METABOLISM OF AZO DYES TO CARCINOGENIC AMINES

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ABSTRACT

The metabolism of the purified benzidine-based azo dye Direct Black 38 (DB-38) and the 3-3'dichlorobenzidine-based Pigment Yellow 12 (PY-12) was studied in the hamster. A single oral dose of DB-38 containing 3.0 ppm benzidine (Bzd), 6.0 ppm 4-aminobiphenyl (4-ABP) and 670 ppm of 2,4-diaminoazobenzene (DiAmAzBz) was administered at 100 mg/kg to 18 male Syrian golden hamsters. Urine specimens collected over a period of 8 days and analyzed by electron capture-gas chromatography and high pressure liquid chromatography showed significant total amounts of Bzd (10 µg), monoacetylbenzidine (MoAcBzd, 535 µg), diacetylbenzidine (DiAcBzd, 28 µg) and 4-ABP (11 µg). Levels of metabolites peaked at 8-16 hours with MoAcBzd, the major metabolite, still quantitated after 7 days. In addition, alkaline hydrolyzable conjugates of Bzd (328 µg) and 4-ABP (613 µg) were found. The level of excreted metabolites far exceeded the levels of Bzd and 4-ABP present as impurities in the dye and represent metabolic breakdown of the dye. Mutagenic potential of DB-38 and the major metabolites evaluated with the Ames Salmonella test indicated: MoAcBzd and DiAcBzd - strong (TA 1538); 4-ABP - moderate (TA 98 and 100); DB-38, Bzd, DiAmAzBz - weak (TA 100); hamster urine, 8-16 hours containing 26 µg MoAcBzd - moderate (TA 1538). No compounds were mutagenic in the absence of S-9 fraction.

In contrast to DB-38, studies with PY-12 (100 mg/kg) did not produce any detectable levels of the hypothetical metabolites indicating either no metabolism or little absorption of the pigment.

ABBREVIATIONS

DB-38, Direct Black 38; PY-12, Pigment Yellow 12; Bzd, benzidine; 4-ABP, 4-aminobiphenyl; DiAmAzBz, 2,4-diaminoazobenzene; MoAcBzd, monoacetylbenzidine; DiAcBzd, diacetylbenzidine; EC-GC, electron-capture gas chromatography; HFB, heptafluorobutyryl; HPLC, high pressure liquid chromatography.

ACKNOWLEDGMENT:

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Epidemiological investigations of workers in the dye industry have shown an increase in bladder tumors over that expected in age-adjusted cohort populations (1,2). The benzidine-based dyes represent one class of dyes and include many of those most commonly used.

Benzidine (Bzd), a structural component of these dyes, is also an impurity and is a known human bladder carcinogen (3). The hypothesis that the dyes may be metabolized back to Bzd prompted studies on the carcinogenicity and metabolism of these dyes.

Studies sponsored by the National Cancer Institute have shown that Direct Black 38, Direct Brown 95, and Direct Blue 6 produced liver tumors in rats as early as five weeks after continuous dosing (4). Urine specimens contained Bzd in the parts per billion (ppb) range. Okajima, et al., found that Direct Black 38 (DB-38) produced bladder, liver, and colon tumors in 46% of male rats given 500 parts per million (ppm) of the dye in drinking water for 60 weeks (5). Bzd was not detected in urine specimens.

The metabolism of DB-38, Direct Brown 95, Direct Blue 6, and Direct Red 28 was studied by Rinde and Troll (6). When single oral doses of the dyes were given to Rhesus mønkeys, Bzd and monoacetylbenzidine (MoAcBzd) were found in the urine. It is known that these commercial dyes contain many impurities, including Bzd.

The discovery that these dyes were carcinogenic in rats, together with the knowledge that they may be metabolized to Bzd in animals and humans, has lead to the recommendation by the National Institute for Occupational Safety and Health that workers no longer be subject to the adverse health effects of these dyes and that these dyes no longer be used (7,8).

Other concerns have been raised on the potential metabolic fate of the dichlorobenzidine-based pigment, Pigment Yellow 12 (PY-12) (9). Previous research on the metabolism and carcinogenesis of benzidine-based azo dyes has been done with commercial dyes containing unspecified impurities. The question of the origin of Bzd in the urine of animals dosed with these dyes has not been answered. This report describes a definitive metabolism study in the hamster of DB-38 containing defined levels of amine impurities. Specific methodologies were developed and used to identify and quantitate dye metabolites in urine. Selected metabolites and the dye were also evaluated for mutagenic potential. Preliminary metabolism studies were also done with PY-12.

DB-38 was purchased from GAF Corporation, New York, N.Y. PY-12 was purchased from the Dry Color Manufacturers' Association, Nutley, N.J. Other chemicals were obtained from commercial sources or synthesized (9). Purification of the dye and pigment was conducted manually by exaustive liquid-liquid extraction. Amine impurities were converted to their heptafluorobutytyl (HFB) derivatives and analyzed by electron-capture gas chromatography (EC-GC). Stability and recovery studies of the dye and potential metabolites in urine were conducted prior to the dosing of animals. Details of these procedures are reported by Nony and Bowman (10).

Potential metabolites of the dye, Bzd, and the pigment were synthesized and HFB derivatives prepared. Structures were confirmed by gas chromatography-mass spectrometry. Urine metabolites were extracted, derivatized and analyzed by EC-GC using a 5% Dexil 300 on Anakrom Q glass column and a Ni^{63} detector. High pressure liquid chromatography (HPLC) was also used to analyze underivatized urine extracts using a reverse phase column (μ Bondapak C_{18}, Waters Associates, Milford, MA.) and an ultraviolet detector at 295 nm. Conjugated metabolites were first hydrolyzed with sodium hydroxide, then extracted and analyzed by EC-GC and HPLC. Details of the metabolite analysis are as reported by Nony and Bowman (11).

Male Syrian golden hamsters obtained from ARS Sprague-Dawley, Madison, WI., weighing between 104-128 g were housed three to a cage with a total of 18

animals (6 cages) used with the dye. Control urines were collected for 24 hours prior to dosing. Urine samples were collected at intervals of 0-8, 8-16, 16-24, 24-32, 32-48, 48-72, and 144-168 hours after dosing. All collections were done in the presence of dry ice. Hamsters received a single oral dose of DB-38 in water at 100 mg/kg body weight.

In a limited experiment, three hamsters were dosed orally with 100 mg/kg of PY-12 in trioctanoin. Urines were collected as above. Details of the experimental protocol are reported by Nony, et al. (12).

Mutagenicity of urinary metabolites was evaluated using the Ames Salmonella test with and without mouse liver microsomal metabolic activation (9).

RESULTS

The Dyes

Figure 1 shows the structures of DB-38 and related substances with their abbreviations. DB-38 was analyzed upon receipt and was found to contain traces of Bzd and significant amounts of 4-aminobiphenyl (4-ABP) and diamino-azobenzene (DiAmAzBz), both known carcinogens. The latter two compounds have not been previously reported as contaminants of azo dyes. Table I shows the levels of impurities before purification and immediately before use. Note that the levels of non-benzidine impurities were significantly reduced while the level of Bzd actually increased.

Figure 2 shows the structures of PY-12 and related substances along with their abbreviations. The pigment was analyzed and contained 89 ppm of 3,3'-dichlorobenzidine. After purification, the level of dichlorobenzidine was 0.3 ppm. No other amine impurities were detected.

Table I

Analysis of Direct Black 38

	Impurity, ppm		
	Benzidine	4-Aminobiphenyl	Diaminoazobenzene
As Received	<0.1	150	9,200
After Purification	3.0	6.0	670

Urinary Metabolites - Direct Black 38

Bzd (see Figure 1), monoacetylbenzidine (MoAcBzd), diacetylbenzidine (DiAcBzd), and 4-ABP were positively identified in the urine of hamsters fed DB-38. These compounds were analyzed by both HPLC and EC-GC of HFB derivatives. Table II shows the results of HPLC analysis of urine from hamsters given DB-38. Bzd excretion peaked at 0-8 hours but fell to control levels by 48 hours. MoAcBzd also peaked at 0-8 hours, but was 100 times more concentrated than Bzd. Its excretion returned to control levels after 168 hours. DiAcBzd and 4-ABP peaked at 16 and 8 hours, respectively, before returning to control levels by 24 hours. No DiAmAzBz was detected.

Table II

HPLC Analysis of Major Metabolites in Urine of Hamsters Fed One Dose (100 mg/kg) of Direct Black 38

Sampling Interval (hr)	Volume (ml) of Urine ($\bar{x}$ ± SD)[b/]	Total Amount Excreted (µg) of Indicated Metabolites ($\bar{x}$ ± SD)[a/]			
		Benzidine	Monoacetyl-Benzidine	Diacetyl-Benzidine	4-Aminobiphenyl
Pretreatment (24 hr)	9.1 ± 5.4	0.093 ± 0.014	0.142 ± 0.044	0.175 ± 0.030	0.473 ± 0.028
0- 8	2.3 ± 0.1	2.33 ± 2.16	196. ± 107.	2.78 ± 1.68	4.28 ± 2.41
8- 16	3.7 ± 3.2	1.89 ± 2.04	174. ± 170.	4.69 ± 1.34	3.75 ± 2.86
16- 24	2.7 ± 0.7	1.89 ± 2.19	98.7 ± 146.	2.39 ± 0.60	2.02 ± 1.01
24- 32	1.9 ± 0.6	0.258 ± 0.000	6.07 ± 4.80	ND	ND
32- 48	6.2 ± 2.6	0.524 ± 0.000	6.17 ± 6.73	ND	ND
48- 72	12. ± 7.8	ND	1.36 ± 1.08	ND	ND
144-168	8.5 ± 1.3	ND	0.710 ± 0.188	ND	ND

a/ Mean and standard deviation from five cages of three hamsters each. Results were corrected for pretreatment sample background and recovery.

b/ Mean and standard deviation from five cages of three hamsters each.

ND None detected above background.

Figure 3 shows an EC-GC chromatogram from urine collected 8-16 hours after dosing with DB-38. The chromatogram clearly shows 4-ABP (1.4 ppm), Bzd (1.7 ppm), and MoAcBzd (78.7 ppm). DiAcBzd and the alkaline hydrolyzable conjugates of Bzd and 4-ABP were analyzed following hydrolysis to free amine metabolites as previously described (9).

Table III shows the composite results of an EC-GC analysis of hamster urine from dosed animals. The results are similar to those from HPLC analysis but, because of higher sensitivities, levels of metabolites have been detected at longer intervals after dosing. Table IV shows the results for alkaline hydrolyzable conjugates analyzed by EC-GC after pretreatment. It can be seen that Bzd conjugates are present at about 30 times the level of free Bzd but that 4-ABP conjugates are present at about the same levels as the free metabolites. No DiAmAzBz was found in hamster urine by either HPLC or EC-GC.

Urinary Metabolites - Pigment Yellow 12

The expected metabolites of PY-12 shown in Figure 2 were not detected in the urine of hamsters dosed with PY-12. The expected metabolites were chemically synthesized, spiked into control urines and analyzed at the ppb level by EC-GC. However, no traces of these expected metabolites were found in dosed hamster urine.

Table III

EC-GC Analysis of Major Metabolites in Urine of Hamsters Fed Direct Black 38

Sampling Interval (hr)	Volume (ml) of Urine ($\bar{x}$ ± SD)[b/]	Total Amount Excreted (µg) of Indicated Metabolites ($\bar{x}$ ± SD)[a/]			
		Benzidine	Monoacetyl-Benzidine	Diacetyl-Benzidine	4-Aminobiphenyl
Pretreatment (24 hr)	9.1 ± 5.4	0.151 ± 0.217	0.361 ± 0.103	0.087 ± 0.061	0.152 ± 0.155
0- 8	2.3 ± 0.1	4.33 ± 2.39	216. ± 133.	10.7 ± 7.1	6.62 ± 1.72
8- 16	3.7 ± 3.2	3.08 ± 2.44	208. ± 177.	10.3 ± 2.5	3.26 ± 3.02
16- 24	2.7 ± 0.7	2.16 ± 2.33	95.8 ± 133.	5.69 ± 5.11	1.17 ± 1.52
24- 32	1.9 ± 0.6	0.259 ± 0.205	5.59 ± 4.88	0.064 ± 0.063	0.162 ± 0.084
32- 48	6.2 ± 2.6	0.470 ± 0.506	7.39 ± 7.82	0.223 ± 0.212	0.236 ± 0.078
48- 72	12. ± 7.8	0.226 ± 0.184	1.56 ± 0.994	0.626 ± 0.000	ND
144-168	8.5 ± 1.3	ND	0.474 ± 0.393	ND	ND

a/ Mean and standard deviation from five cages of three hamsters each. Results were corrected for pretreatment sample background and recovery.

b/ Mean and standard deviation from five cages of three hamsters each.

ND None detected above background.

Table IV

EC-GC Analysis of Alkaline Hydrolyzable Conjugates in the Urine of Hamsters Fed Direct Black 38

	Total Amount Excreted (µg)	
Sampling Interval (hr.)	Benzidine	4-Aminobiphenyl
Pretreatment (24 hrs.)	0.034	1.16
0- 8	103.	2.57
8- 16	154.	2.56
16- 24	45.5	ND
24- 32	5.59	ND
32- 48	13.9	ND
48- 72	6.41	ND
144-168	0.019	ND

ND - None detected above background.

Mutagenicity Testing

The mutagenic potential of the identified metabolites of DB-38 is shown in Table V. The purified synthetic metabolites were assayed with and without S-9 fraction. None of the compounds tested were mutagenic without activation. All compounds showed some degree of mutagenic activity in the presence of S-9 fraction with at least one of the tester strains. The degree of mutagenicity based on the number of revertants per plate were: MoAcBzd and DiAcBzd - strong; 4-ABP - moderate; Bzd and DiAmAzBz - weak. Mutagenic evaluation of DB-38 hamster

urine collected at pretreatment, 0-8 and 8-16 hours after dosing, showed no mutagenic activity without activation but mutagenic activity up to 10 times background in the 0-8 hour urine with activation. These results are consistent with the level of MoAcBzd in the urine, the major metabolite.

Table V

Mutagenic Potential of Direct Black 38 and Metabolites

Compound	Tester Strain	Mutagenic Response -S9	Mutagenic Response +S9
Direct Black 38	TA 98	-	-
	TA 100	-	+
Benzidine	TA 98	-	+
	TA 100	-	+
Monoacetylbenzidine	TA 1538	-	+++
Diacetylbenzidine	TA 1538	-	+++
4-Aminobiphenyl	TA 98	-	++
	TA 100	-	++
Diaminoazobenzene	TA 98	-	+
	TA 100	-	+

\- Negative
\+ Weak (2 x background)
++ Moderate (5 - 10 x background)
+++ Strong (40 x background)

CONCLUSIONS

Purified DB-38, containing low defined levels of free Bzd as an impurity, was extensively metabolized to Bzd, the N-acetylbenzidine metabolites and to

unspecified Bzd conjugates in hamsters. About 8-10% of the Bzd contained in the dye in azo linkage could be accounted for in the urine as Bzd or its metabolites. In addition, 4-ABP was found in amounts much greater than the level of impurity, along with its conjugates. These findings indicate that considerable risk may exist for humans exposed to DB-38 since both Bzd and 4-ABP are well established bladder carcinogens. In addition, MoAcBzd, the major metabolite, was found to be mutagenic with activation using the Ames Salmonella test.

In contrast, PY-12 did not produce detectable levels of expected metabolites, indicating either no metabolism or little absorption of the pigment.

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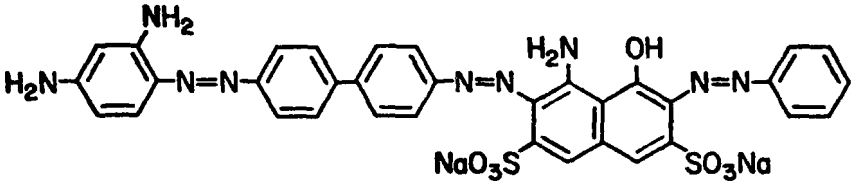
FIGURE LEGENDS

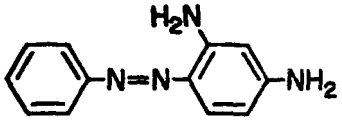
Figure 1 - Direct Black 38 and Related Compounds.

Figure 2 - Pigment Yellow 12 and Related Compounds.

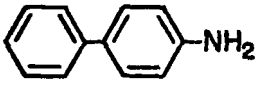
Figure 3 - Electron-Capture Gas Chromatograms of Derivatized Extracts of Urine Collected From Hamsters 8-16 Hours After Treatment With Direct Black 38.



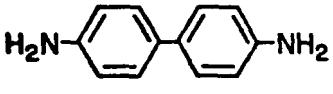
C.I. Direct Black 38



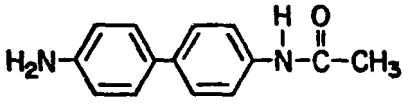
Diaminoazobenzene
(DiAmAzBz)



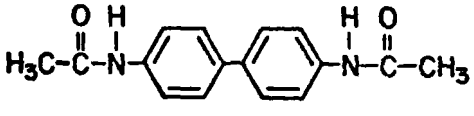
4-Aminobiphenyl
(4-ABP)



Benzidine
(Bzd)

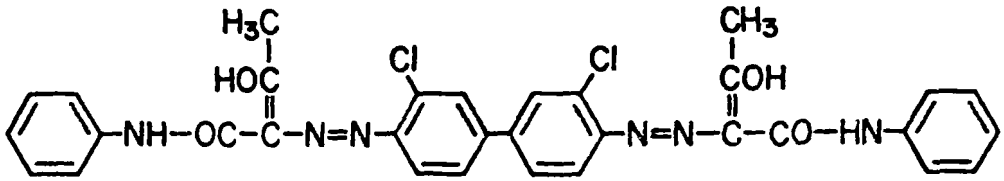


Monoacetylbenzidine
(MoAcBzd)

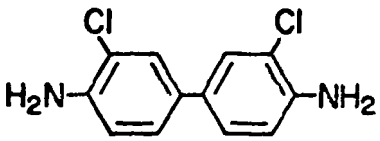


Diacetylbenzidine
(DiAcBzd)

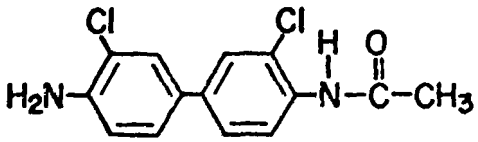
Figure 1



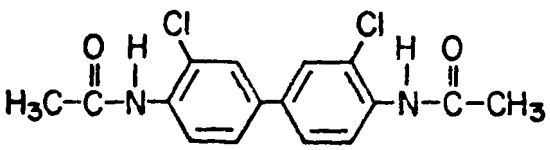
C.I. Pigment Yellow 12



3,3'-Dichlorobenzidine
(DiClBzd)



Monoacetyldichlorobenzidine
(MoAcDiClBzd)



Diacetyldichlorobenzidine
(DiAcDiClBzd)

Figure 2

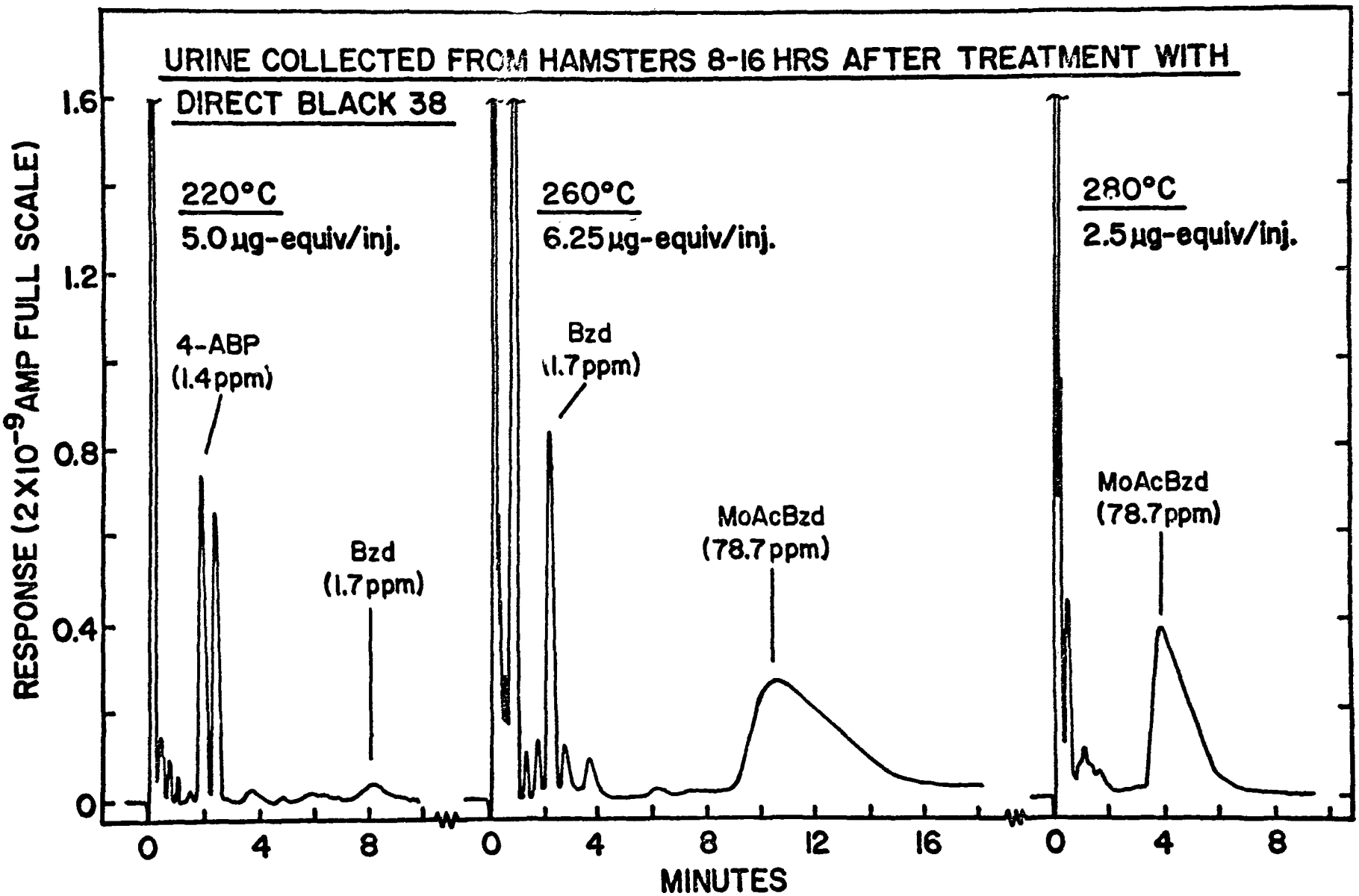

URINE COLLECTED FROM HAMSTERS 8-16 HRS AFTER TREATMENT WITH
DIRECT BLACK 38
220°C
5.0 µg-equiv/inj.
260°C
6.25 µg-equiv/inj.
280°C
2.5 µg-equiv/inj.
4-ABP
(1.4 ppm)
Bzd
(1.7 ppm)
Bzd
(1.7 ppm)
MoAcBzd
(78.7 ppm)
MoAcBzd
(78.7 ppm)
RESPONSE (2X10⁻⁹ AMP FULL SCALE)
1.6
1.2
0.8
0.4
0
0
4
8
0
4
8
12
16
0
4
8
MINUTES

Figure 3

Dr. Jenkins, EPA: I would like to ask one question about the problems you would have in the HPLC analysis of benzidine and I believe you said that you converted that to the appropriate derivative. The reason for my asking would be what other types of compounds would interfere, come out at about the same peak, and what you are converting that to? I am interested in the congeners.

Dr. Lowry, NIOSH: The derivative you mentioned was a heptafluorobutyryl anhydride reaction with an aromatic amine to produce a fluoroelectron capture sensitive group that would be picked up with the electron capture detector of the gas chromatograph.

The HPLC analysis of benzidine was done without derivatization using a reverse phase C_{18} column. The congeners of benzidine are separated from benzidine under these conditions. More details of the methodology can be found in the paper written by C. R. Nony and M. C. Bowman published in the February issue of the Journal of Chromatographic Science (Vol. 18, pages 64-75, 1980).

Dr. Cooper, NCI: It was curious that on the benzidine results in the minus 24 to zero time period you showed excretion of benzidine and yet it fell later to undetectable levels. Where was that benzidine coming from?

Dr. Lowry, NIOSH: It is possible you could call that noise.

Dr. Hegyeli, NCI: Was inhalation considered in this case as most of the workers working with these types of chemicals were inhaling instead of ingesting them.

Dr. Lowry, NIOSH: Inhalation was not considered primarily for reasons of getting the work done with the amount of money that was available to support the work. Further work is being done on two congeners of the benzidine-based dyes, one toludene- based dye, Direct Red 2 and one dianicidine-based dye, Direct Blue 15. NCTR is about to start some work using some radio labeled material to look more thoroughly at metabolism, absorption of the material, and tissue distribution.

NIOSH

PROCEEDINGS OF THE
FIRST NCI/EPA/NIOSH COLLABORATIVE WORKSHOP:
PROGRESS ON JOINT ENVIRONMENTAL AND
OCCUPATIONAL CANCER STUDIES

MAY 6-8, 1980

SHERATON/POTOMAC, ROCKVILLE, MARYLAND

The papers included in these Proceedings were printed as they were submitted to this office.

Appropriate portions of the discussions, working groups and plenary session were sent to the participants for editing. The style of editing varied, as could be expected. To the extent possible, we have attempted to arrive at a consistent format.

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Proceedings were developed from a workshop on the National Cancer Institute's, the Environmental Protection Agency's and the National Institute for Occupational Safety and Health's Collaborative Programs on Environmental and Occupational Carcinogenesis.

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