FINAL

Report on Carcinogens Background Document for

Dyes Metabolized to 3,3'-Dimethoxybenzidine

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

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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'-Dimethoxybenzidine (3,3'-Dimethoxybenzidine Dye Class)

Carcinogenicity

3,3'-Dimethoxybenzidine-based dyes that are metabolized to 3,3'-dimethoxybenzidine are *reasonably anticipated to be human carcinogens* based on the fact that 3,3'-dimethoxybenzidine is carcinogenic in male and female rats (IARC 1974; NTP 1990, 1998) and that metabolism of 3,3'-dimethoxybenzidine-based dyes to release free 3,3'-dimethoxybenzidine 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'-dimethoxybenzidine-based dye, C.I. Direct Blue 15, is carcinogenic in male and female rats (NTP 1992). Further, the pattern of tumors observed with 3,3'-dimethoxybenzidine (NTP 1990) and C.I. Direct Blue 15 (NTP 1992) is similar to that observed with the structurally similar chemical 3,3′-dimethylbenzidine (NTP 1991a) and the 3,3′-dimethylbenzidine-based dye, C.I. Acid Red 114 (NTP 1991b). 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.

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

Other Information Relating to Carcinogenesis or Possible Mechanisms of Carcinogenesis

3,3'-Dimethoxybenzidine is structurally similar to benzidine, a known human carcinogen (IARC 1972, 1982, and 1987; NTP 1998) and 3,3'-dimethylbenzidine, which is reasonably anticipated to be a human carcinogen (NTP 1998). Like benzidine and 3,3'-dimethylbenzidine, 3,3'-dimethoxybenzidine 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'-dimethoxybenzidine-based dyes are chemically equivalent and are readily cleaved by chemical or enzymatic reduction to form free 3,3'-dimethoxybenzidine and the chromophore(s). Reductive cleavage of 3,3'-dimethoxybenzidine and similar dyes 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'-dimethoxybenzidine-based dyes to 3,3'-dimethoxybenzidine also has been shown in studies with rats and dogs (Lynn *et al.* 1980; Bowman *et al.* 1983). By determining the quantities of 3,3'-dimethoxybenzidine and its metabolites excreted following administration of free 3,3'-dimethoxybenzidine versus 3,3'-dimethoxybenzidine-based dyes, Lynn *et al.* (1980) also provided quantitative evidence that each of the two dyes studied was nearly completely metabolized to free 3,3'-dimethoxybenzidine. Metabolism of the dyes to free 3,3'-dimethoxybenzidine in animals is thought to be mediated primarily by bacteria in the gastrointestinal tract (Cerniglia *et al.* 1982; Morgan *et al.* 1994). 3,3'-Dimethoxybenzidine-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'-dimethoxybenzidine, a known bacterial mutagen (Haworth *et al.* 1983).

No information exists to suggest that the mechanism of carcinogenesis of these substances operating in laboratory animals 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 5
2.5 Environmental fate 6
2.5.1 Air 6
2.5.2 Water 6
2.5.3 Soil 6
2.6 Environmental exposure 7
2.7 Occupational exposure 7
2.8 Biological indices of exposure 8
2.9 Regulations 8
3 Human Cancer Studies 11
3.1 Background 11
3.2 IARC reviews 11
3.3 Current studies (Table 3-1) 11
3.4 Discussion 12
4 Studies of Cancer in Experimental Animals 15
4.1 Carcinogenesis studies of DMOB 15
4.1.1 Oral studies in rats 15
4.1.2 Oral studies in hamsters 18
4.1.3 Drinking water studies in mice 18
4.2 Carcinogenesis study of C.I. Direct Blue 15 18
4.3 Transplantability of preputial tumors induced by DMOB, a DMOB-based dye (C.I. Direct Blue 15), or a DMB-based bye (C.I. Acid Red 114) 20
4.4 Oncogene activation induced by DMOB or C.I. Direct Blue 15 21
4.5 Tumorigenic activity of DMOB, DMB, and dyes based on DMOB and DMB 22
4.6 Summary 23
5 Genotoxicity 25
5.1 Prokaryotic systems 25
5.1.1 Induction of mutation in Salmonella typhimurium 25

5.1.2 Mutagenicity in Escherichia coli 26
5.2 Eukaryotic systems 26
5.2.1 Mutagenicity in Drosophila melanogaster 26
5.3 Mammalian systems 27
5.3.1 In vitro assays 27
5.3.2 In vivo assays 28
5.4 Summary 28
6 Other Relevant Data 29
6.1 Mammalian absorption, distribution, metabolism, and excretion of DMOB and DMOB-based dyes 29
6.2 Bacterial metabolism of DMOB-based dyes 31
6.3 Protein adduction 32
6.4 Summary 33
7 References 35
Appendix A: IARC. 1974. *Some Aromatic Amines, Hydrazine and Related Substances, N-Nitroso Compounds and Miscellaneous alkylating Agents.* 3,3′-Dimethoxybenzidine. IARC Monographs on the Evaluation of the Carcinogenic Risk of Chemicals to Man. Lyon, France. World Health Organization. Vol. 4. A-1 – A-10. 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.* C.I. Direct Blue 15. IARC Monographs on the Evaluation of the Carcinogenic Risk to Humans. Lyon, France. World Health Organization. Vol. 57. Pp. B-1 – B-14. 43
Appendix C: Urinary excretion of benzidine, DMB, and DMOB by dogs and rats after oral administration of dye chemicals (Lynn *et al*. 1980) 45
Appendix D: NTP. 1990. Toxicology and Carcinogenesis Studies of DMOB Dihydrochloride in F344/N rats (Drinking Water Studies). NTP Technical Report Series TR-372. Pp. D-1 – D-68. 49
Appendix E: NTP. 1992. Toxicology and Carcinogenesis Studies of C.I. Direct Blue 15 in F344/N rats. NTP Technical Report Series TR-397. pp. E-1 – E-76. 51
Appendix F: Carcinogen Profile for DMOB and DMOB Dihydrochloride (NTP 9th Report on Carcinogens 1999) 53

List of Tables

Table 1-1. Examples of DMOB-based dyes 2
Table 1-2. Physical and chemical properties of DMOB 3
Table 1-3. Physical and chemical properties of some DMOB-based dyes metabolized to DMOB 4
Table 2-1. Production and import values for DMOB-based dyes 5
Table 2-2. National estimates of exposure to DMOB and some DMOB-based dyes 7

Table 2-3. U.S. EPA regulations 9
Table 2-4. OSHA regulations 10
Table 3-1. Cohort studies of workers exposed to DMOB 13
Table 4-1. Tumor incidences in male rats administered DMOB dihydrochloride in drinking water for up to 21 months 16
Table 4-2. Tumor incidence in female rats administered DMOB dihydrochloride for up to 21-months in drinking water 17
Table 4-3. Tumor incidences in Fischer 344/N rats administered C.I. Direct Blue 15 in drinking water for up to 22 months 19
Table 4-4. Detection of activated oncogenes in tumors occurring spontaneously or induced by DMOB or C.I. Direct Blue 15 21
Table 4-5. Identity and frequency of activated *ras* genes within specific tumor types induced by DMOB or C.I. Direct Blue 15 22
Table 4-6. Qualitative tumor responses of rats administered DMOB, a DMOB-based dye, DMB, or a DMB-based dye in drinking water 23
Table 5-1. Mutagenicity of DMOB and dyes metabolized to DMOB in *S. typhimurium* 26
Table 6-1. Urinary excretion of DMOB by dogs after oral administration of DMOB-based dyes 29
Table 6-2. Reduction of C.I. Direct Blue 15 by various anaerobic bacteria 32
Table C-1. Urinary excretion of benzidine, DMB, and DMOB by dogs after oral administration of benzidine-, DMB-, and DMOB-based dyes 1
Table C-2. Urinary excretion of benzidine, DMB, and DMOB by rats after oral administration of benzidine-, DMB-, and DMOB-based dyes (100 mg/kg) 2

List of Figures

Figure 6-1. Proposed metabolic pathways of DMOB 31
Figure 6-2. Formation of DMOB by reductive metabolism of C.I. Direct Blue 15 32

1 Introduction

Dyes metabolized to 3,3'-dimethoxybenzidine (3,3'-dimethoxybenzidine 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'-dimethoxybenzidine (DMOB) as *reasonably anticipated to be a human carcinogen* and the fact that the azo linkages of DMOB-based dyes are chemically equivalent and are readily cleaved by chemical or enzymatic reduction to form free DMOB 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 are able to absorb and reflect light. Most dyes in use today are synthetic organic compounds.

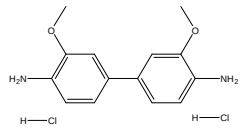
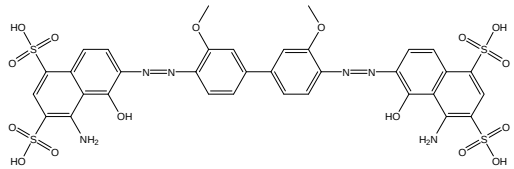
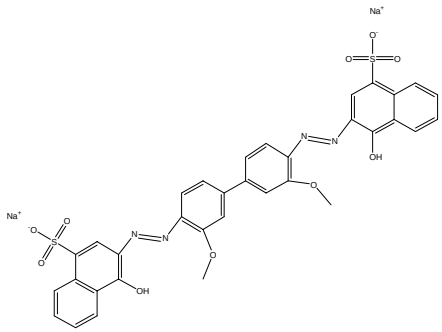
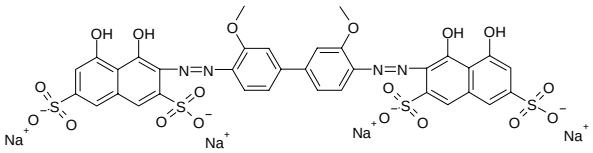
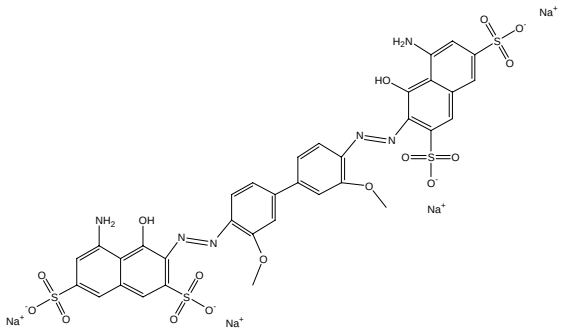
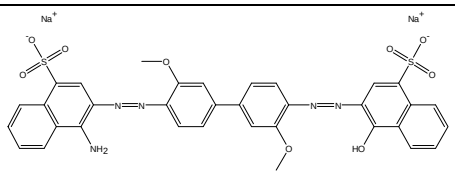
Dyes may be classified according to their chemical structures or their method of application. DMOB-based dyes contain DMOB attached to other substituents by diazo linkages. The dyes evaluated in this report are examples from the class of DMOB-based dyes that have been studied for their potentially carcinogenic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

DMOB ($C_{14}H_{16}N_2O_2$, mol wt 244.29, CASRN 119-90-4) is a methoxylated congener of benzidine and also is known by the following names:

ortho dianisidine
Blue Base
3,3′-dimethoxy-1,1′-biphenyl-4,4′-diamine
4,4′-diamino-3,3′-biphenyldiol dimethyl ether
dianisidine
3,3′-dianisidine
acetamine diazo navy RD
azoene fast blue base
Blue base IRGA B
Blue BN base
Cellitazol B
C.I. azoic diazo component 48
Diacel navy DC
Diazo fast blue B
Fast blue DSC base
Kayaku blue B base
Meisei teryl diazo blue HR
Naphthanil blue B base
Setacyl diazo navy R
Fast Blue
C.I. Disperse Black 6
4,4′-diamino-3,3′-dimethoxybiphenyl
3,3′-dimethoxy-4,4′-diaminobiphenyl
o,o′-dianisidine
acetamine diazo black RD
Amacel developed navy SD
Azogene fast blue B
Blue base NB
Brentamine fast blue B base
Cellitazol BN
Diacelliton fast grey G
Diato blue base B
Fast blue B base
Hiltonil fast blue B base
Lake blue B base
Mitsui blue B base
Neutrosel navy BN
Spectrolene blue B

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

Table 1-1. Examples of DMOB-based dyes

Dye name and formula	CASRN	mol wt	Structure
DMOB-2HCl $C_{14}H_{18}Cl_2N_2O_2$	20325-40-0	317.21	
C.I. Direct Blue 1 C.I. 24410 $C_{34}H_{28}N_6O_{16}S_4$	3841-14-3	904.87	
C.I. Direct Blue 8 C.I. 24140 $C_{34}H_{24}N_4Na_2O_{10}S_2$	2429-71-2	758.68	
C.I. Direct Blue 10 C.I. 24340 $C_{34}H_{24}N_4O_{18}S_4Na_4$	4198-19-0	992.53	
C.I. Direct Blue 15 C.I. 24400 $C_{34}H_{24}N_6O_{16}S_4Na_4$	2429-74-5	992.79	
C.I. Direct Violet 32 C.I. 24150 $C_{34}H_{25}N_5Na_2O_9S_2$	6428-94-0	757.70	
C.I. Direct Black 114	61703-05-7	NA	NA

Source: Chemfinder (1999).
NA: not available.

1.2 Physical-chemical properties

The chemical and physical properties of DMOB are summarized in Table 1-2. DMOB is a colorless, crystalline (sand-like) material that may turn violet upon standing. It is used as an intermediate in making dyes and is sensitive to heat, air, and, prolonged exposure to light (Radian 1991). The U.S. Environmental Protection Agency (EPA) hazardous waste number for DMOB is U091, and its RTECS number is NIOSH/000875000. Table 1-3 summarizes the physical and chemical properties of some DMOB-based dyes (structures are shown in Table 1-1).

Table 1-2. Physical and chemical properties of DMOB

Property	Information	Reference
Molecular weight	244.29	Budavari *et al.* (1996); CRC (1998)
Color	colorless crystals	Budavari *et al.* (1996); CRC (1998)
Physical state	solid crystals	Budavari *et al.* (1996); CRC (1998)
Melting point (^{o}C)	171.5 - 174.5	Budavari *et al.* (1996); CRC (1998)
Boiling point (^{o}C)	NA	Radian (1991)
Vapor pressure (mm Hg at 25°C)	8.8×10^{-9}	HSDB (1991)
Specific gravity	NA	Radian (1991)
Flash point (^{o}C)	206.1	Budavari *et al.* (1996); CRC (1998)
Solubility at 20°C		
Water	slightly soluble, < 0.1 mg/mL	Radian (1991)
95% Ethanol	slightly soluble, < 1 mg/mL	Radian (1991)
Dimethylsulfoxide	soluble, ≥ 100 mg/mL	Radian (1991)
Acetone	soluble, 5 - 10 mg/mL	Radian (1991)
Benzene	soluble	CRC (1998)
Ether	soluble	CRC (1998)
Chloroform	soluble	CRC (1998)

NA: not available.

Table 1-3. Physical and chemical properties of some DMOB-based dyes metabolized to DMOB

Dye name and formula	Color and physical state	Melting point (^oC)	Water solubility
DMOB-2HCl $C_{14}H_{18}Cl_2N_2O_2$	off-white powder	268	1 - 5 g/mL at 20°C
C.I. Direct Blue 1 $C_{34}H_{28}N_6O_{16}S_4$	bright greenish-blue solid	NA	0.1 - 0.5 g/100 mL at 23°C
C.I. Direct Blue 8 $C_{34}H_{24}N_4Na_2O_{10}S_2$	bluish-black powder	NA	0.1 - 1 g/100 mL at 18°C
C.I. Direct Blue 10 $C_{34}H_{26}N_4O_{18}S_4Na_4$	blue solid	NA	5 - 10 g/100 mL at 17°C
C.I. Direct Blue 15 $C_{34}H_{24}N_6O_{16}S_4Na_4$	dark blue, microcrystalline powder	> 300	1 - 5 g/100 mL at 20°C
C.I. Direct Violet 32 $C_{34}H_{25}N_5Na_2O_9S_2$	black solid	294 - 300	0.1 - 1 g/100 mL at 17°C
C.I. Direct Black 114	black powder	NA	1 g/100 mL at 21.1^{o}C

Source: Chemfinder (1999) and Budavari *et al.* (1996).
NA: not available.

1.3 Identification of metabolites

The metabolism of DMOB-based dyes to DMOB, in rats and dogs, results in appreciable levels of the free amine, monoacetyl metabolites, diacetyl metabolites, and alkaline hydrolyzable conjugates (AHCs) of metabolites (see Section 6). AHCs account for the major metabolite fraction, followed by appreciable amounts of diacetyl-DMOB and DMOB, with lesser amounts of monoacetyl-DMOB (Bowman *et al.* 1983).

2 Human Exposure

2.1 Use

The major use of DMOB is as an intermediate in the production of DMOB-based dyes used to color leather, paper, plastic, rubber, and textiles. It also is used as a chemical intermediate in the production of DMOB diisocyanate, used in isocyanate-based adhesive systems and as a component of polyurethane elastomers. DMOB also has been used in assays for metals, thiocyanates, and nitriles (NTP 1990; Radian 1991; Spectrum 1999).

2.2 Production

The United States International Trade Commission (U.S. ITC 1994) reported that DMOB was produced by two companies. DMOB-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 DMOB. Table 2-1 summarizes past total production and import values for those DMOB-based dyes for which information is available.

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

Compound	Value (lb)	Year	Source
DMOB (*o*-dianisidine) (imports)	~554,000	1978	U.S. ITC (1980)
DMOB (*o*-dianisidine) (imports)	~106,000	1983	U.S. ITC (1984)
C.I. Direct Blue 15 (production)	270,000	1982	U.S. ITC (1983)
C.I. Direct Blue 15 (imports)	7,716	1980	U.S. ITC (1981)
Direct Blue dyes (including C.I. Direct Blue 15 and 28) (production)	~1280 (581 kg)	1993	U.S. ITC (1994)
Direct Black dyes (including C.I. Direct Black 114) (production)	~16750 (7,597 kg)	1993	U.S. ITC (1994)

2.3 Analysis

Following human exposure to DMOB-based dyes, urinary DMOB can be detected by hydrolysis of urinary metabolites and isolation of the free diamines through the use of a C_{18} solid sorbent. DMOB is 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 0.9 µg/L, and the limit of quantitation (LOQ) is 3.1 µg/L. For electrochemical detection, the LOD is 0.16 µg/L, while the LOQ is 0.70 µg/L. Recoveries range from 87% to 102% at 2-µg/L, 10-µg/L, and 20-µg/L levels (Neumeister 1991).

2.4 Environmental occurrence

DMOB and DMOB-based dyes may be released into the environment as a result of their production and use. Approximately 99% of waste DMOB is deposited in water, 0.5% in terrestrial soil, and 0.5% in aquatic sediments (U.S. EPA 1988). From 1989 to 1996, four companies reported environmental releases of DMOB; no environmental releases of DMOB were reported for 1996. Seven companies reported releasing DMOB dihydrochloride into the environment, but only one had a release above the threshold reportable amount. A chemical

manufacturing division reported a non-point source release of 2 lb and a point source release of 8 lb of DMOB dihydrochloride into the air. None of the DMOB-based dyes had entries in the Toxic Release Inventory database, because their releases were not subject to reporting under the Emergency Planning and Community Right to Know Act (TRI 1996).

2.5 Environmental fate

Because no information is available about the long-term environmental fate of DMOB *per se*, environmental fate estimates are based on analogies with benzidine (HSDB 1991).

2.5.1 Air

Based upon the vapor pressure of DMOB (8.8×10^{-9} mm Hg at 25° C), it should remain almost entirely in the particulate phase in the ambient atmosphere. DMOB has an estimated half-life of two hours in the vapor phase of the atmosphere due, because it reacts with photochemically produced hydroxyl radicals. No information on photolysis is available; however, because DMOB can absorb light at wavelengths greater than 290 nm, this process may play a role in its degradation (HSDB 1991).

C.I. Direct Blue 15 is an example of a DMOB-based dye that is expected to exist in the particulate phase in the ambient atmosphere, because its ionic state is essentially non-volatile. Particulate-phase C.I. Direct Blue 15 may be removed from the atmosphere by wet and dry deposition (HSDB 1996). No other atmospheric fate information was found for any of the other dyes metabolized to DMOB.

2.5.2 Water

DMOB is moderately persistent in water, with a half-life between 20 and 200 days (U.S. EPA 1988). DMOB released into water binds to humic material in the sediment. Biodegradation of DMOB is an important removal process in water, whereas hydrolysis is not. No information on evaporation was found. DMOB has a slight tendency to bioconcentrate in aquatic organisms, with an estimated bioconcentration factor (BCF) of 13.9 (a BCF greater than 1,000 typically results in significant bioaccumulation in aquatic organisms) (HSDB 1991).

For C.I. Direct Blue 15, the major aquatic fate is adsorption to sediment, which increases with decreasing pH. C.I. Direct Blue 15 is expected to be resistant to aerobic biodegradation. Complete anaerobic biodegradation of C.I. Direct Blue 15 by activated sludge inoculum was reported to take seven days. Volatilization of C.I. Direct Blue 15 from water surfaces is not expected to be an important process, as ionic compounds normally do not readily volatilize (HSDB 1996). No aquatic fate information was found for any of the other dyes metabolized to DMOB.

2.5.3 Soil

When DMOB is released into the soil, the amount of adsorption increases with decreasing pH. DMOB also will react with natural substances in the soil, such as clay minerals and Fe(III) as aromatic amines form covalent bonds with humic materials. DMOB was not shown to biodegrade in the MITI test, and only high levels of yeast extracts enhanced biodegradation (HSDB 1991).

C.I. Direct Blue 15 is retained by the ion-exchange process, particularly on clay surfaces, and by adsorption by mineral surfaces such as geothite, which slow or prevent leaching. Because of its ionic nature, C.I. Direct Blue 15 is expected to be resistant to aerobic biodegradation. Complete anaerobic biodegradation of C.I. Direct Blue 15 by activated sludge inoculum was reported to take seven days. Volatilization of C.I. Direct Blue 15 from the soil is not expected to be an important process (HSDB 1996). No terrestrial fate information was found for any of the other dyes metabolized to DMOB.

2.6 Environmental exposure

Most environmental exposures to DMOB and DMOB-based dyes are through contact with contaminated air, water, and soil (HSDB 1991). General population exposure also may occur via contact with paper, fabrics, and leather products containing these dyes and also as a result of consumer use of these dyes.

2.7 Occupational exposure

The primary modes of potential occupational exposure to DMOB and DMOB-based dyes are by inhalation or dermal contact. Most occupational exposures to DMOB occur in dye manufacturing and processing plants during the production of DMOB, during the use and processing of DMOB to make DMOB-based dyes, or during the application of DMOB-based dyes. 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. While DMOB-based dyes were not specifically included in the survey, the results are considered to be representative of DMOB dye dust levels during weighing. The mean airborne concentration of total dye in the 24 plants randomly monitored was estimated to be 0.085 mg/m^3 (U.S. EPA 1990). Current production processes using DMOB and DMOB-dyes, however, generally are closed systems that minimize worker exposure (HSDB 1991). Occupational exposure also may occur in clinical laboratories through use of DMOB as a detecting reagent.

The National Institute of Occupational Safety and Health (NIOSH) National Occupational Hazard Survey (NOHS) estimated that 204 workers potentially were exposed to DMOB from 1972 to 1974. The National Occupational Exposure Survey (NOES) found that 2,482 workers were exposed to DMOB from 1981 to 1983. Table 2-2 summarizes the exposure data for DMOB and DMOB-based dyes. NIOSH has not recommended any occupational exposure limits for DMOB or DMOB-based dyes.

Table 2-2. National estimates of exposure to DMOB and some DMOB-based dyes

Compound	Potentially exposed workers	
	1980s (NOES)	1970s (NOHS)
DMOB-based dyes	99,783	16,166
Pigment Orange 16	42,046	10,858
Pigment Red 41	1,652	100
C.I. Direct Blue 98	21,079	18

Compound	Potentially exposed workers	
	1980s (NOES)	1970s (NOHS)
C.I. Direct Blue 8	1,450	–
C.I. Direct Blue 15	4,528	68
C.I. Direct Blue 1	7,685	1,138
C.I. Direct Blue 80	7,511	1,500
DMOB (*o*-dianisidine)	2,482	120
DMOB-2HCl (*o*-dianisidine, dihydrochloride)	489	–

–: Not studied.
Provisional data as of January 1, 1990, from the NIOSH NOES (1981–1983) and NOHS (1972–1974), cited in Ruder *et al.* (1990).

2.8 Biological indices of exposure

Exposure to DMOB and DMOB-based dyes can be detected in humans via analysis of urinary metabolites of DMOB (see Section 6.1). DMOB-based dyes are reductively cleaved to DMOB, which is further metabolized and excreted in urine and feces as the parent compound and a number of conjugates. Urine sampling and analysis is done to complement environmental monitoring in assessment of occupational exposure to these compounds.

2.9 Regulations

U.S. EPA regulates DMOB under the Resource Conservation and Recovery Act (RCRA) as a hazardous constituent of waste and under the Comprehensive Environmental Response, Compensation, and Liability Act (CERCLA). U.S. EPA also mandates that industrial releases of DMOB and DMOB dihydrochloride be reported by facilities under the Superfund Amendments and Reauthorization Act (SARA). U.S. EPA regulates DMOB and DMOB dihydrochloride under the Toxic Substances Control Act (TSCA), which requires submission of health and safety information. U.S. EPA also regulates C.I. Direct Blue 15 under TSCA. No regulations (U.S. EPA or FDA) were found for other DMOB-based dyes. The applicable U.S. EPA regulations are summarized in Table 2-3.

OSHA regulates DMOB under the Hazard Communication Standard as a chemical hazard in laboratories. OSHA regulations are summarized in Table 2-4. No FDA regulations were found for DMOB.

Table 2-3. U.S. EPA regulations

Regulatory action	Effect of regulation and other comments
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. DMOB is given the U.S. EPA Hazardous Waste number U091.
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 3,3′-DMOB is 1/1/87, for DMOB dihydrochloride 1/1/92, and for C.I. Direct Blue 218 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 public and the communities surrounding covered facilities about releases of toxic chemicals to assist research, and 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*-toluidine (dimethylbenzidine), dianisidine (DMOB), and C.I. Direct Blue 15 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: The regulations in this table have been updated through the 1998 Code of Federal Regulations 40 CFR, July 1, 1996; 21 CFR, April 1, 1996; 29 CFR, July 1, 1996.

Table 2-4. 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. OSH Act: Final rule for occupational exposure to hazardous chemicals in laboratories.	Dyes metabolized to DMOB classified as carcinogenic (IARC Group 2B) are included as chemical hazards in laboratories. Employers are required to provide employee information and training and to provide a Chemical Hygiene Plan.

Source: The regulations in this table have been updated through the 1998 Code of Federal Regulations 40 CFR, July 1, 1996; 21 CFR, April 1, 1996; 29 CFR, July 1, 1996

3 Human Cancer Studies

3.1 Background

DMOB-based dyes have not been evaluated in human cancer studies as single agents, and most of the epidemiological studies reviewed assessed DMOB 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 DMOB were available. In a subsequent IARC evaluation (IARC 1987), DMOB was placed in Group 2B (*possibly carcinogenic to humans*). The human cancer data, however, was inadequate. IARC also evaluated C.I. Direct Blue 15, a dye that is metabolized to DMOB, in 1993. C.I. Direct Blue 15 was placed in Group 2B (*possibly carcinogenic to humans*); there were no human carcinogenicity data for the dye (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 (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, DMOB, and 3,3'-dimethylbenzidine (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 4 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 can't 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 DMOB exposure may constitute an important component of the exposure. However, since only exposure to total arylamines was evaluated, the study does not directly implicate DMOB in cancer risk.

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

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 cancers for males observed, including 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 cancer 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 studies of DMOB

4.1.1 Oral studies in rats

Several studies of oral administration of DMOB to rats have been reported. In one study, 30 mg of DMOB was administered via gavage in sunflower seed oil to 42 rats, three times a week for three weeks. The dose was reduced to 15 mg after three weeks because of poor survival. The dose of 15 mg was continued for 13 months. Of the18 rats surviving after 14 months, 2 had neoplasms of the Zymbal gland, 1 had a fibroadenoma of the mammary gland, and 1 had an ovarian neoplasm. None of the 50 rats in the control group developed tumors at these sites (Pliss 1963, 1965, cited in IARC 1974 and NTP 1990, 1992).

In another study, male and female Fischer 344 rats (groups of 3 or 14 males and 3 or 15 females) were administered 0.1, 0.3, 1.0, 3.0, 10, or 30 mg of DMOB per rat, five days a week for 52 weeks, followed by a six-month period of observation. A proprietary mixture composed of sodium chloride, sodium carboxymethylcellulose polysorbate 80, and benzyl alcohol in water was used as a vehicle in this study. No controls were reported in this study as cited. Incidences of total neoplasms were increased over those of the 360 pooled vehicle and untreated control rats (no statistical analysis was presented). Tumors were detected at various sites, including the urinary bladder (2 papillomas), mammary gland (3 carcinomas and 2 fibroadenomas), skin (5 carcinomas), intestinal tract (3 carcinomas), and Zymbal gland (8 carcinomas). Tumors appeared as early as day 293, but most were detected upon necropsy, 18 months after the initial DMOB administration (Hadidian *et al*. 1968, cited in IARC 1974 and NTP 1990, 1992).

The carcinogenic potential of DMOB dihydrochloride was evaluated in a drinking-water study in rats of both sexes (NTP 1990). The study was scheduled for a 104-week duration, but was terminated early because of reduced survival of dosed animals attributable to DMOB-associated neoplasms. In this 21-month cancer bioassay, seven-week-old male and female Fischer 344/N rats received drinking water containing DMOB at concentrations of 0, 80, 170, or 330 ppm (corresponding to 0, 6, 12, or 21 mg/kg per day for males and 0, 7, 14, or 23 mg/kg per day for females). The sample sizes for both sexes were 60, 45, 75, and 60 for the control, low-dose, medium-dose, and high-dose groups, respectively. Interim sacrifices of moribund animals or animals bearing large visible tumors were performed throughout the study. The study was terminated at 21 months, when all surviving animals were sacrificed. The tissues and organs of all sacrificed animals were histopathologically examined.

Survival decreased markedly with increasing dosage. Among males, the number of rats surviving at study termination were 44 in the control group and 8 in the low-dose group. None of the male rats in the medium- and-high-dose groups survived the study duration. Among females, 45, 15, and 6 rats survived in the control, low-dose, and medium-dose groups, respectively, and none of the rats in the high-dose group survived.

Tumor incidences and their statistical significance in male and female rats are shown in Tables 4-1 and 4-2. Histopathological examination of the tissues revealed tumors at various sites, including benign and malignant tumors of the skin, Zymbal gland, preputial gland, clitoral gland, mammary gland, uterus, oral cavity, intestine, liver, and mesothelium. An observed increase in the incidence of astrocytomas of the brain may also have been treatment related.

Table 4-1. Tumor incidences in male rats administered DMOB dihydrochloride in drinking water for up to 21 months

	Concentration (ppm) in drinking water			
	0	80	170	330
Tumor type	Tumor Incidence/number examined			
Skin: basal cell or sebaceous gland adenoma or carcinoma	2/60**[a]	33/45**[b]	56/75**[b]	41/60**[b]
Skin: squamous cell papilloma	0/60**[a]	13/45**[b]	28/75**[b]	22/60**[b]
Zymbal gland: adenoma or carcinoma	0/59**[a]	10/45**[b]	25/75**[b]	30/60**[b]
Preputial gland: adenoma or carcinoma	16/60*[a]	12/43	33/73*[b]	29/59*[b]
Oral cavity: papilloma or carcinoma	1/60*[a]	8/45*[b]	10/75*[b]	11/60*[b]
Small intestine: adenocarcinoma	0/60	4/45*[a]	7/75*[a]	5/60*[a]
Large intestine: adenomatous polyp or adenocarcinoma	0/60**[a]	1/45	8/75*[b]	8/60*[b]
Liver: Neoplastic nodule or hepatocellular carcinoma	1/60**[a]	4/45	7/74*[b]	8/60**[b]
Mesothelium: mesothelioma	2/60*[a]	1/45	7/75	6/60
Brain: astrocytoma	0/60	2/44	3/75	1/60

Source: Adapted from NTP (1990).
[a]Statistical significance by Cochran-Armitage Trend Test based on effective rates: *P < 0.05, **P ≤0.001.
[b]Statistical significance by Fisher Exact Test based on effective rates: *P < 0.05, **P ≤0.001.

Table 4-2. Tumor incidence in female rats administered DMOB dihydrochloride for up to 21-months in drinking water

Tumor type	Concentration (ppm) in drinking water			
	0	80	170	330
	Tumor incidence/number examined			
Skin: basal cell adenoma or carcinoma	0/60	4/45*[a]	3/75	2/60
Skin: Squamous cell papilloma	0/60	0/45	3/75	0/60
Liver: Neoplastic nodule or hepatocellular carcinoma	0/60*[b]	1/44	0/75	3/60
Zymbal gland: adenoma or carcinoma	1/60*[b]	12/45**[a]	21/75**[a]	16/60**[a]
Mammary gland: adenocarcinomas	1/60**[b]	2/45	14/75**[a]	20/60**[a]
Oral cavity: papilloma or adenoma	2/60	2/45	6/75	5/60
Large intestine: Adenomatous polyp or adenocarcinoma	0/60*[b]	1/45	1/75	3/60*[b]
Clitoral gland: adenoma or carcinoma	7/58**[b]	27/44**[a]	48/74**[a]	41/45**[a]
Uterus: adenoma or carcinoma	0/60	4/45*[a]	2/75	2/60
Brain: astrocytoma	0/60	1/45	1/75	0/60

Source: NTP (1990).
[a]Statistical significance by Fisher Exact Test based on effective rates: *P < 0.05, **P ≤0.001.
[b]Statistical significance by Cochran-Armitage Trend Test based on effective rates: *P < 0.05, **P ≤0.001.

The incidences of preputial gland and clitoral gland adenomas or carcinomas were significantly increased in the animals administered DMOB hydrochloride. The incidences of preputial and clitoral gland tumors were increased sevenfold and tenfold, respectively, over those in untreated historical controls. Also notable was the earlier appearance of carcinomas in the DMOB-dosed rats (32 weeks in males, 39 weeks in females) than in the controls (87 weeks in males).

DMOB administration also caused significant dose-related increases in the incidences of Zymbal gland adenomas or carcinomas and skin neoplasms. Basal cell or sebaceous gland neoplasms were found in 72% of the DMOB-dosed males, compared with only 3% of the controls. The incidence of these neoplasms was lower in females, but their morphologic type was the same as detected in males; therefore, they were considered to be related to DMOB dihydrochloride exposure. Increased incidences of large and small intestine adenocarcinomas or adenomatous polyps (9% and 5% in high-dose males and females, respectively) also were observed. Large and small intestine adenocarcinomas or adenomatous polyps are rare in rats; none were observed in the 1,601 historical control animals in the National Toxicology Program (NTP) database. The increased incidence of intestinal tumors was considered to be related to DMOB dihydrochloride exposure (NTP 1990).

The NTP concluded that this study provided “clear evidence of carcinogenic activity” of DMOB hydrochloride in male and female Fischer 344/N rats under the conditions of this bioassay (NTP 1990).

4.1.2 Oral studies in hamsters

Groups of 30 male and 30 female Syrian golden hamsters were administered DMOB in the diet at concentrations of 0.1% or 1.0% (1,000 or 10,000 ppm) for 144 weeks. The number of controls was not specified in IARC (1974). The only malignant neoplasm observed was a transitional cell carcinoma of the urinary bladder in one animal after 144 weeks of exposure to DMOB at 1,000 ppm. This neoplasm is rare in hamsters and therefore was attributed to DMOB exposure. Forestomach papillomas were detected in 37% of the high-dose group, compared with 2% of the controls (Saffiotti *et al*. 1967, Sellakumar *et al*. 1969, both cited in IARC 1974).

4.1.3 Drinking water studies in mice

The carcinogenic potential of DMOB dihydrochloride has been evaluated in a drinking-water study in mice of both sexes (Schieferstein *et al*. 1990). In this 112-week cancer bioassay, four-week-old BALB/cStCrlC3Hf/Nctr mice (166 male and 165 female) received drinking water containing DMOB dihydrochloride at 0, 20, 40, 80, 160, 315, or 630 ppm. The animals were scheduled to be sacrificed 13, 26, 39, 52, 78, or 112 weeks after initiation of the bioassay. The high dose level (630 ppm) given over a one-week period is approximately equal to an acute oral bolus dose that would be lethal to half of the animals, assuming that a 30-g mouse drinks 28 g of water per week. Water consumption was depressed in all the dose groups, especially the high-dose group. Although body weight gain was suppressed at the highest dose level during the first year, administration of DMOB dihydrochloride did not affect mortality of either males or females. No increased incidences of neoplasms were observed in any of the tissues examined, which included spleen, Harderian gland, liver, and lung (Schieferstein *et al*. 1990).

4.2 Carcinogenesis study of C.I. Direct Blue 15

The carcinogenicity of C.I. Direct Blue 15, a DMOB-based dye, was evaluated in a 22-month study in 40- to 47- day-old Fischer 344/N rats of both sexes (NTP 1992, IARC 1993). The concentrations of C.I. Direct Blue 15 in distilled drinking water were 0, 630, 1250, or 2500 ppm (corresponding to 0, 45, 90, or 215 mg/kg per day for male rats and 0, 50, 100, or 200 mg/kg per day for female rats). The numbers of male and female rats in the control, low-, medium-, and high-dose groups were 70, 45, 75, and 70, respectively. Ten animals from the control group and the high dose group were sacrificed at the nine-month interim evaluation. Ten additional animals from each group were sacrificed at the 15-month interim evaluation.

The numbers of males surviving the duration of the study were 37 (75%), 8 (24%), 11 (17%), and 2 (4%), respectively, from the 0-, 630-, 1250-, and 2500-ppm dose groups. The numbers of surviving females were 40 (80%), 13 (37%), 22 (35%), and 4 (8%) from the 0-, 630-, 1250-, and 2500-ppm dose groups. Administration of C.I. Direct Blue 15 significantly reduced the survival of both male and female rats. Reduced survival of

dosed animals was attributed to sacrifices of moribund animals necessitated by the appearance of treatment-related neoplasms.

Post-necropsy, histopathological examination of the tissues of the male and female rats revealed benign and malignant neoplasms of the skin, Zymbal gland, preputial gland, clitoral gland, uterus, liver, oral cavity, and small and large intestine. Increased incidences of mononuclear cell leukemia and neoplasms of the brain may also have been treatment-related. Increases in the incidences of Zymbal gland, oral cavity, and intestinal tumors were in the C.I. Direct Blue 15-treated rats were statistically significant and markedly dose-related.

The incidences of squamous cell papillomas or carcinomas of the skin also were significantly increased. A similar increased incidence of basal cell adenomas or carcinomas of the skin was seen in high-dose males (56%, vs. 4% in controls), but not in females. Tumor incidences and their statistical significance are summarized in Table 4-3.

Table 4-3. Tumor incidences in Fischer 344/N rats administered C.I. Direct Blue 15 in drinking water for up to 22 months

Tumor type	Concentration (ppm) in drinking water			
	0	630	1250	2500
	Tumor incidence/number examined[a]			
Males				
Skin: basal cell adenoma or carcinoma	2/50**	9/35**	27/65**	28/50**
Skin: sebaceous gland adenoma	0/50*	1/35	7/65*	3/50*
Skin: squamous cell papilloma or carcinoma	2/50**	4/35	11/65*	19/50**
Zymbal gland: adenoma or carcinoma	1/50**	5/35*	10/65*	20/50**
Preputial gland: adenoma or carcinoma	8/49	5/35	23/64*	9/48
Leukemias	17/50*	19/35*	28/65	20/50*
Liver: neoplastic nodule or hepatocellular carcinoma	0/50**	6/35*	9/65*	11/50**
Oral cavity: squamous cell papilloma or carcinoma	1/50**	10/35**	24/65**	17/50**
Small intestine: adenomatous polyp or adenocarcinoma	0/50	1/35	0/65	2/50
Large intestine: adenomatous polyp or adenocarcinoma	0/50**	1/35	6/65	8/50*
Females				
Skin: basal cell adenoma or carcinoma	1/50	0/35	1/65	0/50

Tumor type	Concentration (ppm) in drinking water			
	0	630	1250	2500
	Tumor incidence/number examined[a]			
Skin: squamous cell papilloma or carcinoma	0/50**	2/35	6/65*	5/50*
Zymbal gland: adenoma or carcinoma	0/50**	4/35	11/65*	17/50**
Clitoral gland: adenoma or carcinoma	7/50**	11/31*	24/64*	27/50**
Leukemias	7/50*	13/35*	27/65**	15/50**
Liver: Neoplastic nodule or hepatocellular carcinoma	0/50**	0/35	2/65	5/50*
Oral cavity: squamous cell papilloma or carcinoma	2/50**	4/35	19/65**	15/50**
Small intestine: adenocarcinoma	0/50*	0/35	1/65	3/50
Large intestine: adenomatous polyp	0/50	0/35	3/65	1/50
Uterus: adenoma or adenocarcinoma	1/50*	0/35	1/65	4/50*

Source: NTP (1992).
[a]Logistic regression tests; this test regards tumors in animals dying prior to terminal kill as nonfatal:
*$P < 0.05$, **$P \leq 0.001$.

Based on the observations in this study, the NTP concluded that there was clear evidence of carcinogenic activity of C.I. Direct Blue 15 in male and female Fischer 344/N rats (NTP 1992). The IARC review of C.I. Direct Blue 15 concluded that it was *possibly carcinogenic to humans* (Group 2B) (IARC 1993).

4.3 Transplantability of preputial tumors induced by DMOB, a DMOB-based dye (C.I. Direct Blue 15), or a DMB-based bye (C.I. Acid Red 114)

Ulland *et al.* (1989) demonstrated the transplantability of preputial gland and epithelial skin neoplasms (epidermal basal cell tumors and epidermal squamous cell carcinomas) induced in Fischer 344/N rats during the lifetime drinking-water studies of DMOB, the DMOB-based dye, C.I. Direct Blue 15, or the DMB-based dye, C.I. Acid Red 114. The neoplasms selected for transformation studies 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 Fischer 344/N rats. 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.

Transplantability also was demonstrated with preputial gland neoplasms induced in Fischer 344/N rats in drinking-water studies of DMOB (NTP 1990) and C.I. Direct Blue

15 (NTP 1992). The transplants were successful, and the transplanted neoplasms appeared to be malignant in nature, growing very rapidly (reaching 3.0 cm in 7 to 9 weeks) with a short latency period. The carcinomas were retrospectively diagnosed, but comparable information was not obtained for preputial gland adenomas. In four serial passages, the transplants did not become less differentiated or anaplastic; however, the transplants maintained their malignant nature despite their well-differentiated morphology. These results confirmed the malignancy of the preputial gland tumors induced by orally administered DMOB and C.I. Direct Blue 15.

4.4 Oncogene activation induced by DMOB or C.I. Direct Blue 15

A study designed to detect activation of *ras* oncogenes in rat tumors induced by DMOB or a DMOB-based dye explored the possibility that their carcinogenic mechanism in rats is the induction of activating point mutations in members of the *ras* gene family (Reynolds *et al*. 1990). Neoplasms obtained from control rats and rats exposed to DMOB or C.I. Direct Blue 15 were assayed for the presence of activated protooncogenes using the NIH 3T3 DNA mouse fibroblast transfection assay (Reynolds *et al*. 1990, NTP 1992). The assay detected activated oncogenes in 21/27 skin, clitoral gland, or preputial gland neoplasms that had been induced by C.I. Direct Blue 15. Activated *ras* oncogenes were detected at a higher frequency (11/13) in tissues of epidermal origin (skin) or histogenetically related tissues (clitoral and preputial gland, 10/14). In comparison, few activated of *ras* oncogenes were detected in spontaneous epithelial neoplasms (1/21) (Reynolds *et al*. 1990). The design and findings of this study are summarized in Tables 4-4 and 4-5.

Table 4-4. Detection of activated oncogenes in tumors occurring spontaneously or induced by DMOB or C.I. Direct Blue 15

Tumor type	Frequency (positive/tested)	Transforming efficiency, foci per µg of DNA	
		Tumor DNA	Transfectant DNA first cycle
Spontaneous[a]			
Benign	0/25	nd	nd
Malignant	1/13	0.03	1.60
Induced by DMOB or C.I. Direct Blue 15			
Benign	1/4	0.01	1.05
Malignant	20/30	0.01 - 0.33	0.20 - 2.00

Source: Reynolds *et al*. (1990)
[a]NIH 3T3 DNA transfection data for 29 spontaneous tumors.
nd: no data.

Table 4-5. Identity and frequency of activated *ras* genes within specific tumor types induced by DMOB or C.I. Direct Blue 15

Tumor type	Frequency (spontaneous tumors)[a]	Activated Oncogene	
		H-*ras*	N-*ras*
Preputial gland adenoma	1/1 (0/1)	1	nd
Preputial gland carcinoma	1/3	1	nd
Clitoral gland carcinoma[b]	8/10 (1/2)	7	1
Basal cell carcinoma	5/6 (np[6])	4	1
Squamous cell carcinoma	6/7 (np)	6	nd
Mammary fibroadenoma	0/2 (0/11)	nd	nd
Mammary adenocarcinoma	0/3 (0/2)	nd	nd
Duodenal adenocarcinoma	0/1 (np)	nd	nd
Subcutaneous. fibroma	0/1 (0/5)	nd	nd

Source: Reynolds *et al*. (1990)
[a] Tumors positive/tumors tested.
[b]One H-*ras* spontaneous activated oncogene observed.
nd: no data; np: not provided.

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

The pattern of the increased tumor incidences observed with C.I. Direct Blue 15 was similar to that seen with DMOB and 3,3′-dimethylbenzidine (DMB), a structural analog of DMOB. Similar exposure to C.I. Acid Red 114, a dye based on DMB, also resulted in a similar pattern of tumors. A comparison of the tumorigenic response in rats for DMOB and DMOB-based dyes with DMB and DMB-based dyes is presented in Table 4-6.

In addition, DMOB, DMB and dyes based on each of these compounds (C.I. Direct Blue 15 and C.I. Acid Red 114, respectively) induced transplantable preputial gland and epithelial gland tumors in F344/N rats (Ulland *et al*. 1989). Activated *ras* oncogenes were also detected in DMOB- and C.I. Direct Blue 15-induced tumors at higher frequencies than those that arose spontaneously (Reynolds *et al*. 1990).

Table 4-6. Qualitative tumor responses of rats administered DMOB, a DMOB-based dye, DMB, or a DMB-based dye in drinking water

Tumor type	Amine/Dye[a]			
	DMOB	DMOB-based C.I. Direct Blue 15	DMB	DMB-based C.I. Acid Red 114
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.

4.6 Summary

Oral administration of DMOB is carcinogenic in rats and hamsters. The pattern of DMOB-induced tumors is similar to that seen with C.I. Direct Blue 15, a dye metabolized to DMOB. The one study of DMOB in mice failed to detect an increased incidence of tumors. The pattern of tumors induced by chronic administration of DMOB, a DMOB-based dye, DMB, and a DMB-based dye is strikingly 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. DMOB, DMB, and a dye based on each of these compounds (C.I. Direct Blue 15 and C.I. Acid Red 114, respectively) induce transplantable preputial gland and epithelial gland tumors in Fischer 344/N rats. In addition, activated *ras* oncogenes are detected in tumors induced by DMOB or C.I. Direct Blue 15 at higher frequencies than in spontaneously occurring tumors.

5 Genotoxicity

5.1 Prokaryotic systems

5.1.1 *Induction of mutation in* Salmonella typhimurium

DMOB has been extensively studied for the induction of gene mutations in *Salmonella typhimurium.* In tests sponsored by the NTP (1992), DMOB dissolved in dimethylsulfoxide (DMSO) was tested at multiple concentrations (0 to 10,000 µg/plate) in three different laboratories, in various *S. typhimurium* strains with and without metabolic activation by S9 liver homogenate from Aroclor-induced rats and hamsters. Overall, DMOB was mutagenic with exogenous metabolic activation in strains TA98 and TA100 (Haworth *et al.* 1983; NTP 1992). One laboratory reported a significant response in strain TA98 without metabolic activation, and another laboratory observed a weakly positive response in strain TA1535 in the presence of hamster S9 liver homogenate.

Another study tested the mutagenic response of various *S. typhimurium* strains (with and without S9 liver homogenate from Aroclor-induced rats or hamsters) to DMOB and the corresponding *N*-monoacetyl and *N,N′*-diacetyl derivatives. In general, TA98 was the most sensitive strain, followed by TA1538; all three compounds were mutagenic in these strains. DMOB was more mutagenic in the presence of S9 liver homogenate from rats than with S9 from hamsters. The *N*-monoacetylated derivative was more mutagenic than DMOB or the *N,N′*-diacetyl derivative (Reid *et al.* 1984, cited in Morgan *et al.* 1994). The mutagenic activity of DMOB and DMOB dihydrochloride was tested in *S. typhimurium* strains TA100 and TA98 with and without rat S9 metabolic activation, at concentrations ranging from 0 to 5 µg/mol. Both the free base and dihydrochloride salt forms of DMOB were mutagenic with metabolic activation, and no appreciable difference in mutagenic activity between the two forms was observed (Messerly *et al.* 1987).

In another gene mutation assay, DMOB at concentrations of 5, 50, or 100 µg/plate was mutagenic in *S. typhimurium* strains TA98 and TA1538 with S9 metabolic activation (Sariaslani and Stahl 1990). Other studies have confirmed that DMOB is mutagenic in strain TA98 with metabolic activation and reported a weak mutagenic response in strain TA100 with activation (You *et al.* 1993).

A number of dyes metabolized to DMOB (C.I. Direct Blue 1, C.I. Direct Blue 8, C.I. Direct Blue 10, C.I. Direct Blue 15, and C.I. Direct Violet 32) are not mutagenic in the absence of conditions that result in the reduction by azo bonds. However, all DMOB-based dyes tested in an azo-reductive system (rat cecal flora mix or flavin mononucleotide [FMN]) were found mutagenic in *S. typhimurium* strains TA98 or TA1538 (Morgan *et al.* 1994). The results of this study are summarized in Table 5-1.

Table 5-1. Mutagenicity of DMOB and dyes metabolized to DMOB in *S. typhimurium*

Compound	% Purity	Metabolic activation			
		None	S9	FMN	Cecal
DMOB	98	-	+	+	+
C.I. Direct Blue 1	-	-	-	+	NT
C.I. Direct Blue 8	30	-	-	+	+
C.I. Direct Blue 10	48	-	-	+	+
C.I. Direct Blue 15	50	-	-	+	+
C.I. Direct Violet 32	-	-	-	+	+

Source: Morgan *et al*. (1994).
S9: standard (aerobic) preincubation test procedure
FMN: FMN-supplemented S9 for reductive metabolism
Cecal: rat cecal flora suspension for anaerobic metabolism
(-): not mutagenic
(+): mutagenic
NT: not tested

C.I. Direct Blue 15, a dye that is metabolized to DMOB, yields DMOB upon metabolic reduction of the azo bonds and is thus considered potentially genotoxic (Ashby and Tennant 1988; Mortelmans *et al*. 1986, both cited in NTP 1992). In the NTP study (NTP 1992), C.I. Direct Blue 15 was not mutagenic in various *S. typhimurium* strains (with or without rat or hamster liver S9 metabolic activation) at various concentrations (100 to 10,000 µg/plate). However, when tested after reductive (rat cecal bacteria or FMN) metabolism, it was mutagenic in *S. typhimurium* strain TA1538 with S9 activation at concentrations of 0.25, 0.50, or 1.00 µM (NTP 1992). Other studies have confirmed that C.I. Direct Blue 15 is mutagenic in *S. typhimurium* strains TA98, TA100, and TA1538 when reductive metabolism precedes incubation (Gregory *et al*. 1981; Brown and Dietrich 1983; Prival *et al*. 1984; Reid *et al*. 1984, all cited in NTP 1992).

C.I. Direct Blue 15 was found to be mutagenic in *S. typhimurium* strain TA98 and the arabinose-resistant tester strain SV50 in the presence of hamster or rat liver S9 metabolic activation. The concentrations tested ranged from 0.10 to 3.00 mg/plate. SV50 was less sensitive than TA98 in detecting mutagenic response (Krishna *et al*. 1986). In this study, DMOB was non-mutagenic in the arabinose-resistant tester strain SV50 and mutagenic in *S. typhimurium* strain TA98.

5.1.2 *Mutagenicity in* Escherichia coli

DMOB failed to induce DNA damage in *Escherichia coli* in the absence of S9 metabolic activation (Fluck *et al*. 1976, cited in NTP 1990).

5.2 Eukaryotic systems

5.2.1 *Mutagenicity in* Drosophila melanogaster

DMOB did not induce sex-linked recessive lethal mutations in adult male *Drosophila melanogaster* administered DMOB in feed at 100 ppm or by injection at 200 ppm (Yoon *et al*. 1985, cited in NTP 1990).

5.3 Mammalian systems

5.3.1 In vitro assays

5.3.1.1 Mouse lymphoma cell mutagenesis assay

In two NTP-sponsored studies (Caspary *et al.* 1988), DMOB dihydrochloride was mutagentic in the L5178Y mouse lymphoma cell mutagenesis assay both with and without metabolic activation by S9 liver homogenate from Fischer 344 rats.

In the first study (Myhr and Caspary 1988), the assays were conducted with no exogenous activation and with activation by S9 liver homogenate from non-induced Fischer 344/N rats. DMOB was mutagenic under non-activation conditions over a narrow concentration range (60 to 90 µg/mL) just below the toxic concentration (≥ 90 µg/mL). Addition of S9 caused a reduction in toxicity. A two-fold increase in mutation frequency was observed at 75 µg/mL. At 250 µg/mL (which exceeded the apparent solubility limit), the mutation frequency increase ranged from 1.6- to 2.2-fold. Insoluble doses of 300 µg/mL induced a three-fold increase in mutation frequency. The primary effect of addition of S9 was to reduce the toxicity of DMOB dihydrochloride without changing the magnitude of the mutagenic activity at toxic doses (Myhr and Caspary 1988).

In the second study (Mitchell *et al.* 1988), the mouse lymphoma assay was conducted with and without S9 liver homogenate from Aroclor-induced Fischer 344 rats. DMOB dihydrochloride was mutagenic in the absence of S9, inducing a three- to four-fold mutation frequency increase at concentrations of 64 µg/mL and 100 µg/mL. In the presence of S9, the toxicity was reduced, and concentration ranges were approximately five times as high. A twofold increase in mutation frequency was observed at concentrations of 328 µg/mL and 437 µg/mL.

5.3.1.2 Chromosomal aberrations (CA)

DMOB dihydrochloride induces CA in Chinese hamster ovary (CHO) cells with and without metabolic activation (Galloway *et al*. 1987, cited in NTP 1990). When first reported, the results of the CA tests were considered negative, but later statistical reanalysis of the data indicated that the results were weakly positive without S9 metabolic activation and positive with S9 activation (Galloway *et al*. 1985, 1987, cited in NTP 1990). Concentrations ranged from 0.005 to 5000 µg/mL.

The DMOB-based dye C.I. Direct Blue 15 failed to induce CA in CHO cells. Concentration ranges for this assay were 1500 to 2250 µg/mL without metabolic activation and 2000 to 2500 µg/mL with metabolic activation. Reductive metabolism was not used in the chromosomal aberrations assay (NTP 1992).

5.3.1.3 Sister chromatid exchanges

DMOB dihydrochloride induced sister chromatid exchanges (SCEs) in CHO cells both with and without S9 metabolic activation. Concentrations ranged from 0.005 to 5000 µg/mL. In one of the two laboratories conducting these cytogenetic tests, the weakly positive SCE result without S9 activation occurred under delayed harvest (3 to 5 hours), because DMOB dihydrochloride induced a delay in the cell division cycle. The positive

results obtained by the other laboratory occurred at lower concentrations of DMOB dihydrochloride that did not affect cell cycle time (Galloway *et al*. 1985, cited in NTP 1990).

C.I. Direct Blue 15 did not induce SCEs in CHO cells. Concentrations ranged from 250 to 750 μg/mL without metabolic activation and 83.3 to 2500 μg/mL with metabolic activation. A 20% increase in SCEs per chromosome of culture would have been classified as a positive result. Reductive metabolism was not used in this assay (NTP 1992).

5.3.2 In vivo *assays*

5.3.2.1 Chromosomal aberrations

A single 100-mg/kg dose of DMOB was injected into four mice. DMSO (2 mL/kg) was used as a negative control, and 7,12-dimethylbenz[*a*]anthracene (DMBA) (100 mg/kg) was a positive control. DMOB caused a statistically significant increase in CA in the bone marrow of mice relative to DMSO controls (aberrant cells = 5.25 ± 0.96%, mitotic indices = 2.05 ± 0.15%). DMOB was not, however, nearly as genotoxic as the positive control, DMBA (You *et al.* 1993).

5.4 Summary

DMOB is mutagenic in *Salmonella typhimurium* with exogenous metabolic activation. C.I. Direct Blue 15, a DMOB-based dye, also is mutagenic in *S. typhimurium* with metabolic activation and in the presence of azo-reductive systems that form DMOB. DMOB induces mutations in the mouse lymphoma cell assay and causes CA and SCE in CHO cells, in the presence or absence of exogenous metabolic activation. DMOB also induces chromosomal aberrations *in vivo* in the bone marrow of mice.

6 Other Relevant Data

6.1 Mammalian absorption, distribution, metabolism, and excretion of DMOB and DMOB-based dyes

In a study of DMOB-based dye absorption, metabolism, and excretion, five female mongrel dogs received single oral doses of 100 mg/kg of C.I. Direct Blue 15 or C.I. Direct Blue 1. Free DMOB, as an impurity in each dye sample, was determined. The urinary excretion of free DMOB also was monitored using gas chromatography. C.I. Direct Blue 15 and C.I. Direct Blue 1 were metabolized to DMOB as indicated by the presence of more amine in the urine than could be accounted for by the presence of free DMOB as an impurity in the dye sample. This is an indication that C.I. Direct Blue 15 and C.I. Direct Blue 1 undergo azo reduction to yield the parent amine, DMOB. There was substantial variation in the percentage of the dose of the dyes excreted as free DMOB. The results of this study are summarized in Table 6-1 (Lynn *et al*. 1980).

Table 6-1. Urinary excretion of DMOB by dogs after oral administration of DMOB-based dyes

Dye	DMOB impurity (ppm)	Dose of DMOB as impurity (µg)	DMOB excreted in urine during 48 hours after dosing (µg)			Percent of dose[a]
			Experiment 1	Experiment 2	Mean	
C.I. Direct Blue 15	46	69	61	168	114	0.03
C.I. Direct Blue 1	18	27	441	119	280	0.08

Source: Lynn *et al.* (1980)
[a]Percent of potential theoretical maximum produced by complete reduction of the azo bonds in the dye.

In a similar study, C.I. Direct Blue 15 and C.I. Direct Blue 1 were administered by gavage (100 mg/kg) to male Sprague-Dawley rats to study the absorption, metabolism, and excretion of the dyes in rats (Lynn *et al.* 1980). The free DMOB impurity in the C.I. Direct Blue 15 and C.I. Direct Blue 1 samples were measured as 1 and <1 µg, respectively. DMOB was excreted in the urine of the rats at concentrations of 13.0 ± 12.9 and 42.5 ± 27.7 µg, respectively, 72 hours after administration of C.I. Direct Blue 15 and C.I. Direct Blue 1. These urinary concentration levels of free DMOB correspond to 0.17 ± 0.18 and 0.55 ± 0.37 percent, respectively, of the potential theoretical maximum produced by complete reduction of the azo bonds of the administered C.I. Direct Blue 15 and C.I. Direct Blue 1 doses. In comparison, the free DMOB was eliminated in the urine of rats in higher concentrations than in the urine of dogs from the same doses of C.I. Direct Blue 15 and C.I. Direct Blue 1. More detailed results from these urinary excretion studies of benzidine-, DMB-, and DMOB-based dyes in rats and dogs are presented in Appendix C (Tables C-1 and C-2).

DMOB-based dyes, including C.I. Direct Blue 15, have been reported to be metabolized to DMOB in humans as indicated by the detection of DMOB in the urine of three of 22 workers who dried and ground two DMOB-based dyes (NIOSH 1981; Rodgers *et al.* 1983, cited in NTP 1992). Azo dyes containing DMOB can also be reduced by rat liver azoreductases to DMOB (Martin and Kennelly 1981, cited in NTP 1992).

Bowman *et al*. (1982) demonstrated the metabolism of C.I. Direct Blue 15, a DMOB-based dye, in male Fischer 344 rats. In this study, the biphenyl portion of the molecule was uniformly labeled.. Approximately 18.8% of the [^{14}C] administered was recovered in the urine of the rats given C.I. Direct Blue 15 (12 mg dye/kg body weight or molar equivalent DMOB by oral gavage). Intact dye in the feces accounted for 12% of the orally administered dose, with 84% of the fecal [^{14}C] resulting from unidentified metabolic products. In comparison, when rats were administered ^{14}C-labeled DMOB, 35% and 74.4% of the [^{14}C] was recovered in the urine and feces, respectively. The excretion of [^{14}C] in the feces and urine peaked at 8 to 16 hours after dosage, although detectable amounts of [^{14}C] were still being excreted 144 to 192 hours after the single oral dose of 12 mg/kg of the ^{14}C-labeled dye . Analysis of urinary metabolites after oral administration of C.I. Direct Blue 15 revealed that radioactivity was excreted in a free amine fraction and in an alkaline hydrolyzable conjugate (AHC) fraction. The free amine fraction was comprised of DMOB (0.22% of the dose), its monoacetylated metabolite (0.27%), and its diacetylated metabolite (0.22%). The AHC fraction contained DMOB (0.48% of the dose). DMOB is more extensively metabolized and excreted than the dye. Diacetylated DMOB was the major metabolite observed following administration of DMOB-based dyes. Following administration of DMOB, most of the dose found in urine was in the AHC fraction (1.56%). Other compounds found in urine were DMOB (1.18%), monoacetylated-DMOB (0.35%), and diacetylated-DMOB (0.93%).

After oral administration of ^{14}C-labeled C.I. Direct Blue 15 to rats, [^{14}C] concentration was initially high in the gastrointestinal tract, with subsequent time-related, widespread distribution of radioactivity to soft tissues.

In further experimentation, Bowman *et al.* (1983) demonstrated the metabolism of several DMOB-based dyes (C.I. Direct Blue 8, C.I. Direct Blue 10, C.I. Direct Violet 32, or C.I. Direct Black 114). In this study, urinary excretion of DMOB and its metabolites was observed in the urine of male Fischer 344 rats up to 96 hours after the oral administration of a single dose of 2 mg of C.I. Direct Blue 8, C.I. Direct Blue 10, C.I. Direct Violet 32, or C.I. Direct Black 114. Sensitive chromatographic analysis (EC/GC) of metabolites in the urine revealed mainly mono- and di-acetylated-DMOB, the parent amine (DMOB), and alkaline hydrolyzable conjugates in concentrations ranging from 15 μg (for alkaline hydrolyzable conjugates derived from C.I. Direct Violet 32) to 0.07 μg (for mono-acetylated DMOB derived from C.I. Direct Blue 8) at the peak excretion period of 12 to 24 hours post-treatment. At the peak excretion period of 12 to 24 hours post-treatment, a total DMOB μg-equivalent of 4.9, 12, 11, and 27 were excreted for C.I. Direct Blue 8, C.I. Direct Black 114, C.I. Direct Blue 10, C.I. Direct Violet 32 doses, respectively. Excretion was essentially complete within 96 hours.

Rodgers *et al.* (1983, cited in NTP 1990) investigated the metabolism of ^{14}C-labeled DMOB administered intravenously to male Fischer 344 rats. Thirty minutes after dosing,

less than 2% of unchanged ^{14}C-labeled DMOB was recovered. Within 72 hours, 70% of the total ^{14}C-labeled dye administered was excreted in the bile. In intact rats, 50% of the dose was found in the intestinal tract after 2 hours. After either oral or intravenous administration, 50% of the dose was excreted in feces and 30 to 40% in urine within three days. Of the [^{14}C] remaining in the animal, 45% was present in the liver in the form of covalently bound metabolites; more than 90% of the urinary radiolabel was in the form of metabolites. Unmetabolized DMOB accounted for 3 to 9% of the dose, and less than 5% was associated with acetyl-DMOB. The proposed metabolic pathway is shown in Figure 6-1.

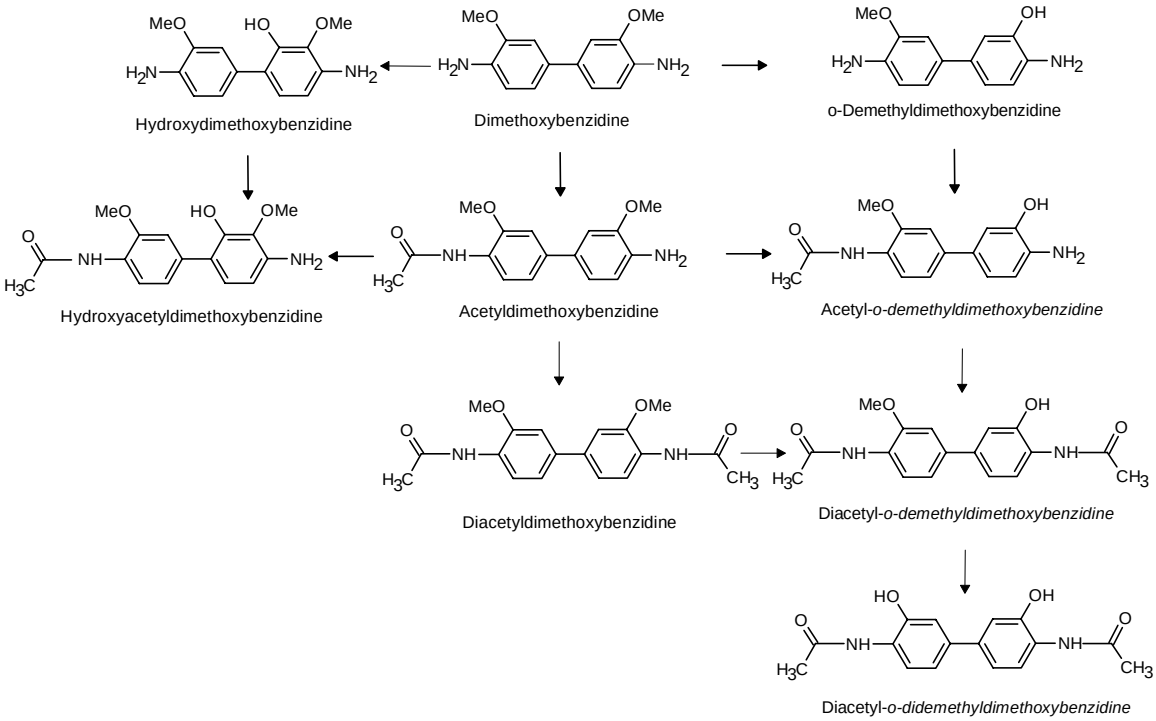
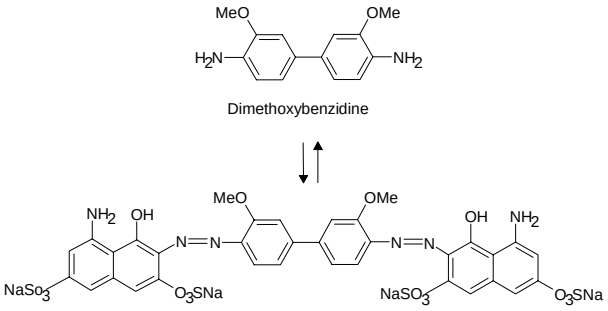


Figure 6-1. Proposed metabolic pathways of DMOB

Source: Rodgers *et al.* (1983, cited in NTP 1992)

6.2 Bacterial metabolism of DMOB-based dyes

The reductive metabolism of DMOB-based dyes (illustrated in Figure 6-2) results in the formation of DMOB. Azo reduction can result from the enzymes present in the liver or azo reductase associated with intestinal bacterial flora. Reductive cleavage of dyes metabolized to DMOB may occur, primarily, through the activity of intestinal bacteria (Martin and Kennelly 1981; Cerniglia *et al.* 1982; Brown and Dietrich 1983; Bos *et al.* 1984, 1986, all cited in NTP 1990). The free DMOB is then absorbed, resulting in systemic exposure, further metabolism (probably in the liver), and subsequent excretion.



Adapted from NTP (1990)

Figure 6-2. Formation of DMOB by reductive metabolism of C.I. Direct Blue 15

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 Blue 15, a DMOB-based dye. This study also investigated 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 (see Table 6-2) varied in the rates at which they reduced C.I. Direct Blue15.

Table 6-2. Reduction of C.I. Direct Blue 15 by various anaerobic bacteria

Organism	C.I. Direct Blue 15 reduction (nmol reduced/mg protein in 8 h)
Bacteroides thetaiotaomicron	26.5
Bifidobacterium infantis	34.8
Citrobacter sp.	110
Clostridium perfringens	315
Clostridium sp.	360
Escherichia coli	10.7
Lactobacillus acidophilus	96.1
Peptococcus anaerobius	113.6
Peptostreptococcus productus	72.7

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

The bacterial isolate from rat intestine was highly efficient in reducing C.I. Direct Blue 15 to DMOB. C.I. Direct Blue 15 (188 nmol) was added to an incubation medium containing 10^{10} bacterial cells. The mixture was assayed for DMOB and C.I. Direct Blue 15 up to 48 hours. Production of DMOB began promptly and was essentially complete (as evidenced by the absence of C.I. Direct Blue 15) within 4 hours.

6.3 Protein adduction

The covalent binding of orally administered DMOB to hemoglobin was studied in female Wistar rats. The results indicated two cleavage products, with amounts of DMOB in excess of or comparable to amounts of the monoacetyl derivative. The hemoglobin

binding index for DMOB was estimated as 2.7 (24.3 for benzidine) (Birner *et al.* 1990). This indicates a potential for binding of these residues to biological macromolecules.

6.4 Summary

The results of a number of studies of the metabolism and elimination of DMOB-based dyes provide evidence that these dyes are subject to *in vivo* metabolism giving rise to the parent amine. The metabolism of DMOB proceeds through *N*-acetylation and excretion in both urine and feces. 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. An assessment of the anaerobic metabolism of DMOB-derived dyes supports this hypothesis. Results indicate that the metabolic conversion of bisazobiphenyl dyes, based on benzidine, DMB and DMOB, to carcinogenic aromatic amines is a general phenomenon and therefore, with few exceptions, should be anticipated for each member of this class of chemicals.

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Appendix A: IARC. 1974. *Some Aromatic Amines, Hydrazine and Related Substances, N-Nitroso Compounds and Miscellaneous alkylating Agents.* 3,3′-Dimethoxybenzidine. IARC Monographs on the Evaluation of the Carcinogenic Risk of Chemicals to Man. Lyon, France. World Health Organization. Vol. 4. A-1 – A-10.

3,3´-DIMETHOXYBENZIDINE*

(o-Dianisidine)

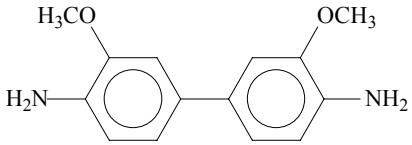
1. Chemical and Physical Data

1.1 Synonyms and trade names

Chem. Abstr. No.: 119-90-4

Bianisidine; 4,4´-diamino-3 ,3´-dimethoxybiphenyl; di-p-amino-di-m-methoxydiphenyl; dianisidine; 3,3´-dimethoxy-4 ,4´-diaminobiphenyl

1.2 Chemical formula and molecular weight



$C_{14}H_{16}N_2O_2$ Mol. wt: 244.3

1.3 Chemical and physical properties of the pure substance

(a) *Description*: Colorless crystals which turn violet on standing

(b) *Melting-point*: 137-138ºC

(c) *Solubility*: Almost insoluble in water, soluble in ethanol, ether, acetone, benzene and chloroform; probably soluble in most organic solvents and lipids

(d) *Chemical reactivity*: A weak base; has the general characteristics of primary aromatic amines

1.4 Technical products and impurities

It is available commercially as the free base (technical and 99% grades) and as its dihydrochloride.

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* Considered by the Working Group in Lyon, June 1973.

2. Production, Use, Occurrence and Analysis

(a) Production and use[1]

3,3'-Dimethoxybenzidine (o-Dianisidine) has been produced commercially for at least 50 years. It is made commercially by reducing the methyl ether of ortho-nitrophenol (ortho-nitro-anisole) to hydrazoanisole, which is subsequently rearranged by acid to o-dianisidine.

Data on production in the United States of o-dianisidine were last reported for the year 1967, when the total production of five companies amounted to 167 thousand kg (US Tariff Commission, 1968). By 1971, only two US companies were producing o-dianisidine. US imports of o-dianisidine through the principal customs districts were reported to have been 124 thousand kg in 1971 (US Tariff Commission, July 1972).

No data are available on the quantity of o-dianisidine produced in countries other than the United States. In 1967, the Federal Republic of Germany was reported to have one producer; Italy was reported to have one producer in 1969; the United Kingdom was reported to have two producers in 1970; and Japan was reported to have three producers in 1972.

o-Dianisidine (or its dihydrochloride) is used principally as a chemical intermediate for the production of dyes. The next most important application is believed to be as an intermediate in the production of o-dianisidine diisocyanate. It has been reported that o-dianisidine has been used for the detection of the presence of a number of metals, thiacyanates, and nitrites, and that o-dianisidine itself was formerly used for dyeing acetate rayon (Lurie, 1964).

o-Dianisidine can be used as an intermediate in the production of 89 dyes (The Society of Dyers and Colourists, 1971). Seven of these dyes, for which 1971 production figures have been reported (US Tariff Commission, October 1972), are listed in the following table:

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

Colour Index No.	Dye	Production (thousands of kg)
24401	Direct Blue 218	479
21160	Pigment Orange 16	153
24410	Direct Blue 1	136
24400	Direct Blue 15	94
24140	Direct Blue 8	64
24411	Direct Blue 76	53
23155	Direct Blue 98	39

In addition, US production of one o-dianisidine-based pigment, Pigment Blue 25 (C.I. 21180), was reported to have been 87 thousand kg in 1971 (US Tariff Commission, August 1972). The o-dianisidine-based dyes and pigments are reportedly useful for dyeing leather, paper, plastics, rubber and textiles (The Society of Dyers and Colourists, 1971).

The phosgenation of the dihydrochloride of o-dianisidine is used in the manufacture of o-dianisidine diisocyanate (also known as 3,3´-dimethoxy-4,4´-diphenylene diisocyanate and 3,3´-dimethoxybenzidine-4,4´-diisocyanate). The amount of o-dianisidine diisocyanate made by the only US producer is not known, but it is estimated that less than 500 thousand kg are produced per year. o-Dianisidine diisocyanate is used in isocyanate based adhesive systems and as a component of polyurethane elastomers.

(b) Occurrence

o-Dianisidine has not been reported to occur as such in nature. It may be present in the waste streams from plants where it is produced or used. o-Dianisidine is listed as a controlled substance in the UK Carcinogenic Substances Regulations 1967 - Statutory Instrument (1967) No. 879.

(c) Analysis

There are many papers which report methods for the separation and subsequent detection of aromatic amines. A brief overview of this general area can be

obtained from the papers on analytical methods cited in the UICC (1970) Monograph. Papers which refer specifically to o-dianisidine usually describe colorimetric detection techniques (Sakai *et al.*, 1960; Glassman & Meigs, 1951). Separation from other amines is normally carried out using thin-layer chromatography (Ghetti *et al.*, 1968).

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

3.1 Carcinogenicity and related studies in animals

(a) Oral administration

Rat: Pliss (1963, 1965) administered doses of 30 mg o-dianisidine dissolved in sunflower seed oil by stomach tube to rats 3 times per week for 13 months (the original number of rats was not stated). Of 18 surviving rats, 4 developed tumours (2 rats had Zymbal gland tumours, 1 an ovarian tumour and 1 a fibroadenoma of the mammary gland). None of the 50 rats in a control group developed tumours at these sites.

Hadidian *et al.* (1968) administered this diamine by stomach tube to 30 male and 30 female Fischer rats in doses ranging from 0.1 to 30 mg/animal in a 'steroid suspending vehicle' (sodium chloride, sodium carboxymethyl cellulose, polysorbate 80, benzyl alcohol and water) 5 days per week for 52 weeks. Tumours appeared in 293 days, but most were found on autopsy at 18 months. Six rats given the 1 mg doses and 6 rats given the 3 mg doses had a total of 8 tumours 29 animals receiving the 10 mg doses had a total of 19 tumours; and 6 rats given the 30 mg doses had a total of 5 tumours. Tumours occurred at various sites including the bladder (2 papillomas), the intestine (3 carcinomas), the skin (5 carcinomas) and the Zymbal gland (3 carcinomas). These tumours were not found in 360 control animals given the vehicle alone.

Hamster: Saffiotti *et al.* (1967) and Sellakumar *et al.* (1969) fed 0.1% and 1.0% o-dianisidine to groups of 30 male and 30 female Syrian golden hamsters. One urinary bladder tumour occurred in the group receiving the lower level of amine. No primary bladder tumours, but forestomach papillomas, occurred in 37% of animals fed 1% of the amine. Of the control group of hamsters, 2% developed stomach papillomas, but none had urinary bladder tumours.

3.2 Other relevant biological data

(a) Animals

Sciarini & Meigs (1961) found that after administration of a dose of 1 g o-dianisidine to two dogs, 0.4% of free diamine and about 5% of a metabolite with properties similar to those of 3,3'-dihydroxybenzidine were excreted in the urine.

(b) Man

o-Dianisidine has been found in the urine of workers exposed to this compound (Meigs *et al.*, 1951, 1954; Ghetti, 1960).

3.3 Observations in man

No epidemiological data on the occurrence of cancer in workers exposed to o-dianisidine alone appear in the literature. Most of the workers exposed to this diamine have also been exposed to related amines such as benzidine, which has been strongly associated with the occurrence of urinary bladder cancer in man (IARC, 1972).

4. Comments on Data Reported and Evaluation[1]

4.1 Animal data

3,3´-Dimethoxybenzidine (o-Dianisidine) was shown to have a carcinogenic effect in rats following oral administration. The findings obtained in the hamster by the same route suggest a similar effect.

4.2 Human data

No conclusive epidemiological studies have been reported concerning the carcinogenicity of o-dianisidine alone in man.

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

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US Tariff Commission (October 1972) Synthetic Organic Chemicals, United States Production and Sales of Dyes, 1971 Preliminary

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.* C.I. Direct Blue 15. IARC Monographs on the Evaluation of the Carcinogenic Risk to Humans. Lyon, France. World Health Organization. Vol. 57. Pp. B-1 – B-14.

CI DIRECT BLUE 15

1. Exposure Data

1.1 Chemical and physical data

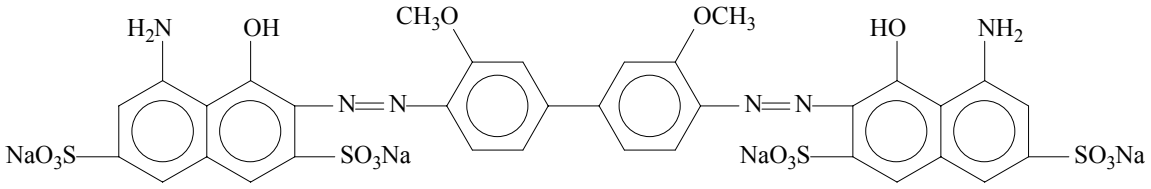
1.1.1 *Synonyms, structural and molecular data*

Chem. Abstr. Serv. Reg. No.: 2429-74-5; replaces 51568-94-6; 95032-75-0

Chem. Abstr. Name: 3,3´-[(3,3´-Dimethoxy[1,1´-biphenyl]-4,4´-diyl) bis(azo)]bis[5-amino-4-hydroxy–2,7-naphthalene disulfonic acid], tetrasodium salt

Colour Index No.: 24400

Synonym: Direct Blue 15



$C_{34}H_{24}N_6O_{16}S_4.4Na$ Mol. wt: 992.85

1.1.2 *Chemical and physical properties*

(a) *Description*: Dark-blue powder (US National Toxicology Program, 1992)

(b) *Melting-point*: 300 °C (decomposes) (US National Toxicology Program, 1992)

(c) *Spectroscopy data*: Infrared and nuclear magnetic resonance spectral data have been reported (US National Toxicology Program, 1992).

(d) *Solubility*: Soluble in water; insoluble in most organic solvents (Society of Dyers and Colourists, 1971a)

1.1.3 *Trade names, technical products and impurities*

Some trade names are: Airedale Blue D; Aizen Direct Sky Blue 5B; Aizen Direct Sky Blue 5BH; Amanil Sky Blue; Atlantic Sky Blue A; Atul Direct Sky Blue; Azine Sky Blue 5B; Belamine Sky Blue A; Benzanil Sky Blue; Benzo Sky Blue A-CF; Benzo Sky Blue S; Cartasol Blue 2GF; Chloramine Sky Blue A; Chloramine Sky Blue 4B; Chrome Leather Pure Blue; Cresotine Pure Blue; Diacotton Sky Blue 5B; Diamine Blue 6B; Diamine Sky Blue; Diaphtamine Pure Blue; Diazol Pure Blue 4B; Diphenyl Brilliant Blue; Diphenyl Sky Blue 6B; Direct Blue 10G; Direct Blue HH; Direct Pure Blue; Direct Pure Blue M; Direct

Sky Blue; Direct Sky Blue A; Direct Sky Blue 5B; Enianil Pure Blue AN; Fenamin Sky Blue; Hispamin Sky Blue 3B; Kayafect Blue Y; Kayaku Direct Sky Blue 5B; Mitsui Direct Sky Blue 5B; Naphtamine Blue 10G; Niagara Blue 4B; Niagara Sky Blue; Nippon Direct Sky Blue; Nippon Sky Blue; Nitto Direct Sky Blue 5B; Oxamine Sky Blue 5B; Paper Blue S; Phenamine Sky Blue A; Pontamine Sky Blue 5BX; Shikiso Direct Sky Blue 5B; Sky Blue 4B;Sky Blue 5B; Tertrodirect Blue F; Vondacel Blue HH.

The raw dye contains about 25% sodium chloride; a desalted preparation (containing ~ 3% salt) contained about 50% CI Direct Blue 15 and about 35 impurities, including 3,3´-dimethoxybenzidine dihydrochloride at 836-1310 ppm (mg/kg). Benzidine was not present at the detection limit of 1 ppm (mg/kg) (US National Toxicology Program, 1992).

CI Direct Blue 15 is available at a purity of 65.5%, containing 15 ppm 3,3´-dimethoxybenzidine (*ortho*-dianisidine; see IARC, 1974, 1987) (Bowman *et al.*, 1982).

1.1.4 *Analysis*

No data were available to the Working Group.

1.2 Production and use

1.2.1 *Production*

CI Direct Blue 15 was first prepared in 1890 (Society of Dyers and Colourists, 1971a). It is produced by coupling 3,3´-dimethoxybenzidine to 1-amino-8-naphthol-3,6-disulfonic acid under alkaline conditions (US Environmental Protection Aency, 1987).

Approximate US production was 108 tonnes in 1972, 241 tonnes in 1977, 98 tonnes in 1981 and 123 tonnes in 1982 (US International Trade Commission, 1974, 1978, 1982, 1983).

1.2.2 *Use*

CI Direct Blue 15 is used to dye cellulose, leather, paper, cotton, silk and wool and to stain biological materials; it is also used to tint cinematographic film (Society of Dyers and Colourists, 1971b). The use pattern for CI Direct Blue 15 in the USA is 65% in textile dyeing, 30% as a paper colourant and 5% for other uses.

1.3 Occurrence

1.3.1 *Natural occurrence*

CI Direct Blue 15 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 Direct Blue 15 was not included in the survey, the results were 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 4527 workers, including 201 women, may have been exposed to CI Direct Blue 15 in seven industries (US National Library of Medicine, 1992).

1.3.3 *Other*

Anaerobic biodegradation of CI Direct Blue 15 gives rise to the amine metabolite, 3,3´-dimethoxybenzidine. 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, derived azo dyes must be handled like the corresponding hypothetical reduction products. CI Direct Blue 15 must therefore be handled like 3,3´-dimethoxybenzidine, which is classified as an A2 compound. Those matrials 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, 40-47 days old, were administered 0, 630, 1250 or 2500 mg/L (ppm) CI Direct Blue 15 (purity, ~ 50%; with ~ 35 impurities, including 3,3´-dimethoxybenzidine) in distilled drinking-water for 96 weeks and were necropsied at 103-104 weeks of age. Survival at 22 months was 37/50, 8/35, 11/65 and 2/50 for male rats and 40/50, 13/35, 22/65 and 4/50 for females in the

control, low-, mid- and high-dose groups, respectively ($p < 0.001$ for both males and females). 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, liver, oral cavity and small intestines and of mononuclear-cell leukaemia in male and female rats, of benign and malignant tumours of the large intestine and preputial gland in males and of the clitoral gland and uterus in females (US National Toxicology Program, 1992).

Table 1. Survival and tumour incidences in male and female Fischer 344/N rats administered CI Direct Blue 15 in the drinking-water for 96 weeks

Survival and tumour types[a]	Dose (mg/L[ppm])				*p*Value[b]
	0	630	1250	2500	
Males					
Survival[c]	37/50	8/35	11/65	2/50	
Skin					
Basal-cell adenoma or carcinoma	2/50	9/35	27/65	28/50	< 0.001
Sebaceous gland adenoma	0/50	1/35	7/65	3/50	= 0.002
Squamous-cell papilloma or carcinoma	2/50	4/35	11/65	19/50	< 0.001
Zymbal gland: adenoma or carcinoma	1/50	5/35	10/65	20/50	< 0.001
Preputial gland: adenoma or carcinoma	8/49	5/35	23/64	9/48	< 0.001[d]
Hepatocellular neoplasms[f]	0/50	6/35	9/65	11/50	< 0.001
Oral cavity: squamous-cell papilloma or carcinoma	1/50	10/35	24/65	17/50	< 0.001
Small intestine: adenocarcinoma	0/50	0/35	0/65	2/50	= 0.078
Large intestine: polyps or adenocarcinoma	0/50	1/35	6/65	8/50	< 0.001
Mononuclear-cell leukaemia	17/50	19/35	28/65	20/50	< 0.001[d]
Females					
Survival	40/50	13/35	22/65	4/50	
Squamous-cell papilloma or carcinoma of the skin	0/50	2/35	6/65	5/50	= 0.001
Zymbal gland: adenoma or carcinoma	0/50	4/35	11/65	17/50	< 0.001
Clitoral gland: adenoma or carcinoma	7/50	11/31	24/64	27/50	< 0.001
Hepatocellular neoplastic nodule or carcinoma	0/50	0/35	2/65	5/50	< 0.001
Oral cavity: squamous-cell papilloma or carcinoma	2/50	4/35	19/65	15/50	< 0.001
Small intestine: adenocarcinoma	0/50	0/35	1/65	3/50	= 0.032
Uterine adenoma or adenocarcinoma	1/50	0/35	1/65	4/50	= 0.004
Mononuclear-cell leukaemia	7/50	13/35	27/65	15/50	< 0.001[d]

From US National Toxicology Program (1992)
[a]Terms used by authors
[b]Logistic regression trend test
[c]At 22 months; reduced survival in exposed groups due to neoplasia
[d]Life-table test

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*

Anaerobic biodegradation of CI Direct Blue 15 gives rise to the amine metabolite, 3,3´-dimethoxybenzidine (Brown & Hamburger, 1987). The dye was cleaved by pure cultures of anaerobic bacteria and by suspensions derived from the intestinal content of rats, with subsequent formation of the amine (Cerniglia *et al.*, 1982).

CI Direct Blue 15 (100 mg/kg) containing 46 ppm (mg/kg) 3,3´-dimethoxybenzidine 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´-dimethoxybenzidine, the potential metabolic product (Lynn *et al.*, 1980). Excretion was found to be 0.03% of the dose of dye administered, which cannot be attributed to the level of impurity. The same dose was also administered once to four male Sprague-Dawley rats by intragastric intubation; after 72 h, 0.17 ± 0.18% of the theoretical maximum was excreted as 3,3´-dimethoxybenzidine and the monoacetyl derivative, the latter constituting a substantial fraction.

When [^{14}C-biphenyl]CI Direct Blue 15 was given as a single dose of 12 mg/kg to six-week-old male Fischer 344 rats by gavage, 74.4% of the dose was excreted in the faeces and 18.8% in urine within 192 h. Only 12% of the dose appeared as intact dye in the faeces within 48 h, the remainder being unidentified metabolic products. Excretion of the free diamine, 3,3´-dimethoxybenzidine, and of its mono- and diacetyl derivatives in urine was determined to be 0.22, 0.27 and 0.22% of the dose, respectively. An equivalent dose of ^{14}C-labelled 3,3´-dimethoxybenzidine was administered for comparison: 52% of the dose appeared in the faeces and 35% in the urine, indicating that the free amine is metabolized to a greater extent than the dye. Only 1.5% of the dose in faeces could be attributed to the free amine fraction, including the acetylated metabolites; in urine, 1.18% of the dose was excreted as the parent compound, 0.35% as the monoacetyl derivative and 0.93% as the diacetyl derivative. Radiolabel was found in all tissues examined from rats dosed with ^{14}C-CI Direct Blue 15. The levels peaked at 4 and 8 h and were highest in the gastrointestinal tract, liver, kidney and lung (Bowman *et al.*, 1982).

4.2 Toxic effects

4.2.1 Humans

No data were available to the Working Group.

4.2.2 Experimental animals

CI Direct Blue 15 binds to albumin, α_1-lipoprotein, β-lipoprotein, haemopexin, prealbumin and α_1-antichymotrypsin, to alter their mobility in crossed immunoelectrophoresis and to degrade C_3 globulin (Emmet *et al.*, 1985). The importance of these findings *in vivo* remains to be established, as it is not known how much unchanged dye is absorbed and transported within the body.

CI Direct Blue 15 was tested for subchronic toxicity in male and female Fischer 344 rats (Morgan *et al.*, 1989; US National Toxicology Program, 1992). Groups of 10 animals of each sex received the dye in drinking-water for 13 weeks at concentrations of 0, 0.063, 0.125, 0.25, 0.50 and 1.0% for females and 0, 0.125, 0.25, 0.50, 1.0 and 3.0% for males. Seven male rats died in the highest-dose group; the first death occurred after three weeks and the last after 13 weeks. Groups given 1% CI Direct Blue 15 gained 17% less body weight than controls, and males treated with 3% of the dye gained 43% less weight than controls. Absolute and relative kidney weights increased in a dose-related manner in males and females. Changes in haematology and clinical chemistry were not observed. Histopathology showed renal and hepatic toxicity in high-dose males that died before termination of the study. In addition to necrosis of hepatocytes and fatty metamorphosis, blue pigment was observed in Kupffer cells. Focal necrosis occurred in proximal tubular epithelial cells. Mild chronic nephropathy was observed in male and female rats given 1% of dye.

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)

Technical-grade CI Direct Blue 15 was not mutagenic to *Salmonella typhimurium* in standard protocols, but it was mutagenic under conditions favouring azo reduction, which would generate 3,3´-dimethoxybenzidine, a known mutagen. CI Direct Blue 15 was reported in an abstract to be mutagenic at the *tk* locus in mouse lymphoma L5178Y cells. It did not induce unscheduled DNA synthesis in rat hepatocytes (abstract) or sister chromatid exchange or chromosomal aberrations in Chinese hamster ovary cells *in vitro*.

Activated *ras* genes were found in 21/34 tumours induced in rats by CI Direct Blue 15 (US National Toxicology Program, 1992) and in 1/38 spontaneous tumours tested (Reynolds *et al.*, 1990; Table 3).

Table 2. Genetic and related effects of CI Direct Blue 15

Test system	Result		Dose[a] (LED/HID)	Reference
	Without exogenous metabolic system	With exogenous metabolic system		
SAF, *Salmonella typhimurium*, forward mutation (arabinose resistance)	0	+[b,c]	100.0000	Krishna *et al.* (1986)
SA0, *Salmonella typhimurium* TA100, reverse mutation	0	–[d]	250.0000	Elliott & Gregory (1980)
SA0, *Salmonella typhimurium* TA100, reverse mutation	0	+[e]	62.5000	Elliott & Gregory (1980)
SA0, *Salmonella typhimurium* TA100, reverse mutation	0	+[f]	150.0000	Brown & Dietrich (1983)
SA0, *Salmonella typhimurium* TA100, reverse mutation	0	(+)[g]	150.0000	Brown & Dietrich (1983)
SA0, *Salmonella typhimurium* TA100, reverse mutation	–	–	5000.0000	Mortelmans *et al.* (1986)
SA5, *Salmonella typhimurium* TA1535, reverse mutation	–	–	5000.0000	Mortelmans *et al.* (1986)
SA7, *Salmonella typhimurium* TA1537, reverse mutation	–	–	5000.0000	Mortelmans *et al.* (1986)
SA8, *Salmonella typhimurium* TA1538, reverse mutation	0	+[d]	125.0000	Reid *et al.* (1984)
SA9, *Salmonella typhimurium* TA98, reverse mutation	0	+[f]	0.0000	Sugimura *et al.* (1977)
SA9, *Salmonella typhimurium* TA98, reverse mutation	0	(+)[d]	500.0000	Elliott & Gregory (1980)
SA9, *Salmonella typhimurium* TA98, reverse mutation	0	+[e]	62.5000	Elliot & Gregory (1980)
SA9, *Salmonella typhimurium* TA98, reverse mutation	0	+[f]	150.0000	Brown & Dietrich (1983)
SA9, *Salmonella typhimurium* TA98, reverse mutation	0	(+)[g]	150.0000	Brown & Dietrich (1983)
SA9, *Salmonella typhimurium* TA98, reverse mutation	0	+[h]	50.0000	Prival *et al.* (1984)
SA9, *Salmonella typhimurium* TA98, reverse mutation	0	+[b]	100.0000	Krishna *et al.* (1986)
SA9, *Salmonella typhimurium* TA98, reverse mutation	–	–	5000.0000	Mortelmans *et al.* (1986)
URP, Unscheduled DNA synthesis, rat primary hepatocytes *in vitro*	–	0	0.0000	Mirsalis *et al.* (1983); abstr.
G5T, Gene mutation, mouse lymphoma L5178Y cells *in vitro*	–	+	0.0000	Rudd *et al.* (1983); abstr.
SIC, Sister chromatid exchange, Chinese hamster ovary cells *in vitro*	–	–	2500.0000	Galloway *et al.* 1987)
CIC, Chromosomal aberrations, Chinese hamster ovary cells *in vitro*	–	–	2500.0000	Galloway *et al.* 1987)

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

[a] µg/ml; 0.0000, not given

[b] Preincubation with hamster or rat liver S9 and flavin mononucleotide supplementation

[c] Rat liver S9 more effective

[d] Anaerobic preincubation or riboflavin supplementation

[e] Plate incorporation and reduction using sodium dithionite

[f] Aerobic preincubation with riboflavin

[g] Anaerobic preincubation with rat caecal bacterial extract, flavin mononucleotide and liver S9

[h] Preincubation with no shaking and hamster liver S9 with flavin mononucleotide

Table 3. Activating *ras* mutations in tumours induced in Fischer 344 rats by CI Direct Blue 15 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										
Preputial gland adenoma	1/1		1					1		
Preputial gland carcinoma	1/3		1			1				
Clitoral gland carcinoma	8/10	1	7	1		4		2		
Basal-cell carcinoma	5/6	1	4			1	1	1		1
Squamous-cell carcinoma (skin)	6/7		6			2		4		
Mammary fibroadenoma	0/2									
Mammary adenocarcinoma	0/3									
Duodenal adenocarcinoma	0/1									
Subcutaneous fibroma	0/1									
Untreated										
Clitoral gland adenoma	1/2		1							1
Preputial gland carcinoma	0/1									
Mammary gland fibro-adenoma 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. Summary of Data Reported and Evaluation

5.1 Exposure data

CI Direct Blue 15, a bis-azo dye derived from 3,3´-dimethoxybenzidine, is used mainly for dyeing textiles and paper. The technical grade contains about 50% of pure dye, in addition to inorganic salts and a mixture of about 35 organic compounds, including 3,3´-dimethoxybenzidine.

5.2 Human carcinogenicity data

No data were available to the Working Group.

5.3 Animal carcinogenicity data

Technical-grade CI Direct Blue 15 was tested for carcinogenicity in one study in rats by administration in the drinking-water. It produced benign and malignant tumours of the skin, Zymbal gland, liver, small intestine and oral cavity as well as leukaemia in animals of each sex, of the large intestine and preputial gland in males and of the uterus and clitoral gland in females.

5.4 Other relevant data

CI Direct Blue 15 caused renal and hepatic toxicity in rats. Reductive cleavage of the azo bonds to yield 3,3´-dimethoxybenzidine was demonstrated *in vivo*.

CI Direct Blue 15 induced mutation in bacteria under conditions that favour reduction. Neither sister chromatid exchange nor chromosomal aberrations were induced in cultured mammalian cells.

5.5 Evaluation[1]

There is *inadequate evidence* in humans for the carcinogenicity of CI Direct Blue 15.

There is *sufficient evidence* in experimental animals for the carcinogenicity of technical grade CI Direct Blue 15.

Overall evaluation

CI Direct Blue 15 *is possibly carcinogenic to humans (Group 2B*).

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

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Table 2. Genetic and related effects of CI Direct Blue 15

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SA0, *Salmonella typhimurium* TA100, reverse mutation	0	(+)[g]	150.0000	Brown & Dietrich (1983)
SA0, *Salmonella typhimurium* TA100, reverse mutation	–	–	5000.0000	Mortelmans *et al.* (1986)
SA5, *Salmonella typhimurium* TA1535, reverse mutation	–	–	5000.0000	Mortelmans *et al.* (1986)
SA7, *Salmonella typhimurium* TA1537, reverse mutation	–	–	5000.0000	Mortelmans *et al.* (1986)
SA8, *Salmonella typhimurium* TA1538, reverse mutation	0	+[d]	125.0000	Reid *et al.* (1984)
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SA9, *Salmonella typhimurium* TA98, reverse mutation	0	(+)[d]	500.0000	Elliott & Gregory (1980)
SA9, *Salmonella typhimurium* TA98, reverse mutation	0	+[e]	62.5000	Elliot & Gregory (1980)
SA9, *Salmonella typhimurium* TA98, reverse mutation	0	+[f]	150.0000	Brown & Dietrich (1983)
SA9, *Salmonella typhimurium* TA98, reverse mutation	0	(+)[g]	150.0000	Brown & Dietrich (1983)
SA9, *Salmonella typhimurium* TA98, reverse mutation	0	+[h]	50.0000	Prival *et al.* (1984)
SA9, *Salmonella typhimurium* TA98, reverse mutation	0	+[b]	100.0000	Krishna *et al.* (1986)
SA9, *Salmonella typhimurium* TA98, reverse mutation	–	–	5000.0000	Mortelmans *et al.* (1986)
URP, Unscheduled DNA synthesis, rat primary hepatocytes *in vitro*	–	0	0.0000	Mirsalis *et al.* (1983); abstr.
G5T, Gene mutation, mouse lymphoma L5178Y cells *in vitro*	–	+	0.0000	Rudd *et al.* (1983); abstr.
SIC, Sister chromatid exchange, Chinese hamster ovary cells *in vitro*	–	–	2500.0000	Galloway *et al.* 1987)
CIC, Chromosomal aberrations, Chinese hamster ovary cells *in vitro*	–	–	2500.0000	Galloway *et al.* 1987)

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

[a] µg/ml; 0.0000, not given

[b] Preincubation with hamster or rat liver S9 and flavin mononucleotide supplementation

[c] Rat liver S9 more effective

[d] Anaerobic preincubation or riboflavin supplementation

[e] Plate incorporation and reduction using sodium dithionite

[f] Aerobic preincubation with riboflavin

[g] Anaerobic preincubation with rat caecal bacterial extract, flavin mononucleotide and liver S9

[h] Preincubation with no shaking and hamster liver S9 with flavin mononucleotide

Appendix C: Urinary excretion of benzidine, DMB, and DMOB by dogs and rats after oral administration of dye chemicals

Table C-1. Urinary excretion of benzidine, DMB, and DMOB by dogs after oral administration of benzidine-, DMB-, and DMOB-based dyes

Benzidine based-dye	Benzidine impurity (ppm)	Dose of Benzidine as impurity (µg)	Benzidine excreted in urine during 48 hours after dosing (µg)			Percent of dose[2]
			Experiment 1	Experiment 2	Mean	
Benzidine	-	-	1161	3147	2154	0.14
Direct Black 4	3	5	320	222	271	0.08
Direct Blue 2	11	17	445	190	317	0.10
Direct Brown 2	24	36	1675	424	1049	0.24
Direct Green 1	< 5	< 8	362	455	408	0.11
Direct Orange 1	5	8	369	238	304	0.07
Direct Orange 8	34	51	545	481	513	0.11
Direct Red 28	3	5	166	316	241	0.06
DMB-based dye	**DMB impurity (ppm)**	**Dose of DMB as impurity (µg)**	**DMB excreted in urine during 48 hours after dosing (µg)**			**Percent of dose[2]**
			Experiment 1	**Experiment 2**	**Mean**	
Direct Blue 25	9	13	62	103	82	0.03
Acid Red 114	< 1	< 1.5	94	175	135	0.04
Direct Red 2	7	11	BLQ[1]	BLQ[1]	-	-
Direct Red 39	2	3	BLQ[1]	BLQ[1]	-	-
DMOB-based dye	**DMOB impurity (ppm)**	**Dose of DMOB as impurity (µg)**	**DMOB excreted in urine during 48 hours after dosing (µg)**			**Percent of dose[2]**
			Experiment 1	**Experiment 2**	**Mean**	
Direct Blue 15	46	69	61	168	114	0.03
Direct Blue 1	18	27	441	119	280	0.08

Source: Lynn *et al*. (1980).
(-) not published.
Dogs weighing 15 kg received 1.5 g of each dye (100 mg/kg). Dogs treated with benzidine received 1.5 g of the free base. Each dye was studied in 2 dogs.
[1] BLQ, below levels of quantitation but the presence of DMB was confirmed by GCMS.
[2] Percent of potential theoretical maximum produced by complete reduction of the azo bonds in the dye.

Table C-2. Urinary excretion of benzidine, DMB, and DMOB by rats after oral administration of benzidine-, DMB-, and DMOB-based dyes (100 mg/kg)

Dye	Benzidine impurity in administered dye (μg)	N-Acetylbenzidine excreted in urine during 72 hours after dosing (μg)		Percent of dose[2]
		Experiment 1	Experiment 2	
Benzidine	34,600	212 ± 34[5]		0.62 ± 0.10
Direct Black 4	< 1	22.8 ± 14.2	26.9 ± 15.3	0.26 ± 0.16
Direct Blue 2	< 1	3.1 ± 3.0	11.6 ± 4.3	0.09 ± 0.08
Direct Brown 2	< 1	18.9 ± 13.2	54.0 ± 33.1	0.34 ± 0.30
Direct Green 1	< 1	17.1 ± 13.0	13.9 ± 6.0	0.17 ± 0.11
Direct Orange 1	< 1	BLQ[1]	BLQ[1]	BLQ[1]
Direct Orange 8	< 1	17.6 ± 9.9	13.2 ± 8.5	0.13 ± 0.08
Direct Red 28	< 1	10.7 ± 7.1	12.4 ± 8.1	0.11 ± 0.07
Dye	**DMB impurity in administered dose (μg)**	**DMB excreted in urine during 72 hours after dosing (μg)[3]**		**Percent of dose[4]**
DMB	25,290	898 ± 278[6]		3.52 ± 0.99
Direct Blue 25	< 1	41 ± 3.0		0.06 ± 0.04
Acid Red 114	< 1	< 0.1		0.01
Direct Red 2	< 1	BLQ[1]		-
Direct Red 39	< 1	BLQ[1]		-
Dye	**DMOB impurity in administered dose (μg)**	**DMOB excreted in urine during 72 hours after dosing (μg)[3]**		**Percent of dose[4]**
DMOB	27250	1247 ± 145[7]		4.59 ± 0.06
Direct Blue 15	1	13.0 ± 12.9		0.17 ± 0.18
Direct Blue 1	< 1	42.5 ± 27.7		0.55 ± 0.37

Source: Lynn *et al.* (1980).
(-) not published.
Mean (± SD) daily urinary excretion of N-acetylbenzidine. Each dye was administered daily (100 mg/kg) for 10 days. Two rats were studied for each dye.
[1] BLQ, below levels of quantitation but the presence of DMB was confirmed by GCMS.
[2] Percentage of the potential theoretical maximum produced by complete reduction of the azo bonds and subsequent mono-N-acetylation.
[3] Total DMB/DMOB excreted in 72 hour following single oral dose. Mean ± SD of four animals .
[4] Percent of potential theoretical maximum produced by complete reduction of the azo bonds in the dye.
[5] Total benzidine excreted in 92 hours following single oral dose. Mean ± SD of 4 animals.
[6] Total DMB excreted in 72 hours following single oral dose. Mean ± SD of 4 animals.
[7] Total DMOB excreted in 72 hours following single oral dose. Mean ± SD of 4 animals.

Appendix D: NTP. 1990. Toxicology and Carcinogenesis Studies of DMOB Dihydrochloride in F344/N rats (Drinking Water Studies). NTP Technical Report Series TR-372. Pp. D-1 – D-68.

NTP TECHNICAL REPORT

ON THE

TOXICOLOGY AND CARCINOGENESIS

STUDIES OF 3,3′-DIMETHOXYBENZIDINE DIHYDROCHLORIDE

(CAS NO. 20325-40-0)

IN F344/N RATS

(DRINKING WATER STUDIES)

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NATIONAL TOXICOLOGY PROGRAM
P.O. Box 12233
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January 1990

NTP TR 372

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CONTENTS

PAGE

ABSTRACT 3
EXPLANATION OF LEVELS OF EVIDENCE OF CARCINOGENIC ACTIVITY 6
CONTRIBUTORS 7
PEER REVIEW PANEL 8
SUMMARY OF PEER REVIEW COMMENTS 9
I. INTRODUCTION 11
II. MATERIALS AND METHODS 21
III. RESULTS 29
RATS 30
GENETIC TOXICOLOGY 54
IV. DISCUSSION AND CONCLUSIONS 55
V. REFERENCES 63

APPENDIXES

APPENDIX A SUMMARY OF LESIONS IN MALE RATS IN THE TWENTY-ONE-MONTH DRINKING WATER STUDY OF 3,3′-DIMETHOXYBENZIDINE DIHYDROCHLORIDE 71

APPENDIX B SUMMARY OF LESIONS IN FEMALE RATS IN THE TWENTY-ONE-MONTH DRINKING WATER STUDY OF 3,3′-DIMETHOXYBENZIDINE DIHYDROCHLORIDE 123

APPENDIX C SENTINEL ANIMAL PROGRAM 165

APPENDIX D WATER AND COMPOUND CONSUMPTION BY RATS IN THE TWENTY-ONE-MONTH DRINKING WATER STUDIES OF 3,3′-DIMETHOXYBENZIDINE DIHYDROCHLORIDE 167

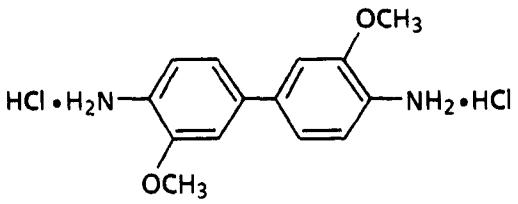
APPENDIX E INGREDIENTS, NUTRIENT COMPOSITION, AND CONTAMINANT LEVELS IN NIH 07 RAT AND MOUSE RATION 171

APPENDIX F ORGAN WEIGHTS IN THE FOURTEEN-DAY DRINKING WATER STUDIES AND RESULTS OF HEMATOLOGY AND SERUM CHEMISTRY ANALYSES IN THE THIRTEEN-WEEK AND NINE-MONTH DRINKING WATER STUDIES OF 3,3′-DIMETHOXYBENZIDINE DIHYDROCHLORIDE 175

APPENDIX G CHEMICAL CHARACTERIZATION, ANALYSIS, AND PREPARATION OF FORMULATED DRINKING WATER MIXTURES OF 3,3′-DIMETHOXYBENZIDINE DIHYDROCHLORIDE FOR THE TOXICOLOGY STUDIES 179

APPENDIX H GENETIC TOXICOLOGY OF 3,3′-DIMETHOXYBENZIDINE 187

APPENDIX I AUDIT SUMMARY 199



3,3′-DIMETHOXYBENZIDINE DIHYDROCHLORIDE

CAS No. 20325-40-0

$C_{14}H_{16}N_2O_2$•2HCl Molecular weight 317.2

Synonyms: *o*-dianisidine dihydrochloride; 3,3′-dimethoxy-(1,1-biphenyl)-4,4′-diamine dihydrochloride; 3,3′-dimethoxy-4,4′-diaminobiphenyl dihydrochloride

ABSTRACT

3,3′-Dimethoxybenzidine dihydrochloride was evaluated in toxicity and carcinogenicity studies as part of the National Toxicology Program's Benzidine Dye Initiative. This Initiative was designed to evaluate representative benzidine congeners and benzidine congener-derived and benzidine-derived dyes. 3,3′-Dimethoxybenzidine 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′-dimethoxybenzidine dihydrochloride (greater than 97.5% pure) in drinking water to groups of F344/N rats of each sex for 14 days, 13 weeks, 9 months, or 21 months. The 21-month studies were intended to last 24 months but were terminated early because of rapidly declining survival due to neoplasia. Studies were performed only in rats because similar studies are being performed in mice at the National Center for Toxicology Research. Genetic toxicology studies were conducted with *Salmonella typhimurium*, Chinese hamster ovary (CHO) cells, and *Drosophila melanogaster*.

Fourteen-Day Studies: All rats receiving drinking water concentrations up to 4,500 ppm lived to the end of the studies. Rats that received water containing 4,500 ppm 3,3′-dimethoxybenzidine dihydrochloride lost weight. Water consumption decreased with increasing concentration of chemical and at 4,500 ppm was less than one-fourth that by the controls. Lymphoid depletion of the thymus in males and hypocellularity of the bone marrow in males and females were seen at the 4,500-ppm concentration, but not at the next lower concentration or in controls.

Thirteen-Week Studies: All rats receiving concentrations up to 2,500 ppm lived to the end of the studies. Final mean body weights of rats given drinking water containing 1,250 or 2,500 ppm 3,3′-dimethoxybenzidine dihydrochloride were 5%-20% lower than those of controls. Water consumption at these concentrations was 40%-60% that consumed by controls. Compound-related effects in rats given water containing 2,500 ppm 3,3′-dimethoxybenzidine dihydrochloride included a mild exacerbation of naturally occurring nephropathy and the presence of a yellow-brown pigment (lipofuscin) in the cytoplasm of thyroid follicular cells. Serum triiodothyronine (T_3) and thyroxin (T_4) concentrations in females receiving 330 ppm or more and T_4 concentrations in males receiving 170 ppm or more were significantly lower than in controls. Thyrotropin (TSH) concentrations were comparable in controls and exposed rats.

Based on the chemical-related nephropathy and reductions in water consumption and body weight gain observed in the 13-week studies, doses for the long-term studies in male and female rats were 0 or 330 ppm 3,3′-dimethoxybenzidine dihydrochloride in drinking water administered for 9 months and 0, 80, 170, or 330 ppm administered for 21 months.

Nine-Month Studies: Ten rats of each sex in the control and 330-ppm groups were evaluated after 9 months. Significant decreases in T_3 and T_4 concentrations were seen in exposed male and female rats. Other lesions seen in exposed rats included foci of alteration in the liver, a carcinoma of the preputial gland in one male, a carcinoma of the clitoral gland in one female, and carcinoma of the Zymbal gland in two males.

Body Weights and Survival in the Twenty-One-Month Studies: The average amount of 3,3′-dimethoxybenzidine dihydrochloride consumed per day was approximately 6, 12, or 21 mg/kg for low, mid, or high dose male rats and 7, 14, or 23 mg/kg for low, mid, or high dose female rats. Mean body weights of male and female rats began to decrease relative to those of controls after about 1 year of exposure at 170 or 330 ppm and were 6%-22% lower for males and 7%-17% lower for females. Survival of rats exposed to 3,3′-dimethoxybenzidine dihydrochloride was reduced because animals were dying with neoplasms or being killed in a moribund condition (survival at 21 months--male: control, 44/60, 73%; low dose, 8/45, 18%; mid dose, 0/75; high dose, 0/60; female: 45/60, 75%; 15/45, 33%; 6/75, 8%; 0/60). Because of these early compound-related deaths, the studies were terminated at 21 months.

Nonneoplastic and Neoplastic Effects in the Twenty-One-Month Studies: Increased incidences of several nonneoplastic lesions were observed in exposed rats, including hematopoietic cell proliferation in the spleen and cystic and centrilobular degeneration and necrosis of the liver. Neoplasms attributed to 3,3′-dimethoxybenzidine dihydrochloride exposure were observed in rats at many tissue sites, including the skin, Zymbal gland, preputial and clitoral glands, oral cavity, small and large intestines, liver, brain, mesothelium, mammary gland, and uterus/cervix. The incidences of these neoplasms in male and female rats are given in the abstract summary table.

Genetic Toxicology: 3,3′-Dimethoxybenzidine was mutagenic in *S. typhimurium* strain TA100 with exogenous metabolic activation and in strain TA98 without activation; a weakly positive response was observed in strain TA1535 with metabolic activation. 3,3′-Dimethoxybenzidine induced sister chromatid exchanges and chromosomal aberrations in CHO cells with and without exogenous metabolic activation. 3,3′-Dimethoxybenzidine did not induce sex-linked recessive lethal mutations in adult male *D. melanogaster* exposed via feeding or injection.

Conclusions: Under the conditions of these 21-month drinking water studies, there was *clear evidence of carcinogenic activity** of 3,3′-dimethoxybenzidine dihydrochloride for male F344/N rats, as indicated by benign and malignant neoplasms of the skin, Zymbal gland, preputial gland, oral cavity, intestine, liver, and mesothelium. Increased incidences of astrocytomas of the brain may have been related to chemical administration. There was *clear evidence of carcinogenic activity* of 3,3′-dimethoxybenzidine dihydrochloride for female F344/N rats, as indicated by benign and malignant neoplasms of the Zymbal gland, clitoral gland, and mammary gland. Increases in neoplasms of the skin, oral cavity, large intestine, liver, and uterus/cervix were also considered to be related to chemical administration of 3,3′-dimethoxybenzidine dihydrochloride.

*Explanation of Levels of Evidence of Carcinogenic Activity is on page 6.
A summary of the Peer Review comments and the public discussion on this Technical Report appears on page 9.

SUMMARY OF THE TWENTY-ONE-MONTH DRINKING WATER STUDIES OF 3,3'-DIMETHOXYBENZIDINE DIHYDROCHLORIDE

Male F344/N Rats	Female F344/N Rats
Drinking water concentration	
0, 80, 170, or 330 ppm 3,3'-dimethoxybenzidine dihydrochloride	0, 80, 170, or 330 ppm 3,3'-dimethoxybenzidine dihydrochloride
Body weights	
Lower than controls	Lower than controls
Survival rates	
44/60; 8/45; 0/75; 0/60 (a)	45/60; 15/45; 6/75; 0/60 (a)
Nonneoplastic effects	
Liver: cystic and centrilobular degeneration and necrosis; spleen: hematopoietic proliferation; lung: histiocytic infiltration; heart: thrombi in the atrium	Liver: cystic and centrilobular degeneration and necrosis; spleen: hematopoietic proliferation; lung: histiocytic infiltration
Neoplastic effects (b)	
Skin--basal cell or sebaceous gland neoplasms: 2/60 (3%); 33/45 (73%); 56/75 (75%); 41/60 (68%)	Clitoral gland: 7/58 (12%); 27/44 (61%); 48/74 (65%); 41/55 (75%)
Skin--squamous cell neoplasms: 0/60; 13/45 (29%); 28/75 (37%); 22/60 (37%)	Zymbal gland: 1/60 (2%); 12/45 (27%); 21/75 (28%); 16/60 (27%)
Zymbal gland: 0/59; 10/45 (22%); 25/75 (33%); 30/60 (50%)	Mammary gland--adenocarcinomas: 1/60 (2%); 2/45 (4%); 14/75 (19%); 20/60 (33%)
Preputial gland: 16/60 (27%); 12/43 (28%); 33/73 (45%); 29/59 (49%)	Skin--basal cell neoplasms: 0/60; 4/45 (9%); 3/75 (4%); 2/60 (3%)
Palate or tongue: 1/60 (2%); 8/45 (18%); 10/75 (13%); 11/60 (18%)	Palate or tongue: 2/60 (3%); 2/45 (4%); 6/75 (8%); 5/60 (8%)
Small intestine: 0/60; 4/45 (9%); 7/75 (9%); 5/60 (8%)	Large intestine: 0/60; 1/45 (2%); 1/75 (1%); 3/60 (5%)
Large intestine: 0/60; 1/45 (2%); 8/75 (11%); 8/60 (13%)	Liver: 0/60; 1/44 (2%); 0/75; 3/60 (5%)
Liver: 1/60 (2%); 4/45 (9%); 7/74 (9%); 8/60 (13%)	Uterus/cervix: 0/60; 4/45 (9%); 2/75 (3%); 2/60 (3%)
Mesothelium: 2/60 (3%); 1/45 (2%); 7/75 (9%); 6/60 (10%)	
Brain--astrocytomas: 0/60; 2/44 (5%); 3/75 (4%); 1/60 (2%)	
Level of evidence of carcinogenic activity	
Clear evidence	Clear evidence

(a) Reduced survival in exposed groups was due to neoplasia.
(b) Number with lesion/total evaluated (percent incidence)

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 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** is demonstrated by studies that are interpreted as showing a marginal increase of neoplasms that may be chemically related.

- **No Evidence of Carcinogenic Activity** is demonstrated by studies that are interpreted as showing no chemically related increases in malignant or benign neoplasms.

- **Inadequate Study of Carcinogenic Activity** is demonstrated by studies that because of major qualitative or quantitative limitations cannot be interpreted as valid for showing either the presence or absence of carcinogenic activity.

When a conclusion statement for a particular experiment is selected, consideration must be given to key factors that would extend the actual boundary of an individual category of evidence. 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.

CONTRIBUTORS

The NTP Technical Report on the Toxicology and Carcinogenesis Studies of 3,3′-Dimethoxybenzidine Dihydrochloride is based on 13-week studies that began in June 1982 and ended in September 1982 and on 21-month studies that began in March 1983 and ended in December 1984 at Hazleton Laboratories America, Inc. (Vienna, VA).

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PEER REVIEW PANEL

The members of the Peer Review Panel who evaluated the draft Technical Report on 3,3′-dimethoxybenzidine dihydrochloride on June 27, 1989, 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: (a) to ascertain that all relevant literature data have been adequately cited and interpreted, (b) to determine if the design and conditions of the NTP studies were appropriate, (c) to ensure that the Technical Report presents the experimental results and conclusions fully and clearly, (d) to judge the significance of the experimental results by scientific criteria, and (e) to assess the evaluation of the evidence of carcinogenicity and other observed toxic responses.

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SUMMARY OF PEER REVIEW COMMENTS ON THE TOXICOLOGY AND CARCINOGENESIS STUDIES OF 3,3′-DIMETHOXYBENZIDINE DIHYDROCHLORIDE

On June 27, 1989, the draft Technical Report on the toxicology and carcinogenesis studies of 3,3′-dimethoxybenzidine 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. 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 21 months because of the rapidly declining survival of exposed animals due to neoplasia.

Dr. McKnight, a principal reviewer, agreed with the conclusions. She commented that the statistical analysis for skin tumors would be more accurate if based on the time at which a tumor first appeared in each animal, rather than the time at which each animal died with a tumor. (In these studies, this change of analysis would not affect the conclusions.)

Dr. Popp, the second principal reviewer, agreed with the conclusions. He pointed out that, because the chemical had previously been shown to be carcinogenic in experimental animals, information could be added to the rationale for doing the current studies. Dr. Popp noted the observation of foci in the liver of rats after dosing for 9 months, which suggested the chemical might be a hepatocarcinogen, yet there was a relatively weak liver tumor response at 21 months. Dr. Morgan speculated that the early animal deaths may have sufficiently shortened the time available for progression of foci to detectable tumors.

Dr. Gold, the third principal reviewer, agreed with the conclusions. She also requested that the rationale for performing the current studies be mentioned in light of findings from earlier studies. She opined that some of the earlier studies were not adequate by current standards. Dr. Morgan said that the rationale for the studies would be stated earlier in the Introduction and that the inadequacies of the earlier studies would be noted. Dr. Gold asked that the National Institute for Occupational Safety and Health data from the current National Occupational Exposure Survey be appended to indicate the estimated number of U.S. workers exposed to the chemical (page 13). Dr. Scala questioned the accuracy of the exposure estimates. Dr. H. Matthews, NIEHS, proposed that the number of workers exposed to 3,3′-dimethoxybenzidine was likely to be greater that the survey estimates because NTP studies have shown, at least in animals, that dyes derived from benzidine or its congeners were metabolically reduced in vivo almost completely to the parent compound. Dr. Gold also suggested that the results from the study in mice conducted at the National Center for Toxicological Research be included in the Report (page 19).

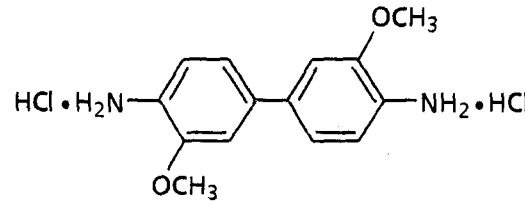
Dr. Mirer said that another rationale for the NTP studies could be that there is no tumor site concordance between humans and animals. Dr. J. Huff, NIEHS, responded that there were no epidemiology studies on this congener to enable determination of concordance. He added that there is a comparable neoplastic site (urinary bladder) in humans and dogs exposed to the parent chemical, benzidine.

Dr. McKnight moved that the Technical Report on 3,3′-dimethoxybenzidine dihydrochloride be accepted with the revisions discussed and the conclusions as written for male and female rats, clear evidence of carcinogenic activity. Dr. Popp seconded the motion, which was accepted unanimously.

I. INTRODUCTION

Use and Production
Exposure
Disposition and Metabolism
Genetic Toxicology
Toxicity and Carcinogenicity Studies
Toxicity and Carcinogenicity of Related Compounds
Study Rationale

I. INTRODUCTION



3,3′-DIMETHOXYBENZIDINE DIHYDROCHLORIDE

CAS No. 20325-40-0

$C_{14}H_{16}N_2O_2 \cdot 2HCl$ Molecular weight 317.2

Synonyms: *o*-dianisidine dihydrochloride; 3,3′-dimethoxy-(1,1-biphenyl)-4,4′-diamine dihydrochloride; 3,3′-dimethoxy-4,4′-diaminobiphenyl dihydrochloride

Use and Production

3,3′-Dimethoxybenzidine dihydrochloride is an off-white powder with a melting point of 274° C. 3,3′-Dimethoxybenzidine is used principally as an intermediate in the production of commercial bisazobiphenyl dyes for coloring textiles, paper, plastic, rubber, and leather (Fishbein, 1981). In the synthesis of the bisazobiphenyl dyes, the amine groups of 3,3′-dimethoxybenzidine are chemically linked with other aromatic amines. A small quantity of 3,3′-dimethoxybenzidine is also used as an intermediate in the production of *o*-dianisidine diisocyanate, which is used in isocyanate-based adhesive systems and as a component of polyurethane elastomers (Woolrich and Rye, 1969; Fishbein, 1981).

3,3′-Dimethoxybenzidine has been produced commercially in the United States for at least 50 years (Fishbein, 1981). 3,3′-Dimethoxybenzidine is synthesized by reduction of *o*-nitroanisole to hydrazoanisole, followed by rearrangement of hydrazoanisole with acid to yield 3,3′-dimethoxybenzidine (IARC, 1974).

Domestic production of 3,3′-dimethoxybenzidine was reduced from 367,000 pounds in 1967 to small quantities in 1978 (USEPA, 1980). No information on more recent production volume is available. Approximately 554,000 pounds of 3,3′-dimethoxybenzidine was imported in 1978 (USEPA, 1980) and 106,000 pounds in 1983 (USITC, 1984). The National Institute for Occupational Safety and Health (NIOSH) reported 33 commercially available (United States) dyes synthesized from 3,3′-dimethoxybenzidine (Boeniger, 1980). Production and importation of 3,3′-dimethoxybenzidine-based dyes were estimated at 1,329,000 pounds (presscake basis) in 1979.

Exposure

Occupational exposure to 3,3′-dimethoxybenzidine may occur during the manufacture of those dyes in which 3,3′-dimethoxybenzidine is an intermediate. Exposure to 3,3′-dimethoxybenzidine may occur by inhalation, ingestion, or skin absorption (Meigs et al., 1951, 1954; El-Hawari et al., 1979). Exposure may also occur indirectly during handling of the finished 3,3′-dimethoxybenzidine-based dyes. Residual amounts of 3,3′-dimethoxybenzidine may be present in the finished dyes due to incomplete dye synthesis or breakdown of the dye after production. As discussed below, there is also evidence to suggest that 3,3′-dimethoxybenzidine-based dyes are metabolized back to the parent compound in vivo, resulting in exposure to 3,3′-dimethoxybenzidine.

Exposure to benzidine, benzidine congeners, and derived dyes has been estimated to include approximately 1,000 workers in dye manufacturing and approximately 10,000 workers in the various application industries (DETO, 1980). Because many of these compounds are found concurrently in the same industry, it is difficult to estimate the number of exposed workers and the extent of exposure to 3,3′-dimethoxybenzidine alone.

Exposure of workers to 3,3′-dimethoxybenzidine may also occur in clinical laboratories (IARC, 1974; Collier, 1974). 3,3′-Dimethoxybenzidine is commonly used for detection of blood and for the quantitation of chlorine in water and of glucose by the glucose oxidase method (Collier, 1974). According to a recent National Occupational Exposure Survey (NIOSH unpublished data), approximately 490 clinical laboratory technologists and technicians are exposed to 3,3′-dimethoxybenzidine.

Nonoccupational exposure to 3,3′-dimethoxybenzidine-based dyes may occur through contact with paper, fabrics, and leather to which these dyes have been applied and through the use of dyes packaged for home use and paints that contain 3,3′-dimethoxybenzidine. No estimates of consumer exposure to 3,3′-dimethoxybenzidine alone were found.

3,3′-Dimethoxybenzidine has been found in samples of commercially produced and imported sneezing powders (Giehl and Salger, 1983; Charles et al., 1984). The commercial material is usually a mixture of black pepper and sawdust; however, in some cases, 3,3′-dimethoxybenzidine or benzidine has been used in place of black pepper. These powders have reportedly caused severe poisoning in children, but the symptoms of 3,3′-dimethoxybenzidine poisoning were not described (Charles et al., 1984).

Disposition and Metabolism

Rodgers et al. (1983) reported that after intravenous administration to male F344 rats, [^{14}C]3,3′-dimethoxybenzidine was rapidly and extensively metabolized; less than 2% of the radiolabel could be recovered unchanged 30 minutes after dosing. Seventy percent of the radiolabel was excreted in the bile within 72 hours, and 50% was located in the intestinal tract after 2 hours. Three days after either oral or intravenous administration, 50% of the radiolabel had been excreted in the feces and 30%-40% excreted in the urine; 45% of the radiolabel remaining in the animal was present in the liver in the form of covalently bound metabolites. Analysis of the pooled urine (days 0-3) demonstrated that more than 90% of the urinary radiolabel was in the form of metabolites. Unmetabolized 3,3′-dimethoxybenzidine accounted for 3%-9% of the urinary radiolabel, and acetyl-dimethoxybenzidine accounted for 5% or less (Figure 1).

Reductive metabolism of 3,3′-dimethoxybenzidine-based dyes may result in formation of 3,3′-dimethoxybenzidine (Figure 2). Azo reduction can be carried out by enzymes in the liver or by azo reductase associated with intestinal bacterial flora. Highly polar compounds are not well absorbed from the gut, and therefore the water-soluble sulfonated dyes would not be expected to be well absorbed by mammals (Walker, 1970). For this reason, reductive cleavage of the benzidine-congener azo dyes is thought to occur primarily through bacterial action in the gastrointestinal tract (Martin and Kennelly, 1981; Cerniglia et al., 1982; Brown and Dietrich, 1983; Bos et al., 1984, 1986). The less polar metabolites could then be absorbed and further metabolized by the liver.

3,3′-Dimethoxybenzidine-based dyes have been shown to be metabolized to 3,3′-dimethoxybenzidine in dogs, rats, and humans. After exposure of dogs and rats to two 3,3′-dimethoxybenzidine-based dyes, 3,3′-dimethoxybenzidine was detected in the urine of both species at levels that were reportedly greater than the amount contributed by 3,3′-dimethoxybenzidine contamination of the dyes (Lynn et al., 1980). Genin (1977) also detected 3,3′-dimethoxybenzidine in the urine of rats exposed to two 3,3′-dimethoxybenzidine-based dyes. In the same study, 3,3′-dimethoxybenzidine was detected in the urine of three workers who dried and ground two 3,3′-dimethoxybenzidine-based dyes. Boeniger (1980) reported finding 3,3′-dimethoxybenzidine in the urine of a person who worked with 3,3′-dimethoxybenzidine-based dyes but not with 3,3′-dimethoxybenzidine itself. The urinary 3,3′-dimethoxybenzidine may have resulted from metabolism of the dyes or from exposure to dyes contaminated with 3,3′-dimethoxybenzidine.

Genetic Toxicology

3,3′-Dimethoxybenzidine has been extensively studied for induction of gene mutations in *Salmonella typhimurium*. The chemical was mutagenic with exogenous metabolic activation in strains TA98, TA100, and TA1538 (Anderson and Styles, 1978; Martin and Kennelly, 1981;

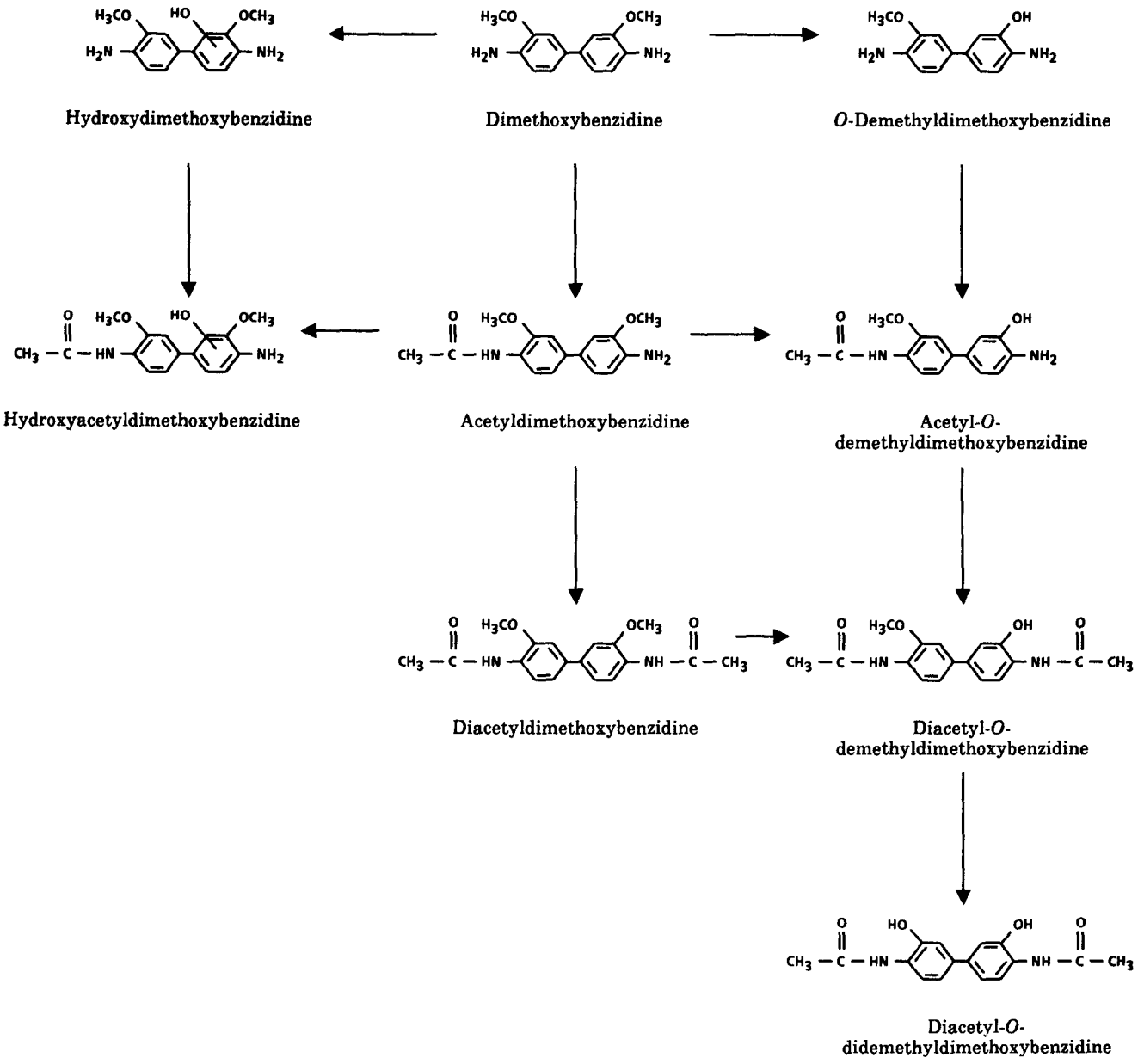


FIGURE 1. PROPOSED METABOLIC PATHWAYS OF 3,3′-DIMETHOXYBENZIDINE
(From Rodgers et al., 1983)

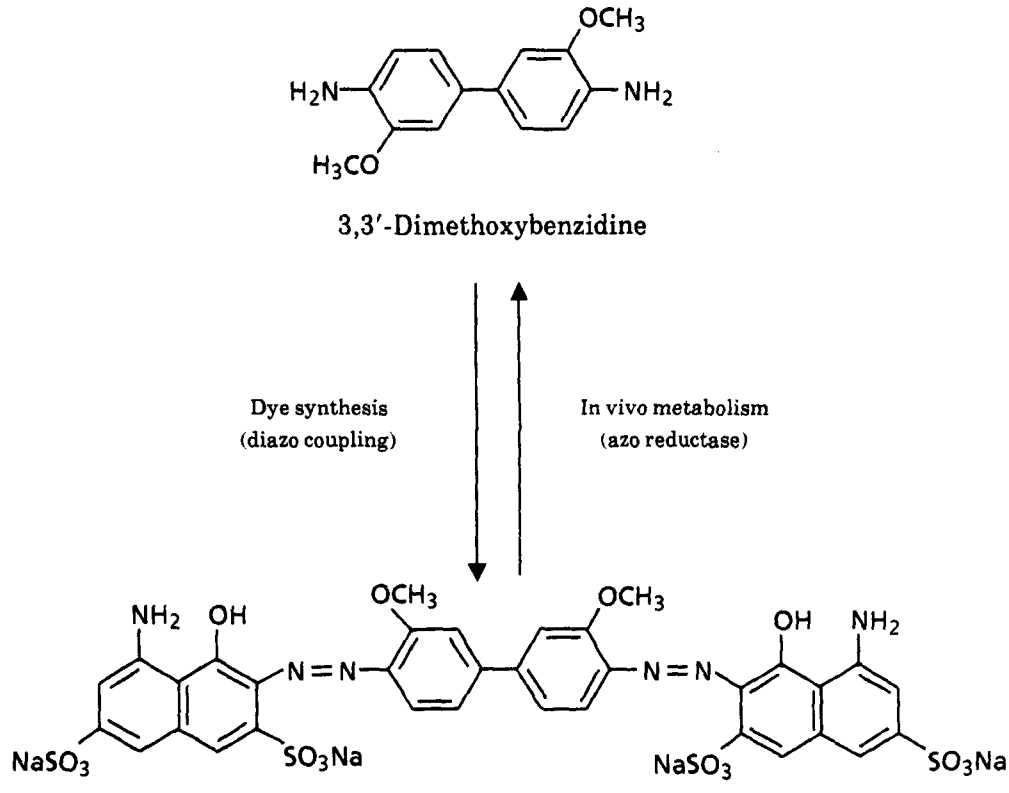


FIGURE 2. FORMATION OF 3,3′-DIMETHOXYBENZIDINE BY REDUCTIVE METABOLISM OF C.I. DIRECT BLUE 15

Probst et al., 1981; Haworth et al., 1983; Rodgers et al., 1983; Reid et al., 1984a,b). Messerly et al. (1987), in a structure-function study of the mutagenic activity of several benzidine derivatives, confirmed the greater activity of 3,3′-dimethoxybenzidine and other substituted aminobiphenyl compounds in *S. typhimurium* TA98 (a strain that mutates via frameshifts) compared with the activity of the chemical in TA100 (a base-substitution strain). The dihydrochloride salt of 3,3′-dimethoxybenzidine also induced gene mutations in *S. typhimurium* TA98 and TA100 (Gregory et al., 1981; Prival et al., 1984; Table H1). Growth inhibition due to induced DNA damage was not observed, however, in *Escherichia coli* treated with 3,3′-dimethoxybenzidine, but this test was performed in the absence of S9 activation (Fluck et al., 1976). Induction of unscheduled DNA synthesis in rat hepatocyte primary cultures treated with 500-1,000 nmol/ml 3,3′-dimethoxybenzidine was reported by Probst et al. (1981). Sister chromatid exchanges were significantly increased in Chinese hamster ovary cells treated with 3,3′-dimethoxybenzidine dihydrochloride with and without S9 metabolic activation (Galloway et al., 1985; Table H2). When originally reported, the results of the chromosomal aberration tests were considered to be negative (Galloway et al., 1985); however, by an updated statistical reanalysis of the chromosomal aberration data (Galloway et al., 1987), the results currently are considered to be weakly positive in the absence of S9 and positive with S9 (Table H3). Negative results were obtained in a *Drosophila melanogaster* sex-linked recessive lethal test in which the chemical was administered by two routes, feeding or injection (Yoon et al., 1985; Table H4).

Mutagenicity data for several metabolites and structural analogs of 3,3′-dimethoxybenzidine are consistent with the positive results in Salmonella and mammalian cell assays seen with 3,3′-dimethoxybenzidine. Benzidine, the parent compound in this series of substituted biphenyls, is positive for induction of gene mutations in *S. typhimurium* TA98, TA100, and TA1538 in the presence of S9 (Ames et al., 1973; Shimizu and Takemura, 1976; Anderson and Styles, 1978; Probst et al., 1981; Baker and Bonin, 1981; Haworth et al., 1983; Reid et al., 1984b) as well as in some strains of *E. coli* with S9 (Venitt and Crofton-Sleigh, 1981; Mohn et al., 1981; Matsushima et al., 1981). Like benzidine, two metabolites of 3,3′-dimethoxybenzidine, *N,N′*diacetyldimethoxybenzidine and *N*-acetyldimethoxybenzidine, were both positive in *S. typhimurium* TA98, TA100, and TA1538 in the presence of S9 activation (Kennelly et al., 1984; Reid et al. 1984b).

Toxicity and Carcinogenicity Studies

In 1980, NIOSH and the Occupational Safety and Health Administration (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 (Boeniger, 1980). In a later report issued to alert workers of 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 preliminary evidence that dyes derived from 3,3′-dimethoxybenzidine may be metabolically converted to the parent compound.

Earlier studies showed that repeated exposure to 3,3′-dimethoxybenzidine results in neoplasms in the gastrointestinal tract, Zymbal 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, the use of toxic doses, and poor animal survival weakened this evidence. In addition, the doses of 3,3′-dimethoxybenzidine administered in earlier feed studies are questionable, since in the current studies, 3,3′-dimethoxybenzidine was shown to be unstable in rodent feed.

Pliss (1963, 1965) reported on the effects of orally administered 3,3′-dimethoxybenzidine (30 mg, three times per week, via gavage in sunflower oil) in rats. This dose was reduced to 15 mg after 3 weeks because of poor survival. Administration at the lower dose was continued for 13 months. The study was started with 42 rats, and 18 survived through month 14. Two of these 18 animals had neoplasms of the Zymbal gland, and 1 had an ovarian neoplasm. None of the 50 control rats developed neoplasms at the same sites as the exposed rats.

Saffiotti et al. (1967) fed diets containing 1,000 ppm 3,3′-dimethoxybenzidine to Syrian golden hamsters (30 males and 30 females per group) in a lifespan study. A transitional cell carcinoma of the urinary bladder was found in one animal after 144 weeks of exposure. This neoplasm is rare in hamsters and was attributed to 3,3′-dimethoxybenzidine exposure. 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 only 2% of the controls, but no urinary bladder lesions were detected. This publication is an abstract and does not detail the experimental design or survival data.

Hadidian et al. (1968) administered 3,3′-dimethoxybenzidine by gavage (0.1, 0.3, 1, 3, 10, or 30 mg per animal per day, 5 days per week) to groups of 3 or 14 (10-mg dose only) male and 3 or 15 (10-mg dose only) female F344 rats. 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 and observed for an additional 6 months; necropsies were then performed. Neoplasms occurred as early as day 293, but most were detected at necropsy 18 months after the initial administration of 3,3′-dimethoxybenzidine. A variety of neoplasms were 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 gland (eight carcinomas). Incidences of neoplasms were significantly increased over those of the 360 pooled vehicle and untreated control rats.

No epidemiologic data on the occurrence of cancer in workers exposed to 3,3′-dimethoxybenzidine in the absence of other compounds suspected of being carcinogenic were found in the literature. No reports on the carcinogenicity of 3,3′-dimethoxybenzidine-derived dyes in animals or humans were found in the literature.

Toxicity and Carcinogenicity of Related Compounds

Benzidine: 3,3′-Dimethoxybenzidine is a congener of benzidine, a known carcinogen for humans (Scott, 1952; Case et al., 1954; IARC, 1972a; 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; Frith and Dooley, 1976). Benzidine has been shown to produce urinary bladder tumors in as many as 90% of workers who have been exposed for up to 30 years (Scott, 1952). Exposure to benzidine may occur directly or by reductive metabolism of benzidine-based dyes. The carcinogenicity of benzidine has been extensively reviewed (IARC, 1972a, 1982, 1987a; Haley, 1975; USEPA, 1980).

Benzidine exposure has been shown to cause urinary bladder tumors in 1/7 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; Littlefield et al., 1983); Zymbal 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). In many of the carcinogenicity studies on benzidine, animal survival was poor, primarily because of administration of toxic doses. These studies, however, leave no doubt that benzidine is carcinogenic for laboratory animals.

3,3′-Dimethylbenzidine: 3,3′-Dimethylbenzidine, a methylated congener of benzidine and a structural analog of 3,3′-dimethoxybenzidine, has been shown to be carcinogenic in laboratory animals. In early studies, Spitz et al. (1950) demonstrated the ability of the compound to induce Zymbal gland neoplasms in rats. In a series of experiments, 3,3′-dimethylbenzidine administered subcutaneously to rats was shown to cause neoplasms of the Zymbal gland, small intestine, and mammary gland (Pliss, 1963, 1965; Pliss and Zabezhinsky, 1970). The IARC (1972b) reviewed the literature on 3,3′-dimethylbenzidine and concluded that it was a systemic carcinogen for rats when given subcutaneously.

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). In 103-week studies, *o*-anisidine hydrochloride was found to be carcinogenic for F344 rats and B6C3F$_1$ mice (NCI, 1978a). Groups of 55 animals of each species and sex received *o*-anisidine in feed at either 5,000 or 10,000 ppm for rats and 2,500 or 5,000 ppm for mice. Controls consisted of 55 untreated animals of each sex and species. Administration of *o*-anisidine hydrochloride resulted in transitional cell carcinomas or papillomas of the bladder in each sex of each species, transitional cell carcinomas of the renal pelvis in male rats, and follicular cell neoplasms of the thyroid gland in male rats. Only one control animal had any neoplasms of the urinary system (a transitional cell papilloma of the renal pelvis in a male mouse).

3,3′-Dimethoxybenzidine-4,4′-diisocyanate: 3,3′-Dimethoxybenzidine is a hydrolysis product of 3,3′-dimethoxybenzidine-4,4′-diisocyanate (dianisidine diisocyanate). Although there is presently no known producer of dianisidine diisocyanate, it was produced by one U.S. manufacturer in the 1970's (IARC, 1986). Dianisidine diisocyanate can be used as a component of polyurethane elastomers and in isocyanate-based adhesives (NCI, 1979; IARC, 1986). In 78-week studies, dianisidine diisocyanate was found to be carcinogenic for F344 rats but not for B6C3F$_1$ mice (NCI, 1979). Dianisidine diisocyanate was administered at either of two concentrations to 50 animals of each species and sex. The compound was administered in feed, with the exception of the first 22 weeks of the study in rats when it was administered by gavage. Controls consisted of 20 animals of each sex and species. The doses of dianisidine diisocyanate administered by gavage to rats were 1,500 and 3,000 mg/kg per day, 5 days per week. Dietary

concentrations for rats and mice were 22,000 and 40,000 ppm. Animals were chemically exposed for 78 weeks, followed by an observation period of 26 weeks for rats and 25 weeks for mice. In rats, administration of dianisidine diisocyanate resulted in neoplasms of the skin in males, endometrial stromal polyps in females, and leukemia and malignant lymphomas in each sex. Dianisidine diisocyanate administration was also associated with the development of a combination of squamous cell carcinomas and sebaceous adenocarcinomas of the Zymbal gland and skin of the ear in rats of each sex. There was no evidence of carcinogenicity of dianisidine diisocyanate for $B6C3F_1$ mice.

Study Rationale

Benzidine is known to cause cancer in humans (IARC, 1972a, 1987a), and 3,3′-dimethoxybenzidine, a benzidine congener, is suspected of possessing carcinogenic potential for humans (Fishbein, 1981). Numerous benzidine and benzidine congener-based dyes have been shown to be metabolized to their parent amines in vivo (Rinde and Troll, 1975; Lynn et al., 1980). Consequently, all benzidine-derived and benzidine congener-derived dyes are logical candidates for carcinogenicity evaluation in laboratory animals.

The National Toxicology Program's (NTP's) Benzidine Dye Initiative is a collaborative effort of the National Institute of Environmental Health Sciences, the National Center for Toxicological Research (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 was to develop an integrated body of data concerning the metabolism and 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 to be impractical, the research program was designed to evaluate representative benzidine congeners and benzidine congener-derived dyes.

3,3′-Dimethoxybenzidine was selected by the collaborating agencies for study in the Initiative to allow comparison of its toxic and carcinogenic effects with those of related chemicals that were studied simultaneously with comparable doses and the same study design. In addition, 3,3′-dimethoxybenzidine was studied to strengthen the evidence for its carcinogenicity. Although results of earlier studies suggested that 3,3′-dimethoxybenzidine was carcinogenic (Pliss, 1963, 1965; Saffiotti et al., 1967; Hadidian et al., 1968), these studies have been criticized because of the use of small groups of animals, the use of toxic doses, poor survival, and the use of parenteral routes of administration (Haley, 1975; DETO, 1980).

TABLE 1. SUMMARY OF THE NATIONAL TOXICOLOGY PROGRAM BENZIDINE CONGENER INITIATIVE

Class/Chemical	Tests (a)
o-Tolidine (3,3′-dimethylbenzidine)	
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
o-Dianisidine (3,3′-dimethoxybenzidine)	
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.

3,3′-Dimethoxybenzidine dihydrochloride is one of five chemicals being evaluated in the 2-year carcinogenicity studies as part of the Benzidine Dye Initiative. The other chemicals currently being studied are C.I. Direct Blue 15 and C.I. Direct Blue 218 (representative 3,3′-dimethoxybenzidine-based dyes), 3,3′-dimethylbenzidine dihydrochloride (a related benzidine congener), and C.I. Acid Red 114 (a representative 3,3′-dimethylbenzidine-based dye). The oral route of administration was selected for the 3,3′-dimethoxybenzidine dihydrochloride, C.I. Direct Blue 15, 3,3′-dimethylbenzidine dihydrochloride, and C.I. Acid Red 114 studies to maximize the chances of detecting systemic effects associated with chemical administration. These four chemicals were studied with the same study design and with staggered starts over a period of 4 months. Because of the instability of 3,3′-dimethoxybenzidine and 3,3′-dimethylbenzidine in feed, all four chemicals were administered in drinking water.

Long-term studies of 3,3′-dimethoxybenzidine dihydrochloride are being conducted in mice at the NCTR as part of the Benzidine Initiative. Male and female (840 each) BALB/c mice were given 0, 20, 40, 80, 160, 315, or 630 ppm 3,3′-dimethoxybenzidine dihydrochloride in drinking water. Animals were killed after exposure for 13, 26, 39, 52, 78, or 112 weeks, and complete necropsies and histopathologic examinations were performed. 3,3′-Dimethoxybenzidine dihydrochloride was not carcinogenic in BALB/c mice (Schieferstein et al., 1989)

II. MATERIALS AND METHODS

PROCUREMENT AND CHARACTERIZATION OF 3,3′-DIMETHOXYBENZIDINE DIHYDROCHLORIDE

CHARACTERIZATION OF FORMULATED DRINKING WATER MIXTURES

FOURTEEN-DAY STUDIES

THIRTEEN-WEEK STUDIES

NINE-MONTH AND TWENTY-ONE-MONTH STUDIES

- Study Design
- Source and Specifications of Animals
- Animal Maintenance
- Clinical Examinations and Pathology
- Statistical Methods

II. MATERIALS AND METHODS

PROCUREMENT AND CHARACTERIZATION OF 3,3′-DIMETHOXYBENZIDINE DIHYDROCHLORIDE

A single lot of 3,3′-dimethoxybenzidine dihydrochloride (lot no. 11F-5034) was obtained from Sigma Chemical Company (St. Louis, MO) in two batches. Purity and identity analyses were conducted at Midwest Research Institute (Kansas City, MO) (Appendix G). The study chemical in both batches was identified as 3,3′-dimethoxybenzidine dihydrochloride by infrared, ultraviolet/visible, and nuclear magnetic resonance spectroscopy. Lot no. 11F-5034 was found to be approximately 98% pure, as determined by elemental analysis, Karl Fischer water analysis, potentiometric titration of the two amine groups, thin-layer chromatography, and high-performance liquid chromatography. Comparison of batch no. 1 and batch no. 2 by high-performance liquid chromatography indicated no significant differences between the two batches.

The identity of the chemical at the laboratory was confirmed by infrared spectroscopy. The stability of the study material was monitored by high-performance liquid chromatography and nonaqueous titration of the amine groups. No deterioration of the study material was seen over the course of the studies.

CHARACTERIZATION OF FORMULATED DRINKING WATER MIXTURES

The stability of 3,3′-dimethoxybenzidine dihydrochloride mixed with NIH 07 Rat and Mouse Ration at 200 ppm and stored for 2 weeks at temperatures ranging from −20° C to room temperature was determined. The feed mixtures were extracted and analyzed by gas chromatography using a 3% OV-17 column and flame ionization detection. The formulated diets were found to be unstable under all storage conditions at or above 5° C. Formulated diets stored open to air and light under simulated animal room conditions lost 12.4% or 18.2% of the chemical after 3 or 7 days, respectively. The same feed mixtures stored in the dark in sealed containers lost 1.6%, 8.9%, or 25.7% of the chemical after storage for 2 weeks at −20° C, 5° C, or room temperature.

Because the feed blends of 3,3′-dimethoxybenzidine dihydrochloride were found to be unstable, drinking water was selected as the route of administration for these studies. The 14-day stability of 3,3′-dimethoxybenzidine dihydrochloride in water at 200 ppm (200 µg/ml), stored at room temperature or at 5° C, was determined. The water solutions were diluted with methanol and analyzed by high-performance liquid chromatography with a C_{18} column and ultraviolet detection at 280 nm. The 3,3′-dimethoxybenzidine dihydrochloride/water solutions were found to be stable for at least 14 days when stored in the dark at room temperature or at 5° C. The water solutions were also stable under simulated dosing conditions for at least 48 hours. Drinking water mixtures were prepared two times per week and were used immediately or, for the 21-month studies, stored at room temperature for up to 7 days before being used.

During the 21-month studies, the drinking water mixtures were analyzed at approximately 4-week intervals. For the 3,3′-dimethoxybenzidine dihydrochloride studies, it was estimated that the mixtures were formulated within ±10% of the target concentrations approximately 99% (103/104) of the time throughout the studies (Table G3). Results of periodic referee analysis performed by the analytical chemistry laboratory indicated good agreement with the results from the study laboratory (Table G4).

FOURTEEN-DAY STUDIES

Male and female F344/N rats were obtained from Frederick Cancer Research Facility and were held for 17 days before the studies began. The rats were 7 weeks old when placed on study.

Groups of five rats of each sex received 0, 200, 350, 750, 1,500, or 4,500 ppm 3,3′-dimethoxybenzidine dihydrochloride in drinking water for 14 days.

Animals were housed five per cage. Water and feed were available ad libitum. The rats were observed two times per day and were weighed on days 1, 7 (males) or 4 (females), and 14. A necropsy was performed on all animals. Organ weight to body weight ratios were determined for brain, lung, heart, liver, kidney, right testis, and thymus. Complete histopathologic

examinations were performed on all controls and animals in the 4,500-ppm groups. The spleen, bone marrow (sternum), and thymus in 1,500-ppm males and bone marrow (sternum) in 1,500-ppm females were examined. Further details are presented in Table 2.

THIRTEEN-WEEK STUDIES

Thirteen-week studies were conducted to evaluate the cumulative toxic effects of repeated exposure to 3,3′-dimethoxybenzidine dihydrochloride and to determine the concentrations to be used in the 2-year studies.

Four-week-old male and female F344/N rats were obtained from Frederick Cancer Research Facility, observed for 14 days, distributed to weight classes, and assigned to dose groups according to a table of random numbers. Rats were 6 weeks old when placed on study.

Groups of 10 rats of each sex received 0, 170, 330, 630, 1,250, or 2,500 ppm 3,3′-dimethoxybenzidine dihydrochloride in drinking water ad libitum for 13 weeks. Rats were housed five per cage. Feed was available ad libitum. Further experimental details are summarized in Table 2.

Animals were observed two times per day; moribund animals were killed. Feed consumption was measured one time per week by cage. Water consumption was measured two times per week. Individual animal weights were recorded one time per week.

Blood was collected from the retro-orbital sinus of all animals at the termination of the studies. Hematocrit values, hemoglobin concentrations, erythrocyte counts, leukocyte counts, and differential leukocyte counts were determined with a Coulter Counter Model S-Plus IV. At the end of the 13-week studies, survivors were killed. A necropsy was performed on all animals. The liver, kidney (right), heart, brain, lung, thymus, and testis (right) were weighed at necropsy. An accumulation of lipofuscin was observed in the thyroid gland after rats were exposed to 3,3′-dimethoxybenzidine for 13 weeks, suggesting a possible chemical effect on thyroid gland function. Thyroid gland function was further evaluated by analyzing the remaining serum samples for changes in triiodothyronine (T_3), thyroxin (T_4), and thyrotropin (TSH). These indices of thyroid gland injury were also investigated in the 2-year studies. T_3, T_4, TSH, blood urea nitrogen, creatinine, lactic dehydrogenase, sorbitol dehydrogenase, and alanine aminotransferase were measured in serum taken from the abdominal aorta at necropsy. T_3 and T_4 were analyzed with the Tri-Tab RIA Diagnostic Kit and the Tetra-Tab RIA Diagnostic Kit (Nuclear Medical Laboratories). TSH analysis was performed by the method of Ridgway et al. (1973). Histopathologic examinations were performed. Tissues and groups examined are listed in Table 2.

NINE-MONTH AND TWENTY-ONE-MONTH STUDIES

Study Design

The 21-month study was originally designed for 24 months using an animal allocation recommended by Portier and Hoel (1984). Additionally, at 9 months, 10 rats of each sex in control groups and 10 rats of each sex in the 330-ppm groups were killed, and at 15 months, 10 rats of each sex in each dose group were to be killed. Animals to be used for the 9- and 15-month studies were designated before the studies were started. Because of the large number of early deaths in the chemically exposed groups, the 15-month interim kill was canceled and these animals were added to the core groups, resulting in 60 rats in the control groups, 45 in the 80-ppm groups, 75 in the 170-ppm groups, and 60 in the 330-ppm groups. The liver, right kidney, heart, brain, lung, thymus, and right testis were weighed at necropsy. Hematocrit values, hemoglobin concentrations, erythrocyte counts, leukocyte counts, and differential leukocyte counts were determined. T_3, T_4, TSH, blood urea nitrogen, creatinine, lactic dehydrogenase, sorbitol dehydrogenase, and alanine aminotransferase were measured in serum taken from the abdominal aorta at necropsy. Histopathologic examinations were performed.

Source and Specifications of Animals

The male and female F344/N rats used in these studies were produced under strict barrier conditions at Simonsen Laboratories. 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

TABLE 2. EXPERIMENTAL DESIGN AND MATERIALS AND METHODS IN THE DRINKING WATER STUDIES OF 3,3′-DIMETHOXYBENZIDINE DIHYDROCHLORIDE

Fourteen-Day Studies	Thirteen-Week Studies	Nine-Month and Twenty-One-Month Studies
EXPERIMENTAL DESIGN		
Size of Study Groups		
5 males and 5 females	10 males and 10 females	9 mo--10 males and 10 females at 0 or 330 ppm; 21 mo--60 males and 60 females at 0 or 330 ppm; 45 males and 45 females at 80 ppm; 75 males and 75 females at 170 ppm
Doses		
0, 200, 350, 750, 1,500, or 4,500 ppm 3,3′-dimethoxybenzidine dihydrochloride in drinking water	0, 170, 330, 630, 1,250, or 2,500 ppm 3,3′-dimethoxybenzidine dihydrochloride in drinking water	9 mo--0 or 330 ppm 3,3′-dimethoxybenzidine dihydrochloride in drinking water; 21 mo--0, 80, 170, or 330 ppm 3,3′-dimethoxybenzidine dihydrochloride in drinking water
Date of First Dose		
3/19/82	6/17/82	3/29/83
Date of Last Dose		
4/2/82	Male--9/16/82; female--9/19/82	9 mo--12/27/83; 21 mo--12/26/84
Duration of Dosing		
14 consecutive d	13 wk	9 or 21 mo
Type and Frequency of Observation		
Observed at least 2 × d; weighed on d 1 and d 7 (male) or d 4 (female) and at the end of the studies; water consumption recorded 1 × wk	Observed 2 × d; weighed 1 × wk; water consumption determined 2 × wk	Observed 2 × d; weighed 1 × wk for 15 wk and then at least 1 × mo
Necropsy, Histologic Examinations, and Supplemental Analyses		
Necropsy performed on all animals; the following tissues examined histologically for control and high dose groups: adrenal glands, brain, cecum, colon, esophagus, heart and aorta, ileum, kidneys, liver, lungs, mammary gland, mandibular and mesenteric lymph nodes, nasal cavity, pancreas, parathyroid glands, pituitary gland, preputial or clitoral gland, prostate/testes or ovaries/uterus, rectum, salivary glands, skin, small intestine, spleen, sternebrae, stomach, thymus, thyroid gland, trachea, urinary bladder, and Zymbal gland. Tissues examined for the 1,500-ppm groups include bone marrow, spleen, sternum, and thymus for males and sternum for females. Organ weights obtained at necropsy	Necropsy performed on all animals; the following tissues examined histologically for control and high dose groups: adrenal glands, brain, cecum, colon, duodenum, epididymis/prostate/testes or ovaries/uterus, esophagus, eyes (if grossly abnormal), gross lesions and tissue masses with regional lymph nodes, heart, ileum, jejunum, kidneys, liver, lungs and mainstem bronchi, mandibular or mesenteric lymph nodes, nasal turbinates, pancreas, parathyroid glands, pituitary gland, preputial or clitoral gland, rectum, salivary glands, spinal cord (if neurologic signs present), spleen, sternebrae including marrow, stomach, thymus, thyroid gland, trachea, urinary bladder, and Zymbal gland. Tissues examined in lower dose groups include kidneys, thymus (male only), and thyroid gland at 1,250 ppm and thyroid gland for both males and females at 630 ppm and females at 330 ppm. Hematologic and serum chemical analyses and thyroid hormone determinations performed; organ weights obtained at necropsy	Necropsy and histologic exams performed on all animals; the following tissues were examined: adrenal glands, brain, cecum, colon, esophagus, heart and aorta, ileum, kidneys, liver, lungs, mammary gland, mandibular and mesenteric lymph nodes, nasal cavity, pancreas, parathyroid glands, pituitary gland, preputial or clitoral gland, prostate/testes or ovaries/uterus, rectum, salivary glands, skin, small intestine, spleen, sternebrae, stomach, thymus, thyroid gland, trachea, urinary bladder, and Zymbal gland. Hematologic and serum chemical analyses, urinalyses, and thyroid hormone determinations performed at 9 mo; organ weights obtained at necropsy

TABLE 2. EXPERIMENTAL DESIGN AND MATERIALS AND METHODS IN THE DRINKING WATER STUDIES OF 3,3'-DIMETHOXYBENZIDINE DIHYDROCHLORIDE (Continued)

Fourteen-Day Studies	Thirteen-Week Studies	Nine-Month and Twenty-One-Month Studies
ANIMALS AND ANIMAL MAINTENANCE		
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)	Simonsen Laboratories (Gilroy, CA)
Study Laboratory Hazleton Laboratories America, Inc.	Hazleton Laboratories America, Inc.	Hazleton Laboratories America, Inc.
Method of Animal Identification Ear tag	Ear punch	Ear tag and ear punch
Time Held Before Study 17 d	14 d	21 d for first shipment and 14 d for second shipment
Age When Placed on Study 7 wk	6 wk	6-7 wk
Age When Killed 9 wk	19 wk	9 mo: 42-43 wk; 21 mo: 98-100 wk
Necropsy Dates 4/2/82	Male--9/17/82; female--9/20/82	9 mo: 12/28/83-1/2/84; 21 mo: 1/3/85-1/4/85 and 1/7/85
Method of Animal Distribution Animals distributed to weight classes and then assigned to cages by one table of random numbers and to groups by another table of random numbers	Same as 14-d studies	Same as 14-d studies
Diet NIH 07 Rat and Mouse Ration (Zeigler Bros., Inc., Gardners, PA); available ad libitum	Same as 14-d studies	Same as 14-d studies
Bedding Hardwood chips (P.J. Murphy Forest Products Corp., Mt. Jewuit, PA)	Same as 14-d studies	Same as 14-d studies
Water Tap or formulated water in glass water bottles (Hazleton Systems, Inc., Aberdeen, MD); available ad libitum	Same as 14-d studies	Same as 14-d studies
Cages Polycarbonate (Hazleton Systems, Inc., Aberdeen, MD)	Same as 14-d studies	Same as 14-d studies
Cage Filters Nonwoven fiber filters (National Paper Co., Wilmington, DE)	Same as 14-d studies	Same as 14-d studies
Animals per Cage 5	5	5
Other Chemicals on Study in the Same Room None	None	None

TABLE 2. EXPERIMENTAL DESIGN AND MATERIALS AND METHODS IN THE DRINKING WATER STUDIES OF 3,3'-DIMETHOXYBENZIDINE DIHYDROCHLORIDE (Continued)

Fourteen-Day Studies	Thirteen-Week Studies	Nine-Month and Twenty-One-Month Studies
ANIMALS AND ANIMAL MAINTENANCE (Continued)		
Animal Room Environment		
Temp--72°-77° F; hum--19%-60%; fluorescent light 12 h/d	Temp--70°-75° F (except for 68° F on 6/19/82); hum--41%-82% (except for 32% on 8/29/82); fluorescent light 12 h/d; 10-12 room air changes/h	Temp--65°-81° F; hum--20%-77%; fluorescent light 12 h/d; 9-17 room air changes/h

parents that were transferred from isolators to barrier-maintained rooms. The rats were shipped to the study laboratory at 3-4 weeks of age and were quarantined at the study laboratory for 2 or 3 weeks. Thereafter, a complete necropsy was performed on five animals of each sex to assess their health status. The rodents were placed on study at 6-7 weeks of age. The health of the animals was monitored during the course of the studies according to the protocols of the NTP Sentinel Animal Program (Appendix C).

Animal Maintenance

The rats were housed five per cage. Feed (Appendix E) and water were available ad libitum. Cages were rotated every 2 weeks during the studies.

Clinical Examinations and Pathology

All animals were observed two times per day. Body weights were recorded one time per week for the first 15 weeks of the studies and then at least one time per month thereafter. Mean body weights were calculated for each group. Animals found moribund and those surviving to the end of the studies were humanely killed. A necropsy was performed on all animals including those found dead. In some cases, a particular organ was autolyzed or lost (e.g., intestine or thymus); thus, the number of animals from which particular organs or tissues were examined microscopically varies and is not necessarily equal to the number of animals that were placed on study. During necropsy, all organs and tissues were examined for grossly visible lesions. All major tissues were fixed and preserved in 10% neutral buffered formalin, processed and trimmed, embedded in paraffin, sectioned, and stained with hematoxylin and eosin for microscopic examination. Tissues examined are listed in Table 2.

When the pathology evaluation was completed by the laboratory pathologist and the pathology data entered into the Toxicology Data Management System, the slides, paraffin blocks, and residual formalin-fixed tissues were sent to the NTP Archives. The slides, blocks, and residual wet tissues were audited for accuracy of labeling and animal identification and for thoroughness of tissue trimming. The slides, individual animal necropsy records, and pathology tables were sent to an independent pathology quality assessment laboratory. The individual animal records and pathology tables were compared for accuracy, slides and tissue counts were verified, and histotechnique was evaluated. All tissues with a tumor diagnosis, all potential target tissues, and all tissues from a randomly selected 10% of the animals were re-evaluated microscopically by a quality assessment pathologist. Target tissues were the oral cavity, intestines, liver, preputial or clitoral gland, Zymbal gland, skin, spleen, bone marrow (male) and mammary gland (female). Nonneoplastic lesions were evaluated for accuracy and consistency of diagnosis only in the potential target tissues, in the randomly selected 10% of animals, and in tissues with unusual incidence patterns or trends.

The quality assessment report and slides were submitted to a Pathology Working Group (PWG) Chairperson, who reviewed microscopically all potential target tissues and any other tissues for which there was a disagreement in diagnosis between the laboratory and quality assessment pathologists. Representative examples of liver, intestine, Zymbal gland, preputial/clitoral gland,

skin, mammary gland, and brain neoplasms and examples of disagreements in diagnosis between the laboratory and quality assessment pathologists were shown to the PWG. The PWG included the quality assessment pathologist and other pathologists experienced in rodent toxicology, who examined the tissues without knowledge of dose group or previously rendered diagnoses. When 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 Pathology Working Group. For subsequent analysis of pathology data, the diagnosed lesions for each tissue type are 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 to be dead from other than natural causes; animals dying from natural causes were not censored. Statistical analyses 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 for a dose-related trend. When significant survival differences were detected, additional analyses using these procedures were carried out to determine the time point at which significant differences in the survival curves were first detected. 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 that 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., in this study, oral cavity) prior to histologic sampling, or when lesions could have appeared at multiple sites (e.g., lymphomas), the denominators consist of the number of animals on which a necropsy was performed.

Analysis of Tumor Incidence: In this study, the large numbers of dosed rats that died or were killed in a moribund condition early in the study were considered to be due primarily to skin, preputial gland, clitoral gland, Zymbal gland, and malignant mammary gland tumors. 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 (i.e., tumors discovered as the 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 exposed animals (due largely to increased incidences of lethal tumors) reduced the power of logistic regression to detect carcinogenic effects in some instances. Hence, although the results of logistic regression analysis are given in the appendixes for informational purposes, in the evaluation of incidental tumors, primary emphasis was given to Cochran-Armitage and Fisher exact tests based on the "effective" number of animals, i.e., the number of animals surviving until observation of the first tumor at that tissue site. These survival-adjusted procedures are recommended by Gart et al. (1979).

Tests of significance include pairwise 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 also were used to evaluate selected nonneoplastic lesions. (For further discussion of these statistical methods, see Haseman, 1984.)

Historical Control Data: Although the concurrent control group is always 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 month 21, control

tumor incidences from the NTP historical control data base for 24-month studies (Haseman et al., 1984, 1985) are included for those 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 by the nonparametric multiple 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.

III. RESULTS

RATS

FOURTEEN-DAY STUDIES

THIRTEEN-WEEK STUDIES

NINE-MONTH STUDIES

TWENTY-ONE-MONTH STUDIES

Body Weights, Water Consumption, and Clinical Signs

Survival

Pathology and Statistical Analyses of Results

GENETIC TOXICOLOGY

FOURTEEN-DAY STUDIES

All rats lived to the end of the studies (Table 3). The final mean body weights of rats that received 4,500 ppm were lower than the initial weights. The final mean body weights of rats that received 1,500 ppm were 4% lower than those of controls. Water consumption decreased as the chemical concentration increased and at 4,500 ppm was less than one-fourth that by the controls. The relative liver and kidney weights were increased, but no microscopic changes were seen in these organs (Table F1). The relative thymus weight for females was significantly lower than that for controls receiving 4,500 ppm, and lymphoid depletion of the spleen in males and females and of the thymus in males was observed. Hypocellularity of the bone marrow was seen at 4,500 ppm (in the groups that lost weight).

THIRTEEN-WEEK STUDIES

All rats lived to the end of the studies (Table 4). Final mean body weights of rats receiving 1,250 or 2,500 ppm were 10% or 20% lower than that of the controls for males and 5% or 11% lower for females. Water consumption at 1,250 or 2,500 ppm was about 60% that by the controls for males and about 45% for females. The relative liver and kidney weights for all groups of dosed male rats, the relative liver weights for females receiving 630 ppm and more, and the relative kidney weights for females receiving 330 ppm and more were significantly greater than those for controls (Table 5). Significant increases in the leukocyte and lymphocyte counts were observed for males receiving 2,500 ppm (Table F2). Segmented neutrophil counts were significantly decreased for males receiving 630 ppm or more and for females receiving 2,500 ppm.

TABLE 3. SURVIVAL, MEAN BODY WEIGHTS, AND WATER CONSUMPTION OF RATS IN THE FOURTEEN-DAY DRINKING WATER STUDIES OF 3,3′-DIMETHOXYBENZIDINE DIHYDROCHLORIDE

Concentration (ppm)	Survival (a)	Mean Body Weights (grams) Initial (b)	Final	Change (c)	Final Weight Relative to Controls (percent)	Water Consumption (d) Week 1	Week 2
MALE							
0	5/5	175	235	+60		21	22
200	5/5	178	241	+63	103	18	19
350	5/5	176	235	+59	100	16	18
750	5/5	175	232	+57	99	15	16
1,500	5/5	177	225	+48	96	13	14
4,500	5/5	177	141	−36	60	4	5
FEMALE							
0	5/5	136	163	+27		32	30
200	5/5	139	163	+24	100	14	15
350	5/5	138	160	+22	98	14	13
750	5/5	138	156	+18	96	12	12
1,500	5/5	141	157	+16	96	13	15
4,500	5/5	139	135	−4	83	7	6

(a) Number surviving/number initially in group
(b) Initial group mean body weight
(c) Mean body weight change of the group
(d) Milliliters per animal per day

TABLE 4. SURVIVAL, MEAN BODY WEIGHTS, AND WATER CONSUMPTION OF RATS IN THE THIRTEEN-WEEK DRINKING WATER STUDIES OF 3,3'-DIMETHOXYBENZIDINE DIHYDROCHLORIDE

Concentration (ppm)	Survival (a)	Mean Body Weights (grams) Initial (b)	Final	Change (c)	Final Weight Relative to Controls (percent)	Water Consumption (d) Week 7	Week 13
MALE							
0	10/10	132	343	+211		21	21
170	10/10	131	337	+206	98	21	22
330	10/10	129	337	+208	98	17	20
630	10/10	132	332	+200	97	16	17
1,250	10/10	129	310	+181	90	13	14
2,500	10/10	129	276	+147	80	12	12
FEMALE							
0	10/10	103	190	+87		27	25
170	10/10	103	186	+83	98	23	21
330	10/10	103	188	+85	99	29	29
630	10/10	103	183	+80	96	16	14
1,250	10/10	105	180	+75	95	13	11
2,500	10/10	103	169	+66	89	10	10

(a) Number surviving/number initially in group
(b) Initial group mean body weight
(c) Mean body weight change of the group
(d) Milliliters per animal per day

TABLE 5. ORGAN WEIGHT TO BODY WEIGHT RATIOS FOR RATS IN THE THIRTEEN-WEEK DRINKING WATER STUDIES OF 3,3'-DIMETHOXYBENZIDINE DIHYDROCHLORIDE (a)

Organ	Control	170 ppm	330 ppm	630 ppm	1,250 ppm	2,500 ppm
MALE						
Necropsy body weight (grams)	326 ± 6.18	319 ± 5.58	325 ± 4.54	318 ± 5.69	**295 ± 5.51	**265 ± 5.45
Liver	25.1 ± 0.20	**27.7 ± 0.19	**27.9 ± 0.21	**29.3 ± 0.30	**31.3 ± 0.35	**32.8 ± 0.58
Brain	5.8 ± 0.10	5.9 ± 0.06	5.8 ± 0.10	6.0 ± 0.09	**6.4 ± 0.11	**6.9 ± 0.11
Heart	2.9 ± 0.04	2.9 ± 0.03	2.8 ± 0.04	2.9 ± 0.06	*3.2 ± 0.10	*3.0 ± 0.06
Right kidney	3.0 ± 0.04	*3.1 ± 0.04	**3.2 ± 0.04	**3.4 ± 0.04	**3.5 ± 0.06	**4.0 ± 0.06
Lungs	3.6 ± 0.09	3.7 ± 0.08	3.5 ± 0.09	3.5 ± 0.05	3.8 ± 0.09	**4.2 ± 0.27
Right testis	4.5 ± 0.10	4.7 ± 0.06	4.6 ± 0.09	4.6 ± 0.08	*4.8 ± 0.08	**5.4 ± 0.07
Thymus	1.1 ± 0.03	*0.9 ± 0.02	**0.9 ± 0.06	**0.9 ± 0.04	**0.8 ± 0.06	**0.8 ± 0.01
FEMALE						
Necropsy body weight (grams)	179 ± 2.20	176 ± 2.22	178 ± 1.65	175 ± 1.46	174 ± 3.44	**164 ± 2.63
Liver	25.9 ± 0.40	26.2 ± 0.36	27.0 ± 0.39	**28.4 ± 0.97	**28.3 ± 0.24	**30.2 ± 0.46
Brain	10.0 ± 0.07	10.1 ± 0.17	9.9 ± 0.07	10.1 ± 0.13	10.2 ± 0.16	**10.6 ± 0.15
Heart	3.2 ± 0.07	3.2 ± 0.03	3.3 ± 0.08	*3.5 ± 0.07	**3.4 ± 0.05	*3.4 ± 0.06
Right kidney	3.2 ± 0.05	3.3 ± 0.05	**3.5 ± 0.05	**3.9 ± 0.06	**4.0 ± 0.09	**4.2 ± 0.05
Lungs	4.7 ± 0.19	4.8 ± 0.13	4.7 ± 0.09	5.0 ± 0.08	4.9 ± 0.08	(b) 4.6 ± 0.06
Thymus	1.3 ± 0.04	1.2 ± 0.04	1.3 ± 0.04	1.4 ± 0.05	1.4 ± 0.03	1.3 ± 0.04

(a) Mean (milligrams per gram) ± standard error for groups of 10 animals, unless otherwise specified. P values are vs. the controls by Dunn's test (Dunn, 1964) or Shirley's test (Shirley, 1977).
(b) Nine animals were weighed.
*P<0.05
**P<0.01

Erythrocyte counts and hematocrit values were significantly decreased by up to 15% in female rats exposed to 630 ppm or more; however, the lack of a concomitant decrease in hemoglobin suggested that these decreases were due to sample hemolysis and were probably not related to chemical exposure. In male rats, a mild increase (<1,000 cells/µl) in total leukocytes was produced by a combination of a mild increase (<1,300/µl) in lymphocytes and a decrease (<400 cells/µl) in neutrophils. None of these changes is biologically relevant. Mild decreases in creatinine (about 20%) were observed in all groups of dosed males and females. These decreases could be produced by loss of muscle mass. Alternatively, decreased concentrations of creatinine can result from substances that interfere with the assay (e.g., bilirubin or hemoglobin).

Compound-related effects seen at 2,500 ppm included mild exacerbation of nephropathy, a condition commonly seen in F344 rats. Nephropathy, characterized by mild tubular regeneration and lymphocytic inflammatory infiltrates, was observed in 10/10 males and 6/10 females. In addition, brown granular pigment was seen in the cytoplasm of the thyroid gland follicular cells of 10/10 males and 10/10 females. The AFIP method for determination of lipofuscin indicated that the pigment was lipofuscin. The mean serum triiodothyronine (T_3) and thyroxin (T_4) concentrations in females receiving 330 ppm or more and the serum T_4 concentrations in males receiving 170 ppm or more were significantly lower than those in controls. The thyrotropin (TSH) concentrations in dosed rats were not significantly different from those in controls (Table F2.)

Dose Selection Rationale: Because of chemical-related exacerbation of nephropathy and decreased water consumption at higher concentrations in short-term studies, drinking water concentrations of 3,3′-dimethoxybenzidine dihydrochloride selected for rats for the 9-month and 2-year (21-month) studies were 80, 170, and 330 ppm.

NINE-MONTH STUDIES

After exposure to 3,3′-dimethoxybenzidine dihydrochloride at 330 ppm for only 9 months, a carcinoma of the preputial gland in one male, focal hyperplasia of the preputial gland in one male, a carcinoma of the clitoral gland in one female, and carcinomas of the Zymbal gland in two males and focal hyperplasia of the Zymbal gland in two males and two females were detected. None of these lesions was observed in control rats. Low dose and mid dose animals were not examined. Other compound-related effects included basophilic and/or eosinophilic foci of altered cells of the liver in 8/10 males and 5/10 females.

The relative kidney and liver weights for males and females receiving 330 ppm were significantly greater than those for controls (Table 6). Significant decreases were seen for T_3 and T_4 concentrations in both male and female rats receiving 330 ppm (Table F3). Decreases in hemoglobin, erythrocyte counts, hematocrit, and mean corpuscular hemoglobin concentrations were observed in exposed rats and were indicative of mild anemia in male rats only. Decreases in lactic dehydrogenase and alanine aminotransferase activity in the 330-ppm groups are not indicative of hepatocellular damage. Urinalysis revealed no evidence of renal damage; there was no apparent effect on the ability to concentrate urine.

TABLE 6. ORGAN WEIGHT TO BODY WEIGHT RATIOS FOR RATS IN THE NINE-MONTH DRINKING WATER STUDIES OF 3,3'-DIMETHOXYBENZIDINE DIHYDROCHLORIDE (a)

Organ	Control	330 ppm
MALE		
Body weight (grams)	390 ± 7.7	373 ± 8.4
Brain	5.2 ± 0.12	5.6 ± 0.11
Kidney	6.1 ± 0.11	**7.0 ± 0.12
Liver	25.5 ± 0.40	**28.7 ± 0.67
FEMALE		
Body weight (grams)	232 ± 3.9	223 ± 3.3
Brain	8.0 ± 0.13	8.3 ± 0.15
Kidney	6.2 ± 0.16	**7.3 ± 0.15
Liver	26.9 ± 0.47	**29.7 ± 0.69

(a) Mean ± standard error in milligrams per gram, unless otherwise specified, for groups of 10 animals; P values vs. controls by Wilcoxon's test (Hollander and Wolfe, 1973).
**$P<0.01$

TWENTY-ONE-MONTH STUDIES

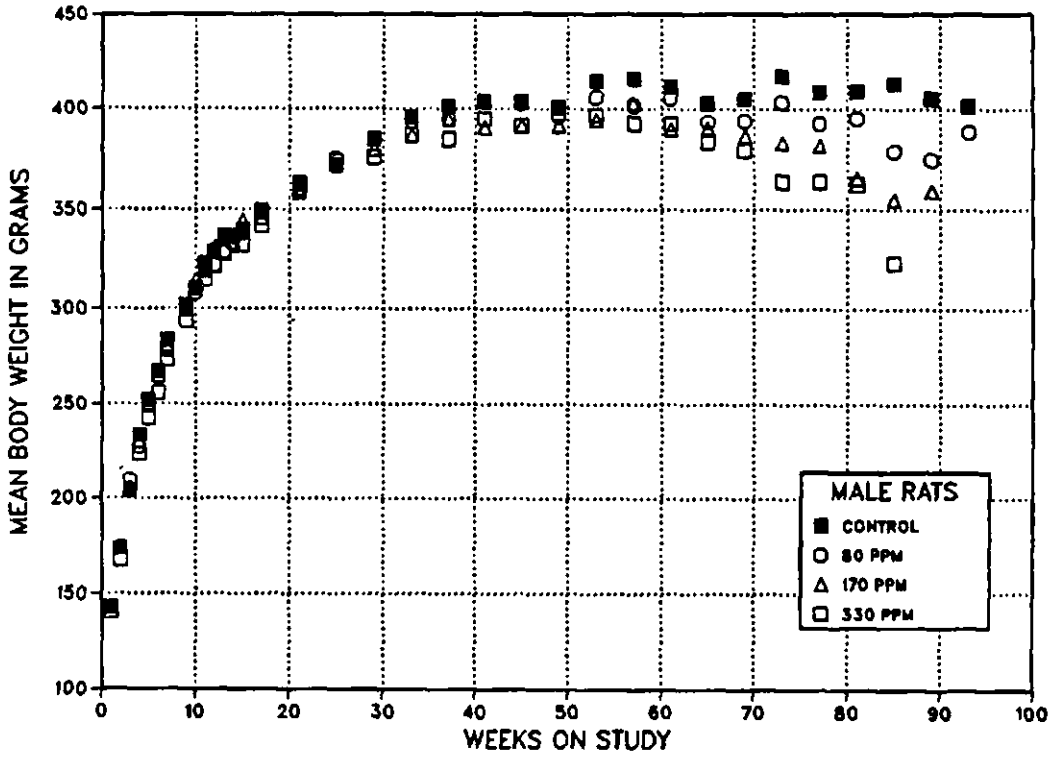
Body Weights, Water Consumption, and Clinical Signs

Mean body weights of high dose male rats were within 6% of those of the controls until week 69 and were 11%-22% lower thereafter; mean body weights of mid dose male rats were within 5% of those of the controls until week 69 and were 6%-14% lower thereafter (Table 7 and Figure 3). Mean body weights of high dose female rats were 9%-11% lower than those of controls after week 53; mean body weights of mid dose female rats were 7%-17% lower than those of controls after week 53. Body weight decreases of 22% for high dose males and 17% for mid dose females occurred in the last week of the studies, and calculations of relative body weights were based on only a few surviving animals. The average daily water consumption per rat by low, mid, and high dose rats was 94%, 97%, and 83% that by controls for males and 99%, 97%, and 78% for females (Tables D1 and D2). The average amount of 3,3'-dimethoxybenzidine dihydrochloride consumed per day was approximately 6, 12, or 21 mg/kg for low, mid, or high dose male rats and 7, 14, or 23 mg/kg for low, mid, or high dose female rats. Clinical signs noted during the studies were limited to increased incidences of tissue masses on the head, over the dorsum, and in the genital area in dosed groups.

TABLE 7. MEAN BODY WEIGHTS OF RATS IN THE TWENTY-ONE-MONTH DRINKING WATER STUDIES OF 3,3'-DIMETHOXYBENZIDINE DIHYDROCHLORIDE

Week on Study	Control Av. Wt. (grams)	Control Number Weighed	80 ppm Av. Wt. (grams)	80 ppm Wt. (percent of controls)	80 ppm Number Weighed	170 ppm Av. Wt. (grams)	170 ppm Wt. (percent of controls)	170 ppm Number Weighed	330 ppm Av. Wt. (grams)	330 ppm Wt. (percent of controls)	330 ppm Number Weighed
MALE											
1	143	70	143	100	45	143	100	75	140	98	70
2	174	70	175	101	45	174	100	75	167	96	70
3	205	70	210	102	45	206	100	75	204	100	70
4	233	70	227	97	45	230	99	75	223	96	70
5	252	70	249	99	45	250	99	75	242	96	70
6	267	70	264	99	45	265	99	75	256	96	70
7	284	70	278	98	45	281	99	75	273	96	70
9	302	70	300	99	45	301	100	75	294	97	70
10	310	70	308	99	45	315	102	75	311	100	70
11	323	70	321	99	45	320	99	75	315	98	70
12	329	69	322	98	45	331	101	75	322	98	70
13	336	69	329	98	45	335	100	75	328	98	70
14	336	69	332	99	45	337	100	75	333	99	70
15	340	69	338	99	45	345	101	75	332	98	70
17	349	69	346	99	(a) 40	346	99	(a) 70	342	98	(a) 65
21	363	69	363	100	45	358	99	75	360	99	70
25	372	69	375	101	45	372	100	75	374	101	70
29	384	69	384	100	45	379	99	75	375	98	70
33	395	69	394	100	45	387	98	75	387	98	70
37	401	69	395	99	45	395	99	75	385	96	70
41	404	(b) 59	404	100	44	391	97	75	395	98	(b) 59
45	404	59	403	100	44	391	97	73	392	97	57
49	401	59	396	99	44	391	98	72	400	100	55
53	414	59	406	98	42	395	95	70	397	96	53
57	416	59	402	97	42	403	97	68	393	94	53
61	411	59	406	99	42	390	95	65	392	95	48
65	403	59	394	98	42	391	97	62	383	95	41
69	405	58	394	97	42	386	95	57	381	94	39
73	417	57	403	97	38	383	92	48	364	87	30
77	409	55	393	96	37	382	93	41	364	89	24
81	409	55	395	97	31	366	89	19	363	89	5
85	413	53	379	92	28	355	86	13	323	78	4
89	405	50	375	93	16	359	89	4	--	--	--
93	403	45	389	97	8	--	--	--	--	--	--
FEMALE											
1	112	70	114	102	45	111	99	75	111	99	70
2	131	70	129	98	45	127	97	75	126	96	70
3	143	70	144	101	45	142	99	75	143	100	70
4	153	70	152	99	45	149	97	75	148	97	70
5	163	70	161	99	45	158	97	(a) 73	155	95	70
6	168	70	168	100	45	166	99	75	163	97	70
7	179	70	174	97	45	170	95	75	169	94	70
9	187	70	184	98	45	180	96	75	176	94	70
10	189	70	186	98	45	187	99	75	183	97	70
11	193	70	190	98	45	185	96	75	183	95	70
12	193	70	192	99	45	193	100	75	188	97	70
13	198	70	196	99	45	193	97	75	192	97	70
14	199	70	198	99	45	197	99	75	196	98	70
15	204	70	201	99	45	199	98	75	197	97	70
17	209	(a) 45	207	99	45	201	96	(a) 70	202	97	(a) 65
21	218	70	215	99	45	208	95	75	208	95	70
25	223	70	222	100	(a) 44	214	96	75	216	97	70
29	225	70	225	100	45	221	98	75	219	97	70
33	232	70	230	99	45	223	96	74	222	96	70
37	237	70	235	99	45	231	97	74	226	95	69
41	243	(b) 60	242	100	45	234	96	73	232	95	(b) 57
45	251	60	251	100	45	243	97	68	241	96	53
49	262	60	257	98	45	252	96	66	249	95	52
53	277	60	271	98	45	255	92	57	253	91	42
57	284	60	275	97	44	263	93	52	256	90	40
61	294	59	290	99	44	269	91	48	264	90	35
65	303	59	292	96	41	277	91	41	269	89	22
69	307	59	295	96	40	284	93	34	276	90	18
73	318	59	307	97	36	282	89	27	289	91	11
77	319	57	307	96	34	285	89	24	285	89	11
81	324	56	306	94	34	281	87	18	293	90	7
85	324	54	304	94	29	288	89	11	295	91	5
89	331	50	298	90	22	285	86	7	--	--	--
93	336	45	307	91	15	280	83	6	--	--	--

(a) The number of animals weighed was lower than the number of animals surviving.
(b) Interim kill



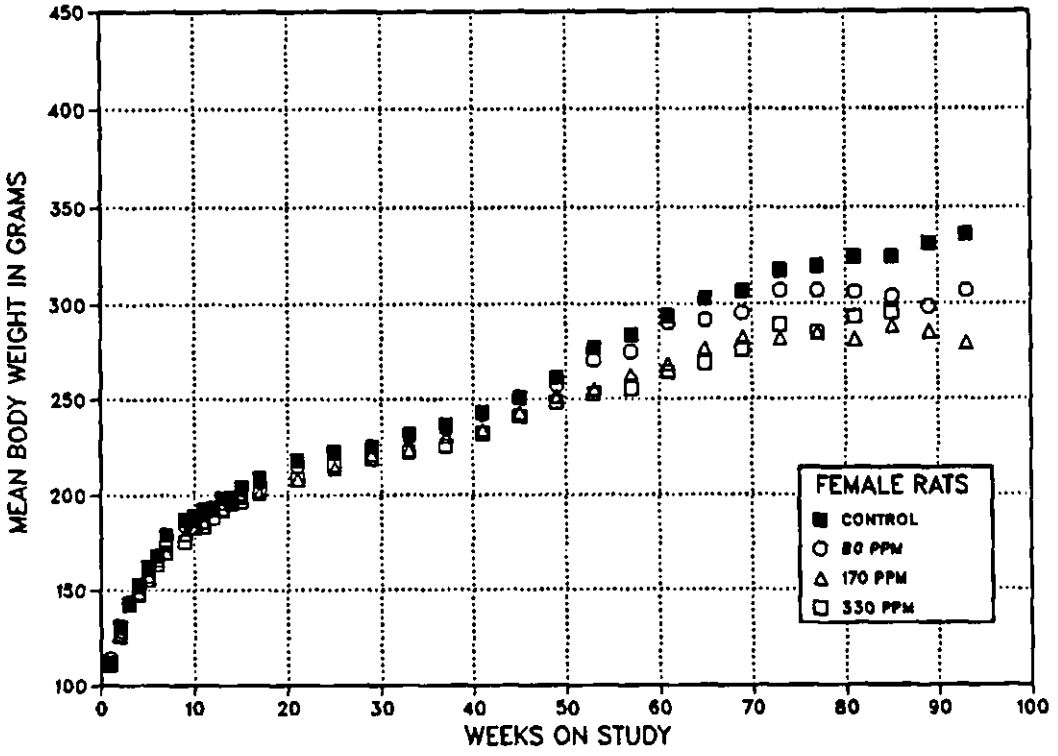


FIGURE 3. GROWTH CURVES FOR RATS GIVEN DRINKING WATER CONTAINING 3,3'-DIMETHOXYBENZIDINE DIHYDROCHLORIDE FOR TWENTY-ONE MONTHS

Survival

Estimates of the probabilities of survival for male and female rats given drinking water containing 3,3′-dimethoxybenzidine dihydrochloride at the concentrations used in these studies and for controls are shown in Table 8 and in the Kaplan and Meier curves in Figure 4. The survival of dosed rats was significantly lower than that of controls after day 552 (low dose), 420 (mid dose), or 401 (high dose) for males and day 483 (low dose), 309 (mid dose), or 304 (high dose) for females.

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 liver, large intestine, small intestine, Zymbal gland, preputial gland, clitoral gland, oral cavity, skin, mammary gland, brain, uterus, mesothelium, spleen, mesenteric lymph nodes, heart, lung, and bone marrow.

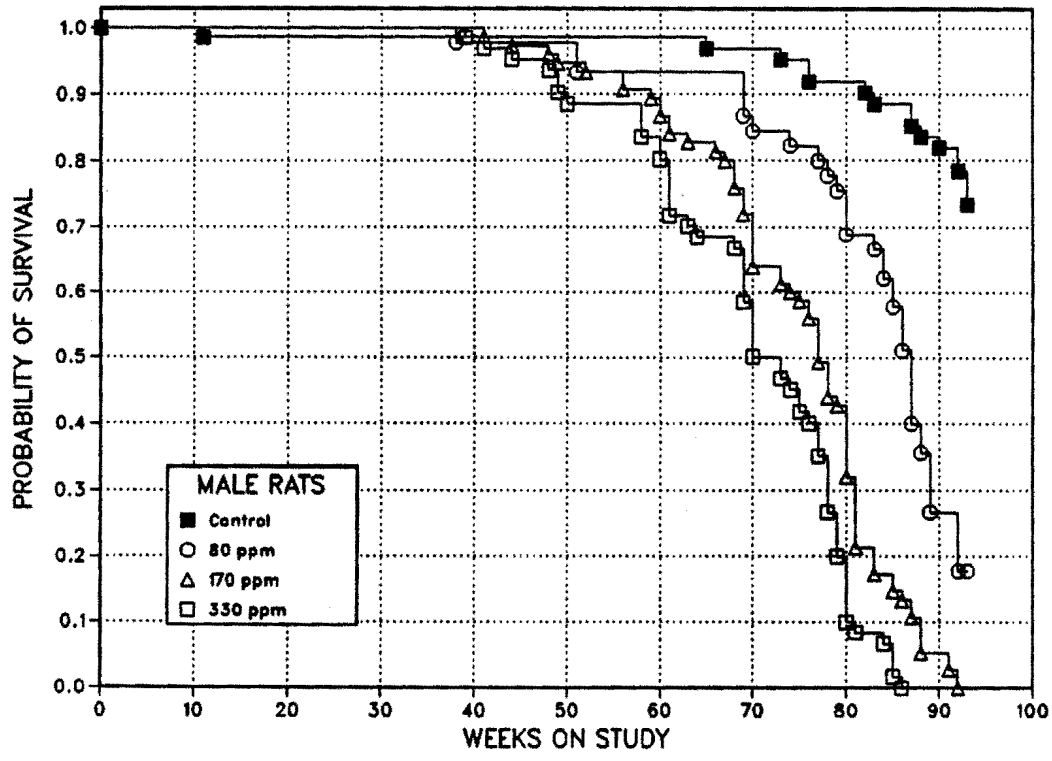
Summaries of the incidences of neoplasms and nonneoplastic lesions, individual animal tumor diagnoses, statistical analyses of primary tumors that occurred with an incidence of at least 5% in at least one animal group, and historical control incidences for the neoplasms mentioned in this section are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

Liver: The administration of 3,3′-dimethoxybenzidine dihydrochloride in drinking water to

TABLE 8. SURVIVAL OF RATS IN THE TWENTY-ONE-MONTH DRINKING WATER STUDIES OF 3,3′-DIMETHOXYBENZIDINE DIHYDROCHLORIDE

	Control	80 ppm	170 ppm	330 ppm
MALE (a)				
Animals initially in study	60	45	75	60
Natural deaths	9	9	25	14
Moribund kills	7	28	50	46
Animals surviving until study termination	44	8	0	0
Survival P values (b)	<0.001	<0.001	<0.001	<0.001
FEMALE (a)				
Animals initially in study	60	45	75	60
Natural deaths	5	3	9	9
Moribund kills	10	27	60	51
Animals surviving until study termination	45	15	6	0
Survival P values (b)	<0.001	<0.001	<0.001	<0.001

(a) First day of termination period: male--647; female--648
(b) The result of the life table trend test is in the control column, and the results of the life table pairwise comparisons with the controls are in the dosed columns.



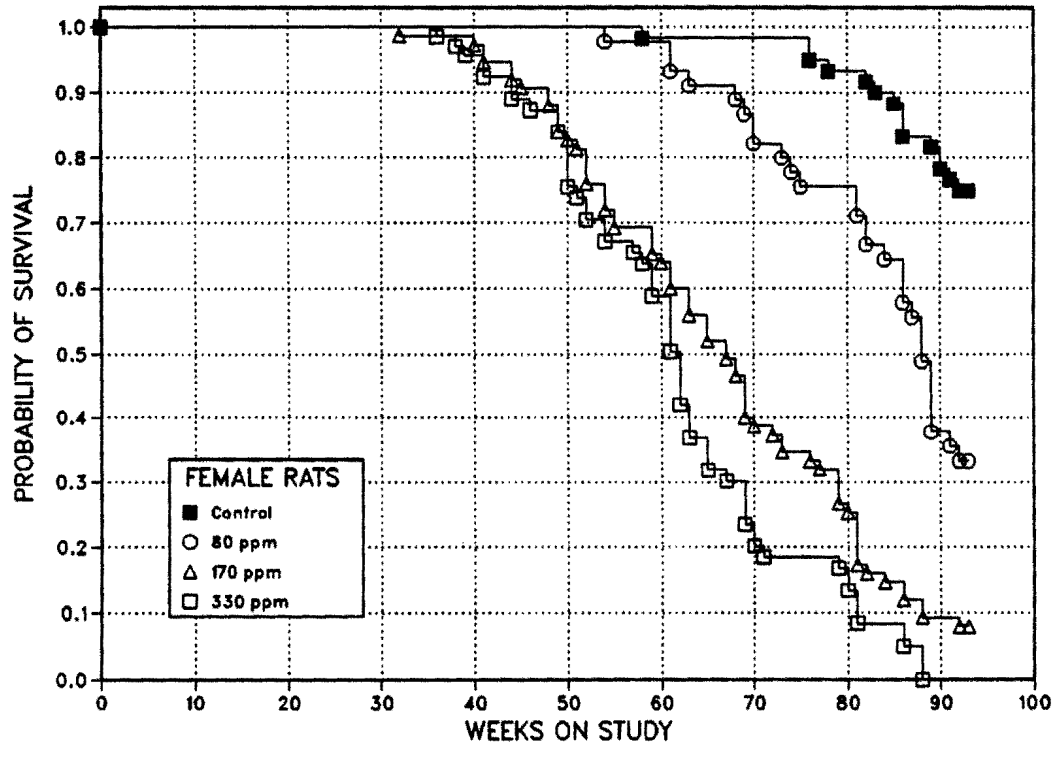


FIGURE 4. KAPLAN-MEIER SURVIVAL CURVES FOR RATS GIVEN DRINKING WATER CONTAINING 3,3'-DIMETHOXYBENZIDINE DIHYDROCHLORIDE FOR TWENTY-ONE MONTHS

male and female rats caused a variety of degenerative and proliferative lesions in the liver (Table 9); the lesions were generally more severe and the incidences were greater in dosed males than in females. The degenerative lesions consisted of clusters of hepatocytes containing cytoplasmic vacuoles (presumably lipid droplets), generalized centrilobular hepatocellular degeneration, randomly distributed single or multiple foci of necrosis, and foci of multilocular cysts containing granular eosinophilic material or erythrocytes (cystic degeneration or spongiosis hepatis). Hepatocellular regeneration, characterized by poorly circumscribed foci of enlarged cells with deeply staining eosinophilic cytoplasm, occurred in livers with the more severe degenerative lesions.

The incidences of clear cell foci were marginally increased in high dose male rats and dosed female rats. Eosinophilic foci were increased in both dosed male and female rats. Clear cell foci consisted of poorly circumscribed clusters of hepatocytes with pale cytoplasm, whereas eosinophilic foci consisted of cells with eosinophilic cytoplasm. These foci were generally smaller than a hepatic lobule and showed little or no compression of the surrounding parenchyma; the hepatic plates in the foci merged imperceptibly with the normal plates. Neoplastic nodules in males and neoplastic nodules or hepatocellular carcinomas (combined) in males and females occurred with significant positive trends; the incidences in mid and high dose males were significantly greater than that in controls (Table 10). Neoplastic nodules were expansile lesions that were generally larger than a hepatic lobule and compressed the surrounding tissue; the hepatic plates within the neoplastic nodule were not arranged in a normal lobular pattern. The hepatocytes showed altered staining properties and slight nuclear pleomorphism and atypia. The hepatocellular carcinomas were larger masses consisting of hepatocytes in solid clusters or trabeculae several layers thick without a lobular pattern; the hepatocytes generally showed greater cellular atypia and pleomorphism than those within the neoplastic nodules.

Large Intestine (Colon, Cecum, or Rectum): Adenomatous polyps or adenocarcinomas (combined) in male and female rats occurred with significant positive trends; the incidences in mid and high dose males and high dose females were significantly greater than those in controls (Table 11).

TABLE 9. NUMBERS OF RATS WITH SELECTED LIVER LESIONS IN THE TWENTY-ONE-MONTH DRINKING WATER STUDIES OF 3,3'-DIMETHOXYBENZIDINE DIHYDROCHLORIDE

Lesion	Male				Female			
	Control	80 ppm	170 ppm	330 ppm	Control	80 ppm	170 ppm	330 ppm
Number examined	60	45	74	60	60	44	75	60
Clear cell focus	19	11	16	28	7	11	18	*15
Cystic degeneration	13	**23	**34	**28	1	2	1	5
Centrilobular degeneration	0	*4	**9	**10	1	3	*8	5
Eosinophilic focus	6	**15	**35	**38	5	7	**20	**28
Hematopoietic cell proliferation	2	**15	**39	**41	1	**18	**43	**41
Necrosis	4	**15	**18	**17	1	3	**13	**18
Regeneration	5	7	**22	**18	6	3	5	4
Cytoplasmic vacuolization	2	2	7	*10	3	1	4	3
Neoplastic nodule	0	3	**7	**6	0	1	0	2
Hepatocellular carcinoma	1	1	0	2	0	0	0	1

*P<0.05 vs. controls
**P<0.01 vs. controls

TABLE 10. LIVER TUMORS IN RATS IN THE TWENTY-ONE-MONTH DRINKING WATER STUDIES OF 3,3'-DIMETHOXYBENZIDINE DIHYDROCHLORIDE (a)

	Control	80 ppm (b)	170 ppm (b)	330 ppm (b)
MALE				
Neoplastic Nodule				
Overall Rates	0/60 (0%)	3/45 (7%)	7/74 (9%)	6/60 (10%)
Effective Rates (c)	0/58 (0%)	3/39 (8%)	7/54 (13%)	6/35 (17%)
Terminal Rates	0/44 (0%)	1/8 (13%)	0/0	0/0
Day of First Observation		538	485	485
Cochran-Armitage Trend Test (d)	P=0.002			
Fisher Exact Test (d)		P=0.062	P=0.005	P=0.002
Hepatocellular Carcinoma				
Overall Rates	1/60 (2%)	1/45 (2%)	0/74 (0%)	2/60 (3%)
Neoplastic Nodule or Hepatocellular Carcinoma (e)				
Overall Rates	1/60 (2%)	4/45 (9%)	7/74 (9%)	8/60 (13%)
Effective Rates (c)	1/58 (2%)	4/39 (10%)	7/54 (13%)	8/35 (23%)
Terminal Rates	1/44 (2%)	2/8 (25%)	0/0	0/0
Day of First Observation	647	538	485	485
Cochran-Armitage Trend Test (d)	P=0.001			
Fisher Exact Test (d)		P=0.083	P=0.024	P=0.001
FEMALE				
Neoplastic Nodule				
Overall Rates	0/60 (0%)	1/44 (2%)	0/75 (0%)	2/60 (3%)
Hepatocellular Carcinoma				
Overall Rates	0/60 (0%)	0/44 (0%)	0/75 (0%)	1/60 (2%)
Neoplastic Nodule or Hepatocellular Carcinoma (f)				
Overall Rates	0/60 (0%)	1/44 (2%)	0/75 (0%)	3/60 (5%)
Effective Rates (c)	0/59 (0%)	1/44 (2%)	0/47 (0%)	3/38 (8%)
Terminal Rates	0/45 (0%)	1/15 (7%)	0/6 (0%)	0/0
Day of First Observation		648		408
Cochran-Armitage Trend Test (d)	P=0.022			
Fisher Exact Test (d)		P=0.427	(g)	P=0.057

(a) For a complete explanation of the entries in this table, see Table A3 (footnotes); the statistical analyses used are discussed in Section II (Statistical Methods).
(b) The estimated dose in milligrams per kilograms per day is given in Section III (Body Weights, Water Consumption, and Clinical Signs) and in Appendix D.
(c) 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 four groups
(d) Based on effective rates
(e) Historical incidence at study laboratory (mean): 7/100 (7%); historical incidence in NTP studies (mean ± SD): 78/1,591 (5% ± 4%)
(f) Historical incidence at study laboratory (mean): 2/100 (2%); historical incidence in NTP studies (mean ± SD): 37/1,643 (2% ± 3%)
(g) No P value is reported because no tumors were observed in the 170-ppm and control groups.

TABLE 11. TUMORS OF THE LARGE INTESTINE IN RATS IN THE TWENTY-ONE-MONTH DRINKING WATER STUDIES OF 3,3'-DIMETHOXYBENZIDINE DIHYDROCHLORIDE (a)

	Control	80 ppm	170 ppm	330 ppm
MALE				
Adenomatous Polyp				
Overall Rates	0/60 (0%)	1/45 (2%)	4/75 (5%)	5/60 (8%)
Effective Rates (b)	0/59 (0%)	1/44 (2%)	4/73 (5%)	5/57 (9%)
Terminal Rates	0/44 (0%)	0/8 (0%)	0/0	0/0
Day of First Observation		644	546	332
Cochran-Armitage Trend Test (c)	P=0.013			
Fisher Exact Test (c)		P=0.427	P=0.090	P=0.026
Adenocarcinoma				
Overall Rates	0/60 (0%)	0/45 (0%)	4/75 (5%)	3/60 (5%)
Effective Rates (b)	0/59 (0%)	0/42 (0%)	4/67 (6%)	3/50 (6%)
Terminal Rates	0/44 (0%)	0/8 (0%)	0/0	0/0
Day of First Observation			485	414
Cochran-Armitage Trend Test (c)	P=0.031			
Fisher Exact Test (c)		(d)	P=0.077	P=0.093
Adenomatous Polyp or Adenocarcinoma (e)				
Overall Rates	0/60 (0%)	1/45 (2%)	8/75 (11%)	8/60 (13%)
Effective Rates (b)	0/59 (0%)	1/44 (2%)	8/73 (11%)	8/57 (14%)
Terminal Rates	0/44 (0%)	0/8 (0%)	0/0	0/0
Day of First Observation		644	485	332
Cochran-Armitage Trend Test (c)	P=0.001			
Fisher Exact Test (c)		P=0.427	P=0.007	P=0.003
FEMALE				
Adenomatous Polyp				
Overall Rates	0/60 (0%)	0/45 (0%)	1/75 (1%)	2/60 (3%)
Adenocarcinoma				
Overall Rates	0/60 (0%)	1/45 (2%)	0/75 (0%)	1/60 (2%)
Adenomatous Polyp or Adenocarcinoma (f)				
Overall Rates	0/60 (0%)	1/45 (2%)	1/75 (1%)	3/60 (5%)
Effective Rates (b)	0/59 (0%)	1/44 (2%)	1/48 (2%)	3/35 (9%)
Terminal Rates	0/45 (0%)	1/15 (7%)	0/6 (0%)	0/0
Day of First Observation		648	424	424
Cochran-Armitage Trend Test (c)	P=0.020			
Fisher Exact Test (c)		P=0.427	P=0.449	P=0.049

(a) For a complete explanation of the entries in this table, see Table A3 (footnotes); the statistical analyses used are discussed in Section II (Statistical Methods).
(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 four groups
(c) Based on effective rates
(d) No P value is reported because no tumors were observed in the 80-ppm and control groups.
(e) Historical incidence at study laboratory: 0/96; historical incidence in NTP studies (mean ± SD): 2/1,541 (0.1% ± 0.5%)
(f) Historical incidence at study laboratory: 0/88; historical incidence in NTP studies: 0/1,601

Adenomatous polyps were exophytic, polypoid masses that protruded into the intestinal lumen. These consisted of glandular structures lined by a single layer of columnar epithelial cells with round nuclei and moderately abundant basophilic cytoplasm. These cells were generally well differentiated, but mucous cells were not present. The adenocarcinomas were similar exophytic masses that showed invasion of the intestinal submucosa. The glandular structures composing the adenocarcinomas were generally more irregular, particularly at the site of invasion, and the epithelial cells were less well differentiated with some atypia.

Small Intestine: The incidences of adenocarcinomas in dosed males were significantly greater than that in controls (Table 12). Adenocarcinomas were seen in 0/60 control, 1/45 low dose, 1/75 mid dose, and 2/60 high dose female rats. The adenocarcinomas invaded the intestinal wall and consisted of glandular structures lined by moderately well to poorly differentiated columnar epithelium. Several of the neoplasms contained mucus-secreting cells forming large dilated spaces filled with mucus (cystic mucinous adenocarcinomas).

Zymbal Gland: The Zymbal glands are specialized sebaceous glands anterior and ventral to the external orifices of the ears. The incidences of adenomas, carcinomas, and adenomas or carcinomas (combined) were significantly greater in the dosed groups than in the control groups (Table 13). Some dosed rats had bilateral neoplasms of the Zymbal gland.

Hyperplasia, adenomas, and carcinomas are part of a morphologic continuum. Hyperplasia was a focal lesion of the glandular epithelium characterized by enlarged cells that distorted the normal acinar arrangement. Adenomas were circumscribed masses consisting of poorly formed acini surrounding ductlike structures lined by squamous epithelium. Sebaceous cell differentiation was evident in the neoplastic acini. Carcinomas were generally larger and invaded adjacent soft tissues. The neoplastic cells demonstrated heterogeneous growth patterns with irregular, poorly formed acinar structures, solid masses, and cords with scattered ductlike structures filled with secretory material and cellular debris. The neoplasms exhibited predominantly sebaceous or squamous differentiation, but some neoplasms had prominent components of each.

TABLE 12. TUMORS OF THE SMALL INTESTINE IN MALE RATS IN THE TWENTY-ONE-MONTH DRINKING WATER STUDY OF 3,3'-DIMETHOXYBENZIDINE DIHYDROCHLORIDE (a)

	Control	80 ppm	170 ppm	330 ppm
Adenocarcinoma (b)				
Overall Rates	0/60 (0%)	4/45 (9%)	7/75 (9%)	5/60 (8%)
Effective Rates (c)	0/59 (0%)	4/44 (9%)	7/75 (9%)	5/60 (8%)
Terminal Rates	0/44 (0%)	0/8 (0%)	0/0	0/0
Day of First Observation		354	417	267
Cochran-Armitage Trend Test (d)	P=0.081			
Fisher Exact Test (d)		P=0.031	P=0.015	P=0.030

(a) For a complete explanation of the entries in this table, see Table A3 (footnotes); the statistical analyses used are discussed in Section II (Statistical Methods).
(b) Historical incidence of adenomatous polyps or adenocarcinomas (combined) at study laboratory (mean): 1/97 (1%); historical incidence in NTP studies (mean ± SD): 5/1,557 (0.3% ± 0.8%)
(c) 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 four groups
(d) Based on effective rates

TABLE 13. ZYMBAL GLAND LESIONS IN RATS IN THE TWENTY-ONE-MONTH DRINKING WATER STUDIES OF 3,3'-DIMETHOXYBENZIDINE DIHYDROCHLORIDE (a)

	Control	80 ppm	170 ppm	330 ppm
MALE				
Hyperplasia				
Overall Rates	1/59 (2%)	**9/45 (20%)	**13/75 (17%)	**14/60 (23%)
Adenoma				
Overall Rates	0/59 (0%)	4/45 (9%)	11/75 (15%)	9/60 (15%)
Effective Rates (b)	0/58 (0%)	4/44 (9%)	11/71 (15%)	9/53 (17%)
Terminal Rates	0/44 (0%)	1/8 (13%)	0/0	0/0
Day of First Observation		353	391	445
Life Table Tests	P<0.001	P=0.011	P<0.001	P<0.001
Cochran-Armitage Trend Test (c)	P=0.002			
Fisher Exact Test (c)		P=0.032	P<0.001	P<0.001
Carcinoma				
Overall Rates	0/59 (0%)	7/45 (16%)	14/75 (19%)	21/60 (35%)
Effective Rates (b)	0/58 (0%)	7/45 (16%)	14/75 (19%)	21/60 (35%)
Terminal Rates	0/44 (0%)	0/8 (0%)	0/0	0/0
Day of First Observation		262	304	284
Life Table Tests	P<0.001	P=0.001	P<0.001	P<0.001
Cochran-Armitage Trend Test (c)	P<0.001			
Fisher Exact Test (c)		P=0.002	P<0.001	P<0.001
Adenoma or Carcinoma (d)				
Overall Rates	0/59 (0%)	10/45 (22%)	25/75 (33%)	30/60 (50%)
Effective Rates (b)	0/58 (0%)	10/45 (22%)	25/75 (33%)	30/60 (50%)
Terminal Rates	0/44 (0%)	1/8 (13%)	0/0	0/0
Day of First Observation		262	304	284
Life Table Tests	P<0.001	P<0.001	P<0.001	P<0.001
Cochran-Armitage Trend Test (c)	P<0.001			
Fisher Exact Test (c)		P<0.001	P<0.001	P<0.001
FEMALE				
Hyperplasia				
Overall Rates	0/60 (0%)	*5/45 (11%)	**14/75 (19%)	**13/60 (22%)
Adenoma				
Overall Rates	0/60 (0%)	3/45 (7%)	4/75 (5%)	3/60 (5%)
Effective Rates (b)	0/59 (0%)	3/44 (7%)	4/48 (8%)	3/35 (9%)
Terminal Rates	0/45 (0%)	0/15 (0%)	0/6 (0%)	0/0
Day of First Observation		424	424	424
Life Table Tests	P<0.001	P=0.036	P=0.010	P=0.005
Cochran-Armitage Trend Test (c)	P=0.054			
Fisher Exact Test (c)		P=0.075	P=0.038	P=0.049
Carcinoma				
Overall Rates	1/60 (2%)	10/45 (22%)	17/75 (23%)	13/60 (22%)
Effective Rates (b)	1/60 (2%)	10/45 (22%)	17/74 (23%)	13/59 (22%)
Terminal Rates	0/45 (0%)	0/15 (0%)	1/6 (17%)	0/0
Day of First Observation	402	424	274	262
Life Table Tests	P<0.001	P<0.001	P<0.001	P<0.001
Cochran-Armitage Trend Test (c)	P=0.006			
Fisher Exact Test (c)		P<0.001	P<0.001	P<0.001

TABLE 13. ZYMBAL GLAND LESIONS IN RATS IN THE TWENTY-ONE-MONTH DRINKING WATER STUDIES OF 3,3'-DIMETHOXYBENZIDINE DIHYDROCHLORIDE (Continued)

	Control	80 ppm	170 ppm	330 ppm
FEMALE (Continued)				
Adenoma or Carcinoma (e)				
Overall Rates	1/60 (2%)	12/45 (27%)	21/75 (28%)	16/60 (27%)
Effective Rates (b)	1/60 (2%)	12/45 (27%)	21/74 (28%)	16/59 (27%)
Terminal Rates	0/45 (0%)	0/15 (0%)	1/6 (17%)	0/0
Day of First Observation	402	424	274	262
Life Table Tests	P<0.001	P<0.001	P<0.001	P<0.001
Cochran-Armitage Trend Test (c)	P=0.002			
Fisher Exact Test (c)		P<0.001	P<0.001	P<0.001

(a) For a complete explanation of the entries in this table, see Table A3 (footnotes); the statistical analyses used are discussed in Section II (Statistical Methods).
(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 four groups
(c) Based on effective rates
(d) Historical incidence at study laboratory (mean): 1/100 (1%); historical incidence in NTP studies (mean ± SD): 19/1,596 (1% ± 2%)
(e) Historical incidence at study laboratory (mean): 1/100 (1%); historical incidence in NTP studies (mean ± SD): 14/1,643 (0.9% ± 2%)
*P<0.05 vs. controls by Fisher exact test
**P<0.01 vs. controls by Fisher exact test

Preputial or Clitoral Gland: The preputial glands of the male rat are modified sebaceous glands bilateral and adjacent to the penis. The clitoral glands of the female are homologous organs located near the base of the clitoris. Ductular ectasia and glandular hyperplasia occurred at increased incidences in dosed male rats but not in the clitoral gland of female rats (Tables 14 and 15). The incidences of carcinomas and adenomas or carcinomas (combined) of the preputial gland in males occurred with significant positive trends; the incidences in the mid and high dose groups were significantly greater than those in the controls. In female rats, the incidences of adenomas, carcinomas, and adenomas or carcinomas (combined) of the clitoral gland were significantly greater in almost all dosed groups than in controls. Bilateral neoplasms of the preputial and clitoral glands occurred in dosed groups of rats.

Hyperplasia, adenomas, and carcinomas of the preputial and clitoral glands are part of a morphologic continuum. Hyperplasia was characterized by clusters of acini consisting of enlarged cells with prominent nuclei. There was some distortion of the acinar arrangement of the cells. Adenomas were circumscribed, expansile lesions exhibiting loss of normal acinar organization. The neoplastic cells were well differentiated and arranged in solid clusters with scattered duct-like structures containing debris. Carcinomas were poorly circumscribed masses with irregular boundaries, often accompanied by inflammation in the surrounding tissue. Overt invasion of the adjacent soft tissue similar to that seen with Zymbal gland carcinomas was generally not observed. The carcinomas exhibited greater heterogeneity of growth pattern and greater cellular pleomorphism and atypia than adenomas.

TABLE 14. PREPUTIAL GLAND LESIONS IN MALE RATS IN THE TWENTY-ONE-MONTH DRINKING WATER STUDY OF 3,3'-DIMETHOXYBENZIDINE DIHYDROCHLORIDE (a)

	Control	80 ppm	170 ppm	330 ppm
Ectasia				
Overall Rates	5/60 (8%)	**12/43 (28%)	**25/73 (34%)	**24/59 (41%)
Hyperplasia				
Overall Rates	2/60 (3%)	*7/43 (16%)	*10/73 (14%)	**12/59 (20%)
Adenoma				
Overall Rates	14/60 (23%)	6/43 (14%)	19/73 (26%)	12/59 (20%)
Effective Rates (b)	14/59 (24%)	6/42 (14%)	19/71 (27%)	12/56 (21%)
Terminal Rates	10/44 (23%)	1/8 (13%)	0/0	0/0
Day of First Observation	531	485	333	423
Life Table Tests	P<0.001	P=0.202	P<0.001	P<0.001
Cochran-Armitage Trend Test (c)	P=0.497			
Fisher Exact Test (c)		P=0.179N	P=0.425	P=0.472N
Carcinoma				
Overall Rates	2/60 (3%)	6/43 (14%)	15/73 (21%)	19/59 (32%)
Effective Rates (b)	2/59 (3%)	6/42 (14%)	15/73 (21%)	19/59 (32%)
Terminal Rates	0/44 (0%)	1/8 (13%)	0/0	0/0
Day of First Observation	603	603	284	267
Life Table Tests	P<0.001	P=0.001	P<0.001	P<0.001
Cochran-Armitage Trend Test (c)	P<0.001			
Fisher Exact Test (c)		P=0.053	P=0.003	P<0.001
Adenoma or Carcinoma (d)				
Overall Rates	16/60 (27%)	12/43 (28%)	33/73 (45%)	29/59 (49%)
Effective Rates (b)	16/59 (27%)	12/42 (29%)	33/73 (45%)	29/59 (49%)
Terminal Rates	10/44 (23%)	2/8 (25%)	0/0	0/0
Day of First Observation	531	485	284	267
Life Table Tests	P<0.001	P=0.003	P<0.001	P<0.001
Cochran-Armitage Trend Test (c)	P=0.003			
Fisher Exact Test (c)		P=0.523	P=0.025	P=0.011

(a) For a complete explanation of the entries in this table, see Table A3 (footnotes); the statistical analyses used are discussed in Section II (Statistical Methods).
(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 four groups
(c) Based on effective rates
(d) Historical incidence at study laboratory (mean): 5/100 (5%); historical incidence in NTP studies (mean ± SD): 117/1,596 (7% ± 5%)
*P<0.05 vs. controls by Fisher exact test
**P<0.01 vs. controls by Fisher exact test

TABLE 15. CLITORAL GLAND LESIONS IN FEMALE RATS IN THE TWENTY-ONE-MONTH DRINKING WATER STUDY OF 3,3'-DIMETHOXYBENZIDINE DIHYDROCHLORIDE (a)

	Control	80 ppm	170 ppm	330 ppm
Ectasia				
Overall Rates	15/58 (26%)	11/44 (25%)	11/74 (15%)	12/55 (22%)
Hyperplasia				
Overall Rates	4/58 (7%)	*9/44 (20%)	8/74 (11%)	6/55 (11%)
Adenoma				
Overall Rates	5/58 (9%)	15/44 (34%)	13/74 (18%)	16/55 (29%)
Effective Rates (b)	5/58 (9%)	15/44 (34%)	13/73 (18%)	16/55 (29%)
Terminal Rates	5/44 (11%)	7/15 (47%)	0/6 (0%)	0/0
Day of First Observation	648	436	358	262
Life Table Tests	P<0.001	P<0.001	P<0.001	P<0.001
Cochran-Armitage Trend Test (c)	P=0.035			
Fisher Exact Test (c)		P=0.002	P=0.102	P=0.005
Carcinoma				
Overall Rates	2/58 (3%)	17/44 (39%)	41/74 (55%)	30/55 (55%)
Effective Rates	2/58 (3%)	17/44 (39%)	41/74 (55%)	30/55 (55%)
Terminal Rates	2/44 (5%)	5/15 (33%)	3/6 (50%)	0/0
Day of First Observation	648	373	220	270
Life Table Tests (d)	P<0.001	P<0.001	P<0.001	P<0.001
Cochran-Armitage Trend Test (c)	P<0.001			
Fisher Exact Test (c)		P<0.001	P<0.001	P<0.001
Adenoma or Carcinoma (d)				
Overall Rates	7/58 (12%)	27/44 (61%)	48/74 (65%)	41/55 (75%)
Effective Rates (b)	7/58 (12%)	27/44 (61%)	48/74 (65%)	41/55 (75%)
Terminal Rates	7/44 (16%)	10/15 (67%)	3/6 (50%)	0/0
Day of First Observation	648	373	220	262
Life Table Tests	P<0.001	P<0.001	P<0.001	P<0.001
Cochran-Armitage Trend Test (c)	P<0.001			
Fisher Exact Test (c)		P<0.001	P<0.001	P<0.001

(a) For a complete explanation of the entries in this table, see Table B3 (footnotes); the statistical analyses used are discussed in Section II (Statistical Methods).
(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 four groups
(c) Based on effective rates
(d) Historical incidence at study laboratory (mean): 8/100 (8%); historical incidence in NTP studies (mean ± SD): 115/1,643 (7% ± 5%)
*P<0.05 vs. controls by Fisher exact test

Oral Cavity (Palate or Tongue): Squamous papillomas and squamous papillomas or squamous cell carcinomas (combined) of the palate or tongue in males occurred with significant positive trends; the incidences in dosed males were significantly greater than those in controls (Table 16). A few squamous cell papillomas occurred in each of the female dosed and control groups, but squamous cell carcinomas occurred only in the mid and high dose groups. The papillomas consisted of branching papillae arising from the mucosal epithelium and extending into the oral cavity. The papillae had a thickened stratified squamous epithelium overlying a thin core of connective tissue. The squamous cell carcinomas often had exophytic papillary structures similar to the papillomas but showed invasion of the underlying submucosa by cords and clusters of neoplastic squamous epithelium.

Skin: A spectrum of epithelial neoplasms of the skin occurred at markedly increased incidences, primarily in male rats given 3,3′-dimethoxybenzidine dihydrochloride (Tables 17 and 18). The incidences of basal cell adenomas, basal cell carcinomas, squamous cell papillomas, and squamous cell carcinomas in males occurred with significant positive trends; except for basal cell carcinomas in low dose males, the incidences in the dosed groups were significantly greater than those in the controls. Small numbers of sebaceous gland adenomas or carcinomas (combined) occurred in dosed male rats. The incidences of keratoacanthomas were significantly increased in low dose male rats and increased ($P = 0.053$) in mid dose male rats.

Small numbers of basal cell adenomas occurred in dosed groups of female rats but not in controls. A basal cell carcinoma was observed in a single low dose female. The incidence of basal cell adenomas or carcinomas (combined) in low dose female rats was significantly greater than that in controls. Squamous cell papillomas were observed in three mid dose female rats.

The basal cell neoplasms consisted of small basophilic cells arranged in branching cords, solid clusters, or nodules with central cavities. Some exhibited features of hair follicles, whereas others showed sebaceous differentiation. Those with predominantly sebaceous differentiation were diagnosed as sebaceous gland adenomas. The basal cell adenomas were circumscribed masses without local invasion, whereas the carcinomas exhibited cellular anaplasia, necrosis, and/or local invasion. The squamous cell papillomas were typical exophytic growths consisting of branching papillae of stratified squamous epithelium, and the squamous cell carcinomas were composed of cords of well to poorly differentiated squamous epithelium that infiltrated the underlying dermis and subcutaneous tissue.

Mammary Gland: Adenocarcinomas in female rats occurred with a significant positive trend; the incidences in the mid and high dose groups were significantly greater than that in the controls (Table 19). The incidence of adenocarcinomas in high dose female rats was four times the highest observed historical incidence in untreated control female F344/N rats. The incidences of fibroadenomas in dosed females were lower than that in controls, probably because of the reduced survival in the dosed groups.

Brain: Malignant astrocytomas were seen in small numbers of dosed, but not control, rats (Table 20). The historical incidence of astrocytomas in untreated control male F344/N rats is 10/1,590 (0.6%) and in female F344/N rats is 15/1,628 (0.9%).

Uterus: Adenomas or carcinomas (combined) of the uterus or cervix were observed in dosed, but not in control, female rats (Table 21). The incidence of adenomas or carcinomas (combined) in low dose female rats was significantly greater than that in controls.

Mesothelium: Mesotheliomas were marginally increased in male rats (Table 22); the historical incidence of mesotheliomas in untreated control male F344/N rats is 47/1,596 (3%), and the highest observed incidence is 5/50.

TABLE 16. ORAL CAVITY SQUAMOUS CELL LESIONS IN RATS IN THE TWENTY-ONE-MONTH DRINKING WATER STUDIES OF 3,3'-DIMETHOXYBENZIDINE DIHYDROCHLORIDE (a)

	Control	80 ppm	170 ppm	330 ppm
MALE				
Hyperplasia				
Overall Rates (b)	0/3 (0%)	0/8 (0%)	2/12 (17%)	0/16 (0%)
Papilloma				
Overall Rates (c)	1/60 (2%)	7/45 (16%)	10/75 (13%)	9/60 (15%)
Effective Rates (d)	1/59 (2%)	7/44 (16%)	10/73 (14%)	9/57 (16%)
Terminal Rates (c)	1/44 (2%)	2/8 (25%)	0/0	0/0
Day of First Observation	647	485	333	402
Cochran-Armitage Trend Test (e)	P=0.029			
Fisher Exact Test (e)		P=0.010	P=0.012	P=0.007
Carcinoma				
Overall Rates (c)	0/60 (0%)	1/45 (2%)	0/75 (0%)	2/60 (3%)
Papilloma or Carcinoma (f)				
Overall Rates (c)	1/60 (2%)	8/45 (18%)	10/75 (13%)	11/60 (18%)
Effective Rates (d)	1/59 (2%)	8/44 (18%)	10/73 (14%)	11/57 (19%)
Terminal Rates (c)	1/44 (2%)	2/8 (25%)	0/0	0/0
Day of First Observation	647	485	333	401
Cochran-Armitage Trend Test (e)	P=0.011			
Fisher Exact Test (e)		P=0.004	P=0.012	P=0.002
FEMALE				
Hyperplasia				
Overall Rates (b)	0/2 (0%)	0/3 (0%)	4/11 (36%)	1/5 (20%)
Papilloma				
Overall Rates (c)	2/60 (3%)	2/45 (4%)	3/75 (4%)	3/60 (5%)
Effective Rates (d)	2/59 (3%)	2/44 (5%)	3/52 (6%)	3/38 (8%)
Terminal Rates (c)	2/45 (4%)	1/15 (7%)	0/6 (0%)	0/0
Day of First Observation	648	644	450	408
Cochran-Armitage Trend Test (e)	P=0.214			
Fisher Exact Test (e)		P=0.574	P=0.440	P=0.299
Carcinoma				
Overall Rates (c)	0/60 (0%)	0/45 (0%)	3/75 (4%)	2/60 (3%)
Papilloma or Carcinoma (g)				
Overall Rates (c)	2/60 (3%)	2/45 (4%)	6/75 (8%)	5/60 (8%)
Effective Rates (d)	2/60 (3%)	2/45 (4%)	6/68 (9%)	5/52 (10%)
Terminal Rates (c)	2/45 (4%)	1/15 (7%)	0/6 (0%)	0/0
Day of First Observation	648	644	331	408
Cochran-Armitage Trend Test (e)	P=0.094			
Fisher Exact Test (e)		P=0.576	P=0.181	P=0.164

(a) For a complete explanation of the entries in this table, see Table A3 (footnotes); the statistical analyses used are discussed in Section II (Statistical Methods).
(b) The denominator is the number of animals examined microscopically; the incidences in the dosed groups are not significantly different from that in the controls by the Fisher exact test.
(c) The denominator is the number of animals examined grossly.
(d) 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 four groups
(e) Based on effective rates
(f) Historical incidence at study laboratory: 0/100; historical incidence in NTP studies (mean ± SD): 7/1,596 (0.4% ± 1.0%)
(g) Historical incidence at study laboratory: 0/100; historical incidence in NTP studies (mean ± SD): 4/1,643 (0.2% ± 0.7%)

TABLE 17. SKIN BASAL CELL AND SEBACEOUS GLAND TUMORS AND KERATOACANTHOMAS IN RATS IN THE TWENTY-ONE-MONTH DRINKING WATER STUDIES OF 3,3'-DIMETHOXYBENZIDINE DIHYDROCHLORIDE (a)

	Control	80 ppm	170 ppm	330 ppm
MALE				
Basal Cell Adenoma				
Overall Rates	1/60 (2%)	31/45 (69%)	47/75 (63%)	35/60 (58%)
Effective Rates (b)	1/59 (2%)	31/42 (74%)	47/67 (70%)	35/50 (70%)
Terminal Rates	1/44 (2%)	7/8 (88%)	0/0	0/0
Day of First Observation	647	480	424	419
Life Table Tests	P<0.001	P<0.001	P<0.001	P<0.001
Cochran-Armitage Trend Test (c)	P<0.001			
Fisher Exact Test (c)		P<0.001	P<0.001	P<0.001
Basal Cell Carcinoma				
Overall Rates	1/60 (2%)	4/45 (9%)	18/75 (24%)	17/60 (28%)
Effective Rates (b)	1/59 (2%)	4/44 (9%)	18/71 (25%)	17/54 (31%)
Terminal Rates	1/44 (2%)	0/8 (0%)	0/0	0/0
Day of First Observation	647	552	417	344
Life Table Tests	P<0.001	P=0.016	P<0.001	P<0.001
Cochran-Armitage Trend Test (c)	P<0.001			
Fisher Exact Test (c)		P=0.104	P<0.001	P<0.001
Basal Cell Adenoma or Carcinoma				
Overall Rates	2/60 (3%)	32/45 (71%)	54/75 (72%)	40/60 (67%)
Effective Rates (b)	2/59 (3%)	32/44 (73%)	54/71 (76%)	40/54 (74%)
Terminal Rates	2/44 (5%)	7/8 (88%)	0/0	0/0
Day of First Observation	647	480	417	344
Life Table Tests	P<0.001	P<0.001	P<0.001	P<0.001
Cochran-Armitage Trend Test (c)	P<0.001			
Fisher Exact Test (c)		P<0.001	P<0.001	P<0.001
Sebaceous Gland Adenoma or Carcinoma				
Overall Rates	0/60 (0%)	2/45 (4%)	3/75 (4%)	2/60 (3%)
Basal Cell Adenoma, Basal Cell Carcinoma, Sebaceous Gland Adenoma, or Sebaceous Gland Carcinoma (d)				
Overall Rates	2/60 (3%)	33/45 (73%)	56/75 (75%)	41/60 (68%)
Effective Rates (b)	2/59 (3%)	33/44 (75%)	56/72 (78%)	41/56 (73%)
Terminal Rates	2/44 (5%)	7/8 (88%)	0/0	0/0
Day of First Observation	647	353	417	337
Life Table Tests	P<0.001	P<0.001	P<0.001	P<0.001
Cochran-Armitage Trend Test (c)	P<0.001			
Fisher Exact Test (c)		P<0.001	P<0.001	P<0.001
Keratoacanthoma (e)				
Overall Rates	1/60 (2%)	5/45 (11%)	7/75 (9%)	1/60 (2%)
Effective Rates (b)	1/59 (2%)	5/42 (12%)	7/70 (10%)	1/53 (2%)
Terminal Rates	0/44 (0%)	0/8 (0%)	0/0	0/0
Day of First Observation	573	556	391	546
Life Table Tests	P=0.006	P=0.003	P=0.002	P=0.370
Cochran-Armitage Trend Test (c)	P=0.457N			
Fisher Exact Test (c)		P=0.044	P=0.053	P=0.725N

TABLE 17. SKIN BASAL CELL AND SEBACEOUS GLAND TUMORS AND KERATOACANTHOMAS IN RATS IN THE TWENTY-ONE-MONTH DRINKING WATER STUDIES OF 3,3'-DIMETHOXYBENZIDINE DIHYDROCHLORIDE (Continued)

	Control	80 ppm	170 ppm	330 ppm
FEMALE				
Basal Cell Adenoma				
Overall Rates	0/60 (0%)	3/45 (7%)	3/75 (4%)	2/60 (3%)
Effective Rates (b)	0/59 (0%)	3/44 (7%)	3/48 (6%)	2/35 (6%)
Terminal Rates	0/45 (0%)	3/15 (20%)	0/6 (0%)	0/0
Day of First Observation		648	423	610
Life Table Tests	P<0.001	P=0.009	P=0.006	P<0.001
Cochran-Armitage Trend Test (c)	P=0.155			
Fisher Exact Test (c)		P=0.075	P=0.087	P=0.136
Basal Cell Carcinoma				
Overall Rates	0/60 (0%)	1/45 (2%)	0/75 (0%)	0/60 (0%)
Basal Cell Adenoma or Carcinoma (f)				
Overall Rates	0/60 (0%)	4/45 (9%)	3/75 (4%)	2/60 (3%)
Effective Rates (b)	0/59 (0%)	4/44 (9%)	3/48 (6%)	2/35 (6%)
Terminal Rates	0/45 (0%)	4/15 (27%)	0/6 (0%)	0/0
Day of First Observation		648	423	610
Life Table Tests	P<0.001	P=0.002	P=0.006	P<0.001
Cochran-Armitage Trend Test (c)	P=0.203			
Fisher Exact Test (c)		P=0.031	P=0.087	P=0.136

(a) For a complete explanation of the entries in this table, see Table A3 (footnotes); the statistical analyses used are discussed in Section II (Statistical Methods).
(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 four groups
(c) Based on effective rates
(d) Historical incidence at study laboratory (mean): 2/100 (2%); historical incidence in NTP studies (mean ± SD): 30/1,596 (2% ± 2%)
(e) Historical incidence at study laboratory (mean): 6/100 (6%); historical incidence in NTP studies (mean ± SD): 39/1,596 (2% ± 4%)
(f) Historical incidence at study laboratory: 0/100; historical incidence in NTP studies (mean ± SD): 7/1,643 (0.4% ± 0.8%)

TABLE 18. SKIN SQUAMOUS CELL TUMORS IN RATS IN THE TWENTY-ONE-MONTH DRINKING WATER STUDIES OF 3,3'-DIMETHOXYBENZIDINE DIHYDROCHLORIDE (a)

	Control	80 ppm	170 ppm	330 ppm
MALE				
Papilloma				
Overall Rates	0/60 (0%)	5/45 (11%)	7/75 (9%)	5/60 (8%)
Effective Rates (b)	0/58 (0%)	5/42 (12%)	7/62 (11%)	5/41 (12%)
Terminal Rates	0/44 (0%)	2/8 (25%)	0/0	0/0
Day of First Observation		515	525	445
Life Table Tests	P<0.001	P<0.001	P<0.001	P<0.001
Cochran-Armitage Trend Test (c)	P=0.032			
Fisher Exact Test (c)		P=0.011	P=0.008	P=0.010
Carcinoma				
Overall Rates	0/60 (0%)	9/45 (20%)	24/75 (32%)	21/60 (35%)
Effective Rates (b)	0/59 (0%)	9/42 (21%)	24/65 (37%)	21/48 (44%)
Terminal Rates	0/44 (0%)	2/8 (25%)	0/0	0/0
Day of First Observation		485	424	445
Life Table Tests	P<0.001	P<0.001	P<0.001	P<0.001
Cochran-Armitage Trend Test (c)	P<0.001			
Fisher Exact Test (c)		P<0.001	P<0.001	P<0.001
Papilloma or Carcinoma (d)				
Overall Rates	0/60 (0%)	13/45 (29%)	28/75 (37%)	22/60 (37%)
Effective Rates (b)	0/59 (0%)	13/42 (31%)	28/65 (43%)	22/48 (46%)
Terminal Rates	0/44 (0%)	3/8 (38%)	0/0	0/0
Day of First Observation		485	424	445
Life Table Tests	P<0.001	P<0.001	P<0.001	P<0.001
Cochran-Armitage Trend Test (c)	P<0.001			
Fisher Exact Test (c)		P<0.001	P<0.001	P<0.001
FEMALE				
Papilloma (e)				
Overall Rates	0/60 (0%)	0/45 (0%)	3/75 (4%)	0/60 (0%)

(a) For a complete explanation of the entries in this table, see Table A3 (footnotes); the statistical analyses used are discussed in Section II (Statistical Methods).
(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 four groups
(c) Based on effective rates
(d) Historical incidence at study laboratory (mean): 3/100 (3%); historical incidence in NTP studies (mean ± SD): 31/1,596 (2% ± 2%)
(e) Historical incidence of papillomas or carcinomas (combined) at study laboratory: 0/100; historical incidence in NTP studies (mean ± SD): 7/1,643 (0.4% ± 0.8%)

TABLE 19. MAMMARY GLAND TUMORS IN FEMALE RATS IN THE TWENTY-ONE-MONTH DRINKING WATER STUDY OF 3,3'-DIMETHOXYBENZIDINE DIHYDROCHLORIDE (a)

	Control	80 ppm	170 ppm	330 ppm
Adenoma				
Overall Rates	0/60 (0%)	1/45 (2%)	0/75 (0%)	2/60 (3%)
Fibroadenoma (b)				
Overall Rates	14/60 (23%)	11/45 (24%)	9/75 (12%)	4/60 (7%)
Effective Rates (c)	14/60 (23%)	11/45 (24%)	9/63 (14%)	4/50 (8%)
Terminal Rates	12/45 (27%)	6/15 (40%)	2/6 (33%)	0/0
Day of First Observation	532	424	476	344
Cochran-Armitage Trend Test (d)	P=0.011N			
Fisher Exact Test (d)		P=0.537	P=0.146N	P=0.026N
Adenocarcinoma (e)				
Overall Rates	1/60 (2%)	2/45 (4%)	14/75 (19%)	20/60 (33%)
Effective Rates (c)	1/60 (2%)	2/45 (4%)	14/73 (19%)	20/57 (35%)
Terminal Rates	1/45 (2%)	0/15 (0%)	2/6 (33%)	0/0
Day of First Observation	648	512	333	284
Life Table Tests	P<0.001	P=0.252	P<0.001	P<0.001
Cochran-Armitage Trend Test (d)	P<0.001			
Fisher Exact Test (d)		P=0.393	P<0.001	P<0.001

(a) For a complete explanation of the entries in this table, see Table B3 (footnotes); the statistical analyses used are discussed in Section II (Statistical Methods).
(b) Historical incidence at study laboratory (mean): 47/100 (47%); historical incidence in NTP studies (mean ± SD): 520/1,643 (32% ± 12%)
(c) 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 four groups
(d) Based on effective rates
(e) Historical incidence at study laboratory (mean): 3/100 (3%); historical incidence in NTP studies (mean ± SD): 49/1,643 (3% ± 2%)

TABLE 20. BRAIN TUMORS IN RATS IN THE TWENTY-ONE-MONTH DRINKING WATER STUDIES OF 3,3'-DIMETHOXYBENZIDINE DIHYDROCHLORIDE (a)

	Control	80 ppm	170 ppm	330 ppm
MALE				
Malignant Astrocytoma (b)				
Overall Rates	0/60 (0%)	2/44 (5%)	3/75 (4%)	1/60 (2%)
Effective Rates (c)	0/58 (0%)	2/37 (5%)	3/48 (6%)	1/30 (3%)
Terminal Rates	0/44 (0%)	1/7 (14%)	0/0	0/0
Day of First Observation		618	536	506
Cochran-Armitage Trend Test (d)	P=0.247			
Fisher Exact Test (d)		P=0.149	P=0.090	P=0.341
FEMALE				
Malignant Astrocytoma (e)				
Overall Rates	0/60 (0%)	1/45 (2%)	1/75 (1%)	0/60 (0%)

(a) For a complete explanation of the entries in this table, see Table A3 (footnotes); the statistical analyses used are discussed in Section II (Statistical Methods).
(b) Historical incidence of astrocytomas at study laboratory (mean): 2/100 (2%); historical incidence in NTP studies (mean ± SD): 10/1,590 (0.6% ± 1%)
(c) 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 four groups
(d) Based on effective rates
(e) Historical incidence of astrocytomas at study laboratory (mean): 2/100 (2%); historical incidence in NTP studies (mean ± SD): 15/1,628 (0.9% ± 2%)

TABLE 21. UTERINE TUMORS IN FEMALE RATS IN THE TWENTY-ONE-MONTH DRINKING WATER STUDY OF 3,3'-DIMETHOXYBENZIDINE DIHYDROCHLORIDE (a)

	Control	80 ppm	170 ppm	330 ppm
Adenoma				
Overall Rates	0/60 (0%)	3/45 (7%)	1/75 (1%)	2/60 (3%)
Carcinoma				
Overall Rates	0/60 (0%)	1/45 (2%)	1/75 (1%)	0/60 (0%)
Adenoma or Carcinoma (b)				
Overall Rates	0/60 (0%)	4/45 (9%)	2/75 (3%)	2/60 (3%)
Effective Rates (c)	0/59 (0%)	4/44 (9%)	2/48 (4%)	2/35 (6%)
Terminal Rates	0/45 (0%)	1/15 (7%)	1/6 (17%)	0/0
Day of First Observation		606	424	563
Cochran-Armitage Trend Test (d)	P=0.230			
Fisher Exact Test (d)		P=0.031	P=0.199	P=0.136

(a) For a complete explanation of the entries in this table, see Table B3 (footnotes); the statistical analyses used are discussed in Section II (Statistical Methods).
(b) Historical incidence at study laboratory: 0/99; historical incidence in NTP studies (mean ± SD): 12/1,632 (0.7% ± 1%)
(c) 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 four groups
(d) Based on effective rates

TABLE 22. MESOTHELIOMAS IN MALE RATS IN THE TWENTY-ONE-MONTH DRINKING WATER STUDY OF 3,3'-DIMETHOXYBENZIDINE DIHYDROCHLORIDE (a)

	Control	80 ppm	170 ppm	330 ppm
Mesothelioma (b)				
Overall Rates	2/60 (3%)	1/45 (2%)	7/75 (9%)	6/60 (10%)
Effective Rates (c)	2/59 (3%)	1/44 (2%)	7/72 (10%)	6/56 (11%)
Terminal Rates	1/44 (2%)	0/8 (0%)	0/0	0/0
Day of First Observation	529	483	339	401
Cochran-Armitage Trend Test (d)	P=0.044			
Fisher Exact Test (d)		P=0.610N	P=0.140	P=0.119

(a) For a complete explanation of the entries in this table, see Table A3 (footnotes); the statistical analyses used are discussed in Section II (Statistical Methods).
(b) Historical incidence at study laboratory (mean): 3/100 (3%); historical incidence in NTP studies (mean ± SD): 47/1,596 (3% ± 3%)
(c) 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 four groups
(d) Based on effective rates

Spleen: Hematopoietic cell proliferation was observed at increased incidences in dosed rats (male: control, 3/60; low dose, 13/42; mid dose, 43/74; high dose, 38/59; female: 3/60; 22/44; 50/75; 47/60).

Mesenteric Lymph Nodes: Reticulum cell hyperplasia was observed at increased incidences in dosed rats (male: control, 0/59; low dose, 3/42; mid dose, 6/73; high dose, 6/56; female: 2/60; 3/44; 18/75; 18/58).

Heart: Thrombi in the atrium were observed at increased incidences in dosed male rats (male: control, 3/60; low dose, 15/44; mid dose, 27/75; high dose, 23/60; female: 0/60; 1/45; 0/75; 1/60). The increased incidences of atrial thrombosis observed in the heart of exposed males may have been related to compound-caused morbidity, which led to impaired circulation and sludging of blood in the atrial chambers. This effect was not observed in exposed female rats, although there was a similar degree of morbidity.

Lung: Histiocytic cellular infiltration was observed at increased incidences in dosed rats (male: control, 0/60; low dose, 3/44; mid dose, 10/75; high dose, 6/60; female: 0/60; 3/45; 4/75; 18/60).

Bone Marrow: Hyperplasia of myeloid cells was observed at increased incidences in dosed rats (male: control, 2/60; low dose, 3/43; mid dose, 14/74; high dose, 7/60; female: 5/60; 8/45; 9/75; 14/60).

3,3′-Dimethoxybenzidine was tested for induction of gene mutations in *Salmonella typhimurium* strains TA98, TA100, TA1535, and TA1537 in each of three laboratories (Haworth et al., 1983; Table H1). In all laboratories, a response ranging from weakly positive to positive was observed with strain TA100 in trials conducted in the presence of Aroclor 1254-induced male Sprague Dawley rat or Syrian hamster liver S9; likewise, positive results were reported for strain TA98 with S9 in all three laboratories, and one laboratory also observed a significant response in TA98 without S9. A weakly positive response was reported by one of the test laboratories with TA1535 in the presence of induced hamster S9. In cytogenetic tests with Chinese hamster ovary cells conducted in two laboratories, sister chromatid exchanges (SCEs) were induced by 3,3′-dimethoxybenzidine both with and without Aroclor 1254-induced male Sprague Dawley rat liver S9; in one of these two laboratories, the positive responses observed in the SCE trials without S9 occurred under conditions of delayed harvest (3-5 hours additional culture time), but the positive results reported by the second laboratory in the SCE test were observed at lower doses of the study chemical which did not affect cell cycle time (Galloway et al., 1985; Table H2). Results of the chromosomal aberration test were reported to be negative (Galloway et al., 1985); however, recent statistical reanalysis (Galloway et al., 1987) of the chromosomal aberration data has resulted in a change in the call from negative to weakly positive without S9 (Litton Bionetics study) and positive with S9 (Columbia University study) (Table H3). 3,3′-Dimethoxybenzidine was negative for induction of sex-linked recessive lethal mutations in adult male *Drosophila melanogaster* exposed to the chemical by feeding (100 ppm) or injection (200 ppm) (Yoon et al., 1985; Table H4). The methods and results are presented in Appendix H.

IV. DISCUSSION AND CONCLUSIONS

Fourteen-Day and Thirteen-Week Studies
Nine-Month Studies
Twenty-One-Month Studies
Nonneoplastic Lesions
Neoplastic Lesions
Tumor Transplant Study
Oncogene Activation
Related Aromatic Amines
Audit
Conclusions

IV. DISCUSSION AND CONCLUSIONS

Consumption of drinking water containing 3,3′-dimethoxybenzidine dihydrochloride led to highly significant incidences of neoplasms at a variety of sites and to mild toxicity in several organs. Unusual neoplasm sites in 3,3′-dimethoxybenzidine-exposed rats include the skin, Zymbal gland, preputial and clitoral glands, intestine, and oral mucosa. Most genotoxic carcinogens are associated with unusual tumor sites, and the short latency and multiple sites of these tumors are most characteristic of potent genotoxic carcinogens, such as benzidine dyes (NCI, 1978b), benzene (NTP, 1986), 1,3-butadiene (NTP, 1984), and glycidol (NTP, 1990). 3,3′-Dimethoxybenzidine and related aminobiphenyls are mutagenic. 3,3′-Dimethoxybenzidine requires S9 for mutagenic activation in the Salmonella assay, indicating that the chemical is metabolized to a mutagenic species, most likely through *N*-hydroxylation.

Fourteen-Day and Thirteen-Week Studies

In the 14-day and 13-week studies, male and female rats were exposed to 3,3′-dimethoxybenzidine dihydrochloride in drinking water at concentrations ranging from 170 to 4,500 ppm. Animal survival was unaffected, and few toxic effects were observed. Water consumption was decreased with increasing 3,3′-dimethoxybenzidine dihydrochloride concentration in both studies. In the 13-week studies, mean body weight gains were decreased in the two top dose groups. Compound-related effects seen in the top dose groups of male and female rats included increases in relative liver and kidney weights, nephropathy, and lipofuscin accumulation in the thyroid gland.

Dose-related decreases in serum triiodothyronine (T_3) and thyroxin (T_4) without a change in thyrotropin (TSH) are not consistent with a toxic effect on the thyroid gland; this effect was probably due to a change in the amount or binding capacity of the protein carrier for these hormones rather than to a direct effect on the thyroid gland. 3,3′-Dimethoxybenzidine is similar in structure to T_3 and T_4, suggesting that the dose-related decreases in serum T_3 and T_4 may be due to competition with 3,3′-dimethoxybenzidine for the carrier protein.

Based on the chemical-induced nephropathy and on reductions in water consumption and body weight gain observed in the 13-week studies, doses for the long-term studies in male and female rats were 0 or 330 ppm 3,3′-dimethoxybenzidine dihydrochloride administered in drinking water for 9 months and 0, 80, 170, or 330 ppm for 21 months.

Nine-Month Studies

Carcinomas of the preputial, clitoral, and Zymbal glands were observed after chemical exposure for only 9 months. Basophilic and/or eosinophilic foci in the liver and hyperplasia of the preputial and Zymbal glands were also detected in exposed rats. These lesions were not observed in control rats. The short latency of these lesions is unusual and indicative of the carcinogenic potency of 3,3′-dimethoxybenzidine dihydrochloride.

In the 9-month studies, hematologic effects were indicative of a mild anemia in male rats. Serum enzyme changes were slight and were not considered indicative of liver injury. Serum T_3 and T_4 were decreased, with no change in TSH, and as in the 13-week studies, these changes were not considered to be a direct effect on the thyroid gland.

Twenty-One-Month Studies

3,3′-Dimethoxybenzidine dihydrochloride studies were terminated at month 21 because of reduced survival in the dosed groups (see Table 8 and Figure 4). The reduced survival of dosed rats first became noticeable in males during months 14-15 and in females during month 11. For humane reasons, animals with large visible masses or those in a moribund condition, usually due to internal neoplasms, were killed rather than allowed to suffer; this program may have influenced the overall survival profile. Mean body weights of high dose male and female rats were 4%-22% lower than those of controls during the second year.

Nonneoplastic Lesions

Increased hematopoietic cell proliferation in the liver and spleen, coupled with bone marrow hyperplasia in exposed groups, are probably related to inflammation and necrosis associated with neoplasms.

3,3′-Dimethoxybenzidine dihydrochloride appeared to stimulate the reticuloendothelial system. This effect was manifested as reticulum cell hyperplasia of the mesenteric lymph nodes. Although this effect may be compound related, it is probably a nonspecific reaction.

Neoplastic Lesions

There was a highly significant association between the consumption of 3,3′-dimethoxybenzidine dihydrochloride and the development of Zymbal gland adenomas and/or carcinomas in dosed male and female rats. With the exception of a carcinoma in one control female (first observed during week 58), Zymbal gland neoplasms were not observed in control groups. Carcinomas were observed at necropsy in exposed males and females as early as week 38. Neoplasms develop at this site infrequently (1%) in historical control rats (Tables A4d and B4d) and usually only late in life (Solleveld et al., 1984). Benzidine, the parent compound of 3,3′-dimethoxybenzidine, also causes Zymbal gland tumors in rats, and it is a known urinary bladder carcinogen in humans (IARC, 1982, 1987a).

3,3′-Dimethoxybenzidine dihydrochloride had a profound effect on the preputial and clitoral glands in exposed male and female rats, giving rise to a high incidence of carcinomas and/or adenomas. The incidences of preputial or clitoral gland neoplasms in high dose male and female rats were 7 and 10 times higher, respectively, than in untreated historical control F344/N rats. In exposed rats, carcinomas were confirmed histologically at necropsy as early as week 32 (females) and week 39 (males), whereas in controls, carcinomas were not observed until week 87 in males or at the end of the study at month 21 in females. Potential precursor lesions (hyperplasia) occurred in small numbers of exposed animals, possibly because most such lesions had already progressed to neoplasms.

Of 350 chemicals evaluated for carcinogenicity in rats and mice by the National Cancer Institute/National Toxicology Program (NCI/NTP), only 12 were associated with skin neoplasms; 11 of these 12 chemicals were administered orally or by inhalation. In the current study, 72% of male rats administered 3,3′-dimethoxybenzidine dihydrochloride in drinking water were found to have basal cell and/or sebaceous gland neoplasms of the skin, compared with only 3% of controls. In exposed male rats, basal cell neoplasms occurred as early as week 50; squamous cell neoplasms occurred as early as week 61. The basal cell neoplasms often showed differentiation to structures associated with sebaceous glands or hair follicles. Epithelial skin neoplasms were observed at low incidences in exposed female rats; however, those detected were of the same morphologic type as those observed in males and were considered to be related to 3,3′-dimethoxybenzidine dihydrochloride consumption.

Few substances induce epithelial neoplasms of the skin unless they are applied directly. Although 3,3′-dimethoxybenzidine dihydrochloride was administered in drinking water, exposure of skin during grooming was likely. The possibility that skin neoplasms resulted from direct exposure of the skin to 3,3′-dimethoxybenzidine dihydrochloride or its metabolites in saliva was considered. However, these neoplasms were more likely a result of systemic exposure to reactive 3,3′-dimethoxybenzidine metabolites, because most aromatic amines require metabolic activation to have carcinogenic activity (Miller and Miller, 1974, 1977) and because many skin neoplasms were present on the backs of the animals, where grooming is minimal. No reports on the carcinogenicity of 3,3′-dimethoxybenzidine after dermal application were found.

3,3′-Dimethoxybenzidine dihydrochloride exposure led to development of neoplasms of the small and large intestine in male rats. Chemically induced neoplasms of the intestine are uncommon in rats; of 350 chemicals studied by the NCI/NTP, only 7--tribromomethane (NTP, 1989), bromodichloromethane (NTP, 1987), captan, (NCI, 1977a), phenazopyridine hydrochloride (NCI, 1978c), proflavin hydrochloride (NCI, 1977b), chrysotile asbestos (NTP, 1985), and Aroclor® 1254 (NCI, 1978d)--were associated with adenocarcinomas, adenomatous polyps, or intestinal carcinomas in rats.

In the current studies, neoplasms were principally cystic mucinous adenocarcinomas of the small intestine and adenomatous polyps and adenocarcinomas of the large intestine. Polyps in the colon were first observed at week 48, whereas adenocarcinomas in the small intestine first occurred after 39 weeks of chemical

exposure. Adenocarcinomas in the large intestine were also observed in the low, mid, and high dose groups of exposed female rats; although not as numerous as in males, these neoplasms were considered to be related to 3,3′-dimethoxybenzidine dihydrochloride exposure because no adenocarcinomas or adenomatous polyps of the large intestine have been observed in 1,601 untreated historical control female F344/N rats.

Squamous cell neoplasms that occurred on the tongue and palate of exposed male rats were strongly associated with exposure to 3,3′-dimethoxybenzidine dihydrochloride. Taken collectively, the observed number of squamous cell papillomas and carcinomas of the oral cavity (16% of dosed animals) represents a large increase in the incidence of relatively rare neoplasms (0.4% in untreated control male F344/N rats). Squamous cell neoplasms of the oral cavity were also detected in dosed female rats, although at lower incidences, but the incidences still markedly exceeded the historical incidence of 0.2%.

3,3′-Dimethoxybenzidine dihydrochloride consumption led to adenocarcinomas in the mammary gland of females receiving the mid and high doses. The incidence of adenocarcinomas in the high dose group (33%) was four times greater than the highest observed historical incidence in untreated control female F344/N rats. The first neoplasm was observed in a high dose female at week 41, whereas in the female controls, the one adenocarcinoma was observed at termination at week 93. The remarkable increase in adenocarcinomas and decreased time-to-tumor were a direct result of 3,3′-dimethoxybenzidine dihydrochloride exposure.

Intake of 3,3′-dimethoxybenzidine dihydrochloride was associated with increased incidences of hepatocellular neoplasms, principally neoplastic nodules (hepatocellular adenoma), in exposed male rats. Although the increased incidences of neoplasms were not as remarkable in the liver as in the other organs, the dose-related increases in hepatocellular neoplasms in the mid and high dose groups of males and in exposed female rats support the conclusion that 3,3′-dimethoxybenzidine dihydrochloride exposure was responsible for these neoplasms. 3,3′-Dimethoxybenzidine dihydrochloride was also associated with an increase in the incidence of eosinophilic foci in male rats. These foci are believed to be reversible changes that may progress to neoplasia (Maronpot et al., 1986). Because of the relatively high incidences of liver foci observed after exposure to 3,3′-dimethoxybenzidine dihydrochloride for 9 months, higher incidences of liver tumors were expected after exposure for 21 months. The low incidence of liver tumors may have been due in part to the early deaths of many animals because of neoplasia at other sites. In addition, early termination of the studies shortened the time available for liver foci to progress to detectable tumors.

Survival of 3,3′-dimethoxybenzidine dihydrochloride-exposed rats was reduced during the 21-month studies primarily because of moribund animals' being killed with the presence of grossly visible neoplasms of the skin, Zymbal gland, and preputial gland in males and of the Zymbal, clitoral, and mammary glands in females. Tumors of these tissues first appeared in males after 32 weeks of exposure (Zymbal gland) and in females after 32 weeks (clitoral gland).

Early deaths from these neoplasms may have reduced the number of male and female rats at risk for development of tumors at other sites. Mesotheliomas in male rats were associated with 3,3′-dimethoxybenzidine dihydrochloride exposure at the two upper doses. Although increased above that observed in controls, the incidences of these lesions were marginal; however, the lesions might have occurred in more animals if these groups had survived longer. Similarly, in dosed female rats, neoplasms of the skin, oral cavity, intestine, liver, and uterus/cervix occurred at incidences that were only marginally increased; however, the survival of exposed female rats was reduced early in the study by neoplasms of the clitoral, mammary, and Zymbal glands. Because of the low spontaneous incidence of most of these tumors and the chemically related early deaths, neoplasms in these tissues were considered to be related to 3,3′-dimethoxybenzidine dihydrochloride exposure.

The association between 3,3′-dimethoxybenzidine exposure and astrocytomas of the brain in male rats is less strong. The incidence of these tumors was only marginally increased and was not dose related. However, in consideration of the reduced survival of exposed rats and of the low spontaneous occurrence of these tumors,

these neoplasms may have been related to 3,3′-dimethoxybenzidine dihydrochloride exposure.

For these later developing or less rapidly lethal tumors, expression of tumor incidence by the standard convention (the number of tumor-bearing animals at a site divided by the number of animals in which this site was examined) might underestimate the tumor incidence that 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 tumor-bearing animals at a particular site divided 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 or low, mid, or high dose) groups. These derived incidences were analyzed statistically with the Cochran-Armitage trend test and the Fisher exact test.

Tumor Transplant Study

Because preputial gland neoplasms are usually 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 rats were transplantable at a higher incidence and with shorter latency periods than benign neoplasms. However, 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 was investigated to provide information on the biologic behavior of these neoplasms (Maronpot et al., 1988; Ulland et al., 1989). All neoplasms selected for transplantation 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. The latency period was short and transplants grew rapidly, reaching 3.0 cm in 7-9 weeks. No differences were observed in morphology or growth of transplants obtained from control or 3,3′-dimethoxybenzidine dihydrochloride-exposed rats. The results of these studies confirm the malignant nature of these preputial gland neoplasms from rats exposed to 3,3′-dimethoxybenzidine dihydrochloride.

Oncogene Activation

Neoplasms obtained from control rats and rats exposed to 3,3′-dimethoxybenzidine dihydrochloride or C.I. Direct Blue 15 (a 3,3′-dimethoxybenzidine-derived dye) were assayed for the presence of activated proto-oncogenes by the NIH 3T3 DNA transfection assay (Anderson et al., 1987). Oncogenes detectable by DNA transfection analysis were present in 21/27 skin, clitoral gland, or preputial gland neoplasms that had been induced by 3,3′-dimethoxybenzidine dihydrochloride or C.I. Direct Blue 15. DNA from both benign and malignant neoplasms was capable of inducing morphologically transformed foci in NIH 3T3 mouse fibroblast cultures.

Thirteen of the chemically induced neoplasm types were of epidermal origin and were classified as basal or squamous cell neoplasms of the skin; activated *ras* oncogenes were detected at a high frequency in these neoplasms (11/13). Histogenetically related neoplasms of the clitoral and preputial glands also had a high frequency of activated *ras* oncogenes (10/14).

It is difficult to compare oncogene activation in spontaneously occurring neoplasms with that in chemical-induced neoplasms because of the substantial difference in the neoplasm types obtained in the two groups. Only 55% (21/38) of the spontaneously occurring neoplasm types were of epithelial cell origin. However, in neoplasms of epithelial cell origin, there is a thirteenfold higher incidence of *ras* gene activation in the chemically induced neoplasms (21/34) than in the spontaneous neoplasms (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′-dimethoxybenzidine or C.I. Direct Blue 15. 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 the 12th, 13th, or 61st codon. The much higher incidence of H-*ras* gene activation and the apparent mutational specificity at codons 13 and 61 of H-*ras* with 3,3′-dimethoxybenzidine exposure suggest that the increased tumor incidence observed in exposed rats is directly related to the genotoxic effect of this chemical.

Related Aromatic Amines

Benzidine and related aromatic amines produce neoplasms 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; IARC, 1987a). In rats, benzidine and other aminobiphenyls cause neoplasms in the Zymbal gland, mammary gland, skin, intestine, and liver. These differences in species and target organ specificity may be related to differences in metabolism.

A number of aromatic amines cause neoplasms in the Zymbal gland (Table 23). The Zymbal gland has been reported to be deficient in sulfotransferase activity (Irving et al., 1971) and transacylase activity (Bartsch et al., 1973), but it is capable of hydroxylating compounds via cytochrome P450-dependent enzymatic pathways (Pohl and Fouts, 1983). Susceptibility of a species to the carcinogenic action of aromatic amines depends 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 additional activation to reactive esters, which act as ultimate carcinogens (Miller and Miller, 1977). Formation of different esters by different species may result in variations in organ specificity (Cohen, 1983).

Of 350 chemicals evaluated for carcinogenicity in rats and mice by the NCI/NTP, only 14 were associated with Zymbal gland neoplasms in rats. Ten of these 14 chemicals are aryl nitrogen derivatives (nitro, amino, or isocyanate), which were mutagenic for *Salmonella typhimurium*, and produced neoplasms in both rats and mice. In a survey of 222 chemicals evaluated by the NCI/NTP, Ashby and Tennant (1988) reported that only 6 were associated with skin neoplasms after systemic administration. Of these six chemicals, five were aryl nitrogen derivatives and five were among the group of nine chemicals that caused Zymbal gland neoplasms. Although not included in this survey, 3,3′-dimethoxybenzidine dihydrochloride, benzidine, and several other aromatic amines (Table 23) also belong to this group of genotoxic carcinogens that cause Zymbal gland and/or skin neoplasms in rodents.

Audit

The experimental and tabulated data for the NTP Technical Report on 3,3′-dimethoxybenzidine dihydrochloride were examined for accuracy, consistency, completeness, and compliance with Good Laboratory Practice regulations. As summarized in Appendix I, the audit revealed no major problems with the conduct of the studies or with collection and documentation of the experimental data. No discrepancies were found that influenced the final interpretation of the results of these studies.

Conclusions

Under the conditions of these 21-month drinking water studies, there was *clear evidence of carcinogenic activity** of 3,3′-dimethoxybenzidine dihydrochloride for male F344/N rats, as indicated by benign and malignant neoplasms of the skin, Zymbal gland, preputial gland, oral cavity, intestine, liver, and mesothelium. Increased incidences of astrocytomas of the brain may have been related to chemical administration. There was *clear evidence of carcinogenic activity* of 3,3′-dimethoxybenzidine dihydrochloride for female F344/N rats, as indicated by benign and malignant neoplasms of the Zymbal gland, clitoral gland, and mammary gland. Increases in neoplasms of the skin, oral cavity, large intestine, liver, and uterus/cervix were also considered to be related to chemical administration of 3,3′-dimethoxybenzidine dihydrochloride.

*Explanation of Levels of Evidence of Carcinogenic Activity is on page 6.
A summary of the Peer Review comments and the public discussion on this Technical Report appears on page 9.

TABLE 23. STRUCTURAL ANALOGS OF 3,3′-DIMETHOXYBENZIDINE WHICH ARE MUTAGENIC CARCINOGENS FOR RAT ZYMBAL GLAND AND SKIN

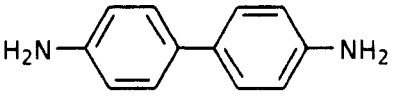
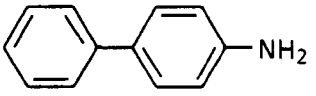
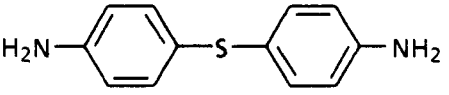
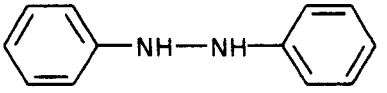
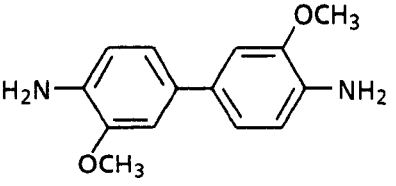
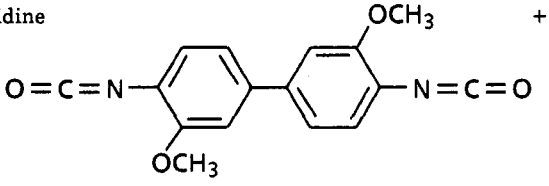
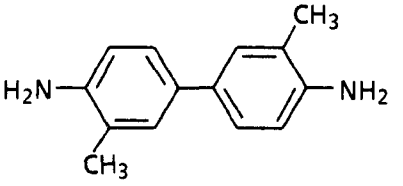
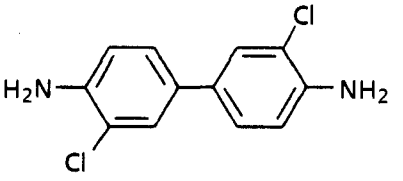
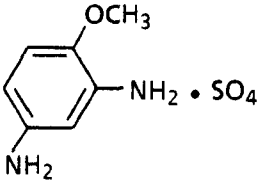
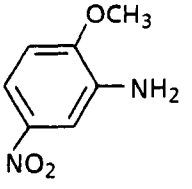
Aromatic Amine	Structure	*Salmonella typhimurium* Assay	Zymbal Gland	Skin	References
Benzidine		+	+	–	IARC, 1987a
4-Aminobiphenyl		+	+	–	IARC, 1987b
4,4′-Thiodianiline		+	+	+	NCI, 1978e
Hydrazobenzene		+	+	–	NCI, 1978f
3,3′-Dimethoxybenzidine		+	+	+	Current studies
3,3′-Dimethoxybenzidine diisocyanate		+	+	+	NCI, 1979

TABLE 23. STRUCTURAL ANALOGS OF 3,3′-DIMETHOXYBENZIDINE WHICH ARE MUTAGENIC CARCINOGENS FOR RAT ZYMBAL GLAND AND SKIN (Continued)

Aromatic Amine	Structure	*Salmonella typhimurium* Assay	Zymbal Gland	Skin	References
3,3′-Dimethylbenzidine		+	+	–	Pliss, 1965
3,3′-Dichlorobenzidine		+	+	+	IARC, 1987c; Lazear and Louie, 1977
2,4-Diaminoanisole sulfate		+	+	+	NCI, 1978g
5-Nitro-*o*-anisidine		+	+	+	NCI, 1978h

V. REFERENCES

V. REFERENCES

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Appendix E: NTP. 1992. Toxicology and Carcinogenesis Studies of C.I. Direct Blue 15 in F344/N rats. NTP Technical Report Series TR-397. pp. E-1 – E-76.

NTP TECHNICAL REPORT

ON THE

TOXICOLOGY AND CARCINOGENESIS

STUDIES OF C.I. DIRECT BLUE 15
(DESALTED INDUSTRIAL GRADE)
(CAS NO. 2429-74-5)

IN F344/N RATS

(DRINKING WATER STUDIES)

NATIONAL TOXICOLOGY PROGRAM
P.O. Box 12233
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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 23

RESULTS 31

DISCUSSION AND CONCLUSIONS 61

REFERENCES 71

APPENDIX A Summary of Lesions in Male Rats in the 22-Month Drinking Water Study of C.I. Direct Blue 15 77

APPENDIX B Summary of Lesions in Female Rats in the 22-Month Drinking Water Study of C.I. Direct Blue 15 139

APPENDIX C Genetic Toxicology 197

APPENDIX D Hematology, Clinical Chemistry, and Urinalysis Results 205

APPENDIX E Organ Weights and Organ-Weight-to-Body-Weight Ratios 211

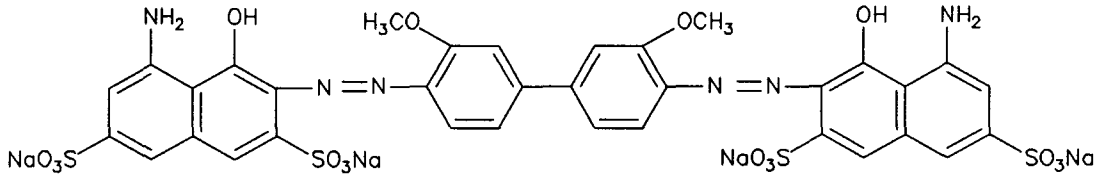
APPENDIX F Chemical Characterization and Dose Formulation Studies 221

APPENDIX G Water and Compound Consumption in the 22-Month Studies 237

APPENDIX H Ingredients, Nutrient Composition, and Contaminant Levels in NIH-07 Rat and Mouse Ration 241

APPENDIX I Sentinel Animal Program 247

ABSTRACT



C.I. DIRECT BLUE 15

CAS No. 2429-74-5

Chemical Formula: $C_{34}H_{24}N_6O_{16}S_4Na_4$ Molecular Weight: 992.8

Synonyms: Airedale Blue D, Aizen Direct Sky Blue 5BH, Amanil Sky Blue, Atlantic Sky Blue A, Atul Direct Sky Blue, Azine Sky Blue 5B, Belamine Sky Blue A, Benzanil Sky Blue, Benzo Sky Blue S, Benzo Sky Blue A-CF, Cartasol Blue 2GF, Chloramine Sky Blue A, Chloramine Sky Blue 4B, Chrome Leather Pure Blue, C.I. 24400, Cresotine Pure Blue, Diacotton Sky Blue 5B, Diamine Blue 6B, Diamine Sky Blue, Diaphtamine Pure Blue, Diazol Pure Blue 4B, 3,3′-[(3,3′-dimethoxy[1,1′-biphenyl]-4,4′-diyl)bis(azo)]bis[5-amino-4-hydroxy-2,7-naphthalenedisulfonic acid] tetrasodium salt, Diphenyl Brilliant Blue, Diphenyl Sky Blue 6B, Direct Blue 10G, Direct Blue HH, Direct Pure Blue, Direct Pure Blue M, Direct Sky Blue (6Cl), Direct Sky Blue A, Direct Sky Blue 5B, Enianil Pure Blue AN, Fenamin Sky Blue, Hispamin Sky Blue 3B, Kayafect Blue Y, Kayaku Direct Sky Blue 5B, Mitsui Direct Sky Blue 5B, Naphtamine Blue 10G, Niagara Blue 4B, Niagara Sky Blue, Nippon Direct Sky Blue, Nitto Direct Sky Blue 5B, Paper Blue S, Phenamine Sky Blue A, Pontamine Sky Blue 5BX, Shikiso Direct Sky Blue 5B, Sky Blue 4B, Sky Blue 5B, Tertrodirect Blue F, Vondacel Blue HH

C.I. Direct Blue 15 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. The dye, industrial grade C.I. Direct Blue 15, was chosen for study as a product to which workers are potentially exposed. Because of the high salt content, the dye was desalted prior to use. The purity was determined to be approximately 50%, with high-performance liquid chromatography indicating one major peak and approximately 35 impurities. Toxicology and carcinogenesis studies were conducted by administering the dye, C.I. Direct Blue 15, in drinking water to groups of F344/N rats of each sex for 14 days, 13 weeks, or 22 months. Planned as 24-month studies, the 22-month studies were terminated early because of rapidly declining animal survival, which was due primarily to neoplasia. 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* and Chinese hamster ovary cells.

14-Day Studies

Rats were given C.I. Direct Blue 15 in drinking water at doses of 1,250, 2,500, 5,000, 10,000, or 30,000 ppm. All control and treated rats survived. Body weight gain in high-dose females was less than that in controls. Water consumption declined as the dose increased. Male and female rats receiving 30,000 ppm had slight degeneration and necrosis of individual hepatocytes in the liver, and females also had mild to moderate renal tubule degeneration and thymic lymphoid depletion.

13-Week Studies
C.I. Direct Blue 15 was administered in drinking water at doses of 0, 1,250, 2,500, 5,000, 10,000, or 30,000 ppm to male rats, and at doses of 0, 630, 1,250, 2,500, 5,000, or 10,000 ppm to female rats. Seven of 10 male rats receiving 30,000 ppm died; all rats in the other groups survived until the end of the studies. Mean final body weights of males receiving 10,000 or 30,000 ppm were 92% and 69% of those of controls, and mean final body weights of females receiving 5,000 or 10,000 ppm were 97% and 94% of those of controls. Tissues from treated animals were stained blue. Compound-related lesions were seen in the kidney and liver of male rats given 30,000 ppm and in the kidney of males and females given 10,000 ppm. The renal lesions included necrosis, degeneration, pigmentation and regeneration of the tubule epithelium, and tubule mineralization. Liver lesions included centrilobular hepatocellular degeneration, fatty metamorphosis, and individual cell necrosis with slight periportal hepatocellular hypertrophy. Lymphoid depletion in the thymus was also seen in the high-dose males. Based on the results of the 14-day and 13-week studies, the high dose chosen for the 22-month studies was 2,500 ppm.

22-Month Studies
At study initiation, 70 rats of each sex were given 0 or 2,500 ppm C.I. Direct Blue 15, 45 rats of each sex were given 630 ppm, and 75 rats of each sex were given 1,250 ppm. Interim evaluations were made at 9 and 15 months. The average amounts of compound consumed per day by the six dose groups after week 52 of the studies were estimated to be 45, 90, and 215 mg/kg for male rats and 50, 100, and 200 mg/kg for female rats.

Survival and Body Weights
The studies were terminated at 22 months due to extensive mortality associated with chemical-related neoplasia. Survival of control, 630, 1,250, and 2,500 ppm males at 22 months was 37/50, 8/35, 11/65, and 2/50; survival of females was 40/50, 13/35, 22/65, and 4/50. At 22 months, the mean final body weights of the 630, 1,250, and 2,500 ppm groups were 95%, 91%, and 81% of those of the control for male rats and 91% of those of the control for all female dose groups.

Histopathologic Effects in the 22-Month Studies
At the 9-month interim evaluations, one adenoma of the Zymbal's gland was seen in a high-dose male rat, and three carcinomas of the clitoral gland were seen in the high-dose females. At the 15-month interim evaluations, Zymbal's gland neoplasms were seen in low- and high-dose males and all treated female dose groups. Mid- and high-dose males and females also had preputial or clitoral gland neoplasms, and a few neoplasms were present in the skin, small and large intestine, liver, and oral cavity of treated animals at 15 months.

At the end of the study, neoplasms related to chemical administration were found in the Zymbal's gland, skin, oral cavity, and the preputial or clitoral gland in both male and female rats. Neoplasms related to chemical administration were also seen at other sites including the small and large intestine, liver, uterus, and brain. The incidence of mononuclear cell leukemia was also increased in treated rats. The incidences of these neoplasms are summarized in the table at the end of this section.

Genetic Toxicology
C.I. Direct Blue 15 was not mutagenic in *Salmonella typhimurium* strains TA100, TA1535, TA1537, and TA98 when tested in a standard preincubation protocol with or without exogenous metabolic activation; however, when a specialized reductive metabolism protocol was used, C.I. Direct Blue demonstrated mutagenic activity in *Salmonella* strain TA1538. C.I. Direct Blue 15 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.

Conclusions
Under the conditions of these 22-month drinking water studies, there was *clear evidence of carcinogenic activity** of C.I. Direct Blue 15 (desalted industrial grade) in male F344/N rats, as indicated by benign and malignant neoplasms of the skin, Zymbal's gland, preputial gland, liver, oral cavity, and small and large intestine. Increased incidences of mononuclear cell leukemia and neoplasms of the brain may have been related to chemical administration.

There was *clear evidence of carcinogenic activity* of C.I. Direct Blue 15 in 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, and uterus, and by mononuclear cell leukemia.

*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 appear on page 11.

Summary of the Carcinogenesis and Genetic Toxicology Studies of C.I. Direct Blue 15

Male F344/N Rats	Female F344/N Rats
Drinking water concentration	
0, 630, 1,250, or 2,500 ppm C.I. Direct Blue 15	0, 630, 1,250, or 2,500 ppm C.I. Direct Blue 15
Body weights	
Dosed groups lower than controls during last 16 weeks of study	Dosed groups lower than controls during last 16 weeks of study
22-Month survival rates	
37/50, 8/35, 11/65, 2/50[a]	40/50, 13/35, 22/65, 4/50[a]
Nonneoplastic effects	
Preputial gland: ectasia: 5/49, 4/35, 15/64, 14/48	Clitoral gland: squamous hyperplasia: 0/50, 2/31, 4/64, 1/50
Liver: cystic degeneration: 1/50, 5/35, 10/61, 7/50	Liver: focal cellular alterations: 34/50, 18/35, 33/65, 23/50
Liver: focal cellular alterations: 27/50, 9/35, 19/61, 21/50	Liver: regeneration: 0/50, 0/35, 9/65, 7/50
Liver: regeneration: 1/50, 5/35, 4/61, 12/50	Zymbal's gland: ectasia: 1/49, 5/35, 13/64, 9/50
Zymbal's gland: ectasia: 2/50, 11/35, 8/64, 12/50	Zymbal's gland: hyperplasia: 0/49, 3/35, 4/64, 5/50
Zymbal's gland: squamous hyperplasia: 0/50, 1/35, 6/64, 5/50	
Neoplastic effects[b]	
Skin basal cell neoplasms: 2/50, 9/35, 27/65, 28/50	Skin squamous cell neoplasms: 0/50, 2/35, 6/65, 5/50
Skin sebaceous cell adenoma: 0/50, 1/35, 7/65, 3/50	Zymbal's gland neoplasms: 0/50, 4/35, 11/65, 17/50
Skin squamous cell neoplasms: 2/50, 4/35, 11/65, 19/50	Clitoral gland neoplasms: 7/50, 11/31, 24/64, 27/50
Zymbal's gland neoplasms: 1/50, 5/35, 10/65, 20/50	Hepatocellular neoplasms: 0/50, 0/35, 2/65, 5/50
Preputial gland neoplasms: 8/49, 5/35, 23/64, 9/48	Oral cavity neoplasms: 2/50, 4/35, 19/65, 15/50
Hepatocellular neoplasms: 0/50, 6/35, 9/65, 11/50	Small intestine adenocarcinoma: 0/50, 0/35, 1/65, 3/50
Oral cavity neoplasms: 1/50, 10/35, 24/65, 17/50	Large intestine adenomatous polyp: 0/50, 0/35, 3/65, 1/50
Small intestine neoplasms: 0/50, 1/35, 0/65, 2/50	Uterine epithelial neoplasms: 1/50, 0/35, 1/65, 4/50
Large intestine neoplasms: 0/50, 1/35, 6/65, 8/50	Mononuclear cell leukemia: 7/50, 13/35, 27/65, 15/50
Uncertain findings	
Mononuclear cell leukemia: 17/50, 19/35, 28/65, 20/50	
Brain neoplasms: 0/50, 1/35, 1/65, 2/50	
Level of evidence of carcinogenic activity	
Clear evidence	Clear evidence
Genetic toxicology	
Salmonella typhimurium gene mutation:	Negative with and without S9 in strains TA100, TA1535, TA1537, and TA 98
Salmonella typhimurium with reductive metabolism:	Positive in strain TA1538
Sister chromatid exchanges	
Chinese hamster ovary cells *in vitro*:	Negative with and without S9
Chromosomal aberrations	
Chinese hamster ovary cells *in vitro*:	Negative with and without S9

[a] Reduced survival in exposed groups was due to neoplasia.
[b] Number of animals 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 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 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 describes studies that are interpreted as showing a marginal increase of neoplasms that may be chemical related.
- **No evidence** of carcinogenic activity describes 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 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 C.I. Direct Blue 15 on November 19, 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.

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SUMMARY OF PEER REVIEW COMMENTS

On November 19, 1990, the draft Technical Report on the toxicology and carcinogenesis studies of C.I. Direct Blue 15 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. Direct Blue 15 by discussing the uses of this chemical, describing the experimental design, and reviewing the 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. Dunnick explained that the studies were intended to last 24 months but were terminated after 22 months because of decreased survival of exposed animals due primarily to neoplasia.

Dr. Klaassen, a principal reviewer, agreed with the conclusions, but he wondered why the incidence of neoplasms rarely reached 50%. He questioned whether there was really an increased incidence of brain neoplasms in dosed female rats. Dr. Klaassen said his major concern was that the chemical was only about 50% pure and suggested that this be indicated in the title and elsewhere.

Dr. McKnight, the second principal reviewer, did not agree with the conclusions. She said the studies should be considered inadequate unless the impurities in the mixture could be characterized and listed and the study labeled as a test of industrial grade C.I. Direct Blue 15. Further, she thought the studies to be relevant only if it could be documented that the impurities were typical of those to which workers were exposed. Dr. McKnight stated that if these issues could be resolved, the highly statistically significant increases in mononuclear cell leukemias in male rats supported these lesions being included under *clear evidence*. She also suggested that the increased incidence of adrenal gland pheochromocytomas might be considered as part of the evidence in males. Dr. Dunnick responded that pheochromocytomas are commonly occurring neoplasms and there was no increase in the incidence of hyperplasias, which suggested a lack of association with chemical exposure. Dr. S.L. Eustis, NIEHS, agreed that mononuclear cell leukemia could be considered part of the evidence in male rats.

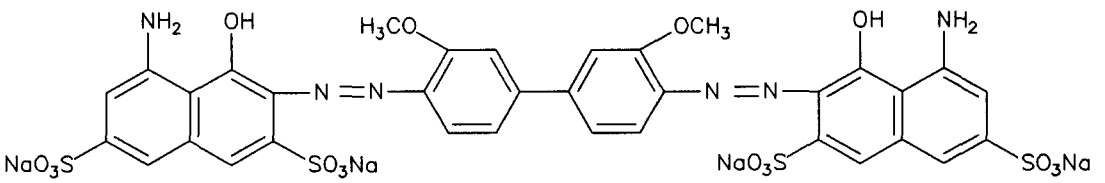
Dr. Zeise, the third principal reviewer, agreed with the conclusions. She shared the concern of the other reviewers about the composition of the chemical and the need to modify the title to reflect what was tested.

Responding to concerns of the reviewers about the purity of the chemical, Dr. Scala noted that the material studied was not industrial or technical grade but just C.I. Direct Blue 15. Dr. Dunnick said the sample was representative of what workers were exposed to, and more descriptive information would be added on the cover and inside. Additionally, information would be added on the two major impurities which are isomers of C.I. Direct Blue 15. Dr. McKnight pointed out that in the conclusions, the name of the chemical was followed by "desalted industrial grade" in parentheses. Dr. Dunnick said this would be added on the title page and cover. Dr. Silbergeld raised the question as to whether 3,3′-dimethoxybenzidine either formed metabolically or present as an impurity could be contributing to the neoplastic effects. Dr. Ashby agreed, noting that the dimethoxy compound would be formed *in vivo* by reductive cleavage, and pointing out that the only positive genetic toxicology finding was in *Salmonella* when reductive metabolism was incorporated.

Dr. Klaassen moved that the Technical Report on C.I. Direct Blue 15 be accepted with the revisions discussed, including wording about the purity and impurities present, and with the conclusions as written for male and female rats, *clear evidence of carcinogenic activity*. Dr. Goodman seconded the motion. Then, three amendments were offered and voted on. Dr. Klaassen moved that the last sentence of the conclusions for female rats be deleted, i.e., "Increased incidences of neoplasms of the brain may have been related to chemical administration." Dr. Goodman seconded the motion, which was accepted by nine yes to two no votes (Drs. Garman, Hayden) with one abstention (Dr. Ashby). Dr. McKnight moved that

mononuclear cell leukemias be added under *clear evidence* in male rats. Dr. Zeise seconded the motion, which was defeated by seven no votes (Drs. Ashby, Carlson, Gold, Goodman, Hayden, and Klaassen, with Chairman Scala breaking the tie) to six yes votes (Drs. Davis, Garman, Longnecker, McKnight, Silbergeld, Zeise). Dr. McKnight moved that adrenal pheochromocytomas be included in the conclusions for male rats as "may have been related to chemical administration." Dr. Silbergeld seconded the motion, which was defeated by nine no votes (Drs. Ashby, Carlson, Davis, Garman, Goodman, Gold, Hayden, Klaassen, Silbergeld) to three yes votes (Drs. Longnecker, McKnight, Zeise). The Panel then accepted the original motion unanimously with 12 votes.

INTRODUCTION



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Synonyms: Airedale Blue D, Aizen Direct Sky Blue 5BH, Amanil Sky Blue, Atlantic Sky Blue A, Atul Direct Sky Blue, Azine Sky Blue 5B, Belamine Sky Blue A, Benzanil Sky Blue, Benzo Sky Blue S, Benzo Sky Blue A-CF, Cartasol Blue 2GF, Chloramine Sky Blue A, Chloramine Sky Blue 4B, Chrome Leather Pure Blue, C.I. 24400, Cresotine Pure Blue, Diacotton Sky Blue 5B, Diamine Blue 6B, Diamine Sky Blue, Diaphtamine Pure Blue, Diazol Pure Blue 4B, Diphenyl Brilliant Blue, 3,3′-[(3,3′-dimethoxy[1,1′-biphenyl]-4,4′-diyl)bis(azo)]bis[5-amino-4-hydroxy-2,7-naphthalenedisulfonic acid] tetrasodium salt, Diphenyl Sky Blue 6B, Direct Blue 10G, Direct Blue HH, Direct Pure Blue, Direct Pure Blue M, Direct Sky Blue (6CI), Direct Sky Blue A, Direct Sky Blue 5B, Enianil Pure Blue AN, Fenamin Sky Blue, Hispamin Sky Blue 3B, Kayafect Blue Y, Kayaku Direct Sky Blue 5B, Mitsui Direct Sky Blue 5B, Naphtamine Blue 10G, Niagara Blue 4B, Niagara Sky Blue, Nippon Direct Sky Blue, Nitto Direct Sky Blue 5B, Paper Blue S, Phenamine Sky Blue A, Pontamine Sky Blue 5BX, Shikiso Direct Sky Blue 5B, Sky Blue 4B, Sky Blue 5B, Tertrodirect Blue F, Vondacel Blue HH

USE AND PRODUCTION

C.I. Direct Blue 15 is a dark blue powder with a melting point of greater than 300° C. A benzidine congener-based dye, it is produced by coupling two moles of 1-amino-8-naphthol-3,6-disulfonic acid with one mole of 3,3′-dimethoxybenzidine dihydrochloride (*Colour Index,* 1956).

Azo dyes based on benzidine and benzidine congeners (3,3′-dimethylbenzidine dihydrochloride and 3,3′-dimethoxybenzidine dihydrochloride) constitute a group of over 90 dyes, all widely used in the United States. C.I. Direct Blue 15 is used as a dye to color textiles, paper, plastic, rubber, and leather (Fishbein, 1981).

The United States Environmental Protection Agency (USEPA) reports that there are seven manufacturers and one importer of C.I. Direct Blue 15 (USEPA, 1988). Although production volumes for three of the manufacturers and for the sole importer are listed as confidential, the remaining manufacturers reported production volumes collectively ranging from 0.1 to 1.1 million pounds. The most recent production volume data show that 270,000 pounds of C.I. Direct Blue 15 were produced in 1982 (USITC, 1983); the United States International Trade Commission (USITC) did not report domestic production volumes of C.I. Direct Blue 15 for 1985 or 1986 (USITC, 1986, 1987). In 1980, 7,716 pounds of the dye were imported (USITC, 1981).

EXPOSURE

From a survey conducted from 1981-1983, the National Institute for Occupational Safety and Health (NIOSH) has estimated that a total of

4,527 workers may be exposed to C.I. Direct Blue 15 (NIOSH, 1989). Industrial exposure to dyes may occur through inhalation of dust or mist, through accidental ingestion, or from direct contact of the dye with skin. The general public may be exposed to C.I. Direct Blue 15 through the use of home dye products or through contaminated water supplies (USEPA, 1980; Fishbein, 1981; NIOSH, 1983).

METABOLISM AND DISTRIBUTION

Reductive metabolism of 3,3′-dimethoxybenzidine-based dyes produces 3,3′-dimethoxybenzidine (Figure 1). Azo reduction can occur either in the liver via 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 well (Walker, 1970). For this reason, reductive cleavage of the benzidine-congener azo dyes is thought to occur primarily through bacterial action in the gastrointestinal 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.

3,3′-Dimethoxybenzidine-based dyes, including C.I. Direct Blue 15, are metabolized to 3,3′-dimethoxybenzidine in dogs, rats, and humans (Lynn *et al.*, 1980; NIOSH, 1981). Azo dyes containing benzidine or one of its congeners can be reduced by mammalian liver azo reductases to form aromatic amines (Martin and Kennelly, 1981); rat intestinal microflora can also, through their azo reductases, metabolize the benzidine-based dyes to their aromatic amines (Cerniglia *et al.*, 1982). Urine recovered from dogs and rats given an oral dose of C.I. Direct Blue 15 contained primarily the N-acetyl derivatives of 3,3′-dimethoxybenzidine and small quantities of free 3,3′-dimethoxybenzidine. Genin (1977) also detected 3,3′-dimethoxybenzidine in the urine of rats exposed to dimethoxybenzidine-based dyes. In the same study, 3,3′-dimethoxybenzidine was detected in the urine of 3 of 22 workers who dried and ground two 3,3′-dimethoxybenzidine-based dyes. The metabolism of C.I. Direct Blue 15 to 3,3′-dimethoxybenzidine and subsequent metabolism of 3,3-dimethoxybenzidine (described by Rodgers *et al.*, 1983) is summarized in Figure 1.

When tissues from rats dosed orally with ^{14}C-C.I. Direct Blue 15 were analyzed for ^{14}C (Bowman *et al.*, 1982), peak tissue concentrations of ^{14}C were found in the brain, heart, lung, and small intestine at 4 hours after dosing, and in the urinary bladder, liver, kidney, lung, and carcass at 8 hours. The highest concentrations of ^{14}C were found in the liver, kidney, lung, and carcass.

Rodgers *et al.* (1983) reported that, after intravenous administration to male F344 rats, ^{14}C-3,3′-dimethoxybenzidine was rapidly and extensively metabolized, with less than 2% of the radiolabel recovered unchanged 30 minutes after dosing. Fifty percent of the radiolabel was located in the intestinal tract after 2 hours, and 70% was excreted in the bile within 72 hours. Three days after either oral or intravenous administration, 50% of the radiolabel had been excreted in the feces and 30% to 40% had been excreted in the urine; 45% of the radiolabel remaining in the animals was present in the liver in the form of covalently bound metabolites. Analysis of the pooled urine (days 0 to 3) demonstrated that more than 90% of the urinary radiolabel was in the form of metabolites, with unmetabolized 3,3′-dimethoxybenzidine dihydrochloride accounting for 3% to 9% of the urinary radiolabel and acetyldimethoxybenzidine accounting for 5% or less.

REPRODUCTIVE TOXICOLOGY

Wilson (1955) studied the teratogenic potential of several benzidine-based dyes in albino rats by injecting pregnant rats 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, followed by Evans blue, which caused abnormalities in 14%, Niagara blue 4B (C.I. Direct Blue 15), which caused abnormalities in 4%, and Niagara sky blue 6B, which caused abnormalities in 3%. 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). The abnormalities reported were generally similar to common spontaneous malformations such as anencephaly, hydrocephalus, and spina bifida. The purity and chemical characterization of the dyes used were not reported.

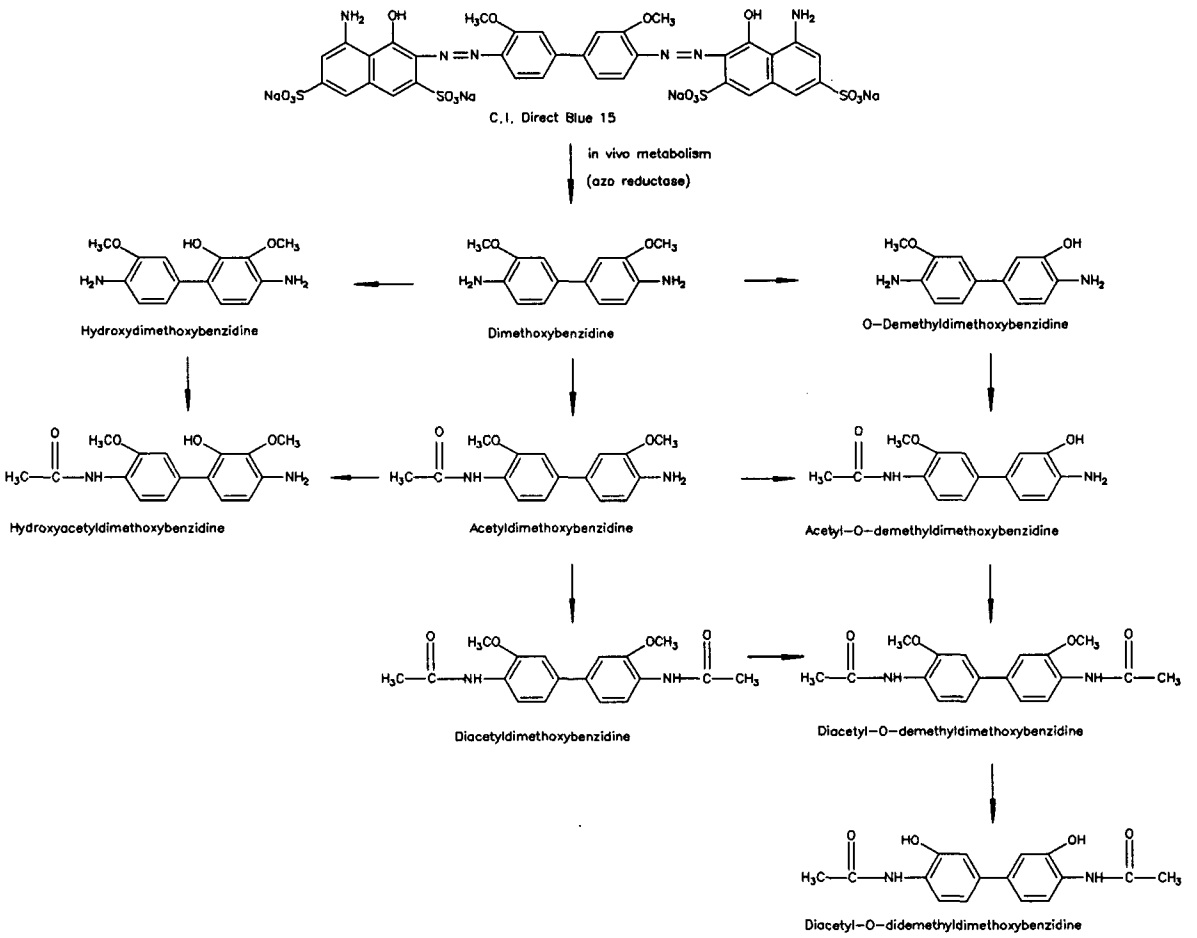


FIGURE 1
Proposed Metabolic Pathways of C.I. Direct Blue 15 and Dimethoxybenzidine (after Rodgers *et al.*, 1983)

Toxicity and Carcinogenicity Studies of Related Compounds

In 1980, NIOSH and the Occupational Safety and Health Administration (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. Direct Blue 15 or 3,3′-dimethoxybenzidine in the absence of other suspected carcinogens were found in the literature.

Benzidine

C.I. Direct Blue 15 is a benzidine congener-based dye. Benzidine is 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). Occupational exposure to benzidine for up to 30 years resulted in urinary bladder neoplasms 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 urinary bladder neoplasms in dogs (Spitz *et al.*, 1950); hepatocellular, harderian gland, and lymphoreticular neoplasms 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.*, 1966). Animal survival was poor in many of the benzidine carcinogenicity studies. Although this was due in most cases to the administration of toxic doses, these studies demonstrated the carcinogenicity of benzidine in laboratory animals.

3,3′-Dimethoxybenzidine

In early rodent studies, repeated exposure to 3,3′-dimethoxybenzidine, the metabolite of C.I. Direct Blue 15, was shown to result in neoplasms of the gastrointestinal tract, Zymbal's gland, skin, and mammary gland of rats and hamsters (Pliss, 1963, 1965; Saffiotti *et al.*, 1966; Hadidian *et al.*, 1968). Although these early studies provided evidence that 3,3′-dimethoxybenzidine is carcinogenic, the use of small numbers of animals, the use of 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 and administration at 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.* (1966) fed diets containing 1,000 ppm 3,3′-dimethoxybenzidine to 30 male and female Syrian golden hamsters. After 144 weeks of exposure, the only neoplasm present 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 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 neo-

plasms occurred as early as day 293, most were detected at 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 in the treated rats was significantly increased over that in the 360 pooled vehicle and untreated control rats.

In the NTP dosed-water studies of 3,3′-dimethoxybenzidine dihydrochloride in rats, neoplasms of the skin, Zymbal's gland, preputial and clitoral glands, oral cavity, intestine, and liver, as well as mesotheliomas and brain neoplasms in males and neoplasms of the mammary gland and uterus in females were present (Table 1) (NTP, 1990a).

BALB/c mice were given 3,3′-dimethoxybenzidine dihydrochloride in drinking water at doses up to 630 ppm for 112 weeks. Body weight gain in mice that received 630 ppm was less than that of controls, but there was no evidence of neoplasms related to chemical administration in either sex (Schieferstein *et al.*, 1990).

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 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, the IARC (1972) concluded that 3,3′-dimethylbenzidine was a systemic carcinogen for rats when given subcutaneously. In the dosed-water studies in rats 3,3′-dimethylbenzidine caused neoplasms of the skin, Zymbal's gland, preputial and clitoral glands, oral cavity, intestine, liver, brain, and lung in male and female rats, and of the mammary gland and hematopoietic system in female rats (Table 1) (NTP, 1990b).

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

o-Anisidine

o-Anisidine (2-methoxyaniline), structurally analogous to one-half the 3,3′-dimethoxybenzidine molecule, is used to manufacture monoazo dyes by diazotization and coupling with other aromatic amines (Noller, 1965). The National Cancer Institute (NCI) found that *o*-anisidine was carcinogenic to F344/N rats and B6C3F$_1$ mice (NCI, 1978). Groups of 55 animals of each sex received 0, 5,000 or 10,000 ppm *o*-anisidine in feed for rats and 0, 2,500 or 5,000 ppm for mice for 103 weeks. Treatment with *o*-anisidine resulted in urinary bladder transitional cell carcinomas or papillomas in both sexes of each species. Male rats also had transitional cell carcinomas of the renal pelvis and follicular cell neoplasms of the thyroid gland. Only one animal in any of the control groups had a urinary system neoplasm, a transitional cell papilloma of the renal pelvis in a male mouse.

o-Toluidine

o-Toluidine (2-aminotoluene) is structurally analogous to one-half the 3,3′-dimethylbenzidine molecule. In NCI (1979) studies, *o*-toluidine hydrochloride was given to groups of 50 F344/N rats and 50 B6C3F$_1$ mice of each sex in feed at concentrations of 3,000 or 6,000 ppm for rats and 1,000 or 3,000 ppm for mice for 101 to 104 weeks. 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 hydrochloride 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.

TABLE 1
Summary of Results of Previous 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 2-Year Drinking Water Studies of C.I. Acid Red 114[c]	
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	
Skin sebaceous cell neoplasms: 1/50, 1/35, 5/65, 6/50	
Skin squamous cell neoplasms: 1/50, 2/35, 11/65, 9/50	
Zymbal's gland neoplasms: 0/50, 0/35, 8/65, 7/50	Zymbal's gland neoplasms: 0/50, 3/35, 18/65, 19/50
	Clitoral gland neoplasms: 11/48, 17/32, 28/62, 23/50
Liver neoplasms: 2/50, 2/35, 15/65, 20/50	Liver neoplasms: 0/50, 0/35, 19/64, 8/50
	Lung neoplasms: 1/50, 2/35, 9/65, 4/50
	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

[a] Dose groups: 0, 80, 170, 330 ppm
[b] Dose groups: 0, 30, 70, 150 ppm
[c] Dose groups: males: 0, 70, 150, 300 ppm; females: 0, 150, 300, 600 ppm

GENETIC TOXICOLOGY

Although information regarding the genotoxicity of C.I. Direct Blue 15 is limited, the available data from the testing of metabolites of C.I. Direct Blue 15 and of structurally related dyes corroborate the mutagenic potential of C.I. Direct Blue 15 after azoreduction and release of active metabolites. C.I. Direct Blue 15 has arylamine groupings, which are considered to be "structural alerts" for genotoxic activity (Ashby and Tennant, 1988), and, as with most benzidine-congener dyes, its activity in *Salmonella typhimurium* is dependent upon the presence of conditions that allow reductive metabolism of the azo bonds to release the parent amine. In standard *S. typhimurium* assays, C.I. Direct Blue 15 was not mutagenic with or without S9 (Mortelmans *et al.*, 1986); however, mutagenic activity was observed when reductive metabolism preceded incubation with the *S. typhimurium* tester strains TA98, TA100, and TA1538 (Gregory *et al.*, 1981; Brown and Dietrich, 1983; Prival *et al.*, 1984; Reid *et al.*, 1984a).

In the absence of specialized protocols for reductive metabolism, C.I. Direct Blue 15 has been tested in mammalian cell systems for induction of gene mutations in mouse L5178Y lymphoma cells (Rudd, 1983), chromosomal aberrations and sister chromatid exchanges in Chinese hamster ovary cells (Galloway *et al.*, 1987), and unscheduled DNA synthesis in Fischer 344/N rat hepatocyte cell cultures. All tests were negative except the gene mutation assay with L5178Y cells, which was positive in the presence of S9.

Results of genotoxicity tests with metabolites of C.I. Direct Blue 15 were largely positive. A key metabolite, 3,3′-dimethoxybenzidine, was positive in a variety of *in vitro* genotoxicity assays (NTP, 1990a). In NTP assays, 3,3′-dimethyoxybenzidine dihydrochloride induced gene mutations in *S. typhimurium* (Haworth *et al.*, 1983; Reid *et al.*, 1984a) and sister chromatid exchanges and chromosome aberrations in Chinese hamster ovary cells (Galloway *et al.*, 1985). It was negative for induction of sex-linked recessive lethal mutations in germ cells of male *Drosophila melanogaster* (Yoon *et al.*, 1985). 3,3′-Dimethoxybenzidine is metabolized by various oxidative pathways to a variety of genotoxic compounds. For example, acetylation pathways produce N-acetyl-3,3′-dimethoxybenzidine, which is a more potent *S. typhimurium* mutagen than the parent compound or the diacetyl derivative (Rodgers *et al.*, 1983; Reid *et al.*, 1984a). Benzidine, the parent compound in this series of substituted biphenyls, was positive for induction of gene mutations in *S. typhimurium* with S9 (Haworth *et al.*, 1983; Reid *et al.*, 1984b), positive for induction of sister chromatid exchanges and chromosome aberrations in Chinese hamster ovary cells (Galloway *et al.*, 1987), and positive for induction of micronuclei, sister chromatid exchanges, and chromosome aberrations in bone marrow cells of mice exposed by intraperitoneal injection (NTP, unpublished data).

STUDY RATIONALE

Benzidine is a known human carcinogen (IARC, 1972, 1987), and the benzidine congeners, 3,3′-dimethylbenzidine dihydrochloride and 3,3′-dimethoxybenzidine dihydrochloride, are known animal carcinogens (NTP, 1990a,b). Since numerous benzidine and benzidine congener-based dyes are metabolized to these parent amines *in vivo* (Rinde and Troll, 1975; NCI, 1978; Lynn *et al.*, 1980; Nony *et al.*, 1980; Bowman *et al.*, 1982), all benzidine- and benzidine congener-derived dyes may be considered possible carcinogens. The dye C.I. Direct Blue 15 (desalted industrial grade) was selected for study as a representative of the dyes derived from 3,3'-dimethoxybenzidine, and the industrial product was used to examine the product to which humans are generally exposed.

NTP's Benzidine Dye Initiative is a collaborative effort of NIEHS, National Center for Toxicologic Research (NCTR), NIOSH, USEPA, the CPSC, and OSHA under the aegis of the NTP. The objective of the Initiative was to develop an integrated body of scientific data concerning the dyes derived from benzidine, 3,3′-dimethylbenzidine, and 3,3′-dimethoxybenzidine (Table 2). 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.

Five benzidine dyes were selected for toxicity and carcinogenicity studies: 3,3′-dimethoxybenzidine dihydrochloride and 3,3′-dimethylbenzidine dihydrochloride, which are benzidine congeners; C.I. Direct Blue 15 and C.I. Direct Blue 218, which are representative 3,3′-dimethoxybenzidine-based dyes; and

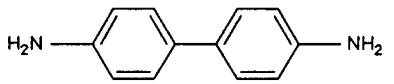
C.I. Acid Red 114, which is a representative 3,3′-dimethylbenzidine-based dye (Figure 2).

The oral route of administration was selected for these studies to mimic potential human exposure in the workplace and in the home. The NTP 2-year rat studies of 3,3′-dimethylbenzidine, 3,3′-dimethoxybenzidine, and C.I. Acid Red 114 have been reported (NTP, 1990a,b; 1992). Long-term mouse studies of 3,3′-dimethylbenzidine, 3,3′-dimethoxybenzidine, and other benzidine-based dyes have been conducted at NCTR. Auxiliary studies involved transplatation of neoplasms (Maronpot *et al.*, 1988 and Ulland *et al.*, 1989) and oncogene activation (Reynolds *et al.*, 1990).

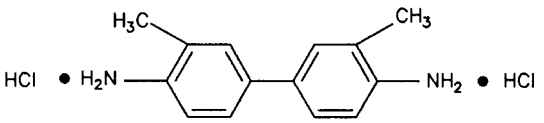
TABLE 2
Summary of the National Toxicology Program Benzidine Congener Initiative

Class/Chemical	Tests[a]
3,3′-Dimethylbenzidine (*o*-toluidine)	
o-Toluidine	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

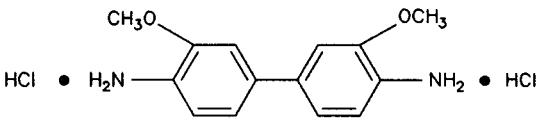
[a] G=genetic toxicology; P=pharmacokinetic studies; M=metabolism studies for detection of carcinogens in urine; B=toxicology and carcinogenicity studies.



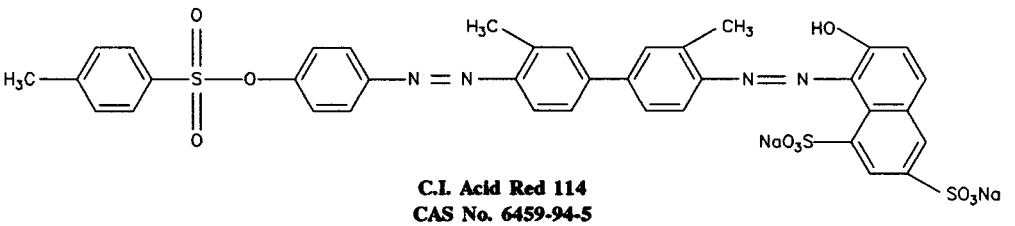
Benzidine
CAS No.92-87-5



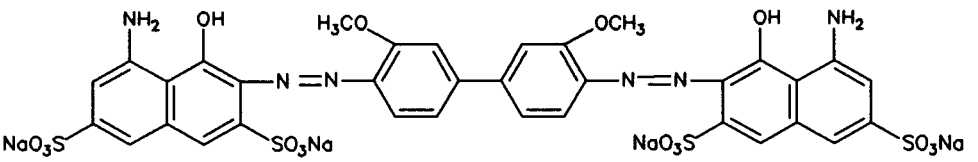
3,3'-Dimethylbenzidine Dihydrochloride
CAS No. 612-82-8



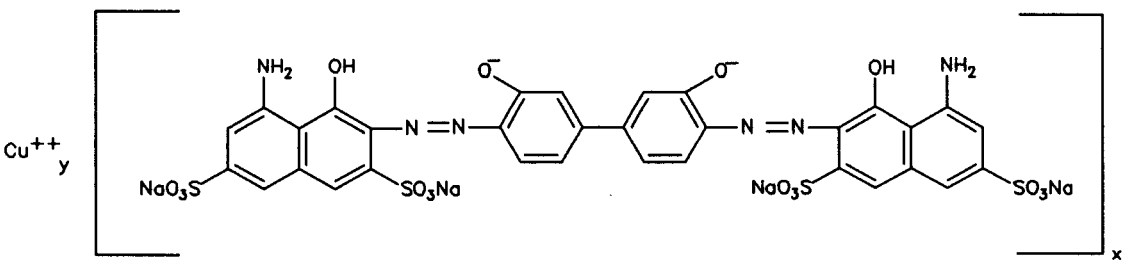
3,3'-Dimethoxybenzidine Dihydrochloride
CAS No. 20325-40-0



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 2
Chemical Structures of Benzidine and Selected Benzidine Congeners and Dyes

MATERIALS AND METHODS

PROCUREMENT AND CHARACTERIZATION OF C.I. DIRECT BLUE 15

The dye, C.I. Direct Blue 15, was obtained from the Atlantic Chemical Company (Nutley, NJ) and supplied to the National Toxicology Program by Dyes Environmental and Toxicology Organization, Inc. (Scarsdale, NY) in two lots (NJ-0-62-611 and A03383-2). Because of the high salt content, the material was desalted by the analytical chemistry laboratory (Midwest Research Institute, Kansas City, MO). Lot NJ-0-62-611 was desalted in two batches, and the desalted material was assigned lot numbers M110481 and M042783. Lot A03383-2 was desalted and assigned lot number M080883. The resultant salt content was about 3%, reduced from approximately 25%. Lot number M110481 was used in the 14-day, 13-week, and 22-month studies, and lot numbers M042783 and M080883 were used in the 22-month studies. Purity, stability, and identity analyses were conducted on all lots at the analytical chemistry laboratory (Appendix F).

The study dye, a dark blue powder, was identified as C.I. Direct Blue 15 by infrared, ultraviolet/visible, and nuclear magnetic resonance spectroscopy. The purity of all desalted lots was determined to be approximately 50% by combining the data from the following analyses: elemental analysis, Karl Fischer water analysis, azo group titrations, thin-layer chromatography, and high-performance liquid chromatography (HPLC). Comparison of the three lots by HPLC showed no significant purity differences. HPLC analysis detected approximately 35 impurities accounting for about 50% of the chromatographic peak area. The two largest imprints as shown by chromatographic analysis were identified by NMR spectrometry as related positional isomers to the major component. Each of the two largest imprints accounted for approximately 10% of the total sample by weight. Two of the lots (M042783 and M080883) were assayed for benzidine and 3,3′-dimethoxybenzidine dihydrochloride content. Benzidine was not detected in either batch at levels greater than 1 ppm, whereas 3,3′-dimethoxybenzidine dihydrochloride was found in lot M042783 at 836 ppm and in lot M080883 at 1,310 ppm. Stability studies performed with HPLC showed that C.I. Direct Blue 15 was stable as a bulk chemical for at least two weeks at temperatures up to 60° C when stored protected from light. Based on the stability study results, the bulk chemical was stored at room temperature in the dark at the study laboratory throughout the study period. The stability of the bulk chemical was monitored by the study laboratory using infrared spectroscopy, 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 appropriate amounts of C.I. Direct Blue 15 with tap water for the 14-day and 13-week studies, and with distilled water for the 22-month studies. Stability tests conducted by the analytical chemistry laboratory showed that solutions of 500 ppm C.I. Direct Blue 15 in water remained stable for at least 21 days when stored at room temperature. Solutions were stable for at least 3 days under simulated dosing conditions, including exposure to normal room light and air.

Dose formulations were prepared twice weekly and made available to the study animals on the day of mixing. The preparation and storage procedures for dosed drinking water in the studies of C.I. Direct Blue 15 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 22-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 92% (104/113) of the formulations were prepared within specifications (Table F3). Results of periodic referee analyses by the analytical chemistry laboratory agreed with those of the study laboratory (Table F4).

14-Day Studies

Male and female F344/N rats were obtained from Frederick Cancer Research Center (Frederick, MD) and observed for 14 days before the studies began. The rats were 50 days old when placed on study. Groups of five rats of each sex received 0, 1,250, 2,500, 5,000, 10,000, or 30,000 ppm C.I. Direct Blue 15 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. 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. All animals were necropsied, and the following organs were weighed: brain, heart, right kidney, liver, lung, right testis, and thymus. Complete histopathologic examinations were performed on all control animals and on males and females receiving 30,000 ppm. Selected tissues were examined from animals in the other dose groups. Further experimental details are presented in Table 3.

13-Week Studies

The 13-week studies were designed to evaluate the cumulative toxic effects of repeated exposure to C.I. Direct Blue 15 and to determine the chemical concentrations to be used in the 22-month studies.

Fischer 344/N rats were obtained from Frederick Cancer Research Center, observed for 21 days, distributed to weight classes, and assigned to dose groups. The rats were 56 days old when placed on study. Groups of ten rats of each sex received 0, 630 (females only), 500, 1,250, 2,500, 5,000, 10,000, or 30,000 (males only) ppm C.I. Direct Blue 15 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 the end of the studies. Erythrocyte counts, leukocyte counts, differential leukocyte counts, hemoglobin concentrations, and hematocrit values were determined from samples drawn from the retro-orbital sinus. Clinical chemistry values for blood urea nitrogen, serum creatinine, lactic dehydrogenase, sorbitol dehydrogenase, and alanine aminotransferase were determined from blood samples collected from the abdominal aorta. Further details are presented in Table 3.

Survivors were killed at the end of the 13-week studies. All study animals were necropsied. The brain, heart, liver, lung, right kidney, right testis, and thymus were weighed at necropsy. Complete histopathologic examinations were performed on all animals in the control groups, all animals in the highest dose groups with a survival rate of 100% (10,000 ppm males and females), and all animals that died or were killed moribund (seven males from the 30,000 ppm group). Target organs were submitted for histopathology for the remaining animals. Tissues examined for each group are listed in Table 3.

22-Month Studies

Study Design

Rats received 0, 630, 1,250, or 2,500 ppm C.I. Direct Blue 15 in distilled drinking water for 96 weeks. There were 70 rats per control group, 45 rats per low-dose group, 75 rats per mid-dose group, and 70 rats per high-dose group. The 22-month studies were originally designed as 24-month studies with an animal allocation proposed by Portier and Hoel (1984). At 9 months, ten rats from the control and 2,500 ppm dose groups were killed, and at 15 months ten rats from each dose group were killed. Because of the high mortality in the dosed groups due to chemical-related neoplasia, the study was terminated at 22 months.

Source and Specification of Animals

Male and female F344/N rats were obtained from Simonsen Laboratories, Inc. (Gilroy, CA) for use in the 2-year studies. The animals were 4 weeks old at receipt. Following a 12- to 19-day quarantine, ten animals of each sex were randomly selected and killed for parasite evaluation and gross observation of disease. Blood samples were collected for viral screens. Study animals were 40 to 47 days 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 five per cage. Feed and water were available *ad libitum*. Feed composition is presented in Appendix H. Further details of animal maintenance are given in Table 3. Information on cage rotation is not available.

Clinical Observations and Pathology

All animals were observed twice daily. Animals were weighed at study initiation, weekly for 16 weeks, and monthly thereafter. Clinical findings were recorded at the time of weighing. Feed consumption was measured weekly, and water consumption was measured twice weekly.

Blood and urine samples were collected from all interim evaluation animals. 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 blood urea nitrogen, creatinine, glucose, alanine aminotransferase, lactic dehydrogenase, sorbitol dehydrogenase, triiodothyronine (T_3), thyroxine (T_4), thyroid stimulating hormone (TSH), and serum osmolality were determined from blood samples collected from the abdominal aorta. T_3 and T_4 levels were analyzed with the Tri-Tab and Tetra-Tab Radioimmunoassay Diagnostic Kits (Nuclear Medical Laboratories). TSH values were determined by the method of Ridgway *et al.* (1973). 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 weights were measured at necropsy. Further details are presented in Table 3.

Animals found moribund, designated for the 9- or 15-month interim evaluations, or surviving to the end of the 22-month studies were killed. All animals were necropsied. 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 dose groups examined are listed in Table 3.

When the pathology evaluation was completed by the study laboratory pathologist, and the pathology data were 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 was evaluated.

A quality assessment pathologist reviewed selected tissues microscopically for accuracy and consistency of lesion diagnosis. All neoplasms and nonneoplastic lesions were reviewed in the following tissues from all male and female rats: liver, lung (males only), small intestine, large intestine, Zymbal's gland, preputial gland, clitoral gland, and uterus. Spleens and livers from all males and females were reviewed for 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 in 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 liver, lung, small and large intestine, spleen, Zymbal's gland, preputial or clitoral gland, skin, pharynx, tongue, and uterus and examples of disagreements in diagnosis between the laboratory and quality assessment pathologist were shown to the PWG. The PWG, which included the quality assessment pathologist and others experienced in rodent toxicologic pathology, 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 Cox's method (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 that were necropsied.

Analysis of Neoplasm Incidence

In the 22-month studies, the deaths of dosed rats and rats killed moribund were considered to be due primarily to neoplasms of the Zymbal's gland, preputial gland, clitoral gland, and skin, and possibly to mononuclear cell leukemia. Consequently, for these lesions, primary emphasis in the analysis of neoplasm incidence was given to the life table test (Cox, 1972; Tarone, 1975), a survival-adjusted procedure appropriate for rapidly lethal neoplasms.

For incidental neoplasms (neoplasms discovered as a result of death from an unrelated cause), the primary statistical method used in these studies was logistic regression, which assumed that the diagnosed neoplasms were discovered as the result of death from an unrelated cause and thus did not affect the risk of death. In this approach, neoplasm 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 neoplasms are incidental, this comparison of the time-specific neoplasm prevalences also provides a comparison of the time-specific neoplasm incidences (McKnight and Crowley, 1984).

In addition to logistic regression, alternative methods of statistical analysis were used, and the results of these tests are summarized in the appendixes. These methods include the Fisher exact test and the Cochran-Armitage trend test (Armitage, 1971; Gart *et al.*, 1979), procedures based on the effective number of animals (i.e., the number of animals surviving until the appearance of the first neoplasm).

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 neoplasm incidence. 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 neoplasm incidence. Although the current studies were terminated at 22 months, neoplasm incidences from the NTP historical control database for 2-year studies (Haseman *et al.*, 1984, 1985) are included for neoplasms appearing to show compound-related effects.

Analysis of Continuous Variables

Clinical chemistry, urinalysis and hematology data, and organ and body weights 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 (Shirley's test) was more appropriate for pairwise comparisons than a test that does not assume a monotonic dose-response trend (Dunn's test). For the 9-month interim evaluations (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, clinical chemistry, and urinalysis data.

Quality Assurance Methods

The 13-week and 22-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 review 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 discrepancies had been resolved or were otherwise addressed during the preparation of this Technical Report.

TABLE 3
Experimental Design and Materials and Methods in the Drinking Water Studies of C.I. Direct Blue 15

14-Day Studies	13-Week Studies	22-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 Center (Frederick, MD)	Frederick Cancer Research Center (Frederick, MD)	Simonsen Laboratories, Inc. (Gilroy, CA)
Time Held Before Study		
14 days	21 days	12-19 days
Average Age When Placed on Study		
50 days	56 days	40-47 days
Date of First Dose		
11 March 1982	1 June 1982	28 February 1983
Duration of Dosing		
14 consecutive days	13 weeks (7 days/week)	96 weeks (7 days/week)
Date of Last Dose		
25 March 1982	31 August 1982	30 December 1984
Average Age at Necropsy		
9 weeks	21 weeks	103-104 weeks 46/47 weeks (9-month interim) 72/73 weeks (15-month interim)
Necropsy Dates		
25 March 1982	1 and 3 September 1982	7-10 January 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 14-day studies	Same as 14-day studies
Animals per Cage		
5	5	5
Method of Animal Identification		
Ear tag	Ear punch	Ear tag

TABLE 3
Experimental Design and Materials and Methods in the Drinking Water Studies of C.I. Direct Blue 15 (continued)

14-Day Studies	13-Week Studies	22-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*	Same as 14-day studies	Distilled water (Polar Water Co., Beltsville, MD) in glass water bottles with stainless steel sippers (Hazleton Systems, Inc., Aberdeen, MD), available *ad libitum*
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 nonwoven polyester fiber filters (DuPont Company, Applied Technologies Division, Wilmington, DE)	Same as 14-day studies	Same as 14-day studies
Animal Room Environment		
Temperature: 71°-74° F Relative humidity: 19%-69% Fluorescent light: 12 hours/day	Temperature: 70°-75° F Relative humidity: 33%-79% Fluorescent light: 12 hours/day Room air changes: 10-12/hour	Temperature: 67°-82° F Relative humidity: 22%-87% Fluorescent light: 12 hours/day Room air changes: 12.1/hour
Doses		
0, 1,250, 2,500, 5,000, 10,000 or 30,000 ppm C.I. Direct Blue 15 in drinking water	0, 630 (females only), 1,250, 2,500, 5,000, 10,000, or 30,000 ppm (males only) C.I. Direct Blue 15 in drinking water	0, 630, 1,250, or 2,500 ppm C.I. Direct Blue 15 in distilled drinking water
Type and Frequency of Observation		
Observed twice daily; body weight initially and weekly; feed consumption weekly; water consumption twice weekly; clinical observation weekly	Observed twice daily; body weight initially and weekly; feed consumption weekly; water consumption twice weekly; clinical observation weekly	Observed twice daily; body weights initially, weekly for 16 weeks, and monthly thereafter; feed consumption measured 1 week every 4 weeks; water consumption measured in a 3- or 4-day segment every 4 weeks; clinical observations at body weight determinations

TABLE 3
Experimental Design and Materials and Methods in the Drinking Water Studies of C.I. Direct Blue 15
(continued)

14-Day Studies	13-Week Studies	22-Month Studies
Necropsy All animals necropsied. Organ weights obtained at necropsy (brain, heart, liver, lung, right kidney, right testis, and thymus).	**Necropsy** All animals necropsied. Organ weights measured were the same as in the 14-day studies.	**Necropsy** All animals necropsied. Organ weights measured at 9-month and 15-month interim sacrifices (brain, kidney, liver).
Histopathology Complete histopathology on male and female control and high-dose (30,000 ppm) animals, including the following organs: adrenal gland, blood smear, bone (sternebrae, femur, or vertebrae, including marrow), 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 turbinates, ovaries, pancreas, parathyroid gland, pharynx (if grossly abnormal), pituitary gland, preputial gland, prostate gland, salivary gland, small intestines (duodenum, ileum, jejunum), spinal cord (if neurological signs present), spleen, stomach, testes, thymus, thyroid gland, trachea, urinary bladder, uterus, Zymbal's gland, and gross lesions. The following organs were examined from 10,000 ppm males: liver; from 10,000 ppm females: kidneys, liver, and thymus; and from 5,000 and 2,500 ppm females: kidneys.	**Histopathology** Complete histopathology on male and female controls, all males and females receiving 10,000 ppm, and all deaths and moribund kills (7 males from the 30,000 ppm group). Tissues examined were the same as in the 14-day studies complete screen. The following organs were examined from 5,000 ppm males: kidney and thymus; from 5,000 ppm females: kidney; and from 1,250 and 2,500 ppm males: kidney.	**Histopathology** Complete histopathology on all animals that died, were killed moribund, were killed at 9 months or 15 months (control and high-dose animals only), or were killed at study termination. Tissues examined were the same as in the 14-day studies complete screen, with the addition of seminal vesicles. Organs for low-dose and mid-dose animals killed at 15 months included the liver, preputial and clitoral glands, and Zymbal's gland.
Clinical Pathology None required	**Clinical Pathology** Clinical pathology studies conducted at the end of the studies. ***Hematology:*** hematocrit, hemoglobin, erythrocytes, leukocyte count and differential ***Clinical chemistry:*** blood urea nitrogen, creatinine, lactate dehydrogenase, sorbitol dehydrogenase, alanine aminotransferase	**Clinical Pathology** Clinical pathology studies 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:*** blood urea nitrogen, creatinine, glucose, serum osmolality, triiodothyronine, thyroxine, thyroid stimulating hormone, lactate dehydrogenase, sorbitol dehydrogenase, alanine aminotransferase ***Urinalyses:*** Protein, glucose, creatinine, pH, specific gravity, urine osmolality, volume, creatinine excretion rate (16 hr), serum/urine osmolality ratio, microscopic exam of sediment

RESULTS

14-DAY STUDIES

All rats survived to the end of the studies (Table 4). The final mean body weight of females receiving 30,000 ppm was 34% lower than that of controls; final mean body weights of other dosed groups were within 11% of the respective control groups. Water consumption declined with increasing dose, and animals in the 30,000 ppm groups consumed less than half that consumed by control animals. The eyes, skin, and feces of all treated rats were stained blue, and high-dose females were thin or emaciated.

There were no notable necropsy findings, although organs and tissues were stained blue in all high-dose animals and in decreasing numbers of lower dose animals. Males receiving 10,000 or 30,000 ppm had increased absolute and relative kidney weights. Females receiving 30,000 ppm showed decreased absolute and relative thymus weights (Tables E1 and E2). Treatment-related histologic changes were seen in the liver and kidney of high-dose male and female rats and in the thymus of high-dose females. Liver lesions included necrosis of individual hepatocytes in males and females and mild degeneration of centrilobular hepatocytes in females. Blue granular pigment was present in renal tubule epithelial cells in both sexes. In addition, mild to moderate renal tubule degeneration was seen in females, affecting multiple scattered tubules and characterized by severe swelling of epithelial cells often accompanied by nuclear pyknosis. Moderate depletion of thymic lymphocytes also occurred in high-dose females.

TABLE 4
Survival, Mean Body Weights, and Water Consumption of Rats in the 14-Day Drinking Water Studies of C.I. Direct Blue 15

Concentration (ppm)	Survival[a]	Mean Body Weights[b] (g)			Final Weight Relative to Controls (%)	Water Consumption[c]	
		Initial	Final	Change		Week 1	Week 2
Male							
0	5/5	162 ± 4.9	207 ± 5.4	+45 ± 2.8		42	39
1,250	5/5	164 ± 4.5	229 ± 3.6	+66 ± 1.8	111	41	33
2,500	5/5	165 ± 4.6	205 ± 4.8	+41 ± 1.9	99	40	42
5,000	5/5	161 ± 4.1	224 ± 3.2	+62 ± 1.7	108	35	34
10,000	5/5	157 ± 3.6	220 ± 5.9	+63 ± 3.5	106	38	38
30,000	5/5	157 ± 4.1	191 ± 5.2	+34 ± 1.9	92	20	20
Female							
0	5/5	128 ± 1.1	149 ± 1.8	+21 ± 2.7		40	36
1,250	5/5	130 ± 1.5	161 ± 3.3	+31 ± 2.2	108	32	29
2,500	5/5	130 ± 1.7	159 ± 2.5	+29 ± 1.1	107	32	30
5,000	5/5	129 ± 2.1	156 ± 2.7	+27 ± 0.9	105	24	28
10,000	5/5	129 ± 2.3	157 ± 3.5	+28 ± 1.7	105	24	29
30,000	5/5	126 ± 2.9	99 ± 7.0	-27 ± 7.7**	66	12	13

** Significantly different (P≤0.01) from the control group by Dunn's test
[a] Number surviving/number initially in group
[b] Weights and weight changes given as mean ± standard error
[c] Milliliters per animal per day, based on average consumption data per group per week for weeks 1 and 2

13-Week Studies

Seven males in the 30,000 ppm group died before the end of the study (Table 5); there were no other deaths. The final mean body weight of surviving males receiving 30,000 ppm was 31% lower than controls. The final mean body weights in the other dosed male and female groups were 92% to 98% of those of the corresponding controls. Clinical findings included hunched posture, rough hair coat, depression, and thin appearance in high-dose males. The skin of dosed animals was stained blue.

Mean absolute and relative kidney weights were significantly increased in both sexes receiving 5,000 and 10,000 ppm, and in surviving males receiving 30,000 ppm (Tables E3 and E4). Significant differences in relative mean organ weights occurred in various dose groups and were considered to be due to the decreased body weights.

Female rats showed statistically significant increases in mean erythrocyte count, hematocrit, absolute lymphocyte count, and blood urea nitrogen (Table D1). These findings were consistent with mild hemoconcentration.

All tissues of males receiving 30,000 ppm were stained blue, and blue staining of the mesenteric lymph nodes and intestinal contents was noted in males and females in all but the lowest dose groups. Treatment-related lesions were seen in the kidneys and livers of males that received 30,000 ppm and in the kidneys of males and females that received 10,000 ppm (Table 6). Renal tubule regeneration occurred in nearly all males, including controls, but occurred with increased severity in males that received 10,000 or 30,000 ppm. Minimal tubule regeneration occurred in treated females, but not in controls, and generally resembled the change seen as a part of the chronic nephropathy that occurs commonly in F344/N rats. Regeneration consisted of tubules lined by a few small cuboidal cells or by increased numbers of cuboidal cells with basophilic cytoplasm and hyperchromatic nuclei; some regenerative tubules were shrunken and surrounded by thickened basement membranes. Lesions of minimal severity involved only scattered tubules, usually affecting less than 10% of all tubules. Mild lesions involved up to 25% and moderate lesions up to 50% of the tubules. Necrosis of individual tubule epithelial cells (tubule necrosis), characterized by nuclear pyknosis, cytoplasmic eosinophilia, and sloughing of necrotic cells into the tubule lumen, was seen in scattered tubules in several 30,000 ppm males. A few males given 30,000 ppm also had bits of mineral within some tubule lumens (mineralized tubules). Blue staining of tubule epithelial cells (tubule pigmentation), presumably due to the presence of the dye, occurred in a few males in the 10,000 and 30,000 ppm dose groups. Tubule degeneration was seen in several females that received 10,000 ppm and was characterized by scattered tubules containing enlarged epithelial cells with abundant finely vacuolated cytoplasm that often filled most of the tubule lumen.

A variety of treatment-related liver changes was observed in six of the high-dose males. These changes included minimal enlargement of periportal hepatocytes (megalocytosis), and degeneration, fatty metamorphosis, or necrosis of centrilobular hepatocytes. Degeneration of centrilobular hepatocytes consisted of individual cells that were smaller with more eosinophilic cytoplasm than normal cells but with normal appearing nuclei. In some animals the degeneration of hepatocytes appeared to proceed to individual cell necrosis. Fatty metamorphosis was characterized by multiple variably sized clear vacuoles (lipid) within the cytoplasm. Blue pigment was seen within Kupffer cells lining hepatic sinusoids adjacent to centrilobular areas. Most of the males in the 30,000 ppm dose group also demonstrated loss of small to moderate numbers of lymphocytes from the thymus gland (lymphoid depletion).

Dose Selection Rationale

Because of the presence of dose-related kidney and liver lesions and increased relative kidney weights, drinking water concentrations of 0, 630, 1,250, and 2,500 ppm C.I. Direct Blue 15 were selected for rats in the 22-month studies.

22-Month Studies

9-Month Interim Evaluation

At 2,500 ppm, males showed a statistically significant increase in relative liver weight and females an increase in absolute kidney weight (Tables E5 and E6). Various hematology and chemistry parameters were significantly different from the

TABLE 5
Survival, Mean Body Weights, and Water Consumption of Rats in the 13-Week Drinking Water Studies of C.I. Direct Blue 15

Concentration (ppm)	Survival[a]	Mean Body Weights[b] (g)			Final Weight Relative to Controls (%)	Water Consumption[c]	
		Initial	Final	Change		Week 1	Week 12
Male							
0	10/10	163 ± 3.2	353 ± 4.5	+190 ± 2.6		23	19
1,250	10/10	164 ± 3.1	329 ± 5.0**	+165 ± 3.2**	93	21	28
2,500	10/10	163 ± 3.5	339 ± 7.5*	+176 ± 5.5**	96	22	23
5,000	10/10	159 ± 3.4	335 ± 3.8*	+176 ± 1.8**	95	18	30
10,000	10/10	169 ± 2.5	325 ± 3.6**	+156 ± 3.5**	92	16	28
30,000	3/10[d]	164 ± 2.9	244 ± 32.1**	+75 ± 30.3**	69	6	16
Female							
0	10/10	130 ± 1.7	205 ± 3.2	+74 ± 2.0		34[e]	21
630	10/10	128 ± 2.7	196 ± 2.5	+68 ± 2.2	96	23	29
1,250	10/10	131 ± 1.3	201 ± 2.0	+70 ± 1.1	98	25	25
2,500	10/10	131 ± 1.7	201 ± 1.9	+70 ± 1.7	98	18	24
5,000	10/10	129 ± 1.7	198 ± 2.3	+69 ± 1.6	97	17	24
10,000	10/10	131 ± 2.4	193 ± 2.2**	+62 ± 1.2**	94	15	19

* Significantly different ($P \leq 0.05$) from the control group by Dunn's test
** $P \leq 0.01$
[a] Number surviving/number initially in group
[b] Body weights and body weight changes given as mean ± standard error. Subsequent calculations are based on animals surviving to the end of the study. Differences from the control group are not significant by Dunn's or Shirley's test.
[c] Milliliters per animal per day, based on average consumption data per group per week for weeks 1 and 12
[d] Week of death: 3, 3, 4, 5, 10, 11, 13
[e] Empty water bottle one weighing

TABLE 6
Incidences of Selected Treatment-Related Lesions in Rats in the 13-Week Drinking Water Studies of C.I. Direct Blue 15

Male	0 ppm	10,000 ppm	30,000 ppm
Kidney[a]			
Tubule regeneration	10/10 (1.0)[b]	10/10 (1.4)	6/7 (2.7)
Tubule necrosis	0/10	0/10	5/7**
Tubule pigment	0/10	4/10*	4/7*
Mineralized tubules	0/10	0/10	3/7
Liver			
Megalocytosis	0/10	0/10	4/7*
Fatty metamorphosis	0/10	0/10	5/7**
Hepatocyte degeneration	0/10	0/10	6/7**
Individual hepatocyte necrosis	0/10	0/10	6/7**
Pigment	0/10	0/10	6/7**
Thymus			
Lymphoid depletion	0/10	0/10	5/7**
Female	**0 ppm**	**5,000 ppm**	**10,000 ppm**
Kidney			
Tubule regeneration	0/10	1/10	9/10** (1.1)
Tubule degeneration	0/10	0/10	5/10*

* Significantly different ($P \leq 0.05$) from the control group by Fisher's exact test
** $P \leq 0.01$
[a] Kidneys of male rats receiving 1,250 ppm or 5,000 ppm were also examined for tubule regeneration, necrosis, pigment, and mineralized tubules; lesion incidences and severity were the same as for the control group.
[b] Severity grade. Severity of 1=minimal, 2=mild, 3=moderate.

control group in males, including a decreased 16-hour mean urine volume with a high mean specific gravity and increased mean osmolality, osmolality ratio, and creatinine (Table D2). Females showed decreases in mean erythrocyte count and hematocrit, indicative of slight anemia. No differences in other parameters were considered biologically significant. Significant histopathologic findings included a Zymbal's gland adenoma in 1/10 high-dose (2,500 ppm) males and clitoral gland carcinomas in 3/10 high-dose (2,500 ppm) females.

15-Month Interim Evaluation

Clinical findings included the appearance of tissue masses beginning at week 40. Mean body weights at necropsy were significantly less than controls in the high-dose males and females (Tables E7 and E8). Males that received 1,250 and 2,500 ppm showed statistically significant increased absolute and relative liver weights. Statistically significant increases in relative organ weight occurred in various organs and dose groups and were considered to be due to decreases in body weight. Few hematology and clinical chemistry parameters varied significantly from controls (Table D3). High-dose males had a decreased hematocrit and mean cell volume, increased absolute segmented neutrophil count, and decreased absolute lymphocyte and eosinophil count; females in the same dose group had a decreased hemoglobin concentration. Both males and females had statistically significant decreased T_4 values. Serum creatinine was decreased in all female dose groups. Urinalysis results for high-dose males were similar to those found in males at the 9-month interim evaluation; these changes were not duplicated in the female dose groups. However, rats in all treated groups showed increased urine pH.

A variety of neoplasms and nonneoplastic lesions related to chemical administration were found in male and female rats administered C.I. Direct Blue 15 for 15 months (Table 7). Lesions sites included the Zymbal's gland, preputial or clitoral gland, skin, oral cavity, intestine, and liver.

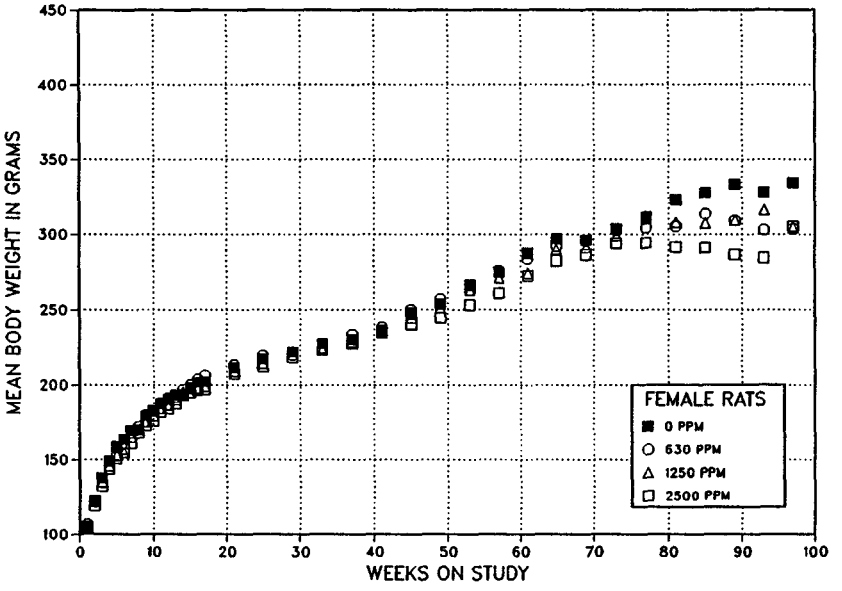
Body Weights, Water Consumption, and Clinical Findings

At 22 months, the final mean body weights of the 630, 1,250, and 2,500 ppm groups were 95%, 91%, and 81% of controls for males and 91% of controls for all female groups (Figure 3 and Tables 8 and 9). From week 52 to 97, the average daily water consumption per male rat in the low-, mid-, and high-dose groups was 9%, 9%, and 24% higher than that by the controls; for exposed female rats the consumption was 15%, 17%, and 12% higher than that by the controls. The average amount of C.I. Direct Blue 15 consumed per rat per day during weeks 51 through 97 was approximately 45, 90, and 215 mg/kg for low-, mid, and high-dose males and 50, 100, and 200 mg/kg for low-, mid-, and high-dose females (Tables G1 and G2). Clinical findings were limited to the appearance of tissue masses and swellings in the ventral body, in the genital region, and on the head, which usually corresponded to skin or mammary lesions, preputial/clitoral masses, and Zymbal's gland neoplasms seen at necropsy. General pallor and emaciation were also noted in treated animals.

TABLE 7
Incidences of Selected Treatment-Related Lesions in Rats at the 15-Months Interim Evaluation of the 22-Month Drinking Water Studies of C.I. Direct Blue 15

	0 ppm	630 ppm	1,250 ppm	2,500 ppm
Male				
Liver				
Hepatocholangiocarcinoma	0/10	0/10	0/10	1/10
Neoplastic nodule	0/10	1/10	0/10	0/10
Eosinophilic focus	0/10	2/10	0/10	6/10**
Zymbal's Gland				
Carcinoma	0/10	1/10	0/10	2/10
Adenoma	0/10	0/10	0/10	1/10
Hyperplasia, focal	0/10	0/10	0/10	1/10
Preputial Gland				
Carcinoma	0/10	0/10	4/10*	1/10
Adenoma	0/10	0/10	0/10	2/10
Hyperplasia, focal	0/10	0/10	2/10	1/10
Oral Cavity (Tongue or Pharynx)				
Papilloma, squamous cell	0/10	3/10	0/10	0/10
Skin				
Basal cell carcinoma	0/10	0/10	2/10	1/10
Papilloma, squamous cell	0/10	1/10	0/10	2/10
Large Intestine				
Adenomatous polyp	0/10	1/10	1/10	2/10
Small Intestine				
Adenocarcinoma	0/10	0/10	0/10	1/10
Female				
Liver				
Neoplastic nodule	1/10	0/10	0/10	0/10
Eosinophilic focus	0/10	0/10	0/10	1/10
Zymbal's Gland				
Adenoma	0/10	2/10	1/10	3/10
Hyperplasia, squamous	0/10	2/10	1/10	0/10
Clitoral Gland				
Carcinoma	0/10	0/10	2/10	1/10
Adenoma	1/10	1/10	1/10	1/10
Hyperplasia, focal	0/10	0/10	1/10	1/10
Oral Cavity (Pharynx)				
Papilloma, squamous cell	0/10	0/10	0/10	2/10
Large Intestine				
Adenomatous polyp	0/10	0/10	0/10	1/10
Small Intestine				
Adenocarcinoma	0/10	0/10	0/10	1/10

* Significantly different ($P \leq 0.05$) from the control group by Fisher's exact test
** $P \leq 0.01$



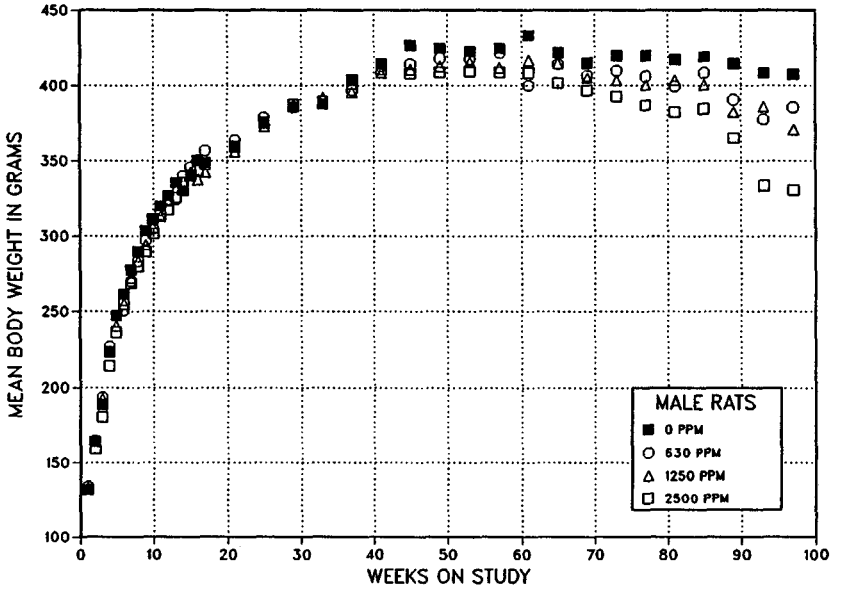


FIGURE 3
Growth Curves for Rats in the 22-Month Drinking Water Studies of C.I. Direct Blue 15

TABLE 8
Mean Body Weights and Survival of Male Rats in the 22-Month Drinking Water Study of C.I. Direct Blue 15

Week on Study	0 ppm		630 ppm			1,250 ppm			2,500 ppm		
	Av. Wt. (g)	Number of Survivors	Av. Wt. (g)	Wt. (% of controls)	Number of Survivors	Av. Wt. (g)	Wt. (% of controls)	Number of Survivors	Av. Wt. (g)	Wt. (% of controls)	Number of Survivors
1	131	50	134	102	35	134	102	65	132	101	50
2	162	50	166	102	35	164	101	65	159	98	50
3	191	50	197	103	35	193	101	65	180	94	50
4	222	50	227	102	35	223	100	65	215	97	50
5	246	50	248	101	35	240	97	65	235	96	50
6	260	50	249	96	35	254	98	65	254	98	50
7	277	50	270	98	35	272	98	65	268	97	50
8	290	50	285	98	35	283	98	65	279	96	50
9	302	50	297	98	35	294	97	65	289	96	50
10	311	50	304	98	35	305	98	65	301	97	50
11	319	50	317	100	35	312	98	65	313	98	50
12	325	50	324	99	35	323	99	65	316	97	50
13	334	50	323	97	35	330	99	65	324	97	50
14	329	50	340	103	35	337	103	65	333	101	48
15	339	50	345	102	35	342	101	65	338	100	48
16	349	50	350	100	35	337	97	65	339	97	48
17	347	50	356	103	35	343	99	65	344	99	48
21	357	50	363	102	35	356	100	65	356	100	48
25	374	50	376	101	35	372	100	65	370	99	48
29	385	50	382	99	35	388	101	65	385	100	48
33	388	50	386	100	35	392	101	65	387	100	48
37	401	50	391	98	35	395	99	65	397	99	47
41	414	50	410	99	35	407	98	65	408	99	47
45	426	50	409	96	34	410	96	65	405	95	47
49	422	49	412	98	33	411	97	64	407	96	46
53	421	48	413	98	33	415	98	64	405	96	46
57	422	48	417	99	33	410	97	63	405	96	43
61	431	48	395	92	32	414	96	62	405	94	42
65	420	47	410	98	31	413	98	62	398	95	42
69	415	47	406	98	31	405	98	59	397	96	36
73	420	45	410	98	31	403	96	56	393	94	31
77	420	45	406	97	29	401	95	54	387	92	27
81	417	44	400	96	28	404	97	52	382	92	20
85	419	43	408	97	23	400	96	44	385	92	14
89	415	42	391	94	20	382	92	40	365	88	10
93	408	40	377	92	15	386	95	22	334	82	8
97	407	37	386	95	8	371	91	13	331	81	2
Mean for weeks											
1-13	259		257	100		256	99		251	97	
14-52	377		377	100		374	99		372	99	
53-97	418		402	96		400	96		382	91	

TABLE 9
Mean Body Weights and Survival of Female Rats in the 22-Month Drinking Water Study of C.I. Direct Blue 15

Week on Study	0 ppm		630 ppm			1,250 ppm			2,500 ppm		
	Av. Wt. (g)	Number of Survivors	Av. Wt. (g)	Wt. (% of controls)	Number of Survivors	Av. Wt. (g)	Wt. (% of controls)	Number of Survivors	Av. Wt. (g)	Wt. (% of controls)	Number of Survivors
1	106	50	106	101	35	105	99	65	104	98	50
2	123	50	122	99	35	122	99	65	120	97	50
3	138	50	134	97	35	135	98	65	132	96	50
4	149	50	149	100	35	146	98	65	144	96	50
5	159	50	159	100	35	153	96	65	151	95	50
6	164	50	155	94	35	156	95	65	157	96	50
7	170	50	169	100	35	167	98	65	161	95	50
8	170	50	172	102	35	171	101	65	168	99	50
9	179	50	181	101	35	176	98	65	173	97	50
10	183	50	184	101	35	180	98	65	176	97	50
11	187	50	189	101	35	185	99	65	182	97	50
12	190	50	192	101	35	187	99	65	185	97	50
13	193	50	192	100	35	191	99	65	188	98	50
14	195	50	198	102	35	196	101	65	193	99	50
15	198	50	201	101	35	198	100	65	195	99	50
16	200	50	205	102	35	198	99	65	196	98	50
17	201	50	206	103	35	199	99	65	197	98	50
21	211	50	213	101	35	210	99	65	207	98	50
25	216	50	220	102	35	214	99	65	213	98	50
29	222	50	220	99	35	223	101	65	219	99	50
33	227	50	225	99	35	224	99	65	224	99	50
37	230	50	234	102	35	229	100	64	228	99	50
41	236	50	239	101	35	236	100	64	235	100	50
45	247	49	250	101	35	245	99	64	241	98	49
49	253	49	258	102	35	252	100	64	245	97	48
53	265	49	263	99	35	264	100	64	254	96	46
57	274	49	276	101	35	272	99	64	262	96	45
61	286	49	283	99	35	273	96	60	274	96	42
65	295	49	291	99	35	291	99	57	285	97	42
69	296	49	295	100	33	291	98	56	286	97	38
73	303	47	302	100	32	299	99	54	294	97	32
77	312	47	304	98	28	310	100	51	294	94	29
81	323	46	305	95	25	308	95	49	292	90	27
85	328	46	314	96	21	307	94	48	291	89	21
89	333	46	309	93	21	309	93	44	287	86	15
93	328	45	303	92	18	316	97	33	284	87	8
97	334	41	303	91	13	304	91	25	305	91	4
Mean for weeks											
1-13	162		162	100		160	98		157	97	
14-52	220		222	101		219	100		216	98	
53-97	306		296	97		295	97		284	93	

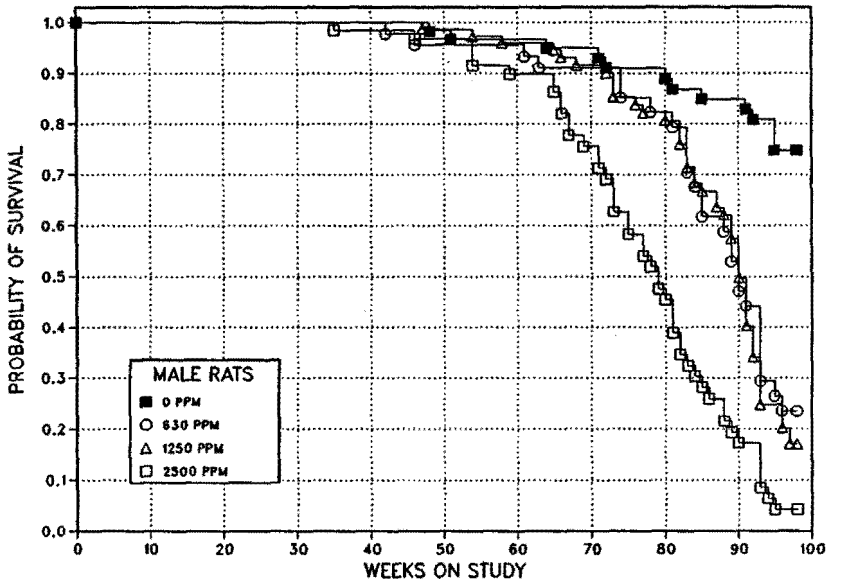
Survival

Estimates of the probabilities of survival for male and female rats given C.I. Direct Blue 15 and for controls are shown in Table 10 and in the Kaplan-Meier curves in Figure 4. By week 81, almost half of the high-dose males and females had been found dead or were killed while moribund from chemical-induced neoplasia.

TABLE 10
Survival of Rats in the 22-Month Drinking Water Studies of C.I. Direct Blue 15

	0 ppm	630 ppm	1,250 ppm	2,500 ppm
Male[a]				
Animals initially in study	70	45	75	70
9-Month interim evaluation	10	0	0	10
15-Month interim evaluation	10	10	10	10
Natural deaths	5	12	24	12
Moribund kills	8	15	30	34
Accidental deaths				2
Animals surviving until study termination	37	8	11	2
Percent survival at end of studies[b]	75	24	17	4
Mean survival (days)[c]	632	565	584	472
Survival analyses[d]	P<0.001	P<0.001	P<0.001	P<0.001
Female[a]				
Animals initially in study	70	45	75	70
9-Month interim evaluation	10	0	0	10
15-Month interim evaluation	10	10	10	10
Natural deaths	4	4	12	15
Moribund kills	6	18	31	31
Animals surviving until study termination	40	13	22	4
Percent survival at end of studies[b]	80	37	35	8
Mean survival (days)[c]	662	577	587	493
Survival analyses[d]	P<0.001	P<0.001	P<0.001	P<0.001

[a] First day of terminal kill: male, 680; female, 682
[b] Kaplan-Meier determinations. Survival rates adjusted for accidental deaths and interim evaluations.
[c] Mean of all deaths (uncensored, censored, terminal kill).
[d] 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.



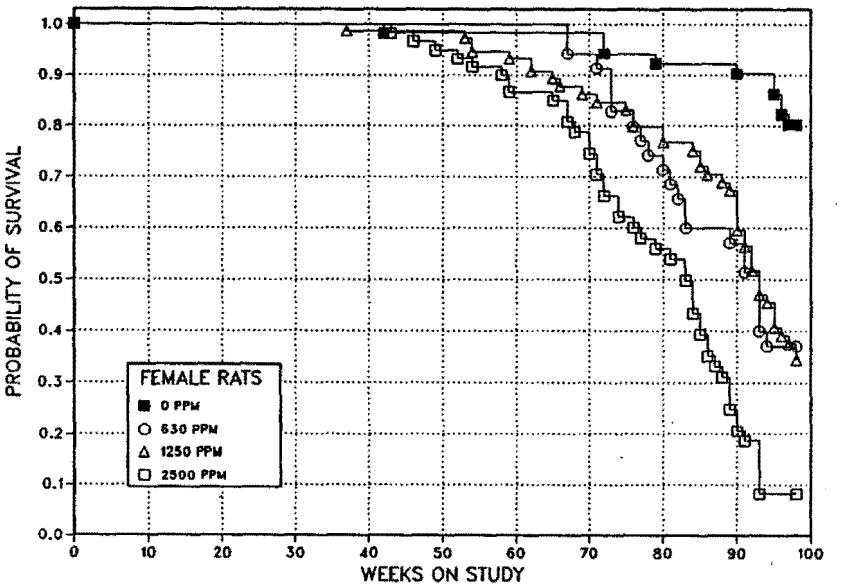


FIGURE 4
Kaplan-Meier Survival Curves for Rats in the 22-Month Drinking Water Studies of C.I. Direct Blue 15

Pathology and Statistical Analyses of Results

This section describes the statistically significant or biologically noteworthy changes in the incidences in rats of neoplasms or nonneoplastic lesions of the skin, Zymbal's gland, clitoral and preputial glands, hematopoietic system, liver, oral cavity (tongue or pharynx), small intestine, large intestine, uterus, brain, kidney, adrenal gland, spleen, bone marrow, and heart.

Summaries of the incidences of neoplasms and nonneoplastic lesions, individual animal tumor diagnoses, statistical analyses of primary neoplasms 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: The incidences of a variety of epithelial neoplasms of the skin were increased in male and female rats treated with C.I. Direct Blue 15 (Table 11). The incidences of basal cell adenomas, basal cell carcinomas, and basal cell adenomas or carcinomas (combined) were moderately increased in low-dose males and markedly increased in mid- and high-dose males. Many of the treated males had multiple basal cell adenomas. There was no increase in the incidence of basal cell neoplasms in treated females. Several sebaceous gland adenomas occurred in treated males, but not in control males (Plate 1); the incidence of this lesion was significantly increased in the mid- and high-dose groups. The incidence of squamous cell papilloma was significantly increased in high-dose males and mid- and high-dose females, while the incidence of squamous cell carcinoma was significantly increased in mid- and high-dose males only. The incidence of squamous cell papilloma or squamous cell carcinoma (combined) was significantly increased in the mid- and high-dose groups of each sex.

Basal cell neoplasms were composed of small, basophilic cells that formed sheets, cords, or solid lobules sometimes containing 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. Some neoplasms consisted solely of sebaceous elements and were diagnosed as sebaceous gland adenoma or carcinoma. Squamous cell papillomas were exophytic growths composed of a pedunculated, highly branched fibrovascular core covered by thickened stratified squamous epithelium. Squamous cell carcinomas were highly invasive neoplasms consisting of irregular cords of disordered pleomorphic squamous cells that projected into the dermis and often showed varying degrees of keratin formation.

Zymbal's Gland: Zymbal's glands are specialized sebaceous glands that lie ventral and anterior to the orifice of the external ear. The incidence of Zymbal's gland neoplasms was markedly increased in treated male and female rats (Table 12). The incidence of adenomas or carcinomas (combined) was significantly increased in all treated groups of males and females. Zymbal's glands from some treated animals of each sex contained nonneoplastic changes, including focal hyperplasia of the glandular cells, squamous focal hyperplasia of the squamous epithelium lining glandular ducts, and dilatation of ducts.

There was a morphologic continuum from adenoma to carcinoma. Adenomas were discrete nodular masses composed of glandular acini of relatively normal-looking 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. Often atypical, neoplastic cells exhibited disordered growth patterns and formed solid masses, irregular acinar structures, and cords with scattered ductular structures and areas of necrosis. Some carcinomas consisted principally of sebaceous cells, while others were composed mainly of stratified squamous epithelium; some neoplasms had prominent components of both. A few of the carcinomas metastasized to the lung or lymph node.

TABLE 11
Skin Proliferative Lesions in Rats in the 22-Month Drinking Water Studies of C.I. Direct Blue 15

	0 ppm	630 ppm	1,250 ppm	2,500 ppm
Male				
Basal Cell Hyperplasia				
Overall rates[a]	1/50 (2%)	1/35 (3%)	3/65 (5%)	3/50 (6%)
Basal Cell Adenoma				
Overall rates	2/50 (4%)	8/35 (23%)	23/65 (35%)	26/50 (52%)
Effective rates[b]	2/48 (4%)	8/33 (24%)	23/62 (37%)	26/43 (60%)
Terminal rates[c]	1/37 (3%)	2/8 (25%)	8/11 (73%)	2/2 (100%)
First incidence (days)	659	632	460	408
Life table tests[d]	P<0.001	P<0.001	P<0.001	P<0.001
Logistic regression tests[d]	P<0.001	P=0.001	P<0.001	P<0.001
Basal Cell Carcinoma				
Overall rates	0/50 (0%)	2/35 (6%)	4/65 (6%)	10/50 (20%)
Effective rates	0/45 (0%)	2/28 (7%)	4/53 (8%)	10/23 (43%)
Terminal rates	0/37 (0%)	0/8 (0%)	0/11 (0%)	2/2 (100%)
First incidence (days)	-[e]	646	637	551
Life table tests	P<0.001	P=0.063	P=0.018	P<0.001
Logistic regression tests	P<0.001	P=0.122	P=0.065	P<0.001
Basal Cell Adenoma or Carcinoma[f]				
Overall rates	2/50 (4%)	9/35 (26%)	27/65 (42%)	28/50 (56%)
Effective rates	2/48 (4%)	9/33 (27%)	27/62 (44%)	28/43 (65%)
Terminal rates	1/37 (3%)	2/8 (25%)	8/11 (73%)	2/2 (100%)
First incidence (days)	659	632	460	408
Life table tests	P<0.001	P<0.001	P<0.001	P<0.001
Logistic regression tests	P<0.001	P<0.001	P<0.001	P<0.001
Sebaceous Gland Adenoma				
Overall rates	0/50 (0%)	1/35 (3%)	7/65 (11%)	3/50 (6%)
Effective rates	0/44 (0%)	1/28 (4%)	7/52 (13%)	3/21 (14%)
Terminal rates	0/37 (0%)	1/8 (13%)	0/11 (0%)	0/2 (0%)
First incidence (days)	-	680 (T)	633	561
Life table tests	P<0.001	P=0.200	P<0.001	P=0.001
Logistic regression tests	P=0.002	P=0.200	P=0.004	P=0.026
Squamous Cell Papilloma				
Overall rates	2/50 (4%)	3/35 (9%)	5/65 (8%)	8/50 (16%)
Effective rates	2/47 (4%)	3/31 (10%)	5/61 (8%)	8/40 (20%)
Terminal rates	2/37 (5%)	1/8 (13%)	2/11 (18%)	1/2 (50%)
First incidence (days)	680 (T)	578	617	460
Life table tests	P<0.001	P=0.087	P=0.018	P<0.001
Logistic regression tests	P=0.001	P=0.258	P=0.107	P=0.005
Squamous Cell Carcinoma				
Overall rates	0/50 (0%)	1/35 (3%)	7/65 (11%)	13/50 (26%)
Effective rates	0/47 (0%)	1/31 (3%)	7/61 (11%)	13/40 (33%)
Terminal rates	0/37 (0%)	0/8 (0%)	2/11 (18%)	1/2 (50%)
First incidence (days)	-	591	460	539
Life table tests	P<0.001	P=0.380	P<0.001	P<0.001
Logistic regression tests	P<0.001	P=0.461	P=0.018	P<0.001

TABLE 11
Skin Proliferative Lesions in Rats in the 22-Month Drinking Water Studies of C.I. Direct Blue 15
(continued)

	0 ppm	630 ppm	1,250 ppm	2,500 ppm
Male (continued)				
Squamous Cell Papilloma or Squamous Cell Carcinoma[g]				
Overall rates	2/50 (4%)	4/35 (11%)	11/65 (17%)	19/50 (38%)
Effective rates	2/47 (4%)	4/31 (13%)	11/61 (18%)	19/40 (48%)
Terminal rates	2/37 (5%)	1/8 (13%)	3/11 (27%)	1/2 (50%)
First incidence (days)	680 (T)	578	460	460
Life table tests	P<0.001	P=0.034	P<0.001	P<0.001
Logistic regression tests	P<0.001	P=0.146	P=0.011	P<0.001
Female				
Basal Cell Adenoma				
Overall rates	1/50 (2%)	0/35 (0%)	0/65 (0%)	0/50 (0%)
Basal Cell Adenoma or Carcinoma[h]				
Overall rates	1/50 (2%)	0/35 (0%)	1/65 (2%)	0/50 (0%)
Squamous Cell Papilloma				
Overall rates	0/50 (0%)	2/35 (6%)	5/65 (8%)	5/50 (10%)
Effective rates	0/47 (0%)	2/28 (7%)	5/51 (10%)	5/28 (18%)
Terminal rates	0/40 (0%)	1/13 (8%)	4/22 (18%)	0/4 (0%)
First incidence (days)	-	535	673	607
Life table tests	P<0.001	P=0.087	P=0.005	P<0.001
Logistic regression tests	P=0.001	P=0.199	P=0.007	P=0.003
Squamous Cell Carcinoma				
Overall rates	0/50 (0%)	0/35 (0%)	1/65 (2%)	0/50 (0%)
Squamous Cell Papilloma or Squamous Cell Carcinoma[i]				
Overall rates	0/50 (0%)	2/35 (6%)	6/65 (9%)	5/50 (10%)
Effective rates	0/47 (0%)	2/28 (7%)	6/51 (12%)	5/28 (18%)
Terminal rates	0/40 (0%)	1/13 (8%)	4/22 (18%)	0/4 (0%)
First incidence (days)	-	535	666	607
Life table tests	P<0.001	P=0.087	P=0.002	P<0.001
Logistic regression tests	P=0.001	P=0.199	P=0.004	P=0.003

(T)Terminal kill
[a] Number of tumor-bearing animals/number of animals necropsied
[b] Number of tumor-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 control incidence are the P values associated with the trend test. Beneath the dosed group incidence are the P values corresponding to pairwise comparisons between the 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] Not applicable; no tumors in animal group
[f] Historical incidence for 2-year NTP studies of untreated control groups (mean ± standard deviation): 21/1596 (1.3% ± 1.9%)
[g] Historical incidence: 29/1596 (1.8% ± 1.7%)
[h] Historical incidence: 6/1643 (0.4% ± 0.8%)
[i] Historical incidence: 7/1643 (0.4% ± 0.8%)

TABLE 12
Zymbal's Gland Proliferative Lesions in F344/N Rats in the 22-Month Drinking Water Studies of C.I. Direct Blue 15

	0 ppm	630 ppm	1,250 ppm	2,500 ppm
Male				
Squamous Hyperplasia				
Overall rates[a]	0/50 (0%)	1/35 (3%)	6/64 (10%)	5/50 (10%)
Adenoma				
Overall rates	0/50 (0%)	2/35 (6%)	2/65 (3%)	4/50 (8%)
Effective rates[b]	0/45 (0%)	2/28 (7%)	2/53 (4%)	4/23 (17%)
Terminal rates[c]	0/37 (0%)	1/8 (13%)	0/11 (0%)	0/2 (0%)
First incidence (days)	–[e]	660	577	551
Life table tests[d]	P<0.001	P=0.023	P=0.228	P=0.004
Logistic regression tests[d]	P=0.024	P=0.054	P=0.316	P=0.041
Carcinoma				
Overall rates	1/50 (2%)	3/35 (9%)	8/65 (12%)	17/50 (34%)
Effective rates	1/50 (2%)	3/33 (9%)	8/65 (12%)	17/46 (37%)
Terminal rates	0/37 (0%)	0/8 (0%)	0/11 (0%)	0/2 (0%)
First incidence (days)	501	583	323	372
Life table tests	P<0.001	P=0.128	P=0.014	P<0.001
Logistic regression tests	P<0.001	P=0.247	P=0.083	P=0.001
Adenoma or Carcinoma[f]				
Overall rates	1/50 (2%)	5/35 (14%)	10/65 (15%)	20/50 (40%)
Effective rates	1/50 (2%)	5/33 (15%)	10/65 (15%)	20/46 (43%)
Terminal rates	0/37 (0%)	1/8 (13%)	0/11 (0%)	0/2 (0%)
First incidence (days)	501	583	323	372
Life table tests	P<0.001	P=0.007	P=0.005	P<0.001
Logistic regression tests	P<0.001	P=0.045	P=0.037	P<0.001

TABLE 12
Zymbal's Gland Proliferative Lesions in F344/N Rats in the 22-Month Drinking Water Studies of C.I. Direct Blue 15 (continued)

	0 ppm	630 ppm	1,250 ppm	2,500 ppm
Female				
Glandular or Squamous Hyperplasia				
Overall rates	0/49 (0%)	3/35 (9%)	4/64 (6%)	5/50 (10%)
Adenoma				
Overall rates	0/50 (0%)	1/35 (3%)	5/65 (8%)	3/50 (6%)
Effective rates	0/49 (0%)	1/35 (3%)	5/60 (8%)	3/42 (7%)
Terminal rates	0/40 (0%)	0/13 (0%)	1/22 (5%)	0/4 (0%)
First incidence (days)	-	495	432	547
Life table tests	P=0.006	P=0.421	P=0.016	P=0.031
Logistic regression tests	P=0.118	P=0.462	P=0.067	P=0.122
Carcinoma				
Overall rates	0/50 (0%)	4/35 (11%)	7/65 (11%)	14/50 (28%)
Effective rates	0/49 (0%)	4/35 (11%)	7/64 (11%)	14/50 (28%)
Terminal rates	0/40 (0%)	0/13 (0%)	1/22 (5%)	1/4 (25%)
First incidence (days)	-	465	432	296
Life table tests	P<0.001	P=0.017	P=0.007	P<0.001
Logistic regression tests	P=0.001	P=0.056	P=0.037	P=0.001
Adenoma or Carcinoma[g]				
Overall rates	0/50 (0%)	4/35 (11%)	11/65 (17%)	17/50 (34%)
Effective rates	0/49 (0%)	4/35 (11%)	11/64 (17%)	17/50 (34%)
Terminal rates	0/40 (0%)	0/13 (0%)	2/22 (9%)	1/4 (25%)
First incidence (days)	-	465	432	296
Life table tests	P<0.001	P=0.017	P<0.001	P<0.001
Logistic regression tests	P<0.001	P=0.056	P=0.004	P<0.001

[a] Number of tumor-bearing animals/number of animals necropsied or examined microscopically for this tumor type
[b] Number of tumor-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 control incidence are the P values associated with the trend test. Beneath the dosed group incidence are the P values corresponding to pairwise comparisons between the 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] Not applicable; no tumors in animal group
[f] Historical incidence for 2-year NTP studies of untreated control groups (mean ± standard deviation): 18/1596 (1.1% ± 1.8%)
[g] Historical incidence: 14/1643 (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 near the penis. There was a marked treatment-related increase in the incidence of clitoral gland neoplasms in female rats (Table 13). The incidence of clitoral gland adenomas or carcinomas (combined) was significantly increased in all treated female groups, and many treated females developed bilateral adenomas or carcinomas. The incidence of preputial gland adenomas or carcinomas (combined) was significantly increased only in the mid-dose male group (Table 14; Plate 2). The incidences of nonneoplastic changes of the clitoral or preputial glands were higher in treated rats than in controls. The incidence of hyperplasia of the stratified squamous epithelium lining glandular ducts was slightly increased in treated females, and the incidence of dilatation of the ducts (ectasia) was moderately increased incidence in mid- and high-dose male rats.

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 with a few duct-like structures, sometimes containing debris, scattered within the neoplasms. Carcinomas were poorly demarcated masses that sometimes invaded adjacent tissues. They were composed of solid sheets and clusters of disorganized pleomorphic cells, and there was often an abundance of small, basophilic basal-like cells (reserve cells). Some carcinomas exhibited marked cellular atypia or contained large areas of necrosis.

Hematopoietic System: The incidence of mononuclear cell leukemia was significantly increased in all treated male and female groups (Table 15) as determined by survival-adjusted analyses. The incidence in the high-dose groups was somewhat less than that in the mid-dose groups, perhaps because of the reduced survival and competing risks from other fatal neoplasms.

TABLE 13
Clitoral Gland Lesions in Female F344/N Rats in the 22-Month Drinking Water Study of C.I. Direct Blue 15

	0 ppm	630 ppm	1,250 ppm	2,500 ppm
Squamous Hyperplasia				
Overall rates[a]	0/50 (0%)	2/31 (6%)	4/64 (6%)	1/50 (2%)
Adenoma				
Overall rates	5/50 (10%)	5/31 (16%)	12/64 (19%)	12/50 (24%)
Effective rates[b]	5/49 (10%)	5/31 (16%)	12/59 (20%)	12/42 (29%)
Terminal rates[c]	4/40 (10%)	3/13 (23%)	5/22 (23%)	2/4 (50%)
First incidence (days)	666	558	432	453
Life table tests[d]	P<0.001	P=0.074	P=0.007	P<0.001
Logistic regression tests[d]	P=0.003	P=0.197	P=0.077	P=0.006
Carcinoma				
Overall rates	2/50 (4%)	6/31 (19%)	12/64 (19%)	15/50 (30%)
Effective rates	2/50 (4%)	6/31 (19%)	12/64 (19%)	15/50 (30%)
Terminal rates	2/40 (5%)	1/13 (8%)	3/22 (14%)	0/4 (0%)
First incidence (days)	682 (T)	506	253	372
Life table tests	P<0.001	P=0.010	P=0.002	P<0.001
Logistic regression tests	P=0.025	P=0.057	P=0.063	P=0.005
Adenoma or Carcinoma[e]				
Overall rates	7/50 (14%)	11/31 (35%)	24/64 (38%)	27/50 (54%)
Effective rates	7/50 (14%)	11/31 (35%)	24/64 (38%)	27/50 (54%)
Terminal rates	6/40 (15%)	4/13 (31%)	8/22 (36%)	2/4 (50%)
First incidence (days)	666	506	253	372
Life table tests	P<0.001	P=0.001	P<0.001	P<0.001
Logistic regression tests	P<0.001	P=0.028	P=0.010	P<0.001

(T)Terminal kill
[a] Number of tumor-bearing animals/number of animals examined microscopically for this tumor type
[b] Number of tumor-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 control incidence are the P values associated with the trend test. Beneath the dosed group incidence are the P values corresponding to pairwise comparisons between the 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 NTP studies of untreated control groups (mean ± standard deviation): 115/1643 (7.0% ± 4.9%)

TABLE 14
Preputial Gland Neoplasms in Male F344/N Rats in the 22-Month Drinking Water Study of C.I. Direct Blue 15

	0 ppm	630 ppm	1,250 ppm	2,500 ppm
Adenoma				
Overall rates[a]	6/49 (12%)	2/35 (6%)	12/64 (19%)	8/48 (17%)
Effective rates[b]	6/47 (13%)	2/33 (6%)	12/63 (19%)	8/44 (18%)
Terminal rates[c]	5/37 (14%)	1/8 (13%)	4/11 (36%)	0/2 (0%)
First incidence (days)	565	660	530	372
Life table tests[d]	P<0.001	P=0.560	P=0.002	P<0.001
Logistic regression tests[d]	P=0.039	P=0.466N	P=0.143	P=0.228
Carcinoma				
Overall rates	2/49 (4%)	3/35 (9%)	11/64 (17%)	1/48 (2%)
Effective rates	2/47 (4%)	3/33 (9%)	11/63 (17%)	1/44 (2%)
Terminal rates	2/37 (5%)	0/8 (0%)	0/11 (0%)	0/2 (0%)
First incidence (days)	680 (T)	578	372	600
Life table tests	P=0.092	P=0.107	P=0.003	P=0.379
Logistic regression tests	P=0.441N	P=0.300	P=0.056	P=0.687
Adenoma or Carcinoma[e]				
Overall rates	8/49 (16%)	5/35 (14%)	23/64 (36%)	9/48 (19%)
Effective rates	8/47 (17%)	5/33 (15%)	23/63 (37%)	9/44 (20%)
Terminal rates	7/37 (19%)	1/8 (13%)	4/11 (36%)	0/2 (0%)
First incidence (days)	565	578	372	372
Life table tests	P<0.001	P=0.141	P<0.001	P<0.001
Logistic regression tests	P=0.121	P=0.547	P=0.019	P=0.232

(T)Terminal kill
[a] Number of tumor-bearing animals/number of animals examined microscopically for this tumor type
[b] Number of tumor-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 control incidence are the P values associated with the trend test. Beneath the dosed group incidence are the P values corresponding to pairwise comparisons between the 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 dose group is indicated by N.
[e] Historical incidence for 2-year NTP studies of untreated control groups (mean ± standard deviation): 117/1596 (7.3% ± 5.2%)

TABLE 15
Leukemias in F344/N Rats in the 22-Month Drinking Water Studies of C.I. Direct Blue 15

	0 ppm	630 ppm	1,250 ppm	2,500 ppm
Male[a]				
Overall rates[b]	17/50 (34%)	19/35 (54%)	28/65 (43%)	20/50 (40%)
Effective rates[c]	17/48 (35%)	19/31 (61%)	28/62 (45%)	20/42 (48%)
Terminal rates[d]	11/37 (30%)	5/8 (63%)	9/11 (82%)	2/2 (100%)
First incidence (days)	445	544	472	452
Life table tests[e]	P<0.001	P<0.001	P<0.001	P<0.001
Logistic regression tests[e]	P=0.004	P=0.018	P=0.053	P=0.012
Female[f]				
Overall rates	7/50 (14%)	13/35 (37%)	27/65 (42%)	15/50 (30%)
Effective rates	7/49 (14%)	13/35 (37%)	27/58 (47%)	15/42 (36%)
Terminal rates	5/40 (13%)	3/13 (23%)	11/22 (50%)	2/4 (50%)
First incidence (days)	624	463	449	453
Life table tests	P<0.001	P<0.001	P<0.001	P<0.001
Logistic regression tests	P=0.006	P=0.025	P<0.001	P<0.001

[a] Historical incidence for 2-year drinking water studies with untreated control groups (mean ± standard deviation): 594/1,596 (37% ± 16%); range 10%-72%
[b] Number of tumor-bearing animals/number of animals necropsied
[c] Number of tumor-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 control incidence are the P values associated with the trend test. Beneath the dosed group incidence are the P values corresponding to pairwise comparisons between the 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 drinking water studies with untreated control groups (mean ± standard deviation): 324/1,643 (20% ± 8%); range 6%-40%

Liver: The incidence of neoplastic nodules or hepatocellular carcinoma (combined) was significantly increased in all treated male groups and in the high-dose female group (Table 16). Livers of a few treated males contained multiple neoplastic nodules. No hepatocellular neoplasms occurred in control animals of either sex. Neoplastic nodule is the term previously used for proliferative hepatocellular lesions currently classified as hepatocellular adenoma. Neoplastic nodules were well-demarcated masses that compressed the adjacent parenchyma and varied in size from several hepatic lobules to nearly an entire liver lobe. The hepatic plates within neoplastic nodules were not organized in a normal lobular pattern and often intersected at nearly right angles with the plates of the adjacent normal liver. In some cases, sinusoids were apparent within neoplastic nodules, but generally the nodules appeared to be more solid than the surrounding parenchyma. Neoplastic hepatocytes were slightly pleomorphic and exhibited increased eosinophilic staining. Hepatocellular carcinomas, in contrast, consisted of highly disorganized cells that formed solid clusters, glandular structures, and broad trabeculae many cell layers thick. Cells within carcinomas were often moderately to markedly pleomorphic and exhibited varying degrees of atypia.

A variety of nonneoplastic liver lesions were present in treated male and female rats (Table 17). The incidence of eosinophilic foci was moderately to markedly increased in all treated groups of males and slightly increased in the high-dose female group. 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. The incidences of hematopoietic cell proliferation and regeneration were slightly to moderately increased in treated male and female rats. Hematopoietic cell proliferation was presumably secondary to inflammation associated with neoplasms in treated animals. Regeneration was characterized by one or more discrete nodular foci consisting of increased numbers of hepatocytes with normal morphology arranged in a lobular pattern. Regeneration represents an attempt by the liver to recover from hepatocellular injury. The increase in the incidence of regeneration in this study was considered secondary to hepatocellular damage caused by mononuclear cell leukemia, which was more common in treated animals. The incidence of degenerative changes was marginally increased in treated males and females. These changes included single or multiple small scattered foci of hepatocyte necrosis (most commonly affecting centrilobular hepatocytes), the presence of clear cytoplasmic vacuoles in scattered clusters of hepatocytes (cytoplasmic vacuolization), and multiple focal clusters of variably sized cysts filled with granular eosinophilic materials or erythrocytes (cystic degeneration).

TABLE 16
Liver Neoplasms in F344/N Rats in the 22-Month Drinking Water Studies of C.I. Direct Blue 15

	0 ppm	630 ppm	1,250 ppm	2,500 ppm
Male				
Neoplastic Nodule				
Overall rates[a]	0/50 (0%)	6/35 (17%)	8/65 (12%)	7/50 (14%)
Effective rates[b]	0/47 (0%)	6/31 (19%)	8/60 (13%)	7/38 (18%)
Terminal rates[c]	0/37 (0%)	3/8 (38%)	2/11 (18%)	0/2 (0%)
First incidence (days)	-[e]	544	579	463
Logistic regression tests[d]	P=0.003	P=0.002	P=0.003	P=0.003
Hepatocellular Carcinoma				
Overall rates	0/50 (0%)	0/35 (0%)	1/65 (2%)	4/50 (8%)
Effective rates	0/45 (0%)	0/28 (0%)	1/53 (2%)	4/24 (17%)
Terminal rates	0/37 (0%)	0/8 (0%)	0/11 (0%)	0/2 (0%)
First incidence (days)	-	-	632	547
Logistic regression tests	P<0.001	-	P=0.540	P=0.009
Neoplastic Nodule or Hepatocellular Carcinoma[f]				
Overall rates	0/50 (0%)	6/35 (17%)	9/65 (14%)	11/50 (22%)
Effective rates	0/47 (0%)	6/31 (19%)	9/60 (15%)	11/38 (29%)
Terminal rates	0/37 (0%)	3/8 (38%)	2/11 (18%)	0/2 (0%)
First incidence (days)	-	544	579	463
Logistic regression tests	P<0.001	P=0.002	P=0.002	P<0.001
Female				
Neoplastic Nodule				
Overall	0/50 (0%)	0/35 (0%)	2/65 (3%)	4/50 (8%)
Effective	0/46 (0%)	0/21 (0%)	2/48 (4%)	4/24 (17%)
Terminal	0/40 (0%)	0/13 (0%)	1/22 (5%)	1/4 (25%)
First incidence (days)	-	-	625	585
Logistic regression tests	P=0.002	-	P=0.246	P=0.016
Hepatocellular Carcinoma				
Overall rates	0/50 (0%)	0/35 (0%)	0/65 (0%)	1/50 (2%)
Neoplastic Nodule or Hepatocellular Carcinoma[g]				
Overall	0/50 (0%)	0/35 (0%)	2/65 (3%)	5/50 (10%)
Effective	0/46 (0%)	0/25 (0%)	2/49 (4%)	5/27 (19%)
Terminal	0/40 (0%)	0/13 (0%)	1/22 (5%)	1/4 (25%)
First incidence (days)	-	-	625	564
Logistic regression tests	P<0.001	-	P=0.246	P=0.010

[a] Number of tumor-bearing animals/number of animals necropsied
[b] Number of tumor-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 control incidence are the P values associated with the trend test. Beneath the dosed group incidence are the P values corresponding to pairwise comparisons between the controls and that dosed group. The logistic regression tests regard tumors in animals dying prior to terminal kill as nonfatal.
[e] Not applicable; no tumors in animal group
[f] Historical incidence for 2-year NTP studies of untreated control groups (mean ± standard deviation): 78/1591 (4.9% ± 4.3%)
[g] Historical incidence: 37/1643 (2.3% ± 2.7%)

TABLE 17
Numbers of F344/N Rats with Selected Nonneoplastic Liver Lesions in the 22-Month Drinking Water Studies of C.I. Direct Blue 15

Lesion	Male				Female			
	0 ppm	630 ppm	1,250 ppm	2,500 ppm	0 ppm	630 ppm	1,250 ppm	2,500 ppm
n	50	35	65	50	50	35	65	50
Eosinophilic focus	2	7 *	15 **	23 ***	2	2	3	6
Hematopoietic cell proliferation	0	4 *	6 *	13 ***	5	5	14	13 *
Regeneration	1	5 *	5	15 ***	0	0	10 **	7 **
Focal/multifocal necrosis	1	0	4	3	0	0	3	2
Diffuse centrilobular necrosis	1	3	4	2	1	0	0	2
Cytoplasmic vacuolization	3	0	6	8	4	6	9	9
Cystic degeneration	1	5 *	10 *	7 *	0	1	0	1

* Significantly different ($P \leq 0.05$) from the control group by logistic regression analysis
** $P \leq 0.01$
*** $P \leq 0.001$

Oral Cavity (Tongue or Pharynx): Squamous cell papilloma and squamous cell carcinoma of the oral cavity epithelium are uncommon neoplasms in untreated F344/N rats, with an average historical incidence in NTP 2-year studies less than 1% (range 0% to 2%; Tables A4d and B4d). In these studies, the incidence of squamous cell papilloma or carcinoma (combined) of the oral cavity was substantially increased in treated males and females (Table 18). In addition, hyperplasia of the epithelium of the palate was seen in two low-dose and four mid-dose female rats, and hyperplasia of the tongue was seen in one high-dose female rat. 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 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 (Plate 3). Fibroplasia and inflammation were sometimes seen with the invasion.

Small Intestine: A few adenocarcinomas of the small intestine occurred in male and female rats treated with C.I. Direct Blue 15, and a single adenomatous polyp was seen in one low-dose male rat (Table 19). These neoplasms occur rarely in untreated F344/N rats (mean <1%, range 0%-2%; Tables A4b and B4b). All of these neoplasms occurred in the high-dose groups except for the single adenomatous polyp in a low-dose male and one adenocarcinoma in a mid-dose female. One high-dose male had two adenocarcinomas of the small intestine. 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. Some adenocarcinomas contained large cystic spaces filled with mucus and debris (cystic mucinous adenocarcinoma). The adenomatous polyp was a pedunculated exophytic mass that consisted of a stalk-like core of fibrous tissue covered by numerous glandular structures lined by a single layer of well-differentiated columnar cells with abundant basophilic cytoplasm.

TABLE 18
Neoplasms of the Oral Cavity in F344/N Rats in the 22-Month Drinking Water Studies of C.I. Direct Blue 15

	0 ppm	630 ppm	1,250 ppm	2,500 ppm
Male				
Squamous Cell Papilloma[a]				
Overall rates[b]	0/50 (0%)	9/35 (26%)	18/65 (28%)	15/50 (30%)
Effective rates[c]	0/50 (0%)	9/34 (26%)	18/65 (28%)	15/47 (32%)
Terminal rates[d]	0/37 (0%)	3/8 (38%)	1/11 (9%)	0/2 (0%)
First incidence (days)	-[f]	316	460	372
Logistic regression tests[e]	P<0.001	P<0.001	P<0.001	P<0.001
Squamous Cell Carcinoma[g]				
Overall rates	1/50 (2%)	1/35 (3%)	6/65 (9%)	2/50 (4%)
Effective rates	1/50 (2%)	1/35 (3%)	6/65 (9%)	2/47 (4%)
Terminal rates	1/37 (3%)	0/8 (0%)	0/11 (0%)	0/2 (0%)
First incidence (days)	680 (T)	293	502	479
Logistic regression tests	P=0.503	P=0.739N	P=0.141	P=0.461
Squamous Cell Papilloma or Squamous Cell Carcinoma				
Overall rates	1/50 (2%)	10/35 (29%)	24/65 (37%)	17/50 (34%)
Effective rates	1/50 (2%)	10/35 (29%)	24/65 (37%)	17/47 (36%)
Terminal rates	1/37 (3%)	3/8 (38%)	1/11 (9%)	0/2 (0%)
First incidence (days)	680 (T)	293	460	372
Logistic regression tests	P<0.001	P=0.001	P<0.001	P<0.001
Female				
Squamous Cell Papilloma[h]				
Overall rates	2/50 (4%)	3/35 (9%)	12/65 (18%)	9/50 (18%)
Effective rates	2/49 (4%)	3/35 (9%)	12/63 (19%)	9/46 (20%)
Terminal rates	2/40 (5%)	0/13 (0%)	3/22 (14%)	1/4 (25%)
First incidence (days)	682 (T)	463	583	372
Logistic regression tests	P=0.015	P=0.491	P=0.008	P=0.035
Squamous Cell Carcinoma[i]				
Overall rates	0/50 (0%)	1/35 (3%)	8/65 (12%)	6/50 (12%)
Effective rates	0/49 (0%)	1/35 (3%)	8/64 (13%)	6/47 (13%)
Terminal rates	0/40 (0%)	1/13 (8%)	0/22 (0%)	1/4 (25%)
First incidence (days)	-	682 (T)	432	359
Logistic regression tests	P=0.015	P=0.277	P=0.023	P=0.023
Squamous Cell Papilloma or Squamous Cell Carcinoma				
Overall rates	2/50 (4%)	4/35 (11%)	19/65 (29%)	15/50 (30%)
Effective rates	2/49 (4%)	4/35 (11%)	19/64 (30%)	15/47 (32%)
Terminal rates	2/40 (5%)	1/13 (8%)	3/22 (14%)	2/4 (50%)
First incidence (days)	682 (T)	463	432	359
Logistic regression tests	P<0.001	P=0.294	P<0.001	P=0.001

TABLE 18
Neoplasms of the Oral Cavity in F344/N Rats in the 22-Month Drinking Water Studies of C.I. Direct Blue 15 (continued)

(T)Terminal kill
[a] Historical incidence for 2-year NTP studies of untreated control groups (mean ± standard deviation): 3/1596 (0.2% ± 0.6%)
[b] Number of tumor-bearing animals/number of animals necropsied
[c] Number of tumor-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 control incidence are the P values associated with the trend test. Beneath the dosed group incidence are the P values corresponding to pairwise comparisons between the controls and that dosed group. The logistic regression tests regard tumors in animals dying prior to terminal kill as nonfatal. For all tests, a negative trend or a lower incidence in a dose group is indicated by N.
[f] Not applicable; no tumors in animal group
[g] Historical incidence: 4/1596 (0.3% ± 0.7%)
[h] Historical incidence: 1/1643 (0.1% ± 0.4%)
[i] Historical incidence: 3/1643 (0.2% ± 0.6%)

TABLE 19
Neoplasms of the Small Intestine in F344/N Rats in the 22-Month Drinking Water Studies of C.I. Direct Blue 15

	0 ppm	630 ppm	1,250 ppm	2,500 ppm
Male				
Adenomatous Polyp				
Overall rates[a]	0/50 (0%)	1/35 (3%)	0/65 (0%)	0/50 (0%)
Adenocarcinoma				
Overall rates	0/50 (0%)	0/35 (0%)	0/65 (0%)	2/50 (4%)
Effective rates[b]	0/47 (0%)	0/31 (0%)	0/59 (0%)	2/36 (6%)
Terminal rates[c]	0/37 (0%)	0/8 (0%)	0/11 (0%)	0/2 (0%)
First incidence (days)	-[e]	-	-	479
Logistic regression tests[d]	P=0.078	-	-	P=0.304
Adenomatous Polyp or Adenocarcinoma[f]				
Overall rates	0/50 (0%)	1/35 (3%)	0/65 (0%)	2/50 (4%)
Effective rates	0/48 (0%)	1/33 (3%)	0/62 (0%)	2/42 (5%)
Terminal rates	0/37 (0%)	0/8 (0%)	0/11 (0%)	0/2 (0%)
First incidence (days)	-	421	-	479
Logistic regression tests	P=0.309	P=0.573	-	P=0.304
Female				
Adenocarcinoma				
Overall rates	0/50 (0%)	0/35 (0%)	1/65 (2%)	3/50 (6%)
Effective rates	0/49 (0%)	0/33 (0%)	1/56 (2%)	3/38 (8%)
Terminal rates	0/40 (0%)	0/13 (0%)	0/22 (0%)	0/4 (0%)
First incidence (days)	-	-	479	578
Logistic regression tests	P=0.032	-	P=0.688	P=0.075

[a] Number of tumor-bearing animals/number of animals necropsied
[b] Number of tumor-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 control incidence are the P values associated with the trend test. Beneath the dosed group incidence are the P values corresponding to pairwise comparisons between the controls and that dosed group. The logistic regression tests regard tumors in animals dying prior to terminal kill as nonfatal.
[e] Not applicable; no tumors in animal group
[f] Historical incidence for 2-year NTP studies of untreated control groups (mean ± standard deviation): 5/1557 (0.3% ± 0.7%)

Large Intestine: Adenomatous polyps and adenocarcinomas of the large intestine mucosa also occur rarely in untreated F344/N rats (mean <1%, range 0% to 2%; Tables A4a and B4a). Several of these neoplasms occurred in treated male and female rats, but none were seen in untreated rats (Table 20). The incidences of adenomatous polyp, adenocarcinoma, and adenomatous polyp or adenocarcinoma (combined) were significantly increased in the high-dose male group. Adenomatous polyps of the large intestine had a histologic appearance similar to that of polyps occurring in the small intestine (Plate 4). Adenocarcinomas resembled polyps except that adenocarcinomas contained areas of invasion of the fibrous tissue core by neoplastic glandular epithelial cells.

Uterus: Uterine epithelial neoplasms occurred with somewhat higher incidence in treated females than in controls (Table 21). The incidence of adenoma or adenocarcinoma (combined) in the high-dose group was significantly increased and exceeded the range of historical control values for untreated female F344/N rats from NTP 2-year studies [4/1632 (0.2%), range 0% to 2%]. The incidence of endometrial stromal polyps from the 15-month interim evaluations and the 22-month studies combined was increased in the low- and mid-dose groups [control, 5/60 (8.3%); low-dose, 13/45 (28.9%); mid-dose, 14/65 (21.5%); high-dose, 7/60 (11.7%)]. Endometrial stromal polyps are commonly occurring neoplasms in untreated female F344/N rats, and all incidences in this study were well within the range of historical control values from NTP 2-year studies [341/1632 (21%), range 8% to 36%]. Because the incidence in the control group from this study is at the low end of the historical control range, the increased incidence of endometrial stromal polyps in the low- and mid-dose groups is not considered to be a treatment-related effect.

Brain: Malignant astrocytomas occurred in a few animals of each sex (males: control, 0/50; low-dose, 1/35; mid-dose, 1/65; high-dose, 2/50; females: control, 1/50; low-dose, 0/35; mid-dose, 2/65; high-dose, 1/50). Astrocytomas are uncommon neoplasms in untreated F344/N rats and are usually late-occurring neoplasms seen at necropsy. In these studies, there was substantial early mortality in treated groups, which greatly reduced the numbers of animals at risk for the occurrence of brain neoplasms. Consequently, malignant astrocytomas in treated animals may have been associated with the administration of C.I. Direct Blue 15.

Kidney: Two adenomas of renal tubule epithelium occurred in the high-dose male group. No primary renal neoplasms occurred in any of the other treated or control groups of either sex. The incidence of this neoplasm lies within the historical range for untreated male F344/N rats from 2-year NTP studies [10/1590 (1%), range 0% to 6%], and there was no treatment-related increase in the incidence of renal tubule epithelial hyperplasia, a lesion generally considered to be the precursor of renal tubule neoplasms. Hyperplasia was seen only in one low-dose and one high-dose male. Therefore, the occurrence of these two adenomas was not considered to be a treatment-related effect.

Adrenal Gland: The incidence of benign or malignant pheochromocytomas (combined) in the high-dose male group was significantly different from controls (control, 16/50; low-dose, 5/35; mid-dose 21/65; high-dose, 17/50). The incidence of adrenal medullary hyperplasia was similar across male dose groups (control, 4/50; low-dose, 5/35; mid-dose, 2/65; high-dose, 5/50). The overall incidences of hyperplasia and pheochromocytoma were essentially identical in the control and high-dose groups, but there was marked early mortality in the high-dose group and the first occurrence of a pheochromocytoma was during week 65 of the study in the high-dose group and during week 91 in the control group. Because the statistical analysis results reflect survival data as well as numbers of neoplasms, and because the neoplasms occurred earlier in high-dose males than in control males, there is a statistically significant difference between the control and high-dose groups. Pheochromocytomas are relatively slow-growing neoplasms that are seldom seen before one year of age. They begin as focal noncompressive proliferative lesions of the adrenal medullary cells, diagnosed as hyperplasias. As the proliferative lesions increase in size, they displace and compress the normal parenchyma, at which point they are diagnosed as pheochromocytomas. The neoplasms continue to grow and can reach a centimeter

TABLE 20
Neoplasms of the Large Intestine in F344/N Rats in the 22-Month Drinking Water Studies of C.I. Direct Blue 15

	0 ppm	630 ppm	1,250 ppm	2,500 ppm
Male				
Adenomatous Polyp				
Overall rates[a]	0/50 (0%)	1/35 (3%)	2/65 (3%)	5/50 (10%)
Effective rates[b]	0/45 (0%)	1/31 (3%)	2/59 (3%)	5/33 (15%)
Terminal rates[c]	0/37 (0%)	0/8 (0%)	0/11 (0%)	0/2 (0%)
First incidence (days)	–[e]	579	559	502
Logistic regression tests[d]	P=0.005	P=0.471	P=0.317	P=0.010
Adenocarcinoma				
Overall rates	0/50 (0%)	0/35 (0%)	4/65 (6%)	3/50 (6%)
Effective rates	0/45 (0%)	0/31 (0%)	4/58 (7%)	3/32 (9%)
Terminal rates	0/37 (0%)	0/8 (0%)	0/11 (0%)	0/2 (0%)
First incidence (days)	–	–	628	505
Logistic regression tests	P=0.034	–	P=0.072	P=0.156
Adenomatous Polyp or Adenocarcinoma[f]				
Overall rates	0/50 (0%)	1/35 (3%)	6/65 (9%)	8/50 (16%)
Effective rates	0/45 (0%)	1/31 (3%)	6/59 (10%)	8/33 (24%)
Terminal rates	0/37 (0%)	0/8 (0%)	0/11 (0%)	0/2 (0%)
First incidence (days)	–	579	559	502
Logistic regression tests	P<0.001	P=0.471	P=0.030	P=0.002
Female				
Adenomatous Polyp				
Overall rates	0/50 (0%)	0/35 (0%)	3/65 (5%)	1/50 (2%)
Effective rates	0/45 (0%)	0/18 (0%)	3/35 (9%)	1/9 (11%)
Terminal rates	0/40 (0%)	0/13 (0%)	2/22 (9%)	0/4 (0%)
First incidence (days)	–	–	640	646
Logistic regression tests	P=0.062	–	P=0.094	P=0.347

[a] Number of tumor-bearing animals/number of animals necropsied
[b] Number of tumor-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 control incidence are the P values associated with the trend test. Beneath the dosed group incidence are the P values corresponding to pairwise comparisons between the controls and that dosed group. The logistic regression tests regard tumors in animals dying prior to terminal kill as nonfatal.
[e] Not applicable; no tumors in animal group
[f] Historical incidence for 2-year NTP studies of untreated control groups (mean ± standard deviation): 2/1541 (0.1% ± 0.5%)

TABLE 21
Neoplasms of the Uterus in Female F344/N Rats in the 22-Month Drinking Water Study of C.I. Direct Blue 15

	0 ppm	630 ppm	1,250 ppm	2,500 ppm
Adenocarcinoma				
Overall rates[a]	1/50 (2%)	0/35 (0%)	0/65 (0%)	3/50 (6%)
Effective rates[b]	1/46 (2%)	0/21 (0%)	0/45 (0%)	3/17 (18%)
Terminal rates[c]	1/40 (3%)	0/13 (0%)	0/22 (0%)	0/4 (0%)
First incidence (days)	682 (T)	-[e]	-	607
Logistic regression tests[d]	P=0.042	P=0.723N	P=0.619N	P=0.132
Adenoma or Adenocarcinoma[f]				
Overall rates	1/50 (2%)	0/35 (0%)	1/65 (2%)	4/50 (8%)
Effective rates	1/46 (2%)	0/21 (0%)	1/45 (2%)	4/17 (24%)
Terminal rates	1/40 (3%)	0/13 (0%)	1/22 (5%)	1/4 (25%)
First incidence (days)	682 (T)	-	682 (T)	607
Logistic regression tests	P=0.004	P=0.723N	P=0.623	P=0.025

(T)Terminal kill
[a] Number of tumor-bearing animals/number of animals necropsied
[b] Number of tumor-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 control incidence are the P values associated with the trend test. Beneath the dosed group incidence are the P values corresponding to pairwise comparisons between the controls and that dosed group. The logistic regression tests regard tumors in animals dying prior to terminal kill as nonfatal. For all tests, a negative trend or a lower incidence in a dose group is indicated by N.
[e] Not applicable; no tumors in animal group
[f] Historical incidence for 2-year NTP studies of untreated control groups (mean ± standard deviation): 4/1632 (0.2% ± 0.7%)

or more in diameter. Thus, pheochromocytomas range in size from minute lesions which can only be diagnosed microscopically to large masses which can replace the entire adrenal parenchyma. Pheochromocytomas occur commonly in aged male F344/N rats and are usually considered incidental findings. The apparent earlier onset of pheochromocytomas in high-dose males in this study is presumably due to the high early mortality in this group, which resulted in a larger number of high-dose animals being examined at an earlier age than was the case for controls. It is likely that some of the control animals may have had pheochromocytomas by ages similar to those of the high-dose males, but they were not detected in control animals until later in life because the control animals lived longer. No difference in the incidence of hyperplasia, the precursor of pheochromocytoma, was found between the control and high-dose groups. Consequently, the statistically significant difference between the control and high-dose male groups is not considered to represent a treatment-related effect.

Spleen: Hematopoietic cell proliferation occurred with increased incidence in the spleens of treated male and female rats (males: control, 1/50; low-dose, 3/35; mid-dose, 10/64; high-dose, 17/50; females: control, 5/50; low-dose, 12/35; mid-dose, 20/65; high-dose, 18/50). This effect was considered to be secondary to the inflammation associated with the neoplasms in treated animals.

Bone Marrow: The incidence of hyperplasia of the bone marrow was markedly increased in treated male rats (control, 1/49; low-dose, 14/35; mid-dose, 20/63; high-dose, 16/50). The hyperplasia was due to an increase in hematopoietic cell proliferation secondary to neoplasm-associated inflammation in treated animals.

Heart: The incidence of thrombus within the atrium of the heart was higher in the mid- and high-dose male groups than in the controls (control, 2/50; low-dose, 3/35; mid-dose, 17/65; high-dose, 12/50).

Genetic Toxicity

C.I. Direct Blue 15 was not mutagenic in *Salmonella typhimurium* strains TA100, TA1535, TA1537, or TA98 when tested in a standard preincubation protocol at concentrations of 100 to 10,000 μg/plate in the presence or absence of Aroclor 1254-induced male Sprague-Dawley rat or Syrian hamster liver S9 (Table C1) (Mortelmans *et al.*, 1986). As do most benzidine congener dyes, this compound requires reductive metabolism of the azo bonds to release the parent amine, which can then be oxidatively metabolized to an active mutagen. When tested in such a reductive metabolism protocol, C.I. Direct Blue 15 was mutagenic in *S. typhimurium* strain TA1538 (Table C2) (Reid *et al.*, 1984a,b). Some mutagenic activity was observed in the presence of rat S9 without prior reduction, but the mutagenicity was greatly increased following reduction. The fact that the mutagenic activity of C.I. Direct Blue 15 was less than expected in the bacterial reduction system, based on the comparative activity of the dimethoxybenzidine control, can be explained by the small proportion of dye that was reduced using this system. In a test system using a flavin mononucleotide reduction and hamster S9 activation protocol, the mutagenic activity of C.I. Direct Blue 15 was greater than expected. This increase in mutagenic activity may have resulted from the formation of additional reduction products in the crude dye mixture that was tested.

In cytogenetic tests with Chinese hamster ovary cells, C.I. Direct Blue 15 did not induce sister chromatid exchanges when tested at concentrations up to 750 μg/ml in the absence of S9, or at concentrations up to 2,500 μg/ml in the presence of Aroclor 1254-induced male Sprague-Dawley rat liver S9 (Table C3) (Galloway *et al.*, 1987). No induction of chromosomal aberrations was observed in Chinese hamster ovary cells treated with up to 2,250 μg/ml C.I. Direct Blue 15 without S9 or 2,500 μg/ml with S9 (Table C4) (Galloway *et al.*, 1987). Reductive metabolism was not used in these cytogenetic tests.

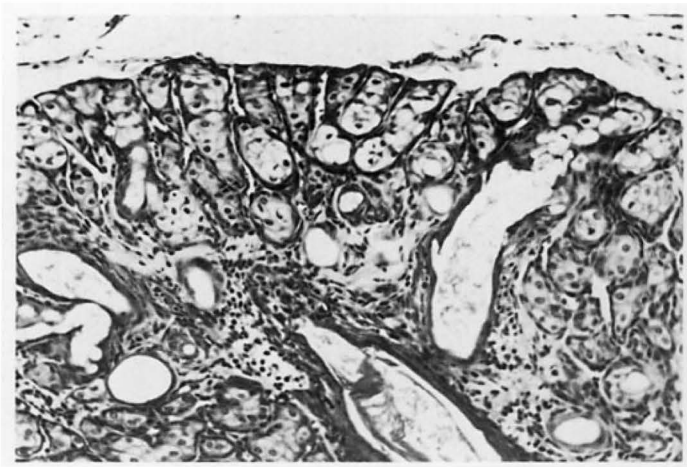


PLATE 1
Sebaceous gland adenoma of the skin in an F344/N rat administered 2,500 ppm C.I. Direct Blue 15 in drinking water for two years. Magnification 150×

PLATE 2
Preputial gland carcinoma in an F344/N rat administered 1,250 ppm C.I. Direct Blue 15 in drinking water for two years. Magnification 150×

PLATE 3
Squamous cell carcinoma of the tongue in an F344/N rat administered 2,500 ppm C.I. Direct Blue 15 in drinking water for two years. Magnification 60×

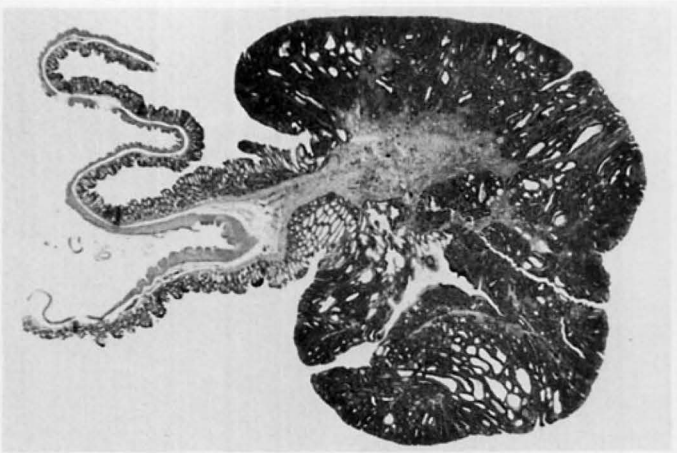


PLATE 4
Adenomatous polyp of the large intestine in an F344/N rat administered 2,500 ppm C.I. Direct Blue 15 in drinking water for two years. Magnification 10×

DISCUSSION AND CONCLUSIONS

The NTP's Benzidine Dye Initiative is a program that was developed to study the toxicology and carcinogenicity of the class of dyes derived from benzidine, 3,3′-dimethylbenzidine dihydrochloride, and 3,3′-dimethoxybenzidine dihydrochloride. The dyes selected for study were representative of the more than 90 benzidine dyes in use. These studies examined the toxic and carcinogenic properties of C.I. Direct Blue 15 (desalted industrial grade). The dye examined was the product to which workers are potentially exposed. The purity of the dye was determined to be about 50%, with approximately 35 impurities.

14-Day Studies

Groups of five rats of each sex were administered C.I. Direct Blue 15 in drinking water at dose levels of 0, 1,250, 2,500, 5,000, 10,000 or 30,000 ppm for 14 days. All control and treated rats survived. Final mean body weights of rats in the 30,000 ppm groups were lower than controls (males, 8%; females, 34%). Water consumption declined with increased dose. Clinical findings included blue-stained eyes, skin, and feces in all treated animals. Organs and tissues of treated rats were also stained blue. Animals receiving 30,000 ppm showed histologic evidence of mild hepatotoxicity; females at this dose level also showed evidence of nephrotoxicity and thymic lymphoid depletion.

13-Week Studies

Male rats received C. I. Direct Blue 15 in drinking water at doses of 0, 1,250, 2,500, 5,000, 10,000, or 30,000 ppm, and female rats received doses of 0, 630, 1,250, 2,500, 5,000, or 10,000 ppm. Seven of ten male rats receiving 30,000 ppm died during the treatment period; animals in all other groups survived until the end of the studies. Final mean body weights were 8% and 31% lower than that of controls in male rats given 10,000 and 30,000 ppm and were 3% and 6% lower than that of controls in female rats given 5,000 and 10,000 ppm. As in the 14-day studies, major organs and tissues from hepatic toxicity was observed in the seven males that died before the end of the studies. An increase in the severity of nephropathy was seen in both males and females receiving 10,000 ppm, and relative kidney weight was higher in rats that received 5,000 ppm or greater than in controls. Because of this kidney toxicity, a high dose of 2,500 ppm was selected for the 22-month studies.

22-Month Studies

The toxicity and carcinogenicity studies of C.I. Direct Blue 15 were designed to last 2 years, but were terminated at 22 months because of extensive early deaths in the treated groups. The allotment of animals to groups was based on study designs recommended by Portier and Hoel (1984): at study initiation, 70 animals per sex received 0 ppm or 2,500 ppm, 45 animals per sex received 630 ppm, and 75 animals per sex received 1,250 ppm. The amount of compound consumed per day by rats at the three dose levels was approximately 45, 90, or 215 mg/kg for male rats and 50, 100, or 200 mg/kg for female rats. Compound consumption calculations were based on average water consumption by groups of animals during these studies. Ten animals in the control and 2,500 ppm groups were evaluated at 9 months and 10 animals from each dose group were evaluated at 15 months. At week 97, the final mean body weights relative to controls of the 630, 1,250, and 2,500 ppm groups were 95%, 91%, and 81% for male rats and 91% for all three dosed female rat groups.

At the 9-month interim evaluation, a Zymbal's gland adenoma was seen in one high-dose male rat, and clitoral gland carcinomas were seen in three high-dose female rats. At the 15-month interim evaluation, two low-dose females, one mid-dose female, three high-dose males, and three high-dose females had Zymbal's gland neoplasms; two mid-dose female rats and one high-dose female rat had clitoral gland carcinomas. At the 9-month and 15-month interim evaluations, lesions were also noted in the skin, preputial gland, intestines, liver, and oral cavity of treated animals, and the percentages of animals with these lesions were higher at 15 months.

In the 22-month studies, chemical-related neoplasms and nonneoplastic lesions were found at many sites, including the Zymbal's gland, skin, oral cavity, intestine, liver, and preputial and clitoral glands in male and female rats; these findings are similar to those observed in the 15-month 3,3′-dimethylbenzidine dihydrochloride and the 21-month 3,3′-dimethoxybenzidine dihydrochloride studies. In the NTP database of over 350 long-term rodent studies, 18 studies include the Zymbal's gland as a site for neoplasm formation in the rat and 16 chemicals caused neoplasms in the skin in the rat; 12 chemicals caused neoplasms in both the Zymbal's gland and skin (Table 22). Many of these chemicals have in common a structure that contains a nitrogen-aromatic bond. Most chemicals that caused Zymbal's gland lesions or skin lesions also caused lesions at other sites in the body and were positive in the NTP *Salmonella typhimurium* mutagenicity assays, as was the case for C.I. Direct Blue 15, 3,3′-dimethoxybenzidine dihydrochloride, and 3,3′-dimethylbenzidine dihydrochloride. The incidences of neoplasms of the epidermis of the skin, the oral cavity epithelium, and the epithelium of the Zymbal's, clitoral, and preputial glands were high in these studies, and neoplasms often occurred at more than one of these sites in the same animal.

The incidences of Zymbal's gland carcinoma or adenoma were markedly increased in rats receiving C.I. Direct Blue 15. The incidences in the treated groups were well above the historical mean for Zymbal's gland lesions in rats in the NTP database for 2-year rodent studies even though the historical rates listed in this report are for 2-year rodent studies, whereas the C.I. Direct Blue 15 study was for 22 months.

The incidences of skin squamous cell neoplasms in male rats (2/50, 4/35, 11/65, 19/50) and in female rats (0/50, 2/35, 6/65, 5/50) were increased above the NTP historical mean for untreated controls (males, 1.8%; females, 0.4%). The incidences of skin basal cell neoplasms were significantly increased in male rats (2/50, 9/35, 27/65, 28/50), but not in female rats (1/50, 0/35, 1/65, 0/50). Exposure of the skin to C.I. Direct Blue 15 may have occurred through the systemic distribution of the chemical or through direct contact with the skin while the animals were grooming.

In female rats, the incidence of clitoral gland adenomas or carcinomas was markedly increased in the treated groups (7/50, 11/31, 24/64, 27/50), and in males the incidence of preputial gland neoplasms was increased in the mid-dose group (8/49, 5/35, 23/64, 9/48). The increase in the incidence of liver neoplasms was more marked in male rats (0/50, 6/35, 9/65, 11/50) than in female rats (0/50, 0/35, 2/65, 5/50), although the incidences of liver neoplasms in both sexes of treated animals were above the mean incidences in the NTP historical database (males, 4.9%; females, 2.3%).

Oral cavity and intestinal neoplasms were seen in male and female rats; these sites may also have been exposed to the chemical either through direct contact or through the systemic circulation. The incidences of squamous cell neoplasms in the oral cavity were significantly increased in male rats (1/50, 10/35, 24/65, 17/50) and female rats (2/50, 4/35, 19/65, 15/50). The incidences of large intestine neoplasms (males: 0/50, 1/35, 6/65, 8/50; females: 0/50, 0/35, 3/65, 1/50) and small intestine neoplasms (males: 0/50, 1/35, 0/65, 2/50; females: 0/50, 0/35, 1/65, 3/50) were increased in male and female rats at 22 months; neoplasms of the large and small intestine were also seen in a few dosed animals evaluated at 15 months. The NTP historical mean for 2-year studies for oral cavity and intestinal neoplasms in rats ranges from 0% to 0.3%.

The incidence of adenoma or adenocarcinoma of the uterus was significantly increased in high-dose females (1/50, 0/35, 1/65, 4/50). These neoplasms occur rarely in untreated rats; the historical mean for NTP 2-year studies is 0.3%.

Neoplasms were found in the brain of males (0/50, 1/35, 1/65, 2/50) and females (1/50, 0/35, 2/65, 1/50). The brain neoplasms were malignant astrocytomas, an uncommon malignant neoplasm of glial cell origin. The incidences of these neoplasms were only marginally increased. However, in view of the reduced survival of treated rats and the low spontaneous occurrence of malignant astrocytomas (historical incidence: males, 0.63%; females, 0.92%), these neoplasms may have been related to chemical exposure. A low incidence of brain astrocytomas was also seen in the rat studies of 3,3′-dimethoxybenzidine dihydrochloride and 3,3′-dimethylbenzidine dihydrochloride.

TABLE 22
Evidence of Zymbal's Gland and Skin Neoplasms in Rats and *Salmonella* Mutagenicity for Selected National Toxicology Program Chemicals

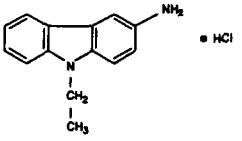
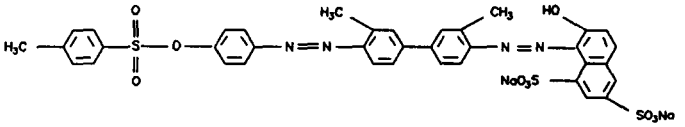
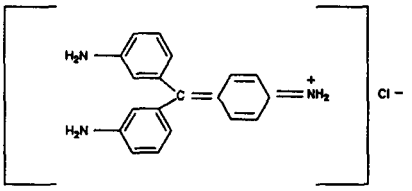
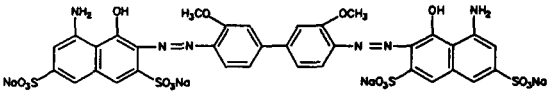
Chemical Name/ Structure	Technical Report Number	Zymbal's Gland Neoplasms M	Zymbal's Gland Neoplasms F	Skin Neoplasms M	Skin Neoplasms F	*Salmonella* Mutagenicity Results
3-Amino-9-Ethylcarbazole HCl 	93	+	+	+		+
Benzene	289	+	+	+		
C.I. Acid Red 114 	405	+	+	+	+	+
C.I. Basic Red 9 Monohydrochloride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

TABLE 22
Evidence of Zymbal's Gland and Skin Neoplasms in Rats and *Salmonella* Mutagenicity for Selected National Toxicology Program Chemicals (continued)

Chemical Name/ Structure	Technical Report Number	Zymbal's Gland Neoplasms M	Zymbal's Gland Neoplasms F	Skin Neoplasms M	Skin Neoplasms 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 22
Evidence of Zymbal's Gland and Skin Neoplasms in Rats and *Salmonella* Mutagenicity for Selected National Toxicology Program Chemicals (continued)

Chemical Name/ Structure	Technical Report Number	Zymbal's Gland Neoplasms M	Zymbal's Gland Neoplasms F	Skin Neoplasms M	Skin Neoplasms F	*Salmonella* Mutagenicity Results
Glycidol	374	+		+		+
Hydrazobenzene	92	+				+
8-Methoxypsoralen	359	+				+
Nithiazide	146				+	+
5-Nitroacenaphthene	118	+	+			+
Nitrofurazone	337				+	+

TABLE 22
Evidence of Zymbal's Gland and Skin Neoplasms in Rats and *Salmonella* Mutagenicity for Selected National Toxicology Program Chemicals (continued)

Chemical Name/ Structure	Technical Report Number	Zymbal's Gland Neoplasms M	Zymbal's Gland Neoplasms F	Skin Neoplasms M	Skin Neoplasms F	*Salmonella* Mutagenicity Results
5-Nitro-*o*-Anisidine	127	+	+	+		+
4,4′-Thiodianiline	47	+	+			+
β-Thioguanidine Deoxyriboside	57		+			
Tris(Aziridinyl)Phosphine Sulfide	58	+	+	+	+	+
4-Vinyl-1-Cyclohexene Diepoxide	362			+	+	+

When analyzed by life table or logistic regression analyses, the incidence of mononuclear cell leukemia was significantly increased in female rats (7/50, 13/35, 27/65, 15/50) and marginally increased in male rats (17/50, 19/35, 28/65, 20/50). A marginal increase in the incidence of mononuclear cell leukemia was also seen in female rats in the studies of 3,3′-dimethylbenzidine. The historical mean for mononuclear cell leukemia in female rats is 13.9%.

The benzidine dyes have been found to be genotoxic agents (Table 23). Available data from metabolism studies indicate that C.I. Direct Blue 15 is metabolized to its parent compound (dimethoxybenzidine), the likely precursor of the active moiety. C.I. Direct Blue 15 was negative in the standard NTP *Salmonella* assays, but was positive in *Salmonella typhimurium* strain TA1538 when reductive metabolism was used. C.I. Direct Blue 15 has aromatic-amine groupings, which are considered to be "structural alerts" for genotoxic activity (Ashby and Tennant, 1988).

Tumor development and formation may occur through several mechanisms. Talaska *et al.* (1987) have shown that benzidine or a benzidine metabolite can form DNA adducts in the mouse liver and have suggested that benzidine may cause tumor formation through somatic mutations, which allow a cell to escape the growth control of the organism and become a neoplasm. Büsser and Lutz (1987) investigated the stimulation of liver DNA synthesis and cell proliferation by DNA-binding carcinogens (benzidine, carbon tetrachloride, and aflatoxin) and hepatic tumor promoters (DDT, phenobarbital, and thioacetamide) and found that the DNA-binding carcinogens did not stimulate liver DNA synthesis, but the tumor promoters did. Similar studies have not been carried out with C.I. Direct Blue 15.

TABLE 23
Comparison of National Toxicology Program Mutagenicity Test Results for Selected Benzidine Dyes[a]

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

[a] Results are presented as result of test without S9/result of test with S9. 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.

[b] Positive in *Salmonella* 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. These compounds are not mutagenic in TA1538 in the absence of a reducing system (Reid *et al.*, 1984a).

[c] Not mutagenic with reductive metabolism.

In other studies, BALB/c mice were treated with 3,3′-dimethylbenzidine dihydrochloride in drinking water at doses ranging from 5 to 150 ppm for up to 116 weeks, or with 3,3′-dimethoxybenzidine dihydrochloride in the drinking water at doses ranging from 20 to 630 ppm for up to 112 weeks. Dose-related increases in the incidence of lung alveolar cell neoplasms were seen in males, but not in females, after 3,3′-dimethylbenzidine dihydrochloride treatment. No increases in neoplasm incidence were seen in the mice treated with 3,3′-dimethoxybenzidine dihydrochloride (Schieferstein *et al.*, 1990). The spectrum of lesions observed in these mouse studies was quite different from that seen in the NTP rat studies.

Benzidine and related aromatic amines produce neoplasms 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, the liver is the major organ affected (Bonser *et al.*, 1956; Vesselinovitch *et al.*, 1975; Littlefield *et al.*, 1983; IARC, 1987), and in rats, benzidine and other aminobiphenyls cause neoplasms in the Zymbal's gland, mammary gland, skin, intestine, and liver. These differences may be related to species-specific and organ-specific differences in metabolism.

A number of aromatic amines cause neoplasms in the Zymbal's gland. Reported to be deficient in sulfotransferase activity (Irving *et al.*, 1971) and transacylase activity (Bartsch *et al.*, 1973), the Zymbal's gland 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 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 additional activation to form reactive esters, which act as ultimate carcinogens (Miller and Miller, 1977). Formation of different esters by different species may result in variations in organ specificity (Cohen, 1983).

Neoplasm Transplant Study

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.

Oncogene Activation

Neoplasms obtained from control rats and rats exposed to 3,3′-dimethoxybenzidine dihydrochloride or C.I. Direct Blue 15 (a 3,3′-dimethoxybenzidine-derived dye) were assayed for the presence of activated protooncogenes by the NIH 3T3 DNA transfection assay (Reynolds *et al.*, 1990). Oncogenes detectable by DNA transfection analysis were present in 21/27 skin, clitoral gland, or preputial gland neoplasms that had been induced by 3,3′-dimethoxybenzidine dihydrochloride or C.I. Direct Blue 15. DNA from either benign or malignant neoplasms was capable of inducing morphologically transformed foci in NIH 3T3 mouse fibroblast cultures.

Thirteen of the chemical-induced neoplasm types were of epidermal origin and were classified as basal or squamous cell neoplasms of the skin; activated *ras* oncogenes were detected at a high frequency in

these neoplasms (11/13). Histogenetically related neoplasms of the clitoral and preputial glands also had a high frequency of activated *ras* oncogenes (10/14). In contrast, the occurrence of *ras* oncogene activation in spontaneous epithelial neoplasms of the F344/N rat is low (1/21).

It is possible that chemical-induced neoplasms were derived from a common epidermal progenitor stem-cell population that was susceptible to electrophilic attack by activated metabolites of 3,3′-dimethoxybenzidine dihydrochloride or C.I. Direct Blue 15. A relatively high percentage (62%) of the chemical-induced rat neoplasms contained activated alleles of either H-*ras* or N-*ras*.

CONCLUSIONS

Under the conditions of these 22-month drinking water studies, there was *clear evidence of carcinogenic activity** of C.I. Direct Blue 15 (desalted industrial grade) in male F344/N rats, as indicated by benign and malignant neoplasms of the skin, Zymbal's gland, preputial gland, liver, oral cavity, and small and large intestine. Increased incidences of mononuclear cell leukemia and neoplasms of the brain may have been related to chemical administration. There was *clear evidence of carcinogenic activity* of C.I. Direct Blue 15 in 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, and uterus, and by mononuclear cell leukemia.

*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 appear on page 11.

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Appendix F: Carcinogen Profile for DMOB and DMOB Dihydrochloride (NTP 8th Report on Carcinogens 1998)

3,3′-Dimethoxybenzidine and 3,3′-Dimethoxybenzidine Dihydrochloride CAS Nos. 119-90-4 and 20325-40-0

First Listed in the *Third Annual Report on Carcinogens*

Carcinogenicity

There is sufficient evidence for the carcinogenicity of 3,3′-dimethoxybenzidine in experimental animals (IARC V.4, 1974; IARC S.4, 1982). When administered by gavage, 3,3′-dimethoxybenzidine induced tumors at various sites including Zymbal gland tumors, intestinal carcinomas, skin carcinomas, and urinary bladder papillomas in rats of both sexes. When administered in the diet, 3,3′-dimethoxybenzidine increased the incidence of forestomach papillomas. When administered in the drinking water, 3,3′-dimethoxybenzidine dihydrochloride increased the incidence of Zymbal gland adenomas and carcinomas, liver neoplastic nodules or hepatocellular carcinomas, large intestine adenomatous polyps or adenocarcinomas, and squamous cell papillomas or carcinomas of the oral cavity in rats of both sexes; preputial gland carcinomas, basal cell adenomas and carcinomas of the skin, adenocarcinomas of the small intestine, and mesotheliomas in male rats; and clitoral gland adenomas and carcinomas, basal cell adenomas or carcinomas of the skin, mammary gland adenocarcinomas, and uterus/cervix adenomas or carcinomas in female rats (NTP 372, 1990).

An IARC Working Group reported that there is inadequate evidence for the carcinogenicity of 3,3′-dimethoxybenzidine in humans. No epidemiological data on the occurrence of cancer in workers exposed to 3,3′-dimethoxybenzidine alone appear in the literature. Most of the workers exposed to this substance were also exposed to related amines, such as benzidine, which are strongly associated with urinary bladder cancer in humans (see Benzidine, Section III.A) (IARC, V.4, 1974; IARC S.4, 1982; IARC S.7, 1987).

Properties

3,3′-Dimethoxybenzidine occurs as colorless crystals which turn violet upon standing. It is virtually insoluble in water and probably soluble in most organic solvents (e.g., ethanol, ether, acetone, benzene, and chloroform). 3,3′-Dimethoxybenzidine is available commercially as the free base (technical and 99% grades) and as its dihydrochloride salt. When heated to decomposition, 3,3′-dimethoxybenzidine emits toxic fumes of nitrogen oxides (NO_x).

Use

3,3′-Dimethoxybenzidine is used principally as a chemical intermediate for the production of azo dyes. The Society of Dyers and Colourists reported its use as an intermediate in the production of 89 dyes in 1971. Among the dyes listed were Direct Blue 218, Pigment Orange 16, Direct Blue 1, Direct Blue 15, Direct Blue 8, Direct Blue 76, and Direct Blue 98. About 30% of the 3,3′-dimethoxybenzidine consumed is used as a chemical intermediate to produce o-dianisidine diisocyanate for use in adhesive systems and as a component of polyurethane elastomers and resins. 3,3′-Dimethoxybenzidine is used as a dye for leather, paper, plastics, rubber, and textiles, and a reagent to detect metals, thiocyanates, and nitrites (IARC V.4, 1974).

Production

The Chem Sources USA directory did not identify any producers of 3,3′-dimethoxybenzidine in 1986, but listed 13 suppliers (Chem Sources, 1986). No other current production data were available. U.S. imports of 3,3′-dimethoxybenzidine were reported by the USITC to be 106,000 lb and imports of 3,3′-dimethoxybenzidine and its dihydrochloride were reported to be 655,000 lb in 1983 (USITCa, 1984). The 1979 TSCA Inventory identified two companies producing an unspecified amount of 3,3′-dimethoxybenzidine and six companies importing 55,500 lb in 1977. The CBI Aggregate was less than 1 million lb (TSCA 1979). U.S. imports of 3,3′-dimethoxybenzidine through the principal custom districts were reported to be 273,000 lb in 1971. Data on domestic production of 3,3′-dimethoxybenzidine were last reported in 1967, when five companies produced 368,000 lb; 3,3′-dimethoxybenzidine has been produced commercially for at least 50 years (IARC V.4, 1974).

Exposure

The primary routes of potential human exposure to 3,3′-dimethoxybenzidine are inhalation and dermal contact. Exposure to 3,3′-dimethoxybenzidine can occur during its use as a chemical intermediate in the production of azo dyes, o-dianisidine diisocyanate formulations, textile processing, and packaging processes. Workers potentially exposed to the chemical include dye makers and o-dianisidine diisocyanate production workers. However, present dye production processes for 3,3′-dimethoxybenzidine and its dye derivatives are generally closed systems with minimal risk to workers. The National Occupational Hazard Survey, conducted by NIOSH from 1972 to 1974, estimated that 200 workers were possibly exposed to 3,3′-dimethoxybenzidine in the workplace (NIOSH, 1976). Potential human exposure may occur as a result of the presence of trace contaminants in end products that are formulated with 3,3′-dimethoxybenzidine (e.g., azo dyes, pigments, adhesives, resins, and polyurethane elastomers). CPSC is concerned that these dyes and pigments contain residual levels or trace impurities of 3,3′-dimethoxybenzidine in the ppm range and that traces may be present in the final consumer products. Presently, no data are available on the actual quantities in the final consumer products. A dermal penetration study in rabbits indicated that a 3,3′-dimethoxybenzidine-based dye was not dermally absorbed in significant amounts.

Regulations

In late 1980, CPSC started collecting data to propose a ban on the use of 3,3′-dimethoxybenzidine-based dyes in mass-merchandised consumer dye products; however, the use of benzidine congener dyes in consumer household dyeing products and in commercial textile applications has been decreased voluntarily. Artists and crafts people have been alerted to potential hazards from inhaling powders of dyes based on 3,3′-dimethoxybenzidine. EPA regulates 3,3′-dimethoxybenzidine under the Comprehensive Environmental Response, Compensation, and Liability Act (CERCLA), Resource Conservation and Recovery Act (RCRA), and Superfund Amendments and Reauthorization Act (SARA). An adjustment of the statutory reportable quantity (RQ) from 1 lb to 100 lb has been established for this chemical under CERCLA. RCRA regulates 3,3′-dimethoxybenzidine as a hazardous constituent of waste. EPA has included 3,3′-dimethoxybenzidine on a list of

priority hazard substances under SARA. OSHA regulates 3,3'-dimethoxybenzidine and its dihydrochloride salt under the Hazard Communication Standard and as chemical hazards in laboratories.