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ABSTRACT

Urinary bladder carcinogenesis in rodents bears numerous similarities to the diseases in humans. In rats, the process progresses through the morphologic stages of simple hyperplasia, papillary and nodular hyperplasia, papilloma, noninvasive, and invasive carcinoma. In mice, the pathogenesis can be similar or can follow a sequence of marked dysplasia with or without hyperplasia, leading to carcinoma *in situ* and ultimately to high-grade invasive carcinoma. Although the papillary and nonpapillary diseases appear to be related in rodents and in humans, they are distinct morphologically, biologically, and molecularly. Numerous classes of genotoxic chemicals have been identified as bladder carcinogens in rodents, and some of these have also been identified as carcinogenic in humans, most notably, aromatic amines, nitrosamines, and cyclophosphamide. In contrast, nongenotoxic chemicals appear to be highly specific with respect to species, strain, diet, agent, dose, and mechanism. For some, it is unclear whether the results at high doses in rodents can be extrapolated to low doses or to humans, e.g., chemicals that cause bladder cancer only at high doses related to the formation of calculi. Numerous observations in rodents can assist in identifying possible mechanisms involved for these nongenotoxic chemicals and therefore can be important for a rational evaluation of human risk.

Keywords. Bladder cancer; genotoxic chemicals; calculi; cell proliferation; hyperplasia

URINARY BLADDER CANCER PATHOGENESIS

Evidence increasingly is accumulating to support the hypothesis that transitional cell carcinoma of the urinary bladder actually represents 2 distinct, albeit related diseases (24, 44). The first type is papillary transitional cell carcinoma, which is usually low grade and noninvasive but tends to recur frequently following treatment. The second disease is the nonpapillary type that tends to be high grade and invasive, and it is frequently lethal to the patient due to local aggressiveness and/or distant metastases. In addition to differing pathogenetic processes, these 2 diseases appear to involve different molecular events (44). In animal models, these 2 diseases are similar to those in humans but tend to occur separately (9).

In rats, bladder cancer is nearly always of the papillary type (9). The pathogenetic process for this disease involves the initial appearance of simple hyperplasia that may be diffuse or focal. This gradually evolves into focal areas of nodular and/or papillary hyperplasia, followed by evolution to a papilloma and ultimately to noninvasive low-grade carcinoma. In contrast to the disease in humans (9, 24), the papillary low-grade tumors eventually evolve into high-grade lesions and invasion, but they only rarely metastasize. Some other distinctions between the rat model and the human disease include the morphologic appearance of the papillomas, particularly the larger ones. In rats, these more closely resemble the inverted type of papilloma of humans rather the typical fronds of papillary transitional cell carcinoma. In addition, as these lesions progress to papillomas and especially to carcinomas, there is an increasingly squamoid appearance, and frequently the carcinomas are a mixture of transitional cell and squamous cell elements. The more advanced lesions frequently have admixtures also of adenocarcinoma and undifferentiated carcinoma in addition to spindle cell elements occasionally being present.

In mice, the pathogenesis of bladder cancer differs between strains and with respect to carcinogen (3). In the Swiss mouse, the most frequent types of lesions progress through pathogenetic changes similar to that in the rat, beginning with simple hyperplasia and evolving into focal nodular and papillary hyperplasia, papillomas, and ultimately, carcinomas. There is a much greater tendency for the focal hyperplastic lesions to be nodular rather than papillary, in contrast to the rat, in which the changes are frequently a mixture of both or a predominance of the papillary type (42). This is particularly true in relationship to calculi, where diffuse papillary hyperplasia (papillomatosis) occurs in the rat, in contrast to diffuse nodular hyperplasia in the mouse.

In contrast to the papillary type of lesions induced in rats and most strains of mice with most carcinogens, a model has been developed that frequently involves the production of the nonpapillary type of transitional cell carcinoma in mice (5). *N*-butyl-*N*-(4-hydroxybutyl)nitrosamine (BBN) is administered either by intragastric gavage or in the drinking water to B6D2F$_1$ mice. The earliest changes in this model are the appearance of urothelial dysplasia with or without simple hyperplasia. This can range from mild nuclear changes to severe dysplasia and carcinoma *in situ*. The characteristic of this model is that it involves high-grade lesions. These *in situ*, high-grade lesions rapidly evolve into deeply invasive transitional cell carcinomas, usually grade 3 or 4 on a scale of 4, and they metastasize frequently (36). This model behaves very similarly to the high-grade, nonpapillary transitional carcinoma in humans. The deeply invasive lesions frequently have squamous or undifferentiated elements present.

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Although I have been describing the pathogenesis of transitional cell carcinomas in the urinary bladder, the pathogenesis appears to be similar throughout the urothelium, including the renal pelvis, ureter, and urinary bladder (24). Papillary transitional cell carcinomas occur much more frequently in the urinary bladder in most rat models compared to their incidence in the ureter or renal pelvis, unless there is obstruction of the urinary tract, which leads to more frequent development of renal pelvic lesions. In contrast, in the high-grade, nonpapillary disease in mice, although lesions occur more commonly in the urinary bladder, they also occur frequently in the renal pelvis.

As in humans, most malignancies of the urinary bladder in animals arise from the epithelium, and most are of transitional cell type (9, 39). As indicated above, squamous elements are frequent in both the rat and mouse models in more advanced lesions, and pure squamous cell carcinomas arise frequently in conditions related to chronic inflammatory disorders, also similar to the situation in humans, such as seen with schistosomiasis. Mesenchymal tumors of the urinary bladder are rare in rats and mice. Lymphomas and leukemias are uncommon in rats, but infiltrates of lymphoma or leukemia are relatively common in many strains of mice.

Human Urinary Bladder Carcinogens

Urinary bladder carcinogenesis has been studied extensively ever since the report in 1895 by Rehn suggesting a relationship between employment in the aniline dye industry in Germany and the development of bladder cancer (19). Subsequent research demonstrated that the class of chemicals referred to as aromatic amines were responsible for the urinary bladder carcinogenicity of these workers, and this has been documented in a variety of other occupational settings (7). The aromatic amine carcinogens include 2-naphthylamine, 4-aminobiphenyl (ABP), and benzidine and related benzidine-derived azo dyes. The experimental model most closely resembling the relationship of aromatic amines to human bladder cancer has been the dog, largely because of similarities in metabolic activation, excretion, and tumor development. Nevertheless, a variety of aromatic amines have been demonstrated as rodent bladder carcinogens; 2-acetylaminofluorene (2-AAF) has been studied most extensively in rodents as a model compound. Although this chemical and other aromatic amines most commonly induce liver cancer in rodents, 2-AAF and several other aromatic amines produce variable incidences of bladder cancer, also.

More recently, additional aromatic amines have been identified as likely human bladder carcinogens, including phenacetin-containing analgesics, ortho-toluidine, and 4,4′-methylene bis (2-chloroaniline) (MOCA) (19). Based on experimental evidence (7), there is also some suggestion that the corresponding nitroaromatic compounds, which are metabolized to a similar intermediate as the aromatic amines, may also be human bladder carcinogens, although the evidence is considerably weaker.

The metabolic activation of these aromatic amines and aromatic nitro compounds is through the formation of the *N*-hydroxylamine (31), which is further activated by various routes to reactive electrophilic *N*-ester metabolites. Depending on the target organ, different ultimate metabolic steps appear to be involved; for the urinary bladder, it appears that *N*-glucuronidation is an obligate step (31). The formation of the *N*-hydroxylamine and reactive metabolites occurs through the P_{450} enzyme system in the liver but also can be accomplished by various other routes of oxidation or peroxidation, including by prostaglandin H synthase (49).

DNA adducts are formed by the reactive metabolites that lead to mutations, and ultimately these result in the generation of cancer. For aromatic amines such as 2-AAF, the formation of DNA adducts appears to be linear with respect to administered oral dose, even extending to extremely low doses (5 ppm of the diet) (40). However, as demonstrated in the ED_{01} megamouse study utilizing 2-AAF administered to more than 24,000 female mice, the dose–response for generation of bladder tumors is nonlinear, following a sigmoidal-shaped curve (45). Although DNA adducts are generated at extremely low doses, tumors do not begin to be detected until the dose reaches 60 ppm of the diet, even with a detection level of 1% above background.. The reason for this appears to be the necessity for generating hyperplasia to produce adequate numbers of DNA replicative events to induce a detectable incidence of tumors (15). Increased proliferation does not occur until the dose level of 60 ppm of 2-AAF in the diet, exactly the same dose at which tumors begin to appear. The mouse bladder epithelium contains a very small number of basal stem cells in the epithelium (estimated at less than 10^5 per mouse bladder).

The interaction between DNA adduct formation and cell proliferation has also been demonstrated utilizing different agents to produce the different events (18). *N*-[4-(5-nitro-2-furyl]-2-thiazoly]formamide (FANFT) is also genotoxic, leading to DNA adduct formation and mutagenic events in the bladder epithelium. At doses of FANFT below 0.01% of the diet, significant incidences of tumors are not detected in the 2-yr bioassay compared to controls. It is only above this dose that a detectable incidence of tumors is produced, and like 2-AAF, it is at these higher doses that a hyperplastic response is produced along with an increased labeling index as detected by incorporation of tritiated thymidine. Administration of FANFT at 0.005% of the diet produces no increase in tumors compared to controls. Similarly, administration of sodium saccharin as 5% of the diet to rats does not produce a detectable incidence of tumors in a 2-yr bioassay (see below), but a mild hyperplastic response is produced. When this low dose of FANFT (0.005%) is coadministered with 5% sodium saccharin, an increased incidence of bladder tumors is produced (34). Thus, there is a strong synergy between the mild genotoxic effect of a low dose of FANFT combined with the cell proliferative effect of sodium saccharin.

The interaction between genotoxic and proliferative inducing agents is common in human carcinogenesis (11). For the urinary bladder, cigarette smoking is the major etiologic factor in industrialized human populations (19). This is partly due to production of ABP and other aro-

matic amines in cigarette smoke that leads to the formation of DNA adducts in the bladder epithelium. However, the amounts present in cigarettes (nanograms per cigarette) are not adequate to produce the high incidences seen in cigarette smokers based on earlier studies involving occupational exposure to ABP. However, cigarette smoking also increases the proliferation of the bladder epithelium, although the specific substance or substances producing this response are unknown (4). Nevertheless, the mild genotoxic exposure due to low levels of ABP and other aromatic amines in cigarette smoke combined with the cell proliferative response in the bladder epithelium of cigarette smoke is synergistic and produces a significant incidence of bladder cancer.

Other chemicals have also been identified as human bladder carcinogens, including cyclophosphamide and related compounds (chemotherapeutic agents) (19), and more recently there has been evidence presented that exposure to high levels of arsenicals also increases the incidence of bladder cancer in humans (1) and in experimental models (48).

In rodent models, the most commonly used bladder-specific carcinogens that have been investigated are BBN and related nitrosamines and FANFT and related nitrofurans. Metabolic activation of these chemicals has not been delineated as thoroughly as for the aromatic amines, but nevertheless, they are clearly genotoxic, are metabolically activated to reactive electrophiles, produce DNA adducts, and ultimately produce mutations. Animals that have been administered these compounds can actually be quantitatively assessed for exposure based on the mutagenicity of their urine. The morphology of the lesions produced by any of these agents is similar in the rat but differs in the mouse, as described above.

Nongenotoxic Bladder Carcinogens

Chemicals that by themselves or through a metabolite do not bind to DNA are referred to as nongenotoxic carcinogens. To produce abnormalities in the genome, the ultimate step in carcinogenesis, errors in DNA need to be produced indirectly by these agents. This occurs during DNA replication because replication does not have 100% fidelity (14, 16). Under usual circumstances, the error rate is extremely rare during DNA replication, but when this is multiplied times the enormous number of DNA replications that occur in a target tissue during a lifetime, an adequate number of mistakes can be generated to lead to an increased incidence of cancer in the target tissue. Importantly, the errors in DNA replication must occur in a pluripotential (stem) cell population, which in the bladder is represented by a fraction of the basel cell population of the urothelium.

It is also important to keep in mind that it is the number of DNA replications that is important, not the rate of replication. This has been misunderstood in numerous publications during the past decade, with an emphasis being placed on labeling index as an indicator of cell proliferation. The labeling index merely reflects the rate of replication, not the actual number. The labeling index is defined as the number of labeled cells (for DNA replication, this is usually the number of cells incorporating tritiated thymidine or bromodeoxyuridine) divided by the total number of cells of that population. By simple arithmetic calculation, the number of labeled (replicating) cells equals the labeling index times the total number of cells. Thus, the total number of cell replications can be increased by either increasing the labeling index and/or increasing the number of cells in the population (hyperplasia). Under most circumstances, both labeling index (rate) and cell number increase together, but circumstances do occur where one or the other increases, or one may actually go down when the other increases. The total number of DNA replications is the important parameter.

The number of DNA replications can be increased either by increasing the number of cell births or decreasing the number of cell deaths. Cell births can be increased either by direct mitogenic stimulation, or more commonly for epithelial tissues in response to chemicals, there is toxicity followed by regenerative proliferation. Decreased cell deaths can be produced by inhibiting apoptosis or inhibiting differentiation (which ultimately is another cell death process). Effects on cell death have only recently begun to be investigated, and interesting data have been forthcoming. Nevertheless, although this is another mechanism, ultimately, it has an affect on the number of cell divisions that occur in a target tissue. By inhibiting either apoptosis or cell differentiation there is an accumulation (increased number) of cells in the stem cell population of the tissue. Even if these are proliferating at the normal rate, the total number of replications is increased because of the increase in number of cells.

The best understood process leading to toxicity and regeneration in the bladder has been related to the production of calculi in the urinary tract (6, 8, 10, 20, 27, 32, 35, 38, 41). Calculi can be produced in the urinary tract by a variety of means, including surgical implantation of pelleted material, alteration of physiological processes, precipitation of the administered chemical or one of its metabolites, or a combination of these. Regardless of the mechanism leading to the formation of calculi in the lower urinary tract, the results are the same. There is abrasion of the urothelium leading to erosion and ulceration of the epithelium with hemorrhage, inflammation, and regeneration. The extent to which this occurs is dependant on the coarseness of the surface of the calculus that is implanted or formed, by its size and multiplicity, and by the ability to retain the material in the rodent bladder without causing complete obstruction of the urinary tract. Because rodents are horizontal quadripeds, in contrast to humans that are vertical bipeds, calculi can lodge in the dome of the bladder without causing complete obstruction and can be present for several months to the entire lifetime of the animal (20).

In response to the severe damage to the epithelium, there is a tremendous regenerative response leading to extensive papillary hyperplasia (papillomatosis) in the rat and diffuse nodular hyperplasia in the mouse. These evolve into formation of papillomas and ultimately carcinomas. In a study (30) involving surgical implantation of paraffin wax pellets into the mouse bladder, the incidence was 10.6% at the end of 1 yr, 26.7% after 18 mo, and 53.8% after 2 yr. The incidence of tumors in rats and

mice in response to the presence of calculi varies considerably for different agents, species, strains, and even from experiment to experiment. All of the variables contributing to this variation have not been delineated. Overall, rats appear to be more susceptible than mice to both the proliferative response as well as the ultimate development of tumors.

An important consideration in extrapolating results from rodent animal studies to humans for chemicals producing bladder cancer via the production of urinary tract calculi is that it is only a high-dose phenomenon (41). An adequate amount of material must be administered to produce a concentration of the critical substances in the urine to lead to precipitation and ultimately formation of calculi. This can be influenced by the overall composition of the urine, including the high osmolality of rodent urine as well as the high urinary concentration of protein and the solubility of different substances affected variably by urinary pH. Regardless, a large amount of the administered substance is usually required to produce an adequate concentration of the material that ultimately leads to the formation of calculi. If less than this amount of material is administered to the test animal, no calculus forms, there is no toxicity or regeneration, and there is consequently no tumor formation. If humans are exposed to only small amounts of the agent, levels that are inadequate for formation of calculi in the urine, then there is no carcinogenic hazard for the human population. This approach to risk assessment for this class of compounds has been accepted by the United States Environmental Protection Agency following extensive investigations and deliberations of a working panel on this subject. The panel's work was published as multiple articles in an issue of *Food and Chemical Toxicology* (September 1995) (6, 8, 10, 20, 27, 32, 35, 38, 41).

In addition to calculus-produced urinary tract toxicity and regeneration, more subtle forms of solid-state carcinogenesis can also occur. Microcrystalluria can be produced by a variety of substances, including silicates (37) and carbonic anhydrase inhibitors (29). These lead to erosion of the bladder epithelium, usually without full thickness ulceration, but nevertheless, this results in regenerative hyperplasia, although usually without inflammation. Ultimately, a low but significant incidence of bladder tumors is produced. This has been studied most extensively with silicates, which can cause urinary tract lesions not only in rodents but also in various domesticated animals, such as sheep (23).

An even more subtle form of solid-state carcinogenesis in the rodent is not only restricted to high doses but is also rat specific. This involves the formation of amorphous calcium phosphate precipitate in the urinary tract following administration of high doses of various sodium salts (12, 22) such as sodium saccharin and sodium ascorbate. Administration of the salts at doses equimolar to 5% sodium saccharin in the diet produces a calcium phosphate-containing precipitate in the urine that is cytotoxic to the urothelium, leading to necrosis of the superficial layer of the bladder epithelium, erosion without inflammation, and a relatively slight regenerative hyperplasia. Administration is generally required beginning at birth and continuing through the lifetime of the rat rather than beginning at 7 or 8 wk of age as is typically done in a 2-yr bioassay.

Because urinary pH of 6.5 or greater is required for the formation of the precipitate, any treatment that leads to acidification of the urine inhibits the formation of the precipitate, inhibits the formation of the proliferation, and inhibits the formation of the tumors. Thus, administration of the parent acid of the sodium salt (such as acid saccharin or ascorbic acid), administration of the sodium salt in AIN-76A semisynthetic diet, or coadministration of the sodium salt with high doses of ammonium chloride produces acidic urine (less than 6.0) and inhibits the formation of the precipitate, the regeneration, and the tumorigenicity of these compounds (22).

For formation of the precipitate, high concentrations of protein in the urine are also required. Male rats with α_{2u}-globulin in the urine in addition to high concentrations of albumin are more susceptible to the effect of sodium salts than are female rats. Administration of sodium saccharin or sodium ascorbate to the NBR rat, which does not excrete large amounts of α_{2u}-globulin, leads to much less of a response than when administered to F344 male rats (26, 47).

Mice do not respond to the sodium salts with a hyperplastic or tumorigenic response, although they also have high concentrations of urinary protein, excrete large amounts of the administered anion in the urine, and produce a urinary pH above 6.5 (22). In contrast to the rat, however, the mouse has significantly lower urinary concentrations of calcium, magnesium, and phosphate, which clearly influences the solubility of calcium phosphate and influences the formation of the precipitate and the responsiveness to these salts (2). Because the sodium salts are effective only in the rat and only at high doses (for sodium saccharin and sodium ascorbate, dietary levels $>1\%$ are required), they pose no carcinogenic hazard to humans. Administration of sodium saccharin to monkeys for 18–23 yr has recently been demonstrated to have no effect on the urinary tract, either with respect to precipitate formation, toxicity, proliferation, or tumorigenicity (46). In addition, numerous epidemiological studies have failed to show any hyperplastic (4) or tumorigenic (21) effect of sodium saccharin on the urinary bladder in humans.

Mechanisms for producing toxicity and regeneration in the urothelium that do not involve formation of calculi, microcrystalluria, or calcium phosphate-containing precipitate have also been demonstrated in rodents.

Administration of high doses of tributyl phosphate (TBP) produces severe erosion and ulceration of the bladder epithelium in rats with marked regenerative hyperplasia and ultimately an increased incidence of bladder tumors (3). The regenerative response is similar to that seen with calculi, with formation of a diffuse nodular and papillary hyperplasia in the rat bladder. This does not occur in the mouse. The regenerative response to ulceration is the same regardless of what the inciting stimulus is, whether calculi, surgery, freezing, or chemical. TBP does not produce calculi, crystalluria or amorphous precipitate but appears to act as a severely toxic (corrosive)

chemical to the bladder epithelium. The proliferative response is similar to that seen following direct intravesical instillation of concentrated acetic acid (25), which produces ulceration and marked regenerative hyperplasia. If the stimulus is removed, whether acetic acid or TBP, the ulcer eventually heals and the epithelium returns to normal. A similar phenomenon can be seen with substances like acetic acid when applied to a variety of epithelia, including the skin. If the stimulus is toxic enough, there is ulceration, regeneration, and ultimately repair and healing if the stimulus is removed.

o-Phenylphenol (OPP) also produces hyperplasia of the rat bladder epithelium and ultimately the production of bladder tumors (28, 43). Neither it nor its sodium salt produces calculi, microcrystalluria, or precipitate formation in the urine, but there is cytotoxicity of the superficial layer of the bladder epithelium and regeneration. This occurs only at high doses (≤8,000 ppm of the diet). It would appear that OPP or, more likely, one or more of its metabolites is cytotoxic to the bladder epithelium leading to urothelial toxicity and regeneration. The details of this have yet to be delineated.

All of the nongenotoxic chemicals described so far produce bladder hyperplasia and tumor formation based on toxicity and regeneration. Another means of increasing proliferation is for a chemical or its metabolites to directly produce a mitogenic response in the target tissue without acting through a toxic process. We recently demonstrated that propoxur administered to rats at high doses produces urothelial hyperplasia and ultimately tumors (13). Somewhat surprisingly, there was no evidence of toxicity in the bladder epithelium when examined by light or scanning electron microscopy. Nevertheless, there was significant proliferation as detected morphologically and by determination of increased bromodeoxyuridine (BrdU) labeling index. Although the mechanism has yet to be determined, mitogenic responses usually occur because of effects directly on growth factors and/or hormones.

Mechanistic Considerations in Evaluating Bladder Carcinogens

Based on the above description and examples, an approach to evaluating the possible mechanisms involved in carcinogenesis by chemicals can be suggested (14, 16, 17).

The most important determination is the evaluation of the genotoxicity of the chemical and, if possible, its metabolites. This can be done utilizing a routine battery of genotoxicity assays but also should involve an evaluation of the structure–activity relationships of the chemical and its metabolites. A variety of computerized programs have been developed for structurally evaluating the mutagenic and carcinogenic activity of chemicals, and this can be highly predictive of the ability of a chemical to react ultimately with DNA and produce mutagenicity and carcinogenicity by that mechanism (33). These programs are specifically designed to detect genotoxic (DNA-reactive) types of carcinogens. If a chemical is genotoxic, an evaluation of human cancer risk can be performed balancing it against potential benefits. The dose–response is likely to be influenced by cell-proliferative effects, such as those described above for 2-AAF and FANFT, but nevertheless, the extrapolation usually implies that DNA adduct formation will occur even at the lowest dose.

If the chemical is suggested as not likely to be DNA reactive based on a structure–activity evaluation and is nongenotoxic based on a standard battery of *in vitro* and *in vivo* assays, it is then classified as nongenotoxic and acts by increasing cell proliferation. This can be produced, as described above, either by increasing cell births or decreasing cell deaths. If increasing cell births is by a mitogenic response, an influence on specific cell receptors is highly likely, although not always the case. Although investigations on inhibiting apoptosis and cell differentiation are beginning to show a relationship of some chemicals to the induction of cancer, this has yet to be demonstrated for the bladder.

Toxicity is most frequently the cause of increased proliferation in the bladder secondary to exposure to nongenotoxic chemical agents. As described above, this can be produced by a variety of mechanisms and can be relatively easily determined for a given chemical.

An approach to evaluating a nongenotoxic chemical that produces bladder cancer in a standard 2-yr bioassay is suggested as follows: If not already performed, evaluation for genotoxicity must be accomplished first. After demonstrating that the chemical is a nongenotoxic compound, the next step is to re-evaluate the histopathology from the 90-day study or from other short-term studies that are available. Examination of the bladder for the presence of calcification, inflammation, erosion, or ulceration can be done on routine hematoxylin and eosin–stained slides from tissues fixed in formalin and embedded in paraffin. Any information available on urinary chemistry, particularly urinary pH, can also be useful in evaluating possible mechanisms. Any data generated by evaluation of urinary chemistries by dipstick methodologies, however, are essentially useless for determination of mechanism (10). Next, an evaluation from the observations made during the short- and long-term bioassays for presence of calculi and/or hematuria should also be investigated. Calculi frequently are passed during the course of an experiment by the animals without being detected by the investigators. This can occur because the calculi are either too small for gross visual detection or they have dissolved in the urine.

After these preliminary investigations have been accomplished, some idea of possible mechanisms may be ascertained and may provide suggestions for further investigations. Otherwise, a repeat of the 90-day study is useful but with specific investigations designed for the urinary tract. Administration of multiple doses is useful, including those that are tumorigenic and nontumorigenic in the chronic bioassay. A dose below which urinary tract hyperplasia occurs is also useful for interpretation. Usually, during the course of administration of the chemical in a repeat 90-day study, urine is collected once or twice prior to the completion of the study. It is best if the urine can be collected as a fresh voided specimen early in the morning shortly after the lights have gone on in the animal room (10). Rodents are nocturnal animals, and the effects of a test chemical administered in the diet or

drinking water on the urine occur during the night while the animals are eating and drinking. Measurement of urinary pH by microelectrode can be accomplished on a drop of urine, and multiple urinary chemistries can be accomplished on a sample as small as 25–50 ml of urine. If essential, urine can be collected over a period of time, but this should be performed during the nighttime hours rather than during the day. Care must be exercised in the collection of the urine to avoid contamination and other difficulties in interpretation. A portion of the urine should then be centrifuged and examined for the presence of calculi, crystals, and amorphous precipitate. This is best accomplished by transferring a portion of the sediment to a filter for examination by scanning electron microscopy with attached X-ray reflective spectroscopy. Thus, an estimate can be made not only of quantitative differences in the amount of crystalluria or precipitate present, but qualitative differences in composition can be determined. At the end of the 90-day study, the bladders are collected following intraperitoneal administration of BrdU 1 hr prior to death. The bladders are fixed in Bouin's fixative that allows for processing for light microscopic evaluation of hematoxylin and eosin–stained pathologic sections, immunohistochemical evaluation of the labeling BrdU labeling index, and scanning electron microscopic examination of the bladder surface. Utilizing these methods, a qualitative and quantitative estimate of bladder epithelial proliferation and evidence of superficial or deeper erosion or evaluation of the urothelium and regeneration can be determined.

Based on these investigations, a reasonable assessment can be developed to determine whether toxicity is occurring, whether calculi, crystals, or precipitate are involved in the toxicity, or whether a direct mitogenic effect is occurring. If toxicity is occurring without the formation of solid materials in the urine, then the chemical or metabolites are likely to be the cause of the toxicity. Alterations in urinary physiology as causatively related to bladder carcinogenesis usually imply formation of solids in the urine. Further delineation of the mechanism can then be pursued, if appropriate, based on the results of these relatively short and inexpensive studies.

By having an assessment of these effects with respect to dose, the potential for extrapolation of a dose–response to humans can also be made and a more rational assessment of carcinogenic risks can be made.

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