Two-Generation Saccharin Bioassays
by D. L. Arnold*

The controversy regarding the safety of saccharin for human consumption started shortly after its discovery over 100 years ago and has yet to subside appreciably. The consumption of saccharin, particularly in North America, began to escalate when the U.S. Food and Drug Administration set new standards of identity which allowed foods containing artificial sweeteners to be promoted as "nonnutritive" or "noncaloric" sweeteners for use by the general public. In 1968, when cyclamates were banned, at least 10 single-generation feeding studies were undertaken with saccharin to more accurately assess the potential toxicological consequences resulting from the anticipated increase in its consumption. None of these studies resulted in any overt regulatory action. Subsequently, the introduction of the two-generation chronic toxicity/carcinogenicity bioassay added a new tool to the toxicologist's arsenal. Three two-generation studies using saccharin have since been conducted. The results from these studies clearly show that when rats were exposed to diets containing 5 or 7.5% sodium saccharin from the time of conception to death, an increased frequency of urinary bladder cancers was found, predominantly in the males. While some study results suggested that impurities in commercial saccharin or the presence of urinary tract calculi may have been responsible for the observed bladder tumors, it now appears that these possibilities are highly unlikely. The mechanism by which saccharin elicited the bladder tumors using the two-generation experiment has not been ascertained.

Introduction
Saccharin was discovered by accident in 1879 by Constantin Fahlberg while working in Ira Remsen's laboratory at Johns Hopkins University (1). Fahlberg was investigating the oxidation mechanisms of toluenesulfonamide, when a compound with which he was working accidentally splattered on his fingers. Fahlberg, seemingly not the most fastidious of individuals, failed to wash his hands adequately and as a consequence, while eating supper that evening he noted that his bread had a sweet taste which he traced to the chemical commonly known as saccharin (2). In the intervening years, saccharin has been alternately castigated and approved for human use by various federal regulatory agencies. The controversy regarding the safety of saccharin for human consumption was well underway by 1890 (3) and has not appreciably abated in the intervening years. This melodrama has involved members of the scientific community, trade associations, legislative and regulatory authorities, the press, the public and even a president of the United States (2). The most recent tempest was precipitated by the findings of a two-generation chronic bioassay which has been dubbed the Canadian Saccharin Study (4). This study, which was the third to use the two-generation bioassay model suggested by the late Dr. Leo Friedman (5), had followed similar saccharin studies conducted at the Wisconsin Alumni Research Foundation (WARF) (6) and at the U.S. Food and Drug Administration (FDA) (7, 8). The ensuing controversy gave rise to the publication of at least two books (9, 10), numerous articles in the popular literature, a plethora of scientific activity, a large media blitz by several trade associations, as well as the convening of various panels and conferences to evaluate and discuss the human consumption of saccharin in light of these experimental results (11-18).

Early History
Some of the first toxicological tests with saccharin were reportedly conducted in France where diabetics received 5 g of saccharin per day for 5 months. No harmful effects were noted (19). However, in 1890, the Commission of the Health Association in France decreed saccharin to be "harmful," and forbade its manufacture or importation. Several scientists, especially French hygienists, protested violently, attempting to refute the assertions that saccharin was a harmful chemical (9).
In 1902, Germany placed a complete ban on the use of saccharin in all foodstuffs except foods intended for diabetics (20). Also in 1902, Dr. Harvey W. Wiley formed his famous "poison squad" which consisted of young volunteers who ingested various foods and food additives to ascertain their effects upon man. One of the chemicals ingested by this group was saccharin (21). Based on the findings of this study, Dr. Wiley attempted without success to block the commercial use of saccharin as a food additive (10).

Subsequently, saccharin was banned in the United States during 1912 for use in soft drinks and foods because it was considered to be an adulterant. However, during World Wars I and II, sugar supplies became so limited that the ban on saccharin use was temporarily lifted (2).

In 1959, under new standards of identity, the FDA allowed artificial sweeteners to be used in a variety of prepared foods which "should be used only by ... those who must restrict ..." their intake of sugar. Subsequently, foods containing artificial sweeteners were sold not only to people who had to either restrict their sugar consumption (diabetics) or who should avoid excessive caloric intake, but were also promoted as "nonnutritive" or "noncaloric" sweeteners for use by the general public (2).

**Toxicology Studies**

Prior to 1950, several toxicological studies of short and intermediate duration had been conducted with different species of laboratory animals and man (3, 22-30). The first chronic/carcinogenic bioassay using saccharin was undertaken by Fitzhugh et al. (31). They conducted a two-year feeding study in which rats received a control diet (7M, 9F) or diets containing 1.0% (10M, 10F) or 5.0% sodium saccharin (9M, 9F). Saccharin had no apparent effect upon mortality, hematology or organ weights (liver, kidney and spleen), but did result in a slight growth depression in the 5% group. The only significant pathological change was observed in the 5% group, where seven animals (sex unspecified) had thoracic lymphosarcomas; four of these seven rats had abdominal lymphosarcomas as well, which the authors stated was unusual even though the strain of rats used were known to have a high spontaneous incidence of lymphosarcomas. The urinary bladder was not examined histologically. The evaluation of the pathological changes observed in this study is even today a matter for debate (13, 19).

During the sixties a second chronic/carcinogenicity bioassay was conducted by Lessel (32), who fed groups of 20 male and 20 female rats diets containing 0, 0.005, 0.05, 0.5 or 5.0% saccharin for 2 years. Another group received 1 mL of a 1% solution of trypan blue SC every 2 weeks for 1 year as a positive control. In the 5% and trypan blue groups, mortality was higher than in the controls. Retardation of growth was observed in both sexes receiving the 5% diet despite a greater feed consumption. One female and four male rats in the 5% saccharin group had bladder calculi and another male had kidney calculi. The female rat with calculi had an extensive transitional cell papilloma of the bladder while another 5% female without calculi had hyperplasia and a papilloma. No evidence of nematodes was found.

In 1969, the use of the artificial sweetener cyclamate as a food additive was banned. It therefore was anticipated that the consumption of saccharin would increase significantly. Consequently, ten additional one-generation chronic/carcinogenic bioassays were undertaken using the rat, mouse and hamster in such countries as Canada, Germany, Great Britain, Japan and the United States (11). None of these studies indicated toxicological consequence of a nature sufficient to initiate regulatory action.

The one-generation rat feeding study performed in Canada was conducted by Munro et al. (35) and to quote the U.S. NRC report dealing with the safety of saccharin and sodium saccharin in the human diet, this "... test was carried out with exceptional care" (34). In this study, groups of 60 male and 60 female weanling Charles River rats were fed semipurified diets with daily dosages of 0, 90, 270, 810 or 2430 mg of sodium saccharin/kg body weight for 26 months (2500 mg/kg body weight is approximately equivalent to 5% in the diet). Additional saccharin was added to each diet to compensate for a moisture content of 1.4%, and the diet was analyzed for several potential contaminants. The animals were observed daily for clinical signs of toxicity and periodic cytological examinations of the urine were conducted for the parasite *Trichosomoides crassicauda* or its ova, of which none were detected.

Five bladder tumors were observed in the test animals. In addition, three animals had grossly visible bladder calculi, and 67 other animals were found to have bladder calculi which were small enough to pass through the urethra. The authors concluded that neither the presence of bladder calculi nor the saccharin treatments were associated with the presence of bladder tumors. Various panels (11, 34) and individuals (10) have concurred with the authors' conclusions, but Reuber (35) concluded from his review of this study that the results suggested "that saccharin is most likely carcinogenic for the urinary bladder."

A rather significant turn of events regarding the toxicological testing of saccharin occurred at the
Eighth Annual Meeting of the Society of Toxicology, when during a presentation at the Society’s Symposium on the Evaluation of the Safety of Food Additives and Chemical Residues, the late Dr. Leo Friedman (5) suggested that “no-effect dose levels be specifically defined to include that period of an animal’s lifetime from conception to sexual maturity.” This suggestion gave rise to the two-generation chronic/carcinogenic bioassay as we know it today.

The first two-generation bioassay in which saccharin was fed, was conducted under contract at the Wisconsin Alumni Research Foundation (WARF). This study (6) had groups of 20 male and 20 female Sprague-Dawley rats being fed diets containing 0.0, 0.05, 0.5 or 5.0% sodium saccharin produced by the Remsen-Fahlberg procedure for 14 weeks prior to mating, as well as during mating and lactation. Following weaning, the F₁ generation was killed and the F₂ generation was weaned onto their parents’ diet, which they consumed until the study was terminated 100 weeks later. Thus, the F₂ generation animals were exposed to saccharin and its impurities during gestation, throughout lactation via the dam’s milk and for the remainder of their lifetime via the diet.

The authors reported that saccharin had no remarkable effects upon the reproductive indices, excepting a depressed pup body weight in the 5.0% group at weaning. However, after the F₂ generation had been on test for 13 weeks post-weaning, the growth curves were similar in all groups for the balance of the study. Neither feed consumption nor survival was affected by treatment, and the observed hematological changes were considered to be the result of age and poor health rather than treatment. The authors suggested that the incidence of tumors was apparently increased by the consumption of the 5.0% diet. The incidence of squamous cell carcinomas of the uterus and transitional cell carcinomas of the urinary bladder, were seen exclusively in the treated groups, with higher incidence at higher dosage levels.

This study apparently was the first chronic study undertaken using the two-generation animal model, which likely enhanced the chances that its results would be critically evaluated and the deficiencies of this experimental model emphatically pointed out. While the U.S. NRC (34) panel recognized the important implications of using an animal model system for testing wherein transplacental exposure was used, experience and information was limited regarding; (a) the dose level to which the fetus was exposed; (b) the developmental stages during organogenesis; (c) the metabolic capacity of the fetus; (d) the immune competency of the fetus; and (e) factors related to fetal pharmacology. Additionally, the panel went on to point out that factors such as urinary tract stones and parasites, which are known to cause bladder tumors in rats, were not ruled out as causative agents in this study.

After the U.S. Food and Drug Administration (FDA) had an opportunity to review the results of this study, saccharin was removed from the Generally Recognized as Safe (GRAS) food additive list. This action had the legal force of allowing a future ban on saccharin if there was any question of saccharin’s safety as a food additive (36).

The second two-generation saccharin feeding study was conducted by the FDA between 1970 and 1972 as a combination three-generation reproduction study and two-generation chronic feeding study. An interim report was presented at the annual Society of Toxicology meeting in 1974 (7), but a final report did not appear until 1980 (8).

The experimental design consisted of groups of 10 male and 20 female Sprague-Dawley rats being fed diets containing 0.0, 0.01, 0.1, 1.0, 5.0 or 7.5% sodium saccharin, or 1.51% sodium carbonate (giving a sodium ion concentration equivalent to 5.0% sodium saccharin). Another group was fed a diet containing 5.0% calcium cyclamate, which was fed as a reference compound. A sufficient number of animals were randomly selected from the F₂ litters to give a total of 48 males and 48 females per test group. Interim sacrifices of four animals/sex/group were made at 14 months and five animals/sex/group at 18 months; but these rats were not part of the original 48 animals/group. The first generation animals were killed after they produced a second litter.

The authors reported that treatment did not have any apparent effect on hematological values, organ weights, or survival and that birth weights of rats ingesting 5.0 or 7.5% saccharin or 5.0% cyclamate were depressed versus controls. Although some of the initial weight differences were overcome, these animals had lower body weights throughout the study. There were several significant decreases in average and relative organ weights, expressed as percentage of body weight, which the authors attributed to decreases in body weights. Additionally, there were no dose- or time-related trends in these results when the data from the serial sacrifices were included in the analysis. The most significant pathological findings included nine urinary bladder neoplasms in the 7.5% group (7 males, 2 females), one in the 5.0% group (male) and one in a control male. Three sections of each bladder (dome, midpoint and bladder neck) from rats that were on test for more than 18 months, were examined histologically for evidence of the bladder parasite Trichosomoides crassicauda. No
parasites were found, and the authors concluded that the parasites did not contribute to the development of the observed bladder neoplasms. Additionally, the authors concluded that the incidence of gross calculi was not associated with the observed neoplasms.

The authors also pointed out that several pathologists or groups of pathologists independently reviewed tissue sections from the bladder, and while some differences in diagnosis did occur, the reviewing groups confirmed that there was a significant increase in urinary bladder neoplasms in the group receiving 7.5% sodium saccharin.

This study was also criticized along the same lines as was the WARF study. In addition, the U.S. NRC (34) panel suggested that the maximum tolerated dose (MTD) may have been exceeded for the 5.0 and 7.5% dose groups due to the body weight suppressions. While the panel commended the inclusion of a group to ascertain what effect the high dietary sodium levels attributable to the inclusion of 5.0% sodium saccharin in the diet might have upon metabolic disposition, they questioned whether or not carbonate was an appropriate anion.

Before getting into the details of the third two-generation study, which was conducted by the Canadian Health Protection Branch, it is appropriate to point out some of the events happening outside the animal laboratory and their role in the design of this study.

The inability of saccharin to induce a significant tumorigenic response in any one of the one-generation feeding studies, including the one conducted by Munro et al. (32), was consistent with the findings of a number of epidemiological studies that were published in the early seventies (37-39). Additionally, the high dosages of saccharin required to elicit bladder tumors in the two-generation studies, as well as the apparent predilection for the second generation males, resulted in more questions than answers for the toxicological problem being studied. These findings suggested that an impurity in saccharin might be responsible for the observed tumorigenic effects. Studies conducted in the Health Protection Branch laboratories indicated that o-toluene sulfonamide (o-TS) was the major impurity in all of the saccharin samples used in the various long-term feeding studies (40-42). The concentrations of o-TS ranged from 118 to 6100 ppm in these samples (43) which were all produced by the Remsen-Fahlberg method. These and other observations led to the hypothesis that o-TS, a known inhibitor of carbonic anhydrase (44, 45), the enzyme involved in the acidification of urine (46), would increase the excretion of bicarbonate. This action could produce an alkaline urine, predisposing the animal to urolithiases in the kidney and bladder. Irritation from these stones over a long period of time could produce hyperplasia and ultimately tumors (43). A correlation between bladder stones and bladder tumors had previously been found for several experimental situations (47).

As a partial test of his hypothesis, a study was conducted wherein groups of 24 to 27 untreated Sprague-Dawley female rats were mated on a one-to-one basis with untreated males. On day one of pregnancy, the pregnant females were given the first of 43 daily doses of o-TS (dosage levels: 0, 40, 100 or 250 mg o-TS/kg/day) in corn oil via gavage. The pups were then weaned onto diets containing sufficient amounts of a o-TS to provide the same dose levels as received by their respective dams. Three interim kills and a final kill, when the pups were 105 days old, were performed, at which time the incidence of renal calculi, bladder calculi and histopathological bladder calculi in 21-day-old rats (unsexed) and in 105-day-old male and female rats (48). These findings lent support to the preceding hypothesis.

Health Protection Branch scientists thus proposed that an additional two-generation chronic feeding study be undertaken to more fully test the preceding hypothesis. The initial protocol for this study called for groups of rats to be fed either a standard commercial chow diet or one containing graded levels of o-TS. In addition, ammonium chloride was to be added to the drinking water of one of the highest o-TS dosed groups to prevent the formation of an alkaline urine (47, 49). Comments on this experimental protocol were solicited from other regulatory agencies and a number of internationally recognized toxicologists, as well as from several industrial organizations.

Prior to the start of this study, a quantity of saccharin produced by the Maumee procedure became available. This method of saccharin production results in no detectable levels of o-TS and the total amount of impurities is less than the Remsen-Fahlberg procedure (4, 50). This saccharin was fed to an additional group of rats at a level of 5% in the low diet, a level comparable to the highest dose used in the WARF two-generation study as well as several conventional one-generation studies (11). In keeping with the above hypothesis, the latter group was included as a "negative" control group.

The final study protocol consisted of groups of 50 male and 50 female (except where noted) Sprague-Dawley rats being fed one of the following dietary treatments: control; 2.5 mg o-TS/kg/day; 25 mg o-TS/kg/day; 250 mg o-TS/kg/day; 250 mg o-TS/kg/day with 1% NH₄Cl in the drinking water (F, group size 40 males, 38 females; F, 49 males, 50 females; or 5% sodium saccharin.
Regarding the conduct of this study (4), the F₀ animals in this two-generation bioassay were 32 days of age when the study commenced. After 90 days on test, these animals were mated on a one-to-one basis within treatment groups. The pups were weaned onto their parents' diet. Unlike the WARB (6) and FDA (7, 8) studies, both the F₀ and F₁ generations were continued on test for their lifetime. Periodically, urine samples were filtered and stained by the Papanicolaou method and examined microscopically for sediment and exfoliated epithelial cells in an attempt to diagnose alterations in bladder epithelium. The filters were also examined for ova and the adult form of Trichosomoides crassicauda. Additionally, hematological evaluations were conducted during the course of the study and a limited water balance study was undertaken with some of the F₁ animals. Great care was taken to ensure that this study was conducted in accordance with the best toxicological practices employed at that time (51, 52).

Rats from both generations fed diets providing 250 mg o-TS/kg or 250 mg o-TS with 1.0% NH₄Cl in the drinking water or containing 5% sodium saccharin had decreased growth rates, but only the former two groups had a lowered feed consumption. There were no treatment related effects upon reproduction, longevity, or hematological parameters. The animals were free of the bladder parasite Trichosomoides crassicauda. While a few animals developed grossly visible bladder and kidney stones, the incidence was not treatment related. The incidence of bladder tumors is shown in Table 1. Tumor diagnosis was by consensus among seven independent pathologists who were not associated with the design or conduct of the study. There was a statistically significant increase in the incidence of bladder tumors in both generations of male rats fed the diet containing 5% sodium saccharin (p < 0.03, F₀; p < 0.002, F₁); compared to their respective control groups. An evaluation of individual feed consumption did not reveal any statistical difference between the amount of feed consumed by animals which had bladder tumors and those that did not. The incidence of bladder tumors in all of the other treatment groups was not significantly different from that of the respective control groups. A limited water balance study indicated that the animals receiving the diets containing sodium saccharin drank approximately twice as much water and voided twice as much urine as did the control animals. The urine was also found to be hypotonic.

The complete findings of this study were not available when several individuals (10, 35) and panels (11) were reviewing the results of the various saccharin studies. Consequently, some of the points:

| Treatment groups | F₀ generation | F₁ generation |
|------------------|---------------|---------------|
|                  | Benign        | Malignant     | Benign        | Malignant     |
| **Males**        |               |               |               |               |
| Control          | 1             | 0             | 0             | 0             |
| 2.5 mg o-TS/kg/day | 1             | 0             | 0             | 0             |
| 25 mg o-TS/kg/day | 0             | 0             | 0             | 0             |
| 250 mg o-TS/kg/day | 1             | 0             | 0             | 0             |
| + 1% NH₄Cl in drinking water | 0             | 0             | 0             | 0             |
| 5% Sodium saccharin | 4²,³         | 3²           | 4²           | 8²,³,⁴       |
| **Females**      |               |               |               |               |
| Control          | 0             | 0             | 0             | 0             |
| 2.5 mg o-TS/kg/day | 1             | 0             | 2             | 0             |
| 25 mg o-TS/kg/day | 0             | 0             | 0             | 0             |
| 250 mg o-TS/kg/day | 0             | 0             | 0             | 0             |
| + 1% NH₄Cl in drinking water | 0             | 0             | 0             | 0             |
| 5% Sodium saccharin | 0             | 0³           | 0             | 2             |

²Number of animals with tumors (i.e., benign plus malignant) was significantly higher (p < 0.03) than control. Statistics were based on numbers of animals surviving until first tumor was observed after 87 weeks on test (No. control = 38F, 36M; No. saccharin = 40F, 38M).

³Additional urinary tract tumors consisted of one animal with a urethral tumor and one animal with a malignant lesion of the kidney pelvis.

⁴Number of animals with malignant tumors (p < 0.002) and total tumors (benign plus malignant; p < 0.01) was significantly higher than control. Statistics were based on number of animals surviving until first tumor was observed after 67 weeks on test (No. control = 45F, 42M; No. saccharin = 49F, 45M).

⁵Additional urinary tract tumors consisted of one animal with a urethral tumor.

⁶Additional urinary tract tumors consisted of two animals with malignant lesions of the kidney pelvis.
raised by these reviewers have subsequently been answered with the publication of this study. Generally speaking, the shortcomings previously referred to regarding two-generation chronic bioassays are also applicable to this study. Unfortunately, many of the criticisms of this experimental model are no closer to being answered today than they were at the time the WARP study was conducted (11, 34, 53). The other major criticism of this study, which was also applicable to the high saccharin dosages used in the FDA study, was that the MTD would appear to have been exceeded. While several guidelines for the conduct of chronic/carcinogenicity bioassays as well as the NCI (54) and IARC (55) guidelines suggest that a weight differential greater than 10% between the control and treated groups indicates that the MTD has been exceeded, it has been pointed out that there was generally a greater growth differential between control and female rats receiving high dosages of saccharin than there was for the male rats in the various saccharin bioassays (11). Furthermore, there has not been any substantial scientific evidence presented to support the contention that a weight suppression of 10% or greater renders the animal more or less susceptible to carcinogenic responses. However, evidence has existed for many years that caloric restriction and overnutrition/obesity appear respectively to decrease and increase the incidence of spontaneous tumors (56).

While the evidence from this two-generation study clearly indicated that o-TS did not have any carcinogenic potential in this model system, Schmähl (57) reported that o-TS may possibly have a weak carcinogenic effect. His study consisted of groups of 38 male and 38 female Sprague-Dawley rats, 3 months of age, fed diets containing 0, 20 or 200 mg o-TS/kg body weight for the remainder of their lifetimes. The incidence of bladder carcinomas and bladder papillomas was 0, 0, 1 and 0, 3, 4, respectively. The carcinoma was observed after 759 days (days on test presumably) and the four benign tumors were found after 861, 877, 878, and 996 days. In the 20 mg/kg group the papillomas were detected after 539, 766 and 873 days.

Since Schmähl (57) did not mention the presence of bladder parasites as he had with a previous study (58), or bladder calculi, it might be presumed that neither of these possibilities was involved in the production of the observed bladder lesions. Consequently, except for the possibility of a difference in purity of the test chemical, it is not possible at this time to determine the cause of this apparently conflicting result.

Recently, as a component of a larger study, Hooson et al. (59) administered o-TS in the drinking water or diet to groups of 63 or 50 female Wistar SPF weanling rats, respectively, at a level equivalent to 70 mg/kg. No evidence of bladder hyperplasia or neoplasia was observed. One possible contributory factor to these variable findings was suggested by the authors when they were discussing interpretation of certain bladder lesions in the rat. They suggested that part of this problem may be attributable to the absence of a universally accepted system for classifying rat bladder neoplasia, assessing invasion of the underlying connective tissue, as well as incomplete knowledge regarding the behaviour of such proliferative lesions. While there is evidence that the bladder neoplasia induced by a variety of carcinogens are often preceded by hyperplastic lesions that are papillary or nodular in their growth pattern, it is possible that such lesions are not progressive, or may even be reversible, in some situations. Unfortunately, reversible or irreversible hyperplasias cannot be distinguished histologically (59).

Observations and Comments

To evaluate and compare studies undertaken with the same substance, requires detailed information concerning the design, conduct, parameters monitored, how monitoring was performed, and analytical and statistical procedures. Very seldom is this information provided with appropriate detail in journal articles. Nevertheless, Taylor and Morgenroth (60) attempted to undertake such an exercise using five different studies in which saccharin was fed to rats. Based on the information available to them, they concluded that: it was not likely that the strain of rat had a role in the increased incidence of bladder tumors; the basal diet did not appear to be a factor in the experimental observations; a dose level of 5% sodium saccharin or greater is needed in addition to in utero exposure to produce bladder tumors; the amount of o-TS contamination was not a factor in the findings of bladder lesions; and the bladder parasite, Trichosomoides crassicauda apparently was not involved in the findings of the various saccharin studies.

Based on the data currently available, Taylor and Morgenworth may be correct in their conclusion that a dose level of 5% sodium saccharin is required to elicit bladder tumors. However, it should be pointed out that until the industrially sponsored two-generation saccharin feeding study, currently in progress at the International Research and Development Corporation, Mattawan, Michigan, was initiated (16), dose levels between 1 and 5% sodium saccharin have never been fed to rats during a chronic/carcinogenicity bioassay. For a substance such as saccharin, which only elicits a relatively low incidence of bladder lesions when fed at a dietary level
of 5% and requires a latent period of approximately two years, one might not expect an appreciable incidence of bladder lesions attributable to saccharin at dietary levels of 1%, unless the number of test animals in the group exceeds 50 and the survivability after two years on test is excellent. The experimental design of the aforementioned industry-sponsored study does include increasing the number of animals in each test group as the doses are lowered (16). Consequently, this study may well provide evidence that saccharin will elicit bladder tumors in male offspring at dose levels below 5% using the two-generation model.

Before proceeding, a few additional comments concerning the conclusions of Taylor and Morgenroth (60) are not impertinent. All three of the two-generation saccharin feeding studies and the two one-generation feeding studies conducted by Munro et al. (53) and by Homburger (61), all used Sprague-Dawley rats. While the latter two studies were considered to be negative, it has been suggested it may be "more prudent to conclude that these studies were not conducted adequately than that they were negative" (11). In retrospect, based primarily on the first-generation findings of the two-generation HPB study, and the observation of Fukushima and Cohen (62), there appears to be some evidence to suggest that the one-generation studies were either not sensitive enough to detect the carcinogenic potential of saccharin, in part attributable to the age of the animal when the study was initiated, or the procedures used to evaluate the pathological changes of the bladder and urinary tract elicited by saccharin were not appropriate. A countering argument is that the statistically significant ($p > 0.03$) increased incidence of bladder tumors seen in the first generation of the two-generation HPB study (4) may have been due to random chance. However, the mean latent period for saccharin to elicit bladder tumors in the rat is approximately 90 to 100 weeks (4, 7, 8, 63), suggesting that the improved survivability of the test animals in the two-generation HPB study (4) enhanced the possibility of observing tumors in the saccharin treated rats. For additional comments and analysis, the reader is referred to the literature (10, 11, 53).

One point which has not been adequately addressed concerns whether the incidence of bladder tumors in the two-generation saccharin studies may have a genetic component as well as a test chemical component. Specifically, in the WARF (6) and HPB (4) studies, the F1 animals were mated on a one-to-one basis, while in the FDA (7, 8) study, one male was mated with two females. Grice et al. (53) have found that the inter-litter variation for styrene, saccharin and amaranth exposure was statistically sign-
ificant whereas the inter-litter differences regarding transplacental exposure were more homogeneous. Based on a limited data base, Grice et al. suggested that as a result of inter-litter differences in both transplacental exposure and/or exposure during lactation as well as genealogical considerations, the litter rather than the individual pup may be the more appropriate unit for purposes of statistical analysis.

Concluding Remarks

Any review of the toxicological data concerning saccharin would convince any skeptic that saccharin is one of the most thoroughly tested food additives ever consumed. However, as the adage goes, the more one knows the more one realizes how much one does not know. One of the biggest questions that still confronts the scientific and regulatory communities regarding saccharin is the precise mechanism by which saccharin elicits bladder tumors in laboratory rats. While the scientific community has dubbed saccharin an epigenetic (64) or secondary carcinogen (65), the North American regulatory agencies currently do not distinguish between electrophilic carcinogens and those which elicit tumors by any other mechanism. However, as pointed out by Truhaut (65), it should be possible to establish a threshold or no-effect level for chemicals that produce tumors by mechanisms other than electrophilic interaction with cellular macromolecules.

The strongest evidence that saccharin is a carcinogen involves the two-generation bioassay model, regarding which there is limited experience and which raises many questions to which answers presently do not exist (18, 34, 59). While some may view the two-generation bioassay as a more sensitive analytical tool for toxicological testing and/or a very practical toxicological testing model, particularly regarding saccharin, our experience with hexachlorobenzene in the two-generation bioassay (Arnold et al., unpublished data) does not suggest that it is more sensitive per se, particularly regarding cancer. However, studies presently underway in the United States regarding the testing of a number of food colors using the two-generation bioassay, will provide a better insight into this question.

The role which impurities may play in the toxicological effects observed as a result of exposure to saccharin, cannot be completely dismissed. However, there is no compelling evidence that the levels of impurities normally found in commercial saccharin (66) contribute to the observed carcinogenicity of saccharin. The fact that several batches of Remsen-Fahlberg-produced saccharin were used in the two-generation WARF (6) and FDA (7, 8) studies, while
Maumee-produced saccharin was used in the HPB study (4), would support such a conclusion. If an impurity were responsible for the carcinogenic effect of saccharin, it would have to be an extremely potent bladder carcinogen.

Saccharin is not metabolized to any significant extent (67-71), if at all (72-76); it is nucleophilic (64) and does not bind to rat liver or bladder DNA (77), but it does suppress primary humoral antibody production in the rat (78). At dosages of 5% and above, several pharmacokinetic changes are also induced in the rat (79). Saccharin, therefore, probably does not act as a typical chemical carcinogen according to the theory that most, if not all, ultimate carcinogens are strong electrophilic agents. (80). Consequently, we had initially proposed that it was possible that saccharin administered at the dietary level used in the two-generation studies may have elicited bladder tumors by some type of physical mechanism. While there was some initial support for this suggestion (4), subsequent studies in our laboratory were not supportive of such a hypothesis (16). Several of the subsequent authors will discuss evidence for other possible mechanisms by which saccharin may give rise to tumors.

The author thanks Mr. A. Peterkin for his assistance in preparing the manuscript, and Mrs. T. Kelly for her typing assistance.

REFERENCES

1. Remsen, L., and Fahlberg, C. On the oxidation of substitution products of aromatic hydrocarbons. IV. On the oxidation of orthotoluenesulfonamide. Am. Chem. J. 1: 426-436 (1879-1880).
2. Munro, I. C., Stavric, B., and Lacombe, R. The current status of saccharin. In: Toxicology Annual 1974 (C. L. Winek, Ed.), Marcel Dekker, New York, 1974, pp. 71-89.
3. de Nito, G. Sull’azione tossica della saccharina. Richerche isto-patologiche. Boll. Soc. Ital. Biol. Sper. 11: 934-935 (1936).
4. Arnold, D. L., Moodie, C. A., Grice, H. C., Charbonneau, S. M., Stavric, B., Collins, B. T., McGuire, P. F., Zawidzka, Z. Z., and Munro, I. C. Long-term toxicity of ortho-tolu-enesulfonamide and sodium saccharin in the rat. Toxicol. Appl. Pharmacol. 52: 113-152 (1980).
5. Friedland, J. Symposium on the evaluation of the safety of food additives and chemical residues: II. The role of the laboratory animal study of intermediate duration for evaluation of safety. Toxicol. Appl. Pharmacol. 16: 498-506 (1970).
6. Tisdal, M. D., Nees, P. O., Harris, D. L., and Derse, P. H. Long-term feeding of saccharin in rats. In: Symposium: Sweeteners (G. E. Inglett, Ed.), Avi Publishing Co., Westport, Conn., 1974, pp. 145-158.
7. Taylor, J. M., and Friedman, L. Combined chronic feeding and three-generation reproduction study of sodium saccharin in the rat. Toxicol. Appl. Pharmacol. 29: 154 (abstract no. 2000) (1974).
8. Taylor, J. M., Weinberger, M. A., and Friedman, L. Chronic toxicity and carcinogenicity to the urinary bladder of sodium saccharin in the in utero-exposed rat. Toxicol. Appl. Pharmacol. 54: 57-75 (1980).
9. Rhein, R. W., Jr., and Marion, L. The Saccharin Controversy: A Guide for Consumers. Monarch Press, New York, 1977.
10. Cranmer, M. F. Saccharin: A report by Dr. Morris F. Cranmer (G. H. Scheer, Ed.), American Drug Research Institute Inc., Tippecanoe, In., 1980.
11. Office of Technology Assessment. Cancer Testing Technology and Saccharin. Congress of the U.S., Stock No. 052-003-00471-2, Washington, DC, 1977.
12. WHO. Evaluation of certain food additives. Twenty-First Report of the Joint FAO/WHO Expert Committee on Food Additives, World Health Organization. Technical Report No. 617, Geneva, 1978, pp. 24-26.
13. Proceedings, Toxicology Forum on Saccharin, Center for Continuing Education, University of Nebraska Medical Center, 42nd and Dewey, Omaha, Nebraska, May 9-11, 1977, Toxicology Forum, Washington, D.C., 1977.
14. Saccharin Update, Special Session, Given Institute, Aspen, Colorado, July 23, 1978, Toxicology Forum, Washington, DC, 1978.
15. Saccharin Working Group of the Toxicology Forum, Given Institute, Aspen, Colorado, July 8, 1979, Toxicology Forum, Washington, DC, 1979.
16. Saccharin Working Group of the Toxicology Forum, Given Institute, Aspen, Colorado, July 27, 1980, Toxicology Forum, Washington, DC, 1980.
17. Guggenheim, B. (Ed.). Health and Sugar Substitutes (Proceedings of the ERGOB Conference on Sugar Substitutes, Geneva, October 30-November 1, 1978). S. Karger, Basel, 1979.
18. Committee for a Study on Saccharin and Food Safety Policy. Saccharin: Technical assessment of risks and benefits. National Research Council, Report No. 1, prepared for Food and Drug Administration under contract 223-78-2145, Washington, DC, 1978.
19. Food and Drug Administration. Saccharin and its salts. Proposed rule making. Federal Register. 42: 19996-20010 (1977).
20. Menden, E. Saccharin in der Ernährung des Menschen. Ernähr. Umsch. 11: 361-364 (1967).
21. Marin, G., and Van Allen, J. Food Pollution: The Violation of Our Inner Ecology. Holt, Rinehart and Winston, New York, 1972, p. 109.
22. Jessen, F. Zur Wirkung des Saccharins. Arch. Hygiene 10: 64-80 (1890).
23. Matthews, H. B., Fields, M., and Fishbein, L. Saccharin: distribution and excretion of a limited dose in the rat. J. Agr. Food Chem. 21: 916-919 (1973).
24. Herter, C. A., and Folin, O. In: Influence of Saccharin on the Nutrition and Health of Man. U.S. Department of Agriculture, Report No. 94, Government Printing Office, Washington, DC, 1911.
25. Becht, F. C. Influence of saccharin on the catalases of the blood. J. Pharmacol. Exppl. Therap. 16: 155-197 (1921).
26. Carlson, A. J., Eldridge, C. J., Martin, H. P., and Foran, F. L. Studies on the physiological action of saccharin. J. Metab. Res. 3: 451-477 (1923).
27. Neumann, R. O. Wird die Ausnutzung des Nahrungseiweisses durch Saccharin beeinflusst? Arch. Hyg. Bakteriol. 96: 265-276 (1926).
28. Neumann, R. O. Wird die Ausnutzung des Nahrungseiweisses durch saccharin beeinflusst? Arch. Hyg. Bakteriol. 97: 275-276 (1926).
29. Staub, H., and Staehelein, R. Saccharin. Med. Press. Circ. 193: 419-422 (1936).
30. de Boer, H. W., and Bosgra, O. Onderzoekingen betref-
1. Lassel, B. Carcinogenic and teratogenic aspects of saccharin. In: Third International Congress, Food Science and Technology, SOS/70 Proceedings, Washington, DC, 1970, pp. 764-770.

2. Munro, I. C., Moodie, C. A., Kreowski, D., and Grice, H. C. A carcinogenicity study of commercial saccharin in the rat. Toxicol. Appl. Pharmacol. 32: 513-526 (1975).

3. National Research Council. Report to FDA on the safety of saccharin and sodium saccharin in the human diet. Food and Drug Administration, Publication No. 239-137, Washington, DC, 1974.

4. Reuber, M. D. Carcinogenicity of saccharin. Environ. Health Perspect. 25: 173-200 (1978).

5. Epstein, S. S. The Politics of Cancer. Sierra Club Books, San Francisco, 1978, p. 199.

6. Armstrong, B., and Doll, R. Bladder cancer mortality in diabetics in relation to saccharin consumption and smoking habits. Brit. J. Prev. Soc. Med. 29: 73-81 (1975).

7. Kessler, I. I. Nonnutritive sweeteners and human bladder cancer: Preliminary findings. J. Urol. 115: 143-146 (1976).

8. Morgan, R. W., and Jain, M. G. Bladder cancer: smoking, beverages and artificial sweeteners. Can. Med. Assoc. J. 111: 1067-1070 (1974).

9. Stavric, B., Laccombe, R., Munro, I. C., By, A. W., Klassen, R., and Ethier, J. Studies on water soluble impurities in commercial saccharins. Paper presented at American Chemical Society National Meeting, Los Angeles, March 31-April 5, 1974, 1974, Anal. No. 171.

10. Stavric, B., Laccombe, R., Watson, J. R., and Munro, I. C. Isolation, identification and quantitation of o-toluenesulfonamide, a major impurity in commercial saccharins. J. Assoc. Off. Anal. Chem. 57: 678-681 (1974).

11. Stavric, B., Klassen, R., and By, A. W. Impurities in commercial saccharin. I. Impurities soluble in inorganic solvents. J. Assoc. Off. Anal. Chem. 59: 1051-1058 (1976).

12. Grice, H. C. Commentary and discussion section on toxicology: report of the National Academy of Science. In: Sweeteners: Issues and Uncertainties. Academy Forum, National Academy of Sciences, Washington, DC, 1975, pp. 114-119.

13. Krebs, H. A. Inhibition of anhydride by sulphonamides. Biochem. J. 43: 525-528 (1948).

14. Miller, W. H., Dessert, A. M., and Roblin, R. O., Jr. Heterocyclic sulphonamides as carbonic anhydride inhibitors. J. Am. Chem. Soc. 72: 4893-4896 (1950).

15. White, A., Handler, P., Smith, E. L., Hill, R. L., and Lehman, I. R. Principles of Biochemistry, 6th ed. McGraw-Hill, New York, 1978, pp. 1068-1069.

16. Clayson, D. B. Bladder carcinogenesis in rats and mice. Possibility of artifacts. J. Natl. Cancer Inst. 52: 1865-1869 (1974).

17. Arnold, D. L., Moodie, C. A., McGuire, P. F., Collins, B. T., Charbonneau, S. M., and Munro, I. C. The effect of o-toluenesulfonamide and sodium saccharin on the urinary tract of neonatal rats. Toxicol. Appl. Pharmacol. 51: 455-463 (1979).

18. Flaks, A., and Clayson, D. B. The influence of ammonium chloride on the induction of bladder tumors by 4-ethylulphoninaphthalene-1-sulphonamide. Brit. J. Cancer 31: 585-587 (1975).

19. Stavric, B. The Canadian saccharin study. In: Toxicology Forum on Saccharin. Center for Continuing Education, University of Nebraska Medical Center, Omaha, Nebraska, May 9-11, Toxicology Forum, Washington, DC, 1977, pp. 73-80.

20. Arnold, D. L., Charbonneau, S. M., Zawadzka, Z. Z., and Grice, H. C. Monitoring animal health during chronic toxicity studies. J. Environ. Pathol. Toxicol. 1: 227-239 (1977).

21. Fox, J. G., Thibert, P., Arnold, D. L., Kreowski, D. R., and Grice, H. C. Toxicology studies. II. The laboratory animal. Food Cosmet. Toxicol. 17: 661-675 (1979).

22. Grice, H. C., Munro, I. C., Kreowski, D. R., and Blumenthal, H. In utero exposure in chronic toxicity/carcinogenicity studies. Food Cosmet. Toxicol. 19: 373-379 (1981).

23. Sontag, J. M., Page, N. P., and Saffiotti, U. Guidelines for Carcinogen Bioassay in Small Rodents (National Cancer Institute Carcinogens Technical Report Series No. 1), DHEW Publication No. NIH 76-801, Washington, DC, 1976.

24. International Agency for Research on Cancer. Long-Term and Short-Term Screening Assays for Carcinogens: A Critical Appraisal. (Monographs on the Evaluation of the Carcinogenic Risk of Chemicals to Humans, Supplement 2), IARC, Lyon, 1980.

25. Tannenbaum, A., and Silverstone, H. Nutrition and the genesis of tumours. In: Cancer, Vol. 1 Part I, Research Into Causation, (R. W. Raven, Ed.), Butterworth, London, 1987, pp. 303-334.

26. Schmähl, D. Experiments on the carcinogenic effect of ortho-tolualsulfonamid (OTS). Z. Krebsforsch. 91: 19-22 (1978).

27. Schmähl, V. D. Fehlen einer kancerogenen Wirkung von Cyclamlat, Cyclohexylamin und Saccharin bei Ratten. Arzneim. Forsch. 23: 1466-1470 (1973).

28. Hopson, J., Hicks, R. M., Grasso, P., and Chowaniec, J. Ortho-toluenesulphonamide and saccharin in the promotion of bladder cancer in the rat. Brit. J. Cancer 42: 129-147 (1980).

29. Taylor, J. M., and Morgen-roth, V. H., III. Comparison of studies on saccharin and sodium nitrite. J. Assoc. Anal. Chem. 62: 883-888 (1979).

30. Homburger, F. Negative lifetime carcinogen studies in rats and mice fed 50,000 ppm saccharin. In: Chemical Toxicology of Food. (C. L. Galli, R. Paololetti and G. Vettorazzi, Eds.), Elsevier/North Holland Biomedical Press, Amsterdam, 1981, pp. 359-373.

31. Fukushima, S., and Cohen, S. M. Saccharin-induced hyperplasia of the rat urinary bladder. Cancer Res. 46: 734-736 (1986).

32. Hicks, R. M., and Chowaniec, J. The importance of synergy between weak carcinogens in the induction of bladder cancer in experimental animals and in humans. Cancer Res. 37: 2943-2949 (1977).

33. Ashby, J., Styles, J. A., Anderson, D., and Paton, D. Saccharin: an epigenetic carcinogen/mutagen? Food Cosmet. Toxicol. 16: 95-103 (1978).

34. Truhaut, R. An overview of the problem of thresholds for chemical carcinogens. In: Carcinogenic Risk Strategies For Interaction (W. Davis and C. Rosenfeld, Eds.), International Agency for Research on Cancer, No. 25, Lyon, 1979, pp. 191-202.

35. International Agency for Research on Cancer. Some Non-Nutritive Sweetening Agents (IARC Monographs on Evaluation of the Carcinogenic Risk of Chemicals to Humans, Vol. 22), IARC, Lyon, 1980, pp. 111-185.

36. Pitkin, R. M., Andersen, D. W., Reynolds, W. A., and Filer, L. J., Jr. Saccharin metabolism in Macaca mulatta. Proc. Soc. Exptl. Biol. Med. 137: 803-806 (1971).

37. Pitkin, R. M., Reynolds, W. A., Filer, L. J., Jr., and Kling, T. G. Placental transmission and fetal distribution of saccharin. Am. J. Obstet. Gynecol. 111: 280-286 (1971).
69. Kennedy, G., Fancher, O. E. and Calandra, J. C. Metabolic fate of saccharin in the albino rat. Food Cosmet. Toxicol. 10: 143-149 (1972).
70. Mathews, S. A., and McGuigan, H. The influence of saccharin on the digestive enzymes. J. Am. Med. Assoc. 45: 544-547 (1905).
71. Lethco, E. J., and Wallace, W. C. The metabolism of saccharin in animals. Toxicology 3: 287-300 (1975).
72. Ball, L. M., Renwick, A. G., and Williams, R. T. The fate of [14C] saccharin in man, rat and rabbit and of 2-sulphamoyl [14C] benzoic acid in the rat. Xenobiotica 7: 189-203 (1977).
73. Byard, J. L., and Golberg, L. The metabolism of saccharin in laboratory animals. Food Cosmet. Toxicol. 11: 391-402 (1973).
74. Byard, J. L., McChesney, E. W., Golberg, L., and Coulston, F. Excretion and metabolism of saccharin in man. II. Studies with 14C-labelled and unlabelled saccharin. Food Cosmet. Toxicol. 12: 175-184 (1974).
75. Minegishi, K.-I., Asahina, M., and Yamaha, T. The metabolism of saccharin and the related compounds in rats and guinea pigs. Chem. Pharm. Bull. 20: 1351-1356 (1972).
76. Sweatman, T. W., and Renwick, A. G. Saccharin metabolism and tumorigenicity. Science 205: 1019-1020 (1979).
77. Lutz, W. K., and Schlatter, C. H. Saccharin does not bind to DNA of liver or bladder in the rat. Chem. Biol. Interact. 19: 253-257 (1977).
78. Luini, W., Mantovani, A., and Garattini, S. Effects of saccharin on primary humoral antibody production in rats. Toxicol. Letters 8: 1-6 (1981).
79. Sweatman, T. W., and Renwick, A. G. The tissue distribution and pharmacokinetics of saccharin in the rat. Toxicol. Appl. Pharmacol. 55: 18-31 (1980).
80. Miller, J. A., and Miller, E. C. Carcinogens occurring naturally in foods. Fed. Proc. 35: 1316-1321 (1976).