11

N98008

SUMMARY OF DATA FOR CHEMICAL SELECTION

Bixin (Annatto)
6983-79-5 (1393-63-1)

BASIS OF NOMINATION TO THE CSWG

The nomination of bixin to the CSWG is based on the high production volume of annatto, which is consumed by virtually every person in the United States. According to data provided by the Food and Drug Administration (FDA), annatto is one of the most highly consumed colorants in the US food supply. Bixin is the ingredient that contributes this color.

Despite annatto's status as an unregulated color additive, surprisingly little is known about the toxicity of bixin or norbixin, which are intentionally concentrated in annatto extracts and oils. Chronic toxicity studies have been conducted on annatto; at first glance, they suggest that annatto is nontoxic. However, these studies do not hold up well under scrutiny. In these studies, the exact concentrations of bixin or norbixin were not reported; these may vary anywhere from 2 to 50%. Also, no attempt has been made to characterize other ingredients in annatto that might have biological activity. Concern about the potential toxicity of bixin is increased because a few short-term tests for genotoxicity of annatto have been described as positive.

Subchronic studies of purified bixin and short-term tests of bixin, norbixin, and technical grade annatto products would alleviate concerns about the inferior data underpinning the widespread use of annatto. Also, mechanistic and metabolism studies would extend scientific knowledge about an important and ubiquitous class of chemicals, the carotenoids. Testing by the NTP would be especially appropriate since the producers of annatto are farmers in third world countries; the revenues from annatto are insufficient for these producers to finance toxicity testing for this product

SELECTION STATUS

ACTION BY CSWG: 12/10/97

Studies requested:

- Subchronic study
- *In vitro* cytogenetics
- *In vivo* micronucleus
- Lung adenomas in Strain-A mouse model

Priority: Moderately high

Rationale/Remarks:

- Widespread human exposure
- Unknown potential for adverse health effects
- Reconsider for carcinogenicity after subchronic study is completed

INPUT FROM GOVERNMENT AGENCIES/INDUSTRY

Dr. Dan Benz, Center for Food Safety and Applied Nutrition (CFSPAN), FDA, and Dr. Ed Matthews, formerly with CFSPAN, provided information on annatto from FDA's Priority-based Assessment of Food Additives (PAFA) database.

Ms. Joellen Putnam, Scientific Project Manager, Flavor and Extract Manufacturers' Association (FEMA), provided a copy of the British Industry Biological Research Association (BIBRA) profile on annatto.

CHEMICAL IDENTIFICATION

CAS Registry No.:

Anatto 1393-63-1
Bixin (cis) 6983-79-5
Bixin (trans) 39937-23-0
Norbixin (cis) 626-76-6
Norbixin (trans) 542-40-5

Chemical Abstract Service Names:

Annatto: None
Bixin: methyl (9-cis)-hydrogen 6,6'-diapo-.psi.,psi-carotenoidioate
Norbixin: 6,6'-Diapo-,psi.,psi.-carotenedioic acid

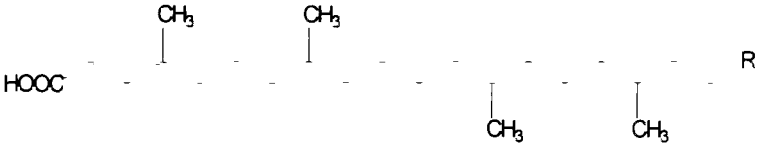
Synonyms and Trade Names:

Achiote, Achiotl, Achote, Urucu, Beni-No-Ki, Bija, Onota, Orleanstrauch, Roucou, Roucouyer, Roucoyer, Uruku (The Raintree Group, 1997); Annotta, Arnatta, Arnatto, Arnotta, CI Natural Orange 4, CI 75120.

Structural Class:

Unsaturated aliphatic hydrocarbon, carboxylic acid derivative; carotenoid

Structure, Molecular Formula, and Molecular Weight:



Bixin: R=$COOCH_3$, Norbixin: R=COOH

Bixin:
Molecular formula: ($C_{25}H_{30}O_4$)
Molecular weight: 394.5

Norbixin:
Molecular formula: ($C_{24}H_{28}O_4$)
Molecular weight: 380.5

Chemical and Physical Properties (bixin):

Description:

Orange crystals (Lewis, 1993)

Melting Point:

198°C (Lide, 1995);
Decomposes at 217°C (Lewis, 1993)

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Solubility: Insoluble in water; soluble in ethyl alcohol and acetone, slightly soluble in ether (Lide, 1995)

Technical Products and Impurities: Annatto is an orange-red colorant obtained through extraction of the seeds of the fruit of the *Bixa orellana* tree. Bixin and norbixin are the principle coloring constituents of annatto (Hallagan *et al.*, 1995)

Annatto seeds contain 40-45% cellulose, 3.5-5.5% sucrose, 0.3-0.9% essential oil, 3% fixed oil, 4.5-5.5% pigments (comprised of 70-80% bixin), 13-16% protein, and other constituents. Annatto seeds also contain tannins, ethereal oils, saponins, mustard oil-like substances and mono- and sesquiterpenes (The Raintree Group, 1997; BIBRA, 1986).

Annatto oil solutions contain bixin. Bixin is present mainly in the cis form, but trans bixin, a thermal degradation product, is also present. Water-soluble annatto solutions contain the alkali salts of norbixin, which results when bixin is saponified and the methylethyl group is removed (TJP Market Development, 1997; BIBRA, 1986; Noonan, 1975).

EXPOSURE INFORMATION

Production Methods: Annatto is an ancient crop of the tropical lowlands of Latin America. The annatto tree is a small, ornamental evergreen that produces burr-like pods containing 5 to 100 seeds. Annatto is mainly traded in the form of sun-dried seeds in burlap sacks. The bixin content of the annatto seed is the major specification; it has to be 2.5 to 3.0% as a minimum. The shelf-life of annatto seeds is up to one year when they are stored in a cool, dry warehouse, but the seeds start losing color in 3-4 months. For longer storage, annatto seeds can be frozen. Annatto is also traded as powders or pastes. Annatto paste is produced after the pulpy exterior of the annatto seeds is dried in the sun. The resulting paste is sold if the bixin content is at least 2.5%. The paste is transported in drums. Annatto powder is transported in polyethylene-lined paper sacks or drums (TJP Market Development, 1997).

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Liquids of various concentrations are prepared by leaching annatto seeds with an extractant prepared from food-grade materials, including organic solvents, edible vegetable oils and fats, and alkaline or alcoholic solutions. The extractant selected depends on the intended end use. Water-soluble extracts are prepared by agitation of the annatto seeds in alkaline solutions. The solution is then filtered, and to refine the product, the pigment is precipitated by addition of hydrochloric acid. This precipitate is filtered, dried, and washed with petroleum solvent to remove unwanted impurities and odors. The norbixin pigment that is produced is then mixed with alkali and dissolved in water. A second method for preparation of water-soluble annatto color is the extraction of bixin pigment from the seeds by an organic solvent, removal of the solvent by distillation and then hydrolysis with aqueous-alkali solution which produces norbixin (TJP Market Development, 1997).

Oil-soluble annatto color is prepared by immersion of annatto seeds in vegetable oil followed by mechanical abrasion. The resulting slurry is heated under a vacuum, and the solution is filtered to remove insoluble material (TJP Market Development, 1997).

Production/Import levels: The major producing countries of annatto are all from South America; Peru is by far the world leader, with Guatemala and Ecuador trailing. Of the African countries, Kenya is the largest exporter, with the Ivory Coast also providing exports. India and Spain are emerging as important exporters to the European market. The United States is an exporter of processed annatto (TJP Market Development, 1997).

Because part of the annatto is processed into liquids of various strength before export, estimating the size of the annatto market is difficult. Annatto seed imports to the United States are estimated to be 2,500-3,000 metric tons (5.5-6.6 million lbs) with a market value of $2.5-3.3 million. Importers estimate that the demand for annatto will grow 4-5% per year for the next 3 years (TJP Market Development, 1997).

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Producers/Importers: *The Directory of World Chemical Producers 1995/96* (Chemical Information Services, Inc., 1994) lists ten annatto producers. Several may be importers who purchase annatto seeds, powder, or paste and process it further to meet the needs of their clients. In the U.S., importers include Kalsec, Inc. of Kalamazoo, and Pfizer Inc., Chemical Division, New York. INEXA Industria Extractora, C.A., in Ecuador, and Parnaiba, in Brazil, are listed as bixin producers. Annatto extract, FEMA 2103, is available from Meer or Penta in New Jersey, or from Enjayes in India; annatto seed, FEMA 2104, is available from Meer (Flavor and Fragrance Materials, 1993).

Use Pattern: Carotenoids are the most widespread color agents occurring naturally in both plants and animals. The carotenoids, bixin, norbixin, β-carotene, and to a lesser extent, canthaxanthin, are widely used in foods for the purpose of imparting color (Noonan, 1975). According to the FDA, annual consumption of annatto seed as a direct food additive is 3.65 million lbs with an additional consumption of 1.5 million lbs of annatto extract (FDA, 1994). Its primary application is in coloring butter, cheese, and other dairy products. Annatto can be used for coloring ingested drugs and as a tracer in food products. Current trends show that annatto oil is used increasingly in body care products, where it adds a rich sunny color to creams, lotions, and shampoos (The Raintree Group, 1997). Other minor uses are in meat, chocolate, popcorn, and fish products (TJP Market Development, 1997); in the manufacture of wood stains and varnishes; and in the dyeing of silk (Budavari, 1996). Additional information is contained in Table 1

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Table 1. Annatto Products and Their Uses

Product	Description	Applications
water-soluble powder	annatto extracted with potassium carbonate and potassium hydroxide	dry mixes, soups, drinks, cakes, cereals, desserts, tablets
water-soluble liquid	annatto extract dissolved in potassium carbonate/hydroxide solution	cheeses, cereals, cakes, drinks, snack foods, ice cream cones, ice cream, sausage casings
oil-soluble liquid	annatto extract in vegetable oil	margarine, fats and oils, shortenings, spreads, salad oils, butters, spice blends, pasta
oil-soluble suspension	annatto extract suspended in vegetable oil	processed cheeses, cake mixes, frostings, candy coatings, salad oil, margarine
emulsion	purified annatto, processed and emulsified with propylene glycol, monoglycerides and potassium hydroxide	beverages, candies, yoghurt, sherbets, butters, margarine, cheeses, shortening, frosting, confectionery coatings, baked goods, snack foods, salad dressings, ice creams

Source: Annatto suppliers (TJP Market Development, 1997)

Human Exposure: The primary exposure of humans to annatto and its components, bixin and norbixin, occurs through its widespread use in foods and its increasing use in personal care products. Occupational exposure in traditional industries appears to be limited. No reports of environmental contamination were found; it is unlikely that this issue has been studied.

The National Occupational Exposure Survey (NOES), which was conducted by the National Institute for Occupational Safety and Health (NIOSH) between 1981 and 1983, estimated that 1,887 employees in one industry were potentially exposed to annatto in the workplace. No information on bixin or norbixin was found. The NOES database does not contain information on the frequency, level, or duration of exposure to workers of listed chemicals (NLM, 1997).

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Regulatory Status: No standards or guidelines have been set by NIOSH or OSHA for occupational exposure or workplace maximum allowable levels of annatto, bixin, or norbixin. Annatto, bixin, and norbixin were not on the American Conference of Governmental Industrial Hygienists (ACGIH) list of compounds for which recommendations for a Threshold Limit Value (TLV) or Biological Exposure Index (BEI) are made.

As a color additive, annatto is subject to very different regulations in the United States and Europe. In the United States, §73.30 of the Color Additive Amendments to the Food, Drug and Cosmetic Act states that "Annatto extract may be safely used for coloring foods generally, in amounts consistent with good manufacturing practice . . . Certification of this color additive is not necessary for the protection of the public health."

In the European Union, an allowed daily intake for annatto was established by the Scientific Committee for Food (BIBRA, 1986; Hallagan, et al., 1995). This European standard is zero to 2.5 mg/kg/day, based on annatto preparations containing 2-6% carotenoids, expressed as bixin. This regulation is based on the acceptable daily intake (ADI) for humans of 0.065 mg/kg/day as bixin established by the Joint FAO/WHO Expert Committee on Food Additives (JECFA, 1982). The Ministry of Agriculture, Fisheries and Food in Great Britain has expressed concern that uses of annatto in the United Kingdom could result in consumers exceeding the ADI for annatto (TJP Market Development, 1997).

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EVIDENCE FOR POSSIBLE CARCINOGENIC ACTIVITY

With few exceptions, toxicological data on natural color additives are not as extensive as the data for synthetic color additives. During the 1960s and 1970s, the FDA required safety studies on the synthetic color additives as a condition for keeping them on the market No similar regulatory initiative has been mounted for natural color additives like annatto (Hallagan *et al.*, 1995).

Human Data: No epidemiological studies or case reports investigating the association of exposure to annatto, bixin, or norbixin and cancer risk in humans were identified in the available literature

Annatto can elicit an allergic response in some individuals. Fifty-six patients with urticaria or angioedema were orally challenged with annatto extract, and 26% had a positive test (Mikkelson *et al* , 1978). In a study of 112 patients with recurrent urticaria, 10% orally challenged with annatto dye, had a positive response and 14% had an uncertain challenge (Juhlin, 1981). Neither of the above studies was double blinded or placebo controlled (Hallagan *et al* , 1995).

A patient developed urticaria, angioedema, and severe hypotension within 20 minutes following ingestion of milk and Fiber One™ cereal. Skin tests to milk, wheat, and corn were negative, but the patient had a strong positive skin test to annatto dye. The nondialyzable fraction of annatto dye displayed two protein staining bands in the 50 kD range. Immunoblotting demonstrated patient IgE specific for one of these bands (Nish *et al* , 1991).

Animal Data: *Acute Studies*: In rats, the oral LD_{50} of oil-soluble annatto extract (bixin content not given) was reported to be >50 g/kg; for water-soluble annatto extract (norbixin content not given), the oral LD_{50} was >35 g/kg (van Esch *et al.*, 1959).

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Subacute/Subchronic Studies. Several old studies involving repeated oral administration were summarized by BIBRA (1986), which cautioned that some were unpublished and probably none were comprehensive in nature

- One of six dogs fed "fat-soluble annatto" (0.67-2 g/kg/day) for 9 weeks, followed by a normal diet for 5 weeks and then half this dose for 38 weeks died of liver damage (Kay & Calandra, 1961, unpublished).

- Mortality, liver and kidney function, blood and the histopathology of major organs were reportedly unaffected in groups of six dogs apparently fed very high doses (up to 20% of the diet) of "an aqueous extract of annatto seed" for one year (Kay & Calandra, 1961, unpublished).

- The microscopic appearance of the liver, kidneys, and spleen, the blood, and the organ weights and growth of six pigs given about 400 mg/kg/day of "fat-" or "water-soluble annatto" in the diet for 21 weeks were unaffected by these treatments (van Esch *et al* , 1959).

- No effects on the blood or major organs were reported in groups of 10 rats of each sex fed 1-2 g/kg/day of "water-" or "fat-soluble annatto" for about 13 weeks or up to about 250 mg/kg/day of "fat-" or "water-soluble annatto" for at least two years (van Esch *et al.*, 1959; Zbinden & Studer, 1958). No information on the effects examined or whether any histopathological exams were conducted was available, so these studies provide little useful information to evaluate the toxicity of annatto.

- No significant adverse effects on reproduction were reported in two- and three-generation studies of 10 pairs of rats fed "water-soluble annatto" in the diet at 250-500 mg/kg/day (van Esch *et al.*, 1959).

Chronic/Carcinogenicity Studies. In the early 1950s, the Government Control of Dairy Products and Eggs in Copenhagen funded rodent experiments on "Danish vegetable Annatto butter-colour." The mice originated from the inbred ST/Eh strain. Rats originated from a non-inbred stock supplied by Lovens kemishe Fabrik, Copenhagen. Evidence of carcinogenicity was not found. These studies by Engelbreth-Holm & Iversen (1955) are summarized below.

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- One hundred female rats were fed 26 mg "Danish vegetable Annatto butter-colour" in soy oil daily, administered dropwise into the mouth. The experiment continued for 26 months, most of the animals' lifetimes. Another 100 female rats served as untreated controls. Histopathology was performed on 86 experimental animals and 98 controls, and the authors concluded that annatto had no toxic effects and was not carcinogenic.

- Fifty male and 50 female mice were fed one drop of 10% "Danish vegetable Annatto butter-colour" in soy oil in a like manner, for 24 months. Untreated controls used for all of the mouse studies consisted of 130 males and 119 females. Annatto did not influence average weight or lifespan. No hepatic degeneration or cirrhosis was observed. A slight increase in the number of pulmonary adenomas in treated females (6/42 vs 3/119) and a decrease in hepatic tumors in the treated males (1/47 vs. 15/130) were observed. Information on historical controls that would facilitate evaluation of the study was not provided. The authors concluded that oral annatto administration to mice proved of no carcinogenic effect

- Fifty male and 50 female mice were painted twice a week for three months at the interscapular region with 0.5 ml of 50% "Danish vegetable Annatto butter-colour" in benzene. The animals were retained throughout their lifetimes. No skin tumors were observed during the treatment period, and the authors concluded that annatto treatment had no carcinogenic effect on mouse skin.

- Once a week for eight weeks, 70 male and 30 female mice were injected subcutaneously on the back with 0.1 ml of "Danish vegetable Annatto butter-colour." Following treatment, the mice were held for their lifetimes. In the females, 22% (6/27) developed lung adenomas vs. 3% (3/119) in the untreated controls. In the males, 12% (15/130) of the untreated controls developed hepatomas vs. 3% (2/66) in the annatto group. Some sarcomas were observed at the site of the injection, but vehicle controls were not employed so that the results are difficult to interpret. The authors concluded that subcutaneous annatto injections "excited occasional sarcomas at the site of injection . . .The treatment had no definite influence on spontaneous tumor development and involved no definite toxic injury. "

Engelbreth-Holm and Iversen (1955) did a statistical analysis of hepatomas, leukemia, and pulmonary tumors in all three groups of mice combined. The only significant finding was for pulmonary tumors in females (17/113 in animals receiving annatto vs 3/119 in

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untreated controls), leading the authors to conclude that "treatment with Annatto increased obviously the number of pulmonary tumours in female mice."

van Esch and coworkers (1959) reported that no increase in tumor incidence was observed in rats injected subcutaneously with 125 mg/kg of "fat-soluble annatto" three times weekly for 9 months and observed for 2 years, or in mice similarly treated with doses of about 500 mg/kg three times weekly, which were also fed about 750 mg/kg/day of "fat-soluble annatto" extracts" throughout life. Critical details, such as the number of animals exposed, strains, treatment of control animals, whether histopathology was conducted, and the tissues and organs examined, are not available, making analysis of the study results difficult.

Short-Term Tests: Table 2 presents data on the genotoxicity of annatto, annatto extracts, pastes, and oils, and bixin.

Overall, the weight-of-evidence of assays for mutagenic activity supports a conclusion that they were negative, but a few assays for genotoxicity were described as weakly positive or positive. Analysis of the information is impeded by the frequent failure to identify bixin/norbixin content in the test materials.

Table 2: *In vitro* genotoxicity of annatto and its components

Test system/strain or cell line (locus)	Dose; study details (activation, solvent, schedule)	Result	References
Endpoint: Mutation			
S. typhimurium TA97 & TA102	Annatto pigment; 1-10 mg/ plate with or without rat liver S9 (DMSO solvent)	-	Fijita *et al*., 1988
S. typhimurium TA98 & TA100	Annatto, with and without rat liver S9	-	Yasui *et al*., 1982

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Test system/strain or cell line (locus)	Dose; study details (activation, solvent, schedule)	Result	References
S. typhimurium TA92, TA94, TA98, TA100, TA1535 & TA1537	Water-soluble annatto, with and without rat liver S9, max dose 10 mg/plate, phosphate buffer solvent	-	Ishidate *et al*., 1984
S. typhimurium TA98 & TA100	Water-soluble annatto, no experimental details given	weak +	Sasaki *et al*., 1980
Gene conversion in *Saccharomyces cerevisiae* strain BZ34	Test substance identified as bixin. No experimental details presented.	-	Murthy, 1979
Gene conversion in *Saccharomyces cerevisiae* strain D4	Test substance identified as annatto; no experimental details presented.	-	Haveland-Smith, 1981
Fluctuation tests in *E. coli* WP2 uvrA and *S. typhimurium* TA1538	Annatto with or without activation with caecal extracts or liver microsomes from rats, 1 mg/ml	-	Haveland-Smith, 1981
	Endpoint: Chromosomal Aberrations		
Chinese hamster fibroblasts	Water-soluble annatto extracts, 10 and 25 mg/ml, and annatto, 16 mg/ml; no activation, physiologic saline solvent	weak + (25 mg/ml); - (10 mg/ml); - (16 mg/ml)	Ishidate *et al*., 1984
Human fibroblasts (HE 2144 cells)	Water-soluble annatto; 0.038 mg/ml; no activation	-	Sasaki *et al*., 1980
	Endpoint: DNA Damage		
Bacillus subtilis	Annatto pigment Liquid rec assay, 1 day cultivation; direct DNA damaging activity at concentrations less than those which were negative in spore rec-assay method.	+	Nonaka *et al*., 1991

-, negative; + positive

Metabolism: Very little information was found on the metabolism of annatto, annatto extracts, bixin, or norbixin. The metabolism of the related substance, β-carotene, differs in rats and humans; humans absorb carotenes unchanged whereas rats do not. The relevance to bixin and norbixin is unclear, however. β-Carotene is largely converted to vitamin A in humans and then esterified and transported in the lymph in the same manner as dietary retinol.

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Bixin and norbixin lack the necessary ring structures to be Vitamin A precursors (Hallagan, *et al* 1995; Goodwin, 1986).

In 1980, the European Economic Community commissioned a survey to determine the effects of annatto in rats, dogs, mice, and humans. This study apparently showed that the principal annatto pigments are metabolized by the liver. The pigments were detectable in the blood after a single oral dose, and within a few hours, the blood levels fell to zero, with only a trace of the pigments found in the feces. Thus, it appears that in humans and rats, the pigments are absorbed from the intestine into the blood and clearance from the blood is quite rapid (EEC, 1980).

Other limited information on the metabolism of annatto, bixin, and norbixin was summarized in the BIBRA profile of annatto. No tissue accumulation was found in groups of 10 rats of each sex fed 1-2 g/kg/day of "water-" or "fat-soluble annatto" for about 13 weeks (Zbinden & Studer, 1958). According to summary reports of an unpublished study by Philp (1981), bixin fed to rats for one year at 10-20 mg/kg/day had no observable effects on growth or the weights of the liver, kidney, or fatty tissue. Bixin was not found in these tissues.

Other Biological Effects: Hyperglycemic effects of "annatto extract" were described in a limited study by Morrison and coworkers in 1987 Two grams of annatto extract were administered daily for 14 days to mongrel dogs weighing between 9 and 16 kg The hyperglycemic effect, as detected by glucose tolerance tests, persisted for four weeks after administration of annatto extract. Tissues from seven dogs were examined microscopically. Hepatic and pancreatic damage observed included disarrangement of internal mitochondrial structure, fusion of mitochondria, and formation of numerous residual bodies.

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Structure/Activity Relationships: One compound, β-carotene, was chosen for structural comparison with bixin. Although there are important structural differences between bixin and β-carotene, namely the lack of a ring structure on either end of bixin, β-carotene is the only member of the carotenoid family that has been thoroughly tested for carcinogenic potential. Literature searches on crocetin [CAS No. 27876-94-4] and canthaxanthin [CAS No. 514-78-3], two carotenoids with a structure more similar to bixin, were conducted, information on anticarcinogenic activity was found but no data on mutagenicity, genotoxicity, or carcinogenicity was found.

Since the National Research Council report on *Diet and Cancer* in 1982, there has been an enormous effort on the part of epidemiologists to evaluate the relation of intake of foods rich in antioxidant nutrients to cancer, and, to a lesser extent, to cardiovascular disease (Machlin, 1995). Epidemiologic interest in dietary factors was also stimulated in 1981 by the publication of a review by Peto and coworkers entitled "Can Dietary Beta-Carotene Materially Reduce Human Cancer Risk?" Additional impetus was given by *The Causes of Cancer*, a book by Doll and Peto that also came out in 1981. In this report, the authors raised the possibility that some nutritional factors might rival abstinence from tobacco in preventing cancer (Comstock *et al.*, 1992).

Much more information is now available with which to test these suggestions. Early evidence suggested an association between serum β-carotene concentrations and a lower incidence of cancer. For example, Comstock and coworkers (1992) reviewed 10 large study populations of persons from Finland and the United Kingdom, workers from Switzerland, persons of Japanese ancestry in Hawaii, and four different populations from mainland United States. In over 70% of the reports, cancer cases had lower levels of β-carotene than did controls. The situation for lung cancer was particularly striking; prediagnostic levels of serum β-carotene were all considerably lower than those of their

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matched controls. (It should be noted, however, that serum β-carotene levels are lower in smokers, so this association could be an artifact.)

The associations between cardiovascular disease, cancer rates, and serum β-carotene have not held up under clinical trial (Mayne, 1996; Greenberg & Sporn, 1996). The Physicians' Health Study followed 22,071 U.S. male doctors treated with 50 mg of β-carotene or placebo every other day, for an average of 12 years. The results unequivocally ruled out the possibility that there is even a slight reduction in incidence of cancer or mortality from cardiovascular disease with such supplementation. Two trials using lung cancer as the primary endpoint have been completed. The Alpha-Tocopherol Beta-Carotene (ATBC) Trial involved 29,133 men age 50-69 from Finland who were heavy smokers at the start of the study Participants receiving β-carotene had a significantly higher incidence of lung cancer and total mortality than participants receiving the placebo. The Beta-Carotene and Retinol Efficacy Trial (CARET) studied more than 18,000 persons at elevated risk for lung cancer because of exposure to asbestos or cigarette smoking. They were treated daily with β-carotene and retinyl palmitate or with placebo for an average of four years. The trial was ended 2 years early when the researchers recognized an elevated risk of death from lung cancer in the group receiving the supplements.

According to Greenberg & Sporn (1996), the disappointing results of the clinical trials of β-carotene reaffirm the importance of obtaining solid scientific knowledge before initiating a clinical trial. Although studies in animals suggested that β-carotene might prevent damage to DNA and perhaps retard the initiation of carcinogenesis, there was little evidence that it suppresses the progression of neoplasia after exposure to carcinogens. There also was no clear evidence that β-carotene inhibits the oxidation of low-density lipoproteins, the principal proposed mechanism of its postulated antiatherogenic properties

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Certain results in animal studies appear to have fueled the interest in β-carotene as the likely chemopreventive agent. Carotenoids are known to protect against photo-sensitization, and β-carotene significantly slowed the growth of UV-induced skin tumors in hairless mice. However, only β-carotene showed the ability to slow growth of skin tumors from DMBA and croton oil, without UV light (Matthews-Roth, 1982; Matthews-Roth, 1983). These studies encouraged investigations of only those carotenoids that could be converted to Vitamin A (Goodwin, 1986).

Key papers on the carcinogenicity and anticarcinogenicity of annatto and β-carotene are summarized in Table 3.

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Table 3. Summary of Information on Annatto and β-Carotene

Carcinogenicity Information	Genotoxicity Information	Anticarcinogenicity Information
ANNATTO		
Rat: oral: " Annatto butter color" given at 26 mg/kg/day for life, produced no increase in tumors (Engelbreth-Holm & Iversen, 1955) Mouse: oral: "Annatto butter color", 1 drop of 10% solution, given daily for life, produced no increase in tumors (Engelbreth-Holm & Iversen, 1955). skin: "Annatto butter color" applied for 3 months as a 50% solution in benzene produced no skin tumors (Engelbreth-Holm & Iversen, 1955). subcutaneous injections: "Annatto butter color" given as 8 weekly injections (0.1 ml), produced a few injection site tumors in animals held for life; control animals did not receive injections of vehicle (Engelbreth-Holm & Iversen, 1955).	generally negative for mutations in *S. typhimurium* TA 92, TA94, TA97, TA98, TA100, TA102, TA1535, TA1537, and TA1538 w/wo S9; compound tested not always clearly defined (Fujita *et al.*, 1988; Ishidate *et al.*, 1984; Sasaki *et al.*, 1980; Yasui *et al.*, 1982) negative for gene conversion in *Saccharamyces cerevisiae* gene conversion (annatto, Haveland-Smith, 1981, bixin, Murthy, 1979) negative for mutations in *E. coli*, annatto (Haveland-Smith, 1981) weakly positive for chromosomal aberrations in Chinese hamster fibroblasts at highest dose, 25 mg/ml; water soluble annatto (Ishidate *et al.*, 1984) negative for chromosomal aberrations in human fibroblasts at 0.038 mg/ml, water soluble annatto (Sasaki *et al.*, 1980) positive for DNA damage in *Bacillus subtilis*, annatto pigment, (Nonaka *et al.*, 1991)	bixin exhibited the same modest cytotoxicity vs. mouse leukemia L5178Y cells *in vitro* as β-carotene (Suzuki *et al.*, 1994) bixin did not increase gap junctional intercellular communication in 10T1/2 cells (Zhang *et al.*, 1991) bixin was a potent inhibitor of lipid peroxidation in 10T1/2 cells (Zhang *et al.*, 1991)

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Carcinogenicity Information	Genotoxicity Information	Anticarcinogenicity Information
BETA-CAROTENE		
Animals: oral: 100-1000 mg/kg/day did not induce cancer in SD rats (510 males, 510 females) or CD-1 mice (600 males, 600 females) (Heywood *et al.*, 1985) Humans: 8 of 8 prospective studies and 18 of 20 retrospective studies found carotenoid and/or fruit and vegetable intake to be associated with reduced lung cancer risk (Mayne, 1996) average intake not related to mortality from lung, stomach, or colorectal cancer in 16 cohorts in Seven Countries Study (Ocke *et al.*, 1995) supplemental β-carotene significantly increased incidence of lung cancer and total mortality in heavy smokers, age 50-69, in Finland (Mayne, 1996; Greenberg & Sporn, 1996) intervention trial, CARET, terminated early because of increased incidence of lung cancer in smokers or asbestos workers receiving supplemental β-carotene (Mayne, 1996; Greenberg & Sporn, 1996) Physicians Health Study found no effect of β-carotene supplements on cancer or cardiovascular disease incidence (Greenberg & Sporn, 1996)	not mutagenic in standard Ames test at 0.1-4.1 mg/plate; negative for chromosome breaks and non-dysjunctions in the *in vivo* micronucleus test (Heywood, 1985) negative in *S. typhimurium*; weakly positive for chromosome aberrations-polyploidization observed (Ishidate *et al.*, 1984) negative in *S. typhimurium* TA97 and TA102 w/wo S9 activation (Fujita *et al.* 1988) reduced micronuclei counts in sputum from heavy smoker asbestos workers in 14-week trial (Mayne, 1996)	delayed appearance and decreased number of skin tumors induced by DMBA in hairless mice (Matthews-Roth, 1982) no influence on mutagenicity of cigarette smoke condensate or B(a)P in *S. typhimurium* TA98 with S9 (Terwel & van der Hoeven, 1985) modulated clastogenicity induced by cyclophosphamide but not mitomycin C in human hepatoma cells (Salvadori *et al.*, 1993) significantly inhibited DNA adduct formation by aflatoxin B_1 *in vitro* (Goswami *et al.*, 1989) did not inhibit development of aberrant crypt foci in SD rats administered MNU (Narisawa *et al.*, 1996) partially protects HepG2 human liver cells against oxidant-induced damage (Martin *et al.*, 1996) modest cytotoxicity vs. mouse leukemia L5178Y cells *in vitro* (Suzuki *et al.*, 1994) increased gap junctional intercellular communication in a dose-dependent manner in 10T1/2 cells (Zhang *et al.*, 1991) weaker inhibitor of lipid peroxidation in 10T1/2 cells than bixin (Zhang *et al.*, 1991)

B(a)P = benzo(a)pyrene; DMBA = dimethylbenzanthracene; MNU = methyl nitrosourea; SD = Sprague-Dawley

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