Summary of the National Toxicology Program Benzidine Dye Initiative

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The benzidine dye initiative is a research program established by the National Toxicology Program to generate an integrated body of scientific information regarding the potential health risks associated with exposure to benzidine- and benzidine-congener-derived dyes. Because an in-depth evaluation of each of the hundreds of benzidine-congener-derived dyes was considered impractical, the research program was designed to study the metabolism and disposition, genetic toxicity, and in vivo toxicity and carcinogenicity of two primary benzidine congeners, 3,3'-dimethylbenzidine and 3,3'-dimethoxybenzidine, and a select group of prototypical dyes derived from those amines. It was anticipated that by applying the basic information generated in these extensive studies, it would be possible to make regulatory decisions about other dyes after conducting only a minimal number of experiments such as studies of disposition and metabolism, and in vitro mutagenicity. This paper summarizes the results of studies conducted to evaluate the metabolism, disposition, mutagenicity, toxicity, and carcinogenicity of representative benzidine congeners and derived dyes. — Environ Health Perspect 102(Suppl 2):63–78 (1994).

Key words: benzidine, 3,3'-dimethylbenzidine, 3,3'-dimethoxybenzidine, C.I. Direct Blue 15, C.I. Direct Blue 218, C.I. Acid Red 114, chemistry, metabolism, carcinogenesis, mutagenicity

Introduction

Haley (1) reviewed the early literature on the toxicologic and carcinogenic effects of benzidine and a new review is currently in progress by the Agency for Toxic Substances and Disease Registry. Since the intent of our communication is to present a body of experimental results, only a brief review of clinical and experimental highlights will be presented.

An increased incidence of cancer of the urinary bladder was first associated with human occupational exposure to dyes and dye chemicals in 1895 (1). These tumors, initially referred to as aniline cancers because of the extensive use of aniline in dye chemistry, were attributed to exposure to either the starting materials or to the finished dyes per se. Over the intervening years several aromatic amines, used as starting materials in the dye manufacturing process, were shown to be animal carcinogens and benzidine was identified as a carcinogen for the human urinary bladder in 1973 (1). Simultaneously the results of cancer epidemiology studies in Japanese Kimono painters implicated benzidine-derived dyes as human carcinogens. In addition, two congeners of benzidine, 3,3'-dimethylbenzidine and 3,3'-dimethoxybenzidine, also employed in the dye industry, were found to increase tumor incidence in rats (1). The results of these early studies on 3,3'-dimethylbenzidine and 3,3'-dimethoxybenzidine were difficult to interpret because of the small groups of animals used and reduced survival.

Although certain benzidine-derived dyes (e.g., Direct Blue 6, Direct Brown 95, and Direct Black 38) were considered to be carcinogenic in rats (2), it remained to be established whether the carcinogenic action was attributable to the dyes per se, trace levels of benzidine present in the dyes as a contaminant, or metabolites of the dyes. Further, in addition to equivocal data concerning the carcinogenicity of 3,3'-dimethylbenzidine and 3,3'-dimethoxybenzidine, dyes derived from either of these benzidine congeners had not been adequately studied for carcinogenic activity.

These questions concerning possible carcinogenicity, combined with the potential for exposure of humans to the benzidine congeners during dye manufacture and to the dyes during their use, prompted the design and conduct of a series of experiments to explore the carcinogenic potential of several benzidine-congener-derived dyes in rats. The US Environmental Protection Agency, the Consumer Protection and Safety Commission, and the Occupational Safety and Health Agency nominated several benzidine dyes for carcinogenicity testing by the National Toxicology Program (NTP). During the planning stages it was recognized that a large number of dyes existed and that should one or more be found to possess undesirable biological activity an alternate, most likely of ill defined toxicologic potential, would be readily available as a substitute. The substitute would then require testing before regulatory decisions relative to its use could be made. Such a circuitous path would have placed an enormous burden on the resources of the NTP.

To circumvent this potential dilemma the collaborating agencies agreed to implement a research program on the benzidine congeners and dyes derived from them, the results of which could be used to predict the carcinogenic potential of other benzidine-congener-derived dyes. The broad objective of the initiative was to generate an integrated body of scientific information regarding the potential health risks likely to be associated with exposure to any benzidine-congener-derived dye. To this end, experiments were designed to study the metabolism and disposition, genetic toxicity, and in vivo toxicity and carcinogenicity of 3,3'-dimethylbenzidine and 3,3'-dimethoxybenzidine, and a select group of prototypical dyes derived from those amines. It was anticipated that by applying the basic information generated in these ex-

The authors thank Drs. G. Boorman, R. Maronpot, and R. Tennant for critically reviewing this manuscript.

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tensive studies it would be possible to make regulatory decisions about other dyes after conducting only a minimal number of experiments such as studies of disposition and metabolism, and in vitro mutagenicity.

Chemistry

Nomenclature and Structure

Nomenclature of colorants can be confusing. The difference between dyes and pigments is in the method used to apply the coloring agent. Dyes are applied as a solution or a vapor while pigments are applied without losing their crystalline structure. The Colour Index (C.I.) (3) names dyes by reference to application class and shade, and by using a sequential number. The dyes used in the current studies belong to the direct and acid application classes which are water-soluble anionic dyes (4). Benzidine- and benzidine-congener- (i.e., chemically related to benzidine) based dyes include those derived from 3,3'-dimethylbenzidine and 3,3'-dimethoxybenzidine, as well as benzidine itself. Synonyms for 3,3'-dimethylbenzidine and 3,3'-dimethoxybenzidine, the benzidine congeners used in the studies reported here, are o-toluidine and o-dianisidine, respectively. Chemical structures, CAS numbers, and C.I. numbers for benzidine, the benzidine congeners, and the related dyes that were studied are presented in Table 1.

Synthesis

Benzidine and its congeners are dye intermediates, i.e., precursors of dyes. A common method used to prepare benzidine and congeners involves the reduction of nitrobenzenes followed by the acid catalyzed intramolecular rearrangement of the resulting hydrazobenzenes. Subsequently, the dyes are produced by the diazotization of the amino groups on the benzidines and azo coupling to reactive aromatic ring systems (other dye intermediates). A generalized synthetic scheme is shown in Figure 1. Metallizing of dyes sometimes is carried out to improve the stability of the azo groups; however, the mechanism by which the added metal chelates with the dye is not always well understood. For the one metallized dye used in our carcinogenesis studies, C.I. Direct Blue 218, the structure has not been completely defined. However, the synthetic route consists of coupling 1 mole of o-dianisidine (3,3'-dimethoxybenzidine) to 2 moles of 4-amino-5-hydroxy-2,7-napthalene disulfonic acid under alkaline pH conditions followed by metallizing and elimination of methyl groups.

Table 1. Benzidine-based dyes studied in the National Toxicology Program Initiative.

| Chemicals          | Tests | Structure                        |
|--------------------|-------|----------------------------------|
| Benzidine derived  |       | ![Structure Diagram]             |
| C.I. Direct Blue 5 | S, M  | ![Structure Diagram]             |
| C.I. Direct Orange 1| M     | ![Structure Diagram]             |
| C.I. Direct Green 1| S, M  | ![Structure Diagram]             |
| C.I. Direct Orange 8| M     | ![Structure Diagram]             |
| C.I. Direct Black 4 | M     | ![Structure Diagram]             |
| C.I. Direct Brown 2| S, M  | ![Structure Diagram]             |
| C.I. Direct Blue 3  |       | ![Structure Diagram]             |
| C.I. Direct Red 19  | M     | ![Structure Diagram]             |
| C.I. Direct Black 26| S     | ![Structure Diagram]             |
| C.I. Direct Brown 16| S     | ![Structure Diagram]             |
| 3,3'-Dichlorobenzidine derived | S | ![Structure Diagram]             |
| Pigment Yellow 12  | S     | ![Structure Diagram]             |

Continued
from the methoxides to form the copper complex (5). Depending on the synthesis methods employed and the needs of the end user, the dyes may be impure "press-cakes" or highly pure crystalline products. The most important property of the dyes to the end user is the color, while purity is of secondary concern. Because humans are exposed to these impure commercial products, commercial sources for the test materials were selected. As was found in our chemical characterization studies, the purity of the dyes varies greatly.

**Chemical Analyses**

Comprehensive purity, identity, and stability studies were performed on the benzidine congeners and dyes used in the 2-year chronic studies. Reports on the analyses performed are on file at the National Institute of Environmental Health Sciences. The study chemicals were identified as 3,3'-dimethylbenzidine dihydrochloride, 3,3'-dimethoxybenzidine dihydrochloride, C.I. Direct Blue 15, C.I. Acid Red 114, or C.I. Direct Blue 218 by infrared, ultraviolet/visible, and nuclear magnetic resonance spectroscopy. The purities of 3,3'-dimethylbenzidine dihydrochloride and 3,3'-dimethoxybenzidine dihydrochloride were determined to be 99 and 98%, respectively, by elemental analysis, Karl Fischer water analysis, titrations (nonaqueous amine and neutralization titrations), thin-layer chromatography, and high-performance liquid chromatography (HPLC). Because of its high salt content, Direct Blue 15 was desalted by dialysis, which reduced the salt content from approximately 25 to about 3%. The purity of the desalted dye was determined to be about 50%. Approximately 35 impurities were detected by HPLC analysis accounting for about 50% of the chromatographic peak area. No attempt was made to identify the chromatographic peaks. However, the chemical was assayed for free benzidine and 3,3'-dimethoxybenzidine content. Benzidine could not be detected at levels greater than 1 ppm in either of the two lots used in the study, whereas 3,3'-dimethoxybenzidine was found at 826 and 1310 ppm in two lots that were sampled.

The dye, Acid Red 114, was desalted by dialysis, and the salt content reduced from approximately 14.9 to about 0.9%. The purity of the desalted dye was estimated at 82 to 85%. There were approximately 15 organic impurities observed by HPLC analysis; these impurities were similar in structure to the major component, with the two largest components estimated at 3%
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each. In addition, there was approximately 1 to 4% water present in the dye. The level of benzidine detected by HPLC did not exceed 1 ppm; whereas, 3,3'-dimethylbenzidine was detected at a concentration of approximately 5 ppm.

Direct Blue 218 contained about 2% salt after desalting by dialysis. The purity of the desalted chemical was determined to be approximately 60%. Over a dozen impurities were detected by HPLC analysis, accounting for approximately 40% of the chromatographic peak area. No attempt was made to identify the chromatographic peaks, however, the concentrations of benzidine and 3,3'-dimethoxybenzidine were determined. Benzidine could not be detected in either lot at levels greater than 1 ppm. 3,3'-Dimethoxybenzidine was found at levels less than or equal to 7 ppm.

Dose Formulation

Dose formulations were characterized and the concentrations confirmed during the toxicology studies. In all cases, except C.I. Direct Blue 218, reverse-phase HPLC-UV systems were used. Detector wavelengths were 280 nm for 3,3'-dimethylbenzidine and 3,3'-dimethoxybenzidine; 546 nm for C.I. Direct Blue 15 and 512 nm for C.I. Acid Red 114. For C.I. Direct Blue 218, visible spectroscopy at 622 nm was employed. Reports on the dose formulation studies are on file at the National Institute of Environmental Health Sciences.

Initially, attempts were made to formulate 3,3'-dimethylbenzidine dichloride and 3,3'-dimethoxybenzidine dichloride in water. However, feed formulations were unstable when stored in the dark in sealed containers at room temperature, 5°C, or 2°C. Drinking water was then investigated as a vehicle for chemical administration. Solutions of 3,3'-dimethylbenzidine dichloride or 3,3'-dimethoxybenzidine dichloride in water remained stable for at least 14 days when stored at either room temperature or 5°C. Solutions were also stable for up to 48 hr under simulated animal dosing conditions. During the studies drinking water formulations were prepared two times/week and used immediately or stored at room temperature for up to 7 days before being used. The drinking water solutions were analyzed at approximately 4-week intervals. Of the dose formulations analyzed, 99% were determined to be within 10% of the target concentration. Results of periodic independent analyses agreed with the results of the toxicology study laboratory.

The drinking water formulations of Direct Blue 15 and Acid Red 114 remained stable for at least 21 days when stored at room temperature. Solutions were stable for at least 3 days under simulated dosing conditions including exposure to normal room light and air. Dose formulations were prepared twice weekly and made available to the study animals on the day of mixing. Dose formulations were analyzed at least once every 4 weeks for the duration of the studies; all were determined to be within +10% of the target concentrations.

Direct Blue 218 was insoluble in water at the required concentrations for the 2-year studies. Because of the solubility limitations, the dose formulations were prepared by mixing Direct Blue 218 with feed. Stability tests showed that the formulations were homogeneous and stable for at least 21 days when stored at room temperature, and for at least 3 days under simulated animal dosing conditions. Dose formulations were prepared once every 2 weeks. The formulations used for dosing were analyzed at least once every 4 weeks; 97% were determined to be within 10% of the target concentration.

Metabolism and Mechanisms of Toxicity

As part of the initiative to characterize the toxicity and carcinogenicity of benzidine and benzidine-congener based dyes, the NTP conducted and sponsored studies of the metabolism of both the dyes and parent compounds. These studies were conducted at the National Center for Toxicological Research (NCTR) and through contractual mechanisms. This section presents a summary of that work, and includes relevant work done by others since that time, to more completely describe the fate and mechanisms of toxicity of these compounds.

Metabolism/Disposition of Dyes

The objectives of metabolism and disposition studies conducted or supported by the NTP were to investigate the degree to which the dyes or parent amines were absorbed from the gastrointestinal tract, and to determine the amount of parent amine eliminated in urine following exposure to the dyes (Figure 2). The presence of the parent amine in urine was taken as evidence of exposure of the target tissue for humans, the urinary bladder. It was further assumed that results obtained with representatives of each dye class would be representative of the respective classes. That is, the absence of the release of benzidine, or the respective congeners, or metabolites of these compounds, would be taken as evidence that the dyes were not metabolized to the parent compounds, and that human risks associated with exposure to the dyes may not be related to exposure to the respective parent amine. Similarly, release of the respective parent amine following exposure to most or all of the dyes would indicate that exposure to the dyes would entail risks similar to those associated with the parent compound. Mixed results would be taken as evidence that more dyes should be tested prior to judging the risks associated with using this class of compounds.

The biological reduction of azo compounds to release aromatic amines had been previously demonstrated by a number of investigators (6–9) prior to the NTP studies of benzidine and related dyes. Further, the metabolism of four benzidine-based dyes by rhesus monkeys to benzidine was described in a brief report by Rinde and Troll (9) in 1975. However, metabolite identification in the studies of Rinde and Troll were limited to thin-layer chromatography versus authentic standards of benzidine and N-acetylbenzidine. Therefore the NTP-sponsored studies were
designed to confirm and extend those observations by studying seven additional benzidine dyes, two dimethoxybenzidine-based and four dimethylbenzidine-based dyes. The NTP studies also more fully characterized the metabolism of these compounds and confirmed metabolite structures by gas chromatography and mass spectral analysis.

Dyes representative of those in use at the time were selected for metabolism and disposition studies. Benzidine-based dyes selected included Direct Orange 1, Direct Green 1, Direct Orange 8, Direct Black 4, Direct Brown 2, Direct Blue 2 and Direct Red 28. The 3,3'-dimethoxybenzidine-based dyes studied were Direct Blue 15 and Direct Blue 1. In addition, Direct Blue 25, Direct Red 2, Direct Red 39 and Acid Red 114, all 3,3'-dimethylbenzidine-based dyes, were studied. In addition to the dyes, these studies also included the parent compounds (the structures of these compounds are presented in Table 1).

The dog was chosen as a test species because, other than humans, it was the only species known to develop bladder tumors as a result of benzidine exposure (1). The rat was chosen for comparative purposes, and because this species was to be used in chronic studies of several of these dyes.

The objective of the initial studies was to confirm the metabolism of benzidine and benzidine-based dyes to the parent amines. In these initial studies the test chemicals were administered to dogs at 100 mg/kg in meat balls, and urine was collected at 24 hr intervals for 3 days. No benzidine or related compounds were detectable in urine by the third day. Rats received similar doses of the dyes by oral gavage in water daily for 10 days. Urine from both species was collected over ice and stored at approximately 20°C. Concentrations of benzidine, the congeners, and known metabolites of these compounds in urine were determined by gas chromatography. Concentrations of benzidine, 3,3'-dimethylbenzidine or 3,3'-dimethoxybenzidine in the starting dyes also were determined by gas chromatography. In analyses of both the urine and the dyes, the identities of the chromatographic peaks were confirmed by mass spectrometry (10).

Results of studies with dogs established that the respective free parent amines were excreted in urine following administration of each of the benzidine and 3,3'-dimethoxybenzidine dyes studied, and following administration of Direct Blue 25 and Acid Red 114, two of the four 3,3'-dimethylbenzidine-based dyes. Administration of two other 3,3'-dimethylbenzidine-based dyes, Direct Reds 2 and 39, did not result in 3,3'-dimethylbenzidine in the urine of dogs. After administration of benzidine-based dyes, the amounts of benzidine excreted were small (about 0.1% of the dose), but they were at least nine times greater than the amounts of benzidine administered as contaminants in the dyes, and were equivalent to the amount excreted following administration of neat benzidine. Since dogs do not readily acetylate aromatic amines such as benzidine, free amines were excreted in the urine. On the other hand, rats readily acetylate these compounds and excreted both the free amines and significant amounts of the N-acetyl metabolites of benzidine and its congeners (10).

Conclusions that could be drawn from these studies were that the azo linkages of most dyes studied were reduced to release free benzidine or the respective congeners, which were subsequently excreted in urine. Azo reduction probably occurred in the intestine and, less probably, in liver. Reduction of dyes to the parent amines by anaerobic intestinal bacteria from humans, monkeys, and rats, was confirmed in a later report (11). More important, based on the amount of benzidine or its congeners excreted in urine, it appeared that most dyes were reduced quantitatively to release the parent amines to result in exposure of the target tissue, the urinary bladder.

As a result of their quantitative reduction to the parent amine, exposure to benzidine-, or benzidine-congener-based dyes appears to result in exposure to concentrations of benzidine (or congener) that are comparable to those that occur following exposure to equivalent amounts of free benzidine (or congener). Furthermore, the consistency of results obtained with benzidine and related dyes implies that their reduction to release the respective parent amine is probably a general phenomenon. That is, similar results and risks associated with exposure to benzidine should be anticipated with most dyes based on benzidine and its congeners (10). These results also imply that benzidine detected in urine from workers exposed to benzidine-based dyes was probably derived from the dyes, rather than from traces of benzidine present in the dyes as a contaminant (12). The significance of this finding is that the production and use of benzidine and related dyes carries inherent risks which cannot be overcome by producing and using dyes which contain less of the respective parent amine as a contaminant.

**Benzidine Metabolism**

Neither benzidine nor its congeners are thought to be carcinogenic prior to metabolic activation. The presence of benzidine or its congeners in urine was considered significant only because it demonstrated that the target tissue in humans, urinary bladder, was exposed to a potential carcinogen following exposure to the dyes. The speculated necessity for metabolic activa-
tion was consistent with the report that benzidine is a bacterial mutagen only when activated by a liver enzyme preparation (13). The first in vitro study of benzidine metabolism demonstrated that benzidine is acetylated to form N-acetylbenzidine, N,N'-diacetylbenzidine, and N-hydroxy-N,N'-diacetylbenzidine (14). These studies further implied that the latter metabolite was somehow involved in the alkylation of DNA.

In an early study of comparative metabolism of benzidine in several species Claysen et al. (15) demonstrated that the dog was the only species studied that did not form N-acetylbenzidine when administered the parent compound. In another early study, Sciarini and Meigs (16) isolated the N- and O-glucuronides as well as the N- and O-sulfate metabolites from dogs and other species administered benzidine. A more thorough study of benzidine disposition and metabolism in dogs and rats by Lynn et al. (17) demonstrated that benzidine is readily absorbed from the gastrointestinal tract, metabolized extensively, and rapidly excreted in urine and feces. Excretion in feces exceeded that in urine by 2- to 3-fold, and less than 10% of the dose remained in the tissues, primarily liver, after 3 days.

The use of radiolabeled benzidine and HPLC analysis permitted Lynn et al. (17) to detect at least 17 distinct metabolite peaks in urine following oral administration to rats. Benzidine accounted for less than 2% of the total radiolabel excreted in urine. The major metabolites excreted in bile and urine were isolated and identified as glucuronide conjugates of benzidine, N-acetylbenzidine, N,N'-diacetylbenzidine, 3-hydroxy-N,N'-diacetylbenzidine, and free N-acetyl- and N,N'-diacetylbenzidine. 3-Hydroxy-N,N'-diacetylbenzidine was the major metabolite excreted in urine and bile. A study of the mutagenicity of these metabolites in the Salmonella/lysis 59 system showed that both the mono- and diacetylated metabolites were approximately 10 times as mutagenic as benzidine. The most mutagenic metabolite isolated in this study, N-hydroxy-N,N'-diacetylbenzidine glucuronide, was approximately 100 times more potent a mutagen than the parent compound when incubated with β-glucuronidase to release the hydroxylated diacetylamine (18).

Lynn et al. (17) measured concentrations of benzidine-derived radioactivity in all the major tissues at 0.5, 1, 2, 4, 8, 24, and 72 hr after dosing. Radioactivity was concentrated only in liver, which contained the highest concentrations at every time point. Extraction and analysis of radiolabel from liver determined that very little of this radioactivity was free benzidine, but approximately half was in the form of two metabolites, N-acetyl- and N,N'-diacetylbenzidine. The remainder was in the form of unidentified polar metabolites. N,N'-Diacetylbenzidine accounted for approximately 80% of the benzidine-derived radioactivity in blood at the early time points; however, concentrations at later time points were too low to permit accurate analysis. Studies with isolated, perfused rat livers demonstrated that all of the metabolites isolated and identified from whole animals could be formed by the liver. Further, some of these metabolites were themselves subject to additional metabolism, including the rapid reduction of the most mutagenic metabolite, N-hydroxy-N,N'-diacetylbenzidine, to the less mutagenic N,N'-diacetylbenzidine (18).

Metabolism and disposition of 3,3'-dimethoxybenzidine in intact rats was similar to that reported for benzidine, except that the 3-methoxy groups provided additional sites for metabolic attack and resulted in more rapid and more extensive metabolism (19). The most mutagenic metabolite isolated in this study was N-acetyltriethoxybenzidine.

**Benzidine Interaction with DNA**

As mentioned above, benzidine is believed to be carcinoogenic only following metabolic activation. Like other carcinogenic aromatic amines, such as 2-naphthylamine and 2-acetylaminofluorene, benzidine and its congeners were presumed to be metabolized to intermediates which reacted with DNA to result in mutations and carcino- genicity. Evidence for the increased mutagenicity of benzidine following metabolic activation was provided by the studies of Morton et al. (14), who reported nucleic acid binding of benzidine; and Lynn et al. (17), who reported that the glucuronide of N-hydroxy-N,N'-diacetylbenzidine was as much as 100 times more mutagenic in the Salmonella/lysis 59 assay than benzidine. Martin et al. (20) studied the metabolism and covalent binding of benzidine and a benzidine metabolite, N-acetylbenzidine, in vitro and in vivo in the rat. They isolated and identified a product of DNA alkylation, N-(deoxyguanosin-8-yl)-N'-acetylbenzidine, which could have been formed from either N-acetylbenzidine or N,N'-di-acetylbenzidine, both of which are formed by rat liver (18) and excreted in urine (17).

Following their isolation of a product of benzidine acetylation of DNA, Martin et al. (21) demonstrated that this adduct is readily formed in vitro by rats dosed with benzidine, N-acetylbenzidine or a benzidine-based dye, Direct Blue 6. Only a trace of binding was detected following administration of N,N'-diacetylbenzidine which implied that this metabolite may not be the precursor to N-hydroxy-N,N'-diacetylbenzidine, previously reported to be a potent bacterial mutagen (17). Subsequent work using enzyme preparations from rat and mouse liver provided data to support the hypothesis that N-hydroxy-N,N'-diacetylbenzidine is the proximate carcinogen in these species, because of its capacity to alkylate DNA. This work also demonstrated that this metabolite is formed from N'-hydroxy-N-acetylbenzidine, rather than N,N'-diacetylbenzidine (22).

The target organ for benzidine carcinogenesis in humans is the urinary bladder, and the best experimental animal to model this lesion is thought to be the dog. Unlike rats and mice, dogs do not acetylate benzidine or other amines known to induce bladder cancer (23). Therefore, the causative agent in benzidine carcinogenesis in the dog was not thought to be the hydroxylacetylated metabolite speculated to account for carcinogenesis in rodent studies.

Beland et al. (24) studied the formation of DNA adducts in urinary bladder of dogs following oral administration of a number of known carcinogenic aromatic amines, including benzidine and N-acetylbenzidine. Their studies demonstrated a positive correlation of the degree of binding of carcinogenic aromatic amines to bladder DNA with the potency of the respective compounds. Further, of the known carcinogens studied, binding to DNA in the dog bladder following administration of benzidine and N-acetylbenzidine was equivalent to that observed following administration of two other potent bladder carcinogens, 2-naphthylamine and 4-nitro-biphenyl. These investigators proposed that N-hydroxyamines account for the carcinogenicity of each of these compounds in the urinary bladder of dogs, and speculated that these reactive intermediates are formed in the liver and transported to the bladder as glucuronide conjugates which hydrolyze in the weakly acidic urine to release the reactive intermediate (24).

A more recent publication has demonstrated that, in the dog, benzidine may also be transported to the bladder as a glucuronide conjugate which could be hy-
drolyzed to release free benzidine (25). Later work by members of this same group, however, demonstrated that benzidine is also metabolized to a reactive intermediate by arachidonic acid-dependent prostaglandin H synthase and that prostaglandin H synthase is present in high concentrations in dog bladder (26, 27). The reactive intermediate formed by prostaglandin H synthase, benzidine diimine, was proposed to react directly with DNA to form an adduct, N-(deoxyguanosin-8-yl)benzidine, which was later isolated and identified (28). In studies of a series of human tissues the urinary bladder was shown to be rich in prostaglandin H synthase, and among the aromatic amines studied, benzidine was shown to be the optimum substrate for this enzyme (29). Thus, the presence of prostaglandin H synthase in dog and human urinary bladder probably accounts for the activation and carcinogenicity of benzidine in this tissue.

Summary

Studies of the metabolism and disposition of dyes derived from benzidine and benzidine congeners established that, following ingestion, the azo linkages of these compounds are reduced, probably by bacteria in the intestines, to release the parent amines. Reduction of the dyes to the respective free amines appears to be nearly complete, therefore, ingestion of most benzidine and related dyes is thought to be comparable to ingestion of an equivalent amount of the respective free amine. The free amines (benzidine and its congeners) are absorbed readily from the gastrointestinal tract, rapidly and extensively metabolized and excreted in both urine and feces. The degree of benzidine excretion in urine by experimental animals exposed to dyes, and the actual detection of benzidine in urine of workers in the dye industry, strongly support the potential for exposure of human urinary bladder to this known bladder carcinogen following exposure to the respective dyes.

The probable reactive intermediate which accounts for the carcinogenicity of benzidine in rodents is N-hydroxy-N,N'-diacetylbenzidine. This reactive intermediate is formed in liver and is probably transported to target tissues in blood or urine. Dogs do not acetylate benzidine and humans acetylate benzidine less readily than rodents; therefore, the reactive intermediates of benzidine vary with species. Metabolism to a reactive intermediate by dogs and humans may also occur in liver, but urinary bladder carcinogenicity is more probably mediated by the action of prostaglandin H synthase in the target tissue. Prostaglandin H synthase forms a diimine metabolite which reacts directly with DNA in the bladder.

Mutagenicity

Salmonella mutagenicity studies were prompted by the fact that benzidine-congener dyes were not uniformly and reliably positive in oxidative in vitro mutagenicity assays. It was believed that the dyes required reductive cleavage to yield the parent amine in order to be mutagenic. The objectives of the mutagenicity portions of the initiative were to establish an optimal protocol for the reductive cleavage of the dyes, and evaluate the mutagenic effects of benzidine-congener-derived dyes. A part of this latter activity was to determine if the mutagenicity of the dyes was attributable to the parent amine metabolites, or if activity was either quantitatively or qualitatively influenced by the chromophore portions of the molecules. In addition, the mutagenicity of the benzidine and benzidine-congener metabolites found in the urine of dosed rats was investigated. It was reasoned that the demonstration of a genotoxic potential for these urinary metabolites would increase the confidence in predictions of carcinogenicity after short-term oral administration of these dyes in laboratory animals. These studies would also provide additional information regarding the metabolic activation of the dyes.

Three Salmonella mutagenesis testing protocols were used. All dyes were first tested (under code) using a standard, aerobic preincubation procedure in Salmonella strains TA98, TA100, TA1535, and TA1537, with and without metabolic activation. S9 fractions derived from the livers of Aroclor 1254-treated male Sprague-Dawley rats and Syrian hamsters were used. Some dyes were also tested using strain TA97. The detailed test procedure and criteria for a positive response are in Mortelmans et al. (30) and Zeiger et al. (31, 32).

The mutagenic and nonmutagenic dyes were also tested following treatment under reducing conditions to liberate the parent amine. Equimolar concentrations of the parent benzidine congeners were tested in parallel with the dyes. The FMN reduction protocol of Prival and Mitchell (33) and the celac flora reduction procedure of Reid et al. (34, 35) were used. In the Prival procedure, the dyes were preincubated using the standard aerobic procedure except that the liver S9 and cofactors were supplemented with NADH and FMN to provide reducing conditions (33, 36). The cecal flora reduction procedure employed a washed suspension of rat cecal flora. The dye was added to this culture and incubated overnight under anoxic conditions. The incubation mixture was extracted with ethyl acetate, dissolved in DMSO, and tested for mutagenicity using the standard aerobic preincubation procedure (34). All reduction experiments were performed using strains TA98 and TA1538, because these strains are more responsive to the mutagenic effects of benzidine, 3,3'-dimethoxybenzidine, and 3,3'-dimethylbenzidine than the other tester strains.

Mutagenicity of the Dyes

With the exception of Direct Blue 218 (30, 34) and Pigment Yellow 12 (31, 34), all dyes tested were mutagenic in Salmonella, and produced frameshift mutations in strains TA98 or TA1538 when tested under conditions that fostered reduction of the azo bonds (Table 2) (30–34). Only Direct Black 38 (30, 34) and Direct Blue 2 (30) were mutagenic without activation. The dyes that were mutagenic with the standard aerobic S9 preparation generally gave weaker responses than they did following azoreduction. The positive responses with the standard S9 mix alone showed that this metabolic activation preparation was able to induce low levels of azoreduction or that the dye sample contained impurities that could be activated to mutagens without azoreduction.

Many of the dyes tested had high levels of impurities, although the specific impurities were not identified. It was not determined to what extent any mutagenicity seen under aerobic conditions was due to bacterial reduction of the dye, and what extent was due to mutagenic impurities.

Mutagenic Potency of Reduced Dyes and Congeners

In the azoreduction protocols, the various dyes were tested in parallel with equimolar concentrations of the parent benzidine congeners (34). This was done to compare the level of mutagenicity induced by the parent amine with the mutagenicity induced by an equimolar concentration of amine released from the dye. Benzidine was more mutagenic with hamster than rat S9; 3,3'-dimethoxybenzidine was more mutagenic when tested with rat than hamster S9; and 3,3'-dimethylbenzidine gave equivalent results with both S9s. 3,3'-
Dihydroxybenzidine, a putative metabolite of Direct Blue 218, was more mutagenic with hamster liver S9 in Salmonella strain TA98 (E Zeiger et al. unpublished data). 3,3'-Dichlorobenzidine was the most potent mutagen, and the only congener that was mutagenic without S9 activation. There did not appear to be a correlation between the mutagenic responses induced by the parent amines and those induced by the reduced dyes. For some dyes, the reduction products were more mutagenic than an equimolar quantity of the amine; for other dyes the opposite was seen. These results may be related to the possible mutagenic or inhibitory effects of the other moieties released from the dye during azoreduction, to mutagenic impurities in the dye preparation, or to incomplete reduction of the dye (34).

**Mutagenicity of Metabolites**

The N-acetylated and the N,N-diacyl-ated urinary metabolites of benzidine, 3,3'-dimethylbenzidine and 3,3'-dimethoxybenzidine were mutagenic in Salmonella strains TA98 and TA1538; the N-acetylated metabolites were also mutagenic in TA100 (35). These results show that, with the exception of Direct Blue 218 and Pigment Yellow 12, all dyes tested that can be reduced to their benzidine congeners, are mutagenic in Salmonella. The mutagenic responses obtained with the rat cecal preparations show that the dyes can be reduced to their constituent benzidine congeners by the gut flora as effectively as by liver preparations under reduced conditions. In addition, the results show that these cecal preparations do not further metabolize the resulting aromatic amines to products that will not be mutagenic upon subsequent aerobic metabolism by liver enzymes.

The azo linkages in Direct Blue 218 also appeared to be reduced by the NADP/FMN-fortified rodent liver preparations and by the cecal extract, as judged by the change in visible color intensity, but the resulting product was not mutagenic. This may indicate that the reduction of this dye does not release the free amine, but a copper complex of this amine with the chromophore or to 3,3'-dihydroxybenzidine. In the Reid et al. study (34), approximately 50% of the Direct Blue 218 was reduced by rat cecal bacteria as determined by spectrophotometric analysis. However, comparison of the mutagenic responses of this congener to the test responses of Direct Blue 218 showed that the highest dose tested of Direct Blue 218 was 0.5 μmole/plate, whereas the mutagenic response of 3,3'-dihydroxybenzidine were seen only at doses above 1.5 μmole/plate. Pigment...
Yellow 12 did not appear to be reduced, probably as a consequence of its insolubility, and was not mutagenic. The question of the mutagenicity of the chromophore moieties of the dyes was not addressed. In a number of instances, however, the mutagenic responses following reduction of the dye and subsequent oxidative metabolism of the reduced products was greater than that obtained following similar treatment of the parent congener amine (33,36). This suggests that the chromophore was also mutagenic.

**Summary**

Mutagenicity studies provided evidence that the benzidine- and benzidine congener-derived dyes require reductive metabolism before they are mutagenic. All soluble dyes tested, with the exception of Direct Blue 218 (copper chelated), produced frameshift mutations in Salmonella when tested under conditions that fostered reduction of the azo bonds. Only Direct Black 38 and Direct Blue 2 were mutagenic without activation, possibly due to the presence of mutagenic impurities in these dye preparations. This information supports the results of metabolism studies relative to the mechanisms of genotoxic action, and increases confidence in predicting the carcinogenicity of these chemicals.

**Carcinogenesis**

**Chronic Carcinogenicity Testing**

The objectives of the in vivo carcinogenicity studies were to: conduct definitive studies of the carcinogenicity of orally administered 3,3'-dimethylbenzidine and 3,3'-dimethoxybenzidine in rats; assess the carcinogenicity of orally administered prototypical benzidine congener derived dyes; develop dose-response relationships for the carcinogenicity of the dyes and the parent amines; determine similarities in the qualitative nature of the responses to the parent amines and the derived dyes; and assess the carcinogenicity of a metallized benzidine congener dye (Direct Blue 218).

Five chemicals were evaluated in the 2-year carcinogenicity studies: 3,3'-dimethoxybenzidine dihydrochloride, and 3,3'-dimethylbenzidine dihydrochloride (benzidine congeners), Direct Blue 15 (a representative 3,3'-dimethoxybenzidine-based dye), Acid Red 114 (a representative 3,3'-dimethylbenzidine-based dye) and Direct Blue 218 a metallized 3,3'-dimethoxybenzidine-based dye). The oral route of administration was selected to maximize the chances of detecting systemic effects associated with chemical administration. 3,3'-Dimethylbenzidine, Acid Red 114, 3,3'-dimethoxybenzidine, and Direct Blue 15 were all studied using the same experimental design. Because of the instability of 3,3'-dimethylbenzidine and 3,3'-dimethoxybenzidine in feed, all chemicals, except Direct Blue 218, were administered in drinking water. Direct Blue 218 was not soluble in drinking water, so it was administered in feed. Because long-term studies of 3,3'-dimethylbenzidine and 3,3'-dimethoxybenzidine in mice were performed at the National Center for Toxicology Research (37,38), only male and female rats were used in the studies described here. Rats and mice of both sexes were used in the Direct Blue 218 study.

All studies utilized untreated controls and three dosed groups of each sex. These experimental groups consisted of "core" animals intended for 24 months of treatment plus additional animals designated for interim evaluations at 9 and 15 months. For all, except the Direct Blue 218 study, the allocation of animals in each group followed a procedure recommended by Portier and Hoel (39). The 9 month interim evaluations were performed in each study; however, because of excessive early cancer-related mortality in the 3,3'-dimethoxybenzidine and 3,3'-dimethylbenzidine studies, the 15 month evaluations were not performed and the animals originally designated for interim evaluation were incorporated into the core group. The Direct Blue 218 studies in both rats and mice followed a standard design with 50 core animals per group and 10 additional animals per group designated for interim evaluation at 15 months; there was no 9 month interim evaluation.

Dose selections for the carcinogenicity studies were based on the results of experiments in which groups of 10 male and 10 female rats received test compound in their drinking water or food for from 2 to 13 weeks. Evaluative criteria used to assess the toxicologic effects of the chemicals during the prechronic studies included body
weight gain, food and water intake, grossly observable signs, mortality, and microscopic evidence of organ toxicity.

Experimental animals had free access to food and water throughout the duration of the experiment. In experiments in which the drinking water was used as the vehicle for the test material, water consumption was measured weekly; weekly food consumption was measured when food was the dosing vehicle. Animals were observed twice daily for morbidity or mortality. Clinical signs and body weights were recorded at regular intervals. All animals were subjected to a complete necropsy. A complete histologic evaluation, consisting of microscopic examination of approximately 40 tissues and all gross lesions, was performed on all animals except the low- and mid-dose animals from the 15 month interim evaluations of Acid Red 114, Direct Blue 15, and Direct Blue 218. Only gross lesions and selected tissues were examined in these groups.

Incidences of neoplasms in dosed and control groups were compared statistically using survival adjusted analyses. The life table test, a procedure appropriate for rapidly lethal tumors, was used for mononuclear cell leukemia and Zymbal's gland tumors, both neoplasms that are rapidly fatal. Incidences of other neoplasms in all but the 3,3'-dimethylbenzidine and 3,3'-dimethoxybenzidine studies were analyzed by using the logistic regression test. Significantly reduced survival in the 3,3'-dimethoxybenzidine and 3,3'-dimethylbenzidine studies compromised the power of logistic regression so the Fisher exact test utilizing the effective number of animals was employed for these two studies. The effective number of animals is the number of animals that survived until the appearance of the initial tumor.

Two- and Thirteen-Week Studies
In general, pronounced effects were not seen in the 2-week or 13-week studies. Dose-related reductions in water consumption were observed in the dosed water studies and reduced feed consumption was seen in the dosed feed study. Both effects presumably were due to poor palatability caused by the presence of the test compound. Reduced body weight gains were observed at the higher dose levels in all studies. This effect was presumed to be secondary to reduced water or food consumption. A few chemical related deaths occurred in rats in the highest dose groups during the 2-week studies of 3,3'-dimethoxybenzidine and during the 13-week studies of 3,3'-dimethylbenzidine and Direct Blue 15.

The most common chemical-related lesions observed in rats treated with each of the compounds for 13 weeks were a mild degree of hepatocyte degeneration and necrosis, and degeneration of renal tubules. Hepatocyte hypertrophy (enlargement) and necrosis were seen in the livers of mice from the 13-week Direct Blue 218 study. The results of these studies have been reported in detail elsewhere (40-46).

Two-Year Studies
Concentrations of 3,3'-dimethylbenzidine, 3,3'-dimethoxybenzidine, Acid Red 114, and Direct Blue 15 added to the drinking water during the chronic study are shown in Table 3. The estimated amounts of chemicals consumed, based on measured water consumption, are also shown in Table 3. In general, doses were highest for Direct Blue 15 followed by 3,3'-dimethoxybenzidine, and with lower consumptions of Acid Red 114 and 3,3'-dimethylbenzidine. Females typically consumed somewhat higher doses of chemicals than did males.

The survivals of male and female rats dosed with either 3,3'-dimethylbenzidine, 3,3'-dimethoxybenzidine, Acid Red 114, Direct Blue 15, or Direct Blue 218 are summarized in Tables 4 and 5. The administration of each of these compounds significantly reduced the survival of both sexes of rats. These significant increases in mortality were considered to be due to increased incidences of chemical-induced, lethal neoplasms. Because of the high incidence of tumors and decreased survivals, the 3,3'-dimethylbenzidine and 3,3'-dimethoxybenzidine studies were terminated at 15 and 21 months, respectively, and the Direct Blue 15 study was terminated at 22 months. Despite significantly reduced survivals in both sexes, the Acid Red 114 study was continued until the scheduled termination (24 months).

Nonneoplastic Lesions
Chemical-related nonneoplastic lesions were observed in the livers and kidneys in some studies. The livers of male and female rats treated with all the benzidine compounds except Direct Blue 218 had increased incidences of foci of cellular alteration, cystic degeneration, hepatocyte degeneration, necrosis, and regeneration. Increased incidences of foci of cellular alteration were observed in mice treated with Direct Blue 218. The severity of nephropathy, a common degenerative change of the kidney in aging F344 rats, was increased in treated males and females from the 3,3'-dimethylbenzidine study, and in treated females from the Acid Red 114 study. In addition, foci of hyperplasia, presumably preneoplastic changes, were seen in the preputial, clitoral, and Zymbal's glands in the 3,3'-dimethylbenzidine, 3,3'-dimethoxybenzidine, Acid Red 114, and Direct Blue 15 studies, and in the lung in the 3,3'-dimethylbenzidine and Acid Red 114 studies.

Neoplastic Lesions
3,3'-Dimethylbenzidine, 3,3'-dimethoxybenzidine, Acid Red 114, and Direct Blue 15 all caused a similar spectrum of neoplastic lesions (Tables 6 to 8). The primary
### Table 6. Treatment-related neoplastic responses: skin, Zymbal’s gland, liver and oral cavity.

| 3,3’-Dimethylbenzidine dihydrochloride | Male rats | Female rats |
|---------------------------------------|-----------|-------------|
| Exposure, ppm | 0 | 30 | 70 | 150 | 0 | 30 | 70 | 150 |
| Skin | | | | | | | | |
| Basal cell | 0/60 | 11/45 | 54/75 | 30/60 | 0/60 | 3/45 | 10/75 | 9/60 |
| Sebaceous gland | 0/60 | 0/45 | 7/75 | 5/60 | 0/60 | 0/45 | 0/75 | 0/60 |
| Squamous cell | 0/60 | 2/45 | 17/75 | 27/60 | 0/60 | 3/45 | 9/75 | 12/60 |
| Keratoacanthomas | 1/60 | 1/45 | 8/75 | 5/60 | 0/60 | 0/45 | 0/75 | 0/60 |
| Zymbal’s gland | 1/59 | 3/45 | 32/75 | 38/59 | 0/57 | 6/44 | 32/73 | 42/60 |
| Liver | 0/60 | 0/45 | 4/75 | 5/60 | 0/60 | 0/45 | 7/74 | 4/60 |
| Oral cavity | 0/60 | 0/45 | 7/75 | 5/60 | 0/60 | 3/45 | 9/75 | 13/60 |

| 3,3’-Dimethoxybenzidine dihydrochloride | Male rats | Female rats |
|---------------------------------------|-----------|-------------|
| Exposure, ppm | 0 | 80 | 170 | 330 | 0 | 80 | 170 | 330 |
| Skin | | | | | | | | |
| Basal cell | 2/60 | 32/45 | 54/75 | 40/60 | 0/60 | 4/45 | 3/75 | 2/60 |
| Sebaceous gland | 0/60 | 2/45 | 3/75 | 2/60 | 0/60 | 0/45 | 0/75 | 0/60 |
| Squamous cell | 0/60 | 13/45 | 28/75 | 22/60 | 0/60 | 0/45 | 0/75 | 0/60 |
| Keratoacanthomas | 1/60 | 1/45 | 8/75 | 5/60 | 0/60 | 0/45 | 0/75 | 0/60 |
| Zymbal’s gland | 0/59 | 10/45 | 25/75 | 30/60 | 1/60 | 12/45 | 21/75 | 16/60 |
| Liver | 1/60 | 4/45 | 7/74 | 8/60 | 0/60 | 1/44 | 0/75 | 3/60 |
| Oral cavity | 1/60 | 8/45 | 10/75 | 11/60 | 2/60 | 2/45 | 6/75 | 5/60 |

C.I. Acid Red 114

| Exposure, ppm | 0 | 70 | 150 | 300 | 0 | 150 | 300 | 600 |
| Skin | | | | | | | | |
| Basal cell | 1/50 | 5/35 | 28/65 | 32/50 | 0/50 | 4/35 | 7/65 | 5/50 |
| Sebaceous gland | 1/50 | 1/35 | 5/65 | 6/50 | 0/50 | 0/35 | – | 0/50 |
| Squamous cell | 1/50 | 2/35 | 11/65 | 9/50 | 0/50 | 0/35 | – | 0/50 |
| Keratoacanthomas | 1/50 | 1/35 | 4/65 | 7/50 | 0/50 | 0/35 | – | 0/50 |
| Zymbal’s gland | 0/50 | 0/35 | 8/65 | 7/50 | 0/50 | 3/35 | 18/65 | 19/50 |
| Liver | 2/50 | 2/35 | 15/65 | 20/50 | 0/50 | 3/35 | 8/65 | 4/50 |
| Oral cavity | 0/50 | 0/35 | 1/65 | 2/50 | 0/50 | 0/35 | 0/55 | 0/50 |

C.I. Direct Blue 15

| Exposure, ppm | 0 | 630 | 1250 | 2500 | 0 | 630 | 1250 | 2500 |
| Skin | | | | | | | | |
| Basal cell | 2/50 | 9/35 | 27/65 | 28/50 | 0/50 | 0/35 | 0/65 | 0/50 |
| Sebaceous gland | 0/50 | 1/35 | 7/65 | 3/50 | 0/50 | 0/35 | 0/65 | 0/50 |
| Squamous cell | 2/50 | 4/35 | 11/65 | 19/50 | 0/50 | 2/35 | 6/65 | 5/50 |
| Keratoacanthomas | 2/50 | 1/35 | 7/65 | 2/50 | 0/50 | 0/35 | 0/65 | 0/50 |
| Zymbal’s gland | 1/50 | 5/35 | 10/65 | 20/50 | 0/50 | 4/35 | 11/65 | 17/50 |
| Liver | 0/50 | 0/35 | 9/65 | 11/50 | 0/50 | 0/35 | 2/65 | 5/50 |
| Oral cavity | 1/50 | 10/35 | 24/65 | 17/50 | 2/50 | 4/35 | 19/65 | 15/50 |

– Not determined. *Concentration in drinking water.

Chemical related neoplasms were tumors of the skin, Zymbal’s gland, oral cavity epithelium, liver, preputial/clitoral glands, and intestines of both males and females. A few of these neoplasms were seen as early as the 9-month interim sacrifices, and the numbers of tumors at the various sites increased as the studies progressed. The microscopic appearance of the chemical-related neoplasms was similar in all studies.

While we found a wide variety of tumors in our studies, Robens et al. (2) reported only liver involvement after administration of the benzidine-derived dyes. It is unlikely that the 13-week studies reported by Robens et al. were long enough for a full expression of toxicity, and therefore a comparison of the studies is difficult to make. By way of contrast, however, we found no evidence of hepatocarcinogenicity during the 13-week studies on the congeners or dyes.

All four chemicals significantly increased the incidence of a variety of epithelial neoplasms of the skin in both sexes of rats. These skin tumors included adenomas and carcinomas of the basal cells and sebaceous glands, squamous cell papillomas and carcinomas, and keratoacanthomas. Neoplasms of the Zymbal’s glands included adenomas and carcinomas, with carcinomas being by far the more common of the two. Carcinomas were highly invasive and occasionally metastasized. Chemical related neoplasms of the oral cavity consisted of squamous cell papillomas and carcinomas originating from the stratified squamous epithelium. Microscopically, these neoplasms resembled the squamous cell neoplasms of the skin. Neoplastic nodules and hepatocellular carcinomas occurred as a chemical related effect in the livers of treated male and female rats (*"neoplastic nodule" was the term used previously for neoplasms now classified as hepatocellular adenomas). Adenomatous polyps and adenocarcinomas of the small and large intestine, neoplasms rarely seen
in untreated F344 rats, occurred in treated male and female rats.

Adenomas and carcinomas of the preputial glands and clitoral glands (female homologue of the preputial glands) occurred frequently in treated rats. Because preputial gland neoplasms are usually not overtly aggressive or invasive and rarely metastasize (47,48), classification of these neoplasms as benign or malignant is difficult (49). The transplantability of preputial gland neoplasms induced by 3,3'-dimethylbenzidine, 3,3'-dimethoxybenzidine and Acid Red 114 was investigated to provide information on the biologic behavior of these neoplasms (49,50). All neoplasms selected for transplantation were retrospectively diagnosed as carcinomas and therefore, comparable information was not obtained for preputial gland adenomas. The transplanted neoplasms did not become anaplastic or less differentiated over four serial passages; however, the transplants behaved biologically as malignant neoplasms in spite of their well-differentiated morphology. The latency period was short and transplants grew rapidly. The results of these studies confirmed the malignant nature of these preputial gland neoplasms from rats exposed to the benzidine congeners and dyes.

In rats treated with 3,3'-dimethylbenzidine, 3,3'-dimethoxybenzidine, Direct Blue 15, and Acid Red 114, the total numbers of neoplasms tended to be greater in males than in females. In addition, tumors of the skin, liver, and oral cavity epithelium were more common in males than females. Some, but not all, of the four chemicals tested caused marginal increases in mononuclear cell leukemia, mesotheliomas, and tumors of the brain, mammary gland, lung, or adrenal gland, which may be related to treatment.

The numbers of animals with primary malignant neoplasms in the 3,3'-dimethylbenzidine, 3,3'-dimethoxybenzidine, Acid Red 114, and Direct Blue 15 studies are shown in Table 9. Malignant tumors were observed in 83 to 98% of all high dose animals; 64 to 91% of mid-dose animals, and 13 to 80% of all low-dose animals. Tumor multiplicity was calculated as the number of primary malignant tumors per animal. Tumor multiplicity was greater than one for all dosed animals except those treated with low and mid doses of 3,3'-dimethylbenzidine, and females in the low-dose group of Direct Blue 15. In male rats, malignant neoplasms were observed earliest in 3,3'-dimethylbenzidine-dosed rats, followed by rats dosed with Direct Blue 15,

### Table 7. Treatment-related neoplastic responses: preputial gland, clitoral gland, uterus, and mammary gland.

| 3,3'-Dimethylbenzidine dihydrochloride | Male rats | Female rats |
|----------------------------------------|-----------|------------|
| Exposure, ppm                          | 0 30 70 150 | 0 30 70 150 |
| Preputial gland                         | 2/60 4/45 6/75 9/60 | - - - - |
| Clitoral gland                          | - - - - | - - - - |
| Mammary gland                          | 0/60 0/45 0/75 0/60 | 1/60 1/45 3/75 6/60 |

| 3,3'-Dimethoxybenzidine dihydrochloride | Male rats | Female rats |
|----------------------------------------|-----------|------------|
| Exposure, ppm                          | 0 80 170 330 | 0 80 170 330 |
| Preputial gland                         | 8/60 12/43 33/75 29/59 | - - - - |
| Clitoral gland                          | - - - - | - - - - |
| Mammary gland                          | 0/60 0/45 0/75 0/60 | 1/60 1/45 3/75 6/60 |

| C.I. Acid Red 114 | Male rats | Female rats |
|-------------------|-----------|------------|
| Exposure, ppm     | 0 70 150 300 | 0 150 300 600 |
| Preputial gland    | - - - - | - - - - |
| Clitoral gland     | - - - - | 11/48 17/32 28/62 23/50 |
| Mammary gland     | 0/50 0/35 0/65 0/50 | 1/50 0/35 1/65 4/50 |

| C.I. Direct Blue 15 | Male rats | Female rats |
|---------------------|-----------|------------|
| Exposure, ppm       | 0 630 1250 2500 | 0 630 1250 2500 |
| Preputial gland      | - - - - | - - - - |
| Clitoral gland       | - - - - | - - - - |
| Mammary gland        | 0/60 0/45 0/75 | 0/60 0/45 0/75 |

-- Not applicable. *Concentration in drinking water.

### Table 8. Treatment-related neoplastic responses: intestine, lung, mesotheliomas, brain, and mononuclear cell leukemia.

| 3,3'-Dimethylbenzidine dihydrochloride | Male rats | Female rats |
|----------------------------------------|-----------|------------|
| Exposure, ppm                          | 0 30 70 150 | 0 30 70 150 |
| Small intestine                        | 0/60 0/45 4/75 8/60 | 0/60 1/45 3/75 5/60 |
| Large intestine                        | 0/60 0/45 6/75 15/60 | 0/60 1/45 3/75 5/60 |
| Lung                                   | 1/60 0/45 8/60 1/60 | 1/45 3/75 4/60 |
| Brain                                  | 0/60 0/45 1/75 2/60 | 0/60 2/45 2/75 1/60 |
| Mononuclear cell leukemia              | 0/60 0/45 0/75 | 0/60 1/45 5/45 6/75 |
| Mesotheliomas                          | 0/60 0/45 3/75 4/60 | 0/45 0/75 0/60 |

| 3,3'-Dimethoxybenzidine dihydrochloride | Male rats | Female rats |
|----------------------------------------|-----------|------------|
| Exposure, ppm                          | 0 80 170 330 | 0 80 170 330 |
| Small intestine                        | 0/60 4/45 7/75 8/60 | 0/60 0/45 0/75 0/60 |
| Large intestine                        | 0/60 1/45 8/75 8/60 | 0/60 1/45 3/75 3/60 |
| Lung                                   | 0/60 2/44 3/75 1/60 | 0/60 0/45 0/75 0/75 |
| Mesotheliomas                          | 2/60 1/45 7/75 6/60 | 0/60 0/45 0/75 0/60 |

| C.I. Acid Red 114 | Male rats | Female rats |
|-------------------|-----------|------------|
| Exposure, ppm     | 0 70 150 300 | 0 150 300 600 |
| Small intestine   | 0/50 0/35 0/65 | 0/50 0/35 1/65 2/50 |
| Large intestine   | 0/50 0/35 0/65 | 0/50 1/35 0/65 3/50 |
| Lung              | 2/50 2/35 2/65 | 3/50 1/50 2/35 9/65 |
| Adrenal medulla   | 12/50 11/35 27/63 | 21/48 1/50 3/35 4/64 |
| Mononuclear cell leukemia | 0/50 0/35 0/65 | 12/50 13/35 18/65 5/60 |

| C.I. Direct Blue 15 | Male rats | Female rats |
|---------------------|-----------|------------|
| Exposure, ppm       | 0 630 1250 2500 | 0 630 1250 2500 |
| Small intestine     | 0/50 1/35 0/65 | 0/50 1/35 0/65 1/50 |
| Large intestine     | 0/50 1/35 0/65 | 0/50 0/35 0/65 0/50 |
| Brain               | 0/50 1/35 0/65 | 0/50 0/35 0/65 0/50 |
| Mononuclear cell leukemia | 17/50 19/35 28/65 | 20/50 7/50 13/35 27/65 15/50 |

*Concentration in drinking water.
Table 9. Number of animals with primary malignant neoplasms in all organs.

|                | Control | Low Dose | Mid Dose | High Dose |
|----------------|---------|----------|----------|-----------|
| Males          |         |          |          |           |
| 3,3'-Dimethylbenzidine.2HCl\(a\) | 2% (1/60) | 13% (6/45) | 64% (48/75) | 83% (50/60) |
| Animals with malignant neoplasms | 1 | 5 | 62 | 90 |
| Total malignant neoplasms | 31 | 59 | 121 | 105 |
| First incidence (days) | 419 | 250 | 229 | 209 |
| 3,3'-Dimethoxybenzidine.2HCl\(b\) | 45% (27/60) | 80% (36/45) | 98% (66/75) | 98% (59/60) |
| Animals with malignant neoplasms | 31 | 59 | 121 | 105 |
| Total malignant neoplasms | 455 | 266 | 287 | 273 |
| First incidence (days) | 114 | 77 | 83 | 64 |
| Acid Red 114\(c\) | 66% (33/50) | 77% (27/35) | 83% (54/65) | 86% (43/50) |
| Animals with malignant neoplasms | 43 | 38 | 76 | 55 |
| Total malignant neoplasms | 1.06 | 0.87 | 1.20 | 1.10 |
| First incidence, days | 131 | 352 | 325 | 377 |
| Direct Blue 15\(d\) | 46% (23/50) | 77% (27/35) | 83% (54/65) | 90% (45/50) |
| Animals with malignant neoplasms | 25 | 37 | 88 | 83 |
| Tumor multiplicity | 0.50 | 1.06 | 1.35 | 1.86 |
| First incidence, days | 445 | 293 | 323 | 243 |
| Females          |         |          |          |           |
| 3,3'-Dimethylbenzidine.2HCl\(a\) | 2% (1/60) | 31% (14/45) | 65% (49/75) | 93% (56/60) |
| Animals with malignant neoplasms | 1 | 19 | 65 | 90 |
| Total malignant neoplasms | 390 | 367 | 184 | 229 |
| First incidence, days | 40 | 373 | 220 | 270 |
| 3,3'-Dimethoxybenzidine.2HCl\(b\) | 45% (27/60) | 71% (32/45) | 91% (68/75) | 93% (56/60) |
| Animals with malignant neoplasms | 31 | 52 | 96 | 78 |
| Total malignant neoplasms | 431 | 411 | 285 | 229 |
| First incidence, days | 40 | 373 | 220 | 270 |
| Acid Red 114\(c\) | 44% (22/50) | 71% (25/35) | 82% (53/65) | 84% (42/50) |
| Animals with malignant neoplasms | 26 | 35 | 87 | 51 |
| Total malignant neoplasms | 0.52 | 1.0 | 1.34 | 1.02 |
| First incidence, days | 431 | 411 | 285 | 229 |
| Direct Blue 15\(d\) | 42% (21/50) | 71% (25/35) | 98% (56/65) | 98% (48/50) |
| Animals with malignant neoplasms | 23 | 29 | 70 | 64 |
| Total malignant neoplasms | 0.46 | 0.83 | 1.08 | 1.28 |
| First incidence, days | 500 | 463 | 253 | 296 |

\[a\]3,3'-Dimethylbenzidine study terminated after 15 months. \[b\]Tumor multiplicity calculated as mean number of malignant tumors per animal. \[c\]3,3'-Dimethoxybenzidine study terminated after 21 months. \[d\]Acid Red 114 study terminated after 24 months. Direct Blue 15 study terminated after 22 months.

3,3'-Dimethoxybenzidine, and Acid Red 114. In females the order in which tumors were first detected was: 3,3'-dimethoxybenzidine, 3,3'-dimethylbenzidine, Acid Red 114, and Direct Blue 15. The results of the Direct Blue 218 study showed a spectrum of neoplastic lesions that was different from that observed for the other four benzidine compounds. Incidences of chemical-related neoplasms were not as dramatic, and were observed primarily in the oral cavity epithelium of rats, and in the livers of mice (Table 10).

Dose-response relationships could not be established for the carcinogenicity of the benzidine congener dyes and the parent amines because of the high incidences of tumors at all doses. These high incidences of tumors after chronic exposure were unexpected because higher doses of the benzidine congeners and dyes produced little or no toxicity in prechronic studies. In early studies of 3,3'-dimethoxybenzidine and 3,3'-dimethylbenzidine, only relatively low incidences of tumors were observed in dosed animals (51-54). However, in those studies, the administration of large, toxic doses of chemical decreased survival and may have resulted in a severe underestimation of the carcinogenic potency of these chemicals.

Quantitative comparisons of the carcinogenic potencies of the four chemicals is difficult because of the lack of dose-response relationships, as well as differences in doses and durations of treatments. Slight differences in the carcinogenic potencies of the two benzidine congener dyes are in part a result of the rate at which they are metabolized and in part by the carcinogenicity of the benzidine congener metabolite. Quantitative differences were observed in the liver and skin of males and females; males in all four studies had a higher tumor incidence at these sites than did females. However, chemical-induced clitoral gland tumors of females occurred more commonly than preputial gland tumors in males.

3,3'-Dimethylbenzidine caused a neoplastic response at an earlier time than did 3,3'-dimethoxybenzidine, suggesting that 3,3'-dimethylbenzidine may be a more potent carcinogen, possibly because it is absorbed to a greater extent, or metabolized to the ultimate carcinogen more efficiently than 3,3'-dimethoxybenzidine. In addition, Acid Red 114, the 3,3'-dimethylbenzidine-derived dye, caused tumors at lower doses than Direct Blue 15, the 3,3'-dimethoxybenzidine derived dye, also suggesting that 3,3'-dimethylbenzidine may be a more potent carcinogen.

Qualitatively, 3,3'-dimethoxybenzidine, 3,3'-dimethylbenzidine, Direct Blue 15, and Acid Red 114 caused a similar spectrum of neoplasms in the Zymbal's gland, skin, liver, oral cavity, clitoral and preputial gland, and to a lesser extent in the intestine of the Fischer F344/N rat. Tumors occur infrequently at these sites, and the high incidences of tumors at these sites in all four studies strongly indicates a common mechanism.

Two uncommon neoplasms that appeared at relatively high incidences in treated rats were Zymbal's gland tumors (males and females) and skin basal cell tumors (males). Most of the chemicals tested by the NTP that induced either Zymbal's gland or skin tumors in rats also caused tumors in other sites. These tumor inducing chemicals (e.g., 4-aminobiphenyl, 4,4'-thiodiianiline) all have in common an aromatic amine functional group that is considered to be a "structural alert" for genotoxic activity, and all are mutagenic in Salmonella. A number of aromatic amines,
or chemicals that are metabolized to aromatic amines, cause neoplasms in the Zymbal's gland of rats (40). Benzidine, the parent compound of this series of chemicals, also causes Zymbal's gland tumors in rats (55), implicating the free amine group of benzidine as a common site for metabolic activation for this related group of chemicals. Although, for the most part, the target sites were similar for the four chemicals, some differences were observed. Acid Red 114 and its parent congeners, 3,3'-dimethoxybenzidine, caused a higher incidence of liver tumors than did Direct Blue 15 and its parent congeners, 3,3'-dimethoxybenzidine. In addition, significantly increased incidences of preputial gland tumors were seen in male rats treated with 3,3'-dimethoxybenzidine, 3,3'-dimethylbenzidine, and Direct Blue 15, but not with Acid Red 114. Some, but not all, of the four chemicals tested caused variable increases in mononuclear cell leukemia, mesotheliomas, and tumors of the brain, mammary gland, lung, and adrenal medulla, which may have been related to chemical treatment. These slight differences in sites of tumor origin among studies are likely a result of differences in dose, the extent of absorption and metabolism of the different chemicals, and possibly the effects of impurities in the dyes. Direct Blue 218, a copper-chelated 3,3'-dimethoxybenzidine-derived dye, was considerably less carcinogenic than the benzidine congeners and nonchelated dyes; it was also nonmutagenic.

Chelation with copper of the hydroxy groups of the benzidine congener is believed to prevent metabolism of the dye to the ultimate carcinogen. The relatively low carcinogenic response to Direct Blue 218 supports the concept that copper chelation inhibited metabolic activation of the dye to a large extent; however, the presence of oral cavity tumors in rats and liver neoplasms in mice suggests that copper chelation does not completely eliminate the carcinogenic effects of the dye. These neoplasms may be a result of incomplete inhibition of metabolism of the chelated dye, and possibly a result of carcinogenic impurities in the dye. The Direct Blue 218 used in these studies was only 60% pure. In addition to organic impurities, the dye also contained free copper which may also be toxic. It is also possible that the oral cavity tumors were a direct effect of the chemical in dosed feed.

The different target sites of Direct Blue 218 in rats and mice suggests potential species differences in metabolism of this dye. Further support for species differences in metabolism of benzidine compounds is provided by studies of 3,3'-dimethoxybenzidine and 3,3'-dimethylbenzidine administered in drinking water to BALB/c mice. Although these compounds are potent, multisite carcinogens in rats, 3,3'-dimethoxybenzidine had no apparent carcinogenic effect (37), and 3,3'-dimethylbenzidine caused only a low incidence of lung tumors in treated mice (38). Metabolic differences may also exist between mouse strains, since liver neoplasms were observed in B6C3F1 mice and lung tumors were observed in BALB/c mice.

**Oncogene Activation**

DNA from neoplasms of skin, preputial and clitoral gland, mammary gland, and intestines of rats exposed to 3,3'-dimethoxybenzidine, 3,3'-dimethylbenzidine, Direct Blue 15, or Acid Red 114, was analyzed for dominant transforming genes using the NIH 3T3 plate transfection assay (56). Oncogenes detectable by DNA transfection analysis were present in 35 of 59 skin, clitoral gland or preputial gland neoplasms that had been induced in F344 rats by the benzidine congeners or derived dyes. DNA from both benign and malignant neoplasms was capable of inducing morphologically transformed foci in NIH 3T3 fibroblasts.

A high percentage of the induced rat neoplasms contained activated alleles of either H- or N-ras. Those neoplasms with activated H-ras contained point mutations in codon 12, 13, or 61. Point mutations at codon 61 accounted for approximately 50% of the H-ras gene activations in both 3,3'-dimethylbenzidine and 3,3'-dimethoxybenzidine induced tumors, with C→A transversions (or G→T transversions in the antisense strand) being the predominant mutation at codon 61 in both groups. The mutations detected at codon 12 were exclusively G→A transitions whereas the mutations at codon 13 were predominantly G→C transversions.

The detection of a high percentage of activated oncoproteins in these chemical-induced neoplasms is in sharp contrast to the relatively low frequency of activated oncoproteins found in spontaneously occurring neoplasms of F344 rats (57). These observations suggest that activation of cellular ras genes by point mutation may be an important step in induction of tumors in rats treated with the benzidine congeners and derived dyes.

A number of epidemiologic studies support the causal relationship between exposure to benzidine and the occurrence of bladder cancer in humans (1). The finding that H-ras gene activation occurs in a portion of human bladder tumors (58,59) and the above results suggesting that ras gene activation is an important step in the induction of tumors in rats treated with the benzidine congeners and derived dyes, suggests that ras activation can be involved in one pathway of human urothelial cell transformation.

**Summary**

Consumption of drinking water containing the benzidine congeners 3,3'-dimethylbenzidine and 3,3'-dimethoxybenzidine, and the prototypical benzidine congener dyes, Acid Red 114 and Direct Blue 15 led to a similar spectrum of neoplasms. The high incidences of tumors at the same sites in all four studies strongly indicates a common mechanism. The detection of a high percentage of activated oncoproteins in these chemical-induced neoplasms suggests that activation of cellular ras genes by point mutation may be an important step in induction of tumors in rats treated with benzidine congeners and derived dyes.

Direct Blue 218, a metallized 3,3'-dimethoxybenzidine-derived dye, was considerably less carcinogenic than the benzidine congeners and nonchelated dyes and was also nonmutagenic. However, the presence of oral cavity tumors in rats and liver neoplasms in mice suggests that copper chelation does not completely eliminate the carcinogenic effects of metallized dyes.

**Conclusions**

The broad objective of the benzidine initiative was to generate a body of scientific information that would facilitate regulatory decision making regarding the toxicologic and carcinogenic risk associated with exposure to benzidine and benzidine congener derived dye materials. The carcinogenicity studies provided clear evidence that the benzidine congeners and prototypical dyes derived from these parent amines are potent carcinogens in animals. Based upon the results of metabolism and mutagenicity studies of other benzidine- and benzidine-congener-derived dyes, one would predict that most, if not all, dyes based on these chemicals are carcinogens.

The metabolism, disposition, mutagenicity, and carcinogenicity data from these studies provide sufficient evidence that the benzidine and benzidine-congener-derived dyes should be regulated as probable human carcinogens.
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