Response of the Isolated Perfused Hepatic Parenchyma to Hypoxia *

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SHOCK and the response of tissues and organs to hypoxia has interested investigators in several fields for many years. With the advent of new disciplines, underlying cellular responses and biochemical phenomena are better understood. Electron microscopy has aided studies of fine structural variations of the cell under these conditions. Although best correlated with biochemical factors, this technic allows an over-all view of the cell, suggesting structural-metabolic relationships that are not evident from biochemical data alone.

In the intact animal, interactions tend to obscure the basic events in the cellular response to injury. The problem is diminished when a single isolated organ can be maintained in a state of relative equilibrium under physiologic conditions, and cellular responses can be associated more closely with specific environmental changes. This condition is provided in the isolated, perfused liver of the rat, since the fine structure of the hepatic parenchymal cell remains essentially unchanged for several hours. The preparation also retains the ability to react to appropriate hormonal stimuli (glucagon and epinephrine) with changes in fine structure consistent with known biochemical events.[2]

This type of information is also valuable in the solution of clinical problems. For example, it is generally agreed that clinical shock is accompanied by inadequate perfusion of tissue and organs resulting in hypoxia.[25] In this study, the changes in hepatic fine structure have been determined when hypoxia and anoxia have been produced both by occlusion of the circulation and by reduction in oxygen content of the perfusate.

Methods

Livers of Sprague-Dawley rats weighing 190 to 320 Gm. were perfused cyclically at 37° C. with 30 ml. of 50 per cent blood obtained from rats and diluted with Ringer's solution. Heparin, 1,000 I.U., and 5,000 units each of buffered penicillin and streptomycin were then added, and the system was flooded with a mixture of 95 per cent O_2 and 5 per cent CO_2, or with nitrogen, depending on the conditions desired. The apparatus and technic of perfusion used have been described previously.[2]

Male rats were given food and water *ad libitum*. Blood was obtained from three to six donors, and another donor provided the liver to be perfused. Each experiment started between 9:00 and 11:00 a.m. in order to insure the use of tissue in a similar nutritional state for all experiments. Rates for production of bile were determined by counting drops from a cannula inserted into the common bile duct. The method of perfusion provided a constant inflow pressure of 14 cm. of water through the portal vein, and the rate of flow was checked for consistency at frequent intervals through-

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out the experiment. Modifications[16, 26] of the method of Gomori[9] were used for determination of acid phosphatase.

Serial biopsy specimens were taken from various lobes of the liver, using one lobe for each biopsy. Blocks 1 × 2 mm. were singly[17] or doubly[24] fixed with glutaraldehyde and/or 1 per cent osmic acid in phosphate or cacodylate buffer at pH 7.4. After dehydration, they were embedded in Epon 812.[14] Sections were cut on a Porter-Blum I ultramicrotome and stained with lead solution.[18] A Siemens Elmiskop I electron microscope was used for studies of fine structure. Corresponding adjacent biopsies were fixed for conventional microscopy with formalin-acetic acid, embedded in paraffin, and stained with hematoxylin and eosin after sectioning.

Experimental Design

Livers were perfused and each was subjected to one of three periods of hypoxia: 30 minutes, 1 hour or 2 hours. Hypoxia was produced by one of two methods: 1) simple ligation of a lobe and 2) flooding of the chamber with nitrogen. Preparations with the shortest period of hypoxia (30 min.) were provided with a new supply of hypoxic blood at the start of the period of hypoxia in order to avoid a short lag in the development of hypoxia. After the assigned hypoxic period, each liver was perfused with oxygenated blood by adding oxygen to the same blood. Each type of experiment was carried out in triplicate with successive perfused livers.

Biopsy specimens were taken after a suitable perfusion had been demonstrated (20–30 min. of perfusion), after the assigned period of hypoxia (two biopsies), and after 30 minutes and 2 hours of recovery. The recovery samples of necessity represented only tissue that had previously been perfused anaerobically.

Perfusions were continued only in those preparations that gave evidence of free flow of blood (12–18 ml./min.) during the control period. During this period, the liver was carefully examined and sites for all five biopsies were selected from tissue with similar efficiency of perfusion. At least five blocks were examined from each biopsy site.

An additional perfusion was carried out when tissue was processed for acid-phosphatase activity; determinations of pO_2, pCO_2, and pH were made. Processing included a 2-hour period of hypoxia and specimens were taken before and after this treatment.

Results

The flow of blood through the isolated, perfused liver was remarkably stable after adjustment occurred. The rate of flow after the institution of hypoxia showed a consistent rise to a maximum of about 25 per cent greater than in other preparations, preceded by a transient decrease in rate. On recovery from hypoxia the rate of blood flow returned to original values except for an occasional preparation stabilizing at a level slightly lower than before hypoxia.

In previous investigations flow of bile continued for many hours with this method of perfusion.[2] Ordinarily, the rate of production of bile in the fourth hour is at least half of that during the first hour of perfusion. A stable rate for production of bile was observed at the time of each control biopsy. During the hypoxic period, the rate of production dropped rapidly. After hypoxia for 30 minutes, it was decreased to 40 to 65 per cent of control values; the flow was 0 to 30 per cent of this value after 1 or 2 hours. On oxygenation of the preparation, transient partial recovery was usually observed for a few minutes, followed by a return to the previous low rate.

Gross Appearance

The appearance of the preparations were observed throughout the period of perfusion, but the standard preparation failed to show any difference that would distin-

guish it from the liver of the rat *in situ*. Often the peripheral tissue showed margination with bluish discoloration that disappeared after perfusion started. This was apparently a surface phenomenon, since light pricking of these areas allowed the escape of well oxygenated blood. During hypoxia of either type, the liver tended to be less richly colored and developed a subtle plexiform pattern, suggesting the outline of lobules. The surface of preparations undergoing the longest periods of hypoxia exhibited a sweating phenomenon.

Light Microscopy

Using the methods of fixation and staining described, significant differences in the appearance of most specimens were not detected. The cells displayed a normal appearance after perfusion and hypoxia induced by 30 minutes of anaerobic perfusion. However, those perfused anaerobically for 1 or 2 hours had a foamy or vacuolated cytoplasm occasionally. These variations in cellular appearance were found to be focal and apparently distributed at random throughout the lobule. Alterations were sufficiently variable so that perfusions lasting 1 and 2 hours could not be distinguished from each other by this method.

Electron Microscopy

Control Perfusion

The features of the hepatic cell after perfusion for 30 minutes did not differ appreciably from that seen in previous studies on the liver of the rat *in vivo*[8] (Fig. 1). Very few changes occurred,[2] even after 4 hours of perfusion under identical circumstances. These consisted of moderate decrease in glycogen, reduction in the size and number of bundles of rough endoplasmic reticulum, slight dispersion of ribonucleoprotein (RNP) particles and moderate increase in the number of lysosomes. These are features that appear under conditions of fasting *in situ*[19] and were not considered related to any adverse condition of perfusion.

Hypoxia (1 and 2 Hours)

Nucleus. The chromatin, usually evenly distributed in well fixed material, was marginated and aggregated (Fig. 2a). The nuclear membrane remained unaltered except for alteration of the spacing of the inner and outer layers of the envelope. This late change, when present, was closely associated with similar changes of the endoplasmic reticulum. The nucleolus was not appreciably altered.

Rough Endoplasmic Reticulum. The rough endoplasmic reticulum (RER) was seen in two forms: the usual array of parallel lamellae studded with ribosomes (Fig. 2a) and homogeneous fields of vesiculated membranes, many of which retained characteristic ribosomes (Fig. 3). These two types occasionally existed in the same cell. The form of rough endoplasmic reticulum did not depend on the integrity of the cell; that is, disrupted cells often displayed the same types of rough ER as did intact cells (Fig. 2b).

Smooth Endoplasmic Reticulum. The lumena of the smooth ER were dilated in all cells although the degree of dilatation varied from cell to cell; in cases with extreme changes in the rough ER, areas of smooth ER were indistinguishable from it (Fig. 3), although in cells with less advanced changes this organelle retained its localization in discrete areas of the cell (Fig. 2a). This structure was also associated with vacuoles (see below).

Glycogen. The total amount of demonstrable glycogen was sharply decreased. This product, ordinarily located in the interstices of the smooth ER was seen with increased frequency in structures that appeared to be lysosomes. The glycogen seen in the cell was compact and appeared in

smaller clumps than are seen usually. This product was depleted in many cells, although there was no discernible correlation between its presence and the retention of normal cellular characteristics or with disruptions of cells. Large quantities of free glycogen were found within the lumina of the sinusoids, and its concentration was proportional to the duration of hypoxia. This was much more evident in ligated specimens, presumably because the debris was not diluted by the circulating blood.

Mitochondria. The mitochondria of all cells displayed changes after lengthy periods of hypoxia. Although the membrane system was relatively unchanged, the matrix was less dense and characteristic granules were absent or decreased in number and size (Fig. 2a). The over-all dimensions of the mitochondria remained the same. Occasionally, however, a mitochondrial structure was found within a dense body, presumably a lysosome.[1] Others had a very dense matrix (Fig. 5b) but showed this change even when apparently free within the sinusoid.

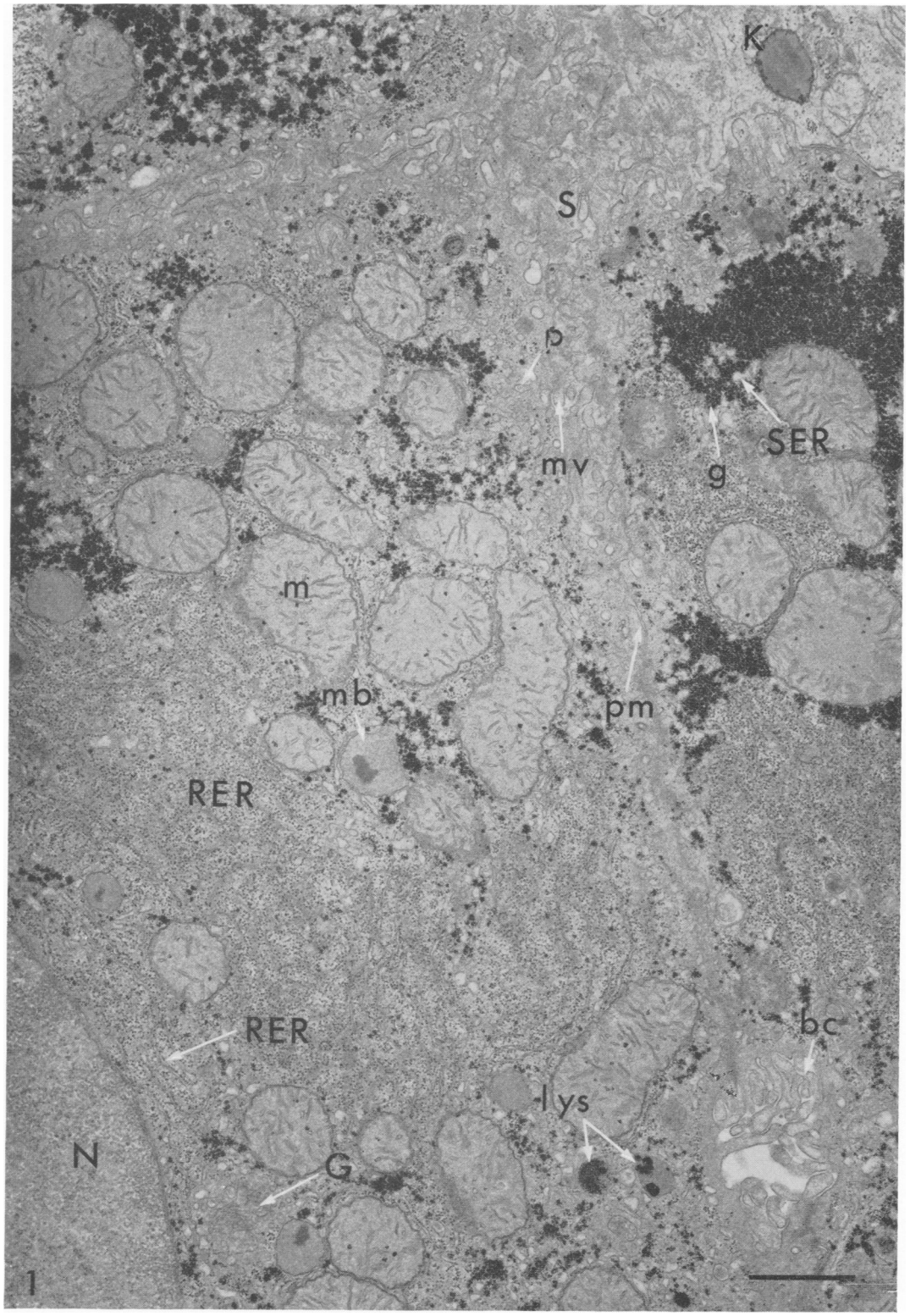
Golgi Apparatus. The golgi apparatus is recognized by a characteristic arrangement of flattened vesicles and cysternae containing small numbers of electron-dense particles. These structures showed little change after hypoxia, although flattened vesicles constituted a lesser proportion of the configuration and electron-dense inclusions were slightly more frequent.

Matrix. The matrix of all cells appeared less dense, but this could be due at least in part to variables in photographic processing. However, the number of mitochondria per unit area of cytoplasm decreased about 25 per cent during prolonged hypoxia. This may have been due partially to swelling of organelles, although vacuoles were excluded from measurement.

Vacuoles. Vacuoles are not reported in the hepatic cytoplasm of the normal rat.[8, 23] During perfusion, small vacuoles were noted in two of over 60 perfusions. In these two instances, the development was minimal but was present to some extent in every block of tissue examined. However, it was noted that these experiments had been recorded as moderately traumatic, with some difficulty experienced in obtaining a free flow of perfusant.

After hypoxia, especially that produced by anaerobic perfusion, cytoplasmic vacuoles were seen in many cells. These varied in size from very small (Fig. 4b) to very large. In the latter instance they virtually replaced the cytoplasm of the cell. Small forms were found only in peribiliary areas near lysosomes. As the size increased a peripheral distribution was often noted that appeared to be associated with the smooth endoplasmic reticulum (Fig. 4a). These structures usually were surrounded by a single membrane, although in some sections the membrane was partially absent. Cellular organelles or cytoplasm often could be seen projecting into the lumen of these structures (Fig. 4b). When tissue was processed to demonstrate acid phosphatase activity by the Gomori method, the resultant lead precipitate was found not only in lysosomes but also in these vacuoles. Cellular

FIG. 1. Electron micrograph of a portion of three hepatic parenchymal cells perfused 30 min (control). Plasma membranes can be seen (pm) approximating each other between the biliary canaliculus (bc) and the sinusoid (S). The sinusoid contains a Küpfer cell (K). Free plasma membrane is thrown into microvilli (mv) and demonstrates pinocytotic pits (p). Dense substance represents glycogen (g) associated with the smooth endoplasmic reticulum (SER). Membrane is obscure when glycogen is plentiful. Rough endoplasmic reticulum (RER) is seen as arrays of parallel particle-studded lamellae when cut in cross section or as field of RNP particles forming rosettes when sectioned tangentially. Granules are plentiful within the matrix of the mitochondria (m). Peribiliary dense bodies (lys) lysosomes are seen near biliary canaliculus. Golgi apparatus (G) is visible, as is a microbody (mb). A nucleus (N) is seen in the lower left corner. Bar represents one micron (× 16,000).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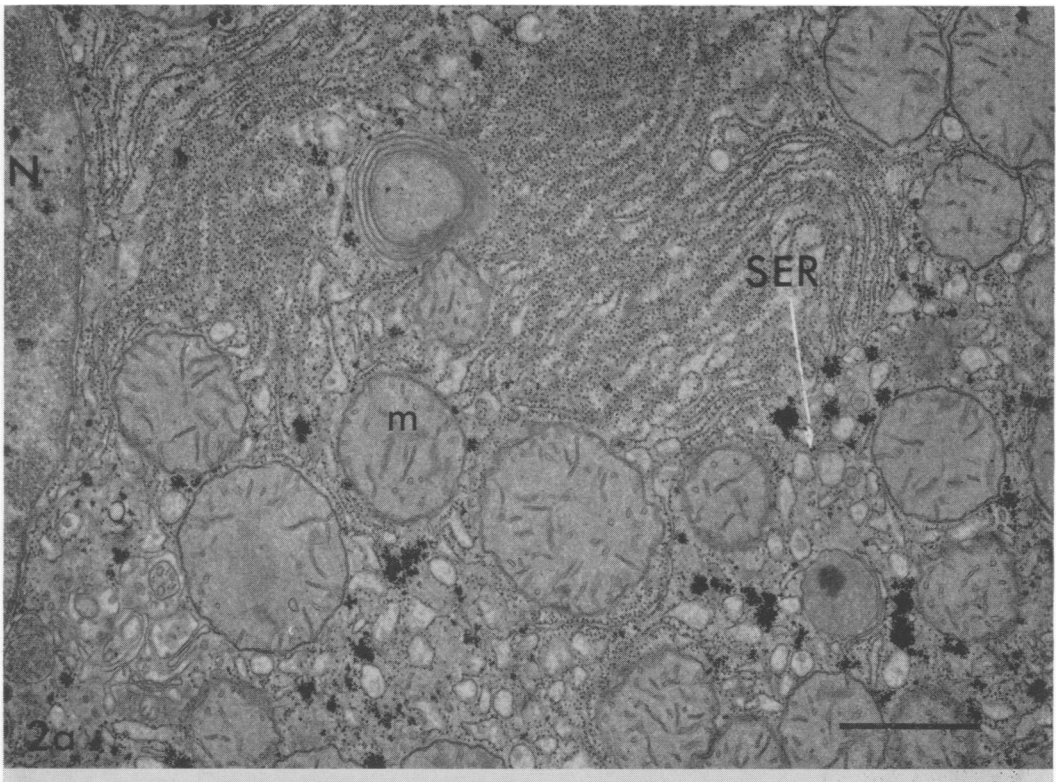
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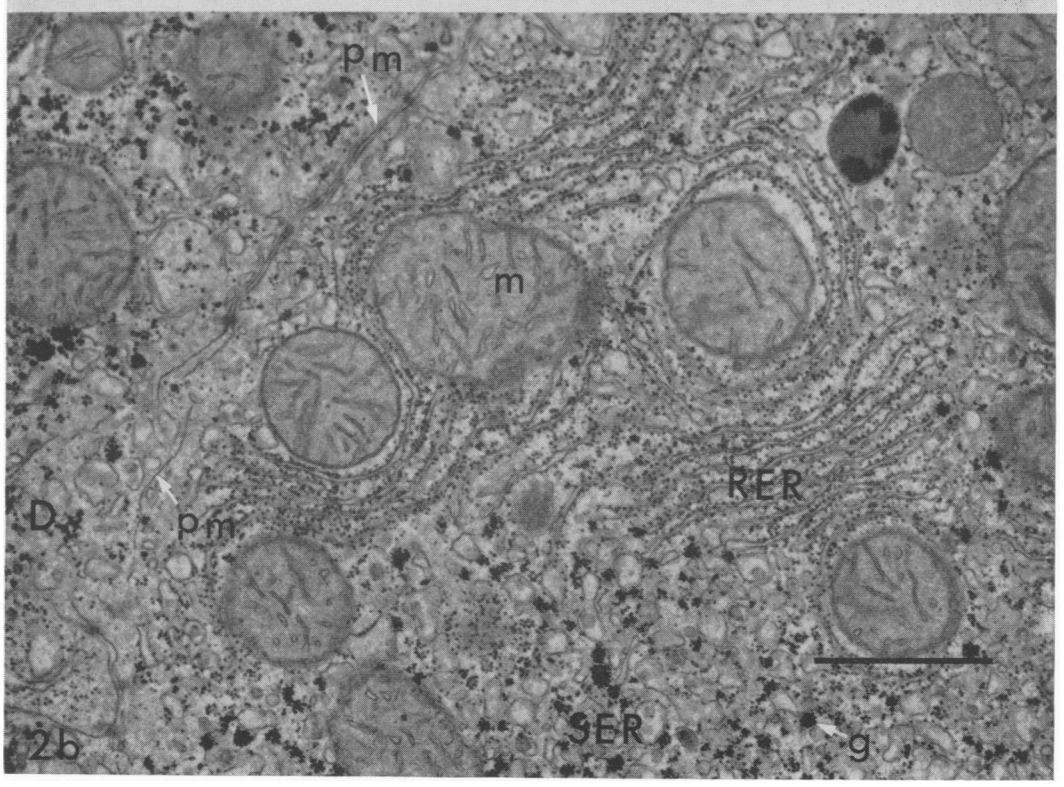
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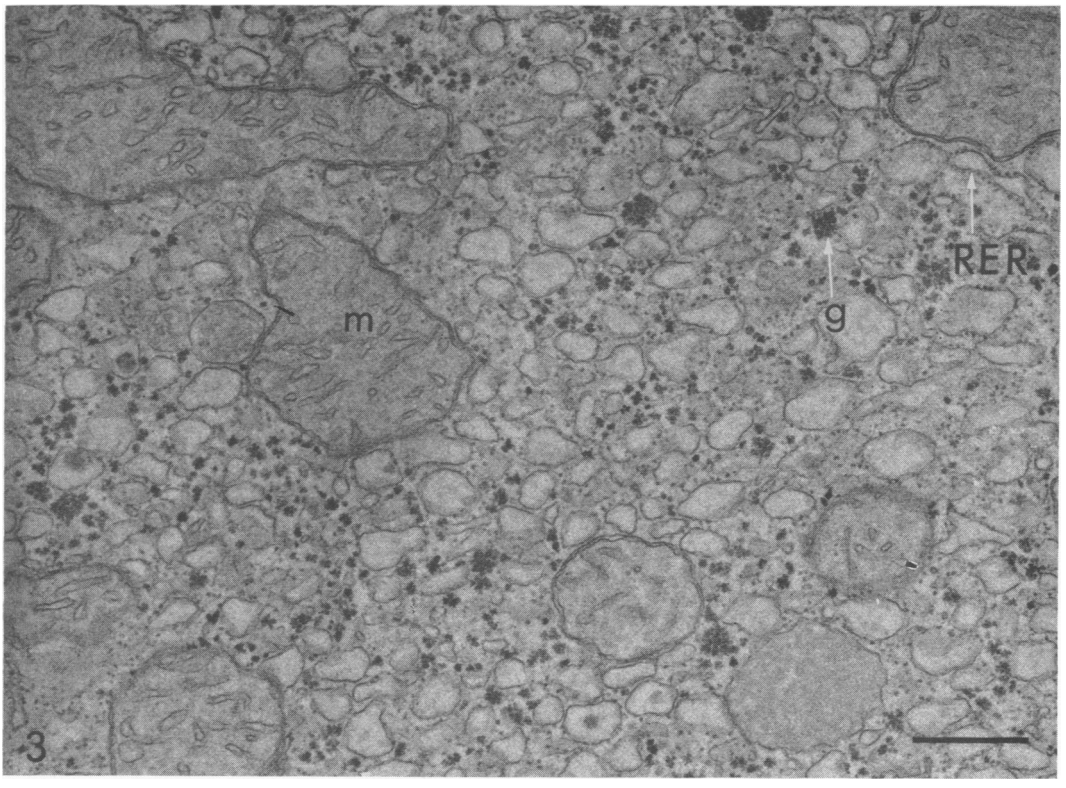


FIG. 3. Portion of a cell hypoxic two hours. Mitochondria (m) are intact, although granules are reduced. Glycogen (g) is generally reduced in amount, although this is not obvious in this cell. Rough endoplasmic reticulum (RER) and smooth ER are vesiculated, but rough ER can be identified by particles clinging to membranes (×15,500).

structures beyond the limits of the vacuole usually were well preserved. Only smaller vacuoles interpreted as very early forms were seen in ligated specimens.

Lysosomes. The peribiliary dense bodies of the hepatic cell have been associated with the lysosomes originally described chemically by deDuve.[5] These bodies were characterized by an heterogeneous collection of amorphous material contained within a single membrane and had a diameter of about 0.5 microns. These structures appeared the same in perfused livers as in specimens from liver *in vivo* (Fig. 1). However after hypoxia they sometimes exhibited a different appearance. When associated with the small (and probably early) vacuoles previously described, they often appeared as a characteristic heterogeneous mass without limiting membrane within a small vacuole. The Gomori method demonstrated lead precipitate in these peribiliary structures (Fig. 5c) and in vacuoles in this region (Fig. 5d). Many that were apparently intact were not as-

FIG. 2a. Section of liver hypoxic for 2 hours. Organelles are intact, although mitochondria (m) lack granules. Nucleus (N) displays mild clumping and margination of chromatin. Smooth endoplasmic reticulum (SER) is slightly dilated (×18,000).

b) Part of an hepatic cell subjected to hypnoxia at 37° C. for 2 hours. Mitochondria (m) are intact but display characteristic granules reduced in number and size. Rough endoplasmic reticulum (RER) is intact, although the smooth endoplasmic reticulum (SER) is slightly dilated. Glycogen (g) is scarce. Plasma membrane (pm) is well preserved between the cells but is quite simple and is disrupted at its free margin. Cellular debris is present in the space of Disse (D) (×23,000).

sociated with vacuoles. In a few cells, changes that might have represented an open or dissolved limiting membrane were not necessarily associated with any change of the surrounding structures. It was suspected that this latter group represented an artifact secondary to fragility or a related phenomenon occurring during fixation or to difficult interpretation of the plane of sectioning. Dense bodies were apparently intact in many of those cells displaying extensive vesiculation of the endoplasmic reticulum or breached plasma membranes (Fig. 6b).

Cell Membrane. The cell membrane of control specimens resembled that seen previously [2] and described by others.[8] The free surface of the cell contained many microvilli and numerous pinocytotic pits, whereas the portion of the membrane facing other cells was simple in contour with the exception of occasional deviations for pseudopodia and the biliary canaliculi (Fig. 1). After hypoxia this appearance was altered. Although portions of the membrane between cells remained unchanged, the free surface of the cell became much flatter. Pinocytotic pits and microvilli were seen less frequently, and the membrane often described a simple line. In many cells the free membrane was discontinous or absent (Fig. 2b). In cells perfused anaerobically, often only half a cell containing constituents with normal appearance persisted. The absent portion of the cell apparently had been eroded by the circulating elements of the blood. Even in these cells most of the plasma membranes between the cells remained intact.

Biliary Canaliculus. During anaerobic perfusion these structures underwent no obvious change, but after ligation of the lobe these structures were found to be closed, accompanied by an increase in the size of the microvilli that may have contributed to occlusion. Occasionally a multilaminated structure (Fig. 5a) was seen, examples of which have been interpreted to be protruding cytoplasmic processes in cross section.[28]

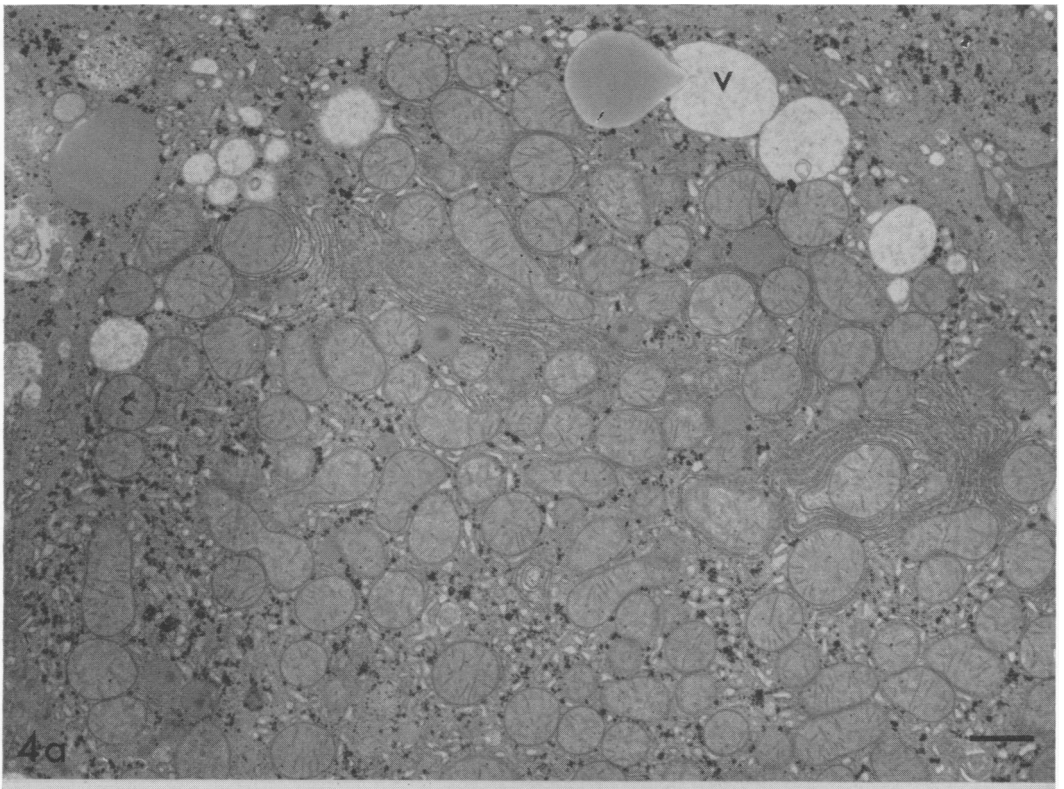
Comparison of Perfused and Ligated Specimens

Most studies have utilized either ligated or incubated hepatic tissue. The two methods for achieving hypoxia used in the present study, anaerobic perfusion and ligation of the lobar vessels, produced similar changes although there were some marked differences in the degree of change produced.

The vacuoles described were seen almost entirely in cells perfused anaerobically. Although they could be identified in ligated specimens, they were very small and not numerous. Glycogen appeared to be less plentiful in the perfused specimens than in those ligated. Apparently the plasma membrane was not ruptured in the one group more than in the other, although the change was much more obvious in the liver perfused anaerobically since these cells were often partially eroded. The biliary canaliculi of the anaerobically perfused specimens remained essentially normal in appearance, whereas they usually appeared to be closed in ligated specimens.

FIG. 4a. Portion of hepatic cell perfused anaerobically for 2 hours. Vacuoles (v) can be seen in the periphery of cell, associated with areas containing glycogen and smooth endoplasmic reticulum (×9,000).

b) Portion of Fig. 4a at higher power. Dilated vacuoles can be seen to coalesce (arrow). Lipid body (L) protrudes into the lumen of one vacuole, as does cytoplasm into the other. Parallel membranes within the cytoplasmic protrusion may represent cristae of adjacent mitochondrion. Smooth endoplasmic reticulum (SER) appears to communicate with vacuole (×35,000).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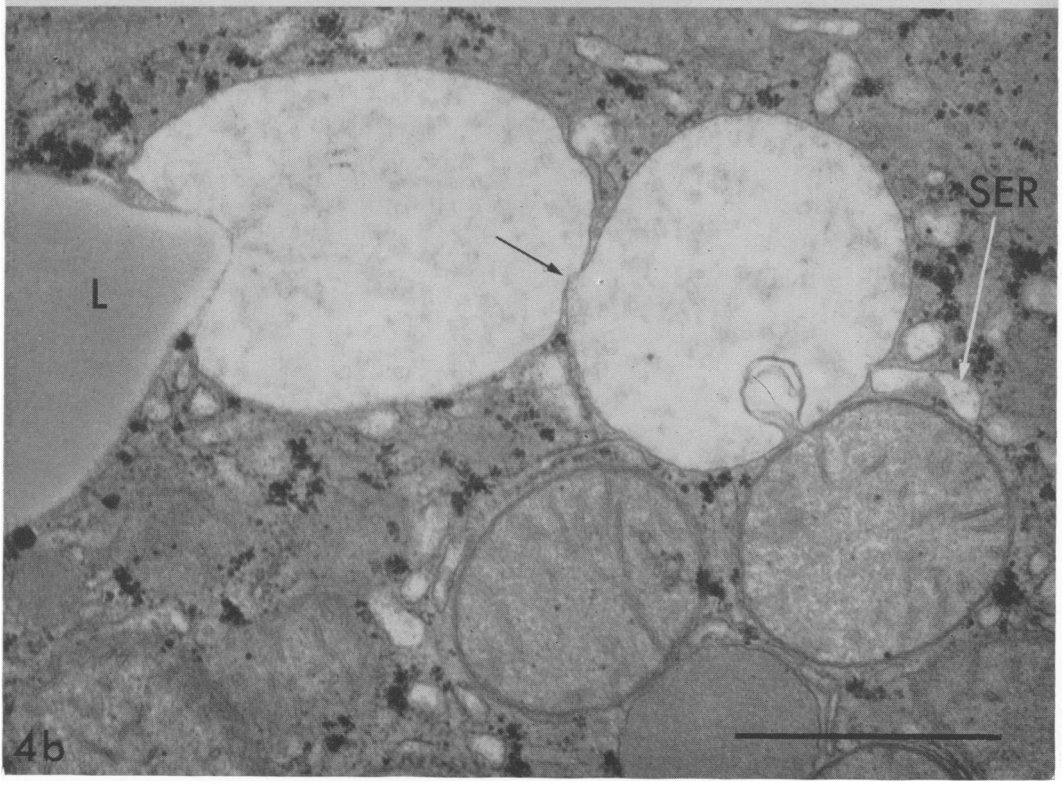
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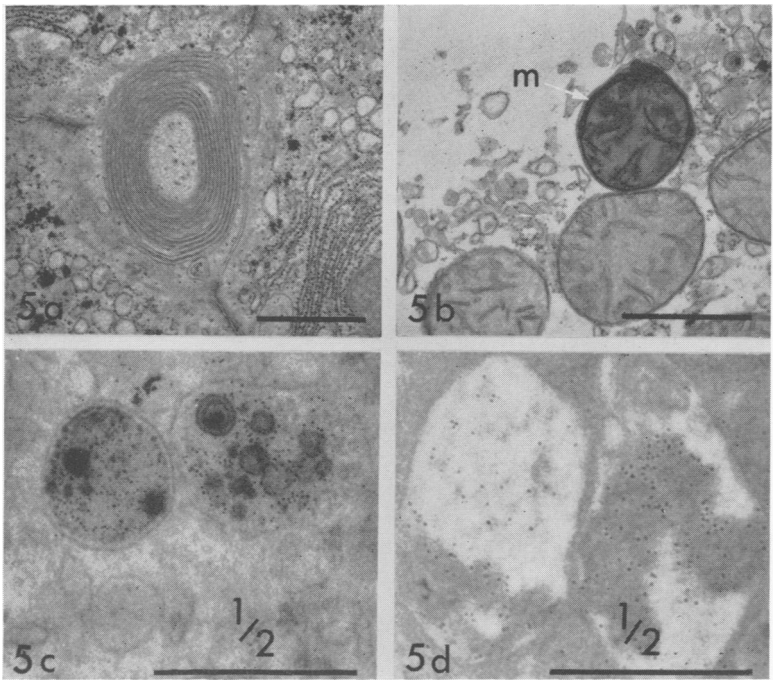


FIG. 5a. Section of an hypoxic cell representing a biliary canaliculus at junction of three cells. Laminated appearance is thought to represent a cross section of protruded cytoplasmic processes (×16,000).

b) Portion of a cell hypoxic for 2 hours. One mitochondrion (m) has become very dense but still displays cristae. Other mitochondria are relatively normal. These organelles are free within the sinusoid (×19,000).

c) Lysosomes in the control material. No stain is used. Fine precipitate of lead within the bodies was deposited by the Gomori reaction for acid phosphatase. Note intact ground substance around these bodies. Bar represents half of one micron (×71,000).

d) These vacuoles from liver hypoxic two hours are not lead stained. Fine lead precipitate results from Gomori reaction for acid phosphatase. Note similarity of precipitate to that in Fig. 4b (×59,000). These vacuoles may represent the results of lysosomal action during hypoxia.

Appearance after Recovery from Hypoxia for 30 Minutes

There was very little evidence of cellular damage after 30 minutes of hypoxia applied by either method. Nearly all cells retained all of the characteristics of the starting material, as did cells seen in the recovery specimens. Some indirect evidence of cellular destruction was deduced from the presence of a small but unusual amount of cellular debris in the lumina of the sinusoids.

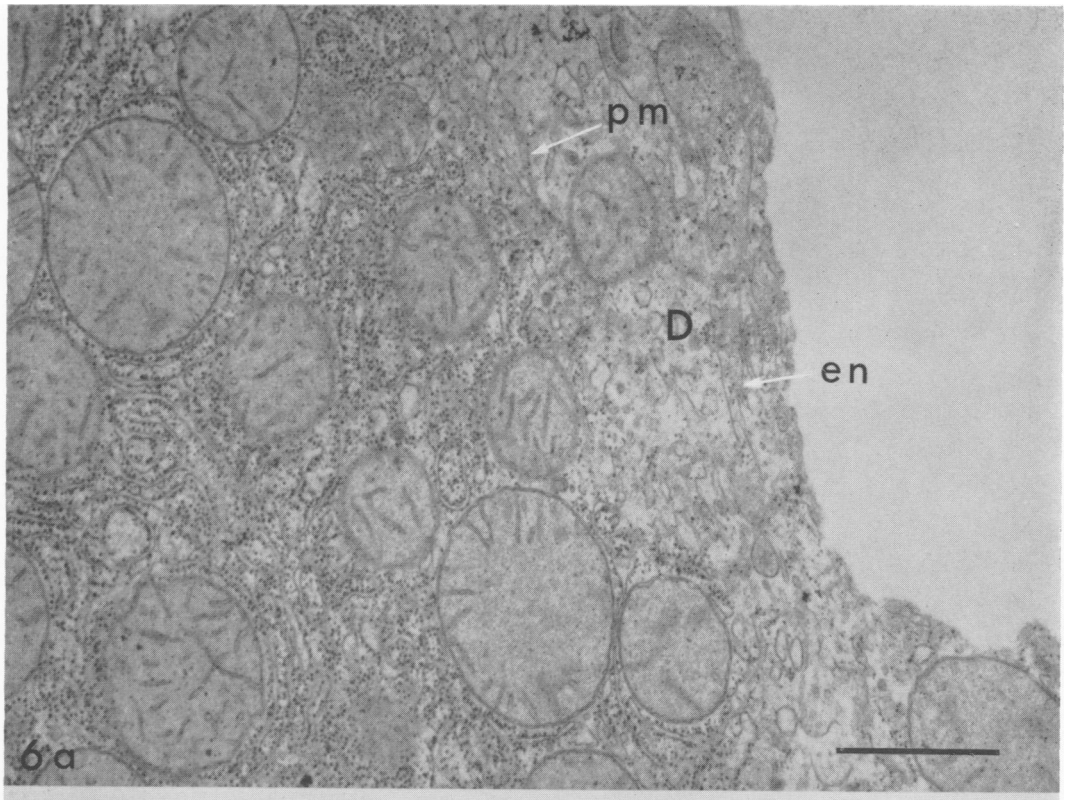
Appearance after Recovery from Hypoxia for 1 to 2 Hours

Changes were generally similar in specimens taken 30 minutes and 2 hours after the restoration of oxygen to the system, although specimens displayed more nearly normal appearance after 2 hours. Cells observed could be classified as one of three types.

1) Many cells had regained nearly all of the features characterizing cells in control reporations. The matrix of the cells appeared slightly denser and the surface structure of the cells appeared to have separated partially. Mitochondrial density per unit area had returned to the control value. Microvilli were particularly well developed and covered the apparently newly exposed surfaces as well as the usual free margin of the cell. The biliary canaliculi were enlarged, appearing slightly distorted.

FIG. 6a. This previously hypoxic cell has not recovered mitochondrial features completely despite perfusion with oxygen for 2 hours. Endoplasmic reticulum is intact. Poorly defined incomplete plasma membrane (pm) and endothelial cell (en) are obscured by debris in the space of Disse (D). Plasma membrane is nearly absent in this cell (×21,500). Space in the upper right corner is the sinusoid.

b) Portion of an hepatic cell after 2 hours of recovery. Rough endoplasmic reticulum is vesiculated (RER). Mitochondria (m) have failed to display normal characteristics. A golgi complex (G) is seen. Apparently intact lysosomes (lys) are near a residual vacuole (V). Vacuoles are seen during recovery period only in those cells that fail to demonstrate normal features (×19,500).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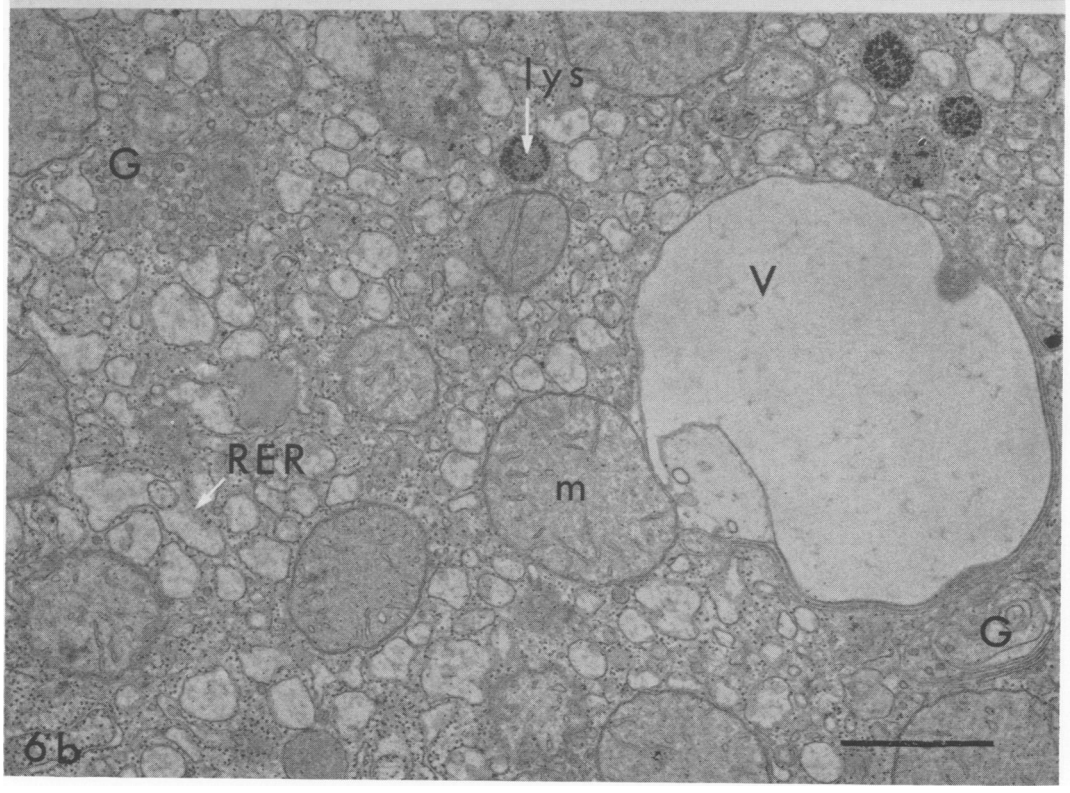
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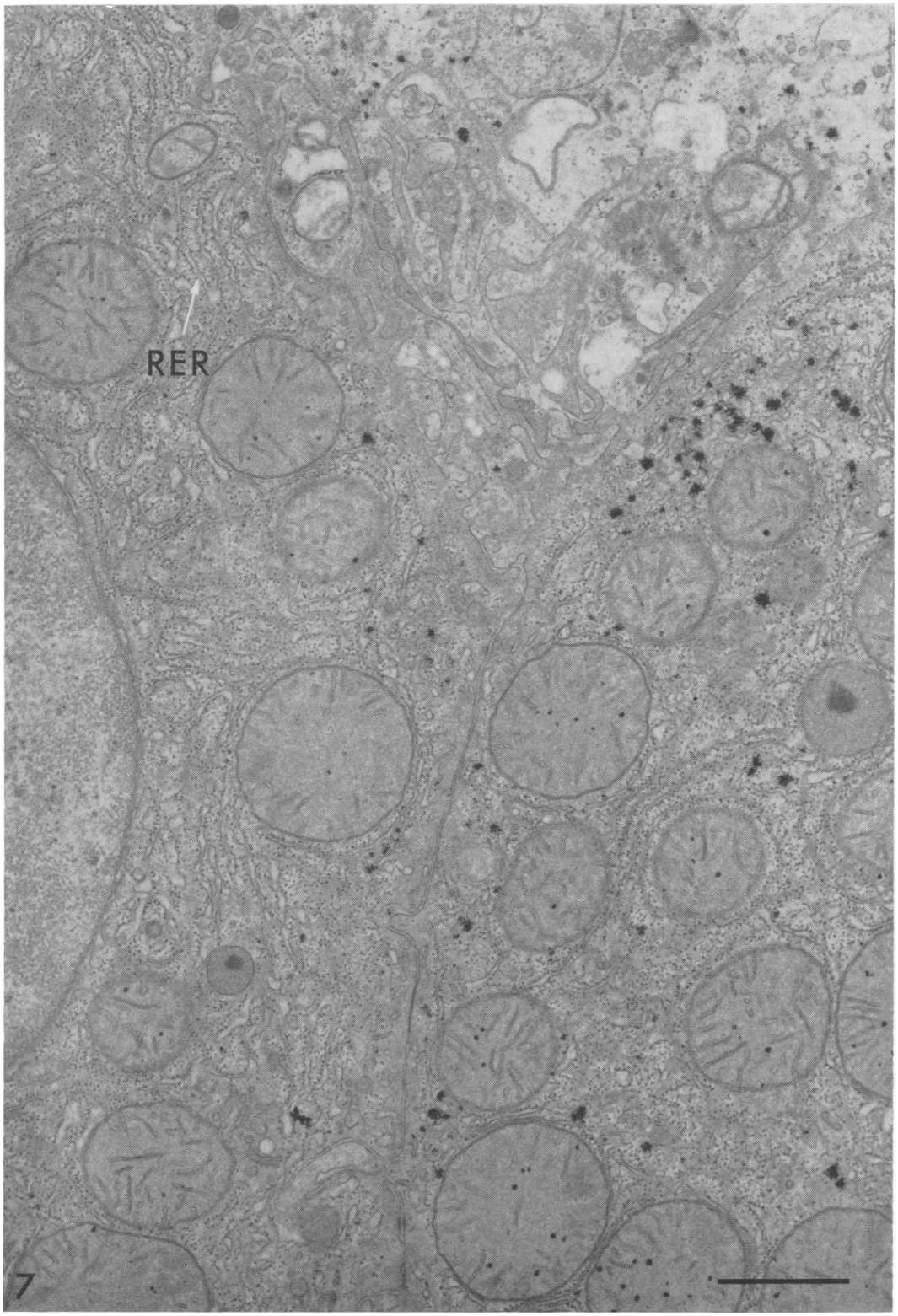
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Vacuoles were nearly absent in these cells, and the number of lysosomes were not measurably increased or decreased. Little glycogen was seen (Fig. 7).

2) Some cells displayed the same appearance as the vesiculated cells seen during hypoxia. The endoplasmic reticulum was completely vesiculated with the loss of zonal organization of the cellular components. The mitochondria remained agranular, and the plasma membrane was a simple structure (Fig. 6b).

3) Other cells displayed the erosion associated with disruption of the plasma membrane and failed to manifest any evidence of recovery (Fig. 6a). Vacuoles were observed in some of these cells.

Discussion

Newer methods of investigation[15] have stimulated re-examination of the responses to injury at the cellular level. Different structural response when the hypoxic state is produced by different methods has been detected by electron microscopy. Several studies have recorded the response of the liver to hypoxia, and there is general agreement that some changes are universal. These include margination and clumping of the nuclear chromatin, loss of mitochondrial granules and thinning of the matrix of this organelle. There is considerable disparity in the time required for these changes to occur, ranging from 5 minutes[10] to many hours under hypothermia.[11, 12] Other observed changes are simplification or discontinuity[5, 10] of the plasma membrane, swelling of the biliary canaliculi and vesiculation of the endoplasmic reticulum.[10, 15, 28] Some observers report vacuolar formation in the cytoplasm, although these changes have been noted only in those experiments in which circulation to the liver continues during hypoxia.[3, 10, 20] These authors are unable to describe a correlation of the vacuoles with an established cellular system. Although Trump *et al.*[28] describe lysosomes that appear open, they conservatively refrain from conclusions, since no surrounding structures are affected and this image may be artifactual.

Since the functions and interrelationships of various components of the cell are not fully understood, it is difficult to determine the criteria of damage within a cell that would indicate loss of viability. A logical approach to this question is observation of fine structure during the recovery period. Any cell that has the ability to re-establish normal characteristics after an alteration during the period of injury presumably is viable. Should the cell fail to revert during recovery, survival is doubtful. Certain characteristics of cellular change can be correlated with an apparent loss of viability, focusing attention upon these changes as a clue to the cause of cellular mortality. In this study these changes are endoplasmic disorganization and disruption of the plasma membrane. Also, any morphologic change that can be correlated with existing biochemical information to indicate cellular injury may be associated with severe cellular injury. In this study the development of cytoplasmic vacuoles containing acid hydrolytic enzymes falls in this category of alterations.

Endoplasmic Reticulum

Although there are many differences in the enzymatic systems associated with the two types of hepatic endoplasmic reticulum (rough and smooth), the visible difference is the possession of ribonucleoprotein particles attached to the membranes of the rough component. The rough ER is con-

FIG. 7. Electron micrograph of cells after recovery for 2 hours. They appear normal in every respect. Rough endoplasmic reticulum (RER) is no longer organized into parallel arrays. This is a normal feature in rat liver perfused for this length of time (4–5 hours). Compare with Fig. 1. Glycogen is depleted (×19,500).

sidered responsible for the manufacture of most of the protein products manufactured in the liver. The two types of membranes differ in their structural response to hypoxia. Whereas the smooth ER is quite labile and tends to become dilated with increased volume of the inner phase, the rough ER is more stable and tends to maintain its usual form for a longer period of hypoxia. However when the period of deprivation is long (1 hour or greater), the rough ER becomes vesiculated, giving the impression of sequestration and disintegration of the large laminar structures that are seen in normal control material. This reaction to injury has been noticed by others in response to carbon tetrachloride[27] and was interpreted as a structural change indicating damage to this organelle. When this change was found in cells during the recovery period there were no signs of viability; that is, the nuclear chromatin remained marginated, mitochondrial granules and density did not return to normal appearance, the cells contained increased amounts of water and those cells retaining an intact plasma membrane possessed one that did not display characteristic pinocytotic pits or microvilli.

This correlation of reticular dissociation with the lack of signs of viability raises the question of its association with the cause of this cellular failure. The importance of the endoplasmic reticulum, especially the rough form, for the functioning of a polyenzymatic process has been demonstrated in studies utilizing differential centrifugation.[4] Enzymatic systems develop increased or decreased activity when treated in such a way as to destroy their chemical union with the membranous system. This could possibly result in the loss of viability of the cell as a whole.

Cellular Disruption

The mechanism of cellular disruption provides another source of possible death of the cell. This process, simplification and disruption of the plasma membrane, has been noted by others in hypoxic cells.[5, 10] Any mechanism causing this phenomenon is obscure, but a likely cause is an increase in the volume of the cell with simple disruption of the distended, free plasma membrane. This view is supported by several findings in this study. Supported membranes (between the cells) remain intact even with the extensive erosion of the cellular contents. The volume of the cell increases, indicated by a decrease in numbers of mitochondria per unit area in the cytoplasma of the parenchymal cell and by the decrease in density of the cytoplasmic matrix. Majno[15] found an increase in the water content of incubated hepatic cells of the rat during the first few hours of autolysis. Osmotic increase in cellular hydration associated with a depression of metabolic activity has been reported[13, 21] to be the mechanism responsible for the change. The absence of a membrane functioning normally, with all it implies in terms of ion exchanges and enzymatic systems, may preclude cellular recovery.

Lysosomes, Vacuoles and Acid Hydrolysis

It has been postulated that the initiating force in death of the cell may be the release of acid hydrolytic enzymes from the lysosomes.[6] These enzymes are found in the peribiliary dense bodies of the hepatic cell which are customarily identified by the demonstration of acid phosphatase activity. Some studies lend experimental support to the association of these hydrolytic enzymes with necrosis. These indicate an increase in the activity of some of these enzymes[5-7, 29] or an increase in acid phosphatase droplets demonstrated by routine histochemical methods[13] during cellular injury.

This study confirms the observation that acid phosphatase is found within the lysosome of the normal parenchymal cell. Dur-

ing extensive hypoxia, we were able to demonstrate the presence of acid phosphatase in newly formed vacuoles originating in the characteristic lysosomal sector of the cell, often abutting lysosomes. This finding suggests the presence of active hydrolysis within the hepatic cell during hypoxia. Since the cellular pH decreases during anaerobic metabolism, the acid enzymes are more active during this period. This phenomenon has many characteristics in common with autolysis. The vacuoles contain not only a liquid phase but also debris representing the remains of most of the types of cellular organelles. Acid hydrolytic enzymes contained within the lysosomes [22] are known to be capable of carrying on the many complex reactions associated with catabolism. Therefore the association of structure and function seems logical. Since this configuration is very common in hypoxic material, but is rarely seen in an apparently normal cell during recovery, this process seems to be reversible, at least within the time limits of this experiment. A continuation of the conditions promoting this vacuolar formation for a longer period of time possibly may cause sufficient cellular damage to preclude reversibility of the process.

Reticular Disorganization, Cellular Disruption and Cytolysis

The prominence of these three changes (reticular disorganization, cellular disruption and cytolysis) appears to vary with different experimental conditions. Both cytolysis and cellular disruption are seen more frequently and in a more advanced state after anaerobic perfusion than after vascular occlusion and stasis. On the other hand there is no detectable difference in the frequency or completeness of reticular disorganization after hypoxia produced by either method. Therefore it appears that the response of the parenchymal cell of the murine liver to hypoxia varies with respect to extent and reversibility of the damage and depends on the manner in which hypoxia is produced. The factors influencing the cells to display more prominent and frequent structural changes of any of the three types described are not clear, although the relative loss of buffering capacity and substrate pool could be more acute in ligated specimens than in those perfused anaerobically. The transient response to restoration of aerobic conditions when the flow of bile was measured, followed by failure to reach control levels, indicates that function may be altered considerably even though many hepatic cells resumed their initial appearance during the recovery period. The complexity of the response, due to variations in conditions of vascular supply and magnitude and duration of original initiating factors, is easily implied from the findings based on experiences with perfusion of the isolated liver. Certainly no single biochemical approach will measure all of the significant cellular responses to hypoxia, making the correlation of biochemical events with morphologic changes the more important to simplify a formidable problem.

Summary

Isolated perfused livers from male rats were subjected to hypoxia by ligation of lobes and by perfusion with hypoxic blood respectively. Specimens for study under the electron microscope were taken before and at the conclusion of periods of hypoxia of both types and during the recovery phase lasting 2 hours.

The rate of flow of bile was reduced by hypoxia—more after 1 to 2 hours than after 30 minutes. Recovery after restoration of access to oxygen was only transient.

Differences were found in the appearance of the hepatic parenchyma when the methods of inducing hypoxia were compared. In ligated specimens, the biliary canaliculi were closed; whereas more and

larger vacuoles were found, the amount of glycogen was less and the cells were more eroded (even though the plasma membrane was not ruptured more frequently) in preparations perfused anaerobically.

Changes noted after 30 minutes of hypoxia were negligible and not consistent, with the exception of more cellular debris in the sinusoids. However the electron microscopy of the liver showed many changes after anaerobic perfusion or ligation for more than 1 hour in contrast to the focal vacuolization of the cytoplasm which was the only change detected by the light microscope after anaerobic perfusion for similar periods of time.

Changes in fine structure included nuclear clumping and margination, loss of mitochondrial granules, alteration in distribution and greater decrease in the amount of glycogen than accounted for by fasting *in situ*, and cellular swelling. Other changes were flattening and disruption of the plasma membrane, vesiculation and disorganization of the endoplasmic reticulum and vacuolization of the cytoplasm. The cytoplasmic vacuoles contained acid phosphatase, an enzyme normally associated only with lysosomes, as well as isolated portions of cellular organelles.

It is postulated that three cellular changes—disorganization of endoplasmic reticulum, disruption and flattening of cellular membrane and vacuolization of cytoplasm—probably contribute to the loss of viability of the cell.

The respective morphologic responses to hypoxia varied independently in extent and reversibility with changes in experimental conditions. Biochemical events correlated with these morphologic changes, although complex, should be followed more easily when the sequence of cellular alterations are used to design experiments for the purpose of elucidating cellular mechanisms of importance in shock or the clinical entities in which hypoxia and/or ischemia are features. They also offer means for assessing therapeutic measures objectively, even though exact quantitation of response is difficult to achieve with electron microscopy.

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