found relative risks between 1.0 and 2.5. At least three more recent case-control studies and one record-linkage cohort study gave a relative risk between 1.2 and 1.4 for a history of urinary tract stones. An interesting feature of these investigations was that the association was stronger in women, with statistically significant relative risks higher than 2. Although there exists a potential for recall bias in the case-control studies and uncontrolled confounding in the cohort study, these findings suggest that the presence of urinary tract stones is associated with bladder carcinoma in humans.

No epidemiological study has addressed the issue of a possible role of persistent crystalluria as a risk factor for urothelial carcinoma in humans. Likewise, quantitative studies on cellular proliferation and possible precursors of bladder carcinoma in relation to the presence of bladder stones and/or urinary infections in humans are lacking.

Overall, epidemiological data do not indicate that saccharin and other artificial sweeteners are related to human bladder cancer, although studies in children are scanty. Occasional observations of increased or decreased risks in some subgroups are to be regarded as fluctuations within a series of multiple comparisons.

***Calculi-, amorphous precipitate- and microcrystalluria-associated irritation, cell proliferation and urinary bladder carcinogenesis in mice and rats***

Many non-genotoxic chemicals have been shown to induce formation of microcrystals, amorphous precipitates and/or calculi in the urine of mice and rats (see Fukushima & Mural, this volume). Such chemicals include uric acid, calcium oxalate, uracil, thymine, melamine and others. The proliferative and tumorigenic effects produced in the bladder of these rodents require the concentration of the chemical in the urine to be sufficiently high to lead to precipitate formation and ultimately to calculi.

The ability of chemicals to produce calculi varies with species, strain and sex, as do the proliferative and tumorigenic responses. Calculus formation is also dependent on specific chemical and physical conditions of the urine (e.g., pH, volume), which have not been completely delineated. Marked increased urothelial cell

proliferation (e.g., papillomatosis in rats) is caused by the presence of a calculus. The same response is observed when chemically inert materials (e.g., glass beads, paraffin wax and cholesterol pellets) are surgically implanted into the bladder of rodents, or when physiological conditions are altered leading to calculus formation (e.g., uric acid metabolism following portacaval shunt). The extent of the proliferation is dependent on the abrasiveness of the surface of the implanted pellet or the number and size of calculi.

These proliferative effects of calculi are commonly sustained in rodents since these species are normally horizontally positioned allowing the object to remain within the lumen of the urinary bladder, with less chance of elimination. If the calculus is removed before a neoplasm is produced, the proliferative changes are rapidly reversed. Bacterial urinary tract infections can enhance the formation of calculi. If the calculus remains in the urinary bladder for an extended period of time, it may lead to cancer formation.

Microcrystalluria is associated with irritation and cell proliferation but its association with bladder carcinomas is less clear.

***Are calculi associated with irritation and cell proliferation in humans and do the mechanisms of carcinogenesis mediated by such effects in rodents operate in humans?***

Urinary bladder calculi, irrespective of composition, cause irritation and cell proliferation in humans (see Fukushima & Mural, this volume). There is some epidemiological evidence that urinary tract cancer in humans is associated with a history of calculi in the bladder (see La Vecchia & Airoldi, this volume). The risk in humans may not be as great as that in rodents because the calculi are usually voided spontaneously or removed by surgical procedures. Thus, although there are quantitative differences in the carcinogenic response to calculi between species, the effect is not species-specific. However, calculus formation is dependent on attainment in the urine of critically high concentrations of the constituent chemicals which form the calculus. The carcinogenic effects are also dependent on reaching a threshold concentration for calculus formation.

### **Urinary bladder carcinogenesis produced by chemicals causing calcium phosphate containing precipitates in the urine of rats**

High doses of several sodium salts of moderate to strong organic acids (see Cohen, this volume) produce a calcium phosphate-containing precipitate in the urine of rats and increased urothelial proliferation. Increased bladder tumour incidences are seen when these sodium salts are administered after treatment with bladder carcinogens such as *N*-[4-(5-nitro-2-furyl)-2-thiazolyl]formamide (FANFT) or *N*-butyl-*N*-(4-hydroxybutyl)nitrosamine (BBN). One exception is sodium hippurate, which reduces the pH of the urine to below 6.5, so that precipitate does not form.

Other sodium salts such as sodium *ortho*-phenylphenate and trisodium nitrilotriacetate which do not cause formation of calcium phosphate-containing precipitates in the urine of rats have other biological properties which are associated with their carcinogenic effects in the urinary bladder of rats.

In the 1970s, sodium saccharin was fed to rats at high doses in the diet in a two-generation protocol (before conception, throughout gestation, lactation and post-weaning, for the lifetime of the animal) and found to be carcinogenic to the urinary bladder. Subsequent studies have demonstrated that if administration begins at birth, tumour response is similar to that observed when exposure began before conception. The male rat is considerably more susceptible than the female rat. Administration beginning before five weeks of age also produced an increased incidence of tumours, although less than when administration began at birth or earlier. Administration of sodium saccharin to rats beginning at six to eight weeks of age, as in a standard two-year bioassay, did not produce a tumorigenic response. Monkeys treated with 25 mg/kg bw/day sodium saccharin starting at birth, and mice, hamsters and guinea-pigs treated with high doses of sodium saccharin in the diet starting at an adult age (doses of the same order of magnitude as those given to rats) did not show proliferation or neoplastic effects in the urinary bladder.

In relation to other sodium salts of moderate to strong organic acids (the most extensively studied being sodium ascorbate), an effect on the urothelium has been identified mainly in male rats using either short-term studies evaluating cell

proliferation in the bladder epithelium or in studies in which high doses of the sodium salt were administered after an initial period of treatment with a bladder carcinogen, such as FANFT or BBN. However, two-generation studies have not been reported for any of these other sodium salts. Only a few of these sodium salts have been studied in mice and no proliferative effect on urothelium has been observed. None of the other sodium salts has been studied in other species.

Saccharin, ascorbate and the other chemicals being discussed are sodium salts of moderate to strong acids. They are nearly completely ionized at physiological pH, and as anionic structures do not interact with DNA. Genotoxicity assays *in vivo* have been nearly entirely negative. Also, the evidence does not support a role of contaminants of these chemicals as the cause of the urinary tract effects in rats. The carcinogenic action of these sodium salts involves the formation of a calcium phosphate-containing precipitate in the urine of the rats fed high dietary doses. This causes cell death of superficial layers of the urothelium, leading to regenerative hyperplasia and ultimately to tumours. Quantitative aspects of the cell proliferation help to explain the necessity for administration beginning at weaning or earlier. The formation of this precipitate requires a urinary pH above 6.5. Administration of the corresponding acids of the sodium salts does not produce a proliferative effect on the rat bladder epithelium. In addition, any treatment which produces acidification of the urine inhibits both formation of the calcium phosphate-containing precipitate and the neoplastic effects of these sodium salts on urothelium. Acidification can be accomplished by co-administration of the sodium salt with ammonium chloride or administration in semi-synthetic diets, such as AIN-76A, which produce markedly acidic urine.

Formation of this precipitate appears to require high urinary concentrations of protein, as found in rats. Male rats are more susceptible than female rats, presumably because of the higher concentration of protein in their urine, secondary to excretion of large amounts of  $\alpha_{2u}$ -globulin. Administration of sodium saccharin or sodium ascorbate to NBR male rats, which do not excrete high concentrations of  $\alpha_{2u}$ -globulin, results in little to no hyperplastic response in the urinary bladder.

Even in male rats, which are the most sensitive animals, high doses are required for formation of the precipitate, induction of the cytotoxicity and induction of proliferative responses in the urothelium. In general, at a level of 1% of the diet, there was no statistically significant effect for either sodium saccharin or sodium ascorbate, and only slight effects were seen at doses of 2.5–3.0% of the diet. Most of the other sodium salts have been evaluated at doses equimolar to 5% sodium saccharin or as 5% of the diet.

Mice are resistant to the proliferative effects of sodium saccharin and the other sodium salts which have been studied, despite also having appropriate urinary pH, high urinary protein concentrations, and high osmolality like the rat. However, urinary concentrations of calcium and phosphate are considerably lower in the mouse than in the rat, and this greatly reduces the potential for formation of a calcium phosphate precipitate.

Studies in monkeys, including one investigation in which sodium saccharin was administered at a dose of 25 mg/kg bw per day for 18–23 years beginning immediately after birth, showed no evidence of formation of the precipitate or cytotoxic or proliferative changes in the urinary tract. These findings may be due to the lower doses of sodium saccharin as well as the much lower concentrations of protein and lower osmolality in the urine of non-human primates than in mice and rats.

***Do the mechanisms of carcinogenesis involving calcium phosphate-containing precipitate formation, cell death and cell proliferation, operate in humans?***

Extensive epidemiological studies in humans have failed to show an increased risk of urinary bladder cancer secondary from use of artificial sweeteners. Healthy humans have very low concentrations of urinary protein and much lower urinary osmolalities than rodents, two of the critical parameters required for the formation of cytotoxic calcium phosphate-containing precipitate. In individuals with nephrotic syndrome, there is proteinuria that can attain protein concentrations nearly as high as those found in rats. However, the osmolality does not increase above normal levels in humans (50–500 mosmol/kg). The highest osmolalities that could be attained in humans have been estimated theoretically at approximately 1200 mosmol/kg,

compared with 1400–2000 mosmol/kg in saccharin-treated rats. Thus the epidemiological, experimental animal and mechanistic data suggest that the tumorigenic response in the urinary bladder of rats generated by these sodium salts is a species- and dose-specific phenomenon that does not occur in humans.

Since epidemiological reports on high dietary intakes of sodium, calcium and vitamin C related to bladder cancer are scattered and the results are inconclusive, no inference on mechanisms of carcinogenesis can be drawn.

***Gaps in mechanistic knowledge***

Few data are available on mechanistic pathways which occur subsequent to calculus formation in the urinary bladder of rodents or humans. *p53* and *H-ras* mutations are not found in urinary bladder tumours in rats treated with calculus-inducing agents. However, the molecular and genetic events involved in cell cytotoxicity, regenerative hyperplasia and tumour formation are unknown. Amorphous precipitates may vary widely in composition and no generalization can be drawn that is universally applicable to all such precipitates. There is little information regarding the reasons for quantitative differences in carcinogenic response to different agents between species and sexes.

Urinary precipitate produced by certain sodium salts consists mainly of calcium phosphate and this precipitate is cytotoxic to the urothelium. Mechanisms leading to urothelial cell death are not known. Critical factors essential for the formation of the urinary precipitate have not been fully identified and the physical and chemical mechanisms for the formation (or the lack of formation) of the precipitate need to be more clearly defined.

***Conclusions***

The overall conclusions of the Working Group were formulated by providing answers to questions concerning how the mechanistic data reviewed in the previous sections can be used in making overall evaluations of carcinogenicity to humans.

***How can mechanistic data on thyroid-stimulating hormone (TSH)-associated follicular-cell neoplasms in rodents be used in making overall evaluations of carcinogenicity to humans?***

Agents that lead to the development of thyroid neoplasia through an adaptive physiological

mechanism belong to a different category from those that lead to neoplasia through genotoxic mechanisms or through mechanisms involving pathological responses with necrosis and repair.

Agents causing thyroid follicular-cell neoplasia in rodents solely through hormonal imbalance can be identified using the following criteria.

- There is a lack of genotoxic activity (agent and/or metabolite) based on an overall evaluation of in-vitro and in-vivo data
- The presence of hormone imbalance has been demonstrated under the conditions of the carcinogenicity assay.
- The mechanism whereby the agent leads to hormone imbalance has been defined.

When tumours are observed both in the thyroid and at other sites, they should be evaluated separately on the basis of the modes of action of the agent.

Agents that induce thyroid follicular tumours in rodents through interference with thyroid hormone homeostasis can, with few exceptions, also interfere with thyroid hormone homeostasis in humans if given at a sufficient dose for a sufficient time. These agents can be assumed not to be carcinogenic in humans at exposure levels which do not lead to alterations in thyroid hormone homeostasis.

***How can mechanistic data on  $\alpha_{2u}$ -globulin-associated renal-cell neoplasms in male rats be used in making overall evaluations of carcinogenicity to humans?***

In making overall evaluations of carcinogenicity to humans, it can be concluded that production of renal-cell tumours in male rats by agents that fulfil all of the following criteria for an  $\alpha_{2u}$ -globulin-associated response is not predictive of carcinogenic hazard to humans:

- Lack of genotoxic activity (agent and/or metabolites) based on an overall evaluation of in-vitro and in-vivo data
- Male rat specificity for nephropathy and renal tumorigenicity
- Induction of the characteristic sequence of histopathological changes in shorter-term studies, of which protein droplet accumulation is obligatory

- Identification of the protein accumulating in tubule cells as  $\alpha_{2u}$ -globulin
- Reversible binding of the chemical or metabolite to  $\alpha_{2u}$ -globulin
- Induction of sustained increased cell proliferation in renal cortex
- Similarities in dose-response relationship of the tumour outcome with the histopathological end-points (protein droplets,  $\alpha_{2u}$ -globulin accumulation, cell proliferation)

In situations where an agent induces tumours at other sites in the male rat or tumours in other laboratory animals, the evidence regarding these other tumour responses should be used independently of the  $\alpha_{2u}$ -globulin-associated tumorigenicity in making the overall evaluation of carcinogenicity to humans.

***How can mechanistic data on calculi- and microcrystalluria-associated urinary bladder neoplasms in mice and rats contribute to making overall evaluations of carcinogenicity to humans?***

For chemicals producing bladder neoplasms in rats and mice as a result of calculus formation in the urinary bladder, the response cannot be considered to be species-specific; thus, the tumour response is relevant to an evaluation of carcinogenicity to humans. There are quantitative differences in response between species and sexes. Calculus formation is dependent on the attainment in the urine of critical concentrations of constituent chemicals which form the calculus; therefore, the biological effects are dependent on reaching threshold concentrations for calculus formation. Microcrystalluria is often associated with calculus formation, but its relevance to species-specific mechanisms cannot be assessed.

***How can mechanistic data on urinary calcium phosphate-containing precipitate-associated bladder neoplasms in rats contribute to making overall evaluations of carcinogenicity to humans?***

Calcium phosphate-containing precipitates in the urine of rats, such as those produced by the administration of high doses of some sodium salts, including sodium saccharin and sodium ascorbate, can result in the production of urinary bladder tumours. This sequence can be considered to be species- and dose-specific and is not known to occur in humans.



In making overall evaluations of carcinogenicity to humans, it can be concluded that the production of bladder cancer in rats via a mechanism involving calcium phosphate-containing precipitates is not predictive of carcinogenic hazard to humans, provided that the following criteria are met:

- the formation of the calcium phosphate-containing precipitate occurs under the conditions of the carcinogenicity bioassay which is positive for cancer induction;
- prevention of the formation of the urinary precipitate results in prevention of the bladder proliferative effect;
- the agent (and/or metabolites) shows a lack of genotoxic activity, based on an overall evaluation of in-vitro and in-vivo data;
- the agent being evaluated does not produce tumours at any other site in experimental animals;
- there is evidence from studies in humans that precipitate formation or cancer does not occur in exposed populations.

In situations where an agent induces tumours at other sites in rats or tumours in other laboratory animals, the evidence regarding these other tumour responses should be used independently of information on tumours associated with calcium phosphate-containing precipitates in making the overall evaluation of carcinogenicity to humans.

# Species differences in chemical carcinogenesis of the thyroid gland, kidney and urinary bladder

E. Dybing and T. Sanner

## Introduction

It is well known that carcinogens show quantitative differences in activity between species (Gold *et al.*, 1984, 1986, 1987, 1990, 1993a, 1995). A classic example is 2-naphthylamine, which appears to be much more potent in humans and dogs than in rats (Kriek, 1969; Radomski, 1979; Gold *et al.*, 1984). Rats, on the other hand, are much more susceptible towards the hepatocarcinogenicity of 2-acetylaminofluorene than mice or hamsters, while guinea-pigs and monkeys appear to be resistant (Miller *et al.*, 1964; Dyer *et al.*, 1966; Miller, 1970; Gold *et al.*, 1984). Aflatoxin B<sub>1</sub> is a very potent liver carcinogen in rats but is less potent in monkeys and hamsters and essentially noncarcinogenic in feeding experiments in mice (Herrold, 1969; Wogan, 1973; Gold *et al.*, 1984; Thorgeirsson *et al.*, 1994). Many of these quantitative species differences in carcinogenic activity may be attributed to differences in carcinogen metabolism (Dybing & Huitfeldt, 1992). In general, however, the quantitative differences in carcinogenic activity between animals are not large. Differences in potency between rats and mice are within a factor of 10 for 74% and within a factor of 100 for 98% of known carcinogens, respectively (Gaylor & Chen, 1986; Gold *et al.*, 1989).

All chemicals for which there is sufficient evidence of carcinogenicity in humans and that have been studied adequately in experimental animals have shown carcinogenic activity in at least one animal species (Wilbourn *et al.*, 1986; Tomatis *et al.*, 1989). Such an association makes it plausible that chemicals which have shown carcinogenic activity in experimental animals also have carcinogenic potential in humans (US Environmental Protection Agency, 1996; IARC, 1998).

Furthermore, animal bioassays have revealed carcinogens that were subsequently found to cause cancer in humans (Wilbourn *et al.*, 1986; Tomatis *et al.*, 1989; Huff, 1992; Tomatis *et al.*, 1997). However, recent evidence indicates that some experimental carcinogens may act through species-specific mechanisms, so that the assumption that all experimental carcinogens are potential human carcinogens may not always be valid (Swenberg *et al.*, 1992; Hard *et al.*, 1993; Cohen & Lawson, 1995).

The present overview considers tumour development in the thyroid gland, kidney and urinary bladder in the context of species-specificity in chemical carcinogenesis. In order to illustrate this, some examples are presented of carcinogens which show species differences in activity at these target sites (Tables 1–3). Such differences may, in principle, be of a quantitative as well as a qualitative nature. In the following presentation and quantitative comparisons, extensive use is made of the data in the Carcinogenic Potency Database (CPDB) of Gold *et al.* (1984, 1986, 1987, 1990, 1993a, 1995). It must be recognized that categorization of carcinogenesis and noncarcinogenicity in the CPDB is based on the evaluation by the authors of the studies included in the database. Thus, there may be discrepancies between the classification of chemicals in this database and that following the more comprehensive evaluation performed by the International Agency for Research on Cancer (IARC).

## General mechanisms of carcinogens

Chemical carcinogenesis is a multistep process involving genetic and epigenetic alterations whereby normal cells are converted into malignant ones (Weinstein, 1988; Boyd & Barrett, 1990;

**Table 1. Mutagenicity of chemicals which have been evaluated in the carcinogenic potency database as carcinogenic in the thyroid gland in rats and/or mice<sup>a</sup>**

Mutagenicity in <i>Salmonella</i>	Rat	Mouse
Mutagenic	<i>ortho</i> -Anisidine.HCl** 2,4-Diaminoaniline sulfate** 1-Ethyl-1-nitrosourea Ethylene thiourea** Glycidol** Iodinated glycerol** 4,4'-Methylenedianiline( <i>N,N</i> -dimethyl)-benzenamine** 4,4'-Methylenedianiline.2HCl** 4,4'-Oxydianiline** <i>para</i> -Rosaniline.HCl** 4,4'-Thiodianiline** Zinc dimethyldithiocarbamate*	3-Amino-4-ethoxyacetanilide* HC Blue No.1** 2,4-Diaminoaniline sulfate** Ethylene thiourea** 4,4'-Methylenedianiline. 2HCl** 1,5-Naphthalenediamine** 4,4'-Oxydianiline** CI pigment red 2** 4,4'-Thiodianiline**
Nonmutagenic	3-Aminotriazole** Chlorinated paraffins (C <sub>12</sub> , 60% chlorine)** <i>N,N'</i> -Diethylthiourea* Malonaldehyde, sodium salt** Methimazole 2,3,7,8-Tetrachlorodibenzo- <i>para</i> -dioxin** Trimethylthiourea* Vinyl acetate	Chlorinated paraffins (C <sub>12</sub> , 60% chlorine)** Diethylstilboestrol** Ethionamide* Sulfamethazine 2,3,7,8-Tetrachlorodibenzo- <i>para</i> -dioxin**
No evaluation	Bemitradine 1-Ethyl-1-nitroso-3-(2-oxopropyl)urea Mirex, photo- <i>N</i> -Nitrosobis(2-oxopropyl)amine Propylthiouracil** Thiouracil**	Oxazepam

<sup>a</sup> From Gold & Zeiger, 1997

\* Tested in both species but positive at some site only in one species

\*\* Tested in both species and positive at some site in both species

Harris, 1992; Barrett, 1993, 1995). The starting point of the carcinogenic process involves a mutational, irreversible cellular change, either induced by a chemical agent or occurring spontaneously. Thereafter, the initiated cell is clonally expanded by epigenetic factors which selectively influence cellular proliferation. Subsequently, multiple genetic changes must occur before the clonally expanded, preneoplastic cells develop into a malignant tumour. These sequential steps have been termed initiation, promotion and progression, respectively, and can be characterized morphologically and biochemically (Bird, 1995; Dragan et

al., 1995; Mehta, 1995). Specific changes in gate-keeper genes, predisposing genes or modifying genes may selectively affect rates of tumour initiation or tumour progression (Kinzler & Vogelstein, 1996).

Barrett (1993) has described three basic mechanisms by which a substance can influence the multistep carcinogenic process: (1) by inducing a heritable mutation in a critical gene; (2) by inducing a heritable, epigenetic change in a critical gene; or (3) by increasing clonal expansion of a cell with a heritable alteration in a critical gene, allowing an increased probability of additional

**Table 2. Mutagenicity of chemicals which have been evaluated in the carcinogenic potency database as carcinogenic in the kidney in rats and/or mice<sup>a</sup>**

Mutagenicity in <i>Salmonella</i>	Rat	Mouse
Mutagenic	Aflatoxin B *	Bromodichloromethane**
	1-Amino-2-methylantraquinone**	1,3-Butadiene**
	2-Amino-4-nitrophenol *	2,4-Diaminophenol.2HCl*
	2-Amino-5-nitrothiazole*	N-Hydroxy-2-acetyl-amino-
	<i>ortho</i> -Anisidine.HCl**	fluorene**
	Azoxymethane	Phenacetin**
	Bromate, potassium*	CI Pigment Red 3**
	Bromodichloromethane**	Streptozotocin**
	Coumarin**	Tris(2,3-dibromopropyl)-
	Formic acid 2-[(5-nitro-2-furyl)-2-thiazolyl]hydrazide**	phosphate**
	1-(2-Hydroxyethyl)-1-nitrosourea**	Vinylidene chloride *
	8-Methoxypsoralen	
	N-[(3-(5-Nitro-2-furyl)-1,2,4-oxadiazol-5-yl)-methyl]-acetamide	
	N-[5-(5-Nitro-2-furyl)-1,3,4-thiadiazol-2-yl]acetamide**	
	<i>ortho</i> -Nitroanisole**	
	1-[(5-Nitrofurfurylidene)amino]hydantoin**	
	N-Nitrosodiethanolamine	
	N-Nitrosodimethylamine**	
	CI Acid Orange 3*	
	Phenacetin**	
	Quercetin*	
	Streptozotocin**	
	1,2,3-Trichloropropane**	
	Vinyl chloride**	
Nonmutagenic	Benzofuran**	<i>ortho</i> -Benzyl- <i>para</i> -
	Caffeic acid**	chlorophenol*
	Captafol**	Caffeic acid**
	Chlorinated paraffins (C <sub>12</sub> , 60% chlorine)**	Chloroform**
	Chloroform**	Daminozide**
	3-( <i>p</i> -Chlorophenyl)-1,1-dimethylurea *	Hydroquinone**
	Chlorothalonil*	Mercurymethylchloride
	Cinnamyl anthranilate**	Nitriotriacetic acid**
	Citrinin	Ochratoxin A**
	1,4-Dichlorobenzene**	
	3,4-Dihydrocoumarin**	
	Dimethyl methylphosphonate*	
	Hexachlorobutadiene	
	Hexachloroethane**	
	Hydroquinone**	
	Isophorone*	
	Lead acetate*	
	$\alpha$ -Limonene*	
	$\alpha$ -Methylbenzyl alcohol*	

Table 2 (contd)

Mutagenicity in <i>Salmonella</i>	Rat	Mouse
Nonmutagenic	Mirex** Nitrotriacetic acid** Nitrotriacetic acid, trisodium salt, monohydrate* Ochratoxin A** Phenazone Phenylbutazone** <i>ortho</i> -Phenylphenate, sodium* Tetrachloroethylene** Tris(2-chloroethyl)phosphate*	
No evaluation	2-Amino-5-(5-nitro-2-furyl)1,3,4-oxadiazole 2-Amino-5-(5-nitro-2-furyl)1,3,4-thiadiazole Barbitol sodium Dichloroacetylene** Diethylacetamide Dimethoxane 4,6-Dimethyl-2-(5-nitro-2-furyl)pyrimidine N-4-(4'-Fluorobiphenyl)acetamide Hexamethylmelamine 2-Hydrazino-4-(5-nitro-2-furyl)thiazole** Lead acetate, basic** 2-Methoxy-3-aminodibenzofuran Z-Methyl-O,N,N-azoxyethane N-(N-Methyl-N-nitrosocarbonyl)-1-ornithine L-5-Morpholinomethyl-3-[(5-nitrofurfurylidene)- amino]-2-oxazolidinone.HCl 3-(5-Nitro-2-furyl)imidazo(1,2- $\alpha$ )pyridine** 1-Nitroso-1-hydroxyethyl-3-chloroethylurea N-Oxydiethylene thiocarbonyl-N-oxydiethylene sulfenamide	1,2-Di- <i>n</i> -butylhydrazine.HCl Dichloroacetylene** 3-Hydroxy- <i>para</i> -butyrophenetidine Lead acetate, basic**

\* From Gold &amp; Zelger, 1997

\*Tested in both species but positive at some site only in one species

\*\*Tested in both species and positive at some site in both species

Table 3. Mutagenicity of chemicals which have been evaluated in the carcinogenic potency database as carcinogenic in the urinary bladder<sup>a</sup>

Mutagenicity in <i>Salmonella</i>	Rat	Mouse
Mutagenic	Allyl isothiocyanate* 2-Amino-4-(5-nitro-2-furyl)thiazole** 4-Amino-2-nitrophenol* <i>ortho</i> -Anisidine.HCl** <i>N</i> -Butyl- <i>N</i> -(4-hydroxybutyl)nitrosamine 4-Chloro- <i>ortho</i> -phenylenediamine** <i>meta</i> -Cresidine** <i>para</i> -Cresidine** Cyclophosphamide** CI Disperse Blue 1* IQ.HCl 2-Naphthylamine** <i>N</i> -[4-(5-Nitro-2-furyl)-2-thiazolyl]formamide** <i>ortho</i> -Nitroanisole** <i>N</i> -Nitroso- <i>N</i> -methyl- <i>N</i> -dodecylamine <i>N</i> -Nitrosodibutylamine** <i>N</i> -Nitrosodiethylamine <i>N</i> -Nitrosopyrrolidine Phenacetin** Quercetin* <i>para</i> -Quinone dioxime* <i>ortho</i> -Toluidine.HCl** Trp-P-2 acetate**	2-Acetylaminofluorene** 4-Aminodiphenyl <i>ortho</i> -Anisidine.HCl** <i>para</i> -Cresidine** <i>N</i> -Hydroxy-2-acetylaminofluorene** <i>N</i> -[4-(5-Nitro-2-furyl)-2-thiazolyl]formamide** Phenacetin** Telone II**
Nonmutagenic	Acetaminophen** 11-Aminoundecanoic acid* Diethylene glycol Melamine* Nitrioltriacetic acid** Nitrioltriacetic acid, trisodium salt, monohydrate* <i>N</i> -Nitrosodiphenylamine* Phenazone <i>ortho</i> -Phenylphenate sodium* <i>ortho</i> -Phenylphenol* Saccharin, sodium* <i>ortho</i> -Toluenesulfonamide Uracil**	Uracil**
No evaluation	Fosetyl Al 2-Methoxy-3-aminodibenzofuran <i>N</i> -Nitroso- <i>N</i> -methyl- <i>N</i> -tetradecylamine <i>N</i> -Nitroso- <i>N</i> -methyldecylamine <i>N</i> -Nitrosobis(2-oxopropyl)amine <i>ortho</i> -Nitrosotoluene <i>N</i> -Oxydiethylene thiocarbamyl- <i>N</i> -oxydiethylene sulfenamide	2-Aminodiphenylene oxide 4-Chloro-4'-aminodiphenylether** 4-Ethylsulfonylnaphthalene-1-sulfonamide

<sup>a</sup> From Gold & Zeiger, 1997

\*Tested in both species but positive at some site only in one species

\*\*Tested in both species and positive at some site in both species

events. Theoretically, chemical carcinogenesis could thus be induced by compounds which act through any one of these mechanisms. Other substances may induce tumours by two or even all three mechanisms. There is ample evidence that mutagens may cause cancer. Most, but not all, human carcinogens are active in a variety of genetic toxicology tests (Bartsch & Malaveille, 1990; Shelby & Zeiger, 1990). However, there are a number of experimental carcinogens which are not mutagens: only about 50% of the experimental carcinogens identified by the US National Toxicology Program are mutagens (Tennant *et al.*, 1987; Zeiger *et al.*, 1990; Ashby & Tennant, 1991). In this review, carcinogenic activity will be related to mutagenicity in the *Salmonella* test (Ames *et al.*, 1975), as reported in the CPDB, although it must be recognized that some carcinogens found to be negative in the *Salmonella* test show clear evidence of genotoxicity in other test systems, such as ochratoxin (Dirheimer, 1996; see Table 2) and hydroquinone (Chen *et al.*, 1994; see Table 2).

There has been much debate about whether nonmutagenic carcinogens can act through stimulation of cell proliferation (Ames & Gold, 1990; Cohen & Ellwein, 1990; Preston-Martin *et al.*, 1990; Weinstein, 1991; Cohen & Ellwein, 1992; Huff, 1992; Melnick *et al.*, 1993). It is proposed that such agents can fix DNA damage occurring spontaneously during cellular replication or that which is induced by other mutagenic agents. In addition, nonmutagenic carcinogens may enhance the probability of additional genetic events during clonal expansion of initiated cells. Mitogenesis is certainly crucial to the carcinogenic process (Ames & Gold, 1990; Preston-Martin *et al.*, 1990; Weinstein, 1991; Barrett, 1993). Mitosis may be induced through cellular signals resulting from the interaction of external agents with cellular receptors. An increase in cell numbers may also be achieved by inhibition of apoptosis (Schulte-Hermann *et al.*, 1993; Goldsworthy *et al.*, 1996). In addition, replicative cell proliferation may be initiated as a secondary event to tissue damage induced after generally high exposures to cytotoxic agents.

A schematic categorization of chemical carcinogens is depicted in Figure 1 (modified from Cohen & Ellwein, 1992). It must be recognized

that such a scheme may be excessively simplistic, since several mechanisms may operate in concert (Barrett, 1993, 1995).

### Cancer of the thyroid gland

There are a number of important species differences in thyroid gland physiology which are important for the development of thyroid tumours (Dohler *et al.*, 1979; Capen, 1994; McClain, 1995). The half-life of thyroxine is much shorter in rats (12–24 h) than in humans (5–9 days) and the serum levels of thyroid-stimulating hormone (TSH) are 25 times higher in rodents than in man (Dohler *et al.*, 1979). Further, rats require about a 10-fold higher production of thyroxine than do humans (Dohler *et al.*, 1979). In addition, the human plasma high-affinity thyroxine-binding globulin is absent in rodents, cats and rabbits (Dohler *et al.*, 1979). This absence results in the blood transport of more free thyroxine in these species compared to humans, and predisposes the former to higher levels of metabolism and excretion. Iodine deficiency readily induces thyroid gland neoplasia in rodents (Axelrad & Leblond, 1955), whereas a clear etiological role for endemic goitre in humans has not been established (Pendergast *et al.*, 1961; Doniach, 1970), although an excess risk of thyroid cancer has, in some studies, been associated with goitre (Ron *et al.*, 1987; Salabè, 1994; D'Avanzo *et al.*, 1995) (see also Franceschi & Dal Maso, this volume).

Thyroid gland neoplasia may be induced both directly by mutagenic carcinogens and indirectly through hormone imbalance (Furth, 1959; Capen, 1994; McClain, 1995). Treatment of rodents with a number of antithyroid substances induces a high incidence of thyroid tumours (Napalkov, 1990). Such hormonal imbalance may occur either through inhibition of thyroid hormone production, secretion or peripheral conversion, or through alteration of metabolism resulting in increased biliary excretion of conjugates. All of these mechanisms lead to a compensatory sustained increase in the synthesis and secretion of TSH through negative feedback on the pituitary gland. Antithyroid agents, such as thiourea, propylthiouracil and methimazole, and sulfonamides, such as sulfadiazine and sulfamethazine, are effective inhibitors of thyroid hormone synthesis in rats, whereas they are much less potent in

monkeys (Takayama *et al.*, 1986). The marked difference in inhibition of thyroid peroxidase between rats and monkeys appears to constitute the biochemical basis for this species difference (Takayama *et al.*, 1986; Swenberg *et al.*, 1992). Among rodents, the rat is more sensitive towards the thyroid neoplastic effect of sulfamethazine than the mouse (Littlefield *et al.*, 1989, 1990).

Many chemicals can, at high doses, alter thyroid function in rodents via enzyme induction leading to increased disposition of thyroxine (Hill *et al.*, 1989; McClain, 1989). In such cir-

cumstances, a compensatory increase in TSH will, if the chemical exposure is prolonged, lead to thyroid gland neoplasia (McClain, 1989). The classical inducer phenobarbital has been shown to act as a thyroid gland tumour promoter in rats (McClain *et al.*, 1988). The promoting effect of enzyme inducers on thyroid gland tumorigenesis is usually greater in rats than in mice, with males generally more sensitive than females (Capen, 1994).

The thyroid gland is a target site for 6% of 354 rat carcinogens and 3% of 299 mouse carcinogens

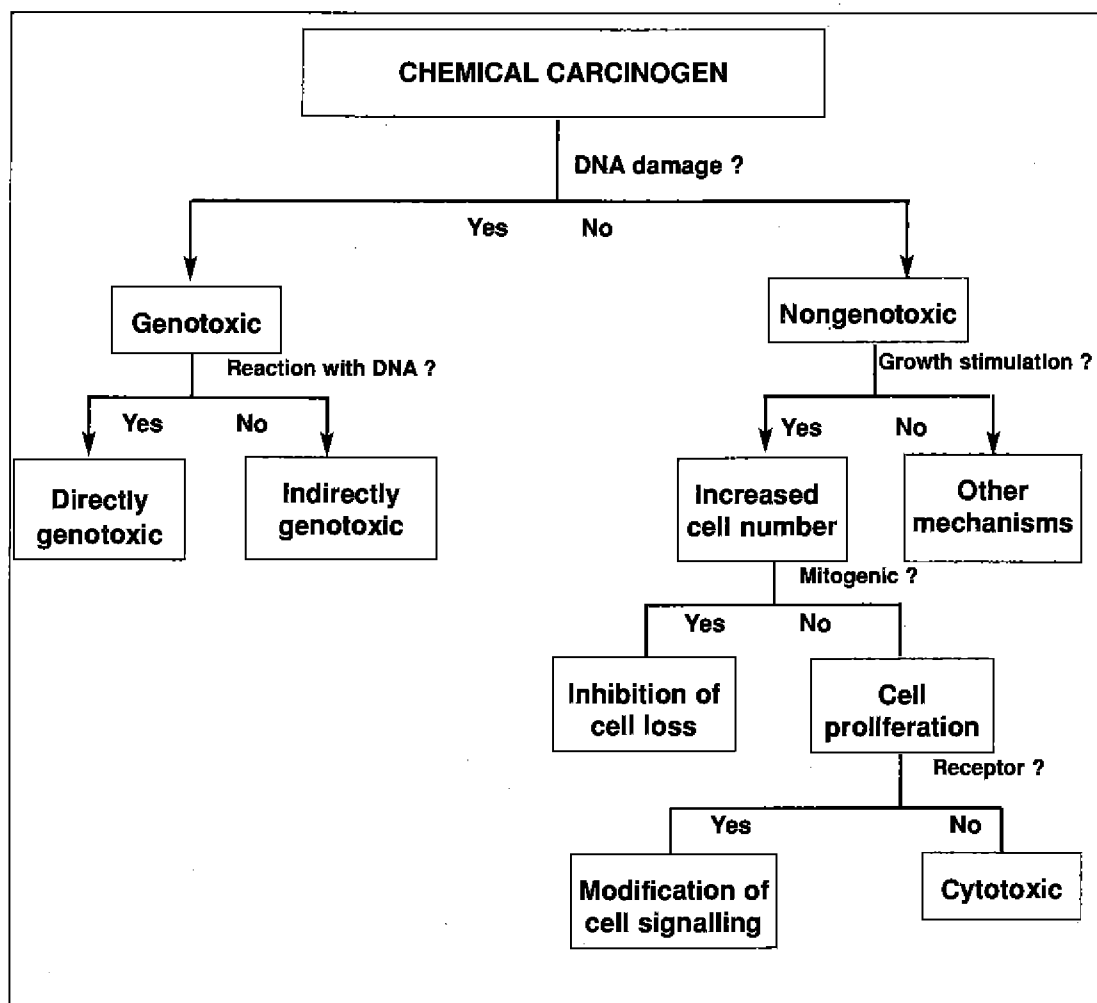


Figure 1. Schematic categorization of chemical carcinogens (modified after Cohen & Ellwein, 1992)



in the CPDB (Gold *et al.*, 1993b). Similar figures were reported for 379 National Cancer Institute/National Toxicology Program (NCI/NTP) long-term studies (Huff *et al.*, 1991). In all, 34 of the CPDB chemicals have been identified as thyroid gland carcinogens in rats and/or mice (Table 1). Additional studies have been added to the CPDB since Gold *et al.* (1993b) calculated the frequencies of tumours at different target sites, the number of substances inducing tumours at a particular site in Tables 1–3 may be higher than those which can be calculated from the percentages given by Gold *et al.* (1993b). Of the 25 thyroid carcinogens which have been tested in rats and mice, 20 (80%) were positive in both species at any site, but only seven were positive in the thyroid gland in both rats and mice (Tables 1 and 4). Five of these seven carcinogens were mutagenic in *Salmonella* and two were not. The thyroid gland carcinogens that were mutagenic were all much more potent in rats than in mice (Table 4).

### Cancer of the kidney

The kidney and ureter together constitute a target site for 13% of 354 rat carcinogens, but only 4% of the 299 mouse carcinogens in the CPDB (Gold *et al.*, 1993b). Huff *et al.* (1991) found that 7% of the carcinogens in the NCI/NTP experiments caused kidney cancer in rats and approximately 1% in mice. In all, 81 of the CPDB chemicals have been identified as kidney carcinogens in rats and/or mice (Table 2). Of the 57 renal carcinogens which have been tested in rats and mice, 38 (68%) were positive in both species at any site, while 11 were positive in the kidney in both rats and mice (Tables 2 and 4). Four of these 11 carcinogens were mutagenic in *Salmonella*, five were nonmutagenic and for two carcinogens no evaluation was reported in the CPDB. Three of the four mutagenic kidney carcinogens were somewhat more potent in the rat compared to the mouse, whereas all of the non-mutagenic kidney carcinogens were more potent in rats than in mice (Table 4).

Some nongenotoxic renal carcinogens are only active in the male rat (Borghoff *et al.*, 1990; Swenberg *et al.*, 1992; Hard *et al.*, 1993). Of the carcinogens listed in Table 2, 1,4-dichlorobenzene, dimethyl methylphosphonate, hexachloroethane, isophorone, *d*-limonene and tetrachloroethylene

cause hyaline droplet nephropathy which leads to replicative tubule cell proliferation via a reversible interaction with the male rat-specific urinary protein  $\alpha_{2u}$ -globulin. Unleaded gasoline and its constituent 2,2,4-trimethylpentane, which also cause hyaline droplet nephropathy, renal cell proliferation and renal tumours (Short *et al.*, 1987, 1989), as well as *d*-limonene, act as tumour promoters in the kidney (Short *et al.*, 1989; Dietrich & Swenberg, 1991a; Hard *et al.*, 1993). No other member of the lipocalin protein superfamily (Flower, 1996), to which the  $\alpha_{2u}$ -globulin protein belongs, binds hyaline droplet nephropathy-inducing agents (Lehman-McKeeman & Caudill, 1992). The uniqueness of the mechanism for this type of renal carcinogen is further substantiated by the fact that in the NCI-Black-Reiter rat strain, which lacks hepatic mRNA for  $\alpha_{2u}$ -globulin (Chatterjee *et al.*, 1989), no hyaline droplets or other aspects of renal disease are induced by chemicals which induce hyaline droplets in males of other rat strains (Dietrich & Swenberg, 1991a,b). Further strengthening the association between the presence of  $\alpha_{2u}$ -globulin and development of hyaline droplet nephropathy is the demonstration that *d*-limonene induces nephropathy in transgenic mice expressing  $\alpha_{2u}$ -globulin (Lehman-McKeeman & Caudill, 1994).

The kidney is unusual compared with other target sites, in that the proportion of carcinogens that are positive in the kidney is greater for *Salmonella*-negative than for *Salmonella*-positive compounds, as recorded in the CPDB file (Gold *et al.*, 1993b). Many of the nonmutagenic kidney carcinogens cause renal tubule necrosis at higher doses. Nitrilotriacetic acid is a more potent nephrotoxicant in the rat than in the mouse (Anderson *et al.*, 1985), which corresponds with its relative tumorigenic potency in the two species (Table 4).

Long-term gavage studies with hydroquinone have shown increases in tubule cell adenomas of male Fischer 344 rats accompanied by renal tubule epithelial degeneration, but these effects were not observed in female rats or B6C3F<sub>1</sub> mice (US National Toxicology Program, 1989; Hard *et al.*, 1997). Although hydroquinone gives negative results in the *Salmonella* test (Gold *et al.*, 1993b), high intraperitoneal doses are reported to cause clastogenic effects in mouse bone marrow cells (Chen *et al.*, 1994). In cell proliferation studies

**Table 4. Carcinogenic potency (TD<sub>50</sub>) values for carcinogens positive at target site in both rats and mice<sup>a</sup>**

Target site	Carcinogen	Mutagenicity in <i>Salmonella</i>	TD <sub>50</sub> rats mg/kg bw/d	TD <sub>50</sub> mice mg/kg bw/d
Thyroid gland	2,4-Diaminoanisole sulfate	Mutagenic	162	1060
	Ethylene thiourea		7.42	82
	4,4'-Methylenedianiline. 2HCl		27.2	128
	4,4'-Oxydianiline		14.3	598
	4,4'-Thiodianiline		5.59	88.4
	Chlorinated paraffins (C <sub>12</sub> , 60% chlorine)	Nonmutagenic	736	372
	2,3,7,8-Tetrachlorodibenzo- para-dioxin		0.10 <sup>b</sup>	1.59 <sup>b</sup>
Kidney	Bromodichloromethane	Mutagenic	152	137
	Phenacetin		547	1100
	Streptozotocin		0.78	2.44
	Tris(2,3-dibromopropyl)- phosphate		1.57	2.56
	Caffeic acid	Nonmutagenic	3930	4700
	Chloroform		119	153
	Hydroquinone		349	5320
	Nitrilotriacetic acid		2530	13800
	Ochratoxin A		0.058	358
	Dichloroacetylene	No evaluation	3.34	0.47
	Lead acetate, basic		107	986
Urinary bladder	ortho-Anisidine.HCl	Mutagenic	31.9	935
	para-Cresidine		88.4	44.7
	N-[4-(5-Nitro-2-furyl)-2-thiazolyl]- formamide		1.31	30.5
	Phenacetin		11700	37000
	Uracil	Nonmutagenic	ND <sup>c</sup>	ND <sup>c</sup>
	None	No evaluation	-	-

<sup>a</sup> From Gold & Zeiger, 1997<sup>b</sup> µg/kg bw/d<sup>c</sup> No data found

with hydroquinone in Fischer 344 rats, bromodeoxyuridine incorporation was increased in the kidney tubules of males but not females (English *et al.*, 1994). The underlying mechanism of hydroquinone-induced nephrotoxicity remains to be fully elucidated (Whysner *et al.*, 1995). However, it has been suggested that the tumour response in

rats linked to the rodent-specific chronic progressive nephropathy may have little relevance to humans (Hard *et al.*, 1997).

Long-term administration of chlorothalonil to rats leads to the development of renal tumours, a phenomenon associated with nephrotoxicity induced by a reactive thiol metabolite generated

by the action of cysteine  $\beta$ -lyase (IPCS, 1996). Chlorothalonil is much less nephrotoxic in mice and dogs than in rats and much less nephrocarcinogenic in mice compared to rats (IPCS, 1996; Wilkinson & Killeen, 1996). The rat has much higher renal levels of  $\gamma$ -glutamyl transpeptidase and  $\beta$ -lyase, enzymes involved in chlorothalonil bioactivation, than other species, including humans (Wilkinson & Killeen, 1996).

However, there does not always appear to be such a good correlation between nephrotoxicity and nephrocarcinogenicity. Chloroform administered to B6C3F<sub>1</sub> mice by gavage did not cause kidney tumours (US National Cancer Institute, 1976), although shorter-term administration of chloroform induced degeneration and necrosis with subsequent cell proliferation in this strain of mice (Larson *et al.*, 1994). In contrast, kidney tumours were seen after chloroform administration either by inhalation in male B6F<sub>1</sub> mice or in an aqueous-miscible toothpaste vehicle in male ICI mice (Roe *et al.*, 1979). Chloroform also causes cytotoxicity and cell proliferation in renal tubules of Fischer 344 rats (Larson *et al.*, 1994), although no renal tumour response was detected in a carcinogenicity bioassay in this strain (Matsushima, 1994). On the other hand, treatment of male Osborne-Mendel rats with chloroform by gavage results in the development of kidney tumours (US National Cancer Institute, 1976). There is a lack of intermediate end-points that may be linked to renal cancer development in this strain of rat.

There may also be sex and species differences for mutagenic nephrotoxic carcinogens. Vinylidene chloride, for example, causes renal necrosis and tumours only in male mice, but not in female mice or rats (WHO, 1990), a phenomenon which has been correlated with specific expression of cytochrome P450 2E1 in the kidney of male mice (Speerschneider & Dekant, 1995). These authors did not find evidence of P450 2E1 catalysis in human kidney microsomes, suggesting that vinylidene chloride-induced renal tumorigenesis may be sex- and species-specific.

### **Cancer of the urinary bladder**

A number of chemicals have been shown to induce urinary bladder tumours associated with formation of urinary calculi after long-term administration of

high doses to rodents (Wolkowski-Tyl *et al.*, 1982; Clayson *et al.*, 1995; Cohen, 1995a,b; Cohen & Lawson, 1995). In general, calculi form more readily in rats than in mice or the formation is similar in the two species. However, calculi-induced proliferation appears to be considerably greater in rats than in mice. This difference is partly due to a primarily papillomatous reaction in rats, whereas in mice it is predominantly a nodular response involving a smaller number of cells (Cohen & Lawson, 1995). The presence of calculi leads to erosion of the bladder surface with subsequent extensive regenerative hyperplasia that ultimately results in tumour formation. In general, these bladder carcinogens form calculi in the urine more readily in rats than in mice or at least to similar extents, whereas the proliferative response appears to be considerably greater in rats than in mice (Cohen & Lawson, 1995). There is also a sex difference, male rodents frequently being affected to a greater extent than females. The process involved in urothelial carcinogenesis related to calculus formation may be influenced by factors such as volume, osmolality, cationic and anionic concentration and quantitative and qualitative differences in the presence of urinary protein (Clayson *et al.*, 1995; Cohen, 1995b).

Epidemiological studies have suggested that calculus formation in humans may be a risk factor, but the association is weak (Matanoski & Elliott, 1981; Burin *et al.*, 1995). However, there are obvious differences in calculus residence time between rodents and humans related to the horizontal versus vertical status, which may make humans less sensitive than rodents towards tumour development caused by calculi-forming agents (DeSesso, 1995). Another factor affecting calculus residence time is the fact that the associated pain motivates humans to seek medical attention. Microcrystalluria produced by silicates also causes severe erosion of the urothelium, regenerative hyperplasia and tumour formation (Okamura *et al.*, 1992). Amorphous calcium phosphate precipitates of a number of sodium salts, including sodium saccharin, ascorbate, glutamate, bicarbonate and chloride, are cytotoxic to the rat urothelium and generate a mild regenerative hyperplasia which is associated with the generation of bladder tumours (see Cohen, this volume). These effects are greater in male than in

female rats and appear not to occur in mice, hamsters or monkeys (Ellwein & Cohen, 1990; Cohen & Lawson, 1995). There are clear cationic differences in the responses to saccharin, in that sodium saccharin produces both proliferative and tumorigenic effects, while potassium saccharin has less proliferative effect and calcium saccharin only a marginal effect (Cohen, 1995b). An involvement of the male-rat-specific  $\alpha_{2u}$ -globulin in the carcinogenic effects of sodium salts in the urinary bladder is indicated by the lack of induction of epithelial cell proliferation by sodium saccharin or sodium L-ascorbate in male NCI-Black-Reiter rats (Uwagawa *et al.*, 1994).

Tributyl phosphate induces urinary bladder hyperplasia and tumours in long-term experiments in rats (Weiner *et al.*, 1997), but not in mice (Kotkoskie *et al.*, 1997). This compound is an example of a nongenotoxic bladder carcinogen which causes urothelial cytotoxicity with marked regenerative hyperplasia, but without accompanying crystalluria, urinary precipitate or calculi (Arnold *et al.*, 1997).

The urinary bladder and urethra are together a target site for 10% of the 354 rat carcinogens and 4% of the mouse carcinogens in the CPDB (Gold *et al.*, 1993b). In contrast, the urinary bladder was reported to be the target organ for only 4% of the carcinogens in the 379 NCI/NTP studies examined by Huff *et al.* (1991). Cancer of the urinary bladder occurred in less than 1% of the NCI/NTP experiments in the mouse. In all, 50 of the CPDB chemicals have been identified as

urinary bladder carcinogens in rats and/or mice (Table 3). Of the 32 bladder carcinogens which have been tested in rats and mice, 20 (63%) were positive in both species at any site, whereas only five were positive in the urinary bladder in both rats and mice (Tables 3 and 4). Four of these five carcinogens were mutagenic in *Salmonella* and one was not. Three of the four mutagenic urinary bladder carcinogens were more potent in the rat than in the mouse (Table 4).

## Discussion

Species differences in chemical carcinogenesis may in principle be either qualitative in nature when there are interspecies differences in pathogenetic mechanisms, or be due to quantitative differences in action (Table 5). For carcinogenic mechanisms that can operate across various species, there may be quantitative differences, either due to variation in carcinogen delivery leading to differences in target dose (kinetic differences) or due to dynamic factors resulting from differences in target sensitivity. A number of such dynamic factors may influence the outcome of the carcinogenic process, including variation in receptor levels and affinities, rates and degrees of cell proliferation or apoptosis, as well as in rates and degrees of DNA repair.

Overall concordance in neoplastic response between rats and mice exposed to a given chemical is in the order of 75% (Haseman & Huff, 1987; Gold *et al.*, 1989; Huff *et al.*, 1991). Among rat carcinogens, 76% are positive in the mouse, while 70% of mouse carcinogens are positive in the rat

**Table 5. Principal types of species differences in chemical carcinogenesis**

### I. Qualitative differences

Species-specific differences in pathogenetic mechanisms

### II. Quantitative differences

#### A. Differences in kinetic factors

Differences in carcinogen delivery leading to differences in target dose

#### B. Differences in dynamic factors

Differences in target sensitivity related to, e.g. receptor level and affinity, cell proliferation, apoptosis, DNA repair

(Gold *et al.*, 1989). Somewhat lower concordance has been found for the limited number of compounds that have been tested in hamsters as well as rats and mice. For these, 64% (21/33) of rat carcinogens are positive in hamsters and 61% (17/28) of mouse carcinogens are positive in hamsters (Gold *et al.*, 1991). There may be lower concordance between rodents and humans than between rodent species. Site-specific prediction between rats and mice is less accurate than overall prediction of positivity, since the likelihood is at most 52% that if a chemical induces tumours at a given site in one species, it will also do so at the same site in the other species (Gold *et al.*, 1991). With respect to mutagenicity, mutagens are more likely than nonmutagens to be carcinogenic, more likely to induce tumours at multiple target sites and more likely to be carcinogenic in two species (Gold *et al.*, 1993b; Tennant, 1993; Gray *et al.*, 1995).

Allen *et al.* (1988) attempted to compare potency estimates from epidemiological data with those from animal carcinogenesis bioassays. For the 23 chemicals that were selected, the bioassay data gave potency estimates that were highly correlated with potencies estimated from human studies. However, sufficient evidence of carcinogenicity according to the IARC criteria was available for only 11 of the 23 chemicals studied (IARC, 1987).

A number of nongenotoxic rodent thyroid carcinogens act through an indirect mechanism involving a sustained increase in TSH levels. Given the marked differences between humans and rodents in thyroid gland physiology, it seems probable that such chemicals will be quantitatively much less active in humans. In order to operate through this type of mechanism in humans, the exposures would need to be so high as to induce evidence of thyroid hormone imbalance before development of neoplasia. In addition to such indirectly acting thyroid carcinogens, a number of genotoxic rodent thyroid carcinogens have been identified. These genotoxic thyroid carcinogens are approximately an order of magnitude more potent in rats than in mice. Such thyroid carcinogens may be considered potential human carcinogens, but no information is available indicating what their tumorigenic potency in humans would be.

Rodent kidney carcinogens act through several

different mechanisms. On the one hand, there are many kidney carcinogens that express mutagenic activity. Some of these, such as vinylidene chloride (WHO, 1990) and tris(2,3-dibromopropyl)phosphate (Söderlund *et al.*, 1980) also cause tubule necrosis, so that, in addition to having initiator potential, these carcinogens may elicit a strong promoting effect. Quantitative differences in tumorigenic potency between rats and mice for these compounds may in many instances be related to metabolic factors.

Chemicals inducing renal tumours in male rats through interaction with  $\alpha_2\mu$ -globulin do so through a mechanism which does not operate in female rats or other species. This mechanism of carcinogenesis is thus in the true sense species-specific and qualitatively different from other mechanisms. However, it must be remembered that chemicals causing tumours in male rats through this mechanism may act as carcinogens in other organs and species through alternative mechanisms. Examples of such kidney carcinogens are unleaded gasoline (IARC, 1989) and 1,4-dichlorobenzene (US National Toxicology Program, 1987), both of which also induce hepatic tumours in mice.

Nongenotoxic rodent renal carcinogens are generally more potent in rats than mice, or are active only in rats. Several such carcinogens cause renal necrosis, resulting in subsequent replicative proliferation. Metabolic differences are often responsible for the observed species differences in necrogenic potency, and may also be of importance for species differences in tumorigenicity. In principle, this type of renal carcinogen could be active in humans provided that exposures are comparable in terms of relative tumorigenic potency.

Many nonmutagenic rodent urinary bladder carcinogens appear to act through an indirect mechanism involving formation of calculi and microcrystalluria, resulting in urothelial lesions with accompanying regenerative hyperplasia. Urinary bladder tumours associated with calcium phosphate-containing precipitates produced by some sodium salts are unique to the rat. On the other hand, there are also a number of rodent bladder carcinogens which are mutagenic. Rats appear generally to be more sensitive than mice towards both mutagenic and nonmutagenic bladder carcinogens. Both of these types of carcinogen could be active in humans. However, at

least with respect to bladder carcinogens acting secondarily through calculus formation, humans appear to be less sensitive than rats.

It is important to recognize that most chemicals which are carcinogenic for the thyroid gland, kidney or urinary bladder are also carcinogenic at some other site (Table 6). It appears that more non-mutagens than mutagens show site-specificity for these target organs; however, only for the urinary bladder is the difference statistically different ( $p = 0.02$ ). For the sites discussed, only uracil is exclusively carcinogenic at the same site (urinary bladder) in both rats and mice.

### Conclusions

1. There are many examples of quantitative differences in carcinogenic activity between rats and mice, whereas the database for quantitative comparisons between rodents and humans is small.
2. Mutagenic thyroid carcinogens are more potent in rats than in mice. Chemicals causing thyroid tumours through an indirect mechanism via sustained elevation of TSH levels are presumably much more potent in rats than in humans due to the large differences in thyroid physiology between these two species.
3. There is strong evidence that chemicals causing renal tumours in male rats via interaction with  $\alpha_2\mu$ -globulin and accompanying hyaline droplet nephropathy act through a sex- and species-specific mechanism which does not occur in female rats or other species.
4. Renal toxicity and replicative proliferation is of importance for the activity of many mutagenic as well as nonmutagenic renal carcinogens. Sex and species differences in renal tumorigenesis may often be related to differences in the generation of toxic metabolites in the kidney.
5. The rat is generally more sensitive than the

**Table 6. Site-specificity of chemicals which have been tested in both rats and mice and evaluated in the Carcinogenic Potency Database as carcinogenic in the thyroid gland, kidney or urinary bladder in either or both species<sup>a</sup>**

Site	Mutagenic	Nonmutagenic
Thyroid gland	13% (2/15) <sup>b</sup> [Zinc dimethyldithiocarbamate (R), 3-amino-4-ethoxyacetanilide (M)] <sup>c</sup>	38% (3/8) [N,N-Diethylthiourea (R), trimethylthiourea (R), ethionamide (M)]
Kidney	8% (2/26) [CI Acid Orange 3 (R), 2,4-diaminophenol.2HCl (M)]	26% (7/27) [Chlorothalonil (R), dimethyl methylphosphonate (R), lead acetate (R), $\alpha$ -limonene (R), $\alpha$ -methylbenzyl alcohol (R), tris(2-chloroethyl)phosphate (R), ortho-benzyl-para-chlorophenol (M)]
Urinary bladder	19% (4/21) [Allyl isothiocyanate (R), 4-amino-2-nitrophenol (R), meta-cresidine (R), CI Disperse Blue (R)]	50% (5/10) [Melamine (R), N-nitrosodiphenylamine (R), ortho-phenylphenol, saccharin sodium (R), uracil (R, M)]

<sup>a</sup> From Gold & Zeiger, 1997

<sup>b</sup> Number of chemicals carcinogenic only for given site versus number of chemicals carcinogenic for given site plus other sites

<sup>c</sup> R, rats; M, mice

mouse towards urinary bladder carcinogenesis. The rat is especially sensitive towards chemicals which cause urothelial toxicity and accompanying regenerative hyperplasia associated with calculus, microcrystalluria or precipitate formation.

7. Most of the carcinogens which are active in the thyroid gland, kidney or urinary bladder in both rats and mice are also active at another site.

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# Hormonal imbalances and thyroid cancers in humans

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## Introduction

In humans, the normal thyroid contains two lobes joined by an isthmus. Fibrous septa divide the gland into pseudolobules which, in turn, are composed of vesicles called follicles or acini. Follicle walls are composed of cuboidal epithelium. The lumen of follicles is filled with a proteinaceous colloid, which contains a protein, thyroglobulin, within the peptide sequence of which L-thyroxine ( $T_4$ ) and 3,5,3-triiodo-L-thyronine ( $T_3$ ) are synthesized and stored.  $T_4$  and  $T_3$ , the active thyroid hormones, influence a diversity of metabolic processes: the growth and maturation of tissues, cell respiration, total energy expenditure, and the turnover of essentially all substrates, vitamins and hormones, including the thyroid hormones themselves (Wartofski, 1994). Thyroid function is regulated by pituitary (thyroid-stimulating hormone, TSH) and hypothalamic (thyrotropin-releasing hormone, TRH) mediators and by the glandular organic iodine content.

In humans, the great majority of thyroid cancers arise from the epithelial elements of the gland, mostly from the follicular cells (Robbins *et al.*, 1984). Such carcinomas fall into two broad groups: differentiated and undifferentiated (anaplastic). The former group is subdivided into two types, papillary and follicular. Medullary carcinoma derives from parafollicular cells (i.e., calcitonin-producing cells) and accounts for 5–15% of most series of thyroid carcinomas (Franceschi *et al.*, 1993). It has quite a separate aetiology from other thyroid carcinomas, inheritance being an important determinant (Ron, 1996) and it is not considered further in the present review.

Radiation is the only well defined risk factor for thyroid carcinoma. A pooled analysis of five cohort studies and two case-control investigations,

cumulating over 3 000 000 person-years of follow-up and 700 thyroid cancers, showed that, for persons exposed to radiation before the age of 15 years, the dose-response relationship was best described by a linear equation, even down to very low doses (0.10 Gy) (Ron *et al.*, 1995). In contrast, risk was not appreciably elevated for exposure to external radiation in adults and no clear excess following diagnostic or therapeutic iodine-131 exposure was detected (Ron, 1996).

The thyroid gland is not obviously related to female sex hormones in the way the breast, ovary and uterus are. However, most thyroid disorders, except endemic iodine-deficient goitre, are several-fold more prevalent in women than men. Overall, thyroid cancer shows a 2–3-fold higher incidence in females than in males in most populations (Franceschi *et al.*, 1993). However, female-to-male ratios vary substantially by histological type, being about 3 for papillary carcinoma, 2 for follicular carcinoma and 1 for medullary and undifferentiated carcinoma. This points to the possibility that events related to reproductive and menstrual history may be determinants of the risk of developing the most frequent tumours at this site (i.e., papillary and follicular carcinomas).

Puberty, pregnancy and oral contraceptives are associated with enlargement of the thyroid gland and with increases in serum levels of total  $T_4$  and  $T_3$  caused partly by a rise in the concentration of thyroxine-binding globulin (TBG) (Surks *et al.*, 1990). One possible mechanism for explaining the hormonal influence in the pathogenesis of thyroid cancer is that increased levels of female hormones may cause elevation of TSH levels, leading in turn to thyroid hyperplasia and finally to cancer. Experimentally, increased TSH secretion induces thyroid tumours in rodents (Williams, 1979). Some authors (Pacchiarotti *et al.*, 1986) reported small

but significant increases of TSH levels in the second and third trimesters of pregnancy, perhaps as a compensatory mechanism to meet the increased demand for thyroid hormones in this period. Furthermore, estrogen receptors have been found in thyroid cancers (Miki *et al.*, 1990).

The main focus of the present review is on epidemiological data regarding the relationship between menstrual and reproductive factors and risk of cancer of the thyroid in women. The role of elevated TSH levels will also be considered briefly, on the basis of circumstances (e.g., benign thyroid disease or residence in areas endemic for goitre) or exposures (e.g., food intake) which may cause and/or indicate enhanced TSH secretion.

### Female sex hormones and thyroid cancers

Of at least ten case-control studies which have provided data on female thyroid cancer and reproductive and menstrual factors, four were conducted in the United States of America: one each in Washington State (McTiernan *et al.*, 1984a; 185 cases and 393 controls, below age 80 years), Los Angeles, California (Preston-Martin *et al.*, 1987; 292 cases and 292 controls below age 55 years), Connecticut (Ron *et al.*, 1987; 109 cases and 208 controls below age 80), and Hawaii (Kolonel *et al.*, 1990; Goodman *et al.*, 1992; 140 cases and 328 controls, no age limit).

Five studies were carried out in Europe, including one each in Italy (Franceschi *et al.*, 1990; D'Avanzo *et al.*, 1995; 291 cases and 427 controls, below age 75) and Switzerland (Levi *et al.*, 1993; 100 cases and 318 controls below age 75). Three studies were performed in Sweden (southern Sweden, Wingren *et al.*, 1993; 149 cases and 187 controls, below age 60; northern Sweden, Hallquist *et al.*, 1993, 1994; 123 cases and 240 controls, below age 70; and Uppsala, Galanti *et al.*, 1995a, 1997; 133 cases and 203 controls, below age 72). One further study was conducted in Shanghai, China (Preston-Martin *et al.*, 1993; 207 cases and 207 controls).

Of 1729 cancer cases examined, 80% had papillary carcinoma and 14% follicular carcinoma. Undifferentiated and medullary carcinomas accounted for only 10 and 32 cases, respectively. Four per cent of the cases had other or unspecified types. Thus the present results apply almost exclusively to differentiated thyroid carcinomas, chiefly papillary ones.

In order to evaluate the association of thyroid cancer risk with hormone-related events and exposures, odds ratios (ORs) and corresponding 95% confidence intervals (CI) are presented. Multiple logistic regression equations conditioned on age and including a term for history of radiation therapy (the best known risk factor for thyroid cancer; Ron *et al.*, 1995) were fitted to the original data. Adjustment for other potential confounding factors was also made, where possible.

### Menstrual factors

Information on age at menarche in relation to thyroid cancer risk can be derived from 10 case-control studies (Table 1). In those of Kolonel *et al.* (1990), Franceschi *et al.* (1990) and Galanti *et al.* (1995a), late age at menarche was associated with non-significantly increased ORs around 1.6 for menarche at age 15 or above compared with age 12 or below. In most investigations, however, the distribution of cases and controls with respect to age at menarche was similar.

Some consistent findings emerged with respect to the influence of menopause (Table 2). In all investigations except one (Ron *et al.*, 1987), a higher proportion of cases compared to controls had undergone menopause, although the age distributions of cases and controls were similar and age was allowed for. Significantly increased risk (over two-fold) was found by McTiernan *et al.* (1987), Kolonel *et al.* (1990) and Levi *et al.* (1993). ORs tended to be somewhat higher for artificial than natural menopause, but the association with early menopause persisted after allowance for type of menopause. Late age at menopause was, thus, associated with reduced thyroid cancer risk, ORs for menopause at age 53 or above compared to below 45 years being 0.34 (95% CI, 0.10–1.21) in Kolonel *et al.* (1990) and 0.50 (95% CI, 0.14–1.74) in Levi *et al.* (1993).

In addition to age at menarche and menopause, some investigations included information on menstrual irregularities. Although in those of Franceschi *et al.* (1990) and Levi *et al.* (1993), women who reported irregularities were at slightly increased risk, most studies did not find any clear association with this characteristic.

### Reproductive factors

In all ten published investigations, women with

**Table 1. Odds ratios and corresponding 95% confidence intervals<sup>a</sup> of thyroid cancer by age at menarche**

Study and location	Age at menarche (years)		
	< 13	13, 14	> 14
McTiernan <i>et al.</i> (1987)	1	1.19 (0.79–1.78)	1.35 (0.76–2.38)
Western Washington, USA			
Preston-Martin <i>et al.</i> (1987)	1	0.94 (0.61–1.46)	0.91 (0.43–1.93)
Los Angeles, USA			
Ron <i>et al.</i> (1987)	1	1.35 (0.80–2.25)	1.03 (0.48–2.20)
Connecticut, USA			
Kolonel <i>et al.</i> (1990)	1	1.03 (0.65–1.63)	1.71 (0.98–2.96)
Hawaii, USA			
Franceschi <i>et al.</i> (1990)	1	1.08 (0.78–1.50)	1.54 (0.94–2.51)
Northern Italy			
Levi <i>et al.</i> (1993)	1	1.42 (0.83–2.44)	1.14 (0.59–2.21)
Vaud, Switzerland			
Wingren <i>et al.</i> (1993)	1	0.83 (0.48–1.44)	0.71 (0.36–1.42)
Southeastern Sweden			
Hallquist <i>et al.</i> (1994)	1	1.48 (0.85–2.57)	1.01 (0.51–1.99)
Northern Sweden			
Galanti <i>et al.</i> (1995a)	1	0.82 (0.47–1.44)	1.67 (0.83–3.36)
Uppsala, Sweden			
Preston-Martin <i>et al.</i> (1993)	1	0.67 (0.22–2.10)	0.59 (0.19–1.82)
Shanghai, China			

<sup>a</sup>Estimates from multiple logistic regression equations conditioned on age and adjusted for radiation history and age.

children seemed to be at somewhat increased risk of thyroid cancer compared with nulliparae (Table 3). However, no clear trend of increasing risk with increasing number of births (Table 3) or pregnancies was generally seen and, in several investigations (Ron *et al.*, 1987; Preston-Martin *et al.*, 1993), ORs tended to peak in women with one or two births.

Incomplete pregnancies showed a pattern similar to that for full-term pregnancies. However, in a few studies (Ron *et al.*, 1987; Kolonel *et al.*, 1990; Levi *et al.*, 1993; Preston-Martin *et al.*, 1993), personal history of miscarriages, especially at first pregnancy attempt, and difficulties in conception (Kolonel *et al.*, 1990) seemed directly associated with thyroid cancer risk.

In a cohort study of all women and men who were born in Norway between 1935 and 1974 (about 2.6 million individuals), number of children was significantly related to thyroid cancer risk in women (ORs, 1.3, 1.4, 1.5 and 1.7 for 1, 2, 3 and

4 or more children, compared with none), but not in men (Glattre & Kravdal, 1994). However, even childless women had a thyroid cancer risk well above that of all men.

Although this aspect has received far less attention than parity, a tendency for thyroid cancer risk to increase slightly with increasing age at first pregnancy emerged from several studies (Table 4). Compared with women who bore their first child below 20 years of age, those who bore their first child at age 30 or later showed ORs above one in virtually all investigations, although in none was the risk significant.

#### **Use of exogenous female hormones**

Results on the relationship between oral contraceptive (OC) use and thyroid cancer risk are shown in Table 5, after allowance for other risk covariates (i.e., radiation history, education, parity, and type of menopause). In four investigations (Preston-Martin *et al.*, 1987, 1993; Levi *et al.*, 1993;

**Table 2. Odds ratios and corresponding 95% confidence intervals<sup>a</sup> of thyroid cancer by menopausal status**

	Pre	Menopausal status	
		Post Natural	Artificial
McTiernan <i>et al.</i> (1987) Western Washington, USA	1	2.17 (0.94–5.01)	2.67 (1.30–5.48)
Preston-Martin <i>et al.</i> (1987) Los Angeles, USA	1	0.26 (0.04–1.50)	1.63 (0.64–4.15)
Ron <i>et al.</i> (1987) Connecticut, USA	1	0.90 (0.33–2.46)	0.80 (0.38–1.86)
Kolonel <i>et al.</i> (1990) Hawaii, USA	1	1.90 (0.78–4.61)	2.03 (0.88–4.66)
Franceschi <i>et al.</i> (1990) Northern Italy	1	1.34 (0.66–2.72)	1.91 (0.92–3.99)
Levi <i>et al.</i> (1993) Vaud, Switzerland	1	1.75 (0.46–6.59)	5.61 (1.68–18.74)
Galanti <i>et al.</i> (1995a) Uppsala, Sweden	1	1.37 (0.30–6.32)	2.37 (0.60–9.45)
Preston-Martin <i>et al.</i> (1993) Shanghai, China	1	2.97 (1.08–8.20)	8.36 (0.79–88.48)

<sup>a</sup> Estimates from multiple logistic regression equations conditioned on age, and adjusted for radiation history, hormonal replacement therapy and age.

Wingren *et al.*, 1993), women who had ever used OCs were at somewhat elevated risk, but the association never attained statistical significance. No clear trend was seen with the number of years of OC use.

Two studies, from Connecticut (Ron *et al.*, 1987) and Italy (Franceschi *et al.*, 1990), showed elevated thyroid cancer risk in OC users for younger women (ORs, 1.8 and 1.4 respectively), but no relationship in middle-aged users. The most interesting findings, however, concern recency of OC use, since, in all these studies, risk was specifically increased for current OC use and declined with increasing time since stopping. ORs above 1.5 in current OC users were found by Preston-Martin *et al.* (1987), Ron *et al.* (1987) and Levi *et al.* (1993).

Data are scantier with reference to menopausal hormone replacement treatment. The ORs associated with ever having had such treatment were 1.4 in the Washington study (McTiernan *et al.*, 1984a), 0.5 in the Connecticut study (Ron *et al.*, 1987), 0.9 in the Hawaii study (Kolonel *et al.*, 1990) and 1.1

in one Swedish study (Hallquist *et al.*, 1994), in the absence, however, of any consistent relationship with duration of use or any other time-related variable.

### Thyroid-stimulating hormone (TSH) and growth factors

By analogy with animal models, situations which cause (e.g., iodine deficiency) and/or indicate (goitre, nodules) enhanced TSH secretion have been associated with increased risk in humans (Williams, 1979). The evidence is not, however, totally consistent.

Since Wegelin (1928) reported a 10-fold higher prevalence of thyroid cancer at autopsy in Bern, an endemic goitre area, than in Berlin, an area non-endemic for goitre, goitre and/or iodine deficiency have been considered as probable risk factors for thyroid cancer. Some of highest incidence (Parkin *et al.*, 1992) and mortality (Levi *et al.*, 1994) rates (Table 6) for thyroid cancer worldwide have indeed been found in endemic goitre areas close to the Andes (Colombia, Cali; Ecuador, Quito) and the

**Table 3. Odds ratios and corresponding 95% confidence intervals<sup>a</sup> of thyroid cancer by number of births**

	Number of births				
	0	1	2	3	≥ 4
McTiernan <i>et al.</i> (1987)	1	1.54 (0.81–2.95)	1.10 (0.58–2.07)	1.54 (0.76–3.12)	1.55 (0.74–3.25)
Western Washington, USA					
Preston-Martin <i>et al.</i> (1987)	1	1.23 (0.62–2.44)	1.14 (0.57–2.28)	1.30 (0.52–3.31)	1.15 (0.41–3.20)
Los Angeles, USA					
Ron <i>et al.</i> (1987)	1	1.95 (0.88–4.32)	2.06 (0.96–4.43)	1.10 (0.44–2.76)	1.30 (0.52–3.24)
Connecticut, USA					
Kolonel <i>et al.</i> (1990)	1	0.79 (0.38–1.63)	0.75 (0.39–1.45)	0.52 (0.24–1.09)	1.64 (0.85–3.14)
Hawaii, USA					
Franceschi <i>et al.</i> (1990)	1	1.13 (0.68–1.89)	1.28 (0.78–2.09)	1.30 (0.71–2.37)	1.18 (0.62–2.24)
Northern Italy					
Levi <i>et al.</i> (1993)	1	1.06 (0.50–2.24)	1.14 (0.58–2.23)	1.59 (0.70–3.61)	0.93 (0.30–2.86)
Vaud, Switzerland					
Galanti <i>et al.</i> (1995a)	1	1.38 (0.64–3.01)	0.81 (0.39–1.68)	0.80 (0.34–1.90)	2.02 (0.57–7.17)
Uppsala, Sweden					
Preston-Martin <i>et al.</i> (1993)	1	1.98 (1.04–3.75)	1.66 (0.60–4.58)	0.50 (0.15–1.61)	0.80 (0.25–2.63)
Shanghai, China					

<sup>a</sup> Estimates from multiple logistic regression equations conditioned on age, and adjusted for radiation history, oral contraceptive use and age.

Alps (Switzerland, and Austria). Iceland and Hawaii, however, have outstandingly high rates of thyroid cancer, despite high levels of iodine intake (Goodman *et al.*, 1988). Correction of iodine deficiency through the introduction of iodized salt (Wynder, 1952) or changed farming practices (Phillips, 1997) has proved successful as far as goitre elimination is concerned. With respect to thyroid cancer, clear declines in mortality have been reported in Switzerland (Wynder, 1952), but not in the United States (Ron, 1996).

Separate consideration of various histological types led to the suggestion that follicular and anaplastic carcinomas may be more frequent in endemic goitre areas, while papillary carcinomas may be enhanced by iodine excess (Williams, 1977; Williams *et al.*, 1977). In Sweden, iodination of the food supply was started in 1936 and enhanced in 1966. Regional patterns of thyroid cancer incidence in the period 1958–81 in relation to the presence of former goitre endemicity have been reported (Pettersson *et al.*, 1996). Incidence rates for all thyroid cancers combined were slightly

lower in formerly iodine-deficient areas than in iodine-sufficient ones. The incidence of papillary cancer was higher in iodine-sufficient areas and higher in urban than in rural areas. Conversely, the incidence of follicular and anaplastic carcinoma was slightly elevated in iodine-deficient areas. However, upward trends in incidence rates of papillary carcinoma were similar in iodine-sufficient and iodine-deficient areas. Conversely, the decreases in incidence rates of anaplastic carcinoma were more marked in areas formerly affected by goitre endemicity.

Only two case-control studies have provided data on risk of thyroid cancer by residence in endemic goitre areas (Table 7). In the Italian study, the RR for having resided in endemic goitre areas was 1.3 for < 20 years of residence and 1.6 for 20 or more years (D'Avanzo *et al.*, 1995). Residence before the age of 25 years seemed more influential (Franceschi *et al.*, 1989). In the study in Sweden, a trend towards an association was found with duration of residence in endemic goitre areas (Galanti *et al.*, 1995b). In neither study was any



Table 4. Odds ratios and 95% confidence intervals<sup>a</sup> of thyroid cancer by age at first birth

	Nulliparae	Age at first birth (years)			
		< 20	20-24	25-29	≥ 30
McTiernan <i>et al.</i> (1987) Western Washington, USA	0.70 (0.31-1.60)	1	0.85 (0.47-1.54)	0.70 (0.34-1.44)	1.45 (0.60-3.51)
Preston-Martin <i>et al.</i> (1987) Los Angeles, USA	6.22 (0.35-112.15)	1	1.73 (0.22-13.38)	46.00 (0.72-2908.75)	2.09(0.10-42.86)
Ron <i>et al.</i> (1987) Connecticut, USA	0.47 (0.13-1.62)	1	1.00 (0.40-2.50)	0.74 (0.26-2.05)	1.42(0.42-4.80)
Kolonef <i>et al.</i> (1990) Hawaii, USA	2.95 (1.09-8.01)	1	0.71 (0.36-1.42)	1.09 (0.52-2.28)	2.01(0.76-5.32)
Franceschi <i>et al.</i> (1990) Northern Italy	1.05 (0.46-2.39)	1	0.87 (0.45-1.69)	1.47 (0.74-2.94)	1.73(0.77-3.89)
Levi <i>et al.</i> (1993) Vaud, Switzerland	1.59 (0.39-6.39)	1	2.15 (0.64-7.19)	1.97 (0.56-6.88)	2.18(0.54-8.88)
Galanti <i>et al.</i> (1995a) Uppsala, Sweden	0.70 (0.21-2.29)	1	0.57 (0.25-1.28)	0.66 (0.28-1.55)	1.20(0.37-3.93)
Preston-Martin <i>et al.</i> (1993) Shanghai, China	0.51 (0.14-1.92)	1	1.20 (0.50-2.88)	1.33 (0.49-3.57)	1.34(0.39-4.66)

<sup>a</sup> Estimates from multiple logistic regression equations conditioned on age, and adjusted for radiation history, parity and age.

clear difference detected between papillary carcinomas and follicular carcinomas with respect to residence history.

Results are far more consistent with respect to the influence of previous benign thyroid diseases. Thyroid nodules (adenomas), when analysed separately, were usually associated with higher relative risks than goitre (Table 8). The possibility that ascertainment and/or recall bias somewhat exaggerated RR estimates must be borne in mind. In case-control studies, lower RRs (approximately equal to 6) associated with a positive history of goitre were reported, whereas no significant elevation of risk was seen for a history of hyperthyroidism or hypothyroidism (McTiernan *et al.*, 1984b; Preston-Martin *et al.*, 1987; Franceschi *et al.*, 1989; Kolonel *et al.*, 1990; Levi *et al.*, 1991).

Regarding Graves' disease and its role in thyroid carcinogenesis, the first report was that of a 2.5% prevalence of thyroid cancer in 2114 hyperplastic thyroid glands (Olen and Klinck, 1966), 50% of which were papillary in type. However, no significant excess of thyroid cancer was reported among patients with Graves' disease in Olmsted County, Minnesota, USA (Munoz *et al.*, 1978), nor among those with thyrotoxicosis in the US

Cooperative Thyrotoxicosis study (Dobyns *et al.*, 1974).

In a retrospective follow-up study of 7338 women with either nontoxic nodular goitre, thyroid adenoma, hyperthyroidism, hypothyroidism, Hashimoto's thyroiditis, or no thyroid disease, treated between 1925 and 1974 at the Massachusetts General Hospital, a significant increase in thyroid cancer risk was observed only in women with thyroid adenoma (Goldman *et al.*, 1990).

Finally, with respect to dietary influences, some foods (notably fish) contain large amounts of iodine, while others (e.g., milk, meat, eggs, etc.) can be important sources of iodine depending upon farming practices (Phillips, 1997). Some vegetables, most notably cruciferous vegetables, and certain staple foods, especially cassava and maize, contain goitrogenous substances. The few case-control studies in which dietary information was collected, however, discarded the possibility of any vegetables having an adverse effect on thyroid cancer risk, at least in affluent countries (Franceschi *et al.*, 1993; Galanti *et al.*, 1997). With respect to fish, they suggested that the association may be different according to the local iodine

**Table 5. Odds ratios and corresponding 95% confidence intervals<sup>a</sup> of thyroid cancer by oral contraceptive use**

	Oral contraceptive use	
	Never	Ever
McTiernan <i>et al.</i> (1987)	1	1.00 (0.60–1.68)
Western Washington, USA		
Preston-Martin <i>et al.</i> (1987)	1	1.45 (0.81–2.59)
Los Angeles, USA		
Rion <i>et al.</i> (1987)	1	1.13 (0.61–2.11)
Connecticut, USA		
Kolonel <i>et al.</i> (1990)	1	0.64 (0.39–1.07)
Hawaii, USA		
Franceschi <i>et al.</i> (1990)	1	0.95 (0.61–1.49)
Northern Italy		
Levi <i>et al.</i> (1993)	1	1.26 (0.70–2.29)
Vaud, Switzerland		
Wingren <i>et al.</i> (1993)	1	1.22 (0.73–2.03)
Southeastern Sweden		
Hallquist <i>et al.</i> (1994)	1	0.82 (0.47–1.42)
Northern Sweden		
Galanti <i>et al.</i> (1995a)	1	0.93 (0.51–1.69)
Uppsala, Sweden		
Preston-Martin <i>et al.</i> (1993)	1	1.57 (0.87–2.84)
Shanghai, China		

<sup>a</sup>Estimates from multiple logistic regression equations conditioned on age, and adjusted for radiation history, education, parity, type of menopause, and age.

availability (i.e., there may be a positive association in the presence of iodine excess, but a negative one with iodine deficiency) (Franceschi *et al.*, 1993; Glatte *et al.*, 1993).

## Discussion

Several menstrual and reproductive factors have been related to thyroid cancer risk, but often subsequent studies have failed to confirm the findings. This is not surprising since investigations on these topics have not included more than a few hundred women and the available data indicate that these factors are only weakly related to thyroid cancer risk, if at all. Even the few consistent findings (i.e., associations with early menopause, late age at first birth, and recent OC use) may well have been missed in smaller data-sets. Indeed, the associations

**Table 6. Highest incidence and mortality rates of thyroid cancer in females (world-standardized) and female to male (F to M) ratios**

Country, area	Rate	F to M Ratio
<i>Incidence<sup>a</sup></i>		
Colombia, Cali	6.6	3.7
Ecuador, Quito	6.3	2.2
US, Los Angeles, Japanese	6.9	4.9
Filipino	7.9	1.7
US, Hawaii, White	8.7	3.2
Japanese	6.3	1.5
Hawaiian	9.6	1.8
Filipino	24.2	3.7
Chinese	11.3	1.4
Philippines, Manila	8.6	2.5
Iceland	8.3	1.3
<i>Mortality<sup>b</sup></i>		
Iceland	0.9	1.0
Hungary	0.9	1.5
Malta	0.8	1.6
Austria	0.8	1.3
Singapore	0.8	1.0
Switzerland	0.7	1.2
Italy	0.7	1.4
Germany, FRG	0.7	1.2
Germany, GDR	0.7	1.4
Finland	0.7	1.4
Czechoslovakia	0.7	1.4
Israel	0.7	1.2

<sup>a</sup>From Parkin *et al.* (1992).

<sup>b</sup>From Levi *et al.* (1994).

observed are often of similar magnitude (e.g., age at first birth), though not always in the same direction (e.g., age at menopause), as those reported for breast cancer (Lipworth, 1995), which required studies of several thousand cases to be adequately assessed (Collaborative Group on Hormonal Factors in Breast Cancer, 1996; Talamini *et al.*, 1996). Thus, these data suggest that menstrual and reproductive factors are weak correlates of subsequent thyroid cancer risk. The apparently stronger effect of most menstrual and reproductive factors on neoplasms occurring at younger age (Franceschi *et al.*, 1993) suggests that hormonal factors have a late-stage (promotional) effect on

**Table 7. Relative risk of thyroid cancer by residence in endemic goitre areas, Italy, 1986-92 and Sweden 1980-92**

Duration of residence (years)	Sex		Age (years)		Type		All
	Male	Female	< 50	> 50	Papillary	Follicular	
<b>Italy<sup>a</sup></b>							
Never	1	1	1	1	1	1	1
< 20	0.9	1.6	1.3	1.1	1.3	1.9	1.3
> 20	1.1	2.2 <sup>b</sup>	2.5 <sup>b</sup>	1.1	1.7	1.9	1.6
<b>Age at 1st residence</b>							
≤ 25	0.7	1.1	—	—	—	—	1.0
> 25	1.4	2.7 <sup>b</sup>	—	—	—	—	2.4 <sup>b</sup>
<b>Sweden<sup>c</sup></b>							
Never	—	—	1	1	1	1	1
≤ 10	—	—	1.3	0.8	0.9	1.8	1.1
11–20	—	—	0.7	0.9	0.9	0.3	0.8
21–40	—	—	0.8	2.3	1.1	1.9	1.3
> 41	—	—	2.0	1.7	1.5	1.2	1.5
<b>Age at 1st residence</b>							
< 1	1.2	1.1	—	—	0.9	1.8	1.1
1–10	0.9	1.0	—	—	0.9	0.3	0.8
11–20	0.5	2.1	—	—	1.1	1.9	1.3
> 21	0.9	0.9	—	—	1.5	1.2	1.5

<sup>a</sup> Franceschi *et al.* (1989) and D'Avanzo *et al.* (1995).

<sup>b</sup> Statistically significant (95% confidence interval does not include unity).

<sup>c</sup> Galanti *et al.* (1995b).

thyroid cancer, as on several other hormone-related neoplasms, and their influence seems to level off soon after exposure ceases (Day, 1983; Bruzzi *et al.*, 1988).

Some of the associations observed, including the association with early or artificial menopause, may well be due to diagnostic or ascertainment bias since, for instance, women undergoing surgical menopause may be more carefully surveyed for any hormonal — including thyroid — imbalance (Franceschi *et al.*, 1993). Likewise, some excess of cancer risk in current OC users could be due to increased surveillance for thyroid masses among OC users. The majority of thyroid carcinomas in young women, in fact, are detected in the absence of symptoms or signs, as a consequence of examination for other reasons (Franceschi *et al.*, 1993). However, these findings may reflect a real association restricted to current or recent use. Again, this

pattern of risk is similar to that described for breast cancer, for which the association with OC use seems also to be exclusive to current or recent users (Collaborative Group on Hormonal Factors in Breast Cancer, 1996; La Vecchia *et al.*, 1996). A better quantification of the roles of reproductive and menstrual factors and, of greater public health importance, OCs and hormone replacement therapy will emerge from a pooled analysis of individual data from 14 studies, partly unpublished, including about 2300 female cases of thyroid cancer and 3700 control women.

With respect to the role of elevated TSH levels, epidemiological findings on thyroid cancer excess in iodine-rich areas as well as in iodine-deficient areas seem contradictory. Both iodine deficiency and iodine excess may lead to enhanced TSH secretion. Endemic goiter due to iodine excess has been demonstrated in Japanese and Norwegian

Table 8. Relative risk of thyroid cancer in females by history of benign nodules and goitre

Reference	Type of case-control study	Benign nodules	Goitre
McTiernan <i>et al.</i> (1987)	Population-based	10.5 <sup>a</sup>	
Preston-Martin <i>et al.</i> (1987)	Population-based	12.0	6.6
Ron <i>et al.</i> (1987)	Population-based	33.3	5.6
Franceschi <i>et al.</i> (1989)	Hospital-based	Infinity <sup>b</sup>	9.6
Preston-Martin <i>et al.</i> (1993)	Hospital-based	16.0	7.0
Goldman <i>et al.</i> (1990)	Historical follow-up	11.7	2.6
Levi <i>et al.</i> (1991)	Hospital-based	18.3	3.8

<sup>a</sup> Benign nodules and/or goitre<sup>b</sup> No control subjects reported the disease.

fishermen who eat large quantities of iodine-rich seaweed (Suzuki *et al.*, 1965; Okamura *et al.*, 1987), and in locations in China where iodine concentration in drinking water is very high (Li *et al.*, 1987), and iodine-rich salt or pickled vegetables (Zhu *et al.*, 1984) are ingested in large quantities. Direct epidemiological evidence is, however, very scanty. Only one study provided information on TSH levels in the preclinical phase of thyroid cancer (Thoresen *et al.*, 1988), in which 43 patients with thyroid cancer were compared with 128 healthy controls, for whom sera had been collected on average four years earlier, in a large Norwegian serum bank. No difference in TSH level was found, whereas levels of TBG were increased among thyroid cancer patients compared with controls. The increased TBG levels were interpreted as either a secretory product from a slowly growing subclinical tumour or a leakage phenomenon from normal follicles (Thoresen *et al.*, 1988).

Although the importance of accurate histological typing in the study of thyroid cancer cannot be overemphasized, the possibility that correction of iodine deficiency can induce an increase in papillary carcinomas (the so-called papillarization hypothesis) (Rolon, 1986) remains difficult to confirm, but seems unlikely (Pettersson *et al.*, 1996). The elimination of iodine deficiency has been accompanied in affluent countries by elevation of diagnostic standards (e.g., use of thyroid scintigraphy, ultrasound, and fine-needle biopsy). Such improvements account in large part for the increased incidence, since a 'pool' of individuals with clinically silent papillary carcinomas is

probably present in most populations, as shown by the high prevalence of such lesions (up to over 30%) in autopsy series (Franceschi *et al.*, 1993; Ron, 1996).

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# Thyroid stimulating hormone (TSH)-associated follicular hypertrophy and hyperplasia as a mechanism of thyroid carcinogenesis in mice and rats

G.A. Thomas and E.D. Williams

## Introduction

The mammalian thyroid is composed of two types of epithelial cell: the follicular cells, which line the lumen of the colloid-filled follicles and are responsible for the production of thyroid hormones, and the C cells, which are derived from the neuroectoderm, are parafollicular in position in most mammals and are responsible for the production of calcitonin. In both humans and rodents, the cells lie between the follicular cells and the basement membrane, while in dogs they form islands that lie in the interfollicular space. In humans there is no great change in either follicular or C cells with age, but laboratory rats show an age-related increase in the number of C cells. This may correlate with the fact that tumours of the C cells are a relatively common finding in aged rats, although the frequency varies with sex and strain from 0% to 47%, with the highest frequency in the male WAG/Rij rat (DeLellis, 1994). In contrast, C cell number does not increase with age in the mouse, and the incidence of C cell tumours is very low (DeLellis *et al.*, 1996). In humans, C cell numbers are relatively much lower than in the laboratory rodent, and spontaneous C cell tumours (medullary carcinomas) form less than 10% of all thyroid malignancies. They can occur as part of a multiple endocrine neoplasia syndrome, which is associated with a germline mutation in the *ret* proto-oncogene. C cell tumours and differentiated tumours derived from the follicular cells can usually be easily distinguished by the use of immuno-cytochemical techniques for calcitonin and thyroglobulin.

In contrast to C cell tumours, spontaneous tumours of the follicular cell are rare in both rats

and mice. In mice, the frequency in all strains studied is of the order of 1% (Thomas & Williams, 1996); in rats, some variation has been noticed with strain, but the incidence is still lower than 3% (Thomas & Williams, 1994), and such tumours usually occur only in aged animals. The majority of spontaneous tumours are benign. However, the incidence of follicular cell tumours (both benign and malignant) is greatly increased when animals are maintained on a diet which is low in iodide or contains substances known to interfere with thyroid hormone homeostasis (goitrogens). Administration of a mutagen followed by long-term goitrogen treatment can increase the incidence of follicular cell tumours still further.

## Pathology of thyroid follicular proliferative lesions

The majority of proliferative lesions found in both man and animals are benign. Two types of benign lesion can be distinguished: the nodule and the adenoma. Adenomas are defined as being solitary, encapsulated and having a relatively uniform internal architecture and cytology, differing from the surrounding thyroid. They commonly compress the adjacent gland. Nodules are typically less well circumscribed, have a more variable architecture and cytology, which may in part be similar to background thyroid, are unencapsulated and do not compress adjacent tissue. In toxicological studies, multiple lesions are often observed in a background of follicular cell hyperplasia, and distinction between nodule and adenoma can be difficult.

Distinction between nodule and adenoma has been made not only on grounds of morphology

but also on differences in clonal origin in a study in mice. Nodules, in addition to the similarities in their morphology to background thyroid, are polyclonal in origin, whereas adenomas, which show distinct morphological changes from background normal or hyperplastic thyroid, are monoclonal in origin (Thomas *et al.*, 1989).

Nodules and adenomas also occur in humans, where the same morphological criteria are applied for their distinction, and the same difficulties occur in making this distinction in some lesions. Multiple nodules are particularly common in areas of iodide deficiency.

Carcinomas of the thyroid follicular cells in man may be differentiated or, more rarely, undifferentiated. The differentiated types fall into two main groups, named after the dominant architectural pattern in the classical lesions — papillary or follicular. The separation of these two types is now recognized to correlate with a different pattern of oncogene change, different clinical behaviour and different epidemiological associations. The diagnostic importance of the architectural pattern is now considered less specific, but the names remain. Attempts have been made to separate carcinomas in rodents into the same groups as in humans, but while there are architectural differences between tumours, the evidence that these correlate with biologically different entities, as they do in humans, is lacking. In practice carcinomas in rodents show morphologically similar characteristics to adenomas, but are distinguished from them by the evidence of invasion through the capsule into veins and surrounding tissue.

### Thyroid hormone homeostasis

The induction of thyroid follicular cell tumours in rodents is greatly influenced by the level of growth stimulation from thyroid-stimulating hormone (TSH). It is therefore important in considering mechanisms of carcinogenesis in the thyroid to bear in mind some of the steps involved in thyroid hormone synthesis, secretion and metabolism.

Thyroid hormone homeostasis utilizes a variety of mechanisms, many of which have been shown to be affected by diet and xenobiotic agents. Synthesis of the thyroid hormones tri-iodothyronine (T<sub>3</sub>) and tetra-iodothyronine (thyroxine, T<sub>4</sub>) is dependent upon the dietary supply of iodine. Briefly, iodide is taken via a

symporter into the thyroid follicular cell, where it is quickly oxidized by the thyroid-specific enzyme thyroid peroxidase and bound at the apical membrane to tyrosyl residues on the thyroid-specific protein, thyroglobulin. The newly iodinated thyroglobulin molecule lies next to the apical membrane, just within the follicular lumen, where the iodide is bound in the form of mono-iodotyrosine (MIT) and di-iodotyrosine (DIT). These two compounds are then coupled (again under the influence of thyroid peroxidase) to give T<sub>3</sub> and T<sub>4</sub>, which remain bound to thyroglobulin in the follicular lumen. Thyroglobulin is taken up from the lumen by a follicular cell by pinocytosis, and MIT, DIT, T<sub>3</sub> and T<sub>4</sub> are released. T<sub>3</sub> and T<sub>4</sub> pass into the circulation, where they are bound to plasma proteins, whereas MIT and DIT are enzymatically deiodinated in the follicular cell to release iodine for more hormone production.

Thyroid-binding globulin (TBG) is the main plasma protein which binds thyroid hormones in humans. It has a greater affinity for T<sub>4</sub> than T<sub>3</sub>. Two other human proteins are known to bind thyroid hormones, TBPA (which is responsible for 15% of bound plasma thyroid hormone) and albumin. In the rat, the majority of thyroid hormone in plasma is bound to albumin. Only low levels of a protein showing 70% homology to human TBG are present (Shuji, 1991). However, the level of TBG in the rat is upregulated by thyroidectomy, suggesting that it may play some physiological role (Nanno *et al.*, 1986). More T<sub>4</sub> than T<sub>3</sub> is released from the thyroid, but T<sub>4</sub> is metabolized peripherally to give local production of T<sub>3</sub>, the more active hormone.

### Metabolism of thyroid hormones

Thyroid hormones are taken up into the liver, where most conversion to T<sub>3</sub> takes place, by an active process (Krenning *et al.*, 1978) and are then metabolized by deiodination. There are three distinct deiodinases which show different tissue distribution. 5'-Deiodinase I is found in most tissues, including thyroid itself, but is most abundant in liver and kidney. It is capable of both outer-ring deiodination to give T<sub>3</sub> and inner-ring deiodination to give rT<sub>3</sub>, which is not produced by the thyroid, and as yet has no known function. 5'-Deiodinase II, a selenium-containing protein, occurs in the central nervous system, anterior



pituitary and in brown adipose tissue. It deiodinates the outer ring only and its preferred substrate is T<sub>4</sub>. 5'-Deiodinase III is found only in the brain, skin and placenta and deiodinates only the inner ring. It shows a high affinity for both T<sub>3</sub> and T<sub>4</sub>. The latter two deiodinases are thought to give rise only to local T<sub>3</sub> and rT<sub>3</sub> production; in the pituitary thyrotroph cells, this locally produced T<sub>3</sub> is very important in regulating the level of TSH secretion.

Apart from local peripheral tissue production of the more active hormone, T<sub>3</sub>, deiodination is the major route of catabolism of the thyroid hormones in humans, resulting ultimately, after stepwise deiodination, in the production of thyronine. Thyroid hormones are also sulfated by the action of phenol sulfotransferases, and this process is believed to facilitate deiodination. In addition, they are subject to glucuronidation. This pathway is less important in humans, but in rat it is the major route for catabolism of thyroid hormones.

The circulating T<sub>4</sub> level is monitored by the thyrotrophs of the anterior pituitary, which are responsible for the production of the major trophic hormone involved in both thyroid function and growth, TSH. T<sub>4</sub> is metabolized to T<sub>3</sub> by 5'-deiodinase II in the thyrotroph: T<sub>3</sub> then binds to nuclear receptors in the cell. A decrease in T<sub>3</sub> receptor occupancy results in increased synthesis of TSH. A higher tier of control exists in the hypothalamus, which secretes thyrotropin-releasing hormone (TRH), which also stimulates release of TSH from the thyrotrophs.

### **Xenobiotic effects on thyroid hormone homeostasis**

The complexity of both the production and the catabolism of thyroid hormones provides many mechanisms with which xenobiotics can interact. Broadly, these agents can be divided into five categories (Table 1). Class 1 comprises directly acting agents, such as those which interfere with thyroid hormone production either by inhibiting iodine uptake or by inhibiting the thyroid peroxidase stimulated organification of iodide. Class 2 compounds stimulate T<sub>4</sub> clearance, predominantly through an effect on the liver. 2A compounds induce hepatic microsomal enzymes, leading to increased biliary clearance of T<sub>4</sub>, while 2B compounds affect the hepatic transport of thyroid

hormones. Class 3 compounds influence the deiodination of thyroid hormones, either by inhibiting 5'-deiodinase II (e.g., iopanoic acid) or by stimulating 5'-deiodinase I (e.g. phenobarbital). Class 4 includes compounds which affect the plasma binding of thyroid hormones and Class 5 certain neurotransmitters including dopamine which have been implicated in the control of TSH output via TRH. Virtually all compounds which induce thyroid follicular tumours in animals in the long term have been shown to interfere with thyroid hormone homeostasis in the short term. The majority are restricted to classes 1 and 2. A more exhaustive list can be found in Atterwill *et al.* (1992).

Interaction of compounds with thyroid hormone homeostasis has been shown by a variety of different methods, both *in vitro* and *in vivo*. Substances belonging to Class 1 lead to thyroid hyperplasia but reduced thyroid uptake of radioiodine, while substances belonging to Classes 2, 3 and 5 induce thyroid hyperplasia and increased uptake of radioiodine. Substances belonging to Class 4 may, like the other groups, lower total serum levels of T<sub>3</sub> and T<sub>4</sub>. This is followed by transient alterations in circulating TSH and thyroid hormone clearance. These transient effects lead to a new equilibrium and complete adjustment in terms of circulating thyroid hormone and TSH usually occurs. Exceptions to this are compounds, such as polychlorinated biphenyls, which have additional effects on thyroid hormone catabolism (Atterwill *et al.*, 1992). Further tests using assessment of radioactive uptake of iodide or organification of iodide can be carried out using cultured thyroid cells, to separate direct effects on thyroid hormone synthesis from those on catabolism. Where a direct effect on thyroid hormone synthesis has been excluded, further tests to study the effect of xenobiotics on the clearance of radiolabelled T<sub>4</sub> *in vivo* can be carried out. The exact mechanism by which compounds increase T<sub>4</sub> metabolism can be elucidated by measuring the activity of deiodinases, of uridine diphosphate glucuronyl transferase (UDT-GT) or of  $\beta$ -glucuronidase *in vitro*. Finally, effects on the active uptake of thyroid hormones into the hepatocyte can be separated from the effects on glucuronidation using cultured hepatocytes. The Gunn rat carries a germline point mutation in the gene that

**Table 1. Classes of xenobiotics which interfere with thyroid hormone homeostasis, divided according to their mode of interaction**

Class	Action	Example	Tumour induction in animals	Reference
1	Direct action on thyroid (inhibition of either iodine uptake or thyroid peroxidase)	Propylthiouracil	Yes	Taurog (1976)
2	Stimulation of T4 clearance	Phenobarbital	Yes	McClain (1989)
3	Affect deiodinase action	Iopanoate	No	Cavallieri & Pitt-Rivers (1981)
4	Affect binding of thyroid hormone to plasma proteins	Salicylates	No	Cavallieri & Pitt-Rivers (1981)
5	Receptor-mediated	Clomiphene	No	Wenzel (1981)

An exhaustive list can be found in Atterwill *et al.* (1992).

encodes a transcript which is preferentially spliced to give rise to UDT-GT I and II, the enzymes responsible for glucuronidation of T4 and rT3. T3 is glucuronidated by the action of UDT-GT III, which is deficient in Waj rats (Matsui & Hakoziaki, 1979). Using hepatocytes isolated from the Gunn or the Waj rat, it is therefore possible to distinguish between effects on glucuronidation of thyroid hormones and active uptake of T3 and T4 into the hepatocyte. The mechanism of antithyroid action of two H2 antagonists, famotidine and ranitidine, was carefully dissected in a series of papers by Atterwill and colleagues, a study involving many of these in-vitro techniques (Brown *et al.*, 1986, 1987, 1988; Atterwill *et al.*, 1989).

#### The role of mutagens in thyroid carcinogenesis

A number of different mutagens have been used in regimes specifically designed to induce thyroid tumours in rodents. Aromatic amines such as acetylaminofluorene (Doniach, 1950), azo dyes such as 4,4'-methylene-bis-(*N,N*-dimethyl)-benzylamine (Murthy, 1980), nitrosamines, e.g. diisopropanolnitrosamine (DHPN) (Mohr *et al.*, 1977), *N*-bis-(2-hydroxypropyl)-nitrosamine (Hiasa *et al.*, 1982) and the nitrosoureas, e.g.,

methylnitrosourea (MNU) (Schäffer & Müller, 1980; Milmore *et al.*, 1982; Tsuda *et al.*, 1983) have all been shown to induce a low frequency of thyroid tumours in rats. In at least two of these studies, an effect of the mutagen concerned on thyroid homeostasis has been identified (DHPN: Hiasa *et al.*, 1982; MNU: Milmore *et al.*, 1982) evidenced by either a transient rise in TSH or a decrease in circulating T4. Whether this is related to a cytotoxic effect of these compounds on follicular cells, with a resultant decrease in T4 production, or to other actions is not clear. Chemical mutagens are rarely used alone in studies of thyroid tumorigenesis; combination regimes of mutagen plus a goitrogen or mutagen plus a low-iodide diet are much more effective than mutagen alone (Milmore *et al.*, 1982; Tsuda *et al.*, 1983; Doniach, 1950). Agents which demethylate DNA, such as 5-azacytidine, can also potentiate goitrogen-induced thyroid carcinogenesis, although such agents do not show the marked effect of the combination of a mutagen and goitrogen (Thomas & Williams, 1992).

Irradiation, whether external (X-ray) or internal (by administration of isotopes of iodine), has been used extensively in experimental studies of

thyroid tumorigenesis. However, it is important to realize that radiation has two effects on thyroid: a mutagenic effect and a growth stimulatory effect. Experiments with rats have shown a bell-shaped rather than a linear dose-response curve for the tumorigenic effect of radiation in the thyroid, with 30  $\mu$ Ci giving the highest frequency of thyroid tumours in adult rats (Doniach, 1953). This dose lies within a range which demonstrably interferes with thyroid function (Maloof *et al.*, 1952). Further evidence of a second component to radiation-induced thyroid carcinogenesis comes from studies in which one thyroid lobe was shielded from X-irradiation. Tumours developed in both lobes, but were more frequent in the unshielded lobe (Nichols *et al.*, 1965). In addition, radiation-induced carcinogenesis can be inhibited by hypophysectomy (Nadler *et al.*, 1970) or by long-term T4 administration (Doniach, 1974), suggesting that release of TSH from the pituitary plays a key role in radiation-induced thyroid carcinogenesis.

The bell-shaped dose-response curve to radiation with respect to thyroid carcinogenesis can easily be explained. Low doses of radiation cause little thyroid cytotoxicity and therefore little if any rise in TSH, and they give a low frequency of mutations in the DNA of follicular cells, and therefore a low rate of thyroid tumorigenesis. As the dose of radiation increases, the cytotoxicity of radiation increases, resulting in a loss of functioning thyroid follicular cells, as well as an increase in mutation frequency in those cells which survive. The increased cytotoxicity results in a decrease in T4 and an increase in TSH, which stimulates the remaining thyroid follicular cells to grow. At the peak combination of mutation frequency and TSH-induced follicular cell growth, follicular tumours occur at a high frequency. Higher doses of radiation reduce the number of cells which retain the ability to divide, both through direct cytotoxic effects and through mutation overload, so that the frequency of tumours drops. At very high doses, all or virtually all follicular cells die, and no tumours occur. The morphological type of carcinoma induced in animals by radiation does not differ from that induced by goitrogens alone, but combination of mutagens and goitrogens leads to an increase in the number of carcinomas compared to adenomas.

The role of radiation exposure in carcinoma of the thyroid has recently become more prominent following the exposure of a large population to isotopes of iodine, in fallout from the Chernobyl nuclear accident which occurred in 1986 (Baverstock *et al.*, 1992; Kazakov *et al.*, 1992; Likhtarev *et al.*, 1995). The age distribution and morphology of the cases of thyroid cancer which have so far occurred in the population of southern Belarus and northern Ukraine, the areas closest to the reactor, provide interesting information regarding the role of factors other than mutagens in thyroid tumorigenesis in humans. Firstly, there is an age-related susceptibility to the development of thyroid carcinoma following radiation exposure, with the youngest children (under four years) at the time of exposure being very much more susceptible than older children or adults (Williams *et al.*, 1996a). This has also been observed in studies of age of exposure to radiation for therapeutic or diagnostic purposes (Schneider *et al.*, 1986; Ron *et al.*, 1987, 1989; Akiba *et al.*, 1991). There are few reports of age-related susceptibility to radiation-induced experimental thyroid carcinogenesis, but these too suggest that exposure to radioiodine early in life increases the risk of thyroid neoplasia (Walinder, 1972; Walinder & Sjöden, 1972). Secondly, in contrast to the results of animal studies, there appears to be a specific link between exposure to radioiodine in fallout and the morphological type of thyroid cancer induced in humans. The majority (> 95%) of thyroid cancers that can be linked to exposure to radioactive fallout have been papillary carcinomas.

### **TSH and the mechanism of induction of thyroid tumours in humans and animals**

There is overwhelming evidence to suggest that TSH-induced growth of the thyroid epithelial cell plays a critical role in thyroid tumorigenesis in animals and in one type of human thyroid carcinoma. The majority of agents which induce experimental thyroid tumours, usually after prolonged administration, interfere with thyroid hormone homeostasis (Hill *et al.*, 1989). The xenobiotic induction of both thyroid hyperplasia and neoplasia can be inhibited by either hypophysectomy or concomitant T4 administration (Nadler *et al.*, 1970; Doniach, 1974). The importance of TSH-induced growth can also be

demonstrated by the observation that multiple tumours occur in a background of generalized thyroid hyperplasia in those types of congenital hypothyroidism where one of the key steps of thyroid hormone synthesis is absent (dyshormonogenesis). Congenital hypothyroidism from this cause is rare, and occurs in both humans and animals most commonly as a result of mutations in either the thyroid peroxidase or the thyroglobulin genes (Falconer *et al.*, 1965; van Jaarsfeld *et al.*, 1972; de Vijlder *et al.*, 1978; Salvatore *et al.*, 1980). In patients with dyshormonogenesis, the elevated TSH is present from birth, and is now used to detect these cases by neonatal screening. In the untreated severe cases, the continuous high level of TSH leads to diffuse hyperplasia, and multiple benign thyroid tumours commonly develop after some years (often 10 years or more). Carcinoma has been described in patients with dyshormonogenesis, but is very rare (Vickery, 1981).

Thyroid tumours are also more common in man in areas of iodide deficiency (Medeiros-Neto, 1995), and can be induced by iodide deficiency in animals (Axelrad & Leblond, 1955; Schaller & Stevenson, 1966). The effect of iodide deficiency can be exacerbated by the presence of goitrogens in either the diet or drinking water (Ekpechi *et al.*, 1966; Gaitan, 1988). Both iodide deficiency and goitrogen administration cause levels of TSH to rise, and TSH controls not only thyroid cell function, but also growth. When the decrease in the level of circulating T4 is small or short-lived, the deficiency may be redressed by an increase in thyroid cell function alone. However, if the decrease is more severe or prolonged and cannot be redressed by an increase in function alone, an increase in number of units of thyroid hormone production (i.e., the follicular cells) is required. This leads to thyroid follicular cell hyperplasia with the development of a goitre. However, the thyroid follicular cell in the rat possesses only a limited growth potential. Sustained high-dose goitrogen administration results in a rapid increase in follicular cell mitotic activity over 7 to 14 days, but this then falls to relatively low levels after about three months, while the level of TSH remains high. If the goitrogen administration is maintained, tumours of the follicular cell are observed after about eight months (Wynford-Thomas *et al.*, 1982). A similar

observation has been made for the mouse (Peter *et al.*, 1991).

The majority of the tumours induced by prolonged goitrogen administration in rodents, in the absence of mutagen, are benign. The finding by several authors (Jemec, 1977; Todd 1986) that follicular tumours regressed on withdrawal of goitrogen administration has led to some confusion over the distinction between hyperplastic and neoplastic lesions. However, more recent studies have demonstrated that monoclonal lesions induced in mice by a combination of radiation and goitrogen (including carcinomas that clearly showed capsular and vascular invasion), regressed on withdrawal of the goitrogen (Thomas *et al.*, 1991). The clonal nature of these lesions suggests that they are truly neoplastic and derive by clonal evolution from a single cell, but their regression suggests that they retain dependence, at least in part, on TSH for growth. One earlier study using serial transplantation of thyroid tissue from rats maintained for 18 months on a low-iodide diet suggested that the tumour phenotype was lost on transplantation to euthyroid rats, but re-emerged when a serial transplantation was made to a hypothyroid rat (Matovinovic *et al.*, 1970). These results suggest that thyroid follicular epithelial cells can undergo a heritable change resulting in tumour formation in animals with sustained high TSH levels, but the heritable change is insufficient to support the tumour phenotype in the absence of the TSH growth stimulus.

The mechanism by which goitrogen-induced thyroid tumours arise is far from clearly understood. We suggest that three key steps are important in spontaneous thyroid carcinogenesis. First, as the normal follicular cell possesses a growth-limiting mechanism, the continued growth of tumour cells implies that any such mechanism must be deactivated in neoplasia. Second, development of TSH-independent growth is likely to be essential for spontaneous thyroid carcinogenesis. Thirdly, cells must acquire the ability to invade – an essential ingredient for malignancy. Each of these steps may involve alteration in more than one gene; for example, loss of tumour-suppressor gene function may require mutation in both copies of the gene, and gain of independent growth could involve

successive mutations in different growth control genes.

The development of these successive defects that lead to neoplasia can be considered as a process of natural selection at a cellular level: manipulation of the environment in which selection takes place may alter the selection process. In experimental thyroid carcinogenesis, the induction of persistently high TSH levels creates an environment in which any cell that suffers a mutation or mutations leading to loss of its growth-limiting mechanism will give rise to a clone of cells which will continue to grow and therefore continue to be at risk of further gene alteration in the progeny of that cell. If the cell is exposed to maximal growth stimulation from a very high TSH level, mutation in growth control genes may not lead to any additional growth, and may not therefore confer any selective advantage. When invasiveness develops in a cell that has not acquired TSH-independent growth, the resulting carcinomas retain TSH-dependence and will regress when TSH stimulation is withdrawn (Thomas *et al.*, 1991).

Further evidence for the requirement of thyroid follicular cell growth in thyroid neoplasia can be found from the increase in thyroid cancer after radiation. The greatest risk appears to be to those who were young children at the time of exposure. There appears to be little or no increased risk after exposure to radiation as an adult. Tumour incidence following radiation in animals can be increased either by exposure of neonatal animals or by combination of radiation exposure with goitrogen administration. Developmental thyroid growth continues in the mouse until about six weeks of age and in the human until adulthood. In addition to the arguments for greater uptake of radioiodine in the immature thyroid, two other factors may contribute to the higher risk of thyroid neoplasia following radiation early in life. Carcinogenesis is a multistep phenomenon and requires the acquisition of multiple mutations in a clone of cells. Mutation is more likely to occur at S phase. The immature thyroid has a higher proportion of dividing cells compared with the adult, and is therefore more likely to be susceptible to the mutational effects of radiation. In addition, the phase of developmental growth which occurs results in cells that have been exposed to radiation

and therefore possibly harbour mutations which are deleterious to growth regulation, undergoing several more rounds of cell division, thus increasing the probability of acquiring additional deleterious mutations. It is therefore not surprising that exposure to a mutagen during thyroid developmental growth carries an increased risk of development of thyroid tumours later in life.

Recent research has thrown light on some of the molecular events involved in thyroid carcinogenesis. However, much of the work has centred on identification of oncogene mutations in human thyroid tumours rather than in those tumours generated by long-term xenobiotic administration. In humans, the two different morphological types of thyroid carcinoma are associated with different molecular biological events. Papillary carcinomas are associated with gene rearrangements involving either *ret* (a tyrosine kinase-linked receptor for glial-derived neurotrophic factor) or *trk* (a tyrosine kinase-linked receptor for nerve growth factor) oncogenes (Greco *et al.*, 1992; Santoro *et al.*, 1992). However, the frequency of these rearrangements varies between studies (Ishizaka *et al.*, 1991; Jhiang *et al.*, 1994; Zou *et al.*, 1994); this may partly reflect different sex ratios in the study groups, as we have recently identified a sex difference in papillary carcinomas associated with a *ret* rearrangement (Williams *et al.*, 1996b), or differences in the methodology used to identify *ret* activation. Follicular carcinomas are more frequently associated with point mutations in the *ras* oncogene family, although the absolute frequency of *ras* mutation may vary with the technique used to identify mutation (Lemoine *et al.*, 1989; Manenti *et al.*, 1994). A proportion of benign nodules, which can be demonstrated to possess higher than normal uptake of radioiodine (so called 'hot' nodules) have been shown to be associated with mutation in the TSH receptor, leading to constitutive activation (Parma *et al.*, 1993). The majority of oncogene studies carried out in animals have been those using thyroid-specific expression of specific oncogenes in transgenic animals. These have provided interesting results, with thyroid-specific expression of one of the *ret* rearrangements associated with papillary carcinoma (PTC1) leading to the induction of discrete tumours which possess some of the features of human papillary carcinoma; these differ morphologically from

other experimental tumours (Jhiang *et al.*, 1996; Santoro *et al.*, 1996). Thyroid-specific expression of mutated *ras* oncogenes has produced two differing pathologies, depending on the species of thyroglobulin promoter used in the transgene construct. Only a low yield of discrete benign tumours was produced in transgenic mice carrying an activated *Ki-ras* gene on a rat thyroglobulin promoter (Santelli *et al.*, 1993), whereas mice carrying a mutated *Ha-ras* on a bovine thyroglobulin promoter showed a series of different thyroid pathologies ranging from papillary carcinomas to hyperplasia (Rocheffort *et al.*, 1996). This latter study also gave rise to mice which presented with primary lung tumours with papillary morphology, which is slightly surprising as the transgene construct would be predicted to be expressed only in thyroid, suggesting that the use of the bovine promoter may lead to ectopic expression of the transgene. The use of viral genes which have been shown to block *p53* and *Rb* gene action, either separately (*p53*: E6, *Rb*: E7), or both together (SV40 large T) has produced a variety of phenotypes, ranging from severe thyroid hyperplasia and neoplasia (SV40; Ledent *et al.*, 1991), or the development of large colloid goitres and malignant poorly differentiated tumours after about one year (E7; Ledent *et al.*, 1995) to nothing more than a slightly enlarged thyroid gland at six months of age (E6; Ledent *et al.*, 1994). This is perhaps in keeping with the observation that, in humans, mutation of *p53* is not involved in differentiated thyroid carcinoma, but only plays a role in the transition from differentiated to undifferentiated thyroid carcinoma. A role for activation of the TSH receptor pathway in potentiation of carcinogenic activity has been shown by crossing yet another transgenic line, which shows constitutive activation of the adenosine A2 receptor (and therefore of the cyclic AMP cascade which is also activated by the TSH receptor) with the E7 line. The offspring show rapid development of thyroid neoplasia with early and frequent metastases (Coppée *et al.*, 1996). However, care must be exercised in generalizing from these transgenic experiments, as the mechanism of tumour production may differ fundamentally from that in normal animals. In transgenic mice, the oncogene is expressed from the time at which the thyroglobulin promoter is active (around day 18 of gestation in rodents), whereas in

xenobiotic-induced neoplasia, oncogenes are activated by somatic rather than germline mutation, and factors which are expressed in different development time frames may also contribute to development of a thyroid tumour. Further information on the molecular biology of xenobiotic-induced thyroid tumours will be required in order to examine mechanistic differences between mouse and man with respect to thyroid tumours.

### Evidence for species specificity

There is an increasing body of evidence that there are differences in key steps of thyroid hormone synthesis and catabolism between species. Biochemical assays of the inhibitory effect of sulfamethoxazole on thyroid peroxidase in various species have shown that, compared with the rat enzyme, monkey thyroid peroxidase shows significantly less inhibition at the same concentration (Takayama *et al.*, 1986).

Clinically significant antithyroid effects in humans following the use of this and structurally related compounds are lacking (McLaren & Alexander 1979), suggesting that, not surprisingly, humans may be more like monkey than rat, at least with regard to the effect of sulfamethoxazole. In addition, other studies have shown that the H2 antagonists temelastine and lupitidine increase thyroid hormone uptake in isolated rat hepatocytes, but not in a human hepatoblastoma cell line (Aylward, 1995). This suggests that there may be differences in the transporter proteins involved in liver T3 and T4 uptake between rat and human. More evidence of this type is required. However, one should always be aware of the genetic diversity of humans and the limits on the availability of human tissues. Established cell lines are usually derived from cancer cells and have been maintained in culture for long periods. It is therefore possible that some of the effects observed with respect to species differences may be epiphenomena due to the carcinogenic process which occurred *in vivo* or artefacts induced by prolonged maintenance in culture. Wherever possible, studies utilizing cell culture should involve some performed on primary cultures, with adequate controls for sex, age and pre-existing disease of the donor. Such studies will provide interesting data to help dissect the mechanisms by which xenobiotics interfere with thyroid hormone

homeostasis and whether such a mechanism is likely to be relevant to the human.

In addition to biochemical differences, differences in exposure of humans to such compounds should be taken into account. A good case has already been made for the existence of a threshold effect in thyroid carcinogenesis (Paynter *et al.*, 1988). The majority of compounds induce thyroid tumours only after prolonged, high-dose administration in animals, and for most compounds it is unlikely that human exposure would reach such levels (even if the human system involved is as sensitive as that in the test animal). Human exposure more frequently involves a small dose of several different compounds rather than prolonged exposure to a large dose of one. However, little work has been done to study the interaction of compounds which affect different steps in hormone synthesis or catabolism.

### General hypothesis for thyroid carcinogenesis

To conclude, we summarize some of the discussion and in particular put forward a specific hypothesis for the development of thyroid neoplasia. Tumours in any stable tissue require a growth rate that is higher than that of the tissue of origin. The increased growth can result from loss of tumour-suppressor genes, gain-of-function mutations in oncogenes or both. Malignant tumours in addition require a genetic defect or defects conferring the ability to invade and/or metastasize. The thyroid, unlike organs such as the skin or intestine, is not a stem-cell tissue; the normal follicular cell has a limited growth capacity, and the normal level of growth stimulation in the adult animal is extremely low. The principal conclusions that we can draw are the following:

- (i) Sporadic tumours of the follicular cells in rodents or humans require both tumour-suppressor gene and oncogene mutations. Such tumours are very infrequent and a significant proportion are malignant or may progress to malignancy.
- (ii) Tumours of the follicular cells induced by exposure to substances inducing a high level of TSH may arise as a result of tumour-suppressor gene mutations, for example in the gene or genes which control the mechanism that desensitizes growth of the thyroid follicular cell in response

to TSH stimulation. Such tumours may develop at a relatively high frequency in long-term exposed animals, but retain at least partial dependence on a high level of TSH for continued growth, and show a low rate of progression to malignancy.

- (iii) Tumours of the follicular cells occur less frequently with xenobiotic exposure at levels that lead to less lowering of thyroid hormone levels, and therefore lesser degrees of TSH elevation. The importance of acquiring oncogene as well as tumour-suppressor gene mutations increases, but the chance of acquiring these decreases as the level of growth stimulation decreases. Under these conditions, exposure to a mutagen becomes increasingly important for thyroid carcinogenesis. The increase in incidence of follicular cell tumours, including malignant tumours, which follows administration of mutagens to rodents, requires follicular cell growth, is enhanced by a sustained stimulus to growth and is reduced or abolished by diminished post-mutagen growth.
- (iv) Nodules, which are polyclonal and not true tumours, occur relatively frequently as a consequence of exposure to high levels of TSH; they do not show any detectable progression to malignancy, retain dependence on TSH for continued growth, and may result from stromal changes, as evidenced by the increased content of stroma in nodules induced in the mouse.
- (v) There is a threshold in the carcinogenic response to xenobiotics which are not mutagens, but lead to thyroid follicular cell tumours through their effect on thyroid hormone synthesis or metabolism. This threshold can be related to either the buffering capacity within the complex system that maintains constant thyroid hormone levels or changes in thyroid hormone levels that are sufficiently small that they can be remedied by a very minor increase in TSH, leading to an increase in follicular cell function, but not growth.

The evidence for the involvement of oncogenes in sporadic adenomas or carcinomas of the follicular cells is derived largely from human studies, where mutations in the three *ras* genes, in *ret* and in *trk* have all been described in either

adenomas or carcinomas. They have not been found in nodules. Mutations in genes of the TSH receptor pathway, including the receptor itself have also been described in human thyroid tumours. Evidence of their involvement in TSH-induced tumours is limited; mutations in the *ras* genes have been found in rat thyroid tumours induced by radiation followed by goitrogen, and in a much smaller proportion of those induced by goitrogen alone (Lemoine *et al.*, 1988). Recent studies have suggested that the frequency of *ras* involvement in human adenomas was overestimated in earlier studies (Manenti *et al.*, 1994); the rat studies need therefore to be repeated.

Evidence for involvement of tumour-suppressor genes in both sporadic and TSH-induced tumours of the follicular cell is circumstantial. Transgenic mice carrying oncogenic mutations in either *ret* or *ras*, with targeted expression in the thyroid, do not show widespread carcinogenic change, only a relatively small number of tumours arising in adult mice. This shows that at least one further event is needed for these genes to lead to thyroid tumour formation.

Evidence that continual follicular cell growth stimulation is required for the carcinogenic process in thyroid follicular cells has been provided. In brief, many studies have shown that the frequency of thyroid tumour formation, including carcinoma formation, after mutagen administration is greatly enhanced by increased growth stimulation by TSH and abolished if TSH stimulation is suppressed by hypophysectomy or suppressive thyroid hormone therapy immediately following administration of the mutagen. The importance of post-mutagen growth is that it allows the possible development of further mutations; these occur much more commonly in dividing than intermitotic cells.

### Comparison of mechanisms of carcinogenesis of follicular cells in rodents and humans

The evidence suggests that essentially the same mechanism of carcinogenesis exists in both rodents and humans, but there are major quantitative differences. In addition, there are species differences in metabolism of some of the xenobiotics which influence thyroid hormone breakdown. The frequency with which long-term prolonged high levels of TSH in rodents leads to nodules, adenomas and carcinomas is well known

and even hemithyroidectomy was associated with an increase in thyroid carcinogenesis (Doniach & Williams, 1962). In contrast, in humans, while nodules and adenomas are relatively common, carcinoma is very rare. Patients with dys-hormonogenesis commonly develop multiple nodules and adenomas; these may occur as early as 7–10 years of age in severe cases, but more commonly they are first noticed in the teens or later. This shows that several years of exposure to very high levels of TSH in infancy will produce benign thyroid tumours. The progression to malignancy, however, is very rare. In a review in 1981, only 17 cases had been reported (Cooper *et al.*, 1981). The development of malignancy after exposure of follicular cells to high levels of growth stimulation in adult life is extremely rare if it occurs at all. Drugs which block thyroid such as carbimazole are very widely used in the treatment of thyrotoxicosis; even when they are used in combination with a mutagen ( $^{131}\text{I}$ ) in patients with Graves disease, no detectable increase in thyroid cancer has been found (Holm *et al.*, 1980, 1988).

Surveys of populations living in areas of iodine deficiency show that nodular goitres and adenomas are significantly more common than in areas with normal iodide intake. The incidence of thyroid cancer is either slightly increased or unchanged. What does change is the type of thyroid cancer, with follicular carcinoma relatively more common in areas of iodide deficiency, while papillary carcinomas are relatively more common in areas of iodide excess (Williams *et al.*, 1977). Here again, exposure to iodide deficiency in the early years of life is probably important.

In our opinion, the risk of development of thyroid carcinoma as a result of exposure to any cause leading to an increase of TSH is very much lower in humans than in animals, requires exposure for several years, is dose-dependent and very probably age-dependent. The risk of human thyroid carcinogenesis following exposure to xenobiotics leading to a small perturbation in thyroid homeostasis is negligible.

One difference between rodents and humans which has been suggested as a cause of different carcinogenic susceptibility is the way in which thyroid hormones are bound in the plasma. In humans, the main circulating protein that binds both thyroxine and tri-iodothyronine is thyroid-



binding globulin (TBG); prealbumin binds a much smaller proportion. In rodents, although TBG is present, prealbumin is the main binding protein; it has a much lower affinity for thyroid hormones than TBG. The level at which the thyroid pituitary axis functions is dependent upon the level of free thyroid hormones; under normal circumstances the system adjusts to maintain a normal free thyroid hormone level. Interspecies variations in binding protein may affect the rate at which adjustment takes place, but we do not think it likely that they will alter the normal steady state, or the long-term response to a consistent change. Humans who have a congenital complete deficiency in TBG appear otherwise normal, with no clinical thyroid abnormalities.

### Gaps in our knowledge

The tumour-suppressor genes involved in the restriction of thyroid follicular cell growth are not known. It seems unlikely that the genes which predispose to retinoblastoma and familial adenomatous polyposis coli play any major role, as thyroid tumours are not a frequent component of the clinical syndromes that accompany human inherited syndromes when these genes are affected. There is more evidence for a link between thyroid and breast cancer, so that the role of *BRCA1* and *BRCA2* and the *PTEN* gene need to be explored, and a further search made for other possibly thyroid-specific tumour-suppressor genes. The *PTEN* gene is of particular interest, as germline mutations of this gene have been shown to be associated with Cowden's syndrome, in which patients present with breast cancers, hamartomas and thyroid disorders (goitre, adenomas and carcinomas), and the gene encodes a receptor tyrosine kinase and is located in a region where loss of heterozygosity has been previously described in thyroid tumours (Li *et al.*, 1997; Liaw *et al.*, 1997). Very little work has been reported on the oncogenes involved in experimentally induced thyroid tumours (as opposed to the use of known oncogenes in thyroid-specific expression in transgenic animals), and this gap needs to be filled using modern methods.

The comparison between the frequency of development of thyroid tumours in response to elevated TSH in humans and animals could be further studied, for example by developing transgenic dys-hormonogenetic mice or rats.

The tumour incidence in these animals, which like human patients with dys-hormonogenesis will have elevated TSH from birth if not before, could then be compared to that observed in humans.

The dose-effect relationship in nongenotoxic thyroid carcinogenesis needs further study, including varying length of time of exposure as well as dose, together with measurements of TSH and careful estimation of the numbers of tumours induced.

In conclusion, we consider that there is effectively no risk of thyroid carcinogenesis in man from limited exposure to low doses of a compound that has been shown to produce thyroid tumours in rodents only when administered for long periods of time at high doses, providing that the substance has been shown not to be mutagenic, and that it has been shown to interfere with thyroid hormone homeostasis by a defined mechanism.

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# A Mechanistic Relationship between Thyroid Follicular Cell Tumours and Hepatocellular Neoplasms in Rodents

R. M. McClain and J. M. Rice

## Introduction

Thyroid follicular cell neoplasms have been reported to be the most common endocrine tumour response observed in carcinogenicity bioassays of pharmaceutical agents, and neoplasms of liver parenchyma and thyroid follicular epithelium often occur together in rodent carcinogenicity studies (Huff *et al.*, 1991; McConnell, 1992; Haseman & Lockhart, 1993). In one analysis of the United States National Toxicology Program (NTP) database, it was noted that chemicals that produced thyroid proliferative changes were more likely than other chemicals to produce hepatocellular neoplasms, in the same or other species. The correlation is not perfect, since many chemicals that produce liver tumours do not produce thyroid tumours, and *vice versa* (McConnell, 1992). For chemicals that do produce tumours at both these organ sites, microsomal enzyme induction has been suggested to be a mechanistic link that connects pathogenesis of thyroid follicular tumours with that of hepatocellular neoplasms (McClain, 1989).

When chemicals are tested at high dosages in subchronic and chronic toxicity tests in rats and mice, increased liver weight and centrilobular hepatocellular hypertrophy are commonly observed. These observations are indicative of microsomal enzyme induction, which can be confirmed by biochemical measurement of tissue levels of enzyme protein, enzyme-specific mRNA, and rates of metabolic conversion of substrates specifically acted upon by these enzymes. Two important classes of enzyme inducers are the phenobarbital-like inducers and the polynuclear aromatic hydrocarbon-like inducers. Phenobarbital and many other substances that comprise the first group induce biosynthesis of a wide variety of enzymes, including P450s CYP2B1 and CYP2B2 (Lubet *et al.*,

1992) and are located principally or exclusively in the liver. The second group includes compounds like 2,3,7,8-tetrachlorodibenzo-*para*-dioxin (TCDD), which act through a cytoplasmic receptor (the Ah receptor), induce predominantly CYP1A1 and CYP1A2 together with other non-P450 genes, and are widely distributed in many tissues and organs (Conney, 1967; Poland & Knutson, 1982). Chemicals that belong to each of these two classes of enzyme inducers have been shown to produce both hepatocellular and thyroid follicular cell neoplasms in rodents.

The short-term consequences of hepatic microsomal enzyme induction in laboratory animals are relatively minor from a toxicologic point of view. Histologic and functional changes may be observed in the liver, and since microsomal enzymes metabolize a variety of endogenous hormones, vitamins and cofactors as well as many xenobiotic substances, reversible, adaptive functional changes in various endocrine organs can occur. However, the long-term consequences of microsomal enzyme induction can be of greater significance, since alterations in tumour incidence in certain organs may result.

## Carcinogenesis and tumour promotion in liver and thyroid gland by phenobarbital

Phenobarbital (PB), the classic example of an inducer of hepatic CYP2B1 and CYP2B2, has been extensively studied as a hepatic tumour promoter following initiating exposures to various DNA-reactive (genotoxic) carcinogens, in rats and to a lesser extent in mice and other rodents and in non-rodent species (Whysner *et al.*, 1996). PB has been shown to promote hepatocarcinogenesis in rats following initiation with 2-acetylaminofluorene (Peraino *et al.*, 1975, 1980), *N*-nitrosodiethylamine (NDEA; Goldsworthy *et al.*, 1984; Pereira *et al.*, 1986;

Pitot *et al.*, 1987), and other agents (Kitagawa *et al.*, 1984; Diwan *et al.*, 1985a). PB is also a hepatocellular tumour promoter in mice (Diwan *et al.*, 1990; Fullerton *et al.*, 1990; Rumsby *et al.*, 1991) and in *Erythrocebus patas* monkeys (Rice *et al.*, 1989) initiated with NDEA, but does not promote in Syrian golden hamsters, at least at doses comparable to those that are effective for tumour promotion in other species (Diwan *et al.*, 1986a).

PB has also been shown to be a tumour promoter for thyroid follicular cell tumours in rats. Hiasa *et al.* (1982a) gave rats *N*-nitrosodihydroxypropylamine (DHPN) for a period of four to six weeks followed by PB at 500 parts per million (ppm) in the diet, and observed a marked increase in thyroid follicular cell neoplasms in male rats given DHPN followed by PB, compared to rats that received DHPN alone. The incidence of thyroid tumours was later shown to increase with duration of treatment with PB (Hiasa *et al.*, 1984) and to be much more pronounced in male than in female rats (Hiasa *et al.*, 1985). This sex difference in susceptibility to thyroid tumour promotion by PB in rats was independently confirmed in another laboratory (McClain *et al.*, 1988). PB has also been shown to promote thyroid tumorigenesis in rats initiated with *N*-nitrosomethylurea (Tsuda *et al.*, 1983; Diwan *et al.*, 1985a) or NDEA (Diwan *et al.*, 1985b).

Prolonged feeding of PB alone, in the absence of an initiating agent, also results in increased hepatocellular tumour incidence in rats (Rossi *et al.*, 1977; Saito *et al.*, 1990) and mice (Peraino *et al.*, 1973; Thorpe & Walker, 1973; Jones & Butler, 1975; Ponomarev *et al.* 1976; Butler & Jones, 1978; Becker, 1982; Evans *et al.*, 1992) that have not received an initiating dose of a genotoxic agent. PB is thus carcinogenic, by the empirical definition of the term. Although PB alone has not been shown to produce thyroid follicular cell tumours in rats, a number of other PB-like inducers have (Hill *et al.*, 1989; McClain, 1989).

### Mechanism of tumour promotion by phenobarbital in thyroid follicular epithelium

It is widely accepted that there are two basic mechanisms whereby chemicals produce thyroid gland neoplasms in rodents (see Thomas & Williams, this volume). One mechanism involves chemicals that exert a direct, genotoxic, carcinogenic effect on the thyroid gland. The other

involves chemicals which, through a variety of mechanisms, disrupt thyroid gland function and produce thyroid gland neoplasms by processes involving persistent hormone imbalance.

The latter was originally formulated to explain pathogenesis of thyroid tumours produced in rodents treated with antithyroid drugs (Furth, 1959, 1969). Antithyroid compounds initially cause hormonal imbalance by interfering with thyroid hormone production. A sustained increase in synthesis and secretion of thyroid stimulating hormone (TSH) to stimulate the thyroid occurs via negative feedback stimulation of the pituitary gland. Increased TSH stimulation produces a variety of morphological and functional changes in thyroid follicular cells including follicular cell hypertrophy and progressing, in the rodent, to enlargement of the gland (goitre) and eventually to neoplasia. That excessive and prolonged secretion of endogenous TSH alone in mice and rats, in the absence of any chemical treatment, will produce a high incidence of thyroid tumours has been clearly established by experiments in which rats were fed diets deficient in iodine (Axelrad & Leblond, 1955; Leblond *et al.*, 1957; Isler *et al.*, 1958; Ohshima & Ward, 1986) or in which TSH-secreting pituitary tumours were transplanted into mice with normal thyroids (Furth, 1954).

The effect of chemicals on various aspects of thyroid hormone metabolism has an important impact on thyroid hormone economy in rodents (Cavalieri & Pitt-Rivers, 1981). The monodeiodinases are quantitatively the most important path in the disposition of thyroxine. These enzymes metabolise T<sub>4</sub> to the active form of thyroid hormone, T<sub>3</sub>, and catalyse subsequent monodeiodinations in the catabolism of thyroid hormone. In addition, thyroxine is glucuronidated by microsomal thyroxine UDP-glucuronyl transferase and the glucuronide is subsequently excreted in the bile (Robbins, 1981). Many chemicals are hepatic microsomal enzyme inducers at high dosages and are known to alter thyroid function in rodents by increasing the rate of hepatic disposition of thyroid hormone (Oppenheimer *et al.*, 1968; Bastomsky & Murthy, 1976; Comer *et al.*, 1985; Sanders *et al.*, 1988; Hill *et al.*, 1989; McClain *et al.*, 1989; Curran & DeGroot, 1991; Bookstaff *et al.*, 1996; Waritz *et al.*, 1996). Decreased serum thyroid hormone due to increased hepatic disposition results in a

compensatory increase in pituitary TSH which can exert a tumour promoting effect in initiation-promotion model systems (Hiasa *et al.*, 1982a,b) or an increase in thyroid gland neoplasia in two-year carcinogenicity studies (Hill *et al.*, 1989; McClain, 1989).

In rats, PB has been shown to induce hepatic microsomal thyroxine UDP-glucuronyl transferase, increase the biliary excretion of thyroxine glucuronide, decrease serum levels of T3 and T4, and produce a compensatory increase in the plasma level of TSH (McClain, 1989; McClain *et al.*, 1989). In an initiation-promotion model, the tumour promoting effect of PB for the rat thyroid gland was found to be directly proportional to the increased plasma level of TSH. Supplemental administration of thyroxine, at dosages that normalized but did not suppress TSH, completely blocked the tumour promoting effect of PB in the thyroid, but had no inhibitory effect on liver hypertrophy resulting from PB administration (Table 1; McClain *et al.*, 1988; McClain, 1989). The tumour-promoting effect of PB in the thyroid gland thus appears to be mediated by pituitary TSH as a compensatory response to the increased rate of hepatic disposition of thyroid hormone, as opposed to a direct tumour-promoting or carcinogenic effect in thyroid follicular epithelium.

### Hepatocellular tumour promotion by phenobarbital

The conclusion that the mode of action for hepatocarcinogenesis by PB is tumour promotion is widely accepted and is based on a large body of evidence accumulated during the last two decades (Pitot & Sirica, 1980; Peraino *et al.*, 1973, 1980). There is no evidence that PB causes DNA damage in the liver, or that it can initiate carcinogenesis. Tumour promoters exert their effect on initiated cells and on preexisting preneoplastic lesions (Schulte-Hermann *et al.*, 1983) and tumour promotion can be operationally defined as preferential enhancement of growth of altered foci of cells that ultimately evolve to become neoplasms. PB has been shown to enhance selectively the nuclear labeling index of cells within eosinophilic hepatocellular foci in mice given NDEA, resulting in development of eosinophilic hepatocellular adenomas (Pereira, 1993). PB had little or no effect on the nuclear labeling index in hepatocytes in surrounding histologically normal liver or in basophilic hepatocellular foci, which corresponded to lack of enhancement by PB of development of basophilic hepatocellular adenomas (Pereira, 1993).

Although the mode of action of PB on development of liver tumours is clearly by promotion of

**Table 1. Effect of dietary thyroxine on promotion by phenobarbital of nitrosamine-induced thyroid follicular tumours in male rats (modified from McClain *et al.*, 1988).**

Treatment group	% Adenomas (rats affected/rats examined)	Number of tumour foci
Control	0% (0/20)	0
DHPN <sup>a</sup>	37% (6/16)	20
DHPN + PB <sup>b</sup>	83% (15/18) <sup>d</sup>	107
DHPN + PB + T4 <sup>c</sup>	25% (5/20)	11
T4	0% (0/20)	0
PB	0% (0/20)	0
PB + T4	0% (0/20)	0

<sup>a</sup>DHPN: *N*-Nitrosodihydroxypropylamine, 700 mg/kg bw/week subcutaneously for 5 weeks (experimental weeks 1-5).

<sup>b</sup>PB: phenobarbital, fed as the sodium salt, mixed in diet at 500 ppm, for 15 weeks (experimental weeks 6-20).

<sup>c</sup>T4: L-thyroxine, fed as the sodium salt pentahydrate, mixed in diet to provide a dose of 50 µg/kg bw/day for 15 weeks (experimental weeks 6-20).

<sup>d</sup>Fisher's exact test vs. DHPN group,  $p \leq 0.05$ .



neoplastic development, its mechanism of action in hepatocytes is not well understood. Evidence is accumulating, however, that the process involves stimulation of expression of certain peptide growth factors that control cell replication in hepatocytes.

Hepatocytes isolated from the livers of rats that had previously received an initiating dose of a genotoxic hepatocarcinogen require PB in the culture medium for selective outgrowth *in vitro* (Kaufmann *et al.*, 1988). Initiated hepatocytes proliferate and form colonies in primary culture in the presence of PB, under conditions where hepatocytes from control animals degenerate and die. It has recently been shown that tumour growth factor- $\alpha$  (TGF- $\alpha$ ) can substitute for PB in culture medium. Either PB or TGF- $\alpha$  can support colony formation by initiated cells; when combined at optimal concentrations, the response appeared to be saturated, and when these factors were tested in combination at suboptimal concentrations, the two substances were additive for supporting colony formation (Kaufmann *et al.*, 1997).

In rats, PB produces a burst of DNA synthesis in hepatocytes followed by increased mitotic activity and hyperplasia. After the initial burst of DNA synthesis, there is a decrease in the rate of cell proliferation and inhibition of apoptosis in normal hepatocytes (Bursch & Schulte-Hermann, 1983). This corresponds to an increase in tumour growth factor- $\beta$  (TGF- $\beta$ ) in periportal hepatocytes. TGF- $\beta$  is a potent inhibitor of cell proliferation and plays a role in apoptosis (Jirtle & Meyer, 1991). After long-term exposure to PB some hepatocytes lose the ability to take up TGF- $\beta$ . The loss of inhibition of cell proliferation by TGF- $\beta$  provides a growth advantage over normal hepatocytes (Jirtle & Meyer, 1991). In addition, the inhibition of apoptosis in preneoplastic foci could cause a rapid expansion of foci without a persistent increase in cell proliferation (Bursch & Schulte-Hermann, 1983).

Recent research suggests that in strains of mice that are sensitive to tumour promotion the balance between liver cell mitosis and programmed cell death (apoptosis) is disrupted (Böhm & Moser, 1976; Bursch & Schulte-Hermann, 1983). Furthermore, altered DNA methylation may play a variety of roles in carcinogenesis (Counts & Goodman, 1995). In mice, there is a marked strain difference in the spontaneous incidence of hepatocellular tumours and susceptibility to hepatic

tumour promotion (Drinkwater & Ginsler, 1986). This correlates with the observation that the relatively resistant strain is better able to maintain the normal methylation status of DNA in the face of a higher level of cell proliferation (Counts *et al.*, 1996).

### **Carcinogenesis and tumour promotion in liver and thyroid gland by TCDD**

TCDD binds with exceptionally high affinity to the Ah receptor (reviewed in IARC, 1997). It causes induction of gene expression of CYP1A1 and many other enzymes and modulates expression of many growth factors, transcription factors, and related peptide factors in many tissues. TCDD has significant effects on the thyroid gland and on blood levels of thyroid hormones. TCDD is not genotoxic.

The effects of TCDD on tumour incidence in treated animals are complex, vary markedly with the genetic background of the subjects, and are not confined to the liver and thyroid in all studies (IARC, 1997). Osborne-Mendel rats of both sexes and female B6C3F<sub>1</sub> mice given TCDD by gavage twice weekly for 104 weeks developed both thyroid follicular cell adenomas and hepatocellular adenomas; the mice also developed lymphomas and soft-tissue sarcomas. Male mice developed liver tumours and lung tumours (United States National Toxicology Program, 1982).

The relationship of enzyme inducers of the TCDD type to tumour promotion in liver and thyroid is much less well studied than that of PB-type inducers.

### **Correlation of microsomal enzyme induction with tumour promotion in liver and thyroid gland**

A correlation has been well established between potency for hepatic microsomal enzyme induction and capacity for tumour promotion in rat liver and thyroid gland by enzyme inducers of the PB type. Studies of a structurally related series of barbiturates that included non-inducers, weak inducers, and strong inducers have shown a highly significant correlation between microsomal enzyme induction and hepatic tumour promotion in rats (Lubet *et al.*, 1989). For example, the strong enzyme inducers PB (5-ethyl-5-phenylbarbituric acid) and barbital (5,5-diethylbarbituric acid) are effective hepatic tumour promoters, while non-inducers such as either 5-ethyl- or 5-phenylbarbituric acid are not. Furthermore, within the

barbiturate series, the potency for enzyme induction correlates quite well with potency for hepatocellular tumour promotion (Lubet *et al.*, 1989; Rice *et al.*, 1994). This observation extends to other species: PB neither induces microsomal enzymes nor promotes hepatocellular tumorigenesis in the Syrian golden hamster (Diwan *et al.*, 1986a).

The same relationship holds true for hydantoin compounds. 5-Ethyl-5-phenylhydantoin (EPH) was an efficient inducer of hepatic CYP2B1 and other microsomal enzymes in rats and was also an effective promoter of hepatocellular tumours induced by NDEA. In contrast, 5,5-diethylhydantoin (EEH) was ineffective either as an enzyme inducer or as a hepatocellular tumour promoter (Diwan *et al.*, 1986b).

An even more striking interspecies comparison is provided by 1,4-bis[2-(3,5-dichloropyridyloxy)]benzene (TCPOBOP), which is quantitatively much more potent (by orders of magnitude) as a microsomal enzyme inducer in mice than in rats. TCPOBOP was shown to be both a potent inducer and a hepatocellular tumour inducer in mice initiated with NDEA, but at 10-fold higher dosage it had neither effect in rats (Diwan *et al.*, 1992). When the dose-response relationship for enzyme induction was fully explored in Fischer 344 rats, however, which required thousand-fold higher dosages, a level of exposure that was effective for hepatic microsomal enzyme induction was established, and at that much higher dosage level, TCPOBOP was also an effective liver tumour promoter (Diwan *et al.*, 1996).

Similar relationships between potency for hepatic microsomal enzyme induction and for tumour promotion in thyroid follicular epithelium have also been established. In addition to PB and several other barbiturates, other PB-like enzyme inducers have been shown to promote carcinogenesis in the thyroid. These include EPH which, like PB, promoted both hepatocellular and thyroid follicular cell tumorigenesis in NDEA-initiated rats at doses that were effective for hepatocellular microsomal enzyme induction, but not its non-enzyme inducing homologue, EEH (Diwan *et al.*, 1988). TCPOBOP also promoted thyroid follicular cell carcinogenesis in NDEA-initiated Fischer 344 rats at doses that caused maximal induction of CYP2B1 (Diwan *et al.*, 1996).

## Conclusions

The physiological consequences of microsomal enzyme induction are important in the pathogenesis of hepatic and thyroid follicular neoplasia in rodent bioassays for carcinogenicity. This is most likely the consequence of the known effects of microsomal enzyme inducers (hepatocellular and thyroid follicular cell hypertrophy and hyperplasia) which under certain experimental conditions exert a tumour promoting effect in hepatic parenchyma and thyroid follicular epithelium in susceptible species and strains of animals. The modes of action that are involved for individual chemicals need to be thoroughly understood and incorporated into the processes of hazard identification and risk assessment for chemical carcinogens.

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# Human renal-cell carcinoma — epidemiological and mechanistic aspects

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## Introduction

Renal-cell carcinoma (RCC), also known as adenocarcinoma of the kidney, hypernephroma or Grawitz tumour, is the most frequent type of kidney cancer. Other frequent types of kidney cancer include tumours derived from the urothelium in the pelvis and ureter and Wilm's tumour in children.

RCC was first described by König (1826). The erroneous assumption that the tumour originated from the fatty adrenal tissue led to the term hypernephroma, but it later became clear that RCC originates in the mature renal tubular epithelium. Early studies of RCC focused on histology, staging and prognostic factors as well as descriptive epidemiology. In the 1960s and 1970s, studies of risk factors with systematic data collection were published. In the 1980s and 1990s, several specific hypotheses were tested in case-control and follow-up studies, including studies of the roles of tobacco, occupation and medication.

This paper summarizes current knowledge on the etiology of RCC and the possible mechanisms of action of the risk factors so far identified. Reference is made to a selection of the studies of RCC that were particularly well executed or provided new insight.

## Descriptive epidemiology of renal-cell carcinoma

The incidence rates of RCC vary greatly from high-incidence areas in northern Europe and North America, where the incidence is up to 10 per 100 000 (standardized to the world standard population) to low-incidence areas in Asia, Africa and South America where the incidence rates are from 1 to 3 per 100 000. RCC accounts for approximately 2% of all non-skin cancer in the western world (Parkin *et al.*, 1997).

The incidence rate of RCC has been increasing in most countries that have population-based cancer registries. In Denmark, where nationwide cancer incidence figures have been available since 1943, the incidence doubled among men between the 1950s and 1970s and has remained stable since then, at about 8 per 100 000. Among Danish women, the incidence increased slightly from 3 to 4 per 100 000 in the same period (Møllemaard *et al.*, 1993). The data from the Connecticut Cancer Registry for the period 1935–89 indicate that rates in the USA increased among men from 1.6 to 9.6 per 100 000 and among women from 0.7 to 4.2 per 100 000. Just in the period 1974–90, the incidence has increased by 38% in the USA (Katz *et al.*, 1994). Throughout this period, the male : female incidence ratio has remained close to 2, a ratio which is similar in all high-incidence areas, and the incidence is uniformly increasing with age. Incidence rates of RCC are consistently higher among urban than rural dwellers.

Two risk factors, cigarette smoking and obesity, have consistently been linked with RCC in epidemiological studies. For a number of other risk factors such as drugs, occupation, diet, medical and reproductive history and socioeconomic status, there is some evidence of an association.

## Cigarette smoking

In a hospital-based case-control study, Wynder *et al.* (1974) found that the relative risk (RR) for RCC among cigarette smokers was 2 compared with nonsmokers. A dose-response relationship was observed for the number of cigarettes smoked per day. Use of other types of tobacco smoking (pipe and cigar) was weakly associated with risk for RCC. In contrast, another hospital-based case-control study found no association between tobacco smoking and RCC, although chewing tobacco

increased the risk four-fold (Goodman *et al.*, 1986). A case-control study, in which cases were restricted to subjects 55 years of age or under and controls were sampled from the neighbourhoods of cases, found an RR of 2 for RCC among men but not among women (Yu *et al.*, 1986). A hospital-based case-control study, in which controls were sampled from patients without cancer or urological disease, found an association and a dose-effect relationship for cigarette smoking (La Vecchia *et al.*, 1990). Statistically insignificant associations were observed in a case-control study in which controls were individuals with non-tobacco-, non-alcohol- and non-hormone-related diseases (Talamini *et al.*, 1990). A number of population-based studies have confirmed the association. In the study by McLaughlin *et al.* (1984), an association among both men and women was found for cigarette smoking, including a dose-response relationship. In a case-control study that included hospital and population-based controls, a weak nonsignificant association was found (Asal *et al.*, 1988a). A two-fold risk was found for smokers in a Canadian study which also investigated passive smoking, for which a nonsignificant association was observed (Kreiger *et al.*, 1993). A study based in Shanghai found an RR of 2.3 for subjects who had ever versus never smoked cigarettes. The risk among heavy smokers of non-filtered cigarettes was particularly high (McLaughlin *et al.*, 1992a). Although use of smokeless tobacco has generally been found not to increase the risk for RCC, a recent study found an RR of more than 3 among men using chewing tobacco (Muscat *et al.*, 1995). An international multicentre study found a significant, but modest, increase in risk among cigarette smokers (RR, 1.4). Furthermore, an increasing risk was found with increased consumption and duration of smoking. Use of other types of tobacco was not associated with RCC (McLaughlin *et al.*, 1995a). Findings in a cohort study confirmed the association, including a dose-response relationship (McLaughlin *et al.*, 1990).

The few studies which have not found this association have either been small or have used hospital controls, which might lead to underestimation of the true relative risk. In most studies, heavy cigarette smoking was associated with a two- to three-fold increase in risk; the attributable fraction may be as high as one-third.

## Obesity

Wynder *et al.* (1974) observed an excess of overweight female cases and prompted subsequent studies to examine the role of weight. Although some case-control studies confirmed the association in women only (Lew & Garfinkel, 1979; McLaughlin *et al.*, 1984; Chow *et al.*, 1996), other studies found the effect to be present in men also (Goodman *et al.*, 1986; Yu *et al.*, 1986; Asal *et al.*, 1988a,b; Maclure & Willett, 1990; McLaughlin *et al.*, 1992b; Benhamou *et al.*, 1993; Kreiger *et al.*, 1993). One study found a nonsignificant reduction in risk for individuals with a high body mass index (BMI) (Talamini *et al.*, 1990). The international multicentre study found a significant effect in both sexes, but the effect was stronger in women. Among the top 5% women, the odds ratio was 3.6 compared with the lowest quartile. The rate of weight change appeared to be an independent risk factor in women but not in men and there was no association with height or physical activity. The association remained after adjustment for socioeconomic status, smoking and diet (protein and fat) (Mellemegaard *et al.*, 1994a). A cohort study of individuals discharged from Danish hospitals with a diagnosis of obesity found an increased risk for RCC in both sexes (Mellemegaard *et al.*, 1991).

Although there is no direct evidence of the mechanism, the differences found between men and women and other indications of a possible role of hormones in RCC (reproductive factors, receptors on tumour cells) are compatible with a mechanistic pathway involving the formation of oestrogens in fatty tissue which then stimulate the malignant cell. The evidence is sufficient to categorize obesity as a likely risk factor for RCC, with increased peripheral oestrogen formation as a possible mechanism.

## Socioeconomic status

Early reports on the epidemiology of RCC were suggestive of an increased mortality in higher socioeconomic strata (Case, 1964). In the majority of case-control studies, no association has been found, but a French study found that the risk for RCC was higher among individuals with longer education (Benhamou *et al.*, 1993). However, recent studies have found an inverse association in some countries, such as the USA (Asal *et al.*, 1988a,b; Muscat *et al.*, 1995) and Denmark

(Mellemgaard *et al.*, 1994b). The increased risk in lower socioeconomic strata is not caused by the suspected occupational risk factors, obesity or cigarette smoking and may indicate the existence of yet unidentified occupational or other lifestyle factors. The current evidence is not strong enough to label socioeconomic factors as a risk factor for RCC and the underlying reason for the effects observed is unknown.

### Medical history

It has been known for many years that the risk for RCC is increased in individuals with polycystic kidney disease and kidney failure (Ishikawa *et al.*, 1990). In case-control studies of medical history, an association has been observed for previous diagnosis of thyroid and kidney disease, particularly infections (McLaughlin *et al.*, 1984; Schlehofer *et al.*, 1996), as well as circulatory disease in men and digestive disease in women (Asal *et al.*, 1988a). A cohort study of diabetics found an increased risk for RCC in women, but findings from case-control studies offer only limited support for this hypothesis (Adami *et al.*, 1991; Schlehofer *et al.*, 1996). The role of hypertension has attracted special attention because diuretics have been suspected of increasing the risk for RCC (see below). A cohort study of individuals discharged with hypertension, heart failure or oedema showed a two-fold increase in relative risk irrespective of diagnosis. Based on a random sample, 70% of the discharged individuals were treated with diuretics (Mellemgaard *et al.*, 1992a). A similar register-linkage study from Sweden confirmed an increased risk in men and women discharged with a diagnosis of heart failure or oedema (Lindblad *et al.*, 1993). The cohort studies, however, were unable to adjust for the effect of other risk factors such as smoking and obesity. In a large follow-up study of Seventh Day Adventists, an increased risk was found for individuals who reported hypertension (RR, 4.5) or taking antihypertensive medications (RR, 2.9) (Fraser *et al.*, 1990). A prospective study of blood pressure and cancer incidence found an increased RR of 1.2–1.5 for kidney cancer among hypertensive Japanese men in Hawaii. No other cancer site showed an elevated RR (Grove *et al.*, 1991). A follow-up study of thyroid disorders revealed significantly increased risks of kidney cancer in women

discharged from a Danish hospital with a diagnosis of myxoedema (RR, 1.8) or thyrotoxicosis (RR, 1.3). The risk among men was unchanged (Mellemgaard, unpublished). Several case-control studies have found an association between RCC and hypertension (Kreiger *et al.*, 1993; Muscat *et al.*, 1995). In the international multicentre study, the odds ratio for hypertension was 1.4 after adjustment for medication (McLaughlin *et al.*, 1995b).

There is thus some evidence that hypertension and polycystic kidney disease are risk factors for RCC, while the evidence for thyroid disorders is limited.

### Drugs

Two kinds of drug have attracted much attention: diuretics and weak analgesics (Mellemgaard *et al.*, 1992a, 1992b). A case-control study found a five-fold increase in risk for RCC among users of diuretics (Yu *et al.*, 1986). The association was noted in both hypertensive and non-hypertensive individuals. Subsequent studies have shown divergent findings. Apart from the findings in cohort studies of users or suspected users of diuretics described above, one case-control study found the association only in non-hypertensive women (McLaughlin *et al.*, 1988) and another in women irrespective of the presence of hypertension (Kreiger *et al.*, 1993). A screening study, in which prescription information was linked with cancer incidence data, did not find a significant association between furosemide, spironolactone or thiazides and RCC (Selby *et al.*, 1989). In contrast, a case-control study nested in a health plan cohort found a three-fold increase in risk for RCC among individuals who had filled prescriptions for diuretics. The association remained after adjustment for hypertension (Finkle *et al.*, 1993). A similarly designed study found an odds ratio of 4 in female users of thiazide, but no dose-response effect was detected. No other diuretics were studied (Hiatt *et al.*, 1994). Finally, a third study nested in a health plan found an association with several types of diuretic such as thiazides (odds ratio, 1.9), loop diuretics (odds ratio, 3.1) and potassium sparing (odds ratio, 2.1). Other antihypertensive agents and hypertension itself were so closely associated with diuretic use that it was difficult to separate the effect of one from the other (Weinmann *et al.*, 1994). The fact that all types of



diuretic collectively have been linked with risk for RCC weakens the plausibility of the association because diuretics have different modes of action.

The other extensively studied drugs are analgesics. It is known that phenacetin causes kidney failure and is involved in urothelial cancers, but the association with RCC is less well established. Several case-control studies have found moderately increased risks among users of phenacetin (McLaughlin *et al.*, 1984; Maclure & MacMahon, 1985; McCredie *et al.*, 1988; Kreiger *et al.*, 1993), as well as non-phenacetin-containing analgesics (Asal *et al.*, 1988a). Two cohort studies of hospital discharge records from patients with painful chronic diseases such as rheumatoid arthritis both found a weak association in women (Mellemegaard *et al.*, 1992b; Lindblad *et al.*, 1993). In these studies, there was no information on the type of drug or other risk factors. A cohort study found a six-fold increase in risk, but the observation was based on only nine cases, all male (Paganini-Hill *et al.*, 1989). The international multicentre study found no effect of any of the commonly used analgesics on the risk of RCC (McCredie *et al.*, 1995). A recent study of acetaminophen (paracetamol), a phenacetin metabolite, found a more than two-fold increase in risk for renal cancer among subjects who filled 40 or more prescriptions compared to no prescriptions (Derby & Jick, 1996).

In conclusion, there is some evidence that phenacetin increases the risk for RCC, while the evidence is insufficient with regard to other analgesics and diuretics.

## Diet

An early correlation study of international consumption figures and cancer mortality data showed significant correlations between RCC and animal fat and protein (Wynder *et al.*, 1974). Later case-control studies have provided some support for the suspected association with animal protein. A particularly strong association was seen in a study from Shanghai, where men with high meat consumption were at a four-fold increased risk for RCC. The effect among women was insignificant and weak (McLaughlin *et al.*, 1992a). A case-control study of diet and RCC found some indication of an effect of a high intake of animal protein and fat, but the findings were not consistent over case groups (incident/prevalent) and generally this

study was hampered by a very low participation rate (Maclure & Willett, 1990). Other US studies have found no association with meat intake (McLaughlin *et al.*, 1984; Yu *et al.*, 1986; Kreiger *et al.*, 1993). An Italian case-control study found a positive association with fat intake, especially oils and margarine (Talamini *et al.*, 1990). The international multicentre study, which included a wide range of dietary questions, found an odds ratio of 1.7 for the highest quartile for total energy consumption. However, it was not possible to identify one energy source as more important than others (Wolk *et al.*, 1996).

A protective effect has been suggested for vegetables (Maclure & Willett, 1990; Talamini *et al.*, 1990; McLaughlin *et al.*, 1992a; Wolk *et al.*, 1996) and fruits (McLaughlin *et al.*, 1992a; Wolk *et al.*, 1996). For micronutrients, the international multicentre study found an increased risk of RCC associated with low intake of vitamin E and magnesium (Wolk *et al.*, 1996). In one of the participating centres in this study (Denmark), a striking association was seen for dairy products, with increased risks particularly among women associated with a high intake of milk with a high fat content and thick butter spread (Mellemegaard *et al.*, 1996). An association with milk had previously been observed (McCredie *et al.*, 1988) but the majority of studies did not confirm this association (Kreiger *et al.*, 1993; Wolk *et al.*, 1996).

An association between intake or exposure to *d*-limonene and risk of RCC in man has not been studied. The primary dietary source of *d*-limonene, citrus fruits and juice, is not associated with an increase in risk (Chow *et al.*, 1994; Wolk *et al.*, 1996).

In spite of the wide international variation in incidence, there is thus little evidence that dietary factors play a role in RCC.

## Reproductive factors

The clinical observation that oestrogens may interfere with the course of RCC and the existence of steroid receptors in RCC cells prompted a search for epidemiological evidence of an association. A role for reproductive factors in RCC has been suggested in several case-control studies. An increased risk was found in women taking oestrogens at menopause (Asal *et al.*, 1988a) and another study found an increased risk among women who

had experienced a fetal loss and also had three or more live births. In women who reported no fetal loss, there was no effect of number of live births (Kreiger *et al.*, 1993). The international multicentre study, which collected information on a number of reproductive variables, found an increased risk (odds ratio, 1.8) among women who reported six or more births compared with those reporting one birth. Furthermore, the data suggested a decrease in risk with increasing age at menarche and age at first birth. There was no association with age at menopause, but women who took oral contraceptives were at half the risk of those who did not (Lindblad *et al.*, 1995).

For reproductive factors, it thus appears that although there is a plausible mechanism, the evidence of an association with RCC is limited.

### Coffee, tea and alcohol

The international consumption figures for coffee and renal cancer mortality have a correlation coefficient of 0.79 (Shennan, 1973), and the association has been evaluated in several case-control studies, without positive findings (Asal *et al.*, 1988a; McCredie *et al.*, 1988; Maclure & Willett, 1990; Talamini *et al.*, 1990; Kreiger *et al.*, 1993) with two exceptions. One case-control study found an association with decaffeinated coffee (Goodman *et al.*, 1986) and another found an increased risk among female coffee drinkers (Yu *et al.*, 1986). In neither study was a dose-response relationship observed. In the international multicentre study, no clear trend was seen, but women with the highest consumption (more than 41 cups per week) had a significantly increased risk of 2.1 (Wolk *et al.*, 1996).

There are very few data on tea use and risk of RCC. McLaughlin *et al.* (1984) found a three-fold increased risk among women who drank more than three cups a day. No effect was seen in men. In contrast, an inverse association was found in another case-control study (Yu *et al.*, 1986). The international multicentre study found no effect of tea drinking (Wolk *et al.*, 1996).

Most studies on alcohol-drinking have found no association with the risk for RCC (Wynder *et al.*, 1974; McLaughlin *et al.*, 1984; Yu *et al.*, 1986; Asal *et al.*, 1988a; Maclure & Willett, 1990; Kreiger *et al.*, 1993; Muscat *et al.*, 1995). However, the international multicentre study found a protective

effect of alcohol among women. The risk for women with a high intake of alcohol, especially wine, was reduced to half the risk of teetotalers (Wolk *et al.*, 1996).

In conclusion, these beverages are probably not risk factors for RCC, and the evidence that alcohol protects against RCC is inconclusive.

### Occupation

Occupational risk factors for RCC have been studied more than any other type of risk factor. Several occupations or exposures are under suspicion.

Human exposure to gasoline is particularly interesting in the light of these agents' ability to induce  $\alpha_2$ -globulin nephropathy and renal tumours in male rats. A number of cohort studies of refinery workers, truck drivers and gas station attendants have found either no increase, or a very moderate increase in risk (Schottenfeld *et al.*, 1981; Hanis *et al.*, 1982; Thomas *et al.*, 1982; Wen *et al.*, 1983; McLaughlin *et al.*, 1987; Poole *et al.*, 1993; Lynge *et al.*, 1997). Cohort studies are usually not able to separate the effect of gasoline from that of other hydrocarbons and other substances which may affect risk of renal cell carcinoma such as asbestos or cigarette smoking. Case-control studies, which can adjust for the effects of other factors, have either given negative results or found moderate and statistically non-significant increases in risk, and a trend with duration of exposure is often absent (McLaughlin *et al.*, 1985; Asal *et al.*, 1988a; Siemiatycki *et al.*, 1988; Sharpe *et al.*, 1989; Partanen *et al.*, 1991; Mandel *et al.*, 1995) (Table 1).

An excess risk has been reported among workers exposed to asbestos, and asbestos fibres have been found in kidneys (Smith *et al.*, 1989). Cohort studies of workers in asbestos production, insulation and shipyards have shown increased risks (Selikoff *et al.*, 1979; Maclure, 1987). The international multicentre study found an increased risk among workers who reported being exposed to asbestos (Mandel *et al.*, 1995), but other studies that have examined the role of asbestos have found no association (McLaughlin *et al.*, 1984; Asal *et al.*, 1988a; Partanen *et al.*, 1991).

Employment in coke-ovens and exposure to aviation fuels and possibly other hydrocarbons were associated with RCC in some studies (Redmond *et al.*, 1972; McLaughlin *et al.*, 1984;

**Table 1. Studies on the association between exposure to gasoline (vapour or engine exhausts) and renal cell carcinoma**

<i>Case-control studies</i>		
McLaughlin <i>et al.</i> (1985)	USA	OR=1.0
Asal <i>et al.</i> (1988a)	USA	OR=4.3 for men, 1.6 for women
Siemiatycki <i>et al.</i> (1988)	Canada	OR=1.2
Sharpe <i>et al.</i> (1989)	Canada	OR=1.1
Partanen <i>et al.</i> (1991)	Finland	OR=1.7
Mandel <i>et al.</i> (1995)	International	OR=1.6
<i>Cohort studies</i>		
Schottenfeld (1981)	USA	95701 petroleum industry employees: SIR =1.0 among refinery workers: SIR=0.9
Hanis <i>et al.</i> (1982)	USA	8866 refinery workers: SMR=1.6
Thomas <i>et al.</i> (1982)	USA	2509 refinery workers: SMR=1.4
Wen <i>et al.</i> (1983)	USA	16880 refinery workers: SMR =1.1
McLaughlin <i>et al.</i> (1987)	USA	7405 gas station attendants/refinery workers: SIR <1.0
Poole <i>et al.</i> (1993) (nested case-control)	USA	100 000 refinery workers: OR =1.0
Lynge <i>et al.</i> (1997)	Scandinavia	19000 gas station attendants: SIR=1.3

OR= Odds ratio, SIR= Standardized incidence rate, SMR= Standardized mortality rate

Siemiatycki *et al.*, 1987; Asal *et al.*, 1988a; Partanen *et al.*, 1991; Mandel *et al.*, 1995) while other studies found no association (McLaughlin *et al.*, 1987; McCredie & Stewart, 1993; Poole *et al.*, 1993).

Laundry and dry-cleaning workers have been found, in both cohort and case-control studies, to be at increased risk for RCC (Blair *et al.*, 1979; Asal *et al.*, 1988a; McCredie & Stewart, 1993). However, two recent large cohort studies have found no effect of employment in laundry and dry cleaning (Blair *et al.*, 1990; Lynge *et al.*, 1995).

Two heavy metals, cadmium and lead, have come under suspicion as risk factors for RCC. Such an association was first suggested in a small case-control study of RCC (Kolonel, 1976), but later studies have not offered much support for an

association between exposure to cadmium and RCC (McLaughlin *et al.*, 1984; Asal *et al.*, 1988a; McCredie & Stewart, 1993). As cigarette smoking was one of the sources of cadmium examined in the original study, it is possible that the association was caused by confounding. Limited support is, however, offered by the international multicentre study, in which an association with self-reported exposure to cadmium was observed (Mandel *et al.*, 1995).

There are several studies of renal tumours in rodents induced by lead in the diet (van Esch & Kroes, 1969). Human data are sparse, and a large register-linkage study of a Danish supplementary pension scheme found no excess risk for RCC in metallic industries (Olsen & Jensen, 1987), while a cohort study of lead smelter workers found an

excess mortality from kidney cancer (Steenland *et al.*, 1992). Lead and cadmium content in renal tissue in RCC patients has been examined, but concentrations of both metals were low (Ala-Opas & Tahvonen, 1995).

With the large number of comparisons in screening studies for occupational risk factors in the work environment, it is not surprising that a number of associations have been suggested but not confirmed in subsequent studies. This is the case with newspaper pressmen, lumberjacks, physicians, architects, paperboard printing workers, firefighters, painters and textile workers (Paganini-Hill *et al.*, 1980; McLaughlin *et al.*, 1987; Sinks *et al.*, 1992; Auperin *et al.*, 1994; Delahunt *et al.*, 1995; Henschler *et al.*, 1995).

### Miscellaneous

Patients with chronic lymphocytic leukaemia, who are known to have decreased immune function, have been found in one study to have a risk for RCC which is twice that of the background population (Mellemgaard *et al.*, 1994c).

Based on the observation of endemic nephropathy in the Balkans, which may be caused by ochratoxin A or other fungal products, and which has been linked to urothelial cancer, there has been concern that ochratoxins may also be involved in the etiology of RCC. RCC has been induced in mice fed a diet containing ochratoxin A (Kanisawa & Suzuki, 1978). However, there are no human epidemiological data supporting this hypothesis, as occupational groups which may be exposed to fungi, such as bakers and handlers of flour and grain, are not at increased risk for RCC (Mellemgaard *et al.*, 1994d).

Marginally increased risks for RCC have been described in individuals who have received radiation therapy for ankylosing spondylitis (Boice *et al.*, 1988), and an increased incidence has been noted in individuals who were exposed to Thorotrast (an  $\alpha$ ,  $\beta$  and  $\gamma$  emitter) used for retrograde pyelography (Kauzlaric *et al.*, 1987). However, recent case-control studies have found no association between radiation and risk for RCC (Mellemgaard *et al.*, 1994d).

In summary, the evidence regarding asbestos and polycyclic aromatic hydrocarbons demonstrates a possible association with RCC, while the evidence regarding exposure to gasoline, lead, cad-

mium, radiation and dry-cleaning products is insufficient.

### Genetic factors

Examination of RCC tumour tissue has often revealed a characteristic chromosomal defect consisting of deletions of distal chromosome 3p (Zbar *et al.*, 1987). The region involved is in the same area as the defects seen in von Hippel-Lindau disease, in which a tumour-suppressor gene has been identified, and as many as one-third of all those affected may develop RCC (Glenn *et al.*, 1991; Latif *et al.*, 1993; Bailly *et al.*, 1995). Studies of families with many cases of RCC have revealed a balanced translocation between chromosomes 3 and 8 (Wang & Perkins, 1984). However, the proportion of cases with a genetic background is probably small (Mellemgaard *et al.*, 1994b).

### Conclusion

Table 2 summarizes the epidemiological evidence concerning the etiology of RCC. It has been established beyond doubt that cigarette smoking causes RCC, and, furthermore, that obesity, in particular in women, is involved in the carcinogenic process, by a process that possibly involves peripheral formation of oestrogens which stimulate tumour cells. The suspected occupational risk factors are responsible for a minor proportion of the RCC cases in the developed countries, but the facts that low socioeconomic status is a risk factor and that the sum of etiological fractions is far from 1.0, particularly among men, suggest that other occupations or exposures which may confer an increased risk are yet to be identified. The effect of diet may be significant but, as for other cancer sites, it is very difficult to identify the responsible food items. A diet rich in protein and fat may lead to RCC, and dairy products may have a special role.

Generally speaking, there is a problem in making inferences from epidemiological data, which by definition are based on observations made in populations rather than individuals (Kaldor, 1992). The difficulties can be illustrated by the very complex nature of the association between reproductive factors and RCC, where the effect of the reproductive factors are intertwined with those of hypertension, obesity and use of oral contraceptives (Chow *et al.*, 1995).

**Table 2. Assessment of evidence concerning risk factors for renal cell carcinoma**

Risk factor	Evidence of association <sup>a</sup>	Mechanism
Cigarette smoking	3	Peripheral oestrogen formation?
Obesity	3	
Alcohol	0	
Phenacetin	2	
Other analgesics	1	Shared risk factors?
Protein/fat	2	
Other dietary factors	0	
Diuretics	1	
Reproductive factors	2	Oestrogens act as promoters?
Socioeconomic status	1	Indicator of occupational factors? Diet?
Asbestos	2	Direct action on tubular epithelium?
Polycyclic aromatic hydrocarbons	2	
Dry cleaners	1	
Lead/cadmium	1	
Medical history (hypertension, thyroid disorders)	1	

<sup>a</sup> Evidence of association:

0, No evidence

1, Little evidence, i.e. positive and negative studies, too little evidence to draw conclusions

2, Positive association in several studies

3, Positive association in most studies, very likely to increase risk for RCC

Although previous studies have provided valuable information, the etiology of RCC remains incompletely understood and many suspected risk factors, including occupational, warrant further investigation. Information on the molecular epidemiology and on the mechanisms of the risk factors is sparse and much work is needed to provide a better understanding of the pathogenesis of RCC.

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# Human renal carcinoma – pathogenesis and biology

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## Introduction

In order to relate any animal model to a given human cancer, the model should resemble its putative human counterpart. Renal carcinoma (RC) in the human is characterized by substantial cytohistological diversity, which has led to classification schemes based primarily on microscopic appearance. It is of fundamental importance to establish whether classification schemes that are in use identify biologically distinct entities. Since distinct entities are likely to result from different pathogenetic mechanisms, a classification scheme that reflects true biological diversity provides an indispensable foundation for interspecies comparisons.

The classification of human RC is at present in an uncomfortable but exciting state of transition. Earlier classification schemes were based solely upon cytological (usually cytoplasmic) characteristics, architectural pattern or putative level of origin within the renal tubule. Recent molecular and cytogenetic findings have provoked a re-examination of these criteria. Some morphological features which pathologists had emphasized for decades have been shown to have little or no fundamental significance. Criteria used to distinguish some RC categories may have been spurious. Lines of definition between entities have been drawn inappropriately, and the names applied to entities are often misleading. Distinct entities are being identified within categories hitherto viewed as homogeneous. Those who wish to relate patho-biology of animal models of RC to their human counterparts must be aware of the present transitional state of classification schemes for these tumours, and the central role now being played by cytogenetic and molecular criteria for classification.

## Histological categories of RC

For decades, RC was perceived as a single entity with somewhat heterogeneous cytological appearances and growth patterns. Certain cytoplasmic appearances (e.g., clear, granular or basophilic) and architectural patterns (e.g., tubular, papillary, solid) were incorporated into popular classification schemes. However, these descriptive designations seemed to lack clinical or biological significance, and were merely assigned adjectival status in WHO and some other classification systems. With the exception of those rare specimens with predominantly spindle cell ('sarcomatoid') features, which were associated with poor prognosis, these cytohistological features seemed not to identify important clinical or biological characteristics. Nuclear features were more closely related to prognosis, and nuclear grading schemes based on nuclear enlargement and atypia were applied to all morphological variants of RC (Fuhrman *et al.*, 1982). Nuclear grade, though important prognostically, was not used to define biologically distinct neoplastic entities.

The existence of clinically and histologically distinct entities within the RC spectrum became generally accepted in the late 1970s, when renal *oncocytoma* was established as a low-grade or benign entity formerly subsumed under RC (Klein & Valensi, 1976). Oncocytomas were initially thought to be derived from proximal tubules, but subsequent studies suggested an origin from one of the intercalated cell types in collecting ducts of the cortex and outer medulla (Störkel *et al.*, 1988). During the same period, *papillary RC* was recognized as a distinct clinical and biological entity (Mancilla-Jimenez *et al.*, 1976). Though this concept was supported by most workers, the quantitative criteria for assigning the term varied, some authors using 50% papillary structures, while

others required up to 100% in order to use this designation (Amin *et al.*, 1997a). Recently, positive staining for cytokeratin 7 has been suggested as a phenotypic feature helpful in the diagnosis of papillary RC (Gatalica *et al.*, 1995). Some authors have suggested that all papillary RC are positive for cytokeratin 7 (Gatalica *et al.*, 1995; Amin *et al.*, 1997a), while others have noted that high-grade tumours within this spectrum are often negative for this marker (Delahunt & Eble, 1997). My own experience supports the latter conclusion.

A series of important morphological studies by Thoenes, Störkel, and colleagues (Thoenes *et al.*, 1986; Störkel & van den Berg, 1995) introduced new concepts and excellent analytical approaches to the subject that have proven to be directly related to distinctive cytogenetic anomalies. They added *chromophobe carcinoma* to the list of apparently distinct entities (Thoenes *et al.*, 1985), also derived from an intercalated cell in the outer collecting duct (Störkel *et al.*, 1989). Their studies reinforced the concept that tumours formerly designated as papillary RC might comprise a distinct clinical and biological category, but suggested that cytological features might be more definitive than architectural pattern. They introduced the term *chromophil RC* for tumours formerly termed papillary RC. This departure from emphasis on papillary architecture was justified. Other RC subtypes can have a papillary growth pattern, and some tumours that otherwise fit the papillary carcinoma profile have a predominantly tubular architecture. However, the term 'chromophil' also lacks precision. Oncocytomas and other renal epithelial neoplasms are characterized by strong affinity for histological stains, and some 'papillary' tumours have abundant clear cells. The designation *papillary RC* retains general popularity despite its imperfections.

Another addition to the list of morphological entities is *Bellini duct (or collecting duct) carcinoma* (Fleming & Lewi, 1986; Rumpelt *et al.*, 1991). The spectrum of Bellini duct carcinoma was more recently expanded by the recognition of a distinctive, highly malignant carcinoma of children and young adults, arising near the papillary end of the collecting duct in patients with the sickle cell trait, and designated as *medullary RC* (Davis *et al.*, 1995a; Eble, 1996). It was proposed that this is triggered by epithelial injury from vascular occlusion in the

renal papillary region by sickled erythrocytes. This does not, however, account for the curious fact that medullary RC has to date been reported only in patients with sickle trait. It does not appear that this tumour has ever been reported in a patient known to have had homozygous sickle cell anaemia.

### Cytogenetic and molecular correlations

Cytogenetic studies of RC have revolutionized the identification of neoplastic entities, and have confirmed that RC is indeed a collection of pathogenetically distinct neoplasms. This has provided a new and potentially more valid basis for distinguishing different diseases that have common morphologic features, which is rapidly becoming incorporated into the definitional criteria for these neoplasms. Cytogenetic and molecular phenotyping is becoming an indispensable diagnostic tool, capable of resolving cytohistological dilemmas and controversies (Kovacs & Hoene, 1987; Störkel & van den Berg, 1995; Bugert & Kovacs, 1996; Motzer *et al.*, 1996; Steiner & Sidransky, 1996; van den Berg *et al.*, 1997a). These techniques show tremendous promise for defining RC subtypes and distinguishing degrees of malignancy within subtypes. However, molecular and cytogenetic studies are now facing the same problem that has bedevilled morphologists, which is the distinction of fundamental changes from those of secondary importance.

Table 1 presents a current cytohistological classification scheme for human RC, and indicates the major cytogenetic characteristics now associated with each entity. This table is necessarily tentative, and subject to revision as new data become available in this rapidly evolving field.

### Conventional (or clear cell) renal carcinoma

This is by far the most common pattern of RC in humans, constituting approximately 75–85% of cases. The term 'clear cell' is potentially misleading, since only about 75% of tumours are composed exclusively or predominantly of cells with clear cytoplasm (Figure 1). Most of the remaining tumours are composed mainly, or exclusively, of cells with granular cytoplasm.

As many as 97% of sporadic conventional RC studied to date have shown deletions, mutations or hypermethylation inactivation involving at

Table 1. Renal carcinoma classification<sup>a</sup>

Category (alternative name)	Proposed origin	Cytogenetic features	
		Major	Minor
Conventional RC (clear cell)	Proximal tubule	3p-	5q+, +7, -Y, 8p-, 9p-, 14q-
Papillary RC (chromophil)	Proximal tubule	+7, +17, -Y	3q+, +12, +16, +20
Xp 11.2 RC	? Proximal tubule		t(Xp11.2)
Oncocytoma	Collecting duct (intercalated cell)	Mitochondrial DNA t(11q13)	
Chromophobe RC	Collecting duct (intercalated cell)	-X, -Y, -1	-2, -6, -10, -13, -17, -21
Bellini duct carcinoma (collecting duct)	Collecting duct (principal cell)	?1q32.1-2	8p-, 13q-
Medullary RC	Collecting duct (distal end)	Unknown	
Miscellaneous, unclassified			

<sup>a</sup> Modified from scheme presented by Motzer *et al.* (1996).

Cytogenetic data derived primarily from Dijkhuizen *et al.*, 1995; Dijkhuizen, 1997; van der Berg *et al.*, 1997a and Bugar & Kovacs (1996). Sarcomatoid RC is not included as an entity, as it apparently represents progression phenomena occurring in most of the other RC subtypes on this table. The designation Xp11.2 RC is suggested here, to emphasize the unique features of this carcinoma, which other authors have included under the heading of papillary RC or conventional RC.

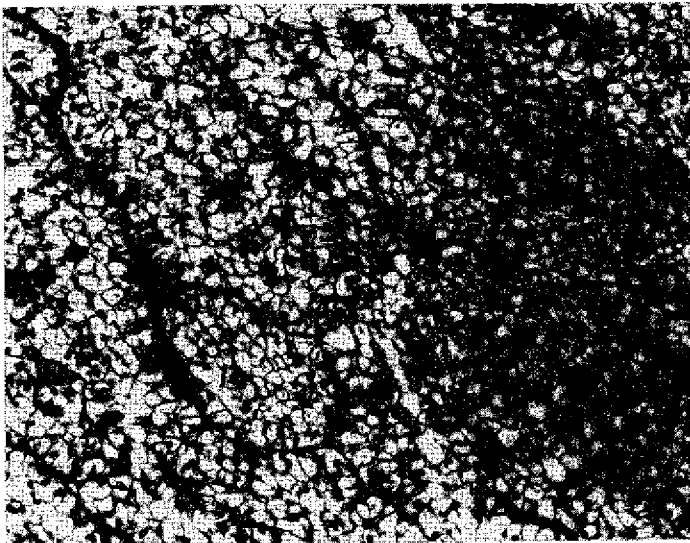


Figure 1. Clear cell renal carcinoma

least one allele of the von Hippel-Lindau (*VHL*) gene at chromosome 3p25 (Linehan *et al.*, 1995; Los *et al.*, 1996; Steiner & Sidransky, 1996). The *VHL* gene is thought to be a classic tumour-suppressor gene that controls gene transcription. The protein it encodes binds to elongins B and C, interfering with the function of RNA polymerase II (Duan *et al.*, 1995; Kibel *et al.*, 1995; Los *et al.*, 1996). Patients with von Hippel-Lindau disease show evidence of loss of the wild-type allele along with retention of the mutated allele not only in established conventional RC, but also in a variety of putative precursor lesions, including microscopic atypical cysts with early epithelial proliferative changes (Lubensky *et al.*, 1996). A recent study of sporadic conventional RC reported a single 3p deletion in renal cell adenomas, suggesting that a second deletion is involved in progression of adenoma to carcinoma (van den Berg *et al.*, 1997b). The potential relevance of this finding to at least one animal model is suggested by the recent report of *VHL* mutations in three conventional RC produced in rats by *N*-nitrosodimethylamine and protein-deprived diet (Shiao *et al.*, 1998).

Because only one allele of *VHL* is altered in some tumours, it is suggested that other genes can be involved in development of sporadic conventional RC, some of which act in coordination with the altered *VHL* allele. Other sites on 3p are involved in many of these tumours. One recently identified site is a breakpoint region centering on 3p14 (van den Berg *et al.*, 1997a). Partial trisomy of 5p is another relatively common finding, and may be involved in progression of adenoma to carcinoma. A variety of other nonrandom chromosomal increases and deletions have been reported in smaller numbers of cases, and probably reflect secondary events in conventional RC (van den Berg *et al.*, 1997a).

The *Rb* gene, often involved in a variety of human neoplasms, is rarely altered or absent in conventional RC (Lai *et al.*, 1997), and *p53* was found to be mutated in only 3/53 RC of unspecified subtypes (Imai *et al.*, 1994). The three cases with mutations were of high grade and stage, suggesting that when present, these mutations were associated with tumour progression. *MDM2* was not amplified in any case in that study. Escape from the growth-suppressing activity of TGF $\beta_1$  was observed in 16/30 human RC cell lines, suggesting

that this mechanism may be an especially important progression factor (Ramp *et al.*, 1997). Many other growth factors have been implicated in the progression of conventional RC (Motzer *et al.*, 1996).

#### **Papillary (chromophil) renal carcinoma**

Papillary RC comprises approximately 10–15% of human RC, and is typically characterized microscopically by prominent papillary architecture with basophilic or acidophilic epithelial cells surfacing the papillations (Amin *et al.*, 1997a; Delahunt & Eble, 1997) (Figure 2). Clinically, this tumour has a propensity for multifocal and bilateral involvement, and is usually less aggressive than conventional RC. Debate as to whether papillary RC comprised a distinct neoplastic entity was silenced by the discovery that the chromosome 3p abnormalities characteristic for most conventional RC, as well as the commonly observed 5q duplications, were not present in papillary RC. Instead, the latter show consistent polysomies for certain chromosomes (Kovacs, 1989; Dijkhuizen *et al.*, 1996). Chromosomes 17 and 7 are the most important, being increased in number in 80–90% of reported tumours. Loss of the Y chromosome from tumour cells is observed in about 85% of papillary RC in males (Dijkhuizen *et al.*, 1996). Numerical gains of certain other chromosomes, especially 12, 16 and 20, and partial loss of 17p were associated with evidence of progression in papillary RC (Dijkhuizen *et al.*, 1996).

A recent report of trisomy 3 in five of eight papillary RC suggests that this trisomy may be associated with prominent haemosiderin deposits in the neoplastic epithelium, and with low-grade, early-stage tumours (Renshaw & Fletcher, 1997).

#### **Xp11.2 renal carcinoma**

This distinctive carcinoma is often characterized by papillary arrangements with prominent clear cell cytological features (Figure 3), showing a consistent chromosomal translocation involving Xp11.2, the most commonly reported one being t(X;1)p11.2;p34 (de Jong *et al.*, 1986; Tomlinson *et al.*, 1991; Meloni *et al.*, 1993; Dijkhuizen *et al.*, 1995; Tonk *et al.*, 1995). Most of the cases showing these features were males whose diagnosis was in early childhood. This tumour probably deserves a separate place in RC classification schemes because of its unique histological and cytogenetic features.

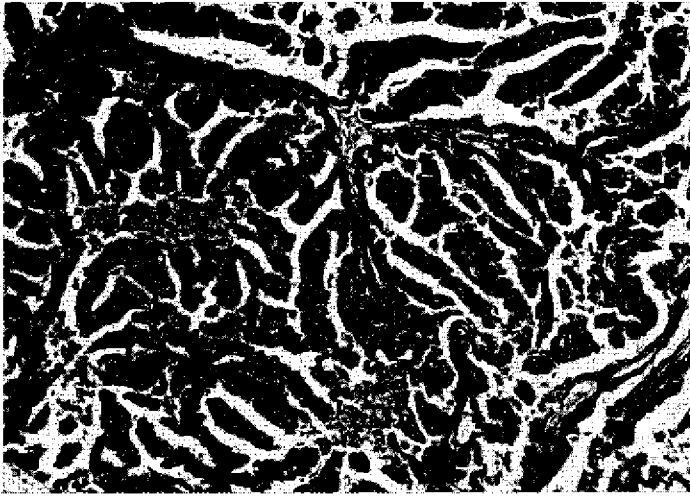


Figure 2. Papillary renal carcinoma



Figure 3. Renal carcinoma associated with Xp11.2 breakpoint. Clear tumour cells identical to those of clear cell renal carcinoma are arranged in prominent papillary formations.

Histologically it shares features of conventional RC and papillary RC, but cytogenetic and clinical distinctions justify its designation as a separate entity.

#### **Chromophobe renal carcinoma**

This recently delineated entity (Thoenes *et al.*, 1985) comprises about 4% of cases (Akhtar *et al.*, 1995; Crotty *et al.*, 1995). It is thought to arise

from intercalated cells, which are found in collecting ducts within the cortex and outer medulla, and in the junctional region connecting distal convoluted tubules with collecting ducts (Störkel *et al.*, 1989; Kim *et al.*, 1994). Cytologically, chromophobe RC has abundant pale cytoplasm with a pale, finely reticulated cytoplasm, instead of the prominent vacuolated or granular cytoplasmic appearance of conventional RC (Figure 4). Colloidal

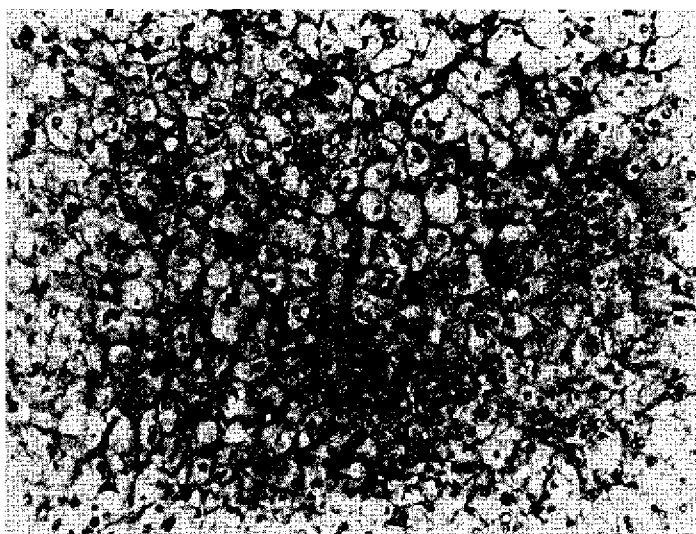


Figure 4. Chromophobe renal carcinoma

iron stains are strongly positive in this entity, in contrast to other recognized variants of RC.

This tumour is distinguished by hypodiploidy, with loss of multiple chromosomes. The most commonly observed deletions are of chromosomes 1, 2, 6, 10, 13, 17, 21 and Y (Bugert *et al.*, 1997). Alterations of mitochondrial DNA have also been described (Horton *et al.*, 1996).

### **Oncocytoma**

Oncocytoma is a distinctive neoplasm with abundant eosinophilic, densely granular cytoplasm and an alveolar growth pattern. Histochemical and ultrastructural studies reveal abundant mitochondria (Amin *et al.*, 1997b). This tumour is thought to arise from an intercalated cell of the distal nephron and outer collecting duct, as described above for chromophobe RC (Störkel *et al.*, 1988). Two categories of intercalated cells are recognized; type A cells have fewer mitochondria than type B (Clapp *et al.*, 1987). Morphological similarities suggest that type A cells may be more closely related to chromophobe RC and type B cells to oncocytoma. Morphological overlap exists between chromophobe RC and oncocytoma, reflecting the closely related nature of the putative cells of origin. Oncocytomas are generally perceived as benign tumours, and it has been suggested that 'metastasizing oncocytomas'

represent other entities (Amin *et al.*, 1997b; Dijkhuizen, 1997). High-grade papillary RC, medullary and collecting duct RC, some chromophobe RC, and possibly other malignant renal epithelial tumours often possess abundant eosinophilic cytoplasm. Nuclear atypia in true oncocytomas do not denote a worse prognosis (Amin *et al.*, 1997b).

Cytogenetic studies indicate considerable heterogeneity among oncocytomas, but suggest the existence of two distinct categories (Neuhaus *et al.*, 1997). One variant is characterized by loss of chromosomes 1 and X/Y, a feature shared with chromophobe RC. It has been suggested that this subset of oncocytomas may in fact belong to the chromophobe category, which could contribute to the problem of 'metastasizing oncocytomas', (Neuhaus *et al.*, 1997). The other subset is characterized by translocations involving 11q13 (Neuhaus *et al.*, 1997). The 11q13 region includes a number of genes potentially involved in oncogenesis. Chromosomes 1 and 11 also contain genes encoding mitochondrial proteins, and oncocytomas contain numerous mitochondria with altered mitochondrial DNA (Welter *et al.*, 1989).

### **Bellini duct (collecting duct) carcinoma**

Bellini duct carcinoma is an uncommon neoplasm, presumably arising from the principal cells of the

inner medullary collecting duct (Fleming & Lewi, 1986; Rumpelt *et al.*, 1991). It is characterized by basophilic or acidophilic cells with variably atypical nuclei, and variably distinct tubular or tubulopapillary growth patterns. Papillary formations may be so prominent as to lead to diagnostic confusion with papillary RC at the light microscopic level. These tumours are often associated with nuclear atypia in adjacent non-neoplastic collecting ducts. Few data concerning cytogenetic features of Bellini duct carcinoma are available. One report suggests that loss of 1q32.1-32.2 may be a consistent finding in these tumours (Steiner *et al.*, 1996). Loss of 8p and 13q is also reported to be frequent in these tumours (Dijkhuizen, 1997).

Medullary renal carcinoma (Davis *et al.*, 1995a; Eble, 1996) is of extraordinarily malignant potential, and death within a few months of diagnosis is the rule. It is characterized by pleomorphic acidophilic cells growing as sheets, tubules, or cribriform nodules. It is highly invasive, and intensely desmoplastic. The strong association of this entity with sickle cell trait, and the known tendency for patients with sickle trait or sickle cell anaemia to suffer focal ischaemic damage to this region of the kidney suggests a possible pathogenetic relationship to distal collecting duct injury. To date, no cytogenetic results have been reported for this entity.

### Precursor lesions as clues to pathogenesis

The studies outlined above suggest that most RC subtypes have a narrow range of cytogenetic anomalies common to most examples, with an increasingly large number of additional changes associated with tumour progression. It is assumed that high-grade RC of most, if not all, subtypes represents the end of a multistep process, as has been so clearly defined for adenocarcinoma of the colon in humans and certain other neoplastic models in animals.

If multiple discrete steps are involved in progression of most RC, then a search for morphological correlates of various stages in initiation, promotion and progression is reasonable. Small epithelial neoplasms are commonly encountered incidentally during autopsy examination of adult patients. In a careful study of 400 autopsies, in which both kidneys were completely sectioned at 1–2 mm intervals and all grossly visible lesions

examined microscopically, a total of 251 tumours were found in 83 patients (Eble & Warfel, 1991; Eble, 1993). There was a linear increase with age in the proportion of patients with these small tumours, from 10% between ages 21–40 to 40% of patients aged 70–90 years. A striking male preponderance (70:13) was observed, but it was not possible to determine whether these lesions were related to potentially carcinogenic exposures. Papillary lesions predominated, and clear cell foci constituted fewer than 10% of these small, presumably early, tumours.

Debate concerning the malignant status of these small lesions has raged for many years, and has been well summarized by Eble (1993). The earlier view that renal tumours below a certain arbitrary size were adenomas, and those above this limit carcinomas has been supplanted by widespread acceptance of the concept that some epithelial nodules are benign adenomas, whereas other lesions of equally small size have already achieved malignant status. Arbitrary definitions based on tumour size are no longer considered valid.

Much of the debate on differentiating adenoma from carcinoma antedates the recognition of cytogenetically defined RC subtypes. In view of their presumably distinct biological nature, it is essential to consider these subtypes individually.

### Conventional (or Clear cell) renal carcinoma

Experimental models of carcinogenesis are valuable for studying early cytological anomalies that precede overt proliferative activity in the pathogenesis of conventional RC. The demonstration of comparable sequences in humans, especially those that might be related to particular carcinogenic exposures, would represent a major advance. However, many difficulties lie in the way of achieving this goal, and little information is available concerning early stages in the development of conventional RC.

Mourad *et al.* (1994) evaluated renal tubular cytological changes in 110 kidneys resected for RC, including 66 cases of conventional RC. They observed intratubular epithelial dysplasia, characterized by cytological abnormalities, including nuclear enlargement, enlargement and eosinophilia of nucleoli, and cellular crowding in 21 cases of conventional RC (32%). Such dysplasia was felt to resemble changes reported in kidneys of



male Syrian hamsters bearing RC induced by high doses of oestrogen. In one case, the abnormal cells had proliferated to obliterate the lumen of proximal tubules, and was felt to represent intratubular carcinoma *in situ*. Intratubular epithelial dysplasia was found mainly in sections near the tumour, and seemed to occur independently of other abnormalities in the kidney such as inflammation or nephrosclerosis.

Patients with von Hippel-Lindau disease, an autosomal dominant disorder due to mutations in the *VHL* gene, which are also found in a majority of conventional RC, provide a unique opportunity to study early stages in the development of conventional RC. Cystic changes are commonly observed in these patients, and cysts are often lined by clear cells resembling those of low-grade conventional RC (Bernstein *et al.*, 1987). These sometimes form intraluminal nodules of proliferating epithelial cells, suggestive of early neoplasia. A recent study of 26 cysts and microscopic clear cell proliferations in kidneys of two von Hippel-Lindau patients revealed loss of the wild-type and retention of the mutated allele in all but one of the lesions studied. These results suggested that *all* clear cell lesions in the kidneys of von Hippel-Lindau patients represent neoplasms, including cysts lined by a single layer of clear epithelial cells (Lubensky *et al.*, 1996).

Other polycystic renal disorders, including adult polycystic kidney disease and tuberous sclerosis, are associated with increased incidence of RC, including conventional RC, and frequently show epithelial hyperplasia of cyst linings (Bernstein *et al.*, 1987). These authors argued effectively that cystic change itself is an early manifestation of epithelial hyperplasia and may represent an early stage in development of conventional RC.

#### **Papillary (chromophil) renal carcinoma**

Putative precursor lesions more commonly encountered in kidneys with papillary RC than with any other category of RC (Kovacs & Kovacs, 1993). A resemblance to nephrogenic rests, the usual precursor of nephroblastoma (Beckwith *et al.*, 1990), was suggested by Kovacs. A potential relationship between papillary RC and nephroblastoma is also supported by our observation (unpublished) that papillary proliferative foci in some nephroblastomas stain positively for cyto-

keratin 7. Monomorphous papillary epithelial neoplasms in infant kidneys may appear identical to tumours deemed to be papillary RC in adults. However, the fact that the incidence of the small papillary lesions encountered incidentally at autopsy increases decade by decade through adult life (Eble & Warfel, 1991; Eble, 1993) suggests that most early papillary 'adenomas' are acquired rather than congenital lesions.

In the last few years, pathologists have become increasingly aware of an adenomatous lesion of the human kidney characterized by basophilic epithelium and variably prominent papillary formations. These lesions are designated 'metanephric adenoma'. It has been suggested that these may be related to nephrogenic rests or nephroblastoma, but some may also be related to papillary RC (Davis *et al.*, 1995b; Jones *et al.*, 1995).

Cytogenetic studies have revealed a relatively consistent pattern (-Y,+7,+17) in papillary adenomas, with additional karyotypic changes being associated with progression to malignancy (Kovacs *et al.*, 1991). Papillary RC would appear to be the subtype most amenable to investigation of early pathogenetic stages in RC.

End-stage kidneys maintained on long-term dialysis are particularly prone to development of papillary RC (Ishikawa & Kovacs, 1993; Hughson *et al.*, 1996). Basophilic epithelial proliferations termed 'embryonal hyperplasia' are frequent in such kidneys (Hughson *et al.*, 1978). The chemical determinants triggering and maintaining the hyperplastic and neoplastic changes in these kidneys have not yet been clearly defined, but may ultimately provide useful clues to chemical induction of papillary RC.

#### **Bellini duct carcinoma and medullary renal carcinoma**

Bellini duct carcinoma is often associated with dysplastic changes in cells of adjacent non-neoplastic collecting ducts (Kennedy *et al.*, 1990). This suggests that cytologically detectable precursors or changes may be commonly present in this form of malignancy. The variant occurring in patients with sickle cell trait, designated medullary RC, has been suggested to be related pathogenetically to sequelae of microvascular injury caused by the underlying haematological disorder (Davis *et al.*, 1995a).

### **Oncocytomas and chromophobe renal carcinoma**

Patients with tuberous sclerosis may develop oncocytomas (Srinivas *et al.*, 1985). The cells of these tumours are identical to those lining cysts that sometimes occur in young infants with tuberous sclerosis (Bernstein *et al.*, 1986). It is possible that these cysts represent early stages in oncocytoma formation. We are aware of no description of comparable findings in chromophobe RC. A distinctive form of RC has been reported in patients with tuberous sclerosis, which were considered by the authors to have a clear or granular cell appearance comparable to conventional RC. Their distinguishing feature is consistent positivity for the melanocyte marker HMB-45, and frequently negative findings for cytokeratin (Bjornsson *et al.*, 1996). Though the cases were described as having clear cell RC, the published photomicrographs suggest a similarity to chromophobe RC. It remains to be determined whether these lesions are related, or represent a renal neoplasm unique to patients with tuberous sclerosis.

### **Transitional cell (urothelial) carcinomas**

Urothelial carcinoma of the renal pelvis, like those of the ureter and urinary bladder, has long been associated with known or suspected carcinogenic exposures. Features supporting this association include a male preponderance, increasing incidence with age, and a tendency for multifocal origin with adjacent cellular dysplasia (Corrado *et al.*, 1991). Specific risk factors identified or suspected to date include tobacco and industrial exposures (McLaughlin *et al.*, 1983), phenacetin (Palvio *et al.*, 1987) and Thorotrast, a thorium-containing radiological contrast material (Christensen *et al.*, 1983).

### **Pathogenetic mechanisms**

Pathogenetic mechanisms can be considered at two levels of resolution, cellular and molecular.

#### **Cellular injury and tumour pathogenesis**

Cellular injury, and hyperplastic reparative response, are early stages before overt tumorigenesis in animal models of RC pathogenesis. Mellempgaard (this volume) has summarized the epidemiological observations suggesting that environmental factors play a role in pathogenesis of human RC. If it could be shown that a putative

human carcinogen was associated with tubular epithelial alterations progressing to neoplasia, powerful support for causal relevance would be established. However, early stages of nephron injury attributable to these putative environmental factors seem not to have been clearly defined to date. A significant difficulty in this type of analysis is that tubular damage in kidneys of ageing adults is extremely common.

The high metabolic rate of most nephron cells predisposes them to injury from many causes. Renal tubular damage and cellular necrosis are common sequelae of anoxia, hypovolaemic shock and a wide variety of metabolic or toxic derangements in patients of all ages. Features specific to individual cell types in the nephron (e.g., protective surface layers, receptors for a given toxic agent, high concentration of organelles that concentrate, detoxify or chemically alter a specific agent) can also determine specific patterns of injury within nephrons.

Renal physiology creates an intriguing scenario for agent-specific patterns of toxic injury to different levels of the nephron, varying with the pattern of excretion, secretion, and reabsorption of the agent in question. The key role of the kidney in excretion of toxins from the circulation exposes renal cells to high concentrations of a wide variety of toxic substances. Some substances are delivered via glomerular filtration, while others are secreted into the lumen, or reabsorbed, at specific levels of the nephron. The concentration of a given toxin will usually change dramatically as it progresses down the nephron. A substance present in the glomerular filtrate that is not reabsorbed by the nephron will be concentrated approximately 100-fold by the time it reaches the distal end of the collecting duct, due to the normal process of urinary concentration. A toxic substance that is filtered by the glomerulus but completely resorbed in the proximal tubule, on the other hand, would be capable of producing direct cellular injury only in the most proximal portion of the nephron. These and other potential scenarios illustrate how different substances might injure specific levels of the nephron. A complete list of therapeutic, environmental and nutritional factors that are known to injure renal tubule cells at some concentration would probably be longer than the list of substances known *not* to injure them. However,

the list of agents or factors known or suspected to be involved in RC pathogenesis is relatively short (see Møller, 1995, this volume).

The critical issue would be to identify the cytological and molecular features of tubular cell injury preceding formation of the various types of RC, and to determine if these features are associated with specific exposures to suspected carcinogenic factors. To date, there seems to be virtually no information of this type for humans. One of the most widely available models for study is the end-stage kidney with long-term dialysis support, discussed above. A variety of early hyperplastic changes are seen in these kidneys before the development of overt neoplasia. Most of the tumours appear to be related to papillary RC.

There is a disappointing paucity, if not complete lack, of information concerning renal changes attributable to hydrocarbon exposure, tobacco usage, or other situations suspected or known to be associated with increased RC incidence in humans. A major obstacle in the way of such analyses is the frequency of renal tubular injury from a variety of causes, making it difficult to establish a relationship of tubular damage to any one factor.

### Molecular mechanisms

Various molecular mechanisms of RC pathogenesis have been proposed. The best characterized single gene defect associated with RC to date is the *VHL* mutations associated with most cases of clear cell RC, as discussed above. The protein encoded by this gene inhibits the elongation of transcription, by binding to elongins B and C. Lack of this protein facilitates uncontrolled proliferation. Thus, the *VHL* gene is a classic tumour-suppressor gene (Linehan *et al.*, 1995; Los *et al.*, 1996; Lubensky *et al.*, 1996).

A long list of cell cycle regulators and growth factors has been implicated in RC pathogenesis or progression (Motzer *et al.*, 1996). In addition, IL6 and other cytokines have been suggested to play a role, though their importance remains uncertain (Kozlowski, 1997). It is probable that identification of specific genes in addition to *VHL* that are involved in the pathogenesis of various types of RC will rapidly lead to a precise understanding of molecular mechanisms underlying development of these tumours.

### Conclusion

Traditional cytohistological criteria for classification of RC are rapidly being supplemented, and in many instances replaced, by definitions based on cytogenetic and molecular features. The distinctive cytogenetic patterns revealed to date show clearly that RC is not a homogeneous entity, and that different mechanisms probably apply to each distinct tumour category. Epidemiological studies of human renal neoplasms and experimental animal models must be related to specific RC phenotypes.

As histological and cytogenetically based tumour definitions become supplanted by specific molecular definitions, it will be possible to address more precisely the fundamental question as to whether the various subtypes of human RC are biologically related to seemingly similar tumours of other species. At the present time this can be addressed only for conventional RC, by inquiring whether similar tumours in experimental models are related to alterations in a gene or gene product comparable to those in the *VHL*-associated tumours in humans.

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# $\alpha_2$ -Urinary globulin-associated nephropathy as a mechanism of renal tubule cell carcinogenesis in male rats

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## Introduction

$\alpha_2$ -Urinary globulin ( $\alpha_{2u}$ -globulin) nephropathy is a renal syndrome that occurs exclusively in male rats. To date, a diverse group of chemicals has been shown to cause acute renal changes manifested by accumulation of protein in phagolysosomes of renal proximal tubule cells. The acute toxicity appears to be caused by the accumulation of a single protein,  $\alpha_{2u}$ -globulin, that is specific to the male rat. The protein overload causes renal cell injury, compensatory cell proliferation and ultimately a low but significant incidence of renal tubule tumours.

The contribution of  $\alpha_{2u}$ -globulin to the species-specificity of the nephropathy has been proven in several ways. It has been determined that rats that do not synthesize  $\alpha_{2u}$ -globulin do not develop renal toxicity or tumours. Furthermore, although mice are resistant to this toxicity, transgenic mice engineered to synthesize  $\alpha_{2u}$ -globulin do develop the nephropathy. Mechanistic studies have demonstrated that the rate-limiting step in development of the syndrome is the reversible, but specific, binding of a chemical (or its metabolites) to  $\alpha_{2u}$ -globulin. A comprehensive survey of structurally-related proteins along with experimental analyses has provided evidence that, although other species including humans synthesize proteins that are similar to  $\alpha_{2u}$ -globulin, differences in ligand-binding properties, physiological function and renal handling of these homologues preclude their involvement in this protein droplet nephropathy.

## Chemical inducers of $\alpha_{2u}$ -globulin nephropathy

The chemicals currently known to produce  $\alpha_{2u}$ -globulin nephropathy, as well as the major molecular events believed to be mechanistically

linked to the syndrome, are summarized in Table 1. Overall, chemical inducers of  $\alpha_{2u}$ -globulin nephropathy represent a diverse and ever-growing class. The nephropathy was originally characterized following exposure of rats to a variety of small, branched-chain petroleum hydrocarbons (Carpenter *et al.*, 1975), but it is now recognized that both aliphatic and aromatic compounds representing a variety of solvents, fuels, pesticides, drugs and naturally occurring compounds can produce this toxicity. As noted in Table 1, many chemicals are known to cause the acute syndrome of renal protein overload, but only a small subset has been tested in chronic carcinogenicity bioassays. However, all such chemicals that induced renal tubule tumours exclusively in male rats (Table 1) have also produced the acute nephropathy, suggesting that there is a link between the nephropathy and the carcinogenic outcome. The only exception to this generalization is gabapentin (Dominick *et al.*, 1991), which produced the nephropathy but did not produce renal tumours.

Collectively, the research that underpins the mechanistic understanding of  $\alpha_{2u}$ -globulin nephropathy is extensive and highly coherent. Several chemicals and mixtures have been studied in great detail. These include *d*-limonene, 2,4,4-trimethylpentane (TMP), unleaded gasoline, isophorone, 1,4-dichlorobenzene, 3,5,5-trimethylhexanoic acid and decalin. Many other chemicals that show male-rat-specific nephropathy have been evaluated for morphological and biochemical parameters of the syndrome, albeit to varying, usually lesser, extents. This can create confusion, and critics of the  $\alpha_{2u}$ -globulin nephropathy hypothesis have frequently cited data on additional chemicals that clearly do not fit the

Table 1. Biochemical and pathophysiological data for chemicals causing  $\alpha_2\mu_1$ -globulin nephropathy<sup>a</sup>

Substance/chemical	Protein droplets	Increased $\alpha_2\mu_1$ -globulin	Binding to $\alpha_2\mu_1$ -globulin	Cell proliferation	Initiation/promotion	Male rat renal tumours
$\alpha$ -Limonene	+	+	+	+	+	+
Unleaded gasoline	+	+	+	+	+	+
2,2,4-Trimethylpentane	+	+	+	+	+	NR
Sodium barbitol	+	+	+	+	+	NR
Diethylacetyl urea	+	+	+	+	+	NR
1,4-Dichlorobenzene	+	+	+	+	NR	+
Isophorone	+	+	+	+	NR	+
3,5,5-Trimethyl hexanoic acid derivatives	+	+	+	NR	NR	NR
Decalin	+	+	NR	+	NR	NR
Tricyclodecane	+	NR	NR	NR	NR	NR
1-Decalene	+	+	+	NR	NR	NR
2-Decalene	+	+	+	NR	NR	NR
Tetrachloroethylene	+	+	NR	+	NR	+
Pentachloroethane	+	+	NR	+	NR	+
C <sub>10</sub> -C <sub>12</sub> isoparaffinic solvent (saturated aliphatic hydrocarbons)	+	+	NR	NR	NR	NR
JP-4, JP-5 jet fuel (mixed distillate hydrocarbons)	+	NR	NR	NR	NR	+
Diesel fuel, marine	+	NR	NR	NR	NR	+
JP-10 synthetic jet fuel (exohexahydro-4,7-methanindan)	+	NR	NR	NR	NR	+
RJ-5 synthetic jet fuel (hydrogenated dimers of norbornadiene)	+	NR	NR	NR	NR	+
JP-7 distillate jet fuel	+	NR	NR	NR	NR	NR
JP-TS distillate jet fuel	+	NR	NR	NR	NR	NR
Stoddard solvent	+	NR	NR	NR	NR	NR
Naphthas	+	NR	NR	NR	NR	NR
60 Solvent	+	NR	NR	NR	NR	NR
Levamisole	+	+	NR	NR	NR	NR
Gabapentin	+	+	NR	NR	NR	NR



Table 1. (contd)

Substance/chemical	Protein droplets	Increased $\alpha_2$ -globulin	Binding to $\alpha_2$ -globulin	Cell proliferation	Initiation/promotion	Male rat renal tumours
Tridecyl acetate	+	+	NR	NR	NR	NR
Isopropylcyclohexane	+	+	NR	NR	NR	NR
t-Butyl cyclohexane	+	NR	NR	NR	NR	NR
Tetralin	+	NR	NR	NR	NR	NR
Hexachloroethane	+	NR	NR	NR	NR	+
Dimethyl methylphosphonate	+	NR	NR	NR	NR	+
Methyl isobutyl ketone	+	NR	NR	NR	NR	NR
Methyl isoamyl ketone	+	NR	NR	NR	NR	NR
Diisobutyl ketone	+	NR	NR	NR	NR	NR
1,3,6-Tricyanohexane	+	NR	NR	NR	NR	NR
4-Chloro- $\alpha,\alpha,\alpha$ -trifluorotoluene	+	NR	NR	NR	NR	NR

<sup>a</sup> Compilation of US Environmental Protection Agency, 1981; Swanberg, 1993; Lehman-McKeeman, 1997

<sup>b</sup> Based on cell counts from urine

NR, not reported

mechanistic requirements, in order to cloud the issue. It is also necessary to understand the criteria that exclude a chemical from being considered to work through the  $\alpha_{2u}$ -globulin nephropathy mechanism. For instance, a chemical that is considered to be genotoxic or that induces a similar response in male and female rats or in other species may also induce  $\alpha_{2u}$ -globulin nephropathy, but the primary mechanism of carcinogenic action would not require  $\alpha_{2u}$ -globulin.

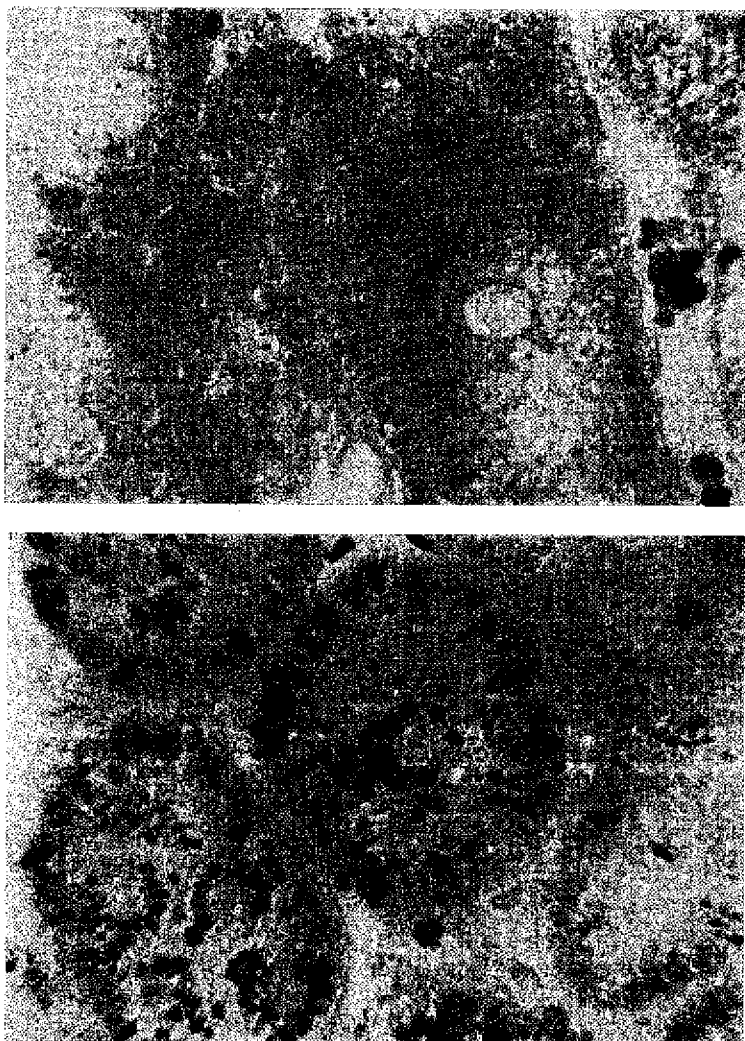
Along with the broad spectrum of chemicals listed in Table 1, it is also apparent that chemicals that cause  $\alpha_{2u}$ -globulin nephropathy differ in their potency. *d*-Limonene is one of the most potent members of this class, inducing significant renal disease with all of the pathognomonic lesions and a 25% incidence of renal tumours only in male rats at the end of two year carcinogenicity bioassays (US National Toxicology Program, 1990). In contrast, other less potent chemicals induce increases in hyaline droplets, but no progression to more advanced lesions (Dominick *et al.*, 1991). Chemicals that are genotoxic or produce renal tumours in female rats or in mice are not included in Table 1. Furthermore, if an alternate mechanism has been established, as in the case of potassium bromate-induced oxidative stress, it is not included. While these chemicals may also cause  $\alpha_{2u}$ -globulin nephropathy, it cannot be considered the sole mechanism for carcinogenesis. The  $\alpha_{2u}$ -globulin nephropathy is likely to change quantitative relationships for dose response. This chapter summarizes the biochemical and pathophysiological mechanisms underlying the renal protein overload and development of renal tubule tumours seen with  $\alpha_{2u}$ -globulin nephropathy.

### Histopathological features of $\alpha_{2u}$ -globulin nephropathy

The acute, pathognomonic feature of  $\alpha_{2u}$ -globulin nephropathy is the rapid accumulation of protein (or hyaline) droplets in the proximal tubule cells following chemical treatment (Alden *et al.*, 1984; Swenberg *et al.*, 1989). Male rats are unique among all species in that they present with a background of spontaneous protein droplets in the proximal tubule, particularly the cells of the P<sub>2</sub> segment. These spontaneous droplets have been attributed to the large amount of filtered protein that is reabsorbed and degraded in the lysosomal com-

partment of these cells (Maunsbach, 1966; Kretschmer & Bernstein, 1974). By light microscopy, these droplets are somewhat difficult to detect with routine haematoxylin and eosin staining, but they can be readily visualized with special stains including the Mallory-Heidenhain stain (Alden *et al.*, 1984; Lehman-McKeeman, 1997), Lee's methylene basic blue fuschin stain (Short *et al.*, 1986) or immunohistochemistry (Burnett *et al.*, 1989; Dietrich & Swenberg, 1991a). Figure 1 (top panel) shows the typical appearance of an untreated male rat kidney prepared with Mallory-Heidenhain staining, in which a few small protein droplets are noted. In contrast, after *d*-limonene treatment (Figure 1, bottom panel), there is an obvious and significant increase in the number, size and staining intensity of the protein droplets. These droplets contain significant amounts of  $\alpha_{2u}$ -globulin, as determined by immunohistochemical detection (Figure 2). At the ultrastructural level (Figure 3), these large droplets are seen to represent phagolysosomes that can be so engorged with protein that they become irregular and polyangular in shape and contain crystalloid inclusions (Stone *et al.*, 1987; Garg *et al.*, 1989).

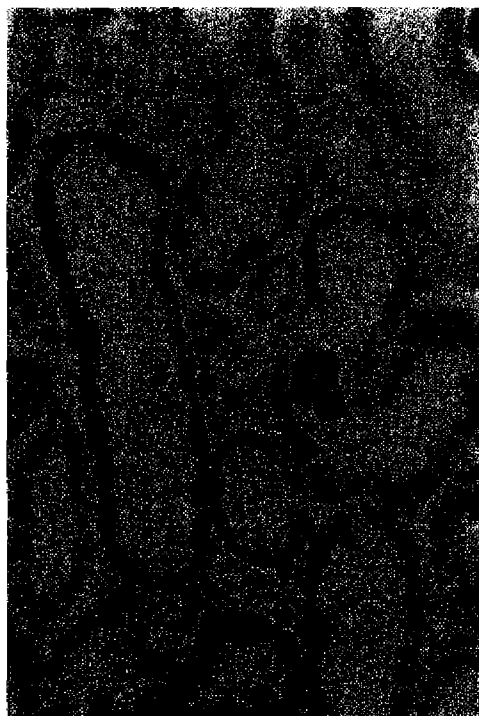
Whereas the accumulation of protein droplets occurs rapidly, continued chemical treatment results in additional histological changes in the kidney. Thus, 3–13 weeks of dosing leads to progressive renal injury, characterized by single cell degeneration and necrosis in the renal proximal tubule. Dead cells are sloughed into the lumen of the nephron, and while moving through the nephron contribute to the development of granular casts at the cortico-medullary junction. These granular casts stain positively for  $\alpha_{2u}$ -globulin, representing a highly specific lesion (Figure 4). As a result of renal cell death and degeneration, there is compensatory cell proliferation in the cortex. This increase in cell proliferation is dose- and time-related and only occurs in male rats (Alden *et al.*, 1984; Trump *et al.*, 1984; Short *et al.*, 1986, 1987, 1989a; Dietrich & Swenberg, 1991b; Umemura *et al.*, 1992; Lehman-McKeeman, 1995). Dramatic reduction in cell proliferation occurs within days of stopping exposure (Short *et al.*, 1987), and typically recovery can occur, and normal renal architecture is restored, if treatment is stopped at this time (Mattie *et al.*, 1991). However, if treatment continues, linear papillary mineralization, caused



**Figure 1.** Histopathological characterization of the spontaneous and exacerbated formation of hyaline droplets, the hallmark of  $\alpha_2$ -globulin nephropathy, in male rat kidneys ( $\times 320$ ). When stained with Mallory-Heidenhain stain, small, lightly-stained eosinophilic droplets are observed in the proximal tubule epithelium of untreated male rats (top). No similar spontaneous droplet formation is observed in the kidneys of any other species. Following a single oral dosage of *d*-limonene to male rats (150 mg/kg), the number, size and staining intensity of the droplets is markedly increased within 24 hours (bottom).

by accumulation of calcium hydroxyapatite in the thin limbs of Henle, is noted after several months of treatment (Figure 5) and there is an accelerated onset of the cortical changes typical of chronic progressive nephropathy typically seen in older male rats (Alden *et al.*, 1984; Trump *et al.*, 1984; Short *et al.*, 1987). With chronic exposure, sporadic foci of atypical hyperplasia, defined as a focal aggregation of morphologically abnormal cells

(Dietrich & Swenberg, 1993), can be observed in the proximal tubules, and the atypical foci progress to renal adenomas and carcinomas on prolonged exposure. Again, these changes are observed only in male rats. No similar renal changes have been observed in female rats or in any other species including mice, dogs or monkeys (US National Toxicology Program, 1983, 1986a, b, 1987a, b, 1990; Kitchen, 1984; Webb *et al.*, 1990).



**Figure 2.** Immunohistochemical localization of  $\alpha_{2u}$ -globulin in the proximal tubule cells. A polyclonal antibody to  $\alpha_{2u}$ -globulin was used to detect the presence of  $\alpha_{2u}$ -globulin within the protein droplets induced following  $\alpha$ -limonene treatment.



**Figure 3.** Electron micrograph of a renal proximal tubule cell showing the appearance of crystalloid inclusions in the cell following the development of  $\alpha_{2u}$ -globulin nephropathy.

A major factor that determines the pathophysiological progression of this syndrome and the carcinogenic outcome is the duration of exposure. For example, if exposures are limited, such as in the 90-day stop study on decalin (Gaworski *et al.*, 1985), the hallmark hyaline droplet response is observed, but the renal changes do not progress to include the chronic sequelae and no tumours are seen. As discussed below, the increased cell turnover and sustained cell proliferation are prerequisites for, and ultimately involved in, the tumour development associated with this syndrome.

The protein droplet nephropathy described above is specific for male rats. If a protein droplet nephropathy is also produced in female rats (exception would be an androgen) or mice of either sex, it cannot be called  $\alpha_{2u}$ -globulin nephropathy. If other dose-related degenerative renal lesions are produced by a chemical in female rats or in mice, establishment of a causal relationship between  $\alpha_{2u}$ -

globulin and the nephropathy and/or carcinogenicity in male rats will require more extensive research. This distinction should not pertain to minor exacerbations of common spontaneous renal disease.

### $\alpha_{2u}$ -Globulin

The histological characteristics of this syndrome were determined well before the role of  $\alpha_{2u}$ -globulin in renal toxicity and tumorigenesis was defined. Given that the droplets observed histologically appeared to contain protein, initial biochemical experiments focused on identifying what protein or proteins had accumulated in the droplets following exposure. Using two-dimensional electrophoresis, Alden *et al.* (1984) demonstrated that only one protein had accumulated in the kidney after chemical treatment, and this protein was subsequently identified as  $\alpha_{2u}$ -globulin. Moreover, immunohistochemical studies have localized the



**Figure 4.** Immunohistochemical localization of  $\alpha_{2u}$ -globulin within the granular casts which appear at the cortico-medullary junction following repeated administration of a compound (TMP) that induces acute  $\alpha_{2u}$ -globulin nephropathy.



**Figure 5.** Chronic exposure to chemicals which cause  $\alpha_{2u}$ -globulin nephropathy leads to the development of papillary mineralization resulting from the accumulation of calcium hydroxyapatite in the thin limbs of the loops of Henle. The mineralization is readily apparent in cross sections (left) of the kidney, and upon microscopic evaluation of the medullary region (right).

accumulation of  $\alpha_{2u}$ -globulin to the protein droplets (Figure 2; Burnett *et al.*, 1989; Dietrich & Swenberg, 1991a).

The identification of  $\alpha_{2u}$ -globulin immediately put into perspective the male rat specificity of this syndrome.  $\alpha_{2u}$ -Globulin is an unusual protein synthesized exclusively by adult male rats (Table 2). It was originally isolated from male rat urine by Roy *et al.* (1966). It is synthesized predominantly in liver (Roy & Neuhaus, 1966; Kurtz, 1981) but extrahepatic sites of synthesis, including the salivary, lachrymal, preputial, meibomian and perianal glands, have been identified (MacInnes *et al.*, 1986; Murty *et al.*, 1987; Mancini *et al.*, 1989). However,  $\alpha_{2u}$ -globulin purified from these accessory glands is electrophoretically distinct from the hepatic form of the protein, and is not male-specific as the protein has been detected in the salivary, lachrymal and preputial glands of female rats (MacInnes *et al.*, 1986; Murty *et al.*, 1987).

$\alpha_{2u}$ -Globulin is encoded by a multi-gene family clustered on chromosome 5 (Kurtz, 1981) and its expression is regulated by a complex hormonal interaction, requiring testosterone, glucocorticoids, insulin, thyroid hormone and growth hormone (Kurtz *et al.*, 1976a,b, 1978a,b; Kurtz & Feigelson, 1978; Roy *et al.*, 1980). It is generally recognized that gene expression is maximal in a hormonally intact, sexually mature male rat, and expression cannot be induced further (Kurtz & Feigelson, 1978).  $\alpha_{2u}$ -Globulin mRNA is very abundant in liver, accounting for the second highest mRNA levels in the male rat liver. Only albumin is more

abundant (Sippel *et al.*, 1976; Kurtz *et al.*, 1978a). Although female rats possess the entire complement of hepatic  $\alpha_{2u}$ -globulin genes, oestrogen is a very effective repressor of their expression (Roy *et al.*, 1975). Masculinization of female rats stimulates expression of  $\alpha_{2u}$ -globulin, but not to the levels seen in males (Roy & Neuhaus, 1967; Chatterjee *et al.*, 1989).

The  $\alpha_{2u}$ -globulin detected in the kidney is considered to be hepatic in origin (Roy *et al.*, 1966; MacInnes *et al.*, 1986). Once synthesized, the protein is rapidly secreted from the liver and hepatic levels are very low at steady state (Roy *et al.*, 1966). The molecular weight of  $\alpha_{2u}$ -globulin is approximately 18.5 kDa, and the protein is freely filtered across the glomerulus (Roy *et al.*, 1966; Neuhaus, 1986). In fact, renal clearance of the protein is so effective that plasma concentrations of  $\alpha_{2u}$ -globulin are low, typically no higher than 3 mg/100 ml (Roy & Neuhaus, 1966). It is estimated that adult male rats synthesize about 50 mg  $\alpha_{2u}$ -globulin per day, and the entire amount synthesized is filtered by the kidney (Roy *et al.*, 1966; Neuhaus *et al.*, 1981; Lehman-McKeeman & Caudill, 1992a). Only about 60% of the filtered  $\alpha_{2u}$ -globulin (or approximately 30 mg/day) is reabsorbed by the kidney (Neuhaus, 1986; Lehman-McKeeman & Caudill, 1992a), in contrast to the complete reabsorption of most other proteins by the proximal tubule cells (Maack *et al.*, 1979). Because it is so abundant, the large amount of  $\alpha_{2u}$ -globulin which is reabsorbed is thought to contribute to the spontaneous formation of protein droplets in the

**Table 2. Characteristics of  $\alpha_{2u}$ -globulin**

Synthesized primarily in the liver of adult male rats; not synthesized by any other species
Complex hormonal regulation of hepatic gene expression
Required for expression: testosterone, glucocorticoids, insulin, thyroid hormone, growth hormone
Inhibits expression: oestrogen
Low molecular weight (18.5 kDa) allows for glomerular filtration
Major urinary protein excreted by adult male rats
Member of the $\alpha_{2u}$ -globulin protein superfamily, also referred to as lipocalins
Unlike other lipocalins, no endogenous ligand is detected, and its function is unknown
Binds hyaline droplet inducing agents in a reversible manner and with a molar ratio of 1
Ligand binding cavity is spherical and has a molecular volume of about 85 Å <sup>3</sup>

male rat kidney. The remaining, non-absorbed protein (15–20 mg/day) is excreted in the urine, where, as the most abundant urinary protein, it accounts for about 35% of the total urinary protein (Roy *et al.*, 1966; Neuhaus *et al.*, 1981; Lehman-McKeeman & Caudill, 1991).

Although male rats synthesize a large quantity of  $\alpha_{2u}$ -globulin, the function of this protein remains unknown. The detection of  $\alpha_{2u}$ -globulin in pheromone-producing glands (preputial, meibomian and perianal glands) suggests a function relating to the transport of pheromones. However, no endogenous ligand for  $\alpha_{2u}$ -globulin has been identified (Roy *et al.*, 1966; Lehman-McKeeman & Caudill, 1992b; Lehman-McKeeman *et al.*, 1998). Other functions which have been suggested for the protein include roles in renal fatty acid binding (Kimura *et al.*, 1989) or in the regulation or modulation of spermatogenesis (Roy *et al.*, 1976).

Whereas no physiological function for  $\alpha_{2u}$ -globulin is known, its role in the pathophysiology of male-rat-specific protein droplet nephropathy and renal carcinogenesis is unequivocal. Only  $\alpha_{2u}$ -globulin accumulates in the protein droplets, only male rats synthesize  $\alpha_{2u}$ -globulin, and only male rats develop this syndrome. The nephropathy is not seen in female rats or any other species (Alden *et al.*, 1984; Swenberg *et al.*, 1989; Borghoff *et al.*, 1990; Hard *et al.*, 1993). Furthermore, the nephropathy does not develop in juvenile male rats (Alden *et al.*, 1984), since synthesis of the protein is not detected until puberty (Roy *et al.*, 1983), nor is it observed in the male NCI-Black Reiter (NBR) rat (Dietrich & Swenberg, 1991a), an unusual strain which does not synthesize  $\alpha_{2u}$ -globulin (Chatterjee *et al.*, 1989). Finally, although mice are refractory to this toxicity, the nephropathy can be produced in transgenic mice engineered to express  $\alpha_{2u}$ -globulin (Lehman-McKeeman & Caudill, 1994).

### Biochemical mechanisms of $\alpha_{2u}$ -globulin nephropathy

A common characteristic of all chemicals that produce  $\alpha_{2u}$ -globulin nephropathy is the ability to bind to the protein, which appears to be the rate-limiting step in the development of the nephropathy (Swenberg *et al.*, 1989; Borghoff *et al.*, 1990;

Lehman-McKeeman, 1993, 1997). In the kidney there is a sex-dependent retention of chemical, with more compound distributing to and being retained by the kidney in male rats than in female rats (Charbonneau *et al.*, 1987; Lehman-McKeeman *et al.*, 1989, 1991), and in the male rat kidney, 20–40% of the chemical is bound specifically to  $\alpha_{2u}$ -globulin (Lock *et al.*, 1987; Charbonneau *et al.*, 1989; Lehman-McKeeman *et al.*, 1989, 1991). The bound chemical can be dissociated by protein denaturation, indicating that the binding is reversible, but the ligand–protein complex survives tissue homogenization, centrifugation and lyophilization, suggesting that the complex is stable and does not dissociate *in vivo* (Lock *et al.*, 1987; Lehman-McKeeman *et al.*, 1989, 1991). The stability of the protein–chemical complex is also suggested by time course studies which show that as the chemical is cleared from the kidney, the percentage of chemical that is protein-bound increases (Lock *et al.*, 1987). Data on equilibrium saturation binding *in vitro* indicate that the dissociation constant for xenobiotic binding to  $\alpha_{2u}$ -globulin is approximately  $10^{-7}$  M (Borghoff *et al.*, 1991; Lehman-McKeeman & Caudill, 1992a,b).

For several chemicals, the moieties that bind to  $\alpha_{2u}$ -globulin have been identified (Table 1). These ligands are as structurally diverse as the class of compounds which produce the syndrome. In some cases, parent compound will bind directly to the protein (isophorone), while in others, oxidative metabolites, including epoxides (*d*-limonene-1,2-epoxide), ketones ( $\gamma$ -lactone of 3,5,5-trimethylhexanoic acid) and hydroxylated metabolites of aliphatic (2,4,4-trimethyl-2-pentanol) or aromatic compounds (2,5-dichlorophenol) have been isolated from  $\alpha_{2u}$ -globulin. It is clear that the ligand-binding site of  $\alpha_{2u}$ -globulin must accommodate very diverse chemical structures. More information on the nature of the binding site in  $\alpha_{2u}$ -globulin and the orientation of the chemical in the binding site is becoming available as the X-ray crystal structure of the protein is solved (see below). However, it is clear that ligands must be hydrophobic in nature and have a molecular volume of no greater than  $100 \text{ \AA}^3$  to fit into the protein binding site (Borghoff *et al.*, 1991; Bocskei *et al.*, 1992; Lehman-McKeeman, 1997).

Although the ligand–protein complex can be isolated from the kidney *in vivo*, it is not known

where the interaction between the xenobiotic and  $\alpha_{2u}$ -globulin occurs. The complex appears to be localized in the phagolysosomal compartment, suggesting that binding does not take place in the kidney. Rather, the interaction probably occurs outside the kidney (in liver or blood), and following glomerular filtration, the complex enters the proximal tubule cells by endocytosis, accumulating in the phagolysosomes after lysosomal fusion.

Following binding of a ligand to  $\alpha_{2u}$ -globulin, the rate of lysosomal degradation of the protein is reduced relative to the native protein. In experiments with renal cortical lysosomal lysates as enzyme source, the rate of degradation *in vitro* of  $\alpha_{2u}$ -globulin to which *d*-limonene-1,2-epoxide, isophorone or 2,5-dichlorophenol was bound decreased by about 30% (Lehman-McKeeman *et al.*, 1990). In contrast, lysosomal degradation of other proteins such as albumin was not altered by the presence of any of these chemicals, and there was no change in lysosomal cathepsin activity towards model substrates in the presence of the chemicals or the  $\alpha_{2u}$ -globulin complexes (Lehman-McKeeman *et al.*, 1990). Using immunoblotting techniques, it has been found that  $\alpha_{2u}$ -globulin exists in two forms in the kidney. One form is the native protein (18.5 kDa), whereas the second is a smaller form (about 16 kDa) representing the native protein from which the first nine amino acids have been cleaved. In general, the quantities of both forms of the protein increase after chemical treatment (Saito *et al.*, 1991; Kurata *et al.*, 1994). Therefore, the increase in amounts of protein droplets seen following chemical treatment occurs because the rate of degradation of  $\alpha_{2u}$ -globulin is reduced by ligand binding, and with chemical binding, protein begins to accumulate in the phagolysosomes.

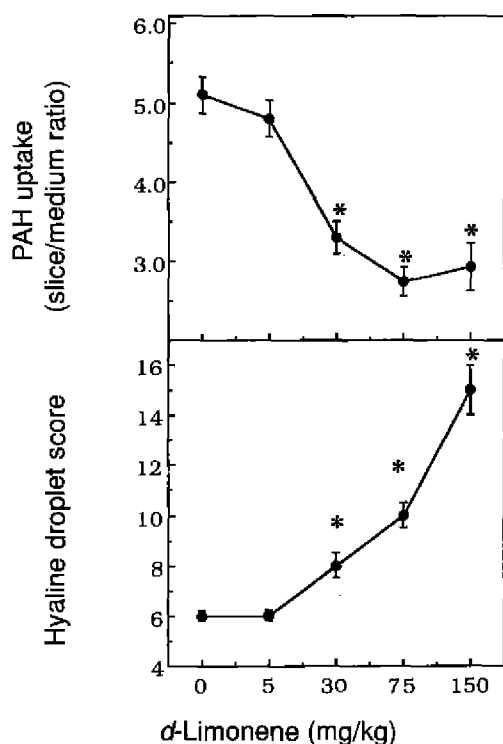
As chemical treatment continues, the lysosomes become enlarged, engorged with protein and polyangular (Figure 3). Although the mechanisms of cell death are not understood for this syndrome, cytotoxicity is evidenced by single cell necrosis in the P<sub>2</sub> segment of the proximal tubule (Short *et al.*, 1986; Swenberg *et al.*, 1989); loss of renal function is dose- and time-dependent. Renal functional perturbations include reduced uptake of organic anions, cations and amino acids and a mild proteinuria resulting from a large increase in the amount of  $\alpha_{2u}$ -globulin excreted in urine (Lehman-

McKeeman, 1995). These functional changes occur only in male rats and only at dosages which exacerbate the protein droplet formation (Figure 6). In response to the cell death and functional changes, there is a compensatory increase in cell proliferation in the kidney, most notably in the P<sub>2</sub> segment of the proximal tubules, the site of protein accumulation (Short *et al.*, 1989a; Umemura *et al.*, 1992; Lehman-McKeeman, 1995). With continued treatment, the cell proliferation persists for as long as exposure continues, but it does not restore renal function. The increase in renal cell proliferation is believed to exert a promotional influence on the kidney, such that sustained cell turnover is mechanistically linked to the development of renal tubule tumours via tumour promotion (Short *et al.*, 1989a,b; Dietrich & Swenberg, 1991b; Kurata *et al.*, 1994). Thus,  $\alpha_{2u}$ -globulin nephropathy begins acutely as protein accumulation, but represents a continuum of changes that ultimately progress to renal tumours (Swenberg *et al.*, 1989; Flamm & Lehman-McKeeman, 1991; Hard *et al.*, 1993; Hard & Whysner, 1994).

### Renal tubule tumour formation in $\alpha_{2u}$ -globulin nephropathy

In bioassays conducted by the National Toxicology Program, renal tubule tumours are uncommon in rats, occurring spontaneously at incidences of 0.96 and 0.07% for male and female Fischer 344 rats, respectively (US National Institute of Environmental Health Sciences, 1996). As noted in Table 1, several chemicals causing renal tubule tumours exclusively in male rats have been identified, and the highest tumour rate (seen with *d*-limonene) was approximately 25% (US National Toxicology Program, 1990). In this respect, these chemicals differ from classical renal carcinogens such as the nitrosamines, for which tumour incidences approach 100%. The renal tumours associated with  $\alpha_{2u}$ -globulin nephropathy are also distinguished from classical renal carcinogens in that they show a much longer latency period, requiring at least 18 months of continued dosing, and the chemicals that cause the male rat-specific renal tumours are not genotoxic (Hard, 1984, 1987; Dietrich & Swenberg, 1993; Hard *et al.*, 1993). For the renal carcinogens listed in Table 1, there is generally no evidence of mutagenicity in *Salmonella*





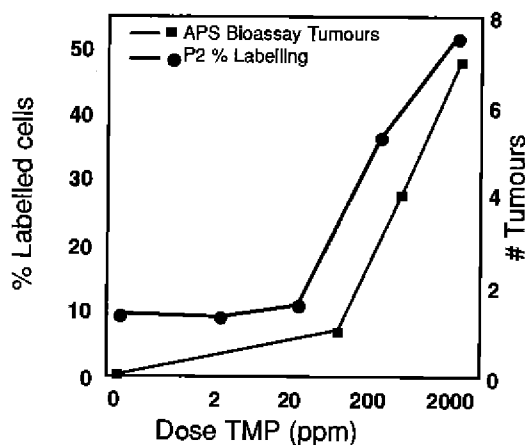
**Figure 6.** Dose-response relationship for *d*-limonene-induced acute (single exposure) exacerbation of hyaline droplet formation and subchronic changes (91 days of repeat dosing) in renal function. Exacerbation of hyaline droplets was evaluated histologically 24 hours after a single oral dosage of *d*-limonene (150 mg/kg). Droplets were graded on a scale from 0-16 (with a maximum score of 16) after staining with Mallory-Heidenhain stain. In this example, renal function was assessed as the *in vitro* uptake of *p*-aminohippurate (PAH) in cortical slices prepared from rats dosed daily with *d*-limonene for 91 days. Asterisks indicate dosages for which both hyaline droplet scores and PAH uptake were statistically different from control ( $p < 0.05$ ). A dosage of 5 mg/kg did not exacerbate hyaline droplet formation and did not change PAH uptake. At dosages of 30 mg/kg and higher, dose-dependent increases in hyaline droplet severity were observed along with a dose-dependent decrease in PAH uptake.

*typhimurium* (with or without metabolic activation) or mouse lymphoma cells, or clastogenicity in Chinese hamster ovary cells (US National Toxicology Program, 1983, 1986a,b, 1987a,b, 1990). The identification of a potentially reactive epoxide intermediate of *d*-limonene that binds to  $\alpha_{2u}$ -globulin might raise some concern that the epoxide was directly involved in the carcinogenicity of *d*-limonene. However, this epoxide, an unusually

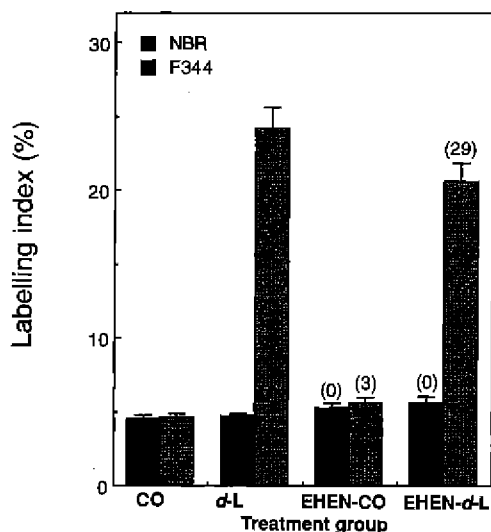
stable chemical, also shows negative results in a battery of mutagenicity tests (Watabe *et al.*, 1980, 1981; Burnett *et al.*, 1989; von der Hude *et al.*, 1989).

The lack of genotoxicity among the chemicals causing  $\alpha_{2u}$ -globulin nephropathy and male-rat-specific renal tumours supports the concept that nongenotoxic mechanisms are involved in the carcinogenic response. The cell proliferation seen with subchronic and chronic exposure to these chemicals is necessary for the tumour formation associated with this syndrome (Short *et al.*, 1989a,b; Swenberg *et al.*, 1989; Dietrich & Swenberg, 1991b; Umemura *et al.*, 1992). Sustained cell proliferation is believed to be a primary mechanism underlying the tumour response to many nongenotoxic chemicals, as accelerated cell turnover can promote clonal expansion of spontaneously initiated cells (Grasso, 1987; Swenberg *et al.*, 1989; Cohen & Ellwein, 1990; Williams & Whysner, 1996). Dose-related and male-rat-specific increases in cell proliferation have been demonstrated with all of the chemicals evaluated, and the dose-response relationships for cell proliferation parallel those for hyaline droplet formation and the induction of renal tumours (Figure 7; Short *et al.*, 1987, 1989a,b). As mentioned earlier, the increased cell proliferation is dependent on continued exposure to the agent inducing the nephropathy. When male and female rats were exposed to unleaded gasoline or TMP in the same inhalation chambers, cell proliferation did not increase in females, but there were clear, concentration-dependent increases in male rats (Short *et al.*, 1989a). Furthermore, the link between the nephropathy and cell proliferation was established in experiments comparing NBR and Fischer 344 rats exposed to *d*-limonene (Dietrich & Swenberg, 1991a,b). Cell proliferation was increased in Fischer 344 rats, but not in NBR rats which lack  $\alpha_{2u}$ -globulin and do not develop  $\alpha_{2u}$ -globulin nephropathy following 4 or 31 weeks of exposure to *d*-limonene (Figure 8). Thus, the increase in cell proliferation was shown to be totally dependent on the presence of  $\alpha_{2u}$ -globulin.

The concept that chemicals inducing  $\alpha_{2u}$ -globulin nephropathy cause renal tubule tumours by secondary, non-genotoxic mechanisms is further supported by the results of several initiation-promotion studies. After unleaded gasoline and *d*-limonene were identified as causing  $\alpha_{2u}$ -globulin



**Figure 7.** Dose-response relationship for TMP-induced proximal tubule cell proliferation and the development of renal tubule tumours.



**Figure 8.** Relationship between  $\alpha$ -limonene induced renal cell proliferation and kidney tumour promotion in male Fischer 344 and NBR rats. No increase in cell proliferation was observed in the NBR rat, which does not synthesize  $\alpha_{2u}$ -globulin, whereas increased cell proliferation and promotion of EHEN-initiated renal tumours was observed in the Fischer 344 rat.

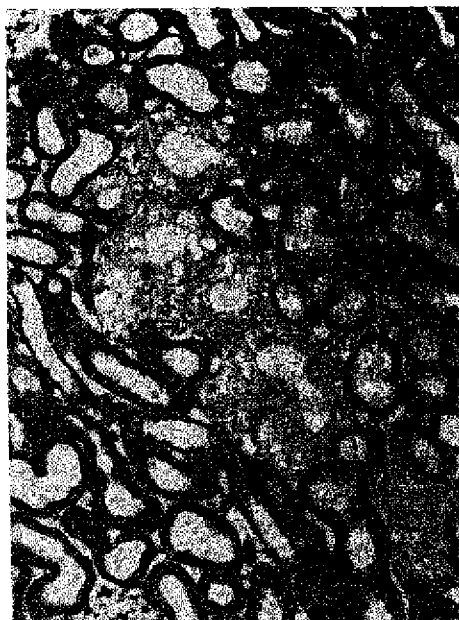
nephropathy, two large initiation-promotion experiments were conducted. The first was a classical initiation-promotion experiment on unleaded gasoline and TMP that included all of the sequence controls and was conducted on male and female rats (Short *et al.*, 1989b). In this study, *N*-nitroso-ethylhydroxyethylamine (EHEN) was used as the initiator and three concentrations of unleaded gasoline or 50 ppm TMP were evaluated for their ability to promote atypical renal foci and renal tumour formation. Parallel studies on cell proliferation were conducted (Short *et al.*, 1989a). Evidence of tumour promotion was present only in male rats, demonstrating that promotion paralleled the findings in the carcinogenicity bioassay on unleaded gasoline (Kitchen, 1984). Promotion was concentration-dependent, with significant increases in renal foci and tumours in nitrosamine-initiated rats exposed to 300 ppm unleaded gasoline, paralleling the results of the carcinogenicity bioassay. Promotion (nitrosamine followed by TMP or unleaded gasoline) was much more effective than administration of the same substances in the reverse sequence, i.e., animals were exposed to TMP or unleaded gasoline for 24 weeks, then given nitrosamine for two weeks and held for an additional 35 weeks (Short *et al.*, 1989b).

A second initiation-promotion study, conducted with EHEN and  $\alpha$ -limonene, compared the response of NBR and Fischer 344 male rats (Dietrich & Swenberg, 1991b) in order to evaluate the requirement for  $\alpha_{2u}$ -globulin in formation of renal foci and tumours. In this study, cell proliferation, atypical tubules, atypical hyperplasia and renal adenomas were evaluated. EHEN was an effective initiator when administered alone to either strain of rat, causing increases in atypical tubules, the earliest preneoplastic lesion (Dietrich & Swenberg, 1993). When EHEN treatment was followed by  $\alpha$ -limonene, marked increases in atypical tubules (Figure 9) and atypical hyperplasia (Figure 10) were induced in male Fischer 344 rats, but no increase was seen in NBR rats. Likewise, incidence of renal adenomas (Figure 11) was significantly increased in Fischer 344 rats, but none occurred in NBR rats. Increased cell proliferation was present at 7 and 32 weeks of promotion in Fischer 344, but never in NBR rats.

The initiation-promotion studies conducted with unleaded gasoline and  $\alpha$ -limonene were

performed after the ability of these chemicals to cause  $\alpha_2$ -globulin nephropathy had been characterized. In contrast, the renal tumour-promoting activity of sodium barbital and a major hydrolysis product, diethylacetylurea, was established well before these two chemicals were shown to cause  $\alpha_2$ -globulin nephropathy in male rats. Briefly, in standard initiation-promotion protocols, both sodium barbital and diethylacetylurea have been shown to promote renal tumour formation in male Fischer 344 rats following initiation by *N*-nitrosodiethylamine (Diwan *et al.*, 1985, 1989a,b). Both agents also produced nephrotoxicity and increased renal cell proliferation (Ward *et al.*, 1991), but only after kidneys were evaluated by Mallory-Heidenhain staining and immunohistochemical analysis was their ability to induce  $\alpha_2$ -globulin nephropathy recognized (Kurata *et al.*, 1994).

Collectively, the initiation-promotion studies provide compelling evidence that unleaded gasoline, TMP, *d*-limonene and sodium barbital act as renal tubule tumour promoters and that this



**Figure 10.** Microscopic appearance of atypical renal cell hyperplasia in a male Fischer 344 rat initiated with EHEN and promoted with *d*-limonene.



**Figure 9.** Microscopic appearance of an atypical tubule in a male Fischer 344 rat initiated with EHEN and promoted with *d*-limonene.

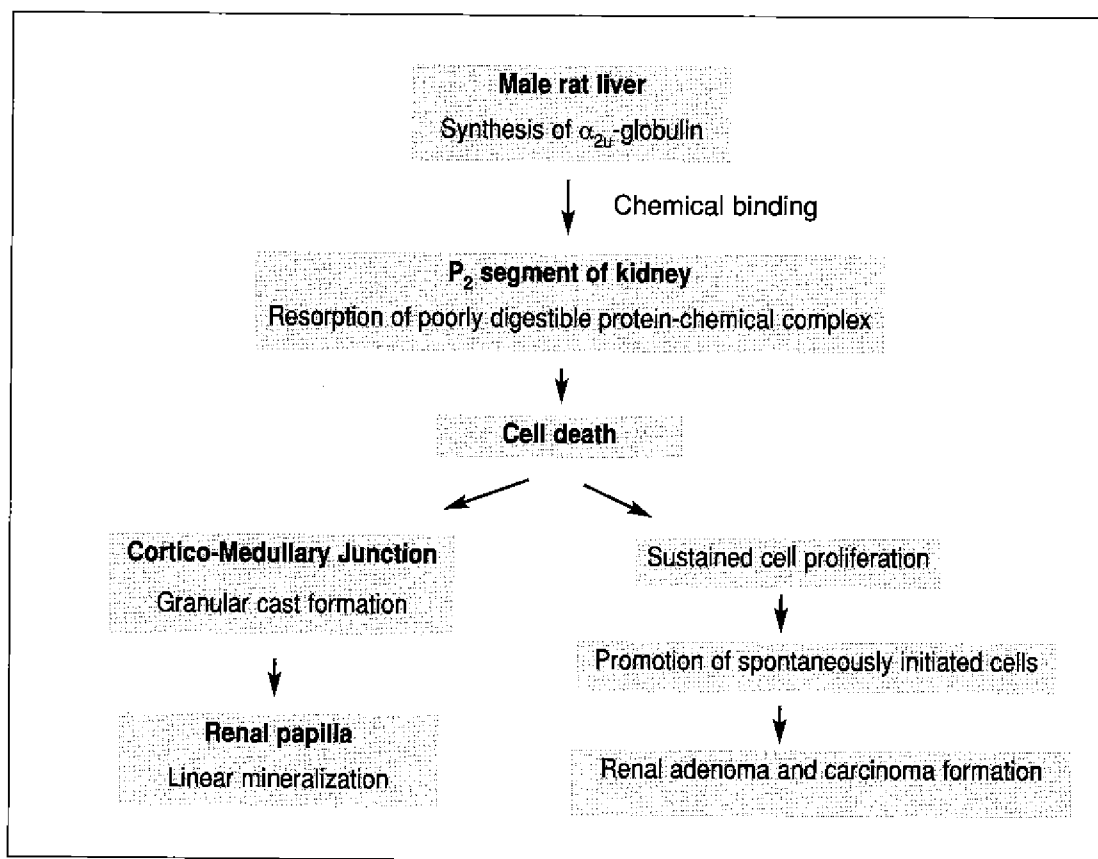


**Figure 11.** Microscopic appearance of a large renal adenoma in a male Fischer 344 rat initiated with EHEN and promoted with *d*-limonene.

promoting effect requires  $\alpha_{2u}$ -globulin. The requirement for  $\alpha_{2u}$ -globulin also explains why the renal tumour induction by these agents is a male-rat-specific effect. The link between the acute development of  $\alpha_{2u}$ -globulin nephropathy and the chronic development of renal tubule tumours is the renal cell injury which stimulates sustained cell proliferation. In this manner, the biochemical and pathophysiological data support the conclusion that  $\alpha_{2u}$ -globulin nephropathy begins as an acute protein overload but represents a continuum of changes that ultimately progress to renal tumours. The major mechanistic events in this continuum are summarized in Figure 12.

#### Human relevance of $\alpha_{2u}$ -globulin nephropathy

Given the compelling and comprehensive evidence that  $\alpha_{2u}$ -globulin is necessary for the development of this syndrome and that the protein is synthesized exclusively by adult male rats, it seems reasonable to conclude that  $\alpha_{2u}$ -globulin nephropathy is unique to the male rat. However,  $\alpha_{2u}$ -globulin is a member of a large protein superfamily (Brooks, 1987; Flower *et al.*, 1993; Flower, 1994) characterized by a very unusual tertiary structure. The proteins in this family, described as lipocalins and often referred to as the  $\alpha_{2u}$ -globulin protein superfamily, are synthesized in many species, including humans. The primary function of the lipocalins is



**Figure 12.** Schematic representation of the continuum of changes initiated by the binding of a xenobiotic to  $\alpha_{2u}$ -globulin. Small droplets form spontaneously in male rat kidneys due to the large amount of  $\alpha_{2u}$ -globulin in the kidney. When a chemical binds to  $\alpha_{2u}$ -globulin, the acute, pathognomonic response is the rapid exacerbation of hyaline droplet formation, and with repeated exposure, the syndrome progresses from protein overload to renal cell injury, cell death and compensatory sustained hyperplasia prior to the development of renal tubule tumours.

to bind and transport small hydrophobic molecules, and for many of them, endogenous ligands have been identified (Table 3). The unique structural motif is a  $\beta$ -barrel with eight anti-parallel  $\beta$ -strands that fold into an orthogonal calyx, or cup-like structure. Ligands are bound within the  $\beta$ -barrel cavity which is lined almost exclusively by hydrophobic amino acids and which form the shape of the pocket and contribute to ligand specificity (Papiz *et al.*, 1986; Brooks, 1987; Godovac-Zimmerman, 1988; Cowan *et al.*, 1990).

The superfamily protein that is most similar to  $\alpha_{2u}$ -globulin is mouse urinary protein (MUP). MUP is the mouse homologue of  $\alpha_{2u}$ -globulin, sharing nearly 90% sequence homology and hormonally controlled in a manner similar to that described previously for  $\alpha_{2u}$ -globulin (Hastie *et al.*, 1979; Shaw *et al.*, 1983). MUP is also the major urinary protein excreted by mice (Finlayson *et al.*, 1963; Lehman-McKeeman & Caudill, 1992a) and represents the most abundant mRNA species in the mouse liver (Hastie *et al.*, 1979). However, unlike  $\alpha_{2u}$ -globulin, MUP expression is not fully repressed by oestrogen, so that female mice synthesize and excrete MUP, albeit at levels that are much lower than in males (Rumke & Thung, 1964; Hastie *et al.*, 1979; Knopf *et al.*, 1983; ). It is estimated that male mice excrete about 15 mg of MUP daily (Lehman-McKeeman & Caudill, 1992a).

If similarities in molecular structure are sufficient for the superfamily proteins to bind to chemicals that cause  $\alpha_{2u}$ -globulin nephropathy, then, because of the very high homology between

$\alpha_{2u}$ -globulin and MUP, mice should be sensitive to developing a similar renal syndrome. However, studies conducted in mice have clearly indicated that no renal changes, including renal tumours, occur following exposure to any of the chemicals known to cause renal tumours in male rats (US National Toxicology Program, 1983, 1986a,b, 1987a,b, 1990; Bomhard *et al.*, 1988; Lehman-McKeeman *et al.*, 1991; Lake *et al.*, 1997). The lack of toxicity in mice treated with agents that induce hyaline droplets is attributed to several major differences between MUP and  $\alpha_{2u}$ -globulin. Specifically, MUP does not bind any of the chemicals known to bind to  $\alpha_{2u}$ -globulin (Lehman-McKeeman & Caudill, 1992a) and MUP is not reabsorbed into the kidney to any significant extent (Larsen *et al.*, 1990; Lehman-McKeeman & Caudill, 1992a). In direct contrast,  $\alpha_{2u}$ -globulin transgenic mice are sensitive to *d*-limonene-induced  $\alpha_{2u}$ -globulin nephropathy although the renal handling of MUP is not affected in any way (Lehman-McKeeman & Caudill, 1994).

There are two other major differences between  $\alpha_{2u}$ -globulin and the lipocalin superfamily proteins that distinguish  $\alpha_{2u}$ -globulin from all of these proteins. The first difference is that for most of the proteins in the superfamily, important endogenous ligands have been identified. In direct contrast, no endogenous ligand has been detected for  $\alpha_{2u}$ -globulin (Table 3; Lehman-McKeeman *et al.*, 1998). For example, it is well established that retinol-binding protein (RBP) binds and transports retinol (Blaner, 1989) and apolipoprotein D transports cholesterol in the circulation (Drayna *et al.*, 1987). Similarly, a variety of ligands have been identified for odorant-binding protein (Pevnsner *et al.*, 1990) and  $\alpha 1$ -acid glycoprotein (Ganguly *et al.*, 1967; Urien *et al.*, 1982). For MUP, 2-sec-butyl-4,5-dihydrothiazole, a compound with established pheromonal properties, has been isolated from the purified protein (Liebich *et al.*, 1977; Bocskei *et al.*, 1992; Lehman-McKeeman *et al.*, 1998). The presence of endogenous ligands not only indicates a function for these proteins, but is also likely to prevent the binding of hyaline droplet-inducing agents to the superfamily proteins. In this regard, it has been shown empirically that other superfamily proteins, particularly those synthesized by humans, do not bind to agents that induce  $\alpha_{2u}$ -globulin nephropathy (Lehman-McKeeman & Caudill, 1992b). In the

**Table 3. Members of the lipocalin or  $\alpha_{2u}$ -globulin superfamily<sup>a</sup>**

Protein	Endogenous ligand
$\alpha_{2u}$ -Globulin	Unknown
Mouse urinary protein	2-(sec-butyl)-4,5-Dihydrothiazole
Retinol binding protein	Retinol
$\alpha 1$ -Acid glycoprotein	Drugs, steroids
$\beta$ -Lactoglobulin	Retinol
Odorant-binding protein	Odorants
Apolipoprotein D	Cholesterol

<sup>a</sup> Abridged from Lehman-McKeeman, 1997

absence of an endogenous ligand for  $\alpha_{2u}$ -globulin, the empty binding pocket allows chemicals to bind to the protein. Furthermore, it also appears that the binding of ligands to other superfamily proteins does not affect the renal handling of the protein, whereas for  $\alpha_{2u}$ -globulin, binding of a chemical clearly alters the renal degradation of the protein (Urien *et al.*, 1982; Blaner, 1989; Lehman-McKeeman *et al.*, 1990). Therefore, although the other superfamily proteins are reabsorbed by the proximal tubule, they do not accumulate in the kidney either spontaneously or following chemical treatment (Alden *et al.*, 1984). This observation also holds true for humans, as no  $\alpha_{2u}$ -globulin-like protein has been detected in human kidney tissue (Borghoff & Lagarde, 1993).

The last and most important difference between  $\alpha_{2u}$ -globulin and the other members of the lipocalin superfamily concerns the nature of the binding cavity in  $\alpha_{2u}$ -globulin. X-ray crystallographic data for  $\alpha_{2u}$ -globulin show that the binding pocket (1) is closed off to water; (2) is lined by extremely hydrophobic amino acid residues, and (3) has a probe-accessible volume of  $84.0 \pm 0.9 \text{ \AA}^3$ , which is very similar to that ( $75 \text{ \AA}^3$ ) of the (also hydrophobic) binding pocket in MUP (Bocskai *et al.*, 1992; Lehman-McKeeman, 1997). However, subtle differences in the amino acids that line the binding pocket markedly affect the shape of the cavities, which differs greatly between MUP and  $\alpha_{2u}$ -globulin (Figure 13). Specifically, two phenylalanine residues in  $\alpha_{2u}$ -globulin (Phe-54 and Phe-103) serve to close off the back of the binding cavity, creating a pocket that is spherical in shape (Lehman-McKeeman, 1997). The large spherical shape of this pocket does not restrict binding, supporting the fact that a diverse array of chemical structures can bind to  $\alpha_{2u}$ -globulin. In contrast, the amino acid residues at positions 54 and 103 in MUP are leucine and alanine, respectively. These amino acids do not close off the pocket, yielding a cavity that is flattened and elongated (Bocskai *et al.*, 1992). In this manner, the MUP cavity is restricted by its elongated shape, and cannot accommodate bulky chemicals such as *d*-limonene-1,2-epoxide (Lehman-McKeeman & Caudill, 1992a; Lehman-McKeeman *et al.*, 1998). Other lipocalins, such as retinol-binding protein also have flattened, elongated binding cavities that overlap only by about  $20 \text{ \AA}^3$  with that of  $\alpha_{2u}$ -globulin when the

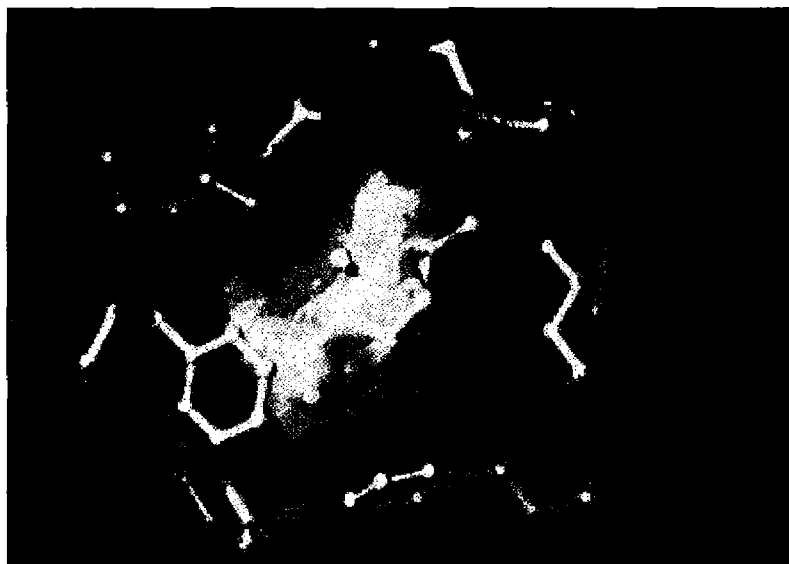
protein structures are superimposed (Cowan *et al.*, 1990). Thus, the specific binding of chemicals to  $\alpha_{2u}$ -globulin is ultimately attributable to the unique nature and shape of its ligand binding site, which does not restrict chemical binding.

Collectively, the present data support the conclusions that only  $\alpha_{2u}$ -globulin contributes to this renal syndrome and that structurally-similar  $\alpha_{2u}$ -globulin superfamily proteins synthesized by humans are not predictive of any risk for humans to develop renal cancer when exposed to agents that induce  $\alpha_{2u}$ -globulin nephropathy. At the same time, however, there is the possibility, albeit remote, that other, unrelated proteins which bind xenobiotics might serve as a surrogate for  $\alpha_{2u}$ -globulin in humans. To directly address this issue, Borghoff and Lagarde (1993) characterized the protein composition of human (male) kidneys and determined whether agents known to bind to  $\alpha_{2u}$ -globulin could bind to human kidney proteins. The results of this work demonstrated that the protein composition of human kidney was very different from that of male rat kidneys and there was no specific binding of  $\alpha_{2u}$ -globulin ligands to human renal proteins. These results provide convincing evidence that there is no protein in humans that can contribute to a renal syndrome like  $\alpha_{2u}$ -globulin nephropathy.

### Alternative hypotheses

During the past 15 years, a significant body of data, including biochemical, cellular and molecular evidence, has been assembled by a number of research groups to support the conclusion that  $\alpha_{2u}$ -globulin plays a central role in the continuum described for the development of male-rat-specific renal tubule tumours (Figure 12). Furthermore, extensive data support the conclusion that the properties of  $\alpha_{2u}$ -globulin are so unique that no other protein can contribute to such a syndrome. Most importantly, even in cases of potentially high human exposure to agents that induce the syndrome in male rats (as in the case of therapeutic uses), there are no data implicating these chemicals as human nephrotoxins or carcinogens. Despite the thorough evaluation of the mechanisms underlying this syndrome, the possibility that other mechanisms may contribute to the toxicity has been suggested.

One alternative proposal is that the chemicals themselves are nephrotoxic and that binding to



**Figure 13.** Depiction of the ligand-binding cavities in  $\alpha_{2u}$ -globulin (blue cloud) and mouse urinary protein (MUP) (red cloud) as determined by X-ray crystallography. For  $\alpha_{2u}$ -globulin, the shape of the binding cavity is essentially spherical, resulting from the orientation of two phenylalanine residues which close off the back of the cavity. The two phenylalanine residues in  $\alpha_{2u}$ -globulin are replaced by leucine and alanine, respectively in MUP, resulting in a binding cavity that is elongated and flattened (red cloud) relative to the binding pocket in  $\alpha_{2u}$ -globulin. Although the binding cavities of the two proteins are similar in volume, the actual overlap in shape (the highlighted, pink region) is very small. The spherical binding cavity in  $\alpha_{2u}$ -globulin is far less restrictive, thereby allowing for a broad spectrum of chemicals to bind to the protein.

$\alpha_{2u}$ -globulin simply serves to concentrate them in the male rat kidney (Melnick, 1992; Melnick & Kohn, this volume). In this manner, toxicity is observed because the delivered dose to the target organ is increased. Accordingly, this proposal is still dependent on the presence of  $\alpha_{2u}$ -globulin, and given the unique properties of the protein, the lack of human relevance still holds. At the same time, however, existing data do not support the contention that direct toxicity of the chemicals themselves is involved in renal toxicity and carcinogenicity. First, as noted earlier, the  $\alpha_{2u}$ -globulin-ligand complex is quite stable, arguing against the notion that the chemical can be released from the kidney without protein denaturation. Second, direct evaluation of TMP metabolites has indicated that they are not nephrotoxic (Borghoff & Lagarde, 1993). Finally, the most compelling data supporting the non-toxic effects of these chemicals exists for *d*-limonene, an agent currently being developed as a cancer chemotherapeutic agent (Crowell & Gould, 1994). In early animal studies to address its

anti-carcinogenic activity, *d*-limonene was fed in the diet to female rats at levels up to 100 000 ppm (10% of diet), and no toxicity was observed (Elegbede *et al.*, 1986; Elson *et al.*, 1988). Furthermore, in a 13-week study conducted by the US National Toxicology Program before the bioassay for *d*-limonene, female rats were given doses ranging from 150 to 2400 mg/kg by gavage and no renal toxicity was detected, whereas in male rats, renal lesions were observed at all dosages (US National Toxicology Program, 1990). Although there are no accompanying toxicokinetic data for these studies, it is reasonable to assume that the renal concentrations of *d*-limonene achieved at the extremely high dosages tolerated by female rats are at least equal to the renal concentrations achieved in male rats at much lower dosages.

It is also important to emphasize that the syndrome characterized by  $\alpha_{2u}$ -globulin nephropathy is specific to male rats and involves non-genotoxic mechanisms. Therefore, if a chemical is deemed to be genotoxic or causes renal injury and tumours in female rats or other species,

mechanisms other than or in addition to  $\alpha_{2u}$ -globulin nephropathy must be contributing. It is possible that for certain chemicals, particularly halogenated hydrocarbons,  $\alpha_{2u}$ -globulin may contribute to nephrotoxic and nephrocarcinogenic responses in male rats, but other mechanisms, including metabolic activation by glutathione conjugation (Dekant *et al.*, 1989; Lock, 1989) may also be involved in the development of toxicity and carcinogenicity.

Another argument put forward as providing evidence in conflict with the existing mechanism of  $\alpha_{2u}$ -globulin nephropathy is that, for several chemicals, there is a discontinuity between the acute nephropathy and the development of renal tumours (Huff, 1996). As discussed earlier, there is a critical level of sustained cell proliferation that is necessary to promote renal tubule tumour formation. If the severity of the acute nephropathy and subsequent regenerative hyperplasia is below that critical level, a chemical (e.g., gabapentin) may not produce renal tumours. The concept of a non-linear dose-response relationship for this syndrome is entirely consistent with the hypothesis that non-genotoxic mechanisms are involved in the development of the renal tubule tumours in male rats (Grasso, 1987; Short *et al.*, 1987; Williams & Whysner, 1996). Furthermore, as emphasized earlier, the cell proliferation that links renal injury to renal foci and tumours must be sustained chronically. Consequently, it is inappropriate to infer carcinogenic outcome based on subchronic regimens such as a 90-day stop study (Gaworski *et al.*, 1985).

Overall, although alternative hypotheses have been suggested, they are not supported by the existing data, and no effort has been made to generate new data in support of these hypotheses.

#### **Risk assessment for species-specific carcinogenicity caused by $\alpha_{2u}$ -globulin nephropathy**

If sufficient data are generated to demonstrate that a chemical causes renal tubule tumours in male rats via a mechanism involving  $\alpha_{2u}$ -globulin nephropathy, the existing evidence implies that the renal tumours are not predictive of a similar risk to humans. Thus, any evaluation of risk is based on a qualitative, rather than a quantitative analysis. However, this is not a determination to be made lightly, and it is critical that the appropriate

scientific evidence is developed to support the conclusion that a chemical does not pose a cancer risk to humans. The US Environmental Protection Agency Risk Assessment Forum has developed criteria which must be met in order to establish that a chemical causes renal tubule tumours via a mechanism involving  $\alpha_{2u}$ -globulin. These criteria, and their impact on risk evaluation are presented here to summarize the extensive review incorporated into the human risk assessment for male rat-specific renal tumours (US Environmental Protection Agency, 1991).

Given some of the current misconceptions that have led to the development of alternative hypotheses, it is important that the criteria used to establish that an agent causes renal tumours in male rats by a mechanism involving  $\alpha_{2u}$ -globulin nephropathy be stringent, clearly communicated and strictly adhered to. The essential criteria which need to be satisfied to conclude that an agent causes kidney tumours in male rats through a response associated with  $\alpha_{2u}$ -globulin are listed in Table 4. The first criterion is that an agent causing renal tumours should be negative in a battery of genetic toxicity tests, thereby supporting a non-genotoxic mechanism of tumour formation. Both the acute renal effects as well as the renal tubule

**Table 4. Criteria for establishing the role of  $\alpha_{2u}$ -globulin nephropathy in male rat renal carcinogenesis**

##### **Essential evidence**

- Renal tumours occur only in male rats
- Acute exposure exacerbates hyaline droplet formation
- $\alpha_{2u}$ -Globulin accumulates in hyaline droplets
- Subchronic histopathological changes including granular cast formation and linear papillary mineralization
- Absence of hyaline droplets and characteristic histopathological changes in female rats and mice
- Negative for genotoxicity in a battery of tests

##### **Additional supporting evidence**

- Reversible binding of chemical (or metabolites) to  $\alpha_{2u}$ -globulin
- Increased and sustained cell proliferation in P<sub>2</sub> segment of proximal tubules in male rat kidneys
- Dose response relationship between hyaline droplet severity and renal tumour incidence



tumours must be male rat-specific. If clear evidence of renal toxicity or an increased incidence of renal tumours are observed in female rats or in other species, these criteria do not apply. Thirdly, the chemical should present with the common histological and biochemical features of  $\alpha_2$ -globulin nephropathy, namely the exacerbated formation of hyaline droplets in proximal tubule cells, the development of subchronic renal changes including granular cast formation and linear papillary mineralization, and the absence of similar histological changes in female rats or in other species. It is also essential to show that  $\alpha_2$ -globulin accumulates in the kidney and that the chemical (or metabolites) can bind reversibly to  $\alpha_2$ -globulin. Data showing that chemical treatment causes sustained increases in renal cell proliferation in the P<sub>2</sub> segment of the proximal tubule are important to link the acute toxicity with tumour development. Lastly, dose-response relationships which characterize toxicity and the carcinogenic outcome should be similar.

In reviewing chemicals listed in Table 1, it is obvious that only a few of the compounds studied meet all these criteria. Those agents for which all criteria have been fulfilled include *d*-limonene, unleaded gasoline, sodium barbital (and its metabolite, diethylacetylurea), 1,4-dichlorobenzene and isophorone. The fact that only a few chemicals meet the described criteria underscores the stringency of the criteria and the breadth of mechanistic evidence that is required to prove this mechanism. Collectively, however, the data supporting these criteria demonstrate a mechanism that is unique to the male rat, a mechanism that is predicated on the unusual features of  $\alpha_2$ -globulin, and a mechanism that has no correlate in humans.

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# Possible mechanisms of induction of renal tubule cell neoplasms in rats associated with $\alpha_{2u}$ -globulin: role of protein accumulation versus ligand delivery to the kidney

R. L. Melnick and M. C. Kohn

## Introduction

In 1991, the US Environmental Protection Agency (EPA) adopted the view that when kidney tumours are induced in male rats, but not in female rats, and  $\alpha_{2u}$ -globulin accumulation is also observed in the kidney, the kidney tumour response may not be applicable to human risk assessment (USEPA, 1991). Three criteria were established by EPA for examining evidence linking  $\alpha_{2u}$ -globulin accumulation with the tumour response, namely:

- (1) increased number and size of hyaline droplets in renal proximal tubule cells of treated male rats,
- (2) identification of the accumulating protein in the hyaline droplets as  $\alpha_{2u}$ -globulin, and
- (3) presence of additional aspects of the pathological sequence of lesions associated with  $\alpha_{2u}$ -globulin nephropathy (single cell necrosis, exfoliation of epithelial cell into the proximal tubule lumen, formation of granular casts, linear mineralization of papillary tubules, and tubule hyperplasia).

Additional information to be considered includes reversible binding of ligand to  $\alpha_{2u}$ -globulin, decreased lysosomal degradation of  $\alpha_{2u}$ -globulin, and sustained cell proliferation in the P<sub>2</sub> segment of the proximal tubule of male rats at doses that induce renal neoplasms. However, demonstration of the latter factors was not necessary, even though they are more mechanistically linked to the  $\alpha_{2u}$ -globulin hypothesis. The EPA specifies that if experimental data do not meet the criteria in any one of the three categories listed

above, the  $\alpha_{2u}$ -globulin process alone is not considered to be responsible and the kidney tumour response may be used for both hazard identification and quantitative risk estimation. If experimental data reasonably fulfil these criteria but some tumours are attributable to other carcinogenic processes, the EPA's cancer risk assessment policy is to include the tumour response for hazard identification but to engage in quantitative risk estimation only if the non- $\alpha_{2u}$ -globulin potency can be estimated. If the tumour response is *solely* attributable to  $\alpha_{2u}$ -globulin nephropathy, the EPA does not use that response in human hazard identification or quantitative risk estimation. In several cases, much debate has ensued on the applicability of these criteria and about the  $\alpha_{2u}$ -globulin hypothesis for evaluation of human cancer risk (Huff, 1996; Dietrich, 1997; Melnick *et al.*, 1997).

A previous review of this topic (Melnick, 1992) identified several inconsistencies and important data gaps in the hypothesis linking  $\alpha_{2u}$ -globulin nephropathy to the induction of kidney tumours in male rats and demonstrated how alternative hypotheses based on the same available information are plausible. Thus the mechanisms of chemically induced  $\alpha_{2u}$ -globulin nephropathy and chemically induced renal cancer remain at the level of operational hypotheses.

When generalized classification schemes cannot accommodate wide variations in response to various toxicants or when quantitative relationships between the supposed early-stage carcinogenic events and cancer outcome cannot be established, then use of hypothetical schemes may

result in excessive reliance on preconceived notions rather than on data from definitive experiments.

The purpose of the previous review (Melnick, 1992) was to stimulate further research that would lead to a better understanding of the mechanisms involved in chemically induced renal carcinogenesis. Research findings that address assumptions in mechanistic hypotheses of chemical carcinogenesis can be valuable in reducing uncertainties in predictions of human risk and lead to improved scientifically based public health decisions. Unfortunately, little progress has been made on some of the fundamental issues that were previously raised. It seems that the creation of imprecisely formulated classification systems based on mechanistic assumptions and qualitative correlations of response stifles the advancement of basic science. Thus in several studies of chemical induction of kidney tumours in male rats, research goals appear to have been focused more on showing that criteria for dismissing positive animal findings had been reached than on improving our understanding of the quantitative relationships in the biological processes leading to the carcinogenic effect.

The development of public health policies for judging the relevance of animal findings in evaluations of human hazard potential demands rigorous and objective scrutiny of the strengths and weaknesses of hypotheses, especially when decisions based on such hypothesis may contribute to widespread exposure to chemicals that would otherwise be restricted. This is particularly important for kidney carcinogens, since the incidence of human kidney cancer (in the USA) has increased by 30% between 1973 and 1989 (Miller *et al.*, 1992), and the incidence is higher in males than in females, both in humans (Miller *et al.*, 1992) and in animals (Barrett & Huff, 1991). Instructive in this respect are reports of increased kidney cancer risk associated with human exposure to gasoline vapours (Siemietycki *et al.*, 1987; Partanen *et al.*, 1991; Lynge *et al.*, 1997), while animal exposure to totally vaporized unleaded gasoline is associated with the induction of  $\alpha_{2u}$ -globulin nephropathy and kidney tumours in male rats (MacFarland *et al.*, 1984; Short *et al.*, 1987, 1989a, 1989b; Gérin *et al.*, 1991). Because of changes in the composition of hydrocarbons and additives in gasoline over the past 50 years (e.g.,

benzene, tetraethyl lead) and differences in the composition of gasoline vapour exposures and totally vaporized gasoline (Halder *et al.*, 1986), it is likely that the animal and human exposures differed significantly; however, the results of these studies indicate that some constituents of gasoline increase the incidence of kidney cancer in animals and in humans.

The extent and quality of data for individual chemicals that induce  $\alpha_{2u}$ -globulin nephropathy and kidney cancer varies greatly. Consequently, Swenberg (1993) cautioned that "this necessitates a case-by-case analysis of the available data when determining the relevance for humans of this chemically induced renal disease in male rats." This paper examines reported relationships between exposure to chemicals that cause  $\alpha_{2u}$ -globulin accumulation in the kidneys of male rats and the induction of renal tubule cell neoplasms.

### The $\alpha_{2u}$ -globulin hypothesis

The hypothesis on the role of  $\alpha_{2u}$ -globulin in chemical induction of kidney cancer was developed based on the observation that protein droplets containing  $\alpha_{2u}$ -globulin accumulate in epithelial cells of the proximal convoluted tubules of male rats exposed to hydrocarbons that had been reported to cause kidney cancer in male rats after long-term exposure.  $\alpha_{2u}$ -Globulin is a protein of low molecular weight (18.7 kDa) synthesized in the liver of mature male rats under androgenic control (Roy & Neuhaus, 1967). It is not synthesized by hepatocytes of female rats, mice of either sex, or several other species including humans. Hydrocarbons or their metabolites that bind reversibly to  $\alpha_{2u}$ -globulin do not appear to cause an increase in the hepatic concentration of this protein (Viau *et al.*, 1986; Olson *et al.*, 1987; Murty *et al.*, 1988).

$\alpha_{2u}$ -Globulin is secreted into the blood, filtered through the glomerulus, and partially reabsorbed (~60%) by endocytosis into renal tubule epithelial cells of the P<sub>2</sub> segment (Neuhaus *et al.*, 1981). The unabsorbed fraction is excreted in the urine, while the reabsorbed portion is presumably hydrolysed to amino acids after fusion of endocytotic vesicles with epithelial cell lysosomes. The accumulation of protein droplets containing  $\alpha_{2u}$ -globulin was suggested to be due to reversible binding of xenobiotic ligands to this protein, rendering it



more resistant to proteolytic degradation by lysosomal enzymes (Swenberg *et al.*, 1989; Lehman-McKeeman *et al.*, 1990). Binding is considered to be critical for  $\alpha_{2u}$ -globulin accumulation; however, since binding affinities for various ligands have been reported to vary by more than three orders of magnitude ( $1.8 \times 10^{-7}$  to  $5.2 \times 10^{-4}$  M), other factors were suggested to be involved in  $\alpha_{2u}$ -globulin accumulation (Borghoff *et al.*, 1991). *In vitro* studies of lysosomal degradation of  $\alpha_{2u}$ -globulin purified from the urine of control male rats showed a 24–33% decrease in the rate of degradation when the protein was incubated with excess amounts of ligands that cause  $\alpha_{2u}$ -globulin accumulation in the male rat kidney (Lehman-McKeeman *et al.*, 1990).

Proteolytic processing of  $\alpha_{2u}$ -globulin during its resorption into renal proximal tubules results in the accumulation of a kidney-type  $\alpha_{2u}$ -globulin ( $\alpha_{2u}$ -K; 17 kDa), which was identified as a kidney fatty acid-binding protein (Kimura *et al.*, 1989; Uchida *et al.*, 1995). Proteolytic processing at the brush border membrane appears to precede the endocytotic uptake of this protein. Treatment of male rats with agents that cause  $\alpha_{2u}$ -globulin accumulation results in large increases in  $\alpha_{2u}$ -K in the male rat kidney (Saito *et al.*, 1992). The major form of the protein excreted in the urine of control rats is the native type (18.7 kDa), whereas in the urine of rats treated with chemicals that induce  $\alpha_{2u}$ -globulin accumulation, dose-dependent increases in  $\alpha_{2u}$ -K excretion have been observed (Saito *et al.*, 1996).

The excessive accumulation of  $\alpha_{2u}$ -globulin is hypothesized to cause lysosomal dysfunction, resulting in cell killing (Swenberg *et al.*, 1989). The actual cause of cell death is not known. Sloughing of necrotic epithelial cells into the tubule lumen has been observed and intratubular granular casts of necrotic cellular debris accumulate at the junction of the P<sub>3</sub> segment of the proximal tubule and the thin loop of Henle. Regenerative proliferation of epithelial cells in the P<sub>2</sub> segment occurs in response to the cell loss (Short *et al.*, 1987, 1989a; Dietrich & Swenberg, 1991a).

Although the mechanistic link between cell proliferation and kidney cancer is unknown, it has been suggested that regenerative hyperplasia causes the tumorigenic response in the male rat kidney by increasing the likelihood of fixing pre-

sumed spontaneous cancer-initiating DNA damage into heritable mutations or by promoting the clonal expansion of spontaneously initiated cells (Short *et al.*, 1989b; Dietrich & Swenberg, 1991a). In support of this hypothesis, neither protein droplet nephropathy nor increases in renal tumours have been observed in female rats or in mice of either sex exposed to chemicals that induce  $\alpha_{2u}$ -globulin accumulation in the kidneys of male rats. Further, protein droplet nephropathy was not induced in NIH-Black-Reiter (NBR) rats (Dietrich & Swenberg, 1991b), a strain that is deficient in hepatic  $\alpha_{2u}$ -globulin synthesis. However, no two-year carcinogenicity study in NBR rats has been reported and renal tumours induced by these chemicals in male rats have not been analysed for genetic alterations.

### Changes in renal $\alpha_{2u}$ -globulin levels and relationships to kidney cancer

#### Unleaded gasoline

Two-year inhalation studies of unleaded gasoline, at exposure concentrations of 0, 67, 292 and 2056 ppm, resulted in dose-related increased incidences of kidney tumours in male Fischer 344 rats (0, 2, 9 and 16%, respectively) and liver tumours in female B6C3F<sub>1</sub> mice (MacFarland *et al.*, 1984). The kidney findings are particularly noteworthy, because renal tubule cell neoplasms are uncommon in untreated male Fischer 344 rats, occurring at a rate of approximately 0.6% (Solleveld *et al.*, 1984).

Halder *et al.* (1985) examined the toxicity of 15 hydrocarbon compounds found in unleaded gasoline, and concluded that the nephrotoxicity of gasoline was due primarily to the alkane components, and that the nephrotoxic potency of these compounds increased with the degree of alkane branching. Consequently, 2,2,4-trimethylpentane (TMP), one of the most active nephrotoxic components in this mixture, has been used as a model compound to study the mechanism of renal toxicity induced by unleaded gasoline. In male Fischer 344 rats treated with TMP for three weeks, the dose-response curve for renal protein droplet accumulation correlated with that for increased replication of epithelial cells in the P<sub>2</sub> segment of renal proximal tubules (Short *et al.*, 1987).

A similar dose-dependence was observed for the kidney tumour response in male Fischer 344 rats exposed to unleaded gasoline for two years and for

the increased replication rates of P<sub>2</sub> epithelial cells after three weeks of inhalation exposure to unleaded gasoline (Short *et al.*, 1987). An enhanced rate of cell proliferation (4–11-fold) in the P<sub>2</sub> segment has been observed through 48 weeks of exposure of male Fischer 344 rats to 300 ppm unleaded gasoline (Short *et al.*, 1989a). Enhanced cell proliferation (4–8-fold) was also observed in P<sub>3</sub> epithelial cells through 22 weeks of exposure to 300 ppm unleaded gasoline. Because no increase in cell replication was seen in the kidney of female rats at any time point, it was hypothesized that chronic cell proliferation associated with  $\alpha_{2u}$ -globulin nephropathy may be responsible for the apparent sex- and species-specificity of the kidney carcinogenic effects of unleaded gasoline in laboratory animals. However, as noted above, elevated risk of kidney cancer associated with human exposure to gasoline vapours has been reported, so it cannot be stated that the carcinogenic effects of complex gasoline mixtures are entirely species-specific.

Selected intermediates of TMP metabolism were administered to male Fischer 344 rats, and kidney sections were examined for evidence of  $\alpha_{2u}$ -globulin accumulation (Charbonneau *et al.*, 1987a). All chemicals studied, including the carboxylic acid metabolites of TMP (Charbonneau *et al.*, 1987b), which do not bind to  $\alpha_{2u}$ -globulin, caused accumulation of  $\alpha_{2u}$ -globulin. This observation suggests that binding to  $\alpha_{2u}$ -globulin may not be the only process associated with TMP-induced renal accumulation of this protein.

2,4,4-Trimethyl-2-pentanol (TMP-2-OH) was

the major metabolite of TMP detected in the male rat kidney and was associated with an increase in the renal concentration of  $\alpha_{2u}$ -globulin; this metabolite was not detected in the female rat kidney (Charbonneau *et al.*, 1987b). TMP-2-OH has a high binding affinity for  $\alpha_{2u}$ -globulin ( $K_d = 0.2 \mu\text{M}$ ; Borghoff *et al.*, 1991). The reason for the sex differences in urinary metabolite profiles and in the renal distribution of TMP-2-OH may be important in understanding sex or species differences in the induction of renal nephropathy by TMP. Charbonneau *et al.* (1987b) also presented data on the kidney concentrations of  $\alpha_{2u}$ -globulin and of TMP-equivalents in male Fischer 344 rats at 24 hours after treatment with [<sup>14</sup>C]TMP by gavage. At doses of TMP that elicited accumulation of  $\alpha_{2u}$ -globulin, the renal molar concentrations of  $\alpha_{2u}$ -globulin were substantially higher than the renal molar concentrations of radiolabelled TMP equivalents. For example, as the dose of TMP was increased from 5 to 50 mg/kg body weight, the increase in renal  $\alpha_{2u}$ -globulin was 3.5 times greater than the increase in TMP equivalents. Thus, at a dose of TMP (50 mg/kg) that has been shown to induce severe protein droplet nephropathy and high levels of regenerative hyperplasia (Short *et al.*, 1987), a large percentage (~70%) of the  $\alpha_{2u}$ -globulin present in the kidney of male rats is unbound. This point is also illustrated in studies with TMP-2-OH (Table 1). Twenty-four hours after administration of a single oral dose of 600 mg/kg, the renal concentration of  $\alpha_{2u}$ -globulin in male rats was approximately 3100  $\mu\text{M}$  whereas the renal concentration of TMP-2-OH was only about 800

**Table 1. Renal stoichiometry of  $\alpha_{2u}$ -globulin and TMP-2-OH in male Fischer 344 rats 24 hours after an oral dose of TMP-2-OH<sup>a</sup>**

Dose (mg/kg)	$\alpha_{2u}$ -globulin ( $\mu\text{M}$ )	TMP-2-OH ( $\mu\text{M}$ )	$\alpha_{2u}$ -globulin: TMP-2-OH (molar ratio)
6	950	400	2.4
60	1900	800	2.4
600	3100	800	3.9

<sup>a</sup> Adapted from Borghoff *et al.* (1995)

$\mu\text{M}$  (Borghoff *et al.*, 1995). This study shows that as the dose of TMP-2-OH was increased, the concentration of  $\alpha_{2u}$ -globulin increased to a greater extent than that of the ligand. Because of the large excess of  $\alpha_{2u}$ -globulin compared to TMP-2-OH and the 1:1 stoichiometry of ligand binding to  $\alpha_{2u}$ -globulin (Lehman-McKeeman & Caudill, 1992), accumulation of  $\alpha_{2u}$ -globulin in the male rat kidney is unlikely to be due simply to ligand binding and inhibited degradation of the ligand-bound protein.

Borghoff *et al.* (1992) compared the accumulation of  $\alpha_{2u}$ -globulin and the level of cell proliferation in the kidney of male Fischer 344 rats administered European high test (EHT) gasoline or unleaded gasoline for 10 days. Unleaded gasoline has a higher concentration of branched hydrocarbons than EHT and produced a greater increase in  $\alpha_{2u}$ -globulin accumulation. At doses containing approximately equivalent amounts of TMP (500 mg/kg body weight of EHT and 16 mg/kg body weight of unleaded gasoline), protein droplet scores and renal concentrations of  $\alpha_{2u}$ -globulin were similar, while the level of cell proliferation in the renal proximal tubules was nearly two times greater with the EHT dose than with the unleaded gasoline dose. Evidently, stimulation of renal cell proliferation in rats administered EHT must have involved mechanisms unrelated to  $\alpha_{2u}$ -globulin accumulation, possibly induced by other components in this complex mixture. Hence, the observation of both  $\alpha_{2u}$ -globulin accumulation and cell proliferation in kidney tubules of male rats does not necessarily indicate that the proliferative response was due solely to  $\alpha_{2u}$ -globulin nephropathy.

Short *et al.* (1989b) observed a dose-related increase in the incidence of atypical cell foci (considered by the authors to be preneoplastic lesions) in the kidneys of male Fischer 344 rats, but not in female rats, initiated with *N*-nitrosoethyl(hydroxyethyl)amine (EHEN) and then exposed for 59 weeks to unleaded gasoline at exposures ranging from 10 to 300 ppm. An increase was similarly observed in EHEN-initiated male rats that were exposed to 50 ppm TMP. Renal lesions were not increased significantly in male rats treated with unleaded gasoline or TMP alone. These studies indicate that unleaded gasoline and TMP can promote the development of chemically induced preneoplastic lesions in the kidney of male rats.

### **Methyl *t*-butyl ether (MTBE) and *t*-butyl alcohol (TBA)**

Inhalation exposure of Fischer 344 rats to 0, 400, 3000 or 8000 ppm MTBE (6 hours/day, 5 days/week, for up to two years) produced increased incidences of renal tubule adenomas and carcinomas and of interstitial cell adenomas of the testes in male rats (Chun *et al.*, 1992). The severity of chronic nephropathy was increased in exposed male and female rats, though no carcinogenic response was observed in females. The severity of nephropathy in male rats was greater than that typically seen with chemicals that induce  $\alpha_{2u}$ -globulin accumulation. The same exposures in CD-1 mice, but for only 18 months, produced increased incidences of hepatocellular carcinomas in males and hepatocellular adenomas and carcinomas in females (Burleigh-Flayer *et al.*, 1992).

Administration of the MTBE metabolite *t*-butyl alcohol in drinking water to Fischer 344 rats for two years produced increased incidences of renal tubule hyperplasias and renal tubule adenomas or carcinomas in male rats but not in female rats. As with MTBE, *t*-butyl alcohol treatment increased the severity of nephropathy in both male and female rats (Cirvello *et al.*, 1995; National Toxicology Program, 1995). Interestingly, a treatment-related increase in hyaline droplet accumulation was observed in the renal proximal tubules of male rats but not in female rats given *t*-butyl alcohol in drinking water for 13 weeks. In addition, an increase in renal tubule cell replication was observed in male rats, but only at exposures that exceeded the carcinogenic doses used in the two-year study (Takahashi *et al.*, 1993).

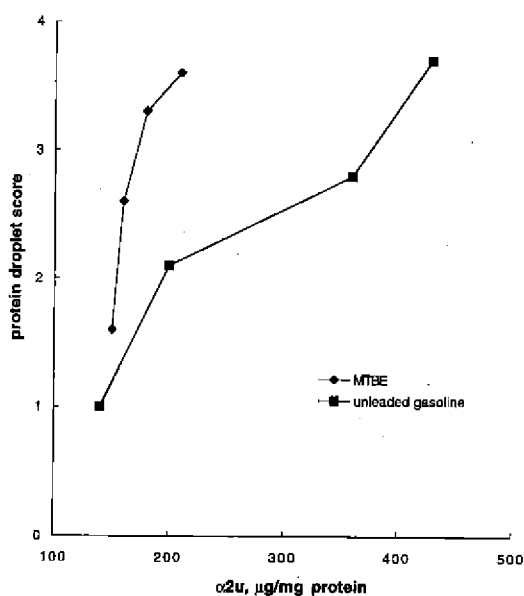
Immunohistochemical studies of  $\alpha_{2u}$ -globulin in kidney sections from male rats exposed to MTBE for 13 weeks did not show an exposure-related increase in staining for this protein (Swenberg & Dietrich, 1991). Because staining was equivalent after exposure to 400, 3000 or 8000 ppm MTBE, yet only the two higher concentrations produced kidney tumours, a clear relationship between  $\alpha_{2u}$ -globulin accumulation and kidney carcinogenesis could not be established. Furthermore, proteinaceous casts localized at the junction of the proximal tubules and the thin loop of Henle did not stain positively for  $\alpha_{2u}$ -globulin. Thus, classical effects of  $\alpha_{2u}$ -globulin nephropathy-inducing agents are not evident in rats exposed to MTBE

(Swenberg & Dietrich, 1991), suggesting that other factors are involved in MTBE-induced nephropathy and renal carcinogenicity in male rats.

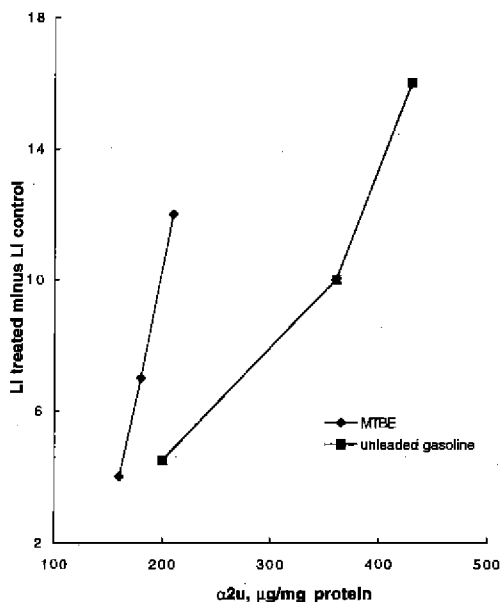
Ten-day exposures to MTBE vapours, at 0, 400, 1500 or 3000 ppm, produced exposure-related increases in protein droplet accumulation and renal epithelial cell proliferation in proximal tubules of male Fischer 344 rats but not in female rats (Prescott-Mathews *et al.*, 1997). Unlike other chemicals that induce  $\alpha_{2u}$ -globulin nephropathy, a very mild exposure-related increase in the kidney concentration of  $\alpha_{2u}$ -globulin was observed in male rats (by the enzyme-linked immunosorbent assay, which is more quantitative than  $\alpha_{2u}$ -globulin immunohistochemical staining), with a significant increase seen only at 3000 ppm.

A comparison between the renal effects of unleaded gasoline and MTBE evaluated in the same laboratory is worth noting (Figures 1 and 2). Exposure of male Fischer 344 rats to unleaded gasoline for 10 days produced dose-related

increases in protein droplets,  $\alpha_{2u}$ -globulin accumulation and renal epithelial cell proliferation (Borghoff *et al.*, 1992). At exposures to unleaded gasoline and MTBE that produced comparable increases in protein droplets and cell proliferation, male rats treated with unleaded gasoline had much greater increases in  $\alpha_{2u}$ -globulin (300%) than those treated with MTBE (40%). Further, the slope of plots of protein droplet score (Figure 1) or elevated cell proliferation (Figure 2) versus concentration of  $\alpha_{2u}$ -globulin in the kidneys of exposed male rats is much steeper for MTBE than it is for unleaded gasoline. These slopes should be invariant with the identity of the  $\alpha_{2u}$ -globulin-inducing chemical if  $\alpha_{2u}$ -globulin accumulation alone is the cause of these effects. The divergence between increases in protein droplets or cell proliferation and  $\alpha_{2u}$ -globulin accumulation suggests that other factors than  $\alpha_{2u}$ -globulin are largely responsible for the protein droplet and cell proliferation responses in the kidneys of male rats exposed to MTBE.



**Figure 1.** Relationship between  $\alpha_{2u}$ -globulin ( $\alpha_{2u}$ ) concentration and protein droplet score in the proximal tubules of male Fischer 344 rats exposed to methyl *t*-butyl ether by inhalation (Prescott-Mathews *et al.*, 1997) or to unleaded gasoline (Borghoff *et al.*, 1992) by gavage for 10 consecutive days.



**Figure 2.** Relationship between  $\alpha_{2u}$ -globulin ( $\alpha_{2u}$ ) concentration and increases in labelling index (LI) in the proximal tubules of male Fischer 344 rats exposed to methyl *t*-butyl ether by inhalation (Prescott-Mathews *et al.*, 1997) or to unleaded gasoline (Borghoff *et al.*, 1992) by gavage for 10 consecutive days.

Poet *et al.* (1996) found that the interaction between kidney proteins and MTBE did not withstand dialysis in buffer or anion exchange chromatography and suggested that binding between MTBE and kidney proteins was either very weak or nonspecific. For other chemicals that induce  $\alpha_{2u}$ -globulin accumulation in the male rat kidney, approximately 20–40% of the ligand remains bound after dialysis in buffer. This finding is true even for chemicals such as 1,4-dichlorobenzene and its metabolite 2,5-dichlorophenol (Charbonneau *et al.*, 1989), which also have weak binding affinities for  $\alpha_{2u}$ -globulin ( $\sim 5 \times 10^{-4}$  M; Borghoff *et al.*, 1991). Thus, interactions between MTBE and  $\alpha_{2u}$ -globulin appear to be different from those of other chemicals that induce the accumulation of this protein.

In spite of the differences in behaviour between MTBE and other chemicals that induce  $\alpha_{2u}$ -globulin nephropathy, a review panel assembled by the US National Research Council (NRC) concluded that results of the 10-day study of MTBE in rats showed that  $\alpha_{2u}$ -globulin was involved in the causation of the kidney tumours observed in the two-year inhalation study and that the kidney cancer findings should not be used for human risk assessment (National Research Council, 1996). The evaluation by the NRC panel shows how reliance on simplistic classification systems applied to renal carcinogenesis can lead to less than rigorous scrutiny of the strengths and weaknesses of available data in the decision-making process.

### ***d*-Limonene**

A two-year study of *d*-limonene administered by gavage in corn oil showed increased incidences of renal tubule neoplasms in male rats (0/50 control; 8/50 at 75 mg/kg; 11/50 at 150 mg/kg), but not in female rats that received 300 or 600 mg/kg or mice that received up to 1000 mg/kg (National Toxicology Program, 1990).

Most chemicals that have been shown to induce protein droplet nephropathy and renal carcinogenesis also increased tumour incidence at other organ sites; mouse liver was the site of the most common tumour response. *d*-Limonene is an exception to this common occurrence. Although *d*-limonene has a weak binding affinity for  $\alpha_{2u}$ -globulin, its metabolite, *d*-limonene oxide has

a strong binding affinity ( $5.6 \times 10^{-7}$  M) for  $\alpha_{2u}$ -globulin (Borghoff *et al.*, 1991; Lehman-McKeeman & Caudill, 1992). In rats dosed with *d*- $[^{14}\text{C}]$ -limonene, the renal concentration of *d*-limonene equivalents was 2.5 times higher in male rats than in female rats, and *d*-limonene oxide was identified associated with  $\alpha_{2u}$ -globulin in the male rat kidney (Lehman-McKeeman *et al.*, 1989).

Dietrich and Swenberg (1991a) reported that administration of *d*-limonene by gavage at 150 mg/kg for 30 weeks promoted renal tumour development in male Fischer 344 rats, but not in male NBR rats, after EHEN initiation. This species difference was attributed to hepatic synthesis and renal accumulation of  $\alpha_{2u}$ -globulin in the Fischer rats and the associated increase in renal tubule cell proliferation. Other differences between these strains of rats might have had an effect on the results. For example, EHEN produced a much higher incidence of atypical hyperplasias in renal tubules and a higher incidence of liver tumours in Fischer 344 rats than in NBR rats. Because no two-year carcinogenicity study has been performed with NBR rats, it is not known whether this strain of rat responds to initiators and promoters of renal carcinogenesis similarly as do Fischer 344 rats.

### ***Gabapentin***

The anticonvulsant drug gabapentin (1-(aminomethyl)cyclohexanecarboxylic acid) caused  $\alpha_{2u}$ -globulin nephropathy, including a moderate to marked increase in hyaline droplets, in male Wistar rats given 2000 mg/kg for 104 weeks, without producing kidney tumours (Dominick *et al.*, 1991). In a 13-week study, this dose of gabapentin caused hyaline droplet accumulation in proximal tubules, granular cast formation in the renal cortex and outer medulla, and tubule epithelial regeneration. Thus, the accumulation of hyaline droplets containing  $\alpha_{2u}$ -globulin and the degenerative/regenerative changes in the proximal tubule epithelium (P<sub>1</sub> segment) that occurred in male rats exposed to gabapentin were not predictive of renal carcinogenesis with chronic exposure. Renal tubule cell proliferation data are not available for rats exposed to this drug.

### ***Lindane***

Administration of lindane by gavage to male Fischer 344 rats (4 days) at 10 mg/kg per day

induced  $\alpha_{2u}$ -globulin nephropathy (Dietrich & Swenberg, 1990); this dose is similar to the doses of lindane used in a long-term feeding study (up to 470 ppm) that did not increase the incidence of renal tumours in male rats (National Cancer Institute, 1977). Thus, this study, like the one above, shows that renal changes associated with  $\alpha_{2u}$ -globulin nephropathy are not always predictive of renal carcinogenicity.

#### **1,4-Dichlorobenzene (1,4-DCB)**

Administration of 1,4-dichlorobenzene by gavage for two years led to increased incidences of renal tubule cell neoplasms in male rats (1/50 control, 3/50 at 150 mg/kg, and 8/50 at 300 mg/kg) and hepatocellular neoplasms in mice of both sexes (National Toxicology Program, 1987); no neoplastic effects were observed in female rats. The severity of nephropathy was greater in dosed male rats than in controls, while dose-related increases in the incidence of nephropathy were observed in female rats and in mice of both sexes.

1,4-DCB and its major metabolite, 2,5-dichlorophenol, bind reversibly to  $\alpha_{2u}$ -globulin (Charbonneau *et al.*, 1989) but with affinities three orders of magnitude lower than TMP-2-OH or *d*-limonene oxide (Borghoff *et al.*, 1991). Cell proliferation studies in rats administered 1,4-DCB by gavage for 4 days showed increases in proliferating cells in the proximal tubule of males at the high dose (300 mg/kg), but not at 150 mg/kg or in female rats at either dose level (Umemura *et al.*, 1992). Increases in cell proliferation were also observed in the livers of rats of both sexes, even though this was not a target site of 1,4-DCB carcinogenesis. Evidently, increases in cell proliferation after short-term exposures are not reliably predictive of carcinogenesis due to long-term exposure.

Species differences in the biotransformation of 1,4-DCB may be important in the organ-specific carcinogenic effects of this chemical. Glutathione conjugates of 2,5-dichlorohydroquinone, a metabolite of 1,4-DCB, have been suggested to contribute to the nephrotoxic effects of 1,4-DCB in the male rat (Klos & Dekant, 1994), while 2,5-dichlorobenzoquinone may be more important in the hepatocarcinogenicity of 1,4-DCB in mice (Hissink *et al.*, 1997).

Relevant to the consideration of a possible role of dichlorohydroquinone in the renal carcino-

genicity of 1,4-DCB are results of studies on its congener, hydroquinone. Hydroquinone is a clastogenic agent that produced kidney tumours in male rats at gavage doses of 25 or 50 mg/kg body weight but no kidney tumours in female rats. The kidney tumour response in male rats was not associated with hyaline droplet accumulation (National Toxicology Program, 1989a). The severity of spontaneous nephropathy commonly seen in aged Fischer 344 rats was increased in high-dose males; however, in comparison to controls, the severity of this lesion was not increased in the low-dose group of male rats. The incidence of liver tumours was also increased in treated female mice. This pattern of tumour response (kidney tumours in male rats but not in female rats and liver tumours in mice) was seen with 1,4-DCB and several other chemicals that induce  $\alpha_{2u}$ -globulin accumulation in the kidney of male rats. Hence, it is evident that factors in addition to  $\alpha_{2u}$ -globulin contribute to the greater susceptibility of male Fischer 344 rats compared with female rats or mice to the development of renal tumours. Because nephrotoxic metabolites of 1,4-DCB are congeners of hydroquinone, these chemicals may produce kidney tumours in male rats by similar mechanisms. In that case, the male rat kidney tumour response to 1,4-DCB would not simply be a consequence of weak binding to  $\alpha_{2u}$ -globulin and subsequent accumulation of this protein.

#### **Tetrachloroethylene**

Tetrachloroethylene, a dry-cleaning agent and industrial degreaser, induced renal tubule cell neoplasms in male rats in a two-year inhalation study at exposure concentrations of 200 and 400 ppm (National Toxicology Program, 1986a). Protein droplets,  $\alpha_{2u}$ -globulin accumulation, and increased cell replication rates were observed in the proximal tubules of male rats dosed with 1000 mg/kg tetrachloroethylene by gavage for 10 days (Goldsworthy *et al.*, 1988); however, hyaline droplets containing  $\alpha_{2u}$ -globulin were not increased in proximal tubules of male rats exposed to 400 ppm tetrachloroethylene by inhalation for four weeks (Green *et al.*, 1990). The latter finding indicates that  $\alpha_{2u}$ -globulin nephropathy was not involved in the induction of the renal tubule cell neoplasms that were observed in the NTP inhalation

study of tetrachloroethylene. A glutathione/ $\beta$ -lyase activation pathway producing a mutagenic intermediate may be involved in the renal carcinogenicity of tetrachloroethylene (Green *et al.*, 1990).

### Potassium bromate (KBrO<sub>3</sub>)

KBrO<sub>3</sub>, an oxidizing agent used as a food additive for treatment of wheat flour, was administered to male and female Fischer 344 rats in drinking water for 110 weeks at concentrations of 250 and 500 ppm. This treatment produced high incidences of renal cell tumours in both sexes (males: 6% controls, 60% low-dose, 88% high-dose; females: 0% controls, 56% low-dose, 80% high-dose; Kurokawa *et al.*, 1990). The renal carcinogenicity of KBrO<sub>3</sub> has been attributed to oxidative DNA damage (Sai *et al.*, 1994; Umemura *et al.*, 1995). Levels of 8-hydroxydeoxyguanosine, a product of oxidative DNA damage formed by oxygen-radical-generating agents, were increased in kidney DNA but not in liver DNA of rats given oral doses of KBrO<sub>3</sub>.

The finding that hyaline droplets containing  $\alpha_{2u}$ -globulin accumulated in male rats treated with KBrO<sub>3</sub> led to the suggestion that  $\alpha_{2u}$ -globulin nephropathy may contribute an additive effect in the renal carcinogenicity of KBrO<sub>3</sub> in male rats (Umemura *et al.*, 1993). However, in the absence of data on binding of bromate to  $\alpha_{2u}$ -globulin and demonstration of consequent reduced proteolytic degradation of the bound protein, it is not possible to determine whether  $\alpha_{2u}$ -globulin accumulation resulted from the  $\alpha_{2u}$ -globulin mechanism described above or perhaps resulted from oxidative damage associated with KBrO<sub>3</sub> treatment. Because the incidences of renal cell tumours were similarly elevated in male and female rats given equivalent concentrations of KBrO<sub>3</sub> in their drinking water and because treatment with KBrO<sub>3</sub> caused increased cell proliferation in the female rat kidney (Umemura *et al.*, 1995), it is likely that  $\alpha_{2u}$ -globulin accumulation was irrelevant to the carcinogenic effects of KBrO<sub>3</sub> in the kidney.

### Ferric nitrilotriacetate (Fe-NTA)

A single administration of Fe-NTA to male rats suppresses proteolytic processing of  $\alpha_{2u}$ -globulin in renal proximal tubules, resulting in an increase in native  $\alpha_{2u}$ -globulin in the kidney and a decrease in  $\alpha_{2u}$ -K levels (Uchida *et al.*, 1995). Repeated administration of Fe-NTA caused renal tubule

necrosis as a consequence of membrane lipid peroxidation and produced a high incidence of renal adenocarcinomas in male rats and mice (Ebina *et al.*, 1986). Interestingly, the corresponding aluminium salt (Al-NTA) produced renal tubule necrosis and regenerative hyperplasia similar to that induced by Fe-NTA, but did not elicit a renal tumour response. KBrO<sub>3</sub> and Fe-NTA induce poly(ADP-ribosyl)ation and DNA double-strand breaks in renal cortical nuclei of exposed male rats (McLaren *et al.*, 1994). Poly(ADP-ribosyl)ation is thought to represent a post-translational modification of nuclear proteins involved in DNA repair. The above findings show that kidney changes unrelated to ligand binding to  $\alpha_{2u}$ -globulin can also affect  $\alpha_{2u}$ -globulin accumulation in the male rat and that nephrotoxicity and renal carcinogenicity can occur as independent phenomena. Hence, renal accumulation of  $\alpha_{2u}$ -globulin may be a consequence rather than a cause of nephrotoxicity.

### Hexachlorobenzene (HCB)

Dietary administration of HCB (75 and 150 ppm), a polychlorinated aromatic fungicide, produced high incidences of liver and kidney neoplasms in rats of both sexes (Ertürk *et al.*, 1986). At equivalent doses, female rats had higher incidences of liver neoplasms and male rats had higher incidences of kidney neoplasms. Administration of HCB (50 or 100 mg/kg) by gavage produced  $\alpha_{2u}$ -globulin nephropathy in male rats but no renal changes in female rats (Bouthillier *et al.*, 1991). HCB was also found to bind reversibly to  $\alpha_{2u}$ -globulin. Because kidney tumours were also induced in female rats treated with HCB, the tumour response in males cannot be attributed simply to the associated increase in renal  $\alpha_{2u}$ -globulin levels.

### Modelling $\alpha_{2u}$ -globulin accumulation and ligand dosimetry in the male rat kidney

The studies described above indicate that renal accumulation of  $\alpha_{2u}$ -globulin in male rats may result from inhibition of renal lysosomal proteinases or from certain degenerative changes (e.g., oxidative damage). Because the relationship between  $\alpha_{2u}$ -globulin accumulation and nephrotoxicity has not been elucidated, the possibility that observed increases in renal  $\alpha_{2u}$ -globulin levels may be a consequence rather than a cause of renal toxicity cannot be excluded. Integrating data on

the relationships between xenobiotic exposure,  $\alpha_{2u}$ -globulin accumulation, and tumour outcome in a comprehensive mathematical model of the involved processes, rather than simply relying on qualitative correlations, would help to distinguish between several possible explanations for the observations.

One attempt to evaluate the toxicokinetics of a specific  $\alpha_{2u}$ -globulin-inducing chemical (2,4,4-trimethyl-2-pentanol, TMP-2-OH) in the male rat kidney has been described (Borghoff *et al.*, 1995). This mechanism-based dosimetry model includes ligand interaction with  $\alpha_{2u}$ -globulin, reduced lysosomal degradation of  $\alpha_{2u}$ -globulin as a result of ligand binding, and renal accumulation of  $\alpha_{2u}$ -globulin. The model was developed using literature values for secretion of  $\alpha_{2u}$ -globulin from the liver, the fraction of  $\alpha_{2u}$ -globulin excreted in urine, the fraction cleared from plasma by glomerular filtration, and the rate of  $\alpha_{2u}$ -globulin degradation. In trying to reproduce experimental data on  $\alpha_{2u}$ -globulin and TMP-2-OH levels in the blood and kidneys, several parameter values were altered but without any clear justifications. The authors found that their model underpredicted the measured renal concentrations of  $\alpha_{2u}$ -globulin and overpredicted renal TMP-2-OH concentrations in treated male rats, even when they tripled the reported rate of  $\alpha_{2u}$ -globulin synthesis in the liver. Furthermore, to simulate the increased level of renal  $\alpha_{2u}$ -globulin following oral administration of TMP-2-OH, the rate of renal degradation of the  $\alpha_{2u}$ -globulin-TMP-2-OH complex had to be reduced to zero; however, this tactic prevented the model from reproducing the observed later decline in renal  $\alpha_{2u}$ -globulin after an oral dose of 600 mg TMP-2-OH/kg body weight. This large decrease in the degradation rate of the ligand-protein complex is not consistent with reported data (as indicated above, the rate of  $\alpha_{2u}$ -globulin degradation *in vitro* was decreased by about 30% in the presence of excess ligand; Lehman-McKeeman *et al.*, 1990).

The lack of correspondence between this model's predictions and the experimental data indicates that the model's structure does not adequately describe the quantitative relationships among the processes involved in  $\alpha_{2u}$ -globulin accumulation in the male rat kidney and/or that parameter values may be incorrect. This

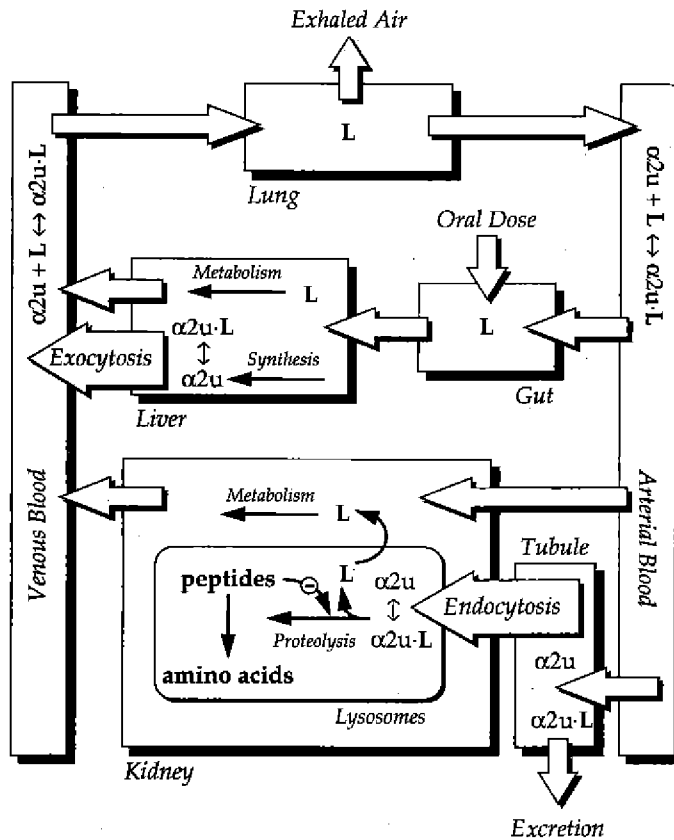
modelling effort, which is the first quantitative test of the  $\alpha_{2u}$ -globulin hypothesis, demonstrates that a 30% reduction in the degradation rate of  $\alpha_{2u}$ -globulin resulting from ligand binding is inadequate to explain the observed accumulation of  $\alpha_{2u}$ -globulin in the male rat kidney. Clearly, processes not included in the  $\alpha_{2u}$ -globulin hypothesis must be involved.

The  $\alpha_{2u}$ -globulin hypothesis is based on several qualitative observations; however, quantitative linkages between the critical biological processes in the hypothesis not only remain unproved, but have not even been tested. Two critical steps in the  $\alpha_{2u}$ -globulin hypothesis are the reversible binding of the xenobiotic ligand to  $\alpha_{2u}$ -globulin and subsequent reduction in the proteolytic degradation of the bound protein. Any model of  $\alpha_{2u}$ -globulin accumulation in the male rat kidney must be consistent with the effects of strong and weak binding ligands. The  $\alpha_{2u}$ -globulin hypothesis focuses largely on effects of the ligand on  $\alpha_{2u}$ -globulin without adequate consideration of the effects of  $\alpha_{2u}$ -globulin on the ligand. The presence of a binding protein such as  $\alpha_{2u}$ -globulin in the male rat can have important consequences on the disposition of the binding ligand. Hence, evaluations of the  $\alpha_{2u}$ -globulin hypothesis must address the mutual effects of the protein and ligand on each other in the male rat; these issues were included in the TMP-2-OH/ $\alpha_{2u}$ -globulin model developed by Borghoff *et al.* (1995).

In male rats exposed to a chemical that binds to  $\alpha_{2u}$ -globulin or that is metabolized to an  $\alpha_{2u}$ -globulin-binding ligand, retention of the ligand in tissues will be affected by the presence and amount of  $\alpha_{2u}$ -globulin, as well as the binding affinity of the ligand for  $\alpha_{2u}$ -globulin. A realistic model of these processes must include descriptions of the absorption, distribution and metabolism of the parent compound, as well as the hepatic secretion, resorption and renal degradation of  $\alpha_{2u}$ -globulin (e.g., Figure 3). If the binding ligand is the parent compound, the kinetics of its metabolism and elimination must be included in the model; if one or more metabolites bind to  $\alpha_{2u}$ -globulin, the kinetics of their formation and elimination must be included.

The presence of  $\alpha_{2u}$ -globulin in the liver, blood and kidney will affect the distribution of  $\alpha_{2u}$ -globulin-binding ligands. Estimations of tissue partition coefficients must account for ligand binding to





**Figure 3.** Partial block diagram of the physiologically based pharmacokinetic model of the disposition of an  $\alpha_{2u}$ -globulin ( $\alpha_{2u}$ ) ligand (L) and its effect on the renal accumulation of  $\alpha_{2u}$ -globulin.

$\alpha_{2u}$ -globulin in homogenates of these tissues. Binding of ligand to  $\alpha_{2u}$ -globulin in the liver may occur if the ligand can cross the secretory vesicle membrane. In that case, the rate of hepatic metabolism of the ligand would be affected by both its rate of transport across the membrane and its binding affinity for  $\alpha_{2u}$ -globulin. If the ligand can cross the lysosomal membrane, it may appear in the cytosol of the renal proximal tubule cells following resorption and partial degradation of bound  $\alpha_{2u}$ -globulin.

A related issue to be considered is whether the presence of ligand affects hepatic secretion of  $\alpha_{2u}$ -globulin. Exposure of mature male rats to unleaded gasoline by gavage with doses that resulted in large increases in the concentration of

$\alpha_{2u}$ -globulin in the kidney did not alter significantly the hepatic concentrations of  $\alpha_{2u}$ -globulin or its mRNA when measured 18 to 24 hours after the last exposure (Olson *et al.*, 1987; Murty *et al.*, 1988). Inhalation exposure of male rats to an isoparaffinic solvent did not significantly increase hepatic levels of  $\alpha_{2u}$ -globulin, but doubled the plasma  $\alpha_{2u}$ -globulin concentration (Viau *et al.*, 1986), suggesting that exposure to isoparaffinic solvents may result in a coordinated elevation in the hepatic synthesis and secretion of  $\alpha_{2u}$ -globulin. The issue of hepatic response to an  $\alpha_{2u}$ -globulin-binding ligand has been only partially addressed for relatively few chemicals. Because potential effects of binding ligands on hepatic synthesis of  $\alpha_{2u}$ -globulin may be largely resolved within 18

hours after exposure, studies are needed that include evaluations at times much closer to the last exposure. Regardless of whether hepatic  $\alpha_{2u}$ -globulin is secreted as the free or ligand-bound form, in the blood the equilibrium between these forms would be established as determined by the binding affinity of the ligand for this protein.

An adequate toxicokinetic model also must reflect the glomerular filtration and resorption of  $\alpha_{2u}$ -globulin followed by its degradation. The filtered fraction that is not reabsorbed (~40%) (Neuhaus *et al.*, 1981) is excreted in the urine. The assumption that free and bound  $\alpha_{2u}$ -globulin are equally resorbed has not been examined, but the data of Saito *et al.* (1996) are consistent with this assumption. Finally, the model must accurately represent the degradation of  $\alpha_{2u}$ -globulin in proximal tubule cells. Lehman-McKeeman *et al.* (1990) reported that degradation of  $\alpha_{2u}$ -globulin by lysosomal proteinases was reduced by about 30% in the presence of excess xenobiotic ligand. Accumulation of  $\alpha_{2u}$ -globulin in the male rat kidney may not be due only to ligand binding; Olson *et al.* (1988) found that treatment of male rats with leupeptin, an inhibitor of lysosomal proteolysis, resulted in renal accumulation of hyaline droplets containing  $\alpha_{2u}$ -globulin. Furthermore, consideration of potential changes in renal lysosomal proteinase activities *in vivo* in response to protein accumulation need additional investigation, because increases of 60% in renal cortical cathepsin B and D activities were seen in male rats four days after administration of unleaded gasoline (Murty *et al.*, 1988).

Because the model by Borghoff *et al.* (1995) was not able to simulate the renal accumulation of  $\alpha_{2u}$ -globulin in male rats dosed with TMP-2-OH, we suggest that several assumptions in the  $\alpha_{2u}$ -globulin hypothesis are incorrect. First, ligand binding to  $\alpha_{2u}$ -globulin may increase the rate of hepatic secretion of this protein. Second, degradation of unbound  $\alpha_{2u}$ -globulin may also be reduced as a consequence of accumulation of the ligand in proximal tubule cells (Lehman-McKeeman *et al.*, 1990). Release of ligand, perhaps early during the degradation of  $\alpha_{2u}$ -globulin, and subsequent binding to formerly free  $\alpha_{2u}$ -globulin may reduce proteolytic degradation of both bound and free  $\alpha_{2u}$ -globulin. Third, increased uptake of protein (e.g.,  $\alpha_{2u}$ -globulin) induces lysosomal cathepsins

(Olbricht *et al.*, 1993; Eisenberger *et al.*, 1995) and would be expected to stimulate proteolysis of  $\alpha_{2u}$ -globulin (Murty *et al.*, 1988). Fourth, liver and kidney are known to possess cytoplasmic fatty acid-binding proteins with a broad range of lipophilic ligands (Maatman *et al.*, 1991). Binding of TMP-2-OH to such proteins would significantly affect the concentration of free ligand. We are testing these hypotheses by comparing a new model's predictions with the published data.

#### Relationships between $\alpha_{2u}$ -globulin accumulation, cell proliferation, and kidney cancer

Foci of chronic progressive nephrosis, characterized by degeneration/regeneration of the proximal tubule epithelium, are common in ageing male and female rats and are even seen to a minimal degree in renal proximal tubules of control Fischer 344 rats by 20 weeks of age. The frequency of these lesions and their severity increase with age. Short *et al.* (1989a) reported that the rates of regenerative cell replication in these lesions in control rats are higher than the cell replication rates of proximal tubule segments affected by chemicals that induce  $\alpha_{2u}$ -globulin accumulation. Konishi and Ward (1989) also observed high levels of cell proliferation associated with chronic progressive nephrosis in control female Fischer 344 rats. In spite of the high frequency of these spontaneous proliferative lesions, the incidence of kidney tumours in untreated male rats is low (<1.0 %), even when they are maintained for up to 146 weeks of age (Solleveld *et al.*, 1984).

High rates of renal tubule cell proliferation in rats are not reliable predictors of kidney cancer. For example, in the bioassay of chloroform in rats, renal tumours were significantly increased in males but not in females (National Cancer Institute, 1976). In contrast to these findings, the labelling index in renal proximal tubules was not increased in male rats given bioassay doses of chloroform by gavage for three weeks (Larson *et al.*, 1995a) but was increased 17-fold and 26-fold in female rats given bioassay doses of this nephrotoxic agent (Larson *et al.*, 1995b).

Although cell proliferation is a basic component of multistage carcinogenesis, there are no data demonstrating that cell division is the limiting factor in kidney cancer (Barrett & Huff, 1991). Cell proliferation data for exposures longer

than three weeks are available for only two chemicals (unleaded gasoline and *d*-limonene) that form  $\alpha_{2u}$ -globulin ligands and induce renal tumours in long-term studies. Cell proliferation data for 48 weeks of exposure to TMP are also available; however, a carcinogenicity study of this chemical has not been reported. Thus, quantitative dose-response relationships between sustained cell proliferation and renal carcinogenesis in male rats have not been established for most chemicals that induce  $\alpha_{2u}$ -globulin nephropathy.

Alternatively, the unbound ligand (e.g., TMP-2-OH) that accumulates in the kidney cytosol by one of the mechanisms suggested above may be subsequently converted to the proximate nephrotoxicant or carcinogen (e.g., the aldehyde or carboxylic acid metabolites). In this case,  $\alpha_{2u}$ -globulin may simply serve as a vector affecting the levels of the renal toxicant/carcinogen or its precursor in the male rat kidney.

### Summary and conclusions

The  $\alpha_{2u}$ -globulin hypothesis is supported by the findings that :

- (1) Several nongenotoxic chemicals that cause  $\alpha_{2u}$ -globulin accumulation and renal carcinogenesis in male rats do not induce kidney tumours in animals that lack the ability to synthesize  $\alpha_{2u}$ -globulin in the liver (e.g., female rats or mice of either sex).
- (2) Chemicals that cause  $\alpha_{2u}$ -globulin accumulation in the kidney of male Fischer 344 or Sprague-Dawley rats do not induce protein droplet nephropathy in male NBR rats (Dietrich & Swenberg, 1991b), a strain deficient in hepatic  $\alpha_{2u}$ -globulin synthesis.
- (3) Chemicals (or their metabolites) that bind reversibly to  $\alpha_{2u}$ -globulin cause  $\alpha_{2u}$ -globulin accumulation in the male rat kidney (Borghoff *et al.*, 1991).
- (4) *In vitro* degradation of ligand-bound  $\alpha_{2u}$ -globulin by lysosomal extracts was decreased in the presence of excess  $\alpha_{2u}$ -globulin ligand (Lehman-McKeeman *et al.*, 1990).
- (5) S-phase labelling index in the  $P_2$  segment of renal proximal tubules was increased in male rats exposed to several chemicals or chemical mixtures that induce  $\alpha_{2u}$ -globulin accumulation, and for unleaded gasoline, the dose-

dependence for the renal epithelial cell labelling index is similar to that of the kidney tumour response (Short *et al.*, 1987, 1989a).

- (6) Unleaded gasoline and *d*-limonene elicited tumour-promoting activity in the kidney of male Fischer 344 rats initiated with EHEN (Short *et al.*, 1989b; Dietrich & Swenberg, 1991a), whereas *d*-limonene did not promote renal carcinogenesis in EHEN-initiated male NBR rats (Dietrich & Swenberg, 1991a).

This review has identified several inconsistencies and critical gaps in the data claimed to prove the  $\alpha_{2u}$ -globulin hypothesis. These deficiencies reveal how limited is our understanding of the proposed linkage between the reversible binding of xenobiotic ligands to  $\alpha_{2u}$ -globulin, the accumulation of  $\alpha_{2u}$ -globulin in the male rat kidney, and male rat-specific kidney carcinogenesis.

- (1) Several contradictory observations have been reported; for example, gabapentin and lindane induce  $\alpha_{2u}$ -globulin accumulation and nephropathy in male rats at doses that did not increase kidney tumour incidence.
- (2)  $\alpha_{2u}$ -globulin accumulation may arise by mechanisms unrelated to ligand binding to this protein; for example, 2,2,4-trimethylpentanoic acid, a metabolite of TMP (Charbonneau *et al.*, 1987a), and leupeptin, an inhibitor of lysosomal proteolysis (Olson *et al.*, 1988), cause  $\alpha_{2u}$ -globulin accumulation without binding. In addition, increases in  $\alpha_{2u}$ -globulin accumulation in male rats treated with potassium bromate or Fe-NTA may occur secondary to oxidative damage rather than protein binding (Sai *et al.*, 1994; Uchida *et al.*, 1995).
- (3) A 30% reduction in the lysosomal degradation rate of  $\alpha_{2u}$ -globulin resulting from ligand binding was inadequate to explain the observed accumulation of  $\alpha_{2u}$ -globulin in the male rat kidney (Borghoff *et al.*, 1995).
- (4)  $\alpha_{2u}$ -globulin accumulation in the male rat kidney has been observed under conditions in which ~70% of the protein was unbound (Charbonneau *et al.*, 1987b; Borghoff *et al.*, 1995).
- (5) Some compounds with weak binding affinity for  $\alpha_{2u}$ -globulin (e.g., MTBE) cause hyaline droplet accumulation and induce kidney

- tumours in male rats but give rise to very small increases in renal concentrations of  $\alpha_{2u}$ -globulin (Chun *et al.*, 1992; Prescott-Mathews *et al.*, 1997).
- (6) Except for *d*-limonene, the chemicals that induce both  $\alpha_{2u}$ -globulin accumulation and kidney carcinogenesis also induce cancer at other sites (see Table 2); mouse liver neoplasms were the most common (Melnick, 1992). This finding suggests that other mechanisms are involved in the carcinogenicity of these chemicals.
  - (7) Several compounds that cause  $\alpha_{2u}$ -globulin nephropathy and induce kidney tumours in male rats also induce renal toxicity and/or renal carcinogenesis in female rats or in mice (e.g., 1,4-DCB, MTBE, HCB, potassium bromate). Kidney toxicity and carcinogenicity induced by these chemicals can certainly not be attributed simply to the presence of  $\alpha_{2u}$ -globulin.
  - (8) The potential contributions of alternative metabolites in the toxic and carcinogenic processes have not been adequately investigated (e.g., 2,5-dichlorohydroquinone or a subsequent metabolite from 1,4-DCB, the glutathione conjugate of tetrachloroethylene, and the acid and aldehyde metabolites of TMP).
  - (9) Foci of chronic progressive nephrosis, which are renal tubule lesions appearing in control Fischer 344 rats by 20 weeks of age, have cellular replication rates that are higher than those of  $P_2$  proximal tubule cells in male rats exposed to chemicals that induce  $\alpha_{2u}$ -globulin accumulation (Short *et al.*, 1989a); however, the incidence of spontaneous kidney tumours in untreated male rats is low (<1.0%) even at 146 weeks of age (Solleveld *et al.*, 1984).
  - (10) Although cell proliferation is a basic component of multistage carcinogenesis, there are no data demonstrating that the carcinogenic outcome in the kidney is determined by the cell division rate (Barrett & Huff, 1991). Furthermore, there is no adequate database

**Table 2. Sites of tumour induction in rats or mice exposed to chemicals that induce  $\alpha_{2u}$ -globulin accumulation, in addition to kidney tumours in male rats**

Chemical	Rats	Mice	Reference
Unleaded gasoline	—	Liver	MacFarland <i>et al.</i> , 1984
Methyl <i>t</i> -butyl ether/ <i>i</i> -butyl alcohol	Testis, haematopoietic system	Liver, thyroid	Chun <i>et al.</i> , 1992; Burleigh-Flayer <i>et al.</i> , 1992; Belpoggi <i>et al.</i> , 1995; Cirvello <i>et al.</i> , 1995
$\alpha$ -Limonene	—	—	NTP, 1990
1,4-Dichlorobenzene	—	Liver	NTP, 1987
Tetrachloroethylene	Haematopoietic system	Liver	NTP, 1986a
Hexachlorobenzene	Kidney (females) liver	Liver	Ertürk <i>et al.</i> , 1986; Cabral <i>et al.</i> , 1979
Potassium bromate	Kidney (females) mesothelium thyroid	Kidney	Kurokawa <i>et al.</i> , 1990
Ferric nitriloacetate	Kidney (females)	Kidney	Ebina <i>et al.</i> , 1986; Li <i>et al.</i> , 1987
Hexachloroethane	—	Liver	NTP, 1989b
Isophorone	Preputial gland	Liver	NTP, 1986b

relating cell proliferation rate to renal tumour response in male rats (Melnick, 1992).

- (11) Dose-response studies do not support a relationship between the increase in hyaline droplets containing  $\alpha_{2u}$ -globulin and kidney carcinogenicity in male rats (e.g., tetrachloroethylene).
- (12) Human exposure to gasoline vapours has been associated with increased kidney cancer risk, indicating that the renal tumorigenicity of gasoline may not be species-specific.

These findings suggest that among the chemicals reviewed in this paper, only *d*-limonene meets the criteria presented in the consensus report in this volume for agents causing kidney tumours through an  $\alpha_{2u}$ -globulin-associated response in male rats.

The hypothesis that kidney tumours in the male rat are caused by promotion of initiated cells by regenerative hyperplasia consequent to cytotoxicity due to  $\alpha_{2u}$ -globulin accumulation is consistent with some observations and inconsistent with others. Therefore, the hypothesis is unproved and, at best, represents only one element in a complex etiology of kidney cancer. To embrace this hypothesis as an adequate explanation of the renal carcinogenic effects of  $\alpha_{2u}$ -globulin ligands would be to elevate hypothesis to the level of data and liable to result in a failure to perform definitive experiments that are needed to identify mechanistic details.

Mechanisms of hydrocarbon-induced nephropathy and renal carcinogenesis are not well understood. Correlative studies do not prove causal relationships, and hypotheses that are styled to influence public health policies need strong scientific support. The "widely accepted hypothesis" links the kidney tumour response in male rats to  $\alpha_{2u}$ -globulin accumulation resulting from the reversible binding of a xenobiotic ligand to this protein, which renders  $\alpha_{2u}$ -globulin less susceptible to proteolytic degradation. However, exceptions have been identified and a test of this hypothesis failed to demonstrate critical quantitative relationships that would be expected between exposure to an  $\alpha_{2u}$ -globulin-binding ligand and  $\alpha_{2u}$ -globulin accumulation in the male rat kidney. Further, the  $\alpha_{2u}$ -globulin hypothesis ignores the fact that the

presence of  $\alpha_{2u}$ -globulin in the male rat affects the concentration of the binding ligand in the kidney, as well as the potential impact of this alteration on target organ dosimetry. Alternative hypotheses based on the same information are plausible. For example,  $\alpha_{2u}$ -globulin accumulation may be a consequence of renal toxicity rather than its cause. Two chemicals that cause  $\alpha_{2u}$ -globulin accumulation (TMP-2-OH and 2,5-dichlorophenol) were toxic to proximal tubule epithelial fragments *in vitro* (Wilke *et al.*, 1994).

We hypothesize that  $\alpha_{2u}$ -globulin may serve to increase the concentration of the carcinogenic agent or its precursor in the male rat kidney. In this case,  $\alpha_{2u}$ -globulin may cause a left-shift in the kidney cancer dose-response curve for  $\alpha_{2u}$ -globulin-binding ligands in male rats relative to responses in female rats or mice. If the effect of  $\alpha_{2u}$ -globulin is largely on the renal concentration of toxicant, then extrapolations across species should adjust for differences in target dose rather than consider the effects in male rats to be irrelevant to humans.

It should be recognized that the mechanisms of chemically induced  $\alpha_{2u}$ -globulin nephropathy and chemically induced renal cancer have yet to become more than operational hypotheses and that the alternative view advanced here must also be considered an unproved hypothesis. It would be inappropriate to accept or reject either hypothesis before the mechanisms of renal carcinogenesis are more fully understood and these or any other reasonable hypotheses have been adequately tested. Further experimental studies and mathematical models that address assumptions in the hypotheses relating exposure to  $\alpha_{2u}$ -globulin-binding chemicals with  $\alpha_{2u}$ -globulin accumulation and kidney cancer in the male rat are needed to better understand the processes involved and to strengthen the scientific basis for any public health decisions.

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# Human bladder cancer: epidemiological, pathological and mechanistic aspects

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## Introduction

Among risk factors that have been associated with bladder carcinoma in humans are cigarette smoking, occupational exposure to various chemicals including aromatic amines, bladder infections by *Schistosoma haematobium* and the use of some drugs such as phenacetin-containing analgesics, chlornaphazine and cyclophosphamide. Associations with coffee drinking and artificial sweeteners, saccharin and cyclamates, have also been suspected, but the epidemiological evidence is now reassuring (Matanoski & Elliott, 1981; La Vecchia & Decarli, 1996; Ross *et al.*, 1996; Silverman *et al.*, 1996).

The possibility that bladder cancer is associated with many different chemical agents is not surprising, since most metabolites and carcinogens are excreted through the urinary tract. For example, tobacco smoking contains various mutagenic substances that, after being absorbed into the circulation, pass into the urine. Consequently, the urine of smokers is mutagenic in the Ames test (Doll & Peto, 1981; Silverman *et al.*, 1996).

The present paper reviews major aspects of the descriptive and analytical epidemiology of bladder cancer. Most data refer to transitional-cell carcinoma of the bladder, which accounts for about 95% of bladder cancer in white populations — but only about 85% in American blacks and even less in Middle East populations (Bedwani *et al.*, 1993). In the latter region, squamous-cell cancers are frequent and have considerably worse prognoses than transitional-cell types.

## Descriptive epidemiology

Between 1973 and 1994 in the United States, bladder cancer incidence increased moderately in both sexes and various races, whereas mortality tended to decline, most noticeably in men (Kosary

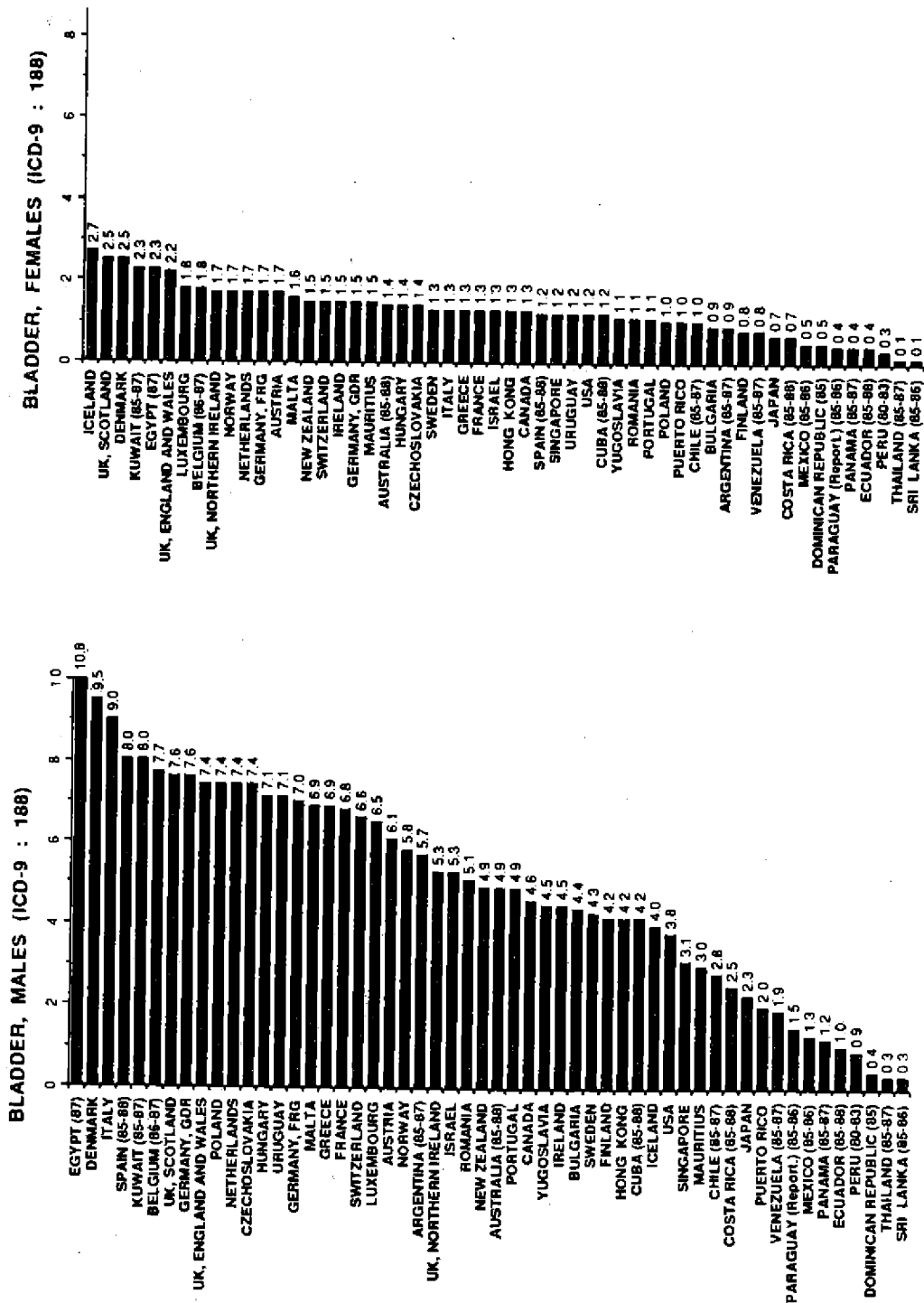
*et al.*, 1996). In Europe, high incidence rates for bladder carcinoma have been recorded in areas with a high concentration of chemical industries (northern Italy, Saarland and Bas-Rhin). Death rates also tend to be high in heavily industrialized countries (Figure 1), as well as in Egypt where bladder schistosomiasis is endemic (Bedwani *et al.*, 1993; Levi *et al.*, 1993, 1994).

Problems of case ascertainment and death certification may affect bladder cancer mortality data. In several countries rates in men have shown increases in the elderly, but stable or declining rates in middle age. Some of these patterns, however, are probably real, and reflect cohort effects in tobacco exposure, since this is one of the smoking-related neoplasms (US Office of Smoking and Health, 1982; IARC, 1985). Within Europe, there are in fact several similarities with the pattern of rates observed for lung cancer, with downward trends over the last one or two decades in several northern European countries, but persistent rises in southern and eastern Europe (La Vecchia *et al.*, 1992; Figure 2). Changes in occupational exposure to carcinogens (which are another major cause of bladder cancer (Matanoski & Elliott, 1981; La Vecchia & Decarli, 1996) in subsequent generations of European men may also have caused earlier rises and subsequent declines in rates, which are particularly large at younger ages.

In the late 1980s, most of the age-adjusted mortality rates for men within Europe fell in the rather narrow range of 5 to 8 per 100 000. Only Denmark (9.5) and Italy (9.1) had rates above 8. Low rates were registered in Sweden and a few southern European countries. Rates for women were between 1 and 3 per 100 000 in most countries, and showed no appreciable trend over time.

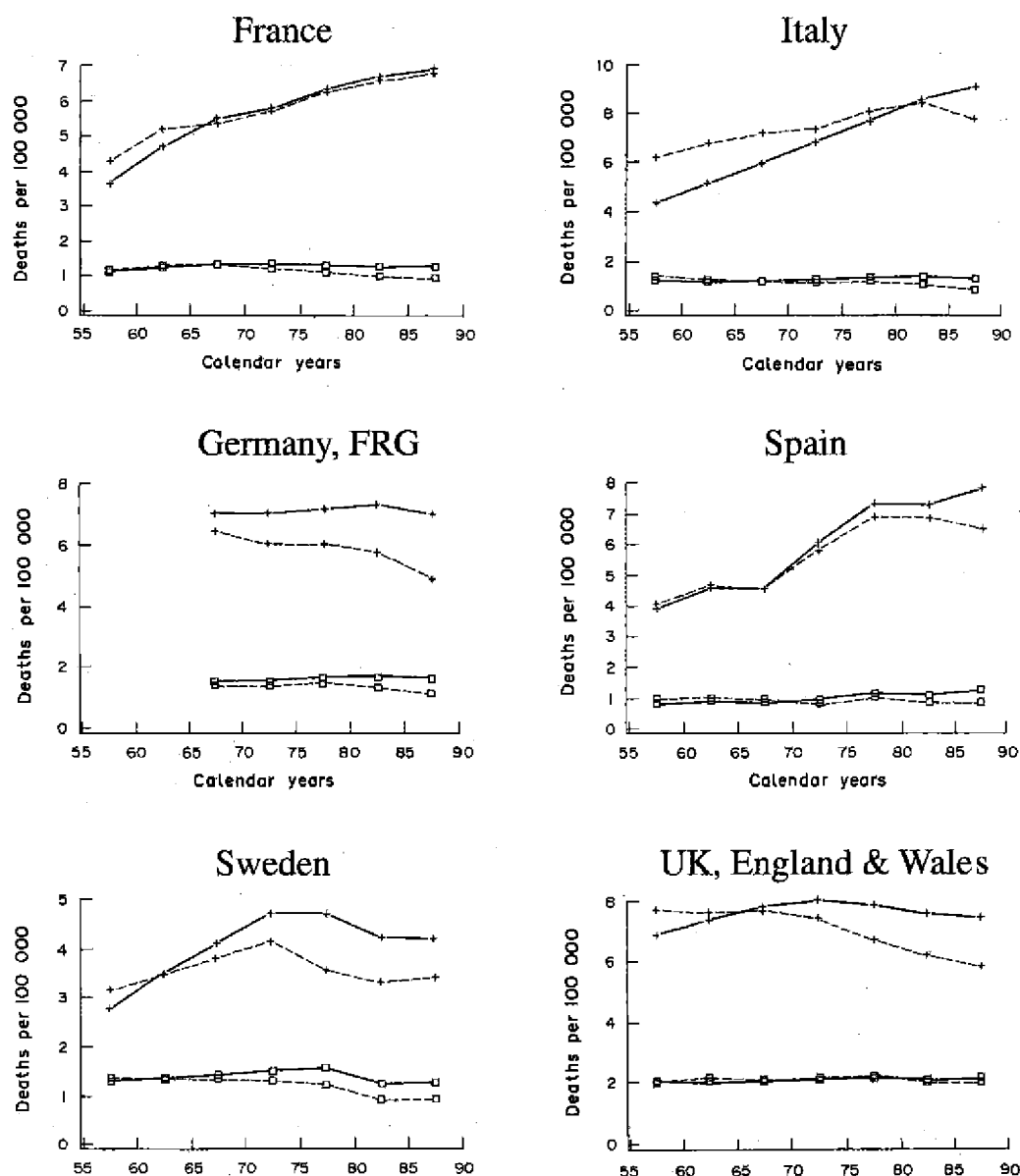
When an age, period and cohort model (Osmond & Gardner, 1982; Decarli & La Vecchia,

Figure 1. Bladder cancer mortality (age-standardized, world standard) in countries providing data to the World Health Organization database (from Levi *et al.*, 1994).



**Figure 2.** Trends in age-standardized mortality rates from bladder cancer in selected European countries (La Vecchia *et al.*, 1992). Males, +; females, □. Solid line: all ages; broken line, ages 35–64.

## Deaths from bladder cancer



1987) was applied to mortality data, cohort values in men in most of western Europe increased up to the generations born around 1920–40, and declined thereafter (Figure 3a). Steady upward trends were however observed in Spain and Hungary. The pattern of cohort and period of death values were similar for women in several countries, although generally less consistent, possibly due to smaller absolute numbers (Figure 3b).

This pattern of cohort trends indicates that the generations with heaviest exposure to tobacco smoking, as well as occupational exposure to aromatic amines and other chemicals in most European countries, were those born in the first decades of the 20<sup>th</sup> century, with subsequent declines in most recent decades (La Vecchia *et al.*, 1998).

### Tobacco smoking

Cigarette smoking is undoubtedly the main recognized cause of bladder cancer not only in developed countries, but also in the few developing ones for which data are available (Bedwani *et al.*, 1993, 1997). Smokers have a two- to four-fold increased risk for bladder cancer as compared to nonsmokers, and the risk increases with the number of cigarettes smoked and the duration of smoking. Furthermore, high-tar, black-tobacco cigarettes are associated with higher bladder cancer risk than low-tar, blond-tobacco types (Matanoski & Elliott, 1981; D'Avanzo *et al.*, 1990) (Table 1).

More than two dozen case-control and several cohort studies have provided data on the smoking-bladder cancer relationship, and have consistently shown an elevated risk among smokers (Dolin, 1991). The largest study, based on 2992 cases and 5782 controls from 10 geographical areas of the United States (Hartge *et al.*, 1987), found a relative risk (RR) of 2.9 for current and of 1.7 for former smokers. The RR declined to 1.4 after 30 or more years since stopping smoking. In another study of over 1800 cases from nine United States cities (Augustine *et al.*, 1988), the RR was 2.5 for male heavy smokers, but the relation was less consistent for women.

Smokers of filtered low-tar cigarettes show a less strong association as compared to smokers of unfiltered high-tar cigarettes, with approximately a 30–40% lower risk. It is unclear, however, how much of this difference is due to the tar yield of cigarettes rather than to the type of tobacco, since

these two factors tend to be correlated. Black tobacco, in fact, has higher levels of aromatic amines, which are one of the major bladder carcinogenic components in tobacco smoking. For instance, in a case-control study from northern Italy (D'Avanzo *et al.*, 1990), the RR was 3.8 for cigarette smokers of black tobacco, as compared to 2.7 for smokers of blond tobacco.

Smokers who have also been occupationally exposed to aromatic amines tend to have an additive component for the overall RR for bladder cancer, i.e. the RR for simultaneous exposure to both factors is closer to the sum than to the product of the individual risks (La Vecchia *et al.*, 1990). This may also be related to the common constituents of genetic susceptibility, since slow acetylators are at increased risk for bladder cancer from both exposures (Bartsch *et al.*, 1990; Vineis *et al.*, 1990).

In terms of population-attributable risk, in Great Britain it was estimated that about 50% of bladder cancer cases could be attributed to cigarette smoking (85% of men and 27% of women, Moolgavkar & Stevens, 1981). In the United States, the estimated proportion of bladder cancer associated with smoking was about half in men and about one-third in women (Hartge *et al.*, 1987). In Italy, the proportion of bladder cancer attributable to tobacco smoking was of similar magnitude, on the basis of the number of smokers and relative risk estimates in case-control studies carried out in Turin and Milan (Vineis *et al.*, 1983; D'Avanzo *et al.*, 1990). In the latter study, the overall population-attributable risk was 50% (66% men, 17% women; D'Avanzo *et al.*, 1995).

### Occupational exposure to aromatic amines

A high risk of bladder carcinoma has been observed in dyestuff factory workers exposed to aromatic amines since the end of the 19<sup>th</sup> century. Involved in bladder carcinogenesis are 2-naphthylamine, benzidine and other aromatic amines, such as fuchsin, auramine and safranin (IARC, 1987).

It had been estimated that 5–10% of bladder carcinomas in Great Britain and North America were of occupational origin (Moolgavkar & Stevens, 1981). The proportion was probably greater in the past and in other heavily industrialized areas of the world, including for instance

**Figure 3a.** Age, period and cohort estimates for bladder cancer mortality among men in selected European countries, from La Vecchia *et al.*, 1998.

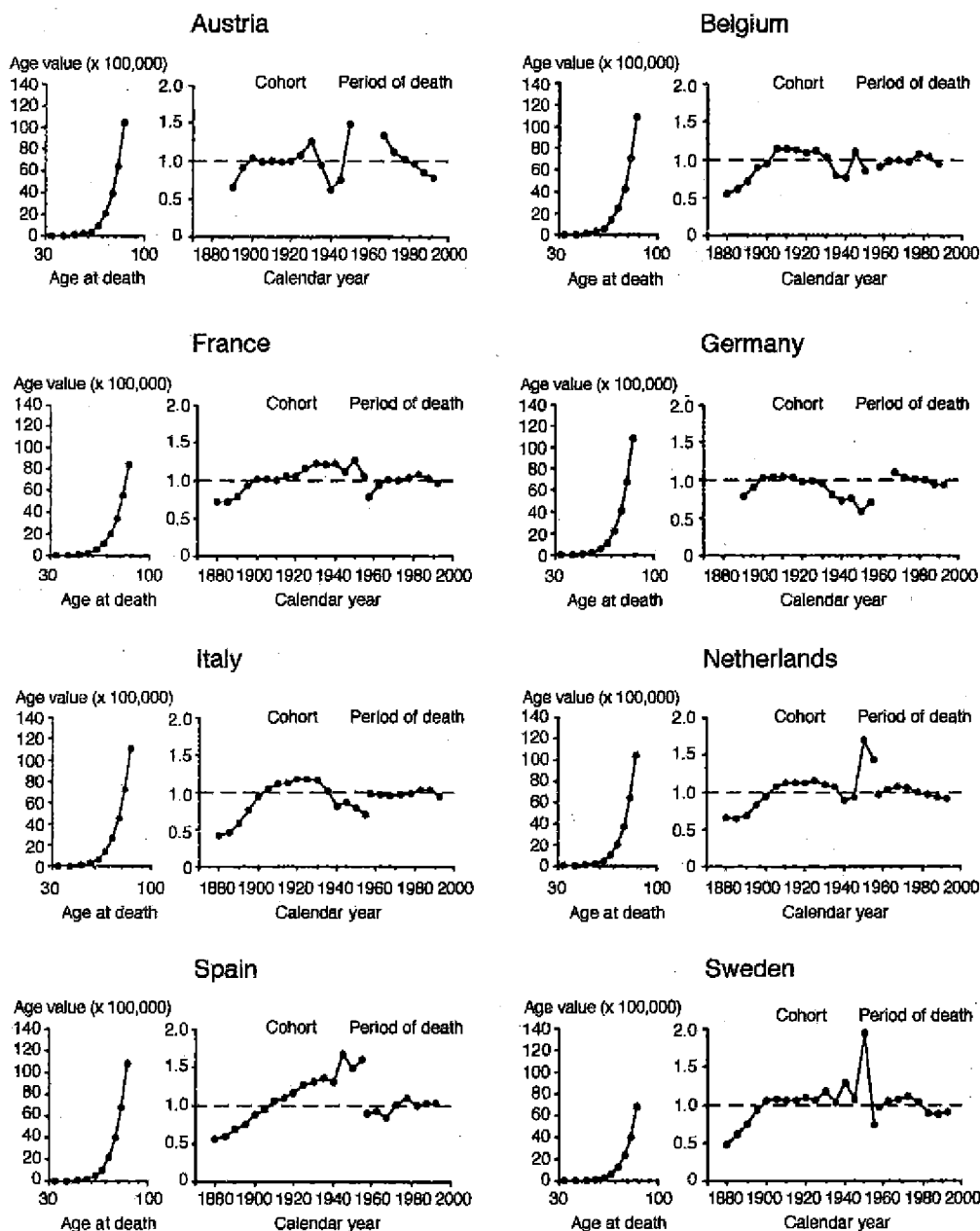
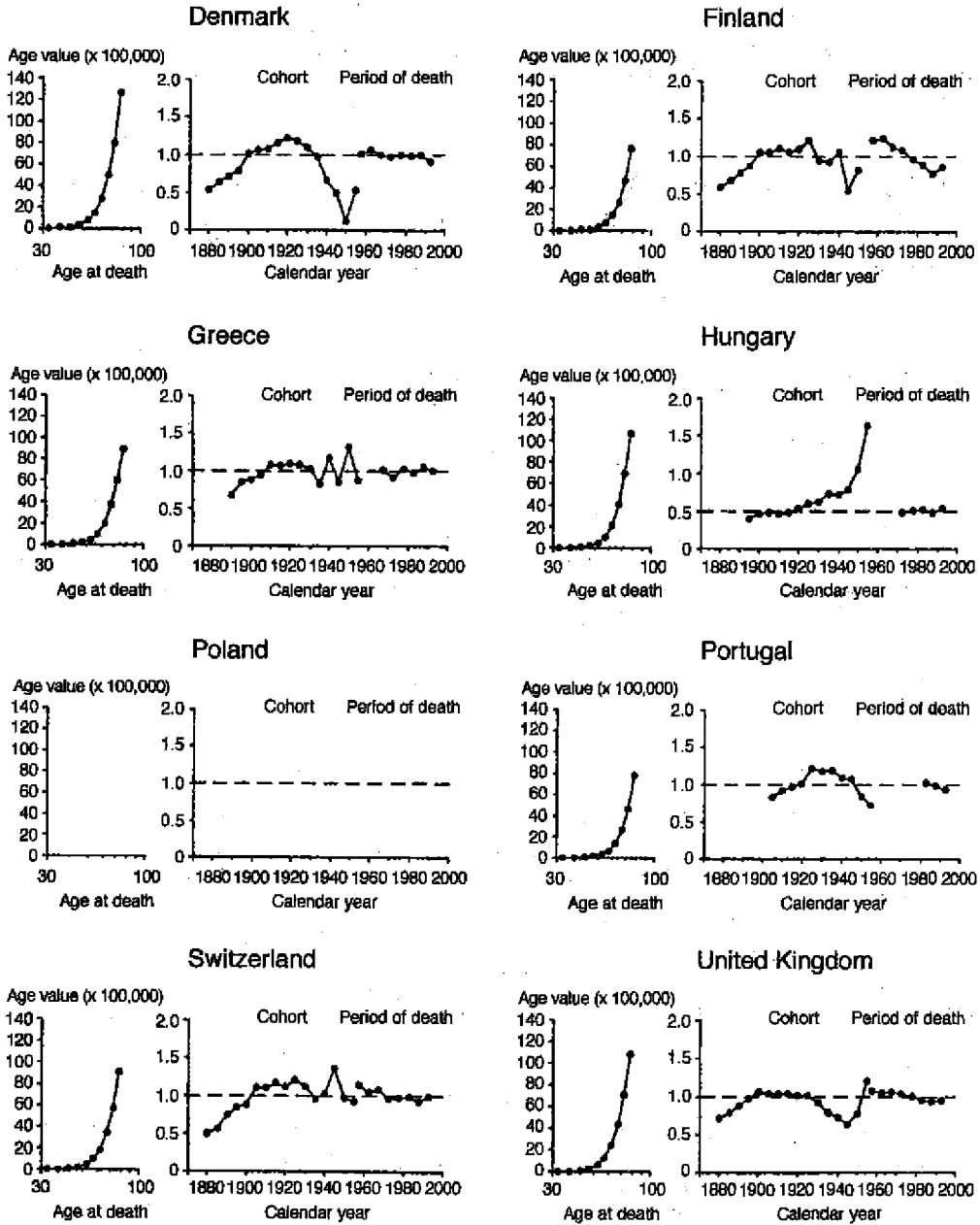


Figure 3a (contd).



**Figure 3b.** Age, period and cohort estimates for bladder cancer mortality among women in selected European countries, from La Vecchia *et al.*, 1998.

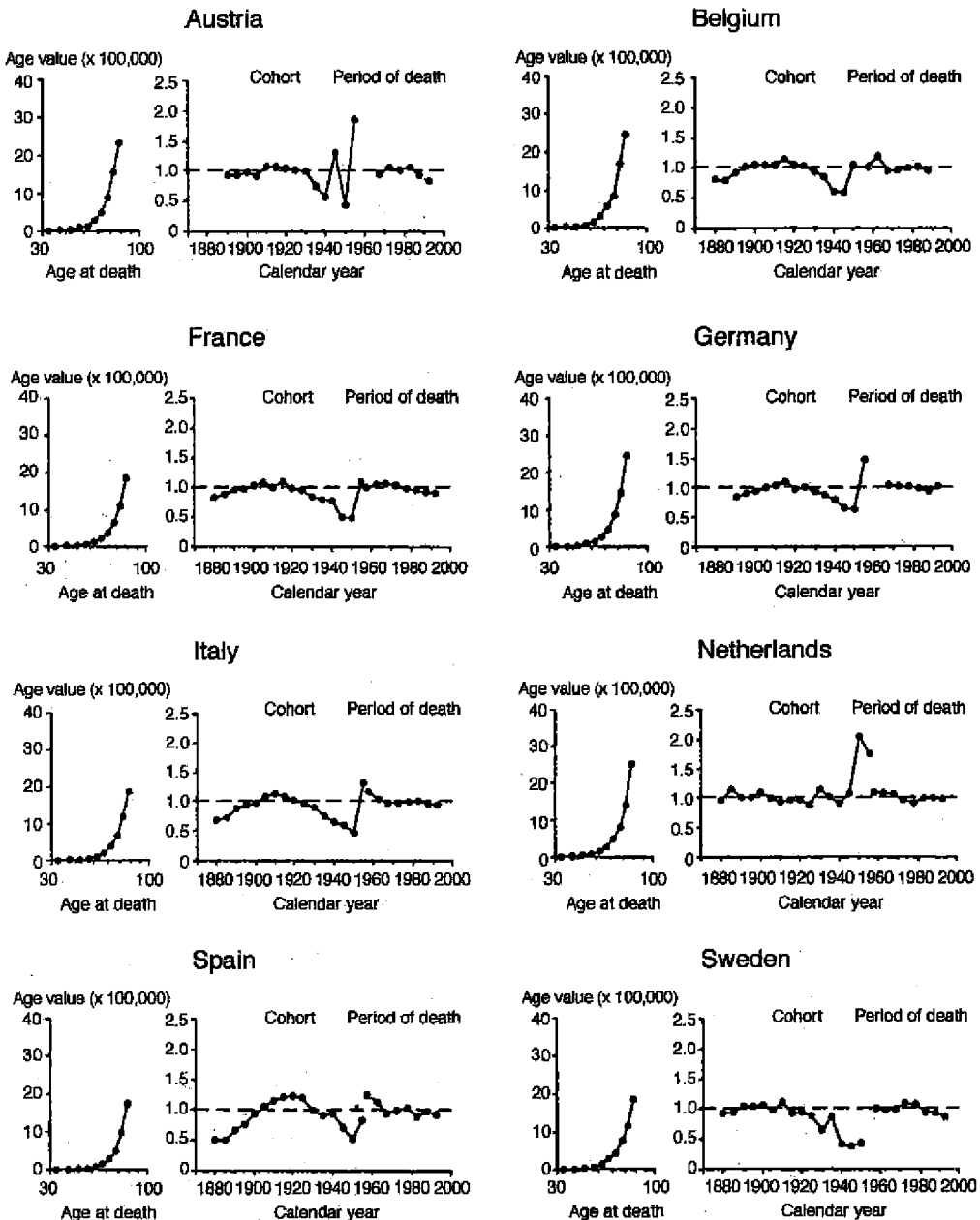
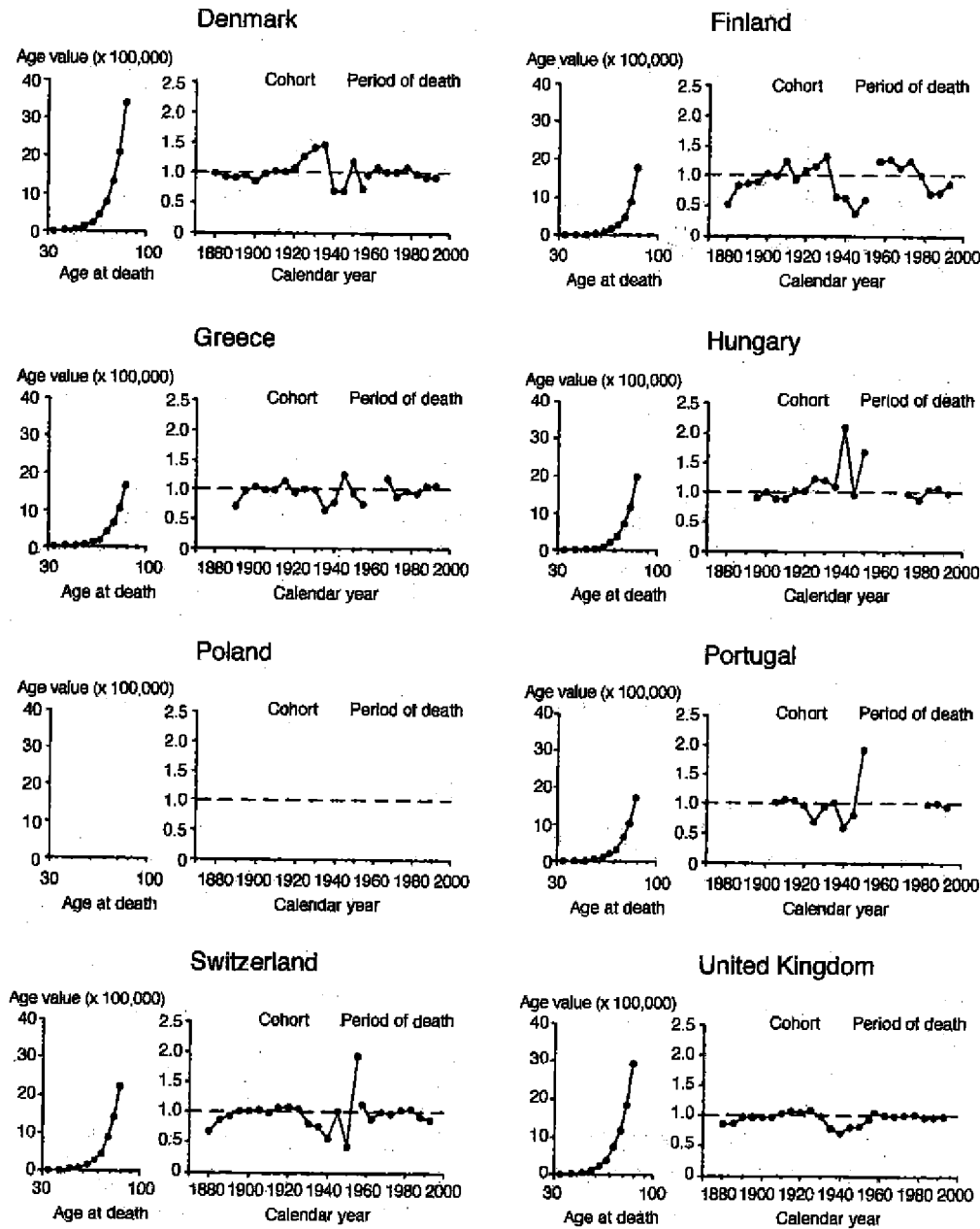




Figure 3b. (contd)



**Table 1. Relative risk (RR) estimates (and 95% confidence intervals [CI]) of bladder cancer in relation to selected measures of exposure to tobacco smoking in a study of 337 cases and 392 controls from northern Italy<sup>a</sup>**

	Bladder cancer	Controls	RR	(95% CI)
<b>Never smokers</b>	75	168	1 <sup>b</sup>	
<b>Ex-smokers</b>	89	95	1.9	(1.2-3.1)
<b>Current smokers</b>				
< 20 cigarettes/day	73	60	2.9	(2.0-4.2)
≥ 20 cigarettes/day	100	69	3.9	(2.4-6.4)
<b>Duration of smoking<sup>c</sup></b>				
< 30 years	56	84	1.8	(1.1-3.0)
≥ 30 years	187	119	3.5	(2.3-5.2)
<b>Type of tobacco<sup>d</sup></b>				
Blond or mixed	205	187	2.7	(1.8-4.0)
Black	38	19	3.8	(2.0-7.4)

<sup>a</sup> From D'Avanzo *et al.* (1990)

<sup>b</sup> Reference category

central Europe (Levi *et al.*, 1993, 1994), and has likely been declining during the last few years (La Vecchia *et al.*, 1998).

Up to 1970, a group of dyestuff factory workers on the outskirts of Turin were exposed to naphthylamine, benzidine and other aromatic amines, and a study of hospital cases identified 23 bladder carcinomas (Rubino & Coscia, 1973). In this same cohort of workers, up to 1981, among 664 workers exposed to aromatic amines, 41 deaths from bladder carcinoma were registered (46 times the expected number). The data from this cohort provide therefore one of the few examples in human carcinogenesis in which the number of cases is so high as to allow the evaluation of duration of exposure, age at first exposure and time since last exposure in different models of carcinogenesis (Decarli *et al.*, 1985; Piolatto *et al.*, 1991).

The results obtained by applying two different models, multiplicative and additive (in other words assuming that the risk related to aromatic amines influences multiplicatively or additively the baseline risk), are shown in Table 2 with reference to categories arbitrarily chosen and showing, respectively, RR estimates and excess absolute risk.

On the basis of both models, the risk was higher in workers directly involved in the manufacture of aromatic amines than for those with irregular exposure. There was no marked effect of age at first exposure on the excess absolute risk of bladder carcinoma, but the RR was strongly and inversely associated with age at first exposure, even when duration and other variables were included in the model. Assuming that the process of carcinogenesis has several stages (the so-called 'multistage' carcinogenesis), this pattern of risk (i.e., an age independence on the excess absolute risk, and an inverse relationship of RR with age at first exposure) indicates an effect on one of the first stages of the carcinogenesis process (Armitage & Doll, 1961; Day & Brown, 1980).

The excess absolute risk increased considerably with duration of exposure, and continued to increase moderately even after exposure had stopped. The relative risk, however, diminished when exposure ceased, suggesting the existence also of an effect on one of the latter stages of the process. The results from the two models do not appear therefore to be contradictory when referred to the 'multistage' theory of carcinogenesis, even though they are not wholly consistent with a

**Table 2. Relative and absolute excess bladder cancer risk estimates in workers exposed to aromatic amines according to different variables: data from a cohort of 664 workers of a dyestuff factory in northern Italy<sup>a</sup>**

Variables	Multiplicative model Relative risk	Additive model Absolute excess risk
<b>Age at first exposure</b>		
< 25 years	1 <sup>b</sup>	1 <sup>b</sup>
25-34 years	0.42	1.05
≥ 35 years	0.17	1.77
<b>Duration of exposure</b>		
< 5 years	1 <sup>b</sup>	1 <sup>b</sup>
5-9 years	1.94	3.84
≥ 10 years	1.80	9.40
<b>Job category</b>		
Naphthylamine or benzidine manufacture	1 <sup>b</sup>	1 <sup>b</sup>
Naphthylamine or benzidine use	0.09	0.09
Intermittent contact with naphthylamine or benzidine	0.08	0.07
Fuchsin or safranin T manufacture	0.41	0.56
<b>Time since last exposure</b>		
During exposure	1 <sup>b</sup>	1 <sup>b</sup>
< 5 years	0.59	0.95
5-9 years	0.39	1.16
≥ 10 years	0.36	2.14

<sup>a</sup> From Decarli *et al.* (1985) and Piolatto *et al.* (1995), modified.

<sup>b</sup> Reference category

single stage effect, early or late, in the carcinogenesis process (Piolatto *et al.*, 1991, 1995).

The results obtained by mathematical model fitting are of theoretical interest in that they provide information on the effects produced by carcinogens on a multistage process. In this model, it is assumed that only cells reaching the stage  $n$  have some likelihood of evolving to the  $n + 1$  stage, and so on. The role of a carcinogen then would be to enhance such a likelihood. Carcinogens affecting early stages of the process (initiators) will influence the clinical appearance of the tumour after long periods (usually decades) since first exposure, due to the number of transitions required for the full process to be completed. Moreover, following the cessation of exposure, cancer incidence will continue to rise (Day & Brown, 1980; Hicks, 1980).

Different indications can be derived from a model that suggests a late-stage effect. This is considered to be a reversible phenomenon in the short-medium time frame (Hicks, 1980). In this case, absolute excess risk would be higher for older subjects, who are likely to have more cells in advanced transition stages. However, a late-stage effect is more readily preventable, since cessation of exposure will cause a substantial reduction in cancer incidence after a short interval.

The dyestuff worker study showed that aromatic amines probably act on more than one stage of the process of carcinogenesis, probably an early and a late one. According to a general role for preventing late-stage effects, removal of subjects known to have been exposed to bladder carcinogens from further exposure to aromatic amines

and other bladder carcinogens, including tobacco, is a priority for prevention. However, since an early-stage effect was also suggested by the model of aromatic amines on bladder carcinogenesis, continuing surveillance of workers exposed to these chemicals in the past is of major importance. Thus, better understanding of the model of bladder carcinogenesis not only holds theoretical interest, but has also immediate implications for cancer prevention and public health.

### **Biomarkers of cancer susceptibility and exposure in risk assessment**

Aromatic amines, like most other chemical carcinogens, require metabolic activation to reactive species that bind to DNA to exert their carcinogenic effect. Polymorphic distribution of the enzymes involved in the activation and/or deactivation of aromatic amines in humans has been regarded as an important determinant of individual susceptibility to their carcinogenic effects. Metabolic polymorphisms, genetically determined or arising from environmental factors, can mediate the formation of aromatic amine-DNA or haemoglobin adducts, that may be used to detect human exposure to these compounds. Biological markers of exposure in conjunction with markers of individual susceptibility may be predictive of bladder cancer risk.

The N-acetylation of aromatic amines and the O-acetylation of their N-hydroxy derivative are catalysed by acetyltransferases. Acetylation may result in detoxification, but, in some cases, acetylation causes activation to DNA-binding metabolites, as observed with the arylamine benzidine (Rothman *et al.*, 1996; Zenser *et al.*, 1996). N-acetyltransferase (NAT) activity in humans is coded by two distinct genes named NAT1 and NAT2 (Grant *et al.*, 1989). The NAT2 enzyme has long been known to be polymorphic. In about 50% of Caucasians, termed slow acetylators, this enzyme activity is reduced. A number of point mutations in the NAT2 gene associated with the slow acetylator phenotype have been identified (Blum *et al.*, 1991).

The relationship between acetylation phenotype and cancer susceptibility was first observed in a case-control study of bladder cancer, in which a large proportion of slow acetylators was detected among subjects occupationally exposed to aromatic amines, but not among smoke-related bladder cancer patients (Cartwright *et al.*, 1982).

The high proportion of slow acetylators among occupationally exposed patients has been a matter of controversy (D'Errico *et al.*, 1996). A significant excess of genotypic slow acetylators has also been reported in bladder cancer patients occupationally exposed to aromatic amines or who were cigarette smokers, thus confirming that the slow NAT2 genotype is a risk factor in bladder carcinogenesis (Risch *et al.*, 1995; Okkels *et al.*, 1997).

The NAT1 enzyme was considered to be monomorphic until some recent studies demonstrated the presence of a variant polyadenylation signal of the NAT1 gene (NAT1\*10 allele) associated with higher enzyme activity (Bell *et al.*, 1995). The activity of NAT1 is reportedly higher than that of NAT2 in the bladder and may increase the formation of DNA binding metabolites of aromatic amines within the target organ (Badawi *et al.*, 1995). However, the NAT1 genotype has not been associated with increased risk of bladder cancer (Okkels *et al.*, 1997).

The aromatic amine 4-aminobiphenyl (4-ABP), which is present in tobacco smoke, is thought to be relevant to bladder carcinogenesis. In line with the risks for bladder cancer observed in epidemiological studies, the levels of 4-ABP-haemoglobin adducts have been associated with smoking status and type of tobacco (Bryant *et al.*, 1988) and with aromatic amine-DNA adducts in exfoliated urothelial cells (Talaska *et al.*, 1991). The NAT2 phenotype is associated with levels of 4-ABP-haemoglobin adducts which were higher in subjects with the slow phenotype than in those with the rapid one (Bartsch *et al.*, 1990; Vineis *et al.*, 1990; Yu *et al.*, 1994). The NAT2 phenotype did not affect DNA-benzidine adducts in exfoliated urothelial cells from exposed workers, suggesting a different activation pathway for aromatic mono- and diamines (Rothman *et al.*, 1996).

The NAT1\*10 genotype and phenotype correlated with higher levels of aromatic amine adducts in DNA from bladder mucosa. As expected, individuals with the slow NAT2 and the rapid NAT1 genotypes showed the highest adduct level (Badawi *et al.*, 1995).

About 50% of Caucasians show an inherited deletion of two copies of the gene for the enzyme glutathione S-transferase M1, which is involved in the detoxification of a number of carcinogens. Several studies have suggested that individuals

homozygous for the deleted genotype (*GSTM1* 0/0) are at higher risk of developing bladder cancer (Bell *et al.*, 1993; Brockmüller *et al.*, 1994). However, it was shown that the *GSTM1* deletion may not be a risk factor for bladder cancer development, but may be related to the bladder cancer patient survival (Okkels *et al.*, 1996).

Although 4-ABP or its metabolites have not been shown to be substrates for glutathione S-transferases, 4-ABP-haemoglobin adduct levels were higher in subjects carrying the *GSTM1* 0/0 genotype who were also slow acetylators (Yu *et al.*, 1995). It is thus evident that 4-ABP-DNA and haemoglobin adduct formation is significantly influenced by metabolic polymorphism, and might modify bladder cancer risk.

### Hair dyes

Studies of occupational exposure to hair dyes up to 1992 were reviewed within the *IARC Monographs* programme (IARC, 1993). At least seven cohort and 11 case-control studies have included data on occupational exposure to hair dyes — among hair-dressers, barbers and beauticians — and subsequent bladder cancer risk. The pooled RR estimate was 1.4 (183 observed versus 129 expected) for cohort studies, and some association was also observed in several case-control studies. These results are compatible with some moderate associa-

tion between past professional exposure to hair dyes and subsequent bladder cancer risk, but errors and biases in observational epidemiological studies could be responsible, particularly since allowance for smoking was lacking or inadequate in most studies. A major open question, moreover, is whether current exposure to modern hair dyes is still related to some excess risk, or whether the selective elimination of carcinogenic compounds over recent years has reduced any such risk to immeasurable levels. Only further surveillance and future studies will provide definite answers.

Five case-control studies included information on personal use of hair dyes and bladder cancer risk. None of these showed any excess bladder cancer risk among users of hair dyes (Table 3). Thus, the overall evidence from epidemiological studies allows the exclusion of any appreciable and measurable risk for bladder cancer from personal use of hair dyes (La Vecchia & Tavani, 1995).

### Dietary factors

A role of diet and nutrition on bladder carcinogenesis is plausible, since most substances and metabolites, including carcinogens, are excreted through the urinary tract. Ecological studies have found positive correlations between fat and oil consumption and bladder cancer, but these are

**Table 3. Summary results from selected case-control studies of bladder cancer providing information on personal use of hair dyes<sup>a</sup>**

Reference; country	Sex	No. of cases	No. of exposed cases	Odds ratio
Howe <i>et al.</i> (1980), Canada	F	152	NR	0.7
	M	480	8	(No exposed control)
Hartge <i>et al.</i> (1982), USA	F	733	443	0.9
	M	2249	172	1.1
Ohno <i>et al.</i> (1985), Japan	F	65	42	1.6
Claude <i>et al.</i> (1986), Germany	F	91	NR	No association
	M	340	NR	
Nomura <i>et al.</i> (1989), USA	F	66	41	1.5
	M	195	1.5	1.3

<sup>a</sup> From La Vecchia & Tavani (1996); modified

F, female; M, male; NR, not reported

only partly reflected in the international differences in bladder cancer rates, which are generally higher in Europe than in North America. At least 10 case-control and three cohort studies of bladder cancer including some information on dietary factors have been published over the last two decades, and these have recently been reviewed (La Vecchia & Negri, 1996).

Of seven studies which considered various types and measures of fruit and vegetable consumption, six found a reduced risk with increasing consumption, which was more consistent for vegetables, with RR estimates of between 0.5 and 0.7 for the highest versus the lowest consumption level. There is, therefore, suggestive evidence that a diet rich in fresh fruit and vegetables is a correlate — or an indicator — of reduced bladder cancer risk. No clear association emerged for other foods investigated, including meat and milk.

With reference to nutrients (Table 4), total fat intake was related to bladder cancer risk in three

case-control studies, with RRs between 1.4 and 1.7 for the highest versus the lowest consumption level. However, no relation between fats and bladder cancer emerged in a cohort study on Japanese Americans in Hawaii (Nomura *et al.*, 1991). Similarly, no consistent association emerged between protein or carbohydrate consumption and bladder cancer risk.

Among micronutrients, vitamin A, and particularly carotenoids, showed a protective effect on bladder cancer risk in four case-control studies (Mettlin & Graham, 1979; La Vecchia *et al.*, 1989; Nomura *et al.*, 1991; Vena *et al.*, 1992), including one in which total calorie intake was measured, but they were not consistently related in two other studies. There were only scattered and inconclusive data on vitamin C and E (Risch *et al.*, 1988; Riboli *et al.*, 1991). One study suggested that calcium intake may be related to bladder cancer risk (Riboli *et al.*, 1991). Another, conducted in New York City, found an RR of 2.1 for the highest sodium consumption level (Vena *et al.*, 1992).

**Table 4. Relationship between intake of various nutrients and bladder cancer risk.**  
Main results from selected studies<sup>a</sup>

Reference, country	Nutrient	Relative-risk estimate for level of intake <sup>b</sup>				
		1 (low) <sup>c</sup>	2	3	4	5 (high)
Steineck <i>et al.</i> (1990), Sweden	Fat	1	1.0	1.4	1.4	1.7
	Protein	1	0.9	1.0	1.2	1.4
	Carbohydrates	1	1.2	1.2	1.6	1.2
	Total energy	1	1.1	1.5	1.7	1.4
Riboli <i>et al.</i> (1991), Spain	Total fat	1	1.4	1.6	1.4	-
	Saturated fat	1	1.7	2.2	2.3	-
	Protein	1	1.1	1.2	1.5	-
	Carbohydrates	1	1.2	1.0	1.4	-
Vena <i>et al.</i> (1992), USA	Fat	1	1.3	1.2	1.4	-
	Protein	1	0.8	0.6	0.6	-
	Carbohydrates	1	1.0	1.0	1.0	-
	Total energy	1	1.3	1.6	1.8	-
Chyou <i>et al.</i> (1993), Hawaii	Fat	1	0.7	0.9	-	-
	Protein	1	0.7	0.9	-	-
	Carbohydrates	1	0.7	1.0	-	-
	Total energy	1	0.8	1.1	-	-

<sup>a</sup> From La Vecchia & Negri (1996), modified

<sup>b</sup> In some cases, summary relative risk is derived from published data.

<sup>c</sup> Reference category.

Thus, available data on diet and bladder cancer remain inconclusive. This is at least partly attributable to the limited number of cohort studies, in which satisfactorily detailed and validated dietary questionnaires were used. Despite these limitations, available data suggest that a diet rich in fresh fruit and vegetables is a correlate of reduced bladder cancer risk. The role of specific nutrients remains open to debate.

### Coffee drinking

Since the early 1970s, the possible association between coffee consumption and bladder cancer risk has been a topic of widespread interest. Cole (1971), in a case-control study from the Boston area reported an RR of 1.2 in men and 2.6 in women who drank coffee compared with non-drinkers. Since then, more than two dozen case-control studies have been published on the topic, the main results of which have been reviewed in detail, among others, in the IARC Monographs programme (1991).

Briefly, compared with non-consumers of coffee, the RR in most studies tended to be elevated in drinkers, but this increase was generally not dose- or duration-related.

This risk pattern was clear in the largest case-control study on bladder cancer based on 2982 cases and 5782 controls interviewed in a collaborative, population-based study in 10 geographical areas of the United States (Hartge *et al.* 1983). The RRs for ever versus never coffee drinkers, after simultaneous allowance for sex, age, race, geographical area and tobacco consumption, were 1.6 (95% confidence interval (CI), 1.2–2.2) for men, 1.2 (95% CI, 0.8–1.7) for women, and 1.4 (95% CI, 1.1–1.8) for both sexes combined. When various consumption levels were considered, the RR was 1.5 (95% CI, 1.1–1.9) for men who drank over 64 cups per week, but no consistent dose-risk relation was evident in either sex. Similarly, there was no association with duration of coffee drinking. No interaction or effect modification was observed with geographical area, race, occupation, artificial sweetener use or history of urinary tract infections. The authors noted that adjustment for smoking reduced the RR for ever versus never coffee drinking from 1.8 to 1.4, and that residual confounding by tobacco (or other correlates of coffee drinking) would only partly explain the apparent relation-

ship between bladder cancer and coffee drinking, which was still present when only subjects who claimed to be lifelong nonsmokers were considered.

On the basis of these findings, together with data from different parts of the world on the coffee-bladder cancer relation, it is possible to exclude confidently a strong association between coffee and bladder cancer, while at the same time it appears that coffee drinking may represent an indicator of risk. Whether this indicator is non-specific or includes some aspects of causality is still open to debate.

In biological terms, caffeine and the large number of other substances contained in coffee may have a wide spectrum of direct as well as indirect metabolic activities. It is therefore conceivable that even small amounts of coffee may induce changes in levels of carcinogens or anti-carcinogens in the bladder epithelium. On the other hand (although it is less likely), coffee drinking may systematically interfere with the likelihood of interview. These and other possible sources of error or bias may at first sight appear to be largely speculative. A considerable amount of epidemiological research, however, has systematically, and with remarkable consistency, shown an association between coffee and bladder cancer. Even apparently less plausible hypotheses should therefore be considered and, if possible, tested in future research (La Vecchia, 1993).

### Saccharin and other artificial sweeteners

Large quantities of saccharin (i.e., a few percent of the diet) have been shown to cause bladder cancer in rodents, when administered after *N*-methyl-*N*-nitrosourea, and when given alone over more than one generation (US Congress Office of Technology Assessment, 1977; Doll & Peto, 1981; Silverman *et al.*, 1996).

A case-control study based on 408 cases (Howe *et al.*, 1977) showed a 60% increased risk for bladder cancer in men (but not women) who used artificial sweeteners. The issue attracted widespread interest at that time, but several subsequent epidemiological studies failed to provide any consistent evidence of association between saccharin or other artificial sweeteners consumed in various forms and human bladder cancer. The RRs were slightly above unity in some of these and slightly below unity in others, with no clear pattern.

The largest and most informative study included 3010 cases of bladder cancer and 5783 population controls from 10 areas of the USA (Hoover & Hartge Strasser, 1980): the overall RR associated with ever having used any form of artificial sweeteners was 1.02 (95% CI, 0.92–1.11). No association was observed in men (RR, 0.99) or women (RR, 1.07), nor according to type or form of artificial sweeteners, and there was no dose-risk relationship. This study also evaluated subgroups based on use of cigarettes and potential exposures to occupational carcinogens. Excesses of borderline significance were observed in nonsmoking women, smoking men, and heavily smoking women.

In contrast, a study from the Greater Boston area (Morrison & Buring, 1980) found an RR of 0.6 in smoking men and 1.5 in smoking women, although none of these estimates was significant. Similarly inconsistent results across strata of sex and smoking habits were observed in a study from Yorkshire, UK (Cartwright *et al.*, 1981). The authors did not interpret these findings as suggestive of human carcinogenicity of saccharin. In another study, no association was observed in separate strata of histological type of bladder cancer (squamous-cell, adenocarcinoma, transitional-cell; Kantor *et al.*, 1988).

In a study on postmortem material, no relation was found between the proportion of atypical nuclei in bladder epithelium and use of artificial sweeteners. Information on the latter was available for only 149 out of 282 patients. Proportions were unadjusted for tobacco smoke (Auerbach & Garfinkel, 1989).

A descriptive study from Denmark (Jensen & Kambi, 1982) provided no evidence of increased bladder cancer rates during the first 30–35 years of life associated with *in utero* saccharin exposure during the Second World War.

It is therefore now clear that saccharin — or other artificial sweeteners — in the doses commonly used, are not relevant human bladder carcinogens on an individual risk or public health level.

### Urinary tract diseases

Infectious agents and other diseases of the urinary tract, which may cause chronic irritation and hence favour the action of specific carcinogens, have a major influence on bladder cancer risk in developing countries such as Egypt and Tanzania.

In these areas, there is a consistent relation between bladder carcinoma and bladder schistosomiasis, and a substantial proportion of bladder cancers are of squamous-cell type (Morrison & Cole, 1982; Kantor *et al.*, 1984; Bedwani *et al.*, 1993, 1997).

Urinary tract infections, moreover, may explain the relatively high bladder cancer rates in some southern Italian provinces where rates of other tobacco-related neoplasms are relatively low (Cislaghi *et al.*, 1986). The proportion of cases in Italy attributable to infection is difficult to estimate, but is probably about 10% (La Vecchia *et al.*, 1991; D'Avanzo *et al.*, 1995). The strength of the association, however, remains open to discussion, because of difficulties in exposure assessment and definition among other factors.

In fact, the role of infectious agents other than schistosomiasis and of nonspecific cystitis or urinary calculi in bladder carcinogenesis is difficult to study epidemiologically, particularly because early symptoms of bladder cancer are similar to those of cystitis, and subjects with urinary tract conditions probably tend systematically to recall episodes of cystitis or other urinary tract conditions more accurately. Not surprisingly, therefore, published evidence is somewhat inconsistent, with RRs for urinary tract infections ranging from 1 (Kjaer *et al.*, 1989) to 5 (Wynder *et al.*, 1963) and for urinary tract stones from 1 (Kjaer *et al.*, 1989) to 2.5 (Dunham *et al.*, 1968).

With reference to urinary tract stones, the RR for bladder cancer was 2.2 in males in an early US study (Wynder *et al.*, 1963), and around 1.5 in the large multicentric US study (Kantor *et al.*, 1984). Three more recent case-control studies (González *et al.*, 1991; La Vecchia *et al.*, 1991; Sturgeon *et al.*, 1994) found RRs between 1.2 and 1.4. Whether this reflects a causal association rather than recall bias is still unclear.

A Swedish record-linkage study, based on a cohort of 61,114 patients hospitalized for kidney or ureter stones and followed for up to 18 years (Chow *et al.*, 1997), based on a total of 46 cases of renal pelvis or ureter cancer and 319 cases of bladder cancer, found a standardized incidence ratio of 2.5 for renal pelvis or ureter cancer and 1.4 for bladder cancer. The association was stronger in women. In a prospective cohort study of Japanese conducted in Hawaii, no relation was observed



between serum uric acid and bladder cancer (Kolonel *et al.*, 1994).

Most findings, therefore, are consistent with a two- to three-fold elevated risk for urinary tract infections, although some of this apparent excess may be attributed to more accurate recall of urinary symptoms by bladder cancer cases. For urinary tract stones the association is moderate, and its causality remains open to discussion; this association may be stronger in women (La Vecchia *et al.*, 1991).

Published data also indicate that the role of urinary tract infections is probably related to one of the later stages of the process of carcinogenesis (Armitage & Doll, 1961).

## Conclusions

There is ample scope for prevention and control of bladder cancer, including (a) avoidance of tobacco smoking; (b) close surveillance and avoidance of occupational exposures to aromatic amines and other related chemicals; (c) dietary changes, which have not yet, however, been satisfactorily defined; (d) stricter control of bladder and other urinary tract infections; and (e) identification of individuals who are susceptible to aromatic amine carcinogenesis.

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# Calculi, precipitates and microcrystalluria associated with irritation and cell proliferation as a mechanism of urinary bladder carcinogenesis in rats and mice

S. Fukushima and T. Murai

## Introduction

A number of environmental agents which lack genotoxic activity act as promoters for urinary bladder carcinogenesis in rodents. Carcinogenesis due to chronic physical irritation has for many years attracted attention of researchers (Brand *et al.*, 1975) and a number of studies of this phenomenon in the urinary bladder have been performed.

In this chapter, the possible mechanisms linking formation of calculi, precipitates and microcrystalluria with urinary bladder cancer induction in rats and mice are discussed, concentrating particularly on new data for uracil and sodium ascorbate.

## Urinary calculi and bladder cancer in rodents

The coexistence of carcinomas and calculi in the urinary bladders of rats and mice has been observed in carcinogenicity studies on a variety of chemical agents. In most strains of rats and mice, the spontaneous incidences of these lesions are low (Goodman *et al.*, 1979; Frith *et al.*, 1982; Maekawa *et al.*, 1983). However, two strains of rat, the Brown Norway and DA/Han, have high incidences of spontaneous bladder tumours that are often associated with the presence of calculi (Boorman, 1975; Deeborg *et al.*, 1985).

A number of older publications describe calculus formation in the urinary bladder following surgical treatment including implantation with cholesterol, paraffin wax or wood pellets, glass beads or chalk powder (Jull, 1951; Bonser *et al.*, 1953; Mobley *et al.*, 1966; Toyoshima & Leighton, 1975; Teelman & Niemann, 1979). In rodents, foreign

bodies are thus associated with urinary bladder carcinogenesis (Clayson, 1974). However, in these studies, cytomorphological and proliferative reactions to the surgery itself led to difficulties in interpretation of the underlying mechanisms. From this point of view, models utilizing endogenous urinary calculus formation by non-genotoxic agents allow more precise investigation of cell proliferation due to physical irritation.

Herz *et al.* (1972) found uric acid urolithiasis in the dilated renal pelvic cavities, dilated ureters and enlarged urinary bladders of rats with surgical portacaval anastomosis (i.e., linking the portal vein to the vena cava). They suggested that this treatment resulted in alterations of renal tubule function, leading to a lowered pH with a consequent decrease in solubility of uric acid. Bladder carcinomas were observed in animals with portacaval shunts and chronic irritation by stones was suggested to be the most likely cause (Engelmann *et al.*, 1987). However, Mori and Yamaguchi (1989) suggested that tumour development after portacaval anastomosis in rats might be due to vitamin A deficiency rather than to bladder calculi or generation of urinary carcinogens, because this condition was correlated with increased urothelial ornithine decarboxylase activity. Schramek *et al.* (1993) reported sex-dependent urolithiasis in rats with portacaval shunts. Heidenreich and Engelmann (1993) suggested that hormonal alterations might contribute to sex-dependent stone formation in rats with portacaval anastomosis, because significantly decreased testosterone levels and increased oestradiol and glucagon levels in males and marked rises in

testosterone and glucagon levels as well as a decrease in oestradiol levels in females were correlated with urolithiasis.

There are many chemicals which cause formation of endogenous urinary calculi or microcrystalluria when administered to rats and mice, as shown in Table 1 (Cohen & Ellwein, 1991). Ethylene glycol and its derivatives are converted to oxalic acid, which is then excreted through the kidney and forms urinary calcium oxalate calculi. Weil *et al.* (1965) reported the presence of calcium oxalate calculi in the urinary bladders of male rats treated with diethylene glycol but not in female rats, and that males but not females developed papillomas. They suggested that the calculi rather than the chemical administered were responsible for the tumorigenesis, because transplantation of the stones also induced urinary bladder tumours. Hueper and Payne (1963) similarly described calculi and tumour induction in the urinary bladders of mice fed polyoxyethylene-(8)-stearate. While

the authors claimed that the chemical or some impurity was the cause of tumour induction (because of the presence of a tumour in one female mouse lacking urinary bladder stones), bladder calculi are likely to be voided more easily by females than males because of the shorter urethra and the lack of curvature (Clayson *et al.*, 1995). Although conflicting views have been presented, it was concluded by Teelman and Niemann (1979), who implanted multiple small glass beads surgically into the bladder lumina of male and female mice, that calculi were associated with tumour induction. Clayson (1979) reported that dietary xylitol and terephthalic acid induced formation of bladder stones and bladder tumours in rodents. Heck and Tyl (1985) reviewed work on terephthalic acid, dimethyl terephthalate and melamine which produce stone formation in the urinary bladder and associated tumours. These chemicals basically induce an epithelial hyperplastic response due to irritative stimulation by calculi (Frith *et al.*, 1984; Oyasu *et al.*, 1984; Heck & Tyl, 1985).

**Table 1. Chemicals which form endogenous urinary calculi or microcrystalluria in mice and/or rats**

Uracil
Thymine
Melamine
Uric acid
Homocysteine
Cysteine oxalates
Calcium oxalate
Calcium phosphate
Ethylene glycol and its derivatives
Biphenyl
4-Ethylsulfonylnaphthalene-1-sulfonamide
Oxamide
Acetazolamide
Terephthalic acid
Dimethyl terephthalate
Nitrotriacetate
Polyoxyethylene-(8)-stearate
Glycine
Orotic acid

Adapted from Cohen & Ellwein (1991)

### Uracil calculi and cancer induction in rats

Lalich (1966) first reported stone formation in the urinary bladder of rats after oral administration of uracil, a component of RNA. Subsequently, Shirai *et al.* (1986) demonstrated that oral administration of uracil induced mucosal papillomatosis, a severe, diffuse papillary hyperplasia of the urinary bladder, associated with yellowish-white and finely granular calculi (Figure 1), with a low incidence of urinary bladder carcinomas also being observed after a relatively short period of treatment. The calculi were found to consist of uracil itself.

In a two-year carcinogenicity test of uracil administered to Fischer 344 rats of both sexes, urinary bladder carcinomas, particularly transitional cell carcinomas, were observed at very high incidence in males, but at a low incidence in females (Fukushima *et al.*, 1992). All the carcinomas were papillary or polypoid, and non-invasive. No metastases to other organs were observed. In addition, all carcinomas were associated with the presence of a large number of urinary bladder calculi. In the renal pelvis, transitional cell carcinomas with formation of calculi were observed in 23% of males and 11% of females.

Dose-dependent increases in the incidence of urinary bladder tumours were observed in male



**Figure 1.** Urinary bladder calculi in a rat treated with 3% uracil in the diet (provided by Dr T. Shirai)

Fischer 344 rats given 3, 1 or 0.3% uracil in the diet for 36 weeks followed by 4 weeks without the chemical, the total observation period being 40 weeks (Okumura *et al.*, 1991). A high incidence (11/15, 73%) of carcinomas was observed in the urinary bladder of 3% uracil-treated rats, but not in the 1% or 0.3% treatment groups, and no calculi were observed with these lower doses. Shirai *et al.* (1987) and Masui *et al.* (1989) previously detected low yields of urinary bladder carcinomas after treatment with 3% uracil for 20 weeks (1/10, 10%) and for 30 weeks (1/5, 20%). Wang *et al.* (1987) observed urinary bladder carcinomas at an incidence of 26% (6/23) after 20 weeks of uracil exposure followed by non-treatment for 20 weeks. These results imply that the induction of carcinomas in the urinary bladder requires both an adequate uracil concentration and time for tumour progression to occur.

The effects of sodium chloride (NaCl) on uracil administration provided clear mechanistic

evidence on the carcinogenicity of uracil in the rat urinary bladder (Fukushima *et al.*, 1992). Male Fischer 344 rats were given diets containing 3% uracil, 3% uracil plus 5% NaCl, 3% uracil plus 10% NaCl or only 10% NaCl for 36 weeks and then diet without chemicals for 4 weeks. Increased amounts of urine were observed in the NaCl-treated groups. The average food and water consumption were higher in groups given uracil plus NaCl, especially 10% NaCl, than in the group given uracil alone. The incidences of carcinomas and calculi of the urinary bladder were 75% and 81% for uracil alone, 6% and 6% for uracil plus 5% NaCl, and zero and zero for uracil plus 10% NaCl. Thus, the induction of carcinomas by uracil was clearly related to the presence of calculi in the urinary bladder.

Thymine (5-methyluracil) is also able to exert a similar effect in the rat urinary bladder (Okumura *et al.*, 1992). Male Fischer 344 rats were administered 3% or 1% thymine in the diet for 36 weeks followed by a 4-week period without the chemical. Exploratory laparotomy at week 36 revealed the formation of fine granuliform calculi in the urinary bladder in the 3% thymine group. Papillomas and carcinomas were induced in 9/20 (45%) rats compared with 1/20 (5%) rats of the 1% group, which had very limited calculi. The results indicate that the calculi induced by thymine are also directly related to urinary bladder carcinomas in rats.

#### Uracil calculi and cancer induction in mice

Sakata *et al.* (1988) reported calculus formation in the urinary bladder of mice following administration of uracil. Both male and female mice of two strains, Swiss and C3H, were given 3 or 1% uracil in their diet for 15 weeks. The higher dose (3%) induced epithelial proliferative changes of the urinary bladder with calculus formation by 10 weeks of administration and a more severe hyperplastic lesion (nodular and papillary hyperplasia) was observed in mice killed at week 15. Mice fed 1% uracil rarely had calculi or proliferative changes. The males tended to be more severely affected than the females, particularly those of the C3H strain. The pattern of proliferation was predominantly nodular type with downward growth.

The carcinogenicity of uracil in mice was confirmed in a long-term study (Fukushima *et al.*, 1992). Male and female B6C3F<sub>1</sub> mice were given a

diet containing 0 (control) or 3% (weeks 1 to 6) and then 2.5% (weeks 7 to 96) uracil. The total observation period was 96 weeks. Gross observation of the urinary bladders revealed thickening of the wall in the uracil-treated groups, but no polypoid or papillary lesions were seen on the luminal surface of the urinary bladder. Transitional cell carcinomas were histologically observed with a high incidence in females, but at a low incidence in males. No papillomas were found in either sex of any group. In addition, no renal pelvic tumours were observed in the uracil-treated mice. The incidences of calculi in the urinary bladder of uracil-treated groups could not be determined exactly at the time of autopsy, and no calculi were found in the urinary bladder in any fixed preparation, because they disappeared during three weeks' fixation with formalin.

#### Melamine calculi and cancer induction in rats

Melamine (2,4,6-triamino-s-triazine) is used in the manufacture of melamine-formaldehyde amino resins, found in household goods such as buttons and electrical equipment. Short-term mutagenicity and related tests have found it to be non-genotoxic. In a two-year carcinogenicity study, Melnick *et al.* (1984) observed transitional cell carcinomas in the urinary bladder at an incidence of 16% (8/49) in male Fischer 344 rats given 0.45% melamine in the diet, but not in females (0%, 0/46). Seven of eight rats with carcinomas had urinary bladder calculi and the overall incidence of calculi (93%) was much higher in the melamine-dosed group than in controls (4%).

Okumura *et al.* (1992) also examined the carcinogenicity of melamine in male Fischer 344 rats administered doses of 0.3, 1 or 3% in the diet for 36 weeks followed by 4 weeks without the chemical. Urinary bladder carcinomas were induced in 79% (15/19) of the 3% group, 5% (1/20) of the 1% group, and 0% (0/20) of the 0.3% melamine-treated rats. A significant correlation between calculus formation estimated by exploratory laparotomy at experimental week 36 and tumour incidence at week 40 was observed.

As was the case with uracil, an inhibitory effect of NaCl on carcinogenicity of melamine for the urinary bladder in rats was clearly shown by Ogasawara *et al.* (1995). In Fischer 344 male rats

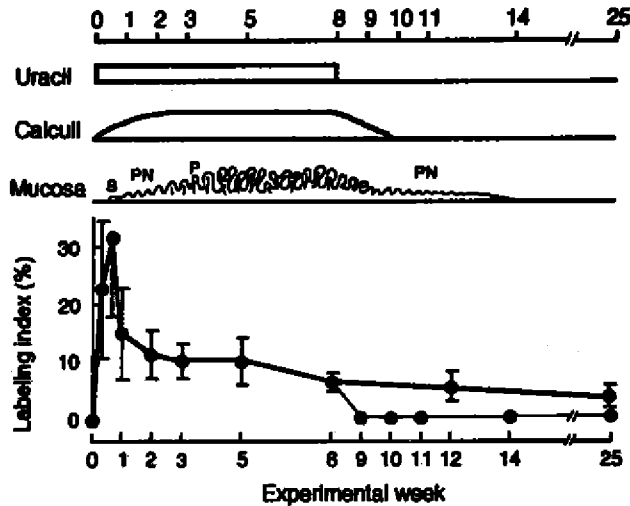
given 3% melamine alone in the diet, the incidence of urinary bladder carcinomas was 90%, whereas no such lesions were evident in animals given 3% melamine plus 10% NaCl. The water intake, used as an index of urinary output, was increased by NaCl treatment and calculus formation resulting from melamine administration was suppressed. The main constituents of the calculi were determined to be melamine itself and uric acid (total content 61–81%) in equal molar ratios. The results from all experiments indicate that carcinoma induction in the rat urinary bladder by melamine is directly due to irritative stimulation by calculi, as with uracil.

#### Urinary calculi and cell proliferation

The initiating mechanism of calculi induced by non-genotoxic agents in the bladder remains uncertain, but some hypotheses have been suggested. In a review by Clayson *et al.* (1995), three possibilities for initiating factors were proposed: (1) the excess mitosis in a normally quiescent tissue may lead to mutation, (2) inflammation through the accretion of phagocytes in the tissue may lead to excessive oxidative DNA damage (Cohen & Ellwein, 1990, 1991), or (3) a urinary constituent derived from the metabolism of natural constituents of the food supply may act as an initiator.

Shirai *et al.* (1986, 1989) found that dietary administration of uracil at a concentration of 3% quickly induced bladder mucosal papillomatosis secondary to the formation of urinary calculi in all treated rats (Figure 2). Surprisingly, although urinary calculi and bladder papillomatosis, which showed papillary projections of epithelial proliferation, were severe and extensive, they disappeared when the treatment with uracil stopped; the bladder mucosa returned to normal. Thus, uracil calculi induce a reversible hyperplasia of the urinary bladder epithelium. 5-Bromo-2'-deoxyuridine (BrdU) or proliferating cell nuclear antigen (PCNA) labelling indices in the epithelium were high during uracil treatment but quickly returned to normal after removal of the chemical insult (Shirai *et al.*, 1989, 1995; Otori *et al.*, 1997). Interestingly, cyclin D1-positive cells were observed immunohistochemically in hyperplastic epithelium of the urinary bladder in rats during uracil administration, but not when the treatment





**Figure 2.** Time sequence in the development and disappearance of calculi and epithelial proliferative lesions. S, simple hyperplasia; PN, papillary or nodular hyperplasia; P, papilloma. (Contributed by Dr T. Shirai)

was stopped (Otori *et al.*, 1997). In addition, PCNA-positive cells were present mainly at the top to middle of the papillary projections in papillomatosis. On the other hand, areas positive for anti-Le<sup>y</sup>(BM-1/JIMRO), an important marker of apoptosis, were mainly located at the bottom to middle of the papillary projections. It is speculated that areas of PCNA-positive cells are in direct contact with the calculi, while BM-1 positive cells are secondarily induced. The results confirm that irritation by the calculi simultaneously increases both cell proliferation and apoptosis in papillomatosis. When the uracil treatment is continued, the level of apoptosis is weaker than that of proliferation, resulting in papillary hyperplasia. On the other hand, when urinary calculi disappeared one week after the uracil treatment was stopped, PCNA-positive cells had almost disappeared and apoptosis was still markedly increased. In disappearing papillomatosis, the surface epithelium was found to be clearly stained with BM-1 and nick-end labelling, markers of apoptosis. Although clear erosion and ulcers are not observed in the urinary bladders of animals given uracil, the hyperplastic response is likely to be regenerative in nature. However, the degree of proliferation due to uracil-induced

calculi appears to be more marked than is generally observed in regenerative processes induced by ulcers (Shirai *et al.*, 1978). Therefore, further studies are required to elucidate the mechanism of the cell proliferation due to calculi. Uracil itself is not cytotoxic for the urinary bladder.

To evaluate the mechanism of the appearance of uracil-induced papillomatosis, Fukushima *et al.* (unpublished data) examined changes in the activity and localization of ornithine decarboxylase (ODC), a key enzyme in polyamine biosynthesis, and epithelial proliferation accompanying the sequential bladder epithelial changes following administration and withdrawal of uracil. ODC activity during uracil administration was maintained at a high level compared to normal epithelium, but this activity sharply decreased after cessation of uracil treatment. Accumulation of ODC protein could be observed in the proliferating bladder epithelium by immunohistochemical examination and Western blotting analysis, and even after cessation of treatment, protein binding to anti-ODC antibody remained mildly elevated. Sequential changes of PCNA-positive cells in the epithelium during the development and disappearance of papillomatosis correlated with ODC activity. ODC

mRNA was also strongly expressed in the proliferating epithelium of rats treated with uracil and weakly in normal epithelium, in accordance with the findings for ODC protein. These data demonstrate that cell proliferation in the development of papillomatosis is closely associated with increased polyamine production, and moreover suggest that ODC activity is up-regulated in a post-translational step.

Sakata *et al.* (1988) examined DNA synthesis in the urinary bladder epithelium of male and female Swiss and C3H mice treated with 3 or 1% uracil in the diet using [methyl-<sup>3</sup>H]thymidine. The labelling indices were significantly increased in both strains and sexes treated with 3% uracil as compared to controls, and in males compared to females at week 10, but not week 15. Clayson and Pringle (1966) reported that the rate of mitosis in the urothelium of the mouse bladder with paraffin wax or cholesterol pellets was 20–30-fold higher than that of normal bladder. The increase in the rate of cellular proliferation is probably the result of the damaging effect of the implanted bladder concretions (Clayson *et al.*, 1995).

#### Gene alterations in urinary bladder carcinomas induced by uracil treatment alone or in two-stage carcinogenesis models

Exposure to a genotoxic carcinogen may cause cell cycle arrest at the G1 check-point involving negative cell cycle regulators such as p53, p21<sup>waf1/cip1</sup> and pRB. Under conditions of continuous exposure to a genotoxic carcinogen, cells with abnormalities in such negative regulators might be expected to enter S phase and thus possess a growth advantage over the cells retained in the G1 phase. In experimental rat urinary bladder carcinomas induced by continuous exposure to the genotoxic carcinogen *N*-nitroso-*n*-butyl(4-hydroxybutyl)amine (BBN), p53 mutations are frequent (Masui *et al.*, 1994, 1996). Chronic stimulation of proliferation increases the fixing of DNA damage in epithelial cells as mutations (Cohen & Ellwein, 1990). This implies that the frequency of spontaneous initiated and putative preneoplastic foci increases with enhanced cell proliferation due to longer or more intense calculus stimulation. However, tumours associated with uracil alone may lack such mutations (Fukushima *et al.*, unpublished data), and indeed, infrequent

alteration of the p53 gene in tumours produced in a two-stage rat urinary bladder carcinogenesis model has been reported by Asamoto *et al.* (1994). These authors also indicated that cells with a p53 mutation may not have any particular advantage under the influence of a promoter as confirmed by our recent results (Lee *et al.*, 1997). Thus p53 mutation is not a critical event. Furthermore, *H-ras* mutations are not observed in uracil-induced urinary bladder carcinomas of rats. The search for alternative control pathways must therefore continue.

#### Promotion of urinary bladder carcinogenesis by sodium and potassium salts

A list of sodium and potassium salts which show promoting effects on urinary bladder carcinogenesis is given in Table 2 (see also Cohen, this volume). The mechanism by which sodium or potassium salts might affect proliferation of urinary bladder epithelial cells remains to be defined.

As summarized in Table 3, there is an apparent relationship between promoting potential and fluctuation in urinary parameters, particularly pH and sodium or potassium concentration. Parent acids of sodium salts, *per se*, do not show

Table 2. Sodium salts which have promoting activity in the second stage of urinary bladder carcinogenesis

Compound	References
Sodium saccharin	Hicks <i>et al.</i> , 1975; Cohen <i>et al.</i> , 1979
Sodium L-ascorbate	Fukushima <i>et al.</i> , 1983a
Sodium <i>o</i> -phenylphenate	Fukushima <i>et al.</i> , 1983b
Sodium erythorbate	Fukushima <i>et al.</i> , 1984
Sodium bicarbonate	Fukushima <i>et al.</i> , 1986a
Sodium citrate	Fukushima <i>et al.</i> , 1986b
Sodium phenobarbital	Diwan <i>et al.</i> , 1989
Sodium barbital	Diwan <i>et al.</i> , 1989
Sodium chloride	Shibata <i>et al.</i> , 1992
Sodium succinate	Otoshi <i>et al.</i> , 1993
Trisodium phosphate	Shibata <i>et al.</i> , 1992
Potassium carbonate	Fukushima <i>et al.</i> , 1987
Tripotassium phosphate	Shibata <i>et al.</i> , 1992

Table 3. Summary of relation between changes in urinary parameters and promoting potential

Test chemicals	Promoting potential	Fluctuation in urinary parameters			Increase of crystals
		pH	Electrolyte	Total ascorbic acid	
Ascorbic acid + NaHCO <sub>3</sub>	+++	↑	Na ↑	↑	++
Ascorbic acid	-	↓	↔	↑	±
NaHCO <sub>3</sub>	+	↑	Na ↑, Ca ↑, P ↓	↔	+ ~ ++
Na ascorbate	+++ ~ +++	↑	Na ↑, Mg ↓	↑	++
Na ascorbate + NH <sub>4</sub> Cl	±	↔	Na ↑, Cl ↑	↑	+ ~ ++
NH <sub>4</sub> Cl	-	↓	Na ↓, Cl ↑	↔	±
K <sub>2</sub> CO <sub>3</sub>	±	↑	K ↑, Cl ↓	↔	ND
Ascorbic acid + K <sub>2</sub> CO <sub>3</sub>	+++	↑	K ↑, Cl ↓	↑	ND
CaCO <sub>3</sub>	-	↑	Ca ↑, Mg ↓	↔	ND
Ascorbic acid + CaCO <sub>3</sub>	-	↔	Ca ↑, Mg ↓	↑	ND
MgCO <sub>3</sub>	-	↔	Ca ↑, Mg ↑, P ↓	↔	ND
Ascorbic acid + MgCO <sub>3</sub>	-	↔	Ca ↑, Mg ↑, P ↓	↑	ND

For further details, see Fukushima *et al.* (1986a) and Fukushima *et al.* (1987)

ND, not detected, but calculus formation was not observed.

promoting activity or increase the urinary pH and sodium ion concentration (Fukushima *et al.*, 1986b). However, a combination of L-ascorbic acid plus sodium bicarbonate or potassium carbonate did reveal co-promoting activity associated with urinary changes (Fukushima *et al.*, 1987). Other cations, such as calcium and magnesium, in the urine did not show any promoting activity (Fukushima *et al.*, 1987). Cohen *et al.* (1991a) also reported that calcium carbonate alone did not exert a promoting effect. Sodium hippurate was negative under increase of urinary sodium ion concentration without elevation of urinary pH in the tumour promotion bioassays, although the mechanism remains to be clarified.

The hypothesis has been suggested that physical irritation by precipitates or microcrystalluria

formed in the urinary bladder of rats given sodium saccharin is related to proliferation of urinary bladder epithelium (Cohen *et al.*, 1991b). Analysis of urine from rats given non-genotoxic sodium or potassium salts has revealed increases in the urinary pH as well as in the concentrations of sodium or potassium ions. These factors could be relevant to the promoting mechanism. However, Cohen *et al.* (1995) have reported the presence of precipitates in urine of rats given not only sodium saccharin but also sodium L-ascorbate, sodium erythorbate, sodium bicarbonate, sodium chloride and sodium citrate, but not sodium *o*-phenylphenate (S.M. Cohen, personal communication).

Cohen and Lawson (1995) paid particular attention to amorphous precipitates, which are formed under relatively high pH in the urine of

rats administered sodium salts, as a significant factor in proliferation of urinary bladder epithelium. This is of considerable importance in the context of the human hazard potential of the artificial sweetener, sodium saccharin. In addition, other salts acting as promoters also induce cell proliferation in the bladder epithelium (Shibata *et al.*, 1989, 1992; Cohen *et al.*, 1995).

### **Influence of physical stimulation of the urinary bladder epithelium as a factor responsible for sex, strain and species differences**

Kurata *et al.* (1986) suggested that interaction between high urinary sodium concentration and the existence of urinary crystals might be an important factor related to promoting activity, since biphenyl induced the formation of characteristic stones and a high sodium concentration in the urine without altering urinary pH. Shirai *et al.* (1987) and Masui *et al.* (1989) similarly found strong promoting activity of urinary calculi in BBN- and N-methyl-N-nitrosourea-initiated urinary bladder carcinogenesis in rats.

A fundamental hypothesis proposed by Cohen *et al.* (1991b) was that a combination of a high pH brought about by ingestion of sodium saccharin plus a high concentration of urinary proteins results in precipitation and formation of microcrystals, including silicate, which may act as microabrasives that injure urinary bladder epithelium, leading to compensatory regenerative hyperplasia. Okamura *et al.* (1992) showed that silicate urolithiasis induced by tetraethylorthosilicate induces microscopic changes in urinary bladder epithelium similar to those observed in rats given sodium saccharin. They pointed to  $\alpha_{2u}$ -globulin, a characteristic urinary protein in male rats as the key substance. This hypothesis fitted the finding that sodium saccharin exerts promoting activity in male rats but not in female rats or in mice. Mice have low levels of urinary protein and do not produce  $\alpha_{2u}$ -globulin (Hard, 1995), while female rats have very much lower levels of this protein than males (Vandoren *et al.*, 1983). Cohen *et al.* (1995) suggested that a similar mechanism might be responsible for the promoting effects of sodium salts including sodium L-ascorbate, glutamate and bicarbonate, after demonstrating that these can also cause precipitate formation. The above hypothesis is strengthened by evidence gained

with NCI-Black-Reiter (NBR) rats, which lack the ability to synthesize  $\alpha_{2u}$ -globulin and are less prone to the urothelial hyperplasia induced by sodium saccharin than intact or castrated male Fischer 344 rats (Garland *et al.*, 1994). This hypothesis might explain the lack of carcinogenic activity of saccharin in humans, which do not produce  $\alpha_{2u}$ -globulin. Recently, Uwagawa *et al.* (1997) demonstrated that sodium L-ascorbate had no promoting effect on urinary bladder carcinogenesis in NBR male rats, which do exhibit a proliferative response to uracil equivalent to that seen with Fischer 344 rats (Uwagawa *et al.*, 1994).

Thus, this hypothesis is very attractive to explain sex and species differences observed in the case of sodium saccharin and possibly other sodium salts, and is especially important with regard to human risk assessment, but there are some difficulties (Clayson *et al.*, 1995; Hard, 1995). Recently Murai *et al.* (unpublished data) found promoting activity of sodium L-ascorbate in bladder carcinogenesis of female rats which lack urinary  $\alpha_{2u}$ -globulin. This result indicates the need for further investigation of promotion mechanisms.

Clayson *et al.* (1995) questioned whether the rate of cellular proliferation induced by sodium saccharin is sufficient to induce genetic changes leading to cancer. Arnold and Clayson (1985) speculated another mechanism for the carcinogenicity of sodium saccharin. They claimed that permeability of cell membranes to sodium saccharin might differ between the neonatal stage and early maturity and its penetration into epithelial cells might induce genomic changes through unequal inhibition of enzymes concerned with DNA synthesis. In addition to the effects of sodium saccharin administered during the neonatal stage on cellular proliferation, endogenous mitogens such as epidermal growth factors whose synthesis is regulated by sex hormones might affect two-generation bioassays of sodium saccharin.

### **Strain, sex or species differences in promoting activity of sodium salts**

The promoting activity of sodium salts described above has been observed in males of a limited number of rat strains. However, strain differences in promotion of urinary bladder carcinogenesis by

sodium saccharin or sodium L-ascorbate have been observed (Ito *et al.*, 1983; Mori *et al.*, 1987, 1991; Uwagawa *et al.*, 1994; Murai *et al.*, 1997a; Uwagawa *et al.*, 1997). Tamano *et al.* (1993) reported that B6C3F<sub>1</sub> mice are resistant to agents that promote bladder tumorigenesis in rats, including sodium L-ascorbate, in spite of significant increases in urinary pH value and sodium ion concentration after four weeks' initiation with BBN. The initiation period seems to be important in relation to promoting activity in other species because after six weeks' administration of BBN to the Mongolian gerbil, 2-*tert*-butyl-4-hydroxyanisole (BHA), which is known to be a promoter of rat urinary bladder tumours, did not show any promoting activity. However, after 20 weeks' administration of BBN, promoting activity of sodium L-ascorbate was found in this species (Mori *et al.*, unpublished observations). Hamsters and monkeys also fail to show a promoting response to sodium saccharin and female rats do not respond to sodium saccharin to the same extent as male rats (IARC, 1980; Ellwein & Cohen, 1990), although sodium L-ascorbate does have promoting activity in female rats.

### Anatomical relationships of bladders in humans versus rats

DeSesso (1995) suggested that the disparity of tumour incidence between humans and rats might be related to anatomical aspects of the bladder rather than to any species difference in tissue susceptibility. Thus, objects within the lumen of the bladder, which are greatly influenced by gravity, will come to rest on the most inferior surface of the bladder and the difference in facility of excretion of such objects could be attributed to the difference in anatomical location of the bladder. In man, the internal urethral orifice is located at the bottom of the bladder in the ordinary standing or sitting position. On the other hand, in rats the bladder is normally parallel to the ground, with the orifice to one side. Thus objects in the bladder are liable to be excreted by humans, while in rats they may persist for longer periods. The ventral dome of the bladder of rats might therefore be expected to be more affected by calculi, precipitates or microcrystalluria. Cohen *et al.* (1990) reported that hyperplasia was observed in the wall of the ventral dome of the bladder of rats

given sodium saccharin. DeSesso (1995) also speculated that the epithelium of the urinary bladder might become damaged if crystals or microcrystals are trapped in the crypts that are formed as the asymmetric unit membrane invaginates into the supranuclear region of the superficial cells.

### Dietary and genetic influences on promoting activity of sodium salts

Since dietary and genetic factors have been reported to have considerable influence on chemical-induced carcinogenesis, Mori *et al.* (1987) investigated the modifying effects of diet and strain on promoting activity of sodium L-ascorbate in two-stage urinary bladder carcinogenesis assays in male F344/DuCrj (F344) and LEW/Crj (LEW) rats after BBN initiation. Two kinds of commercial basal diet (Oriental MF (M) and Clea CA-1 (C)) were used. The relative promoting effects of sodium L-ascorbate on bladder carcinogenesis were: F344 strain-M diet > LEW strain-C diet > F344 strain-C diet = LEW strain-M diet. In both strains and with both diets, increases in urinary pH and in the concentrations of sodium ions and total ascorbic acid showed no obvious correlation with the strength of promotion. Garland *et al.* (1989) observed a similar dietary influence on promotion by sodium saccharin. Moreover, Garland *et al.* (1989) and Okamura *et al.* (1991) showed that the low urinary pH associated with consumption of the AIN-76A diet eliminated the promoting activity of sodium saccharin.

### Possible role of precipitate or crystal formation in promotion of urinary bladder carcinogenesis in rats

As described above (Mori *et al.*, 1987), susceptibility to sodium L-ascorbate promotion differs among strains of rats, with Fischer 344 and LEW animals being sensitive. In contrast, ODS/Shi-od/od (ODS, Osteogenic Disorder Shionogi, genotype: od/od) rats, which lack the ability to synthesize L-ascorbic acid, are not sensitive (Mori *et al.*, 1991), although they are sensitive to BBN (Mori *et al.*, 1990). However, heterozygotes (+/od) and normal (+/+) ODS rats, which can synthesize L-ascorbic acid, are also resistant to sodium L-ascorbate promotion, when compared with Fischer 344 and LEW rats (Mori *et al.*, 1991). Recently, Mori *et al.* (1997)

further showed that ODS rats are resistant to the modifying effects of sodium bicarbonate and/or L-ascorbic acid on two-stage urinary bladder carcinogenesis. Thus other genetic factors may play an important role in promotion by sodium L-ascorbate of two-stage urinary bladder carcinogenesis and genes other than that at the *od* locus in ODS rats might be involved. ODS rats were developed from a sister strain of the WS/Shi (WS) strain, established by full-sibling mating of Wistar rats introduced in 1952 to Aburahi Lab., Shionogi & Co., Ltd., from the Faculty of Agriculture of Tokyo University, Japan. Therefore, some loci of biochemical markers in ODS rats are shared by the WS strain (Makino *et al.*, 1990). Three experiments were performed to examine strain differences in sensitivity of rats to the promoting effects of sodium L-ascorbate on the development of urinary bladder tumours (Murai *et al.*, 1997a). In the first experiment, WS, ODS and LEW rats were given 0.05% BBN in their drinking water and subsequently fed basal M diet with or without a 5% sodium L-ascorbate supplement. In LEW rats, the sodium L-ascorbate treatment increased the induction of neoplastic lesions in the urinary bladder, whereas WS and ODS-*od/od* animals did not show this response. Equivalent increases in urinary pH and sodium concentration were observed in all three strains. In the second experiment, WS and Fischer 344 rats were maintained on two kinds of commercial basal diet (M and C), during the administration of sodium L-ascorbate. In Fischer rats, feeding M diet during the promotion period yielded significantly more neoplastic lesions than the C diet, but in WS rats no influence of diet was apparent. Urinary parameters did not differ between the two strains. In the third experiment, strain differences in biosynthesis of  $\alpha_{2u}$ -globulin were assessed in ODS, WS and Fischer 344 rats. Immunohistochemical analysis of renal tubules and Western blotting analysis of urine revealed the presence of  $\alpha_{2u}$ -globulin without any significant variation between the three strains. Moreover, Murai *et al.* (1997b) observed that female Fischer 344 rats, which have low biosynthesis of  $\alpha_{2u}$ -globulin, are sensitive to the promoting activity of sodium L-ascorbate to the same extent as male Fischer 344 rats. These data suggest that differences in susceptibility to promotion are indeed due to genetic factors, as Mori *et al.* (1991) proposed,

rather than to dietary factors or the ability to synthesize  $\alpha_{2u}$ -globulin. However, further studies are needed to confirm this hypothesis, because other urinary precipitates such as calcium phosphate may exert such effects (Clayson *et al.*, 1995).

Previous reports have suggested that different levels of protein in the urine between male and female rats are related to the proliferative stimulus of sodium saccharin on the rat urinary bladder epithelium, and that the sex-dependence of nephropathy is mostly attributable to the presence of  $\alpha_{2u}$ -globulin, which occurs exclusively in urine of males (Hard, 1995). In the experiment described above, LEW and ODS rats both exhibited mild nephropathy, whereas lesion development was more marked in the WS strain. Moreover, the total protein level in the urine of ODS rats did not differ from those of Fischer 344 or LEW rats, while slightly higher values in WS rats were gained with test sticks, which indicate urinary protein levels. Thus, urinary  $\alpha_{2u}$ -globulin and protein levels in general may not always be critical for the urinary bladder tumour-promoting activity of sodium L-ascorbate. Moreover, Fukushima *et al.* (1983a) reported no correlation between the number of crystals in urinary sediments and the occurrence of neoplastic lesions in experimental rats after BBN initiation. Indeed, from results of our studies, as summarized in Table 3, no clear relationship between increased crystal formation and promoting activity was noted, while simultaneous increases in sodium or potassium ion concentration and pH were positively correlated with promoting activity as long as they occurred together. In contrast, treatments causing an increase in either the urinary pH or the sodium ion concentration alone did not lead to clear promoting activity (Fukushima *et al.*, 1983b).

Imaida *et al.* (1983) indicated that the membrane potential of the epithelium in the early stage of urinary bladder carcinogenesis is significantly increased by sodium saccharin. Since the apical membrane potential of the cell depends largely on the permeability to sodium ions, this presumably reflects the activity of the sodium ion channel, which is essential for sodium ion transport across the urinary bladder epithelium. Moreover, amiloride (an inhibitor of  $\text{Na}^+/\text{H}^+$  exchange) can inhibit both the promoting effects and the increase of BrdU labelling index, which is an indicator of cell

proliferation, induced by sodium L-ascorbate, whereas ouabain (an inhibitor of  $\text{Na}^+/\text{K}^+$  ATPase) does not influence the promoting effects (Murai *et al.*, 1996, 1997c). We therefore speculate that increases of sodium or potassium in urine may stimulate  $\text{Na}^+/\text{H}^+$  exchange activity and bring about a rise in intracellular sodium or potassium concentration and pH, resulting in increased DNA synthesis. However, there is conflicting evidence regarding the membrane potential (Gatzky *et al.*, 1989; Asamoto *et al.*, 1992) and further experiments are needed to clarify this point.

### Genetic factors relating to the promoting mechanisms of microcrystals or precipitates

While microcrystal or precipitate formation could explain the response to sodium saccharin in different species, this hypothesis cannot be universally applied to other compounds. The mechanism underlying promotion by sodium saccharin might also differ from the case with other sodium salts, because in a two-generation study it was itself found to induce bladder tumours (Arnold *et al.*, 1980). Two-generation investigations of other sodium salts have not been reported.

Several reports of race, population and family variation in human urinary bladder cancer have pointed to an important role of genetic factors in urinary bladder carcinogenesis (Fraumeni & Thomas, 1967; Herring *et al.*, 1979; Lynch *et al.*, 1979; Kiemeny & Schoenberg, 1996; Schoenberg *et al.*, 1996).

Mori *et al.* (1992) showed that susceptibility to the promoting effects of sodium L-ascorbate is inherited as a dominant autosomal trait, by examining hybrids between sensitive (Fischer 344 and LEW) and resistant (WS) parents. We are now paying special attention to the information encoded by the inherited genes. These might be related to  $\text{Na}^+/\text{H}^+$  exchange, as Rotin *et al.* (1989) suggested from observation of *in vivo* growth retardation in an  $\text{Na}^+/\text{H}^+$  exchange-deficient mutant-derived human bladder carcinoma cell line. Genetic factors contributing to the sensitivity to sodium salts of organic acids might be essential for promotion to occur.

### Conclusions

Information concerning factors modifying the development of bladder carcinomas has been accumulated through experimental animal studies. However, the mechanisms of promotion by urinary calculi, precipitates and microcrystalluria have still to be elucidated in detail and, as discussed in this paper, there is much conflicting evidence.

Cell proliferation due to uracil treatment is thought to be regenerative hyperplasia due to cell necrosis. However, the degree of proliferation observed with uracil-induced calculi appears to be more marked than that generally apparent in regenerative processes. Indeed, an unknown mitogenic mechanism seems also to act, since gene analysis of tumours induced by uracil alone revealed an absence of *p53* or *H-ras* gene mutation. This result seems to be in conflict with the usually accepted view (Cohen & Ellwein, 1990) that all such chronic stimulation increases the fixing of DNA damage in epithelial cells as mutations.

Clayson *et al.* (1995) pointed out that the cell proliferation induced by sodium saccharin would not be expected to be a major component of cancer induction in the urinary bladder. Indeed, the degree of promoting activity appears to differ between chemicals associated with pronounced urolithiasis and others such as sodium salts, in that the former can induce carcinomas without initiation but the latter usually do not, except for sodium saccharin.

For more reliable evaluation of human risk, the species, sex and strain differences as well as differences in physiology and anatomy must be taken into consideration. As described above, the susceptibility to sodium salts remains to be elucidated for many strains or species. Differences are regulated by DNA and analysis of genes governing responses to non-genotoxic carcinogens in experimental animals clearly warrant further study in order to facilitate extrapolation of animal data to humans.

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# Calcium phosphate-containing urinary precipitate in rat urinary bladder carcinogenesis

S.M. Cohen

## Introduction

In 1970, Bryan *et al.* (1970) reported that sodium saccharin incorporated in cholesterol pellets which were then surgically implanted into the mouse urinary bladder produced an increased incidence of cancer. Thus began a saga, that has already lasted nearly three decades, of attempts to ascertain the potential cancer hazard to humans of this commonly used sweetener (Arnold *et al.*, 1983; Ellwein & Cohen, 1990). The form of saccharin used in animal experiments to produce bladder cancer has been the sodium salt, administered as high doses in the diet. The sodium and calcium salts are consumed by humans.

Tumours formed after ingestion of high doses of sodium saccharin are produced only in rats (Frederick *et al.*, 1989; Ellwein & Cohen, 1990). The tumours produced in mice by implantation of sodium saccharin-containing cholesterol pellets appear to be formed after the rapid leaching of the chemical from the pellet; the coarse pellet, no longer containing saccharin, acts as an abrasive, leading to significant urothelial necrosis, regeneration and ultimately bladder cancer (DeSesso, 1989). Oral administration of high doses of sodium saccharin to mice does not produce a proliferative effect in the urothelium (Fukushima *et al.*, 1983a; Frederick *et al.*, 1989; Arnold *et al.*, 1995).

Administration of sodium saccharin in a standard two-year bioassay in rats, beginning at six to eight weeks of age, has not produced bladder cancer (Arnold *et al.*, 1983; Ellwein & Cohen, 1990). However, increased incidence of bladder cancer has been observed when lifetime administration to rats is begun before conception (two-generation bioassay) (Schoenig *et al.*, 1985; Ellwein & Cohen, 1990), at the time of birth (Schoenig *et al.*, 1985) or before five weeks of age (Arnold *et al.*, 1980). The male rat is significantly more susceptible to the urothelial effects of sodium saccharin than the

female, with female rats showing no effect in most studies (Arnold *et al.*, 1980; Ellwein & Cohen, 1990). The dose-response curve for sodium saccharin carcinogenicity in a two-generation bioassay is extremely steep, with a no-effect level at 1% of the diet (Schoenig *et al.*, 1985).

In contrast to its lack of carcinogenicity even at high doses in a standard two-year bioassay, sodium saccharin administered to mature rats at high doses after a brief treatment with a known bladder carcinogen produces high incidences of bladder tumours. Agents which have been used to pre-treat rats before sodium saccharin administration include intravesical *N*-methyl-*N*-nitrosourea (MNU) (Hicks *et al.*, 1973), *N*-[4-(5-nitro-2-furyl)-2-thiazolyl]formamide (FANFT) (Cohen *et al.*, 1979), *N*-butyl-*N*-(4-hydroxybutyl)nitrosamine (BBN) in the drinking-water (Nakanishi *et al.*, 1980a), cyclophosphamide (CP) by intraperitoneal injection (Cohen *et al.*, 1982) and freeze ulceration (Cohen *et al.*, 1982). Similarly to two-stage carcinogenesis in mouse skin and rat liver, treatment with sodium saccharin may be started after administration of the bladder carcinogen or it may be delayed; the longest interval between the two treatments has been 18 weeks (Hasegawa *et al.*, 1985). As in the two-generation bioassay, the dose-response curve is steep, with a no-effect level of 1% (Nakanishi *et al.*, 1980b). Tests of sodium saccharin in mice using the two-stage model of carcinogenesis and 2-acetylaminofluorene (2-AAF) as the initial carcinogen treatment gave negative results (Frederick *et al.*, 1989). The dietary levels used in mouse experiments were similar to those used in rat studies, resulting in higher doses on a mg/kg bw basis.

Strains of rats have different susceptibilities to the urothelial effects of sodium saccharin and other sodium salts, with Wistar rats being less susceptible than Fischer 344 or Sprague-Dawley rats

(Fukushima *et al.*, 1983a; Mori *et al.*, 1987; Murai *et al.*, 1997), NBR rats being only slightly susceptible (Garland *et al.*, 1994a; Uwagawa *et al.*, 1994) and ACI rats having particularly high sensitivity (Mori *et al.*, 1987). However, it is likely that the ACI strain is not optimal for evaluation of chemicals as bladder carcinogens since there is considerable hyperplasia in the ACI rat bladder even in control animals. It is unclear whether this spontaneous proliferation is due to parasitic contamination, urinary calculus formation or some other unidentified mechanism.

Although other forms of saccharin have not been investigated as extensively as sodium saccharin, it is clear that the acid form of the chemical (acid saccharin) is without effect on the rat urothelium (Hasegawa & Cohen, 1986; West *et al.*, 1986; Cohen *et al.*, 1991a).

### Carcinogenicity of other sodium salts

Following the report by Fukushima *et al.* (1983b) demonstrating that sodium ascorbate also increased bladder tumour incidence in male rats when administered after BBN, the issue of saccharin carcinogenicity was extended to a broader class of chemicals, the sodium salts of organic acids. As in the case of sodium saccharin, the male rat is more susceptible to the effects of sodium ascorbate than the female, the mouse is without urothelial effects (Tamano *et al.*, 1993), the doses required are similar to those for sodium saccharin (Cohen *et al.*, 1995a) and the parent acid, ascorbic acid, is without effect on the urothelium (Fukushima *et al.*, 1983b; Shioya *et al.*, 1994). In the standard two-year bioassay, sodium ascorbate does not produce an increased incidence of bladder cancer; however, as with sodium saccharin, administration of high doses of sodium ascorbate to male rats following a two-generation protocol produces an increased incidence of bladder tumours (Cohen *et al.*, unpublished observations).

Subsequently, several sodium salts of other organic acids (Table 1) have been identified as having an effect on the bladder urothelium of male rats, either increasing urothelial proliferation following administration at high doses or increasing the incidence of bladder tumours when administered after pretreatment with a bladder carcinogen such as FANFT or BBN (Fukushima *et al.*, 1984, 1986; DeGroot *et al.*, 1988; Ellwein & Cohen, 1990; Cohen *et al.*, 1991a; Shibata *et al.*, 1991; Otoshi

**Table 1. Sodium salts producing proliferative effects in the rat bladder when administered at high doses in the diet**

Saccharin	Citrate	Erythorbate
Ascorbate	Succinate	Phosphate
Glutamate	Bicarbonate	
Aspartate	Chloride	

*et al.*, 1993; Lina *et al.*, 1994; Kitamura *et al.*, 1996). Monosodium glutamate has been studied at high doses in the diet, producing an increased incidence of urothelial proliferation (DeGroot *et al.*, 1988), but not bladder cancer in a two-year bioassay (Owen *et al.*, 1978). Sodium and potassium bicarbonates and carbonates produced increased urothelial proliferation in short-term bioassays (Fukushima *et al.*, 1991; Cohen *et al.*, 1995b), with increased incidences of bladder tumours if administered for more than two and a half years rather than the standard two years (Lina *et al.*, 1994). Glutamic acid, in contrast to the corresponding acids of other sodium salts, has shown some evidence of causing increased proliferation of the urothelium (DeGroot *et al.*, 1988).

### Hypotheses concerning mechanisms of action

Any hypothesis that is offered to explain the effects of administering saccharin and other sodium salts on the bladder must be consistent with the findings in a number of animal models (see Table 2).

During the past three decades, several mechanisms have been suggested for the carcinogenicity of sodium salts in rats (Table 3), beginning with the classical approach of possible interaction of

**Table 2. Key observations regarding the carcinogenicity of sodium salts**

Do not react with DNA
Increase bladder cancer in rats, but not in mice or monkeys
Positive in two-generation bioassay but not standard two-year bioassay
Male rats are more susceptible than female rats
High doses are required
Sodium salt positive, parent acid negative

**Table 3. Suggested mechanisms by which sodium salts produce bladder urothelial proliferative effects in rats**

Reaction with DNA
Formation of urinary calculi
Carcinogenic contaminant
Alteration of urinary physiology

saccharin or a possible metabolite with DNA (Arnold & Boyes, 1989). Sodium saccharin, like the other sodium salts, is a salt of a moderately strong acid with a  $pK_a$  of approximately 2.0 (Williamson *et al.*, 1987). Thus, under physiological conditions, essentially all of the administered chemical is ionized and present as a strong anion, not a cation as required for reactivity with DNA. No DNA binding was detected following administration of high doses of sodium saccharin to rats (Lutz & Schlatter, 1977), as expected given the chemistry of the molecule. Furthermore, in rats, as in humans, there is no evidence that the saccharin molecule is metabolized (Sweatman *et al.*, 1981).

In general, assays involving point mutations, such as the Ames assay, have given negative results, even with extremely high doses of sodium saccharin (Arnold & Boyes, 1989). Assays involving evaluation of chromosomal aberrations have shown some positive results, but only at extremely high concentrations. At these concentrations, it is possible that cytotoxicity is involved or, more likely, an osmotic effect (Ashby & Ishidate, 1986), as positive results are also produced with equimolar concentrations of sodium chloride or potassium chloride. A direct evaluation of genotoxicity by sodium saccharin administration in the target tissue, the rat urothelium, has been made using a combination in-vivo-in-vitro assay for unscheduled DNA synthesis (Zukowski *et al.*, 1994). This assay is positive for aromatic amines, which are known bladder carcinogens in humans and in rats, but negative for non-genotoxic compounds such as sodium saccharin.

In summary, the strongly nucleophilic nature of the saccharin anion and the anions of other sodium salts producing similar urothelial effects in rats, the lack of reactivity with DNA of the anion or any potential metabolite and the lack of DNA

binding or DNA damage in the target tissue provide considerable evidence against a genotoxic mechanism for the carcinogenicity of sodium salts such as sodium saccharin or sodium ascorbate.

A second possibility that has been suggested is that the carcinogenicity of sodium saccharin and related salts is due to contamination by a highly carcinogenic contaminant (Ball *et al.*, 1978; Arnold *et al.*, 1980). For sodium saccharin, this has been extensively investigated: a two-generation bioassay evaluation of *ortho*-toluene sulfonamide, the major contaminant of sodium saccharin preparations synthesized by the Remsen-Fahlberg procedure, gave negative results (Arnold *et al.*, 1980). The likelihood of this or other contaminants being responsible for the carcinogenic effects of saccharin or other sodium salts is minimal, not only on the basis of these direct bioassays, but also because the types of contaminant are different when saccharin is synthesized by the Maumee procedure (which does not generate *ortho*-toluene sulfonamide) (Cohen *et al.*, 1979), and the contaminants in sodium ascorbate and other sodium salts are totally unlike those in sodium saccharin.

A third possibility is that the bladder cancer is produced as a result of the formation of urinary tract calculi. Formation of calculi is a well established mechanism by which certain chemicals produce bladder cancer in rodents, especially in rats (Clayson *et al.*, 1995). Administration of extremely high doses of ascorbic acid to humans has been suggested to produce urinary tract calculi composed of calcium oxalate in a relatively small number of individuals, although the evidence is controversial (Wandzilak *et al.*, 1994). Administration of acid saccharin to rats at very high doses has occasionally produced urinary tract calculi composed of saccharin (Cohen *et al.*, unpublished observations). However, the administration to rodents of sodium ascorbate, sodium saccharin or other sodium salts has not been associated with the development of urinary tract calculi. In the bioassays in which sodium saccharin produced bladder cancer in rats, the incidences of calculi in the test groups were similar to those in controls (Arnold *et al.*, 1980; Schoenig *et al.*, 1985; Ellwein & Cohen, 1990).

A major issue regarding the carcinogenicity of sodium salts is whether the anion itself is involved in the carcinogenic process in the target tissue, the

urothelium, or whether the administration of high doses of these sodium salts greatly alters urinary physiology and composition leading to a secondary process by which bladder cancer is produced (Hasegawa & Cohen, 1986; Williamson *et al.*, 1987; Fisher *et al.*, 1989). As is discussed further below, the evidence strongly suggests that after feeding high doses of the salts, tumours are produced indirectly by alteration of the urinary composition rather than by the anion itself. Evidence for this includes the observation that even administration of enormous doses of sodium ascorbate to rats leads to only a slight increase in urinary ascorbate concentrations (total ascorbate, including dehydroascorbate) (Fukushima *et al.*, 1983b; Shioya *et al.*, 1994), in contrast to the situation in humans. Rats are able to synthesize ascorbic acid, unlike humans for whom it is a dietary essential. The metabolism and excretion of ascorbate in rats also differ quantitatively from those in humans.

In addition, the proliferative and tumorigenic effects of these salts have little correlation with the urinary concentration of the anion (Renwick, 1993). For example, administration of acid saccharin or sodium saccharin (or ascorbic acid and sodium ascorbate) produces similar levels of the anion in the urine and yet the sodium salt produces a proliferative effect, whereas the acid does not (Fukushima *et al.*, 1983b; Hasegawa & Cohen 1986; Shioya *et al.*, 1994).

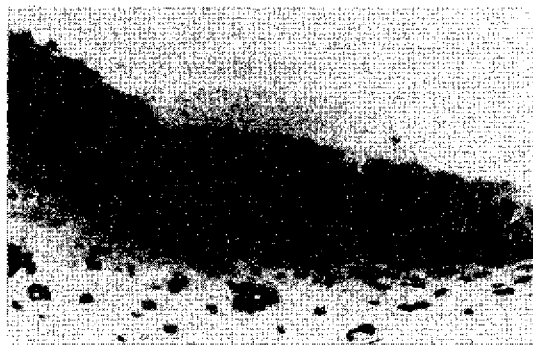
In summary, the evidence suggests that sodium salts produce bladder cancer by an indirect mechanism by altering the composition of the urine as a physiological response to administration of high

levels of a sodium salt. The chemicals do not produce cancer by direct reactivity with DNA or by direct interaction of the chemical or a metabolite with the urothelium.

### Urinary precipitate, cytotoxicity and consequent regeneration

Although administration of high concentrations of sodium salts does not appear to cause genotoxic effects in the bladder epithelium, it does produce an increase in proliferation (Fukushima & Cohen, 1980; Cohen *et al.*, 1990). No statistically significant increase is observed at 1% of the diet for sodium saccharin or sodium ascorbate, the no-effect level for carcinogenicity (Cohen *et al.*, 1995a,c). The extent of proliferation is relatively slight, with an increase in number of cells (hyperplasia) approximately 5–10 times greater than controls. This is evident by light microscopy as simple hyperplasia (Figure 1) which is predominantly present in the dome of the bladder initially, but then becomes more diffuse. Occasionally, nodular and papillary hyperplasia is also present. The rate of cell proliferation is also increased 2–10 times, as detected by labelling index following a one-hour pulse of tritiated thymidine or bromodeoxyuridine. The hyperplastic effect is detectable approximately four weeks after administration of these compounds, but the labelling index is already increased one week after administration has begun (Fukushima & Cohen, 1980).

Increased proliferation was present during the neonatal period in a two-generation type of bioassay, as well as later in life (Cohen *et al.*,



**Figure 1.** (Left) Normal bladder urothelium (arrow) of a control-fed male rat. x 400.

(Right) Simple hyperplasia of the bladder urothelium in a male rat fed 7.5% sodium saccharin in the diet for 10 weeks beginning at 5 weeks of age. x 400.



1995a,c). It is also present if administration begins at 6–8 weeks of age, as in a standard two-year bioassay. This is in apparent contradiction to the need for neonatal exposure for tumour production, since tumours are not produced if administration of the sodium salt begins at 6–8 weeks of age and continues for two years. However, this is a quantitative rather than a qualitative issue (Ellwein & Cohen, 1988). Based on the number of cell divisions that occur, a detectable statistically significant increase in bladder tumour incidence in a standard two-year bioassay with 50 animals per group would not be expected. Instead, the increase in tumour incidence is estimated to be only about 1%.

The requirement that treatment begins during the neonatal period for tumorigenicity to be observed is likely to be due to the difference in proliferation of the normal bladder during this time period compared to the adult bladder epithelium (Cohen *et al.*, 1988). In the adult (4–6 weeks of age) rodent bladder epithelium, as in primates, the labelling index is generally less than 0.1% following a one-hour pulse of the label. In contrast, the bladder epithelium during gestation is a rapidly proliferating tissue with a labelling index of approximately 10%. Nearly every bladder epithelial cell is dividing every day in the prenatal bladder. This rapidly decreases immediately after birth. By seven days of age, the labelling index has decreased to approximately 1–1.5%, and it further decreases to less than 0.1% by approximately four weeks of age. Simple calculations suggest that approximately one-third of the total cell divisions in the normal urothelium of the bladder have occurred by four weeks of age (Ellwein & Cohen, 1988). Thus, administration of high doses of sodium saccharin or sodium ascorbate during this period leads to an increase in the proliferative rate of an already rapidly proliferating urothelium. In addition, since this occurs early in the life span of the animal, there is maximal time for continued expansion during the remainder of the animal's lifetime.

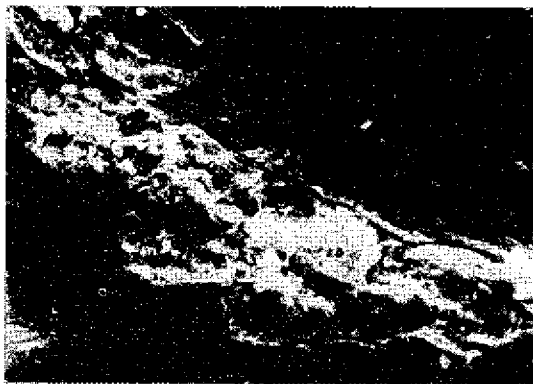
The effect of further increasing the already rapid proliferation during the neonatal period can be replicated by causing a burst of proliferation by simply ulcerating the bladder (Cohen *et al.*, 1982; Murasaki & Cohen, 1983; Hasegawa *et al.*, 1985). If no further treatment is given, the bladder

epithelium returns to normal within 4–6 weeks of ulceration and, after two years, no tumours are produced. However, if sodium saccharin is administered after ulceration, bladder tumours are produced. The number of cell replications occurring following ulceration has been estimated, and it is similar to that achieved by administration of the sodium salt during the neonatal period. Ulceration produced by application of a frozen rod or by administration of cyclophosphamide gives similar quantitative effects on cell replication and tumorigenesis (Cohen *et al.*, 1982).

Extensive cell kinetic analyses and modelling procedures have indicated that increased cell replication can readily explain the quantitative aspects of tumorigenesis with sodium salts, including the requirement for neonatal period treatment in contrast to a standard two-year bioassay (Ellwein & Cohen, 1988).

Increased cell replication can be produced either by direct mitogenesis, which usually involves effects on hormones and/or growth factors, or by toxic effects with consequent regeneration (Cohen & Ellwein, 1990, 1991; Cohen, 1995a). Light microscopic observations of the bladders of animals fed high doses of sodium salts revealed little evidence of toxicity. However, examination by scanning electron microscopy showed necrosis of the superficial cell layer (Figure 2), beginning in the dome of the bladder and then spreading more diffusely (Cohen *et al.*, 1990). However, complete erosion of the bladder epithelium does not occur nor is there ulceration, so there is no accompanying inflammatory response. Nevertheless, to replace the cells which are destroyed and exfoliated, there is regenerative hyperplasia, which, as indicated above, is quite mild, reflecting the mild nature of the toxicity. The extent of hyperplasia and the increase in labelling index are greater in male rats than in female rats (Garland *et al.*, 1994b). The toxicity and regeneration occur only at high doses, with a no-effect level for sodium saccharin and sodium ascorbate of 1% of the diet (Cohen *et al.*, 1990, 1995a,c).

Although calculi are not formed following administration of high doses of sodium salts, recent evidence suggests that a urinary amorphous-appearing precipitate (Figure 3) develops (Arnold *et al.*, 1980; West & Jackson, 1981; Cohen *et al.*, 1991b, 1995b). This is a high-dose



**Figure 2.** Necrosis of the superficial cell layer of the urothelium of a male rat fed 7.5% sodium saccharin in the diet for 10 weeks beginning at 5 weeks of age. x 460.

phenomenon only, with a no-effect level of 1% of the diet in rats fed sodium saccharin or sodium ascorbate. No precipitate forms when the corresponding acids are fed. The prerequisite changes in the urine for precipitate formation include overall high ionic density of the urine (high osmolality), high protein concentration,  $\text{pH} \geq 6.5$  and adequate levels of calcium and phosphate for precipitation of calcium phosphate. Although urinary osmolality and protein concentration in rats fed high levels of sodium salts are decreased compared to controls, they are still significantly higher than those in humans.

The major component of this precipitate is calcium phosphate, as determined by X-ray reflective spectroscopy and micro-analytical chemical analyses. The phosphate appears to be present as the mono- and di-basic forms. Little if any magnesium is present in the precipitate. Magnesium ammonium phosphate crystals are normal constituents of urine. The small amount of magnesium that appears in the precipitate is probably due to contamination by these crystals. In addition, some form of silicon-containing material is also present in the precipitate, along with a relatively small amount of protein, including  $\alpha_{2u}$ -globulin (Cohen *et al.*, 1991b). Relatively large amounts of mucopolysaccharides are present in the precipitate, and the most common mucopolysaccharide appears to be heparan sulfate.

The precipitate in rats administered sodium saccharin contains small amounts of saccharin, as

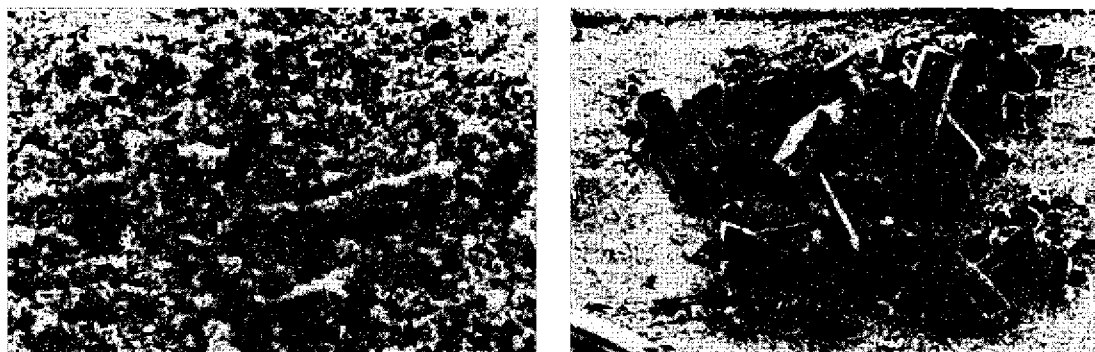
detected by chemical analysis and by infrared spectroscopy. However, there is little ascorbate present in the precipitate in rats fed high doses of sodium ascorbate. This difference between the two sodium salts may be related to the extremely high concentrations of saccharin in the urine following administration of any salt form of saccharin (200 mM after administration of 5% sodium saccharin), in contrast to the relatively low levels of ascorbate (3–4 mM) following administration of 7% sodium ascorbate in the diet (Fukushima *et al.*, 1983b; Hasegawa & Cohen, 1986; Shioya *et al.*, 1994).

*In vitro*, calcium phosphate is an essential ingredient of tissue culture medium for epithelial and other cells, and is usually present at a concentration of approximately 1 mM or less (Cohen *et al.*, 1995d). At these concentrations, the calcium phosphate is entirely soluble. If the concentration is increased to levels at which precipitation occurs (approximately 5 mM), there is cytotoxicity to bladder epithelial cells. Thus, it is likely that the calcium phosphate-containing precipitate in the urine of rats fed high concentrations of the sodium salts is cytotoxic to the urothelium, leading to erosion of the superficial cell layer of the bladder epithelium and consequent regenerative hyperplasia. The overall mechanism for the bladder carcinogenicity of sodium salts is summarized in Figure 4.

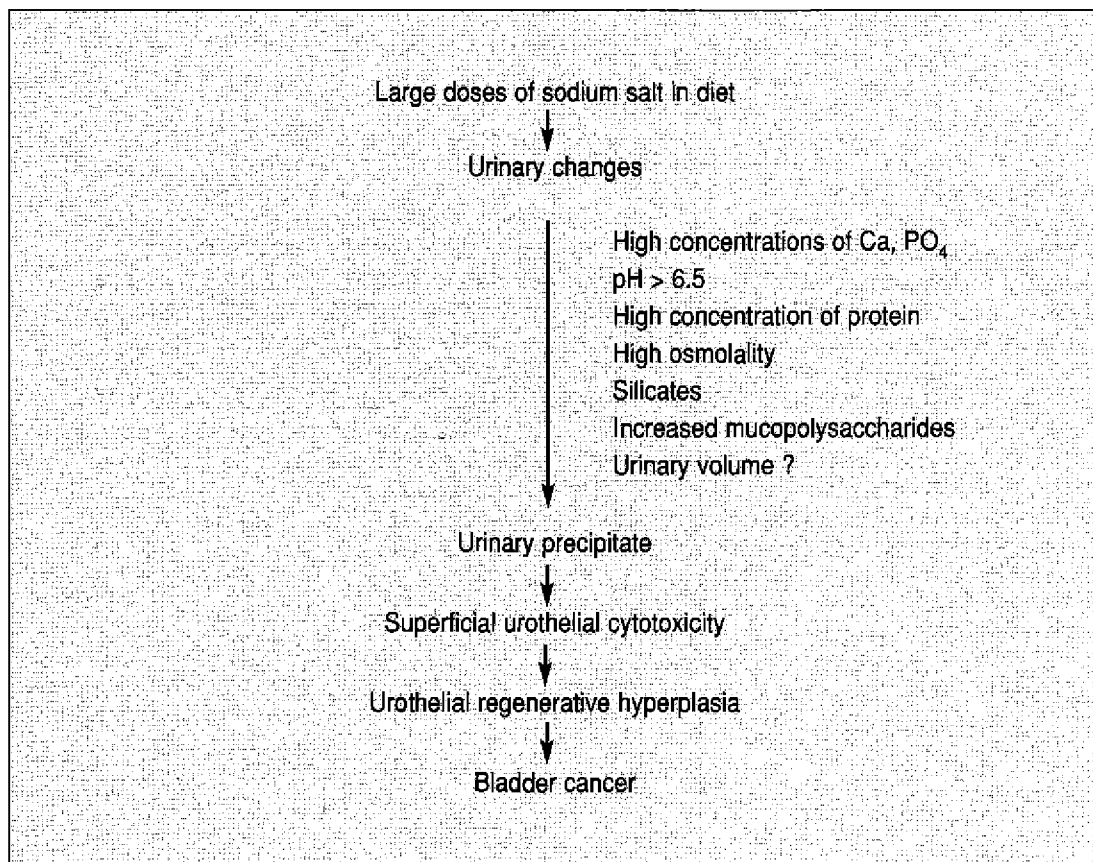
### Influence of urinary protein

The presence of high concentrations of protein in the urine appears to be a prerequisite for the formation of the urinary precipitate and for the urothelial proliferation effects in rats fed high doses of sodium salts. The exact mechanism by which protein contributes to this process is not clear, but may be related to its functioning as a nucleus for initiating formation of the precipitate rather than actually participating in the growth of the precipitate once it has begun. Protein is present in the precipitate, but generally accounts for less than 5% of the total weight of the precipitate (Cohen *et al.*, 1991b).

Rats have extraordinarily high levels of protein in the urine, at mg/mL levels rather than the usual  $\mu\text{g/mL}$  levels occurring in humans (Olson *et al.*, 1990; Hard, 1995). In female rats, the high levels of urinary protein largely consist of albumin. The male rat has even higher concentrations of urinary



**Figure 3.** (Left) A few  $\text{MgNH}_4\text{PO}_4$  crystals with calcium phosphate-containing precipitate from the urine of a male rat fed 7.5% sodium saccharin in the diet for 4 weeks beginning at 5 weeks of age. x 300.  
(Right)  $\text{MgNH}_4\text{PO}_4$  crystals in control urine with their typical shape. x 460



**Figure 4.** Suggested mechanism for the carcinogenicity of sodium salts for rat urinary bladder epithelium. The parameters in the urine must all be at critical levels for the cytotoxic precipitate to form.

protein because of the presence of  $\alpha_{2u}$ -globulin, from the age of approximately six weeks (Hard, 1995).

The anions of the carcinogenic sodium salts have been shown to associate closely with urinary proteins by co-elution in chromatographic systems (Cohen *et al.*, 1991b, 1995b). However, the anions do not appear to be covalently bound, and the association does not appear to be of the same type as occurs with chemicals known to produce kidney tumours in male rats due to binding to  $\alpha_{2u}$ -globulin (Lehman-McKeeman, unpublished observations). The anions associate with albumin, but also to a quantitatively greater extent with  $\alpha_{2u}$ -globulin. It is possible that this close association of these anions with the protein contributes to the formation of the precipitate.

The essential role of urinary protein has been demonstrated by administration of saccharin to strains of rats in which levels of urinary protein, either  $\alpha_{2u}$ -globulin or albumin, are lower. The NCI-Black-Reiter (NBR) strain of male rats does not excrete the large quantities of  $\alpha_{2u}$ -globulin that are seen in other strains of rats, such as Fischer 344 or Sprague-Dawley rats. Administration of high doses of sodium saccharin or sodium ascorbate to NBR male rats produces a significantly lower toxic response than is seen in Fischer 344 rats (Garland *et al.*, 1994a; Uwagawa *et al.*, 1994). Under these circumstances, changes in urinary pH and in levels of urinary components other than proteins in this strain of rat are comparable to those in Fischer 344 rats and the concentration of saccharin excreted by NBR male rats is similar to that of the Fischer 344 rat. There is only slight cytotoxicity or hyperplasia in male NBR rats. The response is similar to that seen after administration of the sodium salts to female Fischer 344 rats.

Similarly, administration of sodium saccharin or sodium ascorbate to albuminaemic rats produces no effect on the urothelium in contrast to administration to normal, congenic strains of rats (Homma *et al.*, 1991).

Thus, although  $\alpha_{2u}$ -globulin appears to contribute to the process quantitatively more than albumin or other urinary proteins (Cohen *et al.*, 1991b), it is not an effect unique to  $\alpha_{2u}$ -globulin, and urothelial toxicity and proliferation can occur, albeit only slightly, in the absence of  $\alpha_{2u}$ -globulin, as seen in female rats or in male NBR rats.

The involvement of high levels of urinary protein, such as albumin, in addition to  $\alpha_{2u}$ -globulin, helps to explain why the proliferative effect of sodium saccharin and sodium ascorbate can be seen in the neonatal male rat before excretion of high concentrations of  $\alpha_{2u}$ -globulin begins, usually at approximately six weeks of age. Essentially, during the first six weeks of life, the male and female rats are similar in their response to these sodium salts (Cohen *et al.*, 1995a,c). Once  $\alpha_{2u}$ -globulin production and excretion begins in male rats, the response becomes significantly greater in males than in females. The effect in female rats is dependent on the high concentrations of albumin rather than the even higher protein concentrations due to  $\alpha_{2u}$ -globulin in the adult male.

A difficulty arises, however, in explaining the lack of urothelial effects of sodium salts in mice, despite urinary protein concentrations similar to those in rats. Mice also have high urinary concentrations of albumin, and males excrete a mouse urinary protein (MUP) that is qualitatively and quantitatively analogous to  $\alpha_{2u}$ -globulin (Olson *et al.*, 1990; Hard, 1995). In chromatographic systems, the association of saccharin with mouse urinary proteins, including MUP, is similar to that of rat urinary proteins. In addition, the urinary concentrations of the administered anion (such as saccharin) in the mouse are similar to or greater than those in the rat.

Although urinary protein is an essential component of the urothelial effect of these sodium salts, it is only one component. Since formation of any precipitate is dependent on the solubility product of the constituent components, a major factor in determining the formation of the precipitate in mouse urine versus the rat is the concentrations of calcium and phosphate. There is also some indication that magnesium concentration can influence the formation of calcium phosphate precipitate, possibly by interaction with other urinary components. Urinary concentrations of calcium are approximately 10–20 times lower in the mouse than in the rat and the concentrations of phosphate and magnesium are approximately two to four times lower (Arnold *et al.*, 1995). Given the multiplicative nature of a solubility product, it is clear that these significant differences in concentrations of calcium and phosphate quantitatively contribute to the lack of precipitate formation and

urothelial proliferative effect in the mouse, in contrast to the rat.

### Effect of urinary pH

For all of the salts, listed in Table 1, for which the corresponding acid has been tested, the latter form is without effect on the urothelium of the male rat, the most sensitive sex-species tested (West *et al.*, 1986; Ellwein & Cohen, 1990; Cohen *et al.*, 1991a; Shioya *et al.*, 1994). With most diets used in rodent bioassays, the urinary pH in the controls ranges from 6.5 to 7.5. Administration of sodium saccharin has little effect on the urinary pH, whereas administration of some of the other sodium salts, such as sodium ascorbate and sodium citrate, leads to a higher urinary pH than the controls (Cohen *et al.*, 1995b). Administration of the saccharin acid leads to a significant decrease in the urinary pH, usually to levels less than 6.0.

It is essential in determining the effect of urinary pH that freshly voided specimens be analysed and that the effect be determined during the night or early in the morning, within 2–3 h after the lights have been turned on in the animal room (Fisher *et al.*, 1989; Cohen, 1995b). Rodents are nocturnal animals, eating and drinking at night and sleeping during the day. During the hours of consumption and shortly thereafter, there is a rise in the urinary pH, which then rapidly decreases once the rats stop eating, becoming quite acidic (pH 5.0–6.0) during the lighted hours.

Formation of the calcium phosphate-containing precipitate in rat urine does not generally occur if the urinary pH is below 6.5. Thus, when the parent acid is administered, producing a urinary pH of 6.0 or less, the precipitate cannot form and, consequently, there is no cytotoxicity, regenerative hyperplasia or tumour formation. This can be demonstrated further by administration of high concentrations of the sodium salt with comparatively high levels of ammonium chloride (Garland *et al.*, 1989; Cohen *et al.*, 1991a; Shioya *et al.*, 1994; Cohen *et al.*, 1995a,b). The resulting marked acidification of the urine completely inhibits the formation of the calcium phosphate-containing precipitate, as well as completely inhibiting the cytotoxic, proliferative and tumorigenic effects of the sodium salt. Similarly, administration of the sodium salt in AIN-76A semi-synthetic diet generally results in a urinary pH of less than 6.0

(Fisher *et al.*, 1989; Garland *et al.*, 1989; Okamura *et al.*, 1991). It appears that the casein, the source of protein in this commonly used semi-synthetic diet, produces a markedly acidic urine, even in controls (pH < 6.0). If albumin is substituted for casein in the diet, the urinary pH attains the levels usually seen in control animals on other diets, such as Purina, Prolab, Oriental M or Clea, and a proliferative response is seen with the sodium salts. It is not the effect of the sodium salts on urinary pH in comparison to controls that is important but rather the specific level of pH that occurs in the urine of the animals administered the sodium salt. Thus, in some experiments, sodium saccharin administration has produced urinary pH slightly lower, rather than slightly higher, than in the controls but the pH is still above 6.5, and a urothelial proliferative response still occurs.

Sodium hippurate is chemically and toxicokinetically similar to the other sodium salts listed in Table 1. However, when it was administered to male rats at high levels in the diet either alone or after exposure to BBN, there was no proliferative response in the bladder (Fukushima *et al.*, 1983c; Schoenig *et al.*, 1985). The doses of sodium hippurate used were comparable to those producing toxic effects with the other sodium salts listed in Table 1, but administration of sodium hippurate at these doses gave a urinary pH less than 6.3. Thus, given the necessity for a urinary pH of at least 6.5, no precipitate can form, and consequently no cytotoxicity, regenerative hyperplasia or tumour formation occurs. Some investigators have claimed that a rise in urinary pH by itself can lead to urothelial proliferation in the rat and ultimately the development of tumours. However, this appears not to be the case, since administration of high concentrations of calcium carbonate in the diet produces a urinary pH of approximately 8.0, and yet there is no proliferative or tumorigenic effect in male rats (Cohen *et al.*, 1991a). Similarly, a rodent diet commonly used in Europe, Altromin 1321, routinely produces a urinary pH of 8.0 or higher, but has no effect on the urothelium of the control-fed rats (Cohen *et al.*, 1994).

### Effect of urinary volume and other factors

A consequence of administration of high levels of sodium salts in the diet is an increase in water excretion accompanied by an increase in water

ingestion (Fisher *et al.*, 1989; Cohen *et al.*, 1995b). This leads to an overall dilution of urinary components, with decreases in osmolality and creatinine levels. Occasionally, however, such as following administration of sodium saccharin, urinary calcium, phosphate and magnesium concentrations remain similar to those in the controls or even increase slightly despite the dilutional effect caused by increased water excretion (Schoenig & Anderson, 1985). A consequence of the increase in urinary excretion, which is usually in the range of two- to three-fold, is that the bladder becomes distended. It remains unclear whether this contributes to the carcinogenicity of these sodium salts or whether it is a coincidental event (Anderson, 1988). Clearly, urinary volume and urinary bladder distension alone are inadequate to explain the effects, since administration of diuretics, such as furosemide (US National Toxicology Program, 1989), produces even greater urinary volumes and distension than do the sodium salts, and yet there is no increase in urothelial proliferation or tumorigenicity.

It has also been suggested that increased sodium by itself leads to the proliferative effects seen with the sodium salts (Otoshi *et al.*, 1993; Shioya *et al.*, 1994). However, this fails to explain the differences between male and female rats, which excrete similar concentrations of sodium in the urine following administration of identical sodium salt levels in the diet (Schoenig & Anderson, 1985) and also fails to explain the differences between rats and mice, again since the urinary sodium concentrations are similar. Nevertheless, it does appear that an increase in sodium ingestion and excretion enhances the proliferative and tumorigenic effects of the sodium salts by unknown mechanisms.

Although the sodium salts have been studied most extensively, a few potassium salts have also been investigated, including potassium saccharin (Hasegawa & Cohen, 1986) and potassium bicarbonate (Fukushima *et al.*, 1991; Lina *et al.*, 1994). In general, the effect on the urothelium is less with the potassium salts than with the sodium salts, despite similar effects on urinary pH, volume and other components of the urine. Administration of high levels of calcium salts tends to produce only a slight proliferative effect on the urothelium (Hasegawa & Cohen, 1986; Cohen *et al.*, 1991a).

This is probably related to the urinary pH usually being 6.5 or lower when these calcium salts are administered. However, if they are co-administered with an agent that increases the urinary pH, there is a corresponding increase in proliferation and tumorigenicity.

As is apparent from the above discussion of the effects of urinary protein, pH, volume, osmolality, calcium, phosphate and possibly sodium, administration of these sodium salts at high levels in the diet produces a pleiotropic effect on the urine and it is the combination of effects that leads ultimately to the production of the calcium phosphate-containing precipitate and the bladder urothelial effects. It is essential that all of these components be present at critical levels for the effect to occur. It appears that mucopolysaccharides also contribute to the effect, as they are the major organic component of the precipitate. Preliminary investigations have shown that there is little mucopolysaccharide in normal rat urine, whereas the amount in the urine of rats ingesting high levels of sodium saccharin is considerable.

Other constituents of the diet that might influence the effect of the sodium salts on the urine include the level of silicates (Cohen *et al.*, 1991b). Administration of high levels of silicates is known to produce highly cytotoxic crystals in the urine of rats and domesticated animals (Emerick *et al.*, 1963). The contribution of these silicates present in the urinary precipitate to the urinary tract effects of sodium salts is unknown.

#### ***ortho*-Phenylphenol and its sodium salt**

Another apparent exception to the generalities above for sodium salts is *ortho*-phenylphenol (OPP). The parent acid, OPP, is a relatively weak acid with a  $pK_a$  of approximately 9. In contrast to acid saccharin, ascorbic acid or glutamic acid, it produces a significant proliferative response in the urothelium of male rats and ultimately produces bladder cancer (Hiraga & Fujii, 1984). This appears to be specific to the male rat, although there is a slight proliferative effect in female rats (Wahle *et al.*, 1997). The carcinogenic effect is seen only at high doses ( $\geq 8000$  ppm of the diet) and there is no effect on the urothelium in mice. Similar doses of the sodium salt of OPP produce a greater effect on the urothelium than does the acid (Fujii *et al.*, 1987). Although this combination of effects bears

some resemblance to those of the other sodium salts mentioned above, the carcinogenicity of the acid OPP itself contrasts with lack of urothelial effects seen with the corresponding acids of the other sodium salts described above.

Administration of OPP or its sodium salt at high levels in the diet produces cytotoxicity and regenerative hyperplasia of the rat urothelium but, in contrast to the salts listed in Table 1, no urinary solids (calculi, microcrystalluria or calcium phosphate-containing precipitate) are produced (Cohen *et al.*, 1997). The toxicity is similar quantitatively to that observed with the sodium salts discussed above, but must be due to the chemical itself or, more likely, its metabolites.

There have been suggestions that, although OPP is not genotoxic, its semiquinone or quinone metabolites might be genotoxic and form DNA adducts. However, *in vivo*, formation of DNA adducts following OPP administration has been observed only in examination of the whole rat bladder by <sup>32</sup>P-postlabelling techniques (Ushiyama *et al.*, 1992) and in the DNA of mouse skin following direct application of extraordinarily high levels of OPP to the skin (Pathak & Roy, 1992). We have investigated the rat urothelium, the target tissue of OPP, following administration of doses as high as 12 500 ppm, and found no evidence of OPP-DNA adducts by <sup>32</sup>P-postlabelling techniques (Cohen *et al.*, 1997).

### Extrapolation to humans

In extrapolating findings in rodents to humans, an evaluation is required of both the effect of dose and the effect of species extrapolation (Cohen & Ellwein, 1990, 1991, 1992; Cohen, 1995a). As described above, the toxicity of sodium salts is a high-dose phenomenon; formation of the urinary precipitate, cytotoxicity, increased proliferation and tumorigenicity are all dependent on dietary levels of sodium saccharin or sodium ascorbate greater than 1%. This is not too surprising given the fact that all of the sodium salts listed in Table 1 except saccharin are natural compounds which are normal components of the diet and/or are formed during intermediary metabolism. Some are essential ingredients of the human diet, such as ascorbate, phosphate and chloride. Based on mechanistic considerations, the effect of the sodium salts appears to be a rat-specific phenomenon, with the effect being greater in males than in

females. The lack of an effect in mice is due to the significantly lower urinary concentrations of calcium, phosphate and possibly magnesium. With respect to primates, both non-human and human, the major differences in the urine compared to rats are in urinary protein concentrations and osmolality (Takayama *et al.*, 1998). Urinary protein concentration is 100–1000 times higher in rodents than in healthy humans, although it can reach much higher concentrations in patients with the nephrotic syndrome, levels approaching those seen in rats. No protein qualitatively or quantitatively similar to  $\alpha_{2u}$ -globulin or MUP occurs in primates (Olson *et al.*, 1990; Hard, 1995). At the urinary protein concentrations seen in primate urine, including humans, there is no co-chromatographic association of these anions with the urinary proteins (Takayama *et al.*, 1998). In the urine of monkeys administered sodium saccharin at a dose of 25 mg/kg bw, we found no evidence of formation of the urinary precipitate (Takayama *et al.*, 1998). Preliminary investigations in humans have also failed to show the formation of this precipitate following administration of sodium saccharin.

In addition to differences in urinary protein concentration, the overall density of the urine in rodents is significantly higher than that in humans (Cohen, 1995b). The usual urinary osmolality in rodents is between 1400 and 2000 mosmol/kg and can reach higher levels. In contrast, humans generally have urinary osmolality of 50–500 mosmol/kg and, theoretically, it has been estimated that it cannot be higher than approximately 1200 mosmol/kg in humans.

Thus, on a purely mechanistic basis, it is expected that primates would not be responsive to the cytotoxic, proliferative or tumorigenic effects of the sodium salts, even at extremely high doses.

A recently completed study involving administration to monkeys of sodium saccharin in the diet at levels of 25 mg/kg bw for 18–23 years showed no cytotoxic or proliferative effect on the urothelium in males or females (Takayama *et al.*, 1998). Similarly, an epidemiological evaluation of the proliferative response in humans ingesting artificial sweeteners failed to show an effect on bladder epithelial proliferation (Auerbach & Garfinkel, 1989).

In addition, there have been many epidemiological evaluations of sodium saccharin in humans and these have failed to demonstrate any significant increased incidence of bladder cancer (Elcock & Morgan, 1993). Thus, based on the epidemiology of tumorigenic or proliferative effects, extensive investigations in other species and an understanding of the mechanisms involved, the urothelial carcinogenicity of sodium salts is a rat-specific high-dose phenomenon. Therefore, there appears to be no carcinogenic hazard associated with human consumption of these sodium salts, despite frequent and commonly high levels of ingestion, especially of those that are naturally occurring.

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# Appendix 1

## Agents that induce epithelial neoplasms of the urinary bladder, renal cortex and thyroid follicular lining in experimental animals and humans:

### Summary of data from IARC Monographs Volumes 1-69

J. D. Wilbourn, C. Partensky and J. M. Rice

#### Introduction

The *IARC Monographs on the Evaluation of Carcinogenic Risks to Humans* are critical summaries of the published scientific evidence for cancer in humans as a result of exposures to selected environmental agents and for carcinogenicity of those agents to animals in bioassays. Other data relevant to carcinogenic hazard identification are also critically reviewed and summarized. Agents are chosen for evaluation on the basis of two criteria: there must be evidence or suspicion of carcinogenicity in humans or in experimental animals, and there must be human exposure. A total of 836 chemicals and mixtures, biological and physical agents, and lifestyle and occupational exposures have been evaluated in volumes 1 to 69 of the *Monographs*. Each *Monograph* concludes with an evaluation, which is the consensus or majority opinion of invited scientific experts as to the strength of the total evidence for carcinogenicity to humans. Criteria for strength of the evidence for carcinogenicity in humans and in animals (*sufficient*, *limited*, *inadequate*, or *evidence suggesting lack of carcinogenicity*), and definitions of the groups (1, 2A, 2B, 3, and 4) that comprise the overall evaluations, are summarized in Tables 1 and 2, respectively. These criteria and definitions are published in the Preamble to the *Monographs* which is printed at the beginning of each volume in the series. The Preamble is revised from time to time, and in 1992 provision was made for inclusion of evidence relating to mechanisms of carcinogenicity in reaching final, overall

evaluations of carcinogenic risk to humans (IARC, 1992).

It must be stressed that the IARC criteria for *sufficient* evidence of carcinogenicity to animals place great emphasis on reproducibility. Evidence for carcinogenicity of any agent that is positive in only a single bioassay, no matter how well conducted, is rarely considered more than *limited* by IARC working groups. Those agents for which evidence of carcinogenicity is *sufficient* in humans or in animals or both, or for which there is *limited* evidence in humans and *sufficient* evidence in animals, and which are associated causally with epithelial tumours of urinary bladder, renal cortex, or thyroid follicular epithelium are listed in Tables 3, 4, and 5, respectively.

These tables were prepared by first utilizing an electronic data base to identify agents associated with tumours at one or more of the selected organ sites, and then cross-checking with the *Monographs* entries for each agent. Where human data are inadequate, only agents that met the criterion of *sufficient* evidence for carcinogenicity in animals were included in the tables. In effect, that restricts the agents included in Tables 3-5 to those in Groups 1, 2A, and 2B of the IARC classification system (Table 2). The resulting tables indicate how often tumours of a given organ site have contributed to IARC evaluations; whether there is or is not concordance between cancer sites in humans and animals for a given agent; and whether more than one organ site played a role in the evaluation of evidence of carcinogenicity. A number of agents

**Table 1. Criteria for carcinogenic hazard evaluation in the IARC Monographs on the Evaluation of Carcinogenic Risks to Humans**

**Degrees of evidence for carcinogenicity in humans**

*Sufficient:*

A positive relation has been observed between the exposure and human cancer in studies in which chance, bias and confounding can be ruled out with reasonable confidence.

*Limited:*

A positive relation has been observed for which a causal interpretation is credible, but chance, bias or confounding could not be ruled out with reasonable confidence.

*Inadequate:*

Available studies are of insufficient quality, consistency or statistical power to permit a conclusion regarding the presence or absence of a causal association; or no data on cancer in humans are available.

*Evidence suggesting lack of carcinogenicity:*

Several adequate studies covering the full range of human exposures exist that are mutually consistent in not showing a positive association between the agent and any cancer studied, at any level of exposure (inevitably limited to the cancer sites, conditions and levels of exposure and length of observation covered by the available studies).

**Degrees of evidence for carcinogenicity in experimental animals**

*Sufficient:*

A causal relationship has been established between the agent and an increased incidence of malignant neoplasms or of an appropriate combination of benign and malignant neoplasms in (a) two or more species of animals or (b) in two or more independent studies in one species carried out at different times or in different laboratories or under different protocols. Exceptionally, a single study in one species may provide sufficient evidence when malignant neoplasms occur to an unusual degree with regard to incidence, site, type of tumour or age at onset.

*Limited:*

Data suggest a carcinogenic effect but are limited for making a definitive evaluation because (a) evidence is restricted to a single experiment; (b) there are unresolved questions regarding the adequacy of the design, conduct, or interpretation of the study; or (c) the agent increases the incidence only of benign neoplasms or of lesions of uncertain neoplastic potential, or of certain neoplasms which may occur spontaneously in high incidences in certain strains.

*Inadequate:*

The studies cannot be interpreted as showing either presence or absence of a carcinogenic effect because of major qualitative or quantitative limitations; or no data in experimental animals are available.

*Evidence suggesting lack of carcinogenicity:*

Adequate studies involving at least two species are available which show that, within the limits of the tests used, the agent or mixture is not carcinogenic. Such a conclusion is inevitably limited to the species, tumour sites and levels of exposure studied.

**Table 2. Overall evaluations<sup>a</sup> of carcinogenicity in the IARC Monographs on the Evaluation of Carcinogenic Risk to Humans**

<b>Group 1</b> Carcinogenic to humans	<i>Sufficient evidence of cancer in humans</i>
<b>Group 2A</b> Probably carcinogenic to humans	<i>Limited evidence of cancer in humans; Sufficient evidence of carcinogenicity in experimental animals</i>
<b>Group 2B</b> Possibly carcinogenic to humans	<i>Inadequate evidence of cancer in humans; Sufficient evidence of carcinogenicity in experimental animals</i>
<b>Group 3</b> Not classifiable as to carcinogenicity to humans	<i>Inadequate evidence of cancer in humans; Less than sufficient evidence of carcinogenicity in experimental animals</i>
<p><b>Exceptionally, agents for which evidence of carcinogenicity is <i>inadequate</i> in humans but <i>sufficient</i> in experimental animals may be placed in this category when there is strong evidence that the mechanism of carcinogenicity in experimental animals does not operate in humans.</b></p>	
<b>Group 4</b> Probably not carcinogenic to humans	<i>Evidence in both humans and experimental animals suggesting lack of carcinogenicity</i>

<sup>a</sup> Overall evaluations of 2A, 2B, and 3 may be adjusted upward or downward on the basis of other relevant data including mechanisms of carcinogenicity. The definitions provided for Groups 1, 2A, 2B, 3 and 4 are the ones most generally used, but additional combinations of human, animal, and mechanistic evidence may also serve to place an agent in Groups 1, 2A, 2B, or 3; for details, see the Preamble to the *IARC Monographs*.

were carcinogenic at a chosen organ site in only one sex of a given species; this information is not captured in the tables. For ease of comparison among the tables, the same set of footnotes is used in all three, although some are not applicable throughout.

A number of chemicals that did not meet the criteria for listing in Tables 3-5 are nonetheless of considerable interest in the context of this volume. These are discussed individually in the text.

### **Epithelial Neoplasms of the Urinary Bladder**

Thirty-eight agents that are carcinogenic to the urinary bladder by systemic exposure in humans, animals, or both are summarized in Table 3. These

comprise 5% of all agents evaluated in *IARC Monographs* Volumes 1-69. Substances that are carcinogenic to the urinary bladder of rats or mice only when incorporated into pellets of inert material and implanted into the lumen of the bladder, such as some of the polynuclear aromatic hydrocarbons and their heterocyclic analogues, are not included in Table 3. For fifteen of these 38 agents, there is *limited* or *sufficient* evidence of carcinogenicity in humans at one or more organ sites including the urinary bladder. Six of the 38 agents are complex mixtures, and for 5 of these evidence for carcinogenicity to the urinary bladder is only from epidemiological studies of exposed humans: diesel engine exhaust, coal tars and coal tar pitches, untreated or mildly treated mineral

Table 3. Some agents (chemicals, mixtures and parasites) producing urinary bladder neoplasms (IARC Monographs, Volumes 1-69)

Agent	Vol., page	Suppl. 7, page	Evidence of carcinogenicity		Target organs						
			H	A	Group	Human	Mouse	Rat	Hamster	Dog	Other
ortho-Aminoazo-toluene	8, 61	56	I	S	2B		Liver <sup>k</sup> Lung Blood vessels/	Liver <sup>k</sup> Bile duct Urinary bladder <sup>o</sup>	Liver <sup>k</sup> Bile duct Urinary bladder <sup>p</sup> Mammary gland	Bile duct <sup>d</sup> Urinary bladder <sup>c</sup>	Rabbit: Urinary bladder <sup>o</sup>
4-Aminobiphenyl	7, 74	91	S	S	1	Urinary bladder <sup>c</sup>	Urinary bladder <sup>c,w</sup> Liver <sup>k</sup> Blood vessels/	Mammary gland intestine		Urinary bladder <sup>p</sup>	Rabbit: Urinary bladder <sup>c,o</sup>
Analgesic mixtures containing phenacetin	24, 135	310	S	L	1	Urinary bladder <sup>c</sup> Kidney/pelvis <sup>c</sup>		Liver <sup>k</sup> Kidney/pelvis <sup>k</sup> Kidney/cortex <sup>k</sup>			
ortho-Anisidine	27, 63	57	I	S	2B		Urinary bladder <sup>w</sup>	Urinary bladder <sup>w</sup> Kidney/pelvis <sup>w</sup> Thyroid <sup>h</sup>			
Arsenic and arsenic compounds	23, 39	100	S	L	1	Skin Liver <sup>p</sup> Lung Urinary bladder <sup>x</sup>	Lung		Lung		
Benzidine	29, 149	123	S	S	1	Urinary bladder <sup>c</sup>	Liver <sup>k</sup>	Liver <sup>k</sup> Bile duct <sup>d</sup> Mammary gland Zymbal gland Large intestine	Liver <sup>k</sup> Bile duct <sup>d</sup>	Urinary bladder <sup>c</sup>	
N,N-Bis(2-chloro-ethyl)-2-naphthyl-amine (Chlornaphazine)	4, 119	130	S	L	1	Urinary bladder <sup>o</sup>	Lung	Connective tissue <sup>u</sup>			

Table 3. Some agents (chemicals, mixtures and parasites) producing urinary bladder neoplasms (IARC Monographs, Volumes 1-69)

Agent	Vol., page	Suppl. 7, page	Evidence of carcinogenicity		Human	Target organs				
			H	A		Group	Mouse	Rat	Hamster	Dog
Bracken fern	40, 47	135	I	S	2B	Lung Small intestine Haematopoietic system <sup>m</sup>	Small intestine Urinary bladder <sup>a</sup> Mammary gland <sup>b</sup>			Guinea-pig: Small intestine Urinary bladder <sup>c</sup>
4-Chloro-ortho-phenylenediamine	27, 81	60	I	S	2B	Liver <sup>k</sup>	Urinary bladder <sup>w</sup>			
para-Chloro-ortho-toluidine	48, 123		L	S	2A	Spleen/ Connective tissue <sup>f</sup>				
Citrus Red No. 2	8, 101	60	I	S	2B	Urinary bladder <sup>a,g</sup> Lung Lymphoid system <sup>g</sup>	Urinary bladder <sup>a</sup>			
Coal-tar pitches	35, 83	174	S	S	1	Skin				
Coal tars	35, 83	175	S	S	1	Urinary bladder <sup>x</sup> Skin Lung Skin Lung Urinary bladder <sup>x</sup> Haematopoietic system <sup>m</sup>				Rabbit: Skin
para-Cresidine	27, 82	61	I	S	2B	Urinary bladder <sup>a,w</sup> Liver <sup>k</sup>	Urinary bladder <sup>a</sup> Liver <sup>k</sup> Bile duct <sup>g</sup>			
Cyclophosphamide	26, 165	182	S	S	1	Lung Mammary gland Lymphoid system <sup>n</sup>	Urinary bladder <sup>a,w</sup> Blood vessels Mammary gland Haematopoietic system <sup>m</sup>			



Table 3. Some agents (chemicals, mixtures and parasites) producing urinary bladder neoplasms (IARC Monographs, Volumes 1-69)

Agent	Vol., page	Suppl. 7, page	Evidence of carcinogenicity		Human	Target organs					Other
			H	A		Mouse	Rat	Hamster	Dog		
3,3'-Dichlorobenzidine	29, 239	193	I	S	2B	Liver <sup>z</sup> Haematopoietic system <sup>m</sup>	Mammary gland Zymbal gland Haematopoietic system <sup>m</sup>	Urinary bladder <sup>w</sup> Liver			
1,3-Dichloropropene (technical grade, 1% epichlorohydrin)	41, 113	195	I	S	2B	Urinary bladder <sup>w</sup> Lung Fore stomach	Liver <sup>z</sup> Fore stomach				
Diesel engine exhaust	48, 41		L	S	2A	Lung Skin	Lung				
3,3'-Dimethoxybenzidine (ortho-Dianisidine)	4, 41	198	I	S	2B		Urinary bladder <sup>a</sup> Zymbal gland Intestine Skin	Fore stomach			
para-Dimethylaminoazobenzene	8, 125	62	I	S	2B	Liver Connective tissue	Liver Skin	Urinary bladder <sup>a</sup>			
Direct Black 38 (technical grade)	29, 295	125	I	S	2A	Liver <sup>k</sup> Mammary gland	Liver <sup>k</sup> Urinary bladder <sup>c</sup> Large intestine				
Disperse Blue 1	48, 139		I	S	2B	Lung Liver <sup>k</sup>	Urinary bladder <sup>a,y</sup>				
2-(2-Formylhydrazino)-4-(5-nitro-2-furyl)thiazole	7, 151	63	I	S	2B	Fore stomach Lung Haematopoietic system <sup>m</sup>	Liver <sup>k</sup> Mammary gland Kidney/cortex <sup>b</sup> Kidney/pelvis <sup>w</sup> Intestine	Fore stomach <sup>a</sup> Urinary bladder <sup>w</sup>			

Table 3. Some agents (chemicals, mixtures and parasites) producing urinary bladder neoplasms (IARC Monographs, Volumes 1-69)

Agent	Vol., page	Suppl. 7, page	Evidence of carcinogenicity		Target organs						
			H	A	Group	Human	Mouse	Rat	Hamster	Dog	Other
4,4'-Methylene bis(2-chloroaniline (MOCA))	57, 271		1	S	2A		Liver <sup>k</sup> Blood vessels <sup>f</sup>	Liver <sup>k</sup> Lung Mammary gland Zymbal gland Blood vessels <sup>f</sup>		Urinary bladder <sup>w</sup>	
N-Methyl-N-nitroso-urea	17, 227	66	1	S	2A		Many sites	Many sites Urinary bladder <sup>o,w</sup>	Many sites		
Mineral oils (untreated or mildly treated)	33, 87	252	S	S	1	Skin GI tract Urinary bladder <sup>b</sup>	Skin				
2-Naphthylamine	4, 97	261	S	S	1	Urinary bladder <sup>b</sup>	Liver Lung	Urinary bladder <sup>c</sup>	Urinary bladder <sup>c</sup>	Urinary bladder <sup>o,p</sup>	Monkey Urinary bladder <sup>w</sup>
Niridazole	13, 123	67	1	S	2B		Fore-stomach Lung Mammary gland Ovary Urinary bladder <sup>o,w</sup>		Fore-stomach Urinary bladder <sup>d</sup>		
Nitriolacetic acid and its di- and trisodium salts	48, 181		1	S	2B		Kidney <sup>b</sup>	Urinary bladder <sup>x,w</sup> Kidney/cortex <sup>s</sup> Kidney/pelvis <sup>w</sup>			
2-Nitroanisole	65, 369		1	S	2B		Liver <sup>k</sup>	Haemato- poietic system <sup>m</sup> Urinary bladder <sup>w</sup> Kidney/pelvis <sup>w</sup> Large intestine <sup>c</sup>			

Table 3. Some agents (chemicals, mixtures and parasites) producing urinary bladder neoplasms (IARC Monographs, Volumes 1-69)

Agent	Vol., page	Suppl. 7, page	Evidence of carcinogenicity		Human	Target organs				
			H	A		Mouse	Rat	Hamster	Dog	Other
N-[4-(5-Nitro-2-furyl)-2-thiazolyl]-acetamide (NFTA)	7, 185	67	I	S	2B	Haemato-poietic system <sup>m</sup> Forestomach	Mammary gland Kidney/-pelvis <sup>w</sup>	Fore-stomach Urinary bladder <sup>w</sup>		
N-Nitrosod-n-butylamine	17, 51	67	I	S	2B	Forestomach Oesophagus Urinary bladder <sup>a,v</sup> Lung Liver	Liver Urinary bladder <sup>a,w</sup> Lung Oesophagus	Fore-stomach Lung/-trachea Urinary bladder <sup>a,c,w</sup> Nasal cavity		Guinea-pig Liver Bile ducts <sup>d</sup> Urinary bladder <sup>a,v</sup> Rabbit Urinary bladder <sup>a,w</sup>
Phenacetin	24, 135	310	L	S	2A	Kidney/-cortex <sup>s</sup>	Kidney/-cortex <sup>s</sup> Kidney/pelvis <sup>s</sup> Nasal cavity Urinary bladder <sup>w</sup>			
Ponceau 3R	8, 199	70	I	S	2B		Liver <sup>s</sup> Urinary bladder <sup>s</sup>			
Saccharin and sodium saccharin	22, 111	334	I	S	2B	Urinary bladder <sup>c</sup> Thyroid <sup>b</sup>	Urinary bladder <sup>s</sup>			
Schistosoma haematobium	61, 45		S	L	1	Urinary bladder <sup>a,v</sup>				Monkey Urinary bladder <sup>p</sup>
Sodium-ortho-phenylphenate	30, 330	382	I	S	2B	Liver	Urinary bladder <sup>p,w</sup> Kidney/pelvis <sup>w</sup>			

Table 3. Some agents (chemicals, mixtures and parasites) producing urinary bladder neoplasms (IARC Monographs, Volumes 1-69)

Agent	Vol., page	Suppl. 7, page	Evidence of carcinogenicity		Target organs						
			H	A	Group	Human	Mouse	Rat	Hamster	Dog	Other
Tobacco smoke	38, 37	359	S	S	1	Lung Urinary bladder <sup>a</sup> Kidney/ pelvis <sup>b</sup> Oral cavity Larynx Pancreas	Skin (condensate)	Lung	Larynx		
<sup>a</sup> Adenoma											
<sup>b</sup> Adenocarcinoma											
<sup>c</sup> Carcinoma											
<sup>d</sup> Cholangioma/cholangiocarcinoma											
<sup>e</sup> Fibroadenoma											
<sup>f</sup> Fibrosarcoma											
<sup>g</sup> Follicular-cell adenoma											

Table 4. Some agents (chemicals and mixtures) producing kidney (renal tubule-cell) neoplasms (IARC Monographs, Volumes 1-69)

Agent	Vol., page	Suppl. 7, page	Evidence of carcinogenicity		Group	Target organs					
			H	A		Human	Mouse	Rat	Hamster	Dog	Other
Aflatoxins Aflatoxin B <sub>1</sub>	56, 245		S	S	1	Liver <sup>k</sup>	Lung Liver <sup>k</sup>	Liver <sup>k</sup> Bile duct <sup>d</sup> Kidney <sup>s</sup>	Bile duct <sup>d</sup>		Monkey: Bile duct <sup>d</sup> Fish: Liver Fish: Liver
Aflatoxin G <sub>1</sub>				S			Liver Kidney <sup>b</sup>				
Analgesic mixtures containing phenacetin	24, 135	310	S	L	1	Urinary bladder <sup>c</sup> Kidney/pelvis <sup>c</sup>		Liver <sup>2</sup> Kidney/pelvis <sup>s</sup> Kidney/cortex <sup>x</sup>			
Benzofuran	63, 431		I	S	2B		Liver <sup>a</sup> Fore stomach <sup>a,c</sup> Lung	Kidney <sup>b</sup>			
Bromodichloro- methane	52, 179		I	S	2B		Liver <sup>k</sup> Kidney <sup>s</sup>	Liver <sup>2</sup> Kidney <sup>s</sup> Large intestine <sup>b</sup>			
Caffeic acid	56, 115		I	S	2B		Fore stomach <sup>a,c</sup> Kidney <sup>b</sup> Lung	Fore stomach <sup>a,c</sup> Kidney <sup>s</sup>			
Captafol	53, 353		I	S	2A		Heart <sup>i</sup> Small intestine <sup>b</sup>	Kidney <sup>s</sup> Liver <sup>2</sup>			
Chloroform	20, 401	152	I	S	2B		Liver <sup>k</sup> Kidney <sup>x</sup>	Liver <sup>2</sup> Kidney <sup>s</sup> Thyroid			
Cycasin	10, 121	61	I	S	2B		Liver Lung Lymphoid system <sup>n</sup>	Liver <sup>k</sup> Bile duct Kidney <sup>b</sup> Large intestine <sup>b</sup> Nervous system	Bile duct Liver Intestine		Guinea-pig Liver <sup>k</sup> Bile duct
Daunomycin	10, 145	61	I	S	2B		Connective tissue <sup>e</sup>	Mammary gland <sup>b</sup> Kidney <sup>s</sup>			
para-Dichlorobenzene	29, 215	192	I	S	2B		Liver <sup>k</sup>	Kidney <sup>b</sup>			

**Table 4. (Contd) Some agents (chemicals and mixtures) producing kidney (renal tubule-cell) neoplasms**  
(IARC Monographs, Volumes 1-69)

Agent	Vol., page	Suppl. 7, page	Evidence of carcinogenicity		Human	Target organs				Rat	Hamster	Dog	Other
			H	A		Mouse	Mouse	Mouse	Mouse				
2,4-Dinitrotoluene	65, 309		I	S	2B		Kidney <sup>s</sup>			Skin Mammary gland <sup>e</sup> Liver <sup>k</sup>			
2-(2-Formylhydrazino)-4-(5-nitro-2-furyl)-thiazole	7, 151	63	I	S	2B		Fore stomach Lung Haemato-poietic system <sup>m</sup>			Liver <sup>k</sup> Mammary gland Kidney/cortex <sup>b</sup> Kidney/pelvis <sup>w</sup> Intestine	Fore stomach <sup>p</sup> Urinary bladder <sup>w</sup>		
Gasoline Unleaded	45, 159		I	L	2B		Liver <sup>k</sup>			Kidney <sup>s</sup>			
Hexachlorobenzene	20, 155	219	I	S	2B		Liver			Liver <sup>k</sup> Bile duct Kidney <sup>r</sup>	Liver <sup>a,d</sup> Thyroid <sup>a</sup>		
Lead and lead compounds: inorganic Lead acetate	23, 325	230	I	S	2B								
Lead phosphate Lead subacetate													
Methyl mercury compounds Methyl mercury chloride	58, 239		I		2B		Kidney <sup>a,c</sup>			Kidney <sup>a,c</sup> Brain Kidney <sup>a,c</sup> Kidney <sup>a,c</sup> Brain			
Nitrotriacetic acid and its di- and tri-sodium salts	48, 181		I	S	2B		Kidney <sup>b</sup> Urinary bladder <sup>w</sup>			Kidney/cortex <sup>s</sup> Kidney/pelvis <sup>w</sup> Urinary bladder <sup>w</sup>			
Nitrobenzene	65, 381		I	S	2B		Lung Thyroid <sup>q</sup>			Liver <sup>k</sup> Kidney Thyroid <sup>b</sup>			



Table 4. (Contd) Some agents (chemicals and mixtures) producing kidney (renal tubule-cell) neoplasms (IARC Monographs, Volumes 1-69)

Agent	Vol., page	Suppl. 7, page	Evidence of carcinogenicity		Target organs			
			H	A	Group	Human	Mouse	Rat
N-Nitrosodietaniline	17, 77	67	I	S	2B			Liver <sup>k</sup> Kidney <sup>a</sup>
								Lung/trachea Nasal cavity <sup>b</sup>
N-Nitrosodiethylaniline	17, 83	67	I	S	2A		Liver <sup>k,j</sup> Oesophagus Forestomach Lung	Liver <sup>k,j</sup> Bile duct <sup>d</sup> Oesophagus Kidney <sup>k</sup>
								Nasal cavity Forestomach
N-Nitrosodimethylamine	17, 125	67	I	S	2A		Liver <sup>k,j</sup> Lung Kidney <sup>a,d</sup>	Liver <sup>k,j</sup> Bile duct <sup>d</sup> Nasal cavity <sup>b</sup>
								Nasal cavity <sup>b</sup>
N-Nitrosomorpholine	17, 263	68	I	S	2B		Liver <sup>k</sup> Lung	Liver <sup>k,j</sup> Nasal cavity Lung Kidney <sup>d</sup> Bile duct <sup>d</sup>
								Nasal cavity
Ochratoxin A	56, 489		I	S	2B		Liver <sup>k</sup> Kidney <sup>e</sup>	Kidney <sup>s</sup>
Potassium bromate	40, 207	70	I	S	2B			Kidney <sup>a,b</sup> Thyroid <sup>k</sup>
Tetrachloroethylene	63, 159		L	S	2A	Lymphoid system <sup>n</sup> Cervix Oesophagus	Liver <sup>k</sup>	Kidney <sup>s</sup> Haemato-poietic system <sup>n</sup>
Tobacco smoke	38, 37	359	S	S	1	Lung Urinary bladder <sup>k</sup> Kidney <sup>d</sup> Oral cavity Larynx Pancreas	Lung Skin (condensate)	Larynx

[illegible]



Table 5. Some agents (chemicals and mixtures) producing thyroid follicular-cell neoplasms  
(IARC Monographs, Volumes 1-69)

Agent	Vol., page	Suppl. 7, page	Evidence of carcinogenicity		Human	Target organs						
			H	A		Group	Mouse	Rat	Hamster	Dog	Other	
Acrylamide	60, 389		I	S	2A		Lung Skin (initiator)	Mammary gland Oral cavity Brain Uterus Clitoral gland Thyroid <sup>h</sup>				
Amitrole (3-amino-1,2,4-triazole)	41, 293	92	I	S	2B		Liver <sup>k</sup> Thyroid <sup>k</sup>	Pituitary gland Thyroid <sup>h</sup>				
ortho-Anisidine and its hydrochloride	27, 63	57	I	S	2B		Urinary bladder <sup>k</sup>	Urinary bladder <sup>k</sup> Kidney/pelvis <sup>w</sup> Thyroid <sup>h</sup>				
Chlordane and heptachlor	53, 115		I	S	2B		Liver <sup>k</sup>	Liver <sup>k</sup> Thyroid <sup>k</sup>				
Chlorendic acid	48, 45		I	S	2B		Liver <sup>k</sup> Lung Thyroid <sup>g</sup>	Liver <sup>k</sup> Lung Pancreas (exocrine)				
Chlorinated paraffins (C12, Cl 60%)	48, 55		I	S	2B		Liver <sup>k</sup> Lung Thyroid <sup>h</sup>	Liver <sup>k</sup> Thyroid <sup>h</sup> Haematopoietic system <sup>m</sup>				
Cl Basic Red 9	57, 215		I	S	2B		Liver <sup>k</sup>	Liver <sup>k</sup> Skin Zymbal gland Thyroid <sup>h</sup>				
2,4-Diaminobenzonitrile	27, 103	61	I	S	2B		Thyroid <sup>h</sup>	Thyroid <sup>h</sup> Skin Preputial/clitoral gland Zymbal gland Mammary gland				

**Table 5. (Contd) Some agents (chemicals and mixtures) producing thyroid follicular-cell neoplasms**  
**(IARC Monographs. Volumes 1-69)**

Agent	Vol., page	Suppl. 7, page	Evidence of carcinogenicity		Target organs						
			H	A	Group	Human	Mouse	Rat	Hamster	Dog	Other
4,4'-Diaminodiphenyl ether (4,4'-oxydianiline)	29, 203	61	I	S	2B		Liver <sup>k</sup> Harderian gland Thyroid <sup>g</sup>	Liver <sup>k</sup> Thyroid <sup>h</sup>			
Ethylene thiourea	7, 45	207	I	S	2B		Liver	Thyroid <sup>i</sup>			
Griseofulvin	10, 153	391	I	S	2B		Liver	Thyroid <sup>k</sup>			
HC Blue No. 1	57, 129		I	S	2B		Liver <sup>k</sup> Thyroid <sup>g</sup>	Lung			
4,4'-Methylene- dianiline dihydro- chloride	39, 347	66	I	S	2B		Liver <sup>k</sup> Thyroid <sup>g</sup>	Liver <sup>z</sup> Thyroid <sup>i</sup>			
Methylthiouraci	7, 53	66	I	S	2B		Thyroid <sup>a</sup>	Thyroid <sup>ac</sup>	Thyroid <sup>e</sup>		
Nitrobenzene	65, 381		I	S	2B		Lung Thyroid <sup>g</sup>	Liver <sup>k</sup> Kidney <sup>r</sup> Thyroid <sup>h</sup>			
Propylthiouraci	7, 67	329	I	S	2B		Thyroid <sup>c</sup> Pituitary gland	Thyroid <sup>ac</sup>	Thyroid <sup>c</sup>		Guinea-pig: Thyroid <sup>a</sup>
Tetrachlorodibenzo- <i>para</i> -dioxin	69		L	S	1	Lung All sites	Liver <sup>k</sup> Thyroid <sup>g</sup>	Liver <sup>k</sup> Thyroid <sup>g</sup> Lung Nasal cavity Oral cavity Tongue			

- Squamous-cell carcinoma
- Transitional-cell carcinoma
- Unspecified
- Neoplastic nodule/hepatocellular adenoma

- ^ Follicular-cell adenoma and carcinoma
  - ^ Follicular-cell carcinoma
- ^ Haemangioma of haemangiosarcoma
  - ^ Hepatoma and/or hepatocellular carcinoma
- ^ Leiomyoma/leiomyosarcoma
  - ^ Leukaemia
  - ^ Lymphoma
- ^ Papilloma
  - ^ Papilloma and transitional-cell carcinoma
- ^ Papilloma, transitional-cell
  - ^ Renal tubule-cell adenoma
  - ^ Renal-tubule cell adenoma and carcinoma
  - ^ Renal-tubule cell carcinoma
- ^ Sarcoma

oils, and tobacco smoke. For these mixtures there are no bioassay data in which tumours of the urinary bladder occurred in exposed animals of any species. Also, chronic infection with the parasite *Schistosoma haematobium* leads to bladder tumours only in humans and some species of non-human primates, not in rodents (IARC, 1994).

Carcinoma of the urinary bladder was one of the first human cancers to be recognized as having an association with occupational exposure to chemicals (Rehn, 1895), especially chemicals used in dyestuff manufacturing. These were much later shown to be aromatic amines. Rats and mice are not uniformly susceptible to carcinogenesis in the urinary bladder by aromatic amines, however, and much research effort was required to identify the dog (Hueper *et al.*, 1938; Bonser, 1943) and the Syrian hamster (Sellakumar *et al.*, 1969) as surrogate species that more closely resemble humans in their response to bladder carcinogens of the aromatic amine family. This is not necessarily the case for bladder carcinogens of other chemical classes. For analgesic mixtures containing phenacetin, human evidence is supported by bioassay data in rats, although the primary tumour site in both humans and rats is the renal medulla. The remaining eight human bladder carcinogens are individual chemicals or groups of chemicals that vary greatly in the extent to which human and animal target organ sites overlap. For 4-aminobiphenyl, benzidine, cyclophosphamide, 2-naphthylamine, and phenacetin, bladder tumours were identified in one or more experimental animal species. 4-Amino-biphenyl is carcinogenic to the bladder in dogs, rabbits, and mice, also producing tumours in mice at other sites; in rats, tumours were only detected at sites other than the bladder. For benzidine, bladder tumours were found only in the dog; benzidine produced tumours only at sites other than the bladder in mice, rats and hamsters. Cyclophosphamide produced tumours in the bladder and at other sites in rats, but only at other sites in mice. 2-Naphthylamine produced bladder tumours in rats, dogs, monkeys and hamsters, but not mice (in which only liver and lung (newborn) tumours were produced). Phenacetin produced tumours of the bladder and also the kidney in rats, but only in

the renal cortex in mice. For arsenic and arsenic compounds, the anti-tumour drug chlornaphazine (a 2-naphthylamine derivative), and for *para*-chloro-*ortho*-toluidine, all of which are associated with increased risk of bladder cancer in humans, no bladder tumours were detected in the available animal bioassays.

Some of the carcinogens listed in Table 3, for which data in humans are *inadequate* but data in animals are *sufficient*, are associated with the formation of urinary calculi (urolithiasis) in rats under the conditions of bioassays in which bladder tumours occurred. These include the dye Disperse Blue 1 and sodium *ortho*-phenyl phenate<sup>1</sup>. Neither chemical produced bladder tumours or stones in mice, in which species each was carcinogenic only at a site other than the bladder. Melamine<sup>2</sup> (2,4,6-triamino-1,3,5-triazine) (not in Table 3, Group 3) was carcinogenic only to the urinary bladder in rats and produced urinary calculi under conditions where tumours developed, but only a single positive bioassay had been reported when this chemical was evaluated (IARC, 1986a) and evidence for carcinogenicity to animals was considered *inadequate* because of the association of tumours with bladder stones. Saccharin<sup>2</sup> and its salts produced only bladder tumours (and urinary precipitate) in rats; evidence for tumours at another site in mice (thyroid) was based on a single experiment.

### Epithelial Neoplasms of the Renal Cortex

Twenty-nine compounds, groups of compounds, or complex mixtures for which there is at least *sufficient* evidence of carcinogenicity in animals are summarized in Table 4. Tumour histology has been footnoted as described in the Monographs. However, adenoma, adenocarcinoma or carcinoma not further specified would refer to tumours of the tubule epithelium. Only one of these, tobacco smoke, is definitely linked to cancer of the renal cortex in humans.

Of these 29 agents, nine are carcinogenic to the renal cortex in both rats and mice: aflatoxins, bromodichloromethane, caffeic acid, chloroform<sup>1</sup>, lead compounds, nitrilotriacetic acid and its salts<sup>1</sup>, *N*-nitrosodimethylamine, ochratoxin A, and tris-(1,3-dibromopropyl)phosphate. Others: benzofuran,

<sup>1</sup> Subsequently re-evaluated in IARC Monographs Volume 73 (October, 1998) with no change in overall evaluation.

<sup>2</sup> Subsequently re-evaluated in IARC Monographs Volume 73 (October, 1998) with a change in overall evaluation.

captafol, cycasin, daunomycin, *para*-dichlorobenzene<sup>1</sup>, 2-(2-formylhydrazino)-4-(5-nitro-2-furyl)thiazole, hexachlorobenzene, nitrobenzene, *N*-nitrosodiethanolamine, *N*-nitrosodiethylamine, *N*-nitrosomorpholine, potassium bromate<sup>1</sup>, tetrachloroethylene, trichloroethylene and 1,2,3-trichloropropane produced tumours of the renal cortex only in rats, indicating that for carcinogenesis at this organ site, the rat is by far the more sensitive species. All these agents except potassium bromate also produced tumours in mice or hamsters or both, but only at sites other than the kidney.

For only one carcinogen in Table 4, methyl mercury chloride, is the kidney the only target organ (in mice only). There are also several compounds for which evidence of carcinogenicity is confined to the kidney, but is considered *limited* in the *IARC Monographs*. Often the evidence was evaluated as *limited* because only a single bioassay had been performed at the time of the evaluation. These include 2-amino-4-nitrophenol (male rats only; IARC, 1993a); chlorothalonil<sup>2</sup> (male and female rats) (IARC, 1983), citrinin (male rats only; females not tested) (IARC, 1986b) and hexachlorobutadiene<sup>1</sup> (male and female rats) (IARC, 1979a); and *d*-limonene<sup>2</sup> (male rats only; IARC, 1993b). Unleaded gasoline, given to rats by inhalation, was carcinogenic only to the kidney and only in males; in mice, it increased the incidence of liver tumours in females only, in which animals a few renal tumours occurred also (IARC, 1989). Hexachloroethane<sup>2</sup> caused a low incidence of kidney tumours in male and female rats, but induced hepatocellular carcinomas in male and female mice (IARC, 1979b).

Oestrogens, whether natural or synthetic and whether steroidal or non-steroidal in structure, are carcinogenic to the kidney in male hamsters, but not in other species. In humans the kidney is not a target organ for medicinal oestrogens, which are carcinogenic to female breast, endometrium and liver under certain conditions of medical use (IARC, 1987), and oestrogens have not been included in Table 4.

### Follicular Cell Neoplasms of the Thyroid Gland

Twenty compounds that cause follicular cell neoplasms of the thyroid in rats and/or mice, and occasionally in other species, are summarized in

Table 5. None of these is unequivocally associated with thyroid cancer in humans. The only compound for which there is some evidence of carcinogenicity to humans at this site is 2,3,7,8-tetrachlorodibenzo-*para*-dioxin (TCDD; Group 1), where the most convincing evidence is for all sites combined (IARC, 1997). Elevated mortality (four deaths) from thyroid carcinoma in persons exposed to TCDD has been recorded in one study (Saracci *et al.*, 1991).

For many compounds, elevated thyroid tumour incidence was accompanied by elevated hepatocellular tumour incidence in the same species (see McClain & Rice, this volume), with or without tumours at any other sites. In some cases, liver tumours but no thyroid tumours were reported in one species, and thyroid tumours but no liver tumours in another, but in some of these cases the thyroid gland (especially in mice) was not examined. Thyroid tumours plus tumours in at least one extrahepatic site were observed for many compounds, in at least one test species, usually the rat (e.g., acrylamide, CI Basic Red 9, 2,4-diaminoanisole, TCDD, 4,4'-thiodianiline, and thiourea). Only a few compounds produced tumours only in the thyroid (e.g., methylthiouracil [rat, mouse, hamster], propylthiouracil [rat, hamster, guinea pig], 2,4-diaminoanisole [mouse]) or in the thyroid plus the pituitary but at no other sites (e.g., amitrole [rat], propylthiouracil [mouse]). However, a number of compounds which have undergone only limited testing and are currently in IARC Group 3 (Table 2) have to date produced tumours only in the thyroid. These include sulfamethoxazole and 4,4'-methylene bis(*N,N*-dimethyl)benzenamine in rats (IARC, 1982).

### Discussion

In *IARC Monographs* evaluations, mechanistic evidence that neoplasms at any site in rodents might not be predictive of a carcinogenic hazard to humans is only applied to the final overall evaluation, and only to the overall evaluation of agents for which there was *sufficient* evidence in bioassays in animals and *inadequate* evidence in humans. When bioassay data are only available from a single study, or there are other deficiencies in the available published studies that in the opinion of an IARC Working Group provide less than *sufficient*

<sup>1</sup> Subsequently re-evaluated in *IARC Monographs* Volume 73 (October, 1998) with no change in overall evaluation.

<sup>2</sup> Subsequently re-evaluated in *IARC Monographs* Volume 73 (October, 1998) with a change in overall evaluation.

evidence of carcinogenicity in animals, such agents would be placed in Group 3 without invoking considerations of carcinogenic mechanism or mode of action (Table 2). Only when there is sufficient evidence of carcinogenicity to animals, inadequate evidence for cancer in humans, and mechanistic evidence that the tumours seen in exposed animals are not predictive of carcinogenicity to humans, might an agent be downgraded from Group 2B to Group 3. In the IARC classification scheme, it is not possible for any agent that causes tumours in animals by any mechanism or mode of action to be classified as *probably not carcinogenic to humans* (Group 4).

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## Appendix 2

# Chemicals associated with tumours of the kidney, urinary bladder and thyroid gland in laboratory rodents from 2000 US National Toxicology Program/National Cancer Institute bioassays for carcinogenicity

James Huff

### Introduction

Since the late 1960s, the US National Cancer Institute (NCI) and since 1978, the US National Toxicology Program (NTP) have studied and evaluated the potential carcinogenicity of nearly 500 chemicals. Most of these were tested in both genders of two species of rodents, typically Fischer 344 inbred rats (and earlier, on occasion, Osborne-Mendel rats) and (C57BL/6 x C3H/HeN MTV-) $F_1$  (B6C3F<sub>1</sub>) hybrid mice. Thus, nearly 2000 individual sex-species bioassays have been accomplished in these last three decades since publication of the first NCI Bioassay Technical Report in 1976.

Long-term (usually two-year) carcinogenesis bioassays continue to be the most appropriate and predictive model for identifying those chemicals with the most likelihood to cause cancer in humans (Montesano *et al.*, 1986; Huff *et al.*, 1991a; IARC, 1997; Huff, 1999). In several instances, chemicals were first shown to be carcinogenic in laboratory animals before evidence of cancer was observed in humans (Tomatis, 1979; Huff, 1993; IARC, 1997). Results from long term experiments in animals should continue to be used judiciously to protect public health, to reduce and prevent cancer risks from these agents, and to establish standards of exposures (Fung *et al.*, 1995). Further, chemical carcinogenesis results can be used to identify and set reasonable priorities for chemicals that should be investigated epi-demiologically (Huff *et al.*, 1991a).

Presented in this paper are lists of chemicals shown by the NCI or the NTP to cause tumours of the kidney, urinary bladder, or/and thyroid gland in rats and/or mice. Examples are given of chemicals that induce tumours in two or more of these target organs, as well as some interesting chemical structure correlations. Chemical carcinogenesis target sites in rodents other than these three have been published (Huff *et al.*, 1991b), and are available on-line: [http://ntp-server.niehs.nih.gov/htdocs/Sites/Site\\_Cnt.html](http://ntp-server.niehs.nih.gov/htdocs/Sites/Site_Cnt.html).

### Materials and methods

The NTP collection of carcinogenesis results contains detailed information on nearly 500 chemicals tested since the early 1970s, involving nearly 2000 gender-specific bioassays. For almost all chemical agents tested, four unique groups of animals were used: male rats, female rats, male mice, female mice. Each grouping consists of 50–60 animals in each of 2–3 exposure groups and a control or unexposed group. Carcinogenic activity was evaluated separately for each gender-specific grouping. Proposed results and conclusions are presented to and peer-reviewed by independent experts in carcinogenesis and related fields in public meetings. Final data, results, evaluations, and conclusions are prepared as detailed technical reports, and these are made publicly available (Huff *et al.*, 1988; Huff, 1998, 1999).

Within the overall NTP tumour data base, organ-specific effects have been recorded and are

available for tabulation and comparisons (Huff *et al.*, 1991b). For this paper, the three target organs selected by the International Agency for Research on Cancer as the subject of a Workshop on carcinogenesis mechanisms in November 1997 form the basis of this compilation: kidney, urinary bladder, thyroid gland.

For a further listing of chemicals that may affect these organs, see also the paper taken from the IARC Monographs (Wilbourn *et al.*, this volume), and other chemical carcinogenesis data collections; these include PHS 149 series (National Cancer Institute, 1999), California EPA Proposition 65 [internet on-line: <http://www.calepa.ca.gov/oehha/docs/9-961stb.htm>], and Gold and Zeiger (1997). Also available on-line are NTP files of long-term bioassay testing results [<http://ntp-server.niehs.nih.gov/htdocs/pub.html>] and the NTP Reports on Carcinogens [[http://ntp-server.niehs.nih.gov/Main\\_Pages/NTP\\_ARC\\_PG.html](http://ntp-server.niehs.nih.gov/Main_Pages/NTP_ARC_PG.html)].

## Results

The numbers of chemicals associated with site-specific neoplasia for the 36 organs and systems routinely evaluated histopathologically are given in Table 1. An abbreviated rank-ordered listing is given in Table 2. Kidney ranks second to liver, thyroid gland ranks seventh between mammary gland and adrenal glands, and urinary bladder ranks 11th between Zymbal gland and intestine. Regarding incidences of human cancers in these organs in the United States (Ries *et al.*, 1997; American Cancer Society, 1998), urinary bladder resides in fourth place for men and eighth for women, kidney ranks eighth for men and 13th for women, and thyroid gland tumours are 18th for men and 12th for women.

For the three target organs emphasized in this paper, using the pathology data on the 500 chemicals tested, the rat kidney appears to be the most responsive, with the male rat predominating. Similar results have been obtained for the urinary bladder, but here the responsiveness seems comparable for both male and female rats. For the thyroid gland, rats again show more positive responses than mice, yet mice do respond more often for this organ site than for kidneys and urinary bladder. Each gender within a species is

approximately equal in numbers of positive responses.

Some have reported correlations among certain chemical-associated organs in rodents. Haseman and Lockhart (1993) reported that certain high incidence tumour combinations do occur: rat liver and rat Zymbal gland, mouse lung and rat/mouse mammary glands, and mouse lung and mouse forestomach. However, the relevance of these empirical observations for the most part allows little insight into rodent carcinogenesis or into human relevance and extrapolation. In fact, however, correspondence between various tumour sites is a less common finding among animals exposed to chemicals. For instance, for the three target sites highlighted in this paper, only one combination is statistically associated: liver and thyroid gland; these rarely occur in the same sex-species group, and even more uncommonly in the same animal. The meaning of this observational combination remains obscure (see McClain & Rice, this volume).

The data selected for Tables 3–5 include: the NCI or NTP Technical Report Numbers; the common chemical names; positive (+), negative (-) or equivocal mutagenicity results in *Salmonella typhimurium* (Ames assay), routes of exposure used for the long-term bioassays; levels of evidence of carcinogenicity for each sex-species group (with parentheses indicating a carcinogenic response in the particular organ of concern); carcinogenic responses that may be related to chemical exposure; and whether tumours were also induced at sites other than the subject organ (yes) or in the subject organ only (no). Results for each of the three organs are listed alphabetically in Tables 3–5, and are summarized below in separate commentaries.

## Kidney

For the kidney, 64 chemicals induced tumours in one or more of the four gender-species experimental groups (Table 3). Most chemically induced tumours of the kidney were renal tubule cell types, with four chemicals causing transitional cell tumours only and seven causing both neoplasms.

For 11 of the 64 chemicals, carcinogenic effects were observed only in the kidney, for nine of the 11, the effect was observed only in a single gender-species group (three of these were equivocal



**Table 1. Numbers of chemicals associated with site-specific neoplasms from nearly 2000 long-term carcinogenesis bioassays**

Organ/tissue site	Ranking	Male rats		Female rats		Male mice		Female mice		Totals		
		POS <sup>a</sup>	EE <sup>a</sup>	POS	EE	POS	EE	POS	EE	POS	EE	POS/EE <sup>a</sup>
Adrenal gland	8	11	11	8	8	6	3	4	1	20	17	37
Bone	29	1	2	0	0	0	0	0	0	1	2	3
Brain	23	2	7	2	4	2	1	1	0	3	9	12
Circulatory system	14	4	0	2	1	8	4	9	2	13	5	18
Clitoral gland	19	0	0	13	2	0	0	0	0	13	2	15
Epididymis	39	0	0	0	0	0	1	0	0	0	1	1
Esophagus	28	3	0	3	0	0	1	0	1	3	1	4
Forestomach	5	19	4	13	5	20	5	21	8	30	12	42
Forestomach/stomach	34	0	1	0	0	0	0	0	0	0	1	1
Glandular stomach	32	0	0	0	2	0	0	0	0	0	2	2
Harderian gland	18	0	0	0	0	10	3	11	2	12	4	16
Heart	27	0	0	0	0	4	0	4	0	4	0	4
Haematopoietic system	4	14	8	12	8	11	6	16	4	34	22	56
Intestines	12	12	3	9	2	1	3	1	0	14	6	20
Kidney	2	41	11	17	4	9	6	1	2	50	14	64
Liver	1	44	10	39	4	76	20	99	10	132	24	156
Lung	3	11	6	12	3	29	6	32	5	46	10	56
Mammary gland	6	4	0	27	7	0	0	11	1	33	7	40
Mesothelium (ac/tv) <sup>b</sup>	22	9	3	2	1	1	0	1	0	9	3	12
Nasal cavity	24	9	2	8	1	3	0	3	1	10	1	11
Oral cavity	20	12	1	12	1	0	1	1	0	14	0	14
Ovary	25	0	0	0	0	0	0	10	0	10	0	10
Pancreas	17	8	8	2	2	0	0	0	0	9	8	17
Parathyroid gland	35	0	1	0	0	0	0	0	0	0	1	1
Pituitary gland	26	0	1	1	2	2	0	3	0	4	3	7
Preputial gland	15	8	5	0	0	4	1	0	0	12	6	18
Seminal vesicle	30	1	0	0	0	1	0	0	0	2	0	2
Skin	9	16	5	8	1	4	3	4	1	18	8	26
Spleen	21	8	2	3	1	1	0	2	0	10	2	12
Subcutaneous tissue	16	7	3	3	2	0	4	4	2	10	8	18
Thyroid gland	7	16	8	15	5	8	6	10	2	25	14	39
Ureter	31	2	0	1	0	0	0	0	0	2	0	2
Urinary bladder	11	14	1	13	4	2	1	3	0	19	4	23
Uterus/cervix	13	0	0	9	0	0	0	8	1	17	1	18
Zymbel gland	10	19	3	17	1	1	2	2	2	21	4	25

<sup>a</sup> POS, positive findings, EE, equivocal evidence, POS/EE, positive findings or equivocal evidence

<sup>b</sup> ac/tv, abdominal cavity/tunica vaginalis

**Rankings** were determined using the POS/EE column totals data. With ties, the site with the most POS was given the higher ranking; e.g., circulatory system [rank 14], preputial gland [15], and subcutaneous tissue [16] had the same number of POS/EE, but circulatory system following by preputial gland had more POS responses.

**Responses** by sex and species:

**Totals** represents the numbers of individual chemicals that caused positive responses [POS], equivocal responses [EE], and combined totals [POS/EE] for each particular organ/system.

The sum of the POS or EE numbers from all of the sex/species columns for an organ site most often is greater than the number in the totals columns, because a given chemical may have caused tumours in more than one sex-species group, but would only be counted once as either a POS or an EE substance.

Testis not listed as no NCI or NTP chemical has caused tumours in this organ.

**Table 2. Rank order of chemically induced site-specific neoplasms from nearly 2000 long-term NCI/NTP carcinogenesis bioassays<sup>a</sup>**

1. Liver	19. Clitoral gland
2. Kidney	20. Oral cavity
3. Lung	21. Spleen
4. Haematopoietic system	22. Mesothelium
5. Forestomach	23. Brain
6. Mammary gland	24. Nasal cavity
7. Thyroid gland	25. Ovary
8. Adrenal gland	26. Pituitary gland
9. Skin	27. Heart
10. Zymbal gland	28. Oesophagus
11. Urinary bladder	29. Bone
12. Intestines	30. Seminal vesicle
13. Uterus/cervix	31. Ureter
14. Circulatory system	32. Glandular stomach
15. Preputial gland	33. Epididymis
16. Subcutaneous tissue	34. Forestomach/stomach
17. Pancreas	35. Parathyroid gland
18. Harderian gland	36. Testes <sup>b</sup>

<sup>a</sup> See details in Table 1.

<sup>b</sup> Listed even though no chemical has been associated with tumours in this organ.

responses). In no case did a chemical induce tumours of the kidney in all four gender-species groups. Several chemicals caused tumours of the kidney in three of the four groups: bromodichloromethane, chloroprene, nitrilotriacetic acid (NTA), tris(2,3-dibromopropyl)phosphate; for one more the findings were equivocal in one of the three positive groups: *ortho*-nitroanisole and tris(2-chloroethyl)phosphate. For these five chemicals, none were mutagenic for *Salmonella*. Considering all 59 chemicals tested for mutagenic activity (5 have not been), 19 (32%) induced mutations in *Salmonella*, whereas 40 did not. What this means mechanistically is uncertain, because one would have to evaluate the full scope of genotoxic activity.

Overall, 50 chemicals were positive or equivocal in male rats, 19 in female rats, 15 in male mice, and only three in female mice (with two of the latter being equivocal). Thus, for the kidney, the female B6C3F<sub>1</sub> mouse is the least sensitive of the species and strains used for carcinogenicity testing

by the NTP, with only a single chemical of the nearly 500 tested being unequivocally positive for the female mouse kidney: NTA. Perhaps much of this gender and species/strain difference – at least for male and female Fischer 344 rats – might decrease if study duration were lengthened to 30 or more months, since female rats seem to develop tumours at this organ site much later than do male rats of this strain.

Consistently, male rats exhibit a higher background incidence of tumours of the kidney at 24 months than do female rats: 1.0% versus 0.11%. This difference extends to step- or multiple sections as well: 4.6% in males versus 0.75% in females (Eustis *et al.*, 1994). In one limited life-span study of 529 male and 529 female Fischer 344 rats, renal tubule cell tumour rates went from 0.34% in males at 24 months to 0.57% at 140–146 weeks (1.7-fold increase); for females the percentages were 0.17% and 0.95% (5.6 fold increase) (Solleveld *et al.*, 1984). More work and some life time chemical exposure studies need to be accomplished to solidify these findings.

### Urinary bladder

For the urinary bladder, 23 chemicals induced tumours in one or more of the four gender-species experimental groups (Table 4). Five chemicals caused tumours only of the urinary bladder: *meta*-cresidine and *N*-nitrosodiphenylamine were positive in both sexes of rats; two were single sex-species carcinogens, one in male rats (melamine) and one in female rats (*para*-benzoquinone dioxime); with another that was positive in male rats yielding equivocal evidence in female rats (4-amino-2-nitrophenol).

Most chemicals caused transitional cell tumours, with both transitional and squamous cell types induced by three chemicals. CI Disperse blue 1, an anthraquinone, was associated with tumours of three cellular types: transitional, squamous, and smooth muscle (mesenchymal). In this case the situation was somewhat confusing because about one-half the animals had bladder stones (National Toxicology Program, 1986); further, one other anthraquinone likewise caused tumours of the urinary bladder in both male and female rats. Squamous cell tumours are thought to arise from transitional epithelial cells (Jokinen, 1990).

**Table 3. Chemicals Associated with Site-Specific Tumour Induction in Kidney [Renal tubule cell, RTC, tumours unless otherwise noted]**

Technical Report No.	Chemical name	Salmonella <sup>a</sup>	Route	Levels of Evidence of Carcinogenicity <sup>b</sup>				Other tumour sites
				MR	FR	MM	FM	
383	1-Amino-2,4-dibromoanthraquinone	+	Feed	[P]	P	N	P	Yes
111	1-Amino-2-methylantraquinone	+	Feed	[P]	P	N	P	Yes
339	2-Amino-4-nitrophenol	+	Gavage	[SE]	NE	NE	NE	No
089	o-Anisidine hydrochloride [T cell] <sup>c</sup>	nd	Feed	[P]	P	P	P	Yes
067	Aspirin, Phenacetin, Caffeine [T & RTC]	nd	Feed	N	[E]	N	N	Yes
469	3'-Azido-3'-deoxythymidine	+	Gavage	nd	nd	[EE]	CE	Yes
370	Benzofuran	-	Gavage	NE	[SE]	CE	CE	Yes
424	o-Benzyl-p-chlorophenol [T & RTC]	-	Gavage	NE	[EE]	[SE]	NE	No
452	2,2-Bis[bromomethyl]-1,3-propanediol <sup>d</sup>	+, -	Feed	CE	CE	[CE]	CE	Yes
321	Bromodichloromethane	-	Gavage	[CE]	[CE]	[CE]	CE	Yes
434	1,3-Butadiene <sup>e</sup>	+	Inhal	nd	nd	[CE]	CE	Yes
436	tert-Butyl alcohol	-	Water	[SE]	NE	EE	SE	Yes
308	Chlorinated paraffins: C12, 60% Cl	-	Gavage	[CE]	CE	CE	CE	Yes
000	Chloroform	-	Gavage	[P]	N	P	P	Yes
467	Chloroprene	-	Inhal	[CE]	[CE]	[CE]	CE	Yes
041	Chlorothalonil	-	Feed	[P]	[P]	N	N	No
335	C.I. Acid Orange 3 [T cell]	+	Gavage	NE	[CE]	NE	NE	No
430	C.I. Direct Blue 218 <sup>f</sup>	-	Feed	SE	NE	CE	CE	Yes
407	C.I. Pigment Red 3	+	Feed	SE	SE	[SE]	NE	Yes
411	C.I. Pigment Red 23	+	Feed	[EE]	NE	NE	NE	No
196	Cinnamyl anthranilate	-	Feed	[P]	N	P	P	Yes
422	Coumarin	+	Gavage	[SE]	[EE]	SE	CE	Yes
463	D & C Yellow No. 11	?, +W	Feed	[SE]	SE	nd	nd	Yes
401	2,4-Diaminophenol dihydrochloride	+	Gavage	NE	NE	[SE]	NE	No
400	2,3-Dibromo-1-propanol	+	Dermal	[CE]	[CE]	CE	CE	Yes
319	1,4-Dichlorobenzene [p-dichlorobenzene]	-	Gavage	[CE]	NE	CE	CE	Yes
423	3,4-Dihydrocoumarin [T & RTC]	-	Gavage	[SE]	NE	NE	SE	Yes
456	1,2-Dihydro-2,2,4-trimethylquinoline	-	Dermal	[SE]	NE	NE	NE	Yes
323	Dimethyl methylphosphonate [T & RTC]	-	Gavage	[SE]	NE	IS	NE	Yes
466	Ethylbenzene	-	Inhal	[CE]	[SE]	SE	SE	Yes
382	Furfural <sup>g</sup>	?, -	Gavage	SE	NE	CE	SE	Yes
356	Furosemide	-	Feed	[EE]	NE	NE	SE	Yes
252	Geranyl acetate <sup>h</sup>	-	Gavage	N	N	N	N	Yes
361 & 068	Hexachloroethane	-	Gavage	[CE]	NE	P	P	Yes
366	Hydroquinone	-	Gavage	[SE]	SE	NE	SE	Yes
291	Isophorone	-	Gavage	[SE]	NE	EE	NE	Yes
347	α-Limonene	-	Gavage	[CE]	NE	NE	NE	No
408	Mercuric chloride	-	Gavage	SE	EE	[EE]	NE	Yes
359	8-Methoxypsoralen	+	Gavage	[CE]	NE	nd	nd	Yes
369	α-Methylbenzyl alcohol	-	Gavage	[SE]	NE	NE	NE	No
348	Methyldopa sesquihydrate	nd	Feed	NE	NE	[EE]	NE	No
313	Mirex [T cell]	-	Feed	[CE]	CE	nd	nd	Yes
266	Monuron	-	Feed	[CE]	NE	NE	NE	Yes

Table 3. (Contd) Chemicals associated with site-specific tumour induction in kidney [renal tubule cell, RTC, tumours unless otherwise noted]

Technical Report No.	Chemical name	Salmonella	Route	Levels of Evidence of Carcinogenicity				Other tumour sites
				MR	FR	MM	FM	
006	Nitrilotriacetic acid	-	Feed	[P]	P	[P]	[P]	Yes
006	NTA-Trisodium <sup>d</sup> [T & RTC]	-	Feed	[P]	[P]	N	N	Yes
416	o-Nitroanisole [T & RTC]	+	Feed	[CE]	[CE]	CE	SE	Yes
341	Nitrofurantoin	+	Feed	[SE]	NE	NE	CE	Yes
358	Ochratoxin A	-	Gavage	[CE]	[CE]	nd	nd	Yes
468 & 443	Oxazepam	-	Feed	[EE]	NE	C	CE	Yes
232	Pentachloroethane	-	Gavage	[E]	N	P	P	Yes
465	Phenolphthalein	?	Feed	[CE]	SE	CE	CE	Yes
367	Phenylbutazone <sup>e</sup> [T & RTC]	-	Gavage	[EE]	[SE]	SE	NE	Yes
333	N-Phenyl-2-Naphthylamine	-	Feed	NE	NE	N	[EE]	No
476	Primidone [Primaclone]	+	Feed	[EE]	NE	CE	CE	Yes
409	Quercetin	+	Feed	[SE]	NE	nd	nd	No
457	Salicylazosulfapyridine [T cell]	-	Gavage	SE	[SE]	CE	C	Yes
311	Tetrachloroethylene	-	Inhal	[CE]	SE	CE	CE	Yes
450	Tetrafluoroethylene	nd	Inhal	[CE]	[CE]	CE	CE	Yes
475	Tetrahydrofuran	-	Inhal	[SE]	NE	NE	C	Yes
243	Trichloroethylene [T & TR 002 & 273]	-	Gavage	[SE]	[SE]	CE	CE	Yes
384	1,2,3-Trichloropropane	+	Gavage	[CE]	CE	CE	CE	Yes
449	Triethanolamine <sup>*</sup>	-	Dermal	[EE]	NE	EE	SE	Yes
391	Tris[2-chloroethyl] phosphate	-	Gavage	[CE]	[CE]	[EE]	EE	Yes
076	Tris[2,3-dibromopropyl] phosphate	nd	Feed	[P]	[P]	[P]	P	Yes
Number of chemicals causing tumours in KIDNEY = 64								

<sup>a</sup> Salmonella results: + = Positive; +W = Weakly Positive; ? = Inconclusive; - = Negative

<sup>b</sup> [ ] = animal group in which tumours of the kidney were observed. MR, male rat; FR, female rat; MM, male mouse; FM, female mouse

CE = Clear evidence of carcinogenicity; P = Evidence of carcinogenicity; SE = Some evidence of carcinogenicity; EE or E = Equivocal evidence of carcinogenicity; NE or N = No Evidence of carcinogenicity; IS = Inadequate study; nd = no NCI or NTP data

<sup>c</sup> T=Transitional cell; RTC = Renal tubule cell

<sup>d</sup> Monohydrate: two studies done on this salt form, with data combined.

<sup>e</sup> Transitional cell tumours are causative in female rats, while renal tubule cell tumours may be related

Note: for tables 3-5, the different abbreviations for levels of evidence of carcinogenicity reflect simply designation changes over time; that is, P became CE and SE; E became EE; N became NE.

<sup>\*</sup> Possible association is one sex of one species

**Table 4. Chemicals associated with site-specific tumour induction in urinary bladder**  
**[transitional cell tumours unless otherwise noted]**

Technical report no.	Chemical name	Salmonella <sup>a</sup>	Route	Levels of Evidence of Carcinogenicity <sup>b</sup>				Other tumour sites
				MR	FR	MM	FM	
234	Allyl isothiocyanate	+W,-	Gavage	[P]	E	N	N	Yes
383	1-Amino-2,4-dibromanthraquinone	+	Feed	[CE]	[CE]	CE	CE	Yes
094	4-Amino-2-nitrophenol	+	Feed	[P]	[E]	N	N	No
218	11-Aminoundecanoic acid	-	Feed	[P]	N	E	N	Yes
089	<i>o</i> -Anisidine hydrochloride	nd	Feed	[P]	[P]	[P]	[P]	Yes
067	Aspirin, Phenacetin, and Caffeine	nd	Feed	N	[E]	N	N	Yes
179	<i>p</i> -Benzoquinone dioxime	+	Feed	N	[P]	N	N	No
452	2,2-Bis[Bromomethyl]-1,3-propanediol	-,+	Feed	[CE]	CE	CE	CE	Yes
458 & 213	Butyl benzyl phthalate	-	Feed	SE	[EE]	NE	NE	Yes
063	4-Chloro- <i>o</i> -phenylenediamine	+	Feed	[P]	[P]	P	P	Yes
467	Chloroprene **	-	Inhal	CE	CE	CE	CE	Yes
299	C.I. Disperse Blue 1 [T,SqC & L] <sup>c</sup>	+	Feed	[CE]	[CE]	EE	NE	Yes
105	<i>m</i> -Cresidine	+	Gavage	[P]	[P]	IS	N	No
142	<i>p</i> -Cresidine [T,SqC]	+	Feed	[P]	[P]	[P]	[P]	Yes
269	1,3-Dichloropropene [Telone II]	+	Gavage	CE	SE	IS	[CE]	Yes
374	Glycidol <sup>d</sup>	+	Gavage	CE	CE	CE	CE	Yes
245	Melamine	-	Feed	[P]	N	N	N	No
006	Nitritotriacetic acid [NTA]	-	Feed	P	[P]	P	P	Yes
006	Nitritotriacetic acid trisodium <sup>d</sup>	-	Feed	P	[P]	N	N	Yes
416	<i>o</i> -Nitroanisole [T,SqC]	+	Feed	[CE]	[CE]	CE	SE	Yes
164	N-Nitrosodiphenylamine	-	Feed	[P]	[P]	N	N	No
457	Salicylazosulfapyridine	-	Gavage	[SE]	[SE]	CE	CE	Yes
153	<i>o</i> -Toluidine hydrochloride	-,+	Feed	P	[P]	P	P	Yes

Number of chemicals causing tumours in **URINARY BLADDER** = 23

<sup>a</sup> Salmonella results: + = Positive; +W = Weakly Positive; ? = Inconclusive; - = Negative

<sup>b</sup> [ ] = animal group in which tumours of the urinary bladder were observed.

CE = Clear evidence of carcinogenicity; P = Evidence of carcinogenicity; SE = Some evidence of carcinogenicity; EE or E = Equivocal evidence of carcinogenicity; NE or N = No Evidence of carcinogenicity; IS = Inadequate experiment; nd = no NCI or NTP data

<sup>c</sup> T=transitional cell; SqC=Squamous cell; L=leiomyoma or leiomyosarcoma

<sup>d</sup> = Monohydrate

\* Possible association in one sex of one species

\*\* Possible association in both sexes of one species

Two chemicals caused tumours of the urinary bladder in each of the four groups: *ortho*-anisidine hydrochloride and *para*-cresidine (isomeric *meta*-cresidine was positive in male and female rats). Fifteen induced tumours of the urinary bladder in male rats, 17 in female rats, three in male mice, and three in female mice. As with the lack of chemically caused kidney tumours in female mice, both male and female B6C3F<sub>1</sub> mice seem considerably less sensitive than are rats to chemical induction of urinary bladder tumours. Thirteen of the 24 chemicals induced mutations in *Salmonella*. There do not appear to be any significant differences between 104 week and 140–146 week studies in background tumour incidences of the urinary bladder in male and female rats (Solleveld *et al.*, 1984).

### Thyroid gland

For thyroid gland, 39 chemicals induced tumours in one or more of the four gender-species experimental groups (Table 5). All but seven chemicals caused follicular cell tumours only; four of these caused C cell tumours, and the other three caused both C cell and follicular cell tumours. Of these 39, seven chemicals induced only tumours of the thyroid gland, with four of these having only equivocal evidence of carcinogenicity; for two of these there were marginal responses in both sexes of one species. Thus, for practical purposes, only three chemicals of the nearly 500 tested induced only thyroid gland tumours: 3-amino-4-ethoxyacetanilide, *N,N'*-diethylthiourea, and trimethylthiourea.

Four chemicals caused tumours of the thyroid gland in each of the tested experimental groups: 2,4-diaminoaniline sulfate, ethylene thiourea (ETU), 4,4'-methylenedianiline dihydrochloride, and 4,4'-thiodianiline. One other chemical – 4,4'-oxydianiline – caused thyroid gland tumours in three of the four groups. Moreover, three of the positive chemicals were anilines and three others were thiourea derivatives.

Regarding gender or species specificity, 24 chemicals were associated with thyroid tumours in male rats, 20 in female rats, 14 in male mice, and 12 in female mice. This represents a more balanced sensitivity than was seen for the kidney or for the urinary bladder. For both C cell and follicular cell tumours in control rats, the incidences increased

considerably between 104 weeks and 140–146 weeks (Solleveld *et al.*, 1984). Twenty of the 39 chemicals were mutagenic to *Salmonella*.

### Comparative target sites

Considering the three tumour-organ sites collectively, rats appear to be more responsive than mice to chemicals inducing tumours at these sites. This is especially true for kidneys and urinary bladder.

In this data set of chemicals, several cause tumours in two or three of these selected target sites (Table 6). *ortho*-Anisidine hydrochloride not only causes tumours of the urinary bladder in each of the four sex-species groups but also causes tumours of the kidney and thyroid gland in male rats. Chloroprene represents the one chemical that causes tumours at each of the three sites in both sexes of rats, and kidney tumours in male mice as well. Still others have some interesting carcinogenic features (Table 7). For example, salicylazosulfapyridine is a goitrogen that does not induce tumours of the thyroid gland, yet does cause tumours of the kidney and urinary bladder (National Toxicology Program, 1997).

### Tumour rates in control animals

Background, naturally occurring, or 'spontaneous' tumour rates for these three target sites are low, compared to other more common spontaneous tumours in laboratory animals (Table 8) (Haseman *et al.*, 1990; Haseman & Elwell, 1996). One exception to these low control rates is C cell tumours of the thyroid gland in Fischer 344 rats.

Of course different species, strains, and genders often vary with respect to tumour incidence at a given organ site. As examples, C57BL/6 mice have a much lower liver tumour rate than do C3H mice, female Sprague-Dawley rats have considerably more mammary tumours than do female Fischer 344 rats, and Fischer 344 rats have a high rate of leukaemias and tumours of the testis. In fact, contra-intuitively perhaps, organ systems with very high control rates of tumours rarely if ever are associated with carcinogenic effects. Interstitial cell tumours of the testis in male Fischer rats, for instance, have never been evaluated by NTP as being a carcinogenic response to a chemical.

Therefore, one needs to know some historical context of background tumour rates before



Table 5. Chemicals associated with site-specific tumour induction in thyroid gland  
[follicular cell tumours unless noted otherwise]

Technical report no.	Chemical name	Salmonella <sup>a</sup>	Route	Levels of Evidence of Carcinogenicity <sup>b</sup>				Other tumour sites
				MR	FR	MM	FM	
021	Aldrin	-	Feed	[E]	[E]	P	N	Yes
112	3-Amino-4-ethoxyacetanilide	+	Feed	N	N	[P]	N	No
089	o-Anisidine hydrochloride	nd	Feed	[P]	P	P	P	Yes
069	Azinphosmethyl	+W,+	Feed	[E]	N	N	N	Yes
452	2,2-Bis[bromomethyl]-1,3-propanediol	-,+	Feed	[CE]	[CE]	CE	C	Yes
436	tert-Butyl alcohol	-	Water	SE	NE	[EE]	[SE]	Yes
308	Chlorinated paraffins: C12, 60% Cl	-	Gavage	CE	[CE]	OE	[CE]	Yes
467	Chloroprene	-	Inhal	[CE]	[CE]	OE	CE	Yes
285	C.I. Basic Red 9 monohydrochloride	+	Feed	[CE]	[CE]	CE	CE	Yes
407	C.I. Pigment Red 3	+	Feed	SE	SE	[SE]	NE	Yes
309	Decabromodiphenyl oxide	-	Feed	SE	SE	[EE]	NE	Yes
084	2,4-Diaminoanisole sulfate [C & F cell] <sup>c</sup>	+	Feed	[P]	[P]	[P]	[P]	Yes
149	N,N'-Diethylthiourea	-	Feed	[P]	[P]	N	N	No
388	Ethylene thiourea [ETU]	-,+W	Feed	[CE]	[CE]	[CE]	[CE]	Yes
374	Glycidol	+	Gavage	[CE]	[CE]	CE	CE	Yes
271	HC Blue 1 <sup>*</sup>	+	Feed	EE	SE	E	E	Yes
009	Heptachlor	-	Feed	N	[E]	P	P	Yes
340	Iodinated glycerol	+	Gavage	[SE]	NE	NE	E	Yes
448	Isobutyl nitrite <sup>*</sup>	+	Inhal	CE	CE	SE	SE	Yes
331	Malonaldehyde, sodium salt	-	Gavage	[CE]	[CE]	NE	NE	Yes
428	Manganese sulfate monohydrate	-	Feed	NE	NE	[EE]	[EE]	No
408	Mercuric chloride <sup>*</sup>	-	Gavage	SE	EE	EE	NE	Yes
186	4,4'-Methylenbis[N,N-dimethyl]-benzenamine	+	Feed	[P]	[P]	E	P	Yes
248	4,4'-Methylenedianiline[DHCl][C & F cell]	+	Water	[P]	[P]	[P]	[P]	Yes
143	1,5-Naphthalenediamine [C & F cell]	+	Feed	N	P	[P]	[P]	Yes
443 & 468	Oxazepam	-	Feed	EE	NE	CE	[CE]	Yes
205	4,4'-Oxydianiline	+	Feed	[P]	[P]	P	[P]	Yes
016	Phosphamidon [C cell]	+	Feed	E	[E]	N	N	Yes
476	Primidone [Primaclone]	+	Feed	[EE]	NE	[CE]	CE	Yes
231	Stannous chloride [C cell]	-	Feed	[E]	N	N	N	No
209	2,3,7,8-Tetrachlorodibenzo-p-Dioxin	-	Gavage	[P]	P	P	[P]	Yes
131	Tetrachlorodiphenylethane [TDE]	-	Feed	[E]	N	N	N	No
033	Tetrachlorvinphos [C cell]	-	Feed	N	[P]	P	P	Yes
446	1-Trans-Delta-9-Tetrahydrocannabinol	-	Gavage	NE	NE	[EE]	[EE]	No
047	4,4'-Thiodianiline	+	Feed	[P]	[P]	[P]	[P]	Yes
037	Toxaphene	+	Feed	[E]	[E]	P	P	Yes
129	Trimethylthiourea	-	Feed	N	[P]	N	N	No
391	Tris[2-chloroethyl] phosphate <sup>*</sup>	-	Gavage	CE	CE	EE	EE	Yes
238	Ziram [C cell]	+	Feed	[P]	N	N	E	Yes

Number of chemicals causing tumours in **THYROID GLAND** = 39

<sup>a</sup> Salmonella results: + = Positive; +W = Weakly Positive; ? = Inconclusive; - = Negative

<sup>b</sup> [ ] = Animal group in which tumours of the thyroid glands were observed, CE = Clear evidence of carcinogenicity; P = Evidence of carcinogenicity; SE = Some evidence of carcinogenicity; EE or E = Equivocal evidence of carcinogenicity; NE or N = No Evidence of carcinogenicity; IS = Inadequate experiment; nd = no NCI or NTP data.

<sup>c</sup> [C cell] = chemical causes C cell tumours of the thyroid gland; F = Follicular cell.

<sup>\*</sup> Possible association in one sex of one species

Table 6. Chemicals causing tumours at two or three of the selected organs: kidney, urinary bladder, thyroid gland

Chemical	Kidney	Urinary bladder	Thyroid gland
<i>o</i> -Anisidine HCl	Male rat	Male rat, female rat, Male mice, female mice	Male rat
2,2-bis [Bromomethyl]- 1,3-propanediol	Male rat [?] Male mice	Male rat	Male rat Female rat
<i>tert</i> -Butyl alcohol	Male rat		Male mice [?] Female mice
Chlorinated paraffins: C12,60%Cl	Male rat		Female rat, Female mice
Chloroprene	Male rat, Female rat, Male mice	Male rat [?], Female rat [?]	Male rat Female rat
C.I. Pigment red 3	Male mice		Male mice
Glycidol		Male mice [?]	Male rat Female rat
Mercuric chloride	Male mice [E]		Male rat [?]
NTA	Male rat Male mice Female mice	Female rat	
NTA trisodium monohydrate	Male rat Female rat	Female rat	
<i>o</i> -Nitroanisole	Male rat Female rat	Male rat Female rat	
Oxazepam	Male rat [E]		Female mice
Salicylazosulfapyridine	Female rat	Male rat Female rat	
Tris[2-chloroethyl]phosphate	Male rat Female rat Male mice [E]		Male rat [?] Female rat [?]

[?], may have been related to chemical; [E], equivocal evidence of carcinogenicity

attempting to compare across species, strains, or genders – including humans. A further caution is selection of appropriate species and strains and genders when planning and designing long term carcinogenesis experiments, especially if one has information from previous long-term experiments, shorter toxicity studies, or structure-effect correlations regarding likely target organs. For instance, B6C3F<sub>1</sub> mice appear to be a relatively insensitive

model to study chemicals thought likely to cause kidney or urinary bladder tumours. Similarly, as other examples, one would be ill-advised to use the Fischer 344 rat to study a potential leukaemogen or a likely testicular tumourigen, or to use female Fischer rats to identify a possible carcinogen for the pituitary gland or mammary gland because of the high background rates of these tumours in this particular strain.



**Table 7. Interesting chemical grouping observations regarding chemicals causing tumours at these selected sites: kidney, urinary bladder, thyroid gland**

<b>Chemical</b>	<b>Observations</b>
1-Amino-2,4-dibromoanthraquinone	<b>Kidney and urinary bladder</b> , male and female rats
1-Amino-2-methylantraquinone	<b>Kidney</b> , male rat
1,4,5,8-Tetraamino-anthraquinone [CI Disperse Blue 1]	<b>Urinary bladder</b> , male and female rats
*2-Amino-4-nitrophenol	<b>Kidney</b> , male rat
*4-Amino-2-nitrophenol	<b>Urinary bladder</b> , male rat; equivocal female rat
o-Anisidine HCl	<b>Kidney</b> , male rat; <b>urinary bladder</b> , male and female rats and mice; thyroid gland, male rat
o-Toluidine HCl	<b>Urinary bladder</b> , female rat
Aspirin, phenacetin, and caffeine	<b>Kidney and urinary bladder</b> , equivocal female rat
Phenacetin	<b>Kidney and urinary bladder</b> , humans and animals
1,3-Butadiene	<b>Kidney</b> , female mice,
Chloroprene	<b>Kidney</b> , male and female rats, male mice; <b>urinary bladder</b> , male [?] and female [?] rats; thyroid gland, male and female rats
Isoprene	<b>Kidney</b> , male rats
*m-Cresidine	<b>Urinary bladder</b> , male and female rats
p-Cresidine	<b>Urinary bladder</b> , both sexes of rats and mice
2-Naphthylamine	<b>Urinary bladder</b> , several species including humans
*N-Phenyl-2-naphthylamine	<b>Kidney</b> , equivocal in male mice; metabolized to 2-naphthylamine
Sallylazosulfapyridine	<b>Kidney</b> , male rats; <b>urinary bladder</b> , male and female rats; goitrogen causing no tumours of thyroid glands
Tetrachloroethylene	<b>Kidney</b> , male rats
Tetrafluoroethylene	<b>Kidney</b> , male and female rats

\*Chemicals causing tumours only at this site.

## Discussion and conclusions

Organ site specificity is a relatively common outcome of carcinogenicity testing (Haseman *et al.*, 1986; Huff *et al.*, 1991b). That is, many chemicals frequently induce cancers in only one, two, or three organ-systems, and in some cases these organs are rodent-specific with no exact counterpart in humans. Conversely, in many other cases, chemicals affect carcinogenesis in multiple organs, in both genders, of multiple strains and of multiple species.

Target site concordance between laboratory animals and humans does occur with significant frequency. Wilbourn *et al.* (1986) and others (Tomatis *et al.*, 1989; Huff, 1994a, 1998, 1999) have shown remarkable tissue or organ concordance of carcinogenicity between those agents causing cancer in humans and that have been tested adequately in laboratory animals.

Correspondence between various tumour sites is not a common finding among animals exposed to chemicals; for instance, for the three target sites

Table 8. Background tumour rates for Fischer 344 rats and B6C3F<sub>1</sub> mice

Male rat	Female rat	Male mice	Female mice
<b>Kidney transitional cell tumours</b>			
2/1352 [0.14%] range = 0-2%	1/1348 [0.07%] 0-2%	1/1351 [0.07%] 0-2%	0/1349 0%
<b>Kidney tubule cell tumours</b>			
13/1352 [0.14%] range = 0-6%	1/1348 [0.07%] 0-2%	3/1351 [0.07%] 0-2%	4/1349 [0.03%] 0-2%
<b>Thyroid gland C cell tumours</b>			
195/1347 [14.5%] range = 4-35%	182/1347 [13.5%] 4-24%	2/1343 [0.15%] 0-2%	0/1340 0%
<b>Thyroid gland follicular cell tumours</b>			
28/1347 [2.08%] range = 0-8%	12/1347 [0.89%] 0-6%	26/1343 [0.15%] 0-6%	26/1340 [1.94%] 0-8%
<b>Urinary bladder squamous cell tumours</b>			
0/1337	0/1332	0/1325	0/1325
<b>Urinary bladder transitional cell tumours</b>			
4/1337 [0.30%] range = 0-2%	4/1332 [0.30%] 0-2%	1/1325 [0.08%] 0-2%	0/1325 0%

highlighted in this paper, only one combination is statistically associated: liver and thyroid gland; these rarely occur in the same sex-species group, and even more uncommonly in the same animal. Other organ combinations do occur with a statistical association, however: rat liver and rat Zymbal gland, mouse lung and rat/mouse mammary glands, and mouse lung and mouse forestomach, as examples (Haseman & Lockhart, 1993). These may be valuable when doing necropsy and histopathology because if one observes a grossly visible liver tumour in rats, then one could look more closely at Zymbal glands; likewise, if a liver tumour in mice is found, then step sections of the thyroid glands could be done. Whether this observational knowledge may lead to other insights remains to be discerned.

Other than ionizing radiation and perhaps dioxins (Huff, 1994b; Huff *et al.*, 1994), no other

chemicals or occupational exposure circumstances are yet known that induce cancer of the thyroid gland in humans. Further, urinary bladder carcinogens appear to be more common in humans than those inducing tumours of the kidney, although kidney tumours often go undiagnosed or undiscovered (Barrett & Huff, 1991; Huff, 1992). In addition, several other agents are suspected of causing cancer of the urinary bladder in humans, including chlorinated drinking water (Morris, 1995), or kidney cancers, including occupational exposures to trichloroethylene (Henschler *et al.*, 1995; Vamvakas *et al.*, 1998) and to gasoline (Lyne *et al.*, 1997).

Thus, in few instances are there compelling findings to support the notion that species-specific carcinogenic effects occur. Arsenic, for example, has been long cited as an example of an exposure circumstance causing cancer in humans (including

cancer of kidney and urinary bladder), and yet has not been shown convincingly to cause cancer in laboratory animals (IARC, 1987). At the same time, one must realize that arsenic has yet to be studied adequately in laboratory animals, and to make a final "exception" conclusion is premature (Chan & Huff, 1997; Huff *et al.*, 1998). Benzene carcinogenicity was another long believed exception to the human-animal paradigm until appropriate and adequate tests were done, showing carcinogenicity in animals in multiple sites, species, strains, and genders carcinogenicity in animals (Huff *et al.*, 1989; Maltoni *et al.*, 1989). Perhaps arsenic will prove to be similar.

More difficult to prove are "species-specific effects" in animals with little or no human data for comparison. In some cases, human data have accrued, dispelling long histories of discounting the initial animal results. Relevant examples include 1,3-butadiene (see Huff *et al.*, 1985 for the first animal data; see Matanoski *et al.*, 1990 for the first human evidence); dioxins (see Kociba *et al.*, 1978 for the animal findings; IARC, 1997 for human data; Huff *et al.*, 1994; Huff, 1994b); vinyl chloride (Viola *et al.*, 1971 and Maltoni & Lefemine, 1974 for first animal data; Creech & Johnson, 1974 for first human data).

Other instances of discovering carcinogenicity in animals and only subsequently in humans (Tomatis, 1979; Huff, 1993; Vainio *et al.*, 1995; IARC, 1997) should give us pause when attempting, by using posed mechanisms or theoretical rodent tumour specificity, to declare any agent that is clearly carcinogenic to animals as being irrelevant to humans. The IARC November 1997 Workshop (this volume) is a tentative beginning of how one might approach these difficult scientific and public health situations.

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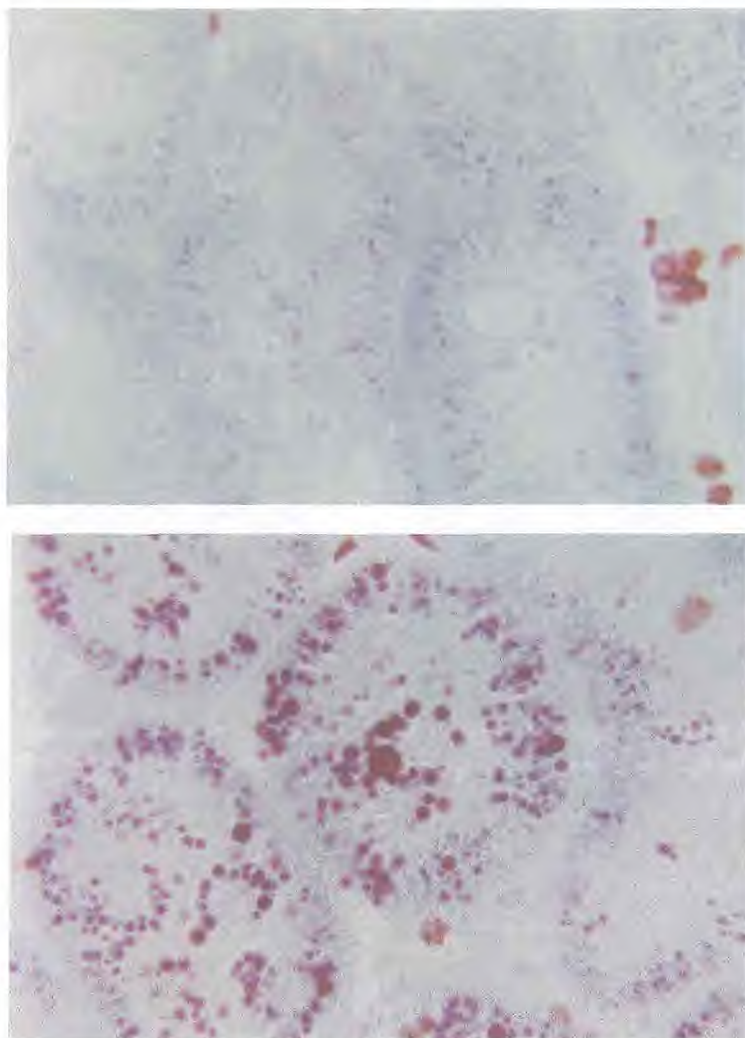
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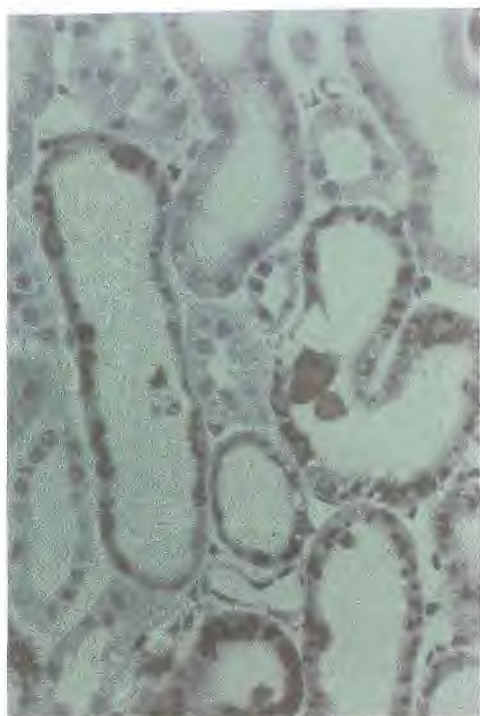


**Figure 1.** Histopathological characterization of the spontaneous and exacerbated formation of hyaline droplets, the hallmark of  $\alpha_2$ -globulin nephropathy, in male rat kidneys ( $\times 320$ ). When stained with Mallory-Heidenhain stain, small, lightly-stained eosinophilic droplets are observed in the proximal tubule epithelium of untreated male rats (top). No similar spontaneous droplet formation is observed in the kidneys of any other species. Following a single oral dosage of  $\alpha$ -limonene to male rats (150 mg/kg), the number, size and staining intensity of the droplets is markedly increased within 24 hours (bottom).

by accumulation of calcium hydroxyapatite in the thin limbs of Henle, is noted after several months of treatment (Figure 5) and there is an accelerated onset of the cortical changes typical of chronic progressive nephropathy typically seen in older male rats (Alden *et al.*, 1984; Trump *et al.*, 1984; Short *et al.*, 1987). With chronic exposure, sporadic foci of atypical hyperplasia, defined as a focal aggregation of morphologically abnormal cells

(Dietrich & Swenberg, 1993), can be observed in the proximal tubules, and the atypical foci progress to renal adenomas and carcinomas on prolonged exposure. Again, these changes are observed only in male rats. No similar renal changes have been observed in female rats or in any other species including mice, dogs or monkeys (US National Toxicology Program, 1983, 1986a, b, 1987a, b, 1990; Kitchen, 1984; Webb *et al.*, 1990).





**Figure 2.** Immunohistochemical localization of  $\alpha_{2u}$ -globulin in the proximal tubule cells. A polyclonal antibody to  $\alpha_{2u}$ -globulin was used to detect the presence of  $\alpha_{2u}$ -globulin within the protein droplets induced following  $\alpha$ -limonene treatment.



**Figure 3.** Electron micrograph of a renal proximal tubule cell showing the appearance of crystalloid inclusions in the cell following the development of  $\alpha_{2u}$ -globulin nephropathy.

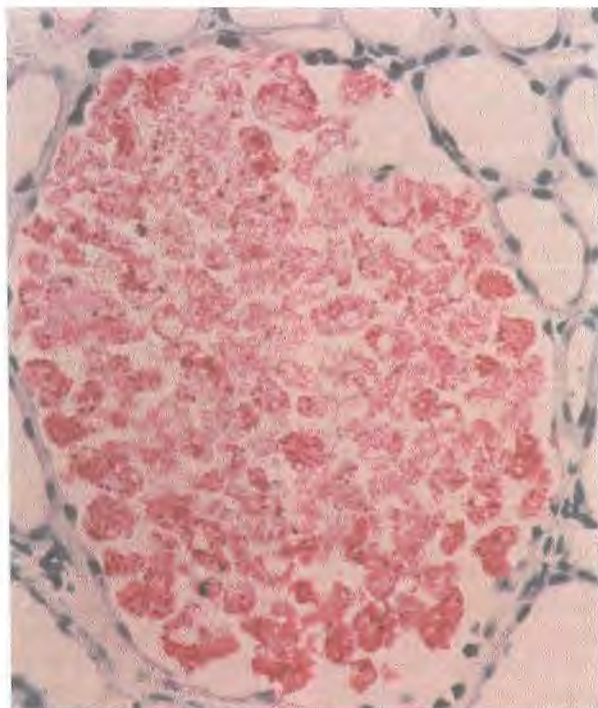
A major factor that determines the pathophysiological progression of this syndrome and the carcinogenic outcome is the duration of exposure. For example, if exposures are limited, such as in the 90-day stop study on decalin (Gaworski *et al.*, 1985), the hallmark hyaline droplet response is observed, but the renal changes do not progress to include the chronic sequelae and no tumours are seen. As discussed below, the increased cell turnover and sustained cell proliferation are prerequisites for, and ultimately involved in, the tumour development associated with this syndrome.

The protein droplet nephropathy described above is specific for male rats. If a protein droplet nephropathy is also produced in female rats (exception would be an androgen) or mice of either sex, it cannot be called  $\alpha_{2u}$ -globulin nephropathy. If other dose-related degenerative renal lesions are produced by a chemical in female rats or in mice, establishment of a causal relationship between  $\alpha_{2u}$ -

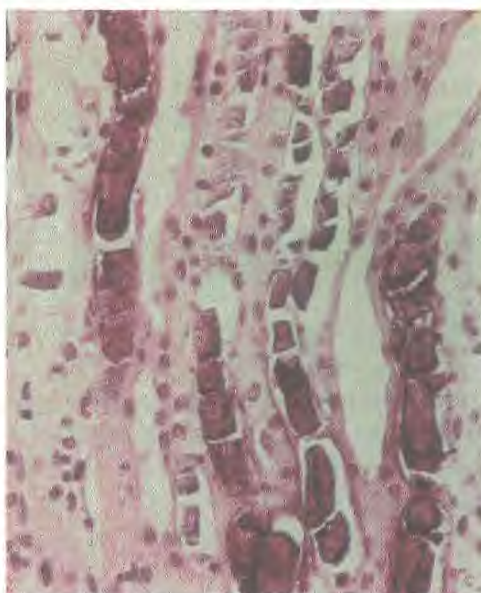
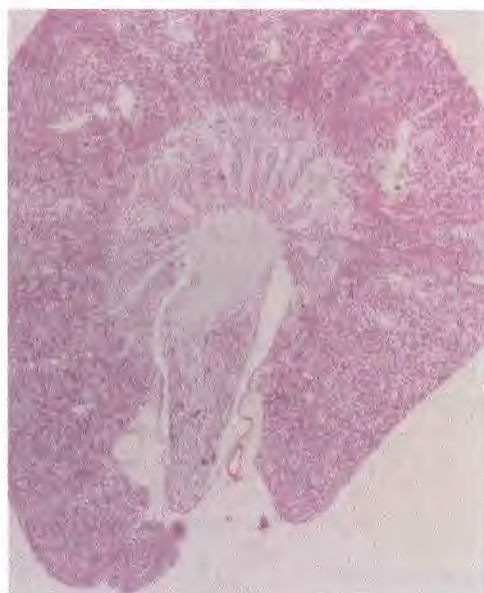
globulin and the nephropathy and/or carcinogenicity in male rats will require more extensive research. This distinction should not pertain to minor exacerbations of common spontaneous renal disease.

#### $\alpha_{2u}$ -Globulin

The histological characteristics of this syndrome were determined well before the role of  $\alpha_{2u}$ -globulin in renal toxicity and tumorigenesis was defined. Given that the droplets observed histologically appeared to contain protein, initial biochemical experiments focused on identifying what protein or proteins had accumulated in the droplets following exposure. Using two-dimensional electrophoresis, Alden *et al.* (1984) demonstrated that only one protein had accumulated in the kidney after chemical treatment, and this protein was subsequently identified as  $\alpha_{2u}$ -globulin. Moreover, immunohistochemical studies have localized the



**Figure 4.** Immunohistochemical localization of  $\alpha_{2u}$ -globulin within the granular casts which appear at the cortico-medullary junction following repeated administration of a compound (TMP) that induces acute  $\alpha_{2u}$ -globulin nephropathy.

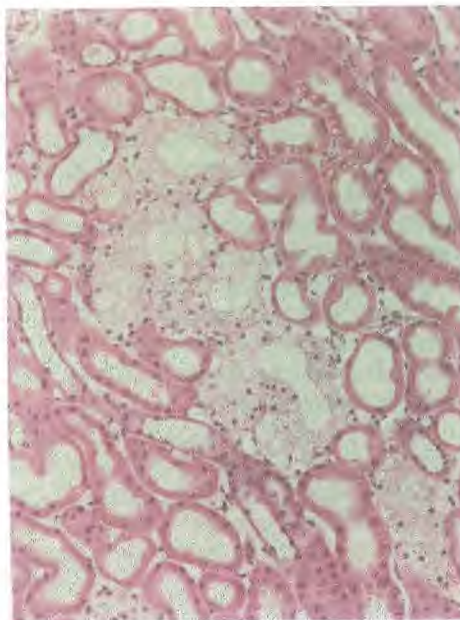


**Figure 5.** Chronic exposure to chemicals which cause  $\alpha_{2u}$ -globulin nephropathy leads to the development of papillary mineralization resulting from the accumulation of calcium hydroxyapatite in the thin limbs of the loops of Henle. The mineralization is readily apparent in cross sections (left) of the kidney, and upon microscopic evaluation of the medullary region (right).

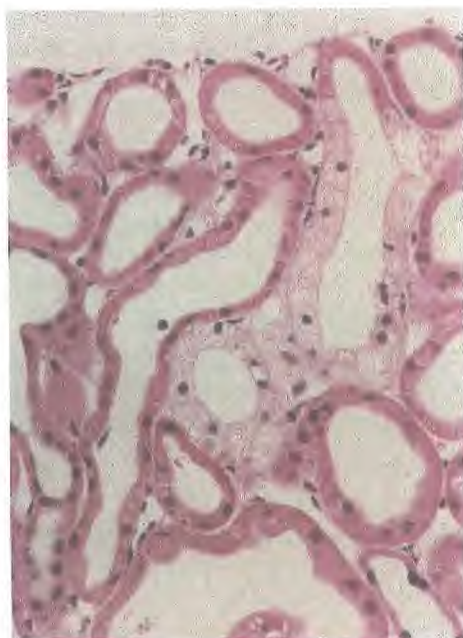


performed after the ability of these chemicals to cause  $\alpha_{2u}$ -globulin nephropathy had been characterized. In contrast, the renal tumour-promoting activity of sodium barbital and a major hydrolysis product, diethylacetylurea, was established well before these two chemicals were shown to cause  $\alpha_{2u}$ -globulin nephropathy in male rats. Briefly, in standard initiation-promotion protocols, both sodium barbital and diethylacetylurea have been shown to promote renal tumour formation in male Fischer 344 rats following initiation by *N*-nitrosodiethylamine (Diwan *et al.*, 1985, 1989a,b). Both agents also produced nephrotoxicity and increased renal cell proliferation (Ward *et al.*, 1991), but only after kidneys were evaluated by Mallory-Heidenhain staining and immunohistochemical analysis was their ability to induce  $\alpha_{2u}$ -globulin nephropathy recognized (Kurata *et al.*, 1994).

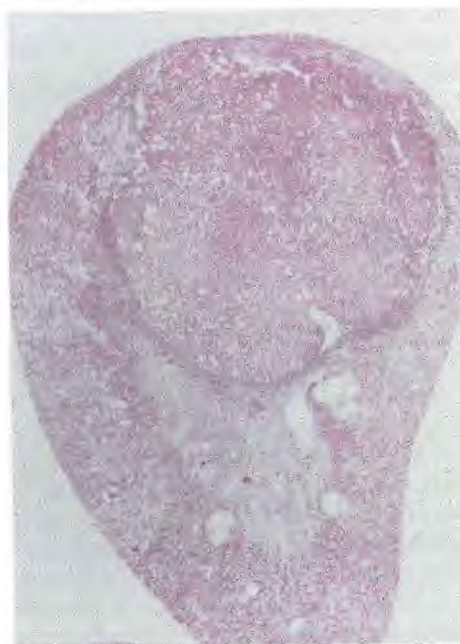
Collectively, the initiation-promotion studies provide compelling evidence that unleaded gasoline, TMP, *d*-limonene and sodium barbital act as renal tubule tumour promoters and that this



**Figure 10.** Microscopic appearance of atypical renal cell hyperplasia in a male Fischer 344 rat initiated with EHEN and promoted with *d*-limonene.



**Figure 9.** Microscopic appearance of an atypical tubule in a male Fischer 344 rat initiated with EHEN and promoted with *d*-limonene.



**Figure 11.** Microscopic appearance of a large renal adenoma in a male Fischer 344 rat initiated with EHEN and promoted with *d*-limonene.

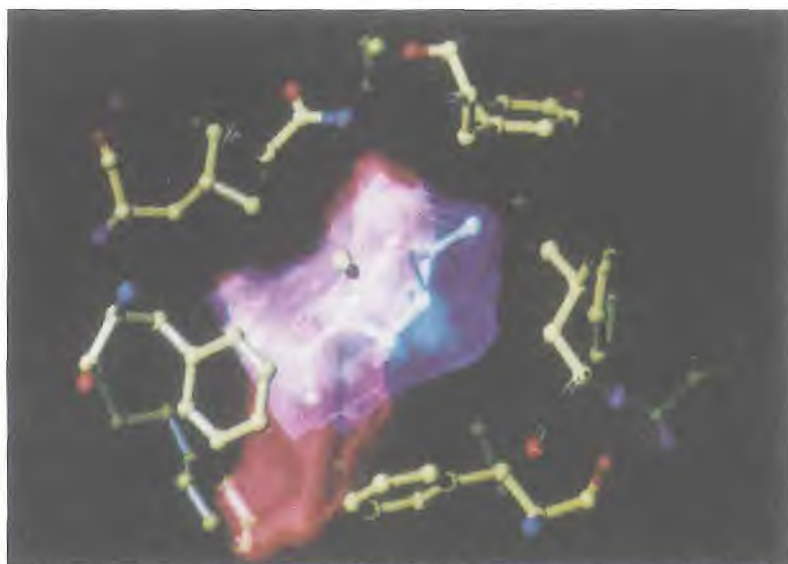


Figure 13. Depiction of the ligand-binding cavities in  $\alpha_{2u}$ -globulin (blue cloud) and mouse urinary protein (MUP) (red cloud) as determined by X-ray crystallography. For  $\alpha_{2u}$ -globulin, the shape of the binding cavity is essentially spherical, resulting from the orientation of two phenylalanine residues which close off the back of the cavity. The two phenylalanine residues in  $\alpha_{2u}$ -globulin are replaced by leucine and alanine, respectively in MUP, resulting in a binding cavity that is elongated and flattened (red cloud) relative to the binding pocket in  $\alpha_{2u}$ -globulin. Although the binding cavities of the two proteins are similar in volume, the actual overlap in shape (the highlighted, pink region) is very small. The spherical binding cavity in  $\alpha_{2u}$ -globulin is far less restrictive, thereby allowing for a broad spectrum of chemicals to bind to the protein.

$\alpha_{2u}$ -globulin simply serves to concentrate them in the male rat kidney (Melnick, 1992; Melnick & Kohn, this volume). In this manner, toxicity is observed because the delivered dose to the target organ is increased. Accordingly, this proposal is still dependent on the presence of  $\alpha_{2u}$ -globulin, and given the unique properties of the protein, the lack of human relevance still holds. At the same time, however, existing data do not support the contention that direct toxicity of the chemicals themselves is involved in renal toxicity and carcinogenicity. First, as noted earlier, the  $\alpha_{2u}$ -globulin-ligand complex is quite stable, arguing against the notion that the chemical can be released from the kidney without protein denaturation. Second, direct evaluation of TMP metabolites has indicated that they are not nephrotoxic (Borghoff & Lagarde, 1993). Finally, the most compelling data supporting the non-toxic effects of these chemicals exists for *d*-limonene, an agent currently being developed as a cancer chemotherapeutic agent (Crowell & Gould, 1994). In early animal studies to address its

anti-carcinogenic activity, *d*-limonene was fed in the diet to female rats at levels up to 100 000 ppm (10% of diet), and no toxicity was observed (Elegbede *et al.*, 1986; Elson *et al.*, 1988). Furthermore, in a 13-week study conducted by the US National Toxicology Program before the bioassay for *d*-limonene, female rats were given doses ranging from 150 to 2400 mg/kg by gavage and no renal toxicity was detected, whereas in male rats, renal lesions were observed at all dosages (US National Toxicology Program, 1990). Although there are no accompanying toxicokinetic data for these studies, it is reasonable to assume that the renal concentrations of *d*-limonene achieved at the extremely high dosages tolerated by female rats are at least equal to the renal concentrations achieved in male rats at much lower dosages.

It is also important to emphasize that the syndrome characterized by  $\alpha_{2u}$ -globulin nephropathy is specific to male rats and involves non-genotoxic mechanisms. Therefore, if a chemical is deemed to be genotoxic or causes renal injury and tumours in female rats or other species,