LOSS AND REAPPEARANCE OF GAP JUNCTIONS IN REGENERATING LIVER

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ABSTRACT

Changes in intercellular junctional morphology associated with rat liver regeneration were examined in a freeze-fracture study. After a two-thirds partial hepatectomy, both gap junctions and zonulae occludentes were drastically altered. Between 0 and 20 h after partial hepatectomy, the junctions appeared virtually unchanged. 28 h after partial hepatectomy, however, the large gap junctions usually located close to the bile canaliculi and the small gap junctions enmeshed within the strands of the zonulae occludentes completely disappeared. Although the zonulae occludentes bordering the bile canaliculi apparently remained intact, numerous strands could now be found oriented perpendicular to the canaliculi. In some instances, the membrane outside the canaliculi was extensively filled with isolated junctional strands, often forming very complex configurations. About 40 h after partial hepatectomy, very many small gap junctions reappeared in close association with the zonulae occludentes. Subsequently, gap junctions increased in size and decreased in number until about 48 h after partial hepatectomy when gap junctions were indistinguishable in size and number from those of control animals. The zonulae occludentes were again predominantly located around the canalicular margins. These studies provide further evidence for the growth of gap junctions by the accretion of particles and of small gap junctions to form large maculae.

KEY WORDS gap junctions · regenerating liver · morphometry · freeze-fracture

Gap junctions are specialized regions of intercellular contact believed to be the sites of passage of inorganic ions between cells (20, 27) and also to participate in the exchange between cells of small molecules, some of which may be important in regulating cellular activities (4, 6, 11, 21, 26). We have examined changes in the number of gap junctions found in a rapidly growing system, the regenerating liver of weanling rats after partial hepatectomy (34). The rapid, clear-cut and reproducible disappearance and reformation of gap junctions we report suggest that the regenerating liver might be an excellent model for the study of gap junction formation. (A preliminary report of this material was presented at the 12th Annual Meeting of the American Society for Cell Biology [33]).

MATERIALS AND METHODS

Experimental Animals

Male, Charles River Breeding Laboratory rats, 3–4 wk old were anesthetized with ether, and a two-thirds

partial hepatectomy was performed according to the method first described by Higgins and Anderson (15). The median and left lateral lobes were ligated close to the hilus before excision. The right lateral lobes and the caudate lobes were minimally disturbed. The animals were sutured and returned to their cages, with no more than three animals occupying each cage. They were given free access to food and water. Animals were sacrificed at 2-h intervals from 8 to 24 h after the operation, at 1-h intervals between 25 and 30 h and between 35 and 40 h. From 40 to 48 h, the animals were sacrificed at 3-h intervals. 15 animals were included in the interval from 28 to 38 h.

Sham-Operated Control Animals

Control, male young weanling rats were ether anesthetized and laparotomized as if a hepatectomy were to be performed. The livers were exposed outside the body walls, manipulated with a Q-tip cotton swab moistened with saline, and then returned to the abdominal cavity. The sham-operated animals were sacrificed within 1 h and between 28 and 30 h after manipulation of the livers.

Unoperated Control Animals

Livers from normal, unoperated male young weanling rats were also included and examined.

Freeze-Fracturing

Small wedges of right lateral lobes were removed, diced into small 1-mm cubes and then placed directly into Karnovsky's paraformaldehyde-glutaraldehyde fixative (17) for 1–2 h at room temperature. The tissues were then equilibrated in 25% glycerol in 0.1 M cacodylate buffer, pH 7.4, for a minimum of 2 h before freezing in liquid Freon held at its freezing point in liquid nitrogen. The tissues were fractured and shadowed in a standard Balzers BAF freeze-etch apparatus (Balzers High Vacuum Corp., Santa Ana, Calif.). The samples were routinely defatted overnight in dimethylformamide (29). The replicas were freed from tissue by digestion in Clorox bleach for 1 h, carefully rinsed in distilled water, placed on uncoated 200-mesh grids, and examined in a Siemens Ia electron microscope operated at 80 kV.

Morphometry

The ratio of junctional to nonjunctional fractured membrane surfaces was obtained by morphometric analysis (30) of our micrographs. A 659 random point test grid was placed over electron micrographs printed at approximately the same magnification and the number of dots falling over junctional and nonjunctional membrane was counted.

Only membrane surfaces that participate in junctional interactions were included in our analysis. The membranes lining the bile canaliculi and the perisinusoidal spaces were excluded from our measurements. The presence of numerous microvilli was considered a characteristic feature of the space of Disse and of the small transitional zone between it and that portion of the hepatocyte membrane which faces other liver cells. We included in our measurements only those membrane surfaces that had few microvilli and were clearly outside the region bordering the space of Disse. With these criteria, the proportion of the membrane in the control animal occupied by gap junctions is 2.9%. The contribution of the bile canaliculi to the total hepatocyte membrane is 13% and that of the space of Disse is 37–40% (14, 31), so that our measurements were made on about one-half of the total actual membrane surface. Gap junctions therefore occupy about 1.5% of the total hepatocyte membrane in normal rats, a figure in close agreement with Bolender's results for the mouse liver.[1]

RESULTS

Control Animals: Unoperated and Sham-Operated

The junctional complex between hepatocytes in the normal and sham-operated rat liver closely resembles those found in freeze-fractured preparations of mouse liver (12, 19) (Fig. 1), the most prominent junctions being zonulae occludentes and gap junctions. The zonula occludens has a typical belt like structure composed of anastomosing ridges on the P-fracture face (the juxtacytoplasmic leaflet of the fractured cell membrane) and a network of grooves on the corresponding E-fracture face (extracellular leaflet of the fractured cell membrane). The zonula occludens forms the boundary of the bile canaliculus and extends along the entire length of each canaliculus; in effect, it isolates the lumen of the bile canaliculus from the intercellular spaces and the space of Disse.

Gap junctions occupy 1.5% of the total hepatocyte membrane (see previous section on Morphometry). They are characterized by a closely packed polygonal array of particles some 6 nm in diameter on the P-fracture face and an array of pits with similar spacing on the corresponding E-fracture face. Small gap junctions (some 0.1 μm in diameter) are often found associated with, and usually completely encircled by, strands of the zonula occludens; numerous large gap junctions, 1–2 μm in diameter, are often, but not always, located close to the zonula occludens near the bile canaliculus margins.

[1] R. Bolender, personal communication.

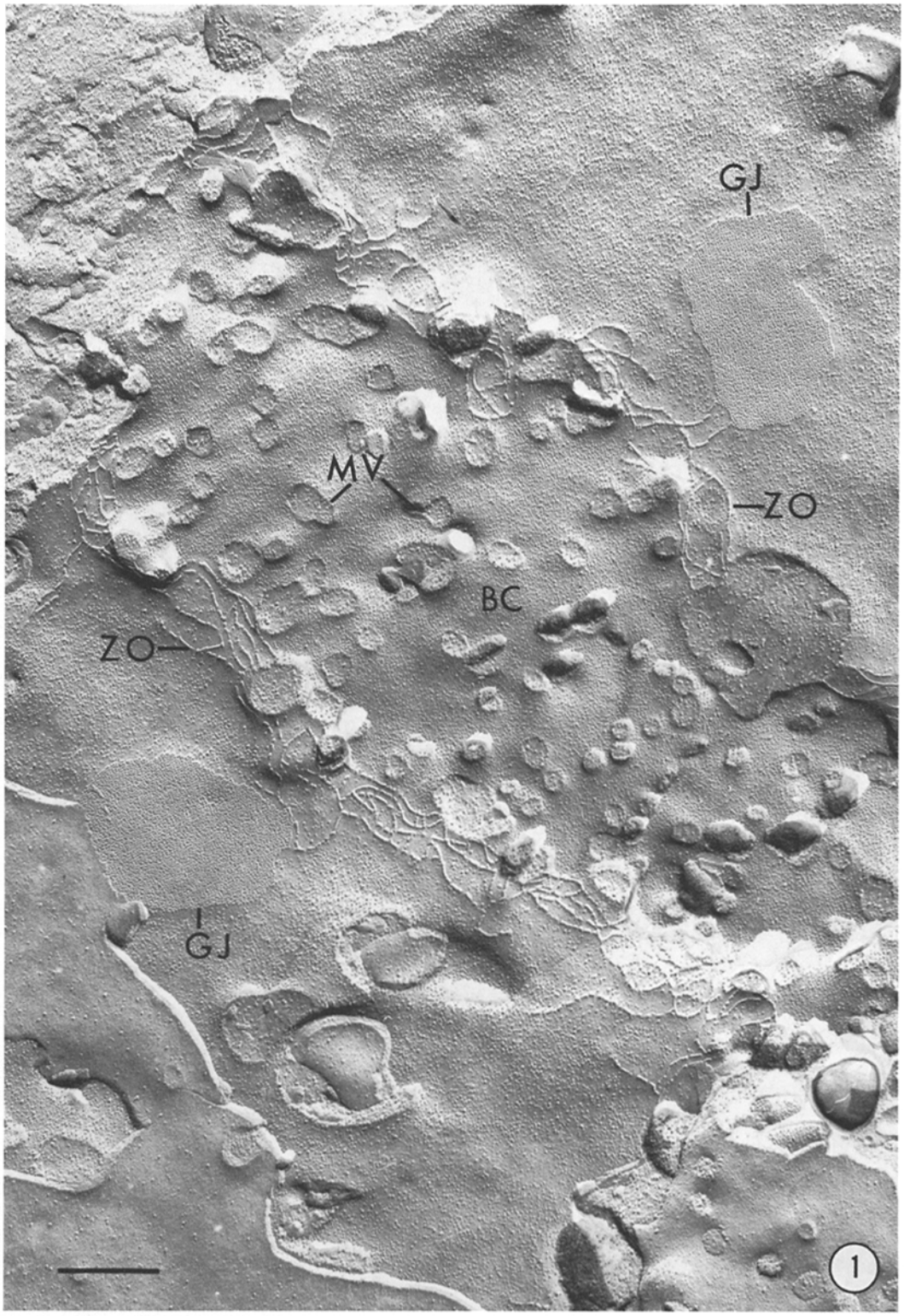

FIGURE 1 Freeze-cleaved replica of the junctional region between hepatocytes from a control rat liver. The bile canaliculus (*BC*) with many cross-fractured microvilli (*MV*) is separated from noncanalicular hepatocyte membrane by the zonula occludens (*ZO*) which extends along the entire length of the canaliculus. Large gap junctions (*GJ*) are located close to the zonula occludens. Bar, 0.25 μm. × 64,700.

Experimental Animals

At times between 0 and 20 h after partial hepatectomy, there are no detectable changes in the structure of the gap junctions found between hepatocytes; the gap junctional maculae are as large as those of the control animals and about as numerous. Small gap junctions are still found within the anastomosing arrays of the zonula occludens. No changes are detected in the appearance of the zonula occludens.

Dramatic changes in the junctional complement occur about 28 h after partial hepatectomy and persist for the next 10–12 h. One of these is a rapid decrease in the number of gap junctions, so that the extensive membrane fracture faces become completely depleted (Fig. 2). Lacking are both the large gap junctions closely associated with the region adjacent to the bile canaliculus and the small gap junctions normally enmeshed within the strands of the zonula occludens. The loss of gap junctions appears to be generalized rather than confined to a specific region of the liver lobule. In the very rare instances when a gap junction could be found during this time period, it was very small, consisting of only a few particles. We could not ascertain the mechanism(s) by which the junctions disappeared but could find no evidence for internalization (3, 5) of gap junctions.

Not only is the gap junction affected 28 h after partial hepatectomy but also there are changes in the organization of the zonula occludens. Although the continuity of the tight junction along the bile canaliculus is maintained, there are many strands of the zonula occludens found away from their usual pericanalicular location. The strands of tight junction farthest from the lumen of the bile canaliculus, instead of remaining generally parallel to the path of the canaliculus, are often seen oriented perpendicular to it. Most striking are the strands of occluding junction found coursing across nonpericanalicular membranes, either single or in an irregular network which occasionally assumes complex configurations occupying large areas of the fractured membrane surface (Fig. 3). Single strands on the P-fracture face appear to be co-linear with grooves on the E-fracture face of the adjacent cell, suggesting that these structures still represent contacts between adjacent cells.

By 40 h after partial hepatectomy (Fig. 4), most of the tight junctional elements found are those of the zonula occludens in its usual pericanalicular location; only a few strands of contact, separate from the main zonular network, remain. Large gap junctions still cannot be found, but examination of the bile canalicular region at high magnification (Fig. 5) reveals the presence of many small gap junctions, so numerous, in fact, that they frequently stud the entire pericanalicular region. The smallest of these consists often of only five to six particles and can be recognized as a gap junction by the presence of the particle-free halo region (23) which offsets the gap junctional particle aggregates from the surrounding nonjunctional membrane particles. Although more difficult to detect, the small gap junctions were also observed on the corresponding E-fracture face; they are characterized as a cluster of some five to six pits or depressions in polygonal array (Fig. 5, *inset*). The presence of both P and E junctional membrane fracture faces suggests that the very small particle aggregates were, in fact, located in regions of intercellular contact. "Formation plaques" described as an early feature of developing gap junctions (2, 7, 8, 16) were not clearly observed in the material described in this communication. Structures resembling formation plaques have been seen, however, in regenerating liver by Yancey and Revel (32).

About 44 h after partial hepatectomy (Fig. 6), the gap junctions are larger and more easily identified. By 44–46 h of regeneration, the total number of gap junctions is decreased compared to the earlier time, but the size of individual gap junctional maculae is significantly increased. The hepatocytes have regained their full complement of gap junctions 46–48 h after partial hepatectomy. At that time, the junctions are indistinguishable in both the size and number from those of control animals (Fig. 7).

The changes in the gap junction complement we have just described are clearly seen by morphometric analysis of the micrographs. The amount of cell surface devoted to gap junctions was estimated by measuring the ratio of junctional to nonjunctional fractured membrane surfaces (as described in Materials and Methods) and plotted vs. time in Fig. 8. From a control value of about 2.9% of the membrane surface (excluding space of Disse and bile canaliculi) occupied by gap junctions, the ratio sharply decreases to virtually zero at 28–30 h after partial hepatectomy and remains there until about 36–38 h. By 44–46 h after partial hepatectomy, the percent of the cell surface with gap junctions increases and becomes almost equal to control levels.

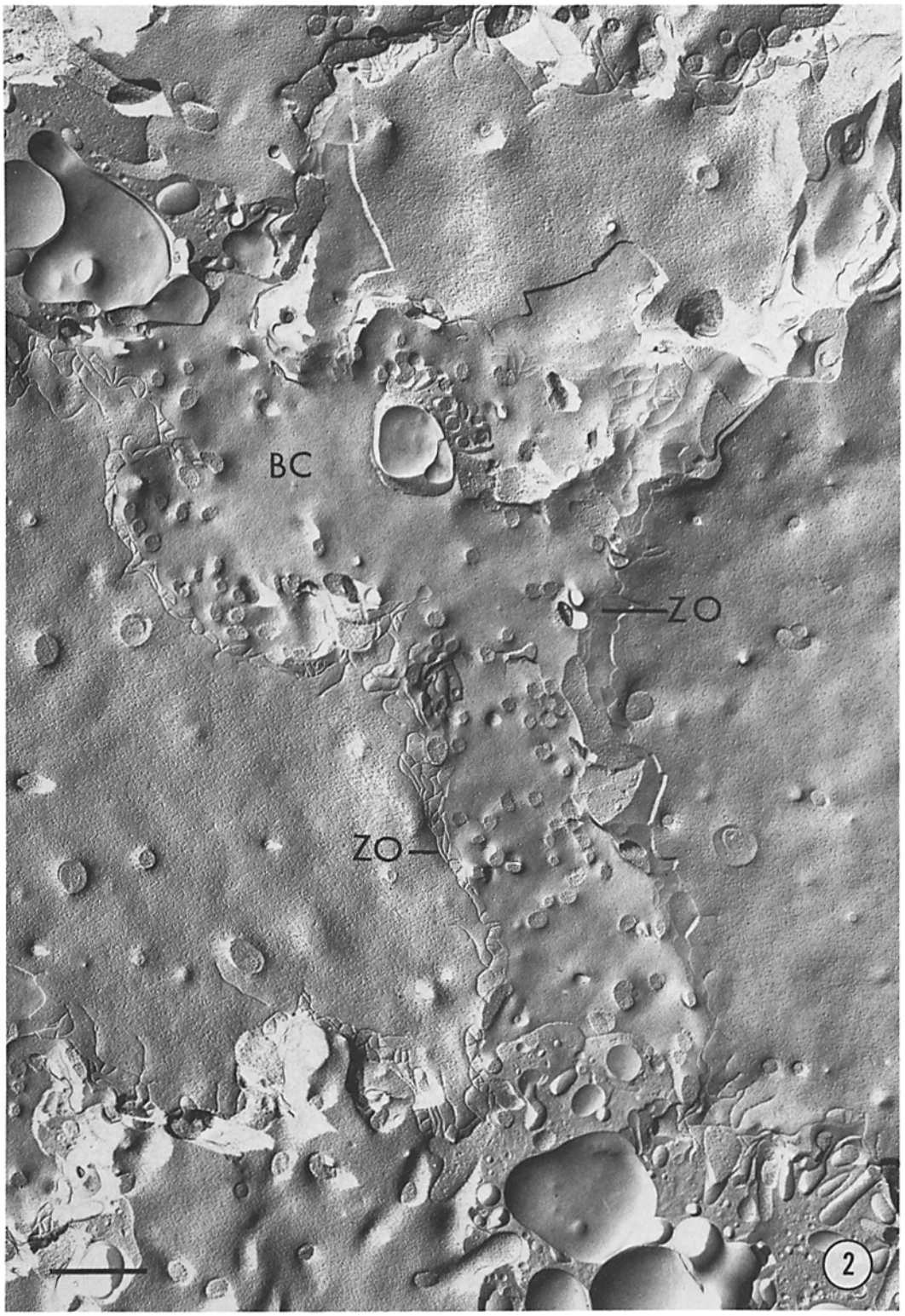

FIGURE 2 Low-magnification view of a freeze-cleaved replica, 28 h after partial hepatectomy showing extensive membrane fracture surfaces. The large gap junctions usually found closely associated with the region adjacent to the bile canaliculus (*BC*) as well as the small gap junctions ordinarily present between the meshes of the zonula occludens (*ZO*) are both missing. Bar, 0.5 μm. × 30,000.

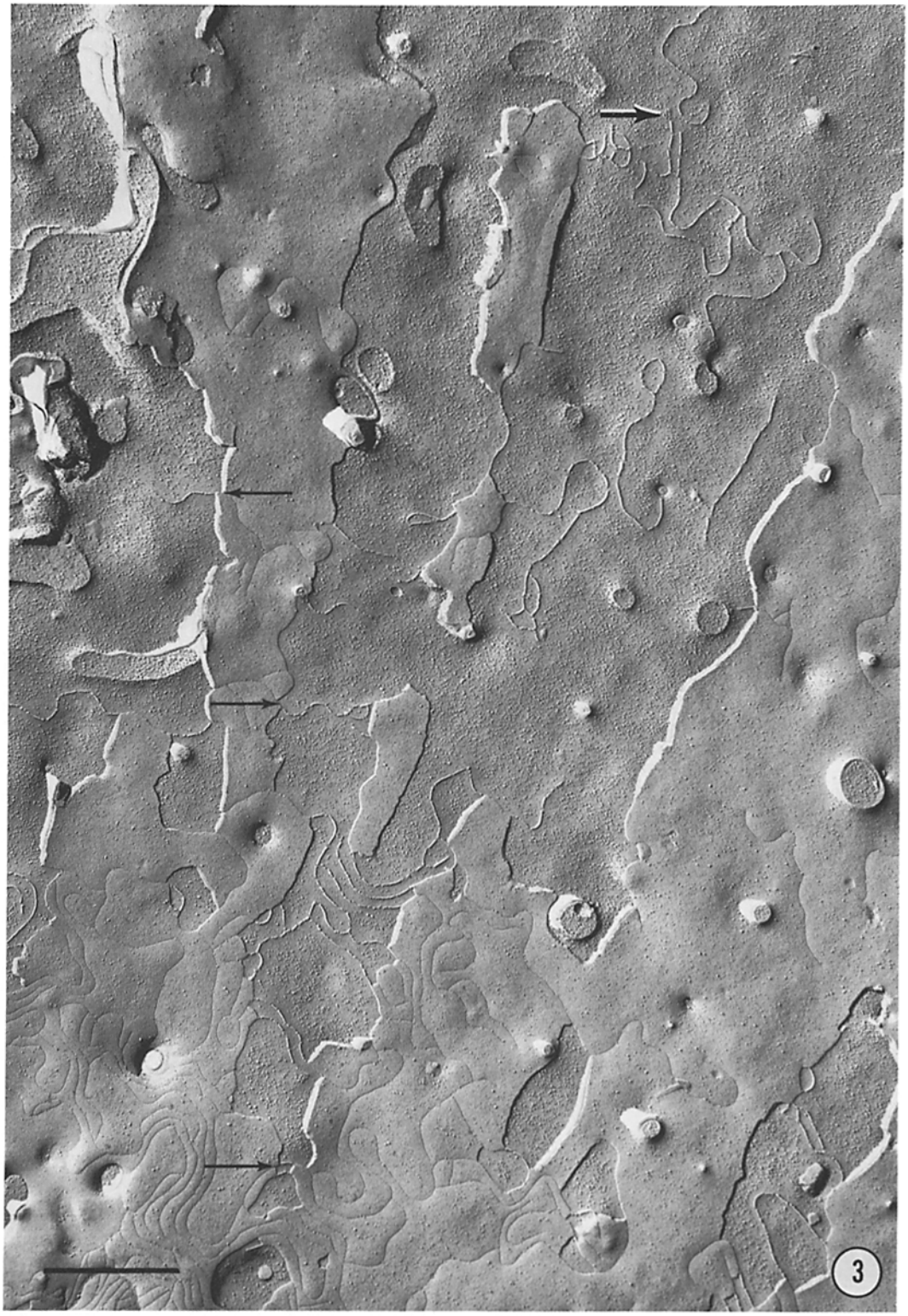

FIGURE 3 Freeze-cleave replica of rat liver 28 h after partial hepatectomy. Numerous strands of the zonula occludens are seen away from the pericanalicular membrane. Some strands are discontinuous and completely isolated from other strands. In some instances, the zonula occludens takes on a very complex appearance and forms a complicated configuration as seen in the lower left half of the figure. The small arrows indicate regions in which the ridges of the P-fracture face appear to be co-linear with the grooves on the E-fracture face. Small particle aggregates can also, on occasion, be seen at the ends of the ridges of the zonula occludens (large arrow). Bar, 0.5 μm. × 43,000.

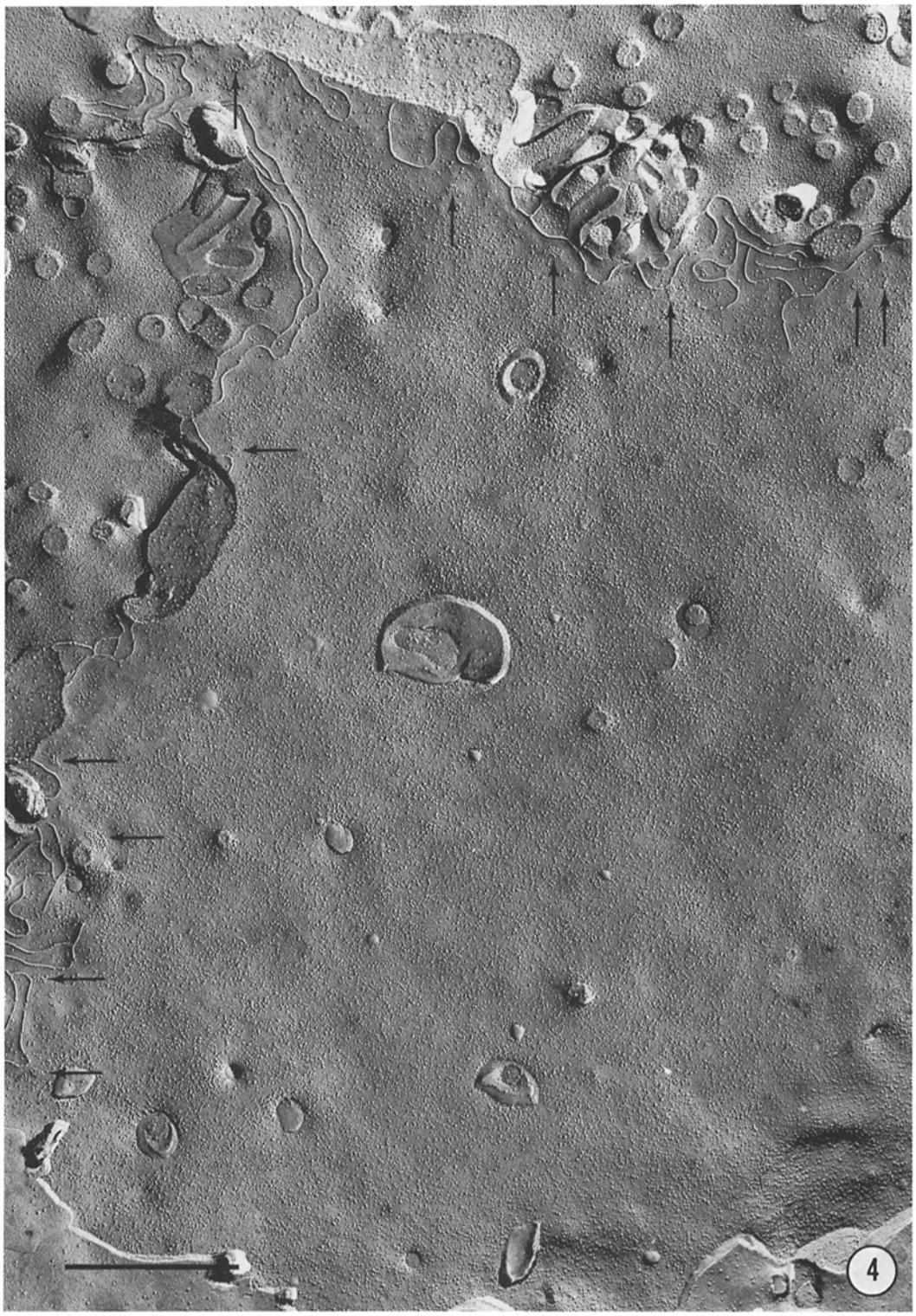

FIGURE 4 Low-magnification view of a rat liver 40 h after partial hepatectomy. Large gap junctions are still absent. Numerous small gap junctions consisting of only a few particles begin to appear closely associated with the zonula occludens (arrows). Bar, 0.5 μm. × 53,000.

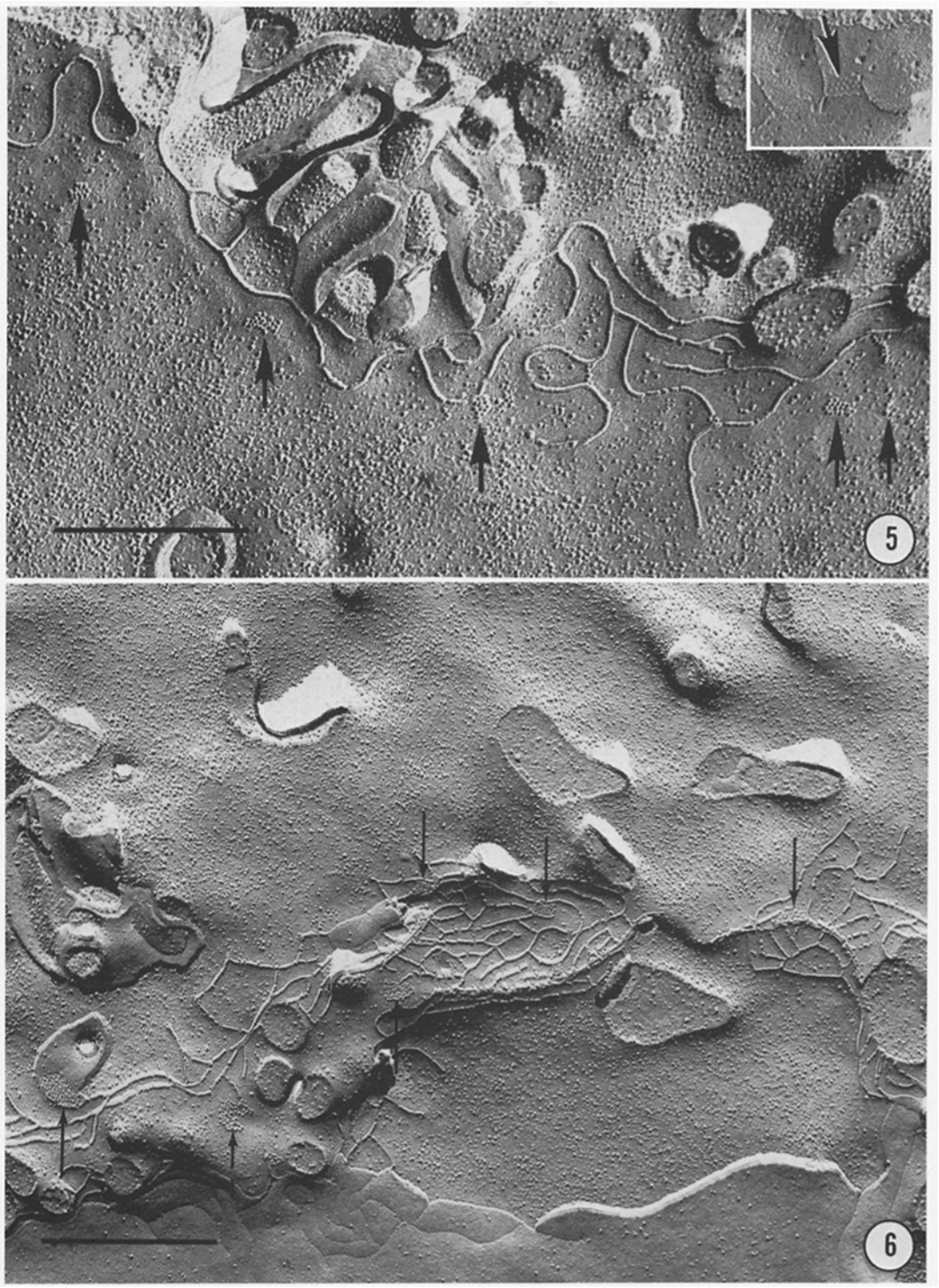

FIGURE 5 Higher-magnification view of the upper portion of Fig. 4 showing numerous gap junctions in the pericanalicular region. Bar, 0.25 μm. × 117,600. *Inset* shows an array of pits from the E-fracture face of one such small junction. × 176,000.

FIGURE 6 Freeze-fracture replica of rat liver 44 h after partial hepatectomy. Gap junctions are still very numerous but larger than those seen in the previous time period (arrows). Bar, 0.5 μm. × 53,600.

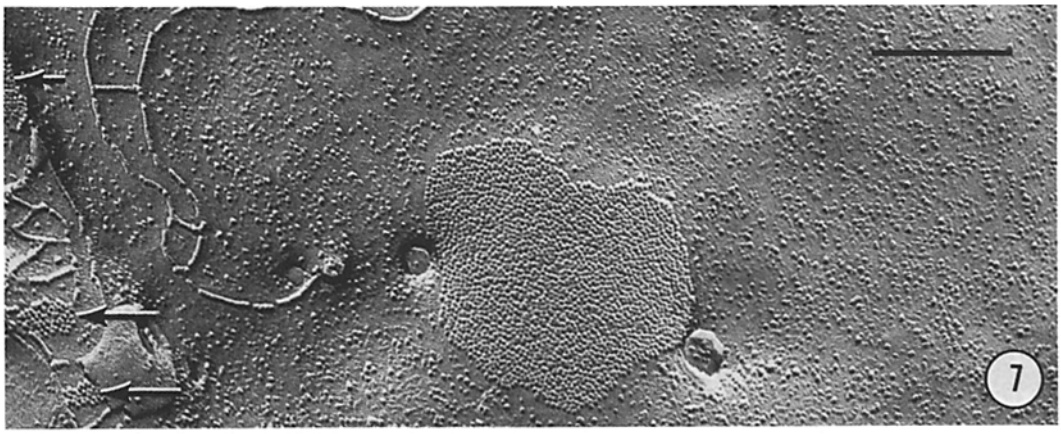

FIGURE 7 Freeze-fracture replica of rat liver 46 h after partial hepatectomy. Gap junctions are now very large and indistinguishable in size and number from those of control animals. In addition, small gap junctions are again found enmeshed within the strands of the zonula occludens (arrows). Bar, 0.25 μm. × 74,000.

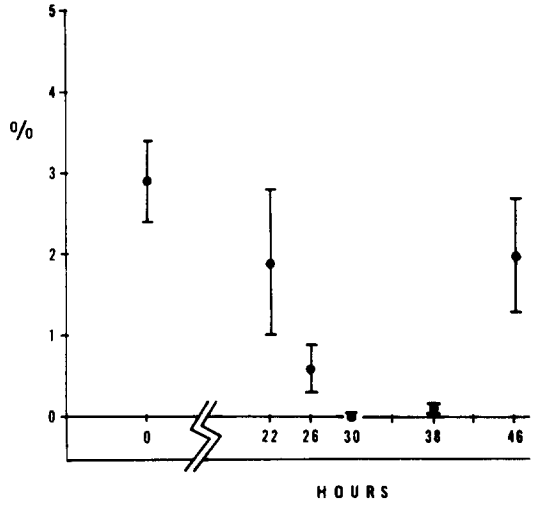

FIGURE 8 A graphic representation of a morphometric analysis of the percentage of cell surface occupied by gap junctions after partial hepatectomy. The abscissa indicates the time after partial hepatectomy and the ordinate represents the percentage of cell surface occupied by gap junctions.

DISCUSSION

We have observed a drastic reduction in the number of gap junctions between hepatocytes in weanling rats starting about 28 h after partial hepatectomy. The loss is temporary; the hepatocytes regain their full complement of gap junctions within 2 days after the operation. Our observations are consistent with previous electrophysiological studies on regenerating liver by Loewenstein and Penn (22), who found that electrical coupling was unimpaired 20 and 48 h after hepatectomy, at times when we find that gap junctions are actually present. It would be of interest to extend the electrophysiological observations to include intermediate stages of regeneration, including the period during which we find that there is a loss of junctions.

The disappearance of gap junctions is temporally correlated with a mitotic peak (18). In hepatectomized weanling rats, the number of mitoses is maximum at about 28 h. This correlation is tantalizing but may be only coincidental. There is evidence from other systems that gap junctions do not disappear during mitosis. Merk and McNutt (24) have shown that dividing granulosa cells retain their gap junctions, and O'Lague et al. (25) have electrophysiological evidence for cell-cell coupling between fibroblasts during mitosis. It is probable that the disappearance of the junctions is related to some of the other changes occurring after hepatectomy.

The disappearance of gap junctions has been postulated to take place either by an internalization of junctions (2, 3, 5, 10) or by a dispersion of junctional particles (10, 13). No evidence for internalization could be found in our material, nor was it possible to follow the dispersion of gap junction particles throughout the membrane, possibly because dispersed junctional particles cannot be distinguished from other intramembrane particles. It is clear, however, that there are specific

mechanisms for removing the gap junction components without drastically affecting the tight junction. Even those gap junctions that are associated with the meshwork of the zonula occludens disappear. This might speak for internalization of the membrane as a mechanism for gap junction removal, since the strands of the zonula occludens would presumably hamper dispersion. Alternatively, one can postulate that the partial disorganization of the zonula occludens which we observe is sufficient to release "entrapped" gap junctions which would then be lost by the same mechanism as the other gap junctions.

In many of the other examples of forming junctions, the assembly process seems to take place in a specialized region of the membrane (2, 7, 8, 16). These areas are characterized by a smooth area of the membrane and contain rather large-appearing intermembrane particles. Such formation plaques are not very prominent but have been observed in regenerating liver (32). The growth of gap junctions by the accretion of particles or by fusion of small gap junctions to form larger ones has been inferred from studies of developing junctions and may be a rather general mechanism. This suggests that lateral association between gap junction particles may play an important role in establishing junctional morphology. Such an accretion mechanism could, in theory, be controlled by cytoplasmic filaments (16), although there is no direct evidence for this at the present time. One could equally well postulate that the specificity for lateral association may reside at the level of the intramembrane particles themselves.

Systems in which one can cause a certain loss and then reappearance of gap junctions at will are likely to be useful in the study of the turnover and the function of these ubiquitous cell junctions (1, 9, 10, 28, 34). In regenerating rat liver, there is a relatively well-synchronized cycle of junctional disappearance and reformation which allows correlative biochemical and morphological studies (32). We are presently trying to identify the membrane protein components which change during the disappearance and reformation of gap junctions.

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