INVESTIGATIONS OF ALLERGIC LIVER INJURY

I. Light, Fluorescent and Electron Microscopic Study of the Effects of Soluble Immune Aggregates

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The immediate or Arthus type allergic reaction has been shown to lead to so-called eosinophilic necrosis of parenchymal liver cells.[1-7] The mechanism of this injury has been attributed to: (a) the formation of particulate complexes of antigen and antibody, resulting in vascular occlusion and ischemic necrosis[6,7]; (b) the direct injury resulting from the interaction of free antigen and cell-bound antibody[3]; and (c) the indirect injury resulting from the interaction of free antigen and antibody within the sinusoidal circulation.[5]

Recent studies have shown that in organs other than the liver, preformed soluble immune aggregates primarily produced an endothelial injury.[8-10] These observations apply to tissues in which an effective barrier separates vascular lumens from the parenchyma. The hepatic lobule has been shown to differ from other organs since it possesses neither a continuous endothelium nor a basement membrane.[11-17] Thus it was the purpose of this study to investigate the response of liver tissue to the injection of such immune complexes.

The investigation by light and fluorescent microscopy was concerned with the over-all features of the resulting lesions. The observations indicated that soluble complexes of antigen and antibody produced an injury morphologically indistinguishable from that resulting from the injection of particulate, precipitated immune aggregates into the portal circulation.[6,7] The electron microscopic observations were confined to the fine structural modulations of the constituents in the space of Disse during the early phases of such injury. The accumulation of an eosinophilic substance in this region, noted by others and designated as one component of the process of serous hepatitis,[18] has been shown to consist in this instance of intracellular edema of Kupffer cells and microvilli of liver cells, leading to distention of the perisinusoidal space.

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Material and Methods

Animals. Female rabbits, weighing 2 to 2.5 kg., of mixed New Zealand and Giant White stock, were used. Animals found to be suffering from hepatic coccidiosis were excluded from the study.

Antigen. Purified human serum albumin (HSA), Connaught Laboratories, Toronto, Canada, was used.

Antiserum. Anti-human serum albumin serum (anti-HSA) was prepared in rabbits immunized by the method of Kabat and Mayer,[19] preceded by two injections of 10 mg. of HSA emulsified with an equal volume of complete Freund's adjuvant.[20] Antiserums were pooled and analyzed for antibody content by the quantitative precipitin technique.[19] The final concentration of the pool was 2.8 mg. of antibody protein per ml.

Immunization Schedule (Experiment I). Rabbits were immunized by repeated courses of injections of HSA, using the method of Kabat and Mayer.[19] Freund's adjuvant was omitted in this instance.

Preparation of Complexes of HSA-Anti-HSA (Experiment II). The point of optimal precipitation was determined by the method of Dean and Webb,[21] using twofold dilutions of the antigen as determined by preliminary trials and a constant dilution of antiserum. Determinations of the point of equivalence were made by visual observation as well as by titrations of the supernatants for excess antigen and antibody respectively. Precipitated complexes were allowed to form overnight at 4° C. After centrifugation and removal of the supernatant, the precipitates were washed 4 times with physiologic saline at 4° C. Soluble complexes were then prepared by the addition of excess antigen (expressed as a multiple of the amount present at equivalence) to the washed precipitate formed at equivalence and incubated for 4 to 6 hours at 37° C. until the precipitate was dissolved. Where total solubilization did not occur after this time, the supernatant was used after centrifugation. Each injection contained 3 times or 25 times excess antigen.

Fluorescein Labeling. Antigen was labeled, where appropriate, with fluorescein isothiocyanate by the modified method of Marshall, Eveland and Smith.[22] This was carried out prior to injection or before incorporation into complexes.

Histologic Methods. Tissues were obtained immediately upon induction of sodium Nembutal anesthesia. Those used for light microscopy were fixed in 10 per cent buffered formalin (pH 7) and embedded in paraffin. Sections were stained with hematoxylin and eosin, Lillie's azure eosin, Unna-Pappenheim's methyl green-pyronin and Masson's trichrome stains. Sections containing fluorescent tracer material were treated by the method of McKinnon, Andrews, Heptinstall and Germuth.[6] They were examined with a Leitz Ortholux microscope fitted with an ultraviolet light source. Preliminary photographs were obtained by means of a Polaroid A.S.A. 3000 film. Definitive photographs were taken on Kodak Panatomic-X film. Tissues intended for electron microscopy were fixed in Palade's cold buffered (pH 7.4) osmium tetroxide in physiologic saline, and kept for ½ hour in the cold at 4° C. and for 1 hour at room temperature. A few fragments of tissue from each specimen were incubated subsequently in a 5 per cent alcoholic solution of uranyl acetate for 1 hour at room temperature. All tissues were embedded in butyl-methyl methacrylate (8:1) after dehydration in graded alcohols. Ultra-thin sections were cut on a Porter-Blum microtome with glass and diamond knives and examined unstained or after flotation on protargol, silver methenamine with and without prior periodic acid oxidation, or on phosphotungstic acid. The sections were mounted on Formvar-coated grids and examined in an RCA EMU 3 electron microscope.

Experiment I

Animals were immunized by the method of Kabat and Mayer.[19] Alum-precipitated HSA, neutralized with N/10 NaOH, was administered intravenously, in quantities

of 100 mg. of protein over 4 weekly periods. Where appropriate, the course was repeated after an interval of 7 days. Upon attaining a titer of 2.8 mg. of antibody protein per ml., animals were challenged with 10 mg. of HSA in the skin of the flank. All rabbits showing a 2+ or better Arthus reaction at the end of 24 hours received injections with 250 mg. of HSA 5 days later directly into the portal vein under sodium Nembutal anesthesia. Two animals dying of anaphylactic shock immediately following injection were not included in the final assessment.

TABLE I

EXPERIMENT I. STUDY OF THE EFFECTS OF A LARGE CHALLENGING DOSE OF ANTIGEN ON THE LIVERS OF HYPERIMMUNE ANIMALS

Rabbit no.	Antibody (mg./ml.)	Amount of HSA * administered systemically (mg.)	Challenging dose (mg.)	Time of sacrifice following challenge (hr.)
11	2.8	500	250	24
12	2.8	400	250	48
13	2.9	600	250	72
25	2.8	500	250 †	24
26	2.9	400	250 †	72

* Human serum albumin.
† Fluorescein-labeled.

Experiment II

Normal rabbits were given direct injections into the portal vein of soluble complexes of HSA-anti HSA formed in 3 × and 25 × antigen excess respectively. Each injection contained approximately 8 mg. of antibody protein.

TABLE II

EXPERIMENT II. STUDY OF THE EFFECTS OF SOLUBLE COMPLEXES FORMED IN ANTIGEN EXCESS ON LIVERS OF NORMAL ANIMALS

Rabbit no.	Complexes formed in	Fluorescent tracer HSA * in complex	Time of sacrifice following injection (hr.)
20	3 × antigen excess	—	24
33	3 × antigen excess	—	48
39	3 × antigen excess	—	72
40	25 × antigen excess	—	24
42	25 × antigen excess	—	72
22	3 × antigen excess	Fluorescent	24
23	3 × antigen excess	Fluorescent	72
2	25 × antigen excess	Fluorescent	24
3	25 × antigen excess	Fluorescent	72

* Human serum albumin.

Controls

Sections of liver from 4 normal animals kept under identical conditions of care and maintenance were examined with the light and electron microscopes. No comparable lesions were found in these.

Two animals were injected with 250 mg. of HSA and two others with 10 ml. of

rabbit anti-HSA respectively directly into the portal vein. No lesions were demonstrable in these at the conclusion of 24 and 48 hours respectively.

The dialysate, containing large quantities of fluorescein isothiocyanate, obtained during preparation of the protein tracer material, was injected directly into the main trunk of the portal vein of two animals in 10 ml. quantities. No lesions were demonstrable in these with light microscopy after 24 and 48 hours respectively. Small quantities of the fluorescent substance were found in hepatic sinusoids, presumably localized within Kupffer cells.

Results

Since the lesions were found to be essentially identical in both experiments, the observations will be described simultaneously.

Gross Observations

In the livers of all animals were found focal and diffuse areas of necrosis situated mainly in subcapsular locations. Petechial hemorrhages were present in the margins of lesions in a number of animals.

Observations with Light Microscopy

The main components of the lesions were maplike areas of midzonal coagulative necrosis with occasional extensions to peripheral and centrilobular regions. The over-all size of these varied from large, diffuse lesions, involving several adjacent lobules in continuity (Fig. 1), to foci involving no more than a dozen liver cells. Peripheral liver plates and cells in the vicinity of central veins were almost invariably spared (Fig. 1). The sinusoids in the very centers of necrotic areas appeared totally occluded by cells and debris (Fig. 2). At the margins of the necrotic foci, narrowing of sinusoids was noted, but further outwards toward portal tracts and central veins, sinusoids were often found to be widely dilated.

At 24 hours Kupffer cells were preserved and were often increased in number in the central portions of the necrotic zones (Fig. 2). In the marginal portions of these regions parenchymal cells were seen in various stages of degeneration, showing cloudy swelling, and hydropic and vacuolar changes.

At 48 and 72 hours the number of surviving Kupffer cells was considerably reduced. There was evidence of resorption of necrotic parenchymal cells. Deeply basophilic, possibly regenerating liver cells made their appearance in such areas (Fig. 3). Degenerative changes were still noted in the marginal zones. The over-all size of the lesions did not increase beyond the limits noted at 24 hours.

The cellular reaction consisted of a scanty infiltration by various mesenchymal cells intermingled with Kupffer cells (Figs. 2 and 3). At 72 hours plasma cells were prominent in hyperimmune animals both in the margins of the necrotic lesions and in portal tracts. In the normal animals in experiment II little portal area reaction was noted at this

time. Cytoplasmic basophilia was seen in many Kupffer cells at 72 hours and was more prominent in the hyperimmune group (experiment I).

Immunohistochemical Observations

Normal liver cells possess a distinct pale blue autofluorescence. Necrotic areas were easily recognizable by the loss of this quality (Figs. 4 and 5). In both experiments where fluorescent tracer-labeled antigen was injected either in its free form (experiment I) or in the form of antibody-bound complexes (experiment II), aggregates of intensely and specifically (apple-green) fluorescent material were found in sinusoids throughout the necrotic zones and their marginal areas (Figs. 4 and 5). The material could not be identified in Kupffer cells with certainty. The quantity of the aggregates remained approximately constant between 24 and 72 hours. The particles ranged from extremely small rounded or elongated bodies to others measuring nearly 7 μ in diameter. The amount of aggregated fluorescent substance was somewhat greater in the hyperimmune animals of experiment I (Fig. 5) than in the normal animals of experiment II (Fig. 4).

No fluorescent tracer was found within liver cells, in the portal tracts or in the radicles of portal veins outside of lobules. Exact localization of the fluorescent tracer in totally necrotic lesions was difficult because of the marked disruption of the architecture.

Electron Microscopic Observations

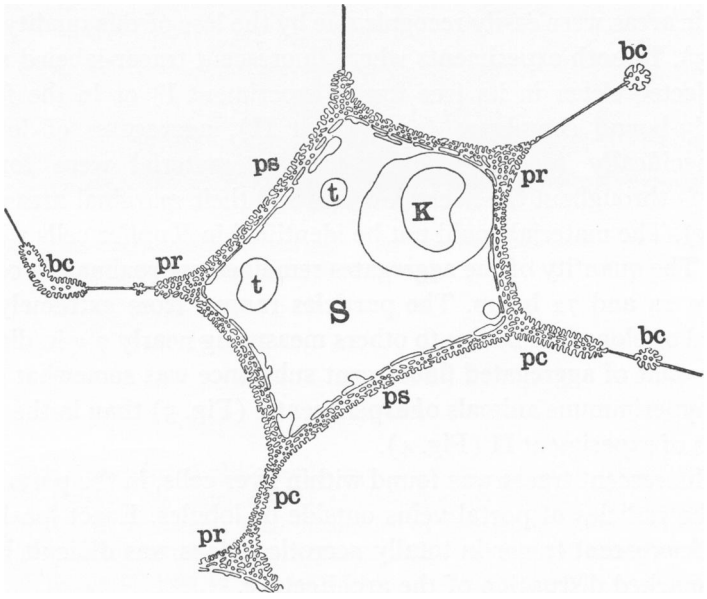
Owing to limitations of space, these observations are limited to the normal and pathologic constituents of the blood-liver barrier. A later publication will deal with the changes in parenchymal liver cells. A further limitation was imposed by restricting observations to the marginal zones of necrotic areas and, in the main, to those remote from portal tracts. This course of action was dictated partly by histologic considerations which will be dealt with anon and partly by knowledge acquired from light and fluorescent microscopy. The latter indicated that cells in prenecrotic phases of the process were most likely to be found in these locations.

The fine structure of the liver has been extensively investigated,[15,23–28] and some reports have dealt more specifically with the sinusoidal lining and the perisinusoidal space.[11–14,17,29,30] Further studies have elucidated certain aspects of the phagocytic function of Kupffer cells and of the transport mechanism of colloidal particles from the blood stream into parenchymal liver cells and vice versa.[24,31,32]

The fine structure of the rabbit liver has not been dealt with in detail in other publications.

Cross sections of sinusoids (Fig. 6) show them to be angular in shape,

the angles occurring at points of contact of neighboring liver cells which form the outer wall of sinusoids. The contact edges of liver cells have rounded contours since such areas are almost invariably sectioned tangentially, producing a pyramid-shaped gap between them; the apex of



TEXT-FIGURE 1. Structure of the blood-liver barrier of the rabbit. An angulated sinusoid (S) is seen. Trabeculae (t) extend around the periphery of the sinusoid from the main body of the Kupffer cell (K). Between the endothelium and the liver cell lie the microvilli of the latter. The intervening area constitutes the perisinusoidal space of Disse (ps). At the points of contact of neighboring parenchymal cells, wedge-shaped perisinusoidal recesses are present (pr). These occasionally extend for some distance outward from the sinusoid to form perisinusoidal canals (pc). Bile canaliculi (bc) form as a result of focal separations of parenchymal cell membranes. Neither perisinusoidal canals nor bile canaliculi possess an endothelial lining.

the wedge points away from the lumen. This is referred to as the perisinusoidal recess.

Kupffer cells tend to be located over these angular recesses (Fig. 6). The shape of the main body of these cells varies from round or ovoid to elongated, depending on the angle of sectioning. The cell is visualized as an octopus-like structure with widely extending flat trabeculae. The trabeculae are wide ribbon-like branches of cytoplasm extending alongside the sinusoidal wall or projecting into the sinusoidal lumen where they are often seen as isolated, cross-sectioned villi. The ultimate branches of the trabeculae form elongated sheetlike profiles which line most of the sinusoidal periphery (Fig. 14). Gaps are clearly demonstrable in the endothelial lining of the sinusoids, bringing liver cells into direct contact with the blood stream (Fig. 14). The sinusoid lining is

visualized as being in continual restless motion, producing intercellular spacings which are transient and of varied sizes. A surfeit of endothelial lining is seen in some areas where terminal trabeculae of Kupffer cells overlap like tiles on a roof, suggesting that they had retracted from neighboring denuded areas. Plasma can be demonstrated in the perisinusoidal space in relation to such gaps while in other areas the endothelium appears to seal off the underlying spaces effectively.

It has been noted previously[15] that the attachment of Kupffer cells to the sinusoid wall is only tenuous if indeed it exists at all. Others[12–14] have stated that the attachment is accomplished by villous processes of the Kupffer cells interlocking with microvilli of the underlying liver cells. This latter concept could not be confirmed in the rabbit (Fig. 8), nor could any attachment be demonstrated between neighboring Kupffer cells.

The structural organization of Kupffer cells normally shows little evidence of their specialized function (Fig. 8). The nucleus, bounded by a nuclear membrane, shows a few infoldings. Closely applied to the membrane is an outer zone of condensed karyoplasm. The nuclear center is less osmiophilic and homogeneous except for denser areas resulting from tangential sections through the peripheral condensed zones. This nuclear configuration is in accord with the usual structure of mesenchymal cell nuclei. The nucleolus is generally inconspicuous. Under conditions of normal activity, cytoplasmic organelles are rather scanty. The Golgi complex is small and the endoplasmic reticulum poorly developed, only occasional elongated or vesicular profiles being found in random locations in the cytoplasm. The ergastoplasmic vesicles are mostly smooth with no identifiable granules of ribonucleoprotein on their external surface although such microsomal fractions are found fairly abundantly through the cytoplasm. Mitochondria are found both in the perinuclear region and in outlying portions of the cytoplasm. Their small numbers and simple structure suggest that the normal energy requirement of the cell is low.

The perisinusoidal space (space of Disse; Figs. 9 and 14), noted by others,[11–14,17,29,30] occupies the position immediately subadjacent to the endothelial lining. Its lumen boundary consists of the cytoplasm of Kupffer cells, the outer one being formed by the parenchymal liver cells. Innumerable, ramified and overlapping microvilli of the latter project into the space (Figs. 9 and 14). In sections stained with protargol, cell membranes stain intensely except in areas where structures are sectioned tangentially. Material can be found in the space of Disse which has the characteristics of condensed plasma (Fig. 9). This is difficult to distinguish from the smudgy outlines of some microvilli. In sections stained

with periodic acid-silver methenamine (PASM) a deeply black, argentophilic material of granular appearance and elongated shape can be seen in the space of Disse (Fig. 7). This corresponds to the periodic acid-Schiff (PAS)-positive substance demonstrated in this location by Wassermann[17] and to the PASM-positive material in this location in thin sections examined with the light microscope (Fig. 10). Although the PASM method is insensitive to small quantities of aldehydes,[33] it nevertheless stains intensely glycoproteins and mucopolysaccharides containing the necessary concentration of 1,2-glycol groups. This substance when present in small quantities is not demonstrable by the protargol method of staining sections for the electron microscope, suggesting some more specific affinity than simple argentophilia. The PASM-positive material is thought to represent ground substance in which the fibrillar components of the sinusoid wall are found (Figs. 11 to 13). Contrary to Wassermann's statement,[17] the fibrils are easily demonstrable in thin sections provided they are stained with phosphotungstic acid or uranyl acetate (Figs. 11 to 13). Their diameter approximates 500 Å, and the striations along their long axes are spaced at intervals of approximately 640 Å (Fig. 11). They are seen mostly in cross section in compact bundles suggesting a circumferential position in relation to the sinusoid. Neither the PASM-positive material nor the fibers were demonstrable within Kupffer or liver cells. In the vicinity of the portal tract the PASM-positive material and fibers are normally abundant, producing a wide perisinusoidal space (Figs. 9 and 13). In such areas caution must be exercised when interpreting lesions since the material in unstained or protargol-stained sections suggests a pathologically distended space of Disse (Fig. 9).

From the perisinusoidal recesses there extend between liver cells for variable distances perisinusoidal canals which appear either to end blindly or to communicate with a neighboring sinusoid. These canals have a lining of parenchymal cell microvilli but no endothelial covering, and they contain few fibrillar and amorphous argentophilic components. Rouiller[28] suggested that communications existed between sinusoids and bile capillaries. This could not be substantiated in the livers of rabbits, although some perisinusoidal canals reached to within a very short distance of the bile canaliculi (Fig. 6). It should be emphasized, however, that the distinction between these structures and the perisinusoidal canals in normal tissues is topographic rather than structural. The main histologic features of the blood-liver barrier are summarized schematically in Text-figure 1.

Pathologic Observations. In the marginal zones of the necrotic areas, perisinusoidal spaces were the seat of marked alterations. These were well developed at 24 hours.

The most striking alteration was the almost total obliteration of the

space of Disse by distended microvilli of liver cells (Figs. 15 to 17). Within 24 hours the tips of these became pear-shaped and their matrices lost their usual electron density. At this stage they still remained attached to their parent cells by thin stalks of intact cytoplasm. By the end of 48 hours many of these structures became rounded and detached from their moorings (Figs. 16 to 18). Some were seen at this time in the gaps between the terminal trabeculae of Kupffer cells. In many instances they were distorted, possibly because of the narrow gap through which they passed (Fig. 17). As free bodies in the lumens of sinusoids, they were rounded. At 72 hours large numbers of such bodies were found within the vascular channels, both on the upstream and downstream sides of the necrotic foci (Fig. 19).

In some areas detachment of edematous microvilli failed to occur, and in these cases the space became markedly distended by huge masses of ballooned cytoplasm (Fig. 15). This was partly the result of fusion of neighboring microvilli, since several pedicles were seen at times entering such masses. In other instances the bases were broad and pedicles could no longer be recognized. The internal structure of the swollen microvilli presented a monotonous, finely granular structure with no detectable cytoplasmic organelles. In areas where detachment did occur, the spaces of Disse appeared markedly narrowed though this was a difficult phenomenon to assess with certainty (Fig. 19).

Some terminal tentacles of Kupffer cells participated in this ballooning transformation. They were difficult to distinguish from swollen microvilli except for their lack of attachment to liver cells (Figs. 16 and 18). At 24 hours the change mainly affected the organelles of these trabeculae. Mitochondria were distended and their matrices clear. The cristae mitochondriales appeared too short and retracted. Pinocytosis vesicles within the cytoplasm were distended, the trabeculae assuming a "Swiss-cheese" appearance (Figs. 16 and 18).

At 48 hours the ballooning often affected the cytoplasmic matrix outside the confines of the organelles (Fig. 18). Rounded trabeculae protruded into the sinusoid lumen, and the intercellular gaps appeared larger than usual. This change probably facilitated the migration of detached microvilli. At 72 hours many large terminal trabeculae were found floating freely in sinusoid lumens, though actual detachment could not be proved. The changes in microvilli and the trabeculae of Kupffer cells did not always occur *pari passu*. The former were seen in all the areas studied, to a greater or less degree; the latter were variable, and the time sequence described above could not be as clearly established as in the case of the microvillous alterations.

Large, electron-dense, crenated masses of material measuring approximately 1 to 3 μ were found free within sinusoidal lumens and in Kupffer

cells (Figs. 20 and 21). These were considered to correspond to the antigenic material visualized with fluorescent microscopy. Similar aggregates could be seen in large numbers in animals into whose portal vein precipitated immune complexes had been injected.[34] A high degree of magnification showed them to consist of fairly evenly spaced, denser particles lying in a lighter matrix. The spacings of 200 to 400 Å were reminiscent of the internal structure of antigen-antibody complexes.[35] The substance was not considered to be lipid since in unstained sections it showed relatively little osmiophilia.

When phagocytosed, the substance tended to become concentrated in large Kupffer cell trabeculae (Figs. 20 and 21). At 48 hours many of these outrunners retracted into perisinusoidal recesses and were often found extending deeply into perisinusoidal canals (Fig. 20). This gave the cells a star-shaped appearance (*Sternzellen*). At 72 hours the aggregates within the trabeculae became surrounded by large, distended endoplasmic cisternas not unlike those found in mature plasma cells (Fig. 20). It was thought possible that this prominence of rough-surfaced vesicles corresponded to the development of cytoplasmic basophilia of Kupffer cells at 72 hours. It is noteworthy that lipid and other particulate matter phagocytosed by Kupffer cells did not evoke such an ergastoplasmic response at this time. This suggested that the reaction might be related to the easily catabolizable nature of this material or to the possible capacity of Kupffer cells to become transformed into antibody-producing cells in the presence of an exogenous foreign protein.

The precipitated complexes occasionally entered the space of Disse (Fig. 22). When this phenomenon occurred in marginal areas of the necrotic zones, the swelling of Kupffer cell trabeculae and of microvilli made interpretation of the effects of the precipitated complexes difficult. However, when entry occurred in areas far removed from the necrotic zones, it became apparent that they behaved as an inert substance in such locations. Thus it was concluded that the mere presence of such precipitated complexes in proximity to the constituents of the space of Disse could not be responsible *per se* for the changes cited above.

Other changes in the region of the blood-liver barrier consisted of phagocytosis by Kupffer cells of released cell debris from necrotic areas, entry of exogenous mesenchymal cells and erythrocytes into the space of Disse and mobilization of Kupffer cells. These phenomena will be dealt with in a later publication.

Discussion

Recent studies have shown that soluble immune complexes formed in antigen excess possess a pathogenic potential. Apart from their ana-

phylactogenic property in guinea pigs[36] and mice,[37] they produced inflammatory changes in the skin.[38–40] Large amounts of such aggregates injected intravenously caused characteristic lesions of serum sickness in mice[9,10] and acute glomerulonephritis in rats.[8] The injury in these experiments was primarily endothelial.

Previous investigations of allergic liver injury have shown that the interaction of antigen and antibody in hepatic sinusoids leads to the development of necrotizing "eosinophilic" lesions.[1–7] It was suggested that such injury in the rabbit was dependent upon the intravascular formation of particulate immune precipitates initiating focal ischemic necrosis.[6,7] This view was based partly on studies of interaction of antigen and antibody *in vivo* where the respective proportions of these constituents were not known,[3,5] and partly on the result of portal vein injections of precipitated immune aggregates prepared *in vitro*.[6,7] In the latter experiments hepatic necroses were anticipated since it was known that even inert particulate matter injected into the portal circulation could produce such lesions.[41]

The purpose of the experiments reported in this paper was to determine the effects of portal vein injections of soluble antigen-antibody complexes formed in the presence of an excess of the former component. This *in vitro* preparation of immune aggregates was supplemented by a study of the result of antigen-antibody reactions *in vivo* when theoretical conditions of antigen excess were created in the portal circulation of hyperimmune rabbits by means of large injections of specific antigen.

Observations by light microscopy showed that the midzonal "eosinophilic" necrosis of liver lobules which resulted was identical with that produced by the injection of precipitated complexes.[6,7] When fluorescent tracer antigen was used (either free or incorporated into complexes), aggregation of this substance at the site of the lesions occurred in a manner similar to that noted when fluorescent tracer-labeled antigen was incorporated into precipitated immune aggregates.[6,7] Thus, "antigenic thrombi" formed even when the presence of an antigen excess medium was expected to lead to solubilization of the complexes.

Two explanations are suggested to account for this phenomenon: (a) as a result of the initial injury by soluble complexes, circulatory stasis develops which favors the aggregation of antigen in its free or antibody-bound form at the site of lesions; or (b) owing to the possible dissociation of the components of the complexes within the lobules[42] and preferential uptake of antigen by liver or Kupffer cells, rapid equilibration or perhaps actual slight antibody preponderance may prevail, favoring precipitation. Thus, formation of precipitated complexes in this manner would present a situation the reverse of that found in serum

sickness where precipitation may be the result of an active increase in endogenous antibody.[43]

The second explanation is at present less acceptable since antigen could not be demonstrated conclusively in liver or Kupffer cells. It should be noted that the precipitation phenomenon in this experiment is in some way related to the development of necrosis since antigenic thrombi were found only in or in the immediate vicinity of the lesions. This was not the case after particulate complexes prepared *in vitro* were injected; in this circumstance their presence was noted even in the larger radicles of the portal venous system.[6,7]

The studies by light and fluorescent microscopy did not resolve the question of the possible ischemic nature of the initial injury. A search was therefore made on an ultrastructural level for evidence of any change which could not be accounted for by either ischemia or the direct contact of precipitated complexes with tissues. It was presumed that if such evidence was found, a justifiable suggestion could be made that the injury might be attributed to the activity (toxicity) of soluble complexes.

The findings may be summarized as follows: Within 24 hours following the intrasinusoidal interaction of antigen and antibody in antigen excess, alterations developed in the constituents of the perisinusoidal space. These consisted of ballooning of the terminal trabeculae of Kupffer cells and of the microvilli of liver cells where these projected into the space (Figs. 15 to 18). The outcome of this process was twofold. At 72 hours in some areas most of the swollen microvilli became detached and extruded into the lumen of sinusoids, leaving a narrowed and collapsed perisinusoidal space. In others, detachment failed to occur and the distended villi formed a wide barrier between the blood stream and liver cells.

Several mechanisms may be operative in the production of these changes. It is possible that the essential alteration was a disturbance of cell membrane permeability with a subsequent imbibition of plasma by the trabeculae and microvilli. It is also possible that the edema was related to a disturbance of water elimination or to changes in intracellular electrolyte values. Finally, the change could have been an expression of faulty distribution of water within the cells. It is noteworthy that similar changes have not been observed in any of the electron microscopic investigations of hepatic injury. They were not found in starvation[44] or radiation injury,[45] and no mention was made of them in papers dealing with histotoxic liver injury,[46,47] Simple anoxia,[48,49] although producing alterations in the width of the space of Disse, did not lead to this type of change. Experimentally induced alterations in the width of the peri-

sinusoidal space by injections of glucose and adrenalin,[13] respectively, were not accompanied by injury to Kupffer cell trabeculae or to the microvilli of liver cells. It is nevertheless possible that these alterations may have been overlooked unless they had been specifically searched for.

Thus, on the basis of the available evidence, it is suggested that the changes noted in the constituents of the space of Disse in these experiments may be a specific manifestation of allergic liver injury due to the activity of soluble complexes of antigen and antibody formed in antigen excess. Large amounts of such soluble immune aggregates may prove rapidly lethal to parenchymal liver cells in midzonal locations as a result of hemodynamic factors which control their concentration and the duration of their contact with such cells. In the periphery of these areas the concentrations of the complexes may be considerably smaller and the injury correspondingly more subtle. The possibility must be borne in mind that the widening of the blood-liver barrier may constitute an obstacle to the diffusion of oxygen and induce secondary ischemic damage to the parenchyma. The alterations of liver cells were found to be in most respects indistinguishable from those due to anoxic and histotoxic anoxia.[34]

Summary

Soluble complexes of antigen and antibody (human serum albumin-rabbit anti-human serum albumin) prepared *in vitro* were injected into the portal circulation of a group of normal rabbits. Theoretical conditions of antigen excess were created in another group of hyperimmune rabbits who received a large injection of specific antigen into the portal vein. The resulting midzonal necrosis of liver cells in both groups of animals was indistinguishable from the injury produced when precipitated particulate immune aggregates were administered. When fluorescent tracer-labeled antigen was used in these experiments, precipitates of this material were found in the necrotic areas even when soluble complexes were injected. The question of a possible ischemic basis for the necrosis remained unresolved.

The electron microscopic investigation disclosed a change in the terminal trabeculae of Kupffer cells and in the microvilli of liver cells, where these protruded into the space of Disse. This consisted of a ballooning of these structures and led to detachment of large numbers of microvilli from their parent cells and to their extrusion into the sinusoidal blood stream. These features were not reported in any examples of anoxic or histotoxic liver injury previously examined by electron microscopy. It was suggested that the alterations might represent a specific com-

ponent of allergic liver injury possibly related to the toxicity of soluble immune complexes.

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[*Illustrations follow*]

Legends for Figures

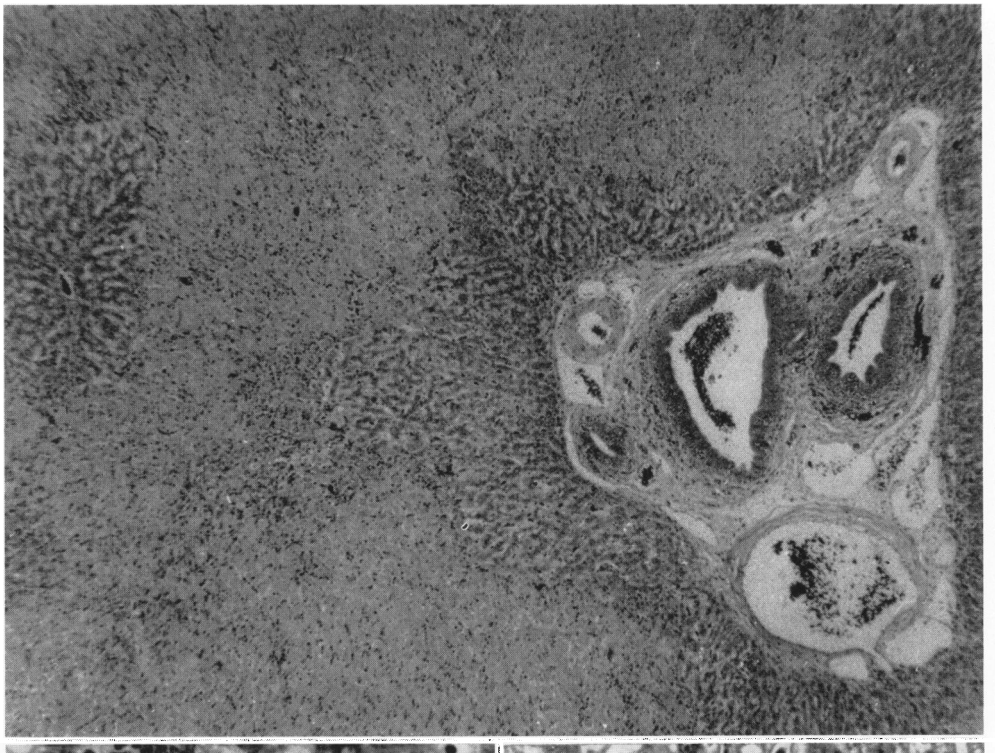
Key to abbreviations used in Figures 6 to 22.

am = argentophilic (PASM-positive) material (ground substance)
bc = bile canaliculus
bd = bile duct
gl = glycogen
LC = parenchymal liver cell
lf = lipofuscin body
m = mitochondrion
mv = microvillus
N = nucleus
ps = perisinusoidal space
S = sinusoid
t = terminal trabecula of Kupffer cell
tr = trabecula of Kupffer cell
v = vacuole

FIG. 1. Rabbit 11 (experiment I). Widespread midzonal necrosis 24 hours after the injection of antigen (250 mg.) into the portal vein of a hyperimmune animal. Note the scanty cellular reaction. Azure eosin stain. × 48.

FIG. 2. Rabbit 20 (experiment II). The margin of a necrotic zone 24 hours after the portal vein injection of soluble complexes formed in 3 × antigen excess. Note the survival of some mesenchymal cells in the center of a necrotic area (right) and the scanty cellular reaction at the periphery. The marginal zone contains cells in various stages of degeneration. Azure eosin stain. × 234.

FIG. 3. Rabbit 39 (experiment II). Seventy-two hours after the portal vein injection of soluble complexes formed in 3 × antigen excess. There is evidence of resorption of necrotic liver cells and several deeply basophilic, possibly regenerating, liver cells appear scattered through the central portion of the lesion. Hematoxylin and eosin stain. × 285.



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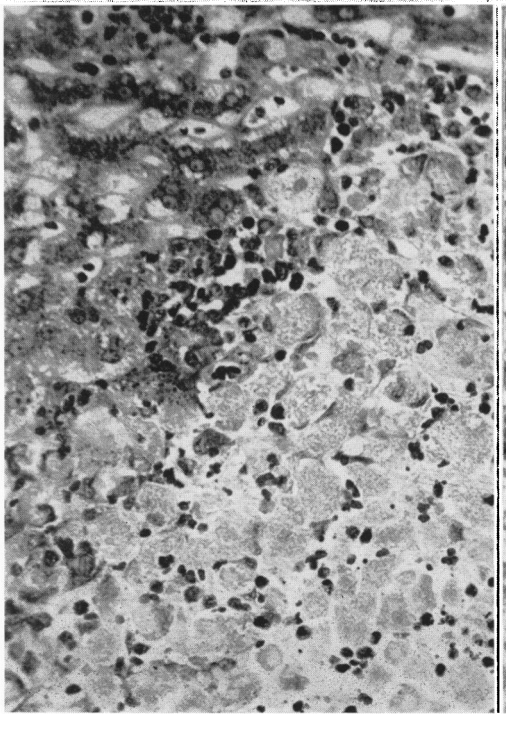

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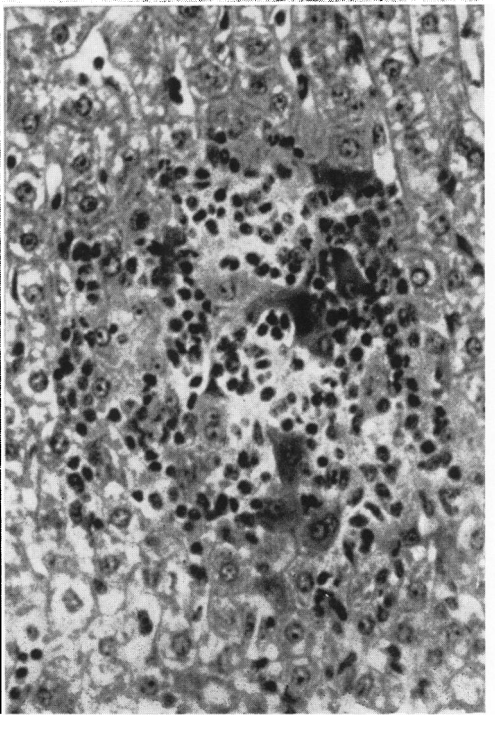

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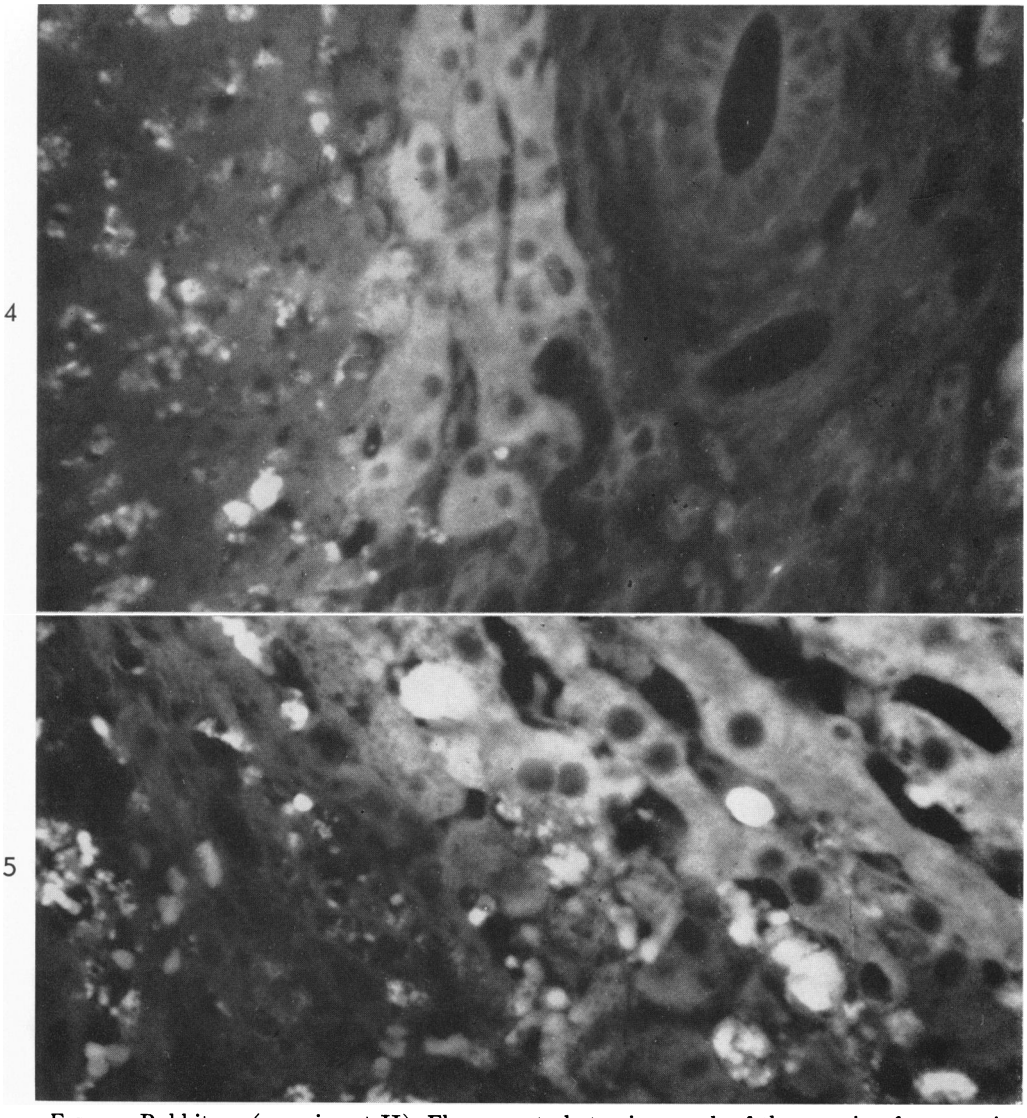

FIG. 4. Rabbit 23 (experiment II). Fluorescent photomicrograph of the margin of a necrotic zone 72 hours after the portal vein injection of soluble complexes formed in 3 × (fluorescent tracer-labeled) antigen excess. A portal tract is on the right. Intact cells of a liver plate maintain their nonspecific autofluorescence (center). The area of necrosis (left) shows loss of this property. Aggregates of antigen (specific apple-green fluorescence; white in photomicrograph) are present in sinusoids but not in the cytoplasm of necrotic parenchymal cells. Their location in Kupffer cells is difficult to determine. Unstained. × 374.

FIG. 5. Rabbit 25 (experiment I). The margin of a necrotic zone 24 hours after the portal vein injection of a challenging (fluorescent tracer-labeled) dose of antigen to a hyperimmune rabbit. Necrotic liver cells (left) show loss of autofluorescence. Large aggregates of antigen (specific apple-green fluorescence; white in photomicrograph) are seen within sinusoids both in the necrotic and marginal areas. Their size is somewhat larger than in Figure 4. Fluorescent photomicrograph, unstained. × 387.

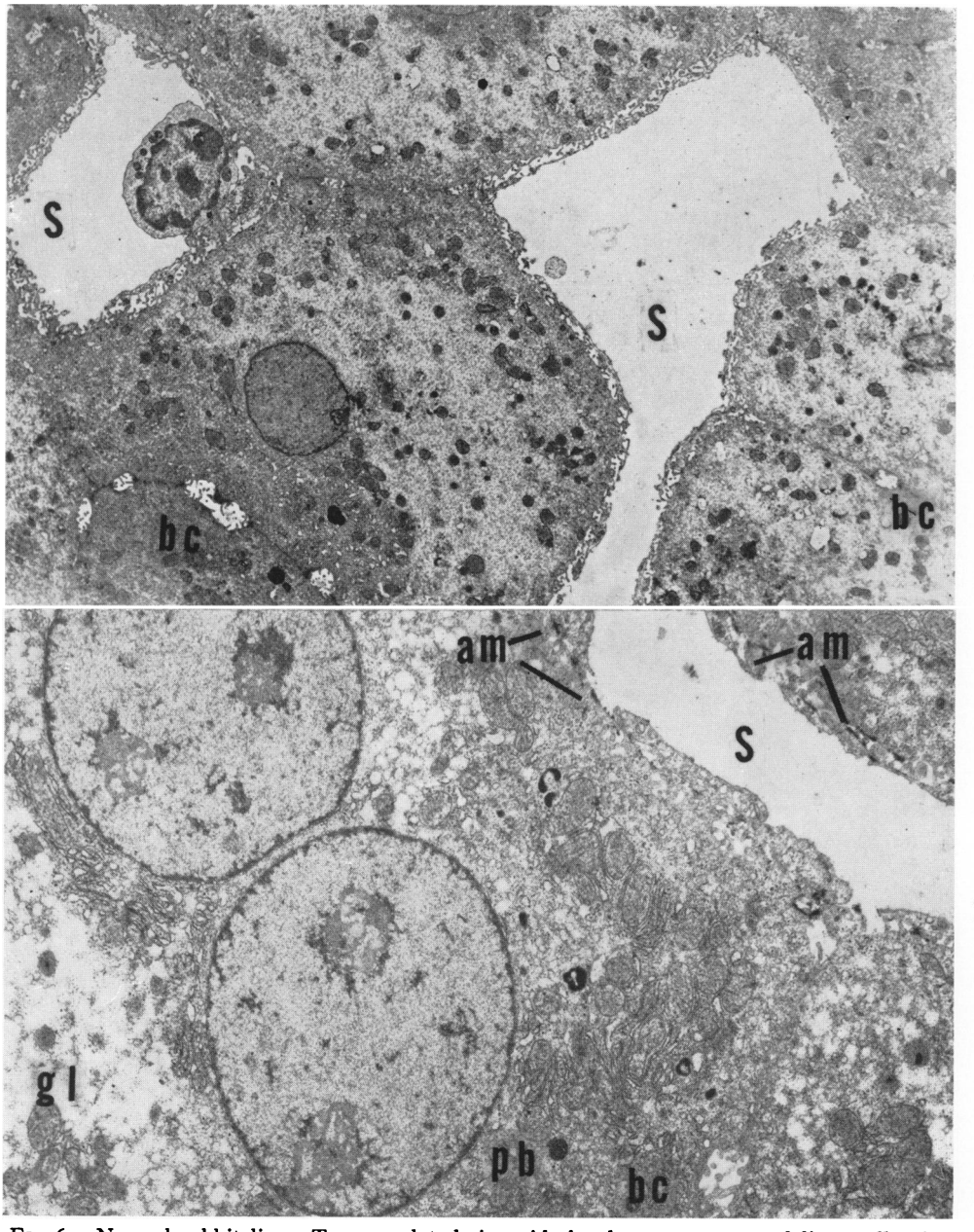

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FIG. 6. Normal rabbit liver. Two angulated sinusoids border upon a central liver cell. The space of Disse surrounding both sinusoids is of even width. Several small perisinusoidal recesses are seen. A Kupffer cell lies in one of these in the left-hand sinusoid. A row of bile canaliculi extends along the intercellular boundary of two liver cells. Protargol. × 4,200.

FIG. 7. Normal rabbit liver. A binucleated liver cell containing the usual complement of finely granular glycogen. The organelles lie in the vicinity of the nuclei and between them and the sinusoid. The space of Disse is difficult to make out, owing to the presence of fine filaments of argentophilic (PASM-positive) material within it. Periodic acid-silver methenamine. × 8,960.

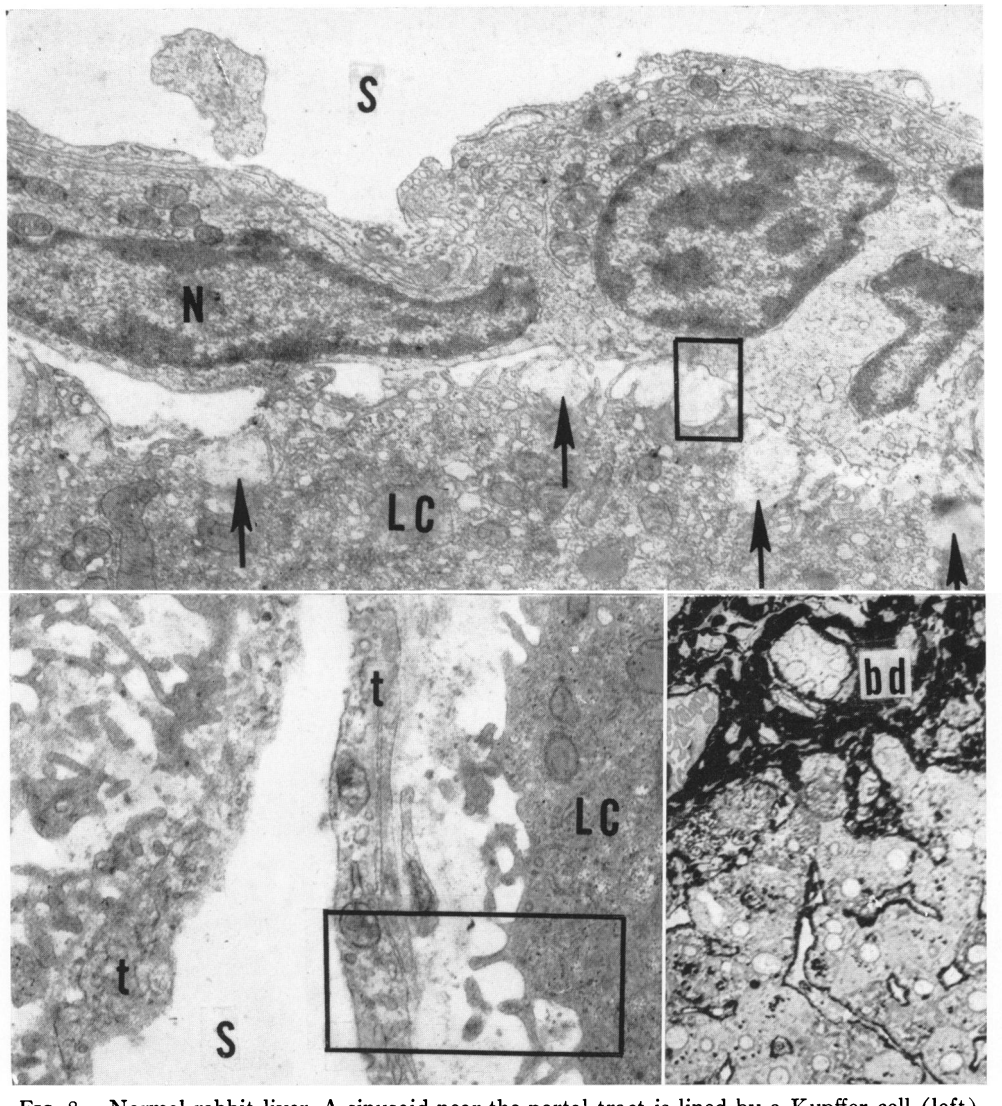

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FIG. 8. Normal rabbit liver. A sinusoid near the portal tract is lined by a Kupffer cell (left) and an exogenous mesenchymal cell which is covered by an outrunner of the former. There is no evidence of an attachment of the Kupffer cell to the liver cell. The microvilli of the latter, projecting into the space of Disse, are separated from each other by a faintly argentophilic material (arrows.) An area similar to that enclosed in a square is seen in Figure 12. Protargol. × 13,200.

FIG. 9. Normal rabbit liver. The angle of a sinusoid in the vicinity of a portal tract. A faintly argentophilic, plasma-like material lies between the endothelium and the microvilli of liver cells. This represents the ground substance with its content of reticulin fibers. These can be seen in Figure 13 in an area corresponding to the one enclosed in a square. Protargol. × 19,040.

FIG. 10. Normal rabbit liver. The sinusoids in the vicinity of a portal tract are lined by a strongly PASM-positive material corresponding to that seen in Figure 9 in the electron micrograph. Thin section (0.5 μ), osmium acid-fixed tissue. Periodic acid-silver methenamine stain. × 360.

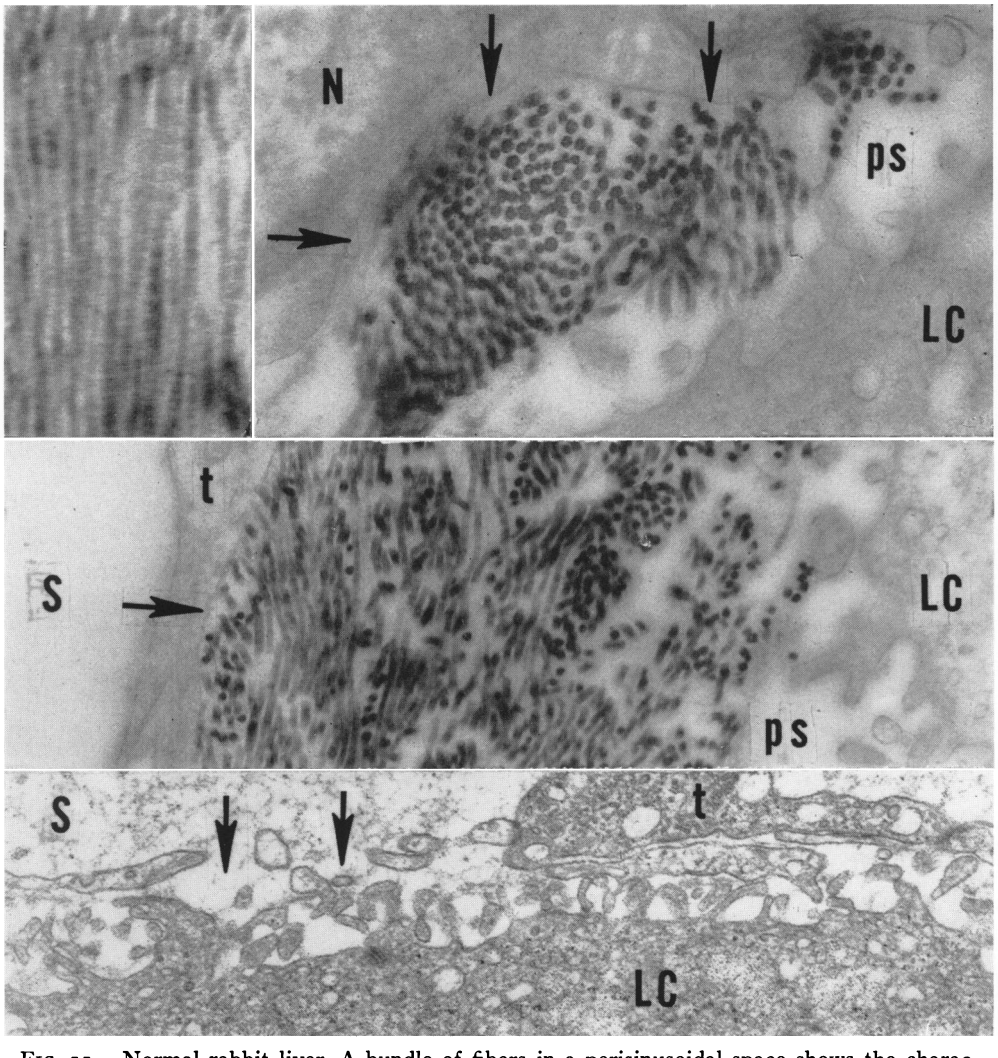
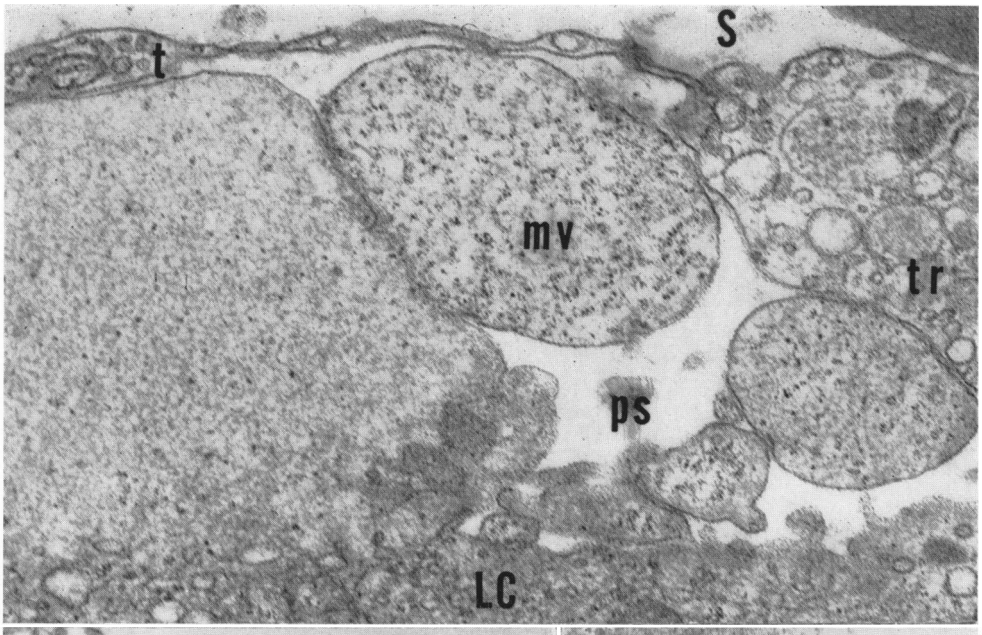


FIG. 11. Normal rabbit liver. A bundle of fibers in a perisinusoidal space shows the characteristic cross-striations of reticulin. Phosphotungstic acid stain. × 36,300.

FIG. 12. Normal rabbit liver. A bundle of cross-sectioned reticulin fibers occupies the space of Disse in an area similar to that enclosed in a square in Figure 8. The arrows point to the cell boundary of the Kupffer cell. The fibers are entirely extracellular in location. Uranyl acetate stain. × 33,000.

FIG. 13. Normal rabbit liver. A space of Disse similar to that seen in the squared area in Figure 9 is occupied by numerous uniformly polarized reticulin fibers. The arrow points to the cell membrane of the terminal trabecula of a Kupffer cell. The fibers have an entirely extracellular location. Phosphotungstic acid stain. × 33,000.

FIG. 14. Normal rabbit liver. A portion of a space of Disse bounded by terminal trabeculae of a Kupffer cell and parenchymal cell microvilli. Arrows point to gaps in the endothelial lining. Protargol stain. × 12,000.



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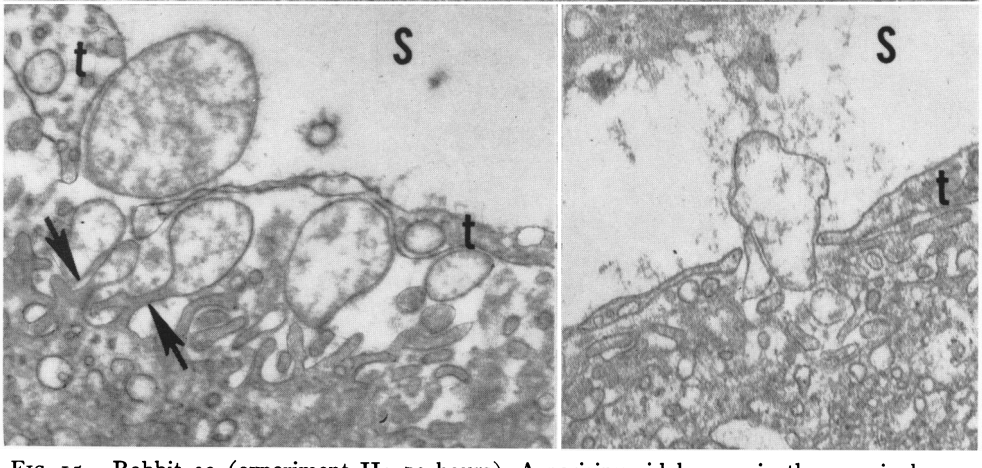

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FIG. 15. Rabbit 39 (experiment II; 72 hours). A perisinusoidal space in the marginal area of a lobular lesion. The space is distended by ballooned, membrane-enclosed microvilli. In their internal structure organelles are absent and there are widely separated RNP granules. The villus on the left of the picture shows a broad base. An endothelial trabecula separates the space entirely from the sinusoid lumen. Note the "Swiss cheese" appearance of a larger trabecula on the right. Protargol stain. × 32,000.

FIG. 16. Rabbit 12 (experiment I; 48 hours). A perisinusoidal space from an area similar to that shown in Figure 15. The pedicles of liver cell microvilli are still unaffected (arrows). The tips are distended, however, and form pear-shaped clubs. The cytoplasm of a neighboring liver cell shows a slight vacuolar change. Protargol stain. × 24,800.

FIG. 17. Rabbit 33 (experiment II; 48 hours). A perisinusoidal space from an area similar to that shown in Figure 10. A ballooned microvillus is in process of being extruded from a space of Disse into a sinusoid lumen. Protargol stain. × 25,600.

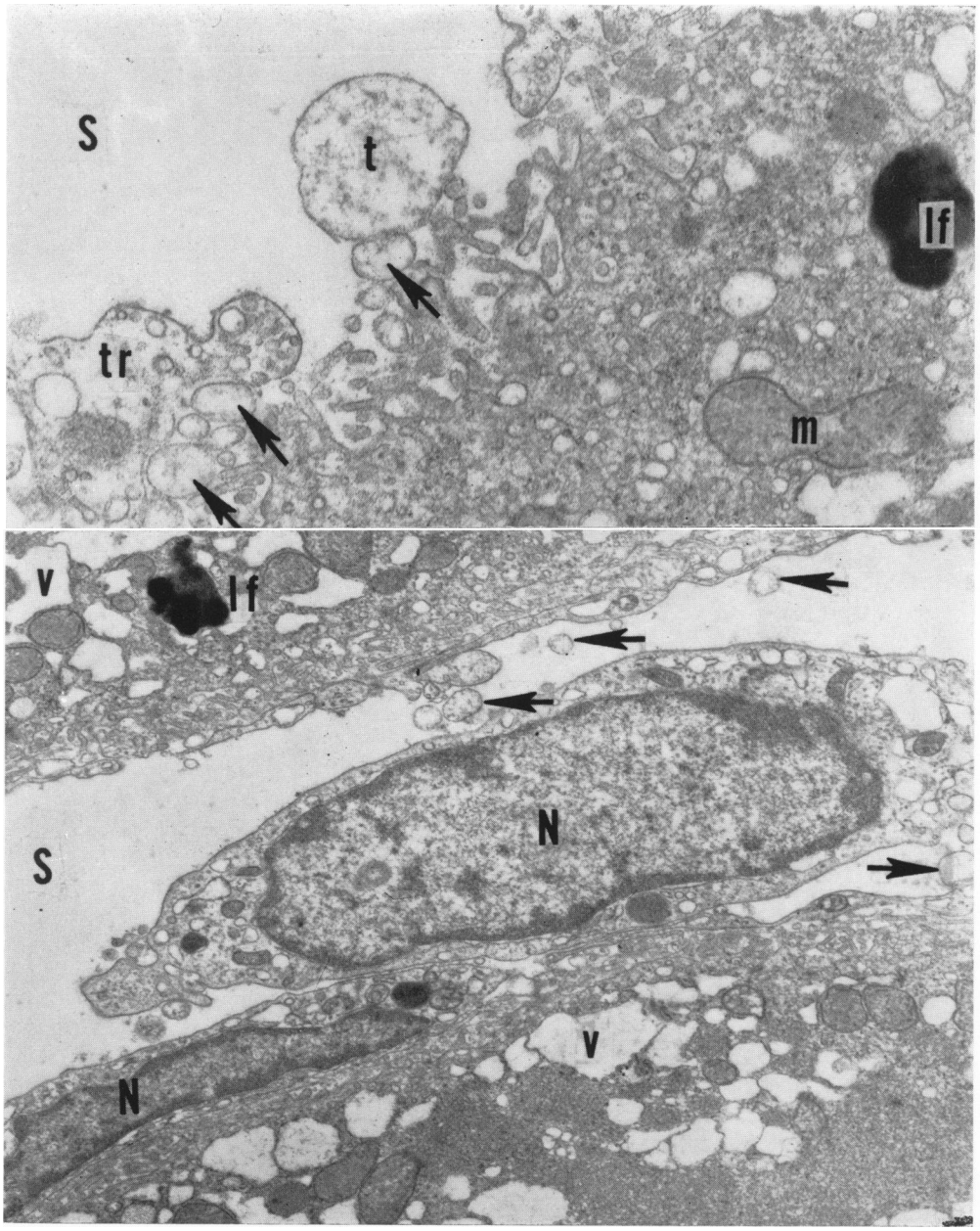

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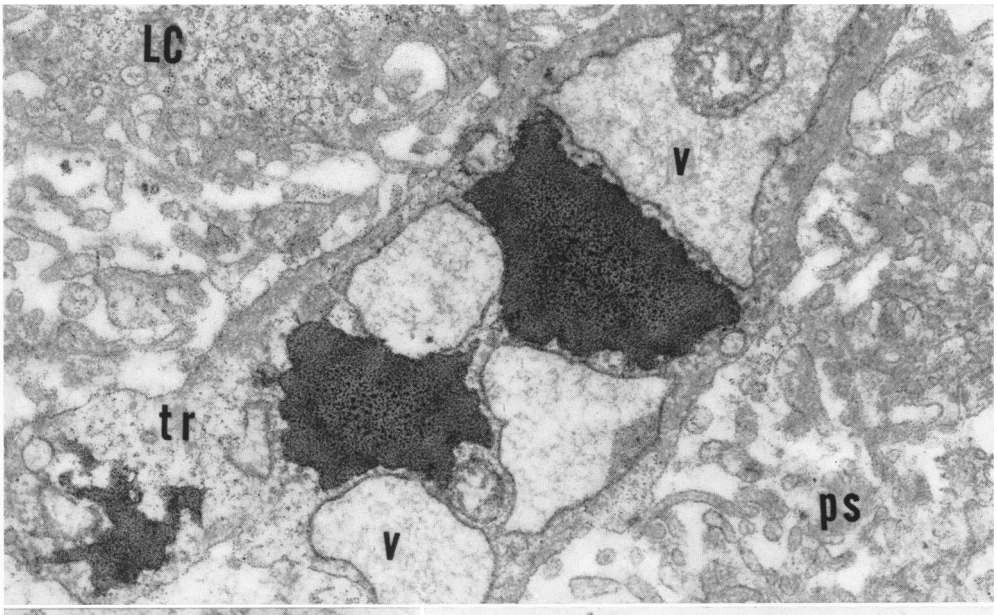
FIG. 18. Rabbit 33 (experiment II; 48 hours). The perisinusoidal space from an area similar to that shown in Figure 15. The trabeculae of Kupffer cells are affected by the ballooning change as well as by distention of microvilli (arrows). There are wide gaps in the Kupffer cell lining and a "Swiss cheese" appearance on the left. The underlying parenchymal cell shows an early vacuolar change. Protargol stain. × 16,800.

FIG. 19. Rabbit 13 (experiment I; 72 hours). A sinusoid in the marginal area of a necrobiotic zone. In the left lower corner a Kupffer cell lies in close apposition to the space of Disse. A second large Kupffer cell is seen almost entirely loose within the sinusoid lumen. Several rounded bodies (arrows) are floating in the sinusoid. These are detached liver cell microvilli. The space of Disse is collapsed. The cytoplasmic matrix between the vacuoles in a parenchymal cell in the right lower corner is markedly condensed. Protargol stain. × 12,000.



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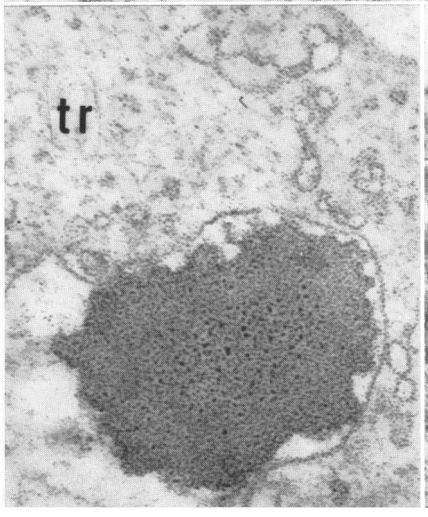

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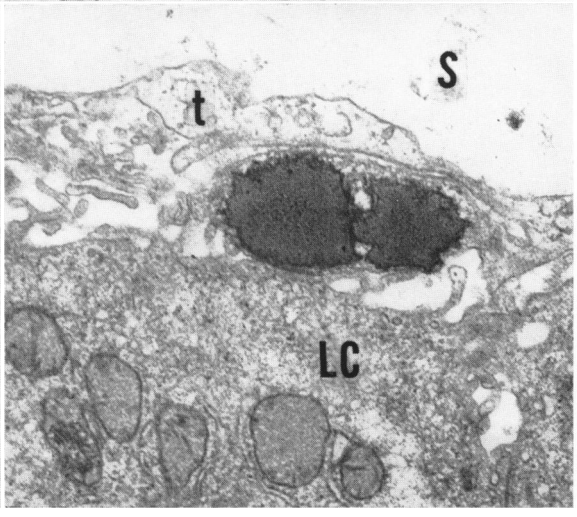

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FIG. 20. Rabbit 3 (experiment II; 72 hours). A trabecula of a Kupffer cell at the margin of a necrobiotic zone. The densely argentophilic masses within the cytoplasm are thought to be precipitated antigen-antibody complexes. Dilated endoplasmic cisternas containing a faintly argentophilic material encompass the masses. The trabecula is retracted into a perisinusoidal recess. The labyrinthine character of the microvilli in the recess is evident in a tangential section on the right of the trabecula. Protargol stain. × 24,000.

FIG. 21. Rabbit 11 (experiment I; 24 hours). A trabecula of a Kupffer cell in process of engulfing an argentophilic mass similar to those seen in Figure 20. Protargol stain. × 44,800.

FIG. 22. Rabbit 42 (experiment II; 72 hours). Argentophilic masses similar to those in Figures 20 and 21 are found in the space of Disse. The Kupffer cell trabecula has closed off the space from the sinusoid lumen. Protargol stain. × 22,400.