A Structural Analysis of Gap and Tight Junctions in the Rat Liver During a Dietary Treatment That Induces Oval Cell Proliferation

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The authors have investigated early changes in liver cell gap and tight junctions that occur when rats are fed a carcinogenic diet. Animals were fed a choline-deficient diet that contained 0.1% ethionine (CDE) for periods up to 6 weeks. Short-term feeding of this diet results in the rapid proliferation of so-called "oval cells" within the liver, which is reversible upon returning the rats to a normal diet. Livers from animals fed the diet were removed at various times during feeding and during recovery from the diet and were analyzed by light and electron microscopy. The freeze-fracture technique was used to produce extended views of the internal structure of liver cell membranes at each stage under study. The characteristic junctional complex surrounding canalicular regions in normal liver disappears after only 2 weeks of the CDE regimen. Gap junctions were not found after 4 weeks of the diet, and tight junctions became increasingly disorganized. Tight junction elements were observed, however, between hepatocytes and oval cells, which indicated that these two cell types do interact directly. Changes occur in the structural complexity of tight junction elements between hepatocytes and between hepatocytes and oval cells. Recovery from the CDE diet results in a rapid increase in junctional complexity, and the large gap junction plaques characteristic of normal liver are visible within 2 weeks after cessation of the CDE regimen. These and other observations demonstrate that reversible alterations in hepatocyte gap and tight junctions occur as a result of administration of a diet that induces oval cell proliferation. The relationship of these changes to those that have been reported during other processes of cell proliferation are discussed. (Am J Pathol 1986, 125:379–392)

GAP JUNCTIONS are integral plasma membrane protein structures that form porelike channels connecting the cytoplasm of contiguous epithelial cells, in nearly all organized tissues. Through these porelike structures, small molecules of less than 1000 daltons can pass freely from cell to cell without entering the interstitial space. Such transfer constitutes one form of intercellular communication and plays a role in processes that include differentiation, development, metabolic cooperation, and growth control.[1–3] Tight junctions, by contrast, serve as intercellular permeability barriers and are particularly important in epithelial tissues.[2]

In normal liver, an extensive network of both gap and tight junctions exists between hepatocytes, which reflects this cell's complex metabolic role and polarity in regard to blood and bile. Alterations in these junctional complexes have been reported in both normal growth processes, which include liver regeneration,[5,6] and in pathologic processes, which include cholestasis[7,8] and hepatomas.[9,10] These studies have concentrated on junctional changes in hepatocytes. Many hepatocarcinogens, however, during the early stages of carcinogenesis before tumors develop, induce the proliferation of another liver epithelial cell type known as the "oval cell."[11] These cells, which are morphologically similar to biliary ductular cells, begin to proliferate in the portal areas and rapidly infiltrate the hepatic parenchyma.[12] When rats are fed a diet deficient in choline and containing 0.1% ethionine (CDE diet) for 4–6 weeks,[13] oval cells may occupy as much as 50% of the liver lobule. Such a radical change in liver cell composition is likely to lead to changes in gap and tight junctions, which

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may have important consequences in liver cell to cell interactions and homeostasis.

We therefore decide to investigate with the use of freeze-fracture electron microscopy, whether changes in hepatocyte tight and gap junctions accompany the oval cell proliferation at the early stages of CDE hepatocarcinogenesis and to determine to what extent any changes in cell-to-cell contact might be reversible. In addition, we wished to establish whether or not oval cells in preneoplastic* liver share intercellular junctions with other oval cells and with hepatocytes. For comparative purposes, we studied intercellular junctions in bile duct-ligated liver because it has been shown by freeze-fracture that ligation of the common bile duct reduces the number of gap junctions and changes the organization of tight junctions in hepatocytes.[7,8]

In this study, we compare junctions in normal rat liver with the cell junctions in livers of rat 1) fed the CDE diet for 2–6 weeks; 2) fed a basal diet from 3 days to 2 weeks after CDE feeding; and 3) after bile duct ligation (BDL).

Materials and Methods

Male Sprague–Dawley rats (Charles River Breeding Laboratories, Wilmington, Mass) were fed *ad libitum* either a standard laboratory chow, a CDE diet or a choline-deficient diet (CD) (Teklad Test Diets, Madison, Wis). For BDL experiments, the common bile duct was ligated and the animal was maintained on normal chow for 4 weeks. After treatments, animals were fasted overnight, livers were removed from the animals while they were under ether anesthesia, and small wedges of liver from the left lateral lobe were diced into small pieces. Specimens included normal liver; liver at 2, 4, and 6 weeks of CDE feeding; liver at 3 days, 1 week, and 2 weeks of feeding normal chow after 6 weeks of CDE feeding ("recovery stage"); liver at 2 weeks of CD feeding; and liver at 4 weeks after bile duct ligation. Tissue samples from two livers were examined extensively for each feeding condition.

Histopathologic examination by light microscopy was carried out on hematoxylin and eosin-stained sections of paraffin-embedded liver. Tissue for thin-sections and freeze-fracture electron miscroscopic examination was fixed in 2% glutaraldehyde in 0.2 M cacodylate buffer (pH 7.5) for 1 hour at room temperature. For thin sectioning, specimens were postfixed in 2% osmium tetroxide for 1 hour, rinsed in distilled water, dehydrated in acetone, and embedded in Spurr's resin. Thin sections were cut on a Sorvall 5000 microtome and stained with uranyl acetate and lead citrate. For freeze-fracturing, the aldehyde-fixed specimens were infiltrated with glycerol to 20% over 1 hour, frozen by immersion in liquid Freon 22, and stored in liquid nitrogen. Replicas were prepared on a Balzers BAF-400 freeze-etching device, at a temperature of −110 C. Metal shadowing was started immediately after the final pass of the fracturing knife at an angle of 40 degrees. Successful replicas were cleaned by two overnight rinses, first, in 100% bleach and, second, in 40% chromic acid; they were then rinsed in distilled water and mounted on copper mesh grids.

All specimens were examined on a Philips 201 electron microscope. The nomenclature of Branton et al[14] has been used for identification of membrane fraction faces.

Results

Light Microscopy

As has been described previously,[13] the CDE diet induces the proliferation of oval cells, which appear within 2 weeks of initiation of the diet and which penetrate the liver lobules beginning in the portal areas (Compare Figures 1A and B). This new population is heterogeneous in size and shape but can be distinguished by their smaller size in comparison with hepatocytes, pale-staining oval nuclei, and scant cytoplasm and their tendency to form lumens.[11] By 6 weeks on the diet, oval cells account for approximately half the cell population of the liver (Figure 1C). Their random dispersal

* The term "preneoplastic" is used only to indicate a temporal sequence. It does not imply a direct link with cell transformation.

Figure 1A—Light micrograph of thick-sectioned normal liver in a portal area. Cords of hepatocytes are separated by sinusoids (and by bile canaliculi not visible at this mangification). A branch of the portal vein in longitudinal section and small ductules are visible (×1000) **B**—After 2 weeks of the CDE diet, scattered oval cells, at the *arrow*, can be found among the hepatocytes. This new cell population is characterized by small, elongate nuclei with scant cytoplasm. (×1000) **C**—At 6 weeks after the CDE diet was begun the liver is barely recognizable because of the proliferation of oval cells. Oval cells can be recognized as small heterogenous cells infiltrating the hepatic parenchyma. The disorder and heterogeneity of this multiplying population is apparent. Many of the hepatocytes appear morphologically abnormal (see *arrow*). (×1000) **D**—At 1 week of recovery after 6 weeks of the CDE diet, the liver is characterized by small hepatocytes with prominent mitochondria and round nuclei. A remaining oval cell cluster is seen in the upper left. (×1000) **E**—At the 2-week recovery stage, hepatocytes appear almost normal, but there are areas where they have not fully separated into regular cords by the sinusoids. A remaining cluster of oval cells can be seen along the lower part of the micrograph. (×1000) **F**—In bile duct-ligated liver, numerous ducts are formed by the proliferating cells. Compare the large size of the ductule cells and lumens here with the oval cells after 6 weeks of the CDE diet (**C**). A connective tissue support surrounding the ductule cells is also visible at the arrow. (×1000)

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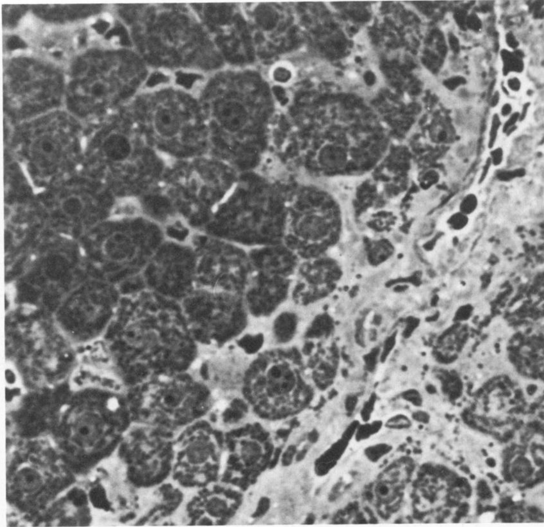

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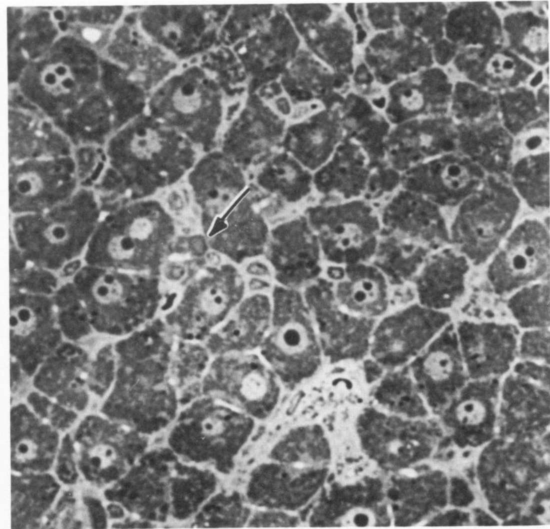

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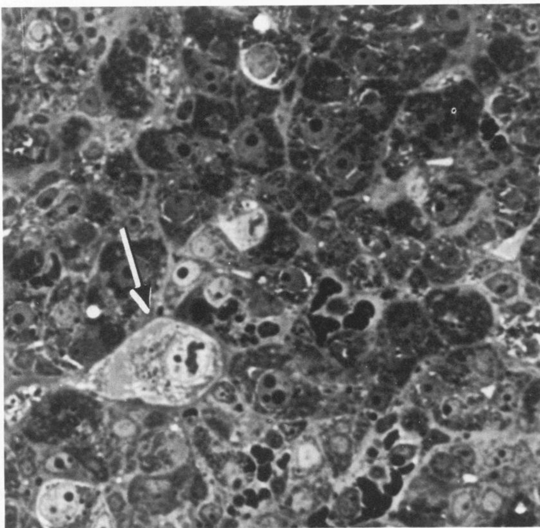

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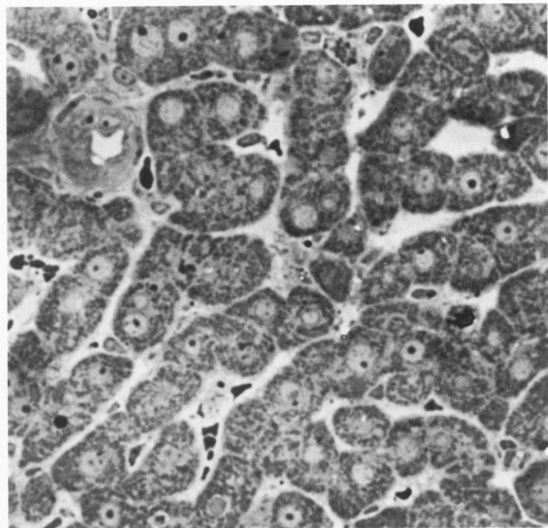

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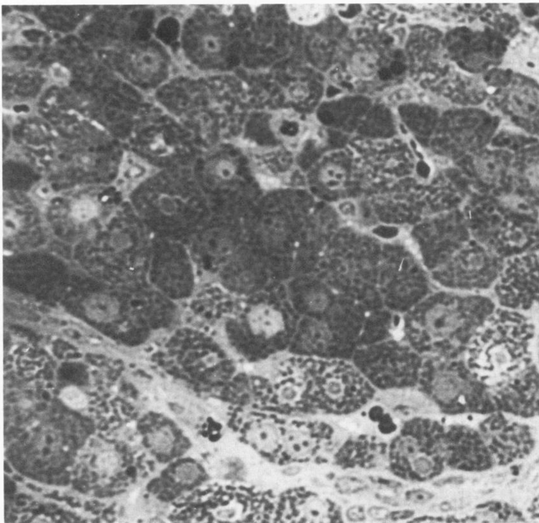

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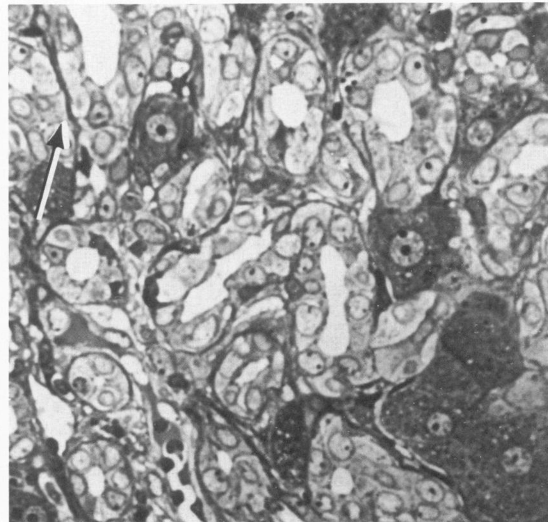


throughout the liver completely disrupts the normal lobular organization of the organ.

Upon removal of the CDE diet from the rats and their return to normal diet, many of these changes are reversible in the rats.[13] When normal chow was fed to rats previously on the CDE diet for 6 weeks, the number of oval cells was significantly reduced after 1 week (Figure 1D). Many small, morphologically normal parenchymal cells were seen in place of the morphologically heterogeneous CDE-affected hepatocytes. This recovery was nearly complete after 2 weeks, but oval cells could still be found and the platelike arrangement of hepatocytes remained somewhat irregular (Figure 1D).

Bile duct ligation results in a proliferation of "ductule cells" in typical acinar arrangement. After 4 weeks this ductular hyperplasia accounts for at least 1 half of the liver mass.[7,8] In contrast to oval cells, these ductule cells constitute a fairly homogeneous population, forming larger and more branching ducts (Figure 1F). Choline deficiency alone causes accumulation of lipid droplets within the hepatocyte cytoplasm but does not produce oval cells. This extensive lipid accumulation distorts the normal appearance of hepatocytes and makes proper fixation and staining difficult. For this reason, comparisons in this study were made primarily between liver tissue from rats on the CDE diet and normal or BDL liver tissue.

Thin-Section Electron-Microscopic Examination

In normal liver, tight junctions can be observed in cross sections of bile canaliculi.[2,4,15] Tight junctions result from "punctate fusions" between adjacent hepatocyte membranes and function as a permeability barrier that seals the canalicular lumen. Tight junctions also exist between the biliary cells (cubodial epithelium) forming the ductules in the portal areas. Gap junctions are somewhat more difficult to locate: In thin section they are represented by a 2–4 nm separation between the apposed cell membranes of adjacent hepatocytes found lateral to the tight junction. Gap junction structure is seen more clearly in freeze fracture.

Normal ultrastructural features of hepatocytes in thin section include round nuclei, prominent nucleoli, numerous mitochondria (with short cristae), and abundant rough endoplasmic reticulum (RER). Bile canaliculi with visible tight junctions can also be seen. The biliary cells of the portal areas are comparatively smaller than hepatocytes and have more elongate nuclei, fewer mitochondria (with long cristae that extend the width of the organelle, see Ogawa et al),[16] and less RER. Bile canaliculi studded with microvilli can be seen between adjacent hepatocytes; they appear either as circular lumens or as more elongate ones, depending on the plane of section.

Within 2 weeks of rats being on the CDE diet, oval cells can be found dispersed among the hepatocytes that thus disrupt hepatocellular connections. By 6 weeks of the rats being on the diet, oval cells are abundant and easily recognized by their oval-shaped nuclei, abundant mitochondria, and cytoplasmic extensions that penetrate the spaces between hepatocytes. Junctional complexes do exist between adjacent oval cells, but their precise nature is unclear (from Figure 5 in freeze-fracture it is clear, however, that at least some of this complex is composed of tight junctions). By the 6-week stage, oval cells form clusters that seem to encircle the hepatocytes—at least in the plane of section—and thereby surround them with a mass of disorganized, smaller cells (see Figure 1C). Bile canaliculi appear distorted, with dilated lumens and irregular microvilli; both tight and gap junctions are difficult to identify after the rats have been on the diet for 2 weeks. As a result, the connections between oval cells and hepatocytes are very irregular. The areas between these cells are filled with microvilli-like processes instead of regular bile canaliculi with junctional complexes; the nature of their membrane interactions cannot be determined in thin section. (As mentioned above, freeze-fracture reveals that in some cases oval cell and hepatocyte membranes do share irregular tight junctions but not gap junctions.) The ultrastructural appearance of hepatocytes after the rats have been on the CDE regimen for 6 weeks is heterogeneous: their nuclei now range in size and shape from very large and round to small and irregular, although cell size and cytoplasmic organelles still distinguish them from oval cells. The mitochondria of hepatocytes are swollen, and hepatocyte cytoplasm is full of lipid and unidentified membraneous material.

Upon removal of the CDE diet from the rats after 6 weeks and their return to a normal diet the liver shows extensive remodeling, which is readily apparent after only 3 days. Many of the oval cells have disappeared; numerous hepatocytes with normal-appearing nuclei, mitochondria, and RER are seen at this time. The overall size of these cells is considerably smaller than that of hepatocytes in normal liver, and the proportion of mitotic figures is high. Both sinusoids and extensive bile canaliculi begin to appear in the areas between hepatocytes formerly occupied by oval cells. Damaged and irregular hepatocytes, in addition to many oval cells, still remain. This recovery progresses rapidly, and by 2 weeks much of the liver appears normal, although scattered oval cells and morphologically abnormal hepatocytes can still be found.

In bile duct-ligated liver, ductule cells proliferate and

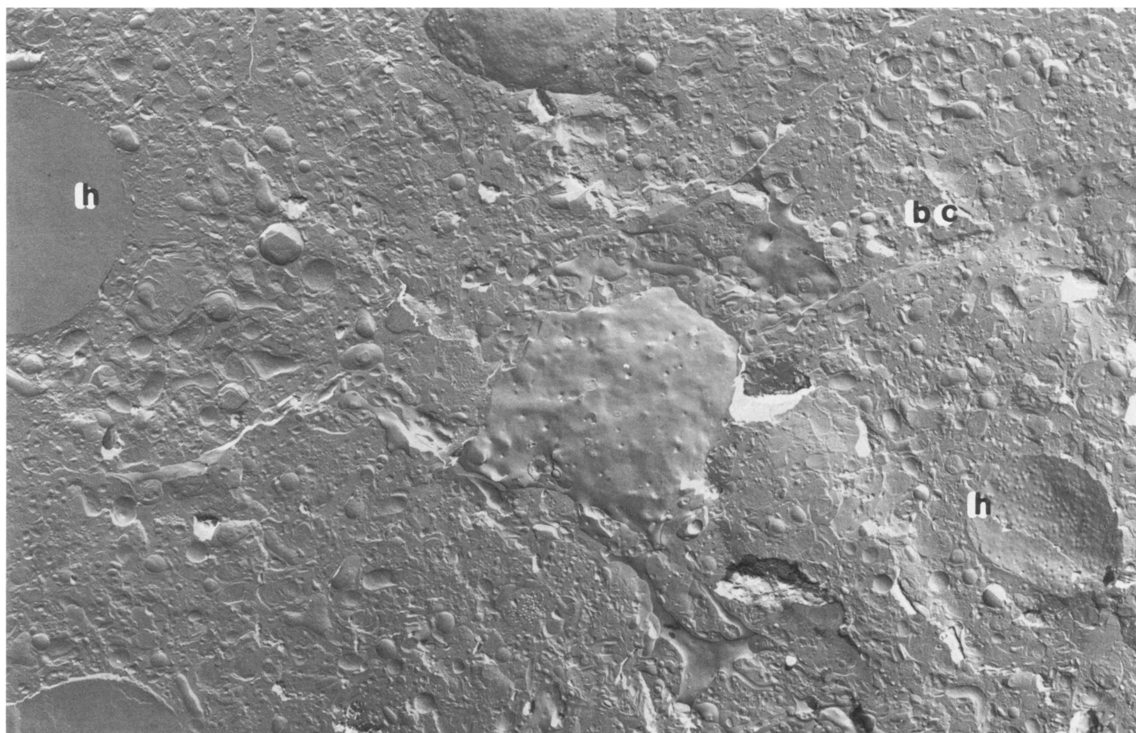


Figure 2—Low magnification of normal liver in freeze-fracture reveals the cellular composition of the organ. Two hepatocytes are identified (*h*), and a bile caniliculus is also shown (*bc*). Hepatocyte nuclei are large and have numerous nuclear pores; mitochondria and RER are visible in their cytoplasm. Note that the cell boundaries are clearly defined. An area of hepatocyte cell membrane appears at center. (×4800)

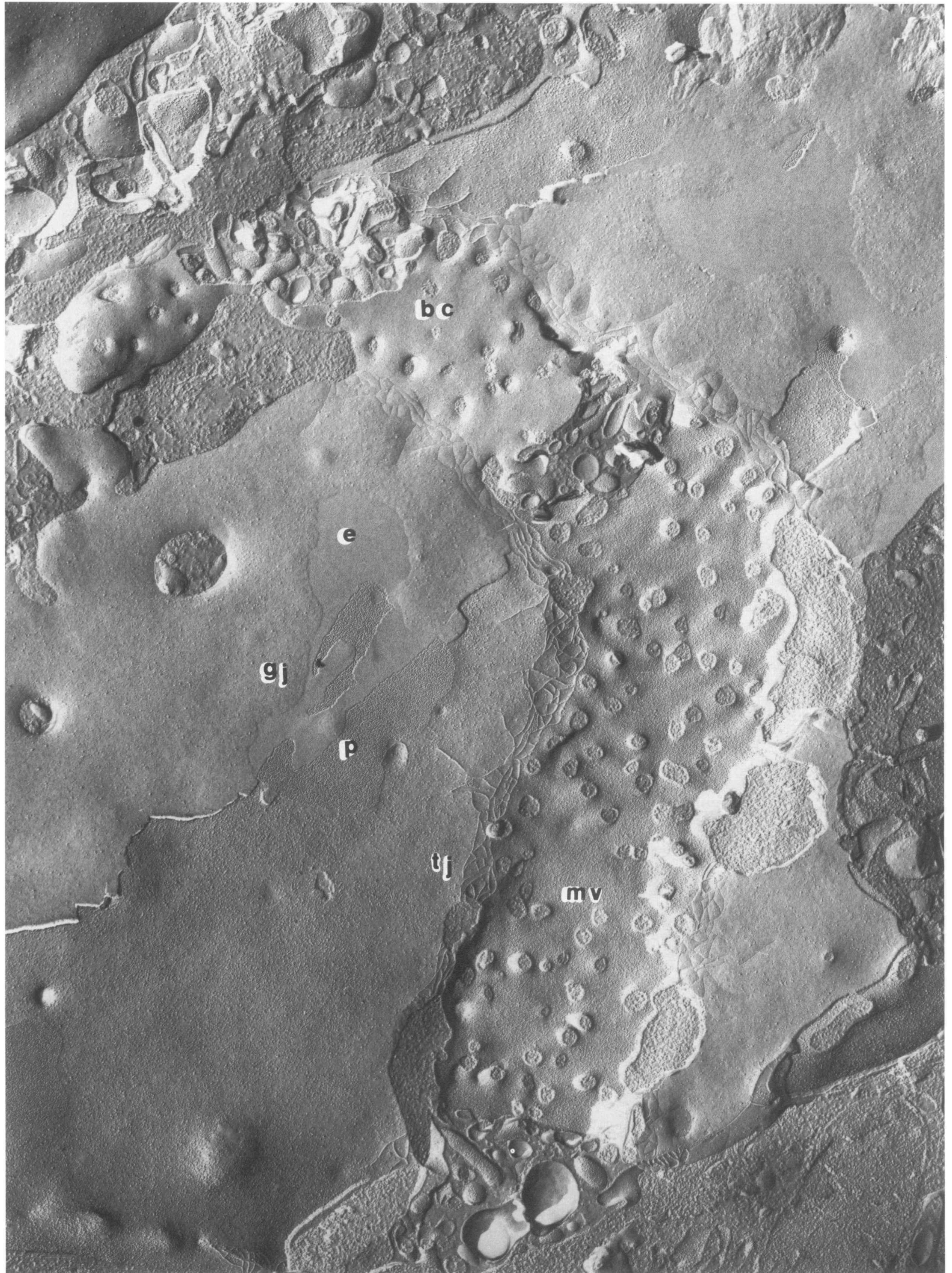
form organized clusters of lumens, most of which are sealed by desmosomes and tight junctions, and are surrounded by a thick basement membrane.* In contrast to liver from rats on the CDE diet, the hepatocytes in this system remain associated with one another and appear unchanged in ultrastructure. However, the bile canaliculi often appear dilated and have either irregular or absent microvilli. As with liver from rats on the CDE diet, it is difficult to locate either tight or gap junctions that belong to hepatocytes.

The CD diet without ethionine caused such excessive lipid accumulation that careful observation of thin-sectioned liver was impossible.

Freeze-Fracture Electron Microscopy

Hepatocytes in freeze-fracture are recognized by their large nuclei with numerous nuclear pores and by their prominent mitochondria (Figure 2). Cell boundaries are well defined and relatively straight; bile canaliculi full of fractured microvilli can be found between adjacent hepatocytes. These canaliculi appear in cross section as round lumens, where the membranes of the two hepatocytes abut, and in longitudinal section as a long lumen surrounded on either side by the lateral membrane surfaces belonging to the two cells. The latter orientation in freeze-fracture reveals the characteristic location of hepatocellular tight and gap junction in the liver (Figure 3). A beltlike array of tight junction strands borders the canalicular lumen, which is studded by microvilli from the adjacent hepatocytes. Gap junction subunits occur on the exposed lateral membranes to either side of the lumen, both as large plaques some distance away from the tight junctions and as small plaques found within the tight junction network. These observations are similar to those reported by other groups on earlier studies of liver cell junction.[6,7]

The tight junction complex forms a network of interconnecting strands, seen as ridges on the E face and as complementary grooves on the P face. The strand number ranges from three to seven in normal liver; the overall width of the belt is consistently narrow. It is well established that the permeability of a tight junction in a given area is determined by several factors, which include strand number, organization, and average depth. Thus, there is a normal variation in these features that allows for different degrees of "tight" or "leaky" junctions that depends on the functional role of the cells possessing the tight junctions.[7,17]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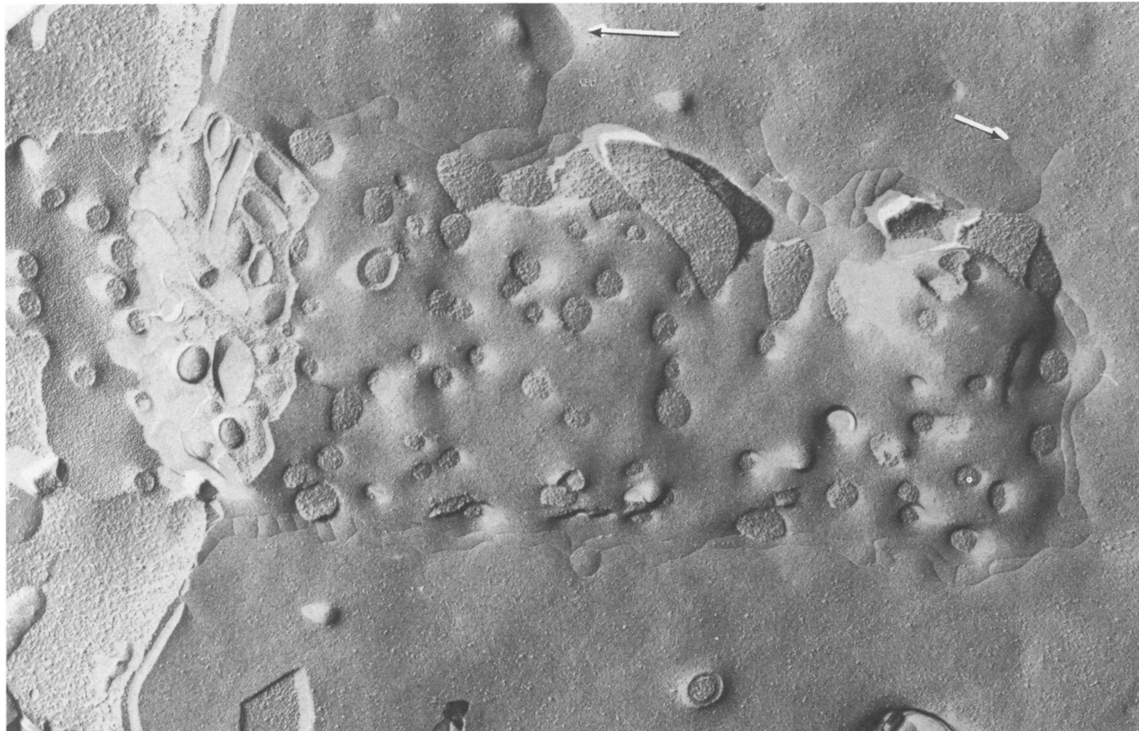

Figure 4—Bile canalicular area at 2 weeks of CDE treatment reveals a significant reduction in the complement of tight and gap junctions when compared with that of the normal liver. The *arrows* point out two plaques of gap junction particles. (×40,000)

Gap junctions appear as irregularly packed particles in the E face and as their complementary pits in the P face. The small gap junctions among the tight junction strands range in number of particles from 4 to 35. The large gap junction plaques vary in size and may be anywhere from 10 to 30 times the size of the small gap junctions; thus, the number of particles ranges from 40 to 1000. These findings are in accord with the extensive quantitative work of Yee and Revel.[5] They estimate the diameter of a single gap junction subunit to be 6 nm, which makes the average diameter of a small gap junction about 0.1 μ and that of a large one between 1.0 and 2.0 μ—a factor of 10 to 20. They also estimate that gap junctional area makes up approximately 1.5% of the total hepatocyte membrane area in normal rat liver.

Use of the CDE diet results in the progressive disorganization and loss of both tight and gap junctions from their characteristic locations on hepatocyte membranes. Two weeks after the CDE diet is started, the tight junction belt can still be found bordering canalicular lumens, but it is reduced in depth and in strand number, now ranging from one to three; the number of wandering strands that project onto the lateral membrane area has increased threefold (Figure 4). The gap junctions at this stage are smaller and decreased in number; the large plaques are found much closer to the tight junction belt, often attached to its loose strands, and the small plaques within the tight junction network are about 1 half as numerous as those in normal liver.

The oval cells just begin to proliferate after the rats have been on the diet for 2 weeks, and, when found, they are readily distinguished from hepatocytes (Figure 5). Oval cells in freeze-fracture are characterized by small, elongate nuclei, about one third the size of hepatocyte nuclei, and with considerably fewer nuclear pores in comparison with hepatocytes. Oval cells are heterogeneous in size and shape, and they have only a small amount of cytoplasm. As seen in thin section, the oval cells are found randomly dispersed between the hepatocytes, and the connections between these two cell types are irregular and difficult to characterize. In some cases, however, tight junctional elements can be clearly observed between hepatocytes and oval cells, as

Figure 3—Hepatocellular junctions in freeze fracture have a characteristic appearance and are readily located in normal liver. Tight junction (*tj*) strands and gap junction (*gj*) particles are positioned around a bile canaliculus (*bc*) with protruding microvilli (*mv*) from the participating hepatocytes. Both E (*e*) and P (*p*) fracture faces of the gap junction region are visible. (×40,000)

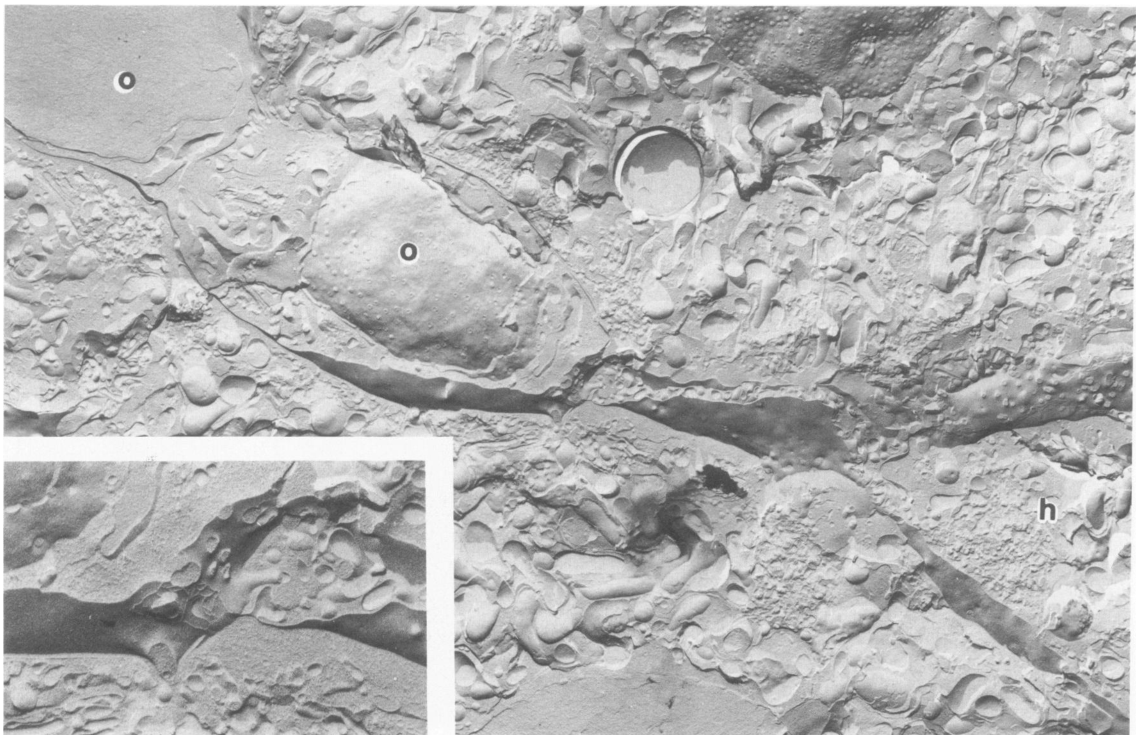

Figure 5—In the liver of rats fed the CDE diet for 2 weeks, tight junctional elements are visible between oval cells and hepatocytes at this junction of four cells in the fracture plane. Oval cells (*o*) are most easily differentiated from hepatocytes (*h*) in fractured liver by their nuclei, which are small and irregularly shaped and have very few nuclear pores. Oval cell nuclei also take up most of the area in the cell, and thus oval cells have little cytoplasm. (×12,500) **Inset**—A higher magnification (×31,000) of a tight junction region at the center of the figure. The cell at the upper left of center is clearly an oval cell, and the cell toward the bottom of the junction has the size and shape of a hepatocyte.

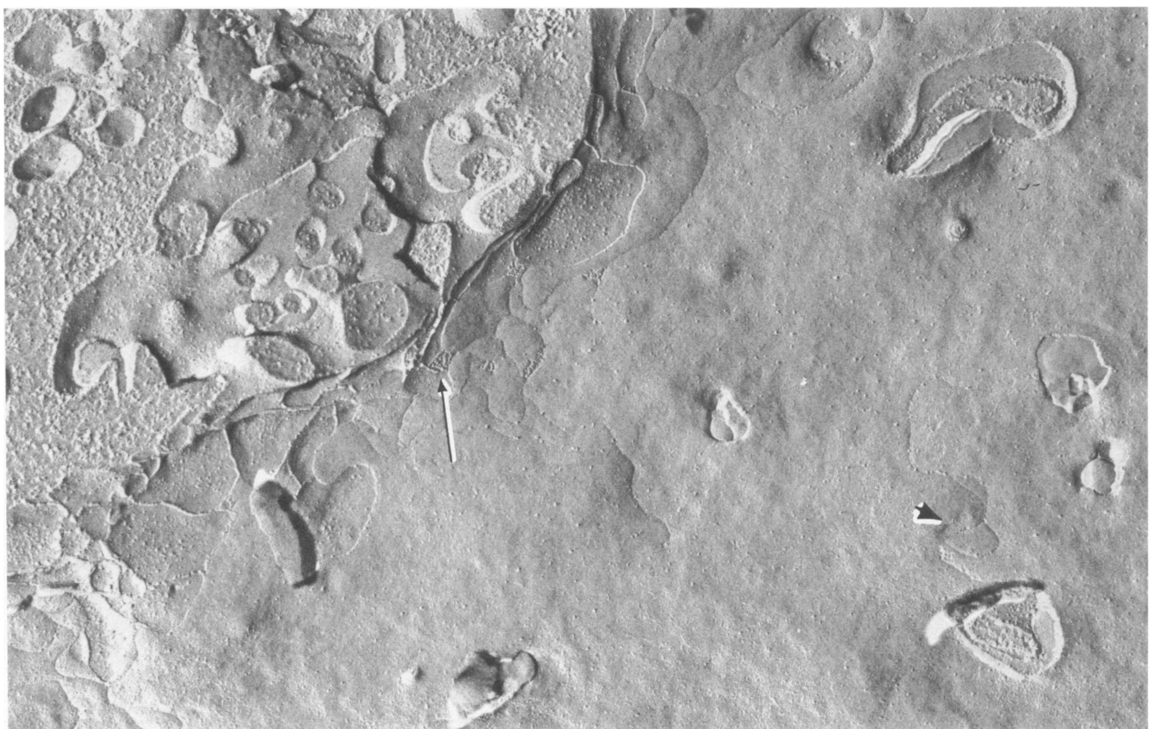

Figure 6—At the 4-week CDE stage, bile canalicular areas are difficult to find. Nonetheless, junctional elements (*large arrow*) can be seen between cells in the liver at this stage. The tight junctions are disorganized, and few gap junctions can be found; of these, some appear to have less densely packed subunits (*arrowhead at right*). (×44,000)

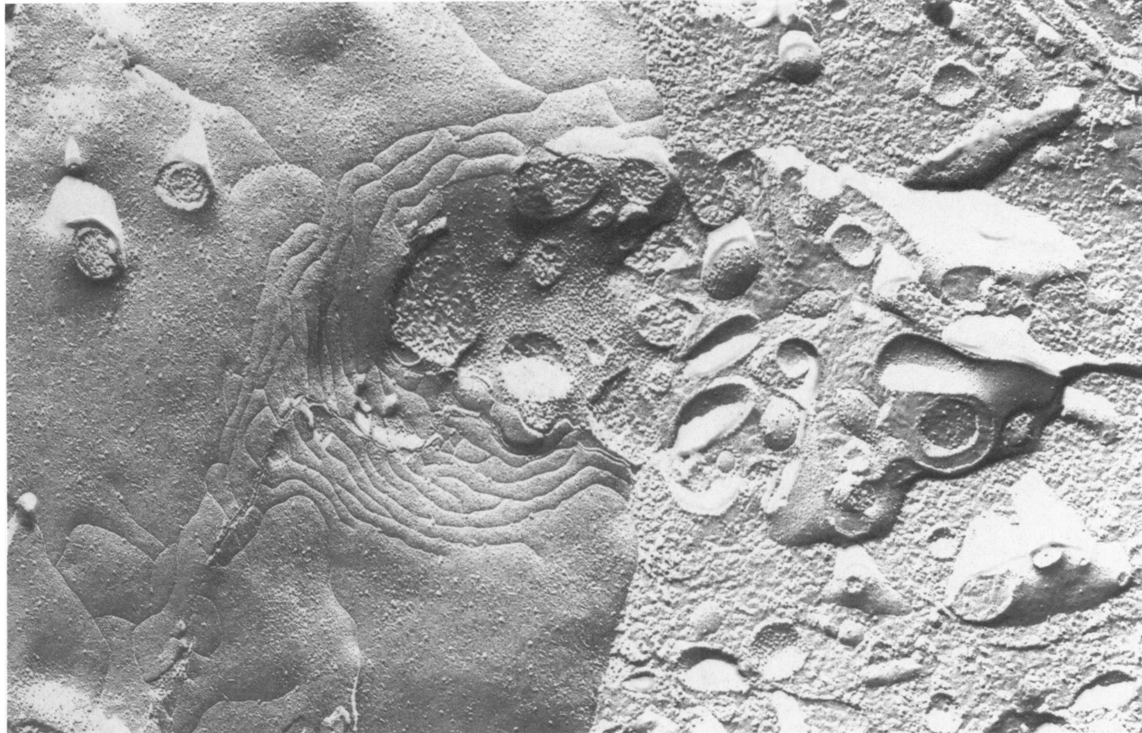

Figure 7—Junctions of any sort after 6 weeks of the CDE diet are difficult to locate. This area appears to be a very small bile canaliculus; the tight junctions strands have virtually no interconnections and no recognizable gap junctions. (×56,000)

in Figure 5, which shows a four-way junction between these two cell populations.

Four weeks after the rats have been on the CDE diet, oval cells are more abundant and are usually found in clusters of two or more arranged in circles (representing the lumens seen in thin section). The characteristic bile canalicular regions of normal liver no longer appear. Instead, disorganized tight junctions are found either on fragments of attached membrane or surrounding large lumens with irregular microvilli. The tight junction strands now range in number from one to three, with fewer interconnections; the number of wandering strands is greater, and these are frequently oriented abluminally or in parallel rows (Figure 6). While the normal bile canaliculi are lost, large gap junctional plaques also disappear at this stage. Small gap junctions are fewer in number and are found farther out into the membrane areas in association with the wandering tight junction strands. These small plaques are often disorganized and have less densely packed subunits (see *arrow* in Figure 6).

Six weeks after the rats have been on the CDE diet, highly irregular tight junctions continue to appear in membraneous areas, where it is difficult to determine which cell types are involved in such junctions; these tight junctions, often discontinuous, contain from two to four strands and have fewer interconnections than those seen between normal hepatocytes. Small bile canaliculi can still be found (Figure 7), but most of the junctional elements observed at the 6-week stage are found on membrane surfaces and resemble those seen at 4 weeks (Figure 6). It is likely that some of these junctions belong to oval cell membranes as well as to hepatocyte membranes, in areas where these two cell types interact (Figure 5). Gap junctions of any size and shape are now rarely found; occasionally, small ones exist in association with the wandering tight junction strands found at random on membrane surfaces. At this stage (figure 8), the cell boundaries between hepatocytes have become irregular and appear to be separated by a space full of irregular microvilli. Oval cells are common but constitute a heterogeneous population that varies in size, shape, and location; some form lumens, and others appear to emerge in the microvilli-filled areas between hepatocytes, thus disrupting the canaliculi and their junctional complexes.

The liver morphologic features undergo rapid recovery when the CDE diet is taken away from the rats and they are fed the normal diet. After 3 days, characteristic bile canaliculi reappear with numerous small gap junctions and a narrow, one-to-three-strand, tight junction network (Figure 9). At 1 week of recovery, extensive "trees" of branching bile canaliculi become a striking feature in the liver (Figure 10). They are lined by

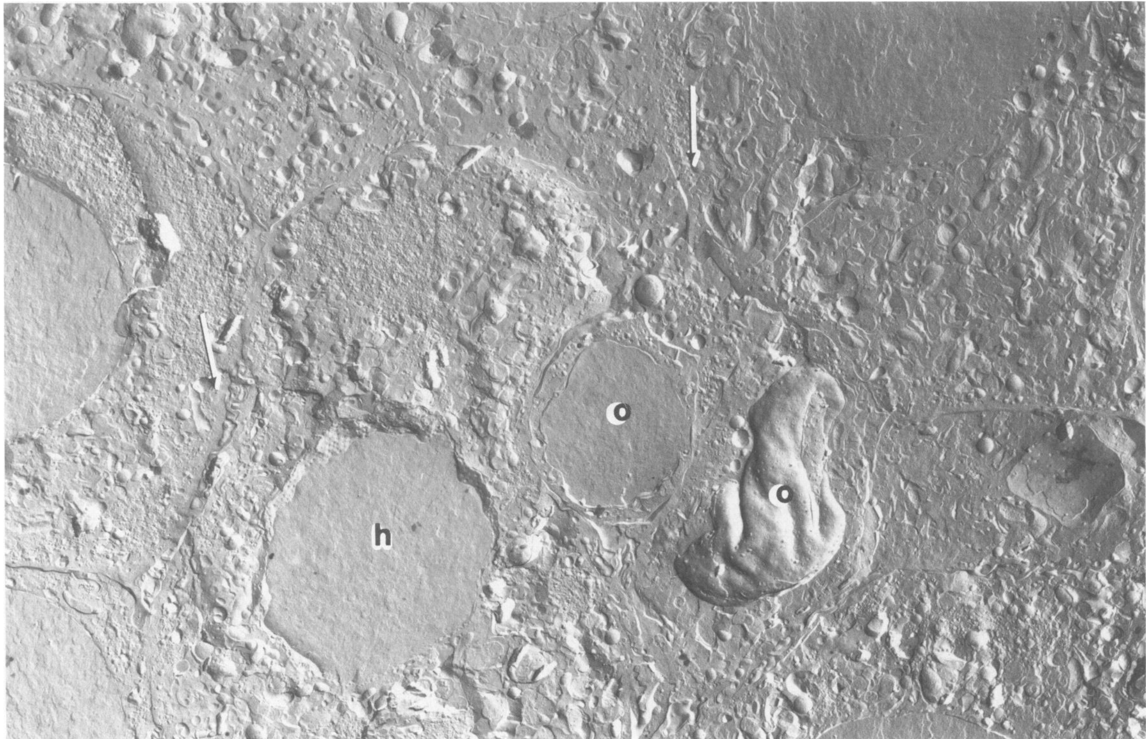

Figure 8—Low magnification of the liver from a rat fed the CDE diet for 6 weeks shows the disorganization of the organ at this early stage of carcinogenesis. (The various cell types seen here can be correlated with those seen in thin section and compared with the freeze-fracture micrograph of normal liver [**Figure 2**].) Cell outlines are visible, but they consist of distinct spaces filled with microvilli or other cytoplasmic protrusions (*at the arrows.*) The very rough areas in the hepatocyte cytoplasm probably correspond to the membraneous material seen in thin section. Oval cells (*o*) and hepatocytes (*h*) are visible. (×4500)

Figure 9—At 3 days of recovery (return to a normal diet after 6 weeks of CDE feeding), the bile canaliculi and the junctional complexes reappear. Once again a tight junction network seals the lumen, and small gap junctions can be found (*arrow*). (×36,000)

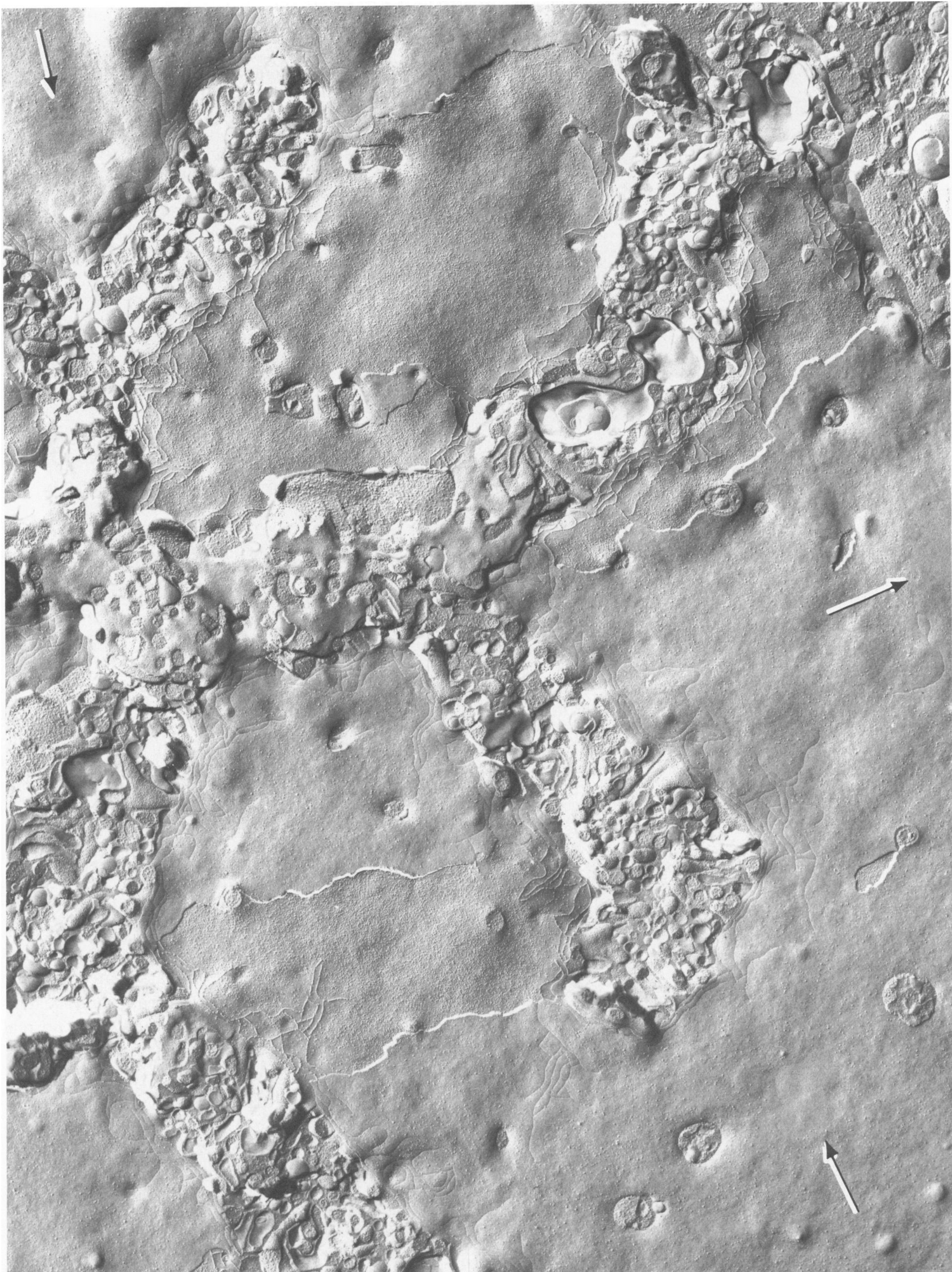

Figure 10—At 1 week of recovery, the junctional complexes are strikingly abundant. Branching bile canaliculi with a full array of junctions, still somewhat irregular, occur throughout the liver at this stage. Gap junction plaques can be seen at the *arrows*. (Original magnification, ×33,000)

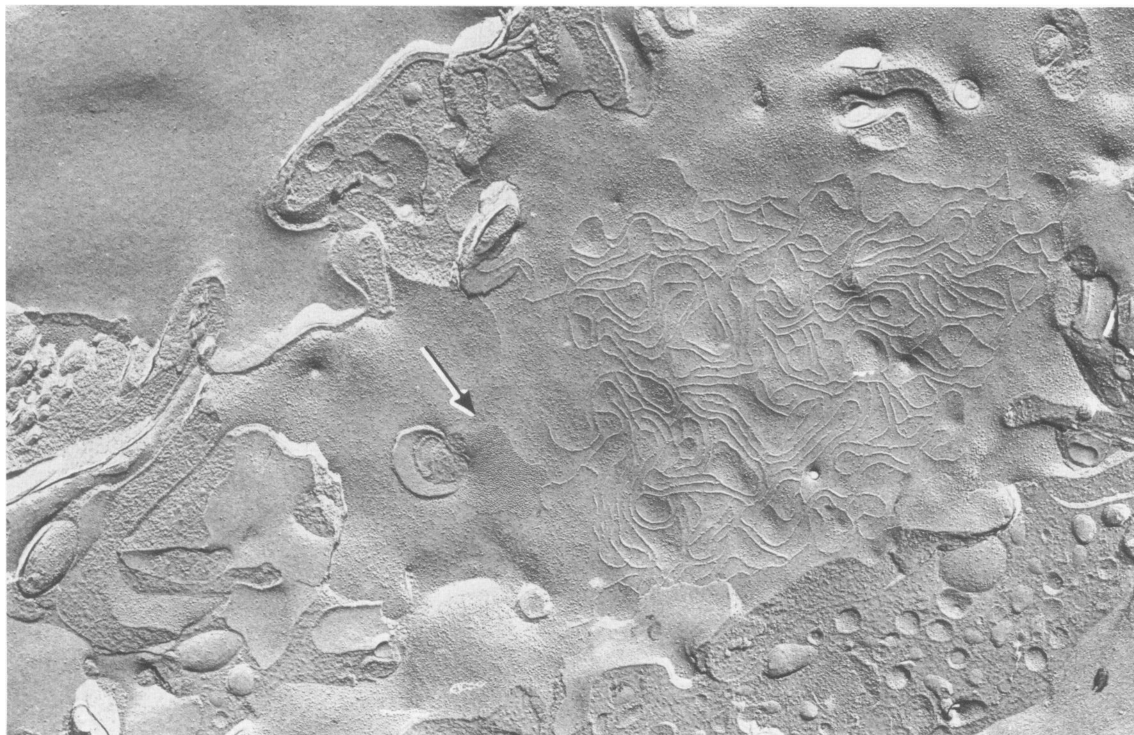

Figure 11—After 2 weeks of recovery, irregular foci of tight and gap junctions (*arrow*) can still be found. (×27,000)

tight junction strands, ranging from two to five in number, and once again contained within a narrow border; there are still numerous wandering strands, but they are considerably shorter than those found in the 6-week CDE stage. Small gap junctions have returned to the tight junction network. Larger plaques in the lateral membrane are rare and, when found, occur closer to the canaliculi than in normal liver. Most of the hepatocytes are also quite small but have characteristic round nuclei and very abundant RER. Oval cells are still common, but many of the boundaries between hepatocytes appear to be restored to normal, except for the unusually numerous bile canaliculi. After 2 weeks of recovery, the hepatocytes appear closer to normal in size, and their bile canaliculi have regained their classic complement of tight junction networks with small and large gap junctions. Local foci of tight junction strands with associated gap junctions can still be found, although their significance is unknown (Figure 11).

The 2-week CD diet without ethionine affects tight and gap junctions only slightly. Tight junction strand number now ranges from two to five, and the width of the belt is somewhat decreased. The large gap junctions are less abundant, but they maintain their distance from the tight junctions.

The effect of bile duct ligation, which produces extrahepatic cholestasis, on the junctional complex is to disorganize tight junctions and eliminate gap junctions. Characteristic bile canalicular areas are not seen, and tight junctions are found as irregular networks on membrane fragments; they range in strand number from zero to six, with frequent discontinuities. It is difficult for one to determine to which cell types these junctions belong, but from the thin sections it is clear that tight junctions occur in some form between the ductule cells arranged in lumens. It seems most likely that the various tight junctional elements found dispersed in ligated liver belong in part to the proliferating ductule cells and in part to the remaining hepatocytes. The only gap junctions observed are very small ones associated with the irregular tight junction strands. These appear to be remnants of the gap junctions of hepatocytes seen in normal liver and their loss related to the cholestatic condition.

Discussion

We designed the present study to determine the effect of the CDE diet on hepatocellular junctions during conditions of oval cell proliferation. We also set out to examine whether junctions were detectable between oval cells as well as between oval cells and hepatocytes. Although the role of oval cells in hepatocarcinogenesis remains unknown,[18,19] most investigators agree that oval cells are a distinct population with its own set of morphologic, biochemical, and functional characteristics.[16,20-24]

In normal liver, adjacent hepatocytes share membrane specializations necessary for the proper liver function. Gap junctions establish cell-to-cell communication, tight junctions regulate canalicular permeability, and desmosomes maintain cell adhesion.[2,4] When viewed in freeze-fracture, tight and gap junctions are easily recognized by their characteristic location and classic appearance (Figure 3). Wherever two hepatocytes abut, they form a bile canaliculus that is encircled by a belt of tight junction strands; plaques of gap junction subunits are found within the tight junction network and in the lateral membranes surrounding the canalicular lumen.

We describe changes in tight and gap junctions in our various samples relative to their appearance in normal liver because it is difficult to locate every possible junctional complex in liver studied with freeze-fracture. Meyer et al[6] have shown that the membrane area exposed in fracturing is only about two fifths of the total membrane area of hepatocytes. Therefore, if the number of junctions is decreased in a given sample, the chance of one's finding these and accurately counting them also decreases because they are less likely to occur in the fraction of membrane exposed. They also conclude that a hepatocyte in normal liver contacts on the average, six other hepatocytes, whereas this number is reduced to one in regeneration of liver after partial hepatectomy. A similar reduction in hepatocyte-to-hepatocyte contacts is produced by the CDE treatment, and, again the chance of fracture across a small number of junctions is reduced.

In CDE-treated liver, as the oval cell population increases, tight and gap junctions appear progressively altered. By 4 and 6 weeks after the rats have been on the diet, few gap junctions are detectable, and the once-extensive hepatocyte tight junctions are reduced and dispersed randomly on membrane areas (Figures 6 and 7). It is apparent that at least some of these irregular tight junctions belong to oval cells, in connection to other oval cells and to hepatocytes (Figure 5). The oval cell tight junctions appear disorganized in comparison with those of normal liver and probably function as a very "leaky" permeability barrier (on Claude and Goodenough's scale).[22] Also unlike hepatocytes, oval cells do not form extensive gap junctions and therefore apparently do not communicate (at least via conventional pathways). These findings correspond to those for regenerating and neoplastic tissues where intercellular junctions are also scarce or absent.[3,9,25-28]

The recovery seen when the diet is returned to normal after rats have been on the CDE diet for 6 weeks is quite impressive. Within 3 days, small hepatocytes with prominent RER have replaced some of the oval cells. The origin of these small hepatocytes is unknown. After a week of recovery, sinusoids reappear between the renewed hepatocytes, which thereby reimposes the platelike arrangement of these cells found in normal liver. Coincident with the cellular recovery is the restoration of hepatocellular junctions surrounding the bile canaliculi (Figures 9 and 10). Within 2 weeks, the liver resembles normal liver in most respects; however, remnant oval cells, abnormal hepatocytes, and junctional proliferations can still be found (Figures 1E and 11).

The reversability of junctional changes associated with oval cell proliferation during the early stages of the CDE diet is similar to the reversibility of junctional changes associated with liver regeneration.[5] In each case, a reversal of the pattern of cell proliferation causes a rapid increase in the number and complexity of gap junctions. Further study is needed before intercellular junctions can be characterized in CDE-induced tumors.

Our BDL results agree with the previous reports on this condition.[7,8] Two features of ligated liver are similar to liver during CDE treatment. First, ligation induces a proliferation of biliary cells and a concomitant disruption of hepatocyte intercellular junctions. Second, these proliferating cells are epithelial in nature and, like oval cells, establish disorganized tight junctions but no gap junctions. However, there are important distinctions between these two systems. Oval cells that proliferate in the early stages of carcinogenesis synthesize alphafetoprotein and its messenger, RNA, while ductule cells produced by ligation of the common bile duct lack these markers.[22,24] Ductule cells in ligated liver are large cuboidal cells that possess a basement membrane, which may serve as a structural framework allowing for the organized proliferation of ductule cells in clustered lumens. In contrast, oval cells are small and irregularly shaped and lack a defined basement membrane, which may account for their essentially random proliferation.

Similarities in the junctional changes taking place are apparent in recovering CDE liver and regenerating liver. The peak in mitotic activity after removal of two thirds of the rat liver occurs at 28 hours, which coincides with the complete absence of gap junctions and the significant disorganization of tight junctions.[5,6] In the next 12 hours, regenerating hepatocytes form extensive branching bile canaliculi sealed by a fairly continuous belt of tight junctions; only the small gap junctions within their border reappear. By 48 hours, all is restored to normal, including the number and arrangement of gap and tight junctions.[5,6] This pattern resembles the sequence of changes seen in the liver after removal of the CDE diet, but the recovery during regeneration occurs on a faster time scale.

It is possible that in the CDE liver loss of gap junctions and deterioration of tight junctions occurs because the hepatocytes respond to the diet by metabolically and physically uncoupling themselves from each other and from the invading oval cells. But whether these hepa-

tocyte junctions are altered directly by the diet or indirectly by the oval cells remains an open question. Hepatocytes in monolayer culture with rat liver epithelial cells similar to cultured oval cells[28] have been shown to form contacts but not intercellular junctions with each other.[29] This intercellular interaction, however, is responsible for maintenance of differentiated function in cultured hepatocytes.[29]

In this study, we have shown that extensive changes occur in hepatocellular junctions in livers of animals fed the CDE diet. We have also shown that oval cells establish their own form of interaction with hepatocytes and with other oval cells, which involves disorganized tight junctions and excludes gap junctions. Oval cells thus share some junctional characteristics with the biliary cells in normal liver, with the biliarylike ductule cells in ligated liver, with regenerating cells, and with tumorigenic cells, but not with the normal hepatocytes or the recovering hepatocytes. The elucidation of oval cell and hepatocyte membrane interactions will be important in the development of an understanding of cellular transformations that occur in eithionine hepatocarcinogenesis.

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