EVIDENCE ON THE CARCINOGENICITY OF

SODIUM SACCHARIN

DRAFT

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Reproductive and Cancer Hazard Assessment Section
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PREFACE

The Safe Drinking Water and Toxic Enforcement Act of 1986 (Proposition 65, California Health and Safety Code 25249.5 *et seq*.) requires that the Governor cause to be published a list of those chemicals "known to the state" to cause cancer or reproductive toxicity. The Act specifies that "a chemical is known to the state to cause cancer or reproductive toxicity…if in the opinion of the state's qualified experts the chemical has been clearly shown through scientifically valid testing according to generally accepted principles to cause cancer or reproductive toxicity." The lead agency for implementing Proposition 65 is the Office of Environmental Health Hazard Assessment (OEHHA) of the California Environmental Protection Agency. The "state's qualified experts" regarding findings of carcinogenicity are identified as the members of the Carcinogen Identification Committee of the OEHHA Science Advisory Board (22 CCR 12301) (referred to hereafter as the Committee).

Sodium saccharin (CAS No. 128-44-9) was added to the Proposition 65 list as a chemical known to the state to cause cancer on January 1, 1988. This chemical was added to the Proposition 65 list as a result of actions taken by the "State's Qualified Experts" at a public meeting held on December 11, 1987.

Since 1987, considerable scientific information has become available relevant to the mode of action of sodium saccharin carcinogenicity in the rat. In April 1994, OEHHA and the U.S. Environmental Protection Agency jointly sponsored the scientific workshop *Assessing the Cancer Risk of Saccharin and Sodium Saccharin*, during which the information available at that time relevant to cancer risk assessment was comprehensively reviewed. In 1999, the International Agency for Research on Cancer (IARC) re-evaluated saccharin and its salts, and re-classified saccharin and its salts as "not classifiable as to their carcinogenicity to humans (Group 3)" based on its conclusion that sodium saccharin produces urothelial bladder tumors in rats by a mechanism not relevant to humans because of critical interspecies differences in urine composition. In 2000, the National Toxicology Program (NTP) released its Ninth Report on Carcinogens in which it removed saccharin from the list of substances "reasonably anticipated to be a human carcinogen."

In light of considerable new relevant information on the carcinogenicity of sodium saccharin published since its consideration for listing by the State's Qualified Experts in 1987, OEHHA is asking the Carcinogen Identification Committee to review the listing of sodium saccharin to determine whether or not it should remain on the Proposition 65 list.

A public request for information relevant to the assessment of the evidence on the carcinogenicity of this chemical was announced in the *California Regulatory Notice Register* on April 6, 2001. OEHHA received submissions from the National Soft Drink Association and from the Calorie Control Council in response to this request. This information has been reviewed and considered by OEHHA in the preparation of this document.

This draft document, *Evidence on the Carcinogenicity of Sodium Saccharin*, was developed to provide the Committee with the available scientific evidence on the carcinogenic potential of this chemical. A public meeting of the Committee to discuss this evidence is scheduled for December 17, 2002. Following discussion and Committee deliberation, the Committee will

determine whether or not sodium saccharin has been "clearly shown through scientifically valid testing according to generally accepted principles to cause cancer," and should remain on the Proposition 65 list or be removed from the Proposition 65 list. (It should be noted that an affirmative vote of a majority of the appointed members of the Committee is required to take any action.) Written public comment on the document should be submitted to OEHHA by December 10, 2002 in order to be considered by the Committee in advance of the meeting. During the December 2002 meeting, the public will have an opportunity to provide verbal comments to the Committee.

TABLE OF CONTENTS

AUTHORS AND REVIEWERS ..... **i**

PREFACE ..... **ii**

TABLE OF CONTENTS ..... **iv**

LIST OF TABLES ..... **vi**

LIST OF FIGURES ..... **vi**

1 EXECUTIVE SUMMARY **1**

2 INTRODUCTION **3**

2.1 1988 Proposition 65 Listing of Sodium Saccharin 3

2.2 IARC and NTP Evaluations Since the Proposition 65 Listing of Sodium Saccharin 3

2.3 Identity of Sodium Saccharin 6

2.4 Occurrence and Use 6

3 DATA ON SODIUM SACCHARIN CARCINOGENICITY **6**

3.1 Carcinogenicity Studies in Humans 7

3.2 Carcinogenicity Studies in Animals 19

3.3 Other Relevant Data 24

3.3.1 Tumor Promotion and Co-Carcinogenesis Studies 24

3.3.2 Genetic Toxicology 28

3.3.3 Pharmacokinetics and Metabolism 31

3.3.4 Urothelial Proliferation and the Relationship Between Urinary Characteristics and the Promoting and Carcinogenic Effects of Sodium Saccharin 34

4 MECHANISM **44**

4.1 Carcinogenicity after Neonatal Exposure and Two-Generation Bioassays 44

4.2 Calcium Phosphate-Containing Precipitate 45

4.3 Other Possible Mechanisms of Action 46

5 SUMMARY AND CONCLUSIONS 48

5.1 Summary of Evidence 48

5.2 Conclusion 50

6 REFERENCES 51

APPENDIX: SACCHARIN (ACID FORM); CAS NO. 81-07-2 63

Evaluations of Saccharin by IARC and NTP 63

IARC 63

NTP 63

Actions by U.S. FDA and U.S. Congress 64

U.S. FDA 64

U.S. Congress 65

Proposition 65 Listing History for Saccharin (CAS No. 81-07-2) 65

APPENDIX REFERENCES 66

LIST OF TABLES

Table 1. Case-Control Studies of Artificial Sweeteners and Bladder Cancer: Selected Characteristics. 9

Table 2. Population-Based Case-Control Studies of Artificial Sweeteners and Bladder Cancer That Incorporated Some Measure of Dose in the Analysis. 13

Table 3. Average Number of Daily Servings of Artificial Sweeteners and Relative Risk of Bladder Cancer, Based on Data Presented in Table III of Hoover and Hartge Strasser (1980). 18

Table 4. Incidences of Urinary Bladder Neoplasms in F_1 Male Sprague-Dawley Rats Exposed to 1 - 7.5% Sodium Saccharin in the Diet (Schoenig *et al.*, 1985).* 21

Table 5. Promotion Studies of Sodium Saccharin in Rats. 26

Table 6. Urinary Concentration of Saccharin in F344 Rats Treated with Several Forms of Saccharin (Hasegawa and Cohen, 1986). 34

Table 7. Comparison of Labeling Indices in Male Fischer Rats Treated for 10 Weeks with Different Forms of Saccharin (Hasegawa and Cohen, 1986). 35

Table 8. Increases in the Labeling Index of Bladder Epithelium in Male and Female Fischer Rats Fed 5% Sodium Saccharin in Their Diets for Ten Weeks (Cohen, 1985)[a]. 36

Table 9. Incidence of Bladder Tumors in Male F344 Rats Initiated with 2% FANFT Followed by Test Chemicals for 72 Weeks (Cohen *et al.,* 1991). 39

Table 10. Incidence of Bladder Tumors in Male F344 Rats Initiated with 0.5% BBN Followed by Test Chemicals for 32 Weeks (Fukushima *et al.*, 1986). 40

Table 11. Urinary pH and Sodium Ion Concentration in Rats Initiated with BBN Then Fed Succinic Acid and Succinate Salts (Otoshi *et al.*, 1993). 40

LIST OF FIGURES

Figure 1. Structure of Sodium Saccharin 6

1 EXECUTIVE SUMMARY

Saccharin is used as a non-nutritive sweetener worldwide. The most common usage of sodium saccharin in the U.S. is in diet beverages and to a lesser extent as a table-top sweetener. It is also used to sweeten personal care products such as toothpaste and mouthwash, and pharmaceuticals. Other products containing sodium saccharin include processed fruits, chewing gum and confections, gelatin desserts, jams and toppings, sauces and dressings, and animal feed. Lesser amounts of saccharin are used in a variety of non-food applications: as a nickel electroplating brightener, and an anaerobic adhesive accelerator (IARC, 1999).

Numerous epidemiology studies have investigated the relationship between artificial sweeteners and bladder cancer risk, although the relationship between neonatal exposure and cancer risk has not been adequately studied. Two of the largest and most well-designed case-control studies have found small increased risks in certain subgroups of the population; however, the subgroups identified as being at elevated risk are inconsistent between the two studies. Increased risks were observed in men in one of the studies, and in men who smoked more than 40 cigarettes daily and in women who never smoked and were never exposed occupationally to known bladder carcinogens in the other. This lack of consistency suggests that either the associations are not causal (probably attributable to chance or recall bias), or saccharin is a weak carcinogen.

The induction of bladder carcinogenesis by sodium saccharin has been shown to occur when rats are chronically exposed to doses at or above 1% to 3% in the diet for periods that include the neonatal period. Small, often non-significant increases in bladder tumor incidence associated with sodium saccharin treatment have been observed in bioassays of weanling rats. Male rats are more sensitive than female rats to the induction of bladder tumors by sodium saccharin. The best study evaluating the carcinogenic effects in rats was conducted by Schoenig *et al.* (1985) and was large enough to detect small increases in tumor incidence (less than 10%). Several other studies have been conducted, but these have limitations including small numbers of animals at end-of-study and limited histopathological evaluation. Multigeneration diet studies in mice observed small, non-significant increases in bladder tumors in sodium saccharin treated animals. Studies in other species have either not been conducted at high enough doses (monkeys) or have not included the neonatal period (guinea pig).

Sodium saccharin has been shown to promote bladder tumors in rats when administered in drinking water or in feed after initiation with chemicals known to cause bladder tumors. Positive findings for bladder tumor promotion by sodium saccharin have been reported at doses at or above 2.5% in the diet following initiation by *N*-methyl-*N*-nitrosourea [MNU], *N*-[4-(5-Nitro-2-furyl)-2-thiazolyl]formamide [FANFT], and *N*-butyl-*N*-(4-hydroxybutyl)nitrosamine [BBN].

High doses of sodium saccharin have induced genotoxic effects *in vitro* and *in vivo*. Sodium saccharin is clearly not mutagenic in *Salmonella typhimurium* (IARC, 1999) and these results are supported by similar mammalian *in vivo* studies in transgenic Big Blue rats (Sweatman and Renwick, 1982) in which mutations were not detected in any organs or tissues. Point mutations in codon 12 of the K-*ras* gene in a human cell line, however, were demonstrated in response to sodium saccharin treatment. Mouse coat spot tests for *in vivo* mutagenicity and tests for dominant lethal mutations have been conflicting, with both positive and negative reports (Ashby, 1985).

Several negative studies of chromosomal aberrations have been reported for sodium saccharin *in vivo,* while most *in vitro* assays reported a positive clastogenic response. Sasaki *et al*. (2002) used the comet assay to demonstrate that sodium saccharin administered to mice *in vivo* is capable of causing DNA damage in tissues of the digestive tract, but not in the bladder. Sodium saccharin did not induce unscheduled DNA synthesis in cultured rat hepatocytes. DNA binding of ^{35}S-labeled saccharin in liver or bladder tissue has not been detected.

Several lines of investigation have attempted to establish a mode of action for sodium saccharin's carcinogenic activity in the rat bladder. Proliferation of the bladder urothelium has been demonstrated in rats after short-term exposures to sodium saccharin. The proliferation rate is significantly greater in sodium saccharin-treated rats compared to rats treated with acid saccharin or other saccharin salts even though all saccharin salts and acid saccharin exist as saccharin anion at urinary pH. Both male and female rat bladder urothelium is responsive to the promoting activity of sodium saccharin. In carcinogenicity bioassays male rats are more responsive than females, and timing of exposure seems critical. Urothelial tumors are readily observed only when exposure includes the neonatal period.

The mechanisms of tumor promotion and carcinogenicity have not been fully elucidated. The hypothesis that has been the most studied proposes that the formation of a cytotoxic calcium phosphate-containing precipitate in the urine is a necessary event, which results in subsequent regenerative hyperplasia, progressing to tumor formation. Other possible mechanisms have not been well studied but include: disruption of normal Na^+/H^+ exchange homeostasis, resulting in increased intracellular pH, and increased cell proliferation; and abnormal expression of urothelial epidermal growth factor receptors (EGFR). Such expression would permit the binding of urinary epidermal growth factor (EGF) to the urothelial EGFR, leading to cell proliferation. While the hypothesis involving formation of the urinary precipitate has been the most studied, and is considered the leading hypothesis to date (IARC, 1999), neither it nor any other hypothesis has been adequately shown to be the operative mode of action of sodium saccharin-induced carcinogenicity in the rat.

In 1999, IARC concluded "that sodium saccharin produces urothelial bladder tumours in rats by a non-DNA-reactive mechanism that involves the formation of a urinary calcium phosphate-containing precipitate, cytotoxicity and enhanced cell proliferation. This mechanism is not relevant to humans because of critical interspecies differences in urine composition." In 2000, NTP concluded "the factors thought to contribute to tumor induction by sodium saccharin in rats would not be expected to occur in humans." Consequently, the Committee must use a "weight-of evidence" approach to evaluate the body of information available to determine whether the mechanism of action of sodium saccharin has been shown not to be relevant to humans.

2 INTRODUCTION

This document reviews the scientific evidence of the carcinogenicity of sodium saccharin. On April 6, 2001, the Office of Environmental Health Hazard Assessment (OEHHA) announced that, in light of the considerable new and relevant information on the carcinogenicity of sodium saccharin available since its consideration for listing by the state's qualified experts in 1987, the listing of sodium saccharin would be reviewed by the Carcinogen Identification Committee to determine whether the chemical should remain on the Proposition 65 list of substances known to cause cancer (*California Regulatory Notice Register,* No. 14-Z, p. 467). This document has been prepared to assist the Committee in its review. This introductory section provides (1) a brief history of the listing of sodium saccharin under Proposition 65, and (2) a brief summary of activity relevant to the carcinogenicity of sodium saccharin by the International Agency for Research on Cancer (IARC) and the National Toxicology Program (NTP) since 1987. This section also provides information on chemical identity, occurrence and use of the compound. Later sections present the scientific evidence on the carcinogenicity of sodium saccharin.

2.1 1988 Proposition 65 Listing of Sodium Saccharin

Sodium saccharin (CAS No. 128-44-9; 1,2-Benzisothiazol-3(2H)-one, 1,1-dioxide, sodium salt) was listed "as known to the state to cause cancer" under Proposition 65 on January 1, 1988 based upon the determination of the State's Qualified Experts (SQE, at that time the Scientific Advisory Panel) at its December 11, 1987 meeting. The agenda for the SQE's December 1987 meeting included the consideration of a number of chemicals listed in the National Toxicology Program's (NTP) Report on Carcinogens, including saccharin, for possible listing under Proposition 65. A description of the evidence supporting the NTP's 1981 listing of saccharin on its list of chemicals *reasonably anticipated to be a human carcinogen*, taken from the NTP's 1985 *Fourth Annual Report on Carcinogens*, was provided to the SQE to assist in their deliberations. The SQE were also aware of the findings of the International Agency for Research on Cancer (IARC) regarding saccharin and sodium saccharin. Specifically, in 1980 IARC concluded "There is *sufficient* evidence that saccharin alone *[not in combination with cyclamates]*, given at high doses, produces tumours of the urinary tract in male rats…" (IARC, 1980); in 1982 IARC included sodium saccharin on its list of chemicals for which there is considered to be sufficient evidence of carcinogenicity in animals. Also in 1982, in contrast to sodium saccharin, IARC concluded that for saccharin there was *limited* evidence of carcinogenicity in animals, and that saccharin was *not classifiable as to its carcinogenicity in humans* (Group 3) (IARC, 1982). In discussion during the December 1987 meeting, the SQE decided to list sodium saccharin, rather than saccharin. The SQE's decision to list sodium saccharin was based on sufficient evidence of carcinogenicity in animals.

2.2 IARC and NTP Evaluations Since the Proposition 65 Listing of Sodium Saccharin

Since the December 1987 listing decision for sodium saccharin by the SQE, additional research has been conducted investigating the mechanism of tumor induction by sodium saccharin; concerns have been raised regarding whether sodium saccharin poses a risk of cancer in humans, and IARC and NTP have completed new reviews.

In 1987 IARC updated and reviewed the evidence for the carcinogenicity of saccharin, classifying saccharin as *possibly carcinogenic to humans* (Group 2B), based on *sufficient* evidence for carcinogenicity in animals and *inadequate* evidence in humans (IARC, 1987). The results of the 1987 IARC review were not available at the time of the December 1987 meeting of the SQE.

In 1999, IARC updated and reviewed the evidence for the carcinogenicity of saccharin and its salts. IARC (1999) concluded:

> "There is *inadequate evidence* in humans for the carcinogenicity of saccharin salts used as sweeteners,"
>
> "There is *sufficient evidence* in experimental animals for the carcinogenicity of sodium saccharin," and
>
> "There is *inadequate evidence* in experimental animals for the carcinogenicity of saccharin (acid form) and calcium saccharin."

IARC determined that "saccharin and its salts are *not classifiable as to their carcinogenicity to humans* (Group 3)." IARC (1999) concluded:

> "that sodium saccharin produces urothelial bladder tumours in rats by a non-DNA-reactive mechanism that involves the formation of a urinary calcium phosphate-containing precipitate, cytotoxicity and enhanced cell proliferation. This mechanism is not relevant to humans because of critical interspecies differences in urine composition. Saccharin and its salts are *not classifiable as to their carcinogenicity to humans (Group 3)*."

In 2000, NTP removed saccharin from the list of substances *reasonably anticipated to be a human carcinogen* in its *Ninth Report on Carcinogens*. The NTP (2000) noted that:

> "There is evidence for the carcinogenicity of saccharin in rats but less convincing evidence in mice. Studies indicate that the observed urinary bladder cancers in rats are related to the physiology of the rat urinary system including urinary pH, osmolality, volume, and the presence of precipitate, and urothelial damage with attendant hyperplasia following consumption of diets containing sodium saccharin at concentrations of 3% or higher with inconsistent findings at lower dietary concentrations. The factors thought to contribute to tumor induction by sodium saccharin in rats would not be expected to occur in humans. The mouse data are inconsistent and require verification by additional studies. Results of several epidemiology studies indicate no clear association between saccharin consumption and urinary bladder cancer. Although it is impossible to absolutely conclude that it poses no threat to human health, sodium saccharin is not reasonably anticipated to be a human carcinogen under conditions of general usage as an artificial sweetener."

and concluded:

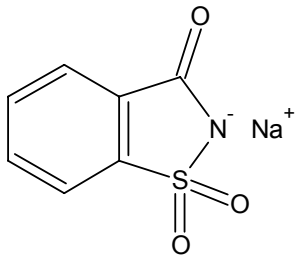
> "Saccharin will be removed from the Report on Carcinogens, because the rodent cancer data are not sufficient to meet the current criteria to list this chemical as *reasonably anticipated to be a human carcinogen*. This is based on the perception that the observed bladder tumors in rats arise by mechanisms not relevant to humans, and the lack of data in humans suggesting a carcinogenic hazard."

The criteria for listing an agent, substance, mixture, or exposure circumstance in the NTP Report on Carcinogens as *reasonably anticipated to be a human carcinogen* were revised in 1996, and currently read as follows (NTP, 2000):

> *"Reasonably Anticipated To Be A Human Carcinogen:*
>
> There is limited evidence of carcinogenicity from studies in humans which indicates that causal interpretation is credible, but that alternative explanations, such as chance, bias or confounding factors, could not adequately be excluded, or
>
> There is sufficient evidence of carcinogenicity from studies in experimental animals which indicates there is an increased incidence of malignant and/or a combination of malignant and benign tumors: (1) in multiple species or at multiple tissue sites, or (2) by multiple routes of exposure, or (3) to an unusual degree with regard to incidence, site or type of tumor, or age at onset; or
>
> There is less than sufficient evidence of carcinogenicity in humans or laboratory animals, however; the agent, substance or mixture belongs to a well defined, structurally-related class of substances whose members are listed in a previous Report on Carcinogens as either known to be a human carcinogen or reasonably anticipated to be a human carcinogen, or there is convincing relevant information that the agent acts through mechanisms indicating it would likely cause cancer in humans.
>
> Conclusions regarding carcinogenicity in humans or experimental animals are based on scientific judgment, with consideration given to all relevant information. Relevant information includes, but is not limited to dose response, route of exposure, chemical structure, metabolism, pharmacokinetics, sensitive subpopulations, genetic effects, or other data relating to mechanism of action or factors that may be unique to a given substance. For example, there may be substances for which there is evidence of carcinogenicity in laboratory animals but there are compelling data indicating that the agent acts through mechanisms which do not operate in humans and would therefore not reasonably be anticipated to cause cancer in humans."

2.3 Identity of Sodium Saccharin

Figure 1. Structure of Sodium Saccharin



Molecular Formula: $C_7H_4NO_3S \bullet Na$

Molecular Weight: 205.2

CAS Registry No. 128-44-9

Chemical Class: benzoic sulfimides

Synonyms: 1,2-Benzisothiazol-3(*2H*)-one,1,1-dioxide, sodium salt; *ortho*-Benzolsulfimide sodium salt; sodium *ortho*-benzosulfimide; sodium saccharide; sodium saccharinate; sodium saccharine, soluble saccharin, succaril, sweeta, sykose, sucra, saxin.

2.4 Occurrence and Use

Saccharin and its salts are not known to occur naturally. Saccharin was discovered by the chemists Ira Remsen and Constantine Fahlberg in 1878 and is used as a non-nutritive sweetener worldwide. Common usage of saccharin salts as a table-top sweetener began in 1917 and consumption has increased steadily since then. The most common usage of sodium saccharin in the U.S. is in diet beverages and to a lesser extent as a table-top sweetener. It is also used to sweeten personal care products such as toothpaste and mouthwash, and pharmaceuticals. Other products containing sodium saccharin include processed fruits, chewing gum and confections, gelatin desserts, jams and toppings, sauces and dressings, and animal feed. Lesser amounts of saccharin are used in a variety of non-food applications: as a nickel electroplating brightener, and an anaerobic adhesive accelerator (IARC, 1999). Worldwide consumption of saccharin and its salts in 1995 was approximately 28,000 tonnes and in the U.S. was approximately 4500 tonnes.

3 DATA ON SODIUM SACCHARIN CARCINOGENICITY

Multiple epidemiology studies have investigated the relationship between consumption of artificial sweeteners, including saccharin, and bladder cancer, most between the years 1974 and 1985. The epidemiologic data available include over twenty case-control studies, a few cohort studies of diabetics, and studies of trends in bladder cancer over time. In animals, four sets of two-generation bioassays of sodium saccharin administered in the diet have been conducted in Sprague-Dawley rats, one set of two-year (single generation) studies of sodium saccharin administered either in diet or drinking water have been conducted in Wistar rats, and a series of one-year dietary studies have been conducted in male ACI, Wistar, Fischer 344, and Sprague-Dawley rats. Multiple sets of single generation bioassays and one set of multigeneration

bioassays of sodium saccharin have been conducted in the mouse. In some mouse studies, sodium saccharin was incorporated into a cholesterol pellet and introduced directly into the bladder. One low-dose dietary study in non-human primates employed small numbers of animals of multiple species that received sodium saccharin from birth up to 24 years of age. Bioassays have also been conducted in guinea pigs. Numerous initiation/promotion studies have been conducted in rats and mice with several different initiators to investigate the promotional activity of sodium saccharin. Sodium saccharin has been tested for genotoxicity in multiple *in vivo* and *in vitro* test systems. Numerous studies of the effects of sodium saccharin on urothelial proliferation have been conducted in rats. Additional relevant data comes from studies of the effects of sodium saccharin on urinary characteristics and from similar studies of other sodium salts.

3.1 Carcinogenicity Studies in Humans

A number of epidemiology studies have investigated the relationship between consumption of saccharin and other artificial sweeteners and the risk of bladder cancer. The majority of these studies were published between 1974 and 1985, and many refer to the consumption of "saccharin" in the form of table-top artificial sweeteners (*e.g.*, tablets or liquid), or dietetic beverages (*e.g.*, soda). Since only salts of saccharin (*e.g.*, sodium saccharin, calcium saccharin) were used in these products (IARC, 1999), these studies are considered to be relevant to the evaluation of sodium saccharin. The relationship between consumption of saccharin and risk of cancers at other sites has not been adequately studied. In one study of limited design, the relationship (among cancer patients) between the use of artificial sweeteners and diet drinks and cancers at sites other than the bladder was investigated, and no evidence of an association at any site was reported (Morrison, 1979). No associations between artificial sweetener use and cancer mortality at sites other than the bladder were reported in studies of limited design in diabetics (Kessler, 1970; Armstrong and Doll, 1975; Armstrong *et al.*, 1976) (see below for discussion of study limitations). Although animal studies indicate that the neonatal period is a critical exposure period for sodium saccharin-induced bladder carcinogenesis (See Sections 3.3 and 4.1), the relationship between neonatal saccharin exposure in humans and risk of cancer has not been adequately investigated. The only study that investigated the relationship between early-in-life exposure to saccharin and bladder cancer was an ecological study of trends in bladder cancer in Denmark in three 5-year birth cohorts for the period 1931-1945, representing two pre-war (unexposed) groups and one war-time (saccharin-exposed, based on usage patterns) group (Jensen and Kamby, 1982). The study analyzed bladder tumors occurring in individuals aged 20-34 and found no increased risk of bladder cancer in the war-time birth cohort; however, the short period of cancer ascertainment in these cohorts (up to age 34) was likely insufficient to account for tumor latency.

The types of epidemiology studies conducted on saccharin and bladder cancer include a number of case-control studies using both matched and unmatched designs, a few cohort studies of diabetics, and studies of the trends in bladder cancer over time. With regard to studies in diabetics, a special group that consumes more saccharin than the general population, mortality trends for bladder cancer were investigated and compared to the general population in a number of studies (Kessler, 1970; Armstrong and Doll, 1975; Armstrong *et al.*, 1976). Morgan and Wong (1985) performed a combined analysis of several studies, with a combined study population of 27,000 diabetics, and derived a combined standard mortality ratio for bladder

cancer in diabetics of 0.70. Several limitations associated with studies of bladder cancer risk in diabetics were identified by IARC (1980), including the possibility that diabetics who do not use artificial sweeteners may have a lower bladder cancer risk than the general population, due to metabolic differences or differences in lifestyle, use of prescription drugs or occupation. Therefore, deriving a risk ratio based on comparison to the general population may conceal the actual risk.

With respect to studies of trends in bladder cancer incidence or mortality over time, no increase was found following the increased use of artificial sweeteners in the U.S. or in Europe (Burbank and Fraumeni, 1970; Armstrong and Doll, 1975; Jensen and Kamby, 1982). However, IARC (1980) noted the following major weaknesses of these time trend studies: first, the proportion of the population exposed to large amounts of artificial sweeteners is small, therefore the effect on the total occurrence of bladder cancer will not be detectable; second, changes over time in exposure to other risk factors, such as smoking and occupation, will also affect the rates; and third, early diagnosis and improvements in medical treatment have led to a high survival rate for bladder cancer (increasing over time), so that mortality rates do not accurately reflect incidence.

More than 20 case-control studies have investigated the relationship between saccharin or artificial sweeteners and urinary bladder cancer (Table 1) (for reviews, see IARC, 1999; Armstrong, 1985; Elcock and Morgan, 1993; Morgan and Wong, 1985). As noted in Table 1, many of these studies suffer from one or more limitations, including small study size, use of hospital or sick controls, low participation rates, or absence of exposure measures.

Table 1. Case-Control Studies of Artificial Sweeteners and Bladder Cancer: Selected Characteristics.

Study	Study Size; Location	Exposure Measure	Comments
Akdas *et al.*, 1990	194 cases 194 controls; Turkey	none	Hospital controls; small numbers of saccharin users; limited reporting
Bravo *et al.*, 1987	406 cases 406 controls; Spain	none	Hospital controls; no participation rates; limited reporting
Cartwright *et al.*, 1981	841 cases 1060 controls; U.K.	none	Hospital controls; participation rate not reported; mixed prevalent and incident cases
Connolly *et al.*, 1978	348 cases 696 controls; Canada	none	Limited reporting
Hoover and Hartge Strasser, 1980; Hoover and Hartge, 1982; Sturgeon *et al.*, 1994 (re-analysis by original investigators); Walker *et al.*, 1982 (re-analysis by separate investigators)	3010 cases 5783 controls; U.S.	Frequency and duration	Publicity may have resulted in differential recall
Howe *et al.*, 1977; Howe *et al.*, 1980; Miller and Howe, 1977; Howe and Burch, 1981	632 cases 632 controls; Canada	Frequency and duration	77% participation rate
Iscovich *et al.*, 1987	120 cases 234 controls; Argentina	none	Hospital and neighborhood controls discussed together; focus on smoking and bladder cancer risk; limited reporting

Study	Study Size; Location	Exposure Measure	Comments
Kessler and Clark, 1978	519 cases 519 controls; U.S.	Exposure groups "low, medium, high' not defined	Hospital controls; prevalent cases (40% of cases died and were excluded)
Miller *et al.*, 1978	265 cases 530 controls; Canada	none	Urology clinic controls; focus on TB treatment and bladder cancer risk
Møller-Jensen *et al.*, 1983	388 cases 787 controls; Denmark	Frequency and duration	Low identification of incident cases by cancer registry (66%)
Momas *et al.*, 1994	219 cases 794 controls; France	Lifetime cumulative exposure	Men only; assessed "use of saccharin as a food additive only," "consumption… from other sources (food and drink) was not considered"
Mommsen *et al.*, 1983	47 cases 94 controls; Denmark	none	Women only; potential observational bias: all interviews conducted by single physician; limited reporting of method of analysis
Morgan and Jain, 1974	231 cases 205 controls; Canada	none	Hospital controls with genitourinary conditions; low participation rates for males (67% cases, 57% controls)
Morrison and Buring, 1980	592 cases 536 controls; U.S.	Frequency and duration	Lower urinary tract cancers (bladder, ureter, renal pelvis, and urethra)
Morrison *et al.*, 1982	848 cases 1324 controls; U.K. and Japan	Frequency and duration	Authors report possible inaccuracies in exposure
Najem *et al.*, 1982	75 cases 142 controls; U.S.	None; mean consumption of cases/controls given	Clinic and hospital controls; some prevalent cases included; small study size

Study	Study Size; Location	Exposure Measure	Comments
Nomura *et al.*, 1991	292 cases 584 controls; U.S.	Duration and cumulative exposure	Lower urinary tract cancers (bladder, renal pelvis, ureter)
Piper *et al.*, 1986	173 cases 173 controls; U.S.	none	Women only, aged 20-49 at diagnosis; possible selection bias: 66% of potential cases included; possible insufficient latency due to young age to observe effects of saccharin
Risch *et al.*, 1988	835 cases 792 controls; Canada	Frequency, cumulative lifetime exposure	Low and differential participation rates: 67% cases, 53% controls
Simon *et al.*, 1975	135 cases 390 controls; U.S.	none	Women only; hospital controls; prevalent cases (excluded 40 cases and110 controls that died)
Wynder and Goldsmith, 1977	163 cases 153 controls; U.S.	Duration	Hospital controls; diabetics "over-represented" among cases
Wynder and Stellman, 1980	367 cases 367 controls; U.S.	Total lifetime intake	Hospital controls: 60% males and 57% females with other cancers; no reporting of participation rates
Zou, 1990	80 cases 160 controls	Frequency and duration	Hospital controls; small study size; limited reporting

Several of the studies listed in Table 1 used hospital or sick controls, which may bias the relative risk towards the null, as noted above, if hospital-based controls have conditions requiring the use of artificial sweeteners, which could lead to an underestimation of cancer risk (IARC, 1999). Silverman *et al*. (1982) compared the findings of Hoover and Hartge Strasser (1980) using hospital and general population controls, and found higher relative risks in the latter comparison; 1.1 using general population controls versus 0.9 using hospital controls for males, and 1.8 using general population controls versus 1.1 using hospital controls for females. This difference disappeared when hospital controls with obesity-related diseases were excluded. Silverman *et al*. (1982) concluded that the magnitude of the bias when obesity-related diseases were not excluded is dependent on the distribution of obesity-related diseases among the hospital controls, and the relation of these diseases to the use of artificial sweeteners.

Low or differential participation rates between cases and controls can be a source of selection bias, if respondents are different than non-respondents with respect to exposure or exposure-related variables. Several studies suffered from some type of participation rate weakness (Howe *et al.*, 1977; Howe *et al.*, 1980, Miller and Howe, 1977; Morgan and Jain, 1974; Piper *et al.*, 1986; Risch *et al.*, 1988).

As noted in Table 1, only nine of the case-control studies incorporated some type of exposure measurement into the analysis. In studies where only users versus non-users were compared, a true effect caused by frequent or heavy use of artificial sweeteners would be diluted, leading to the dismissal of an association, especially if it were a weak one. In addition, the lack of exposure stratification in many of these studies does not permit evaluation of dose-response relationships. In addition, very few of the case-control studies combined exposure to artificial sweeteners from different sources, such as frequency of table-top use (as tablets or drops) and diet drink consumption, and duration of use, to create a measure of cumulative lifetime exposure before time of diagnosis. During the time period covered by these case-control studies, consumption of saccharin in the U.S. was mainly through diet drinks (45% as diet drinks versus 18% as table-top sweeteners, the remainder consumed as additives in prepared food and medications, Armstrong, 1985). Since one diet drink contained two to three times as much saccharin as one regular use of the table-top form during this time period (Hoover and Hartge Strasser, 1980), the desirability of studies that looked at combined consumption of diet drinks and table-top artificial sweeteners is apparent.

Table 2 below compares the results of the population-based case-control studies that investigated the relationship between artificial sweeteners and urinary bladder cancer and that incorporated an exposure measure into the analysis.

Table 2. Population-Based Case-Control Studies of Artificial Sweeteners and Bladder Cancer That Incorporated Some Exposure Measure in the Analysis.

STUDY	MEN			WOMEN		
	Number Cases/ Controls	Exposed Cases/ Control	Odds Ratio (95% CI)	Number Cases/ Controls	Exposed Cases/ Control	Odds Ratio (95% CI)
Hoover and Hartge Strasser, 1980						
Ever/never used artificial sweetener	2258/4277	909/1723	0.99 (0.89-1.10)	742/1499	384/732	1.07 (0.89-1.29)
Low-risk females (i.e., white, non-smoking, not occupationally exposed to dyes, leather, etc.)						
>2 uses of table-top sweeteners/day for 5-9 yr	NA	NA	NA	212/612	13/22	1.8
>2 uses of table-top sweeteners/day for >10 yr	NA	NA	NA	212/612	16/18	2.7
>2 uses of diet drinks/day for 5-9 yr	NA	NA	NA	212/612	3/7	1.4
>2 uses of diet drinks/day for >10 yr	NA	NA	NA	212/612	6/7	3.0
High-risk males (i.e., white smokers: >40 cigarettes/day)						
<1 uses of table-top sweeteners/day	166/226	12/15	1.28	NA	NA	NA
1-1.9 uses of table-top sweeteners/day	166/226	19/14	2.07	NA	NA	NA
2-3.9 uses of table-top sweeteners/day	166/226	16/13	1.96	NA	NA	NA
4-5.9 uses of table-top sweeteners/day	166/226	8/10	1.33	NA	NA	NA
>6 uses of table-top sweeteners/day	166/226	7/7	1.86	NA	NA	NA
<1 diet drink/day	173/248	39/53	1.20	NA	NA	NA
1-1.9 diet drinks/day	173/248	14/19	1.20	NA	NA	NA

STUDY	MEN			WOMEN		
	Number Cases/ Controls	Exposed Cases/ Control	Odds Ratio (95% CI)	Number Cases/ Controls	Exposed Cases/ Control	Odds Ratio (95% CI)
2-2.9 diet drinks/day	173/248	10/5	3.33	NA	NA	NA
>3 diet drinks/day	173/248	6/4	2.62	NA	NA	NA
Howe *et al.*, 1977						
Ever/never used artificial sweetener	480/480	73/47	1.6 (1.1-2.4)	152/152	18/30	0.6
By saccharin use (duration and frequency)						
Non-users	466/469	407/433	1.0	ND	ND	ND
<3 yrs and <2500 tablets/yr	466/469	25/17	1.6 (0.8-3.1)	ND	ND	ND
<3 yrs and >2500 tablets/yr	466/469	6/5	1.3 (0.3-5.3)	ND	ND	ND
>3 yrs and <2500 tablets/yr	466/469	18/12	1.6 (0.7-3.6)	ND	ND	ND
>3 yrs and >2500 tablets/yr	466/469	10/2	5.3 (1.1-50.1)	ND	ND	ND
Howe *et al.*, 1980*						
Frequency of artificial sweetener use						
1-4 tablets/day	480/480	NR	0.9 (0.6-1.4)	152/152	NR	0.3 (0.17-0.59)
5-6 tablets/day	480/480	NR	1.6 (0.95-2.6)	152/152	NR	0.5 (0.2-1.2)
7-8 tablets/day	480/480	NR	1.1 (1.6-2.1)	152/152	NR	1.1 (1.6-2.1)

STUDY	MEN			WOMEN		
	Number Cases/ Controls	Exposed Cases/ Control	Odds Ratio (95% CI)	Number Cases/ Controls	Exposed Cases/ Control	Odds Ratio (95% CI)
>8 tablets/day	480/480	NR	2.8 (1.6-5.1)	152/152	5/0	ND
Møller-Jensen *et al.* 1983 *Ever/never used artificial sweeteners regularly (for at least three months)*	284/583	55/150	0.69 (0.49-0.98)	96/193	26/50	1.06 (0.61-1.85)
Momas *et al.* 1994 *Average lifelong saccharin tablet intake greater/lesser than 365*	161/685	21/44	1.5 (0.8-3.0)	NA	NA	NA
Morrison & Buring 1980 *Ever/never used dietetic beverages*	368/348	144/155	0.8 (0.6-1.1)	143/126	69/46	1.6 (0.9-2.7)
Ever/never used sugar substitutes	325/306	101/113	0.8 (0.5-1.1)	128/119	54/39	1.5 (0.9-2.6)
Morrison *et al.*, 1982						
Manchester (U.K.) *Ever/never used artificial sweeteners or diet drinks*	382/470	140/183	0.9 (0.7-1.2)	142/220	50/87	0.9 (0.6-1.4)
Nagoya (Japan) *Ever/never used sugar substitutes*	223/432	100/238	0.7 (0.5-0.9)	66/144	26/83	0.5 (0.3-0.8)
Nomura *et a.*, 1991						
Diet drinks	195/389	44/65	1.4 (0.9-2.2)	66/132	14/25	1.2 (0.5-2.7)
Table-top	195/389	40/72	1.1 (0.7-1.8)	66/132	10/27	0.7 (0.3-1.5)

STUDY	MEN			WOMEN		
	Number Cases/ Controls	Exposed Cases/ Control	Odds Ratio (95% CI)	Number Cases/ Controls	Exposed Cases/ Control	Odds Ratio (95% CI)
Risch *et al*., 1988**						
Frequency of saccharin use						
Saccharin >0-1 uses/day	NR	NR	1.04 (0.62-1.74)	NR	NR	1.46 (0.77-2.78)
Saccharin >1-3 uses/day	NR	NR	1.17 (0.72-1.91)	NR	NR	0.95 (0.42-2.15)
Saccharin >3 uses/day	NR	NR	0.81 (0.53-1.26)	NR	NR	1.27 (0.61-2.63)
Total lifetime intake	NR	NR	1.01 (0.86-1.18)	NR	NR	0.96 (0.79-1.16)
Diet soda >0-4/wk	NR	NR	0.44 (0.21-0.89	NR	NR	2.05 (0.88-4.77)
Diet soda>4/wk	NR	NR	0.65 (0.34-1.23)	NR	NR	1.94 (0.69-5.40)
Total lifetime intake	NR	NR	1.00 (0.74-1.35)	NR	NR	1.81 (0.83-3.94)

*Results of linear logistic regression adjusted for smoking, coffee, diabetes, bladder infection, and occupation.
**Results of continuous conditional logistic regression adjusted for smoking and diabetes.
NA= Not Available; ND = Not Done; NR = Not Reported

The findings of Howe *et al*. (1977, 1980) showed a significant increasing risk of bladder cancer with increasing exposure to artificial sweeteners for men (relative risk of 1.6, 95% confidence interval 1.1-2.4). The population consisted of urinary bladder cancer cases diagnosed during 1974-1976 in British Columbia, Newfoundland and Nova Scotia. Of 821 eligible patients identified from cancer registries, 632 were interviewed (among those excluded, 43% were not interviewed because they were too ill or dead). Population-based controls were matched individually to the patients by sex, age and neighborhood. Participants answered a detailed questionnaire on use of a variety of artificial sweeteners. They combined frequency and duration of artificial sweetener use to group individuals by lifetime exposure, with two broad categories for exposure level and two for duration. A significant increase in risk ($p=0.007$, trend test) was associated with increased exposure, with relative risks ranging from 1.6 to 5.3. In another analysis, Howe *et al*. divided frequency in more restricted categories and also found a significant increase. The highest exposure group was >8 tablets/day, with a relative risk of 2.8.

Howe *et al*. (1977) also reported that diabetics within the same study population were in fact at a decreased risk compared to non-diabetics, with a relative risk of 0.7. This overall decreased risk could be at least partly explained by the lower frequency of smokers among diabetics. However, when the analysis was restricted to a comparison of male diabetic users of artificial sweeteners to diabetic non-users, the relative risk for users was 1.93 (95% CI 0.65-5.60), which was very similar to the relative risk of 1.7 for non-diabetics in the same study.

The largest investigation was a case-control study conducted by the National Cancer Institute (Hoover and Hartge Strasser, 1980). In this study, 3,010 newly diagnosed cases of bladder cancer from ten U.S. geographical areas were compared to 5,783 geographic area-, age- and sex-matched controls. There were 2258 male patients and 4277 male controls; 742 female cases and 1499 female controls. Patients were interviewed at home about various risk factors. Cases considered to be at low and high risk for bladder cancer were analyzed separately. Low risk white women were defined as those who never smoked and were never exposed occupationally to known bladder carcinogens; high risk men were defined as those who smoked more than 40 cigarettes daily. The data were analyzed with respect to the use of artificial sweeteners, with no distinction made between consumption of saccharin and cyclamates. No overall association was found between the use of artificial sweetener and risk of bladder cancer in men or women. However, an increased risk was observed for heavy use of table-top and diet drinks both in men who smoked more than 40 cigarettes daily, and in low-risk white women (defined above). The authors concluded that this pattern of positive associations was consistent with a weak effect from artificial sweeteners as a whole, but they did not dismiss the possibility of chance as an alternative explanation.

Hoover and Hartge Strasser (1980) presented relative risks for the entire study population of bladder cancer adjusted for age, race, and sex for the combined use of artificial sweeteners in tablet and in diet drink form (Table III in Hoover and Hartge Strasser, 1980). The combined use of sweeteners was tabulated as average number of daily uses of table-top sweeteners (none, <3, 3-5, $\geq$6) by average number of daily servings of diet drinks (none, <2, $\geq$2). Relative risks were 1.56 for individuals who used at least two diet drinks and three to five servings of table-top sweeteners daily, and 1.64 for individuals who used at least two diet drinks and at least six servings of table-top sweeteners daily (Hoover and Hartge Strasser, 1980). The authors noted

that one average serving of diet drink contained two to three times as much artificial sweetener as one average use of table-top artificial sweetener.

Table 3 below presents a re-analysis of the data in Table III of Hoover and Hartge Strasser (1980), in which daily use of table-top sweetener and daily use of diet drinks were combined to derive an average number of (total) daily servings of artificial sweeteners for all individuals in the study population. In combining these data, one diet drink was assumed to be equivalent to 2.5 servings of table-top sweetener, and mid-point estimates for the exposure ranges in Table III were as follows: <2 diet drinks = 2.5 daily servings, ≥2 diet drinks = 10 daily servings, <3 table-top uses = 1.5 daily servings, 3-5 table-top uses = 4 daily servings, and ≥6 table-top uses = 8 daily servings. A significant increasing trend in relative risk was found between consumption levels of 10 to 18 servings/day, with relative risks ranging from 1.23 to 1.70 (test for trend, $p = 0.006$, $\chi^2 = 7.56$) (Table 3). These findings are consistent with a small dose-dependent increased risk of bladder cancer in individuals consuming high levels of artificial sweeteners.

Table 3. Average Number of Daily Servings of Artificial Sweeteners and Relative Risk of Bladder Cancer, Based on Data Presented in Table III of Hoover and Hartge Strasser (1980).

Average Number of Daily Servings[a]	Relative Risk	Number of Cases	Number of Controls
0	1.00	1707	2231
1.5	1.00	189	367
2.5	0.96	314	638
4.0	1.03	292	553
6.5	0.79	59	146
8.0	1.03	18	34
10.0	1.23	38	60
10.5	1.6	28	34
11.5	1.26	35	54
14.0	1.56	20	25
18.0	1.70	7	8

[a] Average number of daily servings of artificial sweetener, where <2 diet drinks = 2.5 daily servings, ≥2 diet drinks = 10 daily servings, <3 table-top uses = 1.5 daily servings, 3-5 table-top uses = 4 daily servings, and ≥6 table-top uses = 8 daily servings.

As discussed by Hertz-Picciotto and Neutra (1994), sodium saccharin is a relatively weak carcinogen in the rat. Using cancer potency estimates derived by Carlborg (1985) based upon bladder tumor incidence data from the Schoenig *et al*. (1985) two-generation bioassays in the rat to predict human risk, Hertz-Picciotto and Neutra (1994) suggested that the relative risk from consumption of 30 mg/kg/day saccharin (equivalent to more than a dozen diet drinks/day) would be on the order of 1.1. These authors concluded "unless typical saccharin consumption is far

higher than what is contained in a few diet sodas per day, the doses to which the general population is exposed will not produce risks that are amenable to epidemiologic study. More bluntly, further observational studies of saccharin carcinogenicity in humans would be unlikely to be fruitful." (Hertz-Picciotto and Neutra, 1994).

In summary, epidemiology studies have not demonstrated a significant risk of bladder cancer associated with the use of artificial sweeteners, including sodium saccharin, although the relationship between neonatal exposure and cancer risk has not been adequately studied. Two of the largest and most well-designed case-control studies have found small increased risks in certain subgroups of the population; however, the subgroups identified as being at elevated risk are inconsistent between the two studies. Increased risks were observed in men by Howe *et al*. (1977, 1980) and in men who smoked more than 40 cigarettes daily and in women who never smoked and were never exposed occupationally to known bladder carcinogens by Hoover and Hartge Strasser (1980). This lack of consistency suggests that either the associations are not causal and are probably attributable to chance or recall bias in certain sub-groups, or saccharin is a weak carcinogen and the lack of association in other subgroups is attributable to confounding from dietary, occupational or other factors, bias from non-differential misclassification of exposure (random errors of recall), or chance association due, in part, to low power to detect a weak effect. As indicated by the analysis of Hertz-Picciotto and Neutra (1994), epidemiology is unlikely to be able to detect a small increase in cancer risk in persons consuming high doses of sodium saccharin, even if humans are equally sensitive as rats to its carcinogenic effects.

3.2 Carcinogenicity Studies in Animals

Studies in experimental animals have been described in detail by IARC (1999). These studies include numerous rodent studies in which experimental animals, primarily rats and mice, have been exposed to saccharin in their diet. The key studies are two-generation bioassays in which animals of each sex of the parent generation (F_0) were administered sodium saccharin in the diet at doses up to 7.5% from weaning or soon after weaning, through mating, pregnancy and weaning of offspring. The offspring (F_1 generation) were then placed on the same diet as their parents for the duration of their lifetime, approximately two years. These studies are briefly described below.

Studies In The Rat

Tisdel et al. (1974) [1973 Wisconsin Alumni Research Foundation (WARF) Studies]

F_1 generation Sprague-Dawley rats (20 animals/sex/group) were fed a diet containing sodium saccharin (0, 0.05, 0.5 or 5%) for up to 100 weeks. Survival was relatively poor among male rats, including the control group. In the 5% sodium saccharin diet group of male rats, seven developed transitional cell carcinomas of the urinary bladder (and one "papillary projection") compared to none in the control group ($p < 0.05$, by Fisher's exact test). The bladder of one male rat in the 0.5% sodium saccharin-treated group developed epithelial hyperplasia with numerous mitotic figures, which the authors considered "precancerous." One "undifferentiated malignancy" of the bladder developed in a low-dose female rat. Four high-dose male rats developed pituitary tumors (two adenomas, one adenocarcinomas, and one carcinoma; $p = 0.053$, by Fisher's exact test) while no control or other treated groups of male rats developed tumors at

this site. Five squamous cell carcinomas of the uterus were observed exclusively in sodium saccharin fed rats, with zero, one, two, and two tumors occurring in the control, low-, mid-, and high-dose female rats, respectively. The small group size limits the power of this study to detect carcinogenic effects.

Arnold et al. (1980) [1977 Canadian National Health and Welfare Ministry Studies]

Thirty-day old Sprague-Dawley rats (50 animals/sex/group) were fed a diet containing either 0 or 5% sodium saccharin continuously for life. Animals were mated after three months. Pups were weaned to their parents' diet, and 50 males and 50 females were randomly selected to constitute the F_1 generation. Of the F_1 generation animals surviving 67 weeks or longer, 8/45 of saccharin-treated males developed transitional cell carcinomas of the bladder compared to 0/42 controls ($p = 0.0037$, by Fisher's exact test). Four treated F_1 males developed transitional cell papillomas. Two of 49 surviving saccharin-treated F_1 females developed bladder cancers, compared to none in the control group.

Bladder tumors (benign and malignant) were observed in 1/36 control F_0 males and 7/38 saccharin-treated F_0 males surviving 87 weeks or more (time to first tumor) ($p = 0.033$, by Fisher's exact test). One treated F_0 male and two treated F_0 females had urothelial tumors of the renal pelvis, and one treated F_0 male had a urethral tumor.

Taylor et al. (1980) [1973 U.S. Food and Drug Administration Studies]

Male and female F_1 generation CD Sprague-Dawley rats (48/sex/group) were fed a diet containing sodium saccharin (0, 0.01, 0.1, 1, 5 or 7.5%) for up to 28 months after their parents had been fed the same diet from weaning. A control group received a diet containing 1.51% sodium carbonate to approximate the amount of sodium excreted by the 5% sodium saccharin-treated group. Interim sacrifices of F_1 animals were performed at 14 months (four/sex/group) and 18 months (five/sex/group). In F_1 rats fed 7.5% sodium saccharin, 6/23 male rats surviving beyond 18 months developed transitional cell papillomas (2) or carcinomas (4) of the bladder (0/29 controls; $p = 0.005$, by Fisher's exact test). The only other transitional cell carcinoma or papilloma occurred in one F_1 female rat fed 7.5% sodium saccharin.

Schoenig et al. (1985) [International Research and Development Corporation (IRDC) studies]

Male F_1 generation Sprague-Dawley rats were fed a diet containing 0 (n = 350), 1% (n = 700), 3% (n = 500), 4% (n = 200), 5% (n = 125), 6.25% (n = 125) or 7.5% (n = 125) sodium saccharin. The incidences of transitional cell tumors of the urinary bladder are presented in Table 4 below. Statistically significant increases in combined incidence of bladder neoplasms occurred at the 3%, 4%, 5%, 6.25%, and 7.5% levels of sodium saccharin in feed. Organs other than those of the urinary system were not examined microscopically unless gross lesions or masses were observed.

Table 4. Incidences of Urinary Bladder Neoplasms in F_1 Male Sprague-Dawley Rats Exposed to 1 - 7.5% Sodium Saccharin in the Diet (Schoenig *et al.*, 1985).*

Bladder Neoplasms	Dose (% in Diet)						
	0	1%	3%	4%	5%	6.25%	7.5%
Transitional Cell Papillomas	0/324	4/658	4/472	***4/189***	***4/120***	***12/120***	***18/118***
Transitional Cell Carcinomas	0/324	1/658	4/472	***8/189***	***11/120***	***8/120***	***19/118***
Transitional Cell Papilloma or Carcinoma	0/324	5/658 (0.8%)	***8/472*** (1.7%)	***12/189*** (6.3%)	***15/120*** (12.5%)	***20/120*** (16.7%)	***37/118*** (37.4%)

* Statistically significant increases in tumor incidence relative to controls are shown in ***bold italics*** ($p < 0.05$, by Fisher's exact test).

Schoenig *et al.* (1985) also investigated the relationship between urinary bladder tumors and *in utero* exposure. Two groups of rats were exposed to 5% sodium saccharin in the diet. In one group, F_0 animals (52 males, 104 females) were placed on the saccharin diet four days prior to mating. Female rats remained on the test diet throughout gestation, but were placed on a control diet after birth (during lactation). After weaning, F_1 male offspring were selected (n = 125) and continued on control diets through the 30 months of age, at which time the surviving animals were killed**.** In the second group, F_0 animals remained on the control diet until the F_1 offspring were born. Lactating dams were then placed on a diet containing sodium saccharin (1%). The concentration of sodium saccharin was increased weekly so that by week three the concentration was 5%. After weaning, F_1 male offspring (n = 125) continued on the 5% sodium saccharin diet through 30 months of age**.** The incidence of the urinary bladder tumors (transitional cell papilloma or carcinoma) in male rats exposed *in utero* was 0/122. In rats exposed from birth (via milk) and throughout life, the incidence was 12/120 ($p = 0.00017$, by Fisher's exact test).

Chowaniec and Hicks (1979)

Eight-week-old Wistar rats were treated with sodium saccharin in either drinking water (75 males and 50 females at 2 g/kg/day, approximately 4% of the diet, according to the authors), or feed (75 males, 75 females at 4 g/kg/day, approximately 7-8% of the diet, according to the authors) for two years. A control group of 55 males and 50 females was not treated. Urothelial tumors of the bladder developed in 3/70 male rats fed sodium saccharin (*vs.* 0/52 in controls; $p = 0.19$, by Fisher's exact test). A single male rat in the drinking water group developed a urothelial tumor of the ureter and a single female rat in the drinking water group developed a urothelial cell tumor of the renal pelvis. Both sexes developed mild urothelial hyperplasia after 85 weeks from sodium saccharin exposure either in drinking water or in feed. Lymphosarcoma or leukemia was found in six saccharin-treated male rats (4 in the drinking water group, 2 in the fed group) compared to none in the control group (for the drinking water group, $p = 0.1$, by Fisher's exact test).

Fukushima et al. (1983)

Several strains of six-week old male rats (ACI, Wistar, F344, and Sprague-Dawley; 40 per strain) were fed diet containing 5.0% sodium saccharin for up to 52 weeks. The experimental design also included control groups (40 per strain, except 45 ACI rats). Interim sacrifices were conducted at 12, 24, and 36 weeks (five per strain, except four ACI rats at week 24). Among ACI rats only, there was an increase in papillary tumors of the urinary bladder (9/32 treated *vs.* 0/28 control, $p = 0.0019$, by Fisher's exact test). Three transitional cell carcinomas of the urinary bladder were observed in sodium saccharin treated ACI rats, but the increase was not statistically significant (3/32 treated *vs.* 0/28 control, $p = 0.14$, by Fisher's exact test). Combined bladder tumor incidence was not presented in the results. The authors reported that "more than half" of both the control and treated ACI rats had *Trichosomoides crassicauda* nematodes in their bladders. The authors stated "the parasite was not associated with the presence of bladder tumors in the ACI rats." No tumors of the urinary bladder were observed in any other strain. Nematode infection was absent in the other strains.

The notable differences in tumorigenicity in studies with different exposure periods supports the hypothesis that critical cancer-related effects of sodium saccharin occur during periods of organ growth, namely cell proliferation or cell division within the urinary bladder, which primarily occurs post-gestationally, during the first three weeks of life in the rat (Cohen and Ellwein, 1990). See Section 4.1 below for additional discussion of the importance of the neonatal period with respect to sodium saccharin-induced bladder cancer.

Animal Data from Species Other Than Rat

Mouse: Several long-term single generation studies of the carcinogenicity of sodium saccharin have been conducted in mice (Homburger, 1978; Fukushima *et al.*, 1983; Frederick *et al.*, 1989; as described in IARC, 1999). Treatments with sodium saccharin began when the mice were six to 19 weeks of age. In one of these studies (Homburger, 1978), two papillomas of the urinary bladder and two cases of papillary hyperplasia were observed among high-dose sodium saccharin treated male mice. A transitional cell carcinoma associated with a bladder stone was found in a control male mouse. Vascular tumors were increased among high-dose male mice in one study (Homburger, 1978), but the increase was not considered to be "significant" (IARC, 1999). IARC (1999) noted limitations to each of these studies including inadequate reporting (Homburger, 1978), small numbers of animals and short duration (Fukushima *et al.*, 1983), and variability in response among control groups (Frederick *et al.*, 1989).

Multigeneration studies were conducted in Swiss mice, exposing $F_0 - F_6$ mice for their lifetimes to 0, 0.2 or 0.5% sodium saccharin in the diet (Kroes *et al.*, 1977; as described in IARC, 1999). Only mice in the F_0, F_{3b}, and F_{6a} generations were examined for tumors. Two male mice (one low-dose F_0 and one high-dose F_{3b}) developed transitional cell carcinomas of the urinary bladder. One female mouse in the control group developed an anaplastic carcinoma of the urinary bladder.

Early experiments by Allen *et al.* (1957) and subsequently by Bryan and Yoshida (1971) demonstrated that bladder tumors could be induced in mice through the implantation of pellets into the lumen of the bladder. Bryan and Yoshida tested 100 chemicals for bladder

carcinogenicity by this method. Saccharin was one of the 15 chemicals which was positive in more than one testing (NAS, 1979). More recently, female Swiss mice (100 per group), aged 60-90 days, received pellets containing either sodium saccharin plus cholesterol or cholesterol alone, inserted into the urinary bladder in two separate experiments (Clayson, 1974; Jull, 1979; DeSesso, 1989; as described in IARC, 1999). IARC (1999) states that 99% of the sodium saccharin had disappeared from the pellet within 1.5 days. In both experiments, significant increases in urinary bladder carcinoma were observed in animals implanted with the saccharin/cholesterol pellet (Trial 1: 31/66 compared to 8/63 in cholesterol pellet only controls; Trial 2: 33/64 compared to 5/43 in controls; $p < 0.001$). IARC (1999) reported that carcinomas in saccharin-exposed mice were more frequently multiple and invasive ($p < 0.009$) and that they were composed of cells with a high mitotic index and exhibited more squamous or glandular metaplasia than that found in tumors in control animals. IARC (1999) also noted a lack of details about these studies and the difficulty in interpreting results of studies involving pellet implantation techniques in mice.

Monkey: Takayama *et al.* (1998) exposed twenty monkeys of three species (seven rhesus, six cynomolgus, six African green, and a rhesus-cynomolgus hybrid) to sodium saccharin in the diet (25 mg/kg body weight for five days per week) beginning within 24 hours after birth and continuing for up to 24 years. Sixteen monkeys (seven rhesus, nine cynomolgus) served as control animals. At necropsy, there was no evidence of urothelial tumor formation and no evidence of increased urothelial proliferation, either by light microscopy or by scanning electron microscopy. There was also no evidence of formation of calcium phosphate-containing urinary precipitate. The dose used in this study was relatively low compared to those that induced bladder tumors in rats. In rats, a dose of sodium saccharin associated with slight increases in bladder tumor incidence was 1% in feed (see Schoenig *et al.*, 1985, above), this corresponds to a daily dose of 400 mg/kg_{bw}-day (calculated assuming male rats consume 4% of their body weight in feed per day). IARC (1999) noted the following limitations to the study: the relatively low dose, the small numbers of animals, and the multiplicity of species.

Small numbers of rhesus monkeys (two to three/sex/dose) were given oral doses of sodium saccharin (0, 20, 100, or 500 mg/kg-day) six days per week for up to 79 months (McChesney *et al.*, 1977 [abstract only]; as described in IARC, 1999). No abnormalities of the urinary bladder were found, although the study is extremely small and considerably less than the lifetime of this species.

Guinea Pig: Thirty six-week-old male Hartley guinea pigs were fed a diet containing 5% sodium saccharin for 52 weeks (Fukushima *et al.*, 1983). Interim sacrifices were conducted at 0, 4, 12, 16, and 20 weeks (three guinea pigs at each time point). The control group consisted of 20 untreated guinea pigs. No bladder lesions were found, although the number of animals at the terminal sacrifice was small (12) and the experiment was considerably less than the lifetime of the animals.

Summary of Bioassay Data

The induction of bladder carcinogenesis by sodium saccharin has been shown to occur when rats are chronically exposed to doses at or above 1% to 3% in the diet for periods that include the neonatal period. Small, often non-significant increases in bladder tumor incidence associated

with sodium saccharin treatment have been observed in bioassays of weanling rats. Male rats are more sensitive than female rats to the induction of bladder tumors by sodium saccharin. The best study evaluating the carcinogenic effects in rats was conducted by Schoenig *et al.* (1985) and was large enough to detect small increases in tumor incidence (less than 10%). Several other studies have been conducted, but these have limitations including small numbers of animals at end-of-study and limited histopathological evaluation. Multigeneration diet studies in mice observed small non-significant increases in bladder tumors in sodium saccharin treated animals. Studies in other species have either not been conducted at high enough doses (monkeys) or have not included the neonatal period (guinea pig).

3.3 Other Relevant Data

3.3.1 Tumor Promotion and Co-Carcinogenesis Studies

Rat Studies

More than twenty studies using initiation/promotion protocols have been conducted evaluating sodium saccharin potential as a tumor promotor in rats. Sodium saccharin has been shown to promote bladder tumors in rats when administered in drinking water or in feed after initiation (IARC, 1999). The promotional properties of sodium saccharin have been observed following initiation with chemicals known to cause bladder tumors. Initiation has been achieved by treatment with relatively low doses over short durations in order to minimize the background incidence of the bladder tumors caused by the initiating agent alone. Drinking water, feed, and instillation directly into the bladder are routes of exposure that have been used in initiation, while promotion has generally been performed with sodium saccharin in the diet. Specific initiators include *N*-methyl-*N*-nitrosourea [MNU], *N*-[4-(5-Nitro-2-furyl)-2-thiazolyl]formamide [FANFT], and *N*-butyl-*N*-(4-hydroxybutyl)nitrosamine [BBN]. Multiple studies have been conducted with each of these initiating agents, with mixed results (see Table 5 below). Positive findings for bladder tumor promotion by sodium saccharin have been reported following initiation by MNU (Hicks *et al.*, 1975; Hicks *et al.*, 1978), FANFT (Cohen *et al.*, 1979; Murasaki and Cohen, 1983 Fukushima *et al.*, 1981; Sakata *et al.*, 1986; Okamura *et al.*, 1991; Cohen *et al.*, 1991), and BBN (Nakanishi *et al.*, 1980b; Hagiwara *et al.*, 1984; Fukushima *et al.*, 1990). Sodium saccharin has also been shown to be cocarcinogenic in male rats when administered concurrently with FANFT for two years (Cohen *et al.*, 1982).

Not all tumor promotion studies of sodium saccharin have produced positive findings. Such studies include those using the initiating agents MNU (Green and Rippel, 1979; Hooson *et al.*, 1980; West *et al.*, 1994), 2-acetylaminofluorene (Ershoff and Bajwa, 1974), FANFT (Imaida and Wang, 1986), and BBN (Sakata *et al.*, 1984; Yu *et al.*, 1992).

Several of the studies have limitations that make interpretation of the results difficult. These include variable dosing within treatment groups due to differing feed and water consumption rates, limited reporting of experimental detail, carcinogenic doses of initiating agents possibly obscuring a promotion effect by sodium saccharin, short experimental duration, and/or small numbers of animals. Many of these study limitations are identified in the IARC review. See Table 5 below for study-specific information.

Sodium saccharin also promotes bladder tumor development after freeze-ulceration of the bladder is performed as an "initiation" event (Murasaki and Cohen, 1983; Hasegawa *et al.*, 1985).

Table 5. Promotion Studies of Sodium Saccharin in Rats.

Initiating Compound*	Study	Sex	Outcome	Comments and Notes
MNU	Hicks *et al.*, 1978	F	+	No MNU dose estimate; possible decay in water
	Green and Rippel, 1979	F	−	Incomplete reporting
	Hooson *et al.*, 1980	F	−/+	Increase in proliferative lesions of the bladder, but not neoplasia; treatment began well after weaning; dose of initiator increased background tumors
	Tsuda *et al.*, 1983	M	−/+	Short exposure duration; increased bladder papillary or nodular hyperplasia, but not neoplasia
	West *et al.*, 1986	F	+	Significant increase at 2.5% sodium saccharin, but not at 0.1, 0.5, 1.0, or 5%. Also decreased time-to-tumor; increased tumor incidence in initiator control group complicated interpretation.
	West *et al.*, 1994	F	−	Carcinogenic dose of MNU (too high); some effects at some doses; short exposure to promoter (4 weeks)
AAF	Ershoff and Bajwa, 1974	F	−	Increase in mucosal hyperplasia of the bladder; inadequate number of animals; no dose estimates
FANFT	Cohen *et al.*, 1979	M	+	Small number of animals
	Murasaki and Cohen, 1983	M	+	Small number of animals
	Fukushima *et al.*, 1981	M	+	—
	Sakata *et al.*, 1986	M	+	Short period of promoter administration (61 weeks)
	Okamura *et al.*, 1991	M	+	Possible confounding by diet; lack of control group for one diet

	Cohen *et al.*, 1991	M	+	Short period of promoter administration
	Imaida and Wang, 1986	M	−	—
BBN	Nakanishi *et al.*, 1980a	M/F	−/+	Hyperplastic lesions increased only (both male and female rats); short promotion period (32 weeks)
	Nakanishi *et al.*, 1980b	M	−/+	Hyperplastic lesions increased only; variable water consumption (dose) among groups; short promotion period (32 weeks)
	Ito *et al.*, 1983	M	+	Limited reporting
	Hagiwara *et al.*, 1984	M	+	Papillomas, hyperplasia only
	Sakata *et al.*, 1984	M	−	Short exposures and experiment duration
	Fukushima *et al.*, 1990	M	+	Short duration; small number of animals
	Yu *et al.*, 1992	M	−/+	Significant increase in papillary or nodular hyperplasia, but not bladder tumors; small number of animals (20-23); short promotion period (36 weeks)
NDBA	Pereira *et al.*, 1983	M	+/−	Carcinomas and hyperplastic nodules of the liver; variable consumption of initiator (dose)

* Abbreviations used in table: MNU = *N*-methyl-*N*-nitrosourea; AAF = 2-acetylaminofluorene; FANFT = *N*-[4-(5-nitro-2-furyl)-2-thiazolyl]formamide; BBN = *N*-butyl-*N*-(4-hydroxybutyl)nitrosamine; NDBA = *N*-nitrosodibutyl-amine.

Mouse Study

A single study examining promotion properties of sodium saccharin in mice was reported by IARC (1999). In this study, female weanling mice were initiated with AAF in their diet, followed by treatment with several concentrations of sodium saccharin in feed from weeks 19 to 135 of life (Frederick *et al.*, 1989). No significant increases in tumors of the bladder or any other site were observed in relation to treatment with sodium saccharin.

3.3.2 Genetic Toxicology

Genotoxicity data have been reviewed by Ashby (1985), Arnold and Boyes (1989), and by IARC (1999).

Chromosomal Alterations (In Vivo and In Vitro)

In vivo clastogenicity studies have mostly been negative although induction of sister chromatid exchanges and single-strand breaks in bone marrow and spermatocytes have been reported in mice after dosing with sodium saccharin at levels up to four g/kg (IARC, 1999). Ashby (1985) also reviewed studies reporting sodium saccharin induced dominant lethal mutations in mice in four of eight studies and none in a rat study. The studies reviewed in Ashby (1985) reported oral, drinking water, diet, i.p. and subcutaneous administration of sodium saccharin at doses up to 10g/kg and there was limited evidence of chromosomal translocations in one study.

In clastogenicity assays *in vitro*, high doses of sodium saccharin induced chromosomal aberrations and sister chromatid exchanges in Chinese hamster ovary, Don and lung cells (IARC, 1999). Ishidate and Odashima (1977) suggested that the clastogenicity in Chinese hamster lung (CHL) cells might be the result of osmotic changes induced by high sodium concentrations. A similar clastogenic response in CHL cells was found for sodium chloride and sodium nitrate when each was tested at elevated dose levels (Ashby, 1985). Ashby and Ishidate (1986) reported equal clastogenicity with different salt forms of saccharin at high dose levels (8-16 mg/ml) in Chinese hamster lung cells and suggested that ionic effects in the assay medium may be critical to clastogenicity. Saccharin salts were not clastogenic *in vitro* at dose levels lower than four mg/ml.

DNA Damage (In Vivo and In Vitro)

Sasaki *et al*. (2002) tested 39 currently used food additives including saccharin and sodium saccharin for the induction of DNA damage in mice using the comet assay. Male ddY mice (four per group) were administered oral doses of sodium saccharin of 100, 1000 or 2000 mg/kg_{bw} and sacrificed three or 24 hours after treatment. No death, morbidity or clinical signs were observed after any treatment. The comet assay was performed on purified nuclei of cells harvested from stomach, colon, liver, kidney, urinary bladder, lung, brain and bone marrow. Sodium saccharin induced DNA damage that was statistically significantly increased above controls ($p < 0.05$) in stomach and colon at doses of 1000 and 2000 mg/kg_{bw} at the three-hour time point. For saccharin (acid form), only the colon at three hours after treatment exhibited statistically significant increases in DNA damage by the comet assay. The authors concluded that sodium saccharin induces DNA damage in gastrointestinal organs at levels below those causing clinical signs. For sake of comparison, the sweeteners sodium cyclamate and sucralose showed weak DNA damaging activity at doses of 2000 mg/kg while aspartame, acesulfame K, and stevia were negative in all organs tested at the same doses (Sasaki *et al.*, 2002). Food dyes such as amaranth, allura red and tartrazine were positive in stomach and gastrointestinal organs at doses as low as 10 mg/kg (Sasaki *et al*., 2002).

Jeffrey and Williams (2000) tested sodium saccharin and other sweeteners in a rat hepatocyte/DNA repair assay that measured unscheduled DNA synthesis (UDS) as an indicator

of DNA damage. For sodium saccharin treatment in a range of 0.05 to 0.2 M in cultured hepatocytes from two different rat strains, F344 and Sprague-Dawley, no unscheduled DNA synthesis was observed in comparison to a high level of UDS induced by the known genotoxicant, 2-aminofluorene. The authors concluded that sodium saccharin does not have DNA-damaging activity.

DNA Binding

Lutz and Schlatter (1977) looked for evidence of genotoxic damage to DNA by assaying the level of ^{35}S-labeled saccharin bound to DNA in male Sprague-Dawley rats treated with saccharin (2.1 mmol/kg body weight) by gavage. No evidence of DNA-saccharin binding was observed in either liver or bladder tissue.

Gene Expression Studies

Yang and Duerksen-Hughes (1998) proposed monitoring the induction of the tumor suppressor gene p53 which is involved in cell cycle progression as an indication of DNA damage. A large number of studies have demonstrated that cells respond to DNA damage by increasing their levels of p53; increased cellular levels of p53 act to prevent the replication of damaged DNA. Thus increased levels of p53 may be an important indicator of DNA damage by direct and indirect DNA reactive agents. An important mechanism leading to increased levels of p53 protein is post-translational modification of the protein, leading to an extension of its normally short half-life. The half-life of p53 protein was extended by treatment with both direct and indirect DNA reactive agents (Yang and Duerksen-Hughes, 1998). Yang and Duerksen-Hughes (1998) analyzed the p53 protein levels in cultured NCTC 929 mouse fibroblast cells by Immunoblot and ELISA (enzyme linked immunosorbant sandwich assay) to investigate the mechanism of sodium saccharin genotoxicity. Direct acting DNA-reactive agents such as *N*-methyl-*N*-nitro-*N*-nitrosoguanidine (MNNG) and *N*-ethyl-*N*-nitrosourea (ENU) induced a quick elevation of p53 levels, while indirect acting agents (requiring metabolic activation) such as aflatoxin B_1 or 2-acetylaminofluorene caused a later peak of p53 induction. Sodium saccharin did not cause an increase in the levels of the p53 protein, supporting the idea that saccharin if it is genotoxic must act via a non-DNA reactive mechanism.

Ogretmen *et al*. (1996) investigated the ability of sodium saccharin to induce the expression of Her2/*neu* and p53 mRNA. As described above, p53 expression is an indicator of DNA damage while Her2/*neu* is an oncogene known to be active in the etiology of breast cancer and many other tumors. Ogretmen *et al*. (1996) utilized the human breast epithelial cell line HBL-100 to test for sodium saccharin mediated induction of Her2/*neu* and p53 expression. When administered to the cell cultures alone, sodium saccharin had no effect on the expression of these two genes. However, when sodium saccharin was administered to HBL-100 cells subsequent to treatment with the known tumor initiating agents MNU or 7,12-dimethylbenz[*a*]anthracene (DMBA), the expression of Her2/*neu* but not p53 was significantly elevated in MNU pretreated cells and slightly elevated in DMBA treated cells. The authors concluded that there is a relatively small effect for sodium saccharin on the transcriptional regulation of Her2/neu.

Mutations (Mammalian Studies)

Mutagenicity studies in rodents *in vivo* and in mammalian cells *in vitro* have been generally negative (IARC, 1999). The few studies reporting positive results are briefly discussed, as well as one study published since the IARC (1999) review. Sodium saccharin was weakly mutagenic in one study in mouse lymphoma L5178Y cells at a dose level of 17-19 mg/ml [Clive *et al.*, 1979]. In two follow-up studies carried out by the Stanford Research Institute, one gave a weakly positive result and the other was negative for dose levels up to 20 mg/ml (LBI/SRI, 1979, as reviewed by Ashby, 1985). Other *in vivo* studies utilized the mouse coat spot test and dominant lethal mutation assay in mice and rats. For both of these assays there were some positive and negative studies and no definitive conclusions regarding mutagenesis in mammalian systems can be drawn (Ashby, 1985; IARC, 1999). Suzuki and Suzuki (1988; 1993) reported that sodium saccharin was mutagenic in RSa cells, a hypermutable cell line derived from human embryos. Sodium saccharin induced point mutations in K-*ras* after six days of treatment (15-30 mg/ml) (Suzuki and Suzuki, 1993) and a dose-dependent increase in the number of oaubain-resistant mutants after a 24-h treatment at doses ranging from 10-22.5 mg/ml. Mutations in codon 12 of the K-*ras* gene were detected in genomic DNA by PCR (polymerase chain reaction) and dot blot hybridization. Equimolar concentrations of NaCl were without effect (Suzuki and Suzuki, 1993). The authors suggested that sodium saccharin was able to induce mutations in RSa cells leading to oubain resistance and point mutations in *K*-ras genes.

Turner *et al.* (2001) assessed the mutagenic potential of sodium saccharin in *lacI* transgenic Big Blue rats. Male rats ($n = 20$) were fed a diet containing 5% sodium saccharin for ten consecutive days and killed 14 days after the final dosing. DNA was isolated and analyzed for the presence of mutations in both liver and bladder tissues. No increases in mutation frequency were observed in sodium saccharin-treated rats.

Mutations (Prokaryotic Studies)

Sodium saccharin was not mutagenic in *Salmonella typhimurium* strains TA100, TA1535, TA1538, TA98, TA92 or TA94 (IARC, 1999). One early study (Batzinger *et al.*, 1977) reported that urine of saccharin-dosed mice was weakly mutagenic to *Salmonella typhimurium* strain TA100. However, further studies found that saccharin caused a marked diuretic effect in mice, which probably resulted in an increase in total urinary histidine concentration. The presence of histidine in the urine might have contributed to false positives since the tester strains of *Salmonella typhimurium* are histidine auxotrophs. No mutagenicity was observed when the urine was diluted to correct for volume change (Ashby, 1985).

Discussion of Genotoxicity Studies

High doses of sodium saccharin have induced genotoxic effects *in vitro* and *in vivo.* The dose levels of sodium saccharin required to induce these effects are up to 10,000 times higher than those required by reference genotoxicants (Ashby, 1985). Ashby (1985) concluded that sodium saccharin elicits the expected toxicities of a sodium salt, but these effects may be more evident than for saline because higher dose levels can be used (LD_{50} for sodium saccharin is 17 g/kg). The evidence for genotoxicity of sodium saccharin is conflicting. Sodium saccharin is clearly not mutagenic in *Salmonella typhimurium* (IARC, 1999) and these results are supported by

similar mammalian *in vivo* studies in transgenic Big Blue rats (Turner *et al.*, 2001) in which mutations were not detected in any organs or tissues. However, this body of evidence is seemingly contradicted by a report from Suzuki and Suzuki (1993) in which point mutations in codon 12 of the K-*ras* gene in a human cell line were demonstrated in response to sodium saccharin treatment. Mouse coat spot tests for *in vivo* mutagenicity and tests for dominant lethals have also been conflicting, with both positive and negative reports (Ashby, 1985).

Several negative studies of chromosomal aberrations have been reported for sodium saccharin *in vivo* while most *in vitro* assays reported a positive clastogenic response (IARC, 1999). Supporting a role for sodium saccharin as a DNA damaging agent is the study of Sasaki *et al*. (2002), which utilized the comet assay to demonstrate that sodium saccharin administered to mice *in vivo* is capable of causing DNA damage in tissues of the digestive tract, but not in the bladder. Jeffrey and Williams (2000) reported that sodium saccharin was not capable of inducing unscheduled DNA synthesis, a measure of DNA repair, in cultured rat hepatocytes. In addition, the report by Lutz and Schlatter (1977) did not detect binding of ^{35}S-labeled saccharin to DNA in liver or bladder tissue.

Evidence that sodium saccharin is not DNA-reactive by either direct or indirect mechanisms comes from the observation that p53, a cell cycle control element that responds to DNA damage, was not induced by sodium saccharin and lends further support for a non-mutagenic action of saccharin (Yang and Duerksen-Hughes, 1998). In addition, studies of the ability of sodium saccharin to induce expression of an oncogene (Her2/*neu*) or a tumor suppressor gene (p53) at the mRNA level in cell cultures of human breast cells were negative (Ogretmen *et al.*, 1996). Treatment with sodium saccharin alone had no effect on expression of Her2/neu or p53. However, a weak induction of Her2/*neu* expression was noted when sodium saccharin treatment was preceded by treatment with the tumor initiating agent MNU (Ogretmen *et al.*, 1996) which provides further support for an indirect activity of sodium saccharin in carcinogenesis. The fact that there were no common endpoints evaluated between any of the studies described above increases the difficulty of drawing a definitive conclusion as to the genotoxicity or mutagenicity of sodium saccharin.

3.3.3 Pharmacokinetics and Metabolism

The majority of reports investigating the pharmacokinetics and metabolism of saccharin were carried out using the acid form of saccharin. Because all forms of saccharin are nearly completely ionized at physiological pH, and because absorption takes place primarily in the small intestine under conditions of neutral pH, the various forms of saccharin are believed to be equivalent in terms of metabolism.

Absorption

Saccharin is a highly polar compound and a strong organic acid. With a pKa of approximately 2.0, saccharin is almost completely ionized at physiological pH whether administered in the acid or salt form. It exists to a greater extent in its un-ionized form in the stomach. However, absorption takes place primarily in the small intestines where, with a neutral pH, saccharin is almost entirely ionized. The rate of absorption of saccharin is slow in both humans and rats. Absorption is about 95% in humans and 80% in rats (Renwick, 1985).

Plasma Protein Binding, Volume of Distribution, Concentration in Tissues

Consistent with its polarity, saccharin has a low volume of distribution. Sweatman and Renwick (1980) reported significant plasma protein binding (69-86%) at all dietary levels in rats. Measurements of steady state-concentrations of saccharin in the tissues of adult male rats fed 1-10% sodium saccharin in the diet showed that the highest concentrations were in the kidney, bladder, and in the gut wall (associated with slow absorption), followed by the plasma. At dietary levels of less than 5%, a linear relationship between the concentration in the diet and that in the plasma and tissues was observed. In animals fed greater than 5% in the diet there was an excessive accumulation of saccharin in the plasma and tissues. Levels of saccharin anion in bladder tissue were relatively constant at concentrations between 1 and 5% in the diet but increased markedly in the tissues of rats fed diets containing 7.5 and 10% saccharin (Sweatman and Renwick, 1980). Female rats fed a diet containing 5% sodium saccharin had higher concentrations in the plasma and tissues compared to male rats, even after allowance for greater saccharin intake in females (Sweatman and Renwick, 1980).

Placental transfer of saccharin to the fetus has been documented in rats, monkeys and humans (West, 1979). Saccharin is transferred to the offspring through the dam's milk, also in ionized form (Sweatman and Renwick, 1982).

Metabolism

There is no evidence that saccharin undergoes biotransformation (Renwick, 1985). Lutz and Schlatter (1977) analyzed the urine of male Sprague-Dawley rats treated with ^{35}S-labelled saccharin by chromatography, and reported that 99.6% of the radioactivity co-migrated with saccharin.

Administration of 7.5% sodium saccharin did not affect levels of cytochrome P-450, cytochrome b_5, NADPH-cytochrome P-450 reductase or activity of aryl hydrocarbon hydroxylase in either single or two-generation rat studies. Sodium saccharin did not alter glutathione levels in the bladder or liver of treated rats. About a two-fold increase in dimethylnitrosamine-N-demethylase activity was observed in saccharin treated rats. The extent of this increase was similar for all salt forms of saccharin (Heaton and Renwick, 1991).

Excretion

Renal elimination is the major route of clearance of saccharin absorbed into the circulation, with about 3% of a dose administered to humans being recovered in feces (Renwick, 1985). Because of the high degree of plasma protein binding, glomerular filtration is not considered an important mechanism of elimination. Renal tubular secretion is the major mechanism of elimination in both rats and humans as indicated by decreased plasma clearance of saccharin when co-administered with probenecid. Pretreatment with probenecid, which inhibits renal tubular secretion, caused a dose-dependent competitive inhibition of saccharin clearance in male rats (63%) and in female rats (53%) at the one dose tested (Sweatman and Renwick, 1980). Renal tubular secretion is a saturable process and plasma concentrations of saccharin greater than 200 µg/ml have been associated with saturation. The increases in plasma and tissue levels in rats given 7.5 and 10% diets are a consequence of reduced elimination of saccharin anion.

In general, sodium saccharin administration in feed does not appreciably change urinary pH. A slight to moderate increase has been reported in male F344 rats in several studies in which rats received 5% sodium saccharin for periods of time from seven days to 52 weeks (Fukushima *et al.*, 1983; Hasegawa and Cohen, 1986; Fisher *et al.*, 1989). Fukushima *et al.* (1983) reported urinary pH of 7.5 in rats treated with sodium saccharin for 52 weeks, compared to 6.9 in control rats. This increase was somewhat larger than that found in Hasegawa and Cohen (1986) and Fisher *et al.* (1989). Fukushima *et al.* (1983) also reported small increases in urinary pH in Sprague-Dawley (sodium saccharin: pH 7.4; control: pH 7.1) and Wistar rats (sodium saccharin, pH 7.1; control, pH 6.8). In contrast, a considerable increase was observed in ACI rats (sodium saccharin, pH 7.6; control, 6.4). However, it should be noted that the number of animals of each strain tested in this study was small (n=5).

Studies in F344 rats have shown that sodium saccharin administration (5% or 7.5% in the diet) results in altered urinary electrolyte concentrations. Increases in urinary sodium concentrations (Cohen *et al.*, 2000; Fisher *et al.*, 1989; Hasegawa and Cohen, 1986) and decreases in urinary potassium (Cohen *et al.*, 2000; Fisher *et al.*, 1989) and chloride concentrations (Cohen *et al.*, 2000; Fukushima *et al.*, 1990) are fairly consistent among studies. Cohen *et al.* (2000) also reported an increase in urinary Mg^{2+} concentration after exposure to 7.5% sodium saccharin for six weeks. For most electrolytes, Cohen *et al.* (2000) found that changes were similar in male and female F344 rats. One exception was urinary Ca^{2+}, which increased in male rats and did not change in female rats after six weeks exposure to 7.5% sodium saccharin in the diet. Reports of urinary Ca^{2+} levels among studies have been inconsistent, with some reports of increased and others of decreased levels after sodium saccharin exposure (Fukushima *et al.*, 1990; Fisher *et al.*, 1989; Hasegawa and Cohen, 1986). Some of the differences in study results are likely due to the time period in which the urine was sampled.

In studies in which 5% sodium saccharin or calcium saccharin was fed to F344 rats for ten weeks, the concentration of saccharin ion excreted was not influenced by the form of the salt (Fisher *et al.*, 1989). These results are consistent with those of Hasegawa and Cohen (1986) who fed rats diets containing 5% sodium saccharin, potassium saccharin, calcium saccharin or acid saccharin or a control diet for to male F344 rats for ten weeks. Results from this study are shown in Table 6.

Table 6. Urinary Concentration of Saccharin in Male F344 Rats Treated with Several Forms of Saccharin (Hasegawa and Cohen, 1986).

Treatment Group		Number of Rats	Saccharin Concentration in Urine (mmol/ml)
Day 7	5% sodium saccharin	6	0.18 ± 0.04[a]
	5% potassium saccharin	6	0.19 ± 0.05
	5% calcium saccharin	6	0.20 ± 0.02
	5% acid saccharin	6	0.21 ± 0.04
	Control	6	0
Day 28	5% sodium saccharin	6	0.17 ± 0.04
	5% potassium saccharin	6	0.14 ± 0.04
	5% calcium saccharin	6	0.14 ± 0.03
	5% acid saccharin	6	0.19 ± 0.02[b]
	Control	6	0

[a] Mean ± standard deviation.
[b] Significantly greater than potassium saccharin and calcium saccharin groups ($p < 0.05$).

3.3.4 Urothelial Proliferation and the Relationship Between Urinary Characteristics and the Promoting and Carcinogenic Effects of Sodium Saccharin

Studies from the 1980s first documented an increase in the rate of proliferation of the bladder urothelium in male rats fed a diet containing high doses of sodium saccharin. This increase is observed soon after sodium saccharin exposure and has been proposed as the mechanism of sodium saccharin induced tumor promotion.

Urothelial Proliferation in Short-Term Studies

In short-term studies, sodium saccharin causes hyperplasia, shown by light and scanning electron microscopy, and by an increase in the rate of DNA synthesis.

Fukushima and Cohen (1980) fed F344 (Fischer 344) male rats a diet containing 5% sodium saccharin for up to 18 weeks. After five weeks of exposure, focal irregularly shaped lesions of the bladder epithelium were observed by scanning electron microscopy. The cells in these areas, apparently areas where superficial cells had exfoliated, had their luminal surfaces covered with ropy rounded microridges rather than peaked microridges seen in normal bladders. At nine weeks, uniform microvilli and some pleomorphic microvilli were observed on cells in these areas. To investigate changes in the rate of DNA synthesis, each rat received a single intraperitoneal (i.p.) injection of [^{3}H]thymidine one hour before sacrifice. The rate of [^{3}H]thymidine uptake, expressed as the labeling index, was measured by autoradiography. Autoradiographs from this study showed that labeled cells were clustered in multiple small foci,

with most of the labeled nuclei observed in cells in the basal layer of the epithelium. The labeling index in the bladder epithelium of treated animals was significantly greater than that in controls after only one week of exposure (sodium saccharin-treated: 0.36 ± 0.18; control rats: 0.05 ± 0.02; $p < 0.05$).

Murasaki and Cohen (1981) fed male Fischer 344 rats (10 rats/group) 0, 0.1, 0.5, 1.0, 2.5, or 5.0% sodium saccharin in their diets for ten weeks. The number of bladder foci (containing ropy microridges, uniform or pleomorphic microvilli) and the number of cells with ropy microridges, uniform microvilli or pleomorphic microvilli were counted by scanning electron microscopy. These lesions increased in a dose-dependent fashion and were significantly greater, for each parameter, in rats fed 5% sodium saccharin compared to other dose groups. To investigate changes in the rate of DNA synthesis, each rat received a single i.p. injection of [^{3}H]thymidine one hour before sacrifice. The rate of [^{3}H]thymidine uptake, expressed as the labeling index, was measured by autoradiography. Autoradiographs from the study demonstrated a dose-dependent increase in the labeling index. These increases were statistically significant at doses of 0.5% sodium saccharin and above.

Hasegawa and Cohen (1986) reported an increase in proliferative lesions of the bladder urothelium in weanling male F344 rats fed a diet containing 5% sodium saccharin for 10 weeks. A significant increase in simple hyperplasia was observed by light microscopy and both uniform and pleomorphic microvilli were observed by scanning electron microscopy. In rats fed a control diet or a diet containing 5% acid saccharin no lesions occurred. Bladders of rats fed diets containing 5% potassium or calcium saccharin showed some evidence of hyperplasia, but pleomorphic microvilli were observed only in sodium saccharin treated rats. In autoradiographs from this study, the labeling index was significantly increased in sodium saccharin fed rats compared to all other groups as shown below in Table 7. Also, the labeling index in rats fed a diet containing 5% potassium saccharin was significantly greater than that in tissue from control animals. Potassium saccharin was clearly less potent than sodium saccharin in inducing cell proliferation, as measured by the labeling index.

Table 7. Comparison of Labeling Indices in Male Fischer Rats Treated for 10 Weeks with Different Forms of Saccharin (Hasegawa and Cohen, 1986).

Diet	Labeling Index (%)
5% Sodium saccharin	0.55 ± 0.20[a]
5% Potassium saccharin	0.18 ± 0.09[b,c]
5% Calcium saccharin	0.12 ± 0.11
5% Acid saccharin	0.07 ± 0.04
Control	0.06 ± 0.04

[a]Significantly different from controls ($p < 0.01$).
[b]Significantly different from controls ($p < 0.05$).
[c]Significantly different from value for sodium saccharin ($p < 0.01$).

Cohen *et al.* (1990) reported both a dose- and time-dependent increase in urinary bladder hyperplasia in male F344N rats (10 animals/group) fed sodium saccharin (0, 3, 5, or 7.5%) in the diet for four, seven or ten weeks, beginning at 28 days of age. Bladders of rats fed a diet containing 7.5% sodium saccharin showed evidence of hyperplasia (by light microscopy) after four weeks of exposure. After seven weeks of exposure, the incidence of hyperplasia was 100%; after 10 weeks of exposure, one rat had a papilloma. By scanning electron microscopy, changes in the urinary bladder were detected in rats exposed for four weeks to 3%, 5% or 7.5% sodium saccharin. After 10 weeks of exposure, 6/10 animals treated with 3% sodium saccharin had areas of necrosis and exfoliation throughout the bladder epithelium. These lesions were more extensive in animals treated for 10 weeks with 5% and especially 7.5% sodium saccharin, of which 6/10 had the most severe grade of lesion exhibiting piling up of small, round cells with uniform microvilli and occasional pleomorphic microvilli. The rate of DNA synthesis of the bladder urothelium was measured for each dose group at four, seven and ten weeks, with each rat administered a single i.p. injection of [^{3}H] thymidine one hour before sacrifice. For each exposure duration, the labeling index was significantly greater in the urothelium of rats fed 7.5% sodium saccharin than those of the other dose groups.

Differences between male and female rats. Cohen (1985) reported significant increases in the labeling index in the bladder epithelium of both male and female rats that received 5% sodium saccharin in their diets for ten weeks, with significantly greater increases in male rats (Table 8).

Table 8. Increases in the Labeling Index of Bladder Epithelium in Male and Female Fischer Rats Fed 5% Sodium Saccharin in Their Diets for Ten Weeks (Cohen, 1985).[a]

Sex	Diet	No. Rats	Labeling Index (%)
Male	Control	6	0.06 ± 0.04
	Sodium saccharin	6	0.55 ± 0.20[b]
Female	Control	6	0.07 ± 0.03
	Sodium saccharin	5	0.26 ± 0.12[c,d]

[a] Animals were five weeks of age at start of experiment (Hasegawa and Cohen, 1986).
[b] Significantly different from controls ($p < 0.01$).
[c] Significantly different from controls ($p < 0.05$).
[d] Significantly different from males fed sodium saccharin ($p < 0.05$).

In contrast, increases in the labeling index of the bladder epithelium were similar in neonatal male and female rats exposed to 5% sodium saccharin. Differences between male and female rats in responsiveness to sodium saccharin are discussed further in sections 4.2 and 4.3.

Differences among species. Fukushima *et al*. (1983) exposed male F344 rats, B6C3F$_1$ mice, Syrian golden hamsters and Hartley guinea pigs to a diet containing 5% sodium saccharin or a control diet for designated periods up to 20 weeks. All animals were six weeks old at the start of the experiments. The rate of DNA synthesis was determined by [^{3}H] thymidine uptake in urothelial cells as described by Fukushima *et al*. (1983) and was measured after four, 12 and 20 weeks of exposure. At 12 weeks, an increase in [^{3}H] thymidine uptake was observed in male rats exposed to sodium saccharin; the increase was significantly different from control animals at the 20 week measurement. No differences were observed in the bladder epithelia of mice, hamsters

or guinea pigs between sodium saccharin-treated and control animals. By scanning electron microscopy, bladder epithelial lesions in the rat were scored as moderate to marked at 20 weeks. However, in a similar experiment described in Fukushima *et al*. (1983), in which various strains of male rats were fed a diet containing 5% sodium saccharin for designated periods up to 52 weeks, only slight lesions of the bladder were observed in F344 rats by scanning electron microscopy (see below).

Differences in rat strains. Fukushima *et al.* (1983) fed male ACI, Wistar, F344 and Sprague-Dawley rats diets containing 0 or 5% sodium saccharin for one year. All rats were six weeks of age at the beginning of the study. At the end of the study, ACI rats had developed bladder tumors (papillomas: 9/32; carcinomas: 3/32). By light microsocopy, no lesions were observed in the other three strains. However, by scanning electron microscopy, lesions were observable in Wistar and F344 rats although these were far less marked than those observed in ACI rats. No changes were seen in Sprague-Dawley rats, the strain tested in most of the sodium saccharin rat bioassays. (It should be noted that the bladder parasite *Trichosomoides crassicauda* was found in some ACI rats whereas the parasite was not found in the other three strains; the parasite was not associated with the presence of bladder tumors [Fukushima *et al.*, 1983].)

Garland *et al.* (1989) also reported that Sprague-Dawley rats were less responsive than F344 rats to the development of bladder epithelial lesions in a 10-week study in which groups of rats were fed either of two separate diets containing 0, 5% or 10% sodium saccharin.

In summary, the above studies showed that the proliferation rate of the bladder urothelium was significantly greater in sodium saccharin treated rats compared to rats treated with acid saccharin or other saccharin salts. Increases in urothelial proliferation were dose-dependent and lesions progressed with time; 0.5% sodium saccharin in the diet for 10 weeks represents the lowest dose tested, and the lowest dose at which a statistically significant increase in the labeling index was observed in Fischer 344 rats. These effects were not seen in mice, hamsters or guinea pigs and changes in the bladder epithelium, as evidenced by an increase in the labeling index, were significantly greater in male compared to female rats.

Relationship Between Alterations in Rat Urinary Characteristics Associated With Sodium Saccharin Treatment and Proliferation and Promotion of the Bladder Urothelium

Acid saccharin has a pK_a of 1.8 and exists as an anion at urinary pH. Similarly, sodium saccharin is ionized at urinary pH with saccharin present as the anion. Because acid saccharin does not appear to induce urinary bladder proliferation in contrast to sodium saccharin, (Hasegawa and Cohen, 1986; Fisher *et al.*, 1989), numerous studies have been conducted to investigate factors that could affect urothelial proliferation and bladder tumor promotion. These include factors that could alter the saccharin molecule, the bladder epithelium's ability to respond to saccharin or the bladder epithelium itself. The various areas studied are summarized below.

Structure of saccharinate ion. Williamson *et al.* (1987) examined the possibility that Na^+ and/or pH might alter the electronic environment of the saccharinate ion and therefore modify its

structure. The effect of physiologically relevant concentrations of cations Na^+, K^+, Ca^{++}, and Mg^{++} as well as the effect of pH on the structure of the saccharinate ion was examined using ^{13}C nuclear magnetic resonance (NMR) spectroscopy. Small shifts were observed in the position of one of the carbon atoms (C-7) in the presence of Na^+ but these shifts were not dose-dependent. pH had no effect on the position of the carbon atoms in the spectrum. The authors commented that the observed shifts were slight (approximately 2-3% of the possible range) and implied that the differences in biological activity between the various saccharin salts could not result from changes in the electronic structure of the saccharinate ion. The structure of the saccharinate ion also appeared unchanged in studies which simulated *in vivo* concentrations of the various cations and in experiments which examined the effect of bicarbonate and urate at various sodium concentrations on the position of the C-7 atom. Thus, the structure of the saccharinate ion in the urinary bladder is thought to be the same, regardless of whether saccharin is administered as the sodium, calcium, or other salt, or as the acid.

Sodium ion concentration. Sodium ion concentration was shown to be a key element in inducing urothelial proliferation of the bladder epithelium (Hasegawa and Cohen, 1986) and also in the promoting activity of sodium saccharin. In an initiation-promotion study, Cohen *et al*. (1991) treated male F344 rats with 2% FANFT for six weeks and then diets containing various doses of sodium or calcium saccharin or acid saccharin for 72 weeks. Sodium saccharin increased the incidence of bladder carcinomas above that observed in the FANFT control group in a dose-dependent manner (Table 9). Acid saccharin, given at 4.2% in the diet, slightly increased the incidence of bladder carcinomas above that seen in the FANFT control, as did calcium saccharin. When sodium chloride was added to the calcium saccharin treatment regimen the incidence of carcinoma was increased from 21/40 to 34/40 ($p = 0.002$). Thus, it appears that addition of NaCl resulted in a progression of the neoplastic response, from papillary or nodular hyperplasia to papilloma, and from papilloma to carcinoma. Simple hyperplasia (not shown in Table 9) was observed in approximately 25% of groups initiated with 2% FANFT and then fed either a control diet or a diet containing calcium saccharin, acid saccharin or ascorbic acid. In groups in which a strong carcinogenic response was observed, simple hyperplasia was observed in few or no animals.

Table 9. Incidence of Bladder Tumors in Male F344 Rats Initiated with 2% FANFT Followed by Test Chemicals for 72 Weeks (Cohen *et al.,* 1991).

Treatment	Papillary or Nodular Hyperplasia	Papilloma	Carcinoma
2% FANFT + 3% sodium saccharin	4/40	5/40	29/40
2% FANFT + 5% sodium saccharin	0/39	1/39	38/39
2% FANFT + 5% sodium saccharin + 1.23% NH_4Cl	12/40	5/40	11/40
2% FANFT + 3.12% calcium saccharin	7/40	2/40	20/40
2% FANFT + 5.2% calcium saccharin	7/40	6/40	21/40
2% FANFT + 5.2% calcium saccharin + 1.34% NaCl	2/40	3/40	34/40
2% FANFT + 2.5% acid saccharin	11/40	6/40	13/40
2% FANFT + 4.2% acid saccharin	5/40	5/40	19/40
2% FANFT + 4.44% ascorbic acid	5/40	5/40	16/40
2% FANFT + 5% sodium ascorbate	3/40	2/40	30/40
2% FANFT + 1.34% NaCl	5/40	5/40	27/40
2% FANFT + control diet	6/39	5/39	12/39
Control + control	0/30	0/30	2/39

Other sodium salts also promote tumors of the rat bladder epithelium (Fukushima *et al.*, 1986; Cohen *et al.*, 1991; Otoshi *et al.*, 1993; Cohen *et al.*, 1998). As part of the study described above (Cohen *et al.*, 1991), sodium ascorbate and ascorbic acid were fed to male rats for 72 weeks after six weeks treatment with 2% FANFT. In rats treated with ascorbic acid, 16/40 developed carcinomas compared to 30/40 in rats treated with sodium ascorbate ($p = 0.002$). The number of carcinomas was also increased in rats treated with 1.34% sodium chloride, after pretreatment with 2% FANFT (27/40).

In another initiation-promotion study, male F344 rats received 0.05% BBN in their drinking water for four weeks and then were fed a control diet or a diet containing ascorbic acid, sodium ascorbate, ascorbic acid with $NaHCO_3$, or $NaHCO_3$ for 32 weeks (Fukushima *et al.*, 1986). As seen in Table 10, incidences of papillomas and carcinomas in rats fed ascorbic acid were dramatically increased when $NaHCO_3$ was added to the diet. Incidences of papillomas and carcinomas were similarly increased in rats fed sodium ascorbate, as compared to rats fed ascorbic acid. Rats fed a diet containing $NaHCO_3$ also had increased incidences of papillomas and carcinomas as compared to rats fed a control diet.

Table 10. Incidence of Bladder Tumors in Male F344 Rats Initiated with 0.5% BBN Followed by Test Chemicals for 32 Weeks (Fukushima *et al.*, 1986).

Treatment	Papillary or Nodular Hyperplasia	Papilloma	Carcinoma
0.5% BBN + 5% ascorbic acid + 3% $NaHCO_3$	20/20	18/20	19/20
0.5% BBN + 5% ascorbic acid	12/20	8/20	4/20
0.5% BBN + 3% $NaHCO_3$	20/20	15/20	16/20
0.5% BBN + 5% sodium ascorbate	20/20	19/20	17/20
0.5% BBN + 5% sodium ascorbate + 1% NH_4Cl	19/20	13/20	9/20
0.5% BBN + 1% NH_4Cl	11/20	5/20	4/20
0.5% BBN	6/20	11/20	5/20

In a study designed to examine the promoting activity of sodium ions under conditions of an equal increase in urinary pH, male F344 rats (16/group) were treated with BBN for four weeks and then fed a control diet or a diet with either 5% succinic acid or 5% sodium succinate or 5% disodium succinate for 32 weeks (Otoshi *et al.*, 1993). Table 11 shows urinary pH and sodium ion concentration for each group of animals. The incidence of urinary bladder tumors was significantly increased in both sodium succinate and disodium succinate treated animals, with 100% incidence of carcinoma in both groups, as compared to 3/16 and 2/16 in succinic acid or control groups, respectively. Bladder tumors in disodium succinate treated animals were significantly larger than those in sodium succinate-treated animals. The labeling index was also significantly increased in disodium succinate treated animals compared to controls. Sodium succinate also increased the labeling index compared to controls, but the increase was not significant.

Table 11. Urinary pH and Sodium Ion Concentration in Rats Initiated with BBN Then Fed Succinic Acid and Succinate Salts (Otoshi *et al.*, 1993).

	Urinary pH	Na^+ (mEq/l)
Control	6.69 ± 0.34	120 ± 42
5% Succinic Acid	6.03 ± 0.13	142 ± 56
5% Sodium succinate	8.06 ± 0.14[b,c]	229 ± 46[b,c]
5% Disodium succinate	8.16 ± 0.22[b,c]	335 ± 68[b,c,d]

[a] Animals were treated for four weeks with 0.05% BBN and then fed a diet containing 5% succinic acid, 5% sodium succinate or 5% disodium succinate for 32 weeks.
[b] Significantly different from control group ($p < 0.05$).
[c] Significantly different from succinic acid treated animals ($p < 0.01$).
[d] Significantly different from sodium succinate treated animals ($p < 0.01$).

In a study which examined the effect of inorganic sodium and potassium salts on bladder tumor promotion, male Fischer 344 rats were treated with 0.05% BBN in drinking water for four weeks and then fed diets containing various sodium and potassium salts for a total of 13 weeks. Treatment was interrupted after the first four weeks, and rats received 3% uracil in their diets for three weeks to accelerate tumor promotion, after which the sodium or potassium salt diets were continued (Shibata *et al.*, 1992). The greatest increase in bladder papillomas and carcinomas occurred in rats treated with 3% Na_3PO_4 in their diets. Rats treated with 3% NaH_2PO_4 also had significant increases in bladder papillomas and carcinomas, compared to controls. The tumor incidence in rats treated with 3% NaH_2PO_4 + 1.7%NaCl was significantly greater than that in rats treated with 3% NaH_2PO_4 alone, suggesting the importance of the sodium ion in tumor promotion. In parallel with these results, the incidence of bladder papillomas and carcinomas in rats treated with 3% K_3PO_4 was significantly greater than that in control animals. Tumor incidence, after treatment with 3% KH_2PO_4 was less than that in the 3% K_3PO_4 group and although increased, not significantly greater than control incidences. However, rats treated with 3% KH_2PO_4 + 1.5% KCl had a significantly increased incidence of bladder papilloma compared control animals.

Thus, multiple studies in F344 rats have shown that the presence of sodium ion in the diet increases bladder tumor incidence in initiation-promotion models. Potassium ion has also been shown to increase tumor incidence.

Urinary pH. Cohen *et al.* (1991) reported that the promoting effect of sodium saccharin was abolished when NH_4Cl was co-administered with 5% sodium saccharin for 72 weeks (after six weeks initiation with FANFT). As shown in Table 9, papillary or nodular hyperplasia was observed, but far fewer lesions progressed to carcinomas when NH_4Cl was added to the diet. Urinary pH was decreased in the presence of NH_4Cl from greater than pH 7 (5% sodium saccharin) to less than pH 6 (NH_4Cl + 5% sodium saccharin) at each of the four measurement periods of the study.

In Fukushima *et al.* (1986), treatment with sodium ascorbate + NH_4Cl (after initiation with BBN) significantly decreased the incidence of papillomas and carcinomas compared to the sodium ascorbate animals ($p < 0.05$) [Table 9].

Shibata *et al.* (1992) investigated the effect of inorganic sodium and potassium compounds on rat bladder tumor promotion in experiments described above. The greatest increase in bladder papillomas and carcinomas occurred in rats treated with 3% Na_3PO_4, which also resulted in alkaline urine (pH 7.1 ± 0.2). However, 3% NaH_2PO_4 also caused a significant increase in bladder tumors but did not result in an alkaline urine (pH, 6.0 ± 0.1). When rats were treated with 3% NaH_2PO_4 + 1.7% NaCl, the tumor incidence increased (compared to promotion with 3% NaH_2PO_4), but urinary pH did not (pH 6.0 ± 0.1).

Sodium chloride (5% or 10%), administered in the diet for 68 weeks to F344 rats who had received 0.01% or 0.05% BBN for four weeks in drinking water, did not significantly increase tumor incidence (Shibata *et al.*, 1986). In a related experiment, rats fed 5% or 10% NaCl in their diets for three weeks did not have a urinary pH greater than that of animals fed a control diet (pH 6.73 ± 0.28 and 6.54 ± 0.41 for 5% and 10% NaCl, respectively compared to 6.84 ± 0.43 in control diet). This result contrasts with that from Cohen *et al.* (1991) where an initiating dose of

2% FANFT followed by a diet containing 1.34% NaCl for 72 weeks resulted in an increase in bladder carcinomas (Table 2). Shibata *et al.* (1992) considered 1.7% NaCl a "weak" enhancer of tumor promotion in rats initiated with BBN/uracil (papillomas per rat, 1.7 ± 1.7 compared to 0.4 ± 0.6 in control rats). Average urinary pH in NaCl treated rats in this study was 6.8 ± 0.3 compared to 6.7 ± 0.3 in control animals.

Administration of high doses of sodium hippurate does not result in urinary bladder cell proliferation or tumor formation or in a urinary pH $\geq$ 6.5 (referenced in Fukushima *et al.*, 1986).

In summary, the promoting activity of sodium saccharin (and sodium ascorbate) is greatly reduced (or eliminated) when NH_4Cl is added to the diet. The addition of NH_4Cl to a diet containing sodium saccharin (or sodium ascorbate) results in acidic urine. Also, studies with inorganic sodium and potassium salts have demonstrated tumor promoting activity of these salts even at an acidic urinary pH. In one study 1.34% NaCl in the diet (administered after initiation with 2% FANFT) caused a significant increase in urothelial bladder carcinoma.

Osmolality and urine volume. Decreased osmolality and increased urine volume have been noted in bladder tumor promotion studies as well as the two-generation bioassays of sodium saccharin (see below).

Dietary factors. Different animal diets have been shown to result in differences in urinary acidity. Such differences are primarily related to the balance between acid- and base-forming dietary constituents. Cations in the diet increase pH; anions decrease pH. The major cations in the diet are Ca^{2+}, Na^{+}, K^{+} and Mg^{2+}. The main acid-forming anions are PO_4^{3-} and Cl^{-}. DeGroot *et al.* (1988) measured total cation and anion content in purified and unrefined rat diets and reported a marked excess of cations in the unrefined diet (compared to the purified diet). This difference between the two diets provided an explanation for the higher urinary pH in animals fed the unrefined diet.

In the case of sodium saccharin, the incidence of bladder tumors was only weakly increased in male rats initiated with FANFT and then fed sodium saccharin in the semisynthetic AIN-76A diet (Imaida and Wang, 1986). Urinary pH in rats fed AIN-76A diet has been measured to be between 5.5-6.5 (Debiec-Rychter and Wang, 1990). The low urinary pH may be due to the smaller amounts of urinary Na^{+}, K^{+} and Ca^{++} in rats fed an AIN-76A diet compared to the unrefined cereal-based Prolab 3200 diet (Fisher *et al.*, 1989). Whether decreased urinary pH or the relatively lower sodium concentration is related to the decreased promoting activity of sodium saccharin in rats fed the AIN-76A diet is unclear.

Salts of other organic acids. Initiation-promotion studies have been conducted with sodium salts of other organic acids. Promotion studies with sodium ascorbate have already been discussed above (Cohen *et al.*, 1991; Fukushima *et al.*, 1986). Results from promotion studies with *o*-phenylphenol (OPP) and its sodium salt (sodium *o*-phenylphenate, SOPP) are similar to those from promotion studies with saccharin and ascorbic acid and their respective sodium salts. In male F344 rats fed diet containing 0.05% BBN (for four weeks) and then diet containing 2% SOPP or 2% OPP (for 32 weeks), significant increases in urinary bladder papillomas (30/30) and carcinomas (27/30) were observed in SOPP-treated rats. In comparison, incidences of papilloma (11/30) and carcinoma (6/30) in OPP-treated rats were not significantly different from 0.05%

BBN controls (papillomas: 7/30; carcinomas: 2/30). In a related experiment, urine pH of rats treated with 2% SOPP was significantly higher than that of both control and 2% OPP-treated rats (Fukushima *et al.*, 1983).

Similar to studies with sodium saccharin, male rats were found to be more susceptible to the promoting effects of sodium salts of other organic acids than female rats. In male and female F44 rats exposed to 1.25% OPP, 2% SOPP, 1.25% OPP + 3% $NaHCO_3$ or 3% $NaHCO_3$ alone in their diets for eight weeks, male rats fed OPP + $NaHCO_3$ developed severe papillary or nodular hyperplasia of the urinary bladder. Female rats developed lesions as well, but these were less severe (Hasegawa *et al.*, 1991).

It should be noted that both OPP and SOPP induce bladder tumors in long-term feeding studies, and, in contrast to sodium saccharin, both OPP and SOPP are metabolized. While OPP and SOPP metabolites are thought to be involved in the mechanism of carcinogenesis, pH and electrolyte changes may be important in their short-term and/or tumor promoting effects.

Relationship Between Alterations in Rat Urinary Characteristics Associated With Sodium Saccharin Treatment and Urothelial Tumor Induction in Bioassays

Sodium ion concentration. In a two-generation bioassay, Taylor *et al.* (1980) fed male and female Sprague-Dawley rats diets containing 0, 0.01, 0.1, 1.0, 5.0 or 7.5% sodium saccharin. In order to control for possible effects of increased sodium ion consumption associated with sodium saccharin treatment, the controls received a diet containing 1.51% sodium carbonate, which resulted in a sodium ion concentration in the control diet equivalent to the sodium ion concentration present in the 5% sodium saccharin diet. One transitional cell polyp was observed in control male rats and one in the 5% sodium saccharin-treated rats. Significant increases in bladder neoplasms were observed in male rats receiving 7.5% sodium saccharin in their diets. Since the sodium ion concentration in control animals was matched to the sodium ion concentration in the 5% sodium saccharin-treated group and not the 7.5% group, this bioassay does not provide information on the effect of the sodium ion concentration.

Urinary pH. In a two-generation bioassay, Chowaniec and Hicks (1979) exposed male and female Wistar rats to 2 g sodium saccharin/kg/day (equivalent to approximately 4% of the diet) in drinking water, to 4 g sodium saccharin/kg/day (approximately 7-8% of the diet) in feed or provided them with a control diet for two years. Measurements in untreated control rats indicated urinary pH averaged between 6.0 and 6.5. In male rats exposed to sodium saccharin via drinking water, an increase in urinary pH became apparent by week 27: average pH was 7.0 with some animals having pH values of 8.5 or 9. Because a marked crystalluria associated with this pH increase occurred in several of the rats, NH_4Cl was added to the drinking water to lower urinary pH, returning it to pH 6.0-6.5. Urinary pH in rats exposed to 4 g sodium saccharin/kg/day in feed was not increased. Although only a few tumors were observed in this study (three urothelial tumors of the bladder in the feed study and one urothelial tumor of the ureter and renal pelvis in a male and female rat in the drinking water study respectively), the incidence of urothelial hyperplasia was statistically significantly increased in the group receiving sodium saccharin in feed. These results suggest that sodium saccharin may cause some effects under conditions of acidic urinary pH. Differences in urinary pH between these Wistar rats and

those studied in Fukushima *et al*. (1983) [5% sodium saccharin-treated rats, pH 7.1; control rats, pH 6.8] may be due, at least in part, to dietary differences.

In contrast to Chowaniec and Hicks (1979), the results of Fukushima *et al.* (1983) suggest that high urinary pH may be related to bladder tumor development. Fukushima *et al.* (1983) observed bladder tumors (papillomas: 9/32; carcinomas 3/32) in ACI rats fed a diet containing 5% sodium saccharin for one year. Urinary pH was also appreciably increased in ACI rats after sodium saccharin treatment (sodium saccharin, pH 7.6; control, pH 6.4). In comparison, similar sodium saccharin exposure resulted in much smaller increases in urinary pH in F344, Sprague-Dawley, and Wistar rats (Section 3.3.3) and no tumors in these strains after one year of treatment.

Salts of other organic acids. In the only two-generation tumorigenicity bioassay conducted with another sodium salt, Cohen *et al.* (1998) fed F344 rats 0, 1, 5 or 7% sodium ascorbate in their diets (F_0 males and females: prior to mating, through gestation and lactation (F_0 dams) and male F_1 offspring: up to 128 weeks). Few bladder tumors were formed. Two urinary bladder carcinomas were observed in the 7% sodium ascorbate group (n = 52), one bladder papilloma and one transitional carcinoma of the renal pelvis were observed in the 5% sodium ascorbate group (n = 45), and one urinary papilloma was observed in the 1% sodium ascorbate group (n=60). Significant increases in simple hyperplasia and papillary or nodular hyperplasia were associated with sodium ascorbate treatment. For papillary or nodular hyperplasia, considered a preneoplastic lesion, these incidences were 1/58, 2/60, 5/45, and 9/52 for 0, 1, 5 and 7% sodium ascorbate diets, respectively.

Osmolality and urine volume. Schoenig *et al.* (1985) reported that an increase in urine volume and a decrease in urine osmolality were highly correlated with the occurrence of urinary bladder tumors in rats treated with sodium saccharin. In the two-generation sodium ascorbate bioassay, Cohen *et al.* (1998) reported a dose-dependent decrease in urine osmolality which was partially inhibited by the addition of NH_4Cl. This decrease was attributed to a concomitant increase in water consumption.

4 MECHANISM

The importance of neonatal exposure in sodium saccharin induced carcinogenicity is discussed in Section 4.1 below. Proposed mechanisms for the effects of sodium saccharin on the bladder urothelium are discussed in Sections 4.2 and 4.3.

4.1 Carcinogenicity after Neonatal Exposure and Two-Generation Bioassays

Schoenig *et al.* (1985) demonstrated that neonatal exposure to sodium saccharin, rather than *in utero* exposure is the exposure period necessary to induce bladder tumors. Cohen and Ellwein (1990) have suggested that administration of sodium saccharin *in utero* does not further increase the proliferation rate of the bladder epithelium since proliferation is essentially at its maximal rate during gestation, with a labeling index of approximately 10% [Cohen *et al.*, 1995]. The labeling index rapidly declines in the three weeks after birth, to <0.1%, a rate typical of the adult rat bladder epithelium. Cohen *et al.* (1995) report that when Sprague-Dawley rats were exposed

to sodium saccharin *in utero*, labeling indices were not significantly higher than in control or acid saccharin-treated rats. But by 21 days after birth, a moderate increase in the labeling index was observed in sodium saccharin treated rats (0.68 ± 0.42) compared to controls (0.12 ± 0.05). Calculations by Ellwein and Cohen (1988) suggested that approximately one-third of the total number of cell divisions in a rat's life-span occur during the first three weeks of life.

Cohen *et al.* (1995) suggested that the increase in the number of initiated cells in rats exposed to sodium saccharin as neonates and the consequent increase in background levels of genetic errors during DNA replication, plus continued administration of sodium saccharin for the remainder of the life-span of the rat, can explain the increased incidence of bladder cancer following administration of sodium saccharin beginning at birth. Thus, Cohen (1985) suggested that the background mutations resulting from increased proliferation of the bladder epithelium (suggested by the increased labeling index in sodium saccharin-exposed neonatal rats) have the same effect as a chemical initiator or as freeze ulceration.

4.2 Calcium Phosphate-Containing Precipitate

Cohen *et al.* (2000) characterized a urinary precipitate in rats administered high doses of sodium saccharin via diet and proposed that the precipitate acts as a microabrasive to urothelial cells, resulting in focal necrosis. It was suggested that the consequent regenerative hyperplasia leads to sodium saccharin-induced urothelial proliferation [and tumorigenesis]. Chemical analysis of the precipitate indicates that it is mainly composed of $Ca_3(P0_4)_2$, with sulfated acid mucopoly-saccharides (mainly chondroitin sulfate), urea, and small amounts of saccharin, silica and protein (Cohen *et al.*, 2000). Two major proteins, α_{2u}-globulin and albumin, were identified. Protein, although a minor component of the precipitate (less than 5%), is theorized to be key to precipitate formation. Observing that anions of sodium salts (*i.e.*, saccharin or ascorbate) associate non-covalently with proteins, such as albumin and to an even greater extent with α_{2u}-globulin, Cohen *et al.* (2000) suggested that these associations alter the protein structure in such a way as to favor the formation of a precipitate.

The hypothesis that the precipitate causes damage to the bladder epithelium and thereby leads to hyperplasia and tumor promotion is supported by several observations. First, calcium phosphate has been shown to be cytotoxic to rat bladder epithelial cells *in vitro* (Cohen *et al.*, 2000). Second, the precipitate is formed shortly after sodium saccharin administration and continues to be present throughout treatment, and its presence is associated temporally with proliferation of the urothelium. Third, the precipitate does not form in acid urine; urothelial proliferation and tumor promoting activity also are not observed under these conditions. Further, precipitate formation is significantly less in female rats than in male rats, in parallel with differences in urinary protein levels between male rats (1.7 ± 0.1 mg/dl) and female rats (0.2 ± 0.1 mg/dl) (females do not produce α_{2u}-globulin) [Cohen *et al.*, 2000]. Also, in contrast to F344 rats, NBR rats (who do not synthesize α_{2u}-globulin) did not have increased labeling indices or scanning electron microscopy findings after an eight-week exposure to 5% sodium saccharin in the diet, although urinary pH and $[Na^+]$ were increased in both strains of rats (Uwagawa *et al.*, 1994).

However, a cause and effect relationship between precipitate formation and tumor promotion or carcinogenicity has not been established. Also, some questions about this theory should be

considered. Male NBR rats lack the male rat specific urinary protein α_{2u}-globulin (and would be expected to form significantly less precipitate than male rats that produce α_{2u}-globulin) and showed no evidence of urothelial proliferation (by SEM or labeling index measurements) following treatment with sodium saccharin. In contrast, female rats, also lacking in α_{2u}-globulin, are responsive to sodium saccharin induced effects. Sodium saccharin causes a significant increase in the labeling index of the bladder epithelium of female F344 rats after 10 weeks of exposure (Cohen, 1985). Female F344 rats form the urinary precipitate although formation is reported to be significantly less than in male F344 rats (Cohen *et al.*, 2000). Also, sodium saccharin has shown promoting activity in initiation-promotion studies in female rats (Chowaniec and Hicks, 1979; West *et al.*, 1986). Thus, the involvement of α_{2u}-globulin in the formation of the urinary precipitate and consequent sodium saccharin-induced urothelial proliferation is unclear. The reported non-responsiveness of male NBR rats to the proliferative effects of sodium saccharin treatment may be due to reasons other than an absence of α_{2u}-globulin.

Another consideration is that most of the studies investigating the relationship between changes in urinary characteristics associated with sodium saccharin treatment and urothelial proliferation have been conducted in F344 rats, while the cancer bioassays have been conducted in Sprague-Dawley (and Wistar) rats. Studies by Fukushima *et al*. (1983), in which 5.0% sodium saccharin was administered in the diet to several strains of mature rats (ACI, Wistar, F344 and Sprague-Dawley) for up to 52 weeks, found that Sprague-Dawley rats were not responsive to the proliferative effects of sodium saccharin, as measured by scanning electron microscopy.

A common mechanism for sodium saccharin induced proliferation of the bladder epithelium and tumor promotion has also not been established. In this regard, West *et al*. (1986) examined the time course of tumor development and the appearance of hyperplasia in female Sprague-Dawley rats administered a single dose of MNU and then treated with 0-5% sodium saccharin and 5% acid saccharin for two years. For doses of sodium saccharin associated with greater tumor development than that seen in the MNU only group, the appearance of hyperplasia coincided with but did not precede tumors.

Cohen *et al.* (1995) postulate that urinary precipitate formation also leads to the early initiating events in sodium saccharin-induced bladder tumors in the rat. Cohen *et al.* (1995) found similar increases in the labeling index in male and female Sprague-Dawley rats after *in utero* and neonatal exposure to 5% sodium saccharin and hypothesized that early morphological changes in the bladder urothelium (observed at 21 days post-birth) were due to precipitate formation. However, no data have been provided to demonstrate the presence of this precipitate in neonatal rat urine. Furthermore, in a two-generation bioassay, sodium saccharin-treated Wistar rats developed urothelial tumors and a significant increase in urothelial hyperplasia under conditions of acidic urinary pH (Chowaniec and Hicks, 1979), conditions in which formation of calcium phosphate-containing precipitate would not be favored.

4.3 Other Possible Mechanisms of Action

Other mechanisms that perturb normal cell function may contribute to sodium saccharin induced tumor promotion and carcinogenicity. Imaida *et al.* (1983) reported increases in membrane potentials of urinary bladder epithelium in male F344N rats administered diets containing 5%

sodium saccharin. These authors speculated that increased epithelial membrane potential could indicate inactivation of membrane Na^+ channels. Involvement of Na^+ transport pathways and Na^+/H^+ exchange have also been raised in Otoshi *et al.* (1993) with the suggestion that increased activation of Na^+/H^+ exchange (and possibly also Na^+-HCO_3^-/Cl^- exchange) might occur in initiated bladder epithelium. An increase in Na^+/H^+ exchange would lead to increased intracellular pH, associated with increased DNA synthesis and cell proliferation (Burns and Rozengurt, 1984; Cameron *et al.*, 1980). Otoshi *et al.* (1993) speculate that the greater promoting activity of disodium succinate compared to sodium succinate might be related to differences in activation of Na^+/H^+ exchange.

Schoenig *et al.* (1985) reported that increased urinary volume was correlated with sodium saccharin induced carcinogenicity. Interestingly, increased bladder volume appears to result in an expansion of the bladder surface with the incorporation of cytoplasmic vesicles into the lumenal membrane. These vesicles contain Na^+ transport pathways (Lewis and de Moura, 1982). If sodium saccharin-induced effects involve activation of Na^+/H^+ exchange, then an increase in the number of Na^+ channels in the bladder epithelium, resulting from increased urine volume associated with sodium saccharin treatment, would suggest the possibility of multiple factors acting in concert to induce tumors.

The molecular mechanisms for activation of Na^+/H^+ exchange are complex and not well understood (Voyno-Yasenetskaya, 1998). Whether urinary changes induced by sodium saccharin treatment (*i.e.*, increased [Na^+], decreased [K^+] or other electrolyte changes, or increased urinary volume) or the activity of saccharin anion under these conditions affects the regulation of Na^+/H^+ exchange or other modulators of cell growth have not been adequately explored.

There is some *in vitro* evidence to suggest that saccharin can affect cell function. In T51B rat liver cells, saccharin stimulates DNA synthesis in calcium-deprived cells, a property saccharin shares with tumor promoters such as 12-tetradecanoyl-13-phorbol-acetate (TPA) and phenobarbital (Boynton and Whitfield, 1980). In these experiments, both sodium saccharin and calcium saccharin elicited rapid DNA synthesis. Sodium saccharin also stimulates protein kinase C in T51B rat liver cells (Kleine *et al.*, 1986). Saccharin significantly increases adenylate cyclase activity in rat skeletal muscle membrane preparations (Striem *et al.*, 1990). In liver membrane preparations, saccharin significantly increases adenylate cyclase activity at high membrane concentrations and decreases activity at low membrane concentrations, although the decrease in activity is not significantly different from that in control preparations (Striem *et al.*, 1990). Adenylate cyclase catalyzes the formation of cyclic AMP, which is involved in a multitude of cell functions. Both cyclic AMP and protein kinase C are involved in intracellular calcium release, modulation of cell membrane ion channels and cell proliferation (Ruddon, 1995).

Vesely and Levey (1978) studied the effect of saccharin (form not specified) on guanylate cyclase activity in a variety of tissues obtained from male Sprague Dawley rats. In these studies, saccharin significantly inhibited guanylate cyclase activity in the bladder, liver, stomach, colon, kidney and lung ($p<0.001$). Inhibition was most profound in the bladder. Guanylate cyclase cataylzes the formation of cyclic GMP. Cyclic GMP is involved in many aspects of cell function, including ion channel regulation and induction of apoptosis.

Whether the effects observed *in vitro* have bearing on the *in vivo* tumor promoting or carcinogenic activity of sodium saccharin is unknown. But the effects of saccharin on adenylate cyclase, on protein kinase C and on guanylate cyclase *in vitro*, do not seem inconsistent with involvement in alteration of Na^+/H^+ exchange. Since studies demonstrating saccharin-induced stimulation of protein kinase C and adenylate cyclase activities were conducted in rat liver cells and liver and skeletal muscle membrane preparations, respectively, it would be important to evaluate the effect of saccharin on these enzymes in rat bladder tissue.

Saccharin (form not specified) has also been reported to inhibit intercellular communication *in vitro* (Trosko *et al.*, 1980). And thus, another hypothesis for its tumor promoting effects is disruption of gap junctional intercellular communication (GJIC). Loss of GJIC can result in cell proliferation (Trosko and Chang, 2001). Inhibition by saccharin is considerably weaker than that observed by the tumor promoter TPA (Trosko *et al.*, 1980).

One suggested mechanism for human bladder cancer is the abnormal expression of epidermal growth factor receptors (EGFR) [Sidransky and Messing, 1992]. Involvement of epidermal growth factor (EGF) has also been suggested in sodium saccharin induced tumor promotion and carcinogenicity. EGF is a normal component in urine, and EGF -EGFR binding stimulates cell proliferation and tumor promotion. Also, EGF-EGFR binding increases at higher pH. Even a marginally higher urinary pH may increase activity. In human bladder tissue, EGFRs are normally found only on the basal urothelium, and therefore, the bladder is largely protected from EGF activity. In premalignant, dysplastic or malignant urothelium, EGFRs are expressed on cells of all epithelial layers, including those directly in contact with urine (Sidransky and Messing, 1992). This expression therefore favors the interaction of premalignant and malignant tissue with urinary EGF (Sidransky and Messing, 1992). If the rat bladder urothelium behaves similarly, then questions about the effects of initiators, of sodium saccharin, and of urinary changes after sodium saccharin treatment on EGFR expression might be investigated as possible contributors to sodium saccharin induced tumorigenesis.

In conclusion, there are multiple hypotheses regarding the mechanism by which sodium saccharin induces bladder tumors in rats. Although the "precipitate" mechanism offers an explanation for the data, certain questions, as discussed above, remain. However, no other hypothesis has been extensively researched.

5 SUMMARY AND CONCLUSIONS

5.1 Summary of Evidence

Numerous epidemiology studies have investigated the relationship between artificial sweeteners and bladder cancer risk, although the relationship between neonatal exposure and cancer risk has not been adequately studied. Two of the largest and most well-designed case-control studies have found small increased risks in certain subgroups of the population; however, the subgroups identified as being at elevated risk are inconsistent between the two studies. Increased risks were observed in men in one study and in men who smoked more than 40 cigarettes daily and women who never smoked and were never exposed occupationally to known bladder carcinogens in the other. This lack of consistency suggests that either the associations are not causal (probably attributable to chance or recall bias), or saccharin is a weak carcinogen.

The induction of bladder carcinogenesis by sodium saccharin has been shown to occur when rats are chronically exposed to doses at or above 1% to 3% in the diet for periods that include the neonatal period. Small, non-significant increases in bladder tumor incidence associated with sodium saccharin treatment have been observed in bioassays of weanling rats. Male rats appear more sensitive than female rats to the induction of bladder tumors by sodium saccharin. The best study evaluating the carcinogenic effects in rats was conducted by Schoenig *et al.* (1985) and was large enough to detect small increases in tumor incidence (less than 10%). Several other studies have been conducted, but these have limitations including small numbers of animals at end of study and limited histopathological evaluation. Multigeneration diet studies in mice observed small non-significant increases in bladder tumors in sodium saccharin treated animals. Studies in other species have either not been conducted at high enough doses (monkeys) or have not included the neonatal period (guinea pig).

Sodium saccharin has been shown to promote bladder tumors in rats when administered in drinking water or in feed after initiation with chemicals known to cause bladder tumors. Positive findings for bladder tumor promotion by sodium saccharin have been reported at doses at or above 2.5% in the diet following initiation by MNU, FANFT and BBN.

High doses of sodium saccharin have induced genotoxic effects *in vitro* and *in vivo.* Sodium saccharin is clearly not mutagenic in *Salmonella typhimurium* (IARC, 1999) and these results are supported by similar mammalian *in vivo* studies in transgenic Big Blue rats (Turner *et al*., 2001) in which mutations were not detected in any organs or tissues. Point mutations in codon 12 of the K-*ras* gene in a human cell line, however, were demonstrated in response to sodium saccharin treatment. Mouse coat spot tests for *in vivo* mutagenicity and tests for dominant lethals have been conflicting, with both positive and negative reports (Ashby, 1985).

Several negative studies of chromosomal aberrations have been reported for sodium saccharin *in vivo,* while most *in vitro* assays reported a positive clastogenic response. Sasaki *et al*. (2002) used the comet assay to demonstrate that sodium saccharin administered to mice *in vivo* is capable of causing DNA damage in tissues of the digestive tract, but not in the bladder. Sodium saccharin did not induce unscheduled DNA synthesis in cultured rat hepatocytes. DNA binding of ^{35}S-labeled saccharin in liver or bladder tissue has not been detected.

Several lines of investigation have attempted to establish a mode of action for sodium saccharin's carcinogenic activity in the rat bladder and key elements have been described. It has been shown that proliferation of the bladder urothelium occurs in rats after short-term exposures to sodium saccharin. The proliferation rate is significantly greater in sodium saccharin-treated rats compared to rats treated with acid saccharin or other saccharin salts even though all saccharin salts exist as saccharin anion at urinary pH. The tumor promoting activity of sodium saccharin appears to require an increased urinary sodium concentration and an alkaline urinary pH. Both male and female rat bladder urothelium is responsive to the promoting activity of sodium saccharin. In carcinogenicity bioassays male rats are more responsive than females, and timing of exposure seems critical. Urothelial tumors are readily observed only when exposure includes the neonatal period.

The mechanisms of tumor promotion and carcinogenicity have not been fully elucidated. The hypothesis that has been the most studied proposes that the formation of a cytotoxic calcium

phosphate-containing precipitate in the urine is a necessary event, which results in subsequent regenerative hyperplasia, progressing to tumor formation. Other possible mechanisms have not been well studied but include: disruption of normal Na^+/H^+ exchange homeostasis, resulting in increased intracellular pH, and increased cell proliferation; and abnormal expression of urothelial epidermal growth factor receptors (EGFR). Such expression would permit the binding of urinary epidermal growth factor (EGF) to the urothelial EGFR, leading to cell proliferation. While the hypothesis involving formation of the urinary precipitate has been the most studied, and is considered the leading hypothesis to date (IARC, 1999), neither it nor any other hypothesis has been adequately shown to be the operative mode of action of sodium saccharin-induced carcinogenicity in the rat.

5.2 Conclusion

There is evidence for the carcinogenicity of sodium saccharin, based on the development of tumors of the urothelium in rats of both sexes. Data from studies in rats indicates that sodium saccharin is a relatively weak animal carcinogen. The evidence from carcinogenicity studies in humans has not demonstrated a strong correlation between saccharin intake and human cancer, although such a response cannot be excluded based on the available data. Several lines of investigation have attempted to establish a mode of action for sodium saccharin's carcinogenic activity in the rat bladder. Thus far, however, the mode of action of sodium saccharin-induced carcinogenicity in the rat has not been fully elucidated.

In 1999, IARC concluded "that sodium saccharin produces urothelial bladder tumours in rats by a non-DNA-reactive mechanism that involves the formation of a urinary calcium phosphate-containing precipitate, cytotoxicity and enhanced cell proliferation. This mechanism is not relevant to humans because of critical interspecies differences in urine composition." In 2000, NTP concluded "the factors thought to contribute to tumor induction by sodium saccharin in rats would not be expected to occur in humans." Consequently, the Committee must use a "weight-of evidence" approach to evaluate the body of information available to determine whether the mechanism of action of sodium saccharin has been shown not to be relevant to humans.

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APPENDIX: SACCHARIN (ACID FORM); CAS NO. 81-07-2

This appendix outlines 1) the evaluations of the carcinogenicity of saccharin (acid form) performed by the International Agency for Research of Cancer (IARC) and by the National Toxicology Program (NTP), 2) actions of the U.S. Food and Drug Administration (U.S. FDA) and the U.S. Congress with regard to saccharin, and 3) the listing and removal of saccharin from the Proposition 65 list of chemicals known to the state to cause cancer.

Evaluations of Saccharin by IARC and NTP

IARC

IARC has reviewed saccharin on four occasions. In 1980, IARC concluded:

> "There is *sufficient* evidence that saccharin alone *[not in combination with cyclamates]*, given at high doses, produces tumours of the urinary tract in male rats…" (IARC, 1980).

In 1982, IARC concluded that for saccharin there was *limited* evidence of carcinogenicity in animals, *inadequate* evidence of carcinogenicity in humans, and that saccharin was *not classifiable as to its carcinogenicity in humans* (Group 3) (IARC, 1982). In contrast at that time IARC concluded the experimental animal evidence to be sufficient for sodium saccharin.

In 1987, IARC updated and reviewed the evidence for the carcinogenicity of saccharin, classifying it as *possibly carcinogenic to humans* (Group 2B), based on *sufficient* evidence for carcinogenicity in animals and *inadequate* evidence in humans (IARC, 1987).

In 1999, IARC updated and reviewed the evidence for the carcinogenicity of saccharin and its salts, concluding:

> "There is *inadequate evidence* in experimental animals for the carcinogenicity of saccharin (acid form) and calcium saccharin."
>
> "There is *inadequate evidence* in humans for the carcinogenicity of saccharin salts used as sweeteners,"
>
> "There is *sufficient evidence* in experimental animals for the carcinogenicity of sodium saccharin," and
>
> IARC (1999) determined that "saccharin and its salts are *not classifiable as to their carcinogenicity to humans* (Group 3)."

NTP

NTP first listed saccharin as *reasonably anticipated to be a human carcinogen* in its *Second Annual Report on Carcinogens* (1981). The listing was based on increases in bladder tumors in experimental animals.

NTP removed saccharin from the list of substances *reasonably anticipated to be a human carcinogen* in the *Ninth Report on Carcinogens* (NTP, 2000) after a request for reevaluation from the Calorie Control Council. During the NTP's re-evaluation process, the NTP's Review Group 1 (RG1; the National Institutes for Environmental Heath Sciences Review Committee for the Report on Carcinogens) and RG2 (the NTP Executive Committee Interagency Working Group for the Report on Carcinogens) voted to delist saccharin (7/3 and 6/2, respectively), while the NTP Board of Scientific Counselors Report on Carcinogens Subcommittee (the External Peer Review Group) voted against delisting and in favor of keeping saccharin listed as *reasonably anticipated to be a human carcinogen* (4/3). The NTP Executive Committee reviewed the recommendations of the RG1, RG2, and the Board subcommittee, and all public comments received by NTP on the proposed delisting of saccharin, and submitted its recommendation to the NTP Director, who made the final decision to remove saccharin from the *Report on Carcinogens*.

In removing saccharin from the list of substances *reasonably anticipated to be a human carcinogen* in its *Ninth Report on Carcinogens,* the NTP (2000) noted that:

> "There is evidence for the carcinogenicity of saccharin in rats but less convincing evidence in mice. Studies indicate that the observed urinary bladder cancers in rats are related to the physiology of the rat urinary system including urinary pH, osmolality, volume, and the presence of precipitate, and urothelial damage with attendant hyperplasia following consumption of diets containing sodium saccharin at concentrations of 3% or higher with inconsistent findings at lower dietary concentrations. The factors thought to contribute to tumor induction by sodium saccharin in rats would not be expected to occur in humans. The mouse data are inconsistent and require verification by additional studies. Results of several epidemiology studies indicate no clear association between saccharin consumption and urinary bladder cancer. Although it is impossible to absolutely conclude that it poses no threat to human health, sodium saccharin is not reasonably anticipated to be a human carcinogen under conditions of general usage as an artificial sweetener."

and concluded:

> "Saccharin will be removed from the Report on Carcinogens, because the rodent cancer data are not sufficient to meet the current criteria to list this chemical as *reasonably anticipated to be a human carcinogen*. This is based on the perception that the observed bladder tumors in rats arise by mechanisms not relevant to humans, and the lack of data in humans suggesting a carcinogenic hazard."

Actions by U.S. FDA and U.S. Congress

U.S. FDA

"In April 1977, the U.S. FDA proposed restrictions on the use of saccharin after a Canadian study confirmed two previous studies that showed that the widely-used artificial sweetener caused cancer in test animals. FDA's proposal called for a ban on saccharin as an additive in food, primarily diet sodas, but would have permitted its continued use as a table-top sweetener

on the condition that medical benefits could be demonstrated for its use. However, a public outcry over the proposal motivated Congress to enact a law which prevented FDA from taking any action to ban saccharin." (http://www.fda.gov/bbs/topics/ANSWERS/ANS00296.htm)

U.S. Congress

In November 1977, Congress passed the Saccharin Study and Labeling Act (Pub. L. No. 95-203, Nov. 23, 1977, 91 stat. 1451) that placed a two-year moratorium on any ban of saccharin while additional safety studies were conducted. The law also required that any foods containing saccharin must carry a label that reads: "Use of this product may be hazardous to your health. This product contains saccharin which has been determined to cause cancer in laboratory animals." Congress extended this legislation several times. On December 15, 2000, President Clinton signed the Saccharin Warning Elimination via Environmental Testing Employing Science and Technology Act ("SWEETEST Act") that removed the previously required warning statements (HR 5668, part of the Health and Human Services Appropriations Bill, HR 4577; 146 Cong Rec E2237-01).

Proposition 65 Listing History for Saccharin (CAS No. 81-07-2)

Saccharin (acid form) (CAS No. 81-07-2) was listed as known to the state to cause cancer under Proposition 65 on October 1, 1989. It was added to the list as a result of a court order enforcing California Health and Safety Code Section 25249.8(a), which requires that the Proposition 65 list "shall include at a minimum those substances identified by reference in Labor Code Section 6382 (b) (1) and those substances identified additionally by reference in Labor Code Section 6382 (d)." Labor Code Section 6382(b)(1) requires inclusion of substances listed as human or animal carcinogens by the IARC. Labor Code Section 6382(d) requires the inclusion on the Proposition 65 list of chemicals identified as carcinogens or potential carcinogens by IARC or the NTP, or chemicals regulated by the Occupational Safety and Health Administration as carcinogens. Saccharin had been identified as causing cancer by IARC in 1987 and by NTP in 1981.

As discussed above, IARC re-evaluated saccharin in 1999, and concluded "There is *inadequate evidence* in experimental animals for the carcinogenicity of saccharin (acid form) and calcium saccharin," and "saccharin and its salts are *not classifiable as to their carcinogenicity to humans* (Group 3)" (IARC, 1999); while in 2000, NTP removed saccharin from the list of substances *reasonably anticipated to be a human carcinogen* in its *Ninth Report on Carcinogens* (NTP, 2000). As a result of these actions by IARC and NTP, the basis for the court ordered listing of saccharin as known to the state to cause cancer under Proposition 65 no longer existed. As a result of the Saccharin Warning Elimination via Environmental Testing Employing Science and Technology Act, signed into law on December 15, 2000, saccharin was no longer formally required to be labeled or identified as causing cancer (Health and Safety Code §25249.8(b) and Title 22, California Code of Regulations Section 12902). OEHHA determined that there was no longer a legal basis for the listing of saccharin and removed it from the Proposition 65 list of chemicals known to the state to cause cancer, effective April 6, 2001.

APPENDIX REFERENCES

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