VENO-OCCLUSIVE DISEASE IN *MACACA SPECIOSA* MONKEYS

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Veno-occlusive disease (VOD) of the liver has been reported in man following the consumption of food and beverages contaminated by the Crotalaria and Senecia plants.[1-3] The seeds and vegetation of these plants contain pyrrolizidine alkaloids,[4, 5] which are potent hepatotoxins. Prior to the reports of Bras *et al.*,[1, 6] VOD of the liver was attributed to a dietary deficiency[2, 3] and was thought to be related to Chiari's syndrome. Attempts to produce VOD in experimental animals have met with only limited success.[7-10] This experiment was conducted to determine if VOD similar to that observed in man could be produced consistently in lower primates.

EXPERIMENTAL PROCEDURE

Fourteen adult *Macaca speciosa* monkeys of both sexes weighing approximately 4 kg. were divided into 2 groups. Seven of the monkeys were anesthetized and given by gastric intubation 1.0 gm. of monocrotaline suspended in 100 ml. of distilled water on Days 1 and 14 of the experiment. The 7 control monkeys received a comparable amount of distilled water by gastric intubation.

The monocrotaline given to the monkeys was extracted from finely ground *Crotalaria spectabilis* seed by the method of Adams and Rogers.[5] The concentration of monocrotaline in the crystalline material obtained from the last extraction was determined colorimetrically as reported by Hayashi.[11]

Complete blood counts,[12] prothrombin times,[13] total serum protein,[14] serum electrophoretic patterns,[15] serum cholesterol,[16] serum bilirubin,[17] and blood urea nitrogen determinations[18] were performed weekly. Bromsulphalein (BSP) retention tests[12] were conducted on the monkeys during the terminal week of life. During the course of the experiment each animal was anesthetized weekly with thiamylal sodium (Parke, Davis) in order to perform a laparotomy. Following this procedure, a triangular biopsy specimen approximately 1.2 × 0.8 cm. was obtained from the lateral margin of the liver. The surgical wound on the liver was subsequently covered with absorbable gelatin (Upjohn) to prevent bleeding.

A portion of the hepatic tissue was placed in Caulfield's[19] and Millonig's[20] fixative for electron microscopy, and the remaining tissue was fixed in buffered neutral formalin. The formalin-fixed tissue was embedded in paraffin, sectioned at 6 μ, and stained with hematoxylin and eosin, Weigert's resorcin-fuchsin stain for elastic fibers, Gomori's reticulum stain, Bennhold's Congo red amyloid stain, and Masson's trichrome stain.[21] Tissues for electron microscopy were dehydrated through a graded series of ethanol, embedded in Epon-Araldite mixture,[22] sectioned on an LKB-8800

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Ultrotome, stained with uranyl acetate and lead citrate, and examined with an RCA EMU-3G electron microscope.

When the monocrotaline-intoxicated monkeys became comatose they were sacrificed by severing the jugular veins. Liver tissue was obtained immediately for electron microscopy. A complete necropsy was subsequently conducted on all of these monkeys and the tissues processed in a manner similar to that previously described for the biopsy tissue.

RESULTS

The mean survival time of the monkeys that received monocrotaline was 21 days, with the first death occurring on Day 14 and the last on Day 38 of the experiment. Before death, 2 liver biopsies were obtained from each of 4 monkeys, 3 from 1 monkey, 4 from 1 monkey, and 5 from 1 monkey. There were 5 liver biopsies taken from each of the control animals.

Following the administration of monocrotaline, the monkeys became quite listless, anorectic, and had a rapid decrease in body weight. There were decreases in the hemoglobin and hematocrit levels of the blood, an increase in the prothrombin time, an elevated BSP retention, and a slight rise in serum bilirubin (Table I). There was also a decline in total serum protein of approximately 3.0 gm./100 ml. A major portion of this decrease in protein was associated with a decline in the albuminous fraction. The terminal serum albumin values of the experimental monkeys averaged 36% while the control values were 62%. There was, however, an absence of any appreciable change in the serum cholesterol and blood urea nitrogen values.

The major gross lesions observed at necropsy were limited to the abdominal cavity. Approximately 200 ml. of ascitic fluid was present in the abdominal cavity of each experimental monkey. The livers were yellowish-green, relatively small, firm, and weighed an average of only 105 gm., while the control livers weighed 148 gm. The capsular surface was slightly granular. On the cut surface the lobular pattern was quite distinct, with a markedly congested central and yellow peripheral area.

When the hepatic tissue was examined microscopically, there was observed extensive centrilobular necrosis along with distinct vascular

TABLE I

HEMATOLOGIC CHANGES IN MONOCROTALINE-INTOXICATED MACACA SPECIOSA MONKEYS (MEAN TERMINAL VALUES)

No. monkeys	*Monocrotaline/ monkey (gm.)*	*Prothrombin (sec.)*	*BSP (30 min.)*	*Serum bilirubin (mg./100 ml.)*	*Serum protein (mg./100 ml.)*	*Albumin (%)*
7	0.0	12.0	<5	0.35	7.5	62
7	2.0	25.0	65	0.90	4.4	36

changes that involved practically all of the central, sublobular, and smaller hepatic veins (Fig. 1). There was also a moderate amount of bile stasis that was particularly obvious in the canaliculi. In the monkeys that survived for longer than 2 weeks, there was an infiltration of connective tissue into the central area of each lobule.

The venous lesions were placed in 3 groups on the basis of their morphologic appearance. One group of vessels was characterized by the accumulation of fluid and blood cells within the subintimal portion of the vessel wall (Fig. 2). The collagenous fibers of this area were widely separated and fragmented. As a result of these subintimal changes, there was a marked reduction in the luminal size of these vessels. In most cases the endothelial surface remained intact. However, in some vessels there were openings in the intimal surfaces that formed direct communications between the medial portion of the vessel wall and the circulating blood.

In the second group of vessels the intima, media, and lumina of the vessels were replaced by a network of fine fibers (Fig. 3). Dispersed between these fibers were a few blood cells and the remains of the endothelial lining cells. Only the collagenous adventitial portion of the vessel wall remained intact. In many sections this network of fibers extended from the smaller veins and sinusoids into the larger veins (Fig. 4). Special stains were employed to determine the identity of the fibers that occluded these vessels. It was found that some of the fibers resembled collagen and reticular fibers while others manifested a fibrin-like staining affinity.

In the third type of vessel the lumina had been obliterated by the collapse of the vessel wall and adjacent stromal tissue. Only the abundant collagen fibers of the adventitia were sufficiently intact to delineate the site of the vessel (Fig. 5). The medial and intimal portions of the vessel wall had completely disappeared or remained only as a few fibers practically devoid of any cellular constituents.

The vascular changes that occurred during the initial week were accentuated during the subsequent 2 weeks. The area immediately surrounding the centrilobular veins was reduced in size. The shrinkage of the area resulted from the loss of parenchymal cells and subsequent reduction in space between the stromal tissue. A few collagen fibers were obvious between the abundant, closely packed reticular fibers. In most of the affected vessels the lumina were occluded by fibrous material. The major differences between the older vascular lesions and those present in the initial biopsy tissue were the increase in collagen fibers and reduction in reticular fibers and fibrin (Fig. 6). Many of the small veins in the centrilobular area had collapsed and were comparable to those observed in the initial biopsy specimens.

When the livers were examined under the electron microscope, distinct changes were observed in the parenchymal cells. Numerous fat droplets, myelin figures, and cytosomes were present throughout the cytoplasm of the affected cells (Fig. 7). The orderly row-like arrangement of the granular endoplasmic reticulum was disrupted and only short, widely dispersed segments remained. Agranular endoplasmic reticulum was infrequently observed in these cells. The mitochondria appeared swollen, with short, sparse cristae and irregular external membranes. The cell membranes were ruptured in many places, particularly along the side adjacent to Disse's space. Large cytoplasmic sequestra as well as individual organelles were observed in Disse's spaces and in sinusoidal spaces. Many of these cellular fragments had been phagocytized by the Kupffer cells of the sinusoids (Fig. 8).

The extensive vascular changes were readily visualized with the electron microscope. The Disse's spaces and the sinusoids were partially or completely filled with fibrin and cellular debris (Fig. 9). Membrane-bound cytoplasmic sequestra and cellular debris were also present within the lumina of the vessels (Fig. 10). The endothelial cells of many vessels were desquamated, leaving the denuded surface exposed. Large quantities of fibrin collected along the internal surface of these vessels. The media, adventitia, and basement membrane of the vessels were difficult to visualize in many cases because of the extensive disruption of the vasculature. By the third week following the administration of monocrotaline there was a distinct proliferation of connective tissue in the space of Disse and in the sinusoids near the centrilobular veins (Fig. 11). In many cases there was complete obliteration of the vascular channels by collagen fibers. There were fragments of cells, solitary hepatocytes, platelets, Kupffer cells, and fibroblasts interspersed between the connective tissue fibers. As a result of the proliferating connective tissue, there was considerable disruption of the architectural pattern of these areas.

Discussion

Considerable difficulty has been experienced in producing VOD in experimental animals. However, in this experiment consistent vascular lesions comparable to those observed in man were produced by the oral administration of monocrotaline to *Macaca speciosa* monkeys. As is frequently the case, there was a decided variation in the response of different species of monkeys to monocrotaline intoxication. VOD has been produced in *Macaca mulatta* monkeys; [23] however, the hepatic lesions were much more difficult to produce and the results less consistent than those obtained with *Macaca speciosa* monkeys.

One of the basic questions regarding VOD concerns the factors that

predispose to its development. The sequence of events can now be postulated with relative surety. Following the oral administration of monocrotaline to *Macaca speciosa* monkeys there is extensive centrilobular necrosis of the liver. Many of the necrotic cell fragments are discharged into the adjacent hepatic sinusoids. An accumulation of cell fragments and fibrin partially or completely occludes the sinusoids and adjacent veins. The veins in the same general area are undergoing distinct morphologic alterations. There is desquamation of the endothelial lining cells, accumulation of fluid in the subintimal portion of the vessel wall, reduction in luminal diameter, and a subsequent development of thrombi from the injured vascular tissue, parenchymal cell fragments, and blood constitutents. Within a short period, if the animal survives, there is an infiltration of fibrous connective tissue into the former vessel lumen and in the adjacent centrilobular stromal tissue.

The data from this experiment indicate that the vascular and parenchymal cell changes occur simultaneously. The degenerative changes that occur in either of these tissues appear to be sufficient to produce venous occlusion; however, this does not appear to be the case. Necrosis of the parenchymal cells as well as degenerative changes within the vessels appear to be equally instrumental in the development of VOD.

The hematologic changes recorded in the monkeys given monocrotaline were readily explained after the hepatic changes were delineated. The extensive centrilobular necrosis would account for the reduction in total serum protein, prolongation of the prothrombin time, increased BSP retention, and shift in the A/G ratio of the serum protein. The ascites observed in all the experimental monkeys could also be attributed at least in part to the marked reduction in serum albumin.

Summary

Seven of 14 adult *Macaca speciosa* monkeys were given 1.0 gm. of monocrotaline by gastric intubation on Days 1 and 14 of the experiment. Hematologic evaluations and liver biopsies were performed at weekly intervals on all the monkeys. The mean survival time of the monkeys given monocrotaline was 21 days. During the course of the experiment there was a decrease in the total serum protein, a shift in the A/G ratio of the serum protein, increase in prothombin time, and a rise in serum bilirubin. Distinct morphologic changes were observed in the liver of all monkeys given monocrotaline. Marked centrilobular necrosis and extensive vascular changes were observed. There was desquamation of the lining epithelium of the centrilobular, sublobular, and smaller hepatic veins, and an accumulation of fluid and blood cells in the subintimal portion of many vessel walls. Thrombi were formed in these vessels by an

accumulation of fibrin, cellular debris, and connective tissue fibers within their lumina. Later, there was a rapid infiltration of connective tissue into the occluded vessels and adjacent necrotic parenchymal tissue. A discussion of the morphologic changes in the liver and their possible correlation with the development of VOD is presented.

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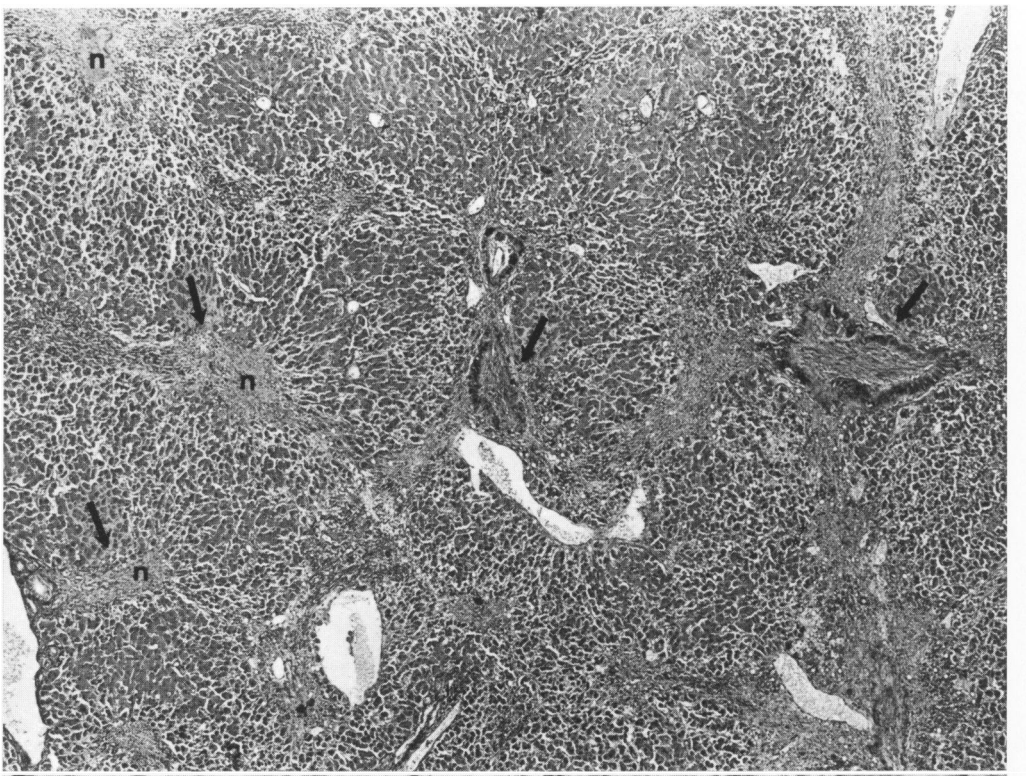
[*Illustrations follow*]

Legends for Figures

Fig. 1. Extensive centrilobular necrosis (n) and vascular involvement (arrow) in liver of monkey given monocrotaline. Masson's trichrome stain. × 40.

Fig. 2. Disruption of intimal lining and accumulation of fluid and blood cells in subintimal area of centrilobular vessel. Note widely separated collagenous fibers in vessel wall. Tissue obtained from liver biopsy taken 1 week after oral administration of monocrotaline. Masson's trichrome stain. × 240.



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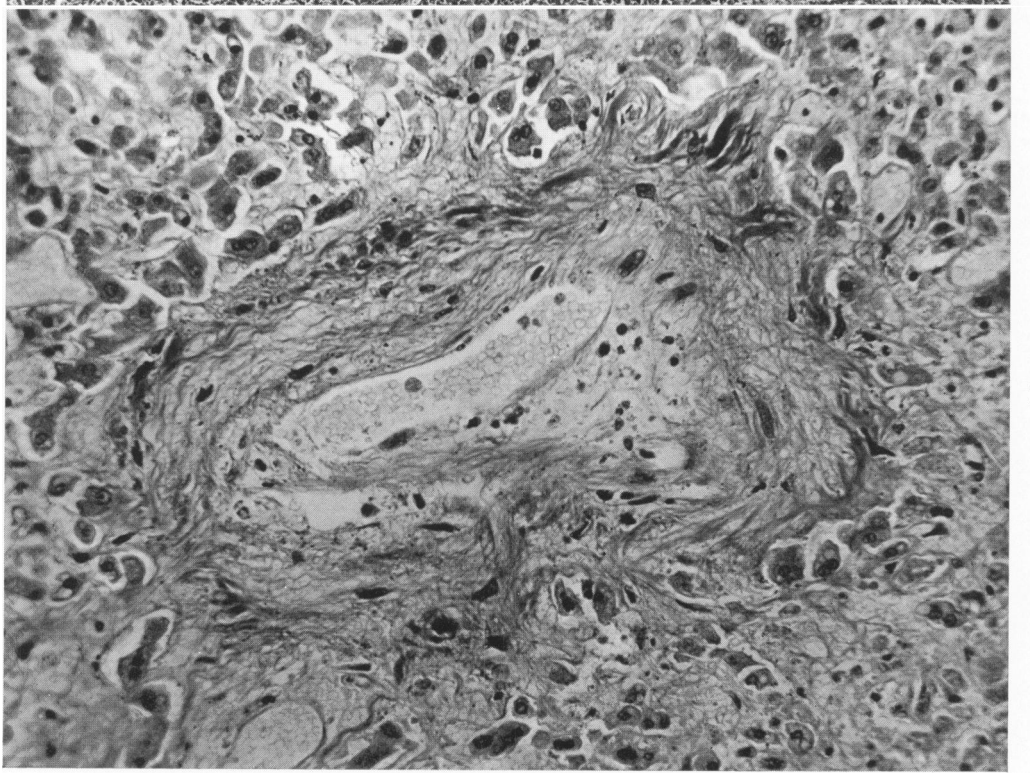

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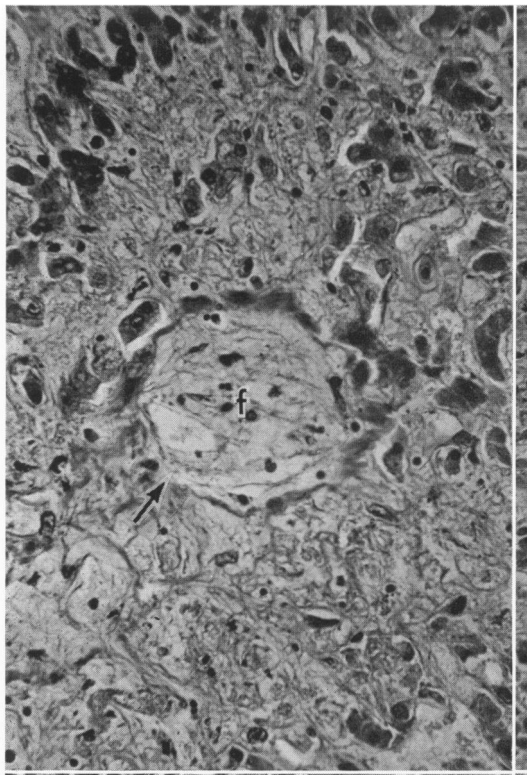
FIG. 3. Fibrin-like strands (f) and a few blood cells within lumen of occluded centrilobular vein. Collagenous adventitia (arrow) is the only identifiable remaining feature of former vessel. Note extensive necrosis and prominent stromal tissue in area surrounding vessel. Liver biopsy taken 1 week after administration of monocrotaline. Masson's trichrome stain. × 240.

FIG. 4. Longitudinal section of occluded centrilobular vessel. Note abundant fibers and scattered cellular elements within occluded lumen (oc). Sinusoids adjacent to parenchymal cells are also occluded (arrow). Masson's trichrome stain. × 200.

FIG. 5. Extensive necrosis of parenchymal cells, close apposition of stromal tissue, and collapsed centrilobular vein (arrow). Collagenous adventitial portion of vessel remains intact, and only a few fibers of the inner wall are visible. Biopsy taken 1 week after monocrotaline administration. Masson's trichrome stain. × 300.

FIG. 6. Medium-sized hepatic vein with abundant collagen (co) within lumen. Adventitial collagen (c″) is the only identifiable tissue of former vessel wall. Liver tissue obtained on Day 21 after oral administration of monocrotaline. Masson's trichrome stain. × 300.



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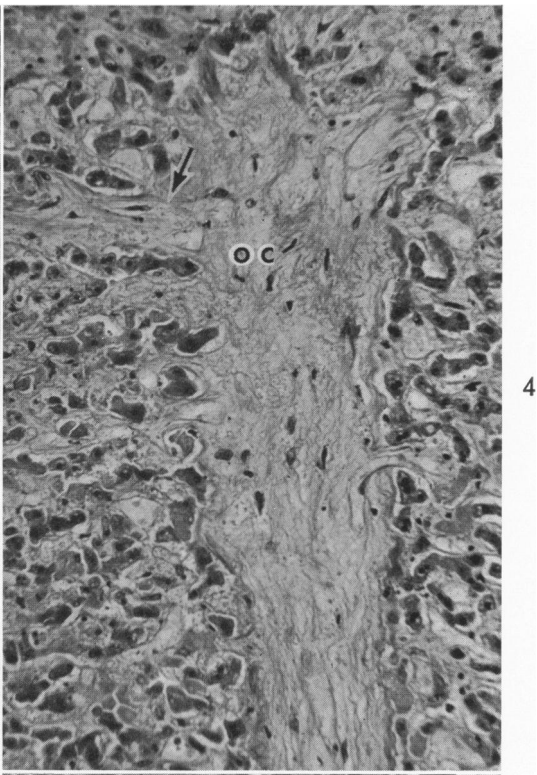

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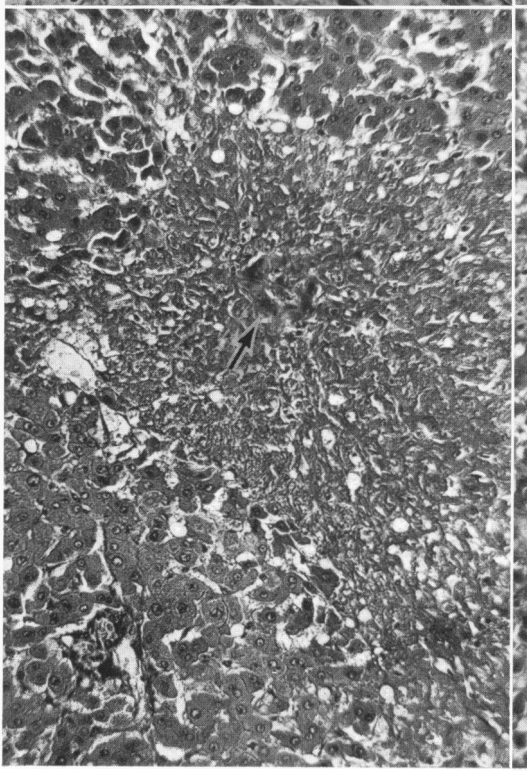

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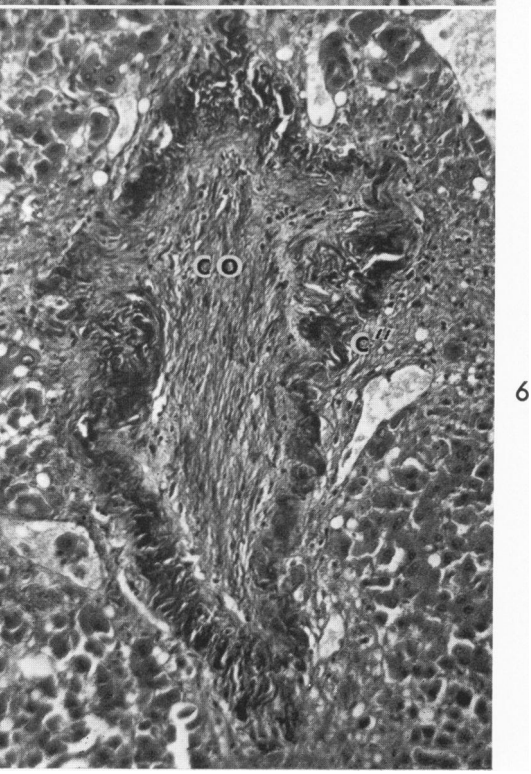

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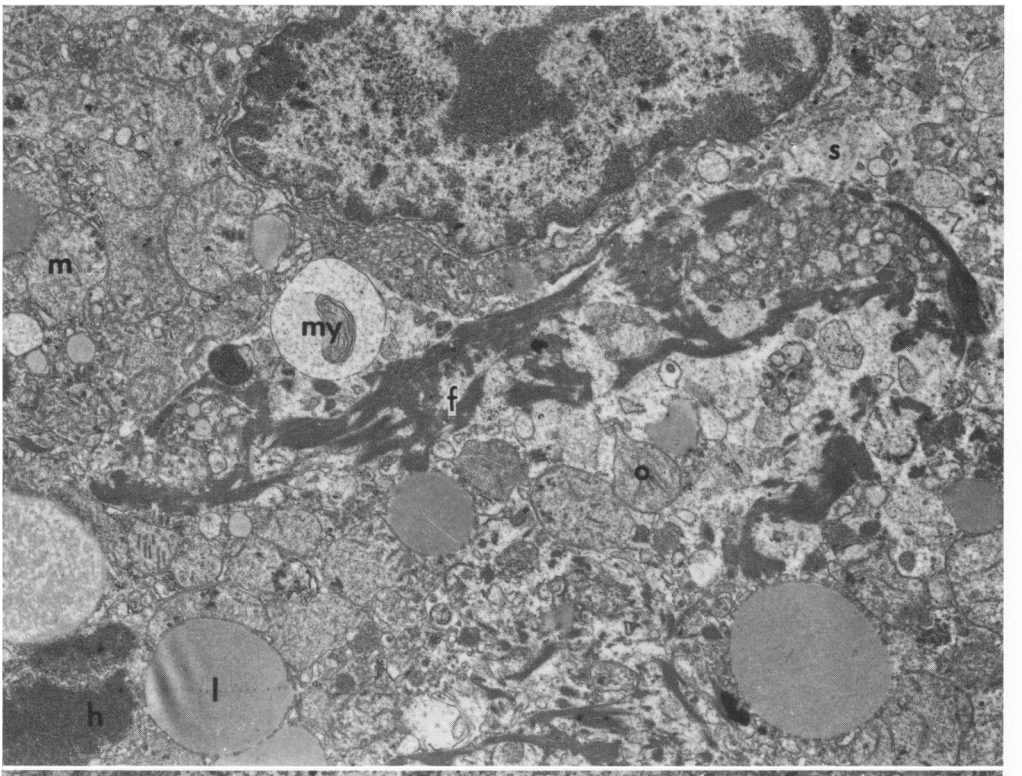
FIG. 7. Liver parenchymal cells from monkey that received monocrotaline 1 week earlier. Note lack of any distinct parenchymal cell plasmalemma separating cytoplasmic organelles from adjacent sinusoidal space (s). Myelin figures (my), fibrin (f), and various cellular organelles (o) are seen within sinusoid. Swollen mitochondria (m), fat droplets (l), hemosiderin-like granules (h), and disrupted endoplasmic reticulum are apparent within cytoplasm of parenchymal cells. Uranyl acetate stain. × 10760.

FIG. 8. Large cytoplasmic sequestrum (sq) of parenchymal cell present within Kupffer cell (k). Note intact membrane (arrow) that separates phagocytized particle from adjacent cytoplasm. Collagen fibers (co) and fibrin (f) are present in sinusoidal space. Cytoplasmic projections, relatively free of organelles, are protruding into adjacent sinusoidal space. Mitochondria (m) are swollen and their external membranes are quite irregular. Cytosomes (c) and fat droplets (l) are abundant in cytoplasm. Uranyl acetate stain. × 6520.



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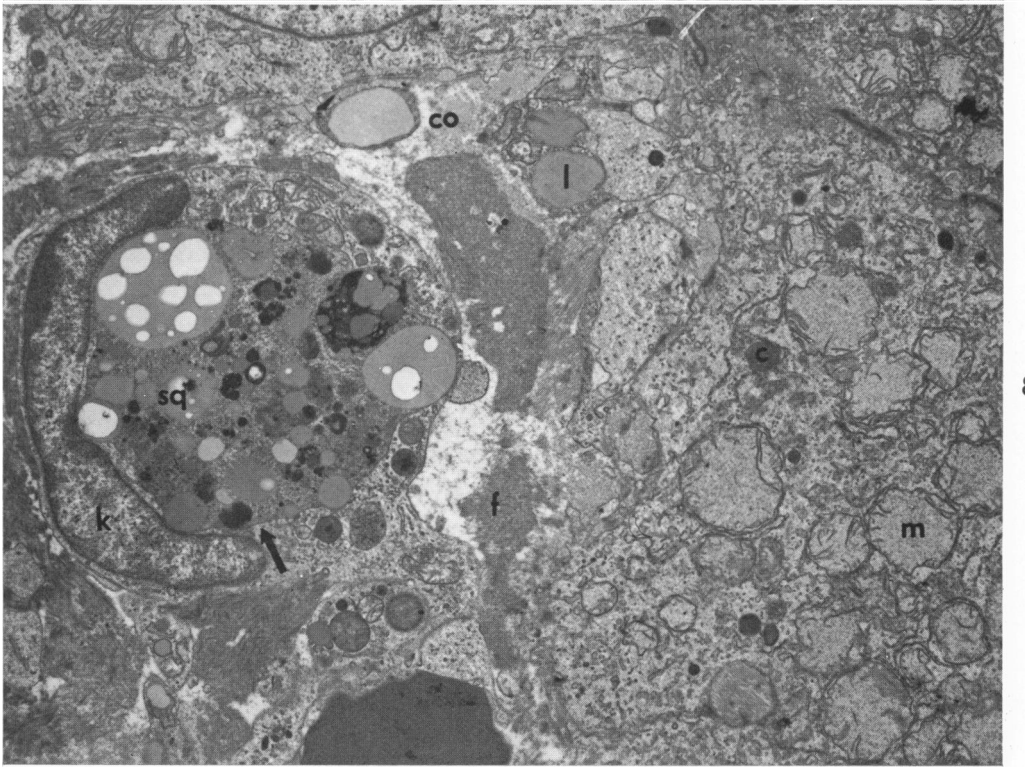

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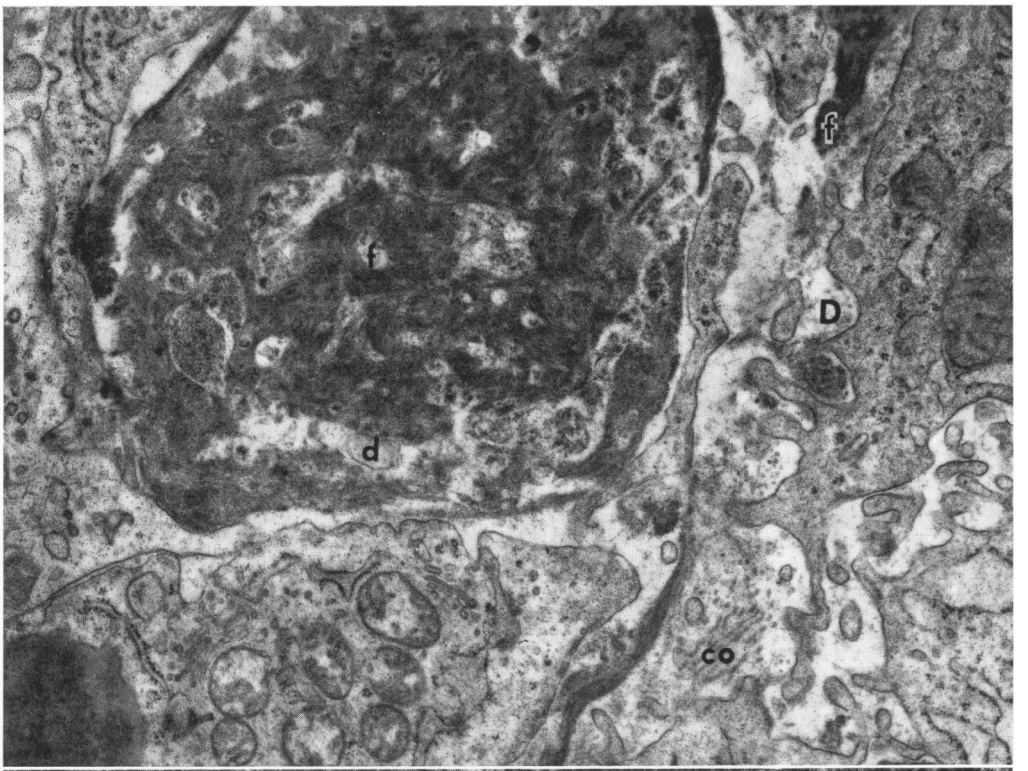

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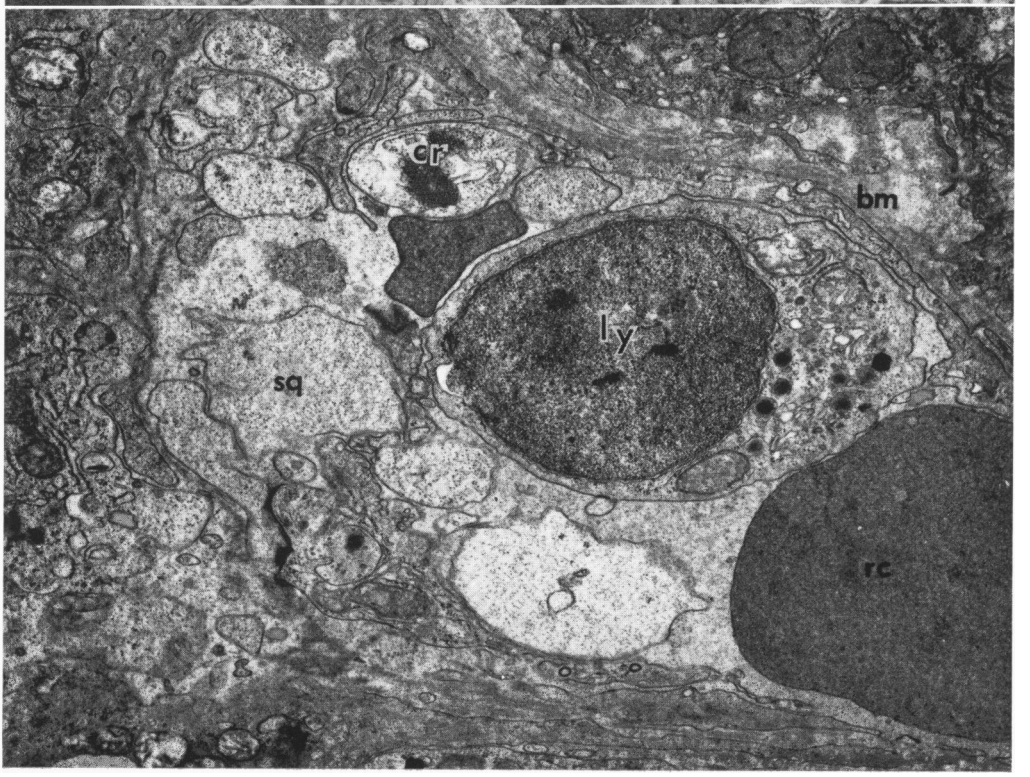

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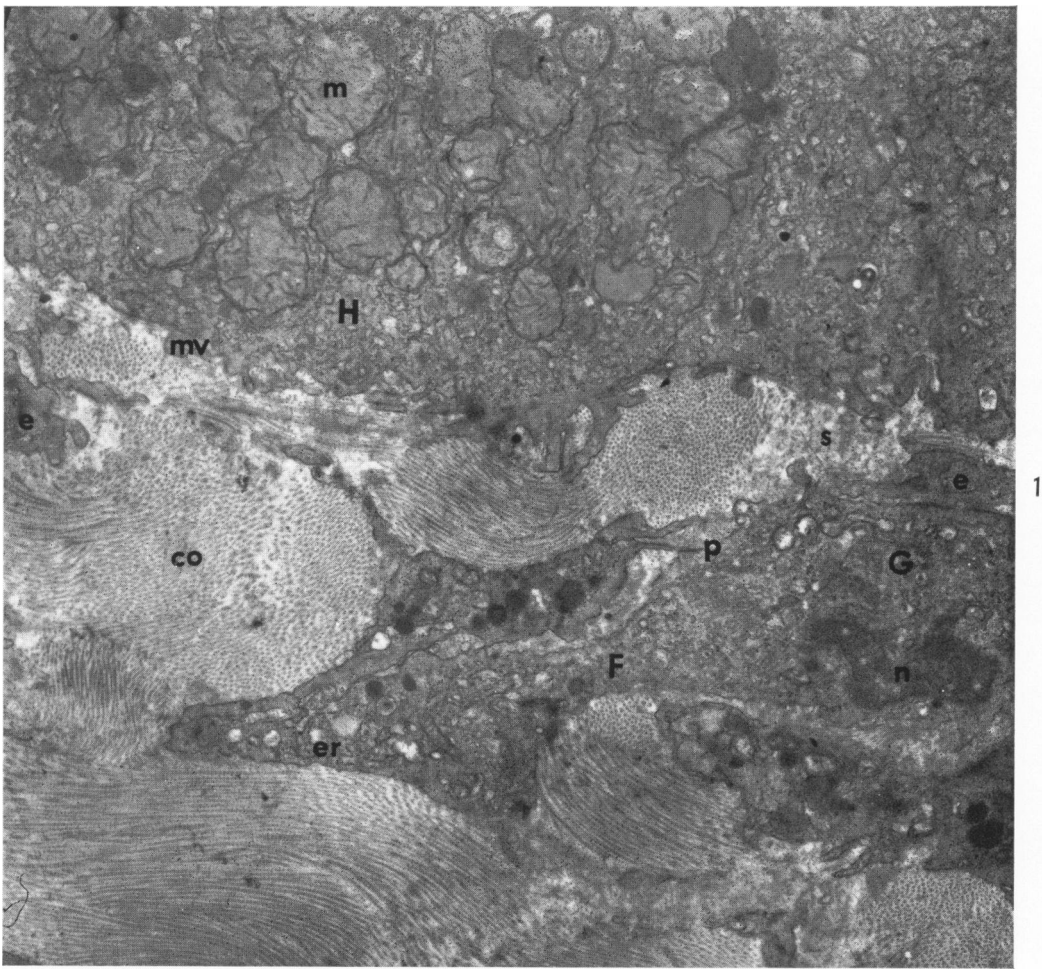

FIG. 9. Sinusoidal space is practically occluded with fibrin (f) and cellular debris (d). Fibrin and collagen (co) also are within Disse's space (D). Similar material was present in many occluded vessels. Uranyl acetate stain. ×26500

FIG. 10. Hepatic vessel of monocrotaline-intoxicated monkey. Note large, electron-lucent cytoplasmic sequestra (sq) within vessel lumen. These sequestra are quite similar to those organelle-free cytoplasmic projections present in parenchymal cells of Fig. 8. Lymphocyte (ly) and red blood cells (rc) also within lumen of vessel. Crystalline material (cr) of undetermined origin is within cytoplasm of endothelial cell. Basement membrane (bm) is relatively distinct along outer surface of endothelial cell. Uranyl acetate stain. × 12500.

FIG. 11. Abundant collagen (co) in Disse's space and adjacent sinusoidal space (s). Fibroblast (F) containing irregular nucleus (n), large Golgi complex (G), distinct granular endoplasmic reticulum (er), and undulating plasmalemma (p) present in sinusoidal space. Small portions of 2 endothelial cells (e) are in close proximity to hepatocytes (H). Hepatocytes contain abundant mitochondria (m) with irregular outer membranes, indistinct cristae, and and apparent absence of intramitochondrial granules. Microvilli (mv) are short, narrow, and parallel rather than perpendicular to cell surface. Uranyl acetate stain. × 9550.