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## Scanning Electron Microscopy of Human Liver Sinusoids

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**Summary.** Hepatic sinusoids of human liver samples obtained by surgical or needle biopsy were examined under the scanning and transmission electron microscopes (SEM and TEM). The surgically obtained tissues were perfused, by the use of a needle and syringe, with Ringer solution and successively with glutaraldehyde solution through the cut ends of blood vessels, while the needle biopsy pieces were perfused by directly puncturing the parenchyme.

Endothelial cells of hepatic sinusoids possessed thin and flat cytoplasm with two types of fenestrations which have already been demonstrated in laboratory animals: smaller ones (less than  $0.1\text{ }\mu\text{m}$ ) occurring in clusters to form "sieve plates" and larger ones ( $0.5\text{--}2\text{ }\mu\text{m}$ ) intermingling among the former.

Kupffer cells were definitely distinguishable from the endothelial cells under the SEM and no graduations were found between either cell type. The Kupffer cell possessed voluminous cytoplasm covered with numerous filopodia, microvillous processes and lamellipodia.

In the space of Disse examined under the TEM, there were a number of attenuated cytoplasmic processes. Because of their contents in fat droplets, caveolae and microfilaments, they were identified as the processes of fat storing cells of Ito. A discontinuous basement membrane-like material was also recognized in the space of Disse.

Although the fine structure of liver sinusoids has been extensively investigated using the transmission electron microscope (TEM), the following points have remained disputable: 1) the size and distribution of endothelial fenestrations (FAWCETT, 1955; HAMPTON, 1958; WASSERMANN, 1958; BENNETT et al., 1959; WOOD, 1963; KUHN and OLIVIER, 1965; BURKEL and LOW, 1966; WISSE, 1970), 2) the question whether Kupffer and endothelial cells are different cells (YAMAGISHI, 1959; CARSTEN, 1961; WOOD, 1963) or mere modifications of a single cell (HAMPTON, 1958; ATERMAN, 1963; BURKEL and LOW, 1966; BLOOM and FAWCETT, 1968; ITO and SHIBASAKI, 1968; NICOLESCU and ROUILLER, 1967), and 3) the identification of the cell processes underlying the endothelial cells (ITO and NEMOTO, 1952; YAMAGISHI 1959; STEINER, 1961; WOOD, 1963; KUHN and OLIVIER, 1965; TANIKAWA et al., 1965; BRONFENMAJER et al., 1966; NICOLESCU and ROUILLER, 1966; TANIKAWA, 1968; COSSEL, 1968; ITO and SHIBASAKI, 1968; WISSE, 1970; WAKE, 1971; WIDMANN et al., 1972). One of the reasons for previous TEM investigators having reached no definite answers to these problems might be that they could obtain the cross-sectional views of only a limited area of the sinusoidal wall.

Recent scanning electron microscope (SEM) studies made on the liver demonstrated that this microscope has advantages over the conventional TEM in that a wide en face view of the tortuous liver cell plates and the sinusoidal wall can easily be obtained. Thus, the SEM studies have contributed much to the elucidation of the tridimensional architectures of hepatocytes (MOTTA and PORTER, 1974; VIAL and PORTER, 1975), bile capillaries (GRISHAM et al., 1975; LAYDEN et al., 1975; MOTTA and

FUMAGALLI, 1975; EGUCHI et al., 1976; GRISHAM et al., 1976), and sinusoids (BROOKS and HAGGIS, 1973; MOTTA and PORTER, 1974; ITOSHIMA et al., 1974; MOTTA, 1975a, b; MUTO, 1975; GRISHAM et al., 1975, 1976; EGUCHI et al., 1976). These investigations, however, have been made by using laboratory animals, while the application of the SEM has not been undertaken for the human liver, because of the difficulties in obtaining a fresh organ or its pieces whose blood should be eliminated by perfusion.

In the present study, we applied newly known methods of perfusion to human biopsy samples and obtained SEM views of the normal structure of the sinusoids, which will provide a base line for future studies on the pathological changes in the human liver sinusoids.

### Material and Methods

Use was made of hepatic tissue from the following patients: 1) a 52-year-old female diagnosed with primary billiary cirrhosis, 2) a 35-year-old female diagnosed with drug induced intrahepatic cholestasis, and 3) a 53-year-old male diagnosed with extrahepatic obstructive jaundice. In those patients, it was considered and later confirmed in the course of the study that pathological changes of the liver were more or less limited to the billiary system and the structure of sinusoids remained intact. Portions of the liver ( $0.5 \times 0.8 \times 1$  cm) were removed at the time of surgery in Cases 1 and 3, while needle biopsy samples ( $0.2 \times 0.2 \times 1.5$  cm) were obtained from Case 2.

Immediately after the tissues were removed, vascular perfusion was made with a needle and syringe. In surgically obtained tissue pieces, the needle was inserted into the cut end of a small blood vessel, while in biopsy cases, the tissue was directly punctured and perfused by the method recommended by MURAKAMI (1976). In order to wash out the blood from the vascular bed of the liver, 20 ml of Ringer solution was first introduced into the tissue. Then 10 ml of 1% glutaraldehyde fixative in Soerensen phosphate buffer solution (pH 7.4) was allowed to flow through the needle.

Pieces of the liver thus treated were further cut into small blocks  $2 \times 2 \times 3$  mm. Most of the blocks were treated for SEM by the tannin-osmium impregnation method by MURAKAMI (1974): The blocks were kept in the same glutaraldehyde fixatives for 6 hrs, immersed in a solution of 2% glycine and 2% glutamate for 2 hrs, then in a 2% tannic acid solution for 12 hrs, osmified in a solution of 2%  $\text{OsO}_4$  for 6 hrs, and washed in several changes of distilled water for 2 hrs. The samples were dehydrated in a graded alcohol series, substituted by isoamyle acetate, and freeze-cracked in liquid nitrogen by mechanical impact according to the method of TOKUNAGA et al. (1976). The samples were transferred to liquid  $\text{CO}_2$  and critical point-dried. Finally, they were coated with a thin layer of gold-palladium in a vacuum-evaporator and examined in a field emission type scanning electron microscope, Hitachi HFS-2, at 10 kV accelerating voltage.

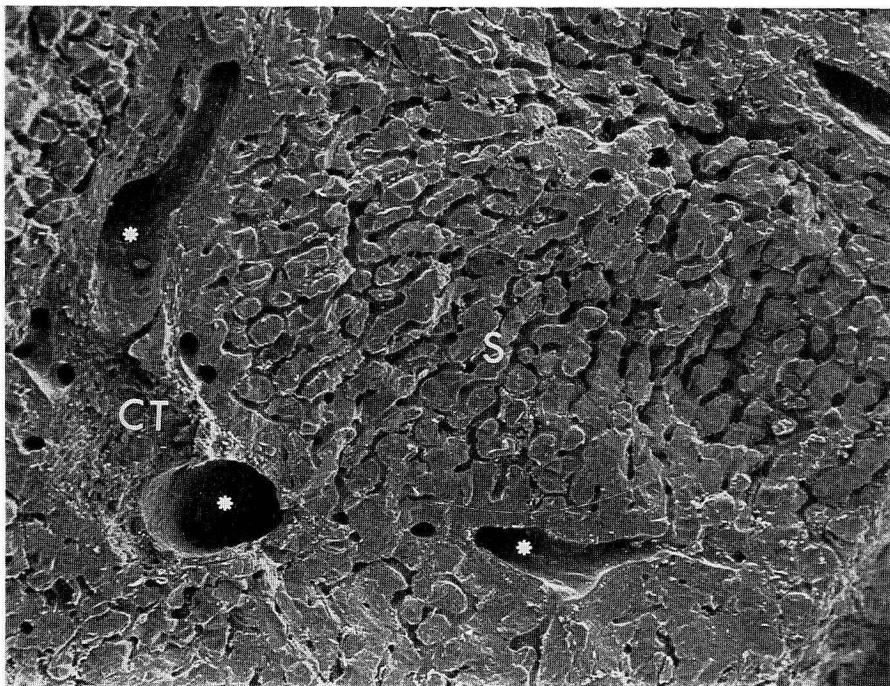
The remaining blocks were processed for conventional TEM study. They were postfixed with 2% osmium tetroxide for 2 hrs. The specimens were dehydrated with a graded series of alcohol and propylene oxide, embedded in LUFT's epoxy resin, and cut into ultra-thin sections with glass knives in a Porter-Blum MT-1 microtome. The sections were doubly stained with uranium acetate and Millonig's lead acetate and examined at 75 kV in a TEM, Hitachi HU 125 DS.

## Results

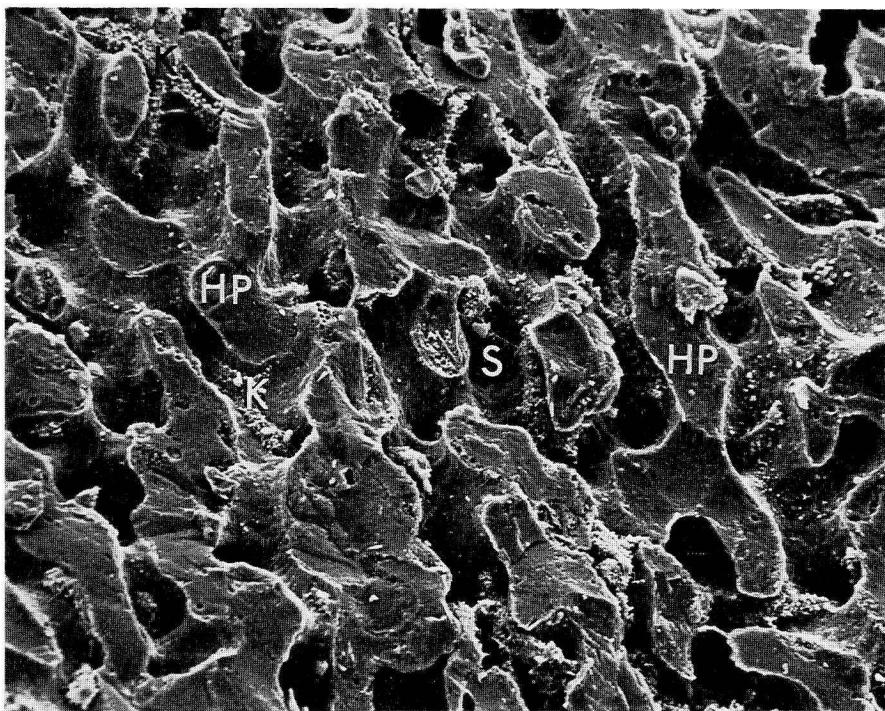
In low power SEM views, the liver parenchyme appears as a labyrinth made up of highly anastomosed cell plates, comprising one-cell-thick muralium of hepatic cells. The spaces between the cell plates are occupied by hepatic sinusoids which represent a specialized vascular bed of the liver (Fig. 1, 2).

After vascular perfusion, whole blood cells in the sinusoidal lumen were completely washed out, so that the luminal surface was well exposed to allow its detailed observation under the SEM. It was thus clear that the sinusoidal lumen was lined by two types of cells morphologically distinguishable from each other: one was the endothelial cell which was smooth and flattened and provided with numerous cytoplasmic fenestrations, and the other was the Kupffer cell, covered by numerous cytoplasmic processes all over its surface (Fig. 2).

Under the TEM, the endothelial cell possessed an attenuated cytoplasm, occasionally bulged into the sinusoidal lumen (Fig. 3). The cell attenuation extended laterally from the nucleus containing part varied in thickness ( $0.1\text{--}3\ \mu\text{m}$ ) in its cross sectional view. The thinnest parts of the cell ( $0.1\ \mu\text{m}$ ) frequently possessed numerous fenestrations of various diameter. By contrast, no fenestration was present in the thickened parts measuring  $0.5\text{--}3\ \mu\text{m}$  (Fig. 4). Under the SEM, the non-fenestrated, thickened parts of the endothelial cell were visualized as branching and anastomosing ridges of cytoplasm radiating from the nuclear portion to the periphery of the cell.



**Fig. 1.** Fractured liver surface showing a liver lobule encircled by a connective tissue septum (CT) conveying larger blood vessels (asterisks). Hepatic sinusoids (S) form a fine network within the lobule.  $\times 75$

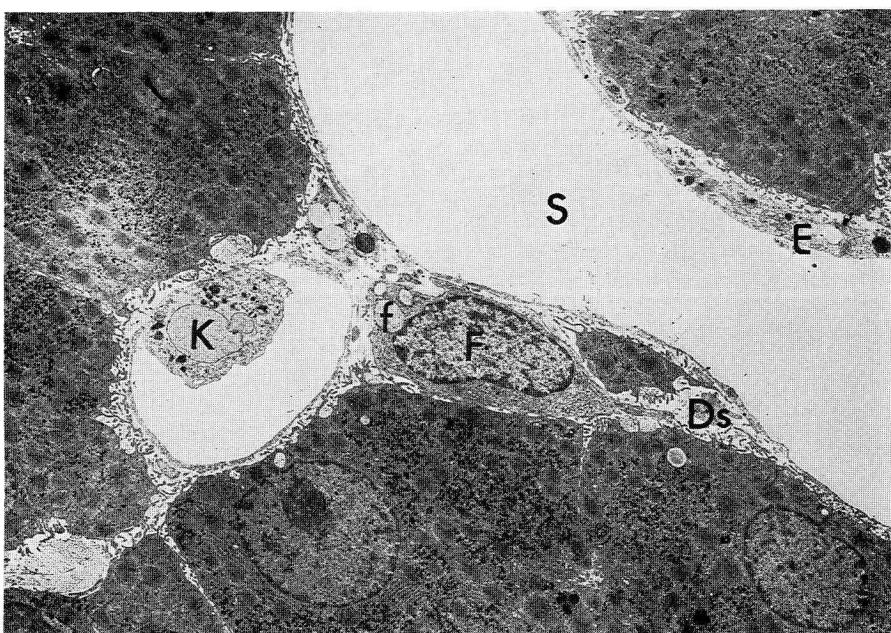


**Fig. 2.** Closer view of a hepatic lobule showing hepatic plates (HP) and sinusoids (S). The hepatic plates anastomose with each other to construct a labyrinthine system. The spaces between the plates are occupied by hepatic sinusoids. The sinusoids are lined by continuous endothelial cells, but occasionally interrupted by Kupffer cells (K) possessing numerous cytoplasmic processes.  $\times 250$

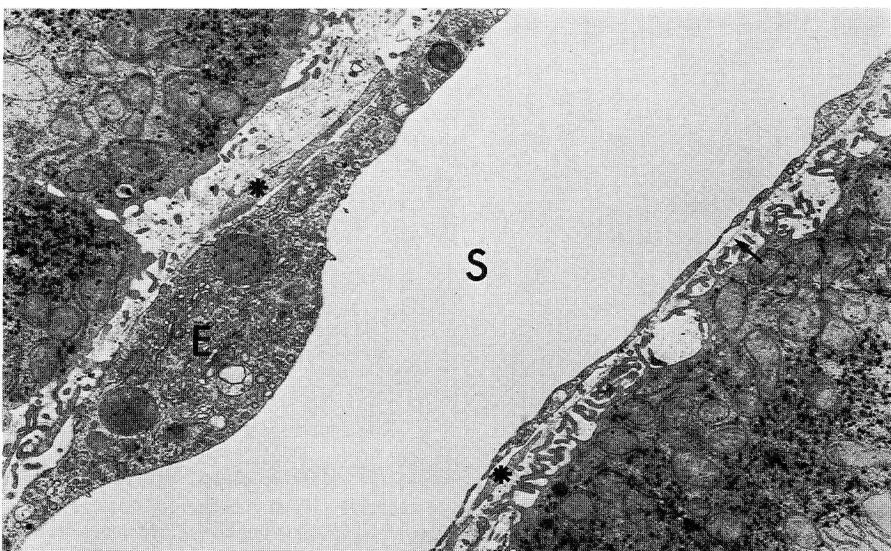
These cytoplasmic ridges bordered fenestrated areas of rounded shapes (Fig. 5). The endothelial cell border clarified with the SEM waved irregularly and touched the neighbor cell by overlapping. Occasionally, the cell border retracted to form small intercellular gaps (Fig. 7).

When examined with the SEM, the endothelial fenestrations could be divided into two types, small and large, according to their size. The small ones were less than  $0.1 \mu\text{m}$  in diameter and round and oval in shape, occurring in clusters (Fig. 5). In the area of their cluster often referred to as "sieve plate," about 25 of them were distributed per  $\mu\text{m}^2$  (Fig. 6). Occasionally in the sections under the TEM, an en face view of fenestrations could be obtained (Fig. 6). With the SEM, the large fenestrations were  $0.5\text{--}2 \mu\text{m}$  in diameter and also showed oval and round shape (Fig. 8). The large fenestrations occurred in many places of sinusoids, intermingling with the small ones, while in some places of sinusoids, the large and small fenestrations occurred separately in different endothelial cells and one could see endothelial cells possessing only small fenestrations (Fig. 5). However, we could not demonstrate such a regular regional difference in the distribution of small and large fenestrations as has been reported to occur between the different zones in the lobule.

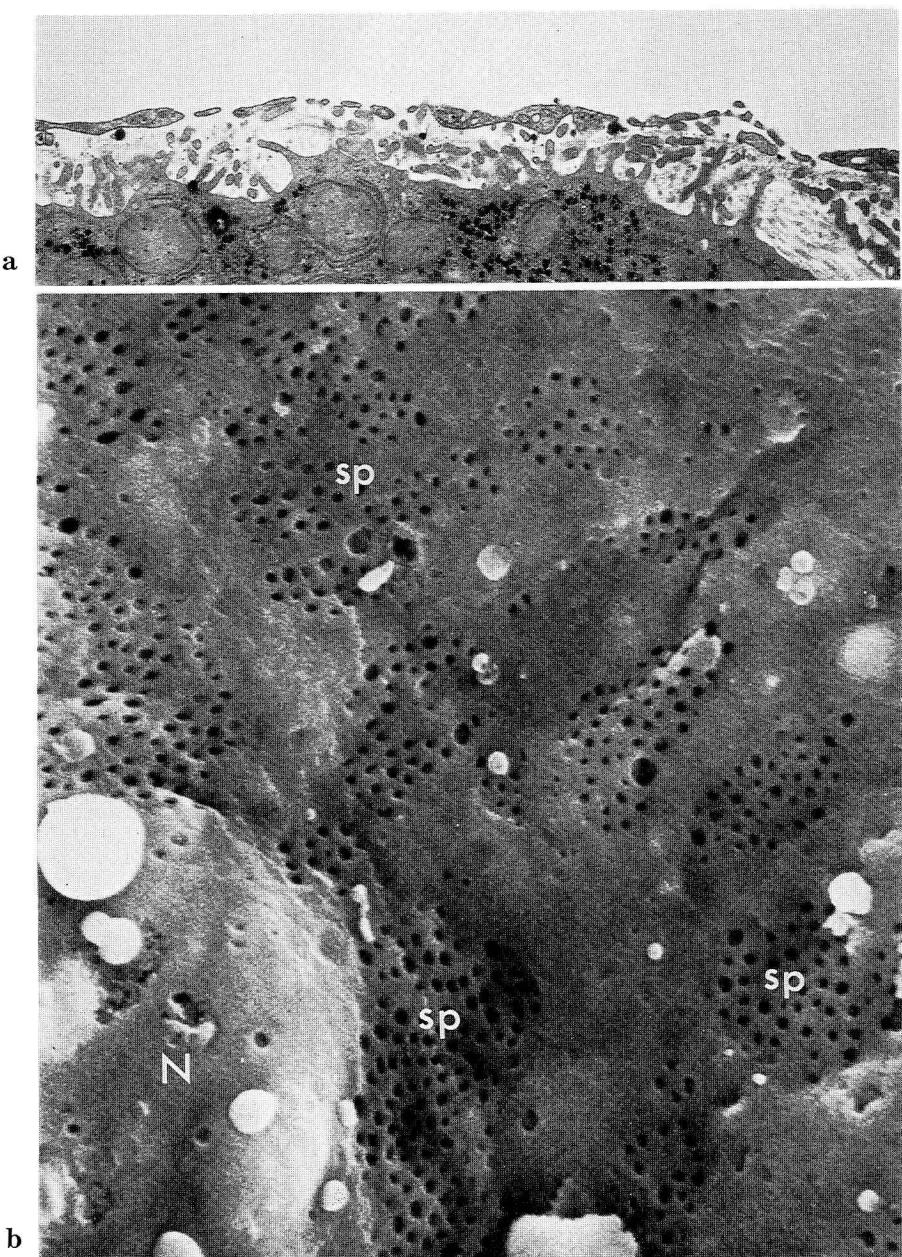
Bulging into the sinusoidal lumen, cells were observed whose surface architec-



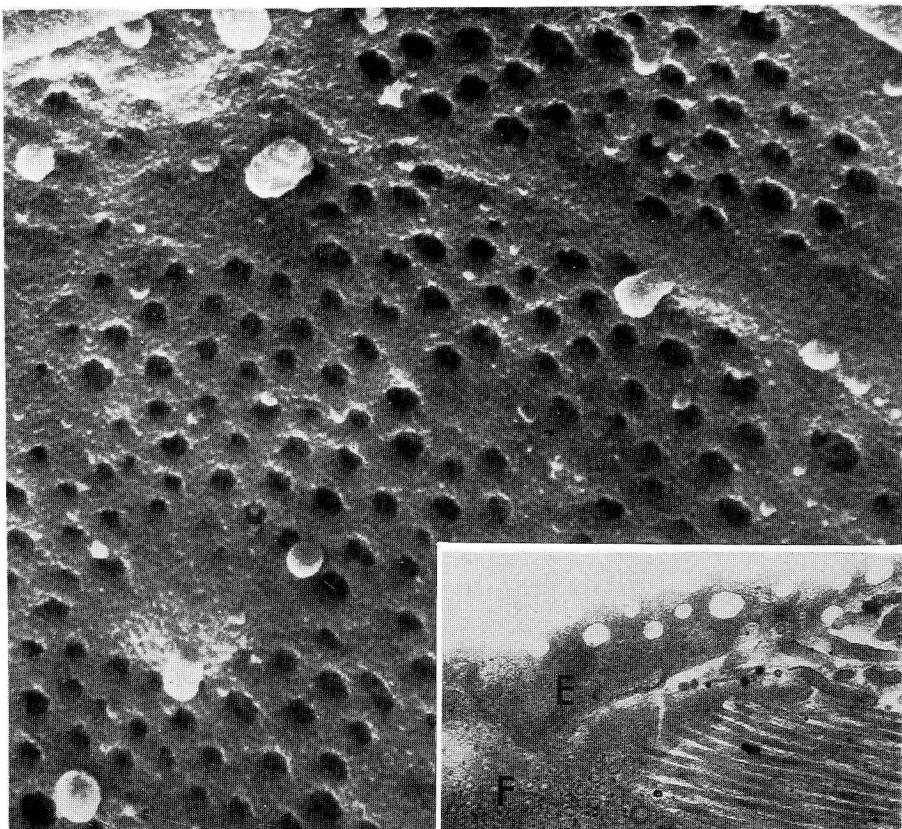
**Fig. 3.** TEM cross sectional image of the sinusoidal wall showing its three cellular components: endothelial cell (*E*), Kupffer cell (*K*) and fat storing cell of Ito (*F*). The Kupffer cell is inserted between the endothelial cells and protruded into the sinusoidal lumen. The cell of Ito is located in the space of Disse (*Ds*) and fat droplets (*f*) are seen in its cytoplasm.  $\times 3,200$



**Fig. 4.** Closer view of endothelial cell (*E*) showing its variety in thickness. The thickened portion contains lysosomes, a Golgi complex and rough endoplasmic reticulum, while the thinned portion is interrupted by small fenestrations. Cytoplasmic processes of Ito cells (asterisks) and basement membrane-like material (arrow) is present in the space of Disse. *S* sinusoid.  $\times 6,500$



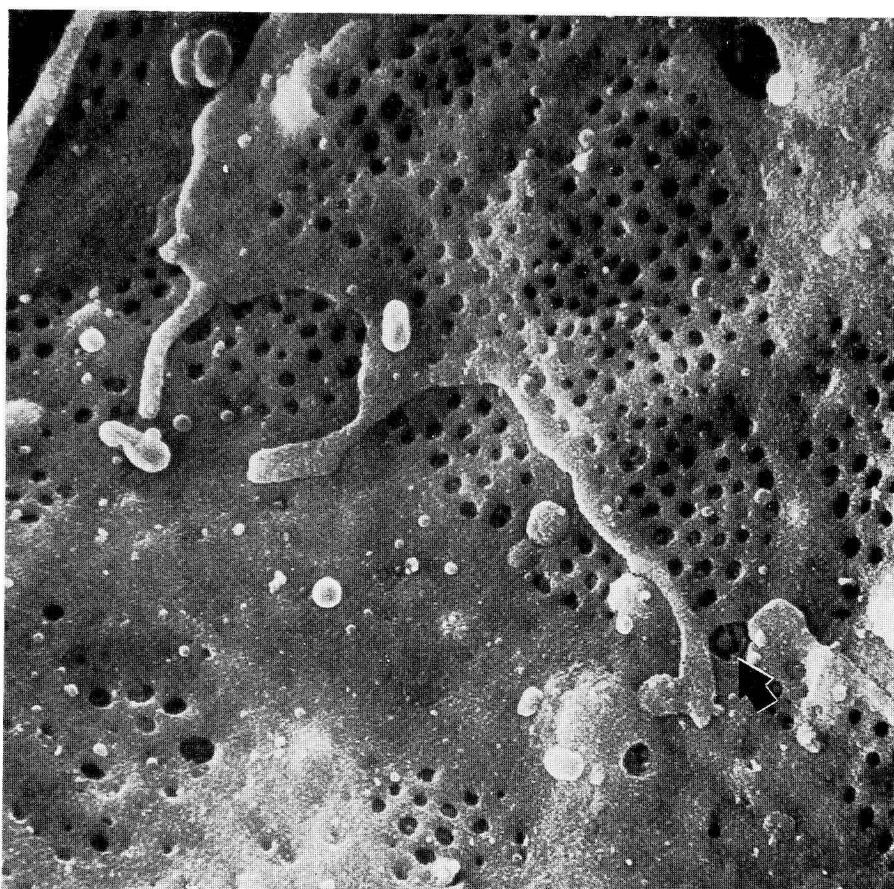
**Fig. 5. a.** TEM cross sectional view showing fenestrated area of endothelial cell attenuation.  
 $\times 14,300$  **b.** Wide luminal view of sinusoidal endothelium showing the relationship between its fenestrated and non-fenestrated areas. The non-fenestrated areas of the endothelial cell radiate from the nuclear swelling (*N*) and separate fenestrated areas. In the latter areas, small fenestrations are typically distributed in uniform spacing to form "sieve plates" (*sp*).  
 $\times 14,000$



**Fig. 6.** Close-up views of a fenestrated area or sieve plate. Small fenestrations with diameter of less than  $0.1 \mu\text{m}$  are shown both in a SEM luminal view and in a TEM grazing section. *E* endothelial cell, *F* tangential section of Ito cell.  $\times 48,000$

ture distinctly differed from that of the fenestrated endothelial cells. From a combined SEM and TEM observation, they were identified as Kupffer cells. Under the SEM, the Kupffer cell was a voluminous form strongly protruded into the sinusoidal lumen. The cell was provided with numerous microvillous processes or filopodia extending around from its surface. Sheet-like processes or lamellipodia were spread out from the cell basis and attached to neighboring endothelial cells. No intercellular gaps between Kupffer cells and endothelial cells could be seen. Under the TEM, the Kupffer cells in many cases took an intercellular location in the endothelium and its basal face directly contacted the hepatocytes without any insertion of endothelial cells (Fig. 3). Only occasionally, profiles of Kupffer cells were found in a supra-endothelial (in the sinusoidal lumen) or a subluminal (within the space of Disse) position.

Through larger fenestrations, one could see hepatocytic microvilli, thicker fibers of apparent collagenic nature, fine and irregular fibrous materials, and, usually taking the most superficial location in the space of Disse, attenuated and slender plates of apparent cytoplasmic nature (Fig. 8, 9). The space of Disse, when observed by TEM, contained numerous hepatocytic microvilli and collagen bundles, the cyto-



**Fig. 7.** Closer SEM view of an endothelial cell border showing adjacent endothelial cells partly overlapping. A fenestrated area extends closely to a wavy endothelial cell margin. A small intercellular fenestration is indicated by an arrow.  $\times 18,000$

plasmic processes extended from the fat-storing cell of Ito (Fig. 3, 4, 10). The last mentioned cell processes, without doubt, corresponded to the attenuated plates in the space of Disse seen under the SEM.

The cytoplasm of the cell of Ito contained numerous microfilaments which ran generally in parallel with the long axis of the process: caveolae or plasma membrane invaginations were numerous in both surfaces of the processes. Fat droplets which characterized this cell were mainly concentrated in the perinuclear region, but occasionally were found in the process (Fig. 10).

No distinct basement membrane was found in the space of Disse under the TEM, but intermittent material resembling it was observed and this seemed to correspond to the fibrous material demonstrated by SEM (Fig. 4, 8, 10).

### Discussion

Perfusion-fixation of small biopsy samples of human liver could be achieved by

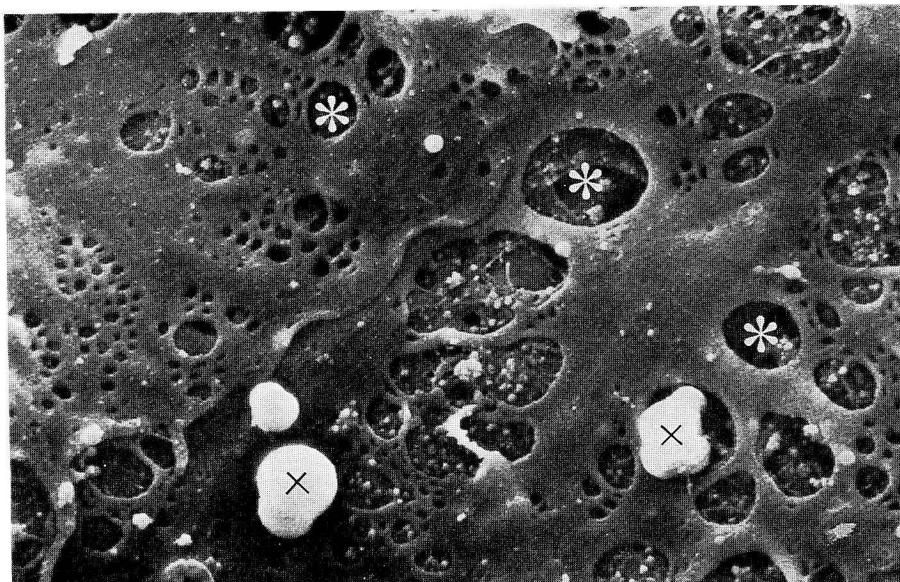
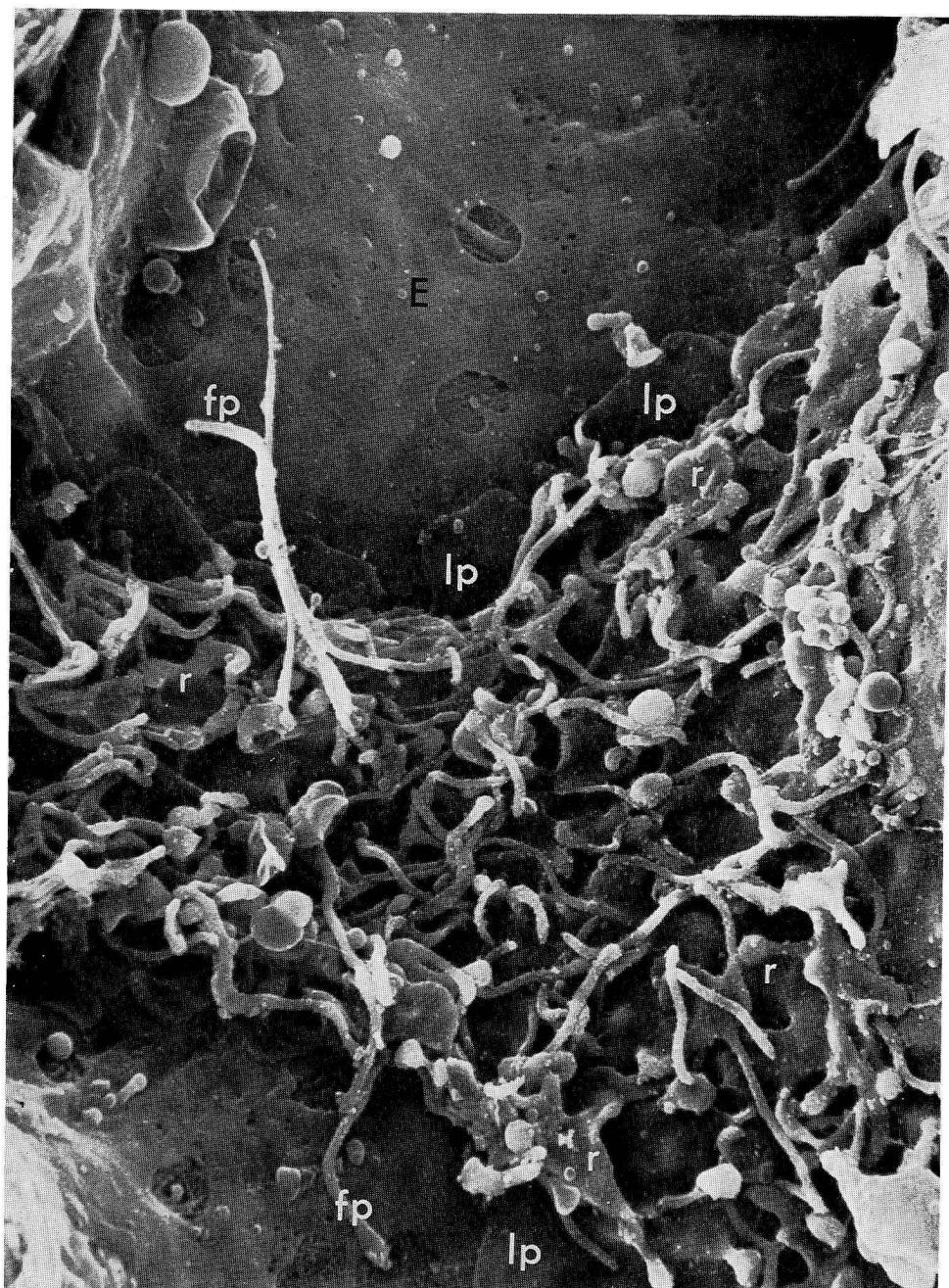


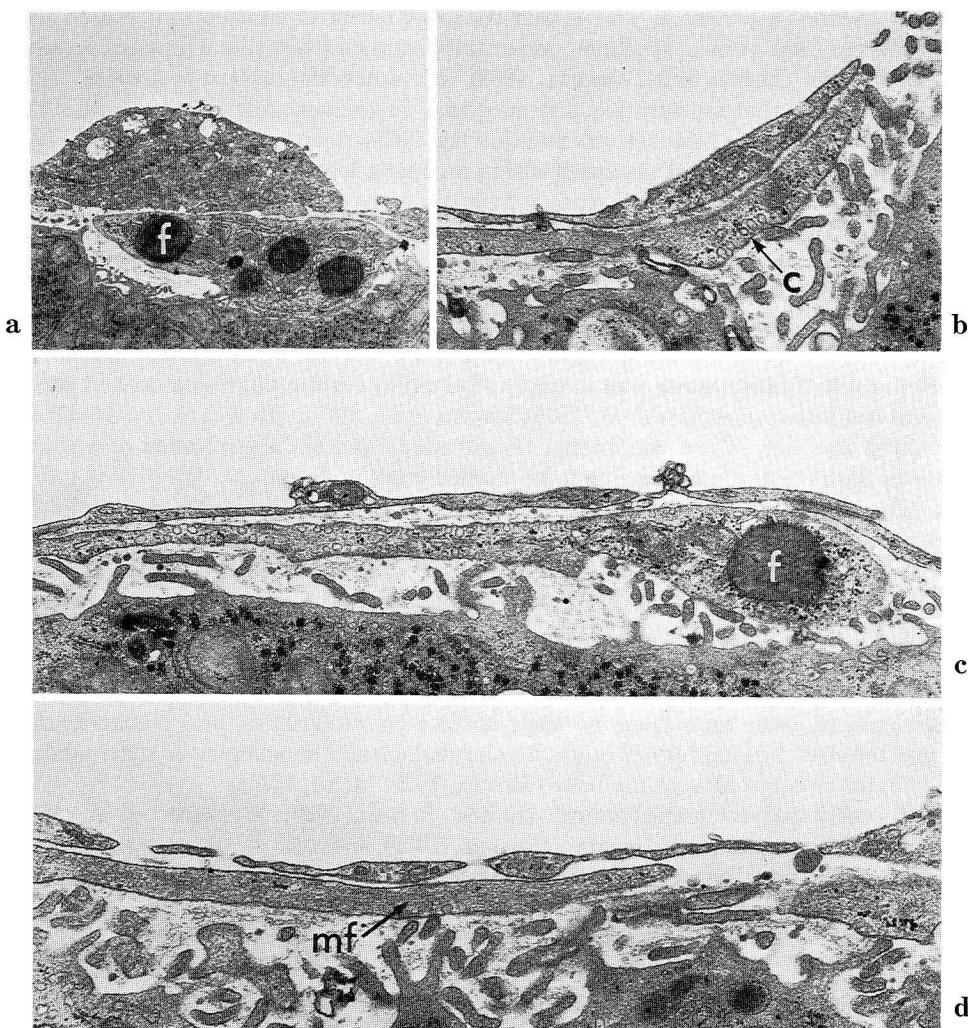
Fig. 8. A portion of sinusoidal wall showing large fenestrations (asterisks), through which granular or fibrous materials are seen in the space of Disse. Plasma precipitates of unknown nature are indicated by  $\times$  marks.  $\times 10,000$

utilizing the puncture perfusion method recommended by MURAKAMI (1976). This simple method together with a puncture into the cut vascular ends of surgically obtained tissues, facilitated not only the complete washing out of blood components unfavorable for SEM observation, but also assured appropriate tissue preservation for TEM, as well as for SEM studies. The first aim of this study is to demonstrate that the difficulty in studying human livers has thus been technically overcome.

The previous SEM observations of the hepatic sinusoidal wall have been done mainly in common laboratory animals: rats (BROOKS and HAGGIS, 1973; MOTTA and PORTER, 1974; MOTTA, 1975; MUTO, 1975; GRISHAM et al., 1976; EGUCHI et al., 1976), guinea pigs (ITOSHIMA et al., 1974) and rabbits and mice (GRISHAM et al., 1975). The previous works showed that the sinusoidal endothelial cell was provided with numerous fenestrations which were located intracellularly (BROOKS and HAGGIS 1973; MOTTA and PORTER, 1974; ITOSHIMA et al., 1974; MUTO, 1975; MOTTA, 1975; GRISHAM et al., 1975, 1976; EGUCHI, 1976) and also partly intercellularly (MUTO, 1975; EGUCHI et al., 1976). The intracellular fenestrations were generally classified into small and large ones (ITOSHIMA et al., 1974; MOTTA and PORTER, 1974; MUTO, 1975; GRISHAM et al., 1975, 1976; EGUCHI et al., 1976). The small fenestrations appeared in groups and were distributed throughout the sinusoids. On the other hand, the large fenestrations differed in distribution from author to author: ITOSHIMA et al. (1974) reported that the large fenestrations were increased in number towards the center of the lobule in the guinea pig, while GRISHAM et al. (1976) reported that they appeared more numerously around the portal tracts in rats. But most other authors failed to demonstrate a specific distribution of fenestrations of different sizes (MOTTA and PORTER, 1974; MOTTA, 1975; MUTO, 1975; EGUCHI et al., 1976). Intercellular gaps, on the other hand, were found



**Fig. 9.** A portion of sinusoidal wall showing a Kupffer cell, which is provided with numerous cytoplasmic processes: filopodia (*fp*), cytoplasmic ruffles (*r*), and lamellipodia (*lp*). The cell is continuously connected with adjacent endothelial cells (*E*).  $\times 8,800$



**Fig. 10.** TEM closer view of sinusoidal walls showing the cytoplasmic processes of Ito cells containing fat droplets (*f*) and microfilaments (*mf*). Note also that the cell is provided with numerous caveolae (*c*) along its surface. **a:**  $\times 4,000$ , **b:**  $\times 18,000$ , **c:**  $\times 20,000$ , **d:**  $\times 25,700$

to occur mainly between endothelial and Kupffer cells in the rat (MUTO, 1975; EGUCHI et al., 1976).

In the present study we confirmed that, also in the human liver, small and large fenestrations are present within the cytoplasm of an endothelial cell and a few small gaps may occur between the cells. But, in the human samples, there was no such large intercellular gap as was reported in the rat liver (MUTO, 1975; EGUCHI et al., 1976).

The occurrence of large fenestrations has been a matter of dispute among the previous TEM workers (see WISSE's review, 1970). Two representative opinions may

be worthwhile to introduce. One is that the large fenestration is nothing but an artifact derived from various specimen preparatory stages such as tissue removal (COSSEL, 1968), fixation (BLOOM and FAWCETT, 1968), and embedding (HAMPTON, 1958). The other opinion is that fenestration size may vary in various normal states: under different nutritional conditions (KUHN and OLIVIER, 1965), among different species (WOOD, 1963), and among different positions within a hepatic lobule (BURKEL and LOW, 1966).

Among SEM studies, the artifact hypothesis may be represented by the paper of BROOKS and HAGGIS (1973), who suggested that the large fenestrations might be produced due to ice crystal damage during the freeze-drying procedure they used. On the other hand, most of the SEM authors have considered that the small and large fenestrations actually co-existed in the living state (MOTTA and PORTER, 1974; IROSHIMA et al., 1974; MUTO, 1975; GRISHAM et al., 1975, 1976). Some authors speculated that the size of fenestration was strongly affected by certain physiological and pathological conditions (GRISHAM et al., 1975; MOTTA et al., 1974). However, the conditions affecting the size of the endothelial fenestrations and the significance of variable sizes of the fenestrations remains to be studied more in detail.

The previous TEM investigators describing the ultrastructure of cells in the liver sinusoidal wall, except for only a few workers (YAMAGISHI, 1959; CARSTEN, 1961; KUHN and OLIVIER, 1965), could not fix a boundary between the endothelial and Kupffer cells (HAMPTON, 1958; ATERMAN, 1963; BURKEL and LOW, 1966; NICOLESCU and ROUILLER, 1967; BLOOM and FAWCETT, 1968; ITO and SHIBASAKI, 1968), and inclined to the view that the two cells are different functional expressions of a single cell type. But the SEM authors have demonstrated that the two cells could always be clearly distinguished from each other by their surface characteristics, and it was further suggested that the Kupffer cell might be morphologically homologous to macrophages distributed in other sites of the body (MOTTA, 1975; MUTO, 1975).

Recently enzyme-histochemical studies (FAHIMI, 1970; WIDMANN et al., 1972; WISSE, 1974), tracer experiments (HOWARD, 1970) and cell labelling experiments (FURTH, 1970) have been attempted to differentiate the endothelial and Kupffer cells and to identify the precursor cell of the latter.

Most of these works, except the suggestion by KELLY et al. (1962) that Kupffer cells are derived from intrahepatic precursor cells, support the assumption that the Kupffer cell is on the line of macrophages of an extrahepatic origin (HOWARD, 1970; FURTH, 1970; WISSE, 1974). The Kupffer cell has been morphologically distinguished (FAHIMI, 1970; WIDMANN et al., 1972) even through various stages of reticuloendothelial stimulations (WISSE, 1974).

The present work confirms that in the human also the Kupffer cell can be clearly discriminated from the endothelial cell under SEM. It is stressed that in survey of a large extent of the sinusoids, no transitional forms could be found, like a cell provided simultaneously with cytoplasmic fenestrations and filo- and lamellipodia.

It has been accepted among the TEM investigators that the sinusoidal wall is composed of double layers of a cellular nature in many places. However, it has been disputed what the underlying cell is like and several possibilities have been proposed concerning that matter. The underlying element has been accounted for as 1) an overlapping endothelial cell (STEINER, 1961; WOOD, 1963; YAMAGISHI, 1959; KUHN and OLIVIER, 1965; TANIKAWA et al., 1965; COSSEL, 1968), 2) as a fat-storing cell (ITO and NEMOTO, 1952; YAMAGISHI, 1959; BRONFENMAJER et al., 1966; NICOLESCU and ROUILLER,

1971; TANIKAWA, 1968; WISSE, 1970; WAKE, 1971; WIDMANN et al., 1972), and 3) as a fibroblast and its related cell (ACKERMAN et al., 1961; WOOD, 1963; KUHN and OLIVIER, 1965; SCHNACK et al., 1966; SAFRAN and SCHAFFNER, 1967), and 4) a migrating Kupffer cell (WASSERMANN, 1958; HAMPTON, 1958; YAMAGISHI, 1959; WISSE, 1972). The cytological features of this cell demonstrated in the present study, especially the occurrence of lipid droplets, microfilaments and caveolae in the cytoplasm, are compatible with the characteristics of the fat-storing cell of Ito as described in Ito and SHIBASAKI's report (1968), and we therefore conclude that the underlying cytoplasmic processes in the space of Disse belong to the cells of Ito.

Although the whole shape of the Ito cell could not be visualized in the present SEM survey, from its fairly dense population in the space of Disse known by light microscopy and TEM, we consider that the cell, besides its probable functions concerning fat and vitamin A metabolism (for review see Ito and SHIBASAKI, 1968), likely contributes to the reinforcement of the sinusoidal wall from the side of the space of Disse.

### ヒト肝類洞の走査型電子顕微鏡による観察

武藤正樹, 西正与, 藤田恒夫

外科的あるいは針生検で得られたヒト肝標本の類洞を、走査および透過型電子顕微鏡(SEMとTEM)で観察した。外科生検で得られた組織は、血管断端より注射針を用いて、リンゲル液とそれに引き続いでグルタールアルデヒド液で灌流した。一方、針生検標本については直接、実質内に針を刺入し、灌流をおこなった。

肝類洞内皮は薄く引き伸ばされた細胞質をもち、すでに実験動物でみられたと同様の二種類の窓をもっている：小さな窓(0.1 μm以下)は集合していわゆる“ふるい板”を作り、大きな窓(0.5–2 μm)は前者の間に混在してみられた。

クッペル細胞はSEMの下では、明かに内皮細胞とは区別される細胞で、両者の間に移行型は認められなかった。クッペル細胞は豊かな細胞質をもち、多くの糸状突起、微絨毛様突起と葉状突起でおおわれていた。

TEMによって観察されたディッセ腔には、多くの薄く伸展した細胞質突起が認められた。脂肪小滴、小窩、マイクロフィラメントの存在から、これは伊東の脂肪摂取細胞の突起と同定された。不連続な基底膜様物質もディッセ腔に認められた。

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