SACCHARIN AND ITS SALTS

These substances were considered by previous working groups, in 1979 (IARC, 1980) and 1987 (IARC, 1987). Since that time, new data have become available, and these have been incorporated into the monograph and taken into consideration in the present evaluation.

1. Exposure Data

1.1 Chemical and physical data

1.1.1 *Nomenclature*

In the literature characterizing exposure to saccharin and its salts, the term 'saccharin' is sometimes used in a generic sense to encompass both saccharin and its salts. It is therefore not always possible to identify clearly the form of saccharin being described.

Saccharin

Chem. Abstr. Serv. Reg. No.: 81-07-2
Deleted CAS Reg. Nos: 474-91-9; 61255-27-4; 126987-83-5
Chem. Abstr. Name: 1,2-Benzisothiazol-3(2*H*)-one, 1,1-dioxide
IUPAC Systematic Name: 1,2-Benzisothiazolin-3-one, 1,1-dioxide
Synonyms: Acid saccharin; anhydro-*ortho*-sulfaminebenzoic acid; 3-benzisothiazolinone 1,1-dioxide; benzoic sulfimide; benzoic sulphinide; benzosulfimide; benzosulfinide; *ortho*-benzoic acid sulfimide; *ortho*-benzoic sulfimide; *ortho*-benzosulfimide; *ortho*-benzoyl sulfimide; 1,2-dihydro-2-ketobenzisosulfonazole; 2,3-dihydro-3-oxobenzisosulfonazole; 1,1-dioxo-1,2-benzisothiazol-3(2*H*)-one; 3-hydroxybenzisothiazole-*S*,*S*-dioxide; saccharimide; saccharin acid; saccharin insoluble; saccharine; saccharinol; saccharinose; saccharol; *ortho*-sulfobenzimide; *ortho*-sulfobenzoic acid imide

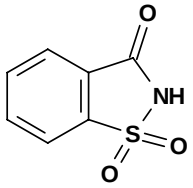
Sodium saccharin

Chem. Abstr. Serv. Reg. No.: 128-44-9
Deleted CAS Reg. No.: 38279-26-4
Chem. Abstr. Name: 1,2-Benzisothiazol-3(2*H*)-one, 1,1-dioxide, sodium salt
IUPAC Systematic Name: 1,2-Benzisothiazolin-3-one, 1,1-dioxide, sodium salt
Synonyms: *ortho*-Benzoylsulfimide sodium salt; saccharin sodium; saccharin sodium salt; saccharin soluble; sodium *ortho*-benzosulfimide; sodium saccharide; sodium saccharinate; sodium saccharine; soluble saccharin

Calcium saccharin

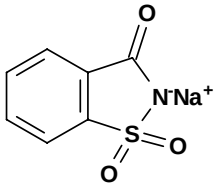
Chem. Abstr. Serv. Reg. No.: 6485-34-3
Deleted CAS Reg. No.: 17105-05-4
Chem. Abstr. Name: 1,2-Benzisothiazol-3(2*H*)-one, 1,1-dioxide, calcium salt
IUPAC Systematic Name: 1,2-Benzisothiazolin-3-one, 1,1-dioxide, calcium salt
Synonyms: Calcium *ortho*-benzosulfimide; calcium saccharinate

1.1.2 *Structural and molecular formulae and relative molecular mass*



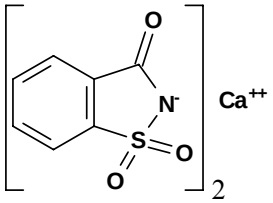
Saccharin

$C_7H_5NO_3S$ Relative molecular mass: 183.19



Sodium saccharin

$C_7H_4NO_3S.Na$ Relative molecular mass: 205.18



Calcium saccharin

$(C_7H_4NO_3S)_2.Ca$ Relative molecular mass: 404.46

1.1.3 *Chemical and physical properties of the pure substance*

(*a*) *Description*: Monoclinic crystals (Budavari, 1996); white crystalline powder [all forms] (Mitchell & Pearson, 1991); exceedingly sweet taste (300 times that of sucrose) [acid] (Bizzari *et al.*, 1996)

(*b*) *Boiling-point*: Acid sublimes (Lide, 1997)

(*c*) *Melting-point*: decomposes at 228°C [acid] (Lide, 1997); > 300°C [sodium and calcium salts] (Mitchell & Pearson, 1991)

(*d*) *Density*: d_4^{25} 0.828 [acid] (Lide, 1997)

(*e*) *Spectroscopy data*: Infrared (prism [322], grating [110]), ultraviolet [15734] and nuclear magnetic resonance (proton [6667], C-13 [4010]) spectral data have been reported [acid] (Sadtler Research Laboratories, 1980).

(*f*) *Solubility*: Acid slightly soluble in water (2 g/L at 20°C) and diethyl ether; soluble in ethanol and acetone; freely soluble in solutions of alkali carbonates (Mitchell & Pearson, 1991; Budavari, 1996; Lide, 1997). Sodium salt soluble in water (1 kg/L at 20°C). Calcium salt soluble in water (370 g/L at 20°C) (Mitchell & Pearson, 1991).

(*g*) *Acid ionization constant*: pK_a, 1.3 (Mitchell & Pearson, 1991)

(*h*) *Stability*: Saccharin solutions buffered at pHs ranging from 3.3 to 8.0 were essentially unchanged after heating for 1 h at 150°C (Mitchell & Pearson, 1991).

(*i*) *Conversion factor*: Saccharin: $mg/m^3 = 7.49 \times ppm$

1.1.4 *Technical products and impurities*

Saccharin is available commercially in three forms: the acid and the sodium (typically the dihydrate [6155-57-3]) and calcium (typically the 3.5 hydrate [6381-91-5]) salts (von Rymon Lipinski, 1995). All are manufactured to meet Food Chemicals Codex specifications, which include: heavy metals (as Pb), not more than 10 ppm (mg/kg); loss on drying, not more than 1%; residue on ignition, not more than 0.2%; selenium, not more than 0.003% [30 mg/kg]; and toluenesulfonamides, not more than 0.0025% [25 mg/kg] (National Academy of Sciences, 1996). Several additional salts of saccharin have been reported, including silver, ammonium, cupric, lithium, magnesium, zinc and potassium salts; although all of these are intensely sweet, none is available commercially. X-ray crystallography has shown that the acid form of saccharin exists as dimers, formed by hydrogen bonding between the imide hydrogen and the keto oxygen (Mitchell & Pearson, 1991).

More than 30 impurities have been reported to occur in saccharin or sodium saccharin produced by either the Remsen or the Maumee process (National Research Council/ National Academy of Sciences, 1978; Riggin *et al.*, 1978). These include in decreasing concentrations: *ortho*- and *para*-toluenesulfonamide, 1,2-benzisothiazol-1,1-dioxide, 1,2-benzisothiazoline-1,1-dioxide, 3-aminobenzisothiazol-1,1-dioxide, 5-chlorosaccharin, 6-chlorosaccharin, methyl saccharin, diphenyl sulfone, ditolylsulfone (various isomers), sulfamoylbenzoic acid, *ortho*-chlorobenzoic acid, *ortho*-sulfobenzoic acid and its ammonium salt, *n*-tetracosane, bis(4-carboxyphenyl) sulfone, toluene-2,4-disulfonamide, saccharin-6-sulfonamide, *N*-methyl-*ortho*-toluenesulfonamide, 4,4′-dibenzoylsulfone, 2- or 3-carboxythiaxanthone-5-dioxide, *ortho*-sulfobenzamide, methyl-*ortho*-sulfamoylbenzoate, methyl-*N*-methylsulfamoyl benzoate and saccharin-*ortho*-toluenesulfoxylimide.

Trade names for saccharin include [Azucaretas], Dulcibona, Garantose, Glucid, Gluside, Hollandia, Maca, Necta Sweet, Sakarin, Saxin, Slim & Sweet, Sucredulcor, [Sucrettes], Sucrosa, Suita, Sukrettine, Suktar-Maro, Sweeta, Sweetex and Syncal. Trade names for sodium saccharin include Cristallose, Cristalosetas, Crystallose, Dagutan,

[Edulcorant Pege], [Gaosucryl], Hermesetas, Kristallose, [Luetta], [Oda], Ril-Sweet, Saccharin Sodium Oral Solution USP 23, Saccharin Sodium Tablets USP 23, [Sanix], Saxin, [Sucromat], Sugarina, Suita Presta, [Sun-Suc], Sweeta, Sykose, Willosetten and Zero. [Names in brackets are for formerly manufactured products] (American Chemical Society, 1998; Reynolds, 1998; Swiss Pharmaceutical Society, 1998).

1.1.5 *Analysis*

Several international pharmacopoeias specify colorimetry and infrared absorption spectrophotometry as the methods for identification, and titration with sodium hydroxide or perchloric acid as methods for assaying the purity of saccharin, sodium saccharin and calcium saccharin. Sodium saccharin in tablets is assayed by ultraviolet spectrophotometry, and sodium saccharin in oral solutions is assayed by liquid chromatography with ultraviolet detection (British Pharmacopoeial Commission, 1993; Council of Europe, 1994; United States Pharmacopeial Convention, 1994; British Pharmacopoeial Commission, 1995; Society of Japanese Pharmacopoeia, 1996; Council of Europe, 1997).

The Food Chemicals Codex specifies titration with sodium hydroxide as the method for assaying the purity of saccharin and its sodium and calcium salts (National Academy of Sciences, 1996).

Gravimetric [method 973.29] and differential pulse polarographic [method 980.18] methods have been described for the determination of saccharin in food, including fruit juices and syrups, alcoholic liquids and solid or semisolid preparations; a colorimetric method [method 934.04] is described for the determination of saccharin in non-alcoholic beverages (Association of Official Analytical Chemists International, 1995). Although several analytical methods for the quantitative determination of saccharin and sodium saccharin in foods and other products are available, high-performance liquid chromatography, which allows simultaneous determination of saccharin, sodium saccharin and other sweeteners, is often preferred (von Rymon Lipinski, 1995).

1.2 Production and use

1.2.1 *Production*

Saccharin was discovered by the chemists Ira Remsen and Constantine Fahlberg in 1878. In 1900, the annual production of saccharin in Germany was reported to be 190 tonnes. In 1902, partly at the insistence of beet sugar producers, saccharin production in Germany was brought under strict control, and saccharin was made available only through pharmacies. Saccharin use increased during the First World War and immediately thereafter as a result of sugar rationing, particularly in Europe. By 1917, saccharin was a common tabletop sweetener in America and Europe; it was introduced to the Far East in 1923. The consumption of saccharin continued between the Wars, with an increase in the number of products in which it was used. The shortage of sugar during the Second World War again produced a significant increase in saccharin usage. In the early 1950s, calcium saccharin was introduced as an alternative soluble form (Mitchell & Pearson, 1991).

A number of companies around the world manufacture saccharin. Most manufacturers use the basic synthetic route described by Remsen and Fahlberg in which toluene is treated with chlorosulfonic acid to produce *ortho*- and *para*-toluenesulfonyl chloride. Subsequent treatment with ammonia forms the corresponding toluenesulfonamides. *ortho*-Toluenesulfonamide is separated from the *para*-isomer (this separation is alternatively performed on the sulfonyl chlorides), and *ortho*-toluenesulfonamide is then oxidized to *ortho*-sulfamoylbenzoic acid, which on heating is cyclized to saccharin (Mitchell & Pearson, 1991). *ortho*-Toluenesulfonamide can occur as a contaminant in saccharin produced by this process, but not in that produced by the Maumee process, described below (Arnold *et al*., 1980; Cohen, 1999).

The only producer in the United States currently uses the Maumee process, in which saccharin is produced from purified methyl anthranilate, a substance occurring naturally in grapes. In this process, methyl anthranilate is first diazotized to form 2-carbomethoxybenzenediazonium chloride. Sulfonation followed by oxidation yields 2-carbomethoxybenzenesulfonyl chloride. Amidation of this sulfonyl chloride, followed by acidification, forms insoluble acid saccharin. Subsequent addition of sodium hydroxide or calcium hydroxide produces the soluble sodium or calcium salt (Mitchell & Pearson, 1991).

China is the world's largest producer of saccharin, accounting for 30–40% of world production, with an annual production of approximately 18 000 tonnes in recent years; its exports amounted to approximately 8000 tonnes. In 1995, the United States produced approximately 3400 tonnes of saccharin and its salts, and Japan produced approximately 1900 tonnes. In the past, several western European companies produced sodium saccharin; however, by 1995, western European production had nearly ceased due to increasing imports of lower-priced saccharin from Asia (Bizzari *et al*., 1996).

Information available in 1995 indicated that saccharin was produced in 20 countries, calcium saccharin was produced in five countries, and sodium saccharin was produced in 22 countries (Chemical Information Services, 1995).

1.2.2 *Use*

Over the last century, saccharin and its salts have been used in a variety of beverages, foods, cosmetics and pharmaceuticals. Its primary function is to provide sweetness without adding calories, and it is used in the following foods and beverages: soft drinks, fruit juices, other beverages and other beverage bases or mixes; table-top sweeteners in tablet, powder or liquid form; processed fruits, chewing-gum and confections; gelatin desserts, jams and toppings; and sauces and dressings. Lesser amounts of saccharin are used in a variety of non-food applications, as a nickel electroplating brightener, chemical intermediate, animal feed sweetener and anaerobic adhesive accelerator (Mitchell & Pearson, 1991).

Worldwide consumption of saccharin and its salts in 1995 was approximately 28 000 tonnes. The consumption pattern of saccharin and its salts in the United States in 1995 (4500 tonnes) was: beverages and table-top sweeteners, 40%; personal-care products (primarily toothpaste and mouthwash), 30%; industrial, 18%; and pharmaceuticals, food, animal feed and tobacco, 12%. In Canada, approximately 142 tonnes of saccharin and its

salts were consumed in 1995 (Bizzari *et al.*, 1996). Western European consumption of saccharin and its salts in 1995 was approximately 4100 tonnes as high-intensity sweeteners and 300–500 tonnes for industrial applications, mainly in electroplating and feed additives.

The largest and only growing application for saccharin in Japan (2510 tonnes in 1995) is as an intermediate in production of the rice fungicide, probenazole. Consumption as a feed additive has been at 140–150 tonnes annually. Japanese consumption of saccharin as a high-intensity sweetener has been limited and has decreased in recent years. Consumption for all uses in 1995 was 2800 tonnes.

1.3 Occurrence

1.3.1 *Natural occurrence*

Saccharin and its salts are not known to occur naturally.

1.3.2 *Occupational exposure*

According to the 1981–83 National Occupational Exposure Survey (National Institute for Occupational Safety and Health, 1998), approximately 225 000, 68 000 and 1000 workers in the United States were potentially exposed to saccharin, sodium saccharin and calcium saccharin, respectively. Occupational exposure to saccharin and its salts may occur during its production and during its use as an intensive sweetener in foods, beverages and pharmaceuticals.

1.3.3 *Dietary intake*

An acceptable daily intake (ADI) of 5 mg/kg bw for saccharin (including its sodium, calcium and potassium salts) was established in 1993 by the WHO/FAO Joint Expert Committee on Food Additives (WHO, 1993a) and in 1995 by the Scientific Committee for Food of the European Union (International Sweeteners Association, 1998). Before these dates, the ADI was 2.5 mg/kg bw (see also section 1.4).

The probable daily intakes of saccharin and its salts in the United States in the early 1980s were estimated from data on food intake derived from a survey in which respondents were asked to record each food consumed at each eating occasion over 14 consecutive days. The results showed that saccharin-sweetened carbonated and non-carbonated soft drinks accounted for a high proportion of the saccharin intake, and table-top and kitchen uses of saccharin as a sugar substitute were also important sources of saccharin in the diet. Other foods did not represent significant sources. The highest average daily intakes of saccharin per kilogram body weight (for saccharin consumers only) were those of men and women aged 18–54 (0.39 mg/kg bw), women in this age group (0.46 mg/kg bw), children aged 2–5 years (0.44 mg/kg bw) and children under two years of age (0.40 mg/kg bw). The average daily intakes for other groups were: 0.36 mg/kg bw for boys and girls aged 6–12; 0.26 mg/kg bw for boys and girls aged 13–17 (0.24 mg/kg bw for boys in this age group) and 0.38 mg/kg bw for men and women aged ≥ 55 years (Calorie Control Council, 1996a).

A survey of intense sweetener consumption in Australia in 1994 consisted of a seven-day survey of high consumers of the main sources of sweeteners, carbonated drinks,

cordials and table-top, with allowance for body weight. Mean intake (expressed as % ADI of 5 mg/kg bw) of saccharin and its salts was 9% for all consumers aged 12–39 (men, 11%; women, 8%); 16% for all consumers aged 12–17; 3% for all consumers aged 18–24; and 9% for consumers aged 25–39 (National Food Authority, 1995).

In a study of the potential intake of intense sweeteners in Brazil in 1990–91, it was found that 72% of the studied population consumed saccharin. The main reasons given for use of intense sweeteners were weight control diet (36%), diabetes (35%) and obesity (23%). Table-top sweeteners were the major source of sweeteners, followed by soft drinks. The median daily intake of saccharin was approximately 16% of the ADI of 5 mg/kg bw (Toledo & Ioshi, 1995).

The use of table-top sweeteners and diet soft drinks and the intake of saccharin were assessed on the basis of the second Dutch National Food Consumption Survey, conducted in 1992. The median daily intake of saccharin by users of intense sweeteners, evaluated from two-day records and a food frequency questionnaire, was 0.2 mg/kg bw. Less than 0.5% of the total population had an intake above the ADI of 2.5 mg/kg bw (Hulshof *et al*., 1995).

The dietary intake of intense sweeteners was evaluated in Germany in 1988-89. In the first part of the study, sweetener intake was evaluated in a representative sample of the population from complete 24-h records of the amounts and types of all foods and drinks consumed by 2291 individuals; 36% of the participants had ingested one or more sweeteners on the examination day. The mean intake of saccharin by users of intense sweeteners was 0.25 mg/kg bw per day; at the 90th percentile of intake, the ingestion of saccharin was about 2.5 times higher. Table-top sweeteners and beverages were the most important sources of sweeteners, contributing more than 80% to the total intake. In the second part of the study, the sweetener intake of the 41 subjects in the one-day study who had ingested any of the sweeteners at levels in excess of 75% of the ADI was further evaluated during a seven-day period. The mean intake of saccharin of this group was 0.42 mg/kg bw per day, which corresponded to 17% of the corresponding ADI value of 2.5 mg/kg bw in the European Union at that time (Bär & Biermann, 1992).

A survey of the intake of food additives in Finland in 1980 included an assessment of the intake of saccharin. The report gave few details of the study design or method and indicated only that the average per-capita daily intake (calculated from consumption figures for various foods and drinks) of saccharin was 5.9 mg/person per day. The ADI at that time was 2.5 mg/kg bw (Penttilä *et al*., 1988). These figures are considerably lower than those found in more recent studies (Renwick, 1995).

1.4 Regulations and guidelines

No national or international occupational exposure limits have been proposed or established for exposure to saccharin in workplace air, and no international guidelines for saccharin in drinking-water have been established (WHO, 1993b).

European Commission regulations and standards stipulate that: (1) saccharin, sodium saccharin or calcium saccharin is allowed for use in non-alcoholic drinks (maximum

useable dose, 80–100 mg/L), in desserts and similar products (100 mg/kg), in confectionery (80–1200 mg/kg) and in vitamins and dietary preparations (1200 mg/kg) (European Commission, 1994a); (2) saccharin, sodium saccharin or calcium saccharin may be added as an aromatic and appetizing substance to animal feedstuffs (European Commission, 1970, 1994b). The Scientific Committee for Food of the European Commission increased the ADI for saccharin from 2.5 mg/kg bw to 5 mg/kg bw in June 1995 (International Sweeteners Association, 1998); however, it has been recommended that intake of saccharin by children should be minimized, and use of saccharin in infant foods should be prohibited (European Commission, 1978; United Nations Environment Programme, 1998).

The Joint FAO/WHO Expert Committee on Food Additives in 1993 re-allocated a group ADI of 5 mg/kg bw for saccharin, sodium saccharin, potassium saccharin and calcium saccharin, singly or in combination (WHO, 1993a). In 1977, it had changed the unconditional ADI for humans of 5 mg/kg bw established for saccharin and its potassium and calcium salts to a temporary ADI of 2.5 mg/kg bw (WHO, 1978).

The regulatory status of saccharin in foods, beverages and table-top and pharmaceutical preparations in a number of countries is presented in Table 1. These data were collected over a period of years and do not necessarily represent the current situation (Calorie Control Council, 1996b).

Regulations and standards in the United Kingdom stipulate that saccharin, sodium saccharin or calcium saccharin is a permitted sweetener for food intended for human consumption. The sale, importation, supply and advertisement of any sweetener, of any food containing a sweetener or of the use of any sweetener as an ingredient in the preparation of foods other than as a permitted sweetener is prohibited. The sale of food for babies and young children which contains an added sweetener is restricted. The use of all permitted sweeteners in jam and similar products intended for diabetic patients and in soft drinks is permitted (Her Majesty's Stationery Office, 1983; United Nations Environment Programme, 1998).

In India, sodium saccharin is permitted in carbonated water up to 100 ppm [mg/L] (as saccharin) and may be sold as a table-top sweetener (Anon., 1981, 1988; United Nations Environment Programme, 1998).

Regulations and standards in Kenya stipulate that saccharin or sodium saccharin is a food additive permitted as a non-nutritive sweetening agent. The food products in or upon which it is permitted and maximum levels of use are listed (Anon., 1978; United Nations Environment Programme, 1998).

In the Russian Federation, regulations and standards stipulate a preliminary safety level for saccharin in ambient air of 0.02 mg/m^3 (Anon., 1983; United Nations Environment Programme, 1998).

In the United States, saccharin is currently approved for use under an interim food additive regulation permitting use for special dietary purposes and in special dietary foods. The food additives saccharin, ammonium saccharin, calcium saccharin and sodium saccharin may be safely used as sweetening agents in food in accordance with the following conditions: if the substitution for nutritive sweeteners is for a valid, special dietary purpose and

Table 1. Regulatory status of saccharin

Country or region	Food[a]	Beverage	Table-top	Pharmaceutical
Afghanistan		+		
Algeria	–	–	–	–
Antigua and Barbuda		+		
Argentina	+	+	+	+
Australia	+	+	+	+
Austria	+	+	+	+
Bahamas	+	+		
Barbados		+		
Belgium	+	+	+	
Bermuda		+		
Bolivia	–	–	+	–
Brazil	+	+	+	
Bulgaria	+	+		
Burundi		+		
Canada	–	–	+	+
Caribbean	+	+	+	
Chile		+	+	+
China	+	+		
Colombia	+	+	+	
Costa Rica	+	+	+	
Cyprus	+	+	+	+
Czech Republic	+	–	+	
Denmark	+	+	+	+
Dominica	+	+	+	
Ecuador	+	+	+	+
Egypt		+		
El Salvador	+	+	+	
Ethiopia		+	+	
Fiji	+	–	–	–
Finland	+	+	+	+
France	+	+	+	+
Germany	+	+	+	+
Greece	+	+	+	
Guam	+	+		
Guatemala	+	+	+	
Guyana	+	+		
Haiti		+	+	
Honduras	+	+		
Hong Kong	+	+	+	
Hungary	+	+	+	
Iceland	+	+	+	–
India		+	+	+
Indonesia	+	+	+	
Iran	+	–	–	–

Table 1 (contd)

Country or region	Food[a]	Beverage	Table-top	Pharmaceutical
Ireland	+	+	+	+
Israel	+	+	+	+
Italy	+	+	+	+
Japan	+	+	+	+
Korea, Republic of	+	+	+	+
Kenya	+	+	+	
Kuwait	+	+	+	
Lebanon	+	+	+	
Luxembourg	+	+	+	–
Malaysia	+	+	+	+
Malta	+	+	+	
Mexico	+	+	+	
Monserrat		+		
Morocco	+	+	+	–
Nassau		+		
Netherlands	+	+	+	+
New Zealand	+	+	+	+
Nicaragua	+	+	+	
Nigeria	+	+	+	
Norway	+	+	+	+
Oman	–	–	+	+
Pakistan	+	+	+	
Panama	+	+	+	
Papua New Guinea	+	+	+	
Paraguay	+	–	+	
Peru	+	+	+	–
Philippines	+	+	+	+
Poland	+		+	
Portugal	+	+	+	+
Puerto Rico	+	+		
Qatar			+	
Russian Federation	+	–	+	+
Rwanda		+		
Samoa	+	+		
Saudi Arabia	+	–	+	
Sierra Leone	+		+	
Singapore	+	+	+	+
Slovakia	+	–	+	
South Africa	+	+	+	
Spain	+	+	+	+
Sri Lanka		+	+	
Surinam	+	+	+	
Sweden	+	+	+	
Switzerland	+	+	+	

Table 1 (contd)

Country or region	Food[a]	Beverage	Table-top	Pharmaceutical
Taiwan	+	+	+	–
Thailand	+	+	+	
Trinidad	+	+		
Tunisia	–	–	–	–
Turkey	+	+	+	+
United Arab Emirates			+	
United Kingdom	+	+	+	+
United States	+	+	+	+
United States Virgin Islands	+	+		
Uruguay	+	+	+	
Venezuela	+	+	+	
Yugoslavia	+	+		
Zaire		+		
Zambia		+		

From Calorie Control Council (1996b); abbreviations: +, permitted; –, prohibited
[a] May not apply to all food categories

is in accord with current special dietary food regulations and policies or if the use or intended use is for an authorized technological purpose other than calorie reduction. The additives are authorized for use as sweetening agents only in special dietary foods, as follows: (1) in beverages, fruit juices and bases or mixes when prepared for consumption in accordance with directions, in amounts not to exceed 12 mg of the additive, calculated as saccharin, per fluid ounce; (2) as a sugar substitute for cooking or table use, in amounts not to exceed 20 mg of the additive, calculated as saccharin, for each expressed teaspoonful of sugar sweetening equivalence; and (3) in processed foods, in amounts not to exceed 30 mg of the additive, calculated as saccharin, per serving of designated size. The additives are authorized for use only for the following technological purposes: to reduce bulk and enhance flavours in chewable vitamin tablets, chewable mineral tablets or combinations thereof; to retain the flavour and physical properties of chewing-gum; and to enhance the flavour of flavour chips used in non-standardized bakery products (Food and Drug Administration, 1998).

2. Studies of Cancer in Humans

2.1 Observational study

The risk for cancer of the urinary bladder was studied among persons in Denmark born in 1941–45, at a time when the use of saccharin had increased by four- to fivefold as a result of a scarcity of sugar. The risk was compared with that of persons born one decade earlier. For people up to age 34, the relative risks were 1.0 (based on 22 observed

cases; 95% confidence interval [CI], 0.7–1.6) for men and 0.3 (3 observed cases; 95% CI, 0.1–1.0) for women (Jensen & Kamby, 1982).

2.2 Case–control studies

Case–control studies of the use of saccharin and other sources of sweeteners and cancer of the urinary bladder are summarized in Table 2. About half of the studies described below involved controls who were hospitalized patients. Use of hospital controls in studies of artificial sweeteners can lead to underestimates of cancer risk, as pointed out by Silverman *et al*. (1983), as hospital controls are more likely than population controls to have a condition that requires them to use artificial sweeteners.

A hospital-based Canadian study included 158 male and 74 female cases of urinary bladder cancer and as many age- and sex-matched controls, who were men with benign prostatic hypertrophy and women with stress incontinence. Patients and controls were interviewed by mail; the response rates were 69% for the patients and 57% for the controls. Despite age-matching, the average age of both female and male controls was two to three years lower than that of cases. There was no difference in the intake of artificial sweeteners between patients and controls when analysed by their smoking history. Use of table-top artificial sweeteners for more than one year was reported by 30 men and 13 women among the patients and 30 men and 28 women among controls. Matched analysis resulted in odds ratios of 1.0 for men and 0.4 ($p < 0.01$) for women (Morgan & Jain, 1974).

A study in 10 hospitals in urban areas of Massachusetts and Rhode Island, United States, addressed the consumption of coffee, tea, artificial sweeteners and other coffee additives by white women, on the basis of previous findings that had suggested an association of a lower incidence of urinary-tract cancer with coffee consumption, which was particularly strong in white women. Two-hundred-and-sixteen histologically verified cases newly diagnosed during 1965 and 1971 were retrospectively identified from clinical records, excluding those reported as dead. Three controls per case were drawn from the discharge registers of the same hospitals and matched to the cases by age, area of residence and hospital; those with urinary tract problems were excluded. Of the 216 eligible cases, 40 had died and 41 did not respond to the questionnaire; the corresponding figures among controls were 110 and 148. The analysis was based on 135 cases and 390 controls. Cases and controls were sent a questionnaire which included separate questions on use of cyclamates and saccharin in coffee and in tea. All of the relevant odds ratios were between 1.0 and 1.2, and their 95% confidence intervals included unity (Simon *et al*., 1975). [The Working Group noted the high proportion of non-participants.]

A hospital-based case–control study carried out during 1969–74 in 17 hospitals in six cities in the United States included 574 male and 138 female cases of urinary bladder cancer and equal numbers of controls matched for age, sex, race and hospital status (private, semiprivate, ward), excluding people with previous or current tobacco-related conditions. Cases and controls were selected from a larger pool assembled for studies on the effects of tobacco and alcohol and were interviewed in hospital. Diabetic patients

Table 2. Case–control studies of the use of saccharin and other sources of sweeteners and cancer of the urinary bladder

Reference	Study type	Sex	No. of cases	No. of controls	Type of sweetener	No. of exposed cases/ no. of exposed controls	Odds ratio	95% CI or *p* value	Additional estimates and comments
Morgan & Jain (1974)	H	M	158	158	SW	30/30	1.0		Not significant
		F	74	74		13/28	0.4	$p < 0.01$	
Simon *et al*. (1975)	H	F	135	390	SA		1.0	0.5–1.7	
Wynder & Goldsmith (1977)	H	M	132	124	SW	13/16	[0.8]	NR	Crude odds ratio
		F	31	29	SW	4/5	[0.8]	NR	
Howe *et al*. (1977, 1980); Howe & Burch (1981)	P	M	480	480	SW	73/47	1.6	1.1–2.4	Dose–response relationship
		F	152	152	SW	18/30	0.6	NR	Risk ratio for 2500 tablets/year
		M	466	469	SA	55/33	1.7	$p < 0.05$	≥ 3 years, 5.3 (10 cases, 2 controls)
Kessler & Clark (1978)	P	M	365	365	TA	85/83	0.9	0.6–1.4	Adjusted odds ratio = 2.6 in non-smoking men; adjusted for confounders
					DB	78/77	1.0	0.6–1.4	
		F	154	154	TA	48/48	0.9	0.5–1.6	
					DB	49/42	1.0	0.6–1.6	
Morrison & Buring (1980)	P	M	469	461	TA	101/113	0.8	0.5–1.1	Adjusted for confounders
					DB	144/155	0.7	0.6–1.1	Adjusted for confounders
					DB ≥ 5 years	44/55	0.7	[0.4–1.2]	
		F	197	165	TA	54/39	1.5	0.9–2.6	Adjusted for confounders
					DB	69/46	1.6	0.9–2.7	Adjusted for confounders
					DB ≥ 5 years	22/6	3.7		
		M NS	44	74	TA	18/31	1.1	NR	Adjusted for confounders
		F NS	39	84	TA	15/28	2.1	NR	Adjusted for confounders
		M NS	47	87	DB	21/44	0.9	NR	Adjusted for confounders
		F NS	43	83	DB	19/27	2.6	NR	

Table 2 (contd)

Reference	Study type	Sex	No. of cases	No. of controls	Type of sweetener	No. of exposed cases/ no. of exposed controls	Odds ratio	95% CI or *p* value	Additional estimates and comments
Wynder & Stellmann (1980)	H	M	302	302	TA	76/80	0.9	0.7–1.3	Current long-term smokers: odds ratio, 0.6 (95% CI, 0.3–1.1)
					DB	45/52	0.9	0.6–1.2	
		F	65	65	TA	14/19	0.6	0.3–1.4	Current long-term smokers: odds ratio, 1.0 (95% CI, 0.2–5.1)
					DB	10/16	0.6	0.3–1.3	
Hoover & Harge-Strasser (1980)	P	M	2258	4277	TA	592/1066	1.0	0.9–1.2	Odds ratio 1.5 for ≥ 3 servings TA and ≥ 2 DB daily or equivalent. All odds ratios adjusted for confounders
					DB	607/1204	1.0	0.8–1.1	
		F	742	1499	TA	236/474	1.0	0.8–1.3	
					DB	262/504	1.0	0.8–1.3	
		F LR	130	402	TA	82/210	1.2	NR	Dose– and duration–response relationships. Odds ratio = 2.7 for ≥ 2 servings for ≥ 10 years (16 cases, 18 controls)
					DB	71/219	1.1	NR	Dose– and duration–response relationships. Odds ratio, 3.0 for ≥ 2 servings for ≥ 10 years (6 cases, 7 controls). Non-smoking men reported to show significantly decreased odds ratios with increasing daily doses.
		M HR	166	226	TA	62/59	[1.7]	$p = 0.01$	Dose–response relationship
						69/81	[1.4]	$p = 0.01$	Dose–response relationship Among heavy female smokers, heavier users of TA and DB reported to give higher odds ratios than no use of AS

Table 2 (contd)

Reference	Study type	Sex	No. of cases	No. of controls	Type of sweetener	No. of exposed cases/ no. of exposed controls	Odds ratio	95% CI or *p* value	Additional estimates and comments
Cartwright (1981)	H	M NS	216	362	SA	33/27	2.2	$p < 0.05$	All adjusted for confounders
		M S	415	427		71/81	0.9		
		F NS	112	181		16/19	1.6		
		F S	99	90		17/14	1.2		
Najem (1982)	H	M/F	65/10	123/19	SA	12/19	1.3	0.6–2.8	
Morrison *et al*. (1982)	P	M	382	470	SW	140/183	0.9	0.7–1.2	No dose–response relationship. Odds ratio for ≥ 10 TA per day, 2.3 for women (9 cases, 8 controls) and 0.6 for men (10 cases, 19 controls). All adjusted for confounders
		F	142	220		50/87	0.9	0.6–1.4	
Manchester		M NS	30	68		11/22	1.6	NR	
		F NS	63	102		24/44	1.2	NR	
Nagoya		M	223	432	SW	100/238	0.7	0.5–0.9	
		F	66	144		26/83	0.5	0.3–0.8	
		M NS	24	76		9/41	0.5	NR	
		F NS	44	129		16/76	0.4	NR	
Møller Jensen *et al*. (1983)	P	M	284	592	SW	54/152	0.7	0.5–1.0	No dose–response relationship; adjusted for confounders; analyses broken down by TA and DB did not alter estimates
		F	98	195		26/50	1.1	0.6–1.9	
		M NS	9	68		4/5	1.9	0.5–7.8	
		M S	267	506		51/127	[0.7]	[0.5–1.0]	
Mommsen *et al*. (1983)	H	F	47	94	SA	6/2	6.7	1.5–30	Adjusted for confounders
Piper *et al*. (1986)	P	F	173	173	SW	77/74	1.1	0.7–1.7	Adjusted for confounders

Table 2 (contd)

Reference	Study type	Sex	No. of cases	No. of controls	Type of sweetener	No. of exposed cases/ no. of exposed controls	Odds ratio	95% CI or *p* value	Additional estimates and comments
Risch *et al*. (1988)	P	M	835	792	TA		1.0	0.7–1.2	No difference between smokers and non-smokers; adjusted for confounders
		F					1.1	0.7–1.8	
		M			SA		1.0	0.9–1.2	
		F					1.0	0.8–1.2	No dose–response relationship for either sex; adjusted for confounders
		M			DB		1.0	0.8–1.3	
		F					1.8	0.8–3.9	No dose-response relationship TA: ever versus never; SA and DB: total lifetime intake as continuous variable
Akdas *et al*. (1990)	H	M	168	168	SW	19/8	[2.5]	$p < 0.05$	
		F	26	26					

H, hospital-based; P, population-based; M, male; F, female; NR, not reported; NS, nonsmoker; S, smoker; SW, sweeteners (unspecified); TA, tablets; DB, dietetic beverage; SA, saccharin; LR, low risk; HR, high risk

represented a slightly higher proportion of cases than controls. Data on use of artificial sweeteners were available only for 132 male cases, 124 male controls, 31 female cases and 29 female controls, as this question was added to the questionnaire only in 1973. Table-top artificial sweeteners were never used by 90% of male cases, 87% of male controls, 87% of female cases and 83% of female controls. The relative risks for three strata of duration of use were all lower than 1.0. The authors noted that the results do not refer to cyclamates, which had entered the United States market too recently to allow any carcinogenic effect to be recognizable (Wynder & Goldsmith, 1977).

A population-based study was carried out in three provinces of Canada (British Columbia, Newfoundland and Nova Scotia) of cases of urinary bladder cancer newly diagnosed during 1974–76 and identified through cancer registries. Of 821 eligible patients, 632 were interviewed (among those excluded, 43% were not interviewed because they were too ill or dead), i.e. 401 in British Columbia, 101 in Newfoundland and 230 in Nova Scotia. Each interviewed patient was matched by sex and age to a randomly selected control living in the same neighbourhood. The percentages of participating controls were 80% of those originally identified in British Columbia, 96% of those in Newfoundland and 100% of those in Nova Scotia. Participants answered a detailed questionnaire on use of a variety of sources of artificial sweeteners. Of the controls, 47/480 men and 30/152 women reported any use of artificial sweeteners. Through matched-pair analysis, relative risks associated with any use of any type of artificial sweeteners of 1.6 (lower limit of 95% CI, 1.1) and 0.6 (not significant) were estimated for men and women, respectively. In men, the relative risks adjusted for level of education, occupational exposures, history of urological diseases, smoking and use of instant coffee were between 1.5 and 1.8. The relative risks for bladder cancer among diabetic patients were 1.0 in men and 0.8 in women. Among diabetic men, the relative risk associated with use of artificial sweeteners was 1.7, based on 20 exposed cases. Of the male users, 82% reported use of brands of table-top artificial sweeteners known to have always contained saccharin only and 94% reported use of one brand only. Thus, for saccharin consumption, men were divided into three categories for both consumption (never, < 2500 and > 2500 tablets per year) and duration (never, ≤ 3 and > 3 years). Relative to people who had never used the preparations, the risks for the two increasing categories of consumption were 1.5 (based on 42 exposed cases; lower 95% CI, 1.0) and 2.1 (16 exposed cases; lower 95% CI, 0.9), respectively. The corresponding findings for duration were 1.4 (based on 30 exposed cases; lower 95% CI, 0.9) and 2.0 (28 exposed cases; lower 95% CI, 1.2). In the highest category of consumption (> 2500 tablets per year for more than three years), the risk relative to that of non-users was 5.3, based on 10 exposed cases and two exposed controls (Howe *et al*., 1977). [The Working Group noted that it is not clearly stated whether the relative risks for consumption and duration were adjusted for potential confounding.] The risks associated with consumption of table-top sweeteners were also estimated in a multivariate analysis, with similar results (Howe *et al*., 1980).

In a study in Baltimore (United States), 519 of 634 surviving patients in whom urinary bladder cancer had been diagnosed in 1972–75 agreed to participate in a study.

Controls, individually matched to each case by sex, age, race and marital status, were randomly chosen from lists in the same hospitals and periods as the patients, but those with diagnoses of cancer or urological conditions were excluded; 75% of the controls were selected at the first sampling. Both male and female patients had a higher level of education than their controls. The study included 365 male and 154 female cases and the same numbers of controls. All were interviewed about smoking habits, occupation and consumption of artificial sweeteners, including table-top sweeteners, dietetic beverages, dietetic foods and total intake in all forms. Artificial sweeteners in any form had been used by 129 male patients and 126 controls and by 77 female patients and 79 controls. The average duration of consumption of various forms of artificial sweeteners was between 3.4 and 8.3 years for male patients, between 5.8 and 7.7 years for male controls, between 2.9 and 7.8 years for female cases and between 5.4 and 8.3 for female controls, depending on the specific type. Any consumption of dietetic beverages was reported by 78 male patients and 77 controls and 49 female patients and 42 controls; and any consumption of dietetic foods by 54 male patients, 39 male controls, 34 female patients and 41 female controls. The odds ratios, adjusted for a series of potential confounders, showed no consistent trend by level of exposure. The relative risks for use of saccharin ranged between 0.7 and 1.1. In matched-pair analyses for 'more than occasional' use of non-nutritive sweeteners (powders, tablets, drops and any table-top sweetener), six types of dietetic beverage plus any dietetic beverage, 10 types of dietetic food plus any dietetic food, the only 95% confidence interval of the estimated odds ratios that excluded unity was that for consumption of dietetic ice cream by women (odds ratio, 3.5; 95% CI, 1.1–11). Stratification by smoking status showed non-significant relative risks associated with consumption of artificial sweeteners of 0.84 for smokers and 1.4 for nonsmokers. In nonsmoking men, the odds ratio was 1.7, which increased to 2.6 (95% CI, 1.2–5.7) after adjustment for a number of confounding factors (Kessler, 1976; Kessler & Clark, 1978). [The Working Group noted the potential bias of including only surviving patients and the possible selection bias represented by the higher level of education of patients than controls.]

The database of a population-based study in the Boston (United States) metropolitan area included 741 histologically confirmed incident cases of benign or malignant cancer of the lower urinary tract diagnosed over 16 months (identified in 65/66 hospitals of the area). A random sample of 677 residents of similar age and sex distribution in the same area during the same period were used as controls. The participation rates were 81% for cases and 80% for controls, leaving 597 cases and 544 controls for the analysis; 98 cases and 15 controls did not participate because they were too ill, dead or their physician declined permission for an interview. A questionnaire and a personal interview addressed a variety of lifetime exposures. In men, any use of dietetic beverages and sugar substitutes was associated with odds ratios of 0.8 (based on 144 exposed cases; 95% CI, 0.6–1.1) and 0.8 (101 exposed cases; 95% CI, 0.5–1.1). The corresponding odds ratios for women were 1.6 (69 exposed cases; 95% CI, 0.9–2.7) and 1.5 (54 exposed cases; 95% CI, 0.9–2.6). Multivariate analyses of the data for men, with adjustment for age,

education, marital status, religion and tobacco consumption, led to a summary estimated relative risk of 0.7 for use of artificially sweetened beverages and 0.8 for use of sugar substitutes. [The Working Group noted that no corresponding multivariate analysis was reported for women.] The frequency or duration of use of either dietetic beverages (four strata), sugar substitutes or dietetic foods was analysed separately for the two sexes. The odds ratios were not consistently associated with increasing use, except for women reporting use of dietetic beverages for longer than five years, with an odds ratio of 3.7 [95% CI, 1.3–10) based on 22 exposed cases. In analyses by smoking status, the odds ratios for women who never smoked were 2.6 for consumption of dietetic drinks (based on 19 exposed cases) and 2.1 for consumption of non-nutritive sweeteners (15 exposed cases). Among men, the odds ratios were no higher among those who had never smoked than in the other groups (Morrison & Buring, 1980).

In a hospital-based study similar to that of Wynder and Goldsmith (1977), 302 male and 65 female bladder cancer patients and equal numbers of controls were interviewed during 1977–79 about lifetime use of table-top artificial sweeteners (mainly saccharin) and of dietetic beverages. Men who had never consumed artificial sweeteners represented 75% of cases and 74% of controls, and the corresponding proportions of women were 79% and 71%; 85% of male patients, 83% of male controls, 85% of female patients and 75% of female controls reported never having drunk dietetic beverages. In a matched analysis, the odds ratios for any use of either artificial sweeteners or dietetic beverages were all lower than 1.0 and all of the 95% confidence intervals included unity. Analyses limited to current long-term (≥ 10 years) cigarette smokers gave relative risks for consumption of sweeteners or dietetic beverages of about 0.6 (95% CI, 0.3–1.1) in men and 1.0 (95% CI, 0.2–5.1) in women (Wynder & Stellman, 1980).

A large population-based case–control study on bladder cancer was specifically designed to address the hypothesis of a role of artificial sweeteners. Newly diagnosed, histologically confirmed cases were identified in 10 areas in the United States; cases of benign papilloma were excluded. Controls represented an age- and sex-matched random sample from the same areas. The participation rates were 87% of eligible cases and 86% of eligible controls, resulting in 2258 male patients, 4277 male controls, 742 female patients and 1499 female controls, who were interviewed at home about a variety of risk factors, including use of table-top sweeteners, dietetic beverages and dietetic foods. All of the odds ratios reported below, unless otherwise specified, were adjusted for age, race, cigarette smoking, coffee drinking and occupational exposure. Any use of any type of artificial sweetener was reported by 909 male patients, 1723 male controls, 384 female patients and 732 female controls, corresponding to odds ratios of 1.0 (95% CI, 0.1–1.1) for men and 1.1 (95% CI, 0.1–1.3) for women. Analyses of consumption were based on six strata of average daily use of table-top sweeteners and five strata of average daily consumption of dietetic drinks. The trend for average daily use of table-top sweeteners by women was statistically significant (p = 0.03, one-tailed), and the highest consumption (six or more daily uses of table-top sweeteners) corresponded to an odds ratio of 1.4, based on 16 exposed cases. In the logistic regression analysis (with adjustment

for sex, region, education and the other variables mentioned above) of combined consumption of dietetic drinks and table-top artificial sweeteners, an odds ratio of 1.5 (95% CI, 1.0–2.1) was estimated for heavy consumers (either at least three servings of table-top artificial sweeteners and at least two dietetic drinks daily or at least some dietetic drinks and six or more servings of table-top artificial sweeteners), with no difference by sex. Individuals considered to be at low and high risk for bladder cancer were analysed separately, as it was hypothesized that an effect of a weak carcinogen would be easier to detect in a population not exposed to bladder carcinogens, and analysis of a high-risk group would reveal any co-carcinogenic effect. The group considered to be at low risk comprised 283 female patients and 831 female controls who were white, did not smoke and were not exposed occupationally to bladder carcinogens. The group considered to be at high risk comprised 235 male patients and 307 male controls who were white and smoked > 40 cigarettes per day. In the low-risk stratum, 130 patients and 402 controls were unexposed, 82 patients and 210 controls used table-top artificial sweeteners, and 71 patients and 219 controls used dietetic drinks. The risk increased with level of intake: for consumption of table-top sweeteners, the odds ratios increased from 0.9 (based on 15 exposed cases) for less than one daily use to 1.8 (based on 22 exposed cases) for three or more daily uses (p for trend < 0.01); for duration of consumption among women reporting two or more daily uses, the odds ratios were 1.3 (based on 14 exposed cases), 1.8 (13 exposed cases) and 2.7 (16 exposed cases) for consumption lasting ≤ 5, 5–9 and ≥ 10 years, respectively (p for trend, < 0.01); for consumption of dietetic drinks, the odds ratios increased from 0.9 (based on 36 exposed cases) for less than one daily use to 1.6 (based on 3 exposed cases) for three or more daily uses (p for trend, not significant); for duration of consumption among women reporting two or more daily uses, the odds ratios were 0.5 (based on 1 exposed case), 1.4 (3 exposed cases) and 3.0 (6 exposed cases) for consumption lasting ≤ 5, 5–9 and ≥ 10 years, respectively (p for trend, < 0.05). In the high-risk stratum, 104 patients and 167 controls were unexposed, 62 patients and 59 controls used table-top artificial sweeteners and 69 patients and 81 controls used dietetic drinks. The odds ratios for the highest consumers were 1.9 (based on 7 exposed cases) for six or more daily uses of table-top artificial sweeteners and 2.6 (based on 6 exposed cases) for three or more daily servings of dietetic drinks (Hoover & Harge-Strasser, 1980). [The Working Group noted that estimates of risk by strata corresponding to duration of consumption were not included for the high-risk population.] An independent analysis gave similar risk estimates (Walker *et al*., 1982).

More recently, the same data were analysed by strata corresponding to tumour stage and histological grade in logistic regression models including the following variables: age, race, education, sex, cigarette smoking, exposures in the workplace, bladder stones, urinary infections, coffee consumption, family history of bladder cancer, use of artificial sweeteners and geographical region. Risks were estimated for persons consuming more than 1680 mg/day of artificial sweeteners in comparison with a group consuming less than that amount. A significantly increased risk was seen only for tumours of histological

grade III/IV (odds ratio, 2.2; 95%, CI 1.3–3.6; based on 23 exposed cases) (Sturgeon *et al*., 1994). [The Working Group noted that the cut-off for consumption corresponded to a very high level and the reason for its choice was not given.]

A large hospital-based case–control study carried out in the United Kingdom in the 1970s included questions on consumption of saccharin as a food additive; a preliminary analysis was reported. The study included 161 newly diagnosed cases in men and 58 in women and 470 prevalent cases in men and 152 in women. Controls were matched by age and sex, with two controls for each newly diagnosed case and one for each prevalent case. Smokers who had quit five years or more previously were considered to be nonsmokers; people who had regularly consumed saccharin for at least one year, five or more years before diagnosis or interview were considered to be saccharin consumers. Of the 631 male patients, 33 were nonsmokers and saccharin consumers, 183 were nonsmokers and did not consume saccharin (reference group), 71 were smokers and saccharin consumers, and 344 were smokers and did not consume saccharin. Among the female cases, the corresponding figures were 16, 96, 17 and 81. The odds ratios for nonsmokers who consumed saccharin were 2.2 (95% CI, 1.3–3.8) for men and 1.6 (95% CI, 0.8–3.2) for women. The other odds ratios ranged between 0.9 and 1.2, and their confidence intervals included unity (Cartwright *et al*., 1981). [The Working Group noted that use of prevalent cases might be associated with bias in the recall of previous exposures and selection bias in relation to survival.]

A hospital-based study among white persons in New Jersey (United States) included 75 cases (65 male) and 142 controls (123 male) matched by age, sex, place of birth, place of residence and hospital. Twelve cases and 19 controls had ever used saccharin (crude odds ratio, 1.3; 95% CI, 0.6–2.8). The average consumption was 3.6 tablets per day among patients versus 2.5 among controls; the average duration of use was 6.3–6.4 years in both groups (Najem *et al*., 1982).

In parallel studies in Manchester, United Kingdom, and Nagoya, Japan, use of artificial sweeteners was compared for patients with newly diagnosed cancer of the lower urinary tract, reported to be all cases in each population, and for a sample of residents in each area. The database included 555 patients and 735 controls in Manchester and 293 patients and 589 controls in Nagoya who were interviewed, out of 577, 817, 348 and 735 subjects, respectively. Questions about consumption of dietetic beverages and foods were more limited in Japan than in the United Kingdom, because product labels used in Japan do not allow assessment of the content of artificial sweeteners. No association between cancer of the lower urinary tract and consumption of artificial sweeteners was detected in either area. Whereas the overall odds ratio in Manchester was 0.9 (95% CI, 0.7–1.2, based on 140 exposed cases) in people of each sex, in Nagoya, the odds ratios for a history of use of sugar substitutes were 0.7 (based on 100 exposed cases; 95% CI, 0.5–0.9) in men and 0.5 (based on 26 exposed cases; 95% CI, 0.3–0.8) in women. The odds ratios estimated by duration of use or current frequency did not suggest a dose–response relationship, except that an odds ratio of 2.3 (based on nine exposed cases) was found for women in Manchester consuming more than 10 tablets per day; the

corresponding odds ratio in men was 0.6. Stratum-specific odds ratios by category of tobacco smoking did not suggest an association, although the age-adjusted odds ratio for use of sugar substitutes by nonsmoking men in Manchester was 1.6 (based on 11 exposed cases) (Morrison *et al*., 1982).

A population-based study of bladder cancer carried out in Copenhagen (Denmark) included 290 male patients, 592 male controls, 98 female patients and 195 female controls aged up to 75. A comparison with data in the Cancer Registry showed that the cases represented two-thirds of those originally eligible, but there were no differences in age, sex, area of residence or occupation between included and excluded cases. Some 99% of the cases had been verified histologically as either invasive or non-invasive. Controls were drawn randomly from among residents in the same municipalities as the cases, and 75% of those originally approached agreed to participate in the study. Any use of artificial sweeteners in coffee, tea or foods was reported by 19% of male patients and 26% of controls and 27% of female patients and 26% of controls. These proportions corresponded to age-adjusted odds ratios of 0.7 (95% CI, 0.5–1.0) for men and 1.1 (95% CI, 0.6–1.9) for women. Analyses restricted to any use of table-top artificial sweeteners or current use in coffee or in tea led to almost identical estimates. For men, analyses by strata corresponding to number of daily uses gave odds ratios lower than 1.0 in all strata; corresponding analyses in women showed no trend in odds ratios, and none of the 95% confidence intervals excluded unity. Analyses restricted to consumption of artificial sweeteners for more than 15 years gave nonsignificant odds ratios of 0.5 (95% CI, 0.2–1.0) for men and 0.8 (95% CI, 0.3–2.5) for women (based on 10 and five exposed cases, respectively). No consistent association emerged from analyses stratified by sex and clinical stage at diagnosis or by sex and histological grade of bladder cancer. The odds ratio for use of artificial sweeteners by men who had never smoked was 1.9 (95% CI, 0.5–7.8, based on four exposed cases), and the odds ratios decreased with increasing average number of cigarettes smoked daily throughout life down to 0.2 (95% CI, 0.1–0.5) among smokers of ≥ 25 cigarettes daily. The corresponding estimates for women were based on small absolute numbers and showed no consistent finding. An analysis restricted to the 70% of users who reported exclusive use of saccharin throughout life gave nonsignificant odds ratios of 0.7 for men and 1.0 for women (Møller Jensen *et al*., 1983).

A study in Aarhus (Denmark) included 47 women with newly diagnosed histologically confirmed bladder cancer attending one hospital and twice as many controls matched by age and area of residence. Six patients and two controls reported saccharin consumption, corresponding to an odds ratio (adjusted for a variety of potential confounders) of 6.7 (95% CI, 1.5–30). The odds ratio for women who had never smoked and were saccharin users was 3.3 (95% CI, 1.4–7.8) [the corresponding odds ratio for women who had ever smoked was not given] (Mommsen *et al*., 1983). [The Working Group noted that the terms 'artificial sweetener' and 'saccharin' appeared to be used synonymously.]

A study in New York (United States) was intended to explore the possible exposure to bladder carcinogens of women aged 20–49, who are commonly considered to be at

low risk for bladder cancer. A total of 259 cases diagnosed in 1975–80 were identified through the cancer registry; 40 were excluded because the diagnosing physician refused to grant permission for the patient to be contacted, and an additional 42 did not participate in a telephone interview for unspecified reasons. Controls identified through random-digit dialling were matched to cases by age and telephone area code. A total of 173 pairs were formed, for eight of which some were data missing. Associations were estimated by analyses of matched pairs with the test of McNemar. Regular use (i.e. 100 or more times used) of table-top artificial sweeteners and/or artificially sweetened beverages was reported by 77 cases and 74 controls, corresponding to an odds ratio of 1.1 (95% CI, 0.7–1.7). It was reported that there was 'no suggestion of a dose–response relationship for the cases', but details were not given (Piper *et al*., 1986). [The Working Group noted that the high proportion of non-participating patients might have biased the selection of study subjects.]

A population-based study was carried out in Alberta and Ontario (Canada) during 1979–82, after saccharin had been banned in Canada in 1978. Patients with newly diagnosed urinary bladder cancer (any degree of histological malignancy) and who were resident in urban centres in the two provinces were individually matched to controls by age, sex and area of residence, identified from a list of residents (some errors in recording demographic data for cases led to an excess of eligible controls). Those interviewed were 835 out of 1251 cases and 792 out of 1483 controls; 32% of cases and 9% of controls were not interviewed because of severe illness or death. The questionnaire included questions on regular consumption of table-top artificial sweeteners and low-calorie foods and drinks. The reported sweeteners were classified as saccharin, cyclamate or both on the basis of brand name and period of use. Conditional logistic regression techniques were used to estimate associations. The odds ratio for a history of and treatment for diabetes mellitus was 1.6 (95% CI, 1.1–2.4, based on 131 subjects with diabetes mellitus) and did not change when variables for sweeteners were included in the model. Twelve series of odds ratios were estimated for people of each sex, i.e. any regular use of table-top artificial sweeteners in all subjects, in nonsmokers only and excluding use in the last 10 years; use of saccharin stratified on three doses and total lifetime intake; use of cyclamate stratified on two doses and total lifetime intake; low-calorie foods stratified on two doses and total lifetime intake; low-calorie foods excluding use within the last 10 years; dietetic soft drinks on two doses and total lifetime intake. Among the 34 odds ratios (l7 for each sex), the only one for which the 95% confidence interval excluded unity was that for total lifetime intake of low-calorie foods (odds ratio, 1.5; 95% CI, 1.0 –2.3) by women; the corresponding odds ratio for men was 1.0 (95% CI, 0.8–1.2). No consistent dose-related trend was seen for use of saccharin (Risch *et al*., 1988).

A hospital-based study in Turkey included 168 male and 26 female newly diagnosed or prevalent cases of histologically confirmed bladder cancer and equal numbers of age- and sex-matched hospital controls. Nineteen patients and eight controls reported use of artificial sweeteners ($p < 0.05$) [odds ratio not presented] (Akdas *et al*., 1990). [The

Working Group noted that the conditions from which the controls were suffering were not reported, but that a large proportion had undergone urological examinations.]

2.3 Cancer occurrence among diabetic patients

Patients with diabetes are known to use artificial sweeteners extensively. They also differ from the general population with regard to a number of lifestyle factors, including smoking habits. The Working Group considered that epidemiological studies of cancer in this population are uninformative with regard to the carcinogenicity of saccharin, since individual data on the use of artificial sweeteners and confounders are not provided. In addition, estimates of the risk for smoking-related cancers, such as those of the urinary bladder and kidney, can be expected to be negatively confounded because of the low smoking rates among diabetic patients.

3. Studies of Cancer in Experimental Animals

Saccharin

3.1 Oral administration

3.1.1 *Single-generation exposure*

Mouse: Groups of 50 female Swiss mice, 9–14 weeks of age, were given 0 or 5% saccharin made by the Remsen-Fahlberg method in the diet for 18 months, at which time the survivors were killed. The average survival rates were not affected, and the tumour incidences were similar in tested and control animals. No pathological alterations were observed macroscopically in the urinary bladder (Roe *et al*., 1970). [The Working Group noted that the urinary bladders were not examined histologically.]

As part of a multigeneration study, two groups of 50 male and 50 female Swiss SPF mice [age unspecified] were fed diets containing 0.5 or 0.2% saccharin (free acid) made by the Remsen-Fahlberg method (containing 0.5% *ortho*-toluenesulfonamide) for up to 21 months. A concurrent control group of 50 males and 50 females received a standard diet. At 18 months, 62, 64 and 66 animals were still alive in the groups receiving 0.5 and 0.2% saccharin and in the control group, respectively. One control female developed an anaplastic carcinoma of the bladder, and one male fed 0.2% saccharin had a noninvasive transitional-cell carcinoma of the bladder (Kroes *et al*., 1977) (see also section 3.1.2).

Groups of 25 male and 25 female Charles River CD mice, eight weeks of age, received diets containing sodium saccharin (containing 345 mg/kg (ppm) *ortho*-toluenesulfonamide) at concentrations of 0, 1 or 5% for up to two years. Animals that died before six months were not examined, and the survival times were not reported. Animals were killed when obvious tumours were seen or when they were moribund; all survivors were killed at two years. All animals that survived six months or longer were examined grossly, and any tissues with abnormal changes were examined histologically; in addition, all vital organs from at least 12 animals in each group were examined histologically. In high-dose males,

two cases of papillary hyperplasia of the bladder and two small papillomas were found. One transitional-cell carcinoma of the bladder associated with a stone was found in male controls. Vascular tumours were seen at increased frequency in male mice at the high dose, while lung tumours, hepatomas and lymphomas occurred with apparently equal incidence in control and treated groups. Any differences in the incidence of tumours were considered not to be significant, and none were found in a duplicate experiment for which no data were given (Homburger, 1978). [The Working Group noted the inadequate reporting of the experiment.]

Fifty male B6C3F_1 mice, six weeks of age, were fed sodium saccharin (purity, 99.5%; with 7 ppm *ortho*-toluenesulfonamide) at a dose of 5% in Oriental M diet for 52 weeks with interim kills of five mice at 0, 4, 8, 16 and 20 weeks after the beginning of the experiment; 20 mice were still alive at the end of the experiment. A control group of 35 mice was fed Oriental MF diet only. There was no effect on growth or survival, and no bladder lesions were detected by autoradiography, histology or scanning electron microscopy (Fukushima *et al.*, 1983a). [The Working Group noted the small number of animals and the short duration of the experiment.]

Groups of 10 male and 10 female inbred ICR Swiss mice, six weeks of age, received 0, 0.5, 1 or 1.5 g/kg bw per day saccharin dissolved in 1 mL distilled water by oral gavage for one year, at which time all remaining mice were killed. No deaths occurred in any of the treated groups. The mice fed 1.5 g/kg bw per day showed slight weight loss when compared with controls (48 versus 55 g); both groups consumed 10 g of food per day per mouse. Five males and three females at the high dose had papillary adenocarcinomas of the thyroid. No thyroid tumours were reported in the other groups, and no tumours of other sites were reported (Prasad & Rai, 1986). [The Working Group noted the inadequate number of mice and the incomplete reporting of this experiment. It also noted that the finding of thyroid tumours has not been replicated in any other study in mice or in other species.]

As part of study of two-stage carcinogenesis, female BALB/c StCrlfC3H/Nctr mice were randomly divided into five groups of 192, 192, 192, 144 and 96 mice and were fed 0, 0.1, 0.5, 1, or 5 sodium saccharin, respectively, in Purina Lab Chow beginning at 19 weeks of age and continuing until 135 weeks of age. A slight but nonsignificant statistically increase in the length of survival was observed in treated mice. No bladder neoplasms were observed in any of the groups, and the incidence of bladder hyperplasia was similar: 8/164 (5%), 10/162 (6%), 9/161 (5%), 7/130 (5%) and 3/79 (4%), respectively. The incidences of Harderian gland neoplasms were 27/163 (17%), 32/172 (19%), 29/160 (18%), 22/132 (17%) and 22/84 (26%), respectively ($p < 0.04$, test for trend). A significant, dose-related reduction in the time to onset of lymphomas was observed, although the incidences were similar among groups (Frederick *et al.*, 1989; see also section 3.4). [The Working Group noted that Harderian gland tumours are common age-related, spontaneous neoplasms, the variable percentage of Harderian glands examined, the variability of the incidence of these tumours in control animals, the unequal numbers of animals per group and the long survival time.]

Rat: Groups of 10 male and 10 female Osborne-Mendel rats, 21 days of age, received diets containing 0, 1 or 5% saccharin [source and purity unspecified] for up to two years. The mortality rate in pooled controls was 14% at one year and 68% at two years; at one year, seven males and nine females in the control group, 10 males and 10 females in the group receiving 1% and nine males and nine females at 5% were still alive; the two-year survival rates were not given. It was reported that 7/18 rats [sex unspecified] at 5% had abdominal lymphosarcomas, and that four of the seven also had thoracic lymphosarcomas. The urinary bladders were not examined (Fitzhugh *et al*., 1951). [The Working Group noted the multiple inadequacies of this study, including the small number of animals in each group.]

Groups of 20 male and 20 female Boots-Wistar rats [age unspecified] were fed 0, 0.005, 0.05 or 5% saccharin made by the Remsen-Fahlberg method [purity unspecified] for two years. At 18 months, 15 male and 14 female controls and 10 male and 10 female rats at the highest dose level were still alive. No statistically significant differences in tumour incidence were found between treated and control animals. Only five bladders, all from animals at the highest dose, were examined histologically. Urothelial hyperplasia was found in one male and one female, and a bladder papilloma was found in another female. Bladder parasites were not found. Bladder calculi were found in four male and one female rats fed 5% saccharin (Lessel, 1971).

Groups of 52 male and 52 female BD rats [age unspecified] were fed 0 (control), 0.2 or 0.5% sodium saccharin made by the Remsen-Fahlberg method [purity unspecified] for up to 30 months starting between 70 and 90 days of age, providing average total doses of 0, 83 and 210 g/kg bw. The survival rates at 18 months were 55/104 controls, 50/104 at the low dose and 41/104 at the high dose; at 24 months, the survival rates were 6/104, 3/104 and 5/104, respectively. Sixteen percent of all animals had parasites (*Strongyloides capillaria*) in the urinary tract. Benign and malignant mesenchymal tumours were found with similar frequency in all groups. No bladder tumours were observed (Schmähl, 1973).

In a study reported in an abstract, groups of Charles River CD male and female rats [number and age unspecified] received saccharin [source and purity unspecified] by an unspecified route (in the diet or by gastric intubation thrice weekly) for 18 months, followed by a six-month period of observation. A high incidence of benign tumours of the pituitary and mammary glands was found in surviving controls and experimental animals. The survival times, types of pathological examination, tumour types and other important experimental details were not reported (Ulland *et al*., 1973). [The Working Group noted the inadequacy of this experiment.]

In a study reported in an abstract, groups of 54–56 male Wistar rats [age unspecified] were fed 0 or 2.5 g/kg bw per day sodium saccharin [source and purity unspecified] for up to 28 months. Ten to 16 rats from each group were killed at 12 months, 11 from each group at 24 months and all survivors [number unspecified] at 28 months. No urinary bladder tumours were observed (Furuya *et al*., 1975). [The Working Group noted the incomplete reporting of this experiment.]

Groups of 60 male and 60 female Charles River CD rats [age unspecified] were fed diets containing sodium saccharin made by the Remsen-Fahlberg method (purity conformed to United States Pharmacoepia, British Pharmacoepia and Food Chemicals Codex specifications) for 26 months, to give daily intakes of 0, 0.09, 0.27, 0.81 or 2.4 g/kg bw. Saccharin treatment did not affect the survival of female rats: at 18 months, approximately 50% of the original animals were alive. The survival of male rats was affected in a dose-related manner: thus, at 18 months, about 80% of male control rats but only about 50% of those at the highest dose were still alive. By 24 months, about 10% of the animals in all groups were alive. Four transitional-cell tumours of the bladder were found, one in a male and one in a female given 0.09 g/kg bw and two in males fed 0.81 g/kg bw; an angiosarcoma of the bladder was found in a male control. Bladder calculi were recorded, but there was no association between the presence of calculi, saccharin treatment and/or bladder tumours. The animals were free from bladder parasites. The combined incidences of lymphomas and leukaemias were 7/54 in males at the highest dose of saccharin and 2/57 in untreated male controls (Munro *et al*., 1975).

Groups of 25 male Charles River CD-1 rats [age unspecified] received sodium saccharin (containing 345 mg/kg (ppm) *ortho*-toluenesulfonamide) in the diet at concentrations of 0, 1 or 5% for up to two years. Animals that died before six months were not examined, and the survival times were not reported. Animals were killed when obvious tumours were seen or when they were moribund; all survivors were killed at two years. All animals that survived six months or longer were examined grossly, and any tissues with abnormal changes were examined histologically; in addition, all vital organs from at least 12 animals in each group were examined histologically. Tumours of the urinary bladder, pituitary, breast and subcutaneous tissue were seen with equal incidence in all groups (Homburger, 1978). [The Working Group noted the inadequate reporting of the experiment].

A group of 75 male and 50 female Wistar SPF rats, eight weeks of age, received sodium saccharin made by the Remsen-Fahlberg method (containing 698 mg/kg (ppm) *ortho*-toluenesulfonamide) in the drinking-water, to give a daily intake of 2 g/kg bw saccharin. Another group of 75 males and 75 females received 4 g/kg bw per day saccharin in the diet. A group of 55 males and 50 females served as controls. The males receiving saccharin in the drinking-water were also given 1% ammonium chloride for four weeks and then 0.5% for life, in order to correct a treatment-associated rise in urinary pH. Of the male controls, 25 were given ammonium chloride at the same concentrations. No treatment-associated change in urinary pH occurred in either of the treated groups of females or in males receiving saccharin in the diet. The experiment was terminated after two years. Survival at 18 months was 49/55 male and 43/50 female untreated controls, 65/75 males and 44/50 females that received saccharin in the drinking-water and 55/75 males and 52/75 females fed saccharin in the diet. At 100 weeks, 37/55 male and 13/50 female controls, 49/75 males and 29/50 females receiving saccharin in the drinking-water and 12/75 males and 16/75 females fed saccharin in the diet were still alive. In control animals, the total tumour incidence was 1/52 males and 9/46 females. In

rats receiving saccharin in the drinking-water at 2 g/kg bw per day, the incidence was 11/71 in males and 10/44 in females; while in rats fed saccharin at 4 g/kg bw per day it was 10/70 in males and 7/68 in females. Transitional-cell carcinomas of the urothelium were not seen in male or female controls, but accounted for 1/71 in males (in the ureter) and 1/44 in females (in the renal pelvis) in rats receiving saccharin in the drinking-water and 3/70 in males (all in the bladder) and 0/68 in females fed saccharin. The incidence of lymphosarcomas and/or leukaemia was 0/52 in male and 0/46 in female controls, 4/71 in males and 1/44 in females given saccharin in the drinking-water, and 2/70 in male and 1/68 in female saccharin-fed rats. One Leydig-cell tumour was found in each of the saccharin-treated groups of males, but none occurred in the testes of untreated male controls. There was a treatment-associated increase in the number of microcalculi within the renal tubules of male (but not female) saccharin-treated rats, with an incidence of 2/52 in controls, 30/71 in males given saccharin in the drinking-water and 16/70 in saccharin-fed males. The animals were free from bladder parasites (Chowaniec & Hicks, 1979). [The Working Group noted the incomplete histopathological examination and the lack of lymphomas and/or leukaemia, a common neoplasm in controls.]

As part of a two-generation study (see section 3.1.2), groups of 50 male and 50 female Charles-River CD (Sprague-Dawley) rats, 30 days of age, were fed either a control diet or a diet containing 5% sodium saccharin prepared by the Maumee process and free of *ortho*-toluenesulfonamide. Survival was not affected by treatment. Bladder tumours (benign and malignant) were observed in 1/36 control males and in 7/38 male rats ($p < 0.03$) fed saccharin which survived 87 weeks or more (the time at which the first tumour was observed). In addition, one treated male and two treated females had urothelial tumours of the renal pelvis, and one treated male had a urethral tumour; no other urothelial tumours were observed in controls. The incidence of bladder calculi was not related to treatment or to tumour incidence. The animals were free of bladder parasites (Arnold *et al*., 1980).

As part of a two-generation study, 50 female Wistar rats [age unspecified] were given 2 g/kg bw per day sodium saccharin made by the Maumee process in the diet for two years. A group of 63 animals served as controls. At week 84, 50/63 controls and 37/50 saccharin-fed rats were still alive. The overall tumour incidences were similar in the two groups, and no bladder neoplasm occurred. Mild focal urothelial hyperplasia was seen in one rat fed saccharin. The animals were free from bladder parasites (Hooson *et al*., 1980). [The Working Group noted that the animals were not started on the test at weaning but had been fed a normal diet for several weeks before the start of the study.]

Groups of 40–48 male ACI, Wistar, Fischer 344 and Sprague-Dawley rats, six weeks of age, were fed 5% sodium saccharin (food additive grade; purity, 99.5%, with 7 ppm *ortho*-toluenesulfonmide) in powdered diet, and surviving rats were killed at the end of 52 weeks of treatment. Interim sacrifices were also performed on five rats of each strain at 12, 24 and 36 weeks. Corresponding control groups of 40–45 rats were fed untreated diet. The treated groups had significant growth retardation, with average body weights at week 52 of treated versus control groups as follows: ACI, 299 versus 327 g; Wistar, 400

versus 447 g; Fischer 344, 403 versus 427 g; and Sprague-Dawley, 593 versus 716 g. There was no apparent effect on survival. At the end of 52 weeks, no urinary bladder lesions were seen in the Wistar, Fischer 344 or Sprague-Dawley rats; of the ACI rats, 1/28 controls had simple hyperplasia and 25/32 (78%) rats treated with sodium saccharin had simple hyperplasia, 20 (62.5%) had papillary/nodular hyperplasia, nine (28.1%) had papillomas and three (9.4%) had carcinomas. It was also reported that one of the ACI rats had a bladder calculus, but more than half of the control and test ACI rats bore the bladder nematode, *Trichosomoides crassicauda* (Fukushima *et al.*, 1983a). [The Working Group noted the limited duration of treatment.]

A group of 68 male Fischer 344 rats, seven weeks of age, were fed 5% sodium saccharin in Oriental MF diet; a control group of 31 rats were fed the basal diet alone. Interim sacrifices were carried out during the course of the experiment, and the remaining rats were killed at the end of 112 weeks of treatment. The body-weight gain was similar in the two groups up to 15 weeks, whereas after 20 weeks the body weight increased more slowly in treated rats than in controls. Simple hyperplasia was seen in the bladders of treated rats as early as eight weeks of treatment, and about two-thirds of rats at all times had simple hyperplasia. Papillary or nodular hyperplasia was observed in the bladders of approximately one-third of rats killed after 8, 12, 20, 80 and 112 weeks of treatment. At 4, 16, 60, 90 and 100 weeks, no rats had papillary or nodular hyperplasia. Simple hyperplasia was occasionally seen in control rats: 2/6 rats at four weeks, 1/5 at 20 weeks, and 1/7 at 100 weeks. No papillary or nodular hyperplasia was seen in the control group, and no papilloma or transitional-cell carcinoma was seen in either group. *Trichosomoides crassicauda* were not present in the bladders. When the stomachs of 20 treated and 11 control rats killed after 80 weeks were examined, all of the sodium saccharin-treated rats had hyperkeratosis at the limiting ridge of the forestomach, and five papillomas of the limiting ridge of the forestomach were reported. Ulcers were seen in the glandular stomach in four animals. No squamous-cell carcinomas or adenocarcinomas were observed in either group (Hibino *et al.*, 1985). [The Working Group noted the incomplete sampling of the stomach between groups. The Group could not agree on the diagnosis of papillomas from the illustrations provided.]

A group of 36 male Sprague-Dawley and 35 male analbuminaemic (a mutant strain derived from Sprague-Dawley rats) rats, six weeks of age, were fed 5% sodium saccharin in powdered CE-2 diet for 80 weeks. Fourteen Sprague-Dawley and 12 analbuminaemic rats served as controls. There was no apparent effect on body weight or on survival. No bladder tumours were present in any of the rats. Simple hyperplasia of the urinary bladder was observed in 2/35 analbuminaemic and 2/36 Sprague-Dawley rats given sodium saccharin (Homma *et al.*, 1991). [The Working Group noted that the treatment period was less than two years.]

Hamster: Groups of 30 male and 30 female random-bred Syrian golden hamsters, eight weeks of age, received saccharin made by the Maumee process at concentrations of 0, 0.156, 0.312, 0.625 or 1.25% in the drinking-water for their natural lifespan. The highest dose used in this study was the maximum tolerated, as determined in an eight-

week study. The average daily consumption ranged from 44 mg/animal given 0.156% to 353 mg/animal given 1.25%. The mean survival time was 50–60 weeks in all groups. The pathological changes observed and the distribution and histological types of neoplasms were within the range of tumours that occur commonly in hamsters in this colony (Althoff *et al*., 1975).

Fifty male Syrian golden hamsters, six weeks of age, were fed 5% sodium saccharin (purity, 99.5%, with 7 ppm *ortho*-toluenesulfonamide) in the diet for 52 weeks. Five animals were killed at 0, 4, 8, 16 or 20 weeks after the beginning of the experiment; 20 hamsters were available at the terminal sacrifice. A group of 35 hamsters served as untreated controls. There was no effect on growth or survival, and no bladder lesions were detected by autoradiography, histology or scanning electron microscopy (Fukushima *et al.*, 1983a). [The Working Group noted the small number of animals and the short duration of the experiment.]

Guinea-pig: A group of 30 male Hartley guinea-pigs, six weeks of age, were fed 5% sodium saccharin (purity, 99.5%, with 7 ppm *ortho*-toluenesulfonamide) in Oriental RC diet for 52 weeks with interim sacrifices of three guinea-pigs at 0, 4, 12, 16 or 20 weeks from the beginning of the experiment; 12 guinea-pigs were available at the terminal sacrifice. A group of 20 guinea-pigs served as untreated controls. Treated guinea-pigs had lower body-weight gain than the controls, but no bladder lesions were detected by autoradiography, histology or scanning electron microscopy (Fukushima *et al.*, 1983a). [The Working Group noted the small number of animals and the short duration of the experiment.]

Monkey: In a study reported in an abstract, one of two batches of sodium saccharin made by the Remsen-Fahlberg method (containing 2.4 and 3.2 mg/kg *ortho*-toluenesulfonamide) (Coulston *et al*., 1975) was given orally at doses of 20, 100 or 500 mg/kg bw per day on six days a week to groups of two, two and three *Macaca mulatta* (rhesus) monkeys of each sex, respectively. Three animals of each sex served as controls. After 79 months on this regimen, 11 monkeys remained in the treated groups, and all monkeys were killed and autopsied. Histopathological examination revealed no abnormal lesions in the urinary bladder, kidneys or testis in monkeys that survived the treatment or in those that died during the test (McChesney *et al*., 1977).

Groups of five female and one male African green (*Cercopithecus aethiops*), two female and five male rhesus (*Macaca mulatta*), three female and three male cynomolgus (*Macaca fascicularis*) and one hybrid (rhesus male cross cynomolgus female) monkey, 0–10 days of age, were given 25 mg/kg bw sodium saccharin (purity, > 99%) in the diet on five days a week for up to 283 months. Eight monkeys died during the course of the experiment, after 103, 128, 157, 168, 170, 192, 214 and 282 months of feeding. The remainder were killed at the end of the experiment, after 207–283 months. Five male and four female cynomolgus and five male and two females rhesus monkeys were available for comparison and were killed at 206–301 months of age. No bladder tumours were detected, and there was no evidence of hyperplasia by light or scanning electron microscopy (Takayama *et al.*, 1998). (Preliminary reports of this experiment were published by

Sieber & Adamson, 1978; Thorgeirsson *et al.*, 1994). [The Working Group noted the relatively small dose of saccharin administered, the relatively small number of monkeys used and the multiplicity of species.]

3.1.2 *Multigeneration exposure*

In these studies, animals of each sex of the parent (F_0) generation were fed saccharin or sodium saccharin from weaning (or very soon after weaning) throughout both pregnancy and before weaning of their offspring. As the offspring were then placed on the same diet as their parents for their lifespan, their exposure to saccharin was greater than that of the F_0 generation by the length of the gestation and suckling periods.

Mouse: Saccharin containing 0.5% *ortho*-toluenesulfonamide was fed to groups of Swiss mice in a multigeneration study for life at concentrations of 0, 0.2 or 0.5% in the diet. The F_0, F_{3b} and F_{6a} generations, consisting of 50 males and 50 females, were used to test the compound for carcinogenicity. The experiments were terminated at 21 months. The survival rates at 18 months were 66, 62, 64 (F_0), 61, 54, 53 (F_{3b}) and 67, 48, 54 (F_{6a}) at 0, 0.2 and 0.5% respectively. Histopathological examination showed that lesions were equally distributed in the control and experimental groups. Two male mice, one of the F_0 generation receiving 0.2% saccharin and one of the F_{3b} generation receiving 0.5% saccharin, developed transitional-cell carcinomas of the bladder at 20.5 months. One female control of the F_0 generation had an anaplastic carcinoma of the bladder at 20.5 months (Kroes *et al*., 1977).

Rat: Groups of 20 male and 20 female weanling Sprague-Dawley rats of the F_1 generation were fed sodium saccharin made by the Remsen-Fahlberg method [purity unspecified] at concentrations of 0, 0.05, 0.5 or 5% of the basal diet for up to 100 weeks. Of the F_1 animals, 12, 10, 11 and 15 males and 16, 14, 14 and 19 females, respectively, survived to 80 weeks. Seven transitional-cell carcinomas of the urinary bladder developed, all in F_1 males on the 5% saccharin diet ($p = 0.001$). The presence or absence of bladder parasites was not recorded. The total numbers of tumour-bearing animals were two males and eight females at 0%, one male and six females at 0.05%, one male and five females at 0.5%, and seven males and 13 females at 5% (Tisdel *et al*., 1974).

Groups of 50 male and 50 female Charles River CD (Sprague-Dawley) rats, 30 days of age, were fed either a control diet or a diet containing 5% sodium saccharin continuously for life. The saccharin was prepared by the Maumee process and was free of *ortho*-toluenesulfonamide. After three months on test, the animals were mated on a one-to-one basis. All litters were culled to eight pups (four males and four females) four days *post partum* in a random manner. The pups were weaned onto their parents' diet, and 50 males and 50 females from each group were randomly selected to constitute the second generation. The survival of the offspring (F_1 generation) was not affected by treatment. Of the F_1 animals surviving 67 weeks or longer, at which time the first tumour was observed, none of the 42 male controls but eight of the 45 saccharin-treated males had developed transitional-cell carcinomas of the bladder [$p = 0.002$], and four had transitional-cell papillomas; two of the 49 surviving females fed 5% sodium saccharin also had bladder

cancers. Although urinary bladder calculi were noted occasionally, their incidence was not related to treatment, nor were they associated with the tumours. The animals were free of bladder parasites (Arnold *et al*., 1980).

Groups of five to seven female Sprague-Dawley were treated by oral gavage with saccharin [form not specified] (containing < 10 ppm *ortho*-toluenesulfonamide) at doses of 0.2, 1 or 5 g/kg bw on days 14, 17 and 20 of pregnancy. Solutions containing 2.5 g/mL of the test compound were given by gavage to the rats, which had received no food or drinking-water overnight. A control group consisted of the offspring of five untreated rats. The numbers of offspring at the low, intermediate and high doses which survived to 28 days of age were 24 males and 27 females, 35 males and 23 females and 32 males and 25 females, respectively; the untreated controls had 25 male and 33 female offspring. Nine of 69 of the offspring of dams treated with the highest dose of saccharin died within the first four days after birth. The F_1 generation offspring were observed for life [time not specified] or killed when found moribund. The mean survival times of males tended to be higher with the higher doses of saccharin but were lower than those of controls. No significant differences in survival were found between groups. There were no lesions of the bladders in any of the treated groups, and there was no increase in the incidence of tumours at other sites. The tumours seen in the treated groups were the same as those seen in controls (Schmähl & Habs, 1980). [The Working Group noted the incomplete reporting of this experiment.]

Groups of 48 male and 48 female Charies River CD (Sprague-Dawley) rats of the F_1 generation were fed dietary levels of 0, 0.01, 0.1, 1, 5 or 7.5% sodium saccharin [method of production and purity unspecified] for 28 months after their parents had been fed the same diet from weaning. There were no significant differences in survival between treated and control animals. Although no difference in bladder tumour incidence was found between F_1 males fed 5% saccharin (1/21) and the F_1 controls (1/25) that survived beyond 18 months, 6/23 F_1 male rats fed 7.5% saccharin developed transitional-cell papillomas or carcinomas of the bladder. This result was significantly different from that in controls. There was no apparent correlation between tumour incidence and presence of bladder stones. The bladders were reported to be 'free of visible parasites' (Taylor *et al*., 1980).

Male Charles River CD rats of the F_1 generation were treated as follows: group 1 (350 rats), untreated controls; group 2 (700 rats), 1% sodium saccharin (produced by the Maumee process; purity, > 99%) in the diet; group 3 (500 rats), 3% sodium saccharin in the diet; group 4 (200 rats), 4% sodium saccharin in the diet; group 5 (125 rats), 5% sodium saccharin in the diet; group 6 (125 rats), 6.25% sodium saccharin in the diet; group 7 (125 rats), 7.5% sodium saccharin in the diet; group 8 (125 rats), 5% sodium saccharin administered through gestation period and then the F_1 rats fed control diet from birth until the end of the experiment; group 9 (125 rats), 5% sodium saccharin in the diet from the time of birth and continuing until the end of the experiment; group 10 (125 rats), 5% sodium hippurate (purity > 98%) in the diet until the age of eight weeks and then as 3% of the diet until the end of the experiment. The parents (the F_0 generation) were given the same diets from six weeks of age and continuing for nine weeks before the beginning of mating; they were then given the same diets through gestation and lactation (except as

noted for groups 8 and 9). Group 8 received sodium saccharin throughout gestation and then received control diet. In group 9, the F_0 generation was given control diet until the birth of the F_1 generation, at which time the dams were given 5% sodium saccharin. There was significant growth retardation in all groups given sodium saccharin at doses of greater than 3% of the diet, including group 9. There were minimal changes in the growth of the rats in group 8, given sodium saccharin until parturition. Survival was comparable in the nine groups, except for a significant increase in survival in groups 5 and 7. The numbers of survivors of the F_1 generation in the nine groups at the time of killing at 30 months of age were: 80/350 (23%), 172/700 (25%), 114/500 (23%), 38/200 (19%), 46/125 (37%), 33/125 (26%), 42/125 (34%), 25/125 (20%), 33/125 (26%) and 38/125 (30%), respectively. The effective numbers of rats were those alive at 15 months of age when the first bladder tumour was detected. The incidences of transitional-cell papillomas were 0/324, 4/658 (0.6%), 4/472 (0.8%), 4/189 (2.1%), 4/120 (3.3%), 12/120 (10%), 18/118 (15%), 0/122, 4/120 (3.3%) and 0/118, respectively. The incidences of transitional-cell carcinomas were 0, 1 (0.2%), 4 (0.8%), 8 (4.2%), 11 (9.2%), 8 (6.7%), 19 (16%), 0, 8 (6.7%) and 0, respectively. It was noted that the background incidence of bladder neoplasia at the study laboratory was 0.8%. In this study, the incidences of bladder neoplasia were significantly increased in groups 3–7 and in group 9. The tumour incidences when 1% sodium saccharin and 5% were fed throughout gestation were not significantly greater than those of controls. There was no difference in the incidence of bladder neoplasms between groups 5 and 9 (5% sodium saccharin throughout gestation and after parturition and 5% sodium saccharin after parturition only) (Schoenig *et al.*, 1985). [The Working Group noted that re-examination of the histopathology of the bladders was performed by Squire (1985), who found a significant increase in the incidence of bladder tumours only at doses of 4, 5, 6.25 and 7.25% sodium saccharin. There were no compound-related effects at 1%, and there was no statistically significant increase in the incidence of transitional-cell carcinomas ($p = 0.25$) at the 3% dose.]

3.2 Intraperitoneal administration

Mouse: In a screening assay of 41 food additives and 22 chemotherapeutic agents in the primary lung tumour bioassay in A/St mice, groups of 20 female mice, six to eight weeks of age, received intraperitoneal injections of sodium saccharin three times a week for the first eight weeks of the experiment (total doses, 15.6 and 78 g/kg bw) followed by an additional 13 weeks of observation. All of the mice were killed at the end of 21 weeks. Fifteen mice at each dose survived until the end of experiment. Lung adenomas were observed in four and eight mice treated with the two doses of saccharin, respectively, and the numbers of lung tumours per mouse were 0.27 ± 0.07 and 0.67 ± 0.17, respectively. Of a group of 30 female mice that received only water, 28 survived until the end of the experiment; 37% developed lung adenomas and the number of tumours per mouse was 0.37 ± 0.07. There was no significant difference between these groups (Stoner *et al.*, 1973). [The Working Group noted that this bioassay is no longer considered a valid screen for the carcinogenic activity of chemicals.]

3.3 Other experimental systems

3.3.1 *Skin application*

A total dose of 0.24 g saccharin [form not specified], made by the Remson-Fahlberg method, was applied as an 8% solution in acetone thrice weekly to the skin of 'S' strain mice. Twenty-five days after the start of the treatment, the animals were given 18 weekly applications of 0.17% croton oil in acetone. At the end of the croton oil treatment, 15 skin papillomas were observed in seven of the 20 saccharin-treated animals, by comparison with four papillomas in four of 19 controls treated with croton oil only. The increase was not statistically significant (Salaman & Roe, 1956).

3.3.2 *Bladder insertion (implantation)*

Saccharin [source and purity unspecified] was mixed with four times its weight of cholesterol, and pellets (9–11 mg) containing 2 mg saccharin were then inserted into the urinary bladder lumina of 20 'stock' mice [sex and age unspecified]. An identical group composed of 28 mice received 9–11-mg pellets of cholesterol. The experiment lasted 52 weeks. Of mice that lived 30 weeks, 4/13 saccharin-treated and 1/24 control animals developed bladder tumours ($p = 0.01$) (Allen *et al*., 1957). [The Working Group noted serious difficulties in interpreting the results of studies conducted with pellet implantation in the bladder because of the significant carcinogenic effect of the pellet itself (Clayson, 1974; Jull, 1979; DeSesso, 1989).]

Sodium saccharin (analytically pure) was mixed at 4–5 mg with four times its weight of cholesterol; pellets weighing 20–24 mg containing sodium saccharin were then inserted into the urinary bladder lumina in two trials with groups each composed of 100 female Swiss mice aged 60–90 days. Ninety-nine percent of the sodium saccharin had disappeared from the pellet within 1.5 days. Identical groups received 20–24 mg pellets of pure cholesterol for 56 weeks. Only the bladders of animals surviving more than 25 weeks were examined microscopically. The first urinary bladder carcinoma was seen in a saccharin-treated animal 42 weeks after surgical insertion. The overall incidences of bladder carcinomas were 31/66 (trial 1) and 33/64 (trial 2) in saccharin-treated mice as compared with 8/63 (trial 1) and 5/43 (trial 2) in animals exposed to pure cholesterol pellets ($p < 0.001$). The carcinomas in saccharin-exposed mice were more frequently multiple and invasive ($p < 0.009$). They were composed of cells with a high mitotic index and exhibited more squamous or glandular metaplasia than was found in tumours in control animals. The incidence of tumours in other tissues were not different from those in control mice (Bryan *et al*., 1970). [The Working Group noted the paucity of details regarding this experiment and the difficulty in interpreting the results of studies involving pellet implantation technique in mice (Clayson, 1974; Jull, 1979; DeSesso, 1989)].

3.4 Administration with known carcinogens

3.4.1 *Benzo[a]pyrene*

Groups of 50 female Swiss mice, 9–14 weeks of age, received an initial single gastric instillation of 0.2 mL polyethylene glycol either alone or containing 50 µg benzo[*a*]pyrene

[purities unspecified]. Seven days later, the test diet containing 5% saccharin [purity unspecified] was fed for 72 weeks. The average survival rates were not different from those of controls. Although mice treated with benzo[*a*]pyrene had an increased incidence of squamous papillomas of the forestomach (20/61), saccharin did not enhance the occurrence (10/32). Hepatocellular adenomas, pulmonary neoplasms and malignant lymphomas occurred at similar frequencies in all groups. No pathological alterations were observed macroscopically in the urinary bladder (Roe *et al*., 1970). [The Working Group noted that benzo[*a*]pyrene is not organotropic for the bladder and that the urinary bladders were not examined histologically.]

3.4.2 *2-Acetylaminofluorene*

Two groups of 12 female Horton Sprague-Dawley rats [age unspecified] were fed a diet supplemented with 300 mg/kg 2-acetylaminofluorene (AAF) for 40 weeks and/or 5% sodium saccharin. Eleven of the controls given AAF and 6/12 rats fed AAF plus saccharin developed palpable mammary and ear-duct tumours. Liver tumours were observed in both groups, but they were smaller and less malignant in the saccharin-fed animals. Microscopic examination of the urinary bladders indicated that the mucosal lining was hyperplastic in all rats fed AAF but more particularly so in those fed AAF plus saccharin; one animal fed AAF plus saccharin had squamous metaplasia and precancerous changes of the mucosal epithelium. No malignant lesions of the urinary bladder were observed (Ershoff & Bajwa, 1974). [The Working Group noted the inadequate number of animals and that the intake of AAF and saccharin could not be assessed since food consumption was not measured.]

Ten groups of weanling female BALB/c StCrlfCrlfC3H/Nctr mice, 21–26 days old, were fed control diet (groups 1–5) or were pretreated with 200 mg/kg diet (ppm) AAF (purity > 97%) (groups 6–10) for 13 weeks, followed by two weeks of control diet, and were then fed 0 (groups 1 and 6), 0.1% (groups 2 and 7), 0.5% (groups 3 and 8), 1% (groups 4 and 9) or 5% (groups 5 and 10) sodium saccharin (purity, > 98%). The saccharin was administered when the mice were 19–135 weeks old. There were 192 mice in each of groups 1–3 and 6–8, 144 mice in each of groups 4 and 9, and 96 mice in each of groups 5 and 10. The mortality rate of mice pretreated with AAF followed by control diet was significantly higher than that of mice that received only the control diet. Addition of sodium saccharin to the diet of AAF-pretreated animals increased their longevity in a dose-related fashion ($p = 0.011$). A less pronounced effect was observed in non-pretreated mice ($p = 0.099$). The incidences of bladder tumours were 2/164 (1%) for the group given AAF plus control diet and 3/165 (2%) for the groups given AAF plus 0.1% saccharin, respectively; no bladder tumours were seen in any of the other groups. The incidences of bladder hyperplasia were similar in the five groups pretreated with AAF and in the groups not pretreated with AAF. There was no dose–related increase in the incidence of neoplasms in any other tissues, including the Harderian gland, but a decreased incidence of lymphomas was seen in the group pretreated with AAF and given 5% saccharin (50% versus 62% in the controls). There was a statistically significant, dose-related reduction in the time to

onset of lymphomas in AAF-pretreated and non-pretreated mice, correlated with the decreased mortality rate observed in saccharin-treated mice. An increase in the incidence of liver neoplasms was seen in AAF-pretreated when compared with non-pretreated mice, but there were no differences between the incidences at the different doses of saccharin and the corresponding controls (Frederick *et al.*, 1989; see also section 3.1.1).

3.4.3 N-*Methyl*-N-*nitrosourea*

A group of 50 female Wistar SPF rats, six to eight weeks of age, were pretreated with a preparation of 1.5 mg *N*-methyl-*N*-nitrosourea (MNU) dissolved in 0.9% sodium chloride (pH 7.0) and instilled into the bladder, and then two days later received 4 g/kg bw per day sodium saccharin (Remsen-Fahlberg, containing an average of 698 mg/kg (ppm) *ortho*-toluenesulfonamide) in the diet for life or up to two years; a further group of 50 females was pretreated with 2 mg MNU and then given 2 g/kg bw sodium saccharin per day in the drinking-water. The control groups consisted of 55 male and 50 female untreated rats, 75 males and 50 females given 2 g/kg bw sodium saccharin per day in drinking-water, 75 males and 75 females fed 4 g/kg bw sodium saccharin per day in the diet, 85 males and females given 1.5 mg MNU, and 50 given 2 mg MNU and maintained on a saccharin-free diet for two years. The incidences of transitional-cell neoplasms of the bladder in surviving animals whose bladders were examined histologically were: 0/52 male and 0/46 female untreated controls, 0/71 males and 0/44 females at 2 g/kg bw per day sodium saccharin in drinking-water, 3/70 males and 0/68 females at 4 g/kg bw day sodium saccharin in the diet, 0/124 in MNU-treated animals, 23/49 females (47%; $p < 0.0005$) given MNU followed by 2 g/kg bw per day of sodium saccharin in drinking-water and 27/47 females (52%; $p < 0.0005$) given MNU followed by 4 g/kg bw per day sodium saccharin in the diet. The first bladder tumour was seen after 95 weeks in the saccharin-fed control group and after eight weeks in the MNU-initiated and sodium saccharin-treated test groups. The animals were free from bladder parasites (Hicks *et al*., 1978; Chowaniec & Hicks, 1979). [The Working Group noted the difficulty in determining the actual dose of MNU because of possible decay in solution over time.]

A single dose of 2 mg MNU was instilled into the urinary bladders of female Wistar rats (AF-Han strain) weighing 195 g. Thereafter, 50 animals were given 2% saccharin [purity unspecified] in the diet, the dose being increased after 10 weeks to 4%, for life, resulting in doses of 1.4–2.5 g/kg bw per day. The control groups consisted of 100 untreated female rats, 50 females receiving MNU alone and 50 females receiving distilled water. A further group of 50 female rats treated with MNU were given 3% calcium carbonate in the diet instead of saccharin. The survival rates at two years were 59/100 untreated controls, 28/50 water controls, 13/50 MNU-treated animals, 15/50 receiving MNU plus calcium and 14/50 given MNU plus saccharin. In the MNU-treated groups, the first tumour of the urinary bladder was found after 14 weeks. Benign and malignant urothelial neoplasms occurred in the renal pelvis, ureter and urinary bladder, the overall incidences of urinary tract tumours being 57% with MNU only (survival, 76 ± 29 weeks), 65% with MNU plus saccharin (survival, 78 ± 25 weeks) and 65% with

MNU plus calcium carbonate (survival, 86 ± 23 weeks). The frequencies were 28, 57 and 43% in the renal pelvis, 17, 12 and 11% in the ureter and 39, 31 and 39% in the urinary bladder, respectively. The frequency of calcification in the urinary tract, including stone formation, was similar in all treated groups, including controls receiving water, and the occurrence did not correlate with that of tumours. One tumour of the urinary tract was found in an untreated control and one in a control receiving a water instillation into the urinary bladder. The presence or absence of bladder parasites was not reported (Mohr *et al*., 1978). [The Working Group noted that many bladder tumours were found and that the animals were heavier than those used in the experiment by Hicks *et al*. (1978).]

Groups of 50 female Wistar/AF-Han rats, average body weight 195 g, were treated with a single intravesicular instillation of water, a single intravesicular instillation of 2 mg MNU in 0.5 mL water with no further treatment, a single intravesicular instillation of 2 mg MNU followed by treatment with 3% calcium carbonate in the diet, a single intravesicular instillation of 2 mg MNU followed by 2% sodium saccharin in the diet for 10 weeks and then increased to 4% of the diet for the remainder of the experiment or a single intravesicular instillation of 2 mg MNU followed by 2% sodium cyclamate in the diet for 10 weeks and then increased to 4% of the diet for the remainder of the experiment. Satellite groups of 12, 15 and 12 rats for the last three groups, respectively, received the same treatments as the other rats in the group but were examined periodically by radiography for the presence of stones. A group of 100 rats was untreated. The incidences of bladder tumours were 0/100 in untreated controls, 1/50 in water controls, 19/49 with MNU, 24/49 with MNU plus calcium carbonate and 25/50 with MNU plus sodium saccharin, indicating no significant increase in tumorigenic activity due to saccharin. Bladder stones were noted in 0, 0, 6, 3 and 10 rats, respectively. No correlation between the presence of stones and the induction of bladder tumours was found (Green & Rippel, 1979). [The Working Group noted the incomplete reporting of this experiment.]

Three groups of 63 female Wistar rats [age unspecified] were pretreated with 0.15 mL of a saturated solution of MNU in saline (10 mg/mL) instilled into the bladder. Two weeks later, rats were given 0 or 2 g/kg bw per day sodium saccharin in the drinking-water for two years; one group received saccharin prepared by the Maumee process and the second group received saccharin prepared by the Remsen-Fahlberg method (containing 40 mg/kg (ppm) *ortho*-toluenesulfonamide). By week 84, 22 controls, 43 animals given 'Maumee' sodium saccharin and 37 rats given 'Remsen-Fahlberg' sodium saccharin had died. An increase in the number of proliferative bladder lesions occurred in animals treated with MNU plus saccharin. The incidence of bladder neoplasia was not significantly different in the saccharin-treated groups, but the mean latent period was shorter (55 and 52 weeks versus 87 weeks). The animals were free from bladder parasites (Hooson *et al*., 1980). [The Working Group noted that the animals were not started on the test at weaning but had been fed a normal diet for several weeks before the start of the study.]

Male Fischer 344 rats weighing 130 g were kept for one week on basal diet and then given twice weekly intraperitoneal injections of 20 mg/kg bw MNU for four weeks

followed by either 32 weeks of 0.05% phenobarbital or 5% sodium saccharin in the diet; given the same four-week treatment with MNU followed by 32 weeks of control diet; or given twice weekly injections of 5 mg/kg bw citrate buffer for four weeks and then either 0.05% phenobarbital or 5% sodium saccharin in the diet. The rats were killed at the end of 36 weeks. No information was provided on growth or survival. MNU followed by sodium saccharin produced papillary or nodular hyperplasia of the bladder in 9/19 (47%) rats, whereas no hyperplasia was seen in the bladders of other animals. Sodium saccharin did not increase the incidences of adenoma or adenocarcinoma of the thyroid, γ-glutamyl transferase-positive foci in the liver or lesions of the forestomach or glandular stomach when administered alone or after MNU. Phenobarbital increased the incidences of thyroid tumours and liver hyperplastic foci but not of neoplasia in the bladder. MNU by itself induced a high incidence of forestomach and glandular stomach foci and tumours and thyroid adenomas (Tsuda *et al.*, 1983). [The Working Group noted the relatively short period of administration and lack of bladder tumour induction with this protocol.]

Female Sprague-Dawley rats, eight weeks of age, were treated with a single intravesicular instillation of 0.5 mg MNU (synthesized at the institute and quantified on the day of use) in 300 µL saline or with an intravesicular instillation of 300 µL of saline. Two days after dosing, the rats were fed diets containing either 0 (120 rats), 0.1% (150 rats), 0.5% (120 rats), 1% (90 rats), 2.5% (60 rats) or 5% (60 rats) sodium saccharin in the diet. Groups of 60 rats were also given 5% saccharin in the diet or sodium saccharin in the drinking-water at a concentration of 2, 3 or 4% to achieve maximum consumption and yet preserve the animals' health. The 2% dose was then used for the remainder of the study. An additional group of 60 rats received four doses of MNU at weekly intervals and were then fed untreated diet for the remainder of the experiment. The experiment was terminated at the end of two years. Administration of MNU did not affect body weight. The average weight of rats receiving saccharin was lower than that of control animals, but the decrease exceeded 10% of the control weight only with 5% sodium saccharin and 5% saccharin in the diet and with 2% sodium saccharin in water. The mortality rates in these groups were not significantly different from each other or from those of the controls. Animals not treated with MNU lived longer than did those treated with MNU, and saccharin appeared to lower the mortality rate substantially throughout much of the study. Animals receiving sodium saccharin in the drinking-water had high, early morbidity and mortality. Two of the 233 rats given sodium saccharin in the diet but not pretreated with MNU developed bladder tumours [specific groups not specified]. The incidences in the group receiving sodium saccharin in the diet after MNU pretreatment were 17/106 (16%) with no sodium saccharin, 15/140 (11%) with 0.1%, 21/107 (20%) with 0.5%, 15/78 (19%) with 1%, 20/50 (40%) with 2.5% and 8/49 (16%) with 5%. The incidence in the group given sodium saccharin in the drinking-water was 9/46 (20%), that in the group given saccharin was 8/49 (16%), and that in the group given four intravesicular instillations of MNU was 24/57 (42%). A significantly increased incidence was seen only in the group given 2.5% sodium saccharin after MNU pretreatment (West *et al.*, 1986). [The Working Group noted the unconventional statistical analysis of the

incidence of bladder tumours, whereby animals dying during the course of the experiment were analysed separately from those killed at the end. The Working Group also noted the lack of an increased incidence of bladder with 5% sodium saccharin, in contrast to other reports with the MNU model. The authors attributed the lack of activity at the highest dose to toxicity, but the degree of toxicity was similar to that seen in other experiments with MNU at a dose of 5% sodium saccharin.]

Groups of 60 female Sprague-Dawley rats, eight weeks of age, were treated with a single instillation of 0.5 mg MNU in 300 µL saline or with 300 µL of saline alone. The MNU was synthesized freshly and quantified on the day of use. Sodium saccharin was administered in the diet by a variety of schedules: 0, 1, 2.5 or 5% was administered in the diet either four weeks immediately preceding, following or on the day of bladder instillation with MNU, and the animals were then maintained on control diet until the end of the experiment at 106 weeks of age; additionally, one group of rats were exposed neonatally through the milk of their mothers, which were given sodium saccharin in the diet for the three weeks of lactation, starting on the day of parturition, and were then fed control diet from weaning until the end of the experiment. An interim sacrifice was conducted at approximately 85 weeks of age, and the experiment was terminated when the rats were 112 weeks of age (106 weeks for the neonatal treatment). There was no apparent effect on body weight or on survival, and deaths were due primarily to the development of mammary tumours. The mortality rate was significantly lower than that of controls for animals given MNU and 1% sodium saccharin at 4–8 and 6–10 weeks, 2.5% sodium saccharin at 8–12 weeks or 5% sodium saccharin at 4–8 and 6–10 weeks. In the groups not given MNU, there were no deaths due to sodium saccharin treatment. Transitional-cell neoplasms of the urinary bladder were diagnosed only in animals treated with MNU; the incidences of bladder tumours ranged from 20 to 41% in the various groups, but there was no statistically significant increase in the incidence of bladder tumours between groups for any treatment period or for any dose of sodium saccharin (West *et al.*, 1994).

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

Groups of 20 male Fischer rats, four weeks of age received 0.2% *N*-[4-(5-nitro-2-furyl)-2-thiazolyl]formamide (FANFT) in powdered diet for six weeks, followed immediately by 5% sodium saccharin (containing < 0.03 mg/kg *ortho*-toluenesulfonamide) in powdered diet for up to 83 weeks, then standard diet up to 104 weeks; pretreatment with FANFT as above, followed by six weeks on standard diet, 5% sodium saccharin in the diet for 77 weeks, then standard diet up to 104 weeks; normal diet for six weeks, followed by 5% sodium saccharin in the diet for 83 weeks, then standard diet up to 104 weeks; or pretreatment with FANFT, followed by standard diet for 98 weeks. Forty-two untreated controls received standard diet for 104 weeks. The experiment was terminated after 104 weeks, at which time 6/20, 9/20, 19/20, 16/20 and 27/42 animals were still alive. The incidences of urothelial carcinomas were 18/19 and 13/18 in the groups given FANFT plus saccharin, 0/20 in the group receiving saccharin alone, 4/20 in the group

receiving FANFT alone and 0/42 in the untreated controls. In addition, 1/19 and 1/18 animals given FANFT plus saccharin had urinary bladder sarcomas, and 1/20 given FANFT only had a bladder papilloma. The presence or absence of bladder parasites was not reported (Cohen *et al*., 1979).

Male Fischer rats, five weeks of age, were given 0.2% FANFT in the diet for four weeks followed by 5% sodium saccharin in the diet for 100 weeks; control diet for four weeks followed by sodium saccharin for 100 weeks; FANFT followed by 2% L-tryptophan; control diet followed by L-tryptophan; FANFT followed by control diet; FANFT for the entire course of the experiment; or control diet for the entire experiment. The incidences of bladder carcinoma were 5/26, 0/26, 2/26, 0/26, 0/25, 8/8 and 0/27, respectively, and the incidences of bladder papilloma were 2, 0, 3, 0, 1, 0 and 0, respectively (Fukushima *et al.*, 1981).

Male Fischer 344 rats, five weeks of age, were treated as follows: 5% sodium saccharin plus 2% L-tryptophan in the diet (13 rats); 5% sodium saccharin plus 0.005% FANFT in the diet (16 rats); 5% sodium saccharin in the diet (20 rats); 2% L-tryptophan in the diet (16 rats); FANFT in the diet (11 rats); or were untreated (23 rats). The respective diets were fed for two years, at which time the remaining rats were killed. A significant decrease in weight gain was seen only in the group given sodium saccharin plus L-tryptophan. Bladder tumours were seen only in the group given sodium saccharin plus FANFT, in which three papillomas and two carcinomas were observed; five rats had nodular or papillary hyperplasia of the bladder. Two of 11 rats in the group given FANFT alone also had nodular or papillary hyperplasia (Murasaki & Cohen, 1983a). [The Working Group noted the small numbers of animals.]

Groups of 30 male Fischer rats, five weeks of age, were treated in two phases, the first lasting for six weeks and the second for 61 weeks with a one-week interval between them. The treatments were as follows: 0.5% aspirin for two days, 0.2% FANFT and 0.5% aspirin in the diet for six weeks, one week of aspirin followed by 5% sodium saccharin up to 68 weeks; 0.2% FANFT, one week of control diet followed by aspirin plus sodium saccharin; 0.2% FANFT, one week of control diet followed by sodium saccharin; 0.5% aspirin for two days, 0.2% FANFT plus aspirin, one week of aspirin followed by control diet; 0.2% FANFT followed by control diet; control diet for seven weeks followed by aspirin plus sodium saccharin; control diet for seven weeks followed by sodium saccharin; aspirin for the entire course of the experiment; or control diet for the entire course of the experiment (40 rats). The experiment was terminated at the end of 68 weeks. The incidences of transitional-cell carcinoma of the urinary bladder were 6 (20%), 8 (28%), 24 (83%), 3 (10%) and 3 (10%) in the five groups given FANFT, respectively, with none in the other groups. The incidences of papillomas were 1, 2, 1, 0 and 3 in the groups given FANFT, respectively, and 1 in the group given only aspirin. The total bladder tumour incidence was significantly lower in the groups given aspirin with FANFT when compared with that given sodium saccharin and FANFT, indicating inhibition by aspirin of the bladder tumorigenic effects of both FANFT and sodium saccharin (Sakata *et al.*, 1986). Johansson *et al.* (1986) reported the effects on the kidneys of the rats in this study separately. The

lesions reported included renal pelvic hyperplasia, metaplasia and atypia, calcification, renal papillary necrosis, vascular changes and rat nephropathy. It was noted that administration of sodium saccharin significantly inhibited the incidence and severity of nephropathy due to ageing but significantly increased the incidence and severity of hyperplasia and calcification in the renal pelvis. Aspirin was noted to increase the incidence and severity of renal papillary necrosis and vascular changes. [The Working Group noted the short period of administration of sodium saccharin.]

Groups of weanling male Fischer rats were treated for five-week pretreatment periods as follows: groups 1–4 (42 rats per group) were fed untreated diet and untreated water; groups 5–8 (42 rats per group) were given 0.2% FANFT in the diet with untreated drinking-water; and groups 9–12 (43, 41, 42 and 42 rats per group) were given untreated diet with 0.005% *N*-nitrosodibutylamine (NDBA) in the drinking-water. For the remaining 95 weeks of the experiment, the rats were all given untreated drinking-water and fed diets containing either no additional chemical (groups 1, 5 and 9), 0.05% phenobarbital (groups 2, 6, and 10), 0.15% phenobarbital (groups 3, 7 and 11) or 5% sodium saccharin (groups 4, 8 and 12). Body-weight gain was decreased by ≥ 10% in groups 3, 4, 5, 6, 7, 11 and 12 relative to their respective controls. There was slightly decreased survival in rats given phenobarbital, but survival was not affected by treatment with FANFT, NDBA or sodium saccharin. The incidences of bladder carcinoma were 0, 0, 0, 0, 8/40 (20%), 15/40 (38%), 19/39 (49%), 15/39 (38%), 5/43 (12%), 2/37 (5%), 9/40 (23%) and 27/40 (18%) for the 12 respective groups. The only group that showed a statistically significant increase in the incidence of bladder carcinomas relative to the respective controls was the group given FANFT followed by 0.15% phenobarbital. Benign papillomas were seen in 0, 0, 0, 0, 10, 10, 6, 3, 2, 2, 2 and 4 rats in the 12 respective groups; again, the only group that showed a significantly increased incidence of papillomas plus carcinomas when compared with the respective controls was group 7. Hepatocellular carcinomas were found in 0, 0, 0, 0, 0, 0, 2.5%, 2.6%, 0, 7%, 27%, 43% and 2.5%, respectively, and oesophageal squamous-cell carcinomas were observed in no animals in groups 1–8 and in 46, 32, 45 and 45% of the rats in groups 9–12, respectively (Imaida & Wang, 1986).

In a later report (Masui *et al.*, 1990), all rats given 5 or 7.5% sodium saccharin in the diet were reported to have squamous-cell hyperplasia and hyperkeratosis at the limiting ridge of the forestomach. Further experiments indicated that administration of saccharin in various diets (AIN-76, Prolab, Purina and NIH-07), either as the acid or the sodium or calcium salt, nearly always produced squamous-cell hyperplasia at the limiting ridge but without progression to papilloma or carcinoma in both Fischer 344 and Sprague-Dawley rats. Control diets, in particular AIN-76 diet, also caused some hyperplasia of the limiting ridge.

Groups of 30 male Fischer 344 rats, five weeks of age, were treated as follows: 0.2% FANFT in pelleted AIN-76A diet (prepared on site) was fed for four weeks followed by an interval of one week of control diet, followed by 5% sodium saccharin in AIN-76A diet up to 104 weeks; 0.2% in AIN-76A diet for four weeks followed by AIN-76A diet

up to 104 weeks; group 3 (only 25 rats), AIN-76A control diet for five weeks followed by 5% sodium saccharin in AIN-76A diet up to 104 weeks; group 4, 0.2% FANFT in Prolab 3200 diet for four weeks followed by one week of control Prolab diet followed by 5% sodium saccharin in Prolab diet up to 104 weeks; group 5, 0.2% FANFT in Prolab diet for four weeks followed by control Prolab diet up to 104 weeks; group 6, 0.2% FANFT in AIN-76A diet for four weeks followed by one week of Prolab control diet followed by 5% sodium saccharin in Prolab diet up to 104 weeks; group 7, 0.2% FANFT in AIN-76A diet for four weeks followed by Prolab control diet up to 104 weeks; group 8, AIN-76A control diet for four weeks followed by one week of Prolab control diet followed by 5% sodium saccharin in Prolab diet up to 104 weeks; group 9, 0.2% FANFT in AIN-76A diet for two weeks followed by one week of control Prolab diet followed by 5% sodium saccharin in Prolab diet up to 104 weeks; group 10, 0.2% FANFT in AIN-76A diet for one week followed by one week of control Prolab diet followed by 5% sodium saccharin in Prolab diet up to 104 weeks; group 11, 0.2% FANFT in AIN-76A diet for two weeks followed by one week of control Prolab diet followed by control Prolab diet up to 104 weeks; group 12, 0.1% FANFT in AIN-76A diet for two weeks followed by one week of Prolab control diet followed by 5% sodium saccharin in Prolab diet up to 104 weeks; group 13, 0.1% FANFT in AIN-76A diet for one week followed by one week of control Prolab diet followed by 5% sodium saccharin in Prolab diet up to 104 weeks; and group 14, 0.1% FANFT in AIN-76A diet for two weeks followed by control Prolab diet up to 104 weeks. The number of rats alive after 50 weeks of the experiment, when the first bladder tumour was detected, was considered to be the effective number of rats. A significant decrease in body weight and a significant effect on survival were seen only in group 3. Transitional-cell carcinomas of the urinary bladder were observed in 2/27 (7.4%), 5/30 (17%), 0/22, 9/30 (30%), 3/30 (10%), 9/30 (30%), 3/29 (10%), 1/30 (3.3%), 0/30, 0/29, 0/30, 2/30 (6.7%), 0 and 1/29 (3.5%) rats, respectively. In addition, papillomas were observed in 1, 2, 0, 3, 2, 1, 0, 1, 0, 0, 1, 0, 0 and 1 rats, respectively. The authors concluded that sodium saccharin promotes bladder cancer when administered to male rats in Prolab diet but not when administered in AIN-76A diet (Okamura *et al.*, 1991). [The Working Group noted the absence of a group administered AIN-76A diet only throughout the experiment. They also noted the lack of bladder tumours in rats given sodium saccharin in AIN-76A diet (group 3), whereas two rats given sodium saccharin in Prolab diet (group 8) had one papilloma and one carcinoma of the bladder.]

Groups of 40 male Fischer 344 rats, six weeks of age, were treated in two phases, the first phase lasting six weeks and the second phase the subsequent 72 weeks. Chemicals were administered in Prolab 3200 diet in groups 1–18 and in NIH-07 diet in groups 19–20. Groups 1–14, 19 and 20 were treated with 0.2% FANFT in the diet and groups 15–18 with control diet in the first phase. The dietary treatments in the second phase were as follows: group 1, 5% sodium saccharin; group 2, 3% sodium saccharin; group 3, 5.2% calcium saccharin; group 4, 3.12% calcium saccharin; group 5, 4.21% saccharin; group 6, 2.53% saccharin; group 7, 5% sodium ascorbate; group 8, 4.4% ascorbic acid; group

9, 5% sodium saccharin plus 1.15% calcium carbonate; group 10, 5.2% calcium saccharin plus 1.34% sodium chloride; group 11, 5% sodium saccharin plus 1.23% ammonium chloride; group 12, 1.15% calcium carbonate; group 13, 1.34% sodium chloride; group 14, control diet; group 15, 5% sodium saccharin; group 16, 5.2% calcium saccharin; group 17, 4.21% acid saccharin; group 18, control diet (untreated controls); group 19, 5% sodium saccharin; and group 20, control diet. The experiment was terminated at the end of 78 weeks. The effective number of rats in each group was considered to be 40, but there were 39 in groups 1, 14, 16 and 18. In comparison with the untreated controls (group 18), all groups except groups 12, 14 and 20 showed decreased body-weight gain. The numbers of rats with urothelial bladder carcinomas were 38, 29, 21, 20, 19, 13, 30, 16, 37, 34, 11, 16, 27, 12, 1, 0, 0, 2, 38 and 21, respectively. The numbers of rats with carcinomas plus papillomas in the various groups were 39, 34, 27, 22, 24, 19, 32, 21, 38, 37, 16, 21, 32, 17, 1, 0, 0, 2, 38 and 26, respectively. Statistically significant enhancement of the incidence of bladder cancer was seen with sodium saccharin, calcium saccharin, sodium ascorbate, sodium saccharin plus calcium carbonate, calcium saccharin plus sodium chloride and sodium chloride. Sodium saccharin enhanced the bladder tumour incidence when given in either the Prolab or the NIH-07 diet. The authors noted that the increased urinary pH resulting from the administration of 0.2% FANFT in the diet remained after FANFT treatment was discontinued. Sodium saccharin, calcium saccharin and acid saccharin by themselves did not significantly increase the incidence of bladder tumours or other lesions, except for simple hyperplasia in the group given sodium saccharin (Cohen *et al.*, 1991a). [The Working Group noted the limited period of administration of the test compound for evaluating single-generation carcinogenic effects.]

3.4.5 *Combinations of* N-*[4-(5-nitro-2-furyl)-2-thiazolyl]formamide, cyclophosphamide, freeze-ulceration and sodium saccharin*

Male Fischer 344 rats, five weeks of age, were treated as follows: group 1 (23 rats), freeze-ulceration of the urinary bladder on day 0, followed immediately by administration of 0.2% FANFT in powdered diet for two weeks and then 5% sodium saccharin for 102 weeks; group 2 (23 rats), freeze-ulceration of the urinary bladder on day 0, followed immediately by 0.2% FANFT in powdered diet for two weeks and then control diet for 102 weeks; group 3 (23 rats), freeze-ulceration of the urinary bladder on day 0, two weeks of control diet and then 5% sodium saccharin in the diet for 102 weeks; group 4 (23 rats), freeze-ulceration on day 0 followed by 104 weeks of control diet; group 5 (21 rats), 0.2% FANFT in powdered diet for two weeks followed by 102 weeks of 5% sodium saccharin in the diet; group 6 (22 rats), 0.2% FANFT in powdered diet for two weeks followed by control diet for 102 weeks; group 7 (21 rats), control diet for two weeks followed by 5% sodium saccharin in the diet for 102 weeks; group 8 (23 rats), 0.2% FANFT in powdered diet for two weeks followed immediately by freeze-ulceration of the urinary bladder and 5% sodium saccharin in the diet for 102 weeks; group 9 (23 rats), 0.2% FANFT in the diet for two weeks followed by freeze-ulceration of the

urinary bladder and control diet for 102 weeks; group 10 (23 rats), freeze-ulceration on day 0 followed by 104 weeks of 5% sodium saccharin in the diet; group 11 (26 rats), intraperitoneal injection of 100 mg/kg bw cyclophosphamide in distilled water on day 0 followed immediately by 0.2% FANFT in the diet for two weeks and then 102 weeks of 5% sodium saccharin in the diet; group 12 (26 rats), intraperitoneal injection of 100 mg/kg bw cyclophosphamide followed immediately by 0.2% FANFT in the diet for two weeks and then control diet for 102 weeks; group 13 (25 rats), intraperitoneal injection of 100 mg/kg bw cyclophosphamide followed by two weeks of control diet and then 5% sodium saccharin for 102 weeks; group 14 (26 rats), intraperitoneal injection of 100 mg/kg bw cyclophosphamide followed immediately by 104 weeks of control diet; group 15 (25 rats), intraperitoneal injection of 100 mg/kg bw cyclophosphamide followed immediately by 104 weeks of 5% sodium saccharin; group 16 (37 rats), 104 weeks of control diet (untreated control group); group 17 (21 rats), 5% sodium saccharin for 104 weeks. The experiment was terminated after 104 weeks. The survival rates of groups 12, 14 and 15 were significantly decreased, with 19, 16, 16, 20, 18, 16, 16, 19, 16, 16, 17, 0, 14, 4, 9, 28 and 14 rats alive at the end of the experiment in the various groups, respectively. The effective number of rats was considered to be the number alive after 76 weeks of the experiment, when the first bladder tumour was detected. The incidences of bladder carcinomas were 4/23 (17%), 0/22, 5/20 (25%), 0/23, 1/21 (5%), 0/19, 0/17, 8/22 (36%), 2/21 (10%), 2/21 (10%), 4/22 (18%), 0/9, 3/17 (18%), 0/7, 3/17 (18%), 0/32 and 0/20, respectively. The numbers of rats with urinary bladder papillomas were 1, 1, 2, 3, 2, 1 and 3, in groups 3, 8, 10, 11, 13, 14 and 15, respectively. Sodium saccharin enhanced the incidence of bladder tumours when administered after FANFT, cyclophosphamide or freeze-ulceration, but sodium saccharin alone administered at a concentration of 5% in the diet to rats for 102 or 104 weeks did not produce benign or malignant tumours of the urinary bladder (Cohen *et al.*, 1982).

3.4.6 N-*Nitrosobutyl*-N-*(4-hydroxybutyl)amine or* N-*nitrosodibutylamine*

Groups of 29–32 male and 29–32 female Fischer 344 rats, six weeks of age, were treated with 0.01% *N*-nitrosobutyl-*N*-4-(hydroxybutyl)amine (NBHBA) in the drinking-water for four weeks (groups 1–5) or received no treatment (groups 6–10), followed by 32 weeks of sodium saccharin in the diet (purity, > 99.5%, containing 7 ppm *ortho*-toluenesulfonamide) at concentrations of 5% (groups 1 and 6), 1% (groups 2 and 7), 0.2% (groups 3 and 8) or 0.04% (groups 4 and 9) or untreated diet (groups 5 and 10), respectively. All rats were killed at the end of 36 weeks. No bladder lesions were seen in the groups without pretreatment with NBHBA. In males, the incidences of papillomas were 6, 10, 8, 11 and 8, respectively, for groups 1–5. The incidences of papillary or nodular hyperplasia were 24, 19, 14, 12 and 11, respectively, which was significant ($p < 0.001$) only at the 5% dose when compared with NBHBA only. The number of hyperplastic lesions per 10 cm of basement membrane was also significantly increased at the 5% dose. Papillomas were seen in two females in group 2 and one in each of groups 1 and 3–5. The incidences of papillary or nodular hyperplasia were 23, 17, 14, 11 and 9

in groups 1–5, respectively; the increases were statistically significant only at 1 and 5%. The authors concluded that sodium saccharin caused dose-related promotion in both males and females, only for hyperplastic lesions and not for bladder papillomas (Nakanishi *et al.*, 1980a). [The Working Group noted the lack of effect on bladder tumours as an end-point.]

Male Wistar rats, eight weeks of age, were treated with 0.01% NBHBA in the drinking-water (groups 1–4) or with untreated drinking-water (groups 5–8) for four weeks and then received the following treatments for 32 weeks: 5% sodium saccharin (purity, > 99.5% with 7 ppm *ortho*-toluenesulfonamide) in the diet (groups 1 and 5); 0.1% caffeine in the drinking-water (groups 2 and 6); 5% sodium saccharin in the diet plus 0.1% caffeine in the drinking-water (groups 3 and 7); or untreated drinking-water and diet (groups 4 and 8). The authors stated that the water consumption was increased by approximately 50% in the groups treated with sodium saccharin when compared with other groups. No difference in growth or survival in the various groups was described. Rats that were not pretreated with NBHBA had no bladder tumours, but 5/26 rats treated with sodium saccharin (group 5) and 7/23 given sodium saccharin plus caffeine (group 7) had papillary or nodular hyperplasia compared with 0/12 in group 8. In group 1, 1/31 rats had a bladder carcinoma; and the incidences of bladder papillomas in groups 1–4 were 9/31 (29%), 6/28 (21%), 4/30 (13%) and 3/23 (13%), respectively; these were not statistically significantly different from each other. There were significant increases in the incidences and numbers of papillary/nodular hyperplasia in groups 1 and 3 compared with group 4. In a second experiment with 0.001% NBHBA, combinations of NBHBA, sodium saccharin and caffeine were administered simultaneously, with NBHBA and caffeine in the drinking-water and sodium saccharin in the diet. The treatments were as follows: group 1, NBHBA plus sodium saccharin plus caffeine; group 2, NBHBA plus sodium saccharin; group 3, NBHBA plus caffeine; group 4, NBHBA; group 5, sodium saccharin plus caffeine; group 6, sodium saccharin; group 7, caffeine; and group 8, no added chemical (untreated controls). The incidences of bladder papillomas were 9/32 (28%) and 10/24 (42%) in groups 1 and 2, respectively, compared with 0/22 in BBN controls, and the incidences of bladder carcinoma were 1/32 (3.1%), 2/24 (8.3%) and 0/22, respectively. No bladder tumours were induced in the other groups (Nakanishi *et al.*, 1980b). [The Working Group noted that the increased water consumption associated with sodium saccharin in the diet resulted in increased exposure of groups 1 and 2 to NBHBA, and this might have accounted for the increased incidences of tumours and other bladder proliferative lesions in these groups.]

Groups of 30 male Fischer 344 rats, six weeks of age, were treated with 0.01% NBHBA in the drinking-water for four weeks followed by either 5% sodium saccharin or 0.5% sodium saccharin in the diet for 34 weeks; a further group received NBHBA followed by basal diet, and additional groups received the same treatments after four weeks of untreated drinking-water. The body-weight gain of rats treated with 5% sodium saccharin with or without NBHBA was slightly decreased, but there was no apparent effect on survival. No bladder lesions were seen in rats that were not pretreated with

NBHBA. The incidences of bladder papillomas were 20% with NBHBA plus 5% sodium saccharin, 33% with NBHBA plus 0.5% sodium saccharin and 33% with NBHBA alone, and the incidences of papillary or nodular hyperplasia were 83%, 47% and 45%, respectively (Fukushima *et al.*, 1983b). [The Working Group noted the lack of effect on bladder tumour incidence and the relatively short duration of the experiment.]

Male Fischer 344 rats, six weeks old, were treated in two phases, the first phase consisting of administration of drinking-water with or without NBHBA for four weeks, followed by treatment with 5% sodium saccharin in the diet or untreated control diet for 32 weeks. The first group received 0.05% NBHBA followed by 5% sodium saccharin; group 2 received 0.05% NBHBA followed by untreated control diet; group 3 received 0.01% NBHBA followed by 5% sodium saccharin in the diet; group 4 received 0.01% NBHBA in the drinking-water followed by untreated control diet; and group 5 received untreated water followed by 5% sodium saccharin in the diet. No bladder tumours were observed in groups 3–5. Twenty of 25 rats in group 1 (80%) and 1/26 (3.8%) rats in group 2 developed bladder carcinomas. The number of papillary or nodular hyperplasias was increased in the group given 5% sodium saccharin after 0.05% NBHBA over that with the same dose of NBHBA alone (100% versus 46%); there were also significant increases in the incidences and numbers of hyperplasias in the rats pretreated with 0.01% NBHBA followed by sodium saccharin (83% versus 39%) (Ito *et al.*, 1983). [The Working Group noted the limited details reported for this experiment.]

Male Fischer 344 rats were treated as follows: group 1, 0.02% AAF in the diet for four weeks followed by 0.05% phenobarbital in the diet for 32 weeks; group 2, AAF followed by 5% sodium saccharin in the diet; group 3, AAF followed by untreated control diet; group 4, 0.01% NBHBA in the drinking-water for four weeks followed by 32 weeks of phenobarbital; group 5, NBHBA followed by 5% sodium saccharin; group 6, NBHBA followed by control diet; group 7, untreated water and diet followed by phenobarbital; group 8, untreated water and diet followed by 5% sodium saccharin in the diet; and group 9, untreated controls. No bladder papillomas were seen in groups 1–3 or 7–9. The incidences of bladder papillomas in groups 4–6 were similar: 9/30 (30%), 6/29 (21%) and 8/28 (29%), respectively. The incidences of papillary or nodular hyperplasia were 14 (47%), 24 (83%) and 11 (39%), respectively, in these groups; additionally, 4/29 (14%) rats in group 2 had papillary or nodular hyperplasia. Liver hyperplastic nodules were observed in 24/24, 27/29, 27/28 and 8/30 rats, respectively, in groups 1–4, with no hyperplastic nodules in groups 5–9. Five rats in group 1 had hepatocellular carcinoma, whereas none were seen in other groups. The authors concluded that phenobarbital enhances liver hyperplasia and sodium saccharin enhances bladder hyperplasia (Ito *et al.*, 1983). [The Working Group noted the lack of effect on bladder tumour incidences and the limited description of the details of this experiment.]

Male Fischer 344 rats, seven to eight weeks of age, received untreated water and control diet (group 1), control diet and 0.02% NDBA in the drinking-water (group 2), 5% sodium saccharin in the diet and untreated drinking-water (group 3) or 5% sodium saccharin in the diet and 0.02% NDBA in the drinking-water (group 4) for a total of 26

weeks with interim sacrifices at four and eight weeks. The end-points of the study were effects on γ-glutamyl transferase-positive foci, hyperplastic nodules and hepatocellular carcinomas in the liver. The number of foci at 26 weeks were 0.31 ± 0.31 (10 rats), 5.03 ± 1.06 (29 rats), 0.23 ± 0.23 (36 rats) and 31.3 ± 4.65 (21 rats), respectively. Similar trends were seen at four and eight weeks. The incidences of hyperplastic nodules were 0/11, 5/29 (17%), 0/36 and 17/21 (81%), respectively, and those of carcinomas were 0, 1 (3%), 0 and 17 (81%), respectively. A major confounding factor in the interpretation of these data is the marked increase in water consumption of rats given sodium saccharin. In group 4, this would have led to a marked increase in the consumption of NDBA, which was administered in the drinking-water, in comparison with group 2; however, the water consumption of these animals was not determined (Pereira *et al.*, 1983). [The Working Group noted the confounding issue of water consumption and therefore the dose of NDBA, suggesting that the effect on liver lesions might have been due to increased NDBA consumption rather than a direct effect of sodium saccharin.]

Groups of 25 male Fischer 344 rats, six weeks of age, were treated for four weeks with 0.01% NBHBA in the drinking-water followed by 32 weeks of 5% sodium saccharin in the diet or control diet for 32 weeks. A third group received no treatment for the first four weeks of the experiment followed by 32 weeks of 5% sodium saccharin in the diet. Rats treated with 5% sodium saccharin had a slight decrease in body-weight gain, but their survival was not affected. Papillomas were seen in five and six rats in groups 1 and 2, respectively, and none in group 3. The incidences of papillary or nodular hyperplasia were 23, 9 and 0 in groups 1–3, respectively (Hagiwara *et al.*, 1984). [The Working Group noted the lack of effect on bladder tumours but the increased incidence of nodular or papillary hyperplasia.]

Male Fischer 344 rats, six weeks old, were treated as follows: groups 1–4 were treated with 0.01% NBHBA in the drinking-water for four weeks; group 5 received no treatment during this period. Group 1 then received consecutive 10-week courses of 5% sodium saccharin, 2% DL-tryptophan and 5% sodium ascorbate in the diet, followed by two weeks of control diet; group 2 received consecutive 10-week courses of sodium saccharin and DL-tryptophan followed by basal diet; group 3 received one 10-week course of sodium saccharin followed by basal diet; and group 5 received consecutive 10-week courses of sodium saccharin, DL-tryptophan and sodium ascorbate followed by basal diet. All rats were killed at the end of 36 weeks. There was an increased incidence of bladder carcinomas, papillomas and papillary or nodular hyperplasia in group 1 but not in the other groups. No bladder lesions were seen in group 5. The incidences of carcinoma were 7/25 (28%), 2/24 (8.3%), 2/25 (8%), 0 and 0, respectively (Sakata *et al.*, 1984). [The Working Group noted the short period of administration of each chemical and the short duration of the experiment.]

Male Fischer 344 rats, six weeks of age, were pretreated with 0.05% NBHBA in the drinking-water for four weeks and then treated with chemicals in the diet for 32 weeks: group 1, untreated controls; group 2, 5% sodium saccharin in the diet; group 3, 5% sodium ascorbate in the diet; group 4, sodium saccharin plus sodium ascorbate in the

diet; group 5, 5% ascorbic acid in the diet; and group 6, sodium saccharin plus ascorbic acid in the diet. All animals were killed at the end of 36 weeks. There was significant (> 10%) reduction in body-weight gain in all treated animals compared with controls. This was particularly marked in groups 4 and 6 in which the body-weight gain was decreased by approximately 20%. The incidences of bladder carcinomas were 0/15, 5/15 (33%), 11/16 (69%), 13/13 (100%), 2/16 (12%) and 1/16 (6.3%), respectively. The authors concluded that there was significant synergy between sodium saccharin and sodium ascorbate and that the significant inhibition seen with ascorbic acid might have been related to the decreased urinary pH (Fukushima *et al.*, 1990). [The Working Group noted the short period of administration and the small number of animals in each group.]

Male Fischer 344 rats, six weeks of age, were treated in two phases. The first phase lasted four weeks and consisted of treatment with either control drinking water (groups 1–4) or drinking-water containing 0.05% NBHBA (groups 5–8). The second phase consisted of 36 weeks of treatment with either control diet (groups 1 and 5), 5% sodium saccharin in the diet (groups 2 and 6), 5% sodium saccharin plus 0.1% nordihydroguaiaretic acid in the diet (groups 3 and 7) or 0.1% nordihydroguaiaretic acid in the diet (groups 4 and 8). The groups fed sodium saccharin plus nordihydroguaiaretic acid had significant growth retardation, but survival was not affected. No bladder hyperplastic lesions or tumours were seen in groups 1–4 (11 rats per group), which were not pretreated with NBHBA. In groups 5–8, the incidences of bladder carcinoma were 1/20 (5%), 2/23 (8.7%), 1/22 (4.5%) and 4/20 (20%), respectively; the incidences of papillomas were 2, 5, 0 and 3, respectively; and the incidences of papillary or nodular hyperplasia were 6, 19, 12 and 7, respectively. The authors concluded that nordihydroguaiaretic acid had anti-tumour promoting activity (Yu *et al.*, 1992). [The Working Group noted the inadequate number of animals, the short duration of the experiment and the lack of effect based on incidences of carcinoma.]

3.4.7 *Urethane*

In a bioassay to screen for lung adenomas, groups of 25 male A/St mice, eight weeks of age, were treated with one of four commercial saccharin preparations (Sweeta tablets, pharmaceutical powder, Sweet 10 liquid or Sweet-n-Low powder). The saccharin preparations were made up freshly each week in distilled water and administered at a dose of 1 g/kg bw daily by gavage on five days a week for a total of 17 weeks. Three groups of controls received distilled water. In the first series, no treatment other than saccharin or water was administered. In a second series, the mice also received an intraperitoneal injection of 0.1 mg/g bw urethane in 0.9% saline; the third group also received an intraperitoneal injection of 1 mg/g bw urethane. The urethane injections were given one week after initiation of saccharin administration. The incidences and numbers of adenomas per lung were determined; a few lungs from each group of mice were examined microscopically to confirm that the adenomas were being counted. The animals generally survived the treatment well, most of the deaths being due to trauma induced by gavage. The animals exposed to saccharin preparations gained somewhat less weight

than the control animals during the course of this experiment, but their weight gain was substantial. The commercial saccharin preparations did not elicit a significant lung tumour response when given alone, with respect to either the incidence or number of lung tumours per mouse. At the low dose of urethane, the only sweetener that produced a lung tumour response greater than that in animals treated with urethane alone was Sweet-n-Low, which produced an approximately twofold increase in the number of lung tumours, although the incidence (47%) was the same as in the control group (52%) and was not statistically significant. At the high dose of urethane, all of the saccharin preparations significantly increased the number of lung tumours per mouse over that in animals treated with urethane alone: 2.13 ± 0.44, 4.36 ± 0.70, 5.95 ± 1.29, 6.08 ± 1.29 and 9.86 ± 3.65 in mice treated with 1 mg/g urethane plus either distilled water, Sweeta tablets, pharmaceutical powder, Sweet 10 liquid or Sweet-n-Low powder, respectively. The tumour incidences, however, were nearly the same and were not statistically different between the groups (Theiss *et al.*, 1980). [The Working Group noted the complex nature of the protocol, the lack of effect at the low dose of urethane and the lack of effect on incidence at the high dose of urethane and that there was no effect when the sweetener preparations were used without administration of urethane.]

3.4.8 N-*Methyl*-N′-*nitro*-N-*nitrosoguanidine*

Groups of 20 treated and 40 control male Wistar rats, seven weeks of age, were given 10 mg/L *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine (MNNG) in the drinking-water and 10% sodium chloride in the diet for eight weeks (groups 1–5); groups 6–10 received neither MNNG nor sodium chloride during the first eight weeks. After these treatments, the rats in groups 1 and 6 were continued as the respective control groups without any further dietary supplementation for the remaining 32 weeks of the experiment (total experimental period of 40 weeks), animals in groups 2 and 7 were fed 10% sodium chloride in the diet, animals in groups 3 and 8 were fed 5% saccharin [form not specified] in the diet, groups 4 and 9 were fed 0.05% phenobarbital in the diet, and groups 5 and 10 received 1% aspirin in the diet. The growth of the animals was significantly retarded by the administration of MNNG plus sodium chloride; these animals showed a compensatory increase in growth rates once the treatments ended, although their weights at the end of the experiment were still lower than those in the groups not treated with MNNG. During the second phase of the experiment, the growth was similar, except for rats receiving the aspirin-supplemented diet, which showed a marked retardation of growth compared with the other groups. No gastric or duodenal tumours occurred in the rats that were not pretreated with MNNG plus sodium chloride. The incidences of gastric adenocarcinomas were 3/39 (7.7%), 4/20 (20%), 3/20 (15%), 1/19 (5.3%) and 0/20, respectively, for groups 1–5, and 3, 3, 1, 1 and 1 rats had duodenal adenocarcinomas, respectively. Only sodium chloride produced a significantly increased incidence in the total number of rats bearing tumours, including those of the stomach plus duodenum. In saccharin-treated rats, diffuse alterations in the fundic mucosa were observed, in which the thickness of the zone of proliferating cells was increased (Takahashi *et al.*, 1984).

3.4.9 *Betel nut*

Male and female C17 mice, 10–12 weeks of age, were either untreated (22 females and 12 males), received a diet containing 10% of a commercial preparation of saccharin-coated betel nut [no information on form or amount of saccharin] (18 females and 14 males), received 0.2 mL of a 0.1% solution of 1, 4-dinitrosopiperazine by oral intubation (14 females and 15 males) or received saccharin-coated betel nut powder in the diet plus 1,4-dinitrosopiperazine by gavage (12 females and 12 males). The mice were treated for 40 weeks and then allowed to live a normal life span on standard diet. There was no significant difference in mortality rate between the groups [overall survival was not indicated]. The incidences of squamous-cell carcinoma of the stomach were 0, 2, 7 and 6 in males and 0, 0, 4 and 3 in females. Reticular-cell neoplasms-type A of the uterus occurred in 2, 3, 5 and 3 female mice in groups 1–4, respectively (Pai *et al.*, 1981). [The Working Group noted the inadequate numbers of animals, the incomplete reporting of the experiment, the lack of information on exposure to saccharin, the lack of a group given saccharin only and the lack of information about the form of saccharin administered.]

3.4.10 *Freeze-ulceration*

Eight groups of 40 male Fischer rats, five weeks of age, were treated as follows: group 1, freeze-ulceration of the urinary bladder followed immediately by 5% sodium saccharin in powdered diet for 104 weeks; group 2, freeze-ulceration of the urinary bladder followed by two weeks of control diet and then 102 weeks of 5% sodium saccharin in the diet; group 3, freeze-ulceration of the urinary bladder followed by four weeks of control diet and then 100 weeks of 5% sodium saccharin in the diet; group 4, freeze-ulceration of the urinary bladder followed by six weeks of control diet and then 98 weeks of 5% sodium saccharin in the diet; group 5, freeze-ulceration of the urinary bladder followed by 18 weeks of control diet and then 86 weeks of 5% sodium saccharin in the diet; group 6, 104 weeks of 5% sodium saccharin in the diet; group 7, freeze-ulceration of the urinary bladder followed by 104 weeks of control diet; and group 8, 104 weeks of control diet (untreated control group). None of the treatments had a significant effect on survival or on body weight. The experiment was terminated after 104 weeks. The incidences of urothelial carcinomas were 11/36, 6/36, 12/40, 7/36, 9/39, 0/39, 1/39 and 0/39 in the eight groups, respectively. In addition, papillomas were found in two rats in group 2 and one rat in group 6. There were no significant differences in the incidences of bladder tumours between groups 1–5. Only one benign papilloma developed in the urinary bladder of rats fed 5% sodium saccharin alone throughout the experiment (group 6) (Hasegawa *et al.*, 1985).

Saccharin/cyclamate mixtures

Oral administration

Single-generation exposure

Rat: Two groups of 52 male and 52 female Sprague-Dawley rats aged 70–90 days, were given a 10:1 mixture of sodium cyclamate:sodium saccharin at a concentration of 2 or 5%

in the diet for up to 30 months. The cyclamate in the mixture contained less than 4 mg/kg cyclohexylamine, but no information on the purity of the saccharin was given. An untreated group of 52 male and 52 female rats served as controls. At 24 months, approximately 10% of the initial number of animals were still alive. The occurrence of bladder parasites (*Strongyloides capillaria*) was noted in 16% of animals. No bladder tumour was found. A similar frequency of benign neoplasms was seen in all groups, consisting of fibromas, fibroadenomas or adenomas of the mammary gland in females and thymomas in males (Schmähl, 1973).

In a study reported in an abstract, two groups of 54–56 Wistar rats [age unspecified] received 0 or 2.5 g/kg bw per day of a mixture of sodium cyclamate:sodium saccharin (10:1) [source and purity unspecified] in the diet for 28 months. Ten to 16 rats of each group were killed at 12 months, 11 at 24 months and all survivors at 28 months. No treated or control animals developed tumours of the urinary bladder (Furuya *et al*., 1975). [The Working Group noted the incomplete reporting of the experiment.]

Groups of 35 male and 45 female weanling FDRL strain Wistar-derived rats were fed a 10:1 mixture of sodium cyclamate:saccharin [purity and method of manufacture unspecified] in the diet at concentrations providing doses of 0, 500, 1100 or 2500 mg/kg bw per day for two years. From week 79, the original groups were split, and 50% of the survivors in each group, except the untreated controls, received cyclohexylamine hydrochloride in the diet: at 25 mg/kg bw for the group receiving 500 mg/kg bw per day of the mixture, at 56 mg/kg bw for the group receiving 1100 mg/kg bw per day of the mixture and at 125 mg/kg bw for those receiving 2500 mg/kg bw per day of the mixture. The mortality rates were similar in control and test groups. Treatment-related pathological changes were seen only in the kidney and bladder. Pelvic hyperplasia was observed more often in the treated groups (8/80, 21/80 and 16/80, as compared with 3/80 in controls). Among animals surviving more than 49 weeks, 9/25 male and 3/35 female rats at 2500 mg/kg bw per day and 0/35 male and 0/45 female controls developed transitional-cell carcinomas of the urinary bladder. Of the treated rats with this tumour, three male and two female rats had received cyclohexylamine. Two of the bladder carcinoma-bearing animals had calculi, and 18 rats at 2500 mg/kg bw per day had non-malignant proliferative bladder lesions. Non-malignant proliferative lesions were found at the lower doses, but their incidence was not significantly higher than that in controls. Renal calcification was seen in 7/12 rats with bladder carcinomas; *Trichosomoides crassicauda* infection was present in one rat with bladder cancer and in four rats with non-neoplastic proliferative lesions at the highest dose, in four given 1120 mg/kg bw per day, in two given 500 mg/kg bw per day and in five control animals (Price *et al*., 1970; Oser *et al*., 1975).

Multigeneration exposure

Mouse: In a multigeneration study, a 10:1 mixture of sodium cyclamate:saccharin was fed at concentrations of 5 and 0.5% or 2 and 0.2%, respectively, in the diet continuously to Swiss mice over six generations. The saccharin contained 0.5% *ortho*-toluenesulfonamide; the cyclamate contained 2.1 mg/kg (ppm) cyclohexylamine. F_0 (parental), F_{3b} and F_{6a}

generations, consisting of 50 males and 50 females each, were used for the studies of carcinogenicity and were treated for 84 weeks. Pathological alterations and urinary bladder calculi occurred with similar frequencies in control and treated groups. Four neoplasms of the urinary bladder occurred: three anaplastic carcinomas (one in a female control of the F_0 generation and two in females of the F_0 and F_{6a} generations fed 2% cyclamate plus 0.2% saccharin) and one papilloma (in a male of the F_{6a} generation given 2% cyclamate plus 0.2% saccharin). The mean latent period was more than 80 weeks (Kroes *et al*., 1977).

Rat: Four groups of 10 male and 20 female Sprague-Dawley rats were treated either with a cyclamate:saccharin (ratio 10:1) mixture at a total concentration of 5% or 2% of the diet, with a diet containing 20% sugar or with untreated diet for three months prior to mating. The F_0 females were continued on these diets throughout weaning of their offspring. Groups of 33–39 male and 34–39 female offspring (F_1 generation) were maintained on the respective diets for lifetime. Their body weights after one year were reduced when compared with controls. The average survival was 532, 480, 323 and 379 days for males and 530, 683, 450 and 464 days for females given 5% and 2% of the mixture or sugar and the control groups, respectively. A single bladder tumour (a papilloma) was found in one female rat given the 2% cyclamate:saccharin mixture, at 308 days of age. No other tumours of the urinary tract were noted, and there were no increased incidence of tumours at other sites. Calculi were observed in the bladder of two of the animals receiving the 5% mixture [sex not specified] and in the renal pelvis of eight of the rats at the high dose [sex not specified]. One rat [sex not specified] at 2% had a kidney stone, but no stones were seen in the sugar-treated animals or in the controls (Schmähl & Habs, 1984). [The Working Group noted the incomplete reporting of the experiment.]

ortho-Toluenesulfonamide

Oral administration

Rat: In a two-generation study, groups of Charles River CD rats, 30 days of age, were fed daily doses of 0 (control), 2.5, 25 or 250 mg/kg bw *ortho*-toluenesulfonamide (purity, > 99.9%) or 250 mg/kg bw *ortho*-toluenesulfonamide plus 1% ammonium chloride in the drinking-water. Each group consisted of 50 males and 50 females, except for the group receiving ammonium chloride in the drinking-water, which comprised 40 males and 38 females. The F_0 animals were started on test at 32 days of age. After three months on test, the animals were mated on a one-to-one basis; all litters were culled randomly to eight pups (four males and four females) four days *post partum*. The pups were weaned onto their parents' diet, and 50 males and 50 females were selected randomly from each group to constitute the second generation (F_1). The two generations remained on test for 30 (F_1) and 32 (F_0) months. The animals were free of bladder parasites. Rats of both generations given 250 mg/kg bw *ortho*-toluenesulfonamide with or without ammonium chloride had lowered feed consumption. There were no treatment-related effects on longevity. The numbers of bladder tumours (all of which were benign) were: in F_0 males, one in a control and one each at 2.5 and 250 mg/kg bw *ortho*-toluenesulfonamide; F_0 females, one at 2.5 mg/kg bw; in F_1 females, two at 2.5 mg/kg bw (Arnold *et al*., 1980).

Groups of 38 male and 38 female Sprague-Dawley rats, three months of age, were given daily doses of 0, 20 or 200 mg/kg bw *ortho*-toluenesulfonamide [source and purity unspecified] for life by adjusting the concentrations added to the diet. The average survival rates were 700 days for controls, 770 days at the low dose and 840 days at the high dose. The total incidences of malignant tumours were not different in treated groups compared with controls. Lymphosarcomas developed in 7/71 controls, 10/75 at the low dose and 10/76 at the high dose. In addition, 3/76 leukoses occurred at the high dose and 5/75 at the low dose, compared with 0/71 in controls. In rats at the high dose, 1/76 carcinoma and 4/76 papillomas of the bladder were found after 759–996 days [$p = 0.03$]; in those at the low dose, 3/75 papillomas of the bladder occurred after 539, 766 and 873 days. No bladder tumours occurred in 71 controls (Schmähl, 1978). [The Working Group noted that the presence or absence of bladder parasites was not recorded, and the sexes of animals with bladder tumours were not specified.]

Three groups of 63, 63 and 50 female Wistar rats [age unspecified] were given pure *ortho*-toluenesulfonamide at a concentration of 0 or 0.1% in the drinking-water or 79 mg/kg bw in the diet for two years. The survival rates were similar in all groups at 84 weeks. No difference in overall tumour incidence was observed between control and test groups. No bladder tumours were observed in any group, although mild diffuse urothelial hyperplasia was found in 1/50 rats fed *ortho*-toluenesulfonamide in the diet (Hooson *et al*., 1980).

Administration with known carcinogens

Three groups of 63 female Wistar rats [age unspecified] were given a single intravesicular dose of 0.15 mL of a saturated solution of MNU in saline. Two weeks later, pure *ortho*-toluenesulfonamide was administered at a concentration of 0 or 0.08 mg/kg bw in the diet or 0.1% in the drinking-water for two years. The survival rates were similar in all groups at 84 weeks. No difference in overall tumour incidence was seen between control and test groups. Neoplasia and hyperplasia of the bladder occurred in 27% and 35%, respectively, of rats given MNU alone, but no statistically significant increase in bladder neoplasia or hyperplasia was observed in groups given MNU and *ortho*-toluenesulfonamide (Hooson *et al*., 1980).

4. Other Data Relevant to an Evaluation of Carcinogenicity and its Mechanisms

4.1 Absorption, distribution, metabolism and excretion

4.1.1 *Humans*

Six women with an average oral daily intake of 100–300 mg saccharin [form not specified] had maximum plasma concentrations after 0.5–1 h and an elimination half-life of 7.5 h (Colburn *et al*., 1981).

In three adult men given an intravenous bolus of 10 mg/kg bw sodium saccharin, the plasma concentration–time curve fitted a two-compartment open model with a terminal half-life of 70 min. There was no indication of metabolism, since the plasma clearance value was slightly less than that for renal clearance and saccharin was recovered in the urine quantitatively. Administration of probenecid decreased the elimination rate, suggesting renal tubular organic anion transport. After oral administration of 2 g sodium saccharin, 85% was adsorbed, as determined by recovery in the urine and by the concentration in plasma (Sweatman *et al*., 1981).

Three groups of five men were given sodium saccharin in single oral doses of 50, 150 or 333 mg/60 kg bw. Peak plasma concentrations occurred between 30 and 60 min after dosing, and 60 and 76% was excreted unchanged in urine at 6 and 24 h, respectively (Pantarotto *et al*., 1981).

Saccharin was detected in the sera and urine of six women aged 19–40 who had consumed saccharin in their diets during the last month of pregnancy, and was also found in the serum of the cord blood of the newborns, at a limit of detection of 20 ng/mL. The daily intake during the last month of pregnancy had been 25–100 mg (Cohen-Addad *et al*., 1986).

After administration of 1-g doses of soluble (sodium) saccharin [form not specified] to three men, saccharin was excreted in the urine quantitatively unchanged by two of the subjects within 48 h. In a subsequent experiment involving six subjects, none excreted the dose quantitatively within 72 h, but no metabolism of saccharin was detected (McChesney & Goldberg, 1973).

Within 48 h, 92% of a dose of 500 mg [^{14}C]saccharin taken by six male volunteers was excreted in the urine and 5.8% in the faeces. Analysis of urine and faeces by high-performance liquid chromatography and thin-layer chromatography revealed only unmetabolized saccharin (Byard *et al*., 1974).

One female and two male volunteers excreted 85–92% of a dose of 1g [3-^{14}C]-saccharin unchanged in the urine within 24 h, before or after taking 1 g saccharin daily for 21 days; no metabolites were found (Ball *et al*., 1977).

4.1.2 *Experimental systems*

(*a*) *Saccharin*

[^{35}S]Sodium saccharin instilled into the bladder of male rats was absorbed into the plasma (Colburn, 1978).

[^{14}C]Saccharin administered by intravenous infusion to five rhesus monkeys at a dose of 4 μg/kg bw per min for 60 min during the last trimester of pregnancy crossed the placenta rapidly and was distributed in all fetal tissues except the central nervous system. During the infusion period, the fetal blood concentrations were approximately 30% those of the mothers. In contrast to the maternal organism, in which radiolabel disappeared quickly after infusion ended, the fetal compartment showed very slow clearance of saccharin and, 2 h after termination of the infusion, the fetal blood concentrations were higher than the maternal ones. The slow rate of fetal clearance suggests that considerable

accumulation might result from repetitive maternal ingestion. No data were available on the penetration of saccharin into the embryonic compartment during organogenesis (Pitkin *et al*., 1971a).

In male Sprague-Dawley rats fed 5% saccharin [about 2500 mg/kg bw per day] in the diet for 66 days, the plasma concentration showed a twofold diurnal variation. In three male rats given diets containing up to 10% saccharin [about 5000 mg/kg bw per day] in the diet for 22 days and one group of females exposed to 5% saccharin, the concentrations of saccharin were higher in the gut wall, kidneys and urinary bladder than in the plasma. There was evidence of decreased clearance at the highest doses. Probenecid was found to inhibit clearance (Sweatman & Renwick, 1980).

The tissue distribution of saccharin was investigated in two-generation feeding studies in Sprague-Dawley rats. After a single oral dose of 50 mg/kg bw [^{3}H]sodium saccharin dihydrate in late pregnancy, the concentrations of saccharin were lower in fetal than in maternal tissues after 6 and 12 h; however, concentrations were higher in fetal urinary bladder and liver after 24 and 48 h. Dams were exposed to 5% sodium saccharin [2500 mg/kg bw per day] in the diet beginning four weeks before mating and were either killed during late gestation or continued on treatment through the F_1 generation until 22 days of age. The concentration of saccharin in both maternal and fetal bladder walls showed wide interindividual variations, but the average concentration in fetal tissue was about twofold greater. The saccharin content of the stomachs of neonatal animals was very low (0.03%) and almost 200 times below the maternal dietary level. The tissue and urinary levels reflected these low dietary levels, but there was a large increase at 23 days, reflecting separation from the mother. In males, the urinary level increased over 10-fold from the neonatal period to 23 days of age, whereas the increase was somewhat less in females. During several time intervals up to 109 days of age, F_1 animals showed only equivocal evidence of accumulation of saccharin in the bladder when compared with other tissues (Sweatman & Renwick, 1982).

[^{35}S]Saccharin was observed to cross the placenta and enter the fetal circulation of Sprague-Dawley rats after treatment by gavage with 100 mg saccharin mixed with the radiolabelled compound (100 μCi, 266 mCi/mmol) on day 19 of gestation of animals that had been receiving 5% saccharin in the diet since gestation day 14. The concentrations in the fetal blood represented about 0.008% of the total dose up to 5 h after exposure. In contrast, the maternal blood levels during this time were 0.03–0.04% of the total dose (West, 1979).

The recovery of unmetabolized sodium saccharin was determined under conditions simulating one of the two-generation bioassays of carcinogenicity in which positive results were obtained (Arnold *et al*., 1980). Male rats that had been exposed to a 5% acid saccharin diet [about 2500 mg/kg bw per day] *in utero* and maintained on this diet throughout the experiment were given 5% [^{3}H]sodium saccharin in the diet for 24 h when they weighed 290 g and were then returned to the unlabelled diet. Urine and faeces were collected at 24-h intervals and analysed. Almost all of the tritium was eliminated within 48 h, with 13–14% in the faeces and the remainder in the urine. Experiments with

single low doses of [^{3}H]sodium saccharin and dosing after administration of 3-methylcholanthrene gave similar results. All of the urinary tritium was found in saccharin by thin-layer chromatography, with a limit of detection of 0.4% of the administered dose (Sweatman & Renwick, 1979).

The renal excretion of sodium saccharin infused into adult male and female Sprague-Dawley rats was found to exceed that of inulin at all plasma concentrations. Maximal tubular secretion was clearly demonstrable at a concentration of 14–20 mg/100 mL, and there was no evidence of tubular reabsorption. Clearance was inhibited when sodium saccharin and *para*-aminohippurate were infused simultaneously, indicating that excretion occurs via the carrier-mediated tubular organic anion transport system. No differences between male and female rats were found in secretory patterns or urinary saccharin levels (Bourgoignie *et al*., 1980).

Purified [^{14}C]sodium saccharin was administered to six-month-old male and female Osborne-Mendel rats as a single oral dose of 5, 50 or 500 mg/kg bw. Between 0.03 and 0.55% of the radiolabelled sodium saccharin was metabolized to carbon dioxide. In groups of Charles River CD rats fed diets containing 0.01, 0.1 or 1% sodium saccharin for one year before administration of a radiolabelled dose of 5, 50 or 500 mg/kg bw per day, recovery of radiolabel in urine and faeces was 80–96% after seven days, which was slightly lower than the recovery (95–100%) from rats that had not been fed any saccharin before the test dose. Identification of metabolites in the urine showed that > 99% of the sodium saccharin remained as saccharin; two metabolites were reported: *ortho*-sulfamoylbenzoic acid and (probably) benzenesulfonamide. [The Working Group noted that the possibility could not be excluded that these represented contaminants of the batch of sodium saccharin administered.] The highest counts of radiolabel after administration of [^{14}C]saccharin were found in the kidney and urinary bladder, and the metabolic profiles in dogs, rabbits, guinea-pigs and hamsters were similar (Lethco & Wallace, 1975).

Two young male SIV50 Sprague-Dawley-derived rats were given single doses of 372 and 390 mg/kg bw [^{35}S]sodium saccharin by gavage and killed 50 h later. At least 99.6% of the radiolabel was found by thin-layer chromatography to correspond to unmetabolized saccharin. No binding of saccharin to DNA of liver or urinary bladder was found (Lutz & Schlatter, 1977).

Saccharin was not metabolized by liver microsomal preparations or by faecal homogenates taken from rats fed 1% hydrated sodium saccharin [approximately 0.2 g per day] in the diet for two years (Ball *et al*., 1977). The accumulation of saccharin by rat renal cortical tissue incubated *in vitro* was dependent on oxygen and was reduced by metabolic inhibitors, suggesting that saccharin is eliminated by active tubular secretion (Goldstein *et al*., 1978).

Saccharin was excreted rapidly and unchanged in the urine by rhesus monkeys (Pitkin *et al*., 1971b). More than 90% of an administered dose was found within 24 h after dosing in the urine of guinea-pigs; about 70% was found in rat urine and the remainder in the faeces (Minegishi *et al*., 1972). Although saccharin is rapidly excreted by rats, administration of multiple doses per day over several days resulted in accumulation in the bladder;

however, it was completely cleared within three days after removal of saccharin from the diet (Matthews *et al*., 1973).

Another study showed that 90% of [^{14}C]sodium saccharin was excreted unchanged in the urine by rats and monkeys of each sex. Pretreatment with phenobarbital to induce mixed-function oxidase activity or prior intake of daily doses of sodium saccharin for over two years did not influence the metabolism of a single oral dose of [^{14}C]saccharin (Byard & Goldberg, 1973).

(*b*) *Impurities of saccharin*

The rates at which seven intragastrically administered impurities of saccharin (radiolabelled *ortho*-toluenesulfonamide, benz[*d*]isothiazoline-1,1-dioxide, 3-aminobenz[*d*]-isothiazole-1,1-dioxide, 5-chlorosaccharin, toluene-2- and -4-sulfonamides and 2- and 4-sulfamoylbenzoic acid) were eliminated in rats were similar. At doses ranging from 20 to 80 mg/kg bw, 80–95% of the impurities were recovered within 24 h in urine and faeces; urinary metabolites of these impurities were identified (Ball *et al*., 1978; Renwick, 1978; Renwick & Williams, 1978; Renwick *et al*., 1978).

In female Wistar rats given single oral doses of 20, 125 or 200 mg/kg bw [^{14}C]*ortho*-toluenesulfonamide, 79, 58 and 36% of the activity was recovered in 24-h urine samples; elimination at 24–48 h was 7, 14 and 33% of the dose, respectively. Within seven days, 4.5, 5.9 and 7% of the activity was recovered from the faeces. The main metabolites in the urine were 2-sulfamoylbenzyl alcohol and its sulfate or glucuronic acid conjugates (80%), *N*-acetyltoluene-2-sulfonamide (6%), saccharin (3%) and 2-sulfamoylbenzoic acid (2%) (Renwick *et al*., 1978).

In a similar study, 50% of administered *ortho*- and *para*-toluenesulfonamides excreted in urine had been metabolized to *ortho*- and *para*-sulfamoylbenzoic acids, respectively (Minegishi *et al*., 1972).

Low oral doses of 0.2–0.4 mg/kg bw [^{14}C]*ortho*-toluenesulfonamide were excreted more slowly in humans than in rats, an average of 56% of the activity being excreted in the urine within 24 h and almost 90% within 48 h. Less than 1% of the activity was found in faeces. The main urinary metabolites were 2-sulfamoylbenzoyl alcohol (unconjugated, 7%; conjugated with glucuronic acid, 11%; conjugated with sulfate, 20%), saccharin (35%) 2-sulfanoylbenzoic acid (4%) and *N*-acetyltoluene-2-sulfonamide (2%) (Renwick *et al*., 1978).

4.1.3 *Comparison between humans and rodents*

A comparison of the rates of urinary excretion showed that the excretory and metabolic pattern of saccharin in humans is very similar to that observed in animals, most of the ingested saccharin being rapidly excreted in unchanged form (McChesney & Goldberg, 1973). These preliminary observations were confirmed in several subsequent experiments in humans, monkeys and rodents. The elimination half-life was longer in humans (70 min) than rats (30 min) (Sweatman & Renwick, 1980; Sweatman *et al*., 1981). With one exception, all of the published studies indicate that saccharin is not

metabolized. [The Working Group noted that in the one exception the possibility could not be excluded that the other urinary substances were contaminants in the batch of sodium saccharin administered.]

4.2 Toxic effects

4.2.1 *Humans*

Anecdotal evidence of effects in humans was related by Oser (1985) from a description of studies in about 12 male volunteers in the United States Department of Agriculture in the early part of this century, who were fed saccharin as a supplement to their diets for several weeks. The men experienced 'digestive disturbances' and, at even smaller doses, a 'renal effect'.

Idiosyncratic effects of saccharin on the liver were reported in a 70-year-old woman. Increased activities of alanine aminotransferase, aspartate aminotransferase, γ-glutamyl transferase and alkaline phosphatase were found after oral administration of three pharmaceutical drugs of which saccharin was the only common constituent. The drugs were lorazepam, dihydroergocristine and chlordemethyldiazepam. Exposure to saccharin alone reproduced the effects (Negro *et al*., 1994).

As incorporation of sodium saccharin into the diet of experimental animals has been reported to have a rapid, pronounced effect on protein digestion in the intestine, which favours microbial metabolism of dietary tryptophan to indole, a urinary bladder co-carcinogen (Sims & Renwick, 1983), the metabolism of tryptophan was studied in 15 human subjects by analysing their daily urinary excretion of indican—a further metabolite of indole—before, during and after ingestion of sodium saccharin. The excretion of indican varied widely in both the control and the experimental groups and was not significantly affected by doses of 1 g sodium saccharin per day for one month (Roberts & Renwick, 1985).

4.2.2 *Experimental systems*

The LD_{50} values for sodium saccharin by oral administration are: mice, 18 g/kg bw; random-bred rats, 17 g/kg bw; Wistar rats, 14 g/kg bw (Taylor *et al*., 1968); and hamster, 8.7 g/kg bw in males and 7.4 g/kg bw in females (Althoff *et al*., 1975). The LD_{50} by intraperitoneal injection is 6.3 g/kg bw in mice and 7.1 g/kg bw in random-bred rats (Taylor *et al*., 1968).

(*a*) *Cell proliferation*

In a two-generation study, the parents of the F_0 generation of Charles River CD rats were fed diets containing 0, 0.01, 0.1, 1, 5 or 7.5% sodium saccharin [0, 5, 50, 500, 2500 or 3750 mg/kg bw per day] from the weanling stage through mating and gestation to weaning of their litters. Of the F_1 generation, 48 animals per dose per sex were exposed to the same concentrations of sodium saccharin for life. The authors reported a statistically significant increase in urinary bladder hyperplasia only in female rats of the F_1 generation exposed to 7.5% sodium saccharin. The hyperplasia found was focal rather

than diffuse and not morphologically precancerous. The incidence of hyperplasia in males was not statistically significantly increased, possibly because of a high incidence of hyperplasia in the male controls. A statistically significant increase in the incidence of urinary bladder neoplasms was found in males fed 7.5% sodium saccharin (Taylor *et al*., 1980).

In a two-generation bioassay of sodium saccharin, 2500 F_1 male Charles River CD rats were divided into groups of 700 to 125 animals, the group at the lowest dose having the largest number. Dietary administration of 1, 3, 4, 5, 6.25 or 7.5% sodium saccharin [500, 1500, 2000, 2500, 3125 or 3750 mg/kg bw per day] was begun at six weeks for males and females of the F_0 generation; the F_1 generation was fed the same diet as the F_0 generation until the terminal kill at 30 months of age. The incidences of simple, papillary and nodular hyperplasia were analysed. Sodium saccharin increased the incidence of the combination of 'any hyperplasia' at 5, 6.25 and 7.5%, and the incidence in the last two groups achieved statistical significance (Schoenig *et al*., 1985; re-examined by Squire, 1985).

No simple, papillary or nodular hyperplasia or papilloma was found among groups of 10-week-old male and female inbred Charles River Fischer 344 rats fed 0.04, 0.2, 1 or 5% sodium saccharin [20, 100, 500 or 2500 mg/kg bw per day] in a stock diet for 32 weeks (Nakanishi *et al*., 1980a). In another experiment, two groups of 10-week-old male Wistar rats were exposed to 5% sodium saccharin in the diet for 36 weeks. In comparison with controls, which had no hyperplasia, sodium saccharin produced simple hyperplasia in 10/26 (38%) of one group and 11/21 (52%) of the other. The incidence of papillary or nodular hyperplasia was 5/26 (19%) and 9/21 (43%) in the two groups, respectively. The discrepancy between these results may be due to a strain difference in susceptibility (Nakanishi *et al*., 1980b).

In a time-course study, six-week-old male Charles River Fischer 344 rats were exposed to 5% sodium saccharin [2500 mg/kg bw per day] in the diet for up to 18 weeks. Three animals per group were killed for histopathological analysis at 1, 3, 5, 7, 9, 12, 15 and 18 weeks, and DNA synthesis was measured at 1, 5, 9 and 18 weeks by [^{3}H]thymidine uptake and autoradiography. In exposed animals, vacuolar degeneration of the epithelial cells was found at three weeks, followed by simple hyperplasia at five weeks. By nine weeks, the degree of hyperplasia had increased, with a display of mitotic figures, hyperplastic foci and pleomorphic microvilli. Increased [^{3}H]thymidine uptake was seen in the exposed rats at all times, and the increase was five- to eightfold the rate seen in controls (Fukushima & Cohen, 1980).

In a study of dose–response, cell proliferation was measured by autoradiography and scanning electron microscopy in groups of three to four five-week-old male Fischer 344 rats after 10 weeks of exposure to 0.1, 0.5, 1, 2.5 or 5% sodium saccharin [50, 250, 500, 1250 or 2500 mg/kg bw/day] in the diet. A dose-related increase in [^{3}H]thymidine labelling index, hyperplasia and the presence of uniform and pleomorphic microvilli were found. At doses ≤ 1% sodium saccharin, there was no statistically significant increase in the incidence of hyperplasia or in the numbers of foci or cells exhibiting

uniform and pleomorphic microvilli. At 0.1% sodium saccharin, there was no statistically significant change in the labelling index (Murasaki & Cohen, 1981). [The Working Group noted the small number of rats in each group and that subsequent studies showed labelling indices in occasional individual, untreated control rats that were similar to those seen in this study in rats treated with 1% sodium saccharin.]

Feeding of 7.5% sodium saccharin [3750 mg/kg bw per day] in the diets of male Sprague-Dawley rats from three weeks of age did not increase ^{3}H-thymidine incorporation in the DNA of urinary bladder epithelium at 1, 2, 3, 6, 10, 15, 20, 30 or 50 weeks after the beginning of exposure. Autoradiographic analysis of the thymidine labelling index at 1, 15 and 50 weeks also did not show statistically significant increases in sodium saccharin-exposed animals when compared with controls (Lawson & Hertzog, 1981).

The effects of 5% sodium saccharin [2500 mg/kg bw/day] in the diet for 20 weeks from six weeks of age were compared in male Fischer 344 rats, B6C3F1 mice, Syrian golden hamsters and Hartley guinea-pigs. Rats developed hyperplasia of the urinary bladder and significantly increased DNA synthesis at 20 weeks, as determined by [^{3}H]-thymidine autoradiography, but the other species did not have similar changes. These results indicated to the investigators that the urothelial-cell proliferative response to high doses of sodium saccharin was specific to the rat. In a comparative study with males of four strains of rats (ACI, Wistar, Fischer 344 and Sprague-Dawley), the ACI strain was reported to be the most susceptible to the effects on the urinary bladder of 5% sodium saccharin in the diet for 52 weeks (Fukushima *et al*., 1983a).

In male Fischer 344 rats, the degree of hyperplasia and microvillus formation induced by freeze-ulceration in the bladder epithelium was similar during the subsequent two-week period whether or not 5% sodium saccharin was administered immediately after the procedure. The group receiving sodium saccharin only did not show hyperplastic changes in this time interval. Two weeks after freeze-ulceration, continuation or initiation of sodium saccharin exposure prolonged the duration of hyperplasia until termination of the experiment at eight weeks. The labelling index in the bladder epithelium of sodium saccharin-exposed groups with or without prior freeze-ulceration was increased above the control value in the last six weeks of the experiment. In another experiment, sodium saccharin given two or eight weeks after freeze-ulceration produced a similar increase in hyperplasia, microvillus formation and labelling index (Murasaki & Cohen, 1983b).

The effect of cation-associated saccharin was studied in five-week-old male Fischer 344 rats given 5% of the diet [2500 mg/kg bw per day] as sodium saccharin, acid saccharin, potassium saccharin or calcium saccharin for 10 weeks. Cell proliferation was measured by [^{3}H]thymidine incorporation, hyperplasia was determined by light microscopy and formation of microvilli by scanning electron microscopy. The labelling index was increased by approximately ninefold when compared with controls by sodium saccharin, threefold by potassium saccharin and twofold by calcium saccharin; there was no increase in labelling index with acid saccharin. Only the changes with the sodium and potassium salts reached statistical significance. Sodium saccharin also caused a statistically significant increase in

the number of animals with simple hyperplasia when compared with controls. Although there was evidence of simple hyperplasia after treatment with potassium saccharin and calcium saccharin, these changes were not statistically significant. Pleomorphic microvilli were observed only in rats given sodium saccharin. Sodium saccharin in the diet increased the urinary excretion of sodium. The urinary concentration of saccharin was similar after administration of each of the four forms of saccharin (Hasegawa & Cohen, 1986).

When seven-week-old male Fischer rats were exposed to 5% sodium saccharin [2500 mg/kg bw per day] in the diet for 21 weeks, [^{3}H]thymidine labelling index and the ornithine decarboxylase activity in the bladder were about fivefold greater than in controls (Tatematsu *et al*., 1986).

The proliferative effects of sodium saccharin were found to vary with diet in five-week-old male Fischer 344 and four-week-old Sprague-Dawley rats exposed to 5 or 7.5% [2500 and 3750 mg/kg bw per day] sodium saccharin. The diets containing sodium saccharin were continued for up to 10 weeks, at which time the animals were killed. The Fischer 344 rats showed statistically significant increases in the [^{3}H]thymidine labelling index with Prolab and NIH-07 diets at four weeks and with the Prolab diet at 10 weeks with 7.5% sodium saccharin. At the other times and doses, increased labelling indices were found, but they were not statistically significant, and no increase was seen in rats fed AIN-76A diet. The hyperplastic and epithelial changes in the groups on the three diets, monitored by scanning electron microscopy, were not remarkably different, except for an increase in the severity of changes with the Prolab diet and 7.5% sodium saccharin fed for 10 weeks. The animals fed AIN-76A diet had lower urinary volumes and higher concentrations of sodium saccharin and calcium, but lower levels of potassium than those on the Prolab and NIH-07 diets. The pH of the urine was also lower in this group (Garland *et al*., 1989).

Weanling male Fischer 344 rats were fed 5% sodium saccharin [2500 mg/kg bw per day] in either a Wayne or an AIN-76A diet for 2, 4, 6, 10 and 16 weeks. Whereas the AIN-76A diet alone or with sodium saccharin resulted in a urinary pH of 5.5–6.5, the Wayne diet with sodium saccharin gave a urinary pH of 7.4. With both diets, sodium saccharin increased the [^{3}H]thymidine labelling index by about fivefold when measured at 2, 4, 6, 10 or 16 weeks. Addition of 2% sodium bicarbonate increased the labelling index for the group on the AIN-76A diet by six- to ninefold; sodium saccharin and sodium bicarbonate in the diet appeared to be additive (Debiec-Rychter & Wang, 1990).

In a dose–response experiment, sodium saccharin was administered in Prolab 3200 diet at concentrations of 0, 3, 5 or 7.5% [0, 1500, 2500 and 3750 mg/kg bw per day] to four-week-old male Fischer 344 rats, which were killed after 4, 7 and 10 weeks of exposure. [^{3}H]Thymidine was injected 1 h before killing, and hyperplasia was measured by light microscopy and scanning electron microscopy. No evidence of increased labelling index or hyperplasia was found with 3% sodium saccharin, but cell necrosis and exfoliation were evident after 10 weeks. A doubling of the labelling index was noted with exposure to 5% sodium saccharin at 10 weeks, with extensive cell damage. With 7.5% sodium saccharin, the labelling index was already increased by threefold after four

weeks, with evidence of increasing hyperplasia over the 4- to 10-week period. In all three groups given sodium saccharin, there was an apparent progression of necrotic changes during treatment (Cohen *et al*., 1990).

In two-generation studies, weanling male and female Sprague-Dawley and Fischer 344 F_0 rats were exposed to either 5% sodium saccharin or acid saccharin [2500 mg/kg bw per day] in Prolab 3200 diet for two weeks and then mated. Offspring were killed 1 h after injection of [^{3}H]thymidine *in utero*, during lactation or after weaning. In a study in which mostly male animals were evaluated, there was no effect of either sodium saccharin or acid saccharin through seven days of age; however, sodium saccharin increased the labelling indices above those of controls at 21, 63 and 91 days of age, while acid saccharin did not. In a study of both male and female Sprague-Dawley rats, increased bladder urothelial cell proliferation was found in animals of each sex at 21 but not at 7 days of age or on days 17 and 21 of gestation. There was also evidence of small foci of superficial necrosis and mild hyperplastic proliferation by 21 days of age in animals of each sex (Cohen *et al*., 1995a).

The effects of exposure to concentrations of up to 6.84% sodium ascorbate [3400 mg/kg bw per day] as a single agent or 5 or 7.5% sodium saccharin [2500 and 3750 mg/kg bw per day] alone or combined with 1.23 or 1.85% ammonium chloride [620 and 920 mg/kg bw per day] in the diet on the urinary bladder of male Fischer 344 rats were measured on the basis of various histopathological and chemical parameters. Rats were placed on the experimental diets and mated, and the offspring were kept on the same diets as the F_0 generation. Female offspring were killed at 21 days of age and males at 16 weeks of age. One hour before death, the male F_1 rats were injected intraperitoneally with bromodeoxyuridine. Sodium saccharin but not sodium ascorbate increased the water consumption significantly. Groups of the F_1 generation at the high dose of sodium saccharin had to be terminated early because of toxic effects, and most of the other experimental groups showed some growth retardation. The urinary pH of rats at 5% sodium saccharin was decreased to less than 6.5 by co-administration of 1.23% ammonium chloride; it remained decreased but above 6.5 on day 37 of exposure to 5% sodium saccharin alone and was similar to that of controls by day 100. All of the groups given sodium ascorbate had urinary pH values significantly greater than those of controls. An amorphous precipitate containing calcium phosphate was present in the urine of male F_1 rats given sodium saccharin or higher doses of sodium ascorbate, but not in the controls, after the lowest dose of ascorbate or in rats given sodium saccharin plus ammonium chloride. The labelling index was increased and there was evidence of hyperplastic changes in the groups receiving 5% sodium saccharin alone or the highest dose of sodium ascorbate; the second highest dose of ascorbate induced evidence of hyperplasia, but the increase in labelling index was not statistically significant. No hyperplasia was observed in the group fed sodium saccharin plus ammonium chloride. In a small, one-generation study, hyperplasia was observed in male rats that had been kept on a diet containing 6.84% sodium ascorbate for 10 weeks but was completely inhibited in a group fed 2.78 or 3.7% ammonium chloride plus 6.84% sodium ascorbate. The caeca of rats given sodium saccharin with or without ammonium chloride were enlarged, but this effect was not seen with sodium ascorbate (Cohen *et al*., 1995b).

Weanling male Fischer 344 male rats were exposed to 5% sodium saccharin [2500 mg/kg bw per day] in Prolab 3200 diet for up to 72 weeks, when their bladder epithelial cells were stained immunohistochemically for uroplakin expression and scored for hyperplastic changes. Simple hyperplastic lesions were induced by sodium saccharin and ascorbate, and the lesions showed identical uroplakin staining patterns (Ogawa *et al*., 1996).

Twenty male and female monkeys (six African green, seven rhesus, six cynomolgus and one rhesus/cynomolgus hybrid) were exposed to 25 mg/kg bw sodium saccharin in the diet on five days per week from 24 h after birth up to 24 years of age. Eight monkeys died between 103 and 282 months of age, and the remainder were killed at between 207 and 283 months of age. At autopsy, none of the monkeys had any abnormalities of the urothelium, including the renal pelvis, ureter, urinary bladder and urethra. Of the monkeys that died, many had multiple abnormalities: two had septic hepatitis, five had lung infections and other abnormalities, one had chronic ulcers of the stomach and oesophagus, two had myocardial degeneration or fibrosis and one had chronic ileitis. The monkeys that were killed had abnormalities similar to those seen in 16 monkeys that served as controls, of which two died. There was no evidence of urothelial hyperplasia in either sodium saccharin-exposed animals or controls. No obvious differences in the chemical composition of the urine were seen in two cynomolgus and two rhesus monkeys of each sex given saccharin and in that of controls less than two years before they were killed. Examination of urine by scanning electron microscopy showed no increase in the frequency of microcrystalluria and no evidence of abnormal microcrystals, precipitate or calculi (Takayama *et al*., 1998).

(*b*) *Physiological and biochemical changes in urine*

Sodium saccharin fed to weanling male Charles River rats at concentrations up to 7.5% [3750 mg/kg bw per day] for four weeks was found to increase the urine volume and decrease the urinary pH. It also increased faecal water content, faecal excretion of sodium and potassium and urinary excretion of calcium, magnesium and phosphorous (Anderson, 1979).

Administration to Wistar rats for two years of 2 g/kg bw per day sodium saccharin in the drinking-water or of 4 g/kg bw per day in the diet reduced the weight gain markedly; fluid intake was increased in the latter group and decreased in the former. The average urinary pH of males in the first group had risen to above 7.0 by 27 weeks, and some animals showed marked crystalluria. These pH changes could be reversed by the addition of ammonium chloride to the diet. The most important exposure-related findings were increased incidences of microcalculi and telangiectasia of the vasa recta in kidneys, of renal pelvic hyperplasia, of extramedullary haematopoiesis and of hepatic zonal necrosis. Hyperplasia of the bladder epithelium occurred earlier in animals of the second group (Chowaniec & Hicks, 1979).

Five-week-old male Fischer 344 rats were fed diets containing 5% sodium saccharin [2500 mg/kg bw/day] until week 104, beginning at week 0 or 4; further groups received

FANFT or L-tryptophan in the diet. The urine was analysed at weeks 1, 2, 4, 5, 6, 7, 8 and 12 and monthly thereafter. Exposure to sodium saccharin increased the water intake, which produced diarrhoea and increased the urinary volume. The concentration of sodium in the urine was not changed, and the only abnormality observed was slightly increased urinary pH during the first three months. There was no increase in crystal formation (Demers *et al*., 1981).

Three groups of four-week-old male Crl:COBS^{R}CD^{R}(SD)BR rats were exposed for 16 weeks to 5% sodium saccharin [2500 mg/kg bw per day] in the diet, 4% sodium saccharin [2000 mg/kg bw per day] in the drinking-water or control diet. Rats receiving saccharin in the diet had increased food and water consumption, urine elimination, precipitate and crystalline urinary sediment and some evidence of mild urothelial hyperplasia. Rats receiving saccharin in drinking-water showed increased urine osmolality (West & Jackson, 1981).

In a two-generation study, seven-month-old Charles River CD rats (F_0) and F_1 rats were exposed to 1, 3, 5 or 7.5% sodium saccharin in the diet [500, 1500, 2500 or 3750 mg/kg bw per day]. Controls were either unexposed or given 5% sodium hippurate, the latter to determine effects attributable to sodium. Sodium saccharin produced several physiological changes in the urine, including increased volume and sodium concentration, decreased osmolality and decreased potassium and zinc concentrations. Increases in bladder weight, hydration and the mineral content of the bladder tissue were also seen. Sodium hippurate caused similar changes, but they were less severe than those produced by sodium saccharin. Similar effects were found in an additional group of animals exposed to sodium saccharin from birth. In a group of animals exposed *in utero*, but not after birth, the measured parameters were similar to those in controls. Some differences in response to sodium saccharin were noted between males and females; for example, caecal weight was increased more in the females, but the mineral and sodium saccharin concentrations in the urine were higher in the males. In addition, at dietary concentrations of sodium saccharin ≥ 5%, the concentrations of sodium, potassium, magnesium and zinc were significantly increased in the bladders of males but not females. The pattern of and dietary concentrations at which these changes occurred suggested to the authors that changes in these mineral constituents in the urine and/or urine volume may play an important role in the etiology of bladder tumours in rats fed high dietary concentrations of sodium saccharin (Schoenig & Anderson, 1985).

Adult male Charles Rivers CD rats were fed either control or a diet containing 7.5% [3750 mg/kg bw/day] sodium saccharin for one month. Increased daily water intake, total urine volume, frequency of urination and average volume per urination were found (Renwick & Sims, 1983).

Histological changes in the urinary bladder related to cell proliferation and urinary chemistry were evaluated in male Fischer 344 rats in relation to dietary exposure to 5% sodium saccharin, acid saccharin, ascorbic acid or sodium ascorbate [2500 mg/kg bw per day]. Urinary pH, sodium, urinary bladder hyperplasia and scanning electron microscopic changes were measured at 8, 16 and 24 weeks. The histological changes seen by

light microscopy were 'slight' or 'very slight' at the earlier times, and none were noted at 24 weeks. Scanning electron microscopy showed slight pleomorphic microvilli at 24 weeks in one of five animals given sodium saccharin and at eight weeks in one of five given sodium ascorbate. Short, uniform microvilli and ropy or leafy microridges were moderately prevalent with sodium saccharin or sodium ascorbate, whereas they were absent in the groups given saccharin or ascorbic acid. The urinary pH was increased by sodium saccharin and sodium ascorbate, was decreased by saccharin and was the same as that in controls with ascorbic acid. Urinary sodium excretion was increased by the sodium salts but not by the acidic forms (Fukushima *et al*., 1986a).

Prolab 3200 or AIN-76A diets containing 5% sodium saccharin or calcium saccharin [2500 mg/kg bw per day] were administered to five-week-old male Fischer 344 rats for 10 weeks. Although the Prolab 3200 diet has a lower pH than the AIN-76 diet, the former contains larger amounts of sodium, calcium, potassium and most other ions than the latter. Exposure to either salt of saccharin resulted in a urinary pH above 6.5 with the Prolab diet and below 6.0 with the AIN-76A diet. The amount of sodium excreted in urine was lowest with the AIN-76A diet with or without calcium saccharin. With sodium saccharin administration, the sodium concentrations in urine were higher with the Prolab diet than with AIN-76A (Fisher *et al*., 1989).

Changes in gastrointestinal and urinary tract physiology in young rats were investigated by a protocol simulating the two-generation bioassay of Schoenig *et al*. (1985; p. 548). Sodium saccharin was administered at 1, 3 or 7.5% [500, 1500 and 3750 mg/kg bw/day] in the diet, beginning with the F_0 generation; males and females in the F_1 generation were killed at approximately 30 days. Rats at 7.5% had decreased urinary pH, potassium and calcium and increased urinary volume, sodium, magnesium, phosphate and ammonia; animals at other doses were not examined for these parameters. In addition, rats fed 7.5% sodium saccharin had anaemia, a 50% increase in serum cholesterol concentration, a 10-fold increase in serum triglyceride concentration and decreased serum and hepatic concentrations of vitamins. These changes were not found at lower doses of sodium saccharin. In another experiment, newborn animals were dosed with sodium saccharin for 90 days, while others were given only control diet after 30 days on sodium saccharin. Some of the observed changes were found to be reversible; however, histopathological examination of the bladder showed no changes at 30 days and only slight changes at 90 days. The authors considered that the observed biochemical and physiological effects were not involved in bladder tumorigenesis. Many of the effects were found to be associated with anaemia due to iron deficiency, and some of them—but not the urinary or bladder effects—could be prevented by dietary iron and/or folate supplementation. This supplementation by itself increased the frequency of bladder hyperplasia (Garland *et al*., 1991a,b, 1993).

In a study involving feeding of various sodium salts at doses equimolar to 7.5% sodium saccharin to male Fischer 344 rats for 10 weeks, urothelial-cell proliferation was studied by light microscopy (hyperplasia), by bromodeoxyuridine labelling and by scanning electron microscopy. The sodium salts of saccharin, ascorbate, glutamate,

aspartate, citrate, erythorbate, bicarbonate and chloride caused various degrees of increase in urothelial proliferation and all resulted in formation of the calcium phosphate-containing urinary precipitate (amounts not quantified). A group of rats that received 1.85% ammonium chloride plus 7.5% sodium saccharin did not show increased proliferation or formation of the precipitate (Cohen *et al*., 1995c).

(*c*) *Urinary precipitate formation and the role of urinary proteins*

In a two-generation lifetime feeding study, groups of 50 male and 50 female Sprague-Dawley rats were exposed to 5% [2500 mg/kg bw per day] sodium saccharin or to various doses of *ortho*-toluenesulfonamide in the diet, beginning when the F_0 generation was 32 days old. Rats were mated on day 90 of the study, and the F_0 generation and F_1 progeny were exposed to the same diets throughout life. The urine of older animals exposed to 5% sodium saccharin, especially the males, contained a flocculent-type precipitate, which dissolved in acetic acid. In 21-day-old pups and in 105-day-old male and female rats receiving *ortho*-toluenesulfonamide in another experiment reported by these investigators, a significant dose–response relationship was found for the incidence of bladder calculi, while sodium saccharin free of *ortho*-toluenesulfonamide did not have a similar effect (Arnold *et al*., 1979, 1980).

Male Fischer 344 rats were fed 7.5% [3750 mg/kg bw/day] sodium saccharin in Prolab 3200 diet beginning at five weeks of age, and urine was collected on Millipore filters two and four weeks later. Whereas control rats had typical phosphate crystals, rats fed sodium saccharin had fewer crystals and approximately one-half contained silicate and were jagged in shape. These silicate crystals were found to result in occasional microabrasion of the urothelial surface. Gel filtration of urine with added radiolabelled sodium saccharin showed two fractions of saccharin-binding proteins: one corresponded to the size of α_{2u}-globulin, while the other corresponded to albumin. The authors postulated that saccharin–protein complexes form a precipitate with silicate in urine that has a pH greater than 6.5 (Cohen *et al*., 1991b).

Support for the role of α_{2u}-globulin in sodium saccharin-induced cell hyperplasia and cell proliferation rates comes from studies with male Fischer 344 and NCI-Black-Reiter (NBR) rats. The latter strain does not produce the male-specific, low-molecular-mass urinary protein α_{2u}-globulin and does not undergo sodium saccharin-enhanced cell proliferation. Male NBR, Fischer 344 and castrated Fischer 344 rats were fed 7.5% sodium saccharin [3750 mg/kg bw per day] in Prolab 3200 diet for 10 weeks. Examination of the bladders by light microscopy revealed that 7/10 sodium saccharin-exposed Fischer 344 rats and 1/10 NBR rats had hyperplastic changes, but none were seen in the unexposed Fischer 344 rats; however, 4/10 castrated Fischer 344 rats also had hyperplastic bladders, although the α_{2u}-globulin urinary content was only 10% of that in normal Fischer 344 males. Examination of the bladder by scanning electron microscopy showed that the most severe changes occurred in castrated and normal sodium saccharin-exposed Fischer 344 rats, whereas the exposed NBR rats showed less severe changes. The increases in caecal weights showed a pattern similar to the changes observed by scanning electron

miscroscopy. The urinary volume of NBR controls was threefold higher than that of Fischer 344 controls, and exposure to sodium saccharin increased the volume by three- to fourfold in NBR, Fischer 344 and castrated Fischer 344 rats (Garland *et al*., 1994).

Six-week-old Fischer 344 and NBR rats were exposed to 5% sodium saccharin [2500 mg/kg bw per day], 5% sodium ascorbate or 3% uracil [1500 mg/kg bw per day] in the diet for eight weeks. Ascorbate produced simple hyperplasia in Fischer 344 rats but not in NBR rats, and uracil produced papillary hyperplasia in both strains. Scanning electron microscopy showed that uracil produced the most severe changes, and sodium saccharin and ascorbate produced some changes only in Fischer 344 rats. Large differences in the bromodeoxyuridine labelling index were found in Fischer 344 rats given sodium saccharin (20-fold) or ascorbate (36-fold) and in both strains exposed to uracil (over 50-fold). In Fischer 344 rats, the urinary pH and sodium concentration were increased by sodium saccharin and ascorbate, and the urine volume was decreased only by ascorbate. In ascorbate-treated NBR rats, a significant increase in urinary volume was observed (Uwagawa *et al*., 1994).

Studies on the appearance of the crystalline and amorphous material in sodium saccharin-exposed male rats are described above (Cohen *et al*., 1995b,c).

(*d*) *Studies primarily on gastrointestinal changes*

Dietary sodium saccharin was tested for its ability to alter caecal microflora in weanling male CD rats exposed to 7.5% sodium saccharin [3750 mg/kg bw/day] in the diet for 10 days. The weights of caecal tissue and its contents were higher than those of controls, which received 7.5% cellulose (Anderson & Kirkland, 1980).

Adult male Charles River (CD)-derived Sprague-Dawley rats (weighing about 300 g) were exposed to 0–10% sodium saccharin [0–5000 mg/kg bw per day], 2% tryptophan [1000 mg/kg bw per day] or 2% tryptophan plus 5% sodium saccharin [2500 mg/kg bw per day] in the diet for one to two months. The presence of sodium saccharin in the diet had a rapid, pronounced effect on the metabolism of dietary tryptophan. The excretion of indican, a metabolite of tryptophan, was increased 3.1-fold per 24 h in animals fed 10% sodium saccharin in the diet, and there was a linear relationship between the dietary concentration and indican excretion. High plasma concentrations of saccharin (200–300 μg/mL) reduced the renal clearance of both saccharin and indican; the authors suggested that this was due to saturation of renal tubular secretion. Indican concentrations in the urine, and the indole concentrations in the caecum were increased, possibly due to accumulation of protein and tryptophan in the caecum. Sodium saccharin produced a dose-related increase in both the weight of the caecal contents and the wall of the caecum. The effects of sodium saccharin and tryptophan were additive. These results are consistent with the concept that sodium saccharin has a major effect on protein digestion in the intestine, which favours increased microbial metabolism of dietary tryptophan to indole, a urinary bladder co-carcinogen (Sims & Renwick, 1983).

Indican and *para*-cresol excretion were found to be increased by three- to fourfold in male Charles River adult rats fed 7.5% sodium saccharin [3750 mg/kg bw per day] in

the diet for 40 days, when compared with controls. As described above, indican is formed from indole, a microbial metabolite of tryptophan, whereas *para*-cresol is formed from tyrosine by the gut flora. The excretion of phenol, another microbial metabolite of tyrosine, was, however, abolished by the same regimen of sodium saccharin, indicating that there is an altered pattern of metabolism in addition to an increase in the amount of metabolites (Lawrie *et al*., 1985).

Three-week-old male Sprague-Dawley rats were fed a diet containing 5% sodium saccharin [2500 mg/kg bw per day] for 4 or 20 weeks. Marked caecal enlargement and decreased β-glucuronidase, nitrate reductase and sulfatase activities were seen in the caecal content; however, there was no change in bacterial concentrations at either exposure interval. Incubation of the caecal contents from control rats with 75 mmol/L sodium saccharin *in vitro* produced similar enzyme inhibitions. The authors surmised that such inhibition may decrease the rate of formation of toxic bacterial products in the hindgut (Mallett *et al*., 1985).

In a two-generation study, 7.5% [3750 mg/kg bw per day] sodium saccharin was fed in the diet to adult male and female Charles River (CD)-derived Sprague-Dawley rats from six weeks before mating. The pups of sodium saccharin-fed dams were found to be exposed to elevated levels of indican via the milk. Sodium saccharin-exposed pups were found to have increased caecal size and caecal protein, decreased caecal tryptophanase activity and increased urine volume and urinary indican excretion. Pups from dams fed sodium saccharin only from the time of parturition showed more variable responses than those from dams fed sodium saccharin from before conception; this was due to variations in tryptophanase activity. These changes were similar in males and females and were greatest during the first month after weaning (Sims & Renwick, 1985).

CD rat dams were fed increasing doses of sodium saccharin: 1% [500 mg/kg bw per day] beginning at parturition, 3% [1500 mg/kg bw] at day 8 and 5% [2500 mg/kg bw] at day 15. Pups were weaned at day 21 and continued on this diet. The pups of dams that had not received sodium saccharin were started on 5% sodium saccharin at weaning. When pups were exposed to sodium saccharin during nursing, no effect was seen on caecal or bladder mass at weaning but the caecal mass after weaning was almost doubled. The caecal mass of pups exposed from the time of weaning was intermediate between that of pups exposed from birth and that of controls. The changes in bladder mass followed a similar pattern but were not as extensive; the increased bladder mass correlated with increased urinary output (Anderson *et al*., 1988a).

Although the studies described above suggest that gastrointestinal changes are implicated in the effects of sodium saccharin, the effects of saccharin on the caecal mass were not found to depend on the chemical form. There was no difference in the mass of the caecum plus contents or caecal tissue after 10 weeks of feeding male weanling CD rats 5% sodium saccharin [2500 mg/kg bw per day] or the molar equivalent of acid saccharin, potassium saccharin or calcium saccharin. Bladder hyperplasia was found only in rats given sodium or potassium saccharin, and increased relative bladder mass was found only with sodium saccharin. Sodium and potassium saccharin were also responsible for an

increased relative urinary mass. The authors concluded that the caecal effects were due to the saccharin anion but that the urinary changes were dependent on the cation. Consequently, caecal mass changes do not appear to be involved in sodium saccharin-induced bladder tumours (Anderson *et al*., 1988b).

The excretion of indican was found not to be responsible for the toxic effects on the bladder of dietary administration of 5% sodium saccharin. Male CD rats were exposed to 5% sodium saccharin [2500 mg/kg bw per day], 1.5% indole [750 mg/kg bw per day] or 5% sodium saccharin plus 1.5% indole added to diet for 10 weeks. Sodium saccharin and indole produced equivalent increases in bladder mass and epithelial hyperplasia; however, in rats exposed to sodium saccharin, the amount of indican excreted in the urine was less than one-tenth the amount produced after dietary exposure to indole. After exposure to indole and sodium saccharin together, the increase in bladder mass was additive, but there was no increase in hyperplasia over that observed with each compound alone (Anderson *et al*., 1989).

(*e*) *Other mechanistic information*

In an assay to detect tumour promoters *in vitro*, sodium saccharin inhibited metabolic cooperation between Chinese hamster V79 cells in culture. A small number of *hprt*$^-$ (6-thioguanine resistant) cells were seeded in the presence of a large number of *hprt*$^+$ cells with 0–5 mg/L sodium saccharin in the culture medium. Sodium saccharin concentrations > 2 mg/L resulted in a dose-related increase in the recovery of 6-thioguanine-resistant cells (Trosko *et al*., 1980).

Sodium saccharin inhibited binding of ^{125}I-labelled mouse epidermal growth factor to 18 cell lines, including HeLa (human carcinoma), MDCK (dog kidney), HTC (rat hepatoma), K22 (rat liver), HF (human foresekin), GM17 (human skin fibroblasts), XP (human xeroderma pigmentosum fibroblasts) and 3T3-L1 (mouse fibroblasts) (Lee, 1981).

Inhibition by sodium saccharin of urease and three proteases *in vitro* was not due to the sodium ion (Lok *et al*., 1982).

Saccharin [type or source not specified] inhibited binding of epidermal growth factor to cultured rat pituitary tumour cells and enhanced prolactin production (Brennessel & Keyes, 1985).

In two initiation-promotion studies, inhibitors of lipid peroxidation were found to inhibit the tumour-promoting effects of sodium saccharin. Both aspirin and the antioxidant nordihydroguaiaretic acid inhibited bladder tumour formation when co-administered with sodium saccharin. These studies indicate that oxidative damage may play a role in the cytotoxic effects of sodium saccharin (Sakata *et al*., 1986; Yu *et al*., 1992).

In cultured bladders from young female Fischer 344 rats, 12 mmol/L sodium saccharin produced urothelial hyperplasia and dysplasia, as detected by histology. Sodium saccharin increased the number of foci of cell proliferation induced by MNU in explant bladder epithelium cultures of young female Fischer 344 rats. Only transient hyperplasia was found with sodium saccharin alone (Knowles & Jani, 1986; Nicholson & Jani, 1988).

Rat bladder explants were exposed continuously to sodium saccharin for up to 28 days. Hyperplastic effects and increased DNA synthesis were reported after exposure to 0.5% (Norman *et al*., 1987), while explants treated with 0.1% resembled control cultures.

Saccharin [type not specified] was found to inhibit intercellular communication in a human urothelial cell-line (JTC-30), which was established from a well-differentiated transitional-cell carcinoma of the urinary bladder. Inhibition measured by dye transfer at 48 and 96 h occurred at a concentration of 3 mg/mL, which was relatively nontoxic. At lower concentrations, there was little or no effect, and higher concentrations were toxic (Morimoto, 1996).

The ionic structure of the saccharinate anion, as studied by two-dimensional nuclear magnetic resonance, was not altered by several ions at the wide ranges of concentrations found in urine after feeding of high doses of various salt forms of saccharin or its acid (Williamson *et al*., 1987).

4.3 Reproductive and developmental effects

4.3.1 *Humans*

The teratogenic potential of saccharin in humans has been reviewed and determined to be minimal; however, the quality of the data considered in the analysis was poor to fair (Friedman & Polifka, 1994). No epidemiological studies of prenatal exposure to saccharin have been reported.

In a case–control study, the use of sugar substitutes was examined in 574 consecutive cases of spontaneous abortions and 320 age-matched controls from three Manhattan hospitals (United States) during 1974–77. Sugar substitutes (assumed to be saccharin) were used by 5.5% of the patients and 5.8% of the controls. There was no significant association between spontaneous abortion and the use of sugar substitutes (Kline *et al*., 1977).

4.3.2 *Experimental systems*

Generally negative results were reported from a one-generation study in mice fed 1% sodium saccharin (Lorke & Machemer, 1975), a multigeneration study in Swiss mice given doses of 0.2 or 0.5% in the diet (Kroes *et al*., 1977), in an abstract reporting a three-generation study in CD rats at 5 or 7% sodium saccharin in the diet (Taylor & Friedman, 1974), in studies for teratogenicity in hamsters and rats at 10 and 100 g per day calcium saccharin (Adkins *et al*., 1972), in a study of exposure of rats before mating and during gestation to 0.4% sodium saccharin in the diet (Luckhaus & Machemer, 1978) and in an abstract of a study in which dogs received 0.5–1.5 g/kg bw of a 10:1 sodium cyclamate:sodium saccharin mixture during pregnancy while their offspring were kept on the same diet until one year of age (Fancher *et al*., 1968). The finding that sodium saccharin was more toxic to fetal mice than to adults was not considered reliable (Tanaka, 1964, cited by Tanaka *et al*., 1973). Morphological abnormalities of the eye and an increased mortality rate of offspring were reported after exposure of pregnant Wistar rats to 0.3 and 3% sodium saccharin in the diet (Lederer & Pottier-Arnould, 1973; Lederer, 1977), but these effects were

attributed to impurities (primarily *ortho*-sulfobenzoic acid) arising from synthesis by the Remsen-Fahlberg method (IARC, 1980).

The studies on reproductive effects and teratogenesis were also reviewed by Arnold *et al*. (1983), who concluded that saccharin is not teratogenic to mice, rats or rabbits. The authors also reviewed the findings of a number of multigeneration studies that were designed primarily to examine effects on bladder tumour formation, i.e. those conducted by the Wisconsin Alumni Research Foundation (Tisdel *et al*., 1974), the Food and Drug Administration (Taylor *et al*., 1980), the Health Protection Branch (Arnold *et al*., 1980) and several other modified multigeneration studies (one involving six generations; Kroes *et al*., 1977). Significant effects on reproductive indices (primarily decrements in body weights) were observed in rats at dietary concentrations of 5–7.5%.

In another review of the teratogenic potential of saccharin in experimental systems (Friedman & Polifka, 1994), it was noted that the frequency of malformations was not increased in offspring of mice, rats or rabbits given up to 800, 1500 and 10 times the human maximum intake, respectively. They noted that a slightly increased incidence of bladder tumours was observed in rats in some studies in which perinatal exposure was to thousands of times the recommended daily intake of saccharin for humans; however, other studies did not show this effect.

Direct exposure of cultured rat embryos to 1 mmol/L saccharin on days 10.5–12.5 of gestation *in vitro* did not affect embryonic growth or morphology, even in the presence of a microsomal activating system (Kitchen & Ebron, 1983). Similar results were reported by Cicurel and Schmid (1988).

The concentration of sodium saccharin that inhibits proliferation of human embryonic palatal mesenchymal cells by 50% was reported to be 5350 µg/mL (26 mmol/L), whereas the authors considered a value < 1 mmol/L to be indicative of teratogenic potential in this assay (Pratt & Willis, 1985). A subsequent interlaboratory comparison of results showed no evidence for any teratogenic effect of saccharin in either an assay for growth of human embryonic palatal mesenchymal cells or an assay for inhibition of mouse ovarian tumour cell attachment (Steele *et al*., 1988).

Exposure of developing *Drosophila* larva to saccharin caused a dose-related increase in the incidence of bent humeral bristles in adults, suggesting potential developmental toxicity in mammals (Lynch *et al*., 1991).

Saccharin [form unspecified] was considered to be inactive in a screening test for teratogenicity in rat embryonic limb buds in culture. The median concentrations that affected chondrogenesis and cell proliferation were 2600 and 4100 µg/mL, respectively (Renault *et al*., 1989). Saccharin at concentrations up to 500 µg/mL did not inhibit mouse embryonic stem cell differentiation (Newall & Beedles, 1996).

Acid saccharin was evaluated for effects on amphibian development in an assay for teratogenicity in frog embryos as part of an interlaboratory validation study. The mean median lethal concentration, the concentration that induced malformations in 50% of the surviving embryos and the teratogenic index resulting from those concentrations in four

laboratories were 16 mg/mL, 16 mg/mL and 1 mg/mL, respectively. The authors concluded that saccharin had little, if any, potential developmental toxicity (Bantle *et al*., 1994).

Saccharin [form unspecified] did not affect offspring viability, growth or morphology when administrated by oral gavage to ICR mice at a minimal maternally toxic dose in a short-term test for teratogenicity (Seidenberg *et al*., 1986).

Pregnant ICR mice (10 controls and five in each treated group) received intraperitoneal injections of 0, 500, 1000 or 2000 mg/kg sodium saccharin on day 10 of gestation, by oral gavage at 0, 5, 10 or 25 mg/kg on days 5–15 of gestation, or via the drinking-water as a 0, 5, 10 or 20% solution on days 0–17 of gestation. Fetuses were examined on day 17 of gestation for viability, growth and gross external and internal malformations. Dams exposed to 25 mg/kg per day by gavage had a slightly elevated incidence of resorptions (5/52 implants versus 7/125 in controls), but this was not significant; the only defect noted in any fetus was an isolated case of cleft palate in this treatment group (Dropkin *et al*., 1985).

In a protocol to assess reproductive toxicity during continuous breeding, CD-1 mice were exposed to 0, 1.25, 2.5 or 5% sodium saccharin in the drinking-water, equivalent to 0, 3.5, 5.9 and 8.1 g/kg per day. The water consumption of the group at the high dose was reduced by 10–20%, whereas it was increased by 20 and 40% at the intermediate and low doses, respectively. Mortality, attributed to dehydration, occurred at a significantly increased rate at the high dose, and the numbers of live pups per litter and pup weight adjusted for litter size were reduced in this group. Exposure to sodium saccharin was maintained for the last litters from both the control and the intermediate-dose groups, and these offspring were used to produce an F_1 generation. There were no treatment-related effects on reproductive function, despite increased fluid consumption (National Toxicology Program, 1997).

4.4 Genetic and related effects

The genetic toxicology of saccharin has been reviewed (Arnold *et al*., 1983; Ashby, 1985; Arnold & Boyes, 1989). Additional information on the genetic and related effects of saccharin is available in a more recent review (Whysner & Williams, 1996).

4.4.1 *Humans*

No data were available to the Working Group.

4.4.2 *Experimental systems* (see Tables 3 and 4 for references)

Saccharin did not induce gene mutation in *Salmonella typhimurium* strains TA100, TA1535, TA1537, TA1538, TA98, TA92 or TA94 in the presence or absence of an exogenous metabolic activation system. It induced aneuploidy in *Saccharomyces cerevisiae* diploid strain D6 but did not induce mitotic recombination.

The results of an alkaline elution assay showed DNA single-strand breaks in rat hepatocytes. The frequency of chromosomal aberrations was not increased in Chinese hamster lung fibroblasts *in vitro*. Saccharin did not induce cell transformation in mouse C3H 10T1/2

Table 3. Genetic and related effects of saccharin

Test system	Result[a]		Dose[b] (LED or HID)	Reference
	Without exogenous metabolic system	With exogenous metabolic system		
Salmonella typhimurium TA100, TA98, TA1535, TA1538, reverse mutation	NT	–	2500 µg/plate	Ashby *et al*. (1978)
Salmonella typhimurium TA100, TA98, TA1535, TA1537, TA1538, reverse mutation	–	–	5000 µg/plate	Rao *et al*. (1979)
Salmonella typhimurium TA100, TA98, TA94, TA92, TA1535, TA1537, reverse mutation	–	–	10000 µg/plate	Ishidate *et al*. (1984)
Salmonella typhimurium TA100, TA98, TA1535, TA1537, reverse mutation	–	–	10000 µg/plate	Mortelmans *et al*. (1986)
Saccharomyces cerevisiae D6, homozygosis	–	NT	500[c]	Parry *et al*. (1981)
Saccharomyces cerevisiae D6, aneuploidy	+	NT	400[c]	Parry *et al*. (1981)
DNA single-strand breaks, rat hepatocytes *in vitro*	(+)	NT	549	Sina *et al*. (1983)
Chromosomal aberrations, Chinese hamster lung cells *in vitro*	–	NT	4000	Ashby & Ishidate (1986)
Cell transformation, C3H 10T1/2 mouse cells	–	NT	100	Saxholm *et al*. (1979)
Cell transformation, RLV/Fischer rat embryo cells	–	NT	50[c]	Traul *et al*. (1981)
Sister chromatid exchange, human lymphocytes *in vitro*	–	–	100	Saxholm *et al*. (1979)
Sister chromatid exchange, human lymphocytes *in vitro*	–	NT	500	Brøgger *et al*. (1979)
Inhibition of gap-junctional intercellular communication, normal and *hprt*-deficient human fibroblasts *in vitro*	–	NT	10000	Mosser & Bols (1983)
Inhibition of gap-junctional intercellular communication, human urothelial carcinoma cells *in vitro*	+	NT	3000	Morimoto (1996)

Table 3 (contd)

Test system	Result[a]		Dose[b] (LED or HID)	Reference
	Without exogenous metabolic system	With exogenous metabolic system		
Inhibition of gap-junctional intercellular communication, Chinese hamster lung V79 cells *in vitro*	+	NT	2000	Umeda *et al*. (1980)
Inhibition of gap-junctional intercellular communication, Chinese hamster lung V79 cells *in vitro*	+	NT	3000	Trosko *et al*. (1980)

[a] +, positive; (+), weakly positive; –, negative; NT, not tested
[b] LED, lowest effective dose; HID, highest ineffective dose; unless otherwise stated, in-vitro tests, µg/mL
[c] Form of saccharin not specified

Table 4. Genetic and related effects of saccharin, sodium

Test system	Result[a]		Dose[b] (LED or HID)	Reference
	Without exogenous metabolic system	With exogenous metabolic system		
Prophage induction	NT	–	250 000[c]	Ho & Ho (1981)
Salmonella typhimurium TA100, TA98, reverse mutation	–	–	800 000 µg/plate[d]	Batzinger *et al*. (1977)
Salmonella typhimurium TA100, TA98, TA1535, TA1537, reverse mutation	–	–	5000 µg/plate	Stoltz *et al*. (1977)
Salmonella typhimurium TA100, TA98, TA1535, TA1538, reverse mutation	NT	–	2500 µg/plate	Ashby *et al*. (1978)
Salmonella typhimurium TA100, TA98, TA1535, TA1537, reverse mutation	–	–	1000 µg/plate	Pool (1978)
Salmonella typhimurium TA100, TA98, TA1535, TA1537, TA1538, reverse mutation	–	–	40 000 µg/plate	Eckhardt *et al*. (1980)
Salmonella typhimurium TA100, TA98, TA1535, TA1537, TA1538, reverse mutation	–	–	10 250 µg/plate	De Flora (1981)
Salmonella typhimurium TA100, TA98, TA1535, TA1537, reverse mutation	NT	–	2500 µg/plate	Herbold (1981)
Salmonella typhimurium TA100, reverse mutation	NT	–	10 000 µg/plate	Imamura *et al*. (1983)
Salmonella typhimurium TA100, TA98, TA94, TA92, TA1535, TA1537, reverse mutation	–	–	10 000 µg/plate	Ishidate *et al*. (1984)
Saccharomyces cerevisiae CM-1293, gene conversion	+	NT	2000	Moore & Schmick (1979)
Saccharomyces cerevisiae CM-1293, homozygosis	+	NT	2000	Moore & Schmick (1979)
Saccharomyces cerevisiae DIS 13, homozygosis	–	NT	18 300[c]	Persic (1986)
Saccharomyces cerevisiae CM-1293, reverse mutation	+	NT	2000	Moore & Schmick (1979)
Saccharomyces cerevisiae DIS 13, aneuploidy	+	–	1830[c]	Persic (1986)
Drosophila melanogaster, sex-linked recessive lethal mutations	+		1140 feed	Rao *et al*. (1971)

Table 4 (contd)

Test system	Result[a]		Dose[b] (LED or HID)	Reference
	Without exogenous metabolic system	With exogenous metabolic system		
Drosophila melanogaster, sex-linked recessive lethal mutations	?		1 µg/fly inj	Kramers (1977)
Drosophila melanogaster, sex-linked recessive lethal mutations	–		82 000 feed	Eckhardt *et al*. (1980)
Drosophila melanogaster, heritable translocations	–		8300 feed	Rao *et al*. (1971)
Unscheduled DNA synthesis, Fischer 344 rat primary hepatocytes *in vitro*	–	NT	10 250	Jeffrey & Williams (1999)
Unscheduled DNA synthesis, Sprague-Dawley rat primary hepatocytes *in vitro*	–	NT	10 250	Jeffrey & Williams (1999)
Gene mutation, mouse lymphoma L5178Y cells, *tk* locus	–	–	12 500[e]	Clive *et al*. (1979)
Sister chromatid exchange, Chinese hamster Don cells *in vitro*	+	NT	206	Abe & Sasaki (1977)
Sister chromatid exchange, Chinese hamster ovary cells *in vitro*	+	NT	5000	Wolff & Rodin (1978)
Sister chromatid exchange, Chinese hamster ovary cells *in vitro*	(+)	NT	100	Ray-Chaudhuri *et al*. (1982)
Chromosomal aberrations, Chinese hamster embryonic lung Cl-1-15 cells *in vitro*	+	NT	100	Kristoffersson (1972)
Chromosomal aberrations, Chinese hamster Don cells *in vitro*	+	NT	1030	Abe & Sasaki (1977)
Chromosomal aberrations, Chinese hamster lung cells *in vitro*	+	NT	8000	Ishidate & Odashima (1977)
Chromosomal aberrations, Chinese hamster ovary CHO-K1 cells *in vitro*	+	NT	20 000	Masubuchi *et al*. (1977)
Chromosomal aberrations, Chinese hamster lung cells *in vitro*	+	NT	4000	Ashby & Ishidate (1986)
Cell transformation, BALB/c3T3 mouse cells	–	NT	500	Sivak & Tu (1980)
Cell transformation, BALB/c3T3 mouse cells	–	NT	5000	Sakai & Sato (1989)
Cell transformation, C3H 10T1/2 mouse cells	–	NT	2000[c]	Mondal *et al*. (1978)
Cell transformation, Fischer 344 rat urothelial cells	–	NT	2200	Knowles & Jani (1986)
Cell transformation, rat bladder explant	–	NT	1230	Nicholson & Jani (1988)
Gene mutation, human embryo RSa cells, Na^+/K^+ ATPase *in vitro*	+	NT	15 000	Suzuki & Suzuki (1988)

Table 4 (contd)

Test system	Result[a]		Dose[b] (LED or HID)	Reference
	Without exogenous metabolic system	With exogenous metabolic system		
Gene mutation, human embryo RSa cells, K-*ras in vitro*	+	NT	15 000	Suzuki & Suzuki (1993)
Sister chromatid exchange, human lymphocytes *in vitro*	+	NT	1000	Wolff & Rodin (1978)
Sister chromatid exchange, human lymphocytes *in vitro*	–	NT	500	Brøgger *et al*. (1979)
Chromosomal aberrations, human lymphocytes *in vitro*	+	NT	2000	Chang & Stacey (1974)
Cell transformation, human neonatal foreskin fibroblasts	–	NT	50	Milo *et al*. (1988)
Body fluids, mouse urine, *Salmonella typhimurium*, reverse mutation	+		2500 po × 1[d]	Batzinger *et al*. (1977)
Body fluids, rat bile, *Salmonella typhimurium*, reverse mutation	–		100 iv × 1	Connor *et al*. (1979)
Host-mediated assay, *Salmonella typhimurium* TA100, TA98 in mouse peritoneal cavity	+		2500 po × 1[d]	Batzinger *et al*. (1977)
DNA single-strand breaks, CD1 mouse liver or renal DNA *in vivo*	+		50 ip	Cesarone *et al*. (1980)
DNA damage, Wistar rat bladder *in vivo*	–		800 iv × 1 [c]	Miyata *et al*. (1980)
Somatic mutation, mice *in vivo*	–		1000 ip × 1	Fahrig (1982)
Somatic mutation, mice *in vivo*	+		750 po × 3	Mahon & Dawson (1982)
Sister chromatid exchange, Chinese hamster bone marrow cells *in vivo*	+		5000 po × 1	Renner (1979)
Sister chromatid exchange, ICR mouse embryo cells *in vivo*	–		2000 ip × 1	Dropkin *et al*. (1985)
Micronucleus formation, C57BL mouse erythrocytes *in vivo*	–		2000 ip × 1	Léonard & Léonard (1979)
Micronucleus formation, NMRI mouse bone marrow cells *in vivo*	–		1025 po/ip × 2	Eckhardt *et al*. (1980)
Chromosomal aberrations, Chinese hamster bone marrow cells *in vivo*	–		1500 po × 3	Van Went-de Vries & Kragten (1975)
Chromosomal aberrations, C57BL mouse bone marrow cells *in vivo*	–		4000 ip × 1	Léonard & Léonard (1979)
Chromosomal aberrations, ICR mouse bone marrow cells *in vivo*	+		1000 diet × 24 w[c]	Prasad & Rai (1987)
Chromosomal aberrations, C57BL mice, spermatogonia *in vivo*, spermatocytes observed	–		2000 ip × 1	Léonard & Léonard (1979)

Table 4 (contd)

Test system	Result[a]		Dose[b] (LED or HID)	Reference
	Without exogenous metabolic system	With exogenous metabolic system		
Chromosomal aberrations, C3H × 101 mice, spermatogonia *in vivo*, spermatocytes observed	–		500 po × 10	Pecevski *et al*. (1983)
Chromosomal aberrations, ICR mice, spermatogonia *in vivo*, spermatocytes observed	+		1000 diet × 24 w[c]	Prasad & Rai (1987)
Chromosomal aberrations, Chinese hamster spermatogonia *in vivo*, spermatogonia observed	–		5000 × 2 po	Machemer & Lorke (1975a)
Chromosomal aberrations, ICR mouse embryo cells *in vivo*	–		2000 × 1 ip	Dropkin *et al*. (1985)
Dominant lethal mutation, Swiss and C17 mice *in vivo*	+		240 × 1 sc	Tezabwala & Gothoskar (1977)
Dominant lethal mutation, CBA mice *in vivo*	+		340 drink 30 d	Rao & Qureshi (1972)
Dominant lethal mutation, NMRI/BOM mice *in vivo*	–		5000 × 5 po	Machemer & Lorke (1973)
Dominant lethal mutation, ICR mice *in vivo*	+		200 × 5 ip	Šrám & Zudová (1974)
Dominant lethal mutation, mice *in vivo*	–		2000 diet 10 w	Lorke & Machemer (1975)
Dominant lethal mutation, mice *in vivo*	–		10 000 × 1 po	Machemer & Lorke (1975b)
Dominant lethal mutation, C57BL mice *in vivo*	–		2000 ip × 1	Léonard & Léonard (1979)
Mouse (C3H x 101) heritable translocations *in vivo*	–		500 × 10 po	Pecevski *et al*. (1983)
Binding (covalent) to SD rat liver or bladder DNA *in vivo*	–		390 × 1 po[c]	Lutz & Schlatter (1977)

Table 4 (contd)

Test system	Result[a]		Dose[b] (LED or HID)	Reference
	Without exogenous metabolic system	With exogenous metabolic system		
Inhibition of gap-junctional intercellular communication, Chinese hamster V79 cells *in vitro*	–	NT	1000	Welsch & Stedman (1984)
Sperm morphology, CBA x BALB/c mice *in vivo*	–		500 × 5 ip	Topham (1980)

[a] +, positive; (+), weakly positive; –, negative; NT, not tested; ?, inconclusive
[b] LED, lowest effective dose; HID, highest ineffective dose; unless otherwise stated in-vitro tests, µg/mL; in-vivo tests, mg/kg bw per day; po, oral; iv, intravenous; ip, intraperitoneal; w, week; d, day
[c] Identification as sodium saccharin is not stated specifically in the reference but is based on solubility in water as identified by Whysner & Williams (1996).
[d] Purified sample
[e] HID is for purified sample; HID for unpurified sample, 19 000 µg/mL

cells, in baby hamster kidney cells or in virally enhanced RLV/Fischer rat embryo cells. It did not increase the frequency of sister chromatid exchange in human lymphocyte cultures. Gap-junctional intercellular communication was inhibited in Chinese hamster V79 lung fibroblasts and in a human urothelial carcinoma cell line, but intercellular communication in human fibroblasts was unaffected.

Sodium saccharin did not induce prophage and was not mutagenic to *S. typhimurium* strains TA100, TA1535, TA1537, TA1538, TA98, TA92 or TA94 in the presence or absence of an exogenous metabolic activation system. In one study, both a purified and an unpurified sample of sodium saccharin induced gene conversions, mitotic recombination and reverse mutation in *Saccharomyces cerevisiae*. It induced sex-linked recessive lethal mutations in *Drosophila melanogaster* treated by injection or feed in two of three studies, but it did not induce heritable translocations in one of the studies after injection of males with sodium saccharin.

Sodium saccharin did not induce unscheduled DNA synthesis or repair as evaluated by net nuclear grain counts in hepatocyte preparations from Fischer 344 and Sprague-Dawley rats. In a single study, neither a purified nor an unpurified sample of sodium saccharin induced gene mutation at the *tk* locus in mouse lymphoma L5178Y cells exposed to the test compounds for 4 h with or without exogenous metabolic activation. Sodium saccharin did increase the frequency of sister chromatid exchanges and chromosomal aberrations in Chinese hamster ovary, lung and Don cells, but did not induce cell transformation in rat bladder explants or urothelial cells, or in BALB/c3T3 mouse or baby hamster kidney cells treated *in vitro*. In a single study, sodium saccharin did not inhibit gap-junctional intercellular communication in Chinese hamster V79 cell cultures.

Sodium saccharin was mutagenic in RSa cells, a cell line derived from human embryos, inducing a dose-dependent increase in the number of ouabain-resistant mutants after a 24-h treatment. Point mutations in codon 12 of K-*ras* were also induced in the RSa cell line after six days of treatment with sodium saccharin. In one study, sister chromatid exchange frequencies were not increased in human lymphocytes obtained from one male donor and cultured with sodium saccharin for 24–48 h, but in a second study, sister chromatid exchanges were induced in lymphocytes that were cultured for 72 h in media containing sodium saccharin. In a single study, chromosomal aberrations were also induced in human lymphocytes cultured with sodium saccharin for 72 h. In another study, human neonatal foreskin fibroblast cultures were not transformed by treatment with sodium saccharin for 12 h.

In a single study, sodium saccharin did not bind covalently to DNA in liver or bladder of Sprague-Dawley rats treated once by gavage. In another study, measurements of DNA sedimentation patterns showed that sodium saccharin did not induce DNA damage in the bladder of Wistar rats after a single intravenous injection. Three of four sodium saccharin samples were mutagenic to *S. typhimurium* strains TA100 and TA98 in a host-mediated assay in which mice received a single oral treatment; only the highly purified sample gave negative results in this assay. Urine samples collected from mice over 24 h after treatment with the same four samples were also mutagenic to *S. typhimurium*. Bile from

Sprague-Dawley rats exposed to sodium saccharin by a single intravenous injection was not mutagenic to *S. typhimurium* in a single study.

Sodium saccharin induced single-strand breaks in liver and kidney DNA of CD1 mice treated by intraperitoneal injection in one study. It induced sister chromatid exchanges in the bone marrow of Chinese hamsters when cells were analysed 26 h after a single oral dose in one study, but not in a second study of ICR mouse embryo cells exposed *in utero* from a single intraperitoneal injection to pregnant dams on day 10 of gestation. Sodium saccharin did not induce micronuclei in C57BL mouse erythrocytes or NMRI mouse bone marrow, nor did it induce chromosomal aberrations in Chinese hamster or C57BL mouse bone marrow after treatment *in vivo*. A single intraperitoneal injection of sodium saccharin to mice did not induce somatic mutation; however, the compound induced somatic mutations when given daily for three days by stomach intubation.

Sodium saccharin induced dominant lethal mutations in mice in three of seven studies. Positive results were reported in CBA mice exposed to sodium saccharin in the drinking-water for 30 days, in ICR mice treated with five daily intraperitoneal injections and in Swiss and C17 mice treated with a single subcutaneous injection. Dominant lethal mutations were not induced in mice treated with two intraperitoneal injections, a single oral dose, five daily oral doses or fed a diet containing sodium saccharin for 10 weeks. The frequencies of chromosomal aberrations in the bone marrow and of spermatogonia of ICR mice were significantly increased after daily doses of sodium saccharin by gavage for 24 weeks. Chromosomal aberrations were not induced by sodium saccharin in ICR mouse embryos treated *in utero* by two intraperitoneal injections to pregnant dams on day 10 of gestation. Sodium saccharin did not induce chromosomal aberrations in spermatogonia of mice treated with a single intraperitoneal injection or with 10 daily oral doses or in spermatogonia of Chinese hamsters treated with two oral doses. In single studies, sodium saccharin did not induce heritable translocations or abnormal sperm morphology in mice given five daily intraperitoneal injections or 10 daily oral doses, respectively.

4.5 Mechanistic considerations

Sodium saccharin fed at high doses to rats increases the incidence of tumours of the urinary bladder if administered throughout the rats' lifetime, beginning before conception, at birth or at 30 days of age, and the effect is greater in male than in female rats. Bladder tumours are produced only sporadically in single rats when feeding is begun later, as in the usual one-generation bioassays. In addition, sodium saccharin increases the incidence of bladder tumours when administered to adult rats after pretreatment with a known genotoxic bladder carcinogen or after freeze-ulceration. As noted in section 3, no significant increase in the incidence of tumours in other organs is produced in rats, and no reproducibly increased incidence of tumours in any tissue, including the urinary bladder, was produced when sodium saccharin was administered to mice, hamsters, guinea-pigs or monkeys, although none of these species has been studied as extensively as the rat.

In all species including humans, about 80–95% of an orally administered dose of sodium saccharin is absorbed and rapidly eliminated in the urine, largely by renal tubular

secretion. Studies of tissue distribution, including two-generation studies, provided no evidence of significant accumulation of saccharin in the bladder. Very small amounts of saccharin were found in the urine of male neonatal rats (up to 18 days of age), presumably from the small amounts of saccharin transferred to the pups during lactation, as evidenced by the low concentration in the stomach contents (Sweatman & Renwick, 1982).

Several mechanisms have been suggested to explain the effects of high dietary doses of sodium saccharin in rats. The possibility that an impurity might be responsible was evaluated extensively in bioassays with *ortho*-toluene sulfonamide, which gave either negative results or involved administration of doses far in excess of those that contaminate saccharin. In addition, *ortho*-toluene sulfonamide is not a contaminant of saccharin synthesized by the Maumee method. The biological responses in short-term studies and in two-stage carcinogenesis studies were similar with sodium saccharin synthesized by the Maumee and by the Remsen-Fahlberg methods. Furthermore, the contaminants are not electrophilic.

Saccharin exists in ionized form under physiological conditions. As the anion is a nucleophile (pKa of saccharin, 2.0), it would not form DNA adducts without prior metabolic alteration. As expected, no binding to DNA was detected after administration to rats *in vivo*. None of the studies in humans provided evidence for biotransformation of saccharin, and several studies in experimental animals showed no metabolism of sodium saccharin at a limit of detection of 0.4% of the administered dose. Trace amounts of metabolic products were found in one study, but the metabolites were not electrophiles; it is not possible to evaluate whether the investigators completely excluded contaminants in their purification process.

The dose-related caecal enlargement that is produced by sodium saccharin led Sims and Renwick (1983) to propose that sodium saccharin changes protein metabolism, leading to accumulation of tryptophan which is converted to indoles by the gastrointestinal microflora. Consequently, indole excretion may promote bladder tumours. Schoenig and Anderson (1985) found, however, that female rats fed sodium saccharin had larger caeca than males. Anderson *et al*. (1988b) found that the caecal enlargement was caused by the potassium, calcium and acid forms of saccharin. Therefore, the cation and sex specificity of sodium saccharin-induced cell proliferation and tumorigenicity cannot be explained by this hypothesis.

Another mechanistic hypothesis relates to sodium saccharin-induced increases in urine volume and sodium excretion. Anderson (1988) proposed that the high urine volume induced increases cell proliferation and bladder mass; however, this concept is unlikely to explain all of the sex-, age- and cation-specific results, and drugs that greatly enhance urine volume have not been associated with induction of bladder tumours.

The most plausible mechanism of the effects of high dietary doses of sodium saccharin in rats is the formation of a calcium phosphate-containing precipitate in the urine which is cytotoxic to the urothelium, resulting in mild regenerative hyperplasia which leads to tumour formation in a small percentage of rats.

Hyperplasia of the urothelium has been demonstrated in various long-term and short-term studies after dietary administration of high doses of sodium saccharin to rats. In a two-generation bioassay, hyperplasia of the bladder epithelium was found in conjunction with bladder tumour formation. At some doses at which tumours were found, a statistically significant increase in hyperplasia was found (Squire, 1985). In a species comparison, male Fischer 344 rats responded to 5% [2500 mg/kg bw per day] sodium saccharin with urothelial cell proliferation, whereas the male $B6C3F_1$ mice, Syrian hamsters and Hartley strain guinea-pigs did not (Fukushima *et al.*, 1983a).

Several other short-term studies have shown that exposure of male rats to sodium saccharin in the diet increases the frequency of cell proliferation in the bladder epithelium secondary to necrosis of superficial cells and exfoliation. The necrosis appears to be limited to the superficial cell layer, without ulceration and without inflammation. In investigations of the time-course of the effects of sodium saccharin on the bladder, vacuolar degeneration of epithelial cells has been demonstrated by light microscopy and superficial cell necrosis by scanning electron microscopy; these lesions are followed by hyperplasia and increased DNA labelling (tritiated thymidine or bromodeoxyuridine). In one study of male Fischer 344 rats, the rate of cell division after 10 weeks of treatment was found to be enhanced at dietary concentrations of 5 and 7.5% sodium saccharin [2500 and 3750 mg/kg bw per day] (Cohen *et al.*, 1990), even after four and seven weeks with 7.5% sodium saccharin, but no effect was found with 3% sodium saccharin [1500 mg/kg bw per day] in the diet. The sodium saccharin dose-related increases in cell proliferation correlated with evidence of cell damage, although cell damage without significant cell proliferation was found with 3% sodium saccharin at 10 weeks. This study indicates a close dose–response relationship between cell damage, cell proliferation, carcinogenesis in two-generation bioassays and tumour promotion. In a two-generation study of exposure to 5% sodium saccharin or acid saccharin [2500 mg/kg bw per day] in the diet, increased bladder epithelial cell proliferation was found only after administration of the sodium salt to males and females of the F_1 generation from 21 days of age.

A comparative study of various forms of saccharin in male rats demonstrated that cell proliferation was increased with sodium and potassium saccharin, but saccharin did not increase the labelling index (Hasegawa & Cohen, 1986); the results with calcium saccharin were equivocal and slight. The urinary concentration of saccharin was the same after administration of any of the four forms. Sodium bicarbonate also increased cell proliferation, and this effect appeared to be additive to that of sodium saccharin (Debiec-Rychter & Wang, 1990). Sodium chloride and sodium ascorbate both increased the thymidine labelling index by about fourfold, and in this experiment sodium saccharin increased the labelling index in bladder epithelium by about sevenfold over that in controls.

Diet has been shown to affect the ability of sodium saccharin to enhance bladder epithelial cell proliferation: animals fed AIN-76A diet (a semisynthetic diet with casein as the protein source, resulting in low urinary pH) did not show sodium saccharin-induced increases in cell proliferation, and diet-specific changes in urinary pH have been proposed

to be an important factor (Garland *et al*., 1989). Sodium ascorbate and sodium saccharin administered during one- and two-generation studies and in two-stage studies of carcinogenesis were found to have similar hyperplastic effects on the urinary bladder. The effects of saccharin and ascorbate were inhibited by co-administration of ammonium chloride, which acidified the urine (Ellwein & Cohen, 1990; Uwagawa *et al*., 1994; Cohen *et al*., 1995a, 1998; Cohen, 1999).

Sodium saccharin and sodium ascorbate are tumorigenic in male rats but usually only when administered in a two-generation protocol rather than in a standard two-year bioassay beginning at six to eight weeks of age (Ellwein & Cohen, 1990; Cohen *et al*., 1998; Cohen, 1999). Several sodium salts administered at high doses in the diet have, however, produced bladder tumours in male rats after prior administration of a known genotoxic bladder carcinogen. These salts include sodium saccharin, sodium ascorbate, sodium aspartate, sodium citrate, sodium erythorbate, sodium succinate, sodium bicarbonate and sodium chloride. All these salts and sodium glutamate and sodium phosphate increased proliferation (hyperplasia) in the bladder in short-term studies in male rats when fed at doses comparable to 5 or 7.5% sodium saccharin (Ellwein & Cohen, 1990; Cohen *et al*., 1995c; Cohen, 1999).

These data indicate that the sequence of events leading to the tumorigenicity of these chemicals is superficial cytotoxicity resulting in regenerative hyperplasia and ultimately in the production of bladder tumours. This effect appears to occur only in rats and more severely in males than in females. Administration from 30 days of age or earlier appears to be necessary to attain a significant incidence of bladder tumours in long-term bioassays.

The requirement for administration beginning early in life appears to be due to quantitative relationships with the total amount of urothelial cell proliferation occurring during the course of the experiment (Ellwein & Cohen, 1988). The urothelium proliferates rapidly during gestation, but the rate quickly decreases after birth, so that the tritiated thymidine labelling index (1-h pulse dose) is 0.1% or less by six weeks of age. Approximately one-third of the lifetime cell divisions of rat urothelium occur by six weeks of age. Feeding sodium saccharin in a two-generation protocol increased proliferation by 21 days of age, substantially increasing the number of cell divisions early in the life of the rat, which can then be sustained by continued administration of sodium saccharin. This process can be reproduced quantitatively by ulcerating the adult male rat bladder, allowing it to heal and then administering 5% sodium saccharin (Cohen *et al*., 1982). The incidence of bladder tumours produced in this ulcer–saccharin protocol after two years is similar to that seen with high doses of sodium saccharin in two-generation studies in male rats.

Although several factors have been associated with the induction of urothelial toxicity, it appears to be due to the formation of a precipitate in the urine of rats after administration of high doses of sodium salts. This effect was first observed by Arnold *et al*. (1980) and subsequently by West and Jackson (1981) and Cohen *et al*. (1991). The effect has been observed in the urine of rats given high doses of sodium salts including those of saccharin, ascorbate, glutamate, aspartate, citrate, erythorbate, chloride and bicarbonate; in the urine of male rats more than in female rats (Arnold *et al*., 1980); and not in mice or monkeys

(Takayama *et al*., 1998; Cohen, 1999). In addition, co-administration of high doses of ammonium chloride with sodium saccharin or sodium ascorbate completely inhibits the appearance of the urinary precipitate (Cohen *et al*., 1995a,c). Thus, there is a close correlation between the presence of this precipitate and the subsequent urothelial toxic and proliferative effects.

The major inorganic constituents of the precipitate appear to be calcium and phosphate, but there are significant amounts of a silicon-containing substance. In addition, the major organic components are mucopolysaccharides and urea, with small amounts of protein and saccharin (Cohen *et al*., 1995b,c; Cohen, 1999). Calcium and phosphate in solution are essential ingredients for cell viability *in vitro* and *in vivo*, but calcium phosphate precipitate *in vitro* is cytotoxic to epithelial cells, including urothelial cells (Brash *et al*., 1987; Cohen, 1999).

Several factors appear to be critical for the formation of the calcium phosphate-containing precipitate: the main ones are high concentrations of calcium, phosphate and protein, pH ≥ 6.5 and high osmolality (Table 5). These factors appear to be critical only in rats (Cohen, 1999). Adequate concentrations of calcium and phosphate are required for precipitation; the rat has approximately 10–20 times more urinary calcium than the mouse and an approximately two to four times higher phosphate concentration (Cohen, 1995). In addition, after being fed high doses of sodium saccharin, rats consume more water, with a consequently increased urinary volume and overall dilution of the urine; however, the urinary concentrations of calcium and phosphate do not decrease (Schoenig & Anderson, 1985).

Urinary precipitate formation is inhibited by a urinary pH below 6.5 (Cohen *et al*., 1995b,c). After administration of high doses of sodium saccharin, the urinary pH may be similar to, lower than or higher than that of controls, but it remains above 6.5 (Ellwein & Cohen, 1990). If the compound is co-administered with ammonium chloride or if it is administered in AIN-76A semisynthetic diet, the urinary pH is below 6.0, no precipitate is found and there is no urothelial toxicity, regenerative hyperplasia or tumour formation. Thus, the parent acids, saccharin and ascorbic acid, do not cause these effects, since the urinary pH remains low after their administration. Sodium hippurate also does not produce these proliferative effects in rats, although the urinary pH remains below 6.3 after its administration (Fukushima *et al*., 1983a; Schoenig *et al*., 1985).

High urinary protein concentrations also appear to be necessary. Although the urinary concentration of protein is high in male and female rats, it is higher in males after sexual maturity because of the excretion of α_{2u}-globulin (Neuhaus & Flory, 1978; Hard, 1995). Studies of sodium saccharin- and sodium ascorbate-induced cell proliferation in NBR rats, which do not have very high urinary levels of the male rat-specific low-molecular-mass protein α_{2u}-globulin, have shown the importance of this protein (Garland *et al*., 1994; Uwagawa *et al*., 1994). Rats that do not produce this protein do not show the enhanced bladder epithelial cell proliferation caused by sodium saccharin or sodium ascorbate and behave similarly to females of other strains, such as Fischer 344. Male Fischer 344 rats given sodium saccharin in the diet had a statistically significantly greater

Table 5. Urinary concentrations of critical variables in various species and strains (control and treated) for the urothelial response to treatment with high doses of sodium saccharin in the diet

Variable	Species								
	Rat					Mouse[e]		Monkey[f]	Human
	Male[a]	Female[a]	Male[a,b]	Male[c]	Male[d]	Male	Female		
High protein[g]	++	+	++	+	±	++	+	–	–
High Ca^{++}, PO_4[g]	+	+	+	+	ND	–	–	+ or –	+ or –
pH ≥ 6.5[g]	+	+	–	+	+	+	+	+ or –	+ or –
High osmolality[g]	+	+	+	+	ND	+	+	–	–
Precipitate formation[h]	+	±	–	±	ND	–	–	–[i]	ND
Hyperplasia[h]	+	±	–	–	–	–	–	–[i]	ND
Tumorigenic effect[h]	+	±	–	ND	–	–	–	–[i]	

+, positive response; ±, equivocal response; –, negative response; + or –, both responses have been observed; ND, not determined; ++, higher protein concentrations in male rats and mice than in females

[a] Fischer 344 and Sprague-Dawley

[b] Rats given a treatment resulting in acidification of the urine, consisting of either acid saccharin, high doses of ammonium chloride with sodium saccharin or sodium saccharin in AIN-76A diet

[c] NBR rats deficient in α_{2u}-globulin

[d] Rats deficient in albumin

[e] Data on urinary parameters and hyperplasia for BALB/c and Swiss strains, data on tumorigenicity for BALB/c mice

[f] Cynomolgus, rhesus and African green

[g] Similar in control animals and in those treated with high doses of sodium saccharin

[h] Urinary precipitate formed in the urine and urothelial hyperplasia or tumours after administration of high doses of sodium saccharin in the diet; the tumorigenic effects include any found in one- or two-generation studies or the two-stage studies in different species

[i] Monkeys given sodium saccharin at a dose of 25 mg/kg bw per day for 18–23 years beginning at birth or within a few days of birth. The Working Group noted the relatively small dose of saccharin administered, the relatively small number of monkeys used and the multiplicity of species.

degree of hyperplasia and much higher cell proliferation rates than control Fischer 344 rats or NBR rats. Therefore, the difference in cell proliferation between these two strains of rats presumably correlates with the differences in urinary excretion of α_{2u}-globulin. Females were found to have no, or at most a very weak, tumorigenic response to sodium saccharin in two-generation studies (Tisdel *et al*., 1974; Arnold *et al*., 1980; Taylor *et al*., 1980), and females do not excrete significant amounts of α_{2u}-globulin.

At three weeks of age, when proliferation increases after dietary administration of sodium saccharin, male and female rats have a similar urinary composition, with high protein concentrations due primarily to albumin. Albumin is present at higher concentrations in male and female rats than in humans and also appears to be effective in contributing to the proliferative effects caused by sodium saccharin. Feeding sodium saccharin to analbuminaemic rats does not produce a proliferative response in the urothelium (Homma *et al*., 1991).

High osmolality also contributes to a greater likelihood of precipitation, and the osmolality of the urine of rats and mice is usually in the range of 1000–2000 mosm/kg or higher. In humans, urinary osmolality is usually < 300, and it has been calculated theoretically not to exceed 1000–1100. Rodents have a higher osmolality mainly because of their greater protein and urea concentrations.

The concentration of the administered anion does not appear to contribute much to formation of the precipitate. Although the urinary saccharin concentration after feeding at 5% of the diet is approximately 200 mmol/L, only small amounts are found in the precipitate. In addition, feeding sodium ascorbate at 5 or 7% in the diet produces the precipitate, but the urinary concentration of ascorbate is less than 20 mmol/L.

Thus, critical levels of multiple factors are required for the precipitate to form. The lower urinary protein concentrations in female rats appear to explain the differences in their response from that of male rats, and the lower urinary calcium and phosphate concentrations in mice appear to explain their lack of both precipitate formation and a urothelial proliferative response. Non-human primates and humans are not expected to develop the precipitate because they have lower urinary protein concentrations and osmolality.

Chemicals that induce urinary calculi, such as melamine and uric acid, produce urothelial toxicity, regenerative proliferation and tumours in rodent bladders. Calculi also appear slightly to increase the risk for bladder cancer in humans (Capen *et al*., 1999). Since bladder tumours are induced only at doses that produce urinary calculi, this process represents a threshold phenomenon. The production of bladder tumours after the feeding of high doses of various sodium salts by a mechanism involving formation of a calcium phosphate-containing precipitate in the urine thus represents a process that is both species-specific (rat) and occurs only at high doses (threshold).

As described in Capen *et al*. (1999), calcium phosphate-containing precipitates in the urine of rats, such as those produced by the administration of high doses of some sodium salts, including sodium saccharin and sodium ascorbate, can result in the production of urinary bladder tumours. This sequence can be considered to be species- and dose-specific and is not known to occur in humans.

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

- the formation of the calcium phosphate-containing precipitate occurs under the conditions of the bioassay in which cancer is induced;
- prevention of formation of the urinary precipitate results in prevention of the proliferative effect in the bladder;
- the agent (and/or its metabolites) has no genotoxic activity *in vitro* or *in vivo*;
- the agent does not produce tumours at any other site in experimental animals; and
- there is evidence from studies in humans that precipitate formation or excess cancer risk does not occur in exposed populations.

Sodium saccharin produces urothelial bladder tumours in rats by a non-DNA-reactive mechanism. The calcium phosphate-containing precipitate occurs in the urine under the conditions of the bioassays. The proliferative effect of sodium saccharin and the formation of the precipitate are prevented by acidification of rat urine. In addition, urine acidification prevents the tumorigenic effect of sodium saccharin in two-stage bioassays. Sodium saccharin does not produce tumours at any other site in experimental animals. Epidemiological studies have not shown that the incidence of cancer is increased in saccharin-exposed populations.

5. Summary of Data Reported and Evaluation

5.1 Exposure data

Saccharin and its salts have been used as sweeteners for nearly a century. Saccharin (acid form), sodium saccharin and calcium saccharin are widely used as non-caloric table-top sweeteners, in beverages and foods, in personal care products and in a variety of non-food applications. The average daily dietary intake is generally less than 1 mg/kg bw.

5.2 Human carcinogenicity data

Case–control studies of the carcinogenicity of artificial sweeteners have been reported only for the urinary bladder or lower urinary tract. Most of the studies were published between 1975 and 1985, so that any association would be to sweeteners that were on the market over 25 years ago. The studies varied widely in the detail with which information on the source and nature of artificial sweeteners was identified, collected and presented. The terms used in the various studies include ‘table-top’, ‘dietetic beverages’, ‘saccharin’ and ‘artificial sweeteners’ with no further characterization; only the salts of saccharin are used in these ways. Eight of the studies considered were hospital-based, which raises uncertainty about the representativeness of the controls’ consumption of artificial sweeteners in relation to the general population. The results of the population-based studies must also be viewed with caution, owing to the sizable proportion of non-

respondents, which might reflect the occurrence of health-related conditions associated with the use of replacements for sugar.

A statistically significant relative risk in the order of 1.6 for the association between use of artificial sweeteners (and saccharin salts as such) and bladder cancer and a dose–response relationship between intake and odds ratio were found for men but not for women in an early study in Canada. In subsequent population-based studies, including a study of several thousand people in the United States, estimates for the entire population of each study did not confirm the existence of an assocation. In some studies, estimates of the strength of the association between consumption of sweeteners and bladder cancer differed between smokers and non-smokers, but the direction of the difference and its distribution between the sexes was inconsistent over the studies.

In spite of the fact that three studies showed high, statistically significant relative risks for small subsets of consumers of very large amounts of artificial sweeteners, the finding was limited to men in one study and to women in the other two. In addition, no consistent pattern of dose–response relationship between use of artificial sweeteners and cancers of the urinary bladder or lower urinary tract is apparent in the available literature.

5.3 Animal carcinogenicity data

Sodium saccharin was tested by oral administration in numerous experiments in rats and mice and in a few studies in hamsters, guinea-pigs and monkeys.

Sodium saccharin produced urinary bladder tumours in male rats in four two-generation studies, in one study in male rats in which administration commenced at birth and in one study commencing at 30 days of age. Sodium saccharin was not carcinogenic for the urinary bladder in several one-generation studies in male and female rats or in mice.

Saccharin (acid form) did not produce tumours in one study in male and female mice, in one study in male rats or in one study in female rats. Calcium saccharin did not produce tumours in one study in male rats.

A few studies with sodium saccharin in hamsters and guinea-pigs also showed no induction of bladder tumours but were considered inadequate. In one long-term (up to 23 years) study in monkeys in which oral administration of sodium saccharin was begun shortly after birth, no bladder tumours were observed, but a relatively low dose (25 mg/kg bw) and relatively few animals were used.

Sodium saccharin has been studied in numerous experiments in adult rats involving administration concurrently or, more frequently, sequentially with other chemicals or treatments. Enhanced bladder tumorigenesis has been observed after prior treatment with known urinary bladder carcinogens. In one study, saccharin (acid form) did not significantly enhance the incidence of bladder carcinogenesis, while calcium saccharin produced a marginal increase.

Thus, the only organ affected by sodium saccharin is the urinary bladder and only in rats exposed for periods including pre- and/or postnatal periods and/or when exposure was begun by 30 days of age.

5.4 Other relevant data

Studies in humans and rodents reveal that, after absorption, saccharin and sodium saccharin are excreted unchanged in the urine. Excretion occurs relatively rapidly with no evidence of accumulation. The strong nucleophilic character of the saccharin anion and the lack of metabolism are consistent with the lack of DNA reactivity. The urinary concentration of the saccharin anion is similar, regardless of the form administered.

Sodium saccharin has been shown to enhance urothelial cell proliferation in rats, primarily in males, resulting in hyperplasia. This regenerative cell proliferation follows urothelial cytotoxic effects. Administration of saccharin (acid form) does not produce these effects in rats. Sodium saccharin at doses that enhance cell proliferation in rats does not do so in other species, including mice, hamsters and guinea-pigs. Hyperplasia was not produced in non-human primates, although the dose used in this study was lower than that used in the studies in rodents.

The cytotoxicity has been shown to result from formation of a calcium phosphate-containing precipitate in rat urine after administration of high doses of sodium saccharin or a variety of other sodium salts. A combination of factors in urine composition appears to be critical for formation of the precipitate, including a pH of 6.5 or greater, high urinary concentrations of calcium phosphate and protein and high urinary osmolality. This combination of critical factors appears to be unique to the rat and is consistent with the species-specific urothelial proliferative and tumorigenic effects in rats.

Saccharin, generally as the sodium salt, has been tested for developmental and reproductive toxicity in mice, rats, hamsters and rabbits. The effects have generally been limited to reductions in body weights at high dietary concentrations. With the exception of a test in *Drosophila* larvae, no effects have been reported in a variety of short-term assays to screen for teratogenicity *in vivo* and *in vitro*.

Saccharin (acid form) was not genotoxic in human or rodent cells *in vitro*. It weakly induced DNA single-strand breaks in rat hepatocyte cultures. It induced aneuploidy in yeast but was not mutagenic to bacteria.

Sodium saccharin induced dominant lethality in three of seven studies in mice *in vivo*; it did not induce heritable translocations, chromosomal aberrations in spermatocytes or embryos or altered sperm morphology in rodents *in vivo*. Negative or conflicting results were obtained in most studies of chromosomal damage in bone marrow, somatic mutation and sister chromatid exchange in rodents *in vivo*. Sodium saccharin was mutagenic in host-mediated and body fluid assays and caused DNA single-strand breaks in hepatic and renal cells of mice; however, bile from rats exposed to sodium saccharin was not mutagenic. Sodium saccharin did not cause DNA damage and did not bind covalently to DNA of rat liver or bladder. It induced genotoxic effects in human and rodent cells and in *Drosophila* and yeast. It was not mutagenic to bacteria.

The positive results for genotoxicity found with sodium saccharin in mammalian cells *in vitro* have been hypothesized to result from increased osmolality (i.e. nonspecific ionic effects). This hypothesis would appear to explain some but not all of the findings of sister chromatid exchange, chromosomal aberrations and gene mutations *in vitro*. The

few positive results seen in mice treated with sodium saccharin *in vivo* would not be readily explained by ionic influences.

Impurities in the test materials could explain the positive results obtained in some studies in mice treated with high doses of sodium saccharin. It is notable that no data are available on the genetic effects of saccharin or its salts in rats; however, the available evidence indicates no binding of sodium saccharin to DNA in rat bladder or liver. Overall, the results of tests for genotoxicity do not support a mechanism for the induction of urothelial-cell tumours in rats involving direct interaction of sodium saccharin with DNA.

5.5 Evaluation

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.

There is *inadequate evidence* in experimental animals for the carcinogenicity of saccharin (acid form) and calcium saccharin.

Overall evaluation

In making its evaluation, the Working Group 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).*

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