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Methylene Dianiline: Acute Toxicity and Effects on Biliary Function

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4,4'-Methylene dianiline (4,4'-diaminodiphenylmethane, DAPM), which is used in the polymer industry, causes hepatobiliary damage in exposed humans. Our objectives were to characterize the acute toxicity of DAPM in liver, particularly on secretion of biliary constituents and on biliary epithelial cell γ-glutamyl transpeptidase (GGT) activity. Biliary cannulas were positioned in Sprague–Dawley male rats under pentobarbital anesthesia. After 1 hr of control bile collection, each rat was given 250 mg DAPM/kg (50 mg/ml) po in 35% ethanol or 35% ethanol only; bile was collected for a further 4 hr. Groups of rats were also examined for liver injury and biliary function at 8 and 24 hr after DAPM. Four hours after DAPM administration, main bile duct cells were severely damaged with minimal damage to peripheral bile ductule cells. Focal periportal hepatocellular necrosis and extensive cytolysis of cortical thymocytes occurred by 24 hr. Serum indicators of liver injury were elevated by 4 hr and continued to rise through 24 hr. By 4 hr, biliary protein concentration was increased 4-fold while concentrations of biliary bile salt, bilirubin, and glutathione were decreased by ~80, 50, and 200%, respectively. DAPM also induced a striking effect on biliary glucose with an ~20-fold increase. Histochemical staining of main bile duct GGT was absent by 8 hr after DAPM. Bile flow was diminished by 40% at 4 hr; three of five rats had no bile flow by 8 hr and none had any bile flow by 24 hr. These results indicate that DAPM rapidly diminishes bile flow and alters the secretion of biliary constituents and is highly injurious to biliary epithelial cells. © 1992 Academic Press, Inc.

4,4'-Methylene dianiline (4,4'-diaminodiphenylmethane, DAPM) is an aromatic diamine used in the production of polyurethane foam, epoxy resins, and polymers. These materials are used for insulation, wire coating, floor coverings, and in aircraft and spacecraft parts (IARC, 1986; Moore, 1978). In addition, polyurethane is currently used in several types of medical equipment, such as artificial dialysis devices, intraaortic balloons, and vascular grafts (Mazzu and Smith, 1984).

Humans exposed to DAPM accidently or occupationally develop jaundice, cholangitis with cholestasis, toxic hepatitis, skin rash, and in one severe case, acute cardiomyopathy (Kopelman *et al.*, 1966; McGill and Motto, 1974; Bastien, 1984; Van Joost *et al.*, 1987; Brooks *et al.*, 1979). Another route of human exposure to DAPM may be through the widespread and increasing use of polyurethane-containing medical devices from which DAPM is released in ppb to ppm amounts during either gamma-ray or autoclave sterilization (Mazzu and Smith, 1984; Shintani and Nakamura, 1989). Therefore, patients who require long-term use of devices such as plasma separators or dialysis machines could be constantly exposed to DAPM and at risk to its adverse effects. In addition, the high elasticity and tensile strength as well as the low density of these resins make them valuable as orthopaedic and odontological implants. The release of DAPM from such implants within biological tissue can be suspected but has only recently begun to be investigated (Berruet *et al.*, 1987).

Chronic studies in rodents have indicated that DAPM is carcinogenic (Weisburger *et al.*, 1984; Lamb *et al.*, 1986). In rats, short-term oral administration causes necrotizing cholangitis with periportal necrosis (Gohlke and Schmidt, 1974) while subchronic exposure elicits inflammatory cell infiltrate, severe bile duct proliferation, and portal fibrosis/cirrhosis (Miyamoto *et al.*, 1977; Fukushima *et al.*, 1979). *In vitro* studies have shown that DAPM is mutagenic (see review, McQueen and Williams, 1990) and genotoxic (Mori *et al.*, 1988). In addition, DAPM induces DNA strand breaks *in vivo* in rat liver and causes an increase in sister chromatid exchanges in mouse bone marrow (Parodi *et al.*, 1981; 1983).

Although DAPM is toxic to humans and animals, virtually nothing is known about the mechanism of its toxicity. Previous subchronic studies have shown that DAPM is selectively injurious to liver and bile ducts but the acute toxic effects of DAPM on biliary epithelial cells and bile formation have not been investigated. Therefore, our objectives were threefold: first, to determine the acute toxicity of a single dose (250 mg/kg) of DAPM on liver and particularly on bile duct cells at early time points; second, to examine the effect of DAPM on enzyme functions in different hepatic locations, i.e., hepatocyte plasma and canalicular membranes, and biliary epithelial cell luminal membranes; and third, to deter-

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mine the early effects of DAPM on bile formation. Hepatotoxicity was assessed by histology and alterations in serum enzyme levels; enzyme function in the different hepatic locations was assessed by histochemistry. Bile formation was evaluated by measuring bile flow and the concentrations of five endogenous constituents of bile. A dose of 250 mg/kg was chosen because the LD_{50} of DAPM in rats is 830 mg/kg (Pludro *et al.*, 1969) and because small foci of periportal necrosis were observed 24 hr after oral administration of 250 mg DAPM/kg to male albino rats (Gohlke and Schmidt, 1974).

METHODS

Chemicals. 4,4'-Methylene dianiline (99% purity) was obtained from Aldrich Chemical Co. (Milwaukee, WI). Taurocholate, 3α-hydroxysteroid dehydrogenase, L-leucyl-4-methoxy-2-naphthylamide, 5'-adenosine monophosphate, γ-glutamyl-4-methoxy-2-naphthylamide, glycylglycine, glutathione reductase, and 5,5'-dithiobis-(2-nitrobenzoic acid) were obtained from Sigma Chemical Co. (St. Louis, MO).

Animals. Male Sprague–Dawley rats from Harlan (Indianapolis, IN), were maintained in wire-bottom cages over absorbent paper in a 12-hr light/dark cycle animal room for 1 week prior to experiments. Rats weighed 325–375 g in each experiment.

Experimental protocols. DAPM was dissolved in absolute ethanol and then diluted to 50 mg/ml in 35% ethanol with distilled water at ~40°C. For the time course studies, rats were given 250 mg DAPM/kg or vehicle alone by gavage between 9 and 10 AM and placed in metabolism cages. Drinking water was available at all times; food was added to the metabolism cages at ~5 PM for animals held overnight.

For the biliary function studies, rats were anesthetized with sodium pentobarbital (50 mg/kg, ip). A ventral midline incision was made and the liver gently retracted to expose the bile duct. A slit was made in the common bile duct ~8 mm distal to its bifurcations and a cannula was inserted ~4 mm into the common bile duct and tied in place. The cannula was a 25-cm length of PE-50 tubing (0.58 mm i.d.) with a beveled tip. The common bile duct was tied off distal to this cannula to avoid leakage of pancreatic fluids. Next the duodenum was punctured ~1 cm distal to the bile duct papilla and a cannula was inserted and sutured in place. The duodenal cannula was made of silastic tubing (0.51 mm i.d.) with a beveled tip of PE-50 (~4 mm). Finally, the midline incision was closed with staples except for ~1 cm to allow exteriorization of the two cannulas. These surgical procedures were generally completed within 30 min.

After surgery, rats were placed on a heating pad at 37°C and taurocholate (36 mM in saline) was infused through the duodenal cannula at a rate (9.2 ml/hr/kg rat) to maintain bile flow (Moslen *et al.*, 1992). Anesthesia was maintained by infusing a dilute solution of pentobarbital (2.5 mg/ml in saline) intraperitoneally. Rats were allowed to equilibrate for 15 to 30 min after surgery before collection of control bile for 1 hr. Bile was collected at 15-min intervals in tared microcentrifuge tubes on ice. Bile to be used for glutathione assays was collected in 50 μl of 5% perchloric acid to prevent thiol oxidation. After 1 hr of control bile collection, DAPM (250 mg/kg) or vehicle was given by gavage. Bile was collected for another 4 hr. Animals were exsanguinated by withdrawal of blood from the inferior vena cava. Liver, spleen, pancreas, and thymus were removed and pieces of each tissue were fixed in 10% neutral buffered formalin for histology. Blood samples were allowed to clot on ice for ~30 min, centrifuged, and serum samples stored at −20°C.

For time course studies, rats were anesthetized with pentobarbital (50 mg/kg, ip) at 6 and 24 hr after DAPM administration. Bile duct cannulas but not duodenal cannulas were positioned and bile was collected by the methods described above. At ~2 hr after cannulation, the experiment was terminated and blood, liver, pancreas, spleen, and thymus were removed. Serum samples were stored at −20°C and tissues were fixed in formalin as described above. From the 6-hr treated animals, small liver cubes (0.5 cm^3) were quick frozen in freon at −90°C for histochemical staining.

Assessment of hepatotoxicity. Serum alanine aminotransferase (ALT), alkaline phosphatase (ALP), and glucose were determined using Sigma reagent kits 57, 10, and 20, respectively. Tissues, fixed in 10% neutral buffered formalin, were processed by standard histological techniques and stained with hematoxylin and eosin for histopathological evaluation. Criteria for determination of alterations in main bile ducts versus bile ductules are based on the descriptions of Yamamoto and Phillips (1984).

Histochemistry. Frozen liver sections (8 μm) were fixed and dilipidated in chloroform:acetone (1/1, v/v) for 5 to 10 min. Staining for leucine aminopeptidase (LAP) was done by a modification of the method of Nachlas *et al.* (1960), using L-leucyl-methoxy-2-naphthylamide as substrate, Fast Blue B salt, and cupric sulfate. γ-Glutamyl transpeptidase (GGT) staining was done by the method of Busachi *et al.* (1981), using γ-glutamyl-4-methoxy-2-naphthylamide as substrate, Fast Blue B salt, and glycylglycine. Staining for 5'-adenosine monophosphatase was done by the method of Hardonk *et al.* (1977), using sodium 5'-adenosine monophosphate as substrate, levamisole, and lead nitrate.

Bile constituents. Bile volumes were determined by weight, assuming a density of 1 g/ml. Total protein was assayed by the method of Lowry *et al.* (1951) using bovine serum albumin as standard. Bile salt was measured enzymatically according to the method of Koss *et al.* (1974) using 3α-hydroxysteroid dehydrogenase and taurocholate as standard. Glucose and total bilirubin were measured using Sigma kits 20 and 550 specific for D-glucose and bilirubin. Total glutathione was assayed by the enzymatic recycling method of Tietze (1969) using glutathione reductase and 5,5'-dithiobis-(2-nitro-benzoic acid).

Statistics. Data on biliary constituents were analyzed by analysis of variance with repeated measures using ABSTAT (Anderson Bell, Boulder, CO), a statistical package for personal computers. If analysis indicated a significant difference between groups, the means for bile data at different times were compared by Scheffe's test or Mann–Whitney *U* test. Serum data for the treated and control groups were compared by Student's *t* test. A *p* value of <0.05 was considered significant.

RESULTS

Table 1 presents the effects of DAPM on serum parameters at 4, 8, and 24 hr. Serum ALT levels progressively increased with time. In contrast, serum ALP values were not elevated until 8 hr after DAPM followed by a marked increase between 8 and 24 hr. DAPM also induced hyperglycemia, with

TABLE 1
Effect of Methylene Dianiline Treatment on Serum Parameters

Parameter	Control[a] 4, 8, and 24 hr	DAPM 4 hr[b]	DAPM 8 hr[c]	DAPM 24 hr[c]
ALT (IU)	41 ± 5	120 ± 11*	306 ± 103**	731 ± 122*
ALP (U/liter)	88 ± 4	98 ± 7	133 ± 9*	848 ± 162**
Glucose (mg/dl)	169 ± 21	251 ± 31*	493 ± 56**	170 ± 20
Bilirubin (mg/dl)	1.28 ± 0.07	1.78 ± 0.09*	1.73 ± 0.08*	1.61 ± 0.11

[a] Means and SEM of eight or nine animals.
[b] Means and SEM of four animals.
[c] Means and SEM of three animals.
* $p < 0.05$ compared to control group.
** $p < 0.01$ compared to control group.

~50 and ~200% increases in serum glucose at 4 and 8 hr, respectively. However, serum glucose values at 24 hr after DAPM were similar to those of the control group. Serum bilirubin was increased at 4 and 8 hr. Since values for the serum parameters of the three control groups were similar at the three time points, the data for all control animals were pooled.

DAPM Effects on Liver Histology and Histochemistry

Histological evaluation of tissues indicated that early injury by DAPM was confined to portal triads of the liver with variable degrees of injury in bile ducts versus ductules. Four hours after DAPM, epithelial cells of the main bile ducts were necrotic and were beginning to separate from each other and from the basement membrane (compare Figs. 1a and 1b), while effects on the small bile ductules at the periphery of portal triads were minimal. By 8 hr, the main bile ducts were denuded of epithelial cells and were generally recognizable by the irregular shape of the underlying interstitial tissue. Epithelial cells of the bile ductules were more disorganized than those observed at 4 hr and ductule lumens were difficult to discern. However, uninjured epithelial cells were seen in the smallest ductules. In addition, the larger portal triads displayed mild to moderate edema (Fig. 1c) with fibrous networks scattered within the transudate. Few inflammatory cells were seen in portal triads 8 hr after DAPM. Periportal parenchymal cells appeared unaffected by DAPM at 8 hr.

At 24 hr after DAPM, moderate to marked edema was present in all portal triads (Fig. 1d). Typical bile duct struc-

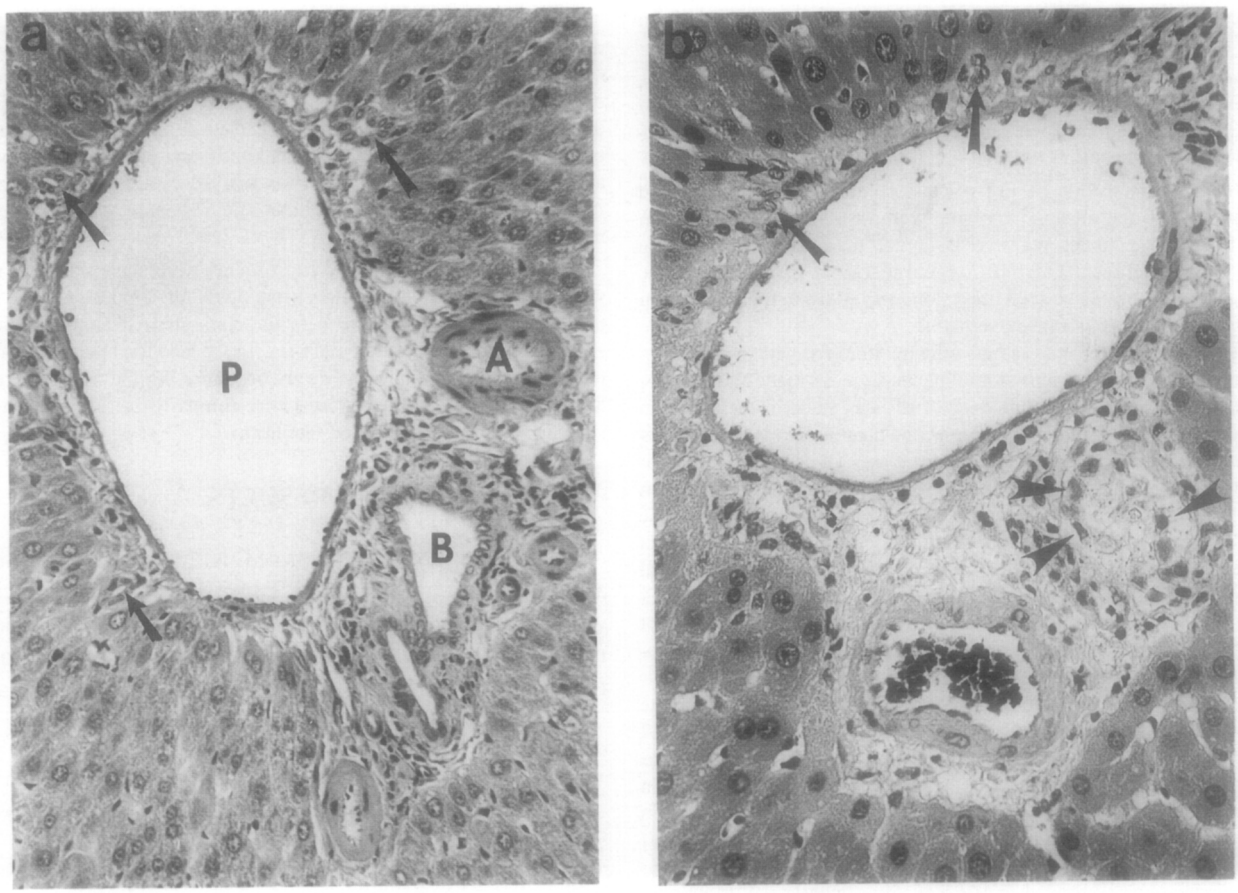
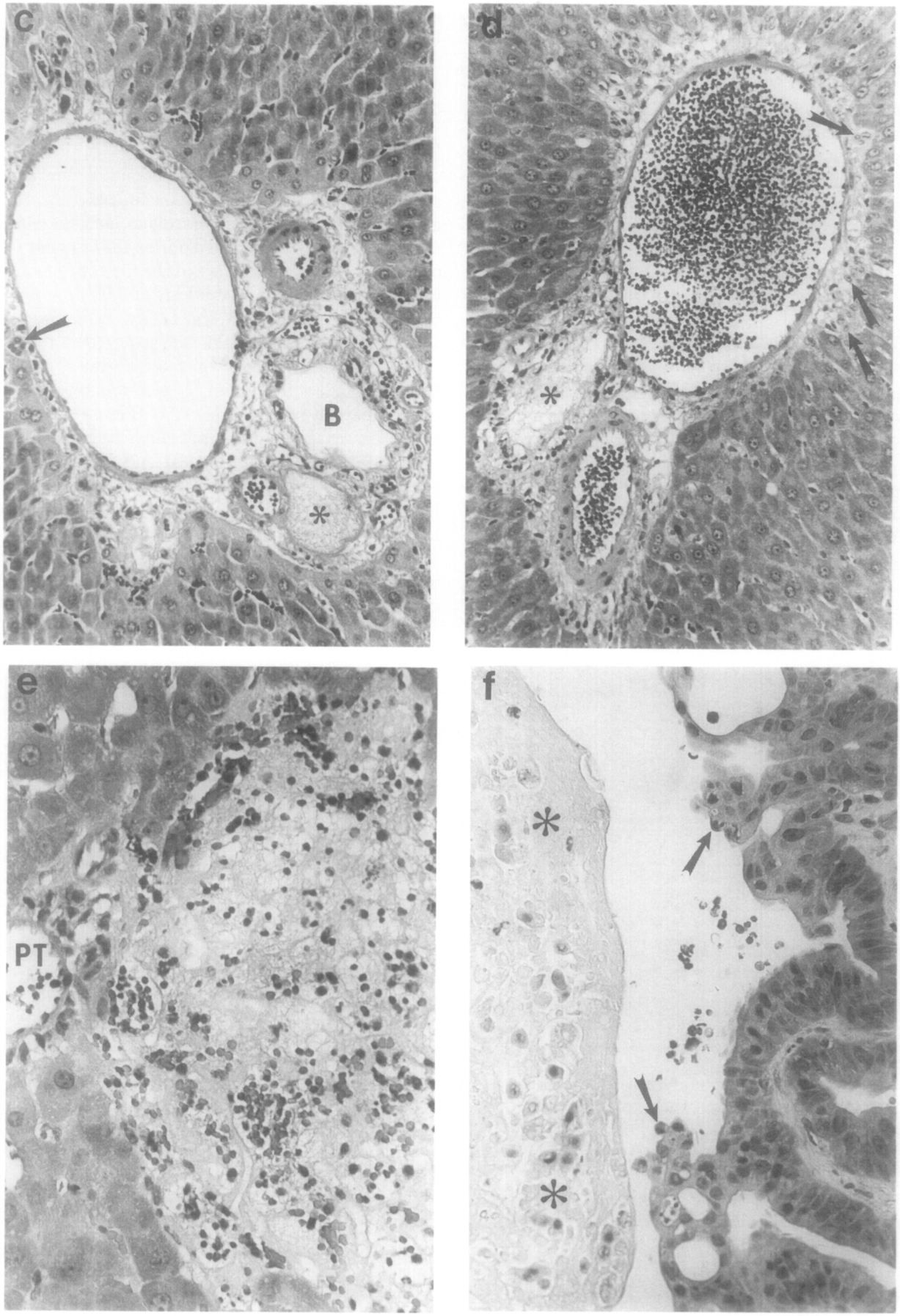


FIG. 1. Light micrographs of the livers of a control rat (a) and rats treated with 250 mg DAPM/kg (b, c, d, e, f). H&E, original magnification (a, c) 220×, (b) 350×, (d) 200×, and (e, f) 330×. (a) In normal liver, main bile ducts (B) are easily identified in the portal triad by their cuboidal epithelial cells and their companion branch of the hepatic artery (A). Bile ductules (arrows) are seen on the periphery of the portal triad adjacent to the hepatic parenchyma. P, portal vein. (b) By 4 hr after DAPM exposure, main bile duct epithelial cells are separating from each other and exfoliating into the bile duct lumen (arrowheads). Peripheral bile ductules (arrows) are unaffected by DAPM. (c) By 8 hr, epithelial cells lining the main bile ducts (B) are destroyed; peripheral bile ductules (arrows) are more difficult to identify. Moderate edema, fibrous material (asterisk), and scattered inflammatory cells are present in this portal triad. (d) At 24 hr, individual biliary epithelial cells can still be identified (arrows) but discrete bile ducts are rare. Marked edema is present and fibrous material is seen within the interstitial tissue of the main bile duct (asterisk). Inflammatory cells continue to be sparse. (e) At 24 hr, an oval region of periportal hepatocellular necrosis is seen extending out from a small portal triad (PT). Numerous inflammatory cells are scattered among these necrotic hepatocytes. (f) At 24 hr, the lumen of the common bile duct in this DAPM-treated rat is nearly filled with amorphous material (asterisks) containing exfoliated and/or necrotic biliary epithelial cells. Cells with pyknotic nuclei (arrows) are also being released from the epithelium lining the common bile duct.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tures were absent, but individual biliary epithelial cells were scattered among the interstitial cells and occasionally appeared to form circular patterns. Similar to the previous report by Gohlke and Schmidt (1974), injury to hepatic parenchymal cells at 24 hr was evident as oval or pear-shaped necrotic regions extending outward from portal triads (Fig. 1e) or as circular areas in midzonal regions. Inflammatory cells, mainly neutrophils, appeared more numerous in areas of necrotic hepatocytes than within injured portal triads. By 24 hr after DAPM, injury had also extended to the common bile duct where exfoliation of necrotic epithelial cells was observed and the lumen of the common bile duct contained cellular fragments surrounded by amorphous material (Fig. 1f).

In addition to liver injury, DAPM induced focal disintegration of cortical thymocytes by 8 hr. At 24 hr, the cortex of the thymus showed prominent cytolysis (Fig. 2). In severely injured animals, ~50% of the cortex was necrotic by this time. No histopathological changes were observed in other organs of DAPM-treated rats.

Histochemical staining demonstrated that functional alterations of liver enzymes were also confined to the portal triad after DAPM treatment. At 8 hr, GGT staining of main bile duct epithelial cells was absent, with staining appearing only in small ducts and ductules (compare Figs. 3a and 3b). In some sections, staining appeared more diffuse, suggesting that GGT was no longer present in discrete duct structures. DAPM had no effect on histochemical staining of the hepatocyte plasma membrane-localized enzyme, 5′-AMPase, or on the canalicular membrane-localized enzyme, LAP, at 8 hr (not illustrated).

DAPM Effects on Biliary Function

DAPM produced marked effects on bile flow and biliary concentrations of endogenous constituents (Fig. 4). DAPM administration induced an immediate choleresis which lasted for 1 hr, but bile flow declined during the last hour to ~40% of basal value by 4 hr (Fig. 4a). In the time–course study, we also found pronounced effects on bile flow at 6 and 24 hr after DAPM; bile flow had ceased in 60 and 100%, respectively, of the animals in each group (data not shown).

The two biliary solutes which are normally concentrated in bile, bile salt and bilirubin, showed striking early decreases during the first hour after DAPM (Figs. 4b and 4c) when choleresis was manifest. Bile salt and bilirubin concentrations then progressively declined to 21 and 50%, respectively, of basal values by 4 hr. Control animals showed a decline in bile salt concentration to ~70% of basal value in the last 2 hr of bile collection ($p < 0.05$). The greater decrease in bile salt concentration in DAPM-treated rats, however, is significantly different from control animals. Total glutathione concentration in bile showed a marked increase of 100%

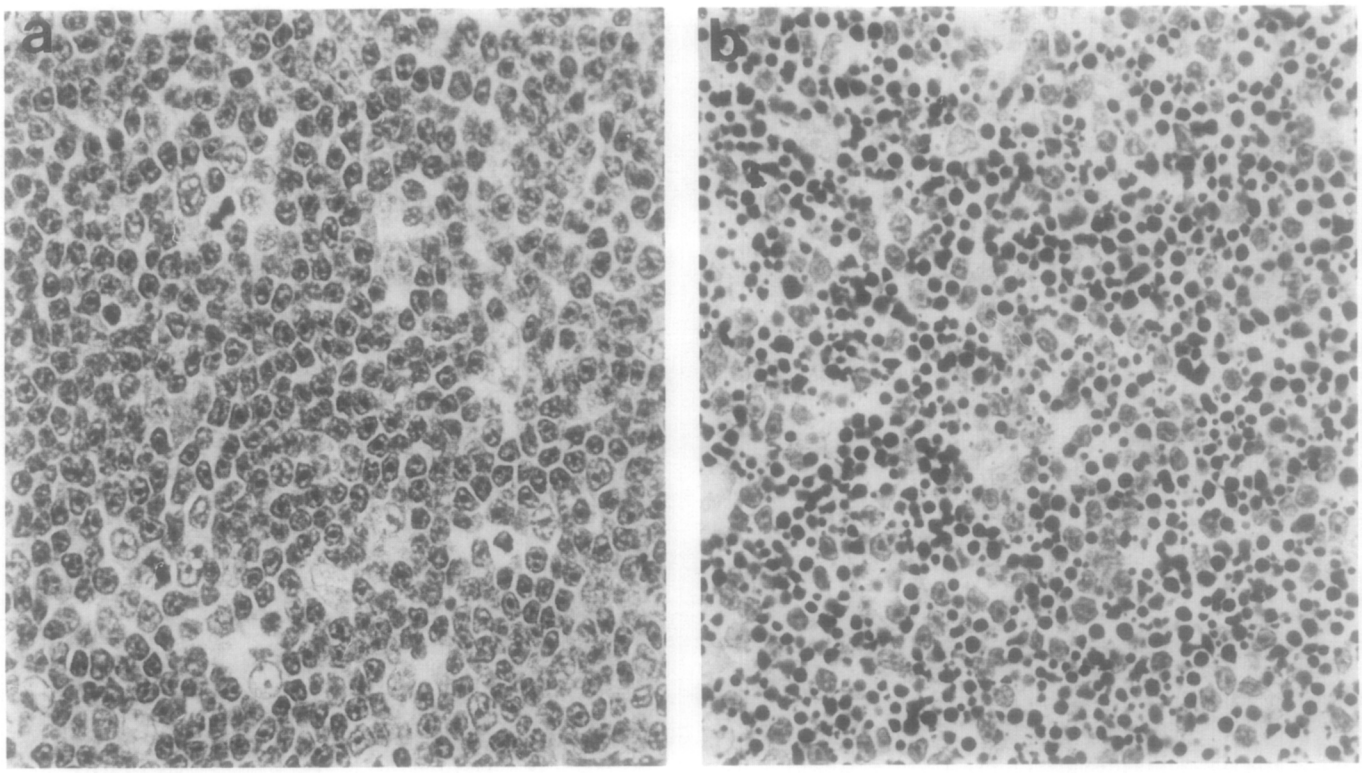

FIG. 2. Light micrographs of the thymus from a control rat (a) and a rat 24 hr after administration of 250 mg DAPM/kg (b). H&E, original magnification, (a, b) 600×. (a) Normal, darkly staining lymphocytes are evenly spread throughout the cortex. (b) Lymphocytes in the cortical area of the thymus are undergoing cytolysis, and pyknotic cells and cell debris are scattered throughout the cortex.

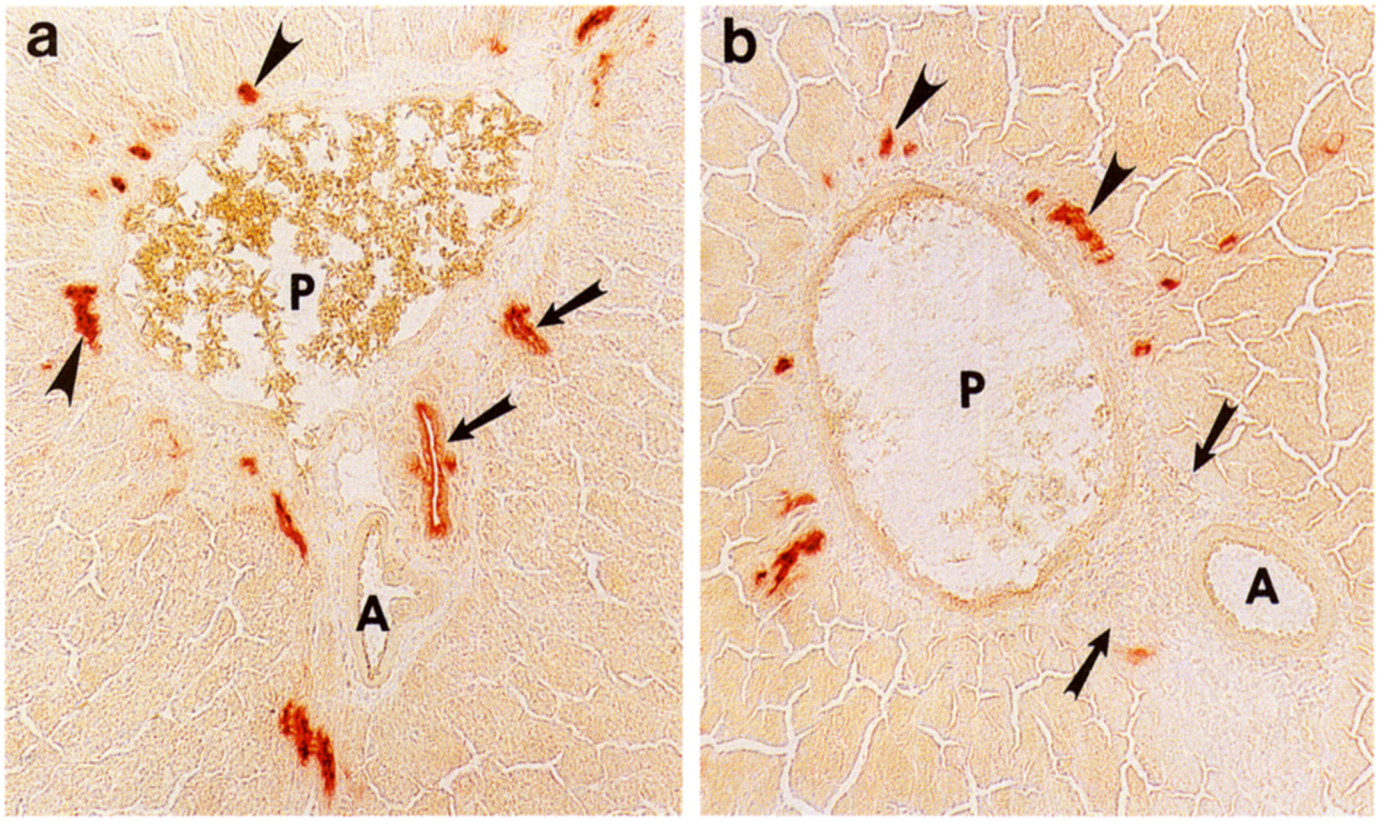

FIG. 3. Light micrographs of frozen sections of livers stained for GGT activity from a control rat (a) and a rat treated with 250 mg DAPM/kg 8 hr earlier (b). Original magnification, (a, b) 175×. (a) Intense GGT activity is present in the epithelial cells of the main bile ducts (arrows) and bile ductules (arrowheads) of normal liver. P, portal vein, A, hepatic artery. (b) In DAPM-treated livers, GGT activity is absent in the region of the portal triad where main bile ducts (arrows) are usually observed. GGT activity in peripheral bile ductules (arrowheads) is typical to slightly diminished.

during the choleretic period after DAPM treatment but by 4 hr, had decreased to <1% of basal value (Fig. 4d). The two biliary solutes which are normally excluded (protein) or removed (glucose) from bile were affected by DAPM with an increase in protein evident by 30 min and an increase in glucose seen by 90 min (Figs. 4e and 4f). By 4 hr, the protein and glucose increases were greater than 4-fold and 20-fold, respectively.

Tables 2 and 3 list the effects of DAPM treatment on biliary excretion of the five endogenous solutes. Since our laboratory and others (Moslen *et al.*, 1992; Ballatori and Truong, 1990) have found considerable animal to animal variation in biliary excretion parameters; we have found it efficient to evaluate the time–course of treatment effects relative to each animals' basal or pretreatment (100%) value. DAPM treatment induced gradual declines in bile salt and bilirubin excretion starting in Hours 2 and 3, respectively, but induced an early increase in glutathione excretion at Hour 1 with a precipitous decline in Hours 3 and 4 (Table 2). In contrast, biliary protein and glucose excretion increased ~5-fold and 3-fold within 1 hr of DAPM treatment (Table 3). After the first hour, protein excretion showed little subsequent change but the early 3-fold increase in glucose excretion was followed by striking ~15- to 20-fold increases in Hours 3 and 4.

DISCUSSION

This time–course study indicates that DAPM is selectively toxic to bile duct epithelial cells in rats after an acute dose and that histologically evident hepatic lesions occur subsequent to biliary lesions. At 4 hr, injury is confined to the main bile ducts where necrotic epithelial cells begin to break down. By 8 hr, injury has developed so rapidly that the main bile ducts are virtually unidentifiable and damaged epithelial cells are seen in bile ductules. The injury is sufficiently severe that edema and fibrous networks are manifest in the larger portal triads by 8 hr; these networks are presumably fibrin produced by the leakage of plasma proteins and fluids into the interstitial spaces. Concurrent with these histological alterations is the loss of GGT activity in bile ducts, an enzyme normally present on the apical membranes of biliary epithelial cells (Hagerstrand and Norden, 1972; Plaa and Priestly, 1977).

We are presently unable to explain the localization of DAPM injury at early times to the main bile ducts, while less severe injury to bile ductules was evident only at later times. Yamamoto and Philips (1984) have suggested that rat bile ductules ("bile ductular plexus") may function to (1) absorb or secrete water or electrolytes, (2) concentrate bile (i.e., function as a gallbladder), or (3) act as a collecting system before bile enters main bile ducts. Therefore, one possible

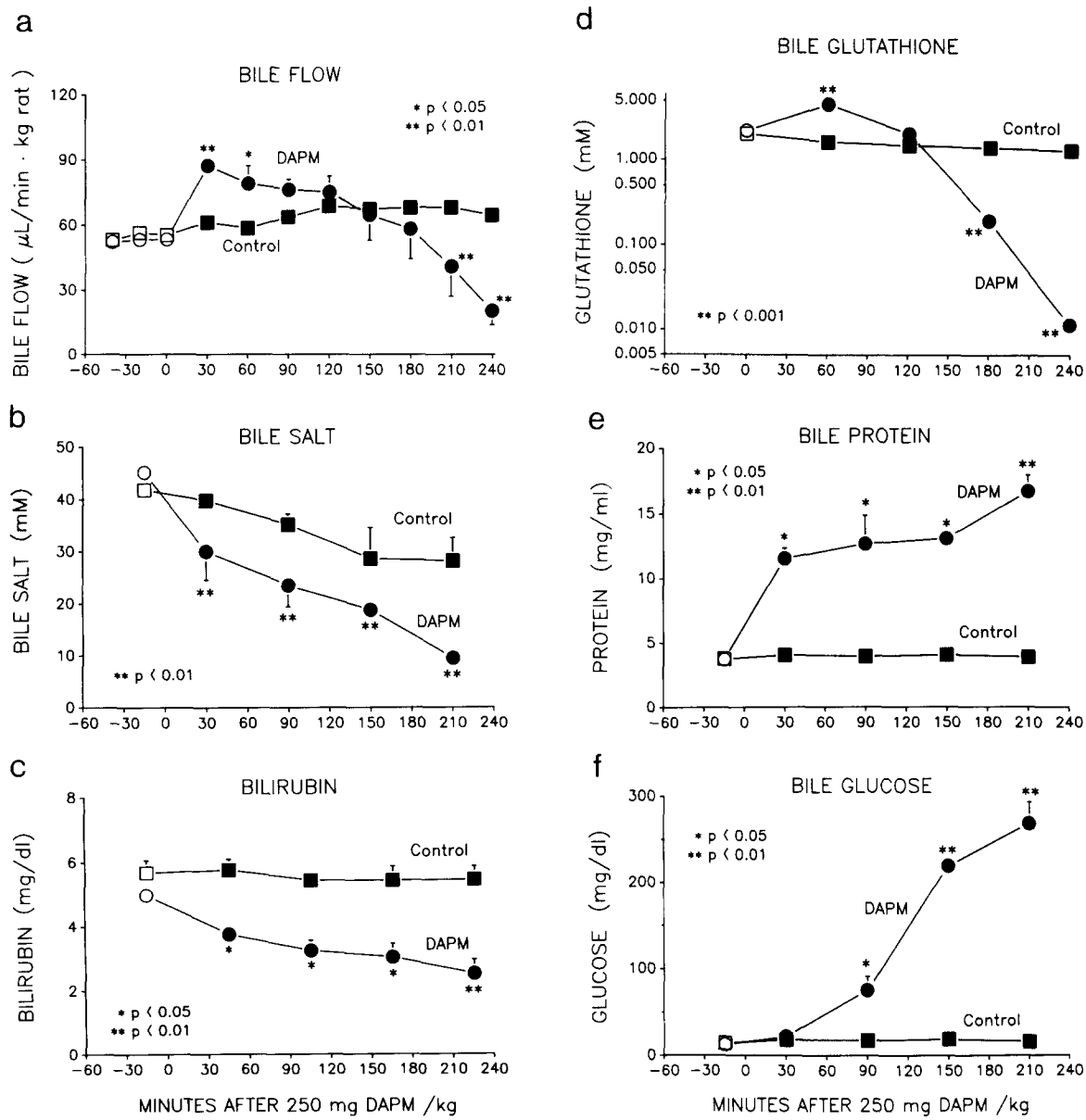

FIG. 4. Time course of changes in bile flow (a) and in biliary bile salt (b), bilirubin (c), glutathione (d), protein (e), and glucose (f) concentrations of control rats and rats treated with 250 mg DAPM/kg. Values are means ± SE of four rats per group. Symbols without error bars indicate the SE lies within the symbol. Asterisks near symbols of DAPM-treated rats denote values significantly higher or lower than values from control rats.

explanation for the difference in localization of DAPM injury between main bile ducts and ductules is that these two structures have different functions or are in contact with more/less concentrated bile.

The severity and rapidity with which DAPM induces injury to biliary epithelial cells is also supported by the time course of alterations in biliary constituents. We measured five different endogenous constituents which are thought to enter, be excluded, or removed from bile by different pathways (Moslen *et al.*, 1992; Coleman, 1987). Of the five biliary constituents measured, only biliary glucose was not significantly altered within the first hour. By 4 hr after DAPM, striking changes had occurred in the concentrations and excretion rates of all biliary constituents examined, especially glucose and glutathione, which indicates profound biliary dysfunction. Interestingly, this study suggests that biliary epithelial cells may play a major role in the unknown site of glucose reabsorption from bile (Guzelian and Boyer, 1974; Lira *et al.*, 1992) since DAPM caused an ~20-fold elevation in biliary glucose levels by 3 hr. Although we cannot explain the early, acute rise in biliary GSH after DAPM administration, we believe this change to be a valid increase because the Tietze (1969) method for glutathione measurement is both specific and highly sensitive.

DAPM-induced injury to biliary epithelial cells is further supported by the significant effect on bile flow which had

diminished by more than 60% at 4 hr. If the dramatic decrease in bile flow between Hours 3 and 4 (Fig. 4a) is representative of injury progression, complete cholestasis would be expected to occur at approximately 6 hr after DAPM administration. This premise is supported by the finding that only two of five animals cannulated at 6 hr were producing bile.

DAPM injury to hepatic parenchyma appears secondary to injury of biliary epithelial cells for several reasons. First, DAPM had no effect on histochemical staining of hepatocyte plasma and canalicular membrane-localized enzymes at 8 hr when GGT staining in biliary epithelial cells was absent or diminished. Second, focal histological alterations in hepatocytes were not observed until 24 hr after DAPM treatment. Third, the modest increases in serum ALT and glucose values at 4 hr (Table 1) suggest a minimal injurious effect of DAPM on hepatic parenchyma. The more slowly manifest increase in serum ALP activity could be related to injury of either biliary epithelial cells or hepatocytes since ALP can be histochemically demonstrated in bile duct cells and less strongly in portal hepatocytes (Desmet *et al.*, 1968; Hagerstrand and Norden, 1972). A more likely explanation for the slow increase in serum ALP activity is that the early cholestasis caused by DAPM elevates bile acid content in hepatocytes which in turn stimulates hepatocellular ALP synthesis with subsequent release of ALP into serum (Ogawa *et al.*, 1990). Because subtle injury is not evident at the light microscope level, ultrastructural studies are required to verify our observations that DAPM induces deleterious effects to biliary epithelial cells before hepatocytes.

A similar pattern of liver injury has also been observed with the hepatotoxicant, α-naphthylisothiocyanate (ANIT), at doses of 100–300 mg/kg. By 6 hr after oral administration of ANIT to rats, edema with mild inflammatory infiltrate is present in some portal triads of the liver and a loss of histochemical staining for GGT is observed in biliary epithelial cells (McLean and Rees, 1958; Connolly *et al.*, 1988). Histological evidence of injury to biliary epithelial cells is observed 8 hr after ANIT (Connolly *et al.*, 1988) while hyperbilirubinemia and elevated serum ALT values occur by 12 hr (Indacochea-Redmond and Plaa, 1971; Drew and Priestly, 1976). Most bile ducts are destroyed by 24 hr and the biliary tracts show moderate to marked degrees of edema and inflammatory infiltrate (Goldfarb *et al.*, 1962; McLean and Rees, 1958). Focal parenchymal injury is also observed at 24 hr (Goldfarb *et al.*, 1962; Connolly *et al.*, 1988).

A major difference between our results with DAPM and previous studies with ANIT is that DAPM causes more rapid alterations in bile flow and effects on biliary constituents than ANIT at a similar dose. When isolated perfused livers from rats treated with 250 mg ANIT/kg were examined at intervals after ANIT administration, a choleretic effect was observed between 2 to 4 hr while bile flow began to decrease at 9 hr (Krell *et al.*, 1982; 1987). Taurocholate and sulfo-

TABLE 3
Effect of Methylene Dianiline Treatment on Biliary Excretion of Protein and Glucose

	% Basal excretion			
	Protein		Glucose	
	Control	DAPM	Control	DAPM
Hour 1	127 ± 8	523 ± 50**	149 ± 8	315 ± 72
Hour 2	125 ± 7	528 ± 92**	157 ± 18	884 ± 182*
Hour 3	145 ± 10	466 ± 85**	168 ± 6	2142 ± 481**
Hour 4	139 ± 15	394 ± 123*	148 ± 27	1593 ± 571**
Basal excretion:	1.9 ± 0.1 μg/min · kg	1.9 ± 0.1 μg/min · kg	7.4 ± 0.8 μg/min · kg	6.5 ± 0.5 μg/min · kg

Note. Values are means and SEM of four animals per group.
* $p < 0.05$ compared to control group.
** $p < 0.01$ compared to control group.

TABLE 2
Effect of Methylene Dianiline Treatment on Biliary Excretion of Bile Salt, Bilirubin, and GSH

	% Basal excretion					
	Bile salt		Bilirubin		GSH	
	Control	DAPM	Control	DAPM	Control	DAPM
Hour 1	113 ± 3	114 ± 25	120 ± 2	133 ± 5	88 ± 5	305 ± 24**
Hour 2	102 ± 9	82 ± 16	120 ± 2	92 ± 6	92 ± 3	127 ± 21*
Hour 3	91 ± 20	55 ± 10**	119 ± 7	69 ± 10**	85 ± 2	10 ± 3**
Hour 4	89 ± 13	19 ± 7**	123 ± 4	35 ± 14**	74 ± 4	0.1 ± 0.1**
Basal excretion:	2.1 ± 0.1 μmol/min · kg	2.3 ± 0.1 μmol/min · kg	3.0 ± 0.3 μg/min · kg	2.6 ± 0.3 μg/min · kg	112 ± 7 nmol/min · kg	117 ± 11 nmol/min · kg

Note. Values are means and SEM of four animals per group.
* $p < 0.05$ compared to control group.
** $p < 0.01$ compared to control group.

bromophthalein secretion rates decreased between 4 and 7 hr and alterations in individual protein secretion rates occurred by 5 hr at doses of 250 and 300 mg/kg, respectively (Krell *et al.*, 1982; Connolly *et al.*, 1988). In *in vivo* experiments with 250 mg ANIT/kg, cholestasis was present in 50% of rats by 16 hr and in all rats by 24 hr (Lock *et al.*, 1982; Indacochea-Redmond and Plaa, 1971). In contrast, our findings indicate that 250 mg DAPM/kg induces a choleretic effect by 30 min, decreases bile flow by 4 hr, and alters biliary solute secretion rates between 1 and 3 hr.

Although we found only moderate numbers of inflammatory cells in portal triads at 24 hr after DAPM, a preliminary report of DAPM hepatotoxicity described marked polymorphonuclear leukocyte infiltrates in portal triads 24 hr after DAPM administration (Bailie and Roth, 1992). Studies by Roth and colleagues indicate that inflammatory cells may play a causal role in the hepatotoxicity of both ANIT and DAPM since reduction of the circulating numbers of neutrophils in rats provided partial protection against ANIT hepatotoxicity (Roth and Hewett, 1990; Dahm *et al.*, 1991) and pretreatment of rats with pentoxifylline, which reduces cytokine activation of neutrophils (Sullivan *et al.*, 1988), provided partial protection against DAPM hepatotoxicity (Bailie and Roth, 1992).

In DAPM-treated rats, we also observed a marked loss of cortical thymocytes over 24 hr. Involution of the thymus can be caused by several mechanisms including direct toxicity, hormonal imbalance, and/or immunosuppression (Dean and Murray, 1991; Knight *et al.*, 1987). The mechanism by which DAPM affects the thymus cannot be proposed until in-depth functional studies of the immune system are completed. One interesting note regarding immunotoxicity, however, is that DAPM, as well as other resins and plasticizers, are known to induce hypersensitivity reactions in humans (Emmet, 1976; Van Joost *et al.*, 1987; Picardo *et al.*, 1990; Dean and Murray, 1991).

The high susceptibility of biliary epithelial cells to DAPM injury suggests that a major route of excretion of DAPM metabolites is into bile. One unsubstantiated study (Kopelman *et al.*, 1966, personal communication to authors) reports that 25% of an ip dose of DAPM to rats appears in bile within 24 hr. In our study, amounts of aromatic amine compounds in bile of DAPM-treated rats were sufficiently high to produce serious artifacts in our attempted measurement of biliary GGT by an assay that monitors the liberation of *p*-nitroaniline from γ-glutamyl-*p*-nitroaniline (Szasz, 1969). Further studies are needed to determine if the biliary epithelial cell injury induced by DAPM is due to enhanced accumulation of the parent DAPM compound or a specific conjugate in bile, similar to a mechanism recently proposed for ANIT injury (Carpenter-Deyo *et al.*, 1991).

In conclusion, this study has demonstrated that DAPM is selectively toxic to epithelial cells of the major bile ducts and thus should be classified, like ANIT, as a cholangiodestructive agent (Cullen and Ruebner, 1991). DAPM rapidly induces profound biliary dysfunction as evidenced by the striking alterations in bile flow and in biliary bile salt, bilirubin, glutathione, protein, and glucose concentrations. Further studies are needed to characterize the hepatic metabolism and biliary excretion of DAPM.

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