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Bromobenzene-Induced Zonal Necrosis in the Hepatic Acinus

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The time course and acinar distribution of bromobenzene-induced hepatic necrosis was studied in the rat. Cellular damage, lipid infiltration, and changes in glycogen deposits were investigated by light microscopy 6, 16, 24, and 48 hr after bromobenzene (BZ) administration. Concomitantly, ultrastructural changes were followed by electron microscopy in each zone of the acinus. To insure accurate orientation in acinar zones, a double embedding technique for electron microscopy was used. Acinar zones were localized by light microscopy and subsequently re-embedded for electron microscopy. Zone 3 was the site of conformational changes in smooth endoplasmic reticulum 6 hr after BZ administration. This condensed, tubular network represented the earliest morphological sign of injury observed by electron microscopy. At 48 hr, cytoplasmic vacuolar degeneration and necrosis were observed in the hepatocytes of acinar zone 3. While no necrosis was observed in the cells of zone 1, other morphological changes occurred. These included progressive lipid accumulation, as well as fluctuations in the amount of rough endoplasmic reticulum and free ribosomes. These latter observations suggested a possible link between protein manufacture and survival of the zone 1 cells. These results established that, following bromobenzene administration, necrosis was restricted to zone 3 hepatocytes.

INTRODUCTION

Bromobenzene (BZ) is a halogenated industrial solvent which induces centrilobular necrosis in the livers of rats and mice (Koch-Weser *et al.*, 1953; Mitchell *et al.*, 1971; Reid *et al.*, 1971; Reid and Krishna, 1973). Tissue injury apparently results from the conversion of BZ to a toxic metabolite, 3,4-bromobenzene oxide, by a cytochrome P-450 system and the subsequent covalent binding of this epoxide to tissue macromolecules (Brodie *et al.*, 1971; Reid, 1973; Reid *et al.*, 1971; Reid and Krishna, 1973). The light microscopic picture (Brodie *et al.*, 1971; Mitchell *et al.*, 1971; Reid *et al.*, 1971) of the resultant zonal necrosis is a striking example of the heterogeneous response of liver cells to toxic injury. Other studies using enzymatic (Shank *et al.*, 1959; Novikoff, 1959) or morphological (Loud, 1968; Jones *et al.*, 1976) criteria have supported the concept that hepatocytes surrounding the terminal portal venule might differ structurally and metabolically from those near the terminal hepatic venule. Rappaport (1973) has defined the areas surrounding these venules as acinar zones 1 and 3, respectively, and has suggested that such liver cell heterogeneity results, in part, from the position of the hepatocytes with respect to the blood supply

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entering this microcirculatory unit. Those studies established the liver acinus as the smallest functional unit of parenchyma. Therefore, the acinus has been chosen as the structural framework for this study.

Many investigators have examined the functional or biochemical aspects of BZ-induced injury. Reid (1973) has used this chemical as a model toxin to determine the mechanisms of drug-induced liver injury. Additional studies in our laboratory (Gumucio *et al.*, 1978a) have employed BZ-damaged livers to establish the role of zone 3 hepatocytes in bile salt secretion. The time course and acinar distribution of liver cell regeneration after toxic injury has also been examined using BZ as a specific zone 3 toxin (Nostrant *et al.*, 1978). In addition, since the epoxide binds covalently to centrilobular cells, BZ has served as a marker for this cell population in isolated hepatocyte preparations (Gumucio *et al.*, 1978b).

In all of these investigations, it was assumed that necrosis was restricted to the centrilobular area. This assumption was based primarily on light microscopic descriptions of BZ-induced damage in the hepatic lobule. However, the acinar distribution of damage and the progression of ultrastructural changes in each zone with time have not yet been systematically studied at the electron microscopic level.

The objective of this study has therefore been to determine the distribution and temporal progression of BZ-induced injury in the hepatic acinus by light (LM) and electron microscopy (EM). To facilitate accurate and reproducible isolation of acinar zones for EM analysis, a double embedding technique has been used. Thus, the ultrastructural events leading to necrosis in zone 3 cells, as well as the morphological changes in the "undamaged" zone 1 hepatocytes, have been examined.

MATERIALS AND METHODS

Experimental Design

Non-fasted female Sprague–Dawley rats weighing 170 to 200 g were used. Animals were injected ip with 0.2 ml BZ (3.8 mmoles/kg, dissolved in corn oil) and sacrificed under sodium pentobarbital anesthesia (0.4 mg/kg, ip) 6, 16, 24, and 48 hr later. Four control rats received corn oil only (0.2 ml, ip). Rats had free access to food and water following the injections. At each time period, three rats were sacrificed for EM and three for LM. A single control rat was also sacrificed. All animals received BZ injections between 9 and 11 AM to minimize the effects of diurnal variations on drug metabolism (Radzialowski and Bousquet, 1967, 1968).

Light Microscopy

Livers were fixed in 10% neutral buffered formalin, and paraffin-embedded tissue sections were stained with PAS. The appearance of cellular damage, inflammation and changes in the intensity of glycogen staining were followed with time in each acinar zone. In addition, the presence and acinar distribution of lipid in 1 μm thick sections of resin-embedded tissue were assessed by LM.

Electron Microscopy

Livers were perfused with 3% glutaraldehyde (Fahimi, 1967) and processed for EM using the double embedding technique described previously (Miller and Gumucio, 1978). In this manner, only terminal portal and terminal hepatic venules less than 40 μm in diameter, the landmarks of acinar zones 1 and 3, respectively, were selected for EM analysis. The criteria used to confirm zone 1 and 3 orientation at the EM level have been described (Rappaport, 1975; Miller and Gumucio, 1978).

One micron thick sections of each venule sampled were stained with 0.1% toluidine blue O for LM examination. For EM, 600 to 800 nm sections were stained with uranyl acetate in 50% ethanol as well as with lead citrate (Reynolds, 1963). Grids were examined and photographed in an AEI Corinth 275 electron microscope using Kodalith 2572 Estar base 70 mm film.

RESULTS

The temporal sequences of morphological changes seen in acinar zones 1 and 3 after BZ administration are summarized in Tables I and II, respectively.

A. Controls

Livers taken at 6 and 16 hr after corn oil administration and stained with PAS revealed slight glycogen depletion in acinar zone 1. However, glycogen deposits in the 24 and 48 hr control livers were equally distributed throughout the cells of the acini. In all control livers, a few scattered lipid droplets were present in the cytoplasm of some zone 3 cells.

As shown in Figs. 1a and b, electron microscopic examination revealed that the relative size and distribution of organelles within each acinar zone were similar in all control livers and appeared to be in agreement with previous observations (Loud, 1968; Jones *et al.*, 1976).

B. Bromobenzene-Treated Rats

1. Light Microscopy

Six hours after the administration of BZ, hepatocytes of all acinar zones contained scattered fat droplets. At 16, 24, and 48 hr, lipid became progressively

TABLE I.

Summary of Ultrastructural Changes Which Occurred in Zone 1 after Bromobenzene Administration

	6 hr	Control	16 hr	Control	24 hr	Control	48 hr	Control
Necrotic cells	0	0	0	0	0	0	0	0
Inflammatory cells	0	0	0	0	0	0	0	0
Lipid	+	0/+	++	0/+	+++	0/+	+++	0/+
Glycogen	++	++	0	0	+	+++	0	+++
RER	++	++	+++	++	++	++	+++	++
Free ribosomes	+	+	++	+	+	+	+++	+

Elements are rated between 0 (none seen) and ++++ (numerous).

TABLE II

Summary of Ultrastructural Changes Which Occurred in Zone 3 after Bromobenzene Administration

	6 hr	Control	16 hr	Control	24 hr	Control	48 hr	Control
Necrotic cells	0	0	0/+	0	++	0	++++	0
Inflammatory cells	0	0	0	0	++	0	++++	0
Lipid	+	0/+	0/+	0/+	0/+	0/+	0/+	0/+
Glycogen	+++	+++	+++	+++	0	+++	0	+++
RER	+++	++	++	++	+	++	+	++
SER	++	++	++	++	+	++	+	++
Free ribosomes	+	+	+	+	+	+	+	+

Elements are rated between 0 (none seen) and ++++ (numerous).

predominant in the cells of zone 1. These periportal accumulations of osmiophilic lipid are demonstrated in Fig. 2a.

The pattern of zone 1 glycogen depletion resembled that of controls at 6 and 16 hr after BZ administration. However, at 24 hr, in contrast to the homogeneous appearance of the control liver, examination of treated livers revealed

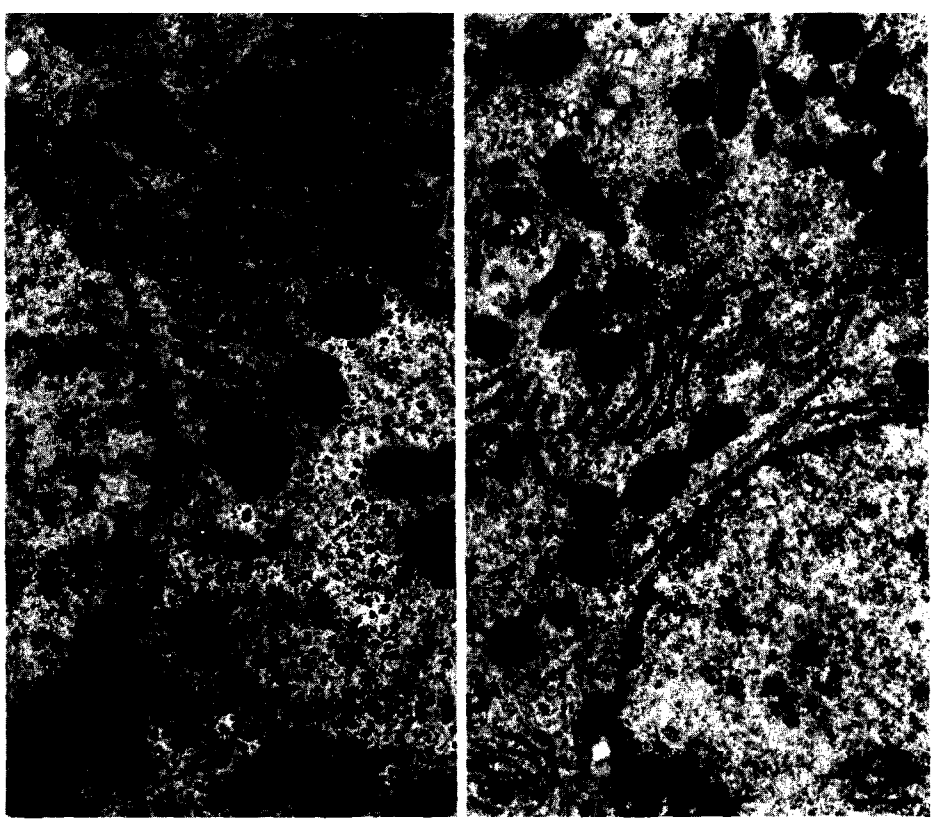

FIG. 1. Control. Electron microscopy. Tissue stained with uranyl acetate and lead citrate. (a) Zone 1 cell, demonstrating characteristically large collections of glycogen rosettes (G). ×9625. Bar = 1 μm. (b) Zone 3 cell. Glycogen rosettes (arrow) are more homogeneously scattered throughout the cytoplasm. Mitochondria appear smaller than in the zone 1 cell. ×9625. Bar = 1 μm.

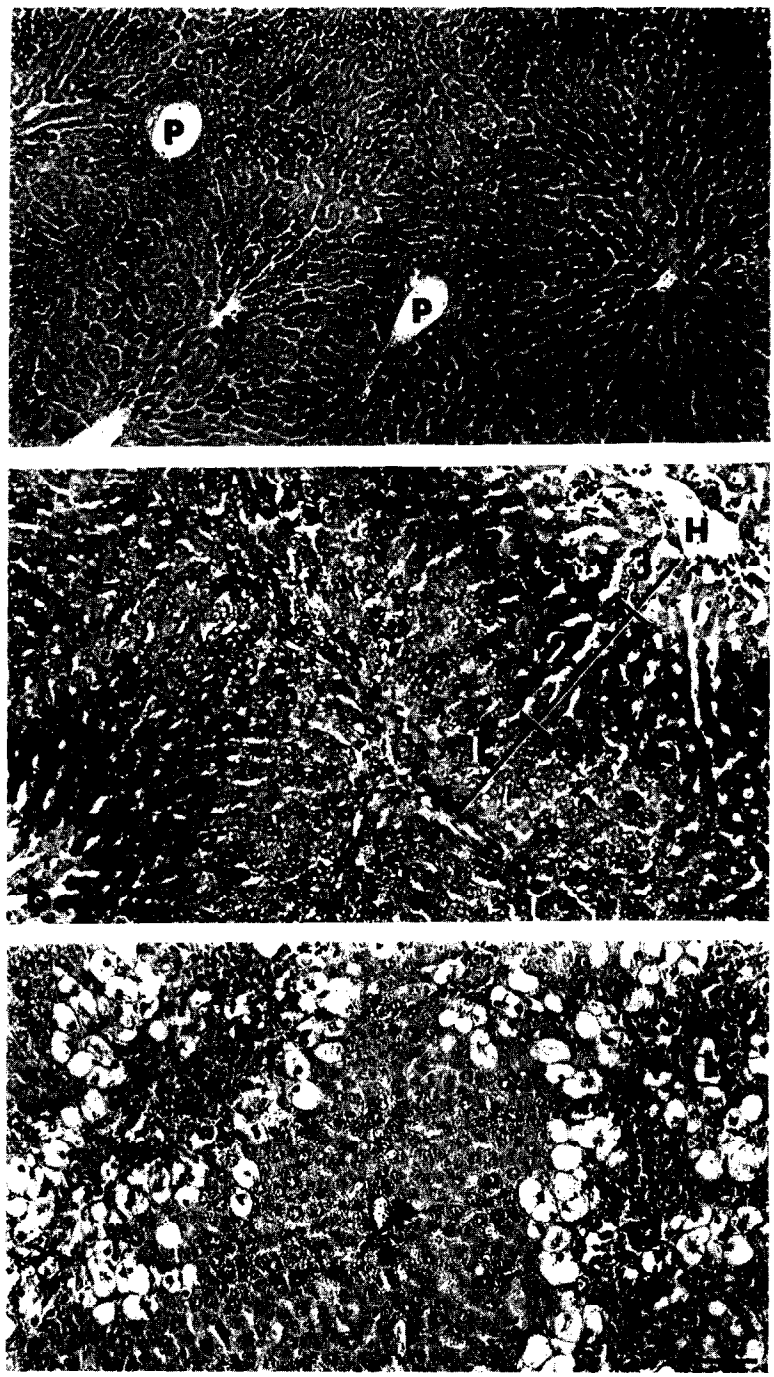

FIG. 2. Light microscopy. (a) Twenty-four hours after BZ administration. A 4 μm thick section of resin-embedded tissue cut on a rotary microtome. The eccentric nature of osmiophilic (black) lipid collections around these larger (100 to 150 μm) portal veins (P) supports the suggestion that areas surrounding these large vessels may be composed of cells from any of the three acinar zones. It is clear that random sampling near such veins may lead to contrasting results (compare the lipid content of areas marked with single and double arrows). No lipid is seen near terminal hepatic venules (H). Unstained tissue post-fixed in osmium

three well-delineated zones (Fig. 2b). Most of the stainable glycogen was restricted to zone 2. While the cells of zone 3 appeared to be completely depleted of glycogen, zone 1 hepatocytes were intermediate in appearance. Forty-eight hours after the administration of BZ, the liver parenchyma was uniformly depleted of glycogen.

Necrotic hepatocytes were first detected by LM 24 hr after the injection of BZ. Damaged cells were observed only in areas immediately surrounding the terminal hepatic venules (zone 3). At 48 hr (Fig. 2c), swelling and vacuolation of zone 3 cells were characteristic. Vacuolated cells often occupied more than one third of the acinus. They were never observed in zone 1. Cytoplasmic vacuoles in the injured cells were not osmiophilic.

2. *Electron Microscopy*

Six hours after BZ administration. As seen in Fig. 3a, the cells of zone 3 contained numerous profiles of rough endoplasmic reticulum (RER). In addition, the smooth endoplasmic reticulum (SER) was tubular and contained electron dense material in the cisternae, lending a mottled appearance to the cytoplasm of these cells near the terminal hepatic venules (Fig. 3b). Zone 1 cells displayed none of these changes (Fig. 3c).

Sixteen hours after BZ administration. Tubular SER was distinct in the cells of zone 3 (Fig. 4a). Occasionally a necrotic hepatocyte bordering the terminal hepatic venule was observed. Glycogen rosettes were present in zone 3 cells, but absent in the hepatocytes of zone 1. Membranes of RER were numerous, and some lipid droplets were seen in the periportal (zone 1) cells (Fig. 4b).

Twenty-four hours after BZ administration. Large, irregular areas of necrosis were seen around terminal hepatic venules (Fig. 5a). Hepatocytes with condensed nuclear chromatin or obvious necrosis were restricted to an area within four to six cells from the venule. Other cells in this zone 3 area exhibited various stages of injury. Those closer to the venule possessed electron dense vacuolated cytoplasm and mitochondria which were thin and elongated. SER was not visible in the dense cytoplasm of these cells. While occasional lymphocytes were seen, macrophages represented the predominant inflammatory element. Surrounding the area of injury and necrosis, hepatocytes with dense, tubular SER and elongated, occasionally doughnut-shaped mitochondria were present (Fig. 5b). Strikingly large collections of glycogen were demonstrated in the cells of zone 2 (Fig. 5c). In zone 1 (Fig. 5d), glycogen rosettes were apparent and the relative number of RER profiles diminished as compared to zone 1 cells of livers examined 16 hours after chemical injection (Fig. 4b).

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tetroxide. ×55. Bar = 100 μm. (b) PAS stained section of paraffin embedded liver tissue 24 hr after BZ administration. Arbitrary division of the parenchyma into three acinar zones is possible. A terminal portal venule (zone 1) courses diagonally through the field. Cells in this area contain moderate amounts of glycogen. Surrounding this are heavy deposits of glycogen in the intermediate or zone 2 cells. Zone 3 cells near the terminal hepatic venules (H) are depleted of glycogen. ×110. Bar = 100 μm. (c) Forty-eight hours after BZ administration. Intact zone 1 cells near a terminal portal venule (P) are nearly encircled by swollen and necrotic cells of nearby zone 3 areas (H). This PAS-stained, paraffin-embedded section shows uniform glycogen depletion across the acinus. ×110. Bar = 100 μm.

Forty-eight hours after BZ administration. Numerous condensed and necrotic cells, cellular debris, and macrophages bordered the terminal hepatic venules of zone 3. Surrounding this area, vacuolated cells were prominent. These vacuoles appeared to arise from dilated RER and nuclear membranes (Fig. 6a). Glycogen rosettes were not detected in hepatocytes of any acinar zone. Numerous profiles of RER membranes and free ribosomes were evident in zone 1 (Fig. 6b). Lipid droplets were also present in these cells. However, no necrosis was observed in the cells of zone 1.

DISCUSSION

The temporal progression and acinar distribution of BZ-induced damage was defined by EM. Certain structural variations noted in each acinar zone at 6 and 16 hr may have been caused, at least in part, by diurnal fluctuations in morphological parameters (Sasse, 1975a; Chedid and Nair, 1972). In preliminary experiments, the time of BZ administration was varied in order to hold constant the time of animal sacrifice. However, this resulted in disproportionately severe necrotic changes and high mortality rates in those animals injected at night. Others have also reported a significant effect of circadian rhythm on drug metabolizing enzyme systems (Radzialowski and Bousquet, 1967, 1968). For this reason, the time of injection was standardized, and the effect of diurnal variations on structure was in part accounted for by the inclusion of a control animal at each experimental point.

Results obtained using this procedure confirmed the zonal distribution of BZ-induced hepatic necrosis. This was confined at all time periods to the cells of zone 3. Some ultrastructural changes were also observed in the hepatocytes of zone 1. Alterations in lipid and glycogen distribution, SER, RER, and free ribosome accumulation, and frank necrotic changes were the significant markers of the tissue response after BZ administration.

Lipid storage increased in zone 1 hepatocytes 16, 24, and 48 hr after BZ administration. Since fat accumulation in this zone was progressive and at all times more prominent than in the corresponding controls, diurnal variations probably did not play a major role in the accumulation of lipid droplets in zone 1. At 6 hr, some lipid was also detected near terminal hepatic venules (zone 3), but its concentration in this area decreased with time. At the time of maximal necrosis, no lipids were observed in zone 3.

Glycogen depletion in acinar zone 1 was seen at 6 and 16 hr in BZ-treated rats as well as in the corresponding controls. These early changes thus probably reflected diurnal variations in glycogen stores (Sasse, 1975a). However, the pattern of glycogen staining differed from the control at 24 hr, when accumulation was noted in zone 2 in treated rats. This was followed by depletion of all acinar zones at 48 hr. Sasse (1975a) has noted that under normal conditions the highest activities of glycogen synthetase and phosphorylase are found in acinar zone 1. Following allyl formate-induced necrosis of acinar zone 1, translocation of these enzyme activities to zone 2 was observed (Sasse, 1975b). The different rates of glycogen depletion and accumulation found in each acinar zone after BZ-induced injury further support the concept of zonal heterogeneity of glycogen metabolism (Sasse *et al.*, 1975).

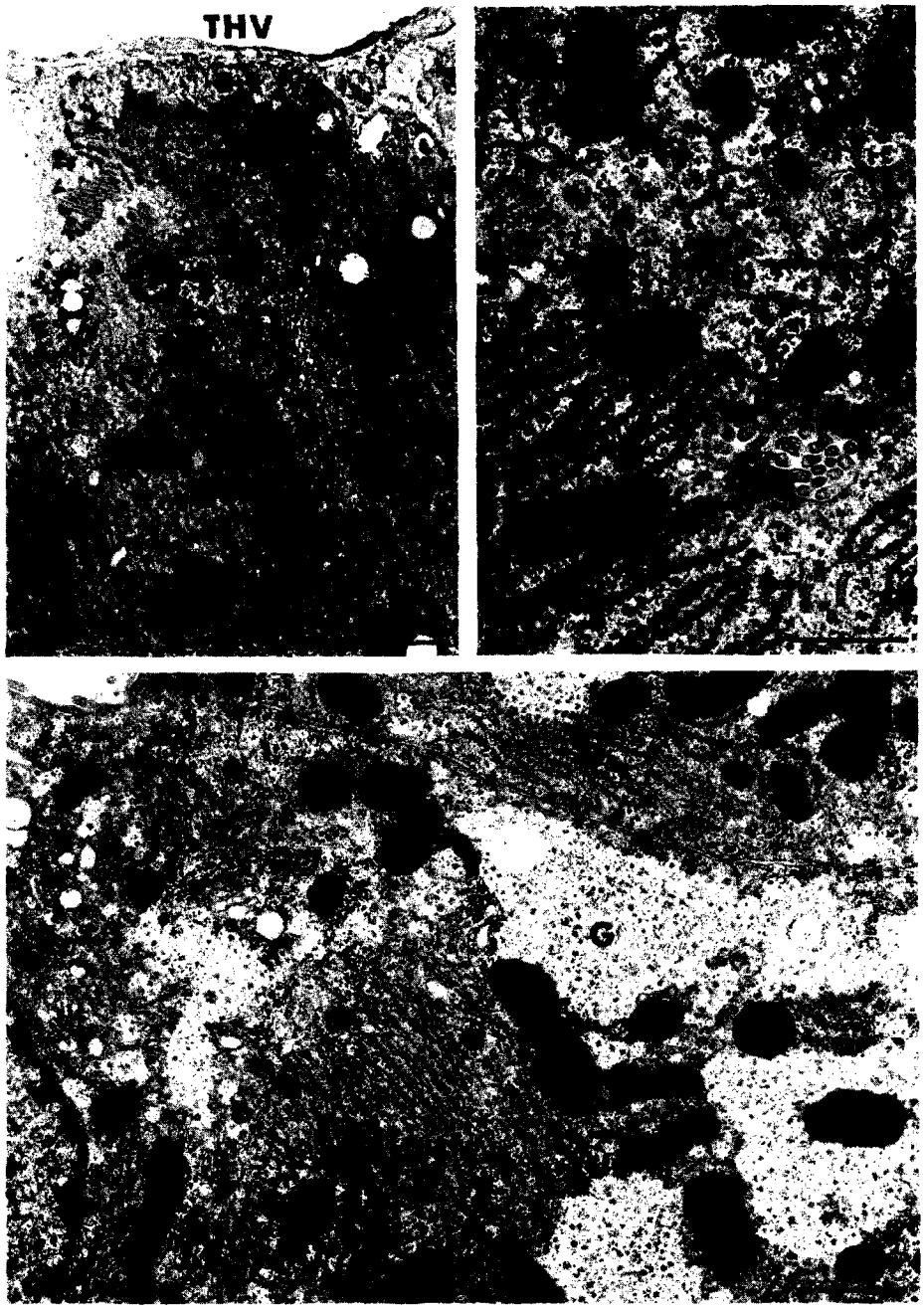

FIG. 3. Electron microscopy. Six hours after BZ administration. Tissue stained with uranyl acetate and lead citrate. (a) Zone 3 cells near the terminal hepatic venule (THV) contain numerous profiles of RER and a few lipid droplets. ×2575. Bar = 5 μm. (b) SER of the zone 3 cells is tubular and condensed, with electron dense cisternal contents (arrow). ×17,160. Bar = 1 μm. (c) Zone 1 cells appear similar to controls. Glycogen collections are intact (G). ×12,400. Bar = 1 μm.

Six hours after BZ administration, SER cisternae of zone 3 cells were tubular and condensed, containing electron dense material. It is proposed that this change is the earliest indicator of hepatocellular injury after BZ administration.

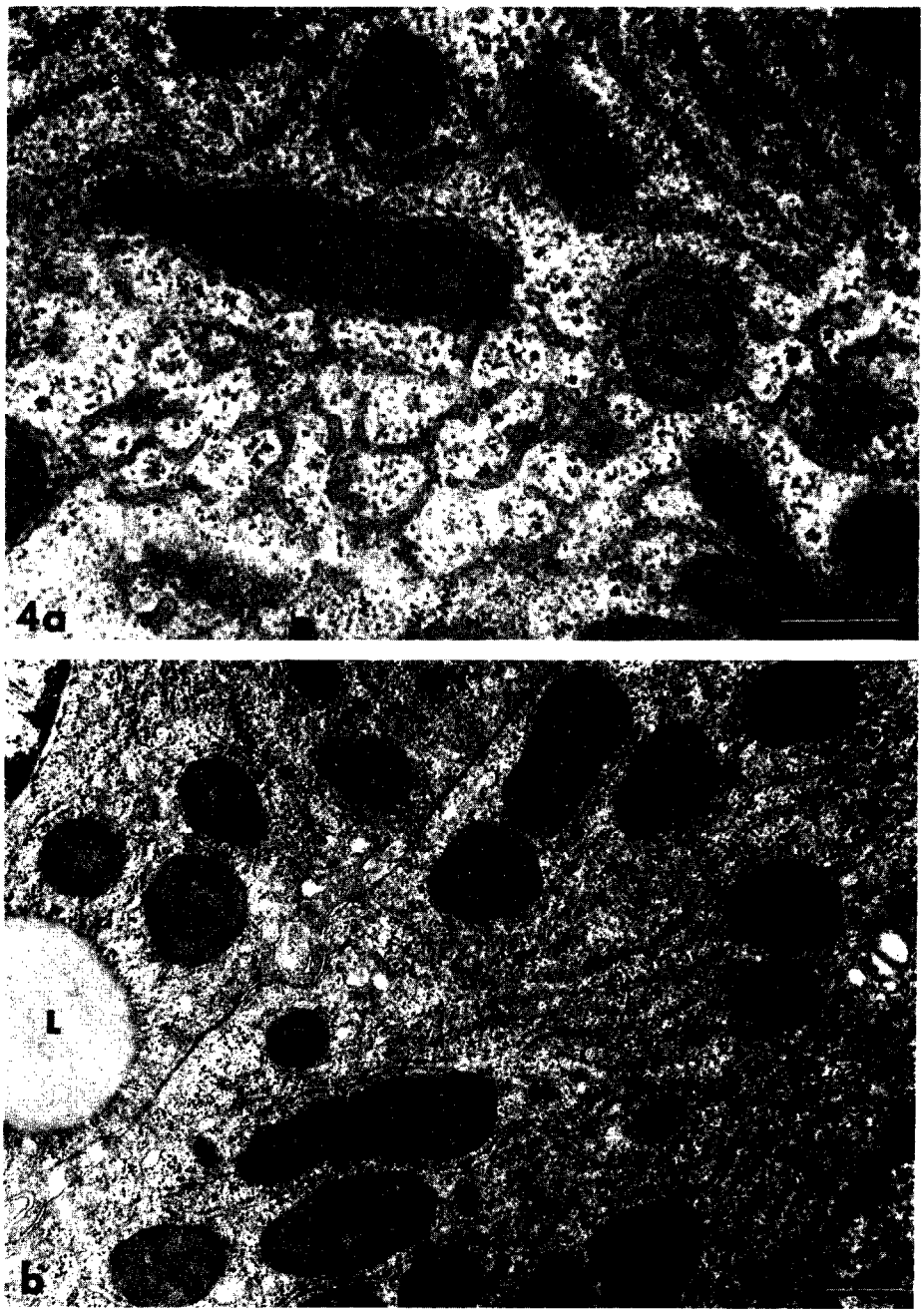

FIG. 4. Sixteen hours after BZ administration. Electron microscopy. Tissue stained with uranyl acetate and lead citrate. (a) Conformational changes in SER characterize the cells of zone 3. Glycogen rosettes (arrow) may be seen associated with condensed SER. Membranes of ER are often closely applied to mitochondria. ×33,000. Bar = 0.5 μm. (b) The cytoplasm of zone 1 cells is filled with RER profiles. Glycogen rosettes are absent. A lipid droplet is present (L). ×22,000. Bar = 0.5 μm.

At 16 hr, alterations in SER involved one third of the acinus. Necrosis and vacuolation were later seen in these cells. In contrast, perturbations in SER conformation were not observed at any time in the cells of acinar zone 1.

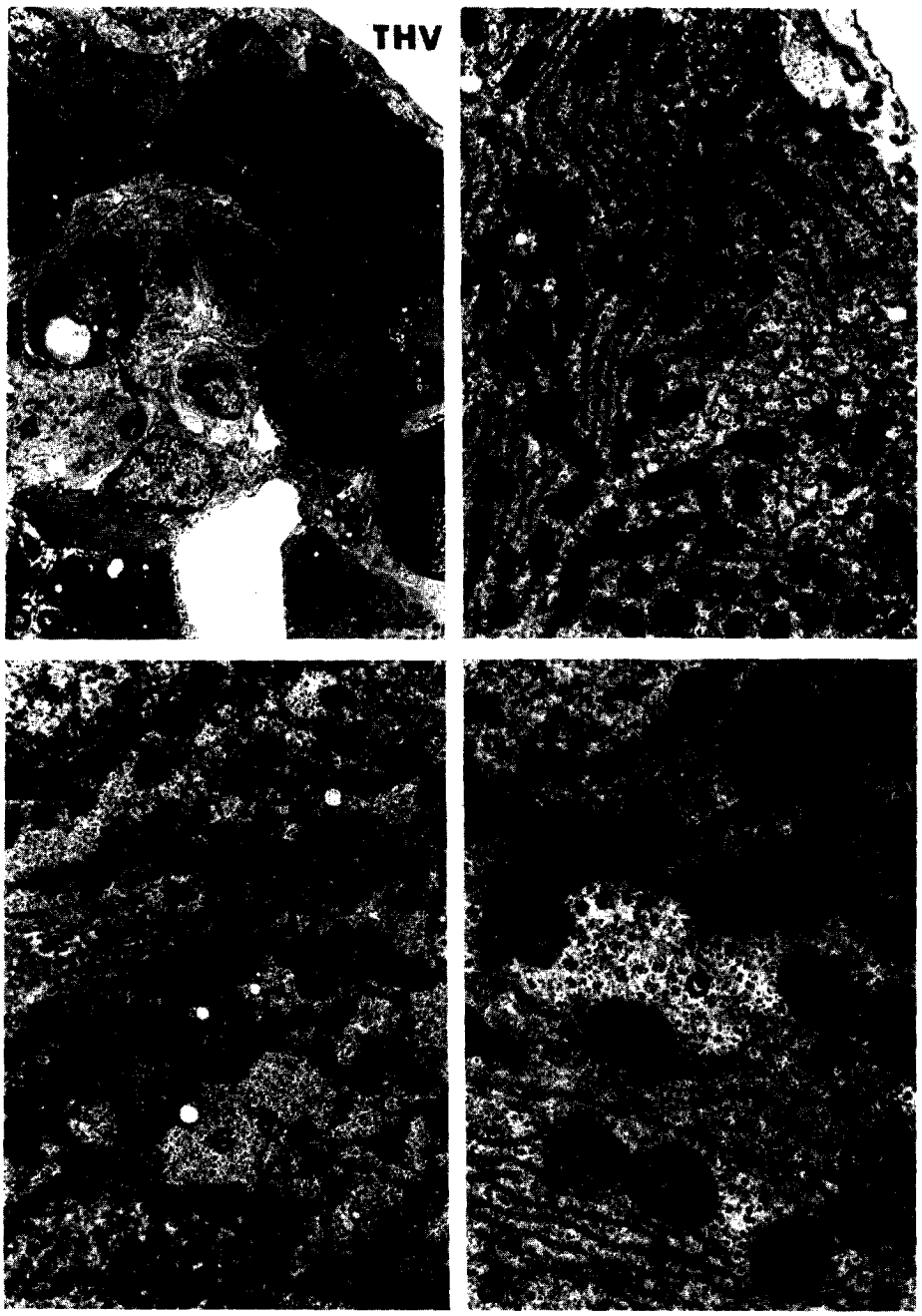

FIG. 5. Twenty-four hours after BZ administration. Electron microscopy. Tissue stained with uranyl acetate and lead citrate. (a) Necrosis around a terminal hepatic venule (THV). Macrophages surround hepatocytic debris (arrow). ×2200. Bar = 5 μm. (b) Cells surrounding the area of necrosis in zone 3 exhibit elongated or doughnut-shaped mitochondria and condensed SER. ×8800. Bar = 1 μm. (c) Large collections of glycogen (G) occupy zone 2 cells. ×5500. Bar = 2 μm. (d) Zone 1 cell. Glycogen (G) is present and the apparent number of RER profiles is comparable to control cells. ×13,200. Bar = 1 μm.

RER accumulation occurred at 6 hr in zone 3 and at 16 and 48 hr in zone 1. Chedid and Nair (1972) have demonstrated that the amount of RER does not vary significantly with respect to the time of day. Thus, these structural findings

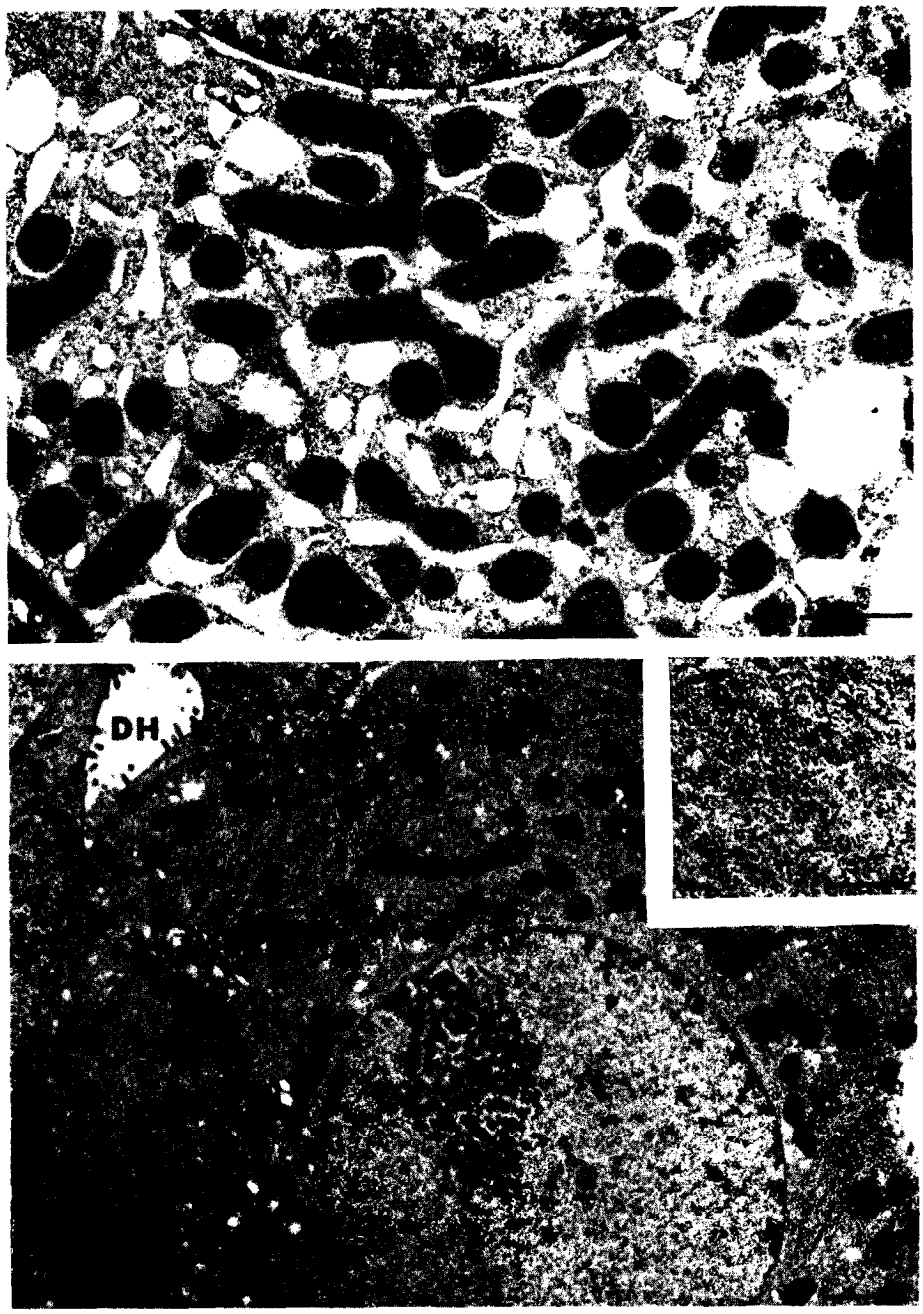

FIG. 6. Forty-eight hours after BZ administration. Electron microscopy. Tissue stained with uranyl acetate and lead citrate. (a) Zone 3 cell. Characteristic vacuoles appear to arise from dilated RER and nuclear membranes. ×15,400. Bar = 1 μm. (b) A zone 1 cell is identified by its membrane contribution to the duct of Hering (DH). Glycogen is absent. The cell is densely filled with RER and free ribosomes (inset). ×7000. Bar = 1 μm. Inset: ×22,200. Bar = 0.5 μm.

suggest that after BZ administration the rates of protein synthesis in each acinar zone might differ.

It was also noted that the number of free ribosomes increased in zones 1 and 2 at 48 hr. Free ribosomes probably participate primarily in the manufacture

of endogenous protein, while the RER is thought to be associated predominantly with the synthesis of proteins destined for transport (Ghadially, 1975). Gillette (1975) has suggested that effective turnover of endogenous protein in zone 1 allows those cells to survive BZ intoxication, while the cells of zone 3, unable to replace damaged intracellular proteins, undergo necrosis. The presence of numerous ribosomes in uninjured cells supports the hypothesis that a successful switch to endogenous protein manufacture may be related to the survival of cells of zones 1 and 2. In addition, Koudstaal (1970) proposed that zone 3 hepatocytes contained higher levels of mono oxygenases than cells of zone 1. This might result in higher concentrations of epoxide in zone 3 hepatocytes and contribute to the location of the damage.

The nature of the vacuoles which accompanied or preceded necrosis in zone 3 is not known. They did not stain with osmium. The appearance of such cells, however, is not specific for BZ, as Oberling and Rouiller (1956) have also described such "balloon" cells in carbon tetrachloride-injured rat livers. Mitchell *et al.* (1973) as well as Dixon *et al.* (1971) has concluded that similar vacuoles induced by acetominophen are the result of hydropic degeneration.

In this study, BZ-induced liver injury was defined in an acinar context and the time course of morphological change followed in each acinar zone. To assure accurate orientation in acinar zones, only venules less than 40 μm in diameter were sampled. As demonstrated in Fig. 2a, the sampling of areas around larger portal veins can lead to confusion since it has been shown that these areas may actually be composed of cells from any of the three zones (Rappaport, 1975). However, the smaller terminal venules are rarely encountered in random minced tissue blocks of the size normally processed for EM. The use of a double embedding technique facilitated their isolation and allowed the systematic analysis of morphological changes in each acinar zone. This is important, since, after zonal injury such as that induced by BZ, the undamaged zone probably maintains liver function. Thus, it is meaningful to describe the morphological progression of damage in the injured zone as well as to determine the ultrastructural changes occurring in the area which is apparently intact.

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