Review
Toxicology of food dyes

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Background: Food dyes, synthesized originally from coal tar and now petroleum, have long been controversial because of safety concerns. Many dyes have been banned because of their adverse effects on laboratory animals or inadequate testing.
Conclusions: This review finds that all of the nine currently US-approved dyes raise health concerns of varying degrees. Red 3 causes cancer in animals, and there is evidence that several other dyes also are carcinogenic. Three dyes (Red 40, Yellow 5, and Yellow 6) have been found to be contaminated with benzidine or other carcinogens. At least four dyes (Blue 1, Red 40, Yellow 5, and Yellow 6) cause hypersensitivity reactions. Numerous microbiological and rodent studies of Yellow 5 were positive for genotoxicity. Toxicity tests on two dyes (Citrus Red 2 and Orange B) also suggest safety concerns, but Citrus Red 2 is used at low levels and only on some Florida oranges and Orange B has not been used for several years. The inadequacy of much of the testing and the evidence for carcinogenicity, genotoxicity, and hypersensitivity, coupled with the fact that dyes do not improve the safety or nutritional quality of foods, indicates that all of the currently used dyes should be removed from the food supply and replaced, if at all, by safer colorings. It is recommended that regulatory authorities require better and independent toxicity testing, exercise greater caution regarding continued approval of these dyes, and in the future approve only well-tested, safe dyes.

Introduction

Synthetic dyes have been used to artificially color foods in industrialized nations for at least a century, and they are used in thousands of foods in the United States.[1] Foods are artificially colored to make unattractive mixtures of basic ingredients and food additives acceptable to consumers. (For a list of all approved synthetic and natural colorings, see FDA, 2007). Added colors can also mask the absence of brightly colored natural ingredients such as fruit.

Dyes are complex organic chemicals that were originally derived from coal tar, but now are made from petroleum. Industrial food producers use synthetic dyes because they are cheaper, more stable, and brighter than most natural colorings. However, they raise significant health concerns. Over the past century, more food dyes have been found to be risky than any other category of food additive. [Banned dyes include: Green 1: liver cancer (animals); Orange 1 and Orange 2: organ damage (animals); Orange B (ban never finalized): contained low levels of a cancer-causing contaminant (it was used only in sausage casings, but is no longer used in the US); Red 1: liver cancer (animals); Red 2: possible carcinogen; Red 4: high levels damaged adrenal cortex of dog;

Red 32: damages internal organs and may be a weak carcinogen (since 1956, it continues to be used as Citrus Red 2 only to color oranges at 2 parts per million); Sudan 1: toxic and carcinogenic (animals); Violet 1: cancer (animals) (was used to stamp the US Department of Agriculture's (USDA) inspection mark on beef carcasses); Yellow 1 and Yellow 2: high dosages caused intestinal lesions (animals); Yellow 3: high dosages caused heart damage (animals); Yellow 4: high dosages caused heart damage (animals). Note, though, that in some cases companies did not bother to go to the expense of re-testing chemicals, which may not have accounted for significant sales, that might have been harmful only at high dosages and not at the lower dosages consumed in foods. http://cspinet.org/reports/chemcuisine.htm.] At this time, consumers' growing preference for natural foods is leading some companies to either avoid colorings or to switch to safe natural colorings, such as beta-carotene (a precursor to vitamin A), paprika, and beet juice. That trend is stronger in Europe than the United States, but some US companies recognize that an ''All Natural'' label can attract customers and may be moving in that direction.

Just three dyes — Red 40, Yellow 5, and Yellow 6 — account for 90% of all dyes used. US Food and

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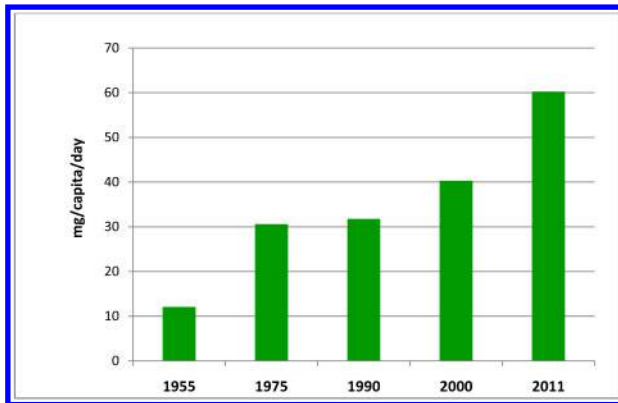
Figure 1   Food dyes marketed per capita per day (mg).

Drug Administration (FDA) data show a dramatic fivefold increase in consumption of dyes since 1955 (see Fig. 1) as people in the United States have increasingly relied on processed foods such as soft drinks, breakfast cereals, candies, snack foods, baked goods, frozen desserts, and even pickles and salad dressings, that are colored with dyes.[2] This paper includes a brief discussion of the laws regulating food dye use, followed by toxicological evaluations of four dyes: Red 3, Red 40, Yellow 5, and Yellow 6. Additional information about those dyes and evaluations of the other food dyes — Blue 1, Blue 2, Citrus Red 2, Green 3, and Orange B — are provided in the Appendix.

Methods
Eligibility criteria
Information about the nine dyes currently approved by the FDA was obtained from published, peer-reviewed studies, as well as unpublished studies and other writings, that were used in the risk assessment and approval process. Studies were not restricted by date, language, or source. Studies judged to be of inadequate and/or unnecessary for this review were excluded.

Search strategy and study selection
Articles and information were found using the following five methods: (1) searches of the US National Library of Medicine's PUBMED; (2) publicly available government documents; (3) internet searches; (4) news articles; and (5) personal correspondence and memoranda in the Center for Science in the Public Interest's (CSPI) files. Relevant articles were identified in PUBMED without restriction on date or source for experimental studies. The primary search strategy was entering each dye as a key word in multiple formats including: "FD&C Yellow #5", "Tartrazine", "Yellow 5", and "FD&C Yellow No. 5". Other key words included: "toxicity", "hypersensitivity", "chronic", "metabolism", and "carcinogenicity". Government documents, predominantly those published in the Federal Register, were obtained from http://www.federalregister.gov or http://www.heinonline.org (subscription required). Other

documents were collected from requests of the authors. Information from websites used the most updated webpage versions; access dates are provided in references. News articles were not restricted by region or date and were obtained from personal collections of the authors or through archival searches, including http://www.nexis.com (subscription required). Personal correspondence and memoranda were between the authors and another party via phone, mail, or email or from meetings. Original letters and emails are saved and are in possession of the authors.

Data extraction
Data used in this review were taken directly from the cited sources. In several cases, information was confirmed with the authors of the original text or with experts in the field.

Food Dyes and the Law
Prior to 1960, US law required that dyes be absolutely "harmless", regardless of dose — a virtual impossibility.[3] Congress passed the 1960 Color Additives Amendment in order to loosen requirements on food dye use, while retaining, along with the FDA, special concerns about the safety of food dyes. James T. O'Reilly, an adjunct professor at the University of Cincinnati College of Law, observed that "Congress felt that … colors deserved greater regulation because of their lesser net benefit to society than such items as food preservatives and common spices".[4] For instance,

- Congress required that each batch of food dyes, but not other colorings (such as from carrots or grape skins), be tested and certified to contain only acceptable levels of contaminants, such as lead and benzidine. Food additives, such as preservatives or flavorings, are not subject to such testing.
- Congress did not permit companies to declare that any dyes are "generally recognized as safe" (GRAS), and thereby not further regulated by the FDA. In contrast, companies are permitted to declare flavorings, emulsifiers, and other such ingredients to be GRAS, even in the absence of toxicity testing.
- The FDA's definition of safety for color additives states that "safe means that there is convincing evidence that establishes with reasonable certainty that no harm will result from the intended use of the color additive".[5] The term "convincing evidence" is a stronger standard of proof than that used for non-color additives.

Members of Congress have emphasized that the safety standard for artificial colorings should be particularly high, because the colorings do not offer any health benefit to offset even small risks. Representative Ted Weiss (D-NY) said, "It doesn't make any difference how much or how little (of a carcinogenic additive) a particular substance contains, especially when you've got a color additive that has no nutrient value and no therapeutic value."[6] Representative King (It is unclear which Rep. King

was quoted in the case: Rep. Cecil King (D-CA) or Rep. David King (D-UT).) said, "The colors which go into our foods and cosmetics are in no way essential to the public interest or the national security …. Consumers will easily get along without (carcinogenic colors)."[7] Unfortunately, as evidenced by the continual approval of dyes for which there is evidence of carcinogenicity, enforcement of the 1960 law has been inadequate.

The FDA has also established legal limits for cancer-causing contaminants in dyes. Those tolerances are intended to ensure that a dye will not pose a lifetime risk of greater than one cancer in one million people.[8] FDA chemists test each batch of dye to confirm that those tolerances are not exceeded. Unfortunately, the FDA's process suffers from several problems. For one thing, those tolerances are based on 1990 dye usage, but per-capita usage has increased by about 50% since then. Second, the FDA did not consider the increased risk that dyes pose to children, who are both more sensitive to carcinogens and consume more dyes per unit of body weight than adults.[9] Third, and most importantly, the tests do not look for "bound" carcinogens (those that occur as parts of larger molecules and are freed during digestion), but generally only "free" contaminants.[10]

Consumer activists have long sought to persuade the FDA to ban dyes. In the early 1970s, CSPI urged the government to ban Violet 1, which was the coloring used in the USDA's meat inspection stamp, because it appeared to cause cancer in animal studies (the dye was banned in 1973). Subsequently, in the 1970s and 1980s, Public Citizen's Health Research Group petitioned and sued the FDA to ban food dyes.[11] In 2008, CSPI petitioned the FDA to ban colors because of their adverse effects on children's behavior.

Even if all color additives were deemed safe, many uses of colorings, both synthetic and natural, still could be considered illegal under the Food, Drug, and Cosmetic Act. Sections 402(b)(3) and (b)(4) of that law stipulate that "A food shall be deemed to be adulterated … (3) if damage or inferiority has been concealed in any manner; or (4) if any substance has been added thereto or mixed or packed therewith so as to … make it appear better or of greater value than it is." Section 403 of the same law says that a food is misbranded "if its labeling is false or misleading in any particular".

Food colorings added to fruit drinks, frozen desserts, gelatin desserts, salad dressings, child-oriented breakfast cereals and snack foods, and countless other products conceal the absence of fruits, vegetables, or other ingredients and make the food "appear better or of greater value than it is". Defenders of colorings would say that consumers could simply read the list of ingredients on the back of the package to detect the presence of colorings and/or absence of nutritive ingredients, but it may be unfair to put that burden on consumers. It is worth noting that the use of artificial flavorings must be declared conspicuously as part of the product names on the front labels.[12] The FDA could require the same of artificially colored foods. A national poll commissioned by CSPI and conducted by Opinion Research Corporation in January 2010 found that 74% of respondents favored such labeling.

Toxicology Review of Individual Dyes

A summary of study results for all dyes are found in Table 1.

The currently approved FD&C dyes are Blue 1 (Brilliant Blue), Blue 2 (Indigo Carmine), Citrus Red 2, Green 3 (Fast Green FCF), Orange B, Red 3 (Erythrosine), Red 40 (Allura Red), Yellow 5 (Tartrazine), and Yellow 6 (Sunset Yellow). Blue 1, Red 40, Yellow 5, and Yellow 6 cause allergic reactions. Blue 1 did not cause tumors in rats and one unpublished study reported kidney tumors in mice; however, the latter study did not include an *in utero* exposure. An *in vitro* study showed that Blue 1 inhibited nerve cell development. Blue 2 did not induce tumors in mice, but neither study was long enough nor included an *in utero* exposure. Blue 2 possibly causes brain and bladder tumors in rats. Citrus Red 2 induced bladder and other tumors in mice and bladder tumors in rats. A study on Green 3 without *in utero* exposure did not induce tumors in mice. However, a rat study possibly induced bladder and other tumors. Orange B was found to be toxic in rats but was not carcinogenic in mice. The mouse studies did not include *in utero* exposure. Red 3 was not carcinogenic in mice, but the only study did not include an *in utero* exposure. Red 3 did induce thyroid tumors in rats. Red 40 is often contaminated with aniline. It possibly induces reticuloendothelial (RE) tumors in mice but did not induce tumors in rats. Yellow 5 has been found to be contaminated with benzidine and 4-amino-biphenyl. It was not carcinogenic in mice, but the only study was too short, did not use the recommended number of animals, and did not include an *in utero* exposure. Yellow 5 also did not induce tumors in rats. Six out of 11 genotoxicity studies were positive. It has also been shown to cause hyperactivity in children. Yellow 6 has been found to be contaminated with benzidine and 4-amino-biphenyl. Yellow 6 did not induce tumors in mice, but these studies did not include an *in utero* exposure. It possibly causes adrenal and testicular tumors in rats.

FD&C Red 3

FD&C Red 3 (Fig. 2), or Erythrosine B, has been used as a food dye since its approval by the USDA in 1907. It is a water-soluble dye with a 58% iodine content.[13] It is used in maraschino cherries, sausage

Table 1 Summary of studies on FD&C dyes

Food dye	Allergic reactions	Carcinogenic contaminants	Tests for cancer*		Other†
			Mouse	Rat	
Blue 1	Yes		No *in utero* studies. One abstract (study not published) reported kidney tumors	No tumors in the only good study	Test tube study found inhibition of nerve cell development
Blue 2			Both studies were too brief and did not include *in utero* exposure	Dosage was likely too low; possible brain and bladder tumors	
Citrus Red 2 (used only on peels of some oranges at 2 parts per million)			Bladder and other tumors	Bladder tumors	
Green 3			The only study did not include *in utero* exposure	Possible bladder and other tumors	
Orange B (no longer used; in 1978 FDA proposed, but never finalized, a ban)			The only two studies did not include *in utero* exposure	Toxic	
Red 3 (FDA has banned it from cosmetics, externally applied drugs, and lakes)			The only study did not include *in utero* exposure.	Thyroid tumors	
Red 40	Yes	Aniline (not all agencies consider aniline to be carcinogenic)	Possible reticuloendothelial tumors of the immune system	No tumors in the only good study	
Yellow 5	Yes	Benzidine, 4-amino-biphenyl	The only mouse study was too brief, used too few mice, and did not include *in utero* exposure	No tumors in the only good study	6 of 13 studies showed genotoxicity. Hyperactivity in children
Yellow 6	Yes	Benzidine, 4-amino-biphenyl	Neither study included *in utero* exposure	Possible adrenal and testicular tumors	

Note: *Tests should be done on both sexes of two rodent species, use sufficient numbers of animals, include *in utero* exposure, last at least 2 years after birth, and use maximally tolerated dosages. Ideally, tests would be conducted by independent labs, but most tests on dyes were conducted by industry.
†In addition, numerous studies have found that mixtures of dyes cause hyperactivity and other behavioral impairments in children.
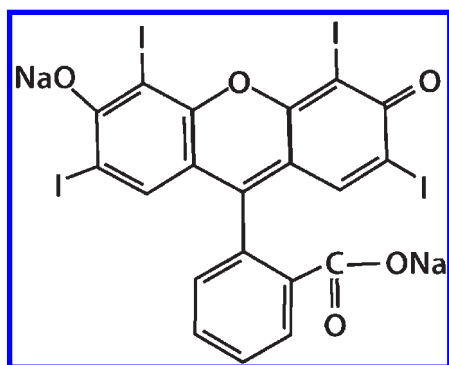
Figure 2   Red 3 (Erythrosine B)

casings, oral drugs, baked goods, and candies. The Acceptable Daily Intake (ADI) for Red 3 is 2.5 milligrams per kilogram of body weight per day (mg/kg bw/day) or 75 mg/day for a 30-kg child.[14] Annual production of Red 3 is equivalent to about 1 mg/person/day (per capita production figures are based on FDA data on the amounts of dyes certified per year).

Metabolism
Osborne–Mendel rats were administered 0.5–500 mg/kg bw Red 3 by stomach tube. Qualitative analysis demonstrated that the dye excreted in the urine or bile was unchanged.[15] In another study, 14 male rats were given one dose (0.5 mg/kg bw) of Red 3. Approximately 55–72% was excreted unchanged in the feces within 3 days. In two bile-duct cannulated rats, 0.44 and 1.67% of the dye was excreted in the bile, indicating that a small amount is absorbed. No color was recovered in the urine. Investigators concluded that "Red 3 is metabolized to some extent in the tissue".[16] Rats administered Red 3 twice weekly for 3 months at doses (according to an industry petition) of 5, 10, 15, and 50 mg/200–250 g bw had elevated serum levels of protein-bound and total iodine.[17] Butterworth et al.[18] also showed that rats administered Red 3 at 0–2% dietary doses over 13 weeks had a dose-related increase in serum levels of protein-bound and total iodine.

In a human study, subjects were orally administered 16 mg of Red 3 for 10 days (more than 15 times typical consumption). Subjects had approximately twice as much protein-bound iodine in their serum compared to levels prior to administration. Levels peaked around days 15–20 and did not return to normal until about 3 months after the beginning of the study.[19]

In vitro effects on neurotransmitters
Red 3 was applied to isolated frog neuromuscular synapses to test its effect on neurotransmitter release using electrophysiological techniques. Concentrations of 10 μmol/l and greater caused an irreversible, dose-dependent increase in acetylcholine release. Investigators concluded that Red 3 may alter the function of more complex systems, but any conclusions regarding its effects on mammalian behavior would be premature given the in vitro nature of the study.[20]

Genotoxicity
Of nine genotoxicity studies on Red 3, four were positive, including one in vivo study, which demonstrated the genotoxic potential of the dye.[21–23] (Table 2 shows numbers of positive and negative genotoxicity tests for each food dye studied.) Of particular concern is that the positive results were in studies using mammalian cells or an in vivo method (comet assay), while most of the negative results came from prokaryotic systems. Some of the key genotoxicity studies are summarized in Table 3.

Chronic toxicity/carcinogenicity
Chronic toxicity studies focusing on the effects of Red 3 on hematology, thyroxine, and protein-bound iodide in Osborne–Mendel rats did not find any adverse effects. Twenty-five rats/sex/group were fed 0 (the only group with 50 rats/sex), 0.5, 1, 2, or 4% Red 3 for 86 weeks or intubated twice weekly with 0, 100, 235, 750, or 1500 mg/kg Red 3 for 85 weeks. The study did not include an in utero phase. At the end of the treatment periods, the rats were fed the control diet until the studies reached the 2-year mark. The studies found no adverse effects in gross or microscopic pathology and no changes in thyroxine-iodide levels. The levels of protein-bound iodide increased, and it was determined that this was due to increased dye levels in the serum.[24]

Table 2   Number of positive and negative genotoxicity studies of FD&C food dyes

FD&C color (generic name)	Total number of positive studies	Positive in vivo studies	Negative studies
Blue 1 (Brilliant Blue)	2	0	7
Blue 2 (Indigo Carmine)	1	0	10
Green 3 (Fast Green)	3	0	6
Red 3 (Erythrosine)	4	1	8
Red 40 (Allura Red)	3	3	7
Yellow 5 (Tartrazine)	6	2	5
Yellow 6 (Sunset Yellow)	2	1	6

Note: The numbers of "Positive in vivo studies" are included in "Total number of positive studies."

The Certified Color Manufacturers Association (CCMA) contracted with Borzelleca *et al.*[25] to conduct a chronic toxicity/carcinogenicity study in Charles River CD-1 mice. The maximum duration of exposure of the mice to 0 (two control groups), 0.3, 1, or 3% Red 3 was 24 months (no *in utero* exposure). All groups consisted of 60 males and 60 females. Investigators reported no statistically significant compound-related effects on behavior, morbidity, mortality, hematology, or general physical observations. A statistically significant increase in the incidence of lymphocytic lymphoma was observed in male mice in the 0.3% low-dose group. However, that effect was not considered compound-related, because there was no dose–response relationship, and the incidence of lymphomas in the high-dose group was similar to that in the controls. The NOAELs (no observed adverse effects levels) were deemed to be 3% (4759 mg/kg bw/day) in males and 1% (1834 mg/kg bw/day) in females.[25]

Borzelleca *et al.*[26] also performed two CCMA-sponsored chronic toxicity/carcinogenicity studies in Charles River CD rats. Unlike the mouse study, these studies included an *in utero* phase. In the F_0 generation of both studies, 60 rats/sex/group were fed 0 (two control groups), 0.1, 0.5, or 1% (original study) and 0 or 4% (high-dose study) Red 3. Random offspring were selected for the F_1 generation and 70 rats/sex/group were given the same dietary levels as the F_0 generation. The maximum exposure was 30 months. Investigators reported no compound-related effects on fertility, gestation, parturition, lactation, pup survival through weaning, or numbers of live and stillborn pups. The most notable effects of the chronic feeding phase were statistically significant increases in the incidences of thyroid follicular cell adenomas in male rats in the 4% treatment group (15 adenomas in the 4% group compared to one in the control groups) and non-significant increases in these tumors in female rats in the 0.5, 1, and 4% treatment groups. High-dose (4%) male rats also showed a statistically significant increase in non-neoplastic proliferative changes of the thyroid. The changes included follicular cell hypertrophy and hyperplasia and follicular cystic hyperplasia. Also, 94% of male rats in the 4% treatment group showed proliferative changes of thyroid follicular cells. Based on the results of the two studies, investigators asserted that Red 3 had NOAELs of 0.5 and 1% in male and female rats, respectively.[26]

Reproductive toxicity

In each generation of a 3-generation study on Red 3 in Sprague–Dawley rats, 25 rats/sex/group received 0, 0.25, 1, or 4% of the dye in their chow. The only significant finding was a statistically significant

Table 3 Summary of genotoxicity studies on Red 3

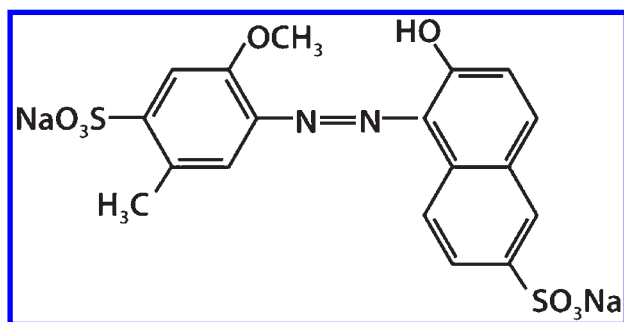| Assay	Mutation type	S9 activation	Dose	Results	Reference
Comet assay	DNA damage	NA	100 mg/kg in glandular stomach and colon; >100 mg/kg in urinary bladder	Positive after 3 hours; negative after 24 hours	23
S. Typhimurium TA1535 and TA100	Base pair	Yes and No	1–10 mg/plate	Negative	13
S. Typhimurium TA1537, TA98 and TA1538	Frameshift	Yes and No	1–10 mg/plate	Negative	
Mouse lymphoma assay (L5178Y/TK$^{+/−}$)	Gene mutation	No	100–600 µg/ml	Negative	
Mouse micronucleus assay	Chromosomal breakage/spindle damage	Yes	24, 80, 240 mg/kg	Negative	
Rec-assay	DNA damage	NA	100–10 000 µg/ml	Negative	
E. coli WP2 uvrA	Base substitution	Yes and No	0.5 mg/ml	Negative	137
In vitro chromosome aberrations in Chinese Hamster fibroblast cells	Chromosome aberrations	No	0.6 mg/ml	Positive	21
Yeast strain D7	Mitotic gene conversion	NA	0–10 mg/ml	Positive	22
Yeast strain XV185-14C	Reverse mutation in eukaryotes	NA	0–10 mg/ml	Positive	
Yeast strain D5	Mitotic recombination	NA	0–5 mg/ml	Negative	

Figure 3   Red 40 (Allura red)

reduction in body weights of parents and pups in all generations at the 4% dietary level, which could have been due to the large consumption of a non-nutritive compound. No compound-related adverse effects on reproductive indices and no gross anomalies were seen. Investigators concluded that the NOAEL for rats was 0.25%, or approximately 149 and 255 mg/kg bw/day for males and females, respectively. That NOAEL was based on the reduced body weight in the 4% group and reduced body-weight gain during gestation in females in the 1 and 4% groups.[27]

FDA efforts to ban Red 3

Red 3 is genotoxic in *in vivo* and *in vitro* assays and is an animal carcinogen. Petitioners seeking Red 3 approval submitted CCMA-sponsored studies after provisional listings in 1960. (Food colorings that were in use when the Color Additives Amendment of 1960 (21 USC § 379e) was passed were 'provisionally listed' pending further testing by industry. Some colorings were subsequently permanently listed, while some were eliminated from the food supply because their safety was not demonstrated, in some cases because industry did not care to market them.)[28] The CCMA studies showed no safety concern, and in 1969 the FDA permanently approved the dye for use in ingested drugs and foods.[29] However, in 1984, FDA's Acting Commissioner, Mark Novitch, said that Red 3 was "of greatest public health concern …. The agency should not knowingly allow continued exposure (at high levels in the case of FD&C Red No. 3) of the public to a provisionally listed color additive that has clearly been shown to induce cancer while questions of mechanism are explored".[11] However, around the same time, Secretary of Agriculture John R. Block was pressing his counterpart, at the Department of Health and Human Services, Secretary Margaret Heckler, not to ban the dye.[30] He wrote, "Some segments of the agricultural community are quite dependent on Red Dye #3 in the processing and marketing of certain commodities, especially canned fruits. I have assured the affected industry that their concerns would be made known to you, as well as my own concern …" In 1989, at the behest of growers and packers, the House of

Representatives told the FDA not to ban the dye until it had done further review of the scientific studies.[31,32] Red 3 petitioners claimed that the color acts as a secondary rather than primary carcinogen and therefore was exempt from the Delaney Clause. However, in 1990, FDA concluded that Red 3 was not proven to be a secondary carcinogen and that "FD&C Red 3 is an animal carcinogen".[33] In 1990, the FDA terminated the provisional listing of Red 3 for use in cosmetics and externally applied drugs; all uses of Red 3 lakes (Lakes are water-insoluble forms of dyes and typically contain aluminum.) were also banned.[33] At the time, the FDA estimated that the lifetime risk of thyroid tumors imposed by Red 3 "was at most 1 in 100,000".[29] Based on today's population, that would indicate that Red 3 is causing cancer in about 3000 people.

Notwithstanding its 1990 finding that Red 3 is an animal carcinogen, the agency still permits Red 3 in ingested drugs and foods, though in 1990 it was reported to have said it would "take steps" to ban those uses, too.[34] As of 2012, the FDA still had not acted.

Conclusion

The harm that Red 3, an acknowledged animal carcinogen, is likely causing far outweighs the effort entailed in banning the dye. It is worth noting that Red 3 has been seen as invaluable by some makers of maraschino cherries, but other brands are dyed with Red 40 or have no added coloring and some brands (Del Monte, Giant) of canned fruit cocktail contain cherries colored with natural colorings (unfortunately, the natural colorings used, carmine or cochineal extract, can cause severe allergic reactions). About 5 million pounds of Red 3 have been used since the FDA's acting commissioner stated that the dye should not be used.

FD&C Red 40

Red 40 (Fig. 3) or Allura Red, is approved for use in beverages, bakery goods, dessert powders, candies, cereals, foods, drugs, and cosmetics and, in terms of pounds consumed, is by far the most-used dye (see Table 4). Red 40 has an ADI of 7 mg/kg bw/day.[35] That ADI translates into 210 mg for a 30-kg child. Companies produce the equivalent of about 25 mg of the dye per person per day, with many children, to whom colorful cereals, candies, snack foods, and dairy products are marketed, may consume several times as much.

Metabolism

In an unpublished report, rats were fed a diet with 5.19% Red 40. While 0.1% was excreted in the urine, 29% of the dye was excreted intact in the feces. The parent dye appears to be broken down by gut flora via azo-reduction into two metabolites, cresidine-4-sulfonic acid and 1-amino-2-naphthol-6-sulfonic acid.[36] In another study, rats and dogs were pretreated daily for

3 days with unlabeled Red 40 followed by ^{35}S-Red 40 for up to 72 hours. Within 72 hours, 92–95% and 76–92% of the radioactivity in feces and 5.7–19.8% and 2.7–3.6% in urine was recovered from dogs and rats, respectively. There was significant retention of radioactivity in the guts of animals.[36]

Genotoxicity

Red 40 was negative in seven genotoxicity assays, but positive in the *in vivo* comet assay in the glandular stomach, lungs, and colon of mice.[23] That indicates that Red 40 can cause DNA damage *in vivo*. Details of the genotoxicity assays are provided in Table 5.

Hypersensitivity

Fifty-two patients suffering from urticaria (hives) and angioedema for more than 4 weeks were placed on a 3-week elimination diet (free of synthetic dyes and other food ingredients or additives that might be allergenic). Red 40 administered orally in doses of 1 or 10 mg induced a hypersensitivity reaction in 15% of the patients who were generally symptom-free at the time of provocation.[37]

Chronic toxicity/carcinogenicity

In the 1970s, Hazleton Laboratories conducted chronic toxicity/carcinogenicity feeding studies of Red 40 in rats and mice, both of which included an *in utero* phase. Using Sprague–Dawley rats, the F_0 generation included 30 rats/sex/group that were administered 0, 0.37, 1.39, and 5.19% of Red 40 in their chow 1 week prior to mating, during mating, gestation, and lactation. F_1 rats, a group of 50 rats/sex/group chosen at random from surviving F_0 offspring were exposed for 118 to 121 weeks. The F_0 and F_1 generations were exposed to the same dose. No compound-related effects of concern were reported, thereby indicating a NOAEL of 5.19% (2829 mg/kg bw/day) for males and 1.39% (901 mg/kg bw/day) for females.[38]

Hazleton Laboratories also performed two chronic toxicity studies of Red 40 in CD-1 mice. In the first study, 50 mice/sex/group (F_0) were administered 0, 0.37, 1.39, or 5.19% Red 40 in their chow 1 week prior to breeding through the gestation and lactation periods. The F_1 generation was randomly selected from surviving pups, and the chronic feeding study used 50 mice/sex/group. The dosages were the same in the F_0 and F_1 generations. At 42 weeks, a total of six RE tumors occurred in the males and females (zero in controls, one each in the low- and mid-dose groups, and four in the high-dose groups). That led the investigators to kill and examine 36% of the animals, reducing each group to 30 mice/sex/group. The remaining F_1 mice were fed Red 40 for a total of 104 weeks. By the end of the study, the investigators concluded that Red 40 did not accelerate the appearance of RE tumors.[39] However, M. Adrian Gross, a senior FDA pathologist, concluded that there was clear evidence to support an acceleration effect on RE tumors, because there was a decreased latency period without a corresponding increase in overall tumor incidence.[40]

A second mouse study was conducted to address the possibility that Red 40 accelerated the appearance of RE tumors, a sign of carcinogenicity.[41] Although the second study used the same dosage groups as the first, the studies differed in several respects. First, the initial study used Ham/ICR (CD-1) mice, while the second used CD-1 outbred mice. Second, the F_0 generation in the second study used 70 mice/sex/group, and the F_1 generation consisted of 100 mice/sex/group. Third, the second study did not include a 42-week interim killing. Fourth, the second study used two control groups instead of one. Finally, the mice in the second study were exposed to Red 40 for 109 weeks — 5 weeks longer than the first study.

The second study, according to the investigators, did not show an early appearance of or increase in RE tumors. However, the difference in RE death rates between the two control groups was statistically significant at the $P=0.008$ level.[42] Only the high-dose males and females experienced a significant increase in relative and absolute thyroid weight. The investigators set a NOAEL of 5.19% in mice or 7300 and 8300 mg/kg bw/day for males and females, respectively.[39]

Limitations of the mouse studies

The first mouse study suggested a reduced latency period for RE tumors, and small numbers of RE-system tumors were seen in all treatment groups prior

Table 4   Food dye certification by the FDA in fiscal year 2011

Food dye	Pounds of total dye (includes lakes)	Percentage of total
Blue 1 (21 CFR 74.101)	706 997	4.7
Blue 2 (21 CFR 74.102)	556 643	3.7
Citrus Red 2 (21 CFR 74.302)	2734	0.0
Green 3 (21 CFR 74.203)	16 746	0.1
Orange B (21 CFR 74.250)	0	0
Red 3 (21 CFR 74.303)	219 560	1.5
Red 40 (21 CFR 74.340)	5 487 226	36.4
Yellow 5 (21 CFR 74.705)	4 221 745	28.0
Yellow 6 (21 CFR 74.706)	3 862 135	25.6
Total	15 073 786	100

to the 42-week killing, the highest incidence being in the high-dose group. The FDA recommended killing 36% of the mice to gain information about the possible acceleration of occurrence of RE tumors, and the killings were done at week 42 of the 2-year study. However, that left a relatively small number of mice available at the end of the study and reduced the ability to analyze tumor incidence.[42]

To better understand the results of the first mouse study, in 1976 the FDA created a working group of scientists from the FDA, National Cancer Institute (NCI), and the National Center for Toxicological Research to monitor the rat and mouse studies being performed for Allied Chemical. Midway through the second mouse study, the working group concluded that the first study did not indicate a risk of carcinogenesis. Following controversy over that conclusion, FDA Commissioner Donald Kennedy appointed four non-governmental statisticians, including Harvard's Frederick Mosteller and Stephen Lagakos, to review the statistical methods used to analyze the studies. Those statisticians were independent and not a part of the FDA working group.[43]

Two problems found with the mouse studies included caging and litter effects.[42] In the second study, the mice housed in the upper row of racks experienced a higher incidence of RE tumors than the mice in lower cages, according to the FDA consultants.[44] The incidence of RE tumors was significantly correlated to the row ($P=0.0005$) and position ($P=0.02$) of the racks.[44] The working group also noted that it was impossible to know if mice were being housed with siblings (litter effect), which might have had an influence on tumor incidence.[43] Confounders such as potential caging and litter effects strongly decrease the credibility of a study.

Also, there was a large variation in RE tumor rates between the two studies. The difference in RE death rates between the two control groups was statistically significant at the $P=0.008$ level.[42] That difference could have been due to the different strains of mice used in the two studies, but does raise questions about the validity of the second study.

Regarding the statistical analyses of the two mouse studies, Lagakos and Mosteller commented that the difference in RE tumor rates between the two studies limited the conclusiveness of the results. They argued that the statistical methods used by the FDA Working Group were not oriented to detecting an acceleration effect.[42] Their analysis concluded that both studies suggested a decreased latency period for, and increased incidence of, RE tumors.[44]

Carcinogenic contaminants

As discussed in this paper with regard to Yellow 5 and Yellow 6, Red 40 has been found to contain

Table 5 Summary of genotoxicity studies on Red 40

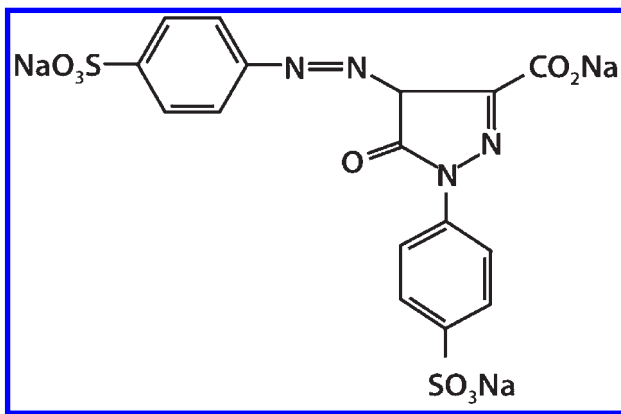| Assay	Mutation type	S9 activation	Dose	Results	Reference
Comet Assay	DNA damage	NA	10 mg/kg in colon; 100 mg/kg in glandular stomach; 1000 mg/kg in lungs	Positive	23
Comet Assay	DNA damage	NA	2000 mg/kg to pregnant mice; 10 mg/kg in male mice	Positive in colon; Positive in colon	23
E. coli WP2 uvrA	Base substitution	Yes and No	10 mg/ml	Negative	141
S. Typhimurium TA1535 and TA100	Base pair	Yes and No	50–500 µg/plate	Negative	138
S. Typhimurium TA98, and TA1537	Frameshift	Yes and No	50–500 µg/plate	Negative	
S. Typhimurium TA1535 and TA100	Base pair	Yes and No	0.2–400 µg/plate	Negative	143
S. Typhimurium TA98, and TA1537	Frameshift	Yes and No	0.2–400 µg/plate	Negative	
S. Typhimurium TA 1535 and TA 1538	Base pair (TA1535) and Frameshift (TA1538)	Yes and No	1, 10, 50, 100, and 250 µg/plate	Negative	142
Yeast strains D-3 and D-5	Mitotic recombination	Yes and No	10 mg/ml	Negative	

Figure 4   Yellow 5 (Tartrazine)

cancer-causing and other contaminants. Health Canada scientists, using a test method that could detect bound and free contaminants, identified small amounts of aniline, *p*-cresidine, and 1-naphthylamine in the dye.[45] *p*-cresidine is "reasonably anticipated to be a human carcinogen", according to the US National Toxicology Program (NTP), and "possibly carcinogenic to humans", according to the International Agency for Research on Cancer (IARC).[46,47] The NCI and the FDA considered aniline to be weakly carcinogenic to rats, though other agencies have not determined that aniline and 1-naphthylamine pose a risk to humans.[46,48,49]

Reproductive toxicity/teratogenicity
To investigate the potential embryotoxicity and teratogenicity of Red 40, pregnant female rats were dosed with 0, 7.5, 15, 30, 100, or 200 mg Red 40/kg bw daily on days 0–19 of gestation through intubation or 0 or 2 mg Red 40/kg bw daily through drinking water on days 0–20 of gestation. No adverse effects on reproduction, embryolethality, or fetotoxicity were reported.[50]

Conclusion
There is evidence, albeit controversial and inconclusive, that Red 40, the most widely used dye, accelerates the appearance of tumors of the RE system in mice. Also, independent consultants (Lagakos and Mosteller) appointed by the FDA raised concerns about the FDA-appointed Working Group's statistical analysis of the data. Considering the positive results in comet genotoxicity assays, the disputed mouse chronic-toxicity studies, causation of hypersensitivity reactions, possible causation of hyperactivity in children, cancer-causing contaminants, and the non-essentiality of the dye, Red 40 should not be used in foods.

FD&C Yellow 5
FD&C Yellow 5 (Fig. 4), also known as Tartrazine, is used in numerous bakery goods, beverages, dessert powders, candies, cereals, gelatin desserts, pet food, and many other foods, as well as pharmaceuticals and cosmetics. After Red 40, it is the most widely used dye (Table 4). The ADI for Yellow 5 is 5 mg/kg bw/day, which equates to 150 mg/day for a 30-kg child.[51] Companies produce the equivalent of 15 mg of the dye per person per day, with many children likely consuming at least several times that much.

Metabolism and metabolic effects
Sulfanilic acid is a metabolite that results from the reduction of Yellow 5 at the N=N azo link. However, when Yellow 5 labeled at the phenylazo group with ^{14}C was administered intraperitoneally in rats and rabbits, no radioactive sulfanilic acid was recovered in the urine.[52] In the same study, when Yellow 5 was administered orally to rats, rabbits, and humans, sulfanilic acid, but little or no unchanged dye, was recovered in the urine. Those results indicate that the reduction of Yellow 5 occurs via the GI flora. Ryan *et al.*[53] confirmed that Yellow 5 is primarily metabolized by the gut microflora of rats after an oral dose.

Apart from the metabolism of the dye, a 50-mg dose of Tartrazine, but not Amaranth (the generic name for the now-banned FD&C Red 2), led to increased or accelerated urinary excretion of zinc in hyperactive children.[54] Whether the effect on zinc is a cause of hyperactivity is not known.

Genotoxicity
Potential genotoxicity of Yellow 5 was tested in 11 studies, with six studies, including two *in vivo* studies, showing positive effects (Table 6). A 1985 report from the US Department of Health and Human Services (HSS) criticized two of the genotoxicity studies and disagreed with their conclusions that Yellow 5 induces chromosomal aberrations.[21,55,56] However, the HHS report stated, "If chromosome aberrations of the type reported for Tartrazine in cultured cells occurred *in vivo*, they certainly would represent a serious adverse effect." In fact, Sasaki *et al.*[23] subsequently demonstrated that Yellow 5 does induce DNA damage *in vivo* in the comet assay.

Chronic feeding/carcinogenicity
The earliest chronic feeding study reported that Yellow 5 was not carcinogenic or toxic in a 2-year study using Osborne–Mendel weanling rats. (Davis *et al.*[57] also tested three groups of two male and two female beagles for 2 years at dosages of 0, 1, and 2% Yellow 5, but that small number of dogs and the brevity of the test do not permit conclusions about the long-term effects of the dye.) The rats were fed 0, 0.5, 1, 2, and 5% Yellow 5. However, that study used only 12 rats of each sex per dosage group. The FDA recommends a minimum of 20 rodents/sex/group for chronic toxicity studies, though many experts consider that far too small a number.[58] Also, the rats were not exposed *in utero*.

Later, in a feeding study sponsored by CCMA, 70 Charles River CD rats/sex/group were exposed to 0, 0.1, 1, 2, or 5% Yellow 5 starting *in utero* for either 30 months or until only 10 rats/sex/group survived.[59] The researchers did not find any compound-related effects on fertility, gestation, parturition, lactation, pup survival, or number of still-born pups. Complete histopathology was performed on all killed animals, and gross necropsies were conducted on animals that died spontaneously, but no adverse effects were reported. This group reported a NOAEL of 5% for both male and female rats.

Borzelleca and Hallagan also performed a chronic toxicity/carcinogenicity study in CD-1 mice.[60] Groups of 60 males and females were fed 0 (two control groups), 0.5, 1.5, or 5% Yellow 5 for 104 weeks. The protocol for this study was similar to Borzelleca and Hallagan's rat study, but the mice were not exposed *in utero*, and were 42 days old at the start of the study — a serious drawback, because infant animals are likely to be more susceptible to toxic or carcinogenic effects than older animals. The investigators claimed that a sufficient number of mice survived until the end of the study (24 months), however half of the groups did not meet the FDA recommendation that in a carcinogenicity study at least 25 mice/sex/group should survive until study termination (see italic numbers in Table 7). In any case, the investigators did not report any significant compound-related effects and concluded that the NOAEL for this study was 5% for both male and female mice (indeed, the lack of any effect at the highest dosage level suggests that a higher dosage should have been used in the chronic feeding studies).

Carcinogenic contaminants

Yellow 5, the second-most widely used dye, may contain up to 13% of other organic and inorganic chemicals.[51] Yellow 5 may be contaminated with several carcinogens, including benzidine and 4-aminobiphenyl. The FDA limits free benzidine to 1 part per billion (ppb), though analytical methods can only detect 5 ppb. Importantly, FDA tests have found that some batches of dye contained as much as 83 ppb of free and bound benzidine, with the latter being liberated in the GI tract.[61] The FDA does not test for bound benzidine when it certifies the purity of dyes. The FDA's 1985 risk assessment (using projections for 1990 consumption levels) calculated a risk for Yellow 5 of 4 cancers in 10 million people, which is slightly smaller than the "concern" level of 1 in 1 million.[48] However, that risk assessment failed to consider the: (1) greater sensitivity of children to carcinogens,[62] (2) greater consumption of Yellow 5 by children than the general population, (3) substantial increase in per capita consumption of Yellow

Table 6 Summary of genotoxicity studies on Yellow 5

Assay	Mutation type	S9 activation	Dose	Results	Reference
Comet Assay	DNA damage	NA	10 mg/kg, >10 mg/kg	Positive (colon) Positive (glandular stomach)	23
Cytogenetics assay	Chromosomal aberrations	NA	NA	Positive	75
S. Typhimurium TA94, TA1537 and TA98	Frameshift	Yes and No	5 mg/plate	Negative	21
S. Typhimurium TA1535, TA100 and TA92	Base pair	No	2.5 mg/ml	Positive	
Chromosomal aberration test, CHL cells	Chromosomal aberrations	No	6 mg/ml	Positive	
In vitro Muntiacus muntjac	Chromosomal aberrations	No	3 µg/ml	Positive	55
S. Typhimurium TA1537, TA1538 and TA98	Frameshift	Yes and No	5 mg/plate	Negative	144
S. Typhimurium TA1535 and TA100	Base Pair			Negative	
S. Typhimurium TA100	Base pair	Yes and No	5 mg/plate	Negative	145
S. Typhimurium TA98	Frameshift	Yes and No		Negative	
rec-assay	DNA damage	Yes and No		Negative	
Chromosomal aberration test, CHL cells	Chromosomal aberrations	Yes and No		Negative	

5 since 1990, (4) possibility that some batches of dye contain large amounts of bound benzidine and other carcinogenic contaminants, and (5) the presence of similar contaminants in Yellow 6. FDA scientists found that one company eliminated benzidine contamination in 1992, suggesting that other companies could do (or might have done) the same.[63] However, with more chemicals being imported from China, India, and other countries, it is important that dyes routinely be tested for bound contaminants.

Hypersensitivity

It is generally accepted that Yellow 5 has hypersensitivity effects. In the 1970s, several cases of Tartrazine sensitivity were reported, most frequently in the form of urticaria and asthma.[64] Neuman *et al.*[65] reported that 26% of patients with a variety of allergic disorders had a positive allergic reaction 10–15 minutes after ingesting 50 mg of the dye. Those reactions included heat-wave, general weakness, blurred vision, increased nasopharyngeal secretions, a feeling of suffocation, palpitations, pruritus, angioedema, and urticaria. An association between aspirin intolerance and Tartrazine sensitivity has been demonstrated in several studies. Stenius and Lemola separately administered aspirin and Yellow 5 to 96 patients and found that about half of the patients with positive reactions to aspirin also had positive reactions to Yellow 5, and about three-fifths of the positive Yellow 5 cases also had positive aspirin reactions.[66] In a double-blind crossover study, Settipane *et al.*[67] found that 0.22 mg of Yellow 5 (much less than is used in most dyed foods) caused a positive reaction in 8% of patients with chronic urticaria and 20% of patients with aspirin intolerance.

In 1986, the Joint Council of Allergy and Immunology, which was established by two major medical organizations, told the FDA that listing Yellow 5 on the label was not sufficiently protective, because reactions could be life-threatening, and urged the agency to ban Yellow 5.

Table 7   Mouse survival at termination of a 24-month study[60]

Dose level (%)	Survival*	
0 (control 1)	30/50	Males
0 (control 2)	28/60	
0.5	31/60	
1.5	*21/60*	
5.0	29/60	
0 (control 1)	*20/60*	Females
0 (control 2)	*24/60*	
0.5	18/60	
1.5	*24/60*	
5.0	33/60	

Note: *No. surviving at termination of study/no. at initiation; italics indicates inadequate numbers of mice surviving.
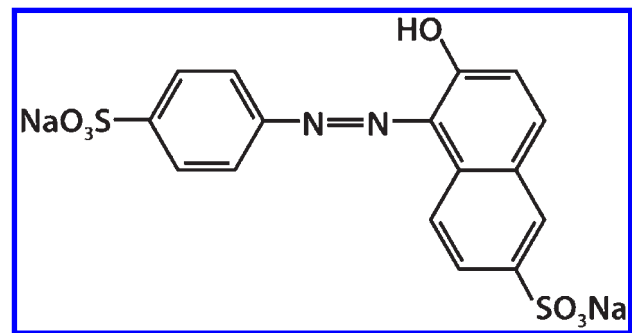

Figure 5   Yellow 6 (Sunset Yellow)

Conclusion

Six out of 11 mutagenicity studies indicated potential health hazards, but Yellow 5 did not appear to be carcinogenic in rats. The chronic feeding study in mice was inadequate and cannot be used to support the dye's safety. In addition, Yellow 5 may be contaminated with significant levels of carcinogens. Tartrazine (the only dye to be tested on its own in hyperactivity studies, instead of in mixtures) has caused hyperactivity in children.[68,69] Yellow 5 can cause sometimes-severe hypersensitivity reactions. Since Yellow 5 poses some risks, has not been adequately tested in mice, and is a cosmetic ingredient that serves no nutritional or safety purpose, it should not be allowed in the food supply.

FD&C Yellow 6

FD&C Yellow 6 (Fig. 5), or Sunset Yellow, is a water-soluble, sulfonated, azo dye used to color baked goods, cereals, beverages, dessert powders, candies, gelatin desserts, sausage, and numerous other foods, as well as cosmetics and drugs. Yellow 6 has an ADI of 3.75 mg/kg bw/day, or 112.5 mg for a 30-kg child.[70] Current average per capita production of Yellow 6 is equivalent to about 14 mg/day, making it the third most widely used dye (Table 4). Considering that the FDA estimates that an average ''high user'' consumes about five times as much dye as an average user over their lifetimes and that per-capita dye consumption has more than doubled in the past three decades, some children may be consuming amounts above the ADI.[2,71]

Metabolism and metabolic effects

Several metabolites were found in the urine of rabbits given a single 0.5 mg/kg oral dose of Yellow 6. Yellow 6 is reduced at the azo linkage primarily in the gut by intestinal microflora to produce sulfanilic acid and 1-amino-2-naphthol-6-sulfonic acid, as well as the *n*-acetylated form of sulfanilic acid, *p*-acetamido-benzene-sulfonic acid. Intact Yellow 6 in the feces accounted for about 2% of the dose.[16] Those findings were confirmed by Honohan *et al.*[72] who dosed five rats with 2.7 mg of ^{14}C-Yellow 6 orally and found only 1–2% of the dose in the form of intact dye in the feces after 24 hours. In another rat study, after a

Table 8   Summary of genotoxicity studies on Yellow 6

Assay	Mutation type	S9 activation	Dose	Results	Reference
Comet assay	DNA damage	NA	2000 mg/kg	Negative (stomach colon, liver, kidney, bladder, lung, or brain)	23
Cytogenetics assay	Chromosomal aberrations	–		Positive	75
S. Typhimurium TA98	Frameshift	Yes and No	300 μg/plate	Negative	146
S. Typhimurium TA100	Base pair	Yes and No	300 μg/plate	Negative	147
Bone marrow micronucleus assay	Chromosomal damage	NA	2000 mg/kg	Negative	74
L5178Y TK+/− mouse lymphoma assay	Forward mutation	Yes	1 mg/ml	Positive	144
S. Typhimurium TA1537, TA1538 and TA98	Frameshift	Yes and No	5 mg/plate; also tested 1 mg/plate sulfanilic acid	Negative	140
S. Typhimurium TA1535 and TA100	Base pair	Yes and No	5 mg/plate; also tested 1 mg/plate sulfanilic acid	Negative	137
Saccharomyces cerevisiae BZ 34	Mitotic gene conversion	No	5 mg/ml	Negative	
E. coli WP2 uvrA	Base substitution	Yes and No	10 mg/ml	Negative	

single oral dose of 100 mg, only 0.8% of intact dye was excreted in the feces, with the rest being the metabolites indicated above. Only 3.6% of the intact dye was absorbed by rats administered 50 mg of Yellow 6 orally.[73]

Apart from the metabolism of the dye, a 50-mg dose of Sunset Yellow (like Tartrazine) led to increased or accelerated urinary excretion of zinc in hyperactive children. Whether the effect on zinc is a cause of hyperactivity is not known.[54]

Genotoxicity

Yellow 6 was negative in six genotoxicity assays, but induced forward mutations and chromosome aberrations in two other assays.[74,75] As shown in Table 8, Yellow 6 did not induce DNA damage in a comet assay or cause frameshift, base pair, or forward mutations; chromosomal aberrations, or mitotic gene conversion.

Chronic toxicity/carcinogenicity

The NTP conducted chronic bioassays using 50 animals/sex/group in F344 rats and B6C3F1 mice. Each group was fed a diet containing 0, 1.25, or 2.5% Yellow 6 for 103 weeks. The control groups consisted of 90 rats or 50 mice of each sex. There was no *in utero* exposure in either study, and both studies were terminated at two years instead of 30 months or the lifetimes of the animals, significantly reducing the sensitivity of the studies. The rat study did not find any statistically significant dye-related neoplastic or non-neoplastic lesions in any of the groups. Low-dose, but not high-dose, male mice had a significantly higher incidence of hepatocellular carcinomas and adenomas compared to controls. Partly because of the lack of a dose-response relationship in the mice, NTP concluded that Yellow 6 was "not clearly related" to a higher rate of carcinogenicity. However, the high rate in the low-dose group certainly raises questions that could only be answered with a new study.[76]

In 1982, Bio/dynamics Inc., under contract to CCMA, conducted two multigeneration, long-term feeding studies in Charles River Sprague–Dawley rats at doses of 0 (two control groups), 0.75, 1.5, and 3% in the first study and 0 (one control group), 0.75, 1.5, and 5% in the second study. The first study was conducted for 30 and 28.5 months for males and females, respectively, and the second study lasted for 25.6 and 27.8 months for males and females, respectively. In the F_1 generation, females in the 3% group in the first study and males in the 5% group in the second study had increased mortality. At termination of both studies, there was an increase in mean absolute and relative kidney weights in females in the 3% groups and 5% groups, as well as an increase in the mean relative and absolute thyroid

weights in males and females in the 5% groups. Females in the 3% group and both males and females in the 5% groups had statistically significant increased incidences of adrenal medullary adenomas compared to controls. Also, males in the 3% group had an increased incidence of testicular interstitial cell adenomas compared to pooled controls. Notwithstanding those findings, the investigators concluded that the studies did not find any evidence of carcinogenicity.[77]

After examining the results of the Bio/dynamics study, the FDA argued that the increased incidence of the tumors was not related to Yellow 6 because of the: (1) lack of dose-response in the 3% and 5% dosage groups (though that is comparing two different studies), (2) lack of precancerous lesions, (3) similar morphology of adrenal medullary lesions in control and treated animals, (4) lack of a difference in the latency periods before tumors occurred, (5) fact that the tumors seen are common spontaneous tumors in older rats, and (6) lack of other studies finding an association between Yellow 6 and this type of tumor.[71]

Bio/dynamics, again under contract to CCMA, performed a chronic toxicity/carcinogenicity study in Charles River CD-1 COBS mice, with 60 mice/sex/group. The study used dosages of 0 (two control groups), 0.5, 1.5, and 5% Yellow 6 in the animals' chow. The study was terminated at only 20 months for the males and 23 months for the females. Another deficiency was that the mice were not exposed *in utero*. Males in the 5% group had significantly higher mortality rates at the end of the study compared to controls. The laboratory concluded that the study did not indicate any concern about carcinogenicity in mice.[78]

In the 1960s, the FDA completed a 7-year feeding study on a small number of beagle dogs. This study was neither large nor long enough to detect carcinogenicity. However, Kent J. Davis, an FDA veterinarian, attributed "tears, eye lid encrustations, pannus [corneal inflammation], and corneal opacity approaching blindness" to ingestion of Yellow 6. He concluded that, because of the eye lesions, "it is apparent that immediate decertification of this color is necessary in order to protect the public health at the recommended level of present safety standards". His recommendation was not followed.[79]

Carcinogenic contaminants

Yellow 6 may be contaminated with several carcinogens, including benzidine and 4-aminobiphenyl. The FDA set a limit of 1 ppb of free benzidine, but Peiperl *et al.*[63] reported that some batches of dye contained a hundred or even a thousand times as much benzidine bound up in other chemical moieties,

which is likely liberated in the colon. The FDA does not test for bound benzidine in the aliquots taken from batches of dyes submitted for certification. The FDA's 1986 risk assessment (using estimates for 1990 consumption levels) estimated a risk of three cancers in 10 million people, which is smaller than the official "concern" level of 1 in 1 million.[71] However, that assessment failed to consider the: (1) greater sensitivity of children,[62] (2) greater consumption of Yellow 6 by children than the general population, (3) substantial increase in per capita consumption of Yellow 6 since 1990, (4) possibility that some batches of dye contain bound forms of benzidine and other contaminants,[62] and (5) presence of similar contaminants in Yellow 5. FDA scientists found that in 1992 one company eliminated benzidine contamination of Yellow 5, suggesting that other companies could do the same for Yellow 6.[63] However, a Health Canada study found that Sunset Yellow FCF (Yellow 6) was still contaminated with benzidine in 1998.[80] With more and more chemicals being imported, it is important that dyes routinely be tested for bound contaminants.

Hypersensitivity

Human hypersensitivity to Yellow 6 was reported as early as 1949.[81] Since then, several cases of hypersensitivity to the color have been reported:

- A 15-year-old pregnant girl experienced anaphylactic shock after receiving an enema that contained Yellow 5 and Yellow 6. The patient was tested via the skin-prick technique for sensitivity to all of the soluble components in the enema. Positive results were observed for both Yellow 5 and Yellow 6.[82]
- A 43-year-old physician was hospitalized for stomach cramps four times over a 2-year period. Double-blind tests confirmed that the cramps were caused by a hypersensitivity to Yellow 6.[83]
- A 53-year-old woman visited the doctor for severe skin lesions. Two days after receiving treatment she was hospitalized for distaste for food, as well as indigestion, retching, belching, severe abdominal pain, and vomiting. When the drugs (administered orally) were discontinued, the symptoms subsided, and when the drugs were administered again the symptoms reappeared. A challenge test confirmed that Yellow 6 was the causative agent.[84]

A study by Michaelsson and Juhlin involved 52 patients with, and a control group of 33 patients without, recurrent urticaria. All subjects were put on a dye-free diet and were free of antihistamines prior to administration of the possible allergen. The researchers tested the effects of several food dyes (including Yellow 6) and preservatives, as well as aspirin, sulfanilic acid (a metabolite of Yellow 6), and a placebo. A dose of 0.1 mg (initial dose for asthma patients) or 1 mg of Yellow 6 was administered to patients with slight or no urticaria symptoms. If no reaction was observed after the initial dose, a higher

dose of 2, 5, or 10 mg was administered to the latter group of patients 1 hour after each previous dose. Symptoms of a hypersensitivity reaction included urticaria, angioedema of lips, eyes, or face, reddening of the eyes, sweating, increased tear secretion, nasal congestion, sneezing, rhinitis (runny nose), hoarseness, wheezing, and a variety of subjective symptoms. Of the 33 control patients, only two with a history of rhinitis showed signs of rhinitis when administered Yellow 5 and Yellow 6. Of the 27 patients with recurrent urticaria who were challenged with Yellow 6, 10 developed urticaria and six experienced subjective symptoms; 11 were negative for symptoms. Eight out of nine patients with positive reactions to Yellow 6 also experienced a positive reaction to aspirin (people sensitive to Yellow 5 also are often sensitive to aspirin).[85]

Michaelsson *et al.*[86] tested seven patients having allergic vascular purpura with oral provocation by 5 mg Yellow 6. One patient had a strongly positive reaction to the dye. That patient was a 32-year-old woman who suffered for 12 years from recurring purpuric lesions. After the patient was put on a diet free from dyes and benzoates (a preservative that has been linked to allergy-like reactions) for 6 months, she was essentially free from lesions.

Conclusion
An NTP study did not detect any problems in chronic feeding studies on rats and mice, though the animals were not exposed *in utero* and the studies were terminated at 2 years. Bio/dynamics concluded that its studies on rats and mice showed that Yellow 6 was not an animal carcinogen, but rats in the two highest dosage groups (3 and 5%) experienced higher incidences of adrenal medullary adenomas. The FDA has given reasons for not considering those tumors significant, but differences between test and control groups should not be rejected on qualitative grounds. A Bio/dynamics mouse study did not report evidence of carcinogenicity, but the study was not as sensitive as it might have been because the mice were not exposed *in utero*. Yellow 6 may be contaminated with significant levels of recognized carcinogens. Whether or not it causes cancer, Yellow 6 raises other, lesser concerns, such as mild to severe hypersensitivity reactions. Because it provides no health benefit whatsoever, Yellow 6 should be removed from the food supply.

Discussion
Our review of the toxicology of the nine dyes used in the US food supply (many of the dyes are used in other countries, as well), identified concerns about the adequacy of the testing of all the dyes. In addition, research indicates that some of the dyes may cause cancer, hypersensitivity reactions, genotoxicity, and hyperactivity (see Table 1).

Most of the studies reviewed in this report suffer from several significant limitations. First, most of the studies were commissioned or conducted by dye manufacturers, so biases could influence the design, conduct, or interpretation of the studies. Ideally, the tests would have been conducted and interpreted by independent scientists. Second, most of the studies lasted no longer than 2 years — some were shorter. Third, many studies did not include an *in utero* phase. Bioassays would be more sensitive if they lasted from conception through 30 months or the natural lives of the rodents (as long as 3 years).[87]

Another consideration of unknown importance is that virtually all the studies evaluated the safety of individual dyes. Many foods, though, contain mixtures of dyes, such as the Blue 1, Blue 2, Red 40, Yellow 5, and Yellow 6 in Kellogg's Hot Fudge Sundae Pop Tarts. Dyes conceivably could have synergistic (or, indeed, antagonistic) effects with one another or with other food additives or ingredients.

One significant limitation of this report is that the authors were restricted to reviewing mostly published studies. Unpublished toxicology studies in the files of the FDA or companies might shed further light on the safety of the dyes.

Neurotoxicity
This report does not explore neurobehavioral toxicity of food dyes in detail but that topic must be touched upon. In the early 1970s, allergist Benjamin Feingold observed that food dyes could cause hyperactivity and other impaired behaviors in child and adult patients. His recommendation that hyperactive children be put on an "elimination" diet generated huge publicity and spurred numerous scientific studies over the years. [See http://cspinet.org/fooddyes/index.html for more detailed information about food dyes and hyperactivity, especially 'Diet, ADHD & Behavior: a quarter-century review — 2009 Update'. Jacobson MF, Schardt D. (Washington: Center for Science in the Public Interest). http://www.cspinet.org/new/pdf/dyesreschbk.pdf; accessed 2010 Feb 20. Also, see CSPI's 2008 petition to the FDA.] A 2004 meta-analysis concluded that there was a cause-and-effect relationship between food dyes and hyperactivity. The authors stated that dyes "promote hyperactivity in hyperactive children, as measured on behavioral rating scales" and that "society should engage in a broader discussion about whether the aesthetic and commercial rationale for the use of [artificial food colorings] is justified".[88]

Two major studies on British children found that mixtures involving six dyes (and the food preservative sodium benzoate) impaired the behavior of even

non-hyperactive children.[89,90] As a result, the British government told the food and restaurant industries to eliminate the dyes tested by the end of 2009, and the European Parliament passed a law that requires a warning notice on all foods that contain one or more of the dyes tested.[91] Between that notice and the fact that dyes were never used as widely in Europe as in the United States, dyes are now rarely used but not eliminated.

Because of those governmental actions and Europeans' aversion to synthetic food ingredients, some products made by McDonald's, Mars, Kraft, PepsiCo, and other major American multinational companies contain dyes in the United States, but natural or no colorings in the United Kingdom. In June 2008, CSPI petitioned the FDA to ban all the widely used food dyes because of their impact on children's behavior (see http://cspinet.org/new/pdf/petition-food-dyes.pdf). Food dyes and all other food additives should be screened in animals and *in vitro* systems for potential behavioral effects before they are allowed into the food supply.

Getting Unsafe Dyes Out of Foods

This review suggests the need for improvements in the FDA's regulation of food dyes and of food additives more generally. Tests of food and color additives are often deficient in terms of duration, number of animals in each dosage group, number of species tested, and dosages used, and fail to consider the cumulative risk of all dyes, rather than of each dye independently. Indeed, the Food, Drug, and Cosmetic Act requires the FDA to consider "the cumulative effect, if any, of such additive … taking into account the same or any chemically or pharmacologically related substance …" [21 USC 379e(b)(5)(A)(ii)]. The FDA should routinely require all carcinogenesis studies to include *in utero* exposure, to last 30 months or the natural lives of the animals, and to be sure that the highest dosage used has some observable effect on the animals. Additives should be evaluated, based on their chemical structures, for potential hypersensitivity reactions and should be monitored after introduction into the food supply. The agency should routinely test for the presence of bound carcinogens, which are not detected in the analytical chemistry tests currently used, and hazardous contaminants should be restricted to safe levels. Approvals should be revoked if unnecessary additives are found to cause serious reactions (e.g. urticaria, anaphylactic reactions) or widespread milder reactions (e.g. nausea, vomiting). The law barring the approval of chemicals that cause cancer in animals should be strictly enforced. Ideally, tests would be conducted and evaluated by independent researchers, but such a reform, initially proposed in legislation (S. 925) in 1975 by Senator Gaylord Nelson, has not been adopted.

In the absence of improved regulations, food processors, and restaurants voluntarily should consider reformulating their foods without dyes (and without natural colorings, including annatto, cochineal extract, and carmine, that cause hypersensitivity reactions). Several major multinational companies have told the authors that they do not use dyes in Europe, because governments have urged them not to, but that they would continue to use dyes in the United States until they were ordered not to or consumers demanded such foods. But there may be a nascent movement away from dyes. Two chain restaurants, Starbucks and Jason's Deli, and snack manufacturer Frito-Lay will be phasing out dyes in the next several years.[92] Also, General Mills has removed dyes from its Trix yogurt.

The FDA, which is charged with protecting the public from unsafe food ingredients, could ban dyes that fail to meet their safety requirements. However, the Food, Drug, and Cosmetic Act makes it even harder for the FDA to revoke previous approvals of food colors than other food additives. (To challenge a proposed ban on a food or color additive, companies can request that the FDA hold a formal public hearing and, if the FDA subsequently still wants to ban the substance, companies can go to court. The process for color additives, though, includes another hurdle, because, if a dye is alleged to cause cancer, companies can request that the FDA create an outside advisory committee to review the matter. Compare 21 USC 379e(b)(5)(C) and 21 USC 371(e)(2) and (f)(2) for colorings with 21 USC 348 409(f) and (h) and 21 CFR 171.130 for other additives.) As one legal analyst stated,

> *Thanks to the foresight and effective lobbying of the cosmetics industry in the 1960s, the proponent of a color additive petition is in an excellent position if the FDA decides to remove [a coloring's] permanent listing. The burdens of proof in a complex process fall on the FDA, and the time required to pass through the procedural maze acts as a disincentive to FDA undertaking any delisting action.[4]*

Ideally, the law would be changed to provide greater consumer protection from dyes that appear to be unsafe. Meanwhile, though, consumers who wish to avoid dyes should carefully read ingredient statements on product labels; it is more difficult to avoid dyes in restaurant foods.

Acknowledgements

Appendix

FD&C Blue 1

FD&C Blue 1 (Fig. A1), or Brilliant Blue, is a water-soluble coloring used in baked goods, beverages, dessert powders, candies, cereals, drugs, and other products. Blue 1 received FDA approval for general use in foods and ingested drugs in 1969. In 1982, the FDA permanently approved the color for use in externally applied drugs and general use in cosmetics excluding the area of the eye. The FDA suggests a maximum ADI for Blue 1 of 12 mg/kg bw/day.[93] For a 30-kg (66-pound) child, that equates to 360 mg/day. Current average dye usege is equivalent to about 3 mg/person/day (based on the entire population, not just children).

Metabolism

In a study of rats, Blue 1 was largely excreted unchanged in the feces (96%) within 36 hours after a 200-mg oral administration. None of the dye was excreted in the urine. In the same study, only 0.7 and 2.8% of a 200-mg oral dose was excreted in the bile of two bile-duct cannulated dogs indicating some intestinal absorption. Investigators calculated that the quantity of absorption of the color from the gastrointestinal (GI) tract was about 10 mg out of a 200-mg dose.[94] Brown *et al.*[95] reported similar results after administering a single 0.27-mg dose of ^{14}C-labeled Blue 1 to female Sprague–Dawley rats. Bile duct-ligated rats excreted the dye in their urine and feces at concentrations of 2.02 and 97.28%, respectively. Given the lower percentage of dye being excreted in the bile, the large amount eliminated through the feces indicates that the dye is poorly absorbed by the GI tract. In this particular study, total intestinal absorption was estimated to be about 2.05 and 0.27% of the total dose in bile duct-ligated and intact rats, respectively. Analysis of the biliary and urinary excretion showed that 95% of the recovered radioactivity was from unchanged Blue 1 while 5% was an unidentified metabolite or degradation product. Blue 1 does not appear to be broken down by intestinal microbiota in rats, but up to 5% is absorbed via the GI tract.[95]

Genotoxicity

Seven studies did not find Blue 1 genotoxic in terms of DNA damage, base pair mutations, base substitutions, or frameshift mutations (see Table A1). However, Blue 1 caused chromosomal aberrations in two studies.[75,96]

Chronic toxicity/carcinogenicity

Hansen *et al.*[97] performed a chronic toxicity study using Blue 1 on rats (another study on dogs was too brief and used too few dogs to provide meaningful results). The rat study lasted 2 years and used 24 Osborne–Mendel rats/sex/group at doses of 0, 0.5, 1, 2, and 5% of the diet. There were no reported
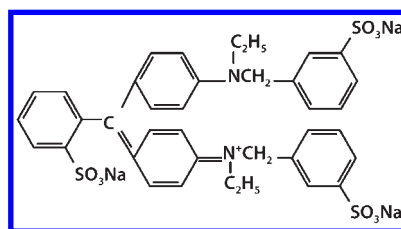

Figure A1   Blue 1 (Brilliant Blue)

compound-related effects in any group on mortality, hematology, or organ weights (heart, liver, spleen, testis, kidney), nor was significant growth inhibition or gross lesions reported. The small numbers of rats in each group renders this study quite insensitive and of marginal value.[97]

The highest-quality carcinogenicity/toxicity studies were performed by Borzelleca *et al.*[98] for the CCMA. The 2-year studies used Charles River CD rats and CD-1 mice. The rat study included an *in utero* phase with 60 rats/sex/group. The rats were fed 0 (two control groups), 0.1, 1, and 2% Blue 1 in the chow for about two months prior to mating. Investigators reported no compound-related effects on reproduction. F_1 generation rats were randomly selected and 70 rats/sex/group were used in the lifetime feeding study (same dosage groups, including two controls, as in the F_0 phase). The maximum exposure times for males and females were 116 and 111 weeks from birth, respectively. F_1 females in the 2% group had a significant decrease in terminal mean body weight (15%) and decreased survival compared to controls. No other compound-related effects were noted. The NOAEL (No Observable Adverse Effect Level) was 1072 mg/kg bw/day (2% group) for males and 631 mg/kg bw/day for females (1% group).[98]
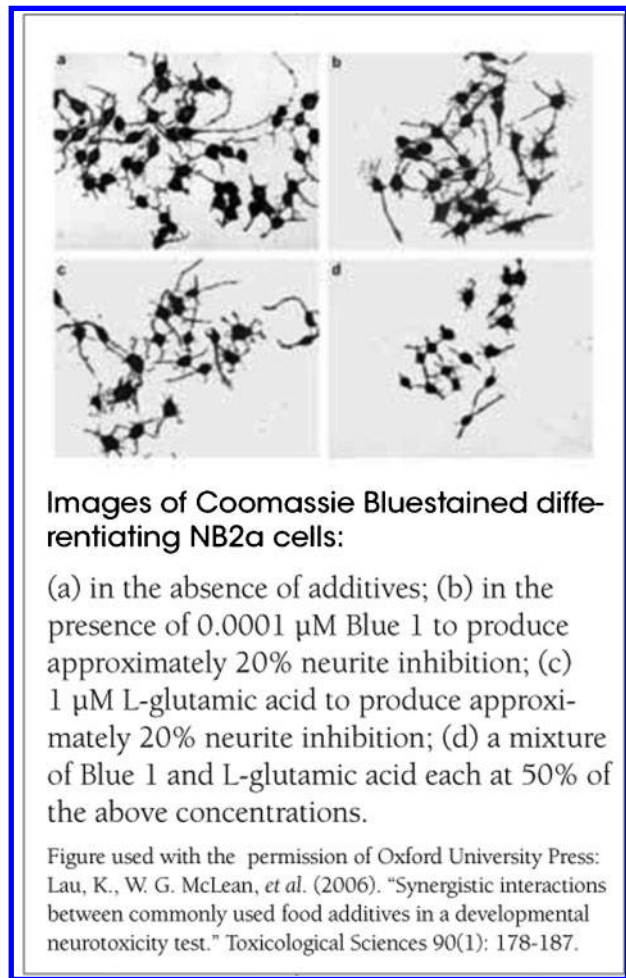
The mouse study did not include an *in utero* phase and used 60 mice/sex/group. Mice were administered 0 (two control groups), 0.5, 1.5, and 5% Blue 1 in their food. The maximum exposure time was 104 weeks for both sexes and the NOAEL was determined to be 5%, or 7354 and 8966 mg/kg bw/day for males and females, respectively. No significant compound-related effects were noted in any of the groups.[98]

Neurotoxicity

Lau *et al.*[99] investigated the individual and potential synergistic effects of Blue 1 and L-glutamic acid (a close relative of the food additive monosodium glutamate) on neuronal development. Investigators used NB2a neuroblastoma cells that were induced to differentiate and grow neurites in the presence or absence of the two food additives. Neurotoxicity was measured in terms of an inhibition of neurite outgrowth. Individually, Blue 1 was found to have an IC_{50} (half-maximal inhibitory concentration) of 0.0514 μmol/l, while L-glutamic acid was found to

Table A1 Summary of genotoxicity studies on Blue 1

Assay	Mutation Type	S9 Activation	Dose	Results	Reference
Comet assay	DNA damage	NA	2000 mg/kg	Negative	23
Cytogenetics assay in Chinese Hamster Cells	Chromosomal aberrations	No	?	Positive	75
S. Typhimurium TA1535 and TA100	Base pair	Yes and No	10 mg/plate	Negative	135, 136
S. Typhimurium TA1538, TA98, and TA1537	Frameshift	Yes and No	10 mg/plate	Negative	
S. Typhimurium TA1538	Frameshift	Yes and No	10 mg/ml	Negative	137
E. coli WP2 uvrA	Base Substitution	Yes and No	10 mg/ml	Negative	
S. Typhimurium TA92, TA1535 and TA100	Base pair	Yes and No	5 mg/plate	Negative	21
S. Typhimurium TA1537, TA94 and TA98	Frameshift	Yes and No	5 mg/plate	Negative	
Chromosomal aberration test, CHL cells	Chromosomal aberrations	No	5 mg/ml	Positive	

Images of Coomassie Bluestained differentiating NB2a cells:

(a) in the absence of additives; (b) in the presence of 0.0001 µM Blue 1 to produce approximately 20% neurite inhibition; (c) 1 µM L-glutamic acid to produce approximately 20% neurite inhibition; (d) a mixture of Blue 1 and L-glutamic acid each at 50% of the above concentrations.

Figure used with the permission of Oxford University Press: Lau, K., W. G. McLean, *et al.* (2006). "Synergistic interactions between commonly used food additives in a developmental neurotoxicity test." Toxicological Sciences 90(1): 178-187.

Figure A2 Synergistic neurite inhibition with addition of Blue 1 and L-glutamic acid[99]

have an IC_{50} of 48.7 µmol/l. When cells were treated with the two additives together, rather than just seeing an additive effect, the two compounds worked synergistically (Fig. A2). A 50:50 mixture of L-glutamic acid and Blue 1 produced 46.1% neurite growth inhibition, which was significantly different from the expected value of 15.8% if the compounds acted additively. On the other hand, the effect on cell viability from the combination of the two additives was increased only in an additive fashion.[99] Without further research it is unknown whether other food dyes might behave similarly.

Feingold suggested that food dyes and additives are associated with hyperactivity disorders in children.[100] The developmental period of synaptogenesis (brain growth-spurt period) occurs in humans from three months before birth to several years after birth.[99] Small amounts of Blue 1 are absorbed by the GI tract in rats, but metabolism studies in children have not been conducted. Blue 1 might possibly have potent effects, and it might take only a small absorbed amount to affect a child's brain development. The blood–brain barrier is not fully developed until 6 months in humans and even after complete
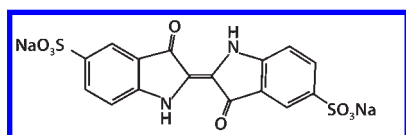
Figure A3   Blue 2 (Indigo Carmine)

development some regions of the brain are never protected by the blood–brain barrier.[101,102] Further neurotoxicity studies need to be conducted on Blue 1 and other dyes.

Conclusions

The most thorough studies of Blue 1, which were sponsored by industry, did not find evidence of carcinogenicity or other toxicity in rats or mice. On the other hand, in an *in vitro* test, Blue 1 inhibited neurite growth and acted synergistically with L-glutamic acid, suggesting the potential for neurotoxicity. That is particularly worrisome for fetuses and babies under the age of 6 months whose blood–brain barrier is not fully developed. Further research needs to be conducted to establish this dye's safety with greater certainty.

FD&C Blue 2

FD&C Blue 2 (Fig. A3) is the approved form of Indigo Carmine. In 1983, the FDA permanently listed Blue 2 for use in foods and ingested drugs.[103] It is widely used to color beverages, candies, pet foods, and other foods and drugs. Blue 2 has an ADI of 2.5 mg/kg bw/day.[104] That ADI is equivalent to 75 mg for a 30-kg child. The FDA certifies an amount of Blue 2 that is equivalent to about 2 mg/person/day.

Metabolism

Studies in rats demonstrated that the majority of Blue 2 and/or its metabolites (including 5-sulfoanthranilic acid, its final breakdown product) are excreted in the feces, with smaller amounts being found in the urine.[52,105] In one bile-duct-cannulated rat given a 20-mg dose of Blue 2, only 0.004% of the dye was excreted in the bile — 125 times as much was found in the urine. The authors concluded that the majority of the small amount of dye that is absorbed intact is excreted through the urine and not the bile, and the dye excreted in the feces is mostly from unabsorbed dye.[105] Those studies show that 5-sulfoanthranilic acid is absorbed more readily by the GI tract than is the intact dye.[105]

Genotoxicity

Details of the genotoxicity studies performed on Blue 2 are provided in Table A2. All of the 11 tests were negative except for a chromosomal aberration assay.[96]

Chronic toxicity/carcinogenicity

Between 1984 and 1986, Borzelleca *et al.*[106] performed several toxicology studies using Blue 2. One

Table A2   Summary of genotoxicity studies on Blue 2

Assay	Mutation Type	S9 Activation	Dose	Results	Reference
Comet assay	DNA damage	NA	2000 mg/kg	Negative	23
S. Typhimurium TA1535 and TA100	Base pair	Yes and No	10 mg/plate	Negative	135
S. Typhimurium TA1538, TA98, and TA1537	Frameshift	Yes and No	10 mg/plate	Negative	
S. Typhimurium TA1538	Frameshift	Yes and No	1 mg/ml	Negative	137
E. coli WP2 uvrA	Base substitution	Yes and No	10 mg/ml	Negative	
S. Typhimurium TA92, TA1535 and TA100	Base pair	Yes and No	5 mg/plate	Negative	96
S. Typhimurium TA1537, TA94 and TA98	Frameshift	Yes and No	5 mg/plate	Negative	
Chromosomal aberration test, CHL cells	Chromosomal aberrations	No	12 mg/ml	Positive	
S. Typhimurium TA1535 and TA100	Base pair	Yes and No	1 mg/plate	Negative	138
S. Typhimurium TA1538, TA98, and TA1537	Frameshift	Yes and No	1 mg/plate	Negative	
rec-assay	DNA damage	No	NA	Negative	139

was a chronic toxicity/carcinogenicity study in rats. The study included an *in utero* phase in which five groups of 60 male and 60 female Charles River CD albino rats were fed 0 (two different control groups), 0.5, 1, or 2% Blue 2 starting at least 2 months prior to mating. F_1 offspring in each dosage group were randomly selected, and 70 rats/sex/group were continued on the same dosages for 29 months in males and 30 months in females. Administration of the dye did not affect the number of pregnant females per group or pup viability at birth. However, there was possible evidence of carcinogenicity.[113]

- *Treated male rats showed a dose-related increase in the incidence of transitional cell neoplasms of the urinary bladder, but the numbers of affected animals were small and the apparent increase was not statistically significant when compared to combined controls (0.8, 1.6, 2.9, and 4.5% of the animals had bladder neoplasms in the control, low-, mid-, and high-dose groups, respectively; there appears to be a dose-related trend but the authors did not do a statistical test).*

- *Male rats in the 2% group had statistically significant increases in malignant mammary-gland tumors and brain gliomas. However, the investigators concluded that the increased mammary-gland tumors were not related to Blue 2. They also concluded that the gliomas were not consistent with several criteria they said were required to classify a compound as a carcinogen. For instance, neither a dose–effect relationship nor a decrease in survival time was seen. They also reported that the incidence of gliomas in treated animals was consistent with historical controls. (Companies [and the FDA] sometimes make comparisons to historical controls when the test group has more tumors than the concurrent controls.) Based on this study, the investigators estimated that the NOAEL for Blue 2 was 2.0%, or 1282 mg/kg bw/day and 1592 mg/kg bw/day for males and females, respectively.*[106]

The FDA's Cancer Assessment Committee concluded that the occurrence of urinary bladder transitional cell neoplasms in the male rats, though apparently dose-related, was not related to treatment with Blue 2 because: (i) historical evidence suggests that this form of cancer is not rare in Charles River CD albino rats; (ii) the number of neoplasms in the high-dose group was small; and (iii) the number of tumors in the high-dose group was not significantly higher than in the control groups.[103]

Regarding the malignant tumors of the mammary gland in the high-dose males, when the Committee combined malignant and benign tumors, there was no longer a statistically significant difference between the controls and high-dose male rats. The Committee concluded that Blue 2 did not cause any significant treatment-related effects in rats.[103]

Although there was a significantly higher incidence of brain gliomas in the high-dose male rats, the FDA's Cancer Assessment Committee was still reluctant to conclude that Blue 2 was the cause because: (i) of a lack of gliosis in the high-dose animals; (ii) the first two observed gliomas of the brain occurred in controls animals; and (iii) data were lacking on the historical incidence of brain gliomas in Charles River albino rats that survive for 30 months. The FDA concluded that 'except for brains of male rats for which the data are equivocal, there is no evidence for carcinogenicity in rats or mice of either sex for all organs examined.' Upon reevaluation of the brain microslides and comparison to controls from a simultaneous study on Green 3, new statistical tests produced P values that were just above 0.05 (the Breslow time-adjusted analysis, produced a P value of 0.053).[103] It is highly questionable to switch a comparison to a different control group after a study is completed. Still, the FDA stated, '… although statistical methods provide insight into the likelihood of being right or wrong in making specific conclusions, they do not provide for certainty as to whether an increase or decrease in tumor incidence is related to treatment.' The Board of Scientific Advisors of the NTP concluded that Blue 2 is safe, citing: (i) no dose-related trend; (ii) lack of non-neoplastic cellular changes in addition to frank neoplasia; (iii) no reduction in latency period; (iv) no varying progression of brain tumors; (v) the inability of Blue 2 to cross the blood–brain barrier; (vi) negative mutagenicity assays; and (vii) lack of evidence in structure-activity analysis.[103]

Borzelleca *et al.*[106] consulted three outside toxicologists on the carcinogenicity issues. Robert Squire, a prominent industry consultant at the Johns Hopkins University School of Medicine, found a lack of persuasive evidence for compound-related carcinogenicity in the glioma and urinary bladder samples.[107] However, Aleksandar Knezevich and Geoffrey Hogan, former vice president of pathology and former vice president of toxicology, respectively, at Bio/dynamics (an industry consulting firm), concluded that the glioma findings 'cannot be dismissed as accidental'. On the other hand, they agreed with the FDA committee that the rates of urinary neoplasms in treated male rats were not clearly different from the controls and were probably not of concern.[108]

After Blue 2 was permanently approved in 1983, the Public Citizen Health Research Group (HRG) filed a formal objection on the grounds that the increase in brain tumors in rats fed Blue 2 was statistically significant. The group argued that the decision to approve Blue 2 violated both the Delaney Clause (which bars cancer-causing food and color additives) and the general safety clause since the dye had not been proven safe.[109]

In a statement to the HRG in 1982, Dr William Lijinsky, a cancer specialist at the NCI's Frederick Cancer Research Center, wrote,

> … *the incidence of these (brain) tumors in the high dose group versus the controls is highly significant… In my own laboratory this would be considered prima facie evidence of carcinogenicity of a treatment. This is especially so because this tumor is so rare, and my conclusion is that Blue 2 is a carcinogen, and should be regulated accordingly.*

Regarding his own evaluation of the histopathology of brain/spinal cord sections in microslides, Dr Benjamin A. Jackson, of the FDA's Division of Pathology in the Color and Cosmetics Evaluation Branch, wrote, '… the possibility cannot be outrightly excluded that the compound (Blue 2) itself, its metabolite(s) or a secondary effect induced by the high dose of the color may have acted to increase the number of brain tumors seen in this study.'[109]

An administrative law judge found that a lack of certain biological factors, such as gliosis, invasiveness of tumors, a clear dose-response relationship, and an increased latency, outweighed the statistically significant incidence of brain gliomas in the rats. The FDA commissioner then concluded that the evidence supported the notion that Blue 2 was not an animal carcinogen and that the permanent listing of Blue 2 was appropriate.[109]

HRG challenged the FDA's decision, contending that the rats may not have been exposed to the maximum tolerated dosage (MTD). According to the FDA's testing guidelines, the highest dosage used in a study 'should be sufficiently high to induce toxic responses in test animals, and should not cause fatalities high enough to prevent meaningful evaluation of the data from the study.' Chronic-study doses '… should be based on results from subchronic studies and other related test substance information.'[58] HRG questioned whether the MTD was used in the chronic toxicity rat study because: (i) no subchronic study was conducted to establish the MTD (the FDA found it acceptable to rely on the results of a previous 1966 study by Hansen); (ii) adult rats in the study did not show alterations typical of animals given the MTD according to the FDA Redbook (FDA's guide for the testing of additives); (iii) 5% was used as the MTD for the chronic mouse study discussed below (as opposed to 2% in the rat study); and (iv) the Hansen study used a high dose of 5%, which led to an increase in the overall number of tumors compared to other groups. HRG argued that allowing a 2% MTD was contradictory to the FDA's own guidelines.[110] Notwithstanding those arguments, the court ruled in favor of the FDA.[111]

In another study, 30 Charles River CD-1 mice/sex/group were fed 0.2, 0.4, 0.8, or 1.6% Blue 2 for 84 weeks.[112] Controls consisted of 60 males and 60 females. The overall death rates in treated mice did not differ significantly from that in the controls. The most common neoplasms seen in both the control and treated mice were generalized lymphoblastomas and pulmonary adenomas. The incidence of lymphoblastomas was not associated with the feeding of Blue 2. There was a significant increase in the incidence of pulmonary adenomas in the lowest-dose treatment group in males compared to controls. That increase was not seen in higher-dosage males or in females and, therefore, was not considered by the authors to pose a risk to humans. In this study the NOAEL was determined to be 0.4% of the diet or approximately 600 mg/kg/day. With a safety factor of 100, that translates into an intake of about 360 mg/day for a 60 kg person.[112] This study was flawed because of its brevity — Charles River CD-1 mice often live to well over 2 years — because the mice were not exposed *in utero*, and because the numbers of mice exposed to each dosage were small.[87]

Borzelleca *et al.*[113] also conducted a carcinogenicity/toxicity study of Blue 2 in mice. That study did not include an *in utero* phase. Blue 2 was fed to 60 Charles River CD-1 mice/sex in 0 (two control groups), 0.5, 1.5, and 5% groups. The study lasted 22 months for males and 23 months for females — longer than the Hooson study discussed above, but still shy of 2 years, let alone the lifetime of the mice. The investigators concluded that Blue 2 did not cause any significant effects on behavior, morbidity, mortality, hematology, or physical observation and considered the NOAEL to be 5%, or 8259 mg/Kg bw/day in male CD-1 mice and 9456 mg/kg bw/day in female CD-1 mice.

Reproductive toxicity and teratogenicity

Borzelleca *et al.*[114] conducted a three-generation reproductive study of Blue 2 in Charles River CD rats. Groups of 10 males and 20 females were fed the dye at levels of 0, 2.5, 25, 75, or 250 mg/kg bw/day. Retinoic acid, a known teratogen in rats, was used as a positive control. Treated parents and pups were normal in terms of general appearance and behavior. The compound was not teratogenic and did not affect fertility, length of gestation, viability, or lactation indices. The compound did not cause anatomical abnormalities in the uteri or ovaries of females given caesarian sections. There were also no compound-related effects on organ weights and gross and microscopic pathological lesions.[114]

Borzelleca *et al.*[115] tested the potential teratogenicity of Blue 2 in Charles River CD rats and Dutch Belted rabbits. Twenty pregnant rats/group received 0.5% methacol (a vehicle control), 7.5 mg/kg/day retinoic acid (a positive control), or 25, 75, or 250 mg/kg/day

Blue 2. Ten pregnant rabbits/group followed the same regimen as the rats, except that 150 mg/kg/day thalidomide was used as a positive control in place of retinoic acid. Investigators reported no compound-related adverse effects on maternal appearance, behavior, body weight, or mortality. There were also no adverse effects on fetal body weight, viability, or abnormalities. The NOAEL for Blue 2, on the basis of this study, was determined to be 250 mg/kg/day in rats and rabbits.

Conclusions

Two chronic toxicity/carcinogenicity studies of Blue 2 in mice did not find any problems, but they were flawed because they did not include an *in utero* phase and were shorter than 2 years. More worrisome was a chronic toxicity/carcinogenicity study in rats that found that males in the 2% group had statistically significant increases in brain gliomas and malignant mammary gland tumors. The FDA found reasons to excuse that evidence of carcinogenesis and neoplasia and approved the continued use of the dye.

Given the statistically significant occurrence of tumors, particularly brain gliomas, in male rats, Blue 2 cannot be considered safe for human consumption. The evidence on Blue 2 certainly does not meet the legal standard for safety: 'that there is *convincing evidence* that establishes with reasonable certainty that no harm will result from the intended use of the color additive …' [emphasis added].[116] Since Blue 2 (parts per million) is a non-nutritive color additive that does not provide any health benefit, and there is hardly 'convincing evidence' of safety, it should not be permitted for human consumption.

Citrus Red 2

Citrus Red 2 (Fig. A4) is an azo dye approved only to color the skins of Florida oranges not used for processing. Amounts are permitted up to 2 parts per million (ppm) in the whole fruit.[117] Only about 1500 pounds of this dye are certified annually, but that is enough to color about two billion oranges.

Metabolism

Radomski *et al.*[118] administered a single oral dose of Citrus Red 2 to rats, dogs, and rabbits. Rats given a single oral dose of 2–20 mg excreted 5–7% of intact dye in their feces over 48 hours. Similar to
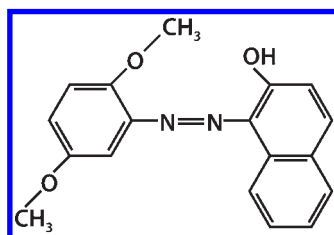

Figure A4 Citrus Red 2

water-soluble azo dyes, this water-insoluble dye is broken down in the GI tract by intestinal bacteria. One breakdown product is 1-amino-2-naphthol, which has been shown to cause bladder cancer in mice.[119] At single doses higher than 5 mg, the dye accumulated in the fat of rats. Small amounts of 1-amino-2-naphthyl sulfate were found in the urine of rats, demonstrating that the 1-amino-2-naphthyl metabolite is absorbed, sulfonated, and then excreted.[118]

Chronic toxicity/carcinogenicity

In one study, 50 mice/sex/group were fed Citrus Red 2 at levels of 0, 0.01, 0.03, 0.1, 0.3, 1, or 3% of their diet. The study lasted up to 80 weeks, an inadequate duration. The study was discontinued in the 0.3, 1, and 3% groups due to increased morbidity and mortality. Mice in the 0.1% group also experienced increased mortality, and females showed degeneration of the liver.[120]

The same researchers conducted a study with 50 mice/sex injected subcutaneously with 10% Citrus Red 2 for 35 weeks, followed by injections every 3 weeks for 15 weeks. The control group received only vehicle injections. Female mice showed an increase in total malignant tumors, which appeared earlier than tumors in the control group. The most common malignant tumors were adenocarcinomas of the lung and lymphosarcomas. There were no injection-site tumors.[120]

Hazleton Laboratories conducted a chronic feeding study in rats. The toxicological data were evaluated by the director of FDA's Division of Pharmacology, A. J. Lehman, who concluded that the synthetic dye is toxic. In this study, 40 rats/sex/group were fed Citrus Red 2 at doses of 0, 0.05, 0.1, 0.5, 1, 3, and 5%. Rats in the two highest dosage groups were sacrificed after 31 weeks because of severe toxicity. The remainder of the rats remained in the study for 104 weeks. Rats in the 0.5 and 1% groups experienced differences from controls in gross appearance, growth, organ weights, and gross and microscopic pathology. At the 0.1% levels, rats showed differences in organ weights, incidence of edema-like swelling, a possible trend toward an increased incidence of fatty metamorphosis (fat droplets in the cytoplasm of cells), and a significant difference in weight gain in females. Researchers did not report an increase in the occurrence of tumors. The NOEL was judged to be 0.05% (500 ppm).[121]

Dacre administered Citrus Red 2 for 24 months to 20 mice and 20 albino rats per dosage group.[122] The dye was given at dosages of 0, 0.05, and 0.25% beginning immediately after weaning, without *in utero* exposure. This study found hyperplasia and a thickening of the urinary bladder wall in both treatment groups in rats and mice. Of greater concern, 2 out of 20 mice that were examined

developed benign papillomas and one male mouse developed a malignant papilloma in the urinary bladder, and four out 28 rats that were examined developed benign papillomas. About the same number of pathological changes were seen in the low- and high-dosage groups in both species and sexes. No problems were seen in control animals.[122]

An internal FDA memo expressed concern about the carcinoma seen in Dacre's mouse study, because benign tumors and hyperplasia also were seen.[79] FDA veterinarian Kent J. Davis wrote, '… this becomes a level of meaningful significance to cancer research workers.' He added,

> *Citrus Red 2 then becomes an intolerable human health hazard if only from the amounts consumed from fingers after peeling oranges treated with this dye. (Some additional dye may be ingested with peel or orange.) The continued certification and use of this color may also be a violation …of the Federal Food, Drug, and Cosmetic Act as amended which prohibits use of any carcinogenic color additive for uses which may result in ingestion of part of such additive.*

Conclusions
Citrus Red 2 is toxic to rats and mice at modest levels and, according to an FDA scientist and the IARC, is a bladder carcinogen.[123] The FAO/WHO Expert Committee on Food Additives stated bluntly: 'This color should not be used as a food additive'.[124]

FD&C Green 3
FD&C Green 3 (Fig. A5), or Fast Green FCF, is a synthetic dye approved for use in food, drugs, personal care products, and cosmetics except for in the area of the eye. It is one of the least-used dyes (Table 4), but may be found in candies, beverages, dessert powders, ice cream, sorbet, and other foods, as well as in ingested drugs, lipsticks, and externally applied cosmetics.[125] The ADI for Green 3 is 2.5 mg/kg bw/day, or 75 mg/day for a 30-kg child.[126] Current usage is equivalent to only 0.1 mg/person/day.

Metabolism
Hess and Fitzhugh studied the metabolism of Green 3 in rats and dogs. Three female and 3 male Osborne–Mendel rats were orally administered a single 200-mg dose of Green 3. An average of 94% of the dye was excreted intact in the feces. No recovery from the urine was reported. Male and female bile duct-cannulated dogs were orally administered a single 200-mg dose of Green 3. None of the color was found in the urine and about 2% of the dye was recovered in the bile of two of three dogs. Hess and Fitzhugh calculated the absorption of the dye from the GI tract of rats and dogs to be ∼5%.[94]

Genotoxicity
As Table A3 indicates Green 3 was mutagenic in the *S.* Typhimurium strain TA100 Ames Assay at 10 mg/
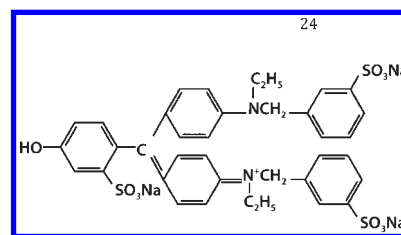

Figure A5   Green 3 (Fast Green FCF)

plate. That assay tests for base-pair mutations, and Green 3 only yielded positive results when tested as a mixture of several batches of dye of varying purity.[21] Green 3 was also positive for mutagenicity in a Fischer rat embryo cell transformation assay.[127] That particular assay tests for malignant cell transformation, an indicator of carcinogenic potential. Green 3 was positive at 1 µg/ml but, surprisingly, produced negative results at higher concentrations. In summary, three of nine studies indicated mutagenicity, but the data overall do not necessarily indicate a human health risk.

Chronic toxicity/carcinogenicity
In 1977, the FDA required that additional chronic toxicity studies be conducted before Green 3 could become a permanently listed food coloring.[128] To fulfill that requirement, the CCMA sponsored chronic feeding studies in mice and rats.

In the first study, Green 3 was administered to 60 Charles River albino rats/sex/group at dosage levels of 0 (two control groups), 1.25, 2.5, and 5% for at least 2 months prior to mating. After reproduction, 2, 3, or 4 pups/sex/litter/group were randomly selected for the long-term study. The same dosage levels used in the *in utero* phase were administered to 70 rats/sex/group for approximately 30 months. No significant effects were noted during the *in utero* phase except that pup mortality was increased in the mid- and high-dose groups of the F_1 generation. In the F_1 generation, a significant decrease in survivorship was seen in all treated groups of males and females, but there was no dose–response trend, making that decreased survivorship difficult to interpret. Urinalysis, hematologic parameters, physical observations, and ophthalmology did not indicate any adverse effects of Green 3.[129]

Histopathological examination revealed that the high-dose group of male rats had increased incidences of urinary bladder transitional cell/urothelial neoplasms, testes Leydig's cell tumors (usually rare and benign in humans), and liver neoplastic nodules. Statistical analyses found that the increased incidences were significant for the urinary bladder transitional cell/urothelial neoplasms ($P=0.04$, Bio/Dynamics analysis) and testes Leydig's cell tumors ($P=0.04$, FDA analysis), when compared to combined

Table A3 Summary of genotoxicity studies on Green 3

Assay	Mutation type	S9 activation	Dose	Results	Reference
Comet assay	DNA damage	NA	2000 mg/kg	Negative	23
S. Typhimurium TA100	Base pair	Yes	10 mg/plate	Positive (in crude sample)	21
S. Typhimurium TA92 and TA1535	Base pair	Yes and No	10 mg/plate	Negative	
S. Typhimurium TA1537, TA94 and TA98	Frameshift	Yes and No	10 mg/plate	Negative	
Chromosomal aberration test, CHL cells	Chromosomal aberrations	No	4 mg/ml	Positive	
Diploid yeast *Saccharomyces Cerevisiea* (BZ 34)	Mitotic gene conversion	No	5 mg/ml	Negative	140
Fischer rat embryo cell transformation	Malignant cell transformation (indicator of carcinogenic potential)	No	1 µg/ml	Positive (Negative at 10 and 100 µg/ml)	127
S. Typhimurium TA1535 and TA100	Base pair	Yes and No	50 µg/plate	Negative	138
S. Typhimurium TA1538, TA98, and TA1537	Frameshift	Yes and No	50 µg/plate	Negative	

controls.[129] Mark Nicolich, a statistician working at the company that conducted the study, stated, 'Therefore, there is statistical evidence that the high dose of the test material increases the occurrence of certain types of tumors in rats'.[130] Nevertheless, FDA scientists concluded that the tumors in the testes were not compound-related because they are common in aged rats (but the concurrent control groups should control for that) and because the numbers of tumors in the low-dose and high-dose groups were comparable (though it is possible that the maximum rate of tumors occurred in the low-dose group). Regarding the urinary bladder neoplasms, the original report submitted by the petitioners stated that the high-dose male rats had a significantly increased incidence of those benign tumors. However, in the final submission, the petitioners submitted an addendum claiming, without any specific justification, lack of statistical significance. The FDA pathologists concluded that neither the incidence nor the severity of the transitional cell hyperplasia of the urinary bladder was treatment related.[125]

In the CCMA-sponsored chronic toxicity/carcinogenicity study on Charles River CD-1 mice, 60 mice/sex/group were fed 0 (two control groups), 0.5, 1.5, or 5% Green 3 in their diet for 24 months. The mice were not exposed to Green 3 *in utero*. No gross or microscopic neoplastic and non-neoplastic observations related to administration of the color were observed. Statistical analysis concluded that Green 3 did not have any negative effect on time-to-tumor, survivorship, or tumor incidence in mice.[131]

Conclusions

Green 3 did not increase tumor rates in CD-1 mice, though the only study did not include *in utero* exposure. Green 3 caused significant increases in bladder transitional cell/urothelial neoplasms and testes Leydig's tumors in high-dose male rats. Despite a last-minute assertion by the testing laboratory that the bladder neoplasms were no longer statistically significant and the FDA's dismissal (based on qualitative considerations, not statistical analyses) of the significance of the testes tumors, Green 3 must remain suspect until further testing demonstrates that it is safe. Evidence of safety is not 'convincing,' as FDA regulations require.[116]

Orange B

Orange B is an azo dye (Fig. A6) that is approved by the FDA for use only in frankfurter and sausage casings up to 150 ppm in the finished food.[132] Batches of Orange B have not been certified for use in the past decade or longer.

Metabolism

Orange B is poorly absorbed in rats. The color is reduced in the gut to form naphthionic acid. That metabolite appears in both the feces and the urine, indicating that some of the metabolite is absorbed.[133]
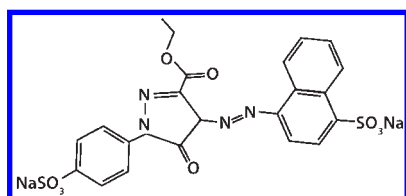
Figure A6   Orange B

Chronic toxicity/carcinogenicity

Orange B was fed to 50 Sprague–Dawley rats/sex/group at doses of 0, 0.5, 1, 2 or 5% for 2 years (an *in utero* phase was not included). By the end of the second year, all of the rats in the 2% group and most in the remaining groups (including the control groups) were dead. Male and female rats in the two highest-dose groups showed lymphoid atrophy of the spleen and bile-duct proliferation. All examined animals in the highest-dose group experienced moderate chronic nephritis, but increased tumor rates were not reported. Investigators gave Orange B a NOAEL of 0.5% for rats.[133]

Orange B was fed to 50 C_3H mice/sex/group and 50 $C_{57}BR/cd$ mice/sex/group at doses of 0, 1, or 5% dietary supplement for their lifespans (the mice were not exposed *in utero*). There was no effect on tumor development or lifespan. The growth rate of the C_3H mouse was depressed in the 5% groups. Investigators assigned a NOEL of 1% to mice.[133]

Conclusion

In 1978, the FDA proposed banning Orange B, but, presumably because companies stopped using it, the FDA never bothered to finalize the ban; it should do so now.[134]

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