III. BIOLOGIC EFFECTS OF EXPOSURE

o-Tolidine is a symmetrical biphenyl compound with an amine group and a methyl group attached to each ring. The amine groups are in the para position to the biphenyl link; the methyl groups are in the ortho position to the amine group [1,2]. The structures of several biphenyl amines, including o-tolidine and benzidine, are similar. These substances are sometimes called biphenyl amines, aminobiphenyl compounds, or simply diamines. Because they have been measured colorimetrically by the formation of colored holoquinone complexes [3], aminobiphenyl compounds have also been referred to as quinonizable substances. Their physical and chemical properties are also similar [1,2]. For example, o-tolidine melts at 129 C, is slightly soluble in water, and is soluble in alcohol and ether, whereas benzidine has a melting point of 126 C, is slightly soluble in water and alcohol, and is soluble in ether. The chemical structures of some aromatic amine compounds are given in Figure III-1 [4,5].

Extent of Exposure

o-Tolidine (3,3'-dimethyl,4,4'-diamino biphenyl, formula weight 212.32) is a crystalline solid. Salient physical and chemical properties of o-tolidine and benzidine are listed in Table XII-1 [1,2,5,6]. o-Tolidine is used both as a dye and as an intermediate in the production of other dyes [4]. It is widely used in small quantities as a laboratory analytical reagent and is a moiety of the commonly used biologic stain, trypan blue [7]. o-Tolidine is used in small quantities in chlorine test kits by water companies and swimming pool owners and in test tapes in clinical laboratories. Although other chemicals are preferred, o-tolidine has been used as a curing agent for urethane resins, in part because some of the other curing agents, eg, 4,4'-methylene bis (2-chloraniline)(MOCA), were proposed for stringent regulation as carcinogens (29 CFR 1910.93).

o-Tolidine is prepared from o-nitrotoluene by reduction to a hydrazo compound and rearrangement to the biphenyl amine in the same manner that benzidine is made from nitrobenzene [4]. Reduction is usually carried out with zinc dust and caustic soda in organic solvents [4]. Other reducing agents used in the manufacture of o-tolidine may also be used to prepare benzidine [4]. If m-nitrotoluene is the starting material, m-tolidine (2,2-dimethyl-4,4'-diamino biphenyl) is formed; dyes made from m-tolidine may be used for dyeing wool, but they have little affinity for cotton [4].

The major US manufacturer of o-tolidine makes an average of 200,000 pounds of o-tolidine salts, eg, hydrochloride, each year. Smaller quantities are produced by other companies. US production data on o-tolidine base are not available. Many chemical companies buy o-tolidine in bulk from other,

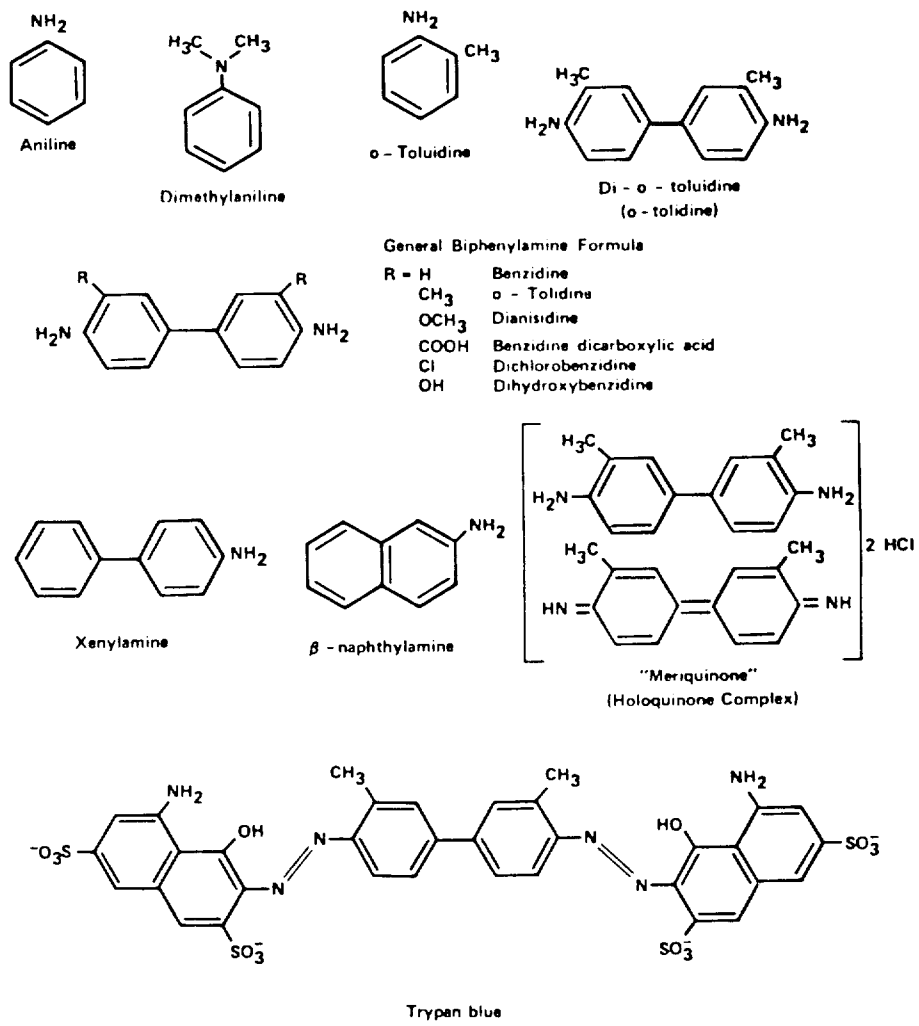

FIGURE III-1

CHEMICAL STRUCTURE OF SOME AROMATIC AMINE COMPOUNDS

principally foreign, manufacturers, repackage it, and sell it in smaller units; they sometimes refine it. About 300,000 pounds of o-tolidine base and 150,000 pounds of o-tolidine salts are imported annually [8,9].

Workers potentially exposed to o-tolidine are listed in Table XII-2 [4]. Workers exposed to the greatest amounts of o-tolidine are probably dye makers, toluene-diisocyanate makers, clinical or analytical chemistry laboratory workers, and repackagers [4,10]. Workers in a variety of occuptions may be exposed to small quantities of o-tolidine for analytical purposes, among them water and sewage plant attendants, sanitarians, forest service chemists, swimming pool service representatives, and chemical test tape or kit makers. It is estimated that less than 100 employees are exposed to large quantities of o-tolidine in the United States, but as many as 200,000 may be exposed to small quantities [11].

Historical Reports

In 1932, Berenblum [12] reviewed the incidence of "aniline cancer." The term "aniline cancer" was a general term used to refer to bladder cancer resulting from exposure to synthetic organic dyes derived from aniline, and to other similar dyes. Aniline was originally thought to cause cancer but more recently has been held to be noncarcinogenic [13]; use of the term "aniline cancer" has since been discontinued [12]. Berenblum concluded that the incidence of bladder tumors in dye-industry workers varied in different factories, districts, and countries. The data, however, did not point to any single reason for this variation. For example, the incidence of bladder tumors was higher in Germany than in other countries, even when the values were corrected for the greater size and age of the German dye industry. The variation in tumor incidence, however, was also found in different factories within Germany. It was observed that the incidence of bladder tumors decreased in one district after various protective measures were instituted in the work practices of these factories. Berenblum [12] suggested that other factors, eg, individual susceptibility, age, race, length of exposure, and type of industrial process for dye making, contributed to variation in tumor incidence.

Berenblum [12] also found reports of tumors localized in other parts of the body. Rehn, in 1895, described how one dye worker had carcinomas in the right ureter and kidney as well as of the bladder with metastases chiefly in the lumbar lymph nodes. Another worker developed a cancer of the renal pelvis of a hydronephrotic kidney. The bladder, however, appeared to be by far the most frequent tumor location. Both benign and malignant tumors were found, the malignant tumors developing as early as the benign tumors. Berenblum found suggestions, dating from 1920, that periodic urine testing and cystoscopic examinations of workers would aid in early diagnosis of bladder tumors.

Until 1932, few experimental studies were conducted to determine which individual chemicals in the dye industry were carcinogenic. The results

reported before then were inconclusive, but it was widely accepted that the causative agents were to be found among the many intermediates used to prepare the dyes [12].

Scott and Williams [14], in 1957, wrote work practice recommendations for the control of bladder cancer caused by occupational exposure. They suggested that large amounts of o-tolidine, dianisidine, and dichlorobenzidine should be treated like benzidine. Benzidine was by then an accepted human carcinogen [13,15].

Effects on Humans

Most investigations regarding o-tolidine have centered on its action on the urinary tract, but they have not developed evidence of chronic effects attributable solely to o-tolidine exposure. There have also been informal comments made to NIOSH regarding nasal irritation. This information is consistent with the observations of one researcher [16]. No other symptoms directly referable to o-tolidine were reported.

Brown and coworkers [17] found no o-tolidine in the urine of one human volunteer exposed to 100 mg of moistened crystalline o-tolidine for 48 hours. It was held against the skin under gauze patches according to the Draize technique [18]. They did detect urinary excretion of 0.113 mg of o-tolidine after 24 hours when 200 mg was applied to the palm of the hand for 8 hours under a rubber glove. No skin irritation was observed.

When one female volunteer was given three oral doses of 100 mg of o-tolidine (65.1% as base), she excreted 5.60-7.47 mg/day [17]. There were no quinonizable substances detected in the urine after 3-4 days.

Rye et al [19] mentioned in a review of diamine toxicity that they had found no cases of human cancer in two decades of experience with workers exposed to o-tolidine, dianisidine, and dichlorobenzidine, but specific data were not given to support this observation. There were, however, 23 cases of urinary system cancer reported in Japanese workers exposed to benzidine, o-tolidine, and dianisidine [20]. The length of exposure and the period between onset of exposure and the development of tumors were not reported, except that 14 workers (61%) had been exposed less than 6 years.

Macalpine [21] described two cases of papilloma of the renal pelvis in workers manufacturing o-tolidine and benzidine. Other chemicals occasionally handled included azobenzol, nitrobenzol, and o-nitrotoluol. The first worker's urine contained red blood cells, and a red, coarsely nodular papilloma was discovered in the vault of the bladder by cystoscopy. It was not necrotic. A smaller papilloma with fronds was located on the left wall of the bladder near the urethra. The man was treated by diathermy, and nine cystoscopic examinations over the next 3.5 years showed him to be tumor free. He then complained of difficulty in urinating, although there was no hematuria. Papillomas found at the neck of the bladder were treated by

diathermy. Several months later, the bladder tumors had grown larger and a large mass was felt in the area of the left kidney. The enlarged left kidney and ureter were removed surgically, and the bladder was treated by diathermy. The patient was discharged, but he was readmitted within 4 months with hematuria. Papillomas were found again at the neck of the bladder; they were larger than before and treated by surgery and diathermy. The patient died suddenly of hemorrhage during the course of treatment; no autopsy was permitted.

The second worker described by Macalpine [21] had a similar occupational and medical history. Eight cystoscopic examinations in 3.5 years were negative, then papillomas recurred in the kidney while the bladder remained free of tumors. He experienced recurrent anuria and died of uremia. At autopsy both kidneys were found to be affected. The right kidney was greatly enlarged by a papillary carcinoma. The left kidney cortex had atrophied; a papilloma obstructed part of the upper calyx. The bladder and both ureters appeared normal. The effects attributable to benzidine could not be distinguished from those of tolidine, and the isomer of tolidine was not identified. This report suggests the need for comprehensive urologic evaluation, including kidney function tests and microscopic examination for red blood cells and abnormal epithelial cells in urine. Repeated cystoscopy did not detect tumors in the upper part of the urinary tract.

In Italy, Barsotti and Vigliani [13] collected data on bladder lesions in 200 workers in the dye industry. Exposure to o-tolidine may have occurred but was not mentioned specifically. The workers had received routine cystoscopic examinations between 1931 and 1948. The authors found that beta-naphthylamine and benzidine were the most dangerous carcinogens of the aromatic amines to which the workers were exposed, whereas aniline had no appreciable carcinogenic potential. Tumors appeared 4-28 years after initial contact with beta-naphthylamine or benzidine.

In 1954, Meigs et al [22] analyzed urine samples from workers over a 5-year period at a plant manufacturing benzidine, dichlorobenzidine, dianisidine, and o-tolidine. About 900 samples from production workers and about 250 samples from non-production controls located in the same building were analyzed by the chloramine-T test. In addition, 17 control urine specimens were obtained from an employee in a different building 500 yards away, and 35 control specimens were obtained from men and women working in a nearby university and hospital. The number of individuals participating was not stated. Workers were required to shower daily after the workshift and provide a clean urine specimen. Air samples from production areas contained "quinonizable" (holoquinone-forming) substances at concentrations of 2-87 μg/cu m, with a mean of 18 ±2 μg/cu m. Air from other areas in the same building contained less than 1 μg/cu m. The chloramine-T test did not distinguish among aminobiphenyl compounds; separation was accomplished by paper chromatography. The results were generally reported in units of benzidine.

Urine samples from workers producing benzidine and substituted benzidines contained quinonizable substances in mean concentrations of 144-1,482 μg/liter and averaged about 500 μg/liter [22]. Samples from workers not making benzidine and related amines contained less than 15 μg/liter of quinonizable substances. Specimens from a worker in another building had a mean of 6 μg/liter, and the control samples from the university and hospital workers contained no detectable biphenyl diamines. Urine specimens from foremen had lower biphenyl diamine concentrations than did those from workers who actually handled the substances. Operators who were directly exposed excreted about 500 μg/liter of o-tolidine and foremen excreted about 50 μg/liter. Biphenyl diamine concentrations in urine were lower in the winter and higher in the summer, although the specific gravity of the urine did not differ appreciably. Higher concentrations of excreted biphenyl diamines were also associated with higher humidity. This is consistent with Meigs' speculation that increased skin moisture from sweating increased diamine absorption. A downward trend was noted in urinary excretion of biphenyl diamines over 3 years. The summer mean declined from 1,482 to 570 μg/liter, the winter mean from 433 to 144 μg/liter. This decrease was related to improved work practices and personal hygiene.

Brief but heavy exposure to a substituted benzidine resulted in a 1-day elevation of excreted quinonizable substance [22]. A worker was accidentally drenched with a slurry containing dichlorobenzidine hydrochloride in acid solution. Although he took a shower and changed his clothes within 5 minutes of the accident, his urinary excretion of diamines went from 43 to 1,130 μg/liter for 1 day and then returned to baseline levels.

Results of urinalyses from other workers led to the discovery of previously unrecognized sources of exposure, such as contaminated boots or gloves [22]. One worker had made only o-tolidine, dianisidine, and dichlorobenzidine, but chromatographic analysis of his urine sample for that day showed benzidine as well as the three diamines to which he was known to have been exposed. Investigation revealed that his boots were contaminated with benzidine. Similarly, o-tolidine was found in urine samples of two workers who had made only dichlorobenzidine on the day in question and in another sample from a worker who had that day made only m-aminophenol.

When both the skin and the clothing were carefully decontaminated, the urinary concentrations of biphenyl amines decreased markedly [22]. Before the initiation of a personal hygiene program at the workplace, one worker's specimens contained quinonizable substances in concentrations averaging 117 μg/liter, the concentration tending to increase slightly as the week progressed. After hygienic work practices were adopted, his average urinary output of quinonizable substances had fallen 80%, to 21 μg/liter, at the end of 14 months [22].

The authors [22] concluded that absorption of aminobiphenyl compounds, such as o-tolidine, through the skin was a more important hazard than was inhalation of airborne particles of these compounds. They based this conclusion on several observations. First, the concentrations of the

compounds in the workplace air were too low to produce the reported urinary concentrations. Also, foremen in the production areas excreted very little quinonizable substance compared with the workers actually handling the substances in the same areas, and the substances excreted corresponded to the substances to which the workers were exposed. Because diamine excretion increased with larger exposures (splashes) and on hot, humid days, Meigs et al suggested that moist skin was conducive to absorption of benzidine and substituted benzidines. They suggested that producing substituted benzidines in the winter or in air-conditioned facilities would reduce absorption through the skin by keeping the skin dry. It should be noted again that the chloramine-T test is not specific and that positive results were followed up by separation by paper chromatography. Whether this detection of unexpected diamines may have been the result of mixed exposures, chromatographic error, or biotransformation is not clear, but the latter is hard to demonstrate. The explanation offered by Meigs et al, that there were occult exposures to other chemicals deposited in boots or clothing, is the most probable. When this study was performed in the early 1950's, skin contact was generally considered the greatest hazard. With the subsequent improvement of engineering controls and work practices, skin contact with o-tolidine could be effectively controlled, and concern shifted to the inhalation hazard of airborne o-tolidine.

A study of occupational bladder tumors in the Japanese dye industry, by Tsuchiya et al [23] in 1975, reported that 1 worker of 107 exposed to "other amines, e.g. orthotolidine, including mixed exposures," excluding benzidine and alpha- and beta-naphthylamine, had a positive urinary Papanicolaou test. The workers' ages and the type and extent of exposure were not reported. Both active and retired workers were tested. Probably all workers exposed to o-tolidine are also exposed to other amines that may be carcinogenic.

Animal Toxicity

While experimental evidence of the effects of exposure to o-tolidine is meager, a few animal studies have attempted to elucidate the toxicologic properties of o-tolidine.

(a) General

Rye et al [19] stated in a review of diamine toxicity that o-tolidine base penetrates the skin more readily than its salts, but no supporting data were given.

In studies of acute toxicity in various species of animals, the toxicity of o-tolidine appears to be similar to, perhaps slightly greater than, that of benzidine. In the rat, the oral LD50 of o-tolidine was 404 mg/kg [24]; for benzidine, it was 566 mg/kg [25]. LD50's of 90.9 mg/kg and 199.4 mg/kg for o-tolidine and benzidine, respectively, were determined after albino mice were given intraperitoneal (ip) injections [17]. Renal toxicity of o-tolidine from a single dose administered orally to the rabbit was said to be greater than

that of benzidine [16], but no quantitative comparison was made.

In 1908, Adler [16] reported that three rabbits weighing about 2 kg each were given o-tolidine suspended in water orally at a daily dose of 1 g. The results, anuria, lethargy, and death within a few days, were said to be similar in all three rabbits tested. According to Adler, effects which he termed "urinary excretion disorders" were produced in rabbits by o-tolidine but not by benzidine.

Brown et al [17] evaluated skin irritation in six albino rabbits. The fur was clipped close to the skin and 0.5 g of o-tolidine, 65.1% base and the rest dihydrochloride, was kept on the skin under small gauze patches for 24 hours. No irritation was noted with intact skin, but very slight erythema appeared when o-tolidine was applied to abraded skin. This suggests that any cutaneous absorption of o-tolidine will probably not be accompanied by significant irritation; thus, it might not be noticeable.

Maruya [26] fed o-tolidine, at a 5% concentration in olive oil, mixed with rice at 20 cc of olive oil solution/kg of rice, to nine adult albino rats for 21-111 days. Control rats were not reported, but a total of 311 other rats were administered 4 other aromatic amines or 14 azo pigments. The ratio of males to females in each group was not reported, but half of the total of 320 rats were male. The kidneys of each rat were microscopically examined when the animal died during, or was killed at the end of, the experiment. Six of the rats exposed to o-tolidine had swelling and thickening of the cells lining the uriniferous tubules (two mild, two moderate, and two severe), with an accumulation of giant cells with abnormally large nuclei in the distal straight portions. The tubular lumen was narrowed. Two other rats showed a mild accumulation of pigment derived from hemolysis in the tubular epithelial cells. The degeneration of tubular cells was generally more severe when pigment granules were found in the cells, but it was not stated whether this finding occurred in rats fed o-tolidine. No results were reported for the ninth rat given o-tolidine. Maruya considered it significant that chemicals like o-tolidine, with a slight tendency to induce hemolysis and pigmentation of the uriniferous tubule cells, strongly provoked swelling of the tubular epithelium and massing of giant cells with heteromorphous giant nuclei.

(b) Carcinogenesis, Mutagenesis, and Teratogenesis

Early attempts to induce tumors of the urinary system experimentally by injecting animals with suspensions of o-tolidine at an unreported dose for 18 months did not succeed [27]. Feeding o-tolidine to dogs similarly yielded no bladder tumors [28]. In an experiment performed by Brown and Franz and reported by Ferber [29], there was one case of bladder cancer in dogs fed o-tolidine. In a brief commentary on the use of hamsters in studying induction of bladder cancer by aromatic amines, Saffiotti and colleagues [30] said they found no carcinogenic activity in hamsters fed o-tolidine (or 2-naphthylamine or benzidine as well as some other aromatic amines) at 0.1% in the diet. No supportive details were given.

Experiments, primarily on dogs, were reported in 1948 by Gehrmann and colleagues [28]. The following compounds were fed to dogs in daily doses 5 times a week for the time period indicated: aniline (300 mg/3.5 years), benzidine (117 mg/5 years), dianisidine (291 mg/3.5 years), alpha-naphthylamine (technical, 330 mg/4.5 years), alpha-naphthylamine (pure, 301 mg/4.5 years), phenyl-alpha-naphthylamine (290 mg/3 years), phenyl-beta-naphthylamine (540 mg/4.5 years), tolidine (isomer not specified, 230 mg/3 years), and beta-naphthylamine (300 mg/50 days). Only beta-naphthylamine was found to produce bladder tumors. No bladder tumors were induced in the three female dogs fed tolidine. The total dosage of tolidine was almost 180 g. Benzidine did not induce detectable bladder tumors or papillomas in these dogs.

As previously mentioned, Ferber [29], in 1970, prepared an unpublished report of an experiment designed to indicate whether bladder cancer could be induced by o-tolidine in dogs and whether it would continue to develop after a normal diet was resumed. Four young mongrel bitches weighing 40-48 pounds (18-22 kg) each were given 200 mg of o-tolidine in gelatin capsules daily for 8-9 months, a total dose of almost 50 g per dog. The findings of cystoscopic examinations before and after o-tolidine administration were negative. One dog died of bladder cancer (papillary tumor and cystitis) 8 years after the last cystoscopic examination. Another dog died of natural causes soon after the first, but no tumors were found at autopsy. The last two dogs were then killed and examined, but no tumors were found. Ferber concluded that o-tolidine may be a weak carcinogen but is not as carcinogenic as benzidine. This conclusion could not be supported statistically, because too few dogs were studied. Old dogs may develop tumors spontaneously; however, bladder tumors in dogs are rare [31].

Spitz et al [15], in 1950, reported the carcinogenic potential of benzidine. Clinical evidence had been lacking until then, because workers exposed to benzidine had frequently also been exposed to beta-naphthylamine, a recognized carcinogen [15]. The experiments of Spitz et al on rats dealt with the individual effects of benzidine; other compounds encountered during its manufacture, such as azoxybenzene, hydrazobenzene, and benzidine sulfate; and those closely allied to it chemically, such as tolidine, azobenzene, and benzidine disulfonic acid. All chemicals were injected subcutaneously (sc). Benzidine sulfate was also fed to rats. Results showed that benzidine did not produce bladder cancer in rats, but it did produce hepatomas and tumors of the Zymbal glands (the specialized sebaceous glands of the rat's auditory canal). o-Tolidine also produced tumors of the Zymbal glands. Technical grade benzidine caused more tumors than pure benzidine; however, the reason for this difference was not discussed. o-Tolidine caused fewer tumors in spite of injection of four times more o-tolidine (60 mg) than benzidine (15 mg) each week, suggesting that o-tolidine is a less potent carcinogen than benzidine. Fifty control rats did not develop tumors.

Holland et al [32] administered o-tolidine sc in peanut oil to 21 Alderly Park strain rats for 241 days (cumulative dose, 5.4 g/kg). The rats survived 94-703 days following the first dose of o-tolidine; tumors were not detected

at necropsy until day 325. A total of 18 rats developed tumors, including tumors of the gastrointestinal tract (11), hepatomas (7), tumors of the bone and associated tissues (including hematopoietic tissues) (4), and Zymbal gland tumors (40. Similarly, 22 rats were administered benzidine sc for 150 days (cumulative dose, 0.75 g/kg). Treated rats survided for 24-387 days following the first dose of benzidine. Tumors were not detected at necropsy until day 84 as compared with day 325 for o-tolidine. A total of 20 of 22 rats developed tumors (compared with 18/21 for o-tolidine), notably hepatomas (19), cholangiomas (18), intestinal tumors (7), and tumors of the Zymbal glands (4).

Holland et al [32] also tested 3,5,3',5'-tetramethylbenzidine in peanut oil administered sc to 24 male rats for 32 weeks at doses varying from 100 to 25 mg/kg during the testing period. A control group of 12 rats was given peanut oil sc only. Gross and microscopic post-mortem examinations revealed a few tumors but none that the authors could definitely relate to 3,5,3',5'-tetramethylbenzidine. Holland et al regarded the tumors associated with the administration of o-tolidine and benzidine as having been induced by these chemicals.

In 1970, Pliss and Zabezhinsky [33] reported the results of sc administration of o-tolidine to rats either in oil suspension or in glycerin pellet implants. The results were also discussed in two other publications [34,35]. White rats of both sexes weighing 100-120 g were used in five experiments. The only general statement about control animals was that 2.5% of an unreported number of untreated old virgin female rats developed fibroadenomas of the mammary glands.

In the first experiment, 27 males and 26 females were injected once a week with 20 mg of o-tolidine in sunflowerseed oil for 13 months [33]. The total dose for each rat was 1.16 g. The appearance of tumors was noted, and necropsies were ultimately performed on all animals. Tumors and abnormal organs were examined microscopically. Tumors appeared in rats of both sexes in 8 months; two males and one female died before the appearance of tumors [33]. Seventeen males and 13 females developed tumors, some more than one [33-35]. Tumors were found in Zymbal glands in 14 males and 6 females in 8-22 months, in preputial glands in 2 males in 20-22 months and in 1 female in 13 months, in the skin in 2 males in 18-23 months and 1 female in 16 months, in the mammary gland in 5 females in 13-22 months, in the forestomach in 3 males in 21-25 months, in the liver of 1 male in 23 months, and in the uterus of 1 rat in 22 months. Four other tumor sites were reported for male rats: one tumor of the small intestine in an unreported induction time, two tumors of the hematopoietic system in 12 and 23 months, one thyroid tumor in 25 months, and one lung tumor in 22 months. Various multiple tumors were found. No sarcomas were found at the site of injection. Only one of 50 control rats injected sc with sunflowerseed oil developed a tumor, a sarcoma associated with a parasitic cyst [34].

In the second experiment, pellets of o-tolidine were used [33]. In this experiment, 20 males and 20 females received weekly sc implants of one pellet containing 20 mg of o-tolidine and 10 mg glycerin for 14 months.

Approximately the same site was used each time for implantation, and the total dose for each rat was 1.22 g. Autopsy procedures were the same as those used in the first experiment. It took 12 months for the first tumor to appear in rats of either sex, at which time 16 males and 20 females were still living. The tumor that appeared earliest and affected the greatest number was, again, in Zymbal glands; six males and five females developed tumors of the Zymbal glands in 12-18 months. Seven females had mammary tumors in 12-23 months. One male and one female showed skin tumors in 17 and 18 months. Three males developed liver tumors in 15-20 months. One male had angiosarcoma in the area of the forelimb in 14 months, another showed neurosarcoma around the ear in 15 months, and a third had a tumor of the hematopoietic system after 20 months. One female had a lymphangioma in the neck after 20 months. Only one rat, a male, showed a tumor, diagnosed as a rhabdomyosarcoma, at the site of implantation in 20 months. In all, neoplasms were found in 23 rats (64%). Six rats had multiple tumors involving chiefly the skin and the liver. By the 18th month, three males and five females were alive.

The third experiment [33] was similar to the second, except that the o-tolidine in the implanted pellet was subjected to ultraviolet irradiation to "oxidize" the o-tolidine photochemically. It was not stated why the authors concluded that application of ultraviolet radiation would oxidize o-tolidine. Twenty-four males and 24 females were used. As in the first and second experiments, the onset and characteristics of tumors were noted and confirmed when necropsies were performed. Tumors were first observed during month 11. Thirty-two rats were then alive, 18 males and 14 females. Six males and two females survived for 18 months. Neoplasms appeared in 25 rats (52%), 15 males and 10 females, and 6 of these had combinations of tumors at different sites. Among the males, nine had tumors of Zymbal glands in 11-19 months, four had tumors of the skin in 16-19 months, one had an intestinal tumor in 15 months, one had a tumor of the submaxillary salivary gland in 18 months, and one had a subcutaneous sarcoma, diagnosed as a rhabdomyosarcoma, at the site of implantation. Among the females, seven had Zymbal gland tumors in 12-16 months, one had a tumor of the mammary gland in 19 months, three had skin tumors in 14-19 months, and one had a tumor of the hematopoietic system in 17 months.

Retention of o-tolidine in the subcutaneous tissues at the sites of injection and implantation was studied in the fourth experiment [33]. Fifteen rats of unreported sex were injected sc once with 20 mg of o-tolidine in sunflowerseed oil; after 1-7 days, the subcutaneous tissue was excised for determination of o-tolidine content. Similarly, a pellet containing 20 mg of o-tolidine was implanted in each of 10 rats (number of each sex not reported), and tissue from the implant site was taken for o-tolidine determination after 7 days. In 2 samples, 0.24-1.65 mg of o-tolidine were found at the site of administration of the oil suspension 24 hours after sc injection; trace amounts were found after 48 hours in 2 samples and 168 hours in 10 samples. The locus of injection was not described. Seven days after implanting a pellet containing 20 mg of o-tolidine, 5-13.5 mg were found at the site of implantation in 10 samples.

In the fifth experiment, the amounts of free and bound aromatic amines were determined by diazotization from samples of the liver, kidneys, spleen, omentum, and Zymbal glands of 12 rats that had received daily sc 20-mg injections of o-tolidine in oil for 8 months [33]. The analytical method was reportedly capable of distinguishing aromatic amines from naturally occurring primary and secondary amines [36]. Eleven rats were used as controls. The highest amine content, expressed as μg/g of organ weight, was found in Zymbal glands, 246 bound and 26 free. The kidneys contained less total amines, 117 bound and 38 free, followed by the omentum with 120 bound and 19 free, the spleen with 90 bound and 35 free, and the liver with 90 bound and 32 free. In the control animals, Zymbal glands contained 31 bound and 11 free, the kidney had 18 bound and 17 free, the omentum had 31 bound and 11 free, the spleen contained 15 bound and 19 free, and the liver had 31 bound and 11 free. The standard error associated with all these weight averages was around 5 μg/g. The low amine content of the omentum suggested to the authors that o-tolidine distribution does not depend primarily on its lipid solubility.

Results of microscopic studies of all tumors found in rats from the first three experiments were discussed together [33]. Most of the tumors of Zymbal glands were diagnosed as squamous-cell carcinomas, some were adenocarcinomas or sarcomas. Lung cancer in one rat was attributed to metastasis from a squamous-cell carcinoma of Zymbal glands. Tumors found in the preputial sebaceous gland were diagnosed as one squamous-cell carcinoma, one adenoma, and one sebaceous carcinoma. Most of the skin tumors were epitheliomas arising from hair follicles, rarely from sebaceous or sweat glands. Tumors of mixed types occurred frequently, and both basal-cell carcinoma and spinocellular carcinoma were reported. Both benign and malignant mammary tumors were found. Most malignant mammary tumors were papillary cystadenocarcinomas. A few carcinomas were cribriform in appearance. Two rats had squamous metaplasia of the glandular epithelium. Five rats from the second experiment had benign fibromas arising from fibroadenomas. One rat in the third experiment had a fibroadenoma of the mammary gland. Tumors of the liver occurred in single or multiple nodules. Both benign hepatomas and hepatocellular carcinomas were found. A few rats had cystadenomas of the bile ducts. In one rat, liver carcinoma metastasized to the lung. The tumors of the forestomach were multiple, squamous-cell, cornified papillomas with a cauliflower appearance. Intestinal tumors appeared in two rats; one developed adenocarcinoma of the small intestine and the other had carcinosarcoma of the large intestine with multiple peritoneal metastases. One mixed tumor of the submaxillary salivary gland occurred. Four rats had tumors of the hematopoietic system, one had reticulosarcoma of the liver with multiple peritoneal visceral metastases, two had reticulosis with multiple visceral foci, and one had myeloid leukemia with enlargement of the liver and spleen. The tumors found at the site of administration were polymorphous subcutaneous neoplasms with giant cells; they were suggested to be rhabdomyosarcomas. Various solitary tumors were found, including pulmonary cystadenocarcinoma, uterine leiomyosarcoma, thyroid adenoma, angiosarcoma in the region of the forelimbs, lymphangioma of the neck, and neurosarcoma of the ear [33]. In another experiment, the one rat tested had a tumor of the heart that the authors suggested was a rhabdomyosarcoma [37].

Pliss and Zabezhinsky [33] concluded that o-tolidine is a carcinogen and suggested that its carcinogenic effects may be caused by unidentified metabolites rather than by the compound itself. The high frequency of tumors in structures of ectodermal origin, they suggested, was related to the accumulation of o-tolidine or its metabolites in excretory organs, such as sebaceous glands. Pliss [34] also concluded that a single methyl group ortho to the amino group reduced the likelihood of liver tumors, but that substituting additional methyl groups in the aminobiphenyl molecule increased the possibility of rats developing liver tumors. The evidence in this paper [34] to support these conclusions is weak. Pliss [35] inferred that the carcinogenic properties of o-tolidine were manifested after its conversion to carcinogenic metabolites because all tumors except one formed in a number of distant tissues and did not form at the sc injection sites. He also observed that more males than females developed tumors, particularly of specialized sebaceous glands, eg, Zymbal glands.

Saffiotti et al [30] and Sellakumar et al [38] conducted similar experiments on hamsters, administering o-tolidine in oral doses of 0.1-1%. Their results were negative.

The papers by Spitz et al [15] and Pliss and Zabezhinsky [33] present considerable evidence of the carcinogenicity of o-tolidine in rats. No bladder tumors were found, but they were not expected because even benzidine does not cause bladder tumors in rats [31].

In 1973, Freeman and coworkers [39] tested the use of cell culture transformation to indicate the carcinogenic potential of several chemicals, one of which was o-tolidine. The chemicals were tested in cell cultures from the F111 pool of rat embryo cells inoculated with Rauscher leukemia virus (RLV). The cultures showed no spontaneous transformation for at least 50 passes by RLV, or by chemical carcinogens alone, but did show transformation with both the virus and the chemical. One set of cultures showing transformation was held indefinitely; another was subdivided at 2-week intervals, with half being held indefinitely and half being subdivided again. When transformation occurred frequently, it could be detected in the original cultures and was more pronounced in daughter cultures. When transformation was rare, the cultures had to be subdivided at least once before it was detected. Transformation was recognized by macroscopic foci of spindle cells without polar orientation or contact inhibition; these cells were tumorigenic when transplanted into newborn Fischer rats.

o-Tolidine was found to be toxic to the cell cultures at 199 μg/ml [39]. At 50 μg/ml, there were well-defined foci of spindle cells. At 10 and 5 μg/ml, transformation was still apparent, but the foci were less well defined. No effects were seen at 1 μg/ml. Although this test uses an in vitro system and is not well established, its usefulness in this case is supported by the findings of tumorigenesis when the transformed cells were transplanted into rats. Similar findings with known carcinogenic chemicals, eg, 4-aminobiphenyl and dichlorobenzidine, lend additional support.

Seiler [40] applied the Friedman-Staub screening test to a variety of chemical mutagens and carcinogens. This procedure uses the amount of tritiated thymidine incorporated into DNA as an indication of DNA damage, and, in turn, of carcinogenicity or mutagenicity. The smaller the amount of radiolabeled thymidine incorporated into DNA, the greater the indication of damage. Adult male mice (23-28 g) were given oral 100 mg/kg doses of o-tolidine. Controls were given only thymidine. o-Tolidine administration resulted in incorporation of 77.6% of the amount of thymidine incorporated by controls, a decrease reported to be statistically significant. Overall, 86% of the known carcinogenic and mutagenic chemicals tested depressed DNA synthesis, while only 10% of noncarcinogenic and nonmutagenic compounds did so. Among the chemicals that demonstrated carcinogenicity in vivo, all the polycyclic hydrocarbons tested, all three azo dyes tested, and six of the seven aromatic amines tested were also transforming agents on this assay. This study [40] supports the finding of Freeman and coworkers [39] that o-tolidine damages DNA in mammalian cells.

In 1976, Shimizu and Takemura [41] reported that o-tolidine at an unreported concentration exerted a positive mutagenic effect on two strains of bacteria used in the Ames test, Salmonella typhimurium TA 98 and TA 100. The Ames test indicates mutation by testing a chemical's ability to change a microorganism's requirement for a specific nutrient, usually histidine. The magnitude of this effect was not reported. The authors compared their results favorably with the positive evidence for carcinogenicity of o-tolidine reported by Pliss in 1970, but did not cite the specific reference.

o-Tolidine base was evaluated for mutagenic activity in another study [42]. Microbial plate assays were performed with Saccharomyces cerevisiae D4 and Salmonella typhimurium TA 1535, TA 1537, and TA 1538, both with and without metabolic activation from tissue homogenate prepared from the lungs, liver, and testes of ICR random-bred mice, Sprague-Dawley rats, and rhesus monkeys. Tests were done in duplicate with positive and negative controls. A test was considered positive if spontaneous reverse mutations in Salmonella and mitotic recombination in Saccharomyces increased tenfold or more above background levels. The initial results were inconclusive, but the nonactivation assays and the activation assays with mouse liver indicated that the number of revertants with Salmonella typhimurium TA 1538 was slightly increased. Additional tests were performed using the same protocol, except that a fivefold or greater increase was considered positive. This time, no mutagenic activity was detected. The experimenters concluded that o-tolidine base was not mutagenic.

In 1977, Ferretti et al [43] tested the mutagenic effects of o-tolidine and several other compounds on Salmonella typhimurium TA 1538. The standard Ames procedure was followed. The results, expressed as a ratio of the average number of revertants/plate with activation by liver microsomal enzymes to the revertants/plate without activation, were 371:18 for benzidine, 422:12 for o-dianisidine, and 80:6 for o-tolidine. The authors judged this to be evidence that benzidine, dianisidine, and o-tolidine were mutagenic. None of the compounds showed mutagenic activity in the absence of microsomal activation;

this is consistent with suggestions that the carcinogenic effect of o-tolidine in rats is due to a metabolite of o-tolidine, rather than to o-tolidine itself [33].

Golub [44] administered o-tolidine to pregnant BALB/c mice at a daily sc dose of 2 mg in 0.1 ml of sunflowerseed oil. The mice were killed after 19-20 days, and the embryonic kidneys were obtained for organ culture. A total of 55 organ cultures were obtained from controls and 25 from mice given o-tolidine. Organ cultures were examined for 20 days. Epithelial hyperplasia and other undescribed cellular changes were noted in the experimental cultures but not in controls, suggesting that o-tolidine had transplacental effects on the embryos of pregnant mice.

Wilson [45], in 1955, reported on the teratogenic effect of o-tolidine and dyes with o-tolidine nuclei, eg, trypan blue. A total of 30 mg of o-tolidine was administered by sc injections on 3 successive days to each of 10 young adult albino rats after pregnancy had proceeded for 1 week. No control group was reported. Pregnancy continued until the 20th day, when the mothers were killed and the offspring examined. A total of 109 implantations occurred in the 10 rats given o-tolidine; of these, no embryos were malformed, but 8% were resorbed. Basing their conclusion on these data, the authors stated that "o-tolidine alone, without azo linkages and naphthalene side rings, was totally ineffective" as a teratogen.

Korotkova and Tokin [46], in 1968, immersed colonial or single sponges in 100 ml of sea water containing 300 mg of o-tolidine for 45 minutes. Control sponges were treated similarly, but India ink was substituted for o-tolidine. All sponges were then observed for 11 days in running sea water. Water temperature was held at 7-9 C at all times. Both living and fixed specimens were studied. Sponges immersed in o-tolidine showed epithelial damage and mitotic activity accelerated to 2-3 times the rate of regenerating tissue. Colonial sponges were more susceptible to o-tolidine than were single sponges, as shown by earlier budding and abnormal growth. Mitotic activity and budding of control sponges were unaltered.

(c) Metabolism

The metabolism of o-tolidine has been investigated in dogs [47,48], rabbits [48], rats [49], and in occupationally exposed workers [48,50]. Although the experimental data are sparse, the available evidence allows a comparison between humans and other mammalian species with respect to the metabolic fate of o-tolidine in vivo.

Urine of workers occupationally exposed to undefined quantities of o-tolidine was reported to contain o-tolidine, N,N'-diacetyl-o-tolidine, and 5-hydroxy-o-tolidine and its conjugates [22,50]. Although N-acetyl-o-tolidine, a known metabolite in animals [48], was not identified in the urine of workers, it probably occurs as an intermediate during the biosynthesis of N,N'-diacetyl-o-tolidine. Benzidine metabolites found in the urine of occupationally exposed workers are reported to include benzidine (3.5-5.6%),

N-acetylbenzidine (5.1-10%), N,N'-diacetyl benzidine (5.1-10.0%), and 3-hydroxybenzidine and conjugates (78.5-89.7%) [51]. These data suggest that the metabolic pathways for o-tolidine (3,3'-dimethylbenzidine) and benzidine are qualitatively similar in the human organism. The first steps in the pathways appear to be acetylation of the amino groups and introduction of the phenolic group on the aromatic ring.

Engelbertz and Babel [48] measured the concentration of aromatic amines after acid hydrolysis in the urine of workers occupationally exposed to o-tolidine. The hydrolysis was carried out to convert any N-acetyl-o-tolidine or N,N'-diacetyl-o-tolidine present into free aromatic amines. The urine samples from eight workers in o-tolidine production units contained 0.050-0.250 mg of aromatic amines, reported as o-tolidine equivalents, per 100 ml. The authors also measured the concentrations of aromatic amines in 12 urine samples collected intermittently from one worker during a 24-hour period. The highest concentrations of aromatic amines were measured within 6 hours after beginning work. The concentration in the last sample was about one-fifth of the maximum value, which indicates that o-tolidine had a half-life of a few hours in this individual. Despite the limited nature of this study, it confirms that o-tolidine was absorbed by occupationally exposed workers and was rapidly metabolized and excreted.

The metabolites of o-tolidine reported to occur in the blood or urine of dogs include o-tolidine, N-acetyl-o-tolidine, N-N'-diacetyl-o-tolidine, 5-hydroxy-o-tolidine or its conjugates, and o-tolidine-5-sulfate or o-tolidine-5-glucuronide [47,48,52]. A pharmacokinetic study of o-tolidine in dogs following exposure by different routes of administration was reported by Engelbertz and Babel in 1956 [52]. The analytical technique they employed was based on colorimetric determination of the diazotization products of the free aromatic amines. Consequently, the method might not distinguish between o-tolidine and any of its aromatic metabolites containing free amino groups.

The maximum color intensity of free aromatic amines in the blood was reached about 2 hours after a 100-mg iv dose was administered [52]. Color intensity then decreased continuously with time. The half-lives of o-tolidine and its metabolites in canine blood appeared to be less than 8 hours. After the dogs received 200 mg in their food, the maximum concentrations of o-tolidine and related amines in the blood also occurred in about 2 hours, and a similar half-life was observed. These data indicate that o-tolidine is rapidly absorbed through the gut following oral ingestion. The measured rates of clearance of aromatic amines and acetylated derivatives from the blood are consistent with a rapid metabolism and excretion of o-tolidine and its metabolites. The analytical techniques employed did not allow for assessment of the fraction of the total dose of o-tolidine or its metabolites in the blood, so that conclusions on the pharmacokinetics of o-tolidine and all of its metabolites are not possible. In parallel experiments, the investigators observed similar rates of absorption, metabolism, and excretion of benzidine and its metabolites.

Engelbertz and Babel [48] also conducted feeding studies to investigate the metabolic fate of N-acetyl-o-tolidine and N,N'-diacetyl-o-tolidine in dogs and rabbits. They devised an extraction procedure to separate the urinary metabolites. They observed that orally administered N-acetyl-o-tolidine was a precursor of both o-tolidine and N,N'-diacetyl-o-tolidine in the urine. Similarly, they reported that N,N'-diacetyl-o-tolidine was a precursor of both N-acetyl-o-tolidine and o-tolidine. The report suggests that a facile metabolic interconversion of o-tolidine and its acetylated derivatives may occur in dogs and rabbits.

An investigation of the metabolism of benzidine in vitro has recently been reported by Morton et al [53,54]. Using extracts prepared from rat, mouse, guinea pig, and hamster livers, the investigators demonstrated the formation of diacetylbenzidine from acetyl CoA and benzidine. Subsequent oxidation of diacetylbenzidine to N-hydroxydiacetylbenzidine and 3-hydroxydiacetylbenzidine was found to be catalyzed by microsomal enzymes. The shift of the acetyl moiety from the nitrogen to the oxygen atom, catalyzed by N-O-acetyltransferase, yielded a highly reactive species which was shown to bind to nucleic acids covalently. This reaction sequence for benzidine parallels the reaction sequence for other carcinogenic aromatic amines, such as 2-aminofluorene [55]. Consequently, these in vitro studies suggest that a fraction of the absorbed o-tolidine will also be metabolized to a similar reactive intermediate.

After the administration of a single ip dose of o-tolidine to a dog, Sciarini and Meigs [47] recovered only about 40% of the dose in the urine as o-tolidine or its known metabolites. The fraction of the dose that remained and was excreted in the feces and the fraction that was covalently bound were not determined. The binding of aromatic amines to tissues during long-term exposure of rats to o-tolidine was investigated by Pliss and Zabezhinsky [33]. After weekly sc injections of 20 mg were given to each animal for 8 months, the authors measured the distribution of aromatic amines using a diazotization method. The content of free and bound aromatic amines in different organs was determined 3 days after the last administration of o-tolidine. Bound aromatic amines were measured after hydrolysis of tissue homogenates in 2N hydrochloric acid. Relative to the control tissues, an increased amount of both free and bound aromatic amines was observed in all examined tissues--the liver, kidneys, spleen, omentum, and Zymbal glands. The largest increase of bound aromatic amines (more than eightfold) occurred in the kidneys and Zymbal glands. Since the increase in aromatic amines was observed 3 days after administration of the last dose and since the in vivo half-life of o-tolidine and its metabolites is only a few hours, these observations are consistent with the formation of a reactive intermediate that covalently binds to cellular macromolecules.

The available evidence [22,47,48,50-52,55,56] indicates that the fates of o-tolidine and benzidine in biologic systems are closely related, probably because of similar metabolic mechanisms. Although quantitative differences in metabolism do occur, these differences are insufficient to allow for postulation of major differences in the mechanisms of action of o-tolidine and benzidine.

Correlation of Exposure and Effect

There is some evidence of carcinogenicity in rats [33,57], possible carcinogenicity in dogs [29], altered enzyme activity in rats [49], and possible evidence of mutagenicity and teratogenicity in mammalian cell cultures. Little information is available on the toxic effects of o-tolidine on humans. The information that is available for humans and animals can only be used as a qualitative index of effects.

Investigators exposed to o-tolidine at unknown concentrations have experienced nasal irritation [16]. Humans absorb o-tolidine through intact skin and excrete o-tolidine and its acetylated and hydroxylated metabolites in urine [17,22,50]. Urinary metabolites of o-tolidine were detected in the urine of a volunteer after an 8-hour application of 200 mg under a glove, but when 100 mg of o-tolidine were applied to the skin for 48 hours there was no detectable urinary excretion of quinonizable substances [17]. Skin irritation has not been reported in humans.

The results of animal experiments have provided some limited data on the uptake of o-tolidine. The half-life of o-tolidine in blood after 100 milligrams of the compound was administered iv, seems to be less than 8 hours [52]. When o-tolidine was ingested with food, maximum blood concentrations of o-tolidine were reached in 2 hours, indicating that o-tolidine is rapidly absorbed through the gut. Similar findings were observed in dogs in parallel experiments conducted with benzidine [52]. After weekly sc injections of o-tolidine in rats [33], the highest observed increase in bound aromatic amines occurred in the kidneys and Zymbal glands. These findings are of interest because of the reports of kidney damage in rats and rabbits [16,26] and carcinoma of the Zymbal glands in rats [16,33] as summarized in the discussion which follows.

The possibility of kidney damage should be considered, but not much information is available on this aspect of o-tolidine toxicity. There is one report of renal failure in rabbits fed o-tolidine at 1 g/day for 3 days [16] and one report of kidney damage, especially in the uriniferous tubules, in rats fed o-tolidine at a 5% concentration mixed in olive oil mixed with rice at 20 cc olive oil solution/kg of rice [26].

Carcinogenicity, Mutagenicity, Teratogenicity, and Effects on Reproduction

Cancer of the urinary bladder [15] has been found in workers making dyes from a variety of hazardous substances, o-tolidine among them, but, because exposures have been to two or more biphenyl amines, there is no strong evidence either to indicate that o-tolidine alone causes cancer in humans or to absolve it as a human carcinogen. Cancer has not been reported in humans exposed to o-tolidine alone, although cancer of the urinary tract has developed in workers exposed to both benzidine and o-tolidine [20,21]. The single report of bladder cancer in one of four dogs fed 200 mg of o-

tolidine/day for 8-9 months [29] does not establish, but is consistent with, the hypothesis that o-tolidine, like benzidine, may induce urinary tract cancer in animals.

Cancer has been found in the Zymbal glands, skin, forestomach, lungs, bone tissues, and hematopoietic system in rats exposed to o-tolidine [32,33], but it has not been demonstrated in hamsters according to a brief commentary by Saffiotti et al [30]. Most of the experiments with rats have used repeated high doses (0.001-1.29 mg) to induce tumors. Single doses (100 mg/kg) have altered enzyme activities in rats [49], but tumors have not been reported.

It has been suggested that a single methyl group ortho to the amino group in the diphenyl diamine molecule reduces the likelihood of liver tumors in rats, but the substitution of additional methyl groups would increase the possibility of developing liver tumors [62]. However, the administration of 3,5,3',5'-tetramethylbenzidine to rats [32] did not produce tumors of the liver.

o-Tolidine resembles benzidine in structure, in physical and chemical properties [1,2,58], and in metabolism [47,48,50-52,55,56,58-60]. The sites and microscopic appearance of tumors observed in rats following o-tolidine administration [33] and dihydroxybenzidine administration [61] are similar. Benzidine, an aromatic amine which is accepted to be a human carcinogen (29 CFR 1910.1010), has not produced bladder cancer in rats [31]. Both o-tolidine and benzidine administration to rats do, however, result in cancer of the Zymbal glands [32,62]. The similarity between o-tolidine and benzidine in chemical structure, in absorption of both compounds by all routes (including the skin), in metabolism to acetylated and hydroxy compounds, in excretion preferentially by the urinary route, and in the induction of tumors of the Zymbal glands in rats suggests that o-tolidine can cause bladder tumors in dogs and humans as does benzidine. In addition, o-tolidine probably is a factor, along with benzidine, in cases of human bladder cancer following mixed exposures to these chemicals, such as those reported by Macalpine [21].

Pliss [62] suggested that the accumulation of o-tolidine or its metabolites in excretory organs, such as sebaceous glands, is related to the high frequency of tumors in structures of ectodermal origin. The results of research by Holland et al [32] lend support to the suggestion.

Although one study [42] did not agree, the indications of mutagenicity of o-tolidine in bacteria [41,43], the influence on DNA synthesis in mouse testes [40], the influence on organ cultures derived from pregnant mice treated with o-tolidine, and in vitro transformation of rat embryo cells [39] support the hypothesis that o-tolidine may adversely affect fundamental cellular control mechanisms.

TABLE III-1

EFFECTS OF EXPOSURE TO o-TOLIDINE ON ANIMALS

Route	Species	Dose	Duration	Observed Effects	Reference
oral	Rat	5%	21-111 d	Changes in uriniferous tubules	26
"	Hamster	0.3%	-	None	30
"	"	"	-	"	38
"	Rabbit	1 g	3 d	Renal failure, death	16
"	Dog	230 mg	3 yr	No bladder tumors	28
"	"	200 mg	9 mo	Bladder cancer in one/four after 8 yr	29
sc (injection)	Rat	100 mg/kg	1 hr	Changes in enzyme activities	49
"	"	"	2 mo	"	49
"	"	5.4 g/kg total	8 mo	Tumors in 85%	32
"	"	20 mg 1.16 g total	13 mo	Tumors in 60%	33, 35
"	Mouse	2 mg	-	Fetal kidney damage	44
sc (implantation)	Rat	20 mg 1.2 g total	14 mo	Tumors in 64% of animals	33
"	"	20 mg oxidized 1.22 g total	"	Tumors in 78%	33
dermal	Rabbit	0.5 g	24 hr	Slight erythema with abraded skin	17