FINAL

Report on Carcinogens Background Document for

Dyes Metabolized to 3,3′-Dimethylbenzidine

Meeting of the
NTP Board of Scientific Counselors
Report on Carcinogens Subcommittee

Prepared for the:
U.S. Department of Health and Human Services
Public Health Service
National Toxicology Program
Research Triangle Park, NC 27709

Prepared by:
Technology Planning and Management Corporation
Canterbury Hall, Suite 310
4815 Emperor Blvd
Durham, NC 27703
Contract Number NOI-ES-85421

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

US 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

Dyes Metabolized to 3,3'-Dimethylbenzidine (3,3'-Dimethylbenzidine Dye Class)

Carcinogenicity

3,3'-Dimethylbenzidine-based dyes that are metabolized to 3,3'-dimethylbenzidine are *reasonably anticipated to be human carcinogens* based on the facts that 3,3'-dimethylbenzidine is carcinogenic in male and female rats (IARC 1972; NTP 1991b, 1998) and that metabolism of 3,3'-dimethylbenzidine-based dyes to release free 3,3'-dimethylbenzidine is a generalized phenomenon, occurring in all species studied (Lynn *et al*. 1980; Bowman *et al*. 1982). Additional evidence of the carcinogenicity of this dye class is the fact that a representative 3,3'-dimethylbenzidine-based dye, C.I. Acid Red 114, is carcinogenic in male and female rats (NTP 1991a). Further, the pattern of tumors observed with C.I. Acid Red 114 (NTP 1991a) and 3,3'-dimethylbenzidine (NTP 1991b) is similar to that observed with the structurally similar chemical 3,3'-dimethoxybenzidine (NTP 1992) and the 3,3'-dimethoxybenzidine-based dye C.I. Direct Blue 15 (NTP 1992). Most notably, each of these four chemicals induces increased incidences of tumors in skin, Zymbal gland, liver, oral cavity, gastrointestinal tract, preputial gland of male rats, and clitoral gland of female rats, among other tissue sites.

No adequate human studies of the relationship between exposure to 3,3′-dimethylbenzidine-based dyes and human cancer have been reported.

Other Information Relating to Carcinogenesis or Possible Mechanisms of Carcinogenesis

3,3'-Dimethylbenzidine is structurally similar to benzidine, a known human carcinogen (IARC 1972, 1979, 1982, and 1987; NTP 1997, 1998) and 3,3′-dimethoxybenzidine, which is reasonably anticipated to be a human carcinogen (IARC 1974; NTP 1992, 1998). Like benzidine and 3,3'-dimethoxybenzidine, 3,3'-dimethylbenzidine is used as a base chemical from which many dyes are synthesized. These dyes are synthesized by linking of various chromophores to the base chemicals via azo linkages. Regardless of the chromophore(s) involved, the azo linkages of 3,3'-dimethylbenzidine-based dyes are chemically equivalent and are readily cleaved by chemical or enzymatic reduction to form free 3,3'-dimethylbenzidine and the chromophore(s). Reductive cleavage of 3,3'-dimethylbenzidine-based dyes to yield free 3,3'-dimethylbenzidine is catalyzed by a number of bacteria, including *Escherichia coli*, found in the human gastrointestinal tract (Cerniglia *et al*. 1982, Morgan *et al.* 1994). Reductive cleavage of 3,3'-dimethylbenzidine-based dyes to 3,3'-dimethylbenzidine also was shown in studies with rats, dogs, and hamsters (Lynn *et al*. 1980, Bowman *et al*. 1983, Nony *et al*. 1983). Metabolism of the dyes to free 3,3'-dimethylbenzidine in animals is thought to be mediated primarily by bacteria in the gastrointestinal tract (Cerniglia *et al.* 1982, Morgan *et al*. 1994). 3,3'-Dimethylbenzidine-based dyes are mutagenic in bacteria when tested with metabolic activation and an azo-reductive preincubation protocol (NTP 1991a). It is

assumed that the reductive system results in the formation of 3,3'-dimethylbenzidine, a known bacterial mutagen (Haworth *et al.* 1983).

No information exists to suggest that the mechanism of carcinogenesis of these substances operating in rats would not also operate in humans.

Table of Contents

Criteria for Listing Agents, Substances or Mixtures in the Report on Carcinogens i
Summary Statement iii
1 Introduction 1
1.1 Chemical Identification 1
1.2 Physical-chemical properties 3
1.3 Identification of metabolites 4
2 Human Exposure 5
2.1 Use 5
2.2 Production 5
2.3 Analysis 5
2.4 Environmental occurrence 6
2.5 Environmental fate 6
2.5.1 Air 6
2.5.2 Water 6
2.5.3 Soil 6
2.6 Environmental exposure 6
2.7 Occupational exposure 6
2.8 Biological indices of exposure 7
2.9 Regulations 7
3 Human Cancer Studies 11
3.1 Background 11
3.2 IARC reviews 11
3.3 Current studies (see Table 3-1) 11
3.4 Discussion 12
4 Studies of Cancer in Experimental Animals 15
4.1 Carcinogenesis study of C.I. Acid Red 114 15
4.2 Carcinogenesis studies of DMB 17
4.2.1 Oral studies of DMB in rats 17
4.2.2 Oral studies of DMB in hamsters 17
4.2.3 Subcutaneous studies of DMB in rats 17
4.2.4 Drinking-water studies of DMB in mice 17
4.2.5 Drinking water studies of DMB in rats 19
4.3 Transplantability of tumors induced by DMOB, a DMOB-based dye, (C.I. Direct Blue), or a DMB-based dye (C.I. Acid Red 114) 21
4.4 Oncogene activation induced by DMB or C.I. Acid Red 114 22
4.4.1 Tumorigenic activity of DMB, DMOB, and dyes based on DMB and DMOB 24
4.5 Summary 24

5 Genotoxicity 27
5.1 Prokaryotic Systems 27
5.1.1 Induction of mutation in Salmonella typhimurium 27
5.2 Eukaryotic systems 28
5.2.1 Mutagenicity in Drosophila melanogaster 28
5.2.2 Mammalian systems in vitro 28
5.2.3 Mammalian systems in vivo 29
5.3 Summary 29
6 Other Relevant Data 31
6.1 Metabolism of DMB-based dyes 31
6.2 Bacterial metabolism of DMB-based dyes 33
6.3 Summary 34
7 References 35
Appendix A: IARC. (1972). *Some Inorganic Substances, Chlorinated Hydrocarbons, Aromatic Amines, N-Nitroso Compounds, and Natural Products.* Monographs on the Evaluation of Carcinogenic Risk of Chemicals to Humans. 3,3'-Dimethylbenzidine (o-Tolidine). Lyon, France. World Health Organization. Vol 1, pp. A-1 – A-8. 41
Appendix B: IARC. (1993). *Occupational Exposures of Hairdressers and Barbers and Personal Use of Hair Colourants; Some Hair Dyes, Cosmetic Colourants, Industrial Dyestuffs and Aromatic Amines.* Monographs on the Evaluation of the Carcinogenic Risk to Humans. C.I. Acid Red 114. Lyon, France. World Health Organization. Vol 57, pp. B-1 – B-12. 43
Appendix C: NTP. (1991). Toxicology and Carcinogenesis Studies of C.I. Acid Red 114 (CAS No. 6459-94-5) in F344/N Rats (Drinking Water Studies). National Toxicology Program Technical Report Series No. 405. pp. C-1 –C-68. 45
Appendix D: NTP. (1991). Toxicology and Carcinogenesis Studies of 3,3'-Dimethylbenzidine Dihydrochloride in F344/N Rats (Drinking Water Studies). National Toxicology Program Technical Report Series No. 390. pp. D-1 –D-74. 47
Appendix E: Carcinogen Profile for 3,3′-Dimethylbenzidine (NTP 9th Report on Carcinogens 1999) PP. E-1 – E-3 49

List of Tables

Table 1-1. Examples of some DMB-based dyes 1
Table 1-2. Physical and chemical properties of some DMB-based dyes 3
Table 1-3. Physical and chemical properties of DMB 4
Table 2-1. Production and import values for some DMB-based dyes 5
Table 2-2. National estimates of exposure to DMB and selected DMB-based dyes 7
Table 2-3. U.S. EPA Regulations 8

Table 2-4. OSHA Regulations 9
Table 3-1. Cohort studies of workers exposed to DMB 13
Table 4-1. C.I. acid red 114 consumption by male and female F-344/N rats 15
Table 4-2. Tumor incidences in F344/N rats administered C.I. Acid Red 114 in drinking water for up to 104 weeks 16
Table 4-3. Average weekly doses of DMB dihydrochloride administered to BALB/c mice for up to 104 weeks 18
Table 4-4. Lung alveolar cell adenomas and adenocarcinomas in BALB/c mice exposed to DMB dihydrochloride in drinking water for up to 104 weeks 19
Table 4-5. Incidence of treatment-related tumors in F344/N rats dosed with 0 or 150 ppm of DMB for 9 months 20
Table 4-6. Tumor incidences in F344/N rats administered DMB hydrochloride in drinking water for 14 months 21
Table 4-7. Detection of activated oncogenes in spontaneous tumors and tumors induced by DMB or a DMB-derived dye 22
Table 4-8. Identity and frequency of activated *ras* genes within specific types of induced and spontaneous tumors 23
Table 4-9. Qualitative tumor responses to DMB, DMB-based dye, DMOB, and DMOB-based dye administered in the drinking water to rats 24
Table 6-1. Urinary excretion of DMB by dogs after oral administration of DMB-based dyes (100 mg/kg) 31
Table 6-2. Urinary excretion of DMB by rats after oral administration of DMB or DMB-based dyes (100 mg/kg) 32
Table 6-3. Reduction of C.I. Direct Red 2 by various anaerobic bacteria 34

1 Introduction

Dyes metabolized to 3,3'-dimethylbenzidine (dimethylbenzidine dyes as a class) were nominated for listing in the Report on Carcinogens (RoC) by the National Institute of Environmental Health Sciences (NIEHS) RoC Review Group (RG1) based on the current RoC listing of the parent compound 3,3′-dimethylbenzidine (DMB) as *reasonably anticipated to be a human carcinogen* and the fact that the azo linkages of DMB-based dyes are chemically equivalent and are readily cleaved by chemical or enzymatic reduction to form free DMB and the chromophore(s).

1.1 Chemical Identification

Dyes are a large and diverse group of organic compounds, many of them water-soluble, that have various applications for coloring numerous products. Dye molecules are colored because they absorb and reflect light. Most dyes in use today are synthetic organic compounds.

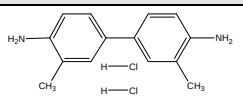
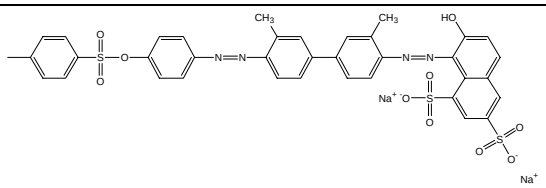
Dyes may be classified according to their chemical structure or their method of application. DMB-based dyes contain DMB attached to other substituents by diazo linkages. The dyes evaluated in this report are examples from the class of DMB-based dyes that have been studied for their potentially carcinogenic properties.

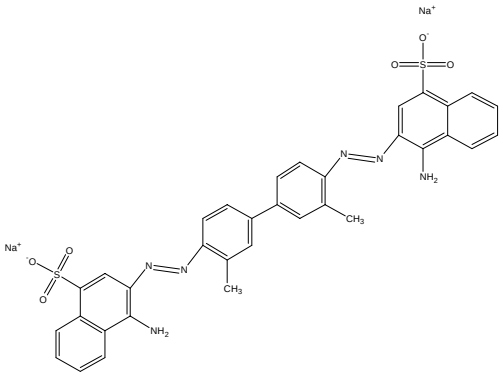
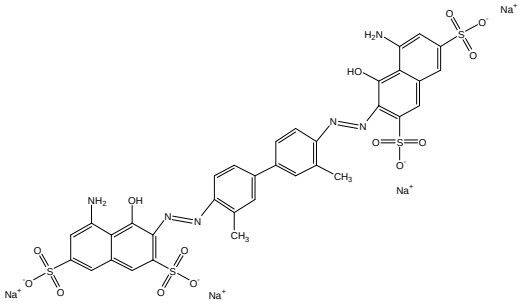
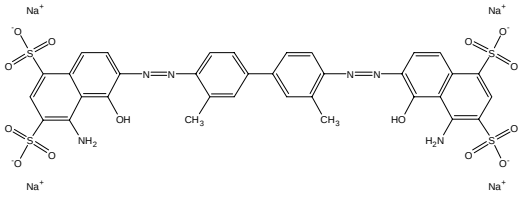
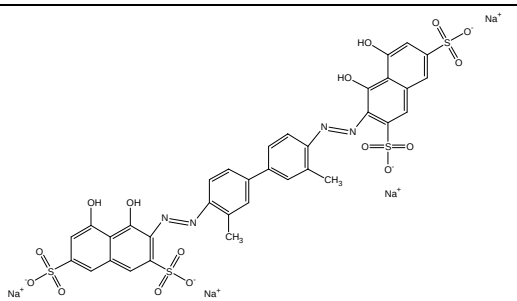
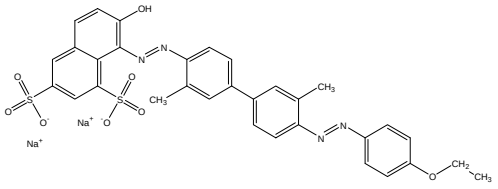
DMB ($C_{14}H_{16}N_2$, mol wt 212.29, CASRN 119-93-7) is a methylated congener of benzidine and also is known by the following names (Chemfinder 1999):

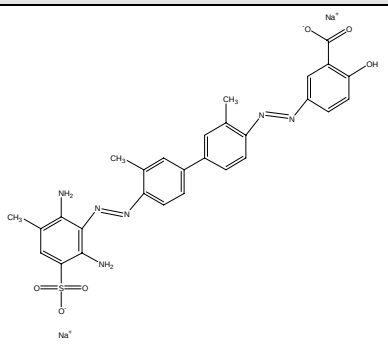
ortho tolidine
fast dark blue base R
3,3'-dimethylbiphenyl-4,4'-diamine
dimethyl benzidine
4,4'-bi-*o*-toluidine
3,3'-dimethyl-4,4'-biphenyldiamine
bianisidine
C.I. 37230
C.I. azoic diazo component 113
3,3'-dimethyl-1,1'-biphenyl-4,4'-diamine
tolidine
3,3'-tolidine
4,4'-diamino-3,3'-dimethylbiphenyl
diaminoditolyl
o,o'-tolidine

The dyes discussed in this report are limited to those containing the DMB moiety and which, upon metabolism, release free DMB. DMB-based dyes for which carcinogenesis and mechanistic studies have been reported in the literature are summarized in Table 1-1.

Table 1-1. Examples of some DMB-based dyes

Dye name and formula	CASRN	Mol wt	Structure
DMB-2HCl $C_{14}H_{18}Cl_2N_2$	612-82-8	285.22	
C.I. Acid Red 114 C.I. 23635 $C_{37}H_{28}N_4O_{10}S_3Na_2$	6459-94-5	830.81	

Dye name and formula	CASRN	Mol wt	Structure
C.I. Direct Red 2 C.I. 23500 $C_{34}H_{26}N_6O_6S_2Na_2$	992-59-6	724.72	
Trypan blue C.I. 23850 $C_{34}H_{24}N_6O_{14}S_4Na_4$	72-57-1	960.79	
Evan's blue C.I. 23860 $C_{34}H_{24}N_6O_{14}S_4Na_4$	314-13-6	960.79	
C. I. Direct Blue 25 $C_{34}H_{22}N_4O_{16}S_4Na_4$	2150-54-1	962.76	
C.I. Direct Red 39 C.I. 23630 $C_{32}H_{26}N_4O_8S_2Na_2$	6358-29-8	704.68	

Dye name and formula	CASRN	Mol wt	Structure
C.I. Direct Orange (disodium salt) $C_{28}H_{24}N_6O_6SNa_2$	6637-88-3	618.57	

Source: (Chemfinder 1999)

1.2 Physical-chemical properties

The chemical and physical properties of the DMB-based dyes listed in Table 1-1 are summarized in Table 1-2. Table 1-3 summarizes the physical and chemical properties of DMB.

Table 1-2. Physical and chemical properties of some DMB-based dyes

Dye name and formula	Color and physical state	Melting Point (°C)	Water solubility (g/100mL)
DMB-2HCl $C_{14}H_{18}Cl_2N_2$	light tan powder	NA	1 – 5 at 22°C
C.I. acid red 114 $C_{37}H_{28}N_4O_{10}S_3Na_2$	dark red powder	250 – 300[a]	< 0.01 at 22.5°C
C.I. Direct Red 2 $C_{34}H_{26}N_6O_6S_2\ Na_2$	brown powder	290	< 0.1 at 16°C
Trypan blue $C_{34}H_{24}N_6O_{14}S_4\ Na_4$	bluish-gray powder	> 300	0.1 – 1 at 20°C
Evan's blue $C_{34}H_{24}N_6O_{14}S_4\ Na_4$	blue crystals with greenish-bronze luster	NA	1 – 5 at 24.8°C
C. I. Direct Blue 25 $C_{34}H_{22}N_4O_{16}S_4Na_4$	deep purple-blue powder	NA	0.1 – 1 at 21°C
C.I. Direct Red 39 $C_{32}H_{26}N_4O_8S_2Na_2$	red powder	285	< 0.1 at 18°C
C.I. Direct Orange (disodium salt) $C_{28}H_{24}N_6Na_2O_6S$	dark brown powder	NA	< 0.1 at 16°C

Source: Chemfinder (1999)
NA: not available
[a](NTP 1991)

Table 1-3. Physical and chemical properties of DMB

Property	Information	Reference
Molecular weight	212.29	Budavari *et al*. (1996); CRC (1998)
Color	white to reddish crystals or crystalline powder	Budavari *et al*. (1996); CRC (1998)
Physical state	solid crystals or crystalline powder	Budavari *et al*. (1996); CRC (1998)
Melting point (°C)	129 - 131	Budavari *et al*. (1996); CRC (1998)
Boiling point (°C)	200	ACGIH (1986)
Specific gravity	1.0	ACGIH (1986)
Flash point (°C)	85	Budavari *et al*. (1996); CRC (1998)
Solubility: at 19°C		
Water	slightly soluble, < 1 mg/mL	HSDB 1991
95% Ethanol	slightly soluble, < 1 mg/mL	HSDB 1991
Dimethylsulfoxide	soluble, ≥ 100 mg/mL	HSDB 1991
Acetone	soluble, ≥ 100 mg/mL	HSDB 1991
Alcohol	soluble	Budavari *et al*. (1996)
Ether	soluble	Budavari *et al*. (1996)
Dilute acids	soluble	Budavari *et al*. (1996)

DMB is white to reddish crystals or crystalline powder. It is used as an intermediate in the manufacture of dyes and is sensitive to light and prolonged exposure to air (Radian 1991). DMB is a strong oxidizer (NIOSH 1994). When heated to decomposition, it emits toxic fumes of nitrogen oxides (SAX 1984). Its U.S. Environmental Protection Agency (EPA) hazardous waste number is U095, and its RTECS number is NIOSH/DD1225000.

1.3 Identification of metabolites

The metabolism of DMB-based dyes in hamsters, rats, and dogs results in appreciable levels of the free amine, monoacetyl metabolites, diacetyl metabolites, and alkaline hydrolyzable conjugates (AHCs) (see Section 6). Generally, for DMB-based dyes, diacetyl-DMB is the major metabolite, followed by monoacetyl-DMB, and smaller quantities of DMB and AHCs (Lynn *et al.* 1980; Bowman *et al.* 1983). Up to 192 hours after a single oral dose of 12 mg/kg of ^{14}C-labeled C.I. Direct Red 2 to F344/N rats, 20.65% was detected in the urine and 73.51% in the feces (Bowman *et al.* 1982).

2 Human Exposure

2.1 Use

According to the Society of Dyes and Colourists, more than 95 dyes are derived from DMB. Approximately 75% of the DMB produced is used as a dye or as an intermediate in the production of DMB-based dyes. These dyes and pigments are used in printing textiles, as biological stains, and in color photography. Approximately 20% of DMB is used in the production of polyurethane-based high-strength elastomers, coatings, and rigid plastics. DMB also is used as a reagent for detecting gold and chlorine in water and as a curing agent for resins (Budavari 1996; HSDB 1991; Spectrum 1999).

2.2 Production

The United States International Trade Commission (U.S. ITC 1994) reported that DMB was produced by two companies and DMB-based dyes were produced by three companies. Current production volumes for individual producers are not reported because they are confidential for both importers and producers of DMB. Table 2-1 summarizes past total production and import values for those DMB-based dyes for which information was available.

Table 2-1. Production and import values for some DMB-based dyes

Compound	Value (kg)	Year	Source
Tolidines and their derivatives, including DMB (*o*-tolidine) (production)	32,014	1993	U.S. ITC (1994)
DMB dihydrochloride (DMB-2HCl) (imports)	34,200	1984	U.S. ITC (1984)[a]
C.I. Acid Red 114 (production)	172,365	1979	U.S. ITC (1980)[b]
C.I. Acid Red 114 (imports)	9,751	1980	U.S. ITC (1981)[b]
C.I. direct dyes, including Direct Blue 25 (production)	11,228	1993	U.S. ITC (1994)
C.I. direct dyes, including Direct Red dyes 2 and 39, Direct Orange 6, and Direct Blue dyes 14 an 53 (imports)	7,597	1993	U.S. ITC (1994)

[a] Cited by NTP 1991b
[b] Cited by NTP 1991a

2.3 Analysis

The analysis of DMB-derived urinary metabolites is based upon measurement of free diamines through the use of a C_{18} solid sorbent. DMB is eluted, concentrated, injected into a high-performance liquid chromatography system and identified and quantified by monitoring of ultraviolet (UV) absorbance (at 280 or 245 nm) and the electrochemical response. The limit of detection (LOD) for UV analysis is < 2 μg/L, and the limit of quantitation (LOQ) is < 6 μg/L. The LOD for electrochemical detection is < 0.3 μg/L, and the LOQ is < 0.9 μg/L. Recoveries range from 87% to 102% at the 2-μg/L, 10-μg/L, and 20-μg/L levels (Neumeister 1991).

2.4 Environmental occurrence

DMB and DMB-based dyes may be released into the environment as a result of their production and use. Approximately 87% of waste DMB is deposited in water, 5% in terrestrial soil, 5% in aquatic sediments, and 3% in the air (U.S. EPA 1986). In 1996, the most recent year for which information was available, one facility reported releasing a total of 31 lb of DMB, 6 lb into air, and 25 lb into water (U.S. EPA 1996). Another company reported releasing DMB dihydrochloride, but no values were given. None of the DMB-based dyes are documented in the Toxic Release Inventory database, because their releases were not subject to reporting under the Emergency Planning and Community Right to Know Act (U.S. EPA 1996).

2.5 Environmental fate

DMB is found in nature only when it is released into the environment from industrial sources. No information on the environmental fate of DMB-based dyes was found.

2.5.1 Air

DMB has a limited half-life (approximately four hours) in the atmosphere's vapor phase, because it reacts with photochemically activated hydroxyl radicals. No information on DMB photolysis was found, but this process could be important, because DMB absorbs sunlight (HSDB 1991).

2.5.2 Water

DMB is moderately persistent in water, with a half-life between 20 and 200 days (U.S. EPA 1986). DMB released into water covalently binds to humic material in the sediment. Biodegradation of DMB is an important removal process in water, whereas hydrolysis is not. No information on evaporation was found. DMB has a slight tendency to bioconcentrate in aquatic organisms with an estimated bioconcentration factor (BCF) of 35 (a BCF of greater than 1,000 typically results in significant bioaccumulation in aquatic organisms) (HSDB 1991).

2.5.3 Soil

Biodegradation is the most important mechanism by which DMB is inactivated in soil, because DMB does not hydrolyze. It covalently binds to humic material in the soil but has only a moderate tendency to be adsorbed by organic matter. DMB leaching is not rapid in soils. No information about DMB volatilization from soil surfaces was found (Baird *et al*. 1977, cited in HSDB 1991).

2.6 Environmental exposure

Most environmental exposures to DMB occur as a result of contact with industrially contaminated air, water, and soil (HSDB 1991). General population exposure also may occur via contact with paper, fabric, and leather products containing DMB-based dyes.

2.7 Occupational exposure

Most occupational exposures to DMB and DMB-based dyes are of workers in dye manufacturing and processing plants. Occupational exposure may occur through inhalation of dust or mist, through accidental ingestion, or direct contact with the skin. In 1986 and 1987, the U.S. EPA, the American Textile Manufacturers Institute, and the Toxicological Association of the Dyestuffs Manufacturing Industry conducted a joint survey to estimate airborne concentrations of dye dust

in dye weighing rooms of plants where powdered dyes were used to dye and print textiles. The mean airborne concentration of total dye in 24 plants randomly selected for monitoring was estimated to be 0.085 mg/m^3 (U.S. EPA 1990).

The National Institute of Occupational Safety and Health (NIOSH) National Occupational Hazard Survey (NOHS) estimated that 418 workers potentially were exposed to DMB from 1972 to 1974. The National Occupational Exposure Survey (NOES) (NIOSH 1990) reported that 9,639 workers were exposed to DMB between 1981 to 1983. Table 2-2 summarizes the exposure survey data for DMB and DMB-based dyes. NIOSH recommended that exposure to airborne DMB be limited to 0.02 mg/m^3, for any 60-minute work period (NIOSH 1978).

Table 2-2. National estimates of exposure to DMB and selected DMB-based dyes

Compound name	Potentially exposed workers	
	1980s (NOES)	1970s (NOHS)
DMB-based dyes	60,595	16,377
C.I. Direct Red 2	1,450	—
C.I. Direct Red 39	1,450	2,136
C.I. Acid Red 114	13,795	2,852
C.I. Direct Blue 14	813	—
C.I. Direct Blue 25	6,004	1,797
C.I. Direct Blue 53	5,353	1,753
DMB (*o*-tolidine)	9,639	418
DMB-2HCl (*o*-tolidine dihydrochloride)	1,179	—

Source: Provisional data as of January 1, 1990, from the NIOSH National Occupational Exposure Survey (1981 - 1983) and National Occupational Hazard Survey (1972 - 1974), cited in Ruder *et al.* (1990).
—, Not available.

Workers in various other occupations also may be exposed to small quantities of DMB and DMB-based dyes. These workers include water and sewage plant attendants, chemical test tape or kit makers, and swimming pool service representatives. Swimming pool water test kits contain 0.5% to 1.0% DMB, and exposure may occur if they are accidentally emptied into the pool. Chemists also may be exposed in the laboratory when using DMB to detect free chlorine or gold (NTP 1998).

2.8 Biological indices of exposure

The primary biomarker for DMB and DMB-based dyes is urinary DMB. DMB-based dyes are reductively cleaved to DMB in the body. Urine sampling and analysis is performed to complement environmental monitoring in assessment of occupational exposure to these compounds.

2.9 Regulations

The American Conference of Governmental Industrial Hygienists (ACGIH) has classified DMB as a suspected human carcinogen (ACGIH 1991).

U.S. EPA regulates DMB under the Resource Conservation and Recovery Act (RCRA) as a hazardous constituent of waste, under the Clean Air Act (CAA) as a hazardous air pollutant that may be released by certain stationary sources, and under the Toxic Substances Control Act (TSCA), which requires submission of health and safety information. Under the Comprehensive Environmental Response, Compensation, and Liability Act (CERCLA) Trypan Blue has a reportable quantity (RQ) of 10 lb. Under the Superfund Amendments and Reauthorization Act (SARA), DMB hydrochloride, C.I. Trypan Blue, and C.I. Acid Red 114 were placed on a list of toxic chemicals subject to reporting requirements and general threshold quantities for reporting of releases have been established for facilities using or producing these compounds. U.S. EPA regulations applicable to DMB and DMB-based dyes are summarized in Table 2-3.

The Occupational Safety and Health Administration (OSHA) also regulates DMB under the Hazard Communication Standard as a chemical hazard in laboratories (see Table 2-4).

Table 2-3. U.S. EPA Regulations

U.S. EPA Regulations	
Regulatory action	Effect of regulation and other comments
40 CFR 63 – PART 63 – NATIONAL EMISSION STANDARDS FOR HAZARDOUS AIR POLLUTANTS FOR SOURCE CATEGORIES. Promulgated: 57 FR 61992, 12/29/92. U.S. Codes: 7401 et seq.; CAA.	Standards that regulate specific categories of stationary sources that emit (or have potential to emit) one or more hazardous air pollutants are listed in this part pursuant to section 112(b) of the CAA.
40 CFR 63.100ff. – Subpart F – National Emission Standards for Organic Hazardous Air Pollutants From the Synthetic Organic Chemical Manufacturing Industry. Promulgated: 59 FR 19454, 04/22/94.	This subpart applies to chemical manufacturing process units that manufacture DMB and are located at a plant site that is a major source as defined in section 112(a) of CAA. Owners and operators of sources subject to this subpart shall comply with the requirements of subparts G and H of this part.
40 CFR 172 – SUBPART B – Table of Hazardous Materials and Special Provisions. Promulgated: 61 FR 50623, 50624, 09/26/96.	The Hazardous Materials Table in this section designates Trypan Blue as hazardous materials for the purpose of transportation of those materials. The reportable quantity for Trypan Blue is 10 lb (4.54 kg).
40 CFR 192.40 ff. – Subpart E – Standards for Management of Thorium Byproduct Materials Pursuant to Section 84 of the Atomic Energy Act of 1954, as Amended. Promulgated: 48 FR 45947, 10/07/83. U.S. Codes: Sec. 275 of the Atomic Energy Act of 1954, 42 U.S.C. 2022, as added by the Uranium Mill Tailings Radiation Control Act of 1978, Pub. L. 95-604, as amended.	This subpart applies to the management of thorium byproduct materials, such as Trypan Blue, under section 84 of the Atomic Energy Act of 1954, as amended, during and following processing of thorium ores, and to restoration of disposal sites following any use of such sites under section 83(b)(1)(B) of the Act.
40 CFR 261 – PART 261 - IDENTIFICATION AND LISTING OF HAZARDOUS WASTE. Promulgated: 45 FR 33119, 05/19/80. U.S. Codes: 42 U.S.C. 6905, 6912(a), 6921, 6922, 6924(y) and 6938.	This part identifies those solid wastes which are subject to regulation as hazardous wastes under parts 262 through 265, 268, and parts 270, 271, and 124 of this chapter and which are subject to the notification requirements of section 3010 of RCRA. Trypan Blue is given the U.S. EPA Hazardous Waste number U236.
40 CFR 302 – PART 302 – DESIGNATION, REPORTABLE QUANTITIES, AND NOTIFICATION. Promulgated: 50 FR 13474, 04/04/85. U.S. Codes: 42 U.S.C. 9602, 9603, and 9604; 33 U.S.C. 1321 and 1361. Trypan Blue has a statutory final RQ of 10 lb (4.54 kg).	This part designates under section 102(a) of CERCLA 1980 those substances in the statutes referred to in section 101(14) of CERCLA, identifies reportable quantities for these substances, and sets forth the notification requirements for releases of these substances. This part also sets forth reportable quantities for hazardous substances designated under section 311(b)(2)(A) of the CWA.

U.S. EPA Regulations	
Regulatory action	Effect of regulation and other comments
40 CFR 372 – PART 372 – TOXIC CHEMICAL RELEASE REPORTING: COMMUNITY RIGHT-TO-KNOW. Promulgated: 53 FR 4525, 02/16/88. U.S. Codes: 42 U.S.C. 11013, 11028. Effective date for DMB dihydrochloride is 1/1/95, for Trypan Blue is 1/1/94, and for C.I. Acid Red 114 is 1/1/95.	This part sets forth requirements for the submission of information relating to the release of toxic chemicals under section 313 of Title III of SARA (1986). Information collected under this part is intended to inform the general public and the communities surrounding covered facilities about releases of toxic chemicals, to assist research, to aid in the development of regulations, guidelines, and standards.
40 CFR 716 – PART 716 – HEALTH AND SAFETY DATA REPORTING. Promulgated: 51 FR 32726, 09/15/86. U.S. Codes: 15 U.S.C. 2607(d). Bisazobiphenyl dyes derived from benzidine and its congeners, *ortho*-tolidine (DMB) and dianisidine (dimethoxybenzidine), have an effective date of 10/04/82 and a sunset date of 10/4/92.	This subpart sets forth requirements for the submission of lists and copies of health and safety studies on chemical substances and mixtures selected for priority consideration for testing rules under section 4(a) of the Toxic Substances Control Act (TSCA) and on other chemical substances and mixtures for which U.S. EPA requires health and safety information in fulfilling the purposes of TSCA.

Source: These regulations in this table have been updated through the 1998 Code of Federal Regulations 40 CFR, July 1, 1998; 21 CFR, April 1, 1998; 29 CFR, July 1, 1998.

Table 2-4. OSHA Regulations

OSHA Regulations	
Regulatory action	Effect of regulation and other comments
29 CFR 1910.1200—Sec. 1910.1200. Hazard Communication. Promulgated 62 FR 42018, 08/04/97.	Requires chemical manufacturers and importers and all employers to assess chemical hazards and to provide information to employees. Hazard Communication program to include labels, material safety data sheets, and worker training.
29 CFR 1910.1450. Promulgated 1/31/90. Amended 58 FR 40191, 7/27/93. OSHA Act: Final rule for occupational exposure to hazardous chemicals in laboratories.	As select carcinogen (IARC Group 2B), dyes that metabolize to DMB are included as chemical hazards in laboratories. Employers required to provide employee information and training and to provide Chemical Hygiene Plan.

Source: These regulations in this table have been updated through the 1998 Code of Federal Regulations 40 CFR, July 1, 1998; 21 CFR, April 1, 1998; 29 CFR, July 1, 1998.

3 Human Cancer Studies

3.1 Background

DMB-based dyes have not been evaluated in human cancer studies as single agents, and most of the epidemiological studies reviewed assessed DMB in chemical mixtures with benzidine derivatives or other arylamines. Benzidine *per se* has been evaluated in a number of epidemiological studies.

3.2 IARC reviews

In IARC (1972), no human carcinogenicity data on DMB were available. In a subsequent IARC evaluation (IARC 1987), DMB was placed in Group 2B (*possibly carcinogenic to humans*). There were, however, no reported human studies. IARC also evaluated C.I. Acid Red 114, a dye that metabolizes to DMB, in 1993. The dye was placed in Group 2B (*possibly carcinogenic to humans*) although the Working Group did not have any human carcinogenicity data (IARC 1993).

Seven arylamines have been classified by IARC. Benzidine-based dyes and 4,4'-methylenebis(2-chloroaniline) (MBOCA) were classified as *probably carcinogenic*, Group 2A, based on a high level of evidence for carcinogenicity in experimental animals. Two industrial chemicals (2-naphthylamine and benzidine), one drug (Chlornaphazine), and two manufacturing processes (manufacture of auramine and magenta) were included in Group 1 on the basis of *sufficient evidence of carcinogenicity in humans*. IARC (1982) concluded that there was sufficient evidence that benzidine is carcinogenic to man. According to IARC (1987), case reports and follow-up studies of workers in many countries had demonstrated that occupational exposure to benzidine is causally associated with an increased risk of bladder cancer; thus benzidine was placed in Group 1 (*carcinogenic to humans*).

3.3 Current studies (see Table 3-1)

In 1996, Ouellet-Hellstrom and Rench (1996) investigated cancer incidence in a cohort of 704 workers employed at a Connecticut chemical plant between 1965 and 1989. The plant produced a variety of chemicals, including arylamines such as dichlorobenzidine, 3,3'-dimethoxybenzidine (DMOB), and DMB. The approximate production volume ratios between 1965 and 1989 were 9:4:1 for dichlorobenzidine, DMOB, and DMB, respectively. Benzidine production stopped before 1965, and only workers never exposed to benzidine at the plant were included in the study. The exposure classification system was developed by a panel of former and current employees based on work processes, potential exposures and job histories, and annual cumulative exposure scores ranging from 0 to 64.4 were calculated for each worker. Cancer cases were identified by three methods: the cohort roster was matched up with cancer cases in the Connecticut Tumor Registry (CTR) through 1990; cancer cases were identified by reviewing death certificates of deceased workers if cancer was a cause or a contributing cause of death; and finally, a mail survey in 1993 was used to determine cancer cases in all members of the cohort who had mailing addresses (potential cases were confirmed by physicians). A total of 27 cancer cases were identified, 23 among male workers and four among female workers. Three of the 23 male cancer cases were non-melanoma skin cancers and were not considered in this study. For men, increased risks were found for cancer of the bladder, with a Standardized Incidence Ratio

(SIR) of 8.3 (95% CI 3.3 - 17.1) and cancer of the testis, with a SIR of 11.4, (95% CI 1.4 - 41.1). For women, breast cancer risk was increased (SIR 1.9, 95% CI 0.4 - 5.6). All bladder cancer cases were potentially exposed to arylamines. Testicular and breast cancer cases were in the non-exposed group. The observed association between bladder cancer and exposure to arylamines increased with increasing exposure (SIRs – 0, 5.5, and 16.4 for no, low, or moderate exposure). All bladder cancer cases were known to be current or former cigarette smokers. Thus, smoking may have contributed to the bladder cancer risk, but probably cannot entirely account for the eight-fold increase in risk (Ouellet-Hellstrom *et al.* 1996).

3.4 Discussion

Arylamines, including benzidine and 2-naphthylamine, have been demonstrated to be human carcinogens. Vineis and Pirastu (1997) reviewed cancer risk in humans resulting from occupational exposure to aromatic amines and tobacco smoking with reference to ecologic, cohort, and case-control studies. Occupational exposures to aromatic amines explain up to 25 percent of bladder cancers. Environmental tobacco smoke as well as occupation may contribute to exposure to aromatic amines. Metabolic polymorphisms, such as the N-acetyltransferase genotype, play a modulating role in the risk of bladder cancer associated with exposure to aromatic amines. The consistent observation of a difference between men and women in bladder cancer risk may indicate gender differences in exposure or in biological determinants of cancer. The study by Ouellet-Hellstrom and Rench (1996) provides additional evidence that arylamine exposure is related to bladder cancer and suggests that DMB exposure may constitute an important component of the exposure. However, since only exposure to total arylamines was evaluated, the study does not directly implicate DMB in cancer risk.

Table 3-1. Cohort studies of workers exposed to DMB

Reference	Population	Exposure	Effects	Potential Confounders
Ouellet-Hellstrom and Rench. (1996) USA. Follow up through 1993	704 workers (585 men and 119 women) first employed at a Connecticut chemical plant between 1965 and 1989. Only workers never exposed to benzidine at the plant were selected. Information on follow-up yielded 8,624 person-years for a follow-up rate of 97% among male employees and 1,660 person-years for a follow-up rate of 97% among women. Expected number based on cancer incidence rates from the State of Connecticut.	Exposure to arylamines established by a committee consisting of four former or current workers knowledgeable about work processes and potential exposures. Scoring system based on intensity of exposure and frequency of contact. Three levels of exposure: none, low, and moderate.	20 Total cancers for males observed. 7 bladder cancers and 2 testicular cancers. **Bladder cancer in men (SIR):** 8.3 (95% CI 3.3 - 17.1). **Bladder cancer in men by exposure level (SIR):** No exposure: 0.0 Low level exposure 5.5 (95% CI 0.7 - 19.8) Moderate level exposure: 16.4 (95% CI 5.3 - 38.2). Smoking and low level exposure 11.6 (95% CI 1.4 - 41.8) Smoking and moderate level exposure: 23.6 (95% CI 7.7 - 55.2). **Testicular cancer in men (SIR):** 11.4 (95% CI 1.4 - 41.1) **Breast cancer in women (SIR):** 1.9 (95% CI 0.4 - 5.6)	All bladder case subjects were known to be current or former cigarette smokers. For other cancers, 37% of male cohort did not indicate smoking status.

4 Studies of Cancer in Experimental Animals

4.1 Carcinogenesis study of C.I. Acid Red 114

The carcinogenic potential of C.I. Acid Red 114, a DMB-based dye, was assessed in male and female Fischer 344/N rats (NTP 1991a). Groups of 50, 35, 65, and 50 male and female rats, 5 weeks old at the time of study initiation, were given drinking water containing C.I. Acid Red 114 at 0, 70 (males only), 150, 300, or 600 (females only) ppm, respectively, for up to 101 weeks. Chemical analysis showed that the dye material was approximately 85% pure and contained 15 organic chemicals closely related to C.I. Acid Red 114 and approximately 5 ppm DMB and < 1 ppm benzidine.

Interim sacrifices of 10 rats per sex were carried out during experimental month 9 (males at 0 and 300 ppm and females at 0 and 600 ppm) and month 15 (males at 1, 10, 150 and 300 ppm and females at 0, 150, 300, and 600 ppm). The number of male rats surviving at termination in the control, 70, 150, and 300 ppm groups were 24/70 (49%), 15/45 (43%), 26/75 (40%), and 1/70 (2%), respectively. The number of female rats surviving at termination in the control, 150, 300, and 600 ppm groups were 36/70 (72%), 13/45 (38%), 6/75 (10%), and 0/70 (0%), respectively. Surviving animals were sacrificed during experimental week 105. Final survival males and females in the high-dose groups was significantly reduced relative to that of concurrent controls ($P < 0.001$). Decreased survival was attributed to sacrifices of moribund rats with treatment-related neoplasms.

Water consumption was monitored to verify palatability of the drinking water solution and to permit determination of daily oral doses of the dye. Water consumption was not affected by the presence of the dye, and estimated daily doses of DMB resulting from the azo reduction of the C.I. Acid Red 114 drinking water doses are summarized in Table 4-1.

Table 4-1. C.I. acid red 114 consumption by male and female F-344/N rats

Experimental week	Drinking water concentration of C.I. Acid Red 114 (ppm)			
	70	150	300	600
	Estimated daily dose of DMB (mg/kg)[a]			
Males				
1 – 13	5.4	11.6	23.0	—
14 – 52	3.9	8.1	16.1	—
53 – 101	3.5	7.6	19.6	—
Females				
1 – 13	—	19.5	35.5	67.1
14 – 52	—	12.8	27.3	47.8
53 – 101	—	9.4	20.5	68.7

Source: NTP (1991a)

—, not tested.

[a] Estimate of DMB molar equivalent doses (mg/kg) based on expected azo reduction of C.I. Acid Red 114 dose.

Chronic oral administration of C.I. Acid Red 114 caused unequivocal dose-related increases in the incidences of benign and malignant tumors of the skin, Zymbal gland, and liver in male and female rats and of the clitoral gland, oral cavity, small and large intestine, and lung in females. Tumor incidences and the results of statistical analyses are summarized in Table 4-2.

Table 4-2. Tumor incidences in F344/N rats administered C.I. Acid Red 114 in drinking water for up to 104 weeks

Tumor type	Tumor incidences/number examined[a]			
	Concentration (ppm)			
	0	70	150	300
Males				
Skin: Basal-cell adenoma or carcinoma	1/50	5/35*	28/65***	32/50***
Sebaceous-cell adenoma or carcinoma	1/50	1/35	5/65	6/50*
Squamous-cell papilloma or carcinoma	1/50	2/35	11/65*	9/50*
Keratoacanthoma	1/50	1/35	4/65	7/50*
Zymbal gland: Adenoma or carcinoma	0/50	0/35	8/65*	7/50*
Liver: Neoplastic nodule or hepatocellular carcinoma	2/50	2/35	15/65*	20/50***
Lung: Alveolar/bronchial adenoma or carcinoma	2/50	2/35	2/65	3/50*
	Concentration (ppm)			
Females	**0**	**150**	**300**	**600**
Skin: Basal-cell adenoma or carcinoma	0/50	4/35*	7/65*	5/50*
Zymbal gland: Adenoma or carcinoma	0/50	3/35	18/65***	19/50***
Clitoral gland: Adenoma or carcinoma	11/48	17/32*	28/62**	23/50*
Liver: Neoplastic nodule or hepatocellular carcinoma	0/50	0/35	19/64***	8/50***
Lung: Alveolar/bronchial adenoma or carcinoma	1/50	2/35	9/65**	4/50
Oral cavity: Squamous-cell papilloma or carcinoma	0/50	3/35	9/65*	6/50*
Small intestine: Polyps or adenocarcinoma	0/50	3/35	1/63	2/50*
Large intestine: Polyps or adenocarcinoma	0/50	1/35	0/64	3/50*

Source: NTP (1991a).
[a]Statistical significance by logistic regression test: $^{*}P < 0.05$; $^{**}P = 0.001$, $^{***}P < 0.001$.

Administration of C.I. Acid Red 114 also was associated with a small but statistically significant increase in the incidence of alveolar/bronchiolar adenomas of the lung in male rats in the high-dose group. Although the incidence of this tumor was low, the researchers noted that administration of the parent amine (DMB) also increased the incidence of pulmonary tumors in male rats (see Section 4.2.4). Based on these tumor incidences, the National Toxicology Program (NTP) concluded that C.I. Acid Red 114 was clearly carcinogenic to male and female F344/N rats under the conditions of this bioassay. Based on these data, the IARC Working Group (IARC 1993) also concluded that there was sufficient evidence to consider C.I. Acid Red 114 to be carcinogenic to experimental animals.

4.2 Carcinogenesis studies of DMB

4.2.1 Oral studies of DMB in rats

Twenty female Sprague-Dawley rats received oral doses of DMB in sesame oil (10 doses at 3-day intervals for a total dose of up to 500 mg/rat). Animals were observed for nine months; of 16 rats that survived nine months, 3 (18%) developed mammary gland carcinomas. Of 132 rats that received only sesame oil, 5 (4%) had a total of 3 mammary gland carcinomas, 1 fibroadenoma, and 5 hyperplasias (Griswold *et al*. 1968, cited in IARC 1972).

4.2.2 Oral studies of DMB in hamsters

DMB was administered to male and female hamsters at dietary concentrations of 0.1% or 0.3%, respectively. These concentrations resulted in total chemical intake of approximately 3.0 and 9.0 g per animal per year, respectively. Animals continued to consume DMB until they died from natural causes (Saffiotti *et al*. 1967, Sellakumar *et al*. 1969, both cited in IARC 1972). Neither DMB dosing regimen increased the incidence of tumors, but dosing of positive control animals with known human and animal carcinogens (including benzidine, dichlorobenzidine, and 2-naphthylamine) increased the incidences of hepatocellular and urinary bladder tumors.

4.2.3 Subcutaneous studies of DMB in rats

DMB suspended in olive oil was administered subcutaneously to Sherman rats (sexes not specified) in weekly doses of 60 mg per animal for a total dose of 5.5 g per animal. No concurrent control groups were used. Five rats developed external auditory canal tumors (most likely Zymbal gland tumors) after day 354 of the study (Spitz *et al*. 1950, cited in IARC 1972).

In another experiment, DMB suspended in sunflower oil was administered to random-bred rats (sex not specified) by subcutaneous injection in weekly doses of 20 mg per rat for 13 months (Pliss and Zabezhinsky 1970, cited in IARC 1972). Of dosed animals that survived for at least eight months (the time of appearance of the initial tumor), 60% (30/50) had 41 tumors with tumors of the Zymbal gland being most frequently observed (66% 20/30). The same investigators implanted pellets of DMB (20 mg in 10 mg of glycerol) into rats. Of 68 animals that survived at least until the appearance of the initial tumor (11 to 12 months), 48 developed 60 tumors, 27 (45%) of which were Zymbal gland carcinomas. Appropriate control groups were not included in these experiments.

4.2.4 Drinking-water studies of DMB in mice

Groups of 120 male and 120 female BALB/c mice were given drinking water containing 0, 5, 9, 18, 35, 70, or 140 ppm of DMB dihydrochloride (Schieferstein *et al*. 1989). Interim sacrifices and histopathological assessments were conducted after 13, 26, 39, 52, 78, or 116 weeks. Water consumption was monitored, and average weekly DMB doses (mg/kg) were determined (Table 4-3).

Table 4-3. Average weekly doses of DMB dihydrochloride administered to BALB/c mice for up to 104 weeks

Drinking water concentration of DMB (ppm)	Average weekly dose of DMB (mg/kg) during the indicated experimental intervals		
	Wk 0 – 4	Wk 48 – 52	Wk 100 – 104
Males			
0	0	0	0
5	3.1	5.0	3.6
9	5.8	9.0	6.4
18	11.3	18.0	13.7
35	22.0	34.6	23.1
70	47.6	70.0	51.8
140	85.4	126.0	95.2
Females			
0	0	0	0
5	4.2	4.2	3.6
9	6.7	6.9	6.8
18	13.7	13.5	11.9
35	27.3	25.9	21.7
70	55.3	52.5	44.1
140	105.0	107.8	109.2

Source: Schieferstein *et al.* (1989)

DMB in oral doses exceeding 100 mg/kg per week was well tolerated by the BALB/c mice of this study, as evidenced by the absence of treatment-related changes in water consumption, body weight gain, or mortality.

Incidences of alveolar-cell adenomas and adenocarcinomas of the lung were increased in a dose-related fashion among males that were either found dead or sacrificed in moribund condition. Similar increases were not observed in females nor in animals randomly selected for interim sacrifice (Table 4-4). The incidences of tumors of the skin, spleen, liver, and Harderian gland were unaffected by the administration of DMB.

Table 4-4. Lung alveolar cell adenomas and adenocarcinomas in BALB/c mice exposed to DMB dihydrochloride in drinking water for up to 104 weeks

Drinking water concentration of DMB (ppm)	Specified sacrifice times (wk)						Animals found dead or moribund
	13	26	39	52	78	112	
	Incidence of lung alveolar cell adenomas and adenocarcinomas/number of animals examined (incidence of adenocarcinomas)						
Males							
0	0/24	0/24	0/8	1/15	11/23	3/10 (1)	5/16 (2)
5	0/24	1/24	0/8	3/16	4/20	5/10 (3)	7/16 (2)
9	0/24	1/24	1/8	1/14	8/18	0/4	5/25 (2)
18	0/24	0/24	0/8	5/14	8/23 (2)	6/10	5/18 (2)
35	0/24	0/24	2/8	2/15	5/18	3/8 (1)	7/24 (6)
70	0/24	0/24	0/8	4/16 (1)	7/21	4/7 (1)	11/20 (5)
140	0/24	0/24	0/8	2/16	8/20	4/7 (1)	13/20 (10)
Females							
0	0/24	0/24	0/8	0/16	4/21	1/7	7/19 (5)
5	0/24	0/24	0/8	1/15	1/23	2/8	4/17 (3)
9	0/24	0/24	1/8	2/16	8/20 (1)	4/9	3/19 (3)
18	1/24	0/24	0/8	1/13	5/21 (1)	4/5 (2)	4/20 (2)
35	0/24	0/24	0/8	3/16	4/20	5/11 (3)	5/17 (2)
70	0/24	1/24	1/8	0/16	2/21	5/10	4/15 (2)
140	0/24	0/24	0/8	4/16 (1)	5/18 (2)	3/11 (1)	4/18 (2)

Source: Schieferstein *et al.* (1989).

4.2.5 Drinking water studies of DMB in rats

Groups of 70, 45, 75, and 70 male and female F344/N rats, five weeks old at the time of study initiation, were given drinking water containing DMB dihydrochloride at 0, 30, 70, or 150 ppm for up to 14 months (NTP 1991b). Although initially planned as a two-year study, this experiment was terminated early because of reduced survival associated with the appearance of treatment-related neoplasms. A scheduled interim sacrifice and histopathological assessment (10 controls and 10 high-dose animals of each sex) was conducted during the ninth month of the study.

Although the incidences of tumors observed in DMB-dosed rats were not significantly elevated at the interim sacrifice, the appearance of any of these neoplasms after only nine months suggested a treatment-associated early onset of some tumors (Table 4-5).

Table 4-5. Incidence of treatment-related tumors in F344/N rats dosed with 0 or 150 ppm of DMB for 9 months

Tumor type	Daily dose (ppm)	
	0	150
	Tumor incidence/number examined	
Males		
Liver: Hepatocellular carcinoma	0/10	2/10
Lung: Alveolar/bronchiolar adenoma or carcinoma	0/10	1/10
Skin: Basal cell carcinoma	0/10	1/10
Preputial gland: Adenoma or carcinoma	0/10	3/10
Small intestine: Mucinous membrane adenocarcinoma	0/10	2/10
Zymbal gland: Carcinoma or adenoma	0/10	3/10
Females		
Lung: Alveolar/bronchiolar adenoma or carcinoma	0/10	1/10
Skin: Squamous cell papilloma	0/10	1/10
Oral cavity: Squamous cell carcinoma (palate)	0/10	1/10
Clitoral gland: Adenoma or carcinoma	0/10	5/10
Zymbal gland: Carcinoma or adenoma	0/10	5/10

Source: NTP (1991b)

Tumor incidences were unequivocally increased in a dose-related manner after 14 months of DMB dihydrochloride administration. Tumor incidences are summarized in Table 4-6. Administration of DMB dihydrochloride increased the incidences of a wide array of malignant and benign tumors in both sexes of F344/N rats. Under the conditions of the experiment, DMB dihydrochloride was clearly carcinogenic to male and female Fischer 344/N rats (NTP 1991b). Further, the array of tumors produced by administration of DMB dihydrochloride was strikingly similar to that produced by administration of C.I. Acid Red 114 (see Table 4-2).

Table 4-6. Tumor incidences in F344/N rats administered DMB hydrochloride in drinking water for 14 months

Tumor type	Daily dose (ppm)			
	0	30	70	150
	Tumor incidences/number examined[a]			
Males				
Skin: Basal cell adenoma or carcinoma	0/60	11/45**	54/75**	30/60**
Sebaceous gland adenoma	0/60	0/45	7/75*	5/60*
Squamous cell papilloma or carcinoma	0/60	2/45	17/75**	27/60**
Keratoacanthoma	1/60	1/45	8/75*	5/60*
Zymbal gland: Adenoma or carcinoma	1/60	3/45	32/75**	36/60**
Preputial gland: Adenoma or carcinoma	2/60	4/45	6/75	9/60*
Liver: Neoplastic nodule or hepatocellular carcinoma	0/60	0/45	35/75**	33/60**
Oral cavity: Squamous cell papilloma or carcinoma	0/60	0/45	4/75	5/60*
Small intestine: Adenomatous polyp or adenocarcinoma	0/60	0/45	4/75	8/60*
Large intestine: Adenomatous polyp or adenocarcinoma	0/60	0/45	6/75*	15/60**
Lung: Neoplasms	1/60	0/45	8/75*	6/60*
Females				
Skin: Basal-cell adenoma or carcinoma	0/60	3/45	10/75**	9/60**
Squamous cell papilloma or carcinoma	0/60	3/45	9/75*	12/60**
Zymbal gland: Adenoma or carcinoma	0/60	6/45*	32/75**	42/60**
Clitoral gland: Adenoma or carcinoma	0/60	14/45**	42/75**	32/59**
Liver: Neoplastic nodule or hepatocellular carcinoma	0/60	0/45	7/74*	4/60*
Oral cavity: Squamous cell papilloma or carcinoma	0/60	3/45	9/75*	13/60**
Small intestine: Adenomatous polyp or adenocarcinoma	0/60	1/45	3/75	5/60*
Large intestine: Adenomatous polyp or adenocarcinoma	0/60	1/45	7/75*	4/60*
Mammary gland: Adenocarcinoma	0/60	1/45	3/75	6/60*

Source: NTP (1991b).
[a] Statistical significance by Fishers exact test: $^{*}P < 0.05$; $^{**}P < 0.001$.

4.3 Transplantability of tumors induced by DMOB, a DMOB-based dye, (C.I. Direct Blue), or a DMB-based dye (C.I. Acid Red 114)

Ulland *et al*. (1989) demonstrated the transplantability of preputial gland and epithelial skin neoplasms induced in Fischer 344 rats during lifetime drinking-water carcinogenesis studies of DMOB, the DMOB-based dye C.I. Direct Blue 15, or the DMB-based dye C.I. Acid Red 114. The neoplasms were retrospectively diagnosed as malignant. Individual neoplasms were not associated with exposure to specific chemicals. Portions of the neoplasms were implanted into the left mammary fat pad of male F344/N rats, and the rate of growth, presence of local invasion and distant metastases, and morphological features were observed following four serial transplants. All transplants were detected early, grew rapidly, and were histomorphologically

similar to the original neoplasms. Metastases were observed with both preputial and skin tumor lines during the serial passages. These results confirmed the malignancy of the preputial gland and skin neoplasms.

4.4 Oncogene activation induced by DMB or C.I. Acid Red 114

A study to detect activation of *ras* oncogenes in tumors induced by DMB or a DMB-based dye explored the possibility that their mechanism of carcinogenesis in rats is the induction of activating point mutations in members of the *ras* gene family (Reynolds *et al.* 1990). Spontaneous tumors and tumors formed in response to the chronic administration of DMB or C.I. Acid Red 114 to rats (as discussed in Sections 4.1 and 4.2) were assayed for the presence of activated oncogenes by the NIH 3T3 DNA mouse fibroblast transfection assay. The results (shown in Table 4-7) confirmed that few activated oncogenes are detected in spontaneous tumors from F344/N rats (1/13 for malignant tumors and 0/25 for benign). In contrast, activated oncogenes were detected in the majority of rat tumors induced by DMB or a DMB-derived dye (5/6 for malignant tumors and 8/10 for benign tumors). The activated oncogene for each tumor is shown in Table 4-8.

Table 4-7. Detection of activated oncogenes in spontaneous tumors and tumors induced by DMB or a DMB-derived dye

Treatment/Tumor type	Frequency (Positive/tested)	Transformation efficiency, foci per μg of DNA	
		Tumor DNA	Transfectant DNA first cycle
Spontaneous[a]			
Benign	0/25		
Malignant	1/13	0.03	1.6
Induced by DMB or C.I. Acid Red 114			
Benign	8/10	0.01 - 0.05	0.03 - 1.05
Malignant	5/6	0.01 - 0.06	0.04 - 0.24

Source: Reynolds *et al.* (1990).
[a] Includes data on 29 spontaneous tumors from F344/N rats reported in an earlier paper.

Table 4-8. Identity and frequency of activated *ras* genes within specific types of induced and spontaneous tumors

Treatment/tumor type	Frequency (positive tumors/tested tumors)	Activated oncogene	
		H-*ras*	N-*ras*
Induced by DMB or C.I. Acid Red 114			
Clitoral carcinoma	4/4	4	—
Basal cell adenoma	4/5	3	1
Basal cell carcinoma	1/1	1	—
Squamous cell carcinoma	3/3	3	—
Trichoepithelioma	1/1	1	—
Fibrosarcoma	0/1	—	—
Mammary fibroadenoma	0/1	—	—
Spontaneous			
Clitoral carcinoma	1/2	1	—
Preputial carcinoma	0/1	—	—
Mammary adenomas	0/11	—	—
Mammary adenocarcinoma	0/2	—	—
Subcutaneous fibromas	0/5	—	—
Lipoma	0/1	—	—
Testicular interstitial cell adenoma	0/5	—	—
Fibrosarcoma	0/2	—	—
Mononuclear cell leukemia	0/3	—	—
Adrenal pheochromocytoma	0/1	—	—
Pancreatic acinar cell adenoma	0/1	—	—
Pancreatic islet cell adenoma	0/1	—	—
Pituitary adenoma	0/1	—	—
Splenic hemangiosarcoma	0/1	—	—
Prostatic adenocarcinoma	0/1	—	—

Source: Reynolds *et al.* (1990).
—, No data reported.

The presence of activated oncogenes in a high percentage of benign tumors induced by DMB or a DMB-based dye suggests that oncogene activation is an early event in the genesis of the tumors. Oligonucleotide hybridization analysis indicated that the H-*ras* oncogenes from the DMB-associated tumors contained mutations at codons 12, 13, or 61. Spontaneously activated H-*ras* genes contained a point mutation at codon 61. The presence of H-*ras* point mutations in DMB-induced benign and malignant neoplasms indicates a probable role for DMB-induced point mutations in the activation of the cellular *ras* genes and in the eventual induction of tumors.

4.4.1 Tumorigenic activity of DMB, DMOB, and dyes based on DMB and DMOB

The pattern of tumors induced by chronic administration of DMB, DMOB, and DMB- and DMOB-based dyes were quite similar (Table 4-9). Such a similar pattern of tumors by the dyes may be taken as evidence of a common mechanism of action for these compounds that would be likely if the dyes were metabolized to the respective amines. In addition, DMB, DMOB, and dyes based on each of these compounds (C.I. Acid Red 114 and C.I. Direct Blue 15, respectively) induced transplantable preputial gland tumors and epithelial gland tumors in F344/N rats (Ulland *et al.* 1989).

Table 4-9. Qualitative tumor responses to DMB, DMB-based dye, DMOB, and DMOB-based dye administered in the drinking water to rats

Tumor type	Amine/Dye[a]			
	DMB	DMB-based C.I. Acid Red 114	DMOB	DMOB-based C.I. Direct Blue 15
Skin				
Basal cell	+	+	+	+
Sebaceous gland	+	+	+	+
Squamous cell	+	+	+	+
Keratoacanthoma	+	+	+	+
Zymbal gland	+	+	+	+
Liver	+	+	+	+
Oral cavity	+	+	+	+
Preputial gland	+	–	+	+
Clitoral gland	+	+	+	+
Mammary gland	+	+	+	+
Small intestine	+	+	+	+
Large intestine	+	+	+	+
Lung	+	+	–	–
Adrenal medulla	–	+	–	–
Brain	+	–	+	+
Mononuclear cell leukemia	+	+	–	+
Mesotheliomas	+	–	+	–

Source: IARC (1993) and NTP (1990, 1991a, 1991b, 1992).

[a]+, Positive tumor response; –, Negative tumor response or not observed.

4.5 Summary

Orally administered DMB is carcinogenic in male and female F344/N rats. Orally administered C.I. Acid Red 114, a DMB-based dye, also is carcinogenic to F344/N rats. The spectrum of tumors induced by C.I. Acid Red 114 was similar to that induced by DMB. The pattern of tumors

induced by chronic administration of DMB, a DMB-based dye, DMOB, and a DMOB-based dye also was similar. Such a similar pattern of tumors is taken as evidence of a common mechanism of action for these compounds, which is likely if the dyes are metabolized to the respective amines. DMB, DMOB, and a dye based on each of these compounds (C.I. Acid Red 114 and C.I. Direct Blue 15, respectively) induce transplantable preputial gland and epithelial gland tumors in Fischer 344/N rats.

5 Genotoxicity

5.1 Prokaryotic Systems

5.1.1 Induction of mutation in Salmonella typhimurium

In tests sponsored by the NTP, DMB dissolved in dimethylsulfoxide was tested at concentrations ranging from 0 to 3333 μg/plate in *Salmonella typhimurium* strains TA100, TA1535, TA1537, and TA98 with and without metabolic activation by S9 liver homogenate derived from rats or hamsters induced with Aroclor 1254. DMB was mutagenic only in *S. typhimurium* strain TA98 in the presence of metabolic activation (Haworth *et al.* 1983).

Similar results were reported in further NTP-sponsored studies by Zeiger *et al.* (1988), who tested DMB dihydrochloride for mutagenicity in *S. typhimurium* using an azo reduction preincubation protocol concentrations ranging from 0 to 666 μg/plate. DMB dihydrochloride was mutagenic only in strain TA98 in the presence of exogenous metabolic activation. It was not mutagenic in strains TA100, TA1535, or TA97 with or without metabolic activation. Elliot and Gregory (1980, cited by IARC 1993) found C.I. Acid Red 114 to be mutagenic in *S. typhimurium* strains TA98, TA100, and TA1538 under reducing conditions.

Another study tested the mutagenic response of *S. typhimurium* strains TA98, TA100, TA1535, and TA1538 (in the presence of S9 metabolic activation) to DMB and its *N*-monoacetyl and *N*, *N*′-diacetyl derivatives. In general, strain TA98 was the most sensitive, followed by TA1538; all three compounds were mutagenic in these strains. In TA100, only the *N*-monoacetylated derivative was mutagenic; DMB produced an equivocal response. None of the compounds was mutagenic in TA1535. The *N*-monoacetylated derivative was more mutagenic than either DMB diamine or the *N*,*N*′-diacetyl derivative in strains TA98 and TA1538 (Reid *et al*. 1984, cited in NTP 1991; Morgan *et al*. 1994).

Urinary metabolites of DMB, obtained from urine extracts from rats orally administered DMB, were more strongly mutagenic in *S. typhimurium* than was DMB itself. Similarly, although the DMB-based dye Evan's blue was not mutagenic in strains TA98 or TA100, its rat urinary metabolites were mutagenic in *S. typhimurium* strain TA98. 3,3′-Dimethyl-*N*-acetylbenzidine and 3,3′-dimethyl-*N*,*N*′-diacetylbenzidine, identified as urinary metabolites of DMB and Evan's blue, were mutagenic in TA98 and TA100 (Tanaka et al. 1982). In another study, DMB also was mutagenic in *S. typhimurium* strains TA98 and TA100 only with S9 metabolic activation (You *et al*. 1993).

Morgan *et al*. (1994) reviewed and summarized the available mutagenicity studies for DMB in *S. typhimurium* strains TA98, TA100, TA1535, and TA1537 and DMB-based dyes (C.I. Acid Red 14, C.I. Direct Blue 25, C.I. Direct Red 2, C.I. Direct Red 39) in *S. typhimurium* strains TA98 and TA1538. These data show that DMB and C.I. Acid Red 114 were mutagenic with metabolic activation by S9 derived from the livers of Aroclor-induced male Sprague-Dawley rats and Syrian hamsters. All dyes tested were mutagenic and produced frameshift mutations in *S. typhimurium* strains TA98 and TA1538 using an azo reduction preincubation protocol (preincubation with flavin mononucleotide [FMN] or rat cecal flora).

5.2 Eukaryotic systems

5.2.1 Mutagenicity in Drosophila melanogaster

Valencia *et al.* (1985) tested DMB dihydrochloride for induction of sex-linked recessive lethal mutations in germ cells of adult male *Drosophila melanogaster*. The compound was administered by injection at a concentration of 2,750 ppm in water or in feed at a concentration of 14,000 ppm. The results were positive in the feeding study, but equivocal in the injection study. In a follow-up study, DMB dihydrochloride in feed at 14,000 ppm did not induce reciprocal translocations in the germ cells.

Woodruff *et al.* (1985, cited by IARC 1993) did not observe induction of sex-linked recessive lethal mutations in *D. melanogaster* by the DMB-derived dye C.I. Acid Red 114 administered in feed at 50,000 ppm or by injection at 1,500 ppm.

5.2.2 Mammalian systems in vitro

5.2.2.1 Mouse lymphoma cell mutagenesis assay

In two NTP-sponsored studies (Caspary *et al.* 1988), DMB was mutagenic in the L5178Y mouse lymphoma cell mutagenesis assay both with and without metabolic activation by S9 liver homogenate from Aroclor-induced F344/N rats.

In the first of these studies (Myhr and Caspary 1988), DMB was mutagenic without activation over a narrow range of concentrations just below those that were excessively toxic. Significant increases in mutation frequency (two- to three-fold) were observed at a concentration of 100 μg/mL without activation. The highest concentration that could be tested, 150 μg/mL, induced seven- to eight-fold increases. With activation, the toxicity of DMB was reduced somewhat, and concentrations of up to 200 μg/mL could be tested. However, higher concentrations were required to induce the same mutagenic response, suggesting that the effect of S9 was a deactivation of DMB. At high toxicity, the maximum increases in mutation frequency were about three- to four-fold.

In the second study (Mitchell *et al.* 1988), DMB induced strongly positive, dose dependent increases in mutation frequency both with and without S9 activation. The lowest effective concentration without activation ranged from 26 to 41 μg/mL, and mutation frequencies were increased 3.8- to 7.9-fold at the highest concentrations tested without activation (64 to 80 μg/mL). As observed by Myhr and Caspary (1988), higher concentrations could be achieved with activation and were required to reach mutation frequencies similar to those found without activation.

5.2.2.2 Chromosomal aberrations and sister chromatid exchange

Galloway *et al.* (1987) examined induction of sister chromatid exchanges (SCEs) and chromosomal aberrations in Chinese hamster ovary (CHO) cells by DMB. Results were positive for both end points with and without S9 metabolic activation. SCEs were induced at concentrations ranging from 5 to 50 μg/mL without activation and 500 to 5000 μg/mL with activation. Concentrations effective in inducing chromosomal aberrations ranged from 125 to 180 μg/mL with activation and 225 to 5000 μg/mL without activation

DMB dihydrochloride induced SCEs and chromosomal aberrations in CHO cells in the absence but not in the presence of S9 metabolic activation (NTP 1991b).

5.2.3 Mammalian systems in vivo

5.2.3.1 Micronucleus test

Morita et al. (1997) evaluated the effect of DMB in five separate rodent micronucleus assays carried out by two laboratories. Groups of 4 or 5 male mice, 8 to 10 weeks of age, were treated by intraperitoneal injection once or twice at various dose levels, and bone marrow and/or peripheral blood was analyzed. The highest dose tested ranged from 40 to 60 mg/kg and was based on mortality. No induction of micronuclei was observed in four studies (three with CD-1 mice and one with MS/Ae mice). Marginal, non-dose-related, but statistically significant micronucleus induction was seen in the fifth study (in MS/Ae mice); the evaluators termed this observation "inconclusive."

According to a summary report of the U.S. EPA Gene-Tox Program, DMB induced micronuclei in the bone marrow of hamsters (upon intraperitoneal injection of 25 mg/kg or more) and of rats (upon oral administration of 50 mg/kg or more) (Mavournin *et al*. 1990).

5.3 Summary

DMB and its *N*- and *N,N′*-diacetyl derivatives were mutagenic in *Salmonella typhimurium*, inducing frameshift mutations with metabolic activation by rat or hamster S9 liver homogenate. Urinary metabolites of DMB and DMB-based dyes also were mutagenic in *S. typhimurium*. Preincubation of dyes metabolized to DMB with azo-reducing FMN or rat cecal flora also resulted in mutagenic activity in *S. typhimurium*. DMB but not intact DMB-based dyes induced mutations in *Drosophila melanogaster*. In mammalian *in vitro* systems, DMB was mutagenic to mouse lymphoma cells and induced chromosomal aberrations and SCEs in CHO cells with or without S9 metabolic activation. In mammalian *in vivo* systems, intraperitoneal administration of DMB induced micronuclei in the bone marrow of hamsters, but results for the bone marrow and peripheral blood of mice were equivocal. DMB administered orally induced micronuclei in the bone marrow of rats.

6 Other Relevant Data

6.1 Metabolism of DMB-based dyes

Lynn *et al*. (1980) reported on the metabolism, in rats and dogs, of a series of DMB-based dyes, C.I. Acid Red 114, C.I. Direct Blue 25, C.I. Direct Red 2, and C.I. Direct Red 39 (chemical structures shown in Table 1-1).

Dogs received single oral doses of 100 mg/kg of the dyes, and the urinary excretion of DMB was monitored with gas chromatography (GC) assays. The results of this experiment are summarized in Table 6-1.

Table 6-1. Urinary excretion of DMB by dogs after oral administration of DMB-based dyes (100 mg/kg)

Dye	DMB impurity (ppm)	Dose of DMB as impurity (μg)	DMB excreted in urine during 48 hours after dosing (μg)			Percent of dose[a]
			Experiment 1	Experiment 2	Mean	
C.I. Direct Blue 25	9	13	62	103	82	0.03
C.I. Acid Red 114	< 1	< 1.5	94	175	135	0.04
C.I. Direct Red 2	7	11	BLQ[b]	BLQ[b]	—	—
C.I. Direct Red 39	2	3	BLQ[b]	BLQ[b]	—	—

Source: Lynn *et al.* (1980).
[a] Percent of potential theoretical maximum produced by complete reduction of the azo bonds in the dye.
[b] BLW: Below levels of quantitation, but the presence of DMB was confirmed by GC mass spectrography.

C.I. Direct Blue 25 and C.I. Acid Red 114 were metabolized to DMB, as evidenced by the presence of more of the amine in the urine than could be accounted for by DMB *per se* in the dye sample as an impurity. These results indicate that C.I. Direct Blue 25 and C.I. Acid Red 114 undergo azo reduction to yield the parent amine. A similar conclusion regarding C.I. Direct Red 2 and C.I. Direct Red 39 could not be made, because DMB was consistently below levels of quantitation in the urine of animals dosed with C.I. Direct Red 2 and C.I. Direct Red 39.

In a similar experiment conducted in male Sprague-Dawley rats (Table 6-2), orally administered C.I. Direct Blue 25 (100 mg/kg) was metabolized to DMB in concentrations comparable to those seen in dogs and, in turn, eliminated in the urine. Similar treatment of rats with C.I. Acid Red 114, C.I. Direct Red 2, and C.I. Direct Red 39 did not result in the urinary excretion of quantifiable concentrations of DMB, although the amine was qualitatively identified by GC/mass spectrometry analyses of urine samples. Metabolism of C.I. Direct Blue 25 to DMB in this study also may be inferred because the concentration of DMB in the urine of the dosed animals was 41-fold (< 1 μg as impurity in C.I. Direct Blue 25 and 41 μg in urine), corresponding to approximately 3.5% of the C.I. Direct Blue 25 dose. DMB was detected in the urine of C.I. Acid Red 114-dosed animals below the level at which it was present as an impurity in the dye. DMB was present in the urine of C.I. Direct Red 2- and C.I. Direct Red 39-dosed animals below levels of quantification (Table 6-2).

Table 6-2. Urinary excretion of DMB by rats after oral administration of DMB or DMB-based dyes (100 mg/kg)

Compound	DMB in administered dose (μg)	DMB excreted in urine during 72 hours after dosing (μg)	Percent of dose[a]
DMB	25,290	898 ± 278[b]	3.52 ± 0.99
C.I. Direct Blue 25	< 1	41 ± 3.0	0.06 ± 0.04
C.I. Acid Red 114	< 1	< 0.1	0.01
C.I. Direct Red 2	< 1	BLQ[c]	—
C.I. Direct Red 39	< 1	BLQ[c]	—

Source: Lynn *et al.* (1990).
[a]Percent of potential theoretical maximum produced by complete reduction of the azo bonds in the dye.
[b]Total DMB excreted in 72 hour following single oral dose (mean ± SD of four animals).
[c] BLQ: Below levels of quantification, but the presence of DMB was confirmed by GC/mass spectrometry.

The metabolism of C.I. Direct Red 2, a DMB-based dye, was demonstrated in F344 rats. For this study, the biphenyl portion of the molecule was uniformly labeled. Up to 192 hours after a single oral dose of 12 mg/kg of the ^{14}C-labeled C.I. Direct Red 2 in rats, 20.65 and 73.5% of the radioactivity was detected in the urine and feces, respectively. Sensitive chromatographic analysis, electrochemical gas chromotography (EC/GC), of radioactive compounds revealed that unchanged dye in the feces accounted for only 10.12% of the orally administered dose, indicating extensive cleavage of C.I. Direct Red 2. The urine contained a free amine fraction and in an alkaline hydrolyzable conjugate (AHC) fraction. The free amine fraction consisted of DMB and its mono- and di-acetylated metabolites. The AHC fraction may have contained DMB conjugates because it was hydrolyzed to yield free DMB. After oral administration to rats, ^{14}C-C.I. Direct Red 2 initially appeared at high concentrations in the gastrointestinal tracts (657 μg equivalent DMB in the small intestine and contents) and urinary bladder (2.14 μg equivalent DMB) of rats. It was widely distributed to soft tissues, including adipose tissue, blood, brain, heart, kidney, liver, lung, muscle, spleen, and testes. Tissue concentrations ranged from 0.045 μg equivalent DMB in brain to 0.963 μg equivalent DMB in the liver (two hours post-administration) and (< 0.002 μg equivalent DMB in brain to 3.64 μg equivalent DMB in the liver (72 hours post administration). The urinary concentration was 34.4 μg-equivalent DMB at 72 hours post administration. In comparison, although the parent base of the dye, DMB, is more extensively metabolized, the metabolism of the C.I. Direct Red 2 dye was similar to DMB, yielding the di-acetyldimethylbenzidine as the major product for both dye and DMB (Bowman *et al.* 1982).

In further experimentation, Bowman *et al.* (1983) demonstrated the metabolism of several DMB-based dyes (C.I. Direct Red 39, C.I. Direct Blue 14, C.I. Direct Blue 53, or C.I. Direct Orange 6) in rats. In the study, urinary metabolites in male Fischer 344 rats were monitored following oral administration of 2 mg dose of C.I. Direct Red 39, C.I. Direct Blue 14, C.I. Direct Blue 53, or C.I. Direct Orange 6. Sensitive chromatographic analysis (EC/GC) of metabolites in the urine revealed mainly mono- and di-acetylated DMB, the parent amine (DMB), and alkaline hydrolyzable conjugates in concentrations ranging from 10 μg (for the diacetylated DMB

derived from C.I. Direct Orange 6) to 0.62 μg (for the alkaline hydrolyzable conjugates derived from C.I. Direct Blue 53) at the peak excretion period of 12 to 24 hours post-treatment. At the peak excretion period of 12 to 24 hours post-treatment, total DMB- equivalent of 5.5, 7.9. 5.3, and 14 μg were excreted for C.I. Direct Red 39, C.I. Direct Blue 14, C.I. Direct Blue 53 or C.I. Direct Orange 6 doses, respectively. Excretion of these metabolites was essentially complete within 96 hours.

In similar experiments on the urinary metabolites of C.I. Direct Red 2, a DMB-based dye, Nony *et al.* (1983) demonstrated the metabolism of the DMB-based dye in rats and hamsters. In these studies, male Fischer 344 rats and Syrian golden hamsters were given single oral doses of 100 mg/kg C.I. Direct Red 2 (determined to be sufficiently pure and stable to be used in metabolism studies). Sensitive chromatographic analytical methods, electrochemical gas chromotography (EC/GC), revealed that both species excreted DMB, its mono- and di-acetylated derivatives, and alkaline hydrolyzable conjugates in the urine following ingestion of C.I. Direct Red 2. At the peak excretion period of 48 to 96 hours, a 20 mL volume urine sample of the rats was found to contain 0.088 ppm DMB, 0.299 ppm mono-acetyldimethylbenzidine, 3.98 ppm di-acetyldimethylbenzidine, and 0.293 ppm acid hydrolyzable conjugates. Excretion of these metabolites was essentially complete within 96 hours.

The results of these studies demonstrate the *in vivo* mammalian metabolism of DMB-based dyes to the parent amine. The results also demonstrate that azo-reductive potential varies according to species and dye. Both dogs and rats excreted DMB following administration of C.I. Direct Blue 25, dogs excreted substantial DMB following administration of C.I. Acid Red 114, rats excreted only a trace DMB following administration C.I. Acid Red 114, and neither dogs nor rats excreted quantifiable amounts of DMB following the administration of C.I. Direct Red 2 or C.I. Direct Red 39. In the rat, substantial quantities of the *N*-acetylated metabolites also were formed, but not in the dog. These differences may be due to variability in metabolism of the various dyes, which may lead to differences in formation of parent amine and, consequently, differences in bioactivation and elimination of the carcinogenic metabolites.

6.2 Bacterial metabolism of DMB-based dyes

Cerniglia *et al.* (1982) assessed the abilities of pure cultures of a variety of anaerobic bacteria to reduce the azo linkages in C.I. Direct Red 2, a DMB-based dye. These investigators also studied the ability of bacterial suspensions from the intestinal contents of rats to carry out the reductive cleavage. Both pure cultures of anaerobes and cultures isolated from rat intestinal contents carried out the reductive cleavage. The known organisms varied in the rates at which they reduced C.I. Direct Red 2 (Table 6-5).

Table 6-3. Reduction of C.I. Direct Red 2 by various anaerobic bacteria

Organism	C.I. Direct Red 2 reduction (nmol reduced/mg protein in 8 hours)
Bacteroides thetaitamicron	110.7
Bifidobacterium infantis	110.7
Citrobacter sp.	249.3
Clostridium perfringens	195.0
Lactobacillus acidophilus	233.6
Peptococcus anaerobius	167.1
Clostridium sp.	118.3
Peptostreptococcus productus	78.4
Esherichia coli	2.5

Source: Cerniglia *et al.* (1982).

The bacterial isolate from rat intestine was highly efficient in reducing C.I. Direct Red 2 to DMB. C.I. Direct Red 2 (233 nmol) was added to an incubation medium containing 10^{10} bacterial cells. The mixture was assayed for DMB and C.I. Direct Red 2 at 1, 2, 4, 8, 12, 24, and 48 hours. Production of DMB began promptly and was essentially complete (as evidenced by the absence of C.I. Direct Red 2) within 4 hours.

6.3 Summary

The results of metabolism and elimination studies of DMB-based dyes provide evidence that the dyes are subject to *in vivo* metabolism giving rise to the parent amine. DMB is further metabolized (via *N*-acetylation) and excreted in the urine and feces. Urinary metabolites are primarily the mono- and di-acetylated derivatives of the parent amine. Because the intact dye molecules are not well absorbed from the gastrointestinal tract, the initial metabolic step, azo reduction, most likely takes place in the gastrointestinal tract. Azo reduction of orally administered chemicals can be mediated by the microflora of the intestinal tract, which contains a variety of anaerobic species. Azo reduction, catalyzed by the action of intestinal bacteria, has been demonstrated in the dog, rat, and hamster, as shown by the appearance of DMB and its acetylated metabolites in the urine following oral administration of DMB-based dyes. Further evidence of the role of anaerobic bacteria in reduction of the azo linkages of DMB-based dyes is seen in the fact that this reaction was effectively catalyzed by a number of bacterial species isolated from the gastrointestinal tracts of rats.

7 References

1. ACGIH. (1986). *Documentation of the Threshold Limit Values*. American Conference of Governmental Industrial Hygienists, Cincinnati, OH. p 577.
2. Baird, R., L. Carmona, and R.L. Jenkins. (1977). Behavior of benzidine and other aromatic amines in aerobic wastewater treatment. *J Water Pollut Control Fed* 49:1609-1615.
3. Bowman, M.C., W.L. Oller, C.R. Nony, K.L. Rowland, and S.M. Billedeau. (1982). Metabolism and distribution of two 14C-benzidine-congener-based dyes in rats as determined by GC, HPLC, and radioassays. *J Anal Toxicol* 6:164-174.
4. Bowman, M.C., C.R. Nony, S.M. Billedeau, J.L. Martin, H.C.J. Thompson, and L.K. Lowry. (1983). Metabolism of nine benzidine-congener-based azo dyes in rats based on gas chromatographic assays of the urine for potentially carcinogenic metabolites. *J Anal Toxicol* 7:55-60.
5. Budavari, S, M. J. O'Neil, A. Smith, and P.E. Heckelman (eds.). (1996). *The Merck Index: An Encyclopedia of Chemicals, Drugs and Biologicals*. 12th ed., p.1498. Whitehouse Station, NJ, Merck & Co., Inc.
6. Caspary, W.J., D.S. Daston, B.C. Myhr, A.D. Mitchell, C.J. Rudd, and P.S. Lee. (1988). Evaluation of the L5178Y mouse lymphoma cell mutagenesis assay: interlaboratory reproducibility and assessment. *Environ Mol Mutagen* 12:195-229.
7. Cerniglia, C.E., J.P. Freeman, W. Franklin, and L.D. Pack. (1982). Metabolism of azo dyes derived from benzidine, 3,3'-dimethyl-benzidine and 3,3'-dimethoxybenzidine to potentially carcinogenic aromatic amines by intestinal bacteria. *Carcinogenesis* 3:1255-1260.
8. Chemfinder. (1999). http://www.chemfinder.com (and type 119-93-7) , CambridgeSoft Corp.
9. CRC. (1998). *Handbook of Chemistry and Physics*. (Lide, D. R. and Frederikse, H. P. R., eds.), New York, NY, CRC Press.
10. Elliot, J. and A.R. Gregory. (1980). Mutagenicity of a series of benzidine congener based dyes. *Vet Hum Toxicol* 22:413-417.
11. Galloway, S.M., M.J. Armstrong, C. Reuben, S. Colman, B. Brown, C. Cannon, A.D. Bloom, F. Nakamura, M. Ahmed, S. Duk, J. Rimpo, B.H. Margolin, M.A. Resnick, B. Anderson, and E. Zeiger. (1987). Chromosome aberrations and sister chromatid exchanges in Chinese hamster ovary cells: evaluations of 108 chemicals. *Environ Mol Mutagen* 10:1-175.
12. Griswold, D.P.J., A.E. Casey, E.K. Weisburger, and J.H. Weisburger. (1968). The carcinogenicity of multiple intragastric doses of aromatic and heterocyclic nitro or amino derivatives in young female Sprague-Dawley rats. *Cancer Res* 28:924-933.
13. Haworth, S., T. Lawlor, K. Mortelmans, W. Speck, and E. Zeiger. (1983). Salmonella mutagenicity test results for 250 chemicals. *Environ Mutagen* 5:3-142.
14. HSDB. (1991). 3,3'-Dimethylbenzidine. http://ntp-server.niehs.nih.gov/Main_Pages/Chem-HS.html (go to H&S database, type 119-93-7) , NTP Chemical Research Depository - Hazardous Substances Data Bank.

15. IARC. (1972). *Some Inorganic Substances, Chlorinated Hydrocarbons, Aromatic Amines, N-Nitroso Compounds and Natural Products*. IARC Monographs on the Evaluation of the Carcinogenic Risk of Chemicals to Man. Vol. 1, 87-91 Lyon, France, World Health Organization.

16. IARC. (1974). *Some Aromatic Amines, Hydrazine and Related Substances, N-Nitroso Compounds and Miscellaneous Alkylating Agents.* IARC Monographs on the Evaluation of the Carcinogenic Risk of Chemicals to Man. Vol. 4. Lyon, France. World Health Organization.

17. IARC. (1979). *Some Monomers, Plastics and Synthetic Elastomers, and Acrolein*. IARC Monographs on the Evaluation of the Carcinogenic Risk of Chemicals to Humans. Vol. 19. Lyon, France, World Health Organization.

18. IARC. (1982). *Some Industrial Chemicals and Dyestuffs.* IARC Monographs on the Evaluation of the Carcinogenic Risk of Chemicals to Humans. Vol. 29. Lyon, France, World Health Organization.

19. IARC. (1987). *Overall Evaluations of Carcinogenicity: An Updating of IARC Monographs Volumes 1 to 42*. IARC Monographs on the Evaluation of the Carcinogenic Risk of Chemicals to Humans. Suppl. 7. Lyon, France, World Health Organization.

20. IARC. (1993). *Occupational Exposures of Hairdressers and Barbers and Personal Use of Hair Colourants; Some Hair Dyes, Cosmetic Colourants, industrial Dyestuffs and Aromatic Amines.* IARC Monographs on the Evaluation of the Carcinogenic Risk of Chemicals to Humans. Vol. 57. pp. 247-257. Lyon, France, World Health Organization.

21. Lynn, R.K., D.W. Donielson, A.M. Ilias, J.M. Kennish, K. Wong, and H.B. Matthews. (1980). Metabolism of bisazobiphenyl dyes derived from benzidine, 3,3'-dimethylbenzidine or 3,3'-dimethoxybenzidine to carcinogenic aromatic amines in the dog and rat. *Toxicol Appl Pharmacol* 56:248-258.

22. Mavournin, K.H., D.H. Blakey, M.C. Cimino, M.F. Salamone, and J.A. Heddle. (1990). The in vivo micronucleus assay in mammalian bone marrow and peripheral blood. A report of the U.S. Environmental Protection Agency Gene-Tox Program. *Mutat Res* 239:29-80.

23. Mitchell, A.D., C.J. Rudd, and W.J. Caspary. (1988). Evaluation of the L5178Y mouse lymphoma cell mutagenesis assay: Intralaboratory results for sixty-three coded chemicals tested at SRI International. *Environ Mol Mutagen* 12:37-101.

24. Morgan, D.L., J.K. Dunnick, T. Goehl, M.P. Jokinen, H.B. Matthews, E. Zeiger, and J.H. Mennear. (1994). Summary of the National Toxicology Program benzidine dye initiative. *Environ Health Perspect* 102:63-77.

25. Morita, T., N. Asano, T. Awogi, Y.F. Sasaki, S. Sato, H. Shimada, S. Sutou, T. Suzuki, A. Wakata, T. Sofuni, and M. Hayashi. (1997). Evaluation of the rodent micronucleus assay in the screening of IARC carcinogens (groups 1, 2A and 2B) the summary report of the 6th collaborative study by CSGMT/JEMS MMS. Collaborative Study of the Micronucleus Group Test. Mammalian Mutagenicity Study Group. *Mutat Res* 389:3-122.

26. Myhr, B.C. and W.J. Caspary. (1988). Evaluation of the L5178Y mouse lymphoma cell mutagenesis assay: intralaboratory results for sixty-three coded chemicals tested at Litton Bionetics, Inc. *Environ Mol Mutagen* 12:103-194.

27. Neumeister, C.E. (1991). Analysis of urine to monitor exposures to benzidine, o-dianisidine, o-tolidine, and 4,4'-methylenedianiline. *Appl Occup Environ Hygiene* 6:953-958.

28. NIOSH. (1978). Criteria for a recommended standard. Occupational Exposure to o-Tolidine DHEW (NIOSH) Publication No. 78-179. U.S. Govt. Printing Office.

29. NIOSH. (1990). National Institute for Occupational Safety and Health. National Occupational Exposure Survey (1981-1983), unpublished provisional data as of July 1, 1990. NIOSH Cincinnati, OH.

30. NIOSH. (1994). *NIOSH Pocket Guide to Chemical Hazards.* DHHS (NIOSH) Publication No.94-116, p. 311, U.S. Govt. Printing Office.

31. Nony, C.R., J.L. Martin, and M.C. Bowman. (1983). Metabolism of a dimethylbenzidine-based dye in rats and hamsters as determined by analysis of the urine for potentially carcinogenic aromatic amines. *J Anal Toxicol* 7:49-54.

32. NTP. (1990). Toxicology and Carcinogenesis Studies of 3,3′-Dimethoxybenzidine Dihydrochloride in F344/N rats (Drinking Water Studies). (TR-372). National Toxicology Program Technical Report Series.

33. NTP. (1991a). Toxicology and Carcinogenesis Studies of C.I. Acid Red 114 (CAS No. 6459-94-5) in F344/N Rats (Drinking Water Studies). (TR-405). National Toxicology Program Technical Report Series.

34. NTP. (1991b). Toxicology and Carcinogenesis Studies of 3,3'-Dimethylbenzidine Dihydrochloride in F344/N Rats (Drinking Water Studies). (TR-390), 231 pp. National Toxicology Program Technical Report Series.

35. NTP. (1992). Toxicology and Carcinogenesis Studies of C.I. Direct Blue 15 in F344/N rats. Technical Report Series 397, 249 pp. National Toxicology Program.

36. NTP. (1997). Benzidine (CAS No. 92-87-5) First Listed in the First Annual Report on Carcinogens. http://ntp-server.niehs.nih.gov/htdocs/8_RoC/KC/Benzidine.html , National Toxicology Program.

37. NTP. (1998). *Eighth Report on Carcinogens.* http://ehis.niehs.nih.gov/roc/eighth/chemicals/dimethylbenzid.pdf, National Toxicology Program Technical Report Series.

38. Ouellet-Hellstrom R. and J.D. Rench. (1996). Bladder cancer incidence in arylamine workers. *J Occup Environ Med.* 38:1239-1247.

39. Pliss, G.B. and M.A. Zabezhinsky. (1970). Carcinogenic properties of ortho tolidine (3,3'-dimethylbenzidine). *J Natl Cancer Inst* 45:283-295.

40. Radian. (1991). *3,3'-Dimethylbenzidine (CAS# 119-93-7).* http://ntp-db.niehs.nih.gov/NTP_Reports/NTP_Chem_H&S/NTP_Chem1/Radian119-90-4.txt , NTP Chemical Repository. Radian Corporation.

41. Reid, T.M., K.C. Morton, C.Y. Wang, and C.M. King. (1984). Mutagenicity of azo dyes following metabolism by different reductive/oxidative systems. *Environ Mutagen* 6:705-717.

42. Reynolds, S.H., R.M. Patterson, J.H. Mennear, R.R. Maronpot, and M.W. Anderson. (1990). Ras gene activation in rat tumors induced by benzidine congeners and derived dyes. *Cancer Res* 50:266-272.

43. Ruder, A.M., L.J. Fine, and D.S. Sundin. (1990). National estimates of occupational exposure to animal bladder tumorigens. *J Occup Med* 32:797-805.

44. Saffiotti, U.F., F. Cefis, R. Montesano, and A.R. Sellakumar. (1967). *Induction of bladder cancer in hamsters fed aromatic amines.* In: *Bladder Cancer: a Symposium* (Deichmann, W. & Lampe K. F., eds.) Aesculapius Publishing Co., pp. 129-135.

45. Sax, N.I. (1984). *Dangerous Properties of Industrial Materials*. (Sax, N. I. and Lewis, S. R., eds.) Van Nostrand Reinhold, New York.

46. Schieferstein, G.J., Y. Shinohara, R.R. Allen, W. Sheldon, D.L. Greenman, and W.T. Allaben. (1989). Carcinogenicity study of 3,3'-dimethylbenzidine dihydrochloride in BALB/c mice. *Food Chem Toxicol* 27:801-806.

47. Sellakumar, A.R., R. Montesano, and U. Saffiotti. (1969). Aromatic amines carcinogenicity in hamsters. *Proc Amer Cancer Res* 10:78.(Abstract)

48. Spectrum. (1999). *Chemical Fact Sheet for o-Tolidine*. Spectrum Laboratories, Inc.

49. Spitz, F., W.H. Mauguigan, and K. Dobringer. (1950). The carcinogenic action of benzidine. *Cancer* 3:789-804.

50. Tanaka, K., T. Mii, S. Marui, I. Matsubara, and H. Igaki. (1982). Some aspects of metabolism and mutagenicity of o-tolidine and an o-tolidine-based azo dye. *Ind Health* 20:227-235.

51. U.S. EPA. (1986). 3,3'-Dimethylbenzidine. http://mail.odsnet.comTRIFacts/55.html .

52. U.S. EPA. (1990). Textile Dye Weighing Monitoring Study. EPA Report No. EPA-560/5-90-009 (main report and site visit reports). Washington, D.C., U.S. EPA Office of Toxic Substances.

53. U.S. EPA. (1996). Toxic Release Inventory for 1996. http://toxnet.nlm.nih.gov/servlets/simple-search?1.15.1.2358 (& type CAS# 119-93-7), U.S. EPA TRIFacts.

54. U.S. ITC. (1980). Synthetic Organic Chemicals -- United States Production and Sales 1979. U.S. International Trade Commission. USITC Publication No. 1099, p.17, U.S. Government Printing Office.

55. U.S. ITC. (1981). Synthetic Organic Chemicals - United States Production and Sales 1980. U.S. International Trade Commission. USITC Publication No. 1163, U.S. Government Printing Office.

56. U.S. ITC. (1984). Synthetic Organic Chemicals - United States Production and Sales 1983. U.S. International Trade Commission. USITC Publication No. 1548, U.S. Government Printing Office.

57. U.S. ITC. (1994). Synthetic Organic Chemicals - United States Production and Sales 1993. U.S. International Trade Commission. USITC Publication No. 2933, U.S. Government Printing Office.

58. Ulland, B.M., R.R. Maronpot, J.K. Lemen, and J.H. Mennear. (1989). Transplantation studies of preputial gland and epithelial skin neoplasms derived from benzidine-based dye carcinogenicity assays in Fischer 344 male rats. *Toxicol Pathol* 17:50-56.

59. Valencia, R., J.M. Mason, R.C. Woodruff, and S. Zimmering. (1985). Chemical mutagenesis testing in Drosophila. 3. Results of 48 coded compounds tested for the national toxicology program. *Environ Mutagen* 7:325-348.

60. Vineis, P. and R. Pirastu. (1997). Aromatic amines and cancer. *Cancer Causes Control* 8:346-355.

61. Woodruff, R.C., J.M. Mason, A.R. Valencia, and S. Zimmering. (1985). Chemical mutagenesis testing in Drosophila. V. Results of 53 coded compounds tested for the National Toxicology Program. *Environ Mutagen* 7:677-702.

62. You, Z., M.D. Brezzell, S. Das, M.C. Espadas-Torre, and B. Hooberman. (1993). ortho-Substituent effects on the in vitro and in vivo genotoxicity of benzidine derivatives. *Mutat Res* 319:19-30.

63. Zeiger, E., B. Anderson, S. Haworth, T. Lawlor, and K. Mortelmans. (1988). Salmonella mutagenicity tests. 4. Results from the testing of 300 chemicals. *Environ Mol Mutagen* 11:1-157.

Appendix A: IARC. (1972). *Some Inorganic Substances, Chlorinated Hydrocarbons, Aromatic Amines, N-Nitroso Compounds, and Natural Products.* Monographs on the Evaluation of Carcinogenic Risk of Chemicals to Humans. 3,3'-Dimethylbenzidine (o-Tolidine). Lyon, France. World Health Organization. Vol 1, pp. A-1 – A-8.

3,3'-DIMETHYLBENZIDINE (o-TOLIDINE)*

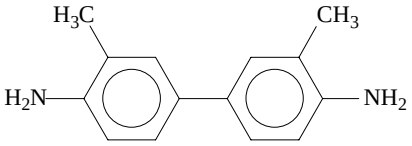
1. Chemical and Physical Data

1.1 Synonyms and trade names

Chem. Abstr. No.: 119937; *Name*: Benzidine, 3,3´-dimethyl-

4,4´-Diamino-3,3´-dimethylbiphenyl; 4,4´-Diamino-3,3´-dimethyldiphenyl; 3,3´-Dimethyl-4,4´-biphenyldiamine; 3,3´-Dimethyl-4,4´-diphenyldiamine; 3,3´-Dimethylbiphenyl-4,4´-diamine; 3,3´-Dimethyldiphenyl-4,4´-diamine; 3,3´-Tolidine; 4,4´-Bi-o-toluidine; 4,4´-Di-o-toluidine; Diaminoditolyl; Fast dark blue base R; C.I. azoic diazo component 113

1.2 Chemical formula and molecular weight



$C_{14}H_{16}N_2$ Mol. wt: 212.28

1.3 Chemical and physical properties of the pure substance

(a) *Description*: White to reddish crystals or crystalline powder

(b) *Melting-point*: 129-131°C

(c) *Absorption spectroscopy*: The ultraviolet absorption spectrum in aqueous solution at different pH and in 2N hydrochloric acid is given by Pickett *et al.* (1950).

(d) *Solubility*: Slightly soluble in water; very soluble in ethanol and ethyl ether

(e) *Chemical reactivity*: A weak base that forms salts with HCl, H_2SO_4, etc.; can be tetrazotized to yield coloured coupling products, and oxidized; its amino groups can be acetylated.

---

* Considered by the Working Group in Geneva, December 1971.

2. Use and Occurrence

(a) Use

Production data for o-tolidine are not available in recent years; however, in 1962 the production in the USA was reported as 243.0 thousand pounds. Imports of o-tolidine into the USA have ranged from a low of 2.3 thousand pounds in 1959 to a high of 134.6 thousand pounds in 1962. Imports in 1969 and 1970 were 80.6 thousand pounds and 97.8 thousand pounds, respectively[1]. o-Tolidine and its salts are widely used in the manufacture of dyestuffs and pigments based on the coupling of the tetrazotized base with phenols and amines. According to the Colour Index, more than 95 dyes are derived from o-tolidine. It is also used as a laboratory agent, e.g., for the detection of blood and for the colorimetric determination of chlorine in air and water.

(b) Analytical methods

Methods for the determination of o-tolidine in the working environment have been described by Meigs *et al*. (1954) and Ghetti *et al*. (1963). Guidelines for the analysis of aromatic amines have been given (UICC, 1970).

3. Biological Data Relevant to the Evaluation of Carcinogenic Risk to Man

3.1 Carcinogenicity and related studies in animals

(a) Oral administration

Rat: Twenty female Sprague-Dawley rats were given, by stomach tube, a suspension of o-tolidine in sesame oil up to a tota1 dose of 500 mg per rat, fractionated in 10 doses at 3-day intervals. Sixteen rats were alive at the end of the observation period, i.e., nine months: three of these showed a total of four mammary carcinomas. In comparison, four out of five animals that survived after being given benzidine (50 mg per rat) showed a total of 17 mammary carcinomas. Among 132 rats that received the solvent only, 5 had a total of three mammary carcinomas, one fibroadenoma and five hyperplasias (Griswold *et al*., 1968). The purity of the material is not stated.

[1] Data from Chemical Information Services, Stanford Research Institute.

Hamster: Commercial o-tolidine at a dietary level of 0.1% (3.0 g per animal per year) fed to groups of 30 male and 30 female hamsters throughout their life-span did not induce bladder or other tumours. Negative results were also obtained at a dietary level of 0.3%, which was the highest tolerated level. No tumours were observed with 1-naphthylamine, 2-naphthylamine, 3,3´-dichlorobenzidine or o-dianisidine at the 0.1% dose level. However, cholangiomas and liver-cell tumours were observed with benzidine and benzidine dihydrochloride at 0.1% and transitional cell carcinomas of the bladder and liver tumours were observed with 3,3´-dichlorobenzidine at 0.3% in the diet; 2-naphthylamine at a concentration of 1.0% in the diet also induced transitional cell carcinoma of the bladder (Saffiotti *et al*., 1967; Sellakumar *et al*., 1969).

(b) Subcutaneous administration

Rat: Commercial o-tolidine in olive oil was given as a weekly s.c. injection, at a dose level of 60 mg per rat per week (total dose: 5.5 g), to 105 Sherman rats. Forty-eight survived more than 300 days and were kept throughout their life-span. In contrast to rats given benzidine, no cirrhosis or hepatomas were observed among those receiving o-tolidine. Five rats developed cancer of the external auditory canal, all tumours appearing after the 354th day. No control group was run at the same time. Out of 56 tumours occurring among 578 untreated rats of the same colony, none was located in the external auditory canal (Spitz *et al*., 1950). Random-bred rats received weekly s.c. injections of purified o-tolidine in sunflower oil for 13 months, at doses of 20 mg per rat per week. Among 50 animals that survived for 8 months (time of occurrence of the first tumour), 30 developed a total of 41 tumours. Twenty carcinomas of Zymbal's gland, and tumours at other sites were seen. Two additional groups of rats (the first comprising 24 and the second 20 animals of each sex) received weekly a subcutaneous implant of a pellet containing 20 mg of purified o-tolidine and 10 mg of glycerol for 14 months. In the second of these groups the o-tolidine had been subjected to ultraviolet irradiation prior to the preparation of the pellet. The difference between the two groups was minimal. Out of a total of 68 animals that were alive at the time of appearance of the first tumour (11-12 months), 48 developed a total of 60 tumours. Among these were 27 Zymbal's gland carcinomas, and tumours at other sites (Pliss & Zabezhinsky, 1970). No control group was run at the same time as these

experiments, but in a preliminary report on these studies it was stated that rats from the same colony did not develop tumours of Zymbal's gland (Pliss, 1965).

3.2 Metabolism in animals and man

(a) Animals

Sciarini & Meigs (1961) after intraperitoneal injection of o-tolidine (70-100 mg/kg bw) into mongrel dogs recovered free o-tolidine from the urine to the extent of 4% and about 40% of a metabolite, probably the 5-ethereal sulfate of o-tolidine, within 3 days. These results are in agreement with the known fact that the dog is unable to acetylate aromatic amines.

(b) Man

Analysing the urine of workers manufacturing o-tolidine, Dieteren (1966) obtained positive evidence of the presence of diacetyl-o-tolidine and of a hydroxyamino metabolite, probably 5-hydroxy-o-tolidine. The possibility of the presence of monoacetyl-o-tolidine was left open.

3.3 Observations in man

o-Tolidine may enter the body by percutaneous absorption, by ingestion or by inhalation (Meigs *et al.*, 1954). Some suspicion of carcinogenicity has been suggested (Scott, 1962), but supporting evidence is not available.

4. Comments on Data Reported and Evaluation[1]

4.1 Animal data

Purified o-tolidine is a systemic carcinogen in the rat when given subcutaneously. The oral experiment in the rat is of doubtful significance because of the small number of animals involved. In feeding experiments, the commercial product failed to produce tumours in hamsters.

4.2 Human data

No epidemiological studies are available.

[1]See also the section ‘Extrapolation from animals to man’ in the introduction to this volume.

5. References

Dieteren, H.M.L. (1966) The biotransformation of o-tolidine. Arch. environm. Hlth, 12, 30

Ghetti, G., Maltoni, C. & Foa, V. (1963) Determinazione di due amine aromatiche (benzidina, o-tolidina) nell'ambiente di lavoro: messa a punto di un metodo. Med. d. Lavoro, 54, 655

Griswold, D.P., Casey, A.E., Weisburger, E.K. & Weisburger, J.H. (1968) The carcinogenicity of multiple intragastric doses of aromatic and heterocyclic nitro or amino derivatives in young female Sprague-Dawley rats. Cancer Res., 28, 924

Meigs, J.W., Sciarini, L.J. & Van Sandt, W.A. (1954) Skin penetration by diamines of the benzidine group. Arch. industr. Hyg., 9, 122

Picket, L.W., Groth, M., Duckworth, S. & Cunliffe, J. (1950) The ultraviolet absorption spectra of substituted bitolyls. J. Amer. chem. Soc., 72, 44

Pliss, G.B. (1965) Concerning carcinogenous properties of ortho-tolidine and dianisidine. Gig. Tr. prof. Zabol., 9, 18

Pliss, G.B. & Zabezhinsky, M.A. (1970) Carcinogenic properties of ortho-tolidine (3,3´-dimethylbenzidine). J. nat. Cancer Inst., 45, 283

Saffiotti, U., Cefis, F., Montesano, R. & Sellakumar, A.R. (1967) Induction of bladder cancer in hamsters fed aromatic amines. In: Deichmann, W. & Lampe, K.F., eds, Bladder cancer: a symposium, Birmingham, Ala., Aesculapius Publishing Co., p. 129

Sciarini, L.J. & Meigs, J.W. (1961) Biotransformation of the benzidines. Studies on diorthotolidine, dianisidine and dichlorobenzidine: 3,3´-disubstituted congeners of benzidine (4,4´-diaminobiphenyl). Arch. environm. Hlth, 2, 584

Scott, T.S. (1962) Carcinogenic and chronic toxic hazards of aromatic amines, Amsterdam & New York, Elsevier, p. 55

Sellakumar, A.R., Montesano, R. & Saffiotti, U. (1969) Aromatic amines carcinogenicity in hamsters. Proc. Amer. Ass. Cancer Res., 10 78

Spitz, S., Maguigan, W.H. & Dobriner, K. (1950) The carcinogenic action of benzidine. Cancer (Philad.), 3, 789

UICC (1970) The quantification of environmental carcinogens (UICC Technical Report Series, Vol. 4)

Appendix B: IARC. (1993). *Occupational Exposures of Hairdressers and Barbers and Personal Use of Hair Colourants; Some Hair Dyes, Cosmetic Colourants, Industrial Dyestuffs and Aromatic Amines.* Monographs on the Evaluation of the Carcinogenic Risk to Humans. C.I. Acid Red 114. Lyon, France. World Health Organization. Vol 57, pp. B-1 – B-12.

CI ACID RED 114

1. Exposure Data

1.1 Chemical and physical data

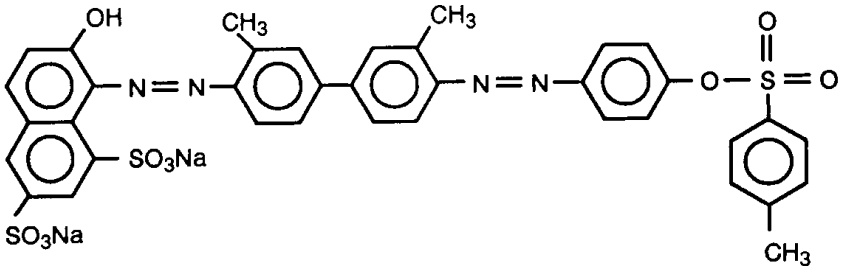
1.1.1 *Synonyms, structural and molecular data*

Chem. Abstr. Serv. Reg. No.: 6459-94-5

Chem. Abstr. Name: 8-[(3,3'-Dimethyl-4'-[(4-[([4-methylphenyl]sulfonyl) oxy]phenyl)-azo][1,1'-biphenyl] -4-yl)azo]-7-hydroxy -1,3-naphthalenedisulfonic acid, disodium salt

Colour Index No.: 23635

Synonyms: Acid Red 114; CI Acid Red 114, disodium salt



$C_{37}H_{28}N_4O_{10}S_3.2Na$ Mol. wt: 830.84

1.1.2 *Chemical and physical properties*

(a) *Description*: Deep-maroon powder (Green, 1990); red powder (US National Toxicology Program, 1991)

(b) *Melting-point*: 250-300 °C (decomposes) (US National Toxicology Program, 1991)

(c) *Spectroscopy data*: Infrared, ultraviolet and nuclear magnetic resonance spectral data have been reported (Pouchert, 1981; Sadtler Research Laboratories, 1988; Green, 1990; US National Toxicology Program, 1991).

(d) *Solubility*: Soluble in water (80 g/l at 80 °C) (International Dyestuffs Corp., 1990); very slightly soluble in ethanol (Green, 1990)

1.1.3 *Trade names, technical products and impurities*

Some trade names are: Acid Leather Red BG; Amacid Milling Red PRS; Benzyl Fast Red BG; Benzyl Red BR; Elcacid Milling Fast Red RS; Erionyl Red RS; Fenafor Red PB; Folan Red B; Intrazone Red BR; Kayanol Milling Red RS; Leather Fast Red B; Levanol

Red GG; Midlon Red PRS; Milling Fast Red B; Milling Red B; Milling Red BB; Milling Red SWB; Polar Red RS; Sandolan Red N-RS; Sella Fast Red RS; Sulphonol Red R; Suminol Milling Red RS; Supranol Fast Red 3G; Supranol Fast Red GG; Supranol Red PBX-CF; Supranol Red R; Telon Fast Red GG; Tertracid Milling Red B; Vondamol Fast Red RS.

Technical-grade CI Acid Red 114 is available in commercial mixtures containing 25-85% pure dye. Other typical ingredients include sodium chloride and mineral oil (see IARC, 1984, 1987) (Crompton & Knowles Corp., 1990; International Dyestuffs Corp., 1990; US National Toxicology Program, 1991; Aldrich Chemical Co., 1992).

1.1.4 *Analysis*

No data were available to the Working Group.

1.2 Production and use

1.2.1 *Production*

CI Acid Red 114, a bright red anionic bisazo dye, is manufactured by converting 3,3'-dimethylbenzidine (*ortho*-tolidine; see IARC, 1972) to the tetraazonium salt, which is then coupled successively to G acid (2-naphthol-6,8-disulfonic acid) and phenol. The phenol hydroxy function is then esterified with *para*-tolylsulfonyl chloride (Green, 1990).

CI Acid Red 114 has been produced commercially since the early 1900s. In the USA, there were six manufacturers and two importers of CI Acid Red 114 in 1977 (US Environmental Protection Agency, 1988). Annual production volume by five manufacturers in 1990 was estimated to be 10-100 tonnes, whereas it was 170 tonnes in 1979 (US International Trade Commission, 1980). In 1980, the USA imported about 7 tonnes of CI Acid Red 114 (US International Trade Commission, 1981).

1.2.2 *Use*

CI Acid Red 114 is used to dye wool (from a weak acid bath), silk (from either a neutral or acetic acid bath), jute and leather. Wool and silk are also printed directly (Green, 1990).

1.3 Occurrence

1.3.1 *Natural occurrence*

CI Acid Red 114 is not known to occur as a natural product.

1.3.2 *Occupational exposure*

No data were available to the Working Group.

The US Environmental Protection Agency, the American Textile Manufacturers Institute and the Ecological and Toxicological Association of the Dyestuffs Manufacturing Industry conducted a joint survey in 1986-87 to estimate airborne concentrations of dye dust in dye weighing rooms of plants where powder dyes are used in the dyeing and printing of textiles.

The survey was based on a sample of 24 sites chosen at random from among textile plants where powder dyes are weighed. Although CI Acid Red 114 was not among the dyes included in the survey, the results are considered to be representative of dye dust levels during weighing of this type of powder dye. The mean airborne concentration of total active colourant in the plants monitored was estimated to be 0.085 mg/m^3 (US Environmental Protection Agency, 1990).

On the basis of a survey conducted in the USA between 1981 and 1983, the US National Institute for Occupational Safety and Health estimated that a total of 18 511 workers, including 352 women, may have been exposed to CI Acid Red 114 at 300 textile and leather goods manufacturing plants (US National Library of Medicine, 1992).

1.3.3 *Other*

Anaerobic biodegradation of CI Acid Red 114 gives rise to the amine metabolites, 3,3'-dimethylbenzidine and 4-methylbenzenesulfonic acid (4'-aminophenyl) ester. Following incubation of 100 mg/l of dyestuff at 35 °C in the presence of anaerobic sludge inoculum, primary degradation was complete within seven days (Brown & Hamburger, 1987).

1.4 Regulations and guidelines

In Germany, CI Acid Red 114 must be handled like the corresponding hypothetical reduction amine, 3,3;-dimethylbenzidine, which is classified as an A_2 compound. Such materials are considered to have been proven to be carcinogenic only in animal experimentation but under conditions comparable to those of possible human exposure at the workplace (Deutsche Forschungsgemeinschaft, 1992).

2. Studies of Cancer in Humans

No data were available to the Working Group.

3. Studies of Cancer in Experimental Animals

3.1 Oral administration

Rat

Groups of 50, 35, 65 and 50 male and 50, 35, 65 and 50 female Fischer 344/N rats, five weeks old, were administered 0, 70, 150 or 300 mg/l (ppm) (males) and 0, 150, 300 or 600 ppm (females) CI Acid Red 114 (purity, 85%; with about 15 organic chemicals of similar structure, including approximately 5 ppm 3,3'-dimethylbenzidine and < 1 ppm benzidine in distilled drinking-water for 104 weeks. Survival at 105 weeks was 24/50, 15/35, 26/65 and 1/50 for male rats and 36/50, 13/35, 6/64 and 0/50 for females in the control, low-, mid- and high-dose groups, respectively ($p < 0.001$ for both males and females). All female rats receiving 600 ppm died by week 89. The decreased survival in the treated groups was due to development of treatment-related neoplasms. As shown in Table 1, there were increased

incidences of benign and malignant tumours of the skin, Zymbal gland and liver in male and female rats, and of the clitoral gland, oral cavity, small and large intestine and lung in female rats (US National Toxicology Program, 1991).

Table 1. Survival and tumour incidences in male and female Fischer 344/N rats administered CI Acid Red 114 in the drinking-water for 104 weeks

Survival and tumour types[a]	Dose (mg/l [ppm]				p Value[b]
Males	0	70	150	300	
Females	0	150	300	600	
Males					
Survival[c]	24/50	15/35	26/65	1/50	
Skin					
Basal-cell adenoma or carcinoma	1/50	5/35	28/65	32/50	<0.001
Sebaceous-cell adenoma or carcinoma	1/50	1/35	5/65	6/50	=0.007
Squamous-cell papilloma or carcinoma	1/50	2/35	11/65	9/50	=0.001
Keratoacanthoma	1/50	1/35	4/65	7/50	<0.001
Zymbal gland adenoma or carcinoma	0/50	0/35	8/65	7/50	=0.005
Liver neoplasms	2/50	2/35	15/65	20/50	<0.001
Females					
Survival	36/50	13/35	6/64	0/50	
Basal-cell adenoma or carcinoma of the skin	0/50	4/35	7/65	5/50	=0.012
Zymbal gland adenoma or carcinoma	0/50	3/35	18/65	19/50	<0.001
Clitoral gland adenoma or carcinoma	11/48	17/32	28/62	23/50	<0.001
Liver neoplasms	0/50	0/35	19/64	8/50	<0.001
Lung adenoma or carcinoma	1/50	2/35	9/65	4/50	=0.007
Oral cavity squamous-cell papilloma or carcinoma	0/50	3/35	9/65	6/50	=0.017
Small intestine polyps or adenocarcinoma	0/50	0/35	1/63	2/50	>0.05
Large intestine polyps or adenocarcinoma	0/50	1/35	0/64	3/50	>0.05

4. Other Relevant Data

4.1 Absorption, distribution, metabolism and excretion

4.1.1 *Humans*

No data were available to the Working Group.

4.1.2 *Experimental systems*

CI Acid Red 114 (100 mg/kg) containing less than 1% 3,3'-dimethylbenzidine as an impurity was administered once in the diet to two female mongrel dogs weighing 15 kg, and

48-h urine was analysed for 3,3'-dimethylbenzidine, the potential metabolic product Lynn et al., 1980). Excretion was found to be 0.04% of the dose of dye administered, which is in excess of what would be expected from the level of impurity; *para*-aminophenyl-*para*-toluenesulfonate was also identified as a urinary metabolite. The same dose of CI Acid Red 114 was also administered once to four male Sprague-Dawley rats by intragastric intubation; after 72 h, only 0.01% of the dose could be identified as 3,3'-dimethylbenzidine.

4.2 Toxic effects

4.2.1 *Humans*

No data were available to the Working Group.

4.2.2 *Experimental animals*

CI Acid Red 114 was tested for toxicity in male and female Fischer 344/N rats in a range-finding study for carcinogenicity testing (US National Toxicology Program, 1991). The purity of the test compound was estimated to be 82-85%; impurities consisted of about 15 organic chemicals of similar structure, with benzidine at less than 1 ppm and 3,3'-dimethylbenzidine at about 5 ppm. In a 13-day study, groups of five rats were dosed with 0, 10 000, 20 000 or 30 000 ppm (mg/l) in drinking-water. Except for one accidental death, all rats survived to the end of the study. Final mean body weights were significantly lower for males in the mid- and high-dose groups (83 and 77%, respectively) and for females in all dose groups (92, 88 and 80%, respectively). Hypocellularity of sternal bone marrow was found in three males and in all females given 20 000 ppm. The marrow was depleted of erythroid and myeloid cells. Lymphocytic depletion of the thymus was observed in four males and one female of the same dose group.

In the 13-week study, groups of 10 rats received 0, 600, 1200, 2500, 5000 or 10 000 ppm (mg/l) CI Acid Red 114 in the drinking-water for 94 days (males) or 95 days (females) (US National Toxicology Program, 1991). All rats survived. Body weights were lower than those in controls in all groups that received 1200 ppm and above (94-85%). Relative liver weights were increased in all dosed males and females; absolute and relative kidney weights were increased in females receiving doses of 1200 ppm and above. Haematocrit, haemoglobin and erythrocyte counts were decreased in dosed females, and the erythrocyte count was reduced at 1500 ppm and above in males. Some enzyme levels were elevated, consistent with mild hepatocellular damage, and minimal-to-mild lesions in liver were seen upon histopathological examination. Kupffer cells in the livers of most treated females contained brown pigment. An increased prevalence of reticulum-cell hyperplasia of the mesenteric lymph node was observed in treated males and females. Tubular regeneration and chronic inflammation of the kidneys occurred more frequently in treated females than in controls. Minimal amounts of brownish pigment were also seen in the tubular epithelial cells of the kidneys.

4.3 Reproductive and prenatal effects

No data were available to the Working Group.

4.4 Genetic and related effects

4.4.1 *Humans*

No data were available to the Working Group.

4.4.2 *Experimental systems* (see also Table 2 and Appendices 1 and 2)

CI Acid Red 114 was mutagenic to *Salmonella typhimurium* strains TA1538 and TA98 under reducing conditions. It did not induce sex-linked recessive lethal mutation in *Drosophila melanogaster* and did not induce unscheduled DNA synthesis in primary cultures of rat hepatocytes (abstract) or sister chromatid exchange or chromosomal aberrations in cultured Chinese hamster ovary cells. It did not induce unscheduled DNA synthesis in the hepatocytes of rats dosed orally (abstract).

Activated *ras* genes were found in 13/16 tumours induced in rats by CI Acid Red 114 (US National Toxicology Program, 1991) and in 1/38 spontaneous tumours tested (a CGA in codon 61 in a clitoral gland adenoma) (Reynolds et al., 1990; Table 3).

5. Summary of Data Reported and Evaluation

5.1 Exposure data

CI Acid Red 114, a bis-azo dye derived from 3,3'dimethylbenzidine, is used to dye wool, silk, jute and leather.

5.2 Human carcinogenicity data

No data were available to the Working Group.

5.3 Animal carcinogenicity data

CI Acid Red 114 was tested for carcinogenicity in one study in rats by administration in the drinking-water. It increased the incidences of benign and malignant tumours of the skin, Zymbal gland and liver in male and female rats, and of the clitoral gland, lung, oral cavity and small and large intestine in female rats.

5.4 Other relevant data

Reductive cleavage of the azo bonds to yield 3,3'-dimethylbenzidine was demonstrated *in vivo*.

CI Acid Red 114 induced gene mutation in bacteria under reducing conditions. It did not induce gene mutation in insects or sister chromatid exchange or chromosomal aberrations in cultured mammalian cells.

Table 3. Activating *ras* mutations in tumours induced in Fischer 344 rats by CI Acid Red 114 and in untreated animals

Tumour type	Frequency	N-*ras*	H-*ras*							
			Total	Codon 12		Codon 13		Codon 61		
				GAA	AGA	CGC	GTC	AAA	CTA	CGA
Treated										
Clitoral gland adenoma	4/4		4			1		1	1	1
Basal-cell adenoma (skin)	4/5	1	3	2	1					
Basal-cell carcinoma (skin)	1/1		1		1					
Squamous-cell carcinoma (skin)	3/3		3					2	1	
Trichoepithelioma (skin)	1/1		1		1					
Fibrosarcoma	0/1									
Mammary fibroadenoma	0/1									
Untreated										
Clitoral gland adenoma	1/2		1							1
Preputial gland carcinoma	0/1									
Mammary gland fibroadenoma or adenoma	0/11									
Mammary adenocarcinoma	0/2									
Subcutaneous fibroma or fibroadenoma	0/5									
Lipoma	0/1									
Testicular interstitial-cell adenoma	0/5									
Fibrosarcoma	0/2									
Mononuclear-cell leukaemia	0/3									
Adrenal phaeochromocytoma	0/1									
Pancreatic acinar adenoma	0/1									
Pancreatic islet-cell adenoma	0/1									
Pituitary adenoma	0/1									
Splenic haemangiosarcoma	0/1									
Prostatic adenocarcinoma	0/1									

Adapted from Reynolds *et al*. (1990)

5.5 Evaluation[1]

There is *inadequate evidence* in humans for the carcinogenicity of CI Acid Red 114.

There is *sufficient evidence* in experimental animals for the carcinogenicity of CI Acid Red 114.

[1] For definition of the italicized terms, see Preamble.

Overall evaluation

CI Acid Red 114 *is possibly carcinogenic to humans (Group 2B*).

6. References

Aldrich Chemical Co. (1992) *Aldrich Catalog/Handbook of Fine Chemicals 1992-1993*, Milwaukee, WI, p. 25

Brown, D. & Hamburger, B. (1987) The degradation of dyestuffs: Part III. Investigations of their ultimate degradability. *Chemosphere*, **16**, 1539-1553

Crompton & Knowles Corp. (1990) *Material Safety Data Sheet: Intrazone Red BR* (*CI Acid Red* 114), Reading, PA

Dellarco, V.L. & Prival, M.J. (1989) Mutagenicity of nitro compounds in *Salmonella typhimurium* in the presence of flavin mononucleotide in a preincubation asssay. *Environ. Mutag*., **13**, 116-127

Deutsche Forschungsgemeinschaft (1992) *MAK- and BAT-Values List 1992. Maximum Concentrations at the Workplace (MAK) and Biological Tolerance Values (BAT) for Working Materials* (Report N. 28), Weinheim, VCH Verlagsgesellschaft, pp. 64, 80-81

Elliot, J. & Gregory, A.R. (1980) Mutagenicity of a series of benzidine congener based dyes. *Vet. hum. Toxicol*., **22**, 413-417

Gren, F.J. (1990) *The Sigma-Aldrich Handbook of Stains, Dyes and Indicators*, Milwaukee, WI, Aldrich Chemical Co., p. 38

IARC (1972) *IARC Monographs on the Evaluation of Carcinogenic Risk of Chemicals to Man, Vol. 1, Some Inorganic Substances, Chlorinated Hydrocarbons, Aromatic Amines, N-Nitroso Compounds, and Natural Products*, Lyon, pp. 87-91

IARC (1984) IARC Monographs on the Evaluation of the Carcinogenic Risks of Chemicals to Humans, Vol. 33, Polynuclear Aromatic Compounds, Part 2: Carbon Blacks, Mineral Oils and Some Nitro-arenes, Lyon, pp. 87-168

IARC (1987) *IARC Monographs on the Evaluation of Carcinogenic Risks to Humans, Suppl. 7, Overall Evaluations of Carcinogenicity: An Updating of IARC Monographs Volumes 1 to 42*, Lyon, pp. 252-254

International Dyestuffs Corp. (1990) *Material Safety Data Sheet: Elcacid Milling Fast Red RS* (Acid Red 114), Clifton, NJ

Lynn, R.K., Donielson, D.W., Ilias, A.M., Kennish, J.M., Wong, K. & Matthews, H.B. (1980) Metabolism of bisazobiphenyl dyes derived from benzidine, 3,3'-dimethylbenzidine or 3,3'-dimethoxybenzidine to carcinogenic aromatic amines in the dog and rat. *Toxicol. appl. Pharmacol*., **56**, 248-258

Mirsalis, J., Tyson, K., Beck, J., Loh, F., Steinmetz, K., Contreras, C., Austere, L., Martin, S. & Spalding, J. (1983) Induction of unscheduled DNA synthesis (UDS) in hepatocytes following *in vitro* and *in vivo* treatment (Abstract No. Ef-5). *Environ. Mutag*., **5**, 482

Mortelmans, K., Haworth, S., Lawlor, T., Speck, W., Tainer, B. & Zeiger, E. (1986) *Salmonella* mutagenicity tests. II. Results from the testing of 270 chemicals. *Environ. Mutag*., **8** (Suppl. 7), 1-119

Pouchert, C.J., ed. (1981) *The Aldrich Library of Infrared Spectra*, 3rd Ed., Milwaukee, WI, Aldrich Chemical Co., p. 1465

Prival, M.J., Bell, S.J., Mitchell, V.D., Peiperl, M.D. & Vaughan, V.L. (1984) Mutagenicity of benzidine and benzidine-congener dyes and selected monoazo dyes in a modified *Salmonella assay*. *Mutat. Res*., **136**, 33-47

Reid, T.M., Morton, K.C., Wang, C.Y. & King, C.M. (1984) Mutagenicity of azo dyes following metabolism by different reductive/oxidative systems. *Environ. Mutag*., **6**, 705-717

Reynolds, S.H., Patterson, R.M., Mennear, J.H., Maronpot, R.R. & Anderson, M.W. (1990) ras Gene activation in rat tumors induced by benzidine congeners and derived dyes. *Cancer Res*., **50**, 266-272

Sadtler Research Laboratories (1988) Sadtler Standard Spectra, Philadelphia, PA

US Environmental Protection Agency (1988) *Computer Printout (CIS): 1977 Production Statistics for Chemicals in the Non-confidential Initial TSCA Chemical Substances Inventory*, Washington DC, Office of Pesticides and Toxic Substances

US Environmental Protection Agency (1990) *Textile Dye Weighing Monitoring Study* (EPA Report No. EPA-560/5-90-009, Main Report and Site Visit Reports), Washington DC, Office of Toxic Substances

US International Trade Commission (1980) *Synthetic Organic Chemicals, United States Production and Sales, 1979* (USITC Publication No. 1099), Washington DC, US Government Printing Office, p. 65

US International Trade Commission (1981) *Imports of Benzenoid Chemicals and Products, 1980* (USITC Publication No. 1163), Washington DC, US Government Printing Office, p. 47

US National Library of Medicine (1992) *Registry of Toxic Effects of Chemical Substances* (RTECS No. QJ6475500), Bethesda, MD

US National Toxicology Program (1991*) Toxicology and Carcinogenesis Studies of CI Acid Red 114 (CAS No. 6459-94-5) in F344/N Rats (Drinking Water Studies*) (NTP TR 405; NIH Publ. No. 92-3136), Research Triangle Park, NC, US Department of Health and Human Services

Venturini, S. & Tamaro, M. (1979) Mutagenicity of anthraquinone and azo dyes in Ames' *Salmonella typhimurium* test. *Mutat. Res*., **68**, 307-312

Woodruff, R.C., Mason, J.M., Valencia, R. & Zimmering, S. (1985) Chemical mutagenesis testing in *Drosophila*. V. Results of 53 coded compounds tested for the National Toxicology Program. *Environ. Mutag*., **7**, 677-702

Table 2. Genetic and related effects of CI Acid Red 114

Test system	Result		Dose (LED/HID)[a]	Reference
	Without exogenous metabolic system	With exogenous metabolic system		
SA0, *Salmonella typhimurium* TA100, reverse mutation	–	–	500.0000	Venturini & Tamaro (1979)
SA0, *Salmonella typhimurium* TA100, reverse mutation	0	–[b]	200.0000	Elliot & Gregory (1980)
SA0, *Salmonella typhimurium* TA100, reverse mutation	0	+[c]	62.5000	Elliot & Gregory (1980)
SA0, *Salmonella typhimurium* TA100, reverse mutation	–	–	167.0000	Mortelmans *et al.* (1986)
SA5, *Salmonella typhimurium* TA1535, reverse mutation	–	–	500.0000	Venturini & Tamaro (1979)
SA5, *Salmonella typhimurium* TA1535, reverse mutation	–	–	167.0000	Mortelmans *et al.* (1986)
SA7, *Salmonella typhimurium* TA1537, reverse mutation	–	–	167.0000	Mortelmans *et al.* (1986)
SA8, *Salmonella typhimurium* TA1538, reverse mutation	–	–	500.0000	Venturini & Tamaro (1979)
SA8, *Salmonella typhimurium* TA1538, reverse mutation	–	+[d]	100.0000	Reid *et al.* (1984)
SA9, *Salmonella typhimurium* TA98, reverse mutation	–	–	500.0000	Venturini & Tamaro (1979)
SA9, *Salmonella typhimurium* TA98, reverse mutation	0	–[b,e]	500.0000	Elliot & Gregory (1980)
SA9, *Salmonella typhimurium* TA98, reverse mutation	0	+[c]	62.5000	Elliot & Gregory (1980)
SA9, *Salmonella typhimurium* TA98, reverse mutation	0	+[f]	40.0000	Prival *et al.* (1984)
SA9, *Salmonella typhimurium* TA98, reverse mutation	–	(+)[g]	500.0000	Mortelmans *et al.* (1986)
SA9, *Salmonella typhimurium* TA98, reverse mutation	0	+[e]	125.0000	Dellarco & Prival (1989)
DMX, *Drosophila melanogaster*, sex-linked recessive lethal mutations	–		1500.0000	Woodruff *et al.* (1985)
DMX, *Drosophila melanogaster*, sex-linked recessive lethal mutations	–		50000.0000 feed	Woodruff *et al.* (1985)
URP, Unscheduled DNA synthesis, rat primary hepatocytes *in vitro*	–	0	0.0000	Mirsalis *et al.* (1983); abstr.
SIC, Sister chromatid exchange, Chinese hamster ovary cells *in vitro*	–	–	50.0000	US National Toxicology Program (1991)

Table 2 (contd)

Test system	Result		Dose (LED/HID)[a]	Reference
	Without exogenous metabolic system	With exogenous metabolic system		
CIC, Chromosomal aberrations, Chinese hamster ovary cells *in vitro*	–	–	50.0000	US National Toxicology Program (1991)
UPR, Unscheduled DNA synthesis, rat hepatocytes *in vivo*	–		0.0000 po	Mirsalis *et al.* (1983); abstr.

+, positive; (+), weakly positive; –, negative; 0, not tested

[a]In-vitro tests, μg/ml; in-vivo tests, mg/kg bw

[b]Anaerobic preincubation or with riboflavin supplementation

[c]Plate incorporation, with reduction using sodium dithionate

[d]Bacterial caecal reduction

[e]Plate incorporation with or without riboflavin

[f]Preincubation with hamster or rat liver S9 and flavin mononucleotide supplementation, no aeration

[g]Hamster liver S9

Appendix C: NTP. (1991). Toxicology and Carcinogenesis Studies of C.I. Acid Red 114 (CAS No. 6459-94-5) in F344/N Rats (Drinking Water Studies). National Toxicology Program Technical Report Series No. 405. pp. C-1 –C-68.

NTP TECHNICAL REPORT

ON THE

TOXICOLOGY AND CARCINOGENESIS

STUDIES OF C.I. ACID RED 114

(CAS NO. 6459-94-5)

IN F344/N RATS

(DRINKING WATER STUDIES)

A desalted commercial dye containing approximately 85% 1,3-Naphthalenedisulfonic acid, 8-((3,3′-dimethyl-4′-((4-(((4-methylphenyl)sulfonyl)oxy)phenyl)azo)(1,1′-biphenyl)-4-yl)azo)-7-hydroxy-disodium salt, 10% structurally related compounds, 1%-4% water, and 1% sodium chloride

NATIONAL TOXICOLOGY PROGRAM
P.O. Box 12233
Research Triangle Park, NC 27709

December 1991

NTP TR 405

NIH Publication No. 92-3136

U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES
Public Health Service
National Institutes of Health

CONTRIBUTORS

National Toxicology Program

C.J. Alden, Ph.D.
G.A. Boorman, D.V.M., Ph.D.
D.W. Bristol, Ph.D.
J.K. Dunnick, Ph.D.
S.L. Eustis, D.V.M., Ph.D.
T.J. Goehl, Ph.D.
R.A. Griesemer, D.V.M., Ph.D.
J.K. Haseman, Ph.D.
M.P. Jokinen, D.V.M.
M.M. McDonald, D.V.M., Ph.D.
G.N. Rao, D.V.M., Ph.D.
D.B. Walters, Ph.D.
K.L. Witt, M.S., Oak Ridge Associated Universities

Hazleton Laboratories America, Inc.

Conducted studies and evaluated tissues

B.M. Ulland, D.V.M., Principal Investigator
R.H. Cox, Ph.D.
B.A. Kulwich, D.V.M.
J.K. Leman, B.S.
K.J. Petrovics
H.A. Rutter, Jr., Ph.D.

Experimental Pathology Laboratories, Inc.

Provided pathology quality assessment

J.F. Hardisty, D.V.M., Principal Investigator
R. Brown, D.V.M., M.S.

Integrated Laboratory Systems

Prepared pathology audits

J.C. Bhandari, D.V.M., Ph.D., Principal Investigator

NTP Pathology Working Group

Evaluated slides and prepared pathology reports on rats (16 November 1989)

S. Grumbein, D.V.M., Ph.D., Chair
Pathology Associates, Inc.
R. Brown, D.V.M., M.S.
Experimental Pathology Laboratories, Inc.
M. Dominick, D.V.M., Ph.D.
Parke-Davis
M.P. Jokinen, D.V.M.
National Toxicology Program
K. Morgan, B.V.Sc., M.R.C.V.S., Ph.D.
CIIT

Biotechnical Services, Inc.

Prepared Technical Report

L.G. Cockerham, Ph.D., Principal Investigator
G.F. Corley, D.V.M.
M.C. Hirrel, Ph.D.
K.D. Mencer, B.A.

CONTENTS

ABSTRACT 5

EXPLANATION OF LEVELS OF EVIDENCE OF CARCINOGENIC ACTIVITY 8

PEER REVIEW PANEL 9

SUMMARY OF PEER REVIEW COMMENTS 10

INTRODUCTION 11

MATERIALS AND METHODS 19

RESULTS 27

DISCUSSION AND CONCLUSIONS 55

REFERENCES 65

APPENDIX A Summary of Lesions in Male Rats in the 2-Year Drinking Water Study of C.I. Acid Red 114 73

APPENDIX B Summary of Lesions in Female Rats in the 2-Year Drinking Water Study of C.I. Acid Red 114 129

APPENDIX C Genetic Toxicology 189

APPENDIX D Organ Weights and Organ-Weight-to-Body-Weight Ratios 201

APPENDIX E Hematology, Clinical Chemistry, and Urinalysis Results 207

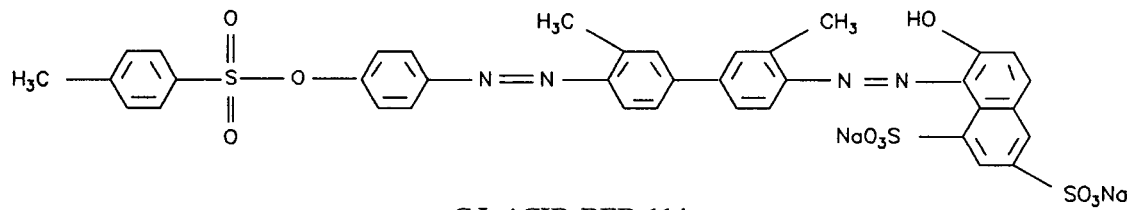
APPENDIX F Chemical Characterization and Dose Formulation Studies 215

APPENDIX G Water and Compound Consumption in the 2-Year Studies 229

APPENDIX H Ingredients, Nutrient Composition, and Contaminant Levels in NIH-07 Rat and Mouse Ration 233

APPENDIX I Sentinel Animal Program 239

ABSTRACT



C.I. ACID RED 114

CAS No. 6459-94-5

Chemical Formula: $C_{37}H_{28}N_4O_{10}S_3Na_2$ Molecular Weight: 830.8

Synonyms: 1,3-Naphthalenedisulfonic acid, 8-((3,3′-dimethyl-4′-((4-(((4-methylphenyl)sulfonyl)oxy)phenyl)azo)(1,1′-biphenyl)-4-yl)azo)-7-hydroxy, disodium salt, Acid Leather Red BG, Acid Red 114, Amacid Milling Red PRS, Benzyl Fast Red BG, Benzyl Red BR, Cerven Kysela, C.I. 23635, Erionyl Red RS, Folan Red B, Kayanol Milling Red RS, Leather Fast Red B, Levanol Red GG, Midlon Red PRS, Milling Red B, Milling Red BB, Milling Red SWB, NCI C61096, Polar Red RS, Sandolan Red N-RS, Sella Fast Red RS, Sulphonol Fast Red R, Supranol Fast Red GG, Supranol Red PBX-CF, Supranol Red R, Telon Fast Red GG, Tertracid Milling Red B, Vondamol Fast Red RS

C.I. Acid Red 114 is one of five chemicals being evaluated in 2-year carcinogenicity and toxicity studies as part of the NTP's Benzidine Dye Initiative. This Initiative was designed to evaluate representative benzidine congeners, benzidine congener-derived dyes, and benzidine-derived dyes. C.I. Acid Red 114 was nominated for study because of the potential for human exposure during production of bisazobiphenyl dyes and because benzidine, a structurally related chemical, is a known human carcinogen.

Toxicology and carcinogenesis studies were conducted by administering desalted, industrial grade C.I. Acid Red 114 in drinking water to groups of F344/N rats of each sex for 13 days, 13 weeks, 9 or 15 months, or 2 years. These studies were performed only in rats because studies of benzidine congeners were being performed in mice at the National Center for Toxicological Research (NCTR). Genetic toxicology studies were conducted in *Salmonella typhimurium*, Chinese hamster ovary cells, and *Drosophila melanogaster*.

13-Day Studies

Rats were exposed to C.I. Acid Red 114 in drinking water at doses of 0, 10,000, 20,000, or 30,000 ppm. All control and dosed rats survived except one male rat in the 20,000 ppm dose group. Final mean body weights in the three dosed groups were 94%, 83%, or 77% of controls for males and 92%, 88%, or 80% of controls for females. Water consumption declined with increased dose. Clinical findings included red stained fur, ears, and tail in all test animals. On gross necropsy, organs and tissues were also stained red.

13-Week Studies

C.I. Acid Red 114 was administered in drinking water at doses of 0, 600, 1,200, 2,500, 5,000, or 10,000 ppm. All control and dosed animals survived until the end of the study. Final mean body weights in the five dosed groups were 97%, 89%, 87%, 87%, or 85% of controls for males and 97%, 94%, 94%, 92%, or 89% of controls for females. Water consumption was decreased in dosed animals. As was seen in the 13-day studies, major organs and tissues from treated animals were stained red. Kidney toxicity characterized by regeneration and karyomegaly of tubule epithelial cells with chronic inflammation was observed in female rats at doses of 1,200 ppm or above. Treatment-related increases in relative liver weights and elevated liver enzyme levels were seen in males and females,

centrilobular pallor in the liver was seen in all male dose groups. Because of these body weight differences, decreases in water consumption, and organ toxicity, the doses chosen for the 2-year studies were 70, 150, and 300 ppm for males and 150, 300, and 600 for females.

2-Year Studies

Male rats received doses of 0, 70, 150, or 300 ppm of C.I. Acid Red 114, and female rats received 0, 150, 300, or 600 ppm. Seventy animals were in the control and high-dose groups, 45 in the low-dose groups, and 75 in the mid-dose groups. Ten animals were evaluated from the control and high-dose groups at 9 months, and ten animals from all dose groups were evaluated at 15 months. The average amount of compound consumed per day was 4, 8, or 20 mg/kg for males and 9, 20, or 70 mg/kg for females.

Survival and Body Weights

Survival at 105 weeks for male rats receiving 0, 70, 150, or 300 ppm was 24/50, 15/35, 26/65, and 1/50; for females receiving 0, 150, or 300 ppm, survival was 36/50, 13/35, and 6/64. All female rats receiving 600 ppm died by week 89. The decreased survival in treated groups was due primarily to the development of chemical-related neoplasms. Of the surviving animals, the final mean body weights for males receiving 70 or 150 ppm were 94% and 90% of control and for females receiving 150 or 300 ppm, 99% and 84% of control. These weight differences began in the second year of the studies and were attributed in part to the development of neoplasms in the dosed groups.

Histopathologic Effects in the 2-Year Studies

At 9 and 15 months, a few neoplasms were seen in the liver, lung, clitoral gland, skin, Zymbal's gland, oral cavity epithelium, and small and large intestine, and the number of neoplasms at these sites increased as the studies progressed. At 2 years, there was a clear carcinogenic response in the skin, Zymbal's gland, and liver of male and female rats, and in the clitoral gland, oral cavity epithelium, small and large intestine, and lung in female rats. Treatment-related increases were also seen in the incidence in neoplasms of the oral cavity epithelium, adrenal gland, and lung of male rats, and in mononuclear cell leukemia and in neoplasms of the mammary gland and adrenal gland in female rats. The incidence of these neoplasms was generally lower, but was significant and considered to be marginally related to chemical treatment. The same neoplastic effects have been previously observed in some or all of the NTP studies with dimethoxybenzidine, dimethylbenzidine, or C.I. Direct Blue 15.

Genetic Toxicology

In a standard preincubation protocol, C.I. Acid Red 114 was mutagenic in *Salmonella typhimurium* strain TA98 in the presence of induced hamster liver S9, and an equivocal response was noted in strain TA100 with hamster liver S9. However, no significant mutagenic activity was noted in strains TA1535 or TA1537 with or without S9 activation. In a modified *S. typhimurium* gene mutation test which employed reductive metabolism followed by oxidative metabolism with S9 liver enzymes, C.I. Acid Red 114 was strongly mutagenic in strain TA1538. C.I. Acid Red 114 did not induce sister chromatid exchanges or chromosomal aberrations in Chinese hamster ovary cells with or without S9 activation; reductive metabolism was not used in these cytogenetic tests. No increase in sex-linked recessive lethal mutations was observed in germ cells of male *Drosophila melanogaster* administered C.I. Acid Red 114 by feeding or injection.

Conclusions

Under the conditions of these 2-year drinking water studies, there was *clear evidence of carcinogenic activity** of C.I. Acid Red 114 for male F344/N rats, as indicated by benign and malignant neoplasms of the skin, Zymbal's gland, and liver. Increased incidences of neoplasms of the oral cavity epithelium, adrenal gland, and lung may have been related to chemical administration. There was *clear evidence of carcinogenic activity* for female F344/N rats, as indicated by benign and malignant neoplasms of the skin, Zymbal's gland, clitoral gland, liver, oral cavity epithelium, small and large intestines, and lung. Increased incidences of mononuclear cell leukemia, mammary gland adenocarcinoma, and adrenal gland pheochromocytomas may have been related to chemical administration.

*Explanation of Levels of Evidence of Carcinogenic Activity appears on page 8. A summary of peer review comments and the public discussion on this Technical Report appears on page 10.

Summary of the 2-Year Carcinogenesis and Genetic Toxicology Studies of C.I. Acid Red 114

Male F344/N Rats	Female F344/N Rats
Drinking water concentration	
0, 70, 150, or 300 ppm C.I. Acid Red 114	0, 150, 300, or 600 ppm C.I. Acid Red 114
Body weights	
Dosed were 9% lower than controls during second year	Dosed were 24% lower than controls during second year
2-Year survival rates[a]	
24/50, 15/35, 26/65, 1/50	36/50, 13/35, 6/64, 0/50
Nonneoplastic effects	
None	None
Neoplastic effects[b]	
Skin basal cell neoplasms: 1/50, 5/35, 28/65, 32/50	Skin basal cell neoplasms: 0/50, 4/35, 7/65, 5/50
Skin keratoacanthoma: 1/50, 1/35, 4/65, 7/50	Zymbal's gland neoplasms: 0/50, 3/35, 18/65, 19/50
Skin sebaceous cell neoplasms: 1/50, 1/35, 5/65, 6/50	Clitoral gland neoplasms: 11/48, 17/32, 28/62, 23/50
Skin squamous cell neoplasms: 1/50, 2/35, 11/65, 9/50	Liver neoplasms: 0/50, 0/35, 19/64, 8/50
Zymbal's gland neoplasms: 0/50, 0/35, 8/65, 7/50	Lung neoplasms: 1/50, 2/35, 9/65, 4/50
Liver neoplasms: 2/50, 2/35, 15/65, 20/50	Oral cavity epithelium neoplasms: 0/50, 3/35, 9/65, 6/50
	Small intestine neoplasms: 0/50, 0/35, 1/65, 2/50
	Large intestine neoplasms: 0/50, 1/35, 0/65, 3/50
Uncertain findings	
Oral cavity epithelium neoplasms: 0/50, 0/35, 1/65, 2/50	Mammary gland adenocarcinoma: 0/50, 3/35, 6/65, 3/50
Adrenal gland pheochromocytomas: 17/50, 11/35, 27/63, 21/49	Adrenal gland pheochromocytomas: 1/50, 3/35, 5/64, 1/50
Lung neoplasms: 2/50, 2/35, 2/65, 3/50	Mononuclear cell leukemia: 12/50, 13/35, 18/65, 5/30
Level of evidence of carcinogenic activity	
Clear evidence	Clear evidence
Genetic toxicology	
Salmonella typhimurium gene mutation:	Positive with S9 in strain TA98; equivocal with S9 in strain TA100; Negative with or without S9 in strain TA1535 or TA1537
Salmonella typhimurium with reductive metabolism:	Positive in strain TA1538
Sister chromatid exchange in Chinese hamster ovary cells *in vitro*:	Negative with or without S9
Chromosomal aberration in Chinese hamster ovary cells *in vitro*:	Negative with or without S9
Drosophila melanogaster germ cell mutation:	Negative by feeding or injection

[a] Reduced survival in exposed groups was due to neoplasia.
[b] Number with lesion/total evaluated

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 because of major flaws cannot be evaluated **(inadequate study)**. These categories of interpretative conclusions were first adopted in June 1983 and then revised in March 1986 for use in the Technical Reports 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 quintet is selected to describe the findings. These categories refer to the strength of the experimental evidence and not to either 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 describes 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. This 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:

- the 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 incidences 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);
- the presence or absence of dose relationships;
- the statistical significance of the observed tumor increase;
- the 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.

PEER REVIEW PANEL

The members of the Peer Review Panel who evaluated the NTP draft Technical Report on C.I. Acid Red 114 on March 11, 1991 are listed below. Panel members serve as independent scientists, not as representatives of any institution, company, or governmental agency. In this capacity, panel members have five major responsibilities in reviewing 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 carcinogenicity activity and other observed toxic responses.

National Toxicology Program Board of Scientific Counselors Technical Reports Review Subcommittee

Daniel S. Longnecker, M.D., Chair
Department of Pathology
Dartmouth Medical School
Hanover, NH

Paul T. Bailey, Ph.D.
Toxicology Division
Mobil Oil Corporation
Princeton, NJ

Jay I. Goodman, Ph.D.
Department of Pharmacology and Toxicology
Michigan State University
East Lansing, MI

Ellen K. Silbergeld, Ph.D.*
University of Maryland Medical School
Baltimore, MD

Ad Hoc Subcommittee Panel of Experts

Louis S. Beliczky, M.S., M.P.H.
Department of Industrial Hygiene
United Rubber Workers International Union
Akron, OH

Gary P. Carlson, Ph.D.
Department of Pharmacology and Toxicology
Purdue University
West Lafayette, IN

Harold Davis, D.V.M., Ph.D., Principal Reviewer
School of Aerospace Medicine
Brooks Air Force Base, TX

Robert H. Garman, D.V.M.
Consultants in Veterinary Pathology
Murrysville, PA

David W. Hayden, D.V.M., Ph.D.
Department of Veterinary Pathobiology
College of Veterinary Medicine
University of Minnesota
St. Paul, MN

Curtis D. Klaassen, Ph.D.
Department of Pharmacology and Toxicology
University of Kansas Medical Center
Kansas City, KS

Barbara McKnight, Ph.D., Principal Reviewer
Department of Biostatistics
University of Washington
Seattle, WA

Lauren Zeise, Ph.D., Principal Reviewer
California Department of Health Services/RCHAS
Berkeley, CA

*Did not attend

SUMMARY OF PEER REVIEW COMMENTS

On March 11, 1991, the draft Technical Report on the toxicology and carcinogenesis studies of C.I. Acid Red 114 received public review by the National Toxicology Program Board of Scientific Counselors' Technical Reports Review Committee and associated Panel of Experts. The review meeting was held at the National Institute of Environmental Health Sciences, Research Triangle Park, NC.

Dr. J. K. Dunnick, NIEHS, introduced the toxicology and carcinogenesis studies of C.I. Acid Red 114 by noting this was one of five chemicals being evaluated as part of the NTP Benzidine Dye Initiative, describing the experimental design, reporting on survival and body weight effects, and commenting on compound-related neoplasms and nonneoplastic lesions in male and female rats. The proposed conclusions were *clear evidence of carcinogenic activity* for male and female F344/N rats.

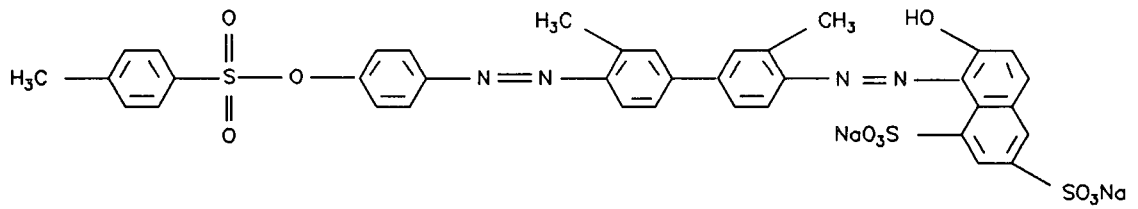
Dr. Zeise, a principal reviewer, agreed in principle with the conclusions. However, she proposed that increased incidences of mononuclear cell leukemia and thyroid follicular cell neoplasms in female rats may have been related to chemical administration and should be cited as such in the conclusions.

Dr. McKnight, the second principal reviewer, agreed with the conclusions. She also thought that the increased incidences of mononuclear cell leukemia and thyroid follicular cell neoplasms in female rats may have been related to chemical administration. In response to Drs. Zeise and McKnight, Dr. Dunnick stated that there was not enough evidence to support even a marginal finding for thyroid tumors in that there was an increase only in the low-dose group, no increase by the trend test, and no increase in precursor hyperplastic lesions in dosed groups. With regard to mononuclear cell leukemia in female rats, Dr. Dunnick commented that incidences in dosed groups were within the historical control range and that high and early mortality in the high-dose group was felt to be due to toxicity of the chemical and not mononuclear cell leukemia. Dr. McKnight pointed out that by the life table test, the test normally used for mononuclear cell leukemia, the pairwise comparison of each of the dose groups with the control group is statistically significant, and the trend test is highly significant.

Dr. Davis, the third principal reviewer, agreed with the conclusions. He asked why the doses in female rats were double those in males since hematologic data and data on kidney degeneration from the 13-week studies suggested females were more sensitive to toxic effects. Dr. Dunnick said apparent liver toxicity in males in the 13-week studies was the primary reason for the different dose levels used in 2-year studies.

Dr. Zeise moved that the Technical Report on C.I. Acid Red 114 be accepted with the revisions discussed and the conclusions as written for male and female rats, *clear evidence of carcinogenic activity*, with the addition of mononuclear cell leukemia to the conclusion for female rats as "may have been related to chemical administration." Dr. McKnight seconded the motion, which was accepted unanimously with ten votes.

INTRODUCTION



C.I. ACID RED 114

CAS No. 6459-94-5

Chemical Formula: $C_{37}H_{28}N_4O_{10}S_3Na_2$ Molecular Weight: 830.8

Synonyms: 1,3-Naphthalenedisulfonic acid, 8-((3,3′-dimethyl-4′-((4-(((4-methylphenyl)sulfonyl)oxy)phenyl)azo)(1,1′-biphenyl)-4-yl)azo)-7-hydroxy, disodium salt, Acid Leather Red BG, Acid Red 114, Amacid Milling Red PRS, Benzyl Fast Red BG, Benzyl Red BR, Cerven Kysela, C.I. 23635, Erionyl Red RS, Folan Red B, Kayanol Milling Red RS, Leather Fast Red B, Levanol Red GG, Midlon Red PRS, Milling Red B, Milling Red BB, Milling Red SWB, NCI C61096, Polar Red RS, Sandolan Red N-RS, Sella Fast Red RS, Sulphonol Fast Red R, Supranol Fast Red GG, Supranol Red PBX-CF, Supranol Red R, Telon Fast Red GG, Tertracid Milling Red B, Vondamol Fast Red RS

PRODUCTION, USE, AND EXPOSURE

C.I. Acid Red 114, an azo dye, is a red powder that decomposes between 250° C and 300° C. It is produced by coupling 2 moles of phenol to *o*-toluidine (3,3′-dimethylbenzidine) followed by coupling this precursor to G acid (2-naphthol-6,8-disulfonic acid) (*Kirk-Othmer*, 1978).

Azo dyes based on benzidine and benzidine congeners (3,3′-dimethylbenzidine and 3,3′-dimethoxybenzidine) constitute a group of over 90 dyes, all widely used in the United States. The United States Environmental Protection Agency (USEPA) reports there are six manufacturers and two importers of C.I. Acid Red 114 (USEPA, 1988). Although annual production volumes are listed as confidential for one of the manufacturers and for both importers, the remaining manufacturers reported collectively that production volumes ranged from 20,000 to 200,000 pounds. The most recent production volume data from the United States International Trade Commission (USITC) show that 380,000 pounds of C.I. Acid Red 114 were produced in 1979 (USITC, 1980). In 1980, 21,497 pounds of the dye were imported (USITC, 1981); the USITC did not report domestic production volumes of C.I. Acid Red 114 for 1985 or 1986 (USITC, 1986, 1987).

From a survey conducted from 1981-1983, the National Institute for Occupational Safety and Health (NIOSH) has estimated that a total of 18,510 workers may be exposed to C.I. Acid Red 114 (NIOSH, 1991). Industrial exposure to these dyes may occur through inhalation of dust or mist, through accidental ingestion, or from direct contact to the skin. The general public may be exposed to C.I. Acid Red 114 from clothes or other products containing the dye or from contaminated water supplies (USEPA, 1980; Fishbein, 1981; NIOSH, 1983).

METABOLISM

Reductive metabolism of 3,3′-dimethylbenzidine-based dyes produces 3,3′-dimethylbenzidine. Azo reduction can occur either in the liver via hepatic enzymes or in the gut by action of azo reductase associated with intestinal bacterial flora. Because highly polar compounds are absorbed from the gut with difficulty, mammals are not expected to absorb the water-soluble sulfonated dyes (Walker, 1970). Thus, reductive cleavage of benzidine-congener azo dyes is thought to occur primarily by bacterial action in the intestinal tract (Martin and Kennelly, 1981; Cerniglia *et al.*, 1982; Brown and Dietrich, 1983; Bos *et al.*, 1984, 1986). Following reductive cleavage, the less polar metabolites are subject to intestinal absorption and further metabolism by the liver.

Metabolism of 3,3′-dimethylbenzidine-based dyes to 3,3′-dimethylbenzidine occurs in dogs and rats (Lynn *et al.*, 1980) and also in humans (NIOSH, 1981). Following exposure to C.I. Acid Red 114, 3,3′-dimethylbenzidine was detected in the urine of dogs and rats (Lynn *et al.*, 1980). Dogs metabolized the dyes Direct Blue 25 and Acid Red 114 to 3,3′-dimethylbenzidine and excreted it in urine. Rats metabolized Direct Blue 25 to 3,3′-dimethylbenzidine and *N*-acetyl-3,3′-dimethylbenzidine, with urine concentrations of 3,3′-dimethylbenzidine comparable to those observed for dogs. However, rats given Acid Red 114 excreted only trace amounts of 3,3′-dimethylbenzidine in urine.

NIOSH (1980) reported the presence of 3,3′-dimethylethylbenzidine in the urine of two employees working in a dye manufacturing plant. The workers were in contact with 3,3′-dimethylbenzidine-based dyes, but not with 3,3′-dimethylbenzidine itself. The presence of 3,3′-dimethylbenzidine in the urine may have resulted from metabolism of the dyes or from exposure to dyes contaminated with 3,3′-dimethylbenzidine. Hartman *et al.* (1978) found that a cell-free extract of *Fusobacterium*, a human intestinal anaerobe, reduced Trypan Blue (C.I. Direct Blue 14), a 3,3′-dimethylbenzidine-derived dye, to 3,3′-dimethylbenzidine.

Tanaka *et al.* (1982) reported that urine extracts from rats treated with 3,3′-dimethylbenzidine or Evans Blue, a 3,3′-dimethylbenzidine-derived dye, contained *N*-acetyl-3,3′-dimethylbenzidine and *N,N*′-diacetyl-dimethylbenzidine, as well as 3,3′-dimethylbenzidine. Urine extracts containing these metabolites were more mutagenic than those containing only 3,3′-dimethylbenzidine. Although Evans Blue was not mutagenic, urine extracts from rats exposed to Evans Blue were mutagenic.

REPRODUCTIVE TOXICOLOGY

Wilson (1955) studied the teratogenic potential of several benzidine-based dyes in albino rats by injecting pregnant females with a 1% aqueous solution of each dye on days 7, 8, and 9 of pregnancy. Trypan Blue was the most potent teratogen, causing malformations in 49% of living offspring compared to 0% in untreated albino rats, followed by Evans Blue, which caused 14% abnormalities, Niagara Blue 4B (C.I. Direct Blue 15), which caused 4% abnormalities, and Niagara Sky Blue 6B, which caused 3% abnormalities. The teratogenic effects of the azo dyes were confirmed in a series of studies by Beaudoin and Pickering (1960), Lloyd *et al.* (1965), Beck and Lloyd (1966), Lloyd and Beck (1966), and Beaudoin (1968). Although the purity and chemical characterization of the dyes used were not reported, the abnormalities were generally similar to common spontaneous malformations such as anencephaly, hydrocephaly, and spina bifida.

TOXICITY AND CARCINOGENICITY STUDIES OF RELATED COMPOUNDS

In 1980, NIOSH and the OSHA issued a health hazard alert stating that persons working with 3,3′-dimethoxybenzidine-, benzidine-, or 3,3′-dimethylbenzidine-based dyes should be aware of the potential health hazards associated with excess exposure (NIOSH, 1981). In a later report issued to alert workers to the hazards of benzidine congener dyes, NIOSH stated that workplace exposure to dyes based on 3,3′-dimethoxybenzidine may pose a carcinogenic risk to workers (NIOSH, 1983). These conclusions were based on evidence from animal studies indicating that 3,3′-dimethoxybenzidine is carcinogenic and on evidence that dyes based on 3,3′-dimethoxybenzidine may be metabolized to the parent compound.

No epidemiologic data on the occurrence of cancer in workers exposed to either C.I. Acid Red 114 or 3,3′-dimethylbenzidine in the absence of other suspected carcinogens were found in the literature.

Benzidine

C.I. Acid Red 114 is a benzidine congener-based dye. Benzidine is a known carcinogen for humans (Scott, 1952; Case *et al.*, 1954; IARC, 1972a,b; Zavon *et al.*, 1973), rats (Spitz *et al.*, 1950; Griswold *et al.*, 1968), hamsters (Saffiotti *et al.*, 1966), and mice (Bonser *et al.*, 1956; Prokofjeva, 1971; IARC, 1972a,b; Frith and Dooley, 1976). Occupational exposure to benzidine for up to 30 years resulted in urinary bladder tumors in as many as 90% of the workers studied (Scott, 1952). Exposure to benzidine may occur directly or by reductive metabolism of benzidine-based dyes. Several reviews address the carcinogenicity of benzidine extensively (IARC, 1972a,b; Haley, 1975; USEPA, 1980; IARC, 1982).

Benzidine exposure caused urinary bladder tumors in dogs (Spitz *et al.*, 1950); hepatocellular, harderian gland, and lymphoreticular tumors in mice (Bonser *et al.*, 1956; Vesselinovitch *et al.*, 1975; Frith and Dooley, 1976); Zymbal's gland, hepatocellular, and mammary gland carcinomas in rats (Spitz *et al.*, 1950; Griswold *et al.*, 1968); and hepatocellular carcinomas, adenomas, and cholangiomas in hamsters (Saffiotti *et al.*, 1967). Animal survival was poor in many of the carcinogenicity studies of benzidine. Although in most cases this was due to the administration of toxic doses, these studies demonstrate that benzidine is carcinogenic in laboratory animals.

3,3′-Dimethylbenzidine

3,3′-Dimethylbenzidine, a methylated congener of benzidine, has been shown to be carcinogenic in rats. In early studies, Spitz *et al.* (1950) demonstrated the ability of the compound to induce Zymbal's gland neoplasms in rats. In a series of experiments, 3,3′-dimethylbenzidine dihydrochloride administered subcutaneously to rats was shown to cause neoplasms of the Zymbal's gland, small intestine, and mammary gland (Pliss, 1963, 1965; Pliss and Zabezhinsky, 1970). From a review of the literature, IARC (1972b) concluded that 3,3′-dimethylbenzidine was a systemic carcinogen for rats when given subcutaneously. In dosed water studies with rats, 3,3′-dimethylbenzidine dihydrochloride caused neoplasms of the skin, Zymbal's gland, preputial and clitoral glands, oral cavity, intestine, liver, brain, and lung in male and female rats, as well as in the mammary gland and hematopoietic system in female rats (NTP, 1991)(Table 1).

BALB/c mice given 3,3′-dimethylbenzidine dihydrochloride in the drinking water at doses up to 140 ppm for 116 weeks showed no evidence of dose-related neoplasms in female mice, but dose-related lung neoplasms were found in male mice (Schieferstein *et al.*, 1989).

3,3′-Dimethoxybenzidine

Repeated exposure to 3,3′-dimethoxybenzidine, the metabolite of C.I. Direct Blue 15, was shown to cause neoplasms of the gastrointestinal tract, Zymbal's gland, skin, and mammary gland of rats and hamsters (Pliss, 1963, 1965; Saffiotti *et al.*, 1967; Hadidian *et al.*, 1968). Although these early studies provided evidence that 3,3′-dimethoxybenzidine is carcinogenic, the use of small numbers of animals, high toxic doses, and poor animal survival weakened the strength of this evidence.

Pliss (1963, 1965) administered 30 mg 3,3′-dimethoxybenzidine in sunflower oil by gavage to rats three times per week. Because of poor survival, this dose was reduced to 15 mg after 3 weeks; administration of this lower dose was continued for 13 months. Of the 42 rats that began the study, 18 survived through month 14. Two of the 18 survivors had neoplasms of the Zymbal's gland; none of the 50 control rats developed neoplasms at this site.

In a life span study, Saffiotti *et al.* (1967) fed diets containing 1,000 ppm 3,3′-dimethoxybenzidine to 30 male and 30 female Syrian golden hamsters. After 144 weeks of exposure, the only neoplastic finding was a transitional cell carcinoma of the urinary bladder in one animal. Sellakumar *et al.* (1969) conducted a similar study in which a higher dietary concentration of 3,3′-dimethoxybenzidine (10,000 ppm) was administered to hamsters. Forestomach papillomas were detected in 37% of the exposed animals and in 2% of the controls, but no urinary bladder lesions were detected. The latter publication is an abstract and does not detail the experimental design or survival data. Hadidian *et al.* (1968) administered 0.1, 0.3, 1.0, 3.0, 10, or 30 mg 3,3′-dimethoxybenzidine per animal per day, 5 days per week, by gavage to groups of three male and

three female F344/N rats (14 males and 15 females in the 10 mg group). The vehicle was a proprietary mixture composed of sodium chloride, sodium carboxymethylcellulose, polysorbate 80, and benzyl alcohol in water. The animals were exposed for 52 weeks, observed for an additional 6 months, and then necropsied. Although neoplasms occurred as early as day 293, most were detected at terminal necropsy. A variety of neoplasms was reported, and pooled results for all dosed male and female groups included neoplastic lesions of the urinary bladder (two papillomas), mammary gland (three carcinomas, two fibroadenomas), skin (five carcinomas), intestinal tract (three carcinomas), and Zymbal's gland (eight carcinomas). The incidence of neoplasms was significantly increased over that of the 360 pooled vehicle and untreated control rats.

The NTP dosed water studies of 3,3′-dimethoxybenzidine dihydrochloride in rats have been reported (NTP, 1990). This chemical caused neoplasms of the skin, Zymbal's gland, preputial and clitoral glands, oral cavity, intestine, and liver, as well as mesotheliomas and neoplasms in the brain of males and neoplasms in the mammary gland and uterus of females (Table 1).

BALB/c mice were given 3,3′-dimethoxybenzidine in drinking water at doses up to 630 ppm. There was a decrease in body weight gain at the 630 ppm dose level relative to the controls, but there was no evidence of treatment-related neoplasms in either sex (Schieferstein *et al.*, 1990).

C.I. Direct Blue 15

The NTP rat dosed-water studies of C.I. Direct Blue 15, a benzidine dye derived from 3,3′-dimethoxybenzidine, have been reported (NTP, in press). This chemical caused neoplasms of the skin, Zymbal's gland, preputial and clitoral gland, oral cavity, large intestine, liver, brain, and uterus as well as mononuclear cell leukemia (Table 1).

o-Anisidine

o-Anisidine (2-methoxyaniline) is structurally analogous to one half of the 3,3′-dimethoxybenzidine molecule and is used to manufacture monoazo dyes by diazotization and coupling with other aromatic amines (Noller, 1965). The National Cancer Institute (NCI) found in bioassays that *o*-anisidine was carcinogenic to F344/N rats and B6C3F$_1$ mice (NCI, 1978). Groups of 55 animals of each species and sex received *o*-anisidine in feed at 5,000 or 10,000 ppm for rats and 2,500 or 5,000 ppm for mice for 103 weeks. Controls consisted of 55 untreated animals of each sex and species. Treatment with *o*-anisidine resulted in transitional cell carcinomas or papillomas of the urinary bladder in both sexes of each species. Male rats also exhibited transitional cell carcinomas of the renal pelvis and follicular cell tumors of the thyroid gland. Only one animal in any of the control groups had a urinary system tumor, which was a transitional cell papilloma of the renal pelvis in a male mouse.

o-Toluidine

o-Toluidine hydrochloride (2-aminotoluene) is structurally analogous to one half of the 3,3′-dimethylbenzidine molecule. In studies performed by NCI (1979), *o*-toluidine hydrochloride was fed to groups of 50 F344/N rats and 50 B6C3F$_1$ mice of each sex for 101 to 104 weeks. The feed contained concentrations of 3,000 or 6,000 ppm for rats and 1,000 or 3,000 ppm for mice. Controls consisted of 20 untreated animals of each sex and species. Exposure of rats to *o*-toluidine hydrochloride resulted in sarcomas of the spleen and other organs in both males and females, mesotheliomas of the abdominal cavity or scrotum in males, and transitional cell carcinomas of the urinary bladder in females. Administration of *o*-toluidine also resulted in increased incidences of fibromas of the subcutaneous tissue in males and fibroadenomas or adenomas of the mammary gland in females. In mice, hemangiosarcomas occurred at various sites in males, and hepatocellular carcinomas or adenomas of the mammary gland occurred in females.

GENETIC TOXICOLOGY

Information regarding the genotoxicity of C.I. Acid Red 114 is limited. The available data from the testing of metabolites of C.I. Acid Red 114 and structurally related dyes corroborate the mutagenicity of C.I. Acid Red 114 by azoreduction and release of active metabolites. As with most benzidine congener dyes, a clearly positive response in the *Salmonella typhimurium* gene mutation assay is dependent on conditions which allow metabolism of the azo bonds to release the parent amine. In

standard *S. typhimurium* assays, only weak mutagenic activity is detected with S9, primarily in frameshift strain TA98, and no activity is apparent in the absence of S9 (Venturini and Tamaro, 1979; Mortelmans *et al.*, 1986). However, when reductive metabolism precedes incubation with the *S. typhimurium* frameshift strains (TA98, TA1538) in the presence of S9, a strong mutagenic response is obtained (Elliot and Gregory, 1980; Prival *et al.*, 1984; Reid *et al.*, 1984a,b).

In mammalian cell systems, C.I. Acid Red 114 has not been tested in protocols allowing reductive metabolism. There was no induction of gene mutations in mouse L5178Y lymphoma cells with and without rat liver S9 (Rudd *et al.*, 1983), or unscheduled DNA synthesis in F344/N rats hepatocytes *in vitro* or *in vivo* (Mirsalis *et al.*, 1983). A key metabolite of C.I. Acid Red 114, 3,3′-dimethylbenzidine, was positive in a variety of *in vivo* genotoxicity assays (NTP, 1991). In the presence of S9 metabolic activation, 3,3′-dimethylbenzidine induced gene mutations in frameshift-sensitive *S. typhimurium* strains TA98 and TA1538 (Shimizu and Takemura, 1976; Hartman *et al.*, 1978; Martin and Kennelly, 1981; Waalkens *et al.*, 1981; Haworth *et al.*, 1983; Reid *et al.*, 1984a,b). Positive results with and without S9 were also obtained for induction of triflurothymidine resistance in mouse L5178Y lymphoma cells (Mitchell *et al.*, 1988; Myhr and Caspary, 1988) as well as sister chromatid exchanges and chromosomal aberrations in Chinese hamster ovary cells (Galloway *et al.*, 1987). 3,3′-Dimethylbenzidine was also positive for induction of unscheduled DNA synthesis (Martin *et al.*, 1978) and DNA repair (Kornbrust and Barfknecht, 1984) in mammalian cells *in vitro* with S9. Two metabolites of 3,3′dimethylbenzidine, *N*-acetyl-3,3′-dimethylbenzidine and *N,N′*-diacetyl-3,3′-dimethylbenzidine, were both positive in *S. typhimurium* strains TA98, TA100, and TA1538 in the presence of S9 (Tanaka *et al.*, 1982; Kennelly *et al.*, 1984; Reid *et al.*, 1984a,b). Benzidine, the parent compound in this series of substituted biphenyls, is positive for induction of gene mutations in *S. typhimurium* with S9 (Haworth *et al.*, 1983; Reid *et al.*, 1984b). Positive induction of micronuclei, sister chromatid exchanges, and chromosomal aberrations was also obtained in bone marrow cells of mice exposed by intraperitoneal injection (NTP, unpublished data).

STUDY RATIONALE

Benzidine is a known human carcinogen (IARC, 1972a,b; 1987a,b), and the benzidine congeners, 3,3′-dimethylbenzidine and 3,3′-dimethoxybenzidine, are known animal carcinogens (IARC, 1972b, 1974). Benzidine and benzidine congener-based dyes have been shown to be metabolized to these parent amines (Rinde and Troll, 1975; NCI, 1978; Lynn *et al.*, 1980; Nony *et al.*, 1980; Bowman *et al.*, 1982).

The National Toxicology Program's (NTP) Benzidine Dye Initiative is a collaborative effort of NIEHS, NCTR, NIOSH, USEPA, OSHA, and CPSC under the aegis of the NTP. The objective of the Initiative was to develop an integrated body of scientific data concerning the metabolism and pharmacokinetics, genetic toxicology, and *in vivo* carcinogenicity of dyes derived from benzidine, 3,3′-dimethylbenzidine, and 3,3′-dimethoxybenzidine. Because studying each of the hundreds of benzidine-based dyes was considered impractical, the research program was designed to evaluate representative benzidine congeners, benzidine congener-derived dyes, and benzidine-derived dyes.

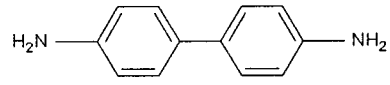
The five benzidine dyes selected for toxicity and carcinogenicity studies were: 3,3′-dimethylbenzidine and 3,3′-dimethoxybenzidine which are benzidine congeners; C.I. Acid Red 114 which is a 3,3′-dimethylbenzidine-based dye; and C.I. Direct Blue 15 and C.I. Direct Blue 218 which are 3,3′-dimethoxybenzidine-based dyes (Figure 1).

The oral route of administration was selected for these studies to mimic potential exposure in the workplace and in the home. Because long-term studies of 3,3′-dimethylbenzidine dihydrochloride and 3,3′-dimethoxybenzidine dihydrochloride were being conducted on mice at NCTR, the NTP 2-year studies of these chemicals used only rats. Results from the studies with 3,3′-dimethoxybenzidine dihydrochloride, 3,3′-dimethylbenzidine dihydrochloride, and C.I. Direct Blue 15 have been reported (NTP, 1990, 1991, in press). Auxiliary studies by Maronpot *et al.* (1988) and Ulland *et al.* (1989) report on transplantation studies of tumors and oncogene activation (Reynolds *et al.*, 1990).

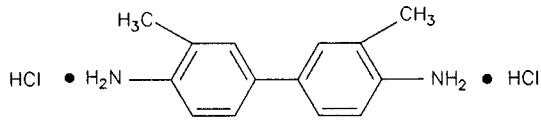
TABLE 1
Incidences of Neoplasms in National Toxicology Program Benzidine Dye Studies

Male F344/N Rats	Female F344/N Rats
Neoplasms in the 21-Month Drinking Water Studies of 3,3′-Dimethoxybenzidine Dihydrochloride[a]	
Skin basal cell or sebaceous gland neoplasms: 2/60, 33/45, 56/75, 41/60	Skin basal cell neoplasms: 0/60, 4/45, 3/75, 2/60
Skin squamous cell neoplasms: 0/60, 13/45, 28/75, 22/60	
Zymbal's gland neoplasms: 0/59, 10/45, 25/75, 30/60	Zymbal's gland neoplasms: 1/60, 12/45, 21/75, 16/60
Preputial gland neoplasms: 16/60, 12/43, 33/73, 29/59	Clitoral gland neoplasms: 7/58, 27/44, 48/74, 41/55
Palate or tongue neoplasms: 1/60, 8/45, 10/75, 11/60	Palate or tongue neoplasms: 2/60, 2/45, 6/75, 5/60
Small intestine neoplasms: 0/60, 4/45, 7/75, 5/60	
Large intestine neoplasms: 0/60, 1/45, 8/75, 8/60	Large intestine neoplasms: 0/60, 1/45, 1/75, 3/60
Liver neoplasms: 1/60, 4/45, 7/74, 8/60	Liver neoplasms: 0/60, 1/44, 0/75, 3/60
Mesotheliomas: 2/60, 1/45, 7/75, 6/60	
Brain astrocytomas: 0/60, 2/44, 3/75, 1/60	
	Mammary gland adenocarcinomas: 1/60, 2/45, 14/75, 20/60
	Uterus or cervix neoplasms: 0/60, 4/45, 2/75, 2/60
Neoplasms in the 15-Month Drinking Water Studies of 3,3′-Dimethylbenzidine Dihydrochloride[b]	
Skin basal cell neoplasms: 0/60, 11/45, 54/75, 30/60	Skin basal cell neoplasms: 0/60, 3/45, 10/75, 9/60
Skin sebaceous cell adenoma: 0/60, 0/45, 7/75, 5/60	
Skin keratoacanthomas: 1/60, 1/45, 8/75, 5/60	
Skin squamous cell neoplasms: 0/60, 2/45, 17/75, 27/60	Skin squamous cell neoplasms: 0/60, 3/45, 9/75, 12/60
Zymbal's gland neoplasms: 1/59, 3/45, 32/75, 36/59	Zymbal's gland neoplasms: 0/57, 6/44, 32/73, 42/60
Preputial gland neoplasms: 2/60, 4/45, 6/75, 9/60	Clitoral gland neoplasms: 0/60, 14/45, 42/75, 32/59
Liver neoplasms: 0/60, 0/45, 35/75, 33/60	Liver neoplasms: 0/60, 0/45, 7/74, 4/60
Oral cavity neoplasms: 0/60, 0/45, 4/75, 5/60	Oral cavity neoplasms: 0/60, 3/45, 9/75, 13/60
Small intestine neoplasms: 0/60, 0/45, 4/75, 8/60	Small intestine neoplasms: 0/60, 1/45, 3/75, 5/60
Large intestine neoplasms: 0/60, 0/45, 6/75, 15/60	Large intestine neoplasms: 0/60, 1/45, 7/75, 4/60
	Mammary gland adenocarcinoma: 0/60, 1/45, 3/75, 6/60
Lung neoplasms: 1/60, 0/45, 8/75, 6/60	Lung neoplasms: 1/60, 1/45, 3/74, 4/60
Mesothelioma: 0/60, 0/45, 3/75, 4/60	
Brain neoplasms: 0/60, 0/45, 1/75, 2/60	Brain neoplasms: 0/60, 2/45, 2/75, 1/60
	Mononuclear cell leukemia: 1/60, 3/45, 6/75, 4/60
Neoplasms in the 22-Month Drinking Water Studies of C.I. Direct Blue 15[c]	
Skin basal cell neoplasms: 2/50, 9/35, 27/65, 28/50	
Skin sebaceous cell adenoma: 0/50, 1/35, 7/65, 3/50	
Skin keratoacanthomas: 2/50, 1/35, 7/65, 2/50	
Skin squamous cell neoplasms: 2/50, 4/35, 11/65, 19/50	Skin squamous cell neoplasms: 0/50, 2/35, 6/65, 5/50
Zymbal's gland neoplasms: 1/50, 5/35, 10/65, 20/50	Zymbal's gland neoplasms: 0/50, 4/35, 11/65, 17/50
Preputial gland neoplasms: 8/49, 5/35, 23/64, 9/48	Clitoral gland neoplasms: 7/50, 11/31, 24/64, 27/50
Liver neoplasms: 0/50, 6/35, 9/65, 11/50	Liver neoplasms: 0/50, 0/35, 2/65, 5/50
Oral cavity neoplasms: 1/50, 10/35, 24/65, 17/50	Oral cavity neoplasms: 2/50, 4/35, 19/65, 15/50
Small intestine neoplasms: 0/50, 1/35, 0/65, 2/50	Small intestine adenocarcinoma: 0/50, 0/35, 1/65, 3/50
Large intestine neoplasms: 0/50, 1/35, 6/65, 8/50	Large intestine adenomatous polyp: 0/50, 0/35, 3/65, 1/50
	Uterine neoplasms: 1/50, 0/35, 1/65, 4/50
Mononuclear cell leukemia: 17/50, 19/35, 28/65, 20/50	Mononuclear cell leukemia: 7/50, 13/35, 27/65, 15/50
Brain neoplasms: 0/50, 1/35, 1/65, 2/50	

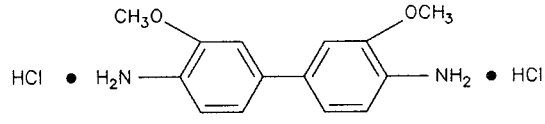
[a] Dose groups: 0, 80, 170, 330 ppm
[b] Dose groups: 0, 30, 70, 150 ppm
[c] Dose groups: 0, 630, 1,250, 2,500 ppm



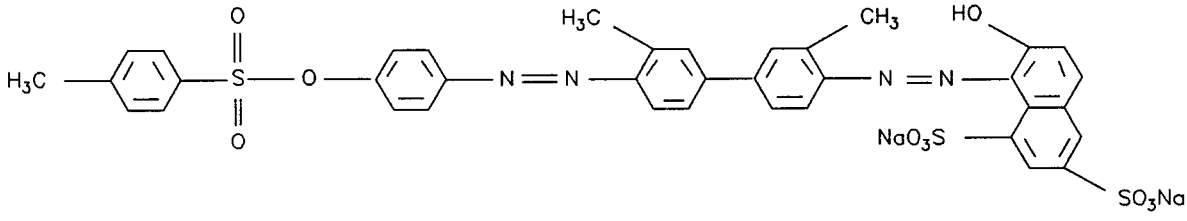
Benzidine
CAS No. 92-87-5



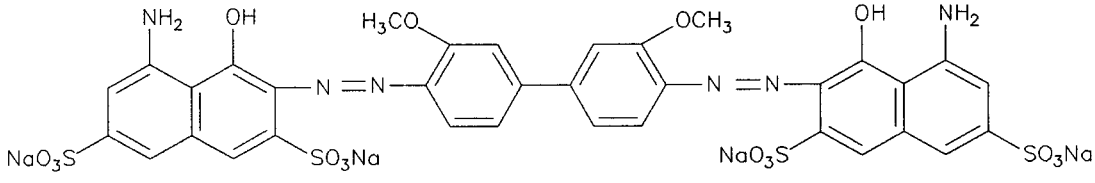
3,3'-Dimethylbenzidine Dihydrochloride
CAS No. 612-82-8



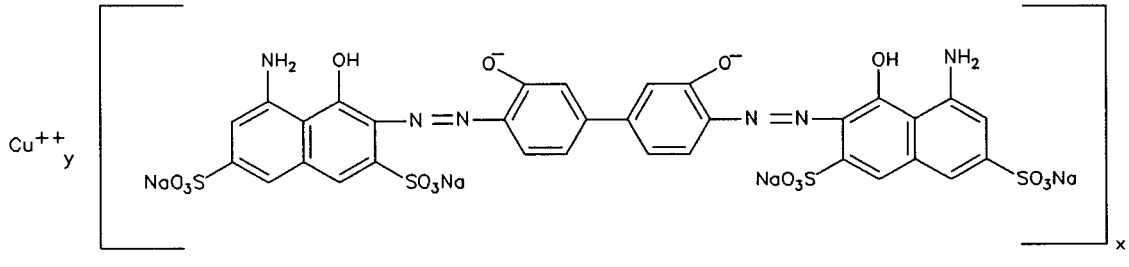
3,3'-Dimethoxybenzidine Dihydrochloride
CAS No. 20325-40-0



C.I. Acid Red 114
CAS No. 6459-94-5



C.I. Direct Blue 15
CAS No. 2429-74-5



C.I. Direct Blue 218
CAS No. 28407-37-6

FIGURE 1
Chemical Structure of Benzidine and Selected Benzidine Congeners and Dyes

MATERIALS AND METHODS

PROCUREMENT AND CHARACTERIZATION OF C.I. ACID RED 114

C.I. Acid Red 114 was obtained in one lot (A101681) from the Atlantic Chemical Company, Nutley, NJ. The dye was desalted in two batches designated as Lots M113081 and M032582. Identity, purity, and stability analyses were conducted by the analytical chemistry laboratory, Midwest Research Institute (MRI), Kansas City, MO (Appendix F). Lot M113081 was used in the 14-day drinking water studies, and Lot M032582 was used in the 13-week and 2-year drinking water studies.

The study dye, a red powder, was identified as C.I. Acid Red 114 by infrared and nuclear magnetic resonance spectroscopy (Appendix F). Purity was evaluated by elemental analysis, water analysis, titration of azo groups, spark source mass spectroscopy, thin layer chromatography (TLC), and high performance liquid chromatography (HPLC). Percent purity was estimated as 82% for Lot M113081 and 85% for Lot M032582.

There were approximately 15 organic impurities observed by HPLC analysis; these impurities were similar in structure to the major component, with the two largest components estimated at 3%. In addition, there was approximately 1% to 4% water and 1% sodium chloride present in the dye. The level of benzidine detected by HPLC did not exceed 1 ppm in either lot; whereas 3,3'-dimethylbenzidine was detected in both lots at a concentration of approximately 5 ppm.

The dye was found to be stable in bulk form when stored protected from light for at least 2 weeks at temperatures up to 60° C. Based on the stability study results, the bulk chemical was stored at room temperature in the dark at the study laboratory. The stability of the bulk chemical was monitored by the study laboratory by HPLC and ultraviolet/visible spectroscopy. No degradation of the study material was detected.

PREPARATION AND ANALYSIS OF DOSE FORMULATIONS

The dose formulations were prepared by mixing the appropriate amount of C.I. Acid Red 114 with tap water (Fairfax Co., VA) for the 13-day studies, or with distilled water (Polar Water Co., Beltsville, MD) for the 13-week and 2-year studies. Stability studies conducted by the analytical chemistry laboratory showed that solutions of 375 ppm C.I. Acid Red 114 in water were stable for at least 3 weeks in the dark at room temperature and for at least 3 days under simulated animal room conditions. Dose formulations were prepared twice weekly for the 14-day and 13-week studies. Dose formulations were prepared in a similar manner for the 2-year studies but were stored 1 week at room temperature prior to use. Preparation and storage procedures for dosed drinking water in the studies of C.I. Acid Red 114 are presented in Appendix F.

Dose formulations were routinely sampled to determine concentration throughout all three studies. In the 13-day studies, dose formulations from the animal room were analyzed prior to test initiation and at the end of the studies (Table F2). In the 13-week studies, dose formulations were analyzed prior to initiation of the studies, at mid-point, and at study end (Table F3). In the 2-year studies, dose formulations were analyzed prior to initiation and at least once every four weeks for the duration of the studies (Table F4). Samples were taken for analysis from the animal room throughout the 13-week and 2-year studies. While the concentrations of the high-dose formulations used in the 13-day studies were outside the ± 10% specifications, all formulations for the 13-week and 2-year studies were within 10% of the target concentrations. Dose formulations were sampled for periodic referee analyses by the analytical laboratory, and results from both laboratories were in agreement (Table F5).

13-DAY STUDIES

Male and female F344/N rats were obtained from Frederick Cancer Research Center (Frederick, MD)

and observed for 14 days prior to the studies. The rats were 7 weeks old when treatment was initiated.

Animals of each sex were weighed and assigned to a weight class; then five animals were randomly placed into each of the dose groups. Groups of five rats of each sex received either 0, 10,000, 20,000, or 30,000 ppm C.I. Acid Red 114 in drinking water for 13 consecutive days. Feed and water were supplied *ad libitum*. Animals were observed twice daily for mortality and once daily for clinical signs. Water consumption by cage was measured twice weekly but recorded as a weekly total. Body weights were measured three times: at the initiation of treatment, at day 7, and at day 13. Complete necropsies were performed on all animals at the end of the studies. The following organs were removed and weighed: brain, heart, liver, lung, right kidney, right testis (males), and thymus. Ratios of organ weight to body weight were determined. Complete histopathologic examinations were performed on all control and the 30,000 ppm dose group animals. In addition, the sternebrae, marrow, and thymus were examined from males and females in 10,000 and 20,000 ppm dose groups. Further experimental details are presented in Table 2.

13-Week Studies

The 13-week studies were conducted to evaluate cumulative toxic effects of repeated exposure to C.I. Acid Red 114 and to determine dose levels for the 2-year studies. The strain and source of rats were the same as the 13-day studies. Rats were randomly assigned by weight class to dose groups and were caged as described for the 13-day studies (Table 2). Rats were observed for 15 days prior to initiation of the studies and were 7 to 8 weeks old when the studies began.

Groups of 10 F344/N rats of each sex received 0, 600, 1,200, 2,500, 5,000, or 10,000 ppm C.I. Acid Red 114 in drinking water. The dosing period was 94 days for males and 95 days for females. Feed and water were supplied *ad libitum*. Animals were observed twice daily for mortality and once daily for clinical signs. Feed and water consumption were measured as in the 13-day studies. Body weights were measured at study initiation and then weekly for the duration of the studies. Complete necropsies were performed on all animals. The age at necropsy was 20 to 21 weeks. Final body weights, selected organ weights, and organ-weight-to-body-weight ratios were measured as in the 13-day studies (Table 2). Complete histopathologic examinations were performed on all control animals and the 10,000 ppm dose group. Organs examined in the lower dose groups were: liver, spleen, mesenteric lymph node, pancreas, and right kidney (females only).

Hematology and clinical chemistry evaluations were performed on blood samples drawn from the abdominal aorta of all animals surviving to the end of the studies. Hematology and clinical chemistry analyses are listed in Table 2.

2-Year Studies

Study Design

C.I. Acid Red 114 was administered in distilled drinking water for 104 weeks. Rats were separated by sex, weighed and grouped by weight class and assigned allocation according to the recommendations of Portier and Hoel (1984). The numbers of animals placed on study for each dose group were 70 controls, 45 low-dose, 75 mid-dose, and 70 high-dose. Male rats received 0, 70, 150, or 300 ppm C.I. Acid Red 114; female rats received 0, 150, 300, or 600 ppm.

Source and Specification of Animals

Strain and species of rats were obtained from the same source as for the 13-day and 13-week studies. The animals were 4 weeks old when received. They were observed for 9 days prior to treatment and were 5 weeks old at the initiation of these studies. Ten animals, five of each sex, were randomly selected and sacrificed prior to the studies and examined for parasites or signs of disease. Serum samples were collected for viral screens. Animal health was monitored throughout the studies according to the protocols of the NTP Sentinel Animal Program (Appendix I).

Animal Maintenance

Animals were housed five per cage as in the other studies. Cages were rotated on the racks from top to bottom and racks of cages within the animal room were rotated every two weeks. Feed and water were supplied *ad libitum*. Distilled water (Polar Water Co., Beltsville, MD) was the vehicle used in administering the chemical to the dosed

animals or the controls. Feed composition is presented in Appendix H. Additional details on animal maintenance are given in Table 2.

Clinical Observations and Pathology

All animals were observed twice daily. Clinical findings were recorded during body weight measurements. Body weights were recorded weekly for the first 14 weeks, again at week 16, and then every four weeks until week 92. After week 92, weights were recorded biweekly up to week 104. Water consumption was recorded twice weekly, except for week 104, and averaged every four weeks.

Organ weights and organ-weight-to-body-weight ratios were determined for control and high-dose groups at 9 months and for all dose groups at 15 months.

Hematology parameters were measured from blood collected from the retroorbital sinus after 39 weeks of chemical exposure for the 9-month evaluation, and after 65 weeks of chemical exposure for the 15-month evaluation. Clinical chemistry analyses were performed on blood sampled from the abdominal aorta on the day of sacrifice. Animals were fasted for 16 hours and anesthetized with sodium pentobarbital just prior to blood collection. Samples for urinalysis were collected by housing ten animals in metabolism cages for 24 hours with only distilled drinking water available.

Ten animals were selected from the high-dose and control groups for the 9-month interim evaluations; an additional 10 animals were selected from each dosed and control group for the 15-month interim evaluations. Complete histologic examinations were conducted on all animals that died or were killed moribund and on all animals necropsied at the end of the studies. Also, selected tissues were evaluated from low- and mid-dose group animals from the 15-month interim evaluations (Table 2). Tissues for microscopic examination were preserved in 10% neutral buffered formalin, then embedded in paraffin, sectioned, and stained with hematoxylin and eosin.

When the pathology evaluation was completed by the study laboratory pathologist and the pathology data entered into the Toxicology Data Management System (TDMS), the microscope slides, individual animal necropsy records, and pathology tables were forwarded to an independent pathology quality assessment laboratory. Individual animal records and pathology tables were compared for accuracy, slides and tissue counts were verified, and histotechnique was evaluated.

A quality assessment pathologist reviewed selected tissues microscopically for accuracy and consistency of lesion diagnosis. All neoplastic and nonneoplastic lesions were reviewed in the following organs: from male rats, liver and Zymbal's gland; from female rats, liver, Zymbal's gland, lung, clitoral gland, and thyroid gland. The adrenal medulla from all males and females also was reviewed for proliferative lesions, and spleen and liver from all females were reviewed to confirm the presence of mononuclear cell leukemia. In addition, all neoplastic diagnoses in tissues other than those already mentioned were reviewed in all animals, and all diagnoses (neoplastic and nonneoplastic) were reviewed from a random 10% of the animals from each control and high-dose group.

The quality assessment report and slides were submitted to the Pathology Working Group (PWG) chair, who reviewed the slides of tissues with chemical-related effects and of any other tissues for which there was disagreement in diagnosis between the laboratory and quality assessment pathologist. Representative histopathology slides of tissues with chemical-related lesions and examples of disagreements in diagnosis between the laboratory and quality assessment pathologist were shown to the PWG. The PWG included the quality assessment pathologist and others experienced in rodent toxicologic pathology who examined the tissues without knowledge of dose group or previously rendered diagnoses. Whenever the consensus diagnosis of the PWG differed from that of the laboratory pathologist, the diagnosis was changed to reflect the opinion of the PWG. This procedure has been described, in part, by Maronpot and Boorman (1982) and Boorman *et al.* (1985). The final pathology data represent a consensus of contractor pathologists and the NTP PWG. For subsequent analysis of pathology data, the diagnosed lesions for each tissue type were separated or combined according to the guidelines of McConnell *et al.* (1986).

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 were censored from the survival analyses at the time they were found dead from other than natural causes. Animals dying from natural causes were not censored. Statistical analysis for a possible dose-related effect on survival used the method of Cox (1972) 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 analysis are two-sided.

Calculation of Incidence

The incidence of neoplasms or nonneoplastic lesions is given as the ratio of the number of animals bearing such lesions at a specific anatomic site to the number of animals in which the site was examined. In most instances, the denominators include only those animals for which the site was examined histologically. However, when macroscopic examination was required to detect lesions (e.g., oral cavity) prior to tissue sampling for histopathology, or when lesions (e.g., lymphomas) could have occurred at multiple sites, the denominators consist of the number of animals on which a necropsy was performed.

Analysis of Tumor Incidence

In the 2-year studies, the deaths of dosed rats and those killed moribund during the studies were considered due to tumors of the skin, Zymbal's gland, and clitoral gland. Consequently, for these particular lesions, primary emphasis in the analysis of tumor incidence was given to the life table test (Cox, 1972; Tarone, 1975), a survival-adjusted procedure appropriate for rapidly lethal tumors.

For incidental tumors (tumors discovered as a result of death from an unrelated cause), the primary statistical method used in this study was logistic regression, which assumed that the diagnosed tumors were discovered as the result of death from an unrelated cause and thus did not affect the risk of death. In this approach, tumor prevalence was modeled as a logistic function of chemical exposure and time. Both linear and quadratic terms in time were incorporated initially, and the quadratic term was eliminated if it did not significantly enhance the fit of the model. The dosed and control groups were compared on the basis of the likelihood score test for the regression coefficient of dose. This method of adjusting for intercurrent mortality is the prevalence analysis of Dinse and Lagakos (1983), further described and illustrated by Dinse and Haseman (1986). When tumors are incidental, this comparison of the time-specific tumor prevalences also provides a comparison of the time-specific tumor incidences (McKnight and Crowley, 1984).

In some instances the reduced survival in dosed animals (due largely to the increased incidence of lethal tumors) reduced the power of logistic regression to detect carcinogenic effects. In these instances, procedures based on effective number of animals (i.e., the number of animals surviving until the appearance of the first tumor of that particular type) were given primary emphasis. These procedures include the Fisher's exact test and the Cochran-Armitage trend test (Armitage, 1971; Gart *et al.*, 1979).

Tests of significance include paired comparisons of each dosed group with controls and a test for an overall dose-response trend. Continuity-corrected tests were used in the analysis of tumor incidence and reported P values are one-sided. The procedures described above were also used to evaluate selected nonneoplastic lesions. For further discussion of these methods, see Haseman (1984).

Historical Control Data

Although the concurrent control group is the first and most appropriate control group used for evaluation, there are certain instances in which historical control data can be helpful in the overall assessment of tumor incidence. Tumor incidences from the NTP historical control data base for 2-year studies (Haseman *et al.*, 1984, 1985) are included for tumors appearing to show compound-related effects.

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 using the multiple comparison procedures of

Dunnett (1955) and Williams (1971, 1972). Clinical chemistry and hematology data, which have typically skewed distributions, were analyzed using the multiple comparison methods of Dunn (1964) and Shirley (1977). Jonckheere's test (Jonckheere, 1954) was used to evaluate the significance of dose-response 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-response trend (Dunnett's or Dunn's test). For the 9-month studies (in which a single dose group was compared with the controls), Wilcoxon's rank sum test (Hollander and Wolfe, 1973) was used to evaluate organ weight, hematology, serum chemistry, and urinalysis data.

Quality Assurance Methods

The 13-week and 2-year studies were conducted in compliance with FDA Good Laboratory Practice Regulations (21 CFR Part 58). In addition, as study records were submitted to the NTP Archives, they were audited retrospectively by an independent quality assurance contractor. Separate audits covering completeness and accuracy of the pathology data, pathology specimens, final pathology tables, and the preliminary draft of this NTP Technical Report were conducted. Audit procedures are presented in the reports, which are on file at the NIEHS. The audit findings were reviewed and assessed by NTP staff so that all had been resolved or were otherwise addressed during the preparation of this Technical Report.

TABLE 2
Experimental Design and Materials and Methods in the Drinking Water Studies of C.I. Acid Red 114

13-Day Studies	13-Week Studies	2-Year Studies
Study Laboratory		
Hazleton Laboratories America, Inc. (Vienna, VA)	Hazleton Laboratories America, Inc. (Vienna, VA)	Hazleton Laboratories America, Inc. (Vienna, VA)
Strain and Species		
F344/N rats	F344/N rats	F344/N rats
Animal Source		
Frederick Cancer Research Center (Frederick, MD)	Frederick Cancer Research Center (Frederick, MD)	Frederick Cancer Research Center (Frederick, MD)
Time Held Before Study		
14 days	15 days	9 days
Age When Placed on Study		
49 days	54 days	35 days
Date of First Dose		
30 March 1982	29 June 1982	10 June 1983
Date of Last Dose		
12 April 1982	31 October 1982 for males 1 November 1982 for females	31 May 1985
Duration of Dosing		
13 consecutive days	94 days for males 95 days for females	104 weeks (7 days/week)
Age at Necropsy		
62 days	21 weeks	109 weeks 45 weeks (9 month interim) 71 weeks (15 month interim)
Necropsy Dates		
12 April 1982	1 and 2 November 1982	10-12 June 1985
Size of Study Groups		
5 males and 5 females	10 males and 10 females	Control: 70/sex Low-dose: 45/sex Mid-dose: 75/sex High-dose: 70/sex
Method of Animal Distribution		
Animals distributed to weight classes and then randomized to test and control groups and position in racks.	Same as 13-day studies	Same as 13-day studies
Animals per Cage		
5	5	5
Method of Animal Identification		
Ear punch	Ear punch	Ear punch then ear tag

TABLE 2
Experimental Design and Materials and Methods in the Drinking Water Studies of C.I. Acid Red 114 (continued)

13-Day Studies	13-Week Studies	2-Year Studies
Diet NIH-07 Rat and Mouse Ration, meal (Zeigler Bros., Inc., Gardners, PA); available *ad libitum*	Same as 13-day studies	Same as 13-day studies
Maximum Storage Time for Feed 120 days after milling	Same as 13-day studies	Same as 13-day studies
Water Tap water (Fairfax County Water Authorities) in glass water bottles with stainless steel sippers (Hazleton Systems, Inc., Aberdeen, MD); available *ad libitum*	Distilled water (Polar Water Co., Beltsville, MD) in glass water bottles with stainless steel sippers (Hazleton Systems, Inc., Aberdeen, MD); available *ad libitum*	Same as 13-week studies
Cages Polycarbonate (Hazleton Systems, Inc., Aberdeen, MD)	Same as 13-day studies	Same as 13-day studies
Bedding Heat-treated hardwood chips (P.J. Murphy Forest Products, Mt. Jewett, PA)	Same as 13-day studies	Same as 13-day studies
Cage Filters Reemay nonwoven polyester fiber filters (DuPont Company, Applied Technologies Division, Wilmington, DE)	Same as 13-day studies	Same as 13-day studies
Animal Room Environment Temperature: 72°-78° F Relative humidity: 27%-69% Fluorescent light: 12 hours/day	Temperature: 70°-75° F Relative humidity: 25%-74% Fluorescent light: 12 hours/day Room air changes: 16/hour	Temperature: 66°-83° F Relative humidity: 25%-77% Fluorescent light: 12 hours/day Room air changes: 11.4/hour
Doses 0, 10,000, 20,000 or 30,000 ppm C.I. Acid Red 114 in drinking water	0, 600, 1,200, 2,500, 5,000, 10,000 ppm C.I. Acid Red 114 in drinking water	0, 70 (males only), 150, 300, or 600 (females only) ppm C.I. Acid Red 114 in drinking water
Type and Frequency of Observation Observed twice/day; body weight initially and once/week; water consumption twice/week; clinical observation daily	Observed twice/day; body weight initially and once/week; water consumption twice/week; clinical observation once/week	Observed twice/day; body weights initially, once/week for 14 weeks to week 16, once/month thereafter to week 92, then every 2 weeks to week 104; water consumption measured in a 3- or 4-day segment twice/week, recorded every 4 weeks; not measured at week 104; clinical observations at body weight determinations

TABLE 2
Experimental Design and Materials and Methods in the Drinking Water Studies of C.I. Acid Red 114 (continued)

13-Day Studies	13-Week Studies	2-Year Studies
Necropsy Necropsy performed on all animals. Organ weights obtained at necropsy (brain, heart, liver, lung, right kidney, right testis, and thymus). **Histopathology** Complete histopathology on male and female control and high-dose (30,000 ppm) animals, including the following tissues: adrenal gland, bone (sternebrae, including marrow), brain, clitoral gland, epididymis, esophagus, heart, kidney, large intestines (cecum, colon, rectum), liver, lymph nodes (mandibular, mesenteric), mammary gland, nasal turbinates, ovary, pancreas, parathyroid gland, pituitary gland, preputial gland, prostate gland, salivary gland, skin, small intestines (duodenum, ileum, jejunum), spleen, stomach, testes, thymus, thyroid gland, trachea, urinary bladder, uterus, Zymbal's gland, and gross lesions. The following tissues were examined from 10,000 and 20,000 ppm males and females: bone (sternebrae, including marrow) and thymus. **Clinical Pathology** None required	**Necropsy** Necropsy performed on all animals. Organ weights measured were the same as in the 13-day studies. **Histopathology** Complete histopathology on male and female controls and all males and females receiving 10,000 ppm. Tissues examined were the same as in the 13-day studies complete screen. Selected tissues were examined from other dose groups as follows: 1,200, 2,500, and 5,000 ppm dose groups, liver, spleen, pancreas, mesenteric lymph node from males and females, kidney from females only; 600 ppm dose group, liver, spleen, pancreas from males and females, mesenteric lymph node from males only, kidney from females only. **Clinical Pathology** Clinical pathology studies were conducted at the end of the studies. ***Hematology:*** hematocrit, hemoglobin, erythrocytes, and leukocyte count and differential. ***Clinical chemistry:*** urea nitrogen, creatinine, alanine aminotransferase, lactate dehydrogenase, and sorbitol dehydrogenase.	**Necropsy** Necropsy performed on all animals. Organ weights measured at 9-month and 15-month interim sacrifices (brain, kidney, liver). **Histopathology** Complete histopathology on all animals from the 9-month interim evaluations, all control and high-dose animals from the 15-month interim evaluations, all animals that died or killed moribund, and all animals killed at the end of the studies. Tissues examined were the same as in the 13-day studies complete screen with the addition of seminal vesicles. In 15-month interim evaluations, tissues examined from low- and mid-dose groups were: 70 and 150 ppm males, liver, kidney, lung, preputial gland, Zymbal's gland, mesenteric lymph node; 150 and 300 ppm females, adrenal gland, liver, kidney, lung, clitoral gland, Zymbal's gland, spleen, pancreas; 300 ppm females only, mesenteric lymph node. **Clinical Pathology** Clinical pathology studies were conducted at 9 and 15 months. ***Hematology:*** hematocrit, hemoglobin, erythrocytes, mean cell volume, mean cell hemoglobin, mean cell hemoglobin concentration, and leukocyte count and differential. ***Clinical chemistry:*** urea nitrogen, creatinine, serum glucose, alanine aminotransferase, lactate dehydrogenase, sorbitol dehydrogenase, triiodothyronine, thyroxine, thyroid-stimulating hormone, serum osmolality, and osmolality ratio. ***Urinalyses:*** urine osmolality, creatinine excretion, urine creatinine, urine volume, specific gravity, and urine pH

RESULTS

13-DAY STUDIES

All rats survived to the end of the studies except for the accidental death of one male in the 20,000 ppm dose group. Final mean body weights were significantly lower for males in the mid- and high-dose groups and for females in all dose groups (Table 3). Overall water consumption decreased with increasing dose. Significantly reduced body weights in the dosed animals made evaluation of the organ weights difficult (Table D1). Organ-weight-to-body-weight ratios were significantly increased for some organs in males and females but were considered secondary to the decrease in body weight. In males, both absolute and relative thymus weights were significantly decreased in mid- and high-dose groups. There were no notable necropsy findings, although organs and tissues were stained red in dosed animals.

Chemical-related histopathologic findings were observed in sternal bone marrow and thymus of both sexes. In males and females receiving 20,000 ppm C.I. Acid Red 114, hypocellularity of sternal bone marrow was found in three males and in all females. In these animals, the marrow was depleted of both erythroid and myeloid cells. This condition was present in only one animal of each sex in the 10,000 ppm dose group. Lymphocytic depletion of the thymus was observed in four males and one female in the 20,000 ppm dose group, but was not observed in the 10,000 ppm dose group.

TABLE 3
Survival and Mean Body Weights of Rats in the 13-Day Drinking Water Studies of C.I. Acid Red 114

Concentration (ppm)	Survival[a]	Mean Body Weight[b] (g) Initial	Final	Change	Final Weight Relative to Controls (%)	Water Consumption[c] Day 1	Day 13
Male							
0	5/5	152 ± 3	218 ± 6	66 ± 3	–	22	21
10,000	5/5	134 ± 1[d]	206 ± 2	72 ± 1	94	21	17
20,000	4/5[e]	154 ± 3	181 ± 3**	29 ± 2**	83	18	18
30,000	5/5	155 ± 3	168 ± 6**	12 ± 3**	77	15	18
Female							
0	5/5	122 ± 3	151 ± 4	29 ± 1	–	20	20
10,000	5/5	119 ± 2	139 ± 2**	20 ± 1**	92	19	13
20,000	5/5	119 ± 2	133 ± 3**	14 ± 2**	88	13	14
30,000	5/5	119 ± 1	121 ± 3**	2 ± 3**	80	12	11

** Significantly different (P≤0.01) from the control group by Williams' or Dunnett's test
[a] Number of animals surviving/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 studies.
[c] Grams per animal per day, based on average consumption data per group per day for days 1 and 13.
[d] A review of the data did not identify the reason for the low initial body weights for this group. At randomization, the mean body weights for all groups was 134 grams. However, 4 days later at study initiation, weights of all groups except for the 10,000 ppm group increased by about 20 grams.
[e] Accidentally killed before day 8

13-WEEK STUDIES

All rats survived to the end of the studies. Final mean body weights for all groups given 1,200 ppm and above were significantly lower than the untreated controls (Table 4). Water consumption for all dose groups was lower than controls.

Absolute and relative organ weights are presented in Table D2. Relative liver weights were significantly increased in all dosed males and females, while absolute and relative kidney weights were significantly increased in females receiving doses of 1,200 ppm and above.

Clinical findings attributed to C.I. Acid Red 114 included discolored fur, urine stains in females, and red crusts around noses in males. Red discolored fur was seen as early as week 2 in high-dose groups and was present in all dose groups by week 9. Discolored, urine-stained fur in females was observed in the two highest dose groups by week 2. Although observed in females by weeks 10 and 11, red crusts around noses were more prevalent in males beginning in week 5.

Hematology and clinical chemistry results for males and females are presented in Table E1. Hematocrit, hemoglobin, and erythrocyte counts were decreased in dosed females and the erythrocyte count was reduced at 1,500 ppm and above in males. These findings are consistent with the reduction in bone marrow cellularity observed in the 13-day studies and suggest that the chemical has a direct effect on hematopoietic cells at high-dose levels. Levels of alanine aminotransferase, lactate dehydrogenase, and sorbitol dehydrogenase were elevated, often significantly, in dosed males, while only sorbitol dehydrogenase levels were significantly elevated in dosed females. These findings are consistent with mild hepatocellular damage.

Slight chemical-related changes were seen in the liver, pancreas, and mesenteric lymph node of treated animals of both sexes and in the kidney of treated females (Table 5). All lesions were minimal to mild in severity. Liver changes in males were minimal and consisted of decreased staining intensity of the cytoplasm of centrilobular hepatocytes

TABLE 4
Survival and Mean Body Weights of Rats in the 13-Week Drinking Water Studies of C.I. Acid Red 114

Concentration (ppm)	Survival[a]	Mean Body Weight[b] (g)			Final Weight Relative to Controls (%)	Water Consumption[c]	
		Initial	Final	Change		Week 1	Week 13
Male							
0	10/10	160 ± 5	343 ± 5	183 ± 4	–	19	22
600	10/10	159 ± 3	333 ± 4	174 ± 4	97	18	19
1,200	10/10	157 ± 3	305 ± 5**	148 ± 4**	89	15	16
2,500	10/10	151 ± 4	298 ± 6**	147 ± 6**	87	14	15
5,000	10/10	159 ± 3	299 ± 5**	140 ± 5**	87	6	15
10,000	10/10	159 ± 4	291 ± 4**	132 ± 5**	85	10	14
Female							
0	10/10	115 ± 3	193 ± 4	78 ± 2	–	16	19
600	10/10	113 ± 3	187 ± 2	74 ± 3	97	19	16
1,200	10/10	110 ± 2	181 ± 2**	71 ± 2	94	11	16
2,500	10/10	116 ± 2	183 ± 3**	66 ± 3**	94	10	11
5,000	10/10	114 ± 3	179 ± 3**	65 ± 4**	92	10	12
10,000	10/10	116 ± 3	172 ± 2**	57 ± 3**	89	7	10

** Significantly different (P≤0.01) from the control group by Williams' or Dunnett's test
[a] Number of animals surviving/number initially in group
[b] Weights and weight changes are given as mean ± standard error.
[c] Grams per animal per day, based on average consumption data per group per week for weeks 1 and 13.

TABLE 5
Incidences of Selected Treatment-Related Lesions in Rats in the 13-Week Drinking Water Studies of C.I. Acid Red 114

Dose	0 ppm	600 ppm	1,200 ppm	2,500 ppm	5,000 ppm	10,000 ppm
Male						
n	10	10	10	10	10	10
Liver						
Centrilobular pallor	0	3	8**	9**	8**	10**
Pancreatic acinar cell						
Degeneration	0	0	9**	8**	7**	8**
Mesenteric lymph node						
Reticulum cell hyperplasia	2	9**	10**	10**	10**	9**
Female						
n	10	10	10	10	10	10
Liver						
Pigment	0	9**	10**	10**	10**	9**
Pancreatic acinar cell						
Degeneration	0	5*	9**	6**	9**	9**
Kidney						
Tubule regeneration	2	1	7*	5	7*	7*
Tubule pigment	0	0	2	5*	3	9*
Chronic inflammation	1	3	5	7**	6*	8**
Karyomegaly	0	0	1	9**	10**	10**
Mesenteric lymph node						
Reticulum cell hyperplasia	3	–[a]	2	10**	10**	9**

* Significantly different (P≤0.05) from the control group by Fisher exact test
** P≤0.01
[a] Tissue not examined at this dose level

(centrilobular pallor). Livers of most treated females contained brown pigment (presumably hemosiderin) within scattered Kupffer cells. Degeneration of scattered individual pancreatic acinar cells was observed in treated males and females and was characterized by the lack of cytoplasmic staining sometimes accompanied by individual cell necrosis. The incidence of reticulum cell hyperplasia of the mesenteric lymph node was increased in treated males and females. This change consisted of multiple small clusters of reticulum cells within the medullary portion of the node.

Tubule regeneration, characterized by slightly dilated tubules lined by small basophilic epithelial cells, and chronic inflammation, consisting of occasional clusters of lymphocytes within the cortical interstitium, occurred more frequently in treated females. These changes are characteristic of the nephropathy commonly observed in aging Fischer rats. In addition, minimal amounts of brownish pigment within tubule epithelial cells and enlargement of scattered tubule epithelial nuclei (karyomegaly) were observed in treated females.

Dose selection rationale: Based on decreases in body weight and in water consumption and organ toxicity, the doses selected for the 2-year studies were 0, 70, 150, and 300 ppm C.I. Acid Red 114 for male rats and 0, 150, 300, and 600 ppm for females.

2-YEAR STUDIES

9-Month Interim Evaluations

Necropsy body weights for females receiving 600 ppm C.I. Acid Red 114 were significantly lower ($P \leq 0.05$) than the controls (Table D3). In the high-dose males and females, absolute and relative liver weights were significantly increased ($P \leq 0.01$). In high-dose males, absolute kidney weight was greater than the controls. In high-dose females, both absolute and relative kidney weights were greater than the controls.

Hematology, clinical chemistry, and urinalysis results are presented in Table E2. Several urinalysis parameters in 300 ppm dosed males and 600 ppm dosed females were significantly increased. Those parameters increased included urine osmolality, osmolality ratio, urine creatinine, and specific gravity, while urine volume was significantly decreased. In dosed females, hematocrit, hemoglobin, and erythrocyte counts were significantly decreased, indicating the development of mild anemia as seen in the 13-week studies. Lactate dehydrogenase and sorbitol dehydrogenase levels were significantly increased, which is consistent with hepatocellular damage. Triiodothyronine and thyroxine levels were significantly decreased and may have been secondary to reduced production of thyroid-binding globulin by the liver.

At 9 months, a few neoplastic lesions were observed including one neoplastic nodule (hepatocellular adenoma) and one alveolar/bronchiolar adenoma in high-dose males, and one clitoral gland carcinoma in a high-dose female (Table 6). In addition, there were a few nonneoplastic changes in treated animals including cytoplasmic vacuolization, clear cell foci, and hepatocyte hypertrophy in the liver; an increase in the severity of nephropathy in the kidneys of high-dose males; and an increase in the incidence and severity of nephropathy in high-dose females.

TABLE 6
Incidences of Selected Treatment-Related Lesions in Rats at the 9-Month Interim Evaluations in the 2-Year Drinking Water Studies of C.I. Acid Red 114

	Male		Female	
	0 ppm	300 ppm	0 ppm	600 ppm
n	10	10	10	10
Liver				
Neoplastic nodule[a]	0	1	0	0
Vacuolization, cytoplasmic	0	10** (1.0)[b]	0	2 (1.5)
Basophilic focus	0	10** (1.4)	0	10** (1.0)
Clear cell focus	0	7** (1.0)	0	0
Hepatocyte hypertrophy	0	0	0	10** (2.0)
Lung				
Alveolar/bronchiolar adenoma	0	1	0	0
Clitoral gland				
Carcinoma	–	–	0	1
Kidney				
Nephropathy	10 (1.1)	10 (1.5)	3 (1.0)	10** (1.5)

** Significantly different ($P \leq 0.01$) from the control group by Fisher exact test
[a] Term previously used for neoplasm now diagnosed as hepatocellular adenoma.
[b] Values in parentheses are average severity grades of lesions in affected animals; 1 = minimal and 2 = mild.

15-Month Interim Evaluations

Absolute and relative liver weights were significantly greater ($P \leq 0.01$) than the controls for males receiving 300 ppm and for females in all dosed groups (Table D4). Relative kidney weights were increased in mid- and high-dose females.

Hematology, clinical chemistry, and urinalysis results are presented in Table E3. Several hematology parameters, including hematocrit, hemoglobin, erythrocyte count, and mean cell volume, were decreased in 300 ppm dosed males and 600 ppm dosed females. These findings were consistent with a poorly regenerative anemia. Segmented neutrophils were significantly increased in the 300 and 600 ppm dosed females, presumably as a result of inflammation associated with neoplasms occurring in these groups. Alanine aminotransferase was significantly increased in 600 ppm dosed females and sorbitol dehydrogenase was significantly increased in all dosed females and is indicative of hepatocellular damage.

Triiodothyronine and thyroid-stimulating hormone (TSH) levels were significantly increased in 600 ppm dosed females; increased TSH levels are consistent with the marginal increase in proliferative thyroid follicular cell lesions seen in the 2-year studies. Urine osmolality, osmolality ratio, urine creatinine, and specific gravity were significantly increased in 300 ppm dosed males, while urine volume was significantly decreased, and these effects are considered to be secondary to decreased water intake.

A variety of lesions were found in male and female rats given C.I. Acid Red 114 in drinking water for 15 months (Table 7). Proliferative lesions included hepatocellular adenoma (neoplastic nodule) and hepatocellular carcinoma in the liver; alveolar/bronchiolar adenoma and alveolar epithelial hyperplasia of the lung; sebaceous gland adenoma and squamous cell carcinoma of the skin; Zymbal's gland adenoma, carcinoma, and hyperplasia; adenoma, carcinoma, and hyperplasia of the clitoral gland; squamous cell papilloma and carcinoma in the oral cavity epithelium (palate and tongue); and adenocarcinoma of the small and large intestine. Additional treatment-related nonneoplastic effects included cytoplasmic vacuolization, cystic degeneration, hepatocyte hypertrophy, and clear and basophilic foci in the liver; an increase in the severity of nephropathy in treated males; and an increase in the incidence and severity of nephropathy in treated females.

TABLE 7
Incidences of Selected Treatment-Related Lesions in Rats at the 15-Month Interim Evaluations in the 2-Year Drinking Water Studies of C.I. Acid Red 114

Male	0 ppm	70 ppm	150 ppm	300 ppm
n	10	10	10	10
Liver				
Neoplastic nodule[a]	0	0	0	1
Vacuolization, cytoplasmic	1	6* (1.2)	5 (1.2)	10** (2.2)
Cystic degeneration	0	1	3 (1.0)	3 (1.3)
Basophilic focus	3 (1.0)[b]	8* (1.1)	10** (1.0)	10** (2.3)
Clear cell focus	1	2 (1.5)	2 (2.0)	7** (1.4)
Hepatocyte hypertrophy	0	0	1	2 (1.0)
Lung				
Alveolar/bronchiolar adenoma	0	1	0	0
Hyperplasia, alveolar epithelium	1	2 (1.5)	3 (2.0)	4 (2.3)
Skin				
Sebaceous gland adenoma	0	0	0	1
Squamous cell carcinoma	0	0	0	1
Tongue				
Squamous cell carcinoma	0	0	1	0
Zymbal's gland				
Adenoma	0	0	1	1
Kidney				
Nephropathy	10 (1.5)	10 (1.7)	10 (2.1)	10 (2.5)

Female	0 ppm	150 ppm	300 ppm	600 ppm
n	10	10	10	10
Liver				
Hepatocellular carcinoma	0	0	0	5*
Neoplastic nodule[a]	0	2	1	6**
Vacuolization, cytoplasmic	2 (1.0)	3 (1.7)	9** (1.6)	9** (3.1)
Cystic degeneration	0	0	2 (1.5)	2 (1.5)
Basophilic focus	6 (1.0)	9 (1.3)	10* (1.9)	10* (1.4)
Hepatocyte hypertrophy	0	4* (1.0)	9** (1.8)	10** (3.6)
Lung				
Alveolar/bronchiolar adenoma	0	0	0	3
Hyperplasia, alveolar epithelium	0	2 (1.0)	3 (1.0)	8** (2.3)

TABLE 7
Incidences of Selected Treatment-Related Lesions in Rats at the 15-Month Interim Evaluations in the 2-Year Drinking Water Studies of C.I. Acid Red 114 (continued)

	0 ppm	150 ppm	300 ppm	600 ppm
Female (continued)				
n	10	10	10	10
Clitoral gland				
Adenoma	0	1	3	4*
Carcinoma	0	0	0	2
Hyperplasia, squamous	0	0	2 (2.0)	2 (3.0)
Oral cavity (palate and tongue)				
Squamous cell papilloma	0	0	0	4*
Skin				
Squamous cell carcinoma	0	0	1	0
Small intestine				
Adenocarcinoma	0	0	1	1
Large intestine				
Adenocarcinoma	0	0	0	1
Zymbal's gland				
Papilloma	0	0	0	2
Carcinoma	0	0	1	1
Hyperplasia, squamous	0	2 (2.0)	2 (1.5)	6** (1.7)
Kidney				
Nephropathy	7 (1.1)	9 (1.1)	10 (2.0)	10 (3.9)

* Significantly different ($P \leq 0.05$) from the control group by Fisher exact test
** $P \leq 0.01$
[a] Term previously used for neoplasm now diagnosed as hepatocellular adenoma.
[b] Values in parentheses are average severity grades of lesions in affected animals; 1 = minimal; 2 = mild, 3 = moderate, and 4 = marked.

Body Weights, Water Consumption, and Clinical Findings in the 2-Year Studies

At week 105 of the 2-year studies, final mean body weights relative to controls for male rats in 70 ppm, 150 ppm, and 300 ppm dose groups were 94%, 90%, and 90%, and for females in 150 ppm and 300 ppm dose groups, relative weights were 99% and 89% (Tables 8 and 9, Figure 2). These body weight decrements began in the second year of the studies and were considered to be due to the development of neoplastic disease in dosed animals. Female rats in the 600 ppm dose group only survived until week 88; their final mean body weight relative to controls was 72%.

Mean water consumption was, in general, similar (±10%) among the dosed and control groups, except in 300 ppm dosed males and 600 ppm dosed females where water consumption was increased during the last year of the studies. This increase was attributed, in part, to an increase in the severity of nephropathy relative to the controls.

The daily dose of C.I. Acid Red 114 during weeks 53 to 101 was approximately 4, 8, and 20 mg/kg for males receiving 70, 150, and 300 ppm; and 9, 21, and 69 mg/kg for females receiving 150, 300, and 600 ppm (Tables G1 and G2). Tissue masses and swellings were the most common clinical findings in dosed rats. Red staining of the fur was noted in all dosed groups.

TABLE 8
Mean Body Weights and Survival of Male Rats in the 2-Year Drinking Water Study of C.I. Acid Red 114

Weeks on Study	0 ppm Av. Wt. (g)	0 ppm No. of Survivors	70 ppm Av. Wt. (g)	70 ppm Wt. (% of controls)	70 ppm No. of Survivors	150 ppm Av. Wt. (g)	150 ppm Wt. (% of controls)	150 ppm No. of Survivors	300 ppm Av. Wt. (g)	300 ppm Wt. (% of controls)	300 ppm No. of Survivors
1	118	70	119	101	45	117	100	75	119	101	70
2	159	70	160	100	45	155	98	75	156	98	70
3	199	70	196	99	45	196	99	75	195	98	70
4	227	70	228	100	45	230	101	75	227	100	70
5	249	70	255	102	45	251	101	75	253	101	70
6	272	70	271	100	45	274	101	75	271	100	70
7	287	70	287	100	45	286	100	75	280	98	70
8	300	70	302	101	45	305	102	75	297	99	70
9	314	70	314	100	45	319	101	75	315	100	70
10	331	70	330	100	45	334	101	75	329	100	70
11	332	70	342	103	45	344	104	75	339	102	70
12	346	70	349	101	45	354	102	75	348	101	70
13	352	70	347	98	45	358	102	75	352	100	70
14	360	70	359	100	45	364	101	75	358	100	70
15	361	70	371	103	45	371	103	75	368	102	70
17	370	70	377	102	45	382	103	75	378	102	70
21	396	69	391	99	45	392	99	75	387	98	70
25	418	69	415	99	45	422	101	75	421	101	70
29	423	69	423	100	45	432	102	75	428	101	70
33	436	69	438	101	45	437	100	75	435	100	70
37	443	69	442	100	45	449	101	75	443	100	70
41	449	59[a]	455	101	45	452	101	75	450	100	60[a]
45	457	59	455	100	45	457	100	75	455	100	59
49	462	59	459	99	45	463	100	74	458	99	59
53	460	58	463	101	44	466	101	74	456	99	59
57	454	58	453	100	44	453	100	74	445	98	57
61	454	58	459	101	44	462	102	73	451	99	56
65	459	58	460	100	43	463	101	73	452	99	56
69[a]	458	45	452	99	32	455	100	62	442	97	43
73	459	45	450	98	32	451	98	61	435	95	42
77	457	44	452	99	29	452	99	60	436	95	32
81	450	44	447	99	28	445	99	60	427	95	29
85	445	42	448	101	28	443	100	59	411	92	27
89	455	39	444	98	28	437	96	54	400	88	26
93	445	37	427	96	26	421	95	48	388	87	20
95	449	35	426	95	22	413	92	45	365	81	17
97	439	34	422	96	21	407	93	41	359	82	12
99	440	32	413	94	21	401	91	38	365	83	11
101	434	30	417	96	18	410	95	30	352	81	6
103	446	25	419	94	16	406	91	26	356	80	3
105	437	24	411	94	15	393	90	26	392	90	1
Terminal sacrifice		24			15			26			1
Mean for weeks											
1-13	268		269	100		271	101		268	100	
14-52	416		417	100		420	101		416	100	
53-105	449		439	98		434	97		408	91	

[a] Interim evaluation occurred.

TABLE 9
Mean Body Weights and Survival of Female Rats in the 2-Year Drinking Water Study of C.I. Acid Red 114

Weeks on Study	0 ppm Av. Wt. (g)	0 ppm No. of Survivors	150 ppm Av. Wt. (g)	150 ppm Wt. (% of controls)	150 ppm No. of Survivors	300 ppm Av. Wt. (g)	300 ppm Wt. (% of controls)	300 ppm No. of Survivors	600 ppm Av. Wt. (g)	600 ppm Wt. (% of controls)	600 ppm No. of Survivors
1	96	70	96	100	45	97	101	75	98	102	70
2	119	70	119	99	45	116	97	75	117	98	70
3	137	70	136	99	45	133	97	75	133	97	70
4	150	70	148	99	45	146	97	75	145	97	70
5	159	70	162	102	45	155	98	75	156	98	70
6	170	70	168	99	45	163	96	75	161	95	70
7	177	70	175	99	45	170	96	75	168	95	70
8	183	70	184	100	45	176	96	75	174	95	70
9	187	70	186	100	45	180	97	74	181	97	70
10	194	70	193	99	45	187	96	74	184	95	70
11	196	70	197	101	45	190	97	74	186	95	70
12	201	70	204	101	45	194	96	74	191	95	70
13	204	70	203	100	45	196	96	74	193	95	70
14	206	70	208	101	45	199	97	73	196	95	70
15	209	70	210	100	45	203	97	73	200	96	70
17	216	70	214	99	45	210	97	73	208	96	70
21	219	70	220	100	45	211	97	73	209	96	70
25	231	70	232	101	45	224	97	73	221	96	70
29	235	70	233	99	45	229	97	73	223	95	69
33	242	70	244	101	45	231	96	73	228	94	69
37	247	70	248	100	45	239	97	73	233	94	68
41	255	60[a]	259	102	45	244	96	73	237	93	67
45	263	60	269	102	45	252	96	72	242	92	56
49	272	60	274	101	45	260	96	72	242	89	56
53	286	60	290	101	45	267	93	71	249	87	50
57	290	60	293	101	45	278	96	71	250	86	44
61	300	60	305	102	43	287	96	69	250	83	33
65	307	59	312	102	43	292	95	68	254	83	29
69[a]	320	49	315	98	32	296	93	57	252	79	14
73	330	48	323	98	31	300	91	52	271	82	8
77	334	47	332	99	30	309	93	45	258	77	8
81	344	45	335	97	29	307	90	41	270	79	5
85	345	42	341	99	27	306	89	34	249	72	3
89	352	42	341	97	25	306	87	27			
93	352	41	346	98	22	300	85	21			
95	354	41	341	96	20	295	84	17			
97	353	40	345	98	17	288	82	17			
99	357	39	348	98	16	294	83	14			
101	358	39	349	98	16	298	83	11			
103	360	36	345	96	14	299	83	6			
105	354	36	350	99	13	296	84	6			
Terminal sacrifice		36			13			6			0
Mean for weeks											
1-13	167		167	100		162	97		161	96	
14-52	236		237	100		227	96		222	94	
53-105	335		330	99		295	88		256	76	

[a] Interim evaluation occurred.

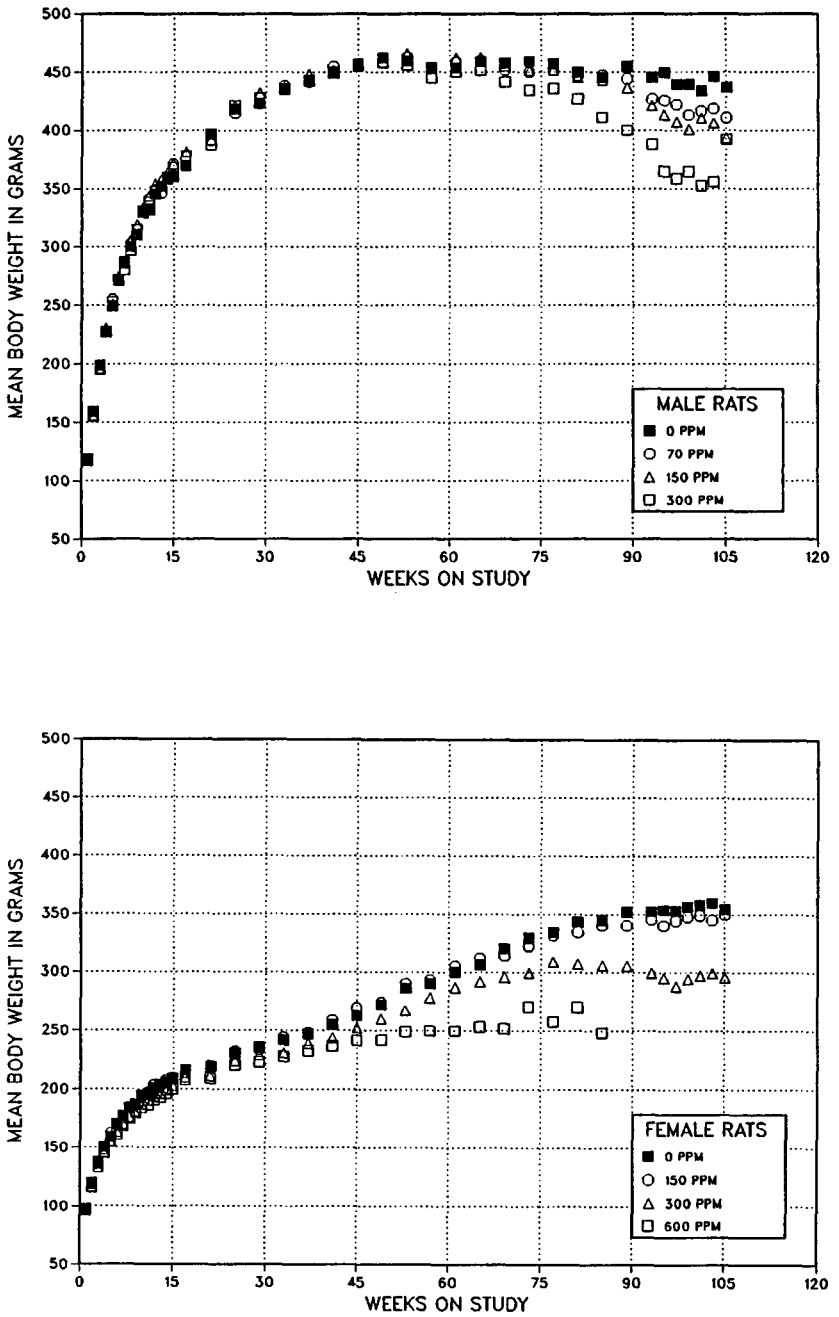


Figure 2
Growth Curves for Male and Female Rats Administered C.I. Acid Red 114 in Drinking Water for 2 Years

Survival

Estimates of the probabilities of survival for male and female rats given C.I. Acid Red 114 and the controls are shown in Table 10 and in the Kaplan-Meier survival curves in Figure 3. Decreases in survival in females receiving 600 ppm began after week 52 and all had died by week 88. Final survival was decreased in males receiving 70 ppm and in females receiving 150 ppm. These decreases in survival were due to the development of neoplasms in dosed animals.

TABLE 10
Survival of Rats in the 2-Year Drinking Water Studies of C.I. Acid Red 114

Male	0 ppm	70 ppm	150 ppm	300 ppm
Animals initially in study	70	45	75	70
Natural deaths	13	11	16	16
Moribund	13	9	23	33
Interim evaluations[a]				
9 month	10	0	0	10
15 month	10	10	10	10
Animals surviving to study termination	24[b]	15	26	1
Percent survival at end of study[c]	49	43	40	2
Mean survival days[d]	579	616	643	527
Survival analysis[e]	P<0.001	P=0.730	P=0.507	P<0.001

Female	0 ppm	150 ppm	300 ppm	600 ppm
Animals initially in study	70	45	75	70
Natural deaths	4	6	17	13
Moribund	10	16	41	37
Accidental deaths[a]	0	0	1	0
Interim evaluations[a]				
9 month	10	0	0	10
15 month	10	10	10	10
Animals surviving to study termination	36	13	6	0
Percent survival at end of study[c]	72	38	10	0
Mean survival days[d]	602	609	562	412
Survival analysis[e]	P<0.001	P=0.002	P<0.001	P<0.001

[a] Censored from survival analyses.
[b] One of these animals was dead on the last day of the studies.
[c] Kaplan-Meier determinations. Survival rates adjusted for accidental deaths and interim evaluations.
[d] Mean of all deaths (uncensored, censored, terminal sacrifice).
[e] The entry under the "0 ppm" column is associated with the life table trend test (Tarone, 1975). Subsequent entries are the results of pairwise tests (Cox, 1972).

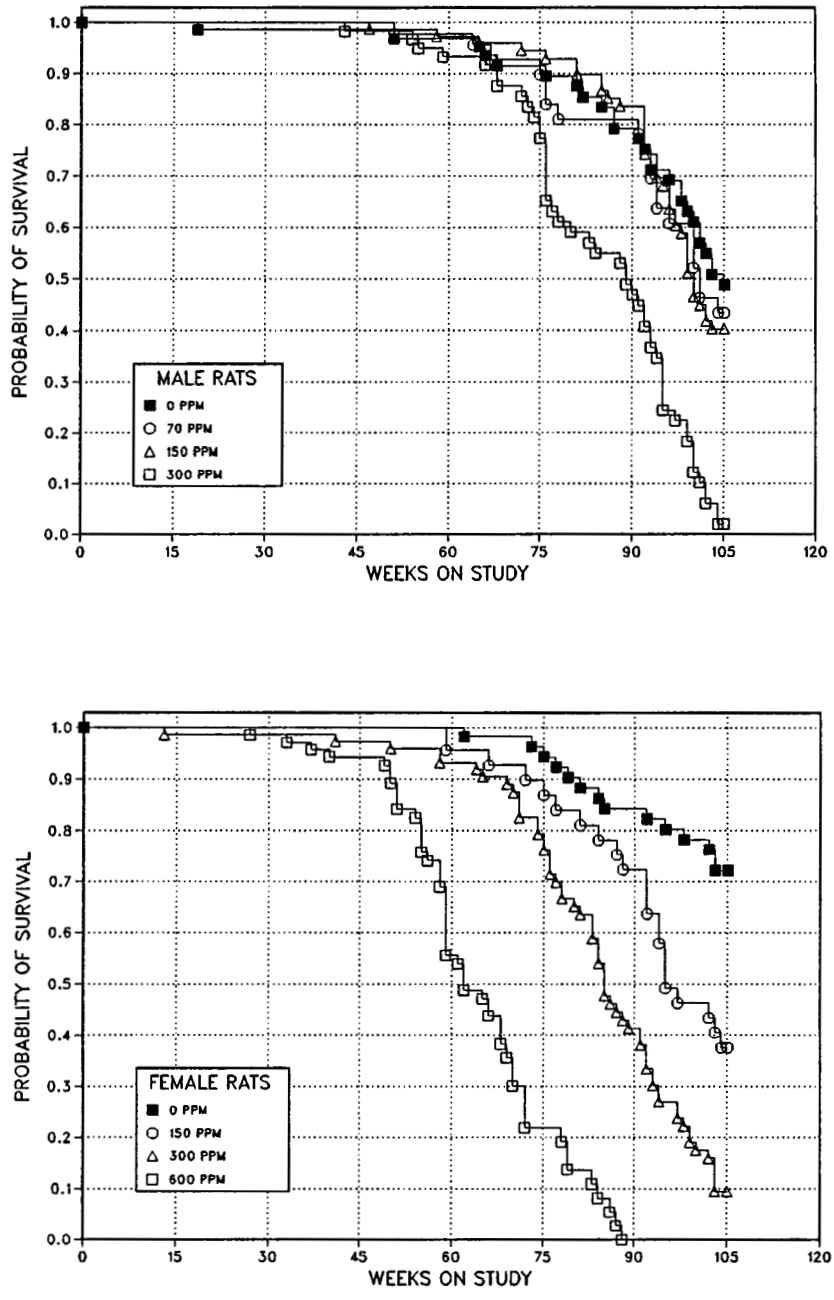


Figure 3
Kaplan-Meier Survival Curves for Male and Female Rats Administered C.I. Acid Red 114 in Drinking Water for 2 Years

Pathology and Statistical Analyses of Results

Summaries of the incidences of neoplasms and nonneoplastic lesions, individual animal tumors, statistical analyses of primary tumors occurring with an incidence of at least 5% in at least one animal group, and historical control incidences for selected neoplasms discussed in this section are presented in Appendixes A and B for male and female rats.

Skin: A variety of epithelial neoplasms of the skin occurred with increased incidences in male and female rats treated with C.I. Acid Red 114 (Table 11). The incidences of basal cell adenomas, basal cell carcinomas, and the combined incidence of basal cell adenomas and carcinomas were moderately increased in low-dose (70 ppm) males and significantly increased in mid- (150 ppm) and high-dose (300 ppm) males. Many of the treated males had multiple basal cell adenomas. There was a statistically significant increase in the incidence of basal cell neoplasms in females from the low- (150 ppm) and mid-dose (300 ppm) groups. The incidence of sebaceous gland adenomas was increased in treated males and was significantly increased in the high-dose group. The combined incidences of squamous cell papilloma and squamous cell carcinoma were significantly increased in mid- and high-dose males, and in mid-dose females. The lower incidence of skin neoplasms in the high-dose (600 ppm) female group was considered secondary to reduced survival in this group due to neoplasms at other sites. The incidence of keratoacanthomas was increased in dosed male groups compared to controls and was significantly increased in the high-dose group. A single keratoacanthoma occurred in one high-dose female.

Basal cell neoplasms were composed of small basophilic polygonal cells that formed sheets, tortuous cords, or solid lobules that often contained central cavities. Adenomas were discrete, well demarcated masses while carcinomas exhibited local invasion and frequently contained areas of necrosis. Many basal cell neoplasms contained areas of squamous, sebaceous, or hair follicle differentiation (Plate 1). Some neoplasms consisted solely of sebaceous elements and were diagnosed as sebaceous gland adenoma or carcinoma. Squamous cell papillomas were exophytic growths that were pedunculated and highly branched, and composed of a fibrovascular core covered by thickened, stratified, squamous epithelium. Squamous cell carcinomas were highly invasive lesions consisting of multiple irregular cords of disordered, pleomorphic, stratified, squamous epithelial cells, often containing foci of keratin formation, which projected into the dermis (Plate 2). Keratoacanthomas were cystic structures lying within the dermis that were lined by a thick, highly folded layer of heavily keratinized, stratified, squamous epithelium.

Zymbal's Gland: The Zymbal's gland is a specialized sebaceous gland that lies ventral and anterior to the orifice of the external ear. Zymbal's gland neoplasms grow very rapidly and generally lead to death of the animal; therefore, the life table analysis is the most appropriate statistical test for the incidences of these neoplasms. There were moderate increases in the incidences of Zymbal's gland neoplasms in treated males and marked increases in treated female rats (Table 12). The combined incidences of adenomas and carcinomas were significantly increased in the mid- and high-dose male groups and in all groups of treated females. Zymbal's glands from some treated animals contained nonneoplastic lesions including focal hyperplasia of the squamous epithelial lining of glandular ducts (hyperplasia, squamous) and dilatation of glandular ducts (ectasia)(Tables A5 and B5). There was a morphologic continuum from adenoma to carcinoma. Adenomas were discrete nodular masses composed of glandular acini of relatively normal appearing sebaceous cells and containing ductular structures lined by stratified squamous epithelium. Occasionally, these ductular structures were dilated and filled with secretory material. Carcinomas were generally larger and invaded adjacent tissues. Neoplastic cells were often atypical. They exhibited disordered growth patterns and formed solid masses, irregular acinar structures, and cords, with a scattering of ductular structures. Occasionally, areas of necrosis were observed. Some carcinomas consisted principally of sebaceous cells while others were composed mainly of stratified squamous epithelium; some neoplasms had prominent components of both.

TABLE 11
Skin Neoplasms in Rats in the 2-Year Drinking Water Studies of C.I. Acid Red 114

Male	0 ppm	70 ppm	150 ppm	300 ppm
Skin: Keratoacanthoma[a]				
Overall rates[b]	1/50 (2%)	1/35 (3%)	4/65 (6%)	7/50 (14%)
Effective rates[c]	1/42 (2%)	1/28 (4%)	4/58 (7%)	7/28 (25%)
Terminal rates[d]	1/24 (4%)	1/15 (7%)	4/26 (15%)	1/1 (100%)
First incidence (days)	732 (T)	732 (T)	732 (T)	582
Life table tests[e]	P<0.001	P=0.654	P=0.200	P<0.001
Logistic regression tests[e]	P<0.001	P=0.654	P=0.200	P=0.005
Skin (Sebaceous Gland): Adenoma or Carcinoma[f]				
Overall rates	1/50 (2%)	1/35 (3%)	5/65 (8%)	6/50 (12%)
Effective rates	1/45 (2%)	1/32 (3%)	5/61 (8%)	6/41 (15%)
Terminal rates	0/24 (0%)	1/15 (7%)	4/26 (15%)	0/1 (0%)
First incidence (days)	706	732 (T)	593	512
Life table tests	P<0.001	P=0.638	P=0.132	P=0.001
Logistic regression tests	P=0.007	P=0.663	P=0.166	P=0.036
Skin: Basal Cell Adenoma				
Overall rates	1/50 (2%)	4/35 (11%)	26/65 (40%)	30/50 (60%)
Effective rates	1/46 (2%)	4/32 (13%)	26/62 (42%)	30/44 (68%)
Terminal rates	1/24 (4%)	3/15 (20%)	19/26 (73%)	1/1 (100%)
First incidence (days)	732 (T)	651	641	473
Life table tests	P<0.001	P=0.071	P<0.001	P<0.001
Logistic regression tests	P<0.001	P=0.073	P<0.001	P<0.001
Skin: Basal Cell Carcinoma				
Overall rates	0/50 (0%)	1/35 (3%)	5/65 (8%)	6/50 (12%)
Effective rates	0/46 (0%)	1/32 (3%)	5/62 (8%)	6/44 (14%)
Terminal rates	0/24 (0%)	0/15 (0%)	4/26 (15%)	0/1 (0%)
First incidence (days)	–[g]	724	673	473
Life table tests	P<0.001	P=0.411	P=0.043	P=0.002
Logistic regression tests	P=0.003	P=0.408	P=0.046	P=0.020
Skin: Basal Cell Adenoma or Carcinoma[h]				
Overall rates	1/50 (2%)	5/35 (14%)	28/65 (43%)	32/50 (64%)
Effective rates	1/46 (2%)	5/32 (16%)	28/62 (45%)	32/44 (73%)
Terminal rates	1/24 (4%)	3/15 (20%)	20/26 (77%)	1/1 (100%)
First incidence (days)	732 (T)	651	641	473
Life table tests	P<0.001	P=0.032	P<0.001	P<0.001
Logistic regression tests	P<0.001	P=0.030	P<0.001	P<0.001
Skin: Squamous Cell Papilloma				
Overall rates	1/50 (2%)	0/35 (0%)	3/65 (5%)	2/50 (4%)
Effective rates	1/35 (3%)	0/23 (0%)	3/48 (6%)	2/18 (11%)
Terminal rates	1/24 (4%)	0/15 (0%)	1/26 (4%)	0/1 (0%)
First incidence (days)	732 (T)	–	654	704
Life table tests	P=0.020	P=0.594N	P=0.368	P=0.012
Logistic regression tests	P=0.093	P=0.594N	P=0.401	P=0.078
Skin: Squamous Cell Carcinoma				
Overall rates	0/50 (0%)	2/35 (6%)	8/65 (12%)	7/50 (14%)
Effective rates	0/45 (0%)	2/32 (6%)	8/61 (13%)	7/41 (17%)
Terminal rates	0/24 (0%)	2/15 (13%)	5/26 (19%)	0/1 (0%)
First incidence (days)	–	732 (T)	565	512
Life table tests	P<0.001	P=0.141	P=0.009	P=0.002
Logistic regression tests	P=0.006	P=0.141	P=0.013	P=0.017

TABLE 11
Skin Neoplasms in Rats in the 2-Year Drinking Water Studies of C.I. Acid Red 114
(continued)

Male (continued)	0 ppm	70 ppm	150 ppm	300 ppm
Skin: Squamous Cell Papilloma or Squamous Cell Carcinoma[i]				
Overall rates	1/50 (2%)	2/35 (6%)	11/65 (17%)	9/50 (18%)
Effective rates	1/45 (2%)	2/32 (6%)	11/61 (18%)	9/41 (22%)
Terminal rates	1/24 (4%)	2/15 (13%)	6/26 (23%)	0/1 (0%)
First incidence (days)	732 (T)	732 (T)	565	512
Life table tests	P<0.001	P=0.336	P=0.007	P<0.001
Logistic regression tests	P=0.001	P=0.336	P=0.010	P=0.011

Female	0 ppm	150 ppm	300 ppm	600 ppm
Skin: Keratoacanthoma[j]				
Overall rates	0/50 (0%)	0/35 (0%)	0/65 (0%)	1/50 (2%)
Skin: Basal Cell Adenoma				
Overall rates	0/50 (0%)	3/35 (9%)	5/65 (8%)	3/50 (6%)
Effective rates	0/49 (0%)	3/33 (9%)	5/58 (9%)	3/19 (16%)
Terminal rates	0/36 (0%)	2/13 (15%)	1/6 (17%)	0/0 (0%)
First incidence (days)	–	663	521	454
Life table tests	P<0.001	P=0.016	P=0.005	P=0.006
Logistic regression tests	P=0.040	P=0.036	P=0.049	P=0.211
Skin: Basal Cell Carcinoma				
Overall rates	0/50 (0%)	1/35 (3%)	2/65 (3%)	2/50 (4%)
Skin: Basal Cell Adenoma or Carcinoma[k]				
Overall rates	0/50 (0%)	4/35 (11%)	7/65 (11%)	5/50 (10%)
Effective rates	0/50 (0%)	4/35 (11%)	7/62 (11%)	5/45 (11%)
Terminal rates	0/36 (0%)	2/13 (15%)	1/6 (17%)	0/0 (0%)
First incidence (days)	–	614	521	344
Life table tests	P<0.001	P=0.006	P<0.001	P<0.001
Logistic regression tests	P=0.012	P=0.020	P=0.013	P=0.071
Skin: Squamous Cell Papilloma				
Overall rates	0/50 (0%)	0/35 (0%)	1/65 (2%)	1/50 (2%)
Skin: Squamous Cell Carcinoma				
Overall rates	0/50 (0%)	0/35 (0%)	3/65 (5%)	0/50 (0%)
Effective rates	0/38 (0%)	0/15 (0%)	3/10 (30%)	0/0 (0%)
Terminal rates	0/36 (0%)	0/13 (0%)	1/6 (17%)	0/0 (0%)
First incidence (days)	–	–	715	–
Life table tests	P=0.003	–	P=0.002	–
Logistic regression tests	P=0.009	–	P=0.007	–
Skin: Squamous Cell Papilloma or Squamous Cell Carcinoma[l]				
Overall rates	0/50 (0%)	0/35 (0%)	4/65 (6%)	1/50 (2%)
Effective rates	0/50 (0%)	0/35 (0%)	4/60 (7%)	1/33 (3%)
Terminal rates	0/36 (0%)	0/13 (0%)	1/6 (17%)	0/0 (0%)
First incidence (days)	–	–	614	403
Life table tests	P<0.001	–	P=0.001	P=0.417
Logistic regression tests	P=0.034	–	P=0.011	P=0.931

TABLE 11
Skin Neoplasms in Rats in the 2-Year Drinking Water Studies of C.I. Acid Red 114
(continued)

(T)Terminal sacrifice
[a] Historical incidence for 2-year studies for untreated control groups in NTP feed and drinking water studies (mean ± standard deviation): 34/681 (5.0% ± 3.0%); range 2%-11%
[b] Number of lesion-bearing animals/number of animals necropsied or examined microscopically for this tumor type
[c] Number of lesion-bearing animals/effective number of animals, i.e., number of animals alive at first occurrence of this tumor type in any of the groups
[d] Observed incidence at terminal kill
[e] Beneath the "0 ppm" column are the P values associated with the trend test. Beneath the dosed group columns are the P values corresponding to pairwise comparisons between the controls and that dosed group. The life table analysis regards tumors in animals dying prior to terminal kill as being (directly or indirectly) the cause of death. The logistic regression tests regard these lesions as nonfatal. For all tests, a negative trend or a lower incidence in a dosed group is indicated by N.
[f] Historical incidence for 2-year studies for untreated control groups in NTP feed and drinking water studies (mean ± standard deviation): 3/681 (0.4% ± 0.9%); range 0%-2%
[g] Not applicable; no tumors in animal group
[h] Historical incidence for 2-year studies for untreated control groups in NTP feed and drinking water studies (mean ± standard deviation): 6/681 (0.9% ± 1.3%); range 0%-6%
[i] Historical incidence for 2-year studies for untreated control groups in NTP feed and drinking water studies (mean ± standard deviation): 17/681 (2.5% ± 1.5%); range 0%-4%
[j] Historical incidence for 2-year studies for untreated control groups in NTP feed and drinking water studies (mean ± standard deviation): 4/680 (0.6% ± 1.0%); range 0%-2%
[k] Historical incidence for 2-year studies for untreated control groups in NTP feed and drinking water studies (mean ± standard deviation): 3/680 (0.4% ± 0.7%); range 0%-2%
[l] Historical incidence for 2-year studies for untreated control groups in NTP feed and drinking water studies (mean ± standard deviation): 5/680 (0.7% ± 0.8%); range 0%-2%

TABLE 12
Zymbal's Gland Neoplasms in F344/N Rats in the 2-Year Drinking Water Studies of C.I. Acid Red 114

Male	0 ppm	70 ppm	150 ppm	300 ppm
Zymbal's Gland: Adenoma				
Overall rates[a]	0/50 (0%)	0/35 (0%)	1/65 (2%)	1/50 (2%)
Zymbal's Gland: Carcinoma				
Overall rates	0/50 (0%)	0/35 (0%)	7/65 (11%)	6/50 (12%)
Effective rates[b]	0/49 (0%)	0/35 (0%)	7/65 (11%)	6/49 (12%)
Terminal rates[c]	0/24 (0%)	0/15 (0%)	2/26 (8%)	0/1 (0%)
First incidence (days)	–[d]	–	325	524
Life table tests[e]	P<0.001	–	P=0.022	P=0.002
Logistic regression tests[e]	P=0.011	–	P=0.013	P=0.018
Zymbal's Gland: Adenoma or Carcinoma[f]				
Overall rates	0/50 (0%)	0/35 (0%)	8/65 (12%)	7/50 (14%)
Effective rates	0/49 (0%)	0/35 (0%)	8/65 (12%)	7/49 (14%)
Terminal rates	0/24 (0%)	0/15 (0%)	2/26 (8%)	0/1 (0%)
First incidence (days)	–	–	325	524
Life table tests	P<0.001	–	P=0.014	P<0.001
Logistic regression tests	P=0.005	–	P=0.008	P=0.009

Female	0 ppm	150 ppm	300 ppm	600 ppm
Zymbal's Gland: Adenoma				
Overall rates	0/50 (0%)	0/35 (0%)	2/65 (3%)	6/50 (12%)
Effective rates	0/50 (0%)	0/33 (0%)	2/59 (3%)	6/27 (22%)
Terminal rates	0/36 (0%)	0/13 (0%)	1/6 (17%)	0/0 (0%)
First incidence (days)	–	–	599	412
Life table tests	P<0.001	–	P=0.060	P<0.001
Logistic regression tests	P<0.001	–	P=0.191	P=0.007
Zymbal's Gland: Carcinoma				
Overall rates	0/50 (0%)	3/35 (9%)	17/65 (26%)	13/50 (26%)
Effective rates	0/50 (0%)	3/35 (9%)	17/61 (28%)	13/42 (31%)
Terminal rates	0/36 (0%)	1/13 (8%)	1/6 (17%)	0/0 (0%)
First incidence (days)	–	411	403	355
Life table tests	P<0.001	P=0.042	P<0.001	P<0.001
Logistic regression tests	P=0.010	P=0.163	P<0.001	P=0.021
Zymbal's Gland: Adenoma or Carcinoma[g]				
Overall rates	0/50 (0%)	3/35 (9%)	18/65 (28%)	19/50 (38%)
Effective rates	0/50 (0%)	3/35 (9%)	18/61 (30%)	19/42 (45%)
Terminal rates	0/36 (0%)	1/13 (8%)	1/6 (17%)	0/0 (0%)
First incidence (days)	–	411	403	355
Life table tests	P<0.001	P=0.042	P<0.001	P<0.001
Logistic regression tests	P<0.001	P=0.163	P<0.001	P<0.001

[a] Number of lesion-bearing animals/number of animals necropsied or examined microscopically for this tumor type
[b] Number of lesion-bearing animals/effective number of animals, i.e., number of animals alive at first occurrence of this tumor type in any of the groups
[c] Observed incidence at terminal kill
[d] Not applicable; no tumors in animal group
[e] Beneath the "0 ppm" column are the P values associated with the trend test. Beneath the dosed group columns are the P values corresponding to pairwise comparisons between the controls and that dosed group. The life table analysis regards tumors in animals dying prior to terminal kill as being (directly or indirectly) the cause of death. The logistic regression tests regard these lesions as nonfatal.
[f] Historical incidence for 2-year studies for untreated control groups in NTP feed and drinking water studies (mean ± standard deviation): 11/681 (1.6% ± 1.7%); range 0%-4%
[g] Historical incidence for 2-year studies for untreated control groups in NTP feed and drinking water studies (mean ± standard deviation): 6/680 (0.9% ± 1.0%); range 0%-2%

Clitoral Glands: The clitoral glands of the female rat are bilateral, modified sebaceous glands located near the base of the clitoris. There was a marked treatment-related increase in the incidence of clitoral gland neoplasms in female rats (Table 13). The combined incidence of adenomas and carcinomas was significantly increased in all treated female groups. A few treated females had bilateral adenomas or carcinomas. Focal glandular cell hyperplasia of the clitoral gland occurred at a slightly increased incidence in the mid-dose female group. Adenomas were discrete, well-demarcated, expansile masses displaying some loss of the normal acinar architecture. They were composed of relatively well-differentiated cells arranged in solid clusters; a few ductlike structures, sometimes containing debris, were scattered within the neoplasms. Carcinomas were poorly demarcated masses that sometimes invaded adjacent tissues (Plate 3). They were composed of solid sheets and clusters of disorganized pleomorphic cells, and often there was an abundance of small basophilic basal-like cells (reserve cells). Some carcinomas exhibited marked cellular atypia or contained large areas of necrosis.

TABLE 13
Clitoral Gland Proliferative Lesions in Female F344/N Rats in the 2-Year Drinking Water Study of C.I. Acid Red 114

	0 ppm	150 ppm	300 ppm	600 ppm
Clitoral Gland: Hyperplasia, glandular	4/48 (8%)	2/32 (6%)	8/62 (13%)	2/50 (4%)
Clitoral Gland: Adenoma				
Overall rates[a]	7/48 (15%)	10/32 (31%)	10/62 (16%)	10/50 (20%)
Effective rates[b]	7/48 (15%)	10/32 (31%)	10/59 (17%)	10/43 (23%)
Terminal rates[c]	5/34 (15%)	4/11 (36%)	3/6 (50%)	0/0 (0%)
First incidence (days)	663	538	403	351
Life table tests[d]	P<0.001	P=0.004	P=0.002	P<0.001
Logistic regression tests[d]	P=0.091	P=0.028	P=0.271	P=0.231
Clitoral Gland: Carcinoma				
Overall rates	4/48 (8%)	9/32 (28%)	19/62 (31%)	15/50 (30%)
Effective rates	4/48 (8%)	9/32 (28%)	19/60 (32%)	15/46 (33%)
Terminal rates	2/34 (6%)	4/11 (36%)	3/6 (50%)	0/0 (0%)
First incidence (days)	641	411	400	339
Life table tests	P<0.001	P=0.003	P<0.001	P<0.001
Logistic regression tests	P=0.003	P=0.030	P=0.001	P=0.022
Clitoral Gland: Adenoma or Carcinoma[e]				
Overall rates	11/48 (23%)	17/32 (53%)	28/62 (45%)	23/50 (46%)
Effective rates	11/48 (23%)	17/32 (53%)	28/60 (47%)	23/46 (50%)
Terminal rates	7/34 (21%)	8/11 (73%)	6/6 (100%)	0/0 (0%)
First incidence (days)	641	411	400	339
Life table tests	P<0.001	P<0.001	P<0.001	P<0.001
Logistic regression tests	P<0.001	P=0.003	P=0.001	P=0.014

[a] Number of lesion-bearing animals/number of animals examined microscopically for this tumor type
[b] Number of lesion-bearing animals/effective number of animals, i.e., number of animals alive at first occurrence of this tumor type in any of the groups
[c] Observed incidence at terminal kill
[d] Beneath the "0 ppm" column are the P values associated with the trend test. Beneath the dosed group columns are the P values corresponding to pairwise comparisons between the controls and that dosed group. The life table analysis regards tumors in animals dying prior to terminal kill as being (directly or indirectly) the cause of death. The logistic regression tests regard these lesions as nonfatal.
[e] Historical incidence for 2-year studies for untreated control groups in NTP feed and drinking water studies (mean ± standard deviation): 74/606 (12.2% ± 5.7%); range 5%-23%

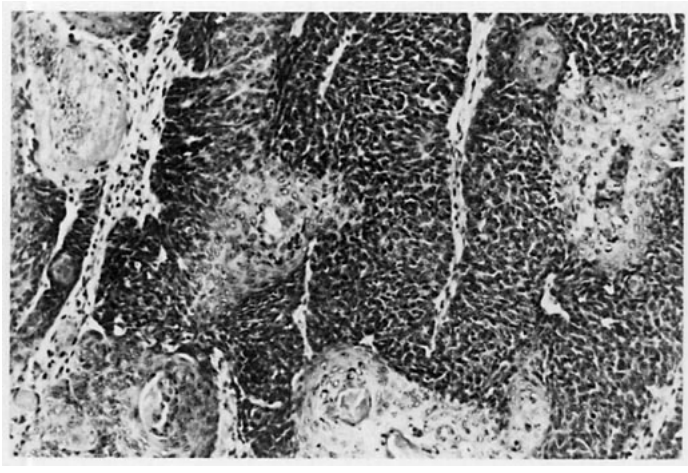


Plate 1
Basal cell carcinoma in the skin of a male rat from the 300 ppm dose group of the 2-year study. Broad cords of neoplastic basal cells have invaded the dermis. Areas of differentiation toward squamous epithelium and hair follicles are present. Magnification 120×

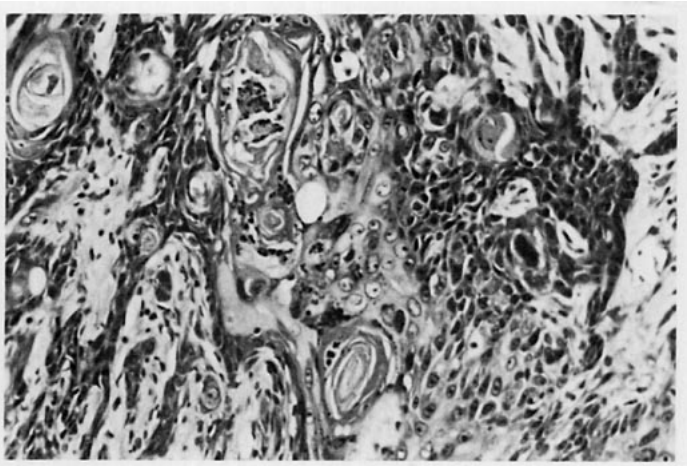


Plate 2
Squamous cell carcinoma in the skin of a male rat from the 300 ppm dose group of the 2-year study. Irregular cords and clusters of neoplastic stratified squamous epithelial cells with foci of keratinization have invaded the dermis. Magnification 190×

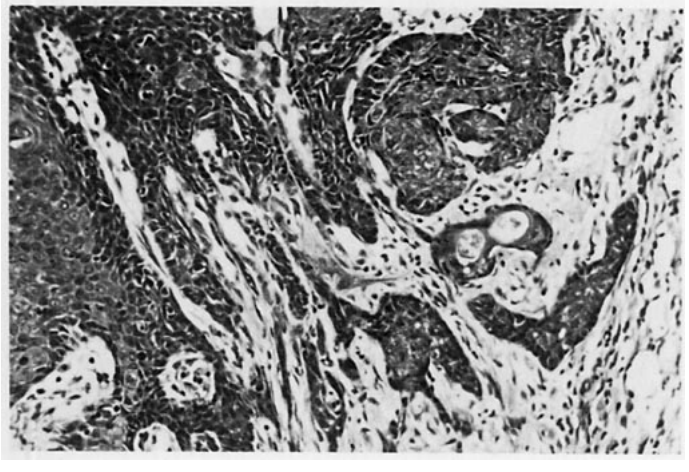


Plate 3
Clitoral gland carcinoma in a female rat from the 600 ppm dose group of the 2-year study. Irregular cords of glandular epithelial cells have invaded the adjacent connective tissue. Magnification 150×

Liver: The combined incidences of neoplastic nodules and hepatocellular carcinomas were significantly increased in mid- and high-dose male and female groups (Table 14). Livers of a few treated animals contained multiple neoplastic nodules. (Neoplastic nodule was the term previously used to refer to neoplasms currently diagnosed as hepatocellular adenomas.) These nodules were well-demarcated, expansile masses that compressed the adjacent parenchyma. Hepatic cords within neoplastic nodules were not organized in a normal lobular pattern and often intersected at near right angles with the cords of the adjacent normal liver cells. Neoplastic hepatocytes were slightly pleomorphic and exhibited increased eosinophilic staining. Hepatocellular carcinomas differed from neoplastic nodules in that neoplastic cells within carcinomas formed solid clusters, glandular structures, and broad trabeculae that were several (generally five or more) cell layers thick. Cells within carcinomas were often moderately to markedly pleomorphic and exhibited varying degrees of atypia.

A variety of nonneoplastic liver lesions, generally of minimal to mild severity, increased in incidence in treated males and females (Table 15). These lesions included eosinophilic foci, mixed cell foci, and hematopoietic cell proliferation, as well as degenerative changes characterized by cystic degeneration, hepatocyte necrosis, and fatty change. Eosinophilic foci consisted of clusters of hepatocytes with abundant, brightly eosinophilic cytoplasm. Foci caused little or no compression and blended smoothly with the surrounding parenchyma. Mixed cell foci were similar in appearance except that they consisted of a mixture of cells with either eosinophilic or clear cytoplasm. Cystic degeneration is a common degenerative lesion of the liver and is characterized by multiple focal clusters of variably sized cysts filled with granular eosinophilic materials or erythrocytes. The increase in hematopoietic cell proliferation was presumably secondary to inflammation associated with neoplasms in treated animals. Hepatocyte necrosis involved single or multiple small scattered foci of hepatocytes, which most commonly affected centrilobular hepatocytes. Fatty change ranged from focal to multifocal to diffuse and consisted of multiple, clear, discrete vacuoles within hepatocyte cytoplasm.

Lung: Alveolar/bronchiolar adenomas and carcinomas (combined) occurred with slightly increased incidences in dosed male rats (Table 16). The incidence in the high-dose male group was significantly increased. The incidence of alveolar epithelial hyperplasia was slightly increased in dosed males, but none of the increases were significant. The incidence of adenomas was significantly increased in the mid-dose female group, and the incidences of adenomas and carcinomas (combined) in the mid- and high-dose female groups exceeded the historical control range for untreated females from NTP 2-year studies (Table B4g). The incidence of focal or multifocal alveolar epithelial hyperplasia was numerically increased in mid- and high-dose males and females; the increase was significant in high-dose females. Alveolar/bronchiolar adenomas were discrete expansile masses that compressed adjacent lung parenchyma. They consisted of alveolar structures usually lined by a single layer of cuboidal to columnar cells. Carcinomas resembled adenomas, but cells within carcinomas showed more cellular atypia and tended to form multiple layers or solid sheets. Alveolar epithelial hyperplasia consisted of clusters of alveoli lined by a single layer of cuboidal cells; borders of hyperplastic areas tended to blend smoothly with the adjacent normal parenchyma.

TABLE 14
Liver Tumors in F344/N Rats in the 2-Year Drinking Water Studies of C.I. Acid Red 114

Male	0 ppm	70 ppm	150 ppm	300 ppm
Liver: Neoplastic Nodule				
Overall rates[a]	2/50 (4%)	1/35 (3%)	10/65 (15%)	15/50 (30%)
Effective rates[b]	2/44 (5%)	1/29 (3%)	10/60 (17%)	15/35 (43%)
Terminal rates[c]	1/24 (4%)	1/15 (7%)	7/26 (27%)	1/1 (100%)
First incidence (days)	607	732 (T)	641	531
Logistic regression tests[d]	P<0.001	P=0.631N	P=0.042	P<0.001
Liver: Hepatocellular Carcinoma				
Overall rates	0/50 (0%)	1/35 (3%)	6/65 (9%)	7/50 (14%)
Effective rates	0/44 (0%)	1/31 (3%)	6/61 (10%)	7/38 (18%)
Terminal rates	0/24 (0%)	0/15 (0%)	5/26 (19%)	0/1 (0%)
First incidence (days)	–[e]	694	687	530
Logistic regression tests	P<0.001	P=0.423	P=0.023	P=0.008
Liver: Neoplastic Nodule or Hepatocellular Carcinoma[f]				
Overall rates	2/50 (4%)	2/35 (6%)	15/65 (23%)	20/50 (40%)
Effective rates	2/44 (5%)	2/31 (6%)	15/61 (25%)	20/38 (53%)
Terminal rates	1/24 (4%)	1/15 (7%)	11/26 (42%)	1/1 (100%)
First incidence (days)	607	694	641	530
Logistic regression tests	P<0.001	P=0.553	P=0.003	P<0.001

Female	0 ppm	150 ppm	300 ppm	600 ppm
Liver: Neoplastic Nodule				
Overall rates	0/50 (0%)	0/35 (0%)	15/64 (23%)	6/50 (12%)
Effective rates	0/50 (0%)	0/35 (0%)	15/60 (25%)	6/33 (18%)
Terminal rates	0/36 (0%)	0/13 (0%)	1/6 (17%)	0/0 (0%)
First incidence (days)	–	–	403	411
Logistic regression tests	P<0.001	–	P<0.001	P=0.009
Liver: Hepatocellular Carcinoma				
Overall rates	0/50 (0%)	0/35 (0%)	6/64 (9%)	3/50 (6%)
Effective rates	0/50 (0%)	0/35 (0%)	6/61 (10%)	3/34 (9%)
Terminal rates	0/36 (0%)	0/13 (0%)	0/6 (0%)	0/0 (0%)
First incidence (days)	–	–	400	501
Logistic regression tests	P=0.003	–	P=0.025	P=0.009
Liver: Neoplastic Nodule or Hepatocellular Carcinoma[g]				
Overall rates	0/50 (0%)	0/35 (0%)	19/64 (30%)	8/50 (16%)
Effective rates	0/50 (0%)	0/35 (0%)	19/61 (31%)	8/34 (24%)
Terminal rates	0/36 (0%)	0/13 (0%)	1/6 (17%)	0/0 (0%)
First incidence (days)	–	–	400	411
Logistic regression tests	P<0.001	–	P<0.001	P<0.001

(T)Terminal sacrifice
[a] Number of lesion-bearing animals/number of animals necropsied or examined microscopically for this tumor type
[b] Number of lesion-bearing animals/effective number of animals, i.e., number of animals alive at first occurrence of this tumor type in any of the groups
[c] Observed incidence at terminal kill
[d] Beneath the "0 ppm" column are the P values associated with the trend test. Beneath the dosed group columns are the P values corresponding to pairwise comparisons between the controls and that dosed group. The logistic regression tests regard these lesions as nonfatal. For all tests, a negative trend or a lower incidence in a dosed group is indicated by N.
[e] Not applicable; no tumors in animal group
[f] Historical incidence for 2-year studies for untreated control groups in NTP feed and drinking water studies (mean ± standard deviation): 22/680 (3.2% ± 3.5%); range 0%-10%
[g] Historical incidence for 2-year studies for untreated control groups in NTP feed and drinking water studies (mean ± standard deviation): 3/680 (0.4% ± 1.0%); range 0%-3%

TABLE 15
Nonneoplastic Liver Lesions in F344/N Rats in the 2-Year Drinking Water Studies of C.I. Acid Red 114

	0 ppm	70 ppm	150 ppm	300 ppm
Male				
Liver: Eosinophilic Focus				
Overall rates[a]	6/50 (12%)	5/35 (14%)	19/65 (29%)	26/50 (52%)
Adjusted rates[b]	23.1%	26.6%	54.7%	100.0%
Terminal rates[c]	5/24 (21%)	3/15 (20%)	12/26 (46%)	1/1 (100%)
First incidence (days)	671	641	641	473
Logistic regression tests[d]	P<0.001	P=0.461	P=0.013	P<0.001
Liver: Mixed Cell Focus				
Overall rates	7/50 (14%)	9/35 (26%)	24/65 (37%)	14/50 (28%)
Adjusted rates	29.2%	50.8%	68.1%	43.8%
Terminal rates	7/24 (29%)	7/15 (47%)	16/26 (62%)	0/1 (0%)
First incidence (days)	732 (T)	530	530	377
Logistic regression tests	P=0.012	P=0.097	P=0.003	P=0.065
Liver: Hematopoietic Cell Proliferation				
Overall rates	1/50 (2%)	2/35 (6%)	6/65 (9%)	14/50 (28%)
Adjusted rates	2.6%	8.0%	19.5%	79.4%
Terminal rates	0/24 (0%)	0/15 (0%)	4/26 (15%)	0/1 (0%)
First incidence (days)	641	651	403	512
Logistic regression tests	P<0.001	P=0.376	P=0.112	P<0.001
Liver: Cystic Degeneration				
Overall rates	6/50 (12%)	13/35 (37%)	33/65 (51%)	31/50 (62%)
Adjusted rates	19.7%	60.0%	85.7%	100.0%
Terminal rates	3/24 (13%)	7/15 (47%)	21/26 (81%)	1/1 (100%)
First incidence (days)	454	530	565	470
Logistic regression tests	P<0.001	P=0.006	P<0.001	P<0.001
Liver: Hepatocyte Necrosis				
Overall rates	3/50 (6%)	3/35 (9%)	10/65 (15%)	22/50 (44%)
Adjusted rates	7.7%	12.6%	26.1%	88.6%
Terminal rates	0/24 (0%)	0/15 (0%)	3/26 (12%)	0/1 (0%)
First incidence (days)	454	447	654	412
Logistic regression tests	P<0.001	P=0.471	P=0.099	P<0.001
Liver: Hepatocyte Fatty Change				
Overall rates	3/50 (6%)	3/35 (9%)	6/65 (9%)	7/50 (14%)
Adjusted rates	8.4%	11.8%	14.9%	43.5%
Terminal rates	1/24 (4%)	0/15 (0%)	1/26 (4%)	0/1 (0%)
First incidence (days)	355	447	567	473
Logistic regression tests	P=0.197	P=0.456	P=0.356	P=0.226

TABLE 15
Nonneoplastic Liver Lesions in F344/N Rats in the 2-Year Drinking Water Studies of C.I. Acid Red 114
(continued)

	0 ppm	150 ppm	300 ppm	600 ppm
Female				
Liver: Eosinophilic Focus				
Overall rates	0/50 (0%)	12/35 (34%)	38/64 (59%)	42/50 (84%)
Adjusted rates	0.0%	63.1%	95.4%	100.0%
Terminal rates	0/36 (0%)	7/13 (54%)	5/6 (83%)	0/0 (0%)
First incidence (days)	–	538	400	258
Logistic regression tests	P<0.001	P<0.001	P<0.001	P<0.001
Liver: Mixed Cell Focus				
Overall rates	2/50 (4%)	9/35 (26%)	8/64 (13%)	15/50 (30%)
Adjusted rates	5.6%	51.8%	53.3%	76.8%
Terminal rates	2/36 (6%)	6/13 (46%)	2/6 (33%)	0/0 (0%)
First incidence (days)	733 (T)	523	538	186
Logistic regression tests	P=0.001	P=0.002	P=0.014	P=0.053
Liver: Hematopoietic Cell Proliferation				
Overall rates	0/50 (0%)	7/35 (20%)	15/64 (23%)	9/50 (18%)
Adjusted rates	0.0%	30.2%	59.1%	49.9%
Terminal rates	0/36 (0%)	2/13 (15%)	2/6 (33%)	0/0 (0%)
First incidence (days)	–	411	400	339
Logistic regression tests	P=0.058	P=0.006	P<0.001	P=0.041
Liver: Cystic Degeneration				
Overall rates	0/50 (0%)	5/35 (14%)	25/64 (39%)	14/50 (28%)
Adjusted rates	0.0%	25.2%	88.3%	71.5%
Terminal rates	0/36 (0%)	1/13 (8%)	4/6 (67%)	0/0 (0%)
First incidence (days)	–	638	521	339
Logistic regression tests	P<0.001	P=0.010	P<0.001	P<0.001
Liver: Hepatocyte Necrosis				
Overall rates	2/50 (4%)	3/35 (9%)	9/64 (14%)	8/50 (16%)
Adjusted rates	4.0%	18.4%	40.3%	53.7%
Terminal rates	0/36 (0%)	1/13 (8%)	1/6 (17%)	0/0 (0%)
First incidence (days)	431	663	496	355
Logistic regression tests	P=0.069	P=0.488	P=0.139	P=0.566
Liver: Hepatocyte Fatty Change				
Overall rates	2/50 (4%)	4/35 (11%)	10/64 (16%)	8/50 (16%)
Adjusted rates	5.6%	15.6%	37.2%	100.0%
Terminal rates	2/36 (6%)	0/13 (0%)	1/6 (17%)	0/0 (0%)
First incidence (days)	733 (T)	462	477	351
Logistic regression tests	P=0.119	P=0.247	P=0.077	P=0.055

(T)Terminal sacrifice
[a] Number of lesion-bearing animals/number of animals necropsied or examined microscopically for this tumor type
[b] Kaplan-Meier estimated tumor incidence at the end of the study after adjustment for intercurrent mortality
[c] Observed incidence at terminal kill
[d] Beneath the "0 ppm" column are the P values associated with the trend test. Beneath the dosed group columns are the P values corresponding to pairwise comparisons between the controls and that dosed group. The logistic regression tests regard these lesions as nonfatal.

TABLE 16
Proliferative Lesions of the Lung in F344/N Rats in the 2-Year Drinking Water Studies of C.I. Acid Red 114

Male	0 ppm	70 ppm	150 ppm	300 ppm
Lung: Alveolar Epithelial Hyperplasia				
Overall rates[a]	2/50 (4%)	4/35 (11%)	9/65 (14%)	8/50 (16%)
Lung: Alveolar/bronchiolar Adenoma				
Overall rates	0/50 (0%)	2/35 (6%)	1/65 (2%)	3/50 (6%)
Effective rates[b]	0/35 (0%)	2/23 (9%)	1/48 (2%)	3/18 (17%)
Terminal rates[c]	0/24 (0%)	1/15 (7%)	0/26 (0%)	1/1 (100%)
First incidence (days)	–[d]	701	654	694
Logistic regression tests[e]	P=0.022	P=0.148	P=0.550	P=0.002
Lung: Alveolar/bronchiolar Carcinoma				
Overall rates	2/50 (4%)	0/35 (0%)	1/65 (2%)	0/50 (0%)
Lung: Alveolar/bronchiolar Adenoma or Carcinoma[f]				
Overall rates	2/50 (4%)	2/35 (6%)	2/65 (3%)	3/50 (6%)
Effective rates	2/35 (6%)	2/23 (9%)	2/48 (4%)	3/18 (17%)
Terminal rates	2/24 (8%)	1/15 (7%)	1/26 (4%)	1/1 (100%)
First incidence (days)	732 (T)	701	654	694
Logistic regression tests	P=0.102	P=0.521	P=0.618N	P=0.017

Female	0 ppm	150 ppm	300 ppm	600 ppm
Lung: Alveolar Epithelium, Hyperplasia				
Overall rates	6/50 (12%)	6/35 (17%)	15/65 (23%)	20/50 (40%)**
Lung: Alveolar/bronchiolar Adenoma				
Overall rates	1/50 (2%)	2/35 (6%)	8/65 (12%)	4/50 (8%)
Effective rates	1/50 (2%)	2/35 (6%)	8/59 (14%)	4/31 (13%)
Terminal rates	1/36 (3%)	0/13 (0%)	1/6 (17%)	0/0 (0%)
First incidence (days)	733 (T)	411	582	454
Logistic regression tests	P=0.013	P=0.508	P=0.003	P=0.120
Lung: Alveolar/bronchiolar Carcinoma				
Overall rates	0/50 (0%)	0/35 (90%)	1/65 (2%)	0/50 (0%)
Lung: Alveolar/bronchiolar Adenoma or Carcinoma[g]				
Overall rates	1/50 (2%)	2/35 (6%)	9/65 (14%)	4/50 (8%)
Effective rates	1/50 (2%)	2/35 (6%)	9/59 (15%)	4/31 (13%)
Terminal rates	1/36 (3%)	0/13 (0%)	1/6 (17%)	0/0 (0%)
First incidence (days)	733 (T)	411	582	454
Logistic regression tests	P=0.007	P=0.508	P=0.001	P=0.120

(T)Terminal sacrifice
** Significantly different (P≤0.01) from the control group by the logistic regression test.
[a] Number of lesion-bearing animals/number of animals necropsied or examined microscopically for this tumor type
[b] Number of lesion-bearing animals/effective number of animals, i.e., number of animals alive at first occurrence of this tumor type in any of the groups
[c] Observed incidence at terminal kill
[d] Not applicable; no tumors in animal group
[e] Beneath the "0 ppm" column are the P values associated with the trend test. Beneath the dosed group columns are the P values corresponding to pairwise comparisons between the controls and that dosed group. The logistic regression tests regard these lesions as nonfatal. For all tests, a negative trend or a lower incidence in a dosed group is indicated by N.
[f] Historical incidence for 2-year studies for untreated control groups in NTP feed and drinking water studies (mean ± standard deviation): 22/680 (3.2% ± 2.6%); range 0%-10%
[g] Historical incidence for 2-year studies for untreated control groups in NTP feed and drinking water studies (mean ± standard deviation): 13/679 (1.9% ± 2.1%); range 0%-6%

Oral Cavity (Tongue or Pharynx): Squamous cell papillomas and carcinomas, uncommon neoplasms in control F344/N rats, were moderately increased in dosed females compared to controls; three squamous cell papillomas occurred in dosed males (Table 17). However, the incidence was significantly increased only in the mid-dose female group. Squamous cell papillomas were exophytic masses arising from the oral mucosa and consisted of a pedunculated, highly branched core of fibrous tissue covered by a thick layer of stratified squamous epithelium. Squamous cell carcinomas were flat, broad lesions of the oral mucosa that consisted of cords and clusters of disorganized, pleomorphic, squamous epithelial cells that invaded deep into the underlying submucosa. Fibroplasia and inflammation sometimes accompanied the invasion.

Small Intestine (Duodenum or Jejunum): A few neoplasms of the small intestine occurred in female rats treated with C.I. Acid Red 114. An adenomatous polyp was found in one mid-dose and in one high-dose female, while an adenocarcinoma occurred in one high-dose female. These neoplasms are rare in F344/N rats. No adenomatous polyps or carcinomas were found in 680 untreated female controls from NTP 2-year studies (Table B4l). A single adenocarcinoma occurred in a high-dose male. Although the incidence of neoplasms in female rats is low, the fact that these neoplasms are rare plus the fact that two occurred in the high-dose group in which there was markedly reduced survival suggest that these neoplasms may have been chemical related. The adenomatous polyps were pedunculated, exophytic masses consisting of a stalk-like core of fibrous tissue and covered by numerous glandular structures and were lined by a single layer of well-differentiated, columnar cells with abundant basophilic cytoplasm. Adenocarcinomas were poorly demarcated and invaded the submucosal and muscular layers of the intestinal wall. They consisted of large, poorly differentiated, columnar cells that formed multiple, irregular, variably sized glandular structures surrounded by abundant fibrous tissue stroma, and contained large cystic spaces filled with mucus and debris (cystic mucinous adenocarcinoma).

Large Intestine (Colon or Rectum): Adenomatous polyps were seen in two high-dose and one low-dose female, and an adenocarcinoma was noted in a single high-dose female. These neoplasms are rare in F344/N rats. No adenomatous polyps or adenocarcinomas of the large intestine were found in 680 untreated female control rats from NTP 2-year studies (Table B4l). Although the number of large intestine neoplasms is small, the fact that these are rare neoplasms plus the fact that three occurred in the high-dose group in which there was markedly reduced survival suggest these neoplasms may have been related to chemical administration. Neoplasms in the large intestine had a similar histologic appearance to those seen in the small intestine.

Mammary Gland: Adenocarcinomas of the mammary gland occurred in treated groups of female rats (Table 18). The incidences fall within the historical control range for untreated female F344/N rats from NTP 2-year studies (Table B4i). The incidence in the mid-dose group compared to controls was significant by the Fisher exact test using effective rates. Mammary gland adenocarcinomas may be fatal neoplasms; however, eight of the 12 dosed females with mammary gland adenocarcinomas also had one or more neoplasms that could have been the cause of death. Thus, for this neoplasm the logistic regression or Fisher exact test were considered more appropriate. Although all incidences fall within the historical control range, the fact that these neoplasms occurred only in dosed females, and that the incidence was significantly increased in the mid-dose group, indicate these neoplasms may have been treatment related. Histologically, adenocarcinomas invaded adjacent tissue and consisted of acinar structures and solid nodules of cuboidal to columnar cells with vacuolated cytoplasm and pleomorphic, deeply basophilic nuclei. The incidence of mammary gland adenoma was increased in the mid-dose group as compared with untreated controls. The increase was not statistically significant, but the incidence in the mid-dose group was slightly above the historical control range for NTP 2-year drinking water studies (Table B4i). Fibroadenomas occurred at decreased incidences in mid- and high-dose females. This may have been a reflection of the decreased survival in these groups.

Adrenal Gland: In the adrenal medulla, the combined incidence of benign or malignant pheochromocytomas was significantly increased in the high-dose males (17/50, 34%; 11/35, 31%; 27/63, 43%; 21/49, 43%). However, these incidences are within the historical control incidence for untreated male F344/N rats from NTP 2-year studies (Table A4i). The incidences of focal or multifocal hyperplasia, the precursor lesion to pheochromocytoma, were significantly ($P<0.05$) increased in mid-dose males (12/50, 24%; 7/35, 20%; 28/63, 44%; 13/49, 27%). Pheochromocytomas occurred in small numbers of females in all groups, but were more frequent in the low- and mid-dose groups. The

TABLE 17
Oral Cavity Neoplasms in F344/N Rats in the 2-Year Drinking Water Studies of C.I. Acid Red 114

Male	0 ppm	70 ppm	150 ppm	300 ppm
Oral Cavity: Squamous Cell Papilloma				
Overall rates[a]	0/50 (0%)	0/35 (0%)	1/65 (2%)	2/50 (4%)
Oral Cavity: Squamous Cell Carcinoma				
Overall rates	0/50 (0%)	0/35 (0%)	0/65 (0%)	0/50 (0%)
Oral Cavity: Squamous Cell Papilloma or Squamous Cell Carcinoma[b]				
Overall rates	0/50 (0%)	0/35 (0%)	1/65 (2%)	2/50 (4%)

Female	0 ppm	150 ppm	300 ppm	600 ppm
Oral Cavity: Squamous Cell Papilloma				
Overall rates	0/50 (0%)	3/35 (9%)	6/65 (9%)	4/50 (8%)
Effective rates[c]	0/50 (0%)	3/35 (9%)	6/61 (10%)	4/44 (9%)
Terminal rates[d]	0/36 (0%)	2/13 (15%)	0/6 (0%)	0/0 (0%)
First incidence (days)	–[e]	567	487	349
Logistic regression tests[f]	P=0.193	P=0.059	P=0.066	P=0.302
Cochran-Armitage test[f]	P=0.076			
Fisher exact test[f]		P=0.066	P=0.025	P=0.045
Oral Cavity: Squamous Cell Carcinoma				
Overall rates	0/50 (0%)	0/35 (0%)	3/65 (5%)	2/50 (4%)
Effective rates	0/50 (0%)	0/35 (0%)	3/61 (5%)	2/40 (5%)
Terminal rates	0/36 (0%)	0/13 (0%)	0/6 (0%)	0/0 (0%)
First incidence (days)	–	–	454	372
Logistic regression tests	P=0.408	–	P=0.295	P=0.795
Cochran-Armitage test	P=0.077			
Fisher exact test		–	P=0.162	P=0.195
Oral Cavity: Squamous Cell Papilloma or Squamous Cell Carcinoma[g]				
Overall rates	0/50 (0%)	3/35 (9%)	9/65 (14%)	6/50 (12%)
Effective rates	0/50 (0%)	3/35 (9%)	9/61 (15%)	6/44 (14%)
Terminal rates	0/36 (0%)	2/13 (15%)	0/6 (0%)	0/0 (0%)
First incidence (days)	–	567	454	349
Logistic regression tests	P=0.156	P=0.059	P=0.025	P=0.225
Cochran-Armitage test	P=0.017			
Fisher exact test		P=0.066	P=0.003	P=0.009

[a] Number of lesion-bearing animals/number of animals necropsied or examined microscopically for this tumor type
[b] Historical incidence for 2-year studies for untreated control groups in NTP feed and drinking water studies (mean ± standard deviation): 4/681 (0.6% ± 1.5%); range 0%-4%
[c] Number of lesion-bearing animals/effective number of animals, i.e., number of animals alive at first occurrence of this tumor type in any of the groups
[d] Observed incidence at terminal kill
[e] Not applicable; no tumors in animal group
[f] Beneath the "0 ppm" column are the P values associated with the trend test. Beneath the dosed group columns are the P values corresponding to pairwise comparisons between the controls and that dosed group. The logistic regression tests regard these lesions as nonfatal. The Cochran-Armitage and Fisher exact tests compare directly the effective incidence rates.
[g] Historical incidence for 2-year studies for untreated control groups in NTP feed and drinking water studies (mean ± standard deviation): 6/680 (0.9% ± 1.0%); range 0%-2%

TABLE 18
Mammary Gland Neoplasms in Female F344/N Rats in the 2-Year Drinking Water Study of C.I. Acid Red 114

	0 ppm	150 ppm	300 ppm	600 ppm
Mammary Gland: Adenocarcinoma[a]				
Overall rates[b]	0/50 (0%)	3/35 (9%)	6/65 (9%)	3/50 (6%)
Effective rates[c]	0/50 (0%)	3/35 (9%)	6/63 (10%)	3/46 (7%)
Terminal rates[d]	0/36 (0%)	0/13 (0%)	1/6 (17%)	0/0 (0%)
First incidence (days)	–[e]	582	285	351
Logistic regression tests[f]	P=0.456	P=0.089	P=0.061	P=0.755
Cochran-Armitage test[f]	P=0.170			
Fisher exact test[f]		P=0.066	P=0.027	P=0.106
Mammary Gland: Adenoma[g]				
Overall rates	1/50 (2%)	1/35 (3%)	4/65 (6%)	0/50 (0%)
Effective rates	1/46 (2%)	1/30 (3%)	4/45 (9%)	0/8 (0%)
Terminal rates	0/36 (0%)	0/13 (0%)	1/6 (17%)	0/0 (0%)
First incidence (days)	711	663	538	–
Logistic regression tests	P=0.433	P=0.672	P=0.166	P=0.999N
Cochran-Armitage test[f]	P=0.363			
Fisher exact test		P=0.637	P=0.174	P=0.852N
Mammary Gland: Fibroadenoma[h]				
Overall rates	19/50 (38%)	13/35 (37%)	12/65 (18%)	1/50 (2%)
Effective rates	19/46 (41%)	13/30 (43%)	12/45 (27%)	1/8 (13%)
Terminal rates	15/36 (42%)	9/13 (69%)	4/6 (67%)	0/0 (0%)
First incidence (days)	683	638	538	603
Logistic regression tests	P=0.394	P=0.194	P=0.466	P=0.641
Cochran-Armitage test[f]	P=0.033N			
Fisher exact test		P=0.524	P=0.105N	P=0.121N

[a] Historical incidence for 2-year studies for untreated control groups in NTP feed and drinking water studies (mean ± standard deviation): 22/680 (3.2% ± 4.0%); range 0%-12%
[b] Number of lesion-bearing animals/number of animals necropsied or examined microscopically for this tumor type
[c] Number of lesion-bearing animals/effective number of animals, i.e., number of animals alive at first occurrence of this tumor type in any of the groups
[d] Observed incidence at terminal kill
[e] Not applicable; no tumors in animal group
[f] Beneath the "0 ppm" column are the P values associated with the trend test. Beneath the dosed group columns are the P values corresponding to pairwise comparisons between the controls and that dosed group. The logistic regression tests regard these lesions as nonfatal. The Cochran-Armitage test and Fisher exact tests compare directly the effective incidence rates. For all tests, a negative trend or a lower incidence in a dosed group is indicated by N.
[g] Historical incidence for 2-year studies for untreated control groups in NTP feed and drinking water studies (mean ± standard deviation): 7/680 (1.0% ± 1.3%); range 0%-4%
[h] Historical incidence for 2-year studies for untreated control groups in NTP feed and drinking water studies (mean ± standard deviation): 235/680 (34.6% ± 13.2%); range 8%-56%

incidence of hyperplasia was significantly ($P<0.05$) increased in mid-dose females as compared with control: for pheochromocytomas, 1/50 (2%), 3/35 (9%), 5/64 (8%), 1/50 (2%); for hyperplasia, 6/50 (12%), 7/35 (20%), 15/64 (22%), 2/50 (4%). The incidence of pheochromocytomas in all dose groups is within the historical control incidence for untreated female F344/N rats from NTP 2-year studies (Table B4k). The low incidence of hyperplasia in high-dose females is presumably a reflection of the decreased survival in this group. The significance of these findings is unclear, but evidence of a significant increase in the high-dose male group despite the decreased survival, and the increases in hyperplasia and neoplastic lesions in low- and mid-dose females, suggest a possible treatment-related effect.

Hematopoietic System: The incidences of mononuclear cell leukemia in dosed female rats were increased relative to controls (12/50, 24%; 13/35, 37%; 18/65, 28%; 5/50, 10%). Mononuclear cell leukemia occurs commonly in control female rats, and the incidence in the low-dose group just exceeds the historical control range for untreated control females from NTP 2-year studies (Table B4n). A similar increase was not observed in the mid- or high-dose groups. The incidences in all dosed groups were significantly different from controls by the life table test. Since mononuclear cell leukemia is considered rapidly lethal, the life table test is usually the most appropriate statistical analysis for these neoplasms. However, in this study there were several other types of malignant neoplasms which could have caused the deaths of the animals. Thus, due to the competing risks from other lethal neoplasms, the life table test may not be the most appropriate analysis in this case. Accordingly, it is unclear whether or not the slight increase in mononuclear cell leukemia in treated females was related to chemical administration.

Thyroid Gland: Follicular cell adenoma or carcinoma (combined) occurred with low incidences in dosed female rats (control, 0/50; low-dose, 3/35, 9%; mid-dose, 3/64, 5%; high-dose, 1/50, 2%). The incidence in the low-dose group was significantly different from controls by logistic regression analysis ($P=0.011$) and fell outside the range of historical controls for follicular cell neoplasms in untreated female rats from NTP 2-year studies (Table B4j). The incidences of follicular cell hyperplasia, a lesion generally considered to be a precursor to follicular cell neoplasia, were similar among female groups (1/50, 2%; 1/35, 3%; 3/64, 5%; 2/50, 4%). Incidences of follicular cell neoplasms or hyperplasia were not increased in dosed male rats. The biological significance of the follicular cell neoplasms in dosed female rats is questionable.

Kidney: Nephropathy, a spontaneous age-related disease, occurred in nearly all female rats. However, the nephropathy was more severe in high-dose females than in controls. Severity was graded by the fraction of renal parenchyma affected, as follows: minimal, usually less than 20%; mild, 20% to 50%; moderate, 50% to 75%; marked, greater than 75%. When expressed on a scale of one to four (1=minimal, 2=mild, 3=moderate, 4=marked); the average severity per group was control, 1.9; low-dose, 1.9; mid-dose, 2.1; and high-dose, 2.8. Nephropathy consisted of a spectrum of lesions including varying degrees of tubule dilatation and distortion with occasional cyst formation; proteinaceous tubule casts; atrophy, regeneration, and hypertrophy of tubule epithelium; thickening of tubule and glomerular basement membranes; interstitial fibrosis; scattered foci of suppurative inflammation, primarily within degenerate tubules; and a scattering of varying numbers of mononuclear inflammatory cells within the interstitium. Karyomegaly (enlargement of tubule epithelial cells), was diagnosed in 11/50 high-dose females; this change may have been secondary to the nephropathy. Parathyroid gland hyperplasia and fibrous osteodystrophy of the bone occurred in some high-dose females secondary to alteration in calcium metabolism caused by the nephropathy.

Uterus: The incidence of endometrial stromal polyps was significantly increased in the low-dose group compared with the controls (4/50, 8%; 8/35, 23%; 8/65, 12%; 2/50, 4%). However, endometrial stromal polyps are common in female F344/N rats and the incidence in the low-dose group is within the historical control range for untreated F344/N females from NTP 2-year studies (Table B4m). Also, the incidence in the control group from this study was at the low end of the historical control range. Consequently, the increased incidence of endometrial stromal polyps in the low-dose group is not considered to be a treatment-related effect.

Spleen: Hematopoietic cell proliferation occurred with increasing incidences in the spleens of treated male and female rats (males: 3/50, 4/35, 3/64, 21/50; females: 3/50, 8/35, 20/63, 16/50). This effect was considered to be secondary to the mild anemia and to the inflammation associated with neoplasms in treated animals.

Heart: The incidence of thrombus within the atrium of the heart was increased in the mid- and high-dose male groups as compared with controls (5/50, 4/35, 18/65, 18/50). Although the biological significance of the thrombi is unclear, it is possible they may have formed as a consequence of debilitation in tumor-bearing animals. Debilitation may have led to impaired circulation which allowed pooling of blood within the atrium, resulting in thrombus formation.

GENETIC TOXICOLOGY

C.I. Acid Red 114 was tested for induction of gene mutations in *Salmonella typhimurium* by a standard preincubation protocol at concentrations of 100 to 10,000 μg/plate in the presence and the absence of Aroclor 1254-induced male Sprague-Dawley rat or Syrian hamster liver S9 (Table C1; Mortelmans *et al.*, 1986). A weakly positive response was observed in TA98 with hamster S9 and an equivocal response was observed in TA100, also in the presence of hamster S9. No significant mutagenic activity was observed in TA1535 or TA1537. This compound, as with most benzidine congener dyes, requires reductive metabolism of the azo bonds to release the parent amine, which then can be oxidatively metabolized to an active mutagen. When tested by such a reductive/oxidative metabolism protocol, C.I. Acid Red 114 was highly mutagenic to *S. typhimurium* strain TA1538 (Table C2; Reid *et al.*, 1984a). Some mutagenic activity was observed in the presence of rat S9 without prior reduction, but the mutagenicity was greatly enhanced following reduction. With both reduction systems (cecal bacteria and flavin mononucleotide) the mutagenic response obtained with C.I. Acid Red 114 was much greater than expected based on the activity of equimolar amounts of the parent diamine (dimethylbenzidine). This may indicate the presence of mutagenic impurities and the formation of additional reduction products in the crude dye mixture that was tested.

In cytogenetic tests with Chinese hamster ovary cells, C.I. Acid Red 114 did not induce sister chromatid exchanges (Table C3) or chromosomal aberrations (Table C4) when tested at concentrations up to 160 μg/mL, with or without Aroclor 1254-induced male Sprague-Dawley rat liver S9. Reductive metabolism was not used in these cytogenetic tests.

No increase in sex-linked recessive lethal mutations was observed in germ cells of male *Drosophila melanogaster* following administration of C.I. Acid Red 114 either in feed (50,000 ppm) or by injection (1,500 ppm) (Table C5; Zimmering *et al.*, 1985).

DISCUSSION AND CONCLUSIONS

The benzidine dyes and their parent congeners are widely used in manufacturing throughout the United States. The potential health hazard in the workplace from excessive exposure to the benzidine congener dyes was considered significant enough that the Benzidine Dye Initiative was established through NTP in cooperation with NIEHS, NCTR, NIOSH, USEPA, OSHA, and the Consumer Product Safety Commission (NIOSH, 1981; NIOSH, 1983). Since there are more than 90 benzidine-based dyes in use, the Initiative focused on studying the metabolism, pharmacokinetics, genetic toxicology, and *in vivo* carcinogenicity of several representative dyes. The five selected for developing a toxicity and carcinogenic activity data base were C.I. Acid Red 114 and its parent congener 3,3′-dimethylbenzidine, and C.I. Direct Blue 15, C.I. Direct Blue 218, and their parent congener, 3,3′-dimethoxybenzidine. The route of chemical administration selected for these studies in male and female rats was through the drinking water to ensure systemic exposure. The toxicology and carcinogenicity studies of 3,3′-dimethylbenzidine, 3,3′-dimethoxybenzidine, and C. I. Direct Blue 15 have been reported (NTP, 1990, 1991, in press). The studies described in this report examine the toxic and carcinogenic properties of C.I. Acid Red 114 (desalted industrial grade), the fourth chemical studied under the Benzidine Dye Initiative.

No treatment-related deaths occurred in either the 13-day or 13-week studies. Body weight reduction, water consumption, organ weight changes, hematologic changes, and significant histopathologic findings in the 13-day and 13-week studies were considered in selecting the dose levels of C.I. Acid Red 114 for the 2-year studies. Body weights were significantly decreased relative to the controls in males at the 20,000 and 30,000 ppm dose levels and in all female dose groups in the 13-day studies. In rats receiving doses of 1,200 ppm or higher during the 13-week studies, decreases in body weights relative to the controls ranged from 11% to 15% for males and from 6% to 11% for females. Water consumption was markedly lower in dosed animals in the 13-day and 13-week studies than in the controls. In the 13-week studies, both absolute and relative liver weights were significantly increased for all male and female dose groups compared to the controls.

Bone marrow of male and female rats receiving 20,000 ppm or higher C.I. Acid Red 114 for 13 days was depleted of erythroid and myeloid cells, suggesting there was a direct cytotoxic effect that resulted in anemia at these dose levels. The mechanism of the cytotoxic effect is unknown but may be due, in part, to the binding of benzidine metabolites to DNA (Zenser *et al.*, 1980; Yamazoe *et al.*, 1988).

In the 13-week studies, treatment-related decreases in hematocrit, hemoglobin, and erythrocyte counts were observed in females and to a lesser extent in males. The parent congener of C.I. Acid Red 114, 3,3′-dimethylbenzidine, showed similar results in high-dose groups after 13 weeks of chemical administration (NTP, 1991). 3,3′-dimethoxybenzidine and C.I. Direct Blue 15 caused more modest changes in the hematology profile than did 3,3′-dimethylbenzidine (NTP, 1990, in press).

Serum levels of alanine aminotransferase, lactate dehydrogenase, and sorbitol dehydrogenase in dosed males were elevated, characteristic of liver damage. In females, sorbitol dehydrogenase was the primary enzyme with elevated serum levels which suggests the liver damage was less severe. This conclusion was confirmed to a limited extent by histopathologic findings in which male livers had a centrilobular pallor while female livers were only pigmented. Elevated levels of sorbitol dehydrogenase also occurred in animals administered 3,3′-dimethylbenzidine for 13 weeks but only a marginal elevation of liver enzyme levels was present in animals administered 3,3′-dimethoxybenzidine or C.I. Direct Blue 15 after 13 weeks (NTP 1990, 1991, in press).

In female rats receiving 2,500 ppm or higher for 13 weeks, kidneys developed degenerative lesions consisting of inflammation, tubule regeneration, and karyomegaly. Similar lesions were observed in male and female rats after 13 weeks of 3,3′-dimethylbenzidine, 3,3′-dimethoxybenzidine, and C.I. Direct

Blue 15 administration (NTP 1990, 1991, in press). Pancreatic acinar cell degeneration was noted in dosed male and female rats as it had been noted in previous benzidine dye studies; however, its occurrence may be secondary as a result of the general debilitation of the study animals.

Based on the results of the 13-day and 13-week studies, the dose levels of C.I. Acid Red 114 selected for males in the 2-year studies were 0, 70, 150, and 300 ppm, and dose levels selected for females were 0, 150, 300, and 600 ppm. After 2 years, treatment-related tumors were found in many sites of male and female rats including skin, Zymbal's gland, liver, oral cavity, lung, and adrenal gland. Tumors occurring only in female rats were found in the clitoral gland, small and large intestines, and mammary gland. At 9- or 15-month interim evaluations, tumors were found in the liver, lung, clitoral gland, skin, Zymbal's gland, oral cavity, and intestine. The unusually early appearance of these tumors demonstrates the carcinogenic potency of C.I. Acid Red 114.

The incidence of skin basal cell neoplasms increased in male rats (control, 1/50; 70 ppm, 5/35; 150 ppm, 28/65; 300 ppm, 32/50) and to a lesser extent in female rats (0/50; 4/35; 7/65; 5/50). The higher incidence of skin basal cell tumors in male rats was also seen in the 3,3′-dimethylbenzidine, 3,3′dimethoxybenzidine, and C.I. Direct Blue 15 studies (NTP 1990, 1991, in press). The mechanism for this sex difference could not be determined but may be due, in part, to metabolic differences between the sexes.

As in the previous three Benzidine Dye Initiative studies, the incidences of Zymbal's gland neoplasms in male and female rats were increased by C.I. Acid Red 114 exposure. In the NTP database of over 350 rodent studies, 17 chemicals induced Zymbal's gland tumors, 14 induced skin tumors, and 11 induced both tumors in rats (Table 19). Most of the chemicals inducing either Zymbal's gland or skin neoplasms also caused neoplasms at other sites. These tumor-inducing chemicals have a common aromatic-amine grouping which is considered to be a "structural alert" for genotoxic activity (Ashby and Tennant, 1988). This is supported by positive mutagenicity results in *Salmonella typhimurium* assays in the C.I. Acid Red 114 studies as well as the 3,3′-dimethylbenzidine, 3,3′dimethoxybenzidine, and C.I. Direct Blue 15 studies.

In female rats, the incidence of clitoral gland adenomas and carcinomas was increased as in the previous three benzidine-congener dye studies. A similar dose-related increase in preputial gland neoplasms was not found in male rats receiving C.I. Acid Red 114, but was found in male rats receiving 3,3′-dimethylbenzidine, 3,3′dimethoxybenzidine, and C.I. Direct Blue 15. The reason male rats were not susceptible to preputial gland neoplasms following 2 years of C.I. Acid Red 114 administration is not known.

The incidence of liver neoplasms was increased in dosed male rats and, to a lesser extent, in dosed female rats. The higher incidences of liver neoplasms in dosed male rats relative to females was also observed in the 3,3′-dimethylbenzidine, 3,3′-dimethoxybenzidine, and C.I. Direct Blue 15 studies. Whether the differences in the incidences of liver neoplasms are related to differences between the sexes in metabolizing benzidine compounds, or are the result of some other mechanism, has not been determined.

There was a marginal but statistically significant increase in the combined incidence of alveolar/bronchiolar adenomas or carcinomas in the treated male rats. While the numbers for these neoplasms are low, dimethylbenzidine, the parent congener, also caused an increase in neoplasms at this site in male rats.

The incidences of neoplasms in the oral cavity and in the small or large intestine of female rats were also above the incidences in the concurrent control and the historical database. In male rats receiving C.I. Acid Red 114, the incidence of oral cavity neoplasms was marginally increased compared to the controls; no statistically significant increases in the incidence of intestine tumors occurred. In the previous benzidine congener dye studies, both male and female rats had increased incidences of oral cavity and intestinal neoplasms.

The combined incidences of benign or malignant pheochromocytomas of the adrenal medulla were marginally increased in male and female rats. The incidences were higher in the low- or mid-dose groups, and this was attributed to the early death of most of the animals at the high dose. Supportive evidence for a neoplastic response in this organ was the increased incidence of focal or multifocal hyperplasia.

TABLE 19
Evidence of Zymbal's Gland and Skin Tumors in Rats and *Salmonella* Mutagenicity for Selected National Toxicology Program Chemicals[a]

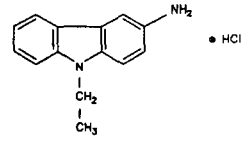
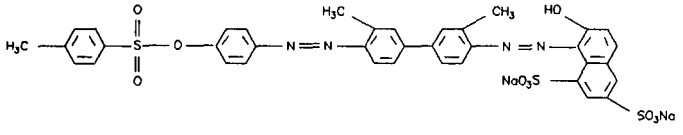
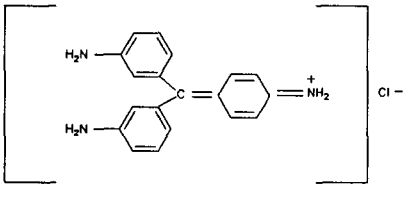
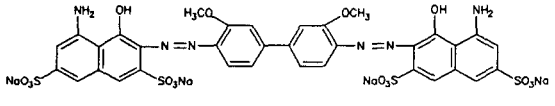
Chemical Name/ Structure	Technical Report Number	Zymbal's Gland Tumors M F	Skin Tumors M F	*Salmonella* Mutagenicity Results
3-Amino-9-Ethylcarbazole HCl 	93	+ +	+	+
Benzene 	289	+ +	+	–
C.I. Acid Red 114 	405	+ +	+ +	+
C.I. Basic Red 9 Monohydrochloride 	285	+ +	+	+
C.I. Direct Blue 15 	397	+ +	+ +	+

TABLE 19
Evidence of Zymbal's Gland and Skin Tumors in Rats and *Salmonella* Mutagenicity for Selected National Toxicology Program Chemicals (continued)

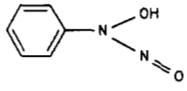
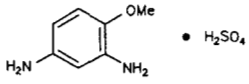
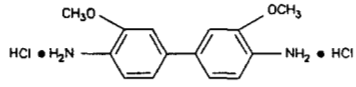
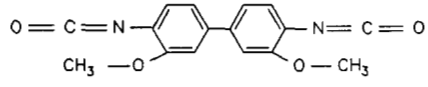
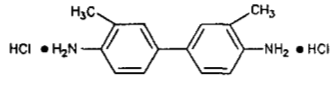
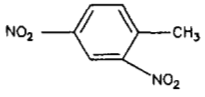
Chemical Name/ Structure	Technical Report Number	Zymbal's Gland Tumors M F	Skin Tumors M F	*Salmonella* Mutagenicity Results
Cupferron 	100	+		+
2,4-Diaminoanisole Sulfate 	84	+ +	+	+
3,3′-Dimethoxybenzidine Dihydrochloride 	372	+ +	+ +	+
3,3′-Dimethoxybenzidine-4,4′-Diisocyanate 	128	+ +	+	+
3,3′-Dimethylbenzidine Dihydrochloride 	390	+ +	+ +	+
2,4-Dinitrotoluene 	54		+	+

TABLE 19
Evidence of Zymbal's Gland and Skin Tumors in Rats and *Salmonella* Mutagenicity for Selected National Toxicology Program Chemicals (continued)

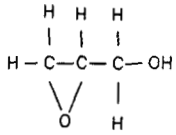
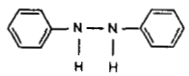
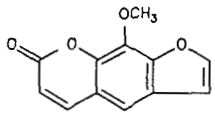
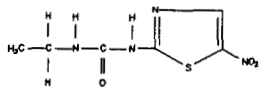
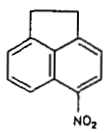
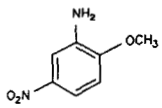
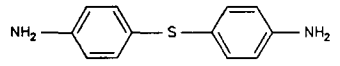
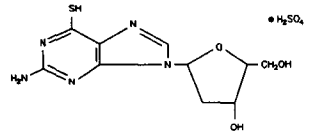
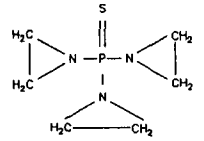
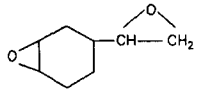
Chemical Name/ Structure	Technical Report Number	Zymbal's Gland Tumors M F	Skin Tumors M F	*Salmonella* Mutagenicity Results
Glycidol 	374	+	+	+
Hydrazobenzene 	92	+		+
8-Methoxypsoralen 	359	+		+
Nithiazide 	146		+	+
5-Nitroacenaphthene 	118	+ +		+
5-Nitro-*o*-Anisidine 	127	+ +	+	+

TABLE 19
Evidence of Zymbal's Gland and Skin Tumors in Rats and *Salmonella* Mutagenicity for Selected National Toxicology Program Chemicals (continued)

Chemical Name/ Structure	Technical Report Number	Zymbal's Gland Tumors M F	Skin Tumors M F	*Salmonella* Mutagenicity Results
4,4′-Thiodianiline	47	+ +		+
				
β-Thioguanidine Deoxyriboside	57	+		
				
Tris(Aziridinyl)Phosphine Sulfide	58	+ +	+ +	+
				
4-Vinyl-1-Cyclohexene Diepoxide	362		+ +	+
				

[a] + = positive evidence or results; − = negative results

The incidence of adenocarcinoma of the mammary gland in female rats was significantly increased in the mid-dose group. Mammary gland neoplasms were also seen in female rats after treatment with 3,3′-dimethoxybenzidine, 3,3′-dimethylbenzidine, and C.I. Direct Blue 15. Mononuclear cell leukemia was also considered to be marginally increased in female rats.

The spectrum of lesions found in the skin, Zymbal's gland, liver, oral cavity, clitoral gland, and to a lesser extent the small and large intestine was similar for C.I. Acid Red 114, 3,3′-dimethylbenzidine, 3,3′-dimethoxybenzidine, and C.I. Direct Blue 15. The incidences of skin and liver tumors were higher in male than in female rats in all four benzidine-congener dye studies. However, the incidences of liver tumors produced by administration of C.I. Acid Red 114 and its parent congener 3,3′-dimethylbenzidine was greater than the tumor incidences produced by C.I. Direct Blue 15 or its parent congener, 3,3′dimethoxybenzidine. Some, but not all of the four, benzidine chemicals studied caused increases in the occurrence of lesions in the brain, mammary gland, lung, and adrenal gland as well as mononuclear cell leukemia and mesotheliomas. Clear evidence for increased incidence of preputial gland tumors was seen in the studies of

3,3′-dimethylbenzidine, C.I. Direct Blue 15, and 3,3′-dimethoxybenzidine, but not in the studies of C.I. Acid Red 114.

Earlier studies showed carcinogenic activity in rats from exposure to the benzidine congeners, 3,3′-dimethylbenzidine and 3,3′-dimethoxybenzidine. Hadidian *et al.* (1968) found evidence for treatment-related neoplasms in the urinary bladder, mammary gland, intestinal tract, and Zymbal's gland of male and female rats after 52 weeks of 3,3′-dimethoxybenzidine administered by gavage at doses ranging from 0.3 to 10 mg/kg. The number of animals treated was small (3 to 15/dose group), so it was necessary to pool the data to determine any treatment-related effects. In hamsters, 3,3′-dimethoxybenzidine induced urinary bladder tumors and forestomach papillomas (Saffiotti *et al.*, 1967; Sellakumar *et al.*, 1969). In previous studies on the carcinogenic effects of 3,3′-dimethylbenzidine, rats developed Zymbal's gland, preputial gland, and mammary gland tumors after receiving subcutaneous injections for 2 to 14 months (Spitz *et al.*, 1950; Pliss and Zabezhinsky, 1970).

The carcinogenic effects of the benzidine compounds have been studied in mice as well. The National Center for Toxicological Research conducted studies on the toxic and carcinogenic activities of 3,3′-dimethylbenzidine dihydrochloride and 3,3′dimethoxybenzidine dihydrochloride in the BALB/c mouse (Schieferstein *et al.*, 1989, 1990). The BALB/c mouse was selected because it is susceptible to chemically induced cancer of the urinary bladder, which is the target organ for benzidine carcinogenicity in humans (Meigs *et al.*, 1986). 3,3′-Dimethoxybenzidine at doses from 20 to 630 ppm or 3,3′-dimethylbenzidine at doses from 5 to 140 ppm was administered to BALB/c mice in drinking water for 112 to 116 weeks. Results showed decreased water consumption and lower body weights in male and female mice given 3,3′-dimethoxybenzidine and in female mice given 3,3′-dimethylbenzidine. Unlike the rat studies, BALB/c mice produced no treatment-related neoplasms in the Zymbal's gland, skin, oral cavity, preputial gland, clitoral gland, intestine, or liver, although increased incidences in lung neoplasms were seen in male mice treated with 3,3′-dimethylbenzidine (Schieferstein *et al.*, 1989, 1990). Vesselinovitch *et al.* (1975) reported that benzidine caused liver tumors, lung adenomas, and harderian gland tumors in B6C3F$_1$ mice. Other studies of benzidine showed treatment-related liver tumors in mice (Littlefield *et al.*, 1983) and in rats (Spitz *et al.*, 1950; Pliss and Zabezhinsky, 1970).

Weisburger (1983) reported that rats were more sensitive than mice to the carcinogenic effects of aromatic amines. Differences in absorption and metabolism and in experimental design might account for the different susceptibility of rats and mice to the carcinogenic effects from benzidine, the benzidine congeners, and benzidine dyes. There is limited information on the absorption of 3,3′-dimethoxybenzidine, 3,3′-dimethylbenzidine, C.I. Direct Blue 15, and C.I. Acid Red 114 in rats after oral administration. Lynn *et al.* (1980) showed that only small amounts of C.I. Direct Blue 15 or C.I. Acid Red 114 (less than 1% of the administered dose) were excreted in the urine after administration of a single oral dose of 100 mg/kg to male Sprague-Dawley rats. Bowman *et al.* (1982) administered 12 mg/kg ^{14}C-labeled C.I. Direct Blue 15 or molar equivalent doses of 3,3′-dimethylbenzidine or 3,3′-dimethoxybenzidine to male F344/N rats by oral gavage. Approximately 19% of the dose was recovered in urine of animals treated with C.I. Direct Blue 15; 35% to 40% of the dose was recovered in urine of animals administered 3,3′-dimethylbenzidine or 3,3′dimethoxybenzidine. The doses of 3,3′-dimethylbenzidine, 3,3′-dimethoxybenzidine, C.I. Direct Blue 15, or C.I. Acid Red 114 used in the NTP 2-year drinking water studies were higher than those used in the absorption studies of Bowman *et al.* (1982). The exact absorption cannot be extrapolated simply from the doses administered in the NTP studies. However, for the doses used in the NTP studies, 3,3′-dimethylbenzidine and C.I. Acid Red 114 appear to affect the liver more than do 3,3′-dimethoxybenzidine or C.I. Direct Blue 15. Such effects may be attributed to enhanced absorption or metabolism.

The benzidine dyes are metabolized to their parent congeners or to *N*-acetyl derivatives of their congeners and are excreted in urine (Lynn *et al.*, 1980, 1984; Bowman *et al.*, 1982; Nony *et al*, 1983; Rodgers *et al.*, 1983). Studies with benzidine have shown that the ultimate carcinogenic moiety is an activated form of benzidine produced via metabolic azo reduction. The sequence of benzidine metabolism is thought to begin first with *N*-acetylation followed by *N*-hydroxylation to form *N*′-hydroxy-*N*-acetylbenzidine which is further metabolized by glucuronidation. Prostaglandin H synthetase can activate hydroxylamines resulting in electrophilic

intermediates which bind to DNA (Wise *et al.*, 1984; Wang *et al.*, 1990).

Susceptibility of a species to the carcinogenic action of aromatic amines depends in part on the ability of the species to *N*-hydroxylate the amine substituent. *N*-Hydroxylation appears to be a necessary step in the metabolic activation of aromatic amines. *N*-Acyl and *N*-acetyl aromatic amine derivatives require activation to reactive esters, which act as ultimate carcinogens (Miller and Miller, 1977). Formation of various esters by different species may result in variations in organ specificity (Cohen, 1983). Although the Zymbal's gland has been reported to be deficient in sulfotransferase activity (Irving *et al.*, 1971) and transacylase activity (Bartsch *et al.*, 1973), it is capable of hydroxylating compounds via cytochrome P_{450}-dependent enzymatic pathways (Pohl and Fouts, 1983). In the case of these benzidine compounds, the Zymbal's gland is particularly susceptible to tumor formation.

Because preputial gland neoplasms usually are not overtly aggressive or invasive and rarely metastasize (Goodman *et al.*, 1979; Reznik and Ward, 1981), classification of these neoplasms as benign or malignant is difficult (Maronpot *et al.*, 1988). Studies by Ward and Lynch (1984) showed that malignant preputial/clitoral gland neoplasms from aging F344/N rats were transplantable at a higher incidence and with shorter latency periods than benign neoplasms. These conclusions were based on a single-passage study with a single carcinoma and four adenomas.

The transplantability of preputial gland neoplasms induced by 3,3′-dimethoxybenzidine dihydrochloride, C.I. Direct Blue 15, or C.I. Acid Red 114 in male F344/N rats was investigated to provide information on the biologic behavior of these neoplasms (Maronpot *et al.*, 1988; Ulland *et al.*, 1989). All neoplasms selected for transplanting were retrospectively diagnosed as carcinomas and therefore comparable information was not obtained for preputial gland adenomas. The transplanted preputial gland neoplasms did not become anaplastic or less differentiated over four serial passages. However, the transplants behaved biologically as malignant neoplasms in spite of their well-differentiated morphology. Transplants grew rapidly, reaching 3.0 cm in 7 to 9 weeks. No differences were observed in morphology or growth of transplants obtained from the controls or animals dosed with benzidine congener or dye. The results of these studies confirm the malignant nature of these preputial gland neoplasms from rats.

Tumor formation occurred at many sites after the administration of C.I. Acid Red 114. The mechanism for this tumor formation is thought to be due in part to genetic toxicity of the benzidine compounds. All of the chemicals tested positive in the *Salmonella* assay (Table 20). Cleavage of the azo bonds by reductive metabolism was necessary before positive results could be obtained for C.I. Acid Red 114 or C.I. Direct Blue 15 in the *Salmonella* tests. Tumors from animals treated with these benzidine compounds were shown to have activated *ras* genes (Reynolds *et al.*, 1990).

There is considerable evidence indicating that the activation of protooncogenes and the loss of specific regulatory substances, such as suppressor genes, may be distinct steps in the process of carcinogenesis (Barrett *et al.*, 1987). Activated oncogenes have been detected in only 3% of the spontaneous tumors of Fischer rats in contrast with the detection of activated H-*ras* or N-*ras* in 68% of epithelial tumors induced by benzidine congeners and derived dyes (Reynolds *et al.*, 1990). Furthermore, the presence of these activated oncogenes in several benign tumors suggests that *ras* activation may be an early event in the induction of neoplasms by these compounds. Thus, the activation of *ras* genes by point mutation is an important step in the induction of tumors, at least in rats, by this class of benzidine derived compounds.

TABLE 20
Comparison of National Toxicology Program Mutagenicity Test Results for Selected Benzidine Dyes[a]

Chemical Name	*Salmonella*[b]	CHO SCE	CHO Abs	*Drosophila* SLRL	*Drosophila* RT
3,3′-Dimethoxybenzidine	+	+	+	–	
3,3′-Dimethylbenzidine	+	+	+	+	–
C.I. Acid Red 114	+	–	–	–	
C.I. Direct Blue 218	–	+w	–	–	
C.I. Direct Blue 15	+	–	–		

[a] CHO SCE = Chinese hamster ovary cell sister chromatid exchange test; CHO Abs = Chinese hamster ovary cell chromosomal aberration test; SLRL = sex-linked recessive lethal test; RT = reciprocal translocation test; + = positive; – = negative; +w = weak evidence for positive response. For description of S9 source and details of experimental technique, see Appendix C. Only the *Salmonella* test was conducted with reductive metabolism.

[b] Positive responses were observed in *Salmonella typhimurium* strain TA1538 after incubation in a bacterial reduction system. Such a protocol allows for *in vitro* reduction of the azo linkages, mimicking the metabolism of these compounds in the human intestinal tract, and release of the parent amine, which can then be oxidatively metabolized using an induced rat or hamster liver S9 system (Reid *et al.*, 1984a). The first three chemicals were also positive when tested in a standard preincubation protocol.

CONCLUSIONS

Under the conditions of these 2-year drinking water studies, there was *clear evidence of carcinogenic activity** of C.I. Acid Red 114 for male F344/N rats, as indicated by benign and malignant neoplasms of the skin, Zymbal's gland, and liver. Increased incidences of neoplasms of the oral cavity epithelium, adrenal gland, and lung may have been related to chemical administration. There was *clear evidence of carcinogenic activity* for female F344/N rats, as indicated by benign and malignant neoplasms of the skin, Zymbal's gland, clitoral gland, liver, oral cavity epithelium, small and large intestines, and lung. Increased incidences of mononuclear cell leukemia, mammary gland adenocarcinoma, and adrenal gland pheochromocytomas may have been related to chemical administration.

*Explanation of Levels of Evidence of Carcinogenic Activity appears on page 8. A summary of peer review comments and the public discussion on this Technical Report appears on page 10.

REFERENCES

Armitage, P. (1971). *Statistical Methods in Medical Research*, pp. 362-365. John Wiley and Sons, New York.

Ashby, J., and Tennant, R.W. (1988). Chemical structure, *Salmonella* mutagenicity and extent of carcinogenicity as indicators of genotoxic carcinogenesis among 222 chemicals tested in rodents by the U.S. NCI/NTP. *Mutat. Res.* **204**, 17-115.

Barrett, J.C., Oshimura, M., and Koi, M. (1987). Role of oncogenes and tomour suppressant genes in a multistep model of carcinogenesis. In *Symposium on Fundamental Cancer Research* (Becker, F., Ed.), Vol. 38, pp. 45-56.

Bartsch, H., Dworkin, C., Miller, E.C., and Miller, J.A. (1973). Formation of electrophilic *N*-acetoxyarylamines in cytosols from rat mammary gland and other tissues by transacetylation from the carcinogen *N*-hydroxy-4-acetylaminobiphenyl. *Biochim. Biophys. Acta* **304**, 42-55.

Beaudoin, A.R. (1968). Teratogenic activity of six disazo dyes in the Wistar albino rat. *Proc. Soc. Exp. Biol. Med.* **127**, 215-219.

Beaudoin, A.R., and Pickering, M.J. (1960). Teratogenic activity of several synthetic compounds structurally related to trypan blue. *Anat. Rec.* **137**, 297-305.

Beck, F., and Lloyd, J.B. (1966). The teratogenic effects of azo dyes. *Adv. Teratol.* **1**, 131-193.

Bonser, G.M., Clayson, D.B, and Jull, J.W. (1956). The induction of tumours of the subcutaneous tissues, liver and intestine in the mouse by certain dye stuffs and their intermediates. *Br. J. Cancer* **10**, 653-667.

Boorman, G.A., Montgomery, C.A., Jr., Eustis, S.L., Wolfe, M.J., McConnell, E.E., and Hardisty, J.F. (1985). Quality assurance in pathology for rodent carcinogenicity studies. In *Handbook of Carcinogen Testing* (Milman, H., and E. Weisburger, Eds.), pp. 345-357. Noyes Publications, Park Ridge, NJ.

Bos, R.P., Groenen, M.A.M., Theuws, J.L.G., Leijdekkers, Ch.-M., and Henderson, P.Th. (1984). Metabolism of benzidine-based dyes and the appearance of mutagenic metabolites in urine of rats after oral or intraperitoneal administration. *Toxicology* **31**, 271-282.

Bos, R.P., Van Der Krieken, W., Smeijsters, L., Koopman, J.P., De Jonge, H.R., Theuws, J.L.G., and Henderson, P.Th. (1986). Internal exposure of rats to benzidine derived from orally administered benzidine-based dyes after intestinal azo reduction. *Toxicology* **40**, 207-213.

Bowman, M.C., Oller, W.L., Nony, C.R., Rowland, K.L., Billedeau, S.M., and Lowry, L.K. (1982). Metabolism and distribution of two ^{14}C-benzidine-congener-based dyes, in rats as determined by GC, HPLC, and radioassays. *J. Anal. Toxicol.* **6**, 164-174.

Brown, J.P., and Dietrich, P.S. (1983). Mutagenicity of selected sulfonated azo dyes in the *Salmonella*/microsome assay: Use of aerobic and anaerobic activation procedures. *Mutat. Res.* **116**, 305-345.

Case, R.A.M., Hosker, M.E., McDonald, D.B., and Pearson, J.T. (1954). Tumours of the urinary bladder in workmen engaged in the manufacture and use of certain dyestuff intermediates in the British chemical industry. Part I. The role of aniline, benzidine, alpha-naphthylamine, and beta-naphthylamine. *Br. J. Industr. Med.* **11**, 75-104.

Cerniglia, C.E., Freeman, J.P., Franklin, W., and Pack, L.D. (1982). Metabolism of azo dyes derived from benzidine, 3,3′-dimethylbenzidine and 3,3′-dimethoxybenzidine to potentially carcinogenic aromatic amines by intestinal bacteria. *Carcinogenesis* **3**, 1255-1260.

Cohen, S.M. (1983). Promotion of urinary bladder carcinogenesis. In *Organ and Species Specificity in Chemical Carcinogenesis*, Basic Life Sciences, Vol. 24. (R. Langenbach, S. Nesnow, and J.M. Rice, Eds.), pp. 253-270. Plenum Press, New York.

Cox, D.R. (1972). Regression models and life tables. *J. R. Stat. Soc.* **B34**, 187-220.

Dinse, G.E., and Haseman, J.K. (1986). Logistic regression analysis of incidental-tumor data from animal carcinogenicity experiments. *Fundam. Appl. Toxicol.* **6**, 44-52.

Dinse, G.E., and Lagakos, S.W. (1983). Regression analysis of tumour prevalence data. *Appl. Statist.*. **32**, 236-248.

Dunn, O.J. (1964). Multiple comparisons using rank sums. *Technometrics* **6**, 241-252.

Dunnett, W. (1955). A multiple comparison procedure for comparing several treatments with a control. *J. Am. Stat. Assoc.* **50**, 1095-1121.

Elliot, J., and Gregory, A.R. (1980). Mutagenicity of a series of benzidine congener based dyes. *Vet. Hum. Toxicol.* **22**, 413-417.

Fishbein, L. (1981). Aromatic amines of major industrial importance: use and occurrence. In *Environmental Carcinogens Selected Methods of Analysis,* Vol. 4 (H. Egan, Ed.), pp 51-74. IARC Publications No. 40. International Agency for Research on Cancer, Lyon, France.

Frith, C.H., and Dooley, K. (1976). Hepatic cytologic and neoplastic changes in mice given benzidine dihydrochloride. *J. Natl. Cancer Inst.* **56**, 679-682.

Galloway, S.M., Bloom, A.D., Resnick, M., Marolin, B.H., Nakamura, F., Archer, P., and Zeiger, E. (1985). Development of a standard protocol for *in vitro* cytogenetic testing with Chinese hamster ovary cells: Comparison of results for 22 compounds in two laboratories. *Environ. Mutagen.* **7**, 1-51.

Galloway, S.M., Armstrong, M.J., Reuben, C., Colman, S., Brown, B., Cannon, C., Bloom, A.D., Nakamura, F., Ahmed, M., Duk, S., Rimpo, J., Margolin, B.H., Resnick, M.A., Anderson, B., and Zeiger, E. (1987). Chromosome aberrations and sister chromatid exchanges in Chinese hamster ovary cells: Evaluations of 108 chemicals. *Environ. Mol. Mutagen.* **10** (Suppl. 10), 1-175.

Gart, J.J., Chu, K.C., and Tarone, R.E. (1979). Statistical issues in interpretation of chronic bioassay tests for carcinogenicity. *J. Natl. Cancer Inst.* **62**, 957-974.

Goodman, D.G., Ward, J.M., Squire, R.A., Chu, K.C., and Linhart, M.S. (1979). Neoplastic and nonneoplastic lesions in aging F344 rats. *Toxicol. Appl. Pharmacol.* **48**, 237-248.

Griswold, D.P., Jr., Casey, A.E., Weisburger, E.K., and Weisburger, J.H. (1968). The carcinogenicity of multiple intragastric doses of aromatic and heterocyclic nitro or amino derivatives in young female Sprague-Dawley rats. *Cancer Res.* **28**, 924-933.

Hadidian, Z., Fredrickson, T.N., Weisburger, E.K., Weisburger, J.H., Glass, R.M., and Mantel, N. (1968). Tests for chemical carcinogens. Report on the activity of derivatives of aromatic amines, nitrosamines, quinolines, nitroalkanes, amides, epoxides, aziridines, and purine antimetabolites. *J. Natl. Cancer Inst.* **41**, 985-1036.

Haley, T.J. (1975). Benzidine revisited: A review of the literature and problems associated with the use of benzidine and its congeners. *Clin. Toxicol.* **8**, 13-42.

Hartman, C.P., Fulk, G.E., and Andrews, A.W. (1978). Azo reduction of trypan blue to a known carcinogen by a cell-free extract of a human intestinal anaerobe. *Mutat. Res.* **58**, 125-132.

Haseman, J.K. (1984). Statistical issues in the design, analysis and interpretation of animal carcinogenicity studies. *Environ. Health Perspect.* **58**, 385-392.

Haseman, J.K., Huff, J., and Boorman, G.A. (1984). Use of historical control data in carcinogenicity studies in rodents. *Toxicol. Pathol.* **12**, 126-135.

Haseman, J.K., Huff, J., Rao, G.N., Arnold, J., Boorman, G.A., and McConnell, E.E. (1985). Neoplasms observed in untreated and corn oil gavage control groups of F344/N rats and (C57BL/6N × C3H/HeN)F_1 (B6C3F_1) mice. *JNCI* **75**, 975-984.

Haworth, S., Lawlor, T., Mortelmans, K., Speck, W., and Zeiger, E. (1983). *Salmonella* mutagenicity test results for 250 chemicals. *Environ. Mutagen.* **5** (Suppl. 1), 3-142.

Hollander, M. and Wolfe, D.A. (1973). *Nonparametric Statistical Methods,* pp. 120-123. John Wiley and Sons, Inc., New York.

International Agency for Research on Cancer (IARC) (1972a). Benzidine. IARC Monographs on the Evaluation of Carcinogenic Risk of Chemicals to Man, Vol. 1, pp. 80-86. IARC, Lyon, France.

International Agency for Research on Cancer (IARC) (1972b). Benzidine. IARC Monographs on the Evaluation of Carcinogenic Risk of Chemicals to Man, Vol. 1, pp. 87-91. IARC, Lyon, France.

International Agency for Research on Cancer (IARC) (1974). 3,3′-Dimethoxybenzidine (*o*-dianisidine). Some Aromatic Amines, Hydrazine and Related Substances, N-Nitroso compounds and Miscellaneous Alkylating Agents. IARC Monographs on the Evaluation of Carcinogenic Risk of Chemicals to Man, Vol. 4. IARC, Lyon, France.

International Agency for Research on Cancer (IARC) (1982). Benzidine and its sulphate, hydrochloride, and dihydrochloride. IARC Monographs on the Evaluation of Carcinogenic Risk of Chemicals to Man, Vol. 29. IARC, Lyon, France.

International Agency for Research on Cancer (IARC) (1987a). IARC Monographs on the Evaluation of Carcinogenic Risk of Chemicals to Humans. Overall evaluations of carcinogenicity: An updating of *IARC Monographs*, Vol. 1-42 (Suppl. 7). IARC, Lyon, France.

International Agency for Research on Cancer (IARC) (1987b). Benzidine (group 1). IARC Monographs on the Evaluation of Carcinogenic Risks to Humans. Overall evaluations of carcinogenicity: An updating of *IARC Monographs* Vol. 1-42 (Suppl. 7). IARC, Lyon, France.

Irving, C.C., Janss, D.H., and Russell, L.T. (1971). Lack of *N*-hydroxy-2-acetylaminofluorene sulfotransferase activity in the mammary gland and Zymbal's gland of the rat. *Cancer Res.* **31**, 387-391.

Jonckheere, A. (1954). A distribution-free k-sample test against ordered alternatives. *Biometrika* **41**, 133-145.

Kaplan, E.L., and Meier, P. (1958). Nonparametric estimation from incomplete observations. *J. Am. Stat. Assoc.* **53**, 457-481.

Kennelly, J.C., Beland, F.A., Kadlubar, F.F., and Martin, C.N. (1984). Binding of N-acetylbenzidine and N,N'-diacetylbenzidine to hepatic DNA of rat and hamster *in vivo* and *in vitro*. *Carcinogenesis* **5**, 407-412.

Kirk-Othmer Encyclopedia of Chemical Technology. (1978). 3rd ed., vol. 3, pp. 399. John Wiley and Sons, Inc., New York.

Kornbrust, D.J., and Barfknecht, T.R. (1984). Comparison of rat and hamster hepatocyte primary culture/DNA-repair assays. *Environ. Mutagen.* **6**, 1-11.

Littlefield, N.A., Nelson, C.J., and Frith, C.H. (1983). Benzidine dihydrochloride: toxicological assessment in mice during chronic exposures. *J. Toxicol. Environ. Health* **12**, 671-685.

Lloyd, J.B., and Beck, F. (1966). The relationship of chemical structure to teratogenic activity among bisazo dyes: a re-evaluation. *J. Embryol. Exp. Morph.* **16**, 29-39.

Lloyd, J.B., Beck, F., and Griffiths, A. (1965). Structure-activity studies for the teratogenic effects of disazo dyes. *J. Pharm. Pharmacol.* **17**, Suppl., 126S-128S.

Lynn, R.K., Donielson, D.W., Ilias, A.M., Kennish, J.M., Wong, K., and Matthews, H.B. (1980). Metabolism of bisazobiphenyl dyes derived from benzidine, 3,3′-dimethylbenzidine or 3,3′-dimethoxybenzidine to carcinogenic aromatic amines in the dog and rat. *Toxicol. Appl. Pharmacol.* **56**, 248-258.

Lynn, R.K., Garvie-Gould, C.T., Milam, F., Scott, K.F., Eastman, C.L., Ilias, A.M., and Rodgers, R.M. (1984). Disposition of the aromatic amine, benzidine, in the rat: Characterization of mutagenic urinary and biliary metabolites. *Toxicol. Appl. Pharmacol.* **72**, 1-14.

Margolin, B., Collings, B., and Mason, J. (1983). Statistical analysis and sample-size determinations for mutagenicity experiments with binomial responses. *Environ. Mutagen.* **5**, 705-710.

Maronpot, R.R., and Boorman, G.A. (1982). Interpretation of rodent hepatocellular proliferative alterations and hepatocellular tumors in chemical safety assessment. *Toxicol. Pathol.* **10**, 71-80.

Maronpot, R.R., Ulland, B., and Mennear, J. (1988). Transplantation characteristics, morphologic features, and interpretation of preputial gland neoplasia in the Fischer 344 rat. *Environ. Health Perspect.* **77**, 33-36.

Martin, C.N., and Kennelly, J.C. (1981). Rat liver microsomal azoreductase activity on four azo dyes derived from benzidine, 3,3′-dimethylbenzidine, or 3,3′-dimethoxybenzidine. *Carcinogenesis* **2**, 307-312.

Martin, C.N., McDermid, A.C., and Garner R.C. (1978). Testing of known carcinogens and noncarcinogens for their ability to induce unscheduled DNA synthesis in HeLa cells. *Cancer Res.* **38**, 2621-2627.

McConnell, E.E., Solleveld, H.A., Swenberg, J.A., and Boorman, G.A. (1986). Guidelines for combining neoplasms for evaluation of rodent carcinogenesis studies. *JNCI* **76**, 283-289.

McKnight, B., and Crowley, J. (1984). Tests for differences in tumor incidence based on animal carcinogenesis experiments. *J. Am. Stat. Assoc.* **79**, 639-648.

Meigs, J.W., Marrett, L.D., Ulrich, F.U., and Flannery, J.T. (1986). Bladder tumor incidence among workers exposed to benzidine: a thirty-year follow-up. *JNCI* **76**, 1-8.

Miller, J.A., and Miller, J.A. (1977). Ultimate chemical carcinogens as reactive mutagenic electrophiles. In *Origins of Human Cancer*, Vol. 4. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.

Mirsalis, J., Tyson, K., Beck, J., Loh, E., Steinmetz, K., Contreras, C., Austere, L., Martin, S., and Spalding, J. (1983). Induction of unscheduled DNA synthesis (UDS) in hepatocytes following *in vitro* and *in vivo* treatment. *Environ. Mutagen.* **5**, 482. (Abstr.)

Mitchell, A.D., Rudd, C.J., and Caspary, W.J. (1988). Evaluation of the L5178Y mouse lymphoma cell mutagenesis assay: Intralaboratory results for 63 coded chemicals tested at SRI International. *Environ. Mol. Mutagen.* **12** (Suppl. 13), 37-102.

Mortelmans, K., Haworth, S., Lawlor, T., Speck, W., Tainer, B., and Zeiger, E. (1986). *Salmonella* mutagenicity tests. II. Results from the testing of 270 chemicals. *Environ. Mutagen.* **8** (Suppl. 7), 1-119.

Myhr, B.C., and Caspary, W.J. (1988). Evaluation of the L5178Y mouse lymphoma cell mutagenesis assay: Intralaboratory results for sixty-three coded chemicals tested at Litton Bionetics, Inc. *Environ. Mol. Mutagen.* **12** (Suppl. 13), 103-194.

National Cancer Institute (NCI) (1976). Guidelines for Carcinogen Bioassay in Small Rodents. Technical Reports Series No. 1. NIH Publication No. 76-801. National Institutes of Health, Bethesda, MD.

National Cancer Institute (NCI) (1978). Bioassay of *o*-Anisidine Hydrochloride for Possible Carcinogenicity (CAS No. 134-29-0). NCI Technical Report Series No. 89. NIH Publication No. 78-1339. National Institutes of Health, Bethesda, MD.

National Cancer Institute (NCI) (1979). Bioassay of *o*-Toluidine Hydrochloride for Possible Carcinogenicity (CAS No. 636-21-5). NCI Technical Report Series No. 153. NIH Publication No. 79-1709. National Institutes of Health, Bethesda, MD.

National Institutes of Health (NIH) (1978). Open Formula Rat and Mouse Ration (NIH-07). NIH Publication No. 11-1335. National Institutes of Health, Bethesda, MD.

National Institute of Occupational Safety and Health (NIOSH) (1981) Health hazard alert--benzidine, *o*-tolidine-, and *o*-dianisidine-based dyes. Reprinted in *Am. Ind. Hyg. Assoc. J.* **42(5)**, A-36-A-40.

National Institute of Occupational Safety and Health (NIOSH) (1983). Preventing health hazards from exposure to benzidine congener dyes. Publication No. 83-105. NIOSH, Cincinnati, OH.

National Institute of Occupational Safety and Health (NIOSH) (1991). National Occupational Exposure Survey (NOES) (1981-1983), unpublished provisional data as of March 1, 1991.

National Toxicology Program (NTP) (1990). Toxicology and Carcinogenesis Studies of 3,3′-Dimethoxybenzidine Dihydrochloride in F344/N Rats and B6C3F$_1$ Mice (Drinking Water Studies). NTP TR No. 372. NIH Publication No. 90-2827. National Institute of Environmental Health Sciences, Research Triangle Park, NC.

National Toxicology Program (NTP) (1991). Toxicology and Carcinogenesis Studies of 3,3'-Dimethylbenzidine Dihydrochloride in F344/N Rats (Drinking Water Studies). NTP TR No. 390. NIH Publication No. 91-2845. National Institute of Environmental Health Sciences, Research Triangle Park, NC.

National Toxicology Program (NTP) (in press). Toxicology and Carcinogenesis Studies of C.I. Direct Blue 15 in F344/N Rats (Drinking Water Studies). NTP TR No. 397. NIH Publication No. 92-2854). National Institute of Environmental Health Sciences, Research Triangle Park, NC.

Noller, C.R. (1965). *Chemistry of Organic Compounds*, pp. 561-565, 743-744, W.B. Saunders Co., Philadelphia.

Nony, C.R., Bowman, M.C., Cairns, T., Lowry, L.K., and Tolos, W.P. (1980). Metabolism studies of an azo dye and pigment in the hamster based on analysis of the urine for potentially carcinogenic aromatic amine metabolites. *J. Anal. Toxicol.* **4**, 132-140.

Nony, C.R., Althaus, J.R., and Bowman, M.C. (1983). Chromatographic assays for traces of potentially carcinogenic metabolites of two azo dyes, Direct Red 2 and Direct Blue 15, in rat, hamster and human urine. *J. Anal. Toxicol.* **7**, 40-48.

Pliss, G.B. (1963). On some regular relationships between carcinogenicity of aminodiphenyl derivatives and the structure of substance. *Acta. Intl. Union Contra Cancer.* **19**, 499-501.

Pliss, G.B. (1965). Carcinogenic properties of ortho-tolidine and dianisidine. *Gig. Tr. Prof. Zabol.* **9**, 18-22.

Pliss, G.B., and Zabezhinsky, M.A. (1970). Carcinogenic properties of orthotolidine (3,3′-dimethylbenzidine). *J. Natl. Cancer Inst.* **45**, 283-289.

Pohl, R.J., and Fouts, J.R. (1983). Cytochrome P-450-dependent xenobiotic metabolizing activity in Zymbal's gland, a specialized sebaceous gland of rodents. *Cancer Res.* **43**, 3660-3662.

Portier, C.J., and Hoel, D.G. (1984). Design of animal carcinogenicity studies for goodness-of-fit of multistage models. *Fundam. Appl. Toxicol.* **4**, 949-959.

Prival, M.J., and Mitchell, V.D. (1982). Analysis of a method for testing azo dyes for mutagenic activity in *Salmonella typhimurium* in the presence of flavin mononucleotide and hamster liver S9. *Mutat. Res.* **97**, 103-116.

Prival, M.J., Bell, S.J., Mitchell, V.D., Peiperl, M.D., and Vaughan, V.L. (1984). Mutagenicity of benzidine and benzidine-congener dyes and selected monoazo dyes in a modified *Salmonella* assay. *Mutat. Res.* **136**, 33-47.

Prokofjeva, O.G. (1971). Induction of hepatic tumors in mice by benzidine. *Vopr. Onkol.* **17**, 61-64.

Reid, T.M., Morton, K.C., Wang, C.Y., and King, C.M. (1983). Conversion of Congo red and 2-azoxyfluorene to mutagens following in vitro reduction by whole-cell rat cecal bacteria. *Mutat. Res.* **117**, 105-112.

Reid, T.M., Morton, K.C., Wang, C.Y., and King, C.M. (1984a). Mutagenicity of azo dyes following metabolism by different reductive/oxidative systems. *Environ. Mutagen.* **6**, 705-717.

Reid, T.M., Wang, C.Y., King, C.M., and Morton, K.C. (1984b). Mutagenicity of some benzidine congeners and their *N*-acetylated and *N,N′*-diacetylated derivatives in different strains of *Salmonella typhimurium*. *Environ. Mutagen.* **6**, 145-151.

Reynolds, S.H., Patterson, R.M., Mennear, J.H., Maronpot, R.R., and Anderson, M.W. (1990). *Ras* gene activation in rat tumors induced by benzidine congeners and derived dyes. *Cancer Res.* **50**, 266-272.

Reznik, G., and Ward, J.M. (1981). Morphology of hyperplastic and neoplastic lesions in the clitoral and preputial gland of the F344 rat. *Vet. Pathol.* **18**, 228-238.

Rinde, E., and Troll, W. (1975). Metabolic reduction of benzidine azo dyes to benzidine in the rhesus monkey. *J. Natl. Cancer Inst.* **55**, 181-182.

Rodgers, R.M., Garvie-Gould, C., Scott, K.F., Milam, D.F., and Lynn, R.K. (1983). Metabolism, distribution, and excretion of the carcinogenic aromatic amine, 3,3′-dimethoxybenzidine in the rat. *Drug Metab. Dispos.* **11**, 293-300.

Rudd, C.J., Mitchell, A.D., and Spalding, J. (1983). L5178Y Mouse lymphoma cell mutagenesis assay of coded chemicals incorporating analyses of the colony size distributions. *Environ. Mutagen.* **5**, 419.

Sadtler Standard Spectra. Sadtler Research Laboratories, Philadelphia, PA.

Saffiotti, U., Cefis, F., Montesano, R., and Sellakumar, A.R. (1966). Induction of bladder cancer in hamsters fed aromatic amines. *Ind. Med. and Surg.* **35**, 564.

Saffiotti, U., Cefis, F., Montesano, R., and Sellakumar, A.R. (1967). Induction of bladder cancer in hamsters fed aromatic amines. In *Bladder Cancer, A Symposium* (Deichmann, W.B. and Lampe, K.F., Eds.), pp. 129-135. Aesculapius Publishing Co., Birmingham, AL.

Schieferstein, G.J., Shinohara, Y., Allen, R.R., Sheldon, W., Greenman, D.L., and Allaben, W.T. (1989). Carcinogenicity study of 3,3′-dimethylbenzidine dihydrochloride in BALB/c mice. *Food Chem. Toxicol.* **27**, 801-806.

Schieferstein, G.J., Sheldon, W.G., Allen, R.R., Greenman, D.L., and Allaben, W.T. (1990). Oncogenic evaluation of 3,3′-dimethoxybenzidne dihydrochloride in BALB/c mice. *J. Am. College Toxicol.* **9**, 71-77.

Scott, T.S. (1952). The incidence of bladder tumours in a dyestuffs factory. *Br. J. Indust. Med.* **9**, 127-132.

Sellakumar, A.R., Montesano, R., and Saffiotti, U. (1969). Aromatic amines carcinogenicity in hamsters. *Proc. Amer. Assoc. Cancer Res.* **10**, 78. (Abstr.)

Shimizu, H., and Takemura, N. (1976). Mutagenicity and carcinogenicity of some aromatic amino and nitro compounds. *Jpn. J. Indust. Health* **18**, 138-139.

Shirley, E. (1977). A non-parametric equivalent of Williams' test for contrasting increasing dose levels of a treatment. *Biometrics* **33**, 386-389.

Spitz, S., Maguigan, W.H., and Dobringer, K. (1950). The carcinogenic action of benzidine. *Cancer* **3**, 789-804.

Tanaka, K., Mii, T., Marui, S., Matsubara, I., and Igaki, H. (1982). Some aspects of metabolism and mutagenicity of *o*-toluidine and *o*-toluidine-based azo dye. *Indust. Health* **20**, 277-235.

Tarone, R.E. (1975). Tests for trend in life table analysis. *Biometrika* **62**, 679-682.

Ulland, B.M., Maronpot, R.F., Lemen, J.K., and Mennear, J.H. (1989). Transplantation studies of preputial gland and epithelial skin neoplasms derived from benzidine-based dye carcinogenicity assays in Fischer 344 male rats. *Toxicol. Pathol.* **17**, 50-56.

U.S. Environmental Protection Agency (USEPA) (1980). TSCA Chemical Assessment Series, Preliminary Risk Assessment, Phase I. Benzidine, its congeners, and their derivative dyes and pigments, No. 560/11-80-019. Office of Pesticides and Toxic Substances, USEPA, Washington, DC.

U.S. Environmental Protection Agency (USEPA) (1988). Computer printout (CIS): 1977 Production Statistics for Chemicals in the Non-confidential Initial TSCA Chemical Substances Inventory. Office of Pesticides and Toxic Substances, USEPA, Washington, DC.

U.S. International Trade Commission (USITC) (1980). Synthetic Organic Chemicals, United States Production and Sales, 1979. USITC Publication No. 1099. Washington, DC.

U.S. International Trade Commission (USITC) (1981). Imports of benzenoid chemicals and products, 1980. USITC Publication No. 1163. Washington, DC.

U.S. International Trade Commission (USITC) (1986). Synthetic Organic Chemicals, United States Production and Sales, 1985. USITC Publication No. 1892. Washington, DC.

U.S. International Trade Commission (USITC) (1987). Synthetic Organic Chemicals, United States Production and Sales, 1986. USITC, Publication No. 2009. Washington, DC.

Venturini, S., and Tamaro, M., (1979). Mutagenicity of anthraquinone and azo dyes in Ames' *Salmonella typhimurium* test. *Mutat. Res.* **68**, 307-312.

Vesselinovitch, S.D., Rao, K.V.N., and Mihailovich, N. (1975). Factors modulating benzidine carcinogenicity bioassay. *Cancer Res.* **35**, 2814-2819.

Waalkens, D.H., Joosten, H.F.P., Yih, T.D., and Hoekstra, A. (1981). Mutagenicity studies with o-tolidine and 4,4′ - tetramethyldiaminodiphenylmethane. *Mutat. Res.* **89**, 197-202.

Walker, R. (1970). The metabolism of azo compounds: A review of the literature. *Food Cosmet. Toxicol.* **8**, 659-676.

Wang, C.Y., Zukowski, K., Yamada, H., Imaida, K., and Lee, M.-S. Production of urothelial tumors in the heterotopic bladder of rat by benzidine derivatives. *Cancer Res.* **50**, 2868-2871.

Ward, J.M., and Lynch, P.H. (1984). Transplantability of naturally occurring benign and malignant neoplasms and age-associated nonneoplastic lesions of the aging F344 rat as biological evidence for the histological diagnosis of neoplasms. *Cancer Res.* **44**, 2608-2615.

Weisburger, E.K. (1983). Species differences in response to aromatic amines. *Basic Life Sci.*. **24**, 23-47.

Williams, D.A. (1971). A test for differences between treatment means when several dose levels are compared with a zero dose control. *Biometrics* **27**, 103-117.

Williams, D.A. (1972). The comparison of several dose levels with a zero dose control. *Biometrics* **28**, 519-531.

Wilson, J.G. (1955). Teratogenic activity of several azo dyes chemically related to trypan blue. *Anat. Rec.* **123**, 313-334.

Wise, R.W., Zenser, T.V., Kadlubar, F.F., and Davis, B.B. (1984). Metabolic activation of carcinogenic aromatic amines by dog bladder and kidney prostaglandin H synthase. *Cancer Res.* **44**, 1893-1897.

Yamazoe, Y., Zenser, T.V., Miller, D.W., and Kadlubar, F.F. (1988). Mechanism of formation and structural characterization of DNA adducts derived from peroxidative activation of benzidine. *Carcinogenesis* **9**, 1635-1641.

Zavon, M.R., Hoegg, U., and Bingham, E. (1973). Benzidine exposure as a cause of bladder tumors. *Arch. Environ. Health* **27**, 1-7.

Zenser, T.V., Mattammal, M.B., Armbrecht, H.J., and Davis, B.B. (1980). Benzidine binding to nucleic acids mediated by the peroxidative activity of prostaglandin endoperoxide synthetase. *Cancer Res.* **40**, 2839-2845.

Zimmering, S., Mason, J.M., Valencia, R., and Woodruff, R.C. (1985). Chemical mutagenesis testing in *Drosophila*. II. Results of 20 coded compounds tested for the National Toxicology Program. *Enrivon. Mutagen.* **7**, 87-100.

Appendix D: NTP. (1991). Toxicology and Carcinogenesis Studies of 3,3'-Dimethylbenzidine Dihydrochloride in F344/N Rats (Drinking Water Studies). National Toxicology Program Technical Report Series No. 390. pp. D-1 –D-74.

NTP TECHNICAL REPORT

ON THE

TOXICOLOGY AND CARCINOGENESIS STUDIES OF 3,3′-DIMETHYLBENZIDINE DIHYDROCHLORIDE

(CAS NO. 612-82-8)

IN F344/N RATS

(DRINKING WATER STUDIES)

NATIONAL TOXICOLOGY PROGRAM
P.O. Box 12233
Research Triangle Park, NC 27709

June 1991

NTP TR 390

NIH Publication No. 91-2845

U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES
Public Health Service
National Institutes of Health

CONTRIBUTORS

National Toxicology Program

C.J. Alden, Ph.D.
D.W. Bristol, Ph.D.
G.A. Boorman, D.V.M., Ph.D.
S.L. Eustis, D.V.M., Ph.D.
R.A. Griesemer, D.V.M., Ph.D.
J.K. Haseman, Ph.D.
C.W. Jameson, Ph.D.
M.P. Jokinen, D.V.M.
D.L. Morgan, Ph.D.
G.N. Rao, D.V.M., Ph.D.

NTP Pathology Working Group

Evaluated slides, prepared pathology report, 13 September 1988

L.H. Brennecke, D.V.M., Chair
Pathology Associates, Inc.
K. Ayers, D.V.M.
Burroughs Wellcome Company
R. Brown, D.V.M., M.S.
Experimental Pathology Laboratories, Inc.
B. Davis, V.M.D., observer
North Carolina State University
D. Dixon, D.V.M., Ph.D.
National Toxicology Program
K. Heider, D.V.M.
Ciba-Geigy, Switzerland
M.P. Jokinen, D.V.M.
National Toxicology Program
M.M. McDonald, D.V.M., Ph.D.
National Toxicology Program

Hazleton Laboratories America

Conducted studies, evaluated pathology findings

B.L. Ulland, Ph.D., Principal Investigator
G.A. Parker, D.V.M.
J. Lemen, M.S.

Experimental Pathology Laboratories, Inc.

Provided pathology quality assessment

R. Brown, D.V.M., M.S.

Integrated Laboratory Systems

Prepared quality assurance audits

J.C. Bhandari, D.V.M., Ph.D.

Biotechnical Services, Inc.

Prepared Technical Report

L.G. Cockerham, Ph.D. Principal Investigator
G.F. Corley, D.V.M.
J.L. Hoskyn, B.S.

CONTENTS

ABSTRACT 5

EXPLANATION OF LEVELS OF EVIDENCE OF CARCINOGENIC ACTIVITY 9

PEER REVIEW PANEL 10

SUMMARY OF PEER REVIEW COMMENTS 11

INTRODUCTION 13

MATERIALS AND METHODS 21

RESULTS 29

DISCUSSION AND CONCLUSIONS 61

REFERENCES 69

APPENDIX A Summary of Lesions in Male Rats in the 14-Month Drinking Water Study. 77

APPENDIX B Summary of Lesions in Female Rats in the 14-Month Drinking Water Study 139

APPENDIX C Genetic Toxicology 197

APPENDIX D Hematology, Clinical Chemistry, and Urinalysis Results in the 13-Week and 9-Month Studies 207

APPENDIX E Organ Weights and Organ-Weight-to-Body-Weight Ratios in the 14-Day, 13-Week, and 9-Month Studies 211

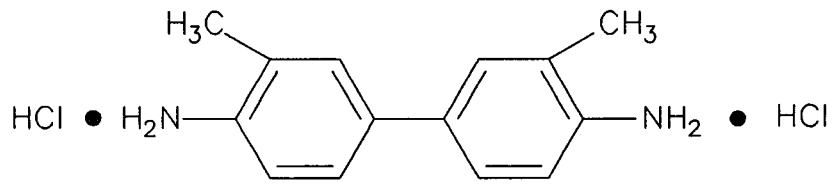
APPENDIX F Chemical Characterization and Dose Formulation Studies 219

APPENDIX G Water and Compound Consumption by Rats in the 14-Month Drinking Water Studies 229

APPENDIX H Ingredients, Nutrient Composition, and Contaminant Levels in NIH-07 Rat and Mouse Ration 233

APPENDIX I Sentinel Animal Program 237

ABSTRACT



3,3′-DIMETHYLBENZIDINE DIHYDROCHLORIDE

CAS No. 612-82-8

$C_{14}H_{16}N_2 \cdot 2HCl$ Molecular Weight 285.2

Synonyms: *o*-Tolidine dihydrochloride; 3,3′-Dimethylbiphenyl-4,4′-diamine dihydrochloride; 3,3′-Dimethylbiphenyl-4,4′-biphenyldiamine dihydrochloride; 4,4′-Diamino-3,3′-dimethylbiphenyl dihydrochloride

3,3′-Dimethylbenzidine dihydrochloride is one of five chemicals being evaluated in 2-year carcinogenicity and toxicity studies as part of the NTP's Benzidine Dye Initiative. This Initiative was designed to evaluate representative benzidine congeners, benzidine congener-derived dyes, and benzidine-derived dyes. 3,3′-Dimethylbenzidine dihydrochloride was nominated for study because of the potential for human exposure during production of bisazobiphenyl dyes and because benzidine, a structurally related chemical, is a known human carcinogen.

Toxicology and carcinogenesis studies were conducted by administering 3,3′-dimethylbenzidine dihydrochloride (approximately 99% pure) in drinking water to groups of F344/N rats of each sex for 14 days, 13 weeks, or 9 or 14 months. The 14-month exposures were planned as 24-month exposures but were terminated early because of rapidly declining animal survival, due primarily to neoplasia. These studies were performed only in rats because similar studies were being performed in mice at the National Center for Toxicological Research (NCTR). Hematologic and serum chemical analyses and thyroid hormone determinations were conducted in conjunction with the 13-week and 9-month studies. Genetic toxicology studies were conducted in *Salmonella typhimurium*, Chinese hamster ovary (CHO) cells, and *Drosophila melanogaster*.

14-Day Studies: Rats were exposed to 3,3′-dimethylbenzidine dihydrochloride in drinking water at doses ranging from 600 to 7,500 ppm. All five males and one female in the 7,500 ppm group and 1/5 males in the 5,000 ppm group died. Final mean body weights were decreased in males receiving 1,250 ppm or more and in all exposed females, and final mean body weights of animals receiving 2,500 ppm or more were lower than initial weights. Water consumption decreased with increasing

chemical concentration. Compound-related effects observed in rats receiving 5,000 ppm or more included minimal to slight hepatocellular necrosis, accumulation of brown pigment (presumably bile) in individual hepatocytes, increased severity of nephropathy relative to controls, and severe lymphocytic atrophy of the thymus. Treated animals also showed an increased severity of atrophy of the bone marrow relative to controls, varying degrees of lymphocytic atrophy of the mandibular and mesenteric lymph nodes and spleen, increased vacuolization and necrosis of cells of the adrenal cortex, focal acinar cell degeneration in the pancreas, and, in males, increased immature sperm forms in the testis and epididymis.

13-Week Studies: 3,3′-Dimethylbenzidine dihydrochloride was administered in drinking water at doses of 300, 500, 1,000, 2,000, and 4,000 ppm. All rats receiving 4,000 ppm and 4/10 males and 1/10 females receiving 2,000 ppm died before the end of the studies. Depressions in final mean body weight relative to controls ranged from 12% to 48% for males and from 9% to 42% for females. Water consumption decreased with increasing dose. At compound concentrations of 300 to 2,000 ppm, mean water consumption was 29% to 83% of control values. Compound-related effects included an increase in the severity of nephropathy relative to controls; hepatocellular necrosis and accumulation of brown pigment (presumably bile) in sinusoidal lining cells; lymphocytic atrophy of the thymus, spleen, and mandibular and mesenteric lymph nodes; atrophy of the bone marrow in the higher-dose groups; degeneration of pancreatic acinar cells; and, in males, immature sperm forms in the testis and epididymis. Decreases in serum triiodothyronine (T_3) values were observed in exposed females, and decreases in mean thyroxin (T_4) concentrations in exposed males and females; no significant changes were observed in thyroid stimulating hormone (TSH) levels in exposed rats.

Based on the decreased survival, reductions in water consumption and body weight gain, and chemical-induced hepatocellular and renal lesions observed in the 13-week studies, the doses selected for the 9- and 14-month drinking water studies of 3,3′-dimethylbenzidine dihydrochloride were 0, 30, 70, and 150 ppm. Seventy rats of each sex were used in the control group, 45 in the low-dose group, 75 in the mid-dose group, and 70 in the high-dose group.

9-Month Studies: Ten rats of each sex in the control and 150 ppm dose groups were evaluated after 9 months. Chemical-related effects observed in exposed animals included alveolar/bronchiolar carcinoma in one male, basal cell carcinoma of the skin in one male, a squamous cell carcinoma of the oral cavity in one female, preputial gland carcinoma in two males, clitoral gland carcinoma in three females, adenocarcinoma of the small intestine in two males, Zymbal's gland carcinoma in two males and three females, hepatocellular carcinoma in two males, and adenomatous polyps of the large intestine in three males. Other effects seen in dosed rats included focal cellular alteration in the liver, lymphoid atrophy in the spleen, and increased severity of nephropathy relative to controls. An increase in serum T_3 values was observed in exposed males, and a decrease in mean T_4 concentrations in exposed males and females. TSH concentrations were increased in exposed male and female rats.

Body Weights and Survival in the 14-Month Studies: The average amount of 3,3′-dimethylbenzidine dihydrochloride consumed per day was approximately 1.8, 4.0, or 11.2 mg/kg for low-, mid-, or high-dose male rats and 3.0, 6.9, or 12.9 mg/kg for low-, mid-, or high-dose female rats. The mean body weight of high-dose males was about 85% of the control value by week 28. By the end of the study, mean body weights of low-, mid-, and high-dose males were 97%, 92%, and 70% of the control values, respectively. Mean body weights of high- and mid-dose females were about 85% of control values at week 32 and week 44, respectively. At the end of the study, mean body weights of exposed females were about 94%, 81%, and 74% of control values for low-, mid-, and high-dose groups, respectively. Because of extensive neoplasia, many exposed males and females were dying or were sacrificed moribund in the first year, and all high-dose males died by week 55. The studies were terminated at weeks 60 to 61, at which time the group survivals were male: control, 60/60; low dose, 41/45; mid dose, 50/75; high dose, 0/60; female: 59/60; 39/45; 32/75; 10/60.

Nonneoplastic Effects in the 14-Month Studies: Increases in nonneoplastic lesions in dosed rats included cystic degeneration and foci of cellular alteration in the liver; exacerbation of nephropathy; and focal or multifocal hyperplasia of the Zymbal's gland, preputial and clitoral glands, and alveolar epithelium.

Neoplastic Effects in the 14-Month Studies: Neoplasms were observed in exposed rats at many sites: skin, Zymbal's gland, preputial and clitoral glands, liver, oral cavity, small and large intestine, mammary gland, lung, brain, and mesothelium. The incidence of these neoplastic effects in male and female rats is summarized in the table at the end of this section.

Genetic Toxicology: 3,3′-Dimethylbenzidine dihydrochloride was mutagenic in *Salmonella typhimurium* strain TA98 with exogenous metabolic activation; it was not mutagenic in strains TA100, TA1535, or TA97 with or without activation. 3,3′-Dimethylbenzidine dihydrochloride induced sister-chromatid exchanges (CHO) and chromosomal aberrations in CHO cells in the absence of exogenous metabolic activation; these effects were not evident in tests with S9 activation. Sex-linked recessive lethal mutations were induced in germ cells of adult male *Drosophilia melanogaster* administered 3,3′-dimethyl-benzidine dihydrochloride in feed or by injection. No reciprocal translocations occurred in *D. melanogaster* germ cells following exposure to 3,3′-dimethylbenzidine dihydrochloride.

Conclusions: Under the conditions of these 14-month drinking water studies, there was *clear evidence of carcinogenic activity** of 3,3′-dimethylbenzidine dihydrochloride for male F344/N rats, as indicated by benign and malignant neoplasms of the skin, Zymbal's gland, preputial gland, liver, oral cavity, small and large intestine, lung, and mesothelium. Increased incidences of neoplasms of the brain may have been related to chemical administration. There was *clear evidence of carcinogenic activity* for female F344/N rats, as indicated by benign and malignant neoplasms of the skin, Zymbal's gland, clitoral gland, liver, oral cavity, small and large intestine, mammary gland, and lung. Increased incidences of neoplasms of the brain and mononuclear cell leukemia may have been related to chemical administration.

* Explanation of Levels of Evidence of Carcinogenic Activity is on page 9. A summary of peer review comments and the public discussion on this Technical Report appears on page 11.

Summary of the 14-Month Drinking Water Studies and Genetic Toxicology of 3,3′-Dimethylbenzidine Dihydrochloride

Male F344/N Rats	Female F344/N Rats
Drinking water concentrations	
0, 30, 70, or 150 ppm 3,3′-dimethylbenzidine dihydrochloride	0, 30, 70, or 150 ppm 3,3′-dimethylbenzidine dihydrochloride
Body weights	
Exposed groups lower than controls	Exposed groups lower than controls
2-Year survival rates	
60/60, 41/45, 50/75, 0/60[a]	59/60, 39/45, 32/75, 10/60[a]
Nonneoplastic effects	
Preputial gland: hyperplasia	Clitoral gland: hyperplasia
Liver: cystic degeneration, focal cellular alterations	Liver: cystic degeneration, focal cellular alterations
Lung: hyperplasia	Lung: hyperplasia
Zymbal's gland: hyperplasia	Zymbal's gland: hyperplasia
Neoplastic effects	
Skin basal cell neoplasms: 0/60, 11/45, 54/75, 30/60	Skin basal cell neoplasms: 0/60, 3/45, 10/75, 9/60
Skin sebaceous cell adenoma: 0/60, 0/45, 7/75, 5/60	
Skin keratoacanthomas: 1/60, 1/45, 8/75, 5/60	
Skin squamous cell neoplasms: 0/60, 2/45, 17/75, 27/60	Skin squamous cell neoplasms: 0/60, 3/45, 9/75, 12/60
Zymbal's gland neoplasms: 1/59, 3/45, 32/75, 36/59	Zymbal's gland neoplasms: 0/57, 6/44, 32/73, 42/60
Preputial gland neoplasms: 2/60, 4/45, 6/75, 9/60	Clitoral gland neoplasms: 0/60, 14/45, 42/75, 32/59
Liver neoplasms: 0/60, 0/45, 35/75, 33/60	Liver neoplasms: 0/60, 0/45, 7/74, 4/60
Oral cavity neoplasms: 0/60, 0/45, 4/75, 5/60	Oral cavity neoplasms: 0/60, 3/45, 9/75, 13/60
Small intestine neoplasms: 0/60, 0/45, 4/75, 8/60	Small intestine neoplasms: 0/60, 1/45, 3/75, 5/60
Large intestine neoplasms: 0/60, 0/45, 6/75, 15/60	Large intestine neoplasms: 0/60, 1/45, 7/75, 4/60
	Mammary gland adenocarcinoma: 0/60, 1/45, 3/75, 6/60
Lung neoplasms: 1/60, 0/45, 8/75, 6/60	Lung neoplasms: 1/60, 1/45, 3/74, 4/60
Mesothelioma: 0/60, 0/45, 3/75, 4/60	
	Brain neoplasms: 0/60, 2/45, 2/75, 1/60
Uncertain findings	Mononuclear cell leukemia: 1/60, 3/45, 6/75, 4/60
Brain neoplasms: 0/60, 0/45, 1/75, 2/60	
Level of evidence of carcinogenic activity	
Clear evidence	Clear evidence

Genetic toxicology

Salmonella typhimurium	
Gene mutation:	Positive with S9 in strain TA98; Negative with or without S9 in strains TA100, TA1535, or TA97
Sister chromatid exchanges	
Chinese hamster ovary cells *in vitro*:	Positive without S9
Chromosomal aberrations	
Chinese hamster overy cells *in vitro*:	Positive without S9
Sex-linked recessive lethal mutations	
Drosophila melanogaster in vitro:	Positive administered by injection or in feed
Reciprocal translocations	
Drosophila melanogaster in vitro:	Negative administered in feed

[a] Reduced survival in exposed groups was due to neoplasia.

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 because of major flaws cannot be evaluated (**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 quintet 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 describes 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 describes studies that are interpreted as showing a chemically 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 describes studies that are interpreted as showing a marginal increase of neoplasms that may be chemically related.

- **No Evidence** of carcinogenic activity describes studies that are interpreted as showing no chemically related increases in malignant or benign neoplasms.

- **Inadequate Study** of carcinogenic activity describes 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 is selected for a particular experiment, consideration must be given to key factors that would extend the actual boundary of an individual category of evidence. This 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 incidences 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.

PEER REVIEW PANEL

The members of the Peer Review Panel who evaluated the draft Technical Report on 3,3′-dimethylbenzidine dihydrochloride on April 25, 1990, are listed below. Panel members serve as independent scientists, not as representatives of any institution, company, or governmental agency. In this capacity, Panel members have five major responsibilities:

- 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.

National Toxicology Program Board of Scientific Counselors Technical Reports Review Subcommittee

Michael A. Gallo, Ph.D., Chairperson
Department of Environmental and Community Medicine
UMDNJ–Rutgers Medical School
Piscataway, New Jersey

Daniel S. Longnecker, M.D.
Department of Pathology
Dartmouth Medical School
Hanover, New Hampshire

Ellen K. Silbergeld, Ph.D.
University of Maryland Medical School
Baltimore, Maryland
Environmental Defense Fund
Washington, DC

Ad Hoc Subcommittee Panel of Experts

John Ashby, Ph.D.
Central Toxicology Laboratory
Imperial Chemical Industries, PLC
Alderly Park, England

Gary P. Carlson, Ph.D.
Department of Pharmacology and Toxicology
Purdue University
West Lafayette, Indiana

Harold Davis, D.V.M., Ph.D., Principal Reviewer
School of Aerospace Medicine
Brooks Air Force Base, Texas

Robert H. Garman, D.V.M.
Consultants in Veterinary Pathology
Post Office Box 68
Murrysville, Pennsylvania

Lois Swirsky Gold, Ph.D.
Lawrence Berkeley Laboratory
University of California
Berkeley, California

David W. Hayden, D.V.M., Ph.D.
Department of Veterinary Pathobiology
College of Veterinary Medicine
University of Minnesota
St. Paul, Minnesota

Curtis D. Klaassen, Ph.D.*
Department of Pharmacology and Toxicology
University of Kansas Medical Center
Kansas City, Kansas

Barbara McKnight, Ph.D., Principal Reviewer
Department of Biostatistics
University of Washington SC-32
Seattle, Washington

Lauren Zeise, Ph.D., Principal Reviewer
California Dept. of Health Services/RCHAS
Berkeley, California

* Unable to attend

SUMMARY OF PEER REVIEW COMMENTS

On April 25, 1990, the draft Technical Report on the toxicology and carcinogenesis studies of 3,3′-dimethylbenzidine dihydrochloride received public review by the National Toxicology Program Board of Scientific Counselors' Technical Reports Review Subcommittee and associated Panel of Experts. The review meeting was held at the National Institute of Environmental Health Sciences, Research Triangle Park, NC.

Dr. D. L. Morgan, NIEHS, began the discussion by reviewing the experimental design, results, and proposed conclusions (*clear evidence of carcinogenic activity* for male and female rats). Dr. Morgan explained that the studies were intended to last 24 months but were terminated after 14 months because of rapidly declining survival of exposed animals, due primarily to neoplasia.

Dr. McKnight, a principal reviewer, agreed with the conclusions.

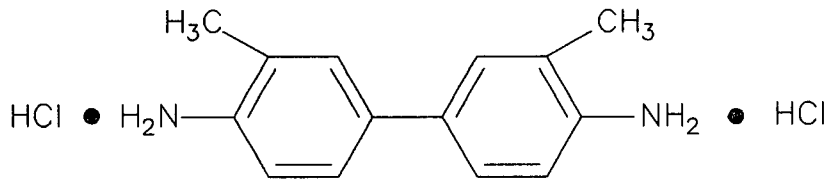
Dr. Zeise, the second principal reviewer, agreed with the conclusions with the exceptions that she felt (1) the marginally increased incidences of benign pheochromocytomas of the adrenal gland medulla in male rats may have been treatment-related, and (2) the marginally increased incidences of mononuclear cell leukemias in female rats may have been treatment-related. Dr. Morgan said pheochromocytomas were commonly occurring tumors in male rats and there was not an increased incidence of hyperplasias. With regard to leukemia, he noted that the study was terminated at 14 months and most leukemias develop after this time. Thus, the rats were not at risk long enough to determine if leukemia was treatment related. Dr. Zeise thought that liver neoplasia in the rat should be reported according to the current classification system, whereby the diagnosis of "neoplastic nodule" is given as either "hepatocellular adenoma" or "hyperplasia." Dr. Morgan explained that "neoplastic nodule" was the accepted terminology when the slides for these liver lesions were read.

Dr. Davis, the third principal reviewer, agreed with the conclusions.

Dr. William Allaben, National Center for Toxicologic Research (NCTR), reported on the 2-year studies of 3,3′-dimethylbenzidine dihydrochloride administrered to BALB/c mice at dose levels ranging from 5 to 140 ppm in drinking water. The only lesions of consequence in these studies were fatal alveolar cell tumors of the lung seen in a dose-related manner in male mice.

Dr. McKnight moved that the Technical Report on 3,3′-dimethylbenzidine dihydrochloride be accepted with the conclusions as written for male and female rats, *clear evidence of carcinogenic activity*. Dr. Davis seconded the motion, which was accepted unanimously with ten votes. Dr. Zeise then moved that mononuclear cell leukemia be added to the conclusion for female rats as "may have been related to chemical administration." Dr. McKnight seconded the motion, which was accepted by nine yes votes to one no vote (Dr. Gold).

INTRODUCTION



3,3′-DIMETHYLBENZIDINE DIHYDROCHLORIDE

CAS No. 612-82-8

$C_{14}H_{16}N_2 \cdot 2HCl$ Molecular Weight 285.2

Synonyms: *o*-Tolidine dihydrochloride; 3,3′-Dimethylbiphenyl-4,4′-diamine dihydrochloride; 3,3′-Dimethylbiphenyl-4,4′-biphenyldiamine dihydrochloride; 4,4′-Diamino-3,3′-dimethylbiphenyl dihydrochloride

USE AND PRODUCTION

3,3′-Dimethylbenzidine dihydrochloride is a yellow crystalline powder that is slightly soluble in water and very soluble in ethanol, ethyl ether, and dilute acids. It is used principally as an intermediate in the production of commercial bisazobiphenyl dyes for coloring textiles, paper, plastic, rubber, and leather. The amine groups of 3,3′-dimethylbenzidine are chemically linked with other aromatic amines in the synthesis of the bisazobiphenyl dyes. The National Institute of Occupational Safety and Health (NIOSH) lists approximately 480 dyes based on 3,3′-dimethylbenzidine, 96 of which were produced in 1981 (NIOSH, 1983). 3,3′-Dimethylbenzidine is also used as a laboratory reagent for the detection of blood and for the colorimetric determination of chlorine in air and water (IARC, 1972).

3,3′-Dimethylbenzidine is manufactured by reducing *o*-nitrotoluene to hydrazotoluene with alkali and then reacting the hydrazotoluene with hydrochloric acid to yield 3,3′-dimethylbenzidine and other by-products (*Kirk-Othmer*, 1978). The production and use of benzidine congeners and benzidine-derived dyes decreased in the United States after reports that benzidine was carcinogenic. According to the Ecological and Toxicological Association of the Dyestuffs Manufacturing Industry, the benzidine congeners currently used in the United States as intermediates are imported (personal communication from T. Helmes to D. Morgan, 1989). No recent United States production data for 3,3′-dimethylbenzidine were found; however, available import data show that approximately 34,200 kg of 3,3′-dimethylbenzidine came through principal U.S. customs districts in 1984 (USITC, 1984).

EXPOSURE

Exposure to 3,3′-dimethylbenzidine may occur by inhalation, ingestion, and skin absorption (Meigs *et al.*, 1951, 1954; El-hawari *et al.*, 1979). Occupational exposure to 3,3′-dimethylbenzidine may occur during the manufacture of dyes of which 3,3′-dimethylbenzidine is a chemical intermediate or during handling of the finished 3,3′-dimethylbenzidine-based dyes, where residual amounts of 3,3′-dimethylbenzidine may be present due to incomplete dye synthesis. There is also evidence to suggest that 3,3′-dimethylbenzidine-based dyes are metabolized back to the parent compound *in vivo*, resulting in exposure to 3,3′-dimethylbenzidine. Exposure of workers to 3,3′-dimethylbenzidine may also occur in clinical and analytical chemistry laboratories when 3,3′-dimethylbenzidine is used for the detection of blood or in the quantitation of chlorine in water and glucose by the glucose oxidase method (IARC, 1972; Collier, 1974).

Approximately 1,000 workers are exposed to benzidine, benzidine congeners, and benzidine-derived dyes in dye manufacturing, and approximately 10,000 workers in the various application industries (DETO, 1980). Since many benzidine compounds may exist simultaneously within the same industry, it is difficult to estimate the numbers of exposed workers and extent of exposure to 3,3′-dimethylbenzidine alone. A recent survey estimates there is a potential for exposure to 3,3′-dimethylbenzidine for approximately 10,000 U.S. employees (NIOSH, 1989).

Nonoccupational exposure to 3,3′-dimethylbenzidine-based dyes may occur through contact with materials colored with these dyes or through the use of packaged dyes and paints containing 3,3′-dimethylbenzidine. No estimates of consumer exposure to 3,3′-dimethylbenzidine alone could be found.

METABOLISM

Reductive metabolism of 3,3′-dimethylbenzidine-based dyes produces 3,3′-dimethylbenzidine (Figure 1). Azo reduction can occur either in the liver, via the hepatic enzymes, or in the gut, by the action of azo reductase associated with intestinal bacterial flora. Because highly polar compounds are absorbed from the gut with difficulty, mammals are not expected to absorb the water-soluble sulfonated dyes (Walker, 1970). Thus, reductive cleavage of benzidine-congener azo dyes is thought to occur primarily by bacterial action in the intestinal tract (Martin and Kenelly, 1981; Cerniglia *et al.*, 1982; Brown and Dietrich, 1983; Bos *et al.*, 1984, 1986). Following reductive cleavage, the less polar metabolites are subject to intestinal absorption and further metabolism by the liver.

3,3′-Dimethylbenzidine-based dyes are metabolized to 3,3′-dimethylbenzidine in dogs and rats (Lynn *et al.*, 1980) and also in humans (Boeniger, 1980). Following exposure to 3,3′-dimethylbenzidine-based dyes, 3,3′-dimethylbenzidine was detected in the urine of dogs and rats at levels greater than the amount that could be accounted for by contamination of the dyes with 3,3′-dimethylbenzidine (Lynn *et al.*, 1980). Dogs metabolized the dyes Direct Blue 25 and Acid Red 114 to 3,3′-dimethylbenzidine and excreted it in urine. Rats metabolized Direct Blue 25 to 3,3′-dimethylbenzidine and N-acetyl-3,3′-dimethylbenzidine, with urine concentrations of 3,3′-dimethylbenzidine comparable to those observed for dogs. However, rats given Acid Red 114 excreted only trace amounts of 3,3′-dimethylbenzidine in urine. Neither dogs nor rats excreted measurable amounts of 3,3′-dimethylbenzidine in the urine after administration of Direct Red 2 or Direct Red 39.

Boeniger (1980) reported the presence of 3,3′-dimethylbenzidine in the urine of two employees working in a dye manufacturing plant. The workers were in contact with 3,3′-dimethylbenzidine-based dyes, but not with 3,3′-dimethylbenzidine itself. The presence of 3,3′-dimethylbenzidine in the urine may have resulted from metabolism of the dyes or from exposure to dyes contaminated with 3,3′-dimethylbenzidine. Hartman *et al.* (1978) found that a cell-free extract of *Fusobacterium*, a human intestinal anaerobe, reduced Trypan Blue (C.I. Direct Blue 14), a 3,3′-dimethylbenzidine-derived dye, to 3,3′-dimethylbenzidine.

Tanaka *et al.* (1982) reported that urine extracts from rats treated with 3,3′-dimethylbenzidine or Evans Blue, a 3,3′-dimethylbenzidine-derived dye, contained N-acetyl-3,3′-dimethylbenzidine and N,N′-diacetyl-dimethylbenzidine, as well as 3,3′-dimethylbenzidine. Urine extracts containing metabolites were more mutagenic than those containing 3,3′-dimethylbenzidine. Although Evans Blue was not mutagenic, urine extracts from rats exposed to Evans Blue were mutagenic.

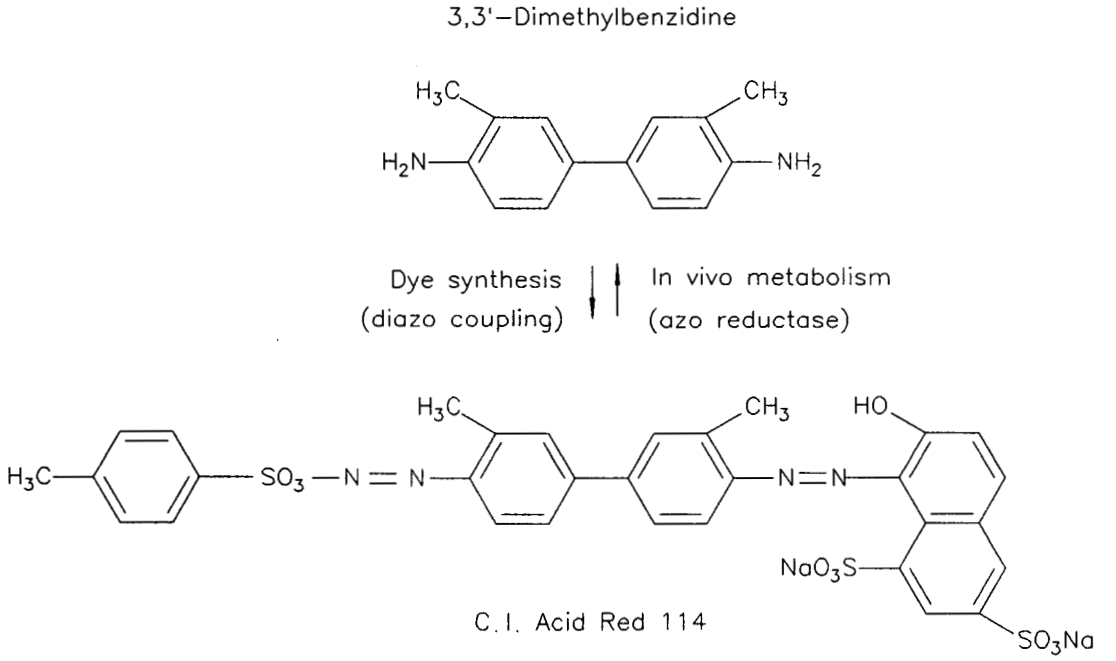


FIGURE 1
Formation of 3,3'-Dimethylbenzidine by Reductive Metabolism of C.I. Acid Red 114

GENETIC TOXICOLOGY

The only available mutagenicity information on the dihydrochloride salt of 3,3'-dimethylbenzidine is included in the NTP test data in this report. Induction of frameshift-type gene mutations occurred in *Salmonella* strain TA98 in the presence of S9 metabolic activation (Zeiger *et al.*, 1988; Table C1). Induction of sister chromatid exchanges (SCE) and chromosome aberrations occurred in cultured Chinese hamster ovary (CHO) cells without S9 metabolic activation (Tables C2 and C3). Induction of sex-linked recessive lethal mutations occurred in germ cells of male *Drosophila* fed or injected with the chemical; however, induction of reciprocal translocations did not occur (Valencia *et al.*, 1985; Tables C4 and C5).

3,3'-Dimethylbenzidine is genotoxic in bacterial and eukaryotic systems. 3,3'-Dimethylbenzidine induced gene mutations in frameshift-sensitive *Salmonella* strains TA98 and TA1538 only in the presence of S9 metabolic activation (Shimizu and Takemura, 1976; Hartman *et al.*, 1978; Martin and Kennelly, 1981; Waalkens *et al.*, 1981; Haworth *et al.*, 1983; Reid *et al.*, 1984a). Two metabolites of 3,3'-dimethylbenzidine, N,N'-diacetyl-3,3'-dimethylbenzidine and N-acetyl-3,3'-dimethylbenzidine, were both positive in *Salmonella* strains TA98, TA100, and TA1538 in the presence of S9 metabolic activation (Tanaka *et al.*, 1982; Kennelly *et al.*, 1984; Reid *et al.*, 1984a). 3,3'-Dimethylbenzidine induced trifluorothymidine resistance in mouse L5178Y lymphoma cells with and without S9 metabolic activation (Mitchell *et al.*, 1988; Myhr and Caspary, 1988). 3,3'-Dimethylbenzidine also gave positive results in *in vitro* mammalian cell assays for the induction of unscheduled DNA synthesis (UDS) (Martin *et al.*, 1978), DNA repair (Kornbrust and Barfknecht, 1984), SCE (Waalkens *et al.*, 1981; Galloway *et al.*, 1987), and chromosomal aberrations (Galloway *et al.*, 1987). The UDS and DNA repair assays were both conducted with S9 metabolic activation. The cytogenetic tests were performed with and without metabolic activation, and positive results were

obtained under both conditions. Another report cites the induction of micronuclei in bone-marrow polychromatic erythrocytes in male Wistar rats given 3,3′-dimethylbenzidine by gavage (Cihak, 1979).

Mutagenicity data for closely related structural analogs of 3,3′-dimethylbenzidine are consistent with the positive results reported above. NTP Technical Report No. 372 (NTP, 1990a) presents a detailed review of the test results for 3,3′-dimethoxybenzidine. This compound tested positive for mutagenic toxicity in *Salmonella* strains TA98, TA100, and TA1535 and induced SCE and chromosomal aberrations in CHO cells, but did not induce sex-linked recessive lethal mutations in adult male *Drosophila.*

Benzidine, the parent compound in this series of substituted biphenyls, induced gene mutations in *Salmonella* strains TA98, TA100, and TA1538 in the presence of S9 metabolic activation (Ames *et al.*, 1973; Shimizu and Takemura, 1976; Anderson and Styles, 1978; Baker and Bonin, 1981; Probst *et al.*, 1981; Haworth *et al.*, 1983; Reid *et al.*, 1984b). Benzidine also induced gene mutations in some strains of *Escherchia coli* in the presence of S9 metabolic activation (Matsushima *et al.*, 1981; Mohn *et al.*, 1981; Venitt and Crofton-Sleigh, 1981). Benzidine and/or its dihydrochloride salt also gave positive results in a variety of *in vitro* eukaryotic genotoxicity assays. It induced mitotic aneuploidy (Parry and Sharp, 1981) and gene conversion (Zimmermann and Scheel, 1981; Sharp and Parry, 1981) in *Saccharomyces*, UDS in mouse primary hepatocyte cultures (Williams, 1978; Probst *et al.*, 1981; Althaus *et al.*, 1982), and gene mutation in mouse L5178Y lymphoma cells (Jotz and Mitchell, 1981; Mitchell *et al.*, 1988; Myhr and Caspary, 1988). Benzidine also induced SCE and chromosomal aberrations in CHO cells (Natarajan and van Kesteren-van Leeuwen, 1981; Galloway *et al.*, 1987) and human lymphoblastoid cells (Tohda *et al.*, 1980). The *in vivo* administration of benzidine induced UDS in rat hepatocytes (Mirsalis *et al.*, 1982) and micronuclei (Salamone *et al.*, 1981; NTP, unpublished), SCE (Parodi *et al.*, 1983; NTP, unpublished), and chromosomal aberrations (NTP, unpublished) in mouse bone marrow cells.

TOXICITY AND CARCINOGENICITY STUDIES

The National Institute of Occupational Safety and Health (NIOSH) and the Occupational Safety and Health Administration (OSHA) issued a health hazard alert in 1980 stating that persons working with 3,3′-dimethylbenzidine-, benzidine-, and 3,3′-dimethoxybenzidine-based dyes should be aware of the potential health hazards associated with excess exposure (Boeniger, 1980). In a later report issued to alert workers to the hazards of benzidine-congener dyes, NIOSH stated that exposure to 3,3′-dimethylbenzidine-based dyes in the workplace may pose a carcinogenic risk (NIOSH, 1983). These health alerts were based on evidence from animal studies indicating that 3,3′-dimethylbenzidine is carcinogenic and on preliminary evidence indicating metabolic conversion of the 3,3′-dimethylbenzidine-based dyes to the parent compound, 3,3′-dimethylbenzidine. These early carcinogenicity studies of 3,3′-dimethylbenzidine have been criticized for their small numbers of study animals, lack of concurrent controls, use of toxic doses, and use of parenteral routes of chemical administration (Haley, 1975; DETO, 1980).

Spitz *et al.* (1950) treated Sherman rats with weekly subcutaneous doses of 60 mg 3,3′-dimethylbenzidine in olive oil. Treatment-related mortality was high, with 43 of 105 animals dying before day 200 and only 48 animals surviving longer than 300 days. The only significant lesions observed were tumors of the auditory canal, probably Zymbal's gland tumors, in five rats; the first of these tumors was observed on day 354. No auditory canal tumors were among the 56 tumors observed in 578 untreated rats of the same colony. This investigation lacked a concurrent control group.

Pliss (1963) gave rats weekly subcutaneous injections of 20 mg 3,3′-dimethylbenzidine for 13 months. An unspecified number of animals died during the first 2 weeks of the study, and the number of animals surviving the treatment was not indicated. A variety of tumors appeared, primarily between months 14 and 22, with Zymbal's gland tumors occurring most frequently. Lesions also appeared in the liver, mammary gland, gastrointestinal tract, and skin.

Griswold *et al.* (1968) treated 20 female Sprague-Dawley rats with a suspension of 3,3′-dimethylbenzidine in sesame oil. The total dose of 500 mg per rat was divided into 10 equal doses given by stomach tube at 3-day intervals. Animals were observed for 9 months. Mammary gland tumors developed in 3/16 survivors, and, among 132 vehicle control rats, five had a total of three carcinomas, one fibroadenoma, and five hyperplasias of the mammary gland. Neoplasms were not seen at other sites.

No tumors were observed in a lifespan study on groups of 30 male and 30 female hamsters given 3,3′-dimethylbenzidine at 0.1% or 0.3% (the highest tolerated level) in the diet (Saffiotti *et al.*, 1967; Sellakumar *et al.*, 1969).

Pliss and Zabezhinsky (1970) gave 27 male and 26 female rats a 4% suspension of 3,3′-dimethylbenzidine (20 mg per rat) in 0.5 mL sunflower oil by subcutaneous injection once weekly for 13 months. Fifty rats survived to 8 months, after which time the first tumor was observed. Thirty rats developed a total of 41 tumors, including 20 Zymbal's gland carcinomas; neoplasms of the mammary gland, preputial gland, forestomach, skin, lung, liver, thyroid, and uterus were also seen. Tumor production at distant body sites after subcutaneous injection is considered a reliable indication of carcinogenicity (IARC, 1986).

In the same study, two groups of rats were subcutaneously implanted with pellets containing 20 mg 3,3′-dimethylbenzidine and 10 mg glycerol. Pellets implanted in the first group (20 per sex) were not ultraviolet irradiated. The pellets implanted in the second group (24 per sex) were ultraviolet irradiated before implantation to investigate the effects of oxidation on the carcinogenicity of 3,3′-dimethylbenzidine. The differences between the two groups were minimal. From a total of 68 rats alive at the first tumor observation (11-12 months), 48 rats developed a total of 60 tumors. Twenty-seven of these were Zymbal's gland carcinomas, with neoplasms of the mammary gland, skin, liver, and hematopoietic system accounting for the remainder. Although control groups were not monitored during these experiments, a preliminary report of these studies states that rats from the same colony did not develop tumors of the Zymbal's gland (Pliss, 1965).

In more recent studies, the National Center for Toxicological Research (NCTR), as part of the Benzidine Dye Initiative, exposed BALB/c mice (120 per sex per group) to 0, 5, 9, 18, 35, 70, or 140 ppm 3,3′-dimethylbenzidine dihydrochloride in drinking water (Schieferstein *et al.*, 1989, 1990). Groups of mice of each sex were killed after 3, 6, 9, 12, 15, or 24 months of exposure. There were no treatment-related effects on body weights or on water or food consumption. Treatment-related increases in the incidence of fatal lung alveolar cell bronchial adenomas, bronchial carcinomas, and combinations of these were observed in males only. Nonfatal lung tumors did not show a significant dose-related trend. Fatal lung tumors appeared around 78 weeks in mice exposed to 140 ppm 3,3′-dimethylbenzidine; a treatment-related decrease in survival resulting from fatal lung neoplasms was also noted.

TOXICITY/CARCINOGENICITY OF RELATED COMPOUNDS

Benzidine

3,3′-Dimethylbenzidine is a congener of benzidine, a known carcinogen for humans (Scott, 1952; Case *et al.*, 1954; IARC, 1972; Zavon *et al.*, 1973), rats (Spitz *et al.*, 1950; Griswold *et al.*, 1968), hamsters (Saffiotti *et al.*, 1966), and mice (Bonser *et al.*, 1956; Prokofjeva, 1971; IARC, 1972; Frith and Dooley, 1976; Littlefield *et al.*, 1983). Occupational exposure to benzidine for up to 30 years resulted in bladder tumors in as many as 90% of workers (Scott, 1952). Exposure to benzidine may occur directly or by reductive metabolism of benzidine-based dyes. Several reviews address the carcinogenicity of benzidine extensively (IARC, 1972; Haley, 1975; USEPA, 1980; IARC, 1982).

Benzidine exposure caused bladder tumors in dogs (Spitz *et al.*, 1950); hepatocellular, harderian gland, and lymphoreticular tumors in mice (Bonser *et al.*, 1956; Vesselinovitch *et al.*, 1975; Frith and Dooley, 1976); Zymbal's gland, hepatic, and mammary gland carcinomas in rats (Spitz *et al.*, 1950; Griswold *et al.*, 1968); and hepatocellular carcinomas, adenomas, and cholangiomas in hamsters (Saffiotti *et al.*, 1967). Animal survival was poor in many of the carcinogenicity studies of benzidine. Although this was due, in most cases, to the administration of toxic

doses, these studies do assert that benzidine is carcinogenic in laboratory animals.

o-Toluidine

o-Toluidine hydrochloride (2-aminotoluene) is structurally analogous to one-half the 3,3′-dimethylbenzidine molecule. In studies performed by the National Cancer Institute, *o*-toluidine hydrochloride was given to groups of 50 F344/N rats and 50 B6C3F$_1$ mice of each sex in feed at dose levels of 3,000 or 6,000 ppm for rats and 1,000 or 3,000 ppm for mice for 101 to 104 weeks (NCI, 1979a). Twenty untreated animals of each sex and species were used as controls.

Exposure of rats to *o*-toluidine hydrochloride resulted in sarcomas of the spleen and other organs in both males and females. *o*-Toluidine hydrochloride induced mesotheliomas of the abdominal cavity and scrotum in males and transitional-cell carcinomas of the urinary bladder in females. Administration of *o*-toluidine also resulted in increased incidences of fibromas of the subcutaneous tissue in males and fibroadenomas or adenomas of the mammary gland in females. In mice, hemangiosarcomas occurred at various sites in males, and hepatocellular carcinomas or adenomas of the mammary gland occurred in females.

3,3′-Dimethoxybenzidine

The Benzidine Dye Initiative included the evaluation of 3,3′-dimethoxybenzidine dihydrochloride for carcinogenicity (NTP, 1990a). F344/N rats of each sex received 3,3′-dimethoxybenzidine dihydrochloride in drinking water at either 80, 170, or 330 ppm for 21 months. These studies used 50 untreated animals of each sex as controls. After 9 months, neoplastic effects attributed to 3,3′-dimethoxybenzidine dihydrochloride exposure were noted. After exposure for up to 21 months, neoplasms were observed at many sites, including the skin, Zymbal's gland, preputial and clitoral glands, oral cavity, small and large intestine, liver, brain, mesothelium, mammary gland, and uterus and cervix.

Pliss (1963, 1965) treated rats with 30 mg 3,3′-dimethoxybenzidine three times per week via sunflower oil gavage. Because of poor survival, the dose level was reduced to 15 mg after 3 weeks and continued at that level for 13 months. Of the 42 rats starting the study, 18 survived for 14 months. Two survivors exhibited tumors of the Zymbal's gland, and one, an ovarian tumor. None of the 50 control rats developed tumors at the same sites as the exposed rats.

In a lifespan study, Saffiotti *et al.* (1967) treated groups of 30 golden hamsters per sex with 1,000 ppm (0.1% w/w) 3,3′-dimethoxybenzidine in the diet. A small transitional-cell carcinoma of the urinary bladder was found in one animal after 144 weeks of exposure. This tumor is rare in hamsters and was attributed to treatment with 3,3′-dimethoxybenzidine. The same investigators conducted a similar study on hamsters that used a higher dose of 3,3′-dimethoxybenzidine (1.0%) (Sellakumar *et al.*, 1969). Forestomach papillomas occurred in 57% of the treated animals and in only 2% of the controls. No bladder lesions were detected at these dietary concentrations. This publication is an abstract and does not detail the experimental design or survival data.

In a gavage study, Hadidian *et al.* (1968) gave 30 male and 30 female Fischer rats 0.1, 0.3, 1.0, 3.0, 10, or 30 mg 3,3′-dimethoxybenzidine per animal per day, 5 days per week. The vehicle was a proprietary mixture composed of NaCl, carboxymethylcellulose, polysorbate 80, and benzyl alcohol in water. Animals received these dose formulations for 52 weeks, after which they were observed for 6 months and necropsied. Tumors occurred as early as 293 days, but most were detected at necropsy. The variety of tumors reported at necropsy included neoplastic lesions of the urinary bladder (two papillomas), mammary gland (three carcinomas, two fibroadenomas), skin (five carcinomas), intestinal tract (two carcinomas), and Zymbal's gland (eight carcinomas). Tumor incidences were significantly increased over those observed for 360 pooled vehicle and nonvehicle control rats.

o-Anisidine

o-Anisidine (2-methoxyaniline) is structurally analogous to one-half the 3,3′-dimethoxybenzidine molecule. *o*-Anisidine is used in the manufacture of monoazo dyes by diazotization and coupling with other aromatic amines (Noller, 1965). The National Cancer Institute (NCI, 1978a) found that *o*-anisidine was carcinogenic to F344/N rats and B6C3F$_1$ mice. Groups of 55 rats of each sex received *o*-anisidine in feed at 5,000 or 10,000 ppm for 103 weeks; similar

groups of mice received 2,500 or 5,000 ppm. Fifty-five untreated animals of each sex and species were used as controls.

Treatment with *o*-anisidine resulted in transitional-cell carcinomas or papillomas of the bladder in both sexes of each species. Male rats also exhibited transitional-cell carcinomas of the renal pelvis and follicular-cell tumors of the thyroid tissue. Only one urinary system tumor was observed in the control groups of rats or mice, a transitional cell papilloma of the renal pelvis in a male mouse.

Study Rationale

Benzidine is a known carcinogen (IARC, 1972; 1987) and 3,3′-dimethylbenzidine, a benzidine congener, is possibly carcinogenic for humans (IARC, 1987). Numerous benzidine and ben- zidine congener-based dyes are metabolized to these parent amines *in vivo* (Rinde and Troll, 1975; Lynn *et al.*, 1980). Thus, all benzidine- and benzidine congener-derived dyes are logical candidates for carcinogenicity testing.

The National Toxicology Program's (NTP) Benzidine Dye Initiative is a collaborative effort of NIEHS, NCTR, NIOSH, the U.S. Environmental Protection Agency, the Consumer Product Safety Commission, and OSHA under the aegis of the NTP. The objective of this Initiative is to develop an integrated body of scientific data concerning the metabolism, pharmacokinetics, genetic toxicology, and *in vivo* carcinogenicity of dyes derived from benzidine, 3,3′-dimethylbenzidine, and 3,3′-dimethoxybenzidine (Table 1). Because studying each of the hundreds of benzidine-based dyes was considered impractical, the research program was designed to evaluate representative benzidine congeners, benzidine congener-derived dyes, and benzidine-derived dyes.

Table 1
Summary of the National Toxicology Program Benzidine Congener Initiative

Class/Chemical	Tests[a]
3,3′-Dimethylbenzidine (*o*-tolidine)	
o-Tolidine	G, P, B
C.I. Direct Red 2	G, M
C.I. Direct Red 39	G, M
C.I. Acid Red 114	G, P, B
C.I. Direct Blue 25	G
C.I. Direct Blue 53	G, M
C.I. Direct Blue 14	G
C.I. Direct Orange 6	G, M
3,3′-Dimethoxybenzidine (*o*-dianisidine)	
o-Dianisidine	G, P, B
C.I. Direct Blue 15	G, P, B
C.I. Direct Blue 218	G, P, B
C.I. Direct Black 114	G, M
C.I. Direct Yellow 68	G, M
C.I. Direct Blue 8	G, M

[a] G=genetic toxicology; P=pharmacokinetic studies; M=metabolism studies for detection of carcinogens in urine; B=toxicology and carcinogenicity studies.

The agencies collaborating in the Benzidine Dye Initiative selected 3,3′-dimethylbenzidine for study to allow comparison of its toxic and carcinogenic effects with those of related chemicals studied simultaneously with comparable doses and study designs. 3,3′-Dimethylbenzidine was also studied to strengthen the evidence for its carcinogenicity. Although results of earlier studies suggest that 3,3′-dimethylbenzidine is carcinogenic (Griswold *et al.*, 1968; Hadidian *et al.*, 1968; Pliss and Zabezhinsky, 1970), these studies were criticized for their use of small numbers of study animals, lack of concurrent controls, use of toxic doses, and use of parenteral routes of chemical administration (Haley, 1975; DETO,1980).

3,3′-Dimethylbenzidine dihydrochloride is one of five benzidine congeners or benzidine congener-derived dyes selected for evaluation in the 2-year carcinogenicity studies as part of the Benzidine Dye Initiative. The other chemicals studied are 3,3′-dimethoxybenzidine dihydrochloride (a related benzidine congener), C.I. Direct Blue 15 and C.I. Direct Blue 218 (representative 3,3′-dimethoxybenzidine-based dyes), and C.I. Acid Red 114 (a representative dimethylbenzidine-based dye). The oral route of administration was selected for these studies to maximize the chances of detecting systemic effects associated with chemical administration. These studies used the same design. The instability of 3,3′-dimethylbenzidine dihydrochloride and 3,3′-dimethoxybenzidine dihydrochloride in feed required administration of these chemicals in drinking water. Because long-term studies of 3,3′-dimethylbenzidine dihydrochloride and 3,3′-dimethoxybenzidine dihydrochloride were being conducted on mice at NCTR, the NTP studies of these chemicals used only rats.

MATERIALS AND METHODS

PROCUREMENT AND CHARACTERIZATION OF 3,3′-DIMETHYLBENZIDINE DIHYDROCHLORIDE

3,3′-Dimethylbenzidine dihydrochloride was obtained from the Taylor Chemical Company in two lots. Lot number T122380 was used in both the 14-day and 13-week studies, and lot number IP22 was used in the 14-month studies. Purity and identity analyses were conducted at the Midwest Research Institute, Kansas City, MO (Appendix F). The study chemical in both lots was identified as 3,3′-dimethylbenzidine dihydrochloride by infrared, ultraviolet/visible, and nuclear magnetic resonance spectroscopy. The purity of both lots was determined to be 99% by elemental analysis, Karl Fischer water analysis, titrations (non-aqueous amine and neutralization titrations), thin-layer chromatography, and high-performance liquid chromatography (HPLC). Comparison of the two lots by HPLC showed no significant purity differences. The test laboratory confirmed the chemical identity by infrared spectroscopy, and the stability, by HPLC and non-aqueous amine titration. No degradation of the study material was detected by these analytical methods.

PREPARATION AND ANALYSIS OF DOSE FORMULATIONS

Initially, attempts were made to formulate 3,3′-dimethylbenzidine dihydrochloride in feed. The 2-week stability of NIH-07 Rat and Mouse Ration formulated with 675 ppm 3,3′-dimethylbenzidine dihydrochloride was determined at storage temperatures ranging from -20° C to room temperature. Results showed that feed formulations were unstable when stored at or above 5° C. Formulated diets stored open to air and light and under simulated animal room conditions lost 18% or 21% of the chemical after 3 or 7 days, respectively. The same formulations stored in the dark in sealed containers at room temperature, 5° C, or -20° C lost 23%, 15%, or 5% of the chemical following storage for 2 weeks.

Drinking water was then investigated as a vehicle for chemical administration. Tests showed that solutions of 675 ppm 3,3′-dimethylbenzidine dihydrochloride in water remained stable for at least 14 days when stored at either room temperature or 5° C. Solutions were also stable for up to 48 hours under simulated dosing conditions, including exposure to normal room light.

Tap water was used for the preparation of dose formulations during the 14-day studies, and distilled water was used during the 13-week and 14-month studies. Dose formulations were prepared twice weekly and made available to the study animals within 7 days of mixing. The preparation and storage procedures for dosed drinking water in the studies of 3,3′-dimethylbenzidine dihydrochloride are presented in Table F1.

The study laboratory analyzed the formulations used for dosing by ultraviolet spectroscopy at least once every 4 weeks during the 14-month studies. Based on the number of times the dose formulations were determined to be within ±10% of the target concentration, it is estimated that 80% of the formulations were prepared within specifications (Table F3). Results of periodic referee analyses by the analytical chemistry laboratory agreed with the results of the study laboratory (Table F4).

14-DAY STUDIES

Male and female F344/N rats were obtained from Frederick Cancer Research Facility (Frederick, MD) and observed for 13 days before the studies began. The rats were 48 days old when placed on study. Groups of five rats of each sex received 0, 600, 1,250, 2,500, 5,000, or 7,500 ppm 3,3′-dimethylbenzidine dihydrochloride in drinking water for 14 consecutive days. Animals were housed five per cage, and water and feed were available *ad libitum*. Animals were observed twice daily. Clinical observation of the animals was conducted daily. The animals were weighed at the start of the study and

on days 7 and 14. Feed consumption was measured once weekly, and water consumption was measured twice weekly. Complete necropsies were performed on all animals. The following organs were weighed: brain, heart, kidney (right), liver, lung, testicle (right), and thymus. Complete histopathologic examinations were performed on all control animals, males receiving 5,000 and females receiving 7,500 ppm. Selected tissues were examined for the other dose groups. Further details are presented in Table 2.

13-WEEK STUDIES

The 13-week studies were conducted to evaluate cumulative toxic effects of repeated exposure to 3,3′-dimethylbenzidine dihydrochloride and to determine the chemical concentrations to be used in the 2-year studies.

Fischer 344/N rats were obtained from Frederick Cancer Research Facility, observed for 16 days, distributed to weight classes, and assigned to dose groups. The rats had a median age of 55 days when placed on study. Groups of ten rats of each sex received 0, 300, 500, 1,000, 2,000, or 4,000 ppm 3,3′-dimethylbenzidine dihydrochloride in drinking water for 13 weeks. Rats were housed five per cage, and water and feed were available *ad libitum*. Animals were observed twice daily, and clinical observations were recorded weekly. Feed and water consumption were recorded by cage once weekly and twice weekly, respectively. Animals were weighed at the start of the study and weekly thereafter.

Blood was collected from all animals surviving to study termination. Erythrocyte counts, leukocyte counts, differential leukocyte counts, hemoglobin concentrations, and hematocrit values were determined on samples drawn from the retro-orbital sinus. Clinical chemistry values for blood urea nitrogen (BUN), serum creatinine, lactic dehydrogenase (LDH), sorbitol dehydrogenase (SDH), alanine aminotransferase (ALT), triiodothyronine (T_3), thyroxine (T_4), and thyroid stimulating hormone (TSH) were determined from blood samples collected from the abdominal aorta. T_3 and T_4 were analyzed with the Tri-Tab RIA Diagnostic Kit and the Tetra-Tab Diagnostic Kit (Nuclear Medical Laboratories). TSH analysis was performed by the method of Ridgway *et al.* (1973). Further details are presented in Table 2.

Survivors were killed at the end of the 13-week studies. Necropsies were performed on all study animals. The brain, heart, liver, lung, kidney (right), testis (right), and thymus of survivors were weighed at necropsy. Complete histopathological examinations were performed on all animals in the control and high-dose groups, all animals in the highest dose groups with a survival rate of 60% or less (2,000 ppm dose group), and on all animals that died or were killed moribund. Selected organs were submitted for histopathology for the remaining animals. Tissues examined for each group are listed in Table 2.

9-MONTH AND 14-MONTH STUDIES

Study Design

The 14-month studies were originally designed for 24 months with an animal allocation recommended by Portier and Hoel (1984). At 9 months, ten rats of each sex in the control and 150 ppm dose groups were killed, and at 14 months ten rats of each sex at each dose level were to be killed; animals were predesignated for the 9- and 14-month sacrifices prior to study start. Because of the large number of early deaths in the exposed groups, the study was terminated at 14 months, and the 14-month interim sacrifice animals were added to the core groups, resulting in 60 rats in the control groups, 45 in the 30 ppm groups, 75 in the 70 ppm groups, and 60 in the 150 ppm groups.

Source and Specification of Animals

Male and female F344/N rats were obtained from Frederick Cancer Research Facility (Frederick, MD) for use in the 2-year studies. Breeding stock for the foundation colony at the production facility originated at the National Institutes of Health Repository. Animals shipped for study were progeny of defined microflora-associated parents and were transferred from isolators to barrier-maintained rooms. The animals were 4 weeks old at receipt. Following a 14-day quarantine, five animals of each sex were randomly selected and sacrificed for parasite evaluation and gross observation of disease. Serology samples were collected for viral screens. Study animals were 6 weeks old at study initiation. The health of the animals was monitored during the course of the studies according to the protocols of the NTP Sentinel Animal Program (Appendix I).

Animal Maintenance

The rats were housed 5 per cage. Feed (Appendix H) and water were available *ad libitum*. Cages were rotated every 2 weeks during the studies. Further details of animal maintenance are given in Table 2.

Clinical Observations and Pathology

All animals were observed twice daily. Animals were weighed at study initiation, weekly for 14 weeks, at week 17, and every 4 weeks thereafter. Clinical findings were noted and recorded at the time of weighing. Feed consumption was measured weekly, and water consumption, twice weekly.

At 9 months, ten rats of each sex from the control and high-dose (150 ppm) groups were killed. Blood and urine samples were collected prior to sacrifice. Hematocrit values, hemoglobin concentrations, erythrocyte counts, total leukocyte counts, leukocyte differential counts, mean cell volume, mean cell hemoglobin, mean cell hemoglobin concentration, and blood cell morphology were determined from blood drawn from the retro-orbital sinus. Clinical chemistry values for BUN, creatinine, glucose, ALT, LDH, SDH, T_3, T_4, TSH, and serum osmolality were determined from blood samples collected from the abdominal aorta. T_3, T_4, and TSH were analyzed with the same methods used in the 13-week studies. Urine measurements included protein, glucose, creatinine, pH, specific gravity, urine osmolality, volume, and creatine excretion rate (16-hour); urine sediment was examined microscopically. Brain, liver, and kidney were weighed at necropsy. Further details are presented in Table 2.

Animals found moribund, designated for the 9-month studies, or surviving to the end of the 14-month studies were killed. Necropsy was performed on all animals. At necropsy, all organs and tissues were examined for gross lesions, and all major tissues were fixed and preserved in 10% neutral buffered formalin, trimmed and processed, embedded in paraffin, sectioned, and stained with hematoxylin and eosin for microscopic examination. The tissues and groups examined are listed in Table 2. In some cases, a particular organ or tissue may have been autolyzed or lost; thus, the numbers of organs and tissues examined microscopically vary and are not necessarily equal to the number of animals placed on study.

When the pathology evaluation was completed by the study laboratory pathologist and the pathology data entered into the Toxicology Data Management System (TDMS), the microscope slides, individual animal necropsy records, and pathology tables were forwarded to an independent pathology quality assessment laboratory. At this laboratory, individual animal records and pathology tables were compared for accuracy, slides and tissue counts were verified, and histotechnique evaluated.

A quality assessment pathologist reviewed selected tissues microscopically for accuracy and consistency of lesion diagnosis. All neoplastic and nonneoplastic lesions were reviewed in the following tissues from all male and female rats: clitoral or preputial gland, liver, lung, kidney, and Zymbal's gland. In addition, all neoplastic diagnoses in tissues other than those already mentioned were reviewed in all animals, and all diagnoses (neoplastic and nonneoplastic) were reviewed from a random 10% of the animals from each control and high-dose group.

The quality assessment report and slides were submitted to the Pathology Working Group (PWG) Chair, who reviewed the slides of tissues with treatment-related effects and of any other tissues for which there was disagreement in diagnosis between the laboratory and quality assessment pathologist. Representative histopathology slides of tissues with treatment-related lesions and examples of disagreements in diagnosis between the laboratory and quality assessment pathologist were shown to the PWG. The PWG included the quality assessment pathologist and others experienced in rodent toxicology who examined the tissues without knowledge of dose group or previously rendered diagnoses. Whenever the consensus diagnosis of the PWG differed from that of the laboratory pathologist, the diagnosis was changed to reflect the opinion of the PWG. This procedure has been described, in part, by Maronpot and Boorman (1982) and Boorman *et al.* (1985). The final pathology data represent a consensus of contractor pathologists and the NTP PWG. For subsequent analysis of pathology data, the diagnosed lesions for each tissue type were separated or combined according to the guidelines of McConnell *et al.* (1986).

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 were censored from the survival analyses at the time they were found dead from other than natural causes. Animals dying from natural causes were not censored. Statistical analysis for a possible dose-related effect on survival used the method of Cox (1972) 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 analysis are two-sided.

Calculation of Incidence

The incidence of neoplastic or nonneoplastic lesions is given as the ratio of the number of animals bearing such lesions at a specific anatomic site to the number of animals in which the site was examined. In most instances, the denominators include only those animals for which the site was examined histologically. However, when macroscopic examination was required to detect lesions (e.g., oral cavity) prior to tissue sampling for histopathology, or when lesions (e.g., lymphomas) could have occurred at multiple sites, the denominators consist of the number of animals on which a necropsy was performed.

Analysis of Tumor Incidence

In the chronic study, the deaths of dosed rats and those killed in moribund condition early in the study were considered due to tumors of the skin, Zymbal's gland, clitoral gland, and preputial gland. Consequently, for these particular lesions, primary emphasis in the analysis of tumor incidence was given to the life table test (Cox, 1972; Tarone, 1975), a survival-adjusted procedure appropriate for rapidly lethal tumors.

For incidental tumors (tumors discovered as a result of death from an unrelated cause), one method of analysis used in this study was logistic regression. This method of adjusting for intercurrent mortality is the prevalence analysis of Dinse and Lagakos (1983), further described and illustrated by Dinse and Haseman (1986). However, markedly reduced survival in dosed animals (due largely to the increased incidence of lethal tumors) reduced the power of logistic regression to detect carcinogenic effects in some instances. Therefore, although the results of logistic regression analysis are given in Appendixes A and B for informational purposes, primary emphasis was given to the Cochran-Armitage and Fisher exact tests based upon the *effective number* of animals. The effective number is the number of animals surviving until the appearance of the first tumor. These survival-adjusted procedures are recommended by Gart *et al.* (1979).

Tests of significance include paired comparisons of each dosed group with controls and a test for an overall dose-response trend. Continuity-corrected tests were used in the analysis of tumor incidence and reported P values are one-sided. The procedures described above were also used to evaluate selected nonneoplastic lesions. For further discussion of these methods, see Haseman (1984).

Historical Control Data

Although the concurrent control group is the first and most appropriate control group used for evaluation, there are certain instances in which historical control data can be helpful in the overall assessment of tumor incidence. Although the current studies were terminated at 14 months, tumor incidences from the NTP historical control data base for 2-year studies (Haseman *et al.*, 1984, 1985) are included for tumors appearing to show compound-related effects.

Analysis of Continuous Variables

Organ-weight-to-body-weight ratios and hematology and serum chemistry data from the 14-day and 13-week studies were analyzed using the nonparametric comparison procedures of Dunn (1964) and Shirley (1977); Jonckheere's test (Jonckheere, 1954) was used to evaluate the significance of dose-response trends and to determine whether Dunn's or Shirley's test was more appropriate for pairwise comparisons. For the 9-month studies (in which a single dose group was compared with the controls), Wilcoxon's rank sum test (Hollander and Wolfe, 1973) was used to evaluate organ weight, hematology, serum chemistry, and urinalysis data.

Quality Assurance Methods

The 13-week and 14-month studies were conducted in compliance with FDA Good Laboratory Practice Regulations (21 CFR Part 58). In addition, as study records were submitted to the NTP Archives, they were audited retrospectively by an independent quality assurance contractor. Separate audits covering completeness and accuracy of the pathology data, pathology specimens, final pathology tables, and preliminary draft of the NTP Technical Report were conducted. Audit procedures are presented in the reports, which are on file at the NIEHS. The audit findings were reviewed and assessed by NTP staff and were resolved or were otherwise addressed during the preparation of this Technical Report.

TABLE 2
Experimental Design and Materials and Methods in the Drinking Water Studies of 3,3′-Dimethylbenzidine Dihydrochloride

14-Day Studies	13-Week Studies	14-Month Studies
Study Laboratory Hazleton Laboratories America, Inc. (Vienna, VA)	Hazleton Laboratories America, Inc. (Vienna, VA)	Hazleton Laboratories America, Inc. (Vienna, VA)
Strain and Species F344/N rats	F344/N rats	F344/N rats
Animal Source Frederick Cancer Research Facility (Frederick, MD)	Frederick Cancer Research Facility (Frederick, MD)	Frederick Cancer Research Facility (Frederick, MD)
Time Held Before Study 13 days	16 days	14 days
Age When Placed on Study 48 days	Median age 55 days	42 days
Date of First Dose 22 March 1982	30 July 1982	30 June 1983
Date of Last Dose 5 April 1982	Males: 2 November 1982 Females: 3 November 1982	21-29 August 1984 (dosed until necropsy)
Duration of Dosing 14 consecutive days	13 weeks (7 days/week)	60-61 weeks (7 days/week)
Age at Necropsy 9 weeks	21-22 weeks	66-67 weeks
Necropsy Dates 5 April 1982	Males: 3 November 1982 Females: 4 November 1982	21-29 August 1984
Size of Study Groups 5 males and 5 females	10 males and 10 females	Control: 70/sex Low-dose: 45/sex Mid-dose: 75/sex High-dose: 70/sex
Method of Animal Distribution Animals distributed to weight classes and then randomized to test and control groups and position in racks.	Same as 14-day studies	Same as 14-day studies
Animals per Cage 5	5	5
Method of Animal Identification Ear tag	Ear punch	Ear punch and ear tag

TABLE 2
Experimental Design and Materials and Methods in the Drinking Water Studies of 3,3′-Dimethylbenzidine Dihydrochloride (continued)

14-Day Studies	13-Week Studies	14-Month Studies
Diet		
NIH-07 Rat and Mouse Ration, powdered (Zeigler Bros., Inc., Gardners, PA); available ad libitum	Same as 14-day studies	Same as 14-day studies
Water		
Tap water (Fairfax County Water Authorities) in glass water bottles with stainless steel sippers (Hazleton Systems, Inc., Aberdeen, MD); available ad libitum	Distilled water (Polar Water Co., Beltsville, MD) in glass water bottles with stainless steel sippers (Hazleton Systems, Inc., Aberdeen, MD); available ad libitum	Same as 13-week studies
Cages		
Polycarbonate (Hazleton Systems, Inc., Aberdeen, MD)	Same as 14-day studies	Same as 14-day studies
Bedding		
Heat-treated hardwood chips (P.J. Murphy Forest Products, Mt. Jewett, PA)	Same as 14-day studies	Same as 14-day studies
Cage Filters		
Reemay polyester nonwoven fiber filters (DuPont Company, Applied Technologies Division, Wilmington, DE)	Same as 14-day studies	Same as 14-day studies
Animal Room Environment		
Temperature: 72°-77° F Humidity: 41%-69% Fluorescent light: 12 hours/day	Temperature: 69°-74° F Humidity: 24%-74% Fluorescent light: 12 hours/day Room air changes: 16.7/hour	Temperature: 65°-92° F Humidity: 25%-80% Fluorescent light: 12 hours/day Room air changes: 10.4/hour
Doses		
0, 600, 1,250, 2,500, 5,000, or 7,500 ppm 3,3′-dimethylbenzidine dihydrochloride in drinking water	0, 300, 500, 1,000, 2,000, or 4,000 ppm 3,3′-dimethylbenzidine dihydrochloride in drinking water	0, 30, 70, or 150 ppm 3,3′-dimethylbenzidine dihydrochloride in drinking water
Type and Frequency of Observation		
Observed twice/day; body weight initially and once/week; feed consumption once/week; water consumption twice/week	Same as 14-day studies	Observed twice/day; body weights initially, once/week for 14 weeks, at week 17, once/month thereafter; feed consumption measured 1 week/month; water consumption measured 1 week/month in 3-day and 4-day segments; clinical observations at body weight determinations

TABLE 2
Experimental Design and Materials and Methods in the Drinking Water Studies of 3,3′-Dimethylbenzidine Dihydrochloride (continued)

14-Day Studies	13-Week Studies	14-Month Studies
Necropsy, Histopathology, and Clinical Pathology Studies		
Necropsy Necropsy performed on all animals. Organ weights obtained at necropsy (brain, heart, liver, lung, right kidney, right testis, and thymus).	**Necropsy** Necropsy performed on all animals. Organ weights measured were the same as in the 14-day studies.	**Necropsy** Necropsy performed on all animals. Organ weights measured at 9-month interim evaluation (brain, kidney, liver).
Histopathology Complete histopathology on male and female controls, 7,500 ppm females, and 5,000 ppm males, including the following organs: adrenal, blood smear, bone (sternebrae, femur, or vertebrae, including marrow), bone marrow (sternum), brain, clitoral gland, epididymis, esophagus, eyes (if grossly abnormal), heart, kidney, large intestines (cecum, colon, rectum), liver, lung with mainstem bronchi, lymph nodes (mandibular, mesenteric), nasal cavity and turbinates, ovaries, pancreas, parathyroid, pharynx (if grossly abnormal), pituitary, preputial gland, prostate, salivary gland, skin, small intestines (duodenum, ileum, jejunum), spinal cord (if neurological signs present), spleen, stomach, testes, thymus, thyroid, trachea, urinary bladder, uterus, Zymbal's gland, and gross lesions. The following target organs were examined from 5,000 ppm females and 2,500 ppm males and females: adrenal, bone marrow (sternum), epididymis, kidneys, liver, lymph nodes (mandibular, mesenteric), pancreas, spleen, testes, and thymus. The following target organs were examined from 1,250 ppm males: adrenal, bone marrow (sternum), liver, lymph nodes (mandibular, mesenteric), kidneys, spleen, testes, and thymus; from 1,250 ppm females: adrenal, bone marrow (sternum), kidneys, pancreas, and thymus; from 600 ppm females: bone marrow (sternum).	**Histopathology** Complete histopathology on male and female controls, all deaths and moribund kills (all 4,000 ppm males and females and 4 males and 3 females from 2,000 group), all males and females from the 2,000 ppm group surviving to termination. Tissues examined were the same as in the 14-day studies complete screen. The following organs were examined from 1,000 ppm males: adrenal, bone marrow (sternum), kidney, liver, lymph nodes (mandibular, mesenteric), pancreas, spleen, testes, and thymus; from 500 ppm males: kidney, liver, and pancreas; from 300 ppm males: liver and pancreas; from 100 ppm females: bone marrow (sternum), kidney, liver, lymph nodes (mandibular, mesenteric), pancreas, spleen, and thymus; from 500 and 300 ppm females: kidney, liver, and pancreas.	**Histopathology** Complete histopathology on all animals that died, were moribund kills, or were killed at 9 months or termination. Tissues examined same as 14-day studies complete screen with the addition of seminal vesicles.
Clinical Pathology None required	**Clinical Pathology** Clinical pathology studies conducted at 13 weeks. ***Hematology:*** hematocrit, hemoglobin, erythrocytes, leukocytes, segmented neutrophils, lymphocytes, monocytes, eosinophils, and erythrocyte and lymphocyte morphology ***Clinical chemistry:*** blood urea nitrogen, creatinine, triiodothryronine, thyroxine, thyroid-stimulating hormone, lactate dehydrogenase, sorbitol dehydrogenase, alanine aminotransferase	**Clinical Pathology** Clinical pathology studies conducted at 9 months. ***Hematology:*** hematocrit, hemoglobin, erythrocytes, leukocytes, segmented neutrophils, lymphocytes, monocytes, eosinophils, and erythrocyte and lymphocyte morphology ***Clinical chemistry:*** blood urea nitrogen, creatinine, glucose, serum osmolality, triiodothryronine, thyroxine, thyroid-stimulating hormone, lactate dehydrogenase, sorbitol dehydrogenase, alanine aminotransferase ***Urinalysis:*** Protein, glucose, creatinine, pH, specific gravity, urine osmolality, volume, creatinine excretion rate (16 hr), serum/urine osmolality, and microscopic exam of sediment

RESULTS

14-DAY STUDIES

All five males and one female receiving 7,500 ppm 3,3′-dimethylbenzidine dihydrochloride and 1/5 males receiving 5,000 ppm died (Table 3). The final mean body weights of rats receiving 2,500 ppm or more were lower than the initial weights. Depressions in final mean body weight relative to controls ranged from 11% to 60% in male rats receiving at least 1,250 ppm and from 6% to 61% in treated females. Water consumption declined with increasing dose and at 7,500 ppm was less than one-sixth that by controls. Clinical findings included urine stains, skin cold to the touch, rough hair coat, ataxia, and reddish discharge at the eyes and nares in the 7,500 ppm group and thinness and/or kyphosis in other groups.

The absence of body fat was the most notable necropsy observation in animals receiving 5,000 and 7,500 ppm. Gross necropsy findings included small thymus glands in 2,500 and 5,000 ppm males and females and small seminal vesicles in 7,500 ppm males. Significant depressions in the absolute weights and increases in the relative weights of several organs (Tables E1 and E2) reflected the marked decreases in necropsy body weight for animals receiving 2,500, 5,000, and 7,500 ppm. Hepatocyte necrosis and brown pigmentation of the cells lining the hepatic sinusoids were present in males receiving as little as 2,500 ppm and females receiving 5,000 and 7,500 ppm. An increase in the severity of nephropathy and bone marrow hypocellu-

TABLE 3
Survival, Mean Body Weights, and Water Consumption of Rats in the 14-Day Studies of 3,3′-Dimethylbenzidine Dihydrochloride

Concentration (ppm)	Survival[a]	Mean Body Weights (g)			Final Weight Relative to Controls (%)	Water Consumption[d]	
		Initial[b]	Final	Change[c]		Week 1	Week 2
Male							
0	5/5	143 ± 2.7	218 ± 4.8	+74 ± 2.9		24	24
600	5/5	147 ± 2.3	218 ± 3.2	+70 ± 2.0	100	15	18
1,250	5/5	148 ± 2.5	195 ± 2.9	+47 ± 2.4	89	16	17
2,500	5/5	147 ± 1.5	105 ± 8.3	-41 ± 7.1	48	7	8
5,000	4/5[e]	147 ± 1.2	88 ± 3.8	-60 ± 5.0	40	4	4
7,500	0/5[e]	146 ± 1.9	–[f]	–	–	3	3
Female							
0	5/5	117 ± 1.3	153 ± 2.6	+36 ± 1.6		19	17
600	5/5	116 ± 2.3	143 ± 2.1	+27 ± 1.9	94	14	13
1,250	5/5	115 ± 1.0	132 ± 0.7	+17 ± 0.8	86	10	10
2,500	5/5	115 ± 1.0	112 ± 1.2	-3 ± 0.6	73	8	9
5,000	5/5	114 ± 1.4	60 ± 1.0	-54 ± 1.2	39	4	3
7,500	4/5[e]	114 ± 0.5	63 ± 1.4	-52 ± 1.2	41	3	3

[a] Number surviving/number initially in group
[b] Initial group mean body weight ± standard error of the mean. Subsequent calculations are based on animals surviving to the end of the study.
[c] Mean body weight change of the survivors ± standard error of the mean
[d] Milliliters per animal per day, based on average consumption data obtained during the 2-week interval
[e] All mortality in these groups occurred by day 13.
[f] No data are reported due to 100% mortality in this group.

larity (atrophy) was associated with exposure to as little as 2,500 ppm 3,3′-dimethylbenzidine dihydrochloride. Treated animals showed moderate to severe lymphocytic atrophy of the thymus and varying degrees of lymphocytic atrophy of the spleen and mandibular and mesenteric lymph nodes. Treated animals also showed necrosis and vacuolation of adrenal cortical cells, focal acinar cell hypertrophy of the pancreas, and, in males, increased numbers of immature sperm forms in the testis and epididymis.

13-WEEK STUDIES

All animals receiving 4,000 ppm 3,3′-dimethylbenzidine dihydrochloride died by week 4 of the study; 4/10 males and 3/10 females receiving 2,000 ppm also died prior to terminal sacrifice (Table 4). The final mean body weight of 2,000 ppm females was lower than the initial weight. Depressions in the final mean body weights of treated rats relative to controls ranged from 12% to 48% in males and from 9% to 42% in females; these depressions were particularly evident in the 2,000 ppm group. By week 7, water consumption by rats in the 2,000 ppm group was about 45% of that by controls. Clinical findings noted during the studies included crusty red exudate on the noses of rats receiving 300 ppm or more, thinness, stains on the fur, and urine stains. These findings appeared as early as week 1 or 2 in animals receiving 4,000 ppm or 2,000 ppm, respectively.

TABLE 4
Survival, Mean Body Weights, and Water Consumption of Rats in the 13-Week Studies of 3,3′-Dimethylbenzidine Dihydrochloride

Concentration (ppm)	Survival[a]	Mean Body Weights (g)			Final Weight Relative to Vehicle Controls (%)	Water Consumption[d]	
		Initial[b]	Final	Change[c]		Week 7	Week 13
Male							
0	10/10	170 ± 2.7	351 ± 7.8	+181 ± 6.5		23	21
300	10/10	168 ± 2.6	307 ± 5.2	+139 ± 4.3	88	19	16
500	10/10	169 ± 2.5	312 ± 4.0	+143 ± 3.6	89	14	14
1,000	10/10	170 ± 2.5	303 ± 3.8	+133 ± 3.4	86	14	14
2,000	6/10[e]	164 ± 4.2	182 ± 16.7	+16 ± 14.1	52	10	10
4,000	0/10[f]	172 ± 4.1	—[g]	—[g]	—[g]	—[k]	—[g]
Female							
0	10/10	120 ± 3.9	198 ± 2.1	+78 ± 4.2		20	24
300	10/10	123 ± 3.6	181 ± 2.2	+58 ± 3.8	91	12	13
500	10/10	126 ± 2.8	180 ± 2.8	+54 ± 2.8	91	10	12
1,000	10/10	118 ± 2.1	166 ± 2.4[j]	+47 ± 2.7	84	8	11
2,000	7/10[h]	122 ± 2.8	115 ± 7.3	-7 ± 9.2	58	9	7
4,000	0/10[i]	123 ± 2.6	—[g]	—[g]	—[g]	—[l]	—[g]

[a] Number surviving/number initially in group
[b] Initial group mean body weight ± standard error of the mean. Subsequent calculations are based on animals surviving to the end of the study.
[c] Mean body weight change of the survivors ± standard error of the mean
[d] Milliliters per animal per day, based on average consumption data obtained during the 13-week interval
[e] Week of death: 6, 7, 11, 13; one animal died before completion of terminal sacrifice
[f] Week of death: 2, 2, 3, 3, 3, 3, 4, 4, 4, 4
[g] No data are reported due to 100% mortality in this group.
[h] Week of death: 13; two animals died before completion of terminal sacrifice
[i] Week of death: 3, 3, 3, 3, 3, 3, 3, 4, 4, 4
[j] Mean based on 9 animals; one female inadvertently not weighed at week 13
[k] Water consumption was 5 mL/animal per day for weeks 1 and 2 and 15 mL/animal per day for week 3.
[l] Water consumption was 4 mL/animal per day for week 1, 5 mL/animal per day for week 2, and 13 mL/animal per day for week 3.

Mean necropsy body weights were significantly decreased in rats at all dose levels, making changes in absolute and relative organ weights more difficult to evaluate. The only consistent effect was a reduction in thymus weight in females at all dose levels (Tables E3 and E4). Significant decreases in hematocrit and erythrocyte count were observed in males receiving 1,000 and 2,000 ppm and in females receiving 500 ppm or more (Table D1). Males and females at all dose levels showed elevated sorbitol dehydrogenase (SDH) levels; alanine aminotransferase was increased only in the 1,000 ppm male and 2,000 ppm female groups. Males receiving 300 or 1,000 ppm showed significant elevations in lactate dehydrogenase. Slight increases in mean blood urea nitrogen (BUN) levels were observed in male rats receiving 2,000 ppm. Decreases in mean creatinine observed in females receiving 300 and 500 ppm were not considered compound related. Triiodothyronine (T_3) values were significantly decreased in exposed females, and thyroxin (T_4) values were significantly decreased in exposed males and females. No significant changes in thyroid stimulating hormone (TSH) levels were observed in exposed rats.

A paucity of body fat and the presence of reddened areas in the glandular mucosa of the stomach were the most notable necropsy findings. Significant histomorphologic alterations were observed in the liver, kidney, bone marrow, lymphoid organs (spleen, mandibular and mesenteric lymph nodes, and thymus), pancreas, and testis of treated rats (Table 5). Hepatic damage, including minimal to moderate hepatocyte necrosis (scattered individual hepatocytes) and brown pigment within the sinusoidal lining cells, was observed in males and females receiving 2,000 and 4,000 ppm and, to a lesser extent, in females receiving 300 ppm and more and in one male receiving 300 ppm. Males and females receiving 1,000 ppm and more and females receiving 500 ppm showed an increased severity of nephropathy over controls; some females receiving 500, 1,000, or 2,000 ppm exhibited karyomegaly of renal tubule epithelial cells. Bone marrow hypocellularity (atrophy) was observed at doses of 2,000 and 4,000 ppm and was consistent with clinical pathology findings. Lymphocytic atrophy of the thymus, spleen, and mandibular and mesenteric lymph nodes was observed in the 2,000 and 4,000 ppm dose groups; females receiving 1,000 ppm also showed lymphocytic atrophy of the thymus. Animals receiving 2,000 and 4,000 ppm showed pancreatic acinar degeneration, and males receiving 1,000, 2,000, or 4,000 ppm showed immature sperm forms in the testis and epididymis; these changes were considered possibly secondary to the general physical debilitation of the study animals.

Dose Selection Rationale

Because of reduced survival in the groups receiving 2,000 or 4,000 ppm, dose-related depressions in weight gain and water consumption, and evidence of compound-related hepatocellular and renal damage and bone marrow hypocellularity (atrophy) in the 13-week studies, drinking water concentrations of 3,3′-dimethylbenzidine dihydrochloride selected for rats in the 9-month and 14-month studies were 30, 70, and 150 ppm.

9-Month Studies

Mean necropsy body weights of animals receiving 150 ppm 3,3′-dimethylbenzidine dihydrochloride were decreased significantly relative to controls. Mean absolute and relative liver and kidney weights and relative brain weights for animals receiving 150 ppm were significantly greater than those for controls (Tables E5 and E6). Moderate decreases in hematocrit, hemoglobin, and erythrocyte counts were observed in high-dose animals (Table D2). Increases in creatinine in males, blood glucose in females, and SDH in both sexes (more pronounced in females) were observed in treated animals. Although T_3 assay results for males and females conflicted, decreases in T_4 and increases in TSH were recorded for high-dose males and females; these alterations were not accompanied by histologic changes in the thyroid gland. Increased urine osmolality, urine/serum osmolality, urine creatinine, urine specific gravity, and protein concentration were observed in high-dose animals. The increase in protein concentration was likely only a reflection of low urine volume.

After exposure to 3,3′-dimethylbenzidine dihydrochloride at 150 ppm for only 9 months, a variety of treatment-related lesions were found, including neoplastic nodules (hepatocellular adenomas) and hepatocellular carcinoma of the liver; proliferative epithelial lesions of the Zymbal's gland, preputial and clitoral glands, and oral cavity, including squamous papilloma and carcinoma, adenomas, carcinomas, and focal hyperplasia; epithelial neo-

TABLE 5
Incidences of Treatment-Related Lesions in Rats in the 13-Week Drinking Water Studies of 3,3′-Dimethylbenzidine Dihydrochloride

Lesion	0 ppm	300 ppm	500 ppm	1,000 ppm	2,000 ppm	4,000 ppm
Male						
Liver						
Individual hepatocyte necrosis	0/10	0/10	0/10	0/10	7/10**	3/10
Pigment	0/10	1/10	0/10	0/10	10/10**	9/10**
Kidney						
Nephropathy	10/10 (1.1)[a]	–[b]	10/10 (1.0)	10/10 (1.6)	10/10 (2.6)	10/10 (2.6)
Thymus						
Lymphocytic atrophy	0/10	–	–	0/10	5/6**	9/9**
Spleen						
Lymphocytic atrophy	0/10	–	–	0/10	5/10*	10/10**
Mandibular Lymph Node						
Lymphocytic atrophy	0/10	–	–	0/10	7/10**	10/10**
Mesenteric Lymph Node						
Lymphocytic atrophy	0/10	–	–	0/10	1/10	2/9
Bone Marrow						
Hypocellularity	0/10	–	–	0/10	8/10**	10/10**
Pancreas						
Degeneration[c]	0/10	–	–	0/10	4/10*	10/10**
Testes						
Immature sperm	0/10	–	–	1/10	3/10	7/10**
Female						
Liver						
Individual hepatocyte necrosis	0/10	1/10	6/10**	4/10*	7/10**	7/9**
Pigment	0/10	10/10**	10/10**	10/10**	9/10**	8/9**
Kidney						
Nephropathy	2/10 (1.0)	5/10 (1.0)	10/10** (1.0)	10/10** (1.0)	10/10** (2.2)	7/9** (2.1)
Karyomegaly[d]	0/10	0/10	0/10	7/10**	9/10**	0/9
Thymus						
Atrophy	0/10	–	–	2/10	7/8**	5/5**
Spleen						
Atrophy	0/10	–	–	0/10	4/10*	9/9**
Mandibular Lymph Node						
Atrophy	1/10	–	–	0/10	5/10	7/7**
Mesenteric Lymph Node						
Atrophy	0/10	–	–	0/10	4/10*	6/7**
Bone Marrow						
Hypocellularity	0/10	–	–	0/10	10/10**	9/9**
Pancreas						
Degeneration	0/10	–	–	–	2/10	8/9**

* Significantly different ($P \leq 0.05$) from the control group by Fisher exact test
** $P \leq 0.01$
[a] Values in parentheses are average severity grades for affected animals; 1=minimal, 2=slight, 3=moderate.
[b] Organ not examined in animals at this dose level.
[c] Terminology preferred by Pathology Working Group for the lesion diagnosed as acinar hypertrophy by the laboratory pathologist.
[d] Terminology preferred by Pathology Working Group for the lesion diagnosed as megalocytosis by the laboratory pathologist.

plasms of the skin, including squamous cell papilloma, sebaceous gland adenoma, and basal cell carcinoma; mucinous adenocarcinoma of the small intestine; adenomatous polyp of the colon; hyperplasia of the alveolar epithelium; invasive alveolar/bronchiolar carcinoma; and lymphoid atrophy of the spleen (Table 6). Nonneoplastic changes, including hepatocellular hypertrophy, basophilic foci, fatty change, and cystic degeneration, occurred in the liver of treated animals. The severity of nephropathy was increased in treated males and females, and the incidence of nephropathy was increased in treated females as compared to controls. Moderate chronic nephropathy was observed in all treated animals and was consistent with observed changes in kidney weights and BUN and creatinine levels.

TABLE 6
Incidences of Treatment-Related Lesions in Rats in the 9-Month Drinking Water Studies of 3,3′-Dimethylbendizine Dihydrochloride

	Male		Female	
	0 ppm	150 ppm	0 ppm	150 ppm
Number of animals examined	10	10	10	10
Liver				
Hepatocellular carcinoma	0	2	0	0
Neoplastic nodule[a]	0	5*	0	1
Hepatocyte hypertrophy	0	10**	0	10**
Basophilic focus	0	10**	0	0
Fatty change[b]	1	10**	0	10**
Cystic degeneration	0	7**	0	0
Lung				
Alveolar/bronchiolar carcinoma	0	1	0	0
Alveolar/bronchiolar adenoma	0	0	0	1
Alveolar epithelium hyperplasia	0	7**	0	1
Skin				
Basal cell carcinoma	0	1	0	0
Sebaceous gland adenoma	0	1	0	0
Squamous papilloma	0	0	0	1
Oral Cavity (Palate)				
Squamous cell carcinoma	0	0	0	1
Preputial/Clitoral Gland				
Adenoma	0	1	0	2
Carcinoma	0	2	0	3
Small Intestine				
Mucinous adenocarcinoma	0	2	0	0
Large Intestine				
Adenomatous polyp	0	3	0	0
Zymbal's Gland				
Carcinoma	0	2	0	3
Adenoma	0	1	0	2
Squamous papilloma	0	3	0	1
Squamous hyperplasia	0	3	0	1
Focal hyperplasia	0	1	0	0
Kidney				
Nephropathy[c]	10 (1.0)	10 (3.4)	3 (1.0)	10 (3.0)
Spleen				
Lymphoid atrophy[d]	0	10**	0	7**

* Significantly different (P≤0.05) from the control group by Fisher exact test
** P≤0.01
a Term previously used for lesions currently classified as hepatocellular adenoma.
b Diagnosed as cytoplasmic vacuolization by the study pathologist.
c Values in parentheses are average severity grades; 1=minimal, 2=mild, 3=moderate, and 4=marked.
d Diagnosed as lymphoid depletion by the study pathologist.

14-MONTH STUDIES

Body Weights, Water Consumption, and Clinical Findings

The mean body weight of males receiving 150 ppm was approximately 85% of that of controls by week 29 and about 70% of the mean control value by study termination. By week 33, mean body weight for females receiving 150 ppm was approximately 85% of the control mean body weight, and by week 45 the same was true for females receiving 70 ppm (Tables 7 and 8 and Figure 2). The average daily water consumption per rat in the low-, mid-, and high-dose groups was 95%, 91%, and 105% that by controls for males and 101%, 96%, and 79% for females (Tables G1 and G2). The average amount of 3,3′-dimethylbenzidine dihydrochloride consumed per day was approximately 1.8, 4.0, or 11.2 mg/kg for low-, mid-, or high-dose males and 3.0, 6.9, or 12.9 mg/kg for low-, mid-, or high-dose females. Clinical findings noted during the study were limited to increased incidences of tissue masses on the head, over the dorsum, and in the ventral posterior area.

TABLE 7
Mean Body Weights of Male Rats in the 14-Month Drinking Water Study of 3,3′-Dimethylbenzidine Dihydrochloride

Week on Study	0 ppm		30 ppm			70 ppm			150 ppm		
	Av. Wt. (g)	Number Weighed	Av. Wt. (g)	Wt. (% of controls)	Number Weighed	Av. Wt. (g)	Wt. (% of controls)	Number Weighed	Av. Wt. (g)	Wt. (% of controls)	Number Weighed
1	141	70	141	100	45	142	101	75	143	102	70
2	173	70	174	100	45	177	102	75	171	99	70
3	214	70	212	99	45	207	97	74	201	94	70
4	236	70	230	97	45	233	99	74	221	94	70
5	262	70	254	97	45	255	97	74	239	91	70
6	278	70	272	98	45	273	98	74	253	91	70
7	292	70	289	99	45	288	99	74	266	91	70
8	305	70	301	99	12	304	100	74	277	91	70
9	320	70	316	99	45	316	99	74	287	90	70
10	327	70	324	99	45	328	100	74	298	91	70
11	345	70	342	99	45	335	97	74	305	89	70
12	352	70	345	98	45	340	97	74	314	89	70
13	354	70	351	99	45	346	98	74	317	90	70
14	362	70	358	99	45	363	100	74	322	89	70
15	365	70	364	100	45	366	100	74	332	91	70
17	380	70	376	99	45	372	98	74	337	89	70
21	401	70	399	100	45	388	97	74	352	88	70
25	412	70	410	100	45	401	97	74	362	88	70
29	423	70	413	98	45	405	96	74	361	86	70
33	436	70	426	98	45	418	96	73	368	84	67
37	447	70	432	97	44	426	95	72	368	82	64
41	447	60	440	94	44	423	95	72	356	80	44
45	459	60	449	98	44	430	94	67	342	75	34
49	454	60	433	98	44	432	95	67	319	70	27
53	453	60	443	98	43	423	93	64	332	73	6
57	447	60	432	97	42	408	92	57	–	–	0
Mean for Weeks											
1-14	283		279	99		279	99		258	92	
15-57	427		418	98		408	96		319	76	

TABLE 8
Mean Body Weights of Female Rats in the 14-Month Drinking Water Study of 3,3′-Dimethylbenzidine Dihydrochloride

Week on Study	0 ppm Av. Wt. (g)	0 ppm Number Weighed	30 ppm Av. Wt. (g)	30 ppm Wt. (% of controls)	30 ppm Number Weighed	70 ppm Av. Wt. (g)	70 ppm Wt. (% of controls)	70 ppm Number Weighed	150 ppm Av. Wt. (g)	150 ppm Wt. (% of controls)	150 ppm Number Weighed
1	114	70	113	100	45	114	100	75	116	102	70
2	130	70	130	100	45	130	100	75	129	99	70
3	145	70	144	99	45	140	96	75	139	95	70
4	154	70	152[a]	99	45	149	96	75	146	94	70
5	164	70	160	98	45	156	96	75	153	94	70
6	173	70	169	98	45	165	96	75	160	92	70
7	179	70	174	97	45	170	95	75	165	92	70
8	184	70	176	96	45	174	94	75	168	92	70
9	188	70	183	97	45	176	94	75	173	92	70
10	193	70	186	96	45	181	94	75	176	91	70
11	196	70	191	98	45	183	94	75	178	91	70
12	198	70	191	97	45	185	94	75	182	92	70
13	199	70	194	97	45	187	94	75	182	91	70
14	203	70	195	96	45	195	96	75	184	90	70
15	207	70	197	95	45	195	94	75	189	91	70
17	210	70	205	97	45	196	93	75	190	90	70
21	217	70	211	97	45	203[b]	94	75	196	90	70
25	225	70	218	97	45	209	93	74	200	89	69
29	228	70	222	97	45	212	93	73	203	89	69
33	238	70	228	96	45	218	92	73	206	87	69
37	247	70	236	96	45	220	89	72	205	83	66
41	251	60	243	97	45	223	89	71	211	84	51
45	264	60	252	96	45	223	85	70	209	79	46
49	271	60	263	97	45	228	84	64	211	78	41
53	280	60	268	96	44	228	82	55	209	75	30
57	287	59	270	94	40	232	81	42	214	74	16
Mean for Weeks											
1-14	173		168	98		165	96		161	93	
15-57	244		234	96		216	89		204	84	

[a] Mean based on 40 animals. One cage inadvertently not weighed.
[b] Mean based on 70 animals. One cage inadvertently not weighed.

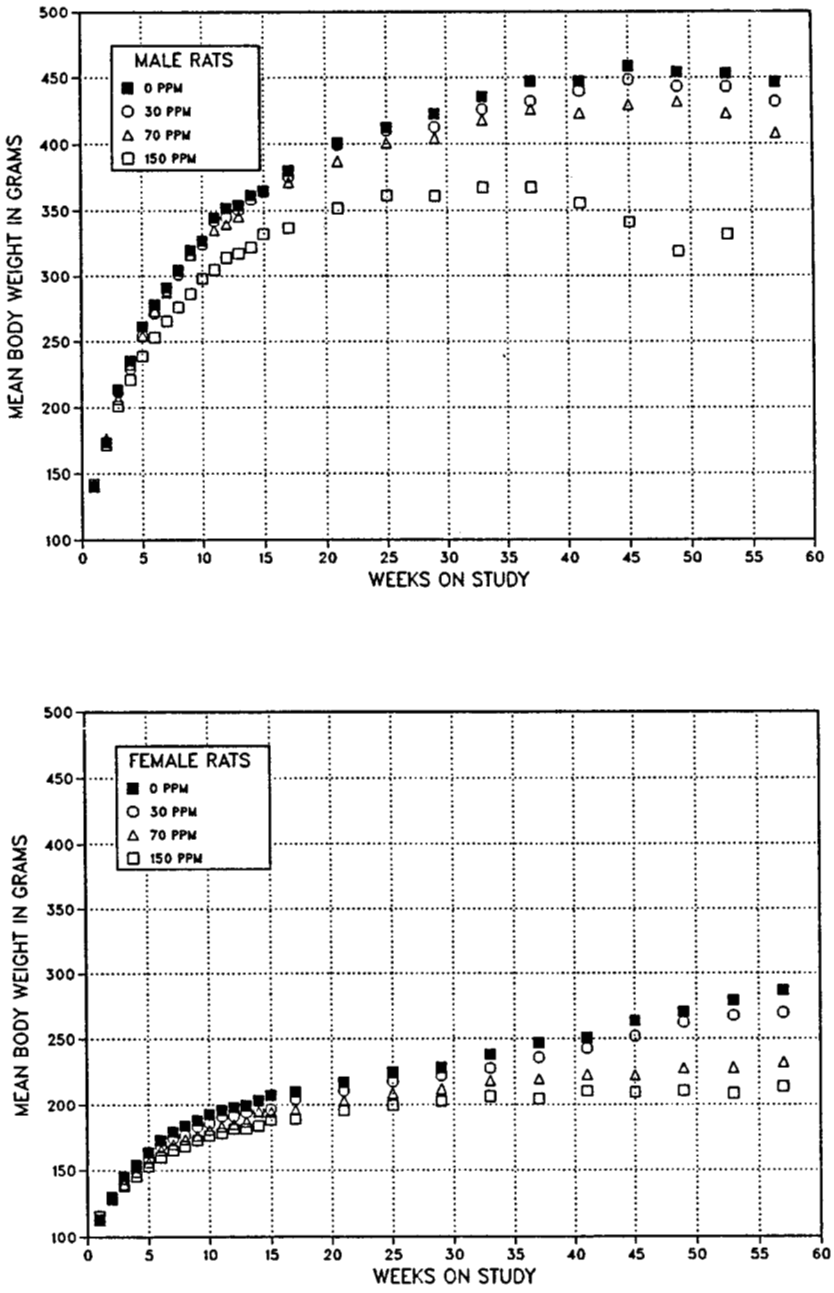


FIGURE 2
Growth Curves for Rats Given Drinking Water Containing 3,3′-Dimethylbenzidine Dihydrochloride for 14 Months

Survival

Estimates of the probabilities of survival for male and female rats given drinking water containing 3,3'-dimethylbenzidine dihydrochloride at the concentrations used in these studies and for controls are shown in Table 9 and in the Kaplan-Meier curves in Figure 3. By week 55, all high-dose males had been found dead or killed moribund; only about 25% of the high-dose females survived to week 56.

TABLE 9
Survival of Rats in the 14-Month Drinking Water Studies of 3,3'-Dimethylbenzidine Dihydrochloride

	0 ppm	30 ppm	70 ppm	150 ppm
Male[a]				
Animals initially in study	70	45	75	70
Interim kill (9 months)	10			10
Natural deaths		2	5	15
Moribund kills		2	19	45
Accidental deaths			1	
Animals surviving until study termination	60	41	50	0
Survival P values[b]	<0.001	0.059	<0.001	<0.001
Female[a]				
Animals initially in study	70	45	75	70
Interim kill (9 months)	10			10
Natural deaths		1	6	5
Moribund kills	1	5	37	45
Animals surviving until study termination	59	39	32	10
Survival P values[b]	<0.001	0.049	<0.001	<0.001

[a] First day of termination period: male, 21 August 1984; female, 23 August 1984
[b] The result of the life table trend test (Tarone, 1975) is in the control column, and the results of the life table pairwise comparisons (Cox, 1972) with the controls are in the dosed columns.

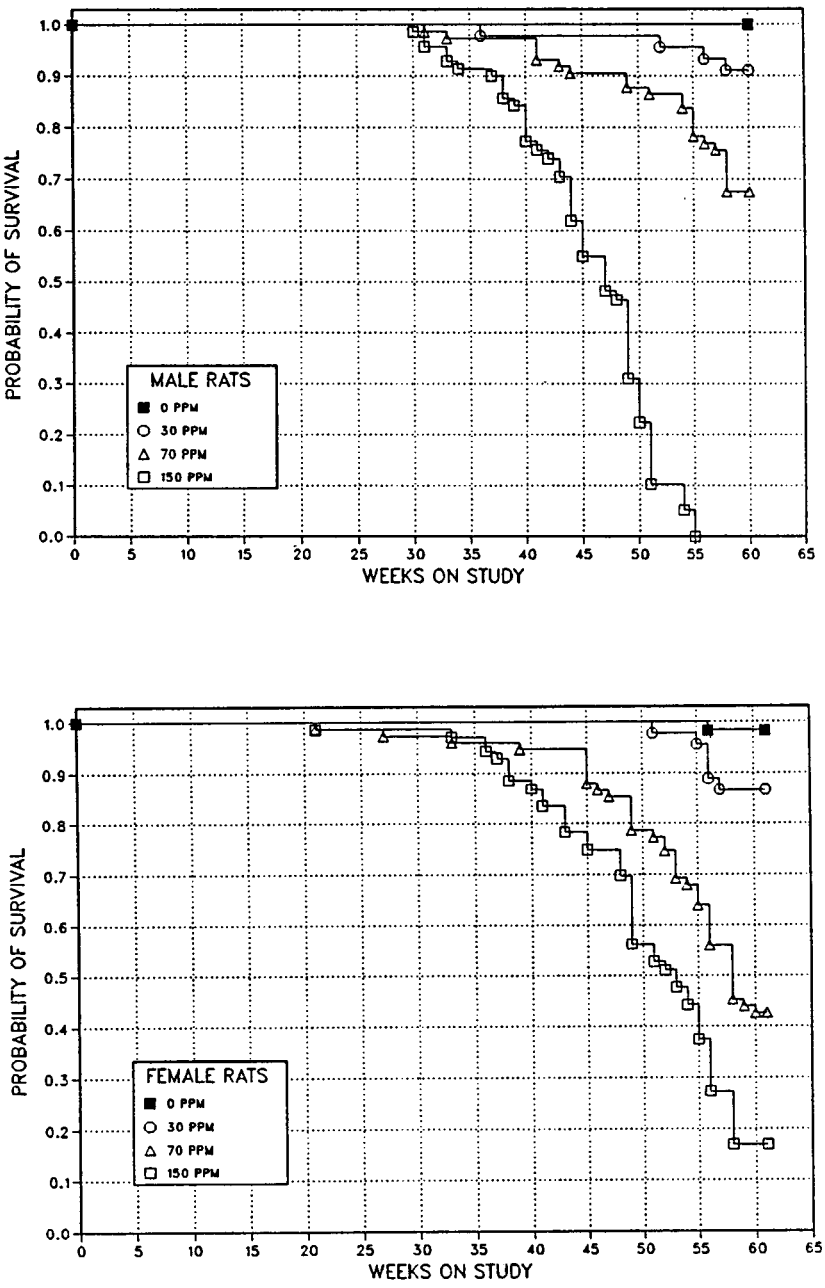


FIGURE 3
Kaplan-Meier Survival Curves for Rats Given Drinking Water Containing 3,3′-Dimethylbenzidine Dihydrochloride for 14 Months

Pathology and Statistical Analyses of Results

This section describes the statistically significant or biologically noteworthy changes in the incidences of rats with neoplastic or nonneoplastic lesions of the skin, Zymbal's gland, preputial and clitoral glands, liver, oral cavity, small intestine, large intestine, mammary gland, lung, mesothelium, brain, adrenal medulla, hematopoietic system, testis, uterus, kidney, parathyroid, heart, glandular stomach, adrenal cortex, spleen, bone marrow, mandibular lymph node, nose, and seminal vesicle.

Summaries of the incidences of neoplasms and nonneoplastic lesions, individual animal tumor diagnoses, statistical analyses of primary tumors occurring with an incidence of at least 5% in at least one animal group, and historical control incidences for selected neoplasms discussed in this section are presented in Appendixes A and B for male and female rats, respectively.

Skin: A variety of epithelial neoplasms of the skin occurred with increased incidence in male and female rats treated with 3,3′-dimethylbenzidine dihydrochloride (Table 10). The increases were often marked in males. A single keratoacanthoma in a male rat was the only epithelial skin neoplasm diagnosed in an untreated animal. The incidence of basal cell adenomas and basal cell carcinomas was increased in treated males and females, and the overall incidence of adenomas in mid- and high-dose males reached 69% and 48%, respectively. The incidence of basal cell adenomas or carcinomas (combined) was significantly increased in all treated males and in mid- and high-dose females. Basal cell adenomas occurred at multiple sites in 47% of mid-dose males and 35% of high-dose males. The incidence of squamous cell papillomas or squamous cell carcinomas (combined) was significantly increased in the mid- and high-dose groups of both sexes. The incidence of keratoacanthomas and sebaceous gland adenomas was significantly increased in mid-and high-dose males. Basal cell neoplasms were locatedbeneath the epidermis and consisted of small polygonal basophilic cells that formed densely packed sheets, branching cords, or solid nodules often containing a central cavity (Figure 4). Adenomas were discrete, circumscribed masses, while carcinomas exhibited local invasion. Many of the basal cell neoplasms contained areas of sebaceous, squamous, or hair follicle differentiation. Squamous cell papillomas were exophytic growths composed of a highly branched fibrovascular core covered by thickened stratified squamous epithelium. Squamous cell carcinomas were plaquelike masses of pleomorphic stratified squamous epithelial cells exhibiting disordered growth and invasion of the underlying dermis. Keratoacanthomas consisted of a central cavity that was often connected to the surface and lined by a thick, highly folded layer of deeply keratinized, stratified squamous epithelium. Sebaceous gland adenomas were composed of multiple glandular structures consisting of nodules of well-differentiated sebaceous cells surrounded by one or more layers of basal cells.

Zymbal's gland: Zymbal's glands are specialized sebaceous glands anterior and ventral to the orifices of the external ear. There was a marked increase in the incidence of Zymbal's gland adenomas and carcinomas in treated male and female rats (Table 11). Some treated rats had bilateral carcinomas of the Zymbal's gland. The incidence of adenomas or carcinomas (combined) was significantly increased in mid- and high-dose males and in all treated females. Carcinomas in some treated rats metastasized to the lung, while others invaded into the brain. Zymbal's glands from some treated animals exhibited nonneoplastic changes, including focal hyperplasia of the glandular cells (hyperplasia, glandular), focal hyperplasia of the stratified squamous epithelium lining the glandular ducts (hyperplasia, squamous), diffuse enlargement of the gland secondary to enlargement of the glandular cells (hypertrophy), and dilation of the glandular ducts (ectasia). There was a morphologic continuum from Zymbal's gland adenoma to carcinoma. Adenomas were discrete masses composed of glandular acini of sebaceous-like cells surrounding ductular structures lined with squamous epithelium. Carcinomas were generally larger and invaded adjacent soft tissue. Neoplastic cells within carcinomas exhibited cellular atypia and disordered growth patterns and formed irregular acinar structures, solid masses, and cords, with a scattering of ductlike structures (Figure 5). Some carcinomas consisted predominantly of sebaceous cells, while others were composed principally of stratified squamous epithelium; some had prominent components of both.

TABLE 10
Skin Tumors in F344/N Rats in the 14-Month Drinking Water Studies of 3,3′-Dimethylbenzidine Dihydrochloride

	0 ppm	30 ppm	70 ppm	150 ppm
Male				
Keratoacanthoma				
Overall rates	1/60 (0%)	1/45 (2%)	8/75 (11%)	5/60 (8%)
Effective rates[a]	1/60 (2%)	1/44 (2%)	8/67 (12%)	5/27 (19%)
Terminal rates	1/60 (2%)	1/41 (2%)	7/50 (14%)	0/0
Day of first observation	419 (T)	419 (T)	379	338
Life table tests	P<0.001	P=0.674	P=0.010	P<0.001
Cochran-Armitage test[b]	P<0.001			
Fisher exact test[b]		P=0.670	P=0.024	P=0.010
Sebaceous Gland Adenoma				
Overall rates	0/60 (0%)	0/45 (0%)	7/75 (9%)	5/60 (8%)
Effective rates	0/60 (0%)	0/44 (0%)	7/72 (10%)	5/49 (10%)
Terminal rates	0/60 (0%)	0/41 (0%)	5/50 (10%)	0/0
Day of first observation			405	280
Life table tests	P<0.001	–[c]	P=0.006	P=0.001
Cochran-Armitage test	P<0.005			
Fisher exact test		–[c]	P=0.012	P=0.016
Basal Cell Adenoma				
Overall rates	0/60 (0%)	10/45 (22%)	52/75 (69%)	29/60 (48%)
Effective rates	0/60 (0%)	10/44 (23%)	52/72 (72%)	29/45 (64%)
Terminal rates	0/60 (0%)	10/41 (24%)	39/50 (78%)	0/0
Day of first observation		419 (T)	307	281
Life table tests	P<0.001	P<0.001	P<0.001	P<0.001
Cochran-Armitage test	P<0.001			
Fisher exact test		P<0.001	P<0.001	P<0.001
Basal Cell Carcinoma				
Overall rates	0/60 (0%)	1/45 (2%)	4/75 (5%)	2/60 (3%)
Effective rates	0/60 (0%)	1/44 (2%)	4/68 (6%)	2/43 (5%)
Terminal rates	0/60 (0%)	1/41 (2%)	4/50 (8%)	0/0
Day of first observation		419 (T)	419 (T)	296
Life table tests	P<0.001	P=0.424	P=0.043	P=0.127
Cochran-Armitage test	P=0.121			
Fisher exact test		P=0.423	P=0.076	P=0.172
Basal Cell Adenoma or Carcinoma[d]				
Overall rates	0/60 (0%)	11/45 (24%)	54/75 (72%)	30/60 (50%)
Effective rates	0/60 (0%)	11/44 (25%)	54/72 (75%)	30/45 (67%)
Terminal rates	0/60 (0%)	11/41 (27%)	41/50 (82%)	0/0
Day of first observation		419 (T)	307	281
Life table tests	P<0.001	P<0.001	P<0.001	P<0.001
Cochran-Armitage test	P<0.001			
Fisher exact test		P<0.001	P<0.001	P<0.001

TABLE 10
Skin Tumors in F344/N Rats in the 14-Month Drinking Water Studies of 3,3′-Dimethylbenzidine Dihydrochloride (continued)

	0 ppm	30 ppm	70 ppm	150 ppm
Squamous Cell Papilloma				
Overall rates	0/60 (0%)	0/45 (0%)	8/75 (11%)	15/60 (25%)
Effective rates	0/60 (0%)	0/45 (0%)	8/72 (11%)	15/55 (27%)
Terminal rates	0/60 (0%)	0/41 (0%)	6/50 (12%)	0/0
Day of first observation			405	238
Life table tests	P<0.001	–[c]	P=0.003	P<0.001
Cochran-Armitage test	P<0.001			
Fisher exact test		–[c]	P=0.006	P<0.001
Squamous Cell Carcinoma				
Overall rates	0/60 (0%)	2/45 (4%)	10/75 (13%)	13/60 (22%)
Effective rates	0/60 (0%)	2/45 (4%)	10/74 (14%)	13/59 (22%)
Terminal rates	0/60 (0%)	1/41 (2%)	9/50 (18%)	0/0
Day of first observation		391	406	211
Life table tests	P<0.001	P=0.165	P<0.001	P<0.001
Cochran-Armitage test	P<0.001			
Fisher exact test		P=0.181	P=0.002	P<0.001
Squamous Cell Papilloma or Carcinoma[e]				
Overall rates	0/60 (0%)	2/45 (4%)	17/75 (23%)	27/60 (45%)
Effective rates	0/60 (0%)	2/45 (4%)	17/74 (23%)	27/59 (46%)
Terminal rates	0/60 (0%)	1/41 (2%)	14/50 (28%)	0/0
Day of first observation		391	405	211
Life table tests	P<0.001	P=0.165	P<0.001	P<0.001
Cochran-Armitage test	P<0.001			
Fisher exact test		P=0.181	P=0.001	P<0.001
Female				
Keratoacanthoma				
Overall rates	0/60 (0%)	0/45 (0%)	0/75 (0%)	1/60 (2%)
Sebaceous Gland Adenoma				
Overall rates	0/60 (0%)	0/45 (0%)	1/75 (1%)	1/60 (2%)
Basal Cell Adenoma				
Overall rates	0/60 (0%)	3/45 (7%)	5/75 (7%)	5/60 (8%)
Effective rates	0/60 (0%)	3/45 (7%)	5/64 (8%)	5/41 (12%)
Terminal rates	0/59 (0%)	3/39 (8%)	3/32 (9%)	2/10 (20%)
Day of first observation		421 (T)	338	370
Life table tests	P<0.001	P=0.060	P=0.013	P<0.001
Cochran-Armitage test	P=0.014			
Fisher exact test		P=0.076	P=0.034	P=0.009
Basal Cell Carcinoma				
Overall rates	0/60 (0%)	0/45 (0%)	5/75 (7%)	4/60 (7%)
Effective rates	0/60 (0%)	0/45 (0%)	5/69 (7%)	4/46 (9%)
Terminal rates	0/59 (0%)	0/39 (0%)	1/32 (3%)	0/10 (0%)
Day of first observation			315	336
Life table tests	P<0.001	–[c]	P=0.015	P=0.010
Cochran-Armitage test	P=0.009			
Fisher exact test		–[c]	P=0.041	P=0.033

TABLE 10
Skin Tumors in F344/N Rats in the 14-Month Drinking Water Studies of 3,3′-Dimethylbenzidine Dihydrochloride (continued)

	0 ppm	30 ppm	70 ppm	150 ppm
Basal Cell Adenoma or Carcinoma[f]				
Overall rates	0/60 (0%)	3/45 (7%)	10/75 (13%)	9/60 (15%)
Effective rates	0/60 (0%)	3/45 (7%)	10/69 (14%)	9/46 (20%)
Terminal rates	0/59 (0%)	3/39 (8%)	4/32 (13%)	2/10 (20%)
Day of first observation		421 (T)	315	336
Life table tests	P<0.001	P=0.060	P<0.001	P<0.001
Cochran-Armitage test	P<0.001			
Fisher exact test		P=0.076	P=0.001	P<0.001
Squamous Cell Papilloma				
Overall rates	0/60 (0%)	1/45 (2%)	6/75 (8%)	5/60 (8%)
Effective rates	0/60 (0%)	1/45 (2%)	6/72 (8%)	5/55 (9%)
Terminal rates	0/59 (0%)	1/39 (3%)	4/32 (13%)	0/10 (0%)
Day of first observation		421 (T)	391	264
Life table tests	P<0.001	P=0.417	P=0.003	P=0.002
Cochran-Armitage test	P=0.015			
Fisher exact test		P=0.429	P=0.024	P=0.023
Squamous Cell Carcinoma				
Overall rates	0/60 (0%)	2/45 (4%)	4/75 (5%)	7/60 (12%)
Effective rates	0/60 (0%)	2/45 (4%)	4/64 (6%)	7/41 (17%)
Terminal rates	0/59 (0%)	2/39 (5%)	3/32 (9%)	3/10 (30%)
Day of first observation		421 (T)	406	338
Life table tests	P<0.001	P=0.153	P=0.016	P<0.001
Cochran-Armitage test	P<0.001			
Fisher exact test		P=0.181	P=0.068	P=0.001
Squamous Cell Papilloma or Carcinoma[g]				
Overall rates	0/60 (0%)	3/45 (7%)	9/75 (12%)	12/60 (20%)
Effective rates	0/60 (0%)	3/45 (7%)	9/72 (13%)	12/55 (22%)
Terminal rates	0/59 (0%)	3/39 (8%)	6/32 (19%)	3/10 (30%)
Day of first observation		421 (T)	391	264
Life table tests	P<0.001	P=0.060	P<0.001	P<0.001
Cochran-Armitage trend test	P<0.001			
Fisher exact test		P=0.076	P=0.003	P<0.001

(T)Terminal sacrifice
[a] Number of tumor-bearing animals/effective number of animals, i.e. number of animals alive at the first occurrence of this tumor type in any of the groups
[b] Based on effective rates
[c] No tumors in dosed group or control group; statistical test not performed
[d] 2-year historical incidence for untreated control groups at study laboratory (mean): 2/100 (2%); historical incidence for untreated control groups in NTP studies (mean ± SD): 21/1,596 (1.3% ± 1.9%)
[e] 2-year historical incidence for untreated control groups at study laboratory (mean): 3/100 (3%); historical incidence for untreated control groups in NTP studies (mean ± SD): 29/1,596 (1.8% ± 1.6%)
[f] 2-year historical incidence for untreated control groups at study laboratory (mean): 0/100 (0%); historical incidence for untreated control groups in NTP studies (mean ± SD): 6/1,643 (0.4% ± 0.7%)
[g] 2-year historical incidence for untreated control groups at study laboratory (mean): 0/100 (0%); historical incidence for untreated control groups in NTP studies (mean ± SD): 7/1,643 (0.4% ± 0.8%)

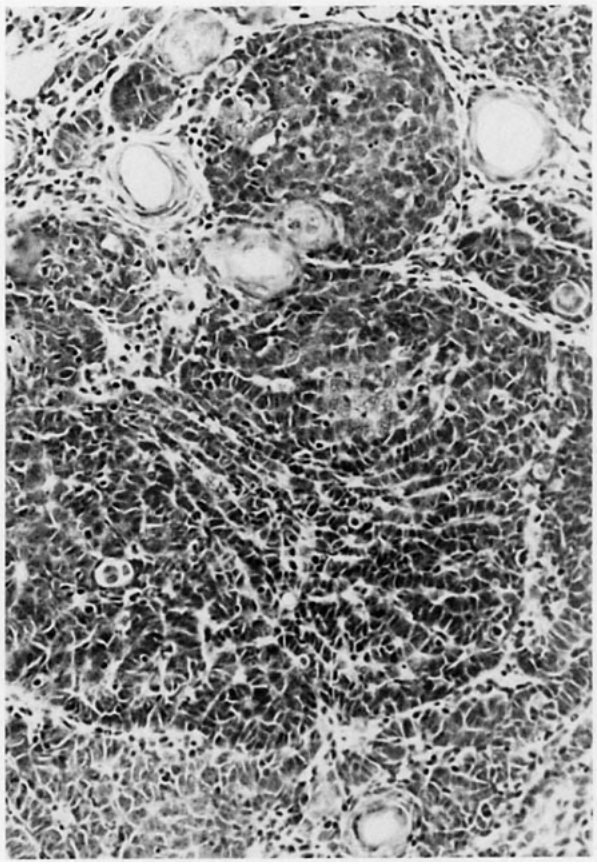


FIGURE 4. Basal cell carcinoma of the skin in a male F344/N rat administered 70 ppm 3,3′-dimethylbenzidine dihydrochloride in drinking water for 14 months. The neoplasm consists of cords and solid clusters of pleomorphic basal cells. At top of figure multiple clusters of neoplastic cells are seen invading the adjacent connective tissue. (×175)

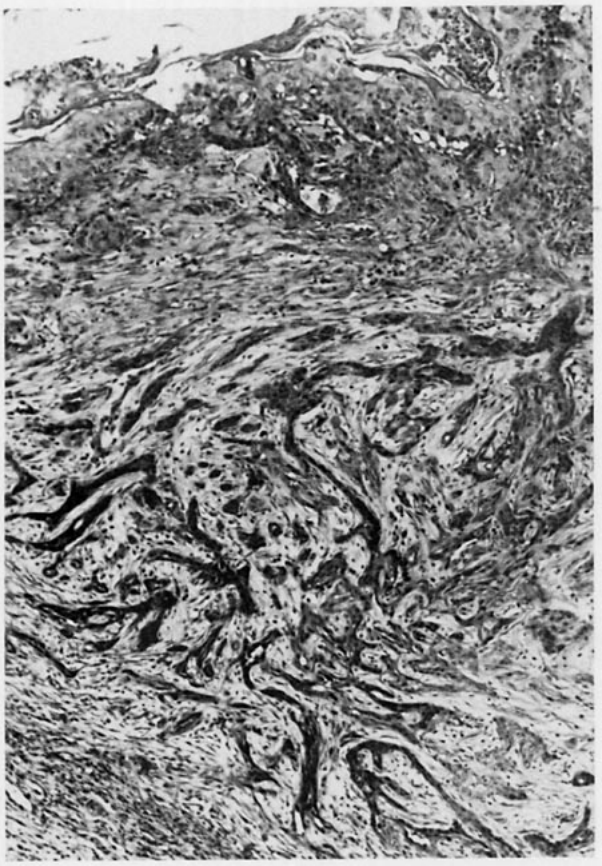


FIGURE 5. Zymbal's gland carcinoma in a female F344/N rat administered 150 ppm 3,3′-dimethylbenzidine dihydrochloride in drinking water for 14 months. This carcinoma consists of stratified squamous epithelium that forms irregular cords and deeply invades the underlying connective tissue. (×75)

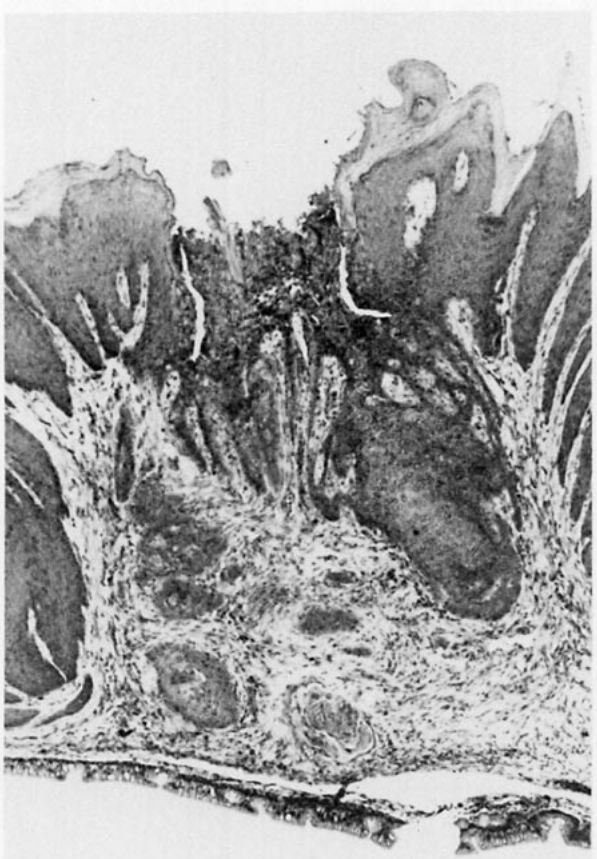


FIGURE 6. Squamous cell carcinoma of the posterior oral cavity in a male F344/N rat administered 150 ppm 3,3′-dimethylbenzidine dihydrochloride in drinking water for 14 months. Cords and clusters of neoplastic epithelial cells have deeply invaded the underlying connective tissue. The layer of respiratory epithelium lining the nasopharynx appears at the bottom of figure. (×70)

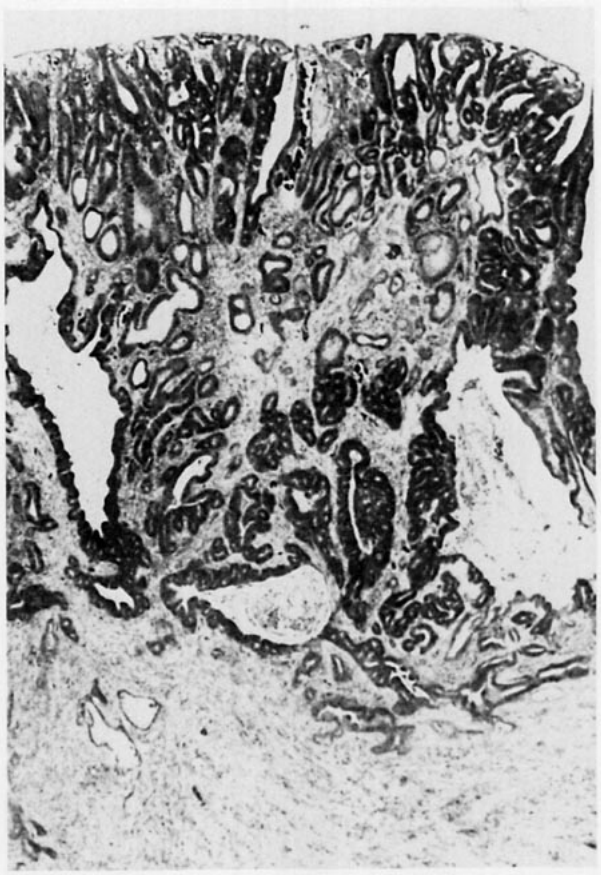


FIGURE 7. Adenocarcinoma of the mucuosal epithelium of the colon in a male F344/N rat administered 150 ppm 3,3′-dimethylbenzidine dihydrochloride in drinking water for 14 months. Multiple irregular glandular structures composed of neoplasitc epithelial cells have invaded the underlying submucosa, causing the mucosa to appear substantially thicker than normal. Some of the glands are dilated and contain mucus and debris. (×35)

TABLE 11
Zymbal's Gland Lesions in F344/N Rats in the 14-Month Drinking Water Studies of 3,3′-Dimethylbenzidine Dihydrochloride

	0 ppm	30 ppm	70 ppm	150 ppm
Male				
Hyperplasia				
Overall rates	0/59 (0%)	0/45 (0%)	1/75 (1%)	8/59 (14%)
Adenoma				
Overall rates	1/60 (2%)	1/45 (2%)	13/75 (17%)	16/60 (27%)
Effective rates[a]	1/60 (2%)	1/44 (2%)	13/72 (18%)	16/54 (30%)
Terminal rates	1/60 (2%)	1/41 (2%)	10/50 (20%)	0/0
Day of first observation	419 (T)	419 (T)	378	254
Life table tests	P<0.001	P=0.674	P<0.001	P<0.001
Cochran-Armitage test[b]	P<0.001			
Fisher exact test[b]		P=0.670	P=0.002	P<0.001
Carcinoma				
Overall rates	0/60 (0%)	2/45 (4%)	21/75 (28%)	23/60 (38%)
Effective rates	0/60 (0%)	2/45 (4%)	21/74 (28%)	23/60 (38%)
Terminal rates	0/59 (0%)	0/41 (0%)	6/50 (12%)	0/0
Day of first observation		359	229	209
Life table tests	P<0.001	P=0.170	P<0.001	P<0.001
Cochran-Armitage test	P<0.001			
Fisher exact test		P=0.181	P<0.001	P<0.001
Adenoma or Carcinoma[c]				
Overall rates	1/60 (2%)	3/45 (7%)	32/75 (43%)	36/60 (60%)
Effective rates	1/60 (2%)	3/45 (7%)	32/74 (43%)	36/60 (60%)
Terminal rates	1/59 (2%)	1/41 (2%)	15/50 (30%)	0/0
Day of first observation	419 (T)	359	229	209
Life table tests	P<0.001	P=0.192	P<0.001	P<0.001
Cochran-Armitage test	P<0.001			
Fisher exact test		P=0.209	P<0.001	P<0.001
Female				
Hyperplasia				
Overall rates	0/60 (0%)	4/44 (9%)	7/73 (9%)	2/60 (3%)
Adenoma				
Overall rates	0/60 (0%)	4/45 (9%)	11/75 (15%)	12/60 (20%)
Effective rates	0/60 (0%)	4/45 (9%)	11/72 (15%)	12/57 (21%)
Terminal rates	0/59 (0%)	4/39 (10%)	5/32 (16%)	3/10 (30%)
Day of first observation		421 (T)	338	251
Life table tests	P<0.001	P=0.024	P<0.001	P<0.001
Cochran-Armitage test	P<0.001			
Fisher exact test		P=0.031	P<0.001	P<0.001

TABLE 11
Zymbal's Gland Lesions in F344/N Rats in the 14-Month Drinking Water Studies of 3,3'-Dimethylbenzidine Dihydrochloride (continued)

	0 ppm	30 ppm	70 ppm	150 ppm
Carcinoma				
Overall rates	0/60 (0%)	2/45 (4%)	22/75 (29%)	35/60 (58%)
Effective rates	0/60 (0%)	2/45 (4%)	22/74 (30%)	35/59 (59%)
Terminal rates	0/59 (0%)	0/39 (0%)	1/32 (3%)	3/10 (30%)
Day of first observation		357	184	229
Life table tests	P<0.001	P=0.176	P<0.001	P<0.001
Cochran-Armitage test	P<0.001			
Fisher exact test		P=0.181	P<0.001	P<0.001
Adenoma or Carcinoma[d]				
Overall rates	0/57 (0%)	6/45 (13%)	32/75 (43%)	42/60 (70%)
Effective rates	0/60 (0%)	6/4[illegible] (13%)	32/74 (43%)	42/59 (71%)
Terminal rates	0/59 (0%)	4/39 (10%)	6/32 (19%)	5/10 (50%)
Day of first observation		357	184	229
Life table tests	P<0.001	P=0.[illegible]	P<0.001	P<0.001
Cochran-Armitage test	P<0.001			
Fisher exact test		P=0.005	P<0.001	P<0.001

(T)Terminal sacrifice
[a] Number of tumor-bearing animals/effective number of animals, i.e. number of animals alive at the first occurrence of this tumor type in any of the groups
[b] Based on effective rates
[c] 2-year historical incidence for untreated control groups at study laboratory (mean): 1/100 (1%); historical incidence for untreated control groups in NTP studies (mean ± SD): 19/1,596 (1.2% ± 1.9%)
[d] 2-year historical incidence for untreated control groups at study laboratory (mean): 1/100 (1%); historical incidence for untreated control groups in NTP studies (mean ± SD): 14/1,643 (0.9% ± 1.5%)

Clitoral and Preputial Glands: The clitoral glands of the female rat are bilateral modified sebaceous glands located near the base of the clitoris. The preputial glands of the male rat are homologous organs located adjacent to the penis. There was a marked increase in the incidence of clitoral gland adenomas and carcinomas in treated females (Table 12) and a moderate increase in the incidence of preputial gland adenomas in treated males (Table 13). A few female rats developed bilateral clitoral gland carcinomas. The increase in incidence of adenomas, carcinomas, and adenomas or carcinomas (combined) was significant in all treated female groups. The increase in preputial gland neoplasms was significant only in high-dose males. Adenomas were discrete expansile masses exhibiting some loss of normal acinar architecture. Neoplastic cells were well differentiated and arranged in solid clusters with a scattering of ductlike structures containing debris. Carcinomas were poorly defined masses composed of disorganized sheets of pleomorphic cells that sometimes invaded the adjacent tissue. Carcinomas commonly contained areas of necrosis and exhibited greater cellular atypia and disordered growth than adenomas.

TABLE 12
Clitoral Gland Lesions in Female F344/N Rats in the 14-Month Drinking Water Study of 3,3'-Dimethylbenzidine Dihydrochloride

	0 ppm	30 ppm	70 ppm	150 ppm
Hyperplasia				
Overall rates	0/60 (0%)	1/45 (2%)	4/75 (5%)	0/59 (0%)
Adenoma				
Overall rates	0/60 (0%)	9/45 (20%)	32/75 (43%)	17/59 (29%)
Effective rates[a]	0/60 (0%)	9/45 (20%)	32/73 (44%)	17/58 (29%)
Terminal rates	0/59 (0%)	5/39 (13%)	14/32 (44%)	5/10 (50%)
Day of first observation		391	229	296
Life table tests	P<0.001	P<0.001	P<0.001	P<0.001
Cochran-Armitage test[b]	P<0.001			
Fisher exact test[b]		P<0.001	P<0.001	P<0.001
Carcinoma				
Overall rates	0/60 (0%)	5/45 (11%)	11/75 (15%)	18/59 (29%)
Effective rates	0/60 (0%)	5/45 (11%)	11/72 (15%)	18/55 (33%)
Terminal rates	0/59 (0%)	5/39 (13%)	3/32 (9%)	3/10 (30%)
Day of first observation		421 (T)	315	254
Life table tests	P<0.001	P=0.010	P<0.001	P<0.001
Cochran-Armitage test	P<0.001			
Fisher exact test		P=0.013	P<0.001	P<0.001
Adenoma or Carcinoma[c]				
Overall rates	0/60 (0%)	14/45 (31%)	42/75 (56%)	32/59 (54%)
Effective rates	0/60 (0%)	14/45 (31%)	42/73 (58%)	32/58 (55%)
Terminal rates	0/59 (0%)	10/39 (26%)	16/32 (50%)	7/10 (70%)
Day of first observation		391	229	254
Life table tests	P<0.001	P<0.001	P<0.001	P<0.001
Cochran-Armitage test	P<0.001			
Fisher exact test		P<0.001	P<0.001	P<0.001

(T)Terminal sacrifice
[a] Number of tumor-bearing animals/effective number of animals, i.e. number of animals alive at the first occurrence of this tumor type in any of the groups
[b] Based on effective rates
[c] Historical incidence for untreated control groups at the study laboratory (mean): 8/100 (8%); historical incidence for untreated control groups in NTP studies (mean ± SD): 115/1,643 (7.0 ± 4.8%)

TABLE 13
Preputial Gland Lesions in Male F344/N Rats in the 14-Month Drinking Water Study of 3,3′-Dimethylbenzidine Dihydrochloride

	0 ppm	30 ppm	70 ppm	150 ppm
Hyperplasia				
Overall rates	0/60 (0%)	0/45 (0%)	1/75 (1%)	5/60 (8%)
Adenoma				
Overall rates	2/60 (3%)	4/45 (9%)	4/75 (5%)	8/60 (13%)
Effective rates[a]	2/60 (3%)	4/44 (9%)	4/72 (6%)	8/49 (16%)
Terminal rates	2/60 (3%)	4/41 (10%)	4/50 (8%)	0/0
Day of first observation	419 (T)	419 (T)	419 (T)	280
Life table tests	P<0.001	P=0.182	P=0.258	P<0.001
Cochran-Armitage test[b]	P=0.018			
Fisher exact test[b]		P=0.206	P=0.430	P=0.022
Carcinoma				
Overall rates	0/60 (0%)	0/45 (0%)	2/75 (3%)	1/60 (2%)
Adenoma or Carcinoma[c]				
Overall rates	2/60 (3%)	4/45 (9%)	6/75 (8%)	9/60 (15%)
Effective rates	2/60 (3%)	4/44 (9%)	6/72 (8%)	9/49 (18%)
Terminal rates	2/60 (3%)	4/41 (10%)	6/50 (12%)	0/0
Day of first observation	419 (T)	419 (T)	419 (T)	280
Life table tests	P<0.001	P=0.182	P=0.086	P<0.001
Cochran-Armitage test	P=0.008			
Fisher exact test		P=0.206	P=0.205	P=0.011

(T)Terminal sacrifice
[a] Number of tumor-bearing animals/effective number of animals, i.e. number of animals alive at the first occurrence of this tumor type in any of the four groups
[b] Based on effective rates
[c] 2-year historical incidence for untreated control groups at study laboratory (mean): 5/100 (5%); historical incidence of untreated control groups in NTP studies (mean ± SD): 117/1,596 (7.3% ± 5.2%)

Liver: A variety of neoplastic and nonneoplastic lesions occurred with increased incidence in male and female rats treated with 3,3′-dimethylbenzidine dihydrochloride (Tables 14 and 15). The incidence of hepatocellular neoplasms was markedly increased in mid- and high-dose males and moderately increased in mid- and high-dose females; no hepatocellular neoplasms occurred in the control or low-dose groups. In treated males there was a marked increase in the incidence of both neoplastic nodules and hepatocellular carcinomas in the mid- and high-dose groups. (Neoplastic nodule was the term used previously for proliferative hepatocellular lesions currently classified as hepatocellular adenoma.) The increase in incidence of neoplastic nodules, hepatocellular carcinomas, and neoplastic nodule or hepatocellular carcinoma (combined) was highly statistically significant. A few males had multiple carcinomas, and many had multiple neoplastic nodules. A single hepatoblastoma occurred in one high-dose male. There was a moderate increase in the incidence of neoplastic nodules in mid- and high-dose females, and one mid-dose and one high-dose female each had a single carcinoma. The incidence of neoplastic nodules or carcinomas (combined) was significantly increased in both the mid- and high-dose female groups. Neoplastic nodules were discrete expansile masses that were larger than hepatic lobules and compressed the adjacent parenchyma. Hepatic plates within the neoplastic nodules were not organized in a normal lobular pattern and often intersected at near right angles with the plates in the adjacent normal liver. Neoplastic hepatocytes exhibited altered staining properties and slight pleomorphism and atypia. Hepatocellular carcinomas were larger than neoplastic nodules and consisted of markedly disorganized hepatocytes that formed solid clusters, glandular structures, or broad trabeculae several cell layers thick. Neoplastic hepatocytes generally showed moderate to marked pleomorphism and atypia. Hepatoblastomas arise within existing carcinomas and are thought to represent a highly undifferentiated form of hepatocellular neoplasm. The hepatoblastoma consisted of irregular clusters and cords of small fusiform cells with scant cytoplasm and deeply basophilic nuclei.

Cystic degeneration, hematopoietic cell proliferation, and foci of cellular alteration (basophilic, eosinophilic, and mixed cell foci) occurred with markedly increased incidence in treated rats of each sex. The incidence of focal and multifocal hepatocellular necrosis was slightly increased in both sexes, and the incidence of fatty change of hepatocytes was slightly increased in high-dose males and mid-dose females. The incidence of bile duct hyperplasia was markedly reduced in both sexes of treated rats as compared with controls; this is probably a reflection of the reduced survival in treated rats. Cystic degeneration is a common degenerative change in the rat liver and consists of focal clusters of variably sized cystic spaces containing granular eosinophilic material or erythrocytes. The increased incidence of hematopoiesis was presumably secondary to inflammation associated with the neoplasms in treated animals. Foci of cellular alteration consisted of poorly demarcated clusters of hepatocytes with altered cytoplasmic staining. Although some were large enough to be seen grossly, many of these foci were smaller than a hepatic lobule and caused minimal or no compression, blending with the adjacent normal parenchyma. Basophilic foci were characterized by cells with basophilic cytoplasm, while eosinophilic foci were composed of cells with cytoplasm that stained more vividly eosinophilic than that of normal hepatocytes. Mixed cell foci consisted of mixtures of cells with eosinophilic cytoplasm and cells with clear cytoplasm.

Oral Cavity (Tongue or Pharynx): Treatment with 3,3′-dimethylbenzidine dihydrochloride resulted in several squamous cell papillomas and carcinomas of the tongue and pharynx in male and female rats (Table 16). No squamous cell neoplasms of the oral cavity occurred in untreated rats of either sex. The incidence of squamous cell papillomas or carcinomas (combined) was significantly increased in the high-dose male and mid- and high-dose female groups. Papillomas were exophytic masses arising from the oral mucosal surface and extending into the oral cavity. They consisted of a stalk-like, highly branched core of fibrous tissue covered by a thickened layer of stratified squamous epithelium. Squamous cell carcinomas were flat, broad-based lesions of the oral epithelium consisting of disorganized clusters and cords of pleomorphic squamous epithelial cells invading the underlying submucosa (Figure 6). Invasion was often accompanied by fibrous tissue proliferation and inflammation.

TABLE 14
Liver Tumors in F344/N Rats in the 14-Month Drinking Water Studies of 3,3′-Dimethylbenzidine Dihydrochloride

	0 ppm	30 ppm	70 ppm	150 ppm
Male				
Neoplastic Nodule[a]				
Overall rates	0/60 (0%)	0/45 (0%)	29/75 (39%)	26/60 (43%)
Effective rates[b]	0/60 (0%)	0/44 (0%)	29/72 (40%)	26/49 (53%)
Terminal rates	0/60 (0%)	0/41 (0%)	23/50 (46%)	0/0
Day of first observation			393	280
Cochran-Armitage test[c]	P<0.001			
Fisher exact test[c]		–[d]	P<0.001	P<0.001
Hepatocellular Carcinoma				
Overall rates	0/60 (0%)	0/45 (0%)	12/75 (16%)	12/60 (20%)
Effective rates	0/60 (0%)	0/45 (0%)	12/72 (17%)	12/55 (22%)
Terminal rates	0/60 (0%)	0/41 (0%)	11/50 (22%)	0/0
Day of first observation			379	238
Cochran-Armitage test	P<0.001			
Fisher exact test		–	P<0.001	P<0.001
Neoplastic Nodule or Hepatocellular Carcinoma[e]				
Overall rates	0/60 (0%)	0/45 (0%)	35/75 (47%)	33/60 (55%)
Effective rates	0/60 (0%)	0/45 (0%)	35/72 (49%)	33/55 (60%)
Terminal rates	0/60 (0%)	0/41 (0%)	28/50 (56%)	0/0
Day of first observation			379	238
Cochran-Armitage test	P<0.001			
Fisher exact test		–	P<0.001	P<0.001
Female				
Neoplastic Nodule				
Overall rates	0/60 (0%)	0/45 (0%)	7/74 (9%)	3/60 (5%)
Effective rates	0/60 (0%)	0/45 (0%)	7/58 (12%)	3/36 (8%)
Terminal rates	0/59 (0%)	0/39 (0%)	6/32 (19%)	0/10 (0%)
Day of first observation			378	343
Cochran-Armitage test	P=0.014			
Fisher exact test		–	P=0.006	P=0.050
Hepatocellular Carcinoma				
Overall rates	0/60 (0%)	0/45 (0%)	1/74 (1%)	1/60 (2%)
Neoplastic Nodule[b] **or Hepatocellular Carcinoma**[f]				
Overall rates	0/60 (0%)	0/45 (0%)	7/74 (9%)	4/60 (7%)
Effective rates	0/60 (0%)	0/45 (0%)	7/58 (12%)	4/36 (11%)
Terminal rates	0/59 (0%)	0/39 (0%)	6/32 (19%)	0/10 (0%)
Day of first observation			378	343
Cochran-Armitage test	P=0.004			
Fisher exact test		–	P=0.006	P=0.018

(T)Terminal sacrifice
[a] Term used previously for lesions currently classified as hepatocellular adenoma.
[b] Number of tumor-bearing animals/effective number of animals, i.e., number of animals alive at the first occurrence of tumors in any of the groups
[c] Based on effective rates
[d] No tumors in dosed group or control group; no statistics performed
[e] 2-year historical incidence for untreated control groups at study laboratory (mean): 7/100 (7%); historical incidence for untreated control groups in NTP studies (mean ± SD): 78/1,591 (4.9% ± 4.3%)
[f] 2-year historical incidence for untreated control groups at study laboratory (mean): 2/100 (2%); historical incidence for untreated control groups in NTP studies (mean ± SD): 37/1,643 (2.3% ± 2.7%)

TABLE 15
Numbers of F344/N Rats with Selected Nonneoplastic Liver Lesions in the 14-Month Drinking Water Studies of 3,3′-Dimethylbenzidine Dihydrochloride

Lesion	Male				Female			
	0 ppm	30 ppm	70 ppm	150 ppm	0 ppm	30 ppm	70 ppm	150 ppm
Number examined	60	45	75	60	60	45	74	60
Basophilic focus	1	31 ***	54 ***	27 ***	0	13 ***	11 ***	3
Eosinophilic focus	0	0	57 ***	53 ***	0	7 **	57 ***	38***
Mixed cell focus	0	37 ***	54 ***	30 ***	0	34 ***	49 ***	32***
Fatty change	1	2	1	7 *	0	0	4	2
Hematopoietic cell proliferation	0	2	27 ***	15 ***	0	7 **	19 ***	8**
Cystic degeneration	0	24 ***	67 ***	51 ***	0	3	12 ***	11***
Focal or multifocal necrosis	3	4	10	5	0	3	7 *	2

* Significantly different ($P \leq 0.05$) from the control group by Fisher exact test; based on effective rates
** $P \leq 0.01$
*** $P \leq 0.001$

TABLE 16
Squamous Cell Tumors of the Oral Cavity in F344/N Rats in the 14-Month Drinking Water Studies of 3,3′-Dimethylbenzidine Dihydrochloride

	0 ppm	30 ppm	70 ppm	150 ppm
Male				
Squamous Cell Papilloma				
Overall rates	0/60 (0%)	0/45 (0%)	3/75 (4%)	2/60 (3%)
Squamous Cell Carcinoma				
Overall rates	0/60 (0%)	0/45 (0%)	1/75 (1%)	3/60 (5%)
Squamous Cell Papilloma or Carcinoma[d]				
Overall rates	0/60 (0%)	0/45 (0%)	4/75 (5%)	5/60 (8%)
Effective rates[a]	0/60 (0%)	0/44 (0%)	4/67 (6%)	5/32 (16%)
Terminal rates	0/60 (0%)	0/41 (0%)	3/50 (6%)	0/0 (0%)
Day of first observation			341	324
Cochran-Armitage test[b]	P<0.001			
Fisher exact test[b]		–[c]	P=0.074	P=0.004
Female				
Squamous Cell Papilloma				
Overall rates	0/60 (0%)	3/45 (7%)	7/75 (9%)	9/60 (15%)
Effective rates	0/60 (0%)	3/45 (7%)	7/73 (10%)	9/59 (15%)
Terminal rates	0/59 (0%)	3/39 (8%)	3/32 (9%)	3/10 (30%)
Day of first observation		421 (T)	363	229
Cochran-Armitage test	P=0.002			
Fisher exact test		P=0.076	P=0.013	P=0.001
Squamous Cell Carcinoma				
Overall rates	0/60 (0%)	1/45 (2%)	2/75 (3%)	4/60 (7%)
Effective rates	0/60 (0%)	1/45 (2%)	2/64 (3%)	4/41 (10%)
Terminal rates	0/59 (0%)	1/39 (3%)	2/32 (6%)	1/10 (10%)
Day of first observation		421 (T)	421 (T)	338
Cochran-Armitage test	P=0.008			
Fisher exact test		P=0.429	P=0.264	P=0.025
Squamous Cell Papilloma or Carcinoma[d]				
Overall rates	0/60 (0%)	3/45 (7%)	9/75 (12%)	13/60 (22%)
Effective rates	0/60 (0%)	3/45 (7%)	9/73 (12%)	13/59 (22%)
Terminal rates	0/59 (0%)	3/39 (8%)	5/32 (16%)	4/10 (40%)
Day of first observation		421 (T)	363	229
Cochran-Armitage test	P<0.001			
Fisher exact test		P=0.076	P=0.004	P<0.001

(T)Terminal sacrifice
[a] Number of tumor-bearing animals/effective number of animals, i.e. number of animals alive at the first occurrence of this tumor type in any of the groups
[b] Based on effective rates
[c] No tumors in dosed group or control group; statistical test not performed
[d] Details for the incidence of these neoplasms in the tongue and pharynx are presented in Tables A1 (males) and B1 (females). Historical incidence in untreated control groups in NTP studies: 7/1,596 for males and 4/1,643 for females.

Small Intestine (Duodenum, Jejunum, or Ileum): Adenocarcinomas and adenomatous polyps of the small intestine mucosa occur rarely in untreated F344/N rats. Several of these neoplasms were found in treated male and female rats in these studies (Table 17). All neoplasms occurred in mid- and high-dose rats, with the exception of a single adenomatous polyp in a low-dose female. The incidence of adenomatous polyps or adenocarcinomas (combined) was significantly increased in the high-dose male and female groups. Adenocarcinomas were poorly demarcated masses that invaded the intestinal wall. They consisted of irregular, variably sized clusters and glandular structures of moderately to poorly differentiated columnar cells, often surrounded by proliferating fibrous tissue containing inflammatory cells. Some adenocarcinomas contained mucus secreting cells that formed large cystic spaces filled with mucus (cystic mucinous adenocarcinoma). Adenomatous polyps were pedunculated masses that projected into the intestinal lumen. They consisted of a stalk-like core of fibrous tissue covered by numerous glandular structures lined by a single layer of moderately well-differentiated tall columnar cells with round nuclei and abundant basophilic cytoplasm.

Large Intestine (Cecum, Colon, or Rectum): Adenocarcinomas and adenomatous polyps of the large intestine mucosa are rarely seen in untreated F344/N rats. Several of these neoplasms occurred in treated male and female rats in these studies (Table 18). All neoplasms occurred in mid- or high-dose animals, except for a single adenomatous polyp in a low-dose female. Two high-dose males had multiple polyps. The incidence of adenomatous polyps or adenocarcinomas (combined) was significantly increased in the high-dose male and female groups.

The histologic appearance of adenocarcinomas (Figure 7) and adenomatous polyps of the large intestine was similar to that of the small intestine.

TABLE 17
Tumors of the Small Intestine in F344/N Rats in the 14-Month Drinking Water Studies of 3,3′-Dimethylbenzidine Dihydrochloride

	0 ppm	30 ppm	70 ppm	150 ppm
Male				
Adenomatous Polyp				
Overall rates	0/60 (0%)	0/45 (0%)	1/75 (1%)	1/60 (2%)
Adenocarcinoma				
Overall rates	0/60 (0%)	0/45 (0%)	3/75 (4%)	8/60 (13%)
Effective rates[a]	0/60 (0%)	0/45 (0%)	3/74 (4%)	8/59 (14%)
Terminal rates	0/60 (0%)	0/41 (0%)	3/50 (6%)	0/0 (0%)
Day of first observation			419 (T)	211
Cochran-Armitage test[b]	P<0.001			
Fisher exact test[b]		–[c]	P=0.165	P=0.003
Adenomatous Polyp or Adenocarcinoma[d]				
Overall rates	0/60 (0%)	0/45 (0%)	4/75 (5%)	8/60 (13%)
Effective rates	0/60 (0%)	0/45 (0%)	4/74 (5%)	8/59 (14%)
Terminal rates	0/60 (0%)	0/41 (0%)	3/50 (6%)	0/0 (0%)
Day of first observation			379	211
Cochran-Armitage test	P<0.001			
Fisher exact test		–	P=0.090	P=0.003
Female				
Adenomatous Polyp				
Overall rates	0/60 (0%)	1/45 (2%)	1/75 (1%)	0/60 (0%)
Adenocarcinoma				
Overall rates	0/60 (0%)	0/45 (0%)	2/75 (3%)	5/60 (8%)
Effective rates	0/60 (0%)	0/45 (0%)	2/72 (3%)	5/57 (9%)
Terminal rates	0/59 (0%)	0/39 (0%)	0/32 (0%)	0/10 (0%)
Day of first observation			309	251
Cochran-Armitage test	P=0.003			
Fisher exact test		–	P=0.296	P=0.025
Adenomatous Polyp or Adenocarcinoma[e]				
Overall rates	0/60 (0%)	1/45 (2%)	3/75 (5%)	5/60 (8%)
Effective rates	0/60 (0%)	1/45 (2%)	3/72 (4%)	5/57 (9%)
Terminal rates	0/59 (0%)	0/39 (0%)	1/32 (3%)	0/10 (0%)
Day of first observation		391	309	251
Cochran-Armitage test	P=0.011			
Fisher exact test		P=0.429	P=0.159	P=0.025

(T)Terminal sacrifice
[a] Number of tumor-bearing animals/effective number of animals, i.e. number of animals alive at the first occurrence of this tumor type in any of the groups
[b] Based on effective rates
[c] No tumors in dosed group or control group; statistical test not performed
[d] 2-year historical incidence for untreated control groups at study laboratory (mean): 1/97 (1%); historical incidence for untreated control groups in NTP studies (mean ± SD): 5/1,557 (0.3± 0.7%)
[e] 2-year historical incidence for untreated control groups at study laboratory (mean): 0/99; historical incidence for untreated control groups in NTP studies (mean ± SD): 0/1,611

TABLE 18
Tumors of the Large Intestine in F344/N Rats in the 14-Month Drinking Water Studies of 3,3′-Dimethylbenzidine Dihydrochloride

	0 ppm	30 ppm	70 ppm	150 ppm
Male				
Adenomatous Polyp				
Overall rates	0/60 (0%)	0/45 (0%)	6/75 (8%)	9/60 (15%)
Effective rates[a]	0/60 (0%)	0/44 (0%)	6/67 (9%)	9/38 (24%)
Terminal rates	0/60 (0%)	0/41 (0%)	5/50 (10%)	0/0 (0%)
Day of first observation			384	308
Cochran-Armitage test[b]	P<0.001			
Fisher exact test[b]		–[c]	P=0.019	P<0.001
Adenocarcinoma				
Overall rates	0/60 (0%)	0/45 (0%)	0/75 (0%)	7/60 (12%)
Effective rates	0/60 (0%)	0/45 (0%)	0/67 (0%)	7/36 (19%)
Terminal rates	0/60 (0%)	0/41 (0%)	0/50 (0%)	0/0 (0%)
Day of first observation				309
Cochran-Armitage test	P=0.001			
Fisher exact test		–	–	P=0.001
Adenomatous Polyp or Adenocarcinoma[d]				
Overall rates	0/60 (0%)	0/45 (0%)	6/75 (8%)	15/60 (25%)
Effective rates	0/60 (0%)	0/45 (0%)	6/67 (9%)	15/38 (39%)
Terminal rates	0/60 (0%)	0/41 (0%)	5/50 (10%)	0/0 (0%)
Day of first observation			384	308
Cochran-Armitage test	P<0.001			
Fisher exact test		–	P=0.019	P<0.001
Female				
Adenomatous Polyp				
Overall rates	0/60 (0%)	1/45 (2%)	6/75 (8%)	4/60 (7%)
Effective rates	0/60 (0%)	1/45 (2%)	6/70 (9%)	4/46 (9%)
Terminal rates	0/59 (0%)	1/39 (3%)	4/32 (13%)	1/10 (0%)
Day of first observation		421 (T)	310	355
Cochran-Armitage test	P=0.020			
Fisher exact test		P=0.429	P=0.022	P=0.033
Adenocarcinoma				
Overall rates	0/60 (0%)	0/45 (0%)	1/75 (1%)	1/60 (2%)
Adenomatous Polyp or Adenocarcinoma[e]				
Overall rates	0/60 (0%)	1/45 (2%)	7/75 (9%)	4/60 (7%)
Effective rates	0/60 (0%)	1/45 (2%)	7/70 (10%)	4/46 (9%)
Terminal rates	0/59 (0%)	1/39 (3%)	4/32 (13%)	1/10 (10%)
Day of first observation		421 (T)	310	355
Cochran-Armitage test	P=0.021			
Fisher exact test		P=0.429	P=0.011	P=0.033

(T)Terminal sacrifice
[a] Number of tumor-bearing animals/effective number of animals, i.e. number of animals alive at the first occurrence of this tumor type in any of the groups
[b] Based on effective rates
[c] No tumors in dosed group or control group; statistical test not performed
[d] 2-year historical incidence for untreated control groups at study laboratory (mean): 0/96 (0%); historical incidence for untreated controls in NTP studies (mean ± SD): 2/1,541 (0.1% ± 0.4%)
[e] 2-year historical incidence for untreated control groups at study laboratory (mean): 0/88 (0%); historical incidence for untreated controls in NTP studies (mean ± SD): 0/1,601 (0% ± 0%)

TABLE 19
Mammary Gland Tumors in Female F344/N Rats in the 14-Month Drinking Water Study of 3,3′-Dimethylbenzidine Dihydrochloride

	0 ppm	30 ppm	70 ppm	150 ppm
Adenocarcinoma[a]				
Overall rates	0/60 (0%)	1/45 (2%)	3/75 (4%)	6/60 (10%)
Effective rates[b]	0/60 (0%)	1/45 (2%)	3/71 (4%)	6/51 (12%)
Terminal rates	0/59 (0%)	1/39 (3%)	1/32 (3%)	3/10 (30%)
Day of first observation		421 (T)	363	285
Cochran-Armitage test[c]	P=0.002			
Fisher exact test[c]		P=0.429	P=0.156	P=0.008

(T)Terminal sacrifice
[a] 2-year historical incidence for untreated control groups at study laboratory (mean): 2/100 (2%); historical incidence in NTP studies (mean ± SD): 44/1,643 (2.7% ± 2.2%)
[b] Number of tumor-bearing animals/effective number of animals, i.e. number of animals alive at the first occurrence of this tumor type in any of the groups
[c] Based on effective rates

Mammary Gland: Mammary gland adenocarcinomas, uncommon neoplasms of the female F344/N rat, occurred with a dose-related increased incidence (Table 19). The increase in the high-dose group was significant as compared with controls and was above the highest overall historical incidence for untreated female F344/N rats from 2-year NTP studies [44/1643 (2.7%), range 0-8%]. There was no increase in the incidence of fibroadenomas (control, 2/60; low dose, 1/45; mid dose, 4/75; high dose 0/60).

Lung: Alveolar/bronchiolar adenomas occurred with a slightly increased incidence in treated males relative to untreated controls (Table 20). The incidence of focal or multifocal hyperplasia of alveolar epithelium was markedly increased in treated rats of each sex, while the incidence of histiocytic cellular infiltration of the lung was markedly increased in females (control, 12/60, 20%; low dose, 23/45, 51%; mid dose, 36/74, 49%; high dose, 29/60, 48%). The incidence of alveolar/bronchiolar adenoma or carcinoma (combined) was significantly increased in male rats in the mid- and high-dose groups. The lower incidence of alveolar epithelial hyperplasia in high-dose males and females relative to that in mid-dose rats may have been due to the markedly decreased survival of the high-dose groups.

TABLE 20
Lung Tumors in F344/N Rats in the 14-Month Drinking Water Studies of 3,3'-Dimethylbenzidine Dihydrochloride

	0 ppm	30 ppm	70 ppm	150 ppm
Male				
Alveolar Epithelial Hyperplasia				
Overall rates	5/60 (8%)	14/45 (31%)	31/75 (41%)	17/60 (28%)
Alveolar/Bronchiolar Adenoma				
Overall rates	1/60 (2%)	0/45 (0%)	7/75 (9%)	6/60 (10%)
Effective rates[a]	1/60 (2%)	0/45 (0%)	7/73 (10%)	6/57 (11%)
Terminal rates	1/60 (2%)	0/41 (0%)	6/50 (12%)	0/0 (0%)
Day of first observation	419 (T)		406	226
Cochran-Armitage test[b]	P=0.012			
Fisher exact test[b]		P=0.571N	P=0.057	P=0.049
Alveolar/Bronchiolar Carcinoma				
Overall rates	0/60 (0%)	0/45 (0%)	1/75 (1%)	0/60 (0%)
Alveolar/Bronchiolar Adenoma or Carcinoma[c]				
Overall rates	1/60 (2%)	0/45 (0%)	8/75 (11%)	6/60 (10%)
Effective rates	1/60 (2%)	0/45 (0%)	8/73 (11%)	6/57 (11%)
Terminal rates	1/60 (2%)	0/41 (0%)	7/50 (14%)	0/0 (0%)
Day of first observation	419 (T)		406	226
Cochran-Armitage test	P=0.013			
Fisher exact test		P=0.571N	P=0.033	P=0.049
Female				
Alveolar Epithelial Hyperplasia				
Overall rates	1/60 (2%)	11/45 (24%)	30/73 (41%)	13/60 (22%)
Alveolar/Bronchiolar Adenoma				
Overall rates	1/60 (2%)	1/45 (2%)	3/74 (4%)	3/60 (5%)
Effective rates	1/60 (2%)	1/45 (2%)	3/63 (5%)	3/41 (7%)
Terminal rates	1/59 (2%)	1/39 (3%)	2/32 (6%)	0/10 (0%)
Day of first observation	421 (T)	421 (T)	338	370
Cochran-Armitage test	P=0.094			
Fisher exact test		P=0.676	P=0.328	P=0.181
Alveolar/Bronchiolar Carcinoma				
Overall rates	0/60 (0%)	0/45 (0%)	0/74 (0%)	1/60 (2%)
Alveolar/Bronchiolar Adenoma or Carcinoma[d]				
Overall rates	1/60 (2%)	1/45 (2%)	3/74 (4%)	4/60 (7%)
Effective rates	1/60 (2%)	1/45 (2%)	3/63 (5%)	4/41 (10%)
Terminal rates	1/59 (2%)	1/39 (3%)	2/32 (6%)	0/10 (0%)
Day of first observation	421 (T)	421 (T)	338	370
Cochran-Armitage test	P=0.033			
Fisher exact test		P=0.676	P=0.328	P=0.086

(T)Terminal sacrifice
[a] Number of tumor-bearing animals/effective number of animals, i.e. number of animals alive at the first occurrence of this tumor type in any of the groups
[b] Based on effective rates
[c] 2-year historical incidence for untreated control groups at study laboratory (mean): 1/100 (1%); historical incidence for untreated control groups in NTP studies (mean ± SD): 44/1593 (2.8% ± 2.3%)
[d] 2-year historical incidence for untreated control groups at study laboratory (mean): 1/100 (1%); historical incidence for untreated control groups in NTP studies (mean ± SD): 25/1639 (1.5% ± 1.5%)

TABLE 21
Mesotheliomas in Male F344/N Rats in the 14-Month Drinking Water Study of 3,3'-Dimethylbenzidine Dihydrochloride

	0 ppm	30 ppm	70 ppm	150 ppm
All Organs: Mesothelioma (Benign, Malignant, and NOS)[a]				
Overall rates	0/60 (0%)	0/45 (0%)	3/75 (4%)	4/60 (7%)
Effective rates[b]	0/60 (0%)	0/45 (0%)	3/67 (4%)	4/38 (11%)
Terminal rates	0/60 (0%)	0/41 (0%)	3/50 (6%)	0/0
Day of first observation			419 (T)	308
Life table tests	P<0.001	–[d]	P=0.092	P<0.001
Cochran-Armitage test[c]	P=0.003			
Fisher exact test[c]		–[d]	P=0.144	P=0.020

(T)Terminal sacrifice
[a] 2-year historical incidence for untreated control groups at study laboratory (mean): 3/100 (3%); historical incidence for untreated control groups in NTP studies (mean ± SD): 47/1596 (2.9% ± 2.6%)
[b] Number of tumor-bearing animals/effective number of animals, i.e. number of animals alive at the first occurrence of this tumor type in any of the dose groups
[c] No tumors in dosed group or control group; statistical test not performed
[d] Based on effective rates

Mesothelium: Malignant mesotheliomas of the testis and/or epididymis occurred in a few mid- and high-dose males (Table 21). The incidence of mesotheliomas showed a positive trend, and the incidence in high-dose males was significantly increased.

Brain: Small numbers of rare malignant neoplasms of glial cell origin (astrocytoma and glioma) or meningeal origin (malignant meningioma and meningeal sarcoma) occurred in treated male and female rats. Gliomas are poorly differentiated glial cell neoplasms often consisting of a mixture of cells with different morphologies; astrocytomas are better differentiated glial cell neoplasms in which neoplastic cells resemble astrocytes. Malignant gliomas occurred in one mid-dose and one high-dose male and in one mid-dose and one high-dose female. Malignant astrocytomas occurred in one low-dose and one mid-dose female. Malignant meningioma occurred in one low-dose female and one high-dose male; a single meningeal sarcoma, a presumably less-differentiated form of malignant meningioma, occurred in a mid-dose male. Brain neoplasms in control animals are most commonly found at the 2-year terminal sacrifice. The earliest occurring brain neoplasm in this study was the malignant meningioma in the high-dose male that died during week 48. The earliest occurring glial cell neoplasm was the malignant glioma in the high-dose male that died on week 50. The occurrence of these neoplasms solely in treated rats, combined with the early occurrences of these neoplasms, indicates they may have been treatment related.

Adrenal Gland Medulla: Benign pheochromocytomas occurred at a marginally increased incidence in treated male rats (control, 0/60; low dose, 2/45, 4%; mid dose, 1/75, 1%; high dose, 3/59, 5%). The incidence in high-dose males was significantly increased relative to controls. Focal hyperplasia of the adrenal medulla, a lesion generally considered a precursor to pheochromocytoma, was seen in only one mid-dose male. The lack of a dose-related increase in the incidence of pheochromocytomas and the absence of a treatment-related increase in the incidence of hyperplasia in this study indicate the marginal increase in incidence in the high-dose group is not a significant treatment-related effect.

Hematopoietic System: Mononuclear cell leukemia in treated female rats occurred with a slightly increased incidence as compared with controls (control, 1/60; low dose, 3/45; mid dose, 6/75; high dose, 4/60). Mononuclear cell leukemias are generally seen late in life; most cases occur after 18 months of age, and the incidence increases with increasing age. In this study there was substantial early mortality in treated animals due to treatment-related neoplasms in a variety of other tissues, necessitating termination of the study at 14 months. Since the majority of leukemias would not be expected to occur prior to 14 months, the animals in this study were not at risk for a long enough period of time to allow for the full development of leukemias in either control or treated animals. The slightly increased incidences of mononuclear cell leukemia suggest there may have been an earlier onset of leukemia in treated female rats, indicating that the increase in leukemias may have been treatment-related.

Testes: The incidence of interstitial cell adenomas, very common neoplasms in male F344/N rats, was significantly increased in low-dose males as compared with controls (control, 24/60, 40%; low dose, 26/45, 58%; mid dose, 26/75, 35%; high dose 2/60, 3%). However, the incidence of interstitial cell hyperplasia was similar in control and low-dose groups (44/60, 73%; 35/45, 78%; 28/75, 37%; 4/60, 7%). The low incidence in the high-dose group is presumably related to the high early mortality. The increase in the incidence of adenomas in the low-dose group was considered unlikely to be treatment-related because there was no apparent treatment effect on the incidence of interstitial cell hyperplasia, the precursor to adenoma. The lack of a treatment-related increase in hyperplasia is not due to decreased survival since the total number of mid-dose males surviving to study termination (50) was comparable to the number of controls living to termination (60). Consequently, the marginal increase in the incidence of adenomas in the low-dose group is not considered to be treatment related.

Uterus:The incidence of stromal polyps showed a significant negative trend in treated female rats (control, 11/60; low dose, 9/44; mid dose, 3/75; high dose, 1/60). The incidence in mid- and high-dose females was significantly reduced as compared with controls, but was considered secondary to the decreased survival in these groups.

Kidney: Treatment with 3,3′-dimethylbenzidine dihydrochloride was associated with an increased incidence of nephropathy in treated females as compared with controls and a notable increase in the severity of nephropathy in high-dose males and in mid- and high-dose females (Table 22). Nephropathy was characterized by tubule epithelial degeneration and necrosis and accompanied by regenerative tubule cell proliferation that was most marked in males. Minimal karyomegaly, a slight increase in the size of renal tubule cell nuclei, was diagnosed in the kidneys of numerous treated females by the study pathologist. The Pathology Working Group (PWG) reviewed this lesion and believed it to be indicative of regenerative tubule cell proliferation occurring as a part of the nephropathy. Thus, the PWG concluded that karyomegaly in treated females did not represent a distinct treatment-related effect. A single renal tubule adenoma occurred in one high-dose female.

TABLE 22
Incidences and Severity of Nephropathy in Male and Female F344/N Rats in the 14-Month Drinking Water Studies of 3,3'-Dimethylbenzidine Dihydrochloride

	0 ppm	30 ppm	70 ppm	150 ppm
Male				
Nephropathy[a]	60/60 (1.7)	43/45 (1.9)	74/75 (2.0)	59/59 (3.4)
Female				
Nephropathy	47/60 (1.3)	44/45 (1.7)	72/74 (2.9)	59/60 (2.9)

[a] Values in parentheses are average severity grades; 1=minimal, 2=mild, 3=moderate, and 4=marked.

Parathyroid Gland: Hyperplasia of the parathyroid gland occurred in 11/58 high-dose males, 3/58 high-dose females, and 5/70 mid-dose females; none was seen in any other group. These lesions are presumed to be secondary to the increased severity of nephropathy in treated rats.

Heart: Minimal to mild cardiomyopathy occurred with increased incidence in treated males (control, 17/60; low dose, 19/45; mid dose, 35/75; high dose 31/59).

Glandular Stomach: Cystic degeneration of the mucosa of the glandular stomach, a common change in aging F344/N rats, consists of atrophy of glandular epithelium and dilatation of the glandular lumens. The incidence of this lesion was increased in treated females (control, 1/60; low dose, 3/45; mid dose, 6/74; high dose, 11/60), but not in males (22/60; 0/45; 4/75; 15/59).

Adrenal Gland Cortex: Angiectasis, characterized by mild dilatation of cortical sinusoids, was seen in treated females (0/60; 0/45; 8/74; 8/60). Cytoplasmic vacuolation of adrenocortical cells occurred with increased frequency in treated males (0/60; 0/44; 5/75; 5/59) and females (2/60; 0/45; 10/74; 6/60).

Spleen: The incidence of hematopoietic cell proliferation was increased in treated males (control, 0/60; low dose, 1/45; mid dose, 22/75; high dose, 11/55) and females (0/60; 6/45; 20/74; 14/60). The increased incidence was considered to be secondary to inflammation associated with neoplasia.

Bone Marrow: Hyperplasia or atrophy of the hematopoietic cell elements of the bone marrow occurred in a few treated males (hyperplasia: mid dose, 1/75; high dose, 3/60; atrophy: high dose, 8/60) and females (hyperplasia: high dose, 7/59; atrophy: mid dose, 1/73; high dose, 4/59). These lesions were not observed in controls.

Mandibular Lymph Node: Plasma cell hyperplasia, characterized by increased numbers of plasma cells within the medullary areas, occurred with increased incidence in treated males (control, 2/60; low dose, 5/45; mid dose, 18/74; high dose, 16/60) and females(1/60; 5/43; 20/72; 18/60). This may have been secondary to the inflammatory response associated with Zymbal's gland neoplasms.

Nose: Suppurative inflammation occurred with increased incidence in mid- and high-dose females (mid-dose, 6/75; high-dose, 5/60).

Seminal Vesicle: The incidence of atrophy was increased in treated male rats (control, 0/60; low dose, 1/44; mid dose, 8/75; high dose, 5/58) and was probably secondary to debilitation in treated males.

GENETIC TOXICITY

3,3′-Dimethylbenzidine dihydrochloride produced positive responses at low doses in several tests for genetic toxicity. 3,3′-Dimethylbenzidine dihydrochloride was tested with a preincubation protocol for induction of gene mutations in *Salmonella typhimurium* strains TA100, TA1535, TA97, and TA98 in the presence and absence of Aroclor 1254-induced male Sprague-Dawley rat or Syrian hamster liver S9. Mutagenic activity was observed only in strain TA98 in the presence of S9 (Zeiger et al., 1988; Table C1). In cytogenetic tests with Chinese hamster ovary cells, 3,3′-dimethylbenzidine dihydrochloride induced sister-chromatid exchanges (SCE) (Table C2) and chromosomal aberrations (Table C3) in the absence of S9. Neither endpoint was elevated in trials conducted with S9 from Aroclor 1254-induced male Sprague-Dawley rat liver. In the SCE tests, positive responses were recorded in each of two trials without S9. In the chromosomal aberration assay, the first of two trials without S9 was negative, but in the second trial three intermediate dose levels produced significant increases in aberrations. 3,3′-Dimethylbenzidine dihydrochloride induced sex-linked recessive lethal mutations in the germ cells of male *Drosophila* when administered either in feed or by injection (Table C4). No induction of reciprocal translocations occurred in *Drosophila* germ cells following exposure of males by feeding (Table C5). Appendix C contains the methods and complete results of all genetic toxicology studies.

DISCUSSION AND CONCLUSIONS

Consumption of drinking water containing 3,3′-dimethylbenzidine dihydrochloride by rats led to highly significant increased incidences of neoplasms at a variety of sites and mild toxicity in several organs. In low-, mid-, and high-dose males, 13%, 64%, and 83%, respectively, were observed to have malignant neoplasms, and many animals in the mid- and high-dose groups had multiple malignant neoplasms. Similarly, malignant neoplasms were found in 31%, 65%, and 93% of low-, mid-, and high-dose females, with many animals in all dose groups having malignant neoplasms at multiple sites. Only 2% of male or female control rats had malignant neoplasms.

The principal sites and organs with neoplasms included the skin, Zymbal's gland, preputial and clitoral glands, liver, oral mucosa, and small and large intestine. In both sexes, the incidence of these neoplasms was dose related, and the tumor latency generally decreased with increasing dose. The occurrence of neoplasms at most of these sites in the F344/N rat is uncommon and often associated with exposure to genotoxic carcinogens. The short latency and multiple sites of these neoplasms are characteristic of genotoxic carcinogens such as the benzidine dyes (NCI, 1978a), 3,3′-dimethoxybenzidine (NTP, 1990a), benzene (NTP, 1986), 1,3-butadiene (NTP, 1984), and glycidol (NTP, 1990b).

14-DAY AND 13-WEEK STUDIES

In the 14-day and 13-week studies, male and female rats were exposed to 3,3′-dimethylbenzidine dihydrochloride in drinking water at concentrations ranging from 300 to 7,500 ppm. In the 14-day studies, all five males and one female receiving 7,500 ppm and 1/5 males receiving 5,000 ppm 3,3′-dimethylbenzidine dihydrochloride died. In the 13-week studies, all animals receiving 4,000 ppm and 4/10 males and 3/10 females receiving 2,000 ppm 3,3′-dimethylbenzidine dihydrochloride died. Mean necropsy body weights showed a dose-related decrease. Histopathologic evidence of hepatic (necrosis and pigment within the sinusoidal lining cells) and renal damage (increased severity of nephropathy) was seen in exposed rats. In addition,lymphocytic atrophy was observed in the thymus, mandibular and mesenteric lymph nodes, and spleen of treated animals, and atrophy of the bone marrow was seen in rats receiving 2,000 and 4,000 ppm. Water consumption was decreased with increasing chemical concentration.

Small dose-related decreases in hematocrit values indicated a slight anemia in males and females; however, the lack of a concomitant decrease in hemoglobin levels suggested that these decreases were a result of hemolysis. The slight increase in serum sorbitol dehydrogenase (SDH) activity in treated rats was indicative of mild liver damage.

Based on the decreased survival, reductions in dosed water consumption and body weight gain, and chemical-induced hepatocellular and renal lesions observed in the 13-week studies, the 9- and 14-month studies were conducted in male and female rats by administering 0, 30, 70, or 150 ppm 3,3′-dimethylbenzidine dihydrochloride in drinking water.

9-MONTH STUDIES

Carcinomas of the preputial and clitoral glands, Zymbal's gland, liver, skin, lung, oral cavity, and small intestine were observed in high-dose animals after exposure to 3,3′-dimethylbenzidine dihydrochloride for only 9 months. Basophilic foci and neoplastic nodules in the liver and hyperplasias of the Zymbal's gland and lung were also detected in exposed rats. These lesions were not detected in control rats. The short latency of these lesions is unusual and indicative of the carcinogenic potency of 3,3′-dimethylbenzidine dihydrochloride.

Hematologic effects indicated a mild anemia in treated male and female rats. Serum SDH levels were increased about tenfold in females and two-fold in males and were indicative of liver injury. A decrease in serum thyroxin (T_4) with an increase in thyroid stimulating hormone (TSH) is indicative of mild hypothyroidism in treated male and female rats.

14-MONTH STUDIES

3,3′-Dimethylbenzidine dihydrochloride studies were terminated at month 14 because of reduced survival in the dosed groups. All high-dose males had been found dead or killed moribund by week 55, and only about 25% of the high-dose females survived to week 56. Survival was influenced, in part, by an aggressive sacrifice program that, for humane reasons and to preclude autolysis, called for removal of animals with large ulcerated neoplasms. Mean body weights of the high-dose male and female groups were approximately 85% of those of corresponding control groups by weeks 28 and 32, respectively, and 70% and 75% by study termination, respectively. By week 44, the mean body weight of the mid-dose female group was 85% of that of the control group.

Clinical Findings

The most important clinical finding in the 14-month studies was the appearance of tissue masses on the head, over the back, and in the genital area. These masses, for the most part, represented the development of Zymbal's gland tumors, epithelial skin tumors, and preputial/clitoral gland tumors, respectively. Tissue masses at these three sites had a relatively short latency, with tissue masses on the head first appearing after 24 weeks of chemical exposure.

Nonneoplastic Lesions

3,3′-Dimethylbenzidine dihydrochloride exposure was associated with increased incidences of several nonneoplastic lesions. Cystic degeneration, a common degenerative change in the rat liver, was observed in the liver of exposed rats and appeared to be treatment related. Increased hematopoiesis in the liver was likely secondary to inflammation associated with neoplasms. A treatment-related increase in the incidence of nephropathy was observed in female rats, and an increase in the severity of nephropathy was observed in high-dose males and mid- and high-dose females. Although treatment related, the increase in alveolar macrophages (histiocytic cellular infiltration) observed in the lung of treated females was probably a nonspecific reaction and possibly a sequela to hyperpnea related to anemia or stress.

Neoplastic Lesions

Of the male rats exposed to 150 ppm 3,3′-dimethylbenzidine dihydrochloride in drinking water, 50% were found to have skin basal cell tumors, and 45% had squamous cell skin neoplasms. These neoplasms were not observed in untreated controls. Epithelial skin neoplasms were composed principally of basal or squamous cells. In treated males, basal cell tumors occurred as early as week 40 (observed at necropsy), and squamous cell tumors as early as week 30. Although the incidence of epithelial skin tumors in treated females was not as remarkable as that in males, the incidence of these neoplasms was significantly increased by 3,3′-dimethylbenzidine dihydrochloride treatment. Skin neoplasms detected in treated females were of the same morphological type as those observed in males and were a result of 3,3′-dimethylbenzidine dihydrochloride exposure.

Skin neoplasms could have been caused by systemic exposure to reactive 3,3′-dimethylbenzidine metabolites or by direct exposure to 3,3′-dimethylbenzidine in the drinking water. Because 3,3′-dimethylbenzidine dihydrochloride was administered in drinking water, exposure of skin during grooming was likely. Skin neoplasms may have resulted from direct exposure of the skin to the compound or to its metabolites in saliva, or from metabolism by the skin of 3,3′-dimethylbenzidine dihydrochloride to a reactive intermediate. No reports on the carcinogenicity of 3,3′-dimethylbenzidine dihydrochloride after dermal administration were found.

There was a highly significant correlation between the consumption of 3,3′-dimethylbenzidine dihydrochloride and the development of Zymbal's gland adenomas and/or carcinomas in treated males and females. With the exception of an adenoma in one control male, Zymbal's gland neoplasms were not observed in control groups. Carcinomas and adenomas were observed at necropsy in treated males as early as weeks 30 and 36, respectively, and in treated females, as early as weeks 26 and 36, respectively. Neoplasms infrequently develop spontaneously at this site (1% of untreated historical control rats) and usually only late in life (Solleveld *et al.*, 1984).

Exposure to 3,3′-dimethylbenzidine dihydrochloride had a profound effect on the clitoral gland in treated female rats, giving rise to a high incidence of adenomas and/or carcinomas. The incidence of

these neoplasms in high-dose females was about 8 times higher than those in untreated historical control F344/N rats in 2-year studies. Adenomas were found in treated female animals as early as week 33, and carcinomas as early as week 36. Potential precursor lesions (hyperplasia) occurred in small numbers in treated animals, possibly because most such lesions had already progressed to neoplasms. An increased incidence of neoplasms was also observed in the preputial gland of treated males. Although not as marked as the incidence of clitoral gland tumors in treated females, the incidence of preputial gland neoplasms in treated males was about three times higher than that of laboratory historical controls. Preputial gland adenomas and carcinomas were confirmed histologically as early as weeks 40 and 44, respectively. Adenomas of the preputial gland were observed in two control rats at 60 weeks (historical incidence in untreated controls 0% at study laboratory, 4.3% in NTP studies).

Intake of 3,3′-dimethylbenzidine dihydrochloride was associated with an increased incidence of hepatocellular neoplasms, principally neoplastic nodules (hepatocellular adenoma), in treated male and female rats. The incidence of hepatocellular neoplasms in treated female rats was considerably lower than in males. The incidence of neoplastic nodules or carcinomas (combined) was significantly ($P \leq 0.05$) increased for mid- (47%) and high-dose (55%) males and mid- (9%) and high-dose (7%) females; no hepatocellular neoplasms occurred in the untreated or low-dose groups of either sex. It was therefore concluded that 3,3′-dimethylbenzidine dihydrochloride treatment was responsible for these neoplasms in male and female rats. 3,3′-Dimethylbenzidine dihydrochloride was also associated with an increase in the incidence of basophilic, eosinophilic and mixed cell foci in male and female rats, which, if these foci are considered precursor lesions, strengthens the conclusion that 3,3′-dimethylbenzidine dihydrochloride is a hepatocarcinogen. The chemical also caused mild hepatotoxicity.

Squamous cell neoplasms occurring in the oral cavity (tongue and palate) of treated females were strongly associated with exposure to 3,3′-dimethylbenzidine dihydrochloride. Significant numbers of squamous cell tumors of the oral cavity were also detected in treated male rats although at a lower incidence. Neoplasms of the oral cavity could have been caused by direct exposure to 3,3′-dimethylbenzidine dihydrochloride in the drinking water or by systemic exposure to reactive 3,3′-dimethylbenzidine dihydrochloride metabolites. Taken collectively, the observed number of squamous cell papillomas and carcinomas of the oral cavity represents a comparatively large increase in the incidence of relatively rare tumors (historical incidence of 4/1,643, 0.2%, in untreated female F344/N rats in 2-year studies). It was concluded that these tumors were caused by 3,3′-dimethylbenzidine dihydrochloride treatment in male and female rats.

3,3′-Dimethylbenzidine dihydrochloride exposure led to development of uncommon epithelial neoplasms of the small and large intestine in male and female rats. Chemically induced neoplasms of the intestine are uncommon in rats. Of 370 chemicals studied by the NCI/NTP, only seven were associated with adenocarcinomas, adenomatous polyps, or carcinomas of the intestine in the rat: 3,3′-dimethoxybenzidine (NTP, 1990a), tribromomethane (NTP, 1989), bromodichloromethane (NTP, 1987), Captan (NCI, 1977), phenazopyridine hydrochloride (NCI, 1978b), chrysotile asbestos (NTP, 1985), and glycidol (NTP, 1990b).

The neoplasms in the current study were principally cystic mucinous adenocarcinomas of the small intestine and adenomatous polyps of the large intestine. Adenocarcinomas of the small intestine first occurred after 30 and 36 weeks of treatment in males and females, respectively, and colonic polyps were first observed at necropsy at week 44 in males and females. Adenocarcinomas were also observed in the large intestine of seven high-dose males and one mid-dose and one high-dose female. Although the increase in incidence of these tumors in females was not as marked as in males, these tumors were considered due to 3,3′-dimethylbenzidine dihydrochloride exposure since no adenocarcinomas or adenomatous polyps have been observed in 1,601 untreated historical control female F344/N rats in 2-year NTP studies.

3,3′-Dimethylbenzidine dihydrochloride consumption led to a dose-related increase in the incidence and a shortened latency of adenocarcinomas of the mammary gland of female rats. The incidence of adenocarcinomas was statistically significant only in the high-dose group; no adenocarcinomas were observed in untreated control rats. This neoplasm was first observed at necropsy in high-dose females at week 41, in mid-dose females at week 52, and in

low-dose females at week 60. Based upon the dose-related increase in incidence of adenocarcinomas and decrease in time-to-tumor, it was concluded that mammary gland neoplasms were a result of 3,3′-dimethylbenzidine dihydrochloride treatment.

The incidence of alveolar/bronchiolar neoplasms of the lung was significantly increased in mid- and high-dose male rats. A dose-related increase in the incidence of these lung tumors occurred in female rats and was significant in the high-dose group. Hyperplasia of the alveolar epithelium occurred in up to 41% of treated male and female rats. Because alveolar/bronchiolar tumors are uncommon in the F344 rat (2.8% or 1.5% in untreated male or female control rats in NTP 2-year studies) and because of the high treatment-related incidences of hyperplasia, alveolar/bronchiolar tumors were considered directly related to 3,3′-dimethylbenzidine dihydrochloride treatment.

A few uncommon malignant neoplasms of glial cell or meningeal origin occurred in the brains of treated male and female rats, but not in controls. The first neoplasms were observed at week 50 and 55 in high-dose males and females, respectively, and at week 60 in mid-dose males and females. The incidence of these tumors was only marginally increased, and was not dose related. However, in view of the reduced survival of treated rats and low spontaneous occurrence of these tumors (historical incidence <1.0% for any of these tumors in NTP 2-year studies), these neoplasms may have been related to 3,3′-dimethylbenzidine dihydrochloride exposure.

An increased incidence of mesotheliomas in male rats was associated with 3,3′-dimethylbenzidine dihydrochloride treatment in the mid-dose (3/75, 4%) and high-dose (4/60, 7%) groups. Mesotheliomas were not detected in untreated male control or low-dose rats. The laboratory control incidence (2/100) was similar to the overall historical incidence of malignant mesotheliomas in male F344/N rats (0.7%, 11/1,596) in 2-year NTP studies. Although the increased incidence of mesotheliomas in the mid- and high-dose rats was not as marked as that of other neoplasms, it is possible that the incidence would have been higher had the animals in these groups survived longer. In consideration of the decreased survival and moderately increased incidence of mesotheliomas in these animals, it was concluded that these tumors were a result of 3,3′-dimethylbenzidine dihydrochloride treatment.

Survival of 3,3′-dimethylbenzidine dihydrochloride-exposed rats was reduced during the 14-month studies, primarily because of the number of moribund sacrifices associated with the presence of grossly visible neoplasms of the skin, Zymbal's gland, and preputial and clitoral glands in male and female rats. Tumors of these tissues first appeared in males after treatment for 30 weeks (Zymbal's gland and skin) and in females after 26 weeks (Zymbal's gland). Early mortality from these tumors may have reduced the number of male and female rats at risk for development of tumors at other sites.

For these later developing or less rapidly lethal tumors, expression of tumor incidence by the standard convention (the number of animals with tumors at a site divided by the total number of animals in which this site was examined) may underestimate the tumor incidence which would have been observed in the absence of early deaths. Therefore, tumor incidence ratios were expressed in terms of the "effective" number of animals actually at risk, i.e., the number of animals bearing a tumor at a particular site by the number of animals alive in each group at the time the first tumor was observed at that site in any of the four (control, low, mid, or high dose) groups. These derived incidences were analyzed statistically with the Cochran-Armitage trend test and the Fisher exact test.

ONCOGENE ACTIVATION

Neoplasms obtained from control rats and rats treated with 3,3′-dimethylbenzidine dihydrochloride or C.I. Acid Red 114 (a 3,3′-dimethylbenzidine-derived dye) were assayed for the presence of activated proto-oncogenes by the NIH 3T3 DNA transfection assay (Anderson *et al.*, 1987; Reynolds *et al.*, 1990). Oncogenes detectable by DNA transfection analysis were present in 13/14 skin or clitoral gland neoplasms induced by 3,3′-dimethylbenzidine dihydrochloride or C.I. Acid Red 114. DNA from both benign and malignant neoplasms was capable of inducing morphologically transformed foci in NIH 3T3 mouse fibroblast cultures. Oncogenes were not detectable in one fibrosarcoma and three mammary fibroadenomas in treated rats.

Fourteen of the 18 chemically induced tumors were of epidermal origin, and activated *ras* oncogenes

were detected at a high frequency in these tumors (12/14). Neoplasms of the clitoral glands had a high frequency of activated *ras* oncogenes (4/4).

It is difficult to compare oncogene activation in spontaneously occurring tumors with that in chemically induced tumors because of the substantial difference in the tumor types obtained in the two groups. Only 55% (21/38) of the spontaneously occurring tumors were of epithelial cell origin. However, in comparing the tumors of epithelial cell origin, there was a 15-fold higher incidence of *ras* gene activation in the chemically induced tumors (13/18) than in the spontaneous tumors (1/21).

It is possible that chemically induced neoplasms were derived from a common epidermal progenitor stem-cell population that was susceptible to electrophilic attack by activated metabolites of 3,3′-dimethylbenzidine dihydrochloride or C.I. Acid Red 114. A relatively high percentage (62%) of the chemically induced rat neoplasms contained activated alleles of either H-*ras* or N-*ras*. Those neoplasms with activated H-*ras* contained point mutations in codons 12, 13, or 61. The much higher incidence of H-*ras* gene activation and apparent mutational specificity at codons 13 and 61 of H-*ras* with 3,3′-dimethylbenzidine dihydrochloride exposure suggest that the increased tumor incidence observed in treated rats is directly related to the genotoxic effect of this chemical.

Related Aromatic Amines

Benzidine and related aromatic amines produce tumors in a wide variety of tissues in experimental animals. In humans, exposure to benzidine is associated with cancer of the urinary bladder (Zavon *et al.*, 1973); in mice, however, the liver is the major target organ (Bonser *et al.*, 1956; Vesselinovitch *et al.*, 1975; Littlefield *et al.*, 1983). In rats 3,3′-dimethylbenzidine, 3,3′-dimethoxybenzidine, benzidine, and other aminobiphenyls cause tumors in the Zymbal's gland, mammary gland, skin, intestine, and liver. 3,3′-Dimethylbenzidine caused tumors in the lung of both rats and mice. Although the mechanism is not entirely clear, these differences in species and target organ specificity appear to be related to differences in metabolism.

A number of aromatic amines cause tumors of the Zymbal's gland; however, the basis for this organ specificity is poorly understood. The Zymbal's gland has been reported to be deficient in sulfotransferase activity (Irving *et al.*, 1971) and transacylase activity (Bartsch *et al.*, 1973), but is capable of hydroxylating compounds via cytochrome P_{450}-dependent enzymatic pathways (Pohl and Fouts, 1983). Susceptibility of a species to the carcinogenic action of aromatic amines depends upon the ability of the species to *N*-hydroxylate the amine substituent. *N*-hydroxylation appears to be a necessary but insufficient step in the metabolic activation of aromatic amines, and subsequent formation of an ester is required, resulting in an active electrophilic agent (Miller and Miller, 1977). Formation of different esters by different species may result in variations in organ specificity (Cohen, 1983).

Of 370 chemicals evaluated for carcinogenicity in rats and mice by the NCI/NTP, only 15 were associated with Zymbal's gland neoplasms in rats. Ten of these 15 are aryl nitrogen derivatives (nitro, amino, or isocyanate) that were mutagenic for *Salmonella typhimurium* and produced neoplasms in both rats and mice. In a survey of 222 chemicals evaluated for carcinogenicity in rats and mice by the NCI/NTP (Ashby and Tennant, 1988), only nine chemicals were associated with Zymbal's gland tumors in the rat. Eight of these chemicals were aryl nitrogen derivatives, were mutagenic for *S. typhimurium*, and produced tumors in both rats and mice. Only six of the 222 chemicals surveyed were associated with skin tumors following systemic administration. Of these six chemicals, five were aryl nitrogen derivatives, and five were among the group of nine chemicals which caused Zymbal's gland tumors. Although not included in this survey, 3,3′-dimethylbenzidine, 3,3′-dimethoxybenzidine, benzidine, and several other aromatic amines also belong to this unique group of genotoxic carcinogens that cause Zymbal's gland and/or skin tumors in rodents (Table 23).

3,3′-Dimethoxybenzidine, a related benzidine congener, was studied simultaneously with 3,3′-dimethylbenzidine using the same study design (NTP, 1990a). The 3,3′-dimethoxybenzidine study was also terminated early (21 months) because of poor animal survival due to neoplasia. Both 3,3′-dimethoxybenzidine and 3,3′-dimethylbenzidine are potent carcinogens affecting principally the skin, Zymbal's gland, clitoral and preputial glands, liver, oral cavity,

TABLE 23
Structural Analogs of 3,3′-Dimethylbenzidine That Are Mutagenic Carcinogens for the Zymbal's Gland and Skin in Rats

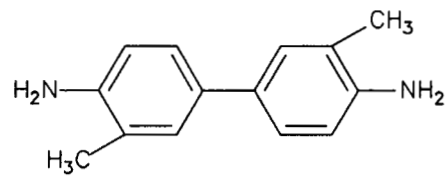
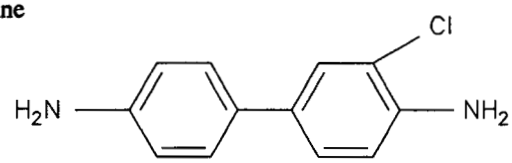
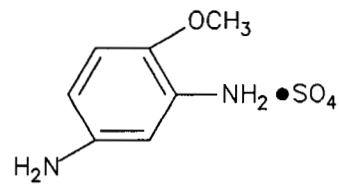
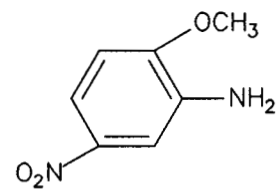
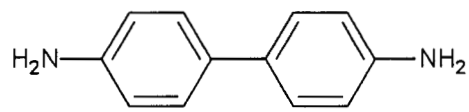
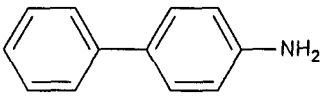
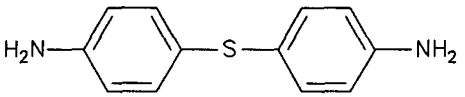
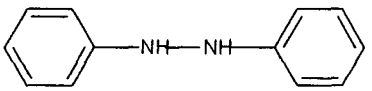
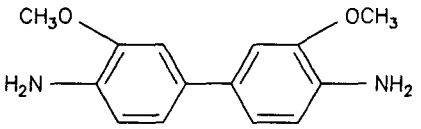
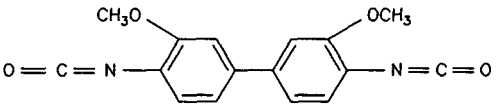
Aromatic Amine	Structure	*Salmonella typhimurium* Assay	Zymbal's Gland	Skin	References
3,3′-Dimethylbenzidine		+	+	+	Pliss, 1965; Current studies
3,3′-Dichlorobenzidine		+	+	+	IARC, 1987; Lazear and Louie, 1977
2,4-Diaminoanisole sulfate		+	+	+	NCI, 1978e
5-Nitro-*o*-anisidine		+	+	+	NCI, 1978f
Benzidine		+	+	-	IARC, 1987

TABLE 23
Structural Analogs of 3,3′-Dimethylbenzidine That Are Mutagenic Carcinogens for the Zymbal's Gland and Skin in Rats (continued)

Aromatic Amine	Structure	*Salmonella typhimurium* Assay	Zymbal's Gland	Skin	References
4-Aminobiphenyl		+	+	–	IARC, 1987
4,4′-Thiodianiline		+	+	+	NCI, 1978c
Hydrazobenzene		+	+	–	NCI, 1978d
3,3′-Dimethoxybenzidine		+	+	+	NTP, 1990a
3,3′-Dimethoxybenzidine diisocyanate		+	+	+	NCI, 1979b

and intestine in the F344/N rat. In addition, both benzidine congeners caused increased incidences in neoplasms in the mesothelium, mammary gland, and brain. Although the increase in the incidence of neoplasms in these organs was less remarkable, the fact that both related chemicals caused lesions at these sites further supports its significance.

3,3′-Dimethylbenzidine caused alveolar/bronchiolar tumors in F344/N rats, whereas 3,3′-dimethoxybenzidine did not. In studies conducted at the NCTR, 3,3′-dimethoxybenzidine was negative for these tumors in BALB/c mice, and 3,3′-dimethylbenzidine caused a low incidence of lung tumors. The reasons for these species and target site differences are not clear.

CONCLUSION

Under the conditions of these 14-month drinking water studies, there was *clear evidence of carcinogenic activity** of 3,3′-dimethylbenzidine dihydrochloride for male F344/N rats, as indicated by benign and malignant neoplasms of the skin, Zymbal's gland, preputial gland, liver, oral cavity, small and large intestine, mesothelium, and lung. Increased incidences of neoplasms of the brain may have been related to chemical administration. There was *clear evidence of carcinogenic activity* for female F344/N rats, as indicated by benign and malignant neoplasms of the skin, Zymbal's gland, clitoral gland, liver, oral cavity, small and large intestine, mammary gland, and lung. Increased incidences of neoplasms of the brain and mononuclear cell leukemia may have been related to chemical administration.

* Explanation of Levels of Evidence of Carcinogenic Activity is on page 9. A summary of peer review comments and the public discussion on this Technical Report appears on page 11.

REFERENCES

Althaus, F.R., Lawrence, S.D., Sattler, G.L., Longfellow, D.G., and Pitot, H.C. (1982). Chemical quantification of unscheduled DNA synthesis in cultured hepatocytes as an assay for the rapid screening of potential chemical carcinogens. *Cancer Res.* **42**, 3010-3015.

Ames, B.N., Durston, W.E., Yamasaki, E., and Lee, F.D. (1973). Carcinogens are mutagens: A simple test system combining liver homogenates for activation and bacteria for detection. *Proc. Natl. Acad. Sci.* USA **70**, 2281-2285.

Ames, B.N., McCann, J., and Yamasaki, E. (1975). Methods for detecting carcinogens and mutagens with the Salmonella/mammalian-microsome mutagenicity test. *Mutat. Res.* **31**, 347-364.

Anderson, D., and Styles, J.A. (1978). An evaluation of 6 short-term tests for detecting organic chemical carcinogens. Appendix 2. The bacterial mutation test. *Br. J. Cancer* **37**, 924-930.

Anderson, M.W., Angerman-Stewart, J., Patterson, R., Maronpot, R.R., and Reynolds, S.H. (1987). *Ras* gene activation in rat tumors induced by various benzidine derived dyes. *Proc. Am. Assoc. Cancer Res.* **28**, 149 (Abstr.).

Ashby, J. and Tennant, R.W. (1988). Chemical structure, Salmonella mutagenicity and extent of carcinogenicity as indicators of genotoxic carcinogenesis among 222 chemicals tested in rodents by the U.S. NCI/NTP. *Mutat. Res.* **204**, 17-115.

Baker, R.S.U., and Bonin, A.M. (1981). Study of 42 coded compounds with the *Salmonella*/mammalian microsome assay. Evaluation of short-term tests for carcinogens: Report of the International Collaborative Program. *Prog. Mutat. Res.* **1**, 249-260.

Bartsch, H., Dworkin, C., Miller, E.C., and Miller, J.A. (1973). Formation of electrophilic *N*-acetoxyarylamines in cytosols from rat mammary gland and other tissues by transacetylation from the carcinogen *N*-hydroxy-4-acetylaminobiphenyl. *Biochim. Biophys. Acta* **304**, 42-55.

Boeniger, M. (1980). National Institute of Occupational Safety and Health (NIOSH) (1980) Health hazard alert---benzidine, *o*-tolidine-, and *o*-dianisidine-based dyes. U.S. Department of Health and Human Services Publication No. 81-106. *Am. Ind. Hyg. Assoc. J.* **42**, A-36-A-60.

Bonser, G.M., Clayson, D.B, and Jull, J.W. (1956). The induction of tumours of the subcutaneous tissues, liver and intestine in the mouse by certain dyestuffs and their intermediates. *Br. J. Cancer* **10**, 653-667.

Boorman, G.A., Montgomery, C.A., Jr., Eustis, S.L., Wolfe, M.J., McConnell, E.E., and Hardisty, J.F. (1985). Quality assurance in pathology for rodent carcinogenicity studies. In *Handbook of Carcinogen Testing* (Milman, H., and E. Weisburger, Eds.), pp. 345-357. Noyes Publications, Park Ridge, NJ.

Bos, R.P., Groenen, M.A.M., Theuws, J.L.G., Leijdekkers, Ch.-M., and Henderson, P.Th. (1984). Metabolism of benzidine-based dyes and the appearance of mutagenic metabolites in urine of rats after oral or intraperitoneal administration. *Toxicology* **31**, 271-282.

Bos, R.P., van der Krieken, W., Smeijsters, L., Koopman, J.P., De Jonge, H.R., Theuws, J.L.G., and Henderson, P.Th. (1986). Internal exposure of rats to benzidine derived from orally administered benzidine-based dyes after intestinal azo reduction. *Toxicology* **40**, 207-213.

Brown, J.P., and Dietrich, P.S. (1983). Mutagenicity of selected sulfonated azo dyes in the Salmonella/microsome assay: Use of aerobic and anaerobic activation procedures. *Mutat. Res.* **116**, 305-315.

Case, R.A.M., Hosker, M.E., McDonald, D.B., and Pearson, J.T. (1954). Tumours of the urinary bladder in workmen engaged in the manufacture and use of certain dyestuff intermediates in the British chemical industry. Part I. The role of aniline, benzidine, alpha-naphthylamine, and beta-naphthylamine. *Br. J. Industr. Med.* **11**, 75-104.

Cerniglia, C.E., Freeman, J.P., Franklin, W., and Pack, L.D. (1982). Metabolism of azo dyes derived from benzidine, 3,3′-dimethylbenzidine and 3,3′-dimethoxybenzidine to potentially carcinogenic aromatic amines by intestinal bacteria. *Carcinogenesis* **3**, 1255-1260.

Cihak, R. (1979). Evaluation of benzidine by the micronucleus test. *Mutat. Res.* **67**, 383-384.

Code of Federal Register (CFR) 21 Part 58.

Collier, H.B. (1974). Are orthotolidine and dianisidine health hazards to laboratory workers? *Clin. Biochem.* **7**, 3-4.

Cohen, S.M. (1983). Promotion of urinary bladder carcinogenesis. In *Organ and Species Specificity in Chemical Carcinogenesis*, Basic Life Sciences, Vol. 24. (R. Langenbach, S. Nesnow, and J.M. Rice, Eds.), pp. 253-270. Plenum Press, New York.

Cox, D.R. (1972). Regression models and life tables. *J. R. Stat. Soc.* **B34**, 187-220.

Dinse, G.E., and Haseman, J.K. (1986). Logistic regression analysis of incidental-tumor data from animal carcinogenicity experiments. *Fundam. Appl. Toxicol.* **6**, 44-52.

Dinse, G.E., and Lagakos, S.W. (1983). Regression analysis of tumour prevalence data. *J. R. Stat. Soc.* **C32**, 236-248.

Dunn, O.J. (1964). Multiple comparisons using rank sums. *Technometrics* **6**, 241-252.

Dyes Environmental and Toxicology Organization, Inc. (DETO) (1980). Comments on the Testing Recommendations of the TSCA Interagency Testing Committee for Benzidine Based, o-Tolidine Based and o-Dianisidine Based Dyes. Document Control No. 80T-30. DETO, Scarsdale, NY.

El-hawari, A., Sawyer, M., and Hainje, M. (1979). Absorption and elimination of ^{14}C-labeled Direct Black 14 and Direct Black 38 following dermal application to male New Zealand rabbits. Midwest Research Institute Report prepared for I.B.M. Corporation, M.R.I. Project No. 4612-B(2). Kansas City, MO.

Frith, C.H., and Dooley, K. (1976). Hepatic cytologic and neoplastic changes in mice given benzidine dihydrochloride. *J. Natl. Cancer Inst.* **56**, 679-680.

Galloway, S.M., Armstrong, M.J., Reuben, C., Colman, S., Brown, B., Cannon, C., Bloom, A.D., Nakamura, F., Ahmed, M., Duk, S., Rimpo, J., Margolin, B.H., Resnick, M.A., Anderson, B., and Zeiger, E. (1987). Chromosome aberrations and sister chromatid exchanges in Chinese hamster ovary cells: Evaluations of 108 chemicals. *Environ. Molec. Mutagen.* **10**(Suppl. 10), 1-175.

Gart, J.J., Chu, K.C., and Tarone, R.E. (1979). Statistical issues in interpretation of chronic bioassay tests for carcinogenicity. *J. Natl. Cancer. Inst.* **62**, 957-974.

Griswold, D.P., Jr., Casey, A.E., Weisburger, E.K., and Weisburger, J.H. (1968). The carcinogenicity of multiple intragastric doses of aromatic and heterocyclic nitro or amino derivatives in young female Sprague-Dawley rats. *Cancer Res.* **28**, 924-933.

Hadidian, Z., Frederickson, T.N., Weisburger, E.K., Weisburger, J.H., Glass, R.M., and Mantel, N. (1968). Tests for chemical carcinogens. Report on the activity of derivatives of aromatic amines, nitrosamines, quinolines, nitroalkanes, amides, epoxides, aziridines, and purine antimetabolites. *J. Natl. Cancer Inst.* **41**, 985-1036.

Haley, T.J. (1975). Benzidine revisited: A review of the literature and problems associated with the use of benzidine and its congeners. *Clin. Toxicol.* **8**, 13-42.

Hartman, C.P., Fulk, G.E., and Andrews, A.W. (1978). Azo reduction of trypan blue to a known carcinogen by a cell-free extract of a human intestinal anaerobe. *Mutat. Res.* **58**, 125-132.

Haseman, J.K. (1984). Statistical issues in the design, analysis and interpretation of animal carcinogenicity studies. *Environ. Health Perspect.* **58**, 385-392.

Haseman, J.K., Huff, J., and Boorman, G.A. (1984). Use of historical control data in carcinogenicity studies in rodents. *Toxicol. Pathol.* **12**, 126-135.

Haseman, J.K., Huff, J., Rao, G.N., Arnold, J., Boorman, G.A., and McConnell, E.E. (1985). Neoplasms observed in untreated and corn oil gavage control groups of F344/N rats and (C57BL/6N × C3H/HeN)F_1 (B6C3F_1) mice. *JNCI* **75**, 975-984.

Haworth, S., Lawlor, T., Mortelmans, K., Speck, W., and Zeiger, E. (1983). *Salmonella* mutagenicity test results for 250 chemicals. Environ. Mutagen. **5** (Suppl. 1), 3-142.

Hollander, M. and Wolfe, D.A. (1973). *Nonparametric Statistical Methods*, pp. 120-123. John Wiley and Sons, Inc., New York.

International Agency for Research on Cancer (IARC) (1972). Benzidine. IARC Monographs on the Evaluation of Carcinogenic Risk of Chemicals to Man, Vol. 1., pp. 80-86. World Health Organization, IARC, Lyon, France.

International Agency for Research on Cancer (IARC) (1982). Benzidine and its sulphate, hydrochloride, and dihydrochloride. IARC Monographs on the Evaluation of Carcinogenic Risk of Chemicals to Man, Vol. 29., World Health Organization, IARC, Lyon, France.

International Agency for Research on Cancer (IARC) (1986). Long-term and short-term assays for carcinogenesis: A critical appraisal. Reports of an ad-hoc Working Group which met in Lyon, 2-6 December 1985. IARC Scientific Publication No. 83, p. 73. World Health Organization, IARC, Lyon, France.

International Agency for Research on Cancer (IARC) (1987). IARC Monographs on the Evaluation of Carcinogenic Risk of Chemicals to Humans. Overall evaluations of carcinogenicity: An updating of *IARC Monographs* Volumes 1 to 42, Supplement 7, pp. 62, 91-92, 123-125, 193-194. World Health Organization, IARC, Lyon, France.

Irving, C.C., Janss, D.H., and Russell, L.T. (1971). Lack of *N*-hydroxy-2-acetylaminofluorene sulfotransferase activity in the mammary gland and Zymbal's gland of the rat. *Cancer Res.* **31**, 387-391.

Jonckheere, A. (1954). A distribution-free k-sample test against ordered alternatives. *Biometrika* **41**, 133-145.

Jotz, M.M., and Mitchell, A.D. (1981). Effects of 20 coded chemicals on the forward mutation frequency at the thymidine kinase locus in L5178Y mouse lymphoma cells. In *Evaluation of Short-Term Tests for Carcinogens.* (F.J. de Serres and J. Ashby, Eds.), pp. 580-593. Elsevier/North-Holland, New York.

Kaplan, E.L., and Meier, P. (1958). Nonparametric estimation from incomplete observations. *J. Am. Stat. Assoc.* **53**, 457-481.

Kastenbaum, M.A., and Bowman, K.O. (1970). Tables for determining the statistical significance of mutation frequencies. *Mutat. Res.* **9**, 527-549.

Kennelly, J.C., Stanton, C.A., and Martin, C.N. (1984). The effect of acetyl-CoA supplementation on the mutagenicity of benzidines in the Ames assay. *Mutat. Res.* **137**, 39-45.

Kirk-Othmer Encyclopedia of Chemical Technology (1978). 3rd ed., Vol. 3, pp. 772-775. John Wiley and Sons, New York.

Kornbrust, D.J. and Barfknecht, T.R. (1984). Comparison of rat and hamster hepatocyte primary culture/DNA repair assays. *Environ. Mutagen.* **6**, 1-11.

Lazear, E.J. and Louie, S.C. (1977). Mutagenicity of some congeners of benzidine in the *Salmonella typhimurium* assay system. *Cancer Lett.* **4**, 21-25.

Littlefield, N.A., Nelson, C.J., and Frith, C.H. (1983). Benzidine dihydrochloride: Toxicological assessment in mice during chronic exposures. *J. Toxicol. Environ. Health* **12**, 671-685.

Lynn, R.K., Donielson, D.W., Ilias, A.M., Kennish, J.M., Wong, K., and Matthews, H.B. (1980). Metabolism of bisazobiphenyl dyes derived from benzidine, 3,3'-dimethylbenzidine or 3,3'-dimethoxybenzidine to carcinogenic aromatic amines in the dog and rat. *Toxicol. Appl. Pharmacol.* **56**, 248-258.

Margolin, B.H., Collings, B.J., and Mason, J.M. (1983). Statistical analysis and sample-size determinations for mutagenicity experiments with binomial responses. *Environ. Mutagen.* **5**, 705-716.

Maronpot, R.R., and Boorman, G.A. (1982). Interpretation of rodent hepatocellular proliferative alterations and hepatocellular tumors in chemical safety assessment. *Toxicol. Pathol.* **10**, 71-80.

Martin, C.N., McDermid, A.C., and Garner R.C. (1978). Testing of known carcinogens and noncarcinogens for their ability to induce unscheduled DNA synthesis in HeLa cells. *Cancer Res.* **38**, 2621-2627.

Martin, C.N., and Kennelly, J.C. (1981). Rat liver microsomal azoreductase activity on four azo dyes derived from benzidine, 3,3'-dimethylbenzidine, or 3,3'-dimethoxybenzidine. *Carcinogenesis* **2**, 307-312.

Matsushima, T., Takamoto, Y., Shirai, A., Sawamura, M., and Sugimura, T. (1981). Reverse mutation test on 42 coded compounds with the *E. Coli* WP2 system. Evaluation of short-term tests for carcinogens: Report of the International Collaborative Program. *Prog. Mutat. Res.* **1**, 387-395.

McConnell, E.E., Solleveld, H.A., Swenberg, J.A., and Boorman, G.A. (1986). Guidelines for combining neoplasms for evaluation of rodent carcinogenesis studies. *JNCI* **76**, 283-289.

Meigs, J.W., Brown, R.M., and Sciarini, L.J. (1951). A study of exposure to benzidine and substituted benzidines in a chemical plant. *Arch. Indust. Hyg. and Occup. Med.* **4**, 533-540.

Meigs, J.W., Sciarini, L.J., and Van Sandt, W.A. (1954). Skin penetration by diamines of the benzidine group. *Arch. Indust. Hyg. and Occup. Med.* **9**, 122-132.

Miller, J.A. and Miller, E.C. (1977). Ultimate chemical carcinogens as reactive mutagenic electrophiles. In *Origins of Human Cancer* (H.H. Hiatt, J.D. Watson and J.A. Winsten, Eds.), Vol. 4. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.

Mirsalis, J.C., Tyson, C.K., and Butterworth, B.E. (1982). Detection of genotoxic carcinogens in the *in vivo-in vitro* hepatocyte DNA repair assay. *Environ. Mutagen.* **4**, 553-562.

Mitchell, A.D., Rudd, C.J., and Caspary, W.J. (1988). Evaluation of the L5178Y mouse lymphoma cell mutagenesis assay: Intralaboratory results for sixty-three coded chemicals tested at SRI International. *Environ. Molec. Mutagen.* **12**(Suppl. 13), 37-101.

Mohn, G.R., Vogels-Bouter, S., and van der Horst-van der Zon, J. (1981). Studies on the mutagenic activity of 20 coded compounds in liquid test using the multipurpose strain *Escherichia coli* K-12/343/113 and derivatives. Evaluation of short-term tests for carcinogens: Report of the International Collaborative Program. *Prog. Mutat. Res.* **1**, 396-413.

Myhr, B.C., and Caspary, W.J. (1988). Evaluation of the L5178Y mouse lymphoma cell mutagenesis assay: Intralaboratory results for sixty-three coded chemicals tested at Litton Bionetics, Inc. *Environ. Molec. Mutagen.* 12(Suppl. 13), 103-194.

Natarajan, A.T. and van Kesteren-van Leeuwen, A.C. (1981). Mutagenic activity of 20 coded compounds in chromosome aberrations/sister chromatid exchanges assay using Chinese Hamster Ovary (CHO) cells, In: Evaluation of Short-Term Tests for Carcinogens: Report of the International Collaborative Program. *Prog. Mutat. Res.* 1, 551-559.

National Cancer Institute (NCI) (1976). Guidelines for Carcinogen Bioassay in Small Rodents. NCI Technical Report No. 1. U.S. Department of Health, Education and Welfare, Public Health Service, National Institutes of Health, Bethesda, MD.

National Cancer Institute (NCI) (1977). Bioassay of Captan for Possible Carcinogenicity. NCI Technical Report No. 15. U.S. Department of Health, Education and Welfare, Public Health Service, National Institutes of Health, Bethesda, MD.

National Cancer Institute (NCI) (1978a). Bioassay of *o*-Anisidine Hydrochloride for Possible Carcinogenicity. NCI Technical Report No. 89. U.S. Department of Health, Education and Welfare, Public Health Service, National Institutes of Health, Bethesda, MD.

National Cancer Institute (NCI) (1978b). Bioassay of Phenazopyridine Hydrochloride for Possible Carcinogenicity. NCI Technical Report No. 99. U.S. Department of Health, Education and Welfare, Public Health Service, National Institutes of Health, Bethesda, MD.

National Cancer Institute (NCI) (1978c). Bioassay of 4,4'-Thiodianiline for Possible Carcinogenicity. NCI Technical Report No. 47. U.S. Department of Health, Education and Welfare, Public Health Service, National Institutes of Health, Bethesda, MD.

National Cancer Institute (NCI) (1978d). Bioassay of Hydrazobenzene for Possible Carcinogenicity. NCI Technical Report No. 92. U.S. Department of Health, Education and Welfare, Public Health Service, National Institutes of Health, Bethesda, MD.

National Cancer Institute (NCI) (1978e). Bioassay of 2,4-Diaminoanisole Sulfate for Possible Carcinogenicity. NCI Technical Report No. 84. U.S. Department of Health, Education and Welfare, Public Health Service, National Institutes of Health, Bethesda, MD.

National Cancer Institute (NCI) (1978f). Bioassay of 5-Nitro-*o*-Anisidine for possible carcinogenicity. NCI Technical Report No. 127. U.S. Department of Health, Education and Welfare, Public Health Service, National Institutes of Health, Bethesda, MD.

National Cancer Institute (NCI) (1979a). Bioassay of *o*-Toluidine Hydrochloride for Possible Carcinogenicity. NCI Technical Report No. 153. U.S. Department of Health, Education, and Welfare, Public Health Service, National Institutes of Health, Bethesda, MD.

National Cancer Institute (NCI) (1979b). Bioassay of Dianisidine Diisocyanate for Possible Carcinogenicity. NCI Technical Report No. 128. U.S. Department of Health, Education, and Welfare, Public Health Service, National Institutes of Health, Bethesda, MD.

National Institutes of Health (NIH) (1978). Open Formula Rat and Mouse Ration (NIH-07). NIH Publication No. 11-1335. National Institutes of Health, Bethesda, MD.

National Institute of Occupational Health (NIOSH) (1983). Preventing health hazards from exposure to benzidine congener dyes. U.S. Department of Health and Human Services Publication No. 83-105. U.S. Department of Health and Human Services, Public Health Services, Centers for Disease Control, NIOSH, Cincinnati, OH

National Institute of Occupational Health (NIOSH) (1989). National Occupational Exposure Survey (NOES), 1981-1983, unpublished data. U.S. Department of Health and Human Services, NIOSH, Cincinnati, OH.

National Toxicology Program (NTP) (1984). Toxicology and Carcinogenesis Studies of 1,3-Butadiene in B6C3F$_1$ Mice. NTP Technical Report No. 288, NIH Publication No. 84-2544, National Institutes of Health, Research Triangle Park, NC.

National Toxicology Program (NTP) (1985). Toxicology and Carcinogenesis Studies of Chrysotile Asbestos in F344/N Rats. NTP Technical Report No. 295, NIH Publication No. 86-2551, National Institutes of Health, Research Triangle Park, NC.

National Toxicology Program (NTP) (1986). Toxicology and Carcinogenesis Studies of Benzene in F344/N Rats and B6C3F$_1$ Mice. NTP Technical Report No. 289, NIH Publication No. 86-2545, National Institutes of Health, Research Triangle Park, NC.

National Toxicology Program (NTP) (1987). Toxicology and Carcinogenesis Studies of Bromodichloromethane in F344/N Rats and B6C3F$_1$ Mice. NTP Technical Report No. 321, NIH Publication No. 88-2577, National Institutes of Health, Research Triangle Park, NC.

National Toxicology Program (NTP) (1989). Toxicology and Carcinogenesis Studies of Tribromomethane (Bromoform) in F344/N Rats and B6C3F$_1$ Mice. NTP Technical Report No. 350, NIH Publication No. 89-2805, National Institutes of Health, Research Triangle Park, NC.

National Toxicology Program (NTP) (1990a). Toxicology and Carcinogenesis Studies of 3,3′-Dimethoxybenzidine Dihydrochloride in F344/N Rats. NTP Technical Report No. 372, NIH Publication No. 89-2827, National Institutes of Health, Research Triangle Park, NC.

National Toxicology Program (NTP) (1990b). Toxicology and Carcinogenesis Studies of Glycidol in F344/N Rats and B6C3F$_1$ Mice. NTP Technical Report No. 374, NIH Publication No. 90-2829, National Institutes of Health, Research Triangle Park, NC.

Noller, C.R. (1965). *Chemistry of Organic Compounds*, pp. 561-565, 743-744. W.B. Saunders Co., Philadelphia

Parodi, S., Zunino, A., Ottaggio, L., De Ferrari, M., and Santi, L. (1983). Lack of correlation between the capability of inducing sister-chromatid exchanges in vivo and carcinogenic potency, for 16 aromatic amines and azo derivatives. *Mutat. Res.* **108**, 225-238.

Parry, J.M., and Sharp, D. (1981). Induction of mitotic aneuploidy in the yeast strain D6 by 42 coded compounds. In *Evaluation of Short-Term Tests for Carcinogens. Report of the International Collaborative Program.* (F.J. de Serres and J. Ashby, Eds.), pp. 468-480. Elsevier/North-Holland, Inc., New York.

Pliss, G.B. (1963). On some regular relationships between carcinogenicity of aminodiphenyl derivatives and the structure of substance. *Acta. Intl. Union Contra Cancer.* **19**, 499-501.

Pliss, G.B. (1965). Carcinogenic properties of ortho-tolidine and dianisidine. *Gig. Tr. Prof. Zabol.* **9**, 18-22.

Pliss, G.B., and Zabezhinsky, M.A. (1970). Carcinogenic properties of orthotolidine (3,3′-dimethylbenzidine). *J. Natl. Canc. Inst.* **55**, 181-182.

Pohl, R.J. and Fouts, J.R. (1983). Cytochrome P-450-dependent xenobiotic metabolizing activity in Zymbal's gland, a specialized sebaceous gland of rodents. *Cancer Res.* **43**, 3660-3662.

Portier, C.J., and Hoel, D.G. (1984). Design of animal carcinogenicity studies for goodness-of-fit of multistage models. *Fundam. Appl. Toxicol.* **4**, 949-959.

Probst, G.S., McMahon, R.E., Hill, L.E., Thompson, C.Z., Epp, J.K., and Neal, S.B. (1981). Chemically-induced unscheduled DNA synthesis in primary rat hepatocyte culture: A comparison with bacterial mutagenicity using 218 compounds. *Environ. Mutagen.* **3**, 11-32.

Prokofjeva, O.G. (1971). Induction of hepatic tumors in mice by benzidine. *Vopr. Onkol.* **17**, 61-64.

Reid, T.M., Morton, K.C., Wang, C.Y., and King, C.M. (1984a). Mutagenicity of azo dyes following metabolism by different reductive/oxidative systems. *Environ. Mutagen.* **6**, 705-717.

Reid, T.M., Wang, C.Y., King, C.M., and Morton, K.C. (1984b). Mutagenicity of some benzidine congeners and their N-acetylated and N,N'-diacetylated derivatives in different strains of *Salmonella typhimurium*. *Environ. Mutagen.* **6**, 145-151.

Reynolds, S.H., Patterson, R.M., Mennear, J.H., Maronpot, R.R., and Anderson, M.W. (1990). *Ras* gene activation in rat tumors induced by benzidine congeners and derived dyes. *Cancer Res.* **50**, 266-272.

Ridgway, E.C., Weintraub, B.D., Cevallos, J.L., Rack, M.C., Maloof, F. (1973). Suppression of pituitary TSH secretion in the patient with a hyper-functioning thyroid nodule. *J. Clin. Invest.* **52**, 2783-2792.

Rinde, E., and Troll, W. (1975). Metabolic reduction of benzidine azo dyes to benzidine in the rhesus monkey. *J. Natl. Cancer Inst.* **55**, 181-182.

Sadtler Standard Spectra. IR No. 46351; UV No. 24237; NMR No. 27767M. Sadtler Research Laboratories, Philadelphia.

Saffiotti, U., Cefis, F., Montesano, R., and Sellakumar, A.R. (1966). Induction of bladder cancer in hamsters fed aromatic amines. *Ind. Med. and Surg.* **35**, 564.

Saffiotti, U., Cefis, F., Montesano, R., and Sellakumar, A.R. (1967). Induction of bladder cancer in hamsters fed aromatic amines. In *Bladder Cancer, A Symposium* (Deichmann, W.B. and Lampe, K.F., Eds.), pp. 129-135. Aesculapius Publishing Co., Birmingham, AL.

Salamone, M.F., Heddle, J.A., and Katz, M. (1981). Mutagenic activity of 41 compounds in the *in vivo* micronucleus assay, In: Evaluation of Short-Term Test for Carcinogens: Report of the International Collaborative Program. *Prog. Mutat. Res.* **1**, 686-697.

Schieferstein, G.J., Shinohara, Y., Allen, R.R., Greenman, D.L., and Allaben, W.T. (1989). Carcinogenic evaluation of 3,3'-dimethylbenzidine (DMB) in BALB/c mice. Presented at the 28th Annual Meeting of the Society of Toxicology, February 27 - March 3, 1989, Atlanta, GA.

Schieferstein, G.J., Shinohara, Y., Allen, R.R., Greenman, D.L., and Allaben, W.T. (1990). Carcinogenicity study of 3,3'-dimethylbenzidine dihydrochloride in BALB/c mice. *Food Chem. Toxicol.* **27**, 801.

Scott, T.S. (1952). The incidence of bladder tumors in a dyestuffs factory. *Br. J. Indust. Med.* **9**, 127-132.

Sellakumar, A.R., Montesano, R., and Saffiotti, U. (1969). Aromatic amines carcinogenicity in hamsters. *Proc. Amer. Assoc. Cancer Res.* **10**, 78.

Sharp, D.C. and Parry, J.M. (1981). Induction of mitotic gene conversion by 41 coded compounds using the yeast culture JD1, In: Evaluation of Short-Term Tests for Carcinogens: Reports of the International Collaborative Program. *Prog. Mutat. Res.* **1**, 491-501.

Shimizu, H., and Takemura, N. (1976). Mutagenicity and carcinogenicity of some aromatic amino and nitro compounds. *Jpn. J. Indust. Health* **18**, 138-139.

Shirley, E. (1977). A non-parametric equivalent of Williams' test for contrasting increasing dose levels of a treatment. *Biometrics* **33**, 386-389.

Solleveld, H.A., Haseman, J.K., and McConnell, E.E. (1984). Natural history of body weight gain, survival, and neoplasia in the F344 rat. *JNCI* **72**, 929-939.

Spitz, S., Mauguigan, W.H., and Dobringer, K. (1950). The carcinogenic action of benzidine. *Cancer* **3**, 789-804.

Tanaka, K., Mii, T., Marui, S., Matsubara, I., and Igaki, H. (1982). Some aspects of metabolism and mutagenicity of o-tolidine and an o-tolidine-based azo dye. *Indust. Health* **20**, 227-235.

Tarone, R.E. (1975). Tests for trend in life table analysis. *Biometrika* **62**, 679-682.

Tohda, H., Horaguchi, K., Takahashi, K., Oikawa, A., and Matsushima, T. (1980). Epstein-Barr virus-transformed human lymphoblastoid cells for study of sister chromatid exchange and their evaluation as a test system. *Cancer Res.* **48**, 4775-4780.

U.S. Environmental Protection Agency (USEPA) (1980). TSCA Chemical Assessment Series, Preliminary Risk Assessment, Phase I. Benzidine, its congeners, and their derivative dyes and pigments. USEPA No. 560/11-80-019. Office of Pesticides and Toxic Substances, USEPA, Washington, D.C.

U.S. International Trade Commission (USITC) (1984). Imports of benzenoid chemicals and products, 1983. Publication No. 1548, p. 17. U.S. International Trade Commission, Washington, DC.

Valencia, R., Mason, J.M. Woodruff, R.C., and Zimmering, S. (1985). Chemical mutagenesis testing in Drosophila: III. Results of 48 coded compounds tested for the National Toxicology Program. *Environ. Mutagen.* **7**, 325-348.

Venitt, S., and Crofton-Sleigh, C. (1981). Mutagenicity of 42 coded compounds in a bacterial assay using *Escherichia coli* and *Salmonella typhimurium*. Evaluation of short-term tests for carcinogens: Report of the International Collaborative Program. *Prog. Mutat. Res.* **1**, 351-360.

Vesselinovitch, S.D., Rao, K.V.N., and Mihailovich, N. (1975). Factors modulating benzidine carcinogenicity bioassay. *Cancer Res.* **35**, 2814-2819.

Waalkens, D.H., Joosten, H.F.P., Yih, T.D., and Hoekstra, A. (1981). Mutagenicity studies with o-tolidine and 4,4′ - tetramethyldiaminodiphenyl-methane. *Mutat. Res.* **89**, 197-202.

Walker, R. (1970). The metabolism of azo compounds: A review of the literature. *Fd. Cosmet. Toxicol.* **8**, 659-676.

Williams, G.M. (1978). Further improvements in the hepatocyte primary culture DNA repair test for carcinogens: detection of carcinogenic biphenyl derivatives. *Cancer Lett.* **4**, 69-75.

Zavon, M.R., Hoegg, U., and Bingham, E. (1973). Benzidine exposure as a cause of bladder tumors. *Arch. Environ. Health* **27**, 1-7.

Zeiger, E., Anderson, B., Haworth, S., Lawlor, T., and Mortelmans, K. (1988). *Salmonella* mutagenicity tests: IV. Results from the testing of 300 chemicals. *Environ. Molec. Mutagen.* **11**(Suppl. 12), 1-158.

Zimmerman, F.K. and Scheel, I. (1981). Induction of mitotic gene conversion in strain D7 of Saccharomyces cerevisiae by 42 coded chemicals, In: Evaluation of Short-Term Test for Carcinogens: Report of the International Collaborative Program. *Prog. Mutat. Res.* **1**, 481-490.

Appendix E: Carcinogen Profile for 3,3′-Dimethylbenzidine (NTP 8th Report on Carcinogens 1998) PP. E-1 – E-3.

3,3′-Dimethylbenzidine
CAS No. 119-93-7

First Listed in the *Third Annual Report on Carcinogens*

Carcinogenicity

There is sufficient evidence for the carcinogenicity of 3,3′-dimethylbenzidine in experimental animals (IARC V.1, 1972). When administered by subcutaneous injection, commercial 3,3′-dimethylbenzidine induced Zymbal gland carcinomas and external auditory canal carcinomas in rats. An IARC Working Group reported that no adequate data were available to evaluate the carcinogenicity of 3,3′-dimethylbenzidine in humans (IARC V.1, 1972; IARC S.7, 1987).

Properties

3,3′-Dimethylbenzidine is a white to reddish crystalline powder that is slightly soluble in water and very soluble in ethanol, ethyl ether, and dilute acids. It is produced as technical-grade dry and paste formulations of various purities. When heated to decomposition, it emits toxic fumes of nitrogen oxides (NO_x).

Use

More than 75% of the 3,3′-dimethylbenzidine consumed is used as a dye or an intermediate in the production of dyestuffs and pigments. According to the Society of Dyers and Colourists, more than 95 dyes are derived from 3,3′-dimethylbenzidine. About 20% of the 3,3′-dimethylbenzidine consumed is used to produce polyurethane-based high-strength elastomers, coatings, and rigid plastics. 3,3′-Dimethylbenzidine is used in small quantities by water companies and swimming pool owners in chlorine test kits, by clinical laboratories in test tapes for the detection of blood, or for the colorimetric determination of chlorine in air or water (IARC V.1, 1972).

Production

The Chem Sources USA directory identified two domestic suppliers of 3,3′-dimethylbenzidine (Chem Sources, 1990). 3,3′-Dimethylbenzidine was imported through the principal United States customs districts in 1989, however, the quantity was unpublished. Imports appear to be the major source of 3,3′-dimethylbenzidine in the United States. In 1986 and 1985, there were three domestic producers of 3,3′-dimethylbenzidine, but no production volumes were reported (USITC, 1987; SRIa, 1986). No producers or production volumes were reported by the USITC in 1984. One producer of 3,3′-dimethylbenzidine hydrochloride was identified in 1983 with no production volume stated (USITC, 1984). The USITC reported imports of 75,000 lb of 3,3′-dimethylbenzidine, and 163,700 lb of its hydrochloride in 1983, compared with the import of more than 5,000 lb of 3,3′-dimethylbenzidine hydrochloride in 1980. Approximately 3.5 million lb of 3,3′-dimethylbenzidine and 240,000 lb of the hydrochloride were imported into the United States in 1979 (USITCa, 1984). The 1979 TSCA Inventory identified one producer of 3,3′-dimethylbenzidine with production volume not specified and four companies importing 115,500 lb in 1977. The CBI Aggregate was between 1 million and 100 million lb (TSCA, 1979). The major company producing 3,3′-dimethylbenzidine in the United States ceased production in 1978; its average annual production was about 200,000 lb.

Exposure

The primary routes of potential human exposure to 3,3′-dimethylbenzidine are inhalation, dermal contact, and ingestion. ACGIH has recommended no threshold-limit value (TLV) time-weighted average (TWA) for 3,3′-dimethylbenzidine because it is regarded as a suspected human carcinogen (ACGIH, 1986). Workers potentially exposed to 3,3′-dimethylbenzidine include dye makers, repackagers of 3,3′-dimethylbenzidine and dimethylbenzidine-based dyes, and personnel in clinical and analytical laboratories. Workers in a variety of occupations may possibly be exposed to small quantities of 3,3′-dimethylbenzidine used for analytical purposes, including water and sewage plant attendants, chemical test tape or kit makers, and swimming pool service representatives. Swimming pool water test kits contain 0.5%-1.0% 3,3′-dimethylbenzidine. Exposure may occur if the test solutions are emptied back into the pool. In 1978, NIOSH estimated that fewer than 100 employees possibly were exposed to large quantities of 3,3′-dimethylbenzidine in the United States, but as many as 200,000 may possibly be exposed to small quantities (NIOSHb, 1979e). The National Occupational Exposure Survey (1981-1983) indicated that 8,676 workers, including 5,383 women, were potentially exposed to 3,3′-dimethylbenzidine (NIOSH, 1984). This estimate was derived from observations of the actual use of the compound (62% of total observations) and the use of tradename products known to contain the compound (38%). The National Occupational Hazard Survey, conducted by NIOSH from 1972 to 1974, estimated that 420 workers were potentially exposed to 3,3′-dimethylbenzidine in the workplace (NIOSH, 1976).

Dimethylbenzidine-based dyes and pigments are metabolized to 3,3′-dimethylbenzidine. Residual levels of 3,3′-dimethylbenzidine may be present in dimethylbenzidine-based dyes and pigments and in the final consumer products. Available data indicate that such contaminants occur in the parts-per-million range. A dimethylbenzidine-based dye was not absorbed dermally to any substantial degree when tested in rabbits.

Regulations

EPA regulates 3,3′-dimethylbenzidine under the Resource Conservation and Recovery Act (RCRA) as a hazardous constituent of waste and has established a reportable quantity (RQ) of 10 lb under the Comprehensive Environmental Response, Compensation, and Liability Act (CERCLA). Under the Superfund Amendments and Reauthorization Act (SARA), 3,3′-dimethylbenzidine was placed on a list of toxic chemicals subject to reporting requirements, and general threshold quantities have been established for facilities using or producing the compound. NIOSH recommended a 20 $\mu g/m^3$ ceiling for 3,3′-dimethylbenzidine exposure in the workplace, with no skin contact. OSHA has set standards limiting occupational exposure to 3,3′-dimethylbenzidine. OSHA regulates this compound under the Hazard Communication Standard and as a chemical hazard in laboratories.