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The Perfused Human Liver Wedge Biopsy: A New *In Vitro* Model for Morphological and Functional Studies

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We developed a simple and inexpensive method of perfusing small specimens of human liver *in vitro* that maintains short-term tissue viability as judged by protein transport function and morphological features. The technique allows investigation of liver function at the cellular level in normal specimens and those with hepatobiliary disease. Electron microscopy of specimens perfused with this system demonstrates the presence of an incomplete basement membrane within the space of Disse and shows that human microbodies contain crystalline-like cores morphologically similar to those found in rat liver.

The lack of an appropriate *in vitro* system for experimentation has markedly hampered our understanding of human liver physiology. The present study was undertaken to develop a procedure for perfusing human liver wedge biopsies which would maintain complete morphological integrity and allow investigation of liver function at the cellular level, in both normal and various pathologic states. The described perfusion method was developed from a procedure used to fix human liver for scanning electron microscopy (1). Preliminary experiments presented here and elsewhere (Burwen et al., unpublished observations) indicate that this *in vitro* system is particularly useful for the study of uptake and disposition of plasma-derived proteins within human hepatocytes.

MATERIALS AND METHODS

After obtaining informed consent from patients (ages 31 to 78), human liver wedge biopsies were obtained at the time of surgical laparotomy. Upon excision of a 1- to 3-gm segment, the biopsy was immediately immersed in cold heparinized Krebs-Ringer bicarbonate buffer. The total elapsed time from excision of the biopsy to onset of perfusion did not exceed 2 min. Nine of these biopsies, judged normal by histologic examination by light microscopy, were used in this study.

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The perfusion system consists of a syringe containing the perfusate, an infusion pump for delivering the perfusate at a given flow rate (3.8 ml per min), a hyperbaric chamber for oxygenation of the perfusate (2), and a heated chamber for maintaining the tissue at a desired temperature (Figure 1). The hyperbaric chamber contains coils of silastic tubing for gas exchange, a gas flow meter, and a pressure gauge and valve to maintain oxygen pressure within the chamber at 50 mm Hg. An 18 gauge needle connected to the chamber via polyethylene tubing is inserted perpendicularly through Glisson's capsule into the biopsy specimen. The insertion of the needle through the capsule is an important step since this layer of connective tissue supports the needle and prevents movement-induced trauma of the tissue.

The tissue is initially perfused with heparinized buffer solution at 37°C to flush blood from the sinusoids. The marked blanching of the perfused relative to the unperfused or poorly perfused areas is used to identify and later remove unperfused tissue. Elimination of unperfused tissue is required to obtain valid data on cellular function.

After the sinusoids are flushed, the tissue is perfused with culture medium 199OR for various periods. Substances whose uptake is to be studied [e.g., epidermal growth factor (EGF)] may be added to the perfusate either continuously or as a bolus. The effluent is collected as it leaves the cut surface of the specimen and can be recirculated or removed (the latter is equivalent to a single-pass perfusion system).

Biopsies to be fixed for either light or electron micros-

copy are perfused with glutaraldehyde (2.5%)-paraformaldehyde (0.7%) in freshly prepared bicarbonate buffer (pH 7.4) for 5 min. The same perfusion apparatus is used after first disconnecting the oxygenator. Any tubing exposed to fixative is discarded to avoid accidently exposing fresh specimens to traces of aldehydes. The tissue is then cut into small pieces and immersion fixed for an additional 1 to 2 hrs. Needle-damaged areas and nonperfused areas are discarded. The tissue is postfixed in 1% osmium tetroxide containing 1.5% KCN, dehydrated in alcohol, and embedded in Epon 812 or LX-112 [embedding medium (a replacement for Epon 812), Ladd Research Industries, Inc., Burlington, Vt.].

Thick sections (0.5 μm) for light microscopic autoradiography were coated with Kodak NTB-3 emulsion, exposed for 2 to 3 weeks, developed in Kodak D-19 developer, stained with toluidine blue, and examined in a Zeiss photomicroscope.

For the transmission electron microscopy studies, thin sections of approximately 800 Å were cut on a Sorvall MT-2 microtome, stained with uranyl acetate and lead citrate, and examined in a Phillips 300 electron microscope operated at 60 KV. For electron microscopic autoradiography, thin sections were placed on parlodion-coated grids, overlayed with Ilford L-4 emulsion, exposed for 3 to 7 weeks, developed in Kodak D-19 developer, and stained and examined as above.

For scanning electron microscopy, pieces of fixed tissue were frozen in liquid nitrogen and shattered before being dehydrated in alcohol. The resulting pieces were critical point dried in carbon dioxide in a Bomar Critical Point dryer, mounted on specimen stubs, coated with gold, and examined in a S-4 Cambridge scanning electron microscope equipped with a lanthanum hexaboride filament.

EGF was a gift from Dr. R. J. St. Hilaire and was labeled with ^{125}I by a modified chloramine T method (3).

RESULTS AND DISCUSSION

Wedge biopsies perfused with culture medium for up to 30 min after excision demonstrated a remarkable degree of preservation (Figure 2). In well-perfused areas, few, if any, blood cell elements were present. There was no evidence of gross vesiculation, endothelial cell sloughing, or hepatocyte swelling, findings frequently observed following hypoxia (4). Liver biopsies taken from patients over age 60 showed numerous lipofuscin granules and autophagic vacuoles (Figure 2).

SINUSOIDAL LINING

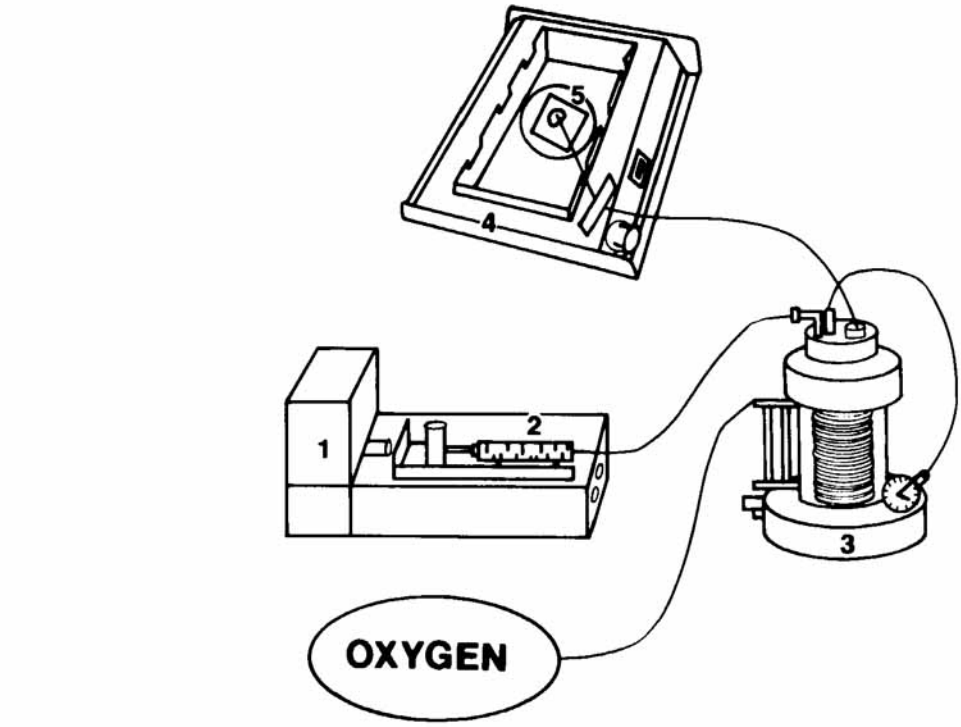
The sinusoidal lining of the human liver appeared remarkably similar to that of the rat by both transmission and scanning microscopy (Figures 3 to 5). Kupffer cells with long extended filipodia and flattened endothelial cells with fenestrae were readily observed (Figure 4). Hepatocyte microvilli protruded through the fenestrae into the sinusoidal space, a finding commonly observed in rat liver (Figures 3 and 4). Although we have not done quantitative analysis of the occurrence of the fenestrae, from qualitative observation, they appear to be much fewer than in rodents, with little evidence of distinct sieve plates (5). Fat-laden cells (6) were well preserved, although not a single pit cell (7) was found in any specimen. Of particular interest was the presence of fragments of basement membrane-like material within the space of Disse (Figure 3). These fine thread-like, discontinuous, electron dense structures appeared to be directly related to the endothelial cells, but were never observed limiting endothelial fenestrae. Similar material suggestive of basement membranes has been reported by others in normal livers (6) and in certain disease states (8). Despite the immunohistochemical demonstration of Type 4 collagen in the space of Disse (Roll et al., unpublished observations) (9), basement membranes within this space have not been reported in any normal animal with the exception of ruminants (10). Although our methods yield excellent morphology, perhaps, with further refinement, the full extent of the basement membrane within the space of Disse will be discernable.

ORGANELLES

Endoplasmic reticulum within normal human liver hepatocytes was particularly well preserved in all of the specimens although its distribution appeared to vary somewhat from cell to cell (Figures 3, 6, and 7). Rough-surfaced endoplasmic reticulum appeared as aggregates of ribosome-studded, flattened cisternae which were arranged in parallel lamellae scattered randomly throughout the cytoplasm (Figure 7). As in other mammals, the cisternae of rough-surfaced endoplasmic reticulum communicated with the smooth-surfaced endoplasmic reticulum (Figure 7). The communicating smooth-surfaced endoplasmic reticulum comprised a typical complex meshwork of branching and anastomosing tubules which, on occasion, could be seen in communication with the Golgi membranes. Neither rough-surfaced endoplasmic reticulum nor smooth-surfaced endoplasmic reticulum was found to have any connection with the plasma membrane. Typically, the smooth-surfaced endoplasmic reticulum was associated with deposits of glycogen (11, 12).

Golgi membranes are relatively numerous in hepatocytes of all mammals studied including man. Claude has estimated that each cell may contain as many as 50 such complexes (13), although it is still unclear whether they are actually a continuation of a single large complex. In the human, the Golgi complex is comprised of three to five closely packed parallel smooth surface cisternae and a variable number of associated vesicles (Figures 6 and 8). The small vesicles, some of which appear coated, were interpreted to be primary lysosomes, although we realize that this interpretation is not certain until the appropriate cytochemical reactions are undertaken. The large bulbous ends of the Golgi cisternae, as well as some of the associated larger vesicles, were often filled with electron-dense particles of about 250 to 800 Å in diameter (Figure 6). Based on studies of rat liver, these structures may be assumed to represent the triglyceride-rich nascent, very low-density lipoproteins that participate in the transport of newly synthesized lipid from the liver to the plasma (14–16).

As in the rat, the normal human liver contains an entire family of structures, thought to be lysosomes, that participate in the indirect or lysosomal pathway for pro-



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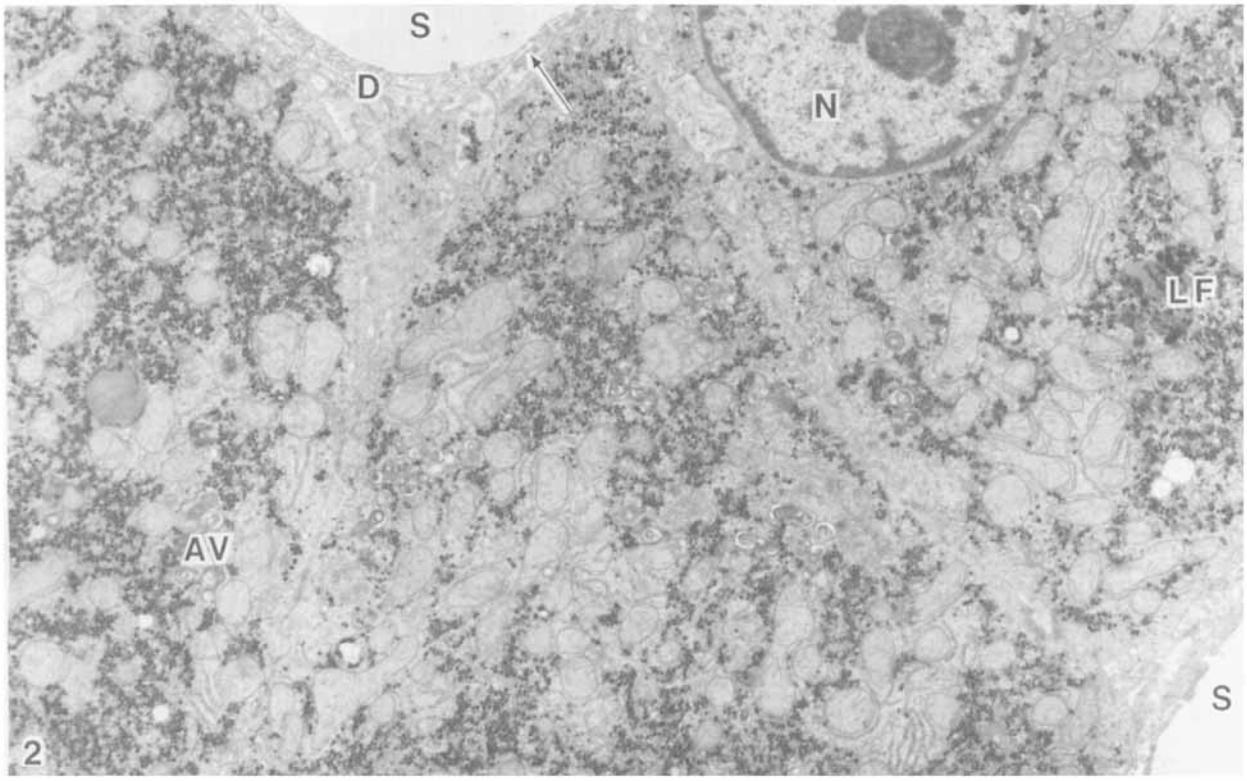
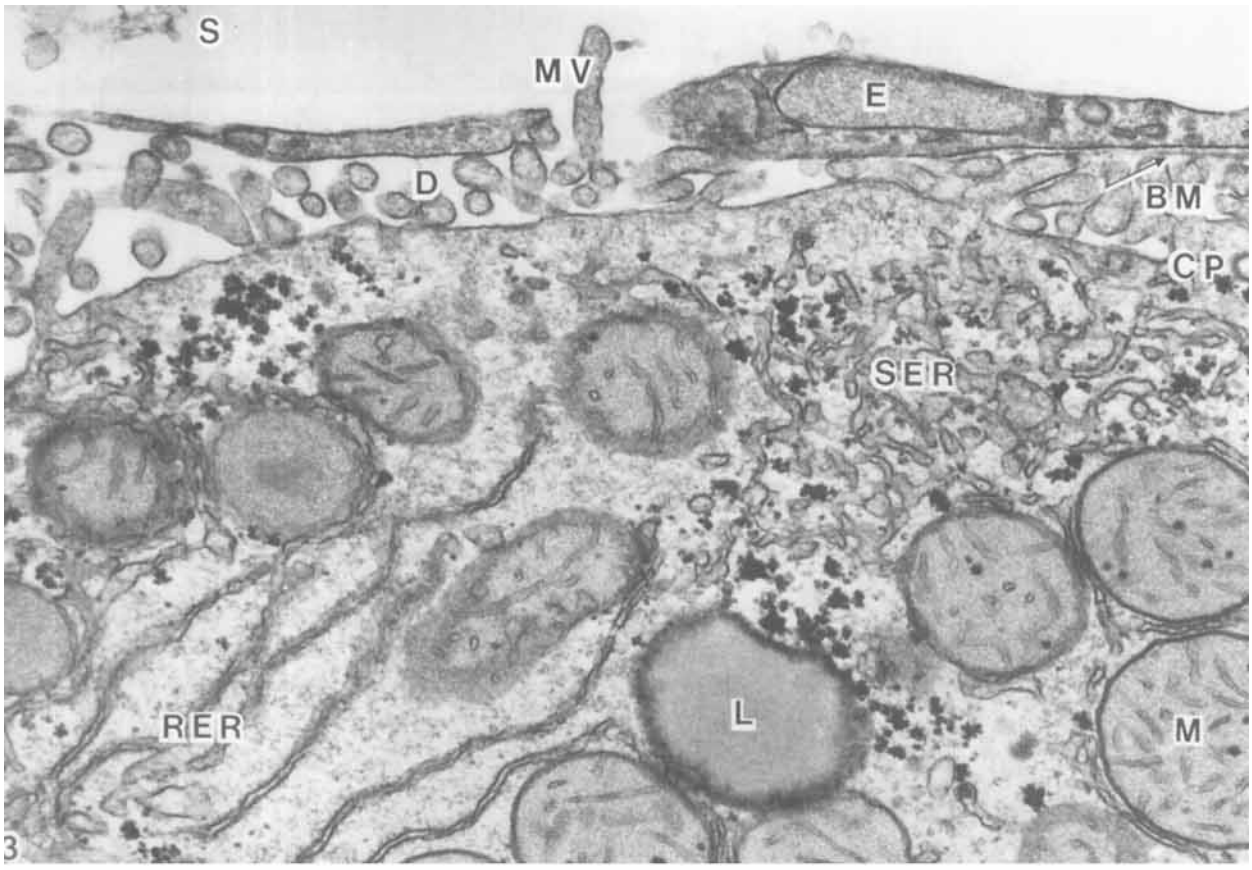
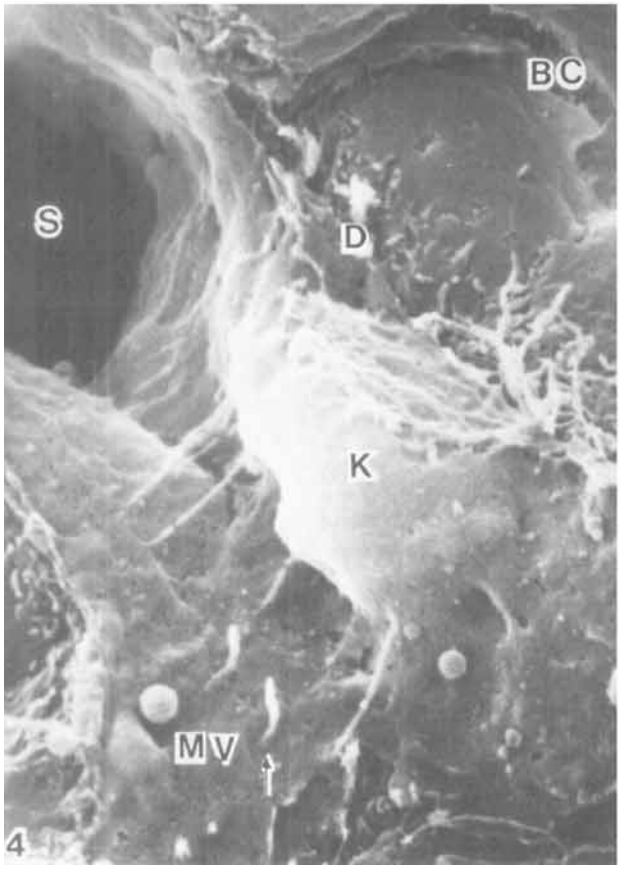


FIG. 1. Diagrammatic representation of the components used in perfusion of isolated human wedge biopsies. (1) Harvard infusion pump calibrated to deliver 3.8 ml per min of perfusate to the biopsy specimen. (2) Syringe containing perfusate. This may be wrapped with temperature-controlled heating coils. (3) Hyperbaric oxygenator (lung). Chamber pressure is maintained at 50 mm Hg and oxygen flow rate is 4 liters per min. (4) Dry heating bath. (5) Wedge biopsy. The specimen rests on a gauze sponge within the Petri dish.

FIG. 2. Portions of three adjacent hepatocytes from a normal human liver wedge biopsy 30 min after surgical excision and perfusion with medium. Sinusoid (S); space of Disse (D); nucleus (N); endothelial fenestrae (*arrow*); lipofuscin (LF); autophagic vacuoles (AV); ~7000×.





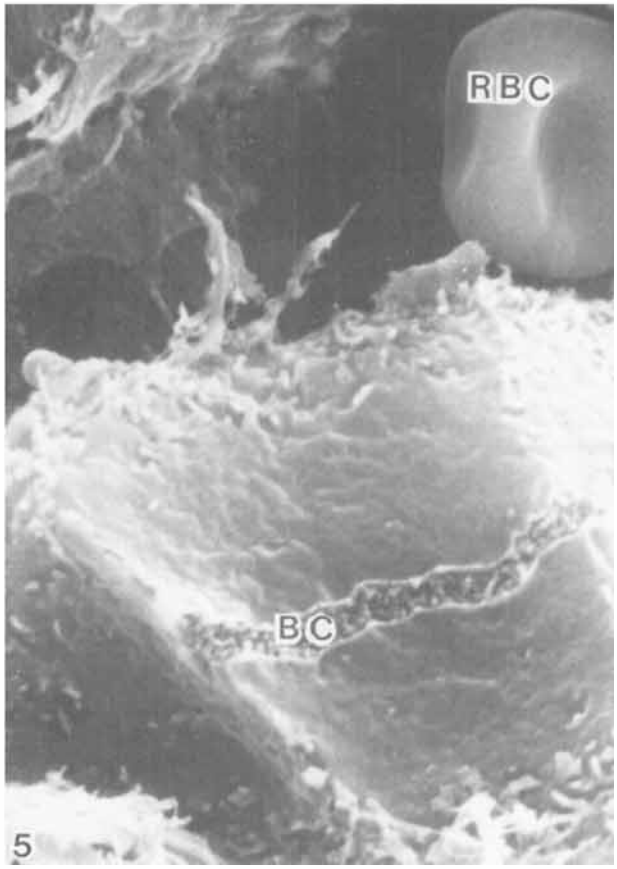


FIG. 3. Thirty minutes postexcision: this micrograph shows the sinusoidal surface of a hepatocyte and the discontinuous endothelial lining (E). A hepatocyte microvillus (MV) protrudes through an endothelial fenestra. Note the small span of basement membrane (*arrow*) just beneath the endothelial cells. Sinusoid (S); space of Disse (D); cytoplasmic lipid droplet (L); mitochondria (M); rough-surfaced endoplasmic reticulum (RER); smooth-surfaced endoplasmic reticulum (SER); coated pit (CP) ~40,000×.

FIGS. 4 and 5. Scanning electron micrographs of fractured surfaces of human wedge biopsies 30 min postexcision. In Fig. 4, a Kupffer cell (K) with filopodia is observed bulging into the sinusoidal space (S). Note the endothelial fenestrae, some of which contain protruding hepatocyte microvilli (*arrow*). Centrally located bile canaliculi (BC) are readily observed in Figs. 4 and 5. Red blood cell (RBC). Fig. 4, ~8,000×; Fig. 5, ~12,000×.

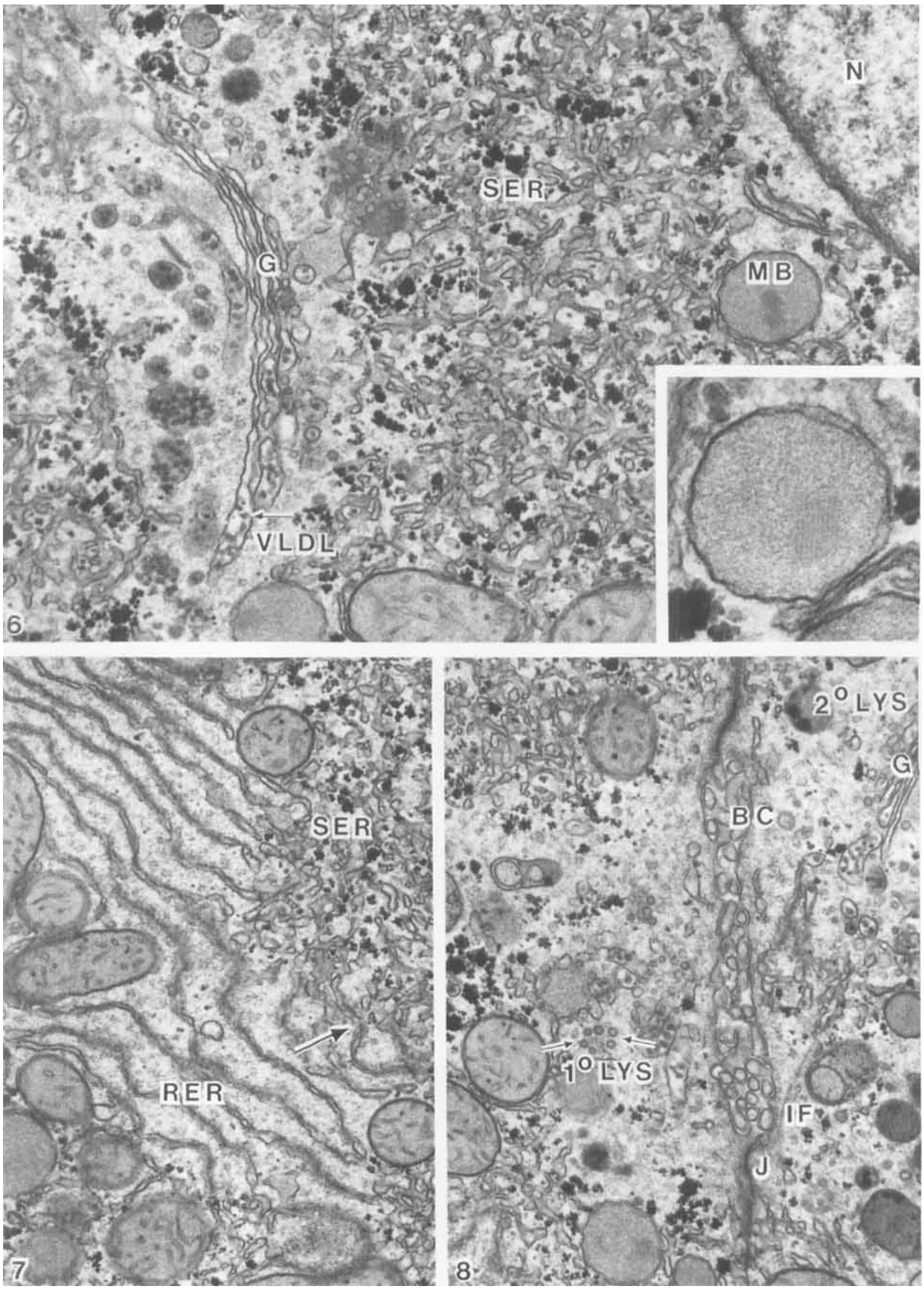


FIG. 6. Thirty minutes postexcision. Ultrastructural details are well preserved. Note the tubular profiles of smooth-surfaced endoplasmic reticulum (SER), Golgi complex (G) containing nascent very low-density lipoproteins (VLDL, *arrow*), microbodies with crystalloid-like inclusions (MB), and nucleus (N). *Insert* shows a detail of the crystalline-like core of a microbody. ~40,000×; *Insert*, ~90,000×.

FIG. 7. Flattened parallel profiles of rough-surfaced endoplasmic reticulum (RER) are well demonstrated on this micrograph from the same biopsy as Fig. 6. Note the continuity between the RER and smooth-surfaced endoplasmic reticulum (SER, *arrow*). ~40,000×.

FIG. 8. Biopsy same as Fig. 6. A bile canaliculus (BC) is centrally located between two adjacent hepatocytes. Junctional complex (J) and intermediate filament (IF). The Golgi complex (G) contains nascent VLDLs. Secondary lysosome (2° LYS); primary lysosomes (1° LYS). ~40,000×.

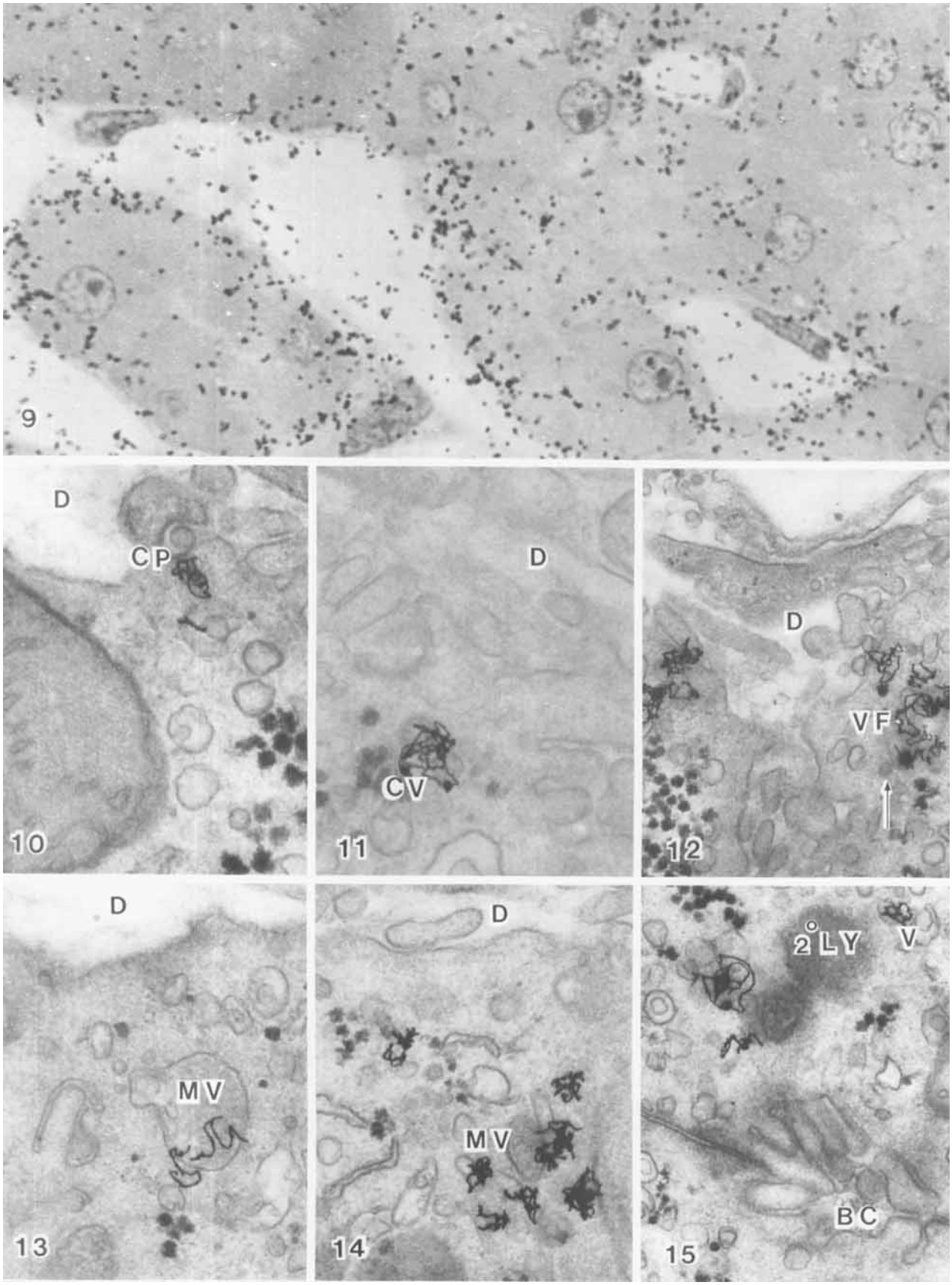


FIG. 9. Light microscopic autoradiograph of normal human liver wedge biopsy following infusion of ^{125}I-EGF. Within 15 min, most of the grains are internalized by hepatocytes and are located at the sinusoidal surfaces. ~500×.

FIGS. 10 through 15. Electron microscopic autoradiographs demonstrating association of ^{125}I-EGF grains with coated pits (CP), coated vesicles (CV), and multivesicular body-like structures (MV). Space of Disse (D), vesicle fusion (VF), shuttle vesicle (V), bile canaliculus (BC), secondary lysosome (2°LY). ~50,000×.

cessing of such ligands as EGF (St. Hilaire et al., unpublished observations), asialoglycoproteins (17), and apo-B peptide of low-density lipoproteins (18). These structures include multivesicular body-like structures, primary, and secondary lysosomes (Figure 8). Coated pits (considered a part of the indirect pathway) were frequently observed along the sinusoidal parenchymal cell surface (Figure 3). In addition, numerous autophagic vacuoles and lipofuscin granules were observed in liver specimens from older patients (Figure 2). Other organelles and inclusions were consistent with those normally observed in rodent liver, including mitochondria, glycogen, and cytoplasmic lipid droplets (Figures 2, 3, 6, 7, and 8). It was not unusual to find mitochondria that contained lamellaform cristae, as has been reported by others (19).

MICROBODIES

Microbodies were observed throughout the hepatocytes. Their matrix was homogeneous and surrounded by a single membrane, similar to those observed in rats and other species. In exceptionally well-preserved areas within hepatocytes, the microbodies contained both peripheral and central crystalline-like cores (Figures 3 and 6). While these have been reported in laboratory animals as distinctive uricase-containing crystalline cores, they have only rarely been observed in human microbodies. The possibility that some human microbodies might contain crystalline cores was first suggested by Biempica (20) in 1966. Peripheral electron-dense or marginal plates similar to those described by Sternlieb were also observed (19). Electron diffraction studies will be needed, however, to definitively establish the crystalline nature of the microbody core.

UPTAKE AND TRANSPORT OF EGF

In preliminary studies with the perfusion system, 0.5 μCi of ^{125}I-EGF (0.2 μCi per ng) was given as a bolus in 0.5 ml of medium. Both light and electron microscopic autoradiography demonstrated the internalization of EGF by human hepatocytes at the sinusoidal surface. At 10 min postinjection, autoradiographic grains in light micrographs were located primarily within hepatocytes near the sinusoidal surface (Figure 9), and electron micrographs demonstrated that these grains were predominantly localized within cytoplasmic vesicles (Figures 10 to 12). At 30 min postinjection, autoradiographic grains were found in association with GERL and secondary lysosomes, as well as in the vicinity of the pericanalicular cytoplasm (Figures 13 to 15). The results indicate that EGF uptake and disposition by human hepatocytes are similar to that observed in rat hepatocytes (St. Hilaire et al., unpublished observations).

CONCLUSIONS

This technique for the short-term maintenance of viable human wedge biopsies will allow experiments to be performed on human tissue that previously were only possible in other species. The cellular viability of the preparations, as judged by electron microscopy, is excellent. Although this report concentrated on the sinusoids and hepatocytes, the connective tissue stroma and biliary tree were also well preserved. This technique is simple and inexpensive and can be easily set up in most laboratories. Although we are currently using it to study ligand binding, the procedure also lends itself to studies of metabolism, lipoprotein secretion and catabolism, hormone effects, and carbohydrate and glycoprotein processing.

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