DYES METABOLIZED TO BENZIDINE

1. Exposure Data

See Monograph on Benzidine in this volume.

2. Studies of Cancer in Humans

The Working Group reviewed available epidemiological studies that evaluated the association between exposure to benzidine derivatives or azo dyes metabolized to benzidine and cancer. Three benzidine derivatives used in the manufacture of azo dyes were reviewed: 3,3′-dimethylbenzidine (*ortho*-tolidine), 3,3′-dimethoxybenzidine (*ortho*-dianisidine), and 3,3′-dichlorobenzidine. These benzidine congeners have been evaluated in previous Monographs and classified in Group 2B (*possibly carcinogenic to humans*) (IARC, 1972, 1974, 1982, 1987). The Working Group also reviewed available human studies on the carcinogenicity of three azo dyes metabolized to benzidine: Direct Brown 95, Direct Blue 6, and Direct Black 38, all classified in Group 2A as benzidine-based dyes (IARC, 1982, 1987). Most studies that analysed the association between benzidine derivatives and cancer were conducted in workers employed in the manufacture of azo dyes. In these studies, confounding by concomitant exposure to the Group-1 carcinogens benzidine and 2-naphthylamine is likely, and precludes the evaluation of the effect of carcinogenicity by the individual congeners (see Monographs on benzidine and 2-naphthylamine in this volume). The Working Group was aware of studies among workers likely to be exposed to azo dyes metabolized to benzidine. One of these occupational groups, hairdressers and barbers, was evaluated in another Monograph in this volume by this Working Group (see Monograph on occupational exposures of hairdressers and barbers). Other occupations, such as shoe and leather workers, textile workers, and painters, have been evaluated in previous volumes (IARC 1987, 1990, Vol 98). For this evaluation, studies on these occupations were reviewed, but only when they specifically listed exposure to benzidine derivatives or azo dyes metabolized to benzidine. In some instances noted below, exposure to a specific azo dye was not mentioned.

2.1 Benzidine derivatives

2.1.1 *Cohort studies* (Table 2.1)

IARC Monograph Volume 29 (IARC, 1982) reported the results of three studies of workers exposed to 3,3′-dichlorobenzidine (Gerarde and Gerarde, 1974; Gadian, 1975; MacIntyre, 1975). At the time, the Working Group noted that these studies examined relatively small cohorts of workers, and that the time since first exposure to 3,3′-dichlorobenzidine was 20 or fewer years for over two thirds of the workers. Also, in the study by Gerarde and Gerarde (1974), follow-up of exposed workers was less than 85% complete. [The Working Group agreed that the significance of these findings is uncertain.]

As part of a notification programme in a dye-intermediary production plant, Schulte *et al.* (1985) identified a cohort of 1385 workers employed from 1940 to 1972. The cohort was potentially exposed to 3,3′-dimethylbenzidine, 2-naphthylamine, benzidine and 1-naphthylamine, with 2-naphthylamine as the major exposure. A questionnaire was used to obtain information on occupational history, particularly relating to working in the 2-naphthylamine-grinding room, use of protective measures, and history of other jobs with potential exposure to bladder carcinogens. Additionally, information was obtained on alcohol, tobacco, coffee and artificial sweetener use, and personal health history. Follow-up was conducted from date of first employment until 1982. Incidence rates in the cohort were compared to estimated incidence rates for bladder cancer in the United States. For the entire cohort, 13 cases of bladder cancer were observed (overall RR, 3.9; 95% CI, 2.2–6.8). Stratified analyses by race and length of employment also showed statistically significant associations. The most remarkable was among black workers with invasive cancer and more than 10 years of employment (RR, 111.1; 95% CI, 35.0–352.5). The authors reported that, in general, black workers had jobs involving greater exposure to 2-naphthylamine.

In a nested case–control study, Schulte *et al.* (1986) evaluated the effect of potential risk factors on the risk for bladder cancer. The analysis included the aforementioned 13 bladder-cancer cases and the remaining 1372 workers in the cohort as controls. Workers were considered exposed if they had more than one year of employment or any employment in two departments with potential 2-naphthylamine exposure. The crude odds ratio between exposure and bladder cancer was 7.0 (95% CI, 3.9–12.4). The odds ratio for the association between bladder cancer and duration of employment, controlling for smoking and source of drinking water, was 4.3 (95% CI, 1.8–10.3).

Sinks *et al.* (1992) evaluated cancer risk at a paperboard-printing manufacturing plant in Georgia, USA. The cohort consisted of 2050 workers employed for more than one day, with duration of employment obtained through company records. Company material safety-data sheets were reviewed and potential carcinogens were identified. From supplier information, the authors determined that pigments were manufactured from 3,3′-dichlorobenzidine and *ortho*-toluidine, but these substances were not identified in laboratory tests. One bladder-cancer death (SMR 2.6; 95% CI, 0.1–14.5) and one renal cell cancer death (SMR, 1.4; 95% CI, 0.0–7.8) were observed. For the incidence analysis, six cases of

Table 2.1. Summary of cohort studies of populations exposed to [benzidine derivatives and] azo dyes metabolized to benzidine

Reference, location, name of study	Cohort description	Exposure assessment	Organ site (ICD code)	Exposure categories	No. of cases/ deaths	Relative risk (95% CI)*	Adjustment factors	Comments
Benzidine derivatives								
Schulte *et al.* (1985); Schulte *et al.* (1986) Georgia, USA	Nested case–control study from cohort of 1385 workers employed in a chemical plant during 1940–72	Workers potentially exposed to 3,3′-dimethylbenzidine, BZ and BNA. Questionnaire used to obtain occupational history and other risk factors; workers considered exposed if employed >1 year or any time in any 2 departments with potential BNA exposure	Bladder			**OR**		
				Exposed	13	7.0 (3.9–12.4)		
				Duration of employment	13	4.3 (1.8–10.3)	Smoking, source of drinking water	

Table 2.1 (contd)

Reference, location, name of study	Cohort description	Exposure assessment	Organ site (ICD code)	Exposure categories	No. of cases/ deaths	Relative risk (95% CI)*	Adjustment factors	Comments
Sinks *et al.* (1992) Georgia, USA	Cohort of 2050 workers (1828 men, 222 women) employed >1 day at a paperboard printing manufacturing plant during 1957–88; mortality follow up 1957–88; incidence follow-up to 1990; vital status 90%; cause of death 82%; nested case–control study of 6 renal cell cancer cases and 48 controls	Length of employment and department from plant records; materials used from review of MSDS and supplier information				**SMR**	Age, sex	Mortality, national reference; incidence, local reference; which workers were exposed to DCB was not determined
			Renal cell	Overall	1	1.4 (0.0–7.7)		
			Bladder	Overall	1	2.6 (0.1–14.7)		
						SIR		
			Renal cell	Overall	6	3.7 (1.4–8.1)		
			Bladder	Overall		1.1 (0.2–3.1)		
						OR		
				Department (>5 years)				
			Renal cell	Finishing	3	16.6 (1.7–453.1)		
				Maintenance	1	5.3 (0.1–223.4)		

Table 2.1 (contd)

Reference, location, name of study	Cohort description	Exposure assessment	Organ site (ICD code)	Exposure categories	No. of cases/ deaths	Relative risk (95% CI)*	Adjustment factors	Comments
Naito *et al.* (1995) Urban area, Japan	Cohort of 442 workers of a BZ production and dye manufacturing plant (437 men, 5 women) during 1935–88; mortality and incidence follow-up 1935–92; vital status 100%	Workers exposed to one or more substance, including 3,3′-dimethoxybenzidine, BZ and BNA. Duration of employment at BZ manufacture or use facility as surrogate of duration of exposure				**SMR**		National reference; incidence rates reported by duration of exposure; PPE reportedly used among all workers
			Urinary tract (188, 189)	Dye manufacture	6	15.8 (5.8–34.3)		
			Bladder	Dye manufacture	5	27.0 (8.8–63.0)		
Ouellet-Hellstrom & Rench (1996) Connecticut, USA	Cohort of 704 workers (585 men, 119 women) first employed at a chemical plant between 1965 (when BZ production was discontinued) and 1989; incidence follow-up 1965–94; vital status 96%	Personnel records for occupational history; exposure scoring for arylamines (3,3′-dimethoxybenzidine, 3,3′-dimethylbenzidine, DCB, *o*-toluidine, *o*-chloroaniline) for each job title based on expert judgement		**Men**		**SIR**		State reference; workers with testicular cancer had no exposure to arylamines
			Bladder	Overall	7	8.3 (3.3–17.1)		
				Annual cumulative exposure score (CES)				
				No exposure	0	0		
				<2.5	2	5.5 (0.7–19.8)		
				2.5+	5	16.4 (5.3–38.2)		
				CES among smokers				
				<2.5		11.6 (1.4–41.8)		
				2.5+		23.6 (7.7–55.2)		
			Testis	Overall	2	11.4 (1.4–41.1)		

Table 2.1 (contd)

Reference, location, name of study	Cohort description	Exposure assessment	Organ site (ICD code)	Exposure categories	No. of cases/ deaths	Relative risk (95% CI)*	Adjustment factors	Comments
Rosenman & Reilly (2004) Michigan, USA	Cohort of 488 white men employed in a chemical manufacturing facility during 1960–77; mortality follow-up 1979–2001; incidence follow-up 1981–2002 (Michigan Tumor Registry)	Workers classified as exposed to BZ and DCB if employed before 1973, those employed during or after 1973 were exposed to DCB only. Time and length of employment estimated from social security records.	Bladder	Overall	3	**SMR** 8.3 (1.7–24.4)		National reference for SMR and SEER for SIR
				Year started work				
				<1973	3	9.6 (2.0–28.1)		
				≥1973	0	0		
			Lympho-haematopoietic cancer	Overall	6	2.8 (1.4–6.2)		
				Year started work				
				<1973	3	1.8 (0.4–5.3)		
				≥1973	3	6.6 (1.4–19.4)		
			Leukemia	Overall	4	5.1 (1.4–12.9)		
						SIR		
			Bladder	Overall	22	6.9 (4.3–10.4)		

Table 2.1 (contd)

Reference, location, name of study	Cohort description	Exposure assessment	Organ site (ICD code)	Exposure categories	No. of cases/ deaths	Relative risk (95% CI)*	Adjustment factors	Comments
Azo dyes metabolized to benzidine								
Stern *et al.* (1987) Minnesota and Wisconsin, USA	Cohort of 9365 tannery production workers (7085 men, 2280 women), employed during 1940–79 at tannery A and 1940–80 at tannery B; mortality follow-up 1940–82; vital status 95%; cause of death 97%	Occupational history from plant records; duration of employment as surrogate for cumulative exposure; BZ measured in 2 samples of bulk dyes: 2.0 & 55 ppm	Bladder			**SMR**		State reference; no increased SMR for other cancers
				Latency (15+ years)	4	0.5 (0.1–1.3)		
				Department				
				Retan, color, fat-liquor	2	1.0 (0.2–3.2)		
				Finishing	3	0.9 (0.2–2.5)		
			Leukemia (ICD-7, 204)	Latency (15+ years)	10	1.0 (0.5–1.9)		
				Department				
				Retan, color, fat-liquor	3	1.0 (0.2–2.9)		
				Finishing	7	1.3 (0.5–2.6)		
			Lymphomas (ICD-7, 200-203, 205)	Latency (15+ years)	12	0.9 (0.5–1.5)		
				Department				
				Retan, color, fat-liquor	1	ND		
				Finishing	7	0.9 (0.4–1.9)		
Costantini *et al.* (1989) Florence and Pisa, Italy	Cohort of 2926 male workers newly employed in tanneries during 1950–81; at least one period of employment of >6 months; mortality follow-up 1950–83; vital status 99%	Employment in the tannery industry from municipal records	Bladder			**SMR**		National reference
				Overall	5	1.5 (0.5–3.5)		
				Latency (years)				
				<15		0		
				15–19		1.6		
				20–24		2.8		
				25–29		3.9		
				p for trend		>0.05		

Table 2.1 (contd)

Reference, location, name of study	Cohort description	Exposure assessment	Organ site (ICD code)	Exposure categories	No. of cases/ deaths	Relative risk (95% CI)*	Adjustment factors	Comments
You *et al.* (1990) Shanghai, China	Cohort of 1210 workers (1060 men and 150 women) employed >1 year in weighing and formulating or dyeing at textile printing and dyeing plants during 1949–83; vital status 98%	Workers divided in 2 groups based on BZ-based dyes usage: high exposure (>500 kg/month) and low exposure (<500 kg/month)	Bladder	Overall	1	ND		Local reference
Montanaro *et al.* (1997) Genoa, Italy	Cohort of 1244 workers at a tannery (870 men, 374 women) employed >6 months during 1955–88; mortality follow-up to 1994; vital status 96%; cause of death 98%	Length of employment from plant records; information on department only for 25% workers				**SMR**		National and regional reference
			Bladder	Overall	10	2.4 (1.2–4.5)		
				Duration of exposure (years)				
				<5	3	4.4 (0.9–12.8)		
				5–14	0	0 (0–2.8)		
				15+	7	3.0 (1.2–6.1)		
			Lymphoma (200-202)	Overall	1	0.4 (0.01–2.0)		
			Leukemia (204-208)	Overall	0	0 (0.0–0.9)		

BNA, 2-naphthylamine; BZ, benzidine; DCB, 3,3'-dichlorobenzidine; MSDS, material safety data sheets; ND, not determined; SIR, standardized incidence ratio; SMR, standardized mortality ratio

renal-cell cancer were seen (SIR, 3.7; 95% CI, 1.4–8.1), but no increase in bladder-cancer risk was observed (3 cases; SIR, 1.1; 95% CI, 0.2–3.1). In a nested case–control study, the risk for renal-cell cancer by duration of employment and department or work process was evaluated. Employment in the finishing department for five or more years was associated with an increased risk for this cancer type (three cases; OR, 16.6; 95% CI, 1.7–453.1). The authors could not determine if workers from the finishing department were exposed to inks.

Naito *et al.* (1995) conducted a retrospective cohort-mortality study of 442 workers (437 men, 5 women) exposed to one or more substances (mainly benzidine, 2-naphthylamine, 1-naphthylamine, and 3,3′-dimethoxybenzidine) at a benzidine production and dye-manufacturing plant in Japan. No industrial hygiene data for the plant were available; therefore, duration of employment at the facility was used as a surrogate for duration of exposure. Nineteen workers were potentially exposed to 3,3′-dimethoxybenzidine during dye manufacture, and of these only three were exposed solely to 3,3′-dimethoxybenzidine. The authors reported that all workers in the factory wore work clothes, gloves, high rubber boots, and a gas mask. An increased risk for bladder cancer was found among workers engaged in dye manufacture (SMR, 27.0; 95% CI, 8.8–63.0). Increased risks for cancer mortality for other organs were observed, but none were statistically significant [results for lymphohaematopoietic cancers were not reported]. Incidence rate ratios of urothelial cancer increased with duration of exposure (*P* for trend, 0.04). [Due to the small number of workers exposed to 3,3′-dimethoxybenzidine alone or in combination with other arylamines, it is not possible to attribute the increased risk for bladder cancer to exposure to this substance. Furthermore, reduced exposures are likely due to the reported use of personal protective equipment].

Ouellet-Hellstrom and Rench (1996) studied workers at the same chemical plant previously studied by Meigs *et al.* (1986) in Connecticut, USA. The cohort consisted of 704 workers (585 men and 119 women) first employed at a plant during the period 1965–89, after benzidine production was discontinued, and were therefore never exposed to benzidine. Workers were presumably exposed to 3,3′-dichlorobenzidine (predominantly used at the plant), 3,3′-dimethoxybenzidine and 3,3′-dimethylbenzidine. The authors used personnel records at the plant and a questionnaire to obtain occupational history and information on other risk factors such as smoking. Each job title was assigned an exposure score to arylamines based on expert judgment. An annual cumulative exposure score to arylamines was calculated for each worker. Expected numbers of cancers were estimated using cancer-incidence rates for the state of Connecticut. Men had elevated SIRs for cancers of the buccal cavity, bladder, kidney, brain and testis. Only for bladder cancer (SIR, 8.3; 95% CI, 3.3–17.1) and testicular cancer (SIR, 11.4; 95% CI, 1.4–41.1) did this increase reach statistical significance. No excess of lymphohaematopoietic cancers was observed (SIR, 1.1; 95% CI, 0.1–4.1). The two workers with testicular cancer had no exposure to arylamines, and one had worked at the plant for only 15 days. Women had a statistically insignificant increase in breast cancer (SIR, 1.9; 95% CI, 0.4–5.6). The SIR for bladder cancer increased with increasing exposure. This excess bladder cancer

occurred among chemical operators (who worked with arylamines over long periods) and mechanics (who had short periods of exposure that was likely intense). The bladder cancer SIRs by cumulative exposure among smokers were higher than for the total cohort. [This study provides strong evidence of the association between exposure to benzidine-based dyes—or benzidine derivatives metabolised to benzidine—and bladder cancer].

Rosenman and Reilly (2004) analysed a cohort of 488 white men employed in a chemical manufacturing facility in Michigan, USA. The facility produced benzidine from 1960 through 1972 and 3,3′-dichlorobenzidine from 1961 to 2001. Workers were identified from social security records. Since no plant records were available, social security data were used to estimate time of first work and years worked. Analyses were conducted for the entire cohort and separately for people who began to work in 1973 or later, after benzidine production was discontinued. For the whole cohort, an excess of bladder-cancer mortality was observed (SMR, 8.3; 96% CI, 1.7–24.4). All cases occurred in those with five or more years of duration of work. There were six deaths from lymphohaematopoietic cancer (SMR, 2.8; 95% CI, 1.04–6.2), including one from non-Hodgkin lymphoma, one from multiple myeloma, two from chronic lymphocytic leukaemia, one from acute leukaemia, and one from chronic myelogenous leukaemia (SMR for leukaemia, 5.1; 95% CI, 1.4–12.9). Only one bladder cancer case (of 22 observed) occurred among workers starting employment after 1972, when the plant was only producing 3,3′-dichlorobenzidine. A statistically significant increase in mortality from lymphohaematopoietic cancer was observed among workers who began work in 1973 or later. [Not enough latency among those exposed only to 3,3′-dichlorobenzidine may have accumulated].

2.2 **Dyes metabolized to benzidine** (Table 2.2)

2.2.1 *Case reports and proportional mortality studies*

Genin (1977) analysed the urine of 22 workers involved in the drying and grinding of azo dyes metabolized to benzidine (e.g. Direct Black 38) and dyes metabolized to 3,3′-dimethoxybenzidine (e.g. Direct Blue 15) (IARC, 1982, 1993). Benzidine was found in the urine of eight workers, and 3,3′-dimethoxybenzidine in the urine of three. A retrospective search of plant records showed five cases of bladder cancer in dryers and grinders.

In a proportional mortality study of 1429 bleachers and dyers presumed to be exposed to dyes metabolized to benzidine in the United Kingdom, Newhouse (1978) showed no excess deaths from cancer of the bladder (14 deaths observed, 13.1 expected) [The Working Group of Volume 29 noted that the study was limited in that no certificates of deaths occurring in the first 20 years after start of exposure were available, and only approximately one third of the workers included in the analysis had actually been exposed to dyes.]

Table 2.2. Summary of case-control studies of populations exposed to azo dyes metabolized to benzidine

Reference, study location and period	Organ site (ICD code)	Characteristics of cases	Characteristics of controls	Exposure assessment	Exposure categories	Relative risk (95% CI)	Adjustment factors	Comments
Yoshida *et al.* (1971), Kyoto, Japan 1954–1971	Bladder	200 men from three Kyoto hospitals	148 men from two of the three hospitals with urologic diseases other than bladder cancer	Interview with patients or their family or extracts from hospital charts to assess exposure to dyes. BZ-based dyes reportedly used	Overall	6.8 (p=0.002)		3 cases reported the habit of licking brushes dipped in dyes
Myslak *et al.* (1991) East Ruhr area, Germany 1984–87	Bladder (malignant and benign)	403 (290 carcinomas, 113 papillomas) (21 painters) from 3 hospitals; aged 69.6±9.3 (mean±SD); response rate 82%; 100% histologically confirmed	426 (8 painters) with benign prostate disease from same hospitals; response rate 84%	Mailed standardized questionnaire for occupational history	Painters overall	2.8 (1.2–6.3)		Benzidine-based dyes heavily manufactured in Germany before 1950, painters prepared paints themselves

BZ, benzidine; SD, standard deviation

2.2.2 *Cohort studies*

Stern *et al.* (1987) conducted a mortality study in a cohort of 9365 tannery production workers (7085 men, 2280 women), employed at two chrome-leather tanneries in Minnesota and Wisconsin. The processes in both tanneries have remained more or less the same since the end of the 19th century. The authors reported detectable concentrations of benzidine in bulk dyes in the dye room [but exposure was likely to be due to benzidine-based dyes]. Chemical sampling was conducted at both plants. Detectable concentrations (2 and 55 ppm) of benzidine in two samples of bulk dyes were found in the retan/colour/fat-liquor department. Occupational history was obtained from plant records; duration of employment was used as a surrogate for cumulative exposure, and cancer mortality analyses for select causes also examined workers ever employed at specific departments. Expected mortality rates were calculated using US and State death rates (no major differences were observed in risk estimates using both rates; only the latter are presented here). The authors did not observe an excess in the risk for bladder-cancer mortality (tannery A: one observed death; tannery B: four observed deaths; SMR, 1.0; 95% CI, 0.3–2.5). Risk was not increased for other primary sites of cancer mortality. Analysis by duration of exposure did not show increased mortality risk for those workers with more than 15 years of latency (SMR, 0.5; 95% CI, 0.1–1.3). The authors reported that of the five deaths from bladder cancer, four occurred among workers of tannery B, and two of these deaths occurred in the retan/colour/fat-liquor department (1.0 expected), but each of those had worked for less than two months at the tannery.

A cohort mortality-study of 2926 male workers newly employed in tanneries between 1950 and 1981 and with at least one period of employment of more than six months was conducted by Costantini *et al.* (1989) in Florence and Pisa, Italy. Known exposures in the plants include dyes metabolized to benzidine, dyes metabolized to 3,3′-dimethylbenzidine and dyes metabolized to 3,3′-dimethoxybenzidine. Employment in the tannery industry was obtained from municipal records, including beginning and end of work. Non-statistically significant increases in mortality were observed for cancers of the kidney, pancreas, lung, bladder (5 deaths; SMR, 1.5; 95% CI 0.5–3.5) and lymphohaematopoietic system. Analyses by latency did not show positive trends, except for bladder cancer, where the SMR increased with latency, although the trend was not statistically significant. All bladder-cancer deaths occurred in workers who entered the cohort between 1950 and 1964.

You *et al.* (1990) conducted a retrospective cohort study among workers in 17 knitting factories, 10 stocking factories, nine silk printing and dyeing factories and seven printing and dyeing factories in Shanghai, China. The cohort of 1210 workers (1060 men and 150 women) had worked for more than a year in weighing and formulating or dyeing, where they had been exposed to the dust of dyes metabolized to benzidine. Fifteen types of benzidine-derived dyes have been used in these factories, and the dyes used in the

largest quantities included Direct Black 38. Cancer mortality and incidence were analysed among exposed workers by dividing them into two groups based on their usage of benzidine-based dyes: high exposure (> 500 kg/month) and low exposure (< 500 kg/month). Only one case of bladder cancer was observed in the exposed group [no risk estimate was provided].

Montanaro *et al.* (1997) studied mortality in a cohort of 1244 workers employed at a chrome tannery in Genoa, Italy, between 1955 and 1988, where workers were exposed to azo dyes metabolized to benzidine and other chemicals used in the tanning process. Length of employment was obtained from plant records; information on department was available only for 25% of the workers. Mortality for all cancers was 12% higher than expected. An excess of bladder-cancer mortality was observed (10 deaths; SMR, 2.4; 95% CI, 1.2–4.5) as well as an excess of colorectal cancer deaths (SMR, 1.8; 95% CI, 1.1–2.9). No excess deaths due to lymphoma or leukaemia were reported (SMR, 0.4; 95% CI, 0.01–2.0 and SMR, 0; 95% CI, 0.0–0.9, respectively).

2.2.3 *Case–control studies*

A hospital-based case–control study of 200 male bladder-cancer cases and 148 male controls of the same age range with urinary disorders in Kyoto, Japan, showed that 17 (8.5%) of the cases and 2 (1.4%) of the controls had worked in the silk-dyeing industry. The odds ratio for employment in the silk-dyeing industry was 6.8 ($P = 0.002$). At least 7 of the 17 patients with bladder cancer who had worked in the dyeing industry were kimono painters, some of whom may have ingested dyes by holding brushes or spatulas in their mouths while working. Reportedly, benzidine-based dyes were used by these kimono painters (Yoshida, 1971). [The Working Group noted that no data on potential confounding factors were provided.]

In a study in a major industrial area of Germany, Myslak *et al.* (1991) selected 403 malignant and benign bladder-cancer cases from three hospitals, and 426 controls with benign prostate disease from the same hospitals. All of the cases were histologically confirmed. Study participants were mailed a questionnaire to obtain information on complete occupational history and smoking habits. The questionnaires were coded for occupational categories, and study participants were classified as painters if they had been employed in this occupation for at least six months and did not have another occupation known to be associated with bladder cancer. Painters were of interest because benzidine-based dyes were manufactured on a large scale in Germany before 1950, and during that time painters usually prepared the paints themselves. Among the cases, 21 were painters and among the controls eight were painters. The overall relative risk for bladder cancer among painters was 2.8 (95% CI, 1.2–6.3).

3. Studies of Cancer in Experimental Animals

3.1 Direct Black 38

3.1.1 *Oral administration*

(a) Mouse

A group of 60 ICR mice [sex unspecified], four weeks of age, weighing 25–30 g, received 3000 mg/L Direct Black 38 [purity unspecified] in their drinking-water for 55–60 weeks, at which time the 59 surviving animals were killed. Hepatocellular carcinomas were found in 46/59 (78%) mice, and mammary carcinomas in 20/59 (34%); nine animals developed both types of tumours. A further 40 mice were given the same concentration of Direct Black 38 in drinking-water, and two mice were killed every two weeks starting from week 16 of treatment. The first liver tumour occurred in a mouse killed 20 weeks after the start of treatment. No liver or mammary tumour was reported in a group of 20 untreated controls (Asada *et al.,* 1981).

(b) Rat

Groups of 10 male and 10 female Fischer 344 rats, six weeks of age, were fed a diet containing 0, 190, 375, 750, 1500 or 3000 mg/kg [ppm] Direct Black 38 and 1.3% corn oil for 13 weeks. The compound was determined by HPLC to be 87.1 ± 3.4% pure, with the following components: water, 7.13 ± 0.54%; NaCl, 7.9%; benzidine, < 0.004%; and traces of at least eight other impurities. The infrared spectrum was as expected. Surviving rats were killed at 13 weeks. All animals that were given 3000 mg/kg Direct Black 38 died before termination of the experiment: male rats survived for less than five weeks and female rats less than 12 weeks. Of the nine surviving males that received 1500 mg/kg, four (44%) had hepatocellular carcinomas and five (55%) had neoplastic nodules. No male receiving another dose exhibited a tumour, although 7/10 (70%) male animals given 375 mg/kg, 9/10 (90%) males given 750 mg/kg, and 5/9 (55%) males given 1500 mg/kg had foci of cellular alteration or basophilic foci in the liver. Of the females, 5/10 (50%) given 1500 mg/kg exhibited neoplastic nodules in the liver at the termination of the experiment, and all females administered 750 or 1500 mg/kg had foci of cellular alteration in the liver (NTP, 1978; Robens *et al.,* 1980). [The Working Group noted the short duration of the experiment, the small number of animals tested, and the impurity of the compound used.]

Groups of 12–15 Wistar rats were administered 100 mg/L or 500 mg/L commercial Direct Black 38 [purity unspecified; direct Deep Black EX; benzidine-free, as shown by HPLC] in their drinking-water. When the eight rats of the 12 that received 100 mg/L were killed at 60 weeks, no tumour was observed. Of 15 rats administered 500 mg/L Direct Black 38, 13 survived until 60 weeks; two (15%) papillomas and three (23%) carcinomas of the urinary bladder, three (23%) carcinomas of the liver and two (15%) adenocarcinomas of the colon were seen in six animals. No tumour was observed in a control

group of nine rats (Okajima *et al.,* 1975). [The Working Group noted the small number of animals.]

A group of 20 male and 25 female rats (strain and age unspecified) were given 400 mg/L Direct Black 38 [source and purity unspecified] in their drinking-water (0.04%) for 14 months, at which time four males and two females were still alive. One of the females had 'breast cancer' [pathological designation not specified]; no other neoplasm was noted (Niitsu, 1973). [The Working Group noted the poor survival of the animals and the short duration of the experiment; in addition, the number of control animals was not specified.]

3.1.2 *Bladder implantation*

Two groups of 50 female dopamine-deficient mice (weight, 20 g) received either a paraffin wax pellet (20 mg) containing 10% Direct Black 38 [purity unspecified] or a wax pellet alone implanted in the bladder. After 40 weeks, when the surviving animals were killed, three bladder carcinomas were observed among the 21 mice still alive. In the control group, one bladder carcinoma was observed in 36 surviving mice (Niitsu, 1973). [The Working Group noted the short duration of the experiment.]

3.2 Direct Blue 6

3.2.1 *Oral administration*

Groups of 10 male and 10 female Fischer 344 rats, six weeks old, were fed a diet containing 0, 190, 375, 750, 1500 or 3000 mg/kg [ppm] Direct Blue 6 and 1.3% corn oil for 13 weeks. The compound was determined by HPLC to be 59.9 ± 1.9% pure, with the following components: water, 9.18 ± 0.51%; NaCl, 20.8%; benzidine, < 0.004%; and traces of at least eight other impurities. Survivors were killed at 13 weeks. All animals that were given 3000 mg/kg Direct Blue 6 and one male rat that received 1500 mg/kg diet died before termination of the study; all males given the highest dose died before five weeks on the study, and all females at that dose were dead by 10 weeks. Liver-cell tumours were seen in eight of 10 (80%) males given 1500 mg/kg; two (20%) were hepatocellular carcinomas and six (60%) were neoplastic nodules. Of animals given 3000 mg/kg, 1/9 (11%) males and 7/9 (78%) females were found to have liver-cell tumours at autopsy before the termination of the experiment: four of the tumours in females were hepatocellular carcinomas and three were neoplastic nodules. No neoplastic lesion was seen in animals of either sex given lower doses. The first tumours appeared after four weeks of feeding in the males and after five weeks of feeding in the females. Almost all animals fed 750 or 1500 mg/kg exhibited foci of cellular alterations in the liver and some basophilic foci were seen in the livers of animals receiving 3000 mg/kg. In the same bioassay, no increased incidence of tumours, compared with that in controls, was found in groups of 10 male and 10 female B6C3F1 mice fed diets containing 750, 1500, 3000, 6000 or 12,500 mg/kg [ppm] of Direct Blue 6 and killed 13 weeks later (NTP, 1978;

Robens *et al.*, 1980). [The Working Group noted the short duration of the experiment, the limited number of animals tested, and the impurity of the compound used.]

Twenty female Wistar rats [age unspecified] were given 400 mg/L Direct Blue 6 [purity unspecified] in their drinking-water (0.04%) for 14 months. At 12 months, 12 animals were still alive, and one (8%) had a squamous-cell carcinoma of the outer ear. No other neoplasm was found (Niitsu, 1973). [The Working Group noted the small number of animals and the lack of a control group.]

3.2.2 *Subcutaneous and/or intramuscular administration*

(a) Rat

A group of 20 male and female rats (strain unspecified), 3–4 months of age and weighing 100–159 g, were treated with doses of 10 mg Direct Blue (purity, 97%) by weekly or bi-weekly subcutaneous injections up to a total dose of 200 mg. Animals survived less than 270 days. No tumours were seen. No control group was included (Fujita *et al.,* 1957). [The Working Group noted the poor survival and lack of proper controls.]

3.2.3 *Bladder implantation*

A group of 50 female dd mice (20 g) had either a paraffin wax pellet (20 mg) containing 10% Direct Blue 6 [purity unspecified] or a wax pellet without dye implanted in the bladder. After 40 weeks, when the surviving animals were killed, bladder carcinomas were found in 3 of 21 (14%) [$P = 0.13$; Fisher exact test] treated mice and in 1 of 36 (3%) controls still alive at that time (Niitsu, 1973).

3.3 Direct Brown 95

3.3.1 *Oral administration*

(a) Rat

Groups of 10 male and 10 female Fischer 344 rats, 6 weeks old, were fed a diet containing 0, 190, 375, 750, 1500 or 3000 mg/kg [ppm] Direct Brown 95 and 1.3% corn oil. The compound was determined by HPLC to be 72.2 ± 7.0% pure, with the following components: water, 4.99 ± 0.22%; NaCl, 14.9%; benzidine, < 0.004%; and traces of at least eight other impurities. Surviving rats were killed at 13 weeks. All male and female animals that received 1500 or 3000 ppm Direct Brown 95 died before termination of the study: male rats survived for less than five weeks, females given the high dose survived less than six weeks on the study, and females fed 1500 ppm up to 12 weeks; two males receiving 750 ppm Direct Brown 95 also died before the end of the study. Among male rats, basophilic foci or foci of cellular alteration were seen in 2/9 (22%) animals given 3000 ppm, in 7/8 (87%) given 1500 ppm and in 8/10 (80%) given 750 ppm. Among

females, 4/8 (50%) given the 1500-ppm dose exhibited neoplastic nodules, and one of these showed a hepatocellular carcinoma; basophilic foci or foci of cellular alteration in the liver were seen in 3/8 (37%) females given 3000 ppm, 6/8 (75%) given 1500 ppm and 3/10 (30%) given 750 ppm. No other relevant findings in relation to neoplastic development were seen in these animals.

(b) Mouse

In the same bioassay, groups of 10 male B6C3F1 mice, 6–7 weeks of age, were fed a diet containing 750, 1500, 3000, 6000 or 12,500 ppm [ppm] Direct Brown 95 and 1.3% corn oil. Groups of 10 female B6C3F1 mice, 6–7 weeks of age, were fed similar diets containing 350, 750, 1500, 3000 or 6000 ppm of the dye. Control diets contained corn oil in amounts equal to that in the diets of groups given the highest doses. The compound was administered for 13 weeks, when all animals were killed. The only suggestive neoplastic lesion observed was foci of basophilic cellular alteration in the liver of one male mouse administered 12,500 ppm Direct Brown 95 (NTP, 1978; Robens *et al.*, 1980). [The Working Group noted the short duration of the experiment, the limited number of animals tested, and the impurity of the compound used. Other samples of the compound may have different impurities.]

3.4 CI Acid Red 114

3.4.1 *Oral administration*

Groups of 45–75 male and female F344/N rats, 5 weeks of age, received doses of 0, 70, 150, or 300 ppm (male) or 0, 150, 300, or 600 ppm (female) of CI Acid Red 114 (purity, 82–85%; 15 organic impurities: two represented ~3%, benzidine < 1 ppm) in the drinking-water. Seventy animals were in the control and high-dose groups, 45 in the low-dose groups, and 75 in the mid-dose groups. Ten animals were evaluated from the control and high-dose groups at nine months, and ten animals from all dose groups were evaluated at 15 months. The average amount of compound consumed per day was 4, 8, or 20 mg/kg for males and 9, 21, or 69 mg/kg for females. Survival at 105 weeks for male rats receiving 0, 70, 150, or 300 ppm was 24/50, 15/35, 26/65, and 1/50; for females receiving 0, 150, or 300 ppm, survival was 36/50, 13/35, and 6/64 (see Table 3.1). All female rats receiving 600 ppm had died by week 89. The decreased survival in treated groups was due primarily to the development of chemical-related neoplasms. Of the surviving animals, the final mean body weights for males receiving 70 or 150 ppm were 94% and 90% of control and for females receiving 150 or 300 ppm, 99% and 84% of control. These weight differences began in the second year of the studies and were attributed in part to the development of neoplasms in the dosed groups.

Table 3.1. Survival and tumour incidences in male and female Fischer 344/N rats administered CI Acid Red 114 in the drinking-water for 104 weeks

Survival and tumour types[a]	Dose (mg/L [ppm])				*p* Value[b]
Males	0	70	150	300	
Females	0	150	300	600	
Males					
Survival[c]	24/50 (48%)	15/35 (43%)	26/65 (40%)	1/50 (2%)	
Skin					
Basal-cell adenoma or carcinoma	1/50 (2%)	5/35 (14%)	28/65 (43%)	32/50 (64%)	<0.001
Sebaceous-cell adenoma or carcinoma	1/50 (2%)	1/35 (3%)	5/65 (8%)	6/50 (12%)	=0.007
Squamous-cell papilloma or carcinoma	1/50 (2%)	2/35 (6%)	11/65 (17%)	9/50 (18%)	=0.001
Keratoacanthoma	1/50 (2%)	1/35 (3%)	4/65 (6%)	7/50 (14%)	<0.001
Zymbal gland adenoma or carcinoma	0/50 –	0/35 –	8/65 (12%)	7/50 (14%)	=0.005
Liver neoplasms	2/50 (4%)	2/35 (6%)	15/65 (23%)	20/50 (40%)	<0.001
Females					
Survival[c]	36/50 (72%)	13/35 (37%)	6/64 (9%)	0/50 –	
Basal-cell adenoma or carcinoma of the skin	0/50 –	4/35 (11%)	7/65 (11%)	5/50 (10%)	=0.012
Zymbal gland adenoma or carcinoma	0/50 –	3/35 (8%)	18/65 (28%)	19/50 (38%)	<0.001
Clitoral gland adenoma or carcinoma	11/48 (23%)	17/32 (53%)	28/62 (45%)	23/50 (46%)	<0.001
Liver neoplasms	0/50 –	0/35 –	19/64 (30%)	8/50 (16%)	<0.001
Lung adenoma or carcinoma	1/50 (2%)	2/35 (6%)	9/65 (14%)	4/50 (8%)	=0.007
Oral cavity squamous-cell papilloma or carcinoma	0/50 –	3/35 (8%)	9/65 (14%)	6/50 (12%)	=0.017
Small intestine polyps or adenocarcinoma	0/50 –	0/35 –	1/63 (16%)	2/50 (4%)	NS
Large intestine polyps or adenocarcinoma	0/50 –	1/35 (3%)	0/64 –	3/50 (6%)	NS

From US National Toxicology Program (1991b)
[a] Terms used by authors
[b] Logistic regression test for trend
[c] At 22 months; reduced survival in exposed groups due to tumour development
NS, not significant

At nine and 15 months, a few neoplasms were seen in the liver, lung, clitoral gland, skin, Zymbal gland, oral cavity epithelium, and small and large intestine, and the number of neoplasms at these sites increased as the studies progressed (see Table 3.1). At two years, there was a clear carcinogenic response in the skin, Zymbal gland, and liver of male and female rats, and in the clitoral gland, oral cavity epithelium, small and large intestine, and lung in female rats. Treatment-related increases were also seen in the incidence in neoplasms of the oral cavity epithelium, adrenal gland, and lung of male rats,

and in mononuclear cell leukaemia and in neoplasms of the mammary gland and adrenal gland in female rats. The incidence of these neoplasms was generally lower, but was significant and considered to be marginally related to chemical treatment. The same neoplastic effects had been previously observed in some or all of the NTP studies with dimethoxybenzidine, dimethylbenzidine, or C.I. Direct Blue 15 (NTP, 1991a,b).

3.5 CI Direct Blue 15

3.5.1 *Oral administration*

At study initiation, 70 F344/N rats of each sex, 40–47 days of age, were given 0 or 2500 ppm CI Direct Blue 15 [purity, ~50%; ~35 impurities, including 3,3′-dimethoxybenzidine (836–1310 ppm) and benzidine (< 1 ppm)], 45 rats of each sex were given 630 ppm and 75 rats of each sex were given 1250 ppm in the drinking-water. Interim evaluations were made at nine and 15 months. The average amounts of compound consumed per day by the six dose groups after week 52 of the studies were estimated to be 45, 90, and 215 mg/kg for male rats and 50, 100, and 200 mg/kg for female rats. The studies were terminated at 22 months due to extensive mortality associated with chemical-related neoplasia. Survival of control, 630-, 1250-, and 2500-ppm males at 22 months was 37/50, 8/35, 11/65, and 2/50 (see Table 3.2); survival of females was 40/50, 13/35, 22/65, and 4/50. At 22 months, the mean final body weights of the 630-, 1250-, and 2500-ppm groups were 95%, 91%, and 81% of those of the control for male rats and 91% of those of the control for all female dose groups. At the nine-month interim evaluations, one adenoma of the Zymbal gland was seen in a high-dose male rat, and three carcinomas of the clitoral gland were seen in the high-dose females. At the 15-month interim evaluations, Zymbal gland neoplasms were seen in low- and high-dose males and in all treated female dose groups. Mid- and high-dose males and females also had preputial or clitoral gland neoplasms, and a few neoplasms were present in the skin, small and large intestine, liver, and oral cavity of treated animals at 15 months. At the end of the study (see Table 3.2), neoplasms related to chemical administration were found in the Zymbal gland, skin, oral cavity, and the preputial or clitoral gland in both male and female rats. Neoplasms related to chemical administration were also seen at other sites including the small and large intestine, liver, uterus, and brain. The incidence of mononuclear cell leukaemia was also increased in treated rats (NTP, 1992a).

3.6 C.I. Direct Blue 218

3.6.1 *Oral administration*

(a) Mouse

Groups of 50 male and 50 female B6C3F1 mice, 7 weeks of age, were administered C.I. Direct Blue 218 [purity approximately 60%; no attempt was made to identify major

Table 3.2. Survival and tumour incidences in male and female Fischer 344/N rats administered CI Direct Blue 15 in the drinking-water for 96 weeks

Survival and tumour types[a]	Dose (mg/L[ppm])				*P* Value[b]
	0	630	1250	2500	
Males					
Survival[c]	37/50	8/35	11/65	2/50	
Skin					
Basal-cell adenoma or carcinoma	2/50 (4%)	9/35 (26%)	27/65 (42%)	28/50 (56%)	<0.001
Sebaceous gland adenoma	0/50 –	1/35 (3%)	7/65 (11%)	3/50 (6%)	=0.002
Squamous-cell papilloma or carcinoma	2/50 (4%)	4/35 (11%)	11/65 (17%)	19/50 (38%)	<0.001
Zymbal gland: adenoma or carcinoma	1/50 (2%)	5/35 (14%)	10/65 (15%)	20/50 (40%)	<0.001
Preputial gland: adenoma or carcinoma	8/49 (16%)	5/35 (14%)	23/64 (36%)	9/48 (19%)	<0.001[d]
Hepatocellular neoplasms nodule or carcinoma	0/50 –	6/35 (17%)	9/65 (14%)	11/50 (22%)	<0.001
Oral cavity: squamous-cell papilloma or carcinoma	1/50 (2%)	10/35 (29%)	24/65 (37%)	17/50 (34%)	<0.001
Small intestine: adenocarcinoma	0/50 –	0/35 –	0/65 –	2/50 (4%)	=0.078
Large intestine: polyps or adenocarcinoma	0/50 –	1/35 (3%)	6/65 (9%)	8/50 (16%)	<0.001
Mononuclear-cell leukaemia	17/50 (34%)	19/35 (54%)	28/65 (43%)	20/50 (40%)	<0.001[d]
Females					
Survival	40/50	13/35	22/65	4/50	
Squamous-cell papilloma or carcinoma of the skin	0/50 –	2/35 (6%)	6/65 (9%)	5/50 (10%)	=0.001
Zymbal gland: adenoma or carcinoma	0/50 –	4/35 (11%)	11/65 (17%)	17/50 (34%)	<0.001
Clitoral gland: adenoma or carcinoma	7/50 (14%)	11/31 (35%)	24/64 (38%)	27/50 (54%)	< 0.001
Hepatocellular neoplastic nodule or carcinoma	0/50 –	0/35 –	2/65 (3%)	5/50 (10%)	<0.001
Oral cavity: squamous-cell papilloma or carcinoma	2/50 (4%)	4/35 (11%)	19/65 (29%)	15/50 (30%)	<0.001
Small intestine: adenocarcinoma	0/50 –	0/35 –	1/65 (2%)	3/50 (6%)	=0.032
Uterine adenoma or adenocarcinoma	1/50 (2%)	0/35 –	1/65 (2%)	4/50 (8%)	=0.004
Mononuclear-cell leukaemia	7/50 (14%)	13/35 (37%)	27/65 (42%)	15/50 (30%)	<0.001[d]

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

impurities. However, benzidine was not detected at levels greater than 1 ppm and dimethoxybenzidine was detected at 7 ppm] in the diet at 0, 1000, 3000 or 10,000 ppm for 103 weeks. Mean body weights of males and females receiving 10,000 ppm were 19% lower than controls for males and 27% lower than controls for females. Mortality was similar to that of the controls. In male mice, the incidence of hepatocellular adenomas or carcinomas (combined) was increased: 21/50 (42%), 20/50 (40%), 23/50 (46%) and 45/50 (90%) ($P < 0.001$, Fisher exact test and Logistic regression trend test) in control, low-, mid- and high-dose groups, respectively. In female mice, the incidence of hepatocellular adenomas or carcinomas (combined) was increased: 10/49 (20%), 15/50 (30%), 21/49 (43%) and 45/49 (92%) ($P < 0.001$, Fisher exact test; $P < 0.001$ trend test) in control, low-, mid- and high-dose groups, respectively (NTP, 1994).

(b) Rat

Groups of 50 male and 50 female Fischer 344 rats, 6–7 weeks of age, were administered C.I. Direct Blue 218 [purity, see study above] in the diet at 0, 1000, 3000 or 10,000 ppm for 103 weeks. Mean body weights of males and females receiving 10,000 ppm were 11% lower than controls for males and 9% lower than controls for females. Mortality among females receiving 10,000 ppm was slightly but not significantly lower than that of the controls. In males, the incidence of squamous cell papillomas or carcinomas (combined) of the pharynx was increased: 0/50, 0/50, 0/50 and 6/50 (12%) ($P = 0.013$, Fisher exact test and $P < 0.001$, Logistic regression trend test) in the control, low-, mid- and high-dose groups, respectively. In females, the incidence of tumours in the treated groups was not significantly different from that in the control groups (NTP, 1994).

4. Mechanistic and Other Relevant Data

4.1 Absorption, distribution, metabolism and excretion

4.1.1 *Humans*

Lowry *et al.* (1980) measured benzidine and monoacetylbenzidine in the urine of workers exposed to benzidine-based azo dyes during their manufacture, or during textile or paper dyeing. A colorimetric screening method, based on reaction of extracted aromatic amines with 2,4,6-trinitrobenzene-sulfonic acid, and a specific electron-capture gas chromatographic (EC-GC) detection method were used. Alkali-labile conjugates of benzidine and 2,4-diaminoazobenzene were found together with free 2,4-diamino-azobenzene and traces of benzidine, 3,3′-dimethylbenzidine and 3,3′-dimethoxy-benzidine.

Dewan *et al.* (1988) used high performance liquid chromatography (HPLC) to analyse the presence of benzidine and mono- and diacetyl-benzidine in post-shift urine samples of 18 workers in a small-scale unit manufacturing Direct Black 38. Acetylated

metabolites were found in all of the urine samples (range, 6–131.8 μg/L for monoacetyl-benzidine), and benzidine was found in all except two (range, 2.4–48.9 μg/L). Two workers who lived on the factory premises excreted very high levels of benzidine (362.5 and 260.3 μg/L) and its metabolites (1117.2 and 660.2 μg/L, for monoacetylbenzidine) in their urine. None of the urine samples showed the presence of 4-aminobiphenyl. Environmental sampling showed high dust levels during powdering of the finished product. Exposure to benzidine by inhalation at this stage was considered unlikely, because no benzidine was detectable in the finished dye.

Beyerbach *et al.* (2006) used gas chromatography/mass spectrophotometry (GC/MS) to identify and quantify, in groups of Indian workers producing benzidine-2HCl or Direct Black 38 from benzidine-2HCl, aniline and other compounds, the following haemoglobin adducts: benzidine-Hb, N-acetylbenzidine-Hb, 4-aminobiphenyl-Hb, aniline-Hb. The latter two were quantitatively the major adducts. The amounts of adducts were highly correlated in all the exposed workers ($n = 33$). The benzidine-exposed group had 10- to 17-fold higher adduct levels than the dye workers. Since 4-aminobiphenyl can be metabolically released from benzidine and the azo dye, and aniline can be released from the azo dye, the presence of 4-aminobiphenyl-Hb and aniline-Hb may be the consequence of exposure to the parent compounds, or of exposure to benzidine and the azo dye after metabolic release of the arylamine moiety.

4.1.2 *Experimental systems*

(a) In-vivo studies

Rhesus monkeys excreted an average of 1.25% benzidine plus monoacetyl-benzidine of the benzidine moiety in Direct Black 38, Direct Blue 6, and Direct Brown 95, respectively, in the urine after receiving two different doses by gavage, whereas gavage with pure benzidine yielded 1.45%. These analyses were done by use of selective extraction followed by thin-layer chromatography (TLC). The authors thus postulated a nearly complete metabolic conversion of these three dyes to benzidine (Rinde & Troll, 1975).

Following oral administration of a single dose of 10 mg/kg bw Direct Black 38 to Syrian golden hamsters, 10.7 μg benzidine, 535 μg monoacetylbenzidine, 27.6 μg diacetylbenzidine, 11.5 μg 4-aminobiphenyl and, as alkali-hydrolysable conjugates, 328.5 μg benzidine and 6.3 μg 4-aminobiphenyl were identified in the urine by parallel electron-capture gas chromatography and HPLC. Peak excretion occurred between 0–8 and 8–16 hours. These results indicate that a total of 10% of the dye is metabolized to benzidine and its metabolic follow-up products (Nony *et al.*, 1980b).

Several bisazobiphenyl dyes derived from benzidine, 3,3′-dimethylbenzidine or 3,3′-dimethoxybenzidine were studied in the dog and rat. The dyes were given orally and the urine was analysed for benzidine, 3,3′-dimethyl- or 3,3′-dimethoxybenzidine by use of a specific gas chromatographic assay. The identity of the peaks was confirmed by GC/MS. Dogs treated acutely (100 mg/kg bw) with benzidine-derived dyes excreted substantial

quantities of benzidine (166–1675 μg) in urine (0–48 hours). No benzidine was detected in urine before treatment. Benzidine present in dog urine following dye administration exceeded by at least ninefold the benzidine present as impurity in the administered dyes, and was comparable to that excreted in urine when pure benzidine was fed (100 mg/kg bw). Rats chronically dosed (100 mg/kg bw/day) with benzidine-based dyes excreted *N*-acetylbenzidine (3–54 μg/day) and traces of benzidine in urine. Bisazobiphenyl dyes derived from 3,3′-dimethylbenzidine and 3,3′-dimethoxybenzidine were metabolized to 3,3′-dimethyl- and 3.3′-dimethoxybenzidine, respectively, in both the dog and rat. The *N*-acetyl derivatives of 3,3′-dimethyl- and 3.3′-dimethoxybenzidine were identified in urine from rats treated with dyes derived from 3.3′-dimethyl- and 3.3′-dimethoxybenzidine, respectively. The results indicate that the metabolic conversion of bisazobiphenyl dyes, derived from benzidine, 3,3′-dimethyl- and 3,3′-dimethoxybenzidine, to carcinogenic aromatic amines is a general phenomenon, and therefore with few exceptions should be anticipated for each member of this class of chemicals (Lynn *et al.*, 1980).

The mutagenic activation *in vivo* of three azo dyes was studied. Wistar rats received solutions of benzidine, Direct Black 38 and Direct Brown 95 orally or by intraperitoneal injection. Urine was collected for 24 hours. For Direct Black 38, significantly higher mutagenicity values were found in the urine after oral administration than after intraperitoneal treatment. Such differences were not observed for benzidine and Direct Brown 95. The results suggest that for some compounds like Direct Black 38, extrahepatic enzymes, most likely present in the intestinal flora, play a substantial role in the azo cleavage (Bos *et al.*, 1984). After oral administration of these dyes to germ-free Wistar rats, no mutagenicity was observed in the urine. A germ-free rat that received benzidine produced urine with mutagenicity comparable to that of a normal rat. These results show that after oral administration, reduction by the intestinal microflora is the first step in the bio-toxification of benzidine-based dyes (Bos *et al.*,1986).

(b) In-vitro studies

Preparations of rat and mouse intestine *in vitro* have been shown to convert Direct Black 38 to benzidine (Niitsu, 1973). After increasing the microbial activity in rats by feeding a meat-based diet, the azo-reductase level was also enhanced (Goldin *et al.* 1978).

Anaerobic bacterial suspensions isolated from human faeces and from the intestinal contents of rhesus monkeys and CD rats were incubated for 48 hours with Direct Black 38 (a benzidine-based azo dye), Direct Red 2 (3,3′-dimethylbenzidine-based), and Direct Blue 15 (3,3′-dimethoxybenzidine-based). The respective free amines, benzidine, 3,3′-dimethylbenzidine and 3,3′-dimethoxybenzidine, formed by azo reduction of the dyes by intestinal bacteria were isolated and identified by GC/MS. Within six hours of incubation, 90–100% of each dye was reduced by all three bacterial suspensions. The results suggest that anaerobic intestinal bacteria may play a significant role in the metabolism of dyes derived from benzidine (Cerniglia *et al.*, 1982a).

The metabolism of Direct Black 38 (a benzidine-based azo dye), Direct Red 2 (3,3′-dimethylbenzidine-based) and Direct Blue 15 (3,3′-dimethoxybenzidine-based) has been

studied both in pure cultures of anaerobic bacteria and in bacterial suspensions derived from the intestinal contents of rats. All of the pure cultures and the rat intestinal bacteria were able to reduce the azo linkages of Direct Black 38, Direct Red 2 and Direct Blue 15 with the subsequent formation of benzidine, 3,3′-dimethylbenzidine and 3,3′-dimethoxybenzidine, respectively. The metabolites were isolated and identified by GC/MS, and had chromatographic and mass-spectral properties similar to those of authentic standards. In-vitro anaerobic incubations of rat intestinal microorganisms are able to reduce and cleave the azo bonds of dyes derived from benzidine, 3,3′-dimethylbenzidine and 3,3′-dimethoxybenzidine to form potentially carcinogenic aromatic amines (Cerniglia *et al.*, 1982b).

Metabolism of the benzidine-based dye Direct Black 38 was examined by use of a semi-continuous culture system that simulates the lumen of the human large intestine. The system was inoculated with freshly voided human faeces, and an active flora was maintained as evidenced by volatile fatty acid and gas production. Within seven days after exposure to the dye, the following metabolites were isolated and identified by GC/MS: benzidine, 4-aminobiphenyl, monoacetylbenzidine, and acetylamino-biphenyl. Benzidine reached its peak level after 24 hours, accounting for 39.1% of the added dye. Its level began to decline, and by day seven the predominant metabolite was acetylaminobiphenyl, which accounted for 51.1% of the parent compound. Formation of the deaminated and *N*-acetylated analogues of benzidine, which are more mutagenic and lipophilic, has not been attributed so far to the intestinal microbiota (Manning *et al.*, 1985).

The role of the rat intestinal flora in the azo reduction of some benzidine-based dyes was studied by measurement of the formation of benzidine after anaerobic incubation of 1 mM Direct Black 38, Direct Blue 6 and Direct Brown 95 in the presence of caecal bacteria *in vitro*. After about six hours, the concentration of benzidine had reached a maximum at 160 μM for Direct Black 38, at 40 μM for Direct Blue 6, and at 30 μM for Direct Brown 95 (Bos *et al.*, 1986).

The azo reduction and acetylation *in vitro* of three azo dyes were studied. In the presence of rat-liver S9, benzidine was released from Direct Black 38 and Direct Brown 95, whereas hardly any benzidine was produced during incubation of Direct Blue 6. Incubation of benzidine with isolated rat hepatocytes resulted in the formation of diacetylbenzidine. No diacetylbenzidine was formed during incubation of benzidine with rat-liver S9, unless the cofactor for the acetylation reaction, acetyl coenzyme A, was added to the incubation medium. Isolated rat hepatocytes were capable of producing diacetylbenzidine from the three test dyes without supplementation with acetyl coenzyme A (Bos *et al.*, 1984).

Ingested azo dyes can be metabolized to aromatic amines by intestinal microorganisms, but hepatic enzymes can also catalyse the reductive cleavage of the azo linkage to produce the parent amines. The intestinal microbial azoreductase may be more important than the liver enzymes in azo reduction. Anaerobic bacteria were isolated from caecal or faecal contents from experimental animals and humans. The significance of the capacity of intestinal bacteria to reduce azo dyes and the conditions of azo reduction were

investigated. The azoreductase(s) that catalyse these reactions have been found to be oxygen-sensitive and to require flavins for optimal activity. The azoreductase activity in a variety of intestinal preparations was influenced by dietary factors such as cellulose, proteins, fibres or antibiotics (Chung *et al.* 1992).

In a plate assay for the detection of anaerobic bacteria that produce azoreductases, ten strains of anaerobic bacteria capable of reducing azo dyes were tested. The strains were isolated from human faeces and identified as *Eubacterium hadrum* (2 strains), *Eubacterium* spp. (2 species), *Clostridium clostridiiforme*, a *Butyrivibrio* sp., a *Bacteroides* sp., *Clostridium paraputrificum*, *Clostridium nexile*, and a *Clostridium* sp. The average rate of reduction of Direct Blue 15 (a dimethoxybenzidine-based dye) in these strains ranged from 16–135 nmol of dye per min per mg of protein. The enzymes were inactivated by oxygen. In seven isolates, a flavin compound (riboflavin, flavin adenine dinucleotide, or flavin mononucleotide) was required for azoreductase activity. In the other three isolates and in *Clostridium perfringens*, no added flavin was required for activity. Each bacterium expressed only one azoreductase isozyme. At least three types of azoreductase were produced by the different isolates. All of the azoreductases were produced constitutively and released extracellularly (Rafii *et al.*, 1990; Rafii & Cerniglia 1995).

4.2 Genetic and related effects

4.2.1 *3,3′-Dimethylbenzidine dihydrochloride*

3,3′-Dimethylbenzidine dihydrochloride was mutagenic in *Salmonella typhimurium* strain TA98 with exogenous metabolic activation; it was not mutagenic in strains TA100, TA1535, or TA97 with or without activation. 3,3′-Dimethylbenzidine dihydrochloride induced sister-chromatid exchange and chromosomal aberrations in Chinese hamster ovary (CHO) cells in the absence of exogenous metabolic activation; these effects were not evident in tests with S9 activation. Sex-linked recessive lethal mutations were induced in germ cells of adult male *Drosophila melanogaster* given 3,3′-dimethylbenzidine dihydrochloride in the feed or by injection. No reciprocal translocations occurred in *D. melanogaster* germ cells following exposure to 3,3′-dimethylbenzidine dihydrochloride (NTP, 1991a).

4.2.2 *3,3′-Dimethoxybenzidine dihydrochloride*

3,3′-Dimethoxybenzidine was mutagenic in *S. typhimurium* strain TA100 with exogenous metabolic activation and in strain TA98 without activation; a weakly positive response was observed in strain TA1535 with metabolic activation. 3,3′-Dimethoxybenzidine induced sister chromatid exchange and chromosomal aberrations in CHO cells with and without exogenous metabolic activation. 3,3′-Dimethoxybenzidine did not

induce sex-linked recessive lethal mutations in adult male *D. melanogaster* exposed via feeding or by injection (NTP, 1990).

4.2.3 *Direct Black 38*

Direct Black 38 was mutagenic in *Salmonella typhimurium* strains TA98 and TA100 when tested in the presence of a mouse-liver metabolic activation system; no mutagenicity was observed in the absence of activation (Lazear & Louie 1978). Monoacetylbenzidine, a major metabolite of benzidine (see above), and urine from hamsters given Direct Black 38 (100 mg/kg bw) were mutagenic in *S. typhimurium* strain TA1538, but only when tested in the presence of metabolic activation (Lazear & Louie, 1978; Nony & Bowman 1980a). Urine from rats given 500 mg/kg bw Direct Black 38 was also mutagenic in *S. typhimurium* strains TA98 and TA100 in the presence of metabolic activation (Tanaka, 1980).

4.2.4 *CI Acid Red 114*

In a standard pre-incubation protocol, CI Acid Red 114 was mutagenic in *Salmonella typhimurium* strain TA98 in the presence of induced hamster liver S9, and an equivocal response was noted in strain TA100 with hamster liver S9. However, no significant mutagenic activity was noted in strains TA1535 or TA1537, with or without S9 activation. In a modified *S. typhimurium* gene-mutation test that employed reductive metabolism followed by oxidative metabolism with S9 liver enzymes, CI Acid Red 114 was strongly mutagenic in strain TA1538. This dye did not induce sister chromatid exchange or chromosomal aberrations in CHO cells with or without S9 activation; reductive metabolism was not used in these cytogenetic tests. No increase in sex-linked recessive lethal mutations was observed in germ cells of male *Drosophila melanogaster* administered CI Acid Red 114 by feeding or injection (NTP, 1991b).

4.2.5 *CI Direct Blue 15*

CI Direct Blue 15 was not mutagenic in *Salmonella typhimurium* strains TA100, TA1535, TA1537, and TA98 when tested in a standard pre-incubation protocol with or without exogenous metabolic activation; however, when a specialized reductive metabolism protocol was used, C.I. Direct Blue demonstrated mutagenic activity in *Salmonella* strain TA1538. C.I. Direct Blue 15 did not induce sister chromatid exchange or chromosomal aberrations in CHO cells with or without S9 activation; reductive metabolism was not used in these cytogenetic tests (NTP, 1992a).

4.2.6 *CI Direct Blue 218*

CI Direct Blue 218 was not mutagenic in *Salmonella typhimurium* strains TA98, TA100, TA1535, or TA1537 tested with and without exogenous metabolic activation

(S9). It was also tested in a modified *Salmonella* test-protocol that employed reductive metabolism supplied by flavin mononucleotide or rat caecal bacteria, followed by oxidative metabolism; results of this test with strain TA1538 were also negative. C.I. Direct Blue 218 induced a small but significant increase in sister chromatid exchange in CHO cells at the highest dose tested, without S9. No increase in chromosomal aberrations was observed in these cells, with or without S9. C.I. Direct Blue 218 did not induce sex-linked recessive lethal mutations in germ cells of male *Drosophila melanogaster* (NTP, 1994).

4.2.7 *CI Pigment Red 23*

CI Pigment Red 23, an azo dye, was mutagenic in *Salmonella typhimurium* strains TA100, TA1537, and TA98, with and without exogenous metabolic activation (S9), but it was not mutagenic in strain TA1535. CI Pigment Red 23 induced sister chromatid exchange in CHO cells in the absence of S9, but not with S9 activation. The pigment did not induce chromosomal aberrations in these cells in the presence or absence of S9 (NTP, 1992b).

4.3 Mechanistic considerations

This section discusses the general problem of how to approach the hazard evaluation of precursors of established carcinogens, e.g., azo-colourants. These are synthesized by coupling aryl amines with single or multiple diazo moieties. Benzidine and the benzidine derivatives 3,3′-dimethyl-, 3,3′-dimethoxy-, and 3,3′-dichlorobenzidine are compounds that can be double-diazotized. The azo group can be cleaved reductively by intestinal bacteria or azo reductases in the liver and in other tissues, and the amines released. Aminonaphthalene and monocyclic aromatic amines are also encountered. In many cases the released amines or their metabolites have been detected in experimental animals as well as in humans. Most of these basic components are carcinogenic in experimental animals, benzidine itself being an IARC Group-1 carcinogen. Hundreds of such azo colourants exist, and they obviously cannot be all tested individually for carcinogenic potential.

As an example, Direct Red 28 was orally administered to rats and the haemoglobin adducts were used as biomarkers to demonstrate that benzidine is released from the dye. The same haemoglobin adducts were found with benzidine itself. Upon hydrolysis of the haemoglobin adduct benzidine, monoacetylbenzidine and 4-aminobiphenyl were identified in the same relative amounts (Birner *et al.*, 1990). This shows that the dye is metabolized to benzidine, and one of its two amino groups is *N*-oxidized to the nitroso-derivative that binds to haemoglobin in the erythrocytes. A radical-based mechanism may also play a role. It shows also that the reactive metabolite is widely distributed and can be expected to produce DNA lesions in most tissues. It should be noted that 4-amino-biphenyl is a metabolite of the dye and of benzidine, which links this exposure to that of

nitrobiphenyl, a combustion product and common environmental pollutant (Neumann, 2001).

Similarly, benzidine and 4-aminobiphenyl are released from Direct Black 38 and Direct Brown 1, commonly used in leather and textile industries. The particular role of skin bacteria for reductive cleavage and the subsequent absorption of the amines from textiles is emphasized (Gnanamani *et al.*, 2004).

It is concluded that all azo colourants whose metabolism can liberate a carcinogenic aromatic amine are potentially carcinogenic. It has therefore been recommended that the colourants be dealt with as if they were classified in the same categories as the corresponding carcinogenic or suspected carcinogenic amine (Deutsche Forschungsgemeinschaft, 2007). There are, however, colourants that have been claimed to be insoluble and that may not contribute to the amine exposure. This can be tested by use of biomarkers. In case of Pigment Yellow 17, a diarylide azo pigment with a 3,3′-dichlorobenzidine component, the expected haemoglobin adducts were identified after intratracheal instillation and oral administration to rats, but the level was very low. In a 4-week feeding study, Zwirner-Baier & Neumann (1994) calculated that 0.6% of the dose was cleaved in the intestine and the 3,3′-dichlorobenzidine absorbed. Others have suggested that the amine component of 3,3′-dichlorobenzidine-based dyes is in general practically not bioavailable (Golka *et al.*, 2004).]

When the contribution of a benzidine-based dye to cancer risk is claimed to be low or negligible, the bio-availability of the carcinogenic component should be excluded, e.g. by use of biomarkers of exposure or biomarkers of effect. However, if this is not the case, it does not seem justified to classify benzidine-based dyes differently from benzidine.

5. Summary of Data Reported

5.1 Exposure data

See the Monograph on Benzidine in this volume.

5.2 Human carcinogenicity data

The epidemiological evidence has been reviewed for workers exposed to benzidine derivatives (3,3′-dimethyl-, 3,3′-dimethoxy-, and 3,3′-dichlorobenzidine) and benzidine-based dyes. Studies of occupations with potential exposure to dyes metabolized to benzidine were reviewed only in those instances in which the authors specifically noted that such an exposure was occurring, including shoe and leather workers, and textile workers.

Consistently elevated risks for bladder cancer were observed among workers exposed to benzidine derivatives. There is also some evidence of increased risks with intensity of exposure and duration of exposure.

The Working Group identified four cohort studies and two case–control studies in populations occupationally exposed to dyes metabolized to benzidine. The risk for bladder cancer for workers exposed to dyes metabolized to benzidine was not consistent across studies, with some reports showing excess risk and others not showing an effect. A limitation of most studies is that while they indicate the potential for exposure to these compounds, no quantitative exposure data are presented.

The difficulty of evaluating the cancer risk from exposure to these compounds arises from concomitant exposures to benzidine and 2-naphthylamine, which are bladder carcinogens in humans. Potential cross-contamination with residual benzidine may also occur. Consequently, it is not possible to establish if the risks observed in certain studies are due to potential confounders or to benzidine precursors of dyes metabolized to benzidine.

5.3 Animal carcinogenicity data

Direct Black 38 was tested for carcinogenicity in mice by oral administration, producing hepatocellular carcinomas and mammary carcinomas. In rats, Direct Black 38 produced hepatocellular carcinomas within 13 weeks after administration in the diet, and carcinomas in the urinary bladder, liver and colon after administration in drinking-water.

In a single well-conducted study, Direct Blue 6 produced hepatocellular carcinomas in male and female rats within 13 weeks after its oral administration.

Direct Brown 95 was tested for carcinogenicity in a single well-conducted study under Good Laboratory Practice. After oral administration, it produced neoplastic nodules in the livers of 4/8 female rats, one of which showed a hepatocellular carcinoma. This study was terminated after 13 weeks. The finding of preneoplastic lesions after such a short exposure period suggests a carcinogenic effect similar to that of Direct Black 38 and Direct Blue 6.

C.I. Acid Red 114 was tested for carcinogenicity in rats by administration in the drinking-water. It produced a clear carcinogenic response in the skin, Zymbal gland and liver of male and female rats, and in the clitoral gland, oral cavity epithelium, small and large intestine, and lung in female rats after two years. Treatment-related increases were also seen in the incidences of neoplasms of the oral cavity epithelium, adrenal gland, and lung of male rats, and of mononuclear cell leukaemia and neoplasms of the mammary gland and adrenal gland in female rats.

C.I. Direct Blue 15 was tested for carcinogenicity in rats by administration in the drinking-water. It produced neoplasms in the Zymbal gland, skin, oral cavity, and the preputial or clitoral gland in both male and female rats. The 24-month study was terminated at 22 months because of rapidly declining animal survival, which was due primarily to neoplasia. Neoplasms related to chemical administration were also seen at other sites including the small and large intestine, liver, uterus, and brain.

C.I. Direct Blue 218 was tested for carcinogenicity in mice and rats by administration in the diet. It produced hepatocellular adenomas and carcinomas in male and female mice,

and squamous cell papillomas or carcinomas of the pharynx in male rats after two years of treatment.

5.4 Other relevant data

See the Monograph on Benzidine in this volume.

6. Evaluation

6.1 Cancer in humans

There is *inadequate evidence* in humans for the carcinogenicity of dyes metabolized to benzidine.

6.2 Cancer in experimental animals

There is *sufficient evidence* in experimental animals for the carcinogenicity of Direct Black 38.

There is *sufficient evidence* in experimental animals for the carcinogenicity of Direct Blue 6.

There is *sufficient evidence* in experimental animals for the carcinogenicity of Direct Brown 95.

6.3 Overall evaluation

Dyes metabolized to benzidine are *carcinogenic to humans (Group 1)*

In reaching this evaluation, the Working Group considered the following:

(1) Benzidine is a Group-1 *human carcinogen,* (2) metabolism of benzidine-based dyes results in the release of free benzidine in humans and in all experimental animal species studied, and (3) exposure to benzidine from exposure to benzidine-based dyes is equivalent to exposure to large doses of benzidine.

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