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Toxicity of an Anthraquinone Violet Dye Mixture Following Inhalation Exposure, Intratracheal Instillation, or Gavage[1]

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Anthraquinone dyes are utilized by the military in colored-smoke grenades. During production, workers in munitions plants may be exposed to fugitive emissions of these dyes or mixtures thereof. The effects of a prototype violet dye mixture (VDM) consisting of Disperse Red 11 (DR11), [1,4-diamino-2-methoxy-anthraquinone] and Disperse Blue 3 (DB3) [1-methylamino-4-hydroxyethylamino-anthraquinone] on F344 male and female rats have been investigated. Acute 1-day inhalation exposures (6 hr) to VDM were conducted at 1000, 300, 100, 70, 40, and 10 mg/m^3, with an additional exposure to 40 mg/m^3 6 hr/day for 5 days; 4.22 ± 2.1 μm (MMAD ± δg). Lung burdens of dye, general histopathology, and/or liver function were evaluated at 0, 3, and 7 days postexposure. Unexpected lethality due to severe liver damage was observed with acute exposures of ≥300 mg/m^3 and in the 5-day 40 mg/m^3 exposures. Centrilobular degeneration and necrosis of liver cells was concentration-dependent with inhalation of VDM ≥40 mg/m^3. In addition, nasal olfactory epithelium exhibited degeneration and necrosis with acute exposures ≥10 mg/m^3. Lung instillations at 250, 500, and 1000 μg of the VDM revealed no lung or liver toxicity. Because per os exposure due to preening was suspected as a major exposure route, a gavage study with the VDM and its two component dyes DR11 and DB3 (800 mg/kg) was undertaken. One day following gavage with DR11 or DB3, serum enzymes indicative of liver toxicity (*LDH*, *SGPT*, *SDH*, and *ICDH*) were slightly elevated (1–6× control). However, rats gavaged with VDM had serum enzyme levels 10–100× control by Day 1 after gavage, indicating acute liver toxicity. Activities of liver enzymes involved in xenobiotic and glutathione metabolism were also acutely affected. All of the dyes caused various degrees of induction of glucose-6-phosphate dehydrogenase, glutathione reductase, glutathione peroxidase, and nonprotein sulfhydryls. The enzymes involved in xenobiotic metabolism (glutathione *S*-transferase, NADPH cytochrome-c reductase, and P450) were also elevated by the two component dyes, in contrast to their significant depression with VDM treatment. The similarity between the liver and olfactory epithelium effects of these compounds and the lack of pulmonary tissue effects is not fully understood, but the interaction of the individual dyes as VDM emphasizes the need to assess chemicals such as the anthraquinones as their likely-to-be-encountered mixtures.

Anthraquinones are a diverse group of naturally occurring and synthetic chemical compounds used widely in industry as colorants in foods, drugs, cosmetics, hair dyes, and textiles and in medicine as purgative, antimicrobial, and antitumor preparations (Sendelbach, 1989). In general, little information is available concerning the health risks of most anthraquinones, and human toxicity data are lacking except for a few clinical reports on allergic contact dermatitis (NIOSH, 1981; Hatch, 1984).

The U.S. Army utilizes various anthraquinone dyes in colored-smoke grenades for marking, signaling, and identification of sites in the field. The dye is purchased from commercial suppliers and is formulated with a strong oxidant and other combustion enhancers into combustible mixtures within grenades in what are described as load, assembly, and pack (LAP) plants. The nature of these dye compounds makes full containment difficult in grenade manufacture and hence incidental worker exposure is virtually unavoidable; contact may be via inhalation or dermal exposure. Additional concern is raised by the potential exposure of ground troops during training and field operations.

Inhalation toxicity studies on two dyes similarly used in colored-smoke munitions, Solvent Yellow 33 (a quinoline dye) and Solvent Green 3 (an anthraquinone dye), have previously been reported (Sun *et al.*, 1987). Both the yellow dye and yellow/green dye mixture exhibited low toxicity, although the yellow/green mix appeared slightly more toxic than the yellow dye alone, suggesting some synergistic in-

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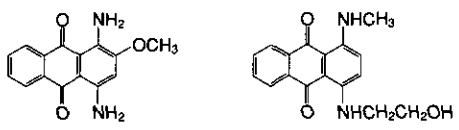
teraction between the two dyes. Another dye mixture, a prototype violet dye, with advantageous physical and chemical properties for use in smoke munitions, was proposed for widespread use and was evaluated in the present study. This binary mixture consisted of two pure anthraquinone dyes, 92.9% Disperse Red 11 (DR11) [1,4-diamino-2-methoxy-anthraquinone] and 7.1% Disperse Blue 3 (DB3) [1-methylamino-4-hydroxyethylamino-anthraquinone] (Fig. 1). The only available toxicity data on these components indicated that the acute oral LD50 values was in the range of 2–3 g/kg (Material Data Safety Sheet: DR11, 86-0608, DB3; 86-0056) and, in the case of violet dye mixture (VDM), 794–1000 mg/kg and 1413–1778 mg/kg for undefined male and female rats, respectively (J. Eaton, USAMRDC, personal communication). The comparability of these LD50 assays for the component and mixed dyes, and hence the comparability of the LD50 values themselves, is questionable. Inhalation data providing evidence of toxicity are not available.

The present study was designed to evaluate the biological impact of this prototype VDM on small laboratory animals in order to assess the potential health risks to humans incidentally exposed in the handling of the dye mixture in the workplace. This report presents the findings of several studies to characterize the toxicity of VDM in rats, including studies which involved exposures by inhalation (whole-body and nose-only), gavage, and intratracheal instillation of VDM and, in selected experiments, the component dyes (DR11 and DB3).

MATERIALS AND METHODS

Animals. Ninety-day-old, Fischer 344 SPF male and female rats (Charles River Laboratories, Raleigh, NC) were used throughout the study. The rats were received at 60 days of age and held for 30 days prior to exposure. The animals were housed by gender 3 per cage and maintained under conditions of constant temperature (72 ± 2°F), humidity (50 ± 10%), and lighting (12:12 light:dark cycle) until they were exposed. Purina Rat Chow 5001 and water were provided *ad libitum* with only water available during exposures. Each study group (concentration) consisted of 18 to 32 rats evenly divided by gender unless otherwise noted in the text or tables. Because the concentration–response studies did not reveal gender-based susceptibility, follow-up studies used only male rats in an attempt to minimize variability in the findings.

VDM aerosol exposure system. A detailed description of the aerosol exposure system, including aerosol generation and monitoring, chamber distribution, and particle characterization, is presented elsewhere (Higuchi and Steinhagen, 1991; Higuchi and Davies, 1990). Briefly, the aerosol exposure system for generation of VDM consisted of modified dry material feeder (AccuRate, Model 106), jet grinding mill (Jet-o-Mizer, Model 0101), single-stage impactor, aerosol neutralizers, and sound-insulating foam. The powder feeder delivered the dye through a venturi on the jet mill grinding chamber, where two high-speed air jets ground the bulk powder material into the finer particles. The ground dye exited the jet mill and entered a single-stage impactor where the aerosol stream was neutralized to eliminate electrostatic charge on the dye particles, mixed with humidified dilution air, and passed through a tubular section of sound-dampening foam, to reduce both high- and low-frequency noise created by the jet mill, before entering the inhalation chambers.



Disperse Red 11 **Disperse Blue 3**

FIG. 1. Chemical structure of Disperse Red 11 (1,4-diamino-2-methoxy-anthraquinone) and Disperse Blue 3 (1-methylamino-4-hydroxyethylamino-anthraquinone).

Four 2-m^3 stainless steel/glass exposure chambers (Hazleton-2000) were used to conduct three concentration and one clear air control exposures simultaneously. The inhalation chambers were monitored continuously for temperature (72 ± 2°F) and relative humidity (60 ± 20%). Additional monitoring included chamber air flow (at least 15 air changes/hr), contaminant flow rate (mg/min), and aerosol concentration (RAM-S; GCA Corp., Bedford, MA). Only one centrally located tier was used in each chamber to hold two, 16-unit (rat) modules to optimize aerosol distribution across all individual animal cages (<12% variation across 12 sampling sites). Actual aerosol concentration for each study exposure was determined each hour gravimetrically by collection of aerosol at 1.0 liters/min on type-A/E, glass-fiber filters (Gelman Sciences, Ann Arbor, MI) just above the holding cages. Once each exposure run, particle size distribution was determined using a seven-stage cascade impactor (Anderson, Atlanta, GA). Single, acute inhalation exposures (6 hr) to VDM were nominally 1000, 300, 100, 70, 40, and 10 mg/m^3 with an additional exposure regime of 40 mg/m^3, 6 hr/day for 5 days. The range of MMADs for the concentration–response studies was 3.9–5.4 μm. Actual exposure concentrations and particle size determinations for each of the studies are reported in Table 1. The 5-day exposure to 40 mg/m^3 exhibited a daily mean (±SD) concentration of 38 ± 2 mg/m^3 with a particle size distribution of 3.0 ± 2.1 μm (MMAD ± σg).

Nose-only exposures were conducted with 20 rats/group restrained in whole-body/nose-protruding plexiglass tubes (sized for 250-g rats) placed inside the H-2000 chambers on top of the rat-holding cages and exposed to 300 mg/m^3 (303 ± 33 mg/m^3; MMAD 4.7 ± 2.0 μm) of VDM for 6 hr. This approach was taken to avoid or minimize the contamination of the rat fur with the VDM aerosol.

Lung instillation. Rats (18 males/group) were dosed via intratracheal instillation with 0.25 ml of the VDM as an aqueous suspension in saline. The low dose (250 μg) was chosen to be 10× greater than the burden of dye found in the lungs of rats immediately following 300 mg/m^3 6-hr inhalation exposure (~25 μg). The other two doses were 2× and 4× this low dose to maximize the chance of observing a toxic effect (500 and 1000 μg); saline sham served as the vehicle control. Time points chosen for observation and analysis were 1, 3, and 7 days postinstillation (6 rats/group). Prior to instillation, the syringe containing the dye suspension was vigorously shaken with a test tube vortex. A 0.5-ml bolus of air which was drawn into the syringe above the instillate fluid prior to vortexing to ensure complete delivery of the material. Previously, we have shown that particles can be reproducibly delivered (93.8 ± 2.0% recovery) using this method (Costa *et al.*, 1986).

Gavage methods. The VDM and the two component dyes (DR11 and DB3) were each suspended in a 25% propylene glycol (PEG)/water solution. PEG was used to ensure dispersal of the particles within the aqueous vehicle and aid in dye absorption since administration would be in the form of a one-time bolus (2 ml) in contrast to the ~24 hr administration via preening. The gavage dose was 800 mg/kg for each of the component dyes and VDM. This dose was based on a calculated estimate of maximal body surface deposition occurring during whole-body exposure to 100 mg/m^3 for 6 hr with subsequent ingestion by preening. The basic assumption for this calculation was that the entire body fur surface of the rat lay exposed cross-sectionally to the particles passing through the chamber cross-

section at 15 air changes/hr for 6 hr. Time points of 1, 3, and 7 days postgavage were chosen for observation and analysis.

Tissue preparation. Rats were anesthetized with Nembutal (50 mg/kg). Serum was obtained by removing 5 ml of blood via the abdominal aorta. The blood was allowed to clot for 30 min and was then centrifuged. The serum was frozen at −20°C for subsequent determination of clinical chemistry endpoints. After exsanguination by cutting the abdominal aorta, the livers and lungs were removed and weighed, and a 0.25-g section from each was excised and homogenized in 3.0 ml of 6% metaphosphoric acid to precipitate tissue protein. The homogenates were centrifuged at 14,000*g* for 20 min at 4°C and the supernatants were decanted and stored at −80°C for determination of whole liver and lung non-protein-sulfhydryl content (NPSH). The remaining liver and lung were homogenized in 50 mM Tris–HCl, 1.15% KCl, pH 7.4, with a weight-to-volume ratio of 1:3 and 1:7, respectively. The homogenates were centrifuged at 20,000*g* for 20 min at 4°C and the supernatants were decanted, recentrifuged at 100,000*g* for 1 hr at 4°C, and stored at −80°C for determination of glucose-6-phosphate dehydrogenase (G6PDH), glutathione reductase (GRD), glutathione peroxidase (GPX), glutathione S-transferase (GTR), and total protein. The microsomal pellets were resuspended in 50 mM Tris–HCl, 1.15% KCl, 25% glycerol, pH 7.4, and stored at −80°C for determination of NADPH cytochrome-c reductase (CYTOC) and cytochrome P450 (P450).

Clinical chemistry. All serum chemistries, alanine aminotransferase (ALT/SGPT), lactate dehydrogenase (LDH), sorbitol dehydrogenase (SDH), and isocitrate dehydrogenase (ICDH), were run on a Centrifichem System 500 centrifugal analyzer (Baker Instruments, Allentown, PA) using commercially prepared kits from Baker Instruments or Sigma Chemical Co. (St. Louis, MO).

Biochemical assays. The following biochemical assays were modified for liver and lung analysis on the Centrifichem System 500 centrifugal analyzer. The assay methods for total GPX and GRD have been reported elsewhere (Jaskot *et al.*, 1983). G6PDH assays were performed using 1.2 mM glucose 6-phosphate, 0.15 mM NADP, and 50 mM triethanolamine–HCl, pH 7.5, as modified from Mustafa *et al.* (1977). GTR assays were performed using 1-chloro-2,4-dinitrobenzene (CDNB) as substrate modified from Habig *et al.* (1974). The activity of CYTOC was assayed using horse heart cytochrome c as substrate as modified from Phillips and Langdon (1962). P450 was determined using a Cary 219 spectrophotometer by the method of Omura and Sato (1964). NPSH content was determined by a modification of the method of Sedlak and Lindsay (1968). The reagent contained 2.0 mM ethylenediaminetetraacetic acid and 0.21 mM 5,5′-dithiobis-2-nitrobenzoic acid (DTNB) in a 0.4 M Tris–HCl buffer, pH 8.9. Sample concentration of NPSH is determined from a standard curve. Protein concentration was determined using the Bio-Rad method (Bio-Rad Laboratories, Richmond, CA) for total protein determinations as modified for use on the centrifugal analyzer. The reagent contained a 1:5 dilution of the commercially prepared Bio-Rad dye reagent in deionized water and filtered prior to use. Sample protein concentration was determined from a standard curve using BSA standards.

Histopathology. All necropsies, histologic preparation of tissues, histopathologic evaluation of tissues, and photographs were provided by Experimental Pathology Labs, Inc., RTP, NC. Routine H + E-stained slides from paraffin-embedded tissues were prepared from the formalin-preserved nasal cavity (three regions), trachea, tracheobronchial lymph node, inflated lung, brain, stomach, duodenum, jejunum, ileum, cecum, colon, liver, spleen, and kidneys. All evaluations were done blind to the extent possible since most samples were tinted purple from the VDM exposures.

Lung burden and blood analysis. Lungs were removed from subgroups of the exposed rats (n = 2 to 6 rats/group as designated in Table 2), were dissected at the carina, and were homogenized in deionized water 1:4 (w:v) and frozen at −20°C until analysis. Frozen samples were thawed, diluted in 10 ml of DI water, and rehomogenized for 1 min (pulsed) using a Tekmar sonic disruptor. The samples were then extracted 3× with 10 ml of methylene chloride with each emulsion diluted to 50 ml and finally concentrated to 1 ml by evaporation under N_2. Each sample was diluted to 2 ml in acetonitrile and filtered through an Alltech PTFE (250 mm × 0.45-μm) syringe filter prior to HPLC analysis. The following conditions for HPLC analysis of the VDM were used: isocratic 95/5 acetonitrile/methylene chloride mobile phase with a flow rate of 1 ml/min; Whatman PAC column (250 mm × 5 mm i.d., 10-μm particle size), 254 nm uv detection, a 40-μl sample injection volume. Under these conditions it was possible to separate the sample components and to quantitate the peak containing the coeluting DR11 and DB3 components by manual integration. The sample values were then adjusted by recovery values (57.9%) previously determined from rat lung samples spiked with varying concentrations of the dyes. Analysis of selected blood samples for VDM was conducted similarly, but with blood diluted in water rather than lung tissue. Attempts to extract the VDM from urine were abandoned when methylene chloride failed, indicating that the colored compound was a water-soluble metabolite and we were not prepared to analyze it.

Statistical analysis. The data were analyzed using a two-way analysis of variance. The two factors were treatment (at various levels of exposure) and time postexposure (days). Significant interaction resulted in subtesting among levels of the variables examining the standardized differences in the least-square means. A p value of less than or equal to 0.01 indicated statistical significance due to multiple comparisons.

RESULTS

Inhalation

There was a trend for particle size to correlate with chamber aerosol concentration (i.e., particle size increased with concentration, Table 1). The reasons for this were likely due to the higher feed rate of the jet mill required to achieve the highest concentrations (300 and 1000 mg/m^3). This would result in less impact time for disruption of particle agglomerates during sonic dispersion as well as increase the chance of reagglomeration of these self-adherent particles in the inlet conduit to the chamber. However, at VDM levels of 100 mg/m^3 and less, particle sizes were stabilized.

Table 2 summarizes the results from the inhalation exposures with VDM. Immediately after the single 6-hr whole-body exposures, all rats showed fur deposition of VDM. As a general observation, the degree of coloration of the fur was concentration-dependent, with all rats exhibiting vigorous and persistent preening activity. By morning, the ani-

TABLE 1
Aerosol Exposure Characteristics

Nominal VDM concentration (mg/m^3)	Actual VDM concentration[a] (mg/m^3)	Particle size[b] (μm)
1000	933 ± 38	5.4 ± 2.1
300	321 ± 25	4.6 ± 2.1
100	105 ± 27	4.0 ± 2.3
70	71 ± 8	3.9 ± 2.1
40	43 ± 4	3.9 ± 2.2
10	13 ± 2	3.9 ± 2.1

[a] Means ± SD.
[b] MMAD ± σg.

TABLE 2
Summary: Inhalation Exposures

Violet dye mix (mg/m^3)[a]	Postexposure recovery	Histopathology	VDM lung burdens ($\mu g/g$ lung)
1000	All rats (18) died by Day 3 postexposure.	ND	Day 0, 57[d] Day 3, 78[d]
300	9/18 rats died by Day 3 postexposure. The remaining rats were moribund and hence killed.	Liver: Severe centrilobular degeneration and necrosis. Nasal: Degeneration and necrosis of olfactory epithelium.	Day 0, 25.5 ± 1.6[e] Blood, 4.61[d,f] Day 3, 18.9 ± 2.1[e] Blood, 2.09[d,f] (moribund or dead rats)
100	All rats lived (30) through the 3- and 7-day holding period.	Liver: (Day 3) Moderate degeneration and necrosis. (Day 7) Normal. Nasal: (Day 3) Degeneration, necrosis, and sloughing of olfactory epithelium. (Day 7) Slight degeneration present.	Day 0, 6.50 ± 1.03[e] Blood, 2.2 ± 0.26[d,f] Day 3, 0.90 ± 0.57[e] Blood, 0.2 ± 0.2[d,f] Day 7, 0.03 ± 0.01[e] Blood, <0.03[d,f]
70	All rats (18) lived through the 3- and 7-day holding period.	Liver: Normal. Nasal: (Day 3) Moderate degeneration and necrosis of olfactory epithelium. (Day 7) Slight degeneration.	Day 0, 3.00[d] Day 3, 0.30[d] Day 7, <0.03
40	All rats (18) lived through the 3- and 7-day holding period.	Liver: Normal. Nasal: (Day 3) Moderate degeneration and necrosis of olfactory epithelium. (Day 7) Minimal degeneration.	Day 0, 6.40[d] Day 3, 0.08[d] Day 7, 0.03[d]
10	16/18 rats lived through the 3- and 7-day holding period.	Liver: Normal. Nasal: (Day 3) Slight degeneration and necrosis of olfactory epithelium. (Day 7) Minimal degeneration.	Day 0, 1.40[d] Day 3, <0.03[d] Day 7, <0.03[d]
40[b]	16/32 rats were dead or moribund by Day 2 postexposure. The remaining rats survived to Day 7 postexposure.	ND	ND
300[c]	18/20 rats lived through the 3- and 7-day holding period.	Liver: Normal. Nasal: (Day 3) Degeneration, necrosis, and sloughing of olfactory epithelium. (Day 7) Slight degeneration present.	Day 0, 64.5 ± 16.4[e] Day 3, ND Day 7, ND

Note. ND, no data.
[a] Whole-body 6-hr exposure unless otherwise noted.
[b] Whole-body 5-day exposure, 6 hr/day.
[c] Nose-only 6-hr exposure.
[d] 2–3 rats.
[e] 6 rats.
[f] μg/ml whole blood.

mals were virtually clean with only a stripe of violet coloring on the cranial–dorsum area, presumably an area out of reach of the animals' preening strokes. Violet-colored fecal material was evident as was violet-colored urine. Rats exposed to the highest VDM concentrations (1000 and 300 mg/m^3) consistently began to deteriorate during Day 2 with most deaths occurring between Days 2 and 3. By Day 3, the mortality incidence at 1000 mg/m^3 was 18 dead/18 and at 300 mg/m^3 9 dead + 9 moribund/18. At lower concentrations, the violet coloring was qualitatively similar, but quantitatively less (by observation), with the physical condition of the rats not visibly affected.

Tissues of the moribund animals (n = 5; 300 mg/m^3 group) were prepared for histopathologic evaluation. Only severe centrilobular liver damage and necrosis of the nasal olfactory epithelium were apparent in the exposed animals

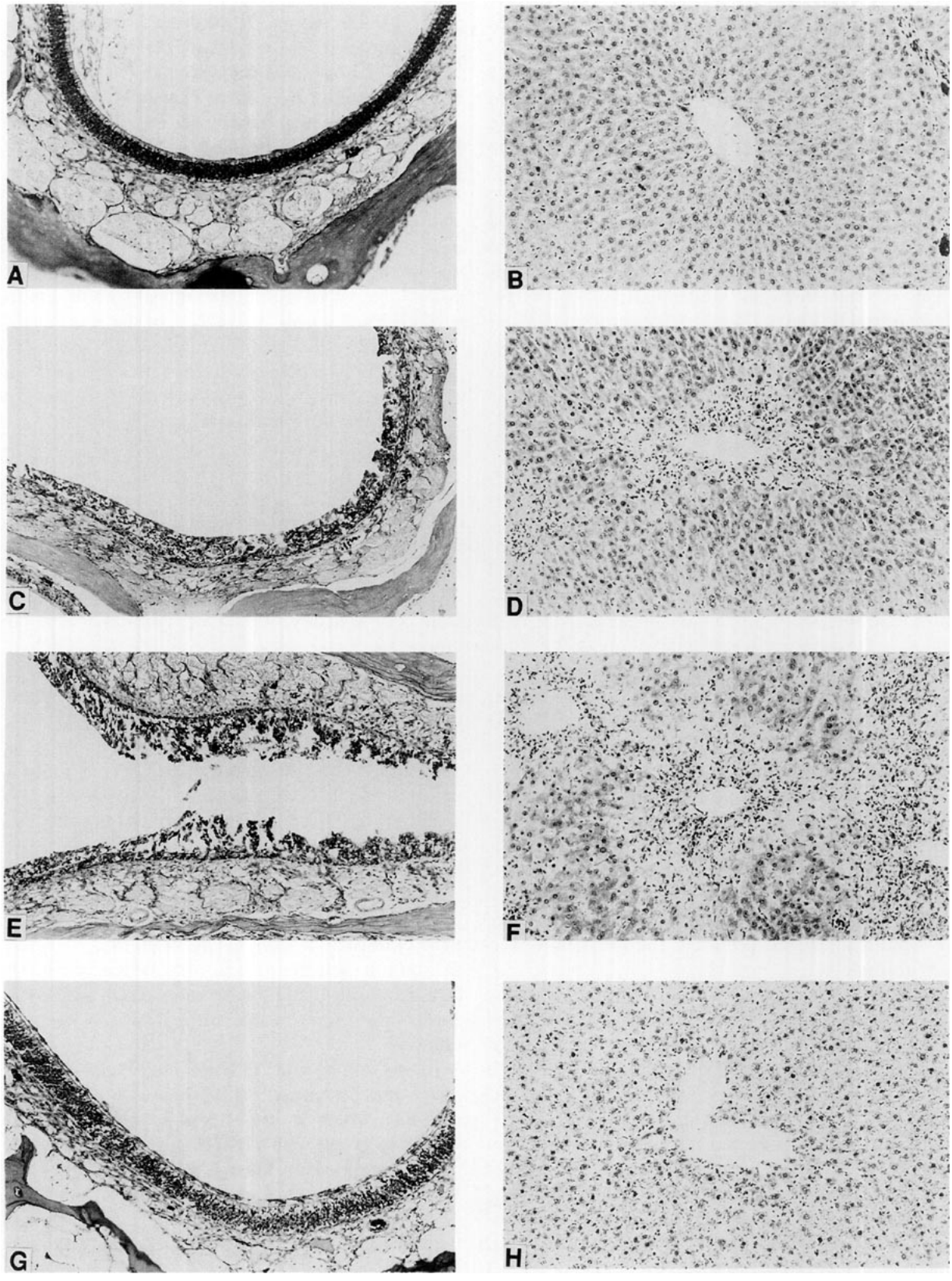


FIG. 2. Olfactory epithelium (left photos) and liver centrilobular region (right photos) following inhalation exposure to air or VDM. Air (A,B), 100 mg/m^3 VDM for 6 hr, 3 days postexposure (C,D), 300 mg/m^3 VDM for 6 hr, 3 days postexposure (E,F), 100 mg/m^3 VDM for 6 hr, 7 days postexposure (G,H). All photos are magnified 250×, except E which is 400×.

(Fig. 2). Pilot assessments of serum SGPT and LDH from the moribund animals indicated 50× and 10× increases, respectively, for these liver injury enzymes as compared to control. Other tissues, including the lung airway and parenchyma, appeared normal. Analogously exposed rats (100, 70, and 40 mg/m^3, Table 2) did not experience mortality up to 7 days postexposure. However, 2 rats/18 in the 10 mg/m^3 group did succumb, somewhat surprisingly, between Days 2 and 3; their tissues were not assessed due to rigor. The tissues affected in the 100 mg/m^3 animals were the same as those of the higher concentrations. Centrilobular liver damage at Day 3 was somewhat moderated and largely regressed to normal by Day 7 (Fig. 2). Pilot serum chemistry data from these same animals revealed correspondingly elevated SGPT and LDH levels (29× and 2×, respectively) at Day 3 also returning to normal by Day 7. Similarly, the nasal olfactory epithelium appeared degenerated and necrotic at 3 days, but, unlike the liver, it was not fully restored at Day 7 (Fig. 2).

Exposures at 70, 40, and 10 mg/m^3 resulted in no apparent hepatocellular pathology at the light microscopic level. However, in contrast, these rats clearly exhibited concentration-dependent, moderate to slight nasal olfactory cell degeneration. These lesions waned considerably by Day 7 postexposure, but evidence of damage persisted, mostly in the form of incompletely restored architecture of the olfactory epithelium.

Eighteen of 20 rats exposed "nose-only" to 300 mg/m^3 survived through 7 days postexposure, in contrast to the whole-body studies. The 2 animals that died were visibly contaminated with VDM by the end of exposure due to failure of the nose seal. Histologic evaluation of the livers of rats killed at Day 3 was unremarkable, while the nasal olfactory epithelium showed degeneration, with characteristic sloughing and necrosis. The damage was extensive, essentially as that seen previously with the whole-body exposures. At Day 7 postexposure, a slight degeneration of the olfactory epithelium again persisted; the livers, however, remained normal.

Fifty percent of the rats exposed to 40 mg/m^3 for 6 hr/day for 5 days were dead or moribund by Day 2 after the 5-day exposure regime. The moribund rats that were killed for evaluation at Day 3 had serum SGPT and LDH levels 5–10 times above control rats. By Day 7 postexposure, the surviving rats had normal serum enzyme levels.

Lung burdens were determined in subgroups of rats from each exposure level ($n = 2 - 6$) at 0, 3, and 7 days after exposure (Table 2). Lung weights were not affected by the VDM exposures. The lungs of rats exposed to 1000 mg/m^3 ($n = 2$) contained 57 μg VDM/g of tissue immediately postexposure. Assay of two dead rats (prior to rigor) on Day 3 indicated that little if any clearance from the lung had occurred (78 μg VDM/g). Although this latter value may have been influenced by the fact that the assay was conducted in dead animals, analogous results were obtained in rats exposed to 300 mg/m^3. Immediately after exposure, these lungs contained 25.5 ± 1.65 μg VDM/g ($n = 6$) as compared to 18.9 ± 2.1 μg VDM/g at 3 days ($n = 6$ live but moribund animals). Interestingly, in the nose-only exposed animals ($n = 6$), the 0-time lung burden was 64.5 ± 16.6 μg VDM/g of lung tissue, ~2.5× the dose of the whole-body exposed lungs (as noted above, no liver, but clear olfactory tissue damage was observed).

At exposures ≤100 mg/m^3, more than 85% of the initial lung burden was cleared by Day 3; virtually all the lung deposited VDM was cleared by Day 7 ($n = 6$ for each time point). The VDM retention in the 100 mg/m^3 group at Day 0 was 6.5 ± 1.0 μg/g, about 26% of the 300 mg/m^3 group. The variation among the other exposure groups at the Day 0 time point probably reflects the small sample sizes ($n = 2$), although the between-animal variability in each group was not particularly variant (<40%).

Lung Instillation

Serum LDH, SGPT/ALT, SDH, and ICDH were not significantly different from control at any VDM concentration. Also, lung and liver G6PDH, GRD, GTR, GPX, NPSH, CYTOC, and P450 were not significantly different from control at any VDM concentration or time postinstillation.

Gavage

The results of serum enzymes of liver injury from rats gavaged with 800 mg/kg of DB3, DR11, or VDM are illustrated in Fig. 3. The DB3 component of the VDM caused a slight but significant elevation in all of the enzymes (3–6× control) by Day 3 post-treatment. By Day 7, these were back to control levels. The DR11 component caused minimal enzyme changes (−1.5 to 2.5×) that were rarely significantly different (exceptions: Day 1 LDH; Day 7 SGPT) from control up to 7 days. The VDM mix, however, caused substantial increases (10- to 100-fold) in SGPT, SDH, and ICDH at both 1 and 3 days postgavage. By Day 7, all enzyme values were near control values. No mortality was observed.

Liver G6PDH and GRD were significantly elevated 1, 3, and 7 days postgavage for all three dyes with the exception of GRD, which at Day 1 was no different from control following gavage with VDM (Fig. 4). Liver GPX was significantly increased on Days 1 and 3 following gavage with DB3 and DR11 and at Day 7 postgavage with DR11. NPSH was significantly increased on Day 3 postgavage with DB3 and Day 7 postgavage with DB3 and VDM (Fig. 4).

As depicted in Fig. 5, liver GTR was significantly elevated on Days 1, 3, and 7 postgavage with DB3 and DR1, while no change from control was seen at Days 1 and 7 postgavage with VDM. In contrast, a significant decrease in

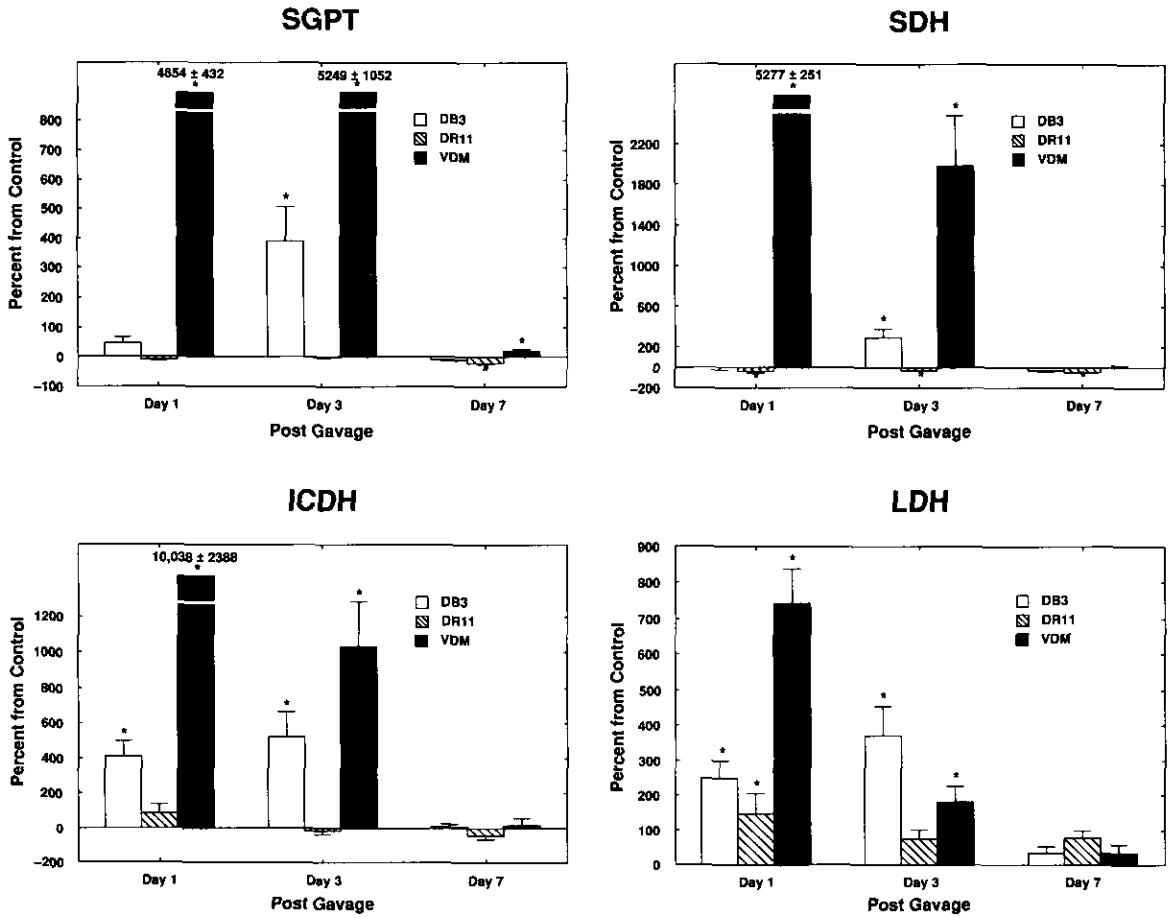


FIG. 3. Percentage from control of serum enzymes 1, 3, and 7 days after gavage with 800 mg/kg of DB3, DR11, and VDM (means ± SE, $n = 6$). *Statistically different from control at $p \leq 0.01$.

GTR was observed at Day 3. The significant increases in CYTOC were observed at Days 1 and 3 postgavage with DB3 and DR11 (50 to 130%) contrasts with the significant decreases (−25 to −50%) in CYTOC due to VDM at Days 3 and 7 postgavage and at Day 7 postgavage with DB3 (Fig. 5). Liver P450 was significantly elevated Day 1 postgavage with DB3 and DR11 and Day 3 postgavage with DR11, but was significantly decreased at Day 3 postgavage with VDM and Day 7 postgavage with DB3 and VDM (Fig. 5).

DISCUSSION

This study evaluated the toxicity of a mixture of two anthraquinone dyes (92.9% DR11 [1,4-diamino-2-methoxy-anthraquinone]: 7.1% DB3 [1-methylamino-4-hydroxyethylamino-anthraquinone]) in rats following a single 6-hr inhalation exposure. In its formulation, this anthraquinone mixture was formed from solubilization of the red and blue dyes and spray-dried to a fine, tenacious violet powder. Aerosol generation for exposure to rats resulted in particles in the 3- to 5-μm range, a particle size range which led to significant surface deposition as well as lung and nasal retention. The pattern of results in the inhalation exposure studies suggested that the systemic toxicity observations were probably not due to inhalation of the dust per se (lung deposition), but rather to the oral ingestion (via preening) of the dye adhering to the fur of the animals. Toxicity was limited to the liver and olfactory epithelial tissues of the nose while the lung itself and the 12 other organ tissues examined appeared to have been spared. Follow-up experiments were conducted utilizing direct intratracheal instillation of the VDM, as well as gavage of the VDM and its constituent dyes to better characterize the nature of the toxicity. It appears that a significant systemic dose of VDM could be achieved via preening behavior of the animals, leading to gut absorption and liver toxicity. The studies with the constituent dyes suggested a likely metabolic interaction between these dyes, with the resultant toxicity being much greater than either dye alone or their additive sum. What specific metabolism was involved is uncertain (apparently unique—in kind or degree—to the liver and, curiously, the olfactory epithelium). The observed nasal olfactory toxicity, itself, was probably the re-

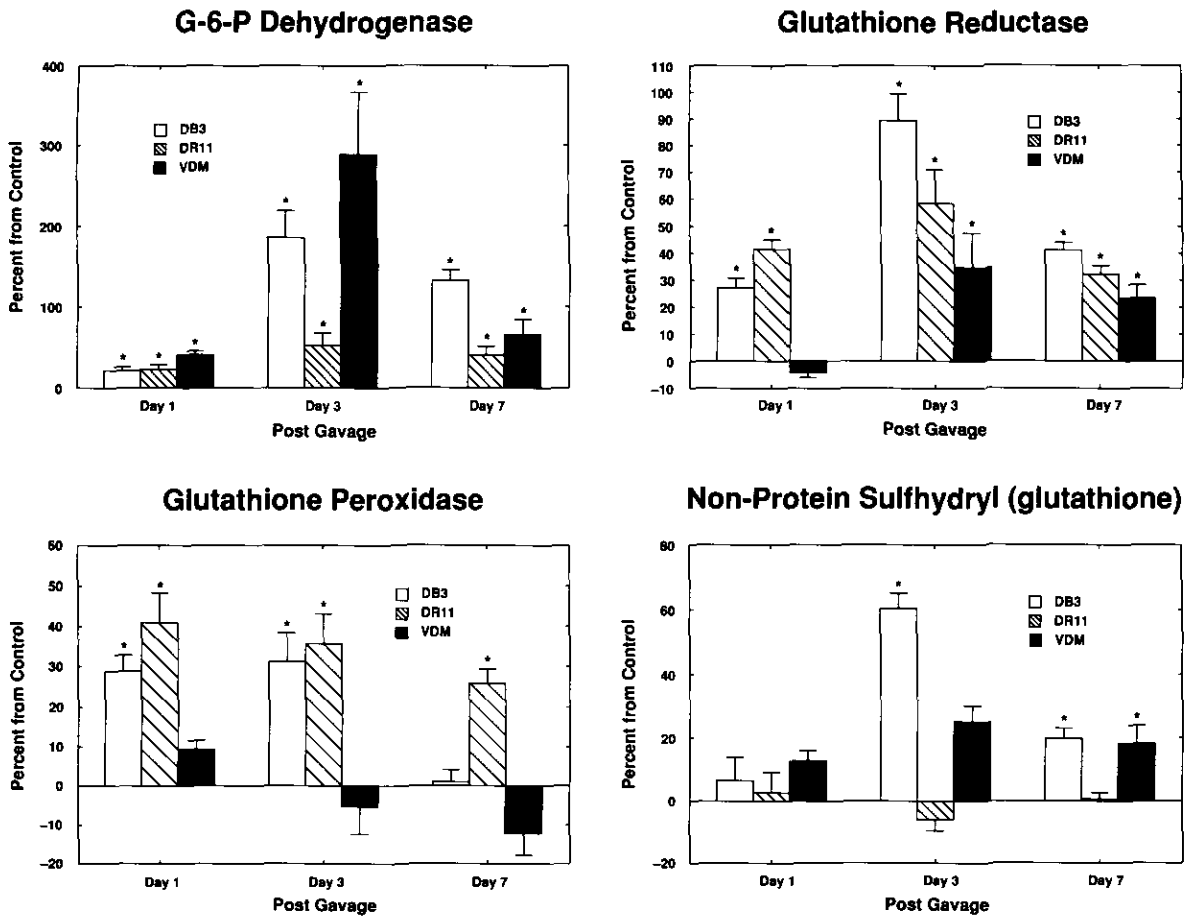


FIG. 4. Percentage from control of liver glutathione-related enzymes 1, 3, and 7 days after gavage with 800 mg/kg of DB3, DR11, and VDM (means ± SE, $n = 6$). *Statistically different from control at $p \leq 0.01$.

sult of direct nasal deposition of VDM during inhalation and not redistribution of other toxic entities.

Whole-body inhalation exposure of male and female rats to high concentrations of VDM (1000 or 300 mg/m^3) was essentially lethal within 3 days postexposure. Death appeared to be due to severe liver necrosis. Lower concentrations also resulted in concentration-dependent liver damage by Day 3 which largely resolved by Day 7. Surprisingly, no pathology was apparent in the lungs of rats at any exposure concentration despite deep lung retention of substantial amounts of dye. Respiratory tract lesions were isolated to the nose where deposition of these large dye particles (~4 μm MMAD) would be expected to be high. Deposition of the dye on the fur of exposed rats was also very high (subjectively appearing to be concentration-dependent), which as is typical was followed by vigorous preening by the animals. When rats were exposed nose-only to 300 mg/m^3 for 6 hr (a lethal whole-body exposure), no mortality or liver injury was observed except in two animals which broke their nose seals and had visibly contaminated upper body areas. Again, no lung pathology was apparent despite the ~3× greater lung burden in these nose-only exposed rats (78:25 μg). Indeed, no evidence of lung toxicity was apparent with lung instillation of up to 1000 μg, almost 15× the dose determined in the lungs of these nose-only exposed animals.

The localization of the respiratory tract pathology to the nasal olfactory epithelium, seen even at 10 mg/m^3, and complete absence of other respiratory damage suggest that these cells were, for some reason, unusually sensitive to the VDM. Several investigators have shown that nasal epithelium contains biotransformation enzymes capable of metabolizing various organic compounds (Bond, 1983; Foster *et al.*, 1986; and Hadley and Dahl, 1982). One explanation for the degeneration and necrosis solely of the olfactory epithelium would involve a specific unusual susceptibility via a critical target attacked by the VDM chemical mix. Perhaps a more credible hypothesis would involve metabolism of the VDM to a toxic metabolite which acts immediately and does not diffuse to neighboring cells (respiratory epithelium) within the nose, which remain unaffected because they lack that specific metabolic activity. The cellular demarcation of toxicity was clear (Fig. 2), despite the fact that the nasal deposition of the VDM particles would certainly

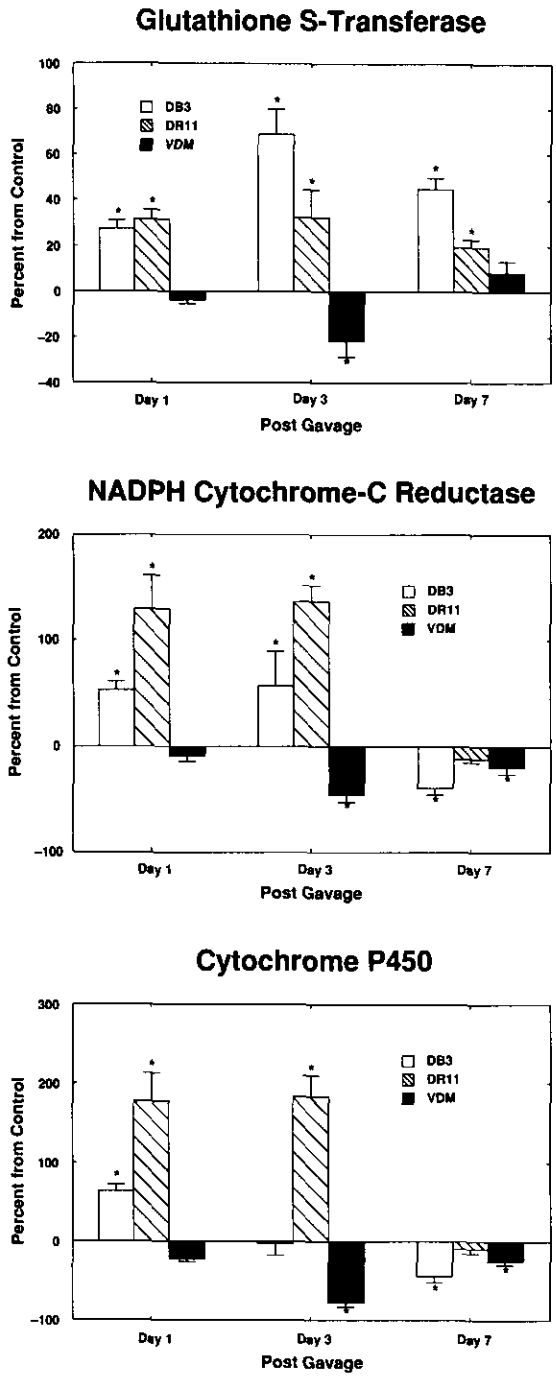


FIG. 5. Percentage from control of liver xenobiotic enzymes 1, 3, and 7 days after gavage with 800 mg/kg of DB3, DR11, and VDM (means ± SE, $n = 6$). *Statistically different from control at $p \leq 0.01$.

have broached the separation between cell types. Because the olfactory and the liver lesions paralleled each other (particularly at the high concentration exposures when dose to the liver was likely substantial), a similarity in the toxic mechanism might be suggested, although we have no direct evidence in support of this hypothesis. Moreover, the presence of olfactory damage at inhaled VDM concentrations which resulted in no liver injury would support the contention that the olfactory cells are not simply being affected by a circulating metabolite originating in the liver.

Anthraquinone dyes deposited within the lung, because of their relatively low lipid and water solubilities, would probably not be readily transported across the air–blood interface into the blood. The particles would only slowly dissolve in the lung fluids, and would thus more likely be available for macrophage/mucociliary clearance. While not assessed directly, the clearance of VDM was consistent with this scenario. Moreover, those animals suffering acute, lethal systemic toxicity appeared to clear little or none of the deposited VDM, probably due to the poor health condition of the animals. Gut transport of food (and dye) consumed immediately postexposure appeared also to be largely inhibited. On the other hand, the lung burdens in animals less affected systemically (100 mg/m^3) were readily cleared (>85% by Day 3) from the lungs. Blood concentrations of dye were generally low (~2 μg/ml; Table 2), but if adjusted for total blood volume (~10 ml), the circulating extractable VDM would exceed the original dose retained by the lung. The violet coloring of the serum and urine appeared to be a solubilized form of VDM (i.e., metabolite) since it was not extractable with methylene chloride. It seems unlikely that mucociliary clearance and swallowing (or even dissolution) of the lung-retained dye could have added significantly to the systemic dose. This was confirmed by the lung instillation studies which revealed no local pulmonary toxicity or systemic effects.

We also exposed rats for 5 consecutive days 6 hr/day to a low concentration of VDM (40 mg/m^3) that had previously shown limited toxic effects after one 6-hr exposure to address the question of whether tolerance to the toxic effect of VDM would occur or whether toxicity would be cumulative. Although the rats did not show untoward effects during the 5-day exposure, 2 days after its completion, 50% (16/32) of the rats had died. These findings led us to conclude that the VDM toxicity observed was cumulative due to repeated daily ingestion of dye from preening. As a simple assessment, the dose accumulated, if assumed to be linear by daily preening with minimal fecal loss, would fall between that resulting from the 100 mg/m^3 (no mortality and moderate liver injury) and 300 mg/m^3 (lethal with severe liver necrosis) exposures.

In the gavage studies with VDM and its constituent dyes (DB3 and DR11), the DR11 component appeared to have negligible liver toxicity (as per serum chemistry) at a dose about that which may have been ingested as VDM during the inhalation exposure, while the DB3 component was only slightly toxic at ~40 times that relative VDM dose. In contrast, VDM itself caused the rapid onset of liver toxicity 1 day following gavage with a hepatotoxicity pattern analogous to that derived from the whole-body inhalation exposure. However, the intensity of effect in terms of serum enzyme responses in the gavaged rats versus "preening

rats" occurred earlier (Day 1, SGPT 47×:1×; LDH 8×:1×) and was higher (Day 3, SGPT 52×:19×; LDH 3×:1×), which may have reflected dosimetric differences relative to the preened dose or dose rate or perhaps the influence of the 25% PEG/aqueous vehicle used in the gavage study. Interestingly, there was no mortality among the gavaged animals although the SGPT and LDH value of these animals approximated those of rats moribund 3 days after 300 mg/m^3. Unfortunately, the lack of histopathological evaluation in the gavaged animals makes uncertain the degree of actual histologic damage in these animals.

These data suggest that a synergistic interaction occurred between the two component dyes (DR11 and DB3) in VDM to elicit the observed toxicity in the liver and olfactory epithelium of exposed rats. Since metabolism was not specifically analyzed in this study, suggested mechanisms remain inferential and therefore speculative. We hypothesize that liver (and olfactory epithelial) cells elaborate a toxic metabolite from the DB3 component by a mechanism which is potentiated via the DR11-induced activity of the P450 (observed to increase by more than 200% as early as 24 hr post-treatment). While an alternative explanation might include the inhibition of a detoxification step, we believe the lack of direct toxic effect of DR11 on the liver itself and similar, although much reduced, toxicity of DB3 as compared to VDM at ~40× the constituent dose, would better support the contention of potentiation. Clearly, direct study of this interaction would be needed to elucidate this issue. Why only olfactory and liver cells are affected remains unclear and may be due to a unique enzyme or P450 isoenzyme found only in these tissues.

In conclusion, the anthraquinone dyes, DR11 and DB3, appear to be minimally toxic when individually administered to rats. However, when combined, they are capable of interacting, perhaps via P450-related metabolic pathways, to specifically damage centilobular hepatocytes and olfactory epithelial cells. The lung itself, which in humans would be a primary portal of entry of VDM under occupational or field conditions, was unaffected by VDM in rats even at very high instilled doses. Exposures to VDM via the digestive tract, however, unveil significant potential for toxicity. This phenomenon reinforces the need to consider realistic exposure situations, including the portals of entry and the exposure mixtures, in the assessment of toxic risks.

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