BENZIDINE

1. Exposure Data

This section includes data on benzidine-based dyes, benzidine congeners and benzidine-congener-based dyes.

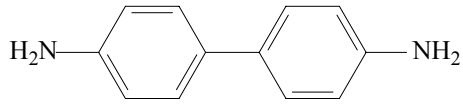
1.1 Benzidine and benzidine-based dyes – Chemical and physical data

1.1.1 *Benzidine*

(a) Nomenclature

Chem. Abstr. Serv. Reg. No.: 92–87–5
CAS Name: [1,1'-Biphenyl]-4,4'-diamine
Synonyms: 4'-Amino-[1,1'-biphenyl]-4-ylamine; 4-(4-aminophenyl)-aniline; benzidine; 4,4'-bianiline; *p,p′*-bianiline; 4,4'-biphenyldiamine; 4,4'-diamino-1,1'-biphenyl; C.I. 37225; C.I. Azoic Diazo Component 112; 4,4'-diaminobiphenyl; *p,p′*-diaminobiphenyl; 4,4'-diaminodiphenyl; *p*-diaminodiphenyl; 4,4'-diphenylenediamine

(b) Structural formula, molecular formula, and relative molecular mass



$C_{12}H_{12}N_2$ Rel. mol. mass: 184.24

(c) Chemical and physical properties of the pure substance

Description: White or slightly-reddish, crystalline powder (O'Neil, 2006)
Boiling-point: 401 °C (Lide, 2008)
Melting-point: 120 °C (Lide, 2008)
Solubility: Slightly soluble in water, diethyl ether, and dimethyl sulfoxide; soluble in ethanol (Lide, 2008)

(d) Trade name

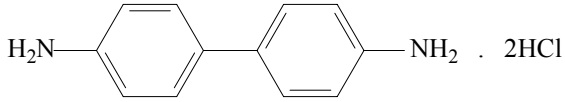
Trade name: Fast Corinth Base B.

1.1.2 *Benzidine dihydrochloride*

(a) Nomenclature

Chem. Abstr. Serv. Reg. No.: 531–85–1
CAS Name: [1,1'-Biphenyl]-4,4'-diamine, hydrochloride (1:2)
Synonym: Benzidine hydrochloride; [1,1'-biphenyl]-4,4'-diamine, dihydrochloride

(b) Structural formula, molecular formula, and relative molecular mass



$C_{12}H_{12}N_2.2HCl$ Rel. mol. mass: 257.16

(c) Chemical and physical properties of the pure substance

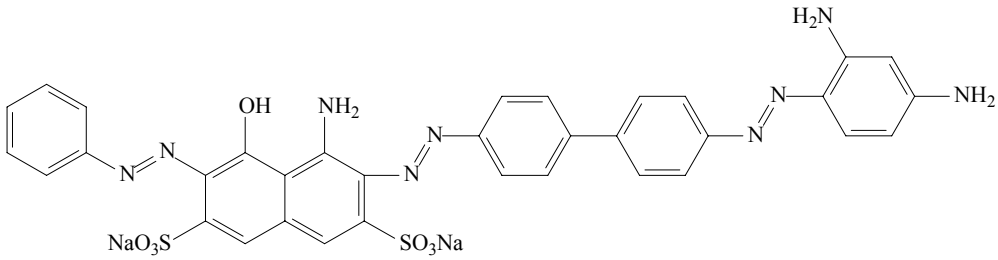
Description: Crystals (O'Neil, 2006)
Solubility: Soluble in water and ethanol (O'Neil, 2006)

1.1.3 *C.I. Direct Black 38*

(a) Nomenclature

Chem. Abstr. Serv. Reg. No.: 1937–37–7
CAS Name: 4-Amino-3-[2-[4'-[2-(2,4-diaminophenyl)diazenyl][1,1'-biphenyl]-4-yl]diazenyl]-5-hydroxy-6-(2-phenyldiazenyl)-2,7-naphthalenedisulfonic acid, sodium salt (1:2)
Synonyms: 4-Amino-3-[[4'-[(2,4-diaminophenyl)azo][1,1'-biphenyl]-4-yl]azo]-5-hydroxy-6-(phenylazo)-2,7-naphthalenedisulfonic acid, disodium salt; C.I. 30235; C.I. Direct Black 38; C.I. Direct Black 38, disodium salt; Direct Black 38; disodium 4-amino-3-[[4'-[(2,4-diaminophenyl)azo][1,1'-biphenyl]-4-yl]azo]-5-hydroxy-6-(phenylazo)-2,7-naphthalenedisulfonate; disodium 4-amino-3-[[4'-[(2,4-diaminophenyl)azo][1,1'-biphenyl]-4-yl]azo]-5-hydroxy-6-(phenylazo)naphthalene-2,7-disulfonate

(b) Structural formula, molecular formula, and relative molecular mass



$C_{34}H_{25}N_9O_7S_2.2Na$ Rel. mol. mass: 781.73

(c) Chemical and physical properties of the pure substance

Description: Grey-black powder (IARC, 1982)
Solubility: Soluble in water; moderately soluble in ethanol and ethylene glycol monoethyl ether; insoluble in other organic solvents (IARC, 1982)

(d) Trade names

Trade names: ATul Direct Black E; Ahco Direct Black GX; Airedale Black ED; Aizen Direct Deep Black EH; Aizen Direct Deep Black GH; Aizen Direct Deep Black RH; Amanil Black GL; Amanil Black WD; Apomine Black GK; Apomine Black GX; Atlantic Black BD; Atlantic Black C; Atlantic Black E; Atlantic Black EA; Atlantic Black GAC; Atlantic Black GG; Atlantic Black GXCW; Atlantic Black GXOO; Atlantic Black SD; Azine Deep Black EW; Azocard Black EW; Azomine Black EWO; Belamine Black GX; Bencidal Black E; Benzamil Black E; Benzanil Black E; Benzo Deep Black E; Benzo Leather Black E; Benzoform Black BCN-CF; Black 2EMBL; Black 4EMBL; Brasilamina Black GN; Brilliant Chrome Leather Black H; Calcomine Black; Calcomine Black EXL; Carbide Black E; Chloramine Black C; Chloramine Black EC; Chloramine Black ERT; Chloramine Black EX; Chloramine Black EXR; Chloramine Black XO; Chloramine Carbon Black S; Chloramine Carbon Black SJ; Chloramine Carbon Black SN; Chlorazol Black E; Chlorazol Black EA; Chlorazol Black EN; Chlorazol Burl Black E; Chlorazol Leather Black ENP; Chlorazol Silk Black G; Chlorazol black; Chrome leather Black E; Chrome leather Black EC; Chrome leather Black EM; Chrome leather Black G; Chrome leather Brilliant Black ER; Coir Deep Black C; Columbia Black EP; Columbus Black EP; Coranil Direct Black F; Diacotton Deep Black; Diacotton Deep Black RX; Diamine Deep Black EC; Diamine Direct Black E; Diaphtamine Black V; Diazine Black E; Diazine Direct Black E; Diazine Direct Black G; Diazol Black 2V; Diphenyl deep Black G; Direct Black A; Direct Black BRN; Direct Black CX; Direct Black CXR; Direct Black E; Direct Black EW; Direct Black EX; Direct Black FR; Direct Black GAC; Direct Black GW; Direct Black GX; Direct Black GXR; Direct Black JET;

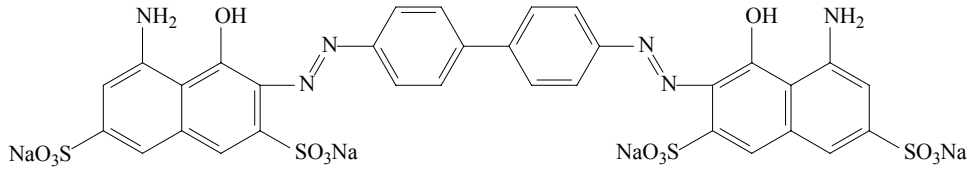
Direct Black Meta; Direct Black Methyl; Direct Black N; Direct Black RX; Direct Black SD; Direct Black WS; Direct Black Z; Direct Deep Black E; Direct Deep Black E Extra; Direct Deep Black EA-CF; Direct Deep Black EAC; Direct Deep Black EW; Direct Deep Black EX; Enianil Black CN; Erie Black B; Erie Black BF; Erie Black GAC; Erie Black GXOO; Erie Black JET; Erie Black NUG; Erie Black RXOO; Erie Brilliant Black S; Erie Fibre Black VP; Fenamin Black E; Fibre Black VF; Fixanol Black E; Formaline Black C; Formic Black C; Formic Black CW; Formic Black EA; Formic Black MTG; Formic Black TG; Hispamin Black EF; Interchem Direct Black Z; Kayaku Direct Deep Black EX; Kayaku Direct Deep Black GX; Kayaku Direct Deep Black S; Kayaku Direct Leather Black EX; Kayaku Direct Special Black AAX; Lurazol Black BA; META Black; Mitsui Direct Black EX; Mitsui Direct Black GX; Nippon Deep Black; Nippon Deep Black GX; Paper Black BA; Paper Black T.

1.1.4 *C.I. Direct Blue 6*

(a) Nomenclature

Chem. Abstr. Serv. Reg. No.: 2602–46–2
CAS Name: 3,3′-[[1,1'-Biphenyl]-4,4'-diylbis(2,1-diazenediyl)]bis[5-amino-4-hydroxy-2,7-naphthalenedisulfonic acid], sodium salt (1:4)
Synonyms: 3,3′-[[1,1'-Biphenyl]-4,4'-diylbis(azo)]bis[5-amino-4-hydroxy-2,7-naphthalenedisulfonic acid], tetrasodium salt; 3,3′-[[1,1'-biphenyl]-4,4'-diylbis(azo)]bis[5-amino-4-hydroxy-2,7-naphthalenedisulfonic acid], tetrasodium salt; 2,2'-(4,4'-biphenylylenebisazo)bis[8-amino-1-naphthol-3,6-disulfonic acid], tetrasodium salt; C.I. 22610; C.I. Direct Blue 6; C.I. Direct Blue 6, tetrasodium salt; tetrasodium 3,3′-[[1,1'-biphenyl]-4,4'-diylbis(azo)]bis[5-amino-4-hydroxynaphthalene-2,7-disulphonate]

(b) Structural formula, molecular formula, and relative molecular mass



$C_{32}H_{20}N_6O_{14}S_4.4Na$ Rel. mol. mass: 932.76

(c) Chemical and physical properties of the pure substance

Description: Blue-violet solid (IARC, 1982)

Solubility: Soluble in water; slightly soluble in ethanol and ethylene glycol monoethyl ether; insoluble in other organic solvents (IARC, 1982)

(d) Trade names

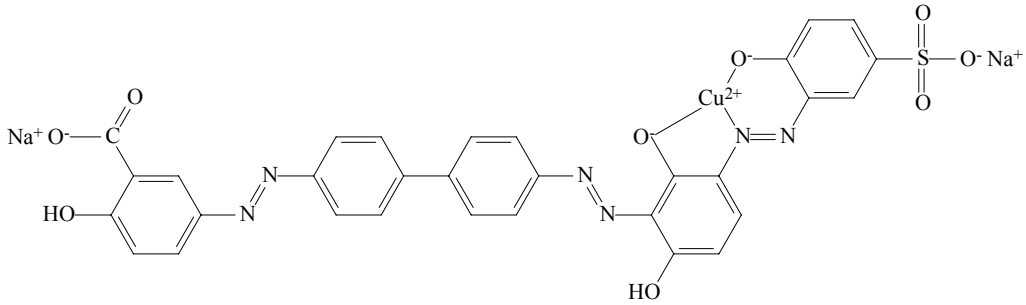
Trade names: Airedale Blue 2BD; Aizen Direct Blue 2BH; Amanil Blue 2BX; Atlantic Blue 2B; Atul Direct Blue 2B; Azocard Blue 2B; Azomine Blue 2B; Belamine Blue 2B; Bencidal Blue 2B; Benzanil Blue 2B; Benzo Blue 2B; Benzo Blue BBA-CF; Benzo Blue BBN-CF; Benzo Blue GS; Blue 2B; Blue 2B salt; Brasilamina Blue 2B; Calcomine Blue 2B; Chloramine Blue 2B; Chlorazol Blue B; Chlorazol Blue BP; Chrome Leather Blue 2B; Cresotine Blue 2B; Diacotton Blue BB; Diamine Blue; Diamine Blue 2B; Diamine Blue BB; Diaphtamine Blue BB; Diazine Blue 2B; Diazol Blue 2B; Diphenyl Blue 2B; Diphenyl Blue KF; Diphenyl Blue M2B; Direct Blue 2B; Direct Blue 2BA; Direct Blue 6; Direct Blue A; Direct Blue BB; Direct Blue GS; Direct Blue K; Direct Blue M2B; Direct Sky Blue K; Enianil Blue 2BN; Fenamin Blue 2B; Fixanol Blue 2B; Hispamin Blue 2B; Indigo Blue 2B; Kayaku Direct; Kayaku Direct Blue BB; Mitsui Direct Blue 2BN; Modr Prima 6; Naphtamine Blue 2B; Niagara Blue 2B; Nippon Blue BB; Paramine Blue 2B; Phenamine Blue BB; Pheno Blue 2B; Pontamine Blue BB; Tertrodirect Blue 2B; Vondacel Blue 2B.

1.1.5 *C.I. Direct Brown 95*

(a) Nomenclature

Chem. Abstr. Serv. Reg. No.: 16071–86–6
CAS Name: [2-hydroxy-5-[2-[4'-[2-[2-(hydroxy-**κ**O)-6-hydroxy-3-[2-[2-(hydroxy-**κ**O)-5-sulfophenyl]diazenyl-**κ**N1]phenyl]diazenyl][1,1'-biphenyl]-4-yl]diazenyl]benzoato(4-)]-cuprate(2-), sodium (1:2)
Synonyms: C.I. 30145; C.I. Direct Brown 95; 5-[[4'-[[2,6-dihydroxy-3-[(2-hydroxy-5-sulfophenyl)azo]phenyl]azo][1,1'-biphenyl]-4-yl]azo]-2-hydroxybenzoic acid, copper complex; [dihydrogen 5-[[4'-[[2,6-dihydroxy-3-[(2-hydroxy-5-sulphophenyl)azo]phenyl]azo]-4-biphenylyl]azo]salicylato(2-)]-copper, disodium salt; [5-[[4'-[[2,6-dihydroxy-3-[(2-hydroxy-5-sulfophenyl)azo]phenyl]azo][1,1'-biphenyl]-4-yl]azo]-2-hydroxybenzoato(4-)]-cuprate(2-), disodium; [5-[[4'-[[2-(hydroxy-**κ**O)-6-hydroxy-3-[[2-(hydroxy-**κ**O)-5-sulfophenyl]azo-**κ**N1]phenyl]azo][1,1'-biphenyl]-4-yl]azo]-2-hydroxybenzoato(4-)]-cuprate(2-), disodium

(b) Structural formula, molecular formula, and relative molecular mass



$C_{31}H_{18}CuN_6O_9S.2Na$ Rel. mol. mass: 760.10

(c) Chemical and physical properties of the pure substance

Description: Reddish-brown powder
Solubility: Soluble in water; slightly soluble in ethanol; insoluble in acetone (IARC, 1982)

(d) Trade names

Trade names: Aizen Primula Brown BRLH; Aizen Primula Brown PLH; Amanil Fast Brown BRL; Amanil Supra Brown LBL; Atlantic Fast Brown BRL; Atlantic Resin Fast Brown BRL; Belamine Fast Brown BRLL; Benzamil Supra Brown BRLL; Benzanil Supra Brown BRLL; Benzanil Supra Brown BRLN; Brown 4EMBL; Calcodur Brown BRL; Chloramine Fast Brown BRL; Chloramine Fast Brown BRLL; Chloramine Fast Cutch Brown PL; Chlorantine Fast Brown BRLL; Chrome Leather Brown BRLL; Chrome Leather Brown BRSL; Cuprofix Brown GL; Derma Fast Brown W-GL; Dermafix Brown PL; Dialuminous Brown BRS; Diaphtamine Light Brown BRLL; Diaphthamine Light Brown BRLL; Diazine Fast Brown RSL; Diazol Light Brown BRN; Dicorel Brown LMR; Diphenyl Fast Brown BRL; Direct Brown BRL; Direct Fast Brown BRL; Direct Fast Brown LMR; Direct Light Brown BRS; Direct Supra Light Brown ML; Durazol Brown BR; DuroFast Brown BRL; Eliamina Light Brown BRL; Enianil Light Brown BRL; Fastolite Brown BRL; Fastusol Brown LBRSA; Fastusol Brown LBRSN; Fenaluz Brown BRL;Helion Brown BRSL; Hispaluz Brown BRL; Ismafast Brown BRSL; KCA Light Fast Brown; KCA Light Fast Brown BR; Kayarus Supra Brown BRS; Paranol Fast Brown BRL; Peeramine Fast Brown BRL; Pontamine Fast Brown BRL; Pontamine Fast Brown NP; Pyrazol Fast Brown BRL; Pyrazoline Brown BRL; Saturn Brown LBR; Sirius Supra Brown BRL; Sirius Supra Brown BRS; Solantine Brown BRL; Solar Brown PL; Solex Brown R; Solius Light Brown BRLL; Solius Light Brown BRS; Sumilight Supra Brown BRS; Suprazo Brown BRL; Suprexcel Brown BRL; Tertrodirect Fast Brown BR; Tetramine Fast Brown BRDN Extra; Tetramine Fast Brown BRP;

Tetramine Fast Brown BRS; Triantine Brown BRS; Triantine Fast Brown OG; Triantine Fast Brown OR; Triantine Light Brown BRS; Triantine Light Brown OG.

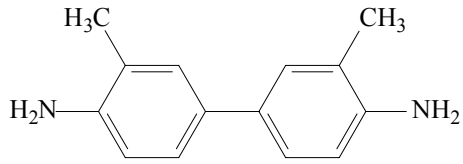
1.2 3,3′-Dimethylbenzidine and 3,3′-dimethylbenzidine-based dyes – Chemical and physical data

1.2.1 *3,3′-Dimethylbenzidine*

(a) Nomenclature

Chem. Abstr. Serv. Reg. No.: 119–93–7
CAS Name: 3,3′-Dimethyl[1,1-biphenyl]-4,4′-diamine
Synonyms: 4′-Amino-3,3′-dimethyl[1,1′-biphenyl]-4-ylamine; 4,4′-bi-*ortho*-toluidine; C.I. 37230; 4,4′-diamino-3,3′-dimethyl-1,1′-biphenyl; 4,4′-diamino-3,3′-dimethylbiphenyl; 4,4′-diamino-3,3′-dimethyldiphenyl; diaminoditolyl; diaminotolyl; 3,3′-dimethyl-(1,1′-biphenyl)-4,4′-diamine; 3,3′-dimethylbiphenyl-4,4′-diamine; 3,3′-dimethyl-4,4′-biphenyldiamine; 3,3′-dimethyl-4,4′-diphenyldiamine; 3,3′-dimethyldiphenyl-4,4′-diamine; 4,4′-di-*ortho*-toluidine; 3,3′-tolidine; *ortho*, *ortho*′-tolidine; 2-tolidine; *ortho*-tolidine

(b) Structural formula, molecular formula, and relative molecular mass



$C_{14}H_{16}N_2$ Rel. mol. mass: 212.29

(c) Chemical and physical properties of the pure substance (O'Neil 2006)

Description: White to reddish crystals or crystalline powder
Melting-point: 129–131°C
Solubility: Slightly soluble in water; soluble in ethanol, diethyl ether, and dilute acids

(d) Technical products and impurities

Trade names: Fast Dark Blue Base R; and C.I. Azoic Diazo Component 113.

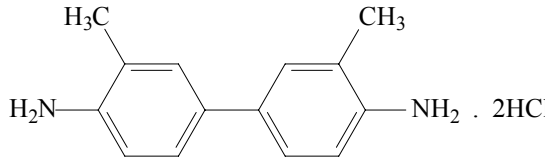
1.2.2 *3,3′-Dimethylbenzidine dihydrochloride*

(a) Nomenclature

Chem. Abstr. Serv. Reg. No.: 612–82–8

CAS Name: 3,3′-Dimethyl[1,1-biphenyl]-4,4′-diamine, hydrochloride (1:2)
Synonyms: 3,3′-Dimethyl[1,1-biphenyl]-4,4′-diamine, dihydrochloride; *ortho*-tolidine dihydrochloride

(b) Structural formula, molecular formula, and relative molecular mass



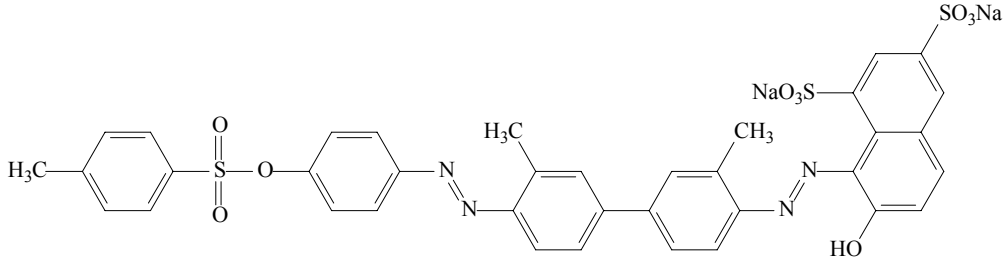
$C_{14}H_{16}N_2.2HCl$ Rel. mol. mass: 285.21

1.2.3 *C.I. Acid Red 114*

(a) Nomenclature

Chem. Abs. Serv. Reg. No.: 6459–94–5
CAS Name: 8-[2-[3,3′-Dimethyl-4'-[2-[4-[[(4-methylphenyl)sulfonyl]oxy]phenyl]diazenyl] [1,1'-biphenyl]-4-yl]diazenyl]-7-hydroxy-1,3-naphthalenedisulfonic acid, sodium salt (1:2)
Synonyms: C.I. 23635; C.I. Acid Red 114; C.I. Acid Red 114, disodium salt; 8-[[3,3′-Dimethyl-4'-[[4-[[(4-methylphenyl)sulfonyl]oxy]phenyl]azo][1,1'-biphenyl]-4-yl]azo]-7-hydroxy-1,3-naphthalenedisulfonic acid, disodium salt; disodium 8-((3,3′-dimethyl-4'-(4-(4-methylphenylsulphonyloxy)phenylazo)(1,1'-biphenyl)-4-yl)azo)-7-hydroxynaphthalene-1,3-disulphonate

(b) Structural formula, molecular formula, and relative molecular mass



$C_{37}H_{28}N_4O_{10}S_3.2Na$ Rel. mol. mass: 830.82

(c) Chemical and physical properties of the pure substance (O'Neil 2006)

Description: Red powder (NTP, 1991a)
Solubility: Soluble in water (NTP, 1991a)

(d) Trade names

Trade names: Acid Leather Red BG; Acid Milling Red BS; Acid Milling Red RS; Acid Red F-RS; Acid Red P-RS; Acid Red RS; Amacid Milling Red PRS; Anadurm Red M-R; Apollo Nylon Fast Red R; Atul Acid Milling Red RS; Benzyl Fast Red BG; Benzyl Red BR; Best Acid Milling Red RS; Colomill Red RS; Concorde Acid Red M-RS; Concorde Leather Red RSN; Covalene Red RS; Covalene Scarlet RS; Covanyl Scarlet RS; Daedo Acid Red RS; Dinacid Milling Red RG; Dycosweak Acid RS; Elcacid Milling Fast Red RS; Eniacid Fast Red R; Erionyl Red RS; Erionyl Red RS 125; Everacid Milling Red RS; Everlan Red RS; Fabracid Red M-RS; Fenafor Red PB; Folan Red B; Indacid Milling Red RS; Intrazone Red BR; Kayanol Milling Red RS; Kayanol Milling Red RS 125; Kenamide Red K 2R; Kenanthrol Red R; Leather Fast Red B; Lerui Acid Red F-RS; Levanol Red GG; Midlon Red PRS; Milling Fast Red B; Milling Fast Red R; Milling Red B; Milling Red BB; Milling Red SWB; Monacid Red RS; Polar Red RS; Sandolan Red N-RS; Sella Fast Red RS; Sulphonol Fast Red R; Sulphonol Red R; Suminol Milling Red RS; Supranol Fast Red 3G; Supranol Fast Red GG; Supranol Red PBX-CF; Supranol Red R; Telon Fast Red GG; Tertracid Milling Red B; Tetracid Milling Red B; Tetracid Milkling Red G; Vondamol Fast Red RS.

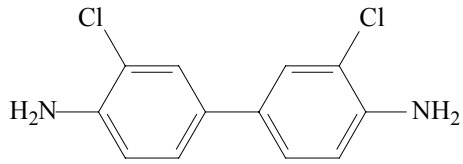
1.3 3,3′-Dichlorobenzidine – Chemical and physical data

1.3.1 *3,3′-Dichlorobenzidine*

(a) Nomenclature

Chem. Abs. Serv. Reg. No.: 91–94–1
CAS Name: 3,3′-Dichloro-[1,1'-biphenyl]-4,4'-diamine
Synonyms: 4'-Amino-3,3′-dichloro[1,1'-biphenyl]-4-ylamine; C.I. 23060; 4,4'-diamino-3,3′-dichlorobiphenyl; 4,4'-diamino-3,3′-dichlorodiphenyl; *ortho*, *ortho*'-dichlorobenzidine; 3,3′-dichloro-*para*, *para*'-bianiline; 3,3′-dichlorobiphenyl-4,4'-diamine; 3,3′-dichloro-4,4'-diamino(1,1-biphenyl); 3,3′-dichloro-4,4'-diaminobiphenyl

(b) Structural formula, molecular formula, and relative molecular mass



$C_{12}H_{10}Cl_2N_2$ Rel. mol. mass: 253.13

(c) Chemical and physical properties of the pure substance

Description: Needles from alcohol (O'Neil, 2006; Lide, 2008)
Melting-point: 132.5°C (Lide, 2008)
Solubility: Insoluble in water; soluble in acetic acid, benzene, and ethanol (Lide, 2008)

(d) Trade names

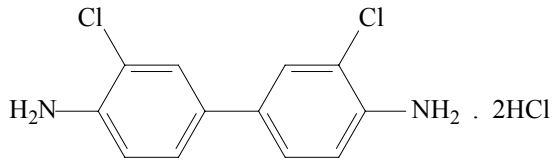
Trade names for 3,3′-dichlorobenzidine include: Curithane C 126.

1.3.2 *3,3′-Dichlorobenzidine dihydrochloride*

(a) Nomenclature

Chem. Abs. Serv. Reg. No.: 612–83–9
CAS Name: 3,3′-Dichloro-(1,1'-Biphenyl)-4,4'-diamine, dihydrochloride
Synonyms: 3,3′-Dichlorobenzidine dihydrochloride; 3,3′-dichlorobenzidine hydrochloride

(b) Structural formula, molecular formula, and relative molecular mass



$C_{12}H_{10}Cl_2N_2.2HCl$ Rel. mol. mass: 326.05

(c) Chemical and physical properties of the pure substance

Description: Leaflets from water (O'Neil, 2006)
Solubility: Insoluble in water; very soluble in ethanol (Lide, 2008)

1.4 3,3-Dimethoxybenzidine and dimethoxybenzidine-based dyes – Chemical and Physical Data

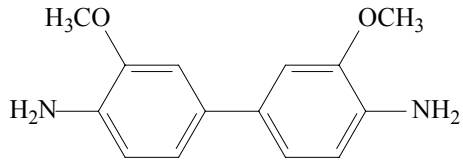
1.4.1 *3,3′-Dimethoxybenzidine*

(a) Nomenclature

Chem. Abs. Serv. Reg. No.: 119–90–4
CAS Name: 3,3′-Dimethoxy-[1,1'-biphenyl]-4,4'-diamine

Synonyms: 4,4′-Bi-*ortho*-anisidine; C.I. 24110; C.I. Disperse Black 6; 4,4'-diamino-3,3′-dimethoxy-1,1'-biphenyl; 4,4'-diamino-3,3′-dimethoxy-1,1'-diphenyl; dianisidine; 3,3′-dianisidine; *ortho*-dianisidine; 3,3′-dimethoxybiphenyl-4,4'-diamine; 3,3′-dimethoxy-4,4'-diaminobiphenyl; 3,3′-dimethoxy-4,4'-diaminodiphenyl

(b) Structural formula, molecular formula, and relative molecular mass



$C_{14}H_{16}N_2O_2$ Rel. mol. mass: 244.29

(c) Chemical and physical properties of the pure substance

Description: Leaflets or needles from water (Lide, 2008)
Melting-point: 137°C (Lide, 2008)
Solubility: Insoluble in water; soluble in acetone, benzene, chloroform, diethyl ether, and ethanol (Lide, 2008)

(d) Trade names

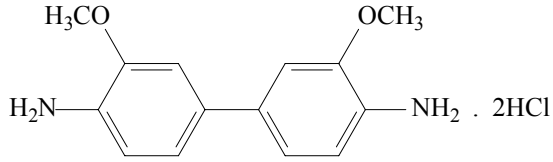
Trade names for 3,3′-dimethoxybenzidine include: Acetamine Diazo Black RD; Acetamine Diazo Navy RD; Amacel Developed Navy SD; Azoene Fast Blue Base; Azofix Blue B Salt; Azogene Fast Blue B; Azogene Fast Blue B Salt; Blue BN Base; Blue Base NB; Blue Base IRGA B; Brentamine Fast Blue B Base; C.I. Azoic Diazo Component 48; Cellitazol B; Cibacete Diazo Navy Blue 2B; Diacel Navy DC; Diacelliton Fast Grey G; Diato Blue Base B; Diazo Fast Blue B; Fast Blue B Base; Fast Blue Base B; Fast Blue DSC Base; Hiltonil Fast Blue B Base; Kayaku Blue B Base; Lake Blue B Base; Meisei Teryl Diazo Blue HR; Mitsui Blue B Base; Naphthanil Blue B Base; Neutrosel Navy BN; Setacyl Diazo Navy R; Spectrolene Blue B.

1.4.2 3,3′-Dimethoxybenzidine dihydrochloride

(a) Nomenclature

Chem. Abs. Serv. Reg. No.: 20325–40–0
CAS Name: 3,3′-Dimethoxy-[1,1'-biphenyl]-4,4'-diamine, hydrochloride (1:2)
Synonyms: C.I. Disperse Black 6, dihydrochloride; *ortho*-dianisidine dihydrochloride; 3,3′-dimethoxy-[1,1'-biphenyl]-4,4'-diamine dihydrochloride

(b) Structural formula, molecular formula, and relative molecular mass



$C_{14}H_{16}N_2O_2.2HCl$ Rel. mol. mass: 317.21

(c) Chemical and physical properties of the pure substance

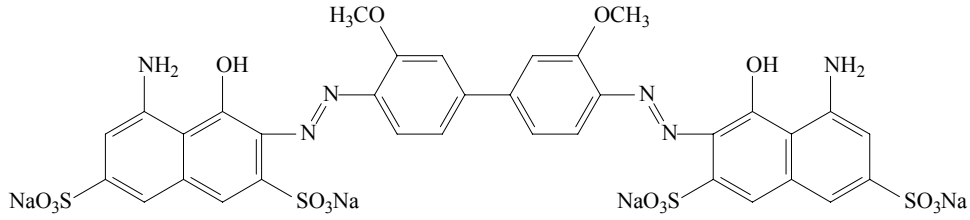
Description: Off-white powder (NTP, 1990)
Melting-point: 274°C (NTP, 1990)
Solubility: Readily soluble in hot water and sparingly soluble in cold water and alcohol (Schwenecke & Mayer, 2005)

1.4.3 *C.I. Direct Blue 15*

(a) Nomenclature

Chem. Abs. Serv. Reg. No.: 2429–74–5
CAS Name: 3,3′-[(3,3′-Dimethoxy[1,1'-biphenyl]-4,4'-diyl)bis(2,1-diazenediyl)]bis[5-amino-4-hydroxy-2,7-naphthalenedisulfonic acid], sodium salt (1:4)
Synonyms: C.I. 24400; C.I. Direct Blue 15; C.I. Direct Blue 15, tetrasodium salt; 3,3′-[(3,3′-dimethoxy[1,1'-biphenyl]-4,4'-diyl)bis(azo)]bis[5-amino-4-hydroxy-2,7-naphthalenedisulfonic acid], tetrasodium salt; tetrasodium 3,3′-[(3,3′-dimethoxy[1,1'-biphenyl]-4,4'-diyl)bis(azo)]bis[5-amino-4-hydroxy-2,7-naphthalenedisulfonate];

(b) Structural formula, molecular formula, and relative molecular mass



$C_{34}H_{24}N_6O_{16}S_4.4Na$ Rel. mol. mass: 992.81

(c) Chemical and physical properties of the pure substance

Description: Dark blue powder (NTP, 1992)
Solubility: Soluble in water (NTP, 1992)

(d) Trade names

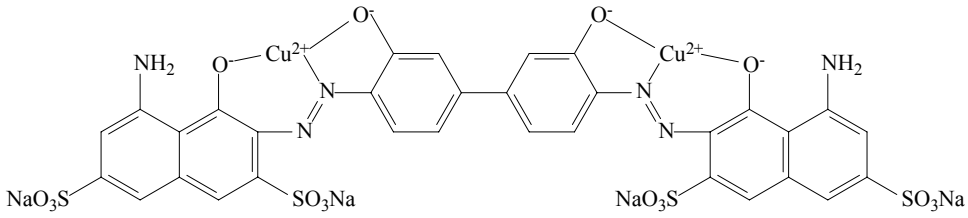
Trade names for C.I. Direct Blue 15 include: Airedale Blue D; Aizen Direct Sky Blue 5B; Aizen Direct Sky Blue 5BH; Amanil Sky Blue; Atlantic Sky Blue A; Atul Direct Sky Blue; Azine Sky Blue 5B; Belamine Sky Blue A; Benzanil Sky Blue; Benzo Sky Blue A-CF; Benzo Sky Blue S; Cartalsol Blue 2GF; Cartasol Blue 2GF; Chloramine Sky Blue 4B; Chloramine Sky Blue A; Chrome Leather Pure Blue; Cresotine Pure Blue; Diacotton Sky Blue 5B; Diamine Blue; Diamine Blue 6B; Diamine Sky Blue; Diamine Sky Blue CI; Diaphtamine Pure Blue; Diazol Pure Blue 4B; Diphenyl Brilliant Blue; Diphenyl Sky Blue 6B; Direct Blue 10G; Direct Blue FFN; Direct Blue FFN-B 15; Direct Blue HH; Direct Lake Blue 5B; Direct Pure Blue; Direct Pure Blue M; Direct Pure Blue N; Direct Sky Blue; Direct Sky Blue 5B; Direct Sky Blue A; Enianil Pure Blue AN; Fenamin Sky Blue; Hispamin Sky Blue 3B; Kayafect Blue Y; Kayaku Direct SKH Blue 5B; 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; Nitsui Direct Sky Blue 5B; Nitto Direct Sky Blue 5B; Oxamine Sky Blue 5B; Paper Blue S; Phenamine Sky Blue A; Pontacyl Sky Blue 4BX; Pontamine Sky Blue 5 BX; Pontamine Sky Blue 5BX; Shikiso Direct Sky Blue 5B; Sky Blue 4B; Sky Blue 5B; Tertrodirect Blue F; Vondacel Blue HH.

1.4.4 *C.I. Direct Blue 218*

(a) Nomenclature

Chem. Abs. Serv. Reg. No.: 28407–37–6
CAS Name: [μ-[[3,3′-[[3,3′-di(hydroxy-κO)[1,1'-biphenyl]-4,4'-diyl]bis(1,2-diazenediyl-κN1)]bis[5-amino-4-(hydroxy-κO)-2,7-naphthalenedisulfonato]](8-)]]dicuprate(4-), sodium (1:4)
Synonyms: C.I. 24401; C.I. Direct Blue 218; 2,2’-(3,3′-dihydroxy-4,4′-biphenylylenebisazo)bis[8-amino-1-naphthol-3,6-disulfonic acid, dicopper derivative, tetrasodium salt; 3,3′-[(3,3′-dihydroxy[1,1'-biphenyl]-4,4'-diyl)bis(azo)]bis[5-amino-4-hydroxy-2,7-naphthalenedisulfonic acid, copper complex; [μ-[[3,3′-[(3,3′-dihydroxy[1,1'-biphenyl]-4,4'-diyl)bis(azo)]bis[5-amino-4-hydroxy-2,7-naphthalenedisulfonato]](8-)]]dicuprate(4-), tetrasodium; Direct Blue 218; [tetrahydrogen-3,3′-[(3,3′-dihydroxy-4,4'-biphenylylene)bis(azo)]bis[5-amino-4-hydroxy-2,7-naphthalenedisulfonato](4-)]dicopper, tetrasodium salt; tetrasodium [μ-[[3,3′-[(3,3′-dihydroxy[1,1'-biphenyl]-4,4'-diyl)bis(azo)]bis[5-amino-4-hydroxynaphthalene-2,7-disulphonato]](8-)]]dicuprate

(b) Structural formula, molecular formula, and relative molecular mass



$C_{32}H_{16}Cu_2N_6O_{16}S_4.4Na$ Rel. mol. Mass: 1087.82

(c) Chemical and physical properties of the pure substance

Description: Dark blue solid (NTP, 1994)
Solubility: Limited solubility in water (NTP, 1994)

(d) Trade names

Trade names for C.I. Direct Blue 218 include: Amanil Supra Blue 9GL; Carta Blue VP; Fastusol Blue 9GLP; Intralite Blue 8GLL; Pontamine Bond Blue B; Pontamine Fast Blue 7GLN; and Solantine Blue 10GL.

1.5 Analysis

Analytical studies on benzidine began in the 1950s. Recent studies include the use of gas chromatography/mass spectrometry (GC/MS) to detect very low (ppm-ppb) levels in water and paint samples. While GC analysis invariably requires derivatization of the amine before the analysis, analysis by liquid chromatography (LC) in combination with mass spectrometry does not. Also, the use of modern LC-MS/MS methods permits the analysis of complex mixtures. Table 1.1 presents a selection of recent studies on the analysis of benzidine and benzidine-based dyes in various matrices.

Table 1.1. Selected methods of analysis of benzidine and benzidine congeners in various matrices

Sample matrix	Sample preparation	Assay method	Detection limit	Reference
Benzidine				
Water & soil	pH adjustment, extraction (dichloromethane), evaporation, residue dissolved in mobile phase	CZE	1 ppm	Bromley & Brownrigg (1994) in Choudhary (1996)
Finger paints	Paint containing amine is applied to an inert surface and dried. Painted sample and modifier (methanol) are placed in SFE cartridge for extraction and GC analysis	SFE/GC	< 0.5 μg/g	Garrigós *et al*. (2000, 2002)
Food polyurethane packaging	Dissolve in ethanol at 500μg/mL; dilute to 5μg/mL; refrigerate up to 5 weeks; protect from light by covering containers with aluminum foil	LC-ESI-MS/MS	0.9 μg/L	Mortensen *et al*. (2005)
Food colourants	Dissolve 100 mg in 5ml of pH9 borate buffer	μLC/ECD	36 pmol/L	Shelke *et al*. (2005)
Water	Dissolve in methanol (1 mmol/L); dilute; add to deionized water	LC/ECD	4.5 nmol/L	Mazzo *et al*. (2006)
Water	Extract from water at pH 8.5 with dichloromethane; evaporate solvent; silylate	GC/MS	4 ng/L	Shin & Ahn (2006)
3,3'-Dimethylbenzidine				
Toys	Sodium dithionite reductive cleavage of azo dye and analysis of resultant amines	HPLC/UV	<20 μg/g	Garrigós *et al*. (2002)
Water	A mixture of 20 amines is dissolved in methanol, diluted to different concentrations for analysis. Other solvents are dichloromethane, ethyl acetate, and methanol/ dichloromethane (50:50)	GC/MS	5 ng/mL	Doherty (2005)

Table 1.1 (contd)

Sample matrix	Sample preparation	Assay method	Detection limit	Reference
3,3'-Dimethylbenzidine (contd)				
Food polyurethane packaging	Dissolve in ethanol at 500µg/mL; dilute to 5µg/mL; refrigerate up to 5 weeks; protect from light by covering containers with aluminum foil	LC-ESI-MS	0.7 µg/L	Mortensen *et al*. (2005)
Water	Dissolve in methanol (1 mmol/L); dilute; add to deionized water	HPLC/ECD	7.69 nmol/L	Mazzo *et al*. (2006)
3,3'-Dicholorobenzidine				
Urine	Urine specimens (100mL) were extracted at pH 6-7 with chloroform; extract were evaporated to dryness after adding *p*-chlorobiphenyl as an internal standard. The residue was dissolved in 100 µL of benzene containing 1% (v/v) 1-aminobutane	GC/MS	10-20 pg	Hurst *et al*. (1981)
Textiles	Extract fabric with citrate buffer; decolorize extract with hydrosulfite; extract with *tert*-butylmethyl ether; concentrate and dilute with methanol	LC-MS/MS	20.1 µg/L	Sutthivaiyakit *et al.* (2005)
Water	Dissolve in methanol (1 mmol/L); dilute; add to deionized water	LC/ECD	5.15 nmol/L	Mazzo *et al*. (2006)
Water	Extract from water at pH 8.5 with dichloromethane; evaporate solvent; silylate	GC/MS	20 nl/L	Shin & Ahn (2006)

Table 1.1 (contd)

Sample matrix	Sample preparation	Assay method	Detection limit	Reference
3,3'-Dimethoxybenzidine				
Toys	Sodium dithionite reductive cleavage of azo dye and analysis of resultant amines	HPLC/UV	<20 μg/g	Garrigós *et al*. (2002)
Water	A mixture of 20 amines is dissolved in methanol, diluted to different concentrations for analysis. Other solvents are dichloromethane, ethyl acetate, and methanol/ dichloromethane (50:50)	GC/MS	5 ng/mL	Doherty (2005)
Textiles	Extract fabric with citrate buffer; decolorize extract with hydrosulfite; extract with *tert*-butylmethyl ether; concentrate and dilute with methanol	LC-MS/MS	47.8 μg/mL	Sutthivaiyakit *et al*. (2005)

CZE, capillary zone electrophoresis; ECD, electro-chemical detection; ESI, electrospray ionization; GC, gas chromatography; HPLC, high-performance liquid chromatography; LC, liquid chromatography; MS, mass spectrometry; SFE, supercritical fluid extraction; UV, ultraviolet

1.6 Production

1.6.1 *Benzidine and benzidine-based dyes*

(a) Benzidine

Benzidine and its substitution products (*ortho*-tolidine [3,3′-dimethylbenzidine], 3,3′-dichlorobenzidine, and *ortho*-dianisidine [3,3′-dimethoxylbenzidine]) represent the group called the diphenyl bases. They are used mainly as intermediates in the production of azo dyes and azo pigments. Symmetrically or asymmetrically coupled products can be produced by simultaneous or successive diazotization (coupling), respectively. The diphenyl bases have been of interest as cross-linking agents, e.g., in polyurethane plastics, in which they can noticeably increase temperature stability. The diphenyl radical has a chain-stiffening effect in polyamides. The ability of the diphenyl bases to react with numerous cations, anions, and organic substances, such as oxidizing agents and blood, is used for analytical and diagnostic purposes (Schwenecke & Mayer, 2005).

Benzidine and the other diphenyl bases are produced in three separate processing stages: 1) reduction of nitro groups to form hydrazo compounds; 2) benzidine rearrangement; 3) isolation of the bases. Benzidine has been produced from nitrobenzene on an industrial scale since about 1880. Commercial production methods include alkaline iron reduction, amalgam reduction, and electrochemical reduction. The resultant hydrazobenzene is rearranged with hydrochloric acid or sulfuric acid during cooling. The base is then isolated in the form of benzidine hydrochloride or benzidine sulfate. The conversion of these salts to the free base is avoided as much as possible because of the chronic toxicity of benzidine (Schwenecke & Mayer, 2005).

The most important reaction commercially is the diazotization of the two amino groups. Reaction with nitrous acid converts benzidine into the tetrazonium compound, in which the first diazonium group is coupled very vigorously whereas the second reacts more slowly. As a result it is possible to produce asymmetrical diazo dyes. Gradual diazotization is also possible (Schwenecke & Mayer, 2005).

The manufacturing of benzidine is prohibited in several countries, e.g., Japan, Republic of Korea, Canada and Switzerland (UN/UNEP/FAO, 2009). According to EU legislation, the manufacture of benzidine has been prohibited in Europe since 1998 (European Commission, 1998).

Benzidine is no longer manufactured for commercial purposes in the USA. All large-scale production was discontinued in 1976, and only small quantities remain available for use in diagnostic testing. Estimated US benzidine production in 1983 was 500 pounds (227 kg) (possibly excluding some captive production), compared with 10 million pounds (4500 tonnes) in 1972 (ATSDR, 2001).

Available information indicates that benzidine was produced and/or supplied in research quantities in the following countries: Germany, Hong Kong Special Administrative Region, India, the People's Republic of China, Switzerland, and the USA

(Chem Sources-International, 2010). Available information indicates that benzidine hydrochloride was produced and/or supplied in research quantities in the following countries: Belgium, Canada, Germany, Hong Kong Special Administrative Region, India, Switzerland, and the USA (Chem Sources-International, 2010).

(b) Benzidine-based dyes

Benzidine-based dyes were produced in commercial quantities in the United States starting no later than 1914. Total production in the USA reached 14 million kg (31 million pounds) in 1948, which dropped to about 2.9 million kg (6.4 million pounds) in 1976 and about 780 000 kg (1.7 million pounds) in 1978 (IARC, 1982). In 1978, Direct Black 38 accounted for about 48% of the production, followed by Direct Blue 2 (12.8%) and Direct Green 6 (6.4%). In 1974, nine manufacturers produced benzidine-based dyes; by 1979, only one manufacturer remained, producing 17 benzidine-based dyes (NTP, 2005a).

Information was collected in Europe from 1996 to 1998 for the IUCLID database for substances with a production or import volume between 10 and 1000 tonnes/year (Low Production Volume Chemicals (LPVCs)). Direct Black 38 was included on the list of LPVCs (Allanou *et al.*, 1999; European Commission, 2008).

Available information indicates that Direct Black 38 was produced and/or supplied in research quantities in the following countries: Germany, Hong Kong Special Administrative Region, India, Japan, the United Kingdom, and the USA (Chem Sources-International, 2010).

Direct Blue 6 was produced and/or supplied in research quantities in the following countries: Germany, Hong Kong Special Administrative Region, India, Japan, and the USA (Chem Sources-International, 2010).

Direct Brown 95 was produced and/or supplied in research quantities in the following countries: India, Japan, and the USA (Chem Sources-International, 2010).

1.6.2 *Dimethylbenzidine and dimethylbenzidine-based dyes*

(a) 3,3′-Dimethylbenzidine

ortho-Nitrotoluene undergoes alkaline reduction with zinc dust, electrolytic reduction, or catalytic reduction to form 2,2′-dimethylhydrazobenzene. This is rearranged in dilute hydrochloric acid or 20% sulfuric acid at 5–50°C. The free base (3,3′-dimethylbenzidine) or the dihydrochloride can be isolated (Schwenecke & Mayer, 2005).

In 1978, the major company producing 3,3′-dimethylbenzidine in the USA ceased production; its annual production had averaged approximately 200 000 pounds (NTP, 2005b). The USEPA (2003, 2007) Inventory Update Rule regulation requires manufacturers and importers of certain chemical substances listed in the TSCA Chemical Substance Inventory to report manufacturing information (aggregate production volumes) for chemicals manufactured (including imported) in amounts of 10 000 pounds or greater (in 1986) or 25 000 pounds or greater (in 2003) at a single site. Table 1.2 presents the

aggregate production volumes that were reported for 3,3′-dimethylbenzidine dihydrochloride. 3,3′-Dimethylbenzidine was included on the list of LPVCs (Allanou *et al.*, 1999; European Commission, 2008).

Available information indicates that 3,3′-dimethylbenzidine was produced or supplied in the following countries: Canada, Germany, Hong Kong Special Administrative Region, India, Japan, the Netherlands, the People's Republic of China, South Africa, Switzerland, and the USA (Chem Sources-International, 2010).

Table 1.2. 3,3′-Dimethylbenzidine dihydrochloride production volumes

Year	Volume (in thousands of pounds)
1986	10–500
1990	10–500
1994	NR
1998	NR
2002	NR
2006	NR

USEPA (2003, 2007)
NR, not reported

(b) Dimethylbenzidine-based dyes

Acid Red 114 can be prepared by coupling *ortho*-tolidine [3,3′-dimethylbenzidine] to phenol, which is then coupled to G-acid (2-naphthol-6,8-disulfonic acid), followed by reaction of the phenolic hydroxyl group with *para*-toluenesulfonyl chloride (Chudgar & Oakes, 2003). Acid Red 114 was included on the list of LPVCs (Allanou *et al.*, 1999; European Commission, 2008).

Table 1.3 presents the aggregate production volumes that were reported for Acid Red 114 by the USEPA.

Available information indicates that Acid Red 114 was produced and/or supplied in research quantities in the following countries: Germany, Hong Kong Special Administrative Region, India, Japan, and the USA (Chem Sources-International, 2010).

Table 1.3. Acid Red 114 production volumes

Year	Volume (in thousands of pounds)
1986	10–500
1990	10–500
1994	NR
1998	10–500
2002	NR
2006	NR

USEPA (2003, 2007)
NR, not reported

1.6.3 *Dichlorobenzidine*

3,3′-Dichlorobenzidine is commercially produced by reduction of *ortho*-nitrochlorobenzene to form a hydrazo compound, which is rearranged in the presence of mineral acids to form 3,3′-dichlorobenzidine (Schwenecke & Mayer, 2005). The commercial product is usually provided in the form of the dihydrochloride salt because of its greater stability. 3,3′-Dichlorobenzidine dihydrochloride was included on the list of HPVCs with a range of 10 000 to 50 000 tonnes (Allanou *et al.*, 1999; European Commission, 2000).

Table 1.4 presents the aggregate production volumes that were reported for 3,3′-dichlorobenzidine by the USEPA.

Table 1.4. 3,3′-dichlorobenzidine production volumes

Year	Volume (in thousands of pounds)
1986	NR
1990	>500–1000
1994	10–500
1998	>1000–10 000
2002	10–500
2006	NR

USEPA (2003, 2007)
NR, not reported

Table 1.5 presents the aggregate production volumes that were reported by the USEPA for 3,3′-dichlorobenzidine dihydrochloride.

The US International Trade Commission reported a production volume of 3,3′-dichlorobenzidine-based dyes of over 18 million pounds in the USA in 1983; 3,3′-dichlorobenzidine is no longer used to manufacture dyes in the USA (ATSDR, 1998).

Available information indicates that 3,3′-dichlorobenzidine was produced and/or supplied in the following countries: Hong Kong Special Administrative Region, India, the People's Republic of China, the United Kingdom and the USA (Chem Sources-International, 2010), whereas 3,3′-dichlorobenzidine dihydrochloride was produced and/or supplied in the following countries: Belgium, Hong Kong Special Administrative Region, India, Japan, the People's Republic of China, Switzerland, the United Kingdom and the USA (Chem Sources-International, 2010).

Table 1.5. 3,3′-dichlorobenzidine dihydrochloride production volumes

Year	Volume (in millions of pounds)
1986	>1–10
1990	>1–10
1994	>10–50
1998	>10–50
2002	>10–50
2006	10–<50

USEPA (2003, 2007)

1.6.4 *Dimethoxybenzidine and dimethoxybenzidine-based dyes*

(a) Dimethoxybenzidine

3,3′-Dimethoxybenzidine has been produced commercially since the 1920s. 3,3′-Dimethoxybenzidine and 3,3′-dimethoxybenzidine dihydrochloride were included on the list of LPVCs (Allanou *et al.*, 1999; European Commission, 2008).

Data on production of 3,3′-dimethoxybenzidine in the USA were last reported in 1967, when five companies produced approximately 368 000 pounds (IARC, 1974). Table 1.6 presents the aggregate production volumes that were reported for 3,3′-dimethoxybenzidine dihydrochloride by the USEPA.

Available information indicates that 3,3′-dimethoxybenzidine was produced and/or supplied in the following countries: Germany, Hong Kong Special Administrative Region, India, Japan, Switzerland, the United Kingdom and the USA (Chem Sources-International, 2010), whereas 3,3′-dimethoxybenzidine dihydrochloride was produced and/or supplied in the following countries: Germany, Hong Kong Special Administrative Region, India, Japan, the People's Republic of China and the USA (Chem Sources-International, 2010).

Table 1.6. 3,3′-dimethoxybenzidine dihydrochloride production volumes

Year	Volume (in thousands of pounds)
1986	10–500
1990	10–500
1994	10–500
1998	>500–1 000
2002	10–500
2006	<500

USEPA (2003, 2007)

(b) Dimethoxybenzidine-based dyes

Direct Blue 15 is prepared by coupling *ortho*-dianisidine (3,3′-dimethoxybenzidine) to two moles of H-acid (4-amino-5-hydroxy-2,7-naphthalenedisulfonic acid) under alkaline conditions. Direct Blue 218 is produced from Direct Blue 15 by metalizing and elimination of methyl groups from the methoxide to form the copper complex (Chudgar & Oakes, 2003). Direct Blue 15 was included on the list of LPVCs (Allanou *et al.*, 1999; European Commission, 2008).

Table 1.7 presents the aggregate production volumes that were reported by the USEPA for Direct Blue 15 and Direct Blue 218.

Available information indicates that Direct Blue 15 was produced and/or supplied in the following countries: Germany, Hong Kong Special Administrative Region, India, Japan and the USA (Chem Sources-International, 2010).

Table 1.7. Production volumes for Direct Blue 15 and Direct Blue 218

Year	Volume (in thousands of pounds)	
	Direct Blue 15	Direct Blue 218
1986	10–500	10–500
1990	>1000–10 000	10–500
1994	>500–1000	10–500
1998	>500–1000	10–500
2002	>500–1000	10–500
2006	NR	<500

USEPA (2003, 2007)
NR, not reported

1.7 Use

1.7.1 *Benzidine and benzidine-based dyes*

(a) Benzidine

Benzidine has been used since the 1850s as the reagent base for the production of a large number of dyes, particularly azo dyes for wool, cotton, and leather. However, because benzidine was found in the 1970s to be carcinogenic to humans, there has been a considerable decline in the use of the benzidine dyes. Benzidine is used for the quantitative determination of sulfuric acid and for the detection and determination of numerous anions and metal ions. The reaction of benzidine with pyridine in the presence of elemental chlorine is suitable for detecting traces of free chlorine or pyridine in drinking-water. The green to blue colouration that occurs when benzidine reacts with hydrogen peroxide in the presence of peroxidases can be used to detect blood. Benzidine still plays a role in many chemical syntheses (Schwenecke & Mayer, 2005).

In the past, benzidine also has been used as a rubber compounding agent, in the manufacture of plastic films, for detection of hydrogen peroxide in milk, and for quantitative determination of nicotine. Most of these uses have been discontinued, although some dyes that may contain benzidine as an impurity are still used as stains for microscopy and similar laboratory applications (ATSDR, 2001).

(b) Benzidine-based dyes

Benzidine-based dyes were used primarily to colour textiles, leather, and paper products and also in the petroleum, rubber, plastics, wood, soap, fur, and hair-dye

industries (NTP, 2005b). Approximately 40% was used to colour paper, 25% to colour textiles, 15% to colour leather, and 20% for diverse applications. By the mid-1970s, most manufacturers started phasing-out the use of benzidine-based dyes and replacing them with other types of dyes (NIOSH, 1980). Access to these dyes for home use is no longer permitted in the US; however, some dyes (particularly direct browns, greens, and blacks) were available as consumer products in the 1970s (ATSDR, 2001).

1.7.2 *Dimethylbenzidine and dimethylbenzidine-based dyes*

(a) 3,3′-Dimethylbenzidine

3,3′-Dimethylbenzidine is a starting material in the production of a large number of azo dyes and pigments. 3,3′-Dimethylbenzidine is used in the determination of oxygen and chlorine in water, and for the colorimetric determination of cations of gold, cerium, and manganese. An important derivative of 3,3′-dimethylbenzidine is its diacetoacetyl compound, 4,4’-bisacetoacetylamino-3,3′-dimethyldiphenyl. It is a coupling agent that is frequently used; in combination with chloroanilines it gives yellow shades. 3,3′-Dimethylbenzidine diisocyanate is used as a cross-linking agent for the synthesis of polymers (Schwenecke & Mayer, 2005).

(b) Dimethylbenzidine-based dyes

Dimethylbenzidine-based dyes and pigments have been used in printing textiles, as biological stains, and in colour photography (NTP, 2005b).

1.7.3 *Dichlorobenzidine*

3,3′-Dichlorobenzidine was introduced in the early 1930s and is an important diphenyl base. It is used as the starting material for pigments with yellow and red shades. These are used for coloring printing inks, paints, plastics, and rubbers. The important diarylide yellow pigments, which are incorrectly known as benzidine yellows, are formed by the combination of 3,3′-dichlorobenzidine with acetic acid arylides. 3,3′-Dichlorobenzidine is also used in the production of polyurethane rubbers (Schwenecke & Mayer, 2005).

Diarylide pigments are important economically, particularly in the production of printing ink. 3,3′-Dichlorobenzidine is by far the most important bisdiazo component. The term “benzidine pigments” is still sometimes used for this group, but this is incorrect because benzidine has never been used to produce diarylide pigments. Diarylide pigments are produced by the bisdiazotization of 3,3′-dichlorobenzidine, followed by coupling with two equivalents of an acetoacetic arylide (Herbst & Hunger, 2004).

1.7.4 *Dimethoxybenzidine and dimethoxybenzidine-based dyes*

(a) Dimethoxybenzidine

3,3′-Dimethoxybenzidine is used almost exclusively as a chemical intermediate for producing dyes and pigments. The Society of Dyers and Colourists reported its use in the production of 89 dyes in 1971. 3,3′-Dimethoxybenzidine is also used as a chemical intermediate to produce *ortho*-dianisidine diisocyanate for use in adhesives and as a component of polyurethanes (IARC, 1974).

(b) Dimethoxybenzidine-based dyes

3,3′-Dimethoxybenzidine-based dyes and pigments have been used as colourants for paper, plastics, rubber, textiles, and leather (IARC, 1974).

1.8 Occurrence

1.8.1 *Natural occurrence*

Benzidine and its congeners are not known to occur naturally.

1.8.2 *Occupational exposure*

Occupational exposure to benzidine, benzidine congeners and their related dyes can occur during the production and use of these substances. Other workers potentially exposed to benzidine include laboratory personnel using benzidine-containing laboratory chemicals. Steinberg (1977) reported the results of a 1974 survey of US forensic laboratories, which showed that 54 of 276 laboratories were familiar with the benzidine test for blood.

Benzidine-based dyes and benzidine congener-based dyes can also be metabolized to benzidine and the respective congener, which may result in additional exposure to the aromatic amine. Exposure studies in benzidine-based dye workers therefore measured benzidine rather than the benzidine-based dyes.

Studies reporting airborne and urine levels and dermal wipes of benzidine in the benzidine and benzidine-based dye industry are listed in Tables 1.8–1.10.

(a) Airborne benzidine

Benzidine concentration in workplace air has been reported for different work settings; the results are summarized in Table 1.8.

In a Moscow aniline-dye factory, benzidine was produced from 1930 to 1988, with a six-year lapse from 1941 to 1947, after which the plant was reconstructed (Bulbulyan *et al.*, 1995). Factory area air-samples were obtained between 1930 and 1971. Benzidine-in-air concentrations of up to 6 mg/m^3 were reported. Levels were lower after the reconstruction

Table 1.8. Benzidine concentration in air in different occupational settings

Reference	Country, year of study	Task	Number of samples		Level benzidine (mg/m^3)
Meigs *et al*., (1951, 1954)	USA, 1948–1952	Benzidine manufacturing – press room	26	mean (max)	0.018 (0.087)
		– other areas	5		<0.001
Zavon *et al*., (1973)	USA, >1958	Benzidine manufacturing – reducers	NR	NR	<0.007
		– conversion tubs	NR	NR	<0.007
		– clarification tub	NR	NR	0.005
		– filter press	NR	NR	0.072–0.415
		– salting-out tub	NR	NR	0.152
		– centrifuge	NR	NR	<0.005
		– location for shoveling benzidine into drums	NR	NR	17.6
Krajewska *et al*., (1980)	Poland, 1976	Benzidine manufacturing – old production process	253	GM +/- SD	0.017 +/- 0.63
		– new, automated production process	275	GM +/- SD	0.0008[a] +/- 0.37 (91% < LOD of 0.0027)
				max	0.031
Bi *et al*., (1992)	China, Tianjin, 1962	Direct dye & benzidine production – transformation	17	mean (max)	0.05 (0.9)
		– deposition	13	mean (max)	0.13 (0.25)
		– filtration	13	mean (max)	0.24 (0.38)
		– oil pump	10	mean (max)	0.16 (0.35)
	China, Jilin, 1965	– packaging	4	mean (max)	0.39 (1.18)
	China, Jilin, 1970	– underground tub	2	mean (max)	0.27 (0.33)

Table 1.8 (contd)

Reference	Country, year of study	Task	Number of samples		Level benzidine (mg/m^3)
Bulbulyan *et al*., (1995)	Russian Federation, 1930–1941	Aniline dye production	NR	range	0.06–1.8
	Russian Federation, 1947–1948		NR	range	0–6 (n undetected unknown)
	Russian Federation, 1956		16	range	0–2.2 (4 undetected)
	Russian Federation, 1957 summer		4	range	0–1.2 (1 undetected)
	Russian Federation, 1957 winter		44	range	0–0.18 (33 undetected)
	Russian Federation, 1971		39	range	0 (all undetected)

Table 1.8 (contd)

Reference	Country, year of study	Task	Number of samples		Level benzidine (mg/m^3)
Kim *et al*., (2007)	Republic of Korea, 1998	Benzidine-based dye manufacture – drying	5		ND
		– packaging	5		ND
		– material treatment	1		ND
		– filtering	5	range	ND–0.65
		– drying	2		ND
		– transport	2		ND
		– maintenance	1		ND
		Benzidine and benzidine-based dyes use	3		ND
		– material treatment			
		– coupling	3		Trace
		– coupling/dissolution	5	range	ND–trace
		– dissolution	3	range	ND–trace
		– filtering	4	range	ND–trace
		– drying	2		ND
		– grinding/packaging	7	mean (range)	0.0417 (ND–0.24)
		– mixing	1		0.1131
		– maintenance	3	range	trace–0.0149

[a] estimated value from probability distribution model
GM, geometric mean; LOD, limit of detection; ND, not detected; NR, not reported; SD, standard deviation

of the plant and airborne benzidine levels were all below the limit of detection in 1971.

In a chemical plant in the USA, benzidine production started in 1929 (Zavon *et al.*, 1973). After an employee had noted haematuria in 1958, concentrations of benzidine in the air at different locations of the plant were assessed. These measurements showed that major exposure occurred at an activated charcoal discard press during hand cleaning as well as during shovelling of dry benzidine into barrels (air concentration, 17.6 mg/m^3).

In another US chemical plant in Connecticut, USA, benzidine was produced between the mid-1940s and mid-1965, while dichlorobenzidine production continued until 1989 (Ouellet-Hellstrom & Rench, 1996). Due to bladder-cancer concerns a permanent biological monitoring programme was instituted in 1949, which continued until 1965. Air concentrations measured in 1948 and 1949 were reported (Meigs *et al.*, 1951, 1954) with a maximum of 0.087 mg/m^3.

In a Polish benzidine-manufacturing plant (Krajewska *et al.*, 1980), air samples showed lower airborne benzidine concentrations after the production process had been changed.

Direct dye-production facilities in three cities in China (Tianjin, Shanghai, Jilin) used imported powdered benzidine until about 1956, when production of benzidine began in Tianjin and Jilin (Bi *et al.*, 1992). Benzidine production ceased in 1977. In addition to the two benzidine-production facilities, in 1971 there were eight benzidine-using facilities in Tianjin and eight in Shanghai. Benzidine was measured in ambient air in the factories in Tianjin and Jilin during 1962–1970, with a maximum of 1.18 mg/m^3, during packaging.

In the Republic of Korea (Kim *et al.*, 2007) benzidine exposure levels in 1998 were available from one benzidine-production facility and two facilities that used benzidine. In many samples benzidine was not detectable, and the highest concentration of 0.65 mg/m^3 was measured during filtering in the benzidine-based dye manufacturing plant.

(b) Biomonitoring of urinary concentrations

Measurements of benzidine and benzidine derivatives in the urine of workers in various factories are summarized in Table 1.9.

In a benzidine production plant in the USA (Zavon *et al.*, 1973) and in a chemical plant in Connecticut, USA (Meigs *et al.*, 1954), urine concentrations were measured pre- and post-shift. In both studies, leves were higher after the workshift than before.

In direct dye-production facilities in three cities of China (Bi *et al.*, 1992), urine levels were determined for selected workers in Tianjin, in 1962. Levels of renal benzidine excretion ranged between non-detectable and 0.77 mg/24 hours.

Table 1.9. Urinary levels of benzidine or benzidine derivatives in exposed workers

Reference	Country, year of study	Task	Number of workers (samples)		Level benzidine
Meigs *et al*., 1954	USA, 1950	Benzidine manufacturing – press room, 6.30 am	12 (36)	mean	0.406 +- 0.080 mg/L
		– press room, 4 pm	12 (81)	mean	1.125 +- 0.213 mg/L
Zavon *et al*., 1973	USA, >1958	Benzidine manufacturing – Monday morning	14	[see graph]	<0.02 mg/L
		– before shift	33	[see graph]	<0.07 mg/L
		– after shift	24	[see graph]	<0.159 mg/L
Lowry *et al*., 1980	USA	dye manufacturing I (Bzd)	7		0
		dye manufacturing I (MoAcBzd)	7	range	0–7 ppb (5 undetected)
		dye manufacturing II (Bzd)	4	mean +- SD	48 +- 46 ppb
		dye manufacturing II (MoAcBzd)	4	mean +- SD	233 +- 257 ppb
		textile dyeing I (Bzd)	4	–	0
		textile dyeing I (MoAcBzd)	4	range	0–4 ppb (3 undetected)
		textile dyeing II (Bzd)	8	range	0–39 ppb (6 undetected)
		textile dyeing II (MoAcBzd)	8	mean +- SD	16.7 +- 18.5 ppb (5 undetected)
		leather dyeing (Bzd)	12	–	0
		leather dyeing (MoAcBzd)	12	–	0
		paper dyeing, only using direct black 38 (Bzd)	47	range	0–1 ppb (45 undetected)
		paper dyeing, only using direct black 38 (MoAcBzd)	47	mean +- SD	3.4 +- 2.1 ppb (38 undetected)
Meal *et al*., 1981	UK	Textile dye houses	20 (114)	range	1.0–25.4 nmol/mmol creatinine (86 undetected)
		Tannery, duestuff quality control laboratories	9 (95)		ND

Table 1.9 (contd)

Reference	Country, year of study	Task	Number of workers (samples)		Level benzidine
Dewan *et al*., 1988	India, NR	Direct Black 38 manufacture	18	range	0.0024–0.3625 mg/L
Bi *et al*., 1992	China, 1962	Direct dye and benzidine production – pressure filter	5	range	0.04–0.77 mg/24h
		– transformation	3	range	0.29–0.44 mg/24h
		– reducer	2	range	ND
Rothman *et al*., 1997	India, 1993	Production of benzidine dihydrochloride and benzidine based dyes	33		
		– free benzidine		mean	1.6 ng/μmol creatinine
		– *N*-acetyl benzidine		mean	19.6 ng/μmol creatinine
		– *N,N'*-diacetylbenzidine		mean	1.0 ng/μmol creatinine
Krajewska *et al*., 1980	Poland, 1976	Benzidine manufacturing, new production process	73	range	0.0004–0.0123 mg/L (64% undetected)

ND, not detected; NR, not reported; ppb, parts per billion; SD, standard deviation

As part of a US National Institute for Occupational Safety and Health (NIOSH) industry-wide study, urine samples were collected from workers exposed to azo dyes during the dye manufacture (two sites) and use (four sites) (Lowry *et al.*, 1980). Levels of benzidine and monoacetylbenzidine were reported for different departments (see Table 1.9). Diacetylbenzidine and 4-aminobiphenyl were not detected in the urine of the workers.

In a Polish benzidine-manufacturing plant, urinary levels were below the detection limit in 64% of the samples. The detected concentrations ranged between 0.004 and 0.0123 mg/L (Krajewska *et al.*, 1980).

In a study in the United Kingdom (Meal *et al.*, 1981), 200 samples from 29 workers exposed to benzidine-derived dyes in three textile-dye houses, two tanneries, and two dyestuff quality-control laboratories were analysed. Of the 29 workers, five (from one woollen-textile industry) had detectable levels of free benzidine in their urine after acid hydrolysis, ranging between 1.0–25.4 nmol/mmol creatinine.

Indian workers in a small-scale unit manufacturing Direct Black 38 provided urine samples. Acetylated benzidine metabolites were detected in all and benzidine in all but two samples (Dewan *et al.*, 1988).

In Indian factories that manufactured benzidine dihydrochloride or benzidine-based dyes, levels of free urinary benzidine and acetyl-benzidine were measured in 33 workers. One subject had non-detectable levels of benzidine, *N*-acetylbenzidine and *N,N′*-diacetylbenzidine in his post-shift urine sample. Mean levels of free benzidine and benzidine metabolites in the remaining 32 workers were reported (Rothman *et al.*, 1997).

(c) Dermal exposure

Three studies reported dermal exposure in workers by measuring levels of benzidine in dermal wipes. They are summarized in Table 1.10.

1.8.3 *Environmental occurrence and exposure of the general population*

Benzidine-based dyes can contain varying amounts of benzidine due to contamination. Twenty-six US-produced dyes based on benzidine were found to contain < 1–20 mg/kg benzidine and one contained 270 mg/kg (IARC, 1982). Eight of 33 benzidine-based dye samples obtained from Belgium, Egypt, India, the Netherlands, Poland, Romania, and the Republic of Korea were found to contain 38–1254 mg/kg of benzidine; the others had 24 mg/kg or less (IARC, 1982).

The general population can be exposed to benzidine when in contact with consumer goods that contain benzidine or benzidine based-dyes, such as leather products (Ahlström *et al.*, 2005), clothes and toys (Garrigós *et al.*, 2002). Some food colours such as tartrazine

Table 1.10. Benzidine concentrations on dermal wipes taken in various industries

Reference	Country, year of study	Task	Number of workers		Level benzidine
Krajewska *et al*., 1980	Poland, 1976	Benzidine manufacturing, old production process – torso	27	mean (range)	4.2 (0.8–28) µg/dcm^2 (7 undetected)
		– right palm	28	mean (range)	444 (4–1800) µg/dcm^2 (2 undetected)
		Benzidine manufacturing, new production process – torso	214	mean (range)	7 (1–300) µg/dcm^2 (75% undetected)
		– right palm	224	mean (range)	13 (1.5–680) µg/dcm^2 (45% undetected)
Bi *et al*., 1992	China, 1962	Direct dye and benzidine production – transformation, handpalm	9	mean (range)	5.7 (0.5–22.2) µg/cm^2
		– transformation, hand back	9	mean (range)	3.3 (0.7–8.6)
		– transformation, front arm	9	mean (range)	5.3 (0.9–19.4)
		– transformation, breast	9	mean (range)	2.6 (0.3–12.3)
		– pressure filter, handpalm	11	mean (range)	11.7 (1.4–51.2)
		– pressure filter, hand back	11	mean (range)	5.8 (0.9–27.6)
		– pressure filter, front arm	11	mean (range)	7.9 (1.1–32.2)
		– pressure filter, breast	11	mean (range)	1.6 (0.7–3.1)
Bulbulyan *et al*., 1995	Russia, 1937–1938	Aniline dye production	NR	range	56.22 mg (sample size unknown)
	Russia, 1947–1948		NR	range	13.16–39.80 mg (sample size unknown)
	Russia, 1957		NR	range	0.22–6.08 mg (sample size unknown)
	Russia, 1971		NR	range	0.013–0.025 mg (sample size unknown)

NR, not reported

and sunset yellow FCF have been reported to contain trace amounts of benzidine (< 5 to 270 ng/g) (Lancaster & Lawrence, 1999).
The general population can also be exposed to benzidine when living near former manufacturing or disposal sites where benzidine and benzidine-based dyes were manufactured or disposed of. Benzidine and benzidine-based dyes have been detected in effluent from plants manufacturing and using dyes. In the effluent of a Brazilian textile-dye processing plant, benzidine was detected at concentrations of 47 µg/L (Alves de Lima *et al.*, 2007). In 1990, benzidine was detected at 240 µg/L (on site) and 19 µg/L (off site) in groundwater at a hazardous-waste site that was the former location of a large dye manufacturer (ATSDR, 2001). Microbial degradation of these benzidine-based dyes may release free benzidine into the environment (ATSDR, 2001).

1.9 Regulations and guidelines

Table 1.11 gives an overview of the regulations and guidelines detailed below.

1.9.1 *Europe*

(a) Council Directives 89/677/EEC and 97/56/EC

According to Council Directive 89/677/EEC, benzidine and its salts are restricted from sale to the general public (EEC, 1989). In Council Directive 97/56/EC, 3,3′dimethylbenzidine, 3,3′-dichlorobenzidine and 3,3′-dimethoxybenzidine are restricted from sale to the general public (European Commission, 1997).

(b) Council Directive 98/24/EC

According to EU regulations, the manufacture of benzidine and its salts has been prohibited since 1998. The Council Directive 98/24/EC in Annex III prohibits the production, manufacture or use at work of benzidine and its salts and activities involving benzidine and its salts. The prohibition does not apply if benzidine and its salts are present in another chemical agent, or as constituents of waste, provided that its individual concentration therein is less than 0.1% w/w (European Commission, 1998).

(c) Directive 2002/61/EC

Directive 2002/61/EC restricts the marketing and use of azocolourants (European Commission, 2002). In this Directive, Annex I to Directive 76/769/EEC is amended. Azodyes which, by reductive cleavage of one or more azo groups, may release one or more of the aromatic amines (benzidine, dimethylbenzidine, dichlorobenzidine, dimethoxylbenzidine) in detectable concentrations, i.e. above 30 ppm in the finished articles or in the dyed parts thereof, according to the testing method established in accordance

Table 1.11. Regulations and guidelines for benzidine, 3,3′-dimethylbenzidine, 3,3′-dichlorobenzidine and 3,3′-dimethoxybenzidine (see references in text)

Country	Directive or regulatory body	Comment	Benzidine	3,3′-dimethyl-benzidine	3,3′-dichloro-benzidine	3,3′-dimethoxy-benzidine
Europe	89/677/EEC	Packaging, labelling Amendment to 76/769/EEC	x			
	97/56/EC	Packaging, labelling Amendment to 76/769/EEC		x	x	x
	98/24/EC	Ban of production and use	x			
	2002/61/EC	Marketing and use of azocolourants; amendment to CD 76/769/EEC	x	x	x	x
	2004/37/EC	Exposed workers	x	x	x	x
	76/768/EEC	Cosmetics directive	x			
	2004/93/EC	Cosmetics directive; amendment to CD 76/768/EEC		x	x	x
	2005/90/EC	List of CMR	x			
Germany	MAK (2007)		skin No BLV	2 No limit	skin No BLV	2 No limit
Japan	JSOH (2007)		1	2B	2B	2B
USA	ACGIH (2001)		A1; skin No TLV	A3; skin No TLV	A3; skin No TLV	
	NIOSH (2005)		x		x	x
	NTP (2005a,b,c,d)		K	RAHC	RAHC	RAHC

x, indicates that the regulation applies to this agent
A1, confirmed human carcinogen; A3, confirmed animal carcinogen with unknown relevance to humans; BLV, biological limit value; K, known to be a human carcinogen; RAHC, reasonably anticipated to be a human carcinogen; skin, potential significant contribution to the overall exposure by the cutaneous route; TLV, tolerable limit value;

with Article 2a of this Directive, may not be used in textile and leather articles that may come into direct and prolonged contact with the human skin or oral cavity.

(d) Directive 2004/37/EC

Benzidine and its salts are regulated by the Directive 2004/37/EC (European Commission, 2004a). The directive applies to activities in which workers are exposed to carcinogens or mutagens of category 1 and 2. Rules are fixed regarding the employers' obligations of reduction and replacement, prevention and reduction of exposure, unforeseen exposure, foreseeable exposure, access to risk areas, hygiene and individual protection, information for the competent authority, information and training of workers, consultation and participation of workers, health surveillance, record keeping and limit values.

(e) Cosmetics Directive (2004/93/EC)

The Commission Directive 2004/93/EC of 21 September 2004 amends Council Directive 76/768/EEC for the purpose of adapting Annexes II and III thereto to technical progress (European Commission, 2004b). In this directive, the following substances are listed in *Annex II* as substances that must not form part of the composition of cosmetic products: benzidine dihydrochloride; benzidine-based azo dyes; dimethylbenzidine (4,4′-bi-*ortho*-toluidine); dimethylbenzidine dihydrochloride; dimethylbenzidine-based dyes (*ortho*-tolidine-based); dichlorobenzidine; dichlorobenzidine dihydrochloride; dimethoxylbenzidine and its salts; and dimethoxylbenzidine-based azo dyes.

(f) Directive 2005/90/EC

In the Directive 2005/90/EC, the list of substances classified as carcinogenic, mutagenic or toxic to reproduction (c/m/r) of Directive 76/769/EEC was amended to include benzidine (European Commission, 2005).

1.9.2 *Germany*

Benzidine and its salts are classified as Category-1 carcinogens by the MAK Commission. The MAK Commission listed benzidine and its salts as substances where percutaneous absorption may significantly contribute to systemic exposure.

3,3′-Dichlorobenzidine is classified as a Category-2 carcinogen by the MAK Commission. The MAK Commission listed 3,3′-dichlorobenzidine as a substance where percutaneous absorption may significantly contribute to systemic exposure.

3,3′-Dimethylbenzidine and 3,3′-dimethoxybenzidine are classified as Category-2 carcinogens by the MAK Commission. No MAK values were set for these substances (MAK, 2007).

1.9.3 *Japan*

The Japan Society for Occupational Health (2007) follows the classification by IARC of benzidine in Group 1; of 3,3′-dimethylbenzidine, 3,3′-dichlorobenzidine, 3,3′-dimethoxybenzidine, C.I. Acid Red 114, and C.I. Direct Blue 15 in Group 2B; and of C.I. Direct Black 38, C.I. Direct Blue 6, and C.I. Direct Brown 95 in Group 2A.

1.9.4 *USA*

(a) ACGIH

Benzidine has been assigned an A1 notation, *Confirmed Human Carcinogen*. No numerical TLV (threshold limit value) is recommended for occupational exposure for agents assigned an A1 notation. A skin notation is recommended based on the skin being a significant route of entry into the body, leading to systemic toxicity. As for any substance with no recommended TLV and an A1 carcinogenicity notification, worker exposure should be carefully controlled to the fullest extent possible (ACGIH, 2001).

3,3′-Dimethylbenzidine (*ortho*-tolidine) and 3,3′-dichlorobenzidine and its dihydrochloride salt, have been assigned an A3 notation, *Confirmed Animal Carcinogen with Unknown Relevance to Humans*. No numerical TLVs are recommended for occupational exposure to these substances. A skin notation is recommended based on the skin being a significant route of entry into the body, leading to systemic toxicity (ACGIH, 2001).

(b) NIOSH

The National Institute for Occupational Safety and Health (NIOSH, 2005) lists benzidine and 3,3′-dichlorobenzidine among 13 OSHA-regulated carcinogens. Exposures of workers to these chemicals should be controlled through the required use of engineering controls, work practices, and personal protective equipment, including respirators. OSHA and NIOSH concluded that benzidine and benzidine-based dyes were potential occupational carcinogens and recommended that worker exposure be reduced to the lowest feasible level. OSHA and NIOSH further concluded that *ortho*-tolidine (3,3′-dimethylbenzidine) and *ortho*-dianisidine (3,3′-dimethoxybenzidine) and dyes based on these compounds may present a cancer risk to workers and should be handled with caution.

(c) NTP

Benzidine and dyes that are metabolized to benzidine are listed in the NTP *Report on Carcinogens* (NTP, 2005a) as *known human carcinogens*.

3,3′-Dimethylbenzidine and dyes that are metabolized to 3,3′-dimethylbenzidine are listed in the NTP *Report on Carcinogens* (NTP, 2005b) as *reasonably anticipated to be human carcinogens*.

3,3′-Dichlorobenzidine and 3,3′-dichlorobenzidine dihydrochloride are listed in the NTP *Report on Carcinogens* (NTP, 2005c) as *reasonably anticipated to be human carcinogens*.

3,3′-Dimethoxybenzidine and dyes that are metabolized to 3,3′-dimethoxybenzidine are listed in the NTP *Report on Carcinogens* (NTP, 2005d) as *reasonably anticipated to be human carcinogens*.

1.9.5 *Other*

(a) GESTIS

Table 1.12 presents some international limit values for benzidine and its congeners (GESTIS, 2007).

Table 1.12. International limit values (2007) for benzidine and its congeners

Country	Limit value – Eight hours		Limit value – Short term		Comments
	ppm	mg/m³	ppm	mg/m³	
Benzidine					
France	0.001	0.008			
Hungary				0.008	
Italy			0.001		
3,3′-Dimethylbenzidine					
Austria	0.003	0.03	0.012	0.12	TRK value (based on technical feasibility)
USA-NIOSH				0.02	Ceiling limit value (60 minutes)
3,3′-Dichlorobenzidine					
Austria	0.003	0.03	0.012	0.12	TRK value (based on technical feasibility)
Hungary				0.03	
Switzerland	0.003	0.03			
3,3′-Dimethoxybenzidine					
Austria	0.003	0.03	0.012	0.12	TRK value (based on technical feasibility)
Switzerland	0.003	0.03			

From: GESTIS (2007)
TRK, technical guiding concentration

2. Studies of Cancer in Humans

2.1 Case reports

Numerous case reports from different countries were reviewed and described in the IARC Monographs Volume 1, Volume 29 and Supplement 7 (IARC, 1972, 1982, 1987). Relevant studies are discussed below.

Vigliani and Barsotti (1962) reported 47 tumours of the urinary bladder (31 carcinomas, 16 papillomas) that occurred between 1931 and 1960 in six Italian dyestuff factories among workers involved in benzidine production and utilization [number of workers at risk not available]. Twenty of the 47 cases occurred between 1931 and 1948 among 83 Italian dyestuff workers. Airborne concentrations of benzidine measured in one of the plants ranged from 0 to 2.0 μg/m^3 (13 samples, mean = 0.3 μg/m^3), and urinary concentrations ranged from 6 to 25 μg/L [the number of specimens analysed was not provided].

Zavon *et al.* (1973) followed for 13 years a group of 25 men occupationally exposed to benzidine during its manufacture in a plant in Cincinnati (USA). All of the workers were exposed to benzidine, three of the workers were also exposed to 2-naphthylamine for about one year, and three to α-toluidine. Airborne benzidine concentrations at various locations within the plant varied from < 0.005 to a maximum of 17.6 mg/m^3 at a location where the workers shovelled benzidine into drums; the approximate mean urinary concentration reached 0.04 mg/L by the end of the workshift. Thirteen men (52%) developed transitional cell bladder carcinoma after a mean exposure of 13.6 years and an average latency (time from first exposure) of 16.6 years. The mean duration of exposure for those who did not develop tumours was 8.9 years. Four renal tumours were observed in three men. [The Working Group of Volume 29 considered that the high incidence of bladder cancer in this cohort was remarkable evidence of the carcinogenic potency of benzidine].

Since the publication of Supplement 7 (IARC, 1987), four case reports have documented the presence of bladder cancer cases among workers exposed to benzidine (Matsushima, 1989; Mason *et al.* 1992; Szeszenia-Dabrowska *et al.* 1997; Miyakawa *et al.* 2001). Based on their observations, Miyakawa *et al.* (2001) suggested that the latency period of occupational bladder cancer after exposure to benzidine could be longer than 40 years.

2.2 Cohort studies (see Table 2.1)

Case *et al.* (1954) studied workers from 21 dyestuff factories in England and Wales. Bladder cancer occurred approximately 15–20 years after exposure to different aromatic amines, including benzidine only, aniline, 1-naphthylamine only, 2-naphthylamine only, magenta, auramine and mixed exposures. They found a total of 127 deaths for which the

Table 2.1. Summary of cohort studies of workers exposed to benzidine

Reference, location, name of study	Cohort description	Exposure assessment	Organ site (ICD code)	Exposure categories	No. of cases/ deaths	Relative risk (95% CI)*	Adjustment factors	Comments
Case *et al.* (1954), England and Wales	Cohort of 4622 dyestuff workers exposed to BZ and other aromatic amines; mortality follow-up 1921–49	Inspection of 21 participating facilities and work history data of cases; workers classified by exposure to different aromatic amines	Bladder	Overall BZ only	127 10	**SMR** 31.1 [25.9–36.9] 13.9 [6.7–25.5]		Reference, England and Wales; 34 cases reported among workers exposed only to BZ
Mancuso & el-Attar (1967), Ohio	Cohort of 639 white men exposed to BZ and/or BNA employed in 1938–39; incidence follow-up through 1965	Based on company records	Bladder and kidney	*Exposure group* BZ only BZ and BNA	 7 18	**Cumulative Incidence per 100 000** 237 1590		
Sun & Deng (1980), China	Cohort of 1601 men exposed to BZ in the chemical dye industry	Duration of employment	Bladder	*Exposure (years)* <5 5–9 10+ *p* for trend	 1 7 13	**Morbidity %** 0.1 1.4 4.7 <0.01		

Table 2.1 (contd)

Reference, location, name of study	Cohort description	Exposure assessment	Organ site (ICD code)	Exposure categories	No. of cases/ deaths	Relative risk (95% CI)*	Adjustment factors	Comments
Morinaga *et al.* (1982), Osaka, Japan	Cohort of 3322 men employed in BZ and BNA manufacture during 1950–78; vital status follow-up 100%	Occupational history from plant records				**SMR**		Among 244 exposed workers with previous genito-urinary cancer 11 developed second primary cancer; SMRs presented for second primary cancer; local reference
			Large intestine	Overall	2	6.9 [0.8–24.9]		
			Liver, gallbladder and bile ducts	Overall	3	8.6 [1.8–25.0]		
			Respiratory system	Overall	4	3.1 [0.9–8.1]		
Rubino *et al.* (1982); Decarli *et al.* (1985); Piolatto *et al.* (1991), Turin, Italy	Cohort of 664 male workers employed >1 year during 1922–70 in a dyestuff manufacturing plant and exposed to arylamines; mortality follow-up 1946–89, 94% complete	Occupational history from plant records included categories of exposure to selected chemicals; overall classification as exposed to aromatic amines	Bladder	Overall	49	**SMR** 30.4 [23.0–40.2]		National reference
				Time since last exposure (years)				
				During exposure	15	100.8 [60.8–167.2]		
				<10	15	39.8 [24.0–66.0]		
				10–19	12	19.5 [11.1–34.3]		
				20+	7	14.8 [7.1–31.0]		

Table 2.1 (contd)

Reference, location, name of study	Cohort description	Exposure assessment	Organ site (ICD code)	Exposure categories	No. of cases/ deaths	Relative risk (95% CI)*	Adjustment factors	Comments
Meigs *et al.* (1986), Connecticut, USA	Cohort of 984 workers (830 men, 154 women) of a BZ manufacturing plant employed ≥1 day during 1945–65; incidence follow-up 1945–78	Occupational history from employment, production and sales records; expert reconstruction of employment histories and estimation of time exposed to BZ	Bladder			**SIR**		State reference; cancer incidence decline[d] >1950, coinciding with measures to reduce exposure
				Overall	8	3.4 [1.5–6.8]		
				BZ exposure in men				
				≤1 day	1	1.8 (0.1–10.1)		
				>1 day–6 months	0	0 (0–4.7)		
				>6 months–<2 years	1	1.9 (0.1–10.7)		
				≥2 years	6	13.0 (4.8–28.4)		
				Employment (years)				
				<1	0	0 (0–3.2)		
				1–5	2	3.4 (0.4–12.4)		
				5+	6	10.0 (0.6–21.7)		
Wu (1988), Shanghai, China	Cohort of 2525 workers (1860 men, 665 women) of BZ manufacturing plants employed >1 year during 1972–81	N/A	Bladder			**SIR**		Local reference
				Overall	30	26.1 (18.8–35.4)		
						RR		
				Interaction analysis				
				BZ -, smoking -		1.0		
				BZ -, smoking +		6.2 (p=0.05)		
				BZ +, smoking -		63.4 (p <0.05)		
				BZ +, smoking +		152.3 (p <0.01)		

Table 2.1 (contd)

Reference, location, name of study	Cohort description	Exposure assessment	Organ site (ICD code)	Exposure categories	No. of cases/ deaths	Relative risk (95% CI)*	Adjustment factors	Comments
Delzell (1989); Sathiakumar and Delzell (2000), New Jersey, USA	Cohort of 3266 workers at a dye and resin manufacturing plant (2859 men, 407 women) employed >6 months during 1952–1995; mortality follow-up 1952–1995; vital status 99%; cause of death 97%	Occupational history from plant records; subjects classified by dates of employment and years worked in 8 major work areas; North Dyes area used BZ from 1959–1970				**SMR**		State reference
			Bladder	Overall	8	1.4 (0.6–2.7)		
				BZ use	4	5.2 (1.4–13.2)		
			Lymphopoietic	Overall	12	0.5 (0.2–0.8)		

Table 2.1 (contd)

Reference, location, name of study	Cohort description	Exposure assessment	Organ site (ICD code)	Exposure categories	No. of cases/ deaths	Relative risk (95% CI)*	Adjustment factors	Comments
You *et al.* (1990), Shanghai, China	Cohort of 736 BZ dyes production workers (550 men, 186 women) employed >6 months; mortality and incidence follow-up from first entry to 1982; vital status 100%; 100% histologically confirmed	Occupational history from plant records; jobs classified into pre-synthesis (chemical changes from BZ to dyes) and post-synthesis (processes leading to finished dyes) groups	Bladder			**SMR**		Local reference
				Men	5	14.7 (p <0.01)		
				Women	0	0		
				Jobs among men				
				Pre-synthesis	5	31.3 (p <0.01)		
				Material treatment	2	66.7 (p <0.01)		
				Synthetic reaction	3	30.0 (p <0.01)		
				Maintenance and others	0	0		
				Post-synthesis	0	0		
						SIR		
				Men	14	19.2 (p <0.01)		
				Women	1	50.0 (p <0.05)		
				Jobs among men				
				Pre-synthesis	14	35.0 (p <0.01)		
				Material treatment	6	75.0 (p <0.01)		
				Synthetic reaction	7	26.9 (p <0.05)		
				Maintenance and others	1	20.0 (p <0.05)		
				Post-synthesis	0	0		

Table 2.1 (contd)

Reference, location, name of study	Cohort description	Exposure assessment	Organ site (ICD code)	Exposure categories	No. of cases/ deaths	Relative risk (95% CI)*	Adjustment factors	Comments
Szeszenia-Dabrowska *et al.* (1991), Poland	Cohort of 6978 male rubber goods production workers employed >3 months during 1945–73; mortality follow-up 1945–85; vital status 90%	Occupational history from plant records; type and concentration of chemical exposure indirectly estimated				**SMR**		National reference; multiple exposures, including BNA
			Bladder	Overall	10	1.2 [0.6–2.2]		
				Subcohort employed during 1945–53	6	2.8 [1.2–6.1]		
			Lympho-haematopoietic (200–208)	Overall	7	0.5 [0.2–1.03]		
Shinka *et al.* (1991); Shinka *et al.* (1995), Wakayama City, Japan	Cohort of 363 workers of 9 dye manufact-uring plants; incidence follow-up to 1964–94; vital status 100%	Occupational history from plant records; workers classified by potential BZ or BNA exposure				**OR**		
			Urothelial	*All factories*				
				BZ exposure	49	8.3 (1.6–42.6)		
				BNA exposure	3	1		
				BZ and BNA	6	4.3 (0.9–19.7)		
				Factory A only				
				BZ exposure	4	12.7 (2.0–81.2)		
				BNA exposure	2	1		
				BZ and BNA	6	6.2 (1.1–35.4)		

Table 2.1 (contd)

Reference, location, name of study	Cohort description	Exposure assessment	Organ site (ICD code)	Exposure categories	No. of cases/ deaths	Relative risk (95% CI)*	Adjustment factors	Comments
Bi *et al.* (1992); Hayes *et al.* (1993); Carreón *et al.* (2006), Tianjin, Shanghai, Jilin, Henan and Chonquin, China	Nested case-control study in cohort of 2515 workers (1850 men, 665 women) employed >1 year during 1945–77 in BZ production and use facilities; 68 cases (diagnosed 1965–1991), 107 controls frequency-matched by 10 year age	Based on knowledge of operations and limited IH data, BZ exposure level assigned to each job and multiplied by duration of exposure	Bladder			**OR**	Lifetime cigarette smoking	
				BZ cumulative level years				
				Low (<30)	32	1.0		
				Medium (30–59)	15	2.7 (1.1–6.3)		
				High (≥60)	17	4.4 (1.8–10.8)		
Bulbulyan *et al.* (1995), Moscow, Russia	Cohort of 4581 aniline dye production workers (2409 men, 2172 women) employed on Jan.1, 1975 and exposed >1 month to BZ or BNA, or employed for >2 years; 514 men, 287 women exposed to BZ or BNA; mortality and incidence follow up 1975–89; ca 90% histologically confirmed cases	Limited industrial hygiene air and environmental measurements; jobs classified into groups based on BZ or BNA exposure	Bladder			**SIR**		Local reference, no lymphohaematopoietic cancer SIR provided for group exposed to BZ or BNA
				Ever exposed to BZ or BNA				
				Men	19	10.8 [6.9–17.0]		
				Women	5	21.0 [8.7–50.4]		
				Ever exposed to BZ				
				Employment (years)				
				<10	6	11.2 (4.1–24.3)		
				10–19	7	17.2 (6.9–35.4)		
				20–29	2	5.7 (0.6–20.6)		
				30–39	2	4.7 (0.1–26.1)		
				40+	1	13.6 (0.2–75.9)		
				p for trend		0.22		

Table 2.1 (contd)

Reference, location, name of study	Cohort description	Exposure assessment	Organ site (ICD code)	Exposure categories	No. of cases/ deaths	Relative risk (95% CI)*	Adjustment factors	Comments
Szymczak *et al.* (1995); Sitarek *et al.* (1995), Poland	Cohort of 10 529 dye production workers (8523 men, 2006 women) employed >3 months during 1945–74; mortality follow-up 1945–91	Workers classified into 4 exposure groups: I- BZ only, II-BZ & other occupational hazards, III -involved in dye production with no BZ exposure, IV- not involved in dye production	Bladder	*Men*		**SMR**	Age, gender, calendar time	National reference
				Exposed to BZ only	9	14.7 [7.6–28.2]		
				Exposed to BZ & other occupational hazards	15	16.3 [9.9–27.1]		
			Lympho-haematopoietic (200–208)	Exposed to BZ only	2	1.7 [0.4–6.7]		
Naito *et al.* (1995), urban area, Japan	Cohort of 442 workers of a BZ production and dye manufacturing plant (437 men, 5 women) during 1935–88; mortality and incidence follow-up 1935–92; vital status 100%	Duration of employment at BZ manufacture or use facility as surrogate of duration of exposure	Urinary tract (188, 189)	BZ manufacture	14	**SMR** 45.1 (24.7–75.7)		National reference; incidence rates reported by duration of exposure; PPE reportedly used among all workers
				BZ use	6	15.8 (5.8–34.3)		
			Bladder	BZ manufacture	10	63.6 (30.5–117.0)		
				BZ use	5	27.0 (8.8–63.0)		

Table 2.1 (contd)

Reference, location, name of study	Cohort description	Exposure assessment	Organ site (ICD code)	Exposure categories	No. of cases/ deaths	Relative risk (95% CI)*	Adjustment factors	Comments
Rosenman & Reilly (2004), Michigan, USA	Cohort of 488 white men employed in a chemical manufacturing facility during 1960–77; mortality follow-up 1979–2001; incidence follow-up 1981–2002 (Michigan Tumour Registry)	Time and length of employment estimated from social security records; workers classified as exposed to BZ or not if employed before or after 1973	Bladder	Overall	3	**SMR** 8.3 (1.7–24.4)		National reference for SMR and SEER for SIR
				Year started work				
				<1973	3	9.6 (2.0–28.1)		
				≥1973	0	0		
			Lympho-haematopoietic	Overall	6	2.8 (1.04–6.2)		
				Year started work				
				<1973	3	1.8 (0.4–5.3)		
				≥1973	3	6.6 (1.4–19.4)		
			Leukemia	Overall	4	5.1 (1.4–12.9)		
			Bladder	Overall	22	**SIR** 6.9 (4.3–10.4)		

ANA, 1-naphthylamine; BNA, 2-naphthylamine; BZ, benzidine; IH, industrial hygiene; ND, not determined; OR, odds ratio; PPE, personal protective equipment; SEER, Surveillance, Epidemiology and End Results Program; SIR, standardized incidence ratio; SMR, standardized mortality ratio

death certificate indicated bladder cancer (4.09 expected). For workers exposed exclusively to benzidine, 10 confirmed bladder-cancer deaths were found (0.72 expected). The authors also reported a total of 34 incident bladder-cancer cases among workers exposed to benzidine only.

In a study of 639 white men exposed to benzidine and/or 2-naphthylamine in a plant in Ohio (USA), Mancuso & el-Attar (1967) reported 14 bladder-cancer deaths and a total of 18 genitourinary cancer deaths. The mortality rate for bladder cancer in the cohort was 78/100 000 versus 4.4/100 000 expected on the basis of Ohio male mortality rates. The cumulative risk for incident bladder cancer in workers exposed to benzidine was reported to be 237 per 100 000. The authors also noted six cases of pancreatic cancer, with a cumulative mortality rate of 39 per 100 000 (*vs* 7.5/100 000 expected).

Among 1601 workers in the chemical-dye industry in China who were exposed to benzidine, methylnaphthylamine and dianisidine, An & Deng (1980) reported 21 cases of bladder cancer. All cases had a history of exposure to benzidine, and no cancer was found among workers exposed to methylnaphthylamine or dianisidine. An exposure-response relationship was suggested, as the percentage of cases among exposed workers increased with length of exposure (test for trend $P < 0.01$).

Morinaga *et al.* (1982) ascertained the incidence of second primary cancers in 3322 workers employed from 1950 to 1978 in industries in Japan that manufactured benzidine and 2-naphthylamine. Of the 244 workers who had developed cancer of the genitourinary organs, 11 men subsequently developed histologically confirmed cancers of the liver, gallbladder, bile duct, large intestine, and lung. An unexposed group of 177 male bladder-cancer patients, assembled from the Osaka Cancer Registry during 1965–1975, showed eight cases of a second primary cancer, five being stomach cancer. No stomach cancer was observed in the study cohort. A statistically significant excess risk for liver, gallbladder, and bile-duct cancer ($P < 0.05$) was found. The number of observed deaths from respiratory cancer was greater than expected, but not statistically significant.

In 1982, Rubino *et al.* reported the results of a retrospective cohort study in dye-manufacturing workers of Turin, Italy. A very high bladder-cancer risk was observed among workers exposed to benzidine (five deaths observed, SMR = 83.3). An extended follow-up conducted by Decarli *et al.* (1985) reported 41 deaths (SMR 46.1; 95% CI, 33.9–62.6) for the total cohort. Piolatto *et al.* (1991) added eight years of follow-up. The cohort included 664 male workers employed more than one year from 1922 to 1970. Occupational history was obtained from plant records and included categories of exposure to selected chemicals including benzidine. The overall bladder-cancer mortality risk was very high (49 deaths, SMR 30.4; 95% CI, 23.0–40.2). The risk for bladder cancer was also found to vary inversely both with age at first exposure and time since last exposure. The authors reported elevated SMRs for upper digestive and respiratory tract cancers.

Meigs *et al.* (1986) reported a statistically significant excess of bladder tumours in a cohort of 984 workers at a benzidine-manufacturing facility in Connecticut. Benzidine-exposure status was determined from information contained in employment, production

and sales records. Eight cases of bladder cancer were observed (SIR 3.4; 95% CI, 1.5–6.8). Risk was greatest among those in the highest exposure category (SIR 13.0; 95% CI, 4.8–28.4). Risk also showed an increasing trend with length of employment: < 1 year, SIR = 0 (1.15 expected; 95% CI, 0–3.2); 1–5 years, SIR = 3.4 (95% CI, 0.4–12.4); and > 5 years, SIR 10.0 (95% CI, 0.6–21.7). The authors report a decline in the overall bladder-cancer incidence among those employed after 1950, coincident with the implementation of major preventive exposure measures, but indicate that this finding is limited by the small number of cases.

Wu (1988) reported the results of studies conducted by The Cooperative Group in China. One of these studies was a retrospective cohort study of 2525 workers exposed to benzidine for at least one year from 1972 through 1981 [the author reports that the production and use of benzidine in China was stopped in 1977]. Twelve deaths and 30 incident bladder-cancer cases were observed. An excess incidence of bladder cancer compared with the Shanghai general population was observed (SIR 26.1; 95% CI, 18.8–35.4). A synergistic effect of smoking on benzidine-associated bladder-cancer risk was also observed. When compared with non-smoking and non-exposed workers, the relative risk for bladder cancer of smoking non-exposed workers was 6.2, compared with a risk of 63.4 for non-smoking exposed workers, and a risk of 152.3 for smoking exposed workers. In addition to the principal findings related to bladder cancer, a slight increase in the incidence of lung and stomach cancers was noted in workers exposed to benzidine. [The Working Group noted that quantitative data were not provided.]

A cohort of workers employed at a New Jersey (USA) dye and resin manufacturing plant was examined from 1952 to 1985 as part of a larger retrospective study of 2642 workers (Delzell *et al.* 1989). Occupational history was obtained from plant records, and department titles were classified into 10 work areas. The azo-dye area involved exposures to dye-related compounds including benzidine. Eighty-nine of the workers had former employment at the Cincinnati Chemical Works (CWW), which had produced or used benzidine and 2-naphthylamine. The 2553 workers who had never worked at the CWW, and therefore had little potential for benzidine exposure, had fewer than expected deaths from all causes combined and from diseases of all major organ systems. The former CWW workers had an excess of cancer, which was due to excess mortality from bladder (SMR 12, $P = 0.004$), kidney (SMR 9.5, $P = 0.04$), and central nervous system (SMR 9.1, $P = 0.04$) cancers. Sathiakumar and Delzell (2000) added 10 years of follow-up by extending the cohort through 1995. The expanded cohort included 3266 workers (2859 men, 407 women) employed more than 6 months. The bladder-cancer mortality excess observed in the earlier study among former CWW workers remained in the expanded cohort. The overall SMR for bladder cancer among all white men employed in the North Dyes area (where benzidine was used from 1959 to 1970) was 5.2 (95% CI, 1.4–13.2). The authors attributed the bladder-cancer excess to exposure to aromatic amines at the CWW, since plant employees that had not worked at the CWW had approximately equal observed and expected deaths from bladder cancer.

You *et al.* (1990) conducted a retrospective cohort study in seven factories producing benzidine-based dyes in Shanghai, China. The cohort included 736 production workers (550 men, 186 women) employed for more than six months. Occupational history was obtained from plant records and included accumulated working time in benzidine production. Men were classified based on their jobs into two groups: pre-synthesis (thought to have been exposed to benzidine) and post-synthesis (exposed mainly to finished benzidine-based dyes). Five deaths and 14 cases of bladder cancer in men were observed, all in the pre-synthesis group. Increased mortality and incidence were observed in the whole cohort and in the pre-synthesis group, particularly among those workers involved in material treatment (SIR 75.0, $P < 0.01$) and synthetic reaction (SIR 26.9, $P < 0.05$).

Szeszenia-Dabrowska *et al.* (1991) studied a cohort of 6978 men employed in rubber goods production, predominantly rubber footwear, in Poland. The cohort included workers employed for more than three months from 1945 to 1973. Occupational history was obtained from plant records. The authors indicated that aromatic amines, including benzidine, are among the chemicals used as additives in rubber factories. The type and level of chemical exposure were indirectly estimated. Ten deaths from bladder cancer were observed in the whole cohort (8.4 expected), and six were among workers employed in production. Among the subcohort employed during 1945 and 1953, and presumably exposed to aromatic amines including benzidine, the SMR for bladder cancer was 2.8 (95% CI, 1.2–6.1). Seven deaths from lymphopoietic cancers were observed (14.2 were expected).

Shinka *et al.* (1991) observed that 105 of 874 Japanese workers of nine dye-manufacturing plants who were engaged in the manufacture and handling of benzidine developed urothelial (primarily bladder) cancer. In a more recent study, Shinka *et al.* (1995) extended the follow-up of 363 exposed workers. Occupational history was obtained from plant records and workers were classified into exposure categories with regard to benzidine or 2-naphthylamine. Workers were also classified according to their work in benzidine manufacture, benzidine use, or both. The risk factors significantly related to tumour occurrence in all nine plants were benzidine as a dye intermediate (OR 8.3; 95% CI, 1.6–42.6) and manufacturing work (OR 4.6; 95% CI, 1.9–11.0).

In a cohort of 1972 benzidine-exposed workers in China between 1972 and 1977, Bi *et al.* (1992) examined bladder-cancer mortality and incidence through 1981. Limited industrial hygiene data on benzidine were available, and were used in combination with knowledge of operations to classify the job held for the longest time as being associated with a high, medium or low exposure to benzidine. The authors reported an overall SMR of 17.5 (eight deaths; 95% CI, 7.5–34.5) and an overall SIR of 25 (30 cases, 95% CI, 16.9–35.7), with risks ranging from 4.8 (95% CI, 1.0–14.1) to 158.4 (95% CI, 67.6–309.0) for low to high exposures. Further, bladder cancer was positively associated with exposure duration. Benzidine-exposed workers who also smoked cigarettes had a 31-fold increased risk for bladder cancer, compared with an 11-fold increased risk observed in

non-smoking workers. Hayes *et al.* (1993) conducted a nested-case control study in 38 bladder cancer cases and 43 controls from this cohort. Using the exposure categories developed by Bi *et al.*, and assigning them a score of 1 for low, 3 for medium and 9 for high, the authors estimated cumulative benzidine exposure as the product of each score times duration of exposure. Cumulative benzidine level-years were categorized into low (< 30), medium (30–59) and high (≥ 60) exposure. Compared with low exposure, the risks for medium and high exposure were OR 2.6 (95% CI, 0.8–8.9) and OR 6.7 (95% CI, 1.7–33.6), respectively. Carreón *et al.* (2006) expanded the previous study to include 68 cases and 107 controls frequency-matched by 10-year age groups. Using the same cumulative benzidine-exposure categories, the risks for medium and high exposure, compared with low, were OR 2.7 (95% CI, 1.1–6.3) and OR 4.4 (95% CI, 1.8–10.8). These risks were adjusted for lifetime cigarette smoking. Additionally, Carreón *et al.* (2006) reported that *NAT2* genotype slow acetylators had a reduced risk for bladder cancer when compared with rapid acetylators (OR, 0.3; 95% CI, 0.1–1.0). The study did not have sufficient power to evaluate the interaction between *NAT2* polymorphisms and benzidine exposure. To overcome this limitation, the authors compared the result presented above with the result of a meta-analysis of eight case–control studies of NAT2 acetylation and bladder cancer in Asian populations not exposed occupationally to aromatic amines. The authors concluded that there was evidence of a gene-environment interaction, as the upper limit of the estimate obtained in their study adjoined the lower limit obtained in the meta-analysis (pooled OR, 1.4; 95% CI, 1.0–2.0).

Bulbulyan *et al.* (1995) evaluated a cohort of 4581 aniline dye-production workers for cancer incidence and mortality. The study included limited industrial hygiene air and environmental measurements of benzidine and 2-naphthylamine dating back to the 1930s; jobs were classified into groups based on exposure to benzidine and 2-naphthylamine. In a group of 514 men and 287 women who had been ever exposed to benzidine or 2-naphthylamine, there were 115 observed cases of all cancers *vs* 62.57 expected cases. Among men ever exposed to benzidine or 2-naphthylamine, increased incidence was observed for cancers of the oesophagus (SIR 3.5; 95% CI, 1.4–8.4), lung (SIR 2.3; 95% CI, 1.5–3.4), and bladder (SIR 10.8; 95% CI, 6.9–17.0). Women had a statistically significant excess of bladder cancer (21.0; 95% CI, 8.7–50.4). Excess cancer rates, including for bladder cancer, were also found in men and women exposed only to "other" chemicals. The risk for bladder cancer did not increase with duration of employment for workers ever-exposed to benzidine, but it did for workers ever-exposed to 2-naphthylamine.

Naito *et al.* (1995) conducted a retrospective cohort mortality study of 442 workers (437 men, five women) exposed to one or more substances (mainly benzidine, 2-naphthylamine, 1-naphthylamine, and *ortho*-dianisidine) at a benzidine-production and dye-manufacturing plant in Japan. No industrial hygiene data for the plant were available; therefore, duration of employment at the facility was used as a surrogate of duration of exposure. The authors reported that all the workers wore work clothes, gloves, high

rubber boots, and a gas mask in the factory, and that it had wide windows in all directions. A significantly increased risk for bladder cancer was found among workers engaged in the manufacture (SMR 63.6; 95% CI, 30.5–117.0) and use (SMR 27.0; 95% CI, 8.8–63.0) of benzidine. Increased risks for cancer mortality for other organs were observed, but these were not statistically significant. [No information was provided for lymphohaematopoietic cancers.] Incidence rate-ratios of urothelial cancer increased with duration of exposure for both benzidine manufacture and use.

Szymczak *et al.* (1995) and Sitarek *et al.* (1995) carried out a mortality study among 10 529 workers in a dye-manufacturing plant in Poland, involving exposure to benzidine. Workers were classified into four groups based on their potential to benzidine exposure. A statistically significant increase in mortality from bladder cancer was observed among men exposed to benzidine only (SMR 14.7; 95% CI, 7.6–28.2) and benzidine and other occupational hazards (SMR 16.3; 95% CI, 9.9–27.1). Increased mortality risks were also observed among men exposed to benzidine and other occupational hazards for pancreatic cancer (SMR = 3.3; 95% CI, 1.2–8.7). No excess mortality for lymphohaematopoietic cancers was observed among men exposed to benzidine alone or in combination with other occupational hazards.

Rosenman and Reilly (2004) analysed a cohort of 488 white men employed in a chemical manufacturing facility in Michigan, USA. The facility had produced benzidine from 1960 through 1972 and 3,3′-dichlorobenzidine from 1961 to 2001. Workers were identified from social security records. Since no plant records were available, social security data were used to estimate time of first work and years worked. Analyses were conducted for the entire cohort and separately for people who began to work in 1973 or later, after benzidine production had been discontinued. For the whole cohort, an excess of bladder-cancer mortality was observed (SMR 8.3; 96% CI, 1.7–24.4). All cases occurred in those with five or more years of duration of work. There were six deaths from lymphohaematopoietic cancer (SMR 2.8; 95% CI, 1.04–6.2) including one from non-Hodgkin lymphoma, one from multiple myeloma, two from chronic lymphocytic leukaemia, one from acute leukaemia, and one from chronic myelogenous leukaemia (SMR for leukaemia 5.1; 95% CI, 1.4–12.9). The SIR for bladder cancer was 6.9 (95% CI, 4.3–10.4); no additional cases of lymphohematopoietic cancer were identified from the cancer registry. All bladder-cancer deaths and 21 of 22 cases of bladder cancer occurred among those employed before 1973. A statistically significant increase in mortality from lymphohematopoietic cancer was observed among workers who began work in 1973 or later.

2.3 **Case-control studies** (see Table 2.2)

Since the publication of Supplement 7, a few case–control studies of benzidine exposure and the risk for bladder and other cancers have been published. These studies have a high potential for misclassification of exposure, as information is collected on past

Table 2.2. Summary of case-control studies of workers exposed to benzidine

Reference, study location and period	Organ site (ICD code)	Characteristics of cases	Characteristics of controls	Exposure assessment	Exposure categories	Relative risk (95% CI)	Adjustment factors & comments
Schumacher and Slattery (1989), Utah, USA	Bladder	417 (332 men, 85 women) from state cancer registry, aged 21-84; response rate 76%	877 (685 men, 192 women) population-based controls, frequency-matched for age and sex in a 2:1 ratio; response rate 79%	Interviewer-administered standardized questionnaire; occupational-exposure linkage system used to identify workers exposed to BZ	*BZ exposure men* Never Ever <10 years 10+ years *BZ exposure women* Never Ever	 1.0 1.2 (0.7–2.1) 1.0 (0.5–2.1) 1.6 (0.6–4.1) 1.0 1.0 (0.4–2.2)	Adjusted for age, smoking, religion, education smoking
You *et al.* (1990), Shanghai, China	Bladder	317 men from Shanghai industry, aged 23–78 years; 41 had occupational exposure to BZ; response rate 100%	317 hospital-based non-cancer controls, matched by hospital, gender, age within 5 years, from same industrial and residential districts as cases	Employment record, workers employed >6 months in the dyestuffs, rubber, cable, ink, dress pressing and cigarette industries were considered exposed	Occupational exposure to BZ	5.7 (p <0.001) ND	(Smoking)
Ugnat *et al.* (2004), British Columbia, Alberta, Saskatchewan, Manitoba, Canada, 1994–97	Bladder	549 men from provincial cancer registries aged 20–75 years; 14 reported BZ exposure; response rate 60%; 100% histologically confirmed	1099 population-based controls (15 reported BZ exposure) frequency-matched by sex, 5-year age group; response rate 59%	Mailed standardized questionnaire, telephone interview to those who failed to return it	Ever exposed to BZ *BZ exposure (years)* Never 1–9 10–19 20+ p for trend	2.2 (1.0–4.9) 1.0 2.7 (0.7–10.5) 0 2.7 (0.7–9.1) 0.26	Adjusted for age, province, education, smoking, exposure years, coffee and tea intake

Table 2.2 (contd)

Reference, study location and period	Organ site (ICD code)	Characteristics of cases	Characteristics of controls	Exposure assessment	Exposure categories	Relative risk (95% CI)	Adjustment factors & comments
Mao *et al.* (2000), British Columbia, Alberta, Saskatchewan, Manitoba, Ontario, Prince Edward Island, Nova Scotia and Newfoundland, Canada, 1994–97	Non-Hodgkin lymphoma	1469 (764 males, 705 females) from provincial cancer registries, aged 20–74 years; 24 (21 men, 3 women) reported BZ exposure; response rate 75%; 100% histologically confirmed	5073 population-based controls (55 reported BZ exposure) frequency-matched by 5-year age group, sex and province; response rate 67%	Mailed standardized questionnaire, telephone interview to those who failed to return it	*Ever exposed to BZ* Men Women *BZ exposure in men (years)* Never 1–3 ≥4 *p* for trend	 1.9 (1.1–3.4) 0.6 (0.2–2.2) 1.0 1.1 (0.3–4.4) 2.2 (1.1–4.0) 0.02	Adjusted for age, province, BMI, milk intake
Hu *et al.* (2002), British Columbia, Alberta, Saskatchewan, Manitoba, Ontario, Prince Edward Island, Nova Scotia and Newfoundland, Canada, 1994–97	Renal cell carcinoma	1279 (691 men, 588 women) from provincial cancer registries, aged 20–70+ years; 32 (28 men, 4 women) reported BZ exposure; response rate 79%; 100% histologically confirmed	5370 population-based controls (66 reported BZ exposure) frequency-matched by 5-year age group, sex and province; response rate 71%	Mailed standardized questionnaire, telephone interview to those who failed to return it	*Ever exposed to BZ* Men Women *BZ exposure in men (years)* Never 1–10 11+ *p* for trend	 2.1 (1.3–3.6) 1.0 (0.3–3.1) 1.0 1.8 (0.8–3.9) 2.5 (1.2–5.0) 0.004	Adjusted for age, province, education, BMI, smoking, alcohol, meat intake

BMI, body mass index; BZ, benzidine; SD, standard deviation

events that may be inaccurately recorded or not available, and information obtained from study participants may be biased. In addition, the level of exposure in these studies is likely to be, on average, much lower than that in the cohort studies, which tend to focus on workers with the highest exposures to benzidine.

In a population-based case–control study in Utah (USA), Schumacher *et al.* (1989) examined the associations between bladder cancer and occupational exposures, including benzidine. The authors included 417 bladder-cancer cases diagnosed between 1977 and 1983, identified from a rapid ascertainment system and the state cancer-registry. Population-based controls were randomly selected and frequency-matched to cases by age and sex in a 2:1 ratio. The number of controls was 877 (685 men, 192 women). Trained interviewers conducted home interviews to obtain complete occupational histories and information on bladder-cancer risk factors. An occupation-exposure linkage system was used to identify workers exposed to suspect bladder carcinogens, including benzidine and 2-naphthylamine. The system graded exposures as "light," "moderate," "heavy" and "unknown". Those with heavy exposures were classified as exposed, and the rest were considered unexposed. The numbers of workers exposed to benzidine and 2-naphthylamine were identical, since the same occupations were linked to both chemicals. Among men, an increased risk for bladder cancer was observed among those exposed to benzidine, even though the association did not reach statistical significance (OR, 1.2; 95% CI, 0.7–2.1). A non-statistically significant risk increase was observed among men with more than 10 years of exposure (OR, 1.6; 95% CI, 0.6–4.1). No increased risk for bladder cancer was observed among women ever-exposed to benzidine. Smoking did not confound these associations.

You *et al.* (1990) selected 317 men with bladder cancer from Shanghai, China, 41 of whom were considered to have been occupationally exposed to benzidine if employed for more than six months in the dyestuffs, rubber, cable, ink, dress pressing or cigarette industries. The study included 317 hospital-based non-cancer controls, matched by hospital, gender, and age within five years. Controls were from the same industrial and residential districts as the cases. Occupational exposure to benzidine was associated with bladder cancer incidence (OR, 5.7; $P < 0.001$).

A series of case–control studies for different cancers and occupations have been conducted in Canada. All of the studies involved the selection of 19 types of cancer (20 755 cases) from eight provincial cancer registries. The cancer registries ascertained cases on the basis of pathology reports. Cancer-free population-based controls (5039) were selected from a random sample of individuals within the provinces. Sampling strategies for controls varied among provinces. Controls were frequency-matched to all cancer cases by age and sex distribution. A standardized questionnaire including occupation and cancer risk factors was mailed to study participants. The questionnaire also recorded information on exposure to 17 different chemicals, including benzidine, for at least a year, and the duration of that exposure. Telephone interviews were conducted with those subjects who failed to return the questionnaire. [These studies have large

sample sizes, are population-based, and control for the known risk factors in each analysis, but have the known limitations of case–control studies, including the low reliability of self-reported exposures and exposure misclassification].

In the most recent analysis of the Canadian series, Ugnat *et al.* (2004) studied 549 men with histologically-confirmed bladder cancer and 1099 male controls. They observed an increased risk for bladder cancer among people who reported exposure to benzidine (adjusted OR, 2.2; 95% CI, 1.0–4.9). The exposure-response relationship was not statistically significant (p for trend = 0.26).

Mao *et al.* (2000) studied 1469 newly diagnosed cases of non-Hodgkin lymphoma and 5073 population-based controls. The authors found increased risks for non-Hodgkin lymphoma among men exposed to benzidine (adjusted OR, 1.9; 95% CI, 1.1–3.4). Among men, an exposure-response effect was observed with increased length of exposure to benzidine (p for trend = 0.02).

Hu *et al.* (2002) analysed the risk for renal cell cancer among 1279 cases and 5370 population-based controls. An increased risk for renal cell carcinoma was observed among men (adjusted OR, 2.1; 95% CI, 1.3–3.6) but not among women (adjusted OR, 1.0; 95% CI, 0.3–3.1). An increased risk with duration of exposure to benzidine was observed among men (p for trend = 0.004).

3. Studies of Cancer in Experimental Animals

Animal bioassays conducted with benzidine were reviewed in IARC Monograph Volumes 1 and 29, and in Supplement 7. The studies mentioned in Volume 1 were all reconsidered in Volume 29. This Section provides a summary of these studies and a more detailed review of more recent ones.

3.1 Benzidine

3.1.1 *Oral administration*

(a) Mouse

Four groups of 50 male B6C3F_1 mice, six weeks of age, were fed 150 ppm benzidine dihydrochloride (certified American Chemical Society (ACS) grade) in the diet for 45 weeks. Groups of 50 mice were killed at 45, 60, 75 and 90 weeks of age, respectively, to evaluate the occurrence of liver-cell tumours. At 45 weeks, 8/50 (16%) mice had tumours, 4% of which were hepatocellular carcinomas. At 60, 75 and 90 weeks the proportions of mice with tumours were 20/50 (40%), 31/50 (62%) and 35/50 (70%). Of these tumours, 10%, 28% and 48%, respectively, were hepatocellular carcinomas. In

historical controls, the incidence of hepatocellular tumours was 1/98 (1%) (Vesselinovitch *et al.,* 1975). [No statistical analysis was applied.]

Three groups of 50 male B6C3F$_1$ mice, six weeks of age, were fed 150 ppm benzidine dihydrochloride (certified ACS grade) in the diet until 45, 60, or 90 weeks of age. All animals were killed at 90 weeks of age. The incidences of mice bearing liver tumours, mostly hepatocellular carcinomas, are given in Table 3.1. A negative relationship was observed between the incidence of liver tumours and the duration of treatment, which may have been related to toxicity (Vesselinovitch *et al.,* 1975). [No statistical analysis was applied.]

Table 3.1. Incidences of liver-cell tumours in male B6C3F$_1$ mice fed benzidine dihydrochloride

Duration of treatment (weeks)	Estimated consumption of benzidine (mg/mouse)	Effective no. animals	Liver-cell tumours	%
39	117	50	35	70
54	162	50	25	50
84	188	50	22	44

From Vesselinovitch *et al.* (1975)

To evaluate the effect of mode of administration in benzidine-induced carcinogenesis, groups of 50 male and 50 female B6C3F1 mice were given doses of 50 or 100 ppm benzidine dihydrochloride (certified ACS grade) in the feed, or twice weekly by stomach tube, at 0.5 or 1.0 mg/treatment. No effects on survival were noted, and all animals were killed at 90 weeks of age. Continuous feeding of benzidine in the diet produced liver-cell tumours in 3/50 (6%) males and in 13/50 (26%) females at the lower dose, and in 11/50 (22%) males and 32/50 (64%) females at the higher dose, which indicates a greater susceptibility of female animals. One liver tumour was seen in 98 male control mice, none in 100 control females. Twice-weekly administration of benzidine by stomach tube seemed to have a weaker hepatocarcinogenic effect than continuous feeding at comparable amounts, especially in female mice: 4/75 (5%) had tumours after intermittent feeding *vs* 13/50 (26%) upon continuous feeding of 50 ppm [no statistical analysis was applied]. Benzidine also caused Harderian gland tumours and lung adenomas in both treatments, and had a marginal effect on the development of lymphoreticular tumours (Vesselinovitch *et al.*, 1975).

Groups of 43–100 B6C3F$_1$ male and female mice [age not specified] were fed a diet containing 150 ppm benzidine dihydrochloride [purity unspecified] (1) from the 12th day of gestation (prenatal) to delivery; (2) to mothers with litters from delivery to weaning; (3) to offspring from weaning to 90 weeks of age; (4) during the pre-natal and pre-weaning period; or (5) prenatally, during pre-weaning and in adulthood. Groups of

untreated controls were also available. Administration pre-natally or during pre-weaning induced a marked increase in the incidence of hepatocellular tumours in male mice (31 and 95%, respectively) but not in females (3 and 5%, respectively). In mice treated from weaning to 90 weeks of age, the tumour incidences were 59% in males and 96% in females. In the group treated both pre-natally and during pre-weaning, the incidences were 100% in males and 25% in females. When mice were treated pre-natally, during pre-weaning and then up to 90 weeks of age, the incidences of hepatocellular tumours were 100% in males and 94% in females [no statistical analysis applied]. The incidences of hepatocellular tumours in untreated controls were 1% in males and 0% in females (Vesselinovitch *et al.,* 1979).

Groups of F_1 (C57BL/6Jf C3Hf/Nctr females × BALB/cStCrLfC3Hf/Nctr males) and mono-hybrid (F_1 females and F_1 males) weanling mice were fed diets containing 0, 30, 60, 120, 200 or 400 ppm of benzidine hydrochloride [purity unspecified]. The 400-ppm dose was chosen on the basis of preliminary tests, the highest dose being probably the maximum tolerated dose. Groups of mice were killed after 40, 60 or 80 weeks of treatment. The incidences of hepatocellular adenomas and carcinomas in the control and treated groups, summarized in Table 3.2, were increased in treated mice (Nelson *et al.,* 1982).

In studies designed to assess the susceptibility of mice to liver tumours at different stages of development, groups of $B6C3F_1$ mice were fed diets containing 150 ppm of benzidine dihydrochloride [purity not specified]. Pregnant female mice were fed from the 12th day of gestation to delivery (group 1), to mothers with litters from delivery to weaning (group 2) and to offspring from weaning through 90 weeks (group 3). Ninety-eight to 100 $B6C3F_1$ male and female mice were killed at 52, 90 and 142 weeks and served as controls. The incidences of hepatocellular adenomas and carcinomas are summarized in Table 3.3. The incidences of carcinomas were increased in the pre-weaning and adult mice (Vesselinovitch, 1983). [No statistical analysis was applied.]

Groups of 72–120 F_1 (BALB/cStCr1fC3Hf/Nctr males × C57BL/6Jf C3Hf/Nctr females) and mono-hybrid cross (MC) (F_1 males and F_1 females) mice, 4–5 weeks of age, weighing 8–15 g were given drinking-water containing 0, 30, 40, 60, 80, 120 or 160 ppm (for males) and 0, 20, 30, 40, 60, 80 or 120 ppm (for females) of benzidine dihydrochloride [purity unspecified] and killed after 33 months of exposure. Dose levels were selected using data from a prior study (Frith & Dooley, 1976). The incidence of hepatocellular carcinomas, along with data on body weights, water consumption, the dose received by animals, the overall mortality, adjusted liver-tumour mortality and the time to liver tumour were reported (Littlefield *et al.*, 1984). Effects on body weight and survival were noted in both strains. The incidences of malignant liver tumours are summarized in Table 3.4. For all four strain/sex combinations, there was a significant dose-related trend for fatal liver tumours, incidental liver tumours, and the pooled estimate using Peto's test [details on statistics not provided]. At the lowest doses, the incidence in Harderian gland

Table 3.2. Incidences of hepatocellular adenomas and carcinomas in mice fed benzidine dihydrochloride

Sacrifice period (weeks)	Dose (ppm)	F1				Mono-hybrid			
		Males		Females		Males		Females	
		%	r/n[a]	%	r/n	%	r/n	%	r/n
40	0	0.0	0/49	0.0	0/48	0.0	0/50	0.0	0/48
	30	0.0	0/98	2.0	2/98	1.0	1/101	0.0	0/97
	60	0.0	0/72	1.4	1/72	0.0	0/71	0.0	0/72
	120	0.0	0/51	0.0	0/49	2.1	1/48	5.9	3/51
	200	6.0	3/50	10.0	5/50	0.0	0/52	12.0	6/50
	400	3.6	1/28	44.8	13/29	3.7	1/27	38.5	10/26
60	0	2.1	1/48	2.1	1/48	0.0	0/48	2.1	1/48
	30	0.0	0/73	4.1	3/74	4.3	3/69	9.7	7/72
	60	8.2	4/49	7.7	4/52	6.5	3/46	22.2	12/54
	120	18.8	9/48	41.4	24/58	16.0	8/50	46.4	26/56
	200	19.1	9/47	88.5	54/61	18.6	8/43	78.3	47/60
	400	52.2	12/23	100.0	41/41	26.9	7/26	86.8	33/38
80	0	0.0	0/46	0.0	0/47	4.4	2/45	0.0	0/48
	30	11.4	5/44	20.9	9/43	4.9	2/41	27.9	12/43
	60	12.8	6/47	53.5	23/43	16.3	7/43	47.6	20/42
	120	28.9	13/45	91.9	34/37	31.8	14/44	96.9	31/32
	200	38.1	8/21	100.0	9/9	36.8	7/19	87.5	7/8
	400	80.0	16/20	0.0	0/1	64.7	11/17	83.3	5/6

From Nelson *et al.* (1982)
[a] r/n = number of animals with tumour / number of animals examined

tumours was also increased compared with that in the controls (4 and 5% in the F_1 females and males, and 5 and 8% in the MC males and females, respectively), and then remained higher at the other dose levels in the males. The incidences in the high doses for F_1 and MC males were 25 and 20%, respectively (dose effect, $P = 0.02$ with a linear and quadratic term). The females of both strains reached a high incidence of 24 to 29% in the mid-doses, which then decreased to about 11 or 12% at the high dose ($P = 0.002$ with a linear and quadratic effect). A dose effect was also observed for angioma of the uterus ($P = 0.07$ with a linear effect). The incidences in the control animals were 3% and 2%, for the F_1 and MC strains of mice, respectively; then it increased with dose to 14% for F_1 mice and 7% for MC mice (Littlefield *et al.*, 1983, 1984).

Table 3.3. Incidences of hepatocellular adenomas and carcinomas in mice fed benzidine dihydrochloride

Group	Treatment Period	Effective Number	Adenoma		Carcinoma		Total	
			No.	%	No.	%	No.	%
Males								
1	Prenatal	36	5	14	3	8	8	22
2	Pre-weaning	52	9	17	26	50	35	67
3	Adult	26	5	19	17	65	22	85
Control	52 weeks	100	0	0	0	0	0	0
	90 weeks	98	1	2	0	0	1	2
	142 weeks	100	4	8	3	6	7	14
Females								
1	Prenatal	56	1	2	1	2	2	4
2	Pre-weaning	43	4	9	5	12	9	21
3	Adult	25	0	0	16	64	16	64
Control	52 weeks	99	0	0	0	0	0	0
	90 weeks	96	0	0	0	0	0	0
	142 weeks	100	1	1	0	0	1	1

From Vesselinovitch (1983)

(b) Rat

Two groups, each of 10 female Wistar rats [age not specified] were fed a diet containing 0.017% benzidine [purity not specified] with casein or a diet containing benzidine with casein hydrolysate-tryptophan. All rats given benzidine plus casein were dead by 224 days after the start of treatment; 2/10 (20%) had liver tumours (1 hepatoma seen at 125 days and 1 bile-duct carcinoma at 178 days). Animals fed benzidine-tryptophan survived longer (424 days): 3/7 (43%) animals examined had hepatocellular tumours (1 carcinoma at 202 days, 1 cholangioma at 236 days and a bile-duct carcinoma at 424 days). None of the animals in the two experimental groups developed bladder tumours (Boyland *et al.*, 1954). [The Working Group noted the small number of animals and the lack of controls.]

Four groups of 10–20 female Sprague-Dawley rats, 40 days of age, were given benzidine [purity was tested, but not described in detail] at doses of 12, 25, 35 or 50 mg/rat in sesame oil by stomach tube for 30 days. A control group was fed the sesame-oil vehicle. At the end of the nine-month period of observation, when the experiment was terminated, 10/10 (100%), 8/10 (80%), 0/20 and 4/20 (20%) animals were still alive in the four treatment groups, respectively. In the vehicle control group, 127/140 (91%) were still alive at nine months. Thus, mortality was high in animals fed the two highest doses of

Table 3.4. Incidence of malignant liver tumours in mice exposed to benzidine dihydrochloride in drinking-water

Dose(ppm)	Male		Female	
	F1	Monohybrid cross	F1	Monohybrid cross
0	14/125 (11%)	17/123 (14%)	3/124 (2%)	10/125 (8%)
20	—	—	51/120 (43%)	54/119 (45%)
30	24/119 (20%)	20/118 (17%)	52/95 (55%)	43/95 (45%)
40	30/96 (31%)	20/95 (21%)	45/72 (63%)	31/71 (44%)
60	23/71 (32%)	23/71 (32%)	55/71 (77%)	37/72 (51%)
80	35/71 (49%)	24/71 (34%)	60/69 (87%)	51/69 (74%)
120	51/71 (72%)	37/71 (52%)	64/72 (89%)	56/72 (78%)
160	49/71 (69%)	32/71 (45%)	—	—

From Littlefield *et al.* (1984)

benzidine, and only five rats (at the highest dose) were autopsied; four of these showed multiple mammary carcinomas. In the groups receiving 12 and 25 mg benzidine/rat, 5/10 (50%) and 7/9 (78%) animals autopsied also showed multiple mammary carcinomas (one rat in the group fed 12 mg had a fibroadenoma). Five of 132 sesame-oil vehicle controls examined had mammary tumours. In the benzidine-treated groups, the first palpable mammary lesions appeared about 60 days after the first treatment. At this point the mean number of mammary masses per rat showed a dose–response relationship [no statistical analysis applied]. No effect was reported in organs other than the mammary gland (Griswold *et al.*, 1968).

(c) Hamster

Groups of 30 male and 30 female random-bred Syrian golden hamsters, nine weeks of age, were fed diets containing 0.1% (w/w) benzidine or benzidine dihydrochloride (certified grade) for life. A control group of the same size was also available. No bladder pathology was seen in either the treated or the control group. In the benzidine-treated group, an increased incidence of liver tumours was observed: 19/22 (86%) males and 6/26 (23%) females developed multiple cholangiomatous tumours, most of which had signs of malignancy; 12 males and three females also developed benign and malignant hepatocellular tumours. In the group fed benzidine dihydrochloride, the liver was also the only target organ: 10/20 (50%) male and 12/27 (44%) female hamsters developed cholangiomas, mostly benign; seven males and four females also developed hepatomas. No liver tumours were seen in females or males of the untreated control group (Saffiotti *et al.,* 1967).

(d) Other animal species

(i) *Rabbit*

An invasive bladder carcinoma was induced in one out of nine rabbits [sex, age and strain not specified] given oral tolerance limit doses of benzidine [purity not specified] (Bonser, 1962). [The Working Group noted the lack of description of experiment or results.]

(ii) *Dog*

Seven mongrel dogs (one male and six females, full grown; ~11.3 kg) were given a total dose of 325 g benzidine [purity not specified] by oral capsules over 5 years (200 mg per day for 15 months and then 300 mg per day for 45 months, on six days a week). One of the female dogs developed bladder carcinomas (Spitz *et al.*, 1950). [The Working Group noted the lack of description of experiment or results.]

(iii) *Frog*

A group of five frogs (*Rana temporaria*), 1–1.5 years of age, received a total oral dose of 60 mg benzidine [purity not specified] and were observed for 20 weeks, after which the experiment was terminated. One liver tumour was observed (Khudoley, 1977). [The Working Group noted the lack of description of experiment or results.]

3.1.2 *Subcutaneous and/or intramuscular administration*

(a) Mouse

Three groups of 12–24 male, albino Delph mice, 10 weeks of age, were given subcutaneous injections of 300 mg benzidine base [purity unspecified] in olive oil, or received olive oil alone, three times a week for 45 weeks. One group served as untreated controls. The survival rates were good in all groups up to 45 weeks when the experiment was terminated. No changes in the bladder were observed in the benzidine-treated animals. In 2/19 (10%) control mice receiving olive oil alone hyperplasia of the bladder was noted. Five of nine (55%) mice given benzidine base had hepatomas, compared with 3/19 (16%) in the olive oil group and 5/17 (29%) in the untreated controls (Baker, 1950). [The Working Group noted the short duration of the experiment.]

A group of 54 male and 13 female C3HA mice [age at start not specified], weighing 18–20 g, were injected subcutaneously with 6 mg/mouse of benzidine [source and purity not specified] dissolved in 0.2 mL of sunflower oil once per week over eight months (total dose, 210 mg/mouse). At the appearance of the first tumour (16 months), 46 mice [sex not specified] were still alive. Liver tumours (hepatocellular carcinomas, adenomas and cholangiomas) developed in 13 mice [sex not specified]; and lung adenocarcinomas were found in two mice. A further group of 114 males were exposed by the same treatment schedule for 13 months (total dose, 336 mg/mouse). At 16 months, 24 mice were still

alive, and 18 developed liver tumours. Hepatomas developed in 1% of historical controls (Prokof'eva, 1971). [The Working Group noted the low survival rates.]

(b) Rat

Groups of Sherman rats, two months of age, average weight 150 g, were given 15 mg of benzidine [technical and purified grades] or benzidine sulfate [technical grade] by subcutaneous injection, once weekly for life. A suitable control group was given the olive oil vehicle. The experimental design and data on survival and tumour incidence are summarized in Table 3.5 (Spitz *et al.*, 1950). [The Working Group noted the poor survival in both treated and control groups.]

Table 3.5. Tumour incidences in rats given subcutaneous injections of benzidine and salts

Compound	Average weekly dose (mg)	Total dose (g)	No. of rats at start	No. of rats surviving more than 300 days	Rats with tumours					
					Liver (neoplasms)		External auditory canal carcinomas		Colon adeno-carcinoma	
					No.	%	No.	%	No	%
Olive oil	910	92.82	50	28	–	–	–	–	–	–
Technical benzidine	15	1.28	233	36	8	3.4	54	23	–	–
Pure benzidine	15	0.96	152	24	6	3.9	32	21	7	1.8
Benzidine sulphate	15	0.94	153	5	1	0.65	16	10.5	–	–

From Spitz *et al*. (1950)

A group of 25 male and 25 female rats [strain and age at start not specified], weighing 100–120 g, were injected subcutaneously with an initial dose of 15 mg benzidine [purity not specified] in 0.5 mL of sunflower-seed oil, once a week for 14 weeks. Due to severe toxicity, a smaller dose of 10 mg per week was then given for the next six weeks to each rat, and finally once every 15 days for six weeks. By six months of treatment, each animal had received a total dose of 300 mg benzidine. Another group of 50 rats [sex unspecified] served as controls: 25 received subcutaneous injections with the solvent for six months while the remaining 25 rats were kept untreated. Of the 15 treated males surviving, 12 developed tumours: two hepatomas, four malignant tumours of the Zymbal gland, six sarcomas at the injection site and two other sarcomas; two of the five surviving treated females developed tumours: one malignant tumour of the Zymbal gland and one myeloid

leukaemia. None of the 25 controls injected with the solvent [sex not specified] developed tumours at the injection site (Pliss, 1964).

A group of 28 rats [strain and sex not specified], 6–8 weeks of age, were given benzidine [purity, source and vehicle not specified] by weekly subcutaneous injections of 5 mg/rat for 32–60 weeks (total dose, 170 mg/rat). When the first tumour appeared at 210 days, 28 rats were still alive. Intestinal tumours developed in four rats between 252 and 318 days (Pliss *et al.*, 1973) [The Working Group noted the lack of available controls and the lack of experimental detail.]

Groups of 16 female and 14 male white non-bred rats [strain and age not specified] were injected subcutaneously with benzidine [source and purity not specified] (5 mg/rat) dissolved in 0.5 mL of sunflower oil, weekly for about 52 weeks (total dose, 160–260 mg/rat). At 219 days, when the first tumour (a skin epithelioma) was detected, 24 rats [sex not specified] were still alive; all animals were killed at 357 days. Tumours were found in 23 rats (95.8%), with an average latent period of 275 days. Nine of 24 rats (39.1%) had multiple primary tumours. Zymbal gland tumours developed in 18 rats (78.3%); five had local fibrosarcomas and one a local rhabdomyosarcoma (Pliss and Iogannsen, 1974). [The Working Group noted that no untreated or solvent controls were available.]

Groups of 18 male and 16 female albino non-inbred rats [age at start not specified] weighing 120–140 g were injected subcutaneously once a week for about 33 weeks with benzidine (5 mg/rat; source and purity not specified) suspended in 0.5 mL of oil [not specified] to give a total dose of 170 mg/rat. At 210 days, when the first tumour appeared, 16 males and 12 females were still alive. A total of 26 tumours developed in 14 males: six local sarcomas, nine tumours of the Zymbal gland, nine liver tumours (cystocholangiomas and hepatocellular carcinomas) and two intestinal tumours (polyposis and adenocarcinoma). A total of 20 tumours developed in 11 females: five local sarcomas, six tumours of the Zymbal gland, four mammary adenocarcinomas, one mammary adenoma, two liver tumours (cystocholangioma and hepatocellular carcinoma) and two intestinal tumours (Pliss & Vol'fson, 1974). [The Working Group noted that no untreated or solvent controls were available.]

(c) Frog

A group of 37 grass frogs (*Rana temporaria*) of both sexes, 1–1.5 years of age, received weekly subcutaneous injections of 0.2–0.5 mL of a 0.5% solution of benzidine [purity not specified] in mineral oil for up to 38 weeks (total dose, 45–114 mg/animal). A group of 120 untreated frogs were observed for 56 weeks (three of these developed skin cystadenopapillomas), and a further group of 67 frogs were given subcutaneous injections of 0.2–0.5 mL mineral oil, weekly for 42 weeks as controls. When the first tumour appeared at 16 weeks, 14 animals in the treated group were still alive, of which six (43%) had tumours of the liver and haematopoietic system [not further specified], with an

average latent period of 24.8 weeks. No tumours were observed in the control group (Khudoley, 1977).

3.1.3 *Intraperitoneal administration*

(a) Rat

Three groups of 30 female CD rats, 30 days of age, were given intraperitoneal injections twice weekly for 4 weeks, of 0, 10 or 30 μmol/kg bw benzidine [purity not specified] as a suspension in trioctanoin. Control rats received trioctanoin only. All survivors were killed 46 weeks after the first injection. No tumours were seen in the kidney or bladder in treated or control groups. In the benzidine-treated groups, a dose-related increase in the incidence of mammary tumours, benign and malignant, was noted: 3/30 (10%) in controls, 7/30 (23%) in the low-dose group and 12/29 (41%) ($P < 0.01$, χ^2 test) in the high-dose group. Zymbal gland tumours (adenomas or carcinomas) were observed in 1/30 (3%) controls, 1/30 (3%) low-dose animals and 7/29 (24%) ($P < 0.05$) high-dose animals. No tumours of the liver were found; however, altered cellular foci in the liver were observed in 9/30 (19%) controls, 14/30 (46%) low-dose animals and 20/29 (76%) ($P < 0.01$) high-dose rats (Morton *et al.,* 1981).

3.1.4 *Inhalation exposure*

(a) Rat

A group of 48 white out-bred rats of both sexes [age at start non specified], weighing 100–120 g, were exposed to an aerosol containing 10–20 mg/m^3 [1.3–2.7 ppm] benzidine [source and purity not specified] in inhalation chambers during four hours/day, for five days a week over 20 months (total dose, 27 mg/rat). Control rats [number not specified] were kept in inhalation chambers and exposed to air during the same period. Animals were kept until moribund. The first myelogenous leukaemia was found in a treated rat 13 months after the start of the experiment, at which time 28 rats were still alive. By the end of the study (28 months), five myeloid leukaemias, two breast fibroadenomas, one squamous-cell cancer of the Zymbal gland, one hepatoma and one breast adenocarcinoma were found in eight animals. Mammary adenomas were found in two of 21 control rats (Zabezhinskiĭ, 1970). [The Working Group noted the lack of information on the size of the aerosol particles and on the survival of controls.]

3.1.5 *Other experimental systems*

(a) Mouse

Following surgical implantation of a 45-mg glass bead in the urinary bladder of female mice (strain 150 ICR) at five weeks of age, the animals were divided into three groups: one group (30 mice) served as controls and was fed a commercial basal diet; the

second group (60 mice) received a diet containing 0.2% benzidine [purity not specified]; the third group (60 mice) was fed a diet containing a mixture of 0.2% benzidine and 2% DL-tryptophan. The experimental groups received their diets starting at six weeks of age for 20 weeks and were then fed the control diet for 40–43 weeks. The experiment was terminated 63 weeks after the start of treatment. Of the group that received benzidine alone, only 19% of the animals were still alive at the end of the experiment, while 65.5% of controls and 49.2% of the group treated with benzidine plus tryptophan were still alive at that time. Hepatomas were observed in 34 of 41 (82.9%) mice treated with benzidine and in 24 of 51 (47.1%) mice treated with the benzidine-tryptophan mixture, indicating an inhibitory effect of tryptophan; no hepatomas were seen in the controls. No bladder tumour was found in any of the animals; however, the authors reported hyperplasia in all bladders observed (Miyakawa and Yoshida, 1980).

(b) Rat

Five groups of 30–40 male Fischer rats (age at start not specified), weighing approximately 200 g, were implanted with a heterotopic bladder, which was then instilled once a week for 20 weeks with 0.5 ml phosphate-buffered saline:dimethyl sulfoxide solution (PBS/DMSO, 4:1) or this solution containing 1 μmol benzidine or the derivatives, *N*′-hydroxy-*N*-acetylbenzidine, the *N*′-glucuronide of *N*′-hydroxy-*N*-acetylbenzidine, or the *N*-glucuronide of *N*-hydroxy-2-aminofluorene [chemicals were synthesized and analysed by authors]. These bladders were then instilled once a week for an additional 30 weeks with PBS without DMSO. The experiment was terminated at the end of 50 weeks. Transitional cell carcinomas were observed in 1 of 39 (3%) of the control group, 1 of 29 (3%) in the benzidine group, 18 of 30 (60%) in the *N*′-hydroxy-*N*-acetylbenzidine group, 28 of 28 (100%) in the *N*′-hydroxy-*N*-acetylbenzidine-*N*′-glucuronide group, and in 24 of 29 (83%) of the *N*-hydroxy-2-aminofluorene-*N*′-glucuronide rats (Wang *et al.*, 1990).

(c) Fish

Benzidine [purity not specified] was mixed into a diet and given to a group of 100 fish (guppies) of both sexes, 10–12 months of age, at a dose of 300 mg/kg dry diet for 56 weeks, at which point the experiment was terminated. The six fish that survived the treatment period had no detectable tumours; however, signs of hepatotoxicity (focal necrosis, fatty dystrophy and diffuse hyperplasia of hepatocytes) were noted. None of the 120 control guppies fed the standard diet developed tumours or preneoplastic changes (Pliss & Khudoley, 1975). [The Working Group noted the high mortality in the treated group.]

3.2 3,3′-Dichlorobenzidine and its dihydrochloride

Studies in experimental animals of carcinogenicity of 3,3′-dichlorobenzidine and its dihydrochloride by oral exposure were previously reviewed by IARC (1982, 1987). Those found to be adequate and/or reported more fully in later publications are included in this evaluation.

3.2.1 *Oral administration*

(a) Mouse

A group of 26 male ICR/JCL mice [age at start not specified] were fed a diet containing 0.1% 3,3′-dichlorobenzidine [purity unspecified, not clear if it was the free amine or the dihydrochloride salt] for up to 12 months. All eight animals killed after six months of treatment (100%) had hepatomas, as did all 18 animals (100%) killed after 12 months of treatment. Of 39 control mice maintained on a normal diet and killed at six, 12 and 18 months, 0/5 (0%), 2/21 (9%) and 5/13 (38%) had hepatomas respectively (Osanai, 1976). [The Working Group noted the absence of information on survival of treated and control animals.]

A group of 22 female and 51 male D mice ($cC_{57}W$ x $C_{57}Bl$ hybrids, age not specified) weighing 12–20 g, received food containing 0.1 ml of a 1.1% suspension of 3,3′-dichlorobenzidine (45.3% 3,3′-dichlorobenzidine, 50% water and 4.7% unspecified impurities) in sunflower oil, which was administered on six days a week for 12 months (total dose, 127–135 mg/mouse). The animals were observed for life. There was no control group but the authors used historical control data. The numbers of mice that survived were: 37 at six months, 34 at 12 months, and 18 mice at the time of appearance of the first tumour (18.5 months). Four of 18 animals (22%) had tumours: 2/18 (11%) had hepatomas, 2/18 (11%) had liver hemangiomas, 1/18 (5%) had a carcinoma of the sebaceous gland, and 1/18 (5%) had a lung adenoma. No liver hepatomas or hemangiomas had been seen in the historical controls (Pliss, 1959). [The Working Group noted the absence of adequate controls].

(b) Rat

A group of 15 female and 35 male outbred Rappolovo rats weighing 110–130 g [age at start not specified] received food containing 0.5–1.0 ml of a 4.4% suspension of 3,3′-dichlorobenzidine (45.3% 3,3′-dichlorobenzidine, 50% water and 4.7% unspecified impurities) in sunflower oil at a dose of 10–20 mg/day, which was administered on six days a week for 12 months (total dose, 4.5 g/rat). The animals were observed for life. The numbers of animals that survived were: 34 at six months and 27 at 12 months. At the time of the appearance of the first tumour (11 months), 29 rats were alive. Twenty-three rats (23/29, 79%) developed tumours, including seven (24%) Zymbal gland tumours, three (10%) skin tumours, seven (24%) mammary gland tumours, two (7%) adenocarcinomas

of the ileum, three (10%) bladder tumours, three (10%) tumours of the haematopoietic system, two (7%) connective tissue tumours, two (7%) salivary gland tumours, one (3%) liver tumour and one (3%) thyroid tumour. Among a group of 130 controls injected with octadecylamine and methylstearylamine, no tumours were found within 23 months (Pliss, 1959). [The Working Group noted the absence of adequate controls.]

Groups of 50 male and 50 female ChR-CD rats, 38 days of age, were given a diet containing 1000 ppm 3,3′-dichlorobenzidine [purity unspecified] for 16 months. An equal number of animals were maintained on a control diet for a period of 24 months. Six rats per group and per sex were killed at 12 months for an interim evaluation. Of the remaining treated rats, six survived up to 16 months, at which time they were killed. A statistically significant ($P < 0.05$) increase in the incidence of tumours was observed in treated compared with control animals for the following target sites in males: granulocytic leukaemias, 9/44 (20%) treated, 2/44 (4%) control; mammary adenocarcinomas, 7/44 (16%) treated, 0/44 control; Zymbal gland carcinomas, 8/44 (18%) treated, 0/44 control. In females, mammary adenocarcinomas were seen in 26/44 (59%) treated, 3/44 (7%) control (Stula *et al.*, 1975).

A group of 20 female Sprague-Dawley rats, 40 days of age, were given 10 doses of 3,3′-dichlorobenzidine dihydrochloride [purity and impurities unspecified] in sesame oil every three days by gastric intubation (total dose, 300 mg/rat, which was the maximum tolerated dose). The observation period was nine months, when the 14 surviving animals were killed. No mammary tumours were observed in 15 treated rats autopsied, while 5/132 (4%) animals treated with sesame oil only had mammary tumours (Griswold *et al.,* 1968).

(c) Hamster

Groups of 30 male and 30 female random bred Syrian golden hamsters, nine weeks of age, were given 0.1% of a technical grade 3,3′-dichlorobenzidine (mixture of 40% as the dihydrochloride and 60% as free base) in powdered diet throughout their life-span (total intake, 3.0 g per animal per year). A similar group of 30 male and 30 female Syrian golden hamsters were fed the powdered diet only throughout their lifetime and served as the control group. No information on survival of the treated or control animals was given. Exposure to this dose level of 3,3′-dimethylbenzidine did not induce any significant carcinogenic effect at any site or bladder pathology (Saffiotti *et al.*, 1967). [The Working Group noted the lack of description of experimental procedures and detailed pathological findings.]

(d) Dog

Six female beagle dogs, one year of age, were each given 100 mg 3,3′-dichlorobenzidine (reported to be 100% pure) in a gelatin capsule three times per week for six weeks, then five times per week continuously for periods up to 7.1 years. Six untreated female beagle dogs served as controls for several studies and were killed after

8.3 to 9.0 years on test. The intake of 3,3′-dichlorobenzidine was between 9.1 and 12.8 mg/kg bw per dose. One dog sacrificed after 3.5 years on test had no tumours. Another sacrificed after 6.6 years on test (total intake, 164 g) had an undifferentiated carcinoma of the urinary bladder. Of the remaining dogs killed at 7.1 years (total intake, 176 g/dog), 4/4 (100%) had papillary transitional-cell carcinomas of the urinary bladder and 3/4 (75%) had hepatocellular carcinomas. None of the six control dogs had these tumours. However, 4/6 (67%) control animals killed at 8–9 years of age had major tumours of the mammary gland (adenocarcinomas and carcinosarcoma) (Stula *et al.*, 1978).

3.2.2 *Subcutaneous and/or intramuscular administration*

(a) Mouse

A group of 15 female and 8 male D mice ($cC_{57}W$ x $C_{57}Bl$ hybrids, age not specified), weighing 12–20 g, received twice weekly 0.1-ml subcutaneous injections of an 11% or a 5.5% suspension of 3,3′-dichlorobenzidine paste (45.3% 3,3′-dichlorobenzidine, 50% water and 4.7% unspecified impurities) in glycerol at a total dose of 265 mg/mouse over 11 months. The numbers of mice that survived were: nine at six months, eight at 12 months, and eight at the time of appearance of the first tumour (12.5 months). The animals were observed for life. Five animals [sex not identified] (62%) had tumours at different sites: 2/8 (25%) had local sarcomas, 3/8 (37%) had liver tumours, 1/8 (12%) had a tumour of the haematopoietic system, 1/8 (12%) had a lung adenoma. Another group of 31 female and 36 male D mice received weekly 0.1-ml subcutaneous injections of an 11% or a 5.5% suspension of 3,3′-dichlorobenzidine paste (45.3% 3,3′-dichlorobenzidine, 50% water and 4.7% unspecified impurities) in glycerol at a dose of 5 to 2.5 mg/mouse. The total dose was 130 mg/mouse over 11 months. The numbers of mice that survived were: 32 at six months, 23 at 12 months, and 20 at the time of appearance of the first tumour (13.5 months). The animals were observed for life. Eight animals [sex not identified] (40%) had tumours at different sites: 1/20 (5%) had local sarcomas, 5/20 (25%) had liver tumours, 1/20 (5%) had a tumour of the haematopoietic system, 2/20 (10%) had a lung adenoma, and 1/20 (5%) had a squamous-cell keratinizing tumour of the lower jaw. No tumour occurred within 23 months in 130 controls injected with octadecylamine or methylstearylamine (Pliss, 1959). [The Working Group noted the absence of adequate controls and poor description of the pathology findings.]

(b) Rat

A group of 61 sexually mature white rats weighing 110–130 g (strain, age, and distribution by sex not specified) initially received twice weekly subcutaneous injections of a suspension of 3,3′-dichlorobenzidine paste (45.3% 3,3′-dichlorobenzidine, 50% water and 4.7% unspecified impurities) in glycerol at a dose of 60 mg/rat. Because of initial high mortality, the dose was reduced beginning from the 6th month to weekly injections of

20 mg/rat. The total dose of 3,3′-dichlorobenzidine was estimated to range from 1.6 to 3 g/rat. The animals were observed for life. A group of 130 rats (strain, age, and distribution by sex not given) served as controls and received subcutaneous injections of octadecylamine or methylstearylamine over a 10-month period and were then observed for life. Thirty-five animals survived to the time of appearance of the first tumour (time not specified). Eighteen animals (51%) had tumours: 9/35 (26%) had Zymbal gland tumours, 7/35 (20%) had mammary tumours, 5/35 (14%) had local sarcomas, 1/35 (3%) had liver tumours, 2/35 (6%) had a tumour of the haematopoietic system, and 1/35 (3%) had a thyroid tumour. No tumour occurred within 23 months in 130 controls injected with octadecylamine or methylstearylamine (Pliss, 1958). [The Working Group noted the inadequate reporting of the experiment.]

A group of rats weighing 100–130 g (strain, age, number and distribution by sex not specified) received subcutaneous injections of 15–60 mg/rat of 3,3′-dichlorobenzidine [purity and impurities unspecified] in sunflower seed oil or glycerol and water at unspecified intervals for 10 to 13 months. No information on survival was provided. Tumours were reported to occur in 74% of animals. No information on number of tumour-bearing animals or time to first tumour was reported. Skin, sebaceous and mammary gland tumours were observed most frequently, and there were also intestinal, urinary bladder and bone tumours. Among 50 control rats injected with the vehicle alone or left untreated, a single sarcoma was reported (Pliss, 1963). [The Working Group noted the inadequate reporting of the experiment.]

3.2.3 *Transplacental exposure*

(a) Mouse

A group of BALB/c mice [number and age not specified] were treated with 5 subcutaneous injections of 3,3′-dichlorobenzidine in sunflower oil (2 mg/injection; total dose, 10 mg/mouse) during the last week of pregnancy. The progeny (13 males and 11 females) were kept with the treated animals throughout lactation and weaning at 3–4 weeks, and were then observed until natural death. Of the offspring that lived 12–20 months, 13/24 (54%) had tumours, compared with 6/30 (20%) of the control progeny. A significant increase in the incidence of lymphoid leukaemias (7/24, 29%) in treated and 0/30 in control animals [sex unspecified]) was observed in the offspring. Lung tumours (5/24 (21%) in treated and 3/30 (10%) in control animals) and mammary tumours (4/11 (36%) in treated and 3/19 (16%) in control animals) were also reported (Golub *et al.,* 1974).

3.2.4 *Administration with known carcinogens*

(a) Rat

Nine groups of 22 and one group of 96 male Wistar rats (age not specified) received the following compounds alone or in sequence for a period of four weeks per compound: *ortho*-*N*-butyl-*N*-(4-hydroxybutyl)nitrosamine (0.01% in drinking water), *N*-[4-(5-nitro-2-furyl)-2-thiazolyl]formamide (0.15% in the diet), *N*-fluorenyl-acetamide (0.025% in the diet) and 3,3′-dichlorobenzidine (0.3% in the diet). An untreated control group consisted of 12 rats. The animals were killed when 40 weeks old. 3,3′-Dichlorobenzidine given in sequence with one or more of the other compounds induced histological changes of the liver (cystic change of bile ducts and oval-cell proliferation) in 44–50% of animals ($P < 0.05$ when compared with groups that did not receive 3,3′-dichlorobenzidine). No change was seen in the liver when 3,3′-dichlorobenzidine was given alone. The incidence of urinary bladder tumours was not significantly increased when 3,3′-dichlorobenzidine was added to the sequence of the chemicals studied (Tatematsu *et al.,* 1977).

3.3 3,3′-Dimethoxybenzidine

Studies in experimental animals of carcinogenicity of 3,3′-dimethoxybenzidine or its dihydrochloride salt by oral exposure were previously reviewed by IARC (1982). Those found to be adequate and/or reported more fully in later publications are included in this evaluation.

3.3.1 *Oral administration*

(a) Mouse

Groups of 166 male and 165 female BALB/c mice, four weeks of age, were given drinking water containing 0, 20, 40, 80, 160, 315, or 630 ppm of 3,3′-dimethoxybenzidine dihydrochloride [purity unspecified] for up to 112 weeks. Interim sacrifices and histopathological assessments were conducted on all dose groups after 13, 26, 39, 52, 78, or 112 weeks. Water consumption was monitored and was depressed in all the groups, especially the high-dose group. Although body-weight gain was suppressed at the highest dose level during the first year, administration of 3,3′-dimethoxybenzidine dihydrochloride did not affect the 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).

(b) Rat

A group of 42 male and female rats (strain, distribution and age not specified) were given 30 mg of 3,3′dimethoxybenzidine [purity unspecified] by gavage in sunflower-seed oil three times a week for three weeks. The dose was then reduced to 15 mg because of

poor survival and continued for an additional 13 months. Eighteen animals survived to 14 months. Two of the 18 animals (11%) that survived to 18 months had neoplasms of the Zymbal gland, one (5.5%) had a fibroadenoma of the mammary gland, and one (5.5%) had an ovarian neoplasm. None of the 50 rats in the control groups (25 injected with sunflower-seed oil subcutaneously and 25 untreated) developed tumours at these sites (Pliss, 1963, 1965). [The Working Group noted the inadequate reporting of the experiment.]

Groups of 3 or 14 (10-mg dose only) male and 3 or 15 (10-mg dose only) female Fischer rats (age not given) were administered 3,3′-dimethoxybenzidine [purity unspecified] by gavage at dose levels of 0, 0.1, 0.3, 1, 3, 10, or 30 mg per animal per day on five days per week. A proprietary mixture composed of sodium chloride, sodium carboxymethylcellulose, polysorbate 80, and benzyl alcohol in water was used as a vehicle in this study. The animals were treated for 52 weeks and then observed for an additional six months. Groups of male and female rats received 0.5 ml of the vehicle five days per week and served as the vehicle controls. In addition, there was a separate group of 90 males and 90 female rats that served as untreated controls. 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 (59 animals) 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 (Hadidian *et al.*, 1968).

Groups of 60, 45, 75, and 60 male and female F344/N rats, seven weeks of age, were given drinking water containing 3,3′-dimethoxybenzidine dihydrochloride at 0, 80, 170, or 330 ppm respectively (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) for up to 21 months. Although initially planned as a two-year study, this experiment was terminated early because of reduced survival in all dose groups associated with the appearance of treatment-related neoplasms. Survival decreased markedly with increasing dose. Among males, the number of rats surviving at study termination was 44 in the control group and eight 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. The tumour incidences in male and female rats are shown in Tables 3.6 and 3.7. Histopathological examination of the tissues revealed tumours at various sites, including benign and malignant tumours of the skin, Zymbal gland, preputial gland, clitoral gland, mammary gland, uterus, oral cavity, intestine, liver, and mesothelium. The observed increase in the incidence of astrocytomas of the brain may also have been related to exposure to 3,3′-dimethoxybenzidine dihydrochloride (NTP, 1990).

(c) Hamster

Groups of 30 male and 30 female, random bred Syrian golden hamsters, nine weeks of age, were given 0.1% 3,3′-dimethoxybenzidine (purity not given) in powdered diet throughout their lifespan (3.0 g per animal per year). A similar group of 30 male and 30 female Syrian golden hamsters were fed the powdered diet only throughout their lifetime and served as the control group. The only malignant neoplasm observed was a transitional cell carcinoma of the urinary bladder in one animal after 144 weeks of exposure to dimethoxybenzidine. This neoplasm is rare in hamsters and therefore was attributed to dimethoxybenzidine exposure (Saffiotti *et al.* 1967). [The Working Group noted the lack of description of experimental procedures and detailed pathological findings.]

Table 3.6. Tumour incidences in male rats given 3,3′-dimethoxybenzidine dihydrochloride in drinking-water for up to 21 months

Tumour type	Concentration (ppm) in drinking-water			
	0	80	170	330
	Tumour incidence/number examined			
Skin: basal cell or sebaceous gland adenoma or carcinoma	2/60**[a] (3%)	33/45**[b] (73%)	56/75**[b] (75%)	41/60**[b] (68%)
Skin: squamous cell papilloma	0/60**[a]	13/45**[b] (29%)	28/75**[b] (37%)	22/60**[b] (37%)
Zymbal gland: adenoma or carcinoma	0/59**[a]	10/45**[b] (22%)	25/75**[b] (33%)	30/60**[b] (50%)
Preputial gland: adenoma or carcinoma	16/60*[a] (27%)	12/43 (28%)	33/73*[b] (45%)	29/59*[b] (49%)
Oral cavity: papilloma or carcinoma	1/60*[a] (2%)	8/45*[b] (18%)	10/75*[b] (13%)	11/60*[b] (18%)
Small intestine: adenocarcinoma	0/60	4/45*[a] (9%)	7/75*[a] (9%)	5/60*[a] (8%)
Large intestine: adenomatous polyp or adenocarcinoma	0/60**[a]	1/45 (2%)	8/75*[b] (11%)	8/60*[b] (13%)
Liver: Neoplastic nodule or hepatocellular carcinoma	1/60**[a] (2%)	4/45 (9%)	7/74*[b] (9%)	8/60**[b] (13%)
Mesothelium: mesothelioma	2/60*[a] (3%)	1/45 (2%)	7/75 (9%)	6/60 (10%)
Brain: astrocytoma	0/60	2/44 (5%)	3/75 (4%)	1/60 (2%)

[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
From NTP (1990)

Table 3.7. Tumour incidence in female rats given 3,3′-dimethoxybenzidine dihydrochloride in drinking-water for up to 21 months

Tumour type	Concentration (ppm) in drinking-water			
	0	80	170	330
	Tumour incidence/number examined			
Skin: basal cell adenoma or carcinoma	0/60	4/45*[a] (9%)	3/75 (4%)	2/60 (3%)
Skin: Squamous cell papilloma	0/60	0/45	3/75 (4%)	0/60
Liver: Neoplastic nodule or hepatocellular carcinoma	0/60*[b]	1/44 (2%)	0/75	3/60 (5%)
Zymbal gland: adenoma or carcinoma	1/60*[b] (2%)	12/45**[a] (27%)	21/75**[a] (28%)	16/60**[a] (27%)
Mammary gland: adenocarcinomas	1/60**[b] (2%)	2/45 (4%)	14/75**[a] (19%)	20/60**[a] (33%)
Oral cavity: papilloma or adenoma	2/60 (3%)	2/45 (4%)	6/75 (8%)	5/60 (8%)
Large intestine: Adenomatous polyp or adenocarcinoma	0/60*[b]	1/45 (2%)	1/75 (1%)	3/60* [b] (5%)
Clitoral gland: adenoma or carcinoma	7/58**[b] (12%)	27/44**[a] (61%)	48/74**[a] (65%)	41/45**[a] (91%)
Uterus: adenoma or carcinoma	0/60	4/45*[a] (9%)	2/75 (3%)	2/60 (3%)
Brain: astrocytoma	0/60	1/45 (2%)	1/75 (1%)	0/60

[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
From NTP (1990)

3.4 3,3′-Dimethylbenzidine

Studies in experimental animals of the carcinogenicity of 3,3′-dimethylbenzidine or its dihydrochloride salt by exposure via oral and subcutaneous administration were previously reviewed by IARC (1982). Those found to be adequate and/or reported more fully in later publications are included in this evaluation.

3.4.1 *Oral administration*

(a) Mouse

Groups of 120 male and 120 female BALB/c mice, four weeks of age, were given drinking-water containing 0, 5, 9, 18, 35, 70, or 140 ppm of 3,3′-dimethylbenzidine dihydrochloride [purity unspecified] for up to 112 weeks. Interim sacrifices and histopathological assessments were conducted on all dose groups after 13, 26, 39, 52, 78, and 112 weeks. Water consumption was monitored, and average weekly 3,3′-dimethylbenzidine dihydrochloride doses (mg/kg) were determined to range from 5 to 126 mg/kg per week. 3,3′-Dimethylbenzidine dihydrochloride in oral doses exceeding 100 mg/kg per week was well tolerated, as evidenced by the absence of treatment-related changes in water consumption, body-weight gain, or mortality. Incidences of alveolar-cell adenomas and adenocarcinomas ($P < 0.0002$, carcinomas; $P < 0.0001$, adenomas and carcinomas combined) of the lung were increased in a dose-related fashion among males that were either found dead or sacrificed in moribund condition. Similar increases were not observed in females or in animals randomly selected for interim sacrifice (Table 3.8). The incidences of tumours of the skin, spleen, liver, and Harderian gland were unaffected by the administration of 3,3′-dimethylbenzidine dihydrochloride (Schieferstein *et al.,* 1989).

(b) Rat

A group of twenty female Sprague-Dawley rats, 40 days of age, were given a suspension of 3,3′-dimethylbenzidine (purity not given) in sesame oil by gavage at a tota1 dose of 500 mg per rat, fractionated in 10 doses at 3-day intervals and then held for an additional eight months. A group of 140 female Sprague-Dawley rats, 40 days of age, served as the controls and received the sesame oil only. Survival was 80% in treated animals versus 90% in the controls. Sixteen rats treated with 3,3′-dimethylbenzidine were alive at the end of the nine-month observation period. Neoplastic responses included significantly increased incidences of mammary tumours. Three of the 16 treated animals (19%) showed a total of four mammary carcinomas. Among 132 surviving control rats, five (4%) had a total of three mammary carcinomas, one fibroadenoma and five hyperplasias (Griswold *et al.*, 1968).

Groups of 70, 45, 75, and 70 male and female F344/N rats, 6 weeks of age, were given drinking-water containing 3,3′-dimethylbenzidine dihydrochloride [purity unspecified] at 0, 30, 70, or 150 ppm, respectively, for up to 14 months. Although initially

Table 3.8. Lung alveolar-cell adenomas and adenocarcinomas in BALB/c mice exposed to 3,3′-dimethylbenzidine dihydrochloride in drinking-water for up to 104 weeks

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

From Schieferstein *et al.*, (1989)

planned as a two-year study, this experiment was terminated early because of reduced survival in all dose groups associated with the appearance of treatment-related neoplasms. A scheduled interim sacrifice and histopathological assessment of 10 controls and 10 high-dose animals of each sex was conducted during the ninth month of the study. Although the incidences of tumours observed in 3,3′-dimethylbenzidine dihydrochloride-dosed rats were not significantly elevated at this interim sacrifice, the appearance of malignant tumours of the liver (male only), lung, mammary gland (female only), skin, preputial gland (male only), oral cavity (female only), small intestine (male only), clitoral gland, and Zymbal gland after only nine months suggested a treatment-associated early onset of some tumours. Tumour incidences, summarized in Table 3.9, were unequivocally increased in a dose-related manner after 14 months of 3,3′-dimethylbenzidine dihydrochloride administration. Administration of 3,3′-dimethylbenzidine dihydrochloride significantly increased the incidences of a wide array of malignant and benign tumours in both sexes of F344/N rats. (NTP, 1991b).

Table 3.9. Tumour incidences in F344/N rats administered 3,3'-dimethylbenzidine hydrochloride in drinking-water for 14 months

Tumour type	Daily dose (ppm)			
	0	30	70	150
	Tumour incidences/number examined[a]			
Males				
Skin: Basal cell adenoma or carcinoma	0/60	11/45** (24%)	54/75** (72%)	30/60** (50%)
Sebaceous gland adenoma	0/60	0/45	7/75*	5/60*
Squamous cell papilloma or carcinoma	0/60	2/45 (4%)	17/75** (23%)	27/60** (45%)
Keratoacanthoma	1/60 (2%)	1/45 (2%)	8/75* (11%)	5/60* (8%)
Zymbal gland: Adenoma or carcinoma	1/60 (2%)	3/45 (7%)	32/75** (43%)	36/60** (60%)
Preputial gland: Adenoma or carcinoma	2/60 (3%)	4/45 (9%)	6/75 (8%)	9/60* (15%)
Liver: Neoplastic nodule or hepatocellular carcinoma	0/60	0/45	35/75** (47%)	33/60** (55%)
Oral cavity: Squamous cell papilloma or carcinoma	0/60	0/45	4/75 (5%)	5/60* (8%)
Small intestine: Adenomatous polyp or adenocarcinoma	0/60	0/45	4/75 (5%)	8/60* (13%)
Large intestine: Adenomatous polyp or adenocarcinoma	0/60	0/45	6/75* (8%)	15/60** (25%)
Lung: Neoplasms	1/60 (2%)	0/45	8/75* (11%)	6/60* (10%)
Females				
Skin: Basal-cell adenoma or carcinoma	0/60	3/45 (7%)	10/75** (13%)	9/60** (15%)
Squamous cell papilloma or carcinoma	0/60	3/45 (7%)	9/75* (12%)	12/60** (20%)
Zymbal gland: Adenoma or carcinoma	0/60	6/45* (13%)	32/75** (43%)	42/60** (70%)
Liver: Neoplastic nodule or hepatocellular carcinoma	0/60	0/45	7/74* (9%)	4/60* (7%)
Oral cavity: Squamous cell papilloma or carcinoma	0/60	3/45 (7%)	9/75* (12%)	13/60** (22%)
Clitoral gland: Adenoma or carcinoma	0/60	14/45** (31%)	42/75** (56%)	32/59** (54%)

Table 3.9 (contd)

Tumour type	Daily dose (ppm)			
	0	30	70	150
	Tumour incidences/number examined[a]			
Females (contd)				
Small intestine: Adenomatous polyp or adenocarcinoma	0/60	1/45 (2%)	3/75 (5%)	5/60* (8%)
Large intestine: Adenomatous polyp or adenocarcinoma	0/60	1/45 (2%)	7/75* (9%)	4/60* (7%)
Lung: Neoplasms	1/60 (2%)	1/45 (2%)	3/74 (4%)	4/60 (7%)
Mammary gland: Adenocarcinoma	0/60	1/45 (2%)	3/75 (4%)	6/60* (10%)

[a] Statistical significance by Fisher exact test: *P <0.05; **P <0.001
From NTP (1991)

(c) Hamster

Groups of 30 male and 30 female random bred Syrian golden hamsters, nine weeks of age, were given 0.1% 3,3′-dimethylbenzidine (purity not given) in powdered diet throughout their life-span (3.0 g per animal per year). A similar group of 30 male and 30 female Syrian golden hamsters were fed the powdered diet only throughout their lifetime and served as the control group. Exposure to this dose level of 3,3′-dimethylbenzidine did not induce any significant carcinogenic effect or bladder pathology (Saffiotti *et al.*, 1967). [The Working Group noted the lack of description of experimental procedures and detailed pathological findings.]

3.4.2 *Subcutaneous administration*

(a) Rat

A group of 105 male and female (distribution not given) Sherman rats, two months of age, were given a mixture of technical grade *ortho*-tolidine (3,3′-dimethylbenzidine) in olive oil by subcutaneous injection for their lifetime. The weekly dose level was 60 mg per rat (maximum cumulated dose, 5.5 g). Of the treated animals, 48 (46%) survived more than 300 days. Five rats (4.8%) developed cancer of the external auditory canal (Zymbal gland), with all tumours appearing after the 354th day. Twenty-eight of the 50 control rats treated with olive oil only (56%) survived more than 300 days. No details on any tumours observed in this control group were reported. While an untreated control group was not run concurrently in this experiment, the authors reported 56 tumours

occurring among 578 untreated rats (490 rats not otherwise incorporated in the study and 88 animals in "diet and vehicle control groups") of the same colony. None of these tumours were located in the external auditory canal (Spitz *et al.*, 1950). [The Working Group noted that this study is limited by poor survival of the animals, which can be attributed to the reported lack of climate control in the animal rooms and widespread disease in the treated and control animals].

Groups of 27 male and 26 female random-bred white rats [strain and age not specified] received weekly subcutaneous injections of a 4% suspension of purified *ortho*-tolidine (3,3′-dimethylbenzidine) in 0.5 ml sunflower oil for 13 months. Doses were 20 mg per rat per week, for a total dose of 1160 mg/rat. Use of control animals was not reported. Twenty-five male and 25 female rats survived for at least eight months (time of occurrence of the first tumour), and 11 males and 5 females lived up to 18 months. Of the animals that survived for at least eight months, 17/25 (68%) males and 13/25 (52%) females developed a total of 41 tumours. Zymbal gland tumours accounted for 14/27 (52%) tumours observed in males and 6/14 (43%) in females. Other sites where tumours were observed included mammary gland (five, female only), skin (three, male and female), preputial gland (three, male and female), and forestomach (one male). An additional group of rats (24 of each sex) received a weekly subcutaneous implant of a pellet containing 20 mg of purified *ortho*-tolidine and 10 mg of glycerol for 14 months. A third group (20 of each sex) received a weekly subcutaneous implant of a pellet containing 20 mg of *ortho*-tolidine that had been subjected to ultraviolet irradiation before the preparation of the pellet. The difference in response to these exposures between the two groups was minimal. Of a total of 68 animals that were alive at the time of appearance of the first tumour (11–12 months), 48 developed a total of 60 tumours. Among these were 27 Zymbal gland carcinomas, and tumours at other sites (Pliss & Zabezhinsky, 1970). [The Working Group noted this study was limited by the lack of a control group. However, in a preliminary report on these studies it was stated that rats from the same colony did not spontaneously develop tumours of the Zymbal gland (Pliss, 1965)].

4. Mechanistic and Other Relevant Data

4.1 Absorption, distribution, metabolism and excretion

4.1.1 *Absorption*

(a) Humans

Benzidine is light and fluffy, but solid and vapour forms can be rapidly absorbed through the skin (Barsotti and Vigliani, 1952; Budavari *et al.*, 1989; Ferber *et al.*, 1976; Meigs *et al.*, 1951, 1954; Zavon *et al.*, 1973). Exposure to benzidine can occur from

breathing contaminated air, wearing contaminated clothing, or by ingestion of contaminated food or water (Meigs *et al.*, 1951). Breathing or ingesting benzidine-based dyes also expose humans to benzidine, because the intestine contains bacteria that can break down these dyes into benzidine (Chung, 1983; Chung *et al.*, 1992).

Absorption follows after exposure by inhalation, or by the oral and dermal routes. Inhalation and skin contact are probably the predominant exposure routes for humans (ATSDR, 2001). It has been reported that benzidine and *3,3′*-dimethylbenzidine can readily penetrate intact skin (Meigs *et al.*, 1951). Industrial workers who handle benzidine and perspire freely were reported to have higher urinary concentrations of benzidine (Meigs *et al.*, 1954).

(b) Animals

Radioactivity was observed in tissues, urine, and faeces following application of 1 mg/kg bw of radioactive benzidine or benzidine derivatives for 1, 8, and 24 hours onto the shaved skin of F344 rats in a well controlled study, in which the animals were prevented from grooming themselves and licking at the site of benzidine application (Shah & Guthrie, 1983). About 25% of the initial dose of benzidine and benzidine derivatives penetrated into rat skin within 8 hours. At 24 hours after dosing, 49% of the radioactivity was recovered from the skin, which indicated that approximately half of the applied benzidine had penetrated into the skin and about 50% of the applied dose had been absorbed.

Aldrich *et al.* (1986) applied radiolabelled Direct Black 38 to the shaved dorsal skin of male F344 rats and New Zealand rabbits that were prevented from licking the site of application. Radioactivity was measured in urine and faeces 24–144 hours after application of the dye. At 144 hours, approximately 3% of the applied radioactivity was detected in the urine and 5% in the faeces of the rabbits. Excretion of radioactive substances was eventually negligible in rats (0.05% in urine and 0.16% in faeces). So skin penetration by the benzidine-based dye was unlikely; the absorbed and excreted radioactive material in the rabbits was presumed to represent benzidine that had been liberated by azo reduction of the dye (ATSDR, 2001). Bos *et al.* (1986) demonstrated transport of benzidine but not benzidine-based dyes across the mucosa of an isolated segment of rat intestine in a perfusion chamber, suggesting that benzidine but not benzidine-based dyes could be absorbed in the intestine.

Qiao *et al.* (1996) and Williams *et al.* (1996) developed a unique approach to study the absorption of chemicals from complex mixtures by use of "mechanistically defined chemical mixtures (MDCM)" applied to pig skin, which is similar in structure and function to human skin. Baynes *et al.* (1996) employed this system to investigate the absorption of mixtures of chemicals consisting of a marker chemical (benzidine), a solvent (acetone or DMSO), a surfactant (0% or 10% sodium lauryl sulfate), a vasodilator (0 μg or 180 μg methyl nicotinate), and a reducing agent (0% or 2% $SnCl_2$). It was found that acetone and DMSO enhanced dermal penetration of benzidine in most of the

mixtures. Compared with other mixtures evaluated, $SnCl_2$ inhibited benzidine absorption irrespective of the solvent present. $SnCl_2$ also inhibited benzidine penetration in DMSO mixtures containing sodium lauryl sulfate only, but not in acetone mixtures. It was proposed that interactions between benzidine and $SnCl_2$ may be the cause of the inhibition of benzidine absorption. The chemical-biological interaction between methyl nicotinate, sodium lauryl sulfate, and the skin may enhance benzidine absorption.

4.1.2 *Distribution*

(a) Humans

There is no information available on the distribution of benzidine in humans.

(b) Animals

In general, there appears to be a rapid plasma clearance of absorbed benzidine, followed by a more gradual metabolism and clearance of its metabolites (Shah & Guthrie, 1983; Lakshmi *et al.*, 1990).

When rabbits were given oral doses of 60–120 mg/kg of benzidine for periods ranging from 42 days to 128 days, the highest concentrations were found in the heart and lungs. Benzidine metabolites were not determined (Oida 1958a,b).

Soloimskaia (1968) reported that benzidine was rapidly absorbed after injection into rats with maximum concentrations of free and bound benzidines found at 2 and 3 hours, respectively. The highest concentrations were found in the blood, followed by liver, kidney, spleen, heart, and lung.

The body distribution of benzidine in various tissues and in urine of rats at 4 and 12 hours after intraperitoneal (i.p.) injection of 100 mg/kg benzidine was as follows: high concentrations were found in the stomach, stomach contents, and small intestine at 4 hours, and in the small intestine and its contents at 12 hours. Tissue concentrations of conjugated metabolites were high at 12 hous, and benzidine concentrations in the liver were high and constant over the 12-hour period (Baker and Deighton, 1953).

When radiolabelled benzidine was applied to the skin of rats, radioactivity was distributed approximately as follows (percentage of applied radioactivity at 1, 8, and 24 hours after application): blood (0.2, 0.3, and 0.7%), liver (1.5, 1.0, and 0.7%), lung (0.09, 0.2, and 0.2%), intestines (1.0, 14.0, and 1.3%), and stomach (0.5, 0.4, and 0.08%). Twenty-four h after dosing approximately half of the applied radioactivity had remained at the site of application (Shah & Guthrie, 1983). In a similar experiment in rats, in which radiolabelled benzidine was injected intravenously, tissues retaining the most radioactivities after three days were muscle, liver, and the stomach. Only a small amount was found in the bladder (Lynn *et al.*, 1984).

Chipman & Mohn (1989) demonstrated that biliary benzidine and benzidine metabolites could be reabsorbed from the intestine and transported again to the liver in both rats and mice. This entero-hepatic re-circulation could contribute to the persistence,

the further metabolism, and presumably the hepatoxicity and carcinogenicity of benzidine and its metabolites.

Sanderson & Clark (1993) showed that intraperitoneal administration of benzidine to pregnant mice resulted in the induction of micronuclei in the maternal bone marrow and in the liver of the fetuses; however, when pregnant mice were orally exposed to benzidine, there was no increase in micronucleated cells either in the maternal livers or the livers of the fetuses (Harper *et al.*, 1989). There was no information on whether benzidine could be stored in maternal tissues and be mobilized during pregnancy or lactation, nor was it known whether benzidine could be excreted in breast milk (ATSDR, 2001).

Kellner *et al.* (1973) studied the distribution of benzidine by injecting 0.2 mg/kg of uniformly labelled [^{14}C]-benzidine into animals of various species. In rats, substantial radio-activity was found after 4 hours in the lung, small and large intestines, the bladder, and the kidney, with smaller amounts in all other tissues and fluids examined. The findings in dogs were generally similar except for the 10- to 15-fold higher levels of radioactivity in bladder tissue, and the much lower activity (about 10% that of rats) in the lung. This was consistent with the high carcinogenicity of benzidine in the bladder of dogs. Approximately 90% of the radioactivity was cleared from the blood during the first 24 hours after dosing, the remainder being cleared more slowly. Half-lives of radioactivity from day one to day six or seven were 68 hours in the rats and 88 hours in the dogs. After seven days, the radioactivity was much reduced in all organs examined from rats, dogs, and monkeys. Highest residual activity was found in the liver for all three species. Expressed as concentration of benzidine in wet tissue, the mean liver concentrations were 0.042 μg/g for rats, 0.087–0.19 μg/g for three dogs, and 0.01 and 0.027 μg/g for the two monkeys (ATSDR, 2001).

In dogs, plasma clearance of benzidine is fairly rapid. Approximatelty 10% remained in the plasma after 5 hours, while metabolism and metabolite clearance occurred more gradually. In a study of four dogs monitored over a 5-hour period following intravenous administration of 1 mg/kg radiolabelled benzidine, Lakshmi *et al.* (1990) found that the initial plasma half-life of benzidine was approximately 30 minutes, while it was about 3 hours for total radiolabel (benzidine and metabolites). Five hours after infusion, 75% of recovered radioactivity was found in the bile (12–25%), urine (23–52%), and carcass muscle (15–30%). Significant amounts of radioactivity were also detected in fat (3–8%), the liver (4–8%), and plasma (2–7%). Small quantities were found in the stomach, intestines, spleen, kidneys, heart, and lungs. The bladder transitional epithelium showed a higher concentration of bound radioactivity than did bladder muscle. In liver, kidney, bladder muscle, and bladder epithelium, the majority of radioactivity was bound to protein, while smaller amounts were bound to DNA.

The differential serum protein-binding of benzidine- and benzidine congener-based dyes and derivatives was studied by use of crossed immuno-electrophoresis (X-IEP) techniques. The binding of these chemicals to certain serum proteins could be observed in

electrophoretic and immunoprecipitation patterns in X-IEP. Benzidine- and dimethylbenzidine-based dyes bound to albumin α_1-lipoprotein, β-lipoprotein, and hemopexin, whereas benzidine and dimethylbenzidine did not produce any electrophoretic shifts. However, autoradiographic analyses with benzidine and 3, 3′-dimethylbenzidine did show binding of benzidine to both α_1- and β-lipoprotein precipitation peaks. Although the physiological or pathological consequences of dye-binding to these serum proteins are not well understood, dyes derived from benzidine and its congeners may be carried by the proteins to different parts of the cell. For example, α_1-lipoprotein delivered the dyes to macrophage lysosomes where they inhibited several lysosomal enzymes, causing prolonged impairment of macrophage function with teratogenic, anti-immune, and potentially carcinogenic consequences (Crowle & May, 1982; Emmett *et al.*, 1985).

4.1.3 *Metabolism*

(a) Humans

Cerniglia *et al.* (1982) studied the metabolism of the azo-dye Direct Black 38 in intestinal bacteria and found that the azo linkage in Direct Black 38 was reduced by azoreductase in these bacteria, resulting in release the carcinogen benzidine. Chung *et al.* (1978) reported that many intestinal bacteria isolated from faeces of patients with polyposis could reduce azo dyes. The bacteria isolated from intestine and/or the skin was also reported to have azoreductase activity (Chung *et al.*, 1992; Chung & Stevens, 1993; Chen, 2006; Platzek *et al.*, 1999; Xu *et al.*, 2007). The azoreductase in different preparations was affected by various dietary factors such as cellulose, proteins, fibres, antibiotics, or supplementation with live cultures of lactobacilli (Chung *et al.*, 1992). Many benzidine congener-based dyes including 3, 3′-dimethylbenzidine, 3, 3′-dimethoxybenzidine, and 3, 3′-dichlorobenzidine were also reported to be reduced by azoreductase in intestinal bacteria and/or other environmental microorganisms to release the benzidine congeners and many other metabolites such as monoacetyl- and diacetylbenzidine and their congeners (Bowman & Nony, 1981; Bowman *et al.*, 1982, 1983; Manning *et al.*, 1985; Cerniglia *et al.*, 1986). These metabolites were also detected in workers exposed to these azo dyes, benzidine or benzidine-based pigments (Vigliani & Barsotti, 1961; Haley, 1975, 1982). Therefore, azo-reduction was considered the first step of azo dye- induced carcinogenesis (Chung, 1983; Chung & Cerniglia, 1992) and control of azoreduction becomes important in azo dye-induced cancer (Chen *et al.*, 2006). It should be pointed out that metabolic conversion of benzidine-, 3, 3′-dimethybenzidine- and 3, 3′-dimethoxybenzidine-based dyes to their respective carcinogenic amine precursors in vivo is a general phenomenon: exposure to benzidine-based dyes has caused bladder cancer in humans. However, studies in which azo pigments based on 3, 3′-dichlorobenzidine such as Pigment Yellow 12 had been orally administered to rats, hamsters, rabbits, and monkeys did not generally show significant amounts of 3, 3′-

dichlorobenzidine in the urine. The aromatic amine components from azo pigments based on *3, 3′*-dichloro-benzidine appear not to be readily bio-available. Therefore, it seems unlikely that occupational exposure to insoluble azo pigments (3, 3′-dichloro-benzidine-based azo dyes such as Pigment Yellow 12) would be associated with a substantial risk for bladder cancer in humans (Golka *et al.*, 2004).

Since benzidine is the major mutagenic and carcinogenic moiety of carcinogenic azo dyes (Chung & Cerniglia, 1992) the mechanisms of activation of benzidine have been extensively studied (Morton *et al.*, 1979).

Rothman *et al.* (1996a) conducted a cross-sectional study of workers exposed to benzidine and benzidine-based dyes, and unexposed controls. Benzidine, *N*-acetyl-benzidine, and *N,N′*-diacetylbenzidine were not detected in the urine of control subjects. Urinary levels of these compounds were low in workers producing benzidine-based dyes and about 17-fold higher in workers manufacturing benzidine. Upon analysis by ^{32}P-postlabelling, four DNA adducts were found to be significantly elevated in urothelial cells of exposed workers compared with controls, the predominant adduct being *N*-(deoxyguanosin-8-yl)-*N′*-acetylbenzidine. This is the only adduct that was significantly associated with the total amount of urinary metabolites of benzidine. These results suggest that *N*-monoacetylation is involved in activation of benzidine, while *N*-diacetylation is likely part of a detoxification pathway. In this study, there was no significant association between *NAT2* genotype and adduct levels.

Zenser *et al.* (1996) assessed *N*-acetylation of benzidine to *N*-acetylbenzidine by use of human recombinant NATs. Km and V_{max} values were higher for NAT1 than for NAT2. The clearance ratios (NAT1/NAT2) for benzidine and *N*-acetylbenzidine were 54 and 535, respectively, suggesting that NAT1 is a more efficient enzyme for *N*-acetyl-benzidine than NAT2. The much higher Km values of NAT1 and NAT2 for *N*-acetyl-benzidine compared with benzidine appear to favour the metabolism of benzidine over that of *N*-acetylbenzidine, for low exposures.

In human liver slices incubated with [^{3}H]-labelled benzidine, the relative amounts of benzidine, *N*-acetylbenzidine, and *N,N′*-diacetylbenzidine were 19 ± 5, 34 ± 4, and 1.6 ± 0.5%, respectively. Similar results were observed if slices were incubated with [^{3}H]-acetylbenzidine instead of [^{3}H]-benzidine (Zenser *et al.*, 1996). Thus, in these studies, conditions in liver slices favour the formation of *N*-acetylbenzidine rather than *N,N′*-diacetylbenzidine. With paraoxon, a deacetylase inhibitor, the formation of *N,N′*-diacetyl-benzidine increased 32-fold. *para*-Aminobenzoic acid, a NAT1-selective substrate, increased the amount of benzidine and decreased the amount of *N*-acetylbenzidine produced, resulting in a decreased ratio of acetylated products. This is consistent with benzidine being an NAT1 substrate. Individuals with rapid *NAT2* genotypes did not form significantly more *N*-acetylbenzidine than did slow acetylators. There was no apparent correlation of *N,N′*-diacetylbenzidine formation with *NAT2* genotype. HPLC analysis of the liver-slice extracts detected *N*-glucuronides of both benzidine and *N*-acetyl-benzidine.

These *N*-glucuronides represent 7 and 16%, respectively, of the total radioactivity recovered by HPLC (Zenser *et al.*, 1996).

Ciotti *et al.* (1999) assessed the capacity of five different human recombinant UDP-glucuronosyltransferases (UGTs) expressed in COS-1 cells. [^{14}C]-Labelled UDP-glucuronic acid was used as a co-substrate. Benzidine, *N*-acetylbenzidine, and *N,N′*-diacetylbenzidine, the *N*-OH derivatives of acetyl- and diacetylbenzidine, the 3-OH derivatives of diacetylbenzidine and benzidine were used as substrates. *N,N′*-diacetyl-benzidine was not a substrate for glucuronidation. UGT1A9 showed the highest relative rate of metabolism, with a preference for the *N*-OH derivatives of acetyl- and diacetyl-benzidine. The overall results suggest the following relative ranking of transferase metabolism: UGT1A9 > UGT1A4 >> UGT2B7 > UGT1A6 ≈ UGT1A1.

Since the *N*-glucuronides of benzidine and *N*-acetyl-benzidine are acid-labile (Babu *et al.*, 1992; 1993), this property was used to indirectly assess these glucuronides in urine from workers in India manufacturing benzidine or benzidine-based dyes, in comparison with those from workers at a construction company. The pH of post-workshift urine was inversely correlated with the proportions of benzidine and *N*-acetylbenzidine present as free (non-glucuronidated) compounds. When controlling for internal dose, individuals with urine at pH < 6 had a tenfold higher level of the deoxy-guanosine adduct of acetylbenzidine in their exfoliated bladder cells, compared with subjects with urine at pH ≥ 7 (Rothman *et al.*, 1997). These results suggest that a low pH of the urine may be a risk factor in bladder cancer.

In both bladder cells and white blood cells, dGp-acetylbenzidine is the major adduct (Zhou *et al.* 1997). The sum of urinary benzidine metabolites (benzidine, *N*-acetyl-benzidine, and *N,N′*-diacetylbenzidine) is an index of internal dose that correlates with the level of the dGp-acetylbenzidine adduct in both peripheral white blood cells and in exfoliated bladder cells. Moreover, adduct levels in human peripheral white blood cells correlate with those in exfoliated bladder cells. Similar mechanisms of adduct formation may exist in both cell types, with white blood cells serving as a surrogate biomarker. Thus, dGp-acetylbenzidine is an important adduct, and human peripheral white blood cells are a relevant cell type for studying this adduct formation.

Lakshmi *et al.* (2000a; 2000b) assessed the metabolic pathways leading to dGp-acetylbenzidine formation in human peripheral white blood cells. Transformation of [^{3}H]-labelled acetylbenzidine was assessed by use of myeloperoxidase (MPO) or hypochlorous acid (HOCl). MPO-mediated metabolism required H_2O_2. While transformation by HOCl was completely inhibited by 10 mM taurine, the metabolism of acetylbenzidine by MPO was only reduced 56%. Transformation by either MPO or HOCl was inhibited by 100 mM 5,5-dimethyl-1-pyrroline *N*-oxide (DMPO), 1 mM glutathione, and 1 mM ascorbic acid. Two previously identified oxidation products of acetylbenzidine, *N′*-hydroxy-*N*-acetylbenzidine and 4′-nitro-4-acetylaminobiphenyl, were not detected. With DNA or dGp present, a new product was observed that corresponded to synthetic dGp-acetylbenzidine. The HOCl-derived adduct was identified by electrospray-ionization mass

spectrometry (ESI/MS) and NMR as dGp-acetylbenzidine. Upon analysis by ^{32}P-postlabelling, dGp-acetylbenzidine increased more than 300-fold if either DNA or dGp was present. Indomethacin (0.1mM) did not alter adduct formation. These results are consistent with human neutrophils forming dGp-acetylbenzidine by a peroxidative mechanism involving MPO.

Studies with many aromatic amines and human liver microsomes (Beland & Kadlubar, 1990) and with acetylbenzidine and rat-liver microsomes have demonstrated CYP-mediated *N*-oxidation (Lakshmi *et al.*, 1997). With rat-liver microsomes, *N′*-hydroxy-*N*-acetylbenzidine is formed. However, *N*-oxidation of acetylbenzidine with human liver microsomes has not been demonstrated.

(b) Experimental systems

N-Acetylation plays an important role in biotransformation of aromatic amines and was assessed for benzidine. In rat-liver slices incubated with 0.05 mM [^{3}H]-labelled benzidine, the acetylated products *N*-acetylbenzidine and *N,N′*-diacetylbenzidine represented 8.8 ± 3.6 and 73 ± 2.5%, respectively, of the total radioactivity recovered after HPLC (Lakshmi *et al.*, 1995a). No unmetabolized benzidine was observed.

Excretion of aromatic amines is facilitated by UDP-glucuronosyltransferases (UGTs). When *N*-glucuronidation of benzidine and *N*-acetylbenzidine was assessed, microsomes from dog and rat produced an identical new HPLC peak, which was dependent upon the presence of UDP-glucuronic acid (Babu *et al.*, 1992, 1993). Whether incubated in the presence or absence of detergents, microsome-catalysed glucuronidation of *N*-acetylbenzidine and *N,N′*-diacetylbenzidine decreased as follows: human > dog > rat. No glucuronidation of *N,N′*-diacetylbenzidine was observed, which is consistent with the lack of glucuronidation of arylamides (Babu *et al.*, 1993). To determine the specificity of the UGT reaction with benzidine and *N*-acetylbenzidine, a wide range of inhibitors (known substrates) was tested. Results were consistent with multiple transferases metabolizing benzidine and *N*-acetylbenzidine.

To correlate results with microsomal glucuronidation to those in intact tissues, dog-liver slices were incubated with 0.05 mM [^{3}H]-labelled benzidine. An HPLC peak corresponding to the glucuronide conjugate of benzidine represented as much as 30% of the total radioactivity recovered (Babu *et al.*, 1992). Neither benzidine nor acetylbenzidine glucuronide was detected under these incubation conditions with rat liver, which rapidly *N*-acetylates benzidine to acetyl- and diacetylbenzidine (Babu *et al.*, 1993; Lakshmi *et al.*, 1995b). This is consistent with *N*-acetylation and *N*-glucuronidation being competing pathways and likely playing a role in benzidine-induced liver cancer in rats and bladder cancer in dogs and humans (Case *et al.*, 1954; Haley, 1975).

The pH of the urine is affected by diet and was considered to be a potential modifier of benzidine-induced bladder carcinogenesis. After 4 or 5 min at pH 5.3 and 37ºC, half of the *N*-glucuronides of benzidine and acetylbenzidine are hydrolysed to their parent amine (Babu *et al.*, 1992; 1993). At pH 7.4, the half-lives of these glucuronides are 104 and 140

min, respectively. The *O*-glucuronides of the hydroxamic acids, *N*-hydroxy-*N*-acetylbenzidine and *N*-hydroxy-*N,N′*-diacetylbenzidine, were not acid-labile. The *N*-glucuronide of *N′*-hydroxy-*N*-acetylbenzidine was acid-labile, with a half-life at pH 5.5 of 3.5 hours, compared with 7.5 min for the *N*-glucuronide of acetylbenzidine. Thus the *N*-glucuronide of *N*-acetylbenzidine is much more likely to be involved in acidic urine-catalysed hydrolysis than is its *N*-hydroxy *N*-glucuronide. The glucuronides of 4-aminobiphenyl and *N*-OH-4-aminobiphenyl were both acid-labile with half-lives of 10 and 32 min, respectively, at pH 5.5 (Babu *et al.*, 1996). In contrast, the *O*-glucuronide of *N*-OH-*N*-acetyl-4-aminobiphenyl was not acid-labile, with half-lives at pH 5.5 and 7.4 of 55 and 68 min, respectively. Thus other *N*-glucuronides of aromatic amines are also acid-labile and may have a shorter half-life than their corresponding *N*-OH *N*-glucuronides. *O*-Glucuronides are not acid-labile (Babu *et al.*, 1996).

To evaluate NADPH-dependent oxidation of benzidine, Lakshmi *et al.* (1996) used liver microsomes from control and β-naphthoflavone-treated rats. Beta-naphthoflavone treatment increased the metabolism of benzidine compared with the control, as judged from the HPLC metabolite-profile and protein/DNA binding. The CYP inhibitors ellipticine and α-naphthoflavone, selective for CYP1A1/1A2, elicited 50% inhibition at approximately 0.2 and 0.5 μM, respectively. Mass spectrometry identified the only metabolite formed as 3-hydroxybenzidine. *N*-hydroxybenzidine formation by CYP has never been reported.

CYP-induced metabolism and activation of acetyl- and diacetylbenzidine was assessed by incubating liver microsomes from control and β-naphthoflavone-treated rats with either substrate (Lakshmi *et al.*, 1997). With β-naphthoflavone-induced microsomes, *N*-hydroxy-*N*-acetylbenzidine formation was eight-fold higher than in the control: a significant formation of ring-oxidation products was demonstrated, and *N′*-hydroxy-*N*-acetylbenzidine formation was at the limit of detection. With control microsomes, more *N′*-hydroxy-*N*-acetylbenzidine was produced than *N*-hydroxy-*N*-acetylbenzidine. While oxidation of diacetylbenzidine was not observed with control microsomes, significant *N*-hydroxy-*N,N′*-diacetylbenzidine formation and ring-oxidation was seen with β-naphthoflavone treatment. Metabolism by β-naphthoflavone-induced microsomes was completely blocked by selective CYP1A1/1A2 inhibitors, α-naphthoflavone and ellipticine. *N′*-Hydroxy-*N*-acetylbenzidine formation by control microsomes was not prevented by these inhibitors. A non-specific CYP inhibitor, SKF-525A, exhibited partial dose-response inhibition of *N′*-hydroxy-*N*-acetylbenzidine. The adduct *N′*-(deoxyguanosin-8-yl)-*N*-acetylbenzidine was detected by ^{32}P-postlabelling in incubations containing DNA and acetylbenzidine, but not diacetylbenzidine. More adduct was detected with control than with β-naphthoflavone-treated microsomes. Thus, while *N*-hydroxybenzidine formation by CYP was not observed, acetyl- and diacetylbenzidine are substrates for these enzymes and form *N*-hydroxy metabolites. These results are consistent with CYP-mediated activation of acetylbenzidine to *N′*-hydroxy-*N*-acetylbenzidine with subsequent binding to

DNA and adduct formation. This is likely a mechanism responsible for the formation of the dGp-acetylbenzidine adduct in humans (Lakshmi *et al.*, 1997).

A study investigating the metabolism of benzidine in slices of the inner renal medulla of rabbits showed that benzidine induced a dose-dependent reversible inhibition of prostaglandin E2 (PGE2) synthesis. Binding of [^{14}C]-labelled benzidine metabolites to medullary tissue was observed. This binding was increased by arachidonic acid, and arachidonic acid-mediated binding was prevented by inhibitors of prostaglandin H synthase. Inhibitors of mixed function oxidase activity (metyrapone and SKF-525A) did not inhibit binding of benzidine metabolite(s). These findings are consistent with previous studies and demonstrate the microsomal co-oxidative metabolism of benzidine by prostaglandin H synthase in the inner medulla (Rapp *et al.*, 1980).

Since the bladder is a possible site for activation of arylamine bladder carcinogens, the dog bladder was studied and found to contain little CYP but substantial prostaglandin H synthase (PHS) activity (Wise *et al.*, 1984). Lakshmi *et al.* (1998) assessed the possible formation of the dGp-acetylbenzidine adduct by peroxidatic activation of acetylbenzidine. Adduct formation was measured by ^{32}P-postlabelling. Ram seminal vesicle microsomes were used as a source of PHS. The peroxidatic activity of PHS induced formation of the adduct, whether DNA or dGp was present. Adduct formation was dependent upon the presence of peroxidase and a specific substrate, i.e. arachidonic acid or H_2O_2. Adduct formation was inhibited by indomethacin (0.1 mM), ascorbic acid (1 mM), and glutathione (10 mM), but not by DMPO (100 mM), a radical scavenger. Since the PHS activity in cultured urothelial cells from humans and dogs is enhanced by bradykinin, calcium ionophore, arachidonic acid, and phorbol ester (Danon *et al.*, 1986; Zenser *et al.*, 1988; 1990), a corresponding increase in benzidine activation could occur.

Zenser *et al.* (1999) examined the mechanism by which PHS from ram seminal vesicle microsomes catalyses the oxidation of the reducing co-factor acetylbenzidine. During the conversion of this compound to its final end product 4′-nitro-4-acetylaminobiphenyl, a new metabolite was detected when 1 mM ascorbic acid was present. Similar results were observed whether arachidonic acid or H_2O_2 was used as substrate. This metabolite co-eluted with synthetic *N*′-hydroxy-*N*-acetylbenzidine but not *N*-hydroxy-*N*-acetylbenzidine. The new metabolite was identified as *N*′-hydroxy-*N*-acetylbenzidine by ESI/MS/MS. It represented as much as 10% of the total radioactivity recovered after HPLC. When *N*′-hydroxy-*N*-acetylbenzidine was substituted for *N*-acetylbenzidine, 4′-nitro-4-acetylaminobiphenyl was formed. Inhibitor studies demonstrated that the metabolism was due to PHS, not CYP. Oxygen-uptake studies did not demonstrate a requirement for molecular oxygen. When [^{18}O]H_2O_2 was used as substrate, [^{18}O] enrichment was observed. These results demonstrate a peroxidative mechanism of oxidation of *N*-acetylbenzidine and *N*′-hydroxy-*N*-acetylbenzidine by PHS and suggest stepwise oxidation of acetylbenzidine to *N*′-hydroxy, 4′-nitroso, and 4′-nitro products (Zenser *et al.,* 1999). In contrast, horseradish peroxidase and myeloperoxidase (MPO) appear to activate acetylbenzidine by a radical mechanism not involving *N*′-hydroxy-*N*-

acetylbenzidine (Lakshmi *et al.*, 1998; 2000a). Thus, dGp-acetylbenzidine is formed by activation of acetylbenzidine with CYP, PHS or MPO, each representing a different mechanism of activation.

To gain more insight into peroxidative activation of acetylbenzidine, glutathione was used to trap the activated intermediate (Lakshmi *et al.*, 2000b; Zenser *et al.*, 2001). Myeloperoxidase, like horseradish peroxidase, metabolizes acetylbenzidine by a mechanism that does not produce 4′-nitro-4-acetylaminobiphenyl. While the thiol conjugate of horseradish peroxidase-activated acetylbenzidine was expected to be similar to that formed with benzidine, *i.e.* 3-(glutathione-*S*-yl)-benzidine (Wise *et al.*, 1985; Lakshmi *et al.* 1994), this was not the case (Lakshmi *et al.*, 2000b). The product was identified by mass spectrometry and NMR as *N*′-(glutathion-*S*-yl)-acetylbenzidine-*S*-oxide. The lack of effect of mannitol and superoxide dismutase suggests that neither the hydroxyl radical nor superoxide is involved in this reaction. Studies also indicated that molecular oxygen is not a source of the sulfinamide oxygen. Methaemoglobin (acting as a peroxidase) catalysed the formation of the same conjugate (Zenser *et al.*, 2001). The proposed mechanism for sulfinamide formation, involving two consecutive one-electron oxidations with subsequent arrangement to a sulfur-stabilized nitrenium ion, suggests that the oxygen may be derived from water. A less active ring-activated intermediate, such as a diimine monocation may be formed, which is a resonance structure of the acetyl-benzidine nitrenium ion. This intermediate may play a role in the activation of acetylbenzidine by horseradish peroxidase (Lakshmi *et al.*, 1998) and MPO (Lakshmi *et al.*, 2000a), leading to formation of the dGp-acetylbenzidine adduct.

Reduction of benzidine-based dyes is a potential source of human exposure to benzidine. Since aromatic amines can be activated to bind haemoglobin, these adducts offer a method for assessing exposure. When female Wistar rats were given an oral dose of 0.5 mmol/kg benzidine and haemoglobin was isolated after 24 hours (Birner *et al.*, 1990; Zwirner-Baier & Neumann, 1998), the haemoglobin binding index (HBI) was 2.4 (benzidine), 18.9 (acetylbenzidine), and 3.0 (4-aminobiphenyl, 4-ABP). Since benzidine is rapidly *N*-acetylated in rats, acetylbenzidine adducts are expected. Diacetylbenzidine is not expected to form haemoglobin adducts, and no adducts were detected after administration of this compound. The presence of 4-ABP adducts was unexpected, and demonstrates an unknown pathway of the metabolism of benzidine. This method was then used to monitor the bioavailability of benzidine and its metabolites following oral administration of Direct Red 28, a benzidine-derived azo-dye, at 1 mmol/kg. The HBI indices for benzidine, acetylbenzidine and 4-ABP were 0.3, 1.8, and 2.2. This demonstrates exposure to these three compounds derived from a benzidine-based dye (Birner *et al.*, 1990). Reactive nitric oxygen species transform benzidine to 4-ABP and 4'-OH-4-amino-biphenyl and this reaction, involving components of the inflammatory response, may be a source of 4-ABP formation from benzidine *in vivo* (Lakshmi *et al.*, 2003).

4.1.4 *Excretion*

(a) Humans

A single oral dose of 100 mg of benzidine in humans resulted in urinary excretion of free benzidine and its mono- and di-acetylated derivatives. However, only less than 1 mg of the initial dose was recovered (Engelbertz & Babel, 1953).

Benzidine and its metabolites were measured in the urine of exposed industrial workers in March and August. The mean urinary concentrations of the compounds after exposure in the spring were as follows: benzidine, 0.28 mg/L; *N*-acetylbenzidine, 0.27 mg/L; *N,N'*-diacetylbenzidine, 0.52 mg/L; conjugated 3-hydroxybenzidine, 3.9 mg/L. During the month of August, a mean overall concentration of 21.8 mg/L was measured for benzidine and its metabolites, the peak excretion being 31 mg/L. Dermal contact with dust containing benzidine was the primary source at the benzidine plants. Daily showers and clean working clothes reduced the quantity of benzidine and its metabolites excreted in the urine of exposed workers (Meigs *et al.*, 1951).

Excretion of 0–363 µg/L benzidine, 6–1117 µg/L acetylbenzidine, and 4–160 µg/L diacetylbenzidine was measured in the urine of workers potentially exposed to several benzidine-based dyes. Exposure was assumed to have been largely by inhalation, but dermal exposure may have been significant as well (NIOSH, 1980; Dewan *et al.*, 1988).

Many workers in dye, printing, warehouse, and colour room shops who were exposed to benzidine-based dyes including Direct Black 4, Direct Blue 2, Direct Brown 2, Direct Green 1, Direct Orange 1, Direct Orange 8, Direct Red 28, Direct Blue 6, Direct 38, and Direct Brown 95 excreted benzidine in their urine (Walker, 1970; Genin, 1977; Lynn *et al.*, 1980; Robens *et al.*, 1980; Haley, 1982).

(b) Animals

(i) *Rat*

Excretion of benzidine after an intravenous dose of 0.2 mg/kg in rats, dogs, and monkeys was 97%, 96%, and 88%, respectively, one week after dosing. Dogs and monkeys excreted benzidine via the urinary route, whereas rats used the biliary route (Kellner *et al.*, 1973). Following intravenous exposure of rats to 0.2 or 2.5 mg/kg radiolabelled benzidine, most of the radioactivity (63–80%) was excreted in the faeces during the first 3 to 7 days, and much less via the urine (17–29%) (Kellner *et al.*, 1973; Lynn *et al.*, 1984). Experiments with bile duct-cannulated rats indicated that virtually all fecal metabolites originated from biliary excretion. Urinary metabolites included 3-hydroxy-*N,N'*-diacetylbenzidine glucuronide (25%), *N,N'*-diacetylbenzidine (12%), and *N*-hydroxy-*N,N'*-diacetylbenzidine glucuronide (4%). Metabolites and relative amounts were similar in bile, except that about half of the 3-hydroxy-*N,N'*-diacetylbenzidine glucuronide was replaced by the 3-glutathion-*S*-yl-*N,N'*-diacetylbenzidine conjugate (Lynn *et al.*, 1984).

After dermal application of radiolabelled benzidine to rats, radioactivity was detected in both urine and faeces as early as one hour after treatment. Excretion was significantly greater (6–8-fold) in urine than in faeces during the first eight hours, but it was comparable for both routes after 24 hours (23% in urine, 19% in faeces) (Shah & Guthrie, 1983).

In rats, the major route of excretion after single oral doses of 0.5, 5 or 50 mg/kg radiolabelled benzidine appeared to be via the faeces. At the lowest dose, 74% of the radioactivity was excreted in the faeces during the first days after exposure and only 17% in the urine. With increasing doses the percentage of radioactive compounds excreted in the faeces decreased, while that in the urine increased. At the low- and mid-level doses (0.5 and 5.0 mg/kg), the major radioactive compounds were identified as 3-hydroxy-*N,N'*-diacetylbenzidine glucuronide (39% and 37%), *N,N'*-diacetylbenzidine (13% and 17%), *N*-hydroxy-*N,N'*-diacetylbenzidine glucuronide (4% and 5%), *N*-acetylbenzidine (3% and 4%), and free benzidine (2% and 2%, respectively). At the high dose (50 mg/kg), the percentage of *N*-hydroxy-*N,N'*-diacetylglucuronide increased substantially to 24%, largely at the expense of *N,N'*-diacetylbenzidine (reduced to 4%). No radioactivity was detected in expired air (Lynn *et al.*, 1984).

The analyses of urine and faeces after intravenous injection of radiolabelled benzidine confirmed that the main excretion route of radioactivity was via the faeces (Kellner *et al.*, 1973; Lynn *et al.*, 1984). However, other studies reported that benzidine, its metabolites, and their conjugates were excreted approximately equally in urine and bile/faeces (Shah and Guthrie, 1983; Lakshmi *et al.*, 1990).

(ii) *Mouse*

After injection of mice with 100 mg/kg benzidine, the following compounds were found excreted in the urine: benzidine (10%), *N*-acetylbenzidine (3.4%), *N,N'*-diacetylbenzidine (2.6%), 3-hydroxy ethereal sulfate (29%), 3-hydroxybenzidine glucuronide (12%), *N*-hydrogen sulfate or glucuronide conjugates (18%), and monoacetylated 3-hydroxy ethereal sulfate or glucuronide-benzidine conjugate (25%) (Sciarini & Meigs, 1961).

(iii) *Dog*

After intraperitoneal injection of benzidine, dogs excreted this compound in the urine, but the fecal excretion was 11 times greater than via the urine (Sciarini & Meigs, 1958). The urinary excretion in dogs after intravenous injection of benzidine was reported to range from 1–2.5 times that found in the bile or faeces (Kellner *et al.*, 1973; Lakshmi *et al.*, 1990). About 30% of the radioactivity excreted in the urine or bile was free benzidine. 3-Hydroxybenzidine was a major metabolite (6%) found in the bile, but not in the urine. *N*-Acetylated metabolites were not found. This is in agreement with the fact that dogs are deficient in *N*-acetyltransferase activity (Lakshmi *et al.*, 1990). The urinary concentration

of free benzidine ranged from 2 to 9%, and the concentration of 3-hydroxybenzidine or its sulfur conjugate ranged from 25 to 50%.

(iv) *Monkey*

When benzidine was intravenously injected in three monkeys (0.2 mg/kg bw), the cumulative excretion during the first seven days varied from 30% to 70% in the urine and from 5 to 36% in the faeces. There were some indications of the presence of *N*-acetylbenzidine, but this was not chemically confirmed (Kellner *et al.*, 1973).

Oral exposure of monkeys to 10 or 100 mg benzidine resulted in urinary excretion of free benzidine and *N*-acetylbenzidine. The combined 72-hour excretion of these two compounds represented only a small fraction (1.5%) of the administered dose (Rinde & Troll, 1975). This contrasts with the 6% reported in the rat as excreted fraction for these two compounds (Lynn *et al.*, 1984).

4.2 Genetic and related effects

4.2.1 *Humans*

Mirkova and Lalchev (1990) studied the cytogenetic effects of occupational exposure to benzidine and benzidine-based dyes (Direct Black 38 and Direct Blue 6) in workers at a manufacturing plant in Bulgaria, who had a recognized high risk for occupational cancer. Twenty-three workers (13 men, 10 women), 47 ± 8.3 years of age and exposed for a mean of 15 years, were compared with 30 controls presumed to have had no exposure. A statistically significant (10-fold) increase in the number of circulating peripheral lymphocytes displaying chromosomal aberrations was observed in exposed workers when compared with controls. The highest frequencies of aberrant lymphocytes were associated with the highest airborne dust concentrations of benzidine (0.42–0.86 mg/m^3) or benzidine-based dyes (7.8–32.3 mg/m^3), and with the highest mean levels of benzidine found in the urine (1.8–2.3 μg/L). The frequency of polyploid lymphocytes was also elevated in workers when compared with controls. No significant association with smoking was observed. A major strength of this study is the monitoring and biomonitoring of benzidine. These data provide clear evidence of benzidine's genotoxicity in humans under occupational exposure conditions, and are in agreement with oral genotoxicity results from animals and in-vitro test systems (see below).

4.2.2 *Experimental systems*

(a) In-vivo studies

There are conflicting reports on the ability of benzidine to induce micronucleated polychromatic erythrocytes in the rat. It was inactive at doses of up to 250 mg/kg bw (Trzos *et al.,* 1978), but was positive (with no dose–response) when tested at comparable

doses (100, 200, 300 mg/kg bw) in another study (Cihák, 1979). It was also reported to be active in inducing micronucleus formation when given at the high dose of 409 mg/kg bw, either dermally or subcutaneously (Urwin *et al.,* 1976).

Several studies have addressed the in-vivo genotoxicity of benzidine in animals following oral or parenteral exposure. Although early studies were conflicting or equivocal, benzidine was clearly demonstrated to induce bone-marrow micronuclei in two strains of male mice (C57BL6 and CBA), 24 and 48 hours after a single administration of 300 mg/kg bw benzidine by oral gavage (Mirkova & Ashby, 1988). The number of micronucleated cells per 1000 normal cells for the test groups (5.75–8.75) was three times that observed in control groups (2.0–2.9). These findings were extended in a subsequent study in which male C57BL6 mice, treated by oral gavage with either a single dose (900 mg/kg bw) or with three consecutive daily doses (150 or 300 mg/kg bw), showed a positive dose–response for bone-marrow micronucleus induction (Mirkova, 1990) [The Working Group noted the high doses used in some of these studies]. Negative results, however, were reported for a different strain of mice (ICR), 6–8 weeks of age, treated with single oral gavage doses of 100 or 200 mg benzidine/kg bw. Harper *et al.* (1989) observed no significant increases in micronucleated cells in the bone marrow of treated male, female, or pregnant female mice (gestation days 16–17), nor in the livers from the fetuses of treated pregnant female mice. When given by single oral gavage to male rats, 200 mg benzidine/kg bw induced unscheduled DNA synthesis in liver cells, which is a repair response to DNA damage (Ashby & Mohammed, 1988). In a study with Swiss-albino mice, 9–13 weeks of age, intraperitoneal administration of benzidine to pregnant dams increased the frequency of micronucleated polychromatic erythrocytes in the liver of fetuses, which suggests that benzidine (or metabolites) can cross the placenta (Sanderson & Clark, 1993).

(b) In-vitro studies

Benzidine has consistently been found to be mutagenic to *Salmonella typhimurium* strain TA1538 when tested in the presence of an exogenous metabolic activation system from Sprague-Dawley rats (see, *e.g.*, Ames *et al.,* 1973; Anderson & Styles, 1978) or humans (see, *e.g.*, Neis *et al.*, 1985). The urine of rats fed benzidine was mutagenic to *S. typhimurium* TA1538, TA98 or TA100 when tested in the presence of a rat-liver metabolic activation system or to *S. typhimurium* TA1538 in the presence of a rat-liver cytosolic fraction; addition of glucuronidase increased the mutagenic activity in TA1538 (Bos *et al.,* 1980).

N-Hydroxy-*N,N'*-diacetylbenzidine was mutagenic to *S. typhimurium* TA1538 in the presence of a partially purified *N,O*-acyltransferase preparation (Morton *et al.*, 1979). Benzidine was negative in the *Escherichia coli* pol A test (Fluck *et al.,* 1976) and in the prophage-induction test (Speck *et al.,* 1978), when tested either in the presence or absence of a rat-liver metabolic activation system. Mutagenic activity on the X-chromosome recessives (visibles and lethals) and RNA genes of *Drosophila melanogaster* has been

reported (Fahmy & Fahmy, 1977). Benzidine (6×10^{-4} M for 30 min) inhibited DNA synthesis in HeLa cells *in vitro* in the absence of activation (Painter, 1978), and *in vivo* in renal and hepatic cells when given intraperitoneally or intragastrically to 14–18-day-old suckling mice in doses of 15–30% of the LD50 (Amlacher & Ziebarth, 1979).

Unscheduled DNA synthesis was induced by benzidine (active dose range, 10^{-7}–10^{-3} M) in HeLa cells in the presence of a phenobarbital-induced rat-liver activation system (Martin *et al.,* 1978) and in rat hepatocytes (Williams, 1978; Brouns *et al.,* 1979). Benzidine, when tested in the presence of a rat-liver metabolic activation system, induced DNA strand-breaks in Chinese hamster V79 cells (Swenberg *et al.,* 1976). When measured by the alkaline elution assay, there was a dose-related increase in DNA strand-breaks in the livers of rats exposed to benzidine *in vivo* (Petzold & Swenberg, 1978). Benzidine (2.5 μg/mL) transformed BHK21 $Cl^{-}13$ cells in the presence of an Aroclor 1254-induced rat-liver metabolic system (Ashby *et al.*, 1978), and was shown to transform Syrian hamster embryo cells (Pienta, 1980).

When tested in many in-vitro assays published since 1982 (IARC, 1982), benzidine has generally shown positive results for reverse mutation in *Salmonella typhimurium* in the presence of exogenous metabolic activation (*e.g.*, liver S-9) (Chung *et al.*, 2000; Dorado & Pueyo, 1988; Duverger-van Bogaert *et al.*, 1995; Gregory *et al.*, 1981; Zeiger *et al.*, 1992); negative for SOS DNA repair in *Escherichia coli* (von der Hude *et al.*, 1988); positive for mutation in yeast (Buchholz *et al.*, 1992; Mitchell & Gilbert 1991); positive (Oberly *et al.*, 1990) or negative (Phillips *et al.*, 1990) for gene mutation in Chinese hamster ovary cells; positive (Fassina *et al.*, 1990; Suter *et al.*, 1992) or negative (Oglesby *et al.*, 1983) for gene mutation in Chinese hamster V79 cells; and positive (*Tk* locus) or negative (*Hgprt* locus) for gene mutation in mouse-lymphoma cells (Henderson *et al.*, 1990; Myhr & Caspary, 1988). Benzidine has also given a positive response when tested for chromosome breakage (Swenberg *et al.*, 1976) and sister chromatid exchange (Grady *et al.*, 1986; Lindahl-Kiessling *et al.*, 1989) in cultured human and animal cells; it was generally positive in cultured hepatocytes for unscheduled DNA synthesis (Kornbrust & Barfknecht 1984a, 1984b; Steinmetz *et al.*, 1988; Williams, 1978); positive for animal cell transformation (Ashby *et al.*, 1978; Pienta, 1980); and negative for DNA-adduct formation in cultured mammalian cells, but positive with calf-thymus DNA, in the absence of exogenous activation (Phillips *et al.*, 1990).

4.2.3 *Effects on cell function*

The results of a study on the expression of mutant p53 protein in workers exposed to benzidine and in bladder-cancer patients (Shen *et al.*, 2005) indicated that the expression level of mutant p53 increased with the exposure-intensity index in exposed workers; the expression was significantly higher in bladder-cancer patients than in the group of workers with the highest exposure-intensity index. Moreover, there was a strong correlation between the Papanicolau grade of exfoliated urothelial cells and the expression

level or the quantity of mutant p53 protein for the higher benzidine-exposure category (Shen *et al.*, 2005).

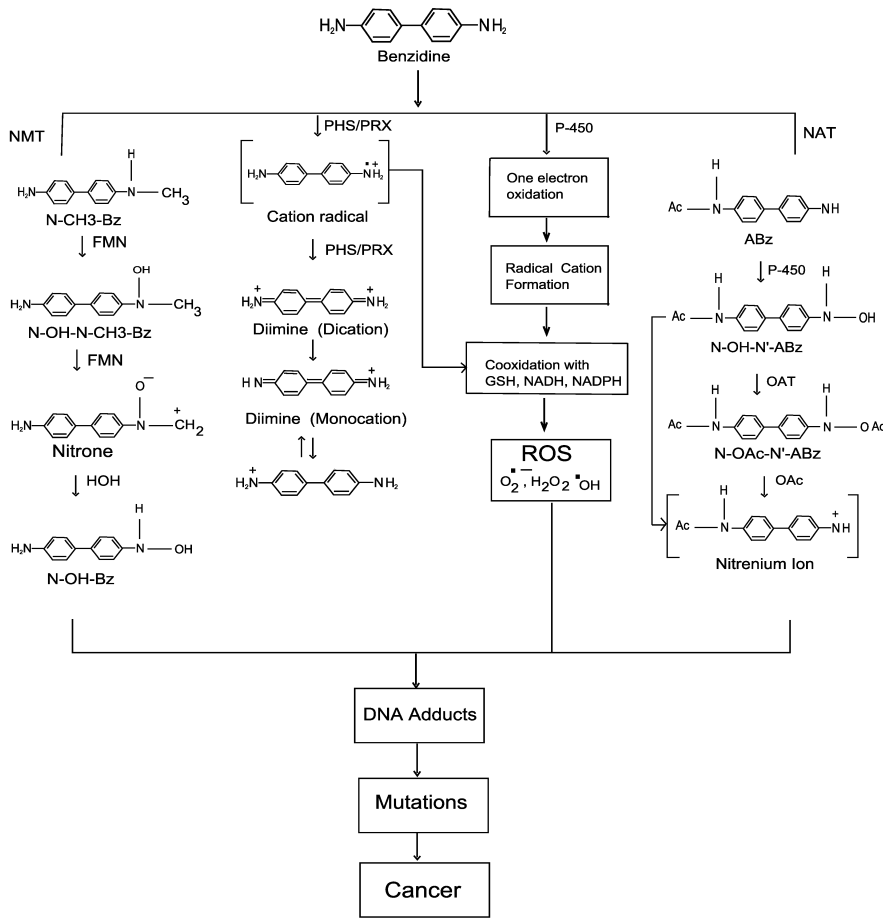
To investigate the expression of mutant p53 protein in relation to benzidine exposure, Xiang *et al.* (2007) analysed mutant p53 protein by use of an immuno-polymerase chain reaction (immuno-PCR) method in the serum of 331 healthy benzidine-exposed workers, while classifying exfoliated urothelial cells in the urine of these workers with Papanicolau's grading (PG). When the workers were divided according to benzidine-exposure level, the amounts of mutant p53 protein in the mid- and high-exposure groups were significantly higher than in the low-exposure group, and also significantly higher in the PG-II and PG-III groups than in the PG-I group (Xiang *et al.*, 2007).

Wu and Heng (2006) found DNA lesions in exon 7 of the *Tp53* gene in rats treated intraperitoneally with benzidine, its major targets being bladder, liver and lung. This suggests that the toxicity of benzidine is probably related to damage in the *Tp53* gene (Wu & Heng, 2006).

4.3 Mechanistic considerations

Benzidine metabolism has been extensively studied (Whysner *et al.*, 1996, Zenser *et al.*, 2002). Pathways involved in benzidine-initiated bladder cancer include the following: benzidine is *N*-acetylated to *N*-acetylbenzidine, which can be *N*-glucuronidated or *N*-hydroxylated in the liver (Zenser *et al.* 2002). *N*-Glucuronides of acetylbenzidine or *N′*-OH-*N*-acetylbenzidine can be transported by the blood and filtered by the kidneys, leading to their accumulation in urine within the lumen of the bladder. The *N*-Glucuronides are acid-labile and are converted back to *N*-acetylbenzidine or *N′*-OH-*N*-acetylbenzidine in acidic urine. Note that while the *N*-glucuronide of acetylbenzidine has an estimated half-life of 7.5 minutes, that for the hydroxylated acetyl-derivative is 3.5 hours. (Babu *et al.* 1995). Thus, *N*-acetylbenzidine is more likely to be hydrolysed than *N′*-OH-*N*-acetylbenzidine during a short transit time of urine in bladder. Within bladder cells, *N′*-OH-*N*-acetylbenzidine could react directly with DNA or, following conversion to the *N*-acetoxy derivative by *N,O*-acetyltransferase, form the dGp-acetylbenzidine adduct. *N*-acetyl-benzidine will require further activation before it can bind to DNA and form this adduct. This activation could involve *N*-oxidation by CYP and/or prostaglandin H synthase (Lakshmi *et al*. 1998). The dGp-acetylbenzidine adduct initiates carcinogenesis by producing mutations that become fixed in the genome and eventually contribute to tumour formation. *N*-Acetylation is both an activation (forming *N*-acetylbenzidine) and inactivation (forming *N,N′*-diacetyl-benzidine) reaction. dGp-acetylbenzidine can be formed from acetylbenzidine by several different enzymatic pathways. Thus, benzidine-induced initiation of bladder cancer is complex, involving multiple organs (i.e. liver, kidney, and bladder) and metabolic pathways (i.e. *N*-acetylation, *N*-glucuronidation and *N*-oxidation by CYP and/or peroxidation) (see Figure 4.1).

Figure 4.1. Metabolic pathways involved in the bladder cancer initiation by benzidine



Adapted from Makena and Chung, 2007

ABz, N-acetylbenzidine; Ac, Acetyl; Bz, Benzidine; P-450, cytochrome P-450; FMN, flavin monooxygenase; GSH, glutathione; NAD(P)H, nicotinamide adenine dinucleotide (phosphate); NAT, N-acetyltransferase; N-CH3-Bz, N-methylbenzidine; N-OAc-N'-ABz, N-acetoxy-N'-acetylbenzidine; N-OH-Bz, N-hydroxybenzidine; N-OH-N'-ABz, N-hydroxy-N'-acetylbenzidine; N-OH-N-CH3Bz, N-hydroxy-N-methylbenzidine; NMT, N-methyltransferase; OAc, acetoxy; OAT, O-acetyltransferase; PHS, prostaglandin H synthetase; PRX, peroxydase; ROS, reactive oxygen species

The results obtained in recent years are compatible with what could be expected from the present understanding of the mode of action of aromatic amines. However, there are still some unanswered questions. Why does benzidine not produce bladder tumours in the rat in contrast to several other species? Although the data are in favour of a genotoxic mechanism, not all species-specific differences can be explained by metabolic activation (Whysner *et al.*, 1996). Interestingly, little is known about the acute toxicity of benzidine, except that it does not stimulate but rather inhibits cell proliferation.

One of the most advanced approaches uses quantitative single-cell proteomics to select biomarkers of effect and to develop profiles of the sequence of events in a complex network of signalling pathways in bladder cancer (Hemstreet & Wang, 2004). A comprehensive view was obtained of the alterations induced by reactive metabolites of 4-aminobiphenyl by gene-expression profiles in human lymphoblastoid TK6 cells. The activity of 2250 genes was altered by treating these cells with *N*-hydroxy-acetylaminobiphenyl. Gene-expression patterns have been linked in this way to phenotypic markers, such as DNA-adduct levels, toxicity and mutagenicity. So far, the results tell us something about the complexity of the responses of a cell exposed to a non-physiological agent, which should caution against searching for monocausal explanations and pathways (Ricicki *et al.*, 2006, Srinivas *et al.*, 2001). The gene-expression profile in livers from mice fed *N*-2-acetylaminofluorene in combination with partial hepatectomy showed that from 2304 cDNA clones 69 were upregulated in comparison with the expression seen after partial hepatectomy alone. The increased gene expression may be associated with the activation of oval cells (Arai *et al.*, 2004).

4.4 Susceptibility

Taking into account that extensive epidemiological studies have indicated a relationship between bladder cancer in populations exposed to arylamines and the slow phenotype for their acetylation, the knowledge of the human acetylator phenotype may be a useful indicator of possible risk for bladder cancer due to exposure to these chemicals. Within the same human liver preparations, benzidine and sulfamethazine acetylation were directly and significantly correlated ($r = 0.672$; $P < 0.05$) (Peters *et al.*, 1990).

In rat-liver slices incubated with [3H]-labelled benzidine, *N,N′*-diacetylbenzidine represented 73 ± 2.5% of the total radioactivity recovered by HPLC, *N*-acetylbenzidine represented 8.8 ± 3.6%, while no unmetabolized benzidine was observed. In human liver slices, benzidine, *N*-acetylbenzidine, and *N,N′*-diacetylbenzidine represented 19 ± 5%, 34 ± 4% and 1.6 ± 0.5%, respectively. Thus in human liver slices the formation of *N*-acetylbenzidine rather than *N,N′*-diacetylbenzidine is favoured. Individuals with rapid *N*-acetyltransferase 2 (*NAT2*) genotypes formed 1.4-fold more *N*-acetylbenzidine than did slow acetylators, but this increase was not significant. These data suggest that in humans the enzyme deacetylase influences hepatic metabolism of benzidine and its subsequent

carcinogenic effects more than *N*-acetyltransferase, and helps to explain the species- and organ-specificity of benzidine-induced carcinogenesis (Lakshmi *et al.*, 1995a).

According to a cross-sectional study among 33 workers exposed to benzidine and 15 unexposed controls (Rothman *et al.*, 1996a), four benzidine-related DNA adducts were significantly elevated in the exfoliated urothelial cells of exposed workers compared with controls. The predominant adduct co-chromatographed with *N*-(3′-phosphodeoxyguanosin-8-yl)-*N*′-acetylbenzidine and it was the only adduct significantly associated with total urinary benzidine metabolites ($r = 0.68$; $P < 0.0001$). This supports the concept that monofunctional acetylation is an activation rather than a detoxification step for benzidine. Almost all benzidine-related metabolites measured in the urine of exposed workers were acetylated among slow acetylators as well as rapid acetylators (95 ± 1.9% *vs* 97 ± 1.6%), and NAT2 activity did not affect the levels of any DNA adduct measured; it is thus unlikely that inter-individual variations in NAT2 function are relevant for benzidine-associated bladder carcinogenesis.

The glutathione *S*-transferase M1-null (*GSTM1-null*) genotype had no impact on DNA adducts in urothelial cells and urinary mutagenicity levels in workers currently exposed to benzidine, and GSTM1 did not conjugate benzidine or its metabolites. These results led to the conclusion that the *GSTM1-null* genotype does not have an impact on bladder cancer caused by benzidine. This is in contrast to studies in the general population suggesting that subjects with the *GSTM1-null* genotype are at a higher risk for bladder cancer (Rothman *et al.*, 1996b).

Studies designed to assess the metabolism of benzidine and *N*-acetylbenzidine by the *N*-acetyltransferases NAT1 and NAT2, conducted with human recombinant NAT1 and NAT2 and human liver slices, indicated that benzidine and *N*-acetylbenzidine are substrates of NAT1. *N*-acetylation of benzidine and *N*-acetylbenzidine did not correlate with the *NAT2* genotype. A higher average acetylation ratio was observed in human liver slices possessing the *NAT1*10* compared with the *NAT1*4* allele, suggesting that *NAT1* may exhibit a polymorphic expression in human liver (Zenser *et al.*, 1996).

The results of studies performed to assess the role of *GSTP1* polymorphism in the development of benzidine-related bladder cancer (Ma *et al.*, 2003) indicated that carriers of the *GSTP1 AG* or *GC* genotypes are found more frequently, but not to a significant extent (OR = 1.95; 95% CI 0.70–5.46), among benzidine-exposed bladder-cancer patients than in benzidine-exposed workers without known disease. Significant differences were found between all benzidine workers without known disease and all workers with known disease with respect to the degree of changes in exfoliated urothelial cells. These findings show the existence of an association between the *GSTP1 AG* or *GC* genotype and higher cytological gradings of exfoliated urothelial cells from formerly benzidine-exposed workers.

Inflammation and infection may play an important role in the activation of benzidine. As a matter of fact, reactive nitrogen/oxygen species (RNOS), which are components of the inflammatory response, were found to react with benzidine forming azo-benzidine.

Glutathione prevented the RNOS-mediated transformation of benzidine (Lakshmi *et al.*, 2003).

A study to evaluate the influence of urinary pH on the levels of free benzidine and *N*-acetylbenzidine and on DNA adducts in urothelial cells (Rothman *et al.*, 1997) demonstrated that individuals with urine at pH < 6 had tenfold higher DNA-adduct levels than did individuals with urine at pH ≥ 7. The pH of the urine was inversely correlated with the proportion of benzidine ($r = -0.78$; $P < 0.0001$) and *N*-acetylbenzidine ($r = -0.67$; $P < 0.0001$) present as free components.

N′-(3′-monophospho-deoxyguanosin-8-yl)-*N*-acetylbenzidine was the major adduct detected in bladder cells from workers exposed to benzidine, and an inverse relationship was observed for the pH of the urine and levels of this adduct, as well as for urinary pH and levels of free (unconjugated) benzidine and *N*-acetylbenzidine (Zenser *et al.*, 1998).

5. Summary of Data Reported

5.1 Exposure data

Benzidine has been used for over a century, mainly for the production of azo dyes and as a rubber-compounding agent. 3,3′-Dimethylbenzidine (*ortho*-tolidine) is produced mainly as an intermediate for dyes and pigments but also for manufacturing polyurethane-base elastomers. 3,3′-Dichlorobenzidine is used primarily in the production of yellow, and some red and orange pigments for the printing ink, textile, paper, paint, rubber, plastic, and related industries. It also has application as a compounding ingredient for rubber and plastics. 3,3′-Dichlorobenzidine is also used with 4,4’-methylenebis(2-chloroaniline) (MOCA) as a curing agent for polyurethane elastomers. 3,3′-Dimethoxybenzidine (*ortho*-dianisidine) is used almost exclusively for the production of azo dyes and azo pigments.

Benzidine and its congeners are not known to occur naturally. Occupational exposure occurs during their production and use. Only studies for benzidine itself are available. Airborne concentrations in the workplace reached maximum values of 6 mg/m^3, measured in Russia in 1947–1948. In more recent studies, from China (1962–1970) and the Republic of Korea (1998), the maximum values were 1.18 and 0.65 mg/m^3, respectively.

Since benzidine-based dyes are known to be metabolized to benzidine, exposure studies in workers have measured the benzidine concentration in urine. The highest reported value was 56 mg/L (Russia 1937–1938). In a dye-manufacturing industry in India, values up to 0.36 mg/L were measured.

The general population can be exposed when living near factories or disposal sites, through plant effluents or groundwater contamination. An additional source of exposure is the use of consumer products containing benzidine- and congener-based dyes, which can be contaminated with the respective amine, and also via uptake of the dyes from those

products and ensuing metabolization. The manufacturing of benzidine is now prohibited in the EU and several other countries, e.g. Japan, the Republic of Korea, Canada and Switzerland.

5.2 Human carcinogenicity data

Many case reports and cohort studies have shown that occupational exposure to benzidine increases the risk for cancer of the urinary bladder among workers in various countries. The studies show consistent positive associations with some indication of dose–response relationships.

In addition, studies that have documented a decreasing bladder-cancer risk in occupational cohorts after removing exposures to benzidine support a causal interpretation of the observed association between benzidine exposure and bladder cancer.

5.3 Animal carcinogenicity data

Benzidine or its dihydrochloride salt was tested in mice, rats, hamsters and dogs by oral administration, in mice and rats by subcutaneous administration and in rats by inhalation and intraperitoneal injection. Following oral administration to newborn and adult mice of different strains and of both sexes, it significantly increased the incidence of benign and malignant liver tumours. In female rats, it markedly increased the incidence of mammary tumours; in male and female hamsters, it increased the incidence of liver tumours; and in dogs it produced bladder tumours. The subcutaneous administration of benzidine or its sulfate to mice produced significant increases in the incidence of benign and malignant liver tumours. In rats, benzidine produced a high incidence of Zymbal-gland tumours; colonic tumours were also reported. The intraperitoneal administration of benzidine to rats resulted in a marked increase in the incidence of mammary and Zymbal-gland tumours. Studies in fish, rabbits and frogs could not be evaluated. The results of the inhalation study in rats could not be interpreted.

3,3′-Dichlorobenzidine or its dihydrochloride salt was tested in mice, rats, hamsters and dogs by oral administration, in mice by transplacental exposure and in mice and rats by subcutaneous administration. When administered in the diet, 3,3′-dichlorobenzidine induced hepatomas in male mice, granulocytic leukaemia and Zymbal-gland carcinomas in male rats, mammary adenocarcinomas in rats of both sexes, and transitional cell carcinomas of the urinary bladder and hepatocellular carcinomas in female dogs. When administered by transplacental exposure, 3,3′-dichlorobenzidine increased the incidence of lymphoid leukaemia in mice. A feeding study in hamsters and the studies with subcutaneous administration in mice and rats could not be evaluated.

3,3′-Dimethoxybenzidine was tested in mice, rats and hamsters by oral administration. When given by stomach intubation to both male and female rats, 3,3′-dimethoxybenzidine caused tumours at various sites, including the Zymbal gland, the

intestine (carcinoma), skin (carcinoma), and urinary bladder (papilloma). When the dihydrochloride salt of 3,3′-dimethoxybenzidine was administered in the drinking-water to male and female rats, increased incidences of Zymbal-gland tumours (adenoma and carcinoma), liver neoplasms and tumours (neoplastic nodules and hepatocellular carcinoma), large intestine tumours (adenomatous polyps and adenocarcinoma), skin tumours (basal-cell adenoma and carcinoma), and oral cavity tumours (squamous-cell papilloma and carcinoma) were observed. Male rats also had increased incidences of tumours of the preputial gland, the small intestine (adenocarcinoma), and mesothelioma, and female rats had increased incidences of tumours of the clitoral gland (adenoma and carcinoma), mammary gland (adenocarcinoma), and uterus or cervix (adenoma and carcinoma). A feeding study in hamsters could not be evaluated.

3,3′-Dimethylbenzidine was tested in mice, rats and hamsters by oral administration and in rats by subcutaneous administration. Oral exposure of mice of both sexes to 3,3′-dimethylbenzidine in the drinking-water as the dihydrochloride salt caused increased incidences of lung tumours (alveolar-cell adenomas and adenocarcinomas). Oral exposure of rats of both sexes to 3,3′-dimethylbenzidine in the drinking-water as the dihydrochloride salt increased the incidence of Zymbal-gland tumours (adenomas and carcinomas), liver tumours (neoplastic nodules or hepatocellular carcinomas), large intestine tumours (adenomatous polyps or adenocarcinomas), skin tumours (basal cell adenomas and carcinomas), and oral cavity tumours (squamous cell papillomas and carcinomas) in both males and females; preputial gland tumours (carcinomas), small intestine tumours (adenocarcinomas) and lung tumours in males; and clitoral gland tumours (adenomas and carcinomas) and mammary gland tumours (adenocarcinomas) in females. In rats, subcutaneous injection of 3,3′-dimethylbenzidine caused significant increases in Zymbal-gland tumours in both sexes and skin, preputial gland and forestomach tumours in males and mammary gland tumours in females. A feeding study in hamsters could not be evaluated.

5.4 Other relevant data

Pathways involved in benzidine-initiated bladder cancer include the following steps in human metabolism: benzidine is *N*-acetylated to acetylbenzidine, which can be *N*-glucuronidated or *N*-oxidized in the liver. *N*-Glucuronides of acetylbenzidine or *N*-hydroxyacetylbenzidine can be transported by the blood and filtered by the kidneys, which results in accumulation in urine within the lumen of the bladder. *N*-Glucuronides are acid-labile and could be converted to acetylbenzidine or *N*′-hydroxy-*N*-acetyl-benzidine in acidic urine. Note that while the *N*-glucuronide of acetylbenzidine has an estimated half-life of 7.5 minutes, that for *N*′-hydroxy-*N*-acetyl-benzidine is 3.5 hours at pH 5.5. Thus, acetyl-benzidine is more likely to be hydrolysed than *N*′-hydroxy-*N*-acetyl-benzidine during a short transit time of urine in the bladder. Within bladder cells, *N*′-hydroxy-*N*-acetylbenzidine could react directly with DNA, or following conversion to the

N-acetoxy derivative by *O*-acetyltransferases, form the adduct *N*-(deoxyguanosin-8-yl)-*N*′-acetylbenzidine (dGp-acetylbenzidine). Acetyl-benzidine requires further activation before it can bind DNA and form this adduct. This activation could involve *N*-oxidation by cytochrome P450 (CYP) enzymes and/or prostaglandin H synthase. The adduct dGp-acetylbenzidine initiates carcinogenesis by producing mutations that become fixed in the genome and eventually contribute to tumour formation. Levels of this DNA adduct in human peripheral white blood cells correlate with those in exfoliated bladder cells. Benzidine can be metabolized to 4-aminobiphenyl and form haemoglobin adducts in rats. Thus, initiation of bladder cancer by benzidine is complex, involving multiple organs (i.e. liver, kidney, and bladder) and metabolic pathways (i.e. *N*-acetylation, *N*-glucuronidation and *N*-oxidation by CYP enzymes or peroxidation). Uroepithelial cells contain substantial prostaglandin H synthase activity along with bladder infiltration with polymorphonuclear leukocytes. Myeloperoxidase activity, an index of infiltration with neutrophils, has also been observed. Both peroxidases could contribute to the activation of acetylbenzidine in bladder epithelium.

Conjugates of benzidine and free benzidine, 3,3′-dimethylbenzidine and 3,3′-dimethoxybenzidine have been measured in urine of workers exposed to benzidine-based azo dyes, and more specifically to Direct Black 38. One study reported formation of haemoglobin adducts derived from benzidine, acetylbenzidine, 4-aminobiphenyl and aniline in workers exposed to Direct Black 38. Likewise, studies in rhesus monkeys, Syrian golden hamsters, dogs and rats exposed to various dyes based on benzidine, 3,3′-dimethylbenzidine and 3,3′-dimethoxybenzidine, e.g., Direct Black 38, Direct Blue 6 and Direct Brown 95, consistently show the presence of the free amines or acetylated amines in the urine. In addition, several studies demonstrated anaerobic bacteria in the intestine of mice, rats and humans to be capable of cleaving the azo-linkage in the dyes, thereby liberating the amine.

Benzidine has been found mutagenic to *Salmonella* when tested in the presence of an exogenous metabolic system from rats as well as from humans. Also *N*-acetylbenzidine and *N*-hydroxy-*N*,*N*′-diacetylbenzidine, which are urinary metabolites of benzidine in the rat, were positive in *Salmonella* in the presence of an activation system. The urine of rats that received benzidine in the food was mutagenic to *Salmonella* in the presence of metabolic activation.

Benzidine consistently showed negative results in *E. coli* tests. Mutagenic activity in the X-linked recessive lethal assay and induction of mutations in RNA genes of *Drosophila* has been reported.

Benzidine was tested in many in-vitro assays in cultured mammalian cells; positive and negative results were observed in gene-mutation tests. Benzidine was active in tests for DNA fragmentation and induction of sister chromatid exchange in cultured human and animal cells; benzidine was generally active in inducing unscheduled DNA synthesis in cultured hepatocytes of rats.

In-vivo tests in animals showed conflicting results with respect to the ability of benzidine to induce micronuclei in polychromatic erythrocytes, but several studies demonstrated micronucleus induction in mice treated orally with a wide range of doses (150 to 900 mg/kg bw).

When benzidine was administered to pregnant female mice, no significant increase in the micronucleus frequency was observed in the liver of the fetuses. Positive results, however, were reported in a different study, in which the frequency of micronucleated polychromatic erythrocytes in the liver was found increased.

There are data on genotoxic effects of benzidine in workers of a manufacturing plant in Bulgaria, who were exposed to benzidine or benzidine-based dyes. A statistically significant (ten-fold) increase in the number of circulating peripheral lymphocytes with chromosomal aberrations was observed in exposed workers. The highest frequencies of aberrant lymphocytes were associated with the highest levels of exposure and correlated with the concentrations of benzidine found in urine. Also, mutant p53 protein was increased in workers exposed to benzidine.

6. Evaluation

6.1 Cancer in humans

There is *sufficient evidence* in humans for the carcinogenicity of benzidine. Benzidine causes bladder cancer in humans.

6.2 Cancer in experimental animals

There is *sufficient evidence* in experimental animals for the carcinogenicity of benzidine.

There is *sufficient evidence* in experimental animals for the carcinogenicity of 3,3′-dichlorobenzidine.

There is *sufficient evidence* in experimental animals for the carcinogenicity of 3.3′-dimethoxybenzidine.

There is *sufficient evidence* in experimental animals for the carcinogenicity of 3,3′-dimethylbenzidine.

6.3 Overall evaluation

Benzidine is *carcinogenic to humans (Group 1)*.

7. References

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