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Promotion in Urinary Bladder Carcinogenesis

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Aromatic amines, including 2-naphthylamine, 4-aminobiphenyl and benzidine, are known urinary bladder carcinogens in man and other species, but in rodents, aromatic amines and amides have usually induced liver tumors, occasionally also with tumors of the bladder and other tissues. Variations in organ specificity are related to differences in metabolism; for the production of bladder tumors, the rates of acetylation and deacetylation appear to be critical. Bladder specific carcinogens in rodents and other species have subsequently been identified, including *N*-butyl-*N*-(4-hydroxybutyl)nitrosamine (BBN) administered in the drinking water, *N*-[(4-(5-nitro-2-furyl)-2-thiazolyl]formamide (FANFT) in the diet and *N*-methyl-*N*-nitrosourea (MNU) instilled intravesically. When low doses of several bladder carcinogens (BBN, FANFT, 2-acetylaminofluorene, and 3,3′-dichlorobenzidine) are administered to rats, either simultaneously or sequentially, a synergistic effect is observed with respect to bladder carcinogenesis. In addition, a multistage carcinogenesis process has been demonstrated for the rat bladder using MNU or FANFT as initiators, and dietary sodium saccharin, sodium cyclamate, or tryptophan as promoters. Calculi (or pellets) appear to enhance the promotion process but are not necessary for it to occur. Recent studies also indicate that urine has a role in the promoting process. The urothelium normally has a very low mitotic rate. If mucosal proliferation is increased, such as during fetal development or during regeneration and repair of an ulcer, the bladder appears to be considerably more sensitive to the effects of promoting substances. For example, if sodium saccharin is administered to rats after ulceration of the bladder, even without prior administration of an initiator, bladder carcinoma develops. Under these conditions, the substance appears as a carcinogen. Human populations with increased bladder epithelial proliferation, such as fetus, infants, patients with bacterial cystitis or men with partially obstructive prostatism, may have increased susceptibility to the action of carcinogenic or promoting stimuli.

Introduction

Urinary bladder carcinogenesis has been associated with exogenous chemicals since the report by Rehn in 1895, describing the association of bladder cancer with the German aniline dye industry (*1*). Hueper and colleagues (*1*) were able to demonstrate that one of the carcinogens to which these workers were exposed was 2-naphthylamine. These experiments were undertaken with dogs and involved long periods of time and great expense. Subsequently, aromatic amines have been of central importance in the study of chemical carcinogenesis in general and bladder carcinogenesis (*1, 2*), specifically. Such studies have been involved in the use of experimental animals and humans, and have resulted in the identification of a number of aromatic amines and amides of significance to human bladder carcinogenesis: 2-naphthylamine, benzidine, 4-aminobiphenyl, phenacetin-containing analgesics (*3*), and chlornaphzine. The discovery of the urinary bladder carcinogenicity of 2-acetylaminofluorene (AAF) in rodents provided an experimental model for studying urinary bladder carcinogenesis in the rat (*1*). However, AAF is not a bladder-specific carcinogen, inducing also tumors of the liver, mammary gland, Zymbal's gland, as well as other organs depending on the species. Metabolic pathways involved in the activation of these compounds have been studied extensively and involves the generation of reactive electrophilic reagents as initially reported by Miller and Miller (*4*). However, it was not until the discovery of bladder-specific carcinogens in rodent species that additional advancements in the mechanisms of bladder carcinogenesis could be achieved. These bladder-specific carcinogens included *N*-butyl-*N*(4-hydroxybutyl)nitrosamine (BBN) administered in the drinking water (*5*), *N*-[4-(5-nitro-2-furyl)-2-thiazolyl]formamide (FANFT) administered in the diet

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(*6*) and *N*-methyl-*N*-nitrosourea (MNU) administered intravesicularly (*7*).

N-[4-(5-Nitro-2-furyl)-2-thiazolyl]formamide (FANFT)

FANFT was initially reported to be a urinary bladder specific carcinogen in Sprague-Dawley female rats (*6*), but was subsequently found to affect male and female rats as well as mice, hamsters, and dogs (*8*). The guinea pig, however, appears to be resistant to the carcinogenic effects of this compound and other nitrofurans. The course of bladder carcinogenesis due to FANFT in these various species is similar. The urinary bladder epithelium is normally three cell layers thick, with a large superficial cell layer, an intermediate cell layer and a basal cell layer resting on a basement membrane separating it from the underlying connective tissue (*2*). During carcinogenesis the normal three-layered urothelium progresses to a diffuse, simple hyperplasia followed by a focal nodular and papillary hyperplasia (*6, 9, 10*). It must be emphasized that through these stages the lesions can be reversible, although with proper stimuli they will progress to cancer (*10, 11*). Eventually, there is the formation of papillomas and noninvasive carcinomas, followed by the development of invasive carcinomas and occasionally metastases. The tumors are usually transitional cell in nature in these species, but squamous cell carcinomas are common in mice and focal squamous cell differentiation within the tumors is common, particularly in rats. Hydronephrosis is uncommon. Nevertheless, FANFT as well as BBN and MNU in these species are sufficiently similar to the human disease to provide considerable useful information. Although the organ specificity of these compounds is dependent to a large extent on their metabolic pathways (*4, 12, 13*), other factors may also be involved, including the presence of urine. Although BBN and FANFT, which are administered orally, are metabolically modified in the liver, it would appear that metabolic activation within the urothelium, the target tissue, may be of critical importance for the activation of these compounds. For example, it has recently been demonstrated (*14*) that a P-450 oxidase system is present in the urinary bladder epithelium which is capable of metabolically activating aromatic amines. More recently, it has also been demonstrated that other peroxidases, such as prostaglandin endoperoxide synthetase can also metabolically activate aromatic amines and nitro compounds, and that it is present in large quantities in the microsomes of the urinary bladder epithelium, including the rat bladder epithelium (*15*).

FANFT in the diet follows a dose response when chronically administered. If administered in the diet at levels of 0.2, 0.1, or 0.05% for 30 weeks, nearly 100% of the rats eventually developed bladder cancer. At the dose of 0.01% only 1 of 40 rats developed a bladder tumor, and at lower doses no bladder lesions were observed through 2 yr of observation (*16*). If administered at a constant dose, 0.2% of the diet, it would also appear that there is a dose effect if the time FANFT is administered is changed (*10, 17*). If it is administered in the diet for 12 or more weeks, progressive nodular and papillary hyperplastic areas are present in the bladder epithelium which eventually develop into carcinomas if the animals are observed for one to two years. If it is fed for 8 to 10 weeks, the nodular and papillary hyperplasia regresses, but the animals eventually develop bladder carcinomas in high incidence. When FANFT was administered in the diet for 4 or 6 weeks, few tumors were present in the bladder after two years (*18, 19*) and none were found in the bladder at earlier times.

Initiation and Promotion in the Urinary Bladder

When low doses of several bladder carcinogens (BBN, FANFT, AAF and 3,3′-dichlorobenzidine) were administered to rats simultaneously, a synergistic effect was observed with respect to the induction of bladder cancer (*20*). If the same chemicals at the same doses were administered sequentially, the results were similar (*21*). However, these experiments involved "complete" carcinogens. A more specific sequential carcinogenesis model involving noncarcinogens or cocarcinogens is the model of initiation and promotion.

Many of the characteristics of initiation and promotion have been demonstrated in the mouse skin model originally described in the 1940s (*22, 23,*). In the past decade, it has become apparent that a similar mechanism is involved in the carcinogenic process in a number of organs including the urinary bladder epithelium (*18, 24, 25*). However, prior to 1970, there was some evidence already available that such a mechanism was involved in urinary bladder carcinogenesis. These experiments involved the pellet implantation technique originally described by Jull (*2*). Clayson and Cooper demonstrated that, although some bladder tumors resulted from the implantation of a pellet into the bladder, the incidence of bladder tumors was greatly increased if the animal was previously treated with diphenylene oxide. Diphenylene oxide administered without subsequent pellet implantation into the bladder did not induce tumors. Bryan and Springberg (*26*) demonstrated that the administra-

tion of the 8-methyl ether of xanthurenic acid administered in a cholesterol pellet significantly increased the incidence of bladder tumors compared to the cholesterol pellet alone. In addition, they demonstrated that if the 8-methyl ether of xanthurenic acid was administered by subcutaneous injection to a mouse that also had a cholesterol pellet in the urinary bladder, a similar incidence of tumors was induced. However, it was not until the report by Hicks et al. (*24*) that a more classical model of initiation and promotion was demonstrated for the urinary bladder. In their experiments, a subcarcinogenic dose of MNU was injected intravesicularly, followed by the oral administration of sodium saccharin or sodium cyclamate in the diet. If administered alone, these compounds did not produce a significant incidence of bladder tumors. However, the sequential administration of MNU followed by sodium cyclamate or sodium saccharin resulted in a markedly increased incidence of bladder tumors.

In our laboratory, we have utilized the FANFT model in inbred Fischer male rats for the study of multistage carcinogenesis of the urinary bladder. FANFT has served as the initiator and is administered in the diet at a level of 0.2%. Promoting substances have included sodium saccharin (5% of the diet) and tryptophan (2% of the diet). Our initial study (*18*) involved the administration of FANFT for 6 weeks, followed by either sodium saccharin or DL-tryptophan, beginning either immediately after the FANFT or after a delay of 6 weeks during which time the rats were fed control diet. The results of this experiment are summarized in Table 1. Both chemicals served as promoting substances in this model, and the 6 week delay did not appear to significantly affect the results. During this 6 week period, the FANFT was completely excreted from the body (*27*) and the mild hyperplasia present after 6 weeks of FANFT administration regressed so that the bladder epithelium appeared histologically normal (*10, 17*). These data suggested that the two stage model of initiation and promotion was applicable to urinary bladder carcinogenesis and that initiation was irreversible, similar to findings in other models (*23*). However, 6 weeks of FANFT followed by control diet for 2 years produced one papilloma and four carcinomas of the bladder in 20 rats. Although these tumors were small and each bladder contained only one tumor (in contrast to the large and multiple bladder tumors with saccharin and tryptophan), this was an unacceptably high level of tumors for the "initiator only group". The experiment was repeated with the use of FANFT for 4 weeks and sodium saccharin or L-tryptophan (instead of the racemic mixture) as promoters (*19*). Results of this experiment are also shown in Table 1. Although the tumor incidence is considerably less than obtained after 6 weeks of FANFT, the combined treatment with FANFT for 4 weeks followed by sodium saccharin induced a significant incidence of bladder tumors. The L-tryptophan treatment, however, was not statistically significant, although with a larger group of animals this may achieve significance.

The promoting activity of sodium saccharin in this model is similar to that originally described in the MNU model (*24*) and subsequently in the BBN model by Ito and his colleagues (*25*). The results with tryptophan, although suggestive of promoting activity, are not conclusive. However, with mice, L-tryptophan administered after FANFT initiation also induced bladder tumors, although, again, the incidence was not statistically significant (*28*). DL-Tryptophan administered to dogs after aromatic amines, 2-naphthylamine and 4-aminobiphenyl, also was suggestive of promoting activity, although the number of dogs used was small (*29*).

Sodium Saccharin

The above experiments are strongly supportive of the initiation and promotion model in urinary

Table 1. Initiation-promotion for the rat urinary bladder using FANFT as initiator.[a]

	6 Weeks of FANFT			4 Weeks of FANFT		
		Rats with bladder tumors			Rats with bladder tumors	
Treatment group	No. of rats	Papilloma	Carcinoma	No. of rats	Papilloma	Carcinoma
FANFT→Saccharin	19	0	18	26	2	5
FANFT→Control→Saccharin	18	0	13			
Control→Saccharin	20	0	0	26	0	0
FANFT→Tryptophan	19	1	10	26	3	2
FANFT→Control→Tryptophan	20	4	10			
Control→Tryptophan	19	0	0	26	0	0
FANFT→Control	20	1	4	25	1	0
FANFT (long-term)	40	0	40	8	0	8
Control	42	0	0	27	0	0

[a]Data of Cohen et al. (*18*) and Fukushima et al. (*11,19*).

bladder carcinogenesis. However, the critical experiment wherein sodium saccharin is administered first followed by FANFT has not yet been completed. Nevertheless, the model of FANFT followed by sodium saccharin has many of the properties usually attributed to initiation and promotion. FANFT is a strongly mutagenic chemical (*30*) in various bacterial and cell culture assays, and the urine of animals administered FANFT contains strong mutagenic activity. Sodium saccharin (*31, 32*) and tryptophan (*33*), in contrast, are not mutagenic in these in vitro assays nor are the metabolites of tryptophan. Also, FANFT administration appears to irreversibly initiate the process since sodium saccharin and tryptophan could be administered with at least a 6-week delay after discontinuing FANFT administration (*18*). Another property of tumor promoters is the induction of cell proliferation in the target tissue regardless of whether the tissue has been initiated or not (*23*). Initial reports with sodium saccharin indicated that hyperplasia was an infrequent result of even high doses of the chemical (*24, 34*). However, using the highly sensitive techniques of scanning electron microscopy and autoradiography, we were able to demonstrate that sodium saccharin does increase the rate of proliferation in the bladder mucosa, but the increase is slight and multifocal (*35*). Multiple serial sections of the urinary bladder mucosa evaluated by light microscopy were able to eventually demonstrate simple hyperplastic areas. The changes induced by sodium saccharin detected by scanning electron microscopy and autoradiography are dose-responsive (*36*), with a range similar to that found by Ito and his colleagues (*25*).

The mechanism of sodium saccharin's effect on the urinary bladder is unknown at this time. It remains unclear as to whether sodium saccharin acts directly on the bladder mucosa or whether it alters the urine or other physiological system in such a way that it indirectly produces its effect. Initially, sodium saccharin was considered to be acting through the production of calculi or an increase in the size and/or the number of crystals in the urine (*32*). In our experiment, using 4 weeks of FANFT as initiator followed by sodium saccharin of L-tryptophan as promoter, we periodically examined the urine of these animals for the presence of calculi, the number and size of the crystals in the urine (predominantly "triple phosphate" crystals) and also the osmolality and various electrolytes (*37*). During the entire course of this experiment, calculi were not found in the urine, and the number and size of the crystals were not increased. The osmolality of the urine in animals fed sodium saccharin in the diet was somewhat less than that in the control animals, and the urinary volume was greatly increased, secondary to the increased fluid intake by these animals. The urinary sodium concentration was slightly increased compared to that in control rat urine, but the total amount of sodium excreted was considerably more than normal. These results are expected following the intake of a large amount of the sodium salt of a weak acid associated with an increased water intake. None of these changes in the urine were found in the rats fed L-tryptophan. However, an interesting finding in the urine of rats fed either sodium saccharin or L-tryptophan was decreased glucose concentration in the urine and subsequently also in the serum.

In addition to the changes in the urinary bladder, we have recently demonstrated that sodium saccharin also has effects on the rat kidney. Male Fischer rats frequently develop an interstitial nephritis with age, particularly after the age of 18 months (*19*). Rats fed sodium saccharin infrequently showed these changes in the kidney even after 2 years (*19*), regardless of other treatment. This inhibition of the formation of interstitial nephritis was not observed in rats fed L-tryptophan.

In contrast to the frequent finding of interstitial nephritis in older male Fischer rats, the appearance of urothelial hyperplasia or tumors of the urinary bladder, ureters, or renal pelvis is unusual in control Fischer rats. In rats fed sodium saccharin, whether FANFT was previously administered or not, approximately half of the rats fed sodium saccharin developed marked hyperplasia of the renal pelvic structures (*38*). The hyperplasia in the renal pelvis was considerably more than in the urinary bladder, and frequently, there was nodular and papillary hyperplasia of the renal pelvic epithelium, particularly at the junction between the papilla and pelvis. FANFT initiation did not appear to alter the incidence of this hyperplastic change, and only one rat administered sodium saccharin (pretreated with cyclophosphamide and FANFT) developed a transitional cell carcinoma of the renal pelvis.

Role of Urine

Urinary bladder carcinogenesis has long been considered to result from exposure of the urinary bladder epithelium to carcinogens in the urine, the so-called urogenous or carrier theory. The ability of the urine to concentrate solutes compared to the level in serum is one of the reasons for this mechanism. Scott and Boyd (*39*) demonstrated that dogs fed 2-naphthylamine and having the urine diverted to the sigmoid colon by ureterosigmoidostomies prevented the induction of bladder tumors. McDonald and Lund (*40*) tied off the top half of the bladder,

preventing exposure to the urine flow but leaving the bottom half exposed to urine. The dogs were fed 2-naphthylamine, and tumors were found only in the half of the bladder exposed to urine. An indication that urine might be more than simply a carrier of carcinogens was suggested by the experiments of Chapman et al. (*41*), in which paraffin wax pellets were inserted into both halves of a bladder which had been divided in half by tying a suture around the mid-portion of the bladder. Again, tumors, although benign, were induced only in the portion of the bladder exposed to urine. The presence of calculi greatly increased the incidence of bladder tumors in the portion of the bladder exposed to urine. However, the addition of 3-hydroxyanthranilic acid to the paraffin wax pellet did not enhance the tumor incidence, possibly because of the very slow release of this chemical from the pellets.

Rowland et al. (*42*) recently provided additional evidence that urine is more than merely a carrier of carcinogens. FANFT was fed at a dose of 0.2% of the diet for 14 weeks. At that time, half of the rats underwent urinary diversion with bilateral ureterosigmoidostomies and the other half were sham operated. Eight of the 19 the sham-operated animals developed bladder tumors within 6 months of the time of operation compared to only one of 18 rats that had undergone urinary diversion. These data would indicate that urine is acting as a promoting substance since it was required for the appearance of tumors despite the administration of an apparently carcinogenic dose of FANFT. Oyasu et al. (*43*) also demonstrated promoting activity for urine using their heterotopic bladder model. In this model, urinary bladders are transplanted subcutaneously into syngeneic rats attached to an Ommaya reservoir through which substances can be injected. If BBN was given to rats and the bladder then transplanted as a heterotopic bladder instilled with 0.9% sodium chloride (saline), no tumors developed in the heterotopic bladder after 38 weeks and two of 13 rats had tumors in the heterotopic bladder after 44 weeks. In comparison, if the BBN was followed by instillation of urine adjusted as to be equiosmolal with the saline solution, eight of 19 animals had bladder tumors in the heterotopic bladder after 38 weeks and eight of 14 had tumors after 44 weeks. If either saline or urine was instilled without previous BBN administration, no tumors appeared in the heterotopic or host bladder. Oyasu and his colleagues also demonstrated (*44*) that urine increased ornithine-decarboxylase activity in an *in vitro* assay of rat bladder tumors, but it was not as potent as the phorbol ester, 12-0-tetradecanoylphorbol-13-acetate (TPA). Although increased activity was also induced by the presence of 3-hydroxyanthranilic acid, a metabolite of tryptophan, most of the activity in urine was present in the fraction with a molecular weight greater than 10,000 daltons.

Although those data would indicate that urine, or some substance(s) in urine has promoting activity, it must be considerably less potent than exogenous promoting substances such as sodium saccharin. For example, FANFT administered as 0.2% of the diet for 6 weeks followed by control diet resulted in the induction of bladder tumors in five of 20 rats, whereas 18 of 19 rats administered 5% sodium saccharin after the FANFT developed bladder tumors (*18*).

In the model of initiation and promotion proposed by Boutwell (*22*), he divided promotion into at least two phases. The first phase of promotion was called conversion and appeared to require something more than mere proliferation of the target tissue after initiation. Thus, croton oil could act as a converting agent, but turpentine, an inducer of cell proliferation on the mouse skin, had considerably weaker activity. The second phase of promotion was referred to as propagation, and this appeared to require only the administration of a substance that increased cell proliferation. Thus, either croton oil or turpentine were active in this phase. Slaga and his colleagues (*45*) have recently refined this experiment considerably using the purified ingredient of croton oil, TPA, as a converting agent and mezerein as the propagating agent. The experiments of Slaga would also indicate that the period required for conversion is relatively short. The propagation phase appears to be the rate limiting phase of tumor induction.

Using this model of promotion, it is possible that urine is acting as a propagating agent rather than as a complete promoter. Thus, in this model, very brief exposure to an initiating agent and exposure to a converting agent for a somewhat longer period of time would be enough to induce bladder cancer, since urine would always be present as an endogenous propagating agent. Without additional exposure to stronger exogenous promoting substances, a low incidence of bladder tumors would be expected despite only brief exposure to carcinogens. A similar model can be formulated for most epithelial tissues with some normally present endogenous agent acting as the propagating substance. This would help to explain induction of tumors in certain instances involving very short exposures to carcinogens and also would partially explain the relatively long periods of time necessary for the induction of most tumors.

Cell Proliferation and Bladder Carcinogenesis

The above experiments provide evidence that the model of initiation-promotion is relevant to urinary bladder carcinogenesis. In the MNU model, the initiating dose of the MNU is applied as a single instillation of chemical into the urinary bladder (*24*) similar to the model of initiation in the mouse skin tumor system. With FANFT, however, administration of an initiating dose requires 4 to 6 weeks of the chemical in the diet at a level of 0.2% (*18*). One of the characteristics for initiating agents in studies in other systems is the necessity of at least one cell cycle being completed to fix initiation irreversibly (*23*). The urinary bladder normally has a very low rate of cell turnover; the labeling index is approximately 4 per 10,000 epithelial cells (*35*). We postulated that administration of FANFT to an animal with a proliferating bladder mucosa might reduce the required time of administration (*38*). The results are shown in Table 2. The marked regenerative hyperplasia of the bladder was induced by the Shirai method (*46*) of freeze ulceration or by cyclophosphamide injection (*1, 2*). In Group 1, ulceration was followed immediately by the oral administration of 0.2% FANFT for 2 weeks and then was promoted by sodium saccharin administered at 5% of the diet for the remaining 102 weeks of the experiment. A similar protocol using cyclophosphamide as the ulcer inducing agent is Group 11. The other groups served as the appropriate control groups. Two weeks of FANFT followed by sodium saccharin was insufficient for the induction of bladder tumors except for one small papilloma (group 5). FANFT (group 6) and saccharin (groups 7 and 17) alone and the ulcer (group 4) alone were insufficient for inducing tumors. The rats administered FANFT after ulceration and then given sodium saccharin (group 1) had bladder tumors as predicted. Similar results occurred with cyclophosphamide (group 11). However, the administration of sodiuim saccharin, either immediately after the ulcer (group 10) or after two weeks of control diet (group 3) resulted in comparable incidences of bladder tumors. A similar result was obtained with cyclophosphamide as the ulcerating agent (groups 15 and 13, respectively). As mentioned above, FANFT and cyclophosphamide produce urine in the rat containing mutagenic activity in *in vitro* assays. In this experiment, however, the presence of FANFT did not increase the incidence of bladder tumors compared to the control if the ulcer was induced before saccharin, and cyclophosphamide did not give significantly greater results than ulceration by freezing, a method which is nonmutagenic. These data suggest that sodium saccharin is carcinogenic when administered to an animal with a proliferating urinary bladder epithelium. They also suggest that mutation, strictly defined as a change in nucleotide content (primarily defined as the result of the Ames assay), is not necessary for the carcinogenic process. Although a point mutation may not be involved in the carcinogenic process, other genetic changes such as recombination or translocation are possible. *In vitro* assays involving Saccharomyces have indicated that sodium saccharin is able to cause these genetic changes (*47*). Additional studies will be required to evaluate this possibility.

Results of the above experiment with sodium saccharin and ulceration are quite consistent with the

Table 2. Effect of ulceration on urinary bladder carcinogenesis.

Group no.	Treatment group[a]	No. of rats	No. of rats with bladder tumors (%)
1	Ulcer→FANFT→Saccharin	22	3 (14)
2	Ulcer→FANFT→Control	22	0
3	Ulcer→Control→Saccharin	19	4 (21)
4	Ulcer→Control	23	0
5	FANFT→Saccharin	21	1 (5)
6	FANFT→Control	19	0
7	Control→Saccharin	17	0
8	FANFT→Ulcer→Saccharin	22	8 (36)
9	FANFT→Ulcer→Control	21	2 (10)
10	Ulcer→Saccharin	21	4 (19)
11	CP→FANFT→Saccharin	22	7 (32)
12	CP→FANFT→Control	9	0
13	CP→Control→Saccharin	16	5 (31)
14	CP→Control	7	1 (14)
15	CP→Saccharin	17	6 (35)
16	Control	32	0
17	Saccharin	24	0

[a]FANFT = 0.2% *N*-[4-(5-nitro-2-furyl)-2-thiazolyl]formamide; saccharin = 5% sodium saccharin; ulcer = freeze ulceration; CP = cyclophosphamide-induced ulceration (100 mg/kg IP); Control = control diet without added test chemical.

results of other experiments with sodium saccharin. Sodium saccharin administration to a single generation of rats induced a few bladder tumors, but the incidence has generally been 1% or less (*24, 32, 34*). However, if administered to pregnant rats and to the offspring during the suckling period and after weaning, a greater incidence of bladder tumors was induced (*34*). *In utero*, the urinary bladder is in a state of active proliferation, whereas almost immediately after the birth the bladder attains its adult slow turnover rate (*2*). Thus, the *in utero* exposure is similar to the exposure after ulceration with the increased proliferation rate.

Sodium saccharin was initially shown to be carcinogenic for the urinary bladder in experiments in which the chemical was administered in a cholesterol pellet inserted into the urinary bladder (*48*). We have demonstrated that the surgical procedure involved in the insertion of the pellet induced the same type of proliferation as that following freeze ulceration with approximately the same kinetics of regeneration and repair (*11*). However, in the pellet experiment, sodium saccharin was leeched from the pellet within a few days of insertion of the pellet. One could envision a process of multistage carcinogenesis in which the saccharin is exposed to a urinary bladder with an increased proliferative rate following the insertion of the pellet. The propagation process would continue if the bladder mucosa was maintained in a state of increased proliferation, such as provided by the presence of the pellet along with the urine itself. In such a model, the early events of carcinogenesis, initiation and conversion (*22*) would be due to the combination of the surgical technique inducing the market increase in cell proliferation and exposure to sodium saccharin. The pellet and urine would then be acting as the propagating agents.

Another possible example presenting a mechanism similar to that illustrated in this experiment is schistosomiasis. Urological schistosomiasis is associated with a greatly increased risk of developing bladder cancer (*1, 2*), particularly squamous cell carcinoma. Recent evidence suggests that nitrosamines are generated during the active inflammation that occurs with schistosomal infections, possibly by in situ generation by the reaction of nitrite with secondary amines in an acid medium. Chronic schistosomiasis is well known to cause increased proliferative processes in the urinary bladder epithelium in humans as well as in the nonhuman primate model.

If applicable to the human situation, these experiments suggest possible populations at increased risk of development of bladder carcinoma if exposed to various environmental agents, whether complete carcinogens or substances such as the type represented by sodium saccharin. It is known that males have an increased risk of developing bladder cancer compared to females (*2*). Could this be related to the development of prostatism in elderly males with consequent proliferation changes in the bladder epithelium? Is exposure to certain chemicals in utero and shortly after birth responsible for an increased risk of development of bladder cancer since the epithelium is proliferating during these times? Are people who have recurrent episodes of acute cystitis with its accompanying regenerative hyperplasia at increased risk if exposed to certain chemicals such as sodium saccharin or cigarette smoking? We obviously have a great deal to learn about bladder carcinogenesis in humans, but hopefully the results in these animal models will provide clues for us to evaluate the human situation.

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REFERENCES

1. Price, J. M. Etiology of bladder cancer. In: Benign and Malignant Tumors of the Urinary Bladder (E. Maltry, Ed.), Medical Examination Publishing Co., Flushing, NY, 1971, 189-251.
2. Clayson, D. B., and Cooper, E. H. Cancer of the urinary tract. Adv. Cancer Res. 13: 271-381 (1970).
3. Johansson, S., Angervall, L., Bengtsson, U., and Wahlquist, L. Uroepithelial tumors of the renal pelvis associated with abuse of phenacetin-containing analgesics. Cancer 33: 743-753 (1974).
4. Miller, J. A., and Miller, E. C. Ultimate chemical carcinogens as reactive mutagenic electrophiles. In: Origins of Human Cancer, Cold Spring Harbor Conferences on Cell Proliferation, Vol. 4 (H. H. Hiatt, J. D. Watson, and J. A. Winsten, Eds.), Cold Spring Harbor Laboratory, Cold Spring Harbor, 1977, pp. 605-627.
5. Druckrey, H., Preussmann, R., Schmahl, D., and Ivankovic, S. Organotrope carcinogene Wirkungen bei 65 verschiedenen *N*-Nitroso Verbindungen an BD-Ratten. Z. Krebsforsch. 69: 103-201 (1967).
6. Erturk, E., Cohen, S. M., Price, J. M., and Bryan, G. T. Pathogenesis, histogenesis, histology and transplantability of urinary bladder carcinoma induced in albino rats by oral administration of *N*-[4-(5-nitro-2-furyl)-2-thiazolyl] formamide. Cancer Res. 29: 2219-2228 (1969).
7. Hicks, R. M., and Wakefield, J. St. J. Rapid induction of bladder cancer in rats with *N*-methyl-*N*-nitrosourea I. Histology. Chem. Biol. Interact. 5: 139-152 (1972).
8. Cohen, S. M. Toxicity and carcinogenicity of nitrofurans. In: Carcinogenesis—A Comprehensive Survey, Vol. 4, Nitrofurans (G. T. Bryan, Ed), Raven Press, New York, 1978, pp. 171-231.
9. Tiltman, A. J., and Friedell, G. H. The histogenesis of experimental bladder cancer. Invest. Urol. 9: 218-226 (1971).
10. Cohen, S. M., Jacobs, J. B., Arai, M., Johansson, S. and Friedell, G. H. Early lesions in experimental bladder can-

cer: Experimental design and light microscopic findings. Cancer Res. 36: 2508-2511 (1976).
11. Fukushima, S., Cohen, S. M., Arai, M., Jacobs, J. B., and Friedell, G. H. Scanning electron microscopy of reversible hyperplasia of the rat urinary bladder. Am. J. Pathol. 102: 373-380 (1981).
12. Lower, G. M., Nilsson, T., Nelson, C. E., Wolfe, H., Gamsky, T. E., and Bryan, G. T. *N*-Acetyltransferase phenotype and risk in urinary bladder cancer. Approaches in molecular epidemiology. Environ. Health Perspect. 29: 71-79 (1979).
13. Kadlubar, F. F., Miller, J. A., and Miller, E. C. Hepatic microsomal N-hydroxy arylamines in relation to urinary bladder carcinogenesis. Cancer Res. 37: 805-814 (1977).
14. Radomski, J. L., Poupko, J. M., and Hearn, W. L. Environmental bladder carcinogens (abstract). National Bladder Cancer Project Abstracts, 1980, p. 35.
15. Cohen, S. M., Zenser, T. V., Murasaki, G., Fukushima, S., Mattammal, M. B., Rapp, N. S., and Davis, B. B. Aspirin inhibition of *N*-[4-(5-nitro-2-furyl)-2-thiazolyl]formamide-induced lesions of the urinary bladder correlated with inhibition of metabolism by bladder prostaglandin endoperoxide synthetase. Cancer Res. 41: 3355-3359 (1981).
16. Arai, M., Cohen, S. M., Jacobs, J. B., and Friedell, G. H. Effect of dose on urinary bladder carcinogenesis induced in F344 rats by *N*-[4-(5-nitro-2-furyl)-2-thiazolyl]formamide. J. Natl. Cancer Inst. 62: 1013-1016 (1979).
17. Jacobs, J. B., Arai, M., Cohen, S. M., and Friedell, G. H. A long-term study of reversible and progressive urinary bladder cancer lesions in rats fed *N*-[4-(5-nitro-2-furyl)-2-thiazolyl]formamide. Cancer Res. 37: 2817-1821 (1977).
18. Cohen, S. M., Arai, M., Jacobs, J. B. and Friedell, G. H. Promoting effect of saccharin and D,L-tryptophan in urinary bladder carcinogenesis. Cancer Res. 39: 1207-1217 (1979).
19. Fukushima, S., Friedell, G. H., Jacobs, J. B., and Cohen, S. M. Effect of L-tryptophan and sodium saccharin on urinary bladder carcinogenesis initiated by *N*-[4-5-nitro-2-furyl)-2-thiazolyl]formamide. Cancer Res. 41: 3100-3103 (1981).
20. Tsuda, H., Miyata, Y., Murasaki, G., Kinoshita, H., Fukushima, S., and Ito, N. Synergistic effect of urinary bladder carcinogenesis in rats treated with *N*-butyl-*N*-(4-hydroxybutyl)nitrosamine, *N*-[4-(5-nitro-2-furyl)-2-thiazolyl]formamide, *N*-2-fluorenylacetamide, and 3,3′-dichlorobenzidine. Gann 68: 183-192 (1977).
21. Tatematsu, M., Miyata, Y., Mizutani, M., Hananouchi, M., Hirose, M., and Ito, N. Summation effect of *N*-butyl-*N*-(4-hydroxybutyl)nitrosamine, *N*-[4-(5-nitro-2-furyl)-2-thiazolyl]formamide, *N*-2-fluorenylacetamide, and 3,3′-dichlorobenzidine on urinary bladder carcinogenesis in rats. Gann 68: 193-202 (1977).
22. Boutwell, R. K. Some biological aspects of skin carcinogenesis. Progr. Exptl. Tumor Res. 4: 207-250 (1964).
23. Scribner, J. D., and Suss, R. Tumor initiation and promotion. Int. Rev. Exptl. Path. 18: 137-198 (1978).
24. Hicks, R. M., Wakefield, J. St. J., and Chowaniec, J. Evaluation of a new model to detect bladder carcinogens or cocarcinogens; results obtained with saccharin, cyclamate and cyclophosphamide. Chem. Biol. Interact. 11: 225-233 (1975).
25. Nakanishi, K., Hirose, M., Ogiso, T., Hasegawa, R., Arai, M., and Ito, N. Effects of sodium saccharin and caffeine on the urinary bladder of rats treated with *N*-butyl-*N*-(4-hydroxybutyl)nitrosamine. Gann 71: 490-500 (1980).
26. Bryan, G. T., and Springberg, P. D. Role of the vehicle in the genesis of bladder carcinomas in mice by the pellet implantation technic. Cancer Res. 26: 105-109 (1966).
27. Swaminathan, S., and Lower, G. M. Biotransformations and excretion of nitrofurans. In: Carcinogenesis—A Comprehensive Survey, Vol. 4, Nitrofurans (G. T. Bryan, Ed.), Raven Press, New York, 1978, pp. 59-97.
28. Matsushima, M. The role of the promotor L-tryptophan on tumorigenesis in the urinary bladder. 2. Urinary bladder carcinogenicity of FANFT (initiating factor) and L-tryptophan (promoting factor) in mice. Japan, J. Urol. 68: 731-736 (1977).
29. Radomski, J. L., Radomski, T., and MacDonald, W. E. Cocarcinogenic interaction between D,L-tryptophan and 4-aminobiphenyl or 2-naphthylamine in dogs. J. Natl. Cancer Inst. 58: 1831-1834 (1977).
30. Wang, C. Y., Benson, R. C., Jr., and Bryan, G. T. Mutagenicity for *Salmonella typhimurium* of urine obtained from humans receiving nitrofurantoin. J. Natl. Cancer Inst. 58: 871-873 (1977).
31. Batzinger, R. P., On, S.-Y. L., and Bueding, E. Saccharin and other sweeteners: mutagenic properties. Science 198: 944-946 (1977).
32. OTA. Cancer Testing Technology and Saccharin. Office of Technology Assessment, United States Government Printing Office, Washington, D.C., 1977.
33. Bowden, J. R., Chung, K.-T., and Andrews, A. W. Mutagenic activity of tryptophan metabolites produced by rat intestinal microflora. J. Natl. Cancer Inst. 57: 921-924 (1976).
34. Arnold, D. L., Moodie, C. A., Grice, H. C., Charbonneau, S. M., Stavric, B., Collins, B. T., McGuire, P. F., Zawidzka, Z. Z., and Munro, I. C. Long-term toxicity of ortho-toluenesulfonamide and sodium saccharin in the rat. Toxicol. Applied Pharmacol. 52: 113-152 (1980).
35. Fukushima, S., and Cohen, S. M. Saccharin-induced hyperplasia of the rat urinary bladder. Cancer Res. 40: 734-736 (1980).
36. Murasaki, G., and Cohen, S. M. Effect of dose of sodium saccharin on the induction of rat urinary bladder proliferation. Cancer Res. 41: 942-944 (1981).
37. Demers, D. M., Fukushima, S., and Cohen, S. M. Effect of sodium saccharin and L-tryptophan on rat urine during bladder carcinogenesis. Cancer Res. 41: 108-112 (1981).
38. Cohen, S. M., Murasaki, G., Fukushima, S., and Greenfield, R. E. Effect of regenerative hyperplasia on the urinary bladder carcinogenicity of sodium saccharin and *N*-[4-(5-nitro-2-furyl)-2-thiazolyl]formamide. Cancer Res. 42: 65-71 (1982).
39. Scott, W. W., and Boyd, H. L. A study of the carcinogenic effect of beta-naphthylamine on the normal and substituted isolated sigmoid loop bladder of dogs. J. Urol. 70: 914-925 (1953).
40. McDonald, D. F., and Lund, R. R. The role of the urine in vesical neoplasm. 1. Experimental confirmation of the urogenous theory of pathogenesis. J. Urol. 71: 560-570 (1954).
41. Chapman, W. H., Kirchheim, D., and McRoberts, J. W. Effect of the urine and calculus formation on the incidence of bladder tumors in rats implanted with paraffin wax pellets. Cancer Res. 33: 1225-1229 (1973).
42. Rowland, R. G., Henneberry, M. O., Oyasu, R., and Grayback, J. T. Effects of urine and continued exposure to carcinogen on progression of early neoplastic urinary bladder lesions. Cancer Res. 40: 4524-4527 (1980).
43. Oyasu, R., Hirao, Y., and Izumi, K. Enhancement by urine of urinary bladder carcinogenesis. Cancer Res. 41: 478-481 (1981).
44. Izumi, K., Hirao, Y., Hopp, L., and Oyasu, R. *In vitro* induction of ornithine decarboxylase in urinary bladder carcinoma cells. Cancer Res. 41: 405-409 (1981).

45. Slaga, T. J., Fischer, S. M., Nelson, K., and Gleason, G. L. Studies on the mechanism of skin tumor promotion: Evidence for several stages in promotion. Proc. Natl. Acad. Sci. (U.S.) 77: 3659-3663 (1980).
46. Shirai, T., Cohen, S. M., Fukushima, S., Hananouchi, M., and Ito, N. Reversible papillary hyperplasia of the rat urinary bladder. Am. J. Path. 91: 33-48 (1978).
47. Moore, C. W., and Schmick, A. Recombinogenicity and mutagenicity of saccharin in *Saccharomyces cerevisiae*. Mutat. Res. 67: 215-219 (1979).
48. Bryan, G. T., Erturk, E., and Yoshida, O. Production of urinary bladder carcinomas in mice by sodium saccharin. Science. 168 : 1238-1240 (1970).