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Methylation of S^{35}-labeled sections demonstrates the methanolytic desulfating action of this procedure on sulfated mucins.

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Manual of Experiments in Pathology

The Intersociety Committee on Research Potential in Pathology would like to evaluate the usefulness of the "Manual of Experiments in Pathology" by Hans Schlumberg. They would appreciate it if those who received the manual would submit their opinions as to its value to them and any comments, criticisms, or possible additions that would help the committee in improving revised issues in the future. Please send your comments to DONALD B. HACKEL, M.D., Duke University Medical Center, Durham, N. C.

Studies on Hepatic Fibrosis

Hans Popper, M.D., Fiorenzo Paronetto, M.D., Fenton Schaffner, M.D., and Victor Perez, M.D.

MOST OF THE STUDIES on hepatic fibrosis have been concerned with the distribution of collagenous fibers in relation to the lobule and particularly with the evolution of cirrhosis.[17, 20, 27, 33] Less attention has been paid to the formation of the individual reticulum and collagen fibers and to the role of the cells in this process. Newer technics including histochemical and electron-microscopic procedures have been applied to fibrogenesis in various structures rich in connective tissue such as skin, rat tail, and tendons,[14, 18, 42] while only few observations covered fiber formation in the liver. Therefore, the fiber distribution in the human liver was studied under normal and abnormal circumstances by light and electron microscopy. This was supplemented with investigation of accelerated new formation of fibers in rats on ethionine-containing diets and after intrahepatic injection of carrageenin.

Among recent studies on parenchymal fibrogenesis, thorough chemical and histological investigations of the kidney, especially of its reticulum, are noteworthy.[36, 41] Extensive light-microscopic examinations in the liver were carried out in carbon tetrachloride intoxication.[1] Accumulation of material giving carbohydrate reactions in beginning hepatic fibrosis was described.[2, 4, 28] Electron-microscopic investigations have demonstrated collagen fibers between the microvilli of the liver cells in the space delineated by Kupffer cells and hepatic cells and apparently close to the latter.[9, 37, 41] The claim was also made that fibrils are visible within the mesenchymal cells in the normal human[9] and mouse[41] liver and the cirrhotic liver.[5]

From the Departments of Pathology and Medicine, The Mount Sinai Hospital, New York.

Supported by the U.S. Army Medical Research and Development Command under Contract DA-49-007-MD-790.

Thanks are due Dr. Leonard Ornstein of the Cell Research Laboratory of The Mount Sinai Hospital, for advice in and support of the electron microscopic investigations.

TABLE 1. Number of Livers Studied for Distribution of Fibers

State	No. of light microscopy sections: 5 μ	No. of light microscopy sections: 1 μ	Electron microscopy
Human			
No hepatic abnormalities	34	3	6
Mild hepatic abnormalities (nonspecific reactive hepatitis)	43	1	0
Granulomatosis (tuberculosis, sarcoidosis)	10	0	0
Schistosomiasis	15	3	0
Gaucher's disease	1	0	0
Viral hepatitis, fatal			
Autopsy	17	1	0
Biopsy	27	2	3
Chronic fatty metamorphosis	6	2	3
Chronic fatty metamorphosis with necrosis	12	2	0
Diffuse septal cirrhosis with fatty metamorphosis	20	6	0
Postnecrotic cirrhosis	30	4	2
Primary biliary cirrhosis	8	2	3
Hemochromatosis	7	1	2
Chronic idiopathic jaundice	6	3	1
Intrahepatic cholestasis ("cholangiolitis")	11	2	3
Extrahepatic biliary obstruction	41	7	5
Primary hepatic carcinoma	26	2	0
Rats			
Normal	20	2	4
Subacute ethionine intoxication	25	2	4
Intrahepatic carrageenin injection	30	2	1

MATERIAL AND METHODS

Liver tissue obtained either at autopsy or by biopsy from patients without evidence of liver injury and with various hepatic disorders, or from rats, form the basis of the study (Table 1). Rats were normal or subjected to either subacute ethionine intoxication, which results in a diffuse increase of hepatic connective tissue,[39] or intrahepatic injection of carrageenin,[45] which produces rapid focal fiber formation. Specimens were fixed in formalin or Carnoy's solution and, after routine embedding sections, were cut 5 μ thick ("routine sections"). These were stained with hematoxylin-eosin, Gomori's silver impregnation,[12] periodic acid–Schiff (PAS) reaction (using an 0.5% aqueous solution of periodic acid for 5 minutes and Schiff reagent for 40 minutes after diastase digestion according to Lillie).[22] Chromotrope aniline blue (CAB),[8] Alcian blue,[20] and Van Gieson stains were also used. In selected instances part of the paraffin block was subsequently doubly embedded with celloidin-paraffin. Sections thinner than 1 μ ("thin sections") were cut, then stained with CAB, Gomori's silver impregnation, and PAS technic, following modified procedures.[13] Minute bits of fresh tissue obtained during liver biopsy were fixed in a buffered 2% aqueous solution of osmium tetroxide. After dehydration they were embedded in butyl methacrylate and cut for electron microscopy on the Porter-Blum microtome. They were examined with a Philips EM100 electron microscope at 100 kv.

RESULTS

The arrangement of fibers and their relation to neighboring cells were studied in normal and abnormal human livers (1) in the immediate vicinity of liver cell plates within the parenchyma, (2) around ductules, and (3) within the portal tracts.

Observations in Normal Livers

Pericellular Fiber Distribution

On the sinusoidal surface of normal human liver cells, a fine layer of PAS-positive nonglycogenic material was noted which seemed to be independent of a PAS-reacting material in the cytoplasm of the Kupffer cells. The latter also was not removed by diastase and was in part diffuse and faint and in part granular. The pericellular layer gave a faint reaction with Alcian blue and stained purple with CAB. In this layer in routine sections a dense framework of argentaffin fibers was noted which formed a continuous line between the liver cell plates and the sinusoids (Fig. 1). These lines occasionally were wavy, and sometimes finer cross fibers seemed to originate from them, running in the tissue spaces. Where liver cells appeared absent, an irregular network of such cross fibers was present. In thin sections (Fig. 2), individual fibers lined up in the perisinusoidal space. The majority appeared as fine points or comma shapes representing cross sections. Less often a continuous longer line was seen. The distances between the different fibers varied from 1 μ to 3 μ except where fine cross fibers appeared closely approximated. Fibroblasts characterized by spindle-shaped nuclei with two nucleoli one third of the long axis of the nucleus apart were not seen within the lobular parenchyma. Under the electron microscope, fibers were rare. When present, they seemed to be uniform in width (250–300 Å) and with a periodicity of about 600 Å (Fig. 3). These fibers were in the tissue space either singly or in small groups of parallel

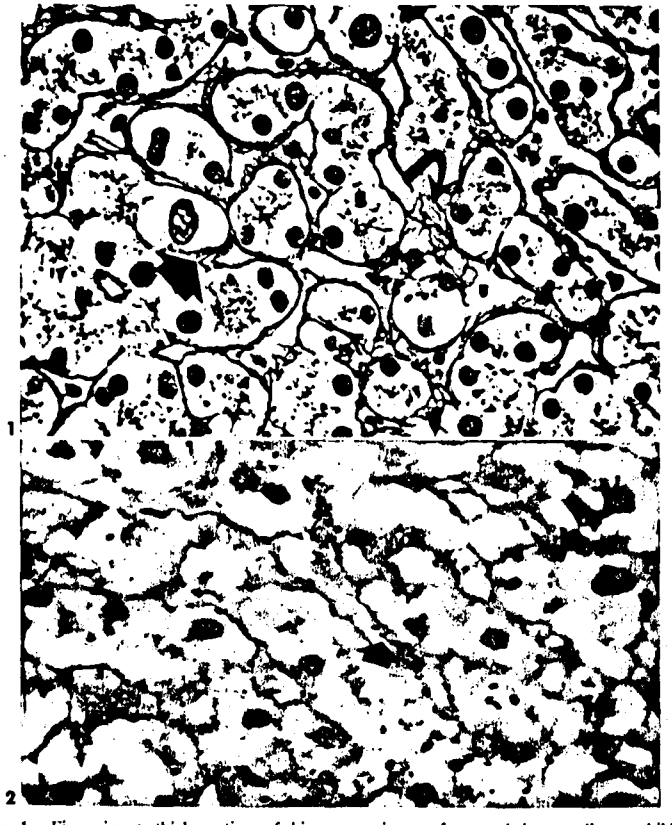


Fig. 1. *Five-micron thick section of biopsy specimen of normal human liver exhibiting* reticulum framework around liver cell plates and appearing as a continuous membrane enveloping them. Finer cross fibers (straight arrow) traverse the interstitial space. Where liver cells are absent, they form a fine network, indicated by the curved arrow. (Gomori's silver impregnation, × 400)

Fig. 2. One-micron thick section of same liver as shown in Fig. *1*, exhibiting individual reticulum fibers cut in cross sections appearing as points along the sinusoidal border of liver cells. Only in a few places do they merge to represent a short line. Few cross fibers are indicated by arrows. (Gomori's silver impregnation, × 400)

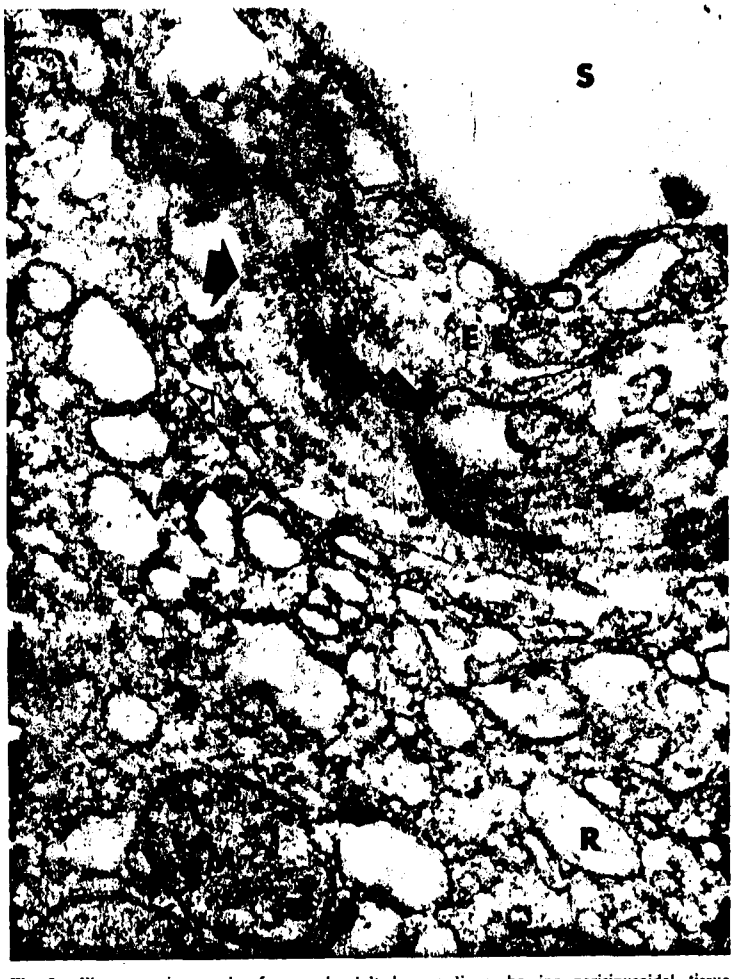


Fig. 3. Electron micrograph of normal adult human liver showing perisinusoidal tissue space containing several parallel reticulum fibers (solid arrows) between a Kupffer cell (*E*) and microvilli of a liver cell (open arrows). *M* is a liver cell mitochondrion, *R*, a small swollen profile of endoplasmic reticulum, and *S*, the sinusoid. (× 90,000)

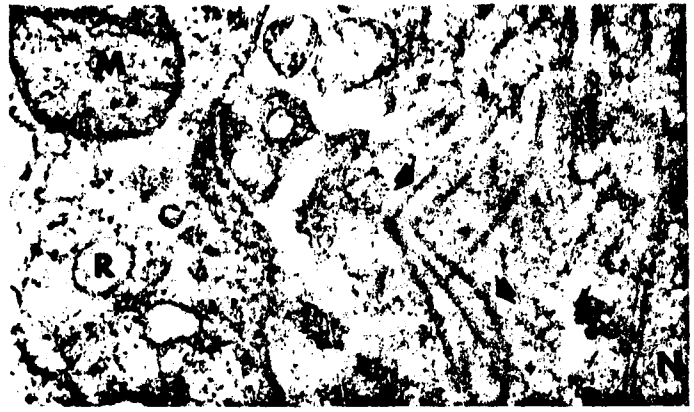


Fig. 4. Electron micrograph of normal adult human liver showing few reticulum fibers (arrows) in somewhat widened tissue space filled with debris between Kupffer cell with nucleus (*N*) and liver cell with mitochondria (*M*) and small swollen endoplasmic reticulum profile (*R*). (× 63,000)

fibrils (Fig. 4). Occasionally, bundles of 10 or more closely approximated parallel fibrils were found in the plane of the section.

Periductular Fiber Distribution

The ductules or cholangioles connect the bile canaliculi within the liver cell plates with the bile ducts in the portal tracts. Around the few ductules on the border of the portal tract or within the parenchyma, no PAS-positive amorphous substance was found. In routine sections a continuous argentaffin membrane also stained darkly with aniline blue and gave a variable PAS staining. Fibroblasts were not noted near the ductules. In thin sections the membrane was composed of individual fibers, as a rule, cut across and closely spaced. Under the electron microscope the ductule, characterized by microvilli projecting into its lumen, was surrounded by a single layer thinner than a collagen fibril and devoid of periodicity (Fig. 5). This basement membrane, not found around liver cell plates, was in turn surrounded by varying numbers of fibrils showing periodicity of about 600 Å. Fibers usually with a periodicity of about 600 Å and in bundles in contact with the outside of the basement membrane were occasionally found on the circumference of the ductules (Fig. 6).

Portal Fiber Distribution

A loose network of thick collagenous bundles and reticulum fibers was present in the portal tracts between vessels and ducts. Between them, little PAS-positive amorphous material was noted which also stained with Alcian blue. A few cells exhibited the spindle-shaped nuclei with polar nucleoli, characteristic of fibroblasts, and their cytoplasm sometimes contained fine PAS-positive granules. Elementary fibrils averaging 250 Å in diameter seen under the electron microscope exhibited little variations and revealed a periodicity of about 600 Å. They were arranged in bundles of approximately 1500 individual fibrils and had a diameter of 1-2 μ. Some elementary fibrils showed branching, and the spaces between fibrils were electron-transparent (Fig. 6 and 7).

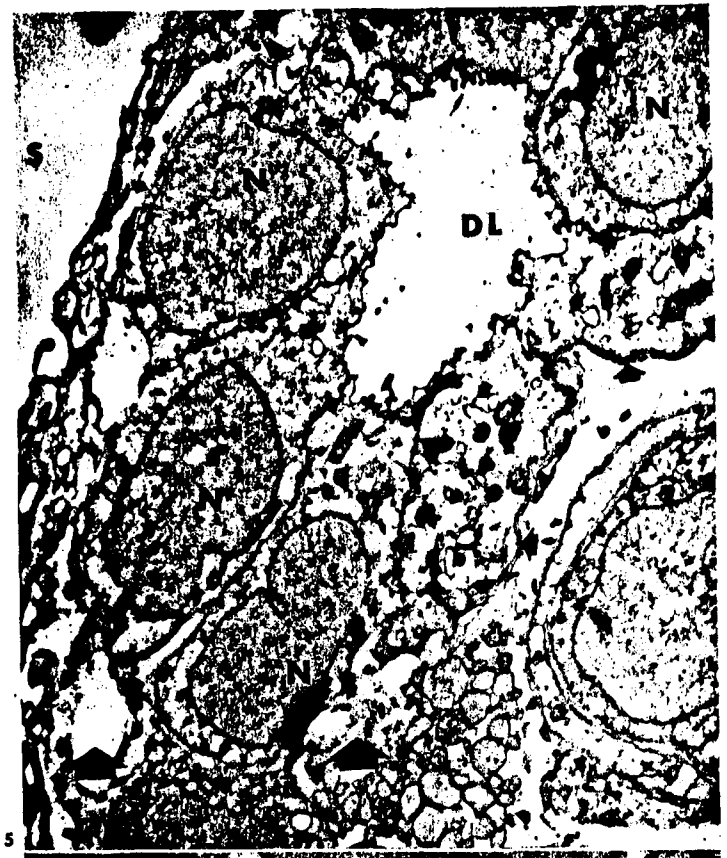
In the normal rat the principal arrangement of the fibers did not vary significantly from that in man except that fewer intralobular reticulum fibers (Fig. 8) and far less PAS-positive material were noted on the borders of the liver cells.

Observations in Abnormal Livers

Pericellular Fibrosis

In mild acute injury to liver cells, as indicated by hydropic swelling, focal cytoplasmic clumping, or bile imbibition, the amount of PAS-positive material around the liver cells was increased, but the arrangement of the fibers did not appear necessarily altered in both routine sections and in thin ones. When the liver cells were considerably enlarged as in fatty metamorphosis, the argentaffin fibers seemed further apart than normal in routine sections and even more so in thin sections, the distance between individual fibers reaching 6 μ. In chronic fatty metamorphosis as seen in gastrointestinal diseases or in chronic alcoholism, the liver cell plates were frequently more than one cell thick and were additionally broadened by fat accumulation so that the fibers appeared even further apart (Fig. 9). The amount of PAS-positive material depended on the degree of liver cell injury. In regenerative nodules of cirrhosis, the reticulum network was sparse while it appeared compressed around the nodules (Fig. 10). This sparcity of fibers was also noted in hepatocellular cancer.

In severe acute liver cell injury as in viral hepatitis or in protracted damage as in chronic fatty metamorphosis with liver cell damage or in cirrhosis, particularly in florid forms, the fibers on the sinusoidal border of the liver cells were increased in number and thickened. This was particularly evident in thin



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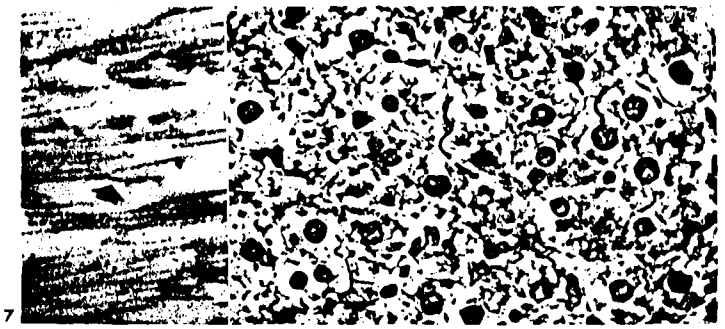

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Fig. 7. Electron micrograph of fibers in a large bundle in portal space of a normal rat, showing (arrow) forking of fibers. (× 38,000)

Fig. 8. Five-micron thick section of normal rat liver. Few reticulum fibers of variable thickness line the sinusoidal border. (Gomori's silver impregnation, × 400)

sections in which the reticulum fibers on the sinusoidal border appeared as closely spaced black dots and comma shapes frequently merging to a continuous membrane of variable width (Fig. 11, 12). Cell loss was not noted in such areas. Under these circumstances, PAS-positive material on the sinusoidal border was considerably increased, and the Kupffer cells were proliferated. Their increased cytoplasm was rich in PAS-positive material. Fibroblasts were usually not seen near epithelial cells surrounded by increased reticulum fibers. Under the electron microscope in acute viral hepatitis or in fatty metamorphosis with liver cell damage, bundles about 0.5 μ in diameter and containing up to 100 fibrils were noted in the tissue spaces in indentations of the sinusoidal borders of the liver cells (Fig. 13). The sinusoidal microvilli in these pockets were flattened against the cell wall by the bundles of fibrils. The normal relationship of Kupffer cells to liver cells was preserved, excluding a loss of liver cells from this location.

In the same conditions, especially in viral hepatitis, single or a few liver cells had disappeared from the other areas. This was indicated in silver impregnations by foci of dense network with heavily interwoven fine fibers sometimes surrounding shrunken or necrobiotic liver cells. These foci were denser and

Fig. 5. Electron micrograph of cross section of ductule of normal rat. *DL* is the ductular lumen showing multiple microvilli; *S* is a sinusoid, and *N* indicates ductular cell nuclei. The ductule is surrounded by a thin basement membrane (small arrows) and by a few small bundles of fibers (large arrows). (× 8,000)

Fig. 6. Detail of area between the two ductular cells in the lower left part of Fig. 5. The fibers (*F*) represented by dots are next to the basement membrane (arrow) which is duplicated in places (open arrow). *N* indicates the nuclei of ductular cells and *L* is the ductular lumen. (× 20,000)

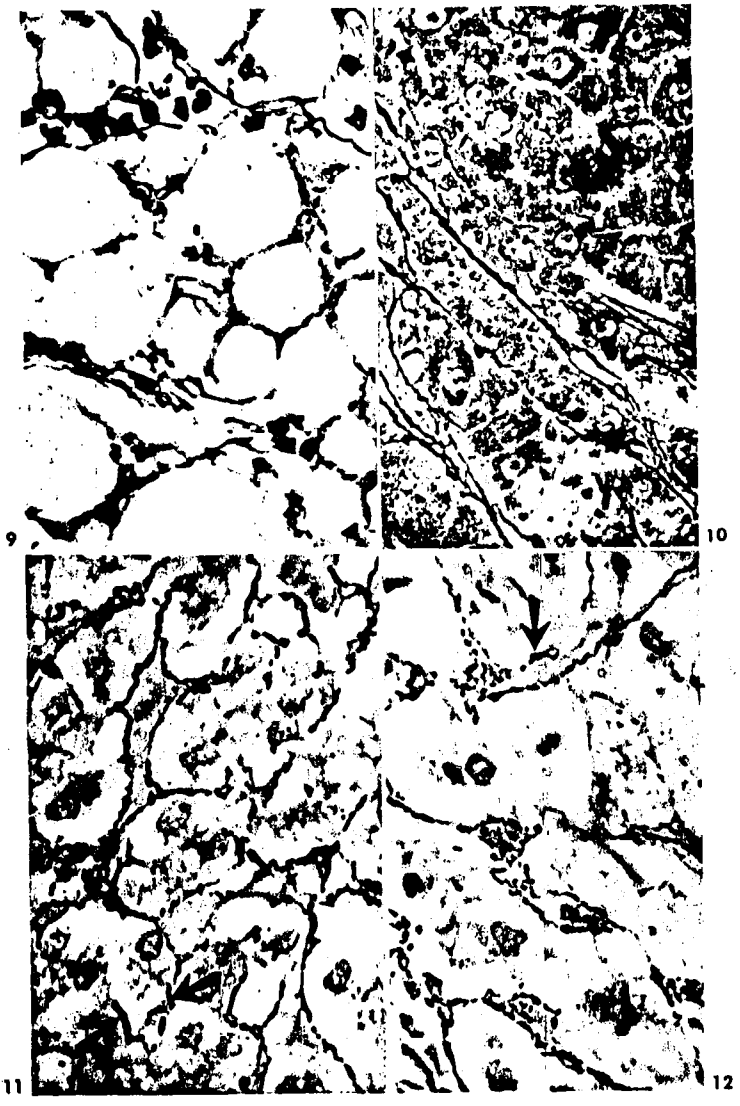


Fig. 13. Electron micrograph of liver from a patient with acute viral hepatitis showing bundles of fibers seen on cross section (solid arrows) in indentations of sinusoidal wall of liver cell, and flattening microvilli (open arrow) against the cell wall. *N* is the nucleus of the liver cell and *M* one of its mitochondria. *NK* is the nucleus of the adjacent Kupffer cell. (× 22,000)

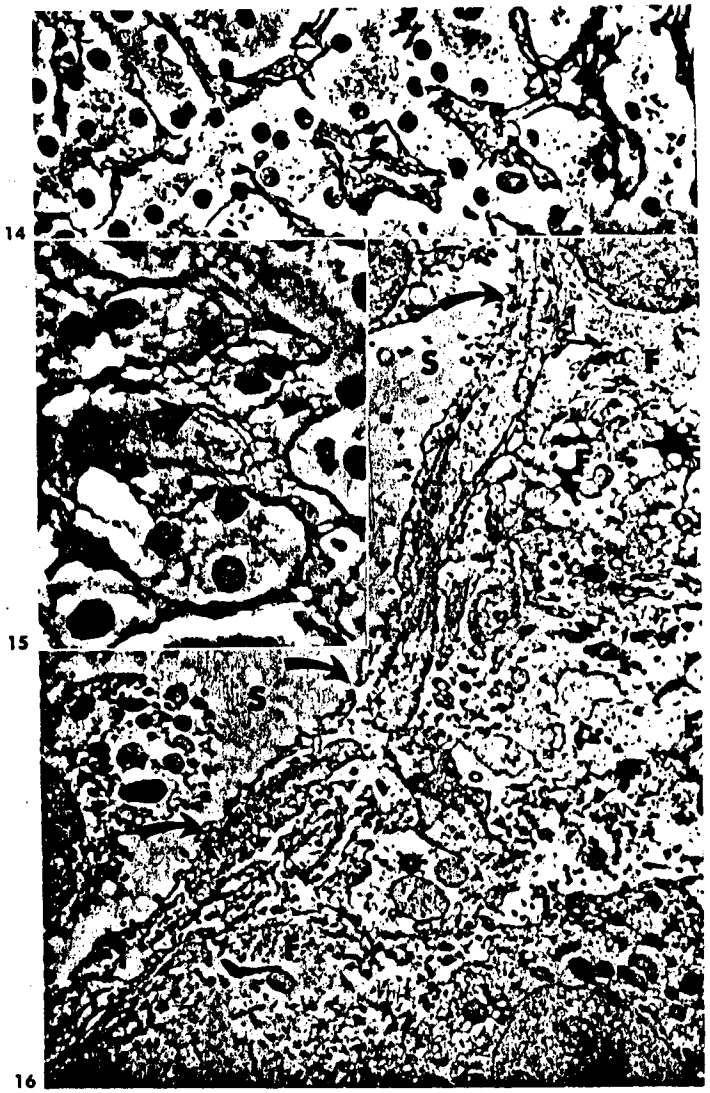
more extensive than the small ones with cross fibers seen in normal livers. In some instances, such scars seemed to persist in the convalescent period when the liver cells had completely recovered (Figs. 14–16). In zones of more extensive parenchymal collapse, sometimes typical fibroblasts were noted. Small foci of collapse were also found in extrahepatic and intrahepatic cholestasis. Under the electron microscope such foci exhibited accumulations of fibrils in parallel or radiating fashion in the extensions of the tissue spaces between two cells or between the liver cells and Kupffer cells. In cholestasis they were in close approximation to bile canaliculi that appeared to be rupturing or had just ruptured (Fig. 17).

Fig. 9. Five-micron thick section of liver biopsy of an alcoholic patient with diffuse fatty metamorphosis. Note scarcity of reticulum fibers which are located around sinusoidal spaces. (Gomori's silver impregnation, × 630)

Fig. 10. Five-micron thick section of liver biopsy of a patient with cirrhosis. Regenerative nodule with almost no reticulum fibers in contrast to increase of fibers from compression around its border. (Gomori's silver impregnation, × 400)

Fig. 11. One-micron thick section of biopsy specimen of a patient with viral hepatitis. Close position of reticulum fibers along the border of liver cells, resulting in their merging in continuous line. In places (arrows) there is conspicuous thickening of the framework (compare with Fig. 2). (Gomori's silver impregnation, × 630)

Fig. 12. One-micron thick section of biopsy specimen of a patient with fatty metamorphosis and liver cell damage. On the sinusoidal border of damaged liver cells (arrow) an increased number of reticulum fibers may be noted, in places appearing thickened. (Gomori's silver impregnation, × 630)



A third type of fiber accumulation was reflected in thick, apparently double layers of reticulum fibers along liver cell plates, frequently associated with brown collagen in silver impregnations (Fig. 18 and 19). The CAB staining was also much heavier, but not necessarily more amorphous PAS material was found. Apparently the reticulum framework of several cells in a plate which had disappeared became displaced toward the wall of the next sinusoid, and under such circumstances, two and sometimes even three rows of fibers were present outside of a single cell. The development was reflected in the aggregation of fibers around shrinking cells (Fig. 20). This increase as a result of collapse differed in arrangement from the thickened framework around damaged liver cells, in which a single row of fibers was maintained. Reduplication of fibers was also noted in both routine and thin silver impregnations and CAB-stained sections in protracted fatty metamorphosis when fatty cysts were present and liver cells had disappeared. Under the electron microscope in the same conditions, occasionally two parallel rows of bundles of fibers, 1–2 μ thick, were seen separated by a layer of necrotic cell debris about 1 μ thick, adjacent to a liver cell (Fig. 21).

In the nodules of both postnecrotic and diffuse septal cirrhosis, focal rarefaction of the reticulum framework as a result of regeneration was intermixed with focal increase from collapse or new formation of fibers.

Periductular Fibrosis

Around proliferating ductules or smallest bile ducts as observed in subacute viral hepatitis or biliary obstruction as well as in almost all types of cirrhosis, both reticulum and collagen fibers were increased, often arranged in an onion peel fashion. Ductular proliferation and periductular fibrosis were particularly conspicuous in postnecrotic cirrhosis (Fig. 22). In thin sections, an almost continuous, silver-impregnated membrane appeared to result from merging of individual fibers (Fig. 23). The proliferated ductules in areas of collapse in acute fulminating viral hepatitis were not surrounded by increased fibers. Where connections between liver cell plates and ductules were frequent in

Fig. 14. Five-micron thick section of liver biopsy specimen of a patient 6 months after severe viral hepatitis. Multiple irregularly shaped aggregations of reticulum fibers indicate area of collapse. (Gomori's silver impregnation, × 400)

Fig. 15. Five-micron thick section of liver biopsy of a patient after recovery from viral hepatitis. Irregular network of reticulum fibers replacing liver cells which have been lost (arrow). (Gomori's silver impregnation, × 900)

Fig. 16. Electron micrograph of liver from a patient after recovery from hepatitis showing liver cell (*LC*) with its nucleus (*N*) in the lower right hand corner. The space above this cell is filled with debris and many bundles of fibers (*F*) while the endothelial lining (arrows) of the sinusoid (*S*) is intact and shows a normal stoma in the lining (middle arrow). *P* is a leukocyte in the sinusoid. (× 16,000)

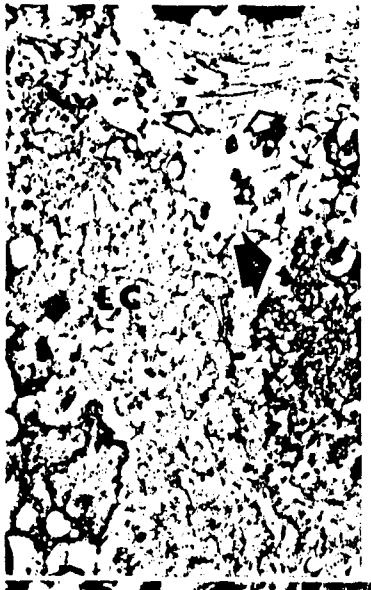


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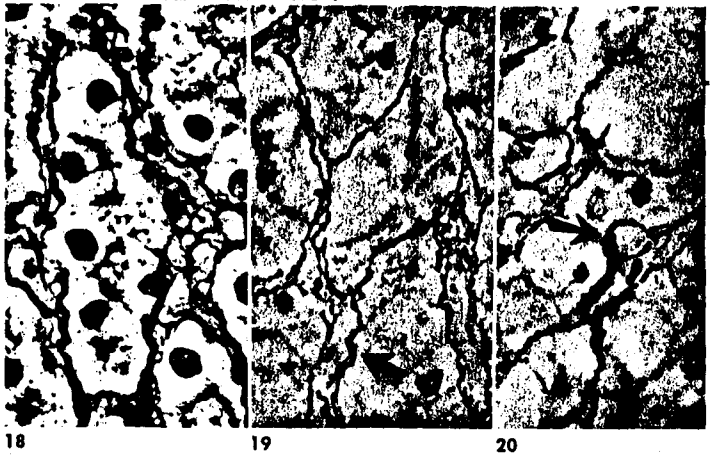
Fig. 17. Electron micrograph of liver of a patient with drug-induced intrahepatic cholestasis, showing fibers (open arrows) in tissue space near rupturing, dilated bile canaliculus filled with bile (solid arrow). *LC* is the liver cell forming part of the dilated canaliculus. (× 20,000)

Fig. 18. Five-micron thick section of liver biopsy specimen of a patient with viral hepatitis exhibiting multiple duplications of reticulum fiber framework around liver cell plates. (Gomori's silver impregnation, × 600)

Fig. 19. One-micron thick section from same case as shown in Fig. 19. Duplication of framework is recognized by two or more rows of single fibers (arrows). (Gomori's silver impregnation, × 630)

Fig. 20. One-micron thick section of same specimen as shown in Fig. 19 and 20, exhibiting approximation of fibers around degenerating liver cell (arrow). (Gomori's silver impregnation, × 630)



18 19 20

Fig. 21. Electron micrograph of liver of a patient with cirrhosis, showing liver cell (*LC*) with its nucleus (*N*) adjacent to two layers of fibers (*A* and *B*) separated by necrotic cell debris (arrow). (× 33,000)

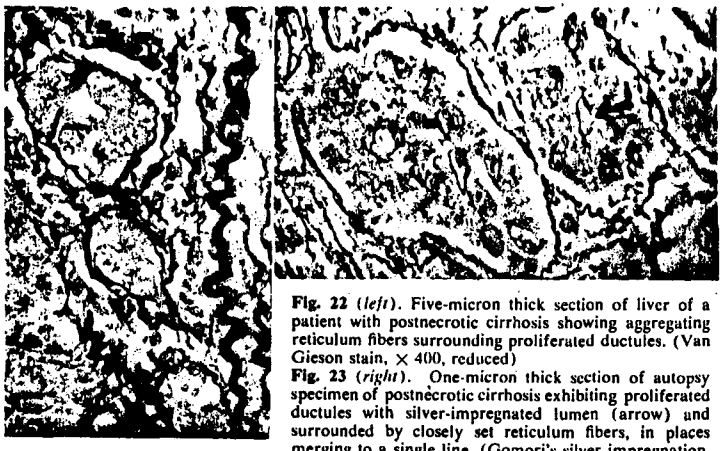


Fig. 22 (*left*). Five-micron thick section of liver of a patient with postnecrotic cirrhosis showing aggregating reticulum fibers surrounding proliferated ductules. (Van Gieson stain, × 400, reduced)
Fig. 23 (*right*). One-micron thick section of autopsy specimen of postnecrotic cirrhosis exhibiting proliferated ductules with silver-impregnated lumen (arrow) and surrounded by closely set reticulum fibers, in places merging to a single line. (Gomori's silver impregnation, × 600, reduced)

chronic conditions as in hemochromatosis or in prolonged biliary obstruction, the liver cell plates near the junction were surrounded by many reticulum fibers, although less than the ductules beyond the junction. Only actively proliferating ductules were surrounded by a PAS-positive amorphous substance. Fibroblasts were not necessarily noted near the connective tissue fibers, but endothelial cells in neighboring capillaries had an increased cytoplasm which was rich in PAS-positive granules. Under the electron microscope, around proliferating ductules characterized by luminal microvilli, many bundles of fibers several microns thick were irregularly intertwined outside the basement membrane, while no fibers were noted inside (Fig. 24).

Portal Tract Fibrosis

Enlargement of portal tracts as a result of fibrosis was produced by one of the following mechanisms: (1) *stellate fibrosis* with collagen membranes extending from the portal tracts into the surrounding parenchyma; the membranes were focally aggregated on the circumference of the portal tracts as seen in chronic fatty metamorphosis and some instances of viral hepatitis; (2) *diffuse fibrosis* with uniform enlargement of the portal tracts by both thin collagen membranes and thick fiber bundles as seen in portal inflammation, viral hepatitis, hemochromatosis, biliary cirrhosis, and schistosomiasis and usually associated with accumulation of inflammatory cells, ductular proliferation, and moderate increase of fibroblasts; (3) *concentric fibrosis* with excess collagen fibers exhibiting a circular arrangement, usually associated with many

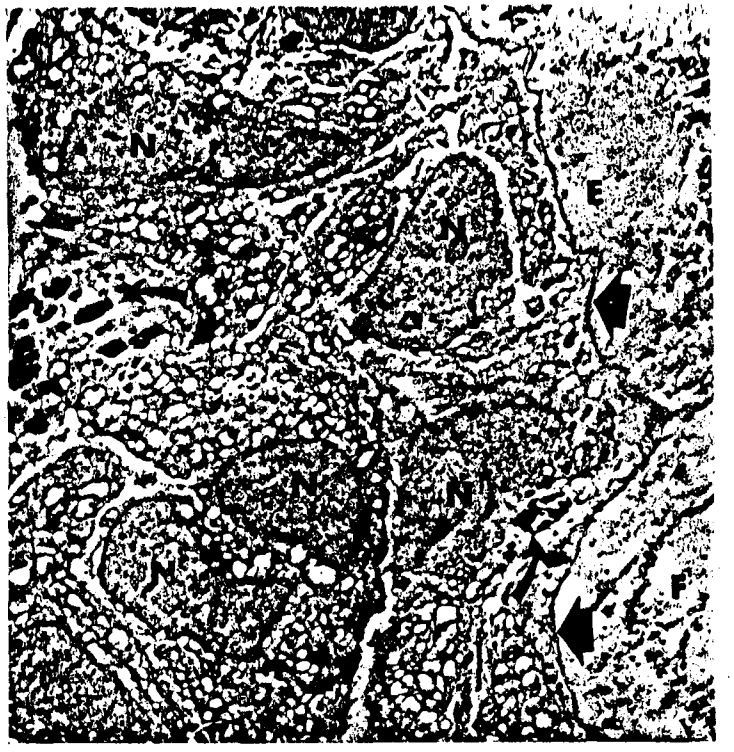


Fig. 24. Electron micrograph of a proliferating ductule from the liver of a patient with primary hemochromatosis. Iron pigment (curved arrows) is present in the cytoplasm of the ductular cells. The nuclei are indicated by *N*. The ductule is surrounded by a thin basement membrane (large arrows) and by very large bundles of fibers (*F*). (× 6,000)

fibroblasts; this type was seen in biliary obstruction, especially in atresia of bile ducts; (4) *fibrosis following collapse*, with aggregation of collapsed reticulum fibers and of wavy collagenous membranes after disappearance of liver cells, as seen in postnecrotic cirrhosis and periportal inflammation; the border between the collapsed zone and the original portal tracts was usually well delineated, and an increase in fibroblasts was not conspicuous; and (5) *irregular fibrosis*, mainly the result of healing granulomas or deposition of extraneous material as in Gaucher's disease and almost always accompanied by accumulation of fibroblasts.

Fig. 25. Electron micrograph of bundles of fibers (*b*) in a portal tract from a patient with portal fibrosis. *M* is the nucleus of a mesenchymal cell. (× 16,000)

In all these conditions, fibers frequently aggregated around proliferated ductules. In accordance with the extent of inflammation as well as the rapidity of fiber formation, variable amounts of PAS-positive interstitial substance were found which usually also gave Alcian blue reaction. The number of portal reticuloendothelial cells with PAS-positive granules appeared also correlated with the activity of the fibrogenesis. Under the electron microscope the striking

difference between the fibrotic portal tract and the normal one was the increase in the number and size of the bundles of fibrils separating the structures by several microns and making the electron-microscopic examination of the portal tract difficult. The fibrils, even in the largest bundles, exhibited the same width and periodicity shown in other locations (Fig. 25).

Observations in Animals

Two types of experimentally produced lesions were studied: the subacute ethionine intoxication,[39] because of its diffuse, intralobular new formation of fibers, and the local transient fibrosis following intrahepatic injection of carrageenin.[45] Detailed descriptions of the changes are being presented elsewhere, and only the observations pertinent to this study are presented here.

In subacute ethionine intoxication of approximately 7 weeks' duration, a conspicuous increase of reticulum fibers was preceded by an increase of perisinusoidal PAS-positive material. The increase of fibers appeared to be the result of several processes; one was new formation around damaged cells; another was collapse after destruction of cells. In fine connective tissue septa thus formed, many PAS-positive histiocytes were seen together with occasional fibroblasts. The most conspicuous increase of fibers was found around the excessively proliferating ductules. It was particularly severe in cholangiofibrosis in which membranes in onion peel-like arrangement around the ductules consisted of merging reticulum fibers. They sometimes predominated without conspicuous increase of fibroblasts. In contrast, within nodules composed of regenerating liver cells, the reticulum fiber framework was very scant, and also the Kupffer cells exhibited few PAS-positive granules. Under the electron microscope the ductules consisted of irregularly arranged cords of epithelial cells around a lumen into which small microvilli projected. The cells in turn were surrounded by a thin basement membrane without periodicity. Collagenous fibrils of usual width and periodicity were seen on the outer surface of the basement membrane and appeared to be either peeling from it or being deposited on it. Some distance from the membranes, several fibrils seemed to combine to form bigger bundles. Sometimes the fibers formed a continuous envelope about 0.5 μ in thickness, surrounding the entire ductule; they did not appear to be attached to any of the mesenchymal cells around the ductules.

Intrahepatic injection of carrageenin in rats was followed by necrosis of liver cells, while in the border zone a PAS- and Alcian blue-positive ground substance was deposited. Proliferation of fibroblasts into this substance was noted within 48 hours. They were mainly derived from mesenchymal cells of the portal tract. Simultaneously ductular cells sprouted. After approximately 3 days, the formation of reticulum fibers became abundant. The fibers were wavy, arranged between cells, and were particularly noted in approximation

Fig. 26. Five-micron thick section of liver of rat which had carrageenin introduced into the liver 4 days before section was made. Extensive proliferation of reticulum fibers can be seen, particularly in approximation to proliferated ductules. (Gomori's reticulum impregnation, × 400, reduced)

to ductules (Fig. 26). Under the electron microscope in the border between the necrosis and the cellular proliferation with ground substance accumulation, collagen fibrils were noted either singly or in small bundles at a time when reticulum fibers were not yet seen under the light microscope. These fibrils had the same width and periodicity as those seen in normal liver. Nowhere, even during the period of intense fibrogenesis, were fibrils noted within the cytoplasmic borders of any cell.

DISCUSSION

Fiber accumulation in the liver, designated as fibrosis, results either from aggregation of preformed fibers after the disappearance of intervening liver cells in the form of collapse, or from formation of new fibers. Fibrosis after extensive collapse, as following centrolobular passive congestion or massive necrosis in postnecrotic cirrhosis, concerns problems of mechanics dealt with especially in studies of cirrhosis and advantageously investigated with three-dimensional reconstruction.[10] New formation of fibers is the more difficult problem. Fibrogenesis, sometimes associated with collapse[21] occurs in three locations: (1) in the portal tract where it is similar to fibrogenesis in connective tissue anywhere else, (2) in the parenchyma around liver cells representing a process probably similar to parenchymal fibrogenesis in other organs and being thus the most interesting aspect of this study, and (3) around proliferating ductules, being a feature shared with such organs as the pancreas, in which similar ducts exist. For all three locations several questions arise. One concerns the fine structure of the hepatic fibers in comparison with those in connective tissue which are characterized under the electron microscope by a periodicity of 640 Å. A second problem involves both the nature of the matrix which surrounds the fibers and the presence of a basement membrane similar to that around renal tubules. The third question regards the contribution of cells in fiber formation, particularly the role of fibroblasts.

Fibrosis in the portal tract shows several well-defined morphologic patterns, in part depending on the etiology of the process. While it results from collapse

in some instances, as in postnecrotic cirrhosis, in most others new fibers are formed. Pre-existing and newly formed fibers consist of the same elementary fibrils as anywhere else in connective tissue. Newly formed fibrils exhibit the same characteristic as the preformed ones being arranged in bundles or groups of bundles. The material surrounding the fibril (matrix) has also the same histochemical character as material anywhere else, consisting mainly of neutral PAS-positive mucopolysaccharides and acid Alcian blue-staining ones. The increase of fibroblasts in the portal tract depends upon the activity of the process and is pronounced in fibrosing granulomas and in children with congenital atresia of the bile ducts. Fibroblasts are conspicuous, but less so in adult extrahepatic biliary obstruction[38] or in chronic inflammation of the portal tract. In the slowly developing portal fibrosis of the Symmer's lesion in schistosomiasis, fibroblasts appear hardly increased.[30] In the very rapid fibrogenesis produced by the intrahepatic injection of carrageenin, large numbers of fibroblasts proliferate from the portal tract.

Within the lobular parenchyma relatively few, mainly isolated, reticulum fibers are demonstrated in thin sections around the sinusoidal border of normal liver cells. The electron microscope shows them to lie in the tissue space between Kupffer cells and liver cells. The diffuse PAS-positive material at the sinusoidal border of the liver cells is very sparse. It was described by Gersh and Catchpole[11] using the freeze drying technic, by Aterman,[2] and by Wassermann.[40] It is mainly of neutral and partly of acid mucopolysaccharide character. The limit of resolution of the light microscope even in very thin sections does not permit one to exclude definitely that this material does not lie within the cytoplasm of Kupffer cells or the peripheral zone of the liver cells. This difficulty cannot be resolved by the electron microscope because of the known lack of electron opacity of mucopolysaccharides. Nevertheless, this material probably represents a ground substance in tissue spaces.[40, 41] It is increased in various types of liver cell damage simultaneously with a widening of the spaces. A basement membrane demonstrable by electron microscopy around glandular and ductular structures in other organs, does not exist around liver cells. The appearance of a continuous membrane in routine silver-impregnated sections is created by overlay of individual fibers.

The reticulum framework is expanded in conditions associated with swelling of the liver cells such as acute liver cell injury and, especially, fatty metamorphosis. This entails distinct separation of individual fibers as particularly evident in thin sections. If regeneration leads to an increase of cells in the individual plates as in fatty metamorphosis[28] or in cirrhotic nodules, or if cancer produces the increase, the sparsity of fibers is even more conspicuous. In contrast, in severe or chronic liver cell injury, as in human viral hepatitis or some cirrhoses, and in subacute ethionine intoxication of the rat, the retic-

ulum framework is increased as the result of three mechanisms. The first is uneven or irregular collapse after necrosis and disappearance of cells, as seen in viral hepatitis and subacute biliary obstruction or after disappearance of fatty cysts in prolonged fatty metamorphosis.[16] It is indicated in silver impregnations by a dense focus composed of an irregular network of reticulum fibers. Larger areas of collapse result in distinct scars sometimes associated with new formation of fibers and the presence of fibroblasts. The second mechanism is expressed in a focal duplication of the reticulum framework seemingly explained by disappearance of whole liver cell plates and subsequent regeneration of neighboring plates compressing the framework of the missing one. This flat collapse may also be accompanied by collagen staining of the reticulum, but typical fibroblasts are not necessarily seen. The third process is an actual increase in fibers around damaged liver cells. This occurs even around swollen cells and differs from the approximation of the framework around cells shrinking during degeneration. In thin sections impregnated with silver, individual reticulum fibers appear far more closely set, and they occasionally merge to imitate a continuous basement membrane, which however, is not demonstrated under the electron microscope. This reveals rather distinct accumulation of elementary fibrils in the tissue space to form large bundles. While in most such instances, the amount of perisinusoidal "ground substance" is increased, cells with the morphologic characteristics of fibroblasts are not necessarily seen in the vicinity. However, the Kupffer cells are usually increased in number and contain much cytoplasmic PAS-positive material, supporting the hypothesis that the Kupffer cells act as fibroblasts forming the ground substance. The combination of increase in fiber from any one of the processes described, together with relative dilution of fibers by regeneration produces the polymorphism of the framework of cirrhotic nodules.

Basement membranes similar to those around proliferating ductules are found around resting ductules. However, the former are surrounded by an increased number of collagen fibers. They appear in contact with this basement membrane in a way similar to that observed with the basement membrane of the cells of the adipose tissue of the rat.[7] PAS-positive amorphous interstitial substance is increased only in active fibrogenesis, in which neighboring reticuloendothelial cells and capillary endothelial cells[28] are implicated as sources, in view of the presence of PAS-positive granules. Typical fibroblasts are recognized with extensive periductular fibrosis. This type of hepatic fiber formation seemed to account for most of the fiber increase in subacute ethionine intoxication of the rat and particularly in cholangiofibrosis. It also contributes in postnecrotic cirrhosis and also in diffuse septal cirrhosis or alcoholic fatty liver. Where connections of liver cells with ductules may be an important feature, as in secondary hemochromatosis associated with anemia or in the frustrated

ductule formation in biliary cirrhosis,[24] this mechanism of fibrosis is of great importance.

The various types of fibrogenesis in the liver follow the same principal pattern whether they take place around hepatic cell plates, around ductules, or in the portal tracts. All three types contribute to the variable manifestations of hepatic fibrosis. The same processes occur which characterize fiber formation anywhere else, modified by the specific circumstances of a parenchymal organ. The elementary fibrils have the characteristic electron microscopic appearance, the periodicity being the same within the limits of a possible error (perhaps up to 20 per cent) imposed by the difficulties inherent in the study of embedded tissue sections under the electron microscope.[34] Hepatic fibrogenesis entails, therefore, reduplication of the same type of elementary fibrils, with formation of bundles and groups of bundles. No electronmicroscopic difference exists between reticulum and collagen fibers as demonstrated tinctorially under the light microscope in so far as individual fibrils are concerned. The collagenous membranes recognized in routine light-microscopic sections and differentiated by their wavy appearance from fibers[10] do not represent different structures but consist of fibers arranged in a sheetlike fashion.

Hepatic fiber formation in all locations seems to be preceded by the deposition of an intercellular amorphous ground substance of mucopolysaccharide character as also indicated by the increased hexosamine content determined chemically in subacute ethionine intoxication of the rat.[39] It is generally accepted that such a ground substance is formed by fibroblasts on the basis of staining reactions and radioautographs.[25, 40] Where pericellular and periductular PAS-positive extracellular material increases without conspicuous presence of cells with the morphologic features of fibroblasts, reticuloendothelial cells—particularly Kupffer cells—might assume fibroblastic function. This is also suggested by the presence of similarly staining material in their cytoplasm. The problem is complicated, however, by similar histochemical reactions of cytoplasmic granules apparently engulfed by phagocytosis.[31]

The role of cells in the formation of collagen fibrils themselves is problematical in all three types of fibrosis. Despite various reports in the literature of intracellular fibrils in hepatic mesenchymal cells,[5, 9, 41] in this study, despite thorough search under the electron microscope, intracellular fibers were never seen not even in the accelerated hepatic fibrogenesis stimulated by carrageenin. However, this may still be a matter of technic requiring further investigation on material more suitable than the liver. To date, the question is not settled whether elementary collagen fibrils or only their precursor (probably in monomeric form[15]) are formed within the cytoplasm of cells.[6, 19, 34] Strong evidence exists that the elementary fibrils form on the outer surface of cells[34, 40] such as fibroblasts. Fiber formation on the surface of epithelial cells has also been

described.[35, 43] In each instance, apparently, stress is a localizing factor. Although no evidence exists that either the hepatic or the ductular cells form fibers, they may act as a stimulus or mold around which the fibers are laid down by mesenchymal cells such as reticuloendothelial cells. In carrageenin fibrogenesis, fibers seem to develop in close approximation to ductules. Altered liver cells or proliferating ductular cells possibly act as a stimulus in a way similar to that of extracellular material like bile or iron in biliary obstruction or hemochromatosis.

These investigations support the viewpoint[9, 27, 32] that fiber formation in hepatic cirrhosis is in most instances not primary but rather secondary to alterations of epithelial cells.

SUMMARY

The various types of hepatic fibrosis resulting from either accumulation of preformed fibers or from new formation of fibers were studied in the human and rat livers by routine microscopic investigation, by observations of ultrathin sections, by histochemical technics, and by electron microscopy. In the normal liver, few collagenous elementary fibrils are silver impregnated as reticulum fibers around hepatic cells. A basement membrane, demonstrable by electron microscope, is absent, and cells with the morphologic characteristics of fibroblasts are not conspicuous. Around bile ductules a basement membrane exists which is in contact with collagen fibrils.

Hepatic fibrosis occurs in the portal tracts or around hepatic cell plates or around proliferated ductules, with varying contributions of each mechanism to the different forms of cirrhosis. Portal fibrosis is associated with various shapes of the tracts, reflecting the etiology of the process, and is usually accompanied by an increase in fibroblasts. Intraparenchymal fibrosis may be the result of new formation of fibrils in the tissue spaces around damaged liver cells or collapse after necrosis of liver cells, which may be accompanied by fiber new formation. Larger scars reveal fibroblasts similar to those in the portal tracts. Duplication of the framework around liver cells may result from disappearance of plates and their replacement by neighboring plates. Around enlarged liver cells and in regeneration and cancer, the reticulum framework appears rarified. Periductular new formation occurs in association with the basement membrane and with varying numbers of fibroblasts. It represents an important pathway in human and experimental fibrosis.

All types of hepatic fibrogenesis follow similar lines which are in principle the same as in connective tissue anywhere. Elementary fibrils have the same width and periodicity throughout within the limits imposed by examination in embedded-tissue sections. New formation of fibers results in aggregation of bundles of fibrils and groups of bundles. No difference exists, from the standpoint of electron microscopy, between the fibers stained under the light microscope with collagen stains and those impregnated as reticulum. Hepatic fibrogenesis in any location is associated with the formation of a similar polysaccharide matrix. It is probably formed by fibroblasts or possibly by reticuloendothelial cells assuming the function of fibroblasts. No evidence was found that the collagen fibrils are formed within the cytoplasm of cells. They seem to form on their outer surface as well as near basement membranes. The nature of the stimulus for the fiber formation around damaged liver cells and proliferated ductular cells is unknown.

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Influence of Age on Transit Time of Cells of Mouse Intestinal Epithelium

I. Duodenum

S. Lesher, Ph.D., R. J. M. Fry, M.B., and Henry I. Kohn, M.D., Ph.D.*

AT THE END of the last century, Bizzozero[1] suggested that the mucosal cells lining the gastrointestinal tract were being constantly replaced. It is now well established that new cells are produced in the crypt, pass to the base of the villus, and thence travel upwards to the tip of the villus, or extrusion zone, from which they drop off into the lumen.[3, 7, 8, 10] Quantitative information concerning the kinetics of this process has been scanty. However, the development of tritiated thymidine for use in autoradiography[6] has provided a technic that has greatly facilitated the study of the genesis, life, and fate of cells. When injected into mice either intraperitoneally or intravenously, tritiated thymidine reaches the cells of the intestinal crypts quickly, is taken up by the cells that are synthesizing DNA, and then remains incorporated in the DNA molecule for the lifetime of the nucleus or its progeny. Most of the labeled thymidine not taken up by the cells is eliminated in the first hour after injection. The only apparent disadvantage of tritiated thymidine is that some damage occurs with doses as low as 1 μc. per gm.[4, 5] Hence, by using this technic it is now possible to determine the rate of cell division in the crypt and the time required for the new cells to pass to the extrusion zone. Such data are of importance in under-

From the Division of Biological and Medical Research, Argonne National Laboratory, Lemont, Ill., and the Radiological Laboratory, University of California School of Medicine, San Francisco, Calif.

The authors acknowledge the technical assistance of A. Sallese and E. Staffeldt.
This work was performed under the auspices of the U. S. Atomic Energy Commission.

* Resident Research Associate from Trinity College, University of Dublin, Dublin, Ireland.