REGENERATION OF THE MAMMALIAN LIVER

I. Auto-Phagocytosis During Dedifferentiation of the Liver Cell in Preparation for Cell Division

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The capacity of lysosomes to cause autolysis of portions of cytoplasm in response to injury has been reported.[1,2] This response results frequently from mild or short-term anoxia and from other forms of cell damage.[1-6] In addition, the appearance of auto-phagocytic lysosomes in fetal tissues or those of the newborn has suggested that these may be involved in the selective removal of organelles whose associated metabolic processes are not required for cell division.[6-10]

One rapidly growing tissue which is well adapted to a study of the association between lysosomes and cell division is the adult mammalian liver responding to partial amputation.[11] Shortly after surgical removal of two-thirds of its mass, this mitotically inactive and highly differentiated tissue begins active synthesis of nucleic acids followed by a burst of cell divisions. The entire process in the rat is largely complete in a period of 72 hours with a 2- to 3-fold increase in the number of cells in the residual liver tissue.[12]

In describing the histologic changes accompanying this mitotic activity, authors have reported that large cytoplasmic bodies appear very soon after partial hepatectomy, and have suggested variously that these are related to increased cell permeability, abnormal protein synthesis or accumulation of lipid.[13-16]

Having noted that these bodies are similar to some previously described structures considered to be lysosomal,[3] that they have a suggestion of inner structure by light microscopy and that they appear concurrently with the onset of increased nucleic acid synthesis,[17] it was decided to study them by electron microscopy and by histochemical analysis. The results indicate that they represent auto-phagocytic lysosomes. The possible relationship of these cytoplasmic organelles to the rapid preparation of the liver for cell division will be discussed.

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Material and Methods

Albino Sprague-Dawley male rats were used throughout the experiment and were obtained from Carworth Farms, Inc., (New City, Rockland County, N. Y.), CFE strain. They were chosen at random from large groups and were fed Purina® Rat Chow and water *ad libitum*. All of the experiments were performed on animals weighing 225 gms ± 25 gm. The operative procedures were performed between 8 a.m. and 11 a.m. to control diurnal variation in the regenerative process, and were all performed by a single individual. Laporotomy and hepatectomy were carried out in a standard manner [12] with particular attention to avoiding contact with the residual lobes or their venous channels. By this method an average of 68 per cent of the liver was removed. Liver tissue was obtained for light microscopy from 10 animals at 3-hour intervals following operation, and of these 10 specimens 3 were selected for electron microscopy. Additional tissue was obtained for light microscopy from each of 5 animals at hourly intervals during the 24-hour period after operation and of these 2 from each group of 5 were selected for electron microscopy. The sequential changes were studied by light microscopy in each of 6 animals and electron microscopic sections were prepared in each of 2 animals in the group of 6 under the following schedules: 1, 3 and 5 hours; 2, 4 and 6 hours and 4, 8 and 12 hours. For performances of each biopsy, the animal was lightly anesthetized with ether, the original incision opened and the lobe of choice freed gently from the surrounding tissues. The usual sequence was the removal of the anterior segment of the caudate lobe, the posterior and then the anterior segment of the right posterior lobe. This removal of additional liver tissue was effected without demonstrably altering the sequence of morphologic changes to be described, as judged by comparison with animals sampled only at the terminal point. All control animals were subjected to a sham-operation in which the length of the incision, the incision of hepatic membranes, manipulation of the liver, and the duration of the ether anesthesia, were equivalent to those procedures in the experimental animals. The animals were sacrificed under light ether anesthesia by cutting first the portal vein and then the thoracic inferior vena cava.

Thin slices of liver were fixed immediately in phosphate-buffered formalin, pH 7.25, for 24 hours, paraffin embedded, sectioned at 4 μ and stained. No differences in the histologic appearance were produced by a wide range of fixatives. The stains utilized were Wilder's silver impregnation method, hematoxylin and eosin, Mallory's phosphotungstic acid hematoxylin (PTAH) and the periodic acid-Schiff (PAS) reaction with and without diastase digestion.[18] These were performed on serial sections in the standard manner save for the PTAH, the details of which are described in the text. Multiple specimens were taken at 1½, 3, 4, 5, 6, 7 and 12 hours for study by electron microscopy. Segments of tissue 3 mm in thickness were fixed at 2° C for 24 hours in 0.01M phosphate-buffered 4 per cent glutaraldehyde at pH 7.2 and then further fixed in unbuffered 1 per cent aqueous osmium tetroxide. The specimens were dehydrated by passage through a series of graded alcohols and embedded in Epon 812. Sections were cut with an LKB ultratome, stained with lead citrate [19] and examined with an RCA EMU 2B electron microscope.

Studies of acid phosphatase activity were carried out on material obtained from the animals used for conventional stains. The tissues were cut at 2 to 3 mm and immediately immersed in a solution of 4 per cent formalin, 1¼ per cent calcium acetate adjusted to pH 7.25 with sodium hydroxide, and were fixed for 18 hours at 0 to 4° C. Slices 5 μ in thickness were then cut on a freezing microtome and collected in cold 0.05M acetate buffer, pH 5. After air-drying on slides at room temperature for 30 minutes they were incubated in a standard Gomori medium [18] 15 to 90 minutes. Control phosphatase sections included material in which phosphatase activity was inhibited by the addition of 0.01M sodium fluoride, by elimination of

glycerophosphate or by elimination of lead nitrate from the medium. All further procedures were performed in a standard manner save for the application of 0.1 per cent safranin (in 1.0 per cent acetic acid) counterstain for 1 minute. This stained the cytoplasm and nucleus lightly. Specimens obtained for acid phosphatase localization by electron microscopy were fixed in cold 0.01M citrate buffered 4 per cent glutaraldehyde or formaldehyde at pH 7.2 containing 7.5 per cent sucrose for 2 to 20 hours. Frozen sections 50 μ in thickness were cut from the fixed tissue and incubated in Gomori lead nitrate-beta glycerophosphate medium for 30 to 120 minutes. The sections were then washed in 7.5 per cent sucrose in 0.05M acetate buffer at pH 5, fixed in unbuffered aqueous 1 per cent osmium tetroxide, dehydrated in alcohols and embedded in Epon 812. Unstained sections were examined with the electron microscope.

Results

Light Microscopy. The histologic alterations noted after partial hepatectomy were generally similar to those previously described.[11] There was an initial congestion of the sinusoids and vacuolization of some cells commencing at about 2 hours. Glycogen diminished rapidly following operation until at 12 hours little or none could be demonstrated, and during this period the normally prominent basophilic granules of the cytoplasm became less evident. All of the time intervals are in reference to the partial hepatectomy which is considered as zero time.

There appeared in the cytoplasm of the liver parenchymal cells following partial hepatectomy a population of spheroid bodies which will be referred to as auto-phagosomes. This group of structures appeared as early as ½ hour in some animals but the average time of first appearance was 2 hours with a maximal number usually developed by 6 to 9 hours; most had disappeared by 24 hours. Many of the cells at the height of the response contained several of these bodies, some of varied sizes (Figs. 1 and 2). There was progression in the number of bodies per section as well as an increase in their size, the earliest measuring approximately 1 μ in diameter and the largest upwards of 25 μ. In some animals there appeared to be a rapid development of bodies with little alteration in the pattern over the next few hours; in others, "crops" of small (1 to 5 μ) bodies appeared throughout the period of observation with a small peak in appearance at 10 to 12 hours. The sequential changes were followed in individual animals subjected to multiple biopsies.

The original location of the bodies was usually in the middle or peripheral cytoplasm. With time some became perinuclear and even indented the nuclei, while others projected more and more into the sinusoid. In preparations stained with hematoxylin and eosin the bodies were a homogeneous and faintly granular pink, with occasional inclusions or vacuoles. They stained a brilliant purple with PTAH, red

with PAS stain and black with reticulin stains. Their staining intensity was slightly diminished by prior diastase digestion. All of the bodies less than 10 μ in diameter and many of the larger ones stained intensely with PTAH in a period of exposure to stain of 2 hours. Exposure for as long as 24 hours, however, failed to stain some of the largest bodies (20 μ or greater), especially those adjacent to sinusoids or those undergoing apparent resorption. Resorption was evidenced by serration of the margins of the bodies and vacuolization of their content.

By use of the Gomori technique for demonstrating acid phosphatase activity, early alterations were found in the normally delicate, finely granular, pericanalicular pattern. One hour after partial hepatectomy there was coarsening of this pattern and apparent aggregation of the granules. With the appearance of bodies, many of the diffuse cytoplasmic and pericanalicular enzyme-positive granules disappeared while the bodies stained, from the time of their appearance, as black spheres (Figs. 3 to 5). Bodies of a diameter greater than 20 μ demonstrated diminished acid phosphatase activity and many contained vacuoles free of reaction. Enzymatic activity was completely lost in the largest bodies or in those projecting into the sinusoidal spaces, especially those undergoing resorption. The Kupffer cells demonstrated an increased enzymatic activity throughout the period of regeneration. The addition of 0.01M sodium fluoride to the substrate eliminated the deposition of lead in all specimens regardless of the duration of exposure.

Electron Microscopy. In control liver specimens vacuolated bodies approximately 0.6 μ in diameter and smaller bodies approximately 0.3 μ in diameter with a dense homogeneous inner matrix were noted in the pericanalicular areas. These structures had appearances consistent with those of lysosomes and microbodies in normal liver.[20,21] As early as 1½ hours after partial hepatectomy another group of spherical bodies bound by a single membrane and measuring approximately 1.0 μ in diameter appeared in the pericanalicular cytoplasm. The inner matrix was somewhat denser than that of the microbody and many contained one or more mitochondria, some of which were surrounded by a membranous whorl (Figs. 6 and 7). These bodies were occasionally in apposition to myelin figures (Figs. 8 and 9). Such organelle-containing structures were never seen in control animals.

By 3 hours the phagocytic bodies were located throughout the cytoplasm of the liver cell. They ranged in size from approximately 10 to 40 μ and contained not only mitochondria but recognizable bundles of granular endoplasmic reticulum and aggregates of glycogen granules. Many of the included mitochondria, although readily identifiable, were undergoing degenerative changes which consisted of breakdown of membrane structure and osmiophilic smudging suggesting auto-

phagocytic activity (Figs. 6 and 7). In the 6- and 7-hour specimens the auto-phagosomes demonstrated extremely variable size and electron density. They were chiefly located in the perinuclear area at this time (Figs. 8 and 9). The largest bodies contained structures thought to represent remnants of organelles and occasionally after 6 hours exhibited little or no electron density.

Acid phosphatase reactions prepared for electron microscopy showed dense staining of the pericanalicular lysosomes in both control and experimental animals. In addition, the auto-phagosomes frequently demonstrated acid phosphatase activity as dense granulations within their limiting membranes and around the encompassed organelles (Figs. 10 to 12). Larger bodies had few focal granular deposits but exhibited a diffuse increase in their lead deposition when compared to controls in which the acid phosphatase activity had been inhibited by fluoride.

Coincident with the appearance of the auto-phagocytic bodies, the cytoplasmic organelles underwent a series of alterations. The mitochondria, normally scattered in large numbers throughout the cytoplasm, were markedly reduced in number. In addition, there was a significant decrease in the amount of the discrete stacked-lamellar forms of granular endoplasmic reticulum present. By the twelfth hour, the granular endoplasmic reticulum reappeared and occupied much of the cytoplasm. The stacked-lamellar structure however, had been replaced by confluent dilated cysternae. Large numbers of mitochondria also reappeared during this period and were located in the interstices of the granular endoplasmic reticulum. Cytoplasmic glycogen was likewise rapidly and markedly diminished after partial hepatectomy (Figs. 13 and 14).

Discussion

It is apparent from these studies that the presence of auto-phagosomes in the rat liver cell following partial hepatectomy is a transient phenomenon. The electron microscopic structure of these organelles, their acid phosphatase activity and their auto-phagocytic function, place them in the class of lysosomes [1] or auto-phagosomes.[6] The largest, exhibiting diminished staining to light microscopy, loss of acid phosphatase activity and decreased matrix density electron microscopically, suggest an end stage of the auto-phagosomes. Final dissolution of their membranes could account for their complete and sudden disappearance.

Although there appears to be a specific predilection for mitochondrial incorporation by these structures, glycogen and endoplasmic reticulum are also identified within them. The loss of these organelles during the early phases of the regeneration of the liver has been previously reported.[11,22-24] The auto-phagocytosis of the organelles may partially account for this loss. The presence of mitochondria and granular endo-

plasmic reticulum in varying stages of degeneration[25] within the bodies and the absence of such degenerative forms elsewhere in the cytoplasm suggest that degradation of the organelles occurs primarily if not solely within auto-phagosomes.

The origin of the auto-phagosomes has been described in only a few tissues, in which they are usually present in limited numbers. Their consistent presence in large numbers in cells of the regenerating rat liver may be related to the rapid transition from a state of inactivity to one of vigorous mitotic activity. The cultured lymphocyte when treated with phytohemagglutinin also undergoes a similar transition from mitotic inactivity to frequent cell divisions. It is interesting to note that recent reports indicate the appearance of large, active lysosomal granules in these cells in a time sequence very close to that of the auto-phagosome in the regenerating liver.[26,27]

The significance of the relationship between alterations in the number and activity of lysosomes in cells and subsequent cell division is as yet unclear. Since the appearance of lysosomal activity has most frequently been associated with the presence of cell injury it is possible that in the residual tissue of the partially excised liver an injury represents the stimulus to cell division. In such an instance the auto-phagosome and cell division might represent parallel but unrelated cell responses.

It has also been reported that damaged portions of cytoplasm can be removed by localized autolysis.[5] Local damage in the regenerating liver may result secondarily from the preferential diversion of substrates from the maintenance of the structure of the cytoplasmic organelles to the needs of cell division. In this instance, the auto-phagocytic lysosomes would represent a disposal mechanism.

Lastly, it has been suggested that lysosomal activation may be the first cell response to a mitotic stimulus.[26,27] The disappearance of the granular endoplasmic reticulum, the synthesis of new ribonucleic acids[17] and the appearance of auto-phagocytic lysosomes are concurrent. If lysosomes are primarily involved in this process, their function may well be to destroy much of the existing organelle structure of the cytoplasm in preparation for the assumption of new synthetic activity. Indeed, the material derived from the digestion of such organelles might serve directly as components for such synthesis.[28]

These findings indicate that auto-phagocytosis of cytoplasmic organelles can be readily studied in the regenerating liver following partial hepatectomy. The consistent temporal pattern of appearance of auto-phagosomes and their presence in large numbers allow description of their development and evolution *in vivo*. Further experiments utilizing this system may offer insight into the nature of the stimulus for cell division and the sequence of cell alterations in preparation for mitosis.

The present study demonstrates that during the preparation of the hepatocyte for cell division there is morphologic dedifferentiation, produced by lysosomal destruction of existing cytoplasmic organelles.

Summary

Amputation of 70 per cent of the mitotically inactive rat liver induces a wave of cell division in the residual tissue. During the early hours of the period between partial hepatectomy and the onset of mitosis, numerous eosinophilic bodies which exhibit acid phosphatase activity have been identified in the hepatic cells. These structures resemble large lysosomes when examined by electron microscopy. They contain numbers of cytoplasmic organelles in various stages of degradation. Acid phosphatase activity within these "auto-phagosomes" is localized mainly around the inclusions although a diffuse reaction product is demonstrable throughout the structure.

It is suggested that this process accounts in part for the observed simplification of the hepatic cell during this period and represents a form of dedifferentiation. These alterations may be an integral feature of the conversion of a cell from a state of mitotic inactivity to that of active division.

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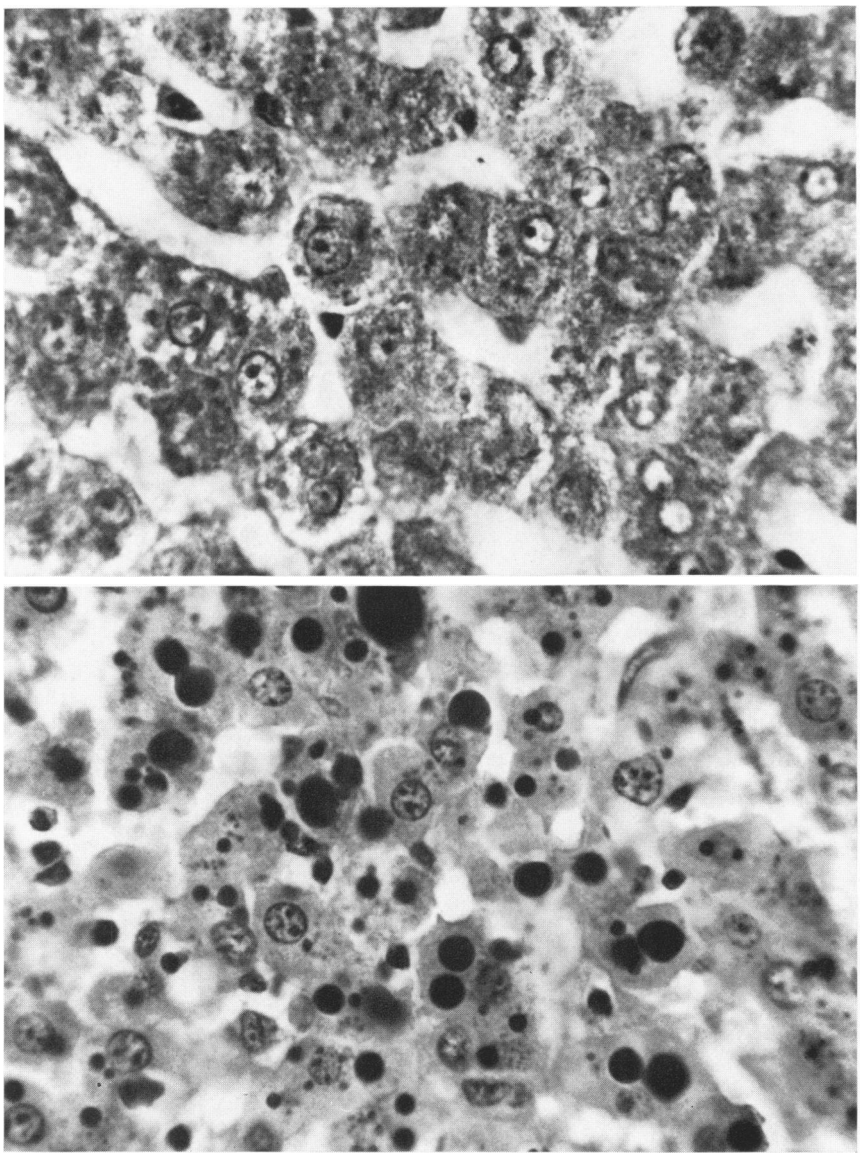


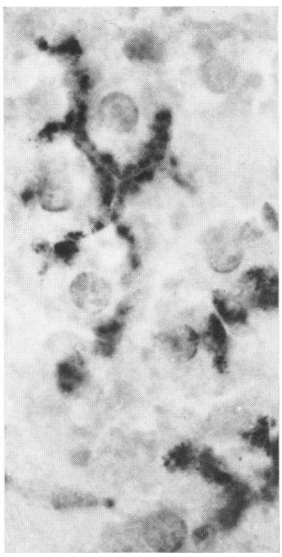
FIG. 1. Normal rat liver stained by the PAS reaction. The diffuse cytoplasmic granules represent glycogen. The staining of these granules was eliminated by prior diastase digestion. No other cytoplasmic structures were so stained. × 450.

FIG. 2. Rat liver 6 hours after excision of 70 per cent of the hepatic mass. PAS staining demonstrates a significant reduction of diffuse granular cytoplasmic staining and the presence of large numbers of intracytoplasmic spherical bodies. These vary in size from 2 to 30 μ and stain intensely. The intensity of the stain was not significantly reduced by prior diastase digestion. × 450.

FIG. 3. Frozen section, normal rat liver, fixed in formol calcium and stained by the standard Gomori lead nitrate-beta glycerophosphate reaction for acid phosphatase activity. Enzymatic activity is demonstrated by the deposition of lead in fine granules, mainly in the pericanalicular areas. No significant aggregates are present. × 450.

FIG. 4. Frozen section, rat liver 3 hours after excision of 70 per cent of the liver mass. The Gomori acid phosphatase reaction demonstrates coarsening of the granular pattern with formation of small intensely positive aggregates. These aggregations of enzymatic activity are still localized in the pericanalicular area. A large Kupffer cell (K) exhibits intense enzyme activity. × 450.

FIG. 5. Rat liver, 6 hours after partial hepatectomy. The tissue was prepared for the Gomori acid phosphatase stain and embedded in Epon 812. The section was then cut at ½ μ and exposed to 2 per cent ammonium sulfide for 2 minutes. The enzymatically active material has now further aggregated into bodies similar in size and distribution to those in the PAS stained sections (Fig. 2). The cytoplasm demonstrates little of the fine granular peribiliary reaction product seen in Figure 3. × 450.



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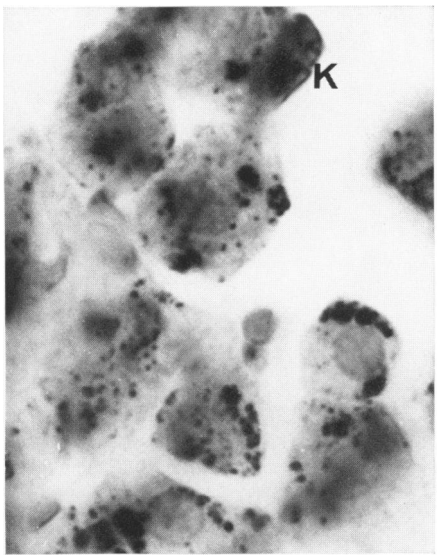

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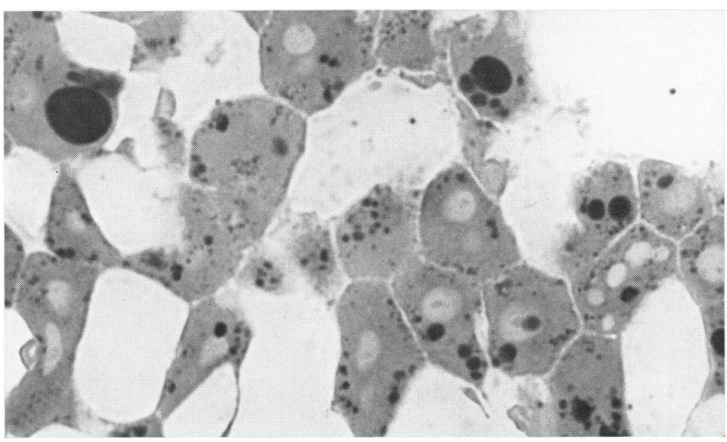

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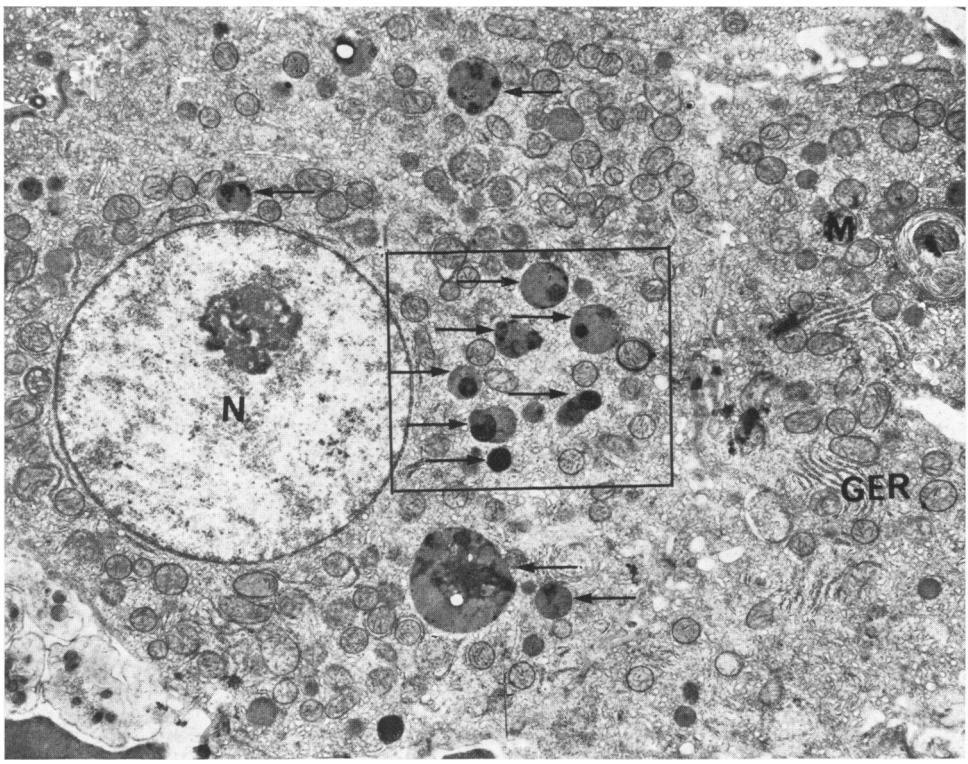
FIG. 6. Rat liver 6 hours after partial excision. The tissue was fixed in glutaraldehyde, embedded in Epon and stained with lead. In the region of the nucleus (N) there is marked diminution of the amount of granular endoplasmic reticulum (GER), glycogen and mitochondria (M) when compared with normal liver (Fig. 13). Located in the middle of the cytoplasm are numerous bodies, the matrix of which is denser than that of the surrounding organelles and within which inclusions can be seen (arrows). × 6,000.

FIG. 7. An enlargement of the area marked in Figure 6. The decrease in cytoplasmic organelles noted is substantiated. The smooth endoplasmic reticulum is intact (SER). Auto-phagosomes with varied content are noted. (a) This membrane bound structure has a homogeneous, granular matrix. It contains a structurally intact mitochondrion. Degeneration of the latter is indicated by smudging of the membranous structure and increased osmophilia. (b) A similar body contains a mitochondrion which has undergone further degeneration with less discreteness of the normal membranes and further smudging. (c) This phagosome contains a well structured membranous system. (d) A glycogen inclusion is present. (e) A mitochondrion is enclosed within a distinct membranous whorl. × 30,000.



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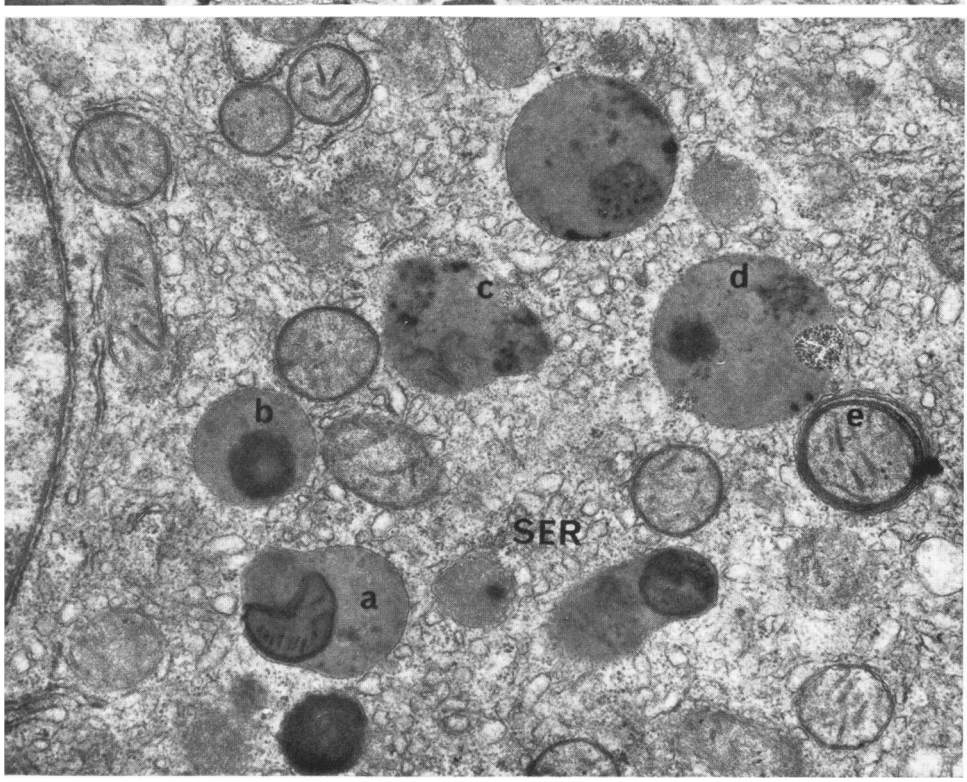

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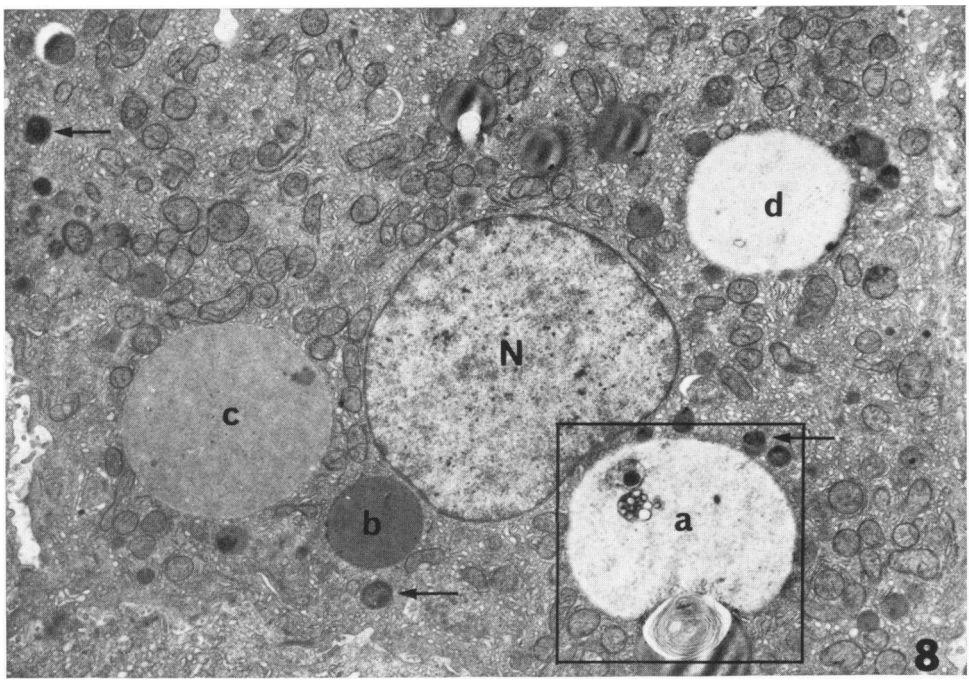
FIG. 8. Rat liver obtained 7 hours after partial hepatectomy; tissue prepared as indicated in legend for Figure 6. The reduction of mitochondria, granular endoplasmic reticulum and glycogen is still evident. Around the nucleus (N) there are cytoplasmic structures of varying sizes and electron density. Several (arrows) are identical in appearance to those auto-phagosomes shown in Figure 7. Larger structures have varying degrees of diminution in electron density. (a-d) A large body with little matrix density (a) indents the nucleus. × 2,500.

FIG. 9. An enlargement of the area delineated in Figure 8. The nucleus (N) and small auto-phagosomes (arrow) are evident. The large body (a) shows a diffuse granular electron density. Within it there are two degenerated membranous inclusions (I). A myelin figure (M) is present at the periphery of the autophagosome (a). × 10,000.



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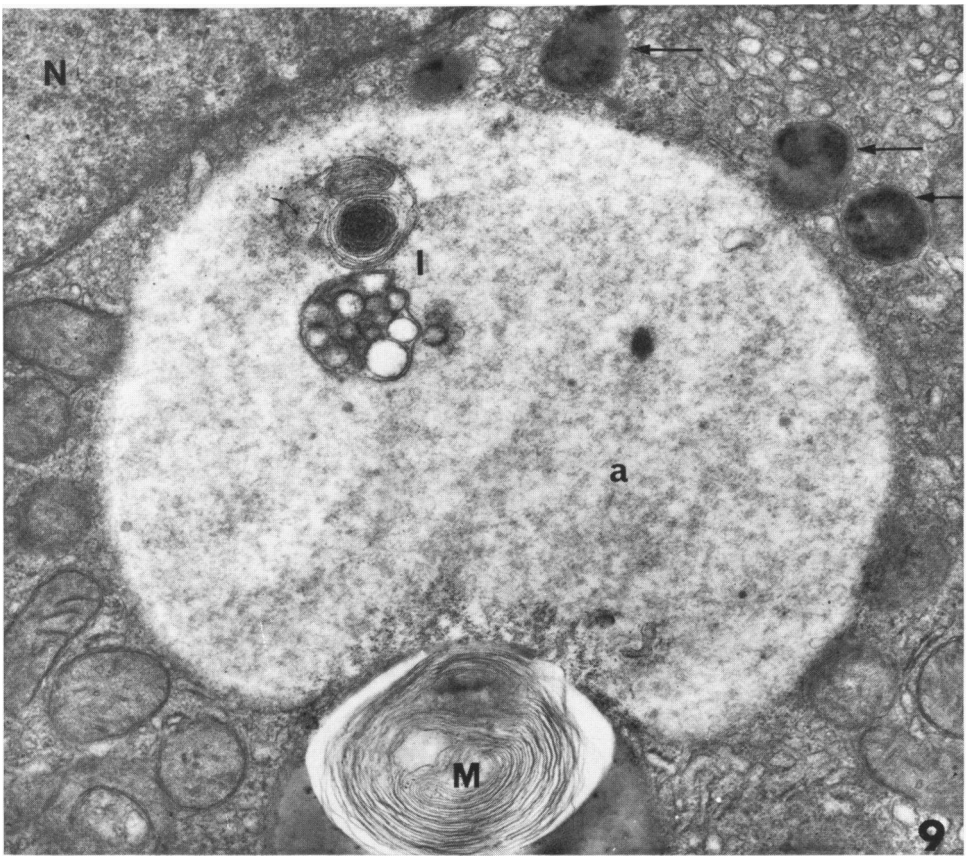

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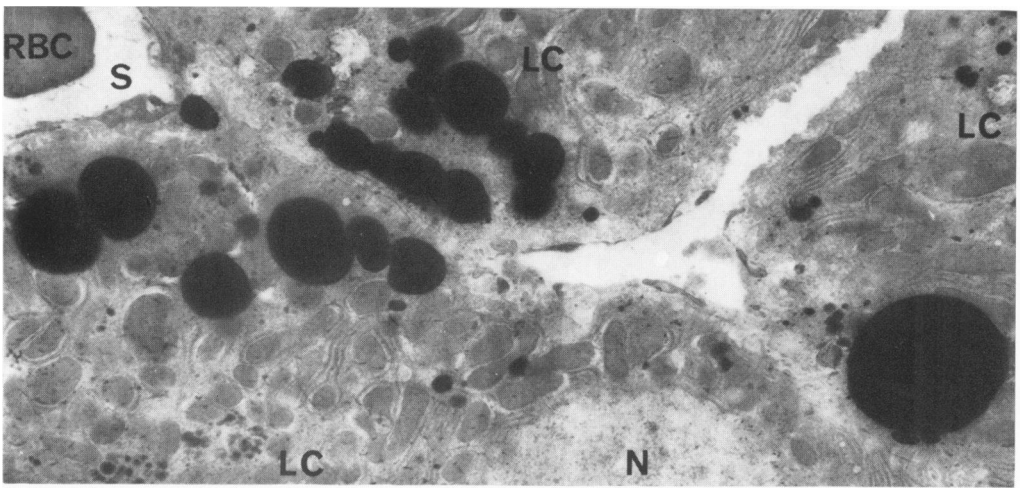
FIG. 10. Rat liver 2 hours after partial hepatectomy. The tissue was fixed in citrate buffered formalin with 7.5 per cent sucrose added. Frozen sections were incubated in the Gomori reaction media, refixed in osmium tetroxide and embedded in Epon. Sections were not lead stained. Multiple bodies of various sizes in several liver cells (LC) demonstrate intense enzymatic activity. Sinusoid (S). Erythrocyte in sinusoid (RBC). Nucleus (N). × 5,000.

FIG. 11. Rat liver 3 hours after partial hepatectomy and prepared as indicated in the legend for Figure 10. An auto-phagosome containing a membrane remnant demonstrates dense acid phosphatase positive granulations. Mitochondria (M). × 30,000.

FIG. 12. Sections prepared as indicated for Figure 10. Several auto-phagosomes of various sizes are located at the sinusoidal border (S). The largest shows dense deposition of lead, indicating enzymatic activity around a membranous inclusion. × 20,000.



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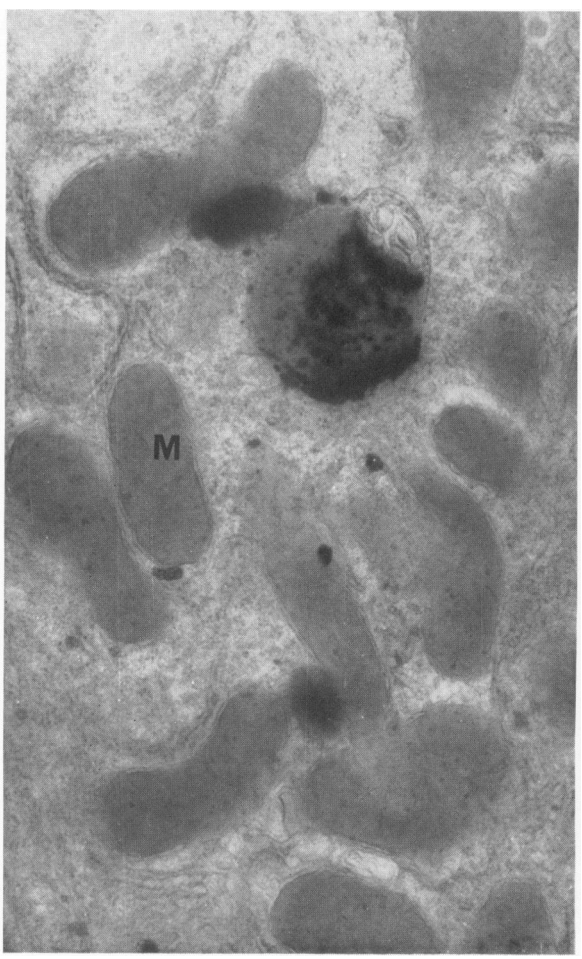

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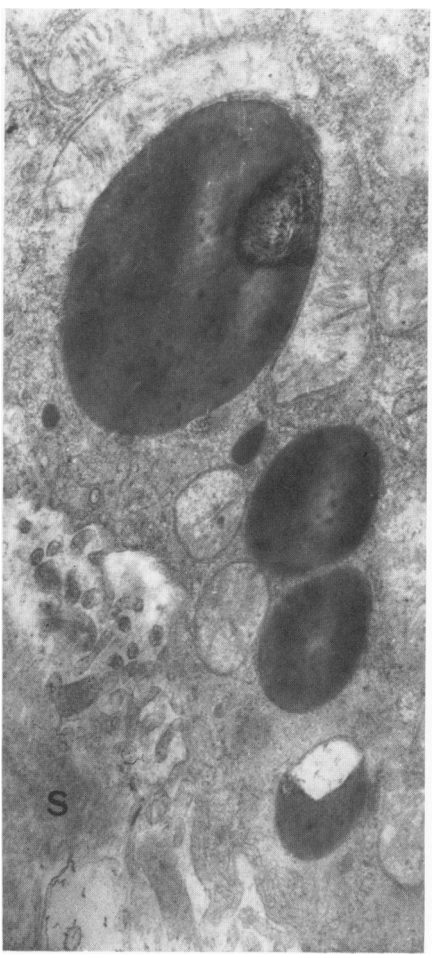

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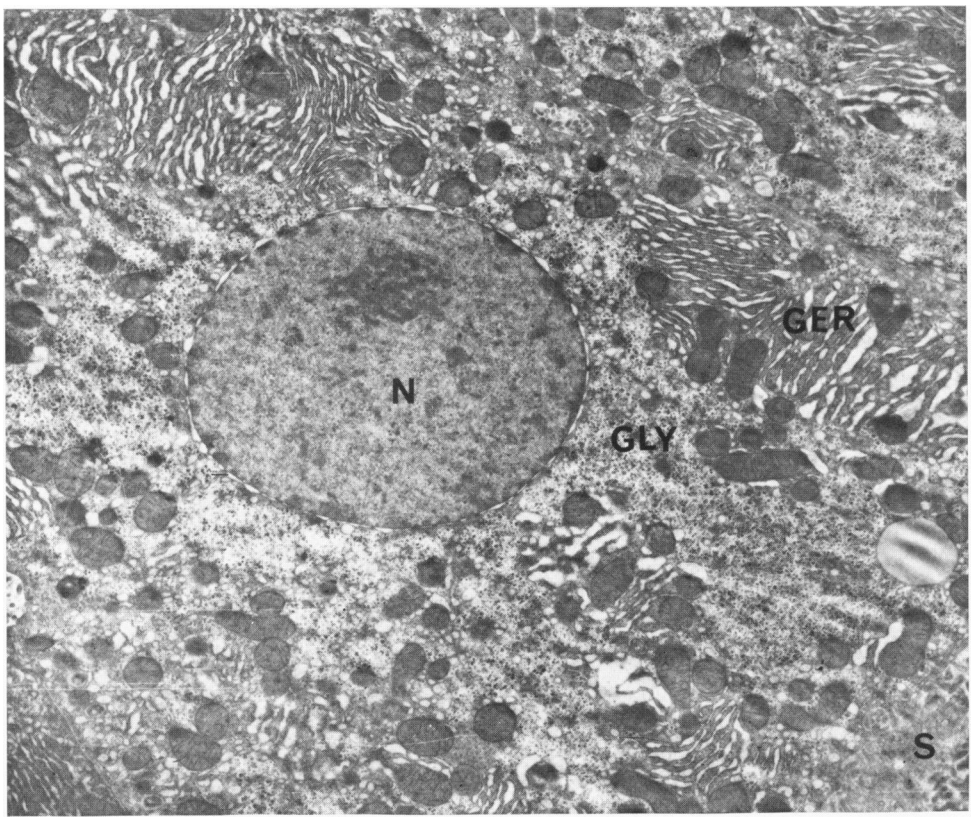
FIG. 13. Control rat liver fixed in glutaraldehyde, embedded in Epon and stained with lead. The granular endoplasmic reticulum (GER) is arranged in discrete stacks of lamellae. Mitochondria (M) are distributed around these lamellae. The peripheral cytoplasm contains abundant glycogen particles (Gly). Nucleus (N). × 6,000.

FIG. 14. Rat liver 9 hours after partial hepatectomy. The granular endoplasmic reticulum is no longer in discrete stacks but forms a continuous network of dilated cisternae. Mitochondria are distributed in the interstices of these lacunae. No glycogen is present in the cytoplasm. Nucleus (N). Sinusoids (S). Bile canaliculus (BC). × 6,000.



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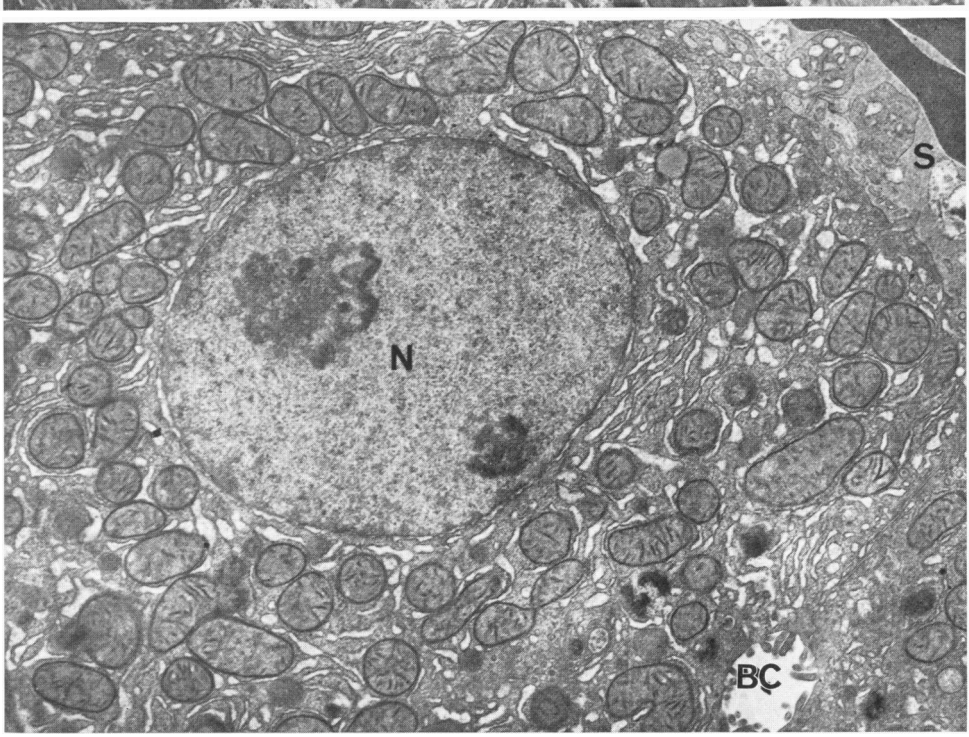

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