Metabolism of Nine Benzidine-Congener-Based Azo Dyes in Rats Based on Gas Chromatographic Assays of the Urine for Potentially Carcinogenic Metabolites

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Abstract

Metabolism experiments were conducted with rats dosed with nine azo dyes based on dimethyl-, dimethoxy-, or dichlorobenzidine to determine whether the free amine congeners, their monoacetyl or diacetyl metabolites, or alkaline hydrolyzable conjugates were excreted in the urine. After preliminary tests of the dyes, 2-mg doses were administered to each animal and urine samples were collected at intervals up to 96 hours. EC/GC procedures were based on the analysis of heptafluorobutyryl derivatives of the free amine congener moieties or their monoacetyl metabolites. Peak levels of metabolites were excreted either 0-12 or 12-24 hours after administration and, in seven of nine instances, no metabolites persisted in the urine after 48 hours. Minimum detectable levels of all metabolites were 12 ppb or less. All nine dyes were shown to be converted to measurable levels of their benzidine-congener-based metabolites in rats.

Introduction

Azo dyes based on benzidine (Bzd) and benzidine-congeners [*i.e.*, 3,3'-dimethylbenzidine (DiMeBzd) and 3,3'-dimethoxybenzidine (DiMxBzd)] constitute a group of some 92 discrete chemicals that are widely used in the United States. For example; during 1978, domestic production and imports amounted to about 4.1 million pounds (1). Exposure of the general public to these materials is through contact with products such as paper, fabrics and leather to which the dyes have been applied; more importantly, packaged dyes for home use and paints contain these chemicals. The Dyes Environmental and Toxicology Organization (DETO) has estimated that about 1,000 workers are exposed to dyes during the manufacturing process and that as many as 15,000 others are exposed in the various dye application industries (1).

Bzd is a known human carcinogen that causes cancer of the urinary bladder (2) and since DiMeBzd and DiMxBzd were reported to induce tumors of the Zymbal's gland and at other sites in rats, the International Agency for Research on Cancer (IARC) classified these compounds as animal carcinogens (3,4). Results from several experiments indicated that several azo dyes based on Bzd, DiMeBzd, and DiMxBzd are metabolized to their parent amines by laboratory animals (5-8) suggesting that the dyes may also be animal carcinogens. Three dyes (Direct Blue 6, Direct Black 38 and Direct Brown 95) known to be metabolized to Bzd, were shown to be carcinogens in rats (9). The National Institute for Occupational Safety and Health (NIOSH) evaluated the evidence and issued a special hazard review concluding that benzidine-based azo dyes are potential human carcinogens, and that exposure to these dyes should be eliminated (10).

As part of the National Toxicology Program (NTP) and in response to the need for scientific information for regulatory decisions in the Environmental Protection Agency (EPA), Consumer Product Safety Commission (CPSC) and Occupational Safety and Health Administration (OSHA), a strategy program was designed to test prototypical dyes to establish basic principles which could be applied to the entire group of dyes. This approach was sought to circumvent the high cost of conducting long-term bioassays of each dye. The scientific objectives and approaches were developed by scientists from the EPA, CPSC, OSHA, National Institutes of Health (NIH), NIOSH, and the Food and Drug Administration (FDA).

The chemical phase of the work related to metabolism of DiMeBzd- and DiMxBzd-based azo dyes to their potentially carcinogenic free amines, as determined by analysis of urine from dosed rats, was conducted at the FDA's National Center for Toxicological Research (NCTR). Results of these tests with four representative dyes from each of the two benzidine-congener bases are presented in this paper. Results from tests with Direct Red 46 [a 3,3'-dichlorobenzidine (DiClBzd)-based dye], a dye of particular interest to the EPA, are also presented.

Experimental

Test Materials

General information concerning the benzidine-congener-based dyes selected for testing in rats is presented in Table I with their structures depicted in Figure 1 (exception: the structure of Black 114 is not known). Samples of these dyes were obtained from

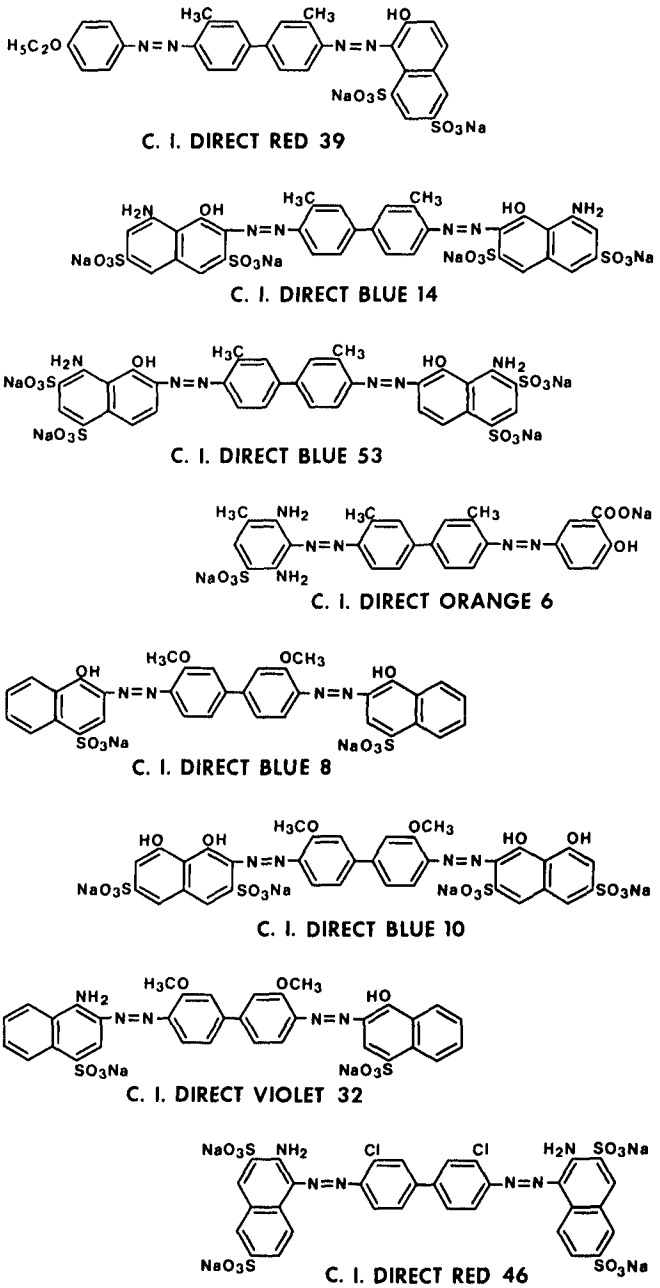

Figure 1. Formulas of eight benzidine-congener-based azo dyes.

the DETO and/or the Midwest Research Institute (MRI) and are described in Table II. A chemical reduction test (11) was used to verify the presence of the correct benzidine-congener base in the dye molecule and to calculate the purity of the dye. In this test, an aqueous solution of the dye was reduced with 0.2 *M* stannous chloride in 12 *N* HCl at 40 °C for about 3 hours (to complete decolorization), made strongly akaline, and the benzidine-congener free amine was extracted with chloroform and analyzed by gas chromatography employing a flame ionization detector (GC/FID). The purity of the dye was calculated on the basis of the amount of benzidine-congener amine obtained from a known amount of dye as compared to the amount theoretically obtainable (Tables I and II). In designing the metabolism tests, it was decided that all dyes should be at least 50% pure and contain no more than 50 ppm of congener amine impurities; five of the nine dyes required purification prior to animal dosing. For purification, the dye was mixed with about twice its weight of cold water (3 °C), filtered on a Buchner funnel and washed with three additional portions of cold water to remove water soluble impurities. The residue on the filter was dissolved in 1 L of water (25 °C), and extracted four times with benzene to remove impurities of aromatic amines. The aqueous phase was freeze dried to recover the purified product. In all cases, this regimen provided the necessary improvement in purity to meet the established criterion; the range of purity for eight

Table II. Purity and Stability of Benzidine-Congener-Based Dyes Administered to Rats

Direct Dye	Purity of Dye (%)* as Received	Purity of Dye (%)* as Dosed	Congener Amine Impurities (ppm)**	Stability at 25° in Urine: Rat	Stability at 25° in Urine: Human
Red 39	70.7	70.7	0.58	U	S
Blue 14	61.9	76.1	21.	U	S
Blue 53	67.4	67.4	1.4	U	S
Orange 6	31.9	61.8	0.31	U	S
Blue 8	29.9	63.8	0.22	U	U
Black 114	8.25†	18.6†	0.39	U	S
Blue 10	47.9	71.6	0.060	S	U
Violet 32	83.4	83.4	0.13	S	S
Red 46	39.7	78.6	7.0	U	U

*Determined by chemical reduction and GC assays.
**Determined by EC/GC of HFB derivatives. Direct Blue 53 and Direct Orange 6 also contained DiMxBzd impurities of 1.6 and 5 9 ppm, respectively
†Purity not determined; MW of dye not known, values are percentages (w/w) of DiMxBzd in the dye.
S = stable, U = unstable.

Table I. General Information for the Benzidine-Congener-Based Azo Dyes Administered to Rats

Direct Dye	Benzidine Congener-base*	Color Index Number	CAS Number	Molecular Formula	Molecular Weight	Bzd-Congener in Pure Dye (%)
Red 39	DiMeBzd	23630	6358-29-8	$C_{32}H_{26}O_6N_4S_2Na_2$	704.65	30.12
Blue 14	DiMeBzd	23850	72-52-1	$C_{34}H_{24}O_{14}N_6S_4Na_4$	960.81	22.09
Blue 53	DiMeBzd	23860	314-13-6	$C_{34}H_{24}O_{14}N_6S_4Na_4$	960.81	22.09
Orange 6	DiMeBzd	23375	6637-88-3	$C_{28}H_{24}O_6N_6SNa_2$	618.55	34.32
Blue 8	DiMxBzd	24140	2429-71-2	$C_{34}H_{24}O_{10}N_4S_2Na_2$	758.65	32.20
Black 114	DiMxBzd	NK	NK	NK	NK	NK
Blue 10	DiMxBzd	24340	4198-19-0	$C_{34}H_{22}O_{18}N_4S_4Na_4$	994.77	24.56
Violet 32	DiMxBzd	24105	6428-94-0	$C_{34}H_{25}O_9N_5S_2Na_2$	757.71	32.24
Red 46	DiClBzd	23050	6548-29-4	$C_{32}H_{18}O_{12}N_6Cl_2S_4Na_4$	969.61	26.11

*DiMeBzd is 3,3'-Dimethylbenzidine (MW 212.28); DiMxBzd is 3,3'-Dimethoxybenzidine (MW 244.28); DiClBzd is 3,3'-Dichlorobenzidine (MW 253 13).
NK = Not known.

of the dyes was 61.8 to 83.4% (Table II). The purity of Direct Black 114 could not be calculated without knowing its molecular weight (MW); however, the material was purified prior to use as described and the DiMxBzd content was increased from 8.25 to 18.6% (w/w).

Nuclear magnetic resonance (NMR) spectra of the dyes were obtained and found to be consistent with their structures except for Direct Blue 10; however, the presence of the DiMxBzd moiety in Direct Blue 10 was confirmed by NMR.

All dyes that met the criterion for purity, either as received or after purification, were subjected to analysis for free aromatic amine impurities (12,13). Briefly, an aqueous solution of dye (1 mg/mL) was extracted with benzene and any extracted congener amine residues were derivatized using heptafluorobutyric anhydride (HFBA) and the derivative was assayed by electron-capture gas chromatography (EC/GC). All dosed dyes met the established criterion for impurities in the metabolism experiments (*i.e.*, ◄ 50 ppm of congener amine impurities as reported in Table II).

Stability tests were conducted on all dyes that met the criteria for purity, impurity and confirmation of structure to determine any *in vitro* contribution by a dye to the metabolite content of urine resulting from bacterial and/or enzyme action in the event that the urine might become contaminated by intact dye. The assumption was made that if a dye was stable in urine at 5 °C, it would also be stable under the conditions employed for collecting urine samples from animals (under dry ice) and in storage prior to assay (−20 °C). The stability tests were conducted as reported (12); 1000 ppm solutions of the dye in human urine or control urine from the test animals were sampled at 0-, 24-, 48-, 72- and 96-hour intervals and analyzed for the appropriate benzidine congener.

Test Animals and Housing Conditions

Male Fischer 344 rats, reared at the NCTR, were housed (2 rats/cage) in s.s. cages (Wahmann Model No. LC-177). An additional 16 mesh s.s. screen was placed on the animal support screen to prevent fecal pellets from contaminating the urine and to allow for facile collection of feces in the event that assays were later required. The rats were seven weeks old, weighed 180 to 220 g at the beginning of the experiments and were kept on a 12-hour light cycle (6 am to 6 pm) for the entire experiment. The animals were allowed free access to water and feed except for a 12-hour fasting period prior to dosing when the feed was withheld and then restored 1 hour after the dose was administered. Urine was collected in a 50-mL graduated glass tube submerged in solid dry ice and positioned under the drain tube of the cage.

Metabolism Tests

Four groups of 4 rats were used in tests with each dye: 3 groups were dosed with the dye and one group, dosed with water, served as a control. Pretreatment samples of urine were collected from all groups during the 36-hour period prior to administration by gavage of a single dose (1 mL) of an aqueous dye solution containing the equivalent of 2 mg of 100% dye. Because of the limited solubility of some dyes in water at ambient temperature, all dyes were weighed and dissolved in water heated to 39 °C (body temperature of the rat) and administered at that temperature. Samples of urine from each group (2 cages) were collected for intervals of 0-12, 12-24, 24-48 and 48-96 hours after the dose was administered and stored at −20 °C until analysis.

Analytical Chemical Methodology

Samples of urine from the metabolism tests with dyes were assayed by EC/GC (13,14). Separate portions of the same sample were assayed for the benzidine-congener free amines and their monoacetyl (MoAc) metabolites, alkaline hydrolyzable conjugates (AHC) or for diacetyl (DiAc) metabolites as shown in Figure 2 (13). The benzidine-congener free amine and its MoAc and DiAc metabolites were extracted from urine under alkaline conditions with benzene; the aqueous phase was subjected to alkaline hydrolysis and again extracted with benzene to remove AHC metabolites as the benzidine-congener amine. The two benzene extracts were then subjected to derivatization with HFBA with trimethylamine as a catalyst and analyzed by EC/GC; any DiAc metabolites present in these extracts were not derivatized or detected. A second sample was made strongly acid with HCl and extracted with chloroform to remove and separate the DiAc metabolite from any free benzidine-congener or MoAc metabolite. The extracted DiAc metabolite was then hydrolyzed to the free amine, derivatized, and analyzed as described. Minimum detectable levels for the three benzidine-congener free amines and their MoAc and DiAc metabolites in rat urine were 12 ppb or less.

The results of assays for benzidine congeners, their MoAc or DiAc derivatives, or congeners excreted as AHC were corrected for pretreatment sample backgrounds and recoveries. Since AHC have not been characterized, recoveries of the individual congener bases were determined through the AHC procedure for use in correcting the analytical results.

The concentrations of the metabolites (ppb or ng/mL) for each of the three groups of test animals were multiplied by their respective sample volumes to determine the amount of each metabolite excreted during each collection interval of the post-treatment period; then the mean and standard deviation (SD) were calculated. These results were converted mathematically to total μg-equivalents of benzidine congener excreted as the free amine, MoAc, DiAc and/or AHC metabolites as follows: DiMeBzd = MoAcDiMeBzd × 0.835, DiAcDiMeBzd × 0.716; DiMxBzd = MoAcDiMxBzd × 0.853, DiAcDiMxBzd × 0.744; DiClBzd = MoAcDiClBzd × 0.858, DiAcDiClBzd × 0.751. No conversion factor was required for the free amines or AHC.

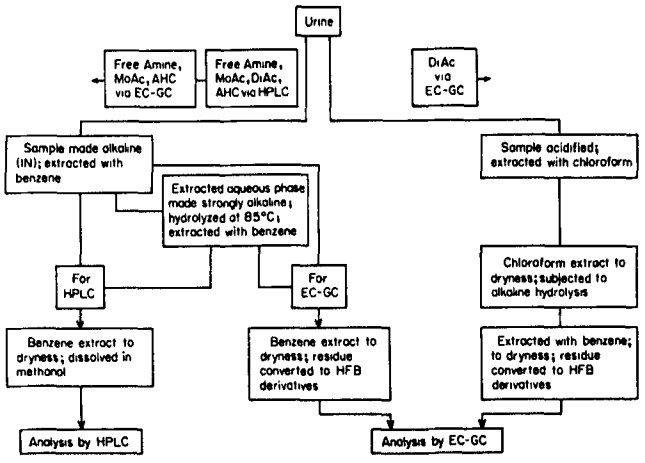

Figure 2. General scheme for extraction, separation, and analysis of benzidine-congener free amines and their monoacetylated (MoAc), diacetylated (DiAc), and conjugated (AHC) products in urine (8).

Results and Discussion

The nine dyes described in Tables I and II were administered to rats as described. None of the animals showed overt symptoms of shock or discomfort except those dosed with Direct Red 39; all 12 rats became lethargic and several indicated signs of shock immediately after the dosing. These rats showed no interest in eating or drinking for several hours after the fasting period; however, they later recovered and began to eat and drink normally. All of the animals survived to the end of the experiment. The weight gained by each rat averaged 18 g during the 96-hour test period; this was comparable to gains observed with control rats.

All dyes were found to be stable in both rat and human urine at 5 °C for 96 hours. However, six of the dyes were shown to liberate high ppm levels of benzidine-congener amines when held in either rat or human urine at 25 °C for 96 hours. This degradation may be due to microbial or enzymatic action and points out the need to collect and store samples of urine at 5 °C or less to avoid erroneous results in the event that the sample is contaminated with dyes. All samples of urine from the test animals were collected under dry ice and stored at −20 °C until analysis; therefore, any amounts of free amines in the urine in excess of the small amount dosed as impurities must be attributed to breakdown of the dye in the animals.

Urine samples from the test and control animals were thawed and assayed in groups of eight using the EC/GC methods described. The data from these assays (ppb or ng/mL) were calculated and the mean quantities of the three metabolites and AHC excreted during each interval of the post-treatment period are presented in Table III. Values for the pretreatment interval (−36 to 0 hour) represent background levels and were used to correct the post-treatment values prior to corrections for recovery. A cursory examination of the excretion values obtained during the post-treatment intervals indicated that all nine dyes were metabolized to yield appreciable levels of the corresponding free amine, MoAc, DiAc and AHC products. Metabolites of six of the nine dyes were excreted at peak levels during the first 12 hours after the dyes were administered. Only Direct Red 39, Direct Orange 6 and Direct Red 46 showed peak excretion levels during the 12- to 24-hour interval. All metabolite levels for all dyes decreased markedly from 24 to 48 hours after dosing and no residues of metabolites were detected above pretreatment levels at the 48- to 96-hour interval, except for Direct Red 39 and Direct Blue 14. Excretion of metabolites derived from the DiMxBzd-based dyes tended to peak and dissipate faster than for those based on DiMeBzd or DiClBzd. Nevertheless, consistent and measurable metabolism of all nine dyes was observed in the rats.

Generally, DiAcDiMeBzd was the major metabolite of those measured from the DiMeBzd-based dyes, followed by MoAcDiMeBzd and smaller quantities of DiMeBzd and AHC. In contrast, AHC were the major metabolites of those from the DiMxBzd-based dyes; appreciable quantities of DiAcDiMxBzd

Table III. EC/GC Assays of Three Metabolites and AHC in Urine from Rats Given a Single Dose (2 mg) of Dye Indicated

	Metabolites (μg) Excreted (x ± SD)*					
	Pretreatment (hr)	Post-treatment Interval (hr)				Congener Excreted as Metabolite**
Metabolite	−36 to 0	0 to 12	12 to 24	24 to 48	48 to 96	(μg-equiv.)
DIRECT RED 39						
DiMeBzd	0.12±0.02	0.98± 0.30	0.70±0.06	0.51±0.16	1.4 ±0.87	3.6
MoAcDiMeBzd	0.46±0.26	0.43± 0.09	1.1 ±0.30	0.62±0.05	ND	1.8
DiAcDiMeBzd	0.02±0.01	3.3 ± 1.5	4.4 ±1.1	2.2 ±0.30	0.58±0.19	7.6
AHC as DiMeBzd	0.13±0.01	0.68± 0.23	0.68±0.27	0.30±0.29	ND	1.7
Total DiMeBzd μg-equiv.	Nil	4.4	5.5	2.9	1.8	15.
DIRECT BLUE 14						
DiMeBzd	0.31±0.03	1.7 ± 0.10	0.66±0.07	0.76±0.28	3.4 ±1.6	6.5
MoAcDiMeBzd	0.17±0.06	4.5 ± 0.29	3.4 ±0.68	1.6 ±0.00	ND	7.9
DiAcDiMeBzd	0.06±0.02	6.6 ± 1.4	5.1 ±1.0	0.23±0.30	ND	8.6
AHC as DiMeBzd	0.07±0.02	1.2 ± 0.29	0.74±0.04	ND	ND	1.9
Total DiMeBzd μg-equiv.	Nil	11.	7.9	2.2	3.4	25.
DIRECT BLUE 53						
DiMeBzd	0.60±0.14	2.9 ± 0.25	1.3 ±0.72	0.58±0.60	ND	4.8
MoAcDiMeBzd	0.23±0.15	4.5 ± 0.32	1.2 ±0.44	1.1 ±0.55	ND	5.7
DiAcDiMeBzd	0.04±0.01	9.6 ± 0.80	3.4 ±0.78	0.50±0.45	ND	9.7
AHC as DiMeBzd	0.03±0.00	2.6 ± 0.89	0.62±0.11	0.08±0.13	ND	3.3
Total DiMeBzd μg-equiv.	Nil	16.	5.3	1.9	Nil	24.
DIRECT ORANGE 6						
DiMeBzd	0.58±0.13	3.1 ± 0.40	1.1 ±0.06	ND	ND	4.2
MoAcDiMeBzd	0.10±0.03	4.1 ± 0.29	3.8 ±0.86	0.94±0.04	ND	7.4
DiAcDiMeBzd	0.10±0.02	3.3 ± 1.4	10. ±4.8	ND	ND	9.6
AHC as DiMeBzd	0.06±0.01	2.5 ± 0.19	2.5 ±0.19	ND	ND	5.0
Total DiMeBzd μg-equiv.	Nil	11.	14.	0.78	Nil	26.
DIRECT BLUE 8						
DiMxBzd	0.08±0.01	9.3 ± 1.7	0.46±0.07	ND	ND	9.8
MoAcDiMxBzd	0.01±0.00	3.0 ± 0.4	0.07±0.01	ND	ND	2.7
DiAcDiMxBxd	0.13±0.03	10. ± 1.	2.5 ±0.4	1.7 ±1.2	ND	10.
AHC as DiMxBzd	0.07±0.01	11. ± 1.	2.5 ±0.2	0.86±0.30	ND	14.
Total DiMxBzd μg-equiv.	Nil	30.	4.9	2.2	Nil	50.

Table III. Continued

Metabolite	Metabolites (μg) Excreted (x±SD)* Pretreatment (hr) −36 to 0	Post-treatment Interval (hr) 0 to 12	12 to 24	24 to 48	48 to 96	Congener Excreted as Metabolite** (μg-equiv.)
DIRECT BLACK 114						
DiMxBzd	0.10±0.02	6.1 ± 1.7	1.0 ± 0.2	ND	ND	7.1
MoAcDiMxBzd	0.03±0.00	8.1 ± 2.4	2.1 ± 0.3	ND	ND	8.7
Di9AcDiMxBzd	0.03±0.01	14. ± 3.	4.8 ± 0.5	0.86±0.20	ND	14.0
AHC as DiMxBzd	0.05±0.01	14. ± 2.	5.4 ± 0.6	0.70±0.24	ND	20.
Total DiMxBzd μg-equiv.	Nil	37.	12.	1.3	Nil	50.
DIRECT BLUE 10						
DiMxBzd	0.19±0.03	10. ± 6.	0.68±0.25	ND	ND	11.
MoAcDiMxBzd	0.07±0.02	4.7 ± 1.1	0.96±0.22	ND	ND	4.8
DiAcDiMxBzd	0.06±0.02	7.5 ± 1.5	5.6 ±1.3	ND	ND	9.8
AHC as DiMxBzd	0.01±0.00	20. ± 5.	4.8 ±1.3	ND	ND	25.
Total DiMxBzd μg-equiv.	Nil	40.	11.	Nil	Nil	51.
DIRECT VIOLET 32						
DiMxBzd	0.09±0.02	8.6 ± 3.2	4.5 ±1.6	0.33±0.05	ND	13.
MoAcDiMxBzd	0.25±0.02	4.2 ± 1.4	3.3 ±1.6	ND	ND	6.4
DiAcDiMxBzd	0.08±0.05	7.3 ± 2.3	5.8 ±1.6	ND	ND	10.
AHC as DiMxBzd	0.02±0.00	23. ±10.	15. ±5.	ND	ND	38.
Total DiMxBzd μ-equiv.	Nil	41.	27.	0.7	Nil	68.
DIRECT RED 46						
DiClBzd	0.06±0.00	0.65 ± 0.07	1.5 ±0.4	1.2 ±0.1	ND	3.4
MoAcDiClBzd	0.11±0.02	1.0 ± 0.2	3.3 ±0.9	1.6 ±0.3	ND	5.1
DiAcDiClBzd	0.04±0.01	1.3 ± 0.3	3.6 ±1.2	ND	ND	3.7
AHC as DiClBzd	0.03±0.01	0.87 ± 0.33	2.3 ±0.7	1.9 ±0.4	ND	5.1
Total DiClBzd μg-equiv.	Nil	3.4	9.3	4.5	Nil	17.

*Mean and SD from three groups of four rats. Results were corrected for background and recovery.
**Mean total amount of metabolites from three groups of four rats expressed as the corresponding amine congener.
ND = No residues detected above pretreatment levels

and DiMxBzd were observed followed by lesser amounts of MoAcDiMxBzd. These results are similar to those reported for ^{14}C-Direct Red 2 (DiMeBzd-based) and ^{14}C-Direct Blue 15 (DiMxBzd-based) (8).

The data from Table III were used to calculate the total amount of congener (μg) found in the urine of 12 rats for each dye and the results are reported in Table IV. These values were then related to the amounts of the corresponding congeners (μg) theoretically available from the doses of dye (24 mg/12 rats) administered and the results were expressed as the percentage of dose converted to the congener (Table IV). An examination of these values, which ranged from 0.62% for Direct Red 39 to 2.6% for Direct Violet 32, indicated that only small quantities of the congeners potentially available from their respective dyes could be found in the urine by the methods employed. These data provide conclusive evidence that all of the dyes were metabolized; small contributions of congeners dosed as impurities in the dyes (0.002-0.50 μg) are also reported in Table IV. It should also be pointed out that the dyes whose metabolites peaked 12 to 24 hours after dosing (*i.e.* Direct Red 39, Direct Orange 6 and Direct Red 46) were least converted to their respective congeners (*i.e.*, 0.62%, 0.95% and 0.81%, respectively) in contrast with the other six dyes that ranged from 1.4 to 2.6%.

This experiment was specifically designed to determine whether the nine dyes were sufficiently metabolized in rats to yield detectable levels of benzidine-congener metabolites in the urine. Although this objective was met, the methodology employed provided little information concerning the extent of absorption, metabolism and distribution of the dyes. However, it has been reported (8,11) that rats dosed with ^{14}C-Direct Red 2 (DiMeBzd-based) or ^{14}C-Direct Blue 15 (DiMxBzd-based) excreted 19 to 21% of the administered dose (2 mg) in the urine and 73 to 74% in the feces; 10 to 12% of the dose in feces was intact dye. In these experiments with ^{14}C-dyes, assays for the free congener amine, MoAc, DiAc and AHC metabolites were also performed as described in the present study. Treatment of data from the ^{14}C-dyes in the manner presented in Table IV indicated that a total of 0.6 and 1.3% of the benzidine-congener moieties available from the dosed dyes were found as the specified metabolites with ^{14}C-Direct Red 2 and ^{14}C-Direct Blue 15, respectively. These results are comparable to those found for the eight DiMeBzd- and DiMxBzd- dyes reported in the pre-

Table IV. Benzidine Congeners in Urine From Twelve Rats Each Given a Single Oral Dose (2 mg) of Dye Indicated

Direct Dye	Base	Congener (μg): Available from Dose*	Dosed as Impurity	Found in Urine	Dose Converted to Congener (%)
Red 39	DiMeBzd	7,200.	0.014	45.	0.62
Blue 14	DiMeBzd	5,300.	0.50	75.	1.4
Blue 53	DiMeBzd	5,300.	0.051	72.	1.4
Orange 6	DiMeBzd	8,200.	0.012	78.	0.95
Blue 8	DiMxBzd	7,700.	0.005	110.	1.4
Black 114	DiMxBzd	8,700.	0.018	150.	1.7
Blue 10	DiMxBzd	5,900.	0.002	150.	2.5
Violet 32	DiMxBzd	7,700.	0.004	200.	2.6
Red 46	DiClBzd	6,300.	0.210	51.	0.81

*Based on complete reduction of dye to its corresponding free amine.

sent study, where DiMxBzd-based dyes generally yielded larger amounts of metabolites measurable by the EC/GC procedures than did the DiMeBzd-based compounds. Although unlabeled dyes were used in the present experiment and only a small percentage of the metabolites were detected, their complete metabolic profiles may be expected to parallel those of ^{14}C-Direct Red 2 and ^{14}C-Direct Blue 15 (8).

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