REGULATORY TOXICOLOGY AND PHARMACOLOGY **16,** 301–317 (1992)

Reevaluating the Carcinogenicity of *ortho*-Toluidine: A New Conclusion and Its Implications[1]

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Received July 13, 1992

The aromatic amine *ortho*-toluidine has been recognized by IARC as an animal carcinogen for the past decade. Three recent epidemiological studies of worker populations have now implicated this chemical as a human bladder carcinogen. In a study by E. Ward, A. Carpenter, S. Markowitz, D. Roberts, and W. Halperin ((1991), *J. Natl. Cancer Inst.* **83,** 501–506), workers definitely exposed to *ortho*-toluidine for at least 10 years experienced a Standardized Incidence Ratio (SIR) of 27.2 (90% CI = 11.8–53.7). The other major exposure was to aniline, which significant epidemiological studies have failed to confirm as a human carcinogen. In retrospect, studies by G. F. Rubino, G. Scansetti, G. Piolatto ((1982) *Environ. Res.* **27,** 241–254) and M. J. Stasik ((1988) *Int. Arch. Occup. Environ. Health* **60,** 21–24) also support the hypothesis that *ortho*-toluidine is a human bladder carcinogen. Animal studies of both *ortho*-toluidine and its possible confounders in these epidemiological investigations further confirm this hypothesis. When evaluated in a suitably comprehensive way, according to the traditional standards for assessing causality outlined by A. B. Hill ((1977) *A Short Textbook of Medical Statistics,* pp. 288–294, Lippincott, Philadelphia) the evidence that *ortho*-toluidine causes human bladder cancer has become much more conclusive. In this case, animal tests have proven a good predictor of human carcinogenicity.

Ortho-toluidine (CAS Registry Number 95-53-4) belongs to a class of organic chemicals known as the aromatic amines. A number of the members of this class of compounds are recognized human carcinogens, and for a decade IARC has recognized *ortho*-toluidine as a carcinogen in animals. Recent evidence points increasingly toward the human carcinogenicity of *ortho*-toluidine.

Originally synthesized in 1844 in Germany, *ortho*-toluidine has been produced commercially in Germany since 1880 and in this country as early as 1922 (IARC, 1982a). The major use of *ortho*-toluidine in the United States is as a chemical intermediate in dye production. *Ortho*-toluidine also is used in rubber production (as an intermediate in the production of accelerators), in pharmaceutical manufacture (to

[1] Research supported in part by NIEHS Training Grant 5 T35 ES07269 and by NIEHS Center Grant 5P30 ES00928.

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0273-2300/92 $5.00

produce sedative hypnotics such as methaqualone and local anesthetics such as prilocaine), in herbicide production (Siduron), and soap production (as an antioxidant), in the manufacture of other chemicals, as well as in processes for textile printing and for rendering colors fast to acids. It also has several uses as a laboratory reagent, such as in clinical tests of blood samples for glucose and of excreta and bodily fluids for blood (OTS, 1984). Though few consumers are exposed, workers in industries and laboratories who are involved in these processes using *ortho*-toluidine may be exposed to this chemical; dye and pigment makers and rubber workers are especially likely to have exposure to *ortho*-toluidine. When NIOSH conducted the National Occupational Exposure Survey from 1981 to 1983, it estimated that 28,500 workers were exposed to this compound in this period (Ward *et al.*, 1991a).

To fully demonstrate the value of the new evidence that has emerged about the carcinogenicity of *ortho*-toluidine, it is necessary not only to review existant data about this chemical in the manner of IARC, but also to go beyond the formal parameters of the carcinogenic risk assessments performed by IARC. Though IARC informally reviews evidence about possible confounding chemical influences during its carcinogen evaluations, it does not include such reviews in its printed reports. Yet this kind of evidence is essential to understanding the significance of recent epidemiological work on *ortho*-toluidine. It has thus seemed crucial to explicitly consider such data in this reevaluation of *ortho*-toluidine's human carcinogenicity.

EVIDENCE FOR ITS CARCINOGENICITY—HUMAN STUDIES

Numerous studies have reported excess rates or numbers of bladder cancers in which *ortho*-toluidine probably plays a contributory role. No case or epidemiological study to date has been able to evaluate the effect of *ortho*-toluidine alone in humans, since workplace exposures have almost invariably been accompanied by exposure to other suspect chemicals. However, the most recent studies provide reasonably strong epidemiologic evidence that *ortho*-toluidine causes bladder cancer in humans.

Several series of case reports of bladder cancer among workers have suggested possible carcinogenic effects of *ortho*-toluidine. Gropp (1958) listed 11 cases of bladder tumors among German chemical factory workers reportedly exposed to aniline and toluidine (presumably *ortho*-) only among a series of 98 cases of bladder tumors. Also, an additional 43 cases had been exposed to 2-naphthylamine and other amines such as benzidine, aniline, and toluidines. In evaluating this evidence, an IARC working group noted that the aniline produced at this time probably also included 4-aminobiphenyl and naphthylamine derivatives—established human carcinogens (IARC, 1978). Lipkin's study (1972) cited 27 cases of bladder cancer in chemical workers exposed to *ortho*- and *para*-toluidine alone, and 21 additional cases among those exposed to these two chemicals as well as 1-napthylamine. Oettel (1968) reported 21 cases of bladder papillomas or carcinomas in workers exposed to toluidine and aniline. These early studies provide impressive numbers of cases of bladder cancer but are limited by imprecise data on exposure and the lack of any estimate of the size of the population from which cases were drawn.

The earliest epidemiological cohort studies provided limited evidence on the carcinogenic effects of *ortho*-toluidine itself. Case and Pearson's study (1954) included a group of 812 men exposed to aniline, many of whom may also have been exposed to

toluidines. Only one of these men had a death certificate specifying bladder cancer as cause of death, compared to the 0.5 cases expected based on the national bladder cancer rate. Uebelin and Pletscher (1954) found no cases of bladder cancer among 35 men who prepared *para*-chloro-*ortho*-toluidine from *ortho*-toluidine, though they did find 3 cases of bladder cancer among 650 workers exposed only to aniline or other unspecified aromatic amines (but not the established carcinogens benzidine or 2-naphthylamine), possibly including *ortho*-toluidine. Somewhat stronger positive evidence came in Khlebnikova *et al.* (1970). In 75 of 81 workers exposed to *ortho*- and *para*-toluidine, two cases of bladder tumors were discovered upon exam; however, one worker with a tumor had only had contact with the *para*- and the other with both *para*- and *ortho*-compounds. These investigators also reported having discovered 6 other cases of bladder cancer among former workers who had been employed for over a decade in the same industry, but the IARC working group found that these researchers had not fully evaluated the other possible chemical exposures or the precise extent and duration of exposure in this group.

More rigorous epidemiological studies addressing the carcinogenicity of *ortho*-toluidine have appeared in the last decade. At least three of these studies provide clearer estimates of the risk from exposure to *ortho*-toluidine, though they, too, face the problem of multiple chemical exposures.

In 1982, an Italian study of dyestuff workers who were employed for at least one month in the manufacture of fuchsin and safranine T reported five bladder cancer deaths between 1946 and 1979 among 53 workers exposed to *ortho*-toluidine and 4,4'-methylene-bis (2-methylaniline) versus the 0.08 expected for a standardized mortality ratio from bladder cancer of 62.5 ($P < 0.001$) (Rubino *et al.*, 1982). The mean latency period for these bladder cancers was 27.4 years (range 12–40) after first exposure. The duration of exposure for these cases was from 12–33 years. This study did not take into account the bladder cancer cases not resulting in mortality, which later studies suggest was substantially greater than the mortality rate. In addition, although all workers with cancer had been exposed to the two above-named chemicals, some were potentially exposed to *ortho*-nitrotoluene, *ortho*-aminoazotoluene, 2,5-diaminotoluene, and aniline as well (see below for epidemiological and toxicological evidence on these chemicals).

In 1988, a large excess of bladder cancers was found among 116 workers exposed to *ortho*-toluidine in the manufacture of 4-chloro-*o*-toluidine between 1929 and 1970 with eight incident bladder cancers observed versus the 0.11 expected for a standardized incidence rate of 72.7 (95% CI = 31.4–143.3) (Stasik, 1988). The median latency period was 27.5 years and the median duration of exposure was 14 years; two workers were employed just 1.5 and 4.0 years, respectively. Workers in the study were possibly exposed to *N*-acetyl-*o*-toluidine and 6-chloro-*o*-toluidine as well. The author attributed the increased incidence of bladder cancers to exposure to 4-chloro-*o*-toluidine, because this was the dominant exposure.

More recently, Ward, *et al.* (1991a) studied workers who were employed at a chemical plant in western New York and exposed primarily to *ortho*-toluidine and aniline in the production of an antioxidant between 1957 and 1988. Among 708 workers "definitely exposed," 7 cases of bladder cancers were observed versus the 1.08 expected for a standardized incidence ratio (SIR) of 6.48 (90% CI = 3.04–12.2). Among 288 workers in the "probably exposed" group, 4 cases of bladder cancers were recorded for a SIR of 3.66 (90% CI = 1.25–8.37). Among the employees ever exposed to *ortho*-

toluidine, a strong dose–response effect was noted with increasing duration of exposure. No excess of bladder cancer was found among employees with <5 years of exposure. However, among employees with 10 years or longer of exposure, 6 cases of bladder cancer were found compared to the 0.22 expected (SIR of 27.2, 90% CI = 11.8–53.7). The test for linear trend was highly significant ($P < 0.001$).

Furthermore, it is likely that the results were conservative estimates. Among former workers living outside New York State, the study could not detect cases of bladder cancer that had not resulted in death. Also, most workers in the exposed group had not yet reached 20 years of latency from onset of exposure–the period of highest risk. The study may thus have come too soon to record the full carcinogenic impact of exposure. Though workers may also have been exposed to small amounts of hydroquinone and 4-aminobiphenyl, findings of *ortho*-toluidine and aniline in the urine of workers who reported exposure to these chemicals further support the causal influence of *ortho*-toluidine and possibly aniline on the elevated bladder cancer rates at this factory.

In 1987, IARC reviewed the evidence for carcinogenicity to humans (IARC, 1987). Without the results of the last two epidemiological studies cited, IARC classified the evidence as "inadequate." It found the studies prior to that of Rubino to be methodologically inadequate and the Rubino study itself to be unable to distinguish between the effects of *ortho*-toluidine and other chemicals. The National Toxicology Program refers almost exclusively to IARC's 1987 conclusions in its own evaluation of this chemical (NTP, 1991); the ACGIH has also followed IARC's evaluation in classifying *ortho*-toluidine as a suspected human carcinogen (ACGIH, 1991).

OTHER RELEVANT HUMAN STUDIES FOR DETERMINING THE CARCINOGENICITY OF *ORTHO*-TOLUIDINE

In order to determine the significance of the above epidemiological data, it is also necessary to review briefly the epidemiological evidence on the chemicals other than *ortho*-toluidine to which the workers in these studies were exposed.

Aniline. While epidemiological studies of exposure to aniline along with other suspect chemicals have shown clusters of bladder cancer, epidemiological studies in which aniline is the primary exposure show, in IARC's words, "little evidence of increased risk" (IARC, 1987). The most conclusive of these studies reported one death from bladder cancer among 1223 workers who had produced or used aniline, compared to 0.83 expected deaths from population rate (Case and Pearson, 1954; Case *et al.*, 1954). Though the results from other studies of aniline alone were also negative, IARC found these difficult to assess because of study design. IARC thus classified the evidence for aniline's carcinogenicity to humans as "inadequate," and concluded that the elevated rates of bladder cancer in studies involving multiple exposures were probably due to chemicals other than aniline (IARC, 1982b).

4-Chloro-o-toluidine. Two retrospective epidemiological reports on workers exposed to this chemical did not reveal any bladder cancer, though the number of subjects was small and the exposure data limited (Ott and Langner, 1983; Uebelin and Pletscher, 1954). Only Stasik's epidemiological study (Stasik, 1988) suggests a link between human bladder cancer and this compound. In 1987, IARC maintained that there was inadequate data on its human carcinogencity (IARC, 1987).

Hydroquinone. IARC found "inadequate data" to classify its carcinogenicity to humans (IARC, 1987).

4-aminobiphenyl. This agent is widely recognized as a human bladder carcinogen (IARC, 1987; NTP, 1991). Salient studies include a case in which in a cohort of 171 men who worked with the chemical, 19 developed bladder tumors (Melick *et al.*, 1955). Among another group of 541 exposed workers followed for 14 years, 43 developed histologically confirmed bladder cancer (IARC, 1971; Melamed, 1972).

4,4'-methylene-bis (2-methylaniline). The Rubino study contains the most impressive evidence for the human carcinogenicity of this substance (Rubino *et al.*, 1982). Yet IARC judged this evidence inadequate for determining human carcinogenicity due to possible confounding effects of *ortho*-toluidine and other chemicals in this study (IARC, 1987). (Ward, *et al.* (1988) identified two cases of bladder tumors in a screening study of workers exposed to a similar chemical, 4,4'methylenebis (2-chloroaniline), but it was not clear if these workers were also exposed to the 4,4'-methylene-bis (2-methylaniline) compound as well.)

O-Aminoazotoluene. IARC found inadequate data to allow any conclusions on its carcinogenicity to humans (IARC, 1987).

2,5-Diaminotoluene. IARC found inadequate data to allow any conclusions about the human carcinogenicity of this chemical (IARC, 1987).

O-Nitrotoluene, N-acetyl-o-toluidine, 6-chloro-o-toluidine. None are rated by IARC for human carcinogenicity; extremely little evidence is available.

EVIDENCE FOR CARCINOGENICITY OF *ORTHO*-TOLUIDINE—ANIMAL STUDIES

Ortho-toluidine has been shown to cause tumors in both rats and mice along with other species of animals and in multiple strains of some species. Table 1 summarizes the available data. In experiments only briefly reported, Russfield *et al.* (1973a, b) induced "significant" rates of subcutaneous tumors (83% versus 16% in controls) in male Charles River CD rats (Sprague Dawley-derived) by feeding them *o*-toluidine for 2 years, probably as a hydrochloride. Male Charles River CD rats fed *ortho*-toluidine HCL for 18 months showed statistically significant increases of subcutaneous fibromas and fibrosarcomas as well as multiple tumors (Weisburger *et al.* 1978). In another experiment of 104 weeks of feeding, with larger groups, exposed male Fisher 344 rats developed significantly elevated rates of multiple-organ sarcomas, fibrosarcomas, angiosarcomas and osteosarcomas; subcutaneous integumentary fibromas; and mesotheliomas of either the tunica vaginalis or multiple organs. Female Fisher 344 rats in the same experiment developed statistically significant increases of spleen sarcomas, osteosarcomas, and mammary tumors (NCI, 1979b). In F-344 male rats, *ortho*-toluidine hydrochloride induced statistically significant excesses of mammary tumors after 72 weeks of feeding and 93 total experimental weeks (Hecht *et al.*, 1982).

Russfield also briefly reported a feeding experiment with Charles River (HA/ICR) CG-1 male and female mice in which *o*-toluidine, probably in HCl form, caused vascular tumors in both sexes (Russfield, 1973a). Albino CD-1 mice fed the same compound for 18 months and observed for 24 months developed statistically significant numbers of vascular tumors in both females and males (Weisburger *et al.*, 1978). B6C3F1 mice fed *ortho*-toluidine HCl for 103 weeks showed statistically significant

TABLE 1

ANIMAL STUDIES OF CARCINOGENICITY OF *o*-TOLUIDINE

Study	Species	Route and duration of exposure	Duration of follow-up	Results: type and number of tumors	Comments
Russfield, 1973a, (abstract)	Rats (Charles River CD) Spraque Dawley Derived 50 males 50 males	Diet Two dose levels of *o*-toluidine, probably HCl (amount not specified)	2 years	Bladder tumors "significant incidence" Subcutaneous fibromas or fibrosarcomas 83% of total exposed, 16% of controls, "significant incidence" (no further figures given)	
Russfield 1973a [abstract]	Mice (Charles River HA/ICR CG-1 males and females (numbers not specified)	Diet Two dose levels of *o*-toluidine, probably HCl (amount not specified)	Not specified	Vascular tumors in both sexes (no further details)	
Weisburger *et al.*, 1978	Mice (albino CD-1) 25 males/25 females 25 males/25 females 25 males/25 females 99 males/102 females	Diet 32,000 mg/kg of *o*-toluidine HCl for 5 months then 16,000 mg/kg for 15 months 16,000 mg/kg for 5 months then 8000 mg/kg for 13 months Matched controls Pooled controls	24 total experimental months	Vascular tumors in males 9/11 high dose ($P < 0.029$) 5/14 low dose ($P < 0.029$) 0/14 matched controls 7/19 pooled controls	
Weisburger *et al.*, 1978	Rats (Charles River CD) 25 males 25 males 25 males	Diet 16,000 mg/kg of *o*-toluidine HCl for 3 months then 8000 mg/kg in 3 months, then 4000 mg/kg for 15 months Matched controls	Not specified	Bladder tumors 4/24 high dose 3/23 low dose 0/16 matched controls 5/111 pooled controls Subcutaneous fibromas and fibrosarcomas 21/24 high dose ($P < 0.025$) 18/23 low dose ($P < 0.025$) 0/16 matched controls 18/111 pooled controls Multiple tumors in males 8/24 high dose ($P < 0.025$)	Bladder tumor results not statistically significant

				6/23 low dose 3/16 matched controls 14/11 pooled controls
NCI, 1979b	Rats (Fisher 344) 50 males/50 females 50 males/50 females 20 females	Diet 6000 mg/kg of *o*-toluidine HCl for 104 weeks 3000 mg/kg for 101 weeks Matched controls	Not specified	Transitional cell tumors of bladder in females 22/47 high dose ($P < 0.001$) 10/45 low dose ($P = 0.018$) 0/20 controls Combined sarcomas, fibrosarcomas, angiosarcomas, osteosarcomas, of multiple organs in males 37/49 high dose ($P < 0.001$) 15/50 low dose ($P = 0.003$) 0/20 controls In females: 21/49 high dose ($P < 0.001$) 3/50 low dose 0/20 controls Spleen sarcomas, osteosarcomas and angiosarcomas in females 12/49 high dose ($P = 0.01$) 9/49 low dose ($P = 0.036$) 0/20 controls Subcutaneous integumentary fibromas in males 27/49 ($P < 0.001$) 28/50 ($P < 0.001$) 0/20 Mammary adenomas and fibroadenomas in females 35/49 high dose ($P = 0.006$) 20/50 low dose 7/20 controls Mesotheliomas of multiple organs or tunica vaginalis in males 9/49 high dose ($P = 0.036$) 17/50 low dose ($P < 0.001$) 0/20 controls

TABLE 1—*Continued*

Study	Species	Route and duration of exposure	Duration of follow-up	Results: type and number of tumors	Comments
NCI, 1979b	Mice (B53Fl)	Diet for 102–103 weeks	103 total experimental weeks	Hepato cellular carcinomas and adenomas in females	
	50 males	3000 mg/kg of *o*-toluidine HCl		13/50 high dose	
	50 females	3000 mg/kg		4/49 low dose	
	50 males	1000 mg/kg		0/20 controls ($P = .007$)	
	50 females	1000 mg/kg		Hemangiosarcomas (all sites) in males	
	20 males	Controls		10/50 high dose ($P = .002$)	
	20 females	Controls		1/50 low dose	
				1/19 controls	
Hecht *et al.*, 1983	Syrian golden Hamsters	Subcutaneous injection for 52 weeks	82 total experimental weeks	No tumors induced	
	15 male	1.9 mmol/kg of *o*-toluidine in	61.3 weeks (mean	0/15	
	15 female	peanut oil by injection weekly	survival time) 57.8 weeks	0/15	
	15 male	Peanut oil only by injection	75.5 weeks	0/15	
	15 female	weekly (controls)	68.7 weeks	0/15	
Hecht *et al.*, 1982	Rats (F-344)	Diet for 72 weeks	93 total experimental weeks		
	30 males (30 autopsied)	0.43 mmol of *o*-toluidine daily		Liver tumors	
	30 males (27 autopsied)	Controls		3/30 exposed, 1/27 controls	
				Bladder tumors	
				4/30 exposed, 0/27 controls	
				Skin fibromas	
				25/30 exposed, 1/27 controls	
				Mammatory tumors	
				13/30 exposed, 0/27 controls	
				Peritoneal tumors	
				14/30 exposed, 2/27 controls	
				Miscellaneous	
				12/30 exposed, 14/27 controls	

Pliss, 1965	Rats (no further information)	Subcutaneous injection 20 mg/wk of *o*-toluidine in sunflower oil	13 months	Tumors at injection site on skin, sebaceous or mammary glands 60% (no other details available)	
Ekman and Strombeck, 1947, 1949	Rats (Albino) 6 males 6 females	Diet 2 g of 7.5% *o*-toluidine in peanut oil daily, halved after 64 days	Up to 91 days	"Epithelial changes with keratosis, metaplasia" (unspecified sex or numbers) "Tendency to incipient papillomatosis" 3/10 examined (unspecified sex)	
Satani *et al.*, 1941	Rats (2) Rabbits (5) Guinea pigs (8) (sex unspecified)	Subcutaneous injection *o*-Toluidine in olive oil or alcohol (dose and duration unspecified)	Not specified	Papillomas of bladder 2/2 rats 4/5 rabbits 5/8 guinea pigs	IARC working group noted "inadequate reporting" (IARC, 1978)
Morigami and Nisimuria, 1940	Rabbits (15) Guinea pigs (10) (sex unspecified)	Subcutaneous injection *o*-Toluidine in olive oil 1 cc/6 weeks for rabbits 0.5 cc/6 weeks for guinea pigs	At least 100 days (not specified)	"Papillomatous changes in epithelial layer of bladder" in some rabbits; guinea pigs died early in experiment	IARC working group noted "inadequate reporting" (IARC, 1978)
Deichmann, 1967	Dogs 5 females	Diet (?) 100 mg *o*-toluidine 5× week	Up to 6 years 1–2 years:2 5 years:1 6 years:2	No abnormalities of urinary bladder observed	

rates of hepatocellular carcinomas in females and hemangiosarcomas in males (NCI, 1979). In earlier years, investigators also produced tumors in other groups of rats, as well as in rabbits and guinea pigs, by subcutaneous injection as well as by diet, although IARC later found these experiments to be "inadequately reported" (Pliss, 1965; Ekman and Strombeck, 1947, 1949; Satani *et al.*, 1941; Morigami and Nisimura, 1940; IARC, 1978).

Of particular relevance for evaluating the epidemiological evidence are the numerous reports of bladder cancer in these animal studies. Four of eight animal studies published since 1970 reported bladder tumors, including two showing a significant incidence of bladder cancer and two showing an elevated incidence that did not meet the standard for significance. Russfield's (1973a, b) study of male Charles River CD rats also showed a "significant incidence" of bladder tumors. The large NCI study of Fisher 344 rats revealed statistically significant rates of transitional cell bladder tumors in females (NCI, 1979). Both Hecht's study of F-344 rats and Weisburger's study of Charles River CD rats demonstrated elevated numbers of bladder cancers in exposed animals in comparison with controls, though these differences did not meet the standard for statistical significance (Hecht *et al.*, 1982; Weisburger *et al.*, 1978). Finally, though they included few animals and limited or no controls, the early work of Ekman and Strombeck as well as that of Satani produced bladder tumors in rats, rabbits, and guinea pigs (Ekman and Strombeck, 1947, 1949; Satani *et al.*, 1941).

By contrast, Hecht's failure to produce any tumors in Syrian golden hamsters may be due to the different route of exposure he employed (subcutaneous injection), the relatively low dose (injections weekly, at 1.9 mmol/kg), the relatively short duration of exposure (only 52 weeks), the brief follow-up period (mean total survival time was 61.3 weeks for exposed males and 57.8 weeks for exposed females—substantially less than for controls), and the small number of animals (15) in each exposure group (Hecht *et al.*, 1983).

In the wake of the Weisburger and NCI studies, IARC determined in 1982 that the evidence on mice and rats was "sufficient" to establish *o*-toluidine as a carcinogen for experimental animals (IARC, 1982a). Hecht's studies only confirmed their conclusions. The National Toxicology Program bases its evaluation of the experimental carcinogenicity of *ortho*-toluidine on these conclusions of IARC (NTP, 1991).

OTHER RELEVANT ANIMAL STUDIES FOR DETERMINING THE CARCINOGENICITY OF *ORTHO*-TOLUIDINE

In addition to the animal data on *ortho*-toluidine, there is also animal evidence relating to materials that may have been present along with *ortho*-toluidine in the workplaces targeted by the more recent epidemiological studies.

Aniline. The evidence for aniline's carcinogenicity to experimental animals is not as extensive as that for *ortho*-toluidine. A single well-designed feeding experiment with rats showed it to cause fibrosarcomas, sarcomas, and hemangiosarcomas of the spleen and peritoneal cavity (NCI, 1978). A similar experiment on mice did not reveal any statistically significant increase in tumors from exposure (NCI, 1978). Other more limited studies on rats, mice, and hamsters have also produced largely negative results. It has not caused bladder tumors in animals. In 1987, IARC rated the evidence for aniline's carcinogenicity to animals as only "limited" (IARC, 1987).

4-Chloro-o-toluidine. A contaminant of the pesticide chlordimeform, this compound has proved carcinogenic in two feeding experiments with mice, producing statistically significant hemangiosarcomas or hemangiomas in both sexes in one strain, and a significant incidence of hemangiomas in both sexes in another strain. Two similar feeding experiments with rats, however, produced negative results in one and apparently significant chromophobe adenomas in the other, but IARC found the design of this last experiment to be flawed (NCI, 1979; Weisburger *et al.,* 1978; IARC, 1983). 4-Chloro-*o*-toluidine has not been known to cause bladder tumors in animals, and the evidence on its animal carcinogenicity is thus more weakly relevant than that for *o*-toluidine.

Hydroquinone. IARC rated the experimental evidence for the carcinogenicity of this chemical to animals as inadequate (IARC, 1987).

4-Aminobiphenyl. This compound has been shown to be carcinogenic in several species by several routes of exposure including oral administration, which induced tumors, including bladder papillomas and carcinomas, in dogs and rabbits. It also has produced tumors in feeding experiments with mice and in subcutaneous injection studies with rats. IARC classifies this evidence, like that for *ortho*-toluidine, as sufficient to establish carcinogenicity in animals (IARC, 1987).

4,4'-Methylenebis (2-methylaniline). The evidence on the animal carcinogenicity of this compound also rates a sufficient from the IARC working group. It has been shown to cause tumors upon oral administration in rats and dogs, but no bladder tumors (IARC, 1987).

O-Aminoazotoluene. IARC rated the experimental evidence on this chemical as sufficient proof of carcinogenicity (IARC, 1987).

O-Nitrotoluene, N-acetyl-o-toluidine, 6-Chloro-o-toluidine. Not rated by IARC; little evidence available.

GENOTOXICITY STUDIES OF *ORTHO*-TOLUIDINE

Genotoxicity studies of this compound have followed no clear-cut pattern. *Ortho*-toluidine induced sister chromatid exchanges, mutation, and unscheduled DNA synthesis in human cells *in vitro* (IARC, 1987). It did not increase the frequency of chromosomal aberrations or micronuclei in bone marrow cells of mice *in vivo*, although it did increase the frequency of sister chromatid exchanges (McFee *et al.,* 1989). An earlier test also failed to produce chromosomal aberrations or micronuclei in bone marrow cells of mice (IARC, 1987). A test for sister chromatid exchanges in Chinese hamsters produced equivocal results (IARC, 1987). *Ortho*-toluidine resulted in transformation, aneuploidy, and chromosomal aberrations in a test of cultured rodent cells, although the test produced conflicting results for sister chromatid exchanges, mutation, and DNA damage (IARC, 1987). Positive results were obtained in a genotoxicity test with the unstable zeste–white system of Drosophila melanogater (Batiste-Alentorn *et al.,* 1991); *ortho*-toluidine also caused somatic mutation in an earlier test in Drosophila (IARC, 1987). A test for micronuclei using erythrocytes of Pleurodeles waltl larvae was positive (Fernandez *et al.,* 1989). A mutagenicity test using Saccharomyces cerevisiae C658-K42 proved only weakly positive (Morita *et al.,* 1989). *Ortho*-toluidine produced conflicting results in tests for mutagenicity to yeasts and positive results under some conditions in tests of bacteria (IARC, 1987). An initiator tRNA acceptance assay was positive (Hradec, 1988).

These studies provide some evidence for a genotoxic effect of *ortho*-toluidine, but the evidence is not sufficiently strong or consistent to characterize it as a cancer initiator or genotoxic carcinogen. Positive results are not consistently confined to either DNA damage, gene mutation or chromosomal aberration. Nor are the overall results sufficiently negative to categorize the substance as a promoter, or epigenetic carcinogen. The evidence thus provides little insight into the mechanism by which *ortho*-toluidine causes cancer (Williams, 1989).

DISCUSSION

Two new epidemiological studies have appeared since IARC last reviewed the evidence for the human carcinogenicity of *ortho*-toluidine in 1987 and decided that this evidence remained inadequate. To reevaluate the evidence as it now stands, we can use as a guide Sir Austin Bradford Hill's eight criteria for establishing whether human disease is related to a specific exposure circumstance (Hill, 1977).

1. *Strength of the association.* Is the effect under consideration a marginal one showing an increased risk that is not statistically significant or is the evidence of a substantial nature? Stasik's (1988) study found a standardized incidence ratio for bladder cancer of 72.7 among workers exposed to *o*-toluidine and some other chemicals. Ward *et al's* (1991a) study discovered a standardized incidence ratio of 27.2 among those exposed for 10 or more years. These studies thus corroborated the high standardized incidence ratio of bladder cancer found in the Rubino (1982) study, of 62.5. Not only are these ratios statistically significant, they are also very high.

2. *Consistency.* How many studies demonstrate the effect in question and are the results comparable? We now have three studies suggesting the carcinogenicity of *o*-toluidine to the human bladder, with high standardized incidence ratios.

3. *Specificity.* Is the action of the agent specific and does it act similarly in different exposure circumstances? Yes, the three recent epidemiological studies link *ortho*-toluidine exposure only to bladder cancer, rather than to any other type of tumor.

In the Rubino study, possible causes of the high rates of bladder cancer other than *o*-toluidine included primarily 4,4′-methylene bis (2-methylaniline). Yet the only epidemiological evidence to clearly implicate this compound comes in this study (studies of 4,4′-methylenebis (2-chloroaniline) notwithstanding), in comparison with the numerous studies that now implicate *o*-toluidine. The limited epidemiological work on 4,4′-methylene bis (2-methylaniline) has much to do with its relatively infrequent use in industry. Virtually no human data was available on the other possible chemical carcinogens to which the study subjects were exposed.

In the Stasik study, suggested causes of the high incidence of bladder cancer other than *o*-toluidine included primarily 4-chloro-*o*-toluidine but also *N*-acetyl-*o*-toluidine and 6-chloro-*o*-toluidine. Stasik's is the only such epidemiological study to suggest a link between 4-chloro-*o*-toluidine and human bladder cancer; a couple of other studies have shown negative results—in contrast to the other positive studies for *o*-toluidine. Exposure to the other two possible carcinogens, *N*-acetyl-*o*-toluidine or 6-chloro-*o*-toluidine, was considerably less than that to either *o*-toluidine or 4-chloro-*o*-toluidine; little or no epidemiological evidence is available on these two additional chemicals. A look at the entire range of epidemiological evidence relevant to this study thus

suggests that *o*-toluidine may be more responsible for the bladder cancers than the study author recognized, despite the greater exposure to 4-chloro-*o*-toluidine.

Lastly, because the other major exposure in the study of Ward *et al.*, (1991a) is to aniline, this study's results provide the most convincing epidemiological evidence for the ability of *o*-toluidine to cause bladder cancer. Significantly more epidemiological evidence is available on aniline than on any other of the chemicals that confound the influence of *o*-toluidine in the two other studies. Though the evidence on aniline in combination with other chemicals suggests some causal influence of this chemical on human bladder cancer—sufficient for Ward *et al.* (1991a) not to rule out any influence on their results—the negative evidence on aniline alone makes *o*-toluidine the most likely cause for the high rates of bladder cancer in the study. Although 4-aminobiphenyl may seem a possible influence given the clear epidemiological evidence on its human carcinogenicity, Ward *et al.* discount its importance since NIOSH analysis of bulk samples of the process chemicals revealed only trace amounts of 4-aminobiphenyl.

Acquavella *et al.*, (1991) have advanced the hypothesis that Ward *et al.*'s (1991a) results were due to 2-naphthylamine and possibly 2-aminofluorene—established carcinogens—which may have been contaminants in the production process during the 1950s using coal-derived benzene (Acquavella *et al.*, 1991). Aside from the inability of Acquavella's hypothesis to account for the elevated bladder cancer rates in the Stasik and Rubino studies, Ward *et al.* (1991b) counter this hypothesis effectively. Ward *et al.* (1991b) report information that suggests the total impurities in benzene used in the 1950s was 0.1% and perhaps less, and that the vast majority of these impurities were probably not carcinogens.

Although some uncertainties still exist about the degree of *o*-toluidine's influence on the bladder cancer rates in each of these studies, the overall pattern of the epidemiological evidence points consistently to *o*-toluidine as a human bladder carcinogen. It is also important to ask whether any more conclusive evidence about *o*-toluidine's human carcinogenicity will be forthcoming. The processes which bring human contact with *o*-toluidine have always involved contact with other suspect chemicals, and they will continue to do so in the foreseeable future. If prevention of important causes of elevated bladder cancer rates is desirable, it is unlikely that epidemiology will yield any more conclusive information than this on which to act. The carcinogenic effects of chemicals like *ortho*-toluidine will always have to be inferred from different combinations of exposures.

4. *Time relationships.* Is the time course of the disease related to the exposure circumstances? Latency periods in all three studies corresponded with those generally found in occupational bladder cancer; they were also consistent among the studies themselves. The mean latency period in the Rubino study was 27.4 years; in the Stasik study it was 27.5 years; and in the Ward *et al.* (1991a) study it was 23 years among those definitely exposed.

5. *Dose–response relationships.* Is there an increasing risk commensurate with an increasing exposure? Ward *et al.*'s (1991a) study was the only one to explicitly test this question. They found a standardized incidence ratio of 0 for those exposed for less than 5 years, compared to 8.8 among those exposed for 5–10 years and 27.2 among those exposed 10+ years; this dose–response curve was highly significant for trend. Durations of exposure in the Rubino study, between 12–33 years, suggested a similar trend. So did the median exposure of 14 years in the Stasik study, although it included two cases who only reported 1.5 and 4.0 years of exposure.

6. *Biological plausibility and coherence of the evidence.* Do animal data support the human epidemiological evidence and is any proposed mechanism of action a plausible one? The animal data on *ortho*-toluidine and the other chemicals involved in the epidemiological studies strongly support the above conclusions about the epidemiological evidence. *Ortho*-toluidine is clearly a carcinogen for numerous species of animals, including rats and mice of different strains; in several cases, it has even proven to be a bladder carcinogen for animals. Among the other chemicals that may confound the influence of *ortho*-toluidine in the epidemiological studies, only 4-aminobiphenyl has produced bladder tumors in animals, and only the latter compound, 4,4′-methylene bis (2-methylaniline), and possibly *ortho*-aminoazotoluene have shown a comparable degree of carcinogenicity in the standard animal models. The first and last of these compounds were only minor exposures in the Ward and Rubino studies, respectively; only those in the Rubino study had exposure to significant amounts of 4,4′-methylene bis (2-methylaniline). Again, the hypothesis of the human as well as animal carcinogenicity of *ortho*-toluidine seems by far the strongest explanation for the available evidence. The genotoxicity studies provide no clear mechanism of carcinogenicity beyond scattered suggestions of genotoxic effects and thus make little contribution to the evidence.

7. *Effect of removal from exposure.* Does a reduction in the exposure lead to a reduction in risk in individuals experiencing improved circumstances? The cohort from the Ward *et al.* (1991a) study offers a natural experiment to test this hypothesis, since workers from the 1980s had significantly decreased exposure to *ortho*-toluidine in comparison with earlier workers, to tell from the available evidence. Due to the long latency period for occupational bladder cancers suggested by the three strongest epidemiological studies, however, we will have to wait at least another decade before this evidence is forthcoming.

8. *Support from analogous experience.* Do similar chemicals act similarly? Aromatic amines including 4-aminobiphenyl (see above), benzidine, and 2-naphthylamine have proven to be both human and animal carcinogens.

Finally, to place these criteria for judging human carcinogenicity within current debates, we should mention that these criteria fall well within the bounds of the most stringent criteria currently being proposed. Even in a conservative classification scheme such as that proposed by Ashby *et al.* (1990) the main difference between categories of "probable" and "known" carcinogens lies in the epidemiological evidence, which is either sufficient, limited, or inadequate. For *ortho*-toluidine, the multiple epidemiological studies, when considered together alongside the evidence for the carcinogenicity of other chemicals to which there was significant exposure, rule out any confounding "with reasonable confidence"—the standard invoked by these authors (Ashby *et al.*, 1990). In the terminology of Feinstein (1988) these studies collectively overcome the vexing problem of isolating "attributable actions" that has plagued epidemiological studies in this area. Indeed, our reevaluation of *ortho*-toluidine's carcinogenicity aims to fullfull Feinstein's prescription for improving the use of epidemiology in these circumstances, through a focus "more on the scientific quality of the evidence, and less on the statistical methods of analysis and adjustment."

CONCLUSION

Recent epidemiological evidence, especially considered alongside the evidence on other chemicals to which workers in the studies were exposed, provides newly conclusive

evidence that *ortho*-toluidine is a human as well as a potent animal carcinogen. IARC as well as the National Toxicology Program and the American Conference of Governmental Industrial Hygienists still classify *ortho*-toluidine as a suspected or probable human carcinogen—a rating that does not take into account more recent data. The new evidence, when evaluated in a suitably comprehensive way, necessitates a rethinking of current regulatory strategies for controlling *ortho*-toluidine exposure in the workplace. *Ortho*-toluidine should be considered a human carcinogen.

Beyond its significance for the regulation and use of *ortho*-toluidine, this case for the chemical's human carcinogenicity also has implications for current debates about the proper criteria for determining carcinogenicity. Though animal tests have come under attack as tools for predicting human carcinogenic effects, *ortho*-toluidine offers one more instance in which significant carcinogenic results in multiple animal species have accurately presaged strong epidemiological evidence for human carcinogenicity. As risk assessors attempt to forge a consensus on the kind and degree of animal evidence that warrants heavy suspicion of human carcinogenicity, they can turn to the toxicological studies of *ortho*-toluidine as an instructive precedent.

Also, though Gori (1991) has recently suggested the need for a precise knowledge about mechanisms in any determination about human carcinogenicity, such an insistence seems inappropriate in the case of a chemical like *ortho*-toluidine. It would be reassuring if we could obtain this knowledge; perhaps it would also alter our strategies for dealing with such outbreaks of cancer. But in the case of *ortho*-toluidine, the mixed results from genotoxicity studies, as well as our imperfect knowledge about how cancer is caused, mean that we cannot expect to demonstrate the mechanism by which *ortho*-toluidine causes cancer in the forseeable future. Likewise, it would also be reassuring if we had results from epidemiological studies of workers exposed to *ortho*-toluidine alone. But the way *ortho*-toluidine is used in industry dictates that we cannot obtain these kinds of results. Realistically, the hypothesis of *ortho*-toluidine's carcinogenicity seems by far the most persuasive explanation for the extremely high cancer rates in the evidence assembled above. Certainly, the evidence for human carcinogenicity is sufficient in this case to justify employing a wider range of the available legal and technical means for protecting workers from proven human carcinogens.

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