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1,1-Dichloroethylene: An Apoptotic Hepatotoxin?

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Within 2 hr after 1,1-dichloroethylene administration, the following phenomena occur in livers of fasted rats: dilation and disruption of bile canaliculi, plasma membrane invagination and loss of microvilli, cytoplasmic vacuolation, and loss of density in mitochondrial matrices. Early, selective loss of enzyme activities was localized by histochemical staining to bile canalicular, and inner and outer mitochondrial membranes. Biliary permeability to inulin increased, a change suggestive of the breakdown of junctions between hepatocytes. Endoplasmic reticulum and lysosomes appeared spared. In addition, scattered, individual hepatocytes exhibited changes characteristic of apoptosis by 2 hr: chromatin aggregation and margination, nucleolar coarse granulation and enlargement, rounded blebs and proturberances on cell surfaces, and the separation of these cells from surrounding parenchyma. In contrast, evidence of plasma membrane leakiness to K^+, Ca^{2+} and soluble cytoplasmic enzymes was not detected until after 2 hr. Based on these observations, we propose that 1,1-dichloroethylene may initiate apoptosis-like cell degradation in selected parenchymal cells prior to or coincident with centrolobular necrosis.

Apoptosis is a rapid type of cell death which is considered by Kerr and colleagues (*1,2*) to differ distinctively from coagulative necrosis. Major morphologic characteristics of the early stages of apoptosis versus necrosis are summarized in Table 1. Unlike coagulative necrosis, cells undergoing apoptosis do not show evidence of either increased plasma membrane permeability or loss of intracellular ATP until after the characteristic early morphologic changes have appeared (*2*). During apoptosis, individual cells condense and form surface blebs containing intact organelles. The blebs are pinched off and the "apoptotic bodies" are phagocytized and digested by neighboring cells (*2*). In necrosis, groups of cells are affected by characteristic swelling prior to the breakdown of plasma and organelle membranes which leads to cell disintegration (*2*). The two processes may both occur in the same tissue but be separated temporally or spatially; for example, after portal vein ligation, Kerr (*3*) found coagulative necrosis in liver centrolobular zones concomitant with apoptosis in scattered cells around the necrotic areas.

Mechanisms underlying the process of apoptosis are uncertain; although there is some evidence for an active initiation of coordinated events within cells destined for apoptosis (*2,4*). Wyllie et al. (*4*) suggest that apoptosis is a selective removal of cells to insure the survival of the organism itself. Apoptosis has been observed in tissues during embryonic development, metamorphosis and neoplasia; as a response of cells or tissues to abrupt hormonal changes; after attachment of "allergized" T lymphocytes and following exposure to ionizing radiation and certain radiomimetic-chemotherapeutic agents (*1,2*). However, morphologic changes characteristic of apoptosis have not previously been described following poisoning with a halogenated hydrocarbon.

1,1-Dichloroethylene (1,1-DCE) is a model hepatotoxin which produces morphological alterations in fasted animals that are strikingly different from those produced by the classic hepatotoxin, carbon tetrachloride (*5*). In this paper we describe structural, functional and biochemical changes that occur in the livers of fasted rats after 1,1-DCE poisoning. Emphasis is given to the early changes found during the first 2 hr after 1,1-DCE administration, since, by 4 hr, centrolobular hepatocytes are largely replaced by masses of ruptured membranes, thrombi and dead cells (*5,6*).

These studies were conducted with fasted male Sprague-Dawley rats (225–375 g) given 200 mg 1,1,-DCE/kg in mineral oil, PO, between 9 and 11 AM. Control rats received only mineral oil.

Ultrastructural Changes

One hour after administration of 200 mg 1,1-DCE/kg, centrolobular hepatocytes show slight morphological alterations (Fig. 1). Nearly all bile canaliculi are enlarged and filled with microvilli, and small vacuoles containing membrane whorls are often present adjacent to or

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Table 1. Morphologic characteristics of apoptosis and coagulative necrosis.[a]

Apoptosis	Coagulative necrosis
Involves scattered cells	Involves groups of contiguous cells
Chromatin marginates in condensed, coarsely granular aggregates which are confluent over nucleus or form large crescentric caps	Chromatin marginates in small, coarsely textured aggregates
Nucleolus enlarges with coarse, scattered granules present	Nucleolus occurs as a compact body until degradation is advanced
Nuclear membrane convolutes, later indents and nucleus disperses into dense masses	Nuclear membrane retains pore structure as degradation occurs
Cytoplasm compacts, translucent vacuoles appear and microvilli disappear	Cytoplasm swells in all compartments
Cytoplasmic organelles retain integrity	Mitochondria and endoplasmic reticulum swell and mitochondrial matrices lose density
Affected cells separate from their neighbors and form surface proturberances which break off to form characteristic apoptotic bodies	Affected cells rupture with breakdown of all membranes

[a]From Wyllie et al. (2).

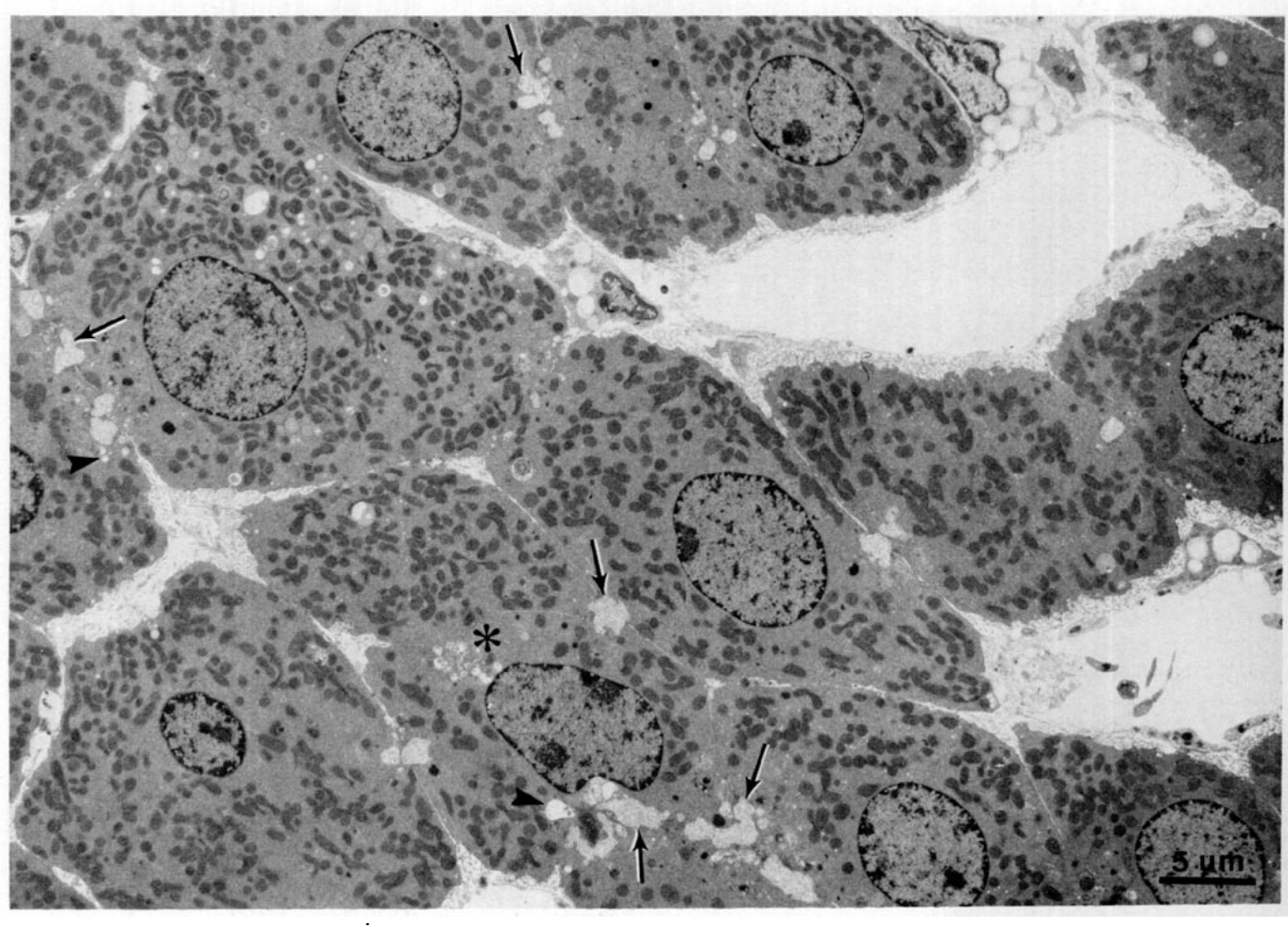

FIGURE 1. Centrolobular liver parenchyma of fasted rat 1 hr after 200 mg 1,1-DCE/kg. Tissue was prepared by *in situ* perfusion first with saline containing 1 mg/L isoproterenol (a vasodilator) at 37°C, followed by perfusion with iso-osmolar 1% glutaraldehyde in 0.1 M PIPES buffer (Sigma), pH 7.4, at 37°C. Cubes of fixed liver were postfixed in 1% osmium tetroxide and saturated uranyl acetate, and embedded in epoxy resins. Early alterations include enlarged bile canaliculi (arrows) with increased numbers of microvilli, small vacuoles (arrow heads) adjacent to or contiguous with bile canaliculi membranes and dilated Golgi (asterisk). Note that mitochondrial matrices are denser than the cytoplasm. ×2800.

continuous with bile canalicular membranes. Occasional hepatocytes exhibit dilated Golgi vesicles and/or vacuoles in the perinuclear region. Typical arrays of smooth and rough endoplasmic reticulum are observed and mitochondria have dense matrices.

Two hours after 200 mg 1,1-DCE/kg, centrolobular to midzonal hepatocytes have retracted cell borders with a loss of microvilli (Fig. 2). Bile canaliculi are dilated, have irregular outlines, and contain membrane fragments and disrupted microvilli. Nearly all mitochondria are swollen, with matrices paler than the adjacent cytoplasm (Figs. 2 and 3). Many hepatocytes contain large, odd-shaped vacuolar spaces within which may be seen cell fragments, vesicles, fibrin, platelets and even red blood cells. Deep invaginations of the plasma membrane into the cytoplasm can be observed confluent with the membranes of the large vacuolar spaces (Fig. 3).

Scattered hepatocytes show striking nuclear alterations with chromatin aggregated against the nuclear envelope (Fig. 2), enlarged nucleoli and in some cells, convolution of the nuclear envelope. These cells may also show blebbing of the cell surface, formation of cell fragments [apoptotic-like bodies (*3*)], and separation of cell boundaries from neighboring parenchyma (Fig. 2). The latter morphologic changes are reminiscent of apoptosis as described by Kerr et al. (*7*) in chronic viral hepatitis, where membrane-bound apoptotic bodies were formed and rapidly phagocytosed within "a few hours."

To clarify the relationship of 1,1-DCE administration to apoptosis, the effects of lower doses of 1,1-DCE on fasted rats are being evaluated. Preliminary sampling of liver sections from animals 2 hr after 50 mg 1,1-DCE/kg indicates the presence of scattered hepatocytes with the typical characteristics of apoptosis listed in Table 1 (Kanz, unpublished observations).

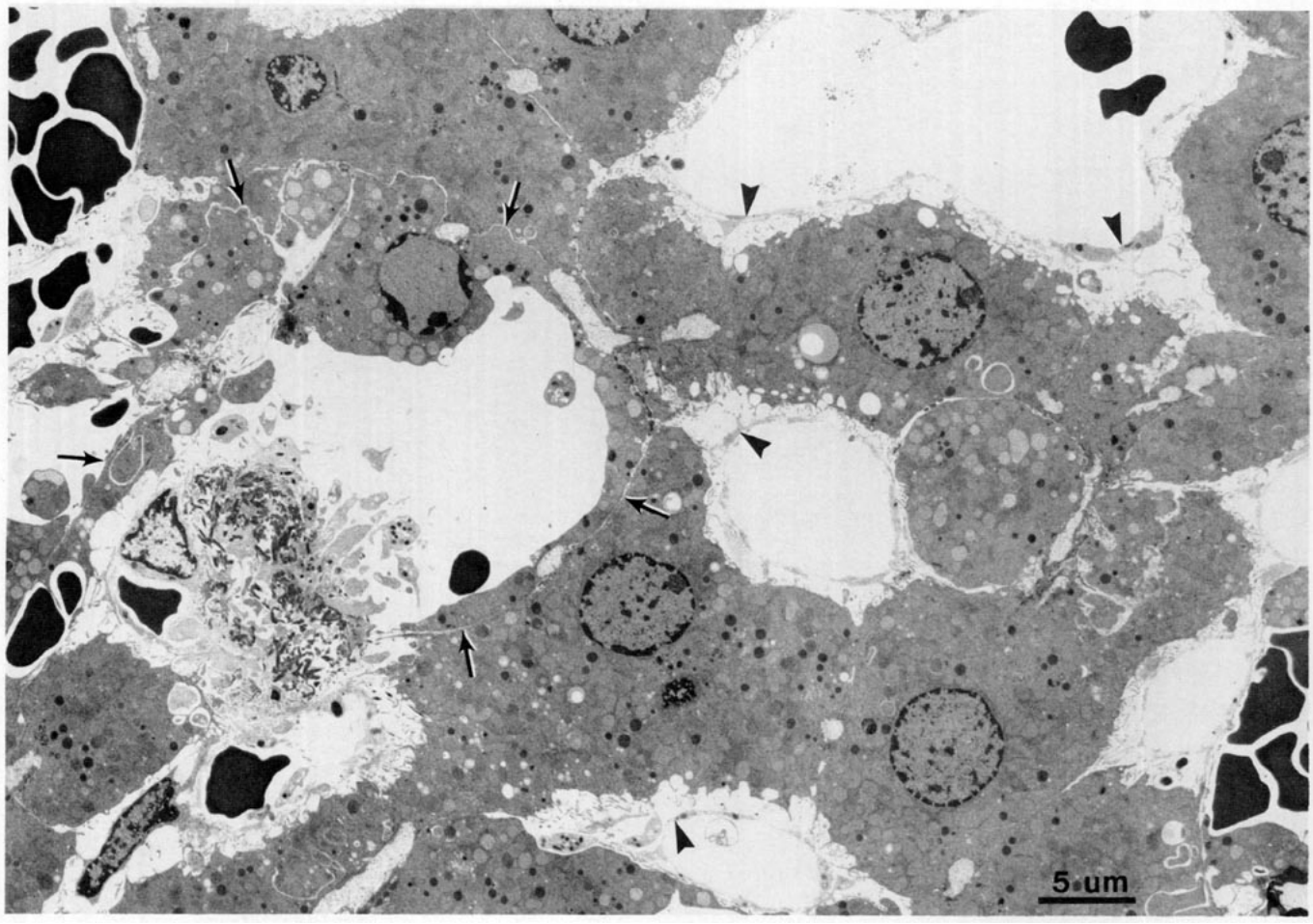

FIGURE 2. Centrolobular liver parenchyma of fasted rat 2 hr after 200 mg 1,1-DCE/kg. Tissue prepared as in Fig. 1. Hepatocytes have fewer microvilli in the space of Disse and cell borders are retracted from the endothelial lining of the sinusoids (arrow heads). The cytoplasm of one hepatocyte (outlined by arrows) is peripherally displaced by a large vacuole whose left margin terminates in a thrombus. The chromatin of this hepatocyte is condensed against the nuclear membrane and the cell surface shows blebbing with the formation of apoptotic-like bodies. A rounded-up hepatocyte separated from its neighbors is at center right. Matrices of many mitochondria are no longer denser than the cytoplasm. ×2700.

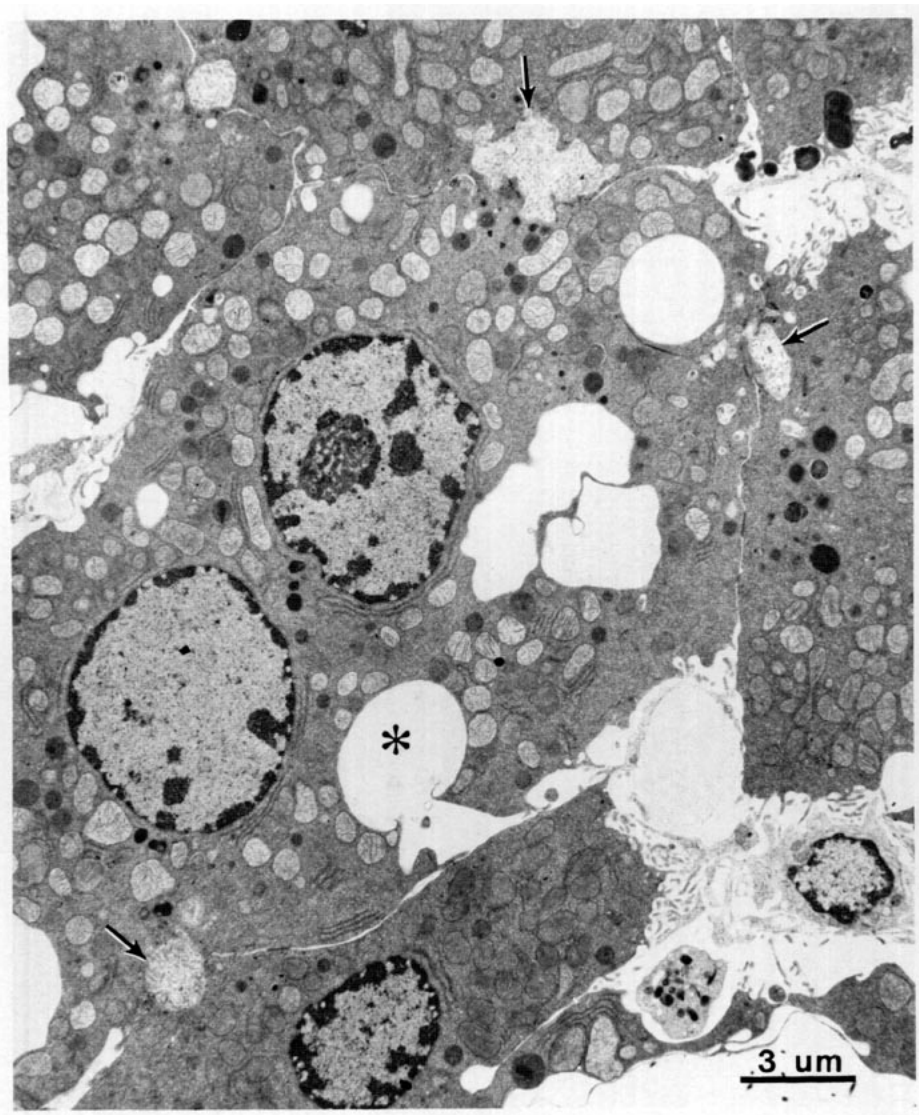

FIGURE 3. Higher power of centrolobular hepatocytes of fasted rat 2 hr after 200 mg 1,1-DCE/kg. Tissue prepared as in Fig. 1. Dilated bile canaliculi (arrows) have irregular outlines with disrupted microvilli. Plasma membrane of hepatocyte in center is deeply invaginated by an irregularly shaped vacuole (asterisk). One nucleus in this cell is slightly convoluted with an enlarged coarsely granulated nucleolus. Rough endoplasmic reticulum is arranged in short, lamellar stacks throughout the cytoplasm. Note that mitochondria are enlarged with translucent matrices. ×5015.

Histochemical Evaluation of Organelle Functional Integrity

Enzymatic histochemistry of quick frozen tissue was used to detect slight or focal alterations in the functional integrity of cell organelles (*6*). Histochemical staining of bile canalicular Mg^{2+}-ATPase and inner mitochondrial membrane succinate dehydrogenase activities (Fig. 4) was diminished 2 hr after 200 mg 1,1-DCE/kg but prior to manifest cell destruction. Staining for outer mitochondrial membrane monoamine oxidase activity was also suppressed by 2 hr. In contrast to these selective changes in canalicular and mitochondrial membrane enzymes, histochemically demonstrable decreases in sinusoidal plasma membrane, mitochondrial matrix, endoplasmic reticulum, and lysosomal and cytosolic enzyme activities were not found until 4 to 6 hr after 1,1-DCE, and then only in regions of gross injury.

Canalicular Permeability and Bile Secretion

Since ultrastructural and histochemical studies indicated prominent early changes in bile canaliculi, the effects of 1,1-DCE on bile volume and biliary permeability to ^{3}H-inulin were investigated (*8*). Inulin is thought to be transferred from plasma to bile primarily by the paracellular route, not the transhepatocyte route (*9*).

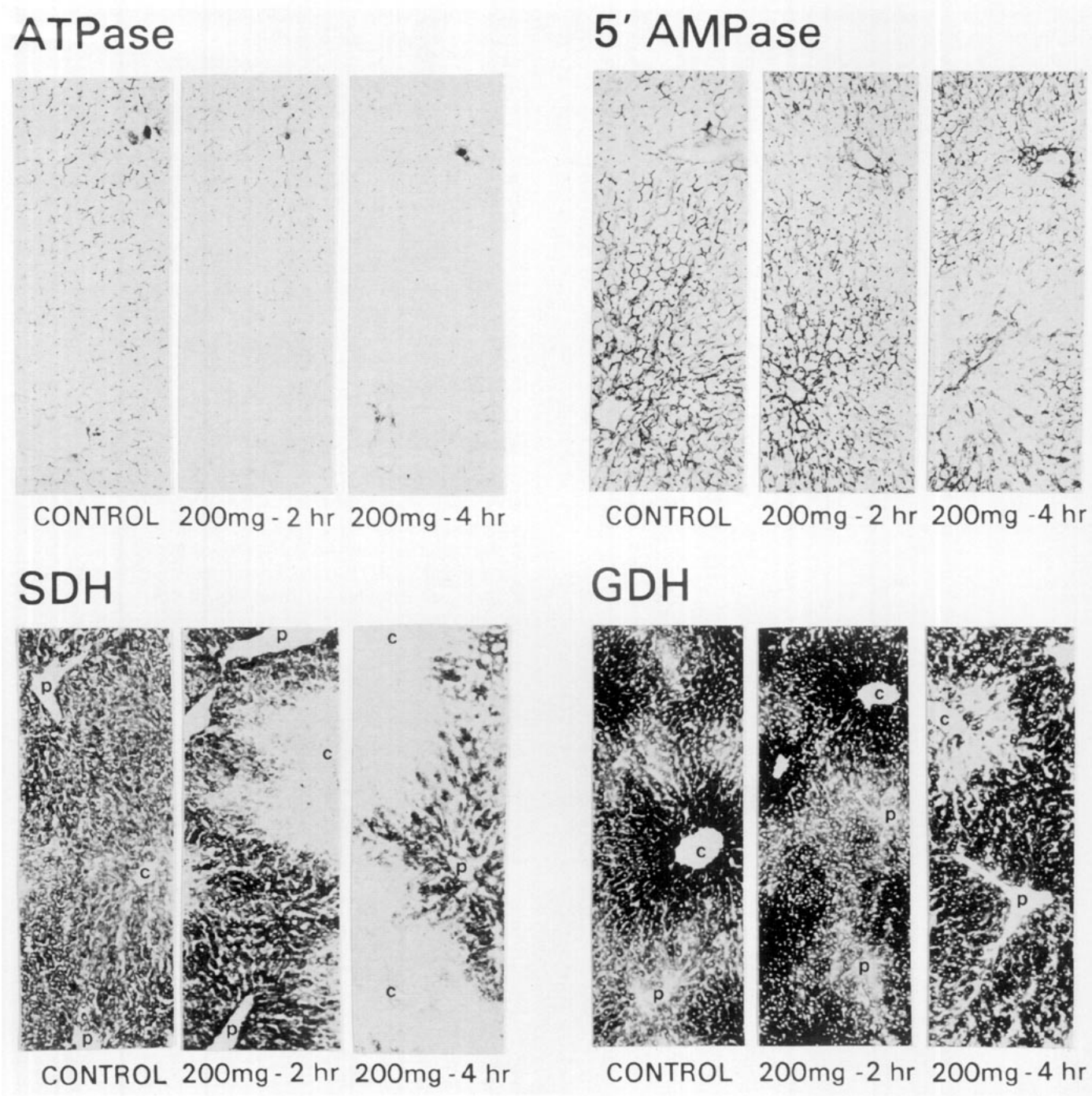

FIGURE 4. Time course of changes in histochemically demonstrable plasma enzymes in fasted rats given 200 mg 1,1-DCE/kg. Staining patterns for (top left) canalicular membrane Mg^{2+}-dependent adenosine triphosphatase (ATPase); (bottom left) sinusoidal plasma membrane 5'-adeosine monophosphatase (5'-AMPase); (top right) mitochondrial inner membrane succinate dehydrogenase (SDH); and (bottom right) mitochondrial matrix glutamate dehydrogenase (GDH). For ATPase and 5'AMPase staining, the portal tract is at upper left and the central vein at lower right of each panel. For SDH and GDH staining, p and c indicate portal tract and central vein, respectively. At 2 hr, the centrolobular and midzonal staining activities of ATPase and the centrolobular activity of SDH were markedly decreased. Decreases in the centrolobular staining for 5'AMPase and for GDH were not seen until 4 hr after 1,1-DCE. ATPase and 5'AMPase, ×70; SDH and GDH, ×35. Modified from Chieco et al. (*6*).

As shown in Figure 5, rates of bile secretion, relatively stable during the hour before 1,1-DCE or mineral oil administration, gradually declined in the control rats while precipitously declining in the 1,1-DCE-treated rats. Biliary inulin clearance, as assessed by the bile/plasma inulin ratio, rapidly increased between 1

and 2 hr after 1,1-DCE. This increased concentration of inulin in the bile of 1,1-DCE-treated animals could be due to an increased transfer of inulin to bile canaliculi through hepatocytes with "leaky" plasma membranes. However, the liver/plasma inulin ratios of the control and 1,1-DCE-treated groups (0.33 ± 0.02 and 0.37 ± 0.02, respectively) were not appreciably different at 4 hr. Alternatively, the increased biliary inulin concentration could be due to an increased transfer of inulin across the paracellular route, possibly due to weakening of the semipermeable junctions that form the boundary between canalicular and lateral intercellular spaces of neighboring hepatocytes. Increased biliary clearance of inulin by this latter route may be related to the observed retraction of cell borders at early times after 1,1-DCE administration (Figs. 1 and 2).

Plasma Membrane Permeability

Serum activities of liver-derived transaminases were not increased above control values (Fig. 6) either by 1 hr after 1,1-DCE administration when morphologic alterations were relatively slight (Fig. 1) or at 2 hr when morphologic alterations became prominent (Fig. 2). The serum transaminase activities increased abruptly between 2 and 3 hr. Prior studies of the time-course of liver metal alterations (*5,6*) indicated that liver sodium content increased during the first hour after 1,1-DCE, whereas liver calcium content did not increase until after 2 hr, concomitant with decreases in liver potassium and magnesium. This early solitary rise in sodium may be due to the observed formation of vacuoles and plasma membrane invaginations (Figs. 2 and 3) which presumably contain sodium-rich plasma. The absence of increases in serum activities of liver-derived enzymes and of alterations in liver metal contents (other than sodium) until times later than 2 hr after 1,1-DCE administration suggests that the plasma membranes of the majority of liver cells retain their integrity during the initial 2-hr period.

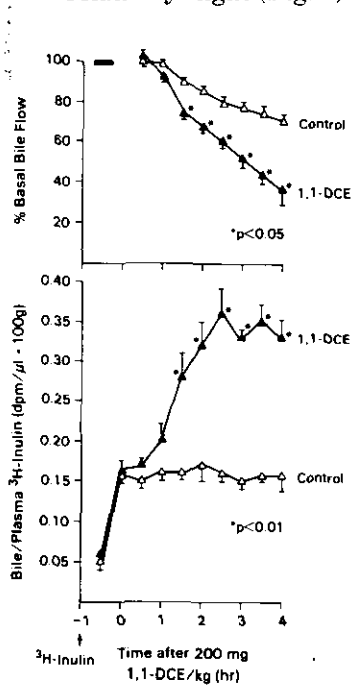

FIGURE 5. Time course of (top) decreases in bile flow and (bottom) increases in bile/plasma ^{3}H-inulin ratio in fasted rats given 200 mg 1,1-DCE/kg or the mineral oil vehicle. Rats were briefly anesthetized with ether and their bile ducts and jugular veins cannulated. Renal pedicles were ligated to prevent renal clearance of inulin. At 1 hr after surgery, freely moving rats were injected with 1 μCi ^{3}H-inulin through the jugular cannula. One hour later, rats were given 200 mg 1,1-DCE/kg or mineral oil. Blood and bile samples were collected at 30-min intervals for ^{3}H-inulin measurement. Changes in the bile flow rate of individual animals were evaluated in comparison to the relatively stable "basal" flow rate during the hour prior to 1,1-DCE or mineral oil treatment. Values are given as the mean ± SE of five to seven animals. Asterisks mark values of 1,1-DCE-treated animals significantly different from control animal values at the same time point.

Biochemical Alterations

Metabolism of 1,1-DCE by the liver apparently occurs by a two-phase process with an initial NADPH–cytochrome P-450-mediated activation of the compound to electrophilic intermediates (*10*), followed mainly by a glutathione (GSH) S-transferase mediated detoxification of these intermediates. Products of GSH conjugation are the major urinary metabolites of 1,1-DCE (*11*). Liver GSH contents rapidly decline during the first 2 hr after 1,1-DCE administration, but, by 4 hr, have begun

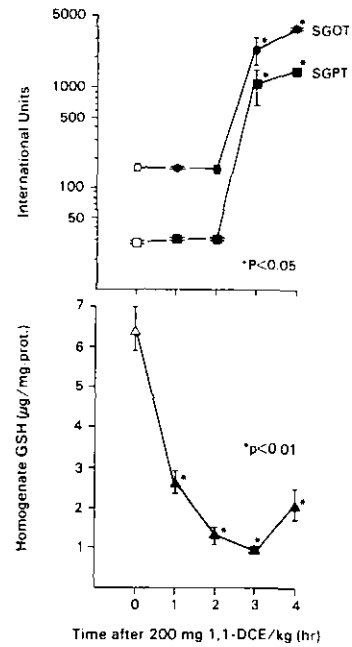

FIGURE 6. Time course of (top) increases in serum activities of glutamate-oxalacetate transaminase (SGOT) and glutamate-pyruvate transaminase (SGPT) and (bottom) decreases in liver reduced glutathione (GSH) in fasted rats given 200 mg 1,1-DCE/kg. Values are means ± SE of four animals. Asterisks mark values significantly different from the zero time control values. Assays were conducted as detailed in Botti et al. (*14*).

to rebound towards control values (Fig. 6). This early loss of liver GSH content is consistent with extensive formation of conjugates between GSH and 1,1-DCE intermediates.

However, the GSH conjugation process does not detoxify all the reactive 1,1-DCE intermediates formed in animals given 200 mg 1,1-DCE/kg. Experiments using ^{14}C-1,1-DCE demonstrated that ^{14}C-label was recovered bound to hepatic proteins and lipids of fasted animals at 2 and 4 hr after 1,1-DCE administration (Table 2). Lesser amounts of bound ^{14}C-label were recovered in fed animals which are markedly less vulnerable to the hepatotoxicity of 1,1-DCE than fasted animals (*12*). Covalent binding of reactive 1,1-DCE intermediates to vital cell constituents is one possible mechanism for the hepatotoxicity of 1,1-DCE.

Table 2. Recovery of covalently bound label derived from ^{14}C-1,1-DCE in protein and lipids of fasted and fed rats given 200 mg 1,1-DCE/kg.

Nutritional status	Time, hr	Covalent binding, nmole ^{14}C/g liver[a]	
		Lipids	Proteins
Fed	4	52 ± 6	89 ± 9
Fasted	2	115 ± 11*	437 ± 105*
Fasted	4	94 ± 14*	460 ± 97*

[a]Values are mean ± SE of four animals.
*$p < 0.05$ compared to fed animals by Duncan's multiple range test.

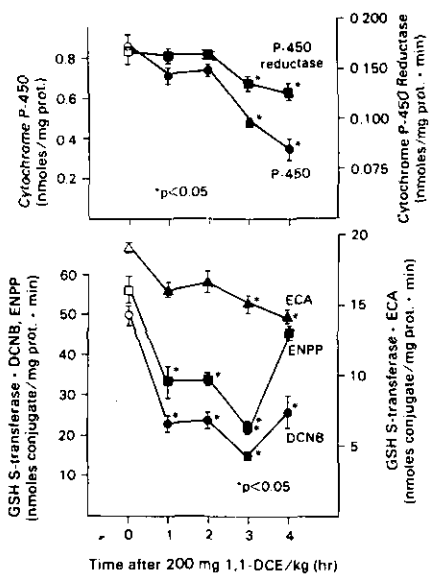

FIGURE 7. Time course of decreases in (top) liver microsomal cytochrome P-450 content and cytochrome P-450 reductase activity and (bottom) in liver cytosolic glutathione S-transferase activities towards 3,4-dichloronitrobenzene (DCNB), 1,2-epoxy-3-(*p*-nitrophenoxy)propane (ENPP), and ethacrynic acid (ECA) in fasted rats given 200 mg 1,1-DCE/kg.

Values are means ± SE of four animals. Asterisks mark values significantly different from the zero time control values. Assays were conducted as detailed in Botti et al. (*14*). Cytosolic glutathione S-transferase activities toward DCNB and ENPP were diminished to about half of control values within 1 hr, while statistically significant changes in cytochrome P-450 and cytochrome P-450 reductase did not occur until 3 hr.

Enzymes potentially involved in the detoxification phase of 1,1-DCE metabolism are deactivated during the first hour after DCE administration (*13*). Liver cytosolic GSH S-transferase activities toward some, but not all, substrates were diminished by 40–55% at 1 hr after 1,1-DCE administration (Fig. 7). Appreciable decreases in cytochrome P-450 content and in the activities of cytochrome P-450 reductase were not seen until 3 hr after 1,1-DCE. The catastrophic hepatotoxicity of 1,1-DCE could be a consequence of the continued capacity of liver cytochrome P-450 enzymes to activate 1,1-DCE to reactive intermediates after liver cells have lost their capability to detoxify 1,1-DCE reactive metabolites through GSH S-transferase-mediated conjugation reactions.

Conclusion

1,1-Dichloroethylene produces cell injury in a characteristic morphologic pattern which proceeds rapidly to classic cell necrosis. In addition, 1,1-DCE appears to elicit a second type of cell death in scattered hepatocytes and at very early times. This selective cell destruction resembles apoptosis with regard to the nuclear changes, surface blebbing and formation of "apoptotic" bodies. The mechanisms by which administration of 1,1-DCE results in different forms of cell death are unknown. The significance of the relationships between the biochemical changes observed during the first 2 hr after 1,1-DCE administration and the appearance of the morphologic phenomena characteristic of apoptosis remains to be delineated.

NOTE ADDED IN PROOF: Recent studies (*15*) indicate that appreciable movement of inulin from blood to bile occurs by rapid vesicular transport across the hepatocyte. Therefore, the increases in biliary inulin observed in the 1,1-DCE-treated animals may not necessarily reflect altered transfer of this marker solute by the paracellular route.

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