FINAL

Report on Carcinogens Background Document for

Methyleugenol

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Prepared for the:
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Criteria for Listing Agents, Substances or Mixtures in the Report on Carcinogens

U.S. Department of Health and Human Services
National Toxicology Program

Known to be Human Carcinogens:

There is sufficient evidence of carcinogenicity from studies in humans, which indicates a causal relationship between exposure to the agent, substance or mixture and human cancer.

Reasonably Anticipated to be Human Carcinogens:

There is limited evidence of carcinogenicity from studies in humans which indicates that causal interpretation is credible but that alternative explanations such as chance, bias or confounding factors could not adequately be excluded; or

There is sufficient evidence of carcinogenicity from studies in experimental animals which indicates there is an increased incidence of malignant and/or a combination of malignant and benign tumors: (1) in multiple species, or at multiple tissue sites, or (2) by multiple routes of exposure, or (3) to an unusual degree with regard to incidence, site or type of tumor or age at onset; or

There is less than sufficient evidence of carcinogenicity in humans or laboratory animals, however; the agent, substance or mixture belongs to a well defined, structurally-related class of substances whose members are listed in a previous Report on Carcinogens as either a *known to be human carcinogen, or reasonably anticipated to be human carcinogen* or there is convincing relevant information that the agent acts through mechanisms indicating it would likely cause cancer in humans.

Conclusions regarding carcinogenicity in humans or experimental animals are based on scientific judgment, with consideration given to all relevant information. Relevant information includes, but is not limited to dose response, route of exposure, chemical structure, metabolism, pharmacokinetics, sensitive sub populations, genetic effects, or other data relating to mechanism of action or factors that may be unique to a given substance. For example, there may be substances for which there is evidence of carcinogenicity in laboratory animals but there are compelling data indicating that the agent acts through mechanisms which do not operate in humans and would therefore not reasonably be anticipated to cause cancer in humans.

Summary Statement

Methyleugenol

CASRN 93-15-2

Carcinogenicity

Methyleugenol is *reasonably anticipated to be a human carcinogen* based on sufficient evidence of its carcinogenicity in experimental animals. Oral administration of methyleugenol to rats increased the incidences of benign and malignant tumors of the liver, stomach, kidney, mammary gland, and skin. Oral administration of methyleugenol to mice increased the incidences of benign and malignant tumors of the liver. Tumors of the stomach in male mice also were considered related to exposure to methyleugenol (NTP 1998). Earlier studies found that methyleugenol and two structurally related allylbenzenes, safrole and estragole, induced liver tumors in mice after intraperitoneal injection (IARC 1976, Miller *et al*. 1983). Safrole is classified by the International Agency for Research on Cancer as *possibly carcinogenic to humans* (Group 2B) and is listed as *reasonably anticipated to be a human carcinogen* in the National Toxicology Program's Report on Carcinogens.

No studies on the potential carcinogenicity of methyleugenol in humans have been reported.

Other Information Relating to Carcinogenesis or Possible Mechanisms of Carcinogenesis

Mechanistic data indicate that liver tumors induced by methyleugenol and structurally related allylbenzenes result from metabolism of these compounds to DNA-reactive intermediates. Methyleugenol may be bioactivated by three different pathways: (1) hydroxylation at the 1′ position of the allylic side chain to yield 1′-hydroxymethyleugenol, followed by sulfation of this intermediate to form 1′-hydroxymethyleugenol sulfate, (2) oxidation of the 2′,3′-double bond of the allylic side chain to form methyleugenol-2,3-oxide, and (3) *O*-demethylation followed by spontaneous rearrangement to form eugenol quinone methide. Formation of protein adducts and DNA adducts in the livers of animals treated with allylbenzenes and induction of liver tumors by these compounds have been attributed to activation via the hydroxylation pathway, because similar effects were produced by the 1′-hydroxy metabolites and because these effects were inhibited by pretreatment with sulfotransferase inhibitors (Miller *et al*. 1983, Boberg *et al*. 1983, Randerath *et al*. 1984, Gardner *et al*. 1996).

Methyleugenol, safrole, and estragole induced unscheduled DNA synthesis in rat hepatocytes, and their corresponding 1′-hydroxy metabolites are more potent genotoxic agents than the parent compounds (Howes *et al*. 1990, Chan and Caldwell 1992). Methyleugenol induced morphological transformation in Syrian hamster embryo cells

(Kerckaert *et al*. 1996), sister chromatid exchanges in Chinese hamster ovary (CHO) cells (NTP 1998), intrachromosomal recombination in yeast (Schiestl *et al*. 1989), and DNA repair in *Bacillus subtilis* (Sekizawa and Shibamoto 1982). Methyleugenol did not induce mutations in *Salmonella typhimurium* (NTP 1998) or *Escherichia coli* (Sekizawa and Shibamoto 1982), chromosomal aberrations in CHO cells (NTP 1998), or micronucleated erythrocytes in peripheral blood of mice (NTP 1998). A higher frequency of *β-catenin* mutations was observed in liver tumors from mice treated with methyleugenol than in spontaneous liver tumors from control mice (Devereux *et al*. 1999). Methyleugenol's lack of mutagenicity in bacteria may be due to the need for sulfation in the metabolic activation of methyleugenol to its ultimate mutagenic or carcinogenic form.

No data are available that would suggest that mechanisms thought to account for tumor induction by methyleugenol in experimental animals would not also operate in humans.

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1 Introduction

Methyleugenol was nominated for listing in the Report on Carcinogens (RoC) by the National Institute of Environmental Health Sciences (NIEHS) -National Toxicology Program (NTP) RoC Review Group (RG1) because two-year studies of methyleugenol conducted by the NTP showed clear evidence of carcinogenic activity in rats and mice of both sexes. In addition, methyleugenol is structurally related to safrole, an agent classified by the International Agency for Research on Cancer (IARC) as *possibly carcinogenic to humans* (Group 2B) and is listed as *reasonably anticipated to be a human carcinogen* in the NTP RoC.

Methyleugenol is used in its natural or synthetic forms as a flavoring agent in foods, an attractant in insecticides, and as a fragrance in perfumes and soaps. Methyleugenol was detected in 98% of 206 adult human serum samples analyzed in the Third National Health and Nutrition Examination Survey (NHANES III). Thus, human exposure is expected to be widespread. This document provides a qualitative evaluation of human exposure to methyleugenol and its potential carcinogenic risk.

1.1 Chemical identification

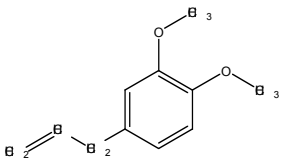
Methyleugenol ($C_{11}H_{14}O_2$, mol wt 178.2304, CASRN 93-15-2) also is known by the following names:

1,2-dimethoxy-4-(2-propenyl)benzene	1-allyl-3,4-dimethoxybenzene
4-allylveratrole	1,2-dimethoxy-4-allylbenzene
1-(3,4-dimethoxyphenyl)-2-propene	1,3,4-eugenol methyl ether
eugenol methyl ether	eugenyl methyl ether
allyl veratrole	veratrole methyl ether
dimethoxy-4-(2-propenyl)benzene	4-allyl-1,2-dimethoxybenzene
o-methyl eugenol ether	3,4-dimethoxyallylbenzene
2-methoxy-4-propenylphenol methyl ether	methyl eugenyl ether

Its RTECS code is CY2450000.

1.2 Physical-chemical properties

The structure of methyleugenol is illustrated in Figure 1-1, and its physical and chemical properties are summarized in Table 1-1. Methyleugenol is a colorless to pale yellow, oily liquid with a boiling point of 254.7°C and a melting point of -4°C. It has a delicate clover-carnation odor and a bitter burning taste. It forms azeotropic mixtures with ethylene glycol, eugenol, and benzoic acid. It slowly darkens and thickens when exposed to air and readily evaporates at room temperature (Lide 1998).



Source: ChemFinder 2000

Figure 1-1. Structure of methyleugenol

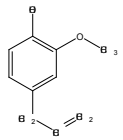
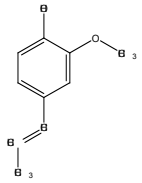
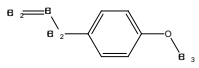
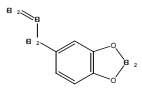
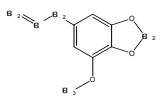
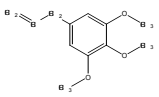
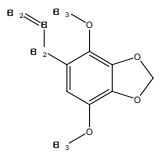
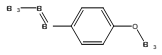
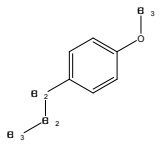
Table 1-1. Physical and chemical properties of methyleugenol

Property	Information	Reference
Molecular weight	178.2304	Budavari *et al.* 1996, ChemFinder 2000
Color	colorless to pale yellow	Budavari *et al.* 1996, Lide 1998, ChemFinder 2000
Odor	delicate clover-carnation odor	Lide 1998, HSDB 1996
Taste	bitter, burning taste	HSDB 1996
Physical state	liquid	Budavari *et al.* 1996, Lide 1998, ChemFinder 2000
Melting point (°C)	-4	Budavari *et al.* 1996, Lide 1998, HSDB 1996
Boiling point (°C)	254.7	Budavari *et al.* 1996, Lide 1998, HSDB 1996
Specific gravity (density) at 20°C or 4°C	1.0396	HSDB 1996
Refractive index	1.532	NTP 1998
Vapor pressure (mm Hg at 85°C)	1	HSDB 1996
Flash point (°C)	117	ChemFinder 2000
Solubility: Water at 19°C Ethanol Ether Chloroform Glycol Propylene glycol	 < 0.1 g/100 L soluble soluble soluble insoluble insoluble	 ChemFinder 2000 HSDB 1996 HSDB 1996 NTP 1998 NTP 1998 NTP 1998

1.3 Identification of structural analogs

Some structural analogs of methyleugenol are listed in Table 1-2.

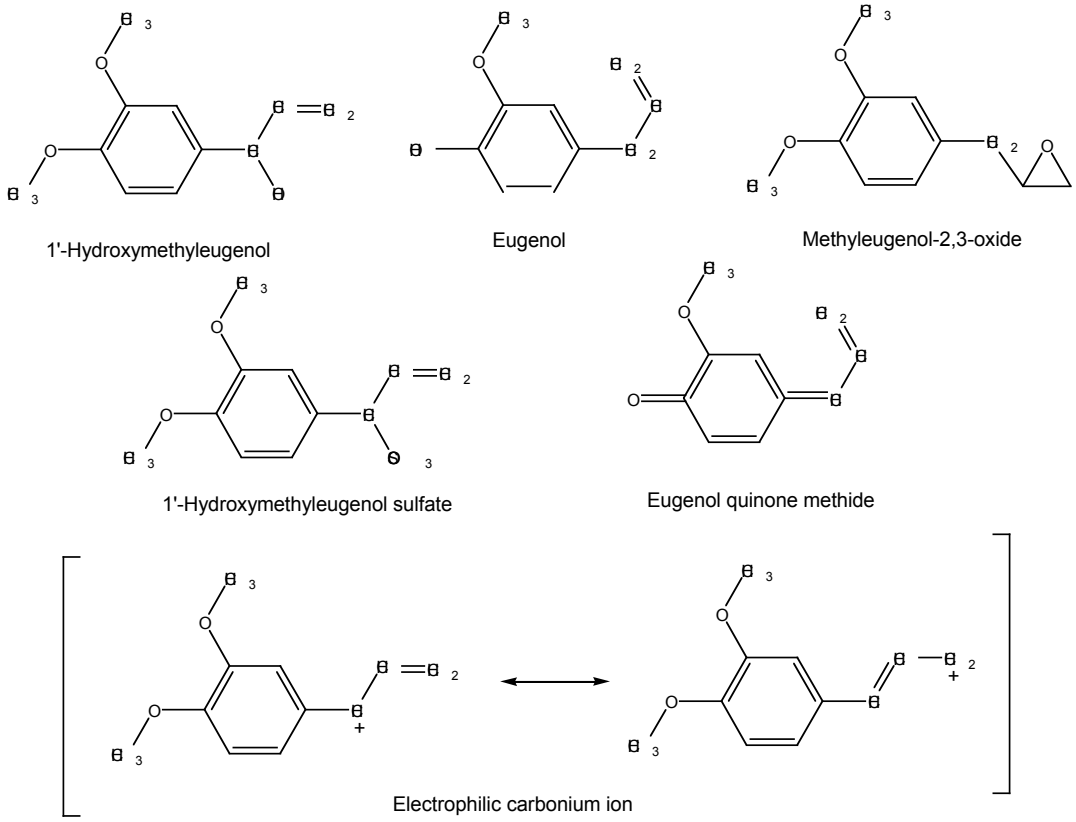
Table 1-2. Certain structural analogs of methyleugenol

Chemical name Formula Molecular weight	CASRN	Structure	Melting point (°C)	Boiling point (°C)	Solubility
Eugenol $C_{10}H_{12}O_2$ 164.20	97-53-0		15.44	487	practically insoluble in water
Isoeugenol $C_{10}H_{12}O_2$ 164.20	97-54-1		64.4	507	slightly soluble
Estragole $C_{10}H_{12}O$ 148.22	140-67-0		NA	216	insoluble
Safrole $C_{10}H_{10}O_2$ 162.19	94-59-7		11.2	232	insoluble in water
Myristicin $C_{11}H_{12}O_3$ 192.21	607-91-0		NA	173 at 40 mm Hg	NA
Elemicin $C_{12}H_{16}O_3$ 208.26	487-11-6		NA	NA	NA
Apiole $C_{12}H_{14}O_4$ 222.24	523-80-8		29.5	294	insoluble in water
trans-Anethole $C_{10}H_{12}O$ 148.20	4180-23-8		23	236	practically insoluble in water
p-Propyl anisole $C_{10}H_{14}O$ 150.22	104-45-0		NA	NA	NA

Sources: Budavari *et al*. 1996, Tice 1999
NA = not available.

1.4 Identification of metabolites

In mammals, metabolites of methyleugenol include 1′-hydroxymethyleugenol, eugenol, methyleugenol-2,3-oxide, and 1′-hydroxymethyleugenol sulfate (Gardner *et al.* 1996, Solheim and Scheline 1976, both cited in NTP 1998; Woo *et al.* 1997). The transient eugenol quinone methide and electrophilic carbonium ions of 1′-hydroxymethyleugenol sulfate also have been identified (Gardner *et al.* 1996). The structures of these metabolites are shown in Figure 1-2.



Source: Gardner *et al*. 1996

Figure 1-2. Structures of mammalian metabolites of methyleugenol

2 Human Exposure

2.1 Use

Methyleugenol is used as a flavoring agent in jellies, baked goods, nonalcoholic beverages, chewing gum, candy, pudding, relish, and ice cream. Methyleugenol has also been used as an anesthetic in rodents (NTP 1998). It also is used as an insect attractant in combination with insecticides (Hays and Laws 1991, cited in NTP 1998). Methyleugenol has been used as an agent in sunscreens (Radian 1991).

2.2 Production

Annual production of methyleugenol in 1990 was estimated at 25,000 lb (SRI 1990, cited in NTP 1998).

2.3 Analysis

The presence of methyleugenol in essential oils is determined by means of negative ion chemical ionization mass spectrometry. High-performance liquid chromatography also can be used to determine the identity, purity, and stability of methyleugenol (NTP 1998). Barr *et al.* (2000) have developed a method for quantifying methyleugenol in human serum through a solid-phase extraction followed by a highly specific analysis by means of isotope dilution gas chromatography/high resolution mass spectrometry. The limit of detection for this analytical method is 3.1 pg/g.

2.4 Environmental occurrence

Methyleugenol is a naturally occurring substance. It is present in many essential oils (Radian 1991). It is a component of rose, pimento, basil, hyacinth, citronella, anise, nutmeg, mace, cinnamon leaves, pixuri seeds, and laurel fruits and leaves. It also has been found in blackberry essence, bananas, black pepper, and bilberries (NTP 1998). Methyleugenol has been detected in the wastewater effluent from a paper mill (Moshonas and Shaw 1978, cited in NTP 1998).

2.5 Environmental fate

2.5.1 Atmospheric Fate

Methyleugenol exists as a vapor in the ambient atmosphere. Vapor-phase methyleugenol reacts with photochemically produced hydroxyl radicals and degrades with an estimated half-life of five hours (HSDB 1996).

2.5.2 Aquatic Fate

Methyleugenol adsorbs to suspended solids and sediments. It volatilizes from water with estimated half-lives of nine days for a model river and 68 days for a model lake. Methyleugenol bioconcentrates in aquatic organisms with a bioconcentration factor (BCF) of 120 (a BCF < 1000 is generally insufficient to result in bioaccumulation in aquatic organisms). Methyleugenol has a half-life of 34 hours in aquatic environments (HSDB 1996).

2.5.3 Terrestrial Fate

Methyleugenol is moderately mobile in soil and was demonstrated experimentally to be immobile in sand, silt clay, and loam. Volatilization may be an important fate process in moist soils, and biodegradation is expected to be the most important fate process in soils. Methyleugenol has a half-life in soil of 16 hours (HSDB 1996).

2.6 Environmental exposure

Although methyleugenol has been identified in various natural substances, no quantitative measurements have assessed nondietary environmental exposure to methyleugenol. The general population is exposed to methyleugenol via ingestion of essential oils and foodstuffs containing the compound (HSDB 1996).

Methyleugenol is used in commercial products as a flavorant at concentrations ranging from 5 ppm to 52 ppm and as a fragrance at concentrations from 0.002% to 0.3%. A subset of serum samples from human adults participating in NHANES III were analyzed for methyleugenol content (Barr *et al*. 2000). Methyleugenol was detected in 98% of the 206 samples analyzed. The mean methyleugenol concentration was 24 pg/g, and the highest concentration was 390 pg/g.

Per capita consumption of methyleugenol in foods was estimated by the World Health Organization to be 0.073 mg/day (WHO 1981, cited in NTP 1998) and, more recently, 0.26 mg/kg body weight (Strofberg and Grundschober 1987, NAS 1989, both cited in NTP 1998).

2.7 Occupational exposure

Occupational exposure to methyleugenol occurs through dermal contact, inhalation, and ingestion. Through the National Occupational Exposure Survey (1981 to 1983), the National Institute for Occupational Safety and Health estimated that 2,824 workers (including 877 females) were potentially exposed to methyleugenol (NTP 1998).

2.8 Biological indices of exposure

Three major pathways describe the metabolism of methyleugenol in humans. These include oxidation of the allylic side chain, formation of the hydroxy acid via epoxidation of the double bonds followed by hydration, and *O*-demethylation and hydroxylation of the benzene ring (NTP 1998). Although there is now a sensitive and accurate method to determine methyleugenol concentration in blood, detailed pharmacokinetic studies will be required in order to determine the relationship between methyleugenol intake and human serum methyleugenol concentrations (Barr *et al.* 2000).

2.9 Regulations

The U.S. Environmental Protection Agency (EPA) regulates methyleugenol under the Federal Insecticide, Fungicide, and Rodenticide Act. EPA allows for exemption from normal tolerances when methyleugenol is used in Oriental fruit fly eradication programs. The U.S. Food and Drug Administration (FDA) regulates methyleugenol, allowing it to be used as a synthetic flavoring substance and adjuvant for direct addition to food for human consumption. Table 2-1 summarizes EPA regulations, and Table 2-2 summarizes FDA regulations.

Table 2-1. U.S. EPA Regulations

Regulatory action	Effect of regulation and other comments
40 CFR 180—PART 180—TOLERANCES AND EXEMPTIONS FROM TOLERANCES FOR PESTICIDE CHEMICALS IN OR ON RAW AGRICULTURAL COMMODITIES. Promulgated: 36 FR 22540, 11/25/71. U.S. Codes: 21 U.S.C. 346a, 371a.	Part 180 provides procedural regulations and specific tolerances for various pesticides. Exemptions from tolerances also are given in this part.
40 CFR 180—PART 180 Subpart D—Exemptions From Tolerances. Promulgated: 47 FR 9002, 03/03/82. U.S. Codes: 21 U.S.C. 321(q), 346(a) and 371.	The insect attractant methyleugenol is exempt from the requirement of tolerances on all raw agricultural commodities when used in combination in Oriental fruit fly eradication programs under the authority of the U.S. Department of Agriculture. The maximum actual dosage per application per acre shall be 28.35 g (one ounce avoirdupois) methyleugenol.

Source: The regulations in this table have been updated through the 1999 Code of Federal Regulations 40 CFR, 1 July 1999.

Table 2-2. U.S. FDA Regulations

Regulatory action	Effect of regulation and other comments
21 CFR 172—PART 172—FOOD ADDITIVES PERMITTED FOR DIRECT ADDITION TO FOOD FOR HUMAN CONSUMPTION. Promulgated: 42 FR 14491 03/15/77. U.S. Codes: 21 U.S.C. 321, 341, 342, 348, 371, 379e	The regulations in Subparts A through I govern the amounts of food additives allowed for human consumption.
21 CFR 172—Subpart F—Flavoring Agents and Related Substances. Promulgated: 61 FR 14245, 04/01/96.	Methyleugenol may be safely used in food provided it is used in the minimum quantity required to produce the intended effect, and otherwise in accordance with all the principles of good manufacturing practice.

Source: The regulations in this table have been updated through the 1999 Code of Federal Regulations 21 CFR, 1 April 1999.

3 Human Cancer Studies

No studies of the relationship of human cancer with exposure to methyleugenol have been reported.

4 Studies of Cancer in Experimental Animals

4.1 Oral administration study in rats

The carcinogenic potential of methyleugenol was evaluated in a cancer bioassay in rats of both sexes (NTP 1998). In this study, five to six week-old F344/N rats (50 per sex) received methyleugenol in 0.5% methylcellulose by gavage at doses of 37, 75, or 150 mg/kg body weight (b.w.), five days per week for 105 weeks. Other groups of F344/N rats (60 per sex) received methyleugenol in 0.5% methylcellulose by gavage at 300 mg/kg b.w., five days per week for 53 weeks, followed by 0.5% methylcellulose (vehicle) only for the remaining 52 weeks. Another group of F344/N rats (60 per sex) was administered 0.5% methylcellulose only and served as controls. At six and 12 months, five control rats and five rats in the 300-mg/kg exposure group were sacrificed. Moribund animals were sacrificed throughout the study. At the end of the study, all surviving animals were sacrificed. The tissues and organs of all animals were examined histopathologically.

Mean body weights of rats administered methyleugenol were lower than those of the vehicle control animals throughout most of the study. All male rats in the 150- and 300-mg/kg groups died before the end of the study. Survival of female rats was slightly lower in the 150-mg/kg group than in the control group. The survival rates are shown in Table 4-1.

Table 4-1. Survival rates of male and female F344/N rats administered methyleugenol by gavage for up to 105 weeks

	Dose level (mg/kg per day)				
Sex	**0[a]**	**37**	**75**	**150**	**300[a,b]**
Males	20/60	16/50	15/50	0/50	0/60
Females	22/60	25/50	22/50	11/50	16/60

Source: NTP 1998

[a]Five rats per group were euthanized at 6 and 12 months of the study.

[b]Stop-exposure group (53 weeks of exposure to methyleugenol followed by 52 weeks of vehicle-only treatment).

Histopathological examination of the tissues revealed benign and/or malignant tumors at various sites, including the liver, glandular stomach, kidney, mammary gland, and skin. The incidences of hepatocellular adenomas, hepatocellular carcinomas, hepatocholangiomas, hepatocholangiocarcinomas, neuroendocrine tumors of the glandular stomach, renal adenomas, malignant mesotheliomas, mammary gland fibroadenomas, and subcutaneous tissue fibromas were significantly increased in rats given methyleugenol. Tumor incidences and their statistical significance are shown in Table 4-2 for male rats and in Table 4-3 for female rats.

Table 4-2. Incidences of neoplastic lesions in male F344/N rats administered methyleugenol by gavage for up to 105 weeks

Tumor type	Tumor incidence/no. rats examined (Poly-3 est. neoplasm rates)[a]				
	Dose level (mg/kg per day)				
	0	37	75	150	300[b]
Liver					
Hepatocellular adenoma	5/50	12/50*	23/50**	38/50**	32/50**
Hepatocellular carcinoma	2/50	3/50	14/50**	25/50**	36/50**
Hepatocellular adenoma or carcinoma	7/50 (17)	14/50 (34)*	28/50 (64)**	43/50 (94)**	45/50 (99)**
Hepatocholangioma	0/50	0/50	0/50	1/50	6/50**
Hepatocholangiocarcinoma (includes multiples)	0/50	0/50	1/50	1/50	7/50**
Hepatocholangioma or hepatocholangiocarcinoma (includes multiples)	0/50 (0)	0/50 (0)	1/50 (3)	2/50 (6)	13/50 (44)**
Glandular stomach					
Malignant neuroendocrine tumor	0/50	0/50	0/50	4/50	2/50
Benign neuroendocrine tumor	0/50	0/50	0/50	3/50*	2/50
Benign or malignant neuroendocrine tumor	0/50	0/50	0/50	7/50**	4/50*
Kidney: Adenoma[c]	4/50 (10)	6/50 (16)	17/50 (44)**	13/50 (37)**	20/50 (65)**
Mammary gland: Fibroadenoma	5/50	5/50	15/50**	13/50**	6/50
Skin (subcutaneous):					
Fibroma	1/50	9/50**	8/50*	5/50	4/50
Fibroma or fibrosarcoma	1/50	12/50**	8/50*	8/50**	4/50
All organs: Mesothelioma	1/50	3/50	5/50	12/50**	5/50*

Source: NTP 1998

*$P \leq 0.05$; significantly different from vehicle controls (Poly-3 test).

**$P \leq 0.01$; significantly different from vehicle controls (Poly-3 test).

[a]Poly-3 estimated neoplasm rates, which adjust for intercurrent mortality, are given in parentheses.

[b]Stop-exposure group (53 weeks of exposure to methyleugenol followed by 52 weeks of vehicle-only treatment).

[c]Combined standard and extended evaluations of renal tubule adenoma.

Table 4-3. Incidences of neoplastic lesions in female F344/N rats administered methyleugenol by gavage for up to 105 weeks

	Tumor incidence/no. rats examined (Poly-3 est. neoplasm rates)[a]				
	Dose level (mg/kg per day)				
Tumor type	0	37	75	150	300[b]
Liver					
Hepatocellular adenoma	1/50	8/50*	11/49**	33/49**	43/50**
Hepatocellular carcinoma	0/50	0/50	4/49	8/49**	22/50**
Hepatocellular adenoma or carcinoma	1/50 (3)	8/50 (20)*	14/49 (33)**	34/49 (77)**	43/50 (97)**
Hepatocholangioma	0/50	0/50	0/49	0/49	8/50**
Hepatocholangiocarcinoma (includes multiple)	0/50	0/50	0/49	3/49	9/50**
Hepatocholangioma or hepatocholangiocarcinoma (includes multiples)	0/50 (0)	0/50 (0)	0/49 (0)	3/49 (8)	17/50 (43)**
Glandular stomach					
Benign neuroendocrine tumor	0/50	0/50	13/50**	9/50**	5/50**
Malignant neuroendocrine tumor	0/50	1/50	12/50**	26/50**	36/50**
Benign or malignant neuroendocrine tumor	0/50 (0)	1/50 (2)	25/50 (59)**	34/50 (80)**	41/50 (82)**
Forestomach: Squamous cell papilloma or carcinoma	0/50	0/50	1/50	3/50	1/50

Source: NTP 1998

*$P \leq 0.05$; significantly different from vehicle controls (Poly-3 test).

**$P \leq 0.01$; significantly different from vehicle controls (Poly-3 test).

[a]Poly-3 estimated neoplasm rates, which adjust for intercurrent mortality, are given in parentheses.

[b]Stop-exposure group (53 weeks of exposure to methyleugenol followed by 52 weeks of vehicle-only treatment).

The NTP concluded that under the conditions of this bioassay, there was *clear evidence of carcinogenic activity* for methyleugenol in male and female F344/N rats. This conclusion was based on increased incidences of liver neoplasms and neuroendocrine tumors of the glandular stomach in male and female rats and increased incidences of kidney neoplasms, malignant mesotheliomas, mammary gland fibroadenomas, subcutaneous fibromas, and fibromas or fibrosarcomas (combined) in male rats (NTP 1998).

4.2 Oral administration study in mice

The carcinogenic potential of methyleugenol was evaluated in a cancer bioassay in mice of both sexes (NTP 1998). In the study, 6- to 7-week-old $B6C3F_1$ mice (50 per sex) received methyleugenol in 0.5% methylcellulose by gavage at doses of 37, 75, or 150 mg/kg b.w. per day, five days per week for 104 weeks. Other groups of $B6C3F_1$ mice (50 per sex) were administered 0.5% methylcellulose vehicle only and served as controls.

The mean body weights of the female mice administered methyleugenol were lower than those of the vehicle control mice by week 17 of the study. In male mice, mean body weights were generally less than those of the vehicle control groups after weeks 81, 41, and 17 for the 37, 75, and 150 mg/kg groups, respectively. Although the survival rate was similar in all male groups (exposed and control), it was significantly lower among exposed females. The survival rates are summarized in Table 4-4.

Table 4-4. Survival of male and female B6C3F$_1$ mice administered methyleugenol by gavage for up to 104 weeks

Sex	Dose level (mg/kg per day)			
	0	37	75	150
Males	38/50	36/50	37/50	35/50
Females	31/50	18/50	18/50[a]	2/50

Source: NTP 1998

[a]Two animals died during the last week of the study

Histopathological examination of the tissues revealed malignant and/or benign tumors in the liver and glandular stomach. The incidences of hepatocellular adenoma and hepatocellular carcinoma were significantly increased in male mice. The incidences of hepatocellular adenoma, hepatocellular carcinoma, and hepatoblastoma were significantly increased in female mice. Two malignant neuroendocrine tumors of the glandular stomach found in the high-dose male mice were considered to be related to methyleugenol exposure because of the rarity of these tumors in control mice.

In addition, a retrospective analysis of tissues from this study, using an assay based on polymerase chain reaction–restriction fragment length polymorphism, found *Helicobacter hepaticus* in 4 of 14 mice in the methyleugenol study. *H. hepaticus* is associated with the development of oval-cell hyperplasia. However, in the female mice administered methyleugenol, the incidence of this tumor exhibited a dose-response pattern (a pattern not seen in nine other studies in which *H. hepaticus* was found in retrospective analyses). In addition, both treatment and control groups were relatively equally affected. Thus, the oval-cell hyperplasia observed in this study was considered to be related to methyleugenol exposure and unrelated to the presence of *H. hepaticus* (Nyska *et al.*1997).

Tumor incidences and their statistical significance are shown in Table 4-5 for male and female mice.

Table 4-5. Incidences of neoplastic lesions in male and female B6C3F$_1$ mice administered methyleugenol by gavage for up to 104 weeks

Tumor type	Tumor incidence/no. mice examined (Poly-3 est. neoplasm rates)[a]			
	Dose level (mg/kg per day)			
	0	37	75	150
Males				
Liver				
Hepatocellular adenoma	26/49	43/50**	38/50**	39/50**
Hepatocellular carcinoma	10/49	20/50*	19/50*	9/50
Hepatocellular adenoma or carcinoma	31/49 (65)	47/50 (97)**	46/50 (96)**	40/50 (86)**
Hepatoblastoma	0/49	0/50	1/50	3/50
Glandular stomach				
Carcinoma	0/49	0/48	0/49	1/50
Malignant neuroendocrine tumor	0/49	0/48	0/49	2/50
Females				
Liver				
Hepatocellular adenoma	20/50	48/50**	46/49**	41/50**
Hepatocellular carcinoma	7/50	37/50**	47/49**	47/50**
Hepatocellular adenoma or carcinoma	25/50 (55)	50/50 (100)**	49/49 (100)**	49/50 (100)**
Hepatoblastoma	0/50	6/50**	11/49**	15/50**
Hepatocholangiocarcinoma	0/50	0/50	0/50	2/50

Source: NTP 1998

*$P \leq 0.05$; significantly different from vehicle controls (Poly-3 test).

**$P \leq 0.01$; significantly different from vehicle controls (Poly-3 test).

[a]Poly-3 estimated neoplasm rates, which adjust for intercurrent mortality, are given in parentheses.

The NTP concluded that under the conditions of this bioassay, there was *clear evidence of carcinogenic activity* for methyleugenol in male and female B6C3F$_1$ mice, based on increased incidences of liver neoplasms in both sexes. Neuroendocrine tumors of the glandular stomach in males also were considered to be related to methyleugenol exposure (NTP 1998).

4.3 Intraperitoneal injection study in mice

Methyleugenol, dissolved in trioctanoin, was administered by intraperitoneal (i.p.) injection to male B6C3F$_1$ mice on days 1, 8, 15, and 22 of age (Miller *et al.* 1983). The total administered dose per mouse was 4.75 μmol (0.85 mg). Livers were examined by laparotomy at 13 months, and the surviving mice were sacrificed at 18 months. In a similar study, 1′-hyroxymethyleugenol was administered to male B6C3F$_1$ mice at a total

dose of 2.85 μmol (0.55 mg). Exposure to methyleugenol or 1′-hyroxymethyleugenol increased liver tumor incidence and multiplicity (Table 4-6).

Table 4-6. Incidences of hepatoma in male B6C3F$_1$ mice administered methyleugenol or 1′-hyroxymethyleugenol by i.p. injections on days 1, 8, 15 and 22 of age[a]

Compound	Total dose (μmol/mouse)	No. of mice examined	Hepatoma-bearing mice (%)	Mean no. of hepatomas/ mouse
Trioctanoin (vehicle control)	–	58	41	0.5
Methyleugenol	4.75	58	96*	3.2*
1′-Hyroxymethyleugenol	2.85	44	93*	3.5*

Source: Miller *et al.* 1983

*$P < 0.001$; significantly different from the vehicle control group (Fisher's exact test).

[a]Mice were observed for up to18 months.

4.4 Summary

Methyleugenol administered orally was found to be carcinogenic in rats and mice. Methyleugenol significantly increased the incidences of liver neoplasms and neuroendocrine tumors of the glandular stomach in male and female rats and the incidences of kidney neoplasms, malignant mesotheliomas, mammary gland fibroadenomas, and subcutaneous fibromas or fibrosarcomas (combined) in male rats. Methyleugenol also significantly increased the incidences of liver neoplasms in mice of both sexes. Neuroendocrine tumors of the glandular stomach in male mice also were considered to be related to methyleugenol exposure. Methyleugenol and its metabolite 1′-hyroxymethyleugenol induced liver tumors in mice that had received four i.p. doses before weaning.

5 Genotoxicity

Though limited, the peer-reviewed published literature indicates both positive and negative results in standard assays for genotoxicity. DNA-damaging effects, as evidenced by unscheduled DNA synthesis in mammalian hepatocyte systems, have been observed under certain conditions. The NTP has performed a battery of genotoxicity evaluations of methyleugenol, including assays for the following effects: gene mutation in *Salmonella typhimurium*, sister chromatid exchange (SCE) in Chinese hamster ovary (CHO) cells *in vitro*, chromosomal aberrations in CHO cells *in vitro*, and micronucleus formation in erythrocytes in mouse blood *in vivo*. This section contains genotoxicity information from the literature and from the NTP Technical Report on the Toxicology and Carcinogenesis Studies of Methyleugenol (CAS No. 93-15-12) in F344/N Rats and B6C3F_1 Mice (Gavage Studies) (NTP 1998).

5.1 Prokaryotic Systems

5.1.1 Gene mutation in Salmonella typhimurium

Methyleugenol, tested up to a maximum concentration of 666 μg/plate, did not induce reverse mutations in *S. typhimurium* strain TA98, TA100, TA1535, TA1537, or TA1538, with or without S9 microsomal metabolic activation (Sekizawa and Shibamoto 1982, Mortelmans *et al*. 1986, Kettering and Torabinejad 1995, NTP 1998).

5.1.2 Gene mutation in Escherichia coli

Methyleugenol did not induce reverse mutations in *E. coli* WP2 *uvr*A *trp*$^-$ either with or without S9 metabolic activation (Sekizawa and Shibamoto 1982).

5.1.3 DNA repair in Bacillus subtilis (rec assay)

At a concentration of 1 mg/disk, methyleugenol tested positive in the *B. subtilis* DNA repair test (rec assay), with a difference of 6.5 mm between the inhibition zones for rec$^-$ and rec$^+$ (Sekizawa and Shibamoto 1982).

5.2 Non-mammalian eukaryotic systems

5.2.1 Intrachromosomal recombination in Saccharomyces cerevisiae

Methyleugenol induced positive, dose-related responses in intrachromosomal recombination studies in the yeast *S. cerevisiae,* both with and without S9 metabolic activation (Schiestl *et al.* 1989, Brennan *et al.* 1996). Methyleugenol at a concentration of 1.0 mg/mL induced a 12.5-fold increase in deletion recombination. The effect was nonlinear, with a threshold between 0.3 and 0.6 mg/mL. The threshold corresponded to the lowest concentration associated with any cytotoxic effect. The authors noted that this type of nonlinear, threshold-dependent response has been reported as characteristic of carcinogens that test negative for gene mutation in *S. typhimurium,* and of some oxidative mutagens (Brennan *et al.* 1996).

5.3 Mammalian Systems

5.3.1 In vitro *assays*

5.3.1.1 Chromosomal aberrations in CHO cells

Methyleugenol did not induce chromosomal aberrations in cultured CHO cells in either the presence or the absence of S9. The methyleugenol concentrations tested were limited by cytotoxicity to a high of 233 μg/mL (NTP 1998).

5.3.1.2 Sister chromatid exchange in CHO cells

Methyleugenol induced SCEs in cultured CHO cells in each of two replicate trials with S9 metabolic activation at concentrations between 17 and 250 μg/mL. The increases in SCEs per chromosome relative to solvent controls ranged from 17.5% to 69.6%. In the absence of S9 activation, no significant increase in SCEs was observed (NTP 1998).

5.3.1.3 Unscheduled DNA synthesis in rat hepatocytes

Methyleugenol induced unscheduled DNA synthesis (UDS) in rat hepatocytes in primary cultures. Dose-related increases in UDS were observed at methyleugenol concentrations from 10^{-4} M to 10^{-3} M, with DNA synthetic activity exceeding control values by as much as 2.7 times (a ratio of 1.5 indicates a positive response) (Howes *et al.* 1990, Chan and Caldwell 1992).

The methyleugenol metabolite 1′-hydroxymethyleugenol was more potent than its parent compound as an inducer of UDS in rat hepatocytes. Dose-related increases in UDS were observed at 1′-hydroxymethyleugenol concentrations from 10^{-5} M to 10^{-4} M, about an order of magnitude lower than methyleugenol concentrations that produced a similar degree of DNA damage and repair. 1′-Hydroxymethyleugenol also was more cytotoxic than methyleugenol (by about an order of magnitude), as measured by the lactate dehydrogenate leakage viability assay (Chan and Caldwell 1992, Gardner *et al.* 1997).

5.3.1.4 Transformation of Syrian hamster embryo (SHE) cells

Methyleugenol at concentrations of 185 to 250 μg/ml for 24 hours induced morphological transformation of cultured SHE cells. The transformation frequency for exposed cultures was approximately four times the frequency for control cultures, and the response was not dose related.

5.3.2 In vivo *assays*

5.3.2.1 Mouse micronucleus test

Methyleugenol administered by gavage to male and female $B6C3F_1$ mice at doses of 10 to 1,000 mg/kg for 14 weeks (at unspecified intervals) did not increase the frequency of micronucleated normochromatic erythrocytes in peripheral blood. In the same study, methyleugenol did not affect the percentage of polychromatic erythrocytes in the blood, indicating no detectable bone marrow toxicity at the doses tested (NTP 1998).

Table 5-1. Genetic and related effects of methyleugenol exposure

Test system	End point	Results	References
S. typhimurium strains TA98, TA100, TA1535, TA1537, TA1538	gene mutation	negative with or without S9	Sekizawa and Shibamoto 1982, Mortelmans *et al.* 1986, Kettering and Torabinejad 1995, NTP 1998
E. coli WP2	gene mutation	negative	Sekizawa and Shibamoto 1982
B. subtilis	DNA repair (*rec*-assay)	positive	Sekizawa and Shibamoto 1982
S. cerevisiae	intrachromosomal recombination	positive	Schiestl *et al.* 1989, Brennan *et al.* 1996
CHO cells	chromosomal aberrations	negative with or without S9	NTP 1998
CHO cells	SCE	positive with S9	NTP 1998
Rat hepatocyte primary cultures	UDS	positive	Howes *et al.* 1990, Chan and Caldwell 1992
SHE cells	morphological transformation	positive	Kerckaert *et al.* 1996
$B6C3F_1$ mice, exposed *in vivo* by gavage	micronucleus test	negative	NTP 1998

5.4 Summary

Table 5-1 summarizes the genetic and related effects of methyleugenol. Methyleugenol did not induce gene mutations in *S. typhimurium* or *E. coli* WP2, either with or without liver S9 metabolic activation. However, methyleugenol tested positive in the rec assay for DNA repair in *B. subtilis* and induced intrachromosomal recombination in *S. cerevisiae*. Methyleugenol did not induce chromosomal aberrations in CHO cells *in vitro,* but it did induce SCEs in CHO cells incubated with rat liver S9. Methyleugenol also induced DNA repair, measured as unscheduled DNA synthesis, in rodent hepatocytes *in vitro* and tested positive in the SHE cell morphological transformation assay. Methyleugenol administered by gavage for 14 weeks did not induce micronuclei in the erythrocytes of $B6C3F_1$ mice.

6 Other Relevant Data

6.1 Absorption, distribution, metabolism, and excretion

Methyleugenol is rapidly absorbed following oral administration to F344/N rats or $B6C3F_1$ mice (NTP 1998). Rats and mice were administered methyleugenol by gavage at dose levels of 36, 75, 150, or 300 mg/kg b.w., either as single doses or for five days per week for 6, 12, or 18 months, or they received single intravenous doses of 36, 75, 150, or 300 mg/kg b.w. The kinetic data are consistent with rapid clearance from the blood, metabolism in the liver, and elimination of metabolites in the urine.

In rats of both sexes, plasma levels of methyleugenol peaked within the first five minutes at all dose levels. Methyleugenol was preferentially distributed to the liver within 72 hours of administration. Elimination of orally administered methyleugenol from the bloodstream was rapid and multiphasic, with terminal half-lives on the order of 15 to 30 minutes in both sexes. Absorbed methyleugenol was rapidly and extensively metabolized, and 85% of its metabolites were eliminated in the urine within 72 hours. The majority of the excreted metabolites were identified as hydroxylated, sulfated, and glucuronidated compounds (NTP 1998).

In mice, plasma levels of methyleugenol peaked within the first five minutes at all dose levels in both sexes. However, methyleugenol was preferentially distributed to the ovaries, stomach, fat, spleen, and liver within 72 hours of administration. Elimination of orally administered methyleugenol from the bloodstream was rapid and multiphasic, with terminal half-lives on the order of 15 to 30 minutes. Absorbed methyleugenol was rapidly and extensively metabolized, and 85% of its metabolites were eliminated in the urine within 72 hours. Although the nature of the major urinary metabolite was unknown, the minority portion contained hydroxylated, sulfated, and glucuronidated compounds (NTP 1998).

6.2 Bioactivation

Methyleugenol is metabolized by the cytochrome P-450 system (Borchert *et al.* 1973, cited in NTP 1998) by three different pathways: *O*-demethylation, side-chain hydroxylation, or side-chain epoxidation. Of the various metabolites formed, 1′-hydroxymethyleugenol and methyleugenol-2′,3′-oxide, were considered to be the ones most likely responsible for the toxic effects of methyleugenol in the liver (Solheim and Scheline 1976, cited in NTP 1998; Woo *et al.* 1997).

The metabolic bioactivation of methyleugenol to its DNA- and protein-reactive intermediates is a two-step process. The first step involves hydroxylation at the 1′ position of the allyl side chain to yield 1′-hydroxymethyleugenol. 1′-Hydroxymethyleugenol is subsequently sulfated to yield 1′-sulfoxy metabolites, which decompose spontaneously in an aqueous environment to electrophilic carbonium ions that can bind covalently to DNA and other cellular macromolecules, including protein (Gardner *et al.* 1996, 1997). A proposed scheme for the bioactivation of methyleugenol is shown in Figure 6-1 (Gardner *et al.* 1996).

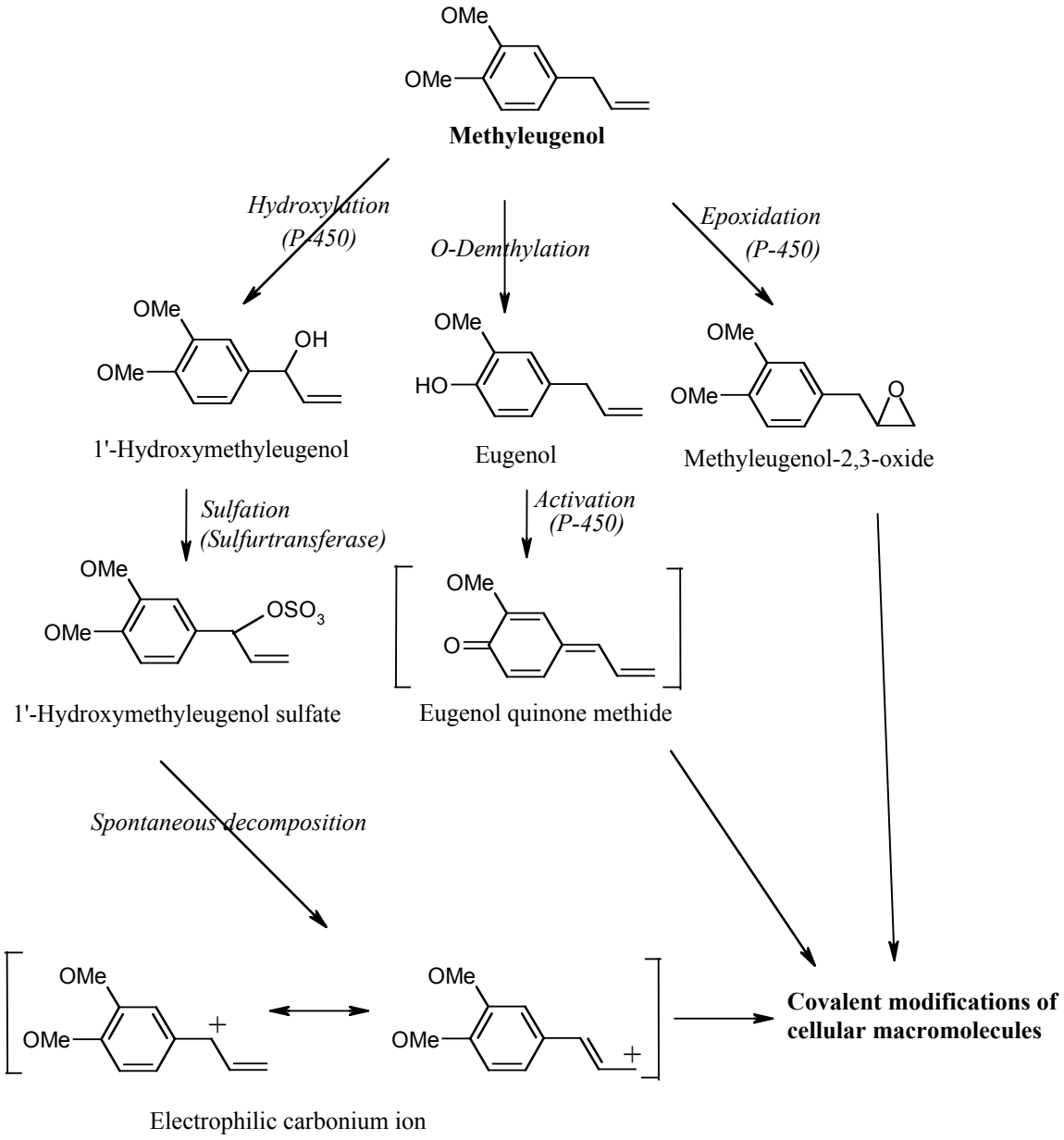
Based on DNA binding data and studies of liver tumor induction by allylbenzenes and their metabolites, it is apparent that bioactivation of methyleugenol via the pathway of side-chain hydroxylation followed by sulfation (Figure 6-1) is an important step in liver tumor induction by this chemical. Bioactivation via the side-chain epoxidation or *O*-demethylation pathways may also contribute to the cancer process.

The lack of correlation between *in vitro* mutagenicity and carcinogenicity of methyleugenol may be due to the requirement of sulfation in the metabolic activation of this compound (Randerath *et al.* 1984, Boberg *et al.* 1983, cited in NTP 1998). In studies with analog allylbenzenes, pretreatment with sulfotransferase inhibitors inhibited the binding of safrole to mouse liver DNA and the tumorigenicity of 1′-hydroxysafrole (Miller *et al.* 1983, Boberg *et al.* 1983, Randerath *et al.* 1984, Gardner *et al.* 1996).

6.3 Formation of protein and DNA adducts

Methyleugenol protein adducts were detected by immunochemical methods (enzyme-linked immunosorbent assay and immunoblotting) in the livers of male F344 rats injected i.p. with methyleugenol (dissolved in tricaprylin) at doses of 10 to 300 mg/kg b.w. per day for five days. A 44-kDa adduct was the major adduct found in the livers of the rats given higher doses of methyleugenol (100 or 300 mg/kg) and the only adduct detected in the livers of rats given low doses of methyleugenol (10 or 30 mg/kg) (Gardner *et al.* 1996).

Methyleugenol-DNA adducts were detected by a modified ^{32}P-postlabeling analysis in the livers of female CD-1 mice injected i.p. with methyleugenol (in trioctanoin) at doses of 2 or 10 mg per mouse. The livers of the mice were sampled at 24 hours and 7, 28, 58, 98, and 140 days after exposure. The major adducts identified were 3′,5′-biphosphate of a N^2-(*trans*-propenylbenzene-3′-yl) deoxyguanosine and a derivative of a N^2-(allylbenzen-1′-yl)deoxyguanosine, and the minor adducts may represent N^6-adenine derivatives. A fourth adduct, which was not characterized, represented 2% to 3% of the total adducts formed (Randerath *et al.* 1984). Similar results were obtained in ^{32}P-postlabeling studies with C57BL/6J and A/J mice (Levy and Weber 1988) and the offspring of C57B1 female mice mated with male C3H mice (B6C3F$_1$) (Phillips *et al.* 1984). Methyleugenol also was shown to bind to protein and DNA *in vitro* in assays using microsomal fractions or rat liver slices in the presence of metabolic activation (Gardner *et al.* 1996, NTP 1998, Williams 1997, Woo *et al.* 1997).



Source: Gardner *et al*. 1996.

Figure 6-1. Pathways of bioactivation of methyleugenol

6.4 Oncogene activation

*6.4.1 Activation of H-*ras *oncogene*

Methyleugenol did not induce any detectable H-*ras* codon 61 mutations in 29 methyleugenol-induced hepatocellular neoplasms in $B6C3F_1$ mice from the NTP two-year gavage study (NTP 1998, Devereux *et al.* 1999).

6.4.2 Activation of β-catenin *oncogene*

Upregulation of Wnt signaling, the result of *β-catenin* activation, is an important event in the development of certain human and rodent cancers (Gumbiner 1997). A study of methyleugenol-induced hepatocellular neoplasms obtained from the NTP two-year carcinogenesis study in $B6C3F_1$ mice (NTP 1998) found point mutations at codons 32, 33, 34, or 41, indicative of *β-catenin* activation, in 20 of the 29 tumors analyzed (Devereux *et al.* 1999). This incidence was significantly greater than the incidence of *β-catenin* mutations in liver tumors from controls (2 of 22). *β-catenin* mutations were detected almost equally in adenomas and carcinomas, indicating that this is an early event in carcinogenesis. The *β-catenin* mutation frequency in these tumors did not appear to be related to methyleugenol dose.

6.5 Structure-activity relationships

Eugenol, safrole, isosafrole, and estragole are allylbenzene compounds that are metabolized via pathways similar to those for methyleugenol to yield analogous side-chain hydroxylation and side-chain epoxidation products (Borchert *et al.* 1973, Stillwell *et al.* 1974, Solheim and Scheline 1976, Delaforge *et al.* 1980a, b, Sims and Grover 1974, Miller *et al.* 1983, all cited in NTP 1998).

Methyleugenol, estragole, and safrole produce similar levels of DNA, RNA, and protein binding. Through ^{32}P-postlabeling analysis, N^2-(estragol-1′-yl)deoxyguanosine and N^2-(*trans*-isoestragol-3′-yl)deoxyguanosine were found to be the major DNA adducts in the livers of CD-1 mice treated with [^{3}H]1′-hydroxyestragole, the proximate carcinogenic metabolite of estragole. N^2-(*cis*-isoestragol-3′-yl)deoxyguanosine and N^6-(*trans*-isoestragol-3′-yl)deoxyguanosine were identified as the minor DNA adducts. DNA adducts that had similar elusion profiles and were assigned analogous structures were obtained from the livers of mice treated with [^{3}H]1′-hydroxysafrole, the proximate carcinogenic metabolite of safrole. The similarity of chromatographic migration (polyethyleneimine-cellulose) maps of the DNA adducts for estragole, safrole, and methyleugenol suggests that the pathway for the activation of all three allylbenzenes may be similar. Anethole, elemicin, parsley and dill apioles, and myristicin were less active in the formation in DNA adducts. No DNA adducts were detected for eugenol (Phillips *et al.* 1984, Randerath *et al.* 1984).

The reduced ability of other structural analogs of methyleugenol (eugenol, elemicin, myristicin, dill apiole, and parsley apiole) to bind DNA or induce carcinogenesis has been suggested to result from the differences in the substitution positions and substituent groups. Thus, methyleugenol, safrole, and estragole, which have methoxy and/or methylenedioxy substitutions at the 4-position or at both the 3-position and 4-position, exhibit the greatest DNA-binding capacity and carcinogenic potential *in vivo*. Elemicin, and myristicin, with substitutions at the 3-position, 4-position, and 5-position, have intermediate DNA-binding capacity, but the levels and/or persistence of the DNA adducts formed apparently are inadequate to induce significant incidences of cancers. Dill apiole and parsley apiole, which have substitutions at the 5-position and at the 2-position, have low DNA-binding capacities and reduced ability to cause cancers. Eugenol, which has a

hydroxy substitution at the 1-position, exhibits no potential to bind DNA (Phillips *et al.* 1984).

6.6 Genotoxicity of some compounds structurally related to methyleugenol

Table 6-1 summarizes genetic and related effects of eugenol, safrole, and estragole. Similarly to methyleugenol, these compounds did not induce mutations in *S. typhimurium* but did induce UDS in rat hepatocytes. In addition, eugenol and safrole induced SCEs in CHO cells, and safrole did not induce chromosomal aberrations.

Table 6-1. Genetic and related effects of allylbenzene compounds structurally related to methyleugenol

Compound	Test system	End point	Results	References
Eugenol	*S. typhimurium*	gene mutations	negative w/ or w/out S9	Sekizawa and Shibamoto 1982, Haworth *et al*. 1983
Safrole			negative w/ or w/out S9	Zeiger and Haworth 1985
Estragole			negative w/ or w/out S9	Zeiger *et al*. 1987
Eugenol	*S. cerevisiae*	intrachromosomal recombination	positive	Schiestl *et al*. 1989
Safrole			–	–
Estragole			–	–
Eugenol	*Drosophila melanogaster*	sex-linked recessive lethal mutation or reciprocal translocation	negative	Foureman *et al*. 1994
Safrole			negative	Zimmering *et al*. 1989
Estragole			–	–
Eugenol	CHO cells	chromosomal aberrations	positive	Galloway *et al*. 1997
Safrole			negative	Gulati *et al*. 1985
Estragole			–	–
Eugenol	CHO cells	SCE	positive	Galloway *et al*. 1997
Safrole			positive	Gulati *et al*. 1985
Estragole			–	–
Eugenol	Rat hepatocyte primary cultures	UDS	positive	Howes *et al.* 1990, Chan and Caldwell 1992
Safrole			positive	Howes *et al.* 1990, Chan and Caldwell 1992
Estragole			positive	Howes *et al.* 1990, Chan and Caldwell 1992
Eugenol	L5178Y mouse lymphoma cells	gene mutation	positive	Myhr and Caspary 1991, Sofuni *et al*. 1996
Safrole			positive	Mitchell *et al*. 1988
Estragole			positive	Myhr and Caspary 1988
Eugenol	mice,	micronucleus test	negative	Shelby *et al*. 1993

Compound	Test system	End point	Results	References
	i.p. injection			
	mice, i.p. injection		negative	Hayashi *et al*. 1988, Maura *et al*. 1989
			positive	Woolverton *et al*. 1986
	rats, oral exposure		negative	Hayashi *et al*. 1988, Maura *et al*. 1989
	rats, gavage		positive	Woolverton *et al*. 1986
Safrole	–		–	–
Estragole	–		–	–
Eugenol	$B6C3F_1$ mice, gavage	chromosomal aberrations	equivocal	NTP 2000
Safrole			–	–
Estragole			–	–

6.7 Carcinogenicity of some compounds structurally related to methyleugenol

6.7.1 Induction of liver tumors

Safrole, estragole, and their 1′-hydroxy metabolites induced liver tumors in preweanling mice when administered via gavage or i.p. injection (Miller *et al.* 1983). Eugenol did not induce tumors in these studies. Dietary administration of safrole, estragole, and 1′-hydroxyestragole to mice for 12 months induced liver tumors. The tumorigenicity of these allylbenzene compounds and some of their metabolites in B6C3F$_1$ and CD-1 mice is summarized in Table 6-2 for i.p. administration and in Table 6-3 for dietary administration.

Table 6-2. Incidences of tumors in male mice administered allylbenzenes or their metabolites by i.p. injection on days 1, 8, 15, and 22 of age

Compound	Total dose (µmol/mouse)	No. of mice examined	Hepatoma-bearing mice (%)	Mean no. of hepatomas/ mouse
CD-1 mice observed for up to 12 months				
Trioctanoin (vehicle control)	–	42	26	0.5
Safrole	9.45	48	67**	1.9**
1′-Hydroxysafrole	4.72	46	65**	2.7*
Safrole 2′,3′-oxide	9.45	44	14	0.3
Estragole	9.45	46	65**	1.7**
Eugenol	9.45	45	24	0.6
B6C3F$_1$ mice observed for up to 12 months				
Trioctanoin	–	32	15	0.2
1′-Hydroxysafrole	3.75	26	92**	2.7*
1′-Hydroxyestragole	1.87	27	93**	2.7*
B6C3F$_1$ mice observed for up to 18 months				
Trioctanoin	–	58	41	0.5
Estragole	4.75	41	83**	2.4**
1′-Hydroxyestragole	1.90	60	98**	5.6*

Source: Miller *et al.* 1983

*$P < 0.05$; significantly different from the vehicle control group (Fisher's exact test).

**$P < 0.001$; significantly different from the vehicle control group (Fisher's exact test).

Table 6-3. Incidences of tumors in female CD-1 mice administered allylbenzenes in the diet for 12 months

Compound	% in diet[a]	No. of mice alive at 10 mo.	Hepatoma-bearing mice (%)	Mean no. of hepatomas/ mouse
Control	0	50	0	0
Safrole	0.25	47	72**	2.1**
Safrole	0.5	49	80**	2.3**
Estragole	0.23	48	56**	1.4**
Estragole	0.46	49	71**	1.8**
1′-Hydroxyestragole	0.25	43	56**	1.2*
Eugenol	0.5	29	0	0

Source: Miller *et al.* 1983

*$P < 0.05$; significantly different from the vehicle control group (Fisher's exact test).

**$P < 0.001$; significantly different from the vehicle control group (Fisher's exact test).

[a]After 12 months of dietary administration of the test compound, animals received control diets until the age of 20 months.

Several studies reviewed by IARC found safrole and its derivatives (isosafrole and dihydrosafrole) to be carcinogenic in rats and mice. Male CFN rats fed 1% safrole developed liver adenomas (Homburger *et al.* 1961, cited in IARC 1976). Hepatocellular sarcomas and cholangiosarcomas were diagnosed in 14 of the 19 liver tumors found in 47 autopsied Osborne-Mendel rats given safrole in the diet at a concentration of 1,000 or 5,000 mg/kg (ppm) (Long *et al.* 1963, Hagan *et al.* 1965, both cited in IARC 1976). Groups of 10 or 25 male and female Osborne-Mendel rats were given dihydrosafrole (Hagan *et al.* 1965, Long and Jenner 1963, both cited in IARC 1976) or isosafrole (Hagan *et al.* 1965, 1967, cited in IARC 1976) in the diet, both at concentrations of 1,000 to 10,000 mg/kg (ppm), for two years. Dihydrosafrole induced tumors of the esophagus at dietary concentrations of 2,500 to 10,000 ppm, and hepatocellular adenomas and carcinomas were reported in five of 50 rats that received isosafrole at a dietary concentration of 5,000 ppm.

Male and female (C57BL/6 x C3H/Anf)F_1 mice (18 per sex) administered safrole by gavage (464 mg/kg) for 4 weeks and then in the diet at a concentration of 1,112 mg/kg (ppm) for up to 82 weeks had higher incidences of liver tumors than controls (Innes *et al.* 1968, 1969, cited in IARC 1976). Increased incidences of hepatocellular carcinomas also were seen in CD-1 male mice given safrole in the diet at concentrations of 4,000 or 5,000 mg/kg (ppm) for 13 months (Borchert *et al.* 1973, cited in IARC 1976). Oral exposure to isosafrole or dihydrosafrole also increased the incidence of liver tumors in male and female (C57BL/6 x C3H/Anf)F_1 mice (Innes *et al.* 1968, 1969, cited in IARC 1976).

Eugenol was not carcinogenic in B6C3F_1 mouse pups when administered in the diet (Miller *et al.* 1983). However, in another study, dietary administration of eugenol to B6C3F_1 mice produced equivocal evidence of carcinogenicity based on marginally increased incidences of liver neoplasms (NTP 1983).

6.7.2 Induction of tumors at sites other than the liver

Neuroendocrine proliferation of the gastric mucosa in humans is an indirect effect of drugs that suppress gastric acid secretion and occurs secondary to hypergastrinemia (Bordi *et al*. 1997, 1998). Because gastrin regulates the function and growth of enterochromaffin-like (ECL) cells, chronic hypergastrinemia can induce ECL-cell hyperplasia and increase the risk of gastric cancer. However, factors that transform ECL cells to the neoplastic phenotype have not been fully determined. Analogous to the induction of gastric endocrine tumors in humans, the induction of benign and malignant neuroendocrine tumors of the glandular stomach in rats and mice exposed to methyleugenol was suggested to be due in part to induction of glandular stomach atrophy and consequent hypergastrinemia (NTP 1998). Mucosal atrophy, characterized by loss of glandular epithelial cells (particularly parietal and chief cells), and neuroendocrine cell hyperplasia were observed in the glandular stomach of rats and mice exposed to methyleugenol. The loss of glandular epithelial cells results in decreased gastric secretion, increased pH in the stomach, increased gastrin production, and gastrin-stimulated proliferation of ECL-like cells (Poynter and Selway 1991, Johnson *et al.* 1993, Thake *et al.* 1995, all cited in NTP 1998). With methyleugenol, the neoplastic conversion may also involve DNA-reactive intermediates formed via the bioactivation pathways shown in Figure 6-1.

Possible mechanisms of tumor induction by methyleugenol in other organs (kidney, mammary gland, and skin) are not known. Furthermore, it is not known whether tumor induction in other organs is affected by methyleugenol-induced alterations in the glandular stomach mucosa.

6.8 Summary

Methyleugenol is rapidly absorbed and cleared from the blood in experimental animals. Metabolism of methyleugenol occurs via the cytochrome P-450 system and involves side-chain hydroxylation, side-chain epoxide diol formation, and *O*-demethylation. Based on DNA binding data and studies of liver tumor induction by allylbenzenes and their metabolites, it is apparent that bioactivation of methyleugenol via the pathway of side-chain hydroxylation followed by sulfation (Figure 6-1) is an important step in liver tumor induction by this chemical. Activated *β-catenin* oncogenes were detected at higher frequencies in methyleugenol-induced mouse liver tumors than in tumors that arose spontaneously. Induction of benign and malignant neuroendocrine tumors of the glandular stomach in rats and mice exposed to methyleugenol may be due in part to induction of glandular stomach atrophy, reduced gastric acid secretion, hypergastrinemia, and gastrin-stimulated proliferation of ECL-like cells. DNA-reactive intermediates of methyleugenol metabolism also may be involved in the neoplastic transformation. Mechanisms of tumor induction by methyleugenol in other organs (kidney, mammary gland, and skin) or induction of mesotheliomas are not known.

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Appendix A: NTP (2000). Technical Report on the Toxicology and Carcinogenesis Studies of Methyleugenol in F344/N Rats and B6C3F$_1$ Mice (Gavage Studies), NTP TR. 491. pp 1-97.

NTP TECHNICAL REPORT

ON THE

TOXICOLOGY AND CARCINOGENESIS

STUDIES OF METHYLEUGENOL

(CAS NO. 93-15-2)

IN F344/N RATS AND B6C3F$_1$ MICE

(GAVAGE STUDIES)

July 2000

NTP TR 491

NIH Publication No. 00-3950

National Toxicology Program

U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES
Public Health Service
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FOREWORD

The National Toxicology Program (NTP) is made up of four charter agencies of the U.S. Department of Health and Human Services (DHHS): the National Cancer Institute (NCI), National Institutes of Health; the National Institute of Environmental Health Sciences (NIEHS), National Institutes of Health; the National Center for Toxicological Research (NCTR), Food and Drug Administration; and the National Institute for Occupational Safety and Health (NIOSH), Centers for Disease Control and Prevention. In July 1981, the Carcinogenesis Bioassay Testing Program, NCI, was transferred to the NIEHS. The NTP coordinates the relevant programs, staff, and resources from these Public Health Service agencies relating to basic and applied research and to biological assay development and validation.

The NTP develops, evaluates, and disseminates scientific information about potentially toxic and hazardous chemicals. This knowledge is used for protecting the health of the American people and for the primary prevention of disease.

The studies described in this Technical Report were performed under the direction of the NIEHS and were conducted in compliance with NTP laboratory health and safety requirements and must meet or exceed all applicable federal, state, and local health and safety regulations. Animal care and use were in accordance with the Public Health Service Policy on Humane Care and Use of Animals. The prechronic and chronic studies were conducted in compliance with Food and Drug Administration (FDA) Good Laboratory Practice Regulations, and all aspects of the chronic studies were subjected to retrospective quality assurance audits before being presented for public review.

These studies are designed and conducted to characterize and evaluate the toxicologic potential, including carcinogenic activity, of selected chemicals in laboratory animals (usually two species, rats and mice). Chemicals selected for NTP toxicology and carcinogenesis studies are chosen primarily on the bases of human exposure, level of production, and chemical structure. The interpretive conclusions presented in this Technical Report are based only on the results of these NTP studies. Extrapolation of these results to other species and quantitative risk analyses for humans require wider analyses beyond the purview of these studies. Selection *per se* is not an indicator of a chemical's carcinogenic potential.

Listings of all published NTP reports and ongoing studies are available from NTP Central Data Management, NIEHS, P.O. Box 12233, MD E1-02, Research Triangle Park, NC 27709 (919-541-3419). The Abstracts and other study information for 2-year studies are also available at the NTP's World Wide Web site: http://ntp-server.niehs.nih.gov.

NTP TECHNICAL REPORT

ON THE

TOXICOLOGY AND CARCINOGENESIS

STUDIES OF METHYLEUGENOL

(CAS NO. 93-15-2)

IN F344/N RATS AND B6C3F$_1$ MICE

(GAVAGE STUDIES)

July 2000

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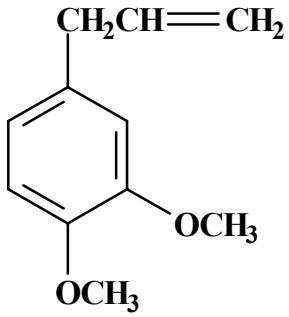
CONTENTS

ABSTRACT



METHYLEUGENOL

CAS No. 93-15-2

Chemical Formula: $C_{11}H_{14}O_2$ Molecular Weight: 178.2

Synonyms: 4-Allyl-1,2-dimethoxybenzene; 4-allylveratrole; 4-allyl-3,4-dimethoxy-benzene; 1,2-dimethoxy-4-allylbenzene; 3,4-dimethoxyallylbenzene ENT 21040; 1-(3,4-dimethoxyphenyl)-2-propene; eugenol methyl ether; 1,3,4-eugenol methyl ether; veratrole methyl ether

Methyleugenol is used as a flavoring agent in jellies, baked goods, nonalcoholic beverages, chewing gum, candy, pudding, relish, and ice cream. It is also used as a fragrance in perfumes, creams, lotions, detergents, and soaps. Methyleugenol has also been used as an insect attractant in eradication programs and as an anesthetic in rodents. Methyleugenol was nominated for testing because of its widespread use and because of its structural resemblance to safrole, a known carcinogen, and isosafrole and estragole. Male and female F344/N rats and B6C3F$_1$ mice received methyleugenol (approximately 99% pure) in 0.5% methylcellulose by gavage for 14 weeks or 2 years. Genetic toxicology studies were conducted in *Salmonella typhimurium*, cultured Chinese hamster ovary cells, and mouse peripheral blood erythrocytes.

14-WEEK STUDY IN RATS

Groups of 9 or 10 male and 10 female F344/N rats were administered 0, 10, 30, 100, 300, or 1,000 mg methyleugenol/kg body weight in 0.5% methylcellulose by gavage 5 days per week for 14 weeks. A water control group of 10 male and 10 female rats received deionized water by gavage. All rats survived until the end of the study. The final mean body weights of 300 and 1,000 mg/kg males and of all dosed groups of females were significantly less than those of the vehicle controls. Erythrocyte microcytosis was demonstrated by decreased mean cell volumes in 300 mg/kg males and 1,000 mg/kg males and females. There was evidence of a thrombocytosis at all time points, demonstrated by increased platelet counts in the 100 mg/kg or greater groups. The serum activities of alanine aminotransferase and sorbitol dehydrogenase were increased in the 100 mg/kg or greater rats at various time points, suggesting hepatocellular injury. Additionally, bile acid concentrations were generally increased in the 300 and 1,000 mg/kg groups at all time points, consistent with cholestasis or altered hepatic function. A hypoproteinemia and hypoalbuminemia, evidenced by decreased total protein and albumin concentrations, occurred in rats in the 300 and 1,000 mg/kg groups at all time points.

Liver weights of 100, 300, and 1,000 mg/kg males and 300 and 1,000 mg/kg females and testis weights of 1,000 mg/kg males were significantly increased. Increased incidences of liver lesions occurred in 300 and 1,000 mg/kg males and females and hepatocellular adenoma occurred in one 1,000 mg/kg male. The incidences of atrophy and chronic inflammation of the mucosa of the glandular stomach were significantly increased in rats administered 300 or 1,000 mg/kg. Increased incidences of adrenal gland cortical hypertrophy and/or cytoplasmic alteration in the submandibular gland occurred in the 100 mg/kg or greater groups.

14-WEEK STUDY IN MICE

Groups of 10 male and 10 female B6C3F$_1$ mice received methyleugenol in 0.5% methylcellulose by gavage at doses of 0, 10, 30, 100, 300, or 1,000 mg/kg, 5 days per week for 14 weeks. A water control group of 10 male and 10 female mice received deionized water by gavage. All but one male and all females receiving 1,000 mg/kg died before the end of the study. The mean body weight gains of mice in the 300 mg/kg groups were significantly less than those of the vehicle controls. The only clinical finding was toxicity manifested as generalized morbidity in mice administered 1,000 mg/kg. Liver weights of 30, 100, and 300 mg/kg males and of 300 mg/kg females were significantly increased. Male mice administered 10 or 30 mg/kg had significantly lower cauda epididymis, epididymis, and testis weights; males receiving 100 mg/kg had significantly lower spermatozoal concentrations. Increased incidences of liver lesions occurred in 1,000 mg/kg males and 300 and 1,000 mg/kg females. The incidences of lesions of the glandular stomach were increased in one or more groups administered 30 mg/kg or greater.

2-YEAR STUDY IN RATS

Groups of 50 male and 50 female rats received methyleugenol in 0.5% methylcellulose by gavage at doses of 37, 75, or 150 mg/kg, 5 days per week for 105 weeks; groups of 60 male and 60 female rats received the 0.5% methylcellulose vehicle only. Stop-exposure groups of 60 male and 60 female rats received 300 mg/kg in 0.5% methylcellulose by gavage for 52 weeks followed by just the 0.5% methylcellulose vehicle for the remaining 53 weeks of the study. Special study groups of 10 male and 10 female rats administered 36, 75, 150, or 300 mg/kg were designated for toxicokinetic studies.

Survival and Body Weights

All 150 and 300 mg/kg males died before the end of the study, and survival of 150 mg/kg females was slightly less than that of the vehicle controls. Mean body weights of all dosed groups of rats were less than those of the vehicle controls throughout most of the 2-year study.

Pathology Findings

Chemical-related liver neoplasms occurred in all dosed groups of rats and included hepatocellular adenoma, hepatocellular carcinoma, hepatocholangioma, and hepatocholangiocarcinoma; at 2 years, there were positive trends in the incidences of hepatocellular adenoma, carcinoma, and adenoma or carcinoma (combined) in core study rats and in the numbers of rats with multiple liver neoplasms. Nonneoplastic lesions included eosinophilic and mixed cell foci, hepatocellular hypertrophy, oval cell hyperplasia, cystic degeneration, and bile duct hyperplasia (females); the incidences of these lesions in dosed groups of male and female rats were increased at 6 months, 12 months, and/or 2 years.

Chemical-related neoplasms and nonneoplastic lesions of the glandular stomach included benign and malignant neuroendocrine tumors in the 150 and 300 mg/kg groups and females in the 75 mg/kg group. In all dosed groups of rats at all time points, the incidences of mucosal atrophy were significantly greater than in the vehicle controls. Neuroendocrine cell hyperplasia was observed in females at 6 months and males and females at 12 months and at 2 years. In core study female rats, there was a positive trend in the incidences of squamous cell papilloma or carcinoma (combined) of the forestomach, and the incidence in the 150 mg/kg group exceeded the historical control range.

The incidences of renal tubule proliferative lesions in male rats were suggestive of a neoplastic effect in the kidney. Therefore, additional step sections of the kidneys of male rats were prepared. The incidences of renal tubule hyperplasia and adenoma in the extended evaluation and the combined incidences of standard and step sections in the 75, 150, and 300 mg/kg

groups were greater than those in the vehicle controls. The incidences of nephropathy were increased in all dosed groups of females, and the increase was significant in the 300 mg/kg group.

In dosed groups of male rats, there was a positive trend in the incidences of malignant mesothelioma, and the incidences were significantly greater in 150 and 300 mg/kg males than in the vehicle controls. The incidences of mammary gland fibroadenoma in 75 and 150 mg/kg males were significantly increased. The incidences of fibroma of the subcutaneous tissue in 37 and 75 mg/kg males and the combined incidences of fibroma or fibrosarcoma in 37, 75, and 150 mg/kg males were significantly increased.

2-YEAR STUDY IN MICE

Groups of 50 male and 50 female mice received methyleugenol in 0.5% methylcellulose by gavage at doses of 0, 37, 75, or 150 mg/kg for 105 weeks. Special study groups of 10 male and 10 female mice administered 37, 75, or 150 mg/kg were designated for toxicokinetic studies.

Survival and Body Weights

Survival of all dosed groups of male mice was similar to that of the vehicle controls. Survival of dosed groups of females was significantly less. Mean body weights of dosed mice were generally less than those of the vehicle controls throughout the studies.

Pathology Findings

Chemical-related increases in the incidences of liver neoplasms and nonneoplastic lesions in mice included hepatocellular adenoma and carcinoma, hepatoblastoma, hepatocholangiocarcinoma, eosinophilic foci, oval cell hyperplasia, bile duct hyperplasia, hemosiderin pigmentation, chronic active inflammation, and hematopoietic cell proliferation. In all dosed groups of males and females, the incidences of hepatocellular neoplasms and the multiplicity of neoplasms were generally greater than in the vehicle controls. The incidences of hepatoblastoma were significantly increased in all dosed groups of females and slightly increased in 150 mg/kg males. Hepatocholangiocarcinoma was observed in 150 mg/kg females. The incidences of eosinophilic foci, oval cell hyperplasia, portal hypertrophy, hepatocyte necrosis, hematopoietic cell proliferation, bile duct hyperplasia, and hemosiderin pigmentation were significantly increased in two or more dosed groups of male and/or female mice.

The incidences of glandular ectasia, mucosal atrophy, chronic active inflammation, epithelial hyperplasia, and neuroendocrine cell hyperplasia of the glandular stomach were increased in one or more dosed groups of male and female mice. In addition, malignant neuroendocrine tumors were observed in the glandular stomach of two 150 mg/kg male mice; one male in this group had a carcinoma.

TOXICOKINETIC STUDIES

Methyleugenol is rapidly absorbed following oral administration to rats and mice. The kinetic data are consistent with rapid clearance from the blood, metabolism in the liver, and excretion of the parent and various metabolites in the urine.

GENETIC TOXICOLOGY

Methyleugenol was not mutagenic in *S. typhimurium* strain TA98, TA100, TA1535, or TA1537, with or without exogenous metabolic activation (S9). In cytogenetic tests with cultured Chinese hamster ovary cells, methyleugenol induced sister chromatid exchanges in the presence of S9, but no induction of chromosomal aberrations was noted in cultured Chinese hamster ovary cells following exposure to methyleugenol, with or without S9. *In vivo*, no increase in the frequency of micronucleated normochromatic erythrocytes was seen in male or female mice administered methyleugenol by gavage for 14 weeks.

PHYSIOLOGICALLY BASED PHARMACOKINETIC MODEL

A physiologically based pharmacokinetic (PBPK) model resulting from intravenous and oral exposure was created to characterize tissue concentrations of methyleugenol in rats and mice. Data used to create the model were obtained from the literature or from current studies. The primary conclusions that can be reached from the PBPK model are: 1) absorption of oral doses of methyleugenol in rats and mice is rapid and complete, 2) distribution of methyleugenol to

tissues is not hampered by capillary permeability, and 3) metabolism of methyleugenol is saturable and must have some extrahepatic component in the mouse. Model-based plasma methyleugenol concentrations were not found to be good dosimeters for evaluating neoplasm dose-response data.

CONCLUSIONS

Under the conditions of these 2-year gavage studies, there was *clear evidence of carcinogenic activity** of methyleugenol in male and female F344/N rats based on the increased incidences of liver neoplasms and neuroendocrine tumors of the glandular stomach in male and female rats and the increased incidences of kidney neoplasms, malignant mesothelioma, mammary gland fibroadenoma, and subcutaneous fibroma and fibroma or fibrosarcoma (combined) in male rats. A marginal increase in the incidence of squamous cell neoplasms of the forestomach may have been related to methyleugenol administration in female rats. There was *clear evidence of carcinogenic activity* of methyleugenol in male and female $B6C3F_1$ mice based on the increased incidences of liver neoplasms. Neuroendocrine tumors of the glandular stomach in male mice were also considered related to methyleugenol administration.

In male and female rats and mice, methyleugenol administration caused significant increases in the incidences of nonneoplastic lesions of the liver and glandular stomach.

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* Explanation of Levels of Evidence of Carcinogenic Activity is on page 14. A summary of the Technical Reports Review Subcommittee comments and the public discussion on this Technical Report appears on page 16.

Summary of the 2-Year Carcinogenesis and Genetic Toxicology Studies of Methyleugenol

	Male F344/N Rats	Female F344/N Rats	Male $B6C3F_1$ Mice	Female $B6C3F_1$ Mice
Doses in methylcellulose by gavage	0, 37, 75, or 150 mg/kg or 300 mg/kg (stop-exposure)	0, 37, 75, or 150 mg/kg or 300 mg/kg (stop-exposure)	0, 37, 75, or 150 mg/kg	0, 37, 75, or 150 mg/kg
Body weights	Dosed groups less than the vehicle control group	Dosed groups less than the vehicle control group	Dosed groups less than the vehicle control group	Dosed groups less than the vehicle control group
Survival rates	20/50, 16/50, 15/50, 0/50, 0/50	22/50, 25/50, 22/50, 11/50, 16/50	38/49, 36/50, 37/50, 35/50	31/50, 18/50, 18/50, 2/50
Nonneoplastic effects	Liver: eosinophilic foci (11/50, 28/50, 43/50, 47/50, 39/50); mixed cell foci (1/50, 7/50, 14/50, 8/50, 2/50); hepatocyte hypertrophy (0/50, 13/50, 25/50, 30/50, 26/50); oval cell hyperplasia (14/50, 17/50, 24/50, 34/50, 27/50); cystic degeneration (4/50, 2/50, 25/50, 38/50, 41/50) Glandular stomach: neuroendocrine cell hyperplasia (0/50, 0/50, 1/50, 8/50, 8/50); atrophy (0/50, 14/50, 32/50, 37/50, 29/50)	Liver: eosinophilic foci (10/50, 20/50, 27/49, 31/49, 37/50); mixed cell foci (6/50, 4/50, 19/49, 9/49, 7/50); hepatocyte hypertrophy (1/50, 13/50, 16/49, 26/49, 31/50); oval cell hyperplasia (1/50, 15/50, 19/49, 35/49, 34/50); bile duct hyperplasia (11/50, 11/50, 17/49, 22/49, 30/50); cystic degeneration (0/50, 0/50, 1/49, 4/49, 29/50) Glandular stomach: neuroendocrine cell hyperplasia (0/50, 5/50, 11/50, 9/50, 3/50); atrophy (3/50, 41/50, 45/50, 39/50, 33/50)	Liver: eosinophilic foci (10/49, 20/50, 25/50, 19/50); oval cell hyperplasia (0/49, 8/50, 27/50, 46/50); hepatocyte hypertrophy (0/49, 1/50, 7/50, 46/50) Glandular stomach: atrophy (0/49, 3/48, 35/49, 45/50); hyperplasia (0/49, 1/48, 15/49, 20/50); ectasia (13/49, 25/48, 40/49, 49/50)	Liver: oval cell hyperplasia (0/50, 46/50, 36/49, 38/50); hepatocyte hypertrophy (0/50, 10/50, 7/49, 23/50); hepatocyte necrosis (5/50, 9/50, 16/49, 17/50); hematopoietic cell proliferation (4/50, 14/50, 23/49, 24/50); bile duct hyperplasia (1/50, 1/50, 11/49, 9/50); hemosiderin pigmentation (0/50, 11/50, 24/49, 19/50) Glandular stomach: atrophy (0/45, 0/49, 10/46, 10/45); ectasia (14/45, 33/49, 31/46, 38/45)

Summary of the 2-Year Carcinogenesis and Genetic Toxicology Studies of Methyleugenol

	Male F344/N Rats	Female F344/N Rats	Male $B6C3F_1$ Mice	Female $B6C3F_1$ Mice
Neoplastic effects	Liver: hepatocellular adenoma (5/50, 12/50, 23/50, 38/50, 32/50); hepatocellular carcinoma (2/50, 3/50, 14/50, 25/50, 36/50); hepatocellular adenoma or carcinoma (7/50, 14/50, 28/50, 43/50, 45/50); hepatocholangioma (0/50, 0/50, 0/50, 1/50, 6/50); hepatocholangiocarcinoma (0/50, 0/50, 1/50, 1/50, 7/50); hepatocholangioma or hepatocholangiocarcinoma (0/50, 0/50, 1/50, 2/50, 13/50) Glandular stomach: benign neuroendocrine tumor (0/50, 0/50, 0/50, 3/50, 2/50); malignant neuroendocrine tumor (0/50, 0/50, 0/50, 4/50, 2/50); benign or malignant neuroendocrine tumor (0/50, 0/50, 0/50, 7/50, 4/50) Kidney: renal tubule adenoma (standard and extended evaluations combined - 4/50, 6/50, 17/50, 13/50, 20/50) Malignant mesothelioma: (1/50, 3/50, 5/50, 12/50, 5/50) Mammary gland: fibroadenoma (5/50, 5/50, 15/50, 13/50, 6/50) Skin (subcutaneous): fibroma (1/50, 9/50, 8/50, 5/50, 4/50); fibroma or fibrosarcoma (1/50, 12/50, 8/50, 8/50, 4/50)	Liver: hepatocellular adenoma (1/50, 8/50, 11/49, 33/49, 43/50); hepatocellular carcinoma (0/50, 0/50, 4/49, 8/49, 22/50); hepatocellular adenoma or carcinoma (1/50, 8/50, 14/49, 34/49, 43/50); hepatocholangioma (0/50, 0/50, 0/49, 0/49, 8/50); hepatocholangiocarcinoma (0/50, 0/50, 0/49, 3/49, 9/50); hepatocholangioma or hepatocholangiocarcinoma (0/50, 0/50, 0/49, 3/49, 17/50) Glandular stomach: benign neuroendocrine tumor (0/50, 0/50, 13/50, 9/50, 5/50); malignant neuroendocrine tumor (0/50, 1/50, 12/50, 26/50, 36/50); benign or malignant neuroendocrine tumor (0/50, 1/50, 25/50, 34/50, 41/50)	Liver: hepatocellular adenoma (26/49, 43/50, 38/50, 39/50); hepatocellular carcinoma (10/49, 20/50, 19/50, 9/50); hepatocellular adenoma or carcinoma (31/49, 47/50, 46/50, 40/50); hepatoblastoma (0/49, 0/50, 1/50, 3/50) Glandular stomach: malignant neuroendocrine tumor (0/49, 0/48, 0/49, 2/50)	Liver: hepatocellular adenoma (20/50, 48/50, 46/49, 41/50); hepatocellular carcinoma (7/50, 37/50, 47/49, 47/50); hepatocellular adenoma or carcinoma (25/50, 50/50, 49/49, 49/50); hepatoblastoma (0/50, 6/50, 11/49, 15/50); hepatocholangiocarcinoma (0/50, 0/50, 0/49, 2/50)
Uncertain findings		Forestomach: squamous cell papilloma or carcinoma (0/50, 0/50, 1/50, 3/50, 1/50)		
Level of evidence of carcinogenic activity	Clear evidence	Clear evidence	Clear evidence	Clear evidence

Summary of the 2-Year Carcinogenesis and Genetic Toxicology Studies of Methyleugenol

Genetic toxicology	
Salmonella typhimurium gene mutations:	Negative in strains TA98, TA100, TA1535, and TA1537, with and without S9
Sister chromatid exchanges	
Cultured Chinese hamster ovary cells *in vitro*:	Positive with S9, negative without S9
Chromosomal aberrations	
Cultured Chinese hamster ovary cells *in vitro*:	Negative with and without S9
Micronucleated erythrocytes	
Mouse peripheral blood *in vivo*:	Negative

EXPLANATION OF LEVELS OF EVIDENCE OF CARCINOGENIC ACTIVITY

The National Toxicology Program describes the results of individual experiments on a chemical agent and notes the strength of the evidence for conclusions regarding each study. Negative results, in which the study animals do not have a greater incidence of neoplasia than control animals, do not necessarily mean that a chemical is not a carcinogen, inasmuch as the experiments are conducted under a limited set of conditions. Positive results demonstrate that a chemical is carcinogenic for laboratory animals under the conditions of the study and indicate that exposure to the chemical has the potential for hazard to humans. Other organizations, such as the International Agency for Research on Cancer, assign a strength of evidence for conclusions based on an examination of all available evidence, including animal studies such as those conducted by the NTP, epidemiologic studies, and estimates of exposure. Thus, the actual determination of risk to humans from chemicals found to be carcinogenic in laboratory animals requires a wider analysis that extends beyond the purview of these studies.

Five categories of evidence of carcinogenic activity are used in the Technical Report series to summarize the strength of the evidence observed in each experiment: two categories for positive results (**clear evidence** and **some evidence**); one category for uncertain findings (**equivocal evidence**); one category for no observable effects (**no evidence**); and one category for experiments that cannot be evaluated because of major flaws (**inadequate study**). These categories of interpretative conclusions were first adopted in June 1983 and then revised in March 1986 for use in the Technical Report series to incorporate more specifically the concept of actual weight of evidence of carcinogenic activity. For each separate experiment (male rats, female rats, male mice, female mice), one of the following five categories is selected to describe the findings. These categories refer to the strength of the experimental evidence and not to potency or mechanism.

- **Clear evidence** of carcinogenic activity is demonstrated by studies that are interpreted as showing a dose-related (i) increase of malignant neoplasms, (ii) increase of a combination of malignant and benign neoplasms, or (iii) marked increase of benign neoplasms if there is an indication from this or other studies of the ability of such tumors to progress to malignancy.
- **Some evidence** of carcinogenic activity is demonstrated by studies that are interpreted as showing a chemical-related increased incidence of neoplasms (malignant, benign, or combined) in which the strength of the response is less than that required for clear evidence.
- **Equivocal evidence** of carcinogenic activity is demonstrated by studies that are interpreted as showing a marginal increase of neoplasms that may be chemical related.
- **No evidence** of carcinogenic activity is demonstrated by studies that are interpreted as showing no chemical-related increases in malignant or benign neoplasms.
- **Inadequate study** of carcinogenic activity is demonstrated by studies that, because of major qualitative or quantitative limitations, cannot be interpreted as valid for showing either the presence or absence of carcinogenic activity.

When a conclusion statement for a particular experiment is selected, consideration must be given to key factors that would extend the actual boundary of an individual category of evidence. Such consideration should allow for incorporation of scientific experience and current understanding of long-term carcinogenesis studies in laboratory animals, especially for those evaluations that may be on the borderline between two adjacent levels. These considerations should include:

- adequacy of the experimental design and conduct;
- occurrence of common versus uncommon neoplasia;
- progression (or lack thereof) from benign to malignant neoplasia as well as from preneoplastic to neoplastic lesions;
- some benign neoplasms have the capacity to regress but others (of the same morphologic type) progress. At present, it is impossible to identify the difference. Therefore, where progression is known to be a possibility, the most prudent course is to assume that benign neoplasms of those types have the potential to become malignant;
- combining benign and malignant tumor incidence known or thought to represent stages of progression in the same organ or tissue;
- latency in tumor induction;
- multiplicity in site-specific neoplasia;
- metastases;
- supporting information from proliferative lesions (hyperplasia) in the same site of neoplasia or in other experiments (same lesion in another sex or species);
- presence or absence of dose relationships;
- statistical significance of the observed tumor increase;
- concurrent control tumor incidence as well as the historical control rate and variability for a specific neoplasm;
- survival-adjusted analyses and false positive or false negative concerns;
- structure-activity correlations; and
- in some cases, genetic toxicology.

NATIONAL TOXICOLOGY PROGRAM BOARD OF SCIENTIFIC COUNSELORS
TECHNICAL REPORTS REVIEW SUBCOMMITTEE

The members of the Technical Reports Review Subcommittee who evaluated the draft NTP Technical Report on methyleugenol on 30 October 1998 are listed below. Subcommittee members serve as independent scientists, not as representatives of any institution, company, or governmental agency. In this capacity, subcommittee members have five major responsibilities in reviewing the NTP studies:

- to ascertain that all relevant literature data have been adequately cited and interpreted,
- to determine if the design and conditions of the NTP studies were appropriate,
- to ensure that the Technical Report presents the experimental results and conclusions fully and clearly,
- to judge the significance of the experimental results by scientific criteria, and
- to assess the evaluation of the evidence of carcinogenic activity and other observed toxic responses.

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SUMMARY OF TECHNICAL REPORTS REVIEW SUBCOMMITTEE COMMENTS

On 30 October 1998, the draft Technical Report on the toxicology and carcinogenesis studies of methyleugenol received public review by the National Toxicology Program's Board of Scientific Counselors' Technical Reports Review Subcommittee. The review meeting was held at the National Institute of Environmental Health Sciences, Research Triangle Park, NC.

Dr. K.M. Abdo, NIEHS, introduced the toxicology and carcinogenesis studies of methyleugenol by discussing the uses of the chemical, describing the rationale for the study and the experimental design, reporting on survival and body weight effects, and commenting on compound-related neoplastic and nonneoplastic lesions in rats and mice. The proposed conclusions for the 2-year gavage studies of methyleugenol were *clear evidence of carcinogenic activity* in male and female F344/N rats and $B6C3F_1$ mice.

Dr. R.A. Herbert, NIEHS, characterized the lesions in the fundic region of the glandular stomach associated with methyleugenol administration in male and female rats and mice. These lesions included atrophy and neuroendocrine cell hyperplasia, as well as benign and malignant neuroendocrine tumors, which are rare in rats and mice as either spontaneous or chemically induced lesions. Dr. Herbert described a series of short-term (14-, 30-, and 90-day) studies providing data that supported the hypothesis that parietal cell cytotoxicity with subsequent mucosal atrophy, increased intragastric pH, and increased circulating gastrin (hypergastrinemia) is probably how methyleugenol produces neuroendocrine tumors in the glandular stomach.

Dr. M.L. Cunningham, NIEHS, presented data from work in progress that described *in vivo* and *in vitro* studies of methyleugenol metabolism in rodents and some recent results from human model systems. He began by describing the more widely studied metabolism of the close structural analogue and hepatocarcinogen, safrole, and contrasted the results with those obtained for methyleugenol. The findings to date indicate that methyleugenol can undergo a variety of Phase 1 oxidation reactions, that metabolites can be further metabolized through Phase 2 conjugations to yield reactive sulfonoxy metabolites, and that human tissue preparations are capable of metabolizing and bioactivating the chemical. The genetic toxicity of methyleugenol is similar to safrole and for both compounds appears to be dependent on both Phase 1 and Phase 2 metabolic activation.

Dr. T.R. Devereux, NIEHS, provided information on molecular alterations in neoplasms from the NTP study, concentrating on the mouse liver and lung neoplasms for which there is a large database of genetic information. She focused on the APC/β-catenin-Wnt signaling pathways that have been implicated in various human and rodent cancers. In neoplasm cells, either a mutation in the APC gene or in β-catenin can upregulate β-catenin and the Wnt signaling pathway, leading eventually to cell proliferation. β-Catenin mutations were found in about half of the methyleugenol mouse liver neoplasms compared with mutations in only 5% of spontaneous neoplasms. Mutations were found at the same sites as those in human hepatocellular carcinomas, suggesting similar carcinogenic pathways. Genetic alterations were not found in H-*ras* or p53, suggesting that these genes are not involved in methyleugenol-induced mouse liver carcinogenesis.

Dr. G.M. Blumenthal, NIEHS, discussed the development of physiologically based pharmacokinetic models to describe and simulate the toxicokinetics of methyleugenol in rats and humans. Animal data were obtained from single-dose administration to rats at 37 mg/kg by intravenous injection and by gavage at 37, 75, and 150 mg/kg. Human data were obtained from an in-house study in which volunteers ate 12 gingersnaps; blood samples were collected prior to exposure and 15, 30, 60, and 120 minutes afterward. Data was also obtained from the NHANES database collected by the Centers for Disease Control and Prevention. The studies to date show that absorption of methyleugenol was rapid in rats and humans with a large first pass effect in rats that was also assumed in humans. Metabolism was saturated at all doses in rats, while a slower metabolism was predicted in humans. Over 90% of the doses were metabolized within 24 hours in rats, and this value was assumed for the human model. More studies are in process and should lead to an entire dose response characterization.

Dr. Hecht, a principal reviewer, agreed with the proposed conclusions. He wondered why, considering the structural similarity to safrole and human exposure to methyleugenol, the NTP had not studied this chemical earlier.

Dr. Cullen, the second principal reviewer, agreed with the proposed conclusions. He thought the study was remarkable because of the presence of two unusual neoplasms. In the liver, unusual mixed neoplasms composed of cholangiocellular and hepatocellular elements suggest a potent carcinogenic effect that affects both biliary and hepatic cell lineage or, possibly, a stem-cell population. He said that gastric neuroendocrine tumors are also rare and thought it prudent that immunohistochemical and histochemical stains were done to establish the cell type. Dr. Herbert noted that some of the liver neoplasms appeared to have a hepatocellular component and a biliary cell component; therefore, the diagnoses of hepatocholangiocellular neoplasms were most descriptive. Dr. Cullen said the dose-related increases in the incidences of oval cell hyperplasia in mice suggested further discussion of the possibility of a synergistic effect with the presence of *Helicobacter* and this lesion.

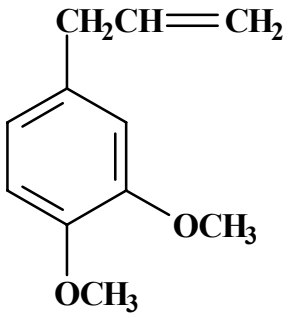
Dr. Bus, the third principal reviewer, agreed with the proposed conclusions. He complimented the NTP on the extensive toxicokinetic and disposition studies including information on how disposition may change with time and with the age of the animals. Noting that the low dose in the rodent studies, 37 mg/kg, was metabolically saturating and likely not a no-observed-effect level, Dr. Bus suggested there were lessons here for future protocol designs to provide data more valuable for future risk assessment purposes. Dr. J.R. Bucher, NIEHS, commented that methyleugenol is listed as a "generally recognized as safe" substance in the United States, and although there is a 5 mg/kg limit in Europe, there are not large differences between concentrations permitted in foods and the 37 mg/kg dose used in rats and mice. Palatability may be the limiting factor.

Dr. Medinsky cautioned that bioavailability of a compound is less relevant when a metabolite is the active/toxic form. Dr. Bailer commented that he was a bit uncomfortable with the possible utility of data for risk assessment purposes when the lowest animal dose is approximately 37,000 times the human dose (the gingersnap study). Dr. G.W. Lucier, NIEHS, pointed out that the blood levels from the NHANES study were only about 1,000-fold greater than the rat blood levels.

Dr. Tim Adams, Flavor and Extract Manufacturers Association (FEMA), stated that actual exposure to methyleugenol has substantially decreased over the last 30 years, with most coming from fruits and spices. Further, FEMA estimates that exposure in the diet exceeds intentional addition by a factor of at least 100. With regard to the neuroendocrine lesions in the stomach, he noted that the agent was given by gavage in a microencapsulated form, perhaps allowing for prolonged stomach exposure. Dr. Bucher said the methyleugenol was in methylcellulose, the gavage vehicle, and not microencapsulated.

Dr. Hecht moved that the Technical Report on methyleugenol be accepted with revisions discussed and the conclusions as written for male and female rats and mice, *clear evidence of carcinogenic activity*. Dr. Cullen seconded the motion, which was accepted unanimously with six votes.

INTRODUCTION



METHYLEUGENOL

CAS No. 93-15-2

Chemical Formula: $C_{11}H_{14}O_2$ Molecular Weight: 178.2

Synonyms: 4-Allyl-1,2-dimethoxybenzene; 4-allylveratrole; 4-allyl-3,4-dimethoxy-benzene; 1,2-dimethoxy-4-allylbenzene; 3,4-dimethoxyallylbenzene ENT 21040; 1-(3,4-dimethoxyphenyl)-2-propene; eugenol methyl ether; 1,3,4-eugenol methyl ether; veratrole methyl ether

CHEMICAL AND PHYSICAL PROPERTIES

Methyleugenol is a colorless to pale yellow, oily liquid with a clove-carnation odor and a bitter taste. It is soluble in ethanol, ethyl ether, chloroform, and most other organic solvents but is insoluble in water, glycol, and propylene glycol. Methyleugenol darkens and thickens slowly when exposed to air and evaporates readily at room temperature (Lide, 1998; *Sax's*, 1992). Methyleugenol has a melting point of −4° C, a boiling point of 254.7° C, a refractive index of 1.532, and a density of 1.0396 at 20° C.

PRODUCTION, USE, AND HUMAN EXPOSURE

Methyleugenol is produced by methylation of eugenol (Opdyke, 1979). The annual production in the United States is estimated at 25,000 pounds (SRI, 1990). Methyleugenol was given generally recognized as safe (GRAS) status in 1965 and is approved by the FDA for use in food (21 CFR 121.1164). Methyleugenol is a natural constituent of a large number of essential oils including rose, basil, hyacinth, pimento, citronella (FEMA, 1978), anise (400 ppm), nutmeg, mace, cinnamon leaves (*Fenaroli's*, 1975), pixuri seeds (520 ppm) (Carlini *et al.*, 1983), and laurel fruits and leaves (*Farm Chemical Handbook*, 1992). The chemical has also been identified in blackberry essence, bananas, black pepper, and bilberries (WHO, 1981). Methyleugenol was detected in the oil (240 ppm) and juice (42 ppb) of oranges treated with abscission chemicals such as cycloheximide (MacGregor *et al.*, 1974). Methyleugenol (0.02 mg/L) has been detected in wastewater effluent from a paper mill (Moshonas and Shaw, 1978).

Methyleugenol is used as a flavoring agent in jellies (52 ppm), baked goods (13 ppm), nonalcoholic beverages (10 ppm), chewing gum, candy (11 ppm), pudding, relish, and ice cream (4.8 ppm) (*Fenaroli's*, 1975). It is also used as a fragrance in perfumes (0.3% to 0.8%), creams and lotions (0.01% to 0.05%), and soaps and detergents (0.02% to 0.2%)

(Opdyke, 1979). One of the major uses for methyleugenol is as an insect attractant. In 1982, methyleugenol was used in combination with malathion to control an outbreak of oriental fruit flies in California (Hays and Laws, 1991). Methyleugenol has also been used as an anesthetic in rodents (Carlini *et al.*, 1981).

The Council of Europe (1974) has listed the acceptable daily intake of methyleugenol as 5 mg/kg. The per capita intake of methyleugenol in foods is estimated at 0.073 mg/day (WHO, 1981). More recent estimates put the daily per capita consumption of methyleugenol at 0.26 μg/kg body weight (Stofberg and Grundschober, 1987; NAS, 1989). The National Occupational Exposure Survey (1981-1983) estimated that 2,824 workers in the United States were potentially exposed to methyleugenol annually (NIOSH, 1990).

ABSORPTION, DISTRIBUTION, METABOLISM, AND ELIMINATION

Experimental Animals

Solheim and Scheline (1976) studied the metabolism of methyleugenol (3,4-dimethoxyallylbenzene) and methylisoeugenol (3,4-dimethoxypropenylbenzene) in male albino Wistar-derived rats. The distribution of metabolites did not change significantly when these two compounds were administered neat by intraperitoneal injection or as a suspension in water by gavage. No unchanged compound was found in either urine or bile.

Humans

The major pathways of metabolism of methyleugenol include the oxidation of the allylic side chain, the formation of the hydroxy acid via epoxidation of the double bond followed by hydration, *O*-demethylation, and hydroxylation of the benzene ring.

The metabolism of allylbenzene compounds including methyleugenol to allyl epoxides has been investigated in several studies (Borchert *et al.*, 1973; Stillwell *et al.*, 1974; Solheim and Scheline, 1976; Delaforge *et al.*, 1980a,b). Epoxides, because of their high reactivity toward cell components, have been implicated as ultimate carcinogens (Sims and Grover, 1974). The epoxides of allylbenzene are relatively stable.

The conversion of safrole epoxide to catechol epoxide demonstrated that the cleavage of the methylenedioxy group may occur with epoxide function intact (Delaforge *et al.*, 1980a,b). The hydroxylation at the C-1′ position and the eventual formation of 3,4-dimethoxycinnamyl alcohol are also thought to represent a key metabolic step that accounts for 60% of the administered dose. The 1′-hydroxy metabolites of safrole and estragole are converted by hepatic sulfotransferase to sulfate conjugates that are thought to be the ultimate carcinogens (Miller *et al.*, 1983). *O*-Demethylation resulting in the formation of phenolic metabolites accounts for only 11% of the total dose of methyleugenol. These phenolic metabolites are likely to be rapidly conjugated and excreted with the allylic group intact (Solheim and Scheline, 1976).

Humans are continually exposed to low levels of allylbenzene compounds that, through their metabolism to epoxides, may be toxic. The concentration of these compounds in humans depends on the rate of synthesis and the rate of deactivation by microsomal enzymes (Delaforge *et al.*, 1980a,b). Ioannides *et al.* (1981) have reported that compounds with intact allyl and methylenedioxyphenyl groups (safrole and isosafrole) were inducers of cytochromes P_{450} and P_{448}. Compounds containing an intact allyl group only (estragole and allylbenzene) or an oxidized allyl and an intact methylenedioxyphenyl group (epoxysafrole) were inducers of P_{448} only.

Results of studies (Gardner *et al.*, 1997) with liver microsome preparations from Fischer 344 rats and humans suggest that the hydroxylation of methyleugenol to the proximate carcinogen 1′-hydroxymethyleugenol is catalyzed by P_{450} isozyme P_{450} 2E1 and by an unidentified isozyme (probably CYP2C6). These researchers also found that methyleugenol caused a dose-dependent autoinduction of this hydroxylation reaction in rats. Hydroxylation of methyleugenol activities varied considerably (37-fold) in the human liver samples tested; the highest hydroxylation activity was similar to that of the liver microsomes of control rats. These results suggest that the risk to humans ingesting methyleugenol is subject to marked inter-individual variability.

PHARMACOLOGY

Methyleugenol is a central nervous system depressant with anesthetic, hypothermic, myorelaxant, and anticonvulsant properties. Jiang *et al*. (1982) studied the pharmacologic effects of methyleugenol in various animals. As a central nervous system depressant, it acted synergistically to prolong the sedative effect of both sodium phenobarbital and sodium thiopental. Rabbits, cats, dogs, and monkeys administered a constant infusion of 50 mg methyleugenol/kg body weight lost pain sensation and the righting and hearing reflexes and had a slowing of respiration and the corneal reflex. Cats had increased salivation, and dogs exhibited vomiting and diarrhea. With slower infusion of massive doses (224 mg/kg total), respiration in the dog could be slowed or stopped altogether, but this effect could be reversed with artificial respiration. Infusion in cats caused a reversible drop in blood pressure. Infusion of 50 mg/kg for 1 minute inhibited the spontaneous electroencephalogram of the rabbit cortex and midbrain reticular activating system for 11 minutes; intraperitoneal injection lowered the body temperature of rats.

Dallmeier and Carlini (1981) found that methyleugenol injected intraperitoneally was one of the most potent of nine congeners tested for anesthetic, hypothermic, and myorelaxant effects in rats and mice. A dose-related anesthetic effect was observed with eugenol and methyleugenol at doses between 1.2 and 2.4 mmol/kg. Both of these chemicals induced hypothermia, causing a 3° C decrease in rectal temperature of rats administered 1.2 mmol/kg by intraperitoneal injection.

Methyleugenol administered in a rectal suppository prevented carbacol-induced spasms of the urinary bladder in humans (Deininger and Wolfe, 1977). The antispasmodic activity of methyleugenol was demonstrated on the isolated guinea pig ileum (Wagner, 1980). Methyleugenol administered by gavage at doses of 50 or 100 mg/kg increased phenobarbital- and ethanol-induced sleep time of mice (Seto and Keup, 1969).

TOXICITY

Methyleugenol is moderately toxic. The median lethal oral doses were 810 to 1,560 mg/kg for rats and 540 mg/kg for mice. The undiluted chemical (98% purity) was neither an eye irritant nor a skin irritant to rats and mice (Beroza *et al*., 1975). No information related to the toxicity of methyleugenol in humans was found in a review of the available literature.

REPRODUCTIVE AND DEVELOPMENTAL TOXICITY

No information describing reproductive or developmental toxicity studies of methyleugenol was found in the literature.

CARCINOGENICITY

Experimental Animals

Miller *et al*. (1983) studied the carcinogenicity of alkylbenzenes, including methyleugenol and 1′-hydroxymethyleugenol, in male B6C3F$_1$ mice administered the chemical prior to weaning. The chemicals were dissolved in tricotanoin and injected intraperitoneally on lactation days 1, 8, 15, and 22. The total dose administered per mouse was 4.75 μmol methyleugenol and 2.85 μmol 1′-hydroxymethyleugenol. The mice were weaned at 4 weeks and maintained on a purified diet for up to 18 months. At week 13, the livers were examined by laparotomy, and mice with extensive liver tumors were sacrificed; remaining animals were sacrificed at 18 months. The results of this study, increased incidences and multiplicity of liver tumors, clearly showed that methyleugenol and the 1′-hydroxy metabolite were carcinogenic to the liver. Other alkylbenzene compounds found to be carcinogenic included safrole and estragole. Eugenol was inactive when administered in the diet for 12 months or injected intraperitoneally to nursing male mouse pups.

Estragole, safrole, isosafrole, and eugenol were investigated for carcinogenicity in long-term studies, and the results were similar to those reported by Miller *et al*. (1983). Safrole and isosafrole administered by stomach tube daily at doses of 464 mg/kg (safrole) and 215 mg/kg (isosafrole) to mice from day 7 to day 28 of age, then in the feed at concentrations of 1,112 ppm and 517 ppm, respectively, have been reported to produce liver tumors (Innes *et al.*, 1969). Osborne-Mendel rats receiving 5,000 ppm isosafrole in the diet for up to 2 years

developed adenomas and carcinomas of the liver (Hagan *et al.*, 1965). Subcutaneous administration of 0.66 to 6.6 mg safrole in tricaprylin on days 1, 7, and 21 after birth produced liver and lung tumors (IARC, 1976). Fennell *et al.* (1984) presented evidence that the sulfate conjugate of 1′-hydroxysafrole may represent the major ultimate electrophilic and carcinogenic metabolite of safrole. Estragole induced hepatomas in male and female Swiss (CD-1®) mice 11 to 14 weeks following administration by gavage twice weekly for 5 weeks (total dose 25 μmol/g) and in female Swiss (CD-1®) mice given 23% or 46% in feed for 12 months. Estragole administered intraperitoneally as a total dose of 4.75 μmol to male $B6C3F_1$ mice for 13 or 18 months and 9.45 μmol to male C3H mice for 12 months produced hepatomas (CCRIS, 1998).

Eugenol administered in feed showed equivocal evidence of carcinogenicity based on the increased incidences of liver neoplasms in 3,000 ppm male and 6,000 ppm female $B6C3F_1$ mice (NTP, 1983).

Humans

No information related to the carcinogenicity of methyleugenol in humans was found in a review of the available literature.

GENETIC TOXICITY

The published data from mutagenicity studies with methyleugenol, although limited, show no activity in most standard assays but show positive results for DNA reactivity, particularly in mammalian hepatocytes. Negative results were reported in *Salmonella typhimurium* gene mutation studies (Sekizawa and Shibamoto, 1982; Mortelmans *et al.*, 1986; Kettering and Torabinejad, 1995), and in the *E. coli* WP2 uvrA gene reversion test (Sekizawa and Shibamoto, 1982). Positive, dose-related responses were observed with methyleugenol in chromosomal recombination studies in the yeast *Saccharomyces cerevisiae* (Schiestl *et al.*, 1989; Brennan *et al.*, 1996). Methyleugenol has been shown in several studies to interact with mammalian hepatocyte DNA, inducing unscheduled DNA synthesis (UDS) in rat hepatocyte primary cultures (Howes *et al.*, 1990; Chan and Caldwell, 1992; Gardner *et al.*, 1997) or forming DNA adducts in rat and human hepatocytes *in vitro* (Gardner *et al.*, 1997) or newborn mouse (Phillips *et al.*, 1984) or rat (Gardner *et al.*, 1997) hepatocytes *in vivo*. Metabolic studies have demonstrated the conversion of methyleugenol by cytochrome P_{450} enzymes to the reactive metabolite 1′-hydroxymethyleugenol (Chan and Caldwell, 1992; Gardner *et al.*, 1997). Testing of 1′-hydroxymethyleugenol for induction of UDS in rat hepatocytes showed the metabolite to be more potent than the parent compound (Chan and Caldwell, 1992). The rate and efficiency of hydroxylation of methyleugenol were shown to be variable among human liver samples tested *in vitro*, and the most efficient conversions noted in human hepatocytes matched the rates observed in control rat hepatocytes (Gardner *et al.*, 1997).

More mutagenicity information is available for eugenol. Eugenol, like methyleugenol, was not mutagenic in *S. typhimurium*, in the presence or the absence of S9 activation (Sekizawa and Shibamoto, 1982; Haworth *et al.*, 1983), but positive, dose-related responses were observed with eugenol in chromosomal recombination studies in the yeast *S. cerevisiae* (Schiestl *et al.*, 1989). Positive results were also reported with eugenol in mammalian cell test systems. Increases in both sister chromatid exchanges and chromosomal aberrations were observed in cultured Chinese hamster ovary cells treated with eugenol (Galloway *et al.*, 1987), and increased mutation frequencies were reported in mouse lymphoma cells treated with eugenol (Myhr and Caspary, 1991; Sofuni *et al.*, 1996).

Results of *in vivo* studies with eugenol were generally negative. Eugenol did not induce sex-linked recessive mutations in germ cells of male *Drosophila melanogaster* (Foureman *et al.*, 1994), and no increase in micronucleated erythrocytes was observed in mouse bone marrow following three intraperitoneal injections of 600 mg eugenol in corn oil/kg body weight (Shelby *et al.*, 1993). Additional negative micronucleus test results were reported in mice after intraperitoneal injection of up to 800 mg eugenol/kg body weight and in female rats after oral administration of up to 1,340 mg eugenol/kg body weight (Hayashi *et al.*, 1988; Maura *et al.*, 1989). Male rats also showed no increase in micronucleated erythrocytes in bone marrow after administration of one-half of the median lethal dose of eugenol (Allavena *et al.*, 1992). In contrast to these negative micronucleus test data,

Woolverton *et al*. (1986) reported positive results in mouse bone marrow micronucleus tests with eugenol dissolved in saline and administered by either intraperitoneal injection (up to 147.9 mg/kg) or gavage (14,794 mg/kg). Data from unpublished NTP *in vivo* studies with eugenol in male $B6C3F_1$ mice showed a small increase in chromosomal aberrations in bone marrow cells that was judged to be equivocal and a positive result in a single, unconfirmed sister chromatid exchange test.

In conclusion, the mutagenicity test data for methyleugenol clearly demonstrated induction of chromosomal damage *in vitro*, but the evidence for chromosomal damage *in vivo* was weak. Results from gene mutation tests with methyleugenol in bacteria were negative.

STUDY RATIONALE

Methyleugenol was nominated by the National Cancer Institute and the FDA for toxicity and carcinogenicity testing by the NTP based on the high potential for human exposure through flavoring agents and its structural resemblance to safrole, a known carcinogen (IARC, 1976), and to eugenol. Oral administration was chosen because it is the most likely route of human exposure; gavage was chosen because of the unpalatability of methyleugenol in feed.

MATERIALS AND METHODS

PROCUREMENT AND CHARACTERIZATION

Methyleugenol

Methyleugenol was obtained from Elan Chemical Company (Newark, NJ) in two lots (8334801 and 9224705). Lot 8334801 was used during the 14-week studies, and lot 9224705 was used during the 2-year studies. Identity, purity, and stability analyses were conducted by the analytical chemistry laboratory, Midwest Research Institute (Kansas City, MO) and the study laboratory (Appendix J). Reports on analyses performed in support of the methyleugenol studies are on file at the National Institute of Environmental Health Sciences.

The chemical, a clear, colorless, or pale yellow liquid, was identified as methyleugenol by infrared, ultraviolet/visible (lot 8334801), and nuclear magnetic resonance spectroscopy and density analysis (lot 8334801). The purity of lot 8334801 was determined by elemental analyses, Karl Fisher water analysis, methoxy group determination, thin-layer chromatography (TLC), and gas chromatography. The purity of lot 9224705 was determined by high-performance liquid chromatography (HPLC). For lot 8334801, elemental analyses for carbon and hydrogen were in agreement with theoretical values for methyleugenol. Karl Fischer water analysis indicated 0.07% ± 0.01% water. Methoxy group determination indicated a purity of 101.4% ± 0.3%. TLC indicated a major spot and a trace impurity by one solvent system and a major spot, six trace impurities, and a slight trace impurity by a second system. Gas chromatography by two systems resolved a major peak and impurities with cumulative areas of 0.38% or 0.47% relative to the major peak area. The overall purity of lot 8334801 was determined to be approximately 99%. For lot 9224705, HPLC indicated one major peak and three impurities with areas greater than 0.1% relative to the major peak area. The overall purity of lot 9224705 was determined to be approximately 99%.

Accelerated stability studies of the bulk chemical were performed by the analytical chemistry laboratory using gas chromatography. These studies indicated that methyleugenol is stable as bulk chemical for 2 weeks when stored protected from light at temperatures up to 60° C. To ensure stability, the bulk chemical was stored at room temperature (22° C, 14-week studies; 25° C, 2-year studies), protected from light in amber glass bottles with Teflon®-lined caps.

Stability was monitored during the 14-week studies with gas chromatography and during the 2-year studies using HPLC. No degradation of the bulk chemical was determined.

Methylcellulose

Methylcellulose (USP/FCC grade) was obtained from Fisher Scientific Company (St. Louis, MO, and Pittsburgh, PA) in three lots. Lot 874544 was used during the 14-week studies, and lots 876672 and 946150 were used during the 2-year studies. Identity, purity, and stability analyses were conducted by the analytical chemistry laboratory and the study laboratory (Appendix J).

Lots 876672 and 946150 were identified as methylcellulose by infrared spectroscopy. Ultraviolet/visible and nuclear resonance spectroscopy were also used to confirm the identity of lot 876672. The purity of lot 876672 was determined by elemental analyses, Karl Fischer water analysis, functional group titration, HPLC, and the complete battery of United States Pharmacopeia (USP) XXI analyses. Elemental analyses for carbon and hydrogen were in agreement with the theoretical values for methylcellulose, assuming 1.8° of substitution and corrected for 1.94% water; elemental analyses also indicated 0.06% sodium. Karl Fischer water analysis indicated 1.94% ± 0.03% water. Functional group titration indicated 30.62% ± 0.08% methoxy group content; this value is consistent with the theoretical value, assuming

1.8° of substitution. HPLC indicated one major peak and no impurities with areas of 0.1% or greater relative to the major peak area. The lot met the USP specifications for methylcellulose for all analyses.

Stability studies of the bulk chemical were performed by the analytical chemistry laboratory using gas chromatography. These studies indicated that methylcellulose was stable as a bulk chemical for 3 weeks when stored protected from light at temperatures up to 60° C. To ensure stability, the bulk chemical was stored at approximately 25° C in sealed containers protected from light. Stability of both lots was monitored during the 2-year studies by monitoring the methoxy group content. No degradation of the bulk chemical was detected.

PREPARATION AND ANALYSIS OF DOSE FORMULATIONS

The dose formulations were prepared by mixing methyleugenol with 0.5% aqueous methylcellulose to give the required concentrations (Table J1). The dose formulations were stored at room temperature in the dark in amber glass bottles for up to 18 days (14-week studies) or 35 days (2-year studies). Homogeneity and stability studies were conducted by the study laboratory using HPLC. Homogeneity of a 200 mg/g formulation was confirmed; stability of a 1.0 mg/g formulation was confirmed for 3 weeks at room temperature and when stored protected from light. Samples stored open to air and light showed methyleugenol losses of 3%. Homogeneity of 0.8 and 60 mg/mL formulations were confirmed; stability of a 0.8 mg/mL formulation was also confirmed for 35 days when the formulation was stored in sealed containers with minimal headspace, at room temperature, protected from light.

Periodic analyses of the dose formulations of methyleugenol were conducted at the study laboratory using HPLC at the beginning, midpoint, and end of the 14-week studies (Table J2). During the 2-year studies, the dose formulations were analyzed with ultraviolet spectroscopy approximately every 8 weeks (Table J3). All dose formulations used in the 14-week and 2-year studies were within 11% of the target concentrations. Results of periodic referee analyses of the 14-week dose formulations performed by the analytical chemistry laboratory agreed with the results obtained by the study laboratory (Table J4).

14-WEEK STUDIES

The 14-week studies were conducted to evaluate the cumulative toxic effects of repeated exposure to methyleugenol and to determine the appropriate doses to be used in the 2-year studies.

Male and female F344/N rats and B6C3F$_1$ mice were obtained from Simonsen Laboratories, Inc. (Gilroy, CA). Upon receipt, the rats were 5 weeks old; mice were 5 (females) or 6 (males) weeks old. Animals were quarantined for 10 or 11 days and were 6 (rats and female mice) or 7 (male mice) weeks old on the first day of the studies. Before initiation of the studies, seven male and five female rats and five male and five female mice were randomly selected for parasite evaluation and gross observation for evidence of disease. At the end of the studies, serologic analyses were performed on five male and five female control rats and mice using the protocols of the NTP Sentinel Animal Program (Appendix L).

Groups of 9 or 10 male and 10 female rats and mice received methyleugenol in 0.5% methylcellulose by gavage at doses of 0, 10, 30, 100, 300 or 1,000 mg/kg, 5 days per week for 14 weeks; groups of 10 male and 10 female rats and mice received deionized water only. Groups of 10 male and 10 female special study rats received the same doses for 22 or 23 days. Feed and water were available *ad libitum*. Rats were housed five per cage; mice were housed individually. Clinical findings were recorded and the animals were weighed weekly and at the end of the studies. Details of the study design and animal maintenance are summarized in Table 1.

Hematology and clinical chemistry analyses were performed on 10 male and 10 female special study rats per group on day 5 and at week 4 and on all core study rats at study termination. At all time points, rats were anesthetized with CO_2, and blood was collected from the retroorbital sinus. Blood samples for hematology analysis were collected in tubes containing EDTA as an anticoagulant. Erythrocyte, platelet, and leukocyte counts, hematocrit values, and hemoglobin concentrations were determined using an Ortho ELT-8 analyzer (Ortho Diagnostic Systems,

Westwood, MA). Leukocyte differentials, reticulocyte counts, and erythrocyte, leukocyte, and platelet morphologies were determined using light microscopy. Mean cell volumes, mean cell hemoglobin, and mean cell hemoglobin concentrations were calculated from the analyses for hemoglobin concentrations, hematocrit values, and erythrocyte counts. Clinical chemistry parameters were determined using the Roche Cobas Fara chemistry analyzer (Roche Diagnostic Systems, Inc., Montclair, NJ). The parameters measured are listed in Table 1.

At the end of the 14-week studies, samples were collected for sperm motility and vaginal cytology evaluations on vehicle control, 30, 100, and 300 mg/kg rats and vehicle control, 10, 30, and 100 mg/kg mice. The parameters evaluated are listed in Table 1. Methods used were those described in the NTP's sperm morphology and vaginal cytology evaluations protocol (NTP, 1987). For 12 consecutive days prior to scheduled terminal sacrifice, the vaginal vaults of the females were moistened with saline, if necessary, and samples of vaginal fluid and cells were stained. Relative numbers of leukocytes, nucleated epithelial cells, and large squamous epithelial cells were determined and used to ascertain estrous cycle stage (i.e., diestrus, proestrus, estrus, and metestrus). Male animals were evaluated for sperm count and motility. The left testis and left epididymis were isolated and weighed. The tail of the epididymis (cauda epididymis) was then removed from the epididymal body (corpus epididymis) and weighed. Test yolk (rats) or modified Tyrode's buffer (mice) was applied to slides, and a small incision was made at the distal border of the cauda epididymis. The sperm effluxing from the incision were dispersed in the buffer on the slides, and the numbers of motile and nonmotile spermatozoa were counted for five fields per slide by two observers. Following completion of sperm motility estimates, each left cauda epididymis was placed in buffered saline solution. Caudae were finely minced, and the tissue was incubated in the saline solution and then heat fixed at 65° C. Sperm density was then determined microscopically with the aid of a hemacytometer. To quantify spermatogenesis, the testicular spermatid head count was determined by removing the tunica albuginea and homogenizing the left testis in phosphate-buffered saline containing 10% dimethyl sulfoxide. Homogenization-resistant spermatid nuclei were counted with a hemacytometer.

A necropsy was performed on all core study rats and mice. The heart, right kidney, liver, lung, spleen, right testis, and thymus were weighed. Tissues for microscopic examination were fixed and preserved in 10% neutral buffered formalin, processed and trimmed, embedded in paraffin, sectioned to a thickness of 5 to 6 μm, and stained with hematoxylin and eosin. A complete histopathologic examination was performed on core study water control, vehicle control, and 1,000 mg/kg rats and water control, vehicle control, and 300 and 1,000 mg/kg mice. Table 1 lists the tissues and organs routinely examined.

2-YEAR STUDIES

Study Design

Groups of 50 male and 50 female rats received methyleugenol in 0.5% methylcellulose by gavage at doses of 37, 75, or 150 mg/kg, 5 days per week for 105 weeks; groups of 60 male and 60 female rats received the 0.5% methylcellulose vehicle only. Stop-exposure groups of 60 male and 60 female rats received 300 mg/kg methyleugenol in 0.5% methylcellulose by gavage for 52 weeks followed by the 0.5% methylcellulose vehicle only for the remaining 53 weeks of the study. Five male and female vehicle control rats and five male and five female rats receiving 300 mg/kg were euthanized at 6 and 12 months for histologic evaluation. Groups of 50 male and 50 female mice received methyleugenol in 0.5% methylcellulose by gavage at doses of 0, 37, 75, or 150 mg/kg for 105 weeks. Groups of 10 male and 10 female rats administered 37, 75, 150, or 300 mg/kg and 10 male and 10 female mice administered 37, 75, or 150 mg/kg were designated for plasma toxicokinetic studies.

Source and Specification of Animals

Male and female F344/N rats and B6C3F$_1$ mice were obtained from Taconic Laboratory Animals and Services (Germantown, NY) for use in the 2-year studies. Rats and mice were quarantined for 11 (males) or 12 (females) days before the beginning of the studies. Five male and five female rats and mice were randomly selected for parasite evaluation and gross observation of disease. Rats were 5 to 6 weeks old and mice were 6 to 7 weeks old at the

beginning of the studies. The health of the animals was monitored during the studies according to the protocols of the NTP Sentinel Animal Program (Appendix L).

Animal Maintenance

Rats were housed three (males) or five (females) per cage; male mice were housed individually and female mice were housed five per cage. Feed and water were available *ad libitum*. Cages and racks were rotated every 2 weeks. Further details of animal maintenance are given in Table 1. Information on feed composition and contaminants is provided in Appendix K.

All animals were observed twice daily. Clinical findings were recorded every 4 weeks; body weights were recorded at the beginning of the studies, every 4 weeks, and at the end of the studies.

Toxicokinetics

Blood samples were collected from the retroorbital sinus of toxicokinetic study rats at 6, 12, and 18 (except 300 mg/kg) months and from toxicokinetic study mice at 12 months. Blood was collected at 7 to 10 post-dosing time points; blood was collected from two or three animals per group at each time point, and blood was collected from each animal at two time points. The time points at which blood was collected from each group are listed in Table 1. At 18 months, 12 to 15 previously undosed male and female rats and mice were given a single dose of 75 mg/kg (mice) or 150 mg/kg (rats) for toxicokinetic studies in aged animals. Blood was collected from three or four rats per group at 9 or 10 time points and from two or three mice per group at five time points. Blood was collected from each rat at two time points, and from each mouse once. The time points at which blood was collected from each group are listed in Table 1. Blood was collected via the retroorbital sinus (rats) or cardiac puncture (mice) into tubes containing EDTA as an anticoagulant. The red cell fraction was separated from the plasma by centrifugation, and the plasma was stored at -20° C until analysis for methyleugenol concentration.

Pathology

A complete necropsy and microscopic examination were performed on all core study rats and mice. At necropsy, all organs and tissues were examined for grossly visible lesions, and all major tissues were fixed and preserved in 10% neutral buffered formalin, processed and trimmed, embedded in paraffin, sectioned to a thickness of 5 to 6 μm, and stained with hematoxylin and eosin for microscopic examination. For all paired organs (e.g., adrenal gland, kidney, ovary), samples from each organ were examined. Neoplasm samples were collected and frozen for oncogene analysis. Tissues examined microscopically are listed in Table 1.

Microscopic evaluations were completed by the study laboratory pathologist, and the pathology data were entered into the Toxicology Data Management System. The slides, paraffin blocks, and residual wet tissues were sent to the NTP Archives for inventory, slide/block match, and wet tissue audit. The slides, individual animal data records, and pathology tables were evaluated by an independent quality assessment laboratory. The individual animal records and tables were compared for accuracy, the slide and tissue counts were verified, and the histotechnique was evaluated. For the 2-year studies, a quality assessment pathologist evaluated slides from all tumors and all primary lesions in potential target organs, which included the adrenal medulla, bone marrow, glandular stomach, kidney, liver, mammary gland, salivary gland, skin, and spleen of male and female rats; the epididymis and testis of male rats; the uterus of female rats; the bone marrow, glandular stomach, liver, and spleen of male and female mice; and the lung of male mice.

The quality assessment report and the reviewed slides were submitted to the NTP Pathology Working Group (PWG) chairperson, who reviewed the selected tissues and addressed any inconsistencies in the diagnoses made by the laboratory and quality assessment pathologists. Representative histopathology slides containing examples of lesions related to chemical administration, examples of disagreements in diagnoses between the laboratory and quality assessment pathologists, or lesions of general interest were presented by the chairperson to the PWG for review. The PWG consisted of the quality assessment

pathologist and other pathologists experienced in rodent toxicologic pathology. This group examined the tissues without any knowledge of dose groups or previously rendered diagnoses. When the PWG consensus differed from the opinion of the laboratory pathologist, the diagnosis was changed. Final diagnoses for reviewed lesions represent a consensus between the laboratory pathologist, reviewing pathologist(s), and the PWG. Details of these review procedures have been described, in part, by Maronpot and Boorman (1982) and Boorman *et al*. (1985). For subsequent analyses of the pathology data, the decision of whether to evaluate the diagnosed lesions for each tissue type separately or combined was generally based on the guidelines of McConnell *et al*. (1986).

TABLE 1
Experimental Design and Materials and Methods in the Gavage Studies of Methyleugenol

14-Week Studies	2-Year Studies
Study Laboratory Southern Research Institute (Birmingham, AL)	Battelle Columbus Laboratories (Columbus, OH)
Strain and Species Rats: F344/N Mice: B6C3F$_1$	Rats: F344/N Mice: B6C3F$_1$
Animal Source Simonsen Laboratories, Inc. (Gilroy, CA)	Taconic Laboratory Animals and Services (Germantown, NY)
Time Held Before Studies 11 days (rats and female mice) or 10 days (male mice)	11 days (males) or 12 days (females)
Average Age When Studies Began 6 weeks (rats and female mice) or 7 weeks (male mice)	Rats: 5 to 6 weeks Mice: 6 to 7 weeks
Date of First Dose Rats: 27 June 1988 Mice: 11 July 1988	Rats: 7 (males) or 8 (females) February 1994 Mice: 25 (males) or 26 (females) October 1993
Duration of Dosing 14 weeks (5 days per week)	Rats: 105 weeks (5 days per week), except 300 mg/kg group, which received only the 0.5% methylcellulose vehicle from week 53 to study completion Mice: 105 weeks (5 days per week)
Date of Last Dose Rats: 26-29 September 1988 Mice: 10-12 October 1988	Stop-exposure rats: 2 (males) or 3 (females) February 1995 Core study animals: Rats: 5 (males) or 6 and 7 (females) February 1996 Mice: 23-25 (males) or 25-26 (females) October 1995
Necropsy Dates Rats: 27-30 September 1988 Mice: 11-13 October 1988	Rats: 6 (males) or 7 and 8 (females) February 1996 (core study) 9 (males) or 10 (females) August 1994 (6-month interim evaluation) 2 (males) or 3 (females) February 1995 (12-month interim evaluation) Mice: 24-26 (males) or 26-27 (females) October 1995
Average Age at Necropsy 20 weeks (rats and female mice) or 21 weeks (male mice)	6-month interim evaluation: 33 weeks 12-month interim evaluation: 58 weeks Terminal sacrifice: Rats: 111 weeks Mice: 110 to 112 weeks
Size of Study Groups Rats: 9 or 10 males and 10 females Mice: 10 males and 10 females	Rats: 60 males and 60 females (vehicle control and 300 mg/kg) 50 males and 50 females (37, 75, and 150 mg/kg) Mice: 50 males and 50 females
Method of Distribution Animals were distributed randomly into groups of approximately equal initial mean body weights.	Same as 14-week studies

TABLE 1
Experimental Design and Materials and Methods in the Gavage Studies of Methyleugenol

14-Week Studies	2-Year Studies
Animals per Cage Rats: 5 Mice: 1	Rats: 3 (males) or 5 (females) Mice: 1 (males) or 5 (females)
Method of Animal Identification Rats: Tail tattoo Mice: Toe clip	Tail tattoo
Diet NIH-07 open formula pelleted diet (Zeigler Brothers, Inc., Gardners, PA), available *ad libitum*, changed weekly	Same as 14-week studies
Water Tap water (Birmingham municipal supply) via automatic watering system (Edstrom Industries, Inc., Waterford, WI), available *ad libitum*	Tap water (Columbus municipal supply) via automatic watering system (Edstrom Industries, Inc., Waterford, WI), available *ad libitum*
Cages Polycarbonate (Lab Products, Inc., Maywood, NJ), changed twice weekly (rats) or once weekly (mice)	Same as 14-week studies, except changed twice weekly (rats and female mice) or once weekly (male mice)
Bedding Sani-Chips® (P.J. Murphy Forest Products Corp., Montville, NJ), changed twice weekly (rats) or once weekly (mice)	Same as 14-week studies, except changed twice weekly (rats and female mice) or once weekly (male mice)
Racks Stainless steel (Lab Products Inc., Maywood, NJ), changed and rotated every 2 weeks	Same as 14-week studies
Animal Room Environment Temperature: 72° ± 3° F Relative humidity: 50% ± 15% Room fluorescent light: 12 hours/day Room air changes: minimum of 10/hour	Temperature: 72° ± 3° F Relative humidity: 50% ± 15% Room fluorescent light: 12 hours/day Room air changes: minimum of 10/hour
Dose Concentrations 0, 10, 30, 100, 300, or 1,000 mg/kg body weight in 0.5% methylcellulose at a volume of 5 (rats) or 10 (mice) mL/kg body weight	Core study: Rats: 0, 37, 75, 150, or 300 mg/kg (Dosing volume=5 mL/kg body weight) Mice: 0, 37, 75, or 150 mg/kg (Dosing volume=10 mL/kg body weight)
Type and Frequency of Observation Observed twice daily; clinical findings were recorded and animals were weighed weekly and at the end of the studies.	Observed twice daily; animals were weighed at the beginning of the studies, every 4 weeks, and at the end of the studies. Clinical findings were recorded every 4 weeks and at the end of the studies.
Method of Sacrifice Carbon dioxide asphyxiation	Carbon dioxide asphyxiation

TABLE 1
Experimental Design and Materials and Methods in the Gavage Studies of Methyleugenol

14-Week Studies	2-Year Studies
Necropsy Necropsy was performed on all core study animals. Organs weighed were the heart, right kidney, liver, lung, spleen, right testis, and thymus.	Necropsy was performed on all animals.
Clinical Pathology Blood was collected from the retroorbital sinus of special study rats on day 5 and at week 4 and from all core study rats surviving to the end of the studies for hematology and clinical chemistry determinations. ***Hematology:*** hematocrit; hemoglobin concentration; erythrocyte and reticulocyte counts; mean cell volume; mean cell hemoglobin; mean cell hemoglobin concentration; platelet count and morphology; leukocyte count and differentials ***Clinical chemistry:*** urea nitrogen, creatinine, total protein, albumin, alanine aminotransferase, alkaline phosphatase, creatine kinase, sorbitol dehydrogenase, and total bile acids	None
Histopathology Complete histopathology was performed on all core study water control, vehicle control, and 1,000 mg/kg rats and on all water control, vehicle control, and 300 and 1,000 mg/kg mice. In addition to gross lesions and tissue masses, the following tissues were examined: adrenal gland, bone with marrow, brain, clitoral gland, esophagus, gallbladder (mice only), heart, large intestine (cecum, colon, rectum), small intestine (duodenum, jejunum, ileum), kidney, liver, lung, lymph nodes (mandibular and mesenteric), mammary gland (with adjacent skin), nose, ovary, pancreas, parathyroid gland, pituitary gland, preputial gland, prostate gland, salivary gland, spleen, stomach (forestomach and glandular), testis (with epididymis and seminal vesicle), thymus, thyroid gland, trachea, urinary bladder, and uterus. In addition, the kidney, liver, salivary gland, and stomach of male and female rats and the adrenal gland and spleen of male rats in the 10, 30, 100, and 300 mg/kg groups, the testis and uterus of rats in the 100 and 300 mg/kg groups, the stomach of male and female mice in the 10, 30, and 100 mg/kg groups, the liver of 100 mg/kg male mice and 10, 30, and 100 mg/kg female mice, and the nose of 100 mg/kg male and female mice were examined.	Complete histopathology was performed on all rats and mice. In addition to gross lesions and tissue masses, the following tissues were examined: adrenal gland, bone with marrow, brain, clitoral gland, esophagus, gallbladder (mice only), heart, large intestine (cecum, colon, rectum), small intestine (duodenum, jejunum, ileum), kidney, liver, lung, lymph nodes (mandibular and mesenteric), mammary gland (with adjacent skin) (except male mice), nose, ovary, pancreas, parathyroid gland, pituitary gland, preputial gland, prostate gland, salivary gland, spleen, stomach (forestomach and glandular), testis (with epididymis and seminal vesicle), thymus, thyroid gland, trachea, urinary bladder, and uterus.
Sperm Motility and Vaginal Cytology At the end of the studies, sperm samples were collected from vehicle control, 30, 100, and 300 mg/kg male rats and vehicle control, 10, 30, and 100 mg/kg male mice for sperm motility evaluations. The following parameters were evaluated: spermatid heads per testis and per gram testis, spermatid counts, and epididymal spermatozoal motility and concentration. The left cauda epididymis, left epididymis, and left testis were weighed. Vaginal samples were collected for up to 12 consecutive days prior to the end of the studies from vehicle control, 30, 100, and 300 mg/kg female rats and vehicle control, 10, 30, and 100 mg/kg female mice for vaginal cytology evaluations. The following parameters were evaluated: the estrous cycle lengths and relative frequency of estrous stages.	None

TABLE 1
Experimental Design and Materials and Methods in the Gavage Studies of Methyleugenol

14-Week Studies	2-Year Studies
Toxicokinetic Studies	
None	Blood was collected from the retroorbital sinus of 10 male and 10 female rats given 37, 75, 150, or 300 mg/kg and 10 male and 10 female mice given 37, 75, or 150 mg/kg for toxicokinetic studies. Blood was collected from rats at 6, 12, and 18 months and from mice at 12 months. Blood was collected at the following time points after dosing: *Rats* 6 and 12 months 37 mg/kg: 5, 15, 30, 60, 90, and/or 120 minutes 75 mg/kg: 5, 30, 90, 120, 240, 360, and/or 450 minutes 150 mg/kg: 5, 30, 90, 240, 360, 480, and/or 600 minutes 300 mg/kg: 5, 60, 120, 240, 360, 540, and/or 780 minutes 18 months Male 37 mg/kg: 5, 15, 30, 60, 90, 120, and 240 minutes 75 mg/kg: 5, 15, 30, 60, 90, 120, 240, and 360 minutes 150 mg/kg: 5, 30, 90, 240, 360, 480, and 600 minutes Female 37 mg/kg: 5, 10, 15, 30, 45, 60, 90, 120, 180, and 240 minutes 75 mg/kg: 5, 15, 30, 60, 90, 120, 240, and 360 minutes 150 mg/kg: 5, 30, 60, 90, 120, 240, 360, 480, and 600 minutes *Mice* Male 37 mg/kg: 5, 10, 20, 30, 40, 60, and 75 minutes 75 mg/kg: 5, 15, 30, 45, 60, 90, and 150 minutes 150 mg/kg: 5, 15, 40, 75, 120, 210, and 300 minutes Female 37 mg/kg: 5, 10, 20, 30, 40, 50, and 60 minutes 75 mg/kg: 5, 15, 30, 45, 60, 90, and 150 minutes 150 mg/kg: 5, 15, 40, 120, 180, and 240 minutes ***Single-Dose Toxicokinetics in Aged Animals*** Blood was collected from the retroorbital sinus of 14 male and 15 female rats and by cardiac puncture from 12 male and 14 female mice after a single gavage dose of 150 mg/kg (rats) or 75 mg/kg (mice) for determination of methyleugenol concentration in plasma, observed maximum plasma concentration (C_{max}), observed time to achieve maximum plasma concentration (T_{max}), and area under the curve plasma concentration time profile (AUC). Blood was collected at the following time points: Male rats: 5, 15, 30, 60, 120, 240, 360, 480, and 600 minutes Female rats: 5, 10, 15, 30, 45, 60, 120, 240, 360, and 480 minutes Mice: 5, 20, 50, 90, and 150 minutes

STATISTICAL METHODS

Survival Analyses

The probability of survival was estimated by the product-limit procedure of Kaplan and Meier (1958) and is presented in the form of graphs. Animals found dead of other than natural causes or missing were censored from the survival analyses; animals dying from natural causes were not censored. Statistical analyses for possible dose-related effects on survival used Cox's (1972) method for testing two groups for equality and Tarone's (1975) life table test to identify dose-related trends. All reported P values for the survival analyses are two sided.

Calculation of Incidence

The incidences of neoplasms or nonneoplastic lesions are presented in Tables A1, A5, B1, B5, C1, C5, D1, and D5 as the numbers of animals bearing such lesions at a specific anatomic site and the numbers of animals with that site examined microscopically. For calculation of statistical significance, the incidences of most neoplasms (Tables A3, B3, C3, and D3) and all nonneoplastic lesions are given as the numbers of animals affected at each site examined microscopically. However, when macroscopic examination was required to detect neoplasms in certain tissues (e.g., harderian gland, intestine, mammary gland, and skin) before microscopic evaluation, or when neoplasms had multiple potential sites of occurrence (e.g., leukemia or lymphoma), the denominators consist of the number of animals on which a necropsy was performed. Tables A3, B3, C3, and D3 also give the survival-adjusted neoplasm rate for each group and each site-specific neoplasm. This survival-adjusted rate (based on the Poly-3 method described below) accounts for differential mortality by assigning a reduced risk of neoplasm, proportional to the third power of the fraction of time on study, to animals that do not reach terminal sacrifice.

Analysis of Neoplasm and Nonneoplastic Lesion Incidences

The Poly-k test (Bailer and Portier, 1988; Portier and Bailer, 1989; Piegorsch and Bailer, 1997) was used to assess neoplasm and nonneoplastic lesion prevalence. This test is a survival-adjusted quantal-response procedure that modifies the Cochran-Armitage linear trend test to take survival differences into account. More specifically, this method modifies the denominator in the quantal estimate of lesion incidence to approximate more closely the total number of animal years at risk. For analysis of a given site, each animal is assigned a risk weight. This value is one if the animal had a lesion at that site or if it survived until terminal sacrifice; if the animal died prior to terminal sacrifice and did not have a lesion at that site, its risk weight is the fraction of the entire study time that it survived, raised to the kth power.

This method yields a lesion prevalence rate that depends only upon the choice of a shape parameter for a Weibull hazard function describing cumulative lesion incidence over time (Bailer and Portier, 1988). Unless otherwise specified, a value of $k=3$ was used in the analysis of site-specific lesions. This value was recommended by Bailer and Portier (1988) following an evaluation of neoplasm onset time distributions for a variety of site-specific neoplasms in control F344 rats and $B6C3F_1$ mice (Portier *et al.*, 1986). Bailer and Portier (1988) showed that the Poly-3 test gave valid results if the true value of k was anywhere in the range from 1 to 5. A further advantage of the Poly-3 method is that it does not require lesion lethality assumptions. Variation introduced by the use of risk weights, which reflect differential mortality, was accommodated by adjusting the variance of the Poly-3 statistic as recommended by Bieler and Williams (1993).

Tests of significance included pairwise comparisons of each dosed group with controls and a test for an overall dose-related trend. Continuity-corrected tests were used in the analysis of lesion incidence, and reported P values are one sided. Values of P greater than 0.5 are presented as 1-P with the letter N added to indicate a lower incidence or negative trend in neoplasm occurrence relative to the control group (e.g., $P=0.99$ is presented as $P=0.01N$). For neoplasms and nonneoplastic lesions detected at the interim evaluation, the Fisher exact test (Gart *et al.*, 1979), a procedure based on the overall proportion of affected animals, was used.

Analysis of Continuous Variables

Two approaches were employed to assess the significance of pairwise comparisons between dosed and control groups in the analysis of continuous variables. Organ and body weight data, which have approximately normal distributions, were analyzed with the

parametric multiple comparison procedures of Dunnett (1955) and Williams (1971, 1972). Hematology, clinical chemistry, plasma concentration, spermatid, and epididymal spermatozoal data, which have typically skewed distributions, were analyzed using the nonparametric multiple comparison methods of Shirley (1977) and Dunn (1964). Jonckheere's test (Jonckheere, 1954) was used to assess the significance of the dose-related trends and to determine whether a trend-sensitive test (Williams' or Shirley's test) was more appropriate for pairwise comparisons than a test that does not assume a monotonic dose-related trend (Dunnett's or Dunn's test). Prior to statistical analysis, extreme values identified by the outlier test of Dixon and Massey (1951) were examined by NTP personnel, and implausible values were eliminated from the analysis. Average severity values were analyzed for significance with the Mann-Whitney U test (Hollander and Wolfe, 1973). Because vaginal cytology data are proportions (the proportion of the observation period that an animal was in a given estrous stage), an arcsine transformation was used to bring the data into closer conformance with a normality assumption. Treatment effects were investigated by applying a multivariate analysis of variance (Morrison, 1976) to the transformed data to test for simultaneous equality of measurements across doses.

Historical Control Data

Although the concurrent control group is always the first and most appropriate control group used for evaluation, historical control data can be helpful in the overall assessment of neoplasm incidence in certain instances. Consequently, neoplasm incidences from the NTP historical control database, which is updated yearly, are included in the NTP reports for neoplasms appearing to show compound-related effects.

QUALITY ASSURANCE METHODS

The 14-week and 2-year studies were conducted in compliance with Food and Drug Administration Good Laboratory Practice Regulations (21 CFR, Part 58). In addition, as records from the 2-year studies were submitted to the NTP Archives, these studies were audited retrospectively by an independent quality assurance contractor. Separate audits covered completeness and accuracy of the pathology data, pathology specimens, final pathology tables, and a draft of this NTP Technical Report. Audit procedures and findings are presented in the reports and are on file at NIEHS. The audit findings were reviewed and assessed by NTP staff, and all comments were resolved or otherwise addressed during the preparation of this Technical Report.

GENETIC TOXICOLOGY

The genetic toxicity of methyleugenol was assessed by testing the ability of the chemical to induce mutations in various strains of *Salmonella typhimurium*, sister chromatid exchanges and chromosomal aberrations in cultured Chinese hamster ovary cells, and increases in the frequency of micronucleated erythrocytes in peripheral blood of mice. The protocols for these studies and the results are given in Appendix E.

The genetic toxicity studies of methyleugenol are part of a larger effort by the NTP to develop a comprehensive database that would permit a critical anticipation of a chemical's carcinogenicity in experimental animals based on numerous considerations, including the molecular structure of the chemical and its observed effects in short-term *in vitro* and *in vivo* genetic toxicity tests (structure-activity relationships). These short-term genetic toxicity tests were originally developed to elucidate mechanisms of chemical-induced DNA damage growing out of the earlier electrophilicity/mutagenicity relationship proposed by Miller and Miller (1977) and the somatic mutation theory of cancer (Straus, 1981; Crawford, 1985). Therefore, the information obtained from these tests applies only to mutagenic carcinogens.

For mutagenic carcinogens, there is a strong correlation between a chemical's potential for DNA reactivity, mutagenicity in *Salmonella*, and carcinogenicity in rodents. The combination of DNA reactivity and *Salmonella* mutagenicity is highly correlated with the induction of carcinogenicity in multiple species and genders of rodents and at multiple tissue sites (Ashby and Tennant, 1991). Data from NTP studies show that a positive response in *Salmonella* is the most predictive *in vitro* test for rodent carcinogenicity (89% of the *Salmonella* mutagens are rodent carcinogens) and that there is no complementarity among the *in vitro* genetic toxicity tests (Tennant *et al.*, 1987; Zeiger

et al., 1990). That is, no battery of tests that included the *Salmonella* test improved the predictivity of the *Salmonella* test alone. Although other *in vitro* genetic toxicity tests correlate less well with rodent carcinogenicity compared with the *Salmonella* test, these other tests can provide useful information on the types of DNA and chromosomal effects induced by the chemical under investigation.

The predictivity for carcinogenicity of a positive response in bone marrow chromosome aberration or micronucleus tests appears to be less than the *Salmonella* test (Shelby *et al.*, 1993; Shelby and Witt, 1995). Positive responses in long-term peripheral blood micronucleus tests have not been formally evaluated for their predictivity for rodent carcinogenicity. However, because of the theoretical and observed associations between induced genetic damage and adverse effects in somatic and germ cells, the determination of *in vivo* genetic effects is important to the overall understanding of the risks associated with exposure to a particular chemical.

RESULTS

RATS

14-Week Study

All rats survived until the end of the study (Table 2). Final mean body weights and body weight gains of 300 and 1,000 mg/kg males and all dosed groups of females were significantly less than those of the vehicle controls; final mean body weights of 1,000 mg/kg males and females were 30% and 16% less than the vehicle controls, respectively (Table 2). Clinical findings possibly related to chemical administration included emaciation and urine staining in 100, 300, and 1,000 mg/kg female rats.

Data for the hematology and clinical chemistry variables are listed in Table F1. At week 14, there was a minimal treatment-related decrease in hemoglobin concentrations and hematocrit values in the 300 mg/kg females and 1,000 mg/kg males and females. The hematocrit and hemoglobin decreases would be consistent with an anemia, but there was no corresponding decrease in erythrocyte counts. There was, however, an erythrocyte microcytosis demonstrated by decreased mean cell volumes in the 300 mg/kg males and 1,000 mg/kg males and females; this change also occurred on day 5 and/or week 4. Animals in the 300 and 1,000 mg/kg groups also had decreased mean cell hemoglobin values; this would be consistent with the decreases in mean cell volumes. This suggests that, while there were equal numbers of circulating erythrocytes for animals in the 300 and 1,000 mg/kg groups at week 14, the erythrocytes were smaller, resulting in lower hematocrit and hemoglobin values. There was evidence of a thrombocytosis at all time points, demonstrated by increased platelet counts in the 100 mg/kg or greater groups. The serum activities of alanine aminotransferase and sorbitol dehydrogenase were increased in the 100 mg/kg or greater male and female groups at various time points and would be consistent with hepatocellular injury or leakage. Additionally, bile acid concentrations were increased in the 300 and 1,000 mg/kg males at all time points and 300 and 1,000 mg/kg females at weeks 4 and 14 and would be consistent with cholestasis or altered hepatic function. However, serum alkaline phosphatase activity, another marker of cholestasis, was either unaffected or decreased in the same animals. A hypoproteinemia and hypoalbuminemia, evidenced by decreased total protein and albumin concentrations, occurred in rats in the 300 and 1,000 mg/kg groups at all time points. There were minimal increases of creatinine concentrations in the 300 and 1,000 mg/kg female rats at all time points. However, urea nitrogen, another marker of renal function, was either unaffected or decreased in the same animals.

TABLE 2
Survival and Body Weights of Rats in the 14-Week Gavage Study of Methyleugenol

Dose (mg/kg)	Survival[a]	Mean Body Weight[b] (g)			Final Weight Relative to Vehicle Controls (%)
		Initial	Final	Change	
Male					
Water Control	10/10	114 ± 4	327 ± 6	214 ± 4	99
Vehicle Control	10/10	118 ± 4	331 ± 7	214 ± 5	
10	10/10	114 ± 4	331 ± 5	218 ± 5	100
30	10/10	117 ± 5	332 ± 8	216 ± 4	100
100	10/10	119 ± 4	322 ± 7	203 ± 4	97
300	9/9	116 ± 5	304 ± 4**	188 ± 4**	92
1,000	10/10	112 ± 4	231 ± 4**	119 ± 5**	70
Female					
Water Control	10/10	96 ± 2	192 ± 3	96 ± 3	98
Vehicle Control	10/10	97 ± 2	196 ± 4	99 ± 5	
10	10/10	94 ± 2	184 ± 1*	89 ± 2*	93
30	10/10	98 ± 3	187 ± 4*	89 ± 3*	95
100	10/10	97 ± 2	183 ± 2**	86 ± 2**	93
300	10/10	97 ± 3	180 ± 2**	83 ± 3**	92
1,000	10/10	95 ± 3	164 ± 4**	69 ± 3**	84

* Significantly different ($P \leq 0.05$) from the vehicle control group by Williams' or Dunnett's test
** $P \leq 0.01$
[a] Number of animals surviving at 14 weeks/number initially in group
[b] Weights and weight changes are given as mean ± standard error.

Liver weights of 100, 300, and 1,000 mg/kg males and 300 and 1,000 mg/kg females and testis weights of 1,000 mg/kg males were significantly greater than those of the vehicle control groups (Table G1). Thymus weights in all dosed males and in 1,000 mg/kg females were significantly less than those of the vehicle control groups. The decreases in thymus weights and differences in other organ weights were likely secondary to body weight differences.

No significant differences in sperm motility or in vaginal cytology parameters between dosed and vehicle control groups were observed (Tables H1 and H2).

Chemical-related lesions were observed grossly in the liver of male and female rats, the testis of male rats, and the uterus of female rats administered 1,000 mg/kg. Liver foci were observed in 8 of 10 males and in 1 of 10 females. The testes were enlarged in all dosed male rats; female rats had small (3/10) or thin (3/10) uteri.

No significant differences were observed between the vehicle and water control groups. Compared to the vehicle control groups, there were significant chemical-related increases in the incidences of microscopic lesions in the liver, glandular stomach, adrenal cortex, submandibular salivary gland, testis, and uterus in dosed rats (Table 3). Incidences of hepatic lesions were significantly increased in rats administered 300 or 1,000 mg/kg, and the lesions were generally more severe in males than in females. Hepatic lesions with increased incidences included cytologic alteration, cytomegaly, Kupffer cell pigmentation, bile duct hyperplasia, and foci of cellular alteration. Cytologic alteration was the term used to describe individual hepatocytes with altered tinctorial staining qualities (eosinophilic or basophilic) and increased mitotic activity and, in general, was of minimal to moderate severity. Cytomegaly was characterized by minimal to moderate enlargement of periportal hepatocytes. Kupffer cell pigmentation was a minimal change characterized by the accumulation of yellow-gold to green pigment within the cytoplasm of periportal hepatocytes. Bile duct hyperplasia consisted of minimal to moderate proliferation of small bile ductules within portal areas. Foci of cellular alteration consisted of discrete foci of hepatocytes with altered cytoplasmic staining (eosinophilic, basophilic, or mixed) and were morphologically similar to those that occur spontaneously or with chemical exposure. One hepatocellular adenoma was present in a male rat administered 1,000 mg/kg.

The incidences of atrophy and chronic inflammation of the mucosa of the glandular stomach were significantly increased in rats administered 300 or 1,000 mg/kg. Lesions were generally of minimal to mild severity in the 300 mg/kg groups and mild to moderate in the 1,000 mg/kg groups. Atrophy consisted of a decrease in the thickness of the gastric mucosa due to generalized loss of glandular epithelial parietal and chief cells accompanied by condensation of the lamina propria. Inflammation was of mild severity and consisted of fibrosis and a diffuse infiltration of the lamina propria by lymphocytes, neutrophils, and macrophages. In addition, there was mild glandular dilatation and increased mitotic activity in the glandular epithelial cells.

The incidences of cortical hypertrophy of the adrenal cortex were significantly increased in 100, 300, and 1,000 mg/kg males and 1,000 mg/kg females. The incidences of cytoplasmic alteration of the submandibular salivary glands were increased in rats administered 30 mg/kg or greater.

Cytoplasmic alteration of the submandibular salivary gland consisted of a loss of cytoplasmic zymogen granules with reduction in the size of serous cells and their ducts. Male rats administered 1,000 mg/kg had significantly increased incidences of moderate dilatation of the seminiferous tubules and testicular degeneration characterized by diffuse loss of spermatogenic cells within the seminiferous tubules. Spermatogonia remaining within the seminiferous and epididymal tubules were morphologically normal. The incidences of mild uterine atrophy were significantly increased in female rats administered 300 or 1,000 mg/kg.

Dose Selection Rationale: Based on lower mean body weights and increased incidences of nonneoplastic liver and glandular stomach lesions in 300 and 1,000 mg/kg rats in the 14-week study, methyleugenol doses selected for rats in the 2-year gavage study were 37, 75, and 150 mg/kg. Stop-exposure groups were given 300 mg/kg for 12 months to provide additional data on progression and regression of liver lesions.

TABLE 3
Incidences of Selected Neoplasms and Nonneoplastic Lesions in Male and Female Rats in the 14-Week Gavage Study of Methyleugenol

	Water Control	Vehicle Control	10 mg/kg	30 mg/kg	100 mg/kg	300 mg/kg	1,000 mg/kg
Male							
Liver[a]	10	10	10	10	10	9	10
Cytologic Alteration[b]	0	0	0	0	0	9** (1.2)[c]	10** (3.0)
Cytomegaly	0	0	0	0	0	9** (1.7)	10** (2.8)
Pigment, Kupffer Cell	0	0	0	0	0	0	10** (1.0)
Basophilic Focus	0	0	0	0	0	0	3 (2.3)
Mixed Cell Focus	0	0	0	0	0	2 (1.5)	9** (2.2)
Hyperplasia, Bile Duct	0	0	0	0	0	6** (1.0)	10** (2.1)
Hepatocellular Adenoma	0	0	0	0	0	0	1
Glandular Stomach	10	10	10	10	10	9	10
Atrophy	0	0	0	0	0	7** (1.7)	10** (3.0)
Inflammation, Chronic	0	0	0	0	0	9** (1.2)	10** (2.6)
Adrenal Cortex	10	10	10	10	10	9	10
Hypertrophy	0	0	0	0	4* (1.0)	9** (1.0)	10** (1.0)
Submandibular Salivary Gland	10	10	10	10	10	9	10
Cytoplasmic Alteration	0	0	0	3 (1.0)	10** (1.0)	9** (1.0)	10** (1.0)
Testis	10	10	10	10	10	9	10
Dilatation	0	0	0	0	0	0	10** (2.7)
Degeneration	0	0	0	0	0	0	10** (2.8)
Female							
Liver	10	10	10	10	10	10	10
Cytologic Alteration	0	0	0	0	0	0	10** (2.3)
Cytomegaly	0	0	0	0	0	9** (1.1)	10** (2.2)
Pigment, Kupffer Cell	0	0	0	0	0	0	9** (1.0)
Mixed Cell Focus	0	0	0	0	0	2 (1.0)	8** (1.9)
Hyperplasia, Bile Duct	0	0	0	0	0	0	9** (1.2)
Glandular Stomach	10	10	10	10	10	10	10
Atrophy	0	0	0	0	0	9** (1.0)	10** (2.9)
Inflammation, Chronic	0	0	0	0	6** (1.0)	10** (1.9)	10** (2.3)
Adrenal Cortex	10	10	10	10	10	10	10
Hypertrophy	0	2 (1.0)	0	0	0	0	9** (1.0)
Submandibular Salivary Gland	10	10	10	10	10	10	10
Cytoplasmic Alteration	0	0	0	7** (1.0)	10** (1.0)	10** (1.0)	10** (1.0)
Uterus	10	10	10	10	10	10	10
Atrophy	0	0	0	0	0	4* (2.5)	10** (2.0)

* Significantly different ($P \leq 0.05$) from the vehicle control group by the Fisher exact test
** $P \leq 0.01$
[a] Number of animals with tissue examined microscopically
[b] Number of animals with lesion
[c] Average severity grade of lesions in affected animals: 1=minimal, 2=mild, 3=moderate, 4=marked

2-Year Study

Survival

Estimates of survival probabilities for male and female rats are shown in Table 4 and in the Kaplan-Meier survival curves (Figure 1). All 150 and 300 mg/kg males died before the end of the study, and survival of 150 mg/kg females was slightly less than that of the vehicle controls.

Body Weights and Clinical Findings

Mean body weights of dosed males and females were less than the vehicle controls throughout most of the study (Figure 2 and Tables 5 and 6). There was little suggestion of recovery in mean body weights in the 300 mg/kg groups when dosing was discontinued for 12 months. There were no clinical findings that could be attributed to methyleugenol administration.

TABLE 4
Survival of Rats in the 2-Year Gavage Study of Methyleugenol

	Vehicle Control	37 mg/kg	75 mg/kg	150 mg/kg	300 mg/kg (Stop-Exposure)
Male					
Animals initially in study	60	50	50	50	60
6-Month interim evaluation[a]	5	0	0	0	5
12-Month interim evaluation[a]	5	0	0	0	5
Accidental deaths[a]	0	1	3	1	1
Moribund	15	18	15	20	23
Natural deaths	15	15	17	29	26
Animals surviving to study termination	20	16	15	0	0
Percent probability of survival at end of study[b]	40	33	32	0	0
Mean survival (days)[c]	678	659	646	615	538
Survival analysis[d]	P<0.001	P=0.389	P=0.294	P<0.001	P<0.001
Female					
Animals initially in study	60	50	50	50	60
6-Month interim evaluation	5	0	0	0	5
12-Month interim evaluation	5	0	0	0	5
Accidental deaths	0	0	1	1	0
Moribund	17	16	14	26	25
Natural deaths	11	9	13	12	9
Animals surviving to study termination	22	25	22	11	16
Percent probability of survival at end of study	44	50	45	23	32
Mean survival (days)	659	672	675	647	638
Survival analysis	P=0.015	P=0.640N	P=0.807N	P=0.053	P=0.343

[a] Censored from survival analyses
[b] Kaplan-Meier determinations
[c] Mean of all deaths (uncensored, censored, and terminal sacrifice)
[d] The result of the life table trend test (Tarone, 1975) is in the vehicle control column [the 300 mg/kg (stop-exposure) group was excluded from the trend test], and the results of the life table pairwise comparisons (Cox, 1972) with the vehicle controls are in the dosed group columns. A lower mortality in a dose group is indicated by **N**.

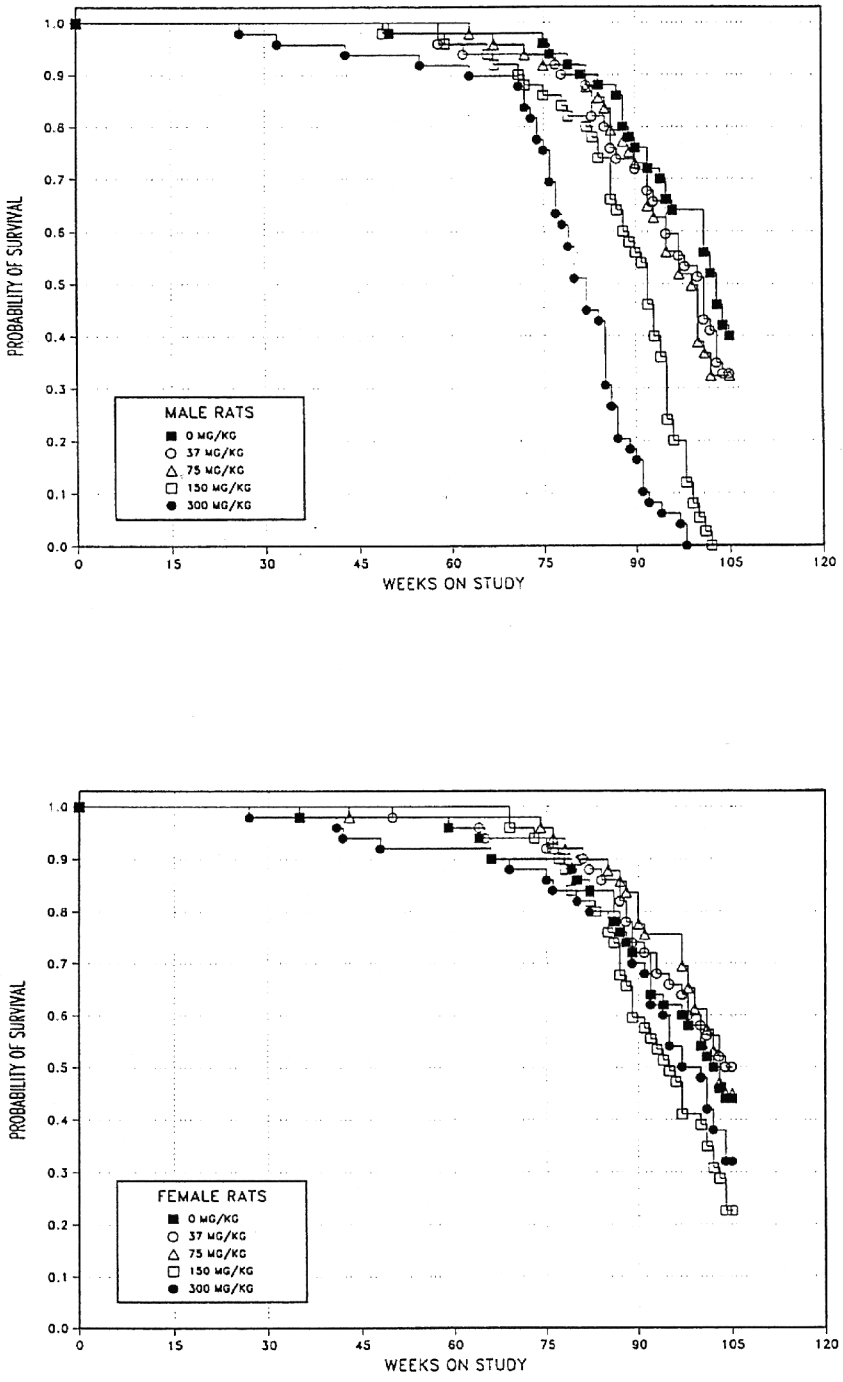


FIGURE 1
Kaplan-Meier Survival Curves for Male and Female Rats Administered Methyleugenol by Gavage for 2 Years

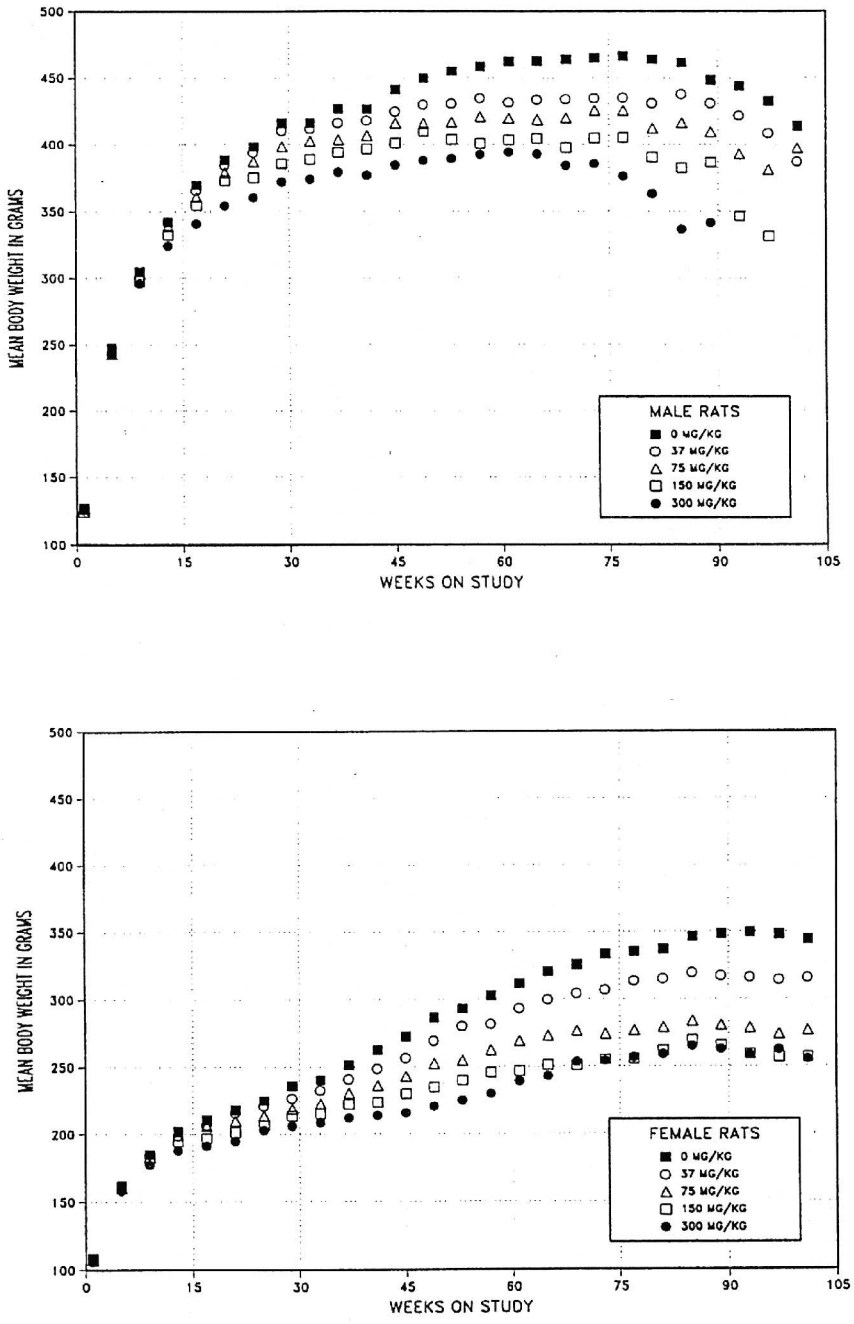


FIGURE 2
Growth Curves for Male and Female Rats
Administered Methyleugenol by Gavage for 2 Years

TABLE 5
Mean Body Weights and Survival of Male Rats in the 2-Year Gavage Study of Methyleugenol

Weeks on Study	Vehicle Control		37 mg/kg			75 mg/kg		
	Av. Wt. (g)	No. of Survivors	Av. Wt. (g)	Wt. (% of controls)	No. of Survivors	Av. Wt. (g)	Wt. (% of controls)	No. of Survivors
1	127	60	126	100	50	127	100	50
5	247	60	244	99	50	243	99	49
9	304	60	300	99	50	303	100	49
13	341	60	342	100	50	339	99	49
17	368	60	366	99	50	361	98	49
21	386	60	384	99	50	379	98	49
25	396	60	394	100	50	387	98	49
29	415	55[a]	410	99	50	398	96	49
33	414	55	412	99	50	402	97	49
37	426	55	416	98	50	403	95	49
41	427	55	418	98	50	407	95	49
45	441	55	425	96	50	416	94	49
49	449	55	430	96	50	416	93	49
53	455	49[a]	431	95	50	416	92	49
57	458	49	435	95	50	421	92	49
61	462	49	431	93	48	420	91	49
65	463	49	434	94	47	419	91	48
69	464	49	434	94	47	420	91	47
73	465	49	435	94	47	425	92	46
77	466	47	435	93	47	425	91	44
81	464	46	431	93	45	412	89	44
85	461	44	438	95	41	416	90	41
89	448	39	431	96	36	409	91	37
93	444	36	422	95	33	393	89	31
97	433	32	408	94	28	381	88	25
101	414	32	387	94	25	397	96	18
Mean for weeks								
1-13	255		253	99		253	99	
14-52	414		406	98		397	96	
53-101	454		427	94		412	91	

TABLE 5
Mean Body Weights and Survival of Male Rats in the 2-Year Gavage Study of Methyleugenol

Weeks on Study	150 mg/kg			300 mg/kg (Stop-Exposure)		
	Av. Wt. (g)	Wt. (% of controls)	No. of Survivors	Av. Wt. (g)	Wt. (% of controls)	No. of Survivors
1	124	98	50	126	100	60
5	245	100	50	243	99	59
9	298	98	50	297	98	59
13	332	97	50	325	95	59
17	355	96	50	341	93	59
21	373	97	50	355	92	59
25	375	95	50	360	91	59
29	385	93	50	371	90	53[a]
33	389	94	50	373	90	52
37	394	92	50	379	89	52
41	397	93	50	376	88	52
45	401	91	50	384	87	51
49	409	91	49	387	86	51
53	404	89	49	389	86	46[a]
57	401	87	49	392	86	45
61	403	87	48	394	85	45
65	404	87	48	393	85	44
69	398	86	46	384	83	44
73	405	87	44	386	83	41
77	405	87	43	376	81	33
81	390	84	41	363	79	25
85	382	83	37	337	73	19
89	386	86	30	341	76	10
93	346	78	23			
97	331	77	10			
101						
Mean for weeks						
1-13	250	98		248	98	
14-52	386	93		370	89	
53-101	388	85		376	82	

[a] Interim evaluations occurred during weeks 27 and 52 for the vehicle control and 300 mg/kg (stop-exposure) groups.

TABLE 6
Mean Body Weights and Survival of Female Rats in the 2-Year Gavage Study of Methyleugenol

Weeks on Study	Vehicle Control		37 mg/kg			75 mg/kg		
	Av. Wt. (g)	No. of Survivors	Av. Wt. (g)	Wt. (% of controls)	No. of Survivors	Av. Wt. (g)	Wt. (% of controls)	No. of Survivors
1	108	60	106	98	50	107	99	50
5	162	60	161	99	50	160	99	50
9	186	60	185	100	50	183	99	50
13	203	60	199	98	50	199	98	50
17	211	60	207	98	50	206	98	50
21	219	60	216	99	50	210	96	50
25	226	60	221	98	50	214	94	50
29	236	55[a]	226	96	50	219	93	50
33	241	55	233	97	50	223	92	50
37	252	54	241	95	50	231	91	50
41	264	54	249	94	50	236	90	50
45	274	54	257	94	50	243	89	49
49	288	54	269	94	50	252	88	49
53	293	49[a]	280	96	49	255	87	49
57	303	49	282	93	49	262	87	49
61	312	48	293	94	49	269	86	49
65	321	47	300	94	48	273	85	48
69	326	45	304	93	47	276	85	48
73	334	45	307	92	47	274	82	48
77	336	45	314	93	46	277	83	46
81	337	43	315	94	46	279	83	44
85	346	42	320	92	43	284	82	44
89	348	37	317	91	39	280	81	41
93	350	32	316	91	36	279	80	37
97	348	31	314	90	32	274	79	37
101	344	27	316	92	29	277	80	30
Mean for weeks								
1-13	165		163	99		162	99	
14-52	246		235	96		226	92	
53-101	331		306	93		274	83	

TABLE 6
Mean Body Weights and Survival of Female Rats in the 2-Year Gavage Study of Methyleugenol

Weeks on Study	150 mg/kg			300 mg/kg (Stop-Exposure)		
	Av. Wt. (g)	Wt. (% of controls)	No. of Survivors	Av. Wt. (g)	Wt. (% of controls)	No. of Survivors
1	107	99	50	107	99	60
5	161	99	50	158	97	60
9	183	98	50	178	96	60
13	195	96	50	189	93	60
17	197	93	50	192	91	60
21	201	92	50	195	89	60
25	206	91	50	203	90	60
29	213	90	50	205	87	54[a]
33	215	89	50	208	86	54
37	222	88	50	212	84	54
41	223	85	50	213	81	53
45	230	84	50	215	78	52
49	235	82	50	219	76	51
53	240	82	50	225	77	46[a]
57	246	81	50	230	76	46
61	247	79	50	239	77	46
65	251	78	50	243	76	46
69	251	77	50	254	78	44
73	255	77	48	255	76	44
77	256	76	46	257	77	42
81	262	78	42	259	77	41
85	269	78	39	265	77	40
89	266	76	32	263	76	37
93	259	74	27	259	74	31
97	257	74	23	263	76	27
101	257	75	19	256	74	24
Mean for weeks						
1-13	162	98		158	96	
14-52	216	88		207	85	
53-101	255	77		251	76	

[a] Interim evaluations occurred during weeks 27 and 52 for the vehicle control and 300 mg/kg (stop-exposure) groups.

Toxicokinetic Studies

Detailed methods and results for the single-administration intravenous and gavage studies in young rats are presented in Appendix M, and results for the core study animals that received methyleugenol by gavage 5 days per week for 6, 12, or 18 months and for the single-administration gavage studies in aged animals are presented in Appendix I. Additional chemical distribution and metabolism data are presented in an unpublished absorption, distribution, metabolism, and elimination study conducted for the NTP (Appendix N).

Absorption: Absorption from oral doses was rapid, with peak plasma levels achieved within the first 5 minutes for all doses in males and females.

Distribution: Methyleugenol and its metabolites were distributed preferentially to the liver 72 hours after gavage or intravenous administration of [^{14}C]-methyleugenol to males. Tissue:blood ratios of methyleugenol-derived radioactivity were 2 to 3 in the liver, 0.9 to 1.4 in the kidney, and significantly less than 1 in all other tissues examined after 72 hours.

Metabolism: Methyleugenol was rapidly metabolized. Approximately 85% of methyleugenol orally administered to males was eliminated in urine as metabolites by 72 hours after dosing. Bioavailability of methyleugenol was low in both males and females, with less than 6% bioavailability at 37 mg/kg. This increased to approximately 13% at 75 mg/kg and 15% to 20% at 150 mg/kg. These findings suggest a strong, but saturable, first-pass metabolic effect, leading to a nonlinear relationship between dose and parent chemical dosimetry. No parent methyleugenol was found in urine from males dosed with methyleugenol orally or by intravenous injection. Hydroxylated, sulfated, and glucuronidated metabolites constituted the majority of metabolites detected in urine.

Elimination: Approximately 85% of methyleugenol administered orally to males was eliminated in urine as parent or metabolites. Elimination of methyleugenol from the bloodstream was rapid and multiphasic, with initial half-lives on the order of 5 minutes and terminal half-lives on the order of 1 to 2 hours in males and females. No difference in the elimination of the parent compound between naive males and females was apparent with either young or aged animals. Male core study animals eliminated methyleugenol more rapidly at 6 and 12 months, with areas under the concentration versus time curve (AUCs) generally less than those for the naive animals. Females at all time points and males at 18 months had AUCs similar to those of naive animals. This suggests that metabolic induction may occur to a greater extent in males than in females. Plots of AUC versus dose were sublinear in males at 6 and 12 months, indicative of metabolic saturation at the higher doses at these time points, but approximately linear at 18 months. The increase in AUCs with age in the core study males and females is suggestive of an age-related decrease in methyleugenol metabolic capability.

Necropsy Observations

Gross lesions were observed in the liver and stomach of males and females. Focal areas of discoloration, nodules (raised lesions less than 5 mm in diameter), and masses (raised lesions greater than 5 mm in diameter) were observed in the livers of dosed rats. The incidences and multiplicity of focal areas of discoloration, nodules, and masses increased with increasing dose. Grossly, there was diffuse thickening of the entire glandular portion of the stomach with or without the presence of one or more nodules and masses.

Pathology and Statistical Analyses

This section describes the statistically significant or bi-ologically noteworthy changes in the incidences of malignant mesothelioma and neoplasms and/or non-neoplastic lesions of the liver, glandular stomach, forestomach, kidney, mammary gland, skin, bone marrow, salivary gland, adrenal medulla, and spleen. Summaries of the incidences of neoplasms and non-neoplastic lesions, individual animal tumor diagnoses, statistical analyses of primary neoplasms that occurred with an incidence of at least 5% in at least one animal group, and historical incidences for the neoplasms mentioned in this section are presented in Appendix A for male rats and Appendix B for female rats.

Liver: Chemical-related lesions included hepatocellular adenoma; hepatocellular carcinoma; hepatocholangioma; hepatocholangiocarcinoma; eosinophilic, basophilic, and mixed cell foci; oval cell and bile duct hyperplasia; cystic degeneration; and hepatocyte hypertrophy in core study and 300 mg/kg (stop-exposure) rats (Table 7 and Appendixes A and B).

In 300 mg/kg rats at the 12-month interim evaluation, four males had hepatocellular adenomas (including two with multiple hepatocellular adenomas), one male had a hepatocholangiocarcinoma, and one female had a hepatocellular carcinoma. At 2 years, one 150 mg/kg male had a hepatocholangioma, and one 75 mg/kg male, one 150 mg/kg male, and three 150 mg/kg females had hepatocholangiocarcinomas. The incidences of hepatocellular adenoma, hepatocellular carcinoma, hepatocholangioma, and hepatocholangiocarcinoma in the 300 mg/kg male and female groups were significantly greater than those in the vehicle controls. At 2 years, there were positive trends in the incidences of hepatocellular adenoma, carcinoma, and adenoma or carcinoma (combined) in core study rats, and the incidences of multiple hepatocellular carcinomas and/or adenomas were also increased in most dosed groups of males and females. The incidences of hepatocellular adenoma or carcinoma (combined) in all groups of males and females were equal to or exceeded the upper end of the historical range for corn oil vehicle controls (Tables 7, A4a, and B4a). No incidences of hepatocholangioma or hepatocholangiocarcinoma were listed in the historical control database. Additionally, a cholangioma was present in two males, and a cholangiocarcinoma was present in one female in the 300 mg/kg groups.

At 6 and 12 months, basophilic, eosinophilic, and mixed cell foci were observed in most 300 mg/kg rats (Tables 7, A1, and B1). At 2 years, the incidences of eosinophilic foci in all dosed groups of rats and of mixed cell foci in the 37, 75, and 150 mg/kg males and in 75 mg/kg females were significantly greater than those in the vehicle controls. All 300 mg/kg males and females had hepatocyte hypertrophy and oval cell hyperplasia at 6 and 12 months. In dosed groups at 2 years, the incidences of hepatocyte hypertrophy and oval cell hyperplasia (except 37 mg/kg males) were significantly increased. All 300 mg/kg males had cystic degeneration at 12 months, and the incidences in 75 mg/kg or greater males and 300 mg/kg females were significantly increased at 2 years. At 2 years, the incidences of bile duct hyperplasia were significantly increased in 150 and 300 mg/kg females; however, the incidences in all dosed groups of male rats were decreased. Focal atypical bile duct hyperplasia was present in males administered 75 mg/kg or greater.

Hepatocellular adenomas were single or multiple usually discrete nodular lesions which compressed the adjacent parenchyma, and in many instances, protruded from the liver surface. Adenomas were characterized by altered tinctorial staining characteristics and loss of normal hepatic lobular architecture with hepatocytes abruptly intersecting the adjacent parenchyma (Plate 1). They were composed of well-differentiated, eosinophilic, basophilic, or vacuolated hepatocytes or mixtures thereof. Minimal to mild hepatocyte pleomorphism and/or hypertrophy were evident. Many adenomas contained areas of cystic degeneration and/or vacuolation. Hepatocellular carcinomas were large well- to poorly demarcated invasive masses which frequently obliterated the hepatic architecture. Carcinomas were pleomorphic, organizing in trabecular, solid or less commonly adenoid growth patterns; multiple growth patterns occasionally occurred within the same neoplasm. The trabecular pattern was most common and was characterized by hepatic plates that were more than one cell layer thick, irregular, and composed of well- to poorly differentiated hepatocytes (Plate 2). Incidences of metastasis, most commonly to the lung, occurred with a positive trend in males (vehicle control, 0/50; 37 mg/kg, 1/50; 75 mg/kg, 2/50; 150 mg/kg, 10/50; 300 mg/kg, 22/50) and females (0/50, 0/50, 1/49, 4/49, 4/50) (Tables A1 and B1).

TABLE 7
Incidences of Neoplasms and Nonneoplastic Lesions of the Liver in Male and Female Rats in the 2-Year Gavage Study of Methyleugenol

	Vehicle Control	37 mg/kg	75 mg/kg	150 mg/kg	300 mg/kg (Stop-Exposure)
Male					
6-Month Interim Evaluation					
Number Examined Microscopically	5				5
Oval Cell, Hyperplasia[a]	0				5** (1.8)[b]
Hepatocyte, Hypertrophy	0				5** (3.0)
Basophilic Focus	0				3
Eosinophilic Focus	0				3
Mixed Cell Focus	0				5**
12-Month Interim Evaluation					
Number Examined Microscopically	5				5
Oval Cell, Hyperplasia	0				5** (1.4)
Hepatocyte, Hypertrophy	0				5** (3.0)
Degeneration, Cystic, Focal	0				5** (1.0)
Basophilic Focus	1				3
Eosinophilic Focus	0				5**
Mixed Cell Focus	0				5**
Hepatocellular Adenoma, Multiple	0				2
Hepatocellular Adenoma (includes multiple)	0				4*
Hepatocholangiocarcinoma	0				1
2-Year Study					
Number Examined Microscopically	50	50	50	50	50
Bile Duct, Hyperplasia	36 (2.0)	17** (1.2)	16** (1.6)	17** (1.8)	28 (1.7)
Bile Duct, Hyperplasia, Atypical, Focal	0	0	1 (3.0)	2 (1.5)	1 (2.0)
Oval Cell, Hyperplasia	14 (2.0)	17 (1.5)	24* (1.5)	34** (1.7)	27** (1.6)
Hepatocyte, Hypertrophy	0	13** (1.7)	25** (2.7)	30** (2.8)	26** (2.5)
Degeneration, Cystic, Focal	4 (1.3)	2 (1.0)	25** (1.3)	38** (2.2)	41** (2.2)
Basophilic Focus	23	21	22	7	6
Eosinophilic Focus	11	28**	43**	47**	39**
Mixed Cell Focus	1	7**	14**	8**	2
Hepatocellular Adenoma, Multiple	0	5*	14**	24**	24**
Hepatocellular Adenoma (includes multiple)	5	12*	23**	38**	32**
Hepatocellular Carcinoma, Multiple	0	0	1	11**	23**
Hepatocellular Carcinoma (includes multiple)	2	3	14**	25**	36**
Hepatocellular Adenoma or Carcinoma[c]					
Overall rate[d]	7/50 (14%)	14/50 (28%)	28/50 (56%)	43/50 (86%)	45/50 (90%)
Adjusted rate[e]	16.6%	34.4%	64.4%	94.0%	99.4%
Terminal rate[f]	3/20 (15%)	7/16 (44%)	9/15 (60%)	0/0	0/0
First incidence (days)	529	431	502	467	437
Poly-3 test[g]	P<0.001	P=0.049	P<0.001	P<0.001	P<0.001

TABLE 7
Incidences of Neoplasms and Nonneoplastic Lesions of the Liver in Male and Female Rats in the 2-Year Gavage Study of Methyleugenol

	Vehicle Control	37 mg/kg	75 mg/kg	150 mg/kg	300 mg/kg (Stop-Exposure)
Male (continued)					
Cholangioma	0	0	0	0	2
Hepatocholangioma, Multiple	0	0	0	0	3
Hepatocholangioma (includes multiple)	0	0	0	1	6**
Hepatocholangiocarcinoma, Multiple	0	0	0	0	1
Hepatocholangiocarcinoma (includes multiple)	0	0	1	1	7**
Hepatocholangioma or Hepatocholangiocarcinoma (includes multiple)					
Overall rate	0/50 (0%)	0/50 (0%)	1/50 (2%)	2/50 (4%)	13/50 (26%)
Adjusted rate	0.0%	0.0%	2.6%	6.2%	44.4%
Terminal rate	0/20 (0%)	0/16 (0%)	0/15 (0%)	0/0	0/0
First incidence (days)	—[h]	—	582	457	502
Poly-3 test	P=0.049	—[i]	P=0.482	P=0.186	P<0.001
Female					
6-Month Interim Evaluation					
Number Examined Microscopically	5				5
Oval Cell, Hyperplasia	0				5** (1.0)
Hepatocyte, Hypertrophy	0				5** (1.0)
Basophilic Focus	0				2
Mixed Cell Focus	0				3
12-Month Interim Evaluation					
Number Examined Microscopically	5				5
Oval Cell, Hyperplasia	0				5** (1.8)
Hepatocyte, Hypertrophy	0				5** (3.0)
Basophilic Focus	5				3
Eosinophilic Focus	0				3
Mixed Cell Focus	0				5**
Hepatocellular Carcinoma	0				1
2-Year Study					
Number Examined Microscopically	50	50	49	49	50
Bile Duct, Hyperplasia	11 (1.3)	11 (1.3)	17 (1.3)	22** (1.5)	30** (1.8)
Oval Cell, Hyperplasia	1 (1.0)	15** (1.3)	19** (1.5)	35** (2.2)	34** (2.1)
Hepatocyte, Hypertrophy	1 (2.0)	13** (2.1)	16** (3.1)	26** (3.2)	31** (3.3)
Degeneration, Cystic, Focal	0	0	1 (1.0)	4 (1.5)	29** (1.9)
Basophilic Focus	36	36	29	17**	10**
Eosinophilic Focus	10	20**	27**	31**	37**
Mixed Cell Focus	6	4	19**	9	7
Hepatocellular Adenoma, Multiple	0	0	5*	23**	36**
Hepatocellular Adenoma (includes multiple)	1	8*	11**	33**	43**
Hepatocellular Carcinoma, Multiple	0	0	0	2	9**
Hepatocellular Carcinoma (includes multiple)	0	0	4	8**	22**

TABLE 7
Incidences of Neoplasms and Nonneoplastic Lesions of the Liver in Male and Female Rats in the 2-Year Gavage Study of Methyleugenol

	Vehicle Control	37 mg/kg	75 mg/kg	150 mg/kg	300 mg/kg (Stop-Exposure)
Female (continued)					
Hepatocellular Adenoma or Carcinoma[j]					
Overall rate	1/50 (2%)	8/50 (16%)	14/49 (29%)	34/49 (69%)	43/50 (86%)
Adjusted rate	2.5%	19.6%	33.4%	76.6%	96.5%
Terminal rate	1/22 (5%)	8/25 (32%)	7/22 (32%)	11/11 (100%)	16/16 (100%)
First incidence (days)	730 (T)	730 (T)	609	508	459
Poly-3 test	P<0.001	P=0.017	P<0.001	P<0.001	P<0.001
Cholangiocarcinoma	0	0	0	0	1
Hepatocholangioma	0	0	0	0	8**
Hepatocholangiocarcinoma, Multiple	0	0	0	0	2
Hepatocholangiocarcinoma (includes multiple)	0	0	0	3	9**
Hepatocholangioma or Hepatocholangiocarcinoma					
Overall rate	0/50 (0%)	0/50 (0%)	0/49 (0%)	3/49 (6%)	17/50 (34%)
Adjusted rate	0.0%	0.0%	0.0%	8.2%	43.0%
Terminal rate	0/22 (0%)	0/25 (0%)	0/22 (0%)	2/11 (18%)	6/16 (38%)
First incidence (days)	—	—	—	534	609
Poly-3 test	P=0.010	—	—	P=0.105	P<0.001

* Significantly different (P≤0.05) from the vehicle control group by the Fisher exact test (interim evaluations) or Poly-3 test (2-year study)
** P≤0.01
(T)Terminal sacrifice
[a] Number of animals with lesion
[b] Average severity grade of lesions in affected animals: 1=minimal, 2=mild, 3=moderate, 4=marked
[c] Historical incidence for 2-year gavage studies with corn oil vehicle control groups (mean ± standard deviation): 16/400 (4.0% ± 3.5%); range, 0%-10%
[d] Number of animals with neoplasm per number of animals with liver examined microscopically
[e] Poly-3 estimated neoplasm incidence after adjustment for intercurrent mortality
[f] Observed incidence at terminal kill
[g] Beneath the vehicle control incidence are the P values associated with the trend test [the 300 mg/kg (stop-exposure) group was excluded from the trend test]. Beneath the dosed group incidence are the P values corresponding to pairwise comparisons between the vehicle controls and that dosed group. The Poly-3 test accounts for differential mortality in animals that do not reach terminal sacrifice.
[h] Not applicable; no neoplasms in animal group
[i] Value of statistic cannot be computed.
[j] Historical incidence: 1/401 (0.3% ± 0.7%); range, 0%-2%

Cholangiomas and cholangiocarcinomas are neoplasms of the biliary system and are uncommon in rats as spontaneous or induced neoplasms. They have been induced in rats following exposure to hepatocarcinogens. Cholangiomas were well-circumscribed, expansive, multilocular masses that consisted of multiple empty cystic spaces of variable size (Plate 3). Cysts were separated by scant fibrous connective tissue septa and were lined by flattened or cuboidal proliferative bile duct epithelium. Cholangiocarcinomas were larger, locally invasive neoplasms composed of atypical pleomorphic proliferative biliary epithelium forming glandular or ductular structures (some of which contained mucous and cellular debris) surrounded by abundant fibrous or collagenous connective tissue stroma (Plate 4).

In addition to the hepatocellular and biliary neoplasms, neoplasms composed of both well- to poorly differentiated hepatocellular and biliary components were observed. In these cases, the diagnosis of hepatocholangioma or hepatocholangiocarcinoma was used (Plates 5 and 6). In some neoplasms, the distinction

between component cells was clear; in others, component cells occurred within less well differentiated glandular structures, and the distinction was less obvious. In the glandular structures, component cells were flattened to low cuboidal (resembling biliary epithelial cells) or were larger resembling hepatocytes.

Bile duct hyperplasia consisted of periportal proliferation of well-differentiated biliary epithelium which formed small, well- to poorly delineated ducts. Bile duct hyperplasia is a common, age-related lesion and is often seen with exposure to hepatotoxins and hepatocarcinogens. However, the lesions are thought to be reparative rather than preneoplastic, and there is no clear evidence that this change progresses to neoplasms. Atypical bile duct hyperplasia consisted of irregularly shaped, dilated ducts lined by pleomorphic atypical cuboidal to columnar cells surrounded by abundant collagenous tissue (Plate 7). In some studies, similar lesions have been diagnosed as cholangiofibrosis or cholangiofibroma. The biologic nature of these lesions is uncertain. In contrast to simple bile duct hyperplasia, they are considered by some to be preneoplastic lesions from which cholangiocellular neoplasms and even hepatocellular neoplasms may develop. In one study, after treatment was discontinued, similar lesions progressed to malignant neoplasms and continued to grow after transplantation.

Oval cell hyperplasia was characterized by the presence of small, oval, slightly basophilic cells that proliferated in rows along the sinusoids adjacent to portal tracts (Plate 8). Oval cell hyperplasia does not occur spontaneously and is often seen with chemicals that are hepatotoxic. Based on ultrastructural and antigenic studies, it has been suggested that oval cells are undifferentiated cholangiolar cells or a stem cell population for hepatocytes and biliary epithelium and that they may be the origin of chemically induced neoplasms of both cell lineages.

Cystic degeneration occurred as focal to multifocal, multilocular lesions composed of multiple irregular variably sized cystic spaces that often contained finely granular or flocculent pale eosinophilic material (Plate 9). In many of the exposed animals, cystic degeneration was extensive and often occurred within neoplasms. These lesions are thought to arise from the perisinusoidal fat-storing (Ito) cells of the liver, and large lesions are considered by some to be benign Ito cell neoplasms. Small foci of cystic degeneration may occur spontaneously in old rats. However, well-developed lesions have been observed with prolonged exposure to hepatocarcinogens and are thought to result from excess accumulation of proteoglycans and/or proteins due to overproduction or impaired degradation of these substances.

Eosinophilic foci were focal lesions up to four hepatic lobules in diameter with distinct margins and minimal to no compression of the adjacent parenchyma. Hepatocytes within the foci were larger and more eosinophilic than the adjacent hepatocytes. Hepatocyte hypertrophy involved individual mid-zonal hepatocytes and was characterized by increased cell size and cytoplasmic eosinophilia.

Glandular Stomach: Chemical-related neoplasms and nonneoplastic lesions occurred in the glandular stomach of male and female rats (Table 8 and Appendixes A and B). Lesions were confined to the fundic region and were more prevalent and severe in females than males. Lesions included benign and malignant neuroendocrine tumors, glandular epithelial atrophy, and neuroendocrine cell hyperplasia.

In all dosed groups of rats at 6 and 12 months and 2 years, the incidences of mucosal atrophy were significantly greater than in the vehicle controls. Neuroendocrine cell hyperplasia occurred in females at 6 months and in males and females at 12 months, and the incidences in 150 and 300 mg/kg males and in 37, 75, and 150 mg/kg females at 2 years were significantly increased. The incidences of benign and malignant neuroendocrine tumors were increased in 150 and 300 mg/kg males, and the incidence of malignant neuroendocrine tumors in 150 mg/kg males was significantly increased compared to the vehicle control group. There was a positive trend in the incidences of benign or malignant neuroendocrine tumors (combined) in females, and the incidences in females administered 75 mg/kg or greater were significantly greater than those in the vehicle controls (Table 8). Benign or malignant neuroendocrine tumors have not been observed in the glandular stomach of male or female historical corn oil gavage vehicle controls.

TABLE 8
Incidences of Neoplasms and Nonneoplastic Lesions of the Glandular Stomach in Male and Female Rats in the 2-Year Gavage Study of Methyleugenol

	Vehicle Control	37 mg/kg	75 mg/kg	150 mg/kg	300 mg/kg (Stop-Exposure)
Male					
6-Month Interim Evaluation					
Number Necropsied	5				5
Atrophy[a]	0				5** (2.0)[b]
12-Month Interim Evaluation					
Number Necropsied	5				5
Atrophy	0				5** (2.0)
Neuroendocrine Cell Hyperplasia	0				2 (1.0)
2-Year Study					
Number Necropsied	50	50	50	50	50
Atrophy	0	14** (1.6)	32** (1.7)	37** (2.2)	29** (2.2)
Neuroendocrine Cell, Hyperplasia	0	0	1 (1.0)	8** (1.5)	8** (1.8)
Benign Neuroendocrine Tumor	0	0	0	3	2
Malignant Neuroendocrine Tumor	0	0	0	4*	2
Benign or Malignant Neuroendocrine Tumor					
Overall rate[c]	0/50 (0%)	0/50 (0%)	0/50 (0%)	7/50 (14%)	4/50 (8%)
Adjusted rate[d]	0.0%	0.0%	0.0%	21.3%	16.5%
Terminal rate[e]	0/20	0/16 (0%)	0/15 (0%)	0/0	0/0
First incidence (days)	—[g]	—	—	642	517
Poly-3 test[f]	P<0.001	—[h]	—	P=0.002	P=0.032
Female					
6-Month Interim Evaluation					
Number Necropsied	5				5
Atrophy	0				5** (3.0)
Neuroendocrine Cell, Hyperplasia	0				1 (1.0)
12-Month Interim Evaluation					
Number Necropsied	5				5
Atrophy	0				5** (3.0)
Neuroendocrine Cell, Hyperplasia	0				1 (1.0)

TABLE 8
Incidences of Neoplasms and Nonneoplastic Lesions of the Glandular Stomach in Male and Female Rats in the 2-Year Gavage Study of Methyleugenol

	Vehicle Control	37 mg/kg	75 mg/kg	150 mg/kg	300 mg/kg (Stop-Exposure)
Female (continued)					
2-Year Study					
Number Necropsied	50	50	50	50	50
Atrophy	3 (1.0)	41** (1.6)	45** (2.1)	39** (2.4)	33** (2.2)
Neuroendocrine Cell, Hyperplasia	0	5* (1.0)	11** (2.0)	9** (2.2)	3 (2.0)
Benign Neuroendocrine Tumor	0	0	13**	9**	5*
Malignant Neuroendocrine Tumor	0	1	12**	26**	36**
Benign or Malignant Neuroendocrine Tumor					
Overall rate	0/50 (0%)	1/50 (2%)	25/50 (50%)	34/50 (68%)	41/50 (82%)
Adjusted rate	0.0%	2.4%	59.2%	80.3%	93.9%
Terminal rate	0/22 (0%)	0/25 (0%)	16/22 (73%)	10/11 (91%)	16/16 (100%)
First incidence (days)	—	718	676	548	477
Poly-3 test	P<0.001	P=0.508	P<0.001	P<0.001	P<0.001

* Significantly different (P≤0.05) from the vehicle control group by the Fisher exact test (interim evaluation) or the Poly-3 test (2-year study)
** P≤0.01
[a] Number of animals with lesion
[b] Average severity grade of lesions in affected animals: 1=minimal, 2=mild, 3=moderate, 4=marked
[c] Number of animals with neoplasm per number of animals necropsied
[d] Poly-3 estimated neoplasm incidence after adjustment for intercurrent mortality
[e] Observed incidence at terminal kill
[f] Beneath the vehicle control incidence are the P values associated with the trend test [the 300 mg/kg (stop-exposure) group was excluded from the trend test]. Beneath the dosed group incidence are the P values corresponding to pairwise comparisons between the vehicle controls and that dosed group. The Poly-3 test accounts for differential mortality in animals that do not reach terminal sacrifice.
[g] Not applicable; no neoplasms in animal group
[h] Value of statistic cannot be computed.

Mucosal atrophy was an early change observed at the 6- and 12-month evaluations and was also present at 2 years. It was confined to the epithelium of the fundic region of the glandular stomach and did not appear to involve the epithelium of the foveolar (gastric pits) region of the mucosa. Atrophy began near the limiting ridge (margo plicans) and invariably involved the entire fundic glandular mucosa. It was characterized by decreased height of the fundic mucosa due to loss of all glandular epithelial cell types, particularly parietal and chief cells (Plate 10).

Neuroendocrine proliferative lesions of the glandular stomach are extremely rare both as spontaneous and chemically induced lesions. In this study, the spectrum of proliferative neuroendocrine lesions observed seemed to constitute a morphologic continuum that ranged from hyperplasia to benign to well- to poorly differentiated malignant neoplasms. For diagnostic purposes, specific criteria were used to separate these lesions based on morphology alone.

Neuroendocrine cell hyperplasia consisted of proliferations of cells morphologically consistent with enterochromaffin-like (ECL) cells of the glandular epithelium. These proliferations were confined to the mucosa. They tended to be focal and did not markedly expand or efface the mucosa, but did displace the adjacent fundic glands (Plate 11a). Component cells were round to polygonal, with eosinophilic to amphophilic finely granular cytoplasm and had round to oval single nuclei and usually a single

nucleolus. The cells occurred in nests, clusters, or often as infiltrations between the glandular epithelial cells extending toward the mucosal surface (Plate 11b).

Similar to neuroendocrine cell hyperplasia, benign neuroendocrine tumors were confined wholly to the mucosa and did not invade the muscularis mucosa. In contrast to hyperplasia, benign tumors were more extensive and frequently caused moderate to marked thickening of the mucosa and sometimes occurred as nodular expansions. Neoplastic cells were arranged in sheets or clusters which displaced and/or replaced the adjacent glands. The cells were mildly pleomorphic but essentially retained their ECL cell features (Plate 12). While some of these neoplasms were quite locally expansive, their biologic behavior is uncertain, and therefore, based on morphology, were considered to be benign.

Malignant neuroendocrine tumors were generally large expansive masses that morphologically exhibited malignant behavior. These neoplasms were diffuse and/or well-demarcated nodular mucosal masses that obliterated the normal mucosal architecture and frequently caused marked thickening of the mucosa (Plate 13). Invasion through the muscularis mucosa with focal nodular and/or expansive infiltrative growth within the submucosa were common. The latter characteristic was the most consistent criterium of malignancy. Most malignant tumors were solid masses consisting predominantly of ECL cells variably mixed with areas of poorly differentiated polygonal cells that had moderate to abundant amounts of eosinophilic cytoplasm. In the more malignant neoplasms, a heterogenous cell pattern was often evident and striking. These neoplasms consisted of sheets of ECL cells mixed with focal to extensive areas of atypical polygonal and sometimes spindle-shaped cells among which were occasionally interspersed variably sized nodular expansions of large polygonal cells that had brightly eosinophilic coarsely granular cytoplasm (Plate 14). A few tumors had a large component of the latter cells. Cellular anaplasia or a high mitotic rate were not characteristic of most of these neoplasms. Blood vessels distended with emboli of neoplastic cells was evidence of the potential for metastases. In female rats, metastases primarily to the liver (vehicle control, 0/50; 37 mg/kg, 0/50; 75 mg/kg,0/49; 150 mg/kg, 3/49; 300 mg/kg, 10/50) and lung (0/50, 0/50, 0/49, 0/49, 4/50) were observed (Table B1).

Immunohistochemical staining of glandular epithelial lesions with chromagranin A (CG-A) and neuron specific enolase (NSE) and the Sevier-Munger histochemical stain for argyrophylia was heterogeneous. Focal areas of neuroendocrine cell hyperplasia stained positively with CG-A and NSE, and the Sevier-Munger stain demonstrated their argyrophilic positivity. Benign neuroendocrine tumors were moderately to strongly positive with CG-A, NSE, and Sevier-Munger staining. Chromagranin-A, NSE, and Sevier-Munger staining in the more malignant tumors was variable. In general, malignant cells that retained morphologic characteristics of ECL cells often exhibited positive staining. However, staining characteristics among and within malignant tumors were heterogeneous and varied in intensity. Areas of negative staining were interspersed with areas of moderately to strongly positive staining. Areas of decreased staining intensity or negative staining were usually those composed of the more atypical cells.

Forestomach: In core study female rats, there was a positive trend in the incidences of squamous cell papilloma or carcinoma (combined) (0/50, 0/50, 1/50, 3/50; Table B3a); however, by pairwise comparisons, the incidences in the dosed groups were not significantly greater than that in the vehicle control group. The incidence in the 150 mg/kg group exceeded the historical control range for corn oil gavage vehicle controls (Table B4b).

Kidney: In the standard evaluation in core study male rats, there was a positive trend in the incidences of renal tubule adenoma (Tables 9 and A3a), and the incidence in the 300 mg/kg males was significantly greater than that in the vehicle controls. The incidences in all groups exceeded the historical control range for corn oil gavage vehicle controls (Tables 9, A3a, A3b, and A4b). The incidences of renal tubule proliferative lesions in male rats were suggestive of a neoplastic effect in the kidney. Therefore, additional step sections of the kidneys of male rats were prepared using the residual formalin-fixed wet tissue. During this evaluation, additional rats with renal tubule proliferative lesions were

TABLE 9
Incidences of Neoplasms and Nonneoplastic Lesions of the Kidney in Male and Female Rats in the 2-Year Gavage Study of Methyleugenol

	Vehicle Control	37 mg/kg	75 mg/kg	150 mg/kg	300 mg/kg (Stop-Exposure)
Male					
6-Month Interim Evaluation					
Number Examined Microscopically	5				5
Nephropathy[a]	4 (1.0)[b]				5 (1.0)
12-Month Interim Evaluation					
Number Examined Microscopically	5				5
Nephropathy	5 (1.0)				5 (2.0)
2-Year Study					
Single Sections (Standard Evaluation)					
Number Examined Microscopically	50	50	50	50	50
Nephropathy	49 (2.8)	46 (2.4)	48 (2.8)	50 (3.0)	47 (3.3)*
Renal Tubule, Hyperplasia, Focal	5 (2.2)	1 (4.0)	7 (2.9)	4 (3.5)	3 (2.0)
Renal Tubule, Hyperplasia, Oncocytic	0	0	0	2 (1.5)	1 (4.0)
Renal Tubule Adenoma, Bilateral	0	0	1	0	0
Renal Tubule Adenoma (includes bilateral)[c]					
Overall rate[d]	3/50 (6%)	2/50 (4%)	6/50 (12%)	6/50 (12%)	8/50 (16%)
Adjusted rate[e]	7.2%	5.2%	15.8%	18.4%	31.2%
Terminal rate[f]	1/20 (5%)	0/16 (0%)	4/15 (27%)	0/0	0/0
First incidence (days)	712	673	596	598	528
Poly-3 test[g]	P=0.046	P=0.531N	P=0.195	P=0.135	P=0.018
Renal Tubule Carcinoma	1	0	0	0	0
Renal Tubule Adenoma or Carcinoma[h]	4	2	6	6	8*
Renal Tubule Benign Oncocytoma	0	0	1	0	1
Step Sections (Extended Evaluation)					
Number Examined Microscopically	50	50	50	50	50
Renal Tubule, Hyperplasia, Focal	10 (3.2)	13 (1.8)	20** (2.7)	20** (2.9)	21** (2.7)
Renal Tubule, Hyperplasia, Oncocytic	0	0	1	2	3
Renal Tubule Adenoma, Multiple	0	1	2	1	0
Renal Tubule Adenoma (includes multiple)					
Overall rate	2/50 (4%)	5/50 (10%)	14/50 (28%)	11/50 (22%)	13/50 (26%)
Adjusted rate	4.8%	12.9%	36.7%	31.9%	45.9%
Terminal rate	1/20 (5%)	3/16 (19%)	8/15 (53%)	0/0	0/0
First incidence (days)	712	718	673	575	537
Poly-3 test	P<0.001	P=0.186	P<0.001	P=0.002	P<0.001
Renal Tubule Benign Oncocytoma	0	0	0	1	1

TABLE 9
Incidences of Neoplasms and Nonneoplastic Lesions of the Kidney in Male and Female Rats in the 2-Year Gavage Study of Methyleugenol

	Vehicle Control	37 mg/kg	75 mg/kg	150 mg/kg	300 mg/kg (Stop-Exposure)
Male (continued)					
Single Sections and Step Sections (Combined)					
Number Examined Microscopically	50	50	50	50	50
Renal Tubule, Hyperplasia, Focal	13 (2.9)	13 (2.0)	21* (2.9)	20* (3.0)	22** (2.8)
Renal Tubule, Hyperplasia, Oncocytic	0	0	1	4	4
Renal Tubule Adenoma, Multiple	1	2	4	3	1
Renal Tubule Adenoma (includes multiple)					
Overall rate	4/50 (8%)	6/50 (12%)	17/50 (34%)	13/50 (26%)	20/50 (40%)
Adjusted rate	9.6%	15.5%	43.9%	37.3%	64.6%
Terminal rate	2/20 (10%)	3/16 (19%)	10/15 (67%)	0/0	0/0
First incidence (days)	712	673	596	575	528
Poly-3 test	P<0.001	P=0.325	P<0.001	P=0.003	P<0.001
Renal Tubule Benign Oncocytoma	0	0	1	1	2
Female					
12-Month Interim Evaluation					
Number Examined Microscopically	5				5
Nephropathy	4 (1.0)				2 (1.0)
2-Year Study					
Number Examined Microscopically	50	50	50	50	50
Nephropathy	35 (1.2)	42 (1.2)	41 (1.2)	44 (1.3)	45* (2.2)*

* Significantly different (P≤0.05) from the vehicle control group by the Poly-3 test (incidences) or Mann-Whitney U test (severities)
** P≤0.01
[a] Number of animals with lesion
[b] Average severity grade of lesions in affected animals: 1=minimal, 2=mild, 3=moderate, 4=marked
[c] Historical incidence for 2-year gavage studies with corn oil vehicle control groups (mean ± standard deviation): 3/400 (0.8% ± 1.0%); range, 0%-2%
[d] Number of animals with neoplasm per number of animals with kidney examined microscopically
[e] Poly-3 estimated neoplasm incidence after adjustment for intercurrent mortality
[f] Observed incidence at terminal kill
[g] Beneath the vehicle control incidence are the P values associated with the trend test [the 300 mg/kg (stop-exposure) group was excluded from the trend test]. Beneath the dosed group incidence are the P values corresponding to pairwise comparisons between the vehicle controls and that dosed group. The Poly-3 test accounts for differential mortality in animals that do not reach terminal sacrifice. A lower incidence in an exposure group is indicated by **N**.
[h] Historical incidence: 5/400 (1.3% ± 1.5%); range, 0%-4%

identified. The incidences of renal tubule proliferative lesions identified in the step sections and the combined incidences of standard and step sections in male rats are presented in Tables 9, A3a, and A3b. The incidences of renal tubule hyperplasia and adenoma in the extended evaluation and the combined incidences of standard and step sections in the 75, 150, and 300 mg/kg groups were greater than those in the vehicle controls. Additionally, there was a slight positive trend in the incidences of renal tubule benign oncocytoma in the extended evaluation and the combined incidences of standard and step sections.

The incidences of nephropathy were increased in all dosed groups of females, and the increase was significant in the 300 mg/kg group (Tables 9 and B5). The incidences of nephropathy in dosed groups of males were similar to that in the vehicle controls (Tables 9 and A5). In dosed males, the severity of nephropathy increased with increasing dose; the severity in 300 mg/kg males was significantly greater than that in the vehicle controls.

Hyperplastic tubules varied in size from slightly greater than the diameter of normal tubules to approximately two to three times greater than a normal tubule. The hyperplastic cells usually formed solid clusters and were slightly more pleomorphic and more basophilic. Renal tubule adenomas consisted of cells that frequently were morphologically similar to those of renal tubule hyperplasia but were distinguished from hyperplasia by their larger size and more complex structure. Adenomas were discrete to well-circumscribed lesions, most of which were approximately 1 mm or greater in diameter (greater than five or more tubule diameters in size). They consisted of multiple, variably sized, tubule-like structures or solid clusters of neoplastic renal tubule epithelial cells separated by fine bands of connective tissue.

Malignant Mesothelioma: In core study males, there was a positive trend in the incidences of malignant mesothelioma (vehicle control, 1/50; 37 mg/kg, 3/50; 75 mg/kg, 5/50; 150 mg/kg, 12/50; Table A3a); the incidence was significantly greater in 150 mg/kg males than in the vehicle controls. The incidence in 300 mg/kg males (5/50; Table A3b) was also significantly greater than that in the vehicle controls (Table A3b). The incidences in the 75, 150, and 300 mg/kg groups exceeded the historical control range for corn oil gavage studies (Table A4c). Mesotheliomas were disseminated along the peritoneal surface of several organs in the abdominal cavity and/or the serosa of the testis and epididymis. The histomorphology of malignant mesotheliomas varied. Typically, most consisted of single or multiple papillary projections lined by one to several layers of mesothelial cells covering cores of fibrous stroma. Others occurred as florid papilliferous proliferations of stroma covered by several layers of proliferating cuboidal to polygonal mesothelial cells (Plate 15). The more malignant mesotheliomas consisted of pleomorphic mesothelial cells arranged in sheets or clusters or forming tubular structures surrounded by abundant amounts of fibrous tissue stroma.

Mammary Gland: Mammary gland fibroadenoma occurred with a positive trend in male rats. The incidences of mammary gland fibroadenoma in 75 and 150 mg/kg males were significantly greater than in the vehicle controls (5/50, 5/50, 15/50, 13/50, 6/50; Tables A3a and A3b). The incidences of mammary gland fibroadenoma in all groups of males exceeded the historical control range for corn oil gavage vehicle controls [23/402 (5.7% ± 1.3%); range, 4%-8%]. Fibroadenomas were expansive nodules/masses composed of well-differentiated ducts, ductules, and/or alveoli lined by a single layer of uniformly cuboidal cells and surrounded by variable amounts of mature fibrous connective tissue. In some masses, the connective tissue was abundant and formed the predominant component. The lumens of many alveoli and ducts contained proteinaceous secretion, and alveolar epithelial cells frequently contained lipid.

Skin (subcutaneous tissue): The incidences of fibroma in 37 and 75 mg/kg males (1/50, 9/50, 8/50, 5/50, 4/50) and combined incidences of fibroma or fibrosarcoma in 37, 75, and 150 mg/kg males (1/50, 12/50, 8/50, 8/50, 4/50; Tables A3a and A3b) were significantly increased; however, the incidences did not increase with increasing dose. The incidences of these lesions in these groups exceeded the historical range for corn oil gavage vehicle controls (Table A4d). Fibromas were well-demarcated, solid, expansive masses composed of well-differentiated fibrous connective tissue. Fibrosarcomas were expansive, locally invasive masses composed of anaplastic spindle cells.

Bone Marrow: Bone marrow hyperplasia was observed at 6 months in one male and four females administered 300 mg/kg and at 12 months in one vehicle control male and five 300 mg/kg males (Tables A5 and B5). At 2 years, the incidences of bone marrow hyperplasia were increased in all groups of dosed females (4/50, 15/50, 11/49, 20/50, 25/50; Table B5), and the increases in the 37, 150, and 300 mg/kg groups were significant. Bone marrow hyperplasia consisted of a marked increase in the density of erythroid or myeloid cells or a mixture of both, frequently accompanied by proliferation of megakaryocytes. Bone marrow hyperplasia was

considered a reactive response secondary to significant hepatic and gastric pathology.

Salivary Gland: The incidences of cytoplasmic alteration of the submandibular salivary gland of all dosed male and female rats were significantly greater than those in the vehicle controls (males: vehicle control, 4/50; 37 mg/kg, 50/50; 75 mg/kg, 49/50; 150 mg/kg, 48/48; 300 mg/kg, 48/48; females: 1/50, 48/48, 49/49, 49/49, 49/50; Tables A5 and B5). This change was also present in all 300 mg/kg rats at 6 and 12 months. Cytoplasmic alteration consisted of a loss of the eosinophilic granules within the striated ducts of the submandibular salivary glands.

Adrenal Medulla: At 2 years, there was a negative trend in the incidences of benign pheochromocytoma in core study males, and the incidences were significantly decreased in the 75 and 150 mg/kg groups compared to the vehicle controls (24/50, 17/50, 11/50, 10/50, 9/50; Tables A3a and A3b). However, the incidence in the concurrent control group (48%) is high compared to the historical control rate for corn oil gavage studies (23%); the incidence in the concurrent controls is also high compared to the historical control rates for dosed feed (26%) and inhalation (32%) studies (Table A4e). The incidences of benign pheochromocytoma in the 75, 150, and 300 mg/kg groups of male rats are also within the historical control range for these routes of administration. Furthermore, there was a significant increase in the incidence of benign pheochromocytoma in 300 mg/kg females at 2 years (1/50, 1/50, 2/50, 2/49, 6/50; Tables B1 and B3b). Although 6/50 is just outside of the historical control range for female rats in corn oil gavage and dosed feed studies, as many as 6/50 have been seen in chamber control groups from inhalation studies (Table B4c). Incidences of hyperplasia did not differ between vehicle control and dosed groups of either male or female rats. The decreased incidences of benign pheochromocytoma in male rats and the increased incidence in female rats were considered variations due to chance rather than to effects associated with methyleugenol administration.

Spleen: The incidences of splenic fibrosis in 150 and 300 mg/kg females at 2 years were significantly increased (3/50, 3/50, 5/50, 12/49, 15/50; Table B5).

MICE

14-Week Study

All mice administered 1,000 mg/kg, except for one male, died before the end of the study (Table 10). Additionally, one 300 mg/kg male died during week 3 and one 10 mg/kg female died during week 12. The mean body weight gains of males and females in the 300 mg/kg groups were significantly less than those of the vehicle controls; the final mean body weights and body weight gains of other groups of mice surviving until the end of the study were similar to those of the vehicle controls (Table 10). The only clinical finding was toxicity manifested as generalized morbidity in male and female mice administered 1,000 mg/kg.

The liver weights of 30, 100, and 300 mg/kg males and of 300 mg/kg females were significantly greater than those of the vehicle controls (Table G2).

Male mice administered 10 or 30 mg/kg had significantly lower cauda epididymis, epididymis, and testis weights than did the vehicle controls (Table H3). Additionally, 100 mg/kg males had significantly decreased spermatozoal concentrations. There were no significant differences in vaginal cytology parameters between dosed and vehicle control mice (Table H4).

TABLE 10
Survival and Body Weights of Mice in the 14-Week Gavage Study of Methyleugenol

Dose (mg/kg)	Survival[a]	Mean Body Weight[b] (g)			Final Weight Relative to Vehicle Controls (%)
		Initial	Final	Change	
Male					
Water Control	10/10	22.7 ± 0.5	32.1 ± 1.2	9.4 ± 1.1	96
Vehicle Control	10/10	22.3 ± 0.4	33.4 ± 0.7	11.1 ± 0.5	
10	10/10	22.9 ± 0.4	33.4 ± 0.8	10.5 ± 0.5	100
30	10/10	22.3 ± 0.3	32.6 ± 0.8	10.3 ± 0.6	98
100	10/10	23.2 ± 0.5	33.7 ± 0.9	10.5 ± 0.7	101
300	9/10[c]	22.3 ± 0.4	30.7 ± 0.6	8.4 ± 0.5**	92
1,000	1/10[d]	21.1 ± 0.7	27.3	6.2	82
Female					
Water Control	10/10	19.0 ± 0.4	30.9 ± 1.1	11.8 ± 0.9	104
Vehicle Control	10/10	19.0 ± 0.3	29.6 ± 0.6	10.7 ± 0.5	
10	9/10[e]	18.5 ± 0.3	29.2 ± 0.7	10.7 ± 0.7	99
30	10/10	19.4 ± 0.4	29.3 ± 0.7	9.9 ± 0.6	99
100	10/10	19.5 ± 0.2	29.2 ± 0.5	9.6 ± 0.5	99
300	10/10	19.4 ± 0.2	27.5 ± 0.6	8.1 ± 0.4**	93
1,000	0/10[f]	18.4 ± 0.3	—	—	—

** Significantly different (P≤0.01) from the vehicle control group by Williams' or Dunnett's test
[a] Number of animals surviving at 14 weeks/number initially in group
[b] Weights and weight changes are given as mean ± standard error. Subsequent calculations are based on animals surviving to the end of the study. No standard error was calculated for groups with high mortality. No final mean body weights or weights changes were calculated for groups with 100% mortality.
[c] Week of death: 3
[d] Weeks of death: 1, 1, 1, 1, 1, 5, 8, 9, 12
[e] Week of death: 12
[f] Weeks of death: 1, 1, 1, 1, 1, 1, 3, 4, 4, 6

Significant chemical-related gross lesions were observed in the liver of male and female mice administered 1,000 mg/kg. One male and two females had enlarged livers, one male had a liver nodule, and one female had a pale liver.

Chemical-related nonneoplastic lesions occurred in the liver, glandular stomach, and nose of male and female mice (Table 11). Hepatic changes were generally only observed in mice that survived beyond the first week of the study. Cytologic alteration, necrosis, bile duct hyperplasia, and focal subacute inflammation occurred in the liver of all 300 mg/kg females. The incidences of these lesions were also significantly increased in 1,000 mg/kg males and females (except subacute inflammation). Female mice administered 1,000 mg/kg had lower incidences of these lesions due to early mortality. Significant differences were not observed between the water and vehicle control mice. Cytologic alteration was observed primarily in periportal sites and was the term used to describe a variety of hepatocellular alterations that included mild nuclear and cytoplasmic enlargement (hypertrophy) and increased cytoplasmic eosinophilia. Necrosis of scattered individual hepatocytes occurred throughout the hepatic lobules. Bile duct hyperplasia consisted of proliferation of immature biliary cells within portal areas. Inflammation consisted of multiple small foci of primarily mononuclear inflammatory cells randomly scattered throughout the liver.

The incidences of atrophy, degeneration, necrosis, edema, mitotic alteration, and cystic glands of the fundic region of the glandular stomach were increased in one or more groups of male and female mice administered 30 mg/kg or greater compared to the vehicle controls (Table 11); these lesions were generally of minimal to mild severity. In males, the incidences of cystic glands in the 30 mg/kg group and degeneration and mitotic alteration in the 300 mg/kg group were significantly increased. In female mice administered 300 mg/kg, the incidences of atrophy, degeneration, edema, mitotic alteration, and cystic glands were significantly increased. In the 1,000 mg/kg mice, early death and subsequent autolysis significantly precluded adequate histopathologic evaluation of gastric lesions. Lesions were generally of minimal to mild severity in the 30, 100, and 300 mg/kg groups and of mild to marked severity in the 1,000 mg/kg groups. Atrophy consisted of a generalized decrease in the thickness of the mucosal epithelium due to loss of parietal and chief cells and shortening of the mucosal glands. Necrosis in the glandular epithelium was characterized by necrosis of parietal cells primarily, and to a lesser extent, chief cells. Degeneration consisted of dilated glands lined by dysplastic atypical epithelial cells and cellular detritus. Cystic glands were dilated and lined by flattened epithelium. Increased numbers of morphologically normal mitotic figures were present in regenerative areas of the glandular epithelium. Edema was of minimal to mild severity and occurred in the lamina propria.

Minimal to mild focal degeneration of the olfactory epithelium of the nose occurred in 30, 300, and 1,000 mg/kg males, 30 mg/kg or greater females, and two water control females. The incidences and severities of this lesion were not dose dependent. Degeneration consisted of unilateral or bilateral focal loss and/or disruption of the sensory olfactory epithelial cells in the dorsal meatus.

Dose Selection Rationale: Based on the high mortality rate of the 1,000 mg/kg group, the lower mean body weight gains of the 300 mg/kg groups, and the hepatotoxic effects in the 300 and 1,000 mg/kg groups, the highest dose selected in the 2-year gavage study in mice was 150 mg/kg.

TABLE 11
Incidences of Selected Nonneoplastic Lesions in Male and Female Mice in the 14-Week Gavage Study of Methyleugenol

	Water Control	Vehicle Control	10 mg/kg	30 mg/kg	100 mg/kg	300 mg/kg	1,000 mg/kg
Male							
Liver[a]	10	10	10	10	10	10	10
Cytologic Alteration[b]	0	0	0	0	0	0	5* (4.0)[c]
Necrosis	0	0	0	0	0	0	5* (2.0)
Hyperplasia, Bile Duct	0	0	0	0	0	0	5* (2.2)
Inflammation, Subacute	0	0	0	0	0	0	4* (1.8)
Glandular Stomach	10	10	10	10	10	10	10
Atrophy	0	0	0	0	0	1 (1.0)	3 (4.0)
Degeneration	0	0	0	1 (1.0)	1 (1.0)	9** (1.0)	3 (2.0)
Necrosis	0	0	0	0	0	0	3 (3.3)
Edema	0	0	0	0	0	0	3 (1.3)
Mitotic Alteration	0	0	0	1 (1.0)	0	10** (1.1)	0
Cystic Glands	0	0	0	6** (1.0)	0	2 (1.0)	1 (2.0)
Nose							
Epithelial Cell, Degeneration	0	0	0	4* (1.5)	0	1 (1.0)	5* (1.8)
Female							
Liver	10	10	10	10	10	10	10
Cytologic Alteration	0	0	0	1 (1.0)	0	10** (2.3)	4* (3.8)
Necrosis	0	0	0	0	0	10** (1.0)	4* (1.8)
Hyperplasia, Bile Duct	0	0	0	0	0	10** (2.0)	4* (1.5)
Inflammation, Subacute	0	0	0	0	0	10** (1.0)	1 (2.0)
Glandular Stomach	10	10	10	10	10	10	10
Atrophy	0	0	0	0	0	10** (1.3)	3 (4.0)
Degeneration	0	0	0	4* (1.0)	3 (1.0)	10** (1.7)	3 (2.0)
Necrosis	0	0	0	0	0	0	3 (4.0)
Edema	0	0	0	0	0	6** (1.0)	3 (1.7)
Mitotic Alteration	0	0	0	4* (1.0)	5* (1.0)	10** (1.2)	0
Cystic Glands	0	0	0	4* (1.5)	0	7** (1.0)	0
Nose							
Epithelial Cell, Degeneration	2 (1.0)	0	0	8** (1.0)	4* (1.0)	5* (1.0)	3 (1.3)

* Significantly different ($P \le 0.05$) from the vehicle control group by the Fisher exact test
** $P \le 0.01$
[a] Number of animals examined microscopically
[b] Number of animals with lesion
[c] Average severity grade of lesions in affected animals: 1=minimal, 2=mild, 3=moderate, 4=marked

2-Year Study

Survival

Estimates of 2-year survival probabilities for male and female mice are shown in Table 12 and in the Kaplan-Meier survival curves (Figure 3). Survival of all dosed groups of male mice was similar to that of the vehicle controls. Survival rates of all dosed groups of female mice were significantly less than that of the vehicle controls. The decreased survival in female mice may have been due to the increased incidences of neoplasms and nonneoplastic lesions; these increases were greater in female mice than in males.

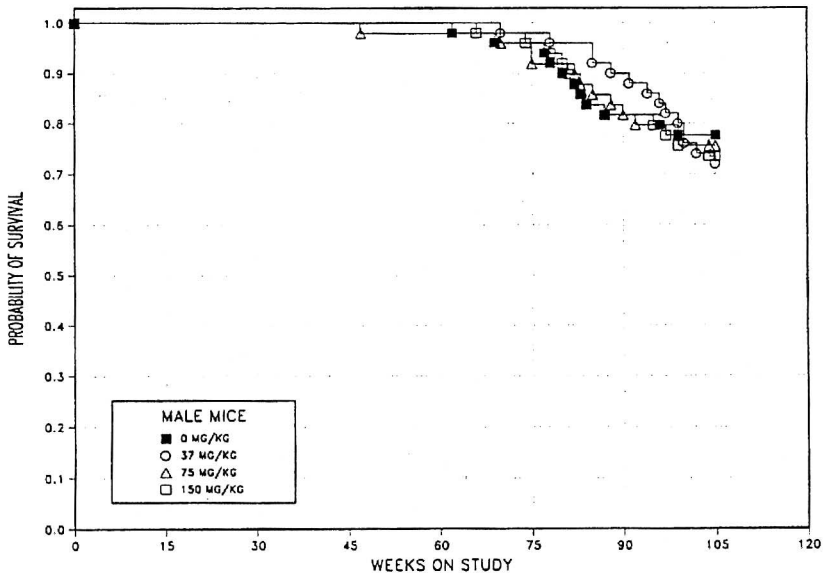
Body Weights and Clinical Findings

Mean body weights were generally less than those of the vehicle controls after weeks 81, 41, and 17 in males and after weeks 33, 17, and 5 in females for the 37, 75, and 150 mg/kg groups, respectively (Tables 13 and 14 and Figure 4). There were no clinical findings that could be attributed to methyleugenol administration.

TABLE 12
Survival of Mice in the 2-Year Gavage Study of Methyleugenol

	Vehicle Control	37 mg/kg	75 mg/kg	150 mg/kg
Male				
Animals initially in study	50	50	50	50
Accidental deaths[a]	0	0	1	2
Missing[a]	1	0	0	0
Moribund	5	6	5	9
Natural deaths	6	8	7	4
Animals surviving to study termination	38	36	37	35
Percent probability of survival at end of study[b]	78	72	76	73
Mean survival (days)[c]	690	705	679	686
Survival analysis[d]	P=0.815	P=0.825	P=1.000	P=0.829
Female				
Animals initially in study	50	50	50	50
Accidental deaths	0	1	1	0
Moribund	5	7	8	13
Natural deaths	14	24	23	35
Animals surviving to study termination	31	18	18[e]	2
Percent probability of survival at end of study	62	38	37	4
Mean survival (days)	696	662	664	637
Survival analysis	P<0.001	P=0.009	P=0.013	P<0.001

[a] Censored from survival analyses
[b] Kaplan-Meier determinations
[c] Mean of all deaths (uncensored, censored, and terminal sacrifice).
[d] The result of the life table trend test (Tarone, 1975) is in the vehicle control column, and the results of the life table pairwise comparisons (Cox, 1972) with the vehicle controls are in the dosed group columns.
[e] Includes two animals that died during the last week of the study



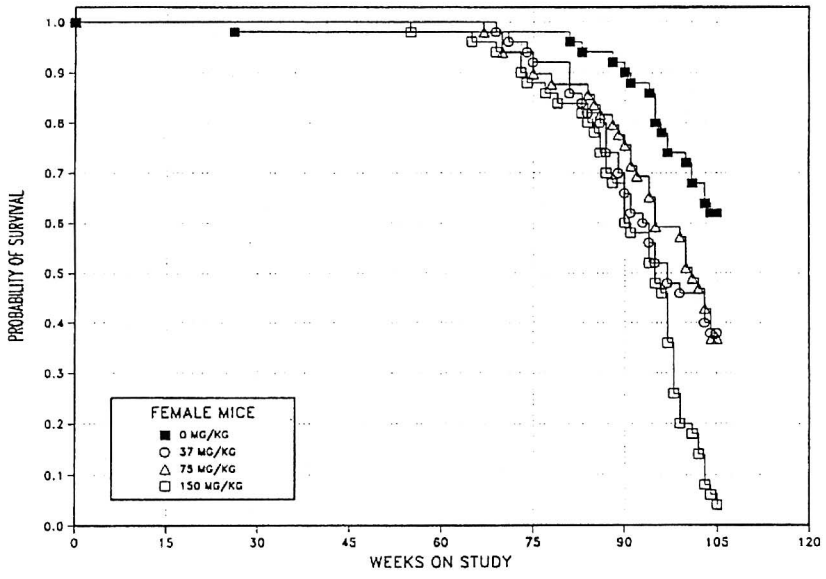


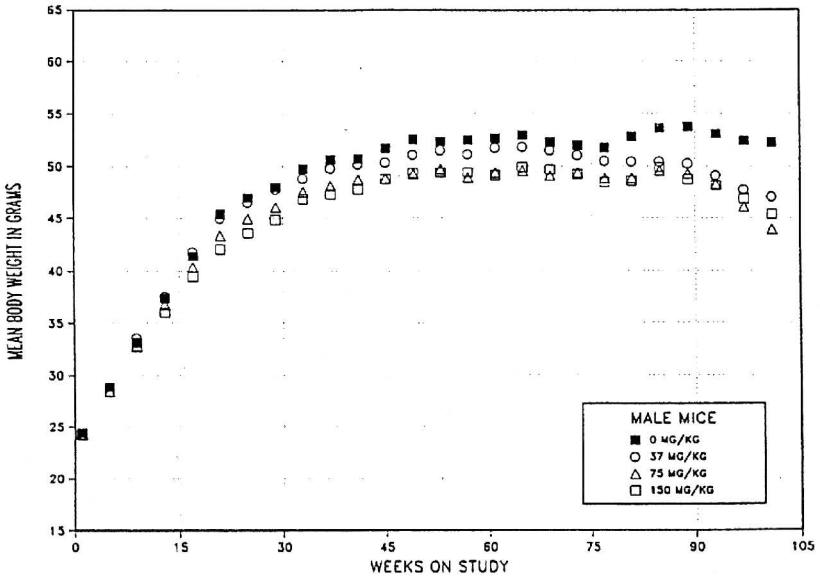
FIGURE 3
Kaplan-Meier Survival Curves for Male and Female Mice Administered Methyleugenol by Gavage for 2 Years

TABLE 13
Mean Body Weights and Survival of Male Mice in the 2-Year Gavage Study of Methyleugenol

Weeks on Study	Vehicle Control		37 mg/kg			75 mg/kg			150 mg/kg		
	Av. Wt. (g)	No. of Survivors	Av. Wt. (g)	Wt. (% of controls)	No. of Survivors	Av. Wt. (g)	Wt. (% of controls)	No. of Survivors	Av. Wt. (g)	Wt. (% of controls)	No. of Survivors
1	24.4	50	24.2	99	50	24.2	99	50	24.2	99	50
5	28.9	50	28.9	100	50	28.4	98	50	28.5	99	50
9	33.2	50	33.6	101	50	32.9	99	49	32.7	99	50
13	37.4	50	37.6	101	50	36.8	98	49	36.0	96	50
17	41.4	50	41.8	101	50	40.4	98	49	39.4	95	50
21	45.4	50	45.0	99	50	43.4	96	49	42.1	93	50
25	47.0	50	46.5	99	50	45.0	96	49	43.6	93	50
29	48.0	50	47.8	100	50	46.1	96	49	44.8	93	50
33	49.8	50	48.8	98	50	47.5	95	49	46.8	94	50
37	50.6	50	49.8	98	50	48.1	95	49	47.3	94	50
41	50.7	50	50.2	99	50	48.7	96	49	47.8	94	50
45	51.7	50	50.3	97	50	48.8	94	49	48.7	94	50
49	52.5	50	51.0	97	50	49.3	94	48	49.3	94	50
53	52.3	50	51.5	99	50	49.7	95	48	49.4	95	49
57	52.4	50	51.1	98	50	48.9	93	48	49.4	94	49
61	52.6	50	51.7	99	50	49.4	94	48	49.1	93	49
65	52.9	49	51.8	98	50	49.6	94	48	49.9	94	49
69	52.2	48	51.5	99	50	49.1	94	48	49.7	95	48
73	51.9	48	51.0	98	49	49.4	95	47	49.2	95	48
77	51.7	47	50.5	98	49	48.9	95	45	48.5	94	47
81	52.8	44	50.4	96	48	48.9	93	45	48.6	92	44
85	53.6	41	50.5	94	48	49.6	93	42	49.9	93	41
89	53.7	40	50.3	94	45	49.3	92	41	48.8	91	40
93	53.1	40	49.1	93	44	48.3	91	39	48.2	91	40
97	52.4	39	47.8	91	41	46.1	88	39	46.9	90	39
101	52.2	38	47.1	90	38	44.0	84	38	45.4	87	37
Mean for weeks											
1-13	31.0		31.1	100		30.6	99		30.4	98	
14-52	48.6		47.9	99		46.4	96		45.5	94	
53-101	52.6		50.3	96		48.6	92		48.7	93	

TABLE 14
Mean Body Weights and Survival of Female Mice in the 2-Year Gavage Study of Methyleugenol

Weeks on Study	Vehicle Control		37 mg/kg			75 mg/kg			150 mg/kg		
	Av. Wt. (g)	No. of Survivors	Av. Wt. (g)	Wt. (% of controls)	No. of Survivors	Av. Wt. (g)	Wt. (% of controls)	No. of Survivors	Av. Wt. (g)	Wt. (% of controls)	No. of Survivors
1	19.1	50	19.2	101	50	19.2	101	50	19.3	101	50
5	23.0	50	22.8	99	50	22.6	98	50	22.5	98	50
9	26.8	50	25.9	97	50	25.6	96	50	24.8	93	50
13	29.4	50	29.6	101	50	28.8	98	50	26.6	91	50
17	34.7	50	34.3	99	50	33.0	95	50	28.8	83	50
21	38.6	50	37.6	97	50	35.6	92	50	31.0	80	50
25	40.5	50	39.7	98	50	37.6	93	50	32.9	81	50
29	43.6	49	42.5	98	50	40.1	92	50	35.0	80	50
33	47.1	49	44.7	95	50	42.4	90	50	37.1	79	50
37	49.2	49	45.4	92	50	43.1	88	50	36.8	75	50
41	50.1	49	46.6	93	50	43.4	87	50	37.9	76	50
45	51.6	49	47.7	92	50	44.6	86	50	39.5	77	50
49	53.9	49	50.1	93	50	46.2	86	50	41.1	76	50
53	54.0	49	50.0	93	50	45.6	84	50	39.7	74	50
57	56.9	49	51.0	90	50	46.6	82	49	40.6	71	49
61	58.3	49	52.3	90	50	47.0	81	49	40.8	70	49
65	59.0	49	52.4	89	50	45.6	77	49	39.0	66	49
69	60.1	49	52.2	87	49	44.0	73	48	37.9	63	47
73	59.0	49	51.7	88	48	42.5	72	46	35.3	60	47
77	60.1	49	49.7	83	46	40.8	68	44	36.1	60	44
81	61.7	49	47.1	76	46	38.5	62	43	35.6	58	42
85	63.2	47	44.0	70	41	36.8	58	42	34.7	55	40
89	62.7	46	43.0	69	36	34.6	55	39	34.6	55	34
93	61.7	44	40.5	66	31	34.1	55	34	33.7	55	29
97	61.1	39	38.0	62	26	34.8	57	29	32.9	54	23
101	60.9	36	37.4	61	23	34.2	56	25	33.0	54	10
Mean for weeks											
1-13	24.6		24.4	100		24.1	98		23.3	96	
14-52	45.5		43.2	95		40.7	90		35.6	79	
53-101	59.9		46.9	79		40.4	68		36.5	61	



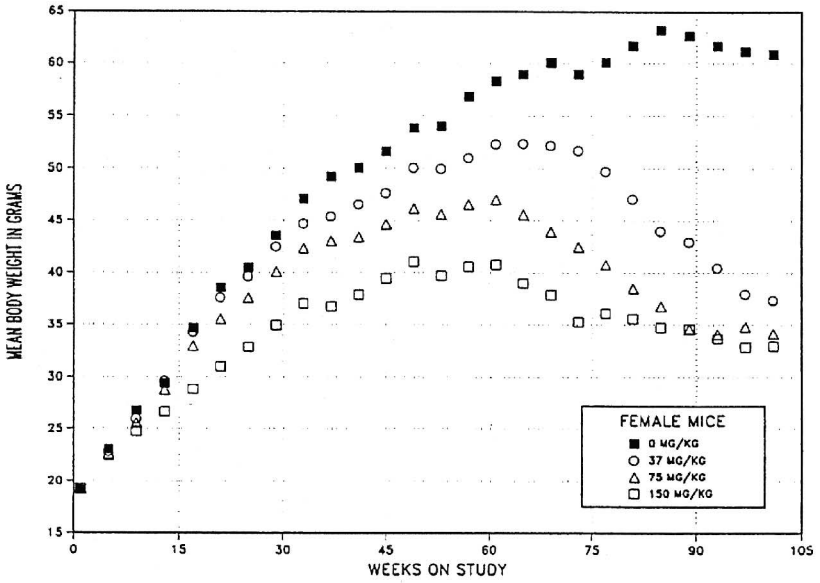


FIGURE 4
Growth Curves for Male and Female Rats
Administered Methyleugenol by Gavage for 2 Years

Toxicokinetic Studies

Detailed methods and results for the single-administration intravenous and gavage studies in young mice are presented in Appendix M, and results for the core study animals that received methyleugenol by gavage 5 days per week for 12 months and for the single-administration gavage studies in aged animals in Appendix I. Additional chemical distribution and metabolism data are presented in an unpublished absorption, distribution, metabolism, and elimination study conducted for the NTP (Appendix N).

Absorption: Absorption from oral doses was rapid, with peak plasma levels achieved within the first 5 minutes for all doses in males and females.

Distribution: Methyleugenol and its metabolites were distributed preferentially to the ovaries, stomach, fat, spleen, and liver 72 hours after oral administration of [^{14}C]-methyleugenol to males. Tissue:blood ratios of methyleugenol-derived radioactivity were approximately 5 in the liver, 5 to 9 in the stomach, 7 in the fat and the spleen, and over 100 in the ovaries after 72 hours. Many other tissues had elevated ratios; this may represent residual binding of metabolites rather than tissue solubility.

Metabolism: Approximately 85% of methyleugenol orally administered to females was eliminated in urine as parent or metabolites by 72 hours after dosing. Bioavailability of methyleugenol was low, with 3% to 5% bioavailability at 25 mg/kg. This increased to approximately 12% at 50 mg/kg and 13% to 19% at 75 mg/kg. These findings suggest a strong, but saturable, first-pass metabolic effect, leading to a nonlinear relationship between dose and parent chemical dosimetry. No unchanged methyleugenol was found in urine from females dosed with methyleugenol orally. Hydroxylated, sulfated, and glucuronidated metabolites constituted a minority of the metabolites detected in urine, with the majority unknown.

Elimination: Elimination of methyleugenol from the bloodstream was rapid and multiphasic, with terminal half-lives on the order of 15 to 30 minutes. No difference in the elimination of methyleugenol between naive males and females was apparent with either young or aged animals. Aged females exhibited a significantly higher AUC; this may be due to differences in the amount of body fat or an age-related decrease in metabolic capability. Core study animals eliminated methyleugenol with AUCs similar to those of the naive animals. Exceptions were for the low-dose females. The AUCs increased linearly with dose in females at 12 months and sublinearly in males at 12 months. The latter finding is indicative of metabolic saturation at the higher doses in males at this time point.

Pathology and Statistical Analyses

This section describes statistically significant and biologically noteworthy changes in the incidences of neoplasms and/or nonneoplastic lesions of the liver, glandular stomach, and bone marrow. Summaries of the incidences of neoplasms and nonneoplastic lesions, individual animal tumor diagnoses, statistical analyses of primary neoplasms that occurred with an incidence of at least 5% in at least one animal group, and historical incidences for the neoplasms mentioned in this section are presented in Appendix C for male mice and Appendix D for female mice.

Liver: Chemical-related increases in the incidences of hepatic neoplasms and nonneoplastic lesions occurred in males and females. Lesions were frequently multifocal and coexisted within the liver. Chemical-related proliferative lesions included hepatocellular adenoma and carcinoma, hepatoblastoma, hepatocholangiocarcinoma (females), eosinophilic focus (males), oval cell hyperplasia, and bile duct hyperplasia (females). Chemical-related nonproliferative lesions included chronic active inflammation in males, and hepatocyte necrosis, hemosiderin pigmentation, and hematopoietic cell proliferation in females (Table 15, Appendixes C and D).

In all dosed groups of male and female mice, the incidences of hepatocellular adenoma and hepatocellular adenoma or carcinoma (combined) were significantly greater than in the vehicle controls. In 37 and 75 mg/kg male mice and in all dosed groups of females, the incidences of hepatocellular carcinoma were significantly increased. Additionally, the multiple incidences of hepatocellular adenoma in dosed males and females and of hepatocellular carcinoma in dosed females were significantly increased. The incidences of hepatocellular adenoma or carcinoma (combined) in all dosed groups of males and females exceeded the historical vehicle control ranges for corn oil gavage studies (Tables 15, C4, and D4).

The incidence of hepatoblastoma in 150 mg/kg males was slightly greater than that in the vehicle controls. In females, there was a significant, dose-related increase in the incidences of hepatoblastoma. The incidences of hepatoblastoma in 150 mg/kg male mice and in all dosed groups of female mice exceeded the historical vehicle control range for corn oil gavage studies (Tables C4 and D4). Hepatocholangiocarcinomas were observed in two 150 mg/kg females (Table 15), and the incidence exceeded the vehicle historical control range for corn oil gavage studies (Tables 15 and D4).

The incidences of eosinophilic foci were significantly increased in dosed groups of male mice. In all dosed groups of males and females, the incidences of oval cell hyperplasia were significantly increased; in males, the incidences increased with increasing dose (Tables 15, C5, and D5). In 75 and 150 mg/kg males and in all dosed groups of females, the incidences of periportal hypertrophy were significantly greater than in the vehicle controls; in males, the incidences increased with increasing dose. The incidences of bile duct hyperplasia in 75 and 150 mg/kg female mice and of hemosiderin pigmentation in all dosed groups of female mice were significantly increased. In females, there was a dose-related increase in the incidences of hepatocyte necrosis, and the increases in the 75 and 150 mg/kg groups were significant. In females, there was also a significant, dose-related increase in the incidences of hematopoietic cell proliferation (Tables 15, C5, and D5).

Hepatocellular adenomas and carcinomas were morphologically similar to those observed in the rat study. Hepatoblastomas were generally well-demarcated masses that frequently arose within hepatocellular adenomas and/or carcinomas (Plate 16). They consisted of sheets of poorly differentiated round to spindle-shaped cells that were frequently aligned radially along numerous small blood vessels. The lung was a frequent site of metastases for hepatocellular carcinomas and hepatoblastomas. Hepatocholangiocarcinomas, bile duct hyperplasia, oval cell hyperplasia, and hepatocyte hypertrophy were histologically similar to those observed in the rat study.

Based on retrospective analyses, *Helicobacter hepaticus* was determined to have infected mice in 12 recent NTP 2-year studies (Appendix S). Of the 12 studies, mice (primarily males) from nine studies had *H. hepaticus*-associated hepatitis. Qualitatively, the hepatitis and silver-staining organisms (*H. hepaticus*) within the liver were similar among the nine studies. Using an assay based on polymerase chain

TABLE 15
Incidences of Neoplasms and Nonneoplastic Lesions of the Liver in Male and Female Mice in the 2-Year Gavage Study of Methyleugenol

	Vehicle Control	37 mg/kg	75 mg/kg	150 mg/kg
Male				
Number Examined Microscopically	49	50	50	50
Oval Cell, Hyperplasia[a]	0	8** (1.1)[b]	27** (1.2)	46** (1.5)
Hepatocyte, Hypertrophy	0	1 (3.0)	7** (2.3)	46** (2.8)
Eosinophilic Focus	10	20*	25**	19*
Inflammation, Chronic Active	19 (1.3)	21 (1.3)	28* (1.3)	28* (1.3)
Hepatocellular Adenoma, Multiple	13	33**	33**	29**
Hepatocellular Adenoma (includes multiple)	26	43**	38**	39**
Hepatocellular Carcinoma, Multiple	1	3	3	2
Hepatocellular Carcinoma (includes multiple)	10	20*	19*	9
Hepatocellular Adenoma or Carcinoma[c]				
Overall rate[d]	31/49 (63%)	47/50 (94%)	46/50 (92%)	40/50 (80%)
Adjusted rate[e]	65.4%	97.4%	96.4%	85.6%
Terminal rate[f]	23/38 (61%)	36/36 (100%)	36/37 (97%)	30/35 (86%)
First incidence (days)	430	593	486	512
Poly-3 test[g]	P=0.018	P<0.001	P<0.001	P=0.016
Hepatoblastoma (includes multiple)[h]	0	0	1	3
Hepatocellular Carcinoma or Hepatoblastoma				
Overall rate	10/49 (20%)	20/50 (40%)	20/50 (40%)	11/50 (22%)
Adjusted rate	21.8%	41.8%	42.2%	24.6%
Terminal rate	5/38 (13%)	10/36 (28%)	11/37 (30%)	7/35 (20%)
First incidence (days)	477	593	486	554
Poly-3 test	P=0.490N	P=0.030	P=0.027	P=0.471
Hepatocellular Adenoma, Hepatocellular Carcinoma, or Hepatoblastoma				
Overall rate	31/49 (63%)	47/50 (94%)	46/50 (92%)	41/50 (82%)
Adjusted rate	65.4%	97.4%	96.4%	86.7%
Terminal rate	23/38 (61%)	36/36 (100%)	36/37 (97%)	30/35 (86%)
First incidence (days)	430	593	486	512
Poly-3 test	P=0.012	P<0.001	P<0.001	P=0.011

TABLE 15
Incidences of Neoplasms and Nonneoplastic Lesions of the Liver in Male and Female Mice in the 2-Year Gavage Study of Methyleugenol

	Vehicle Control	37 mg/kg	75 mg/kg	150 mg/kg
Female				
Number Examined Microscopically	50	50	49	50
Hematopoietic Cell Proliferation	4 (1.3)	14** (1.8)	23** (1.7)	24** (1.6)
Pigmentation, Hemosiderin	0	11** (1.6)	24** (1.8)	19** (1.6)
Bile Duct, Hyperplasia	1 (2.0)	1 (2.0)	11** (1.5)	9** (1.8)
Hepatocyte, Necrosis	5 (2.0)	9 (2.3)	16** (2.3)	17** (2.0)
Oval Cell, Hyperplasia	0	46** (1.9)	36** (1.9)	38** (1.9)
Hepatocyte, Hypertrophy	0	10** (2.1)	7** (2.3)	23** (3.0)
Hepatocellular Adenoma, Multiple	8	39**	38**	32**
Hepatocellular Adenoma (includes multiple)	20	48**	46**	41**
Hepatocellular Carcinoma, Multiple	0	24**	29**	38**
Hepatocellular Carcinoma (includes multiple)	7	37**	47**	47**
Hepatocellular Adenoma or Carcinoma[i]				
Overall rate	25/50 (50%)	50/50 (100%)	49/49 (100%)	49/50 (98%)
Adjusted rate	54.8%	100.0%	100.0%	99.7%
Terminal rate	18/31 (58%)	18/18 (100%)	18/18 (100%)	2/2 (100%)
First incidence (days)	665	477	465	450
Poly-3 test	P<0.001	P<0.001	P<0.001	P<0.001
Hepatoblastoma, Multiple	0	0	0	4
Hepatoblastoma (includes multiple)[j]	0	6**	11**	15**
Hepatocellular Carcinoma or Hepatoblastoma				
Overall rate	7/50 (14%)	38/50 (76%)	48/49 (98%)	49/50 (98%)
Adjusted rate	15.5%	81.0%	98.0%	99.7%
Terminal rate	3/31 (10%)	13/18 (72%)	17/18 (94%)	2/2 (100%)
First incidence (days)	665	493	465	450
Poly-3 test	P<0.001	P<0.001	P<0.001	P<0.001
Hepatocellular Adenoma, Hepatocellular Carcinoma, or Hepatoblastoma				
Overall rate	25/50 (12%)	50/50 (100%)	49/49 (100%)	49/50 (98%)
Adjusted rate	54.8%	100.0%	100.0%	99.7%
Terminal rate	18/31 (58%)	18/18 (100%)	18/18 (100%)	2/2 (100%)
First incidence (days)	665	477	465	450
Poly-3 test	P<0.001	P<0.001	P<0.001	P<0.001
Hepatocholangiocarcinoma[k]	0	0	0	2

* Significantly different (P≤0.05) from the vehicle control group by the Poly-3 test
** P≤0.01
[a] Number of animals with lesion
[b] Average severity grade of lesions in affected animals: 1=minimal, 2=mild, 3=moderate, 4=marked
[c] Historical incidence for 2-year gavage studies with corn oil vehicle control groups (mean ± standard deviation): 267/514 (52.1% ± 14.9%); range, 25%-72%
[d] Number of animals with neoplasm per number of animals with liver examined microscopically
[e] Poly-3 estimated neoplasm incidence after adjustment for intercurrent mortality
[f] Observed incidence at terminal kill
[g] Beneath the vehicle control incidence are the P values associated with the trend test. Beneath the dosed group incidence are the P values corresponding to pairwise comparisons between the vehicle controls and that dosed group. The Poly-3 test accounts for differential mortality in animals that do not reach terminal sacrifice. A negative trend is indicated by **N**.
[h] Historical incidence: 2/514 (0.3% ± 1.0%); range, 0%-3%
[i] Historical incidence: 138/511 (26.8% ± 14.1%); range, 8%-58%
[j] Historical incidence: 0/511
[k] Historical incidence: 1/511 (0.2% ± 0.6%); range, 0%-2%

reaction-restriction fragment length polymorphism (PCR-RFLP), an organism compatible with *H. hepaticus* was identified in three other studies from which adequately preserved (frozen) liver tissue was available, including tissues from 4 of 14 mice in the methyleugenol study (Malarkey *et al.*, 1997; Fox *et al.*, 1999). However, mice from these three studies did not have *H. hepaticus*-associated hepatitis. In the methyleugenol study, the incidences of one of the lesions typically associated with *H. hepaticus* infection, oval cell hyperplasia, increased with increasing dose, which is not typical of the incidences in the other nine studies. In those studies, all groups, including control animals, were relatively equally affected. Also in the methyleugenol study, dose-related increases in the incidences of oval cell hyperplasia occurred in female mice and in the rats. F344/N rats have not been shown to be infected with *H. hepaticus*, and liver lesions in infected female B6C3F$_1$ mice from NTP studies are minimal to nonexistent; therefore, oval cell hyperplasia was attributed to administration of methyleugenol and was not considered to be related to the presence of *H. hepaticus*.

In the nine studies in which the hepatitis was present, spiral organisms consistent with *H. hepaticus* were identified within the livers of animals with liver lesions using the Warthin-Starry silver stain or Steiner's modification. However, not all animals, and especially not all females, within these nine studies had liver lesions, and organisms were not identified using these stains when hepatic lesions were not present. This was true despite positive findings with the PCR-RFLP-based assay in some of the animals. In addition, using Warthin-Starry silver stains on the livers of 12 male mice (two vehicle controls and ten 150 mg/kg males) from the methyleugenol study, no organisms consistent with *H. hepaticus* were identified. While mice from this study of methyleugenol were considered to be infected with *H. hepaticus* based on the PCR-RFLP-based assay, liver disease associated with the infection was not apparent, and the infection was not considered to have compromised the outcome of the toxicology and carcinogenesis studies of methyleugenol.

Glandular Stomach: Nonneoplastic lesions observed in male and female mice included glandular ectasia, mucosal atrophy, chronic active inflammation, epithelial hyperplasia, and neuroendocrine cell hyperplasia. In addition, malignant neuroendocrine tumors were observed in two 150 mg/kg male mice; one male in this group had a carcinoma. Lesions coexisted and were confined to the fundic region of the glandular stomach, affecting both the more superficial foveolar epithelium of the gastric pits and the deeper epithelium of the gastric glands.

A significant, dose-related increase in the incidences of mucosal atrophy of the glandular stomach occurred in male mice; the incidences of mucosal atrophy in 75 and 150 mg/kg female mice were also significantly greater than that in the vehicle controls (Tables 16, C5, and D5). The incidences of hyperplasia in 150 mg/kg males and 75 mg/kg males and females were significantly increased. The incidences of ectasia in all dosed groups of males and females were significantly increased, and the incidences in males increased with increasing dose. There was a dose-related increase in the incidences of chronic active inflammation in male mice, and these incidences in 75 and 150 mg/kg males were significantly greater than that in the vehicle controls; the incidences of this lesion were not increased in dosed females. Four males in the 150 mg/kg group had neuroendocrine cell hyperplasia.

Neuroendocrine cell hyperplasia was similar to that observed in the glandular stomach of rats and consisted of focal proliferations of ECL cells in small nests or clusters. These proliferations were confined to the glandular mucosa infiltrating between and displacing glandular epithelial cells.

Malignant neuroendocrine tumors were locally expansive invasive masses which caused moderate to marked thickening of the mucosa (Plate 17a). The neoplastic cells formed clusters, cords, or poorly defined glandular-like structures displacing and/or replacing the normal architecture of the glandular mucosa. The cells were large, polygonal to spindle-shaped with scant to abundant finely granular amphophilic cytoplasm and large pleomorphic, mostly vesicular nuclei that had one or more prominent nucleoli (Plate 17b). Invasion into the submucosa was extensive and occurred as nests of neoplastic neuroendocrine cells surrounded by moderate amounts of fibrous tissue. Infiltrates of lymphocytes, neutrophils, and macrophages were diffusely interspersed among the tumor cells. The carcinoma was an ulcerated,

TABLE 16
Incidences of Neoplasms and Nonneoplastic Lesions of the Glandular Stomach in Male and Female Mice in the 2-Year Gavage Study of Methyleugenol

	Vehicle Control	37 mg/kg	75 mg/kg	150 mg/kg
Male				
Number Examined Microscopically	49	48	49	50
Atrophy[a]	0	3 (1.0)[b]	35** (1.5)	45** (2.6)
Ectasia	13 (1.0)	25* (1.0)	40** (1.4)	49** (1.7)
Hyperplasia	0	1 (1.0)	15** (1.7)	20** (1.8)
Inflammation, Chronic Active	10 (1.0)	11 (1.1)	25** (1.2)	33** (1.3)
Neuroendocrine Cell, Hyperplasia	0	0	0	4 (3.0)
Carcinoma	0	0	0	1
Malignant Neuroendocrine Tumor	0	0	0	2
Female				
Number Examined Microscopically	45	49	46	45
Atrophy	0	0	10** (1.6)	10** (2.0)
Ectasia	14 (1.0)	33** (1.1)	31** (1.3)	38** (1.4)
Hyperplasia	0	1 (1.0)	5* (1.6)	2 (1.5)
Inflammation, Chronic Active	17 (1.1)	21 (1.0)	12 (1.0)	14 (1.1)
Neuroendocrine Cell, Hyperplasia	0	1 (1.0)	0	0

* Significantly different ($P \le 0.05$) from the vehicle control group by the Poly-3 test
** $P \le 0.05$
[a] Number of animals with lesion
[b] Average severity grade of lesions in affected animals: 1=minimal, 2=mild, 3=moderate, 4=marked

locally extensive, invasive mass that obliterated the mucosal architecture extending into and replacing the submucosa and much of the muscle layers of the stomach. The neoplastic cells occurred as sheets, clusters, and poorly defined cords and were anaplastic, polygonal to spindle-shaped cells with scant eosinophilic cytoplasm and large pleomorphic hyperchromatic nuclei.

One lesion observed in mice but not in rats consisted of focal to multifocal hyperplasia of the columnar foveolar epithelium of the gastric pits (Plate 18). This hyperplasia often extended toward the base of the mucosa with displacement of parietal and chief cells of the mucosal glands and was often accompanied by minimal to mild chief cell hyperplasia. Ectatic mucosal glands were sometimes present at the base of hyperplastic lesions. Ectasia was multifocal and consisted of variably dilated glands which were lined by flattened, often degenerate, epithelial cells (Plate 19). Many ectatic glands contained necrotic cells and cellular debris.

Atrophy of the glandular mucosa was histologically similar to that observed in the rat. It was characterized by loss of glandular epithelial cells (chief and parietal cells) in the fundic region of the stomach with concomitant reduction in the height of the fundic mucosa. The severity of atrophy was variable. Minimal atrophy consisted of the focal to multifocal loss of primarily chief cells in an area equivalent to the width of three glands. Mild atrophy consisted of multifocally extensive to diffuse loss of chief cells, whereas moderate to marked atrophy was characterized by diffuse loss of parietal and chief cells accompanied by a significant reduction in the height of the fundic mucosa. Minimal to mild chronic active inflammation was a consistent change associated with the gastric lesions.

It consisted of infiltrates of mononuclear leukocytes and variable numbers of neutrophils within the deep mucosa, lamina propria, and the submucosa of the glandular stomach.

Bone Marrow: In male and female mice, the incidences of bone marrow hyperplasia were significantly greater than those in the vehicle controls (males: vehicle control, 14/49; 37 mg/kg, 26/49; 75 mg/kg, 33/50; 150 mg/kg, 35/50; females: 18/50, 47/50, 46/48, 50/50; Tables C5 and D5); the incidences in male mice increased with increasing dose. Bone marrow hyperplasia was considered to be secondary to the changes in hepatic and gastric pathology.

GENETIC TOXICOLOGY

Methyleugenol, tested up to a maximum concentration of 666 μg/plate, did not induce mutations in *Salmonella typhimurium* strain TA98, TA100, TA1535, or TA1537, with or without induced hamster or rat liver S9 activation enzymes (Mortelmans *et al.*, 1986; Table E1). In tests for induction of chromosomal effects in cultured Chinese hamster ovary cells, methyleugenol induced sister chromatid exchanges in each of two replicate trials conducted with induced rat liver S9; no significant increase in sister chromatid exchanges was observed without S9 (Table E2). Also in cultured Chinese hamster ovary cells, no significant induction of aberrations occurred following incubation with methyleugenol in either the presence or the absence of S9 (Table E3). The doses tested in the aberrations test were similar to those used in the sister chromatid exchange test and were limited by toxicity to 233 μg/mL. Methyleugenol, administered in doses of 10 to 1,000 mg/kg by gavage to male and female B6C3F$_1$ mice for 14 weeks, did not increase the frequency of micronucleated normochromatic erythrocytes in peripheral blood and did not alter the percentage of polychromatic erythrocytes among total erythrocytes (an indication of bone marrow toxicity) (Table E4).

In conclusion, methyleugenol was not mutagenic in *S. typhimurium* and did not induce chromosomal damage in rodent cells *in vitro* or *in vivo*. A potential for methyleugenol-induced DNA damage was indicated by the positive results seen in the *in vitro* sister chromatid exchange test with Chinese hamster ovary cells in the presence of S9 activation enzymes.

PHYSIOLOGICALLY BASED PHARMACOKINETIC MODEL

A physiologically based pharmacokinetic (PBPK) model was developed to represent mathematically the absorption, distribution, metabolism, and elimination of methyleugenol in rats and mice (Appendix O). Data from plasma concentration time courses following intravenous and gavage administration of methyleugenol to rats and mice were used to develop the model (Appendix M). The model contains compartments for the blood, the gastrointestinal tissue, lumen, and capillary space, as well as capillary space and tissues for liver, kidney, fat, slowly perfused tissues (skin, muscle, and bone) and rapidly perfused tissues (heart, lungs, brain, and viscera). Methyleugenol entered the blood stream either directly from intravenous injection or from absorption from the gastrointestinal lumen into the portal circulation, with equilibration into gastrointestinal tissues. Plasma concentrations were assumed to be identical with blood concentrations. The absorption of methyleugenol was modeled as occurring from a single gastrointestinal lumenal compartment into the gastrointestinal capillary space, equilibrating with gastrointestinal tissues, and then entering the liver capillary space via the portal blood flow. The distribution of methyleugenol between each tissue and its capillary bed was explicitly modeled, a departure from the more common practice of assuming instantaneous equilibration of capillary and arterial blood in PBPK models. Tissue:blood partition coefficients were estimated from the octanol-water partition coefficient of methyleugenol. Other chemical-specific parameters were estimated from the plasma concentration time course data. The metabolism of methyleugenol in rats was confined to the liver and represented as a Michaelis-Menten process. The metabolism of methyleugenol in mice was confined to the liver and kidney (as a representative tissue with significant phase 1 metabolic capacity) and was also represented as a Michaelis-Menten process, with the same K_m for both tissues. The lack of a need for extrahepatic metabolism in the rat model does not lead to the conclusion that extrahepatic metabolism of methyleugenol is not important, only that the blood flow and metabolic capacity modeled for rats was sufficient to act as a surrogate for all methyleugenol metabolism.

The rat and mouse models were used to predict methyleugenol pharmacokinetic behavior from single oral doses of 37, 75, and 150 mg/kg, doses used in the chronic study. The maximum concentration (C_{max}) and the integrated concentration of methyleugenol in the liver (AUC_{liver}) over the 24-hour period following oral dosing were simulated, as was the integrated rate of hepatic metabolism (AUC_{met}) over the same 24-hour period. In both rats and mice, over 90% of administered methyleugenol was determined to have been metabolized within 24 hours. In both rats and mice, C_{max} and AUC_{liver} increased more rapidly than administered dose, while AUC_{met} increased linearly with administered dose. These results indicate that metabolism of methyleugenol is saturating at doses used in the chronic study.

Methyleugenol, NTP TR 491

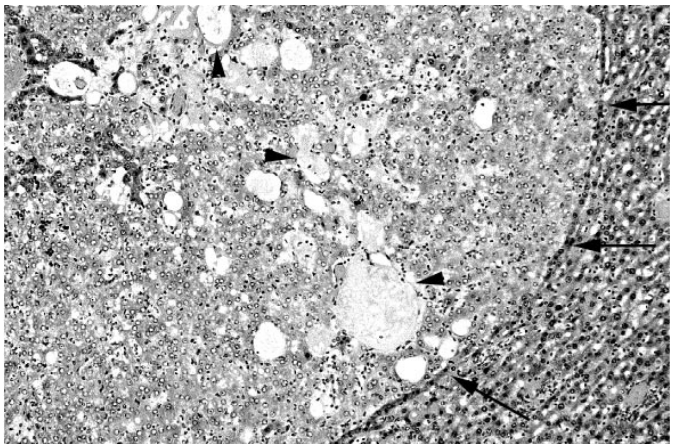


PLATE 1
Hepatocellular adenoma from a male rat exposed to 300 mg/kg methyeugenol. Note sharp demarcation from the adjacent hepatic parenchyma (arrows), loss of normal architecture and areas of cystic degeneration (arrowheads) within the adenoma. H&E; 25×.

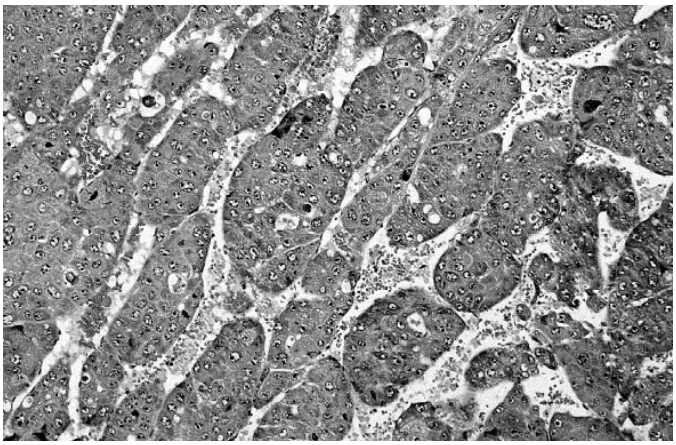


PLATE 2
Hepatocellular carcinoma from a male rat exposed to 300 mg/kg methyeugenol. Note distinct trabecular pattern characterized by thick trabeculae composed of three or more cell layers of neoplastic hepatocytes. H&E; 33×.

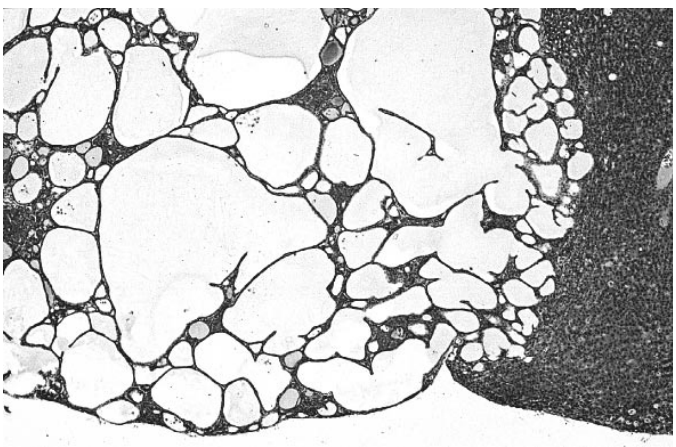


PLATE 3
Cholangioma from a male rat exposed to 300 mg/kg methyeugenol. The multilocular cholangioma is well-demarcated from the surrounding hepatic parenchyma and is composed of multiple cystic spaces of variable sizes. H&E; 8×.

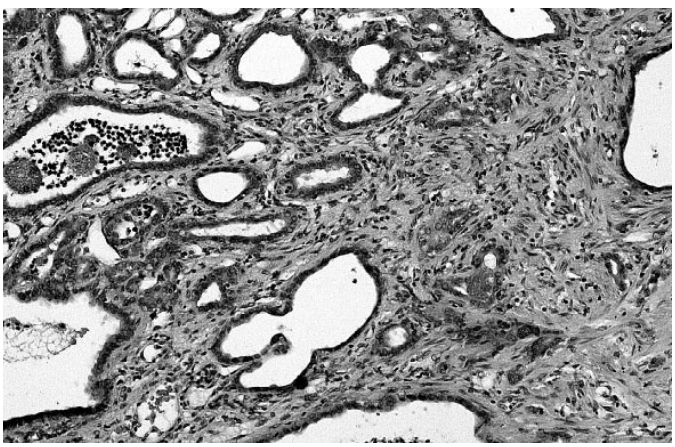


PLATE 4
Cholangiocarcinoma from a female rat exposed to 300 mg/kg methyeugenol. Note irregular ductular structures lined by cuboidal biliary-like epithelial cells and surrounded by abundant connective tissue stroma. H&E; 40×.

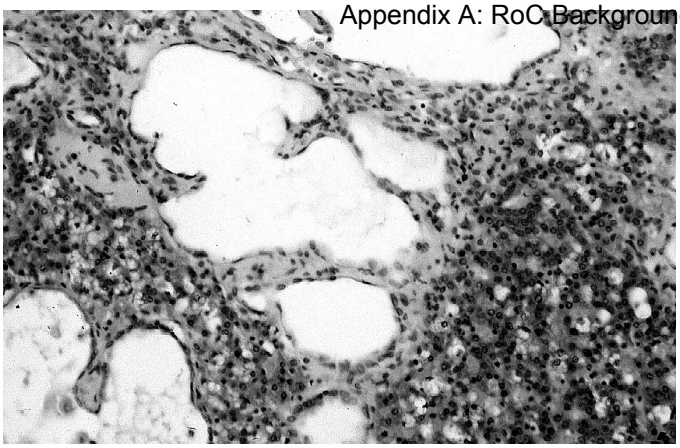


PLATE 5
Hepatocholangioma from a male rat exposed to 300 mg/kg methyeugenol. H&E; 50×.

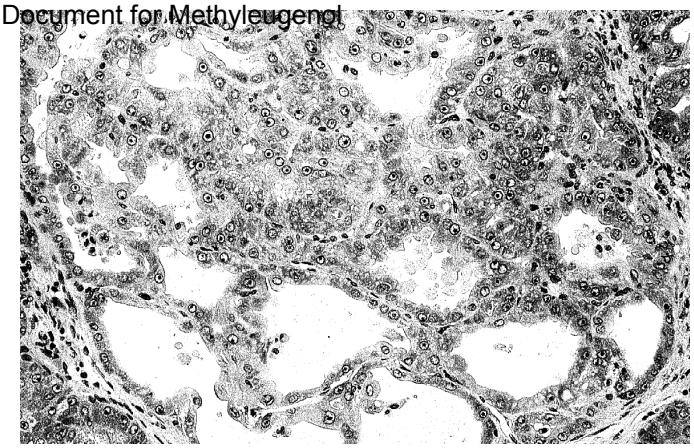


PLATE 6
Hepatocholangiocarcinoma from a male rat exposed to 300 mg/kg methyeugenol. Poorly-differentiated hepatocytes and biliary epithelium form glandular structures. H&E; 50×.

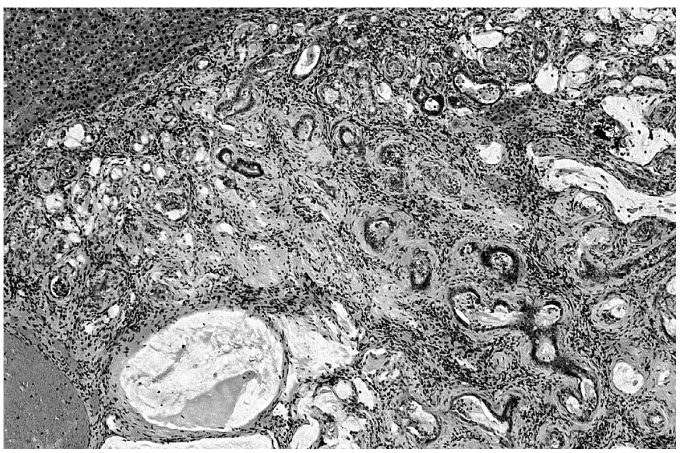


PLATE 7
Atypical bile duct hyperplasia from a male rat exposed to 3000 mg/kg. Note multiple atypical bile ducts surrounded by connective tissue stroma. H&E; 20×.

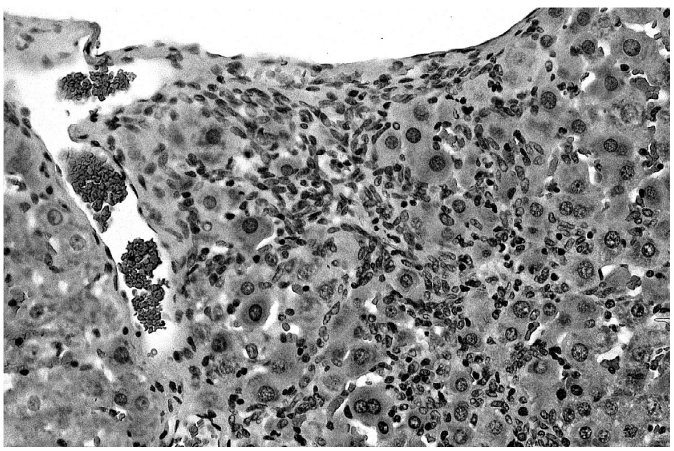


PLATE 8
Oval hyperplasia in a male rat exposed to 300 mg/kg methyleugenol. Note small ovoid cells proliferating within the hepatic sinusoids. Note also nuclei of enlarged (hypertrophic) hepatocytes. H&E; 66×.

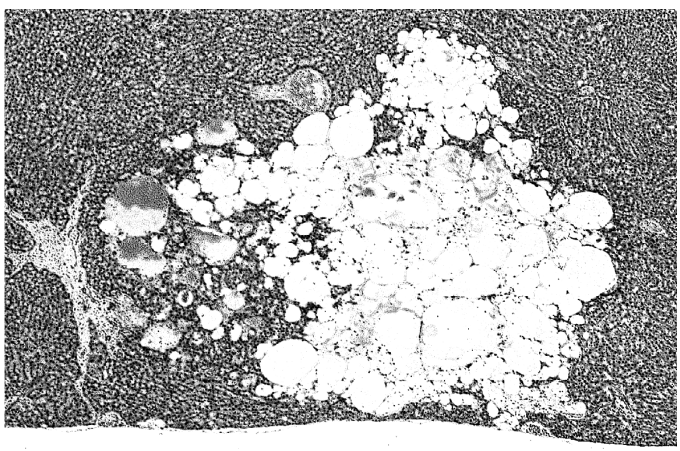


PLATE 9
Focal cystic degeneration from a male rat exposed to 300 mg/kg methyleugenol. Note characteristic multiple cyst-like spaces which contain flocculent material. H&E; 13×.

Methyleugenol, NTP TR 491

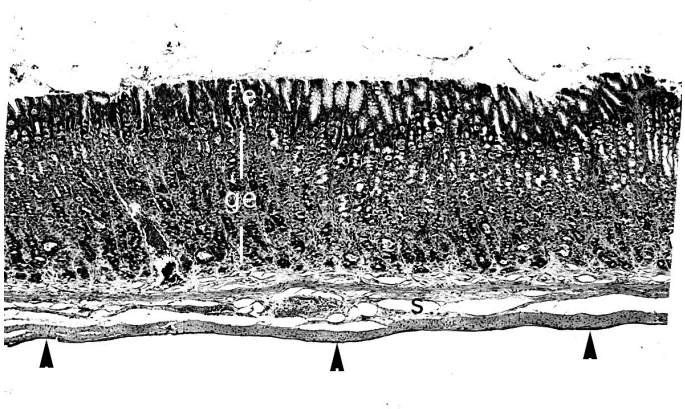


PLATE 10a
Control rat, fundic region of the glandular stomach. Foveolar epithelium of the gastric pits (fe), mucosal glandular epithelium (ge), submucosa (s), tunica muscularis (arrowheads). H&E; 16×.

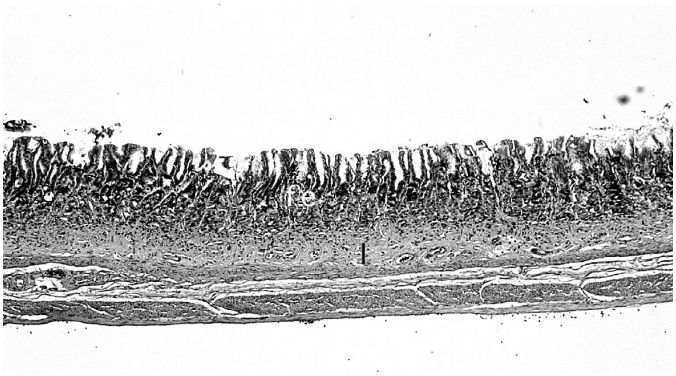


PLATE 10b
Atrophy in the glandular stomach of a female rat exposed to 300 mg/kg methyleugenol. Note decrease in the height of the mucosa due to loss of the mucosal glands with condensation and fibrosis of the lamina propria (l). H&E; 16×.

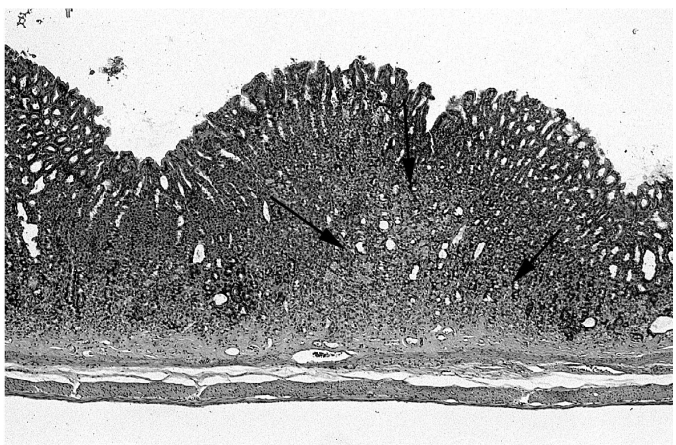


PLATE 11a
Focal neuroendocrine cell hyperplasia (arrows) in the glandular stomach of a male rat exposed to 300 mg/kg methyleugenol. H&E; 13×

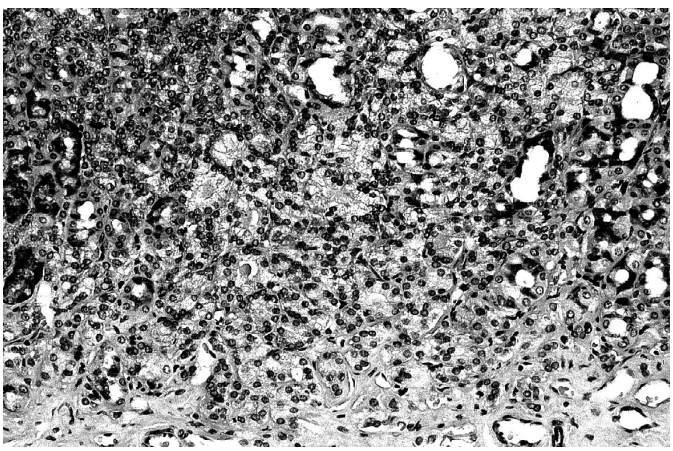


PLATE 11b
Detail of Plate 12. Hyperplastic cells have pale finely granular cytoplasm, small round nuclei and form small poorly-delineated glandular structures. H&E; 50×.

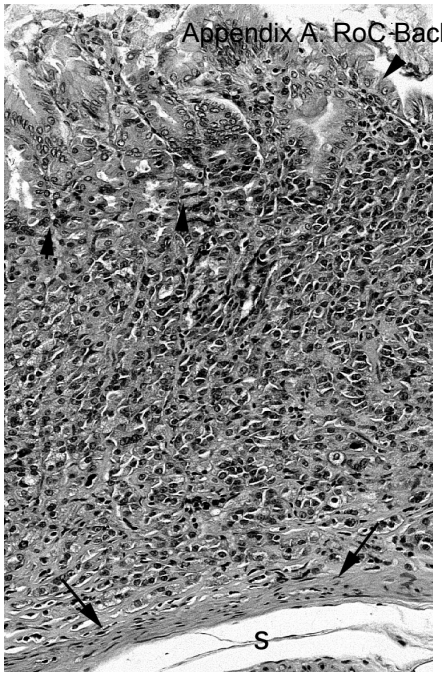


PLATE 12
Benign neuroendocrine tumor in the glandular stomach of a female rat exposed to 300 mg/kg methyleugenol. Neoplastic neuroendocrine cells have displaced and replaced glandular elements but are confined to the mucosa. Note the foveolar epithelium of the gastric pits (arrowheads) and the muscularis mucosa (arrows) separating the mucosa from the submucosa (s). H&E; 50×.

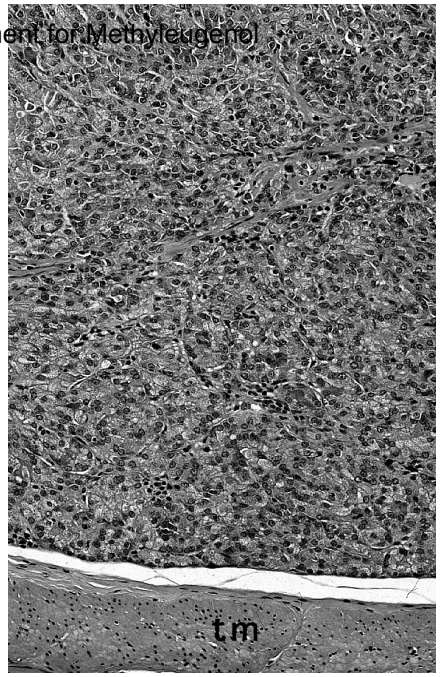


PLATE 13
Malignant neuroendocrine tumor in the glandular stomach of a female rat exposed to 150 mg/kg methyleugenol. Neoplastic neuroendocrine cells have completely replaced the gastric glands, muscularis mucosa and submucosa. Tunica muscularis (tm) H&E; 40×.

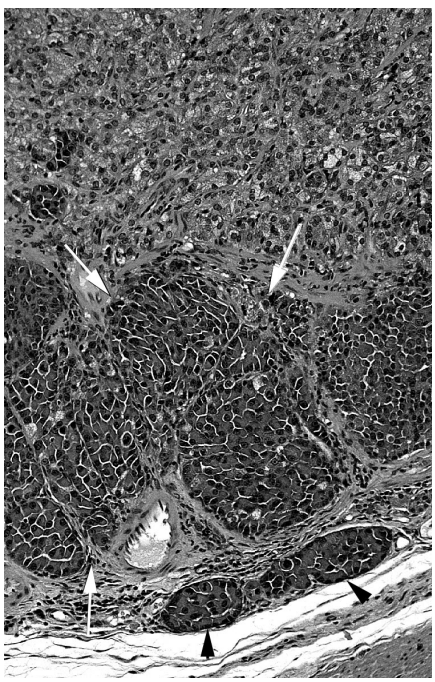


PLATE 14
Malignant neuroendocrine tumor in the glandular stomach of a female rat exposed to 300 mg/kg methyleugenol. Neoplasm is composed of a mixture of neoplastic neuroendocrine cells and nodular aggregates of atypical polygonal cells (arrows). Note neoplastic cell emboli within submucosal blood vessels (arrowheads). H&E; 40×.

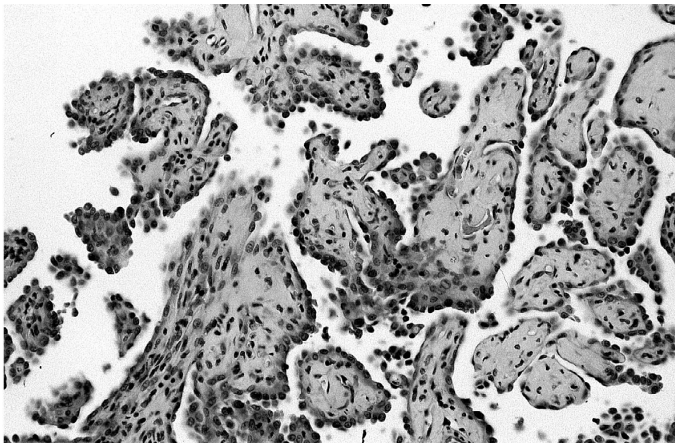


PLATE 15
Malignant mesothelioma from the mesentery of a male rat exposed to 300 mg/kg methyleugenol. Mesothelioma is composed of papilliferous proliferations of connective tissue stroma lined by cuboidal mesothelial cells. H&E; 50×.

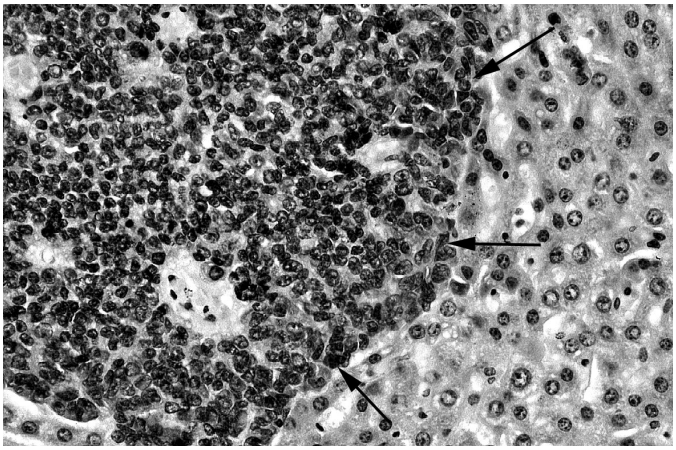


PLATE 16
Hepatoblastoma from a female mouse exposed to 150 mg/kg methyleugenol. Note sharp demarcation from the adjacent hepatic parenchyma (arrows), and characteristic cords of small round to spindyloid tumor cells with dark staining nuclei. H&E; 80×.

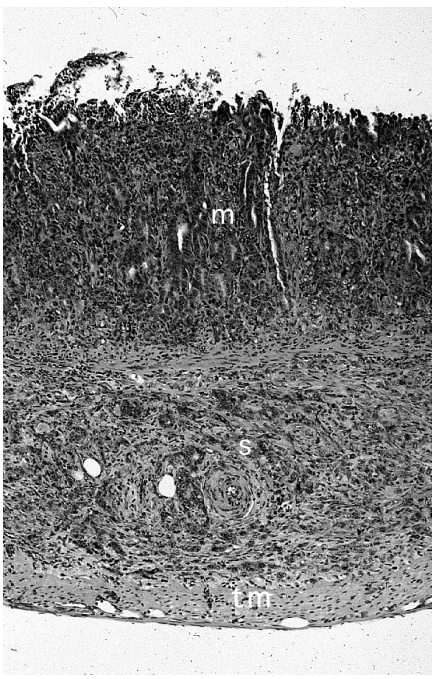


PLATE 17a
Malignant neuroendocrine tumor from the glandular stomach of a male rat exposed to 150 mg/kg methyleugenol. a) The neoplasm has effaced the mucosa (m) and has invaded and expanded the submucosa (s). Tunica muscularis (tm) H&E; 25×.

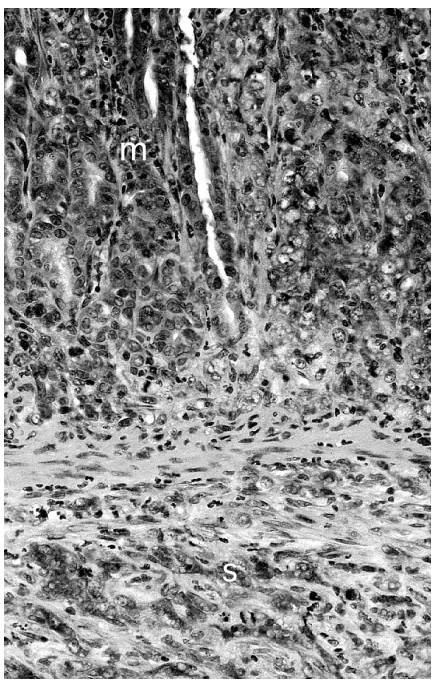


PLATE 17b
Detail of (a) showing neoplastic neuroendocrine cells within the mucosa (m) and invading submucosa (s). H&E; 66×.

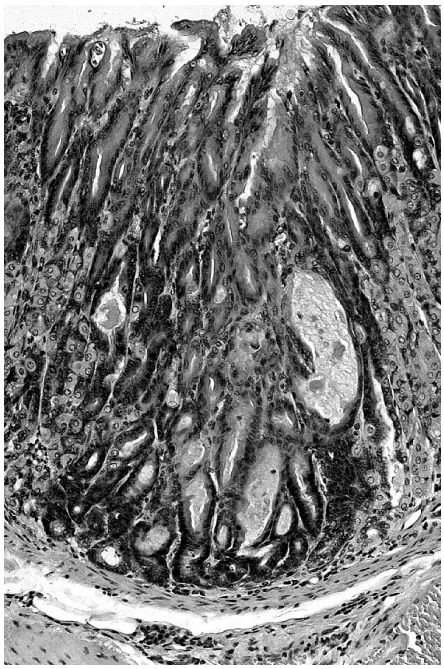


PLATE 18
Focal epithelial hyperplasia in the glandular stomach of a male rat exposed to 150 mg/kg methyleugenol. The hyperplastic epithelium extends from the mucosal surface to the muscularis mucosa. Note dilated (ectatic) glands within the hyperplastic focus. H&E; 40×.

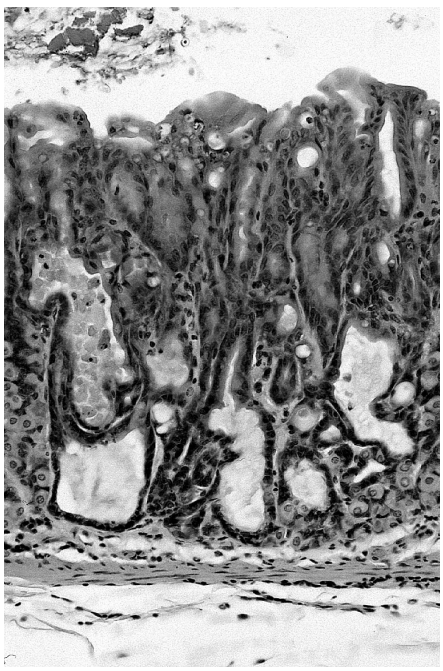


PLATE 19
Multifocal glandular ectasia in stomach of a male rat exposed to 150 mg/kg methyleugenol. The ectatic glands contain necrotic cellular debris. H&E; 50×.

DISCUSSION AND CONCLUSIONS

Toxicology and carcinogenesis studies of methyleugenol were conducted because of potential widespread human exposure and because of its structural resemblance to safrole, a known rodent carcinogen (IARC, 1976). Fourteen-week and 2-year toxicology and carcinogenicity studies were conducted by administering methyleugenol in 0.5% aqueous methylcellulose by gavage to male and female F344/N rats and $B6C3F_1$ mice. Although the principal route of human exposure is via food, the gavage route of administration was used because results of preliminary NTP studies showed methyleugenol in feed to be unpalatable to rats and mice (Battelle, 1997).

Principal findings in the 14-week and 2-year studies of methyleugenol included reduced survival rates (except in 14-week rats), depressed mean body weight gains, and marked liver and glandular stomach lesions in rats and mice at the higher doses. In the 2-year studies, the survival rates of male rats administered 150 or 300 mg/kg and of all dosed groups of female mice were reduced relative to that of the vehicle controls. However, because the majority of the deaths occurred late in the study (after week 85) and were due to liver and glandular stomach neoplasms in rats and liver neoplasms in mice, the doses used for the 2-year studies were considered adequate for evaluating the carcinogenicity of methyleugenol.

The dose-related decreases in mean body weight gain of rats and mice were likely related to the toxicity of methyleugenol to the liver and the glandular stomach. Toxic effects in the liver may cause a derangement in protein, carbohydrate, or fat metabolism with subsequent depression in body weight gain. The hypoproteinemia and hypoalbuminemia observed in dosed rats in the 14-week study suggest that methyleugenol impaired hepatic protein synthesis. Decreases in serum protein concentrations can be caused by several factors, including impaired hepatic protein synthesis (Kaneko, 1989; Nguyen, 1989). The effect of methyleugenol on body weight appears to be irreversible as indicated by the lack of recovery in rats in the stop-exposure groups when dosing was discontinued for 12 months. Methyleugenol-induced glandular stomach atrophy, characterized by loss of parietal and chief cells, may also have contributed to depression in body weight gain. Parietal cells produce gastric acid (hydrochloric acid), and G cells produce proenzymes such as pepsinogen (Guyton and Hall, 1997). The loss of parietal cells results in hypochlorhydria, increased pH, and decreased activities of acid-activated digestive enzymes such as pepsin, trypsin, and chymotrypsin, which in turn lead to inefficient utilization of food and depression in body weight. In addition, the loss of chief cells results in reduced pepsinogen (pepsin precursor) production, which will also lead to inefficient food utilization. Methyleugenol administration caused a significant increase in the stomach pH of female F344/N rats receiving 1,000 mg/kg by gavage for 30 days or 150 mg/kg or greater for 90 days (Appendix P).

In the 14-week studies, the increases in liver weights of rats and mice and the increase in testis weight of rats administered 1,000 mg/kg were the only organ weight differences considered to be related to methyleugenol administration. The increases in liver weights may have been due in part to the induction of cytochrome P_{450} and P_{448} enzyme systems. Allylbenzenes, including methyleugenol, are potent inducers of these enzyme systems (Ioannides *et al.*, 1981; Gardner *et al.*, 1997). The increase in liver and testis weights of dosed animals may also have been caused by toxicity related to the formation of methyleugenol adducts with proteins. A protein adduct was detected in the microsomal fraction of the liver of F344 rats administered methyleugenol intraperitoneally (Gardner *et al.*, 1996).

Hepatocellular injury caused by methyleugenol administration was evidenced by increases in serum alanine aminotransferase and sorbitol dehydrogenase activities and bile acid concentration in dosed rats in the 14-week study. Liver lesions induced by methyleugenol in rats and mice included cytologic alteration, bile duct hyperplasia, necrosis (mice), foci of hepatocellular alteration, hepatocyte hypertrophy, and oval

cell hyperplasia. The oval cell hyperplasia and hepatocellular hypertrophy observed in rats at 6 and 12 months persisted until the end of the stop-exposure study, suggesting that these lesions are irreversible.

Methyleugenol caused increased incidences of liver neoplasms in rats and mice. The increases were marked, particularly in male rats and female mice, which had high rates of metastasis of these neoplasms to the lungs. The vast majority of the neoplasms that occurred were primarily composed of neoplastic hepatocytes (hepatocellular adenomas and carcinomas). Additionally, in rats, a large group of neoplasms consisting of an admixture of what appeared to be hepatocytes and biliary epithelium were classified as hepatocholangioma or hepatocholangiocarcinoma. These are uncommon spontaneous neoplasms in F344/N rats. From a histomorphologic and biologic standpoint, classification of liver neoplasms into this particular category is not always straightforward. Some prefer to classify most neoplasms with this morphology as glandular variants of hepatocellular neoplasms. In addition, three neoplasms composed of neoplastic biliary epithelium were identified in the 300 mg/kg rats (two males with cholangioma and one female with cholangiocarcinoma). These are uncommon spontaneous neoplasms in F344/N rats.

While studied extensively, the exact histogenesis of liver neoplasms remains controversial. Some recent literature suggests that a putative stem cell is likely to play an important role in the development of some liver neoplasms (Sell and Dunsford, 1989). Oval cells are considered to represent or arise from the stem cell and are thought to have the potential to develop into hepatocytes and/or biliary epithelial cells (Factor *et al.*, 1994). Oval cell proliferation was increased in dosed animals in the studies of methyleugenol. Some rat liver tumorigens cause an increase in the incidence of a relatively pure population of hepatocellular neoplasms, while others cause increased incidences of both hepatocellular and cholangiolar neoplasms (Maronpot *et al.*, 1991). It is possible that the spectrum of neoplasms induced by methyleugenol represents a combination of these effects. The proliferative lesions observed in the studies are considered related to administration of methyleugenol.

As is frequently observed in many NTP studies in which robust liver neoplasm responses occur in mice, the incidences of hepatoblastoma in the liver were increased. While the increases are usually most pronounced in male mice, in the current study the increase was more pronounced in female mice. Hepatoblastomas in the mouse are uncommon neoplasms which occur spontaneously or may be chemically induced in the liver of several strains (Turusov *et al.*, 1973; Nonoyama *et al.*, 1988), including the $B6C3F_1$ mouse used in NTP studies. It is considered a malignant neoplasm, and in NTP studies, its metastatic potential appears similar to that of hepatocellular carcinomas. Hepatoblastomas are easily diagnosed because of their distinctive morphology on hematoxylin- and eosin-stained sections and were typical in this study.

Hepatoblastomas almost always occur within an existing proliferative lesion, most often within a hepatocellular carcinoma, and when that occurs in NTP studies, the entire proliferative lesion is diagnosed as a hepatoblastoma. The cell of origin of the hepatoblastoma has not been clearly defined in rodents or humans, but it may be a very primordial cell (Abenoza *et al.*, 1987; van Eyken *et al.*, 1990; Stocker, 1994). Although the histogenesis is not fully understood, the hepatoblastoma is considered to be part of the spectrum of liver neoplasms that occurs both spontaneously and as a result of chemical treatment. Whereas individual analyses are informative, the NTP considers the combinations of hepatocellular carcinoma or hepatoblastoma and of hepatocellular adenoma, hepatocellular carcinoma, or hepatoblastoma to be the most important in evaluating the carcinogenic potential of an agent on the liver.

In the stop-exposure groups, the incidences of liver neoplasms observed after the administration of methyleugenol was discontinued were greater than those observed at 12 months or in the vehicle control groups at 2 years. Similarly, the increased incidences of oval cell hyperplasia and hepatocellular atrophy observed in rats at 6 and 12 months persisted until the end of the stop-exposure study. These results suggest that the effect of methyleugenol on the liver is irreversible and that the preneoplastic lesions produced are likely to progress and produce neoplasms in the absence of continued chemical exposure.

The finding that methyleugenol induced hepatic neoplasms is consistent with findings observed for other chemicals with structures resembling that of

methyleugenol; these chemicals include estragole, safrole, and isosafrole (IARC, 1976; Miller *et al.*, 1983; CCRIS, 1998). Methyleugenol also was found to induce hepatic neoplasms in mice (Miller *et al.*, 1983). Eugenol was not a hepatocarcinogen in the Miller *et al*. (1983) studies and gave equivocal results in the NTP (1983) studies. Miller *et al*. (1983) showed that the 1′-hydroxy metabolites of these chemicals are more potent hepatocarcinogens than the parent compounds. Studies from the same laboratory showed the importance of sulfation in the hepatotumorigenicity of the 1′-hydroxy metabolites (Boberg *et al.*, 1983). These researchers found that the tumorigenicity of 1′-hydroxysafrole can be inhibited by sulfotransferase inhibitors and can be markedly reduced in brachymorphic mice (mice with diminished sulfation capacity).

There were increased incidences of neuroendocrine tumors of the glandular stomach in dosed male and female rats in the 2-year study. Malignant neuroendocrine tumors of the glandular stomach also occurred in two male mice in the 150 mg/kg group in the 2-year study. Because these neoplasms have not been observed previously in NTP gavage studies and because the incidences of these neuroendocrine tumors occurred with a positive trend in rats and male mice and were significantly increased in dosed rats, these neoplasms in both rats and male mice were considered to be related to methyleugenol administration.

The increased incidences of neuroendocrine tumors were also generally associated with significant dose-related increases in the incidences of glandular stomach atrophy and neuroendocrine cell (enterochromaffin-like cell) hyperplasia. Gastric atrophy was observed in both the 14-week and 2-year studies. The atrophy was characterized by the thinning of the fundic mucosa due to a loss of parietal cells and/or chief cells. As mentioned previously, the loss of parietal cells results in decreased gastric acid secretion. Reduced acid production and increased pH in the stomach are known to lead to gastrin production. Increases in stomach pH and serum gastrin levels occurred in female F344/N rats receiving up to 150 mg/kg and increases in serum gastrin levels occurred in male $B6C3F_1$ mice receiving 75 mg/kg or greater (Appendix P). Many reports have shown that long-term exposure to inhibitors of gastric acid secretion leads to induction of enterochromaffin-like cell tumors (Poynter and Selway, 1991; Johnson *et al.*, 1993; Thake *et al.*, 1995); the magnitude of the proliferative response varies with the compound. For the most part, compounds in these reports are considered nongenotoxic, and there is a direct inhibitory effect on gastric parietal cells which results in decreased hydrochloric acid production.

In a study of butachlor (Thake *et al.*, 1995), atrophy of the fundic mucosa with a loss of parietal cells occurred in rats along with hypochlorhydria and hypergastrinemia. Thake *et al*. (1995) postulated that the neuroendocrine tumors of the glandular stomach produced by butachlor were the result of long-term gastrin stimulation of the enterochromaffin-like cells. Because fundic mucosal atrophy was observed in the current studies of methyleugenol, it is probable that hypochlorhydria and hypergastrinemia played a role in the neuroendocrine cell proliferative response. In the 14-week studies of methyleugenol, there was evidence of inflammation, cellular degeneration, and necrosis in addition to gastric mucosal atrophy, suggesting that parietal cell cytotoxicity may have preceded mucosal atrophy. In additional studies of methyleugenol performed by the NTP (Appendixes P and Q), there were chemical-related increases in stomach pH, serum gastrin, and cell proliferation in the fundic glands. These changes suggest that the mechanisms for neuroendocrine tumor induction by methyleugenol and butachlor are similar. However, methyleugenol has some genotoxic activity that should also be considered when attempting to determine the pathogenesis of the neuroendocrine proliferative response.

The high lipophilicity and the extremely rapid absorption of methyleugenol may explain the toxicity of this chemical to the liver and the stomach of rats and mice. The octanol-water partition coefficient for methyleugenol was estimated to be 800, indicating that the chemical is lipophilic and can pass cell membranes easily (Battelle, 1998). Furthermore, the toxicokinetic data for rats and mice showed that the time to achieve maximum concentration in the blood was short (approximately 5 to 15 minutes). Because maximum blood concentrations were reached long before the stomach could have emptied, it is concluded that the chemical was absorbed from the stomach. This conclusion is supported by the damaging effects of

methyleugenol administration to the glandular stomach. The rapid absorption of methyleugenol suggests that the chemical was transported in a bolus dose via the portal vein to the liver, thus causing the severe hepatic effects observed in dosed rats and mice. Methyleugenol is metabolized by the cytochrome P450 system (Borchert *et al.*, 1973). According to Solheim and Scheline (1976), the metabolism involves *O*-demethylation, side-chain hydrolysis, and diol formation. Of the metabolites formed, the two reactive metabolites 1′-hydroxymethyleugenol and the epoxide diol were most likely to be responsible for the toxic effects at these two sites. Like the hepatocarcinogen safrole, methyleugenol showed DNA-binding activity in *in vitro* rat liver slices only in the presence of metabolic activation (unpublished NTP data). The adduct-forming activity of methyleugenol may have been a contributing factor to its hepatotumorigenic activity. Devereux *et al.* (1999) investigated the somatic mutations of β-catenin (a regulator of the cadherin-mediated cell-adhesion system in the Wnt signal transduction pathway) in hepatocellular adenomas and carcinomas in methyleugenol-treated and control $B6C3F_1$ mice (Appendix R). The majority of methyleugenol-induced neoplasms showed β-catenin mutations. Identical mutations have been found in human hepatocellular neoplasms (de la Coste *et al.*, 1998), suggesting similar pathways of carcinogenesis in both species. Accumulations of cytoplasmic β-catenin, which result from mutations of the β-catenin gene, are causatively associated with colon cancer (Takahashi *et al.*, 1998).

In the 2-year study, in addition to liver and glandular stomach neoplasm induction, methyleugenol caused increases in the incidences of other neoplasms in dosed rats including kidney neoplasms (males), malignant mesothelioma, mammary gland fibroadenoma, subcutaneous fibroma (males), and subcutaneous fibroma or fibrosarcoma (combined). Because the incidences of these neoplasms occurred with dose-related trends or were markedly increased in certain dosed groups at rates greater than the historical control rates for corn oil gavage studies, the increases were considered to be related to methyleugenol administration. The marked liver and stomach neoplasm responses may have limited the expression of these additional neoplasms in groups administered 75 mg/kg or greater. The possible mechanisms of tumorigenesis of methyleugenol in these organs are unknown.

The incidence of forestomach squamous cell papilloma or carcinoma (combined) in the 150 mg/kg female rats was not significantly different from that in the vehicle controls. However, because the incidence in this group exceeded the historical vehicle control range for corn oil gavage studies, the increase in the incidence of these neoplasms may have been related to methyleugenol administration.

The increased eosinophilic granularity (cytoplasmic alteration) in the submandibular salivary gland in dosed rats is considered secondary to the toxic effects of methyleugenol on the glandular stomach. Dietary factors such as protein starvation are known to cause loss of zymogen granules (McBride *et al.*, 1987). Methyleugenol administration in rats may have created such a condition due to loss of parietal and chief cells of the glandular stomach. Acid conditions and production of protein digesting enzymes are the functions of these two types of cells, respectively. Loss of these cells could inhibit proper protein utilization and essentially lead to protein deficiency.

There were increases in the incidences of bone marrow hyperplasia in dosed rats and mice and hematopoietic proliferation in the liver of mice. These increased incidences were considered to be secondary to the increased incidences of necrosis and inflammation associated with the large and multiple liver neoplasms in dosed animals.

Uterine atrophy observed in rats administered 300 or 1,000 mg/kg in the 14-week study is likely due to the depressed mean body weights of these animals. Testicular dilatation observed in all male rats in the 1,000 mg/kg group was considered to be related to chemical administration. However, the underlying mechanism of the induction of these lesions by methyleugenol is not known. None of the lesions observed in dosed rats or mice were observed in similar NTP studies in which rats and mice were given up to 12,500 ppm eugenol (a structurally related chemical) in feed (NTP, 1983). No reports indicating that the glandular stomach, adrenal gland, testis, or uterus of rats were sites of toxicity for alkenylbenzenes with structural resemblances to methyleugenol were found in the literature.

The limited genotoxicity data for methyleugenol include negative results in *Salmonella typhimurium*

gene mutation assays (Sekizawa and Shibamoto, 1982; Mortelmans *et al.*, 1986; Kettering and Torabinejad, 1995), with and without liver S9 activation enzymes, and negative results in mammalian cell chromosome damage tests *in vitro* and *in vivo*. However, there was induction of unscheduled DNA synthesis (DNA repair) in human and rodent hepatocytes exposed *in vitro* and *in vivo* (Phillips *et al.*, 1984; Howes *et al.*, 1990; Chan and Caldwell, 1992; Gardner *et al.*, 1997). This may be important in some of the neoplastic responses observed in dosed male and female rats and mice in these studies of methyleugenol. The no-observed-effect level for methyleugenol for rats and mice was not reached in either the 14-week or the 2-year studies but could be well below 37.5 mg/kg, the lowest dose used in these studies.

CONCLUSIONS

Under the conditions of these 2-year gavage studies, there was *clear evidence of carcinogenic activity** of methyleugenol in male and female F344/N rats based on the increased incidences of liver neoplasms and neuroendocrine tumors of the glandular stomach in male and female rats and the increased incidences of kidney neoplasms, malignant mesothelioma, mammary gland fibroadenoma, and subcutaneous fibroma and fibroma or fibrosarcoma (combined) in male rats. A marginal increase in the incidence of squamous cell neoplasms of the forestomach may have been related to methyleugenol administration in female rats. There was *clear evidence of carcinogenic activity* of methyleugenol in male and female $B6C3F_1$ mice based on the increased incidences of liver neoplasms. Neuroendocrine tumors of the glandular stomach in male mice were also considered related to methyleugenol administration.

In male and female rats and mice, methyleugenol administration caused significant increases in the incidences of nonneoplastic lesions of the liver and glandular stomach.

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* Explanation of Levels of Evidence of Carcinogenic Activity is on page 14. A summary of the Technical Reports Review Subcommittee comments and the public discussion on this Technical Report appears on page 16.

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