Safety Assessment of BHT as Used in Cosmetics

Status:	Re-Review for Panel Review
Release Date:	May 10, 2019
Panel Meeting Date:	June 6-7, 2019

The 2019 Cosmetic Ingredient Review Expert Panel members are: Chair, Wilma F. Bergfeld, M.D., F.A.C.P.; Donald V. Belsito, M.D.; Ronald A. Hill, Ph.D.; Curtis D. Klaassen, Ph.D.; Daniel C. Liebler, Ph.D.; James G. Marks, Jr., M.D., Ronald C. Shank, Ph.D.; Thomas J. Slaga, Ph.D.; and Paul W. Snyder, D.V.M., Ph.D. The CIR Executive Director is Bart Heldreth, Ph.D. This safety assessment was prepared by Alice Akinsulie, Scientific Analyst/Writer.

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Memorandum

To: CIR Expert Panel Members and Liaisons
From: Alice Akinsulie Scientific Analyst/Writer
Date: May 10, 2019
Subject: Re-Review of the Safety Assessment of BHT (Butylated Hydroxytoluene)

The CIR Expert Panel first published an assessment of BHT in 2002 with the conclusion "safe as used in cosmetic formulations" (*BHT062019origrep*). Minutes from is the original proceedings are included in this packet (*BHT062019min*).

Because it has been at least 15 years since the report was published, in accord with CIR Procedures, the Panel should consider whether the safety assessment of BHT should be re-opened. An exhaustive search of the world's literature was performed for studies dated 1997 forward. A brief synopsis of the relevant data is enclosed (*BHT062019newdata*).

Also included for your review are current and historical use data (*BHT062019usetbl*). The frequency of use has increased significantly since the initial review. According to VCRP data, BHT was reported to be used in 1709 formulations in 1998. In 2019, the VCRP data indicate that BHT is used in 9485 formulations (*BHT062019fda*). The current maximum concentration of use in leave-on products (0.5%) is the same as reported in 1999 (0.5%; *BHT062019data1-data2*). A data profile is included for the original report and for the new data discovered (*BHT062019prof*)

If, upon review of the new studies and updated use data, the Panel determines that a re-review is warranted, a full draft amended report will be presented at an upcoming meeting.

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Butylated Hydroxytoluene (BHT) Data Profile* – June 6th-7th 2019 – Alice Akinsulie																													
	Use				Toxico-kinetics			Acute Tox			Repeated Dose Tox			DART		Genotox		Carci		Dermal Irritation			Dermal Sensitization				Ocular Irritation		Clinical Studies	
	New Rpt	Old Rpt	Method of Mfg	Impurities	log P	Dermal Penetration	ADME	Dermal	Oral	Inhalation	Dermal	Oral	Inhalation	Dermal	Oral	In Vitro	In Vivo	Dermal	Oral	In Vitro	Animal	Human	In Vitro	Animal	Human	Phototoxicity	In Vitro	Animal	Retrospective/ Multicenter	Case Reports
BHT-final report 2002		**X**	**X**	X			X	X	X		X	X			X	X	X	X	X		X	X		X		X			X
BHT-Re-review 2019	**X**			X					X	X		X				X	X											X	X

* "X" indicates that new data were available in this category for the ingredient

[Butylated Hydroxytoluene (BHT)]

Ingredient	CAS #	InfoB	SciFin	PubMed	TOXNET	FDA	EU	ECHA	IUCLID	SIDS	ECETOC	HPVIS	NICNAS	NTIS	NTP	WHO	FAO	NIOSH	FEMA	Web
Butylated Hydroxytoluene (BHT)	128-37-0	X		18/778	X	X	LISTED/ NO RESTRICTIONS	X	X	N/A	N/A	X	N/A	N/A	N/A	X	X	N/A	X	X

X" indicates that data were available for the ingredient
N/A" indicates that no new data was found from >1997

Search Strategy
[*document search strategy used for SciFinder, PubMed, and Toxnet*]

[*identify total # of hits / # hits that were useful*]

Typical Search Terms
Butylated hydroxytoluene OR 128-37-0 [rn]
2,6-di-tert-butyl-4-methylphenol

1,3-di-teri-butyl-2-hydroxy-5-methyl benzene

2,6-DITERTIARY-BUTYL-p-CRESOL; 4-METHYL-2,6-DITERTIARY-BUTYL-PHENOL

LINKS

Search Engines
- Pubmed (- http://www.ncbi.nlm.nih.gov/pubmed)
- Toxnet (https://toxnet.nlm.nih.gov/); (includes Toxline; HSDB; ChemIDPlus; DART; IRIS; CCRIS; CPDB; GENE-TOX)
- Scifinder (https://scifinder.cas.org/scifinder)

appropriate qualifiers are used as necessary
search results are reviewed to identify relevant documents

Pertinent Websites
- wINCI - http://webdictionary.personalcarecouncil.org

- FDA databases http://www.ecfr.gov/cgi-bin/ECFR?page=browse
- FDA search databases: http://www.fda.gov/ForIndustry/FDABasicsforIndustry/ucm234631.htm;,

- EAFUS: http://www.accessdata.fda.gov/scripts/fcn/fcnnavigation.cfm?rpt=eafuslisting&displayall=true
- GRAS listing: http://www.fda.gov/food/ingredientspackaginglabeling/gras/default.htm
- SCOGS database: http://www.fda.gov/food/ingredientspackaginglabeling/gras/scogs/ucm2006852.htm
- Indirect Food Additives: http://www.accessdata.fda.gov/scripts/fdcc/?set=IndirectAdditives
- Drug Approvals and Database: http://www.fda.gov/Drugs/InformationOnDrugs/default.htm
- http://www.fda.gov/downloads/AboutFDA/CentersOffices/CDER/UCM135688.pdf
- FDA Orange Book: https://www.fda.gov/Drugs/InformationOnDrugs/ucm129662.htm
- OTC ingredient list: https://www.fda.gov/downloads/aboutfda/centersoffices/officeofmedicalproductsandtobacco/cder/ucm135688.pdf
- (inactive ingredients approved for drugs: http://www.accessdata.fda.gov/scripts/cder/iig/

- HPVIS (EPA High-Production Volume Info Systems) - https://ofmext.epa.gov/hpvis/HPVISlogon
- NIOSH (National Institute for Occupational Safety and Health) - http://www.cdc.gov/niosh/
- NTIS (National Technical Information Service) - http://www.ntis.gov/
- NTP (National Toxicology Program) - http://ntp.niehs.nih.gov/
- Office of Dietary Supplements https://ods.od.nih.gov/
- FEMA (Flavor & Extract Manufacturers Association) - http://www.femaflavor.org/search/apachesolr_search/

- EU CosIng database: http://ec.europa.eu/growth/tools-databases/cosing/
- ECHA (European Chemicals Agency – REACH dossiers) – http://echa.europa.eu/information-on-chemicals;jsessionid=A978100B4E4CC39C78C93A851EB3E3C7.live1
- ECETOC (European Centre for Ecotoxicology and Toxicology of Chemicals) - http://www.ecetoc.org
- European Medicines Agency (EMA) - http://www.ema.europa.eu/ema/
- IUCLID (International Uniform Chemical Information Database) - https://iuclid6.echa.europa.eu/search
- OECD SIDS (Organisation for Economic Co-operation and Development Screening Info Data Sets)- http://webnet.oecd.org/hpv/ui/Search.aspx
- SCCS (Scientific Committee for Consumer Safety) opinions: http://ec.europa.eu/health/scientific_committees/consumer_safety/opinions/index_en.htm
- NICNAS (Australian National Industrial Chemical Notification and Assessment Scheme)- https://www.nicnas.gov.au/

- International Programme on Chemical Safety http://www.inchem.org/
- FAO (Food and Agriculture Organization of the United Nations) - http://www.fao.org/food/food-safety-quality/scientific-advice/jecfa/jecfa-additives/en/
- WHO (World Health Organization) technical reports - http://www.who.int/biologicals/technical_report_series/en/

- www.google.com - a general Google search should be performed for additional background information, to identify references that are available, and for other general information

Fragrance Websites, if applicable

- IFRA (International Fragrance Association) – http://www.ifraorg.org/
- Research Institute for Fragrance Materials (RIFM)

Minutes from the 70th meeting, March 3-4, 1999

BHT (Butylated Hydroxytoluene)

Dr. Schroeter recalled that, at the December 2, 1998 Team meetings, his Team concluded that this ingredient is safe up to a concentration of 1% in cosmetic products and that Dr. Belsito's Team concluded that the following data are needed: (1) Current concentrations of use, (2) Photoirritation and photosensitization, and (3) Ocular irritation, if available.

Dr. Schroeter noted that current concentration of use data have been provided. Concerning the issues of photoirritation and photosensitization, he noted that the section on Photoprotective Effects in the report text contains a number of studies indicating that BHT increases the MED and may provide some degree of photoprotection. However, after evaluating these studies, he determined that though BHT increases the MED, whether or not it precludes photoallergenicity is not known.

Dr. Schroeter also noted that a section on Ocular Effects is included in the report text, but that the study does not contain information on the ocular irritation potential of BHT.

Dr. Belsito said that his Team eventually determined that BHT is safe as used, but had expressed concern over the production process yielding up to 93.5% BHT, meaning that impurities may account for up to 6.5% of its composition. However, he noted that data (received on preceding day) indicating that cosmetic grade BHT is more than 99% pure alleviates his Team's concern about impurities and will be incorporated into the report text.

Dr. Belsito indicated that the issue of photoirritation was addressed in the studies by Roshcupkin in the CIR report section on Photoprotective Effects. He noted that the MED doses would have induced photoirritation/photosensitization in these studies if this were a problem, and, therefore, determined that photoirritation/photosensitization data are not needed for completion of the Panel's safety assessment on BHT.

Dr. Schroeter said that the observations that would have been necessary for determining photoallergy induction were not made. In other words, this issue was not addressed.

Dr. Belsito said that the assumption is that the issue of photoallergy was not addressed because photoallergic reactions did not occur, and acknowledged that this may be a false assumption. He also said that had this reaction occurred, this would have made it difficult for the investigators to say that they did not have an MED, because the MED would be interpreted as redness at the exposure site (which is also seen with allergies). Therefore, he said his assumption that BHT raised the MED was based on the fact that if BHT had induced allergy, then the investigators would have detected it.

Dr. Belsito also said that, clinically, no problems that are related to the photoallergenicity of BHT have been identified. He noted that BHT is a widely used antioxidant, and, if there were any problems associated with its use, many clinical cases would be reported in the published literature. Concerning his Team's request for ocular irritation data, Dr. Belsito acknowledged that a safe as used conclusion could be reached in the absence of these data.

Dr. Schroeter said that the reason why photoirritation and photosensitization data are not needed should be stated in the report discussion.

Dr. Andersen wanted to know how the issue of ocular irritation should be addressed in the report discussion, considering that these data were requested by Dr. Belsito's Team.

Dr. Belsito recalled that his Team had requested ocular irritation data (if available), and that these data were not received. He also noted that BHT is widely used and that its ocular irritation potential has not been a noticeable concern.

Furthermore, Dr. Belsito noted that because BHT is not irritating to the skin, it is not likely that it would be irritating to the eye, and that it is used at a concentration of 0.5% max. in cosmetics.

Dr. Bailey stated that BHT is listed as being used in indoor tanning products as well as other tanning preparations. Therefore, he said the issue relating to the photoirritation/photosensitization potential of BHT is relevant.

Dr. Belsito said that one would expect to see many case reports in the literature if BHT were a photosensitizer.

The Panel voted unanimously in favor of issuing a safe as used conclusion on BHT. The Panel also agreed that concerns about the photoirritation/photosensitization potential and ocular irritation potential of BHT will be addressed in the report discussion.

The 70th meeting of the CIR Expert Panel was adjourned.

Minutes from the 72nd meeting, September 9-10, 1999

BHT (Butylated Hydroxytoluene)

Dr. Schroeter stated that a Tentative Report with the following conclusion was issued at the March 3-4, 1999 Panel meeting: On the basis of the animal and clinical data included in this report, the CIR Expert Panel concludes that BHT is safe for use in cosmetic formulations. He also noted that his Team recommended revision of the conclusion to include the terminology “safe as used” rather than “safe for use”.

Dr. Belsito noted that the section on Method of Manufacture indicates that BHT is synthesized with yields up to 93.5%, but that the Panel received new information from CTFA indicating that BHT is 99% pure. He requested that the new information be incorporated into the report text. Additionally, Dr. Belsito requested that the study on the induction of differentiation in mouse N1E-115 neuroblastoma cells be deleted from the report.

Dr. Carlton agreed that the neuroblastoma study should be deleted because it gives the wrong impression (that BHT is a teratogen) and because is not a defined test for teratogenicity.

The Panel voted unanimously in favor of issuing a Final Report with the following conclusion on BHT: On the basis of the animal and clinical data included in this report, the CIR Expert Panel concludes that BHT is safe as used in cosmetic formulations.

Current and historical frequency and concentration of use of BHT according to duration and exposure

	# of Uses		*Max Conc of Use (%)*	
	BHT			
	2019[1]	**1998[2]**	**2018[3]**	**1999[2]**
Totals*	**9485**	**1709**	**0.0000007 – 0.5**	**0.0002 – 0.5**
Duration of Use				
Leave-On	*7367*	*1460*	*0.0000007 – 0.5*	*0.002 – 0.5*
Rinse-Off	*2044*	*196*	*0.000001 – 0.5*	*0.01 – 0.5*
Diluted for (Bath) Use	*74*	*53*	*0.00024 – 0.15*	*0.05 – 0.1*
Exposure Type				
Eye Area	976	610	0.00009 – 0.3	0.0002 – 0.5
Incidental Ingestion	981	261	0.000001 – 0.29	0.03 – 0.5
Incidental Inhalation-Spray	1708; 847[a]; 1581[b]	146; 84[a]; 108[b]	0.0000035-0.21;0.0003-0.09[a]; 0.0000007-0.5[b]	0.02-0.5; 0.008-0.5[a]; 0.02-0.5[b]
Incidental Inhalation-Powder	226; 847[a]	42; 84[a]	0.0021-0.3; 0.0003-0.09[a]; 0.00005-0.5[c]	0.05-0.5; 0.008-0.5[a]
Dermal Contact	8071	1385	0.000001-0.5	0.008-0.5
Deodorant (underarm)	140[b]	10[b]	0.000001-0.4; 0.012-0.19[b]	NR
Hair - Non-Coloring	270	40	0.0000007 - 0.5	0.02 - 0.5
Hair-Coloring	13	6	0.0015 - 0.005	0.05
Nail	37	8	0.0005 - 0.25	0.02 - 0.5
Mucous Membrane	2654	404	0.000001 - 0.31	0.03 - 0.5
Baby Products	6	5	0.0013 – 0.031	0.1

* Because each ingredient may be used in cosmetics with multiple exposure types, the sum of all exposure types may not equal the sum of total uses.

[a.] Not specified whether a powder or a spray, so this information is captured for both categories of incidental inhalation.

[b.] It is possible these products may be sprays, but it is not specified whether the reported uses are sprays.

[c.] It is possible these products may be powders, but it is not specified whether the reported uses are powders.

References

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New Data – BHT

STRUCTURE

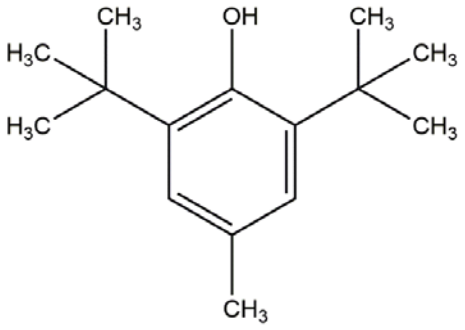

Figure 1. BHT (Butylated Hydroxytoluene)

IMPURITIES

Specifications for BHT indicate that the ingredient should not contain more than 0.5% phenolic impurities and not more than 2 mg/kg lead.[1]

ACUTE TOXICITY

Oral

In an acute oral toxicity study, mice (number/sex not specified) were administered 2000 mg/kg of BHT in olive oil.[2] The LD_{50} was 2000 mg/kg.

Inhalation

In an inhalation study, 6 groups of 4 male Swiss Webster mice were exposed to BHT at concentrations of 4.54, 16, 32, 42.9, 66.6, and 82.6 ml/m^3 (head only exposure) for 30 minutes.[3] The respiratory rate observed (RD_{50}) was calculated to be 59.7 ml/m^3. No indication of sensory irritations was observed at concentrations of 4.54 and 16 ml/m^3.

SHORT-TERM TOXICITY

Oral

In a 28-day dietary exposure study, the effects of BHT on plasma and tissue concentrations were studied in a group of 8 male Sprague-Dawley rats (starting body weight 60 g) and a control group also consisting of 8 males.[4] The rats were fed a restricted diet containing 350 mg/kg bw/day at the beginning of the experiment and 165 mg/kg bw/day at the end. BHT did not affect feed intake, but decreased the body weight ($p < 0.0005$), the amount of liver lipids and liver cholesterol ($p < 0.0001$), and increased the weight of livers ($p < 0.0001$) and lungs ($p < 0.05$) relative to body weight. Also, α-tocopherol was elevated ($p < 0.0001$) and γ-tocopherol was reduced ($p < 0.005$) in blood plasma and liver.

In a 28-day oral study in Sprague Dawley rats, the ability of BHT to induce liver changes (e.g. increase in relative liver weight and induction of gene expression of Phase I and II enzymes in the liver and increased serum cholesterol) was evaluated using male rats (6/group).[5] Animals were administered BHT at doses of 27.8, 87.8, 166.9, 321.4, and 1158.8 mg/kg-day in the diet for 4 weeks. Body weight gain was decreased in the highest dose group, relative liver weight was increased dose-dependently at doses of 87.8 mg/kg bw/day and above. Also, histopathological investigation revealed enlargement of hepatocytes, hepatocellular hypertrophy with nucleolar enlargement in 1158.8 mg/kg dose group. The no-observed-adverse-effect-level (NOAEL) for effects to the liver was 28 mg/kg bw/day.

SUBCHRONIC TOXICITY

Oral and Parenteral

In a study to evaluate the nephrotoxic and pneumotoxic effects of BHT, adult albino rats were administered doses of BHT for 12 weeks.[6] Animals (15/groups) were divided into a negative control group, two vehicle control groups (administered olive oil orally or intraperitoneally (i.p).), a group receiving 15 mg/kg orally, and another group was administered BHT by i.p. injection at a dose of 400 mg/kg bw/week. BHT exposure resulted in both nephrotoxicity and pneumotoxicity. (No further details provided.)

GENOTOXICITY

In Vitro

In a chromosome aberration test, BHT alone induced a significant increase in the frequency of chromatid and isochromatic breaks in relation to the control dimethyl sulfoxide).[7] However, the increase in aberrant cells did not show a dose-response relationship at concentrations of 1, 2.5, and 5 µg/ml.

The genotoxic potential of BHT was assessed in vitro in a mouse lymphoma gene assay (MLA).[8] BHT was assessed at concentrations up to 40 µg/ml in a 3-hour short-term treatment, and up to 50 µg/ml in a 24-hour long-term treatment in the absence and presence of metabolic activation. BHT showed no induction of gene mutations nor of chromosomal damage in the L5178Y mouse lymphoma assay.

In Vivo

BHT was tested in an in vivo micronucleus assay with mouse peripheral reticulocytes.[8] In this study, ICR mice (6/sex/dose) were administered a single dose of 17.3, 34.5, and 69.0 mg/kg BHT by i.p. injection. BHT did not show any clastogenic potential in the in vivo mouse micronucleus assay.

In another study, genotoxicity was evaluated using a comet assay.[2] BHT, at doses between 10 and 1000 mg/kg, was administered in olive oil to groups of 4 male mice. No death, morbidity, or clinical signs were observed after treatment, at any dose. BHT induced DNA damage in the glandular stomach and colon 3 hours after administration. BHT also produced genotoxic effects in the urinary bladder and brain of mice in the100 mg/kg bw group. No effects were seen in the liver, kidney, lung, or bone marrow.

CARCINOGENICITY STUDY

Oral

The association between dietary intake of BHT and stomach cancer risk was investigated in the Netherlands Cohort Study in 1986 among 120,852 men and women aged 55-69 years.[9] Mean intake of BHT was 351 ug/day; mean intake of butylated hydroxyanisole (BHA) was 105 µg/day. After 6.3 years of follow-up, no association with stomach cancer risk was observed for consumption of mayonnaise and other creamy salad dressings with BHT. A statistically non-significant decrease in stomach cancer risk was observed with increasing BHA and BHT intake.

CLINICAL STUDIES

Retrospective and Multicenter Studies

The allergenicity to preservatives was studied from January 1990 to December 1994 in 11,454 patients with suspected allergic contact dermatitis.[10] Patients were patch tested with 2% BHT and readings were made 72 hours after application. BHT at 2% gave a positive reaction in 11 patients, and 51 patients showed a questionable/irritative response.

To evaluate the allergic response to BHT, 244 patients were patch-tested between 1997 and 2000.[11] An occlusive patch containing the test substance was applied to the back of each subject. Patches were removed after 2 days, and readings made on day 2 and day 3. Positive allergic response was noted in 5/224 patients.

Case Study

A 37-year old woman, with no past history of allergy, had developed an itchy inflammatory edema of her scalp and face with occipital lymph node swelling one day after the second application of a new hair-coloring preparation.[12] Patch tests were then performed with the 20 ingredients of the hair dye at their use concentration. BHT at 1% produced positive reactions on day 2 and 4.

Other Clinical Studies

Incidence of contact allergy to monomers in p-*tert*-butylphenol-formaldehyde resin (PTBP-F-R) and potential cross reacting substances, including BHT, was studied.[13] Twelve patients were patch-tested on the back for 2 days using the Finn Chamber technique. Reaction to 1.77% BHT was negative in the patch-test with 12 patients.

From April 2001 to December 2002, a group of 514 patients (178 men, 336 women) suffering from chronic eczema were patch tested in an occlusive epicutaneous test for contact hypersensitivity to selected auxiliary substances of dermatological external and cosmetic preparation.[14] BHT at 2% induced a positive allergic reaction in 2/514 patients. In another patch test study, 2/900 patients showed a positive allergic reaction in a patch test with 2% BHT.[15]

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Final Report on the Safety Assessment of BHT[1]

BHT is the recognized name in the cosmetics industry for butylated hydroxytoluene. BHT is used in a wide range of cosmetic formulations as an antioxidant at concentrations from 0.0002% to 0.5%. BHT does penetrate the skin, but the relatively low amount absorbed remains primarily in the skin. Oral studies demonstrate that BHT is metabolized. The major metabolites appear as the carboxylic acid of BHT and its glucuronide in urine. At acute doses of 0.5 to 1.0 g/kg, some renal and hepatic damage was seen in male rats. Short-term repeated exposure to comparable doses produced hepatic toxic effects in male and female rats. Subchronic feeding and intraperitoneal studies in rats with BHT at lower doses produced increased liver weight, and decreased activity of several hepatic enzymes. In addition to liver and kidney effects, BHT applied to the skin was associated with toxic effects in lung tissue. BHT was not a reproductive or developmental toxin in animals. BHT has been found to enhance and to inhibit the humoral immune response in animals. BHT itself was not generally considered genotoxic, although it did modify the genotoxicity of other agents. BHT has been associated with hepatocellular and pulmonary adenomas in animals, but was not considered carcinogenic and actually was associated with a decreased incidence of neoplasms. BHT has been shown to have tumor promotion effects, to be anticarcinogenic, and to have no effect on other carcinogenic agents, depending on the target organ, exposure parameters, the carcinogen, and the animal tested. Various mechanism studies suggested that BHT toxicity is related to an electrophillic metabolite. In a predictive clinical test, 100% BHT was a mild irritant and a moderate sensitizer. In provocative skin tests, BHT (in the 1% to 2% concentration range) produced positive reactions in a small number of patients. Clinical testing did not find any depigmentation associated with dermal exposure to BHT, although a few case reports of depigmentation were found. The Cosmetic Ingredient Review Expert Panel recognized that oral exposure to BHT was associated with toxic effects in some studies and was negative in others. BHT applied to the skin, however, appears to remain in the skin or pass through only slowly and does not produce systemic exposures to BHT or its metabolites seen with oral exposures. Although there were only limited studies that evaluated the effect of BHT on the skin, the available studies, along with the case literature, demonstrate no significant irritation, sensitization, or photosensitization. Recognizing the low concentration at which this ingredient is currently used in cosmetic formulations, it was concluded that BHT is safe as used in cosmetic formulations.

Received 6 June 2002; accepted 6 June 2002.

[1]Reviewed by the Cosmetic Ingredient Review Expert Panel. This report was prepared by Rebecca S. Lanigan and Torill A. Yamarik, former CIR staff. Address correspondence to F. Alan Andersen, CIR Director, 1101 17th Street, NW, Suite 310, Washington, DC 20036, USA.

INTRODUCTION

BHT (Butylated Hydroxytoluene) is a substituted toluene used as an antioxidant in cosmetic product formulations. BHT is a generally recognized as safe (GRAS) food additive and preservative.

The Cosmetic Ingredient Review (CIR) Expert Panel reviewed the safety of the chemically related (see Figure 1) BHA (Butylated Hydroxyanisole) and reached the conclusion that BHA is safe as a cosmetic ingredient in the present practices of use (Elder 1984).

CHEMISTRY

Definition and Structure

BHT (CAS No. 128-37-0) is a substituted toluene conforming generally to the formula in Figure 1.

Synonyms for BHT include Hydroxylbutyltoluene (Haesen et al. 1987); Butylated Hydroxytoluole (Mersch-Sundermann et al. 1994); 2,6-Bis(1,1-Dimethyl)-4-Methylphenol; DPBC; 2,6-Di-t-Butyl-p-Cresol; Phenol, 2,6-Bis(1,1-Dimethylethyl)-4-Methyl-; Dibutylhydroxytoluene (Wenninger and McEwen 1997); Butylhydroxytoluene; DBMP; Dibunol; Dibutylated Hydroxytoluene; 2,6-Di-*tert*-Butyl-1-Hydroxy-4-Methylbenzene; 3,5-Di-*tert*-Butyl-4-Hydroxytoluene; 2,6-Di-*tert*-Butyl-p-Methylphenol; 2,6-Di-*tert*-Butyl-4-Methylphenol; 4-Hydroxy-3,5-Di-*tert*-Butyltoluene; Methyl-di-*tert*-Butylphenol (Registry of Toxic Effects of Chemical Substances [RTECS] 1997); 4-Methyl-2,6-Di-*tert*-Butylphenol (Mallette and Von Haam 1952; RTECS 1997); 2,6 ditertiary butyl 4-methyl phenol (Kumar et al. 1979); and Dibutyl Hydroxy Toluene (Shiba et al. 1974).

Physical and Chemical Properties

The chemical and physical properties of BHT are given in Table 1.

Reactivity

Phenolic antioxidants such as BHT form stable free radicals that interrupt the propagation step of the oxidation process (Applewhite 1985).

BHT reacts slowly with many radical species (Lambert, Black, and Truscott 1996). Its antioxidant activity can involve the "quenching" of reactive oxygen species, including singlet oxygen, hydroxyl radicals, superoxide, and peroxyl radicals, as

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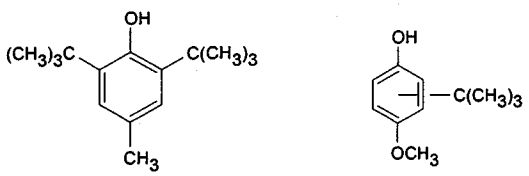


BHT BHA

FIGURE 1
BHT and BHA (Wenninger and McEwen 1997)

well as lipid-soluble radicals. BHT reacts faster in polar solvents, where smaller fractions of the antioxidant are destroyed by the reaction.

BHT is classified as a chain-breaking antioxidant; these antioxidants break the autoxidation chain reaction by donating a hydrogen atom to a lipid radical, producing a stable product and an antioxidant free radical. The antioxidant free radical has adequate stability and is incapable of initiating or propagating the chain reaction (Papas 1993).

Method of Manufacture

BHT is prepared by the addition reaction of p-cresol and either 2-methylpropene (Gennaro 1990) or isobutylene (Hazardous Substances Data Base [HSDB] 1997). When isobutylene was bubbled through p-cresol using sulfuric acid, alkylaluminum halide, or a cation-exchange resin as a catalyst, BHT was synthesized with yields up to 93.5% (Shipp, Data, and Christian 1973). A chemical description of BHT indicated that BHT has a 99% pure chemical composition (CTFA 1999a).

Analytic Methods

BHT has been analyzed using high-performance liquid chromatography (HPLC), gas chromatography (GC), mass spectrometry (MS), GC-MS, infrared spectrometry, ultraviolet (UV) spectrometry, and gel permeation chromatography (Grote and Kupel 1978; Doeden, Bowers, and Ingala 1979; Takahashi and Hiraga 1980; Yamamoto et al. 1991; Irache, Vega, and Ezpeleta 1992).

TABLE 1
Chemical and physical properties of BHT

Property	Description	Reference
Appearance/odor	White to yellowish-white, crystalline solid with a faint, characteristic odor	Grant 1972; CTFA 1999a; Lewis 1993a, 1993b; Nikitakis and McEwen 1990
	Odorless, tasteless	NIPA Hardwicke, Inc. 1998
Boiling point	265°C	Lewis 1993a, 1993b; Budavari 1989
Flash point	260–275°F	Lewis 1993a, 1993b; Budavari 1989
	135°C (combustible)	Lewis 1993b
Density	1.048 (20°/4°C)	Lewis 1993a, 1993b
Melting point	68–70°C	Lewis 1993a; Budavari 1989
Freezing point	69–70°C	NIPA Hardwicke, Inc. 1998; CTFA 1999a
Solubility	Soluble in alcohol, toluene, methyl ethyl ketone, acetone, ether, petroleum ether, benzene, naphtha, methanol, ethanol, isopropanol, cellosolve, and other hydrocarbon solvents; insoluble in water, propylene glycol, and 10% sodium hydroxide	Lewis 1993a, 1993b; Budavari 1989; Nikitakis and McEwen 1990; CTFA 1999a
	400 mg/liter H_2O (20–25°C)	Geyer, Scheunert, and Korte 1986
	Solubility in alcohol: 1 g/4 ml	Gennaro 1990
	Solubility in chloroform or ether: 1 g/1.1 ml	Gennaro 1990
	Solubility in liquid petrolatum: 0.5%	Budavari 1989
Molecular weight	220.24–220.36	Grant 1972; Gennaro 1990; Budavari 1989; U.S. Pharmacopeial Convention, Inc. 1995
Assay	97.0% minimum (cosmetic-grade), 99.0% minimum (cosmetic-grade)	Nikitakis and McEwen 1990; CTFA 1999a
Sulfated ash	0.002% maximum, 0.1% maximum	Nikitakis and McEwen 1990; NIPA Hardwicke, Inc. 1998
Arsenic (as As)	3 ppm maximum	Nikitakis and McEwen 1990
Lead (as Pb)	20 ppm maximum	Nikitakis and McEwen 1990
Viscosity	3.47 centistrokes (0°C) 1.54 centistrokes (120°C)	Lewis 1993b
Refractive index	1.4859 (75°C)	Lewis 1993b
log K_{ow}	5.11–5.20	Geyer, Scheunert, and Korte 1986

Impurities

The typical range of impurities for BHT are ≤10 ppm heavy metals and ≤3 ppm arsenic (CTFA 1999a).

UV Absorption

The absorbance peaks of a 25 ppm BHT solution were both ~0.28 at wavelengths of ~275 and 285 nm (Doeden, Bowers, and Ingala 1979). The light absorption in the range of 230 to 300 nm of a 1-cm layer of a 0.005% *w/v* solution in absolute alcohol was a maximum at 278 nm. The absorbance (1%, 1 cm) at the maximum at 278 nm was 80 to 90 (NIPA Hardwicke, Inc. 1998).

USE

Cosmetic

BHT functions as an antioxidant in cosmetic formulations (Wenninger and McEwen 1997). Data submitted to CIR by the Food and Drug Administration (FDA) in 1998 based on industry reports indicated that BHT was used in 1709 formulations, which are described in Table 2 (FDA 1998).

Concentration of use data are no longer submitted to the FDA. Historical data from 1984 indicated that BHT was used at concentrations up to 1% (FDA 1984). Concentration of use data submitted by industry in 1999 are given in Table 2.

Wilkinson and Moore (1982) reported that the optimum use concentration was 0.01% to 0.1% for cosmetics containing unsaturated materials, with the addition of a sequestering agent such as EDTA or citric acid. Flyvholm and Menné (1990) reported that BHT was used typically in "toiletries" at concentrations of 200 to 1000 ppm, and Bardazzi et al. (1988) reported use concentrations of 0.01% to 0.1% for cosmetics.

When various cosmetics were analyzed using reverse-phase HPLC, BHT was detected (mean recoveries = 84%–86%) at amounts of 208 to 251 μg/g in sun protectors, 189 to 195 μg/g in lip protectors, 251 μg/g in hand cream, 61 to 336 μg/g in "anti-aging" products, and 63 μg/g in an ointment (Irache, Vega, and Ezpeleta 1992).

Noncosmetic

BHT (minimum purity = 99%) was approved for use as a food additive and preservative by the FDA in 1954. It has been GRAS for use in foods since 1959, and is one of the most commonly used antioxidants in fat-containing foods (FDA 1972, 1973; National Toxicology Program [NTP] 1979; Wilkinson and Moore 1982). In 1984, the average daily consumption of BHT was ≤0.5 mg/kg/person (Gosselin, Smith, and Hodge 1984). The current Food and Agriculture Organization of the United States/World Health Organization (FAO/WHO) acceptable daily intake (ADI) of BHT is 0 to 0.125 mg/kg (FAO/WHO 1996a, 1996b), and the European Economic Community limit is 0 to 0.05 mg/kg (Verhagen et al. 1990).

BHT is a weak antioxidant in vegetable oils and is often added to meat fats. Phenolic antioxidants act synergistically and, therefore, are more effective when used in combination (Applewhite 1985); synthetic antioxidants are often used to supplement natural antioxidants such as the tocopherols (Swern 1982). Smolinske (1992) reported that FDA regulations state that antioxidants (as chemical preservatives) can be added either singly or in combination at concentrations up to 0.02% based on the fat or oil content of finished food products and that flavorings and essential oils can contain up to 0.5% BHT. BHT has been used for "carry-over protection" to foods that are processed at high temperatures (Applewhite 1985).

As cited in the Code of Federal Regulations (CFR), when BHT is added to enriched parboiled rice as an optional ingredient at concentrations up to 0.0033%, the statement "Butylated Hydroxytoluene added as a preservative" must be placed prominently on the label. In chewing gum bases and defoaming agents, the antioxidant concentration limit is 0.1%. BHT is an indirect food additive that can be added to food-packaging materials, including adhesives, resinous and polymeric coatings, polyethylene film used for irradiated food packaging, and rubber articles. It is also used in fiber finishing of resin-bonded filters. The relevant citations are 21CFR§ 137.350; 166.110; 172.615; 173.340; 175.015; 175.125; 175.300; 177.2260; 177.2600; 178.3570; 179.45; 181.24; and 182.3173.

BHT functions as an antioxidant, stabilizer, and "antiskinning" agent for petroleum products, jet fuels, rubber, plastics, food packaging, animal feeds, paints and lacquers, adhesive hardeners, cleaning agents, printing products, and thinners (Budavari 1989; Smolinske 1992; Lewis 1993b). BHT has been added to health food supplements for the treatment of herpes infections. Topical pharmaceutical products can contain BHT at concentrations up to 0.2% (Smolinske 1992). Anderson et al. (1994) reported that BHT is used in sperm extenders to sustain sperm viability and prevent cold shock during freezing and thawing.

GENERAL BIOLOGY

Absorption and Distribution

An in vitro dermal absorption study tested BHT on the skin of 6- to 8-week-old pigs. The samples of dermatomed skin with an area of 0.79 cm^2 were mounted in glass diffusion cells that had been rinsed previously with 2% dimethyldichlorosilane in 1,1,1-trichloromethane. Membranes with a permeability coefficient of $<4.5 \times 10^{-3}$ cm/h were used for the study. A preliminary experiment performed to assess barrier function of the skin membrane did not establish significant differences in barrier properties of the skin when contacted with either saline or 50% ethanol/saline for 72 hours (Central Toxicology Laboratory 1998).

BHT (1 mg/ml in corn oil containing 1.5 Mbq [^{14}C]-BHT/ml) was applied to the skin membranes at a dose rate of 200 μl/cm^2 and left covered. After 30 minutes, the skin surface was flushed with 3% Teepol, then distilled water at 32°C and left uncovered for 72 hours after dosing. Samples (0.1 ml) of the receptor fluid (50% *v/v* ethanol in physiological saline) were taken at 0.5, 1, 2,

TABLE 2
Uses of BHT in cosmetics

Product category	Formulations in category (FDA 1998)	Formulations with ingredient (FDA 1998)	Concentration of use (CTFA 1999a, 1999b)
Baby lotions, oils, powders, and creams	53	3	—
Other baby products	29	2	0.1%
Bath oils, tablets, and salts	124	18	0.05%–0.1%
Bubble baths	200	11	0.5%
Bath capsules	2	—	0.1%
Other bath preparations	159	24	0.05%
Eyebrow pencil	91	63	0.2%
Eyeliner	514	400	0.06%–0.5%
Eye shadow	506	119	0.05%–0.5%
Eye lotion	18	1	0.05%
Eye makeup remover	84	5	—
Mascara	167	9	0.0002%–0.5%
Other eye makeup preparations	120	13	0.03%
Colognes and toilet waters	565	77	0.05%–0.2%
Perfumes	195	29	0.02%–0.2%
Powders	247	8	—
Sachets	28	1	—
Other fragrance preparations	148	26	0.05%–0.2%
Hair conditioners	636	14	—
Hair sprays (aerosol fixatives)	267	—	0.05%
Shampoos (noncoloring)	860	18	0.02%–0.1%
Tonics, dressings, and other hair-grooming aids	549	6	0.03%–0.5%
Other hair preparations	276	2	—
Hair dyes and colors	1572	6	—
Blushers (all types)	238	66	0.02%–0.5%
Face powders	250	30	0.05%–0.5%
Foundations	287	40	0.01%–0.5%
Leg and body paints	4	—	0.2%
Lipstick	790	261	0.05%–0.5%
Makeup bases	132	6	0.05%
Rouges	12	3	—
Makeup fixatives	11	1	—
Other makeup preparations	135	25	0.1%
Basecoats and undercoats	48	1	—
Cuticle softeners	19	4	0.02%
Nail creams and lotions	17	1	0.05%
Nail polish and enamel	80	1	—
Other manicuring preparations	61	1	0.02%
Dentifrices	38	—	0.03%
Bath soaps and detergents	385	61	0.05%–0.1%
Deodorants (underarm)	250	10	0.01%–0.1%
Feminine hygiene products	4	—	0.1%
Other personal cleanliness products	291	29	0.1%
Aftershave lotion	216	16	0.07%–0.1%
Men's talcum	8	1	—

(Continued on next page)

TABLE 2
Uses of BHT in cosmetics *(Continued)*

Product category	Formulations in category (FDA 1998)	Formulations with ingredient (FDA 1998)	Concentration of use (CTFA 1999a, 1999b)
Preshave lotions (all types)	14	3	0.05%
Shaving cream	139	17	0.02%–0.1%
Shaving soap	2	—	0.5%
Other shaving preparation products	60	5	—
Skin cleansing preparations	653	29	0.02%–0.1%
Face and neck (excluding shaving) preparations	263	29	0.008%–0.1%
Body and hand (excluding shaving) preparations	796	54	0.02%–0.5%
Foot powders and sprays	35	1	—
Moisturizing preparations	769	67	0.05%–0.5%
Night preparations	188	21	0.02%–0.05%
Paste masks (mud packs)	255	8	0.01%
Skin fresheners	184	1	0.2%
Other skin care preparations	692	35	0.05%–0.1%
Suntan gels, creams, and liquids	136	14	0.02%–0.5%
Indoor tanning preparations	62	9	0.03%–0.1%
Other suntan preparations	38	4	0.1%
1998 totals/ranges for BHT		**1709**	**0.0002%–0.5%**

3, 4, 8, 12, 24, 48, and 72 hours. Repeated application of adhesive tape strips (up to 21 strips) were used to assess the exposure of the stratum corneum to BHT. The remaining membrane was analyzed for dermal absorption.

The mean amount of penetration prior to 24 hours after application was 0.08 to 0.13 $\mu g/cm^2$ BHT. The penetration rate between 24 to 72 hours and the average penetration rate for 0 to 72 hours was 0.002 $\mu g/cm^2/h$. The total amount of BHT that penetrated the skin was 0.13 $\mu g/cm^2$ or 0.07% of the applied dose. The total recovery of BHT from the test system was 114%. A mean of 0.49% remained in the test system following decontamination at 0.5 hour. Although insufficient data were obtained for the distribution of BHT in the stratum corneum (the amount recovered from the tape strips), a mean total of 0.05% was calculated. The amount of BHT associated with the epidermis/dermis was 0.29% of the applied dose. No BHT was extracted from the receptor chamber after removal of the receptor fluid. A small amount of BHT (0.07%) was extracted from the donor chamber at the end of the experiment.

The percutaneous absorption of 10% BHT (in Labrafil) after daily dosing for 21 days was determined using guinea pigs by Courtheoux et al. (1986). Once a week, the animals were dosed with [^{14}C]-BHT. The test site, the retroauricular bald area, was either washed with liquid soap-water or not washed 1 hour prior to dosing. Absorption of BHT was calculated from the amount of radioactivity excreted in the urine and expressed as a percentage of the applied dose. The amount was not corrected for excretion by other routes or for retention in the body. After 8 days of treatment, BHT absorption increased and reached a plateau (<4% of the radioactivity was excreted). Daily washing of the skin did not significantly modify the total amount absorbed. The investigators suggested that either BHT was eliminated slowly after administration of a single dose, BHT had a pharmacologic action in the skin, or repeated application of the vehicle modified the lag time and diffusion rate.

Bronaugh et al. (1989, 1990) performed in vitro skin absorption/metabolism studies on radiolabelled BHT and other compounds using excised skin of female fuzzy rats. The test compound (~5 $\mu g/cm^2$ skin in 15 $\mu l/cm^2$ acetone) was applied to a 200-μm area of the skin of the back, which had been removed, dermatomed, and assembled in a diffusion cell. The diameter of exposed skin was 0.64 cm^2. The amount absorbed into the skin and receptor fluid (Eagle's modified minimal essential medium with 10% fetal bovine serum) during a 24-hour period was determined at 6-hour intervals, and the flow rate was 1.5 ml/h. The skin was washed three times with a 1% detergent solution and rinsed with distilled water. The skin was removed from the diffusion cell and homogenized using a 50:50 buffer-methanol solution. The test compound and any metabolites were extracted from the receptor fluid and skin homogenates in ethyl acetate; 56.0% of the BHT was extracted. The extracts were reduced in volume under nitrogen and analyzed using thin-layer chromatography (TLC), and radioactivity was measured on the TLC plates.

The absorbed dose of BHT remained primarily in the skin at the end of the study. In the receptor fluid, 2.3% ± 0.1% of the applied dose that penetrated the skin was absorbed and 26.8% ± 0.2% of this was metabolized. In the skin, 11.1% ± 0.9% was absorbed and 2.4% ± 0.2% was metabolized. When the skin and receptor fluid values were combined, 13.5% of the applied dose

was absorbed, of which 6.6% was metabolized. During tape-stripping studies, the fraction of the skin radioactivity present in the stratum corneum was 13.8%. TLC had two peaks of radioactivity in addition to the BHT peak: one peak chromatographed with the hydroxy-BHT standard, and the other could not be identified (Bronaugh et al. 1989, 1990).

Metabolism and Excretion

Table 3 lists the common metabolites of BHT as reported in the studies in this section. Table 4 describes quantitative data on excretion of metabolites in rabbits, rats, and humans. Figure 2 presents the pathways through which BHT is metabolized according to Daniel (1986).

Collings and Sharratt (1970) reported that the concentrations of BHT in the subcutaneous adipose tissue of 11 residents of the United Kingdom and 12 residents of the United States were 0.23 ± 0.15 ppm and 1.30 ± 0.82 ppm, respectively. These amounts were 15 to 80 times less than those found in rats fed 50 mg/kg/day BHT.

Conacher et al. (1986) reported that adipose tissue from six Canadian subjects contained 0.12 ppm BHT, although direct extrapolation from rat data predicted an amount of 0.01 ppm. They concluded that the antioxidants accumulated to a greater concentration in the fatty tissues of humans than rats when compared on a dose/body weight basis.

In rabbits, BHT was stored in body tissues upon multiple dosing and was released slowly after a "pseudoequilibrium" that took ~5 days, such that >16-fold accumulation was possible after daily exposure (El-Rashidy and Niazi 1980).

Holder et al. (1970b) administered 100 mg BHT to eight men on two occasions over a 4-day interval. Urine was collected 24 hours after dosing and analyzed. The metabolites were separated and identified using TLC and/or infrared spectrometry, nuclear magnetic resonance spectrometry, and melting point. As shown in Table 3, only BHT-COOH was detected in the first ether extract, but benzoylglycine was identified during the second extract. Subsequent urinalyses were made in duplicate on the pooled 24-hour urine of two adults treated with 1.0 g BHT. The TLC indicated the presence of a very polar compound that was "most probably" BHT-COOH ester glucuronide. The major metabolites in human urine were, therefore, the carboxylic acid of BHT and its glucuronide.

In two male humans, 63.2% to 66.9% of the radioactivity was excreted in the urine after ingestion of a capsule containing 40 mg [^{14}C]-BHT (~0.5 mg/kg; specific activity = 2.4 μCi/mg). Rapid excretion occurred in which 49.5% to 50.7% of the dose was detected in the urine during the first 24 hours after dosing. On the second day after dosing and daily through day 11, 5.5% to 5.6% and 0.5% to 2.8%, respectively, were excreted in the urine. Daily fecal output was 0.02% to 0.3% of the dose per day, as determined from measurements of radioactivity in the feces on days 10, 18, and 31 after dosing (Daniel et al. 1967).

Metabolism studies were performed using rats, rabbits, and humans. The comparative metabolic pathways of BHT are depicted in Figure 2 and quantitative data are listed in Table 4. In these species, oxidation occurred at the *para*-methyl group and one or both of the *tert*-butyl substituents (Daniel 1986).

Metabolism of BHT by humans differed from that of the rat. Free and conjugated BHT were minor urinary constituents and mercapturate was "virtually absent" in humans. BHT was excreted primarily as the glucuronide of a metabolite in which the ring methyl group and a methyl group of one of the *tert*-butyl groups were oxidized to carboxylic acid and a methyl group of the other *tert*-butyl group was oxidized, most likely to an aldehyde (Daniel, Gage, and Jones 1968).

Verhagen et al. (1989) fed single doses of BHT to rats and humans. Male Wistar rats (n = 2–10) were treated with 20 to 200 mg/kg BHT or 200 mg/kg of both BHT and BHA. Human subjects were treated with 0.5 mg/kg BHT (seven nonsmoking males) or 0.25 mg/kg of both BHT and BHA (five females, one smoking). In rats, kinetic parameters increased dose-dependently and plasma BHT concentrations were approximately four times greater than those reported for BHA. Rats excreted ~10% of the high dose as unchanged BHT in the feces, mostly on day 1. Urinary excretion of BHT-COOH was little more than 1%, in decreasing amounts, on days 1 to 4. When BHT and BHA were coadministered, absorption of BHT from the gastrointestinal (GI) tract was decreased in the first few hours after treatment. The plasma kinetics of BHA were not affected by BHT. In humans, the mean plasma concentration-time profile was decreased as compared to that of rats and closely followed plasma BHA kinetics. Unchanged BHT was not detected in the feces, and urinary excretion of BHT-COOH was 0% to 5.5%. After women ingested both antioxidants, no alterations in plasma BHT or BHA profiles occurred.

Rats metabolized the methyl group of BHT to carboxylic acid and excreted it either unbound or as the glucuronide. A mecapturate conjugate was excreted in the urine, but free BHT was the main component excreted in the feces. When administered repeatedly to rats, BHT accumulated in the fatty tissues such that the concentration in subcutaneous adipose tissue peaked in 2 days and decreased over the next 7 days; doses of 500 mg/kg/day produced a final concentration of 100 ppm in fat, and doses of 250 mg/kg/day resulted in a concentration of 30 ppm (Daniel and Gage 1965; Gilbert and Golberg 1965; Wright et al. 1965; Daniel, Gage, and Jones 1968).

BHT was administered intraperitoneally (IP) to 10 male Wistar rats at doses of 100 and 500 mg/kg body weight. The metabolite 2,6-di-*tert*-butyl-p-benzoquinone was the primary metabolite; however, 2,6-di-*tert*-butylhydroquinone and 2,6-di-*tert*-butyl-4-[(methylthio)methyl]phenol were also identified. The urinary and fecal excretion of 2,6-di-*tert*-butyl-p-benzoquinone were 0.048% and 1.52% of the dose, respectively, and the urinary excretion of 2,6-di-*tert*-butyl-4-[(methylthio)-methyl]phenol was 0.003% of the dose 5 days after dosing (Yamamoto, Tajima, and Mizutani 1979).

TABLE 3
Common metabolites of BHT[a]

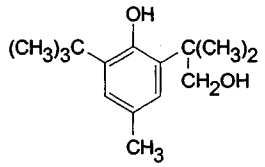
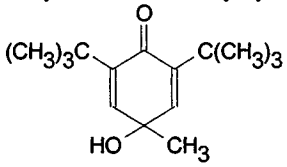
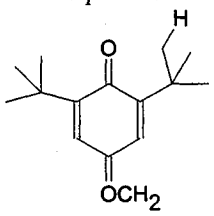
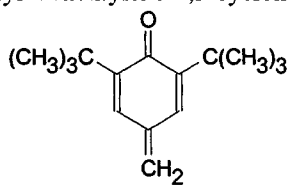
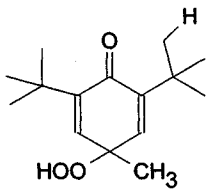
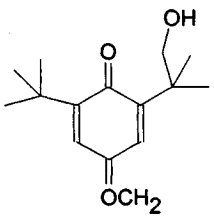
Abbreviation	Full name and/or formula
BHT-acid (BHT-COOH)	3,5-di-*tert*-butyl-hydroxybenzoic acid
BHT-aldehyde	3,5-di-*tert*-butyl-hydroxybenzaldehyde
BHT-alcohol (BHT-OH)	
BHT-3°OH	4-hydroxy-4-methyl-2,6-di-*tert*-butylcyclohexa-2,5-dienone 
BHT-hydroperoxide (BHT-OOH)	2,6-di-*tert*-butyl-4-hydroperoxy-4-methyl-2,5-cyclohexadienone or 4-hydroperoxy-4-methyl-2,6-di-*tert*-butylcyclohexadienone
BHT-quinol	2,6-di-*tert*-butyl-4-hydroxy-4-methyl-2,5-cyclohexadione
BHT-quinone	2,6-di-*tert*-butyl-4-benzoquinone 
DBP	2,6-di-*tert*-butylphenol
BHT-SCH_3	2,6-di-*tert*-butyl-4-[(methylthio)methyl]phenols
BHT-quinone methide (BHT-QM)	2,6-di-*tert*-butyl-4-methylene-2,5-cyclohexadienone 
BHT-peroxyquinol	
BHT-OH-peroxyquinol	

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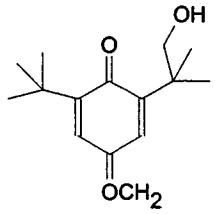
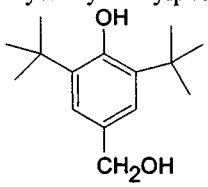
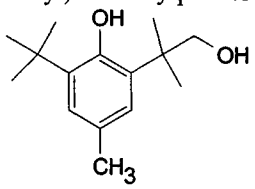
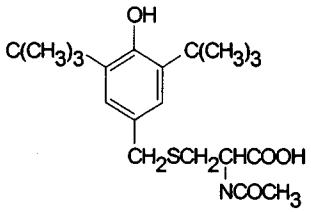
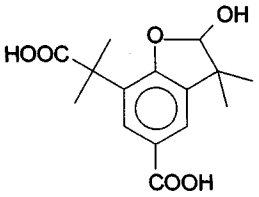
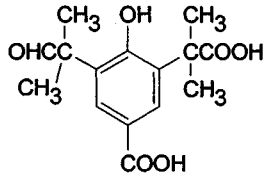
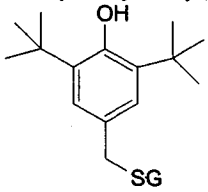
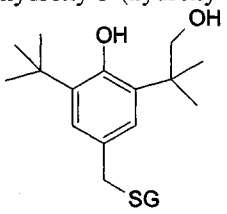
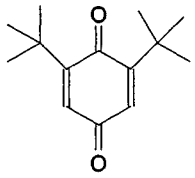
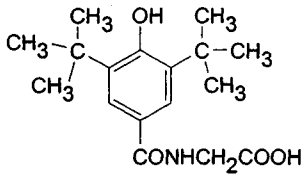
TABLE 3
Common metabolites of BHT[a] *(Continued)*

Abbreviation	Full name and/or formula
BHT-OH-QM	
BHT-MeOH (BHT-1°OH)	2,6-di-*tert*-butyl-4-hydroxymethylphenol 
BHT-BuOH	3,5-di-*tert*-butyl-hydroxybutyl alcohol or 6-*tert*-butyl-2-(hydroxy-*tert*-butyl)-4-methylphenol 
BHT-mercapturic acid	
BHT-(acid)$_2$(hemi)	
BHT-(acid)2(aldehyde)	
BHT-SG	*S*-(3,5-di-*tert*-butyl-4-hydroxybenzyl)glutathione 

(Continued on next page)

TABLE 3
Common metabolites of BHT[a] *(Continued)*

Abbreviation	Full name and/or formula
BHT-OH-SG	*S*-[3-*tert*-butyl-4-hydroxy-5-(hydroxy-*tert*-butyl)benzyl]glutathione 
DBQ	
BHT-BzOH	2,6-di-*tert*-butyl-4-hydroxymethylphenol
BHT-gly	
BHQ	2,6-di-*tert*-butylhydroquinone

[a]See text for discussion.

When [^{13}C]-BHT was administered to humans, two urinary metabolites were 3,5-di-(1-methyl-1-methylethyl)-4-hydroxybenzoic acid and 5-carboxy-7-(1-carboxy-1-methylethyl)-3,3-dimethyl-2-hydroxy-2,3-dihydrobenzofuran (Wiebe, Mercer, and Ryan 1978).

Conning and Phillips (1986) reported that BHT cleared less rapidly than BHA from most species due partly to enterohepatic circulation. Tissue accumulation was greater for BHT than for BHA. The major route of oxidative metabolism was mediated by a microsomal monooxygenase system. The ring methyl group was predominantly oxidized in the rat, rabbit, and monkey, whereas humans oxidized the *tert*-butyl groups. In contrast, other phenolic antioxidants (i.e., gallates and 2-*tert*-butylhydroquinone) were metabolized mainly by nonoxidative pathways, such as methylation or conjugation with sulfate and glucuronic acid.

Thompson et al. (1987) determined that two main metabolic processes occur in microsomal fractions of hepatic and pulmonary tissues from several strains of mice and Sprague-Dawley rats. One process was the hydroxylation of alkyl substituents; the metabolites were a 4-hydroxymethyl product and BHT-BuOH. The 4-hydroxymethyl product was then oxidized to the corresponding benzaldehyde and benzoic acid derivatives, and BHT-BuOH was hydroxylated at the benzylic methyl group to produce a diol that was further oxidized to a hydroxybenzaldehyde derivative. The second process was the oxidation of the aromatic π electron system, which produced BHT-quinol, BHT-quinone, BHT-quinone methide (BHT-QM), and derivatives of the quinol and quinone with a hydroxylated t-butyl group. In the study, microsomes from mice produced large quantities of the 4-hydroxymethyl product and BHT-BuOH; however, the principal metabolite in rat tissues was the former. Mouse pulmonary microsomes produced more quinone relative to other metabolites than hepatic cells. In addition, quantitative differences in BHT metabolites were observed among different strains of mice (C3H/21BG, C57BL/6J, SWR/J, 129/J, and A/J). Other researchers investigated the metabolism of BHT in rodents and reported similar pathways (Matsuo et al. 1984).

Rat liver microsomes and hepatocytes metabolized BHT either by hydroxylation of alkyl substituents or by oxidation of the π-electron system. The latter pathway generated a phenoxy radical that partitioned between two reactive products: BHT-OOH and BHT-QM (Thompson et al. 1990a). QMs are structurally analogous to quinones, with the exception that one of the carbonyl oxygens is replaced by a methylene group (Thompson et al. 1992).

After a single oral dose of 800 mg/kg BHT, the plasma concentration in rats of BHT-QM peaked 18 hours after dosing.

TABLE 4
Metabolism and excretion of BHT

Species	Metabolite	Percentage of dose excreted		Reference
		Urine	Bile	
Rabbit	BHT-acid (BHT-COOH)	8		Dacre 1961
	BHT-acid-glucuronide	16		
	BHT-gly	2		
	BHT-BuOH-glucuronide	19		
	Sulfate	8		
Rabbit	Glucuronide(s)	37		Akagi and Aoki 1962
	Sulfate	17		
	Free phenols	7		
Rat	BHT-alcohol (BHT-OH)	8		Ladomery, Ryan, and Wright 1967b
	BHT-aldehyde	3		
	BHT-acid	5		
	Unidentified	6		
Rat	BHT-OH		1	Ladomery, Ryan, and Wright 1967a
	BHT-aldehyde		2	
	BHT-acid		19	
	BHT-dimer		2	
	Unidentified		18	
Rat	BHT-acid	9	0.8	Daniel, Gage, and Jones 1968
	BHT-acid-glucuronide	15	14	
	BHT-mercapturic acid	11	3	
	Sulfate	13.5	—	
	Other glucuronides	4	2	
	Free phenols	1	0.7	
Human	BHT-acid	1		Daniel, Gage, and Jones 1968
	BHT-acid-glucuronide	2		
	BHT-$(acid)_2$(aldehyde)-glucuronide	35		
	BHT-mercapturic acid	Trace		
	Other glucuronides	5		
	Free phenols	0.5		
Human	BHT-acid	0.3		Wiebe, Mercer, and Ryan 1978
	BHT-$(acid)_2$(hemi)-glucuronide	21		
	BHT-mercapturic acid	Trace		

The amount of BHT in the GI tract was constant from 0.5 to 12 hours, and began to decrease at 18 hours after dosing. The maxima for BHT in both epididymal and subcutaneous adipose tissues were attained at 18 hours. The volumes and weights of the stomach and contents were two to three times greater than the control values 4 to 7 hours after treatment with BHT. The values did not differ from controls at 17 to 24 hours. When 18-hour starved rats were given 800 mg/kg BHT, the rats retained the ingested material in the stomach. When BHT was administered intraduodenally rather than orally to anesthetized rats, the concentration of BHT in portal vein plasma was 0.4 to 1.4 μg/ml; BHT was not found in plasma from the aorta descendens and BHT-QM was not detected in portal vein plasma. The concentrations of BHT alone and BHT and/or BHT radical were 7 to 20 μg/g wet weight in the liver and 25 to 40 μg/g in epididymal adipose tissue. The results of this study indicated that most BHT did not enter lymph but was instead absorbed into portal blood. In addition, BHT at high doses had inhibitory effects on gastric function, leading to its retention in the stomach (Takahashi 1990).

Shaw and Chen (1972) incubated BHT with washed rat liver microsomes, NADPH, and air to give the compounds depicted in Figure 3. Unlike step 2, step 1 required NADPH and air and was inhibited by SKF 525-A and carbon monoxide. Added reduced glutathione (GSH) "augmented" step 2, but the reaction was not inhibited by the addition of ATP. Step 3 required NADPH. The metabolites identified were BHT-OOH, BHT-3°OH, and BHT-1°OH (see Table 3).

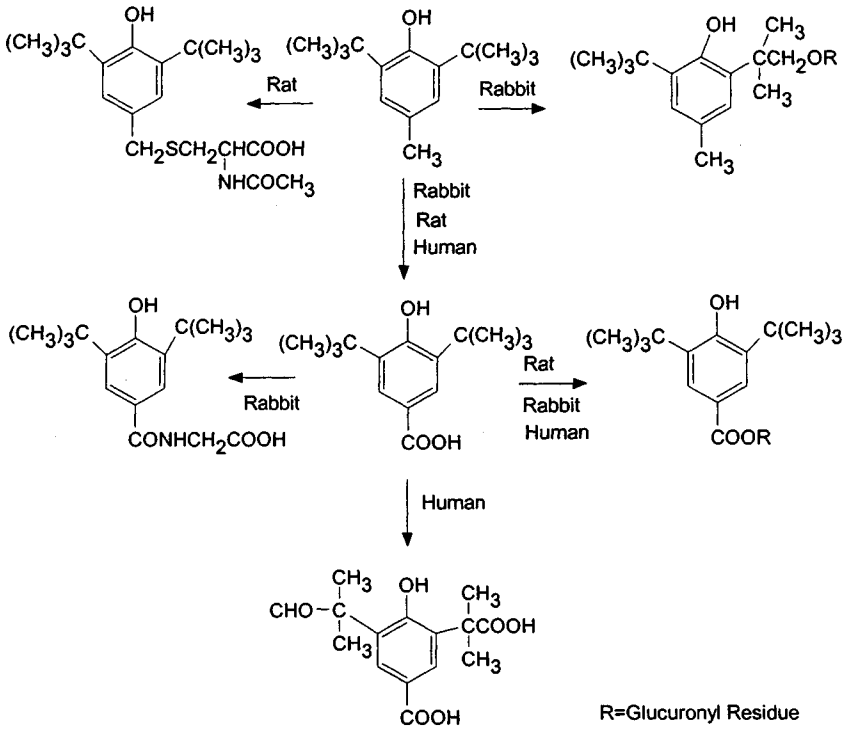


FIGURE 2

Metabolism of BHT. The compounds are (*top row, left to right*) BHT-mercapturic acid, BHT, BHT-BuOH-glucuronide; (*second row, left to right*) BHT-gly, BHT-acid, BHT-acid-glucuronide; and (*third row*) BHT-(acid)$_2$(aldehyde) (Daniel 1986). See Table 4 for the full names.

Chen and Shaw (1974) proposed that BHT was first oxygenated to BHT-OOH, which was converted to BHT-3°OH by either the cytochrome P450 system, a GSH peroxidase, a reducing enzyme system, or by a chemical reducing agent in the enzyme preparation. In addition, BHT-OOH could undergo spontaneous homolytic and/or heterolytic fission to the BHT-3°OH radical or a BHT-3°O$^-$ anionic species that acquired an ion or hydrogen atom to yield BHT-3°OH. This compound could then be oxygenated.

When male Wistar rats were treated IP with 200 mg/kg of a metabolite of BHT, BHT-COOH, DBP, and BHT-quinone were identified by GC and GC-MS in the feces. Biliary excretion of BHQ, its glucuronide, and BHT-COOH glucuronide were also confirmed using GC-MS and HPLC. Twenty-four hours after

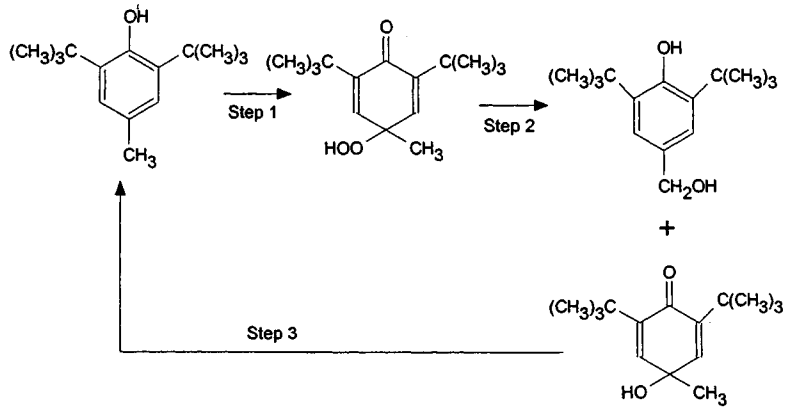


FIGURE 3

Metabolism of BHT (Shaw and Chen 1972).

dosing, the excretion rate of BHQ-glucuronide was approximately ninefold greater than after dosing with BHT. After the rats were treated with BHT-COOH, DBP was detected in the urine (Tajima, Yamamoto, and Mizutani 1983, 1984; Yamamoto et al. 1991).

The biliary metabolism of BHT, BHT-COOH, BHT-OH, and BHT-aldehyde was compared after IP or intravenous (IV) administration to male Wistar rats. For all four test compounds, the major metabolites in enterohepatic circulation were BHT-COOH and its ester glucuronide. When BHT-OH was administered, traces of the alcohol, aldehyde, and BHT-dimers were also detected. The rate of excretion after IV dosing with BHT-COOH was greater that that after IP dosing; however, the total percentage excreted was independent of the route of administration. Total biliary excretion after treatment with BHT or BHT-aldehyde was less after IV dosing than after IP dosing. Biliary excretion of BHT-OH was ~70% of the dose, and was unaffected by the route of administration (Holder et al. 1970a).

Ladomery, Ryan, and Wright (1967a, 1967b) investigated metabolism and excretion of BHT from IV and IP doses of [^{14}C]-BHT using male white rats. At 6 hours, radioactivity equivalent to 95% (IV) and 52% (IP) of the dose was excreted in bile. The metabolites identified were BHT-OH, BHT-aldehyde, and BHT-COOH. Small amounts of BHT diphenyl ethane were also detected. Excretion of BHT-COOH was relatively small in urine compared to bile. This difference was due to a selective reabsorption of BHT-COOH after biliary excretion. Within 4 hours of IP dosing, 37.9% of the dose was detected in the feces and 31.2% was excreted in the urine. The small intestine, colon, and intestinal wall had 7.4%, 2.1%, and 1.6% of the dose, respectively. The liver, spleen, and kidneys each had 0.9% to 0.14% of the dose.

Male Sprague-Dawley rats were fed a diet containing 1.2% BHT for 2 weeks. Using GC, the major metabolite was identified as BHT-QM. Nonconjugated, unchanged BHT was identified in samples of lungs, kidneys, pancreas, brain, and epididymal adipose tissues, but was not detected in hepatic tissue (Takahashi and Hiraga 1979a).

Takahashi and Hiraga (1980) fed 1.00% BHT to male Sprague-Dawley rats for 10 days. Daily consumption was 0.19 to 0.3 mmol/rat for the first 2 days and 0.5 to 0.7 mmol/rat for the remainder of the study. The cumulative dose was approximately 5.0 mmol/rat. The total amount of phenols excreted in both the urine and feces was 3.65 mmol/rat, or 73% of the administered dose. Of this amount, 48% was detected in the urine and 25% was found in the feces. Thirty percent and 11% of the dose was as unconjugated phenols in the urine and feces, respectively. When the urine was analyzed using mass, UV, and infrared spectrometry, the major metabolite was identified as BHT-COOH.

Bianchi et al. (1997) measured the amount of antioxidants in human plasma and omentum tissue homogenates using HPLC with coulometric electrochemical detection. The homogenates were prepared from specimens of 20 female and 30 male patients; however, the analysis of BHT was performed using 42 of the 50 samples. Detectable homogenate concentrations of BHT were obtained in 27 of 42 patients (64%). The sensitivity was 0.4. The estimated amount of BHT was 3.0 ng/ml of intestine homogenates. In addition, 45 plasma samples from the hospital blood bank contained nondetectable amounts of BHT.

Tye, Engel, and Rapien (1965) gave young, adult Nelson rats (one per sex per group) one to five oral doses of ~0.2 mmol/kg [^{14}C]-BHT (~44 mg/kg) on alternate days from days 0 to 9. The rats were killed 24 hours after administration of the final dose, with the exception of the rats given five doses, which were killed 8 days after dosing.

As shown in Table 5, 0.05% was exhaled from the lungs of two males during the 24-hour observation period. "Substantial amounts" of radioactivity were excreted in the urine and feces, moderate amounts were retained in the GI tract, and small amounts were retained elsewhere in the body. The fate of BHT was the same, regardless of the number of doses administered, and total output of radioactivity did not decrease with the increasing number of doses. Although females absorbed more BHT, both sexes readily eliminated the antioxidant from the body. By day 17, up to 92% (males) and 97% (females) of the total dose had been eliminated. In general, female rats retained more radioactivity in the tissues than males, particularly in the gonads. The concentrations were not related to the number of doses administered; therefore, radioactivity did not accumulate in the tissues upon repeated administration. Rats killed 8 days after the final dose had less [^{14}C]-BHT in the tissues than rats killed 24 hours after dosing (Tye, Engel, and Rapien 1965).

The same investigators also administered BHT via the subcutaneous route. Four female Charles River CD rats were given single injections of 0.003 to 0.22 mmol/kg BHT (in peanut oil).

TABLE 5
Elimination and distribution of BHT given orally in the rat (Tye, Engel, and Rapien 1965)

	Amount excreted or retained				
Sex	Urine	Feces	LKLsFS[a]	HBBrSM[b]	Reproductive organs
Male	3%–15%	58%–83%	0.003%–0.10%	0.0004%–0.017%	0.0003%–0.007%
Female	19%–43%	—	0.007%–0.162%	0.001%–0.185%	0.016%–0.15%

[a]LKLsFS = liver, kidneys, lungs, fat, spleen.
[b]HBBrSM = heart, blood, brain, skin, muscle.

Two and 4 days after dosing, urinary concentrations of [^{14}C]-BHT were determined. The rats eliminated 29% to 77% of the dose in the feces and 6% to 14% in the urine. At the lower doses, the relative rate of excretion decreased; this effect was reflected equally in both the feces and urine (Tye, Engel, and Rapien 1965).

In another study, a single dose of [^{14}C]-BHT in olive oil was administered to male Wistar (SPF) rats via a stomach tube. Each rat was treated with 5 mg/rat (11 μCi/ml) or 15 mg/rat (33 μCi/ml) of the radioactive compound. The radioactivity reached maximum concentrations in the liver and serum 6 hours and 6 to 12 hours, respectively, after treatment. The concentration in the liver at that time was 5.1% of the dose. In a subcellular analysis, ~60% of the radioactivity was detected in the supernatant fraction, although it migrated to the microsomal fraction over time. Radioactivity in the microsomal fraction increased "about twice with the decrease of radioactivity in the supernatant fraction." Approximately 25% of the total radioactivity was detected in the microsomal fraction after 48 hours. During submicrosomal analysis, radioactivity was detected in the smooth endoplasmic reticulum (ER) 3 to 6 hours after administration. The maximum specific radioactivity in the smooth ER was reached 6 hours after treatment, and the maximum in the rough ER was reached 12 hours after treatment (Nakagawa, Ikawa, and Hiraga 1978). BHT-induced smooth ER proliferation was related to the increased activity of aminopyrine demethylase, and did not persist after exposure to the antioxidant was halted (Botham et al. 1970).

Terao et al. (1985) used reverse-phase HPLC to determine the amounts of BHT in the tissues and sera of rats fed the antioxidant. One group of female Sprague-Dawley rats was fed a semipurified, high–polyunsaturated fat diet containing no antioxidant for 1 week, then was fed a low-fat diet containing 0.3% BHT for 1, 2, or 3 weeks. Animals of the control group were fed the low-fat diet without antioxidants. A second group of rats was fed the high-fat diet for 1 week, then was fed the low-fat/BHT diet for 2 or 17 weeks. BHT was not detected in the serum or serum lipids. The amount of BHT in hepatic tissue was greater after 2 weeks of feeding than after 3 weeks; the amounts were 0.64 ± 0.26 μg/g tissue and 0.35 ± 0.09 μg/g tissue, respectively, which corresponded to 15.22 ± 2.10 μg BHT/g lipid and 10.80 ± 2.71 μg BHT/g lipid, respectively. BHT was found in much greater concentrations in mammary gland tissue than in the liver and serum of the treated rats, but the concentration was greater after 2 weeks of feeding than after 3 weeks or more. The amount of BHT in mammary gland tissue was 6.37 ± 1.37 μg/g tissue after 2 weeks and 4.52 ± 1.77 μg/g tissue after 3 weeks. These values corresponded to concentrations of 20.08 ± 7.94 μg/g lipid and 14.40 ± 5.48 μg/g lipid, respectively. The amount of BHT found in mammary gland tissue was related to the lipid content of the tissue and to the lipophilic character of BHT. The metabolite 4,6-di-*tert*-butyl-4-[(methylthio)methyl]phenol was detected in mammary gland tissue, but BHT-COOH and BHT-OH were not.

Cytotoxicity

Babich and Borenfreund (1990) performed a neutral red assay using four human cell lines. The assay determined the cytotoxic potential of BHT by quantitating the number of viable, noninjured cells via the uptake and lysosomal accumulation of neutral red dye. The cells used were normal human epidermal keratinocytes and melanocytes, foreskin fibroblasts, and a melanoma, SK-Mel/27. The concentrations of BHT that decreased the absorbance (at 540 nm) of extracted neutral red dye by 50% (NR_{50}) of the control value was determined during 1- and 2-day exposure periods. After 1 day, the NR_{50} values for the foreskin fibroblasts, keratinocytes, melanocytes, and melanoma cells were 46, 53, 65, and 65 μM, respectively. After 2 days, the respective values were 34, 37, 45, and 57 μM. The fibroblasts and keratinocytes, therefore, were more sensitive to BHT cytotoxicity than the melanocytes and melanoma cells, and cytotoxicity was time dependent.

In another study, hepatocytes from male Fischer 344 rats were treated with BHT in dimethyl sulfoxide (DMSO, final concentration <1%). Control cells were treated with vehicle alone. Aliquots of cell suspensions were analyzed for cell death and quantification of the concentrations of glutathione and adenine nucleotides. In addition, the rate of oxygen consumption and respiration control index were determined using isolated hepatic mitochondria. When added to the hepatocytes, 0.5 to 1.0 mM BHT caused concentration-dependent acute cell death that was accompanied by the loss of cellular ATP and GSH and increased AMP. The high concentration reduced the total adenine nucleotides pool to ~60% of the control value. BHT did not react with ATP without hepatocytes; therefore, the depletion of ATP was likely due to inhibition of adenine nucleotide synthesis and/or activation of ATP hydrolysis. At a concentration of 0.5 mM, BHT increased the rate of state 4 oxygen consumption, indicating partial uncoupling of oxidative phosphorylation during mitochondrial respiration. At this concentration, BHT also inhibited the respiratory control index, an indicator of mitochondrial impairment, due to inhibition of state 3 respiration and stimulation of state 4 respiration (Nakagawa, Yaguchi, and Suzuki 1994).

Male Wistar rats dosed with 500 or 1000 mg/kg BHT by stomach tube had remarkable changes in their hepatic GSH concentrations. GSH concentrations were depleted and induced in a time-dependent manner; the period of depletion was dose dependent. In both dose groups, GSH depletion was maximum at 6 hours after BHT administration and by 48 hours it had increased about twofold over control concentrations. The activity of GSH *S*-transferase was not affected until 24 hours after BHT treatment and reached 2.3-fold of the control activity at 48 hours after treatment. This response, accompanied by an increase in GSH concentration, indicated that the mercapturic acid conjugation system was induced by BHT. No significant changes occurred with regard to hepatic lipid peroxide. The activity of glutamic-oxaloacetic transaminase (GOT) and glutamic-pyruvic transaminase (GPT) reached a maximum 24 hours after BHT

administration at 38- and 65-fold of the control rats, respectively. The livers of the high-dose group had centrilobular necrosis and hemorrhage (Nakagawa et al. 1984b).

When assayed in equimolar concentrations using hepatocytes from male Sprague-Dawley rats, BHT was more cytotoxic than BHA (Thompson and Moldéus 1988). Cytotoxicity for both was concentration dependent. If the cells were incubated in medium containing both antioxidants, the observed cytotoxicity was greater than that of either compound and appeared to be additive. For example, the combination of 250 μM BHT and 250 μM BHA produced similar cytotoxic effects as 500 μM BHT or 750 μM BHA alone. The concentrations of reduced thiols decreased concomitant with cell death, and both antioxidants inhibited respiratory control in isolated mitochondria. These effects were reflected by a rapid decrease in ATP concentrations in intact hepatocytes prior to cell death.

BHT-OOH, a metabolite of BHT formed by the combination of a peroxy radical with molecular oxygen, was approximately 20-fold more toxic than BHT to rat hepatocytes, which partially metabolized the compound using cytochrome P450. The mechanism of cytotoxicity was determined using structural analogs of BHT-OOH and was related to the expulsion of the 4-alkyl group as an alkyl free radical. When BHT-OH was metabolized by π oxidation, the resulting BHT-OH-OOH was less toxic than its precursor. Additional details were not available (Thompson et al. 1990a).

BHT and BHT-MeOH had little toxicity for Sprague-Dawley rat hepatocytes when the antioxidants were added to the medium at a concentration up to 1 mM. BHT-OH at a concentration of 0.2 mM killed all of the cells within 60 minutes, and a structural analog was nearly as toxic (Table 6). BHT-OH was oxidized by cytochrome P450 to BHT-OH-QM, a metabolite more electrophilic and toxic than BHT-QM. The analog also formed a QM that was activated by intramolecular hydrogen bonding. The investigators concluded that QM formation was responsible for BHT-OH–induced cytotoxicity for rat hepatocytes (Thompson et al. 1990b).

Bolton et al. (1990) reported that BHT-OH-QM was sixfold more reactive than BHT-QM during studies of the compounds with GSH. Using infrared spectra, nuclear magnetic resonance spectra, and electrochemical measurements, it was determined that the enhanced electrophilicity of BHT-OH-QM was caused by intramolecular hydrogen binding of the ring oxygen with the side-chain hydroxyl.

The concentration of BHT that caused a 50% decrease in the number of viable cells (ID_{50}) after 72 hours was 43.0 μg/ml for KB cells of human epidermoid carcinoma. In contrast, the ID_{50} values for the antioxidants BHA, propyl gallate, and *dl*-α-tocopherol were 12.5, 1.3, and 110.0 μg/ml, respectively (Mochida, Goto, and Saito 1985). BHT (74 μg/ml) also inhibited growth of human maxillary cancer cells in vitro such that the survival rate decreased by 50% (Yamamoto et al. 1996).

Results were similar when rhesus monkey kidney cells were used. In one study, the monolayer cell cultures were treated with 0.035 to 0.136 mmol BHT in 0.9% DMSO. Growth inhibition was concentration and time dependent, and was reversed by removal of BHT from the culture medium. Inhibition of the rate of RNA synthesis was 50% in cells from males and 95% in cells from females treated with the high dose. Protein synthesis was inhibited in male cells by 15%, and by 100% in female cells. DNA synthesis in females was inhibited by ~65% to 75% and in males by ~30% to 35%. The same concentrations of BHT-COOH and BHT-OH did not inhibit RNA synthesis. The data indicated that cells from males metabolized BHT more rapidly than cells from females, thus resulting in decreased cell multiplication in cultures of cells from females (Milner 1967).

A second study using monkey cells was performed by Metcalfe (1971). Concentrations up to 30 mg/g cells (in 1% DMSO) did not cause visible cytopathologic changes, although this dose inhibited the rate of cell multiplication in a dose-dependent manner. Within 30 minutes of exposure, BHT decreased DNA, RNA, and protein synthesis; the inhibition was reversible within 1 hour.

TABLE 6
Cytotoxicity of phenols and peroxyquinols in rat hepatocytes (Thompson et al. 1990b)

Compound	Concentration (mM)	% dead cells in 60 min
Control	0	18
	Phenols	
BHT	3.0	32
BHT-OH	0.20	100
isoBHT-OH[a]	0.30	97
	Peroxyquinols	
BHT-peroxyquinol	0.25	84
BHT-OH-peroxyquinol	0.40	60
isoBHT-OH-peroxyquinol[a]	0.50	70

[a] A structural analog with the same relationship as BHT-OH between the side-chain hydroxyl and the ring oxygen favoring intramolecular hydrogen bonding, with the exception that it is a secondary rather than a primary alcohol, so oxidation to an aldehyde cannot occur.

Nievel (1969) reported that liver microsomes from female Wistar rats fed 450 mg/kg BHT for 7 days had increased incorporation of radioactive amino acids into protein in vitro, mostly into proteins of the ER.

BHT did not inhibit tumor necrosis factor–induced cytotoxicity and growth enhancement when evaluated using L929 and WEHI 164 fibrosarcoma cells (Brekke et al. 1992, 1994).

BHT at a concentration of 100 ppm (0.45 mM) caused marked leakage of lactate dehydrogenase from cultured myocardial and "endothelioid" cells of neonatal rats into the culture medium. The antioxidant significantly decreased the beat rate of the heart cells such that maximum inhibition occurred within 1 hour of exposure to BHT. The cells were morphologically similar to control cells, but BHT at 1000 ppm (4.5 mM) caused cell lysis after a 1-hour exposure period (Leslie, Gad, and Acosta 1978).

At concentrations of 1 to 500 mg/l, BHT decreased the frequency and amplitude of contractions of atrial preparations in vitro. Concentrations of 10 to 500 mg/l BHT decreased methacholine-induced contractions of rat and rabbit ileal cultures. Within 20 and 72 minutes of treatment of rabbit and rat preparations, respectively, the high dose stopped spontaneous atrial contractions. All concentrations of BHT also increased creatine phosphokinase leakage into perfusion solutions in a dose-dependent manner (Gad, Leslie, and Acosta 1979).

Jayalakshmi and Sharma (1986) treated erythrocytes from male and female Wistar rats with BHT at concentrations of 0.02% to 1.00%. For males, the percentage hemolysis values for concentration of 0.02%, 0.10%, 0.20%, 0.40%, 0.50%, 0.75%, and 1.00% were 24, 49, 70, 74, 85, 94, and 90, respectively. For females, the values were 22, 52, 68, 73, 84, 94.5, and 92. Hemolysis peaked at 60% to 65% within 12 minutes of exposure to BHT and resulted from extensive damage to the erythrocyte membrane that resulted in the release of hemoglobin into the suspensions.

When rat erythrocytes were treated with 100 mg BHT in 0.5 ml peanut oil, BHT induced hemolysis via marked modification of membrane integrity and the simultaneous lowering of the concentrations of acetylcholine esterase and ATPase, both membrane-bound enzymes (Kumar et al. 1979).

BHT and other phenolic antioxidants protected cultured human umbilical vein endothelial cells from linoleic acid hyperoxide-induced cytotoxicity. The protection by antioxidants and their antioxidant activity was due primarily to the incorporation rate of the antioxidants into cells because of their lipophilicity, and their orientation in biomembranes (Kaneko, Kaji, and Matsuo 1994).

BHT did not induce permanent morphologic changes in normal human diploid cells during or after a 3-day treatment period. Chinese hamster cells, however, had morphologic changes that persisted for several subcultures. One of these variants had an increased multiplication rate and had different fine structural details compared to the parent cells. Additional details were unavailable (Rajaraman and Rounds 1973).

Concentrations of 0.001 to 1.5 mM BHT were toxic to normal adult rat hepatocytes, which had progressive morphologic degeneration after a 1-day attachment period. This degeneration was observed as degranulation of the cytoplasm, increased ratio of nucleus:cytoplasm and multinucleation, and decreased number of cells with time in culture (Miyazaki, Bai, and Sato 1990). Degranulation also occured after rat basophilic leukemia (RBL-2H3) cells were treated with 50 μM BHT and 12-*O*-tetradecanoyl phorbol-13-acetate (TPA) or antigen (Akasaka et al. 1996).

Takenaga, Honma, and Hozumi (1981) investigated the effects of BHT on lysozyme and phagocytic activity and morphological changes of mouse myeloid leukemia cells (M1). The maximum tolerated concentrations of BHT (data not given) for the cells did not induce lysozome and phagocytic activity in the cells. However, BHT at 10 μg/ml cultured in the presence of 10^{-7} M dexamethasone significantly inhibited the phagocytic activity of the M1 cells. BHT also inhibited the induction of lysozyme activity and morphological changes by dexamethasone without inhibiting the growth of the M1 cells.

Ohno et al. (1984) observed that, 3 days after culturing, 30 μg/ml BHT induced maximum cell differentiation in murine erythroleukemia cells. Concentrations of 5 to 25 μg/ml BHT did not induce differentiation in this cell line. The cooperative effect of DMSO and BHT on the differentiation of erythroleukemia cells were also investigated. BHT dosed at 5 to 25 μg/ml and combined with DMSO produced a greater degree of differentiation than that produced by each individual inducer.

Membrane Effects

Natural and synthetic antioxidants such as BHT stabilized biomembranes, including the membranes of human erythrocytes, against lipid peroxidation (Kagan et al. 1986; Dwight and Hendry 1996). Lipid peroxidation occurred when lipid molecules, primarily unsaturated fatty acids, underwent a chain reaction in which a free radical attacked a lipid molecule to form a lipid radical. Lipid radicals reacted with oxygen to form peroxides, which reacted with other fatty acids to form hydroperoxides and new fatty acid radicals. The rate of reaction accelerated in a geometric manner, and was accelerated by the presence of metal ions, a high pH environment, enzymes, heat, UV light, and pigments, or by a high degree of unsaturation (Papas 1993). BHT prevented free radical–induced changes of ER membrane fluidity in vesicles isolated from rabbit cerebrum (Kaplán et al. 1995).

BHT decreased the temperature at which lipid chains "melted." In addition, the antioxidant reduced the increase of ^{22}Na permeability and Na efflux in vesicles of saturated phospholipids that occurred at temperatures near the phase transition temperature. The maximum reduction was caused by an aqueous concentration of 0.22 mM BHT (Singer and Wan 1977).

Sokolove and Haley (1996) reported that, under some conditions, BHT increased permeability of mitochondria from

livers of Sprague-Dawley rats. The threshold concentration was ~60 nmol BHT/mg mitochondrial protein; below this concentration, no increase occurred, but at concentrations >120 nmol/mg, permeability was decreased.

In a cytotoxicity assay using Sprague-Dawley rat hepatocytes, BHT and BHA acted as membrane uncouplers and dissipated membrane potential across the mitochondrial membrane, released calcium, and caused mitochondrial swelling (Thompson and Moldéus 1988). Similar results were reported by Fusi et al. (1991). BHT and BHA uncoupled mitochondrial oxidative phosphorylation by making the inner membrane more permeable to protons. The antioxidants also interacted directly with electron transport, therefore inhibiting respiration.

BHT stimulated oxygenation of mitochondrial membranes and erythrocyte ghosts by 15-lipoxygenase in rabbits, as determined by increased oxygen consumption, increased loss of polyenoic fatty acids, and the formation of specific lipoxygenase products in membrane phospholipids (Schnurr et al. 1995).

Shertzer et al. (1991) performed a red blood cell osmotic fragility assay using BHT. At concentrations <60 nmol/mg protein, BHT protected against osmotic fragility; however, total osmolysis occurred when 135 nmol/mg protein was added. BHT-mediated changes in fragility were correlated with changes in membrane fluidity: the membrane was less fluid when protection occurred and more fluid when fragility increased. High concentrations of BHT (>77 nmol/mg protein) permeabilized the plasma and mitochondrial membranes, causing enzyme leakage; these changes were also accompanied by increased membrane fluidity.

Experiments with rat liver mitochondria-lysosome suspensions indicated that 10 μmoles BHT in the presence of 26.8 mg protein did not produce a significant protein leakage or increase L-glutamic acid dehydrogenase activity. BHT (1 mM) caused almost complete lysis of human, guinea pig, and rat erythrocytes (Sgaragli et al. 1977).

Synaptic vesicles isolated from the cerebral cortex and striatum of male Sprague-Dawley rats were incubated in the presence of micromolar amounts of BHT. Preincubation with BHT caused a time-dependent decrease in the rate of Mg^{2+}-ATP dependent norepinephrine (NE) accumulation. Under the conditions of this study, about 50% of the BHT added to the vesicle suspension was associated with membrane lipid regions. A significant alteration in membrane fluidity of phospholipid molecules within the bilayer of the vesicles was observed in vesicles incubated with 4 μM BHT compared to those incubated without BHT. BHT could have exerted its effects by decreasing the mobility of a NE carrier protein within the membrane (Gould and Saxer 1982).

Microsomes isolated from rat livers pretreated with phenobarbital were used to investigate the effects of BHT on lipid peroxidation during enzymatic iodination in the endoplasmic reticulum. Low concentrations of BHT (0.0001%) completely inhibited lipid peroxidation while doubling the amount of iodine incorporated into microsomes. In the presence of BHT, iodination caused the loss of 15% of cytochrome P450 and 35% of aminopyrine demethylase activity. The absence of BHT resulted in the loss of 75% cytochrome P450 and 65% aminopyrine demethylase activity. Greater concentrations of BHT (0.005%) prevented lipid peroxidation and preserved cytochrome P450 (Welton and Aust 1972).

Antimicrobial and Antiviral Effects

The minimum inhibitory concentrations (MICs) of BHT against *Escherichia coli, Pseudomonas aeruginosa, Streptococcus mutans* and *agalactiae, Staphylococcus aureus, Norcardia asteroides, Saccharomyces cerevisiae*, and *Candida albicans* were >5000 μg/ml. A concentration of 500 μg/ml was the MIC for *Corynebacterium* species (Kabara 1980).

BHT was a potent inactivator of lipid-containing mammalian and bacterial viruses (Snipes et al. 1975). BHT at a concentration of 50 μg/ml caused 92% inactivation of Newcastle disease virus, inhibited virion adsorption onto chicken-embryonated cells by 32%, inhibited synthesis of intracellular hemagglutinin by 29%, and caused virion envelope damage. At a concentration of 25 μg/ml, BHT inhibited viral replication by 65% (Winston, Bolen, and Consigli 1980). Kim et al. (1978) reported that 40 μg/ml BHT inactivated human and murine cytomegaolvirus by >90% and Semliki forest virus by ~75%. Vaccinia virus was less sensitive to BHT and polio virus was not affected by treatment with the antioxidant.

Low concentrations of BHT (11 to 22 μg/ml) markedly reduced the infectivity of Newcastle disease virus in embryonated chicken eggs. When immature chickens were fed 2000 ppm BHT and exposed to terminal dilutions of the virus, seroconversion percentages of the BHT-treated groups were inversely related to dietary concentrations of BHT. Survival of BHT-treated chickens was proportional to BHT concentration (Brugh 1977).

Sixteen patients with recurrent herpes simplex labialis were treated topically with 15% (*w/v*) BHT in mineral oil and 14 received the vehicle alone. The time from lesion onset to dry crust formation and the duration of the vesicle-ulcer stages were decreased among BHT-treated subjects compared to controls. Virus excretion from lesions appeared to be decreased, but this result was not considered significant. Clinical and laboratory evidence of toxicity of BHT was not observed (Freeman, Wenerstrom, and Spruance 1985).

The antiviral activity of BHT against herpes simplex virus type 1 (HSV-1) was studied in 42 hairless mice. Mice were infected with the virus by making two parallel scratches on the right midthoracic area with a 27-gauge hypodermic needle. The three groups consisted of 14 mice each, included a control group, a group receiving only mineral oil, and a group receiving BHT (2 mg in a 5% solution) in mineral oil. BHT was applied to the infected sites of mice with a dropper two times per day for 3 days starting 24 hours after infection with HSV-1. BHT-treated groups had significantly fewer lesions than control groups 7 to 10 days after infection (Keith 1982).

In a second experiment, mice once infected with HSV-1 were cleared and then reinfected in the same manner except on the left side. Mice were γ-irradiated for 4 minutes in a ^{60}Co γ source at a dose rate of 156 rad/min and reinfected 40 days after primary infection. Treatments with the same dose of BHT cleared mice of lesions much earlier than untreated controls.

In a third experiment, mice were also injected with 50 μl of human serum γ-globulin prior to infection with HSV-1. Treatment with 15% BHT in mineral oil applied with a cotton-tipped applicator twice daily for 5 days resulted in fewer lesions than controls treated with mineral oil only. The treatment procedure changed from the drop procedure to the cotton-tipped applicator because the former caused erythema and some skin sloughing, the latter eliminated this problem.

BHT and BHA were both effective at concentrations of 200 to 400 μg/ml at inhibiting *C. botulinum* growth. BHT appeared to be slightly more effective than BHA at inhibiting *C. botulinum* growth and toxin production. BHT retarded growth and toxin production of *C. botulinum* for up to 2 days at a concentration of 50 μg/g (Reedy, Pierson, and Lechowich 1982).

Robach and Pierson (1979), however, determined that BHA was more successful than BHT at inhibiting growth and toxin formation of various strains of *C. botulinum*. The addition of 50 ppm of BHA was inhibitory to the spores of strain 10755A for 21 days. BHA was completely inhibitory at 25 ppm to strains 62A and 213B for 7 days. When the spores of *C. botulinum* of strain 62A were exposed to 25 ppm of BHT, growth and toxin formation were delayed for 3 days, whereas 100 ppm BHT inhibited spores for 7 days. The addition of 25 and 50 ppm BHT inhibited spores for 3 days in strain 213B, and 100 and 200 ppm inhibited spores in the same strain for 7 days. Similar results were observed for strain 10755A.

Miscellaneous Effects

Alkylphenols such as BHT formed reactive QM intermediates when oxidized by cellular enzymes (Reed and Thompson 1997). QMs also reacted with other biological nucleophilic compounds, including alcohol, water, proteins, and nucleic acids. The reactivity of QMs was influenced strongly by pH, and could be affected by the presence of electron-donating or -withdrawing components on the ring. For example, *ortho*-alkyl groups stabilized *para*-QMs and lessened their reactivity. In general, *ortho*-QMs were more reactive than *para*-QMs. Nucleophilic addition to a QM typically eliminated reformation of the compound (Thompson et al. 1992).

When BHT was oxidized by cytochrome P450, the QM intermediate reacted with nucleophilic protein residues to covalently bind the proteins. Such binding of BHT to protein was metabolism dependent and occurred in tissues of both rats and mice. During an immunochemical visualization assay, detection of the adducts was inhibited by cytochrome P450 inhibitors, deuterated BHT, and the omission of NADPH. When rat liver microsomes were treated with synthetic BHT-QM, similar protein alkylation patterns were observed as in enzyme-mediated incubations. When Sprague-Dawley rats were treated with up to 1000 mg/kg BHT by gavage, protein alkylation peaked at 24 hours after dosing and was dose dependent. The proteins were isolated and identified as a mitochondrial β-oxidation enzyme (enoyl–coenzyme A [CoA] hydratase) and a plasma membrane/cytoskeletal linker protein (Reed and Thompson 1997).

Nakagawa, Hiraga, and Suga (1981a) reported that the addition of thiol compounds such as cysteine or GSH to the incubation mixture inhibited the binding of [^{14}C]-BHT to rat liver microsomes. The metabolites BHT-QM and BHT-OH were "trapped" during thioether binding between the 4-methyl groups of both metabolites and the sulfhydryl group of the thiol compounds, and the water-soluble conjugates formed were then excreted in the urine. In an earlier study (Nakagawa, Hiraga, and Suga 1980a), oral pretreatment of male Wistar rats with 50 or 500 mg/kg/day nonradioactive BHT (in olive oil) for 3 days decreased the in vitro binding of [^{14}C]-BHT to lung microsomal macromolecules after the animals were killed. This effect was accompanied by a decrease in the activity of cytochrome P450 and BHT oxidase. In contrast, IP pretreatment with 80 mg/kg/day phenobarbital (in saline) for 5 days did not alter binding of [^{14}C]-BHT, P450 content, or enzymatic activity.

In another study by Nakagawa, Hiraga, and Suga (1980b), radioactivity from [^{14}C]-BHT (50 mg fed to male Wistar rats; 50 μCi/kg) became bound to DNA, RNA, and protein in the liver. Total radioactivity in the protein fraction peaked within 6 hours and decreased gradually afterwards. Total radioactivity in the nucleic acid fraction increased with time and remained constant for 1 week. The specific radioactivity in RNA increased with time, and was ~18 or 35 times that in DNA or protein after the first week. In addition, pretreatment with phenobarbital increased radioactivity in RNA, DNA, and protein by 170%, 153%, and 154%, respectively. After ion-exchange chromatography, Nakagawa, Hiraga, and Suga (1981b) reported that the radioactivity in hepatic RNA had an "especially high affinity" for AMP and GMP; 41% of the radioactivity recovered was in the AMP fraction and 58.1% was in the GMP fraction (total recovery = 96% of the dose). Phenobarbital treatment produced values of 44.3% in AMP and 55.1% in GMP. The investigators concluded that radioactivity was, therefore, not bound to a moiety of the nucleotides.

Microsomes of the brain, kidneys, and spleen had no BHT-binding capacity, but lung microsomes had binding capacity ~40% of hepatic microsomes (Nakagawa, Hiraga, and Suga 1979).

In cortical slice homogenates and synaptosomes, BHT inhibited malondialdehyde (MDA), a product of lipid peroxidation, by 56% and 70%, respectively (Chan, Yurko, and Fishman 1982).

Male weanling Sprague-Dawley rats were fed low-fat, high–saturated fat, or high–polyunsaturated fat diets with or without 0.3% BHT or 0.05% 2-acetylaminofluorene (2-AAF) for 2 weeks. Liver microsomes were prepared from the treated rats.

Both compounds consistently produced significant decreases in NADPH–cytochrome P450 activity, regardless of the amount and type of dietary fat. When the compounds (in methyl alcohol) were added at concentrations of 1.0 and 1.1 mM to hepatic microsomes from untreated rats, reductase activity was not inhibited (Rikans et al. 1981).

In a later study, BHT-treated rats had increased nuclear envelope cytochrome P450 (Carubelli, Graham, and McCay 1992). Male F344 rats were fed a high–polyunsaturated fat diet for 2 weeks prior to initiation with 100 mg/ml diethylnitrosamine (DEN) in 0.9% NaCl, IP, at a dose of 200 mg/kg/body weight. Two weeks later, the rats were fed 0.02% 2-AAF for the 2-week promotion period. BHT was added to the basal diet at a concentration of 0.3% during the entire 8-week study or during the initiation or promotion period only. The P450 content was greater in rats treated during promotion than in those treated during initiation, and the greatest effect was observed in rats treated with BHT throughout the study period.

Dietary BHT (0.2%) increased the concentrations of serum thiobarbituric acid–reactive substances, high-density lipoprotein (HDL) cholesterol, and hepatic phospholipid after feeding to male Wistar rats for 30 days. Incorporation of BHT increased hepatic microsomal lipid peroxidation (Yamamoto et al. 1995). In another study, the addition of 0.5% BHT to the feed of female CD-1 mice enhanced epoxide hydratase activity, glucuronide conjugation, and glutathione oxidation and reduction conjugation in hepatic microsomes (Cha and Heine 1982). Concentrations of 0.1% to 0.5% BHT in feed increased activity of pulmonary and hepatic epoxide hydratase, an enzyme involved in drug metabolism, in male Sprague-Dawley rats (Kahl and Wulff 1979; Kahl 1980). No increases of cytochrome P450 content or aryl hydrocarbon hydroxylase activity were observed. In a third study (Yang, Strickhart, and Woo 1974), BHT inhibited the mixed function oxygenase system in hepatic microsomes, possibly via binding to cytochrome P450.

BHT at a dietary concentration of 1.20% increased the content of phosphatidylethanolamine or decreased phosphatidylinositol and lysophophatidylcholine in the livers of male Sprague-Dawley rats fed the antioxidant for 1 week. BHT also increased the content of cholesteryl esters and phospholipids, but decreased the amount of triglycerides, nonesterified fatty acids, and diglycerides. The investigators suggested that BHT decreased the activity of fatty acid desaturase in the liver of rats fed the antioxidant (Takahashi and Hiraga 1981a).

Malkinson (1979a) reported that male C3H/Umc and Swiss-Webster mice treated IP with 400 mg/kg BHT had increased phosphorylation of lung proteins that correlated with the time of transient lung enlargement induced by BHT. BHT slightly increased cyclic AMP–dependent protein kinase activity, but did not affect cyclic AMP–binding activity. In another study, however, weekly IP injections of 400 mg/kg BHT for 5 weeks decreased p36 phosphorylation due to a BHT-induced decrease in protein kinase C activity in lung and splenic tissues (Malkinson et al. 1985).

Oral administration of BHT (500 mg/kg) and BHT-OH daily for 3 days increased the total GSH concentration in the rat liver, as well as the activities of GSH *S*-transferase and other enzymes (Nakagawa, Hiraga, and Suga 1981c).

BHT (1000 nM) significantly increased percentage cell viability of cholestantriol-treated newborn rat kidney cells above that of cells treated with >10 μM cholestantriol alone, but viability of BHT/cholestantriol-treated cells was decreased compared to controls. Cells treated with 750 or 1000 nM BHT had reductions in the extent of lipid peroxidation ($p < .05$), and BHT-treated cells had normal superoxide dismutase and catalase activities. The investigators concluded that BHT and other antioxidants could be beneficial in the prevention of in vitro toxicity of products of cholesterol oxidation (Wilson, Sisk, and O'Brien 1997).

When added to feed containing 1% cholesterol, 1% BHT protected rabbits from atherosclerosis (Björkhem et al. 1991). The mean atherosclerotic involvement was 18.6% ± 4.4% for animals fed the cholesterol diet alone and 5.9% ± 1.7% for rabbits fed both cholesterol and BHT. The addition of BHT also gave greater concentrations of total cholesterol, triglycerides, low-density lipoprotein, and very-low-density lipoprotein in plasma. In another study, BHT prevented early cholesterol-induced microcirculatory changes in rabbits, but did not alter the concentrations of blood lipids. Blood flow velocity decreased and arteriolar diameter increased, although the latter parameter did not differ from non–cholesterol-treated controls (Xiu et al. 1994).

Kuzuya et al. (1991) reported that antioxidants, including BHT, increased proliferation of endothelial cells in vitro, possibly via the prevention of lipid peroxidation. In a study by Vainio (1974) using rat liver microsomes, 0.2 mmol/l BHT inhibited enzymatically induced lipid peroxidation in vitro, therefore inhibiting the decrease of monoxygenase activity and increase of UDP-glucuronosyltransferase activity normally caused by peroxidation. At a concentration of 10 mmol/l, BHT inhibited peroxidation, but induced membrane-bound UDP-glucuronosyltransferase activity threefold. The latter change also occurred at a concentration of 1 mmol/l.

Male Sprague-Dawley rats fed 0.25% BHT had increased biliary excretion of IV injected compounds such as sulfobromophthalein and ouabain. The increases appeared to be correlated with the BHT-induced bile flow, which was due to increased canalicular bile production. BHT-treated rats had decreased biliary bile acid concentration and total biliary excretion of bile acids compared to control rats. The results suggested that the increased bile flow was due to the osmotic choleresis related to the secretion of BHT and its metabolites into bile (Choe, Kim, and Yang 1984).

BHT had a protective effect against hexachlorophene (HCP) toxicity in rats. Pretreatment for 3 days with 400 mg/kg BHT orally decreased mortality to 14% from the 100% in the group that received HCP only. A significant decrease also occurred in the number of animals with signs of toxicity. Treatment with BHT at 400 mg/kg did not cause any noticeable toxicity;

however, pretreatment with 600 mg/kg BHT did produce toxicity and mortality, although the protective effect against HCP still occurred. The cerebrospinal fluid pressure also decreased to control values in animals pretreated with BHT. BHT treated animals also had increased liver weights. When BHT was administered after HCP, mortality was not reduced (Hanig, Yoder, and Krop 1984).

Both BHT and BHA accelerated retinoic acid–induced myelocytic differentiation and dihydroxyvitamin D_3–induced monocytic differentiation in human HL-60 myeloblastic leukemia cells, whereas other antioxidants and eicosanoid synthesis inhibitors did not modify cell differentiation (Burns and Petersen 1990).

At a concentration of 1 mM, BHT significantly inhibited cellular respiration in renal explants from Osborne-Mendel rats (Braunberg, Gantt, and Friedman 1982). Cultures incubated with 0.10 to 0.50 mM BHT had increased respiration, compared to controls. Respiration was measured by determining the amount of $^{14}CO_2$ evolved during [^{14}C]-glucose metabolism. The changes were not attributed to the antioxidant properties of BHT, as other antioxidants (e.g., ascorbic acid and tocopherol) did not inhibit respiration, but the structurally similar compound BHA did.

When combined with ethanol, BHT potentiated the depletion of hepatic vitamin A in male Sprague-Dawley rats, such that the vitamin content was <5% of the control value. Retinol and retinyl ester content were decreased similarly in the liver. In the lungs, however, the concentrations of retinol and retinyl esters did not differ from controls after treatment with BHT (Leo, Lowe, and Lieber 1987).

BHT did not stimulate mitogenesis in spleen cells of CBA/J mice fed corn oil diets with or without tocopherol, which was a mitogen itself (Corwin and Shloss 1980).

In male hamsters, vitamin A–induced pigment cholelithiasis was prevented by treatment with BHT (Cardenas, Estañol, and Galicia 1996).

BHT given orally at 600 mg/kg at least 48 hours before carbon tetrachloride (CCl_4) significantly increased survival compared to controls. Three daily oral doses of 400 mg/kg BHT increased survival as effectively as the same dose given once 72 hours before exposure to CCl_4. A single oral dose of 600 mg/kg BHT given 6 or 24 hours prior to CCl_4 had no effect on survival. Intraperitoneal dosing at 1, 24, or 48 hours was ineffective in preventing acute CCl_4 toxicity (Cawthorne et al. 1970).

Male Wistar rats pretreated with 60 mg/kg cobaltous chloride, an inhibitor of cytochrome P450, by subcutaneous injection for 2 days received 1000 mg/kg BHT 12 hours later. The group that received BHT alone had significantly decreased concentrations of GSH. However, in the group that received BHT and cobaltous chloride, the GSH concentration was depressed less than that of the BHT-treated rats. The elevated GOT and GPT activities in serum induced by BHT were decreased when rats were pretreated with cobaltous chloride. It may also be concluded that hepatic change, present in BHT-treated rats in the form of hepatic congestion, was prevented by cobaltous chloride (Nakagawa et al. 1984b, 1985).

Oral doses of BHT (10 and 100 mg/kg) protected the gastric mucosa against the damaging effects of indomethacin (6 to 20 mg/kg) in a dose-dependent manner (Krupińska et al. 1980).

When BHT was added to the incubation medium of enzymatically dispersed corpora lutea from pseudopregnant Wistar rats, the antioxidant increased progesterone secretion in a dose-dependent fashion, but did not increase the concentration of intracellular cyclic AMP (Carlson et al. 1995).

Rao et al. (1994) reported that BHT was a poor inhibitor of arachidonic acid–mediated platelet activation although, at concentrations of 500 and 1000 μM, it inhibited arachidonic acid metabolism to thromboxane B_2 by 62.5% ± 8.3% and 73.1% ± 7.0%, respectively, compared to control platelets.

During in vitro human platelet aggregation tests, BHT, at concentrations of 2×10^{-4} M, inhibited platelet aggregation induced by arachidonic acid sodium salt by 100% and by 42% when aggregation was induced by collagen. BHT inhibited the arachidonic acid cascade 50% after 2 minutes of incubation at concentrations of 9×10^{-3} M (Agradi et al. 1981).

Panganamala et al. (1977) reported that BHT, at concentrations of 4×10^{-5} and 4×10^{-4} M did not inhibit prostaglandin biosynthesis in bovine vesicular glands. At concentrations of 9×10^{-3} M, BHT inhibited arachidonic acid and ADP-induced platelet aggregation 95% and 60%, respectively, in blood samples taken from human volunteers. BHT effectively inhibited soybean lipoxidase 58%, 73%, and 90% at concentrations of 1×10^{-8}, 1×10^{-7}, and 6×10^{-7} M, respectively.

BHT, at a concentration of 500 μM, inhibited by 83% the arachidonate-dependent benzo[a]pyrene (BP) oxidation in enzyme preparations from sheep seminal vesicles (Marnett, Reed, and Johnson 1977).

BHT had only minor effects on nuclear size and DNA content distribution in liver biopsies of male Sprague-Dawley rats given the antioxidant by gavage at a dose of 250 mg/kg/day in 0.5% methyl cellulose for 112 days (Romagna and Zbinden 1981).

BHT and BHA at concentrations of 10 mM decreased the amount of free radicals that could be trapped in male *lac a* mouse keratinocytes and reduced radical-adduct formation (Timmins and Davies 1993).

Lippman (1980) studied the antioxidative effects of BHT and BHA in vitro in rat liver mitochondria isolated from female Sprague-Dawley rats. BHT (1 μmole) was incubated with DL-carnitinyl maleate isoluminol (CML), a reagent known to emit light in the presence of free radicals, in order to determine the chemiluminescence (CL) values of BHT and BHA. The CL values of BHT and BHA indicated they were strong antioxidants. In vitro CL experiments indicated that these antioxidants were most effective from 0.5 to 1.5 hours after incubation with liver mitochondria. The antioxidants were oxidized sufficiently to be considered prooxidants 3.2 to 6.3 hours after incubation. Highly concentrated mixtures of ascorbic acid incubated with the antioxidants >3 hours added to the prooxidizing

effect. The rapid injection of 50 μmoles of DMSO demonstrated that BHT and BHA had some protective strength (log of relative intensity = 1.73 ± 0.08). The addition of ascorbic acid (vitamin C) increased this value.

ANIMAL TOXICOLOGY

Acute Toxicity

The IP LD_{50} for rats of BHT was 8.00 g/kg and no clinical signs or pathologic changes of the organs were observed; BHT was classified as nontoxic (Mallette and Von Haam 1952). Hasegawa et al. (1989) determined that the oral LD_{50} for BHT in ddY male mice (10 mice/group) was 650 mg/kg body weight. Signs of toxicity included tremor and staggering gait. Lung congestion was the only tissue change reported. The LD_{50} for BHT in rats was 2.36 and 0.81 g/kg after single and multiple oral doses, respectively (Stanford Research Institute 1972).

Swiss-Webster mice were treated with 400 mg/kg BHT via IP injection and some were killed days 1, 3, or 5 after injection. Either 24 or 48 hours before the mice were killed, 4 mg/kg diquat (an herbicide) was injected IP as a "challenge to the ongoing cellular events in the pulmonary alveoli." Diquat enhanced epithelial and endothelial damage if administered before the onset of BHT-induced damage. If diquat was administered 1 or 3 days after BHT injection, interstitial and inflammatory reactions were decreased; these reactions were increased if diquat was instead administered on day 4 (Coulombe, Lassonde, and Côté 1987).

Daugherty and Khurana (1985) induced skin necrosis in male Swiss mice via intradermal injections of 0.5 mg (1 mg/ml 0.9% NaCl) doxorubicin. The area of skin ulceration was decreased by 57% and 84%, respectively, after topical and intradermal treatment with 1 to 16 mg/mouse BHT, immediately after injection of doxorubicin.

Male Wistar rats (9–10/group) fed 0.5% BHT for 1 day had increased liver weight that was observed 2 days after treatment and persisted for 3 days. Microsomal biphenyl 4-hydroxylase activity was not affected by treatment with the antioxidant (Creaven, Davies, and Williams 1966).

When male Wistar rats were given one or two doses of 800 mg/kg BHT (route of administration not specified), 24 hours apart, the liver weights increased 20% within the first 24 hours and up to 33% by 48 hours. Hepatic cell proliferation was increased such that the mitotic indices were increased to 2.4%. After a single dose of BHT, disorganization of the cytoplasm of the centrilobular hepatocytes was observed. Cells around central veins had the first apparent degenerative lesions. These lesions were vacuolated or necrotic hepatocytes, accompanied by an abundant macrophage infiltrate. In severely affected rats, the hepatocyte of the entire centrilobular and midzonal regions were necrotic (Powell and Grasso 1988).

Single oral doses of 500 and 1000 mg/kg BHT in corn oil were given to Fischer 344 rats (Nakagawa and Tayama 1988). In males, the high dose caused renal and hepatic damage, decreased the accumulation of *p*-aminohippuric acid in renal slices, and proteinuria and enzymuria. When the rats were previously dosed IP with 80 mg/kg phenobarbital (in saline) for 4 days, rats of the high dose had slight renal tubular necrosis. The changes were dose dependent to a maximum at 24 hours after feeding of BHT; the changes had reversed by 48 hours. Female rats, however, were more resistant to the effects of BHT than males. Kidney slices from females did not accumulate as much *p*-aminohippuric acid, and females had fewer signs of nephro- and hepatotoxicity.

Male Sprague-Dawley rats were given a single, oral dose of 800 mg/kg BHT and some were killed 0.5, 3, 6, 12, 24, 48, 60, and 72 hours after dosing. The concentrations of blood coagulation factors and the hepatic concentrations of BHT and BHT quinone methide were determined. Body weight gain was reduced 24 to 72 hours after BHT administration, but relative liver weights were significantly larger than controls 36 to 72 hours later. No necrotic changes were observed. Coagulation factors II, VII, IX, and X were decreased 36 hours after BHT administration and all reached a minimum 48 to 60 hours after BHT administration. Hepatic concentrations of BHT peaked at 3 hours, whereas BHT quinone methide peaked at 24 hours (Takahashi 1987).

In separate experiments, Takahashi (1987) also administered phylloquinone, phenobarbital, dietary cysteine, cobaltous chloride, and SKF 525A with 800 mg/kg BHT to investigate the effects on plasma coagulation factors. Phylloquinone (1 mg/rat) injected 24 hours after 800 mg/kg BHT prevented the decrease in concentrations of factors VII and X, partially prevented decreases in factor IX, and did not significantly alter concentrations of factor II. Pretreatment with phenobarbital (75 mg/kg for 3 days) and cysteine (1% daily for 3 days) did not prevent the BHT-dependent decrease in vitamin K–dependent factors. Pretreatment with cobaltous chloride (60 mg daily for 2 days) prevented decreases in factors II and VII and partially prevented decreases in factors IX and X. Administration of SKF 525A 30 minutes before or 12 hours after BHT administration partially prevented the decrease in factors II, VII, and X, or all four factors, respectively. Liver enlargement was completely inhibited by the administration of SKF 525A 12 hours after BHT.

Mizutani et al. (1987) treated male ddY mice with 200 to 800 mg/kg (oral) BHT in combination with 4 mmol/kg buthionine sulfoximine (BSO; IP), a chemical that inhibits GSH synthesis. The mice had increased GPT activity and centrilobular necrosis of the hepatocytes. The high dose of BHT alone did not produce hepatotoxicity. A number of metabolism inhibitors such as carbon disulfide prevented the toxic effect, whereas compounds such as cedar wood oil and phenobarbital increased hepatoxicity.

Short-Term Toxicity

BHT and BHA at doses of 50 to 500 mg/kg were administered orally daily for 1 week to male and female SPF Carworth rats. Both antioxidants caused an increase in liver weight at doses of 100, 200, and 500 mg/kg in males and at 200 and 500 mg/kg in

females. BHA did not effect glucose-6-phosphatase or glucose-6-phosphate dehydrogenase activity in the liver. BHT decreased the glucose-6-phosphatase activity in females at doses of 100, 200, and 500 mg/kg and increased glucose-6-phosphate dehydrogenase activity in both sexes at a dose of 500 mg/kg. A significant decrease also occured in males at 200 mg/kg BHT. No significant hepatic fatty change was produced by BHA and any fatty change that occurred with BHT were not considered significant. In either sex, no significant changes in enzyme activity occurred after a 28-day rest period, liver weights returned to normal by day 14 and fatty change was normal by day 28 (Feuer et al. 1965).

Male Sprague-Dawley rats were fed a basal diet or a diet containing 1.2% BHT for 1 week. The platelet-particle concentrations, platelet volume, mean platelet volume, and platelet phospholipids, cholesterol, and cholesteryl esters did not differ significantly between control rats and BHT-treated rats. Platelet distribution was slightly decreased, the number of erythrocytes was increased, and the average corpuscular volume was significantly decreased in BHT-treated animals. BHT also significantly decreased epinephrine-induced platelet aggregation in platelet-rich plasma (PRP) and washed platelets. BHT-treated rats had decreased platelet aggregation microscopically when induced in PRP by ADP, arachidonic acid, or fibrinogen. BHT significantly increased linoleic acid and eicosatrienoic acid and decreased arachidonic acid in total platelet lipid. Plasma triglycerides, diglycerides, and lipid hydroperoxides were significantly decreased. A significant decrease in plasma zinc concentration was also observed in BHT-treated animals (Takahashi and Hiraga 1984).

Male Wistar rats (9–10/group) fed 0.01% to 0.5% BHT for 12 days had increased liver weight, but body weight was unaffected. The activity of microsomal biphenyl 4-hydroxylase was increased for all doses except the lowest dose. If the rats were fed high-fat diets with or without 0.01% BHT for 12 days, liver weight was not affected, but increased activity of the enzyme was induced (Creaven, Davies, and Williams 1966).

BHT did not induce hyperplasia of the nonglandular stomach epithelium after the antioxidant was added to the feed of five male F344 rats at a concentration of 0.7% for 4 weeks (Hirose et al. 1987a).

Powell et al. (1986) dosed male Wistar rats with 25 to 500 mg/kg/day BHT IP for up to 28 days or with 1000 to 1250 mg/kg/day for up to 4 days. Rats given 500 mg/kg/day had progressive periportal hepatocyte necrosis accompanied by proliferation of the biliary ducts, alterations of drug metabolism enzyme activities, and persistant fibrous and inflammatory cell reactions. After treatment for 7 or 28 days, the rats treated with 25 to 500 mg/kg/day BHT had dose-related increase in size of liver. Rats treated with 1000 to 1250 mg/kg/day for 4 days had centrilobular necrosis within 48 hours of treatment.

At a dose of 500 mg/kg/day for 14 days, BHT (10% in arachis oil; dose volume = 5 ml/kg) caused only slight, if any, growth reduction when administered by gavage to weanling, female SPF rats (Gaunt, Gilbert, and Martin 1965a). The difference between the treated group and controls was apparent during the 14-day recovery phase, but was not statistically significant ($p > .05$). BHT-treated rats fed a restricted diet had decreased feed consumption during the first week of treatment and when restored to full diet. Liver enlargement was observed, but absolute liver weights were decreased during the treatment phase when the rats were maintained on the restricted diet. At the end of the recovery period, hepatic parameters were similar to controls. BHT had no effect on the weight or ascorbic acid content of the adrenal glands, even when the rats were deprived of feed. BHT stimulated the activities of liver processing enzymes hexobarbitone oxidase, nitroanisole demethylase, and aminopyrine demethylase during the treatment phase. The activity of hepatic glucose-6-phosphatase was severely reduced after the treatment period, but returned to the control activity after the recovery period. The hepatic concentrations of phospholipid, free fatty acid, and cholesterol were unaffected by treatment with BHT, with or without feed deprivation. Serum concentrations of cholesterol and phospholipid were increased by exposure to BHT, but returned to normal during recovery.

In two studies, BHT induced specific isozymes of drug-metabolizing enzymes that could modify the toxicity of other compounds. Sun et al. (1996) administered BHT in the feed of male ddY mice and Chinese hamsters at concentrations of 0.05% and 0.15% for 14 days. In mice, the low dose increased the concentration of cytochrome P450 and increased the activities of UDP-glucuronosyltransferase and pentoxyresorufin-*O*-dealkylase. The high dose increased the activities of testosterone 6α-, 16α-, and 16β-hydroxylases. In hamsters, both doses enhanced the activities of ethoxycoumarin-*O*-deethylase and glutathione *S*-transferase and the 0.15% BHT increased the activity of testosterone 15α-hydroxylase. Yang et al. (1995) reported that BHT and BHA, 0.5% and 0.75%, respectively, induced hepatic UDP-glucuronosyltransferases when added to the feed of male Wistar rats for 2 weeks; glucuronidation was a "critical pathway for the detoxification of a wide variety of endogenous and exogenous compounds including carcinogens such as aromatic amines and polycyclic aromatic hydrocarbons."

BHT was fed (0.8 to 2.8 mg/cal) for 24 days to 62 rats, hepatic weight was increased after day 1 that remained constant after day 4. The livers were intact and there was no evidence of cell necrosis. The average increase in hepatocyte size was 30% and the nuclei were enlarged and cytoplasm of the cells was increased. During days 1 to 4, BHT caused an increase in [H^3]-thymidine incorporation into DNA with a concomitant rise in mitotic activity; however, these parameters returned to normal thereafter. A single intragastric dose of BHT (1.4 g/kg) increased hepatic weight 40% to 50% after 1 to 2 days (Kerr et al. 1966).

When 1% BHT was fed to female WIST (SPF) rats for 35 days in a diet containing either 20% sodium caseinate or 24% lactalbumin as the protein source, BHT induced nephropathy; however, only rats fed the former protein source had nephrocalcinosis (Meyer, Kristiansen, and Würtzen 1989).

Investigators estimated the maximum tolerated dose (MTD) of BHT by feeding the antioxidant for 7 weeks to F344 rats and B6C3F$_1$ mice at doses of 6200 to 50,000 and 3100 to 50,000 ppm, respectively. Each treatment and control group was comprised of five animals per sex. Control mice were given basal feed only. For males, one rat fed 12,500 ppm BHT, all five rats of the high-dose group, and one mouse of the high-dose group died during the treatment period. For females, all rats of the high-dose group, one mouse of the 25,000-ppm group, and four mice of the high-dose group died during the treatment period. Dose-related decreases in body weight were observed in both female and male animals. Rats of both sexes of the 12,500-ppm dose group had a slight increase in hematopoiesis. Male mice fed 25,000 ppm had slight centrilobular cytoplasmic vacuolation of hepatocytes. The MTD was estimated as 6000 ppm for both rats and mice (NTP 1979).

Nera et al. (1984) added BHT to the feed of male F344 rats for 9 days at concentrations of 0.5% to 2.0%. At the low dose, BHT increased the [^{3}H]-methylthymidine labeling index of the fundic region of the nonglandular stomach, and at the high dose, the index was decreased compared to controls. Slight increases occurred in the 1% to 1.5% treatment groups; the ratio between the test and control groups was 0.74 to 2.03. Microscopically, two rats treated with 2% BHT had thin squamous epithelium of the entire nonglandular stomach. In affected animals, the epithelium was two to three cell layers thick, compared to the normal five or six in controls. The stomachs of the other treated rats appeared normal.

The effects of BHT on plasma and hepatic components were investigated by Saheb and Saheb (1977). For 7 weeks, 96 weanling Sprague-Dawley rats (6/sex/group) were fed a diet supplemented with 20% lard and 0.01%, 0.1%, and 0.5% BHT (dry weight). The low dose had no significant deleterious effects. The middle dose increased weight gain in male rats. The high dose significantly decreased body weight gain of both sexes; however, at this concentration, weight gain was decreased by 31% in males and by 24% in females, compared to controls. Rats of the high-dose group had increased relative liver weights and enlarged livers. The hepatic concentrations of total lipids and total and esterified cholesterol were increased, as were the plasma concentrations of total and esterified cholesterol and phospholipids. High-dose rats had alopecia of the top of the head that sometimes extended to the flanks.

BHT was fed to male and female Norway Hooded rats at doses of 0.1%, 0.2%, 0.3%, 0.4%, and 0.5% for 6 weeks. Rats within each group were littermates. BHT reduced the weight of male and female rats, but the weight reduction was greater in males. Liver weight–body weight ratios were increased in both sexes, as well as the absolute weight of the liver. The weight of the left adrenal gland of male rats relative to the body weight was increased in male rats, but no consistent change occurred in female rats. The serum cholesterol was increased directly proportional to the dietary dose of BHT. The absolute amounts of cholesterol and total lipid per rat liver were increased as a result of the increased weight of the liver caused by BHT (Johnson and Hewgill 1961).

Briggs et al. (1989) fed BHT to male Wistar rats at concentrations of 0.1% to 1.0% for 30 days. Three of eight rats fed 0.75% BHT died. Concentrations $\leq$0.75% did not induce cellular proliferation, as determined using the [^{3}H]-thymidine labeling and mitotic indices. In the liver, the [^{3}H]-thymidine labeling index decreased as the dose increased, but the mitotic index was not affected. No significant changes occurred in the labeling index of the urothelium of the urinary bladder or follicular cells of the thyroid gland. The investigators reported dose-dependent increases of liver size. In a second study, 8 rats/group were fed 0.5% BHT for 2, 4, 8, 10, or 14 days before being killed. The labeling index increased in a time-limited manner, with the largest increases at 2 days. The index decreased from 2 to 8 days and was constant for the remainder of the study. The mitotic index peaked similarly at 2 days, then decreased to below controls. Hypertrophy of hepatic cells, but no other lesions, were observed at microscopic examination.

When male Sprague-Dawley rats were fed various antioxidants and substituted phenols for 3 weeks, the investigators concluded that the hemorrhagic effect of BHT was related to its chemical structure, not its antioxidant properties. The dose was 5.45 mmol/100 g diet (the LD_{50} of 99% pure BHT) for each test compound, and each group consisted of 5 to 10 rats. Three of the five antioxidants, including BHA, did not induce bleeding, and only one (ethoxyquin) induced hemorrhages similar to that caused by BHT. 2,6-di-*tert*-Butylphenol and BHT-OH induced hemorrhages, but BHT-COOH, BHT-aldehyde, and 2,4,6-trimethylphenol did not (Takahashi and Hiraga 1978a).

The oral LD_{50} of BHT was 760 mg/kg/day during a 40-day feeding study using male Sprague-Dawley rats (Takahashi and Hiraga 1978b). The rats were fed a 24% casein diet supplemented with 0.58%, 0.69%, 0.83%, 1.00%, 1.20%, and 1.44% BHT. The mean intakes were 436, 526, 663, 713, 774, and 874 mg/kg/day BHT. Rats dosed with >526 mg/kg/day (0.69%) died. All dead rats had massive hemorrhages in the pleural and peritoneal cavities and some had external hemorrhages. Survivors of all treatment groups had decreased prothrombin indices and hemorrhages of the epididymis, testis, and pancreas. Within 3 to 4 days of the start of the study, all treated animals had mild diarrhea and red urine. Some rats of the low-dose group had a "reddish halo about the mouth," and rats of the high-dose group had rough hair coat after 4 days. Body weight, feed and water consumption, and splenic weight were decreased, and relative liver weight was increased by treatment; these effects were dose dependent.

In another study (Takahashi, Ichikawa, and Sasaki 1990), male Sprague-Dawley rats were treated IP with BHT in olive oil for 7 days. Dose-related decreases in the prothrombin and kaolin-activated partial thromboplastin time indices were reported; these values were ~10% that of controls after treatment with the high dose. In the low-dose group (1.14 mmol/kg/day), one hemorrhagic death occurred and two survivors had

hemorrhaging of the abdominal cavity and epididymis. In the middle-dose group (1.82 mmol/kg/day), no rats died, but five had hemorrhages into the abdominal cavity, epididymis, and cecum. In the high-dose group (2.91 mmol/kg/day), four hemorrhagic deaths occurred and two survivors had hemorrhages of the epididymis and blood in the abdominal cavity. The rats that died had hemorrhages into the abdominal cavity, epididymis, muscle, testis, cranial cavity, nasal cavity, and gastric blood pooling. The investigators also fed BHT to the rats at a concentration of 0.32% (mean intake = 1.49 mmol/kg/day) for 7 days. None of the rats died as a result of treatment, but six had hemorrhages of the epididymis.

Hypoprothrombinemia appeared to have a central role in BHT-induced hemorrhages (Takahashi and Hiraga 1978c). Within 1 week of feeding ≥0.017% BHT to male Sprague-Dawley rats, a significant decrease was observed in the prothrombin index. This change was still evident at week 4 in rats of the group treated with 0.50% BHT. The same investigators (Takahashi and Hiraga 1979b) also reported that phylloquinone oxide (0.68 μmol/kg/day, orally or 10 nmol, IV) prevented hypoprothrombinemia and hemorrhages induced by the feeding of 1.20% BHT. The data suggested that BHT-induced hemorrhagic death was due to the direct or indirect depletion of vitamin K, a conclusion that was also reported by Suzuki, Nakao, and Hiraga (1979) and Cottrell et al. (1994).

Male rats (30/group, 4 groups) were fed either 0.25% BHT diet or the basal diet for 2 weeks. A single injection of 20 μg/kg vitamin K was given to each of these groups 6 hours before the rats were killed. Two groups did not receive the vitamin K injection. Liver weights were increased in groups receiving only BHT and BHT plus vitamin K. The concentration of vitamin K present in the livers of rats fed BHT was not detected (detection limit = 0.03 μg). However, the vitamin was easily detected in the livers of control rats not fed BHT(Suzuki, Nakao, and Hiraga 1983).

In a separate study, vitamin K concentrations were determined in rat feces after treatment with 0.25% BHT in the diet for 2 weeks. Vitamin K at 0.25 and 0.5 μg/g was added to the diets containing BHT and the diets were fed to two groups. The vitamin K concentrations in the feces were greater in the group fed BHT compared to the group not fed BHT. These data suggested that BHT could inhibit the uptake of vitamin K by the liver and/or BHT inhibited intestinal absorption of vitamin K while increasing fecal excretion of vitamin K, causing decreased vitamin K concentrations in the liver.

Five to 10 male and female rats per group were fed BHT, BHA, and other antioxidants for 4 to 8 weeks by Altmann et al. (1985) to determine their effects on the induction of lesions of the nonglandular stomach. BHT (1%) did not induce visible lesions.

Takahashi and Hiraga (1980) performed a metabolism study using male Sprague-Dawley rats fed 1.00% BHT for 10 days (see "General Biology—Absorption and Distribution" and "General Biology—Metabolism and Excretion"). BHT induced hypoprothrombinemia and hemorrhages in 8 of 10 rats of the treatment group, and one rat died. None of the four rats of the control group had hemorrhages. The mean prothrombin index was 102% ± 8% for the control group and 17% ± 4% for the test group. The latter value was significantly different from the control value, $p < .001$. Rats fed BHT also had decreased body weight gain and aciduria (Takahashi and Hiraga 1980).

In a follow-up study, male Sprague-Dawley rats were fed 5.45 mmol/100 g diet BHT or 2,6-di-*tert*-butyl-4-hydroxymethylphenol in either a standard or purified diet for 1 week (Takahashi and Hiraga 1983). At the end of the treatment period, the livers were assayed for unconjugated BHT metabolites and blood clotting times were determined. BHT-QM was detected at concentrations of 6 to 9 μg/g liver in the livers of rats fed BHT, and the hepatic weights were increased. The prothrombin index was decreased to 23% and 70% that of the control value in rats fed BHT and the methylphenol metabolite, respectively, in purified diet. The prothrombin index of rats fed BHT and its metabolite in standard diet did not differ from controls. Hepatic concentrations of BHT in rats fed the metabolite varied according to the diet and the major metabolite in the liver was hydroxy-BHT. For 2,6-di-*tert*-butyl-4-hydroxymethylphenol–treated rats of this group fed the standard diet, the major metabolites were hydroxy-BHT and BHT. The parent molecule itself was not detected in the liver of any of the rats. In addition, rats treated IP with 140 mg 2,6-di-*tert*-butyl-4-hydroxymethylphenol had biliary excretion of BHT-QM and hydroxy-BHT. The investigators concluded that BHT-QM was the metabolite responsible for hemorrhages.

Takahashi (1986) fed male Sprague-Dawley rats 1.2% BHT for 1 to 7 days. Rats fed the antioxidant for 2 to 7 days had time-dependent decreases of blood coagulation factors II (prothrombin), VII, IX, and X. Rats fed for 4 to 7 days had hemorrhages of the epididymis. For these rats, the frequency of bleeding increased generally in a time-dependent manner. Thrombin-induced and calcium-required aggregation of washed platelets did not differ from controls throughout the study. The results were that the concentration of blood coagulation factors rapidly decreased after ingestion of BHT, which could cause the hemorrhagic disease observed in other feeding studies. In other studies (Takahashi 1991), $>10^{-3}$ M BHT and BHT-QM inhibited ADP- and collagen-induced platelet aggregation in heparinized platelet-rich plasma. JCl:SD rats fed 1.20% BHT for 7 days had decreased arachidonic acid (3.9 mM)-induced aggregation, whereas ADP-, collagen-, and arachidonic acid (0.5 to 2.0 mM)-induced aggregation were normal. It was concluded that BHT did not inhibit platelet aggregation when rats were fed the antioxidant, and the aggregation capacity in vitro could have been unimportant in BHT-induced hemorrhages.

BHT-QM was the active metabolite of BHT that induced hemorrhages in Sprague-Dawley rats after oral administration (Takahashi 1988a). BHT was administered at a concentration of 160 mg in 4 ml soybean oil. BHT-QM was administered at doses of 75 and 150 mg/kg, at a concentration of 150 mg/4 ml soybean

oil. Twenty-four or 48 hours after dosing with BHT-QM, the plasma concentrations of blood coagulation factors II (prothrombin), VII, IX, and X were decreased in a dose-dependent manner. A similar dose of BHT (160 mg/4 ml soybean oil) did not induce decreases in the coagulation factors. BHT-QM–treated rats also had hemorrhages of the epididymis and/or thymus. Male ICR mice were resistant to the hemorrhagic effect of BHT. BHT was administered at a dose of 300 mg/kg and BHT-QM was administered at doses of 150 and 300 mg/kg. Both were administered at concentrations of 300 mg/10 ml soybean oil. Twenty-four hours after treatment with BHT-QM (150 or 300 mg/kg), only the concentration of factor VII was decreased. After 48 hours, the concentrations of factors II and X were decreased. The investigators concluded that the anticoagulant effect of BHT was less than that of BHT-QM, and that the difference in toxicokinetics between species could explain the difference in resistance to occurence of hemorrhages. In a 1-week study using male Sprague-Dawley rats (Takahashi and Hiraga 1981b), 1.2% BHT in feed decreased concentrations of plasma coagulation factor concentrations, inhibited ADP-induced platelet aggregation, and increased significantly the leakage of blood into the epididymis.

Male Slc:ddY mice (5–10/group) were fed 1.35% to 5.00% BHT for up to 21 days and male Crj:Hartley guinea pigs (5–6/group) were fed the antioxidants at concentrations of 0.125% to 2.0% for up to 17 days (Takahashi 1992). Of the mice treated with 0.5%, 1.0%, and 2.0% BHT, one, one, and two died, respectively, due to lung hemorrhages. In the other mice and guinea pigs, hemorrhages did not occur. Prothrombin- and kaolin-activated partial thromboplastin time were decreased by up to 30% and 40%, respectively, in mice kept on wood-chip bedding, and 40% and 60% in mice maintained in wire-bottomed cages. The prothrombin index was decreased only in the group treated wtih 1.0% BHT. The investigator concluded that BHT-induced lung hemorrhages in mice was not caused by a severe reduction in the coagulation process as it was in rats. Concentrations of 1.35% to 5.00% BHT caused nephrosis. The dose causing nephrotoxicity in 50% of the test animals was 2.3 g/kg body weight after 1 month of treatment.

Thirty-six Sprague-Dawley rats were fed BHT in a liquid diet for 1 to 24 days. The rats were given 1.7 mg/calorie the first day, 2.2 mg/calorie the second day, and 2.8 mg/calorie for each day thereafter. A group of 18 rats were given a single dose of BHT (1.4 g/kg body weight) by stomach tube, then were fed a control liquid diet. Liver specimens were obtained for electron microscopic examination on days 1, 2, 3, 6, 7, and 24 for the rats fed multiple doses, and at 16, 24, and 48 hours for the rats given the single dose. Hepatocytes from all rats given BHT had proliferation of the smooth-surfaced ER. Proliferation was observed in 20% to 50% of cells of rats killed on days 1 to 2; 70% to 80% of cells of rats killed on days 3 to 7; and 50% in rats killed on day 24. For rats given the single dose, proliferation was observed in 20% to 50% of hepatocytes from rats killed at 24 or 48 hours, and 15% of cells from rats killed at 16 hours. Mitotic activity was also increased after treatment with BHT; two thirds of the liver samples from rats killed after 1 to 4 days of feeding had increased mitotic activity (Lane and Lieber 1967).

BHT-induced lipid and enzyme changes in the blood and liver were investigated using juvenile rhesus (*Macaca mulatta*) monkeys, each weighing approximately 2.5 kg. Monkeys of group 1 ($n = 2$) received no corn oil or antioxidants. Of the monkeys of group 2 ($n = 8$), two were given corn oil, three received 500 mg/kg/day BHA and corn oil, and three were treated with 500 mg/kg/day BHT plus corn oil. Two monkeys of group 3 ($n = 9$) were given corn oil, four received 50 mg/kg/day BHA, and three were treated with 50 mg/kg/day BHT. The monkeys were treated by intubation for 28 days. The antioxidant concentration was 25% (*w/w*), so monkeys of group 2 were given 2 ml/kg/day corn oil and monkeys of group 3 were given 0.2 ml/kg/day corn oil. Feed and water were available ad libitum. Blood samples were drawn prior to the start of the study and weekly from monkeys of group 2 and at the end of the study from monkeys of group 3. Liver biopsies were obtained from group 2 monkeys after 2 weeks of treatment, following a 24-hour fast. At the end of the 4-week treatment period, the monkeys were fasted for 24 hours and killed. Liver and blood samples were obtained and analyzed. BHT and BHA induced few significant alterations in lipid and enzyme concentrations. At the high dose, BHT decreased the total cholesterol concentration in plasma. At the low dose, BHT decreased the concentrations of hepatic and plasma cholesterol. After 2 weeks of treatment, monkeys given the high dose of BHT had a cholesterol:lipid-phosphorus ratio of 5.8, compared to 10.8 for the untreated controls and 11.9 for corn oil controls (Branen et al. 1973).

Rhesus monkeys were given 25% BHT (gastric intubation) in corn oil for 28 days at doses of 50 and 500 mg/kg/day. The high dose caused no notable clinical abnormalities, but infant monkeys treated with the high dose were less responsive than adults given similar doses. Liver hypertrophy, proliferation of the hepatic ER, and modified hepatic enzyme activity were observed. Many of the hepatic nuclei had nucleolar fragmentation and the presence of large, intranucleolar fibrils. The low dose caused less pronounced changes of the hepatocyte cytoplasm, and no nucleolar changes were observed (Allen and Engblom 1972).

Powell and Connolly (1991) administered 500 mg/kg/day BHT in corn oil orally to male Sprague-Dawley rats (10/group), with or without IP pretreatment with an inhibitor of GSH synthesis (800 mg/kg phenobarbitone or 900 mg/kg BSO). The controls were the vehicle alone (5 ml/kg) or with pretreatment with 0.9% saline. The rats were killed 36 hours after treatment with BHT. No signs of toxicity were observed except for a slight decrease in body weight gain in BHT- and BSO-treated rats. No gross lesions were observed at necropsy and no significant microscopic lesions were observed in the kidneys or lungs. No acute hemorrhagic effect was noted after treatment with BHT. Also, the activities of lactate dehydrogenase, aspartate aminotransferase, and alanine aminotransferase were increased

3- to 10-fold. BHT alone did not cause hepatic necrosis or alter the cytochrome P450 activity, but it did increase ethoxycoumarin-*O*-deethylation, which implied an alteration in the ratio of P450 isoenzymes. BHT/phenobarbitone did not change the P450 activity, but did alter the isoenzyme ratio. Pretreatment with BSO or phenobarbitone caused hepatic necrosis in approximately 50% of the animals. Most of the lesions were centrilobular and ranged from "focal necrosis of small clusters of hepatocytes adjacent to terminal venules with mononuclear/macrophage infiltrates to more widespread coagulative necrosis in some areas forming confluent lesions." Absolute and relative liver weights were increased by ~15% in rats treated with BHT alone or BHT/BSO and by ~23% in BHT/phenobarbitone-treated animals. BHT increased mitotic activity, which was consistent with liver hypertrophy and enzyme induction, decreased the distribution of glycogen, and caused vacuolation of a few scattered centrilobular hepatocytes, which was consistent with lipid accumulation. The investigators concluded that a single oral dose of 500 mg/kg BHT was below the threshold for acute hepatotoxicity.

Dore, Atzori, and Congiu (1989) investigated the relationship of hepatic GSH to BHT-induced necrosis. A dose of 500 mg/kg BHT (in olive oil) was administered orally (PO) to male Sprague-Dawley rats for 1 or 3 days. Rats of the control group were treated with the vehicle alone. The concentration of hepatic GSH decreased 6 hours after BHT exposure and returned to normal 24 hours later. Serum GOT concentration and liver morphology were normal. When BHT was administered for 3 days, the serum GOT concentration was decreased 24 hours after the last dose. Liver had extensive perilobular necrosis. Hepatic GSH increased at 24 hours and remained so to 96 hours. The investigators concluded that a single dose of BHT did not induce hepatotoxicity, although it decreased hepatic GSH. When BHT intoxication was produced by three doses, an increase of GSH occurred due to regeneration of hepatocytes following necrosis.

The combination of protein deprivation and treatment with BHT caused significantly greater responses than the sum of the responses of the two individual treatments (Wong and Rao 1983). Groups of female Wistar rats were fed diets containing 24%, 8%, or 4% protein for 5 weeks. During the fifth week, half of the rats were treated intragastrically with 250 mg/kg/day BHT for 7 days. Reduction of protein intake (especially to the 4% concentration) caused significant decreases in body weight gain, relative liver weight, hepatic microsomal cytochrome P450, total liver protein, and serum albumin concentration. In addition, hepatic glucose-6-phosphatase activity, relative heart weight, and serum globulin concentration were increased. Treatment with BHT caused significant reductions in body weight gain and glucose-6-phosphatase activity, liver enlargement, induction of hepatic microsomal protein and cytochrome P450, and an increase in serum total cholesterol. Rats treated with 4% protein and BHT had decreased relative spleen weights and hepatic necrosis. The decreased body weights of BHT-treated rats differed from controls by 214% in the 8% protein group and by 30% in the 24% protein group. Similar results were reported by Nikonorow and Karlowski (1973).

The species differences in the hemorrhagic response to BHT was investigated by Takahashi, Hayashida, and Hiraga (1980). ICR, ddY, DBA/c, C3H/He, BALB/cAn, and C57BL/6 mice were fed 1.2% BHT for 1 week and Sprague-Dawley, Wistar, Donryu, and Fischer rats were fed the same concentration for 3 weeks. Japanese quail were fed 1% BHT for 17 days. New Zealand White-Sat rabbits were fed 177 or 760 mg/kg/day BHT for 2 weeks. Beagle dogs were fed 173, 440, or 760 mg/kg/day for 2 weeks. For the study using dogs, male Sprague-Dawley rats were used as positive controls and were fed 1.2% BHT for 10 days; the mean intake was 876 mg/kg/day. For 3 days, Syrian golden hamsters were treated IP with 380 or 760 mg/kg/day BHT and Hartley guinea pigs were treated IP with 190 or 380 mg/kg/day. BHT was administered in solution with acetone:soy bean oil (1:9; 200 mg BHT/ml solution). Male Sprague-Dawley rats were used as positive controls and were given the same dose as the hamsters.

In these studies, hemorrhagic death occurred and the prothrombin index was decreased in all strains of rats treated with 1.2% BHT. The prothrombin index was also decreased significantly in ddY, BALB/cAn, and C57BL/6 mice fed 1.2% BHT, and intraepididymal and subcutaneous hemorrhages were observed in ddY mice. Guinea pigs injected IP with 380 mg/kg/day BHT had cerebral hemorrhages. Hemorrhages were not observed in hamsters, rabbits, dogs, or quail treated with BHT. The livers of all strains of treated rats contained the BHT-QM metabolite, but nonconjugated phenol metabolites were detected in the livers of the other species. The investigators concluded that a species difference was involved in hemorrhagic effects induced by BHT (Takahashi, Hayashida, and Hiraga 1980).

Simán and Eriksson (1996) reported that 0.5% to 1.0% BHT decreased the hepatic concentration of α-tocopherol after feeding of both compounds (tocopherol dose = 0.4%) to female rats for 4 weeks. BHT did not affect tocopherol concentration in abdominal adipose tissue, which lacked the cytochrome P450 system.

Subchronic Toxicity

In a 16-week feeding study using Carworth SPF rats, 0.1% BHT increased the daily urinary elimination of ascorbic acid and induced liver enlargement, particularly in females. These parameters returned to control values by the end of the study. BHT also increased renal weight and it did not induce hepatotoxicity (Gaunt et al. 1965b). In a study using SPF rats, IP BHT also increased ascorbic acid excretion in the urine (Gaunt, Gilbert, and Martin 1965a). BHT increased the amount of ascorbic acid in the urine and liver of male Wistar rats fed BHT for 14 days, indicating that ascorbic acid synthesis was enhanced via the induction of activities of hepatic UDP-glucose dehydrogenase and UDP-glucuronyltransferase (Horio, Kimura, and Yoshida 1983).

Søndergaard and Olsen (1982) fed 500 and 5000 ppm BHT to male Mol:WIST SPF rats for 90 days to investigate the antioxidant's effect on the thyroid gland. Rats fed the high dose had increased iodine uptake, but the half-life of thyroxine was unchanged or increased slightly. Rats of both groups had increased thyroid gland weight and rats of the high-dose group had increased liver weight. The low dose, which was the lowest-effect level (LEL), corresponded to 25 mg/kg.

Adult, male Wistar Carworth Farms rats were fed a diet containing 13% proteins and 11% lipids (peanut oil) for 21 weeks. Over that period, the concentration of BHT in the feed was increased weekly from 0.1% to 2.1%. When the diet was supplemented with 0.1% BHT, feed intake was limited, but stabilized as long as the concentration was $<1.2\%$. When the rats were treated with $\geq 1.3\%$ BHT, feed intake decreased as a decreasing hyperbolic function of the increased dose. Body weights increased when the rats were treated with $\leq 0.5\%$ and were stable from 0.6% to 0.9% BHT. At concentrations of 1% to 1.2% BHT, body weights decreased rapidly. These results indicated that 1.3% BHT (300 mg/kg/day) was the maximum amount the male rat could "process" when fed 13% protein and 11% lipids. When the protein concentration was increased to 19.5% to 26%, the feed intake and body weight of rats fed 2.1% BHT increased and stabilized; rats fed 26% protein could process up to 630 mg/kg/day BHT.

In a follow-up study, male weanling rats of the same strain were fed the same basal diet and either 0.1% or 0.5% BHT for 68 days. Feed intake and growth rate decreased when the rats were fed 0.1% BHT, but these effects weren't significant until the rats weighed 200 g. At the high dose, both feed intake and growth rate decreased, such that rats ate 570 mg/kg/day when they weighed 80 g and ate 300 mg/kg/day when they weighed 250 g. Based on this and the previous study, the investigators concluded that, regardless of the protein content of the diet, 0.2% BHT decreased feed intake, diet efficiency, growth, and the protein efficiency ratio (Pascal, Durand, and Penot 1970; Durand and Pascal 1973; Pascal 1974).

Halladay et al. (1980) reported that the feeding of 250 mg/kg/day BHT to five male S/A Simonsen albino rats for up to 60 days decreased the concentration of hepatic and intestinal cytochrome P450 and the activities of hepatic *p*-nitroanisole-*O*-demethylase and aminopyrine-*N*-demethylase. BHT decreased the activity of BP hydroxylase by 49% in hepatic preparations on day 15, but the activity was similar to controls on days 30 and 60; intestinal BP hydroxylase activity was also decreased somewhat at all sampling periods. Relative liver weights were increased significantly ($p < .05$).

Chronic Toxicity

When female Sprague-Dawley rats were dosed simultaneously with 125 to 250 mg/kg diet retinyl acetate and 2500 to 5000 mg/kg diet BHT for 120 to 180 days, the incidence of biliary hyperplasia was significantly increased and hepatic fibrosis was greater than that induced by either compound alone. Retinyl acetate alone (high dose) produced a low incidence of hepatic fibrosis. Both doses of BHT decreased the content of total hepatic vitamin A and caused hepatocellular hypertrophy and dose-related increases in liver weight (McCormick, Hultin, and Detrisac 1987).

Crampton et al. (1977) fed 0.4% BHT or 0.25% phenobarbitone to female Wistar rats for up to 80 weeks. Four treated and four control rats were killed for biochemical, histochemical, and morphological analyses after 1, 8 (BHT only), 16, 32, and 80 weeks. Groups of four rats were killed 18 (BHT) or 30 (phenobarbital) days after the 80-week treatment period, and were examined for the reversibility of hepatic changes. In addition, a group of both BHT-treated and untreated rats were injected IP with 80 mg/kg phenobarbitone on days 15, 16, and 17 after cessation of the 80-week treatment period. After treated with BHT, relative liver weight, the activities of ethylmorphine-*N*-demethylase, aniline 4-hydroxylase, biphenyl 4-hydroxylase, and NADPH–cytochrome *c* reductase; the contents of cytochromes P450 and b_5; and the amount of microsomal protein were increased by 35%, 95%, 91%, 15%, 32%, 83%, 78%, and 50%, respectively. Similar increases occurred in phenobarbitone-treated rats. These changes occurred throughout the study, beginning after 1 week of treatment. Centrilobular cell enlargement and hypertrophy of the smooth ER were the only observed morphologic changes, and a "centrilobular depression" of glucose-6-phosphatase was determined during the histochemical analysis. The observed changes were reversible upon cessation of treatment. The investigators concluded that phenobarbitone had greater stimulatory effects on drug-metabolizing enzyme activity than did BHT.

Gray et al. (1972) reported increases of hepatic enzyme concentrations by 50% to 100% after female rats were given oral doses of 250 mg/kg/day BHT for 32 weeks. These increases occurred by the first week. The enzymes affected were ethylmorphine-*N*-demethylase, biphenyl 4-hydroxylase, aniline 4-hydroxylase, cytochrome P450, and cytochrome b_5. The microsomal protein concentration was also increased, as was hepatic weight. No lesions (necrosis) of the liver were observed at microscopic examination.

The feeding of BHT to Norway hooded rats (6–12/sex/group) at a concentration of 0.5 % (*w/w*) for up to 2 years had no adverse effects on reproduction, the microscopic morphology of the spleen, kidneys, liver, testes, or skin, or the ratio of total body weight to the weight of the heart, spleen, and kidneys (Brown, Johnson, and O'Halloran 1959).

Clapp, Satterfield, and Bowles (1979b) fed 0.75% BHT to male and female BALB/c mice from either 8 or 11 weeks of age (47 males, 49 females) for their lifetime. The 11-week group had 49/sex and the 8-week group had 89 males, 98 females. The control group (100 males, 97 females) was given basal feed only. All BHT treatment groups had increased mean survival times compared to controls. Males survived for 726 to 890 days and females survived for 759 to 875 days. Male and female

controls survived for 894 and 701 days, respectively. Although the mean survival time for females was less than that of males, the maximum life spans did not differ between sexes. The number of deaths between 350 and 600 days decreased in groups treated with BHT. Throughout the study, treated mice were heavier, had smoother hair coats, and appeared healthier than controls. At necropsy, treated mice had enlarged livers.

Skin Irritation and Sensitization

BHT (100%) was applied to the skin of animals (species and number not specified) during a 48-hour patch test, and produced moderate irritation but not sensitization (Mallette and Von Haam 1952).

During a topical study of the pulmonary effects of BHT (see "Pulmonary Toxicity"), the antioxidant caused epidermal hyperplasia that was occasionally associated with small ulcer formation (Miyakawa et al. 1986). In this study, 5, 10, 20, or 30 mg BHT in 0.1 ml DMSO was applied to the shaved skin of 10 CD-1 mice per sex, three times weekly for 4 weeks.

Pulmonary Toxicity

General Findings

The potential of BHT to cause pulmonary toxicity has been well documented. In general, BHT caused hemorrhages of the lungs when administered via IP injection or topical application. Signs of toxicity included thickened, inflammed interalveolar septa, and increased numbers of pyknotic nuclei and enlarged cells. Lung tissue also had hyperplasia, hypertrophy, and general disorganization of the cellular components. Edema, marked capillary engorgement and congestion, "blebbing" of the alveolar epithelium, and increased septal cells and macrophages in the alveolar spaces were observed (Marino and Mitchell 1972).

In another study, mice had well-developed gross lesions within 3 days of treatment. Lung weight was increased, and the lungs were enlarged and plum-colored. Microscopic changes were present within 1 day of treatment. The alveolar septa appeared swollen, some perivascular edema was observed, and "blood stasis" occurred in capillaries and small blood vessels. No edema fluid or inflammatory cells were observed, however, within the alveoli. Within 3 days, the investigators observed diffuse thickening of the alveolar septa (such that the alveoli appeared "obliterated"), increased cellularity, and markedly enlarged cells and pyknotic nuclei. Five days after treatment, the alveolar walls were less edematous, but were still thickened in others. Occasional small foci of enlarged cells were observed. The high dose also caused widespread tissue damage that was interpreted as epithelial and macrophage proliferation. Smaller doses caused similar, non–dose-related and non–duration-related changes (Saheb and Witschi 1975).

In another study, BHT was applied to the skin of CD-1 mice (Furukawa, Takahashi, and Hayashi 1985). The test compound was applied to the shaved skin of the back three times per week for 4 weeks. After the second or third application, many of the mice had dose-related respiratory distress and died. Congestion and enlargement of the lungs were observed at necropsy, as well as "oozing of froth from the trachea." During a microscopic examination, the investigators observed collapsed alveoli, dilatation of the alveolar ducts, necrosis of type I cells, infiltration of macrophages into peripheral air space, and increased numbers of type II alveolar cells and interstitial cells.

Miyakawa et al. (1986) applied BHT to the skin of 10 CD-1 mice/sex at doses of 5 to 30 mg BHT/0.1 ml DMSO, three times weekly for 4 weeks. The treated mice had respiratory distress with subsequent, dose-dependent mortality. When necropsied, the mice had congestion and enlargement of the lungs with "oozing of froth from the trachea." The alveolar walls and septa had capillary congestion and edema, and the air spaces contained serofibrinous exudates, macrophages, and desquamated epithelial cells. Degeneration and necrosis of type I alveolar epithelial cells were observed in electron micrographs. Type II cells were increased in number and had unusually elongated pseudopodia near denuded basement membrane that apparently covered the damaged areas.

Other investigators have reported similar findings as shown in Table 7.

The highest dose of BHT that caused no significant increase in lung weight in male CD-1 mice was 175 mg/kg. Doses greater than 175 mg/kg rapidly increased the lung/body weight ratio that reached a plateau at doses of 400 mg/kg. Subcutaneous injections of 50 or 250 mg/kg BHA given with 100, 175, and 250 mg/kg BHT significantly increased the lung/body weight ratio compared to animals receiving only BHT. When higher doses of BHT were given, BHA did not enhance further this ratio above 1.5%. Administration of BHA by IP injection increased the BHT-induced lung injury (Thompson and Trush 1986).

Effects on Macromolecules

BHT modified DNA synthesis in various studies. In one, male C57, C57 × C3H, C3H, Swiss-Webster, DBA/2, BALB/c, and BDF_1-Sch. hybrid mice were injected IP with 400 mg/kg BHT. Within 2 days of treatment, thymidine incorporation and the activities of thymidine kinase, 5′-nucleotidase, DNA polymerase, and glucose-6-phosphate dehydrogenase were increased in the lungs. The results suggested that the activity of thymidine kinase could serve as a marker to quantitate BHT-induced pneumocyte proliferation (Witschi et al. 1976). In a similar study, Witschi, Williamson, and Lock (1977) reported that BHT increased DNA synthesis by lung nuclei of treated Swiss-Webster mice, whereas equimolar amounts of other antioxidants (ascorbic acid, α-tocopherol, propyl gallate, BHA, etc.) did not affect cell proliferation.

At an IP dose of 400 mg/kg in 0.2 ml corn oil, BHT stimulated DNA synthesis in pneumocytes of male Swiss-Webster mice, as determined by the incorporation of [^{14}C]-thymidine into DNA (Witschi and Saheb 1974). Treated mice had ~1.6 times the amount of total DNA per lung as control mice. DNA synthesis was not stimulated in organs other than the lungs. Mice treated

TABLE 7
Studies showing adverse respiratory effects of BHT in mice

Strain (sex)	No. animals/ group	Dose(s)[a]	Reference
Ha/ICR, Swiss non-inbred (F)	5	2500 mg/kg in 1 ml olive oil	Marino and Mitchell 1972
Ha/ICR, Swiss non-inbred (F)	3	250, 750, 1250, 2500 mg/kg in 1 ml olive oil	
DBA/2 (F)	1	4, 40, 400 mg/kg in 0.5 ml olive oil	
DBA/2 (F)	10	28, 830 mg/kg in 0.5 ml olive oil	
Swiss-Webster (M)	12	62.5, 125, 250, 500 mg/kg in 0.2 ml corn oil	Saheb and Witschi 1975
C57 (M) C57 × C3H (M) Swiss-Webster (M) DBA/2 (M) C3H (M) BALB/c (M) BDF_1-Sch. hybrid (M)	6–8	400 mg/kg in 0.2 ml corn oil	Witschi et al. 1976
BALB/c (M)	4–14	250, 500 mg/kg in 0.2–0.3 ml corn oil	Smith 1984
Swiss albino (M)	20	200, 400, 800 mg/kg in olive oil	Waseem and Kaw 1994
Swiss-Webster (M)	10	400 mg/kg in 0.1 ml corn oil/10 g body weight	Coulombe, Filion, and Côté 1985
Swiss-Webster (M)	5–6	125, 250, 400, 500 mg/kg in 0.2 ml corn oil	Witschi and Saheb 1974
Swiss-Webster (M)	16–24	62.5, 215, 500 mg/kg in 0.5 ml tocopherol-stripped corn oil	Omaye, Reddy, and Cross 1977
Swiss-Webster (M)	6	400 mg/kg in 0.1 ml corn oil	Adamson et al. 1977
BALB/cBY (NR) CXB H (NR)	NR[b] NR	200 mg/kg weekly for 4 weeks	Dinsdale, Malkinson, and Miller 1993
BALB/c (M) ICR (M) C57B2/6NHsd (M) SSIn (F)	6–9	300, 400 mg/kg in 0.1 ml corn oil/10 g body weight	Kehrer and DiGiovanni 1990
CD-1 (M, F)	10/sex	5, 10, 20, 30 mg/0.1 ml DMSO (dermal)	Furukawa et al. 1985
CD-1 (M, F)	10/sex	5, 10, 20, 30 mg/0.1 ml DMSO; 3 × weekly for 4 weeks (dermal)	Miyakawa et al. 1986

[a] Unless stated otherwise, the route of administration was IP injection.
[b] NR, not reported.

with 125 to 500 mg/kg BHT had thickened alveolar septa with abnormally big cells and nuclei. In some cells, the nuclei were stained deeply basophilic, but in others they were "pale and almost devoid of chromatin, but with intensely stained nucleoli." Mitotic figures were rarely observed.

Larsen and Tarding (1978) also reported that BHT stimulated DNA synthesis in the lungs of NMRI mice and Wistar rats. A single dose of 500 mg/kg BHT (in soybean oil) was administered IP or by gavage, and [^{14}C]-thymidine was given 4 days later. No sex difference occurred after treatment with BHT, and the degree of DNA sythesis induction was the same for both routes of administration. An increase of total DNA/lung was also reported, and was attributed to increased lung weight and induced hyperplasia; however, the amount of DNA/g lung tissue was not increased.

BHT-induced microscopic changes were accompanied by increases in the total amounts of DNA, RNA, and protein. The incorporation of thymidine into DNA and leucine into protein was increased within 2 days of treatment. The incorporation of orotic acid into total pulmonary RNA was decreased compared to controls (Saheb and Witschi 1975).

Kuo et al. (1978) reported that a single IP dose of 400 mg/kg BHT modified parameters of the cyclic nucleotide system in male C57B1/6Sp inbred mice, as well as producing enlarged lungs and cell proliferation. The activities of cyclic GMP, phosphodiesterases, and protein kinases for cyclic GMP and

cyclic AMP, and the stimulatory modulator of cyclic GMP–dependent enzyme increased. DNA polymerase activity was also increased. These changes occurred prior to or concurrently with pulmonary hypertrophy and hyperplasia, suggesting that cyclic GMP–mediated processes were crucial for BHT-induced cell proliferation after toxic injury of the lungs.

The IP injection of 62.5 to 500 mg/kg BHT caused dose-dependent increases in lung weight of adult, male Swiss-Webster mice, as well as increased lung DNA and nonprotein sulfhydryl concentrations, enzyme activities, and the incidence of lung damage. The enzymes involved were GSH peroxidase, GSH reductase, superoxide dismutase, and glucose-6-phosphatase. The changes in enzyme activity were due to inflammatory and proliferative pulmonary changes from acute lung cell injury and necrosis. These changes were not observed when mice were treated with other antioxidants, including BHA, vitamin E, and ethoxyquin; therefore, the gross anatomical and/or biochemical lung changes were not related to BHT's antioxidant properties (Omaye, Reddy, and Cross 1977).

When 200 to 800 mg/kg BHT (in olive oil) was injected IP into male Swiss mice, the total number of cells detected in the bronchoalveolar lavage fluid increased in a time- and dose-dependent manner at 24 to 48 hours and 7 days after dosing. Total protein content and lactate dehydrogenase activity were increased above control values. These changes were accompanied by microscopic alterations: congestion of capillaries and small blood vessels, increased cellularity, and diffuse thickening of alveolar septa. The thickened septa "obliterated" the alveolar lumen at places (Waseem and Kaw 1994).

Within 1 day of BHT administration (250 to 500 mg/kg; IP) using male BALB/c mice, the activity of bronchoalveolar lavage (BAL) angiotensin-converting enzyme increased fourfold (Smith 1984). On the second day, it increased ninefold and the BAL protein content increased fourfold. The enzyme's specific activity to BAL protein increased threefold. Cellular damage of type I cells and endothelial cell damage were observed in electron micrographs. BAL lactose dehydrogenase activity increased 334% and the BAL cell count doubled. A small increase of polymorphonuclear leukocyte numbers was reported, but the count peaked on day 7.

Kehrer (1982) investigated the effects of collagen production caused by acute lung damage produced by BHT. Female, CD-1 mice were dosed IP with 50 to 400 mg/kg BHT. The control group received corn oil at a volume of 0.1 ml/10g. Collagen synthesis had a linear dose-response relationship when BHT was dosed at 200 mg/kg or greater ($r = .999$). A toxic threshold dose of 160 mg/kg was extrapolated from this relationship. A significant relationship existed between doses of 200 and 400 mg/kg BHT ($p > .05$) and both net hydroxyproline synthesis and the percentage of protein synthesis devoted to collagen 7 days after dosing. Mice of the high-dose group had significantly increased amounts of hydroxyproline 2 to 3 weeks after exposure to BHT compared to the control group. Increased mortality occurred among mice treated with ≥300 mg/kg BHT. The synthesis of pulmonary DNA was significantly increased at doses of ≥200 mg/kg 2, 3, and 7 days after dosing. Maximum thymidine incorporation occurred at doses of 400 mg/kg. The correlation coefficient between thymidine incorporation in DNA and BHT doses of ≥200 mg/kg was .930.

Effects of Other Treatments

General. Diethyldithiocarbamate (DTC) and carbon disulfide (CS_2) were administered, respectively, at 100 and 300 mg/kg orally to SPF-grade mice 1 hour before an IP dose of 400 mg/kg BHT. Both chemicals prevented the increase in lung weight and total DNA content observed in mice given this dose of BHT. At the same doses, DTC and CS_2, administered concurrently, prevented the early loss of lung GSH caused by BHT. Pretreatment with large doses of CS_2 (300 mg/kg) suppressed the covalent binding of BHT to lung macromolecules in vivo by about 23% to 30% between 6 and 24 hours. Lung microsomes isolated from CS_2-treated mice had a dose-dependent decrease in NADPH-dependent binding of BHT in vitro. When low doses of CS_2 (10 mg/kg) were tested in parallel with BHT (100 mg/kg), a rapid dose-dependent reduction in some drug-metabolizing enzymes was observed in lung microsomes. The cytochrome P450 content was also reduced (Masuda and Nakayama 1984).

BHA. The pulmonary toxicity of BHT was increased by the coadministration of BHA, although the latter compound itself did not cause lung damage (Yamamoto et al. 1988). The antioxidants were dissolved in olive oil and administered to male ddY mice IP at a dose volume of 0.1 ml/10 g body weight. Mice of the control group were treated with vehicle alone. Four days after treatment with 200 or 400 mg/kg BHT, the mice were killed and lung damage was assessed by determining the wet lung weight. Body weight changes were also recorded. At a dose of 100 mg/kg BHA, signs of enhanced toxicity of BHT were not observed. When 200 to 400 mg/kg BHA was coadministered, the increased lung weight and decreased body weight were comparable to those after administration of 400 mg/kg BHT alone. A dose of 200 mg/kg BHA caused nearly maximal enhancement of BHT-induced toxicity. The effect of BHA occurred when it was administered 2 hours, but not 4 hours, after inhalation of BHT. Coadministration of BHA significantly increased the radioactivity covalently bound to lung macromolecules 4 to 8 hours after treatment with [^{14}C]-BHT. The area under the curve for unchanged BHT in the lungs 24 hours after coadministration was ~140% that after treatment with BHT alone. The amount of unchanged BHT in plasma increased by 40% to 85%, but the amount of total BHT (mostly its metabolites) in plasma was decreased by 40% to 60%.

A different finding was reported by Mizutani (1985). Dietary administration of 0.5% BHA to mice for 3 days completely protected against BHT-induced lung toxicity. In addition, daily IP injections of 200 to 400 mg/kg BHA to mice for 3 days prevented lung toxicity induced by up to 1000 mg/kg BHT. In both studies where BHA was administered, the investigators concluded that the effect of BHA on BHT toxicity was due to

different periods of exposure rather than from different routes of administration (Mizutani 1985; Yamamoto et al. 1988).

Thompson and Trush (1986) investigated the ability of BHA to enhance metabolism of BHT to BHT-QM, a potentially toxic metabolite, in two enzyme systems. In the presence of BHA and prostaglandin H synthase, covalent binding of BHT to microsomal protein was greatly enhanced. This enzyme also catalyzed the formation of BHT-QM in the presence of BHA. Horseradish peroxidase plus BHA greatly enhanced the formation of BHT-QM, however, in the absence of BHA, this metabolite was not detected.

In another experiment, these researchers investigated the ability of peroxidase enzymes from various mammalian tissues to catalyze the interaction of BHA with BHT. The presence of BHA and arachidonic acid stimulated the covalent binding of BHT to the microsomes from sheep, guinea pig, dog, and human lungs, dog bladder, and rabbit renal medulla. When hydrogen peroxide was used as a substrate, microsomes from rat, mouse, and human lungs and rat intestine enhanced the binding of BHT to BHA. These results suggest that peroxidase enzymes from various mammalian tissues can metabolize BHT to a reactive intermediate that covalently binds to protein. This covalent binding was enhanced by BHA through the increased formation of BHT-QM.

Oxygen. Pulmonary toxicity was reported by other investigators after IP injection of BHT. In a study by Filion (1983), male Swiss-Webster mice were injected with 400 mg/kg BHT dissolved in corn oil prior to exposure to room air or 95% oxygen for the final 24 hours of the incubation period (1, 3, 5, 7, or 15 days). BHT/air induced general cytoplasmic swellings of type I pneumocytes, epithelial injury, and acute inflammation of type II pneumocytes. BHT/oxygen induced similar signs of toxicity with earlier onset and prolonged cellular necrosis, such that the type I pneumocytes were "stripped" from the regenerative epithelium faster than they could be replaced. The type II cells were more susceptible to oxygen, such that a maintained, hypercellular population was absent.

BHT at a dose of 400 mg/kg caused pulmonary fibrosis in male BALB/c mice that were exposed immediately to 80% oxygen for 3 days after IP administration of the antioxidant (Kehrer and Witschi 1981). The treatment resulted in 47% mortality and surviving mice had significant accumulations of pulmonary collagen, observed as increases in total lung hydroxyproline concentrations. The administration of various corticosteroids 1 to 6 days after treatment with BHT generally decreased mortality and/or the accumulation of pulmonary collagen.

Margaretten, Tryka, and Witschi (1988) made hyperoxia-sensitive, male BALB/c mice tolerant to 100% oxygen after IP treatment with 400 mg/kg BHT. The mice survived longer periods in oxygen when exposed to it on days 7, 14, and 21, but not 2, after BHT injection. Tolerance was most pronounced on day 7: the time of death for 50% of controls was 4.5 days and the time of death for 50% of the treated mice was 9.6 days. Oxygen tolerance decreased with time to 7.7 days on day 14 and 7.3 days on day 21. BHT alone induced lung lesions and BHT plus oxygen caused additional damage to the lungs. Within 1 week of exposure, the lungs had diffuse alveolar damage (exudative phase), manifested by marked interstitial edema, modest infiltrate of interstitial mononuclear cells, and prominent hyaline membranes. This phase was followed by the proliferative phase at weeks 2 to 6, in which marked interstitial thickening, infiltrate of interstitial macrophages, some interstitial edema, type II pneumocyte hyperplasia, numerous alveolar macrophages, and foci of organization of alveolar material by fibroblasts were observed.

Haschek and Witschi (1979) reported that the in vivo incorporation of thymidine into mouse pulmonary DNA was significantly inhibited by 70% oxygen exposure 2 to 6 days after IP administration of 400 mg/kg BHT. BALB/c mice exposed to oxygen after BHT had extensive interstitial fibrosis by day 14. In contrast, mice that were not treated with BHT or that were exposed to oxygen 7 days after BHT injection had no fibrosis. When the mice were pretreated for 7 days with oxygen, the development of fibrosis was not enhanced after BHT injection. The investigators suggested that the observed effects were due to an interaction between an agent causing acute pneumotoxicity and a second compound that compromised reepithelialization.

Witschi and Côté (1977) injected male Swiss-Webster mice with 250 to 400 mg/kg BHT, with or without treatment with 100% oxygen. In vivo incorporation of thymidine into pulmonary DNA was measured on days 1 to 7 after treatment with BHT. When oxygen was administered for 24 hours, 2 to 4 days after BHT injection, DNA synthesis was inhibited; however, synthesis was not decreased if the mice were exposed to oxygen on days 5 to 7 after dosing with BHT. Similar results were reported for the incorporation of leucine into protein. The investigators concluded that the type II alveolar cells, which proliferated early after treatment with BHT, were more susceptible to oxygen-induced cytoxocity than interstitial or capillary endothelial cells.

Witschi et al. (1980) also reported that the combination of BHT administration (IP; 500 mg/kg in corn oil) and exposure to either 70% oxygen or low doses of x-rays (average dose rate = 265 rad/min) resulted in an "abnormal and persistent accumulation of collagen" in the lungs of male BALB/c mice. Lesions indicative of diffuse interstitial fibrosis also developed. BHT caused increased collagen, whereas the other two treatments did not. These changes did not occur if oxygen exposure or irradiation was delayed for 6 days after BHT treatment or if they were administered prior to BHT.

Kehrer and Witschi (1980a) studied the effects in BALB/c mice of a single IP injection of 400 mg/kg BHT and subsequent exposure to 70% oxygen for 6 days (group A) or exposure to room air (group B). The two control groups were given an IP injection of corn oil and exposed to either 70% oxygen for 6 days (group C) or room air (group D). Mice of groups treated with BHT had a significant increase in total pulmonary hydroxyproline within 3 days. After removal from oxygen, group A mice

had a rapid increase in total pulmonary hydroxyproline. Mice of group B had significantly less pulmonary hydroxyproline than mice of group A. Control groups did not have increased amounts of pulmonary hydroxyproline.

The synthesis of hydroxyproline was also determined by injecting [^{3}H]-proline in some of the mice from the four different groups. The amount of free proline present in the lungs of group A mice after the injection was not statistically significant. Acid insoluble [^{3}H]-hydroxyproline rapidly accumulated and reached a constant concentration by 2 hours, whereas acid soluble [^{3}H]-hydroxyproline, an index of the degradation of newly synthesized collagen, reached a maximum concentration at 2 hours after injection and then began to decline. The amount of acid soluble [^{3}H]-hydroxyproline in group A mice was significantly lower than control groups. Mice of group B had similar amounts of acid insoluble [^{3}H]-hydroxyproline but the amounts returned to the control values. The amount of free proline in lung tissue from mice of group A was not statistically significant from the amount in mice of group D. Group A mice accumulated significantly greater amounts of hydroxyproline than mice of groups C and D on all days examined. Group B mice also had increased hydroxyproline accumulation compared to controls, but the concentration returned to control values. Collagen synthesis as a percent of total protein synthesis increased in mice of groups A and B; however, in group B, the amounts returned to control values.

Bleomycin. Pulmonary histopathologic changes induced in hamsters by the antitumor agent bleomycin was inhibited by BHT. These changes included fibrosis, macrophage aggregation, epithelial proliferation, and/or the accumulation of type III collagen. Three of 20 male Syrian golden hamsters treated with the compounds died. The hamsters were treated intratracheally with 2.5 U/kg body weight bleomycin for 14 days and then were fed 1% of the antioxidant for 41 days. BHT also reduced the increase in lung weights observed after treatment with bleomycin ($p < .05$) (Ikezaki et al. 1996).

Radiation. Irradiation of the thorax of female BALB/c mice 2 days after an IP injection of 400 mg/kg BHT markedly reduced their survival time. Animals irradiated 2 days after BHT treatment died 18 to 40 days later, whereas animals without BHT pretreatment died 140 to 180 days later. Microscopic lesions of the lungs were similar between both groups. The cause of death in both groups appeared to be a result of radiation pneumonitis. Survival time for the group irradiated 6 days after BHT treatment was similar to the control group. The $LD_{50/180}$ decreased when mice were irradiated 2 days after BHT treatment compared to irradiated controls. Irradiation 6 days after BHT treatment resulted in an $LD_{50/180}$ estimate that was significantly higher than irradiated controls (Ullrich and Meyer 1982).

Male and female BALB/c mice were irradiated with 525 to 750 rad of x-ray with and without pretreatment of 0.75% BHT in the feed for 28 days. BHT demonstrated protection against radiation lethality at 525 to 675 rad through increased survival percentages, which were greater in males than females. Mean survival times were not altered by BHT. The LD_{50} in females was 628.2 ± 4.2 and 642.9 ± 3.9 rad for radiation alone and BHT pretreatment, respectively. The LD_{50} for males was 580.9 ± 5.6 and 622.2 ± 7.5 rad for radiation alone and pretreatment with BHT, respectively. These values were statistically significant suggesting that BHT modified radiation lethality (Clapp and Satterfield 1975).

Species, Strain, and Sex Differences

Acute Toxicity

Strain differences were reported for male mice in BHT-induced toxicity. DBA/2N mice had the lowest IP LD_{50} (138 mg/kg), and the dose-mortality curve had a steep slope in the regression line. AKR, C57BL/6N, and ICR-JCL mice had LD_{50} values of 538, 917, and 1243 mg/kg, respectively, and the slopes of their regression lines were "undramatic." In contrast, the LD_{50} in BALB/c AnN mice was 1739 mg/kg, and the regression line paralleled that of the DBA/2N strain. Irrespective of strain or dose, the deaths occurred within 4 to 6 days of treatment and were accompanied by massive edema and hemorrhage in the lungs (Kawano, Nakao, and Hiraga 1981). For BALB/c, ICR, C57BL/6NJsd, and SSIn mice, the LD_{50} values were 1739, 1243, 917, and ~350 mg/kg, respectively (Kehrer and DiGiovanni 1990).

In male ddY, ICR, and NIH-Swiss mice, the IP LD_{50} values of BHT and its metabolites BHT-OOH, BHT-quinone, and 2,6-di-*tert*-butyl-4-[(methylthio)methyl]phenol were 3550, 190, >1600, 2270, and 1840 mg/kg, respectively; strain differences were not reported (Yamamoto, Tajima, and Mizutani 1980).

Pulmonary Toxicity

BHT-induced pneumotoxicity occurred generally in mice. Female mice were more susceptible to topical BHT-induced lung damage than males (Miyakawa et al. 1986).

In a study using CXB H and BALB/cBy mice, IP BHT (200 mg/kg/week for 7 weeks; in corn oil) caused endothelial injury and damage to type I pneumocytes that resulted in their desquamation into the alveolar lumen. CXB H mice were less susceptible to BHT than BALB/cBy mice, and had decreased mesenchymal proliferation. BALB/cBy mice had an increased number of mesenchymal cells and the influx of monocytes increased septal thickening and focal consolidation. The majority of alveolar macrophages were vacuolated and distended. The observed pneumotoxicity was also accompanied by increased lung weight and alterations in the calcium second messenger pathway (Miller et al. 1994).

Malkinson (1991) reported that mouse strains that were susceptible to lung carcinogenesis had a greater turnover rate of alveolar type II cells and bronchiolar Clara cells than more resistant strains. Subchronic IP injection of 200 mg/kg/week BHT for 4 weeks resulted in sustained lung damage in promotion-sensitive mice (BALB/cBy) compared to promotion-resistant mice (CXB H) and untreated controls (Dinsdale, Malkinson,

and Miller 1993). Damage was observed in type I alveolar epithelial cells, as well as endothelial cells of the capillaries and larger vessels. Many large, proliferating epithelial cells (including type II pneumocytes) were observed in the alveolar wall. In electron micrographs, myofibroblasts, and monocytes had proliferated around all blood vessels, and large fibroblasts were present throughout the alveolar interstitium.

Malkinson and Beer (1983) dosed several strains of mice IP with 400 mg/kg BHT for 6 weeks after a single injection of urethane. BHT increased the tumor number threefold to fourfold in BALB mice, 50% in A-mice (strains sensitive to urethane-induced adenomas: A/WySnJ, A/J, A.BY/SnJ, SWR/J), and did not increase tumor incidence in resistant strains. When the urethane dose was increased, the same strain-dependence on adenoma induction was shown as before. Lethality was also dose-dependent in BALB mice, which was the only strain in which BHT dose varied.

Male and female F-344 rats and male Syrian golden hamsters were topically treated at doses of 240 and 480 mg BHT/0.1 ml DMSO, respectively. In these species, pulmonary alterations were not observed (Miyakawa et al. 1986). BHT, however, produced toxicity in male Jcl-SD rats at IP doses of 640 to 1024 mg/kg/day for 7 days (Sakamoto and Takahashi 1985) and in male Wistar rats at a single dose of 800 mg/kg (Tamizhselvi, Smikkannu, and Niranjali 1995).

Hepatic Toxicity

Male and female Wistar-JCL rats and male C57BL/6N mice were fed 0.5% BHT for 6 consecutive days. None of the animals died. Both rats and mice had increased hepatic weight, microsomal protein content, cytochrome P450 activity, and drug oxidation enzyme activities. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis of microsomes established that BHT induced 46,000 (cytochrome $P450_a$; "with nature of a hemoprotein"), 50,000, and 54,000 ($P450_b$) molecular weight polypeptides in rats; induction of the first did not occur in mice. When BHT was administered to DBA/2N mice (which did not respond to 3-methylcholanthrene, an inducer of drug-metabolizing enzymes), hepatic enlargment and induction of cytochromes did not occur, although 5 of 10 of these mice died (Kawano, Nakao, and Hiraga 1980).

Ocular Effects

BHT inhibited the formation of cataracts in the rat lens after radical-induced damage from 4-hydroxynonenal. BHT pretreatment increased the lens capacity to detoxify 4-hydroxynonenal through the conjugation of the aldehyde with GSH (Srivastava et al. 1996). In addition, Ahmad et al. (1992) reported that dietary BHT increased concentrations of GSH and related enzymes (GST, GSH peroxidase, GSH reductase, γ-glutamylcysteine synthetase, and glucose-6-phosphate dehydrogenase) in the rat lens, retina, and cornea.

REPRODUCTIVE AND DEVELOPMENTAL EFFECTS

The Food and Drug Research Laboratories (FDRL 1972) evaluated the teratogenic potential of BHT using pregnant CD-1 mice. On gestational days (GDs) 6 to 15, the dams were treated with 180 mg/kg/day of the test material via oral intubation. Body weights were recorded on GDs 0, 6, 11, 15, and 17. Offspring were delivered on GD 17 via cesarean section, and the numbers of implantation sites, resorption sites, and live and dead fetuses, as well as the body weights of the live pups, were recorded. The urogenital tract of each dam was also examined for lesions of toxicity, and the fetuses were examined for gross, skeletal, and visceral abnormalities. Under the conditions of this study, BHT had no adverse reproductive or developmental effects.

In another teratogenicity study, 12 to 18 pregnant Dutch-belted rabbits per group were treated with BHT via oral intubation on GDs 6 to 18 (FDRL 1974). After dilution in anhydrous corn oil, the doses were 3.20, 14.9, 69.1, and 320.0 mg/kg BHT. The positive control was 2.5 mg/kg 6-aminonicotinamide, which was administered on GD 9. Body weights were recorded on GDs 0, 6, 12, 18, and 29, and the dams were observed daily for appearance and behavior. On GD 29, the offspring were delivered by cesarean section, and the numbers of corpora lutea, implantation sites, resorption sites, and live and dead fetuses were determined. One dam of the control group and three, four, and three dams of the 14.9-, 69.1-, and 320.0-mg/kg dose groups, respectively, died or aborted before GD 29. The live pups were weighed, killed, and examined for external, visceral, and skeletal abnormalities. The ratio of resorptions to the number of implantation sites per dam was increased in the test groups compared to the sham control group, but the number of skeletal and visceral abnormalities was not significantly different. The investigators concluded that BHT was nonteratogenic, although it caused systemic toxicity.

During a chronic study, six female rhesus monkeys were fed a diet containing 50 mg/kg/day BHA and 50 mg/kg/day BHT (total feed intake = 300 g/day; antioxidant content = 0.1% of diet) for 2 years. At 1 year, the monkeys were bred (one animal of the test group failed to conceive). Six control animals were fed a diet without antioxidants. The monkeys were observed daily for clinical signs of toxicity. The monkeys were weighed monthly. Hematologic studies were performed monthly prior to mating, then were performed on GDs 40, 80, 120, and 160, and days 30 and 60 postpartum. The maternal animals had no abnormalities during dosing and hemograms, blood chemistry parameters, feed consumption, and body weights did not differ from controls. Total body weights of the test offspring were slightly decreased compared to those of untreated monkeys; however, body length and head circumference were not affected. None of the experimental offspring had behavioral abnormalities during the first month after birth. All of the maternal monkeys and four of the offspring (the fifth died at 4 months of age from an illness unrelated to the exposure to BHA and BHT) were observed for 2 years after antioxidant exposure. None of these animals had abnormalities that were attributed to the test compounds, and

the adult females all gave birth to normal offspring during this period (Allen 1976).

A multigeneration reproductive and developmental toxicity study using Crj:CD-1 mice was performed by Tanaka, Oishi, and Takahashi (1993). Ten mice per sex per group were fed 0.015%, 0.045%, 0.135%, or 0.405% BHT or a control diet from 5 weeks of age of the F_0 generation to the weaning of the F_2 generation. During the second week of lactation, one dam died in each of the 0.015% (F_0), 0.045% (F_0), and 0.015% (F_1) test groups. No consistent significant effect was observed on the number of litters, sex ratio, litter size, or litter weight at birth for each generation. Increased litter weights were reported at birth and during lactation for pups of the 0.015% groups and during lactation for F_2 pups of the other treatment groups. Neurobehavioral parameters were not statistically different from controls with the exceptions of increased incidences of surface righting at postnatal days (PNDs) 7 and 4 in the F_1 and F_2 generations, respectively, and negative geotaxis at PND 7 in the F_2 generation. The investigators concluded that 0.015% to 0.405% BHT had little adverse effect on reproductive and neurobehavioral parameters in mice.

Meyer and Hansen (1980) fed 99.5% pure BHT to Wistar rats at a dose of 500 mg/kg/day (0.5% to 0.9% of the diet) from 6 weeks of age of the F_0 to the weaning of the F_1 generation. The F_0 generation consisted of 46 rats per sex; 40 litters/group were produced. Body weights of F_0 males and females were decreased significantly compared to controls. During the first 7 days of gestation, test females had significantly decreased body weight gain that persisted nonsignificantly throughout gestation. The duration of pregnancy, average birth weight, and average litter size did not differ among groups, but a slightly decreased proportion of females/litter was reported. Half of the litters were cofostered such that nondosed dams nursed pups of dosed dams, and vice versa. Pups of dosed dams suckled by nondosed dams had a slightly, relatively slower development than pups of the control group. Pups nursed by dosed dams had delayed development, decreased body weight, and decreased weight gain, as well as "hyperactive behavior" when handled during the first week of lactation. At microscopic examination of the brain, slightly increased incidences of the average number of dead cells were observed in the cerebellum of pups suckled by dosed dams. The investigators suggested that the effect on F_1 offspring was "either by a decreased nutrition due to BHT's influence on the lactation of the F_0 females, BHT's influence on the suckling ability of the F_1 rats or both in combination" (Meyer and Hansen 1980).

In a dose-finding study, Wistar rats were fed 500 to 1000 mg/kg/day BHT (99% pure) from 5 weeks of age of the F_0 to weaning of the F_1 generation. The F_0 consisted of 8 males and 64 females. At birth, the body weights of the pups did not differ from controls, but the weights decreased during lactation. Four weeks after the pups were weaned, body weights were still significantly decreased compared to that of controls. In the main study, rats of the F_0 generation were fed 25 to 500 mg/kg/day BHT. The F_1 offspring were weaned and fed for 22 weeks diets with the same concentrations as the respective parents, with the exception of the high-dose group, which was fed 250 mg/kg/day BHT. The only significant finding was decreased weight gain of pups during lactation, and the investigators suggested that the retardation of body weight gain occurred due to inadequate milk production (McFarlane et al. 1997).

Telford, Woodruff, and Linford (1962) reported that a diet containing 0.5 g BHT decreased the incidence of resorptions compared to a control diet, when fed to pregnant Walter Reed–Carworth Farms rats. In this study, 11 dams of the test group produced 121 normal fetuses (124 implantations), and had 2.4% resorptions in 18.2% of the litters. In the control group, 40.8% of the 126 dams had one or more resorptions, and 1278 normal fetuses (1430 implantations) were produced. The incidence of resorptions was 10.6%.

At a concentration of 0.0125% to 1.55% in low–vitamin E feed, BHT did not prolong gestation or increase mortality in pregnant rats (11/group) compared to a control diet. Concentrations up to 0.313% did not adversely affect the mean litter size, viability index, and lactation index. Dams fed the high dose had drastic weight loss that was associated with fetal deaths. Litter efficiencies were 100% for the control, 0.0125%, and 0.0625% treatment groups, 91% for the 0.313% group, and 0% for the 1.55% group (Ames et al. 1956).

Hinton et al. (1990) fed 25 to 1000 mg BHT/kg body weight daily to male and female rats prior to mating and during gestation and lactation. The high dose caused a slight decrease in number of pregnant females, but BHT had no other effects on dams or pups up to the time of birth. Pups of dams fed $\geq$500 mg/kg/day BHT had marked weight loss at weaning and when fed control diet for 4 weeks after weaning, this weight loss was "markedly ameliorated" in smaller litters. No pathologic changes were observed in the pups, and dams had no evidence of malnutrition.

Dietary BHT (0.1% to 0.5%) did not cause anophthalmia in offspring of six treated albino mice (Johnson 1965). In the high-dose group, the length of gestation was increased and mean pup weight, mean total weight of the litters, and the mean number of live pups were decreased compared to controls. At a concentration of 0.1%, BHT had no adverse effects on the above parameters.

In a multigeneration study using Sprague-Dawley rats, the reproductive and developmental no-observable-effect level (NOEL) was 1000 ppm BHT, when the antioxidant was added to a high-fat diet from 100 days of age of the F_0 generation to weaning of the F_2 generation. For each generation, 16 females and 8 males were mated. After treatment with 3000 ppm BHT, growth rates were decreased by 10% to 20% for both dams and offspring, and serum cholesterol and relative liver weight were both increased by 20% for dams (Frawley et al. 1965).

BHT was nonteratogenic when fed to female Evans No. 1 and ICI SPF mice, or Tuck albino, Benger hooded, Carworth SPF albino, and Porton SPF albino rats at doses of 250 to 1000 mg/kg/day both prior to mating and during gestation, or during gestation alone (Clegg 1965). Pregnant diabetic Sprague-Dawley rats fed 1% BHT had decreased incidences of fetal

malformations compared to offspring of diabetic rats fed the control diet. In the same study, malformations did not occur in offspring of normal rats fed BHT (Eriksson and Simán 1996).

Han et al. (1993) reported that 300 mg/kg/day BHT in corn oil caused an increased incidence of sternebral retardation in offspring of Sprague-Dawley rats after treatment via gavage on GDs 7 to 17. At concentrations up to 400 mg/kg/day, BHT did not affect maternal performance, fetal development, maternal body weight gain, or feed consumption. BHT also did not induce significant external or visceral abnormalities in the offspring, which were examined on GD 20. The antioxidant did, however, increase the relative weight of the liver at high doses.

In a study using rat testis microsomes, phenolic plasticizing agents/antioxidants inhibited Ca^{2+}-ATPase activity (intracellular Ca^{2+} pumps), thereby affecting testicular development via the disruption of homeostatic processes. The IC_{50} of BHT was 0.6 μM. For other phenolic agents, the IC_{50} values ranged from 8×10^{-4} μM for thapsigargin to 10.6 μM for vanadate (Michelangeli et al. 1996).

Behavioral changes were observed in offspring of Swiss-Webster mice fed 0.5% BHT or BHA (Stokes and Scudder 1974). Offspring exposed to BHT both pre- and postnatally had decreased sleeping, increased social and isolation-induced aggression, and a severe deficit in learning during avoidance conditioning studies.

BHT at a concentration of 0.5% in the diet of Sprague-Dawley rats decreased body weights of both dams and pups. Eyelid opening, surface-righting development, and limb coordination in swimming males were delayed, and open-field ambulation was decreased in females. Smaller doses of BHT caused irregularities in maternal body weight, but did not affect the weights of offspring. Pre- and periweaning mortality was 23% in the 0.25% dose group, but doses of 0.125% to 0.25% BHT had no effect on physical or behavioral development or postweaning behavior (Vorhees et al. 1981).

When pregnant CD-1 mice (2–4/group) were injected IP with 1 mg/kg *N*-methyl-*N*-nitrosourea (MNU; a carcinogen, mutagen, and teratogen) on GD 16, the offspring had progressive retinal and cerebellar degeneration from 4 weeks or 12 to 16 weeks of birth, respectively. The progression of retinal degeneration was accelerated by constant fluorescent light exposure and retarded by constant darkness. When 0.75% BHT was added to the feed from GD 17, so that the offspring were exposed both pre- and postnatally, degeneration was reduced by week 8, compared to that observed after treatment with MNU and the control diet. BHT, however, induced sporadic morphologic changes; the incidences of such changes were independent of MNU exposure. The retinas had circular configurations of ganglion cells and arcades of nuclear and plexiform layers. A hyperplastic nodule was observed in one mouse that was treated with feed supplemented with BHT, but was not injected with MNU. The cerebellar regions of the brain had cellular proliferation and prolonged developmental activity after treatment with BHT (Smith and Yielding 1987; Smith, Cooke, and Yielding 1988, 1989).

Microsome-mediated embryotoxicity induced by superoxide radicals was decreased by the addition of BHT (Iannaccone 1986). The embryos were BALB/c or ICR mice and the microsomes were isolated from the livers of Sprague-Dawley rats. An amount of 0.001 mg/ml BHT resulted in 100% viability in the presence of microsomes and cofactors after 2 hours, and 33% viability after 18 hours. At a concentration of 0.025 mg/ml, BHT was toxic to the blastocyst-stage embryos, and a concentration of 0.075 mg/ml killed all of the embryos after a 2-hour incubation period.

IMMUNOLOGIC EFFECTS

The in vivo immunotoxicity of BHT was investigated using male mice (Moon et al. 1987). The mice were treated with BHT at doses of 5 and 50 mg/kg, dissolved in soybean oil, that were administered orally for 10 days. ICR mice were used to determine the blood count for circulating leukocytes; actual and relative body, spleen, thymus, and liver weights; in vivo intravascular phagocytosis by macrophages and Kupffer cells; and delayed hypersensitivity and arthus reactions. For the latter assays, the mice were sensitized with subcutaneous (SC) injections of bovine serum albumin (BSA) emulsified in Freund's complete adjuvant. Seven days later, the mice were challenged with heat aggregated BSA in saline, and the degree of footpad swelling thickness was measured. CBA/J mice were used for an immunoglobulin M (IgM) plaque-forming cells assay. BHT decreased significantly the circulating leukocyte count by 83.6% and 81.8%, respectively, for the two doses. The antioxidant also caused significant liver hypertrophy. Relative spleen and thymus weights were not affected significantly, although decreases were observed. The amount of splenic IgM plaque forming cells was decreased by 80.4% and 61.1%, respectively. The investigators concluded that direct cytotoxicity was not responsible for the depressed antibody response, as splenic cellular populations were not affected by treatment. BHT had no effect on the arthus reaction, phagocytosis, or delayed hypersensitivity.

Male C57BL/6 mice were treated topically on 5 consecutive days with 50 μl of a 0.001 g BHT/dl 95% ethanol solution (0.5 μg/application). The treatment sites were either the shaved back or the dorsal surface of the external ear. Skin biopsies were obtained on day 6, and the population density (cells/mm^2) of Langerhans cells and Thy-1^+ dendritic epidermal cells was determined. Treatment did not produce significant alterations in the number of I^a+ Langerhans cells, although BHT increased the density of dendritic epidermal cells by 49% compared to vehicle-treated controls. BHT enhanced expression of the Thy-1 antigenic marker on keratinocytes. No evidence of tissue injury, inflammation, or mononuclear infiltrate was observed at the treatment sites. When the BHT-treated mice were sensitized to dinitrofluorobenzene and challenged using an ear-swelling assay, no significant differences were noted in contact hypersensitivity compared to controls (Rheins et al. 1987).

The effect of antioxidants, including BHT, on the immunoglobulin production regulation by arachidonic acid was

investigated by Yamada et al. (1996) using cultured rat mesenteric lymph node lymphocytes. Arachidonic acid stimulated IgE production, which was also enhanced by the presence of unsaturated fatty acids. In this study, BHT inhibited the stimulation of IgE production by arachidonic acid, thereby partially "annulling" the adverse effect of the fatty acids.

Lymphocytes were obtained from peripheral blood of 32 donors. When the cells were incubated in 60.0 μg/ml BHT, no change in the viability of the cells was observed; however, the addition of 100 μg/ml decreased viability significantly ($p < .01$). BHT alone at concentrations of 0 to 60.0 μg/ml did not affect lymphocyte stimulation (determined by thymidine uptake); but at a concentration of 50 μg/ml, it suppressed a mixed lymphocyte reaction. A synergistic effect was observed with regard to suppression of phytohemagglutinin-stimulated lymphocytes when the cells were incubated with BHT and either cortisol or prednisolone (Klein and Bruser 1992).

The suppression of in vitro T cell–dependent humoral immunity by antioxidants was investigated by Kim et al. (1996) using spleen cells from SPF BALB/c mice. BHT was dissolved in 1, 10, and 100 mM DMSO and diluted in medium for final concentrations of 0.1, 1, and 10 μM. Using a modified suspension hemolytic assay, the investigators found that BHT suppressed the T cell–dependent B-cell response, but not the T cell–independent and polyclonal B-cell responses. The data indicated that BHT did not directly suppress B cells; rather, humoral immunity was inhibited by suppression of regulation of T cells or by the action of macrophages on B cells.

Female C3HeB/FeJ mice were fed a diet with 0.25% BHT. After approximately 30 days, the mice were injected IP with 0.1 cc of a 20% suspension of sheep red blood cells (SRBCs). The mice were killed 5 days after the injection and the spleen assayed for the cells secreting anti-SRBC antibody. BHT enhanced significantly this humoral immune response compared to control groups (Harman, Heidrick, and Eddy 1977).

In the Mishell-Dutton system, the dose of BHT that caused $\geq$90% suppression of the plaque-forming cell response (to sheep erythrocytes) was 50 μg/culture. The dose that caused a 50% reduction in viability at the end of the 5-day culture period was $\simeq$150 μg/culture (Archer 1978).

BHT (50 μg/ml) was added to a culture of splenic lymphocytes from BDF_1 mice. The cultures were exposed to SRBCs and direct plaque-forming cell (PFC) assays were performed on day 5 of the culture. BHT suppressed the in vitro PFC response to the thymus-dependent antigen SRBC. A partial reversal of this suppression occurred when 1 to 2 mM of N^2,O^2-dibutyryl quanosine 3′:5′-cyclic monophosphoric acid (dbc-GMP) and 3 to 10 mM of Ca^{+2} were added (Wess and Archer 1982).

PHOTOPROTECTIVE EFFECTS

Although they did not prevent UV-induced cytotoxicity in Chinese hamster ovary (CHO) cells (Chan and Black 1977), antioxidants protected against UV-induced skin erythema in various studies.

In one study, 55 SKH-Hr-1 hairless mice per group were fed basal diet, with or without supplementation with 0.5% BHT. At the end of the 2-week feeding period, eight of those mice/group were used in an optical transmission study to investigate the effect of BHT on the transmission of UV radiation through the stratum corneum. The remaining 42 mice were used in the ornithine decarboxylase (ODC) assay to determine the relative contribution of stratum corneum absorbance to the inhibitory effect of BHT upon ODC activity induction. When the stratum corneum was irradiated with UV light at 280 to 320 nm, ~65% more erythemic radiation was required to produce a similar response in BHT-treated mice as that of controls. BHT inhibited UV-induced ODC activity by ~70% in the intact (nonstripped) stratum corneum of BHT-fed mice. When the stratum corneum was stripped prior to UV irradiation, however, ODC activity did not differ among test animals and nonstripped controls. ODC activity did not differ significantly between stripped stratum corneum from BHT-fed and control mice (Koone and Black 1986).

BHT (0.5%) was fed to female, albino hairless mice for 2 weeks and then the mice were irradiated with a QA-450N mercury lamp. The lamp was positioned 50 cm from the dorsum of the animals and delivered 0.23 mJ/m^2. BHT was effective in allaying the erythemic response to UV light. The protective index (PI) for BHT was approximately 2.0. BHT provided a PI of 1.26 when 200 μl (1% solution) was applied to the dorsal skin of mice 30 minutes before irradiation. BHT was more effective at preventing UV light–induced erythema when administered systemically as opposed to topical administration (De Rios et al. 1978).

In another study (Black et al. 1980), the maximum amount of BHT in the skin of female, hairless, SKH-Hr-1 mice was determined after feeding of 0.5% BHT for 12 weeks (recovery = 83%). Continuous feeding of BHT resulted in increased BHT in the skin until week 6, after which the concentration decreased. The greatest amount in the skin was 44 μg/g wet skin. To determine the effects of BHT on UV absorbance, two groups of mice were fed either a control diet or BHT-supplemented diet for 2 weeks. The mice were killed and epidermis samples were taken from 5- to 10-cm^2 pieces of dorsal skin. Forward scattering scans from 250 to 400 nm were obtained with a recording spectrophotometer (with a diffuse reflectance sphere). The average difference spectrum was determined by subtracting control absorption values from those of the BHT group at 5-nm intervals. The dose reduction factor was calculated using the following formula:

$$\frac{\sum_{I=1}^{N} \int_i S(\lambda) I_0(\lambda)\, d\lambda}{\sum_{I=1}^{N} \int_i S(\lambda) I_0(\lambda) 10^{-A(\lambda)}\, d\lambda} = \text{DRF}$$

where A = reference spectral absorbance curve for 10^{-4} M BHT (in ethanol) from 240 to 320 nm for greatest [BHT] in skin; I_0 = relative spectral intensity of radiation source (based on manufacturer's specifications); S = erythema action spectrum.

This was done for both a GE UA-3 source and a solar simulator spectrum. The investigators concluded that, based on the dose reduction factor, BHT in the skin would absorb no more than 1% of the incident UV. The forward scattering transmission by the epidermis from BHT-treated mice, however, was significantly less than that of the epidermis from control mice. Significant increases of absorption occurred at week 2 in BHT-fed mice when the skin was irradiated with 250- to 300-nm UV light, when the amount of BHT in the skin was approximately one-half that found at week 6. Despite the increases, 33% more radiation was required to produce a similar erythematous response in BHT-fed animals compared to controls.

Black et al. (1984) used female, albino, hairless mice (SKH-Hr-1) in studies to determine if BHT promoted epidermal proliferation, which would result in inhibition of UV-induced ODC activity. First, metabolism studies were performed after feeding of 0.5% BHT for 17 days, and the amount of BHT in the skin was determined using TLC and autoradiography. UV-dose dependence of ODC induction was determined after the mice were fed either a control diet or BHT-supplemented diet for 2 weeks. The mice then received graded doses of UV from two FS-20 sunlamps. At 28 hours after irradiation, ODC activity was "linearly responsive" from 0.3 to 0.6 J/cm^2/dose. Next, the time relationship of BHT inhibition was determined. A dose of 400 mg/kg BHT was administered IP at various times prior to UV induction. The animals were killed 28 hours after irradiation and the ODC assay was performed. In these studies, both dietary and IP administration of BHT resulted in inhibition of ODC activity; the degree of inhibition was approximately constant over the above UV dose range. The greatest inhibition occurred when BHT was administered (IP) at least 46 hours prior to irradiation, as the inhibitory events required >20 hours to become effective. In other assays, however, BHT had no effect on the incorporation of [^{3}H]-thymidine or leucine into DNA or protein, respectively, during this time interval. BHT also did not affect the epidermal labeling index. The data indicated that BHT did not induce epidermal proliferation. Instead, the antioxidant properties of the compound could have retarded catabolic processes of the epidermis, therefore changing the optical properties of the stratum corneum.

BHT provided protection against UV-induced erythema, giving a sun protection factor (SPF) of 2 for the skin of hairless mice. Spectral absorbance was increased in the skin of BHT-treated mice compared to controls. BHT did not affect unscheduled DNA synthesis, protein synthesis, or the cell labeling index, suggesting that the antioxidant did not induce cell proliferation. The photoprotective effects of BHT, therefore, were unrelated to increased epidermal thickness. The investigator concluded that the amount of UV radiation reaching the target site was decreased. Spectral transmission measurements of isolated stratum corneum from control mice was ~65% greater than that of BHT-fed mice. When the stratum corneum was first removed by tape-stripping prior to irradiation with UVB, ODC activity in BHT-treated mice did not significantly differ from that of the control group. In contrast, ODC activity was decreased by ~70% in the epidermis of BHT-fed, non–tape-stripped mice (Black 1988).

Roshchupkin, Pistsov, and Potapenko (1979) studied the inhibition of light-induced erythema by BHT in rabbits. The skin was irradiated for 12 to 60 seconds in the 280- to 365-nm wavelength region. The UV light source was a super-high-pressure mercury vapor lamp SVD-120A with two liquid filters, composed of aqueous solutions of $NiSO_4$ and $CoSO_4$ and a 10% aqueous solution of KI. BHT applied 60 minutes before and 2 minutes after irradiation inhibited the erythemal response in skin to UV light, increasing the minimal erythemal dose (MED) by 50%.

In a psoralen photochemotherapy (PUVA) tumorigenesis study using SKH-Hr-1 mice, dietary BHT (0.5%) reduced induction of ODC activity by 40%. Edema increased in skin treated with both 8-methoxypsoralen (8-MOP) and UVA and reached a peak at 40 hours, and erythema peaked at 48 to 72 hours post irradiation. BHT had no detectable effect on skinfold thickness, and no apparent effect on 8-MOP/UVA-induced erythema (Black, Young, and Gibbs 1989). See "Carcinogenicity" for study details.

Väänänen and Hannuksela (1989) investigated the photoprotective and antierythematous effects of BHT and other compounds using four female and six male subjects. The test substances were incorporated in *o/w* creams that contained 65% water, 8% glycerol, and 27% cetearyl alcohol/sodium cetearyl sulfate; 50 mm^3 doses were used to fill large Finn Chambers, which were fixed to the back for 1, 4, or 24 hours prior to UVB irradiation (Waldmann UV 6002; maximum output 311 nm). Just before irradiation with three times the MED, 0.29 to 1.14 J/cm^2, the test substances were removed. The creams were reapplied 1 and 4 hours after irradiation, and removed after 19 and 16 hours, respectively. The test sites were graded visually 24 hours after irradiation, from no reaction (−) to intensive erythema and edema (+++). The sites were also measured using a laser Doppler flowmetry device both before and 24 hours after irradiation. Under the conditions of this study, 0.1% and 1.0% BHT did not reduce UVB-induced erythema. When BHT was applied 24 hours prior to irradiation, the intensity of erythema was >120% of the control value, and when applied up to 4 hours before irradiation, the intensity was approximately the same as the control value. The visual evaluations were not reported.

Embryonic human diploid lung firbroblasts were exposed to BHT a week prior to irradiation. The cells were washed 24 hours prior to irradiation with a tungsten filament source (G.E. PAR-64). The light was passed through a water bath 0.58 m long and cells were exposed to >300 nm. Cells grown in the presence of 2.5 to 100 μg/ml BHT had decreased photosensitivity (increased percent survival) at a light dose of 3 kW/m^{2}h (Pereira, Smith, and Packer 1976).

GENOTOXICITY

Bomhard, Bremmer, and Herbold (1992) reviewed numerous genotoxicity studies on BHT and concluded that BHT was not "a relevant mutagenic/genotoxic risk to man." BHT was

nonmutagenic in *Salmonella typhimurium, Escherichia coli*, with or without metabolic activation, and *Drosophila melanogaster*. Host-mediated assays in mice using *S. typhimurium* were negative. BHT did not induce forward mutations in mouse lymphoma cells, rat liver epithelial cells, or EUE cells (derived from human heteroploid epithelial-like cells). In Chinese hamster V79 fibroblasts, BHT was mutagenic only at cytotoxic doses. Point mutations were not induced in germ cells of mice fed the antioxidant. BHT was typically nonclastogenic in mammalian and plant cells, and did not induce dominant lethals, sex-linked recessive lethals, or heritable translocations. During rec-assays using *Bacillus subtilis*, DNA damage occurred only after addition of S9 rat liver homogenate. BHT did not cause gene conversion in *Saccharomyces cerevisiae*, with or without metabolic activation using lung, testes, and/or liver homogenates from rats, mice, and monkeys. The antioxidant did not affect the mitotic recombination frequency in a host-mediated assay in mice using *S. cerevisiae*, and did not induce DNA repair in *E. coli* during SOS chromotests. BHT inhibited DNA repair synthesis after human lymphocytes were irradiated with UV, and inhibited semiconservative DNA synthesis in nonirradiated cells. It did not affect excision repair and postreplication repair of DNA in an aneuploid clonal cell line. BHT did not induce sister-chromatid exchanges in CHO cells or lung fibroblasts. The antioxidant also increased DNA synthesis in the urinary bladder of treated rats and in isolated rat hepatocytes. These studies and others are described in Table 8.

EFFECT ON GENOTOXICITY OF OTHER AGENTS

BHT and its derivatives also modified the genotoxicity of other agents (Table 9), including X-rays and various chemicals (Stich 1991). Data from several studies suggested that the antimutagenic activity of BHT occurred via alterations of mutagen metabolism by liver microsomes, the combination with free radicals to form stable complexes, or by a reduction of peroxidative damage of DNA (Shamberger 1974; McKee and Tometsko 1979; Wei, Whiting, and Stich 1981; Hrelia et al. 1987).

McKee and Tometsko (1979) reported that BHT reduced the frequency of reversion mutations caused by mutagens that required metabolic activation, but not by direct-acting mutagens. Examples included mouse lymphoma cells which were made radiosensitive by treatment with >0.0001 g% BHT. BHT-treated rats given DEN and 2-AAF had an increase in the number of diploid nuclei after 3 to 5 months of treatment (Kamra et al. 1973). In Ames and modified Ames assays, BHT increased the frequency of revertant colonies induced by 2AAN; decreased the mutagenicity of 4NQO, AF-2, MMS, 2-AAF, anthraquinones, γ-rays, pyrolyzates of albumin, cigarette smoke, nitrogen oxides, DMBA, and other mutagens; increased the mutagenicity of DCB; and both enhanced and inhibited the mutagenicity of aflatoxin B_1 (AFB_1) and benzo[a]pyrene (BP). BHT protected against EMS, but not against x-rays, in a study using mice. In other studies, BHT inhibited the mutagenic and clastogenic effects of DMBA and sodium cyclamate in human cells, decreased the frequency of chromosome aberrations in plant seeds treated with γ-rays and CHO cells treated with bleomycin, protected against EMS-induced mutagenesis in *Drosophila*, and prevented 2-AAF–induced DNA damage in human and rat hepatocytes (McKee and Tometsko 1979).

Rahimtula, Zachariah, and O'Brien (1977) investigated the effects of BHT and BHA on NADPH-dependent menadione reduction and benzo[a]pyrene hydroxylation. Both antioxidants inhibited benzo[a]pyrene hydroxylation, but did not have any effect on the flavoprotein, indicating that BHT and BHA have a direct effect on cytochrome P450. These investigators also found that BHA, 100 μM in the presence of the microsomal fraction and NADPH, in *S. typhimurium* strain TA98 inhibited the mutagenicity of benzo[a]pyrene.

CARCINOGENICITY

During an oral carcinogenesis assay, 1% and 2% concentrations of BHT were added to the feed of $B6C3F_1$ mice for 104 weeks. The mice were examined for signs of tumorigenesis 16 weeks after the BHT feeding period. The control group and two treatment groups were each comprised of 50 mice/sex. The average daily intakes of BHT were 59 and 57 mg/day for males and females fed 1% BHT, respectively, and were 116 and 118 mg/day for males and females of the high-dose group. Males had a dose-related change in survival throughout the study, but no difference in survival occurred between treated and control females until week 88, when the survival of BHT-treated mice increased compared to controls. At week 104, the percent survivals for males were 74% (high-dose), 64% (low-dose), and 40% (control). For females, the values were 89%, 81%, and 58%, respectively. Of the mice, 89% of BHT-treated males, 64% of control males, 91% of BHT-treated females, and 82% of female controls were considered effective and incorporated into the results. Females treated with BHT had a significantly decreased incidence of neoplasms compared to controls, and the survival time of mice with neoplasms was increased. Survival times of males did not differ between treatment and control groups. The control incidences of neoplasms were 84% in males and 85% in females, and mice of this group survived for an average of 108 to 109 weeks. For mice of the low-dose group, 86% and 75% of males and females had neoplasms, respectively. The mean survival times were 110 and 114 weeks, respectively. For males and females of the high-dose group, 81% and 55% had neoplasms, respectively. The mean survival times were 114 and 118 weeks, respectively. Neoplasms of the liver, lungs, hematopoietic system, integumentary system, reproductive system (females), pancreas, esophagus, and small intestine were detected; however, only the incidence of hepatocellular adenomas in males of the high-dose group was significant (Inai et al. 1988).

Shirai et al. (1982) fed $B6C3F_1$ mice (50/sex/group) 200, 1000, or 5000 ppm BHT for 96 weeks, followed by basal feed for 8 weeks. Females fed the two highest doses and males given the high dose had decreased body weight gain, although feed consumption by both sexes did not differ from controls. BHT

TABLE 8
BHT genotoxicity

Assay type	Result	Reference(s)
	In vitro	
DNA cleavage assay, activated by copper	Genotoxicity	Li and Trush 1994
DNA cleavage assay		
BHT	No genotoxicity	Nagai, Ushiyama, and Kano 1993
BHT-COOH	No genotoxicity	
BHT-OH	No genotoxicity	
BHT-QM	No genotoxicity	
BHT-quinol	No genotoxicity	
BHT-quinone	Genotoxic	
Excision repair and semiconservative synthesis inhibition assays	Genotoxic	Daugherty, Davis, and Yielding 1978
	In vitro bacteria	
SOS chromotest, ±S9, in *E. coli* PQ37	No genotoxicity	Brams et al. 1987
	No genotoxicity	von der Hude et al. 1987
	No genotoxicity	Potenberg et al. 1988
	No genotoxicity	von der Hude et al. 1988
Spot test, TA98, TA100, TA1535, TA1537		
With S9	Genotoxic	Rao and Aiyar 1975
Without S9	No genotoxicity	
Host-mediated assay *S. typhimurium* G-46 and TA-1530 and *S. cerevisiae* D-3, BHT at 5% *w/v*	No genotoxicity	Stanford Research Institute 1972
E. coli reversion assay, ±S9, strains Sd-4-73, WP2 hcr trp	No genotoxicity	Szybalski 1958; Ohta et al. 1980
S. typhimurium/mammalian microsome assay, ±S9, strains	No genotoxicity	Litton Bionetics, Inc. 1975
TA92, TA94, TA97, TA98, TA100, TA102, TA104,	No genotoxicity	Fujita, Kojima, and Hiraga 1977
TA1535, TA1537, TA1538	No genotoxicity	Joner 1977
	No genotoxicity	Bruce and Heddle 1979
	No genotoxicity	Bonin and Baker 1980
	No genotoxicity	Kawachi et al. 1980
	No genotoxicity	Shelef and Chin 1980
	No genotoxicity	Kinae et al. 1981
	No genotoxicity	Morita, Ishigaki, and Abe 1981
	No genotoxicity	Reddy et al. 1983
	No genotoxicity	Ishidate et al. 1984
	No genotoxicity	Mortelmans et al. 1986
	No genotoxicity	Hageman, Verhagen, and Kleinjans 1988
	No genotoxicity	Williams, McQueen, and Tong 1990a
	No genotoxicity	Yoshida 1990
Rec-assay, *B. subtilis*	No genotoxicity	Fujita, Kojima, and Hiraga 1977
	No genotoxicity	Ohta et al. 1980
	No genotoxicity	Kinae et al. 1981
	No genotoxicity	Morita, Ishigaki, and Abe 1981
Rec-assay, *B. subtilis* , +S9	Genotoxicity	Hirano et al. 1978
	Genotoxicity	Kawachi et al. 1980
	In vitro mammalian	
S. cerevisiae/mammalian microsome assay, ±S9,	No genotoxicity	Litton Bionetics, Inc. 1975
strain D4 micronucleus test, mice	No genotoxicity	Bruce and Heddle 1979
	No genotoxicity	Paschin, Bakhitova, and Benthen 1986

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TABLE 8
BHT genotoxicity *(Continued)*

Assay type	Result	Reference(s)
HGPRT assay, Chinese hamster V79 lung cells	Same response as TPA	Trosko et al. 1981
HGPRT assay, Chinese hamster V79 cells	Genotoxic; at cytotoxic doses	Paschin and Bahitova 1984
HGPRT assay, F344 rat hepatocytes	No genotoxicity	Williams, McQueen, and Tong 1990a
DNA repair test, F344 rat hepatocytes	No genotoxicity	Williams, Mori, and McQueen 1989
	No genotoxicity	Williams, McQueen, and Tong 1990a
Immunologic DNA synthesis–inhibition assay,[a] HeLa S3 cells	$DI_{50} = 500\ \mu M$[b]	Heil and Reifferscheid 1992
Metabolic cooperation assay, Chinese hamster V79 cells	—	Iwase et al. 1991
Metabolic cooperation assay, Chinese hamster V79 cells	—	Bohrman et al. 1988
Forward mutation assay, ±S9, L5178Y tk^+/tk^- mouse lymphoma cells	Genotoxic	McGregor et al. 1988
Chromosome aberration and sister-chromatid exchange assays, ±S9, CHO cells	No genotoxicity	Galloway et al. 1987
Chromosome aberration assay, CHO cells	Weak mutagen	Patterson, Keith, and Stewart 1987
Chromosome aberration assay, rat bone marrow	No genotoxicity	Kawachi et al. 1980
Cytogenetic assay, rat bone marrow; 30, 900, 1400 mg/kg	No genotoxicity	Stanford Research Institute 1972
Human embryonic lung cells WI-38; 2.5, 25, 250 μg/ml BHT and 0.05 μg/ml TEM	Genotoxic	
Chromosome aberration assay, CHO cells	Genotoxic; at cytotoxic doses	Grillo and Dulout 1995
Anaphase-telophase alteration assay, CHO cells	No genotoxicity	
Sister-chromatid exchange assay, CHO cells and human lymphocytes	No genotoxicity	
Chromosome aberration assay, LAF_1 mice	No genotoxicity	Harman, Curtis, and Tilley 1970
DNA synthesis assays, rat urinary bladder (treated in vivo)	DNA synthesis induced	Marigo et al. 1985
and isolated rat hepatocytes (treated in vitro)	DNA synthesis induced	Shibata et al. 1989
Sister-chromatid exchange assay, DON hamster cells,	No genotoxicity	Abe and Sasaki 1977
CHO cells, Chinese hamster lung fibroblasts	No genotoxicity	Kawachi et al. 1980
	No genotoxicity	Shelby and Stasiewicz 1984
	No genotoxicity	Williams et al. 1984
	No genotoxicity	Ennever and Rosenkranz 1986
	In vitro plant	
Chromosome aberration assay, onion	No genotoxicity	Tandon and Kaul 1961
	No genotoxicity	Alekperov, Abutalybov, and Bagirova 1975
	No genotoxicity	Kaul and Zutshi 1977
	No genotoxicity	Kaul 1979
Chromosome aberration assay, onion (exposed to vapors for 10 days)	Genotoxic	Sax and Sax 1968
	In vivo–in vitro	
In vivo–in vitro replicative DNA synthesis assay, F344 rats		
450 mg/kg for 24, 48 h	No genotoxicity	Uno et al. 1994
450 mg/kg for 39 h	Equivocal	
900 mg/kg for 48 h	No genotoxicity	
900 mg/kg for 24, 39 h	Genotoxicity	

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TABLE 8
BHT genotoxicity *(Continued)*

Assay type	Result	Reference(s)
In vivo–in vitro replicative DNA synthesis assay, B6C3F$_1$ mice		
500 mg/kg for 24, 48 h	No genotoxicity	Miyagawa et al. 1995
500 mg/kg for 39 h	Genotoxicity	
1000 mg/kg for 24, 39 h	No genotoxicity	
1000 mg/kg for 48 h	Genotoxicity	
In vivo		
Dominant lethal assay, Sprague-Dawley rats	Genotoxic	Sheu et al. 1986
Dominant lethal assay, hybrid mice[c]	No genotoxicity	
Heritable translocation assay, three strains of mice	No genotoxicity	
His G46, C207, C3076 host-mediated assay, Swiss mice	No genotoxicity	Rao and Aiyar 1975
DNA synthesis assay, F344 rats	No genotoxicity	Shibata et al. 1991
Dominant lethal assay, ICR/Ha and CD-1 Swiss mice	No genotoxicity	Epstein and Shafner 1968
	No genotoxicity	Epstein et al. 1972
Dominant lethal gene test, rats, single and multiple doses of 30 mg/kg, 0.9 g/kg, 1.4 g/kg BHT; +control = TEM	No genotoxicity	Stanford Research Institute 1972
Sex-linked recessive lethal test, chromosome translocation assay, *D. melangaster*	No genotoxicity	Kamra 1973
	No genotoxicity	Kamra and Rajaraman 1973
	No genotoxicity	Prasad and Kamra 1974
	No genotoxicity	Mazar-Barnett and Muñoz 1980
	No genotoxicity	Sankaranarayanan 1983
	No genotoxicity	Dellarco, Mavourin, and Tice 1985
	No genotoxicity	Ennever and Rosenkranz 1986

[a]Inhibition can lead to accumulation of DNA single- and double-strand breaks, chromosome aberrations, mutations in cells, DNA adducts, etc. (Heil and Reifferscheid 1992).
[b]DI_{50} = concentration that inhibited DNA synthesis by 50% (Heil and Reifferscheid 1992).
[c](101 × C3H)F$_1$ mated with (C3H × C57BL)F$_1$ or (SEC × C57BL)F$_1$ (Sheu et al. 1986).

had no adverse effect on survival rates, did not cause changes in hematologic parameters, and had no effect on features of serum and urine. Neoplasms were observed in the lungs, liver, lymph nodes, and spleen in both test and control mice, but were considered unrelated to BHT treatment.

In another study, investigators fed 3000 and 6000 ppm doses of BHT (purity = 99.9%) to F344 rats and B6C3F$_1$ mice. Each group consisted of 50 animals per sex per species. Control groups were 20 untreated animals per sex and species. The rats were treated for 105 weeks and the mice were treated for 107 to 108 weeks. All surviving animals were killed at the end of the dosing period. The mean body weights of the treated rats and mice were decreased compared to those of the corresponding control groups; the decreases were dose related. Survival was not affected significantly by treatment. In the treated rats, tumor incidences were not increased significantly compared to the corresponding control groups. The investigators concluded that BHT was not carcinogenic under the conditions of this study (NTP 1979).

Six of 18 male BALB/c mice fed 0.75% BHT for 12 months had marked hyperplasia of the hepatic bile ducts with associated subacute cholangitis. In the same study, proliferation of the bile duct epithelium did not occur in 64 untreated or 19 BHT-treated control mice given DEN in drinking water (total dose = 490 mg/kg body weight) for 7 weeks (Clapp, Tyndall, and Cumming 1973).

Würtzen and Olsen (1986) fed food additive–grade BHT to Wistar rats of both sexes. F_0 rats were fed 25, 100, and 500 mg/kg/day BHT, respectively, from 7 weeks of age to weaning of the F_1 generation. F_0 rats were given the control diet alone. The rats were mated after 13 weeks of dosing and the male F_0 rats were discarded. The female F_0 rats were discarded after weaning. In a previous study (Meyer, Blom, and Olsen 1978), BHT had an adverse effect on the kidneys of F_0 females, so the high dose for the F_1 generation was decreased to 250 mg/kg/day. Body weight and feed consumption were recorded regularly, analysis of blood samples were performed, and all F_1 rats were examined for the presence of neoplasms. The rats were killed at 141 to 144 weeks of age and were examined for gross and microscopic lesions.

No differences in feed consumption were observed between BHT-treated and control rats of the F_0 generation. Females of the

TABLE 9

BHT effect on genotoxicity of other agents

Test system	Other agent	Results	Reference
EL_4 lymphoma cells (C57BL mice)	γ-rays	0.001 g% BHT: increased radiosensitization; <0.0001 g% BHT: no significant effect	Kamra, Ghose, and Mammen 1973
Male Wistar rats fed BHT for 7 days to 5 months	*Induction:* DEN[a]; *selection:* 2-AAF	BHT increased frequency diploid nuclei from 3–5 months, then decreased frequency	Haesen et al. 1987
Modified Ames plate incorporation assay in *S. typhimurium* TA98, +S9, 10 μg/plate	2-AAN	BHT increased revertant colonies	Dertinger, Torous, and Tometsko 1993
S. typhimurium and hepatic microsomes from male ddY mice fed 0.1% BHT ± flavonoids	AFB_1	BHT and BHT/flavone increased activation of AFB_1	Sun and Fukuhara 1997
	BP	BHT and BHT/flavone increased activation of BP	
Male (C3H × 101)F_1 mice fed 0.75% BHT for 30 days	EMS	BHT had no effect on chromosome number; decreased the frequency of normal, mature spermatids; increased the frequency of translocation	Cachiero, Russell, and Swartout 1974
Mice pretreated with BHT (oral)	X-rays	BHT had no significant protective effect	Cumming 1970; Cumming et al. 1976
	EMS	BHT decreased the induction of dominant lethals and heritable translocations	
S. typhimurium treated with 200 μg/plate; *E. coli* WP-2 hcr$^-$ treated with 500 μg/plate BHT derivative[b]	4NQO	BHT decreased the frequency of revertants induced by 4NQO	Yoshida 1990
	AF-2	BHT decreased the frequency of revertants induced by AF-2	
	MMS	BHT decreased the frequency of revertants induced by MMS	
	MNNG	BHT decreased the frequency of revertants induced by MNNG	
Ames test	Anthraquinones	BHT decreased the frequency of revertants induced by various anthraquinones	Brown and Brown 1976
Modified Ames test	BP	BHT caused weak inhibition of mutagenesis	Calle and Sullivan 1982
SOS chromotest, +S9	BP	BHT decreased BP metabolism	Hennig, Dobrzański, and Bębenek 1987
EUE cells pretreated with 10^{-4} M BHT	DMBA	BHT decreased the effect of DMBA	Ferreri et al. 1986
Human leukocytes treated with 0.2 μM BHT	DMBA	Frequency of chromosome breaks	Shamberger et al. 1973
Ames test	DMBA	BHT decreased the mutagenicity of DMBA	Brown et al. 1981
	BP	BHT decreased the mutagenicity of BP	

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TABLE 9
BHT effect on genotoxicity of other agents *(Continued)*

Test system	Other agent	Results	Reference
Crepis capillaris L. (Wallr.) seeds	γ-rays	BHT decreased the frequency of chromosome aberrations	Alekperov, Abutalybov, and Bagirova 1975
	Ethylenimine	BHT decreased the frequency of chromosome aberrations	
Human leukocytes	DM(γ)BA	BHT decreased the frequency of chromosome breakage	Shamberger 1974
Salmonella pretreated with BHT	EMS	BHT decreased the mutagenicity	Ben-Hur et al. 1981
CHO cells	Bleomycin	BHT decreased frequency of chromosome aberrations in G_0/G_1 and G_2 cells and chromatid aberrations in G_2 cells; but increased frequency of chromatid aberrations in G_0/G_1 cells	Grillo and Dulout 1997
Senescence-prone mice (SAMR1/Fky, SAMP1/Fky) fed 300 ppm BHT for 12 weeks	Paraquat	BHT decreased rate of hepatic DNA single strand breaks	He and Yasumoto 1994
Chinese hamster V79 cells	BP	BHT decreased the mutagenicity	Katoh et al. 1980
Drosophila	EMS	BHT decreased the mutagenicity	Ives and Demick 1974
Salmonella	—	BHT decreased the frequency of spontaneous point mutations	DeFlora et al. 1994
	Nitric oxides	BHT decreased the mutagenicity	Arroyo et al. 1992
	Pyrolyzates of albumin	BHT decreased the mutagenicity	Fukuhara, Yashida, and Gotó 1981
	4NQO	BHT decreased the mutagenicity	Shamberger et al. 1979
	Cigarette smoke	BHT decreased the mutagenicity	
	Malonaldehyde	BHT decreased the mutagenicity	
	β-Propiolactone	BHT decreased the mutagenicity	
Unscheduled DNA synthesis assay, rat and human hepatocytes (treated in vivo and/or in vitro)	2-AAF	BHT decreased DNA damage; inhibited unscheduled DNA synthesis	Chipman and Davies 1988
	N-hydroxy-2-AAF	BHT decreased DNA damage; inhibited unscheduled DNA synthesis	
Intrasanguineous host-mediated assay, *S. cerevisiae* D7 and male Swiss albino mice treated with 50 mg/kg BHT IP or orally for 5 days (mice injected with yeast)	Azanidazole	*Azanidazole, IP BHT:* decreased liver- and kidney-mediated gene conversion and point mutation frequency	Hrelia et al. 1987
		Azanidazole, oral BHT: decreased reversion frequency	
	Metronidazole	*Metronidazole, oral BHT:* decreased reversion frequency	

Barley seeds, pre- or post-treated with BHT	Propane sultone	*BHT pretreatment:* increased seedling injury, frequency of chromosome aberrations and mutagenicity *BHT post-treatment:* decreased spike sterility and frequency of mutations	Kaul and Tandon 1981
EcoRII cytosine DNA-methyltransferase induction assay	Spingomyelin	BHT increased in vitro DNA methylation	Romanenko, Alessenko, and Vanyushin 1995
Rats fed BHT for 6 days, ±hydrogenated fats	2-AAF	BHT increased mutagenicity of 2-AAF	Ponder and Green 1985
	2-Aminofluroene	BHT increased mutagenicity of 2-aminofluorene	
Ames test	AFB_1	BHT increased number of revertants	Shelef and Chin 1980
Ames test	AFB_1 and BP	BHT decreased mutagenicity and DNA damage	Soni et al. 1997
	AFB_1 and BP	Little to no effect on metabolites of BP	Calle et al. 1978
Primary rat hepatocytes pretreated with BHT	AFB_1	Decreased cytotoxicity and covalent binding of AFB_1 to DNA	Salocks, Hsieh, and Byard 1981
Salmonella TA98, +S9	DCB	BHT increased mutagenicity and binding to DNA; altered metabolism	Ghosal and Iba 1992
	2-AAF	BHT decreased mutagenicity	
	DNPH	BHT had no significant effect	
S. typhimurium TA98, +S9	AFB_1	BHT decreased mutagenicity, no significant effect on DNA binding; increased metabolism	Fukayama and Hsieh 1984
D. melanogaster sperm cells treated with 0.015–0.05M BHT	EMS	BHT had no significant effect on frequency of sex-linked lethal mutations	Goncharova and Kuzhir 1989
Human lymphocytes 1 μM, 10 μM BHT	PMA[a]	BHT decreased chromosomal aberrations	Emerit, Levy, and Cerutti 1983
CHO cells	$CdCl_2$[a]	BHT decreased chromosomal aberrations	Ochi and Ohsawa 1985
Chinese hamster V79, cells, +S9 HGRPT assay	BP	No change in mutation frequencies at molar ratio of 1:1	Paschin and Bahitova 1984

[a]2-AAF = 2-acetylaminofluorene; 2-AAN = 2-aminoanthracene; AFB_1 = aflatoxin B_1; BP = benzo[a]pyrene; EMS = ethyl methanesulfonate; 4NQO = 4-nitroquinoline-1-oxide; AF-2 = 2-(2-furyl)-3-(5-nitro-2-furyl)acrylamide; $CdCl_2$ = cadmium chloride; MMS = methyl methanesulfonate; MNNG = *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine; DMBA = 7,12-dimethylbenz[a]anthracene; DM(γ)BA = 7,12-dimethylbenz[γ]anthracene; DCB = 3,3'-dichlorobenzene; DNPH = 2,4-dinitrophenylhydrazine; PMA = phorbolmyristate acetate.
[b]3-Acetyl-2,5-di-*tert*-cyclopenta-2,4-dienone.

high-dose group, however, had reduced body weight (up to 7%) compared to controls. The Armitage-Cochran test for linear trend in proportions established that the fraction of litters with ≥10 pups decreased in a dose-related manner. The mean birth weights of the F_1 rats in the middle and high-dose groups were slightly decreased compared with controls. The dams had a dose-related decrease in body weight gain during lactation, although feed consumption was not reduced. The body weights of F_1 rats fed 250 mg/kg/day BHT differed from control values by 21% and 16% for males and females, respectively. Survival of BHT-treated rats of both sexes was greater than that of the controls. At two years of age, 86% of males and females of the high-dose group survived, compared to 70% of male controls and 69% of female controls. At study termination, 44% of males and 39% of females survived, compared to 16% and 17% of control males and females, respectively. When all groups were tested for heterogeneity or analyzed for trend, the F_1 males had statistically significant, dose-related increases in the numbers of hepatocellular adenomas and carcinomas (Table 10). For F_1 females, the increases in these neoplasms were statistically significant only when analyzed for trend. All hepatocellular neoplasms were detected when the F_1 rats were more than two years old.

Using the same procedure, Olsen et al. (1986) treated Wistar rats with 25 to 500 mg/kg/day BHT to assess the carcinogenic effects after in utero exposure. BHT had no adverse effects on the appearance or behavior of the rats, with the exception of slight red discoloration of the urine in high-dose males. Feed consumption did not differ between BHT and control groups. The fraction of litters with ≥10 pups decreased ($p < .001$) after treatment with BHT. The antioxidant did not significantly decrease viability during lactation. BHT-treated F_1 rats survived longer than controls. At week 104, 86% of high-dose males and females survived compared to 69% to 70% of controls. At weeks 141 to 144, 39% to 44% survived compared to 16% to 17% of controls. In the high-dose group (F_1), the incidences of hepatocellular adenomas and carcinomas were increased in males and the incidence of hepatocellular adenomas was increased in females compared to controls. Males of the high-dose group ($n = 99$) had 18 adenomas and 8 carcinomas, and control males ($n = 100$) had 1, and 1, respectively. Of the high-dose females ($n = 99$), 12 had adenomas and 2 had adenomas, compared to 2 and none of the controls ($n = 100$). Treatment with 25 to 100 mg/kg/day BHT did not cause significant increases in neoplasm incidence, and the number of high-dose rats with nodular hyperplasias did not differ from controls.

TABLE 10
Incidence of hepatic proliferative lesions after feeding BHT to rats (Würtzen and Olsen 1986)

Dose	No. rats	No. rats with: Hepatic proliferative lesions	Adenoma	Carcinoma
		Males		
0	100	2	1	1
25	80	0	1	0
100	80	2	5	1
250	99	2	18[a]	8[b]
		Females		
0	100	2	2	0
25	79	0	3	0
100	80	4	6	0
250	99	5	12[c]	2[d]

[a]Overall test for heterogeneity, $p < .001$, $\chi^2 = 18.17$; test for trend, $p < .001$, $\chi^2 = 17.97$.
[b]Overall test for heterogeneity, $p < .05$, $\chi^2 = 11.12$; test for trend, $p > .01$, $\chi^2 = 9.40$.
[c]Overall test for heterogeneity, not significant, $\chi^2 = 5.20$; test for trend, $p < .05$, $\chi^2 = 4.99$.
[d]Overall test for heterogeneity, not significant, $\chi^2 = 2.87$; test for trend, not significant, $\chi^2 = 2.59$.

In a chronic study, the feeding of 100 to 12,000 ppm BHT to male F344 rats for 76 to 100 weeks did not significantly increase the incidence of neoplasms at any site. The BHT used in the studies was >99% pure. In contrast, the feeding of 12,000 ppm BHA (95% pure) resulted in a small increase in the incidence of squamous cell papilloma of the nonglandular stomach (Williams, Wang, and Iatropoulos 1990b).

BHT was noncarcinogenic in a 104-week feeding study using Wistar rats (Hirose et al. 1981). The treatment groups consisted of 57 rats/sex, and were treated with 0.25 or 1% BHT. The control group had 36 rats/sex. Treated rats of both sexes decreased body weight gain, relative spleen weight, and white blood cell count, as well as increases in relative liver weight and total blood cholesterol. Treated males had decreased serum triglyceride concentration and increased activity of γ-glutamyl transaminase. No significant microscopic changes were observed in the liver or hematopoietic system. Although neoplasms were detected in the liver, pancreas, mammary glands, uterus, pituitary gland, and adrenal glands of some treated rats, these findings were nonsignificant compared to controls.

When given via stomach tube following IP injection of a single 1-mg/g dose of urethane (ethyl carbamate), 300 mg/kg BHT in saline enhanced the formation of pulmonary adenomas in male Swiss-Webster mice, compared to controls (Witschi and Lock 1978). In an initial study, the mice were treated with 400 mg/kg [^{14}C]-BHT in corn oil by stomach tube. Radioactivity was measured in plasma, lungs, liver, and kidneys from 30 minutes to 10 days later. Radioactivity was greatest in plasma and all tissues examined between 1 and 12 hours after dosing. After 24 hours, less than 1% of the dose remained in the lungs, kidneys, or liver. On days 3 to 5, DNA synthesis in the lungs increased to six to eight times as great as that of the controls and DNA content of the lungs almost doubled. The synthesis and net increase of pulmonary DNA were dose dependent. In the carcinogenesis assay, urethane alone produced an 80% incidence in pulmonary adenoma in all animals treated. The average number of

neoplasms per mouse was 14.74 ± 2.82 (n = 23). After 13 weekly BHT treatments, the incidence of adenomas at the lung surface was 96%, but the average number of neoplasms per mouse was 5.50 ± 1.20 (n = 22). Of the 18 mice treated with 0.9% saline and BHT, one had a neoplasm; however, this was the normal spontaneous incidence of pulmonary adenoma for Swiss-Webster mice. None of the 20 mice treated with 0.9% saline and corn oil had neoplasms. The investigators concluded that doses greater than 100 mg/kg BHT induced extensive cell proliferation in the lungs and promoted adenoma development.

BHT (0.25% to 1.0%) was noncarcinogenic in a 104-week feeding study using groups of 57 Wistar rats of each sex (Hirose et al. 1981). BHT also did not induce neoplasms of the nonglandular stomach in a 16-week feeding study using 21 male Syrian hamsters (Hirose et al. 1986a).

Davies et al. (1993) reported that 0.5% BHT had no effect on ploidy when added to the diet of male F344 rats for 4 to 6 weeks. BHT also did not induce γ-glutamyl transpeptidase activity to the same extent as another antioxidant, ethoxyquin, did.

Rády et al. (1980) investigated the effect of BHT on the activities of four glycolytic enzymes in the lungs of female Lati:CFLP mice: hexokinase, phosphofructokinase, pyruvate kinase, and lactate dehydrogenase. The effects were determined 28 days after IP injection of 400 mg/kg BHT, and were compared to those after injection of the carcinogens DMBA, AFB_1, BP, 3-methylcholanthrene, urethane, and dimethylnitrosamine. The carcinogens enhanced enzyme activity and changed the ratios of lactate dehydrogenase subunits. Under the same conditions, BHT had no effect on the enzyme activities or the isozyme pattern of lactate dehydrogenase. Similar results were reported by Rády et al. (1981) after transplacental exposure of Lati:CFLP mice to 200 mg/kg BHT on GD 18.

Effect of BHT on the Carcinogenicity of Other Agents

The many studies presented in Table 11, in general, demonstrate that BHT can act as a tumor promoter and/or anticarcinogen, depending on the target organ, whether the antioxidant was administered before, during, or after initiation with a carcinogen, and the type of animal used (age, sex, strain, species).

General Findings

In adult A/J mice, for example, BHT had a chemoprotective effect when given prior to urethane and was a tumor promoter when administered chronically after urethane. In neonatal mice, however, the latter treatment had no effect on urethane-induced carcinogenesis. BHT was a cocarcinogen when administered to preweanling mice before urethane (Malkinson 1985).

Numerous studies have been performed on the inhibition of chemical-induced carcinogenicity by antioxidants such as BHT. Potential mechanisms of action are (1) alteration of the metabolism of the carcinogen, (2) scavenging of the active species of the carcinogen, (3) induction of changes in cell permeability or transport, and (4) competition for target-binding sites (Wattenberg 1980; Williams 1993; Slaga 1995).

Witschi (1986b) reported that BHT enhanced the development of spontaneously occurring hepatic neoplasms in C3H mice, but not in BALB/c mice and noted previous work (Witschi, Williamson, and Lock 1977) in which BHT decreased spontaneous development of papillary adenomas in male Swiss-Webster mice.

Additional Specific Studies

Grantham, Weisburger, and Weisburger (1973) reported that prefeeding of BHT (6600 ppm for 4 weeks) increased the urinary elimination of two carcinogens in male and female CD rats, thereby decreasing the amounts available for activation. The rats were injected IP with N-2-fluorenylacetamide (FAA) and N-hydroxy-FAA. The carcinogens were excreted primarily as glucuronic acid conjugates. BHT also decreased radioactivity in blood, the liver, and bound to hepatic DNA at 48 hours after injection of the carcinogens. The investigators concluded that BHT protected against carcinogenicity via the induction of drug metabolism enzymes.

In a study using (C3H × 101)F_1 hybrid (C31F_1) mice, prefeeding of 1% BHT for 4 weeks decreased mortality in males caused by ethyl methanesulfonate, n-propyl or isopropyl methanesulfonate, ethylene dibromide, diethylnitrosamine, and cyclophosphamide. Females were protected against the effects of methyl methanesulfonate, but none of the mice were protected against x-rays, N-methyl-N'-nitro-N-nitrosoguanidine (MNNG), or dipropylnitrosamine (Cumming and Walton 1973).

In another study, BHT shortened the cell cycle of surviving phytohemagglutinin-stimulated human leucocytes, delayed mitosis, uncoiled the chromosomes, and caused damage to the cell membrane of transformed cells (Sciorra, Kaufmann, and Maier 1974).

Piekarski and Konkiewicz (1975) reported that 8 to 60 days after being added to CHO cells, BHT caused a slight reduction in the frequency of transformation via the inhibition of BP-induced hydroxylation. The carcinogen was tested at a concentration of 20 nM/ml and BHT was added to the cells at concentrations up to 1 μM/ml.

BHT interfered with the DEN-induced alterations of plasma esterase activities in BALB/c mice, although the antioxidant caused changes in different plasma esterases. The enzymatic alterations preceeded overt histopathologic lesions induced by either compound, particularly DEN-induced tumorigenesis (Tyndall, Colyer, and Clapp 1975).

Slaga and Bracken (1977) treated shaved skin sites of female CD-1 mice with topical BHT (1000 μg) 5 minutes prior to initiation with 2.56 μg DMBA and promotion with 10 μg TPA, twice weekly. Of the 30 mice that survived to week 28, 57% had papillomas (1.6/mouse). When mice were treated with BHT 6 hours before, 5 minutes before, and 6 hours after initiation, 35% of the 29 survivors had papillomas (0.9/mouse). Of the 30 surviving mice not pretreated with BHT, 82% had papillomas (3.4/mouse). The investigators concluded that BHT inhibited DMBA-initiated neoplasms in mouse skin. BHT did not induce aryl hydrocarbon

TABLE 11
Effect of BHT on the carcinogenicity of other agents

BHT treatment	Carcinogen treatment	Results	Reference
200 mg in olive oil, 1 h prior to DMBA[a]; female Sprague-Dawley rats	DMBA, 12 mg in olive oil	Decreased incidence of mammary tumors	Wattenberg 1972
	DMBA, 30 mg in olive oil	Complete protection against adrenal necrosis	
5 mg/g of diet; female A/HeJ mice	BP 1.0 g/g of diet	Decreased incidence of tumors in forestomach	
6600 ppm in feed for 24–32 weeks; male and female CD SPF rats	DEN, 51 ppm for 24 weeks	No significant effect	Ulland et al. 1973
	223 ppm FAA for 24–32 weeks	Decreased incidence of hepatoma in males	
	239 ppm *N*-hydroxy-FAA for 24–32 weeks	Decreased incidence of hepatic and mammary neoplasms	
0.75% in feed for 12–18 months, from 8 weeks of age; male BALB/c mice	DEN, 4 mg/100 ml drinking water, for 7 weeks from 11 weeks of age	*After 12 months:* increased incidence of reticulum-cell carcinomas; increased incidence of hepatic cysts; *after 18 months:* increased number of papillary adenomas/mouse; decreased incidence of reticulum-cell carcinomas; decreased incidence of papillomas of nonglandular stomach; increased incidence of SCCs[b] of nonglandular stomach	Clapp et al. 1974
0.75% in feed for 18 months, from 8 weeks of age; female BALB/c mice		Increased survival; decreased incidence of SCCs of nonglandular stomach; decreased number of tumor-bearing mice; different gastric tumors (grade II carcinomas → papillomas)	
0.5% in feed, 7 days prior to initiation; male Sprague-Dawley rats	2-AAF, 0.05% in feed for 1 week	*Liver:* decreased 2-AAF binding to DNA, no effect on excision or postreplication repair	Goodman, Trosko, and Yager 1976
		Overall: rats fed 2-AAF alone had no body weight gain, rats fed both had 50% weight gain of negative controls	
6600 ppm in feed for up to 12 weeks, during or after azoxymethane; male F344 rats	Azoxymethane, 7.4 mg/kg, SC	*Concurrent dosing:* decreased incidence of gastrointestinal and ear duct neoplasms; *BHT post-dosing:* no significant effect	Weisburger, Evarts, and Wenk 1977
100–800 mg/kg, IP, 6–7 days prior to urethane; female CFLP mice	Urethane, 1 mg/g, IP	Increased incidence of lung tumors	Boján, Nagy, and Herman 1978
0.75% in feed from 8–12 weeks for 28 days; male and female BALB/c mice	X-rays, 525–750 R (whole body), at 11 weeks of age	Increased survival	Clapp 1978
	DMN, 10–20 mg/kg; DEN, 150–200 mg/kg; MMS, 100–200 mg/kg; or EMS, 400–600 mg/kg, IP at 12 weeks of age	Increased survival after treatment with DMN, DEN, and EMS	

0.75% in feed from 8–12 weeks of age through entire life; male and female BALB/c mice	X-rays, 525–750 R (whole body), at 11 weeks of age	No significant effect on leukemia or neoplasm incidence; increased survival at 18 months	
	DEN, 150–200 mg/kg, in drinking water, 3 weeks after BHT through 18 weeks	No significant effect on leukemia incidence; decreased incidence of SCCs of nonglandular stomach, increased survival at 18 months	
6600 ppm in feed for 10 weeks; male Sprague-Dawley rats	DMH, 30 mg/kg/week, orally for 10 weeks	No significant effect	Barbolt and Abraham 1979
0.75% in feed for life from 8 weeks of age; male and female BALB/c mice	1,2-DMH, 20 mg/kg body weight from 8 weeks of age	*Males:* decreased in tumor-bearing mice when given BHT and DMH as opposed to only DMH; *females:* no protective effect of BHT observed; *both sexes:* decreased number of tumors in the colon in tumor bearing mice	Clapp et al. 1979a
300 mg/kg/week, IP, for 14 weeks; male Swiss-Webster mice	Urethane, 50–1000 mg/kg, IP, 7 days prior to BHT	*100-1000 mg/kg urethane*: increased number of adenomas/mouse *1000 mg/kg:* increased mean tumor diameter *250–1000 mg/kg urethane:* increased number of adenomas/mouse	Witschi and Lock 1979
300 mg/kg/week, IP, for 24 weeks; male Swiss-Webster mice	Urethane, 50–1000 mg/kg, IP, 7 days prior to BHT		
300 mg/kg/week, IP, then 200 mg/kg/ week for 11 weeks or 300 mg/kg/week, IP, for 13 weeks; male C57BL, C3H, and BALB/c mice	Urethane, 1000 mg/kg, IP, 1 week prior to BHT	No significant effect	
300 mg/kg/week, IP, for 13 weeks; male Swiss-Webster mice	Urethane, 1000 mg/kg, IP, 2–6 weeks prior to BHT	Increased number of adenomas/mouse	
	Urethane, 1000 mg/kg, 1 week after BHT	No significant effect	
300 mg/kg/week, IP, for 1–8 weeks; male Swiss-Webster mice	Urethane, 1000 mg/kg, IP, 1 week prior to BHT	Increased number of adenomas/mouse	
400 mg/kg, IP, male BALB/c mice	Urethane, 500 mg/kg, IP, 12–50 h after BHT	Decreased number of lung adenomas/mouse	
400 mg/kg, IP, male BALB/c mice	Urethane, 0.15%–0.5% in drinking water from 18 h to 5 days after BHT	*0.3%–0.5% urethane:* decreased number of lung adenomas/mouse	
0.5% in feed for 9 weeks; male Sprague-Dawley rats	3′-Me-DAB, 0.05% in feed for 9 weeks	Decreased incidence of hepatic neoplasms	Daoud and Griffin 1980
0.66% in feed for 2 weeks prior to DMH male Sprague-Dawley rats	DMH, 20 mg/kg, SC	No significant effect	Bull, Burd, and Nigro 1981

(Continued on next page)

TABLE 11
Effect of BHT on the carcinogenicity of other agents *(Continued)*

BHT treatment	Carcinogen treatment	Results	Reference
0.3% in feed for 30 weeks; female Sprague-Dawley rats	DMBA, 10 mg/ml corn oil, by gavage	*DMBA:* decreased formation of mammary gland tumors	King, McCay, and Kasanke 1981
	NMU, 5 mg/100 g, i.v.	*NMU:* no significant effect	
0.75% in feed, 1 week prior to DMH; male Sprague-Dawley rats	DMH, 20 mg/kg weekly for up to 8 weeks, SC	Inhibition of immunoactivity in colon chromatin	Gabryelak, Pumo, and Chiu 1981
300 mg/kg/week for 8 weeks, IP; male A/J mice	Urethane, 500 mg/kg, IP, 2 weeks prior	Increased number of adenomas/mouse	Witschi, Hakkinen, and Kehrer 1981
	MCA, 10 mg/kg/week for 2 weeks, SC	Increased number of adenomas/mouse	
	DMN, 7 mg/kg, SC	Increased number of adenomas/mouse; increased neoplasm incidence	
300 mg/kg/week for 8 weeks, IP; male A/J mice	Urethane, 500 mg/kg, IP	Increased number of adenomas/mouse	Witschi 1981
0.75% in feed for 24 h/week for 8 weeks (total intake = 104 mg/mouse); male A/J mice	Urethane, 500 mg/kg, IP	Increased number of adenomas/mouse	
	Urethane, 250–500 mg/kg, IP	Increased number of adenomas/mouse	
	Urethane, 1000 mg/kg	No significant effect	
	Urethane, 250–500 mg/kg	Increased number of adenomas/mouse	
1% in feed for 32 weeks; male F344 rats	BBN, 0.01 or 0.05% in drinking water for 4 weeks prior to BHT	*0.01% BBN:* increased incidence of PNH of urinary bladder; increased number of PNH per 10 cm BM[c]; *0.05% BBN:* increased number of PNH and papilloma of bladder per 10 cm BM; increased incidence of papilloma of bladder; increased incidence of bladder carcinomas	Imaida et al. 1983
1% in feed for 32 weeks; male F344 rats	DEN, 200 mg/kg, IP, 2–8 weeks prior to BHT	Decreased number of γ-glutamyltranspeptidase-positive foci of liver	
1.0% in feed for up to 25 weeks; male Wistar rats	MNNG, 50 μg/ml, in drinking water for 25 weeks	*BHT + MNNG:* decreased incidence of gastric cancers by week 40, but no significant effect on the histologic type of adenocarcinomas	Tatsuta, Mikuni, and Taniguchi 1983
		BHT alone: induced slight glandular hyperplasia	
6000 ppm BHT in feed for 25 weeks; male F344 rats	N-2-FAA, 200 ppm in feed for 25 weeks	*Liver:* decreased incidence of neoplasms	Williams, Maeura, and Weisburger 1983
		Urinary bladder: increased incidence of papillomas and carcinomas	

300, 1000, 3000, 6000 ppm NIH-07 diet for 28 days; female Sprague-Dawley rats	DMBA, 5 and 15 mg/rat	Decreased in mammary tumor development at all four doses of BHT with 5 mg/rat DMBA; decreased mammary tumor development at 6000 ppm BHT with 15 mg/rat DMBA; decreased adrenocortical nodules at all doses of BHT and 5 and 15 mg/rat DMBA—type of diet influenced BHT inhibition	Cohen et al. 1984
10–400 mg/kg in 100 μl corn oil, IP, 6 h prior to urethane; male and female strain A mice	Urethane, 1 mg/g body weight in NaCl, IP	*200–400 mg/kg BHT:* decrease in mean number of lung adenomas	Malkinson and Beer 1984
400 mg/kg, IP, 6 h or 7 days before urethane; male and female A mice	Urethane, 1 mg/g, IP	Decrease in mean number of lung adenomas	
200 mg/kg/week, IP, after urethane; male and female A and BALB/cBy mice	Urethane, 1 mg/g, IP	Increase in mean number of lung adenomas	
200 mg/kg/week, IP; male and female A, SWR, BALB/cBy, RIIIS, 129 mice	Urethane, 1 mg/g, IP prior to BHT	Increase in mean number of lung adenomas	
200 mg/kg/week, IP; male and female C57BL/6By, BALB/cBy, RI lines D, E, G-K mice	Urethane, 1 mg/g, IP prior to BHT	*RI lines E, J:* increase in mean number of lung adenomas	
1.0% in feed for 32–34 weeks; male F344 rats	BBN, 0.01%–0.05% in drinking water for 4 weeks prior to BHT	*0.01% BBN:* increased incidence of urinary bladder PNH *0.05% BBN:* increased incidence of urinary bladder papillomas	Ito et al. 1984
300, 1000, 3000, 6000 ppm in feed for 6–25 weeks; male F344 rats	200 ppm FAA for 6–25 weeks	Dose-dependent decrease in number of hepatic tumors per rat in all groups; dose-dependent increased incidence of bladder tumors in high-dose groups	Maeura, Weisburger, and Williams 1984
0.5% in drinking water throughout entire study or when 2-AAF omitted; male Sprague-Dawley rats	2-AAF, 0.05% in feed[d]	Decreased formation of hepatic nodules; no signs of cirrhosis or liver enlargement	Daoud and Griffin 1985

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TABLE 11

Effect of BHT on the carcinogenicity of other agents *(Continued)*

BHT treatment	Carcinogen treatment	Results	Reference
0.5% in feed for 10 days, 24 h prior to AFB_1; male F344 rats	AFB_1, 0.0625 mg/kg orally (total dose = 12.5 μg/rat)	Increased excretion of AFB_1 in urine and feces at 24 h; increased excretion of metabolites in urine, feces, and large intestine; decreased binding of AFB_1 to hepatic DNA	Fukayama and Hsieh 1985
0.25%–1.0% in feed for 6 weeks, feeding initiated 2 weeks after DEN injection; male F344 rats	DEN, 200 mg/kg, IP	No significant inhibitory effect on development of preneoplastic hepatic lesions	Thamavit et al. 1985
300 mg/kg/week, IP, for 5 weeks; male A/J mice	MCA, 10 mg/kg, IP, 1 week prior to BHT	Increased average number of lung adenomas at 4 months	Witschi and Morse 1985
300–6000 ppm in feed for 210 days; female Sprague-Dawley rats	DMBA, 5–15 mg/rat	Decreased incidence of mammary adenocarcinomas; decreased number of tumors/rat (semipurified diet only)	Cohen et al. 1986
0.7% in feed for 33 weeks; female Sprague-Dawley rats	DMBA, 0.25 mg/kg, by gavage	*Mammary gland:* decreased incidence of fibroadenomas; *ear duct:* decreased incidence of adenomas and carcinomas	Hirose et al. 1986a
0.1%–0.5% in feed for 7 months; male F344 rats	NMU, 5 mg, instilled directly into proximal colon prior to BHT	No significant effect	
0.05% or 0.5% in feed for 12 months 3–5 days after carcinogen; male BALB/c mice	DMH, 4–6 × 30 mg/kg, SC	*DMH, 6×:* increased incidence of colon neoplasms; no significant effect on liver	Lindenschmidt et al. 1986
	MNU, 3 × 1.5 mg, intrarectal	*MNU:* no significant effect	
1% in feed, from weeks 8–30, + vitamin K in drinking water; male F344 rats	DHPN, 1000 mg/kg, IP, then 3 × 250 mg/kg starting 3 weeks later, every fortnight	Decreased incidence of hepatic foci; increased incidence of thyroid adenomas	Moore et al. 1986
0.5% in feed for up to 14 weeks; male Wistar rats	*Initiation:* DEN, 200 mg/kg, IP *Selection:* 2-AAF, 0.03% in feed for 2 weeks (+2 ml/kg CCl_4 after 1 week)	Increased incidence and no./cm^2 of γ-glutamyltransferase positive lesions in liver within 3 weeks, but no increase in the incidence of hepatocarcinogenesis	Préat et al. 1986
1% in feed for 32 weeks; male Wistar rats	MNNG, 100 mg/l in drinking water for 8 weeks prior to BHT	No significant effect	Takahashi et al. 1986
1% in feed + 7 ppm vitamin K for 32 weeks; male F344 rats	DBN, 0.05% in drinking water for 4 weeks prior to BHT	*Liver:* increased incidence of hyperplastic nodules *Esophagus:* increased incidence of papillomas and carcinomas *Urinary bladder:* increased incidences of PNH and papillomas; increased number of tumors/10 cm basement membrane *Nonglandular stomach:* no significant effect	Fukushima et al. 1987

0.5% in feed for 5 months; male F344 rats	DMH, 2 or 4 injections at 40 mg/kg, SC, prior to BHT	*Two DMH injections:* increased incidence of colon neoplasms; *four DMH injections:* increased incidence of neoplasms of jejunum and ileum	Lindenschmidt, Tryka, and Witschi 1987
0.1%–0.5% in feed for 6 months; male F344 rats	DMH, 2 injections at 40 mg/kg, SC, prior to BHT	*0.1% BHT:* increased incidence of neoplasms of GI tract (all sites) and large intestine; *0.5% BHT:* increased incidence of neoplasms of small intestine (all sites), jejunum, and ileum	
0.5% in feed for 7 months; male F344 rats	NMU, 90 mg/kg orally prior to BHT	No significant effect	
0.7% in feed for 16 weeks; co-administered to male F344 rats	DBN 0.05% in drinking water for 16 weeks	*Liver:* increased primary HCC Metastases, and hyperplastic nodules *Nonglandular stomach:* decreased hyperplasia *Esophagus, urinary bladder:* no significant effect	Imaida et al. 1988
In feed every 2 weeks for 5 months, dose not stated; 7-day-old Wistar rats	*N*-bis(2-hydroxypropyl) nitrosamine, dose and route not stated	Increased papillary adenocarcinomas at 24 months, increased morphologic changes of pneumocytes	Sugiyama et al. 1989
1%–2% in feed, weeks 3–10; male F344 rats	*Initiation:* DEN, 200 mg/kg, IP, weeks 1–2 *Promotion:* 0.02% 2-AAF or 3′-MeDAB, in feed, weeks 3–10	Decreased area of hepatic glutathione *S*-transferase–positive foci, decreased number of 2-AAF–induced foci	Asamoto et al. 1990
1% + 7 ppm vitamin K in feed for 30 weeks; male F344/DuCrj rats	DHPN, 0.1% in drinking water for 2 weeks prior to BHT	Decreased incidence and area of lung carcinomas and adenomas; increased incidence of thyroid adenomas	Hasegawa et al. 1990
0.6% in feed for 25–57 days; female Sprague-Dawley rats	DMBA, 55.5 mg/kg, gastric intubation	*Urine*: increased DMBA excretion	Singletary 1990
1% in feed for 11 weeks; male F344 rats (4- and 54-weeks-old)	DMAB, 50 mg/kg/week, SC, for 10 weeks	*Blood:* increased DMBA and metabolite content *Mammary gland:* decreased DMBA content *Liver:* decreased DMBA-DNA binding *Young rats:* increased incidence of urinary bladder papillomas and carcinomas; *older rats:* increased incidence of urinary bladder papillomas, carcinomas, and invasive neoplasms	Shirai et al. 1991
5000 ppm/week in feed, weeks 15–39; male B6C3F$_1$ mice	DEN, 100–200 μmol/kg/week for 10 weeks, IP	No hepatic tumor promoting effect	Tokumo, Iatropoulos, and Williams 1991
100–6000 ppm in feed for 76 weeks; male F344 rats	2-AAF, 50 ppm in feed for 76 weeks	*100–6000 ppm:* decreased multiplicity of adenomas and hepatocellular carcinomas of liver; *3000 ppm:* increased incidence of nodular hyperplasia of urinary bladder; *6000 ppm:* increased incidence of nodular hyperplasia and papillomas of urinary bladder; increased number of bladder tumors/rat	Williams et al. 1991

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TABLE 11
Effect of BHT on the carcinogenicity of other agents *(Continued)*

BHT treatment	Carcinogen treatment	Results	Reference
250 mg/kg, in feed, daily for 2 weeks, then 3× weekly for 18 months; male Wistar and BD_6 rats	DMH, 20 mg/kg, SC, weekly, 2 weeks after BHT	No significant effect	Balansky et al. 1992
0.7% in feed for 36 weeks, 3 days after carcinogen; male F344 rats	MNNG, 100 mg/kg, or EHEN, 750 mg/kg, IG[c]; MBN, 2 × 0.5 mg/kg/6 days, SC; DMH, 4 × 40 mg/kg/3–4 days, SC; DBN, 0.05% in drinking water for 4 weeks; DHPN, 0.1% in water for 2 weeks	Increased incidence of thyroid hyperplasias, adenomas, and carcinomas; decreased incidence and multiplicity of colon adenocarcinomas; decrease in multiplicity of renal neoplasms	Hirose et al. 1993
1 g/kg in feed for 16 weeks; female A/J mice	NNK, 1.54 mmol/kg in drinking water for 7 weeks (total = 9.1 mg/mouse)	Increased number of lung adenomas/mouse	Matzinger et al. 1994
200 mg/kg/week for 6 weeks, IP, 7 days after urethane; male and female C × B H and BALB/cBy mice	Urethane, 1 mg, IP	*C × B H mice:* no significant effect *BALB/cBy mice:* increased lung tumor multiplicity; increased inflammatory response	Miller et al. 1994
0.5% BHT in feed on days 28–58; male F344 rats	*Initiation:* DEN, 200 mg/kg, IP, weeks 1–2 *Promotion:* 2-AAF, 20 mg/kg by gavage on days 14–17	Increased area of γ-glutamyltranspeptidase–positive foci; increased number, size, percent section area of glutathione *S*-transferase–positive foci	Lok et al. 1995
5–125 ppm in feed for 42 weeks, starting 2 weeks prior to AFB_1; male F344 rats	AFB_1, 2.5 μg/kg/week, IG, for 40 weeks	*125 ppm BHT:* decreased multiplicity of hepatocellular altered foci	Williams and Iatropoulos 1996
200 mg/kg/week, IP, for 6 weeks; female BALB/cBy mice	MCA, 10–25 μg/g, IP	Increased lung tumor incidence; increased number of tumors/mouse	Malkinson et al. 1997

[a]DEN = diethylnitrosamine; DMN = *N*-nitrosodimethylamine; MCA = 3-methylcholanthrene; DMBA = 7,12-dimethybenz[a]anthracene; AFB_1 = aflatoxin B_1; 3′-Me-DAB = 3′-methyl-4-dimethylaminoazobenzene; 2-AAF = 2-acetylaminofluorene; urethane = ethyl carbamate; BP = benzo[a]pyrene; NNK = 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone; DMAB = 3,2′-dimethyl-4-aminobiphenyl; 3′-Me-DAB = 3′-methyl-4-dimethylaminoazobenzene; NMU = nitrosomethylurea; BBN = *N*-butyl-*N*-(4-hydroxybutyl)nitrosamine; FAA = *N*-2-fluorenylacetamide; DMH = dimethylhydrazine; MNNG = *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine; EMS = ethyl methanesulfonate; MMS = methyl methanesulfonate; DHPN = *N*-bis(2-hydroxypropyl)nitrosamine; EHEN = *N*-ethyl-*N*-hyroxyethylnitrosamine; MBN = *N*-methylbenzyl-nitrosamine; BBN = *N*-butyl-*N*-(4-hydroxybutyl)nitrosamine.
[b]HCC = hepatocellular carcinoma; SCC = squamous-cell carcinoma; PNH = papillary or nodular hyperplasia.
[c]IG = intragastric; BM = basement membrane.
[d]Added to feed during weeks 1–3, 5–6, 9–11.

(BP) hydroxylase when the antioxidant was applied to the skin of mice or added to isolated epidermis in vitro. Although BHT inhibited epidermally mediated, covalent binding of [^{3}H]-BP and [^{3}H]-DMBA to DNA after mice were treated topically with the antioxidant, inhibition of binding did not occur when BHT was added in vitro.

In male Swiss-Webster mice, BHT (300 mg/kg in corn oil; IP) did not influence the number of developing neoplasms induced by the injection of 1 mg/g urethane before, during, or after cell proliferation in the lungs was stimulated by the antioxidant. In this study, BHT was administered 1 hour or 1 to 7 days before injection of the carcinogen. In a study using male A/J and Swiss-Webster mice, however, weekly injections of BHT after injection of urethane enhanced tumor formation compared to controls. The mice were given the carcinogen IP at a dose of 1 mg/g. One week after treatment, one group of carcinogen-treated mice was injected IP with 250 mg/kg BHT. Mice of other groups were injected with urethane and corn oil or saline, 0.9% saline alone, saline plus BHT, or corn oil alone. The injections were repeated weekly. The mice were killed 9 to 13 weeks after injection of urethane. Mice treated with either BHT or corn oil had a similar number of neoplasms per lung at 9 to 11 weeks. BHT induced significantly more neoplasms by 13 weeks, especially after treatment with urethane. Multiple injections of BHT in carcinogen-treated A/J mice caused more neoplasms per lung than mice treated with urethane and corn oil (Witschi, Williamson, and Lock 1977).

Berry et al. (1978) investigated the tumor-promoting activity of BHT in a two-stage skin carcinogenesis assay using female CD1 mice. At a dose of 1 mg in 0.2 ml acetone, twice weekly, topical BHT did not promote DMBA-initiated neoplasms after 30 weeks of treatment.

BHT (0.5%) did not alter ODC activity in the livers or lungs of male Sprague-Dawley rats fed the antioxidant for 3 days. In the liver, it increased the incorporation of [^{3}H]-thymidine into DNA and increased thymidine kinase activity 4- to 10-fold; in lungs, however, BHT did not alter thymidine kinase activity, and thymidine incorporation was decreased. The investigators concluded that "it seems unlikely that BHT would promote carcinogenesis in rat lung" (Saccone and Pariza 1978). In a later study, however, 700 mg/kg BHT increased ODC activity by 190% in the livers of female Sprague-Dawley rats fed the antioxidant, the hepatic cytochrome P450 concentration was increased by 30%, and hepatic DNA damage occurred. A dose of 140 mg/kg, BHT caused no significant changes (Kitchin and Brown 1987).

Male Sprague-Dawley rats were injected twice with 8 mg 3-methylcholanthrene (MCA) IP 24 hours apart to induce activity of aryl hydrocarbon hydroxylase. Tissues (liver, lungs, adrenal glands, kidneys, stomach, colon, and small intestine) were collected from the rats 24 to 30 hours after the second injection. BHT (25 and 125 μM) was incubated for a few minutes with various homogenated tissues, followed by the addition of 70 nmol of the carcinogen BP and a 10-minute incubation period. BHT was added to systems with and without MCA pretreatment. BHT was the least effective of the antioxidants tested at inhibiting BP 3-hydroxylase activity. Compared to control values (no addition of MCA + treatment with BHT), BHT did not decrease significantly the activity of BP 3-hydroxylase in the adrenal glands, stomach, colon, and small intestine. In the lungs, treatment with BHT caused a decrease in enzyme activity when control values are compared to tissues that were pretreated with MCA. In the liver and kidneys, BHT increased enzymatic activity when control values are compared to tissues that were pretreated with MCA (Rahimtula, Zachariah, and O'Brien 1979).

Nakagawa, Hiraga, and Suga (1980b) reported that BHT became bound to DNA, RNA, and protein in the liver of the rat after feeding. It was suggested that BHT competitively inhibited binding of carcinogens or their metabolites to macromolecules, therefore inhibiting tumor formation by certain chemical carcinogens.

Swiss mice (30/sex/group) were fed 5000 ppm BHT or BHA to investigate their effects on the formation of lung neoplasms induced by isoniazid (INH) and hydrazine sulfate (HS). The carcinogens were fed to mice of each group at a dose of 1.1 mg/day. Continuous administration of both antioxidants did not inhibit tumor formation in those groups receiving HS. However, those groups receiving either antioxidant and INH had a significant ($p < .05$) decrease in incidence of lung neoplasms compared to groups treated only with INH, suggesting a prevention or delay in the formation of lung neoplasms. The group fed only BHT diet had a greater incidence of lung neoplasms compared to animals fed the control diet (Maru and Bhide 1982).

Malkinson (1983) injected (IP) male and female mice of two strains, A/J and BALB/cBy, with 1 mg urethane, with or without 0.4 mg/g BHT or 80 μg/g cedrene (cedar-derived sesquiterpene) plus 0.4 mg/g BHT, 6 hour prior to the urethane dose. The mice were then treated with 0.4 g BHT weekly for 6 weeks, beginning 1 week after treatment with urethane, with or without coadministration of cedrene. The number of lung adenomas was determined 14 weeks after dosing with the carcinogen. In A/J mice, a single dose of BHT prior to injection of urethane significantly decreased tumor multiplicity. Cedrene had no effect on the carcinogenicity of urethane, but the sesquiterpene-induced hepatic microsomal mixed-function oxidase activity and inhibited the protective effect of BHT. This suggested that a metabolite of BHT was responsible for its propylactic activity. When urethane administration was followed by multiple injections of BHT, the number of adenomas increased by ~50%. The effect of cedrene on BHT was not significant. Multiple injections of BHT increased the number of neoplasms nearly threefold in BALB/cBy mice, but this effect was "nearly abolished" by coadministration of cedrene.

Witschi and Morse (1983) evaluated the dose-time relationships and cell kinetics in the enhancement of urethane-induced lung tumor formation by BHT. In one study, 15 to 25 female A/J mice were injected IP with 5 to 1000 mg/kg urethane in 0.9% saline and, starting 24 hours later, were fed 0.75% BHT for 12 weeks. The average amount of BHT consumed was 1.13 g,

which corresponded to a dose of 52 g/kg. BHT had no affect on body weight gain, although feed intake was decreased during the first 2 weeks of feeding. BHT did not modify the spontaneous tumor incidence or tumor multiplicity in noncarcinogen-treated mice injected with saline. The tumor incidence for controls was 48%. At 4 months, the tumor incidence in mice given 1000 mg/kg urethane alone was 100%, and for mice given the high dose and BHT, the incidence was 88%. The control incidence was 48%, and was similar to the incidence in mice treated with 50 mg/kg BHT for 9 months. At 4 to 9 months, mice treated with both 1000 mg/kg urethane and BHT had more neoplasms/lung than mice treated with the carcinogen or vehicle alone. The incidence did not differ between groups given 5 to 25 mg/kg urethane, with or without BHT; therefore, BHT did not enhance tumor formation by low doses of the carcinogen.

In a second study by the same investigators, male mice were fed 0.75% BHT from 1, 2, 3, 4, or 8 weeks prior to injection of urethane and for 4 months after treatment with the carcinogen. The 2-week cumulative dose was 7.3 g/kg. Feed consumption was decreased in mice fed BHT 1 week prior to injection of urethane. Tumor multiplicity increased in 4-week animals compared to 8-week animals, but the difference was less than that of 3-week mice compared to 4-week mice. It was concluded that feeding of BHT for 2 weeks prior to injection of urethane was enough to significantly enhance tumor development.

In a third study, male mice were injected IP with 2.5 to 25 mg/kg of the carcinogen and were fed 0.75% BHT for 4 weeks. At the end of this period, the mice were injected with urethane a second time. The mice were killed 4 months later. No difference was observed in tumor multiplicity or incidence compared to controls.

The same investigators injected mice with 1000 mg/kg urethane prior to feeding of 0.10%, 0.25%, or 0.50% BHT for 8 weeks. The cumulative intake was 4.8 to 20.3 g/kg BHT. All three concentrations increased tumor multiplicity in a dose-dependent manner regardless of whether the neoplasms were counted 4 or 9 months after injection.

Males fed 0.75% BHT from 1, 2, or 4 weeks prior to injection of 1000 mg/kg urethane and for 4 weeks after dosing had no enhancement of tumor formation. In a BHT tumor promotion study by these investigators, three to five males and females per group were injected with 5 mg/kg MCA, 300 mg/kg BP, or 7 mg/kg *N*-nitrosodimethylamine in 0.1 ml corn oil/10 g body weight or 0.9% saline. The mice were then fed 0.75% BHT for 8 weeks. BHT increased the proliferation of type II alveolar cells during the first 2 weeks of feeding, and increased tumor multiplicity from 4- to 10-fold over positive controls (Witschi and Morse 1983).

Balansky et al. (1986) treated male and female BD-VI rats with a single oral dose of MNNG in corn oil. Males were treated with 250 mg/kg MNNG by intubation (group 1M), MNNG plus 400 mg/l vitamin C in drinking water (group 2M), MNNG plus vitamin C and 100 g milk broth containing 40,000 IU vitamin A, 0.5 g vitamin E, and 0.1 g BHT three times weekly (group 3M). The antioxidant treatment was started 7 days prior to administration of MNNG and continued throughout the study. Rats of group 4M were treated IP with a daily 1.0 mg/kg dose of oxyferriscorbone, from the week before to the week after MNNG exposure, then three times weekly to the end of the study. Female rats were treated with 333 mg/kg MNNG by intubation (group 1F) or MNNG and 100 mg/rat GSH intragastrically, 1 hour prior to MNNG dosing and 5, 24, 48, and 72 h after dosing (group 2F). The results, after 15 months of treatment, are listed in Table 12.

Vitamin C and GSH were the most effective inhibitors of MNNG-induced carcinogenesis. Modification by BHT was not apparent, probably due to the low dose used. No obvious interaction was observed between vitamin C and the other vitamins (Balansky et al. 1986).

Malkinson and Thaete (1986) investigated the effects of strain and age on the anticarcinogenic and cocarcinogenic effects of BHT. An IP injection of 200 mg/kg BHT in corn oil, 6 hours prior to injection of 1 mg/g urethane in 0.9% saline, was given to strains of mice with high (A/J, SWR/J) or low (BALB/cByJ, 129/J, or C57BL/6J) susceptibility to urethane-induced lung tumorigenesis. Each group had 10 to 16 mice per strain. In adult A/J mice, average lung tumor multiplicity decreased by 32% after treatment with BHT/urethane. In contrast, BHT increased the number of neoplasms by 48% in adult SWR/J mice, 240% in adult C57BL/6J mice, 655% in adult 129/J mice, and 38% in 14-day-old A/J mice. The results were similar for the numbers of type II alveolar cell–derived and bronchiolar Clara cell–derived lung adenomas. Pretreatment with BHT had no effect on adenoma multiplicity in either young or adult BALB/cByJ mice. In a second study, mice were given a single urethane injection followed by six weekly injections of BHT. BHT did not affect the multiplicity of urethane-induced adenomas in young BALB/cByJ mice, but the antioxidant increased tumor multiplicity in adults of this strain.

In tumor promotion studies using mice, BHT decreased spontaneous tumor development compared to vehicle controls (Witschi, Williamson, and Lock 1977). Dietary BHT enhanced the chemoprotective effects of other compounds, including retinyl acetate and indomethacin, against DMBA-induced mammary carcinogenesis in Sprague-Dawley rats (McCormick et al. 1986; McCormick and Wilson 1986).

Male F344 rats were given 0.05% dibutylnitrosamine (DBN) in drinking water for 16 weeks, and were fed 0.7% BHT simultaneously. The rats were then fed basal diet and tap water for another 11 weeks. Most rats given DBN-BHT developed hepatocellular carcinomas. Five DNA samples isolated from the carcinomas were tested for transforming activity using a transfection assay with NIH3T3 cells. Only one sample, from RK118 tumor induced by DBN-BHT, had transforming activity. RK118 produced two independent primary transformants, which formed nine secondary transformants. The primary transformants were highly tumorigenic in nude mice, and formed visible neoplasms within 2 weeks after SC injection (10^6 cells/mouse). DNA from the transformants was digested with restriction endonucleases,

TABLE 12
Effect of antioxidants on MNNG-induced gastric carcinogenesis (Balansky et al. 1986)

No. rats (no. survived)	No. rats with neoplasms	No. cases with gastric neoplasms[a]								
		Nonglandular stomach				Glandular stomach				Other localizations
		SP[b]	P	SCC	S	SA	MA	AC	S	
Group 1M—250 mg/kg MNNG (control)										
40 (34)	28 (82.4%)	7	7	3	2	3	—	6	2	1[c]
Group 2M—MNNG + Vitamin C										
47 (30)	12 (40.0%; $p < .001$)	4	3	—	—	2	2	5	—	1[d]
Group 3M—MNNG + Vitamin C, Vitamin A, Vitamin E, BHT										
47 (27)	11 (40.7%; $p < .01$)	4	4	1	—	1	—	2	—	—
Group 4M—MNNG + Oxyferriscorbone										
47 (20)	10 (50.0%; $p < .05$)	4	3	—	1	1	1	—	—	1[e]
Group 1F—333 mg/kg MNNG (control)										
47 (11)	8 (72.7%)	4	4	2	—	—	1	1	—	—
Group 2F—MNNG + GSH										
53 (25)	9 (36.0%; $p < .05$)	4	1	1	1	—	—	1	1	5[f]

[a] In some cases, multiple neoplasms with different histologic characteristics were detected.
[b] SP = solitary papilloma; P = papillomatosis; SCC = squamous cell carcinoma; S = sarcoma; SA = solitary adenoma; MA = multiple adenoma; AC = adenocarcinoma.
[c] Carcinoma.
[d] Fibrosarcoma.
[e] Mesothelioma.
[f] Fibroadenoma of mammary gland, AC of mammary gland, leiomyoma, lymphosarcoma.

and the activated oncogene was identified as rat N-*ras* using Southern blot analysis. N-*ras* is a transforming gene (via a point mutation) that was found previously in a human neuroblastoma cell line and several human neoplasms. Activated N-*ras* has been associated frequently with mouse thymomas induced by methylnitrosourea (MNU) (Funato et al. 1987).

Hirose et al. (1987b) reported a study in which BHT (0.7%) had no synergistic effect on the formation of proliferative lesions of the nonglandular stomach when administered in feed to male F344 rats with 1% BHA. Two of the 10 treated rats died of internal hemorrhages prior to the end of the study, and liver weights were increased significantly in the surviving rats. In addition, no gross changes were observed in rats treated with a 2% (total) mixture of BHT, BHA, α-tocopherol, propyl gallate, and sodium ascorbate, although mild hyperplasia in the mid region of the stomach was observed in 20% of the rats. This treatment was "far less potent" for the induction of hyperplasia of the nonglandular stomach than 1% to 2% BHA alone.

Using electron spin resonance, Mikuni, Tatsuta, and Kamachi (1987) determined the scavenging effect of BHT on the hydroxy free radicals produced by the reaction of hydrogen peroxide with MNNG. The investigators also examined MNNG-derived free radicals. BHT was added to the H_2O_2-MNNG system (containing the spin-trapping agent 5,5-dimethyl-1-pyrroline-1-oxide, 50% acetonitrile, and NaCl) at concentrations of 2.5 to 50 mM. The systems were transferred to quartz cells which were exposed for 3 minutes to irradiation from a tungsten-halogen lamp with an intensity of 0.3 mW/cm^2, and were examined using an electron spin resonance spectrometer. Free-radical formation from this spectrum was compared to those of systems without H_2O_2 or MNNG that had 50 mM BHT instead of the spin-trapping agent, and of systems that contained no BHT. When BHT was added to the system, the amount of trapped OH and MNNG radicals decreased and reached constant concentrations as the concentration of the antioxidant increased. In the H_2O_2-MNNG-BHT system, the signal of the BHT free radical appeared, indicating that BHT acted as a scavenger. BHT apparently formed the stable peroxyl free radical after the labile phenol radical was abstracted by the OH and MNNG radicals. The OH radical has been known to damage cell membranes and nucleic acids, and the investigators proposed that BHT's scavenging action could prevent MNNG-induced carcinogenesis.

Sato et al. (1987) investigated the potential of BHT to initiate carcinogenesis using a two-stage skin carcinogenesis model where 12-o-tetradecanoyl phorbol-13-acetate (TPA) was the promoter. BHT was dissolved in DMSO at a concentration of 10 mg/ml and was applied to the shaved, dorsal skin of female CD-1 mice twice weekly for 5 weeks (total initiating dose = 100 mg; 20 mice/group). The mice were then treated with TPA for 47 weeks. The positive control compound was DMBA. Topical applications of BHT caused pulmonary toxicity that resulted in the death of ~25% of the treated mice, such that only 15 and 19 mice treated with BHT/TPA and BHT, respectively, survived beyond 26 weeks. Two of the BHT/TPA-treated mice (13%)

had one papilloma each; the average number of neoplasms per mouse was 0.13. No skin neoplasms were observed when mice were treated with BHT only. The investigators concluded that the tumor initiating activity of BHT was nonsignificant.

Taffe and Kensler (1988) applied BHT-OOH to the skin of SENCAR mice twice weekly for 50 weeks after initiation with DMBA. Doses of 2, 8, and 20 μmol BHT-OOH gave maximal papilloma responses of 0.1, 0.6, and 3.6 neoplasms/mouse, respectively. At 60 weeks, the incidences of carcinomas were 0%, 17%, and 28% for the low-, intermediate-, and high-dose groups, respectively. In the two higher-dose groups, the carcinoma: papilloma ratios were 0.08 and 0.40, respectively. Uninitiated mice treated only with the metabolite had no neoplasms. The investigators concluded that BHT-OOH was an effective tumor promoter in mouse skin and that it increased the incidence of carcinomas.

When added to the feed of five F344 rats at a concentration of 1%, BHT increased DNA synthesis in the urothelium of the urinary bladder. DNA synthesis was determined using the incorporation of 5-bromo-2′-deoxyuridine into the nucleic acid. In addition, the bladder epithelial surface of three of five rats had leafy or ropy microridges when examined using scanning electron microscopy. These changes were considered "characteristic of the complete range of bladder promoters investigated" (Shibata et al. 1989).

Taffe et al. (1989) examined the metabolism of BHT-OOH to determine the role of reactive intermediates in the mediation of tumor promotion. This study was performed using isolated neonatal mouse keratinocytes or a cell-free hematin system using electron paramagnetic resonance spectroscopy. Nonradical products were characterized using HPLC separation coupled with UV spectroscopy or MS. When BHT-OOH was incubated with keratinocytes or hematin, a BHT phenoxyl radical was generated; formation of the radical was prevented by heat inactivation of the cells prior to exposure to the BHT metabolite. One nonradical metabolite of BHT-OOH, BHT-quinol, was detected in keratinocytes, whereas incubation with hematin produced BHT-QM, BHT-quinone, BHT, BHT-stilbenequinone, and 2,5-di-*tert*-butyl-5-(2′-oxopropyl)-4-oxa-2-cyclopentenone. When the stable metabolites of BHT-OOH were evaluated for possible promoter activity using ODC activation in mouse skin, topical application of equimolar doses of BHT-quinol, BHT-quinone, BHT-stilbenequinone, and BHT did not induce epidermal ODC activity. The investigators concluded that reactive metabolites such as the BHT phenoxyl radical and BHT-QM could be involved in the molecular mechanisms of tumor promotion by BHT-OOH.

BHT caused a fivefold induction of proliferin gene expression when the antioxidant was added to C3H/10T1/2 cultures at a concentration of 20 μM (Parfett 1992). The proliferin gene family is prolactin and growth hormone related, and produces mitogen-regulated protein, an antagonistic regulator of muscle-specific transcription (Taffe et al. 1989).

Billington, Chard, and Clayton (1990) reported that BHT increased the permeability of tight junctions to horseradish peroxidase in the isolated perfused liver of rats, and decreased the transfer of the protein from the perfusate to bile.

BHT inhibited the activity of poly ADP-ribose transferase by 18% in human epidermoid carcinoma HeP_2 cells also treated with phorbol-12-myristate-13-acetate or benzyl peroxide. Poly ADP-ribosylation "appears to play a role in several facets of chromatin structure and function" (Singh 1990).

Singletary and Nelshoppen (1991) reported that, when female Sprague-Dawley rats were fed 0.4% or 0.8% BHT prior to intubation with 31.6 mg/kg DMBA in 0.5 ml corn oil (~5 mg/rat), the binding of DMBA to DNA decreased by 41.5% and 35.6%, respectively, compared to controls. The decreases in total binding, however, were not due to uniform inhibition of the formation of all individual adducts. The formation of two adducts from the binding of *anti*-dihydrodiolepoxide of DMBA to deoxyguanosine was decreased by 51.5% (combined average) in rats fed BHT. The formation of *syn*-derived DMBA-DNA adducts was not consistently inhibited. The decrease in binding of *syn*-dihydrodiolepoxide to deoxyadenosine was significant only after feeding with the low dose of BHT, and the formation of the *syn*-deoxyguanosine adduct was not affected by feeding of BHT. The investigators concluded that dietary BHT inhibited in vivo mammary DMBA-DNA binding and formation of *anti*-derived DMBA-DNA adducts, the latter of which could contribute to the inhibitory effect of BHT on initiation of DMBA-induced mammary tumorigenesis.

In a study in which female Sprague-Dawley rats were treated with DMBA, BHT, and BHT-quinone inhibited mammary tumorigenesis when administered 2 weeks before to 1 week after the carcinogen. BHT and BHT-quinone (200 mg/kg each) decreased carcinogenicity by 39% and 25%, respectively. An equivalent amount of BHT-BzOH did not have a chemoprotective effect (Singletary et al. 1992).

The effect of BHT on gap junctional intercellular communication was investigated by Chaudhuri et al. (1993) and Guan et al. (1995) using nontumorigenic mouse lung epithelial and rat liver epithelial cell lines. In both studies, BHT was added to the culture medium at a concentration of 0.1% (*v/v*) in DMSO. Inhibition of gap junctional intercellular communication occurred at concentrations ≥62.5 μM in rat liver cells and ≥150 μM in mouse lung cells after 4 hours of treatment. Inhibition occurred within 15 to 30 minutes and was reversed by removing BHT from the culture medium. This initial effect could have been due to gap junction channel closure or blockage, as no changes in the number of junctions, connexin 43 concentration, or the degree of connexin 43 phosphorylation were observed until 2 to 4 hours after treatment. The metabolite 6-*t*-butyl-2-(hydroxy-*t*-butyl)-4-methylphenol was a more potent inhibitor than BHT, whereas another metabolite, 2,6-di-*t*-butyl-4-hydoxymethylphenol was ineffective against the function of gap junctions. Cell viability was determined by lactate dehydrogenase release and gap

junction function was assayed using fluorescent dye microinjection 4 hours after treatment. The gap junctions were stained for microscopy using indirect immunofluorescence via polyclonal rabbit antibodies against three gap junctional proteins. BHT caused dose-dependent inhibition of dye coupling in the cells. The investigators suggested that inhibition of gap junctional intercellular communication could contribute to the tumor promoting activities of BHT.

BHT and other phenolic antioxidants induced expression of c-*fos* and c-*jun* protooncogenes in quiescent human hepatoma HepG2 and JEG-3 choriocarcinoma cells. This effect was determined by measuring the concentrations of mRNA from the two genes. BHT had no effect on the content of glyceraldehyde-3-phosphate dehydrogenase mRNA. This response was dose dependent and reached a maximum at 3 to 6 hours after treatment. In these studies, the cells were treated with 100 or 150 μM BHT in 0.1% ethanol (Choi and Moore 1993).

Inoue et al. (1993) investigated the protective effects of antioxidants on epithelial cell carcinogenesis of the tongue in male Sprague-Dawley rats. The rats were given 0.001% 4NQO in drinking water for 4 or 7 months, with or without BHT (5000 mg/kg feed), which was added to basal feed alone or in combination with sodium selenite and/or vitamin A acetate. At 4 months, rats treated with antioxidants had fewer lesions and less advanced carcinomas than rats treated with the carcinogen alone (Table 13), but the latter modification was not considered significant. Chemoprotection by BHT plus vitamin A acetate was the most effective. At 7 months, the incidence of carcinomas was decreased by treatment with the antioxidants. In addition, the mean macroscopic depth of the invasive carcinomas was reduced from approximately 20.0 mm to 9.8 to 13.9 mm and the mean microscopic depth was decreased from 4.40 to 1.68 to 2.76 mm, respectively; these parameters were considered significantly different from controls, $p < .01$ or $p < .05$.

In a followup study, a 50% decrease in the survival rate of the human tongue cancer cell line was reported after the cells were treated with 2.6 μg/ml selenium, 60 μg/ml vitamin A, and/or 38 μg/ml BHT. The 50% cytotoxicity concentrations were 0.74, 48, and 30 μg/ml of the three compounds, respectively. When BHT was used, the number of G2/M-phase cells increased and the number of S-phase cells decreased; the investigators concluded that BHT inhibited G1-phase cells and blocked their transition to the DNA synthesis phase (Inoue et al. 1995).

The dietary addition of 0.6% BHT for 2 weeks enhanced excretion of DMBA conjugates by >100% during a 15-hour collection period. The investigators suggested that increased biliary excretion of the conjugates was likely related to the reported decrease of DMBA binding to mammary cell DNA to inhibit DMBA-induced carcinogenesis (Liu, Zhang, and Milner 1994).

When tumor oncogenes from 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK)-treated, female A/J mice were characterized, all 19 NNK-induced lung neoplasms (no BHT) had activated Ki-*ras* genes with GC → AT transitions at the second base of codon 12. In contrast, only 11 of 34 (32%) of lung neoplasms from NNK-BHT–treated mice had this Ki-*ras* alteration. The data suggested that the NNK-initiated tumorigenesis pathway was altered to a predominantly non-*ras* mechanism during promotion by dietary BHT (Matzinger et al. 1994).

Wang and Witschi (1995) reported that 43% of male Swiss-Webster mice and 94% of male A/J mice had mutations of the Ki-*ras* protooncogene in lung neoplasms initiated by 20 mg/kg MCA after six weekly injections of 300 mg/kg BHT. In comparison, 44% of A/J mice and 13% of Swiss-Webster mice treated with the carcinogen alone had Ki-*ras* mutations.

TABLE 13
Modification of tongue carcinogenesis by antioxidants (Inoue et al. 1993)

Treatment (effective no. rats)[a]	Incidence % (no. rats)				
	Intact	Hyperplasia	Dysplasia	Carcinoma in situ	Invasive carcinoma
		4NQO for 4 months			
Control (9)	0 (0)	0 (0)	55.6 (5)	11.1 (1)	33.3 (3)
BHT (9)	0 (0)	11.1 (1)	77.7 (7)	0 (0)	11.1 (1)
BHT + Na selenite (9)	0 (0)	22.2 (2)	22.2 (2)	11.1 (1)	44.4 (1)
BHT + vitamin A (8)	50.0 (4)	0 (0)	37.5 (3)	0 (0)	12.5 (1)
BHT + Na selenite + vitamin A (9)	0 (0)	33.3 (3)	33.3 (3)	11.1 (1)	22.2 (2)
		4NQO for 7 months			
Control (10)	0 (0)	0 (0)	0 (0)	0 (0)	100 (10)
BHT (7)	0 (0)	0 (0)	28.6 (2)	28.6 (2)	42.9 (3)[b]
BHT + Na selenite (10)	10.0 (1)	0 (0)	30.0 (3)	0 (0)	60.0 (6)
BHT + vitamin A (7)	0 (0)	0 (0)	0 (0)	14.3 (1)	85.7 (6)
BHT + Na selenite + vitamin A (10)	0 (0)	0 (0)	10.0 (1)	20.0 (2)	70.0 (7)

[a]Number of surviving rats.
[b]Significantly different from controls, $p < .05$.

Guyton et al. (1996) reported that BHT-OOH stimulated activation of extracellular signal–related kinase (ERK) in vivo and in vitro by 14- and 20-fold, respectively, above controls. In these studies, the skin of female CD-1 mice was treated with 20 μmol BHT-OOH during the resting phase of the hair growth cycle, or cultured mouse keratinocytes were incubated with the metabolite. Maximal ERK activation occured within 10 to 15 minutes of treatment in both studies. In vitro, the metabolite activated c-*jun* *N*-terminal kinase and 38-kDa mitogen-activated protein kinase–related protein by fivefold. BHT-OOH–induced ERK activation was prevented by treatment with *N*-acetylcysteine and *o*-phenanthroline. In contrast, an analog of BHT-OOH that produced less BHT-QM was much less effective at ERK activation. These results suggested that activation depended on the formation of specific reactive intermediates, particularly BHT-QM.

Smith and Gupta (1996) reported that, when incubated with VHT/ht mouse liver microsomes, 800 nmol BHT inhibited the formation of BP-DNA adducts by 45.7%, the metabolism of the carcinogen by 26.5%, and the formation of 7,8-BP-diol by 39.4%, compared to controls (Colovai et al. 1993). BHT (10 to 150 μM) also prevented the formation of BP-DNA adducts by 38% when added to Aroclor 1254–induced rat liver microsomes.

In male Wistar rats, 0.75% BHT (in feed for 15 days) decreased the binding of AFB_1 to DNA by 48% and increased GSH *S*-transferase activity. When added to feed for 6 months, 0.06% BHT did not inhibit AFB_1-DNA binding and had little effect on enzyme activity (Allameh 1997).

Photocarcinogenicity Protection

Antioxidants were also reported to protect against photocarcinogenicity. Specifically, BHT inhibited UV induction of epidermal ODC activity, "considered by some to be a requisite step in carcinogenesis" (Black 1988). In one study using hairless mice (Black et al. 1980), BHT decreased the amount of UV radiation that reached the target site.

Black, Young, and Gibbs (1989) performed a PUVA tumorigenesis study using SKH-Hr-1 mice. The mice were treated topically with 8-methoxypsoralen (8-MOP) at a dose of 1 mg/300 μl ethanol, then were exposed to UVA three times weekly for 31 weeks, with or without 0.5% BHT in feed or 100 μl topical BHT. Thirty minutes after topical application, the mice were irradiated using two Sylvania FS40 T12/BL PUVA lamps positioned 14 cm above the dorsa and filtered through window glass (6 mm thick). The dose was 10 kJ/m^2, which did not produce erythema or epidermal damage. Mice of three groups were treated for 31 weeks (cumulative UVA dose = 930 kJ/m^2), and mice of one group was fed BHT throughout the post-treatment evaluation period. Topical BHT had no significant inhibitory effect on UV- induced ODC activity, but dietary BHT decreased induction by 40%. Mice that received 8-MOP and UVA had a "typical tumorigenic response" (incidence = 100%) at 31 weeks. The mean numbers of neoplasms, mostly papillomas, per mouse were 5.8 (8-MOP alone), 6.0 (topical BHT and 8-MOP), and 5.6 (dietary BHT and 8-MOP). BHT had no significant effect on tumor incidence or multiplicity when administered in feed or via topical application. In a feeding study, 0.5% dietary BHT inhibited the initiation and development of actinic lesions and neoplasms in 150 female hairless mice after UV exposure (Black, Chan, and Brown 1978).

In another study, BHT (35 μl/cm^2, in ethanol) was applied to the shaven skin of the backs of female Wistar rats. After 20 minutes, labeled 8-MOP (35 μl/cm^2, in ethanol) was applied to the same site. Twenty minutes after treatment with 8-MOP, the rats were exposed to UVA for 1 hour from five Philips TL 80/10R lamps (spectral intensity = 30 W/m^2). In a second procedure, the same dose of BHT was applied to the skin of the backs of five rats on each of 4 consecutive days. Five rats were treated with 5×10^{-3} M ethanol (the vehicle) alone. Five hours after the last dose, ethanolic 8-MOP was applied to the skin. After 20 minutes, the rats were irradiated as above, with the exception of two rats, which instead were kept in a dimly lit environment. After both procedures, the rats were killed and their skins isolated. BHT decreased the photobinding of 8-MOP to epidermal DNA and proteins by "quenching" reactive 8-MOP intermediates. The mechanism involved the phenolic hydroxyl group of the antioxidant. BHT also decreased binding of 8-MOP to lipid (Schoonderwoerd, Beijersbergen van Henegouwen, and Persons 1991).

Role of Metabolites in BHT-Induced Toxicity

General Toxicity

The effects of BHT and BHT-QM on phylloquinone epoxide reductase activity were investigated using rat liver homogenates from male Sprague-Dawley rats. BHT (0.45 and 4.5 mM) did not inhibit this enzyme with and without dithiothreitol (DTT). However, BHT-QM did inhibit this enzyme in the presence of DTT with and without NADPH. Because the active metabolite BHT-QM inhibited phylloquinone epoxide reductase, this prevented post-translational modifications of blood factors leading to the bleeding and hemorrhagic deaths observed in rats (Takahashi 1988b).

BHA facilitated the formation of QMs by model peroxidases, and enhanced BHT-induced pneumotoxicity when administered SC to mice. The peroxidase activity of cytochrome P450 generated the BHA phenoxy radical that directly oxidized BHT to BHT-QM. Coadministration of BHA enhanced the covalent binding of BHT to mouse lung protein and increased lung weight of treated mice, but did not decrease mouse lung GSH concentration. Deuteration of BHT "abolished" the toxicity from the combination of both antioxidants. The investigators proposed that the pneumotoxicity was due to BHT-QM, and that BHA either depleted a protective mechanism in pulmonary cells or enhanced the biotransformation of BHT into the QM metabolite (Thompson and Trush 1988a, 1988b, 1989).

Compounds that inhibited BHT metabolism via P450 monoxygenase decreased or completely abolished the enhancement of tumor formation by BHT (Malkinson and Beer 1984; Witschi 1986a). In a multistage mouse skin carcinogenesis assay, hematin or keratinocyte cytosol converted BHT-OOH to

the BHT phenoxyl radical, which formed BHT-QM (Taffe et al. 1989; Guyton et al. 1991; Guyton, Thompson, and Kensler 1993). BHT-OOH increased epidermal ODC activity and enhanced DMBA-initiated skin tumorigenesis (Taffe and Kensler 1988), whereas a 4-trideuterated methyl analog of BHT-OOH decreased both changes in ODC activity and tumor promotion (Guyton et al. 1991). In a study using MA/MyJ mice, BHT-BuOH was at least fourfold more potent than BHT in the promotion of urethane-induced lung neoplasms (Thompson et al. 1989).

BHT-OOH was converted to BHT-QM under oxidizing conditions (Tajima, Yamamoto, and Mizutani 1981; Guyton et al. 1991). Of the BHT metabolites tested, only BHT-QM and BHT-OOH had anticoagulant activity, and both were more potent than the parent molecule (Takahashi and Hiraga 1978b; Takahashi, Hayashida, and Hiraga 1980). BHT-QM and BHT-OH-QM can damage DNA through alkylation of exocyclic amino groups of base residues (Lewis et al. 1996). Yamamoto, Tajima, and Mizutani (1980) reported that BHT-OOH was likely the most toxic metabolite of BHT when administered to mice via IP injection, and this metabolite caused cytotoxicity in isolated rat hepatocytes (Thompson, Bolton, and Malkinson 1991).

Cytotoxic effects of the metabolites BHT-OH and BHT-OH-QM in mouse Clara cells were determined in vitro. BHT-OH was 10-fold more toxic than BHT to Clara cells. However, a cytochrome P450 inhibitor protected against the cytotoxic effects of both phenols. The pulmonary effects of BHT were mainly due to the BHT-OH-QM metabolite. Additional details were not available (Bolton, Malkinson, Thompson 1992).

Toxicity corresponded to the heat of formation of QM (see Table 4) intermediates and parent BHT analogs, as well as the electronic charge on the carbonyl oxygen atom of the QMs. The metabolic activation of BHT analogs to their respective QMs was "energetically dependent on the oxidation of the aromatic π-electron system," and the toxic potential of BHT analogs was controlled by protonation of the QM oxygen atom (Yamamoto et al. 1997).

Pneumotoxicity

BHT-induced pneumotoxicity was decreased by agents that influenced biotransformation, such as phenobarbital (Williamson, Esterez, and Witschi 1978), and was enhanced by compounds that lowered GSH concentrations, such as BSO (Mizutani et al. 1984). BHT-induced pulmonary DNA synthesis was diminished by the drug metabolism inhibitors piperonyl butoxide and 2-diethylaminoethyl-2,2-diphenylvalerate, hydrochloride (i.e., SKF 525-A) (Kehrer and Witschi 1980b).

The data indicated that an electrophilic metabolite of BHT was responsible for its pulmonary effects. The covalent binding of BHT to lung protein was inhibited by cysteine and other compounds (Kehrer and Witschi 1980b; Nakagawa, Hiraga, and Suga 1984a).

Bolton and Thompson (1991) reported that lung microsomes catalyzed metabolism of BHT to BHT-QM. In a study of the pneumotoxicity in mice of various alkylphenols, only phenols that could yield QMs were active toxicants (Mizutani et al. 1982). When the 4-methyl group of BHT was replaced with a trideuteromethyl group, decreases occurred in both BHT-QM formation and pneumotoxicity. The analog generated 30% to 40% less BHT-QM (Mizutani, Yamamoto, and Tajima 1983). Therefore, alterations in the structure of BHT that hindered QM formation or reactivity decreased lung damage by the antioxidant.

Verschoyle, Wolf, and Dinsdale (1993) reported that bioactivation of cytochrome P450 2B isoenzymes were responsible for BHT-induced pulmonary toxicity in mice, as pretreatment with cytochrome inhibitors decreased toxicity by a factor of 20.

Hepatotoxicity

Hepatotoxicity has varied with species and sex; such that rats were susceptible, but mice and hamsters were not, and male rats were more sensitive than females (Takahashi, Hayashida, and Hiraga 1980). Mice were resistant to the hemorrhagic effect of BHT, but had extensive centrilobular necrosis and/or hemorrhages when injected with BHT-QM (Takahashi, Hayashida, and Hiraga 1980; Takahashi 1988a). Rats were resistant to BHT-induced pneumotoxicity. Hepatic microsomes of both rats and mice generate similar amounts of BHT-QM and rats produced sixfold more BHT-QM than mice. Mice, however, also produced BHT-OH, a metabolite that was not formed significantly in rats. Mice injected with compounds that inhibited conversion of BHT to BHT-OH were resistant to BHT lung toxicity (Malkinson 1979b; Thompson et al. 1987; Bolton and Thompson 1991). When the pulmonary effects of BHT-OH were evaluated in mice, the metabolite BHT-OH-QM was 4- to 20-fold more potent than BHT. When reaction rates with nucleophiles were compared for BHT-OH-QM and BHT-QM, the hydroxylated metabolite reacted sixfold faster with the thiol group of GSH and 20-fold faster with the methanol hydroxyl group; these results were suggestive that the BHT-OH-QM would more readily bind cellular components and would react with a greater variety of nucleophiles than BHT-QM (Malkinson et al. 1989; Bolton et al. 1990; Thompson et al. 1990b; Bolton et al. 1993). Pulmonary microsomes from mice hydroxylated BHT to BHT-BuOH. This metabolite, compared to BHT, was severalfold more effective as a lung tumor promoter, more pneumotoxic in vivo, and more cytotoxic to isolated rat hepatocytes and mouse bronchiolar Clara cells in vitro (Thompson, Bolton, and Malkinson 1991).

CLINICAL ASSESSMENT OF SAFETY

Skin Irritation and Sensitization

Predictive

Mallette and Von Haam (1952) performed a 48-hour patch test of 100% BHT using "not less than 15 individuals." Of the subjects, 71% had negative irritancy reactions and 29% had slight irritancy reactions. Sensitization reactions were observed in 19% of the subjects. The investigators concluded that BHT was a mild irritant and a moderate sensitizer.

Provocative

Subjects with eczematous dermatitis (360) were patch-tested with 5% BHT or BHA. When placed on the back for 48 hours none of the subjects reacted to BHT and only one subject reacted to BHA (Meneghini, Rantuccio, and Lomuto 1971).

Fisherman and Cohen (1973) treated seven patients with suspected sensitivity to BHA/BHT; 30 patients with either atopic rhinitis, atopic asthma or emphysema, or a nonimmunologic disease of the respiratory tract (control group); and 7 patients with known aspirin intolerance with gelatin capsules containing BHA/BHT. Positive sequential vascular response test results were reported by the antioxidant sensitive patients after challenge with BHA/BHT; however, these patients did not react upon challenge with niacin or aspirin.

When 112 patients with eczematous dermatitis were patch tested with 2% BHT or BHA in petrolatum, 2 reacted to both antioxidants, 1 reacted to BHT alone, and 1 reacted to BHA alone. The patients who reacted to both were asymptomatic when the antioxidants were added to food, and both had acute flares of vesicular eczema on the fingers after oral administration of small amounts. In another study, 83 patients did not react after treatment with 5% BHT or BHA in alcohol (Roed-Petersen and Hjorth 1976).

When 43 patients with perennial asthma were challenged with oral BHA/BHT (maximum dose = 250 mg), none of the patients had signs of bronchoconstriction after exposure to the antioxidants (Weber et al. 1979).

Patients with recurrent urticaria were patch-tested with BHT-BHA (1 to 50 mg). Of the 156 patients tested, 15% reacted positively, 12% had "uncertain" reactions, and 73% did not react to the test material (Juhlin 1981).

Meynadier et al. (1982) investigated sensitivity to BHT 2% in vaseline in 465 individuals (235 males and 230 females) that "most often" suffered from eczema. BHT caused three positive patch-test reactions. No additional details were available.

Over a 2-year period, 1096 patients with facial eczema were routinely patch-tested with a standard series of allergens, an additional set of facial allergens, including the antioxidants (at 1% in petrolatum) BHT, BHA, and BHQ, and ingredients of each patients' cosmetics. Of the patients, seven were allergic to BHA, one reacted to BHT, and five reacted to BHQ (White, Lovell, and Cronin 1984).

Peroral challenge tests were performed with BHA-BHT (1:1; 50 mg/capsule) and other common food additives in 44 cases of chronic urticaria, 91 cases of atopic dermatitis, and 123 cases of contact dermatitis. Two patients each with urticaria or atopic dermatitis had equivocal reactions to BHA-BHT (Hannuksela and Lahti 1986).

Depigmentation

A topical cream was prepared from a food additive containing 20% BHT and 18% BHA in edible oil (final concentrations of test material 0.5% and 3% BHT). The cream was applied to the backs of 50 African-American, 36 females and 14 males, during 48-hour closed patch tests and normal use tests. In the patch test, none of the subjects had reactions at patch removal. Six and nine subjects had positive reactions (slight erythema and desquamation) to 0.5% and 3% BHT, respectively, 7 days after patch removal; these reactions were considered unimportant. In the use study, 3% BHT was rubbed into the skin of the right side of the back for 30 seconds each morning and evening for 7 weeks; the test area was ~5 cm in diameter. No evidence of skin lightening was observed, but one volunteer had mild erythema and folliculitis at 5 weeks, and one had erythema and desquamation of the treatment site that closely resembled lesions of pityriasis rosea (Bentley-Phillips and Bayles 1974).

Maibach, Gellin, and Ring (1975) applied a cream containing 0.2% to 3.3% BHT to the skin of the lower back of 16 adult darkly pigmented males. The creams were applied daily for 60 days on a 1 inch × 1 inch gauze square and were covered with occlusive tape. The test sites were evaluated for signs of depigmentation weekly and at two 1-month intervals after the treatment period. BHT did not cause depigmentation under the conditions of this study.

Case Reports

Two African-American children, one with psoriasis and one with eczema, had hypopigmentation after treatment with polyethylene film applied over a corticosteroid ointment. The area of depigmentation was limited to the treatment site. The film contained BHT as an antioxidant. In one child, the skin returned to normal 8 weeks after treamtent was stopped, but repigmentation did not occur in the second child (Vollum 1971).

An infant with lumbosacral meningomyelocele had BHT, acetone, and other volatile organic compounds present in umbilical cord blood, whereas the mother had a normal blood profile. This suggested a possible selective, one-way transfer of certain constituents to the fetus (Dowty, Laseter, and Storer 1976).

Nitzan, Volovitz, and Topper (1979) reported that nine infants in a pediatric ward who had recoved from acute gastroenteritis had signs of methemoglobinemia, particularly cyanosis. Each patient was being fed a soybean hypoallergenic infant formula containing an antioxidant mixture. The investigators concluded that the antioxidants (BHT, BHA, and propyl gallate) were likely the toxicants. The outbreak ceased when the antioxidants were eliminated from the formula. BHT and BHA are both known to react with oxyhemoglobin to form methemoglobin (Stolze and Nohl 1992), which does not function reversibly as an oxygen carrier (Taylor 1988).

A female patient with contact urticaria (to jewelry and plastics) had urticarial reactions during a 20-minute patch test with several articles of plastic (polyethylene and polyvinylchloride), 1% BHT in ethanol, and 0.1% oleylamide in ethanol. The latter test compound was tested using a 20-minute open test. BHT and oleylamide were used as an antioxidant and slipping agent, respectively, in the plastic samples (Osmunsen 1980).

A female patient with a cutaneous urticarial disseminated eruption with signs of vasculitis after daily consumption of a

chewing gum containing BHT (concentration not stated). Within 1 week after she stopped using the gum, the lesions disappeared. One month later, a series of oral provocation tests were performed at 4-day intervals. Several hours after ingestion of BHT, similar cutaneous lesions appeared (Moneret-Vautrin, Faure, and Bene 1986).

Two female patients, one with chronic psoriasis and contact dermatitis of the legs and feet, and one with chronic hand eczema, reacted to 2% BHA in petrolatum, but not to 2% BHT during a patch test (Tosti et al. 1987).

A 77-year-old male with an 18-month history of statis ulcers of the leg was treated with paste bandages supported by Lestreflex or Secure forte bandages. Upon treatment, the leg again became eczematous. The patient was patch-tested with the European standard series and with BHT (used in the bandages). In the first test, the patient had a (+) reaction to the antioxidant. On further testing, the patient had a (++) reaction to BHT (Dissanayake and Powell 1989).

Goodman et al. (1990) reported that chronic urticaria in two patients was exacerbated by ingestion of 125 or 250 mg BHT and BHA; the lesions subsided when the patients were placed on dye- and preservative-free diets.

Contact leukoderma was reported after an African-American male used a gel for 6 months to darken hair of the scalp and mustache. Depigmentation occurred in the hair and skin of the nuchal and mustache areas, and was attributed to the BHT in the hair dye. A 48-hour patch test to the colors and antioxidant gave negative results, but depigmentation occurred after a 1-month closed patch test (Fisher 1994).

SUMMARY

Butylated Hydroxytoluene (BHT) is a substituted toluene used in cosmetic product formulations as an antioxidant. The chemically related ingredient Butylated Hydroxyanisole (BHA) has already been reviewed by the CIR Expert Panel and is recognized as safe in present practices of use. Data submitted to CIR by the FDA in 1998 based on industry reports indicate that BHT was used in 1709 cosmetic formulations. Historically, BHT was used in concentrations up to 1%, but recent information suggested cosmetic formulations have concentrations at 0.0002% to 0.5%. According to FAO/WHO, the current ADI for BHT is 0 to 0.125 mg/kg.

In vitro and in vivo studies demonstrated that <4% of BHT penetrated the skin. BHT accumulated in adipose tissue at a greater concentration in humans than in rats when compared on a dose/body weight basis. The carboxylic acid of BHT and its glucuronide were the major metabolites present in human urine. The two main metabolic processes occurred in microsomal fractions of hepatic and pulmonary tissue of rats and mice. BHT metabolites differ quantitatively in different strains of mice. Single oral doses of 800 mg/kg BHT in rats resulted in peak plasma concentrations 18 hours after dosing, with inhibitory effects on gastric function. BHT and its metabolites were excreted primarily in the urine in humans, whereas in rats, the metabolites of BHT were seen in both the urine and bile, irrespective of the route of administration.

BHT cytotoxicity was concentration and time dependent and appeared to have a greater effect on female cells because female cells metabolize BHT more slowly. BHT demonstrated cytotoxicity using various mammalian cells lines in vitro, as well as some additive toxic effects when incubated with BHA. BHT decreased the cellular ATP and hepatic GSH, GOT, and GPT levels. BHT is more cytotoxic than BHA and appears to exert its cytotoxic effects in a dose-dependent manner. BHT induced hemolysis in the erythrocytes of rats. Using various mammalian cell lines, some metabolites were more toxic than the parent compound in vitro.

BHT functions to stabilize biomembranes against lipid peroxidation, but at high concentrations (>60 nmol/mg), it increased membrane permeability and acted as a membrane uncoupler. It also interacted directly with electron transport and inhibited respiration. BHT demonstrated antimicrobial and antiviral inactivation at various concentrations. In microsomes isolated from rats, binding to cytochrome P450 was suggested because BHT inhibited the mixed function oxygenase system and decrease in NADPH–cytochrome P450 activity was observed. Although microsomes of the brain, kidney and spleen had no BHT-binding capacity, lung microsomes had a binding capacity of ~40% of hepatic microsomes. BHT inhibited the arachidonic acid cascade and platelet aggregation in vitro. Ascorbic acid incubated with BHT added to the prooxidizing effect observed in liver mitochondria.

BHT was classified as nontoxic in rats at 8.0 mg/kg IP in an acute toxicity study. Acute doses of 800 mg/kg BHT caused increased liver weights in rats. An acute dose of 500 to 1000 mg/kg BHT caused some renal and hepatic damage in male rats; however, the female rats were more resistant to the effects of BHT at this dose. Plasma coagulation factors were decreased after administering 800 mg/kg BHT to rats; however, cobaltous chloride, phylloquinone, and SKF 525A prevented the decrease in coagulation factors.

In a short-term toxicity study, the administration of 0.01% BHT to rats resulted in no significant deleterious effects, 0.1% stimulated growth in male rats, 0.5% decreased body weight, increased liver weights and enlarged livers. The decreased body weight appears to be more evident in male than female rats. A single dose of BHT at 500 mg/kg/day was not sufficient to induce hepatotoxicity. However, repeated doses of 100 to 500 mg/kg BHT and BHA resulted in increased liver weights in male and female rats. High doses of BHT, 1000 to 1250 mg/kg/day, in rats for 4 days resulted in hepatic toxic effects. Oral doses of 0.05% to 0.15% BHT induced changes in drug metabolizing enzymes in mice, hamsters, and rats. The oral LD_{50} of BHT in rats was 760 mg/kg/day and the MTD of BHT in mice and rats was 6000 ppm. Dosing BHT at 1.2% resulted in time-dependent and dose-dependent decreases in blood coagulation factors. The hemorrhagic effect of BHT observed in rats has been reported to be related to the chemical structure of BHT

and not its antioxidant properties. BHT fed for 7 days at 0.32% to 1.2% induced hypoprothrombinemia and depleted vitamin K, resulting in hemorrhage in rats. Hemorrhagic deaths in rats occurred at doses of 1.14 to 2.91 mmol/kg/day. Hemorrhaging occurred in rats fed a metabolite of BHT (BHT-QM) dosed at 75 to 150 mg/kg, which decreased coagulation factors in a dose-dependent manner. Other effects of treatment with BHT resulted in dose-dependent decreases in feed and water consumption, spleen weight, and body weight, as well as alterations in hepatic and plasma cholesterol and increases in liver weight. In short-term studies, species differences were found with respect to hemorrhagic death due to BHT administration.

In subchronic feeding and IP studies, BHT increased ascorbic acid secretion. Increased liver weight occurred at doses of 0.1% and 5000 ppm when BHT was fed for 16 weeks and 90 days, respectively. A diet of 0.2% BHT decreased feed intake, diet efficiency, growth, and the protein efficiency ratio. BHT decreased the activity of several hepatic enzymes when fed to rats at 250 mg/kg/day for 60 days.

Rats dosed with BHT and retinyl acetate simultaneously for 120 to 180 days demonstrated a synergistic effect relative to increases in biliary hyperplasia and hepatic fibrosis. Female rats fed 0.4% BHT for 80 weeks or orally dosed 250 mg/kg/day of BHT for 32 weeks had increased activities of some hepatic enzymes. Mice fed 0.75% BHT (684 to 890 days) had increased mean survival times and increased liver size at necropsy. BHT (100%) produced moderate irritation but not sensitization during a 48-hour patch test.

BHT applied to the skin of CD-1 mice three times per week for 4 weeks caused congestion, enlargement of the lungs, necrosis of type I alveolar epithelial cells, and an increased number of type II alveolar cells. BHT increased DNA synthesis and enzymatic activity in the lungs of mice in various studies. Doses of BHT above 175 mg/kg rapidly increased the lung/body weight ratio. Mice and rats had increased DNA synthesis in the lungs as a result of treatment with 400 or 500 mg/kg BHT.

The pulmonary toxicity of BHT increased with the coadministration of BHA, although the latter compound itself did not cause lung damage. Pulmonary toxicity in mice occurred earlier and additional damage to the lungs was observed when BHT was administered with oxygen exposure. These effects were modified by exposure to oxygen before or 7 days after BHT treatment. The pulmonary histopathologic changes induced by the antitumor agent bleomycin were inhibited by BHT. BHT plus irradiation treatment has been reported to increase and decrease the survival time of mice. Female mice were more susceptible to topical BHT-induced lung damage than male mice. Species and strain differences have been reported in BHT-induced pulmonary toxicity.

In one study, BHT inhibited the formation of cataracts in the rat lens after damage induced by 4-hydroxynonenal.

BHT was nonteratogenic in mice, rabbits, and rhesus monkeys when fed at $\leq$0.405% or $\leq$320 mg/kg/day. BHT fed to rats at 500 to 1000 mg/kg/day caused a decrease in the body weights of their pups due to decreased nutrition or suckling ability. In one study, litter efficiency was decreased when BHT was fed to rats with a diet low in vitamin E content. The reproductive and developmental NOEL in rats was 1000 ppm BHT. In various strains of mice and rats, BHT was nonteratogenic at doses of 250 to 1000 mg/kg/day. Behavioral changes were observed in the offspring of rats and mice fed 0.5% BHT.

BHT decreased significantly the leukocyte count in immunotoxic studies with mice. The density of dendritic epidermal cells increased and the expression of the Thy-1 marker on keratinocytes was enhanced when mice were treated topically with BHT. The addition of 100 μg/ml BHT decreased significantly the viability of lymphocytes in vitro. BHT has been found to enhance and inhibit the humoral immune response. The PFC response has been inhibited by at least 50 μg/ml.

BHT inhibited the transmission of UV radiation through the stratum corneum of mice and inhibited ODC activity in the intact stratum corneum. In various studies, BHT protected against UV-induced skin erythema in mice and rabbits. In human subjects, 0.1% and 1.0% BHT did not reduce UVB-induced erythema when applied before or after irradiation.

In general, BHT did not induce mutations when tested in many genotoxicity studies. However, BHT did modify the genotoxicity of many other agents.

BHT added to the diet of mice caused significant hepatocellular adenomas in males. Female mice had increased survival time and decreased incidence of neoplasms. BHT was noncarcinogenic in studies performed on rats and mice. However, 300 mg/kg BHT given to male mice enhanced the formation of pulmonary adenomas and pulmonary DNA content was increased dose dependently.

BHT and some of its metabolites generally acted as a tumor promoter, anticarcinogen, or had no effect depending on target organ, route of administration, duration of exposure, time of exposure to BHT and the carcinogen, and the age, sex, strain, or species of test animal.

The metabolites of BHT were significant in the toxicity of BHT and some of the metabolites were more potent than the parent molecule. BHT-induced pulmonary toxicity in mice has been attributed to an electrophilic metabolite of BHT. Hepatotoxicity has varied with species depending on the specific metabolite and the amount of metabolites formed. Compounds that inhibited BHT metabolism via P450 monoxygenase decreased or completely abolished the enhancement of tumor formation by BHT.

In predictive tests, 100% BHT was a mild irritant and a moderate sensitizer. In provocative tests, BHT (2% to 5%) produced positive reactions in a small number of patients. BHT produced depigmentation in a few clinical cases but not in provocative testing.

DISCUSSION

The Expert Panel recognized that there is an extensive literature base examining antioxidants, including BHT. Among these are oral toxicity studies that report positive and negative effects

of BHT in the diet. Although dermal application of BHT results in some absorption into the skin, BHT appears to remain primarily in the skin or passes through the skin only slowly, and dermal application does not produce systemic exposures to BHT or its metabolites of the magnitude seen in oral studies.

There were only limited animal and human tests, however, that evaluated the effects of BHT on the skin. Studies in which topical application of BHT was used to moderate the effect of ultraviolet radiation, however, were considered. Although these studies clearly documented the absence of a photosensitization effect (most studies actually show some level of photoprotection), there also was no sensitization or significant irritation reported. Additionally, the Panel noted that BHT is used in suntan preparations and that there have been no clinical cases of irritation, sensitization, or photosensitization that would suggest any safety concerns.

CONCLUSION

On the basis of the animal and clinical data included in this report, the CIR Expert Panel concludes that BHT is safe as used in cosmetic formulations.

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CATEGORY	MAINTERM	CPIS_COUNT
01B - Baby Lotions, Oils, Powders, and Creams	BHT	2
01C - Other Baby Products	BHT	4
02A - Bath Oils, Tablets, and Salts	BHT	28
02B - Bubble Baths	BHT	31
02D - Other Bath Preparations	BHT	15
03A - Eyebrow Pencil	BHT	64
03B - Eyeliner	BHT	355
03C - Eye Shadow	BHT	279
03D - Eye Lotion	BHT	69
03E - Eye Makeup Remover	BHT	5
03F - Mascara	BHT	113
03G - Other Eye Makeup Preparations	BHT	91
04A - Cologne and Toilet waters	BHT	890
04B - Perfumes	BHT	663
04C - Powders (dusting and talcum, excluding aftershave talc)	BHT	28
04E - Other Fragrance Preparation	BHT	149
05A - Hair Conditioner	BHT	93
05B - Hair Spray (aerosol fixatives)	BHT	6
05C - Hair Straighteners	BHT	13
05E - Rinses (non-coloring)	BHT	5
05F - Shampoos (non-coloring)	BHT	43
05G - Tonics, Dressings, and Other Hair Grooming Aids	BHT	54
05I - Other Hair Preparations	BHT	56
06A - Hair Dyes and Colors (all types requiring caution statements and patch tests)	BHT	5
06B - Hair Tints	BHT	1
06D - Hair Shampoos (coloring)	BHT	1
06G - Hair Bleaches	BHT	1
06H - Other Hair Coloring Preparation	BHT	5
07A - Blushers (all types)	BHT	123
07B - Face Powders	BHT	196
07C - Foundations	BHT	130
07D - Leg and Body Paints	BHT	4
07E - Lipstick	BHT	980
07F - Makeup Bases	BHT	29
07G - Rouges	BHT	19
07H - Makeup Fixatives	BHT	5
07I - Other Makeup Preparations	BHT	163
08A - Basecoats and Undercoats	BHT	3
08B - Cuticle Softeners	BHT	8
08C - Nail Creams and Lotions	BHT	5
08E - Nail Polish and Enamel	BHT	6

08G - Other Manicuring Preparations	BHT	15
09C - Other Oral Hygiene Products	BHT	1
10A - Bath Soaps and Detergents	BHT	1266
10B - Deodorants (underarm)	BHT	140
10D - Feminine Deodorants	BHT	3
10E - Other Personal Cleanliness Products	BHT	330
11A - Aftershave Lotion	BHT	105
11D - Preshave Lotions (all types)	BHT	5
11E - Shaving Cream	BHT	27
11F - Shaving Soap	BHT	4
11G - Other Shaving Preparation Products	BHT	18
12A - Cleansing	BHT	180
12B - Depilatories	BHT	19
12C - Face and Neck (exc shave)	BHT	330
12D - Body and Hand (exc shave)	BHT	514
12F - Moisturizing	BHT	1363
12G - Night	BHT	104
12H - Paste Masks (mud packs)	BHT	22
12I - Skin Fresheners	BHT	7
12J - Other Skin Care Preps	BHT	239
13A - Suntan Gels, Creams, and Liquids	BHT	19
13B - Indoor Tanning Preparations	BHT	32
13C - Other Suntan Preparations	BHT	2

Concentration of Use by FDA Product Category – BHT

Product Category	Maximum Concentration of Use
Baby shampoos	0.01-0.031%
Other baby products	0.0013%
Bath oils, tablets and salts	0.09-0.15%
Bubble baths	0.00024-0.064%
Eyebrow pencils	0.0012-0.1%
Eyeliners	0.0004-0.056%
Eye shadows	0.0002-0.17%
Eye lotions	0.0004-0.11%
Eye makeup removers	0.015-0.1%
Mascara	0.00009-0.3%
Other eye makeup preparations	0.002-0.1%
Colognes and toilet waters	0.00014-0.21%
Perfumes	0.21%
Powders (dusting and talcum)	0.0021%
Other fragrance preparations Leave-on Rinse-off	 0.0021% 0.016%
Hair conditioners	0.00042-0.5%
Hair sprays Aerosol Pump spray	 0.00005-0.006% 0.000007-0.00012%
Hair straighteners	<0.01%
Shampoos (noncoloring)	0.000007-0.5%
Tonics, dressings and other hair grooming aids	0.0000007-0.5%
Wave sets	0.000007%
Other hair preparations (non coloring)	0.000008-0.1%
Hair dyes and colors	0.0015%
Hair tints	0.005%
Hair bleaches	0.0025%
Other hair coloring preparations	0.004%
Blushers	0.00013-0.081%
Face powders	0.0076-0.3%
Foundations	0.001-0.1%
Lipstick	0.0076-0.29%
Rouges	0.02-0.03%
Basecoats and undercoats (manicuring preparations)	0.0005%
Cuticle softeners	0.0006-0.09%
Nail polish and enamel	0.2%
Nail polish and enamel removers	0.25%
Other manicuring preparations	0.05%
Dentifrices	0.03%
Mouth washes and breath fresheners	0.000001-0.0015%
Other oral hygiene products	0.27%

Bath soaps and detergents	0.009-0.31%
Deodorants	
Not spray	0.012-0.19%
Aerosol	0.00006-0.4%
Pump spray	0.000001-0.0031%
Vaginal douches	0.0006%
Feminine hygiene deodorants	0.09%
Other personal cleanliness products	
Hand soap	0.098%
Aftershave lotions	0.051-0.099%
Preshave lotions	0.00017%
Shaving cream	0.0003-0.09%
Shaving soap	0.0001-0.1%
Skin cleansing (cold creams, cleansing lotions, liquids and pads)	0.00084-0.3%
Depilatories	0.1%
Face and neck products	
Not spray	0.0005-0.5%
Spray	0.0000035-0.0012%
Body and hand products	
Not spray	0.00005-0.33%
Spray	0.01-0.09%
Foot powders and sprays	0.0003-0.06%
Moisturizing products	
Not spray	0.001-0.2%
Night products	
Not spray	0.003-0.051%
Paste masks and mud packs	0.01-0.09%
Other skin care preparations	0.001-0.1%
Suntan products	
Not spray	0.05-0.1%
Indoor tanning products	0.01-0.2%
Other suntan preparations	0.05-0.1%

Information collected in 2018

Table prepared October 22, 2018