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Aspartame: A Safety Evaluation Based on Current Use Levels, Regulations, and Toxicological and Epidemiological Studies

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Aspartame is a methyl ester of a dipeptide used as a synthetic nonnutritive sweetener in over 90 countries worldwide in over 6000 products. The purpose of this investigation was to review the scientific literature on the absorption and metabolism, the current consumption levels worldwide, the toxicology, and recent epidemiological studies on aspartame. Current use levels of aspartame, even by high users in special subgroups, remains well below the U.S. Food and Drug Administration and European Food Safety Authority established acceptable daily intake levels of 50 and 40 mg/kg bw/day, respectively. Consumption of large doses of aspartame in a single bolus dose will have an effect on some biochemical parameters, including plasma amino acid levels and brain neurotransmitter levels. The rise in plasma levels of phenylalanine and aspartic acid following administration of aspartame at doses less than or equal to 50 mg/kg bw do not exceed those observed postprandially. Acute, subacute and chronic toxicity studies with aspartame, and its decomposition products, conducted in mice, rats, hamsters and dogs have

The authors dedicate this article to Dr. Robert M. Kroes, who actively participated in the preparation of this paper and approved the final version before he died from rapidly progressing lung cancer.

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consistently found no adverse effect of aspartame with doses up to at least 4000 mg/kg bw/day. Critical review of all carcinogenicity studies conducted on aspartame found no credible evidence that aspartame is carcinogenic. The data from the extensive investigations into the possibility of neurotoxic effects of aspartame, in general, do not support the hypothesis that aspartame in the human diet will affect nervous system function, learning or behavior. Epidemiological studies on aspartame include several case-control studies and one well-conducted prospective epidemiological study with a large cohort, in which the consumption of aspartame was measured. The studies provide no evidence to support an association between aspartame and cancer in any tissue. The weight of existing evidence is that aspartame is safe at current levels of consumption as a nonnutritive sweetener.

Keywords Aspartame, Sweetener, Consumption, Toxicology, Safety

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1. INTRODUCTION

An independent panel of recognized experts (hereafter referred to as the Expert Panel), qualified by their scientific training and relevant national and international experience in evaluating the safety of food and food ingredients, assessed the safety status of aspartame as a nonnutritive sweetener. The selection of experts was based on achieving international representation from various areas of toxicology relevant to aspartame including carcinogenesis, toxicokinetics, metabolism, pathology, food toxicology, biostatistics, epidemiology, neurotoxicology, and general toxicology. Dr. William Waddell chaired the Expert Panel.

A comprehensive search of the scientific literature for safety and toxicity information, on aspartame and its breakdown products, was conducted between January and September 2006 by the authors. Studies on the consumption of aspartame were reviewed and a current consumption analysis of aspartame was conducted using the most recent NHANES database. The toxicology and safety-in-use associated with aspartame were critically evaluated, and a summary review document was prepared by the primary author, Dr. Bernadene Magnuson, in consultation and with contributions from the Expert Panel. There were no exclusion criteria. To the best of our knowledge, all studies on the safety of aspartame conducted using scientific methodology and published in the peer-reviewed literature have been included in this review. Unpublished studies and reports were also included provided that methodology and results were available for review and that study conclusions were data-driven.

The review, literature, and supporting documentation were made available to the Expert Panel for comments. The Panel reviewed several modified versions until each panelist agreed that all pertinent issues were carefully considered. In addition, the Expert Panel independently evaluated other materials deemed appropriate and necessary. No preset evalutation criteria for assessing the weight of various pieces of evidence were given to the experts. In the field of toxicology weight of evidence, for causality of toxicity and the agent in question, is assessed by examining the relationship between dose and the following factors: nature and frequency of a toxic effect, the species- and sex-specific nature of the response, the relevance of the effect to humans, the route(s) of exposure causing the effect, the consistency or reproducibility of the findings, and biological plausibility of the effect. The expert panelists judged the weight of evidence of studies on the safety of aspartame using their knowledge, skills and experience.

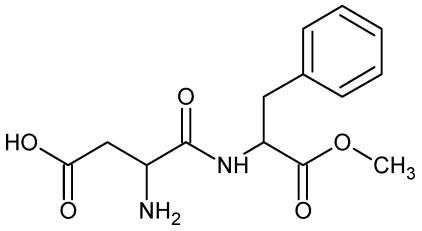

FIG. 1. Structure of aspartame (*N*-*L*-α-aspartyl-*L*-phenylalanine 1-methyl ester).

Throughout the period of review and evaluation, the identity of the sponsor of this review was unknown to the panelists, and the identities of the panelists were unknown to the sponsor. Following this independent, critical evaluation, the Expert Panel conferred and unanimously agreed to the decision herein.

1.1. Description

Aspartame, a synthetic nonnutritive sweetener, is a methyl ester of a dipeptide composed of aspartic acid and phenylalanine. The chemical structure is shown in Figure 1. There are two forms of aspartame, an alpha and a beta form. Only the α form is sweet and unless specified, "aspartame" herein will always refer to the alpha form. Aspartame is a white crystalline powder having no odor, but is intensely sweet. Aspartame is approximately 200 times sweeter than sucrose, the accepted standard for sweetness. Although aspartame yields the same caloric intake as sugar on a weight to weight basis (i.e., 4 kcal/g), it can be added at almost 200 times lower levels and achieve the same sweetness, thereby providing a far lower net caloric intake. This attribute has resulted in the use of aspartame as a low calorie or nonnutritive sweetener in foods and beverages worldwide.

1.2. History of Use

Aspartame was discovered accidentally in 1965 by James Schlatter, a scientist in the G. D. Searle research laboratories, while working to synthesize a tetrapeptide as an inhibitor of the gastrointestinal secretory hormone, gastrin (Mazur, 1984). While preparing the tetrapeptide, he was working to crystallize an intermediate, aspartylphenylalaninemethyl ester, and some of the solution accidentally was spilled onto his hands. Against all good safety practices, Schlatter licked his fingers to pick up a piece of paper and discovered the intense sweet taste (Mazur, 1984).

Cloninger and Baldwin (1970) published a report in *Science* in 1970, comparing the sweetness and other flavor attributes of aspartame in comparison with sugar and other sweeteners (saccharin and cyclamate) and proposed its use as an artificial sweetener.

2. DESCRIPTION, SPECIFICATIONS, AND MANUFACTURING PROCESS

2.1. Description and Specifications

Aspartame is a white powder with no odor, but is extremely sweet to humans, in contrast to other species (Section 0). Aspartame is slightly soluble in water and ethanol. It is not soluble in fats or oils. In dry form, aspartame is very stable, but does degrade at high temperatures and in aqueous solutions over time. The general description and specifications for aspartame are provided in Table 1 and Table 2.

2.2. Manufacturing Process

Aspartame is manufactured by the coupling of amino acids *L*-phenylalanine methyl ester and *L*-aspartic acid to produce the

TABLE 1
General description of aspartame (Budavari et al., 1999; Sweetman, 2002; FCC, 2003; Burdock, 2005)

Appearance	White, crystalline powder
CAS No.	22839-47-0
Chemical formula	$C_{14}H_{18}N_2O_5$
EINECS No.	245-261-3
Functionality in food	Sweetener; sugar substitute; flavor enhancer
INS No.	951
Molecular weight	294.31
NAS No.	1013
Odor	Odorless
Synonyms	3-Amino-*N*-(α-carboxyphenethyl)succinamic acid *N*-methyl ester, stereoisomer; APM; Aspartylphenylalanine methyl ester; *N*-*L*-α-Aspartyl-*L*-phenylalanine 1-methyl ester; Nutrasweet®; Equal®; Canderel® ; Sanecta™; Tri-Sweet™; E951; Dipeptide sweetener; Methyl aspartylphenylalanate; 1-Methyl *N*-*L*-α-aspartyl-*L*-phenylalanine; *L*-Phenylalanine, *N*-*L*-α-aspartyl-, 1-methyl ester (9CI); Succinamic acid, 3-amino-*N*-(α-carboxyphenylethyl)-,*N*-methyl ester, stereoisomer; Sweet dipeptide; Aspartamum; Аспартам

Note. CAS = Chemical Abstracts Service; EINECS = European Inventory of Existing Chemical/Commercial Substances; FCC = Food Chemical Codex; INS = International Numbering System; NAS = National Academy of Sciences.

TABLE 2
Specifications of aspartame

	FCC (2003)	JECFA (1992)
Appearance	White, crystalline powder	White, crystalline powder
Arsenic	NR	Not more than 3 mg/kg
Assay	Not less than 98% and not more than 102% of $C_{14}H_{18}N_2O_5$, calculated on the dried basis	Not less than 98% and not more than 102% of $C_{14}H_{18}N_2O_5$ on the dried basis
5-Benzyl-3,6-dioxo-2-piperazineacetic acid	Not more than 1.5%	Not more than 1.5%
Heavy metals	NR (see lead)	Not more than 10 mg/kg
Identification	Infrared	Solubility; Passes positive test for amino group; Passes positive test for ester group
Lead	Not more than 1 mg/kg	NR (see heavy metals)
Loss on drying	Not more than 4.5%	Not more than 4.5% (105°C, 4 h)
Odor	NR	Odorless
Other optical isomers	NR	Passes test
Other related substances	Not more than 2%	NR
Packing and storage	Store in well-closed containers in a cool, dry place	NR
pH	8% solution = 4.5–6.0	4.5–6.0 (1 in 125 solution)
Residue on ignition	Not more than 0.2%	NR
Solubility	Sparingly soluble in water; Slightly soluble in alcohol	Slightly soluble in water and in ethanol
Specific rotation	$[\alpha]_D^{20^\circ}$: Between +14.5° and +16.5°, calculated on the dried basis	$[\alpha]_D^{20^\circ}$: +14.5° to +16.5° calculated on the dried basis
Sulfated ash	NR	Not more than 0.2%
Transmittance	NR	Passes test (equivalent to an absorbance of not more than approximately 0.022

Note. FCC = Food Chemical Codex; JECFA = Joint FAO/WHO Expert Committee on Food Additives; NR = not reported.

dipeptide methylester (Burdock, 1997). If the coupling is done chemically, both the sweet α form and the nonsweet β form of aspartame are produced, requiring separation to obtain only the α form. The enzymatic process yields only α-aspartame (Rowe et al., 2003). The process is illustrated in the simplified flowchart in Figure 2. Esterified *L*-phenylalanine (Phe) is reacted with N-protected *L*-aspartic anhydride (N-protected-Asp O) to form N-protected *alpha*-aspartame. After the coupling reaction, a series of steps of protection removal, crystallization, decolorization, filtration, sterilization,[1] fine crystallization, and drying, the final product aspartame (*L*-aspartyl-*L*-phenylalanine methyl ester) is obtained.

2.3. Stability

Aspartame is very stable under dry conditions, but degrades during prolonged heat treatment in aqueous solutions. Furda et al. (1975) identified five degradation products of a 1% aqueous solution of aspartame following storage at 37°C for 2 months at pH 4.6. The compounds were 3-carboxymethyl-6-benzyl-2,5-diketopiperazine (DKP), L-aspartyl-phenylalanine, L-aspartic acid, L-phenylalanine, and L-phenylalanine methyl ester (Furda et al., 1975). None of the breakdown products are sweet.

The rate of degradation in aqueous solutions depends on the pH and the temperature (Rowe et al., 2003). Aspartame is most stable between pH 4-5, with a half-life of over 250 days at 25°C (Rowe et al., 2003). The pathway of breakdown of aspartame in solution also depends upon the pH of the solution (see Figure 3) (Bell and Labuza, 1991; Prodolliet and Bruelhart, 1993). At neutral and alkaline pH, aspartame can cyclize to DKP, or hydrolyze to α-aspartylphenylalanine and methanol. DKP and α-aspartylphenylalanine can interconvert, but do not revert to aspartame. In addition to these reactions, at acidic pH (less than 4.5), aspartame can undergo rearrangement to β-aspartame. In addition, the peptide bond in aspartame can be cleaved, producing phenylalanine methyl ester and aspartic acid. β-Aspartame can be cleaved to produce β-aspartylphenylalanine. Lastly, phenylalanine may be formed from hydrolysis of

[1]The sterilization process involves heat treatment at 105°C for 5 s using a heat plate exchanger (personal communication).

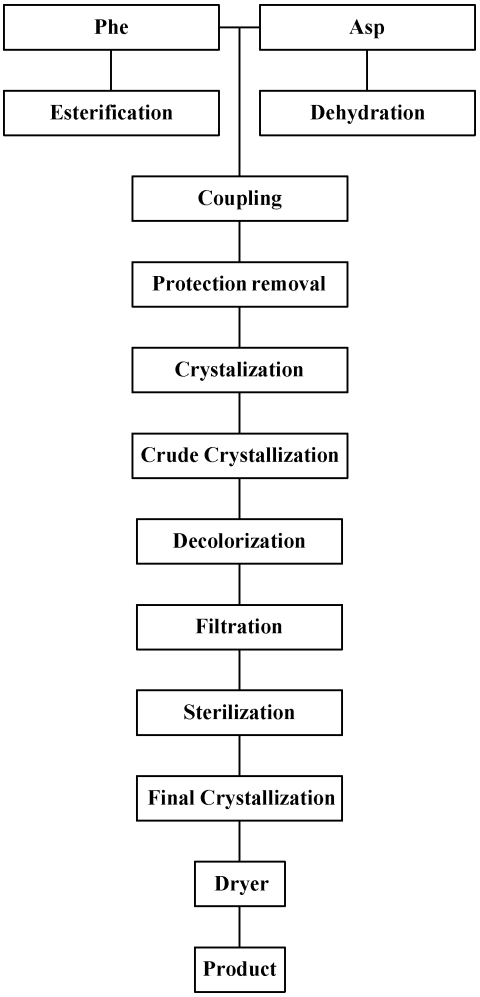

FIG. 2. Flow chart for the production of aspartame.

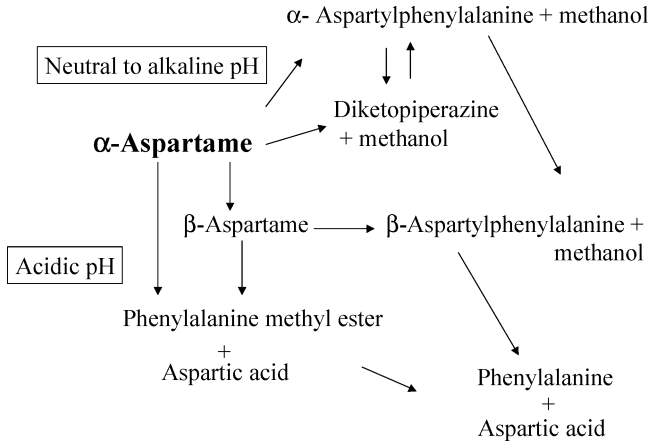

FIG. 3. Decomposition of aspartame. Adapted from Bell and Labuza (1991) and Prodolliet and Bruelhart (1993).

aspartylphenylalanine or phenylalanine methyl ester (Bell and Labuza, 1991).

Another parameter of foods that greatly affects the stability of aspartame is water activity[2] (Bell and Labuza, 1991). Water activity is known to affect the kinetics of many chemical reactions. The effect of water activity on the breakdown of aspartame under various pH conditions was investigated using a model system of agar and microcrystalline cellulose equilibrated to water activities ranging from 0.3 to 0.7. Dry foods are generally defined as those with a water activity below 0.6 as no known microbes grow at this level. The rate of degradation of aspartame increased with increasing water activity. For example, at 30°C and a pH of 5, approximately 60% aspartame was remaining in the model system after 100 days when the water activity was 0.34, whereas this dropped to less than 25% when water activity was 0.57, and to less than 10% at a water activity of 0.66 (Bell and Labuza, 1991). At a temperature of 45°C and a pH of 5, the half-life of aspartame dropped from 6.3 days at a water activity of 0.61, to 5.4 days at a water activity of 0.65 (Bell and Labuza, 1991).

Pattanaargson et al. (2000, 2001) assessed the effect of pH on aspartame degradation in phosphate–citrate buffer solutions ranging from pH 2 to pH 12. They report that the major decomposition product at pH 2 to 6 was phenylalanine methyl ester; in the pH range of 7 to 10, it was DKP; and at pH 12, the major breakdown product was α-aspartylphenylalanine. Thus, this study is in agreement with the pH dependent pathway of aspartame breakdown described earlier (Bell and Labuza, 1991). Sabah and Scriba (1998) reported similar results.

In water, the half-life of aspartame was reported to be 300 days at 20°C, pH 3 (EC, 2000). The stability of aspartame in commercially sterilized dairy beverages was shown to be most dependent upon pH and storage temperature, while the type and concentration of the buffer and the addition of vanilla flavor had little effect (Bell and Labuza, 1994). The half-life of aspartame dropped from 24–54 days at 4°C to 1–4 days at 30°C in reduced calorie dairy beverages, illustrating increased breakdown with increased temperature. A change in pH from 6.7 to 6.4 resulted in a doubling of the half-life, illustrating the effect of even small changes in pH.

Prodolliet and Bruelhart (1993) determined the amount of aspartame and its major decomposition products in a variety of commercial foods purchased in Switzerland and France using high-performance liquid chromatography (HPLC). The 24 foods included tabletop sweeteners, beverages, creams, yogurt, fruit pulp, candies, cheese, and muesli. Breakdown products of aspartame were present at the highest amounts in dairy products, including commercially prepared fruit cream,[3] milk chocolate, malted beverages, and fruit yogurt; however, most foods contained within 90% of the amount of aspartame claimed on the

[2]Water activity is a measure of the amount of water available for microbial growth. It is defined as the ratio of the vapor pressure of water in a solution to the vapor pressure of pure water (Vaclavik and Christian, 2003).

[3]Fruit cream is a custard dessert containing fruit and cream.

label (Prodolliet and Bruelhart, 1993). Gibbs et al. (1996) developed a rapid HPLC method for determination of aspartame and breakdown products in liquid and solid foods. They also report excellent agreement between amount of aspartame reported by the food manufacturer and the amount detected in foods.

DKP, the cyclization product of aspartame, is one of many ubiquitous cyclic dipeptide derivatives found in nature and protein-rich foods. Widely varied foods contain naturally occurring diketopiperazines, ranging from cereal grains to bread, hydrolyzed vegetable protein, cheeses, processed meats such as ham and hotdogs, fish, fish sauce, dried shrimp, dried squid, and beverages including cocoa, coffee, beer, and milk (Hilton et al., 1992; Gautschi and Schmid, 1997; Ginz and Engelhardt, 2000). Concentrations range from 6 pmol/g in milk to 6576 pmol/g in dried shrimp (Hilton et al., 1992). The amount of DKP in aspartame-containing foods depends on the type of food or beverage, the pH, and the time and temperature of storage (Homler, 1984). Under dry conditions, the conversion of aspartame to DKP is slow, at a rate of 5% per 100 h at 105°C (Homler, 1984). DKP levels in commercial foods purchased from stores, including soft drinks, juices, and dairy products, ranged from less than 0.3 to 14% of the aspartame concentration (Prodolliet and Bruelhart, 1993; Kotsonis and Hjelle, 1996). Natural sources also include marine sponges and fungi (Hilton et al., 1992).

The rate of degradation is also affected by the composition of the food product, such as the presence of oil, which enhances the rate of aspartame degradation (Bell and Labuza, 1991). The stability of aspartame is greatly enhanced (up tp 42%) in the presence of β-cyclodextrin (Garbow et al., 2001).

Aspartame hydrochloride, the chloride salt of aspartame, has been marketed under the trade name of Usal (Prudel et al., 1986). Aspartame hydrochloride, stored at pH 2.2 and 37°C for 2 months, was degraded to a higher degree than aspartame stored at pH 4.6 (Furda et al., 1975). As with aspartame, the main decomposition products of aspartame hydrochloride in solution were α-aspartyl-phenylalanine and DKP (Prudel et al., 1986).

Aspartame is not recommended for use in baked or fried food products, due to degradation upon exposure to high temperatures. Aspartame can also react with reducing compounds in the Maillard reaction (Bell and Labuza, 1991). Thermal degradation products of aspartame in the temperature range of 80–200°C were studied using isothermal thermogravimetry, Fourier Transform Infrared (FTIR) and high-performance liquid chromatography (Conceicao et al., 2005). Under these conditions, the only product of degradation is 5-benzyl-3,6-dioxo-2-piperazineacetic acid, which is an enantiomer of the DKP formed in aqueous solutions.

Only α-aspartame has sweetness, therefore breakdown results in loss of sweetness in the food product. This was clearly illustrated by Quinlan et al. (1999), who evaluated the effect of storage at 20°C on the flavor of cola drinks sweetened with various sweeteners. Colas sweetened with aspartame or aspartame/acesulfame-K blends were significantly less sweet after 6 months of storage, due to breakdown of aspartame, whereas colas sweetened with either sucrose or sucralose had little change in sweetness. The loss of sweetness with the breakdown of aspartame results in reduced likelihood of consumption of the products containing the breakdown products, as consumers will reject the product if it does not have the anticipated sweetness. Therefore, consumption of breakdown products of aspartame is self-limiting by the lack of sweetness.

Thus, aspartame is a stable food ingredient in dry form, or in acidic aqueous conditions. Degradation will occur in foods that are more basic and with high temperatures. Therefore, the use of aspartame in baked or heated foods is limited, and best-before dates are utilized on products such as aspartame-sweetened soft drinks to discourage long-term storage.

2.4. Current Uses

Aspartame is approved for use as a sweetening agent and a flavor enhancer. Gram for gram, aspartame has approximately 200 times the sweetness of sucrose, and does not have a bitter or metallic aftertaste (characteristic of saccharin). Aspartame also extends and intensifies a variety of flavors, especially fruit flavors (Prodolliet and Bruelhart, 1993). Aspartame is sold as a tabletop sweetener under the brand name of Equal®, and as an ingredient in food products as NutraSweet®, Canderel®, Sanecta™, Tri-Sweet™, and E951 (Baines, 1985; Thomas-Dobersen, 1989; Arcella et al., 2004). In the United States, the largest use of aspartame is for sweetening low-calorie drinks. Aspartame is also used as a sweetener in some pharmaceuticals (FDA, 2006).

In a study of the worldwide market of intense sweeteners, Fry (1999) illustrated that the majority of aspartame sales is in the Americas and Europe, with Asia and Africa and Oceania accounting for only a small proportion. In contrast, Asia is the largest market for saccharin and cyclamates. Therefore, the market for aspartame is primarily the highly developed countries, principally due to the higher cost of aspartame as compared to other intense sweeteners (Fry, 1999).

2.5. Regulatory Status

Aspartame has been approved for use in food in more than 90 countries (Health Canada, 2005), including the United States, Canada, countries in the European Union, Japan, Australia, and New Zealand. (Table 3) Aspartame has been added to over 6000 products worldwide (Butchko and Stargel, 2001). The following foods are examples of aspartame-sweetened products available in the United States: breath mints, carbonated soft drinks, cereals, chewing gum, flavored syrups for coffee, flavored water products, frozen ice, frozen ice cream novelties, fruit spreads, sugar-free gelatin, hard candies, ice cream toppings, no-sugar-added or sugar-free ice creams, iced tea (powder and ready to drink), instant cocoa mix, jams and jellies, juice blends, juice drinks, maple syrups, meal replacements, mousse, no-sugar-added pies, noncarbonated diet soft drinks, nutritional bars, powdered soft drinks, protein nutritional drinks, puddings, candy chews, sugar-free chocolate syrup, sugar-free cookies,

TABLE 3
Summary of the regulatory status of aspartame by select agencies

Agency	Identification	Permitted functionality	Use limits	Reference
EC/JECFA	E951	Sweetening agent; Flavor enhancer	NR	JECFA (2003); EFSA (2002)
FDA	Food additives permitted for direct addition to food for human consumption. Multipurpose additives. Aspartame	Sweetening agent; Flavor enhancer	cGMP	21 CFR§§ 172.804[4]
FSANZ	General food standards. Substances added to food. Schedule 1- Permitted uses of food additives by food type. Aspartame	Intense sweetener; Flavor enhancer	Confectionery-10,000 mg/kg; Electrolyte drink and electrolyte drink base-150 mg/kg; Brewed soft drinks-1000 mg/kg	Standard 1.3.1[5]
Health Canada	Food additives that may be used as sweeteners. Aspartame	Sweetener	Table-top sweetener-cGMP; Breakfast cereals-0.5%; Beverages, beverage concentrates and mixes-0.1% as consumed; Desserts (and mixes), toppings (and mixes), fillings (and mixes)-0.3% as consumed; Chewing gums, breath freshener products-1%; Fruit spreads, purees and sauces, table syrups-0.2%; Salad dressings, peanut and other nut spreads-0.05%; Condiments-0.2%; Confectionery glazes for snack foods, sweetened seasonings or coating mixes for snack foods-0.1%; Confections and their coatings-0.3%	Food and Drug Regulations, Part B, Division 16, item number A1[6]
The Ministry of Health and Welfare, Japan	Standards for use, according to use categories	Nonnutritive sweetener	No limits cited	JFCRF (2000)

CFR = Code of Federal Regulations; cGMP = current Good Manufacturing Practices; EC = European Commission; FDA = U.S. Food and Drug Administration; FSANZ = Food Standards Australia New Zealand; JECFA = Joint FAO/WHO Expert Committee on Food Additives; NR = not reported.

[4]Title 21 of the U.S. Code of Federal Regulations (CFR), section 172.804, 2005.
[5]Australia New Zealand Food Standards Code, Standard 1.3.1, 2005.
[6]Consolidated Statutes and Regulations of Canada online at (http://laws.justice.gc.ca/en/F-27/C.R.C.-c.870/124334.html). Visited March 20, 2006.

sugar-free ketchup, table-top sweeteners, vegetable drinks, and yogurt (drinkable, fat-free, and sugar-free) (Lim et al., 2006a).

In an update on the safety in use of aspartame, the European Union's Scientific Committee on Food maintained the established acceptable daily intake (ADI) of aspartame by humans at 40 mg/kg of body weight (bw) (EC, 2002). The U.S. Food and Drug Administration (FDA) established an ADI of 50 mg/kg bw for aspartame (CFR, 2005). Recently, the European Food Safety Authority (EFSA, 2006) evaluated a new long-term carcinogenicity study on aspartame and confirmed the previously established ADI for aspartame of 40 mg/kg bw/day. There are no countries known in which approval of aspartame for food use was requested and denied.

Aspartame is approved by the FDA for use under CFR 172.804 as a flavor enhancer and general-purpose sweetening agent for foods in which standards of identity do not preclude such use. Any food containing the additive must bear on its label the following statement: "Phenylketonurics: contains phenylalanine." In addition, when aspartame is used as a sugar substitute for table use, the label must bear instructions not to use it in cooking or baking. The food groups as defined by the FDA (21 CFR 170.3(n)) are listed in Table 4. Aspartame is also approved for use as an inactive ingredient in drug formulations (FDA, 2006).

3. ESTIMATED DAILY INTAKE

The evaluation of safety of adding a food component to the food supply requires consideration of the amount of the component that will be added to food, the amount of its metabolites that will be in the foods, and the amount of these components that are already present or naturally occurring in the food supply. Therefore, the sources and estimated daily intake of aspartame, its constituent amino acids, and methanol are discussed next.

3.1. Consumption of Aspartame

Aspartame does not occur naturally in foods, so estimates of consumption are based only on amounts intentionally added to foods.

3.1.1. Projected Consumption of Aspartame

Estimates of the projected maximum consumption of aspartame calculated by the FDA, the Market Research Corporation of America, and various researchers in the 1970s ranged from 22 to 34 mg/kg bw/day (Stegink et al., 1977, 1981b). These estimates assumed that aspartame would be used as the sweetener to replace all sucrose in the typical American diet and then calculated the maximum possible consumption (i.e., 99th percentile). Therefore, they are much higher than the estimates based on actual consumption data that are described later in this document. Based on the metabolism of aspartame, 22 to 34 mg aspartame/kg bw/day would be equivalent to ingesting 12–19 mg phenylalanine/kg bw/day, 10–15 mg aspartic acid/kg bw/day, and 2.4–3.7 mg methanol/kg bw/day (Stegink, 1987). These amounts were converted into measures more commonly understood by the public by Franz (1986), who calculated that 34 mg/kg bw/day (the expected 99th percentile) would be equal to 57 packets of sweetener or 10 cans of diet soda per day. Another comparison was that an average intake of 500 mg per person would provide a similar amount of aspartic acid and phenylalanine as 6 oz of milk or 3 oz of beef, and as much methanol as 8 oz of vegetable juice or 2 oz of gin (Franz, 1986). Renwick (1990) has made similar comparisons.

Filer and Stegink (1989) estimated that based on the amount of aspartame sold to food and beverage processors, aspartame replaced 15 lb of the ~132 lb of total sweeteners consumed per person per year, which would be equivalent to 38 g/person/year. This is further calculated to be 108 mg/person/day or 1.5 mg/kg bw/day for a 70-kg person. This estimate, however, averages intakes over all individuals whether they are consumers of aspartame or not, so it is an underestimation of intakes by consumers only.

Consumption of aspartame can also be projected based on sales data, which was reported to be 8000 tons/yr in the United States (Hazardous substances database 2005). According to the CEH Marketing Report (Bizarre et al., 2006) the annual U.S. sales of aspartame have steadily increased from 3300 metric tons in 1985 to 10,100 in 2002. Another estimate (personal communication) is that the current global production is 18,000 metric tons, with approximately 9600 tons being consumed in the United States in 2006.

Diet carbonated soft drinks represent the major source of exposure of aspartame in the U.S. diet, so changes in exposure to aspartame may also be inferred from changes in consumption of diet soft drinks. The USDA Economic Research Service[7] has reported the annual per capita consumption of diet carbonated soft drinks from 1984 to 2004. These data, shown in Figure 4, illustrate that consumption of diet soft drinks was level during the 1990s, but started to increase in 2002. The ounces per day per person rose from approximately 4.8 during the 1990s to 5.55 ounces per day in 2004. These data all indicate that consumption of aspartame has increased in recent years.

3.1.2. Reported Studies on the Consumption of Aspartame

The consumption of aspartame has been studied worldwide, in the general population and in special population subgroups, by calculating food intake and multiplying intake of specific foods by the concentration of aspartame in those foods. The outcome of these studies depends on the methods used for the selection of participants, the estimation of food intake and the determination of the amount of aspartame in foods. A summary of reported studies on the consumption of aspartame is provided in Table 5. On average, intake of aspartame was below 3 mg/kg bw per person per day. The strengths and weaknesses of several of these studies have been discussed in a recent review article by Renwick (2006).

[7]http://www.ers.usda.gov/ (site visited May 10, 2006).

TABLE 4
Food groups approved for aspartame supplementation (Burdock, 1997) and use limits per 21 CFR §172.804

Food category	Use limit
Baked goods and baking mixes, including all ready-to-eat and ready-to-bake products, flours, and mixes requiring preparation before serving	When aspartame is used in baked goods and baking mixes, the amount of the additive is not to exceed 0.5% by weight of ready-to-bake products or of finished formulations prior to baking. Generally recognized as safe (GRAS) ingredients or food additives approved for use in baked goods shall be used in combination with aspartame to ensure its functionality as a sweetener in the final baked product
Breakfast cereals, including ready-to-eat and instant and regular hot cereals	NR
Breath mints, hard and soft candy	NR
Chewable multivitamin food supplements	NR
Chewing gum	NR
Dry bases for: beverages, instant coffee and tea beverages; gelatins, puddings and fillings; dairy product analog toppings	NR
Frostings, toppings, fillings, glazes and icings for precooked baked goods	NR
Frozen dairy and nondairy frostings, toppings and fillings	NR
Frozen desserts	NR
Frozen, ready-to-thaw-and-eat cheesecakes, fruit and fruit toppings	NR
Frozen stick-type confections and novelties	NR
Fruit (including grape) wine beverages with ethanol contents below 7% volume per volume	NR
Fruit juice based drinks (where food standards do not preclude such use); fruit-flavored drinks and ades; imitation fruit-flavored drinks and ades	NR
Fruit spreads, fruit toppings, and fruit syrups	NR
Malt beverages of less than 7% ethanol by volume and containing fruit juice	NR
Nonnutritive sweeteners	When used as a sugar substitute for table use, the label shall bear instructions not to use in cooking or baking. Packages of the dry, free-flowing additive shall prominently display the sweetening equivalence in teaspoons of sugar. When used as a sugar substitute tablets for sweetening hot beverages including coffee and tea, the L-leucine may be used as a lubricant in the manufacture of such tablets at a level not to exceed 3.5% of the weight of the tablet.
Ready-to-serve nonalcoholic flavored beverages, tea beverages, fruit-juice-based beverages, and their concentrates or syrups	NR
Refrigerated flavored milk beverages	NR
Refrigerated ready-to-serve gelatins, puddings, and fillings	NR
Yogurt-type products where aspartame is added after pasteurization and culturing	NR
Aspartame may be used as a flavor enhancer in chewing gum, hard candy, and malt beverages containing less than 3% alcohol by volume	NR

Note. 21 CFR §172.804 = Title 21 of the U.S. Code of Federal Regulations (CFR), section 172.804, 2005; NR = not reported.

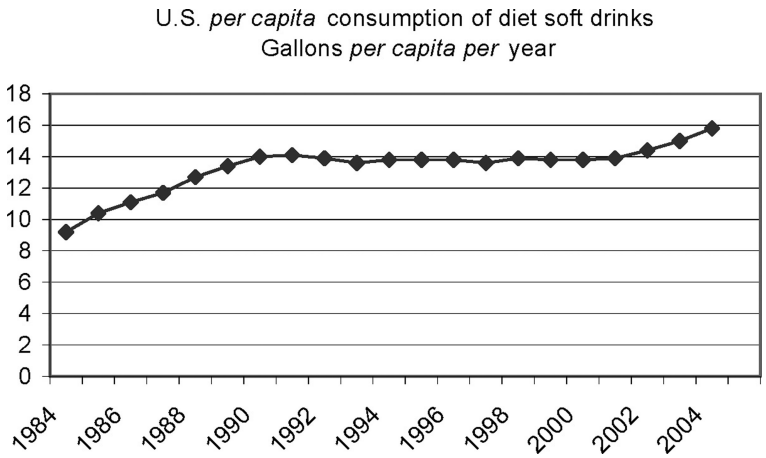

FIG. 4. U.S. per capita consumption of diet soft drinks (USDA Economic Research Service).

3.1.3. Analysis of Estimated Consumption in the United States

The National Health and Nutrition Examination Surveys (NHANES) commenced collecting data on the nutritional intake of Americans in the early 1970s. Three of these surveys were conducted between then and the 1990s. In 1999, NHANES became a continuous survey. Phase one of NHANES III was conducted between 1988 and 1991 (Alaimo et al., 1994), whereas NHANES 1999–2000 is the first of the 2-year data releases planned for the newer continuous survey (Ervin et al., 2004). This new annual survey format is based on a nationally representative sample, but 2 or more years of data are combined to generate adequate sample sizes for subgroup analyses.

A consumption analysis of aspartame was conducted by Burdock Group using the most recent NHANES database. This nationwide dietary intake survey was conducted during 2001–2002, and comprised 2 days of data that were collected for all respondents in the food survey ($n = 9701$ individuals). A full description of the database, the analysis and the results is provided in the consumption report in Appendix I. This analysis is dependent upon two input variables chosen by the investigator: the selected food codes and estimated concentration of aspartame in these foods. Food codes included in NHANES were searched using the keywords aspartame, diet, and sweetener. All foods codes from these keyword searches that could possibly contain aspartame were included. Foods that are baked (i.e., diet cookies) or heated (i.e., extruded breakfast cereals) were eliminated following confirmation (personal communication) that aspartame is not used in these products. The list of food codes used in this analysis is provided in Appendix I. A number of sources was used to determine the concentrations of aspartame assigned to food codes for the consumption analysis (Anonymous, 1991; Leclercq et al., 1999; Butchko and Stargel, 2001; Anonymous, 2005; Anonymous, 2006). When more than one concentration was reported for a food type, the highest reported concentration of aspartame was used. The results of this analysis are shown in Table 6.

Assuming aspartame is added to the selected foods at the levels specified in Table 30 in Appendix I, the mean, 90th and 95th percentile aspartame consumption is estimated at 330, 705, and 940 mg/day, respectively. On a per kilogram body weight basis, this corresponds to mean, 90th and 95th percentile intake of 4.85, 10.43, and 13.29 mg/kg bw/day, respectively. This estimate is higher than previously reported for the United States (Table 5). This is not surprising considering the following factors: (1) This analysis used the dietary survey data from 2001–2002, thus incorporating the changes in dietary consumption described earlier such as increased consumption of diet soda; (2) the highest reported concentration of aspartame in products was used in the calculation although this may not be the actual amount in all the products in that food code; and (3) no correction for the use of other artificial sweeteners or blends of artificial sweeteners was attempted. In the case of diet soft drinks in the United States, the sweetener may be aspartame, acesulfame K, saccharin, or sucralose or a combination, depending on the brand. This assumption was shown to have little impact on the estimation of artificial sweetener consumption of average consumers. But including information on soft drink brand loyalty in probabilistic models such as Monte Carlo could alter the estimated consumption of high consumers (95th percentile) by about twofold (Leclercq et al., 2003). These factors would result in a consumption estimate that is considerably higher than would be determined if further refinements in the concentrations of aspartame in the food products had been attempted. The important point is that, even in the light of increased sales of aspartame and increased consumption of diet products, the average consumption of aspartame remains at approximately one tenth of the ADI, and even the highest consumers are consuming only about 25% of the established ADI levels. This is in agreement with the finding of Renwick (2006).

Another source of aspartame is in pharmaceutical preparations, especially in tablets and sugar-free formulations (FDA, 2006). The amount of aspartame allowed as an inactive ingredient in various drug products is defined by the Center for Drug Evaluation and Research of the FDA (2006). However, the amount actually used in pharmaceuticals is not known as

TABLE 5
Summary of studies to estimate aspartame consumption

Population	Method	Mean (mg/kg bw/day)	90th percentile of consumers (mg/kg bw/day)	95th percentile of consumers (mg/kg bw/day)	Reference
		National/general			
United States, 1989	MCRA survey 14-day averaged, eaters only	1.2	2.2 all ages, eaters only 2.6 dieters 2.6 diabetics	N/A	Butchko and Kotsonis (1991); Kotsonis and Hjelle (1996)
Canada, 1989	N/A	N/A	5.5–5.9 all ages, eaters only 6.0–7.3 dieters 5.5–11.4 diabetics	N/A	Heybach and Ross (1989)
Germany, 1988–1989	24-h records ($n = 2291$)	0.15	2.75 all ages, eaters only	N/A	Bar and Biermann (1992)
England, 1987	7-day diary ($n = 681$)	0.25–1.0 all consumers 1.2–5.3 diabetics	N/A	1.0–6.2 all consumers 1.9–16.6 diabetics	Anonymous (1991)
Great Britain, 1988	9-day diary ($n = 647$)	1% of ADI (~0.4 mg/kg bw/day)	4% of ADI (~1.6 mg/kg bw/day)		Hinson and Nicol (1992)
Brazil, urban residents, 1991	Food frequency questionnaire ($n = 673$)	1.17 consumers, 1.02 diabetics, 1.28 dieters	N/A	N/A	Toledo and Ioshi (1991, 1995)
New Zealand, 2004	Telephone survey ($n = 1015$) 7-day food diary ($n = 137$) by Asp consumers	1.53 all respondents 1.69 consumers	3.93	5.38	FSANZ (2004)
Australia, 2004	Telephone survey ($n = 2514$) 7-day food diary ($n = 263$) by Asp consumers	2.47 all respondents 2.56 consumers	5.30	7.46	FSANZ (2004)
Korea, general population 2005	National Nutrition Survey ($n = 11{,}525$)	0.14 all respondents	4.6	6.4	Chung et al. (2005)
		Specific subgroups			
France, young diabetics, 1997	5-day diary ($n = 227$); calculated TMDI	1.9	N/A	7.8 (97.5th percentile); 15.6 maximum	Garnier-Sagne et al. (2001)
Sweden, diabetics, 1999	Mail questionnaire ($n = 243$ children + 547 adults)	*Less than 10	*Less than 30 for children, less than 10 for adults	*Less than 40 for children, less than 20 for adults	Ilback et al. (2003)
Italy, teenagers, 1996	14 day food diaries ($n = 212$, 56 males, 156 females), age 13–19	0.03 for all teenagers	N/A	0.13	Leclercq et al. (1999)
Italy, teenagers, 2001	4-day food diaries ($n = 362$), 2 subsets of female high consumers ($n = 75$, $n = 79$)	0.04 for all teenagers, 0.09 for females consuming soft drinks, 0.17 for females consuming table-top sweetener	N/A	0.3–0.9 for females subsets	Arcella et al. (2004)
New Zealand, diabetics 2004	Diabetics ($n = 298$)	1.46 all respondents 1.66 consumers	4.51	6.68	FSANZ (2004)
Australia, diabetics 2004	Diabetics ($n = 298$)	2.41 all respondents 2.52 consumers	5.48	7.86	FSANZ (2004)

Note. ADI = acceptable daily intake; ASP = aspartame; bw = body weight; MRCA = Market Research Corporation of America; N/A = not applicable; n = number; TMDI = theoretical maximum daily intake. *Actual values not given, estimated from published histogram.

TABLE 6
Per capita aspartame consumption estimate from target foods

	Eaters only[+] (total individuals that consume targeted foods = 130,357,042)	
	mg/day	mg/kg bw/day
Mean (weighted)	330.2	4.9
90th Percentile (weighted)	704.8	10.4
95th Percentile (weighted)	939.0	13.3

[+]Estimate is based on the consumption of aspartame by individuals in the NHANES sample population who reported consuming a food that contained aspartame at least once; bw = body weight.

much information is sequestered in Drug Master Files and, as such, is inaccessible. Also, for those pharmaceuticals in which aspartame is permitted, the amount actually used may be considerably less than what was permitted. For example, the amount listed by FDA as the maximum allowed in orally disintegrating tablets is 36 mg, whereas the amount reported in two formulations of orally disintegrating tablets was only 0.4 mg (Mishra et al., 2006). In addition, aspartame is not a common sweetener in drug formulations, even though it is approved for many, likely because of the requirement of a label warning that consumption is not suitable for phenylketonurics. A survey of ingredients in 102 chewable tablet and liquid drug preparations found that the most common sweeteners used were saccharin and sucrose followed by sorbitol, which may require a statement on the label indicating their presence, but do not possess the degree of hazard of that of aspartame to a specific population. Only 2/102 formulations listed aspartame as sweetener (Kumar et al., 1993). Therefore, considering the high sweetening power of aspartame, the small quantities of aspartame used in drug formulations, and small percentage of drug formulations sweetened with aspartame, only a trivial amount of aspartame is consumed through pharmaceuticals.

3.1.4. Estimated International Consumption of Aspartame

In New Zealand and Australia, consumption of aspartame was assessed in individuals in age groups of: 12 to 17, 18 to 24, 25 to 39, 40 to 59, and over 60 years. Highest consumption was by individuals aged 25 to 39 (mean, 3.4 mg/kg bw/day and 95th percentile, 9.89 mg/kg bw/day). Consumption by children (12 to 17) was approximately half of these values (mean, 1.75 mg/kg bw/day and 95th percentile, 4.86 mg/kg bw/day). Additionally, the study found that aspartame intake had not increased significantly since the last survey in 1994 (FSANZ, 2004).

In Brazil, the consumption of aspartame is primarily due to tabletop sweetener use rather than soft drinks as in the United States. Cyclamates and saccharin are the most commonly used sweeteners in diet drink formulations in Brazil due to their lower cost (Toledo and Ioshi, 1995).

3.1.5. Estimated Consumption of Aspartame by Special Population Subgroups

Among the general population in Italy, female teenagers, especially those on a weight-reducing diet, have the highest consumption of sugar-free products (Arcella et al., 2004). Similarly, a survey in Australia and New Zealand, reported that there were several population groups who were more likely than others to have consumed products containing intense sweeteners. These subgroups included females, people with diabetes and those on a weight control diet (FSANZ, 2004).

In addition to estimating current consumption rates among Italian teenagers, Arcella et al. (2004) performed a worst case scenario calculation for each artificial sweetener considering that the artificial sweetener would be the source of sweetness in all sweetened food products at the highest concentrations. The highest predicted consumption was for teenage girls consuming high amounts of soft drinks, with an average consumption of 3.1 mg/kg bw/day, the 95th percentile of 7.4 and a maximum of 13.6 mg/kg bw/day. Thus even under these worst-case scenarios, the consumption of aspartame is well below the ADI.

A subgroup of the population that has been identified as high consumers of artificial sweeteners is young diabetics (Butchko and Kotsonis, 1991; Garnier-Sagne et al., 2001). Two studies (Garnier-Sagne et al., 2001; Ilback et al., 2003) estimated the intake of artificial sweeteners by children and adult diabetics (type I and II) and conducted a worst-case scenario determination. Garnier-Sagne et al. (2001) predicted that aspartame intake would never exceed the ADI even for high-intake consumers. Ilback et al. (2003) surveyed 1120 diabetics and reported that average daily intakes of aspartame by children and adult diabetics were below the ADI, for both the top 5% and 10% consumers. Worst-case intakes were calculated for the 10 diabetic children consuming the highest amounts of artificially sweetened foods and using the highest concentrations of sweetener allowed in food products. The worst-case calculation was based on the following assumptions: (1) All sweeteners found in the diet were replaced by aspartame; (2) the level of aspartame in foods was the maximum allowed by regulations, not the concentration actually used in the foods; and (3) the intake was based on the highest reported consumption of 10 diabetic children consuming the highest amounts of artificially sweetened foods.

These worst-case calculations showed that consumption by this small subpopulation would exceed the ADI by only a small percentage (about 114% of the ADI), and thus the authors concluded that there is a sufficient safety margin for aspartame, even in high consuming diabetics in a single time point (Ilback et al., 2003). It must be emphasized that the worst-case calculations do not represent "current use levels" of any population group and will not in the future for several reasons. First, the use of blends of sweeteners, rather than a single sweetener, is increasingly becoming the practice in the food industry. Aspartame is one of the more expensive sweeteners, and using a blend of aspartame in combination with other sweeteners can lower costs for the food manufacturers. Also, each artifical sweetener has a different

sensory profile in terms of how quickly the sweet taste is percieved, how long it lingers, and whether there is an aftertaste. As a result, the food product developer can achieve a taste that is more appealing when blends of sweeteners are used. Therefore, there is no incentive for all sweeteners to be replaced with aspartame. In addition, other new sweeteners, like sucralose and neotame, are being approved and introduced into the market, reducing still further exposre to the existing sweeteners like aspartame. Second, there is no incentive for producers to add more sweetener than is needed, even though the amount approved by food authorities is higher, thus the use of regulatory levels is also an overestimation. Last, the calculations were based on intake of $n = 10$.

The conclusion that even worst-case intake calculations for aspartame provide no evidence of potential risk is supported by the report by Larsen and Richold (1999). Evaluation of the significance of intakes above the ADI for short time periods must consider the imprecise nature of the establishment of the ADI, the extrapolation from lifetime chronic animal studies, and the safety factors in the ADI for species differences and individual differences. Therefore, a short period of exposure slightly above the ADI is not considered to pose any significant risk (Larsen and Richold, 1999).

Overall, the studies have very similar conclusions. Although the intake of aspartame has increased in recent years, the change is not dramatic, remaining well below the ADI, and worst-case scenario predictions suggest that chronic intakes will not reach the ADI.

3.2. Consumption of Constituent Amino Acids

The average consumption of milligram quantities of aspartame contributes very little to the overall total consumption of constituent amino acids in the general population. During the establishment of dietary reference intakes, the consumption of specific amino acids was calculated from NHANES III data collected from 1988 to 1994 (Institute of Medicine, 2005). The mean daily intake from food and supplements for all ages and gender groups ($n = 29{,}015$) is 6.5 g/day for aspartic acid and 3.4 g/day for phenylalanine. Mean and percentiles for specific age and gender groups were also determined (Institute of Medicine, 2005). Therefore, the amino acids in aspartame are consumed in much greater quantities as amino acids derived from foods such as meat and milk. For example, Butchko and Kotsonis (1991) estimated that milk provides approximately 6 times more phenylalanine and 13 times more aspartic acid than an equivalent volume of an aspartame-sweetened beverage.

The tolerable upper intake level (UL) of a nutrient is defined as the highest average daily nutrient intake level that is likely to pose no risk of adverse health effects to almost all individuals in the general population (Institute of Medicine, 2005). The Institute of Medicine (2005) concluded that there are not sufficient data on adverse effects of aspartic acid to develop a UL; however, it noted that supplemental doses of up to 8 g/day (equivalent to about 120 mg/kg bw/day) have not resulted in documented adverse effects. Similarily, there are no studies providing evidence of dose-related adverse effects of phenylalanine in healthy individuals which could be utilized to determine a UL. Single acute doses as high as 10 g of phenylalanine did not result in adverse effects in healthy men. Thus there is no established UL for either aspartic acid or phenylalanine (Institute of Medicine, 2005).

3.3. Consumption of Methanol From Aspartame and Other Sources

The generation of methanol from the breakdown and metabolism of aspartame has been raised as in issue regarding the safety of aspartame. However, this needs to be reviewed in the context of the total amount of methanol consumed in the diet. The general population is exposed to low levels of methanol from a variety of sources and methanol occurs naturally, as a constituent in blood, urine, saliva and expired air, in foods such as fresh citrus fruits and juices, vegetables, and fermented beverages (Francot and Geoffroy, 1956), and from endogenous production from compounds such as pectin (Barceloux et al., 2002).

The amount of methanol that consumers would generate following consumption of aspartame-sweetened beverages has been estimated as 55 mg of methanol/L beverage consumed (Stegink, 1987). Less well known is the fact that methanol is present naturally in foods, and the amount of methanol from aspartame-sweetened beverages would be considerably less than the methanol present in fruit juice (up to 680 mg/L) and citrus fruits (up to 180 mg/L) (Anonymous, 1991). Similarly, Butchko and Kotsonis (1991) estimated that tomato juice provides about six times as much methanol as an equivalent volume of an aspartame-sweetened beverage. The contribution of methanol from natural sources and from aspartame would clearly vary with the food consumption patterns of individuals. For children, consumption of fruit and vegetable juices continue to be greater than consumption of soft drinks containing aspartame (Sperber et al., 1995). In this case, exposure to methanol from aspartame will be very low in comparison to other sources. However, in adults, the consumption of soft drinks tends to replace consumption of fruit and vegetable juices. Therefore, the average contribution of methanol from aspartame to the diet may increase and in specific cases, even exceed the contribution of methanol from natural sources, although the total consumption of methanol may actually be reduced.

An estimate of the amount of methanol consumed from occurrences in natural foods was determined using an analysis of daily intake via natural food occurrence (DINFO). DINFO is a method used to determine consumption of a substance with respect to its natural presence in food. Obviously, DINFO could not be applied in a circumstance where the ingredient was artificial and only DINFO would apply when a substance was not added to food, but occurred only in the natural state.

To calculate the amount of a substance that is consumed from foods naturally containing that substance, the concentration of the substance in food and the amount the food consumed must be known (see Figure 5 for the basic DINFO equation). The

$$\text{Consumption (mg/day)} = \Sigma\,[\text{Concentration in a Specific Food (mg/g)} \times \text{Amount of the Food Consumed (g/day)}]$$

FIG. 5. Basic DINFO equation.

concentration of substances naturally occurring in food is available from commercial databases, such as the Volatile Compounds in Food (VCF) database. Where there is a range of reported values, the average value is generally used for the calculation (unless circumstances or data dictate otherwise). Food consumption data is available from the USDA Continuing Survey of Food Intake by Individuals (CSFII 1994–96, 98) database (2000). The CSFII 1994–96, 98 data is derived from a survey in which approximately 30,000 subjects dutifully recorded the weight, brand and type of food eaten at each occasion over a 1- or 2-day period. The survey is controlled for population groups, length of survey time, and other relevant factors. Weighting analysis is employed to estimate the number of individuals consuming food for the entire U.S. population.

Careful consideration of the population of interest must be made when doing DINFO analysis. For instance, eaters-only DINFO analysis is the amount of substance naturally present in food consumed only by those individuals that actually consume the food. Per capita DINFO analysis is the total amount of substance naturally present in food consumed per the entire U.S. population. Because the number of individuals actually consuming the food is much smaller than the entire U.S. population, eaters-only DINFO analysis always results in a consumption estimate that is higher than per capita DINFO.

Data from the CSFII 1994-96, 98 database (2000)[8] and the VCF database (1999) were used to calculate the eaters-only and per capita DINFO consumption of methanol from natural foods, based on foods reported to contain quantifiable amounts of methanol (Appendix II).[9] This does not include any foods that may have methanol from aspartame. The average DINFO consumption of methanol from foods naturally containing methanol by eaters-only is 10.7 mg/day or 0.178 mg/kg bw/day (for a 60-kg individual). Consumption of methanol by eaters in the 95th percentile is 33.3 mg/day. Per capita DINFO consumption for methanol is 5.187 mg/day or 8.65×10^{-2} mg/kg bw/day (see Table 7).

This analysis suggests that consumption of methanol from aspartame is greater than consumption of naturally occurring methanol; however, several important differences in these two estimates must be taken into consideration. As described in Section 3.1.3, the assumptions used in the estimate of aspartame consumption err on the side of overestimation. For the DINFO analysis, the estimate is likely under-estimating methanol consumption from foods. This is because: (1) only foods with reported quantified amounts of methanol are used in the calculation, which eliminated 92/139 foods reported to contain methanol; (2) the average rather than the highest amount of methanol measured in those foods was used; and (3) there is no adjustment for the amount of methanol that may be produced from the pectin in foods. Frenkel et al. (1998) demonstrated that the production of methanol in pectin-containing plants is regulated by pectin methylesterase, an enzyme that catalyzes demethoxylation of pectins. Recently, Anthon and Barrett (2006) investigated the effect of thermal processing of vegetables on the activity of pectin methylesterase and report that low temperature thermal processing of vegetables, such as occurs in blanching increases the activity of pectin methylesterase and the methanol content. For example, the methanol content of tomato slices increased from 3 to 7 nmol/mg following heating to 65°C for about 5 min. Similar results were reported for green beans. In green beans, the pectin methylesterase activity increased 88-fold from 25 to 65°C (Anthon and Barrett, 2006). Interestingly, this is due to an increased rate of de-esterification of pectin with increased temperature, and to an irreversible change in the enzyme by heating to over 45°C, such that it remains active and methanol production continues at room temperature after blanching (Anthon and Barrett, 2006). Slicing and homogenizing green beans also resulted in methanol accumulation at room temperature, whereas whole beans did not accumulate methanol (Anthon and Barrett, 2006). Therefore, one would predict that thermally processed and/or commercially prepared fruits and vegetables will contain much higher levels of methanol than have been reported for fresh whole foods. These are not included in the DINFO analysis.

In summary, the total methanol consumption from natural sources is estimated to average 10.7 mg/day. For individuals in the 95th percentile of methanol-containing foods the estimated methanol consumption figures are 33.3 mg/day or 0.55 mg/kg bw/day for a 60-kg individual. However, these figures underestimate the total exposure due to lack of data for several exposures. The actual amount of methanol in processed foods is not available for inclusion in the DINFO analysis. The average content of methanol in some commonly consumed foods, such as potatoes, onions, and celery, was not reported and therefore methanol from these foods was not included in the total estimated methanol consumption. Also, methanol produced from the pectin found in fruits and vegetables was not included. Taucher et al. (1995) estimated that humans produce approximately 1000 mg of methanol daily from fruits and vegetables.

Methanol generated from aspartame is estimated to average 33 mg/day or 0.55 mg/kg bw/day for a 60-kg individual. For individuals in the 95th percentile the estimated methanol consumption figures are 93.9 mg/day or 1.57 mg/kg bw/day

[8]CSFII data from 1996 was used for the analysis (U.S. population of 265,462,901 taken from the U.S. Census Bureau, Department of Commerce, www.census.gov/cgi-bin/ipc/idbrank.pl).

[9]Not all food products have been reported to be consumed in the CSFII survey database. Therefore, the "Eaters only" consumption may be based on a subset of the foods found in Appendix II.

TABLE 7
Methanol: Current intake from natural foods, intake from aspartame consumption and total intake (natural + aspartame-derived) for individuals

Methanol intake from	Eaters only (mg/day)	
	Mean	95th Percentile
Consumption from natural foods based on DINFO[1]	10.7	33.3
Consumption from aspartame (10% of estimated aspartame consumption)[2]	33.0	93.9

Note. [1]DINFO = daily intake via natural food occurrence; [2]based on the mean and 95th percentile intake calculated from NHANES (Table 6).

for a 60-kg individual. Since the figures for methanol from aspartame are derived from worse case assumptions, these are likely to overestimate methanol consumption from aspartame.

In conclusion, the amount of methanol contributed to the diet from aspartame-containing products consumption is likely to be less than that from natural sources.

3.3.1. Exposure to Formaldehyde From Methanol in Aspartame

As is described later, methanol is metabolized to formaldehyde, which is rapidly further metabolized. There are many sources of exposure to formaldehyde. Formaldehyde is a constituent of many foods (see Appendix III and Table 33) (Owen et al., 1990; Clary and Sullivan, 1999), and it is produced in the body during the endogenous demethylation of many compounds, including many foods and drugs. For example, the demethylation of the caffeine found in one cup of coffee produces 30 mg of formaldehyde (Imbus, 1988). Formaldehyde is essential in one-carbon pool intermediary metabolism. The metabolite of formaldehyde, formic acid, is a substrate for purine nucleotide synthesis (Sheehan and Tully, 1983). It can be calculated that more than 50,000 mg of formaldehyde is produced and metabolized in an adult human body daily and that an adult human liver will metabolize 22 mg of formaldehyde per minute (Clary and Sullivan, 1999).

Consequently, it is quite clear that the formaldehyde from aspartame provides a trivial contribution to total formaldehyde exposure and metabolism in the body.

4. ABSORPTION, DISTRIBUTION, METABOLISM, AND ELIMINATION

The intestinal absorption and metabolism of aspartame have been intensively studied, in rodents, pigs, primates and humans. Several excellent reviews have been published (Stegink, 1987; Butchko et al., 2002a). In all species examined, aspartame is metabolized in the gastrointestinal tract by esterases and peptidases into three components: the two constituent amino acids, aspartic acid and phenylalanine, and methanol (Figure 6). Aspartame may be completely hydrolyzed to these three components in the gastrointestinal lumen and absorbed into the general circulation, or may be hydrolyzed to methanol and aspartylphenylalanine dipeptide. In this case, the dipeptide is absorbed into the gastrointestinal mucosa cells and then cleaved into amino acids (Stegink, 1987). Aspartame can also be absorbed into the mucosal cells prior to hydrolysis, and be cleaved within the cell, to its three components, which then enter circulation (Matthews, 1984). The available evidence indicates that aspartame does not enter the circulation prior to hydrolysis. Metabolism of the three cleaved components of aspartame has been shown to be identical to the metabolism of the components given individually (Stegink, 1987). Consequently, studies in which aspartame is administered parenterally are not representative of oral administration, which is how aspartame is always consumed by humans.

The metabolism of the sweetener provides approximately 4 kcal/g of energy (Gougeon et al., 2004). The contribution of energy to the diet overall, however, is negligible as the high intensity sweetening power of aspartame (approximately 200 sweeter than sucrose by weight) means that little is needed to be added to foods to achieve sweetness.

4.1. Animal Studies

Studies in the 1970s by Opperman and coworkers (Oppermann et al., 1973b; Ranney et al., 1976; Ranney and Oppermann, 1979; Oppermann and Ranney, 1979) using radiolabeled aspartame demonstrated that aspartame was first hydrolyzed to aspartylphenylalanine and methanol by intestinal esterases, possibly chymotrypsin. Aspartylphenylalanine dipeptide is metabolized to phenylalanine and aspartic acid. Phenylalanine enters the plasma free amino acid pool from the portal blood after partial conversion to tyrosine by hepatic phenylalanine hydroxylase. Aspartate is metabolized within the enterocyte via transamination producing oxaloacetate, thereby reducing the concentration of aspartate entering the portal circulation and plasma free amino acid pool (Filer and Stegink, 1989; Burgert et al., 1991).

Methanol is not subject to metabolism within the enterocyte and rapidly enters the portal circulation. The methanol is oxidized in the liver to formaldehyde. Enzymes involved depend on species: In the rat, the metabolism of methanol to formaldehyde is mediated though a catalase-peroxidase system, whereas in primates and humans, an alcohol dehydrogenase is responsible. Formaldehyde is further oxidized to formic acid by formaldehyde dehydrogenase. This conversion is very rapid,

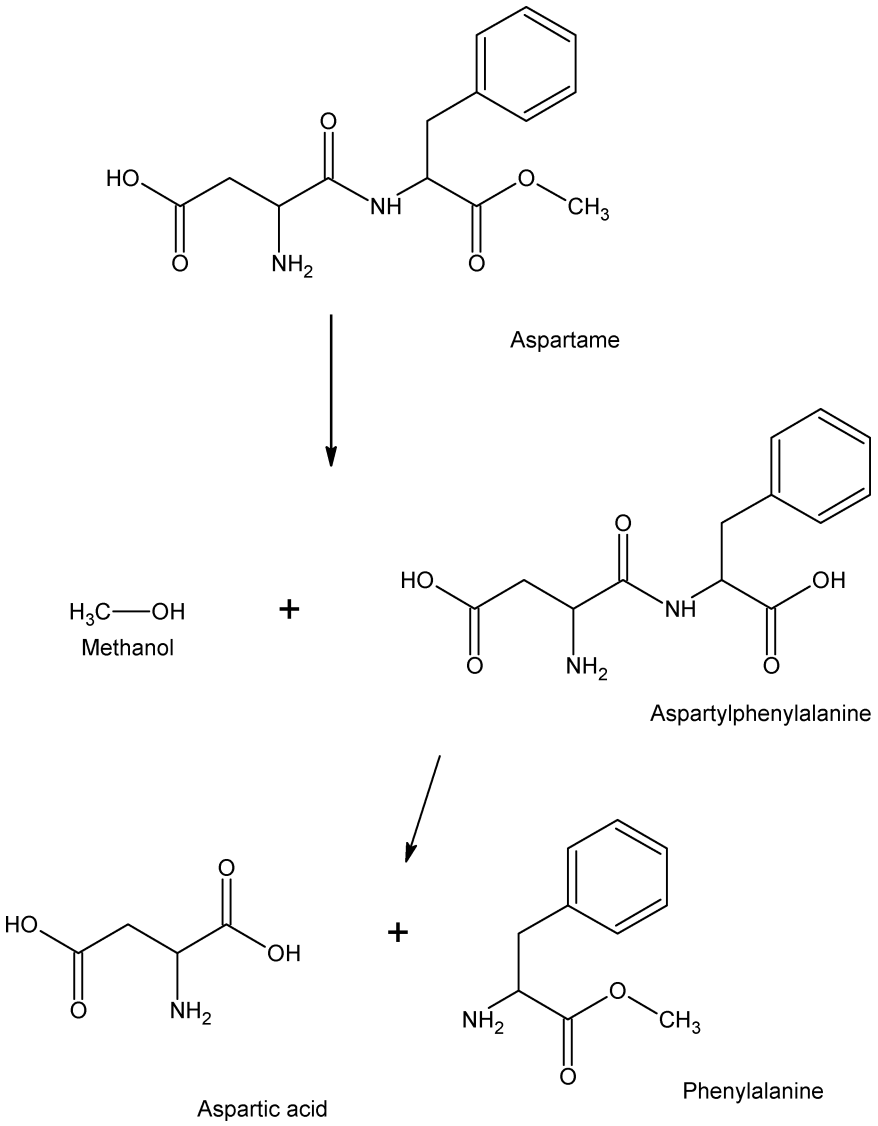

FIG. 6. Metabolism of aspartame (Oppermann et al., 1973b; Ranney et al., 1976; Ranney and Oppermann, 1979; Oppermann and Ranney, 1979).

with formaldehyde having a half-life of only one to 2 min, so there is no accumulation of formaldehyde. Formic acid is ultimately converted to CO_2 and water, via the formation of 10-formyl tetrahydrofolate (Barceloux et al., 2002).

The metabolism of aspartame has been studied in mice (Oppermann and Ranney, 1979; Hjelle et al., 1992), rats (Fernstrom, 1989; Hjelle et al., 1992), rabbits (Ranney et al., 1975), pigs (Burgert et al., 1991), dogs (Karim and Burns, 1996), monkeys (Oppermann et al., 1973b; Ranney et al., 1976; Reynolds et al., 1980), and humans (Filer and Stegink, 1989; Burgert et al., 1991; Karim and Burns, 1996) following administration orally or parenterally. Several reviews conclude that the compound is digested in all species in the same way as any peptide (Ranney et al., 1976; Ranney and Oppermann, 1979). Reynolds et al. (1980) reported that the metabolism of phenylalanine is faster in monkeys than in humans, as the rise in blood phenylalanine levels following administration of an acute oral dose of aspartame was lower in infant monkeys than expected in humans. This has been attributed to a higher rate of catabolism of amino acids due to a higher protein requirement.

The metabolism of phenylalanine was similar in female rhesus monkeys given 0, 15, or 60 mg/kg bw aspartame orally ($n = 3$ per dose group) for 10 days (Oppermann et al., 1973a). No effect of aspartame on absorption of phenylalanine, conversion of phenylalanine to tyrosine, or incorporation of phenylalanine into proteins was detected.

The effect of age on the absorption and metabolism of aspartate was investigated by comparing plasma levels of

aspartate following oral and ip[10] dosing of either 15-day-old or adult Charles River CD-1 mice (sex not specified) with 0, 10, 100, or 1000 mg/kg bw monosodium aspartate (Oppermann and Ranney, 1979). This study demonstrated that plasma levels of aspartate rose to higher levels in infant mice than in adult mice following oral doses of 1000 mg/kg bw of aspartate, indicating a lower rate of metabolism of aspartate in mice and greater sensitivity of infant mice to high doses of aspartate.

To elucidate the peptidase enzymes responsible for the hydrolysis of the dipeptide of aspartame, Hooper et al. (1994) incubated human and pig intestinal, and pig kidney microvillar membrane preparations, with aspartame and three decomposition products. The decomposition products were β-aspartame, α-aspartylphenylalanine, and DKP. Specific inhibitors of four peptidases were used to identify the primary peptidase. In all assays, the concentration of aspartame compounds was 1 mM and the concentration of inhibitors was 0.1 mM. In the absence of inhibitors, aspartame and α-aspartylphenylalanine were rapidly metabolized by human intestinal, pig intestinal and pig kidney microvillar membrane preparations. As expected, β-aspartame and DKP were not metabolized by any of the preparations. The greatest impact on the metabolism of aspartame and α-aspartylphenylalanine occurred with inhibitors or enhancers of aminopeptidase A, indicating that this is the major peptidase responsible for aspartame hydrolysis (Hooper et al., 1994).

The absorption of the decomposition product of aspartame, phenylalanine methyl ester, was examined in rhesus monkeys ($n = 2$) following intragastric and intraduodenal administration of 20 mg of radiolabeled L-[^{14}C]phenylalanine methyl ester (Burton et al., 1984). Blood samples were collected at 0.17, 0.33, 0.5, 0.67, 1.0, 1.5, 2, 3, 4, and 5 h after administration. Less than 0.2% of the phenylalanine methyl ester dose was absorbed intact into portal blood in both routes of administration, as the majority of the phenylalanine methyl ester is hydrolyzed prior to entry to portal blood. In vitro hydrolysis of phenylalanine methyl ester by human blood and plasma, by intestinal homogenates from monkey, dog, and rat, was also demonstrated in this study. Thus, the absorption of this breakdown product of aspartame prior to hydrolysis is very low in monkey and likely would be as low or lower in humans based on in vitro hydrolysis rates (Burton et al., 1984).

Lipton et al. (1991) compared the absorption and metabolism of aspartame decomposition products using in situ perfusion of the jejunum in adult rats. The α-aspartylphenylalanine isomer was rapidly absorbed, but β-aspartylphenylalanine and DKP were slowly absorbed, and to a much lesser degree. In addition, only α-aspartylphenylalanine was metabolized into amino acids by rat liver and intestinal mucosal cell homogenates. These results are in agreement with observations by others of poor absorption and subsequent elimination of β-aspartylphenylalanine and DKP in the urine (Cho et al., 1987; Kotsonis and Hjelle, 1996; Butchko et al., 2002b). In addition, Burton et al. (1989) demonstrated that β-aspartylphenylalanine is a normal constituent of human plasma and urine, as it was isolated from these fluids from individuals who had not consumed aspartame.

Since aspartame is hydrolyzed before it enters the bloodstream, there is no distribution of aspartame outside of the gastrointestinal tract. Stegink et al. (1979) were unable to detect aspartame in blood, erythrocytes or human milk following dosing with up to 50 mg/kg bw when the limit of detection was 0.5 μmol/100 ml. Since aspartame is completely metabolized, there are no excretion data.

4.2. Human Studies

Studies demonstrating the metabolism of aspartame to aspartate, phenylalanine, and methanol in humans, including healthy infants, children, adolescents, and adults, were conducted by Stegink et al. (1981a, 1983a, 1983b, 1984, 1987) and Ranney et al. (1976). No aspartame or aspartylphenylalanine was detected in the blood of humans administered a single oral bolus dose of 200 mg/kg bw or repeated oral doses of 10 mg/kg bw/h for 8 h, demonstrating that similar to rats, rabbits, dogs, and monkeys (Ranney and Oppermann, 1979), aspartame in humans is completely and rapidly hydrolyzed as a dietary dipeptide. The studies on metabolism of aspartame have been reviewed (Renwick, 1986; Butchko et al., 2002a).

Questions that have been raised and investigated surrounding the absorption and metabolism of aspartame include: elevation of plasma concentrations of individual amino acids, the potential for imbalance of ratios of serum amino acids, and effect on blood levels of methanol and formate metabolites. These are discussed in the following sections.

5. BIOCHEMICAL EFFECTS

5.1. Effects of Aspartame on Plasma Amino Acids

The effect of consumption of aspartame on the plasma levels of component amino acids was extensively investigated due to several potential adverse effects of very high levels of aspartic acid and phenylalanine. These include: early observations of adverse effects of subcutaneous injections of L-aspartic acid in neonatal mice; neurotoxicity observed in patients with the disease phenylketonuria (PKU)[11] resulting in high plasma phenylalanine; and potential behavioral effects due to competitive inhibition of brain uptake of tryptophan, a precursor of serotonin (Groff and Gropper, 2000).

[10]ip = intraperitoneal or within the peritoneal cavity (*Stedman's Medical Dictionary*, 1995c).

[11]Phenylketonuria (PKU) is also called Folling's disease. It is congenital deficiency of phenylalanine 4-monooxygenase causing inadequate formation of L-tyrosine, elevation of serum L-phenylalanine, urinary excretion of phenylpyruvic acid and other derivatives, and accumulation of phenylalanine and its metabolites, which can produce brain damage resulting in severe mental retardation, often with seizures, other neurologic abnormalities such as retarded myelination, and deficient melanin formation leading to hypopigmentation of the skin and eczema; it follows autosomal recessive inheritance (*Stedman's Medical Dictionary*, 1995k).

TABLE 8
Range of plasma amino acid concentrations (μmol/dl) in humans (Stegink et al., 1979; Filer and Stegink, 1989; NLM and NIH, 2005)

Amino acid	Children	Adults	PKU patients
Aspartic acid	0 to 2.6	0 to 0.6	NR
Phenylalanine	2.6 to 8.6 (fasting) 12.0–15.0 (postprandial)	4.1 to 6.8 (fasting) 12.0 (postprandial)	Classic: 120–300 (fasting) Heterozygous: 6.0–12.0 (fasting)
Tyrosine	2.6 to 10.0	4.5 to 7.4	NR

Note. NR = not reported.

Many studies have been conducted on various species, and in humans of different ages (young, mature, old) with different health status, using varying dosages, single versus chronic administrations, following fasting and in combination with carbohydrates, protein, and fat. To provide a basis for interpretation of the changes in plasma concentrations reported in these studies, the normal ranges of plasma amino acids in children and adults are given in Table 8.

A summary of the studies that have been conducted to assess changes in plasma amino acids following consumption of aspartame is provided in Table 9. An early study (Stegink et al., 1977) measured levels of free amino acids in the plasma and erythrocytes of 12 healthy adults (6 males; 6 females) given one oral dose of 34 mg aspartame/kg bw. The dose of 34 mg/kg bw was based on calculations that this would represent the 99th percentile of intake if aspartame replaced all dietary sucrose. The experiment was a crossover design. Aspartame, administered after a 24-h fast, did not increase plasma aspartate or phenylalanine levels beyond normal postprandial concentrations (Stegink et al., 1977).

The effect of aspartame on plasma amino acids in lactating women was assessed in six healthy women with well-established lactation (Stegink et al., 1979). In a crossover design, fasting women were given a single dose of 50 mg/kg bw of aspartame or lactose and blood samples were obtained at 0, 0.25, 0.5, 0.75, 1, 1.5, 2, 3, and 4 h after dosing. Breast milk samples were collected at 0, 1, 2, 3, 4, 8, 12, and 24 h post dose. Women consumed a normal diet after the 4-h sampling. No difference was observed in plasma amino acid concentrations between aspartame and lactose administration, except for phenylalanine and tyrosine, which increased following aspartame consumption. Changes in amino acid levels in breast milk following administration of 50 mg/kg aspartame were less than changes observed postprandially. Therefore, they were not considered to be meaningful in terms of effect on overall intake of these amino acids by the suckling infant (Baker, 1984).

When very high single-bolus doses of aspartame (100–200 mg/kg bw) were administered orally to adults ($n = 18$, 9 males and 9 females), plasma levels of aspartic acid increased but did not exceed levels normally observed postprandially (Table 9) (Stegink et al., 1981b; Fernstrom et al., 1983). Plasma phenylalanine levels increased in a dose-dependent manner, as mean peak levels were 20.3, 35.1, and 48.7 μmol/dl in individuals consuming 100, 150, and 200 mg/kg bw, respectively. These levels were higher than average postprandial levels of 12.3 μmol/dl, but well below levels associated with toxicity. Levels returned to normal 8 h after dosing with 100 and 150 mg/kg bw, and after 24 h in participants receiving 200 mg/kg bw. Plasma tyrosine levels were also increased from an average fasting level of 5.7 μmol/dl to peak values of 9.6, 11.0, and 13.7 μmol/dl in individuals consuming 100, 150, and 200 mg/kg bw, respectively, reflecting a conversion of phenylalanine to tyrosine (Stegink et al., 1981b; Fernstrom et al., 1983).

Clinical studies on the blood levels of phenylalanine, aspartic acid and methanol in normal subjects, children and known phenylketonuric heterozygotes have been reviewed (Filer and Stegink, 1989; Stegink and Filer, 1996). Based on the results from numerous studies, Filer and Stegink (1989) calculated that the projected average steady-state concentrations of phenylalanine resulting from daily intake every 2 h of the 99th percentile daily intake (34 mg/kg bw) would be 12 μmol/dl in normal adults. The authors indicate that this is far below the concentration of 60 μmol/dl considered the safe upper limit for plasma phenylalanine in phenylketonurinic patients (Filer and Stegink, 1989).

5.1.1. Effects of Aspartame on Plasma Amino Acids in Developing Animals and Children

The potential for aspartic acid to induce neuronal effects, especially in the developing brain, is another important issue in the assessment of the safety of aspartame. Aspartic acid is a normal brain constituent and, like glutamate, may serve as a central nervous system (brain, spinal cord) excitatory neurotransmitter. Molecules that increase excitatory drive have the potential to cause degeneration of nerve cells with synapses bearing excitatory amino acid receptors. The concentration of aspartic acid and glutamate in fluid bathing nerve cells must therefore be controlled to prevent excessive stimulation of nerve cells that can lead rapidly to excitotoxic neuronal degeneration. Unlike phenylalanine, both aspartic acid and glutamate are nonessential amino acids synthesized in the brain from glucose and other precursors, such that entry of these amino acids from the bloodstream may not be required for normal function.

TABLE 9
Summary of studies evaluating the effect of aspartame on plasma amino acid levels

Species/subject	Dose (based on bw)	Plasma aspartic acid (μmol/dl)	Plasma phenylalanine (μmol/dl)	Other parameters	Reference
		Acute bolus			
Humans, 2 normal and 2 with PKU children	34 mg/kg ASP or Phe (equimolar to 34 mg/kg ASP)	Not measured	Not measured	Urinary excretion of Phe metabolites	Koch et al. (1976a)
Humans, 6M, 6F, healthy adults, fasting	34 mg/kg ASP	[1]No change at 0, 0.25, 0.5, 0.75, 1, 1.5, 2, 3, 4, 8 or 24 h	[1]Fasting = 5–6, ↑to 12 ± 3	Leucine, isoleucine, and valine ↓, return to normal after 4 h	Stegink et al. (1977)
	13 mg/kg Aspartate (~ 34 mg/kg ASP)	[1]No change at 0, 0.25, 0.5, 0.75, 1, 1.5, 2, 3, 4, 8 or 24 h	↓ 1.5 h, then return to normal	Leucine, isoleucine, and valine ↓, return to normal after 4 h	
Humans, 6F adults lactating, fasting	50 mg/kg ASP or lactose	[1]No change over 4 h	[1]Postprandial = 5–6, ↑to 16 ± 5	↑plasma tyrosine, but normal within 4 h No change in asparagine, glutamine or glutamate Breast milk: trend for increase in Phe for 24 h, aspartic acid, and tyrosine for 4 h. Level of increase was less than 1% total average intake of amino acids.	Stegink et al. (1979)
Humans, 9M, 9F healthy adults, fasting	100, 150, 200 mg/kg ASP	[1]Fasting = 0.16-0.27 ↑ 0.43–1.0 at 0.5 h Postprandial = 2.2	[1]Fasting = 5.3–6.7, postpropandial = 12.3 ↑to 20.3–48.7 at 2 h, ↓ normal at 24 h	↑glutamate with 150 and 200 dose	Stegink et al. (1981b)
Humans, 10M, 10F healthy adults, fasting	20 mg/kg ASP in either solution or capsule	Fasting = 0.52 (mean) No change over 8 h, measured at 0.25, 0.5, 0.75, 1, 1.5, 2, 2.5, 3, 4, 6 and 8 h	Fasting = 6.5 (mean) ↑ to maximum (max) of 11.9 with solution, and to max of 10.3 with capsule	Phe/LNAA ratio at baseline = 0.10, increased to 0.18 at 30 min with sol'n, and to 0.15 at 90 min with capsule.	Burns et al. (1990)
Humans, 1-year-old infants	34, 50, 100 mg/kg ASP	No change with 34 or 50 mg/kg. Increase with 100. Similar to results in adult	Fasting = 4.9 – 5.7; 34 mg/kg ↑ to max of 9.4 50 mg/kg ↑ to max of 11.6; 100 mg/kg ↑ to max of 22.3.	Erythrocyte aspartic acid, glutamic acid, phenylalanine and tyrosine. Similar responses as seen previously in adults for all parameters.	Filer et al. (1983)

(Continued on next page)

TABLE 9
Summary of studies evaluating the effect of aspartame on plasma amino acid levels *(Continued)*

Species/subject	Dose (based on bw)	Plasma aspartic acid (μmol/dl)	Plasma phenylalanine (μmol/dl)	Other parameters	Reference
Humans, 10 normal, 15 with PKU, 10 high phenylalaninemia, 14 PKU carriers	10 mg/kg ASP, evaluated 1 h after dose	Not measured	Normal: 4.5↑ to 5.8 PKU: 137 no change Hyper Phe: 41 no change PKU carrier: 6.9↑ to 8.2	Similar to Phe levels, the Phe/LNAA rations increased about 30% in normal and PKU carriers, but not individuals with PKU or hyperphenylalaninemia	Caballero et al. (1986)
Humans, 6 normal, 8 moderate heterozygous PKU, 4 severe heterozygous PKU	100 mg/kg ASP evaluated 30 min after dose	Not measured	Normal: 5.3–6.6 ↑ to 6.4–8.4 Moderate: 9.4–11.6 ↑ to 8.5–14.9 Severe: 7.0–10.8 ↑ to 9.4–12.4	Tryosine plasma levels Normal: 7.3 no change 8.2 Moderate: 7.1 no change 6.6 Severe: 6.4 no change 6.6	Da Silva et al. (2000)
Monkeys, infants *Macaca mulatto, M. fascicularis* and *M. arctoides*. Ages ranged from 1 to 22 days	2000 mg/kg ASP (n = 8) or 2000 mg/kg ASP and 1000 mg/kg monosodium glutamate (MSG) (n = 6) or control (n = 11)	Fasting = 0.3–2.5 ↑ to 21–92 within 4 h dosing with either ASP alone or ASP plus MSG	Fasting = 4–10.2 ↑to 37–215 following ASP alone, and to 45–380 following ASP plus MSG	↑plasma glutamate levels in both levels ↑plasma tyrosine from 6.7 ± 2.6 to 35.5 ± 6.7 in 4 h No evidence of brain pathology	Reynolds et al. (1980)
Mice, CD-1	0, 100, 200, 500, 1000 and 2000 mg/kg, oral ASP (n = 35 mice/dose)	Not reported. Measured Phe/LLNA ratios	Dose-related increase in plasma levels with max of 20 μmol/dl 1 h following 2000 mg/kg. Normal after 4 h.	Phe/LNAA increased 15-fold 1 h after dosing with 2000 mg/kg ASP	Hjelle et al. (1992)
Mice, 15-day-old	0, 10, 100, 1000 mg/kg, oral and ip monosodium aspartic acid	Fasting = 3.5-5.1 μg/ml 10 mg/kg - no change with oral or ip 100 mg/kg – ↑ to 7.1–8.7 at 0.5 h, then drop, oral and ip 1000 mg/kg - ↑ to 554 at 0.5 h with oral, drop at 2 h to normal, ↑ to 718 at 0.5 h with ip drop at 2 h			Oppermann and Ranney (1979)

TABLE 9
Summary of studies evaluating the effect of aspartame on plasma amino acid levels *(Continued)*

Species/subject	Dose (based on bw)	Plasma aspartic acid (μmol/dl)	Plasma phenylalanine (μmol/dl)	Other parameters	Reference
Mice, adults	0,10,100,1000 mg/kg, oral and ip monosodium aspartic acid	Fasting = 3.0–6.8 μg/ml 10 mg/kg–no change 100 mg/kg–no change 1000 mg/kg–↑ to 141 at 0.5 h with oral, normal at 2 h, ↑to 1435 at 0.5 h with ip drop at 2 h			Oppermann and Ranney (1979)
Rats, Sprague-Dawley	0, 50, 100, 200, 500, and 1000 mg/kg, oral ASP (n = 5 rats/dose)	Not reported. Measured Phe/LLNA ratios	Dose-related increase in plasma levels with max of 11 μmol/dl 1 h following 1000 mg/kg. Normal after 4 h.	Phe/LNAA increased 10-fold 1 h after dosing with 1000 mg/kg ASP	Hjelle et al. (1992)
		Short-term/repeated dose			
Humans, 4M, 4F health adults	3 doses of 10 mg/kg ASP, 2 h apart	No change over 6 h	Fasting = 5.1–6.5, ↑ 1.6 to 2.1 above fasting but not above normal postprandial	↑ Phe/LNAA from 0.1 to 0.161, but still within normal postprandial values	Stegink et al. (1988)
Humans, children and adolescents, n = 126	30–77 mg/kg/day ASP; 13 wk	No change	No change	Eye exam, physical, liver and renal function, hematological	Frey (1976)
Humans, heterozygous PKU adults, 25F, 20 M	1800 mg/day ASP; 21 wk	Not measured	No change	No change in plasma tyrosine, hematology, urinanalysis or blood chemistry	Koch et al. (1976b)
Monkey, female adults	0, 15 or 60 mg/kg/day ASP (n = 3 per dose) for 10 days then ^{14}C -Phe ip	Not measured	No effect on Phe metabolism or incorporation in to plasma proteins	No change in expired $^{14}CO_2$ No change in ^{14}C-tyrosine levels indicating no accumulation of Phe	Oppermann et al. (1973a, 1973b)
Rabbits, pregnant females	0 or 1600 mg/kg/day ASP, from day 6–20 of pregnancy, n = 15/group	Not measured	Maternal plasma phenylalanine ↑ from 1.7 mg/dl to 4.9 mg/dl at day 9, return to normal by day 20	No effect on fetal weight or litter size Maternal plasma tryosine ↑ from 3.2 mg/dl to 9.9 mg/dl at day 9, return to normal by day 20 Fetal tryosine ↑ at day 20, so lower Phe/Tyr ratio No change in Phe hydroxylase	Ranney et al. (1975)

Note. ASP = aspartame; bw = body weight; /d = /day; F = female; h = hour(s); ip = intraperitoneal; M = male; MSG = monosodium glutamate ; Phe = phenylalanine; PKU = phenyketonuria; wk = week; LNAA = Large neutral amino acids;

[1]changes in plasma amino acid levels that were also observed with either vehicle (orange juice) or control (lactate, aspartic acid) were not included in tabulation of results.

Most parts of the mature brain, spinal cord, and peripheral nervous system are supplied with blood vessels that block the unimpeded entry of chemicals from the bloodstream. The extracellular fluid bathing neural tissue is separated from the vascular compartment by endothelial cells with tight junctions that constitute a barrier to the free passage of substances from blood to neural tissue. To gain access to most parts of the nervous system, molecules in the bloodstream must traverse the capillary endothelium and, in the central nervous system, the plasma membranes of astrocyte foot processes that abut the outer surface of capillaries. This system, the "blood–brain barrier," has a major role in maintaining the microenvironment of the brain, both by excluding ionized substances greater than 500 daltons and by transporting molecules required for physiological function. D-Glucose, the key energy substrate and important precursor for brain aspartate and glutamate (O'Kane et al., 1999), is transported by stereospecific, insulin-independent transporters that are enriched in brain capillary endothelium. These cells also contain a dicarboxylic acid transporter that, together with astrocytic and neuronal transporters, functions to maintain low levels of glutamate (and probably aspartic acid) in brain extracellular fluid (Easterby-Smith et al., 1994). The blood–brain barrier and its counterpart in the peripheral nervous system thus have crucial roles in the maintenance of neural and hence whole-body homeostasis. The system forms and seals during late development, rendering the developing brain less protected. In addition, after development certain physical (stress), biologic (hypertension), chemical (anesthesia), and disease (meningitis) states may compromise the blood–brain barrier and promote leakage of blood-borne molecules into the brain (Spencer, 2000).

Some parts of the normal adult nervous system possess permeable fenestrated capillaries that readily allow the passage of blood-borne substances, including aspartic acid. Most relevant here are midline structures bordering the third and fourth ventricles of the brain and known as the circumventricular organs. These structures represent small clusters of neurons that have contact with blood and an intrinsic neuronal connection with the hypothalamus, but are outside of the blood–brain barrier. These organs have a central role in regulating diverse physiological functions serving as signal transducers between blood, neurons, and cerebrospinal fluid, and permitting both the release and sensing of hormones without disrupting the blood–brain barrier. The circumventricular organs regulate autonomic functions, including cardiovascular function and body fluid regulation; the central immune response; fever generation in response to pyrogens; and reproductive and feeding behavior (Cottrell and Ferguson, 2004; Biddle, 2006).

The circumventricular organs also permit rapid communication between the brain and peripheral organs via blood-borne molecules. Through these organs, substances in the blood, such as aspartic acid, have free and immediate access to nerve cells in blood–brain barrier-free regions such as the hypothalamus. Aspartame safety with regard to aspartic acid hinges on whether blood levels of this and related amino acids, notably glutamate and cysteine, can reach potentially neurotoxic levels in the human subject, with special attention to the neonate and young children. Transient increases of aspartic acid are also highly relevant, given the acute nature of excitotoxic neurodegeneration. While there may possibly be redundancy of neurons in circumventricular organs as in other areas of the brain, the loss of a nerve cell is an irreversible and thus unacceptable event.

Studies on the effect of aspartame on plasma amino acid levels in animals during development and in children are reviewed here. In addition, biochemical studies on the effects of consumption of asparatame on brain neurotransmitter levels are reviewed in Section 5.2. Toxicological research on the possible neurotoxicity of aspartame in animals is reviewed in Section 6.4 and studies in humans are in Section 6.9. The effects of aspartame consumption during gestation, lactaction and development are reviewed in Section 6.5 and 6.9.2, as part of the overall safety evaluation.

Reilly et al. (1989a) conducted a series of studies on the effect of chronic aspartame exposure on brain amino acid levels, brain neurotransmitter levels, and the binding kinetics of transmitter receptors. The first experiment employed adult male Sprague-Dawley rats exposed to aspartame in drinking water for 30 days at concentrations sufficient to result in consumption of 0 ($n = 14$), 50 ($n = 8$), or 500 ($n = 18$) mg aspartame/kg bw per day. All rats were killed between 7:00 and 8:00 a.m., except for 10 rats in the 500-mg/kg group, which were killed between midnight and 1:00 a.m., following the maximal aspartame consumption period. No differences were found among groups in plasma levels of tyrosine, phenylalanine, alanine, valine and methionine. In a second experiment, brain amino acid levels were measured in rats exposed to 0 or 500 mg/kg bw/day for 15 or 30 days. No change in brain concentrations of tyrosine, phenylalanine, valine, methionine, aspartic acid, or glutamic acid were found in rats consuming 500 mg aspartame/kg/day for 15 or 30 days. The concentration of alanine was lower in rats fed 500 mg/kg bw for 30 days and killed at 7:00 a.m., as compared to the other three groups that included control rats, rats fed the same dose for 15 days, and rats fed the same dose for 30 days, but killed at maximal exposure time. Therefore, this observation is not considered to be a result of aspartame exposure. The results on brain neurotransmitter levels and transmitter receptor kinetics from these experiments are discussed in later sections.

Recognizing the potential increased susceptibility of the developing brain due to incomplete formation of the blood–brain barrier, these authors extended their investigations to evaluate neurotransmitter systems of weanling rats exposed to aspartame during gestation and lactation (Reilly et al., 1990). Immediately following successful breeding, female Sprague-Dawley rats were exposed to aspartame in the drinking water at concentrations to deliver 0 or 500 mg/kg/day. Consumption of aspartame was continued during gestation and lactation, until pups were 20–22 days old. There was no effect of aspartame consumption on plasma or brain concentrations of tyrosine, phenylalanine, alanine, valine, leucine, isoleucine, methionine, aspartic acid, or glutamic acid in the weanling rats. There was also

no difference in body weight changes, litter size, or weanling weights between rats exposed to 500 mg/kg aspartame and control rats. The results on brain neurotransmitter levels and transmitter receptor kinetics from these experiments are discussed in later sections.

Amino acid levels were evaluated in pregnant rabbits (n = 15/group) that received 0 or 1600 mg aspartame/kg bw/day, from day 6 to 20 of pregnancy (Ranney et al., 1975). Maternal plasma phenylalanine levels increased from 1.7 mg/dl to 4.9 mg/dl at day 9, and returned to normal by day 20. Maternal plasma tryosine levels also increased from 3.2 mg/dl to 9.9 mg/dl at day 9, but returned to normal by day 20. The ratios of fetal/maternal plasma amino acids were unchanged, and the authors concluded that aspartame had no effect on the transport of phenylalanine and tyrosine across the placental membrane. No effect on fetal weight or litter size was observed.

Reynolds et al. (1980) studied the effect of aspartame alone or in combination with monosodium glutamate (MSG) on plasma amino acids in infant monkeys. Many previous studies with primates had failed to replicate the findings of neuronal damage in an infant monkey due to monosodium glutamate (MSG) that had been first reported by Olney and Sharpe (1969). The study by Reynolds et al. (1980) was undertaken to confirm uptake and elevation of plasma amino acids in response to aspartame and MSG administration. Infant monkeys ranging from 1 to 22 days old were dosed with 2000 mg/kg bw aspartame (n = 8) or aspartame (2000 mg/kg) and 1000 mg/kg MSG (n = 6). Blood samples were taken after 0, 20, 40, 60, 90, 120, 180, and 240 min for amino acid analysis, and then monkeys were perfused with glutaraldehyde to fix the brain for histopathological analysis. The mean plasma amino acid levels are summarized in Table 10 (the reported standard deviations were omitted in this summary for brevity).

This experiment demonstrated that plasma levels of aspartic acid, glutamic acid, phenylalanine, and tyrosine were significantly elevated in response to administration of these large doses of aspartame and the combination of aspartame and MSG. As discussed in Section 6, no evidence of neuronal pathology was subsequently observed in the brains of these monkeys. The authors state that their results confirm the findings of previous researchers who have not found neuronal damage in a total of 59 infant monkeys given large doses of MSG (Reynolds et al., 1980). They suggest that the hypothalamic damage reported by Olney and Sharp (1969) may have represented artifact.

Filer et al. (1983) compared the effect of acute doses of aspartame (34, 50, and 100 mg/kg bw) on plasma amino acid levels in 1-year old infants to the results obtained with adults. Four blood samples were collected from each infant, one at fasting prior to dose administration and three after the dose. In one half of the infants, the three doses were collected at 0, 30, 60, and 120 min. Samples were collected from the other half of the infants at 0, 45, 90, and 150 min. Erythrocyte amino acid levels were also evaluated. Plasma aspartic acid levels did not change in infants with doses of 34 or 50 mg/kg, but did rise when the high dose of 100 mg/kg was consumed. Plasma phenylalanine levels increased dose-dependently to the same levels observed in adults given the same dose. Similarly, erythrocyte amino acids responses were the same as adults, suggesting that infants absorb and metabolize aspartame in the same manner as adults. In infants, mean plasma phenylalanine concentrations increased from a baseline value of $46 \pm 8\ \mu M$ to a high of $223 \pm 114\ \mu M$ at 30 min following administration of 100 mg/kg bw. The highest plasma concentrations observed with this abusive dose of aspartame were well below the high end of the range of phenylalanine levels observed in children with benign hyperphenylalaninemia (with plasma levels up to 420 μM). These children do not display any mental impairment or other adverse effects, even when not maintained on a phenylalanine-restricted diet (Stegink and Filer, 1996).

TABLE 10
Plasma amino acid levels (μmol/dL) in infant monkeys following administration of aspartame or aspartame and monosodium glutamate (Reynolds et al., 1980)

		Time after administration of dose (min)							
Amino acid	Test compound	0	20	40	60	90	120	180	240
Aspartic acid	ASP[1]	0.69	4.46	22.6	23.0	19.8	17.3	9.95	10.1
Aspartic acid	ASP + MSG[2]	1.36	5.87	25.3	31.4	38.6	49.3	26.9	17.1
Glutamic acid	ASP	9.38	15.8	20.3	23.4	25.7	31.5	30.2	27.3
Glutamic acid	ASP + MSG	8.01	25.9	55.5	113.6	149.6	168.1	137.7	112.8
Phenylalanine	ASP	5.93	67.3	81.9	87.7	94.5	84.3	59.2	52.5
Phenylalanine	ASP + MSG	6.76	48.9	61.2	80.8	100.3	120.0	113.0	35.6
Tyrosine	ASP	6.69	13.2	19.6	22.6	29.6	33.9	34.0	35.5
Tyrosine	ASP + MSG	6.59	12.4	15.2	16.4	19.3	23.9	26.5	27.9

[1]ASP = aspartame (dose = 2000 mg/kg body weight).
[2]ASP = aspartame (dose = 2000 mg/kg body weight) in combination with MSG = monosodium glutamate (dose = 1000 mg/kg body weight).

The effect of repeated consumption of aspartame on plasma amino acid levels in children was assessed in 126 healthy children who were randomly assigned to receive aspartame or sucrose incorporated into a variety of foods for 13 weeks (Frey, 1976). The study was double-blinded. Participants ranged from 2 to 21 years, and included 61 females and 65 males. Dosages were dependent on age and weight, and aspartame consumption ranged from 27 to 77 mg/kg bw/day. Plasma phenylalanine and tyrosine levels were compared for aspartame and sucrose-consuming children by the following age groups: 2–3 yr, 4–6 yr, 7–9 yr, 10–12 yr, and 13–21 yr. No differences in plasma amino acid levels were observed between diet groups after 1, 3, 5, 7, 9, 11, or 13 weeks in any age group (Frey, 1976). Other parameters evaluated in this study are discussed later in the safety evaluation section, Section 7.

In summary, bolus doses of aspartame up to 34 mg/kg bw do not alter amino acid levels, even in infants. In repeated-exposure experiments, which more closely represent the likely pattern of human exposure of large amounts of aspartame consumed in several servings over the course of the day, a dose of 500 mg/kg/day had no effect on amino acid levels in weanling or adult rats. In lactating women, a single dose of up to 50 mg/kg bw had little effect on milk phenylalanine and aspartic acid concentrations. Consumption of aspartame up to 500 mg/kg during pregnancy or lactation did not alter plasma or brain amino acid levels in the developing rat.

Consumption analysis by specific age groups (see Table 28 in Appendix I) determined that the average intake of children up to age 11 years was approximately 5 mg/kg bw/day. This analysis included only children consuming aspartame products and, as such, is an "eaters-only" value. The highest aspartame consumption reported in children was in teenage diabetics, where the maximum intake was 15.6 mg/kg bw (see Table 5) Therefore, dietary consumption of aspartame, even in high-consuming children, is unlikely to affect plasma amino acid levels. Consumption of large bolus abusive doses of aspartame can result in changes in plasma amino acid levels, but even under these conditions, plasma levels do not reach those associated with toxic effects.

5.1.2. *Effects of Aspartame on Plasma Amino Acids in Individuals With Gastrointestinal Disease*

One question raised during this review was whether aspartame would affect plasma levels of phenylalanine or aspartic acid to a greater degree in individuals with gastrointestinal diseases, which affect amino acid absorption, as compared to normal individuals. This would include individuals with celiac disease, cystic fibrosis, or tropical sprue. No study was found in the scientific literature that evaluated plasma levels of amino acids following aspartame in this special population. An expert in dietary management of celiac disease was contacted, and confirmed that no information on this question was available, and that consumption of aspartame was considered to not pose any concerns or adverse effects among celiac patients (personal communication, Shelley Case[12]). This may be because studies indicate that these individuals display impaired, rather than enhanced, absorption of amino acids and peptides. Clark et al. (1977) reported that absorption of amino acids from a mixture of free amino acids was 40% lower in celiac patients ($n = 7$) than in normal individuals ($n = 8$). Absorption of amino acids from dipeptides was also impaired by about 40% in celiac patients (Clark et al., 1977). Similar findings were reported by others (Hellier et al., 1976).

The absorption of free glycine and a glycine-glycine dipeptide in patients with sprue disease ($n = 5$) and healthy individuals ($n = 5$) was compared using a marker perfusion technique (Adibi et al., 1974). Absorption of free glycine was 15-fold lower in sprue patients, whereas absorption of the dipeptide was only 4-fold lower, suggesting dipeptide absorption is superior to free amino acid in this condition. Phenylalanine absorption was evaluated in children with celiac disease ($n = 7$) and healthy children ($n = 3$) using free phenylalanine and a glycine-phenylalanine dipeptide (Nutzenadel et al., 1981). Similar to the findings of Adibi et al. (1974), jejunal absorption of phenylalanine was reduced in celiac children compared to normal children, but the reduction was less severe for the dipeptide. Surprisingly, the increases in blood levels of phenylalanine were not significantly different between the celiac and normal children (Nutzenadel et al., 1981). The authors suggest that additional blood collection times may be needed to explain this observation.

Morin et al. (1976) measured the intestinal mucosa tissue concentrations of radiolabelled phenylalanine in intestinal tissues from children with cystic fibrosis, celiac disease and normal intestinal function following incubation with radiolabelled phenylalanine. Uptake of phenylalanine was approximately 35% lower in tissues from children with cystic fibrosis ($n = 18$) and 45% lower in children with celiac disease ($n = 8$) compared with tissues from normal children ($n = 7$) (Morin et al., 1976). Loss of absorptive function in cystic fibrosis patients, who do not display the loss of absorptive mucosal surface seen in celiac disease, was attributed to lower peptidase activity seen in both the cystic fibrosis and celiac disease children. Monoamine precursor amino acids tyrosine and tryptophan concentrations were evaluated in 5 adult celiac patients and 5 normal adults, following an oral dose of 25 g casein (Hallert et al., 1982). The ratios of tyrosine and tryptophan to large neutral amino acids (discussed below) were either similar to or higher in celiac patients compared to normal adults, indicating that availability of these precursors is not impaired in this population (Hallert et al., 1982).

In summary, the absorption of amino acids and peptides is lower in individuals with gastrointestinal diseases, such as celiac

[12]Shelley Case, a registered dietitian, is a leading North American nutrition expert on celiac disease and the gluten-free diet. She is the author of *The Gluten-Free Diet*, and a member of the Medical Advisory Boards of the Celiac Disease Foundation and Gluten Intolerance Group in the United States and the Professional Advisory Board of the Canadian Celiac Association. http://www.glutenfreediet.ca, Site visited October 1, 2006.

disease, as compared to normal individuals. Therefore, although there are no specific studies assessing the change in plasma amino acids in these individuals in response to consuming aspartame, previous studies suggest plasma amino acid levels would be either similar or lower than reported in normal individuals.

5.1.3. Phenylalanine to Large Neutral Amino Acid Ratio

Concerns have been voiced regarding possible effects of aspartame on brain amino acid levels and brain function (Olney, 1980). Evaluation of changes in the plasma levels of amino acids individually in response to aspartame may be of limited value in assessing the potential effect on brain uptake of the amino acids. The cells of the blood–brain barrier contain high levels of 20 or more specific transport systems that regulate the flux of key solutes from blood into brain interstitial fluid and cerebrospinal fluid and back out again. There are four types of transporters of amino acids. These are: (1) System L, which mediates high-affinity, sodium-independent uptake of zwitterionic amino acids with "large, neutral" side chains, including *L*-leucine, *L*-phenylalanine, *L*-tryptophan, *L*-tyrosine, *L*-isoleucine, *L*-methionine, and *L*-valine; (2) System y^+, which mediates moderate-affinity, sodium-independent uptake of amino acids with cationic side chains, including *L*-arginine, *L*-lysine, and *L*-ornithine; (3) System T, which mediates high-affinity, low-capacity transport of thyroid hormones (T_3 and T_4); and (4) System x^-, which mediates sodium-independent, high-affinity uptake of amino acids with anionic side chains, including *L*-glutamate and *L*-aspartic acid. Kinetic calculations for System L reveal a saturation percentage of >95% when all nine or so amino acid substrates are included. Due to transport saturation, individual amino acids must compete for transport. Transport saturation makes the brain amino acid delivery selectively vulnerable to large imbalances in plasma amino acid concentration such as those that occur in the hyperaminoacidemias, e.g., phenylketonuria and maple syrup disease (Smith, 2000). Thus, the brain uptake of any one amino acid increases only when the ratio of this amino acid to the other amino acids is increased.

Wurtman and Maher (1987) proposed that phenylalanine levels are reduced in the brain following the consumption of protein (although they rise in plasma), due to the competition for uptake by other large neutral amino acids (LNAA) at the transport site. In contrast, when phenylalanine is administered either as a pure amino acid or in aspartame, brain phenylalanine levels may increase. Furthermore, it was hypothesized that increased phenylalanine, in combination with reduced tyrosine and tryptophan, would result in altered neurotransmitter concentrations (Wurtman, 1983). Studies on the effects of aspartame on neurotransmitter levels are reviewed in Section 5.2.

To evaluate the biological significance of changes in amino acid levels and ratios of amino acid levels induced by aspartame, it is necessary to consider the normal variation that occurs in these parameters due to the daily fluctuations in food consumption and varying amino acid composition of dietary protein sources. Plasma amino acid levels fluctuate significantly over a 24-h period during consumption of a normal dietary pattern, with highest amino acid concentrations occurring during the evening and lowest during the morning. Consumption of a protein-free diet markedly alters the plasma amino acid levels, as compared to levels following consumption of 75 or 100 g/day of protein, although the changes in amino acid levels are more dramatic for some amino acids than others (Fernstrom et al., 1979). The ratios of plasma trytophan, tyrosine, and phenylalanine to the LNAA fall as the protein content of the diet is increased. In adults consuming a protein-free diet, the plasma phenylalanine: LNAA ratio was approximately 0.12 ± 0.03 standard deviation, whereas with consumption of 100 g protein/day, the ratio dropped to between 0.06 and 0.07 with little deviation. Therefore studies of the effect of aspartame on plasma amino acids, which are conducted in the morning following a fast and without additional dietary protein, produce more dramatic changes in plasma amino acid ratios than would likely occur due to consumption of asparatame-containing products along with a normal diet.

Fernstrom et al. (1983) evaluated the effect of aspartame on brain levels of LNAA and monoamine transmitters in Sprague-Dawley rats. Thirty minutes after gavage with 200 mg aspartame/kg bw, brain levels of phenylalanine, tyrosine and tryptophan were 116 ± 4, 224 ± 7, and 14 ± 1 nmol/g, respectively, compared to 94 ± 8, 169 ± 8 and 19 ± 1 nmol/g, respectively in rats gavaged with water only. Levels remained elevated for 60 min, and then decreased (Fernstrom et al., 1983). Similar results were obtained with dose-response studies with 0, 50, 100, and 200 mg/kg confirming that aspartame dose-dependently increased brain concentrations of phenylalanine and tyrosine, but not tryptophan (Fernstrom et al., 1983). Aspartame, at levels up to 200 mg/kg bw, did not affect rates of tyrosine or tryptophan hydroxylation. Most importantly, these authors (Fernstrom et al., 1983) also evaluated the concentrations of brain monoamine transmitters. Rats ($n = 6$/group) receiving 200 mg aspartame/kg bw or water were killed after 60 min, and brain levels of serotonin, 5-hydroxyindoleacetic acid, dopamine, and norepinephrine, dihydroxyphenylacetic acid, and homovanillic acid were measured. No differences in brain transmitter concentrations were observed in rats receiving 200 mg/kg bw despite differences in blood and brain amino acid levels.

However, Wurtman and Maher (1987) stated that the large increase in plasma and brain tyrosine following consumption of phenylalanine does not occur in humans, as it occurs in rodents, which have very active phenylalanine-hydroxylation rates. Wurtman and Maher (1987) suggested that a 60-fold higher dose of aspartame would be needed in rodents to obtain comparable elevations in phenylalanine in humans.

The assumptions and calculations of these authors were challenged by Fernstrom (1989), and later tested experimentally by Hjelle et al. (1992). Sprague-Dawley rats ($n = 5$/dose) received an oral dose of aspartame of 0, 50, 100, 200, 500, or 1000 mg/kg bw and CD-1 mice ($n = 5$/dose) were dosed with 0, 100, 200,

500, 1000, or 2000 mg/kg bw. Blood samples collected at 0, 0.25, 0.5, 1, 2, 4, and 8 h post dose were analyzed for amino acid levels and phenylalanine/LNAA ratios were determined. Maximal plasma concentrations for phenylalanine and tyrosime were reached within 0.25 to 1 h and returned to baseline within 4 to 8 h for all doses in both species. To assess the dose of aspartame needed in the rodent to achieve similar changes in the ratio of amino acids to LNAA in man, the phenylalanine/LNAA ratios in this study were compared to those observed in human studies following administration of aspartame. In contrast to the 60-fold difference calculated by Wurtman and Maher (1987), the data from this study clearly indicate that rodents require doses only two to six times higher than humans to achieve similar increases in phenylalanine/LNAA ratios (Hjelle et al., 1992). Therefore, the lack of effect observed with the dose of 200 mg/kg bw utilized by Fernstrom et al. (1983) would be predictive of a lack of effect in humans consuming the highest predicted 99th percentile dose of 34 mg/kg bw.

Ratios of phenylalanine/LNAA in normal adults were not affected by consumption of aspartame alone when the dose was 4 mg/kg or 10 mg/kg bw, but 3 successive doses of 10 mg/kg or a single dose of 34 mg/kg resulted in ratios of 0.16 and 0.23, respectively (Stegink, 1983; Filer and Stegink, 1989).

Curtius et al. (1994) reported that the mean ratio of phenylalanine/LNAA of normal individuals ($n = 4$) was 0.19 ± 0.02 prior to consuming a protein-rich meal. The ratio dropped to a low of 0.09 at 3 h after the meal. When 85 μmol aspartame/kg bw (approximately 25 mg/kg) was added to the protein-rich meal, the ratio in normal subjects ($n = 10$) changed from 0.111 ± 0.02 (baseline) to a peak of 0.132 ± 0.02. Therefore, fluctuations in phenylalanine/LNAA occur during normal variations in dietary patterns, and the consumption of aspartame, even at levels above the 90th percentile of consumption, do not alter this ratio outside the range of normal variation. Additional studies with PKU heterozygotes consuming 25 mg aspartame/kg bw demonstrated that ratios remain in the range considered to be normal for this population (Curtius et al., 1994).

Burns et al. (1991) compared the change in phenylalanine/LNAA ratios in fasting healthy adults (4 males, 4 females) given a drink containing either 500 mg aspartame (approximately 7.5 mg/kg bw) or 100 g sucrose. Baseline ratios were increased by a maximum of 26% following aspartame dosing, and 19% following sucrose dosing. There was no significant difference at any timepoint between the two groups.

Using positron emission tomography (PET), the effect of aspartame on LNAA uptake in the brain was evaluated in humans using [^{11}C]aminocyclohexanecarboxylate (ACHC) (Koeppe et al., 1991). ACHC is a nonmetabolized synthetic amino acid that crosses the blood–brain barrier with similar kinetics as many LNAAs. Healthy male volunteers ($n = 15$) received an intravenous injection of [^{11}C]-ACHC and underwent a baseline scan, and then consumed either an unsweetened drink ($n = 7$) or a drink sweetened with aspartame to achieve a dose of 34 mg/kg bw ($n = 8$). Blood samples were taken before, during and following the scans, and analyzed for amino acid content. The significant finding of this experiment is that although plasma phenylalanine increased 4-fold (56 to 211 μmol/L) in subjects consuming this large dose of aspartame, only small (11.5%) alterations in the patterns of uptake of [^{11}C]-ACHC were observed. The authors conclude that under conditions of normal use, aspartame is unlikely to cause changes in brain amino acid uptake (Koeppe et al., 1991).

5.2. Effects of Aspartame on Brain Neurotransmitters

The effect of aspartame on the rate of tryptophan hydroxylation in the brain was evaluated in Sprague-Dawley rats (Fernstrom et al., 1986). Increased tryptophan hydroxylation follows a carbohydrate-rich meal, resulting in increased levels of serotonin. The addition of aspartame to the carbohydrate meal at levels of 0, 56, 144, 267, and 656 mg aspartame/kg bw did not alter the carbohydrate-induced increase in 5-hydroxytrypophan in rat brain ($n = 6$ rats/dose). Additional experiments with doses up to 1440 mg/kg bw were conducted and inhibition was observed at doses greater than 500 mg/kg bw, but not when doses were less than 400 mg/kg. Therefore, the threshold level for the ability of aspartame to interfere with carbohydrate-induced changes in tryptophan hydroxylation was considered to be 500 mg/kg bw in the rat (Fernstrom et al., 1986).

Changes in neurotransmitters in specific areas of the brain following aspartame dosing of mice were assessed by Coulombe and Sharma (1986). Groups of four male CD-1 mice received a single oral dose of 0, 13, 130, or 650 mg/kg bw aspartame in corn oil and were killed 3 h later. Tissues from various regions of the brain were analyzed for catecholamines, indoleamines and metabolites using HPLC.[13] The greatest effect was observed in the hypothalamus, where the concentrations of norepinephrine and dopamine increased dose-dependently with doses of 13 and 130 mg/kg bw, but no further increases were observed at the 650 mg/kg bw dose. In addition, in the hypothalamus, the concentration of 3-methoxy-4-hydroxy mandelic acid was increased in the highest dose group and the concentration of homovanillic acid was increased in the mid-range (130 mg/kg bw) dose group. No changes in these neurotransmitters were observed in other regions of the brain in aspartame-treated mice. Brain concentrations of serotonin and 5-hydroxyindoleacetic acid were not affected by aspartame in this experiment (Coulombe and Sharma, 1986).

Similarily, Yokogoshi and Wurtman (1986) reported that brain norepinephrine levels were increased in rats 2 h after gavage with a single dose of aspartame (200 mg/kg bw) compared with those of saline controls. Animals receiving oral aspartame also exhibited higher plasma tyrosine and phenylalanine ratios (i.e., the ratios of their plasma concentrations to the summed concentrations of other large neutral amino acids that compete with them for uptake into the brain), than animals receiving saline.

[13] HPLC, high-performance liquid chromatography.

These findings are in contrast to the studies described above by Reilly et al. (1989a) in which administration of 50 or 500 mg aspartame/kg bw/day in the drinking water for 30 days did not significantly alter the following brain amine levels: serotonin, 5-HIAA, dopamine, or norepinephrine in the cortex and hippocampus, and dopamine, DOPAC, and HVA in the striatum. This was true regardless of the time of day of killing and sample collection. These authors also reported that there was no effect on the binding kinetics of dopaminergic, adrenergic and serotonergic receptors in the brain following chronic aspartame ingestion (Reilly et al., 1989b). Importantly the same result was obtained in developing animals. Weanling rats whose mothers had received 500 mg aspartame/kg bw/day during pregnancy and lactation had similar levels of brain neurotransmittors as controls (Reilly et al., 1990). No effects of aspartame exposure were observed in brain adrenergic, serotonergic, and dopaminergic D1 and D2 receptor binder activities (Reilly et al., 1990) or glutamatergic receptor kinetics (Reilly and Lajtha, 1995).

One key difference between these studies is the method of administration of aspartame. When large doses of aspartame (up to 650 mg/kg) are provided as bolus gavage dose, changes in brain neurotransmittors have been observed (Yokogoshi and Wurtman, 1986; Coulombe and Sharma, 1986). However, administration of large doses (500 mg/kg) over time, such as aspartame provided in the drinking water, did not affect brain levels of neurotransmittors or kinetics of those transmitter receptors, even in young animals (Reilly et al., 1989a, 1989b, 1990; Reilly and Lajtha, 1995).

5.2.1. Effect of Co-Ingestion of Carbohydrates on Plasma Amino Acid and Brain Neurotransmitter Levels

Wurtman (1983) proposed that when aspartame-containing beverages are consumed along with dietary carbohydrates, the effect of aspartame on brain composition is increased because the carbohydrates stimulate insulin release, which results in a drop of plasma branched-chained amino acids. The loss of these amino acids, which normally compete with phenylalanine and tyrosine for transport across the blood brain barrier, would result in a higher uptake of these amino acids into brain (Wurtman, 1983). To test this hypothesis, rats (strain and sex undefined) were given water; water plus 3 g/kg bw glucose; water, glucose, and 200 mg/kg bw aspartame; or water plus aspartame, and killed 2 h later. Tyrosine and phenylalanine levels in brains from aspartame plus glucose-treated rats were increased 2- to 3-fold compared to rats receiving only glucose. Similar results were reported by Yokogoshi et al. (1984), who dosed male Sprague-Dawley rats with the same doses of aspartame and glucose. In rats fed glucose and water, serotonin (5-HT) and its metabolite 5-hydroxyindoleacetic acid (5-HIAA) increased, but no increase occurred in rats treated with water, water and aspartame, or glucose and aspartame (Wurtman, 1983). Levels of 5-HT and 5-HIAA in aspartame-treated rats remained the same as those in water-treated rats. Although the author speculates that these observations suggest that high doses of aspartame may cause behavioral changes, no evidence is provided to support these contentions.

To evaluate the effect of aspartame on plasma and brain amino acids, male Sprague-Dawley rats were fasted overnight and then allowed to consume diets containing high or low sucrose levels, moderate, low, or no added casein, with or without 5% aspartame (Torii et al., 1986). The seven experimental groups were: (1) control, no diet offered; (2) casein, low sucrose, no aspartame; (3) casein, low sucrose, aspartame; (4) no casein, high sucrose, no aspartame; (5) no casein, high sucrose, aspartame; (6) low casein, high sucrose, no aspartame; and (7) zero time control (no diet, killed at time zero). Rats ($n = 12$ per group) were killed 2 h after introduction of the diet. Plasma and brain tyrosine and phenylalanine levels were increased in aspartame-fed rats regardless of dietary composition. Brain levels of dopamine and norepinephrine were not altered in any groups compared to controls. The authors report that brain levels of 5-HT were lower in rats fed aspartame-containing diets (groups 3 and 5) as compared to the respective aspartame controls (groups 2, 4, and 6); however, there are no significant differences between aspartame-fed rats and the two control groups (groups 1 and 7) (Torii et al., 1986). Data are presented in small graphs so actual values are difficult to ascertain, but it appears that the differences are due to an increase in 5-HT in rats fed the aspartame-free diets, as levels in aspartame-fed groups are very close to values observed in control groups. These data suggest that the differences between the rats fed aspartame and rats fed the aspartame-free diets are within a normal physiological range. This was confirmed in a subsequent experiment, which demonstrated that rats fed 5% aspartame in the diet for 3 weeks showed no evidence of change in behavior, and the authors concluded that the change in brain monoamines was not physiologically important (Torii et al., 1986).

To assess the possible effect of simultaneous consumption of glucose on the metabolism of aspartame, plasma amino acid, glucose, and insulin levels were evaluated in 12 normal adult subjects in a crossover design following a single dose of aspartame (40 mg/kg bw) with or without glucose (1.2 g/kg bw) in a beverage (Stegink et al., 1990). As expected, plasma glucose and insulin levels rose to higher levels following consumption of the drink with aspartame and glucose, as compared with aspartame alone. The co-consumption of glucose with aspartame did not significantly affect plasma phenylalanine, but it resulted in lower peak plasma aspartic acid, higher alanine and branched chain amino acid levels, and a delay in the time of the peak ratio of phenylalanine to large neutral amino acids. However, these changes were considered small and unlikely to result in adverse effects. Therefore, the authors conclude that co-consumption of glucose has little effect on metabolism of aspartame, especially considering the high dose of aspartame used in this study.

5.3. Effects of Aspartame on Plasma Lipids

Singleton et al. (1999) compared the change in serum triglycerides in 22 humans (12 male, 10 female) following consumption of a high fat dairy drink sweetened with 1 g aspartame, 17.5 g glucose, 30 g fructose, or no sweetener. This was a crossover design, with each participant being tested with each treatment. Blood samples were collected at baseline, and then 2, 4, 6, and 8 h after ingestion of the drink. Serum triglycerides rose after consumption of the drink sweetened with glucose and fructose, but not aspartame, indicating that unlike glucose and fructose, aspartame does not enhance postprandial lipemia following lipid loading.

5.4. Effects of Aspartame on Plasma Glucose

To assess the effect of a single dose of aspartame or saccharin on blood glucose homeostasis, Horwitz et al. (1988) compared the changes in plasma glucose, insulin and glucagon in normal and diabetic individuals. Twelve normal subjects (female) and 10 subjects (5 males, 5 females) with non-insulin-dependent diabetes mellitus consumed beverages sweetened with 400 mg aspartame, 135 mg saccharin, or no sweetener at intervals over 1 week, in random order. The aspartame dose was equivalent to 5–7 mg/kg based on reported body weights of the participants. Plasma samples were taken 15, 30, 45, 60, 75, 90, 120, and 180 min after consumption. No differences were observed in peak plasma insulin, plasma glucose or glucagon levels. Although the area under the curve of plasma insulin was higher after consumption of aspartame, the difference was not considered physiologically important and the authors concluded that at these levels, ingestion of aspartame or saccharin by normal and diabetic individuals has no effect on blood glucose homeostasis. Similar findings were reported in other studies with individuals with diabetes (Nehrling et al., 1985; Shigeta et al., 1985; Horwitz et al., 1988; Carlson and Shah, 1989; Colagiuri et al., 1989).

5.5. Effects on Gastrointestinal Secretions

The effect of aspartame on the gastrointestinal tract was evaluated in a series of experiments by Bianchi et al. (1980) using Charles River rats. The effect of an acute dose of aspartame on food consumption, gastric secretions and gastric ulceration was evaluated. A single intragastric dose of 200 mg/kg bw did not significantly affect the subsequent day's food consumption by rats ($n = 10$/group) that had been trained to consume their food ration within 2 h. To test the effects of aspartame on gastric secretions, pyloric ligation was performed on another group of rats after being anesthetized with ether, followed by intragastric administration of 250 mg/kg bw aspartame in water or water alone. Gastric secretions collected for 5 h from both treatment groups ($n = 6$) did not differ in volume, acid concentration, acid output, or proteolytic activity. To test the effects of aspartame on gastric ulceration, rats were fasted, anesthetized with ether, subjected to pyloric ligation and administered 250 mg/kg bw aspartame in water or water alone. One group was dosed with propantheline bromide (2.5 mg/kg bw) as a positive control. Aspartame was shown to have no effect on gastric ulceration in the stomachs of rats ($n = 6$/group) evaluated after 19 h of exposure to aspartame.

Aspartame had no effect on pepsin activity when tested in vitro at a concentration of 143 μg/ml or pancreatic lipase activity when tested at 1.25 mg/ml (Bianchi et al., 1980). Aspartame, tested at concentrations up to 5.0 mg/ml, did not inhibit isolated rabbit ileal muscle contractions induced in vitro by acetylcholine (Bianchi et al., 1980).

Similarly, incubation of isolated rat pancreatic islets with aspartame at concentrations of 1.0 to 10 mM failed to induce increased insulin release, whether in the presence or absence of D-glucose (Malaisse et al., 1998).

5.6. Effects on Metabolizing Enzymes

To determine whether aspartame may impact the activity of enzymes responsible for the metabolism of other xenobiotics, Tutelyan et al. (1990) fed groups of 30 male Wistar rats diets containing 0, 40, or 4000 mg aspartame/kg/day. Fifteen animals per group were killed after feeding for either 45 or 90 days, and livers were collected. The following parameters were evaluated in liver microsomes: the activities of 7-ethoxycoumarin deethylase, epoxide hydrolase, carboxylesterase, *p*-nitrophenol UDP-glucuronosyltransferase, 1-chloro-2,4-dinitrobenzene glutathione *S*-transferase, and *trans*-4-phenyl-3-buten-2-one glutathione *S*-transferase, and the cytochrome P-450 content. Although at the 45-day timepoint the activities of three enzymes (epoxide hydrolase, carboxylesterase, *p*-nitrophenol UDP-glucuronosyltransferase) were elevated in the high-dose group, these differences were no longer present after 90 days. The authors concluded that consumption of aspartame does not substantially alter the function of liver microsomal enzymes (Tutelyan et al., 1990).

The effect of aspartame on rat brain xenobiotic-metabolizing enzymes was recently reported (Vences-Mejia et al., 2006). Male Wistar rats ($n = 8$ per dose group) were gavaged daily with either distilled water (0 mg aspartame/kg bw), 75 mg aspartame/kg bw, or 125 mg aspartame/kg bw for 30 days. The liver, cerebrum and cerebellum were collected from each animal at necropsy. The major limitation with this study is that the authors then pooled all the tissues together for preparation of microsomes, resulting in only one sample for each group. Therefore, although they measured protein levels and activities of a variety of microsomal enzymes, and presented data suggesting that there were differences in the activity of enzymes between groups, there is no basis for drawing this conclusion as there is no way to assess whether the difference between groups is greater than the difference within a group. The authors used replicated measures of the pooled sample as if they were measures of variation within a group; this is clearly an improper application of statistics. This should have not been published in a peer-reviewed journal. Other points to consider in this paper were a lack of dose response and

lack of correlation between changes in protein level and enzyme activity, which are not addressed by the authors. In summary, due to the major flaws in the experimental design, the information produced has no probative value.

5.7. Sensory Effects

The biochemistry of how aspartame, a dipeptide, has the ability to elicit a taste sensation of sweetness has been reported (Xu et al., 2004), but is not reviewed in this safety monograph. One point that may, however, be an important consideration is the question of the palatability of diets with high concentrations of aspartame that have been used in many of the studies described in this review. Measurements of electrophysical responses and taste preference tests indicate that rodents do not taste aspartame as sweet. Marmosets, rhesus monkeys, and chimpanzees, which are physiologically more similar to humans, show a preference for aspartame and acesulfame-K. In contrast, the gray mouse lemur primate (*Microcebus murinus*), which is more distant phylogenetically, shows no preference for aspartame, acesulfame-K, saccharin, and several other sweet compounds (Schilling et al., 2004). Therefore, there are significant species differences in taste sensation, and aspartame may not affect palatability of the diet to the degree expected based on the human sensation of sweetness of this compound.

In a study with 21 young and 21 elderly human participants, Mojet et al. (2003) reported that perceived intensity of the taste of aspartame decreased with age, but the ability to taste differences in concentrations of aspartame were not decreased. There was no evidence of gender differences in taste perception of aspartame.

5.8. Other Biochemical Effects

The effect of aspartame on the distribution of zinc in rat tissues was investigated due to observations that aspartame can form complexes with zinc in vitro (Kovatsi and Tsouggas, 2002). Male Wistar rats were given either no treatment ($n = 8$) or 40 mg/kg bw/day per os ($n = 8$) for 60 days. Before treatment, and after 14, 28, 42, and 56 days of treatment, rats were kept in metabolic cages for 24 h to collect urine and feces. At the end of the experiment, tissues were collected for determination of zinc concentrations using flame atomic absorption spectroscopy. The effect of aspartame on excretion of zinc in urine and feces was not consistent, with an increased zinc concentration occurring in aspartame-treated rats in urine after 14 days, and decreased zinc in feces after 42 days of aspartame, but no differences at any other time points. At the end of the experiment, heart, liver, spleen, stomach, jejunum, adrenal and hair tissues from aspartame-treated rats had higher zinc concentrations as compared to controls, whereas aspartame-treated rats had lower bone zinc concentrations (Kovatsi and Tsouggas, 2002). As all the data are reported in one bar graph, it is not clear what the actual tissue concentrations were. In addition, no measures of variability around the means are given in the figures. Most changes appear on the order of approximately 10% or less, with the exception of the adrenals. The zinc concentration in the adrenals of rats treated with aspartame appears to be at least twice as high as the level in controls. There was no information as to whether the changes in zinc concentrations observed affected any functions of the tissues. Similarly, this group (Kovatsi and Tsouggas, 2001) reported that aspartame altered the accumulation of Mg in rat tissues, but no functional significance was demonstrated.

Using an in situ internal carotid artery perfusion technique, Wall and Pardridge (1990) demonstrated a dose-dependent reduction in cerebral protein synthesis when plasma phenylalanine concentrations were 200 μM or higher. The inhibition of protein synthesis by high levels of phenylalanine is due to inhibition of the transport of amino acids across the blood brain barrier, resulting in an imbalance that is corrected either by reduction of protein synthesis or an increase in brain proteolysis, or both (Pardridge, 1998). However, as discussed in Section 5.1, the plasma levels of phenylalanine derived from realistic uses of aspartame would not approach these concentrations.

A short-term study was conducted with male albino (age and strain not specified) rats to assess the nutritional and biochemical effects of aspartame, cyclamate and saccharin (Osfor and Elias, 2003). Groups of 20 rats were given daily oral intubations of 100 mg/kg bw aspartame (source not given), 100 mg/kg bw cyclamate, or 50 mg/kg bw saccharin. A group of 10 rats that was not gavaged was used as controls. Rats were 150–200 g at the start of the experiment and were fed an undefined basal diet. Ten rats from each of the treatment groups were killed after 6 weeks, and the remaining 10 were killed after 12 weeks of sweetener exposure. The time at which the control group rats were killed was not defined. Body weight was measured at the time of death. Blood, liver, kidney, and testes were collected. Measurements from blood samples included hemoglobin, red blood cell (RBC) count, white blood cell count, liver function enzymes, urea, creatinine, serum lipids, glucose, insulin, growth hormone and alpha-fetoprotein. Parameters that were significantly different in the aspartame group compared to the control group included reduced body weight gain, reduced RBC count, elevated activity of liver function enzymes (aspartate aminotransferase, alanine aminotransferase and alkaline phosphatase), reduced serum triglycerides, cholesterol, LDL,[14] and VLDL,[15] and elevated insulin and growth hormone levels (Table 11). No histological differences between control rats and aspartame-treated rats were observed in the liver or testes; however, congestion of the blood vessels in the interstitium of kidneys and cloudy swelling in the renal tubules were observed in rats given aspartame. No details on the frequency or degree of these histological changes were provided (Osfor and Elias, 2003). Furthermore, only one dose level of aspartame was administered. The findings in this study are difficult to interpret due to the vague description of the control animals. These animals were initially of lower body weight than treatment groups, were not gavaged with the vehicle to take into

[14]LDL, low-density lipoprotein.

[15]VLDL , very-low-density lipoprotein.

TABLE 11
Biochemical parameters in control rats and rats given aspartame for 6 or 12 weeks (Osfor and Elias, 2003)

Parameter	Control[1]	Aspartame 6 wk, 100 mg/kg bw/day	Aspartame 12 wk, 100 mg/kg bw/day
Body weight	263 ± 2 g	201 ± 6 g*	207 ± 6 g*
RBC count	7.77 ± 0.13 × 10^6/mm^3	7.77 ± 0.11 × 10^6/mm^3	7.13 ± 0.21 × 10^6/mm^3*
Aspartate aminotransferase	73.9 ± 3.0 U/ml	75.7 ± 10.4 U/ml	84.8 ± 2.3 U/ml*
Alanine aminotransferase	94.5 ± 3.0 U/ml	142.6 ± 10.4 U/ml*	135.1 ± 7.7 U/ml*
Alkaline phosphatase	66.6 ± 2.3 U/ml	65.8 ± 4.2 U/ml	106.3 ± 10.5 U/ml*
Serum triglycerides	47.1 ± 1.4 mg/dl	41.1 ± 1.3 mg/dl*	33.7 ± 1.9 mg/dl*
Cholesterol	78.0 ± 3.1 mg/dl	65.8 ± 3.1 mg/dl*	66.6 ± 3.8 mg/dl*
LDL	36.4 ± 2.2 mg/dl	26.6 ± 2.3 mg/dl*	25.4 ± 1.6 mg/dl*
VLDL	9.4 ± 0.3 mg/dl	7.2 ± 0.5 mg/dl*	5.7 ± 0.4 mg/dl*
Insulin	82.4 ± 3.3 μIU/ml	112.1 ± 3.4 μIU/ml *	119.4 ± 6.5 μIU/ml *
Growth hormone	5.41 ± 0.3 μIU/ml	6.84 ± 0.58 μIU/ml*	6.96 ± 0.39 μIU/ml*

[1]Control animals were not well defined, age unknown. *Statistically significantly different from control ($p < .05$); bw = body weight; LDL = low-density lipoprotein; RBC = red blood cells; U = units; IU = International units ; VLDL = very-low-density lipoprotein; wk = week.

account the effect and stress of daily handling and gavaging, and the time of killing or whether they were indeed part of the current experiment is not known. Body weights of rats in all treatment groups were lower than control rats after 6 weeks, regardless of the dose and compound. Food consumption is not reported. The significantly reduced body weight gains of rats fed aspartame are in contrast to other studies, as effects of aspartame on body weight are usually only observed in much longer studies, when doses are in excess of 2000 mg/kg bw/day. It is difficult to determine whether the biochemical changes are due to aspartame, or secondary to the body weight loss. As discussed previously, Singleton et al. (1999) reported no change in serum triglycerides in humans following consumption of aspartame.

In summary, consumption of large doses of aspartame in a single bolus dose will have an effect on some biochemical parameters, including plasma amino acid levels and brain neurotransmitter levels, although some fluctuation in these parameters is a normal response to dietary intake of amino acids. The rises in plasma levels of phenylalanine and aspartic acid levels following administration of aspartame at doses less than or equal to 50 mg/kg bw do not exceed those observed postprandially. The typical diet provides much higher levels of these amino acids than obtained in aspartame-sweetened beverages (see Section 3.2). The question is whether consumption of aspartame at levels expected for humans will result in fluctuations to the extent that there are negative consequences or adverse effects on health. This question is addressed in Section 6.

6. SAFETY EVALUATION

Studies investigating the toxicity of aspartame and the cyclization product of aspartame, DKP, include acute, subchronic, and chronic bioassays, neurotoxicity studies, immunotoxicity studies, and reproductive, teratogenic, and multigenerational bioassays in animals, as well as carcinogenic bioassays in three transgenic mice models. Bacterial studies and in vitro cell culture studies have also been utilized to assess the toxicity of aspartame. As aspartame is completely hydrolyzed following intake, studies employing either intraperitoneal administration or direct exposure of cells in vitro to intact aspartame do not reflect human exposures and therefore, must be carefully interpreted. The safety of aspartame has also been addressed in human case studies, clinical studies and epidemiological studies. These studies are summarized and critically evaluated in this section.

6.1. Acute Studies

Acute toxicity studies with aspartame have been conducted using oral and intraperitoneal exposure routes with mice, rats and rabbits. These are summarized in Table 12. No deaths or adverse effects were observed with oral dosages as high as 10 g/kg bw. Acute toxicity studies were also conducted with DKP in mice, rats and rabbits (Table 13). No deaths or adverse effects were observed with oral dosages as high as 5 g/kg bw. These were the highest doses tested.

The acute toxicity of β-aspartame was evaluated in mice and rats with oral doses of 2500 and 5000 mg/kg bw (Kotsonis and Hjelle, 1996). As no deaths or adverse effects were observed, the LD_{50} of β-aspartame in mice and rats is greater than 5000 mg/kg.

6.2. Subchronic Studies

Subchronic toxicity studies of aspartame in mice, rats and dogs are summarized in Table 14. No adverse effects due to aspartame were observed in mice, rats, or dogs given doses up to 13, 10, or 6 g/kg bw/day, respectively. These were the highest doses tested in these studies (Kotsonis and Hjelle, 1996).

Subchronic toxicity studies with DKP included a gavage 2-week mouse and rat study and a dietary 5-week rat study. The

TABLE 12
Acute toxicity of aspartame

Test species	Route	Test	Dose (mg/kg bw)	Effect	Source
Mouse[1]	Oral	LD_{50}	>10,000	None reported	ChemIDplus Advance (2004)
Mouse, male, Sprague-Dawley (Ha/ICR)	Oral	LD_{50}	>5000	None reported	Andress et al. (1973b)
Rat[1]	Oral	LD_{50}	>10,000	None reported	ChemIDplus Advance (2004); Unpublished report cited in (EC, 2000)
Rat, male, Sprague-Dawley	Oral	LD_{50}	>5000	None reported	Andress et al. (1973b)
Rabbit, male, New Zealand	Oral	LD_{50}	>5000	None reported	Andress et al. (1973b)
Mouse[1]	IP	LD_{50}	>5000	None reported	ChemIDplus Advance (2004)
Mouse, male, Sprague-Dawley (Ha/ICR)	IP		>1000		Andress et al. (1973b)
Rat[1]	IP	LD_{50}	>5000	None reported	ChemIDplus Advance (2004)
Rat, male, Charles River	IP		>1562		Andress et al. (1973b)

Note. [1]Strain and sex not defined; bw = body weight; LD_{50} = the dose that produces 50% lethality in the test population; IP = intraperitoneal.

TABLE 13
Acute toxicity of DKP (Andress et al., 1973a)

Test species	Route	Test	Dose (mg/kg bw)	Effect
Mouse, male, Sprague-Dawley (Ha/ICR)	Oral	LD_{50}	>5000	None reported
Rat, male, Charles River	Oral	LD_{50}	>5000	None reported
Rabbit, male, New Zealand	Oral	LD_{50}	>5000	None reported
Mouse, male, Sprague-Dawley (Ha/ICR)	IP	LD_{50}	>1577	None reported
Rat, male, Charles River	IP	LD_{50}	>1562	None reported

Note. bw = body weight; LD_{50} = the dose that produces 50% lethality in the test population; IP = intraperitoneal.

TABLE 14
Summary of subchronic studies with aspartame

Test species	Duration	Route	Dose (mg/kg bw/day)	Effects	Source
Mice male, Sprague-Dawley (Ha/ICR)	28 days	Oral	≤13,000	None reported at highest dose	Searle E-002 cited in Kotsonis and Hjelle (1996)
Rat, male, Charles River	63 days	Oral	≤10,000	None reported at highest dose	Searle E-004 cited in Kotsonis and Hjelle (1996)
Dogs, male and female, beagle	8 weeks	Oral	≤6000	None reported at highest dose	Searle E-021 cited in Kotsonis and Hjelle (1996)
Rat, male and female, Wistar	90 days	Oral	0, 0.5, 1.5, 5% in feed (food consumption measurements indicated doses were approx. 0, 300, 1000, 3200)	None reported at highest dose. NOAEL = 5% in diet or 3200 mg/kg bw/day	Jonker et al. (1987)

Note. bw = body weight; NOAEL = no-observed-adverse-effect level.

highest doses were 1 g/kg bw/day for mice and 6 g/kg bw/day for rats. No adverse effects were observed in any of the studies (Kotsonis and Hjelle, 1996). Subchronic studies for 4 and 26 weeks were conducted with rats and dogs with β-aspartame at doses of 0, 250, 500, and 1000 mg/kg bw/day. No adverse effects were observed and thus the NOAEL was 1000 mg/kg bw/day for both species (Kotsonis and Hjelle, 1996).

6.3. Chronic and Carcinogenesis Studies

6.3.1. Two-Year Bioassay Studies

Aspartame was first approved by FDA as a nonnutritive sweetener in 1974, based on the toxicity studies that were conducted by the Searle Laboratories. Chronic toxicity studies with aspartame, and its decomposition product, DKP, were conducted in mice, rats, and dogs. A 46-week study with aspartame was also performed with hamsters. Table 15 provides a summary of these studies. The carcinogenic potential of these compounds is discussed in the next sections.

Following the approval of aspartame, a formal objection was submitted to the FDA (FDA, 1981) questioning the conclusions from the rodent studies on aspartame conducted by Searle, and proposing that aspartame may have the potential to cause brain tumors in humans. This objection resulted in FDA staying the regulation approving the marketing of aspartame in 1975, and the establishment of a Public Board of Inquiry to reexamine the studies submitted by Searle to the FDA. Prior to the evaluation by the Board, the 15 studies submitted by Searle were thoroughly audited by the Universities Associated for Research and Education in Pathology, Inc. (UAREP) and by the FDA. The findings of the UAREP, the FDA, and the Public Board of Inquiry were considered and evaluated by the Commissioner of Food and Drugs, resulting in the issuance of the commissioner's Final Decision that at projected levels of consumption, aspartame would not pose a risk of brain damage and will not cause brain tumors (FDA docket, 75F-0355, 1981) (FDA, 1981). This decision resulted in FDA vacating the stay of the original 1974 regulation. Objections to the of the use of aspartame were again filed with the FDA in 1983; however, the regulations approving the use of aspartame was not stayed following these objections, as the FDA stated that they failed to create sufficient doubt about the safety of aspartame. A response to the objections and a denial for a hearing was issued in 1984 by the Acting Commissioner of Food and Drugs (FDA docket 75F-0355 and 82F-0305) (FDA, 1984; Wurtman and Maher, 1987).

Koestner (1984, 1997) reviewed the data on aspartame and tumor incidence using specific criteria (Table 16) that have been established for evaluation of a neurocarcinogen. None of the criteria was fulfilled by aspartame, in comparison to all of them being applicable to nitrosoureas and other neurocarcinogens.

Therefore, the conclusion reached in the mid-1980s was that there is no relationship between aspartame and rodent tumor development, and aspartame remained approved for use in foods by numerous national regulatory agencies as described in Section 2.5. In 1996, the question of the carcinogenic potential of aspartame was raised again by Olney et al. (1996) based on an analysis of epidemiological data (discussed in Section 6.9.3). Subsequently, additional studies on aspartame were conducted in rats (Soffritti et al., 2005, 2006) and in transgenic mice (NTP, 2005).

6.3.2. Lifetime Studies

Soffritti et al. (2005, 2006) recently published two reports on a single lifetime carcinogenicity study in rats conducted by the European Ramazzini Foundation of Oncology and Environmental Sciences. In this study, aspartame was added to a "Corticella" diet[16] at levels of 0, 80, 400, 10,000, 50,000, and 100,000 ppm fed to groups of 150 Sprague-Dawley rats/sex/dose. The authors state that this corresponded to doses of 0, 4, 20, 100, 500, 2500, and 5000 mg/kg bw/day. Rats were housed five per plastic cage, containing white wood-shavings as bedding and maintained for their lifespan. At necropsy, organs and tissues were preserved in 70% ethanol. These included skin and subcutaneous tissues, mammary glands, the brain (three sagittal sections), pituitary glands, Zymbal glands, salivary glands, Harderian glands, cranium (five sections), tongue, thyroid, parathyroids, pharynx, larynx, thymus and mediastinal lymph nodes, trachea, lung and mainstem bronchi, heart, diaphragm, liver, spleen, pancreas, kidneys, adrenal glands, esophagus, stomach, intestine, urinary bladder, prostate, gonads, interscapular brown fat pad, and subcutaneous and mesenteric lymph nodes. Animals were allowed to live until spontaneous death, although the cause of death was not reported.

6.3.2.1. Results Reported by Authors. In the first report (Soffritti et al., 2005), the only results were reduction of weight gain, reduced food consumption, and a greater incidence of the combined incidence of lymphomas and leukemias in the treated animals. In addition, the authors described a nonstatistically significant increase in brain tumors. Food consumption decreased significantly with increasing concentrations of aspartame. Although the data were presented only in graphic form, there appears to be a difference of about 4 to 5 g/day for males and 3 to 4 g/day for females between the control diet and the 10% aspartame diet. This represents a decrease of approximately 20% based on average intake of 25 g/day for males and 18–20 g/day for females. In spite of the fact that the authors reported no difference in body weight among either females or males in the first report, they indicated there was "a slight decrease in body weight of females" in the second report. No statistics were reported. The survival of animals in the control groups was much poorer than the survival rate of treated rats as in some treated groups animals lived 16 to 32 weeks longer. As the background

[16]The Corticella diet is used exclusively by the European Ramazzini Foundation of Oncology and Environmental Sciences. The composition was not reported.

TABLE 15
Chronic oral toxicity studies with aspartame and diketopiperazine

Author	Test species	N	Treatment			Results			Conclusion(s) and statistical significance (*p*value)
			Dose (g/kg/day)	Duration	Endpoints	Dose g/kg	Male	Female	
Searle, E33/34 (Trutter and Reno, 1973; Trutter and Reno, 2006)	Rats, Charles River (CD), Sprague-Dawley	40/sex/dose 60/sex/ control	ASP = 1, 2, 4, and 6–8	2 yr	Brain tumors, evaluated with 2 coronal sections	0	0/58	0/59	$p > .05$
						1	0/4	0/4	No evidence of tumors due to compound.
						2	0/3	0/40	
						4	0/1	1/4	
						6–8	1/40	3/39	Conclusion challenged, data re-evaluated (see next row)
Searle, E87: Reevaluation of tissues from E33/34					Reevaluation of tumors from E33/34 - Brain tumors, evaluated with 8 coronal sections	0	1/59	0/59	$p > .05$
						1	2/36	2/40	No evidence of tumors due to compound
						2	1/40	0/40	
						4	4/40	1/40	
						6–8	0/39	2/38	
Searle, E70 (Trutter and Reno, 1973; Trutter and Reno, 2006)	Rats, Charles River (CD), Sprague-Dawley	40/sex/dose 60/sex/control	ASP = 2, 4	In utero, lactation, and 2 yr	Brain tumors, evaluated 8 coronal sections	0	3/58	1/57	$p > 05$
						2	2/36	1/39	No evidence of tumors due to compound.
						4	1/40	1/40	Conclusion challenged, data reevaluated (see next row)
Searle, E87: Reevaluation of E70 (McConnell, 1973)					Reevaluation of tumors from above studies	Same results as above			$p > .05$ No evidence of tumors due to compound
Searle Laboratories, E75 (Searle Laboratories, 1974)	CD-1 mice	36/sex/dose 72/sex/control	ASP = 1, 2, 4	2 yr	Tumor incidence, with focus on bladder and brain tumors (evaluated 5 coronal sections),	0	17/65	21/66	$p > .05$
						1	4/32	8/31	No evidence of tumors due to compound. NOEL = 4 g/kg/day
						2	6/35	7/31	
						4	8/27	12/31	
Ishii et al. (1981)	SLC Wistar rats	86/sex/dose 86 control/sex	ASP = 1, 2, 4 and ASP:DKP (3:1) = 4 added to CE-7 powdered basal diet	2 yr	Brain tumors, evaluated 6 slices of brain, under dissecting magnifying glass, and 2 sections histologically	0	1 F atypical astrocytoma		$p > .05$
						1 ASP	1 M oligodendroglioma		No evidence of tumors due to compounds. NOEL = 4 g/kg/day Data reevaluated by other pathologists (see next row)
						2 ASP	1 F ependymoma		
							1 F astrocytoma		
						4 ASP	1 M astrocytoma		
						4 ASP+DKP	1 F oligodendroglioma		
An-Pyo Center (2006)	As above	As above	ASP = 1, 2, 4	As above	Reevaluation of tissues from Ishii (1981) study	0	1 F malignant mengioma		Similar results as initially reported.
						1	1 M glioma		No evidence of tumors due to compound. NOEL = 4 g/kg/day
						2	1 F malignant reticulosis; 1F, glioma		
						4	1 male, glioma		
Ishii et al. (1981)	SLC Wistar rats	86/sex/dose 86/sex/control	ASP = 1, 2, 4 and ASP:DKP (3:1) = 4 added to CE-7 powdered basal diet	2 yr	Body weight, food, water, urine analysis Blood CBC and biochemical parameters, heart, spleen, pituitary, adrenal, liver, kidney, testis and ovary in formalin, pathology	No data tables. Dose-dependent depression of body weight gain and food consumption. Also increased urinary calcium and decreased pH, increased relative spleen weight, focal mineralization in renal pelvis			No evidence of toxicity due to compound. NOAEL = 4 g/kg/day

(Continued on next page)

TABLE 15
Chronic oral toxicity studies with aspartame and diketopiperazine *(Continued)*

Author	Test species	N	Treatment			Results		Conclusion(s) and statistical significance (*p*value)
			Dose (g/kg/day)	Duration	Endpoints	Dose g/kg Male	Female	
E72, Bryan (1984a, 1984b)	Female Swiss albino mice	100/group	Bladder pellet[1] with 0, 4.0 mg ASP, 4.0 mg DKP, or 4.0 mg XAE	26 weeks	Urinary bladder tumorigenicity with intravesical pellet implants	No statistically significant increase in any tumor type in treated groups as compared with control		Lack of increased tumors in positive control XAE group suggested insufficient study duration
E72, Bryan (1984a, 1984b)	Female Swiss albino mice	200/group	Bladder pellet with 0, 4.0 mg ASP, 4.0 mg DKP, or 4.0 mg XAE	56 weeks	Urinary bladder tumorigenicity with intravesical pellet implants	Significant increase in bladder tumors in XAE-treated mice, but not in ASP or DKP treated mice		ASP and DKP do not promote bladder tumors
Soffritti et al. (2005, 2006)	Sprague-Dawley rats.	150/sex/dose for 0, 4, 20, and 100 mg/kg bw. 100/sex/dose for 500, 2500 and 5000 mg/kg bw	ASP = 0, 0.004, 0.02, 0.1, 0.5, 2.5, 5	Lifetime until death	Tumorigenicity when added to the diet at levels of 0, 80, 400, 2000, 10,000, 50,000 and 100,000 ppm	Increased in combined lymphoma/leukemia in females, renal carcinomas, malignant schwannomas of peripheral nerves		Authors conclude ASP has "multipotential carcinogenic effects". See discussion for conclusions by expert reviews.
Searle, E77/78 (1974)	Rats, Charles River (CD), Sprague-Dawley	36/sex/dose 72 control /sex	DKP = 0, 0.75, 1.5, 3.0	115 week	Brain tumors	0 (M+F)	2/123 (1.6)	No evidence of tumors due to compound. NOAEL for DKP = 3 g/kg/day
Searle, E27 (1972a)	Hamsters	5/sex/dose	ASP = 1, 2, 4, 12	46 weeks	Neoplastic lesions	Lesions attributed to unidentified infection in colony. No tumors reported		No evidence of tumors due to compound; infection compromised study
Searle, E28 (1972b)	Dogs (purebred Beagles)	5/sex/dose	ASP = 0, 1, 2, 4	2 years	Physical exams every 4 weeks, periodic urine analysis, blood CBC and biochemical parameters	No treatment related changes in body weight, food consumption, physical or clinical parameters, or postmortem examination		No evidence of toxicity. NOAEL for ASP = 4 g/kg/day
Promotional studies								
Ito et al. (1983)	Male F344 rats pretreated for 4 weeks with or without 0.01% BBN in water	Control group = 60, aspartame group = 36	0 or 5% ASP in diet. ASP was one of 16 test chemicals	36 weeks	Urinary bladder pathology	No difference between groups in incidence or severity of urinary bladder papillary or nodular hyperplasia		ASP does not promote urinary bladder papillary or nodular hyperplasia
Hagiwara et al. (1984)	Male F344 rats pretreated for 4 weeks with or without 0.01% BBN in water	25–30/group	0 or 5% ASP, stevioside, or saccharin in diet	32 weeks	Body weight, food, water, urine analysis, liver, kidney, and urinary bladder lesions	No abnormalities in rats treated with aspartame, stevioside or saccharin alone.		Saccharin, but not ASP, promoted bladder carcinogenesis NOEL = 5 g/kg/day

Note. [1]Bladder pellet = intravesical pellets implanted in the bladder, containing cholesterol with or without aspartame. ASP = aspartame; BBN = *N*-butyl-N-(4-hydroxybutyl)nitrosamine; bw = body weight; CBC = complete blood count; DKP = diketopiperazine; F = female; M = male; NOAEL = no-observed-adverse-effect level; NOEL = no-observed-effect level; ppm = parts per million; XAE = xanthurenic acid 8-methyl ester; yr = year.

TABLE 16
Biological criteria for evaluation of neurocarcinogens (Koestner, 1984)

1. Increased incidence beyond expected control levels
2. Shift of brain tumor appearance to a younger age (decreased survival time)
3. Demonstration of dose-response relationship
4. Higher tumor incidence after transplacental exposure
5. Trend towards anaplasia
6. Presence of preneoplastic lesions
7. Multiplicity of tumors in individual animals
8. Tumor occurrence also in peripheral nervous system
9. Tumor induction outside the nervous system
10. Genotoxicity, mutagenicity, chromosomal aberrations

incidence of cancer greatly increases with age, increased survival to older age in treatment groups increases likelihood of cancer development independent of the diet treatment.

The results from the second report (Soffritti et al., 2006) were stated as follows:

> An increase in lymphomas and leukemias with a positive statistically significant trend in both males ($p < 0.05$) and females ($p < 0.01$), in particular in females treated at doses of 100,000 ($p < 0.01$), 50,000 ($p < 0.01$), 10,000 ($p < 0.05$), 2,000 ($p < 0.05$), and 400 ($p < 0.01$) ppm;
>
> A statistically significant increased incidence, with a positive significant trend ($p < 0.01$) of transitional cell carcinomas of the renal pelvis and ureter and their precursors (dysplasias and pappilomas) in females treated at 100,000 ($p < 0.01$), 50,000 ($p < 0.01$), 10,000 ($p < 0.01$), 2,000 ($p < 0.05$), and 400 ppm ($p < 0.05$);
>
> An increased incidence of malignant schwannomas of peripheral nerves with a positive trend ($p < 0.05$) in males;
>
> A significant positive trend of increased incidence of hyperplasia of the olfactory epithelium in both sexes, and sparse adenomas in treatment groups but not the control; and
>
> An increased incidence of malignant tumor-bearing animals with a positive significant trend in males ($p < 0.05$) and in females ($p < 0.01$), in particular those females treated at 50,000 ppm ($p < 0.01$).

6.3.2.2. Evaluation of Lifetime Study by Food Safety Authorities. Several aspects of this study were unclear or not provided in the publication, resulting in requests by several food safety authorities for additional information to adequately evaluate the validity of these findings. Additional information was subsequently provided by the Ramazzini Foundation to the National Toxicology Program (NTP) and the European Food Safety Authority (EFSA). The EFSA (2006) published the results of their evaluation of the findings of the lifetime study, with consideration of the published data and unpublished report by Ramazzini, and the unpublished report of NTP on its evaluation of pathological lesions in the Ramazzini study. The NTP Pathology Working Group conducted a review of a small portion of the slides from the Ramazzini study, as not all slides were provided. Evaluation of the slides from the mammary gland, renal pelvis, Zymbal gland, nasal cavity, oral cavity, and stomach indicated a tendency of the Ramazzini Foundation to overdiagnose epithelial lesions, frequently classifying hyperplastic lesions as malignancies. Specific examples are also discussed in later sections.

6.3.2.3. Study Design. The EFSA and other experts reviewing this study have identified numerous shortcomings of the study design and conduct. The authors' claim of Good Laboratory Practice (GLP) compliance could not be confirmed and numerous deviations from the Organization for Economic Cooperation and Development (OECD) Test Guidelines 451 (OECD, 1981) for carcinogenicity studies were found.

1. Animals.
   a. Animals were not randomized. The large number of animals used in studies conducted by the Ramazzini Institute often necessitate that different dose groups are in different rooms, each having its own microclimate and rate of disease. When animals from various treatment groups are not randomized among the various rooms, different background pathologies per group can occur, including tumor incidence and survival. Although the animals were allowed to live to natural death, survival rates were calculated at specific time points as the percentage of animals still living at that time point. This lack of randomization may have led to unusually low survival rate of the female control group at 104 weeks (27.3%) compared to the survival rate of highest dose group (45%) (EFSA, 2006).
   b. Randomly bred rats of the institute's own strain were raised and maintained under conventional nonbarrier conditions, and histopathology reports revealed a very high incidence of infection in both treated and nontreated rats, likely to be linked to the poor survival at 104 weeks of the rats ranging from 22% (controls) to 31% in the males and from 27.3% (controls) to 45% in the females (EFSA, 2006).
2. Diet.
   a. No information is provided in the published report on the composition of the Corticella diet. It not known whether this is a pelleted or powdered diet. This information was also not provided to the EFSA.
   b. No details on the storage conditions or stability of aspartame, which depends on moisture, temperature, and pH, in the diet were provided. The presence of contaminants in the feed was not evaluated.
   c. According to OECD Guidelines and required by GLP, the incorporation of the substance in diet needs to be tested for homogeneity and range of variation from the target figure. This was not reported.
   d. It is not clear how the aspartame was added to the diet formulation. At a public presentation of the results, in response to questioning, Dr. Soffriti stated that the

aspartame was simply added to the diet (personal communication). A dose level of 100,000 ppm represents 10% of the diet. When compounds are added to this level without adjustment, the nutritional composition of the diet is also altered, as the vitamin and mineral content would be reduced by 10%.

3. Animal housing conditions.
   a. Rats were housed five per plastic cage, containing white wood shavings as bedding. The floor area of the cages was 1025 cm^2. The National Research Council (2006) recommends a maximum of three weanling or two adult rats in this size of cage.
   b. High-density housing may have contributed to the unusually high incidence of widespread infection in a wide variety of tissues in this study as described later.
   c. Housing individual groups separately in different rooms may have contributed to differences in survival among groups.
4. Length of the study.
   a. Animals were allowed to live until spontaneous death, with the rationale that this protocol increases the sensitivity of the assay to detect carcinogens.
   b. Disadvantages of lifetime treatment to natural death as compared to termination of the study after a specified duration, such as 104 or 110 weeks, include an increase in age-related background lesions and higher probability of autolytic tissue changes in animals found dead. The NTP Pathology Working Group review of the slides from the Soffritti study confirmed the presence of autolytic changes in tissues examined.
   c. It is possible that moribund animals were put to death to avoid autolytic tissues damage, which would affect statistical analyses based on the assumption of natural death.
5. Data collections.
   a. Blood collection was not reported. No hematological blood smear assays were performed.
   b. The causes of deaths were not reported.
   c. The absence of individual data and the identification of different states of disease among individuals hinder interpretation.
   d. Although standard histopathology was performed on a comprehensive range of organs and tissues, tabulated histopathological findings in the report were limited to a few tissues and tumor pathology.
6. Statistical analyses.
   a. The incidences of the combined lymphoma and leukemia were identical in the first and second report; however, the conclusions and reported statistical significances are not. In the first report (Soffritti et al., 2005), a significant increase in lymphomas and leukemias occurred only in females, whereas in the second report (Soffritti et al., 2006), the authors state that there was a significant positive trend in males as well. The data are shown in Figure 7.
   b. There is no indication that trend analysis procedures were preformed to support the contention that trends occurred.
   c. A time-to-tumor statistical analysis was not presented. The poly-K statistical test is not appropriate.

In addition to examining the conduct of the study, the interpretations of the data leading to the results described above have been reevaluated (EFSA, 2006). This reevaluation was based on information provided in the publication, the Ramazzini Foundation, unpublished reports, and other existing scientific literature. It also included the results of unpublished transgenic mice studies conducted on aspartame by NTP and an unpublished epidemiological study of aspartame by the National Cancer Institute.

The very low survival rates at 104 weeks, which are likely due to the very high incidence of infection in both treated and control rats, are an important factor that affects the interpretation of the study. According to the EFSA report (2006), these rates were as follows:

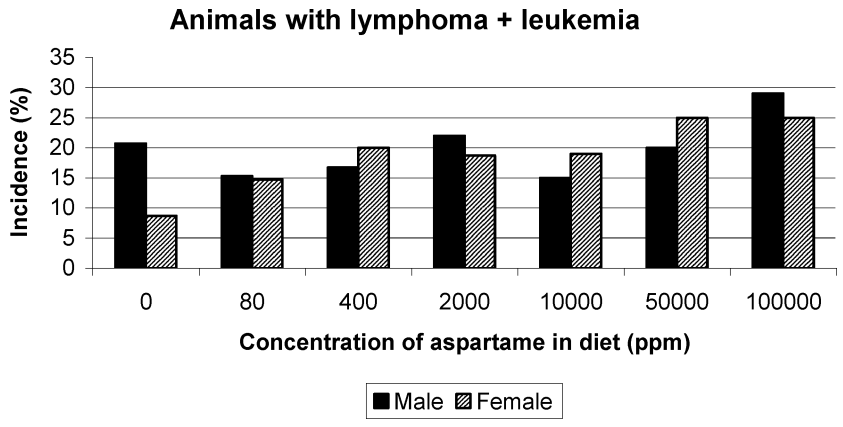

FIG. 7. Combined incidence of rats with lymphoma and leukemia fed varying levels of aspartame in the diet based on data from Soffritti et al. (2005).

- Survival rates ranged from 22% (controls) to 31% (treated) in males and 27.3% (controls) to 45% (treated) in females.
- Brain abscesses ranged from 7 to 11% in males, 4–20% in females, in controls and treated.
- Incidence of pyelonephritis[17] ranged from 23 to 62% in males, 31–83% in females.
- Incidence of pleuritis[18] ranged from 22 to 71% in males, 47–94% in females.
- Incidence of bronchopneumonia ranged from 81–95% in males, 69–97% in females. Other high-incidence observations in all groups included: peritonitis,[19] liver abscesses and hepatitis, and pericarditis[20] and meningitis.[21]

6.3.2.4. Interpretation of Results.

6.3.2.4.1. Lymphomas/Leukemias. In the first publication (Soffritti et al., 2005), an increase in the combined incidence of lymphomas and leukemias was reported for female rats treated with aspartame. From the graph (Figure 7) generated from data provided in the publication, it is clear that there are no differences in incidence between males and females fed aspartame; but there is a noticeable difference between the male and female control groups. The low background incidence in the female controls, probably a result of the poor 2-year survival of this group, is what creates the significant differences in the treatment groups for females and not males, despite very similar incidences in aspartame-treated groups. The inherent confounded interpretation of results when there is an abnormally low background tumor incidence in a control group is not unique to animal studies. This enigmatic feature of exposure-response analyses created by inordinately low baseline rates has been observed in major occupational epidemiology studies, such as the cohort studies of formaldehyde (Blair et al., 1986; Hauptmann et al., 2003, 2004) and acrylonitrile (Blair et al., 1998) workers conducted by the National Cancer Institute, and has stimulated reanalyses and reinterpretation of the NCI cohort data (Marsh et al., 2001, 2004, 2005).

Although there was an increasing dose-response in the first two dose groups compared with control for the female rats, no further increase occurred over the range of 400 to 10,000 ppm, despite consumption of diets containing up to 250 times as much aspartame, which puts into question the issue of a true dose response. Furthermore, it is important that for all groups of females, the incidence rates of lymphoma and leukemia were within the range of historical controls. Based on data provided to EFSA by the Ramazzini Foundation, which included years 1984 to 1991 only, the average incidence of lymphomas/leukemias for females is 13.3% with a range of 4 to 25%. For males, the incidence of lymphomas/leukemias in all groups was also within the range of the reported historical controls (8–30.9%). Although other historical control incidences of tumors for Sprague-Dawley rats have been reported (Giknis and Clifford, 2004; Baldrick, 2005), the data from the Ramazzini Foundation is the best comparison, due to the fact that they use their own in-house bred animals and do not maintain a pathogen-free environment.

The combination of the hemolymphoreticular tumor types, that is, lymphomas and leukemias, for statistical purposes was not justified in the view of the EFSA panel (2006), because tumors of different cellular origin were aggregated. It is noteworthy that Soffritti's group has combined these tumors previously and have been the only group to report high incidences of lymphoma/leukemia in association with exposure to methanol (Soffritti et al., 2002) and formaldehyde (Soffritti et al., 1989, 2002) while others have found no such association. The pooling of tumor types methodology was noted as a limitation in the WHO review of the Soffritti formaldehyde study (CICAD, 2002). Ward et al. (1990) reported that naturally occurring lymphomas and leukaemias can and should be differentiated from induced tumors in chronic rodent bioassays based on organ distribution, cytology, antigen markers, incidence of pneumonia, and age of appearance. No such analyses have been reported by Soffritti (Soffritti et al., 1989, 2002).

The high incidence of bronchopneumonia observed in this study is unusual for Sprague-Dawley rats, and is likely related to the high incidence of lymphoma/leukemias (EFSA, 2006). The progression of chronic murine pneumonia in rats has been shown to result in lymphoid neoplasmas (Innes et al., 1967; Nelson et al., 1967). Furthermore, elimination of chronic respiratory disease in rat colonies reduces the indicence of pulmonary lymonoid neoplasias to close to zero (Innes et al., 1967; Nelson et al., 1967).

The causative agent of many infections in non-specific pathogen-free studies was reported to be *Mycoplasma pulmonis* (Lindsey et al., 1985). Affected sites of *M. pulmonis* include lungs, nasopharynx and middle ears, along with causing shortened life span, altered xenobiotic metabolism and responses to carcinogens.[22] Rats with pulmonary infections develop lesions in multiple sites earlier than rats free from pulmonary disease (Simms, 1967). Based on observations and studies in the 1950s and 1960s, toxicologists recommended that rats with chronic pulmonary disease should not be used for long-term experimental work or toxicity studies (Innes et al., 1967; Nelson et al., 1967; Simms, 1967), which ultimately led to the establishment of pathogen-free animal suppliers for toxicity research. Therefore,

[17]Pyelonephritis = inflammation of the renal parenchyma, calyces, and pelvis, particularly due to local bacterial infection (*Stedman's Medical Dictionary*, 1995m).

[18]Pleuritis = inflammation of the pleura (*Stedman's Medical Dictionary*, 1995l).

[19]Peritonitis = inflammation of the peritoneum (*Stedman's Medical Dictionary*, 1995j).

[20]Pericarditis = inflammation of the pericardium (*Stedman's Medical Dictionary*, 1995i).

[21]Meningitis = inflammation of the membranes of the brain or spinal cord (*Stedman's Medical Dictionary*, 1995e).

[22]http://ccm.ucdavis.edu/cpl/index1.htm

these factors render the results of the Ramazzini studies of doubtful validity.

It is impossible to interpret the lymphoma/leukemia data reported in the Ramazzini study because of the high and highly variable incidences of lung infections (Soffritti et al., 1989, 2002). Accordingly, the overall value of the study is open to question (EFSA, 2006).

Based on these factors, the EFSA (2006) and this panel have concluded that the slight increase in incidences of lymphomas/leukemias in the rats fed aspartame were an incidental finding and should be dismissed.

6.3.2.4.2. Transitional-Cell Carcinomas of the Renal Pelvis and Ureter and Their Precursors. The relevance of observations of tumors of the kidney, ureter and bladder in rat studies to potential risk of tumors to humans has been studied extensively, due to the fact that these tumors are commonly observed in the rat with high doses of a variety of chemicals (Cohen, 1995a, 1995b). It is well established that nongenotoxic chemicals can induce tumors in these tissues when the doses of these chemicals exceed a threshold level (typically 1% of the diet), through a number of mechanisms such as cytotoxicity, formation of urinary precipitates, and increased cell proliferation due to regernerative hyperplasia. The association of ureter foreign bodies with chronic irritation leading to urothelial cell proliferation and urothelial carcinogenesis have been well documented and reviewed (Capan et al., 1999). Thus dose-related urinary tract calcification may be involved in the observations of urothelial hyperproliferation and neoplasms. An exposure of 1% in the diet has been calculated to be equivalent to a human exposure of 30,000 mg/day for a 60-kg individual. Second, the rat has a much greater susceptibility to these tumors than humans, due to differences in urinary protein levels (Cohen, 1995a, 1995b).

As aspartame has been clearly shown to be nongenotoxic, it is likely that the observation of these changes in rats fed high levels of aspartame are also the result of one of these imbalances that are specific for the rat. Therefore, although this finding is probably treatment-related in the rat, it is of no relevance for human risk due to the fact that it occurs only at high doses and is rat-specific (EFSA, 2006).

6.3.2.4.3. Malignant Schwannomas of Peripheral Nerves. The incidence of malignant Schwannomas of peripheral nerves reported in the second publication (Soffritti et al., 2006) is shown in Table 17. The biological relevance of the incidence of peripheral nerve Schwannomas is unclear due to the low number of tumors, the relatively flat dose-response curve over a very wide dose range, and uncertainty about the diagnosis of the tumors (EFSA, 2006).

6.3.2.4.4. Hyperplasia and Adenomas of the Olfactory Tissues. This observation is not indicative of likely risk of nasal tumors in humans from oral consumption of aspartame for several reasons. First, the route of exposure in humans is strictly oral, whereas in the rats, which are obligate nasal breathers, inhalation of the suspected powdered diet containing high levels of aspartame may have caused an inflammatory response, which played a role in the hyperplasia. Specific details of the composition and form of the diet were not provided; however, it is assumed that the diet was in powder form, as pelletting would lead to a loss of aspartame. Second, there is the disagreement regarding the pathological classification of the nasal adenomas. Two cases of adenomas provided by the Soffritti group to the NTP Pathology Working Group were unanimously diagnosed by NTP as hyperplasia. Therefore, severe pathological grading of hyperplasia may have contributed to the observations of adenomas. Lastly, but perhaps most important, there is extensive data demonstrating significant species differences in susceptibility to the development of nasal tumors due to exposure from many compounds (Kai et al., 2006; Jeffrey et al., 2006). Jeffery et al. (2006) reviewed the effects of a variety of organic chemicals demonstrated to produce toxicity and carcinogenicity in rodents following oral administration and systemic distribution. None

TABLE 17
Incidence of Schwannomas in male and female rats fed aspartame (Soffritti et al., 2006)

Dose (mg/kg bw/day)	Male rats		Female rats	
	Cranial	Other sites	Cranial	Other sites
0	1/150[1] (0.7%)	0/150 (0%)	0/150 (0%)	0/150 (0%)
4	1/150 (0.7%)	0/150 (0%)	1/150 (0.7%)	1/150 (0.7%)
20	1/150 (0.7%)	2/150 (1.3%)	0/150 (0%)	0/150 (0%)
100	2/150 (1.3%)	0/150 (0%)	1/150 (0.7%)	2/150 (1.3%)
500	2/100 (2%)	0/100 (0%)	1/100 (1%)	0/100 (0%)
2500	3/100 (3%)	0/100 (0%)	1/100 (1%)	0/100 (0%)
5000	3/100 (3%)	1/100 (1%)	1/100 (1%)	1/100 (1%)

Note. [1]Number of rats with Schwannomas/number of rats in group (percent); bw = body weight.

of the known rodent nasal carcinogens that lack DNA reactivity are considered to represent any cancer hazard, including nasal tumors, in humans. In addition, DNA-reactive rodent nasal carcinogens have not been associated with nasal tumors in humans, although they may be associated with tumors at other sites. Humans have much lower levels of biotransformation enzymes in the nasal mucosa, and usually much lower levels of exposure, which accounts for species differences in susceptibility. Therefore, the observation of hyperplasia, and possibly adenomas in rats fed aspartame in a powdered diet does not provide any evidence for increased tumor risk in humans consuming aspartame orally.

6.3.2.4.5. Total Malignant Tumor-Bearing Animals. According to EFSA (2006), the unpublished report of the NTP Pathology Working Group described several cases of discrepancies regarding the diagnosis of lesions from this study, in which the NTP classification was less severe than the diagnosis assigned by the Soffritti group. Given the high incidence of infection that likely led to the lymphomas/leukemias and that the mechanism of renal and ureter tumors is irrelevant to humans, the EFSA stated that these tumors should have been excluded from the total tumor analysis. The observation of a total of 12 brain tumors in the treatment groups was not dose-related and therefore, although none was observed in the control groups, aspartame cannot be considered responsible for the brain tumors. Considering these observations, the data on total tumors as presented in the Soffritti et al. (2006) publication cannot be accepted as evidence for the carcinogenic potential of aspartame (EFSA, 2006).

Interestingly, in 2002, Soffritti and colleagues (2002) reported a review of 200 compounds that had been evaluated by the Ramazzini Foundation using the same protocol as in the recent studies. Aspartame was listed in this review. The only details mentioned in the review article were that the study included 1800 rats and that the route of exposure was ingestion. Aspartame was *not* listed in the summary as 1 of the 47 agents that showed "clear evidence" of carcinogenicity. It is not known whether this was a separate study or is part of the experiment reported in 2005. As the EFSA report (2006) states that the study reported in 2005 was conducted from June 1997 to May 2000, it is most likely that these are the same studies; however, the number of animals is half of the 3600 rats reported to be in the 2006 studies. If the study was conducted in two stages, this should have been reported to ensure all groups were included in both stages and the appropriate statistics were employed for such a design.

In addition to the reevaluation of data and scientific evidence for each of the conclusions described above, EFSA also took into consideration the negative findings in recent NTP studies of aspartame using transgenic mice (described below in Section 6.3.3) and the negative findings in a recent National Cancer Institute epidemiological study (described below in Section 6.9.3.1). The conclusion of the EFSA evaluation was that "there is no need to further review the safety of aspartame nor to revise the previously established ADI for aspartame (40 mg/kg bw/day). The panel also noted that intakes of aspartame in Europe, with levels up to 10 mg/kg bw/day, are well below the ADI."

In summary, the Soffritti reports alleging carcinogenicity are contradicted by many publications and every scientific consideration. Many potential flaws have been suggested in this report; whether these or some other unidentified flaw is responsible for their incorrect allegations is not known. Nevertheless, it can be confidently stated that these reports provide no credible evidence that aspartame is carcinogenic.

6.3.3. Carcinogenicity Studies in Transgenic Mice

The National Toxicology Program (NTP) completed three carcinogenicity and toxicity studies of aspartame and recently posted their report on their website (NTP, 2005). Three different transgenic mouse models were used, but the protocols for each study were identical. The three models used were the heterozygous p53-deficient (+/–) mouse (sensitive for spontaneous lymphomas and sarcomas), the Cdkn2a-deficient mouse (claimed to be sensitive for suspected brain carcinogens), and the Tg.AC mouse (detection of both genotoxic and nongenotoxic carcinogens and in particular sensitive for development of forestomach tumors). The six concentration levels used were 0, 3125, 6250, 12,500, 25,000, and 50,000 ppm aspartame in NTP 2000 feed. The highest concentration was equivalent to a dose of 7500 mg/kg bw/day. There were 15 mice/sex/group, totaling 180 mice for the heterozygous p53-deficient (+/–) and the Cdkn2a-deficient mice studies. For the Tg.AC mouse study, there was also an additional positive control group, which was treated dermally with 1.25 μg 12-*O*-tetradecanoylphorbol 13-acetate (TPA)/mouse 3 times per week in acetone. The duration of the studies was 39 weeks (or 9 months). All studies were done according to GLP guidelines. These studies have not been published in the peer-reviewed literature, although the report was peer-reviewed within the NTP and data were taken into account in the recent EFSA evaluation of the carcinogenicity of aspartame (EFSA, 2006).

6.3.3.1. Description of the Transgenic Mice Models. Brief descriptions of the three transgenic models used in the NTP study (2001) are given next. The pathways illustrating the role of the proteins that are deleted or mutated in these models in cell cycle regulation are provided in APPENDIX III. The performance of these transgenic assays, the classic NTP 2-year rodent assays and combinations of these assays is discussed and compared in APPENDIX IV.

6.3.3.1.1. p53 Model. The heterozygous p53 mouse model was designed on extensive evidence that p53 is commonly a mutation tumor suppressor gene in a wide variety of human tumors. The function of a tumor suppressor gene is to "guard the genome" and expression of the wild-type gene

product is necessary for proper function and suppression of oncogenic events. Loss of functional protein, either due to mutations in the protein or loss of the gene, results in greatly increased susceptibility to tumor development. Therefore, mice that are completely deficient in p53 because of homozygous null allele (−/−) will spontaneously develop tumors (primarily lymphomas and sarcomas) within the first 3 to 6 months of life. The heterozygous $p53^{+/-}$ mouse model has one copy of the functional wild type, and one null allele, which is not transcribed or translated, resulting in a lower level of p53 protein (French et al., 2001). These mice develop tumors spontaneously as well, but at a much lower incidence and longer latency time (approximately 9 months) as compared to the homozygous null mice.

As heterozygous p53 mice already carry one mutation in their germline, they should be more susceptible to induction of tumors by genotoxic carcinogens. Thus, one explanation for the increased sensitivity of heterozygous p53 model to tumor development is that the carcinogen causes mutation or loss of the second copy of p53, completely depleting the animal of the tumor suppressor gene product. Alternatively, even without a direct hit on the second p53 gene, the lower amount of p53 protein may cause an acceleration of tumor development initiated in other genes. This is termed a gene dosage effect (Venkatachalam et al., 2001). The limitation of this model is that it does not detect most nongenotoxic carcinogens in a short (6-month) protocol; however, a longer 9-month protocol was used in the NTP study.

Storer et al. (2001) reviewed the available data from carcinogenicity studies with the $p53^{+/-}$ transgenic mouse model to assess its usefulness as a short-term carcinogenicity assay. Forty-eight different compounds had been tested, some in multiple studies. In all cases, the mice were $p53^{+/-}$ heterozygotes; however, the background strain varied, and included C3H, CBA, MIH, and C57BL6. The duration of the study was 26 weeks. Overall, 42 of the 48 compounds gave results that were concordant with expectations. In general, nongenotoxic compounds were negative, and most genotoxic carcinogens were positive. *p*-Cresidine gave positive results in 18/19 studies for bladder cancer.

In addition to use in studies for regulatory carcinogenicity assessments, the $p53^{+/-}$ transgenic model has been widely used in the cancer research community to assess dietary, exercise and pharmaceutical chemopreventive agents for their promotional or inhibitory effects on cancer development. Although a review of these studies is beyond the scope of this monograph, Hursting et al. (2004) provides an excellent example of the use of p53-deficient mice models by the National Cancer Institute to investigate diet-gene interactions.

Of the three models used by the NTP to evaluate aspartame, the p53 is the most well accepted and investigated model.

6.3.3.1.2. Cdkn2a-Deficient Model. This model is not widely used and, therefore, has not been as well characterized or evaluated as the p53 or Tg.AC models. It was developed in 1996 by Serrano et al. (1996), based on the frequent detection of mutations and deletions in the Cdkn2a gene in a wide variety of tumors. Of special importance is the evidence that genetic alterations in this gene play an important role in human brain cancers (Ohgaki et al., 2004), making use of this model particularly relevant for suspected brain carcinogens.

The role of the Cdkn2a gene is complex. This gene codes for a number of proteins that function as cyclin-dependent kinase (CDK) inhibitors. Depending on the exon, open reading frame, and polyadenylation sites used, the resulting Cdkn2a gene transcript will be the $p16^{Ink4a}$ variant or the $p19^{Arf}$ variant. The $p16^{Ink4a}$ variant codes for a protein (called p16) which functions to inhibit CDK4, and the $p19^{Arf}$ variant protein product (called p19) functions to stabilize p53 protein. The presence of p16 results in inhibition of CDK4, preventing phosphorylation of pRb, which in turn blocks the transition from G1 to the S phase. The presence of p19 results in sequestration of the protein Mdm2, which prevents Mdm2 from binding to p53 and targeting p53 for degradation. Thus, p53 is available to prevent the G2 to M phase transition. Thus both proteins from the Cdkn2a gene play a critical role in cell cycle regulation (Lubet et al., 2005).

The net result of these actions is that the Cdkn2a gene is acting as a tumor suppressor gene, and loss of the functions of this gene, as in the transgenic Cdkn2a-deficient model, increases susceptibility to tumor formation. This model is also referred to as the *INK4a*/*ARF* model because of the two variants described earlier that are affected. Although the model has not been widely used in studies assessing carcinogenicity of chemicals, it is a well-established model for cancer research due to the prevalence of mutations in this locus in commonly occurring cancers such as breast cancer (D'Amico et al., 2003). A simple search on Pubmed of "INK4a/ARF and cancer" gave nearly 400 hits, indicating the use of this model in cancer research.

6.3.3.1.3. Tg.AC Model. This strain is bioengineered to contain the v-Ha-*ras* oncogene that has been activated with two mutations. The expression of the product of the gene is regulated by a promoter and is not normally expressed in adult tissues. Exposure to ultraviolet (UV) light, specific chemicals and full-thickness wounding induces expression of the transgene, which is necessary to invoke tumor development (Tennant et al., 1995, 1998). The model can detect both genotoxic and nongenotoxic carcinogens, but was not positive for chemicals which have shown a strain-specific or species-specific response in a 2-year bioassay (Tennant et al., 2001). Thus, the initial concerns that this model would have many false-positives have proven to be invalid. In reviews of over 40 studies with this model, the Tg.AC model was more prone to false negatives (Eastin et al., 2001; Pritchard et al., 2003).

This model is most widely accepted for testing of dermal applications as the skin of this genetically-altered mouse acts as if already initiated with a carcinogen, that is, skin papillomas

will develop within 12 weeks following the application of classic promoter compounds (such as TPA) without a prior application of a carcinogen (Jacobson-Kram et al., 2004).

The oral route of administration of carcinogens also generates tumors, including squamous cell papillomas and carcinomas of the forestomach (Tennant et al., 1998). Although this model has also been used with oral exposure, it has been more fully evaluated with dermal applications.

6.3.3.2. Results of the Transgenic Mice Models. In all three studies, there were no neoplasms attributed to exposure to aspartame in either sex at any dose tested. The conclusion of each study was that "there was no evidence of carcinogenicity of aspartame" in the transgenic mice exposed to aspartame in the diet at levels of 0, 3125, 6250, 12,500, 25,000, and 50,000 ppm. As the p53 model is the most widely accepted model, a summary of the neoplasms in the p53 mice fed various levels of aspartame is given in Table 18.

Other nonneoplastic lesions were recorded in these studies, but none occurred with higher incidence in aspartame-fed versus control animals. The reported observations in the p53 study included cellular lymphocytic infiltration of liver (∼4/15 animals per group including control). Similar findings were recorded for salivary glands (5/15 for control and 8/15 for highest dose). Another common lesion was hyperplastic foci in the adrenal cortex, ranging from 80% to 100% in all groups. In females, hyperplastic cystic endometrium was observed in 93–100% of all mice in all groups. Spleen hematopoietic cell proliferation was observed in 3/15 control mice, 4/15 low dose mice, and 1/15 mice in higher doses. Dilation of renal tubules was observed in 6/15 control mice, and between 2/15 (6250 ppm) to 4/15 (50,000 ppm) in female and male mice. In males, 4/15 control mice displayed nephropathy of kidney, but this was only observed in highest aspartame dose group where 3/15 mice also had kidney nephropathy. All other lesions occurred randomly among control and aspartame-treated mice, and were not observed in more than 2/15 mice per dose group (NTP, 2005).

In conclusion, no evidence of carcinogenicity was observed in these transgenic mouse model studies with dietary levels of aspartame equivalent to 7500 mg/kg bw/day. The National Toxicology Program (NTP, 2005) has stated that they have not published these results due to a "current lack of appreciation of the scientific or public health value of negative cancer findings in these models." This is contrary to the views of other regulatory agencies, and a review of statements on the value of these models in risk assessment of carcinogenicity is provided in the next section.

6.3.3.3. Use of Transgenic Models in Regulatory Evaluations. The NTP studies have not been published in the peer-reviewed literature, although the report was peer-reviewed within the NTP. The purpose of this review was to assess whether there is general agreement in the literature that there is a "current lack of appreciation of the scientific or public health value of negative cancer findings in these models." This has been stated as the rationale for not making public the findings of these studies.

6.3.3.3.1. ILSI-ACT. The p53 and Tg.AC transgenic mice models used in the NTP study were also evaluated during a workshop organized by the International Life Sciences Institute Alternative Carcinogenicity Testing (ILSI-ACT) committee. Cohen (2001) summarized the results of assays conducted with 21 chemicals using the standard mouse and rat long-term assay and compared the results of testing with the same chemicals in nine transgenic models and short-term assays. The mechanistic basis for these models and data obtained from studies with a number of chemicals, were reviewed and summarized by workshop participants. These reports were then provided to a group of international cancer experts from government, industry and academic organizations. Following their evaluation, a panel discussion was held with these individuals and workshop participants to address the appropriateness of the alternative models to human cancer risk assessment (Pettit, 2001; Popp, 2001). The consensus in this group was that models had significant value as part of a weight-of-evidence approach to assessing human carcinogenic risk. All of the models were expected to detect genotoxins, with differing responses depending on mechanism of action of the carcinogen in question. For example, the carcinogen may act primarily to activate tumor oncogenes or to cause mutation or deletion of tumor suppressor genes. Although all transgenic models have limitations and are not designed to be stand-alone assays, they are considered to be valuable for consideration in an overall risk assessment (Cohen, 2001; Goodman, 2001).

6.3.3.3.2. FDA. Jacobson-Kram and colleagues (2004) recently reviewed the use of transgenic mice in carcinogenicity hazard assessment by the FDA. Use of transgenic models in carcinogenicity testing protocols was formalized in FDA's 1997 adoption of the Guidance for Industry S1B Testing for Carcinogenicity of Pharmaceuticals,[23] "opening the door" for use of transgenic models in regulatory toxicology assessment. The FDA paradigm is that combining a traditional rat bioassay with a 6-month transgenic study for an overall assessment of the weight-of-evidence results in fewer false positives and no increase in false negatives. Analyses to assess the sensitivity (no false negatives) and specificity (no false positives) to give an overall accuracy score for specific transgenic models and traditional rodent bioassays, as compared to a combined approach, provide support for this paradigm (Pritchard et al., 2003; Jacobson-Kram et al., 2004). The NTP classification as a human carcinogen, in the NTP Report on Carcinogens, was used to assign compounds the classification of carcinogen or noncarcinogen for the analyses in Table 19 (Jacobson-Kram et al., 2004).

6.3.3.3.3. European Regulatory Authorities. In a short opinion paper, van der Laan et al. (2002) stated that similar to long-term rodent bioassays, transgenic models have less-than-perfect accuracy as there are some reports of false negatives and

[23] http://www.fda.gov/cder/guidance/1854fnl.pdf

TABLE 18
Neoplasms observed in p53 mice fed various levels of aspartame in the diet (adapted from NTP, 2005)

	Dietary levels of aspartame in parts per million (ppm)					
Male rats	0	3125	6250	12,500	25,000	50,000
Alimentary:						
Small intestine—polyp	0 (15)					1 (15)
Malignant lymphoma (liver)	0 (14)	0 (15)	2 (15)	0 (15)	0 (14)	0 (15)
Malignant lymphoma (salivary gland)	0 (14)	0 (1)	1 (1)	NR	0 (1)	0 (15)
Sarcoma (prostate)	0 (15)	0 (15)	0 (15)	0 (14)	1 (14)	0 (15)
Cardiovascular—nothing reported						
Endocrine—nothing reported						
General body —nothing reported						
Hematopoietic system:						
Lymph node (Mandibular malignant, lymphoma)	0 (14)	0 (15)	1 (14)	0 (15)	0 (14)	0 (15)
Lymph node (Mesenteric sarcoma)	0 (14)	0 (14)	1 (15)	0 (13)	0 (14)	0 (15)
Lymph node (Mediastinal malignant lymphoma)	0 (13)	0 (14)	2 (14)	0 (13)	0 (12)	0 (13)
Spleen, malignant lymphoma	0 (14)	0 (15)	2 (15)	0 (14)	1 (14)	0 (15)
Thymus, malignant lymphoma	0 (14)	0 (15)	2 (15)	0 (14)	0 (15)	0 (14)
Lymphoma (lung)	0 (15)	0 (15)	2 (15)	0 (15)	0 (14)	0 (15)
Skin, subcutaneous, fibrosarcoma	0 (15)					1 (15)
Lymphoma (kidney)	0 (14)	0 (15)	1 (15)	0 (15)	0 (14)	0 (15)
Lymphoma (multiple sites)	0 (15)	0 (15)	2 (15)	0 (15)	0 (15)	0 (15)
Total						
Animals with primary neoplasms			2		1	2
Number of neoplasms			2		1	2
Benign			2		1	1
Malignant						1

	Dietary levels of aspartame in parts per million (ppm)					
Female rats	0	3125	6250	12,500	25,000	50,000
Genital system—nothing reported						
Hematopoietic system:						
Lymph node (pancreatic malignant, lymphoma)	NR	NR	NR	NR	1 (1)	NR
Lymph node (renal malignant, lymphoma)	NR	NR	NR	NR	1 (1)	NR
Lymph node (mandibular malignant, lymphoma)	0 (15)	0 (14)	0 (14)	0 (14)	1 (15)	0 (14)
Lymph node (mesenteric sarcoma)	0 (15)	0 (14)	0 (14)	1 (14)	0 (15)	0 (15)
Lymph node (mediastinal malignant, lymphoma)	0 (12)	0 (13)	0 (13)	0 (12)	1 (13)	0 (15)
Spleen, malignant lymphoma	1 (15)	0 (15)	0 (15)	0 (15)	1 (15)	0 (15)
Thyroid, malignant lymphoma	1 (14)	0 (14)	0 (14)	0 (15)	0 (15)	0 (15)
Integumentary:						
Mammary gland—carcinoma	1 (13)	0 (14)	0 (14)	1 (15)	0 (12)	0 (15)
Skin, subcutaneous, fibrosarcoma	0 (15)	NR	1 (15)	NR	NR	0 (15)
Bone—femur osteosarcoma	1 (15)	NR	NR	NR	NR	1 (15)
Bone—vertebra osteosarcoma	NR	NR	1 (15)	NR	NR	NR
Nervous system—nothing reported						
Lymphoma (lung)	1 (15)	0 (15)	0 (14)	0 (15)	0 (15)	0 (15)
Lymphoma (kidney)	1 (15)	0 (14)	0 (15)	0 (15)	0 (15)	0 (15)
Lymphoma (multiple sites)	1 (15)	0 (15)	0 (15)	0 (15)	1 (15)	0 (15)
Alimentary: Malignant lymphoma (gallbladder)	1 (14)	NR	NR	NR	NR	0 (15)
Malignant lymphoma (cecum lymphoid tissue)	NR	NR	NR	NR	1 (15)	0 (15)
Malignant lymphoma (liver)	1 (15)	0 (15)	0 (15)	0 (15)	0 (15)	0 (15)

(Continued on next page)

TABLE 18
Neoplasms observed in p53 mice fed various levels of aspartame in the diet (adapted from NTP, 2005) *(Continued)*

	Dietary levels of aspartame in parts per million (ppm)					
Female rats	0	3125	6250	12,500	25,000	50,000
Sarcoma (liver)	NR	NR	NR	1 (15)	NR	NR
Cardiovascular - nothing reported						
Endocrine:						
Pituitary gland (pars distalis, adenoma)	1 (9)	0 (14)	0 (13)	0 (14)	0 (11)	0 (14)
Thyroid (follicular cell, adenoma)	0 (14)	0 (13)	0 (14)	0 (14)	1 (13)	0 (15)
Total animals with primary neoplasms	4	NR	2	2	1	1
Number of neoplasms	4	NR	2	3	2	NR
Benign	1	NR	NR	NR	1	NR
Malignant	3	NR	2	2	1	1

false positives when compared to other supporting data for a specific chemical. With that acknowledgment, the conclusion of a review of the current state of knowledge was that "regulatory authorities cannot neglect the outcome of such studies, but need to be cautious in their interpretation of the data from such models, and the application in risk assessment problems."

More recently, the ILSI Alternatives to Carcinogenicity Testing Committee held a workshop to reassess data from genetically modified mouse model studies that have been evaluated by international regulatory agencies. The perspectives of the U.S. FDA, the European Committee for Proprietary Medicinal Products Safety Working Party, and the Japanese Ministry of Health Labor and Welfare were published (MacDonald et al., 2004). Overall agreement was expressed that the alternative assays can have an important role in regulatory carcinogen safety assessment. However, specific concerns were raised.

1. For the $p53^{+/-}$ model, a study duration of 9 months is recommended, whereas a 6-month duration is considered adequate for the Tg.AC and Tg.rasH2 models. The number of animals per group should be 25, rather than the originally proposed size of 15 per group, to provide adequate statistical power. This model is considered appropriate for carcinogens that have been shown to be positive in genotoxicity tests, but may not be adequately sensitive to detect nongenotoxic carcinogens.
2. It is considered advantageous to include positive controls for all models. For the $p53^{+/-}$ model, the positive control that

TABLE 19
Summary of performance of transgenic assays, NTP rodent assays and combinations (adapted from Pritchard et al., 2003)

Strategy	+ for carcinogen	−for noncarcinogen	False positive	False negative	Overall accuracy
$p53^{+/-}$	21	27	1	10	48/59 (81%)
$p53^{+/-}$ (genotoxic)	16	6	0	4	22/26 (85%)
Tg.AC	17	29	10	6	44/62 (74%)
RasH2	21	18	5	7	39/51 (76%)
$p53^{+/-}$(genotoxic) and Ras H2	30	14	5	4	44/53 (83%)
$p53^{+/-}$ genotoxic) and Tg.AC	25	22	10	4	47/61 (77%)
NTP rodent bioassay	23	17	18	0	40/58 (69%)
NTP rodent bioassay and $p53^{+/-}$ (genotoxic) and Tg.AC (nongenotoxic)	35	13	9	0	48/57 (84%)
NTP rat bioassay and $p53^{+/-}$ (genotoxic) and H2 (nongenotoxic)	33	12	8	0	45/53 (85%)

Note. Definitions: positive for carcinogens, positive assay results for NTP human carcinogens; negative for noncarcinogens, negative assay results for NTP human noncarcinogens; positive for noncarcinogens, positive assay results for NTP human noncarcinogens; negative for carcinogens, negative assay results for NTP human carcinogens. Classification of NTP human carcinogens is based on a comprehensive evaluation of all relevant human, animal and genotoxicity data.

has been used is *p*-cresidine, however an alternative one is being sought due to lack of consistent results with *p*-cresidine. For the Tg.AC models, TPA administered 3 times per week is recommended. The Tg.AC model is most appropriate for testing compounds where dermal exposure is relevant. The usefulness of this model for testing compounds where the human exposure will be orally is not well established, regardless of whether the exposure to the mice is dermal or oral.

3. The genetic background of the parental strain will influence a model's tumor spectrum and may result in "blind spots" or tumor-resistant organs. This needs to be further investigated in all models. Wild-type parental strains should be included in determinations of maximum tolerated dose levels for the genetic models.
4. Lastly, the generally recognized incidence of tumors at rare sites that should be considered treatment-related was 2 out of 15 animals when the group size is 15. More data will be needed to establish the significant incidence level with larger groups (MacDonald et al., 2004).

In conclusion, the acceptance and usefulness of transgenic mouse models in risk assessment is well established. The three transgenic mouse studies on aspartame provide further supportive evidence of the lack of carcinogenic potential of aspartame.

6.3.3.4. Chronic Toxicity and Carcinogenicity Studies on Breakdown Products of Aspartame. The chronic toxicity and carcinogenicity of DKP was assessed individually and in combination with aspartame in several studies as summarized in Table 15. The NOAEL of these studies ranged from 1 to 3 g/kg/day. Observations of benign endometrial hyperplasia in rats fed the highest doses of DKP, but not in rats fed 750 mg/kg bw/day were the basis for the ADI of DKP set by JECFA at 7.5 mg/kg bw/day (JECFA, 1980b). DKP usually is present at the level of 1% of aspartame (JECFA, 1980b). Therefore, based on the consumption analysis of aspartame performed for this evaluation (Section 3.1), the current consumption of DKP for consumers of aspartame (eaters only) is estimated at 0.05 mg/kg bw/day for the mean per capita consumption, and 0.13 mg/kg bw/day for the 95th percentile consumers.

Safety studies have also been conducted on the nonsweet β-aspartame, which may be present in very low levels in aspartame-sweetened foods (Kotsonis and Hjelle, 1996). The NOEL for β-aspartame was at least 500 mg/kg bw/day in all studies, which is at least 10,000 times the estimated 90th percentile of intake of β-aspartame (0.05 mg/kg bw/day) (Kotsonis and Hjelle, 1996).

6.3.3.5. Summary of Chronic Toxicity and Carcinogenicity Studies. The long-term studies on the toxicity of aspartame now include: seven chronic studies of at least 2 years duration with mice or rats to assess carcinogenic potential and chronic toxicity, and 2 studies of 32 to 36 weeks duration to evaluate the tumor promotional activity of aspartame in rats. Extensive peer review and reevaluation of data and study protocols has occurred for several studies in different parts of the world. In all cases, the conclusions of the reviews by authoritative agencies and this panel have been that aspartame does not have carcinogenic or cancer-promoting activity. Furthermore, no toxic effect of aspartame that is relevant to humans consuming aspartame orally has been consistently demonstrated.

One adverse effect that has been noted in several chronic studies is lower weight gain in animals chronically fed extremely high doses of aspartame. As was discussed in Section 6.3.2 on the Soffritti et al. (2006) study, the nutritional composition of the diet will be altered when aspartame replaces dietary constituents at levels exceeding 5%, and no adjustment of the nutritional composition is made. It is not known whether this factor, or the palatability of the diet, affects weight gain. No effect on weight gain is noted at lower doses. These observations do argue against the concerns that aspartame use may lead to weight gain, which will be discussed further in Section 6.9.2.6.

In summary, long-term animal studies with aspartame have been comprehensive and results support the safety of aspartame at doses representing human consumption levels.

6.4. Neurotoxicity Studies

The question of whether aspartame has the potential to be a neurotoxin is one that has been highly contentious (Olney, 1980). Concern with the potential neurotoxicity of aspartame arose from reports of hypothalamic damage in Swiss albino infant mice 5 h after being given large oral doses (1 g/kg bw) of aspartic acid ($n = 4$), glutamate ($n = 19$), or 3 g/kg bw cysteine ($n = 4$) (Olney and Ho, 1970). Subcutaneous injections of aspartic acid into day-old Swiss albino mice (n not defined) for 4 days at a dose of 15 mmol/kg bw resulted in high mortality rates (data not provided), and hypothalamic lesions (incidence not given) and obesity in those who survived for 7 months (10 males and 7 females) (Schainker and Olney, 1974). Subcutaneous injections of aspartic acid into mice on days 2 to 11 of life, of doses starting at 2.2 g/kg bw and increased to 4.4 g/kg bw, resulted in increased body weight, behavioral changes and impairment in reproductive function when mice were 180 days old (Pizzi et al., 1978). To determine the lowest dose of aspartic acid that was neurotoxic, infant mice (8-day old Swiss-Webster strain) were gavaged with doses equivalent to 0, 250, 500, 650, 750, and 1000 mg/kg bw and killed 5 h later. The highest dose that did not result in neuronal necrosis was 500 mg/kg bw (Finkelstein et al., 1983).

Experiments with infant rodents ($n = 4$/group) treated orally with 1 g/kg bw aspartic acid or glutamate developed excitotoxic nerve cell degeneration (Olney and Ho, 1970). Unlike the obvious weakness associated with damage of CNS motor nerve cells, dicarboxylic amino acid-induced neurodegeneration is clinically silent. However, infant rodents with hypothalamic neurodegeneration induced with subcutaneous injections (dose not reported) of monosodium glutamate developed obesity in adult life (Olney, 1969). This observation has raised the question of a possible role of aspartame in development of obesity. The effect of aspartame on body weight is addressed in Section 6.7.2.

The neurotoxic effects of glutamate and aspartic acid on the circumventricular organs are most pronounced in but not restricted to infant animals. According to Olney, 4-fold higher levels of aspartic acid or glutamate administered orally are required to destroy neurons in adult as compared to infant rodent brain. Susceptibility to aspartic acid- (and glutamate-) induced brain damage in rodents appears to decrease with age (Okaniwa et al., 1979). While disagreement exists as to differential species vulnerability to excitotoxic circumventricular organ damage, there is no a priori reason to expect the human brain to be refractory to this phenomenon.

These reports led to intensive investigation for evidence of neurologic biochemical, behavioral and/or morphological changes following exposure to aspartame. Studies conducted in vitro and using animal models are reviewed in Sections 6.4.1 and 6.4.2, respectively. Studies involving human subjects, including infants and children, are reviewed in Section 6.9.2.

6.4.1. In Vitro Studies

The in vitro hippocampal slice preparation was used to evaluate aspartame and several other compounds as neuroexcitatory agents (Fountain et al., 1992). Exposure of slices of the hippocampus to 0.01, 0.1, 1, and 10 m*M* aspartame resulted in potentiation of the response of hippocampal CA1 pyramidal cells, but had no apparent effect on local inhibitory systems. Aspartame exposure did not block the establishment of induction of long-term potentiation at any dose despite the potentiation of pyramidal cell response (Fountain et al., 1988). The limitations of this model include inability to predict effects on specific behaviors, the absence of the effect of the blood–brain barrier, and the absence of the effect of biotransformation (Fountain et al., 1992).

When mouse NB2a neuroblastoma cells are grown in culture, their differentiation can be induced with the addition of dibutyryl cyclic AMP and the removal of serum from culture media, leading to the growth of neurite-like extensions from the cell body (Lau et al., 2005). Inhibition of the growth of these amygdalae was employed as a measure of potential neurotoxicity of four food additives: aspartame, *L*-glutamic acid, brilliant blue, and quinoline yellow. Concentrations of these additives which, when added to the cell growth media, were reported to inhibit induction of neurite growth. The authors attempted to calculate potential plasma concentrations of additives by multiplying amounts in foods by percent absorption reported for the additives (Lau et al., 2005). However, the authors failed to account for the effect of metabolism. Due to complete and rapid hydrolysis in the gut, aspartame has not been detected in plasma following administration of oral doses (Oppermann et al., 1973b; Ranney et al., 1976; Ranney and Oppermann, 1979; Oppermann and Ranney, 1979), and thus neurons will not be exposed to intact aspartame due to consumption of aspartame in food. Therefore, the data from this study cannot be considered to be evidence of neurotoxicity of aspartame and will not be considered in this safety evaluation.

Tsakiris et al. (2006) recently reported that in vitro incubation of human erythrocyte membranes in the presence of combinations of methanol, aspartic acid and phenylalanine resulted in a dose-dependent reduction of acetylcholinesterase activity. Erythrocyte membranes were incubated for 1 h with concentrations that authors thought to likely represent blood levels of these metabolites following dosing with 10, 18, 34, 150, or 200 mg/kg bw aspartame. This assumption is discussed in the next paragraph. Five concentrations of methanol (0, 0.07, 0.14, 0.6, or 0.8 m*M*), aspartic acid (0.82, 1.4, 2.8, 7.6, or 10.0 m*M*), or phenylalanine (0.07, 0.08, 0.14, 0.35, or 0.5 m*M*), or a combination of all three were tested. When tested individually, the three highest concentrations of methanol, the three highest concentrations of aspartic acid, and the two highest concentrations of phenylalanine resulted in significant inhibition of erythrocyte acetylcholinesterase activity, in a dose-dependent manner. Five combinations of all three metabolites were prepared with the corresponding lowest to highest five doses described above for each compound. When five combinations were tested, erythrocyte acetylcholinesterase activity was inhibited 0, 7, 33, 41, and 57%, respectively.

To assess relevance of the findings by Tsakiris et al. (2006), the likelihood that the concentrations of metabolites used in the cell cultures actually represent the blood levels of metabolites following aspartame consumption needs to be evaluated. The only citation given by the authors for the estimated blood concentrations was a study by Stegink et al. (1988), in which only one dose of aspartame was used, and blood methanol levels were not measured. Therefore, the authors do not provide evidence that their concentrations represent likely blood levels of metabolites from aspartame consumption. On the contrary, as discussed in Section 6.9.2, the blood methanol levels were below detection limits in individuals and infants consuming doses of 34 mg/kg bw aspartame, which is much higher than the 95th percentile estimated intake (Table 6). Therefore, concentrations used in this study do not represent the likely blood concentrations following consumption of aspartame even in the 95th percentile consumers. Furthermore, it is well established that acetylcholinesterase activity can be inhibited below normal levels without functional effects. For example, mild poisoning from acetylcholinesterase inhibitors do not manifest until plasma pseudocholinesterase activity is inhibited by 50% or more (Ecobichon, 2001). Therefore, it is highly unlikely that consumption of aspartame will result in inhibition of acetylcholinesterase activity to the level that would have biological significance.

In summary, when in vitro studies employ physiologically relevant concentrations of aspartame metabolites, effects indicative of neurotoxicity are not observed.

6.4.2. Animal Studies

6.4.2.1. Pathological Lesions. Hypothalamic neuronal necrosis was reported following administration of aspartame to young mice given oral doses of 1–2 g/kg bw (Reynolds et al., 1976) and given drinking water containing a mixture of

aspartame, glutamate and aspartic acid (Olney and Ho, 1970). The actual dose received from drinking the aspartame solution was not provided.

In one study (Reynolds et al., 1976), neonatal mice (strain and sex not defined) received oral doses of a slurry of 10% aspartame at levels of 0.5 ($n = 27$), 1.0 ($n = 15$), 1.5 ($n = 17$), and 2.0 ($n = 18$) g/kg bw or monosodium glutamate (MSG) at levels of 0 to 4.0 g/kg bw. Hypothalamic lesions were encountered at dose levels equal to or exceeding 1.0 g/kg bw aspartame and 0.5 g/kg bw MSG. Aspartame administration resulted in a much smaller hypothalamic lesion than did equal dosages of MSG. Infant [macaque] monkeys (sex not defined) received aspartame (2 g/kg bw) ($n = 8$) or MSG (1–4 g/kg bw, $n = 19$) by stomach tube. Hypothalamic morphology in all monkeys remained normal at the microscopic and ultrastructural level. Plasma levels of amino acids were not measured.

Reynolds et al. (1980) conducted another study and found no evidence of brain neuronal necrosis in infant monkeys treated orally with water, or 2000 mg/kg bw aspartame, or 2000 mg/kg bw aspartame plus 1000 mg/kg bw monosodium glutamate. Monkeys (both sexes, *Macaca mulatto, M. fascicularis,* and *M. arctoides)* were sedated and dosed by stomach tube. Blood samples were taken after 0, 20, 40, 60, 90, 120, 180, and 240 min for amino acid analysis (results were presented in Table 10) and then monkeys were perfused with glutaraldehyde to fix the brain for histopathological analysis. The entire hypothalamic region was examined, using both light microscopy and electron microscopy. Due to difficulty in uniform fixation of large tissues such as the brain, local areas of poor perfusion, present in all treatment groups, exhibited abnormal morphology. However, there was no evidence of hypothalamic neuronal necrosis despite evidence of elevation of plasma levels of phenylalanine and aspartic acid. Therefore, the authors (Reynolds et al., 1980) stated that this study demonstrates the differences between rodent and primate brains in terms of susceptibility to neuronal necrosis following exposure to aspartame. Similar differences in the susceptibility of rodents and primates to neuronal necrosis induced by large acute doses of glutamate have been demonstrated (Reynolds et al., 1980). These results support the contention that the immature rodent brain is uniquely susceptible to amino acid-induced neuronal damage and is not a good model for humans. There is also a strong age-dependence in sensitivity to glutamate in rodents with neonatal animals being more sensitive than weanlings, which are in turn, more sensitive than mature animals, indicating that sensitivity decreases with age and maturation of the blood–brain barrier.

The effect of water restriction on the ability of aspartame and monosodium *L*-aspartate monohydrate (MSA) to induce brain lesions was evaluated in 22-day-old ICR mice (Takasaki et al., 1981). Following deprivation of water, or both food and water, for 14 h, mice rapidly consumed hyperosmolar solutions of either aspartame or monosodium *L*-aspartate monohydrate to a volume normally consumed over a period of 24 h. The mice were given a solution of 1.26% aspartame, which was supersaturated, as the solubility of aspartame is 1.03% at 25°C. Thus, the water deprivation of young mice impaired their ability to control intake of hyperosmolar solutions. The authors speculate that dehydration results in conditions that increased susceptibility of neuronal damage, as animals deprived of water, but not animals deprived of food, exhibited induction of neuronal damage following consumption of MSA despite consuming the same amount (one group via water, the other group via food). No hypothalamic lesions developed in animals consuming aspartame by water or food, regardless of water or food deprivation, indicating that dehydration did not increase susceptibility to brain lesions in mice, which are considered to be the most sensitive species (Takasaki et al., 1981). This study also implied that mice, as had been reported for rats, do not respond to the sweetness of aspartame and are able to tolerate levels of sweetness that are unacceptable for humans.

Finkelstein et al. (1988) reported that in young mice (8-day-old Swiss-Webster) given 750 or 1000 mg/kg bw aspartame to induce neuronal necrosis, simultaneous administration of carbohydrates (1 g/kg bw) or prior injection of insulin had a protective effect despite little effect on plasma amino acid concentrations.

6.4.2.2. Susceptibility to Seizures. The ability of aspartame to potentiate the induction of chemically or electrically induced seizures in rats was assessed as a measure of neurotoxicity (Guiso et al., 1988). Male Sprague-Dawley rats were fasted for 16 h, and then given aspartame by oral gavage, followed 1 h later with metrazol,[24] quinolinic acid,[25] or electroshock to induce seizures. Plasma levels of phenylalanine and tyrosine and changes in the ED50[26] of the treatment were measured. Aspartame lowered the ED50 of metrazol-induced convulsions from 65.9 mg/kg bw to 50.7 mg/kg bw when administered in a bolus dose of 1 g/kg bw to fasted rats ($n = 20$/group). No effect was observed when the same dose of aspartame was divided into 3 doses and delivered to fasted rats equally spaced over 120 min or when the dose was delivered to nonfasted rats. Similar results were found with administration of equimolar amounts of phenylalanine, but aspartic acid, methanol and leucine were inactive. Aspartame (1 g/kg bw) did not affect electroencephalographic seizures induced by quinolinic acid or tonic hindlimb extensions induced by electroshock. Concentrations of phenylalanine and tyrosine increased in plasma and brain following dosing with either 1 g/kg bw aspartame or 0.5 g/kg bw phenylalanine in a bolus to fasted rats, but not when rats were fed or the dose was divided. The ratio of phenylalanine to tyrosine was not significantly modified by any treatment (Guiso et al., 1988). These data suggest that aspartame cannot be regarded as a general proconvulsant agent.

[24]Metrazol is a convulsant which induces clonic-tonic convulsions (Guiso et al., 1988).

[25]Quinolinic acid is 2,3-pyridinedicarboxylic acid; a catabolite of tryptophan and a precursor of nicotinic acid. When administered into the hippocampus, this compound causes as seizure similar to human epilepsy of the temporal lobe (Guiso et al., 1988; *Stedman's Medical Dictionary*, 1995n).

[26]ED50 = dose that induced convulsions or seizures in 50% of the animals.

Pinto and Maher (1988a) treated CD-1 mice with aspartame followed by administration of proconvulsant agents, pentylenetetrozole[27] or fluorothyl,[28] to induce seizures. Doses of aspartame were 0, 250, 500, 750, 1000, 1500, and 2000 mg/kg bw given orally as a bolus 1 h before seizure induction. Group sizes were 24/group for pentylenetetrozole and 14/group for fluorothyl experiments. Similar to the findings above, doses of 1000 mg aspartame/kg bw or more given as a bolus increased the percentage of animals convulsing in response to pentylenetetrozole or fluorothyl. The CD50[29] for mice treated with both pentylenetetrozole and aspartame (1 g/kg bw) was reduced to 59 mg/kg bw as compared to 66 mg/kg bw for mice treated only with pentylenetetrozole. Plasma levels of phenylalanine and tyrosine were increased, and the ratio of phenylalanine to large neutral amino acids was increased with doses of 1000 mg/kg bw or more. Using equimolar doses of phenylalanine, aspartic acid, and methanol, the authors demonstrated that the phenylalanine component mediates this activity. Coadministration of 1000 mg/kg bw valine, a large neutral amino acid that competes with phenylalanine for uptake, inhibited the ability of 1 g/kg bw aspartame to potentiate the effect of fluorothyl-induced seizures, further illustrating the need for high levels of phenylalanine for this effect (Pinto and Maher, 1988a, 1988b).

As discussed previously, Pinto and Maher (1988a) suggested that very large bolus doses (>1 g/kg bw) in rodents are needed to be comparable to smaller human exposure doses. Dailey et al. (1989) pointed out that this argument is based on three observations. Doses of aspartame in humans increase plasma phenylalanine levels greater than plasma tyrosine, whereas small doses in rodents result in approximately equal increases in these two amino acids. Very large doses of aspartame are needed in rodents to achieve the imbalance of higher phenylalanine than tyrosine. Dailey et al. (1989) tested this argument by determining the effect of high dose aspartame boluses on brain catecholamines and serotonin concentrations in the pentylenetetrozole-induced seizure model. Male CD-1 mice were gavaged with 0, 1500, 2000, and 2500 mg/kg bw aspartame and treated with pentylenetetrozole 1 h later to induce seizures as in the study by Pinto and Maher (1988a). Despite observing similar changes in plasma amino acids, no change in norepinephrine or dopamine concentrations and no change in seizure induction occurred with any dose of aspartame. In rats administered 1500 mg aspartame/kg bw, serotonin levels were decreased to 77% the levels observed in control rats, but this was not associated with alterations in seizure threshold, suggesting that moderate modifications in neurochemicals can occur without altering seizure susceptibility. The authors state that in previous experiments using tryptophan hydroxylase inhibitors, a decrease of serotonin levels down to 13% of normal was needed to enhance seizures in epilepsy-prone rats (Dailey et al., 1989). The authors discuss potential factors that may be responsible for the difference in response in their study compared to the similar study by Pinto and Maher (1988a), but are unable to resolve this question.

A lack of effect of aspartame on pentylenetetrozole-induced seizures in Swiss albino mice was also reported by Helali et al. (1996) following oral doses of either an acute bolus of 1 g/kg or a subacute dose of 100 mg/kg bw/day aspartame for 14 days ($n = 10$/group). Spontaneous locomotor activity was also assessed and was not affected by aspartame. However, mice receiving 100 mg/kg bw/day aspartame for 14 days required significantly higher doses of three anticonvulsant drugs, ethosuximide, valproate and phenytoin, to protect against pentylenetetrozole-induced seizures, suggesting that aspartame antagonized the activity of the antiepileptic drugs. Decreased levels of adrenalin and noradrenalin, and increased levels of γ-aminobutyric acid (GABA) in the brain tissue from subacute aspartame-treated mice were proposed as a possible mechanism.

Cain et al. (1989) used the kindling[30] model of epilepsy in male hooded rats to assess the effect of aspartame on seizures. Kindling was achieved through electrical stimulation of the basolateral amygdale ($n = 6$) or ventral hippocampus ($n = 6$). Amygdala-kindled rats were gavaged with 0, 25, 50, and 200 mg aspartame/kg bw and hippocampal-kindled rats were gavaged with 0, 200, 1000, and 2000 mg/kg bw 1 h prior to seizure induction. Lower doses of aspartame were chosen for amygdale-kindled rats because this structure is the most sensitive; the dose of 200 mg/kg bw in rats was expected to increase the amino acid levels to the same degree as with 34 mg/kg bw aspartame in humans (the 99th percentile of use as determined by the FDA). Hippocampal-kindled rats more closely mimic human epilepsy, and were tested at 10-fold higher doses. Amino acid levels were not measured. Aspartame had no effect on seizure induction or seizure strength in both models (Cain et al., 1989).

Oral administration of an acute dose of 1000 mg/kg bw aspartame or an equimolar dose of phenylalanine to fasted rats had no effect on onset of seizures following infusion of the methylxanthine, theophylline, in female Lewis rats ($n = 10$/treatment group) (Zhi and Levy, 1989).

Tilson et al. (1989) used a variety of protocols shown in Table 20 to assess aspartame's proconvulsant activity in adult and developing F344 rats. No effects of aspartame at doses up to 2 g/kg bw/day were observed.

Genetically epilepsy-prone rats (GEPR) are considered a good model for testing the effects of compounds on interference with the central nervous system (CNS) because they harbor a

[27]Pentylenetetrozole is a powerful stimulant to the central nervous system; used to cause generalized convulsion in the shock treatment of emotional states and as a respiratory stimulant (*Stedman's Medical Dictionary*, 1995h).

[28]Fluorothyl is bis (2,2,2-trifluoroethyl) ether; a convulsant primarily used in experimental animals. It was formerly used to induce convultions as a alternative to electroshock therapy; http://www.online-medical-dictionary.org/omd.asp? = Fluorothyl.

[29]CD50 is the dose that induces convulsions in 50% of the animals (Pinto and Maher, 1988a).

[30]Kindling is long-lasting epileptogenic changes induced by daily subthreshold electrical brain stimulation without apparent neuronal damage (*Stedman's Medical Dictionary*, 1995d).

TABLE 20
Protocols for assessing proconvulsant activity of aspartame (Tilson et al., 1989)

Induction of convulsions	Protocol	Dose (oral, based on bw)	F344 rats	Outcome
Kindled every hour by stimulation of prepyriform cortex	Acute dose 2 h before and 6 h after stimulation	0 or 1000 mg/kg each time (Total = 2000 mg/kg); n = 15 for control, 13 for aspartame	Adult, male	No effect on rate of kindled seizures
Kindled every hour	Two doses per day for 14 days prior to stimulation	0 or 1000 mg/kg each time (Total = 2000 mg/kg/day); $n = 15$ for control, 13 for aspartame	Adult, male	No effect on rate of kindled seizures
Kindled twice a day	Two doses per day for 14 days, stimulated 2 h after every dose	0 or 1000 mg/kg each time (Total = 2000 mg/kg/day); $n = 22$ for control, 29 for aspartame	Adult, male	No effect on rate of kindled seizures, but aspartame-treated rats lost weight
Electroconvulsive shock	Acute dose 1 h before stimulation	0 or 1000 mg/kg; $n = 26$ for control, 31 for aspartame	Adult, male	No effect on induction of seizures
Pentylenetetrozole (PTZ), at doses of 35, 40, 45 or 50 mg/kg	Acute dose 1 h before stimulation	0 or 1000 mg/kg; $n = 6$ per dose group for all but 50 mg/kg PTZ + aspartame ($n = 12$)	Adult, male	No effect on occurrence or duration of seizures
Kindled twice a day starting at age 90 days	One dose daily from age 3 to 13 days	0 or 1000 mg/kg	Pups, male and female	No effect on rate of kindled seizures in adulthood
Kindled twice a day starting at age 90 days	One dose daily from age 21 to 35 days	0 or 1000 mg/kg	Pups, male and female	No effect on rate of kindled seizures in adulthood

Note. bw = body weight; h = hour.

broad spectrum of CNS noradrenergic and serotonergic deficits, and GEPR have increased sensitivity to seizures produced by a number of stimuli including sound, hyperthermia, electroshock, and chemicals (Dailey et al., 1991). GEPR from two colonies were used to assess the effect of acute and subacute oral doses of aspartame on seizure susceptibility. These were GEPR-3, which exhibit clonic[31] convulsions, and GEPR-9, which exhibit tonic[32] extensor convulsions, in response to acoustical stimuli. Measures of indicating increased susceptibility to seizures in this model include an increase in the audiogenic response score, and/or a decrease in the time from acoustic stimulation to onset of running or onset of clonic or tonic convulsions. GEPR-3 and GEPR-9 were gavaged with a single acute aspartame dose of 1, 50, 100, 250, 500, 1000, 1300, or 2000 mg/kg bw (n = 10–11 rats/dose). In the subacute study, GEPR-3 and GEPR-9 received aspartame in drinking water to achieve doses of approximately 0, 40, 85, 250, 450, and 800 mg/kg bw/day for 28 days (n = 9–10 rats/dose, except for controls where n = 20). Aspartame, in the absence of acoustic stimulation, did not induce seizures at any acute or subacute dose, and did not alter the indices described above indicative of increased susceptibility to seizure induction. Concentration of eight amino acids was measured in plasma and the midbrain to determine ratios of tyrosine, phenylalanine and tryptophan to large neutral amino acids (LNAA). Levels of aspartic acid, tyrosine and phenylalanine, and the ratio of tyrosine and phenylalanine to LNAA all increased in both GEPR-3 and GEPR-9 given acute doses of 1000 mg/kg bw or greater. Neurotransmitter levels (norepinephrine, dopamine, serotonin, and 5-hydroxyindoleacetic acid) were measured in four areas of the brains of acutely dosed rats. No aspartame-induced decrements in norepinephrine or dopamine were observed, rather norepinephrine levels were increased in GEPR that received 500 mg/kg bw aspartame or higher. Decreased levels of serotonin were observed only in the hypothalamus/thalamus region of GEPR-3 given 1000 mg/kg bw or higher aspartame doses. The authors (Dailey et al., 1991) conclude that despite substantial alterations in plasma and brain amino acids, the hypothesized consequences of changes on brain neurotransmitters and increased susceptibility to seizures by aspartame were absent even though very large doses were used in this study.

Similarly, Zhang et al. (1990) reported in a study to determine the effect of precursors on catecholamine synthesis and neurotransmission in the superior cervical ganglion of rats, that the effect of aspartame was less than hypothesized based on predicted changes in amino acids. Aspartame (dose = 306 mg/kg bw) did not cause a significant change in norepinephrine and had less effect on ganglionic transmission than tyrosine (dose = 100 mg/kg bw) in male Sprague-Dawley rats (n = 6/group) following electrical stimulation.

[31]Clonic = a rapid succession of alternating contractions and partial relaxations of a muscle occurring in some nervous diseases (Merriam Webster, 2006a).

[32]Tonic = marked by prolonged muscular contraction (Merriam Webster, 2006b).

Sperber et al. (1995) assessed the effect of prenatal exposure of aspartame on seizure induced by fluorothyl in guinea pigs. Pregnant Duncan Hartley guinea pigs (number not stated) were either untreated or gavaged with 0, 500, or 750 mg/kg bw/day aspartame from the first day of pregnancy until parturition about 65 days later. When pups were 23 days old, they were administered either no treatment, vehicle, 500 or 750 mg/kg bw/day aspartame (n = 5, 3, 6, and 6, respectively) for 1 week, then tested for the time to onset of forelimb clonus and time to onset of tonic seizures induced by exposure to fluorothyl via inhalation. There was no effect of aspartame on gestation length, litter size, birth weight, or subsequent growth of pups to age 30 days. Although the pups that received no treatment had higher seizure threshold than pups that were gavaged, there no were no differences in any measure of seizure susceptibility among pups gavaged with either vehicle or aspartame. Based on their previous studies showing that 500 mg/kg bw/day aspartame during guinea pig pregnancy resulted in impaired ability of pups to perform learning tests at day 15, the authors conclude that aspartame consumption throughout pregnancy has no effect on growth and only subtle effects on neurobehavior. However, the data and experimental protocol used in the learning study were not published (authors refer to abstract only).

Diomede et al. (1991) compared the ability of aspartame to enhance metrazol-induced seizures in two strains of mice (CD1 and DBA/2J), COBS guinea pigs and Sprague-Dawley rats. At doses up to 2000 mg/kg bw, no potentiation of seizures by aspartame was observed in either mice strain or guinea pigs. Rats given 1000 mg/kg bw had a higher incidence of metrazol-induced seizures (18/20) than rats given vehicle alone (12/20). The authors suggest that species differences in the rate of conversion of phenylalanine to tyrosine may have been responsible as at the high doses used, saturation of hepatic phenylalanine hydroxylase may occur. Additionally, as stress, hydration, disturbance in plasma osmolarity and many other factors, influence seizure susceptibility, the authors indicate that the large volumes of solvent needed to deliver high doses of aspartame, may affect seizure thresholds. This group (Guiso et al., 1991) also assessed the effect of co-administration of tyrosine on potentiation of metrazol-induced seizures in male Sprague-Dawley rats by aspartame and phenylalanine. Fasted rats were given tyrosine (0, 500, and 1000 mg/kg bw) in combination with 0 or 500 mg/kg bw phenylalanine, or 0 or 1000 mg/kg bw aspartame. As reported earlier, an acute dose of aspartame (1000 mg/kg bw) and phenylalanine (500 mg/kg bw) increased the percentage of rats having clonic-tonic seizures in response to metrazol. Tyrosine reduced potentiation of metrazol-induced seizures by large acute doses of phenylalanine and aspartame (Guiso et al., 1991).

In addition to rodents, the effect of aspartame on induction of seizures has been studied in primates. Genetically photosensitive baboons from Senegal are an excellent model for epilepsy and are highly sensitive to manipulations of brain monoamines (Meldrum et al., 1989). This model was used to test the effect of aspartame and phenylalanine on photically induced

myoclonus.[33] Four baboons (2 males, 2 females) were tested with phenylalanine doses of 0, 50, 150, and 450 mg/kg bw and four (3 females, 1 male) were tested with aspartame doses of 0, 300, or 1000 mg/kg bw in random order over a 3 month period at weekly intervals. Animals were then exposed hourly to photic stimulation and myoclonic responses were scored for 7 h. Plasma amino acid levels were evaluated during one of the three replicate dosing trials. Despite a 30-fold increase in the plasma phenylalanine to large neutral amino acid ratio following administration of 1000 mg/kg bw aspartame, there was no proconvulsant or anticonvulsant effect of any treatment in baboons with photosensitive epilepsy (Meldrum et al., 1989). Reynolds et al. (1984) reported that infant monkeys receiving 3000 mg/kg bw/day aspartame or 1650 mg/kg bw/day phenylalanine for 9 months displayed no change in EEG, seizures, or behavior.

Sze (1989) reviewed studies on aspartame and seizure susceptibility, and when studies that were published as abstracts or book chapters were included in the evaluation, studies with nine different animal models were identified. When doses of aspartame less than 1000 mg/g were administered, the studies agreed that aspartame and equimolar doses of phenylalanine are without effect on seizure susceptibility in animals. Once doses above 1000 mg/kg bw were used, the results become inconsistent and appear to be highly dependent upon the experimental protocol used (i.e., fasting, dehydration, bolus versus divided doses). Fisher (1989) reached the same conclusions in his review. He further stated that the data indicate that although rats and mice require several fold higher doses of aspartame than humans to produce similar increases in plasma phenylalanine/large neutral amino acid ratio, there is little evidence to justify use of megadoses of aspartame representing 10–40 times the recommended human allowance. Fernstrom (1994) reviewed the role of dietary amino acids and brain function and concluded that a substantial body of evidence clearly indicates that the brain is not affected by ingestion of aspartame, especially in the small amounts consumed by humans.

In summary, some studies using bolus doses of 1000 mg aspartame/kg bw have reported an enhancement of chemically-induced seizure incidence; however, this is not a consistent observation as others have reported that aspartame doses up to 2000 mg/kg bw have no effect on seizure induction. Consumption of aspartame at doses less than 1000 mg/kg bw has no effect on seizures even when administered during gestation. Therefore, as even the highest human consumption levels are more than 50 times lower than this NOAEL, it can be concluded that aspartame does not contribute to seizures in humans.

6.4.2.3. Effects on Behavior and Learning. A key question is "Are the biochemical changes observed following exposure to aspartame, such as changes in brain amino acid levels or neurotransmitters, sufficient to have a functional consequence?"

The effect of adding aspartame at levels of 0, 2, 4, or 6%, or phenylalanine at 3% of the diet through conception and postnatally on behavior was evaluated in Sprague-Dawley rats (Brunner et al., 1979). Rats were fed the diets for at least 14 days before breeding, during breeding, pregnancy and lactation, and for 90 days postnatally. Food consumption measurements determined that these diet levels of aspartame resulted in doses of approximately 0, 1.6, 3.5, and 5 g/kg bw/day during prebreeding and gestation, approximately 0, 4, 7, and 9.6 g/kg bw/day during lactation, and 0, 3, 6, and 9 g/kg bw/day postweaning. The day of age that pups acquired the reflexes of surface righting, auditory startle, forward locomotion and front limb usage was, on average, 1 day later for rats fed the highest dose of aspartame or the phenylalanine diets; this difference was statistically significant. Cliff avoidance and pivoting behavior was not altered by diet. Swimming performance was impaired at 16 days of age in rats fed diets containing either 6% aspartame or 3% phenylalanine, but improved with time and no difference in swimming performance among groups was detected at 20 days of age. Rats fed aspartame at levels at 4% or lower displayed no effect on any behavioral measures.

Similarly, no change in spontaneous behavior was observed in male Sprague-Dawley rats fed 5% aspartame in the diet for 3 weeks (Torii et al., 1986). Plasma tyrosine levels were increased in rats fed aspartame, but no effect on behavior or eating pattern was detected.

Potts et al. (1972; 1980) conducted an elaborate series of experiments to evaluate the effect of aspartame on the central nervous system in CD-1 mice and Fisher rats treated acutely with aspartame, and in rats chronically fed aspartame. The studies and results are summarized in Table 21. No biologically significant adverse effect on a variety of behavioral parameters was observed in rats or mice given acute doses of aspartame ranging from a maximum of 100 to 1000 mg/kg bw. Male rats fed 9% aspartame in the diet from weaning (day 21) for 90 days had impaired learning behavior as assessed by conditioned avoidance to electroshock; however, no significant effects on behavior were observed in rats fed 4.5% aspartame (Potts et al., 1980).

Dow-Edwards et al. (1989) reported that guinea pigs receiving 500 mg/kg bw/day aspartame throughout pregnancy produced offspring with impaired ability to perform a test of odor-associated learning at age 15 days. Pups were injected with either saline or lithium chloride and immediately placed in a chamber with vanilla scent for 30 min. Twenty-four hours later, pups were placed in a U-shaped maze divided with vanilla scent in one end and lemon scent in the other. The pup's preference for the vanilla scent was scored according to how much time it spent in each chamber. The expectation is that lithium chloride-treated pups would have learned an aversion to vanilla and have reduced preference for this scent as compared to saline-treated pups. Pups from aspartame-treated mothers did not show a significant aversion to the vanilla scent, which was observed in pups from vehicle- and nontreated mothers. The authors interpreted this as impairment of learning, and demonstration of a developmentally toxic effect of aspartame. No other measures of pups and mothers were affected by aspartame, including number of pups born, size and growth of pups, physical development, and maternal

[33] Myoclonus is clonic spasm or twitching of a muscle or group of muscles (*Stedman's Medical Dictionary*, 1995f).

TABLE 21
Summary of studies on behavior conducted by Potts and coworkers (1980)

Test	Dose (mg/kg bw)	Species and sample size	Endpoints	Result/NOEL (mg/kg bw)
Motor coordination	Acute: 0, 50, 100, and 200	Mice, $n = 50$, 20, 20, and 10, respectively	Number of mice to fall off rod rotating for 1 min	No effect. NOEL = 200
Hexabarbital potentiation	Acute: 0, 250, 500, and 1000	Mice, $n = 16$/dose group	Minutes of sleep time	No effect. NOEL = 1000
Analgesia	Acute: 0, 50, and 100	Mice, $n = 10$, 30 and 30, respectively	Time to react to thermal stimulus	In one experiment, effect at mid-dose, but not high dose. Also not seen in two replicates of experiment. NOEL = 100
Analgesia	Acute: 0, 50, and 100	Mice, $n = 10$/dose group	Time to react to tail clip	No effect. NOEL = 100
Anticonvulsant	Acute: 0, 50, and 100	Mice, $n = 50$, 20 and 10, respectively	Hind limb tonic extensor response to electroshock	No effect. NOEL = 100
Anticonvulsant	Acute: 0, 100, and 200	Mice, n = 50, 10 and 10, respectively	Clonic convulsions in response to metrazol	No effect. NOEL = 200
Antidepressant activity	Acute: 0, 25, and 200	Mice, n = 10/dose group	Ptosis[1] score	No effect. NOEL = 200
Learning behavior	Acute: 0, 50, 100, and 200	Rats, n = 12/dose group	Conditioned avoidance to electroshock	No effect. NOEL = 100
Motor activity	Chronic, 0, 4.5% or 9% aspartame or 2.5% and 5% phenylalanine in the diet for 90 days[2]	Rats, 21 days old. n = 25-35/group	Counts of activity in activity cages	Increased activity in males fed 9% aspartame, initial increased activity in females fed 2.5% phenylalanine, but effect abated over time
Learning behavior	Chronic, 0, 4.5% or 9% aspartame or 2.5% and 5% phenylalanine in the diet for 90 days[2]	Rats, 21 days old. n = 25-35/group	Conditioned avoidance to electroshock	Male rats fed 9% aspartame or 5% phenylalanine had significantly impaired responses. No differences were observed in females or males treated with lower doses. NOEL for aspartame = 4500, NOEL for phenylalanine = 2500
Learning behavior	Chronic, 0, 4.5% or 9% aspartame or 2.5% and 5% phenylalanine in the diet for 90 days[2]	Rats, 21 days old. n = 25-35/group	Nondiscriminated avoidance response to electroshock (Sidman avoidance)	No significant effects. NOEL for aspartame = 9000, NOEL for phenylalanine = 5000

[1]Ptosis = drooping of the upper eyelid; [2]equivalent to doses of 0, 4.5, and 9 g/kg/day for aspartame; 2.5 and 5 g/kg/day for phenylalanine (PAFA, 1993); bw = body weight; n = number; NOEL = no-observed-effect level.

behavior. The authors speculated that the loss of learned odor aversion was due to an imbalance in maternal and fetal amino acid profiles, but no measures of amino acid levels were reported. Limitations of this study are that behavior was tested only once with one test, only one dose was used, and that a small number of pups from aspartame-treated mothers were tested ($n = 11$ for saline, 6 for LiCl), as compared to the number of pups in other treatments ($n = 14$ for saline, 8–10 for LiCl). As a similar number of pups were born to all mothers, this cannot be attributed to lack of subjects. The authors indicated that the aspartame-treated pups were used in other tests, but none were reported (Dow-Edwards et al., 1989). These inconsistencies reduce confidence in the reported findings. The authors also indicated that subsequent studies were underway to test other doses, but none were published.

Taste aversion tests were also used to assess the effect of aspartame on behavior in a series of six experiments with Sprague-Dawley rats (Holder and Yirmiya, 1989). In contrast to the study just described, these authors used three dose levels, evaluated multiple time points, and conducted several types of behavioral tests. Rats were administered 0, 176, 352, or 704 mg/kg bw aspartame either ip or via gavage and evaluated for acquisition and extinction of taste aversion to saccharin-flavored water over 6 days. Rats developed a taste aversion following ip injections of 352 or 704 mg/kg bw, but not with 176 mg/kg bw dose or oral administration of all doses. Voluntary consumption of an aspartame-containing drink, providing approximately 400 mg/kg bw aspartame, also had no effect on conditioned taste aversions. Similar to taste aversion, high doses (352 or 704 mg/kg bw) of aspartame delivered ip affected wheel running behavior, but this effect was not observed when rats were provided the same dose of aspartame orally, either via gavage or voluntary consumption of an aspartame-containing drink (Holder and Yirmiya, 1989). Analysis of the plasma amino acid levels support the behavioral observations as ip injection of 176 mg/kg bw aspartame led to significant elevations of plasma aspartic acid, glutamate, tyrosine and phenylalanine whereas the same dose given intragastrically did not significantly alter any of the amino acid levels (Holder and Yirmiya, 1989).

Holder (1989) also evaluated the effect of perinatal exposure to aspartame on the development and memory of rat pups. Adult female Sprague-Dawley rats and their pups were given aspartame in their drinking water in concentrations of 0, 0.007%, 0.036%, 0.18%, or 0.9% w/v. An additional group was given phenylalanine in drinking water (0.45%). Adult females ($n = 10$/dose) were exposed to the aspartame and phenylalanine for 12 days, then switched to plain water during a 6-day mating period, and then back to the assigned treatment during pregnancy, parturition and lactation. Pups were then maintained on the same solutions as their mothers received. Based on water consumption, the overall exposure levels were 14.3, 67.8, 346.8, and 1614.3 mg/kg bw/day aspartame and 835.3 mg/kg bw/day phenylalanine for mothers and 32, 154, 836, and 3566 mg/kg bw/day aspartame and 1795 mg/kg bw/day phenylalanine for pups. Developmental measures in pups included body weight, time of pinnae detachment, eye opening, righting reflex, and negative geotaxis (measured as time for pup to turn around when placed head down on an inclined surface). Mothers were tested for maternal retrieval skills by placing pups around the cage and measuring time for mother to retrieve all pups to a common area. Memory skills of pups were evaluated using a radial-arm maze and a milk maze. No differences between mothers or pups drinking aspartame or phenylalanine as compared to rats drinking plain water were found in any parameters measured (Holder, 1989). The total number of pups born to mothers was not reported, but it is stated that litters were culled to remove runts and culled to a maximum of 15 pups. Additionally, the pH of the solutions of aspartame was not reported, or the temperature of the animal room, so it is difficult to assess the stability of aspartame solutions. However, solutions were refreshed three times per week.

Mullenix et al. (1991) evaluated spontaneous behavior in rats dosed orally with aspartame, phenylalanine and tyrosine. Sprague-Dawley male rats were fasted overnight then given equimolar doses of aspartame (500 or 1000 mg/kg bw), *L*-phenylalanine (281 or 562 mg/kg bw), and *L*-tyrosine (309 or 618 mg/kg bw). Each dose group included 15 pairs of rats, 15 treated and 15 controls. In addition, rats treated with 1.0 or 0.5 mg/kg bw *d*-amphetamine were included as positive controls. One hour after treatment, the spontaneous behavior of each rat was quantitatively assessed for 15 min using a computerized pattern recognition system. After behavior testing, blood was collected for amino acid analysis. Plasma phenylalanine and tyrosine levels were increased approximately 5- and 10-fold, respectively, in rats treated with the highest doses of either aspartame or phenylalanine. Only tyrosine plasma levels were increased in rats treated with tyrosine. Despite these documented changes in plasma amino acid levels and exposure to very high doses, there was no change in the spontaneous behavior of rats treated with aspartame, phenylalanine, and tyrosine, whereas behavior measures were significantly increased in rats treated with *d*-amphetamine.

Tilson et al. (1991) conducted a series of experiments to assess the effect of aspartame on behavior and learning (Table 22). Acute and repeated intragastric doses of aspartame up to 2000 mg/kg bw /day had no effect in male Fisher F344 rats ($n = 8$ rats/dose group) on motor activity, acoustic startle reflex, or memory tests. NOAELs were at the highest levels tested (1000 to 2000 mg/kg bw).

The effect of combining aspartame with ethanol on behavior was evaluated in male CD-1 mice (LaBuda and Hale, 2000). Mice were injected ip with 1000 or 2000 mg aspartame/kg bw followed by an ip injection of saline or saline plus 1.6 g/kg bw ethanol. Behavior was evaluated in an open field and an elevated plus-maze designed to evaluate anxiety. Neither aspartame alone nor in combination with ethanol affected anxiety-related behaviors.

Recently, Christian et al. (2004) used a T-maze to test for memory loss in male Sprague-Dawley rats receiving aspartame

TABLE 22
Summary of experiments on effect of aspartame on behavior and learning by Tilson and coworkers (1991)

Protocol	Intragastric dose of aspartame (mg/kg bw)	Test	Result
Motor activity			
Acute—not fasted	0, 250, 500, 1000, 2000	Spontaneous motor activity	No effect on activity
Acute—fasted 24 h	0, 1000 (also tested following ip injection)	Spontaneous motor activity	No effect on spontaneous activity
Short term –14 days	0, Sham, 1000 per day	Motor activity	No effect on spontaneous activity
Acute—not fasted	0, Sham, 1000	Motor activity in rats treated with colchicine-induced hippocampal lesions	No effect on induced hyperactivity
Acute—not fasted	0, 1000	Motor activity in rats treated with *d*-amphetamine sulfate	No interaction between aspartame and stimulant
Reflex response			
Acute—not fasted	0, 250, 500, 1000	Acoustic startle reflex	No effect on reactivity
Short term –14 days	0, Sham, 1000 per day	Acoustic startle reflex	No effect on reactivity
Acute—not fasted	0, 500, 1000	Prepulse inhibition of startle response	No effect on prepulse inhibition
Short term –14 days	0, 1000 per day	Prepulse inhibition of startle response	No effect on prepulse inhibition
Memory			
Acute—not fasted	0, 500, 1000	Passive avoidance predose, train, retest	No effect on acquisition or retention of avoidance
Acute—not fasted	0, 500, 1000	Passive avoidance postdose, train, retest	No effect on acquisition or retention of avoidance
Acute—not fasted	0, 500, 1000	Two-way shuttle	No effect on avoidance or crossings in shuttle
Short term—14 days	0, Sham, 1000 per day	Two-way shuttle	No effect on avoidance or crossings in shuttle
Short term—14 days	0, 1000 per day	Morris water maze	No effect on time to escape

(250 mg/kg bw/day) in drinking water for 120 days. Twelve rats per group were tested periodically during the treatment period for the time required to find a reward (chocolate) in the T-maze. Rats were tested every 2 weeks. Although no differences were noted during the measurements occurring approximately every 2 weeks up to about 72 weeks, rats receiving aspartame took significantly longer to find the reward after 90 days. The next measurement shown is at 120 days, when rats again took longer to find the reward. Rats were then killed and levels of brain cholinergic receptors and NaK-ATPase levels were determined. Muscarinic cholinergic receptor density, measured using radiolabeled quinuclindinyl benzilate, was significantly higher in several areas of the brain of rats receiving aspartame compared to rats receiving only water. NaK-ATPase levels were similar in all areas of the brain, except for the midbrain where levels were higher in aspartame-treated rats. The authors (Christian et al., 2004) speculate that the increase in time to find a reward in the T-maze indicates that chronic aspartame consumption results in memory loss and this was mediated by the change in cholinergic receptor density. However, they also acknowledge that the relationship between memory and receptor density is not well understood. The authors suggest that an alternative explanation for the increased time in the T-maze test is that rats treated with aspartame may have a reduced desire for the chocolate reward. As rats do not recognize aspartame as a sweet substance, it is not clear how this may occur. One possibility is that as aspartame is also used to modify the flavors of other substances (when given below the sweetness threshold in humans), it is possible that while the rats might not taste aspartame as sweet, aspartame could have modified the taste of the chocolate (e.g., making it more bitter). Major limitations of this study are the use of only one tool or task to measure memory and the use of only one dose of aspartame.

The majority of studies designed to detect an effect of aspartame on learning or memory have used multiple doses and multiple tests to evaluate these parameters. These studies report no effect, at doses as high as 4% aspartame in the diet, even when exposed from conception to 90 days postnatally. Higher doses have been reported to affect various indices of learning behavior. There are two studies that, when using only one dose

and only one measure of learning, interpreted their findings to indicate an impairment of learning by aspartame at doses of 250 and 500 mg/kg/day. In summary, well-designed studies using a range of approaches to evaluate learning and memory consistently demonstrate no effect of aspartame consumption at levels up to 4000 mg/kg/day, indicating little likelihood that aspartame will have an effect on memory or learning in humans.

6.4.2.4. Effects on Aggression. Male Long-Evans rats received single intraperitoneal injections of 0, 200, 400, and 800 mg/kg bw aspartame in vehicle (saline plus Tween 80) and then were exposed to an intruder rat and observed for aggression (Goerss et al., 2000). Eleven rats were tested with all aspartame doses delivered in ascending then descending order with a minimum of 1 day between tests. Contrary to an expected enhancement of aggressive behavior based on the hypothesis that aspartame is neuroexcitatory, when rats received aspartame doses of 200 mg/kg bw or higher, the time to attack an intruder rat increased, the number of bites decreased, and fewer rats engaged in aggressive attacks. Rats treated with 200 and 400 mg/kg bw aspartame had higher levels of serotonin (5-hydroxytryptamine) and its metabolite, 5-hydroxyindolacetic acid, in the brain compared to rats receiving vehicle, but this effect was not evident in rats treated with 800 mg/kg bw aspartame. Dopamine and its metabolites, dihydroxyphenylacetic acid and homovanillic acid, were not affected by aspartame treatments. The authors (Goerss et al., 2000) were unable to explain how aspartame administration would result in increased serotonin levels in these groups. The rationale for intraperitoneal administration of aspartame was not given. An intraperitoneal exposure is an irrelevant route of administration when aspartame is extensively metabolized prior to absorption, and the only human exposure is via oral administration.

In summary, there has been extensive investigation of the possibility of neurotoxic effects due to consumption of aspartame. The data from these studies, in general, do not support the hypothesis that aspartame in the human diet will affect neuronal function, learning or behavior. Lajtha et al. (1994) reviewed the effect of aspartame on neural function, and suggest that the lack of changes in neural function in response to changes in plasma and brain amino acid levels, following even large doses of aspartame, is a reflection of the protection of brain function from the great nutritional variation that is present in the diversity of human diets and dietary intakes.

6.5. Teratogenesis and Reproductive Studies

Reproductive studies were conducted by Searle on aspartame and submitted to the Food and Drug Administration and World Health Organization for a food additive petition and safety evaluation. These studies were reviewed by JECFA (1980a) and are summarized in Table 23.

In addition, there are several studies published in the peer-reviewed literature, which are summarized next.

The effect of aspartame on reproduction was evaluated in the study by Brunner et al. (1979) described earlier in

TABLE 23
Unpublished teratogenic and reproductive studies with aspartame cited in (JECFA, 1980a)

Species	Dosage of aspartame and protocol	Results
Chick embryos	0.25 mg ASP/egg, injected into yolk sac, hatched chicks visually evaluated, then necropsied 23 days postinjection	No effect on mortality. No abnormalities observed.
Rats, Charles River, $n = 30$ females/dose, 48 control females, 12 males/dose	2000 or 4000 mg ASP/kg bw/day in the diet during premating, gestation and lactation, by gavage during mating. Pups sacrificed after 21 days.	No effect on parental survival, mating, fertility or paternal weight gain. Maternal weight gain was lower in midgestation in high dose group. No adverse effects.
Rats, strain unknown; $n = 12$ males and 24 females/dose	2000 or 4000 mg ASP/kg bw/day; two-generation study	No effect on fertility, gestation, litter size or survival, behavior, and gross necropsy. Growth of pups was unaffected at low dose, but lower at high dose.
Rats, $n = 24$ females/dose	2000 or 4000 mg ASP/kg bw/day during gestation days 6–15	No effect on resorption, litter size, viability, growth and development.
Rabbits, New Zealand, 3 studies	1000, 2000 or 3000 mg/kg bw/day intragastric, 3:1 ASP:DKP during days 6–18 of gestation	No effect on survival, abortion, premature delivery, or malformations. In one study, conception rate was lower in treated animals compared to controls, but not a consistent finding.
Rabbits, 3 studies	3.28 or 6.08% ASP in the diet during days 6–18 of gestation	No major compound-related embryotoxic or teratogenic effect.

Note. ASP = aspartame; bw = body weight; DKP = diketopiperazine.

Section 7.4.2.3. Sprague-Dawley Rats were fed the diets containing aspartame at levels of 0, 2, 4, or 6% for at least 14 days before breeding, during breeding, pregnancy and lactation, and for 90 days postnatally. Phenylalanine added at a level of 3% was also tested. Food consumption measurements determined that these dietary levels of aspartame resulted in doses of approximately 0, 1.6, 3.5, and 5 g/kg bw/day during prebreeding and gestation, approximately 0, 4, 7, and 9.6 g/kg bw/day during lactation, and 0, 3, 6, and 9 g/kg bw/day postweaning. Breeding pairs were enrolled in the study until 18 litters were available for each group. The number of animals used for various measures and parameters varies; therefore, the total number of animals used in the study is not known. There was no effect on body weights during prebreeding or in maternal weight gain during gestation, but rats fed 6% aspartame during lactation lost more weight than other dietary groups. Increased offspring mortality was observed in rats fed the 6% aspartame and 3% phenylalanine diets. Gestation length and litter size was not affected by diet, however pups fed aspartame weighed significantly less than controls by 30 days and remained lighter throughout the study (Brunner et al., 1979). Eye opening was delayed by 1 day in pups in the 6% aspartame and 3% phenylalanine diet groups, but timing of pinnae detachment and incisor eruption were not affected. Similar findings of no effect of maternal exposure to aspartame up to doses of 1614 mg/kg bw/day, and pup exposure of 3566 mg/kg bw/day on pup development were reported by Holder (1989). The details of this experiment were described earlier in Section 7.4.2.3.

To assess potential postcoital antifertility effects of aspartame, groups of five female Charles River rats were administered 300 mg/kg bw/day aspartame in corn oil or corn oil alone intragastrically for 7 days following mating (Lennon et al., 1980). A similar experiment was conducted in female hamsters with a group of 15 treated with corn oil only, and a group of 5 given daily doses of 300 mg/kg bw aspartame in corn oil (Lennon et al., 1980). There were no differences noted in the number of rats that became pregnant, or the implantation and regression of corpora lutea in hamsters. However, the number of animals used in these studies was very small.

Lennon et al. (1980) conducted a larger experiment with 60 female Charles River rats to assess the effect of adding aspartame to the diet on lactation. After mating and delivery of pups, rats were allocated in body weight matched pairs to treatment groups for a pair-feeding experiment. Groups of 6 rats were fed diets containing 1, 2, 4, 7.5, or 14% by weight aspartame for 21 days. Pair-matched controls were fed the same amount of food as their match consumed ad libitum the previous day, but without added aspartame. This allowed for evaluation of the effect of addition of aspartame to the diet, but removed a possible effect of reduced food consumption. Dam and pup body weights and pup survival were measured. At the end of the 21-day lactation period, mammary glands were removed from dams for histological evaluation. Food consumption and body weights were significantly lower in dams fed 7.5 and 14% aspartame diets on day 1 and throughout the experiment. Similar body weight losses were observed in pair-fed controls indicating that this effect was due to the lower consumption of the diet. Pup body weight was also significantly reduced in these diet groups. Pup survival was significantly reduced in the highest dose group and pair-matched controls. Dams fed the highest dose of aspartame and pair-matched controls had a higher incidence of resting or inactive mammary glands, which were likely due to lack of suckling pups and severe food restriction. There was no adverse effect of adding aspartame to the diet at levels of 1, 2, or 4% on food consumption, dam and pup body weight, pup survival, and mammary gland histology. Based on measured food consumption, the actual doses of aspartame in this study in groups consuming diets with 1, 2, 4, 7.5, or 14% by weight aspartame were 1.87, 3.68, 7.12, 9.11, and 8.83 g/kg/day, respectively (Lennon et al., 1980). Therefore, the NOEL in this study was 7120 mg/kg bw/day.

The effect of aspartame added to feed during gestation in rabbits was assessed (Ranney et al., 1975). Thirty mature New Zealand female rabbits were inseminated, and starting on day 6 of pregnancy were fed either the control rabbit diet or the diet containing 6% aspartame (15/group) until autopsy on day 20. Fetal and maternal tissues were collected. The effect on amino acid levels in tissues was discussed previously (Section 7.4.2.3). No effect on maternal body weight, litter size or fetal weights was observed. As the ratios of fetal/maternal plasma amino acids were unchanged, the authors concluded that aspartame had no effect on the transport of phenylalanine and tyrosine across the placental membrane. Liver phenylalanine hydroxylase activity, which converts phenylalanine to tyrosine, was not affected by treatment. Based on food consumption measurements, the dose and NOEL in this study were 1600 mg/kg bw/day.

Lederer et al. (1985) conducted a study in which female Wistar rats were mated and then fed a commercial rat diet containing 10% aspartame ($n = 14$), 3% DKP ($n = 10$), 1.0% DKP ($n = 15$), 0.3% DKP ($n = 18$), or the diet only ($n = 27$) for the next 20 days of gestation. Rats were then killed and embryonic development evaluated. There was no effect of 10% aspartame in the diet on maternal weight gain, number of implantations or fetal resorptions. Rats fed the high levels of DKP had reduced number of implantations and lower fetal weight. No effects on pregnancy outcomes were observed in rats fed 0.3% DKP. The evaluation of teratogenic effects was focused on ocular lesions, as was previously reported by these authors (Colson et al., 1984) for evaluation of the teratogenic effects of saccharin. Lesions evaluated included cataracts, retinal coloboma,[34] retinal dysplasia, major microphthalmos,[35] anophthalmos,[36] aberrant nerve fibers, and anarchic globes.[37] The incidence and severity of ocular lesions observed in embryos were tabulated to establish a

[34]Coloboma: Any defect, congenital, pathologic, or artificial, especially of the eye (*Stedman's Medical Dictionary*, 1995a).

[35]Microphthalmos: Abnormal smallness of one or both eyeballs.

[36]Anophthalmos: Congenital absence of all tissues of the eyes.

[37]Anarchic globes: Globes of the eye lacking order, regularity, or definiteness.

teratology index for each litter. The teratology indices of litters of rats fed 10% aspartame or 0.3% DKP were similar to controls, but were higher in litters of rats fed 1.0% and 3.0% DKP. Details on the specific lesions that contributed to the teratology indices were not provided. The authors conclude that the presence of 3% DKP in aspartame, which would result in 0.3% DKP in a diet containing 10% aspartame, is an acceptable level. One problem with this study is that in several places, the dietary content of aspartame is listed as 1% instead of the 10%, creating some concern regarding which is correct. Based on the doses of DKP tested, one would predict the 10% aspartame concentration is correct, as it would not be logical to test a higher concentration of a breakdown product than of aspartame itself. Attempts to verify the dose used by looking for published corrections and contacting the authors were unsuccessful. The reproductive toxicity and teratogenic potential of DKP was also evaluated (reviewed in Kotsonis and Hjelle, 1996). The NOAEL of these studies ranged from 1000 to 2500 mg/kg bw/day. Similarily, the reproductive toxicity and teratogenic potential of β-aspartame have been evaluated in rats and rabbits. No teratogenic, embryotoxic, or fetotoxic effects were observed, with the highest dose tested being 750 mg/kg bw/day (Kotsonis and Hjelle, 1996).

Aspartame was evaluated for possible estrogenic, androgenic, progestational, and glucocorticoid activities (Saunders et al., 1980). Doses of 300 mg/kg bw/day (number of days not defined) given orally to female SCH: ARS(ICR)*f* mice did not affect mouse uterine weight in either an estrogenic or estrogen antagonism assay. There was no evidence of progesterone-like activity or progesterone antagonism in female New Zealand rabbits dosed with 300 mg/kg bw/day for 5 days. Castrated male Sprague-Dawley rats administered 300 mg/kg bw/day for 7 days did not display evidence of androgen activity by aspartame, or when treated with testosterone, evidence of androgen antagonism of aspartame. Adrenalectomized male Sprague-Dawley rats dosed with 300 mg/kg bw did not have increased liver glycogen, indicating aspartame does not have cortisone-like activity. One ovary was removed from female Sprague-Dawley rats, and then rats were dosed with vehicle or aspartame (300 mg/kg bw/day) for 14 days. The finding that the weight of the remaining ovary was similar in both groups, was interpreted as evidence of lack of an effect of aspartame on pituitary regulation (Saunders et al., 1980).

The estrogenic activity of more than 90 chemicals, including aspartame, was examined using the estrogen receptor-dependent breast cancer cell line, MCF-7 (Okubo and Kano, 2003). No evidence of estrogenic activity was observed with aspartame at concentrations up to 1000 μg/ml.

In summary, the effect of aspartame during reproduction, development and lactation has been evaluated in rats, mice, hamsters and rabbits. No-effect levels of exposure during reproduction and gestation have been reported to range from 1600 mg/kg bw/day in rabbits to 4000 mg aspartame/kg bw/day in rodents. Adverse effects on pup development were observed in rat studies when doses exceeded 5000 mg/kg bw/day during reproduction and gestation. Consumption of up to 7000 mg aspartame/kg bw/day during lactation had no effect on pup development or maternal health, but higher doses affected body weights. Studies evaluating the estrogenic potential of aspartame have consistently been negative. Therefore, aspartame is considered to have no reproductive or teratogenic activity, and no effect on lactation. In these studies, effects have been observed at exceedingly high doses, and were secondary to reduced body weights.

6.6. Genotoxicity

6.6.1. In Vitro Studies

The genotoxic potential of aspartame has been extensively evaluated in microbial, cell culture and animal models. A summary of these studies is provided in Table 24. In general, aspartame is nongenotoxic, even at high concentrations, and at doses several orders of magnitude higher than those attained through the consumption of aspartame in foods. This conclusion was recently reaffirmed in a review of current data on aspartame safety by the EFSA (2006).

In the Ames/*Salmonella typhimurium* reversion assay, aspartame was found to be nonmutagenic at concentrations up to 5000 μg/plate in tester strains TA98, TA100, TA1535, TA1537, and TA1538 (Molinary, 1978). This was in both the presence and absence of metabolic activation with rat hepatic S9 fraction. A later study by Rencuzogullari et al. (2004) also reported an absence of genotoxicity in *S. typhimurium* strain TA98 at a maximum concentration of 2000 μg/plate. However, these authors reported a small, but statistically significant, increase in the mutations in strain TA100 in the absence of metabolic activation, although the effect was not concentration-dependent. Sheppard et al. (1991, 1993) reported that aspartame was mutagenic in *S. typhimurium* strains TA98 and TA100 following its nitrosation. However, the mutagenicity was positively correlated to the extent of its nitrosation, but this effect was decreased by the incubation of the nitrosated aspartame with rat hepatic S9 enzymes. In this regard, aspartame does not differ from other nitrosatable peptides.

Phenylalanine, a metabolite of aspartame, was found to be mutagenic to *Escherichia coli* K012 *uvrB*, but only at 2 m*M* concentrations (Sargentini and Smith, 1986). In contrast, phenylalanine was nonmutagenic in the wild type *E. coli* strains *uvrB*, *uvrB umuC*, and*uvrB lexA*, suggesting that this amino acid may cause the production of excisable mutations in DNA.

Gebara et al. (2003) reported that aspartame induced somatic segregation in diploid cells of *Aspergillus nidulans*, although this effect was not concentration-dependent. The maximal effect occurred at concentrations of 0.8 and 1.0 mg/ml, with higher concentrations showing significantly reduced segregations as compared to untreated controls.

Aspartame was reported to induce chromosomal aberrations in isolated peripheral human blood lymphocytes at concentrations of 500–2000 μg/ml (Rencuzogullari et al., 2004). However, this effect was not concentration dependent and was less than

TABLE 24
Summary of genotoxicity studies

Test organism	Strains tested	Concentration/dose	Results	Reference
S. typhimurium	TA98, TA100, TA1535, TA1537, TA1538	10–5000 μg/plate	Negative	Molinary (1978)
S. typhimurium	TA98, TA100, TA1535, TA1537, TA1538	10–5000 μg/plate	Negative	Molinary (1978)
S. typhimurium	TA98 and TA100	1–40 m*M* nitrosated aspartame	Positive without S9, negative with S9	Sheppard et al. (1991; 1993)
S. typhimurium	TA98	50 – 2000 μg/plate	Negative	Rencuzogullari et al. (2004)
S. typhimurium	TA100	50–2000 μg/plate	Weak positive	Rencuzogullari et al. (2004)
E. coli	*uvrB*, *uvrB umuC*,*uvrB lexA* and K012 *uvrB*	2 m*M* phenylalanine	Positive in K012 *uvrB*	Sargentini and Smith (1986)
A. nidulans	Diploid UT448/UT196	0.1–12.0 mg/ml	Positive but not dose-dependent	Gebara et al. (2003)
Human lymphocytes		50–2000 μg/ml	Induced chromosomal aberrations, but not conc-dependently	Rencuzogullari et al. (2004)
Human lymphocytes		50–2000 μg/ml	Induced micronucleus formation at high conc	Rencuzogullari et al. (2004)
Human lymphocytes		50–2000 μg/ml	No change in sister chromatid exchanges. Decreased mitotic index	Rencuzogullari et al. (2004)
Primary rat hepatocyte cultures	Sprague-Dawley	0, 5 or 10 m*M*	Negative	Jeffrey and Williams (2000)
Mice	C57B1/6	0, 40 and 400 mg/kg bw	Nonclastogenic	Durnev et al. (1995)
Mice	Swiss albino male	3.5, 35, 350 mg/kg aspartame plus 1.5, 1.5, 150 mg/kg bw acesulfame-K	Nonclastogenic	Mukhopadhyay et al. (2000)
Mice	Male ddY mice	2000 mg/kg bw	Negative in Comet assay	Saski et al. (2002)
Mice		0, 1000, 2000, 4000 and 8000 mg/kg bw/day aspartame for 5 days	Negative in host-mediated assay	Searle report to FDA [#E81]
Mice		0, 1000, 2000, 4000 and 8000 mg/kg bw/day DKP for 5 days	Negative in host-mediated assay	Searle report to FDA [#E82]
Rats	Male Holtzman	0, 400, 800, 1200, 1600 mg/kg bw/day for 5 days	Negative in in vivo cytogenetics	Bowles (1970)
Rats		0, 500, 1000, 2000, 4000 mg/kg bw/day for 5 days	Negative in in vivo cytogenetics	Searle report to FDA [#700-266]
Rats	CR-CD male	0 or 2000 mg/kg bw	Negative in dominant lethal assay	Schroeder (1973)
Rats		0, 500, 1000, 2000, and 4000 mg/kg bw/day for 5	Negative in host-mediated assay	Searle report to FDA [#700-268]

Note. bw = body weight; DKP = 3-carboxymethyl-6-benzyl-2,5-diketopiperazine; FDA = Food and Drug Administration.

the number of chromosomal aberrations induced by mitomycin-C. Similarly, aspartame induced micronucleus formation at the highest concentration; however, there was no increase in the incidence of sister chromatid exchanges in these cells. The replication index of the cells was not affected by aspartame exposure, but it did decrease the mitotic index of the lymphocytes. This effect was concentration dependent.

There was no evidence of DNA-damaging activity by aspartame in primary rat hepatocyte cultures (Jeffrey and Williams, 2000). Cells were derived from Sprague-Dawley rats and incubated with 5 or 10 m*M* aspartame for 20 h prior to assessment of DNA repair.

In conclusion, there is no evidence to indicate that aspartame will induce genotoxic effects under condiditons that are in any way relevant to consumption under typical conditions of use in food. To the contrary, the evidence indicates that aspartame is nongenotoxic, even at high concentrations, and at doses several orders of magnitude higher than those attained through the consumption of aspartame in foods. This conclusion was recently reaffirmed in a review of current data on aspartame safety by the EFSA (2006).

6.6.2. In Vivo Studies

Using an in vivo cytogenetics assay, Bowles (1970) reported no effect of oral doses of aspartame on the chromosomes of bone marrow or spermatogonial cells of male Holtzman rats treated with 0, 400, 800, 1200, or 1600 mg/kg bw/day for 5 days ($n = 10$ rats/dose). In a second study, rats were treated with doses of 0, 500, 1000, 2000, and 4000 mg/kg bw/day for 5 days. Normal aberrations frequencies were observed in bone marrow cells from all groups.

Similarly, no evidence of mutagenic potential of aspartame was found using a host-mediated assay in male albino rats administered aspartame orally for 5 days at dose levels of 0, 500, 1000, 2000, and 4000 mg/kg bw/day [report 700-268, 1972]. Rats were then inoculated with an intraperitoneal injection of *S. typhimurium*, and mutation frequency evaluated 3 h later. The same assay conducted in mice treated with 0, 1000, 2000, 4000, and 8000 mg/kg bw/day aspartame (Searle E81) and DKP (Searle E82) for 5 days also reported no evidence of mutagenic potential.

A dominant lethal assay was conducted with CR-CD male rats orally administered 2000 mg/kg bw aspartame on 1 day (Schroeder, 1973). Each rat was then mated with two female rats per week for 8 weeks. Paternal growth, maternal pregnancy rate, uterine and ovarian examination data and fetal death incidence were reported. Fertility was reduced in males in the fourth week. The number of corpora lutea in females was lower during the third week of mating, but higher during the eighth week of mating to aspartame-treated males, and unchanged all other weeks. The pregnancy rate, number of implantations per female and fetal death rate were unaffected by aspartame (Schroeder, 1973).

A Russian study (Durnev et al., 1995) found no evidence of clastogenic activity when C57B1/6 mice were dosed orally with aspartame (40 and 400 mg/kg bw), cyclamate (11 and 110 mg/kg bw), saccharin (5 and 50 mg/kg bw), acesulfame (15 and 150 mg/kg bw), or sucralose (15 and 150 mg/kg bw) for 5 days.

The combination of aspartame and acesulfame-K was not genotoxic in vivo (Mukhopadhyay et al., 2000). Male Swiss albino mice ($n = 5$/group) were dosed intragastrically with: (1) 3.5 mg/kg bw aspartame plus 1.5 mg/kg bw acesulfame-K, (2) 35 mg/kg bw aspartame plus 15 mg/kg bw acesulfame-K, (3) 350 mg/kg bw aspartame plus 150 mg/kg bw acesulfame-K or (4) vehicle. There were no differences among groups in chromosomal aberrations in bone marrow cells isolated from the femora of mice 18 h after dosing, indicating the combination of aspartame and acesulfame-K is nonclastogenic.

Sasaki et al. (2002) assessed the genotoxicity of 39 food additives using the comet assay of tissues collected from male ddY mice administered 1 oral dose at up to one half of the LD_{50} or 2000 mg/kg bw. For aspartame, a dose of 2000 mg/kg bw was used and mice ($n = 4$/group) were killed after 3 and 24 h. Nuclei were obtained from glandular stomach, colon, liver, kidney, urinary bladder, lung, brain, and bone marrow. No changes were found in the comet lengths of nuclear DNA from tissues from aspartame-treated mice. Thus, aspartame was found to be nongenotoxic in this assay.

Extensive in vitro and in vivo studies provide ample evidence that aspartame is nongenotoxic.

6.7. Additional Studies

6.7.1. Immunological Effects

Aspinall et al. (1980) evaluated the effect of aspartame on inflammatory responses. Male Sprague-Dawley rats were dosed intragastrically with 300 mg/kg bw aspartame or 80 mg/kg bw hydrocortisone ($n = 8$) and injected 1 h later with carrageenan to induce swelling in the paw. There was no difference in swelling between rats treated with aspartame or vehicle, but rats treated with hydrocortisone had significantly reduced swelling. Similarly, in the same study, 325 mg/kg bw aspartame had no effect on the amount of granuloma tissue induced by subcutaneously implanted cotton pellets or the degree of arthritic swelling induced by intradermal injection of killed *Mycobacterium butyricum* in rats. Thus, aspartame did not impair inflammatory responses.

Anti-inflammatory and analgesic effects of aspartame in Wistar rats and Laca mice given oral doses of up to 16 mg/kg bw have been recently reported (Sharma et al., 2005); however, the experimental protocol was not well described, the number of animals used was not reported, and no evidence for a possible mechanism was provided. Therefore, the significance of this report cannot be assessed.

6.7.2. Effect on Body Weight

Induction of neuronal necrosis in newborn mice with subcutanenous injections of monosodium glutamate (0.5 g/kg bw) was followed by marked obesity in later life (Olney, 1969). This report rasied the question of whether exposure to neuroexcitory

compounds may be associated with altered body mass homeostasis and obesity. Therefore, the possible effect of aspartame on development of obesity was considered in this safety evaluation.

Rockhold et al. (1990) used the excitotoxin, *N*-methyl-D-aspartic acid (NDMA), to induce lesions in the paraventricular hypothalamus and assessed effects on cardiovascular function, blood pressure, and body weight changes. Male Sprague-Dawley rats were given bilateral injections of NDMA (12.6 nmol/injection) ($n = 12$) or sham injections ($n = 17$) into the paraventricular hypothalamus. Body weight, food and water consumption, and urine output of rats were monitored for 26 days. Rats injected with NDMA exhibited a significant reduction in food intake and body weight gain.

Chronic studies with feeding of aspartame in animals have not provided support for a possible role of aspartame in obesity as the more commonly seen change in weight is a reduction in food consumption and/or weight gain by animals fed high doses of aspartame. This question is addressed again in Section 6.9.2.6 on human observations.

6.7.3. Cytotoxicity

To assess the effect of the peak blood methanol concentrations of 2 mg/dl reported in adults consuming abusive amounts of aspartame (Stegink et al., 1981a), rat thymocytes were incubated with formaldehyde and assessed for cell shrinkage using flow cytometry as an indicator of early apoptosis and cytotoxicity. No effect was observed on thymocytes incubated with 10 or 30 μM (3 or 9 μg/ml) formaldehyde for 24 h. At higher concentrations (100 and 300 μM, or 30 and 90 μg/ml), after incubation for 24 h with formaldehyde increased cell shrinkage was observed (Nakao et al., 2003). However, as formaldehyde is rapidly metabolized to formic acid, and these levels are not found in the blood following exposure to aspartame, these findings are not relevant to the safety of orally ingested aspartame.

Wang (1980) incubated two mouse cell lines with saccharin, cyclamate and aspartame to determine if these compounds would induce cells to release RNA C-type viruses. The cell lines were Kbalb II, a Kirsten sarcoma virus-transformed Babl/3T3 cell line and JLS V9, a fibroblastic cell line derived from BALB/*c* mice. Both cell lines readily release RNA C-type viruses into culture fluid after exposure to a variety of compounds, including amino acid analogs. Incubation of both cell lines with 50 μg/ml of the sweeteners added to culture media for 24 h resulted in release of viruses, which were characterized as RNA C-type particles. Incubation with sucrose or xylitol resulted in similar viral particle release as observed in vehicle controls (Wang, 1980).

6.7.4. Bacterial Studies

The abilities of 13 species of oral anaerobic bacteria to grow in the presence of aspartame were evaluated to assess the potential effect of consuming aspartame on the diversity and relative levels of various microbial strains in the oral cavity (Wyss, 1993). All but one strain (*Eubacterium timidum*) was able to grow in the presence of 1 m*M* aspartame, when aspartic acid and phenylalanine were eliminated from growth media, including four species auxotrophic for phenylalanine (*Capnocytophaga, Fusobacteriu, Porphyromonas, Treponema)* (Wyss, 1993).

6.8. Intentional Omissions

The use of aspartame for relief and/or treatment of chronic inflammation, arthritis (LaBuda and Fuchs, 2001), hypertension (Maher and Wurtman, 1983), and the prevention of ochratoxin A toxicity (Creppy et al., 1998; Galvano et al., 2001) has been reported. Discussion of these studies was not included in this report as they are not relevant to the evaluation of safety of aspartame.

6.9. Observations in Humans

6.9.1. Human Case Studies

6.9.1.1. Allergic Reactions. Kulczycki (1986) reported two cases of adult females developing urticaria[38] following consumption of aspartame-containing beverages. In one case, a challenge with aspartame resulted in urticaria on arms and neck within 1 h. As it is unlikely that the metabolites of aspartame (aspartic acid, phenylalanine and methanol) are antigenic, the DKP degradation product of aspartame was suspected, but not confirmed, as the causative agent (Kulczycki, 1986).

There has been one case report of lobular panniculitis[39] in diabetic individual consuming large quantities of aspartame (McCauliffe and Poitras, 1991). The subject had recently begun subcutaneous injections of insulin, and was taking five additional medications. He had begun consuming six to seven servings of aspartame as well as diet sodas. He discontinued use of aspartame-containing products, and symptoms subsided. Following a subsequent challenge with pure aspartame, he developed another panniculitis nodule and then completely avoided aspartame products. As pointed out by Geha (1992), many questions are not addressed in this single subject case, such as other changes in diet, the possibility that the panniculitis was a cyclical event or simply a chance occurrence. These same issues surround all single case reports, and highlight the need for well-controlled clinical studies.

Numerous reports of allergic reactions to aspartame to the Centers for Disease Control prompted two double-blind placebo-controlled studies on hypersensitivity to aspartame (Garriga et al., 1991; Geha et al., 1993). Both studies recruited patients that believed they had previously experienced an allergic adverse reaction to aspartame, and conducted double-blind

[38] Urticaria: An allergic disorder marked by raised edematous patches of skin or mucous membrane and usually intense itching and caused by contact with a specific precipitating factor (as a food, drug, or inhalant) either externally or internally (*Stedman's Medical Dictionary*, 1995o).

[39] Panniculitis: Inflammation of subcutaneous adipose tissue (*Stedman's Medical Dictionary*, 1995g).

challenges with aspartame under controlled conditions. No difference was observed in the incidence of hypersensitivity reactions following challenges with placebo or aspartame. However, both studies reported difficulty in recruiting subjects despite recruiting for nearly 3 (Garriga et al., 1991) or 4 years (Geha et al., 1993), so the number of subjects tested was 12 (Garriga et al., 1991) and 21 (Geha et al., 1993). Kulczycki (1995) suggested that the method of subject recruitment, inadequate protection of subject safety, and the challenge design of the study conducted by Geha et al. (1993) may have been flawed and the cause of low recruitment and response rates.

Studies conducted in patients who believed themselves to have an allergic reaction to aspartame did not reveal any differences in incidences of hypersensitivity reactions when exposed to aspartame as compared to exposure to a placebo, although there were some limitations in experimental design.

6.9.1.2. Neurotoxic Reactions. There are numerous allegations on the Internet and consumer magazines of neurotoxicity of aspartame, including seizures, memory loss, and headache. Reviewed here are peer-reviewed studies, book chapters, and articles or comments published in scientific journals.

Tollefson et al. (1988, 1988, 1992) stated that by 1988, the FDA had received 3326 consumer complaints that were reported to the FDA adverse reaction monitoring system, G. D. Searle, or the Centers for Disease Control describing adverse reactions allegedly due to consumption of aspartame. Of these, the majority was by women (77%) between the ages of 20 and 59 (76%) and the most common complaint was headache. However, the authors concluded that the information provided to the FDA did not establish reasonable evidence that the consumption of aspartame was associated with a consistent or unique pattern of symptoms that can be causally linked to its use. The authors further stated that the information from the consumer complaints are difficult to assess because they are accepted without documentation, are anecdotal and not accompanied by complete medical records.

However, others (Johns, 1988) viewed the data from the adverse reaction monitoring system as consistent with the thesis that there is a susceptible subset of the population in which aspartame, like chocolate or red wine, provokes "chemical" headaches. The author indicated that pathophysiology of chemical headaches is not understood and further data are needed to verify or refute the purported association between aspartame and neurological adverse effects. Walton (1988) described eight cases of individuals who believed their seizures were related to aspartame consumption. In all cases, individuals discontinued consumption of aspartame and felt they experienced a cessation of a variety of different symptoms, but none of these associations was confirmed by a subsequent challenge with aspartame.

The incidence of various symptoms of 551 "aspartame reactors," who were identified by three groups including "Aspartame Victims and Their Friends." the Community of Nutrition Institute, and Dr. Monte of Arizona State University, was reported (Roberts, 1988). No information on the actual amount or duration of aspartame consumption was provided. No details regarding the selection process of reactors were provided. The most common were neurological symptoms including headache, dizziness, confusion and convulsions. Other symptoms were psychiatric (depression, irritability, anxiety) or visual and auditory disturbances. No data were provided regarding whether symptoms were self-diagnosed or were ever confirmed by a medical expert.

By 1991, the FDA had received 251 reports of aspartame-associated seizures in their passive surveillance monitoring system for adverse effects (Tollefson and Barnard, 1992). All complaints of seizures were investigated because these are considered severe reactions. Cases were classified according to the strength of the association between consumption of aspartame and probability of that seizure being due to consumption. Group A classification indicates the strongest association, and was assigned when symptoms recurred each time an individual consumed different products containing aspartame. Group B classification was assigned when symptoms recurred following consumption of one aspartame-containing product. If the individual did not rechallenge, a Group C classification was assigned, and a Group D if it was highly unlikely that the symptoms were associated with consumption of aspartame. For example, all complaints in which the seizure occurred before, or more than 24 h after, aspartame consumption were classified as Group D. The largest proportion of complaints (124/251 or 49%) was Group D. Further data obtained by investigation of complaints did not provide sufficient evidence of an association between seizures in humans and aspartame consumption to warrant recommendation of a double-blind case-controlled study by the FDA (Tollefson and Barnard, 1992). The authors state that because of the widespread use of aspartame in the general public, the fact that some people have reactions, including seizures, shortly after consuming aspartame, does not indicate a causal relationship.

A case report of an individual who experienced cessation of dizziness upon discontinuation of use of aspartame in coffee and diet soda prompted an attempt to study the relationship between aspartame consumption and dizziness (Gulya et al., 1992). However, only 7/53 patients experiencing dizziness were willing to complete a questionnaire. Six of the seven people completing the questionnaire reported consuming aspartame and only one experienced an improvement in dizziness upon cessation of aspartame use (Gulya et al., 1992). Therefore, the study offers little support for an association between aspartame and vertigo or light-headedness.

Cases of two individuals who believed that aspartame was a trigger for migraine headaches experienced a worsening of headache rather than relief when an aspartame-containing wafer form of the migraine drug, rizatriptan, was used in place of the tablet form of rizatriptan devoid of aspartame (Newman and Lipton, 2001). Provocation of migraine headaches by aspartame-containing chewing gum in three individuals was reported (Blumenthal and Vance, 1997).

Although a report by Strong (2000) was described as a double-blind study to identify triggers of migraine headaches, the only participant in the study was the author. Aspartame was added to orange juice and consumed, and then the author reported whether and how quickly he experienced a headache. The author did experience a headache after each dose of aspartame. Neither the other foods nor the placebos were delivered in orange juice so it is not clear how this study could be considered a double-blind study. As it involved only one person, this report was considered a case-report.

One hypothesis that has been suggested as a possible cause of individuals experiencing adverse reactions to aspartame is a deficit of α-aspartyl-phenylalanine hydrolase activity (Gougeon et al., 2004). In a study with 48 children who were described as sensitive to sugar and who were fed diets high in sucrose, aspartame, or saccharin for three successive 3-week periods, blood levels of α-aspartyl-phenylalanine were undetectable and α-aspartyl-phenylalanine hydrolase activities did not differ among groups (Stegink et al., 1995). No study was identified that provided support to this hypothesis.

In conclusion, in a number of human case studies, no neurotoxic reactions could be provoked with aspartame challenge. In some studies with limitations regarding design and number of patients, individuals have felt that their migraine headaches and seizures were related to exposure to aspartame. In studies designed to test the hypothesis that aspartame induces neurotoxic symptoms, no reactions would be provoked and/or clearly attributed to aspartame consumption.

Despite levels of plasma dicarboxylic amino acids reaching levels at least 10-fold higher than those observed in neonatal mice, which were associated with neuronal necrosis (Schainker and Olney, 1974), there have been no observed adverse effects of large doses of aspartic acid in studies with humans (see reviews: Meldrum, 1993; Institute of Medicine, 2005) or nonhuman primates (Reynolds et al., 1976, 1980). Therefore, the Institute of Medicine concluded that there remains uncertainty regarding the relevance of the newborn rodent model for assessing the neuronal necrosis potential of aspartic acid in humans (Institute of Medicine, 2005). There are currently insufficient data to develop an upper limit for either aspartic acid or phenylalanine, although dietary supplement doses of up to 8 g/day aspartic acid (equivalent to 120 mg/kg bw/day) and 10 g/day phenylalanine in healthy individuals have not resulted in documented adverse effects (Institute of Medicine, 2005).

6.9.1.3. Other Adverse Effect Reports. There are many unsubstantiated single case reports of adverse effects of aspartame. For example, in a letter to the editor, Robbins and Raymond (1999) report that three individuals suffering from carpal tunnel syndrome had complete resolution of their symptoms upon avoidance of aspartame, despite no changes in physical activity. A possible association between aspartame and Graves' disease was reported (Roberts, 2004); however, others (Virani et al., 2003) have found no such association. Requests for publication of the data that allegedly supports these associations have not been fulfilled; therefore, it is not possible to scientifically assess the validity of such reported associations.

The Centers for Disease Control (MMWR, 1984) interviewed 517 of the 592 complainants who had reported problems with aspartame before mid-1984. Most complaints were received from women between the ages of 21 and 60 living in Arizona. One explanation given for the concentration of complaints in Arizona was the extensive press coverage of possible adverse health effects that occurred in that area. The majority (67%) of symptoms were neurological/behavioral symptoms including headaches, dizziness and mood alterations. Other common symptoms were gastrointestinal (24%), allergic type/dermatologic (15%) and alterations in menstrual cycle (6%). The conclusions of the report were that the complaints were of a wide variety, mild in nature, and are symptoms that are commonly found in the public. Although certain individuals may have an unusual sensitivity to aspartame, the data from this study did not provide evidence for the existence of serious widespread adverse health consequences attributable to use of aspartame.

Some patients with chronic fatigue syndrome have reported intolerance for aspartame (Anonymous, 2006). Relief of four individuals from symptoms of fibromyalgia following elimination of monosodium glutamate and aspartame from the diet was reported (Smith et al., 2001).

Aspartame has been associated with urinary incontinence (Bottomley, 2000), which has been attributed to reports of diuretic effects and increased urinary calcium excretion (Nguyen et al., 1998; Leon, 1999).

6.9.2. Human Clinical Studies

A detailed study of the effects of chronic aspartame consumption conducted by Leon et al. (1989) included 108 healthy adult volunteers, ages 18 to 62 years. This was a randomized, double blind, placebo-controlled, parallel-group design. Participants included 57 women and 51 men, randomly assigned to either the aspartame ($n = 53$) or placebo ($n = 55$) groups. Exclusion criteria were well defined and included body weight greater than 20% above normal range. Subjects consumed three capsules per day containing either 300 mg aspartame or 300 mg microcrystalline cellulose plus 0.9 mg silicon dioxide for 24 weeks. Compliance was monitored. The approximate dose of aspartame was 75 mg/kg bw/day. Extensive clinical laboratory measurements were conducted at baseline, and after 3, 6, 9, 12, 18, and 24 weeks. Measurements included body weight, vital signs, complete blood cell count, serum chemistry of 18 standard parameters, and plasma lipids following a 12-h fast. In addition, metabolic studies were conducted to evaluate levels of plasma amino acids, blood methanol and formate, and urinary formate and creatinine. The results indicated no differences between groups in body weight, vital signs, blood lipid levels, urinalysis results, or incidence of complaints (headache was the most frequent complaint). One individual suspected headaches were due to aspartame and withdrew from the study; however, subsequent challenges with aspartame or placebo capsules under

controlled conditions found no evidence that aspartame induced headaches in this individual. No difference was found in fasting plasma levels of 22 amino acids, including aspartic acid and phenylalanine, or the ratio of phenylalanine to large neutral amino acids. Plasma tyrosine levels were higher in the aspartame group during weeks 3 and 24 than in the control group, but were still within the normal values range. Blood methanol concentrations were usually below detection limits (0.31 mmol/L); however, one individual in the aspartame group had a methanol concentration of 1.0 mmol/L and one in the control group had a level of 0.84 mmol/L. Serum folate, urinary and blood formic acid, and urinary calcium levels were unchanged in both groups. The authors concluded that daily doses of 75 mg/kg bw/day of aspartame were not associated with any significant changes in clinical measures or adverse effects (Leon et al., 1989).

6.9.2.1. Effect of Aspartame on Methanol, Formaldehyde, and Formic Acid. As one of the products of metabolism of aspartame is methanol, the possibility of adverse effects of aspartame due to methanol and methanol metabolites has been suggested in many websites on aspartame. The toxicokinetics and mechanisms of methanol toxicity in humans have been reviewed by the American Academy of Clinical Toxicology (Barceloux et al., 2002). In humans, methanol is metabolized in the liver by alcohol dehydrogenase to formaldehyde, which is rapidly converted to formic acid, with no accumulation of formaldehyde. The metabolism of formic acid is a two-step process involving tetrahydrofolate to form 10-formyl tetrahydrofolate, which in turn is metabolized to CO_2. Formic acid accumulates in the blood because its half-life ($t_{1/2}$ = 3.4–6 h) is very much longer than is that of formaldehyde ($t_{1/2}$ = 1.5 min) (Hantson et al., 2005). Formic acid accumulation is considered the mechanism of toxicity of high doses of methanol, which induces metabolic acidosis, ophthalmic toxicity and central nervous system depression (Barceloux et al., 2002). The lowest blood methanol concentration that has been documented to be associated with metabolic acidosis was 126 mg/dl (Kostic and Dart, 2003).

The effect of consumption of aspartame on blood levels of methanol and formic acid has been examined in a number of studies (see review, Stegink and Filer, 1996). These are summarized in Table 25. Methanol metabolism was evaluated in normal human subjects administered aspartame at doses of 34, 100, 150, and 200 mg/kg bw (Stegink et al., 1981a). Blood methanol levels were below the limits of detection (0.4 mg/dl) in subjects given 34 mg/kg bw aspartame. In adults given higher doses, blood methanol was detected following the aspartame dose in a dose-dependent manner, reaching a peak of 2.5 mg/dl at about 2 h in subjects given 200 mg/kg bw. As formate is the ultimate toxicant, the levels of formate in blood and urine of subjects given a 200 mg/kg bw dose of aspartame were determined (Stegink, 1984). Blood formate levels did not change significantly, but urinary excretion increased, indicating that the formation of formate did not exceed metabolism and excretion rates.

In general, the methanol levels remain below the limit of detection even in individuals consuming a single dose of 34 mg aspartame/kg bw. This dose represents nearly three times the amount eaten by consumers in the 95th percentile. The tolerable

TABLE 25
Methanol and formate levels in blood after aspatame exposure

Species/subject	Dose (based on bw)	Blood methanol and formate levels (mg/dl)	Reference
Humans, 30 healthy adults, fasting, 15 M, 15 F	(1) 34 mg/kg ASP (n = 12) (2) 100 mg/kg ASP (n = 6) (3) 150 mg/kg ASP (n = 6) (4) 200 mg/kg ASP (n = 6)	(1) methanol below LOD (2) methanol peak 1.27, below LOD at 8 h (3) methanol peak 2.14, below LOD at 24 h (4) methanol peak 2.58, below LOD at 24 h No change in blood formic acid levels in subjects given 200 mg/kg ASP	Stegink et al. (1981a)
Humans, infants	(1) 34 mg/kg ASP (n = 10) (2) 50 mg/kg ASP (n = 6) (3) 100 mg/kg ASP(n = 8)	(1) methanol below LOD (2) methanol below LOD (3) methanol peak of 1.02 reached at 90 min, then dropped to ~0.45 at 2.5 h	Stegink et al. (1983a)
Humans, 6 healthy adults, fasting, 3 M, 3 F	600 mg ASP per hour for 8 h	No change in blood methanol or formic acid levels	Stegink et al. (1989)

Note. ASP = Aspartame; bw = body weight; F = female; h = hours; M = male; n = number; LOD = limit of detection = 0.35 mg/dl.

("safe") dose of methanol has been calculated to be 2 g for an adult human (Paine and Dayan, 2001), and the toxic dose reported to cause blindness is 8 g for an adult (Kostic and Dart, 2003). This information indicates that hypothetically, the "safe" dose of aspartame, from the methanol released, would be 18.4 g for an adult (307 mg/kg bw for a 60-kg adult).

Although these experiments demonstrated the ability of healthy adults to metabolize and excrete aspartame metabolic products, there is concern regarding potentially more susceptible subjects. One-year-old infants (Stegink et al., 1983a) were given 34, 50, and 100 mg/kg bw aspartame and blood samples were analyzed for methanol levels. Blood methanol concentrations in children were similar to those in adults consuming the same amount of aspartame, indicating that children were not at increased risk of methanol from aspartame. The 307 mg/kg bw calculation for a "safe" dose of aspartame, from the methanol produced, as shown earlier, therefore may also apply to 1-year-old infants.

In a study to evaluate repeated exposure to aspartame, 126 healthy children were randomly assigned to receive aspartame or sucrose incorporated into a variety of foods for 13 weeks (Frey, 1976). The study was double-blinded. Participants ranged from 2 to 21 years, and included 61 females and 65 males. Dosages were dependent on age and weight, and aspartame consumption ranged from 27 to 77 mg/kg bw/day. Physical examinations, plasma amino acid and methanol measurements, complete blood counts, urine analysis, and ophthalmoscopic examinations were conducted before and after treatment. Adverse effect complaints were also reported. No significant differences in any clinical measurements (blood and urine analyses) were found, no changes were found in physical status (body weight, blood pressure), all methanol values were below the limits of detection, and complaints were similar for the two groups and not clinically significant (Frey, 1976).

Shahangian et al. (1984) calculated that consumption of the equivalent of 3 L of beverages containing 670 mg aspartame per liter would result in a maximal increase in plasma formate levels of 0.9 mg/dL, if all aspartame was absorbed, hydrolyzed and distributed. As the range of plasma formate levels in healthy subjects not consuming aspartame was found to be 7–63 mg/L, the authors concluded that any increase in blood formic acid ascribable to metabolism of aspartame would be insufficient to result in formic acid toxicity.

In conclusion, the expected highest human consumption levels of aspartame did not result in increased blood levels of methanol or formic acid.

6.9.2.2. Effect of Aspartame on Headaches. A double-blind crossover trial with 40 individuals (12 males, 28 females; ages 19–69) who had reported having headaches each time they consumed aspartame was a well-controlled study with patients being housed and monitored in an inpatient unit (Schiffman et al., 1987). Participants were monitored for 2 days, and then challenged with capsules of aspartame (30 mg/kg bw) or placebo (microcrystalline cellulose) on days 3 and 5, with day 4 being a washout day. Diet and extraneous variables were controlled. There was no evidence of an effect of aspartame, as incidence of headache after consumption of aspartame (35%) was similar to after the placebo (45%) (Schiffman et al., 1987).

In contrast, the study by Koehler and Glaros (1988) had volunteers who suffered with migraines stay in their normal environments during a double-blind crossover study with three phases: a 4-week baseline phase, two 4-week experimental phases, and a 1-week washout phase between treatments. Participants ($n = 11$; 2 males, 8 females; ages 18 to 47 years) consumed capsules of aspartame (300 mg) or placebo (microcrystalline cellulose) and self-recorded headaches and diets. The incidence of headaches did not differ from baseline during the placebo phase. Five of the 11 participants reported a higher number of migraines during the aspartame phase as compared to during the baseline or placebo phases. The mean number of headaches reported was 1.72, 1.55, and 3.55 during the baseline, placebo, and aspartame phases, respectively. No differences were reported in the intensity or duration of migraine headaches. Dietary records did not show any substantial changes in diet among phases. One concern is the small number of individuals and high dropout rate from 25 down to 11 participants in this study.

Patients at a headache unit ($n = 190$) were asked to complete a survey regarding whether they felt that alcohol, aspartame or carbohydrate intake were triggers of headaches (Lipton et al., 1989). The limitation of this study is the power of suggestion by having aspartame listed as a possible trigger and then asking for a response. Of the 171 patients who completed the survey, 8.3% reported aspartame as a trigger of headaches, and often a migraine headache. As this was significantly higher than the response to carbohydrates (2.3%), the authors concluded that aspartame might be a migraine headache trigger for some individuals. The study would have been more convincing if the question of dietary triggers had been open-ended and patients identified aspartame on their own.

In the most recent study to assess whether the consumption of aspartame is associated with headaches, Van den Eeden et al. (1994) conducted a double-blind randomized crossover trial with 32 subjects who self-reported sensitivity to aspartame. Only 18 participants completed the full protocol, as other subjects withdrew for various reasons including adverse effects. Subjects took capsules containing either aspartame or placebo (microcrystalline cellulose) 3 times a day to achieve a dose of 30 mg/kg bw/day for 7 days. A significantly higher ($p = .04$) occurrence of self-reported headaches was reported following exposure to aspartame (33% of days) as compared to placebo (24% of days). The subjects who had excess headaches following aspartame dosing were those who had, at the beginning of the study, indicated they were "very sure" that they were susceptible to aspartame-induced headaches. In contrast, those subjects who classified themselves as "somewhat or not sure" reported similar headache incidence during aspartame and placebo exposure periods. The authors conclude that these results indicate that a small subset of the population are

susceptible to aspartame-induced headaches (Van den Eeden et al., 1994).

Studies designed to evaluate the possible effect of aspartame on headaches have reported conflicting results, with some reporting no effect and others suggesting a small subset of the population may be susceptible to aspartame-induced headaches. The number of studies is small and several have high participant dropout rates, making interpretation of results difficult.

6.9.2.3. Effect of Aspartame on Behavior and Cognitive Function. The suggested mechanism for possible effects of aspartame and high phenylalanine on behavior relates to the competition of phenylalanine with tryptophan and other large neutral amino acids for a common carrier across blood brain barriers (Groff and Gropper, 2000). Phenylalanine has higher affinity for the carrier (lower K_m) as compared to tryptophan, so increased concentrations of phenylalanine hypothetically could cause a reduction in brain tryptophan, leading to lower serotonin levels and behavioral effects.

6.9.2.3.1. Studies in Children. Mental retardation resulting from hyperphenylalaninemia in PKU is observed in children having plasma concentrations in the range of 1200–6000 μmol/L, and the plasma threshold is considered to be near 1000 μmol/L (Groff and Gropper, 2000). Seizures and other neurological abnormalities are also symptoms of PKU. Whether adverse effects occur in the lower range concentrations of hyperphenylalaninemia is less clear. Children with classic PKU who maintain chronic levels of serum phenylalanine levels in the range of 240 to 480 μmol/L do not experience mental retardation. Therefore, most investigators believe that some elevation of serum phenylalanine above normal levels, even if chronic, is without adverse effect (Yost, 1989).

Eleven hyperactive boys with attention deficit disorder (ages 6–12 years) were evaluated by parents and teachers for changes in behavior and cognitive measures during 2 weeks of consuming phenylalanine (20 mg/kg bw/day) or a placebo (Zametkin et al., 1987). Plasma phenylalanine levels were increased during the phenylalanine-dosing phase, but no difference was found in urinary phenylethylamine. The authors concluded that their findings support the safety of aspartame, but do not support the hypothesis that precursor loading will affect hyperactivity.

Kruesi et al. (1987) evaluated the effect of sugar and aspartame on aggression and activity in preschool boys (ages 2 to 6 years) who were identified as sensitive to sugar or "sugar responders". The study was a double-blind cross-over challenge with aspartame (30 mg/kg bw), sucrose (1.75 g/kg bw), saccharin (amount not specified) and glucose (1.75 g/kg bw) to sugar-responsive ($n = 14$) and age-matched control boys ($n = 10$). The sweeteners were given in a lemon-flavored drink once in a laboratory setting, and once 4 days later in a home setting. Children were scored for activity and aggression by researchers during the laboratory playroom challenge and by their parents in the days following to detect any delayed reaction, and during the home challenge. Washout periods of 5–7 days occurred between challenges. There was no significant difference in scores of aggression among sweeteners, and lower activity scores during the aspartame challenges (Kruesi et al., 1987). Therefore, this study does not support the hypothesis that aspartame is associated with disruptive behavior in children.

Similar negative results were reported by Roshen and Hagen (1989) and Sarvais et al. (1990) in studies evaluating the effect of aspartame on the learning, behavior and mood of children.

A double-blind controlled trial of 48 preschool children fed diets containing a daily intake of 38 mg/kg bw/day aspartame for 3 weeks showed no adverse effects attributable to aspartame or dietary sucrose on children's behavior or cognitive function (Wolraich et al., 1994). Shaywitz et al. (1994b) assessed the effect of aspartame on behavior and cognitive function of children with attention deficit disorder using a randomized, double blind, and placebo-controlled crossover study design. The dose of aspartame was 34 mg/kg bw/day. Children ($n = 15$, 11 males, 4 females, ages 5 to 13 years) were given capsules of either aspartame or placebo (microcrystalline cellulose) each morning for a 2-week period. Parents were instructed to provide an aspartame-free diet during the study. No effect was found on cognitive, attentive or behavioral testing or on urinary levels of neurotransmitters (norepinephrine, epinephrine, dopamine, HVA, and 5HIAA), although plasma tyrosine and phenylalanine levels were higher 2 h after the aspartame treatment. Plasma phenylalanine levels were reported graphically, and increased from approximately 6 μmol/dl at baseline to about 8.5 μmol/dl 2 h after aspartame dosing. Plasma tryosine level values were not provided (Shaywitz et al., 1994b). Krohn (1994) criticized this study for the small number of participants, stating that such as study does not demonstrate that all children with ADD would be insensitive to aspartame.

Several studies have used aspartame as a negative control compound in investigations into the effect of sucrose on behavior. For example, no differences were found in behavior of 16 hyperactive boys following challenges with a single oral dose of sucrose (57 g) and aspartame (197 mg) in two studies conducted by Wolraich et al. (1985). The problem with these studies is that both dietary sucrose and aspartame have been reported to affect behavior, so it is difficult to use one or the other as a negative control. Wolraich et al. (1994) subsequently addressed this concern in a very well controlled study to assess the behavioral effects of sucrose and aspartame using saccharin as a negative control. This was a 9-week study with all food being provided to families of participants throughout the study to control for other dietary components that may impact behavior. Participants were preschool children ($n = 25$) described as normal and school age children ($n = 23$) described as sensitive to sugar. Experimental diets were provided during 3-week periods in a Latin-square design. The diets provided participants with sucrose (4500–5600 mg/kg bw/day), aspartame (32–38 mg/kg bw/day), or saccharin (10–12 mg/kg bw/day) as the sweetener. Behavior and cognitive measures were assessed weekly. This study also included measures of diet compliance using biochemical tests. No effect

of sucrose or aspartame on behavior or cognitive function was observed in either age group of children (Wolraich et al., 1994). However, the use of glucose or sucrose as a placebo needs to be viewed with caution as other studies have shown improvement of memory and cognitive function following their administration (Sunram-Lea et al., 2001; Sunram-Lea et al., 2002; 2004).

6.9.2.3.2. Studies in Adults. A double-blind randomized crossover trial with 10 healthy volunteers (6 men, 4 women, ages 21–36 years) evaluated the effect of a single dose of aspartame (15 mg/kg bw) or placebo capsules on mood, cognitive function, and reaction time. No effect was observed on hunger, headache, memory, reaction time, or cognition during the study despite elevation of plasma phenylalanine levels following consumption (data values not reported). The percentage of total LNAA that was phenylalanine increased from approximately 11% to a peak of about 18 at the 2-h time point after dosing, but dropped to normal after 8 h (Lapierre et al., 1990).

A single dose of aspartame had no effect on food consumption, mood or alertness in healthy males ages 20 to 35 years. Subjects ($n = 13$/group) were given capsules containing placebo or aspartame (5 or 10 g) in a randomized crossover design (Ryan-Harshman et al., 1987). Blood phenylalanine levels increased from a mean of 7.1 μmol/dl at baseline, by 5.5 and 19.3 μmol/dl at 90 min after consumption of 5 and 10 g aspartame, respectively. Blood tyrosine levels increased by 2.5 and 4.7 μmol/dl, from a baseline of 7.7 μmol/dl at 90 min after consumption of 5 and 10 g aspartame, respectively. These differences were statistically significant, but no behavioral effects were observed.

Pivonka and Grunewald (1990) compared the effect of water, and aspartame- and sugar-containing beverages on mood in 120 young women and found no effect on self-reported surveys of mood.

A highly publicized case report of adverse effects of aspartame consumption in a military pilot led to two controlled studies on the effect of acute (Stokes et al., 1991) and chronic (Stokes et al., 1994) aspartame ingestion on cognitive performance in pilots. The first study involved 12 healthy certified pilots (4 females and 8 males). The study was double-blinded with each subject being tested 5 times, with at least 1 week between treatments given in random order among the 12 participants. Participants were pretested for baseline values, then given placebo capsules, aspartame (50 mg/kg bw), or ethyl alcohol (positive control, estimated dose to raise blood alcohol 0.1%), followed by a posttest with no treatment. For all treatments, participants consumed orange juice with either a trace or the test dose of alcohol, and capsules with either placebo (dextrose) or aspartame. Cognitive performance was tested using the SPARTANS cognitive test battery, which is a sensitive test to detect changes in performance of complex tasks required for aircraft operations. As has been discussed previously, concerns have been voiced regarding the possible potentiation of the effects of aspartame by consumption concurrently with carbohydrates. Therefore, all participants consumed a small carbohydrate meal prior to treatments. Consumption of other foods, aspartame, and alcohol was controlled prior to testing. Blood levels of amino acids were not measured. Cognitive impairment was detected in several tasks following consumption of the low dose of alcohol but not aspartame or placebo treatments (Stokes et al., 1991).

A follow-up study employed a more complex battery of tests (SPARTANS Version 2), chronic exposure to aspartame and measurement of blood phenylalanine (Stokes et al., 1994). Twelve subjects (college students, sex not defined) received placebo capsules or aspartame capsules (50 mg/kg bw/day) for 9 days, or an acute dose of ethyl alcohol to achieve 0.1% blood ethanol levels as described earlier. All participants received the placebo and ethanol treatments once and the aspartame treatment twice with a 7-day interval. Blood phenylalanine and breath alcohol levels were measured. On the last day of treatment periods, when subjects completed the cognitive testing, blood alcohol levels were 0.0% during all treatments except following the alcohol treatment when it averaged 0.09%. Plasma phenylalanine levels averaged 59.08 μmol following placebo treatments and 121.5 μmol following aspartame consumption. Forty-seven task variables were measured and significant differences between pre- and post-test results and aspartame treatment were detected for three tasks. However, unexpectedly, an improvement, rather than impairment, of function was observed in participants following the aspartame treatments. Following ethanol treatments, participants scored lower on 14 tasks. The authors attribute the finding of enhanced performance following aspartame treatment to chance, and conclude that although aspartame given at high doses (50 mg/kg bw/day) approximately doubled plasma phenylalanine levels, there is no evidence of impaired cognitive performance (Stokes et al., 1994).

Cognitive, neurophysiologic and behavioral effects of consuming aspartame for 20 days were evaluated by Spiers et al. (1998) in a group of 48 healthy volunteers (24 men, 24 women, ages 18–34 years). This was a three-way crossover double-blind study with treatments consisting of aspartame, sucrose and placebo. Twenty-four participants received a high dose of aspartame (45 mg/kg bw/day) and the remaining received 15 mg/kg bw/day. Acute effects were evaluated on day 10 of each treatment arm, with testing starting 90 min after consumption of test material. Chronic effects were evaluated on day 20. Plasma phenylalanine levels increased dose-dependently with aspartame consumption, but no other effects were observed.

Concerns exist that the only studies done that show no effect of aspartame are those which use healthy adults and people used to high intakes of aspartame such as diabetics and people on weight-loss regimes (Tsakiris et al., 2006). However, the effect of acute high-dose aspartame was also evaluated in a double-blinded study of 18 patients with Parkinson's disease, as this was considered a susceptible target population for adverse effects (Karstaedt and Pincus, 1993). Ten patients received a dose of 600 mg, and eight received 1200 mg aspartame in capsules or placebo capsules in random order. Motor scores and plasma levels of phenylalanine were measured every hour for 4 h. Although

plasma phenylalanine levels were significantly increased following aspartame consumption, there was no significant difference in motor or disability scores following aspartame as compared to following placebo ingestion (Karstaedt and Pincus, 1993).

Another group considered possibly vulnerable to aspartame were patients suffering from depression (Walton et al., 1993). Participants were adults (ages 24–60 years) undergoing treatment for depression ($n = 8$, 3M, 5F) or nondepressed volunteer controls ($n = 5$, 3M, 2F) of which three considered themselves susceptible to adverse effects of aspartame. Subjects received aspartame (30 mg/kg bw/day) or placebo (sucrose) in capsules for a period of 7 days. Subjects were asked to self-monitor and score the severity of a list of symptoms, which included headache, nervousness, dizziness, nausea, feeling blue or depressed, temper outbursts, and others. One patient experienced pain in his eye, which was followed by retinal detachment. This occurred during the placebo phase, but the authors speculated that consumption of aspartame prior to placebo might have been responsible. Another patient experienced a conjunctival hemorrhage during the aspartame phase. These events lead to a halt of the study. Only 11 participants completed the study, but total points for adverse effects were compared for all 13 participants. Higher point values were recorded by depressed patients during consumption of aspartame (mean = 40) as compared to during consumption of placebo (mean = 10), but nondepressed participants recorded similar points during aspartame (mean = 13) and placebo (mean = 15) phases. The authors concluded that individuals with mood disorders should be discouraged from consuming aspartame (Walton et al., 1993). The limitations of this study include the small number of participants, the provision of a suggested list of adverse effects that may have influenced self-diagnosis, and the lack of control of other dietary factors during the study. The statistics used were also questionable as multiple *t*-tests were used to compare nonparametric measures.

The effect of aspartame on behavior and cognitive function has been studied extensively in animals, and in healthy children, hyperactive children, sugar-sensitive children, healthy adults, individuals with Parkinson's disease, and individuals suffering from depression. The only study to suggest that aspartame may be associated with behavior was the study in depression patients. The limitations of this study were discussed above. Overall, the weight of the evidence indicates that aspartame has no effect on behavior or cognitive function.

6.9.2.4. Effect of Aspartame on Seizures. Children who had been recently diagnosed with generalized absence seizures were enrolled in a double-blind controlled study to ascertain whether aspartame would exacerbate occurrence of absence seizures (also called petite mal seizures) (Camfield et al., 1992). After eating their own choice of breakfast, children ($n = 10$) drank orange juice sweetened with either aspartame (40 mg/kg bw) or sucrose (1 g sucrose for every 25 mg aspartame) to achieve similar sweetness. However, it is unlikely that this amount of sucrose used actually achieved a similar level of sweetness, as the sweetness of aspartame is approximately 200 times that of sucrose. The number and length of spike-wave bursts, indicative of an absence seizure, were determined using EEG[40] recordings for 6 h following consumption of the juice. Each child was tested once with each substance, on two consecutive days, in random fashion. No information was provided regarding whether lunch or snacks were given. There were no significant differences in the frequency or duration of spike-wave bursts; however, when the two factors were combined, the total time spent in spike-wave per observation hour was significantly higher in children on the day aspartame was consumed as compared to when sucrose was consumed (Camfield et al., 1992). The major limitation of this study is the lack of control of food and beverage intake before and after dosing with aspartame or sucrose because fasting and dehydration can affect the susceptibility to seizures (Tollefson and Barnard, 1992). In addition, the effect of sucrose on EEG spike waves, and the brief baseline measurements used in this study was questioned (Shaywitz and Novotny, 1993).

In contrast, a dose of 34 mg/kg bw aspartame had no effect on induction of seizures in a randomized, double-blind, placebo-controlled, crossover study of 10 epileptic children reported by Shaywitz et al. (1994a). The study consisted of two 2-week periods of administration of either placebo or aspartame. Measurements prior to and following treatments included seizure incidence, overall activity and behavior, EEG recordings, adverse experiences, liver function, urine analysis, and plasma levels of amino acid, methanol, formate, glucose, and monoamines and metabolites. Children were allowed to eat their normal diet, but excluding foods on a list of aspartame-containing products. In this study, children experienced seizures, including generalized convulsions and absence seizures, but there was no difference in the occurrence of seizures during the aspartame or placebo treatments. The only differences in any biochemical parameters were increased levels of phenylalanine and tyrosine during aspartame treatments as expected (Shaywitz et al., 1994a).

Rowan et al. (1995) designed a randomized, double-blind, placebo-controlled, crossover study to assess seizure induction following aspartame ingestion in self-reported aspartame-sensitive individuals. Targeted recruitment was conducted of individuals who had complained of experiencing a seizure in response to aspartame to government officials or the manufacturer, and of nearly 9000 neurologists. After 4 years of recruitment, only 16 adults and 2 children participated in the study. Continued recruitment for another 5 years did not locate additional subjects despite reports of large numbers of individuals experiencing seizures. In this study, subjects received 50 mg/kg bw aspartame or an identical placebo in three divided doses throughout the day, on days 2 and 4. EEG recordings were preformed for 5 consecutive days. All meals were uniformly standardized on treatment days. No clinical seizures were observed in subjects during the study. Electrographic seizures were recorded in 2 subjects on days consuming the placebo. Sleep variables were also measured, but no effect of aspartame was observed. Plasma

[40]EEG = electroencephalogram (*Stedman's Medical Dictionary*, 1995b).

phenylalanine levels, and the ratio of phenylalanine to large neutral amino acids, were increased on the day of aspartame dosing, but returned to normal on the following day (Rowan et al., 1995). The major limitation of this study is the small number of participants. Therefore, even in the presence of documented elevation of plasma phenylalanine levels, aspartame was no more likely than placebo to induce seizures in susceptible individuals.

Reviews of the research on aspartame and seizures (Jobe and Dailey, 1993) and aspartame and neural function in general (Lajtha et al., 1994) have concluded that consumption of aspartame does not have significant effects on seizures. The findings of this review concur with this conclusion.

6.9.2.5. Effects of Aspartame in Diabetic Individuals. The fact that aspartame would likely be attractive to diabetics as a glucose-free sweetener was recognized at the inception of development and testing in diabetic animal models and diabetic individuals was included in the studies submitted for aspartame approval. These studies were reviewed by Butchko et al. (2002a). No adverse effects of aspartame in diabetics were observed. Subsequent studies in diabetic individuals were summarized in Section 5.4. Aspartame does not affect glycemic response in normal or diabetic individuals, and does not affect metabolic control or insulin release.

As diabetics with renal disease have altered amino acid and protein metabolism, Gupta et al. (1989) conducted a randomized double-blind study with 23 diabetic patients undergoing maintenance hemodialysis. Following consumption of a dose of 10 mg/kg bw, increases in the levels of phenylalanine and tyrosine were within the normal postprandial range. The authors concluded that aspartame is safe for use by diabetics, including those with chronic renal failure.

The American Diabetes Association (ADA, 2002) and the Canadian Diabetes Association (Gougeon et al., 2004) have also evaluated the use of aspartame for diabetics and have concluded that aspartame has no adverse effects in diabetics, and may aid diabetics in adhering to a sugar-free dietary regime.

6.9.2.6. Effect of Aspartame on Hunger and Weight Gain. Claims that aspartame may increase hunger and food consumption (Rogers et al., 1988) raised questions regarding the use of aspartame by individuals on weight reduction regimes. Studies conducted in the 1970s, prior to the approval of aspartame, included randomized double-blind placebo-controlled clinical studies with obese individuals for 6 and 21 weeks. These studies found no effect of aspartame on body weight (Butchko et al., 2002a). However, the issue of effect of aspartame on eating behavior was raised, leading to a reevaluation of this question. Several studies have been conducted and are reviewed by Butchko et al. (2002a). In one of the well-conducted studies, Drewnowski et al. (1994) fed 12 obese and 12 lean women one of four breakfast preloads sweetened with 50 g sucrose, 500 mg aspartame, or aspartame plus 50 g maltodextrin. All subjects were tested with all treatments. Subsequent food intake and calorie consumption during lunch, snack, and dinner were not affected by the sweetener consumed in the preload (Drewnowski et al., 1994).

Recently, a meta-analysis of 16 randomized controlled trials assessing the effect of aspartame consumption on weight loss, weight maintenance and energy intake was conducted (de la Hunty et al., 2006). The studies that have addressed the question of the effect of aspartame on appetite and body weight, that have actually measured food consumption, have shown that aspartame does not increase caloric intake. In contrast, significant reduction of energy intake with consumption of aspartame compared to other controls was observed, except when the control was a nonsucrose control such as water. In these cases, there was no significant difference (de la Hunty et al., 2006).

In summary, there is no evidence to support an association between consumption of aspartame and the development of obesity. On the contrary, when used in multidisciplinary weight control programs, aspartame may actually aid in long-term control of body weight.

6.9.3. Epidemiological Studies

6.9.3.1. Epidemiological Studies on Aspartame. Concerns regarding an association between aspartame and brain tumors were again raised in statements by Roberts (1990) and by Olney et al. (1996) in a report of an evaluation of data from the National Cancer Institute's Surveillance, Epidemiology and End Results (SEER) program from nine different areas of the United States. These areas comprise approximately 10% of the population. The authors generated graphs of the SEER reported incidence of various brain tumor types from 1975 to 1992. Based on the shape of the curves in the graphs, the authors suggested that brain tumor incidence was increasing, and that the increase was related to the introduction of aspartame into the market. Olney et al. (1996) suggested that the incidence of brain tumors in the FDA studies and the report of Sheppard et al. (1991, 1993) stating that nitrosated aspartame was mutagenic was evidence supporting the hypothesis that aspartame was responsible for the rising brain tumor rates.

However, the conclusions of this study (Olney et al., 1996) were subsequently questioned and criticized by many scientists. Levy and Hedeker (1996) pointed out several problems with the study including: the inclusion of certain tumor types originating outside of the brain, and omission of others types that do; misleading graphing by starting the ordinate at greater than zero to exaggerate the change; and omission of data from 1973 and 1974 to create an illusion of a dramatic increase after aspartame introduction. In letters to the editor, Flamm (1997) and Koestner (1997) challenged many of the statements in the paper by Olney et al. (1996) regarding previous studies on aspartame and the FDA's judgment of these studies. Legler et al. (1999) evaluated the SEER data according to age groups and concluded that brain tumor incidence had stabilized during the 1990s for all age groups except the elderly. The increased incidence in this age group was attributed to improvements in diagnosis. In a review of studies on brain tumors and aspartame, Butchko et al. (2002a) further discuss the implausibility of the assumptions

made by Olney et al. (1996) and also present a reevalutation of the SEER data. Ross (1998) and Seife (1999) explained that the linking of two events, the introduction of aspartame and an increase in brain tumor incidence that incidentally occurred during roughly the same time, is not admissible in epidemiology and is called "ecological fallacy." There was no information provided indicating that individuals with brain tumors actually consumed aspartame. Furthermore, Olney et al. (1996) did not take into account the latency period for development of brain tumors, or that the increasing consumption after introduction of aspartame was not associated with a proportionate increase in brain tumor incidence. Combined, these reports and omissions provide sufficient evidence to raise serious doubt regarding the validity of the Olney study. In 1997, the EFSA also evaluated the purported connection between aspartame and the increased incidence of brain tumors and concluded that the data did not support an association and did not justify a reevaluation of the safety of aspartame (EFSA, 2006).

Trichopoulos (1999) pointed out that Swartz's (1999) hypothesis that aspartame was linked to the increase in breast tumors similarly was an ecological fallacy with no supporting evidence.

A case-control study, conducted to examine the hypothesis that aspartame may act as a human brain carcinogen, examined aspartame consumption by 56 case patients with primary brain tumors and 94 age- and sex-matched control subjects (Gurney et al., 1997). Cases were children in the United States who were diagnosed with a brain tumor between the ages of 0 and 19 during 1984 to 1991. Exposure of both the mother and the child to environmental and nutritional factors, including aspartame, had been collected by personal interviews with the mothers of the children prior to diagnosis. No evidence to support a causal association between increased consumption of aspartame by case children or by mothers of case children was found. There was no suggestion of a dose response based on years or frequency of consumption. Thus, there was no indication that aspartame is a brain carcinogen in this study.

In a case-control study primarily designed to investigate the relationship between risk of brain tumors and exposure to ionizing radiation and cellular telephones, Hardell et al. (2001) also assessed intake of aspartame by questioning participants about consumption of low-calorie drinks. The study included 209 brain tumor cases and 425 age- and sex-matched controls from the Stockholm area in Sweden recruited from 1994 to 1996. The ratio of male to female participants was not defined. The authors report a "nonsignificantly increased" risk of malignant brain tumors (OR = 1.7, 95% CI = 0.84–3.44) associated with low-calorie beverage consumption, but no association when benign or combined brain tumors were evaluated. There are several limitations to this study. No data are given with respect to the level of consumption of drinks by either group. There is no validation that self-reported drink consumption is an accurate measure of aspartame consumption. There is no explanation for why the number of participants included in the aspartame section is only 30 cases and 45 controls. Lastly, the fact that the confidence interval (CI) includes the value of 1.0 indicates that no association exists.

Weihrauch and Diehl (2004) reviewed reports on artificial sweeteners and carcinogenic risk in 2004 and concluded the possible risk of artificial sweeteners to induce cancer is negligible.

Medulloblastoma/primitive neuroectodermal tumors are the most common brain tumors in children. A case-control study of the diets of mothers of children diagnosed with medulloblastoma was conducted to identify whether the maternal diet provided any protective and/or causative dietary factors for this brain tumor type (Bunin et al., 2005). The study participants included 315 mothers of children diagnosed before age 6 years, and 315 mothers of control children. Mothers completed a food frequency questionnaire modified to include specific foods that were of particular interest based on a previous study. One such item was diet soda. Two time periods were assessed, periconception and midpregnancy. An increasing trend was observed for diet soda during the periconception period, with an odds ratio of 1.6 (confidence interval 1.0–2.3) for mothers consuming two or more diet sodas per day, but this trend was not evident in the midpregnancy phase. In addition, following adjustment for confounders, the periconception trend was no longer statistically significant. Therefore, the authors conclude that their data do not support an association of this brain tumor type and aspartame.

Very recently, the National Cancer Institute investigated the relationship between aspartame consumption and the incidence of brain and hematopoietic cancers in a prospective study (Lim et al., 2006b). The study participants were 340,045 men and 226,945 women aged 50–69 years in the NIH-AARP Diet and Health Study from eight locations in the United States. Responses to a baseline food frequency questionnaire regarding consumption frequency of aspartame-containing beverages (soda, fruit drinks, and iced tea) and the addition of aspartame to coffee and hot tea were used to compute daily consumption of aspartame. Most participants consumed between 2 and 3 mg aspartame/kg bw per day. All participants with preexisting cancer were eliminated and remaining participants (n = 473,984) were followed up to 5 years for development of histologically confirmed hematopoietic cancers (n = 2106) and brain cancers (n = 376). Relative risks (RR), estimated using Cox proportional hazards regression, adjusted for age, sex, ethnicity, body mass index, history of diabetes and smoking were computed. There were no significant associations between increasing levels of aspartame consumption and risk of hematopoietic cancers (adjusted RR for $\geq$600 mg/day vs. none = 0.98, 95% CI = 0.76–1.27, p = .56 for trend) or gliomas (RR for $\geq$400 mg/day vs. none = 0.73, 95% CI = 0.46–1.15, p = .05 for trend), in men and women combined. Specific main subtypes of lymphoid cancers (Hodgkin lymphoma, non-Hodgkin lymphoma, and multiple myeloma) and the non-Hodgkin lymphoma subtypes (small lymphocytic lymphoma and chronic lymphocytic leukemia, immunoblastic lymphoma and lymphoblastic lymphoma/leukemia) and nonlymphoid leukemias

were evaluated. No significant associations were found between these cancers and aspartame consumption. These findings did not change with adjustments for education, family history of cancer, physical activity, alcohol, caffeine, and other dietary and lifestyle risk factors. The authors indicated that the large sample size and use of histologically confirmed cancers were strengths of the study, whereas having only a small percentage (less than 1%) of participants in a high consumption category (>1200 mg/day) was a limitation. Overall, the authors conclude that the findings from this epidemiologic study suggest that consumption of aspartame-containing beverages does not raise the risk of hematopoietic or brain malignancies (Lim et al., 2006b). The authors also state that although they will update their findings as more cases develop in the cohort, it is unlikely their conclusions will change because they found no evidence of a stronger effect of aspartame with age and no difference in association between aspartame consumption and cancer in young as compared to older subjects. This large study is considered to provide definitive information.

These studies have been summarized in Table 26. In conclusion, case-control studies and one well-conducted prospective epidemiological study with a large cohort, in which the consumption of aspartame was measured, provide no evidence to support an association between aspartame and brain or hematopoietic tumor development.

6.9.3.2. Epidemiological Studies on Formaldehyde. Soffritti et al. (2005, 2006) postulate that aspartame may be carcinogenic due to formation of methanol during metabolism, which is subsequently metabolized to formaldehyde. Their contention is based on the classification of formaldehyde as a carcinogen. However, this classification is currently being reevaluated. In their recent 1994 follow-up of the National Cancer Institute (NCI) 10-plant cohort mortality study of 25,619 workers exposed to formaldehyde, Hauptmann et al. (2003) suggested a causal association between formaldehyde exposure and leukemia, particularly myeloid leukemia (Hauptmann et al., 2003) and nasopharyngeal cancer (NPC) (Hauptmann et al., 2004). These findings, particularly those for NPC, led IARC in June 2004 to classify formaldehyde as a known human carcinogen (Group 1). The validity of the causal associations suggested by Hauptmann et al. (2003, 2004) was challenged in a series of subsequent reports and letters (Marsh and Youk, 2005) and led to three extensive reanalyses of the NCI cohort data conducted at the University of Pittsburgh (Kerns II et al., 2002; Marsh and Youk, 2004, 2005).

As shown by Marsh and Youk (2005) and (Marsh et al., 2006), the suggestion of Hauptmann et al. (2003; 2004) of a causal association with formaldehyde and NPC was driven entirely by anomalous findings in 1 of the 10 study plants (Plant 1). This key finding was not reported by Hauptmann et al. (2003, 2004).

TABLE 26
Summary of epidemiological studies investigating the association between aspartame and tumor development

Author	Type of study and number of participants	Aspartame consumption	Conclusions
Olney et al. (1996)	Evaluation of SEER data from 9 areas of the United States	Not measured	Incidence of brain tumors increased after the introduction of aspartame to the market
Gurney et al. (1997)	Case control study with 56 brain tumor cases and 94 age- and sex-matched controls	Personal interview of mothers for dietary recall of aspartame consumption of children	No association between consumption of aspartame and risk of brain tumor development
Hardell et al. (2001)	Case control study with 30 brain tumor cases and 45 controls	Recall of low calorie soft drinks. No data of consumption provided	No significant association between brain tumors and low-calorie soft-drink consumption
Bunin et al. (2005)	Case control study with 315 children with medulloblastoma and 315 matched controls	Food frequency questionnaires completed by mothers of children	No association between consumption of aspartame during pregnancy and brain tumor risk
Lim et al. (2006b)	Prospective study $n =$ 473,984 followed for 5 years. Cases included hematopoietic cancers ($n =$ 2,106) and brain cancers ($n = 376$)	Food frequency questionnaires. Consumption ranged from none to over 1200 mg/kg/day in high consumers	No significant associations were found between hematopeoitic or brain cancers and aspartame consumption

Note. $n =$ number.

In the previous update of their Plant 1 study, Marsh et al. (2002) found little evidence of an association with formaldehyde and NPC and suggested that the observed overall NPC excess was most likely due to factors outside the Plant 1 environment. In fact, in the most recent 2003 mortality follow-up of Plant 1 that included an updated nested case-control study, Marsh et al. (2007) found that the large nasopharyngeal cancer mortality excess in Plant 1 may be due to previous employment in the ferrous and nonferrous metal industries of the local area. These jobs entailed possible exposures to several risk factors for upper respiratory tract cancer, including sulfuric acid mists, mineral acid, metal dust, and heat. The uncertainties about the NCI's conclusions created by the reports and reanalyses prompted the NCI to initiate a 2003 follow-up of its formaldehyde worker cohort. This update is now underway and is scheduled for completion in 2007. The uncertainties also prompted the U.S. EPA to postpone its scheduled update of their IRIS document on formaldehyde until the completion of the NCI update.

Therefore, in addition to the issues surrounding the conduct and interpretation of the studies by Soffritti et al. (2005, 2006) that are discussed in Section 6.3.2, there are uncertainties regarding the carcinogenic potential of formaldehyde, even in highly exposed workers. Therefore, it is highly unlikely that formaldehyde formed from the small amount of methanol from consumption of aspartame poses any carcinogenic risk to humans.

7. EVALUATION SUMMARY

Aspartame is a methyl ester of a dipeptide used as a synthetic nonnutritive sweetener in over 90 countries worldwide in over 6000 products. Aspartame is approximately 200 times sweeter than sucrose, and can therefore add sweetness with less weight and consequently fewer calories than sugar.

Aspartame is completely hydrolyzed into three metabolites: aspartic acid, phenylalanine, and methanol. Phenylalanine enters the plasma free amino acid pool from the portal blood after partial conversion to tyrosine by hepatic phenylalanine hydroxylase. Aspartic acid is metabolized within the enterocyte. Methanol rapidly enters the portal circulation and is oxidized in the liver to formaldehyde, which is further oxidized to formic acid and then to CO_2. Formaldehyde has a half-life of about 1.5 min, so there is no accumulation in the tissue. Phenylalanine, aspartic acid, methanol and formaldehyde are all naturally present in foods and the contribution to the total daily intake of each of these from aspartame is small to trivial.

Using the most current food consumption data available for the U.S. population, the average intake of aspartame among individuals consuming aspartame was determined to be 4.9 mg/kg bw/day (95th percentile 13.3 mg/kg bw/day in the United States). The U.S. Food and Drug Administration (FDA) established an ADI of 50 mg/kg bw for aspartame and the European Food Safety Authority recently confirmed its previously established ADI for aspartame of 40 mg/kg bw/day. Therefore, current use levels of aspartame, even by high users in special subgroups, remains well below ADI levels.

Aspartame is very stable under dry conditions, but degrades during prolonged heat treatment in aqueous solutions. Breakdown results in loss of sweetness as the breakdown products are not sweet.

The biochemical effects of aspartame consumption have been extensively studied. Parameters measured following aspartame consumption include plasma amino acid concentrations, the ratio of amino acids to other large neutral amino acids, brain neurotransmitters, plasma lipids, plasma glucose, gastrointestinal secretions, metabolizing enzymes, and other blood and urine components. The effect of co-ingestion of carbohydrates on plasma amino acid and brain neurotransmitter levels has also been studied. These biochemical changes have been measured in animal studies using various species and developmental stages, and in healthy adults and children. In addition, effects in special populations such as individuals with gastrointestinal disease, diabetes, and kidney disorders have been evaluated. In summary, consumption of large doses of aspartame in a single bolus dose will have an effect on some biochemical parameters, including plasma amino acid levels and brain neurotransmitter levels. The rise in plasma levels of phenylalanine and aspartic acid levels following administration of aspartame at doses less than or equal to 50 mg/kg bw do not exceed those observed postprandially.

Acute toxicity studies with aspartame have been conducted using oral and intraperitoneal exposure routes with mice, rats and rabbits. No deaths or adverse effects were reported with oral doses as high as 10,000 mg/kg bw.

In subchronic toxicity studies of aspartame, no adverse effects due to aspartame were reported in mice, rats, or dogs given doses up to 13000, 10000, or 6000 mg/kg bw/day, respectively. Chronic toxicity studies with aspartame, and its decomposition products, have been conducted in mice, rats, hamsters and dogs. The conclusions of these studies were consistent in that no adverse effect of aspartame was found with doses up to 4000 mg/kg bw/day.

The National Toxicology Program (NTP) completed three carcinogenicity and toxicity studies of aspartame using different transgenic mouse models. In all three studies, there were no tumors attributed to exposure to aspartame in either sex at any dose tested. In conclusion, no evidence of carcinogenicity was observed in these transgenic mouse model studies with dietary levels of aspartame equivalent to 7500 mg/kg bw/day.

Recently, Soffritti et al. (2006) reported a study of lifetime aspartame exposure in rats. The authors interpreted their findings as evidence of a carcinogenic effect. Furthermore, the authors speculated that metabolism of the methyl group in aspartame to formaldehyde may account for the carcinogenicity they claim to have observed in their experiments, but this conclusion fails to take into account the fact that the body routinely metabolizes methyl groups from many other sources. In addition, recent reevaluation of epidemiological studies of formaldehyde has revealed uncertainties in the classification of formaldehyde as a human carcinogen. Numerous expert reviews of the Soffritti reports and this panel have identified numerous potential flaws in

the studies, and have concluded that the study does not provide evidence of carcinogenicity of aspartame.

The long-term studies on the toxicity of aspartame now include seven chronic studies of at least 2 years duration with mice or rats to assess carcinogenic potential and chronic toxicity, and two studies of 32 to 36 weeks duration to evaluate the tumor promotional activity of aspartame in rats. Extensive peer review and reevaluation of data and study protocols has occurred for several studies in different parts of the world. In all cases, the conclusions of the reviews by authoritative agencies, including the present Expert Panel, have been that aspartame does not have carcinogenic or cancer-promoting activity. In conclusion, it can be confidently stated that there is no credible evidence that aspartame is carcinogenic.

In addition to carcinogenicity studies, the investigation for evidence of neurologic, biochemical, behavioral and/or morphological changes following exposure to aspartame has been intensive. Studies that mimic human exposure do not show any evidence of neurological effects. The effect of aspartame on induction of seizures has been studied in rodents and primates. The studies agreed that aspartame and equimolar doses of phenylalanine are without effect on seizure susceptibility in animals, even when administered prenatally.

The majority of studies designed to detect an effect of aspartame on learning or memory have used multiple doses and multiple tests to evaluate these parameters. These studies report no effect at doses as high as 4% aspartame in the diet, even when exposure extends from conception to 90 days postnatally. Higher doses have been reported to affect various learning behaviors. In summary, well-designed studies using a range of approaches to evaluate learning and memory consistently demonstrate no effect of aspartame consumption at levels up to 4000 mg/kg bw day, indicating little likelihood that aspartame will have an effect on memory or learning in humans.

In summary, there has been extensive investigation of the possibility of neurotoxic effects due to consumption of aspartame. The data from these studies, in general, do not support the hypothesis that aspartame in the human diet will affect nervous system function, learning or behavior. It is likely that the lack of changes in neural function in response to changes in plasma and brain amino acid levels, following even large doses of aspartame, is a reflection of the protection of brain function. This may be due to the great nutritional variation that is present in the diversity of human diets and dietary intakes.

In summary, the effect of aspartame during reproduction, development and lactation has been evaluated in rats, mice, hamsters, and rabbits. No-effect levels of exposure during reproduction and gestation have been reported to range from 1600 mg/kg bw/day in rabbits to 4000 mg aspartame/kg bw/day in rodents. Adverse effects on pup development were observed in rat studies when doses exceeded 5000 mg/kg bw/day during reproduction and gestation. Consumption of up to 7000 mg aspartame/kg bw/day during lactation had no effect on pup development or maternal health, but higher doses affected body weights. Studies evaluating the estrogenic potential of aspartame have consistently been negative. Therefore, aspartame is considered to have no reproductive or teratogenic activity, and no effect on lactation. In these studies, effects have been observed at exceedingly high doses, and were secondary to reduced body weights.

Human clinical studies with daily doses of 75 mg/kg bw/day (more than 15 times the estimated daily average intake and 1.5 times the established ADI by the FDA) of aspartame for 24 weeks were not associated with any significant changes in clinical measures or adverse effects. The effect of aspartame on behavior, cognitive function, and seizures has been studied extensively in animals, and in healthy children, hyperactive children, sugar-sensitive children, healthy adults, individuals with Parkinson's disease, and individuals suffering from depression. Overall, the weight of the evidence indicates that aspartame has no effect on behavior, cognitive function, neural function, or seizures in any of these groups.

As aspartame is attractive to diabetics as a glucose-free sweetener, testing in diabetic animal models and diabetic individuals was included in the studies submitted for aspartame approval. The conclusion of these studies is that aspartame is safe for use by diabetics, including those with chronic renal failure, and may aid diabetics in adhering to a sugar-free dietary regime.

Recently, a meta-analysis of 16 randomized, controlled trials assessing the effect of aspartame consumption on weight loss, weight maintenance, and energy intake was conducted. There is no evidence to support an association between consumption of aspartame and the development of obesity. On the contrary, when used in multidisciplinary weight control programs, aspartame may actually aid in long-term control of body weight.

Epidemiological studies on aspartame include several case-control studies and one well-conducted prospective epidemiological study with a large cohort ($n = 473{,}984$), in which the consumption of aspartame was measured. The studies provide no evidence to support an association between aspartame and brain or hematopoietic tumor development.

This evaluation summary highlights the major issues regarding aspartame safety. Several other potential effects associated with consuming aspartame, which have been described since its introduction to the market, have also been reviewed and evaluated in this document. These other potential effects were considered to be either of little biological significance (such as a small change in enzyme activity), or the available evidence available at this time is not adequate to conclude that the reported effect actually exists or is due to aspartame; therefore, these other effects are not included in the evaluation summary. The reader is encouraged to refer to the body of this review to obtain a comprehensive understanding of the science relating to aspartame safety.

The current weight of evidence is that aspartame is safe at current levels of consumption as a nonnutritive sweetener. Consumption remains well below established ADI levels, even among special high user sub-populations. No credible evidence was found that aspartame is carcinogenic, neurotoxic, or has

any other adverse effect on health when consumed even at levels many times the established ADI levels.

8. CONCLUSIONS

Aspartame's metabolism is well understood and follows that of other common foods. Aspartame consumption, even at levels much higher than that expected under typical circumstances, has virtually no impact on levels of other blood constituents such as amino acids, methanol or glucose.

Aspartame is a well-studied sweetener whose safety is clearly documented and well established through extensive laboratory testing, animal experiments, epidemiological studies, and human clinical trials.

Controlled and thorough scientific studies confirm aspartame's safety and find no credible link between consumption of aspartame at levels found in the human diet and conditions related to the nervous system and behavior, nor any other symptom or illness.

Aspartame is well documented to be nongenotoxic and there is no credible evidence that aspartame is carcinogenic.

Aspartame does not increase hunger in those who use it; to the contrary, studies indicate it might be an effective tool as part of an overall weight management program.

Aspartame is a well-characterized, thoroughly studied, high-intensity sweetener that has a long history of safe use in the food supply and can help reduce the caloric content of a wide variety of foods.

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This safety review was conducted by the Burdock Group, at the request of the sponsor. Dr. William Waddell was selected as the chair of the review expert panel, and had a free hand in selection of the panelists. As described in the introduction, panelists were chosen to achieve representation of the complete spectrum of toxicological expertise relevant to aspartame. The identity of the sponsor of the review was unknown to the chair and the expert panelists throughout the conduct and completion of the review. The identity of panelists also remained unknown to the sponsor. The Burdock Group managed reimbursement of panelists. There were no known conflicts of interest or potential biases of the authors. This review represents the professional views of the authors.

Note added in proof. The identity of the sponsor of this review was also unknown to peer reviewers and the editor until acceptance of manuscript. The sponsor was Ajinomoto Company, Inc.

ADDENDUM

Since submission of this review, two major studies on aspartame have been published. One paper describes an integrated network of case-control studies to investigate potential association between consumption of artificial sweeteners, including aspartame, and cancer risk (Gallus et al., 2007). This report included studies conducted in Italy between 1991 and 2004, with a total of 8976 cancer cases and 7028 controls. Cancer cases included cancers of the oral cavity and pharynx, esophagus, colon, rectum, larynx, breast, ovary, prostate and renal cell carcinoma. Dietary intake of sugar and artificial sweeteners was assessed using a food-frequency questionnaire. Although the intake of aspartame specifically was not evaluated, the study found no evidence that artificial sweeteners increase cancer risk at these common sites in humans (Gallus et al., 2007).

Results of a second study on aspartame by Soffritti et al. (2007) were released online. This is a follow-up of the Soffritti et al. (2006) study, which was discussed in detail in Section 6.3.2. In the recent study, aspartame at 0 (control, C), 400 (low dose, LD) or 2000 ppm (high dose, HD) was added to the Cesare Maltoni Cancer Research Center (CMCRC) standard diet and fed to female Sprague-Dawley breeders from the colony of CMCRC from the 12th day of pregnancy until weaning of pups. At 4-5 weeks of age, experimental animals were assigned to groups according to the treatment of their mothers. Groups consisted of 70 treated (LD, HD) and 95 control (C) male and female rats. The in-life phase ended at 147 weeks with the death of the last surviving animal. The authors reported no effect of aspartame on feed consumption, no difference in mean body weights among groups, and a slight decrease in survival in treated groups (no statistics given). The incidence of lymphoma/leukemia in male rats was C, 9.5%; LD, 15.7%, HD 17.1%; mainly involving the lung and mediastinal/peripheral nodes. The incidence of lymphoma/leukemia in female rats was C, 12.6%; LD, 17.1%; HD 31.4%; mainly involving thymus, lung, spleen and peripheral nodes. The incidence of mammary carcinomas in female rats was C, 5.3%; LD, 7.1%; HD, 15.7%. The authors also stated that comparison of incidence of lymphoma/leukemia in female rats from current experiment (prenatal exposure) to results from postnatal exposure (Soffritti et al., 2006) demonstrates that when lifespan exposure to APM begins during fetal life, carcinogenic effects are increased.

Several comments regarding the methodology of this study are warranted. Doses were based on an "estimate" assuming a constant food consumption of 20 g/day and a constant body weight of 400 g. Although food consumption and body weight were measured throughout the experiment, the data are only provided after week 16, when the rats would have reached full body weight. Thus the authors' main conclusions are built on the exposure period for which they provide no data.

Other important details of a study designed to assess prenatal exposure have not been addressed in this report. These include: (1) the number of pregnant dams per dose group; (2) whether all dams were primaparous or of equivalent age/maturity; (3) the growth and food consumption for mothers during pregnancy and lactation, (4) pregnancy outcome; (5) how investigators were able to get equal number of males and females for each treatment group, as there was no description of method of culling or assignment of pups from each mother to treatment group; and (6) the body weights of pups at birth and during lactation, which

are needed to assess whether there were differences in treatment groups before postnatal exposure began.

Several points regarding the results also need to be made to assess the validity of the authors' conclusions. The lymphoma/leukemia incidences in the HD group were within (males 17.1%) or close to (females 31.4%) the reported historical control ranges (males 30.9%, females 25.0%). Infection incidences were not reported, despite evidence of high and variable lung infections in the colony (these data were provided in the EFSA report on the first study, see Section 6.3.2.4.1). The authors indicate that in this study, the lung was often the site of lymphoma and, as discussed in Section 6.3.2.4.1, chronic lung infections are associated with development of lymphoma/leukemia. Therefore, it is highly likely that, as in their first study, the incidences of lymphomas/leukemias are associated with infection rates rather than aspartame consumption. The mammary gland carcinoma incidence in HD females (15.7%) was also close to that of historical controls (14.2%). Causes of death were also not reported.

No data are given on the actual prenatal exposure, or evidence of *in utero* exposure to aspartame that could provide a biologically plausible explanation for the authors' contention that prenatal exposure increases cancer risk from aspartame. Aspartame is completely digested by the gastrointestinal tract, into amino acids and methanol, which is subsequently metabolized to carbon dioxide and water. Previous studies with aspartame using the doses in this range have not resulted in significant changes to blood amino acid or methanol levels (reviewed in Section 5.0).

In summary, considering the lack of significant differences between high dose groups and historical control cancer rates, plus the many deficits in the study design and data, it is the opinion of this expert panel that this study (Soffritti et al., 2007) fails to provide convincing evidence of aspartame carcinogenicity.

In conclusion, the findings by Gallus et al. (2007) are in agreement with the findings of this review, and the report by Soffritti et al. (2007) does not provide sufficient evidence to alter the overall conclusions of this review.

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APPENDIX I: ESTIMATED MAXIMUM DAILY INTAKE OF ASPARTAME FROM REQUESTED FOODS[41]

Consumption Analysis Report

In order to determine a hypothetical maximum daily ingestion of aspartame as an added ingredient by humans, a consumption analysis database[42] was analyzed for consumption of aspartame when added to specific foods. This nationwide dietary intake survey was conducted during 2001–2002, and was comprised of 2 days of data that were collected for all respondents in the food survey ($n = 9701$ individuals). Comprehensive and detailed questions were posed to the participants, and the results were used to code individual foods and portion sizes consumed. The results were weighted to place more strength on foods that were consumed by more individuals, and extrapolated to the U.S. population.

All categories designated by the food codes have been utilized in the calculations as appropriate; however, certain categories may contain foods under which a standard of identity exists, prohibiting the addition of ingredients not identified under statues for the standard of identity for that food. Therefore, addition of aspartame to a food for which a standard of identity exists would demand that the food product be named other than that as indicated under the standard of identity.

The maximum concentration of aspartame suggested was utilized in the consumption analysis, to ensure that the estimate includes any individuals that consume larger amounts of the foods.

[41]The food categories contained in this report were identified by the client and the most reasonable food codes fitting these categories were selected by the Burdock Group.

[42]Source: HHS What We Eat in America, National Health and Nutrition Examination Survey (NHANES) 2001–2002, USDA.

TABLE 27
Per capita consumption estimate from target foods

	Eaters only+ (total individuals that consume targeted foods = 130,357,042)	
	mg/day	mg/kg bw/day
Mean (weighted)	330.17	4.85
90th Percentile (weighted)	704.8	10.43
95th Percentile (weighted)	938.97	13.29

Note. Estimate is based on the consumption of aspartame by individuals in the NHANES sample population who reported consuming a food that contained aspartame at least once, i.e., Eaters only; bw = body weight.

The concentrations were obtained from the literature when available (Anonymous, 1991; Leclercq et al., 1999; Butchko and Stargel, 2001; Anonymous, 2005; Anonymous, 2006), or were obtained through personal communication from manufacturers, and converted to milligrams per gram concentrations.

The results of the per capita consumption analysis are provided in Table 27. The resulting mean per capita consumption of aspartame in the selected food codes is 330.17 mg/day (4.85 mg/kg bw/day), and the 90th percentile per capita consumption is 704.8 mg/day (10.43 mg/kg bw/day). Additional analysis of the consumption of aspartame when consumed in the requested foods found that the 30–39 years age group consumed the greatest amount of aspartame (Table 28), while males consumed more aspartame than females (Table 29).

TABLE 29
Consumption of aspartame by gender from the selected foods

Gender	Aspartame consumption (mg/day)	Aspartame consumption (mg/kg body weight/day)
Male	383.69	5.16
Female	291.94	4.64

The average weights for males and females were derived from the U.S. Environmental Protection Agency *Exposure Factors Handbook*, Volume I: *General Factors*.[43] Therefore, the consumption of the food is broken down to the age of the person that ate the food, then the mg/kg bw/day value is calculated based on the average weight for that age group and gender. The weights of the males and females that consumed the ingredient are then compiled based on gender, and utilized to determine the consumption of aspartame on a milligrams per kilogram body weight per day basis (Table 29).

TABLE 28
Consumption of aspartame from the selected foods, broken down by age group

Age group	Average weight (kg)		Aspartame consumption (mg/day)	Aspartame consumption (mg/kg body weight/day)
	Males	Females		
1–2	12.55	11.75	62.08	5.10
3–5	17.6	17.0	97.67	5.66
6–11	28.52	30.4	161.71	5.51
12–19	57.4	53.95	209.56	3.80
20–29	73.7	60.6	349.68	5.43
30–39	78.7	64.2	423.87	5.99
40–49	80.8	67.1	372.21	5.05
50–59	81.0	67.9	382.00	5.12
60–69	78.8	67.9	254.86	3.59
70+	74.8	66.6	182.90	2.60

[43]http://risk.lsd.ornl.gov/homepage/EFH_Final_1997_EPA600P95002Fa.pdf; site visited July 27, 2006.

TABLE 30
Foods selected for the consumption analysis of aspartame

Food code	Description	Concentration (mg/g)
11424000	yogurt, Vanilla, Lemon, Coffee, Nonfat Milk, Low Cal Sweet	0.551
11433500	yogurt, fruited, nonfat milk, low cal sweetener	0.551
11460400	yogurt, frz, chocolate, nonfat milk, w/ low-cal sweet	0.551
11460410	yogurt, frz, not choc, nonfat milk, w/ low-cal sweet	0.551
11514300	cocoa w/ nf dry milk, lo-cal sweetener, water added	0.294
11514500	cocoa w/ whey, lo-cal sweetnr, fortifd, water added	0.294
11515400	cocoa w/ nfdm, low calorie, hi calcium, water added	0.294
11516000	cocoa, whey, lo-cal sweetner mix, lowfat milk added	0.294
11518000	milk bev w/ nf dry mlk, lo-cal sweet, water, choc	0.441
11518050	milk bev w/nf dry milk, lo-cal sweet, water, not choc	0.441
11518100	milk bev w/nfd milk, lo-cal sweet, hi calcium, choc	0.441
11613000	instant bfast, pwdr, swt w/ lo-cal swt, milk added	0.441
11622010	diet beverage,pwdr, reconst w/skim (incl carnation)	0.441
11830970	meal replacement, protein type, milk-base, powder	0.441
13130330	light ice cream, no sugar add, not chocolate	1
13130340	light ice cream, no sugar add, chocolate	1
13160150	fat-free ice cream, no sugar add, choc	1
13160160	fat-free ice cream, no sugar add, flavors other than choc	1
13161520	milk dessert sandwich bar, frz, w/low-cal sweet,lofat	1
13161600	milk des bar, frozen, lo-fat milk & lo-cal sweetener	1
13161630	light ice cream, bar/stick, w/ low-cal sweetener, choc coat	1
13210250	pudding, choc, rte, lo-cal, w/ art swtner, ns dry/can	0.22
13210290	pudding, not choc, rte, lo-cal, w/ art swtner	0.22
13220210	pudding, not choc,from dry, low cal,artificial sweet, w/milk	0.22
13220220	pudding, choc,from dry, low cal,artificial sweet, milk added	0.22
13230120	pudding, canned, low cal, w/artificial swtnr, not choc	0.22
13230140	pudding, canned, lo-cal, w/ art swtner, choc	0.22
28401200	gelatin drink, flavored, w/ low calorie sweetener	0.529
91106000	sugar substitute, sugar–aspartame blend, dry pwd	35
91201010	sugar substitute, aspartame-based, dry powder	35
91351010	syrup, dietetic	2
91351020	topping, dietetic	1.34
91405000	jelly, dietetic, all flavors,sweetened w/ artificial sweeten	2
91406000	jam, marmalades, artificially sweetened	2
91510100	gelatin powder, dietetic, dry	0.838
91511010	gelatin dessert, dietetic, w/ lo-cal sweetener	0.838
91511020	gelatin dessert, diet, w/ fruit, lo-cal sweetner	0.838
91511030	gelatin dessert, dietetic, w/ whipped topping	0.838
91511050	gelatin dessert, dietetic, w/ cream cheese	0.838
91511060	gelatin dessert, dietetic, w/ sour cream	0.838
91511070	gelatin dessert, dietetic, w/ fruit & sour cream	0.838
91511080	gelatin dessert, dietetic, w/ fruit & cream cheese	0.838
91511090	gelatin dessert, dietetic, w/ fruit & vegetables	0.838
91511100	gelatin dessert, dietetic, w/ vegetables	0.838
91511110	gelatin dessert, dietetic, w/ fruit & whip topping	0.838
91611100	ice pop, sweetened w/ low calorie sweetener	0.529
91770000	dietetic or low calorie candy, nfs	0.8
91770010	dietetic or low calorie gumdrops	0.8
91770020	dietetic or low calorie hard candy	3

TABLE 30
Foods selected for the consumption analysis of aspartame *(Continued)*

Food code	Description	Concentration (mg/g)
91770030	dietetic or low calorie candy, chocolate-covered	3
91770050	mints, dietetic or low calorie	1
91802000	chewing gum, sugarless	3.67
92121030	coffee & cocoa, from mix, w/whitener, low cal sweet	0.441
92121040	coffee, from powder, w/ whitener & lo-cal sweetener	0.441
92121050	coffee & cocoa, from pwdr,w/white & low cal sweet,decaf	0.441
92192030	coffee & cocoa (mocha) mix,w/whitener, low cal, dry	0.441
92192040	coffee & cocoa mix,dry,w/whitener & low cal sweet,decaf	0.441
92193020	coffee, dry mix, w/ whitener & low cal sweetener	0.441
92301080	tea, presweetened w/ low calorie sweetener	0.529
92301160	tea, decaffeinated, w/ sugar, nfs	0.529
92301180	tea, decaffeinated, low calorie sweetener, nfs	0.529
92400100	soft drink, nfs, sugar-free	0.661
92410250	carbonated water, sugar-free	0.529
92410320	soft drink, cola-type, sugar-free	0.529
92410350	soft drink, cola-type, decaffeinated, sugar-free	0.529
92410370	soft drink, pepper-type, sugar-free	0.529
92410400	soft drink, pepper-type, decaffeinated, sugar-free	0.529
92410420	cream soda, sugar-free	0.529
92410520	soft drink, fruit-flav, sugar-free, caffeine-free	0.529
92410560	soft drink, fruit-flavored, w/ caffeine, sugar-free	0.529
92410620	ginger ale, sugar-free	0.529
92410720	root beer, sugar-free	0.529
92410820	chocolate-flavored soda, sugar-free	0.529
92411610	cola w/ fruit or vanilla flavor, sugar-free	0.529
11622010	diet beverage,pwdr,reconst w/skim (incl carnation)	0.441

TABLE 31
Consumption of aspartame added to target foods

Food code	Description	mg/day	mg/kg/day
11424000	yogurt, vanilla, lemon, coffee, nonfat milk, low-cal sweet	81.72	1.55
11433500	yogurt, fruited, nonfat milk, low-cal sweetener	98.57	2.05
11460400	yogurt, frz, chocolate, nonfat milk, w/ low-cal sweet	105.2	1.56
11460410	yogurt, frz, not choc, nonfat milk, w/ low-cal sweet	98.56	1.42
11514300	cocoa w/ nf dry milk, lo-cal sweetener, water added	66.26	0.96
11514500	cocoa w/ whey, lo-cal sweetnr, fortifd, water added	219.62	7.7
11516000	cocoa, whey, lo-cal sweetner mix, low-fat milk added	109.48	2.45
11830970	meal replacement, protein type, milk-base, powder	28.85	0.4
13130330	light ice cream, no sugar add, not chocolate	126.65	2.29
13130340	light ice cream, no sugar add, chocolate	24.19	0.36
13160150	fat-free ice cream, no sugar add, choc	48	1.68
13160160	fat-free ice cream, no sugar add, flavors other than choc	76.44	1.09
13161600	milk des bar, frozen, lofat milk & lo-cal sweetener	41	0.61
13161630	light ice cream,bar/stick, w/ low-cal sweetener, choc coat	73.35	1.37
13210250	pudding, choc, rte, lo-cal, w/ art swtner, ns dry/can	26.05	0.76
13210290	pudding, not choc, rte, lo-cal, w/ art swtner	8.37	0.12

(Continued on next page)

TABLE 31
Consumption of aspartame added to target foods *(Continued)*

Food code	Description	mg/day	mg/kg/day
13220210	pudding,not choc, from dry, low cal, artificial sweet, w/ milk	25.98	0.38
91201010	sugar substitute, aspartame-based, dry powder	102.48	1.42
91351010	syrup, dietetic	59.8	0.87
91351020	topping, dietetic	38.03	0.59
91405000	jelly, dietetic, all flavors, sweetened w/ artificial sweeten	28.87	0.39
91406000	jam, marmalades, artificially sweetened	10.55	0.25
91510100	gelatin powder, dietetic, dry	1.78	0.03
91511010	gelatin dessert, dietetic, w/ lo-cal sweetener	107.44	2.08
91511020	gelatin dessert, diet, w/ fruit, lo-cal sweetner	129.18	2.45
91511080	gelatin dessert, dietetic, w/ fruit & cream cheese	227.94	3.36
91511100	gelatin dessert, dietetic, w/ vegetables	152.73	2.29
91611100	ice pop, sweetened w/ low-calorie sweetener	99.88	1.97
91770020	dietetic or low-calorie hard candy	20.02	0.29
91770030	dietetic or low-calorie candy, chocolate-covered	72.96	1.13
91802000	chewing gum, sugarless	13.01	0.26
92121030	coffee & cocoa, from mix, w/ whitener, low cal sweet	131.42	1.94
92121040	coffee, from powder, w/ whitener & lo-cal sweetener	125.61	1.8
92121050	coffee & cocoa,from pwdr, w/ white & low-cal sweet, decaf	131.71	1.79
92192040	coffee & cocoa mix,dry, w/ whitener & low-cal sweet, decaf	2.91	0.04
92301080	tea, presweetened w/ low-calorie sweetener	250.32	3.96
92301160	tea, decaffeinated, w/ sugar, nfs	216.29	3.28
92301180	tea, decaffeinated, low-calorie sweetener, nfs	165.1	2.54
92400100	soft drink, nfs, sugar-free	235.39	3.39
92410250	carbonated water, sugar-free	216.46	4.78
92410320	soft drink, cola-type, sugar-free	376.08	5.47
92410350	soft drink, cola-type, decaffeinated, sugar-free	289.26	4.14
92410370	soft drink, pepper-type, sugar-free	360.48	5.23
92410400	soft drink, pepper-type, decaffeinated, sugar-free	360.59	5.06
92410420	cream soda, sugar-free	234.57	4.58
92410520	soft drink, fruit-flav, sugar-free, caffeine-free	275.72	4.13
92410560	soft drink, fruit-flavored, w/ caffeine, sugar-free	274.75	3.8
92410620	gingerale, sugar-free	163.97	2.48
92410720	root beer, sugar-free	220.18	3.42
92411610	cola w/ fruit or vanilla flavor, sugar-free	284.63	3.63

Note. Not all foods suggested in Table 30 may have been consumed by the sample population; therefore, a consumption analysis for only those foods consumed by the sample population (i.e., "eaters only") could be conducted. Eaters only are individuals in the NHANES sample population who reported consuming a food that contained aspartame at least once.

APPENDIX II: METHANOL CONTENT OF FOODS AND BEVERAGES

TABLE 32
Methanol content occurring in foods

Product	Level (ppm)	Reference
Anise brandy, Ouzo	Trace–192	VCF (1999)
Anise brandy, Pastis	48	VCF (1999)
Anise brandy, Raki	300–563	VCF (1999)
Apple brandy	NR	VCF (1999)
Apple fresh	NR	VCF (1999)
Apple juice	NR	VCF (1999)
Arctic bramble (*Rubus arcticus* L.)	<0.05	VCF (1999)

TABLE 32
Methanol content occurring in foods *(Continued)*

Product	Level (ppm)	Reference
Arrack	25.6–750	VCF (1999)
Banana (*Musa sapientum* L.)	15	VCF (1999)
Bantu beer	NR	VCF (1999)
Beans (heated)	NR	VCF (1999)
Beans (raw)	1.3	VCF (1999)
Beef (boiled, cooked)	NR	VCF (1999)
Beef (canned)	NR	VCF (1999)
Beer	NR	VCF (1999)
Black currants—berries	4	VCF (1999)
Blackberry brandy	320	VCF (1999)
Blue cheeses	NR	VCF (1999)
Blueberry, rabbiteye (*Vaccinium ashei* Raede)	NR	VCF (1999)
Brandy	181–2425	Filer and Stegink (1989)
Brussels sprouts (*Brass. oleracea* var. *gemmifera*)	NR	VCF (1999)
Butter	<0.009	VCF (1999)
Cabbage (cooked)	0.6	VCF (1999)
Calamondin juice (*Citrus mitis* Blanco)	9	VCF (1999)
Carrot (*Daucus carota* L.)	Trace-0.05	VCF (1999)
Cauliflower (cooked)	NR	VCF (1999)
Cauliflower (raw)	NR	VCF (1999)
Cheddar cheese	NR	VCF (1999)
Cheese, Manchego cheese	NR	VCF (1999)
Cheese, Parmesan cheese	NR	VCF (1999)
Cheese, Provolone cheese	NR	VCF (1999)
Cheese, sheep	NR	VCF (1999)
Cheeses, other	NR	VCF (1999)
Cherry brandy	NR	VCF (1999)
Cherry, sweet (*Prunus avium* L.)	NR	VCF (1999)
Chicken (cooked)	NR	VCF (1999)
Cider	NR	VCF (1999)
Cloudberry (*Rubus chamaemorus* L.)	NR	VCF (1999)
Cocoa	NR	VCF (1999)
Coffee	NR	VCF (1999)
Corn, sweet	NR	VCF (1999)
Crab	NR	VCF (1999)
Dalieb, palmyra palm fruit (*Borassus aethiopum* L.)	28	VCF (1999)
Durian (*Durio zibethinus*)	NR	VCF (1999)
Fig dried	<1	VCF (1999)
Fig fresh	5	VCF (1999)
Filbert, hazelnut (roasted)	NR	VCF (1999)
Fish, fatty (smoked)	NR	VCF (1999)
Fish, lean (raw)	NR	VCF (1999)
Gin	76	VCF (1999)
Grape brandy, Armagnac	200–332	VCF (1999)
Grape brandy, Cognac	150–290	VCF (1999)
Grape brandy, other types	NR	VCF (1999)
Grape brandy, Weinbrand	240–2360	VCF (1999)
Grape juice	12–680	Filer and Stegink (1989)

(Continued on next page)

TABLE 32
Methanol content occurring in foods *(Continued)*

Product	Level (ppm)	Reference
Grapefruit juice (*Citrus paradisi*)	0.2–40	VCF (1999)
Grapefruit peel oil	NR	VCF (1999)
Guava fruit (*Psidium guajava* L.)	NR	VCF (1999)
Honey	NR	VCF (1999)
Katsuobushi (dried bonito)	NR	VCF (1999)
Kiwifruit (*Actinidia chinensis*, syn. *A. deliciosa*)	NR	VCF (1999)
Kumazasa (*Sasa albo-marginata*)	NR	VCF (1999)
Laurel (*Laurus nobilis* L.)	NR	VCF (1999)
Lemon juice (*Citrus limon* Burm. f.)	NR	VCF (1999)
Lemon peel oil	NR	VCF (1999)
Lime juice (*Citrus aurantifolia*, *C. latifolia*)	NR	VCF (1999)
Mandarin, Satsuma, peel oil	NR	VCF (1999)
Mango fresh (*Mangifera indica* L.)	NR	VCF (1999)
Melon	NR	VCF (1999)
Mushroom, other varieties	NR	VCF (1999)
Mutton (boiled)	NR	VCF (1999)
Olive (*Olea europae*)	NR	VCF (1999)
Onion (*Allium cepa* L.)	NR	VCF (1999)
Orange juice (*Citrus sinensis* L. Osbeck)	0.8–80	VCF (1999)
Orange peel oil	NR	VCF (1999)
Papaya (*Carica papaya* L.)	NR	VCF (1999)
Parsley leaves	NR	VCF (1999)
Parsnip root cooked	NR	VCF (1999)
Parsnip root raw	NR	VCF (1999)
Passiflora edulis Sims juice (purple)	4	VCF (1999)
Passion fruit wine	NR	VCF (1999)
Peanut (raw)	NR	VCF (1999)
Peanut (roasted)	NR	VCF (1999)
Peanut butter	NR	VCF (1999)
Pear brandy	4300–9300	VCF (1999)
Peas (*Pisum sativum* L.)	1	VCF (1999)
Plum brandy	NR	VCF (1999)
Plum fresh (*Prunus domestica* cultivars)	NR	VCF (1999)
Plum, Japanese (*Prunus salicina* Lindl cultivars)	NR	VCF (1999)
Pork liver	NR	VCF (1999)
Pork, cured (boiled, cooked)	NR	VCF (1999)
Pork, uncured (boiled, cured)	NR	VCF (1999)
Potato baked	NR	VCF (1999)
Potato cooked or boiled	NR	VCF (1999)
Potato raw	NR	VCF (1999)
Quince (*Cydonia oblonga*, Mill.) (Marmelo)	NR	VCF (1999)
Raspberry brandy	NR	VCF (1999)
Raspberry (*Rubus idaeus* L.)	7–16	VCF (1999)
Rice bran	NR	VCF (1999)
Rice unprocessed	NR	VCF (1999)
Rum (qualitative data of all categories of rum)	NR	VCF (1999)
Rum category I (total volatiles > 3600ppm)	36	VCF (1999)
Rum category II (total volatiles 1100-3600 ppm)	0.08-25	VCF (1999)
Rum category III (total volatiles 240-1100 ppm)	8-25	VCF (1999)

TABLE 32
Methanol content occurring in foods *(Continued)*

Product	Level (ppm)	Reference
Sage, Clary (*Sal*via*sclarea* L.)	NR	VCF (1999)
Sake	NR	VCF (1999)
Sesame seed (roasted)	NR	VCF (1999)
Sherry	89–163	VCF (1999)
Shoyu (fermented soya hydrolysate)	NR	VCF (1999)
Shrimps (raw)	NR	VCF (1999)
Soft drinks containing aspartame	55	Filer and Stegink (1989)
Southernpea (*Vinga unguiculata* L.)	NR	VCF (1999)
Soybean	0.02–6	VCF (1999)
Soyprotein (hydrolyzed)	NR	VCF (1999)
Spearmint oil (*Mentha spicata* L.)	NR	VCF (1999)
Spearmint, Scotch, oil (*Mentha cardiaca* Ger.)	NR	VCF (1999)
Strawberry fruit	0.01–5	VCF (1999)
Sweet potato (heated)	NR	VCF (1999)
Tangerine juice	NR	VCF (1999)
Tea, black	0.01–1.8	VCF (1999)
Tomato	64–229	VCF (1999)
Tomato Juice	180–218	Filer and Stegink (1989)
Vinegar	NR	VCF (1999)
Vitis species, other	0.032	VCF (1999)
Vitis vinifera L.	0.04	VCF (1999)
Whiskey, bourbon	60–106	VCF (1999)
Whiskey, Irish	15–56	VCF (1999)
Whiskey, Canadian	48–60	VCF (1999)
Whiskey, malt	<20–160	VCF (1999)
Whiskey, Scotch blended	<20–72	VCF (1999)
Wine, Botytised	25–72	VCF (1999)
Wine, Port	230	VCF (1999)
Wine, red	0.09–209	VCF (1999)
Wine, red	99–271	Filer and Stegink (1989)
Wine, rose	48–101	VCF (1999)
Wine, sparkling	0.086–119	VCF (1999)
Wine, special	NR	VCF (1999)
Wine, white	8.1–116	VCF (1999)
Wine, white	20–36	Filer and Stegink (1989)

Note. NR = not reported, constituent was detected but not quantified; ppm = parts per million.

APPENDIX III: FORMALDEHYDE CONTENT OF COMMON FOODS

TABLE 33
Formaldehyde content of common foods (Owen et al., 1990)

Food source	Formaldehyde content (mg/kg)
Dairy	NR
Milk	0.3–3.3
Yogurt	0.3–3.3
Butter	0.1
Cheese	0.3–1.2
Eggs	0.2–1.2
Yolks	5.5
Provolone cheese	3.14–56.84
Fruit	NR
Apples	1.7–22.3
Pears	6.0–38.7
Grapes	2.9–3.3
Tomatoes	5.7–16.7
Breads and cereals	NR
Crust, crumbs	NR
Method of baking	NR
Straight	2.0–9.9
Sponge	2.0–9.8
No time	1.7–8.6
Preferment	1.4–10.2
Vegetables	NR
Carrots	0.3–10.0
Cucumber	2.3
Cauliflower	6.6
Cabbage	4.7–5.3
Radish	3.7–4.4
Spinach	3.3–25.0
Onions	13.3–26.3
Meat and fish	NR
Raw sausage	NR
Filling	2.0–30.6
Skin	34.0–214
Raw marine fish	6.5–13.6
Raw freshwater fish	0.7–0.8
Fresh haddock	20
Kipper	50–1000
Raw meats[a]	NR
Beef	0.7–3.4
Veal	0.7–3.4
Pork	0.7–3.4
Mutton	0.7–3.4
Chicken	2.3–5.7
Smoked foods	NR
Wurst	1.26
Herring	0.65
Meats	Trace
Vacuum-packed meats	0.7–2.8
Smoked bacon	NR
Inner layer	0.8–11.5
Outer layer	3.5–52.0
Smoked ham, outer layer	224–267
Boiled smoked sausage	0.7–32.2
Smoked marine fish	3.5–20.0
Smoked freshwater fish	1.5–8.8
Other	NR
Maple syrup	Up to 2
Beer	0.009
Coffee	1.00
Soy bean	Trace–0.1

[a]Calabrian sausage, smoked pork fat, speck, lard, Hungarian salami, puriser; NR = not reported.

APPENDIX IV: TRANSGENIC MICE MODELS USED BY THE NATIONAL TOXICOLOGY PROGRAM FOR THE EVALUATION OF THE CARCINOGENICITY OF ASPARTAME

The use of genetically altered or transgenic models for cancer research is a result of years of research into the genetic mechanisms for cancer development. It is now generally accepted that there are at least two major classes of genes involved in carcinogenesis, tumor suppressor genes and oncogenes. Loss of function (inactivation) of tumor suppressor genes or gain of function (activation) of oncogenes has been identified in the majority of human cancers. Advances in molecular biology have provided tools to inactivate or insert these specific genes.

According to the Knudson two-hit hypothesis (Knudson et al., 1975; Knudson, 1996) of carcinogenesis, genetically altered models should be more susceptible to induction of tumors by genotoxic carcinogens because they already carry one mutation in their germline. Thus, these models should represent a more sensitive and rapid detection system for genotoxic carcinogens than the classic 2-year chronic rodent bioassays using wild-type animals. The most commonly used models are the $p53^{+/-}$, Tg.AC, Tg*ras*H2 and the $XPA^{-/-}$ (Jacobson-Kram et al., 2004). Only the three models that have been used for assessment of aspartame carcinogenicity are discussed here. These are the $p53^{+/-}$ mouse, the Tg.AC mouse, and the Cdkn2a deficient or *INK4a*/*ARF* model.

Description of Models

p53 Model

The heterozygous p53 mouse model was designed based on extensive evidence that p53 is a commonly mutated tumor suppressor gene in a wide variety of human tumors. The function of a tumor suppressor gene is to "guard the genome" and expression of the wild-type gene product is necessary for proper function and suppression of oncogenic events. Loss of functional protein, either due to mutations in the protein or loss of the gene, results in greatly increased susceptibility to tumor development. Thus mice that are completely deficient in p53 because of homozygous null allele (−/−) will spontaneously develop tumors (primarily lymphomas and sarcomas) within the first 3 to 6 months of life. The heterozygous $p53^{+/-}$ mouse model has one copy of the functional wild type, and one null allele, which is not transcribed or translated, resulting in a lower level of p53 protein (French et al., 2001). These mice develop tumors spontaneously as well, but at a much lower incidence and longer latency time (approximately 9 months) as compared to the homozygous null mice.

As heterozygous p53 mice already carry one mutation in their germline, they should be more susceptible to induction of tumors by genotoxic carcinogens. Thus one explanation for the increased sensitivity of heterozygous p53 model to tumor development is that the carcinogen causes mutation or loss of the second copy of p53, completely depleting the animal of the tumor suppressor gene product. Alternatively, even without a direct hit on the second p53 gene, the lower amount of p53 protein may cause an acceleration of tumor development initiated in the other gene. This is termed a gene dosage effect (Venkatachalam et al., 2001). The limitation of this model is that it does not detect nongenotoxic carcinogens in a short (6-month) protocol. A longer 9-month protocol, as was used in the NTP study, was recommended.

Storer et al. (2001) reviewed the available data from carcinogenicity studies with the $p53^{+/-}$ transgenic mouse model to assess its usefulness as a short-term carcinogenicity assay. A total of 48 different compounds had been tested, some in multiple studies. All studies were conducted using a standardized protocol approved by ILSI. In all cases, the mice were $p53^{+/-}$ heterozygous mice; however, the background strain varied, including C3H, CBA, MIH, and C57BL6. The duration of the study was 26 weeks. Overall, 42 of the 48 compounds gave results that were concordant with expectations. In general, nongenotoxic compounds were negative, and most genotoxic carcinogens were positive. *p*-Cresidine gave positive results in 18/19 studies for bladder cancer.

In addition to use in studies for regulatory carcinogenicity assessments, the $p53^{+/-}$ transgenic model has been widely used in the cancer research community to assess dietary agents, exercise and pharmaceutical chemopreventive agents for their promotional or inhibitory effects on cancer development. Although a review of these studies is beyond the scope of this monograph, Hursting et al. (2004) provides an excellent example of use of p53-deficient mice models by the National Cancer Institute to investigate diet–gene interactions.

Tg.AC Model

This strain contains the v-Ha-*ras* oncogene, which has been activated with two mutations. The expression of the product of the gene is regulated by a promoter and is not normally expressed in adult tissues. Exposure to UV light, specific chemicals, and full-thickness wounding induces expression of the transgene, which is necessary to invoke tumor development (Tennant et al., 1995, 1998). The model can detect both genotoxic and nongenotoxic carcinogens, but was not positive for chemicals that have shown a strain-specific or species-specific response in a 2-year bioassay (Tennant et al., 2001). Thus, the initial concerns that this model would have many false positives have proven to be invalid. In reviews of over 40 studies with this model, the Tg.AC model was more prone to false negatives (Eastin et al., 2001; Pritchard et al., 2003).

This model is most widely accepted for testing of dermal applications as the skin of this genetically-altered mouse acts as if already initiated with a carcinogen, i.e., skin papillomas will develop within 12 weeks following the application of classic promoter compounds (such as TPA) without a prior application of a carcinogen (Jacobson-Kram et al., 2004).

The oral route of administration of carcinogens also generates tumors, including squamous cell papillomas and carcinomas of the forestomach (Tennant et al., 1998). Although this model has also been used with oral exposure, it has been more fully evaluated with dermal applications.

Cdkn2a-Deficient Model

This model is not widely used, and therefore has not been as well characterized or evaluated as the two already described. It was developed in 1996 by Serrano et al. (1996), and is based on the frequent detection of mutations and deletions in the Cdkn2a gene in a wide variety of tumors. Of special importance is the evidence that genetic alterations in this gene play an important role in human brain cancers (Ohgaki et al., 2004), making use of this model particularly relevant for suspected brain carcinogens.

The role of the Cdkn2a gene is complex. This gene codes for a number of proteins that function as cyclin-dependent kinase (CDK) inhibitors. Depending on the exon, open reading frame, and polyadenylation sites used, the resulting Cdkn2a gene transcript will be the $p16^{Ink4a}$ variant or the $p19^{Arf}$ variant. The $p16^{Ink4a}$ variant codes for a protein (called p16) that functions to inhibit CDK4, and the $p19^{Arf}$ variant protein product (called p19) functions to stabilize p53 protein. The presence of p16 results in inhibition of CDK4, preventing phosphorylation of pRb, which in turn blocks the transition from G1 to the S phase. The presence of p19 results in sequestration of the protein Mdm2,

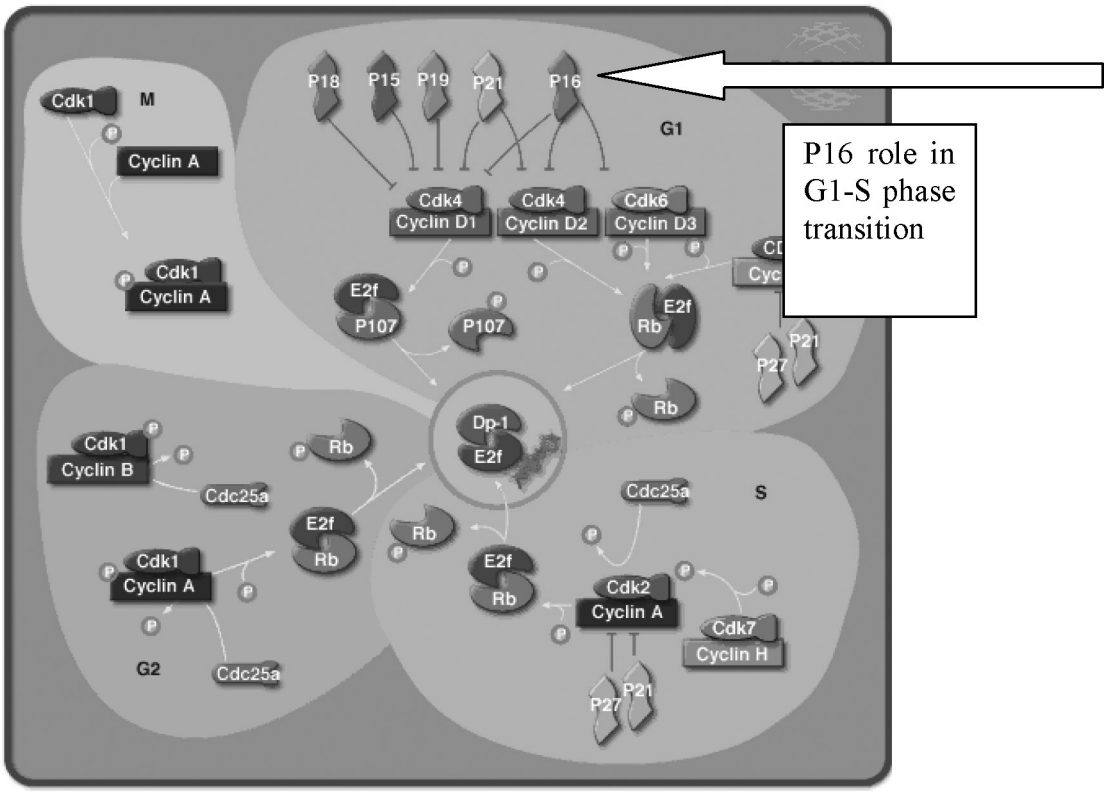

FIG. 1. The role of p16 in the G1-S phase transition of the cell cycle (http://www.biocarta.com/pathfiles/m_cellcyclePathway.asp).

which prevents Mdm2 from binding to p53 and targeting p53 for degradation. Thus, p53 is available to prevent the G2 to M phase transition. Thus both proteins from the Cdkn2a gene play a critical role in cell cycle regulation (Lubet et al., 2005). The pathways illustrating the role of these proteins in cell cycle regulation from Biocarta[44] are shown in Figure 1 and Figure 2.

The net result of these actions is that the Cdkn2a gene is acting as a tumor suppressor gene, and loss of the functions of this gene, as in the transgenic Cdkn2a deficient model, increases susceptibility to tumor formation. This model is also referred to as the *INK4a*/*ARF* model because of the two variants described above that are affected. Although the model has not been widely used in studies assessing carcinogenicity of chemicals, it is a well-established model for cancer research due to the prevalence of mutations in this locus in commonly occurring cancers such as breast cancer (D'Amico et al., 2003).

APPENDIX V: USE OF TRANSGENIC MODELS IN REGULATORY EVALUATIONS

The NTP studies have not been published in the peer-reviewed literature, although the report was peer-reviewed within the NTP. The purpose of this review was to assess whether there is general agreement in the literature that there is a "current lack of appreciation of the scientific or public health value of negative cancer findings in these models" as stated by Dr. David Schwartz, Director of NIEHS, on September 27, 2005. This has been stated as the rationale for not making public the findings of these studies.

ILSI-ACT

The p53 and Tg.AC transgenic mice models used in the NTP study were also evaluated during a workshop organized by the International Life Sciences Institute Alternative Carcinogenicity Testing (ILSI-ACT) committee. Cohen (2001) summarized the results of assays conducted with 21 chemicals using the standard mouse and rat long-term assay and compared with the results of testing the same chemicals in nine transgenic models and short-term assays. The mechanistic basis for these models, and data obtained from studies with a number of chemicals, were reviewed and summarized by workshop participants. These reports were then provided to a group of international cancer experts from government, industry and academic organizations. Following their evaluation, a panel discussion was held with these individuals and workshop participants to address the appropriateness of the alternative models to human cancer risk assessment (Pettit, 2001; Popp, 2001). The general consensus in this group was that models had significant value as part of a weight-of-evidence approach to assessing human carcinogenic risk. All of the models

[44]http://www.biocarta.com/pathfiles/m_cellcyclePathway.asp.

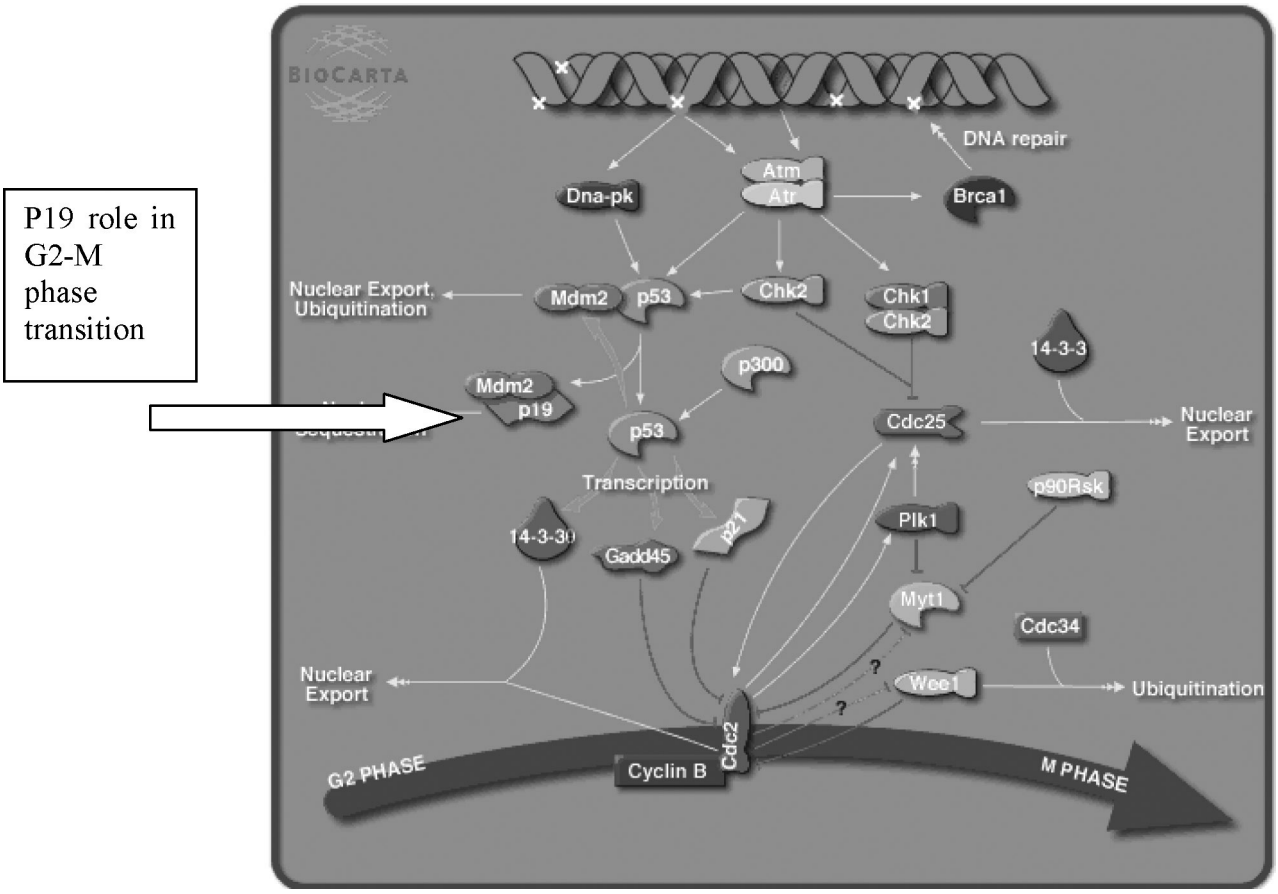

FIG. 2. The role of p19 in the G2-M phase transition of the cell cycle (http://www.biocarta.com/pathfiles/m_cellcyclePathway.asp).

were expected to detect genotoxins, with differing responses depending on mechanism of action of the carcinogen in question. For example, the carcinogen may act primarily to activate tumor oncogenes or to cause mutation or deletion of tumor suppressor genes. Although all transgenic models have limitations and are not designed to be stand-alone assays, they are considered to be valuable for consideration in an overall risk assessment (Cohen, 2001; Goodman, 2001).

FDA

Jacobson-Kram and colleagues (2004) recently reviewed the use of transgenic mice in carcinogenicity hazard assessment by FDA. Use of transgenic models in carcinogenicity testing protocols was formalized in the FDA's 1997 adoption of the Guidance for Industry S1B Testing for Carcinogenicity of Pharmaceuticals (http://www.fda.gov/cder/guidance/1854fnl.pdf), "opening the door" for use of transgenic models in regulatory toxicology assessment. The FDA paradigm is that combining a traditional rat bioassay with a 6-month transgenic study for an overall assessment of the weight-of-evidence results in fewer false positives and no increase in false negatives. Analyses to assess the sensitivity (no false negatives) and specificity (no false positives) to give an overall accuracy score for specific transgenic models and traditional bioassays, as compared to a combined approach, provide support for this paradigm (Table 34) (Pritchard et al., 2003; Jacobson-Kram et al., 2004).

European Regulatory Authorities

In a short opinion paper, van der Lann et al. (2002) stated that similar to long-term rodent bioassays, transgenic models have less-than-perfect accuracy as there are some reports of false negatives and false positives when compared to other supporting data for a specific chemical. With that acknowledgment, the conclusion of a review of the current weight of evidence was that "regulatory authorities cannot neglect the outcome of such studies, but need to be cautious in their interpretation of the data from such models, and the application in risk assessment problems."

More recently, the ILSI Alternatives to Carcinogenicity Testing Committee held a workshop to reassess data from genetically modified mouse model studies that have been evaluated by international regulatory agencies. The perspectives of the U.S.

TABLE 34
Summary of performance of transgenic assays, NTP rodent assays, and combinations (adapted from Pritchard et al., 2003)

Strategy	+ for carcinogen	− for noncarcinogen	+ for noncarcinogen	− for carcinogen	Overall accuracy
$p53^{+/-}$	21	27	1	10	48/59 (81%)
$p53^{+/-}$ (genotoxic)	16	6	0	4	22/26 (85%)
Tg.AC	17	29	10	6	44/62 (74%)
RasH2	21	18	5	7	39/51 (76%)
$p53^{+/-}$(genotoxic) and Ras H2	30	14	5	4	44/53 (83%)
$p53^{+/-}$(genotoxic) and Tg.AC	25	22	10	4	47/61 (77%)
NTP rodent bioassay	23	17	18	0	40/58 (69%)
NTP rodent bioassay and $p53^{+/-}$ (genotoxic) and Tg.AC (nongenotoxic)	35	13	9	0	48/57 (84%)
NTP rat bioassay and $p53^{+/-}$ (genotoxic) and RasH2 (nongenotoxic)	33	12	8	0	45/53 (85%)

Note. Definitions: positive for carcinogens, positive assay results for NTP rodent carcinogens; negative for noncarcinogens, negative assay results for NTP rodent noncarcinogens; positive for noncarcinogens, positive assay results for NTP rodent noncarcinogens; negative for carcinogens, negative assay results for NTP rodent carcinogens.

FDA, the European Committee for Proprietary Medicinal Products Safety Working Party, and the Japanese Ministry of Health Labor and Welfare discussed at that workshop were published (MacDonald et al., 2004). Overall agreement was expressed that the alternative assays can and do have an important role in regulatory carcinogen safety assessment. However, specific concerns were raised.

For the $p53^{+/-}$ model, a study duration of 9 months is recommended, whereas a 6-month duration is considered adequate for the Tg.AC and Tg.rasH2 models. The number of animals per group should be 25, rather than the originally proposed size of 15 per group, to provide adequate statistical power. This model is considered appropriate for carcinogens that have been shown to be positive in genotoxicity tests, but may not be adequately sensitive to detect nongenotoxic carcinogens.

It is considered advantageous to include positive controls for all models. For the $p53^{+/-}$ model, the positive control that has been used is *p*-cresidine; however, an alternative one is being sought due to lack of consistent results with *p*-cresidine. For the Tg.AC models, TPA administered three times per week is recommended. The Tg.AC model is most appropriate for testing compounds where dermal exposure is relevant. The usefulness of this model for testing compounds where the human exposure will be oral is not well established, regardless of whether the exposure to the mice is dermal or oral.

The genetic background of the parental strain will influence a model's tumor spectrum and may result in "blind spots" or tumor-resistant organs. This needs to be further investigated in all models. Wild-type parental strains should be included in determinations of maximum tolerated dose levels for the genetic models.

Lastly, the generally recognized incidence of tumors at rare sites that should be considered treatment-related was 2 out of 15 animals when the group size is 15. More data will be needed to establish the significant incidence level with larger groups (MacDonald et al., 2004).