American Journal of Pathology, Vol. 146, No. 3, March 1995

Catalase-Negative Peroxisomes: Transient Appearance in Rat Hepatocytes during Liver Regeneration after Partial Hepatectomy

Ikuo Oikawa and Phyllis M. Novikoff

From the Department of Pathology, Albert Einstein College of Medicine, Bronx, New York

Using light microscopy enzyme cytochemistry to localize catalase activity in peroxisomes, a population of peroxisome-negative hepatocytes was detected in livers of rats during liver regeneration induced by two-thirds partial hepatectomy. However, examination by electron microscopy revealed that this population of hepatocytes contained peroxisomes with a delimiting membrane and a nucleoid, but no cytochemically demonstrable catalase activity within their matrix. Regenerating livers 6, 18, 24, 36, 48 and 72 hours, and 1 week after partial hepatectomy showed hepatocytes without catalase activity. However, their numbers varied, with the most numerous appearing at 24 hours after partial hepatectomy. Mitosis of catalase-negative hepatocytes were seen along with mitosis of hepatocytes containing the normal complement of catalase-positive peroxisomes. The catalase-negative hepatocytes did not show evidence of apoptosis or necrotic cell death. Lysosomal acid phosphatase activity and bile canalicular ATPase activity were present in hepatocytes with catalase-negative peroxisomes. Another population of hepatocytes with a small number of catalase-positive peroxisomes appeared and were more numerous at 36 hours after partial hepatectomy; ultrastructurally, these hepatocytes contained both catalase-negative peroxisomes, which appeared to undergo dissolution, and catalase-positive peroxisomes, which were smaller in size. After complete restoration of the liver, all hepatocytes displayed essentially uniform numbers of catalase-positive peroxisomes. These studies indicated that during liver regeneration there is a transient loss of catalase in peroxisomes of some hepatocytes. These cells proliferate and with time acquire new catalase-positive peroxisomes. The observations are discussed in relation to peroxisome biogenesis, hepatocellular carcinogenesis, and oxidative stress during liver regeneration. (Am J Pathol 1995, 146:673–687)

Several chemical-induced hepatocarcinogenesis animal models designed to investigate cellular origins of hepatomas employ partial hepatectomy (PH) as a proliferative stimulus.[1–4] PH procedures have also been used in studies of a number of biological processes during liver regeneration (growth control, receptor function, gene expression, biogenesis of organelles, etc.; see reviews[5–7]). After two-thirds PH, the liver returns to normal mass and cell number by a compensatory hyperplasia of the remaining lobes through extensive proliferation of differentiated hepatocytes. Under certain conditions, stem cell, oval cell, and bile ductule cell compartments proliferate and expand to restore liver mass. A stem cell lineage pathway has been invoked not only to explain the origin of precursor cells in liver regeneration[8–11] but also to explain the development of hepatomas in rats administered chemical carcinogens.[12–16] Hepatocytes located in different zones of the hepatic lobule (ie, periportal *versus* pericentral) display biochemical, morphological, enzymatic, and functional heterogeneities. A stem cell pathway has also been considered to account for zonal hepatocyte heterogeneity by the proliferation of stem cell-derived hepatocytes and their migration along the hepatic plate, with the establishment of functional heterogeneity depending on their maturation state and zonal location within the hepatic plate.[17,18] However, recent studies using retroviral vectors to analyze cell lineage pathways in regenerating livers demonstrated proliferation of new

Supported by National Institutes of Health grant CA06576.

Accepted for publication November 20, 1994.

Address reprint requests to Phyllis M. Novikoff, Ph.D., Department of Pathology, Albert Einstein College of Medicine, 1300 Morris Park Avenue, U-423, Bronx, NY 10461.

hepatocytes from mature hepatocytes to replace liver mass, challenging the idea that hepatocytes are derived from periportal stem cells.[19,20]

In our experiments on livers from rats subjected to the Solt-Farber hepatocarcinogenesis protocol,[3] we included parallel studies on control rats in which only two-thirds PH was performed (ie, no exposure to diethynitrosamine or 2-acetylaminofluorene). Liver sections from rats 6 to 168 hours after PH revealed hepatocyte heterogeneity within the hepatic lobule based on the expression of catalase activity, a marker enzyme for peroxisomes.[21–23] In rat liver, peroxisomes are uniformly distributed in all hepatocytes within the hepatic cord. Hepatocyte peroxisomes appear as spherical structures with an average diameter of 0.6 μ, are delimited by a single membrane, and have a finely granular matrix and a characteristic nucleoid. The peroxisome matrix contains oxidases, which produce H_2O_2, and catalase, which decomposes H_2O_2, as well as enzymes for β-oxidation of long-chain fatty acids, cholesterol metabolism, plasminogen biosynthesis, and bile acid synthesis; the peroxisome core is composed of urate oxidase.[24] Human genetic diseases, of which Zellweger's cerebrohepatorenal syndrome is the most characterized, have shown loss or impairment of hepatocyte peroxisomal function.[25]

Using light microscopy enzyme cytochemistry to detect catalase in peroxisomes[26] and methyl-green pyronin to detect nuclear and chromosomal DNA and nucleolar and ribosomal RNA,[27] we found three populations of hepatocytes differing in peroxisome numbers in 4- to 168-hour regenerating rat liver. One population was without peroxisomes (Px-neg), one contained a small number of peroxisomes (Px-neg/pos), and one had the normal number of peroxisomes (Px-pos). All three populations exhibited mitotic activity and were present at all times after PH but not in the same numbers. The Px-pos hepatocytes were the most numerous at each time after surgery. Px-neg hepatocytes were most abundant at 24 hours, whereas Px-neg/pos were most abundant at 36 hours of PH. The Px-neg and Px-neg/pos hepatocytes were distributed randomly throughout the hepatic lobule, although they were more frequently encountered near the portal triad. Electron microscopy revealed that the Px-neg hepatocytes possessed organelles with characteristic morphological features of peroxisomes; however, they did not demonstrate cytochemically the matrix enzyme, catalase. The Px-neg/pos hepatocytes showed two different populations of peroxisomes, one catalase negative (cat-neg), which appeared to undergo dissolution, and the other catalase positive (cat-pos), which were smaller than the cat-neg ones and appeared to be newly formed peroxisomes. Several investigators have employed a rat regenerating liver system to study peroxisomal biogenesis.[28–31] However, no *in situ* studies have been reported on the appearance of cat-neg hepatocytes during liver regeneration.

The absence of catalase activity in peroxisomes is transient because after 2 weeks, all hepatocytes show catalase activity within peroxisomes. The functional consequences for the liver, which contains proliferating populations of hepatocytes with either no or deficient levels of catalase, an enzyme that protects cells against H_2O_2 damage to DNA and membranes, remain to be established. These findings may have implications for establishing hepatocyte heterogeneity of the hepatic plate, development of hepatomas by chemical carcinogens, and peroxisome biogenesis. Parts of this study have been published in abstract form.[32]

Materials and Methods

Animals and Treatment

Young male Fisher 344 rats (Charles River Laboratories, Bloomington, MA) weighing 120 to 130 g were used in all experiments. The rats were maintained in a central animal care facility with a 12-hour light/dark cycle for at least 1 week and were fed standard laboratory chow *ad libitum*. Forty-two rats were subjected to two-thirds PH[33] under ether anesthesia. Rats were sacrificed at the following times after PH: 4, 6, 18, 24, 36, 48, and 72 hours; and one week. Six rats were studied at each time point, except for 4 and 6 hours, in which three were studied. Sham-operated rats served as controls. All procedures were performed between 9 a.m. and 11 a.m., because circadian cycles are thought to cause changes in liver cell organelle distribution.[34] The animal care facilities are under the supervision of veterinarians who follow federal guidelines and all procedures were approved by the Albert Einstein College of Medicine Animal Care Committee.

Tissue Preparation and Sectioning

In all experiments, slices of liver (2 to 3 mm thick) were taken from the right lobe. Livers were prepared as follows for either immunocytochemical and enzyme cytochemical light microscopic studies or enzyme cytochemical ultrastructural studies: 1) unfixed slices were frozen unfixed immediately in liquid nitrogen and stored at −70 C; 2) fixation by immersion or perfusion in either cold 4% paraformaldehyde in 0.1 mol/L phosphate buffer, pH 7.4, 7.5% sucrose or a cold 4%

paraformaldehyde-2.5% glutaraldehyde mixture[35] in 0.1 mol/L cacodylate buffer, pH 7.4, 5% sucrose for a total fixation of 3 hours with continuous shaking in a Dubnoff metabolic shaking incubator Precision Scientific Co., Chicago, IL; and 3) post-fixation with 1% osmium tetraoxide for 1 hour after aldehyde mixture fixative. After the initial fixation, the slices were cryoprotected by infusion with increasing concentrations of cold sucrose (7.5%, 1 hour; 10%, 1 hour; 75%, 18 hours) before sectioning at 10 to 25 μ with a Sartorius freezing microtome (Leitz Co., Rockleigh, NJ); the sections were collected into 7.5% sucrose. Unfixed frozen sections were cut at 8 to 10 μ with a cryostat (Lab-Tek Instruments Co., Fanwood, NJ) and fixed in cold acetone for 15 minutes before light microscopic immunocytochemistry was performed. For electron microscope studies, aldehyde-fixed sections were embedded in Epon after ethanol and propylene oxide dehydration. Ultrathin sections 50 to 80 nm thick were stained with lead citrate and examined with a Philips 300 electron microscope (Philips, Mahwah, NJ) equipped with a goniometer stage.

Enzyme Cytochemistry

Freely floating paraformaldehyde-glutaraldehyde fixed liver sections were incubated for the following enzymes: 1) catalase activity using an incubating medium containing 3,3′diaminobenzidine tetrahydrochloride (DAB-HCl) as substrate at pH 9.7 to demonstrate peroxisomes and microperoxisomes[26] and at pH 7.4 to demonstrate peroxidase;[36] incubation time was 15 to 30 minutes at 37 C; 2) acid phosphatase activity using an incubating medium containing cytidine monophosphate, sodium salt, as substrate at pH 5.0 to demonstrate lysosomes and other acid phosphatase structures;[37] incubation time was 20 to 40 minutes at 37 C; 3) adenosine triphosphatase (ATPase) activity using an incubating medium containing adenosine triphosphate, sodium salt, as substrate at pH 7.4 to visualize bile canaliculi;[38] incubation time 25 to 40 minutes at 37 C. Colocalizations of peroxisomes and ATPase were also performed on aldehyde-fixed liver sections. Sections were also stained for nucleic acids (DNA and RNA) using the methyl-green procedure of Lillie[27]; this procedure reveals nuclei, nucleoli, chromosomes, ribosomes, and cytoplasmic basophilic clumps (which correspond to the endoplasmic reticulum) as well as overall liver histology and for the presence of cytoplasmic lipid spheres using Oil Red O (0.5% Oil Red O in 60% triethylphosphate for 10 minutes, room temperature).

Immunocytochemistry

Paraformaldehyde-fixed frozen sections were exposed to polyclonal rabbit anti-rat glutathione-S-transferase, Yp isoform (GST-Yp) (Upstate Biotechnology, Lake Placid, NY) diluted 1:50 with phosphate-buffered saline (PBS), rinsed in PBS and subsequently exposed to goat anti-rabbit IgG-peroxidase, diluted 1:50 with PBS for 1 hour at room temperature, rinsed in PBS and then incubated in DAB medium at ph 7.6 for 10 to 20 minutes at room temperature to reveal sites of peroxidase activity.[36] Sections exposed to anti-cytokeratin 19 (Amersham, UK) diluted 1:10 with PBS were treated as above except second antibody exposure consisted of a goat anti-mouse polyvalent IgG-peroxidase diluted 1:100 for 2 hours at room temperature. Exposure time for both antibodies was 18 to 24 hours at 4 C. Before antibody exposure, liver sections were treated with 0.3% hydrogen peroxide in methanol to block endogenous peroxidase and with nonspecific goat antiserum. Controls included omitting the exposure to specific antisera and exposing to nonspecific antisera with subsequent steps identical to those used for the specific antisera. Sections were counterstained with hematoxylin for light microscopic studies. *In situ* nuclear DNA fragmentation, a morphological indicator of apoptosis, was detected using the Oncor Apotag-peroxidase kit (Oncor, Gaithersburg, MD).

Results

Light Microscopic Studies

From 4 hours to 1 week after PH, we found hepatocytes that did not demonstrate catalase activity in structures known to contain this enzyme, namely peroxisomes. We also found hepatocytes with fewer cat-pos peroxisomes than normal. The proportion of hepatocytes without cat-pos hepatocytes varied, depending on the time after PH. Figure 1a illustrates the presence of several hepatocytes from a section of 24-hour regenerating rat liver that do not demonstrate peroxisomes when light microscopic cytochemistry is used to detect catalase activity. In this figure, most of the hepatocytes have the normal complement of cat-pos hepatocytes; however, several hepatocytes are found with a complete absence of cat-pos hepatocytes (cat-neg), and a few hepatocytes are found with both cat-neg peroxisomes and a small number of cat-pos peroxisomes (cat-neg/pos hepatocytes). The three populations of hepatocytes were also seen in different stages of mitosis, indicating that all the hepatocytes regardless of their catalase activity were

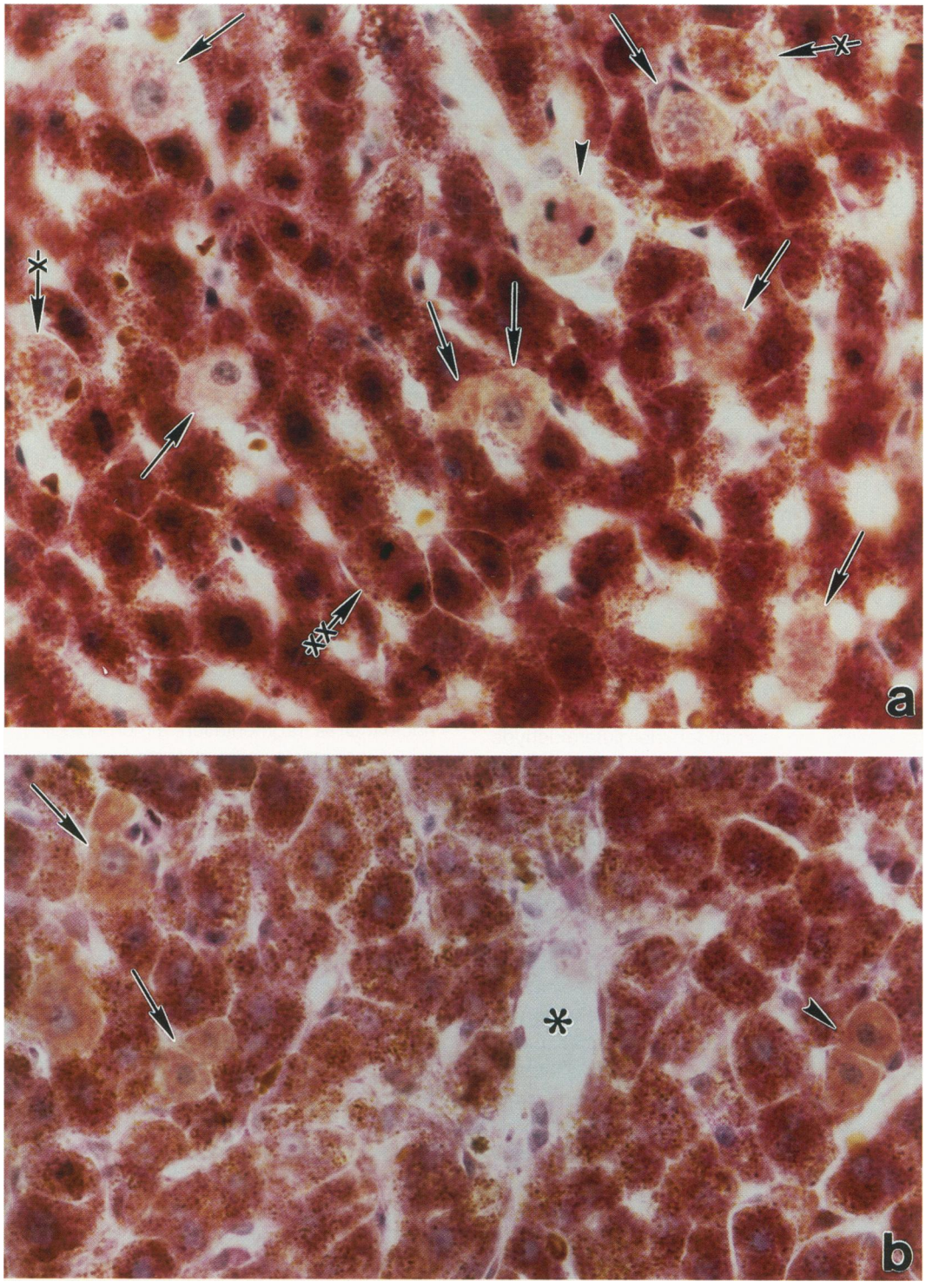

Figure 1. *Rat liver section incubated for catalase activity and stained with methyl-green pyronin:* (a) *24 hours after PH. Several cat-neg hepatocytes* (*arrows*) *are distributed midzonal in the liver acinus. Mitosis of a cat-neg hepatocyte* (*arrowhead*) *and a cat-pos hepatocyte* (*double crossed arrow*) *are evident; both are in the same stage of mitosis. Hepatocytes with few cat-pos peroxisomes are also present* (*single crossed arrows*). *600×.* (b) *36 hours after PH. Doublets of cat-neg hepatocytes* (*arrows*) *are evident in the vicinity of a central vein* (***). *One of hepatocytes of a doublet contains a few cat-pos hepatocytes* (*arrowhead*). *600×.*

proliferating. Figure 1a also shows chromosomes in cat-neg and cat-pos hepatocytes, both of which appear in the same stage of mitosis (ie, telophase). Some of the cat-neg hepatocytes appear rounder than the surrounding cat-pos hepatocytes; these hepatocytes may be about to enter the mitotic cycle.

In this small region of a liver section seven cat-neg, two cat-neg/pos, and ~200 cat-pos hepatocytes are evident. However, in other areas of the same liver section occupied by ~200 cat-pos hepatocytes none to few cat-neg hepatocytes were found. The cat-neg and cat-neg/pos hepatocytes were distributed randomly throughout the hepatic lobule either singly (Figure 1a), in doublets (Figure 1b), or in groups of four or more hepatocytes; however, many more were encountered near portal triad regions. In a liver section from another rat after 24 hours incubated for both catalase and ATPase activities, we observed four contiguous hepatocytes without peroxisomes (Figure 2a), one of which is in mitosis. Hepatocytes that contain a few cat-pos peroxisomes increase after 36 hours PH when compared with other times after PH. Figure 2b illustrates several hepatocytes with a few cat-pos peroxisomes, one of which is in the same stage of mitosis as a hepatocyte with many more peroxisomes. Several hepatocytes with less than the normal peroxisome distribution can be found clustered near portal triad regions. Figure 2c shows one cat-neg hepatocyte and a cluster of several cat-neg/pos hepatocytes close to a portal triad area in a rat liver 36 hours after PH. By 1 week after PH, very few hepatocytes are seen without peroxisomes. Because of the absence of the brown-black reaction product resulting from incubating sections with the diaminobenzidine method, pH 9.7, to detect catalase activity, cat-neg hepatocytes and cat-neg/pos hepatocytes were clearly distinguished from the surrounding cat-pos hepatocytes. Thus far, we have not seen cat-neg or cat-neg/pos hepatocytes in liver sections from sham-operated rats. The cat-neg and cat-neg/pos hepatocytes are integrated within the hepatic cords because we have demonstrated ATPase-positive bile canaliculi between adjacent catalase-negative hepatocytes (Figure 2a), adjacent cat-neg/pos hepatocytes (Figure 2c) and between cat-neg and cat-neg/pos hepatocytes (Figure 2a); both of these populations of hepatocytes form bile canaliculi with cat-pos hepatocytes (Figure 2, a and d). Some cat-neg and cat-neg/pos hepatocytes also showed less pyronin staining within the cytoplasm (Figure 1). We also determined the presence of acid phosphatase activity, a lysosomal enzyme, and found acid phosphatase-positive lysosomes present in both cat-neg and cat-neg/pos hepatocytes with a similar distribution and profiles seen in cat-pos hepatocytes (not illustrated). Immunolocalization of GST-Yp and cytokeratin-19, both bile ductule cell antigens, were limited to bile ductule cells at times after PH. Nuclear chromatin fragmentation and/or cytoplasmic apoptotic bodies, morphological criteria for apoptosis, was not detected in hepatocytes during liver regeneration. We also did not find any morphological changes that were associated with necrosis, such as formation of autophagic vacuoles.

Quantitative Studies

Cat-neg and cat-neg/pos hepatocytes were found at all time points studied after PH and ranged in size between 21.0 and 37.5 μ (26.7 ± 6.1, mean ± SD). Figure 3 illustrates the number of cat-neg and cat-neg/pos hepatocytes observed per 5000 hepatocytes during liver regeneration. Both cat-neg and cat-neg/pos hepatocytes were found as early as 4 hours after PH. It is evident that there is an inverse relationship between the two populations of hepatocytes. The cat-neg hepatocytes were most numerous 24 hours after PH (23.0 ± 3.1 per 5000 hepatocytes), whereas the cat-neg/pos hepatocytes peaked at 36 hours (13.3 ± 3.3/5000 hepatocytes). With time both cat-neg and cat-neg/pos hepatocytes decreased in number. The cat-neg hepatocytes were the most numerous until 36 hours after PH compared with the cat-neg/pos hepatocytes (ratio of 2.27:1.00 at 18 hours, 2.30:1.00 at 24 hours, and 1.17:1.00 at 36 hours), whereas the cat-neg/pos hepatocytes became more numerous after 36 hours when compared with the cat-neg hepatocytes (ratio of 1.29:1.00 at 48 hours, 1.36:1.00 at 72 hours and 1.71:1.00 at 1 week). However, after 2 weeks after PH, neither cat-neg nor cat-neg/pos hepatocyte populations were evident. The appearance of increased cat-neg/pos hepatocytes after the decrease in cat-neg hepatocytes may indicate that cat-neg/pos hepatocytes are derived from the cat-neg hepatocytes, which then develop into hepatocytes with normal numbers of peroxisomes.

Electron Microscopic Observations

Light microscopic studies indicated that hepatocytes without peroxisomes appeared during liver regeneration. To further substantiate this finding, we examined at the ultrastructural level hepatocytes during regeneration that were incubated for catalase and peroxidase activities. We found that hepatocytes that did not show catalase activity after incubation with the diaminobenzidine pH 9.7 method by light microscopy contained peroxisomes with characteristic morphological features, namely a delimiting membrane and a crystalline nucleoid, but did not contain the enzyme catalase in the matrix of the peroxisomes. Figure 4 shows the appearance of peroxisomes after incubation for catalase activity in hepatocytes 24 hours after

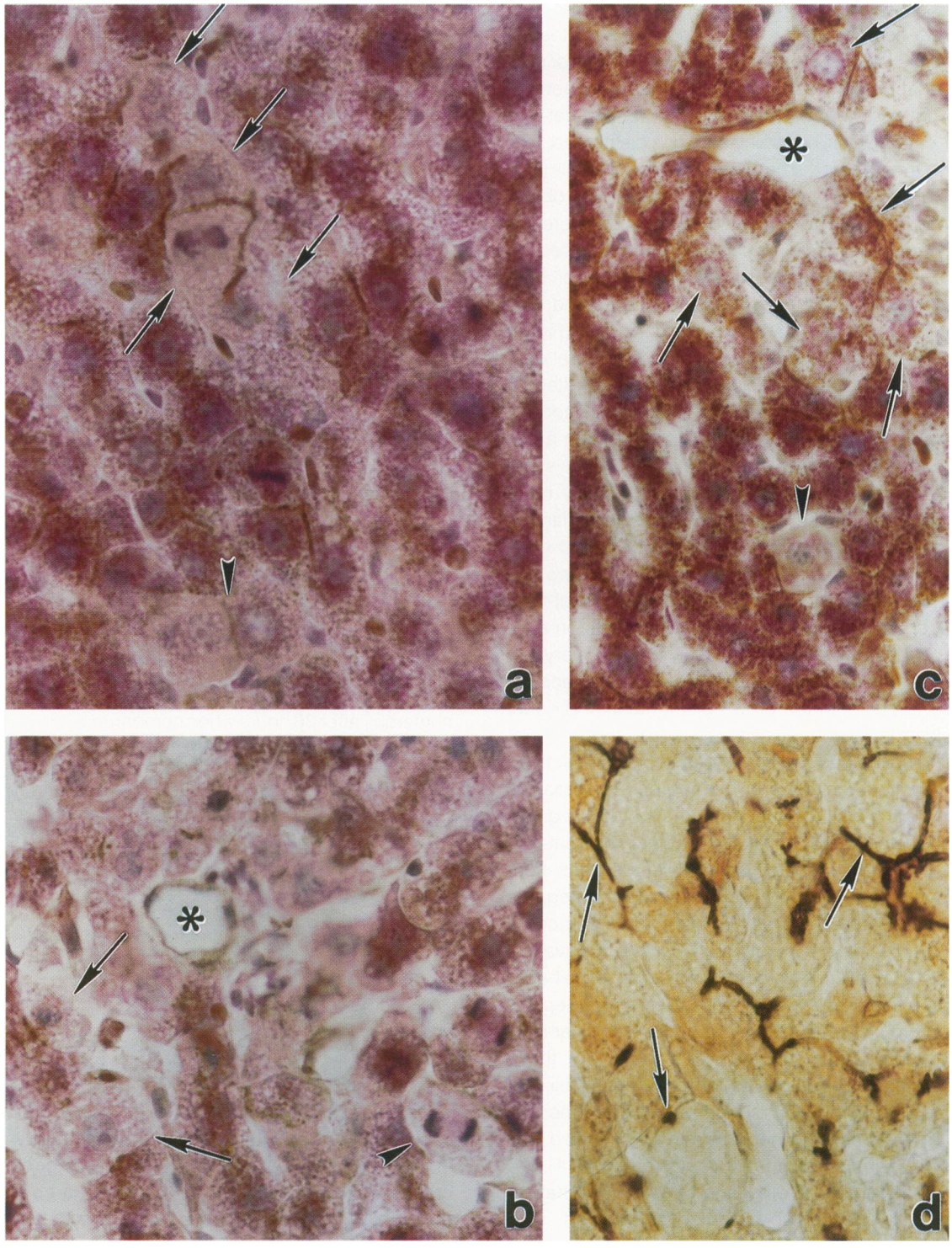

Figure 2. *Rat liver sections:* (a) *24 hours after PH, stained for catalase activity, ATPase activity, and methyl-green pryonin. Arrows indicate four contiguous cat-neg hepatocytes, one of which is in mitosis; note ATPase-positive bile canaliculi between them and hepatocytes with normal complement of peroxisomes. Arrowhead shows ATPase-positive bile canaliculus between a cat-neg hepatocyte and a cat-neg/pos hepatocyte. 600×.* (b) *36 hours after PH, stained for catalase activity and methy-green pyronin. Hepatocytes with fewer cat-pos peroxisomes (arrows) than normal hepatocytes are seen near the portal triad region (*). One of the cat-neg/pos hepatocytes (arrowhead) is in the same stage of mitosis as a hepatocyte with the normal complement of peroxisomes. 600×.* (c) *36 hours after PH, stained for catalase activity, ATPase activity, and methyl-green pyronin. Several hepatocytes with fewer peroxisomes (arrows) than normally found in hepatocytes are evident near the portal triad (*). A hepatocyte without peroxisomes (arrowhead) is evident. Note ATPase-positive bile canaliculi between hepatocytes with normal numbers of hepatocytes and with hepatocytes with none or few peroxisomes. 600×.* (d) *24 hours after PH, stained for catalase activity and ATPase activity. ATPase-positive bile canaliculi (arrows) are present between cat-neg hepatoyctes and cat-pos hepatocytes. 600×.*

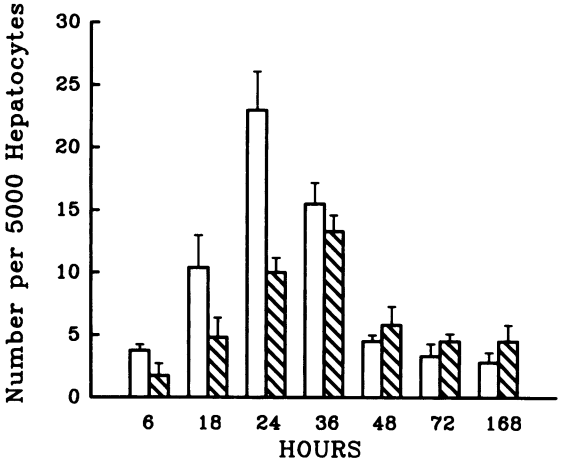

Figure 3. *The numbers of cat-neg hepatocytes (open bars) and cat-neg/pos hepatocytes (slashed bars) per 5000 hepatocytes was determined in liver sections 6, 18, 24, 36, 48, 72, and 168 hours after PH. Values are the mean ± SD for six rats (except 6-hour values, which are for three rats).*

PH. Catalase activity is not present in the matrix of the peroxisomes, although the characteristic core is evident. The matrix appears less dense when compared with the cat-pos peroxisomes present in the adjacent hepatocyte. The overall density of hepatocytes with a normal complement of cat-pos peroxisomes is greater than that of hepatocytes with cat-neg peroxisomes. Figure 4 also demonstrates the presence of a bile canaliculus and desmosomes between both hepatocytes. The cat-neg peroxisomes appeared smaller than the cat-pos peroxisomes (Figures 4 and 5). The crystalline cores were seen free in the cytoplasm (Figure 5). Clusters of smaller core-free peroxisomes were also seen (Figure 5). No other organelle showed catalase activity, including mitochondria; differences in the density of mitochondria were seen occasionally in cat-neg hepatocytes. Lipid spheres were found in all hepatocytes, and did not appear to correlate with the catalase activity of hepatocytes. At 24 hours after PH, as well as at later times, hepatocytes containing both cat-neg and cat-pos hepatocytes are seen. Figure 6, a and b illustrates both cat-neg and cat-pos peroxisomes in the same hepatocyte after 24 and 36 hours, respectively. Figure 6a shows a hepatocyte in which most of the peroxisomes lack catalase activity, whereas in Figure 6b most of the peroxisomes show catalase activity. These figures also illustrate that most peroxisomes are close to cisternae of endoplasmic reticulum. As liver regeneration proceeds, the cat-neg and cat-neg/pos hepatocytes develop new cat-pos peroxisomes. Figure 7 illustrates a hepatocyte in which many new cat-pos peroxisomes have developed; they vary in size but are smaller and more spherical than peroxisomes from normal hepatocytes. This figure also reveals one cat-neg core-containing peroxisome and several isolated nucleoids. Cat-neg/pos and cat-neg hepatocytes are not only contiguous with each other but are also contiguous with hepatocytes containing normal peroxisomes and have bile canaliculi formed between the hepatocytes (Figure 8). Figure 8 also shows one hepatocyte in which essentially all peroxisomes have catalase activity, while the other has no catalase activity in any of the peroxisomes. However, the cat-pos peroxisomes differ in size and shape from those cat-pos peroxisomes in the surrounding hepatocytes. Figures 4, 5, 7, and 8 also show that there are no morphological changes associated with apoptosis or necrosis.

Discussion

As early as 4 hours after PH, a population of hepatocytes lacking catalase activity in peroxisomes appeared during the regeneration of the liver. The proportion of these hepatocytes continued to increase in number peaking at 24 hours, after which it declined, becoming undetectable by 2 weeks. In parallel, another population of hepatocytes that contained few cat-pos peroxisomes appeared and peaked at 36 hours. This population also declined and became undetectable after 2 weeks. The temporal appearance of both populations suggests that the 36-hour peaking hepatocytes may represent a later stage of the 24-hour peaking hepatocytes. Cat-pos peroxisomes may continue to increase in number and size in these partially cat-pos hepatocytes until the peroxisomes become indistinguishable from those found in normal hepatocytes. An alternate explanation is that early in regeneration two different populations of cat-neg hepatocytes, one in which there is complete absence of catalase in peroxisomes and another in which there is a relative absence of cat-neg peroxisomes, appear with each population forming new peroxisomes until the normal complement of peroxisomes is reached. However, ultrastructural studies do not support this latter interpretation. Hepatocytes are seen either totally without cat-pos peroxisomes, with various numbers of cat-neg and positive peroxisomes, or with various sizes of cat-pos, although smaller and uniformly spherical peroxisomes. Moreover, it appears that there is a dissolution and replacement of the cat-neg peroxisomes by newly-formed peroxisomes. Within the same hepatocyte we have detected nucleoid-containing cat-neg peroxisomes that have less dense matrix, have shrunken in size, have extruded the core, and show breaks in the delimiting

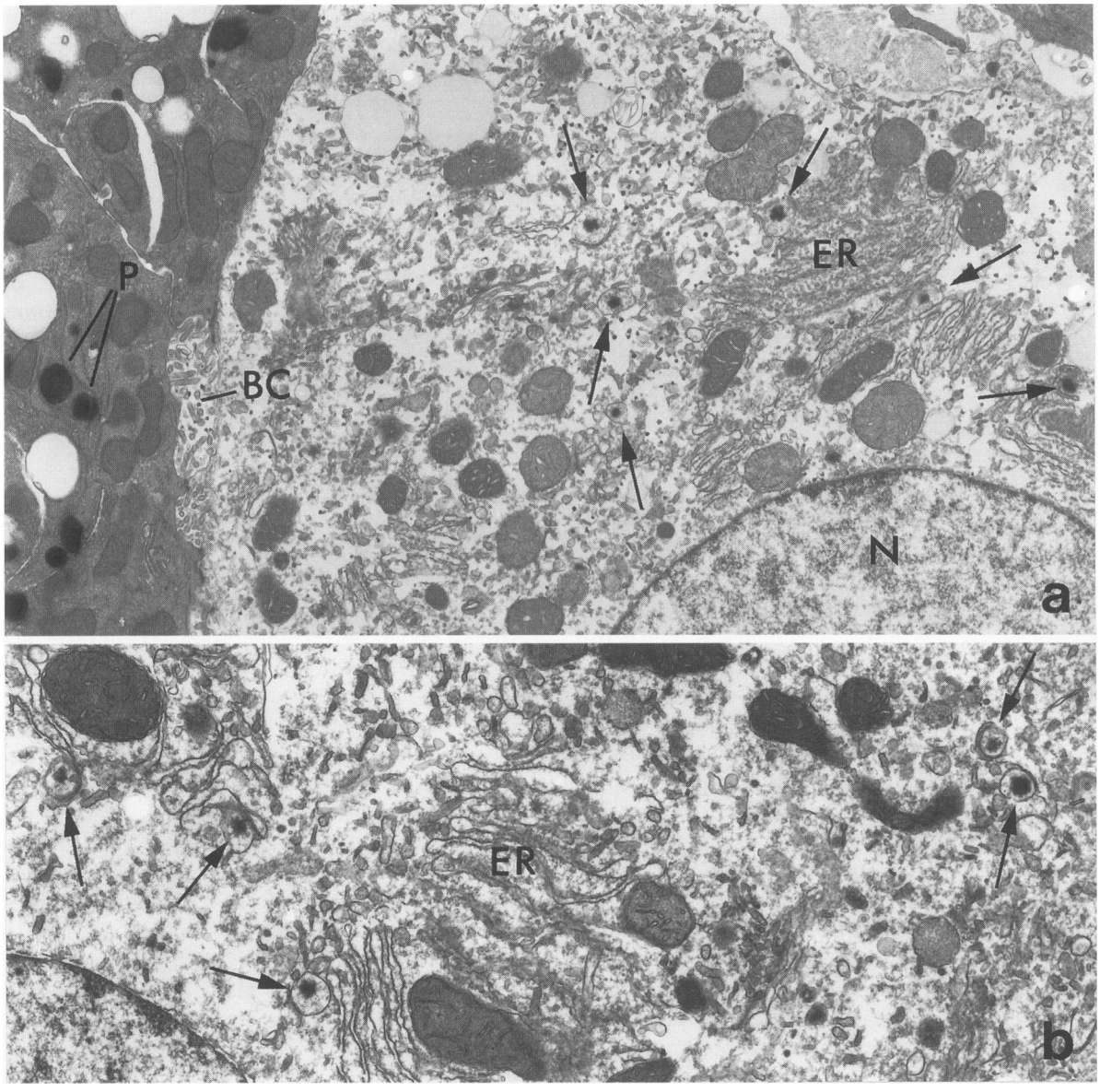

Figure 4. *Liver section incubated for catalase activity.* (**a**) *Several cat-neg peroxisomes (arrows) are evident in a hepatocyte contiguous with a hepatocyte containing cat-pos peroxisomes (P). Cat-neg peroxisomes contain typical crystalline-like nucleoids; the delimiting membrane of some of the cat-neg peroxisomes appears incomplete. The density of the peroxisome matrix varies but appears generally less dense than matrix of peroxisomes in hepatocytes with normal complement of peroxisomes not incubated for catalase. The overall density of the cat-neg hepatocyte is less than the hepatocytes with cat-pos peroxisomes. Note the presence of a bile canaliculus (BC) between the hepatocytes. Nucleus (N) and endoplasmic reticulum (ER) are labeled. 9100×.* (**b**) *Nucleiod-containing cat-neg peroxisomes (arrows) are seen with smaller matrices. Smooth-surface cisternae lie close to the peroxisomes. 15,000×.*

membrane along with cat-pos peroxisomes that vary in size but are uniform in shape. The newly formed peroxisomes that appear in the cat-neg hepatocytes are smaller and more spherical than the peroxisomes of normal hepatocytes.

Earlier studies have described the biogenesis of peroxisomes from either the endoplasmic reticulum, preexisting peroxisomes, or a peroxisomal reticulum in both normal and regenerating livers.[39–43] However, none of those studies reported on the presence of hepatocytes with peroxisomes without catalase activity distributed within the hepatic acinus. Although we have not determined the origin of the newly-formed peroxisomes seen in the cat-neg/pos hepatocytes, the ultrastructural images do not indicate that they derive from preexisting ones by budding or from a peroxisomal reticulum. We have observed small peroxisomes close to either smooth or rough endoplasmic reticulum. It appears that the origin of peroxisomes in the cat-neg hepatocytes differs from those hepatocytes with the normal complement of peroxisomes. *Saccharomyces cerevisiae* mutants

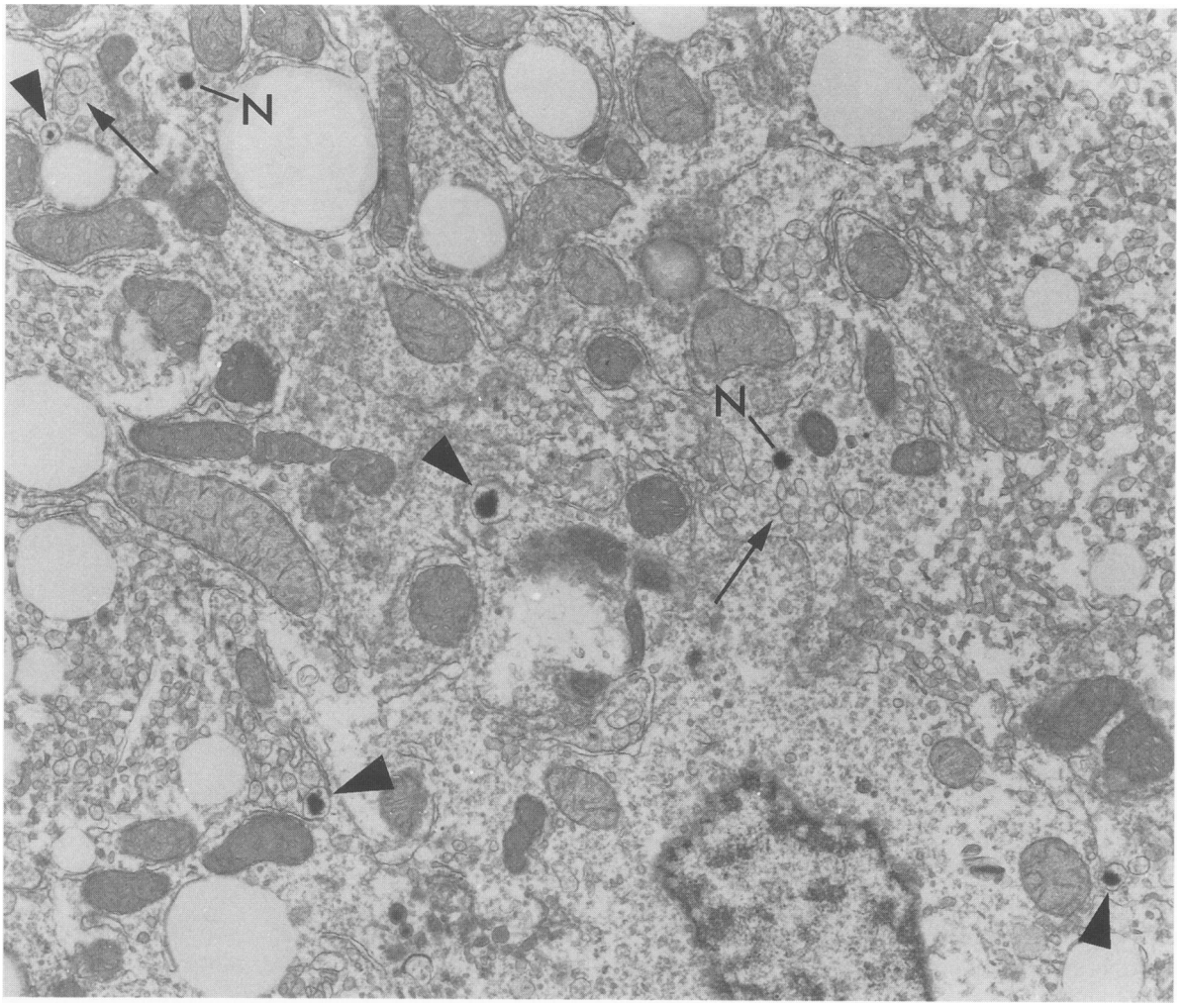

Figure 5. *Liver section incubated for catalase activity:* (**a**) *Clusters of cat-neg peroxisomes are found without nucleoids (arrows). Isolated nucleoids are seen adjacent to the clusters (N). Nucleoid-containing cat-neg peroxisomes are also evident (arrowheads). 10,700×.*

were found to contain clusters of unconnected cat-pos peroxisomes and peroxisomal membrane ghosts.[44] The peroxisomal images in the mutants were similar to those seen in cat-neg and cat-neg/pos hepatocytes. Although we did not perform serial sectioning studies on clusters of peroxisomal ghosts or on newly formed small spherical peroxisomes to determine whether interconnections are present, we did examine many thin sections of cat-neg hepatocytes. We cannot state unequivocally that the newly formed peroxisomes are not part of a reticulum. However, if they are, they do not form a reticulum resembling that reported for cat-pos hepatocytes from regenerating livers.[43]

Heterogeneity in the peroxisome population of regenerating liver has been recently reported. "Light" and "heavy" fractions of peroxisomes were isolated from regenerating rat livers;[30] the "light" fraction contained peroxisomes smaller in size, with a lower content of catalase and uricase and incorporating new proteins faster than the "heavy" fraction. The morphology of the cat-neg and the newly-formed peroxisomes that we observed resembled the "light" fraction isolated from regenerating livers in some respects but not all. Additional heterogeneity in the peroxisome population may exit in liver regeneration. We do not know in which fraction the peroxisomes that we observed in the cat-neg and cat-neg/positive hepatocytes will be found. We observed variation in peroxisome matrix density; this may reflect loss or diminution of matrix-associated enzymes. In our studies, catalase was the only peroxisomal enzyme localized. Studies are in progress to localize other matrix enzymes. Some cat-neg peroxisomes exhibited a clear matrix, whereas others had a denser matrix but not as dense as the matrix of peroxisomes from normal hepatocytes, suggesting differences in the content of matrix enzymes. Peroxisomal ghosts similar to those

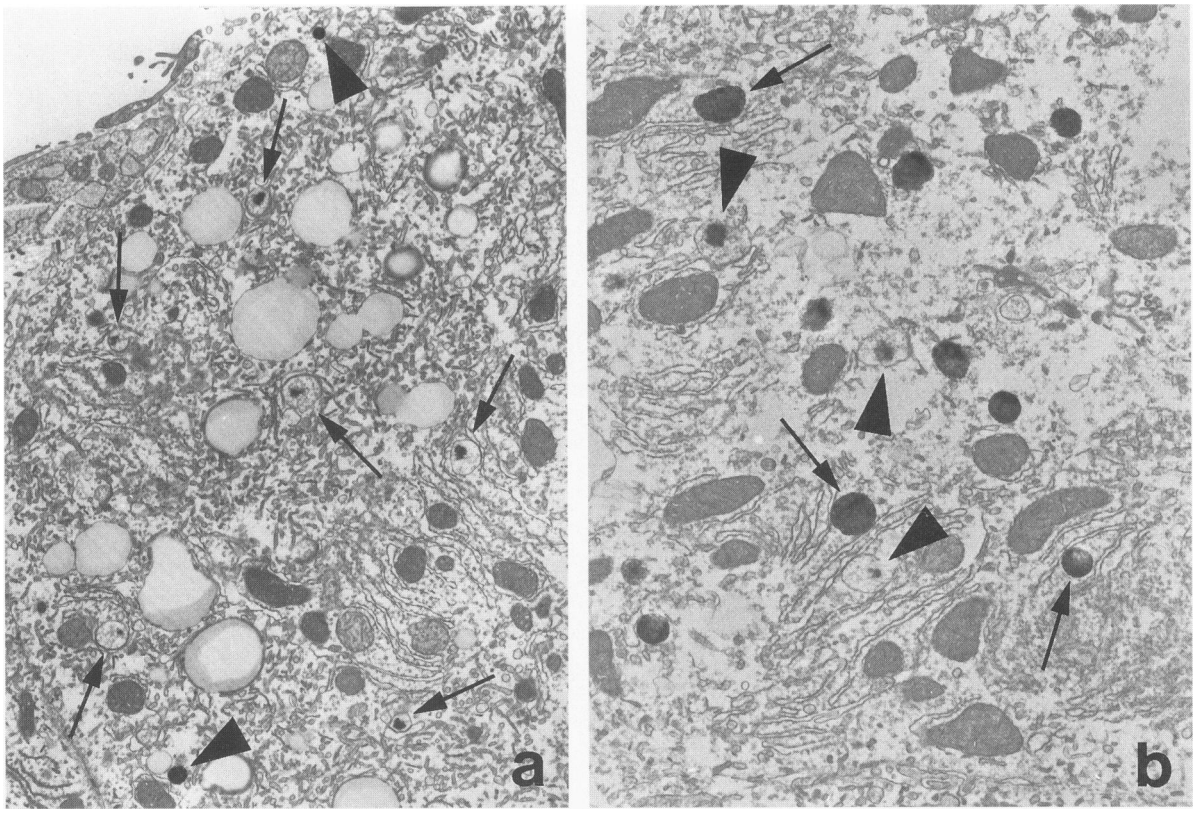

Figure 6. *Liver section incubated for catalase activity.* (a) *Hepatocytes are found which contain many cat-neg peroxisomes (arrows) and few cat-pos peroxisomes (arrowheads). 8000×.* (b) *More spherical-shaped cat-pos peroxisomes (arrows) are seen than nucleoid containing cat-neg peroxisomes. Some of the peroxisomes are in close proximity to endoplasmic reticulum. 10,000×.*

seen in patients with Zellweger's syndrome were also found in some cat-neg hepatocytes; however, they differed from Zellweger's peroxisomes in that nucleoids were encountered near clusters of peroxisomal ghosts.[45] Peroxisomal morphology of other inherited human diseases has also been studied.[46]

Even though hepatocytes have basically similar structural organization, they exhibit structural, functional, enzymatic, and biochemical heterogeneity depending on their position within the hepatic acinus.[47–51] The earliest *in situ* studies demonstrating enzymatic and structural heterogeneity showed higher levels of ATPase, glucose-6-phosphatase, and cytochrome oxidase in hepatocytes near the portal triad along with correlated structural changes in bile canaliculi, endoplasmic reticulum, and mitochondria.[52,53] The factors that have been considered to regulate the expression of individual hepatocytes within the different acinus zones have included the dynamics of the hepatic microcirculatory systems and/or the composition of the extracellular matrix.[54–57] More recently, a stem cell-derived proliferating hepatocyte compartment has been proposed to explain heterogeneity within the hepatic plate. Cells from this compartment proliferate near the portal triad, migrate, or stream along the hepatic cord and develop different functions depending on their maturation state and their ultimate location within the acinus.[17,18] However, recent studies using *in vivo* retroviral-mediated β-galactosidase gene transfer have provided evidence that hepatocytes divide throughout the acinus, refuting the existence of stem cells in liver and hepatocyte streaming across the acinus.[19,20] Our studies demonstrate a different kind of heterogeneity, although transient, than previously reported. The hepatocytes without catalase in peroxisomes proliferate during regeneration along with the proliferation of hepatocytes with normal levels of catalase; this proliferation also occurs throughout the liver acinus. It would be of interest to know the relationship of the hepatocytes we observed without catalase to the hepatocytes that integrate the β-galactosidase gene. Our cat-neg cells were most numerous between 24 and 36 hours after PH. The integration of retroviral vectors was most efficient between 24 and 27 hours. Moreover, hepatocytes without catalase or deficient in catalase are temporarily exposed to increased levels of H_20_2 at the same time that the vector appears to be most efficiently integrated. At no time after PH have we observed necrotic or apoptotic

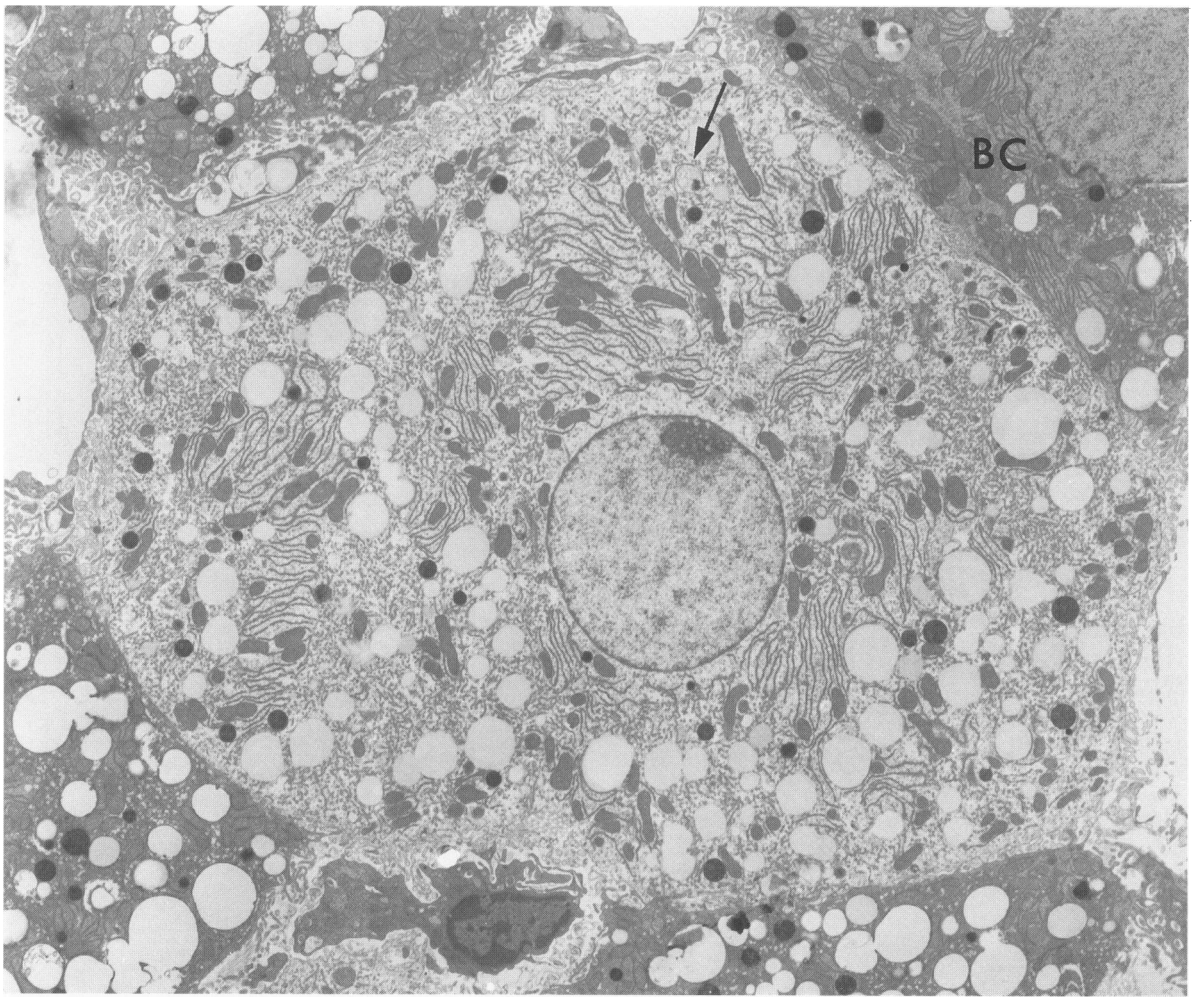

Figure 7. *Liver section incubated for catalase activity. A hepatocyte with many newly formed cat-pos peroxisomes is interspersed among four hepatocytes with normal complement of peroxisomes; even though the hepatocyte has many cat-pos peroxisomes, its overall density is lighter than the other hepatocytes. A cat-neg peroxisome is present (arrow). 3800×.*

hepatocytes,[58] although excess oxidants have been shown to cause DNA and other macromolecular damage in cells.[59] Cells have DNA repair mechanisms and inducible enzyme systems (glutathione transferases (synthetase, reductase, and peroxidase), superoxide dismutase) in addition to catalase to protect against the deleterious effects of oxygen radicals.[60–64] We have not determined whether cat-neg hepatocytes show differences in the localization of glutathione transferases or superoxide dismutase.

Oxidative stress has been implicated as a causative agent in a number of diseases, including tumorigenesis.[65–69] It has been suggested that oxidative damage plays a role in the development of liver tumors in a hepatoma rodent model induced by peroxisomal proliferators, a non-mutagenic class of hepatocarcinogens. In this model peroxisomes proliferate, resulting in increases in fatty acid β-oxidation enzymes and H_2O_2 levels. However, catalase levels do not increase proportionately to sufficiently degrade H_2O_2. The sustained higher levels of H_2O_2 oxidatively damage DNA, mediating the formation of hepatomas.[70] In a diethynitrosamine-phenobarbital hepatoma rat model, oxidative damage to cell foci is considered to be a determining factor in hepatoma formation.[71] The Solt-Farber rat model of hepatocarcinogenesis we use in our experiments employs carcinogens and PH to produce preneoplastic foci and hepatomas.[3] Other chemical-induced hepatocarcinogenesis rodent animal models also use a combination of chemical carcinogens and PH to produce preneoplastic foci and hepatomas.[1,2,4] Our findings have revealed hepatocytes without catalase, which presumably have increased levels of H_2O_2. This may result in the exposure of DNA and other cellular macromolecules to oxidative damage during a time of liver regeneration at which DNA replication is maximal. Although the cat-neg hepatocytes are transiently

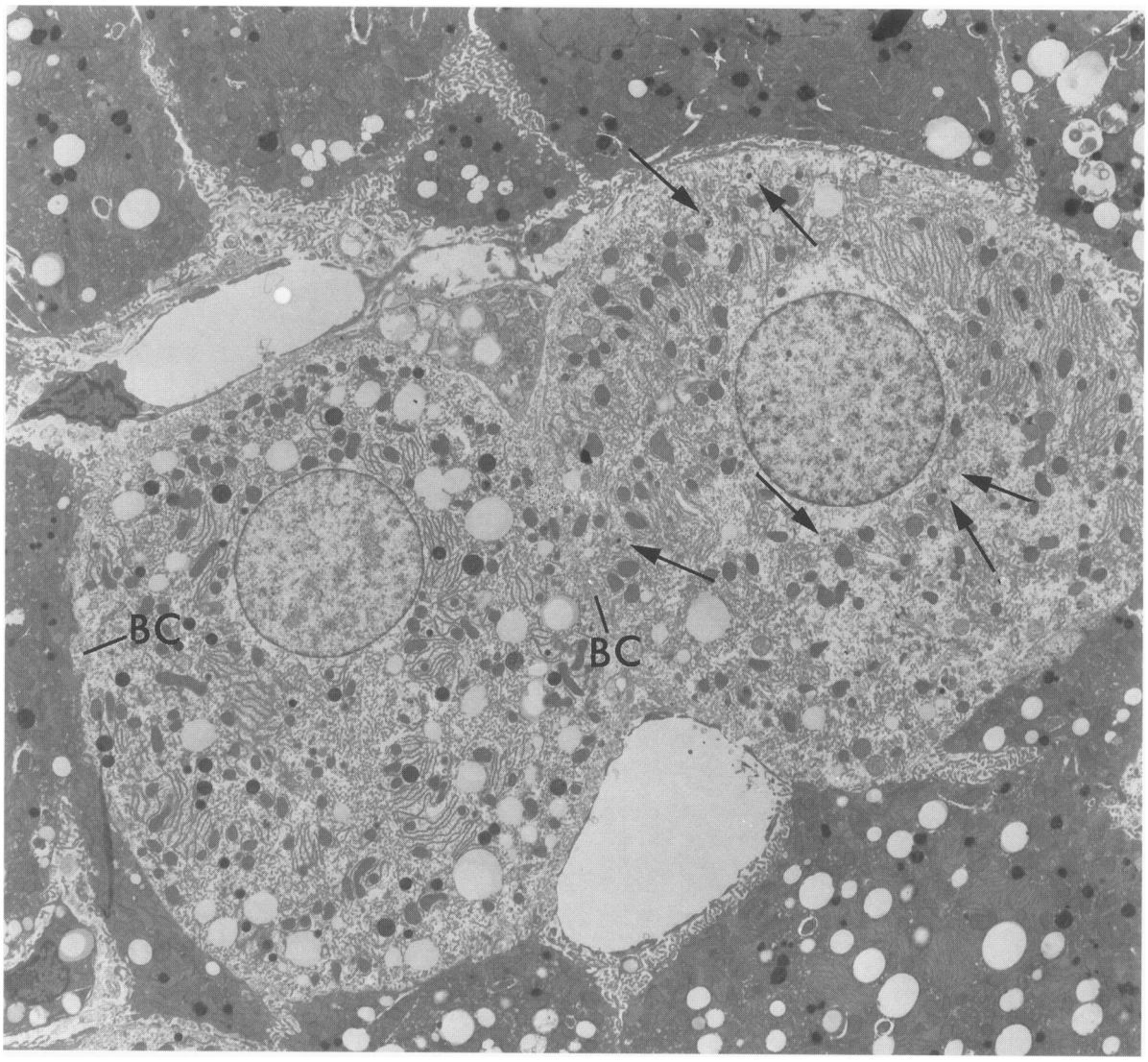

Figure 8. *Liver section incubated for catalase activity. A hepatocyte with cat-neg peroxisomes (arrows) is contiguous with a hepatocyte with newly formed cat-pos peroxisomes. Each hepatocyte is also contiguous with hepatocytes with the normal complement of peroxisomes. Bile canaliculi (BC) are seen between hepatocytes. 3000×.*

present, they divide under conditions of presumed oxidative stress. Alterations could conceivably develop in their genomic apparatus affecting their response to carcinogens. The response of hepatocytes with no or reduced levels of catalase to carcinogens at a time during regeneration when mitosis is prevalent may affect how and which hepatic cells enter the lineage pathways in the development of hepatomas.[72,73] These hepatocytes may also respond differently to changes in blood flow, growth factors, oncogenes, and/or extracellular matrix, influencing the functional differentiation of hepatocytes across the acinus zones. The relationship of our observations to cell lineage pathways involved in the development of preneoplastic foci and hepatomas as well as to the establishment of hepatocyte heterogeneity within the hepatic acinus remain to be established.

Acknowledgments

The authors greatly appreciate the expert assistance of Ms. Ana Yam in the performance of the ultrastructural studies. We also thank Dr. Allan Wolkoff for critical reading of the manuscript.

References

1. Peraino C, Fry RJM, Staffeldt E, Kisieleski WE: Effects of varying the exposure to phenobarbital on its en-

hancement of 2-acetylaminofluorene-induced hepatic tumorigenesis in the rat. Cancer Res 1973, 33:2701–2707
2. Scherer E, Emmelot P: Kinetics of induction and growth of precancerous liver-cell foci, and liver tumour formation by diethylnitrosamine in the rat. Eur J Cancer 1975, 11:689–696
3. Solt D, Medline A, Farber E: Rapid emergence of carcinogen-induced hyperplastic lesions in a new model for the sequential analysis of liver carcinogenesis. Am J Pathol 1977, 88:595–610
4. Goldsworthy TL, Hanigan MH, Pitot HC: Models of hepatocarcinogenesis in the rat: contrasts and comparisons. CRC Crit Rev Toxicol 1986, 17:61–89
5. Bucher NLR, Strain AJ: Regulatory mechanisms in liver regeneration. Liver and Biliary Disease: Pathophysiology, Diagnosis and Management. Edited by GH Millward-Sadler, R Wright, MJP Arthur. Philadelphia, WB Saunders, 1992, pp 258–274
6. Fausto N, Webber E: Liver regeneration. The Liver: Biology and Pathobiology. Edited by IM Arias, JL Boyer, N Fausto, WB Jakoby, DA Schachter, DA Shafritz. New York, Raven Press Ltd, 1994, pp. 1059–1084
7. Michalopoulos GK: Liver regeneration: molecular mechanisms of growth control. FASEB J 1990, 4:176–187
8. Wilson JW, Luduc E: Role of cholangioles in restoration of the liver of the mouse after dietary injury. J Pathol Bacteriol 1958, 76:441–449
9. Sirica AE, Mathis GA, Sano N, Elmore LW: Isolation culture transplantation of intrahepatic biliary epithelial cell oval cells. Pathobiology 1990 58:44–64
10. Lemire JM, Shiojiri N, Fausto N: Oval cell proliferation and the origin of small hepatocytes in liver injury induced by D-galactosamine. Am J Pathol 1991, 139: 535–552
11. Sigal SS, Brill S, Fiorino AS, Reid LM: The liver as a stem cell and lineage system. Am J Physiol 1992, 263: G139–G148
12. Lombardi B: On the nature, properties and significance of oval cells. Recent Trends in Chemical Carcinogenesis, vol. 1. Edited by P Pani, F Feo, A Columbano, ESA Cagliari. 1982, pp 36–49
13. Sell S: The role of determined stem cells in the cellular lineage of hepatocellular carcinoma. Int J Dev Biol 1993, 37:189–201
14. Fausto N: Hepatocyte differentiation and liver progenitor cells. Curr Opin Cell Bio 1990, 2:1036–1041
15. Hixson DC, Faris RA, Yang L, Novikoff PM: Antigenic clues to liver development, renewal and carcinogenesis: an integrated model. The Role of Cell Types in Hepatocarcinogenesis. Edited by AE Sirica. Boca Raton, FL, CRC Press, 1992, pp 151–182
16. Thorgeirsson SS: Hepatic stem cells. Am J Pathol 1993, 142:1331–1333
17. Reid LM: Stem cell biology, hormone/matrix synergies and liver differentiation. Curr Opin Cell Biol 1990, 2:121–130
18. Arber N, Zajicek G, Ariel I: The streaming liver. II. Hepatocyte life history. Liver 1988, 8:80–87
19. Bralet MP, Branchereau S, Brechot C, Ferry N: Cell lineage study in the liver using retroviral mediated gene transfer. Evidence against the streaming of hepatocytes in normal liver. Am J Pathol 1994, 144:896–905
20. Grisham JW: Migration of hepatocytes along hepatic plates and stem-cell fed hepatocyte lineage. Am J Pathol 1994, 144:849–854
21. DeDuve C, Baudhuin P: Peroxisomes (microbodies related particles). Physiol Rev 1966, 46:323–357
22. Novikoff AB: The endoplasmic reticulum: a cytochemist's view (a review). Proc Natl Acad Sci USA 1976, 73: 2781–2787
23. van den Bosch H, Schutgens RBH, Wanders RJA, Tager JM: Biochemistry of peroxisomes. Annu Rev Biochem 1992, 197:61:157
24. Lazarow PB: Peroxisomes: The Liver Biology and Pathobiology, ed 3. Edited by IM Arias, JL Boyer, N Fausto, WB Jakoby, DA Schachter, DA Shafritz. New York, Raven Press, 1994, pp 293–307
25. Goldfischer SL: Peroxisomal diseases. The Liver Biology and Pathobiology, ed 2. Edited by IM Arias, WB Jakoby, H Popper, D Schachter, DA Shafritz. New York, Raven Press, 1988, pp 255–267
26. Novikoff AB, Novikoff PM, Davis C, Quintana N: Studies of microperoxisomes II. A cytochemical method for light and electron microscopy. J Histochem Cytochem 1977, 20:1006–1023
27. Lillie RD: Histopathologic Technic and Practical Histochemistry, ed 3. New York, McGraw-Hill Book Co., 1965, pp 1–715
28. Goldenberg H, Huttinger M, Bock P, Kramer R: Influence of subtotal hepatectomy on peroxisomes and peroxisomal enzymes of rat liver and isolated liver cell fractions. Histochem 1975, 44:47–56
29. Yamamoto K, Fahimi HD: Biogenesis of peroxisomes in regenerating liver. I. Sequential changes of catalase and urate oxidase detected by ultrastructural cytochemistry. Eur J Cell Biol 1987, 43:293–300
30. Luers G, Hashimoto T, Fahimi HD, Volkl A: Biogenesis of peroxisomes: isolation and characterization of two distinct peroxisomal populations from normal and regenerating liver. J Cell Biol 1993, 121:1271–1280
31. Baumgart E, Volkl A, Hashimoto T, Fahimi HD: Biogenesis of peroxisomes: immunocytochemical investigation of peroxisome membrane proteins in proliferating rat liver peroxisomes and in catalase-negative loops. J Cell Biol 1989, 108:2221–2231
32. Oikawa I, Novikoff PM, Yam A: Transient appearance of catalase-negative peroxisomes in rat hepatocytes during liver regeneration after partial hepatectomy. J Histochem Cytochem 1993, 41:1127
33. Higgins GM, Anderson RM: Experimental pathology of the liver. I. Restoration of the liver of the white rat following partial surgical removal. Arch Pathol 1931, 12: 186–202
34. Uchiyama Y, Asari A: A morphometric study of the

variations in subcellular structures of rat hepatocytes during 24 hours. Cell Tissue Res 1984, 236:305–31535
35. Karnovsky MJ: A formaldehyde-glutaraldehyde fixative of high osmolarity for use in electron microscopy. J Cell Biol 1965, 27:137A
36. Graham RC, Karnovsky MJ: The early stages of absorption of injected horseradish peroxidase in the proximal tubules of mouse kidney: ultrastructural cytochemistry by a new technique. J Histochem Cytochem 1966, 14:291–302
37. Novikoff AB: Lysosomes in the physiology and pathology of cells: contributions of staining methods. CIBA Found Symp 1963, 36–73
38. Wachstein M, Meisel E: Histochemistry of hepatic phosphatases at a physiological pH. Am J Clin Pathol 1957, 27:13–18
39. Novikoff AB, Shin WY: The endoplasmic reticulum in the Golgi zone and its relation to microbodies: Golgi apparatus and autophagic vacuoles in rat liver cells. J Microsc Paris 1964, 3:187–206
40. Rigatuso JL, Legg PG, Wood RL: Microbody formation in regenerating rat liver. J Histochem Cytochem 1970, 18:893–900
41. Lazarow PB, Fujiki Y: Biogenesis of peroxisomes. Annu Rev Cell Biol 1985, 1:489–530
42. Reddy JK, Svobada D: Microbodies in experimentally altered cells. VIII. Continuities between microbodies and their possible significance. Lab Invest 1971, 24: 74–81
43. Yamamoto K, Fahimi HD: Three-dimensional reconstruction of a peroxisomal reticulum in regenerating rat liver: evidence of interconnections between heterogeneous segments. J Cell Biol 1987, 105:713–722
44. Zhang JW, Han Y, Lazarow PB: Novel peroxisome clustering mutants and peroxisome biogenesis mutants of *Saccharomyces cerevisiae.* J Cell Biol 1993, 123:1133–1147
45. Santos MJ, Imanaka T, Shio H, Lazarow PB: Peroxisomal integral membrane proteins in control and Zellweger fibroblasts. J Biol Chem 1988, 263:10502–10509
46. Sternlieb I, Quintana N: The peroxisomes of human hepatocytes. Lab Invest 1977, 36:140–149
47. Jungermann K, Katz N: Functional specialization of different hepatocyte populations. Physiol Rev 1989, 69:708–764
48. Traber PG, Chianale J, Gumucio J: Physiologic significance and regulation of hepatocellular heterogeneity. Gastroenterology 1988, 95:1130–1143
49. Rosenberg E, Spray DC, Reid LM: Transcriptional and post-transcriptional control of connexin mRNAs in periportal and pericentral rat hepatocytes. Eur J Cell Biol 1992, 59:21–26
50. Kren BT, Kumar NM, Wang S, Gilila NB, Steer CJ: Differential regulation of multiple gap junctions transcripts and proteins during liver regeneration. J Cell Biol 1993, 123:707–718
51. Le Hir M, Dubach UC: The activities of peroxisomal oxidases in periportal and perivenous zones of the rat liver acinus. Histochem 1980, 69:95–99
52. Novikoff AB: Cell heterogeneity within the hepatic lobule of the rat (staining reactions). J Histochem Cytochem 1959, 7:240–244
53. Novikoff AB, Essner E: The liver cell. Some new approaches to its study. Am J Med 1960, 29:102–131
54. Rappaport AM: Physioanatomical basis of toxic liver injury. Toxic Injury of the Liver. Edited by Farber E, Fisher MM. New York, Marcel Decker, Inc., 1979, pp 1–57
55. Coleman WB, Wennerberg AE, Smith GJ, Grisham JE: Regulation of the differentiation of diploid and some aneuploid rat liver epithelial (stemlike) cells by the hepatic microenvironment. Am J Path 1993, 142:1373–1382
56. Martinez-Hernandez A: The hepatic extracellular matrix. I. Electron immunohistochemical studies in normal rat liver. Lab Invest 1984, 51:57–74
57. Martinez-Hernandez A, Martinez Delgardo F, Amenta PS: The extracellular matrix in hepatic regeneration. Localization of collagen types I, III, IV, laminin and fibronectin. Lab Invest 1991, 64:157–166
58. Wylie AH: Cell death: the significance of apoptosis. Int Rev Cytol 1980, 251–305
59. Farr AB and Kogoma T: Oxidative stress responses in *Escherichia coli* and *Salmonella typhimurium.* Microbiol Rev 1991, 55:561–565
60. Boyer TD, Kenny WC: Preparation characterization properties of glutathione-S-transferases. Biochemical Pharmacology and Toxicology. Edited by D Zakim, DA Vessy. New York, John Wiley, 1985, pp 297–363
61. Demple B, Daikh Y, Greenberg J, Johnson A: Alkylation and oxidative damages to DNA: constitutive and inducible repair systems. Antimutagenesis and Anticarcinogenesis: Mechanisms. Edited by DM Schenkel, PE Hartman, T Kada, Hollaender A. New York, Plenum Publishing Corp., 1985, pp 205–218
62. Ketterer B, Meyer DJ: Glutathione transferases: a possible role in the detoxification and repair of DNA and lipid hydroperoxides. Mutat Res 1989, 214:33–40
63. Teebor GW, Boorstein RJ, Cadet J: The repairability of oxidative free radical mediated damage to DNA: a review. Int J Radiat Biol 1988, 54:131–153
64. Fridovitch I: Superoxides dismutases. J Biol Chem 1989, 246:7761–7764
65. Saul RL, Gee P, Ames BN: Free radicals, DNA damage and aging. Modern Biological Theories of Aging. Edited by HR Warner, RN Butler, RL Sprott, EL Schneider. New York, Raven Press, 1987, pp 113–129
66. Cerutti P: Oxidant promoters. Tumour Promoters and Cancer Genes. Edited by N Colburn, H Moses, E Stanbridge. New York, Alan R Liss, 1988, pp 239–247
67. Halliwell B, Gutteridge JMC: Role of free radicals and catalytic metal ions in human disease: an overview. Methods Enzymol 1990, 186:1–85

68. Floyd RA: Role of oxygen free radicals in carcinogenesis and brain ischemia. FASEB J 1990, 4:2587–2597
69. Frenkel K: Carcinogen-mediated oxidant formation and oxidative damage. Pharmacol Ther 1992, 53:127–166
70. Reddy JK, Rao MS: Oxidative DNA damage caused by persistent peroxisome proliferation: its role in hepatocarcinogenesis. Mutat Res 1989, 214:63–68
71. Stark AA, Russell JJ, Langenbach R, Pagano DA, Zeiger E, Huberman E: Localization of oxidative damage by a glutathione-γ-glutamyl transpeptidase system in preneoplastic lesions in sections of livers from carcinogen-treated rats. Carcinogenesis 1994, 15: 343–348
72. Farber E: On cells of origin of liver cell cancer. The Role of Cell Types in Hepatocarcinogenesis. Edited by AE Sirica. Boca Raton, FL, CRC Press, 1992, pp 1–28
73. Sell S, Dunsford HA: Evidence for the stem cell origin of hepatocellular carcinoma and cholangiocarcinoma. Am J Pathol 1989, 134:1347–1363