Table 3-2. Levels of Significant Exposure to Benzidine - Oral

Key to[a] figure	Species/ (Strain)	Exposure/ Duration/ Frequency (Specific Route)	System	NOAEL (mg/kg/day)	LOAEL Less Serious (mg/kg/day)	LOAEL Serious (mg/kg/day)	Reference
	ACUTE EXPOSURE						
	Death						
1	Rat (NS)	NS (G)				309 (LD_{50})	DOT 1972
2	Mouse (NS)	NS (G)				214 (LD_{50})	DOT 1972
	INTERMEDIATE EXPOSURE						
	Systemic						
3	Rat (Sprague-Dawley)	6 wk (W)	Hepatic		5.3 (bile duct hyperplasia)		DePass and Morris 1981
			Renal		5.3 (proteinaceous casts in tubules)		
			Bd Wt			5.3 (31% decrease in weight gain)	
4	Mouse (BALB/c)	6 wk (W)	Hepatic	32			DePass and Morris 1982
			Bd Wt	32			
	Cancer						
5	Rat (Sprague-Dawley)	9 mo 1x/3d (GO)				3.4 F (CEL: mammary carcinoma)	Griswold et al. 1968

Table 3-2. Levels of Significant Exposure to Benzidine - Oral (continued)

Key to[a] figure	Species/ (Strain)	Exposure/ Duration/ Frequency (Specific Route)	System	NOAEL (mg/kg/day)	LOAEL Less Serious (mg/kg/day)	LOAEL Serious (mg/kg/day)	Reference
	CHRONIC EXPOSURE						
	Systemic						
6	Mouse (BALB/c) M (C57BL/6) F	Lifetime (W)	Hemato	7.2	10.7 (hemosiderosis of the spleen)		Littlefield et al. 1983, 1984
			Hepatic	3.7 F	5.6 F (bile duct hyperplasia)		
			Bd Wt			1.8 F (28% reduction in body weight gain)	
7	Mouse (BALB/c) M (C57BL/6) F	40-80 wk (W)	Bd Wt		2.5 (decreased body weight gain greater than 10%)		Schieferstein 1982
8	Dog (NS)	5 yr 6d/wk (C)	Renal		17.6 (recurrent cystitis)		Spitz et al. 1950
	Neurological						
9	Mouse (BALB/c) M (C57BL/6) F	Lifetime (W)				1.8 F (spongiform leukoencephalopathy)	Morgan et al. 1981; Littlefield et al. 1983
	Reproductive						
10	Mouse (BALB/c) M (C57BL/6) F	lifetime				7.2 F (atrophy of the uterus)	Littlefield et al. 1983
	Cancer						
11	Mouse (BALB/c) M (C57BL/6) F	Lifetime (W)				1.8 F (CEL: Harderian gland adenoma)	Littlefield et al. 1983
12	Mouse (BALB/c) M (C57BL/6) F	Lifetime (W)				1.8 F (CEL: liver carcinomas)	Littlefield et al. 1984

Table 3-2. Levels of Significant Exposure to Benzidine - Oral (continued)

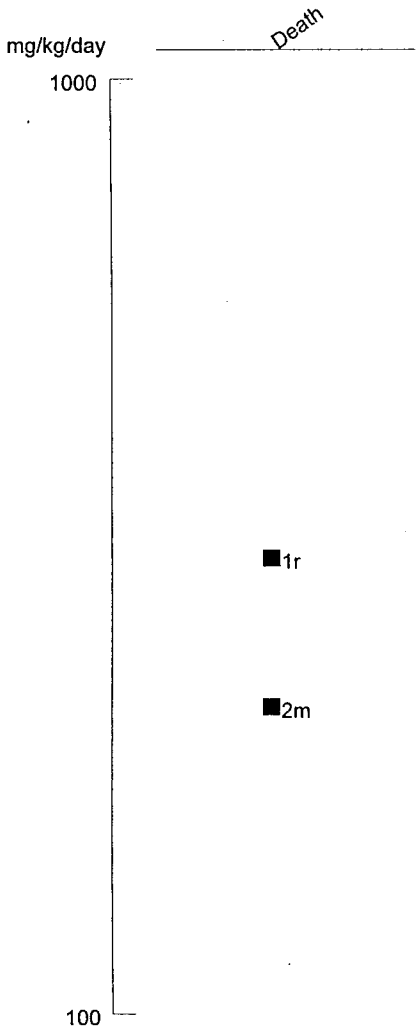
Key to[a] figure	Species/ (Strain)	Exposure/ Duration/ Frequency (Specific Route)	System	NOAEL (mg/kg/day)	LOAEL			Reference
					Less Serious (mg/kg/day)	Serious (mg/kg/day)		
13	Mouse (BALB/c) M (C57B/6) F	40-80 wk (W)				2.5 F	(CEL: liver adenomas, carcinomas)	Nelson et al. 1982
14	Hamster (NS)	Lifetime (F)				61	(CEL: bile duct and liver adenomas, carcinomas)	Saffiotti et al. 1967
15	Dog (NS)	5 yr 6d/wk (C)				24.2	(CEL: bladder carcinomas)	Spitz et al. 1950

[a]The number corresponds to entries in Figure 3-2.

Bd Wt = body weight; (C) = capsule; CEL = cancer effect level; d = day(s); (F) = feed; F = female; (G) = gavage; (GO) = gavage in oil; Hemato = hematological; kg = kilogram(s); LD_{50} = lethal dose, 50% kill; LOAEL = lowest-observed-adverse-effect level; M = male; mg = milligram(s); mo = month(s); NOAEL = no-observed-adverse-effect level; NS = not specified; (W) = water; wk = week(s); x = times; yr = year(s)

Figure 3-2. Levels of Significant Exposure to Benzidine - Oral

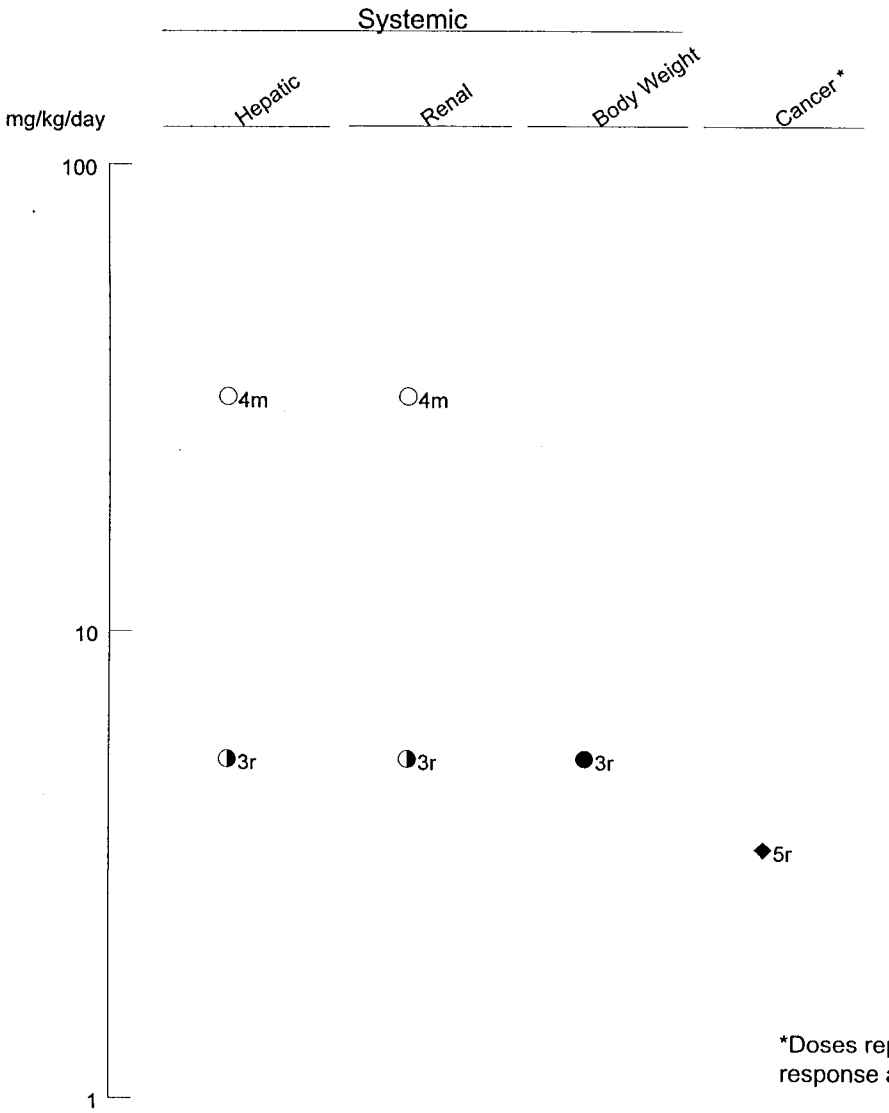
Acute (≤14 days)



c-Cat	-Humans	f-Ferret	n-Mink	◆ Cancer Effect Level-Animals	▼ Cancer Effect Level-Humans	■ LD50/LC50
d-Dog	k-Monkey	j-Pigeon	o-Other	● LOAEL, More Serious-Animals	▲ LOAEL, More Serious-Humans	Minimal Risk Level for effects other than Cancer
r-Rat	m-Mouse	e-Gerbil		◑ LOAEL, Less Serious-Animals	◭ LOAEL, Less Serious-Humans	
p-Pig	h-Rabbit	s-Hamster		○ NOAEL - Animals	△ NOAEL - Humans	
q-Cow	a-Sheep	g-Guinea Pig				

Figure 3-2. Levels of Significant Exposure to Benzidine - Oral (*continued*)

Intermediate (15-364 days)

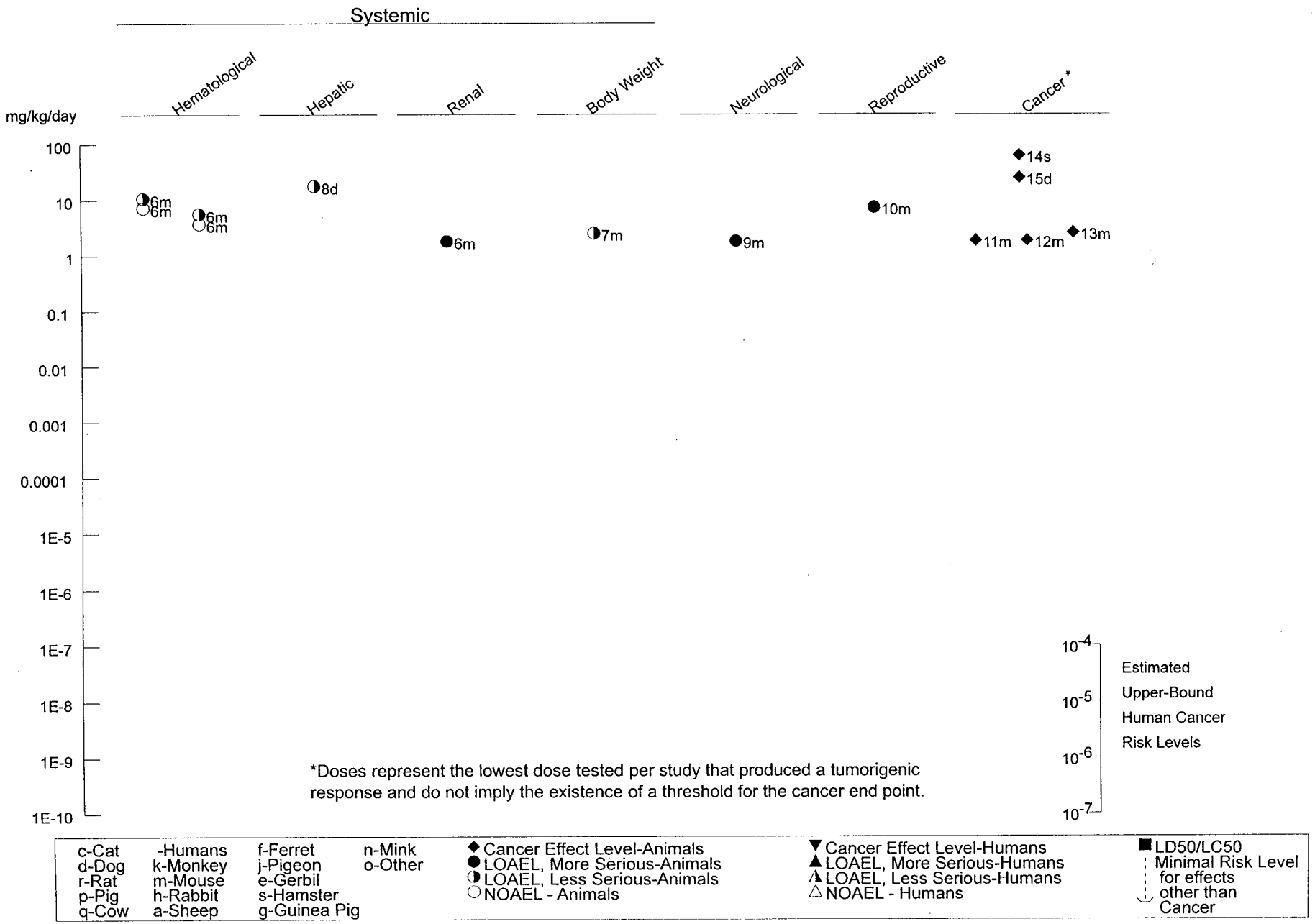


*Doses represent the lowest dose tested per study that produced a tumorigenic response and do not imply the existence of a threshold for the cancer end point.

c-Cat	-Humans	f-Ferret	n-Mink	◆ Cancer Effect Level-Animals	▼ Cancer Effect Level-Humans	■ LD50/LC50
d-Dog	k-Monkey	j-Pigeon	o-Other	● LOAEL, More Serious-Animals	▲ LOAEL, More Serious-Humans	Minimal Risk Level for effects other than Cancer
r-Rat	m-Mouse	e-Gerbil		◑ LOAEL, Less Serious-Animals	◭ LOAEL, Less Serious-Humans	
p-Pig	h-Rabbit	s-Hamster		○ NOAEL - Animals	△ NOAEL - Humans	
q-Cow	a-Sheep	g-Guinea Pig				

Figure 3-2. Levels of Significant Exposure to Benzidine - Oral (*continued*)

Chronic (≥365 days)



pigment in the spleen was found in significant amounts for both sexes of the two crosses examined (F_1 hybrid of C57BL and BALB/C3H strains and subsequent monohybrid cross) at the 40- and 60-week terminations. The increase in the level of pigment was also significant at the 80-week termination in the males. Erythropoiesis in the spleen was significantly increased in females (but not males) for both strains at the 60-and 80-week terminations. The doses at which these effects were first observed were not reported. In another mouse study, macrophages containing hemosiderin pigment were observed in the spleen after lifetime exposures to benzidine at doses of 10.7–12.0 mg/kg/day, but not at 0–7.2 mg/kg/day (Littlefield et al. 1983, 1984). Though typical hematological examinations were not conducted in these studies, the results suggest that benzidine may be capable of damaging erythrocytes, with a resulting deposit of hemosiderin in the spleen.

Hepatic Effects. No studies were located regarding hepatic effects in humans after oral exposure to benzidine.

Cirrhosis of the liver was found in rabbits (perhaps also in dogs, but this was not clearly indicated) orally exposed to approximately 13 or 26 mg/kg/day of benzidine, 1 day/week over a period of 20–128 days (Oida 1958a, 1958b). However, five of the six rabbits died during the study and the cause of death was not reported. Also, no controls were used. This study is not considered reliable for the assessment of hepatic effects and, therefore, is not included in Table 3-2. Cirrhosis of the liver was observed in another rabbit study 8 months following treatment with benzidine (0.5–7.0 grams total dose over periods of up to 3.5 years) (Bonser 1959). The cirrhosis was frequently associated with changes in bile duct tissue. No further details were provided in this abstract; thus, this report is also not included in Table 3-2. Bile duct hyperplasia was observed in rats administered approximately 5.3 mg benzidine/kg/day in the drinking water for 6 weeks (De Pass and Morris 1981). Bile duct hyperplasia was also observed in a lifetime mouse study (Littlefield et al. 1983) at benzidine doses of 5.6–12.0 mg/kg/day, but not at lower doses of 0–3.7 mg/kg/day. The effect was more prevalent in females (21% at 10.7 mg/kg/day) than in males (8% at 12 mg/kg/day). The authors hypothesized about a possible hormone-mediated component of the response. No morphological alterations were observed in the livers from mice administered approximately 32 mg benzidine/kg/day in the drinking water for 6 weeks (DePass and Morris 1982).

Renal Effects. No studies were located regarding renal effects in humans after oral exposure to benzidine.

Nephrosis, nephritis, hematuria, and proteinuria were reported in rabbits orally exposed to approximately 13 or 26 mg/kg/day of benzidine, 1 day/week over a period of 20–128 days (Oida 1958a, 1958b). However, five of the six rabbits died during the study and the cause of death was not reported. Also, no controls were used. This study is not considered reliable for the assessment of renal effects and, therefore, is not included in Table 3-2. Proteinaceous casts were observed in the renal tubules from rats dosed with approximately 5.3 mg benzidine/kg/day in the drinking water for 6 weeks (DePass and Morris 1981). Although not statistically significant ($p<0.10$), Nelson et al. (1982) observed proteinaceous casts in the kidneys of F_1 hybrid (C57BL x BALB/c) female mice exposed to benzidine for 60–80 weeks, but the particular doses (between 0 and 26.2 mg/kg/day) causing the effect were not identified. Hematuria and proteinuria were reported in two dogs within 2–5 weeks of being orally exposed to 7.9 mg/kg/day of benzidine, 1 day/week over a period of 20–128 days (Oida 1958a, 1958b). Even though no other visible signs of toxicity were noted, recurrent episodes of cystitis were observed in the bladders of seven dogs fed benzidine for 5 years (Spitz et al. 1950). The dogs were exposed by capsule to doses of approximately 17.6 mg/kg/day for the first 15 months, then 26.4 mg/kg/day for the remaining 45 months. Histologically, focal collections of lymphocytes were observed, often forming follicles. There was no notable reaction of the mucosa, and the lesions improved without therapy.

Body Weight Effects. No studies were located regarding body weight effects in humans after oral exposure to benzidine.

No significant treatment-related changes in body weight were reported in mice administered approximately 32 mg benzidine/kg/day in the drinking water for 6 weeks (DePass and Morris 1982). Schieferstein (1982) reported decreased body weight gain (greater than 10%) in mice exposed to benzidine at 2.5 mg/kg/day in the drinking water for 80 weeks. Also, Littlefield et al. (1983, 1984) reported that mice administered benzidine in the water for 2 years at dose levels of about 1.8 mg/kg/day gained approximately 28% less weight than controls.

3.2.2.3 Immunological and Lymphoreticular Effects

No studies were located regarding immunological effects in humans after oral exposure to benzidine.

Two animal studies have been reported that address the immunotoxicity of benzidine following oral exposure. In a study by Luster et al. (1992), an unspecified number of female $B6C3F_1$ mice were exposed by gavage for 5 days to three unspecified doses of benzidine, and were then subjected to a battery of

immune response assays used to screen for potential immunotoxicity. Based on induced alterations in spleen/body weight ratio, natural killer cell activity, T-cell response to mitogen and mixed leukocyte antigens, and on a delayed hypersensitivity response, benzidine was classified as an immunotoxicant. Spleen cellularity, thymus/body weight ratio, number of B and T lymphocytes as quantified by cell surface markers, and number of splenic lymphocytes producing IgM antibody were not altered by benzidine exposure. Rao et al. (1971) reported hyperplasia of the lymphoid cells in the spleen and thymic cortex in mice exposed to benzidine dihydrochloride in the feed for 6 weeks; however, the lowest dose at which this effect occurred could not be determined from the study.

3.2.2.4 Neurological Effects

No studies were located regarding neurological effects in humans after oral exposure to benzidine.

Spongiform leukoencephalopathy (central nervous system damage resulting in vacuolization of white matter) was observed in mice after lifetime exposure to 20 ppm of benzidine dihydrochloride in drinking water (Littlefield et al. 1983; Morgan et al. 1981). These vacuoles may represent swollen glial cells and/or intramyelinic vacuoles between the larger axons. Using average weekly dose rate data provided by the authors in another publication concerning this study (Littlefield et al. 1984), 20 ppm was estimated to be equivalent to a benzidine dose of 1.8 mg/kg/day. The LOAEL value (1.8 mg/kg/day) from this study for neurological effects is recorded in Table 3-2 and plotted in Figure 3-2.

3.2.2.5 Reproductive Effects

No studies were located regarding reproductive effects in humans after oral exposure to benzidine. The only information regarding reproductive effects in animals is that from a lifetime study in mice, which reported a 31% incidence of atrophy of the ovaries in animals given benzidine in the drinking water at a dose level of approximately 7.2 mg/kg/day (80 ppm) compared to 11% in control mice (Littlefield et al. 1983).

3.2.2.6 Developmental Effects

In a 5-year retrospective study of residents living near a Superfund site contaminated with benzidine, β-naphthylamine, and benzene, no significant increase in incidence rate was detected for any of 37 most common birth defects (only 4 of which were observed), or in the rate for all combined birth defects

(Budnick et al. 1984). However, the authors noted that the number of births was small, and that birth defects are often under-reported on birth certificates. The extent of actual exposure (if any) to benzidine was not determined.

No studies were located regarding developmental effects in animals after oral exposure to benzidine.

3.2.2.7 Cancer

A retrospective study of residents who lived, during the 1970s near a Superfund site, that was contaminated with benzidine, β-naphthylamine, and benzene detected an excess of bladder cancer, leukemia, and other lymphomas, as well as cancers at several other sites (salivary gland, larynx, bone and jaw, uterus and chorion, rectum, and breast) (Budnick et al. 1984). The principal routes of potential exposure were not identified, but are likely to have included oral exposure to contaminated water and soil. The specific contribution of environmental benzidine (if any), or of possible occupational exposure to benzidine or other carcinogens, could not be ascertained. A retrospective study of Japanese kimono painters who were exposed to benzidine-based dyes (Direct Black 38, Direct Red 28, Direct Red 17, and Direct Green 1) by licking their brushes and spatulas indicated an increased risk for bladder tumors (Yoshida 1971; Yoshida and Miyakawa 1973). Of 200 painters who worked for up to 50 years, 17 (8.5%) contracted bladder cancer. When compared with 148 controls having urinary disease, this represented a relative risk of 6.8 for these cohorts. The average age-adjusted death rate from bladder cancer for the general population in Japan during 1965 was reported to be less than 3 deaths per 100,000 persons. Although direct exposure to benzidine was not described, these data are consistent with the concept that benzidine-based dyes may be metabolically reduced to free benzidine, and thus cause the same cancer effects.

A number of animal studies indicate that oral exposure to benzidine can increase the incidence of a variety of tumors. In female rats given 3.4 mg/kg/day once every 3 days for 30 days, 5 out of 10 animals developed mammary carcinomas within 9 months, compared to 5 out of 132 in the controls (Griswold et al. 1968). In hamsters fed either 0.1% benzidine base (61 mg/kg/day) or 0.1% benzidine dihydrochloride (equivalent to 44 mg/kg/day benzidine base) in the lifetime diet, malignant and benign multiple cholangiomas, hepatomas, and/or liver carcinomas were reported in more than 50% of the surviving hamsters (Saffiotti et al. 1967). No bladder pathology was found in these animals.

In a total of 13 rabbits fed a total dose of 0.5–7.0 grams of benzidine base in the diet for up to 3.5 years, one female developed a transitional cell carcinoma of the bladder after 2 years, while another female developed adenocarcinoma of the gallbladder with widespread metastasis at 6 years of age (Bonser 1959). The author concluded that two tumors occurring in organs of excretion in 13 rabbits is probably significant, and that it is possible that higher dosage levels would have caused more tumors. However, the incidence of tumors in control animals was not stated, making it difficult to assess the findings. No bladder tumors or other significant neoplasia were noted in rabbits intermittently given 13 or 26 mg/kg/day (50 or 100 mg weekly doses over 20–128 days; total doses of 0.1–1.4 grams) (Oida 1958a, 1958b). These reports were lacking in experimental details. The weekly dosing is difficult to evaluate and histopathological procedures were not adequately described, making the data of limited quantitative value.

Spitz et al. (1950) administered benzidine to 1 male and 6 female dogs by capsule for 60 months at a dose of 17.6 mg/kg/day (200 mg daily, 6 days/week for 15 months), followed by 26.4 mg/kg/day (300 mg daily, 6 days/week for 45 months). Dosing may have been suspended for brief periods because of recurring cystitis in dogs. One dog developed a urinary epidermoid carcinoma of the bladder at 19 months. It was later reported that three dogs (the longest survivors) of the Spitz et al. (1950) study developed papillomas and carcinomas of the bladder after 7, 8, and 10 years (Bonser 1959).

The most thorough animal study of oral carcinogenicity of benzidine was conducted at the National Center for Toxicological Research (NCTR) using two strains of male and female mice (a total of 3,456 animals) at five dose levels of benzidine dihydrochloride (Frith et al. 1979, 1980; Nelson et al. 1982; Schieferstein 1982). Doses (in water) were 30–400 ppm benzidine dihydrochloride (equivalent to benzidine doses of 2.5–26.2 mg/kg/day). The mice were sacrificed at 40, 60, and 80 weeks for pathological evaluations. Statistical analysis of the pathology data and the data pertaining to animal weights, water consumption, survival, and tumor incidences was performed. A series of publications resulted from this study. Hepatocellular alterations, hepatocellular adenomas, and hepatocellular carcinomas were all significantly elevated in one or more of the sex-strain combinations, even at the lowest dose level of 30 ppm (2.5 mg/kg/day). The primary target organ observed in the studies was the liver, where dose-response relationships could be observed. The data suggest that the lesions may first have appeared as foci of cellular alteration (acidophilic, basophilic, or vacuolated cellular foci), which may then have given rise to hepatocellular adenomas, which in turn progressed to hepatocellular carcinomas, the main cause of death among treated animals. These altered foci are thus considered preneoplastic lesions, an early stage in the progression from normal tissue to frank cancer.

Hepatocellular carcinomas were also observed in mice given as little as 20 ppm of benzidine dihydrochloride in drinking water for about 33 months (Littlefield et al. 1983, 1984). From experimentally derived average dose rates in mice (Littlefield et al. 1984), this equates to a dose of 1.8 mg/kg/day which is presented in Table 3-2. At this same dose, there was also a substantial increase in the fraction of mice displaying Harderian gland adenomas (18–24%) when compared with controls (4–8%). The response generally leveled off at the mid and high doses. Other tumors that occurred at significantly greater rates than in controls were angioma of the uterus, lung tumors, and reticulum cell sarcomas. This study also provided information on latency and sex and strain differences. Female mice were significantly more susceptible to these adverse effects than male mice, as was the genetically more homogeneous F_1 stock (BALB/c males x C57BL/6 females) when compared with the more heterogeneous F_2-monohybrid cross stock (F_1xF_1).

CEL values from each reliable study for cancer in each species and duration category are recorded in Table 3-2 and plotted in Figure 3-2.

EPA has derived an oral cancer potency factor of $2.3x10^{2}$ $(mg/kg/day)^{-1}$ using the linearized multistage model (EPA 1999a). This factor was calculated from inhalation exposure data reported by Zavon et al. (1973) regarding incidence of bladder tumors in humans occupationally exposed to benzidine. The lifetime average doses that would result in risks of $1x10^{-4}$, $1x10^{-5}$, $1x10^{-6}$, and $1x10^{-7}$ are $4.4x10^{-7}$, $4.4x10^{-8}$, $4.4x10^{-9}$, and $4.4x10^{-10}$ mg/kg/day, respectively, as indicated in Figure 3-2.

Several benzidine-congener-derived dyes during dye manufacture and dye use have been tested for possible carcinogenicity. The need for conducting such bioassays was based on the known carcinogenicity of benzidine in humans and the results of cancer epidemiology studies in Japanese kimono painters exposed to benzidine-derived dyes. Also, 3,3'-dimethylbenzidine and 3,3'-dimethoxybenzidine, benzidine congeners employed in the dye industry, were found to increase tumor incidence in rats (Haley 1975). Five chemicals were evaluated in 2-year carcinogenicity studies: 3,3'-dimethoxybenzidine dihydrochloride (benzidine congener), 3,3'-dimethylbenzidine dihydrochloride (benzidine congener), Direct Blue 15 (a representative 3,3'-dimethoxybenzidine-based dye), Acid Red 114 (a representative 3,3'-dimethylbenzidine-based dye), and Direct Blue 218 (a metallized 3,3'-dihydroxybenzidine-based dye) (NTP 1990, 1991a, 1991b, 1992, 1994). Rats and mice were used in the Direct Blue 218 study, and only rats were used in the other four bioassays. Direct Blue 218 was administered in the feed and the other chemicals in the drinking water. The following brief summary of the results regarding neoplastic lesions has been taken from Morgan et al. (1994). 3,3'-Dimethoxy-

benzidine, 3,3'-dimethylbenzidine dihydrochloride, Direct Blue 15, and Acid Red 114 all caused a similar spectrum of neoplastic lesions. The primary chemical-related neoplasms were tumors of the skin, Zymbal's gland, oral cavity epithelium, liver, preputial/clitoral glands, and intestines of both males and females. Direct Blue 218 induced an entirely different spectrum of neoplastic lesions. Tumor incidences were not as dramatic, and were seen primarily in the oral cavity epithelium of rats, and in the livers of mice. Morgan et al. (1994) noted that quantitative comparisons of the carcinogenic potencies of the chemicals other than Direct Blue 218 is difficult because of the lack of dose-response relationships, as well as differences in doses and durations of treatments.

3.2.3 Dermal Exposure

3.2.3.1 Death

No studies were located regarding death in humans or animals after dermal exposure to benzidine.

3.2.3.2 Systemic Effects

No studies were located regarding respiratory, cardiovascular, gastrointestinal, hematological, musculoskeletal, hepatic, renal, or ocular effects in humans or animals after dermal exposure to benzidine.

Dermal Effects. One human case of severe, recurrent, allergic eczematous dermatitis from benzidine use has been reported (Baer 1945), but data on exposure levels were not available. Of 4,600 patients tested over a 5 year period, 231 (5%) showed dermal sensitization to benzidine (Grimalt and Romaguera 1981). Occupational allergic contact dermatitis was clinically diagnosed in 88.5% (208 cases) of such dermally sensitized patients.

No studies were located regarding dermal effects in animals after dermal exposure to benzidine.

3.2.3.3 Immunological and Lymphoreticular Effects

As noted above, allergic contact dermatitis has been observed in workers occupationally exposed to benzidine (Baer 1945; Grimalt and Romaguera 1981). No other studies were located regarding immunological effects in humans or animals after dermal exposure to benzidine.

No studies were located regarding the following health effects in humans or animals after dermal exposure to benzidine:

3.2.3.4 Neurological Effects

3.2.3.5 Reproductive Effects

3.2.3.6 Developmental Effects

3.2.3.7 Cancer

No studies were located regarding the carcinogenic effects in humans or animals after dermal exposure to benzidine. Although dermal exposure was identified in many of the occupational studies, the relative contribution to the carcinogenic effect of benzidine could not be quantified.

3.3 GENOTOXICITY

The cytogenetic effects of occupational exposure to benzidine and benzidine-based dyes (Direct Black 38 and Direct Blue 6) were studied in workers at a manufacturing plant in Bulgaria having a recognized high risk of occupational cancer (Mirkova and Lalchev 1990). Twenty-three workers 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).

Several studies have addressed the *in vivo* genotoxicity of benzidine in animals following oral or parenteral exposure (Table 3-3). 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 benzidine by oral gavage (Mirkova and Ashby 1988). The number of micronucleated cells per 1,000 normal cells for test groups (5.75–8.75) was

Table 3-3. Genotoxicity of Benzidine *In Vivo*

Species (test system)	End point	Results	Reference
Nonmammalian cells:			
Drosophila melanogaster	Gene mutation	+	Fahmy and Fahmy 1977
D. melanogaster	Gene mutation	+	Graf et al. 1990
Mammalian cells:			
Rat	Micronucleus test	+	Urwin et al. 1976
Rat	Micronucleus test	–	Trzos et al. 1978
Rat	Micronucleus test	+	Cihak 1979
Mouse	Micronucleus test	+	Tice et al. 1990
Mouse	Micronucleus test	+	Mirkova and Ashby 1988
Mouse	Micronucleus test	+	Mirkova 1990
Mouse	Micronucleus test	–	Harper et al. 1989
Mouse	Micronucleus test	+	Sanderson and Clark 1994
Mouse	Chromosomal aberrations[a]	+	Talaska et al. 1987
Mouse	Chromosomal aberrations	+	Sinsheimer et al. 1992
Mouse	Chromosomal aberrations	+	Das et al. 1994
Mouse	DNA adduct formation	+	Phillips et al. 1990
Rat	DNA damage (alkaline elution)	+	Petzold and Swenberg 1978
Rat	DNA damage (alkaline elution)	+	Parodi et al. 1981
Rat	Unscheduled DNA synthesis	+	Ashby and Mohammed 1988
Rat	Unscheduled DNA synthesis	+	Ashby et al. 1990

[a]Liver cells evaluated in partially hepatectomized mice.

– = negative results; + = positive results; DNA = deoxyribonucleic acid

3 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) or with three consecutive daily doses (150 or 300 mg/kg), evidenced a positive dose response for bone marrow micronuclei induction (Mirkova 1990). Negative results, however, were reported for a different strain of mice (ICR) treated with single oral gavage doses of 100 or 200 mg benzidine/kg. No significant increases in micronucleated cells were observed 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 (Harper et al. 1989). Noting that adult liver is more sensitive than bone marrow to the chromosome damaging effects of benzidine, and that fetal liver can be more sensitive than adult liver for certain chemicals, the authors had speculated that micronuclei might be detectable in fetal liver. Lower doses, nonoptimum sampling time, and/or strain differences may have contributed to the negative findings in this study. When administrated by single oral gavage to male rats, 200 mg benzidine/kg induced unscheduled DNA synthesis in liver cells, which is a repair response to DNA damage (Ashby and Mohammed 1988; Ashby et al. 1990). In a study in mice, intraperitoneal administration of benzidine to pregnant dams increased the frequency of micronucleated polychromatic erythrocytes in the liver of fetuses, which suggested that benzidine (or metabolites) can cross the placenta (Sanderson and Clark 1993).

When tested in many *in vitro* assays (Table 3-4), benzidine has generally tested positive for reverse mutation in *Salmonella typhimurium* in the presence of exogenous metabolic activation (e.g., liver S-9) (Ames et al. 1973; Chung et al. 2000; Dorado and 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 and 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 (O'Donovon 1990; 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 and Caspary 1988). Benzidine has also tested positive for chromosome breaks (Swenberg et al. 1976) and sister chromatid exchange (Grady et al. 1986; Lindahl-Kiessling et al. 1989) in cultured human and animal cells; generally positive in cultured hepatocytes for unscheduled DNA synthesis (Kornburst and Barfknecht 1984a, 1984b; Steinmetz et al.1988; Williams 1978); positive for animal cell transformation (Ashby et al. 1978; Pienta 1980); and negative in cultured mammalian cells (but positive with calf thymus DNA) in the absence of exogenous activation for DNA adduct formation (Phillips et al. 1990).

Table 3-4. Genotoxicity of Benzidine *In Vitro*

Species (test system)	End point	Activation system	Results: With activation	Results: Without activation	Reference
Prokaryotic organisms:					
Salmonella typhimurium	Reverse mutation	Human S-9	–	No data	Phillipson and Ioannides 1983
S. typhimurium	Reverse mutation	Human Hep G2	+	No data	Duverger-van Bogaert et al. 1995
S. typhimurium	Reverse mutation	Human S-9	+	–	EPA 1978b
S. typhimurium	Reverse mutation	Human S-9	+	–	Haworth and Lawlor 1978
S. typhimurium	Reverse mutation	Rat S-9	+	–	Chung et al. 2000
S. typhimurium	Reverse mutation	Rat S-9	+	–	Ames et al. 1973
S. typhimurium	Reverse mutation	Rat S-9	+	–	McCann et al. 1975
S. typhimurium	Reverse mutation	Rat S-9	+	No data	Duverger-van Bogaert et al. 1995
S. typhimurium	Reverse mutation	Rat S-9	+	–	Garner et al. 1975
S. typhimurium	Reverse mutation	Rat S-9	+	–	Ferretti et al. 1977
S. typhimurium	Reverse mutation	Rat S-9	+	–	Anderson and Styles 1978
S. typhimurium	Reverse mutation	Rat S-9	+	–	Simmon and Shephard 1981
S. typhimurium	Reverse mutation	Rat S-9	+	–	Messerly et al. 1987
S. typhimurium	Reverse mutation	Rat S-9	+	No data	Dorado and Pueyo 1988
S. typhimurium	Reverse mutation	Rat S-9	+	No data	Bos et al. 1983
S. typhimurium	Reverse mutation	Rat S-9	–	No data	Phillipson and Ioannides 1983
S. typhimurium	Reverse mutation	Rat S-9	+	–	Zeiger et al. 1992
S. typhimurium	Reverse mutation	Hamster S-9	+	–	Zeiger et al. 1992
S. typhimurium	Reverse mutation	Modified rat S-9	(+)	No data	Prival and Mitchell 1982

Table 3-4. Genotoxicity of Benzidine *In Vitro (continued)*

Species (test system)	End point	Activation system	Results: With activation	Results: Without activation	Reference
S. typhimurium	Reverse mutation	Modified hamster S-9	+	No data	Prival and Mitchell 1982
S. typhimurium	Reverse mutation	Modified hamster ± FMN	+	No data	Prival et al. 1984
S. typhimurium	Reverse mutation	Mouse S-9	+	No data	Phillipson and Ioannides 1983
S. typhimurium	Reverse mutation	Hamster S-9	+	No data	Phillipson and Ioannides 1983
S. typhimurium	Reverse mutation	Pig S-9	–	No data	Phillipson and Ioannides 1983
S. typhimurium	Reverse mutation	Rat hepatocytes	+	No data	Bos et al. 1983
S. typhimurium	Reverse mutation	Bovine bladder urothelial cells	+	No data	Oglesby et al. 1983
S. typhimurium	Reverse mutation	RSV+NAT(B)	+	–	Petry et al. 1988
S. typhimurium	Reverse mutation	RSV–NAT(B)	–	–	Petry et al. 1988
S. typhimurium	Reverse mutation	Rat S-9+NAT(H)	+	–	Grant et al. 1992
S. typhimurium	Reverse mutation	Rat S-9–NAT(H)	–	–	Grant et al. 1992
Escherichia coli	SOS DNA repair	Rat S-9	–	–	Von der Hude et al. 1988
E. coli pol A	DNA repair	Rat S-9	–	–	Fluck et al. 1976
E. coli	SOS DNA repair	Hamster S-9	–	–	Von der Hude et al. 1988
E. coli	Prophase induction	Rat S-9	–	–	Speck et al. 1978
E. coli PQ37	SOS DNA repair	Rat S-9	–	No data	Quillardet et al. 1985
Bacillus subtilis	Rec assay	Rat S-9	(+)	–	Kada 1981
Eukaryotic organisms:					
Streptomyces griseus	Reverse mutation	No data	No data	+	Bucholz et al. 1992
Saccharomyces cerevisiae	Forward mutation	Rat S-9	+	No data	Mitchell and Gilbert 1991

Table 3-4. Genotoxicity of Benzidine *In Vitro (continued)*

Species (test system)	End point	Activation system	Results: With activation	Results: Without activation	Reference
Mammalian cells:					
Chinese hamster ovary HGPRT^{+} cells	Forward mutation	S-9	–	–	Phillips et al. 1990
Chinese hamster ovary HGPRT^{+} cells	Forward mutation	S-9	+	–	Oberly et al. 1990
Chinese hamster V79 HGPRT^{+} cells	Forward mutation	Rat S-9 Mouse S-9	– +	– –	Fassina et al. 1990
Chinese hamster V79 HGPRT^{+} cells	Forward mutation	Rat S-9	–	–	O'Donovan 1990
Chinese hamster V79 HGPRT^{+} cells	Forward mutation	Rat hepatocytes	–	–	Suter et al. 1992
Chinese hamster V79 PTA^{+} cells	Forward mutation	Rat hepatocytes	+	–	Suter et al. 1992
Chinese hamster V79 cells	Forward mutation	Bovine bladder urothelial cells	–	–	Oglesby et al. 1983
L5178YTK$^{+/-}$ Mouse lymphoma cells	Forward mutation	Rat S-9	+	No data	Oberly et al. 1984
L5178YTK$^{+/-}$ Mouse lymphoma cells	Forward mutation	S-9	+	+	Clay and Cross 1990
L5178YTK$^{+/-}$ Mouse lymphoma cells	Forward mutation	Rat S-9	+	+	Riach et al. 1990
L5178YTK$^{+/-}$ Mouse lymphoma cells	Forward mutation	Ram PHS	+	(+)	Sarkar et al. 1990
L5178YTK$^{+/-}$ Mouse lymphoma cells	Forward mutation	Rat S-9	+	+	Myhr and Caspary 1988
L5178YTK$^{+/-}$ Mouse lymphoma cells	Forward mutation	Rat S-9	+	+	Mitchell et al. 1988

Table 3-4. Genotoxicity of Benzidine *In Vitro (continued)*

Species (test system)	End point	Activation system	Results: With activation	Results: Without activation	Reference
L5178YTK$^{+/-}$ Mouse lymphoma cells	Forward mutation	Rat S-9	+	+	Henderson et al. 1990
L5178Y HGPRT^{+} Mouse lymphoma cells	Forward mutation	Rat S-9	–	–	Kennelly et al. 1990
L5178Y HGPRT^{+} Mouse lymphoma cells	Forward mutation	Rat S-9	–	–	Henderson et al. 1990
TK6 human lymphoblastoid TK$^{+/-}$ cells	Forward mutation	Rat S-9	(+)	(+)	O'Brien et al. 1990
TK6 human lymphoblastoid HGPRT^{+} cells	Forward mutation	Rat S-9	–	–	O'Brien et al. 1990
Chinese hamster V79	Chromosome breaks	Rat S-9	+	–	Swenberg et al. 1976
Mouse lymphocytes	Chromosomal aberrations	Mouse S-9	+	No data	Das et al. 1994
H4IIE	Sister chromatid exchange		No data	+	Grady et al. 1986
HEP G2	Sister chromatid exchange		No data	+	Grady et al. 1986
Chinese hamster V79	Sister chromatid exchange		No data	–	Grady et al. 1986
IMR-90	Sister chromatid exchange		No data	–	Grady et al. 1986
Human lymphocytes	Sister chromatid exchange	Rat liver cells	(+)	+	Lindahl-Kiessling et al. 1989
HeLa Cells	Unscheduled DNA synthesis	Rat S-9	+	–	Martin et al. 1978
Rat hepatocytes	Unscheduled DNA synthesis		No data	+	Brouns et al. 1979; Kornbrust and Barfknecht 1984a, 1984b; Williams 1978
Hamster hepatocytes	Unscheduled DNA synthesis		No data	+	Kornbrust and Barfknecht 1984a, 1984b
Rat hepatocytes	Unscheduled DNA synthesis	No data	No data	(+)	Harbach et al. 1991

Table 3-4. Genotoxicity of Benzidine *In Vitro (continued)*

Species (test system)	End point	Activation system	Results		Reference
			With activation	Without activation	
Rat hepatocytes	Unscheduled DNA synthesis	No data	No data	+	Steinmetz et al. 1988
Mouse hepatocytes	Unscheduled DNA synthesis	No data	No data	–	Steinmetz et al. 1988
Hamster hepatocytes	Unscheduled DNA synthesis	No data	No data	+	Steinmetz et al. 1988
Monkey hepatocytes	Unscheduled DNA synthesis	No data	No data	–	Steinmetz et al. 1988
Human hepatocytes	Unscheduled DNA synthesis	No data	No data	+	Steinmetz et al. 1988
Human diploid fibroblasts	Unscheduled DNA synthesis	No data	No data	–	Snyder and Matheson 1985
Rat hepatocytes	Unscheduled DNA synthesis	No data	No data	+	Barfknecht et al. 1988
BHK21 C1-13	Cell transformation	Rat S-9	+	–	Ashby et al. 1978
Syrian hamster embryo cells	Cell transformation	No data	No data	+	Pienta 1980
Chinese hamster ovary cells	DNA adduct formation	No data	No data	–	Phillips et al. 1990
Human cervical carcinoma cells	DNA adduct formation	No data	No data	–	Phillips et al. 1990
L5178Y $TK^{+/-}$ mouse lymphoma cells	DNA adduct formation	No data	No data	–	Phillips et al. 1990
L5178Y $TK^{+/-}$ mouse lymphoma cells	DNA adduct formation	No data	No data	–	Phillips et al. 1990
Mouse lymphoma cells	DNA adduct formation	No data	No data	–	Phillips et al. 1990
Human lymphoblastoid TK6 cells	DNA adduct formation	No data	No data	–	Phillips et al. 1990
Chinese hamster V79 cells	DNA adduct formation	No data	No data	+	Phillips et al. 1990

Table 3-4. Genotoxicity of Benzidine *In Vitro (continued)*

Species (test system)	End point	Activation system	Results		Reference
			With activation	Without activation	
Calf thymus DNA	DNA adduct formation	No data	No data	+	Phillips et al. 1990
Calf thymus DNA	DNA adduct formation	RSV	+	–	Petry et al. 1988

– = negative result; + = positive result; (+) = weakly positive result

DNA = deoxyribonucleic acid; FMN = flavin mononucleotide; HGPRT = hypoxanthine guanine phosphoribosyl transferase; NAT(B) = bacterial N-acetyltransferases; NAT(H) = human N-acetyltransferases; PHS = prostaglandin H synthase; PTA = *Pseudomonas* toxin A; RSV = rat seminal vesical microsomes; S-9 = liver homogenate fraction; SOS = inducible bacterial error-prone DNA repair pathway; TK = thymidine kinase

3.4 TOXICOKINETICS

Many studies have been devoted to clarifying the mechanism and etiology of bladder and other cancers caused by benzidine in animals. Both benzidine toxicity and the elimination of benzidine from the body are substantially mediated by metabolic transformation. While some metabolites are detoxication products, others are precursors of proximate and ultimate carcinogens. The latter form adducts with nucleic acids and is presumed to initiate carcinogenesis. The differences in target organs among rodents, dogs, and humans are related to species differences in their metabolic pathways and enzyme activities.

No studies have been reported that indicate that benzidine is absorbed by any process other than passive diffusion. Absorption follows inhalation, oral, and dermal routes of exposure, with the inhalation and dermal routes probably being the most likely for humans. In general, there appears to be a rapid plasma clearance of absorbed benzidine, followed by a more gradual metabolism and clearance of benzidine metabolites (Lakshmi et al. 1990a; Shah and Guthrie 1983). Enterohepatic circulation may contribute to the persistence and toxicity of metabolites of benzidine in the bile (Chipman and Mohn 1989; Percy et al. 1989). Benzidine metabolism involves multiple and complex enzymatic pathways, the relative importance of which may vary according to both species and tissue (Babu et al. 1992, 1993a, 1994a, 1994b; Josephy et al. 1989; Kadlubar et al. 1982; Lakshmi et al. 1990a, 1990b, 1995a, 1995b; Naidu et al. 1992; Wang et al. 1988; Zenser et al. 1996). In the liver, benzidine is thought to be N-acetylated and then N-hydroxylated by the cytochrome P-450 and/or flavin monooxygenase systems, whereas in extrahepatic tissues, peroxidation by prostaglandin H synthase or oxidation by lipoxygenases may play a significant role in benzidine metabolism. Excretion of benzidine, its metabolites, and their conjugates appears approximately equally divided between the urine and the bile/feces (Lakshmi et al. 1990a; Shah and Guthrie 1983).

Some studies also indicate that benzidine-based dyes can be metabolized to benzidine, and that human exposure to the dyes is associated with bladder cancer (Genin 1977; Yoshida and Miyakawa 1973). After oral administration, benzidine dyes can be metabolized to benzidine by microflora of the gastrointestinal tract (Bos et al. 1986). These investigators detected mutagenicity in the urine of ordinary (intestinal microflora-containing), but not germ-free rats after oral administration of two benzidine-based dyes, whereas benzidine produced mutagenic urine in both germ-free and ordinary rats. It is generally agreed that because of the size of the molecule, benzidine-based dyes are unlikely to be absorbed intact; therefore, azo reduction occurs most likely in the intestine and less likely in the liver.

3.4.1 Absorption

3.4.1.1 Inhalation Exposure

No quantitative studies were located regarding absorption in humans or animals after inhalation exposure to benzidine. However, mean urinary levels of benzidine in workers exposed to 0.005–17.6 mg/m^3 benzidine in air increased 4-fold (from 0.01 to 0.04 mg/L) during the course of a workshift, suggesting significant and relatively rapid absorption and excretion. The available data are highly variable, and it is not clear whether high air concentrations correlated with high urinary levels. The relative contribution of dermal or oral absorption was also not rigorously examined in this study (Zavon et al. 1973). Similarly, urine from workers exposed to the dust of Direct Black 38 was found to contain benzidine (2.4–362.5 µg/L), monoacetylbenzidine (6–1,172 µg/L), and diacetylbenzidine (4.2–160 µg/L), suggesting that inhalation absorption had occurred (Dewan et al. 1988; NIOSH 1980c). In addition, it is possible that significant oral exposure to the dyes occurred since inhaled particles are frequently coughed up or brought up by ciliary action and then swallowed. However, the relative contributions of pulmonary and dermal absorption were not discussed in these publications. More recent studies have also provided evidence of inhalation absorption in workers exposed to benzidine (DeMarini et al. 1997; Rothman et al. 1996a).

3.4.1.2 Oral Exposure

No quantitative studies were located regarding absorption in humans or animals after oral exposure to benzidine.

Qualitative evidence of absorption after oral administration of ^{3}H-benzidine to mice in their drinking water was indicated by covalent binding of a benzidine metabolite to DNA in the liver (Martin et al. 1982). A similar experiment with dogs that were fed ^{3}H-benzidine by capsule resulted in covalent binding to DNA in the epithelium of the bladder (Beland et al. 1983). Another study 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 as such, can be absorbed in the intestines (Bos et al. 1986).

3.4.1.3 Dermal Exposure

It is generally agreed that benzidine bases and salts can be readily absorbed through the intact human skin (Ferber et al. 1976; Meigs et al. 1951, 1954; Zavon et al. 1973). The salts are produced by strong acids acting on a weak base, and consequently, readily hydrolyze in aqueous media to yield some free base at equilibrium. The log 10 octanol-water partition coefficient of the base is reported to be 1.36 (Lu et al. 1977). This is considered sufficiently high to allow significant dermal absorption, particularly during long term exposures or when exposed populations are subject to warm, humid weather and/or strenuous working conditions that result in excessive perspiration.

Levels of benzidine and its metabolites were measured in the urine of industrially exposed workers in March and in August of 1958 (Sciarini and Meigs 1961). During the cooler weather of March, a mean of 5 mg/L of benzidine and its metabolites was reported, compared to a mean of 21.8 mg/L during the warm weather of August. The highest excretion was 31 mg/L in August. Given the conditions observed at this plant, these urinary levels are probably largely due to dermal contact with dust containing benzidine. This conclusion is supported by earlier data showing that daily showers and fresh work clothes reduced the quantity of benzidine and its metabolites excreted in the urine of exposed workers (Meigs et al. 1951). In animals, radioactivity was observed in tissues, urine, and feces following application of 1 mg/kg of radioactive benzidine for 1, 8, and 24 hours to the shaved skin of male 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 and Guthrie 1983). At 24 hours, 49% of the radioactivity was recovered from the skin site, indicating that approximately half of the applied benzidine had penetrated the skin. Since almost 50% of the radioactivity was recovered in the excreta, the results suggested that 50% of the applied dose was absorbed. A preliminary report submitted to EPA under Section 8(e) of the Toxic Substances Control Act (TSCA) stated that nearly all of the benzidine-based dye, Direct Black 38, is absorbed directly through the intact skin of rabbits (International Business Machines 1979). However, this was later attributed to probable ingestion (licking of the skin) (Aldrich 1993). In contrast, Aldrich et al. (1986) applied radiolabeled Direct Black 38 to the shaved dorsal skin of the male F344 rats and New Zealand rabbits that were prevented from licking the site of application, and then measured radioactivity in urine and feces 24–144 hours following dye application. Approximately 3% of the administered radioactivity was detected in the urine and 5% in the feces of rabbits at 144 hours. Excretion of radioactivity was negligible in rats (0.05% in urine and 0.16% in feces). Because skin penetration by the whole dye was considered very unlikely, the absorbed and excreted radioactivity in the rabbits was presumed to represent benzidine liberated by azo-reduction of the dye.

3.4.1.4 Other Routes of Exposure

In an *in vitro* study designed to assess the percutaneous absorption and penetration of ^{14}C-benzidine in combination with various other chemical mixtures which might be found in occupational settings, isolated perfused porcine skin flaps were topically treated with benzidine+solvent (acetone or DMSO), benzidine+surfactant (sodium lauryl sulfate), benzidine+vasodilator (methyl nicotinate), benzidine+reducing agent ($SnCl_2$), or various combinations of these mixtures (Baynes et al. 1996). Flow-through diffusion call systems were also utilized. Absorption was defined as the total amount of radioactivity detected in the perfusate for the entire 8-hour perfusion period. Skin penetration was defined as the sum of total absorption and total radioactivity in the skin. Maximum absorption (and maximum penetration over an 8-hour exposure period were approximately 3 and 22%, respectively, for a mixture of benzidine+DMSO+surfactant+vasodilator. It was noted that DMSO enhanced absorption more than acetone, and that the reducing agent inhibited benzidine absorption. It was suggested that chemical-biological interactions between the surfactant and vasodilator might enhance benzidine absorption while chemical-chemical interactions between benzidine and $SnCl_2$ might inhibit benzidine absorption. The percentage of the dose absorbed in this study is much less than the approximately 50% dermal absorption reported by Shah and Guthrie (1983) in rats *in vivo*.

3.4.2 Distribution

There is no information regarding pharmacokinetics of benzidine in children nor is it known whether benzidine can be stored and excreted in breast milk. Although there have been no direct measurements to determine whether it can cross the placenta, there is some indirect evidence that benzidine and/or its metabolites do. This evidence is based on the results of a study in which intraperitoneal administration of benzidine to pregnant mice resulted in the induction of micronuclei in the liver of fetuses (Sanderson and Clark 1993); however, when pregnant mice were orally exposed to benzidine, there was no increase in micronucleated cells either in their livers or the livers of their fetuses (Harper et al. 1989). There is no information on whether benzidine can be stored in maternal tissues and be mobilized during pregnancy or lactation.

3.4.2.1 Inhalation Exposure

No studies were located regarding distribution in humans or animals after inhalation exposure to benzidine.

3.4.2.2 Oral Exposure

No studies were located regarding distribution in humans or animals after oral exposure to benzidine.

3.4.2.3 Dermal Exposure

No studies were located regarding distribution in humans after dermal exposure to benzidine.

One study was found that reports data on the distribution of benzidine in rats following dermal exposure (Shah and Guthrie 1983). When radiolabeled benzidine was applied to the skin of rats, radioactivity was distributed approximately as follows (percentage of applied radioactivity 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%), stomach (0.5, 0.4, and 0.08%), carcass (1.9, 4.1, and 6.9%), urine (0.08, 4.1, and 22.8%), and feces (0.01, 0.7, and 18.7%). Twenty-four hours after administration, approximately half of the applied radioactivity remained at the site of application.

3.4.2.4 Other Routes of Exposure

Following intravenous injection of 0.2 mg/kg of radiolabeled benzidine, radioactivity was distributed rapidly throughout the body in rats, dogs, and monkeys (Kellner et al. 1973). In rats, substantial activity was found after 4 hours in the lung, the small and large intestines, the bladder, and the kidney, with smaller amounts in all other tissues and fluids examined. Findings were generally similar in dogs, except that there was a 10- to 15-fold higher level of radioactivity in the bladder tissue (consistent with benzidine bladder carcinogenicity in dogs), and much lower activity (about 10% that of rats) in the lung. Approximately 90% of the radioactivity was cleared from the blood during the first 24 hours after dosing, with the remainder being cleared more slowly. Half-lives of radioactivity in the blood from day 1 to day 6 or 7 were 65 hours in rats and 88 hours in dogs. After 7 days, activity 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 value for rats was

0.042 µg/g, and values were 0.087–0.19 µg/g for three dogs, and 0.01 and 0.027 µg/g for the two monkeys.

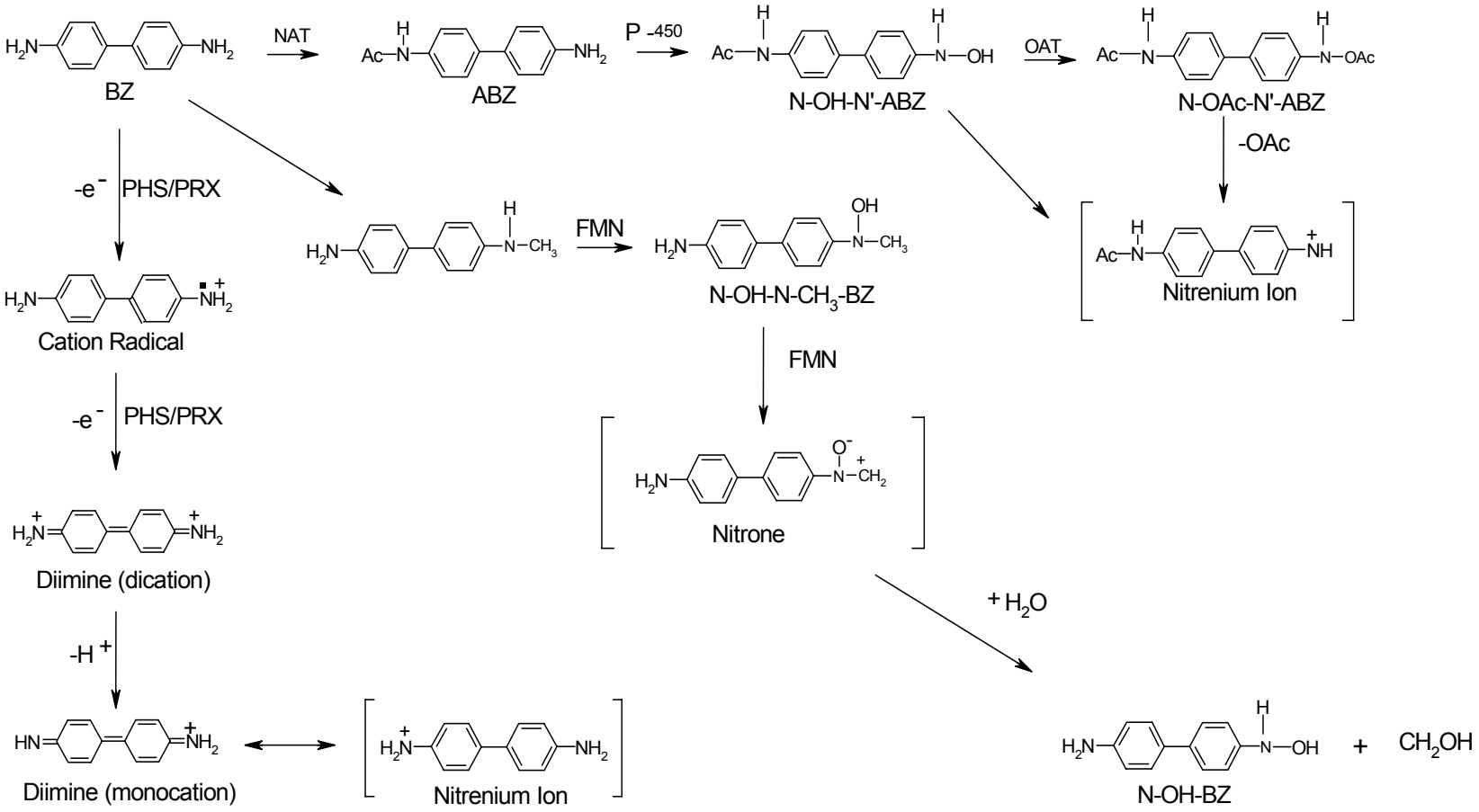
Plasma clearance of benzidine in dogs has been found to be fairly rapid (of the amount present 5 minutes after infusion, approximately 10% remained after 5 hours), while metabolism and metabolite clearance occur more gradually. In a study of four dogs over a 5-hour period following intravenous administration of 1 mg/kg radiolabeled benzidine (Lakshmi et al. 1990a), the initial plasma half-life of benzidine was reported to be approximately 30 minutes, while that for total radiolabel (benzidine plus metabolites) was approximately 3 hours. Five hours after infusion, 75% of recovered radioactivity was found collectively 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%). Smaller quantities were found in the stomach, intestines, spleen, kidney, heart, and lungs. While the bladder contained the lowest fraction of recovered radioactivity (0.1%), the bladder transitional epithelium exhibited a consistently higher concentration of bound radioactivity than bladder muscle. In liver, kidney, bladder muscle, and bladder epithelium, the majority of radioactivity was bound to protein. Smaller amounts were also bound to DNA. Lynn et al. (1984) performed a similar study in rats after intravenous injection of radiolabeled benzidine. Tissues retaining the most radioactivity after 3 days were muscle and liver. A higher amount was found in the stomach as well. Activity was low in the bladder; this is consistent with the liver, but not the bladder, being a target organ for benzidine-induced carcinogenesis in rats.

The importance of enterohepatic circulation has been demonstrated in both rats and mice, where biliary benzidine and benzidine metabolites can be reabsorbed from the intestines and transported again to the liver (Chipman and Mohn 1989). This enterohepatic recycling contributes to the persistence, the further metabolism, and presumably the hepatotoxicity of benzidine and its metabolites.

3.4.3 Metabolism

There is substantial information available from human, animal, and *in vitro* studies regarding various aspects of benzidine metabolism. Numerous *in vivo* metabolites have been reported in a variety of species and a brief discussion of the major ones can be found in earlier EPA documents (EPA 1980a, 1986c) and in more recent publications (Whysner et al. 1996; Zenser et al. 1998). As a general guide, Figure 3-3 presents some of the principal features of several metabolic pathways thought to be involved or potentially involved in the metabolism of benzidine. Although there is no evidence suggesting that the metabolism of benzidine is substantially influenced by route of exposure, there are clear differences in

Figure 3-3. Metabolic Schemes for Benzidine



ABZ = monoacetylbenzidine; BZ = benzidine; FMN = flavin monooxygenase system; N-CH_3-BZ = N-methylbenzidine; N-OAC-N'-ABZ = N'acetoxy-N'-acetylbenzidine; N-OH-N'-ABZ = N-hydroxy-N'-acetylbenzidine; N-OH-N-CH_3-DZ = N-hydroxy-N-methylbenzidine; N-OH-BZ = N-hydroxybenzidine; NAT = N'acetyltransferase; NMT = N-methyltransferase; Oac = acetoxy; OAT = O-acetyltransferase; P-450 = cytochrome P-450 system; PHS = prostaglandin H synthase; PRX = peroxidase

metabolism among species or organs. It is not the purpose of this section to present an exhaustive review of all of the studies that investigated the metabolism of benzidine, but to summarize some basic information from the main contributors to this topic and to provide a foundation for the understanding of the mechanism of carcinogenicity of benzidine, which is further discussed in Section 3.5.2.

The urine of humans exposed to benzidine has been reported to contain free benzidine, N-acetyl-benzidine, N,N'-diacetylbenzidine, 3-hydroxybenzidine, and 3,3'-dihydroxybenzidine (EPA 1980a, 1986c; Sciarini and Meigs 1961). Following oral or intravenous administration of benzidine to rats, at least 17 metabolites were identified in the urine or bile, principally the mono and di-acetyl derivatives, benzidine-N-glucuronide, N-acetylbenzidine glucuronide, N-hydroxy-N,N'-diacetylbenzidine glucuronide, 3-hydroxy-N,N'-diacetylbenzidine glucuronide, and an N,N'-diacetylbenzidine glutathione conjugate (Lynn et al. 1984). Similar findings were reported using isolated perfused rat liver (Lynn et al. 1983). Results from these and also from more recent studies suggest that three main chemical reactions are involved in the metabolism of benzidine: N-acetylation, N-oxidation, and N-glucuronidation. Because primary amines are easier to oxidize than the acetylated amide products, N-acetylation is considered a detoxification reaction and competes with N-oxidation. Also, because amides do not form glucuronides, N-glucuronidation and N-acetylation are also competing pathways. The prevalence of any one of these reactions is species-specific and will ultimately determine the potential development of organ-specific cancer. These three pathways are discussed below.

N-Acetylation is a reaction that involves the transfer of acetate to the nitrogen or oxygen atom of aromatic amines, hydrazines, and N-hydroxylamines, catalyzed by N-acetyltransferase (NAT), a cytosolic enzyme that exhibits polymorphism. Two NAT isozymes, NAT1 and NAT2, have been reported to differ significantly in their intrinsic stabilities and acceptor substrate selectivity. In several species, including humans, acetylator polymorphism results in rapid, intermediate, and slow acetylator phenotypes. The acetylation of benzidine was studied in liver slices from rats and humans (Lakshmi et al. 1995a) and dogs (Lakshmi et al. 1995b). In rat liver slices incubated for 1 hour with [H^3]benzidine, N-acetylbenzidine and N,N'-diacetylbenzidine represented 8.8 and 73% of the total radioactivity recovered, respectively. No unmetabolized benzidine was detected, indicating that rats favor N,N'-diacetylbenzidine formation. In contrast, of the total radioactivity recovered from human liver slices, 19% was benzidine, 34% N'-acetylbenzidine, and only 1.6% N,N'-diacetylbenzidine. When the deacetylase inhibitor paraoxon was included in the experiment with human liver slices, 2% of the radioactivity was recovered as benzidine, 24% as N-acetylbenzidine, and 51% as N,N'-diacetylbenzidine. These findings suggested that

humans favor N-acetylbenzidine formation, and that a deacetylase influences hepatic metabolism of benzidine more than specific NAT genotype (this issue is further discussed in Sections 3.5.2 and 3.10).

Similar experiments conducted with dog liver slices showed that benzidine was converted to metabolites more polar than benzidine; no acetylated metabolites were detected (Lakshmi et al. 1995b). This suggests that the dog is a nonacetylator with respect to benzidine and, as explained further in Section 3.5.2, this is consistent with the fact that no acetylated DNA adduct was observed in this species. In contrast, N'-(deoxyguanosin-8-yl)-N-acetylbenzidine was the major DNA adduct detected following administration of benzidine to rats and mice and of N-acetylbenzidine to hamsters (Kennelly et al. 1984; Martin et al. 1982), in rat liver *in vitro* (Frederick et al. 1985), and in exfoliated urothelial cells of exposed humans (Rothman et al. 1996a). Further studies of the acetylation of benzidine using recombinant NAT1 and NAT2 showed that NAT1 had higher K_m and V_{max} values than NAT2 and that N-acetylbenzidine was a preferred substrate for NAT1 (Zenser et al. 1996). Moreover, a higher acetylation ratio was observed in liver slices possessing the NAT1*10 compared to NAT1*4 allele, indicating that NAT1 may exhibit a polymorphic expression in human liver.

The NADPH-dependent oxidation of benzidine has been studied by Lakshmi (1996b) who showed that microsomes of β-naphthoflavone-treated rats (β-naphthoflavone is a P-4501A1/1A2 inducer) converted benzidine to 3-hydroxybenzidine. Experiments conducted with inhibitors of cytochrome P-450 (families 1–3) confirmed that benzidine was metabolized by cytochrome P-4501A1/1A2. This metabolite had been found to be the most prominent urinary metabolite in workers exposed to benzidine (Sciarini and Meigs 1961). An earlier study had demonstrated that N-oxidation of N-acetylbenzidine and N,N'-diacetylbenzidine occurred in rat and mouse liver subcellular fractions fortified with NADPH and NADH (Frederick et al. 1985). In both species, N-oxidation of N-acetylbenzidine to N'-hydroxy-N-acetylbenzidine was faster than the formation of N-hydroxy-N-acetylbenzidine. Further studies by Lakshmi (1996b) showed that both control and β-naphthoflavone-treated rat liver microsomes oxidized N-acetylbenzidine and N'N'-diacetylbenzidine and that the relative rate of formation of oxidized compounds was N'-hydroxy-N-acetylbenzidine > N-hydroxy-N-acetylbenzidine >> N-hydroxy-N,N'-diacetylbenzidine. Lakshmi (1996b) also presented evidence suggesting that P-4501A1 activity was more involved than P-4501A2 and that, under the conditions of the experiment, N-oxidation exceeded ring oxidation.

Glucuronidation is a major metabolic pathway for generating water soluble substances from toxic substances. Glucuronidation of aromatic amines is carried out by UDP-glucuronosyltransferases, which

are membrane-bound enzymes of the endoplasmic reticulum and that exist in multiple forms. The role of glucuronidation in the metabolism and carcinogenicity of benzidine has been evaluated in numerous studies (Babu et al. 1992, 1993a, 1994a, 1994b, 1995). In the study of Babu et al. (1992), the influence of glucuronidation in the metabolism and carcinogenicity of benzidine was determined using dog liver slices and microsomes. This study demonstrated that the half lives of purified N-benzidine glucuronide in dog urine at 37 EC were 99, 25, and 3 minutes at pH 7.3, 6.3, and 5.3, respectively. It was also shown by Babu et al. (1992) that the N-glucuronide of benzidine was stable in dog plasma at pH 9.3. While the N-glucuronide was found bound to plasma, the binding was weaker than that shown by benzidine. Glucuronide formation of the N-hydroxy metabolites of N-acetylbenzidine (Babu et al. 1995) and N-acetyl-N'-glucuronidation by human, dog, and rat liver (Babu et al. 1993a) was also studied. Human liver slices glucuronidated benzidine and N-acetylbenzidine and the proportion of benzidine and N-acetylbenzidine glucuronidated was affected by the extent of acetylation (Babu et al. 1994a). In contrast to dogs, N-glucuronidation appeared to represent a major pathway for the metabolism of benzidine in humans. Furthermore, results from experiments with inhibitors of glucuronidation in human liver microsomes suggested that more than one UDP-glucuronosyltransferase metabolizes benzidine (Babu et al. 1994a). Recently, the same group of investigators studied the capacity of five different human recombinant UDP-glucuronosyltransferases in COS-1 cells to glucuronide benzidine and its metabolites and found that UGT1A9 exhibited the highest relative rate of metabolism followed by UGT1A4 > UGT1A6 > UGT2B7 >UGT1A1 (Ciotti et al. 2000). UGT1A9 was particularly effective in glucuronidating the two hydroxamic acids, N-hydroxy-N-acetylbenzidine and N-hydroxy-N,N'-diacetyl benzidine, but UGT1A4 showed a 2.3-fold higher rate for N'-hydroxy-N-acetylbenzidine than UGT1A9.

Additional studies by the same group of investigators showed that incubation of N-acetylbenzidine with human liver slices produced a significant amount of N-acetylbenzidine-N'-glucuronide and that rat liver slices needed a much higher concentration of N'-acetylbenzidine to produce the glucuronide (Babu et al. 1993a). Human liver slices incubated with N-acetylbenzidine also formed benzidine (suggesting deacetylation), benzidine glucuronide, and N,N'-diacetylbenzidine (Babu et al. 1994b). Dog liver slices did not produce N'-acetylglucuronide. Liver microsomes from human, rat, and dog produced N-acetyl-benzidine-N'-glucuronide and the relative glucuronidation rate of acetylbenzidine was human >> dog > rat. The fact that glucuronidation of N-acetylbenzidine occurred in dog microsomes but not in dog liver slices suggests that dogs rapidly metabolized N-acetylbenzidine, and that deacetylation to benzidine is a major pathway in this species; lack of glucuronidation of N-acetylbenzidine was essentially due in part to lack of substrate.

Human liver microsomes were also found to glucuronidate N-hydroxymetabolites of N-acetylbenzidine (Babu et al. 1995). The relative rate of glucuronidation was N'-hydroxy-N,N'-diacetylbenzidine >> N'-hydroxy-N-acetylbenzidine = benzidine > N-acetylbenzidine > N-hydroxy-N-acetylbenzidine. At pH 5.5 and 37 °C, the half-lives of the conjugates of N-acetylbenzidine, N'-hydroxy-N-acetylbenzidine, and N-hydroxy-N-acetylbenzidine were 7.5 minutes, 3.5 hours, and 1.8 hours, respectively. Much longer half-lives were obtained for the glucuronides of acetylbenzidine and N'-hydroxy-N-acetylbenzidine when the incubation was carried out at pH 7.4. Compared with N-acetylbenzidine, glucuronides of its N-hydroxymetabolites are more stable at acidic pH. Thus, in acidic urine, it would be more likely that the glucuronide conjugate of N-acetylbenzidine would be hydrolized than those of its N-hydroxy-metabolites.

It is not known whether metabolism in adult humans differs qualitatively or quantitatively from metabolism in children or fetuses. However, the expression of two enzyme families (NAT2 and glucuronosyltransferase [UGT]) that contribute to benzidine metabolism is known to vary developmentally (Leeder and Kearns 1997). There is some NAT2 activity present in 16-week-old fetuses. Almost all infants show the slow metabolizer phenotype between birth and the age of two months. NAT2 reaches the adult phenotype distribution in infants at 4–6 months, and by 1–3 years, adult activity is present. For UGT, ontogeny is isozyme-specific, and adult activity is reached at about 6–18 months. The expression of P-450 enzymes varies developmentally as well, but the precise P-450 isozymes involved in metabolism of benzidine are not known.

The relevance of metabolism to the carcinogenic properties of benzidine is summarized in Section 3.5.2.

3.4.4 Elimination and Excretion

3.4.4.1 Inhalation Exposure

No quantitative studies were located regarding excretion in humans or animals after inhalation exposure to benzidine. However, excretion of 0–363 µg/L benzidine, 6–1,117 µg/L monoacetylbenzidine, and 4–160 µg/L diacetylbenzidine have been demonstrated in the urine of workers who were potentially exposed to a number of benzidine-based dyes (Dewan et al. 1988; NIOSH 1980c). These dyes can be reduced to free benzidine by azoreductases of the liver and intestinal microflora. Exposure is assumed to have been largely by inhalation, but dermal exposure may also have been significant. Conversely, inhalation exposure was likely a factor in several occupational studies showing urinary excretion where

dermal exposure to benzidine was reported as the principal route (Meigs et al. 1951, 1954; Sciarini and Meigs 1961).

3.4.4.2 Oral Exposure

Information regarding excretion in humans after oral exposure is extremely limited. After a single oral dose of 1.4 mg/kg/day, a human was found to excrete free benzidine, monoacetylbenzidine, and diacetylbenzidine (Engelbertz and Babel 1953), while in another study (Troll et al. 1963), six humans excreted N-hydroxyacetyl amino compounds when dosed with 2.9 mg/kg/day benzidine.

The major route of excretion in rats after a single oral administration of 0.5–50 mg/kg radiolabeled benzidine appeared to be via the feces (Lynn et al. 1984). At the lowest dose studied, 74% of the radiolabel was excreted in the feces during the first 3 days after exposure, as opposed to only 17% in the urine. With increasing dose the percentage of radiolabel excreted in the feces decreased, while that in the urine increased. At the low and mid-level doses (0.5 and 5.0 mg/kg), the major radiolabeled 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%). As the dose was increased (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. The analysis of urine and feces after intravenous injection of benzidine confirmed that feces was the main excretory route of radioactivity (Section 3.4.4.4) (Kellner et al. 1973; Lynn et al. 1984).

Oral exposure of monkeys to 10 or 100 mg of benzidine resulted in the urinary excretion of free benzidine and N-acetylbenzidine (Rinde and Troll 1975). The combined 72-hour excretion of these two compounds represented only a small fraction (1.5%) of the administered dose in contrast to the approximately 6% for these two compounds reported by Lynn et al. (1984) in rats.

3.4.4.3 Dermal Exposure

Urinary excretion of benzidine and its metabolites was found to occur in humans during workshift exposure, which was determined to be primarily dermal, although inhalation may also have been a contributory route (Meigs et al. 1951, 1954; Sciarini and Meigs 1961). Mean concentration of urinary compounds detected after exposure in the spring were: benzidine (0.28 mg/L), N-acetylbenzidine

(0.27 mg/L), N,N'-diacetylbenzidine (0.52 mg/L), and conjugated 3-hydroxybenzidine (3.9 mg/L) (Sciarini and Meigs 1961). When determined during hot, humid summer weather, these values increased 1.5- to 5-fold, presumably due to enhanced dermal contact and subsequent absorption. Fecal excretion was not determined.

When radiolabeled benzidine was applied dermally to rats, radiolabel was detected in both the urine and feces as early as 1 hour after treatment (Shah and Guthrie 1983). Excretion was significantly greater (6- to 8-fold) in urine than in feces during the first 8 hours, but by 24 hours, it was virtually the same for both routes (23% in urine, 19% in feces). Identification of the excreted products was not performed.

3.4.4.4 Other Routes of Exposure

The excretion of benzidine after other routes of exposure has been studied in several animal species. Following intravenous exposure of rats to 0.2 or 2.5 mg/kg radiolabeled benzidine, the majority of radiolabel was excreted in the feces during the first 3–7 days (63–80%), as opposed to in the urine (17–29%) (Kellner et al. 1973; Lynn et al. 1984). Experiments with bile duct cannulated rats indicated that virtually all fecal metabolites originate via biliary excretion (Lynn et al. 1984). Urinary metabolites included 3-hydroxy-N,N'-diacetylbenzidine glucuronide (25%), N,N'-diacetylbenzidine (12%), and N-hydroxy-N,N'-diacetylbenzidine glucuronide (4%). Metabolites and levels were similar in bile, except that about half of the 3-hydroxy-N,N'-diacetylbenzidine glucuronide was replaced by the 3-glutathione-N,N'-diacetylbenzidine conjugate.

In contrast to the findings in rat studies, urinary excretion in dogs after intravenous benzidine injection has been reported to range from 1.0 to 2.5 times that found in the bile or feces (Kellner et al. 1973; Lakshmi et al. 1990a) up to about 10 times in an older report (Sciarini and Meigs 1958). About 30% of the radiolabel excreted in the urine or bile was identified as free benzidine. 3-Hydroxybenzidine was a major metabolite (6%) in the bile, but not in the urine, where it apparently appeared as various conjugates. No acetylated metabolites were excreted, in keeping with the lack of N-acetylase activity in dogs. The earlier study reported that urinary concentrations of free benzidine ranged from 2 to 9%, and concentrations of 3-monohydroxybenzidine or its sulfur-conjugate ranged from 25 to 50% (Sciarini and Meigs 1958).

Excretion patterns in three monkeys following intravenous injections of 0.2 mg/kg benzidine varied too substantially to make generalizations (Kellner et al. 1973). Cumulative excretion during the first 7 days

varied from 30 to 70% in the urine, and from 5 to 36% in the feces. Thin-layer chromatography of urine samples was thought to indicate the presence of N-acetylated benzidine metabolites; however, these were not identified.

Intraperitoneal injection of benzidine in rats led to the biliary excretion of bioactivated metabolites as indicated by the bile's induction of nuclear anomalies in intestinal epithelial cells (Percy et al. 1989), or bacterial mutagenicity in a host-mediated assay (Chipman and Mohn 1989). Following intraperitoneal injection of mice with 100 mg/kg benzidine, benzidine and its metabolites were found in the urine in the following percentages: free benzidine (10%), N-acetylbenzidine (3.4%), N,N'-diacetylbenzidine (2.6%), 3-hydroxy ethereal sulfate (29%), 3-hydroxy-benzidine glucuronide (12%), N-hydrogen sulfate or glucuronide conjugates (18%), and monoacetylated 3-hydroxy ethereal sulfate or glucuronide benzidine conjugates (25%). Fecal excretion was not monitored (Sciarini and Meigs 1958).

3.4.5 Physiologically Based Pharmacokinetic (PBPK)/Pharmacodynamic (PD) Models

Physiologically based pharmacokinetic (PBPK) models use mathematical descriptions of the uptake and disposition of chemical substances to quantitatively describe the relationships among critical biological processes (Krishnan et al. 1994). PBPK models are also called biologically based tissue dosimetry models. PBPK models are increasingly used in risk assessments, primarily to predict the concentration of potentially toxic moieties of a chemical that will be delivered to any given target tissue following various combinations of route, dose level, and test species (Clewell and Andersen 1985). Physiologically based pharmacodynamic (PBPD) models use mathematical descriptions of the dose-response function to quantitatively describe the relationship between target tissue dose and toxic end points.

PBPK/PD models refine our understanding of complex quantitative dose behaviors by helping to delineate and characterize the relationships between: (1) the external/exposure concentration and target tissue dose of the toxic moiety, and (2) the target tissue dose and observed responses (Andersen et al. 1987; Andersen and Krishnan 1994). These models are biologically and mechanistically based and can be used to extrapolate the pharmacokinetic behavior of chemical substances from high to low dose, from route to route, between species, and between subpopulations within a species. The biological basis of PBPK models results in more meaningful extrapolations than those generated with the more conventional use of uncertainty factors.

The PBPK model for a chemical substance is developed in four interconnected steps: (1) model representation, (2) model parametrization, (3) model simulation, and (4) model validation (Krishnan and Andersen 1994). In the early 1990s, validated PBPK models were developed for a number of toxicologically important chemical substances, both volatile and nonvolatile (Krishnan and Andersen 1994; Leung 1993). PBPK models for a particular substance require estimates of the chemical substance-specific physicochemical parameters, and species-specific physiological and biological parameters. The numerical estimates of these model parameters are incorporated within a set of differential and algebraic equations that describe the pharmacokinetic processes. Solving these differential and algebraic equations provides the predictions of tissue dose. Computers then provide process simulations based on these solutions.

The structure and mathematical expressions used in PBPK models significantly simplify the true complexities of biological systems. If the uptake and disposition of the chemical substance(s) is adequately described, however, this simplification is desirable because data are often unavailable for many biological processes. A simplified scheme reduces the magnitude of cumulative uncertainty. The adequacy of the model is, therefore, of great importance, and model validation is essential to the use of PBPK models in risk assessment.

PBPK models improve the pharmacokinetic extrapolations used in risk assessments that identify the maximal (i.e., the safe) levels for human exposure to chemical substances (Andersen and Krishnan 1994). PBPK models provide a scientifically sound means to predict the target tissue dose of chemicals in humans who are exposed to environmental levels (for example, levels that might occur at hazardous waste sites) based on the results of studies where doses were higher or were administered in different species. Figure 3-4 shows a conceptualized representation of a PBPK model.

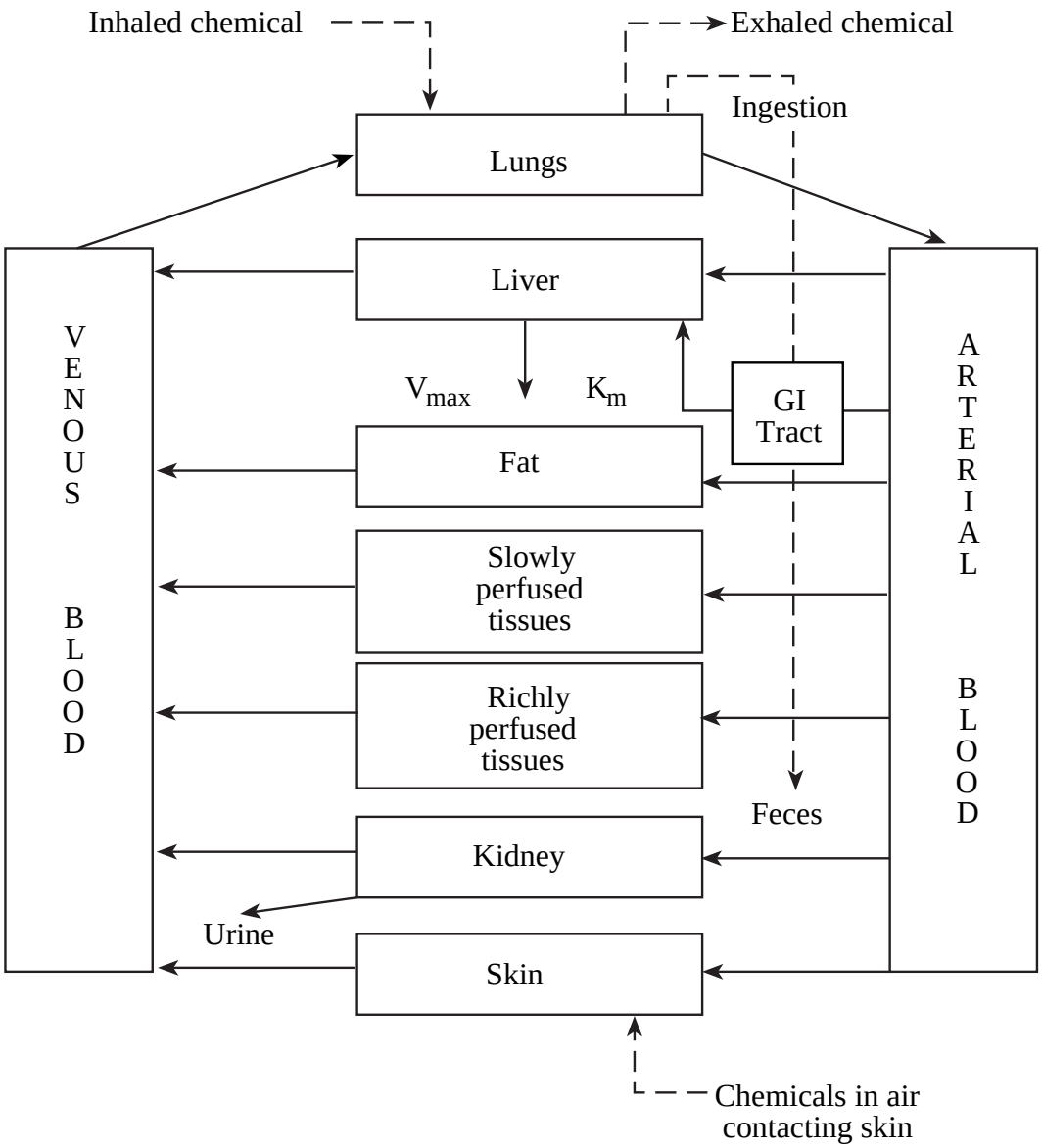
No PBPK modeling studies were located for benzidine.

3.5 MECHANISMS OF ACTION

3.5.1 Pharmacokinetic Mechanisms

There have been no reports that provide any data that benzidine is absorbed by any process other than passive diffusion, regardless of the route of exposure. Benzidine appears readily absorbed across the

Figure 3-4. Conceptual Representation of a Physiologically Based Pharmacokinetic (PBPK) Model for a Hypothetical Chemical Substance



Note: This is a conceptual representation of a physiologically based pharmacokinetic (PBPK) model for a hypothetical chemical substance. The chemical substance is shown to be absorbed via the skin, by inhalation, or by ingestion, metabolized in the liver, and excreted in the urine or by exhalation.

Source: adapted from Krishnan et al. 1994

intestinal mucosa (Bos et al. 1986), through the skin (Aldrich et al. 1986; Meigs et al. 1951; Shah and Guthrie 1983), and probably by pulmonary tissue as well (Zavon et al. 1973).

Benzidine and/or its metabolites are distributed throughout most of the body after dermal exposure in rats (Shah and Guthrie 1983), and after injection in rats and dogs (Kellner et al. 1973). No evidence has been reported to indicate that distribution is mediated by any carrier or binding protein, although conjugation of partially bioactivated benzidine metabolites with glucuronide may aid in their transport to target sites (e.g., from liver to bladder). In dogs administered benzidine intravenously, benzidine-glucuronide was a major metabolite in plasma (Babu et al. 1992). There is evidence in rats (patterns of genotoxic effects induced in the intestine by bile) to suggest that enterohepatic circulation may be involved in the persistence and/or further bioactivation of benzidine reactive metabolites (Chipman and Mohn 1989; Percy et al. 1989).

3.5.2 Mechanisms of Toxicity

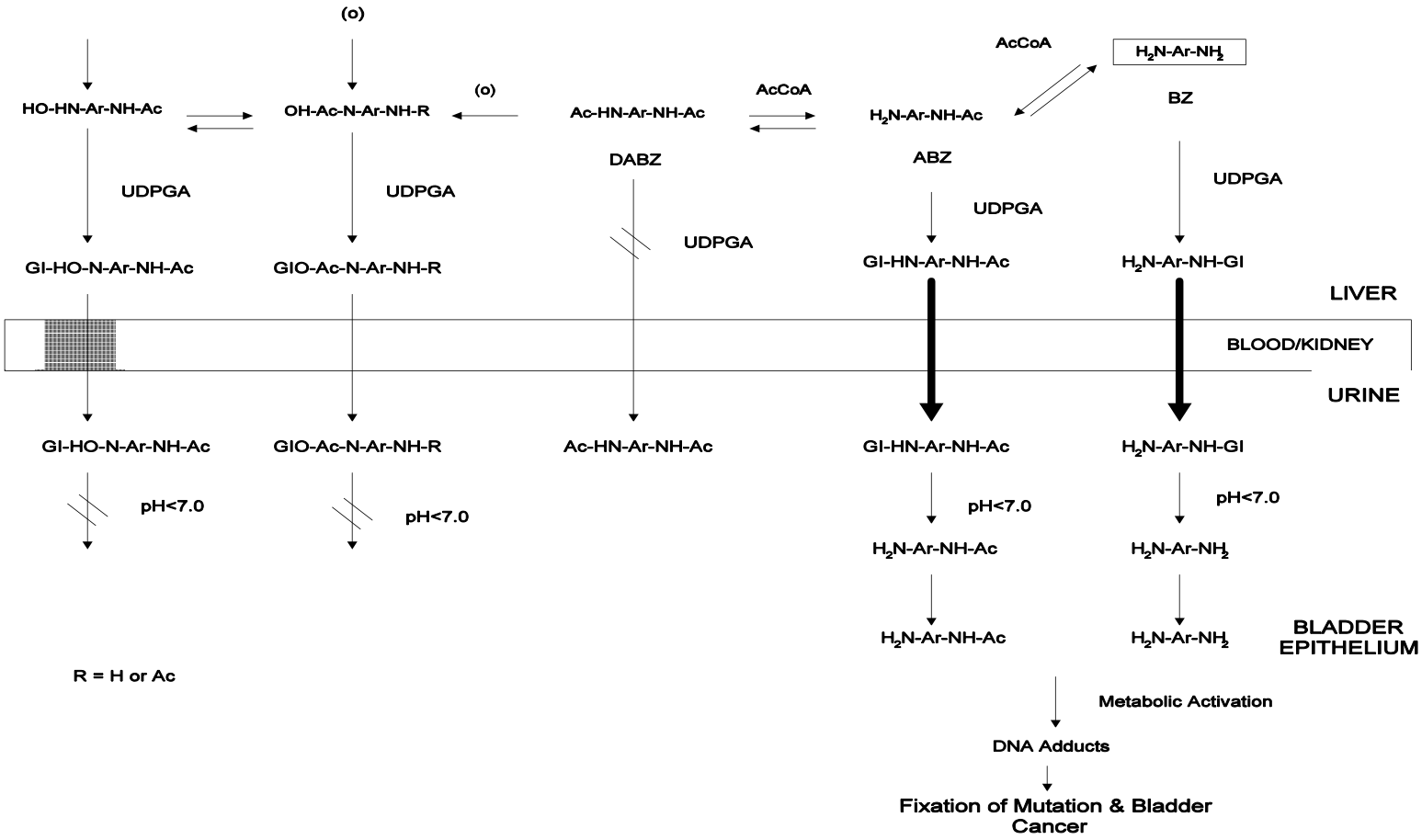
Cancer is the principal and best documented toxic effect of benzidine in both humans (Bi et al. 1992; Bulbulyan et al. 1995; Delzell et al. 1989; Goldwater et al. 1965; Mancusco and El-Attar 1967; Mason et al. 1986; Montanaro et al. 1997; Naito et al. 1995; Piolatto et al. 1991; Shinka et al. 1991; Wu 1988; Xue-Yun et al. 1990; Zavon et al. 1973) and animals (Bonser 1959; Frith et al. 1979, 1980; Griswold et al. 1968; Littlefield et al. 1984; Nelson et al. 1982; Saffiotti et al. 1967; Schieferstein 1982; Spitz et al. 1950; Vesselinovitch et al. 1975; Vorce and Goodman 1989; Zabezhinskii 1970). Like many other arylamines, benzidine is postulated to require metabolic activation to electrophilic derivatives in order to manifest its carcinogenicity and genotoxicity through covalent binding with DNA (Beland et al. 1983; Brouns et al. 1982; Iba 1987; Lang and Iba 1988). One metabolic scheme for bladder cancer, summarized by Wang et al. (1990), involves N-acetylation, N-hydroxylation in the liver, transport of the glucuronide conjugate of the resulting intermediate to the bladder, then hydrolysis of the conjugate followed by absorption and ultimate activation via O-acetylation. This scheme was based on results from experiments in rats with implanted heterotrophic bladders (Wang et al. 1990). In order to circumvent systemic circulation and nonbladder-associated metabolism, benzidine and several metabolites were instilled once a week for 20 weeks directly into these heterotrophic bladders. Heterotrophic bladder cancer was observed in 1 of 39 control animals, 1 of 29 treated with benzidine, 18 of 30 treated with N'-hydroxy-N-acetylbenzidine, and in all 28 treated with N'-hydroxy-N-acetylbenzidine N'-glucuronide. This, however, did not entirely explain the fact that benzidine induces primarily liver cancer in rats, whereas dogs and humans develop bladder cancer as a result of exposure to benzidine. Studies by Zenser and coworkers have provided a

hypothesis that is supported by experimental data. Based on the fact that rats favor acetylation of benzidine and N-acetylbenzidine (Lakshmi et al. 1995a), it was proposed that N-acetylated-N'-glucuronide would not accumulate in rat urine because (1) little N-acetylbenzidine is available and (2) rat hepatic UDP-glucuronosyltransferase activity for N-acetylbenzidine is much lower than in humans. Transient increases in hepatic N-acetylbenzidine that might occur because deacetylation could lead to N-oxidation to hydroxylamine, or N,N'-diacetylbenzidine could undergo N-oxidation and subsequent O-acetylation or N,O-transacetylation to yield mono or diacetylated DNA adducts seen in rat liver. Undetectable or low levels of urinary benzidine or N-acetylbenzidine and/or their glucuronides is consistent with the low incidence of bladder cancer in rats. Results from Morton et al. (1980) demonstrated that in rodents, N-hydroxy-N,N'-diacetylbenzidine can be esterified to an electrophilic reactant by hepatic sulfotransferases and suggested the involvement of this metabolite in the liver carcinogenicity of benzidine.

In humans, Lakshmi et al. (1995a) proposed that benzidine and N-acetylbenzidine are glucuronidated in the liver and transported to the bladder lumen where they are hydrolyzed by acidic urine. Activation in the bladder could include peroxidation by prostaglandin H synthetase (Flammang et al. 1989; Wise et al. 1984c), oxidation by cytochrome P-450 (Butler et al. 1989), and O-esterification by O-acetyltransferase (Frederickson et al. 1992), or N,O-acetyltransferase (Hatcher et al. 1992). N-Acetylbenzidine can be further hydroxylated before glucuronidation. Dogs are nonacetylators, but their ability to form benzidine-N-glucuronide (Babu et al. 1992, 1993a) may contribute to the high incidence of bladder rather than liver cancer. The glucuronides of benzidine and N-acetetylbenzidine were found to be much more stable at pH 7.4 than 5.3 (Babu et al. 1992, 1995). The proposed role of metabolism in benzidine-induced cancer in liver and urinary bladder is presented in Figure 3-5; this figure has been adapted from Babu et al. (1995).

Regardless of the pathway involved, DNA adducts with N-acetylated benzidine derivatives have been observed in rodents and humans, principally that of N-(deoxyguanosin-8-yl)-N'-acetylbenzidine (Kennelly et al. 1984; Martin et al. 1982; Rothman et al. 1996a; Yamazoe et al. 1988). This DNA adduct is presumed to be formed by O-acetylation of N'-hydroxy-N-acetylbenzidine and subsequent binding to a DNA base. As previously mentioned, acidic urine is thought to release the amine from the glucuronides; the amine can then be activated, for example, by prostaglandin H synthase to initiate carcinogenesis. The predominant reactive intermediate generated by prostaglandin H synthase and related peroxidases has been identified as benzidinediimine, and it reacts primarily with the C8 position of deoxyguanosine (Yamazoe et al. 1988). Lakshmi et al. (1994) suggested that at acid pH, benzidinediimine is in

Figure 3-5. The Proposed Role of Metabolism in Benzidine-Induced Cancer



ABZ = N'-acetylbenzidine, AcCoA = Acetylcoenzyme A, BZ = Benzidine, DABZ = N,N'-diacetylbenzidine, HO-HN-Ar-NH-Ac = N'-hydroxy-N-acetylbenzidine, OH-Ac-N-Ar-NH-R = N-hydroxy-N-acetylbenzidine, UDPGA = UDP-glucuronic acid

Source: Babu et al. (1995)

equilibrium with nitrenium ion, the species proposed to bind to bladder DNA. Furthermore, they showed that the *in vitro* formation of benzidinediimine could be prevented by conjugation with glutathione to generate 3-(glutathion-S-yl)-benzidine. The formation of the conjugate was dependent upon diimine and not benzidine, suggesting that conversion of benzidine to diimine occurs before formation of the conjugate.

Susceptibility to bladder cancer has been linked to the slow acetylator type of the polymorphic NAT2 N-acetyltransferase gene (Blum et al. 1991; Ohsako and Deguchi 1990). A study of Chinese workers with high exposure to benzidine demonstrated a 100-fold increased risk for bladder cancer (Bi et al. 1992). However, when members of the benzidine-exposed cohort were screened for their acetylator phenotype and genotype, no positive association was found between N-acetylation phenotype or genotype and bladder cancer risk (Hayes et al. 1993). The authors suggested that the difference between their results and those of the previous investigators may be due to the fact that their subjects were only exposed to benzidine, while those in earlier studies were also exposed to other aromatic amines (monoamines). This was consistent with the fact that N-acetylmonoamines undergo much less oxidation than the monoamine; therefore, for monoamines, acetylation is a detoxification reaction. In contrast, N-acetylbenzidine is relatively reactive and still susceptible to N'-oxidation or N'-glucuronidation; thus, acetylation of benzidine is an activation process potentially leading to bladder cancer. Further research conducted in recent years has shown that slow acetylators are not at increased risk for bladder cancer relative to fast acetylators (Lakshmi et al. 1995a; Rothman et al. 1996a; Zenser et al. 1996). These data are consistent with the observation that benzidine can be acetylated by NAT1 for which genotypic differences in acetylation rate have not been observed. NAT1 in humans may have a dominant role in acetylation of benzidine (Zenser et al. 1996). In addition, the deacylase could have a dominant effect on the profile of benzidine metabolites formed (Lakshmi et al.1995a). These studies are discussed in more detail in Section 3.10.

The genotoxicity of benzidine is also mediated through bioactivated, electrophilic metabolites. This has been shown in: mutagenicity assays in many prokaryotic and eukaryotic systems (Buchholz et al. 1992; Dorado and Pueyo 1988; Duverger-van Bogaert et al. 1995; Henderson et al. 1990; Myhr and Caspary 1988; Sarkar et al. 1990; Zeiger et al. 1992); *in vivo* and *in vitro* unscheduled DNA synthesis tests (Ashby et al. 1990; Steinmetz et al. 1988); chromosomal aberrations assays (Das et al. 1994; Sinsheimer et al. 1992), assays for micronucleus formation (Harper et al. 1989; Mirkova and Ashby 1988; Sanderson and Clark 1993; Tice et al. 1990); and in tests for sister chromatid exchanges (Lindahl-Kiessling et al. 1989). Benzidine metabolites also bind to protein and RNA (Kadlubar et al. 1986b; Lakshmi et al. 1990a; Zenser

et al. 1983), and hemoglobin (Birner et al. 1990; Zwirner-Baier and Neumann 1998), although specific toxicological responses are not yet attributable to these effects.

When male mice were administered 86 ppm benzidine as benzidine dihydrochloride in drinking water for 1 year, 13 of 22 (59%) observed liver tumors were found to contain a mutation in codon 61 of the H-*ras* oncogene (Fox 1990). Although a similar frequency of oncogene mutations were observed in spontaneous liver tumors, much lower frequencies (7–21%) were observed in tumors induced by several nongenotoxic agents. These results are consistent with genotoxicity being at least a component of benzidine's observed carcinogenicity. Epigenetic mechanisms may also be involved, however, as H-*ras* and K-*ras* oncogenes in benzidine-induced liver tumors were found to be hypomethylated in comparison to adjacent nontumor tissue (Vorce and Goodman 1989). Hypomethylation of a gene is thought to enhance its transcription, and thus, benzidine may be capable of facilitating aberrant expression of genes that are involved in carcinogenesis.

3.5.3 Animal-to-Human Extrapolations

Relatively little information is available on noncancer effects of benzidine. Therefore, an attempt to discuss potential interspecies differences or similarities in benzidine noncancer toxicity based on the limited information available is speculative at this time. Benzidine is a bladder carcinogen in humans and dogs, and primarily a liver carcinogen in rodents. The differences in target site are attributed to the species-specific metabolic pathways for activation of benzidine to a reactive intermediate. Extrapolation of carcinogenicity data for benzidine from any one animal species to humans is inappropriate unless there is information suggesting that the particular animal species and humans share common metabolic pathways that produce similar carcinogenic chemical species.

3.6 ENDOCRINE DISRUPTION

Recently, attention has focused on the potential hazardous effects of certain chemicals on the endocrine system because of the ability of these chemicals to mimic or block endogenous hormones, or otherwise interfere with the normal function of the endocrine system. Chemicals with this type of activity are most commonly referred to as endocrine disruptors. Some scientists believe that chemicals with the ability to disrupt the endocrine system are a potential threat to the health of humans, aquatic animals, and wildlife. Others believe that endocrine disrupting chemicals do not pose a significant health risk, particularly in light of the fact that hormone mimics exist in the natural environment. Examples of natural hormone

mimics are the isoflavinoid phytoestrogens (Adlercreutz 1995; Livingston 1978; Mayr et al. 1992). These compounds are derived from plants and are similar in structure and action as endogenous estrogen. While there is some controversy over the public health significance of endocrine disrupting chemicals, it is agreed that the potential exists for these compounds to affect the synthesis, secretion, transport, binding, action, or elimination of natural hormones in the body that are responsible for the maintenance of homeostasis, reproduction, development, and/or behavior (EPA 1997). As a result, endocrine disruptors may play a role in the disruption of sexual function, immune suppression, and neurobehavioral function. Endocrine disruption is also thought to be involved in the induction of breast, testicular, and prostate cancers, as well as endometriosis (Berger 1994; Giwercman et al. 1993; Hoel et al. 1992).

No studies were located regarding endocrine disruption in humans or animals after exposure to benzidine.

3.7 CHILDREN'S SUSCEPTIBILITY

This section discusses potential health effects from exposures during the period from conception to maturity at 18 years of age in humans, when all biological systems will have fully developed. Potential effects on offspring resulting from exposures of parental germ cells are considered, as well as any indirect effects on the fetus and neonate resulting from maternal exposure during gestation and lactation. Relevant animal and *in vitro* models are also discussed.

Children are not small adults. They differ from adults in their exposures and may differ in their susceptibility to hazardous chemicals. Children's unique physiology and behavior can influence the extent of their exposure. Exposures of children are discussed in Section 6.6 Exposures of Children.

Children sometimes differ from adults in their susceptibility to hazardous chemicals, but whether there is a difference depends on the chemical (Guzelian et al. 1992; NRC 1993). Children may be more or less susceptible than adults to health effects, and the relationship may change with developmental age (Guzelian et al. 1992; NRC 1993). Vulnerability often depends on developmental stage. There are critical periods of structural and functional development during both prenatal and postnatal life and a particular structure or function will be most sensitive to disruption during its critical period(s). Damage may not be evident until a later stage of development. There are often differences in pharmacokinetics and metabolism between children and adults. For example, absorption may be different in neonates because of the immaturity of their gastrointestinal tract and their larger skin surface area in proportion to body weight (Morselli et al. 1980; NRC 1993); the gastrointestinal absorption of lead is greatest in infants

and young children (Ziegler et al. 1978). Distribution of xenobiotics may be different; for example, infants have a larger proportion of their bodies as extracellular water and their brains and livers are proportionately larger (Altman and Dittmer 1974; Fomon 1966; Fomon et al. 1982; Owen and Brozek 1966; Widdowson and Dickerson 1964). The infant also has an immature blood-brain barrier (Adinolfi 1985; Johanson 1980) and probably an immature blood-testis barrier (Setchell and Waites 1975). Many xenobiotic metabolizing enzymes have distinctive developmental patterns. At various stages of growth and development, levels of particular enzymes may be higher or lower than those of adults, and sometimes unique enzymes may exist at particular developmental stages (Komori et al. 1990; Leeder and Kearns 1997; NRC 1993; Vieira et al. 1996). Whether differences in xenobiotic metabolism make the child more or less susceptible also depends on whether the relevant enzymes are involved in activation of the parent compound to its toxic form or in detoxification. There may also be differences in excretion, particularly in newborns who all have a low glomerular filtration rate and have not developed efficient tubular secretion and resorption capacities (Altman and Dittmer 1974; NRC 1993; West et al. 1948). Children and adults may differ in their capacity to repair damage from chemical insults. Children also have a longer remaining lifetime in which to express damage from chemicals; this potential is particularly relevant to cancer.

Certain characteristics of the developing human may increase exposure or susceptibility, whereas others may decrease susceptibility to the same chemical. For example, although infants breathe more air per kilogram of body weight than adults breathe, this difference might be somewhat counterbalanced by their alveoli being less developed, which results in a disproportionately smaller surface area for alveolar absorption (NRC 1993).

There are no studies that specifically addressed the health effects of exposure to benzidine in children or immature animals; therefore, it is unknown whether children differ from adults in their susceptibility to health effects from benzidine. Data in adults are mostly derived from occupational studies and, despite the inherent limitations of this type of study, there is unequivocal evidence that benzidine is a bladder carcinogen in humans (Bi et al. 1992; Ferber et al. 1976; Goldwater et al. 1965; Mancuso and El-Attar 1967; Mason et al. 1986; Meigs et al. 1986; Shinka et al. 1991; Tsuchiya et al. 1975; Vigliani and Barsotti 1962; Xue-Yun et al. 1990; Zavon et al. 1973). There is also suggestive evidence of carcinogenicity at other tissue sites as well (Delzell et al. 1989; Morinaga et al. 1982; Okubo et al. 1985; Piolatto et al. 1991; Wu 1988; Zavon et al. 1973).

The only information regarding possible adverse developmental effects in humans is that provided by a study by Wilkins and Sinks (1990) which suggested an association between parental exposure to benzidine and childhood brain tumors. However, results and conclusions of this study are largely speculative due to lack of sufficient validation of the job exposure matrix used to estimate exposure levels. Also, a somewhat limited epidemiological study failed to detect any evidence of elevated rates for birth defects in residents living near a Superfund site contaminated with benzidine (Budnick et al. 1984). However, the extent of actual exposure (if any) to benzidine was not determined. There is considerable evidence that benzidine or its metabolites can be genotoxic, but it is unknown whether parental exposure could result in benzidine metabolites reaching parental germ cells, or whether this might affect childhood development or cancer incidence.

There is no information regarding pharmacokinetics of benzidine in children nor it is known whether benzidine can be stored and excreted in breast milk. Although there have been no direct measurements to determine whether benzidine can cross the placenta, there is some indirect evidence that it or its metabolites do. The evidence is based on the results of a study in which intraperitoneal administration of benzidine to pregnant mice resulted in the induction of micronuclei in the liver of fetuses (Sanderson and Clark 1993); however, when pregnant mice were orally exposed to benzidine, there was no increase in micronucleated cells either in their livers or the livers of their fetuses (Harper et al. 1989). There is no information on whether benzidine can be stored in maternal tissues and be mobilized during pregnancy or lactation.

There is no information on the metabolism of benzidine in children. Analysis of urine and bile from humans exposed to benzidine revealed the presence of metabolites derived from phase I and phase II enzymatic reactions (EPA 1980a, 1986c; Sciarini and Meigs 1961). Biotransformation of benzidine begins with N-acetylation. N-Acetylation in humans is likely done by one of two families of N-acetyltransferases. One of these families, NAT2, is developmentally regulated (Leeder and Kerns 1997). Some enzyme activity can be detected in the fetus by the end of the first trimester. Almost all infants exhibit the slow acetylator phenotype between birth and 2 months of age. The adult phenotype distribution is reached by the age of 4–6 months, whereas adult activity is found by approximately 1–3 years of age. Results from earlier studies had suggested that humans possessing a slow acetylator phenotype may be at a higher risk for developing bladder cancer (Hanke and Krajewska 1990). However, more recent data suggest that the acetylation rate may not be an important risk factor (Hayes et al. 1993; Rothman et al. 1996a). It was also shown that in humans, NAT1 rather than NAT2 plays a more important role in the acetylation of both benzidine and N-acetylbenzidine (Zenser et al. 1996). UGT also

contributes to benzidine metabolism and is known to vary developmentally as well (Leeder and Kearns 1997). Ontogeny is isozyme-specific and adult activity is reached at the age of 6-18 months.

It is generally accepted that benzidine, like many other arylamines, requires metabolic activation by the cytochrome P-450 system in order to manifest genotoxicity and carcinogenicity. The expression of some P-450 enzymes does vary developmentally (Leeder and Kearns 1997). However, for benzidine, the precise cytochrome P-450 isozymes involved in its metabolism by humans are not known.

There are no biomarkers of exposure or effect for benzidine that have been validated in children or in adults exposed as children. No studies were located regarding interactions of benzidine with other chemicals in children or adults.

No information was located regarding pediatric-specific methods for reducing peak absorption following exposure to benzidine, reducing body burden, or interfering with the mechanism of action for toxic effects. In addition, no data were located regarding whether methods for reducing toxic effects of benzidine in adults might be contraindicated in children.

3.8 BIOMARKERS OF EXPOSURE AND EFFECT

Biomarkers are broadly defined as indicators signaling events in biologic systems or samples. They have been classified as markers of exposure, markers of effect, and markers of susceptibility (NAS/NRC 1989).

Due to a nascent understanding of the use and interpretation of biomarkers, implementation of biomarkers as tools of exposure in the general population is very limited. A biomarker of exposure is a xenobiotic substance or its metabolite(s) or the product of an interaction between a xenobiotic agent and some target molecule(s) or cell(s) that is measured within a compartment of an organism (NAS/NRC 1989). The preferred biomarkers of exposure are generally the substance itself or substance-specific metabolites in readily obtainable body fluid(s), or excreta. However, several factors can confound the use and interpretation of biomarkers of exposure. The body burden of a substance may be the result of exposures from more than one source. The substance being measured may be a metabolite of another xenobiotic substance (e.g., high urinary levels of phenol can result from exposure to several different aromatic compounds). Depending on the properties of the substance (e.g., biologic half-life) and environmental conditions (e.g., duration and route of exposure), the substance and all of its metabolites may have left the

body by the time samples can be taken. It may be difficult to identify individuals exposed to hazardous substances that are commonly found in body tissues and fluids (e.g., essential mineral nutrients such as copper, zinc, and selenium). Biomarkers of exposure to benzidine are discussed in Section 3.8.1.

Biomarkers of effect are defined as any measurable biochemical, physiologic, or other alteration within an organism that, depending on magnitude, can be recognized as an established or potential health impairment or disease (NAS/NRC 1989). This definition encompasses biochemical or cellular signals of tissue dysfunction (e.g., increased liver enzyme activity or pathologic changes in female genital epithelial cells), as well as physiologic signs of dysfunction such as increased blood pressure or decreased lung capacity. Note that these markers are not often substance specific. They also may not be directly adverse, but can indicate potential health impairment (e.g., DNA adducts). Biomarkers of effects caused by benzidine are discussed in Section 3.8.2.

A biomarker of susceptibility is an indicator of an inherent or acquired limitation of an organism's ability to respond to the challenge of exposure to a specific xenobiotic substance. It can be an intrinsic genetic or other characteristic or a preexisting disease that results in an increase in absorbed dose, a decrease in the biologically effective dose, or a target tissue response. If biomarkers of susceptibility exist, they are discussed in Section 3.10 “Populations That Are Unusually Susceptible”.

3.8.1 Biomarkers Used to Identify or Quantify Exposure to Benzidine

As indicated by a number of studies previously discussed in Section 3.4.3 (Meigs et al. 1951, 1954; Sciarini and Meigs 1961), a variety of benzidine compounds can be detected in the urine of humans exposed to benzidine or benzidine-based dyes. These include benzidine, hydroxybenzidine, N-acetyl-benzidine, and N,N'-diacetylbenzidine, and various glucuronic acid conjugates of these compounds. For example, Dewan et al. (1988) used a high performance liquid chromatography instrument equipped with an absorbance detector to monitor benzidine and its mono- and diacetylated derivatives in the urine of workers exposed at a dye-manufacturing facility. Detection limits were 0.25 µg/L for the former two compounds, and 0.5 µg/L for the latter. These biomarkers are specific for benzidine or benzidine-derived compounds, and can be confidently identified with the use of appropriate standards, especially in situations where exposure to such compounds seems reasonable. Although there are no background levels of such biomarker compounds, their urinary levels are likely to fall below detection limits within several days to 2 weeks due to relatively short biological half-lives.

With respect to biomarkers that can indicate or quantify exposure, covalent binding of benzidine and some of its congeners to hemoglobin has been demonstrated as a promising approach (Birner et al. 1990; Neumann 1988; Zwirner-Baier and Neumann 1998). After oral treatment of rats with 142 mg/kg of benzidine, hemoglobin adducts were hydrolyzed under alkaline conditions, extracted, and then analyzed by high performance liquid chromatography with an electrochemical detector (Birner et al. 1990). The method is based on the cleavage of sulfinic acid amides formed *in vivo* with hemoglobin by the reaction of metabolically generated nitrosoarenes and the SH-group of cysteine. With benzidine, three cleavage products were identified: N-acetylbenzidine (the major component), benzidine itself, and 4-amino-biphenyl (suggesting the occurrence of a previously unknown metabolic pathway for benzidine). The authors proposed using the analysis of hemoglobin adducts in human blood to monitor for exposure to carcinogenic compounds such as benzidine. The same cleavage products (benzidine, N-acetylbenzidine, and 4-aminobiphenyl) were found in a ratio of 1:17:1 in a more recent study by the oral route in rats (Zwirner-Baier and Neumann 1998).

Adducts of benzidine and its metabolites with DNA can also be an extremely sensitive biomarker for detecting and quantifying exposure (Levy and Weber 1988). Hepatic DNA was isolated from mice injected intraperitoneally with 90 mg/kg of benzidine, hydrolyzed, and then solvent-treated to enhance the relative concentration of nucleotide-benzidine adducts. Following ^{32}P postlabelling, these adducts were resolved by reverse-phase ion-pair high performance liquid chromatography. The major benzidine adduct appeared to be acetylated. This method detected adducts after as little as 3 hours of exposure, and required less than 1 µg of mouse hepatic DNA. A similar approach has detected attomoles (10^{-18} moles) of benzidine-DNA adduct in human peripheral lymphocytes exposed *in vitro* to benzidine (Gupta et al. 1988). The use of standards and controls are important to this methodology of DNA adduct characterization, which is extremely sensitive and relatively rapid. Benzidine-DNA adducts have also been found *in vivo* in peripheral white blood cells (WBC) and exfoliated urothelial cells of workers occupationally exposed to benzidine (Rothman et al. 1996a; Zhou et al. 1997). The most predominant adduct (N-acetylated) co-chromatographed with a synthetic N-(3'-phosphodeoxyguanosin-8-yl)-N'-acetylbenzidine standard; the median level (range) of this adduct in WBC DNA was 194.4 (3.2–975) RAL (relative adduct labeling) x 10^9 in exposed workers and 1.4 (0.1–6.4) in the control subjects. There was a striking correlation between levels of this DNA adduct in WBCs and exfoliated urothelial cells; additionally, the combined levels of urinary benzidine, N-acetylbenzidine, and N,N'-diacetylbenzidine correlated with the levels of the major DNA adduct found in both WBCs and exfoliated urothelial cells (Zhou et al. 1997). A recent study reported that the mutagenicity of urine from workers to Salmonella strain YG1024 was exposure related (DeMarini et al. 1997). That is, the mean urinary mutagenicity

(revertants/µmol creatinine) of unexposed controls was 2.8 compared to 8.2 in low-exposure workers and 123 in high-exposure workers. In addition, urinary mutagenicity showed a strong positive correlation with benzidine urinary metabolites and the level of the presumptive urothelial DNA adduct. Adducts of benzidine with either hemoglobin or DNA are specific for exposure to benzidine or to any substance that, as a result of biotransformation in the body, may give origin to benzidine.

The possibility that mutations in specific genes can be used as biomarkers of exposure (and/or effect) to benzidine (and other arylamines) has also been examined. For example, Taylor et al. (1996) compared the frequency and pattern of p53 (a well-known tumor suppressor gene) mutations in 34 bladder tumors from American workers with past high-level occupational exposure to arylamines to those in 30 bladder tumors from people without such exposure. Their analysis revealed no differences for p53 mutations between the two groups suggesting that exposure to arylamines does not leave a mutational "footprint" in the p53 gene. In contrast, Yasunaga et al. (1997) found that bladder lesions from 26 Japanese workers occupationally exposed to aromatic amines exhibited different patterns of p53 mutations from nonoccupational bladder lesions. Cytosine to thymidine transitions were the most common mutations. The reason for this discrepancy is not apparent. The results of Yasunaga et al. (1997) also suggested that the multifocality noted in most occupational bladder cancer arises both from multiple clonal lesions and from the dissemination of a single clone. Sorlie et al. (1998) analyzed the pattern of mutations in four genes (p53, $p16^{MTS1}$, $p21^{WAF1}$, and H-*ras*) in 21 cases of bladder cancer among western European workers exposed to aromatic amines and found that only p53 had a high frequency of mutations. The spectrum of mutations found was highly suggestive of an involvement of exogenous carcinogens and was identical to the spectrum of p53 mutations detected in bladder cancers of the general population. In the exposed workers, p53 mutations were associated with tumor grade and with high occupational and tobacco exposure. The data further suggested that the same carcinogens may be responsible for the development of bladder cancer in workers exposed to aromatic amines and in the general population. An additional study of workers exposed to 2-naphthylamine and benzidine reported over expression of the *ras* oncogene in bladder biopsies relative to unexposed cancer patients, patients with benign conditions of the bladder, and healthy subjects (Novara et al. 1996). c-erB-2 (an oncogene whose expression is associated with invasiveness and prognosis) was over expressed in both unexposed and exposed bladder cancer cases and, therefore, was not associated with occupational exposure.

3.8.2 Biomarkers Used to Characterize Effects Caused by Benzidine

Early studies (Meigs et al. 1986; Vigliani and Barsotti 1962; Zavon et al. 1973) used bloody urine and cytoscopy as a biomarker for observation of bladder carcinomas. More recent studies have focused on the binding of benzidine's activated metabolites to cellular macromolecules. As discussed in the previous section, hemoglobin and DNA adducts derived from benzidine and its metabolites have been identified and quantified in cells of the blood and liver. Following intravenous administration of radiolabeled benzidine in dogs, significant amounts of DNA binding were observed in the liver, kidney, and bladder (Lakshmi et al. 1990a). The highest concentration of binding was found in the bladder transitional epithelium, the principal target tissue for benzidine's carcinogenic action in this species. DNA adducts were also identified and quantified in bladder epithelial and liver tissues of dogs exposed orally to benzidine and N-acetylbenzidine (Beland et al. 1983). Arylamine substitution at the C8 position of deoxyguanosine was the dominant adduct, and the level of adduct formation correlated with the bacterial mutagenicity of these compounds.

As evidence of genotoxicity, human exposure to benzidine and benzidine-based dyes has been shown to induce chromosomal aberrations and polyploidy in circulating peripheral lymphocytes (Mirkova and Lalchev 1990). Mutagenicity has also been detected in the urine and bile of rodents treated with benzidine (Bos et al. 1980, 1981; Chipman and Mohn 1989). The formation of nuclear anomalies (micronuclei, pyknotic, and karyorrhectic nuclei) in intestinal epithelial cells of rats treated with benzidine is consistent with its intestinal carcinogenic effect in this species (Percy et al. 1989), as is the induction of DNA repair synthesis in explanted rabbit bladder tissue exposed to benzidine (McQueen et al. 1987). However, these and other manifestations of benzidine's genotoxicity are not specific to benzidine or even arylamine exposure.

The liver cytosol activity of arylsulfotransferase was found to be reduced in rats treated with 120 ppm of benzidine in drinking water (approximately 0.26 mg/kg/day) (Ringer and Norton 1988). In rats, this pattern may be characteristic of, though not unique to, liver carcinogens which can utilize the arylsulfotransferase pathway for bioactivation. Its sensitivity, relative specificity, and relevance as a human biomarker are not currently known. The same uncertainties exist for extrapolation to humans of most of the other toxic effects noted in animals.

In humans, detection and examination of exfoliated bladder cells by urine cytology may be useful in detecting bladder cancer (Crosby et al. 1991; Mason et al. 1986; Tsuchiya et al. 1975). Although relevant

to benzidine's principal toxicological effect of concern in humans, this biomarker would not be specific to benzidine exposure. It has been reported that bladder cancer patients who have been exposed to benzidine are at increased risk of developing multiple primary tumors at other sites in comparison with nonexposed bladder cancer patients (Morinaga et al. 1982; Okubo et al. 1985). This clinical presentation might suggest that a possible benzidine exposure has taken place.

Tanigawa et al. (1990) observed an unusual and persistent natural killer cell activity pattern toward a target human cancer cell line in individuals who had been exposed to benzidine or β-naphthylamine. Although overall activity was apparently normal, the unusual natural killer cell pattern was achieved by an increase in the number of circulating natural killer cells, each of which possessed a lower than normal per cell activity. This effect was observed even 20 years after exposure, and the mechanisms are currently unknown. Should this observation be confirmed and verified as a benzidine effect, investigation of its scientific basis, reproducibility, and specificity might allow for its development as a sensitive biomarker. This study also reported an increased proportion of Leu11a-(CD-16) positive circulating peripheral lymphocytes in workers exposed to benzidine or β-naphthylamine. A follow-up study (Araki et al. 1993) reported that the total and relative numbers of T lymphocyte subpopulations (CD4+ and CD3+) in the high-exposure group were significantly lower than those found in the control group. The authors concluded that measurement of CD4+ lymphocytes provides a useful biological marker of past exposure to aromatic amines, including benzidine. The more recent studies of Sung et al. (1995) and Tanigawa et al. (1996) also found changes in the numbers of T lymphocyte subpopulations in high-exposure groups of dye workers exposed to aromatic amines. Tanigawa et al. (1996) suggested that the observed changes in T lymphocytes resemble those noted in patients receiving immunosuppressive therapy.

Additional information concerning biomarkers for effects on the immune, renal, and hepatic systems can be found in the CDC/ATSDR Subcommittee Report on Biological Indicators of Organ Damage (CDC/ATSDR 1990), and on the neurological system in the Office of Technology Assessment Report on Identifying and Controlling Poisons of the Nervous System (OTA 1990). A more detailed discussion of the health effects caused by benzidine can be found in Section 3.2.

Absorption, Distribution, Metabolism, and Excretion. No quantitative studies were located regarding the rate or extent of absorption in humans or animals after inhalation or oral exposure to benzidine. However, relatively rapid absorption can be inferred from an occupational study in which urinary levels of benzidine increased 4-fold during a workshift involving primarily inhalation exposure (Zavon et al. 1973). Similarly, significant absorption of benzidine-based dyes was indicated by the detection of benzidine and its acetylated metabolites in the urine of workers exposed to the dust of Direct Black 38 (Dewan et al. 1988). However, the relative contributions of pulmonary, oral, and dermal absorption were not addressed. Absorption after oral exposure to benzidine in mice (Martin et al. 1982) and dogs (Beland et al. 1983) was indirectly indicated by covalent binding of benzidine metabolites to DNA in liver and bladder tissue. Benzidine produced by microfloral reduction of benzidine-based dyes in the intestines was indirectly determined to be absorbable, and benzidine (but not the dyes) was demonstrated to be transported across the mucosa of an isolated segment of rat intestine in a perfusion chamber (Bos et al. 1986). Benzidine, in combination with acetone or DMSO, sodium lauryl sulfate, and/or methyl nicotinate was shown to be absorbed by isolated perfused porcine skin flaps; the addition of the reducing agent $SnCl_2$ resulted in an inhibition of benzidine absorption (Baynes et al. 1996). No quantitative studies were located regarding absorption in humans after dermal exposure to benzidine, but benzidine and its metabolites were detected in the post-workshift urine of workers exposed to benzidine (Ferber et al. 1976; Meigs et al. 1951, 1954; Sciarini and Meigs 1961). After applying radiolabeled benzidine to the shaved skins of rats, the percentages of applied radioactivity detected in the blood were 0.2, 0.3, and 0.7% after 1, 8, and 24 hours, respectively (Shah and Guthrie 1983). Benzidine-derived radioactivity was distributed throughout the body, with approximately half still remaining at the site of application after 24 hours. A study of dermal application of radiolabeled benzidine to the shaved skin of rabbits and rats reported 3 and 5% of the radioactivity detected in the urine and feces of the rabbits, respectively, and 0.05 and 0.16% in the urine and feces of the rats, respectively (Aldrich et al. 1986). Although sparse, the collective data suggest that benzidine can be absorbed by all three major routes, with absorption and distribution occurring fairly rapidly after inhalation or oral exposure. The relative contribution of inhalation, oral, and dermal routes to typical occupational exposures remains one area where further studies could be useful. More recent studies have also provided evidence of inhalation absorption in workers exposed to benzidine (DeMarini et al. 1997; Rothman et al. 1996a).

No studies were located regarding distribution in humans after inhalation, oral, or dermal exposure to benzidine.

tissues to activate benzidine-based dyes to a mutagenic/clastogenic form. This research is sponsored by the Department of Health and Human Services (DHHS).

Dr. T.R. Devereux, from the National Institute of Environmental Health Sciences (NIEHS), National Institute of Health, plans to examine a set of human bladder tumors from a cohort of people occupationally exposed to benzidine by genomic hybridization for gains or losses of genetic material. The research is sponsored by NIEHS.

Dr. G.P. Hemstreet at the University of Oklahoma Health Science Center, Oklahoma City, Oklahoma, plans to continue screening studies in a cohort of workers occupationally exposed to benzidine both to develop a bladder cancer screening approach and to reduce the number of deaths and serious morbidity associated with bladder cancer in this cohort. The research is sponsored by NIOSH.

Dr. G.M. Lower, from BC Research, Gay Mills, Wisconsin, will use sulfamethazine phenotyping to examine well-defined Japanese bladder cancer populations with and without histories of cigarette smoking, and with and without known occupational exposure to benzidine or β-naphthylamine. The results are expected to allow assessment of the relationship between arylamine-induced bladder cancer and the slow acetylator phenotype as a determinant of susceptibility. This research is sponsored by the DHHS.

Dr. T.K. Rao, from Integrated Laboratory Systems, Research Triangle Park, North Carolina, proposes to conduct cell-mediated metabolism/mutagenesis studies to examine cell/tissue/species specificity for a number of bladder carcinogens. Several factors that affect induction and promotion of bladder carcinogenesis will be investigated. This research is sponsored by the DHHS.

Dr. T.V. Zenser at the Veterans' Administration Medical Center, St. Louis, Missouri, is conducting research aimed at elucidating the pathways involved in benzidine metabolism and DNA-adduct formation in humans exposed to benzidine. This research is sponsored by the National Cancer Institute. Under the sponsorship of the Department of Veterans Affairs, Dr. Zenser is also assessing the possible formation of the DNA-adduct, N'-(3'-monophospho-deoxyguanosin-8-yl)-N-acetylbenzidine, by peroxidatic activation of N-acetylbenzidine in workers exposed to benzidine.