Extracellular matrix synthesis in the mammalian liver.

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SUMMARY

The role of the extracellular matrix in the liver is complex, and this thesis will study both the structure and role of normal hepatic extracellular matrix, and how these are affected in liver disease. In order to do this, it was necessary to develop new techniques as well as using methods already available.

In Chapter 1, the basic structure of the liver is described. It also identifies the different types of liver cells which are present, and their known functions. Liver injury is examined in Chapter 2 and this covers both the possible responses of the hepatic cells to injury, and the patterns of injury found in different disease entities.

Chapter 3 identifies the different component groups of the extracellular matrix, and their general structure and function. The largest of these groups is the collagen family, and this is studied in greater detail in Chapter 4. The remaining components of the extracellular matrix are individually described in Chapter 5.

The previous three chapters looked at the components found in all extracellular matrices, and in Chapter 6 the hepatic extracellular matrix is described in greater detail. This chapter covers both the composition of the extracellular matrix and the cells which are believed to be responsible for their synthesis. It summarises the knowledge of the hepatic extracellular matrix that was available at the beginning of this study.

In Chapter 7, the different techniques available for the ultrastructural examination of liver tissue are described and compared. This chapter also includes the development of novel techniques for the ultrastructural study of the hepatic extracellular matrix. The materials and methods are detailed in Chapter 8, including the types of liver tissue used.
The techniques described in Chapters 7 and 8 were used to map the distribution of the extracellular matrix in normal human liver in Chapter 9. It was necessary to identify the normal distribution of the extracellular matrix in a number of biopsies in order to identify any changes in the distribution which may occur during disease processes. A range of components were examined, including collagen types I, III, IV, V, and VI, laminin, vitronectin and fibronectin. At this stage, it was decided which antibodies used in the study of normal liver were suitable for continuing studies in diseased liver.

It was decided to examine the effects of liver disease on the distribution of collagen types I, III, IV, and

VI, as well as on fibronectin. These are illustrated in Chapter 10.

In the last chapter, a number of points arising from this study are discussed. These include the validity of the techniques used, technical problems with the immunolabelling techniques used, the altered distribution of the extracellular matrix in diseased liver, the novel description of type VI collagen distribution and the possible cellular origin of the extracellular matrix proteins. Finally, the overall, and extremely complex role of the extracellular matrix is examined, both in normal and diseased liver.

The most important aspects of this work fall into two categories. The first is the development of the ultracryomicrotomy techniques which were necessary in order to carry out the immunocytochemical studies. Once this part of the work had been completed, it was possible to study new aspects of the hepatic extracellular matrix in great detail, including the distribution of type VI collagen, and the development of basement membranes in the space of Disse .

CHAPTER 1 STRUCTURE OF THE LIVER

1.1 Liver architecture

The liver is one of the most important organs in the body, and has several major functions including the processing of nutrients present in portal blood, removal and degradation of toxins such as bacterial endotoxin and endogenous substances (e.g. hormones), and the phagocytosis of immune complexes and other particulate matter.

The structure of the liver reflects the complex range of functions and metabolic demands placed upon it. The microcirculatory architecture allows almost free access of blood plasma to the hepatocytes. In the adult liver of most species, the hepatocytes form plates, or cords, of single cell thickness. The capillaries which lie adjacent are known as the sinusoids, and are lined by endothelial cells. The sinusoids are specialised capillaries in that not only are they fenestrated but they lack any identifiable basement membrane when examined ultrastructurally. Evidence that some form of basement membrane is present has come from immunohistochemical studies which have purported to demonstrate the presence of basement membrane components at that site, i.e. type IV collagen and laminin forming an amorphous material (Hahn et al, 1980; Griffiths, Keir and Burt, 1991). This is however controversial and will be referred to later in the text. The structure of the endothelium imparts upon the sinusoid its porosity, this is important for facilitating transport of materials between the sinusoidal lumen and the hepatocytes. The sinusoidal blood flow is of low

(a) Structure of the acinus

1. Acinar agglomerate
2. Three complex acini
3. Simple acini
4. Acinuli

The acinar agglomerate is supplied by a portal vein and hepatic artery branch. This sub-divides into three branches, each supplying a complex acinus.

(b) Comparison of acinar structure and lobular structure

PS - portal space ThV - terminal hepatic vein

1, 2, 3 - microcirculatory zones

- - - - - outline of classic lobule

(c) Comparison of terminologies

Lobular	Acinar
Central, centrilobular, centrizonal	Perivenular, acinar zone 3
Mid-zonal	Acinar zone 2
Peripheral, periportal acinar zone 1	Periportal,
Multilobular	Multiacinar
Panlobular	Panacinar
Central/central (complex)	Peri-acinar
Central/portal	Peri-acinar (simple) peripheral acinar
Portal/portal	Portal/portal

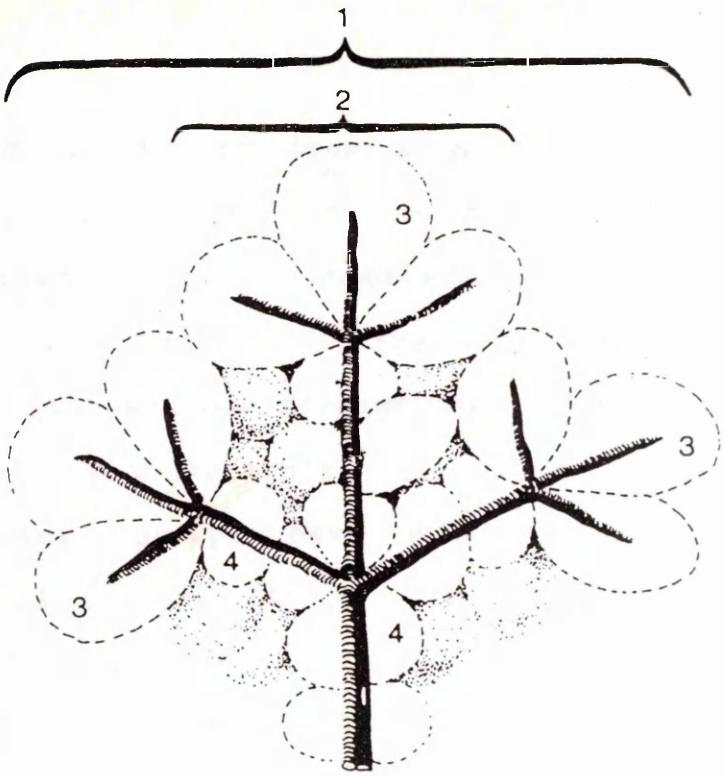

Figure 1.1 (a)

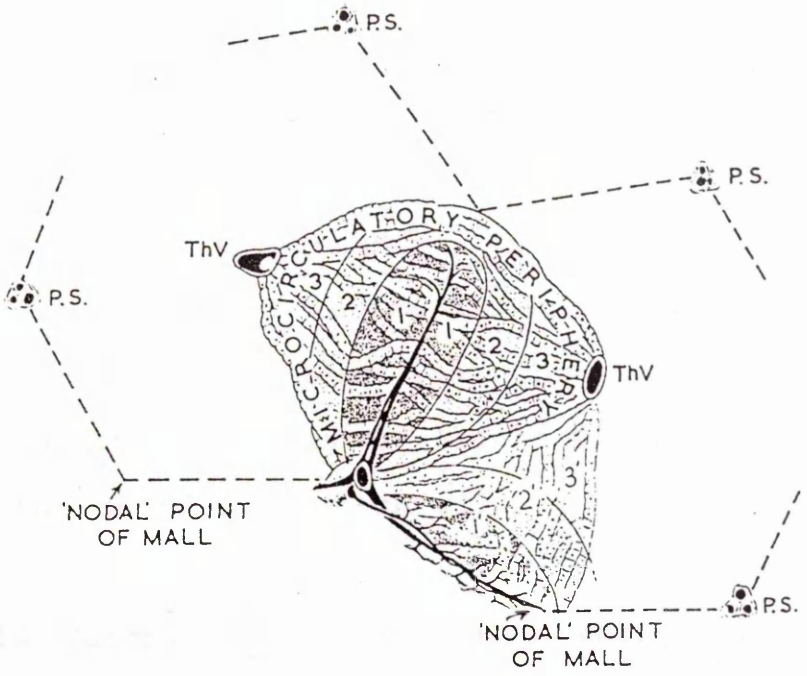

Figure 1.1 (b)

Illustration reproduced from Pathology of the Liver, 2nd edition
(Eds MacSween RMN, Anthony PP and Scheuer PJ.)

pressure and is derived from splanchnic blood from the portal vein, and blood from the hepatic artery. The splanchnic blood contains absorbed nutrients and toxins, while the hepatic artery contains mainly nutrients. The sinusoids carry the blood through the liver, and outflow is via the hepatic vein branches.

Several groups have attempted to define the functional unit of the liver. The concept of the liver lobule was defined by Elias in 1949. It is a polyhedral structure and is usually represented as a hexagon with a terminal hepatic vein at the centre, and portal tracts lying at the corners of the lobule (see Fig 1.1). The liver increases in size by growth and division of the liver lobules. This involves an increase in the number and size of hepatocytes and a reduction in the size of the liver sinusoids. An alternative structural arrangement of the liver is the so-called acinus. Rappaport et al described this as the microcirculatory unit of the liver which can be split arbitrarily into three zones (Fig 1.1). Zone 1 is the periportal zone, zone 2 is the mid-zone, and zone 3 is the perivenular zone. These zones are often used to describe the location of cells or lesions within the liver, and this is the definition which will be used throughout this study.

There is functional heterogeneity among the three zones, and this may be due to the unidirectional flow of blood in the liver. Hepatocytes in zone 1 receive the highest concentration of solutes in the blood, whereas blood entering zone 3 has already been modified by the

hepatocytes in zones 1 and 2. Examples of zonal heterogeneity include hepatocyte uptake of galactose, this being highest in zone 1 (Goresky, Bach and Nadeau, 1973). It has been suggested that sinusoids within zone 1 are narrow and tortuous to allow slow passage of solutes and maximum interaction with hepatocytes, while zone 3 sinuoids are straighter and wider to allow easy drainage of the acinus (Miller et al, 1979). Cytochrome P-450 isoenzymes, one of the key hepatic drug metabolising systems are found in increasing amounts from zone 1 to zone 3 (Gumucio and Chianale, 1980). The distribution of these enzymes means that zone 3 also contains the highest concentration of toxic metabolites. This is consistent with the susceptibility of zone 3 hepatocytes to toxic injury. The metabolism of glucose also has a zonal distribution (Agius et al, 1990). Gluconeogenesis predominates in the periportal zone as does glycogen formation. The same cells are active in glycogenolysis when the appropriate hormonal signal is received. The cells in zone 3 are most active in glycolysis. The location of the enzymes involved in these metabolic processes is also zonal. When rat liver is perfused in a retrograde direction the metabolic zonation is reversed suggesting that the hepatocytes can quickly alter the expression of their enzymes if there is a change in physiological conditions.

The liver is encapsulated in a fibrous sheath called Glissons capsule. There are two tracts of connective tissue within the liver. The larger one extends into the liver at the porta hepatis, and constitutes the fibrous

tissue of the portal tracts throughout the liver. It contains the afferent blood vessels, bile ducts nerves and lymphatics. The smaller tract surrounds the hepatic veins. The two tracts do not connect, and are physically separated by the parenchyma, although the vascular structures do connect by way of the sinusoids.

1.2 Cellular components of mammalian liver

Hepatocytes (also referred to as the parenchymal cells) represent 60% of the liver cells. Because they are larger in size than the other cell types which are collectively referred to as the non-parenchymal cells, they occupy almost 80% of the liver by volume. Five percent of liver volume is taken up by the sinusoidal cells, which comprise four distinct cell populations: sinusoidal endothelial cells, fat-storing cells (also called Ito cells, perisinusoidal cells or lipocytes), Kupffer cells (hepatic macrophages) and pit cells (large granular lymphocytes, or liver associated lymphocytes). The remainder of the liver is composed of (1) other cell types (vascular endothelium, portal tract fibroblasts, bile duct epithelium, neurones and associated Schwann cells), (2) extracellular spaces such as the sinusoidal lumen and the space of Disse, and (3) the extracellular matrix.

1.3 Hepatocytes.

The hepatocytes are large polygonal epithelial cells, which have three distinct membrane domains. These are: (1) the basolateral surface with microvilli,

(perisinusoidal and pericellular), (2) the straight or contiguous domain, and (3) the bile canalicular surface, where the adjacent cell membranes form narrow channels, the canaliculi, for the transport of bile. The plasma membrane of the hepatocyte contains a relatively large amount of glycosylated protein and lipids and a high cholesterol:phospholipid ratio (Arias,1988). The different domains have characteristic features. The sinusoidal surface is involved in phagocytosis and endocytosis, as indicated by the formation of clathrin-coated vesicles and the presence of a wide range of membrane receptors. The canalicular membrane makes up about 15% of the total surface and has different receptors to the basolateral surfaces. Bile acid and other organic anions secreted by the liver cells are transferred into the canaliculus, and its transport along the canaliculus may in part be due to the contractile capability of the canalicular membrane. The hepatocyte contains a large nucleus, and has a well developed endoplasmic reticulum and Golgi apparatus, indicating active protein synthesis. It also contains large numbers of mitochondria.

There are a few metabolic processes which are unique to hepatocytes, such as

i. The urea cycle, which regulates ammonia and amino acid metabolism.

ii. The regulation of cholesterol metabolism

iii. Formation and conjugation of bilirubin.

iv. Formation of glycogen rosettes.

In contrast, there are a number of processes where the hepatocyte is the principal, but not exclusive site i.e. plasma protein synthesis.

1.4 Non-parenchymal cells.

1.4.1 Bile Duct Epithelial cells.

Bile ducts are lined by epithelial cells, which have smaller nuclei and mitochondria than hepatocytes. They are tall columnar cells with basally situated nuclei, and have a basement membrane. In the small intrahepatic bile ducts, the cells may be cuboidal. They can also be distinguished from hepatocytes because they lack the enzyme glucose-6-phosphatase which is characteristic of hepatocytes. They do contain gamma-glutamyl transpeptidase. Bile duct epithelial cells have a characteristic cytokeratin profile, with types 7, 8, 18 and 19 all present (Van Eyken et al, 1987).

1.4.2 Fat-storing cells.

These cells were first described morphologically in 1869 by Boll, but it was not until 1973 that Ito described them as a separate cell type. Fat-storing cells are localised underneath the endothelial lining, within the space of Disse, and processes of the cell can be found in gaps between adjacent hepatocytes. They are identified ultrastructurally by a range of features. These include fat droplets in the cytoplasm, which range in both size and number between species (Ito, 1973). The droplets may be up to 2μm in diameter (Yamamoto and Ogawa, 1983), and

display a rapidly fading green auto-fluorescence with light of wavelength of 328nm.

The lipid droplets are the most prominent feature of fat-storing cells, and contain vitamin A metabolites such as retinyl esters (Brouwer et al 1986), high concentrations of retinol binding proteins and retinoic acid binding protein, and cholesterol. The number of fat droplets varies with age and diet. Fat-storing cells contain 75% of the liver retinoid content, and the parenchymal cells contain 21% (Goodman and Blaner 1984).

The composition of the remaining cytosolic component is similar to that of fibroblasts, with well developed endoplasmic reticulum, Golgi complex, multivesicular bodies and some small lysosomes. Many groups have stated that these are in fact the precursors of the myofibroblast-like cells found in hepatic fibrosis (Friedman, 1990; Mak and Lieber, 1986).

There are few phenotypic markers for fat-storing cells in normal human liver. Although the presence of cytoplasmic lipid can be used for their identification in semi-thin sections, these are not readily identifiable by routine microscopy. They can be distinguished from Kupffer cells and endothelial cells, as they lack the markers for these cell types, but positive identification is difficult. Rat liver fat-storing cells express the intermediate filament protein desmin and this can be used in immunohistochemical studies (Burt et al, 1986). Desmin can be demonstrated

in isolated cell preparations of human fat-storing cells (Friedman et al, 1992) but cannot be readily demonstrated in immunocytochemical studies of human liver (Ahmed et al, 1991). Activated human fat-storing cells appear to express the α-smooth muscle isoform of actin (Matthews and Burt, 1993).

1.4.3 Kupffer cells

These account for about 10% of all liver cells and 80-90% of fixed macrophages in the mononuclear phagocyte system (MPS). They are anchored to the endothelial cells by cytoplasmic processes, with almost 50% of all Kupffer cells found in the periportal zone. The rest are evenly divided between the perivenular and midzones suggesting that the major site of endocytosis is where the blood enters the sinusoid. The contacts with endothelial cells do not involve structures such as desmosomes, but they are strong enough to prevent blood flow dislodging the Kupffer cells. Endocytosis can either occur as pinocytosis (internalisation of liquids), or phagocytosis (internalisation of particulate matter, including bacteria and cell debris). As well as endocytosis, the functions of Kupffer cells include secretion of mediators, processing of antigens and their presentation to immunocompetent cells, and enzyme catabolism (Jones and Summerfield, 1988).

Kupffer cells have morphological appearances typical of macrophages. The cell body contains numerous blebs, microvilli and lamellipodia (Smedsrod et al, 1985), accounting for its stellate appearance in the sinusoid.

Within the cytoplasm there are many lysosomes, pinocytotic vesicles and cisternae, indicative of the role of Kupffer cells in phagocytosis. Ultrastructurally this can be demonstrated by the presence of micropinocytotic vesicles and both endotoxin and lysosomal enzymes may be cleared in this way (Stahl and Schlesinger, 1980)

If the material is particulate and greater than 10 nm in diameter, then it is taken up by phagocytosis. Particulate matter can be taken up in two ways -

(i) non-specific opsonin-mediated phagocytosis, using substances such as fibronectin (Cardarelli et al, 1984).

(ii) immunospecific opsonin-mediated phagocytosis, using Fc receptors or C3 receptors (Munthe-Kaas, Kaplan and Seljelid, 1976).

Phagocytosis can also occur without opsonisation, e.g. as occurs with erythrocyte membranes (Vomel, Muller & Platt,1985) and tumour cells (Roos & Dingemans,1977), and this is due to Kupffer cells recognising cell-surface components on the target cells via a range of specific and non-specific receptors.

Kupffer cells can be activated by a range of signals including zymosan, endotoxin and lymphokines. Activation is associated with production of superoxides, uptake of calcium and release of lysosomal enzymes and is often accompanied by increased proliferation of the cells.

There is no known primary role for Kupffer cells in the production of extracellular matrix components, but they are known to produce growth factors such as TGFß which stimulate the production of extracellular matrix protein components by other cell types such as fat-storing cells.

(Gressner and Meyer, 1991; Friedman and McGuire, 1991)

1.4.4 Sinusoidal endothelial cells

Endothelial cells separate the sinusoidal lumen from the hepatocytes, between which lies the space of Disse. They form a tenuous and selective barrier, as the cells are fenestrated and allow the passage of fluids and solutes from the sinusoidal lumen to the space of Disse (DeZanger and Wisse, 1982). Ultrastructurally, the cell consists of a cell body with a nucleus, and slender extended processes. It contains a large number of pores which are arranged in sieve plates. The size of the fenestrae is important in determining access to the space of Disse and the hepatocytes. The diameter of the fenestrae can be altered by hormones, drugs and alcohol, probably via the cytoskeletal fibrils (McGuire et al, 1992).

Sinusoidal endothelial cells also contain membrane invaginations and lysosomal vesicles which indicate that these cells are capable of endocytosis. They can take up substances such as immune complexes, denatured albumin, collagen, heparin, colloidal carbon, colloidal silver and transferrin (Brouwer, Wisse and Knook, 1988).

The most reliable method for identification of endothelial cells is by their ultrastructural features, and this is preferable to the use of markers such as factor VIII related antigen (Irving et al, 1984) and the Fc and mannose membrane receptors (Shaw et al, 1984). A

combination of these markers may be necessary for definite identification.

1.4.5 Pit cells.

These were first described in 1970 by Wisse and Daems, and are now thought to represent tissue-associated large granular lymphocytes (Bouwens et al, 1987; Bouwens and Wisse, 1987). They are located in the lumen of the sinusoid, and are often adherent to endothelial cells. They have been characterised as non-phagocytic, non-adherent, peroxidase-negative and acid phosphatase positive. In humans they are also identifiable as asialo GM1 positive, CD56 positive CD16 negative.

The ultrastructural features are those associated with lymphocytes, but the cytoplasmic volume is greater than in most circulating lymphocytes. Characteristic organelles include electron-dense cytoplasmic granules and rod-cored vesicles (diameter of 0.2μm). It has been proposed that these cells act as natural killer (NK) cells within the liver, and may play an important role in defence against hepatitis viruses, and against hepatic neoplasia (Geerts, Bouwens and Wisse, 1990; Vanderkerken, Bouwens and Wisse, 1990).

CHAPTER 2 PATTERNS OF LIVER INJURY

2.1 Liver injury

2.1.1 Sub-lethal injury

2.1.2 Necrosis

2.2. Responses to liver injury

2.2.1 Inflammation

2.2.2 Regeneration

2.2.3 Fibrosis and cirrhosis

2.2.4 Carcinogenesis

2.3 Specific forms of liver injury

2.3.1 Viral

2.3.2 Toxins

2.3.3 Cholestasis

2.3.4 Auto-immune disease

2.1 Liver injury

The response of the liver to injury can vary widely. Each response can be regarded as part of a continuous spectrum which ranges from transient acute inflammation to chronic hepatitis and cirrhosis. This chapter will examine the possible responses to injury, as well as some of the more common causes of injury. Hepatocytes bear the brunt of most forms of liver injury and some, indeed most, of the tissue response involves the non-parenchymal cells

2.1.1 Sub-lethal injury

There are a number of sub-lethal injuries which can be found in hepatocytes. One common example is fatty liver or steatosis. The hepatocyte accumulates fat within its cytoplasm which may be present as a single droplet (macrovesicular steatosis) or as a number of small droplets (microvesicular steatosis). The fat is mainly in the form of triglycerides. Steatosis often accompanies obesity and is an early manifestation of alcoholic liver injury. There is rarely any functional impairment associated with simple steatosis per se, and there is little evidence that it progresses to cirrhosis.

Degeneration of hepatocytes often precedes necrosis and can take several forms including the feathery degeneration found in cholestasis or the appearance of the ballooned cell, where the cytoplasm appears empty apart from a few wisps surrounding the nucleus which is enlarged as seen in alcoholic hepatitis.

2.1.2 Necrosis

This can be the result of many types of liver damage. It can involve individual cells or groups of cells within the hepatic acinus or may in some instances affect the entire liver. Different morphological patterns of necrosis are identified as follows.

(a) Focal or spotty necrosis. This can be found in any zone of the acinus. It is common in viral hepatitis.

(b) Zonal necrosis. This can affect any of the three zones of the acinus and each pattern may indicate the underlying cause. Perivenular necrosis is typical of ischaemic injury, and may also be found in drug or chemotoxic reactions. Midzonal necrosis is very unusual and is associated with yellow fever. Periportal necrosis is found in cases of eclampsia and in some types of poisoning e.g. phosphorus.

(c) Submassive (panacinar) necrosis. This is clinically referred to as fulminant hepatitis. There are a number of different causes, with some of the more common ones including acute viral hepatitis, drug toxicity and chemical toxicity.

(d) Piecemeal necrosis. This affects the cells at the interface between connective tissue and parenchymal tissue. It characteristically comprises a predominantly lymphocytic infiltrate, and can be found around portal tracts, the septa of fibrotic liver and the necrotic bridges of viral hepatitis. It was initially described by Popper et al (1965) who emphasised its immunological aspects.

2.2 Response to liver injury.

The first response to almost all types of cell injury is inflammation. It is a necessary first step in the repair of damaged tissue, but it is also a potentially harmful process. Inflammation can result in complete resolution of the injury, or it may lead to a number of other outcomes.

2.2.1 Inflammation

Inflammation can be divided into acute and chronic patterns.

Acute inflammation is a short lived response lasting from several minutes to several days which is characterised by (i) the change in vascular flow and size of the blood vessels;(ii) change in vascular permeability, and (iii) the margination and movement of leukocytes from the intravascular space to the interstitial tissues. This is the most important feature of the inflammatory response and can be divided into stages- first the leukocytes come in contact with the endothelial cells instead of flowing freely along the centre of the vessel. The leukocytes now adhere firmly to the endothelial surface via specific adhesion molecules. After adhesion, the leukocytes move through the gaps between the endothelial cells into the space between the endothelial cells and basement membrane, before crossing the basement into the extracellular space. The cells are directed by a process known as chemotaxis, in which the cells move along a chemical gradient. The next stage is phagocytosis : this is the ingestion of

particulate matter by the leukocytes. Neutrophils and macrophages recognise "foreign" particles, then engulf the particles by encircling them with cytoplasmic processes and enclosing them within a phagosome. This fuses with a lysosome and the contents are degraded. When lysosomal enzymes are released during phagocytosis, they are powerful mediators of inflammation, and amplify the inflammatory process.

Although this is a simplified description of acute inflammation, the basic process is similar, independent of the initial stimulus, and the histological pattern can be easily identified. The process of acute inflammation is seen in some forms of biliary damage when there is obstruction to biliary flow and accompanying infection. It can also be seen when there is sepsis in extrahepatic sites. It may completely resolve (often in mild injury) or heal after the process of scarring has occurred. It may also progress to chronic inflammation.

A more common response to injury in the liver is chronic inflammation which may occur de novo i.e. without an initial acute phase, or may follow acute inflammation. The causes of chronic inflammation include persistent infection by virus, infection by tubercle bacillus, prolonged exposure to foreign matter, and autoimmune disease. There are several histological markers of chronic inflammation including infiltration of macrophages, lymphocytes and plasma cells; proliferation of fibroblasts and small blood vessels; an increase in connective tissue (fibrosis); and tissue destruction (necrosis). The

difference between acute and chronic inflammation is not always clear-cut.

2.2.2 Regeneration

Cells can be divided into three groups based on their ability to regenerate. Hepatocytes are stable cells i.e. under normal conditions they have a low mitotic rate with constant low level turnover. After injury such as partial hepatectomy or viral infection, the stable cells (i.e. hepatocytes) are stimulated to undergo cell proliferation.

The replication of such cells does not guarantee restoration of normal tissue structure and function. In order for this to occur, there must be an intact underlying framework. If the connective tissue framework and basement membrane is damaged, regeneration may be haphazard and result in scarring.

The factors which control hepatic regeneration have not yet been fully identified. Signals or mediators are necessary to switch on rapid cell proliferation after injury, and also to switch off the proliferation when the liver is restored to its normal size. Possible mechanisms include functional overload, and circulating substances such as hormones, nutrients and growth factors.

Growth factors which have been implicated in hepatic regeneration include hepatocyte growth factor (HGF) and transforming growth factors α and ß (TGFα and TGFß). In experimental models of liver regeneration such as partial hepatectomy or CCl_4 intoxication of rats, a rapid increase in serum HGF can be demonstrated within several hours.

The levels of HGF measurable exceed the level known to be mitogenic for hepatocytes in culture (Michalopoulos and Zarnegar, 1992). Mead and Fausto (1988) have shown that TGFα is produced by regenerating hepatocytes and may act in an autocrine manner to promote hepatic regeneration. In contrast, TGFß acts as an inhibitor of cell proliferation.

2.2.3 Fibrosis and Cirrhosis

Fibrosis is the accumulation of extracellular matrix proteins as a result of alterations in the balance of synthesis and degradation. Fibrotic septa in the liver can be either active (formed by new connective tissue), or passive (produced by the collapse of pre-existing connective tissue after liver cell damage). In the early stages, fibrosis is reversible, if the stimulus is removed. The pattern of fibrosis may be focal - scarring around a localised injury, or may extend over large areas of the acini.

In some cases, fibrosis may proceed to cirrhosis. This is a well defined process which involves the formation of regenerative nodules. There are several pre-requisites for the histological diagnosis of cirrhosis. The architecture of the liver must be disrupted; the scarring divides the parenchyma into nodules, and the hepatic vascular architecture is reorganised because of this and is associated the development of new arteriovenous connections.

Conventionally there are two categories of cirrhosis- micronodular, where the majority of the parenchymal nodules

are less than 3mm in diameter; and macronodular, where the nodules are greater than 3 mm. In micronodular cirrhosis, the septa tend to be slender and regular in width, although wider septa may be seen. The pattern is to some extent dependent on the aetiology of the disease. Thus alcoholic cirrhosis is classically micronodular. Portal tracts or hepatic vein branches may disappear, due to portal-central bridging fibrosis. Macronodular cirrhosis may have wide or thin fibrous septa, and the nodules often contain portal tracts and central veins, although they may be unusually close to each other. Because of the larger nodules, diagnosis can be difficult with needle biopsies. It is generally the case that macronodules arise from regenerative micronodules, although some cirrhoses are macronodular from an early stage.

The clinical effects of cirrhosis include portal hypertension, splenomegaly, ascites and hepatic encephalopathy.

2.2.4 Carcinogenesis

Hepatocellular carcinoma (HCC) accounts for over 90% of primary liver malignancies, and worldwide, between 60 and 90% of HCCs are associated with cirrhosis. There is evidence linking protracted infection with hepatitis B, and to a lesser extent hepatitis C, with HCC. This helps to explain the high incidence of HCC in Africa and South-East Asia when compared with Europe and the United States. This subject is reviewed by Okuda (1992)

2.3 Types of liver injury

There a number of possible causes of liver injury. The causative agent may affect both the pattern of injury and the eventual outcome. A number of different agents are described below. It should be remembered that there may be some overlap. This is not an exhaustive list, there are also metabolic forms of liver injury whereby enzyme deficiencies lead to accumulation of amino acids, proteins and sugars in the liver and this may result in liver cell necrosis (Ishak and Sharp, 1987).

2.3.1 Viral

There are a number of viruses which specifically infect humans and are associated with liver cell necrosis. The most common of these are hepatitis A, B and C (previously known as non-A,non-B hepatitis). In addition some viruses which infect predominantly extrahepatic tissues can also be associated with hepatocyte injury. These include Epstein-Barr virus and Cytomegalovirus. Hepatitis lasting less than six months is known as acute hepatitis, if it lasts longer, it is known as chronic hepatitis.

Hepatitis A "infectious hepatitis" is caused by a 27nm RNA enterovirus which is spread by the oral-faecal route and may occur in epidemics. The disease is usually mild and is brief in duration. The mortality rate is extremely low, and there are few long-term complications.

Hepatitis B "serum hepatitis" is transmitted parenterally and requires direct innoculation through skin

or mucous membrane. Hepatitis B is a partially double stranded DNA virus which is found as a 42nm particle. The clinical disease is more severe than hepatitis A, and about 10% of cases are complicated by chronic infection. These patients may become asymptomatic while still retaining the infectious virus particles (carriers), or may develop chronic hepatitis and ultimately cirrhosis and hepatocellular carcinoma.

Much of the hepatocellular damage is caused by associated T-cell mediated immunological mechanisms rather than direct viral toxicity and there is a wide variation in the outcome of HBV infection (Bianchi, Spichtin and Gudat, 1987).

Hepatitis C is a single-stranded RNA virus, responsible for >90% of transfusion related non-A, non-B hepatitis, and a substantial proportion of sporadic non-A, non-B hepatitis. It results in a clinically mild, or even asymptomatic illness but in about 50% of cases, it progresses to a chronic hepatitis and subsequent cirrhosis. There are several histological features which are typical of hepatitis C infection, including dense focal lymphocytic portal infiltrates, steatosis, Kupffer cell hyperplasia and abnormal bile duct epithelium (Underwood, 1990)

There are other hepatitis viruses. Hepatitis D or delta agent only occurs in patients who also have hepatitis B and superinfection in such patients is associated with exacerbation of liver disease. Hepatitis E is a calicivirus. Both of these have been sequenced. Two other recently described but poorly characterised hepatitis

viruses are hepatitis F, a togavirus associated with fulminant acute hepatitis; and hepatitis G, possibly a paramyxovirus involved in giant cell hepatitis.

Acute viral hepatitis has a typical picture of diffuse liver cell injury, liver cell necrosis, hypertrophy of Kupffer cells, inflammatory infiltrate in the portal tract, and regeneration of hepatocytes in the recovery period. In more severe cases, the necrotic areas may connect, (bridging necrosis) and this may be portal-portal, central-central or portal-central.

In chronic hepatitis, there are two main patterns. In chronic persistent hepatitis (CPH) there is minimal hepatocyte necrosis, and the inflammatory infiltrate is limited to the portal tracts. This condition however forms part of a spectrum which also includes chronic active hepatitis (CAH) in variable amounts of both necrosis and fibrosis occur. The portal infiltrate spills out of the portal tract with the development of both piecemeal and bridging necrosis. Fibrosis which results from this rsponse extends from the portal tracts into the parenchyma and may progress to cirrhosis.

2.3.2 Toxins.

There are many toxins and drugs which can produce hepatic injury. This is reviewed in Zimmerman and Ishak, 1987. One of the commonest hepatotoxins is alcohol, which has been linked with liver damage for centuries. Alcohol (ethanol) is metabolised within the hepatocytes, and the resultant intermediate metabolite , acetaldehyde, is toxic

to liver cells. The resultant alteration of the redox potential of these cells leads to altered lipid metabolism and the accumulation of cytoplasmic triglycerides. This explains one of the early sublethal forms of alcohol-induced liver injury. Steatosis, often an early feature of alcoholic liver disease, is reversible if alcohol is withdrawn but may be associated with the formation of lipogranulomas. If alcohol intake continues, there may be progress to alcoholic hepatitis although this is influenced by factors involving individual susceptibility. Most of the changes of alcoholic hepatitis are found in the perivenular zones, and there is a distinctive histological pattern. The hepatocytes are swollen, with Mallory bodies (alcoholic hyalin) present, and there is a neutrophil polymorph infiltrate present. Fine pericellular fibrosis is also a common finding, as well as early septum formation. Steatosis is still present in many cases.

This in turn may progress to alcoholic cirrhosis, when there is widespread disruption of normal liver architecture. Nodules are surrounded by the connective tissue septa, and regeneration occurs within these nodules. The septa are formed by active fibrogenesis, rather than by the collapse of pre-existing structures. The first areas of fibrosis are around the hepatic veins, with eventual spread to the portal areas, resulting in portal-portal or portal-central bridging.

There are a number of ultrastructural changes which are typical of alcohol-induced injury. Mitochondria are swollen and enlarged, with the number of cristae reduced,

leading in some cases to the formation of so-called giant mitochondria. The endoplasmic reticulum becomes swollen and disrupted, with the formation of vesicles.

There are many other drugs which are hepatotoxic, and these involve one of the following mechanisms -

a) The drug, or one of its metabolites, may be directly hepatotoxic e.g. paracetamol or carbon tetrachloride, and have a physicochemical affect on the hepatocyte or on its organelles.

b) The drug is indirectly hepatotoxic and interferes with a metabolic pathway. The structural lesion is secondary and there are a number of different lesions which can occur. (Zimmerman and Ishak, 1987)

2.3.3 Cholestasis

The term cholestasis means a disturbance of the normal bile secretory mechanisms. It can be diagnosed by the accumulation of bile pigment within the liver tissue, and this can be done at an early stage using electron microscopy. The bile constituents can also be found in the blood. The cholestasis can be termed intra- or extra-hepatic, depending on where the causal lesions are found.

Extra-hepatic cholestasis indicates blockage of large bile ducts outside the liver, or within the porta hepatis. Causes include gallstones, tumours and benign strictures. The early microscopic changes consist of fine intracellular bilirubin granules, and bile thrombi with portal oedema and inflammation. The bile ducts start to dilate, and this is followed by ductular proliferation. In the later stages,

these changes become more pronounced and feathery degeneration of hepatocytes affects groups of cells before they are lysed (cholate stasis). Subsequent ductular proliferation is apparent, often associated with progressive portal fibrosis towards, and affecting linkages between portal tracts. One possible complication is that of secondary biliary cirrhosis. The inflammatory reaction leads to scarring and subsequently to cirrhosis.

The second type of cholestasis is intra-hepatic cholestasis. This can be further divided into diseases in which the abnormality is in the hepatocytes or canaliculi ; and those where the damage is in the intrahepatic bile ducts. The causes of the former include viral hepatitis, drug-induced cholestasis, alcoholic hepatitis, cholestasis of pregnancy and α_1-antitrypsin deficiency. So-called extralobular cholestasis is found in primary biliary cirrhosis and primary sclerosing cholangitis.

2.3.4 Autoimmune disease

There are a number of liver diseases which are believed to have an underlying auto-immune cause. Two disorders classically regarded as auto-immune diseases are primary biliary cirrhosis (PBC) and the so-called lupoid chronic active hepatitis. Primary biliary cirrhosis is usually found in middle-aged women, and may be accompanied by other auto-immune diseases such as scleroderma, Sjogrens syndrome and the sicca syndrome. The initial lesion is autoimmune damage to the bile ducts, with cirrhosis

developing later on. It is divided into 4 stages by histological diagnosis (Portmann and MacSween, 1987).

Stage 1. Inflammation of portal tracts and damage to medium-sized ducts.

Stage 2. Reduction in the number of medium-sized ducts, and proliferation of small bile ducts. Piecemeal necrosis in the periportal region.

Stage 3. Inflammation starts to resolve, but fibrotic septa formation begins. Bile ducts are reduced or absent.

Stage 4. Cirrhosis is now found, along with regenerative hyperplastic nodule formation.

Chronic active hepatitis is also believed to have an auto-immune basis, and may overlap with PBC in some patients.

CHAPTER 3 EXTRACELLULAR MATRIX

3.1 Components of the extracellular matrix.

The extracellular matrix contains 4 distinct groups of macromolecules, and these groups are present in all extracellular matrices, although the amount and composition varies depending on site. The four groups are (i) Collagens, (ii) Structural glycoproteins, (iii) Proteoglycans / glycosaminoglycans and (iv) Elastin. These have been reviewed by a number of authors including Miller and Gay (1987) and Labat-Robert et al (1990)

3.1.1 Collagens

At present, 14 different types of collagen have been isolated and at least partially characterised (Labat-Robert, Bihari-Varga and Robert, 1990), and the genes for several have been fully sequenced. The property common to all the collagens is the presence of the classical triple helix within the molecule, although the extent of the helical formation varies between the collagen types. The triple helix is formed from the repetitive sequence (Gly-Xaa-Yaa)n, with many of the X positions filled by proline, and the Y positions by hydroxyproline. Each collagen is believed to be a product of a different gene.

Little is known of the function of the most recently identified collagens, as these were discovered by probing genomic libraries for the repeating sequences which are typical of the collagen triple helix. The commoner collagens act as the "scaffolding" of the matrix. This can be in the form of collagen fibres which are relatively

rigid structures (i.e. types I, II and III) , or forming the mesh-like framework of the basement membrane (type IV)

3.1.2 Glycoproteins

These extracellular matrix components play a major role in cell-matrix interactions. Members of the family include fibronectin, vitronectin, laminin, undulin, nidogen and thrombospondin. One of the best characterised in terms of structure and function is fibronectin, which has been fully sequenced, and which has many isomers; these are derived from differential splicing of fibronectin mRNA.

The cell-matrix interactions are often mediated by a specific amino acid sequence called the RGD or Arg-Gly-Asp sequence, which can interact with integrins (Ruoslahti and Pierschbacher, 1988). This sequence has been identified in fibronectin, vitronectin, laminin, thrombospondin, tenascin and nidogen. Integrins are a family of cell-membrane receptors which interact with cytoskeletal components such as actin, talin and vinculin; this system can therefore transmit signals from the extracellular matrix proteins to the interior of the cell. In such a complex system it seemed unlikely that the RGD sequence was the only recognition sequence, and laminin has already been shown to contain another recognition sequence Tyr-Ile-Gly-Ser-Arg (Martin, 1987)

As well as these cell-recognition sequences, glycoproteins can recognise other binding sites. In the case of fibronectin, there are binding sites present for collagen, heparin, hyaluronic acid, actin and the plasma

membrane (Hynes, 1985). Laminin has similar sites for collagen type IV, heparan sulphate, entactin, endothelial and epithelial cell membrane receptors (Aumailley et al, 1987). This indicates that the glycoprotein molecules are not only involved in cell-matrix interactions, but also in matrix-matrix interactions,

3.1.3 Proteoglycans

Proteoglycans (originally called acid mucopolysaccharides) consist of a protein backbone with glycosaminoglycan chains covalently attached. The only exception is hyaluronic acid which has no protein backbone. The glycosaminoglycans consist of repeating disaccharide units (an amino sugar and a uronic acid molecule). The most abundant proteoglycans are heparan sulphate, dermatan sulphate, chondroitin sulphate, keratan sulphate and heparin (Ruoslahti, 1988). They can be found in loose connective tissue and basement membranes, with the basement membrane proteoglycan most likely to be heparan sulphate proteoglycans. They occupy a large volume in relation to their mass as they are too inflexible to form compact globular structures. Instead, they form extended random-coiled structures which are highly hydrophilic, and which form hydrated gels. It is these gels which resist compressive forces within the extracellular matrix, in contrast to collagen which resists stretching forces.

3.1.4 Elastin

Elastic fibres consist of elastin surrounded by an amorphous glycoprotein matrix. The matrix consists of a

number of microfibrils including fibrillin (Sakai, Keene and Engvall, 1986), but the total number and proportion of different glycoproteins present has not been determined. There are at least 11 mRNA isomers for elastin formed by splicing of the gene (Parks and Deak, 1990). Elastin is synthesised as the soluble precursor tropoelastin, which is then cross-linked to form the mature elastin fibres. Mature elastin has rubber-like properties, but during aging the fibres are attacked by proteases and gradually lose their elasticity.

3.2 Structure of the extracellular matrix

Assembly of the extracellular matrix is a complex process, both for individual components and for the final matrix. The chemical sequence of each of the matrix components dictates the structure of that component, and the components used for each tissue-specific matrix determine the final characteristics of the matrix. The matrix is normally assembled immediately at the cell surface, but this depends on the tissue and cell type involved.

Due to the sequential assembly, it is likely that the first components to be laid down act as templates for later matrix assembly. One such early component is fibronectin. In wound healing and embryological development, fibronectin matrices act as a cell adhesion and guidance system (Vartio et al, 1987; Sternberger and Kimber, 1986).

3.3 Roles of the extracellular matrix.

For many years, the extracellular matrix was regarded as an inert scaffolding which was only used as a physical support for the tissue. As the complex nature of the extracellular matrix protein components has been discovered, many new functions have been proposed for the extracellular matrix. Extracellular matrix proteins are now known to be involved in the regulation of cell migration, development, shape, metabolism and growth (Buck, 1987).

The role of the extracellular matrix in determining the phenotype of cells has been shown in tissue culture, where the choice of culture substrate has a major effect on the cell phenotype (Bucher , Robinson and Farmer, 1990).

Most cells interact in vitro with collagens and glycoproteins. This can be demonstrated by coating culture dishes with purified matrix proteins and plating cells on the surface. This may involve individual components such as type I collagen, or the use of a complex mixture such as that obtained from the Engelbreth-Holm-Swarm sarcoma (EHS) (Schuetz et al, 1988). EHS contains a mixture of basement membrane components such as laminin, type IV collagen and heparan sulphate proteoglycan, as well as growth factors such as TGFß.

Most studies of gene expression by liver cells have been carried out using hepatocyte cultures under different culture conditions (Bucher, Robinson and Farmer, 1990). Hepatocytes grown under classical tissue culture conditions (i.e. on plastic tissue culture dishes) only survive for a

few days, and rapidly lose all tissue specific gene expression. In comparison, common genes such as those for actin and tubulin are transcribed at normal levels. The use of type I collagen rafts as a substrate led to prolongation of viability and of tissue-specific gene expression (Michaelopolis and Pitot, 1975). As the cultures age, the gene expression gradually changes to an embryonic pattern. It has been suggested that this pattern resembles that found in human cirrhosis (Reid, Abreu and Montgomery, 1988). Other substrates tested include type III collagen (poor growth, gene expression similar to quiescent liver (Narita et al,1985); type IV collagen (grow rapidly with gene expression similar to regenerating liver); and the adhesion proteins, fibronectin and laminin (which improve cell adhesion and life span of cultures but do not prolong liver-specific gene expression, Bissell et al, 1987).

More recently several groups have used complex native substrates for hepatocytes. Using EHS gel as a substrate, the life of the cultured cells was increased, and they maintained a rounded shape throughout culture, unlike those grown on collagen. When gene expression was examined, the transcription of liver specific genes was maintained at a level similar to that of normal liver (Bucher et al, 1990). The difference was that the total DNA synthesis was greatly inhibited, and this may be due to the presence of TGFß within the EHS matrix.

These studies have helped to explain one of the functional roles of the extracellular matrix. If the

ability of the hepatocyte to produce liver-specific proteins is affected by the substrate to which it is attached , alterations in the pattern of extracellular matrix protein synthesis and composition of the matrix will lead to changes in liver function.

If the pattern of extracellular matrix protein deposition affects both structural and functional aspects of the liver, then localisation of the different types of matrix proteins may explain some of the alterations in cell function during fibrosis.

CHAPTER 4 COLLAGEN

4.1 Collagen

The term collagen encompasses a large family of glycoproteins. Collagen forms the most abundant group of proteins in mammals, and is often found in the form of insoluble fibres. The role of collagen fibres is primarily structural, but depending on their location the fibres may be rigid or flexible. The location within the tissue also determines the type of collagen found. This chapter will describe the different types of collagen and their classification, with particular reference to those which may be present in liver.

4.2 The chemical structure of collagen.

All collagen molecules consist of three polypeptide chains, or α-chains. The repeated sequence (Gly-Xaa-Yaa)n is present in the helical regions of the chains. The glycine residues are compact, and lack side-chains, and this allows the formation of the tight triple-helical structure which is characteristic of collagen. The X position is filled by proline, leucine, phenylalanine, glutamic acid or aspartic acid, and the Y position by proline, 4-hydroxyproline, glutamine, arginine or threonine. The imino acids are randomly distributed between X and Y positions, but have increased concentrations at the -NH2 and -COOH regions. This may help prevent the unravelling of the helix in these regions (Miller and Gay, 1988; Kivirikko and Mylylla, 1984). The collagen chains are synthesised within the cell, to form the helical procollagen molecules, and then undergo

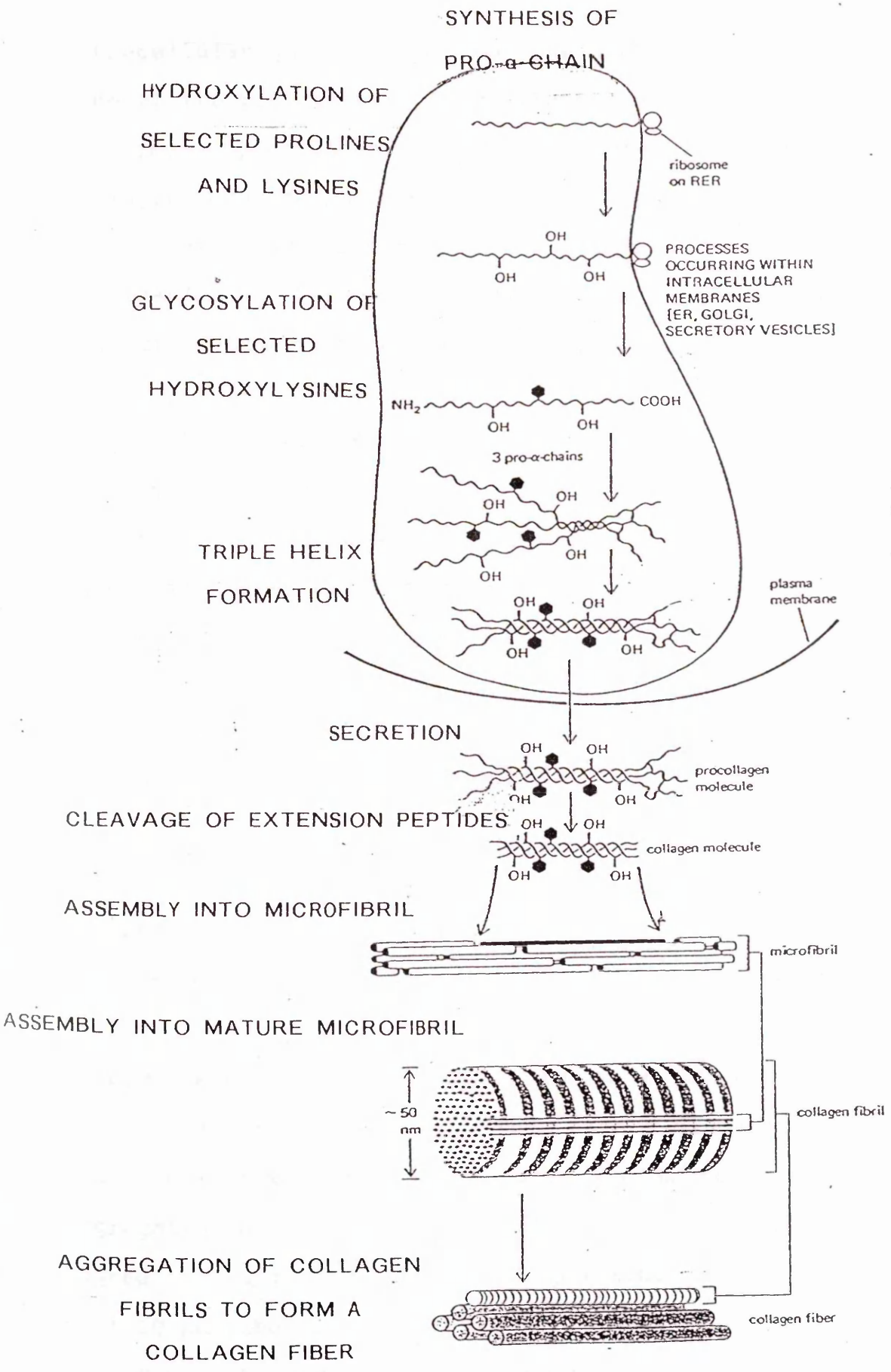

Figure 4.1 Collagen synthesis

Illustration adapted from Molecular Biology of the Cell, 1st edition
(Edited by Alberts et al)

intracellular processing. In the endoplasmic reticulum, some of the residues are hydroxylated by prolyl hydroxylase or lysyl hydroxylase. This is essential for further collagen processing and requires O_2, ferrous iron, α-ketoglutarate and ascorbic acid. The subsequent oxidative deamination of these sugars to active aldehydes helps to bond the three α-chains into a tight helix (Kivirikko and Myllyla, 1984).

Five specific enzymes are required: prolyl-3-hydroxylase, prolyl-4-hydroxylase, lysyl-hydroxylase, galactosyl transferase and glucosyl transferase. There is also some degree of glycosylation of asparagine residues at the -COOH terminal via the dolychol pathway. This occurs while the collagen is in the Golgi apparatus.

Procollagen is processed through the rough endoplasmic reticulum and Golgi apparatus and exported to the extracellular space, via secretory vesicles. Once secreted, these procollagen molecules have their C and N extension peptides removed by procollagen peptidases. This occurs to a variable extent - some such as type III collagen may retain the propeptide molecules. The tropocollagen molecules formed by this process are also called collagen filaments. They have a diameter of 1.5nm and are approximately 300nm long. The tropocollagen then aggregates to form collagen fibrils. Finally, the fibrils aggregate together to form collagen fibres with diameters of 1-10 μm (Ghadially, 1990).

Covalent bonding between the filaments is followed by irreducible cross-links between fibrils. As collagen

COLLAGEN TYPE	NORMAL	CIRRHOSIS
I	40 - 50	60 - 70
III	40 - 50	20 - 30
IV	1	1 - 2
V	2 - 5	5 - 10
VI	0.1	1

Table 4.1 Approximate percentage of principal collagen types in normal and cirrhotic human liver. (after Schuppan, 1990)

matures, it becomes progressively less soluble, and the mature fibres have high tensile strength. These mature fibres are deposited in tissue as loosely associated bundles. In all collagens, coiling of the three α-chains (which may be discontinuous in places) takes place within the cell, then the molecule is secreted to form tropocollagen fibrils. After extracellular processing, a number of these tropocollagen fibrils associate laterally and form collagen fibres. The collagen α-chain can be divided into three main regions

i) the amino terminal peptide (pN)

ii) the carboxy terminal peptide (pC)

iii) the triple helix

4.3 Classification of collagens

Until 1969, it was thought that there was only one type of collagen. The criterion for designation as a collagen molecule is the presence of a sizeable domain in which the triple helical structure is present By 1990, fourteen different proteins with this characteristic feature had been described (Labat-Robert, Bihari-Varga and Robert, 1990). The current classification of collagens is based on physico-chemical properties, and gives three separate groups of molecules.

4.4 Group 1 collagens

Collagens in this group have chains of at least 95,000 Da, and contain uninterrupted triple-helical regions of at least 300nm; they are also referred to as the interstitial

Collagen	Chains	Chain M weight Procollagen	Collagen	Molecular species
Type I	α1(I)	140,000	95,000	$\alpha1(I)2\alpha2(I)$
	α2(I)	125,000	95,000	$\alpha1(I)_3$
Type II	α1(II)	140,000	95,000	$\alpha1(II)_3$
Type III	α1(III)	140,000	95-110,00	$\alpha1(III)_3$
Type V	α1(V)	240,000	115,000	$\alpha1(V)_2\alpha2(V)$
	α2(V)	160,000	?	$\alpha1(V)_3$
	α3(V)	?	?	$\alpha1(V)\alpha2(V)\alpha3(V)$

TABLE 4.2 Group 1 collagen molecules

collagens. Collagens in this group display a characteristic banding pattern with a 67 nm periodicity on electron microscopy. This is due to the collagen molecules associating laterally, but with a relative stagger of 67 nm. This arrangement alternates gap and overlap zones within the fibre. At the present time, there are five collagens in group 1 (Table 4.2). Of these, types II and XI are only found in cartilage and will not be discussed here.

It has recently been suggested that most, if not all banded fibres consist of more than one type of collagen (Burgeson 1988). This has been demonstrated in several tissues - banded fibres in chick cornea are co-polymers of types I and V (Adachi and Hayashi, 1986; Birk et al, 1988) ; skin fibrils and aorta contain types I and III and possibly type V (Fleischmajer et al, 1985) ; tendon fibrils contain types I and III (and possibly type XI). The different types of collagen play an important role in regulation of collagen fibre size, and the types of collagen present are determined by the type of tissue. The ratio of collagen types present may control indices such as fibril diameter and fibril orientation. In vitro studies of fibrils formed from mixtures of type I and type III collagen show an inverse correlation between the proportion of type III present and the fibril size (Lapiere, Nusgens and Pierard, 1977). This may be due to the fact that type I procollagen tends to be completely processed, while type III procollagen may keep a variable proportion of its propeptide. If the propeptides are still present on the

surface of the collagen bundle, it will not continue to increase in diameter (Schuppan, 1990). This is contrary to previous reports about the relationship between type III collagen and fibril diameter (Keene et al, 1987). Fibre diameter may also be affected by the degree of glycosylation (Miyahara et al, 1984).

Type I collagen.

This is the predominant collagen found in vertebrates. There are two different chains, and two molecular species. $\alpha1(I)_2$ $\alpha2(I)$ is the most common species, and $\alpha1(I)_3$ is found infrequently. The pro-α chains are cleaved at the N-terminal peptide then at the C-terminal peptide (Davidson, McEneany and Bornstein, 1975). After cleavage, the α-chain consists of a large triple-helix (1014 amino acid residues, 300nm) with short non-helical ends, and the fibres then aggregate to form thick bundles. Type I collagen has a low percentage of hydroxylysine-linked carbohydrate (0.5-1%), and the ultimate fibre diameter is inversely linked to the content of neutral hexose. Physically, it can be regarded as a semi-flexible rod and this flexibility may be required during fibrillogenesis.

Type I collagen can be identified in almost all interstitial connective tissues (including skin, bone, cornea and liver).

Type III collagen.

There is only one chain type and the molecular species is a trimer of $\alpha1(III)$, often found in association with type I collagen. Extracellular processing is not always

complete, and chains retaining the N-terminal peptide are often present in tissue. It is believed that these terminal peptides must be removed from the fibril surface before the fibrils can grow. High levels of the cleaved terminal pIIIP can often be measured in serum, and this may be clinically useful for measurement of type III collagen turnover although there is some debate about this (Mutimer et al, 1989).

Type III collagen is also found in interstitial connective tissues, but is unusual in fully calcified tissues such as bone. In distensible structures such as arteries it is relatively abundant in comparison to type I collagen

Type V collagen.

Three different chains and three molecular species have been described - $\alpha1(V)_3$; $\alpha1(V)_2$ $\alpha2(V)$; $\alpha1(V)$ $\alpha2(V)$ $\alpha3(V)$ (Miller and Gay, 1987; Fessler and Fessler, 1987). In earlier literature it is referred to as AB collagen. The procollagen form is similar to that of types I and III, with a 300nm triple helix and globular domains at either end. The larger mass of type V collagen is due to an extra sequence at the N-terminal (Burgeson, 1988). Extracellular processing is slow and not fully understood, but it is believed that type V retains part of the N-terminal sequence after processing. All of the C-terminal is removed at this stage.

Once it is processed, type V collagen forms fine fibrils by end-to-end interactions. These have a periodic

banding pattern similar to that of types I and III (Adachi and Hayashi, 1985; Adachi, Hayashi and Hashimoto, 1989). The fibres are often located in a pericellular distribution and are thought to connect basement membranes with the interstitial collagens. They have a high specific binding affinity to heparan sulphate which may be involved in the binding to basement membranes (LeBaron et al, 1989).

This is a minor component of most tissues, where it is found in the pericellular interstitium, and in close proximity to basement membranes. Because of this, it was originally classified as a basement membrane component (Burgeson et al, 1976). It can be co-localised with other interstitial collagen fibres, and it has been suggested that fine type V fibres act as core fibrils to initiate growth of large fibres

There is a large increase in both absolute and relative amounts of this collagen in granulation tissue, healing wounds (Kurita et al,1985) and atherosclerotic vessels (Morton and Barnes, 1982).

4.5 Group 2 collagens

Collagens in group 2 also have chains with a molecular weight of greater than 95,000 Da, but the triple-helical domain is interspersed with non-helical domains. There are five collagens in group 2 (Table 4.3); in contrast to groups 1 and 3, none of these appear to be present in cartilage. They have end-to-end associations rather than lateral ones, and therefore do not form the striated fibres characteristic of group 1 collagens. Although the

Collagen	Chains	Chain M weight Procollagen	Collagen	Molecular Species
Type IV	α1(IV)	185,000	185,000	$\alpha1(IV)_2\alpha2(IV)$
	α2(IV)	170,000	170,000	$\alpha1(IV)_3$
				α2(IV)3
Type VI	α1(VI)	240,000	140,000	α1(VI)α2(VI)α3(VI)
	α2(VI)	240,000	140,000	$\alpha1(VI)_3$
	α3(VI)	240,000	140,000	
Type VII	α1(VII)	?	>170,000	$\alpha1(VII)_3$
Type VIII	?	180,000	180,000	?

Table 4.3 Group 2 collagen molecules

helical regions are interrupted, the molecules still have pN and pC termini.

Type IV collagen.

Two different chains have been isolated, α1(IV) and α2(IV), giving rise to three molecular species - $\alpha1(IV)_3$; $\alpha2(IV)_3$; $\alpha1(IV)_2$ $\alpha2(IV)_1$. Type IV collagen is a key component of basement membrane in all tissues. The proportions of α1(IV) and α2(IV) may vary in different basement membranes (Miller and Gay, 1988). Type IV collagen forms a highly flexible, thread-like molecule with a globular region towards the carboxy terminal (NC1 domain), and a compact helical zone towards the amino terminal (7S domain). The three chains run co-linearly throughout the length of the molecule. Dimers are formed by the NC1 domains binding together, and when the 7S domains of these dimers bind they form multimers. Although most association is end-to-end, some lateral association may also occur. Within the triple helix there are short breaks in the structure, where glycine residues are missing. This allows hyper-flexibility, but also gives an increased sensitivity to pepsin,and thus increases the likelihood of collagen degradation by non-specific proteases. When the sequence of type IV collagen is examined,there is a very low degree of homology with the interstitial collagens such as collagen type I and type III.

The collagenous component of basement membranes in all tissues is believed to consist exclusively of type IV

collagen. The monomers of type IV collagen link to form a loose meshwork, which acts as the structural frame for the basement membrane.

Type VI collagen.

Type VI collagen has three chains and one molecular species, the heterotrimer α1(VI), α2(VI), α3(VI). No obvious proteolytic processing is carried out, and the molecular weights of the chains are α1= 150,000 , α2= 140,000 , α3=240,000 (Colombatti et al, 1987; Bonaldo et al,1989). The different chain lengths mean that there is incomplete matching within the monomer - one of several unusual features of type VI collagen (Chu et al, 1988). The monomer is a triple helix of 105nm, with a globular domain at each terminus. There are two short interruptions within the helix, which confer flexibility. The globular domains are of approximately equal size, and make up 2/3 of the entire molecule (Timpl and Engel, 1987).

The monomers are assembled into dimers by an anti-parallel staggered alignment of the helical domains, and tetramers then form from laterally aligned dimers crossed in a scissor-like fashion with the non-staggered portion of the helical rod. The tetramers are formed within the cell, and the outer globular domains interact extracellularly to form long fibrillar structures (Von der Mark et al, 1984; Engvall, Hessle and Klier, 1986).

The structure of this collagen allows the formation of microfilaments, 3nm in diameter. These microfilaments

have an axial periodicity of approximately 100-120nm, with the periodicity arising from the placement of two alternating zones. One is composed of the thread-like portions of individual molecules, while the other consists of closely packed globular domains. This has lead to the description of type VI collagen as a "beaded filament" (Keene, Engvall and Glanville, 1988).

The turnover of type VI collagen appears to be high, as measured by levels of type VI antigen in serum , but it is a minor component in most tissues (Schuppan, Hahn and Riecken, 1988). There is little homology with other fibre-forming or basement membrane collagens, and type VI collagen has many unique features, some of which have been described above. To date it is the only collagen found in both cartilage and non-cartilagenous tissue, and it has the smallest triple-helical domain of any collagen. It also has 11 Arg-Gly-Asp sequences which indicate an involvement in cell attachment (Chu et al, 1988).

Although present in relatively low concentration, it is a ubiquitous component of the interstitial extracellular matrix, and has been described in a range of tissues including bone and dentine (Becker et al,1986), lung (Amenta, Gill and Marinez-Hernandez, 1988), brain vessels (Roggendorf, Opitz and Schuppan, 1988), neural connective tissue (Peltonen, Jaakola, Hsiao, Timpl, Chu and Uitto 1990), kidney, skin, muscle and liver (Von Der Mark et al, 1984). It has been proposed that it functions as a flexible network involved in the organisation of the connective tissue (Keene, Engvall and Glanville, 1988).

Type VII collagen

This is the largest collagen described to date. There is only one chain type, and the molecular species is the homotrimer of α1(VII). The molecular weight is 1050 KDa, with the triple helix accounting for 150 KDa. The helix is 424nm with at least one sizeable discontinuity. The molecule appears as a long triple helix with a globular domain at one end, and three smaller structures radiating from the globule. Dimer formation is by anti-parallel overlap of the amino-terminals, followed by the excision of the NC2 domain. This then forms unstaggered lateral aggregates by interacting with other molecules, rather than by lateral growth. Type VII collagen is the sole or major component of anchoring fibrils between basement membranes and the underlying connective tissue matrix (Miller and Gay, 1987). Sakai et al (1986) did not detect any type VII collagen within the liver, and this correlates with the absence ultrastructurally of anchoring fibres. In tissues which did contain the protein e.g. skin, chorioamnion, eye and cervix, type VII was found within the basement membrane zone of some epithelial cell layers (Leigh et al, 1988).

Type VIII collagen

There are three unique chains present in type VIII collagen (previously called EC [endothelial collagen]). The chains have molecular weights of 180,000, 125,000 and 100,000 Da. The overall structure is one of three

triple-helical domains separated by two non-helical regions (Miller and Gay, 1987). It is a minor component of most mesenchymal tissues and may be closely associated with the cell surface (Sage and Bornstein,1987).

Type XII collagen

This was first described by Gordon et al in 1987, when they screened a cDNA library from tendon fibroblasts. The predicted amino acid sequence is homologous, but not identical to, the cartilage collagen type IX. The collagen has now been isolated from chick tendon. The homology with type IX collagen suggests that type XII may associate with the surface of banded fibrils.

4.6 Group 3 collagens

These are the collagens with chains of less than 95,000 Da. To date, two members of this group have been described - type IX and type X (Table 4.4). Both are cartilage-specific collagens and not relevant to this study.

Collagen	Chains	Chain M weight Procollagen	Collagen	Molecular species
Type IX	α1(IX)	80,000	70,000	
	α2(IX)	80,000	70,000	
	α3(IX)	80,000	70,000	
			α1(IX)α2(IX)α3(IX)	
Type X	α1(X)	59,000	49,000	α1(X)3

Table 4.4 Group 3 collagen molecules

CHAPTER 5 NON-COLLAGENOUS MATRIX PROTEINS

5.1 Fibronectin

5.2 Vitronectin

5.3 Laminin

5.4 Tenascin

5.5 Undulin

5.6 Thrombospondin

5.7 Proteoglycans

5.1 Fibronectin

Fibronectin was discovered in the early 1970's and was originally called cold insoluble globulin (Mossesson and Umfleet, 1970). It is a dimer consisting of two similar but non-identical chains, and has a molecular weight of 440,000 daltons. The two chains are joined by a disulphide bond at the C-terminal end (Hynes, 1985). It not only forms part of the extracellular matrix, but is found in many body fluids and on the surface of many cells. It is unique among matrix proteins in that it exists in two different forms: plasma and cellular fibronectin. The majority of the plasma form is produced by hepatocytes (Tamkun and Hynes, 1983) and the cellular form by fibroblasts (Hahn et al, 1980). A total of twenty different isoforms are found and these are produced by differential splicing of mRNA (Hynes, 1985). Plasma fibronectin contains three types of homologous repeat sequences (I, II and III), while cellular fibronectin lacks one of the type III repeats called the EIIIA domain.

Fibronectin contains functional regions which bind to other components of the extracellular matrix, such as heparan and collagen (MacDonald, 1988), but there is no self assembly mechanism in solution, unlike the collagen components of the extracellular matrix. Fibronectin can also act as a mediator of cell adhesion to collagens, fibrin and heparan via specific domains (Hynes, 1985). Plasma fibronectin acts as a non-specific opsonin which coats circulating debris and helps uptake by mononuclear phagocyte system (Cardarelli et al, 1984). Fibronectin is

a powerful chemo-attractant and can act as a growth factor for mesenchymal cells.

5.2 Vitronectin

Vitronectin is an adhesion protein with a molecular weight of 70,000 daltons. It is from the same family of adhesion proteins as fibronectin i.e it contains an RGD binding site, but it is biochemically different. It is synthesised as a single chain polypeptide but may form a dimer in the circulation (Preissner, 1991). It is present in plasma, amniotic fluid and urine as well as on cell surfaces (Preissner et al, 1985). Plasma vitronectin is mainly synthesised by hepatocytes but also by platelets, megakaryocytes and macrophages (Preissner, 1991). Several studies have shown that vitronectin is distributed at locations distant to the site of synthesis (Hayman et al, 1983). When vitronectin was sequenced it was found to contain the RGD sequence and a number of binding sites including heparan, collagen and complement components (Cardin and Weintraub, 1989; Preissner, 1991 and Preissner et al, 1985).

Vitronectin has a number of important biological functions including cellular adhesion (Underwood and Bennett, 1989), immune defence against bacteria (Fillipsen et al. 1990), and the regulation of coagulation, fibrinolysis and plasminogen activation (Preissner, 1991).

5.3 Laminin

This is the major non-collagenous structural glycoprotein of basement membranes, and is a cross-shaped molecule formed from three chains. There are three short arms of the cross (37nm) with a globule-rod-globule-rod structure. The fourth arm (77nm) is a long rod ending in a large globular domain. Laminin is a large molecule with a molecular weight of 900,000 daltons, the three chains being A (440,000 Da), B1 (220,000 Da) and B2 (210,000 Da). The three short arms of the cross are formed from the N-terminal segments of the three chains. The chains then align in parallel to form the rod portion of the long arm. It is the C-terminal of the A chain which forms the globule of the long arm.

Laminin is a multifunctional molecule present in all basement membranes. It has binding sites for type IV collagen, heparan sulphate, entactin/nidogen and cell membrane receptors on endothelial and epithelial cells. It is the pentapeptide sequence in the B1 chain (Tyr-Ile-Gly-Ser-Arg or Y-I-G-S-R) which is responsible for cell binding. Laminin has a role in anchoring cells to the basement membrane. The molecule can be broken down proteolytically into a number of fragments, each with different properties. These include fragments 120kD and 60kD, E8 (general cell binding), P1 (cell binding, including hepatocytes and tumour cells) and E3 (heparin binding). Laminin acts on epithelial cells and regulates growth, morphology and differentiation (Martin, 1987).

Laminin is one of the first extracellular matrix proteins to be produced during embryogenesis and interacts with many other cells and components of the extracellular matrix, indicating a major structural and functional role for laminin.

5.4 Tenascin

Tenascin was first distinguished from fibronectin in 1984 by Erickson and Iglesias. It is a six armed oligomer, which they called a hexabranchion, and which has also been called myotendinous antigen, cytotactin, J1 glycoproteins and GMEM (Chiquet-Ehrismann, 1990). Part of the tenascin molecule consists of type III fibronectin repeats and there are also sequences homologous to fibrinogen and EGF. It is a very large molecule with a molecular weight of over 1000 kDa and unlike fibronectin is not present in a circulating form but is found only in tissues.

Tenascin is secreted by fibroblasts and glial cells in tissue culture (Erickson and Bourdon, 1991). The only extracellular matrix molecule known to bind to tenascin is chondroitin sulphate although there are conflicting reports on binding to fibronectin (Lightner et al, 1988; Chiquet-Ehrismann et al, 1988) It has been shown that the binding of cells to tenascin differs from that with laminin or fibronectin in that the cells do not flatten and spread on a tenascin substrate as they do on fibronectin (Chiquet-Ehrismann, 1990). The cell attachment to tenascin is weaker and not all cells can adhere to tenascin. RGD

sequences in tenascin were identified by Bourdon and Ruoslahti (1989); non-RGD receptor sequences may also be present but have not yet been characterised.

Tenascin is a major extracellular matrix protein in many developing organs (Chiquet-Ehrismann et al,1986; Mackie et al,1987; Vaughan et al,1987), is transiently present during wound healing (Mackie et al, 1988) and has been described in malignant tumours (Chiquet-Ehrismann et al,1986, Ichida et al, 1991). Strong tissue expression of tenascin is usually associated with growth and differentiation i.e tumour development.

5.5 Undulin

Undulin is a large glycoprotein composed of three different chains which are products of differential splicing of one gene (Schuppan, 1990). These chains are 175, 180 and 270 kD. Ultrastructurally, it can be visualised as a dumbell shaped monomer, or as an interconnected polymer. Using immunolabelling, the fibres are seen as a parallel display within dense connective tissues. It appears to be produced by mesenchymal cells, and plays a role in the organisation of collagen fibrils. It is usually associated with collagen types I and III.(Schuppan, 1990)

5.6 Thrombospondin

Thrombospondin is another large glycoprotein (450kD) made up of three chains with identical molecular weight (Lawler, 1986). The chains also have identical N-terminal

sequences and enzyme cleavage sites. It is a multi-functional protein and is known to interact with heparin, fibrinogen, fibronectin, plasminogen and type V collagen. It is produced by platelets, megakaryocytes, endothelial cells, smooth muscle cells, fibroblasts, monocytes and macrophages. Thrombospondin has been localised in kidney, skin and lung and it has a similar distribution to the basement membrane in these tissues (Lawler, 1986)

5.7 Proteoglycans

Individual glycosaminoglycans bind to a protein backbone via carbohydrate linkages to form proteoglycans. The only exception to this is hyaluronic acid, which does not bind to a protein backbone. It is now believed that the protein backbone (or core protein) may play an important role in a number of functions (Gressner and Bachem, 1990).

The intact proteoglycans of the extracellular matrix are involved in matrix assembly, where the proteoglycans may help assemble the matrix by linking together other components of the matrix, and may also be involved in regulation of the collagen fibril thickness (Vogel et al, 1984).

CHAPTER 6 THE HEPATIC EXTRACELLULAR MATRIX

6.1. The hepatic extracellular matrix proteins in light microscopic studies.

There have been a large number of studies within the last fifteen years which examined the distribution of the hepatic extracellular matrix (reviewed by Schuppan, 1988, 1990, Rauterberg et al, 1981). Unfortunately, many of these studies concentrated on one or two components of the extracellular matrix proteins, or examined a narrow range of diseases. The earlier studies concentrated on the presence of different extracellular matrix protein components in normal tissue, and once this was established, diseased liver was examined and the distribution patterns compared (Biagini and Ballardini, 1989). Collagen types which have been found in normal liver include types I, III and IV (Clement et al, 1985, Grimaud et al, 1980, Hahn et al, 1980, Voss, Rautenberg and Allam, 1980), V (Rojkind, Giambrone and Biempica, 1979; Schuppan et al, 1986) and VI (Von der Mark et al, 1984).

6.1.1 Methods used in previous studies

These studies used a range of techniques such as indirect immunofluorescence (Bianchi et al, 1984; Schuppan et al, 1986; Geerts et al, 1990), peroxidase-antiperoxidase (Martinez-Hernandez,1984; Clement et al, 1984) - both of these techniques localised the protein; and in-situ hybridisation for localisation of mRNA (Milani et al, 1990; Yamada et al,1989). This can make a direct comparison of studies difficult as some of the techniques are more sensitive than others, and the distribution of mRNA for a

protein may differ from that of the protein produced. In general, most interstitial collagens have a similar pattern of distribution in the normal liver, i.e they are found lining the sinusoids, in portal tracts and around hepatic veins (Schuppan, 1990). In diseased liver, interstitial collagens may also be present in any fibrotic septa. Basement membrane components, such as type IV collagen, laminin and heparan sulphate, are found around the hepatic veins and sinusoids (Hahn et al, 1980; Bianchi et al, 1984; Maher et al,1988). There is no evidence for laminin within the space of Disse in normal liver using light microscopy although another component of the basement membrane, type IV collagen, was present. This changes during hepatic fibrosis, when laminin and type IV collagen are co-distributed in the space of Disse (Hahn et el, 1980, Rauterberg et al, 1981).

Once the tissue distribution has been determined, the next step is to identify the cells of origin of the hepatic extracellular matrix proteins. Using immunocytochemical techniques, the limited resolution of light microscopy techniques means that it can be difficult to determine the precise location of the cells responsible for the production of extracellular matrix components.

In-situ hybridisation techniques have recently been developed for use on frozen tissues, but these are not yet widely available (Yamada et al, 1989; Milani et al, 1990). Studies by Milani et al describe the localisation of mRNA for collagen types I, III and IV in non-parenchymal cells of rat liver. This is in contrast to Yamada et al who

identified mRNA for type III procollagen within hepatocytes, as well as mesenchymal cells of cirrhotic human liver.

In order to overcome the difficulties involved in identification of the cells responsible for hepatic extracellular matrix synthesis, several groups adapted immunocytochemical methods for use in electron microscopy.

6.2 Description of hepatic extracellular matrix protein ultrastructural studies.

Using ultrastructural post-embedding techniques, it was shown that in normal liver, all of the sinusoidal staining previously identified by light microscopy and immunocytochemistry was in fact localised to the space of Disse (Martinez-Hernandez, 1984). This used the immunoperoxidase method to examine normal rat liver, but did not identify any intracellular labelling. In contrast, he was able to immunolocalise laminin within fat-storing cells in regenerating liver (1991). Clement et al (1988) used the same technique to examine human liver, and identified intracellular labelling of laminin in fat-storing cells as well as in vascular endothelial cells, bile duct cells and endothelial cells. They also examined fibrotic human liver and found laminin within hepatocytes.

A more complex relationship between the individual collagen types has recently been described with the discovery of collagen fibrils which were composed of more than one type of collagen (Henkel and Glanville, 1982; Geerts et al, 1990). The mixed fibrils of types I and

III, or I and V, mean that the use of single antibodies to identify the collagen species present may give a misleading picture.

6.3 Hepatic extracellular matrix proteins produced in tissue culture.

As all cells have the same genetic make up, they are all theoretically capable of synthesising matrix components. In practice however, cell types vary in both the type and the amount of matrix proteins that they produce. This is true of both in vivo, and in vitro systems. One approach is to examine each of the cell types individually, and this is most often done using tissue culture systems. The liver cells which have been studied in this way are hepatocytes, fat-storing cells, endothelial cells and Kupffer cells.

6.3.1 Cell isolation.

The first step in tissue culture is to isolate the individual cell types from the liver. The liver tissue is disrupted by perfusion with a solution of collagenase, giving a cell suspension. It is then necessary to separate out the differential cell types, and this is done by centrifugation. If only non-parenchymal cells are required, the hepatocytes can be removed with a pronase solution, and a mixture of non-parenchymal cells is left. This mixture of cells can be separated by density gradient centrifugation which isolates one cell type at a time (Friedman and Roll, 1987), or by centrifugal elutriation

which separates all the components in one step, giving individual fractions (Brouwer, Barelds and Knook, 1984). Once the cells have been isolated, the homogeneity of the population must be determined by examining morphological or biochemical characteristics.

6.3.2 Cell culture techniques.

The regulation of cellular collagen synthesis can be manipulated by the choice of substrate. Most of the earlier studies used tissue culture plastic as the substrate whereas in later studies the plastic culture dishes were coated with collagen type I, type IV or with basement membrane components isolated from the Engelbreth-Holm-Swarm sarcoma (Bissell et al,1987; Bucher, 1990).

6.3.3. In vitro assays.

This approach, has both advantages and drawbacks. Pure populations of each cell type can be examined and the regulation of extracellular matrix protein synthesis examined by the manipulation of the cellular environment. It is also possible to compare the extracellular matrix protein synthesis of the different cell types under similar, but not identical, conditions. This can help to identify the major extracellular matrix protein producers. As well as individual populations, co-cultures can also be used, or cells can be treated with medium conditioned by other cell types in order to examine the possible relationships found in vivo (Meyer, Bachem and Gressner, 1990).

6.4 Extracellular matrix protein synthesis.

Early tissue culture studies of extracellular matrix protein production examined collagen synthesis in hepatocytes (Bissell and Guzelian, 1980). Since then, other cell types, such as fat-storing cells have been examined (DeLeeuw et al, 1984). This showed that several different cell types synthesise the extracellular matrix, and it is not simply a case of identifying the one cell type which is responsible for hepatic extracellular matrix synthesis. If the relationship between the cell types is disturbed, alterations in the amount and composition of the extracellular matrix may occur.

6.4.1 Hepatocytes.

Hepatocytes were the first liver cells to be successfully cultured. Hepatocytes are the most abundant cell type in the liver, and it would not require a high level of collagen synthesis from individual cells to produce the amount of collagen required for physiological processes. Studies by Maher et al (1988) suggested that although hepatocytes were capable of synthesising collagen under cell culture, much of the collagen synthesis observed was in fact due to contamination of the hepatocyte cultures by fat-storing cells

Another approach to identifying the collagen producing cells was to isolate hepatocytes and look for messenger RNA within the cells. Saber et al (1983) reported that mRNA for collagen was detectable in hepatocytes. Recently,

Milani et al (1989,1990) used in-situ hybridisation to identify collagen producing cells in the rat liver, but could not detect any mRNA for laminin or collagen types I, III, or IV within hepatocytes. When Maher and McGuire measured the level of type I collagen in the hepatocytes of C Cl_4 treated rats, there was less than a two-fold increase in the mRNA in the stimulated cells as measured by Northern blot analysis.

The proteoglycan produced by hepatocytes is mainly in the form of heparan sulphate, and is usually cell-associated (Gressner and Schafer 1989). Cultured hepatocytes produce laminin as well as large amounts of plasma fibronectin (Clement et al, 1986; Clement et al, 1988)

6.4.2 Fat-storing cells.

In 1985, Friedman et al described fat-storing cells (lipocytes) as the main source of collagen in normal liver. When fat-storing cells are initially cultured, they are "normal" or "quiescent" and are similar to normal hepatic fat-storing cells. When these cells are cultured for any length of time on uncoated plastic, they become "activated" (Geerts et al, 1989). During this process, they lose their characteristic lipid droplets and become more fibroblast-like (Callea and Desmet, 1985). This corresponds with the transitional cells found in fibrotic liver (Mak and Lieber, 1988). In culture, activated fat-storing cells display increased levels of collagen synthesis.

Fat-storing cells are very sensitive to their culture substrate. If fat-storing cells are maintained on EHS extract, they are non-proliferative (quiescent) and maintain a compact appearance. The cells have low levels of collagen synthesis and the collagen produced is mainly type III. In comparison, fat-storing cells which are maintained on plastic or type I collagen spread out on the substrate, proliferate and synthesise large amounts of type I collagen (Friedman et al, 1989; Senoo et al, 1986; Weiner et al, 1990). This activation of fat-storing cells occurs after several days of culture. These results suggest that in order to obtain data on matrix production of fat-storing cells, it is advisable to use early cultures, or cultures grown on basement membranes. As well as type I and type III collagen, fat-storing cells can also produce type IV collagen.

Fat-storing cells also synthesise large amounts of proteoglycans, up to six times that of hepatocytes (Arenson et al, 1988). The majority of this is dermatan or chondroitin sulphate, which may be involved in collagen fibrillogenesis (Witsch et al, 1990). Gressner and Haarmann (1988) showed that fat-storing cells also synthesise hyaluronic acid. Primary cultures of fat-storing cells produce both fibronectin and laminin in considerable quantities (Ramadori et al, 1987; Maher et al, 1988), as well as tenascin (De Bleser et al, 1991). The amount produced increases as the cells become activated.

The ability of the fat-storing cell to synthesise the components of the extracellular matrix, especially when it

is in the activated state, is consistent with its proposed role as the major cell type involved in hepatic fibrosis.

6.4.3 Endothelial cells.

The endothelial cells produce relatively small amounts of collagen, which Irving et al (1985) identified as type IV collagen. This is consistent with the proposed role of endothelial cells in the deposition of a sub-endothelial matrix which forms an atypical basement membrane. In contrast, endothelial cells produce large amounts of non-collagenous matrix proteins. Cultured endothelial cells produce the cellular form of fibronectin (Rieder et al 1987; Clement et al, 1986), and this has been demonstrated by immunocytochemistry as well as by cell culture. The other major basement membrane glycoprotein is laminin, which both Maher and McGuire ,(1990), and Tsutsumi et al (1988) identified in cultured endothelial cells. Laminin mRNA has also been found in endothelial cells by Milani et al (1989).

6.4.4 Kupffer cells

To date, no collagen production has been identified in these cells by any method. Their role in collagen production apppears to be limited to the production of soluble factors which act as fibrogenic mediators. These factors activate fat-storing cells in cell cultures and in fibrotic liver (Friedman 1990). Like all other macrophages, Kupffer cells synthesise fibronectin

(Tsukamoto 1981) and also have fibronectin surface receptors which play a part in phagocytosis.

Due to the complex relationships between the individual cell types found in the liver, the examination of cell cultures *in vivo* yields limited information regarding the synthesis of extracellular matrix proteins. The results are further complicated by the effects of the tissue culture substrate used. Results obtained by these methods must be regarded with caution. As this is an *in vitro* system the results cannot always be extrapolated to an *in vivo* situation, where there are far more factors involved. With an *in vivo* situation, there are complex interactions between different cell types, which can rarely be duplicated exactly in an *in vitro* model. These interactions result in the release of growth factors and other mediators such as TGFß, which affect the synthesis of extracellular matrix proteins. The use of an *in vitro* system defines the extracellular matrix proteins which the individual cell types are capable of producing under certain circumstances, while an *in vivo* system identifies the components which are being manufactured in the tissue by a range of cell types. This means that *in vivo* techniques are of limited use in examining the synthesis of extracellular matrix proteins.

6.5 Overall knowledge of hepatic extracellular matrix proteins.

The distribution of some components of the extracellular matrix proteins have been well established,

while others have not been studied in detail. The major collagen components are type I and type III, which make up ≥ 80% of the total collagen content (Schuppan, 1990) in both normal and cirrhotic liver. Type I is mainly localised in the portal tracts, around terminal hepatic veins and in Glissons capsule, with some type I collagen present in the space of Disse where it helps form a scaffold throughout the liver. Type III has a similar distribution to that of type I, but it is more abundant (Voss, Rauterberg, Allam et al, 1980; Rauterberg et al, 1981).

The distribution of these two collagens has been examined in a number of studies (Schuppan et al, 1990) and it is now known that one of the the major alterations in fibrotic liver is in the ratio of the two collagens. In normal liver the ratio of type I:type III is 1:1, and in cirrhotic liver this increases to 3:1 (Seyer et al, 1977; Rojkind et al, 1979). In early stages of fibrosis it is type III which is the predominant collagen.

The interstitial collagens which are known to be present in the liver include type V collagen which was found in the portal tracts, and surrounding hepatic veins. It appears to co-localise with type I and III collagen in normal liver, and it is known that they can form hybrid fibrils. In cirrhotic liver, the absolute and relative amount of type V collagen is greatly increased (Rojkind, Giambrone and Biempica, 1979). This results in the hepatocytes being encased in a layer of type V collagen.

Type VI collagen is also found in normal liver (Von der Mark et al 1984).

The components of the basement membrane have also been examined, especially type IV collagen and laminin. Basement membranes are found around the hepatic arteries, portal veins, lymphatic vessels, bile ducts and nerve axons (Vracko, 1982).

Fibronectin is found around portal tracts and hepatic veins, as well as in the space of Disse (Schuppan,1988). The fibronectin can be associated with basement membranes, or with the interstitial collagens (Geerts et al, 1986). It has also been found in the space of Disse unattached to ant other ECM components, where it appears as an amorphous clump.

Other components of the extracellular matrix which are known to be present in liver are undulin, glycosaminoglycans and thrombospondin.

6.6 Aspects of hepatic extracellular matrix proteins yet to be studied.

At the commencement of this study, the abundant interstitial collagens which were first identified had been used in many tissue localization studies, including liver. In contrast, the hepatic distribution of novel collagens such as type VI have not been studied in detail (Von der Mark, 1984).

Few localisation studies have been extended to include ultrastructural aspects, and this imposed certain limitations. In order to identify the cells of origin of

the hepatic extracellular matrix proteins by in vitro methods, it is necessary to identify the different sinusoidal cells. This can done most accurately with electron microscopy techniques.

Previous studies of hepatic extracellular matrix have concentrated on the distribution of the most common ECM components, and the majority of the studies used normal human liver.

As it was impractical to examine all of the extracellular matrix proteins components in detail, the first stage of this study was to choose which components of the extracellular matrix were to be used. As the bulk of the hepatic extracellular matrix proteins consists of collagen, we decided to examine collagen types I, III, IV, V and VI. This allowed us to examine both interstitial and basement membrane collagens in detail. The study of types I, III and IV allowed comparison with previous work using a wide range of methods, while the distribution of types V and VI had not been examined in detail before now.

As well as collagen, we examined other extracellular matrix proteins, in particular, fibronectin, laminin and undulin.

It was possible to use similar techniques for both light microscopy and ultrastructural studies, thus maximising the small amounts of tissue which were available. The deposition of extracellular matrix protein components can be detected by immunocytochemical techniques, in order to identify the pattern of the extracellular matrix within the liver. Using the tissue

which was prepared for ultrastructural studies, it should be possible to detect intracellular labelling. This would identify the cells which are synthesising and secreting the extracellular matrix proteins.

6.7 Aims of this study.

This study was intended to examine two main aspects of the hepatic extracellular matrix.

1. To localise a number of the hepatic extracellular matrix proteins components at both light microscopic and ultrastructural levels.

2. To identify the cell types which were responsible for the production of these components in normal and fibrotic liver.

CHAPTER 7 ULTRASTRUCTURAL IMMUNOCYTOCHEMISTRY

7.1 Immunocytochemistry

Immunocytochemistry has been defined as the use of labelled antibodies as specific reagents for the detection of tissue constituents or antigens "in situ" (Polak and Van Noorden, 1987). Labelling can be carried out on specimens prepared in various ways, and many different labels can be used, but there are some requirements which should be fulfilled for optimal immunolocalisation.

1. The antibodies to be used (either polyclonal or monoclonal) should be specific for the antigen which is to be identified.

2. All epitopes should be accessible to the antibody and secondary label.

3. The antigen should be minimally altered by any fixative used.

4. The original morphology should be preserved whenever possible.

The first immunocytochemical studies of the extracellular matrix were carried out on 3μm sections of tissue, which were either unfixed frozen sections, or paraffin-embedded,fixed material. The antibodies were visualised by any one of a number of systems, including indirect immunofluorescence, peroxidase-antiperoxidase and alkaline phosphatase. These were mostly "sandwich" techniques, and were only of limited use in the localisation of extracellular matrix proteins (Fig 7.1).

The use of unfixed frozen tissue gave poor morphology, and made identification of individual cell types difficult. While the use of fixed, embedded tissue gave better morphology, it was still limited, mainly by the fact that the antibodies can only penetrate the upper part of the section.(Stirling, 1990)

The different second layer systems all had their own advantages and disadvantages. Indirect immunofluorescence gave a strong signal which was suitable for photography, but it was not permanent and the tissue morphology was difficult to examine when normal illumination was used as the sections were lightly fixed and not counterstained. The avidin-biotin labelling system was difficult to use in the liver, as there are large amounts of endogenous biotin liver. It is possible to block endogenous biotin in the liver before immunocytochemistry is carried out. This involves blocking the biotin within the section with avidin or streptavidin before using biotinylated antibodies. In a similar manner, any endogenous peroxidase can be blocked with methanol/hydrogen peroxide to allow peroxidase/anti-peroxidase labelling techniques The use of enzymes such as peroxidase and alkaline phosphatase give good labelling of extracellular tissue components, but have limited use in studying the intracellular components as there can be difficulties in identifying the labelled cells at the light level.

One solution to both of these problems is to use immuno-electron microscopy. This technique uses very thin sections of tissue, which require less penetration, and

also gives better resolution. Identification of intracellular labelling may allow identification of the individual cells which are producing the extracellular matrix.

7.2. The electron microscope

The limit of resolution of a microscope is determined by the wavelength of the energy source, and this limit can be increased by using an electron beam instead of light. Problems of specimen preparation and radiation damage effectively limit the resolution to 2nm.

There are two main types of electron microscopy and these are transmission (TEM) and scanning (SEM). With transmission electron microscopes, the tissue is cut into ultrathin sections, which can be examined in the microscope as transverse sections. All the specimens used in this study were examined by transmission electron microscopy.

7.3 Specimen preparation

In order to study ultrathin sections of biological material in the electron microscope, careful specimen preparation is required. There are several problems unique to electron microscopy, and special fixation, embedding, cutting and staining techniques have been developed.

The first critical step is to obtain and fix the tissue, in order to maintain the original structure and protect the tissue from further damage during processing. The specimen must be fixed as soon as possible. For

successful fixation the tissue blocks must be small enough to allow penetration throughout the entire tissue. It is critical to choose a suitable fixative. This consists of a fixing agent in a vehicle (usually a buffered salt solution). The vehicle must be of physiological osmolarity (or higher) and ionic constitution for the tissue and be able to maintain a constant pH during fixation, in order to prevent the cells from shrinking and disrupting the tissue. Fixatives suitable for light microscopy are not necessarily useful for electron microscopy, as they may not prevent damage to the tissue which is only apparent at the ultrastructural level.

For many biological specimens, two fixatives are used. The primary fixative is usually an aldehyde - glutaraldehyde, paraformaldehyde or a mixture of the two. Paraformaldehyde has a higher rate of penetration, but the reaction with tissue proteins is slow and reversible. Glutaraldehyde reacts very rapidly with proteins and cross-links them before any significant extraction of cytoplasmic components can occur. There is no significant fixation of the lipid component or membranes in the tissue, and there is no contrast in the tissue when viewed in the electron microscope. For secondary fixation an aqueous solution of osmium tetroxide is normally used to fix lipids and proteins. As this is electron dense, it contributes to the staining of the tissue.

7.4 Embedding tissue in resin.

Once fixation for transmission electron microscopy has been completed, it is necessary to embed the tissue in a support medium in order to cut ultrathin sections. Paraffin wax is far too soft for this, and new resins were developed for ultramicrotomy. Most of these resins are water-immiscible, and the tissue must be rinsed in buffer to remove excess fixative, then dehydrated in alcohol or acetone. The tissue is then infiltrated with the liquid resin, placed in capsules and the resin is polymerised. Polymerisation may be induced by heat or ultraviolet irradiation.

7.5 Sectioning of resin embedded tissue

Factors determining the quality of the sections such as the embedding material, block trimming, quality of the knife and the cutting speed will be briefly discussed here. The resin used determines the plasticity and cutting characteristics of the block.

Although knives can be made from sapphire or diamond, the majority of ultrasectioning is carried out with glass knives. These are usually made immediately prior to sectioning. With the introduction of commercial knife-making machines, it is possible to make knives of reproducible quality. Glass is broken to produce a perfect square, which is then broken diagonally to produce two knives with a cutting edge of 45° (Griffiths et al, 1983). The quality of the knives can be determined by

examination in the ultramicrotome, and the best area for sectioning chosen.

The trimmed block is placed in the ultramicrotome and ribbons of sections of approximately 100nm thickness are cut at slow speeds (<1mm/sec) to prevent excess vibration. The ribbons of ultrathin sections are collected on either copper, nickel or gold grids and these are left to dry before they are stained.

7.6 Staining for transmission electron microscopy.

Sections of biological materials lack contrast when they are examined in the electron microscope. This is partially overcome by secondary fixation with osmium tetroxide before resin embedding, and staining of the ultrathin sections with a combination of heavy metal salts, such as uranyl acetate followed by lead citrate. The use of secondary fixatives increases the amount of cross linking in the tissue specimens. This can affect the accessibility of tissue antigens: the use of secondary fixatives is therefore not always suitable for tissues which are to be immunolabelled.

7.7 Ultracryomicrotomy : Advantages for immunolocalisation studies

Immunocytocytochemical studies can be performed with ultrathin sections obtained by the above methods (Beesley, 1989; Stirling, 1990). The limiting factor is preservation of the antigen of interest. During the fixation and embedding, the tissue is treated with a wide

range of chemicals, some of which may damage the antigens present to such an extent that they cannot be identified by conventional immunocytochemical methods. In order to overcome this problem new resins were developed, which did not require the complete dehydration of the tissue by 100% alcohol, and which could be polymerised at lower temperatures with the use of ultraviolet light. Several such resins are now available, and include L.R.White, L.R.Gold and Lowicryl. Although these resins provide an excellent embedding medium for immunocytochemical studies of a number of antigens, some are so sensitive to chemical treatment that they cannot withstand even this degree of processing. An alternative technique was developed to overcome these difficulties (Bernhard, 1965, Tokayasu, 1973). Instead of using a synthetic embedding material, tissue was briefly fixed in low concentrations of fixative and then frozen in small blocks. The frozen tissue could be cut in a specially adapted ultramicrotome, and then immunolabelled in the same way as for resin embedded material. This approach was facilitated by the use of a a cryoprotectant which prevented damage by ice-crystal formation during freezing (Skaer, 1977).

7.8 Development of ultracryomicrotomy

The first attempts at ultracryomicrotomy involved placing conventional ultramicrotomes into a deep freezer (Bernhard, 1965). Using this approach, it was difficult to change the temperature, limiting the types of material which could be cut as there is an optimum temperature for

ultracryomicrotomy of each tissue. This arrangement was also impossible for personnel to use for prolonged periods. Soon after this, cryochamber attachments for conventional ultramicrotomes were designed which used an insulated "box" or cryochamber around the knife stage and specimen arm of the ultramicrotome. Most of these attachments use the Christensen design, where the specimen arm traverses the wall of the cryo-enclosure with an insulated U-shaped bridge (Christensen, 1971). Only the area within the cryochamber is cooled - an economical method when compared to a cryostat. The temperature of the knife and specimen can be regulated independently of each other , and the chamber temperature can also be regulated. All of the cutting controls operate at room temperature and are thus more reliable.

All the ultracryomicrotomy described here was carried out using the Reichert Jung FC4D attachment for the Ultracut E ultramicrotome.

Once the specimen is frozen it is essential to use a good knife. Glass knives are normally used (Griffiths (1984); Stang (1988)), although some diamond and sapphire knives are specially designed for use at low temperatures. The hardness of the tissue block can be varied by altering the concentration of the cryoprotectant, and the sectioning temperature. Sucrose, one of the most common cryoprotectants, is normally used in the concentration range 1.2M to 2.6M. The lower the concentration, the lower the temperature required for sectioning, and the harder and more brittle the block. The concentration

used depends on the type of tissue to be cut, and must be determined for each tissue. The cutting speeds required for this method are usually slow (≤1mm/sec), to prevent chatter and energy transfer to the specimen, and the sections are approximately 100nm thick.

At this stage it is possible to determine the amount of ice-crystal formation which has occurred during the freezing stage. If there has been a lot of ice-crystal formation the sections will be white and crumbly. Well frozen sections will be clear and cellophane like. Sections of approximately 90nm thickness will be blue/green in appearance. These sections are then transferred to a grid and used for immunocytochemical studies.

7.9 Preparation of tissue for immunocytochemistry.

The two main approaches to ultrastructural immunocytochemistry involve either pre-embedding or post-embedding labelling of tissues.

a) Pre-embedding labelling

This involves permeabilization of aldehyde fixed cells or tissues with polar solvents or detergents. The permeabilized structures are then exposed to the primary antibody and the secondary marker, refixed and processed for conventional resin embedding and sectioning. The secondary marker should be a substance which is electron-dense, i.e. it will show up as a dark area when examined under the electron beam. This method is particularly suitable for studying cell suspensions rather than tissue

blocks and has been used successfully to identify antigens on the outer membranes of cells or micro-organisms (Beesley, 1989), but it is not particularly suitable for the localisation of intra-cellular structures (Stirling, 1990). As this study aimed to identify both the cells responsible for the production of extracellular matrix proteins and the pattern of matrix protein deposition within the liver, the usefulness of pre-embedding labelling was very limited.

b) Post-embedding labelling

There are several variations of this method. Conventional Epon embedding can be used as discussed above, but the sensitivity is reduced due to the loss of antigen during dehydration and embedding (Beesley, 1989). There may also be a high degree of non-specific binding to the resin itself. Some of these problems can be at least partially overcome using hydrophilic resins such as L.R.White, and which do not require total dehydration of the tissue (Newman, Jasani and Williams, 1983). Low temperature resins such as Lowicryl have also been developed (Carlemalm, Garavito and Villiger, 1982), which preserve the morphology while greatly improving the sensitivity. The newest post-embedding technique is to use thawed ultra-thin cryosections (Tokuyasu, 1973, 1986). Using this system does have several disadvantages. The blocks have to be stored in liquid nitrogen, and the maximum storage time of the frozen blocks has not yet been determined. The tissue has to be obtained fresh and the

fixative carefully chosen (Burt et al, 1990; Griffiths, Keir and Burt, 1991), and most archival material which may have been frozen cannot therefore be used.

Pre-embedding techniques were not suitable as discussed above, but in the early stages of this study post-embedding labelling using tissue embedded in LR White and LR Gold was tried. The degree of labelling obtained was very low, even with extracellular matrix components which were present in large amounts such as collagen types I and III. As a result the rest of the study concentrated on ultracryomicrotomy. The technique was adapted from that described by Geerts et al (1889) using rat liver. Different fixation regimes were used, and polyvinyl alcohol was used for the post-labelling embedding of sections rather than methyl cellulose. This allowed better visualisation of the sections and also improved the stability of the tissue sections.

7.9.1 Primary antibody layer

The antibody can be against one epitope of the antigen (monoclonal), or against a range of epitopes (polyclonal). The tissue is incubated with non-immune serum in order to block any non-specific binding of antibodies, then a range of antibody dilutions are applied to tissue sections to determine the optimum conditions for labelling. The antibodies are left on overnight at 4°C or at room temperature for several hours. Any unbound antibodies are then washed off, and the bound antibodies are visualised.

7.9.2 Visualisation of antigens.

There are a wide range of labelling systems currently used for immunocytochemistry, but the most common marker used for ultrastructural immunocytochemistry is colloidal gold. This is a spherical electron-dense marker which can be conjugated to many different labelling systems including the following -

Label	Target
Enzymes (e.g. alkaline phosphatase)	Specific enzyme substrates. (NBT/BCIP)
Enzymes (e.g. peroxidase)	Antibodies to enzyme (peroxidase-antiperoxidase)
Lectins	A specific sequence of sugar residues (no requirement for antibody).
Streptavidin	Biotinylated primary antibodies
Protein A	Immunoglobulin (e.g. primary antibody)
Protein G	Immunoglobulin (e.g. primary antibody)
Antibodies	Primary antibody from a different species.

Table 7.1 Labelling systems for immunocytochemistry.

The majority of the labelling in this study used protein A-gold linked to the detection system, with a minority of experiments using IgG-gold. Protein A-gold can be applied to antibodies from a wide range of species (table 7.2), and reacts with the antibody in a 1:1 ratio. This allows quantitative studies, but is a disadvantage if

signal amplification is required. The gold particles form perfect spheres and can easily be identified in sections of biological material. The size of the sphere can be altered to suit the procedure. This is in contrast to the other major ultrastructural immunolabelling technique, peroxidase-antiperoxidase labelling (PAP). The PAP label appears as a diffuse and slightly fuzzy particle. It is not possible to alter the size of the complex. Despite these difficulties, some groups have claimed that it is a more sensitive technique and prefer it to immunogold. Immunogold labelling was chosen for this study because of its clear labelling and acceptable sensitivity.

The efficiency of protein A as a secondary system varies between species (table 7.2). Protein A-gold has been reported to have low sensitivity for goat antibodies, and in part of this study, rabbit anti-goat immunoglobulin conjugated to gold particles was used as a labelling system.

SPECIES	IgG SUBCLASS	PROTEIN A
MAN	IgG1	+++
	IgG2	+++
	IgG3	+/-
	IgG4	+++
	IgA	+/-
	IgM	+
	IgD	-
GOAT	Ig	+/-
RABBIT	Ig	+++

TABLE 7.1 Affinity of immunoglobulin subclasses with protein A

+++ Strong affinity
+ Poor affinity
± Very weak affinity
- No affinity

CHAPTER 8 MATERIALS AND METHODS

8.1. Sources of human tissue

8.1.1 Diagnostic needle biopsies

8.1.2 Normal liver

8.1.3 Liver resections

8.1.4 Diagnosis of liver disease

8.2 Processing of biopsies for light microscopy

8.3 Processing of biopsies for electron microscopy

8.3.1 Processing for routine TEM

8.3.2 Processing for immunocytochemistry

8.4 Immunostaining techniques

8.4.1 Light microscopy

8.4.2 Electron microscopy

8.1. Sources of human tissue

All biopsies were obtained at either the Western Infirmary or Gartnavel General Hospital, Glasgow. As specific fixation regimes were required, it was not possible to use archival material for any of the electron microscopy studies. Some archival material was used for the light microscopy studies and this was obtained from the files of the Western Infirmary. This material had been collected from patients undergoing investigation for liver disease. Some of the PBC biopsies were obtained from the Royal Victoria Infirmary, Newcastle-Upon-Tyne.

8.1.1. Diagnostic needle biopsies.

All diagnostic biopsies were carried out using either the Menghini needle or the Tru-cut needle technique. The majority of the biopsy was fixed in 10% buffered formalin and used for routine histopathology. The remainder of the biopsy was split into small pieces (approx 2mm x1mm) and placed in one of three different fixatives.

i) 2% paraformaldehyde

ii) 2% paraformaldehyde with 0.1% glutaraldehyde

iii) 2% glutaraldehyde

The fixatives used were prepared in 0.1 M phosphate buffer. The paraformaldehyde buffers were stored frozen and thawed imediately prior to use, and the glutaraldehyde was freshly prepared.

8.1.2. Wedge biopsies of normal liver (n=15)

Wedges of liver were removed from cadaveric renal transplant donors. Whenever possible, the liver was perfused with buffer through surface vessels immediately after removal, in order to remove blood from the sinusoids, and to prevent collapse of the sinusoids. Once the tissue had been sufficiently perfused, it was cut into smaller pieces (1x1mm for EM, 2x2cm for light microscopy) and immersion fixed in either formalin (light microscopy) or one of the EM fixatives previously described.

8.1.3 Liver resections (n=5)

These were obtained from patients with hepatic tumours. When the liver was resected, a section of liver which was tumour-free by gross examination was removed by wedge biopsy and placed in fixative (as above). Sections of this tissue were examined to confirm the absence of any significant abnormality.

8.1.4. Diagnosis of liver disease.

All biopsies in this study were examined by Professor RNM MacSween and Dr AD Burt in the Pathology department of the Western Infirmary. The diagnoses are summarised in table 8.1.

8.2 Processing of biopsies for light microscopy

After overnight fixation, the biopsies were processed through graded alcohols to paraffin wax in a VIP tissue processor, and embedded in individual blocks before

DIAGNOSIS	NUMBER OF CASES
NORMAL [1]	20
NON-SPECIFIC HEPATITIS	10
ALCOHOLIC LIVER DISEASE	7
PRIMARY BILIARY CIRRHOSIS	6
CRYPTOGENIC CIRRHOSIS	5
NODULAR REGENERATIVE HYPERPLASIA	4
METASTATIC TUMOUR	3
PRIMARY SCLEROSING CHOLANGITIS	3
CHRONIC ACTIVE HEPATITIS	3
NON A - NON B HEPATITIS	3
STEATOSIS	2
SPACE OCCUPYING LESION	2
PRIMARY BILIARY CIRRHOSIS/ CHRONIC ACTIVE HEPATITIS	2
GRANULOMA	1
HEPATITIS A	1
HEPATITIS B	1
CROHNS DISEASE	1
HAIRY CELL LEUKAEMIA	1
PANCREATITIS	1
CHOLESTASIS	1
CHOLANGITIS	1
SIDEROSIS	1
TOTAL NUMBER	81

TABLE 8.1 Biopsies used in this study

1 Includes cadaveric transplant donors.

sectioning. Five micron thick sections were placed on poly-L-lysine coated slides for further staining.

8.3 Processing of biopsies for electron microscopy

The method used for processing the liver biopsies for electron microscopy depended on whether it was to be used for morphology or immunocytochemistry.

8.3.1 Processing for routine TEM

The tissue was fixed in 2% glutaraldehyde and embedded in araldite resin (see appendix B). The polymerised blocks were then trimmed before cutting in a Reichert-Jung ultramicrotome. Semi-thin sections (2μm) were stained with toluidine blue and the area of interest was chosen for ultrasectioning. After further trimming, 100nm thick sections were cut and placed on copper grids (100 mesh, Agar Scientific, England). All grids were stained with uranyl acetate and lead citrate (see appendix C) and viewed in a Philips CM10 transmission electron microscope.

8.3.2 Processing for immunocytochemistry

When the tissue was used for ultrastructural immunocytochemistry, two different methods were tested.

A. LR White embedding.

Blocks of tissue (0.5mm^2) were immersion fixed in 2% paraformaldehyde ± 0.1% glutaraldehyde for several hours before dehydration through graded alcohols. The block was immersed in a 1:1 mixture of ethanol and LR White resin for

1 hour then placed in LR White resin for 1 hour. The tissue was then placed in a mould containing fresh LR White resin. The block was cured overnight in an anaerobic chamber at 55°C. Sections were cut on a conventional microtome and placed on formvar-coated nickel grids (Agar Scientific, England).

B. Ultracryomicrotomy.

Blocks of tissue (0.5mm^2) were immersion fixed in 2% paraformaldehyde ± 0.1% glutaraldehyde for several hours, before saturation with 2.3M sucrose. The blocks were placed on aluminium stubs (Reichart, Germany) and plunge frozen in liquid nitrogen before storage in liquid nitrogen. The frozen blocks were then trimmed and cut in a Reichert-Jung FC4D ultracryomicrotome, using a dry glass knife as described above. Sections were removed with a wire loop containing saturated sucrose and transferred to carbon/formvar-coated copper grids (Agar Scientific, England) before being placed face down in PBS to remove the sucrose.

In the early stages of the study the biopsies were fixed in paraformaldehyde both with and without glutaraldehyde, and both the immunolabelling and morphology were compared. It was found that in most cases the addition of 0.1% glutaraldehyde improved the morphology of the sections without affecting the immunolabelling. There were however some antibodies which did not immunolabel successfully in the presence of glutaraldehyde, and tissue

fixed in paraformaldehyde alone was used for these antibodies (see Chapter 9).

8.4 Immunostaining techniques

Although the same antibodies were used for investigation at the light and electron microscopic level, the methods used were different for each technique

8.4.1 Light microscopy

The first method was indirect immunofluorescence (IIF), using frozen sections of acetone-fixed tissue.

1. Sections were placed in 0.1M glycine\PBS for 3 x 5 minutes to block any free aldehydes in the tissue, then incubated with 2% BSA\PBS for 15 minutes to block to any sites which could bind antibody in a non-specific manner.
2. Excess BSA was blotted off and 100μl of the primary antibody was applied. The antibodies used are described in table 8.2. Primary antibody was diluted in 1:25 normal swine serum (NSS)/ phosphate buffered saline (PBS), with 0.1% saponin (a detergent which helps the antibody penetrate the section).
3. The sections were incubated at 37°C for 2 hours or at 4°C overnight.
4. Sections rinsed in PBS (2x15 minutes).
5. Appropriate secondary antibodies (fluorescein isothiocyanate (FITC)-goat anti-rabbit or FITC rabbit anti-goat immunoglobulin, Hoescht, UK) were diluted 1:50 in PBS/NSS and applied for 30 minutes at 37°C.

6. Sections were thoroughly rinsed with PBS, then mounted in Citifluor and examined in a Leitz Orthoplan microscope.

The second method used was peroxidase-antiperoxidase (PAP).

1. 5μm thick tissue sections were placed on poly L-lysine coated slides, and the sections were dewaxed in xylene before rehydrating in graded alcohols.
2. The tissue sections were incubated in 1.67% H_2O_2 \ methanol for 30 minutes to block any endogenous peroxidase present.
3. The sections were digested for 30 minutes at 37°C with 0.1% trypsin in tris buffered saline (TBS) containing 0.1% $CaCO_3$. This partially digests the tissue and helps expose the antigens present.
4. They were then rinsed with PBS and blocked with 1:5 NSS:PBS to reduce background staining.
5. All the primary antibodies were diluted in 1:25 NSS:PBS (see table 8.2) and 100μl of antibody was applied to each slide before incubation at 4°C overnight in a moist chamber.
6. The sections were washed in PBS containing 1.8% NaCl for 30 minutes.
7. They were incubated for 30 minutes with the secondary antibody (diluted in 1:25 NSS:PBS). This was either swine anti-rabbit (1:100) or rabbit anti-goat immunoglobulin (1:50) conjugated to horseradish peroxidase (Dakopatts, Denmark).

8. The sections were rinsed with PBS, then incubated with peroxidase-antiperoxidase complex (PAP) for 30 minutes.
9. The resulting complex was visualised with 0.5% 3'3'diaminobenzidine tetrahydrochloride (DAB)/ methanol.
10. The sections were counterstained with Harris's haematoxylin, dehydrated, and mounted with Histamount.

8.4.2 Electron microscopy

Ultracryosections were cut as described in 8.3.2.

1. Sections were blocked with 1% immunoglobulin-free BSA (Sigma, UK) in PBS.
2. They were incubated at 4°C overnight with primary antibody diluted in 1% BSA\PBS (see table 8.2).
3. The grids were then washed several times in PBS.
4. The secondary antibody was diluted in 1% BSA\PBS and incubated for 2 hours at room temperature. (In most cases this was Protein A conjugated with gold particles of 5 or 10nm (Biocell,Wales) although goat anti-rabbit IgG or rabbit anti-goat IgG conjugated to gold were also used).
5. The sections were thoroughly rinsed with PBS and briefly rinsed with water.
6. Sections were lightly counterstained with 2% uranyl acetate before embedding in 2% uranyl acetate: 3% polyvinyl alcohol (10:1 v/v) and drying before examination in the electron microscope. In the early stages of this work, 1.3% methyl cellulose (Tylose,

Fluka, Germany) was used but it was then found that polyvinyl alcohol gave a more even and stable film.

The extracellular matrix consists of many different components including hyaluronic acid. Immunolabelling for collagen types I, III and IV, gave far better results if the sections were pre-incubated with 150 units/ml hyaluronidase (Koch, Germany) for 30 minutes at 37°C to remove some of the hyaluronic acid which appeared to mask the antigens. This technique is similar to the use of trypsin for partial digestion of tissue prior to immunolabelling for light microscopy.

ANTIGEN	RAISED IN	DILUTION
COLLAGEN TYPE I	Rabbit	1:25
Pro COLLAGEN I	Rabbit	1:80
COLLAGEN TYPE III	Goat	1:50
Pro COLLAGEN III	Rabbit	1:80
COLLAGEN TYPE IV	Rabbit	1:100
TYPE IV (NC1 region)	Rabbit	1:50
TYPE IV (7s region)	Goat	1:40
COLLAGEN V [2]	Goat	1:50
COLLAGEN VI [1]	Rabbit	1:160
FIBRONECTIN	Rabbit	1:50
VITRONECTIN [2]	Mouse	1:50
UNDULIN	Rabbit	1:50
LAMININ [1]	Rabbit	1:100
LAMININ P1	Rabbit	1:50
LAMININ 120	Rabbit	1:50
LAMININ 60	Rabbit	1:50

Table 8.2 Antibodies used in study

All antibodies were obtained from Dr D Schuppan, Free University of Berlin, except those marked.

1. Heyl, Germany
2. Sera laboratories, U.K.

CHAPTER 9. EXTRACELLULAR MATRIX COMPONENTS IN NORMAL LIVER.

9.1. Immunocytochemistry at light microscopy level

9.2. Collagen

9.3. Fibronectin

9.4. Vitronectin

9.5. Undulin

9.6. Laminin

9.7. Immunocytochemistry at electron microscopy level

9.8. Collagen

9.9. Fibronectin

9.10. Vitronectin

9.11. Laminin

9.12. Ultrastructural immunolabelling of normal liver

9.1. Immunocytochemistry at light microscopy level.

A total of 81 liver biopsies were obtained during this study, comprising 20 normal samples and 61 samples of diseased tissue. The range of diseases is outlined in table 8.1. While the indirect immunofluorescent technique was used initially to confirm the presence of extracellular matrix components in the tissue, the peroxidase-antiperoxidase (PAP) technique provided better resolution and more intense labelling. The majority of illustrations in this section will therefore be of antigens localised using the PAP technique. In some cases the immunostaining was improved if the sections were digested with trypsin before the antibody was applied, as this helped to break down the crosslinks formed during tissue fixation. Pre-treatment with pepsin was also tested but this did not give any improvement and the use of detergents such as saponin as a pretreatment or in the buffers was also ineffective. In order to test the effectiveness of the different pretreatments, serial sections of normal liver were used. The different pretreatments were carried out and all of the sections were then immunostained under identical conditions. All of the slides were examined to see if there was improved immunolabelling in comparison with sections which had not been pre-treated. Similar comparisons were used to determine the effectiveness of detergents in the buffer.

With some of the antibodies used in this study it was necessary to use tissue which was fixed in Bouins fixative

or in paraformaldehyde. This is consistent with the study of Bedossa et al (1987) on immunolocalisation of collagen types I, III and IV in the liver. As all archive tissue was fixed in either buffered formalin or in formal sublimate, these antibodies could only be used on the wedge biopsies, where there was enough tissue to use a range of fixatives.

9.2 Collagen

Type I collagen. This component was evenly distributed throughout the zones of the liver. It was present in the portal tracts, Glissons capsule, around terminal hepatic veins, and in the space of Disse (Fig 9.1). Occasional staining of hepatocytes was seen, but this was the only intracellular labelling for type I collagen. Labelled hepatocytes were distributed randomly and were present in all zones. Type I could be immunolocalised in acetone-fixed frozen material or in fixed-tissue (paraformaldehyde, buffered formalin or Bouins fixative). Optimal labelling was obtained with Bouins fixed material.

Type III collagen. The distribution of type III collagen was similar to that of type I collagen, although the intensity of labelling within the space of Disse was often stronger. There was an even distribution throughout different acinar zones in normal liver (Fig 9.2). Again, there was focal staining of hepatocytes staining with a similar pattern of distribution to that of type I collagen (Fig 9.3). Type III collagen could also be

immunolocalised in unfixed frozen material or in fixed tissue (paraformaldehyde, buffered formalin or Bouins fixative).

Type IV collagen. This was immunolocalised around blood vessels and bile ducts in portal tracts (Fig 9.4). It was also found within the space of Disse, in a linear distribution. There was no difference in the intensity of labelling within the different zones. The intensity of staining was dependent on the antibody used. Some of the antibodies available gave weak labelling (such as the antibody to the NC1 region) and the strongest labelling was against the 7s component of type IV collagen. Although all the type IV antibodies worked on frozen or Bouins fixed material, only the anti-7s antibody gave labelling on material fixed in paraformaldehyde or buffered formalin. None of the antibodies gave intracellular labelling.

Type V collagen. This collagen was present in the space of Disse in a linear pattern. There was weak staining in the portal tracts and stronger staining around the hepatic vein. There was no zonal distribution and no intracellular staining was seen (Fig 9.5). Staining was only observed in Bouins fixed material.

Type VI collagen. There was immunostaining for type VI collagen in the space of Disse, with a linear pattern along the sinusoidal walls. Staining was weakest in the

FIG 9.1

Distribution of type I collagen in normal liver (peroxidase-antiperoxidase labelling).

Labelling is seen within the space of Disse for type I collagen, and more intensely around a terminal hepatic vein (THV).

FIG 9.2

Distribution of type I collagen in normal liver (peroxidase-antiperoxidase labelling).

Type I collagen is also found around the portal tracts (PT). This figure illustrates the even distribution of type I collagen throughout the liver.

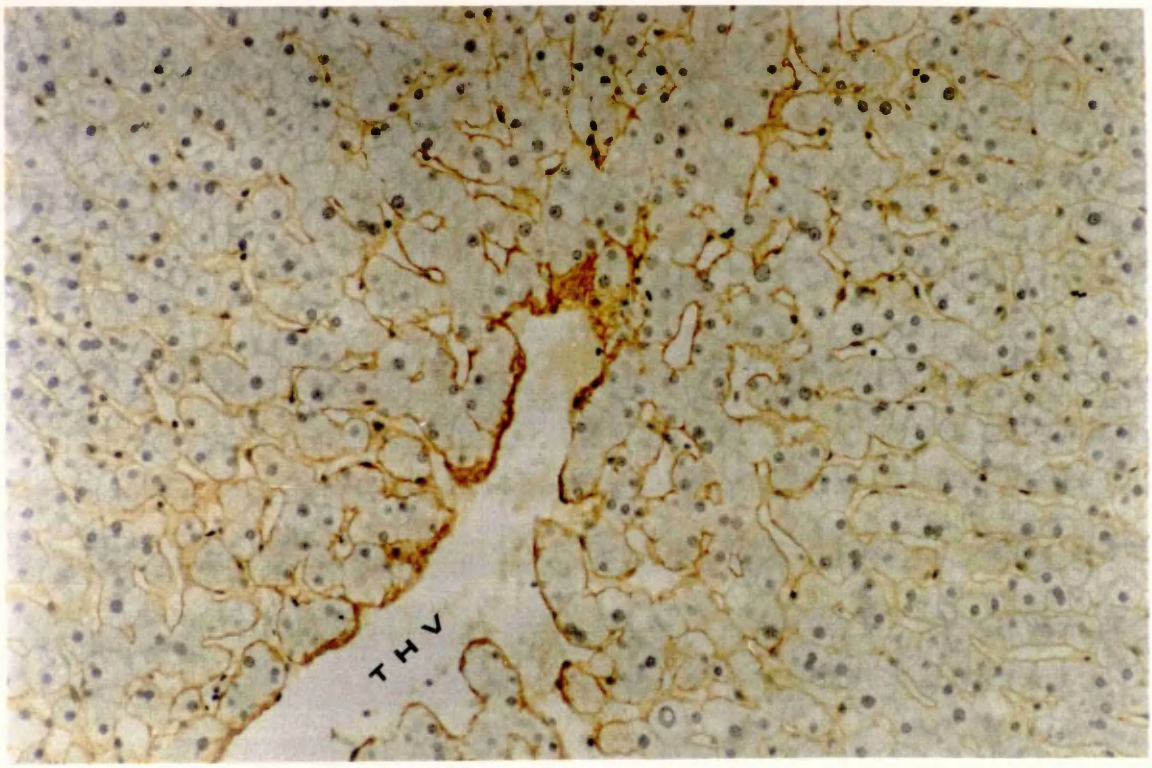

FIG 9.1

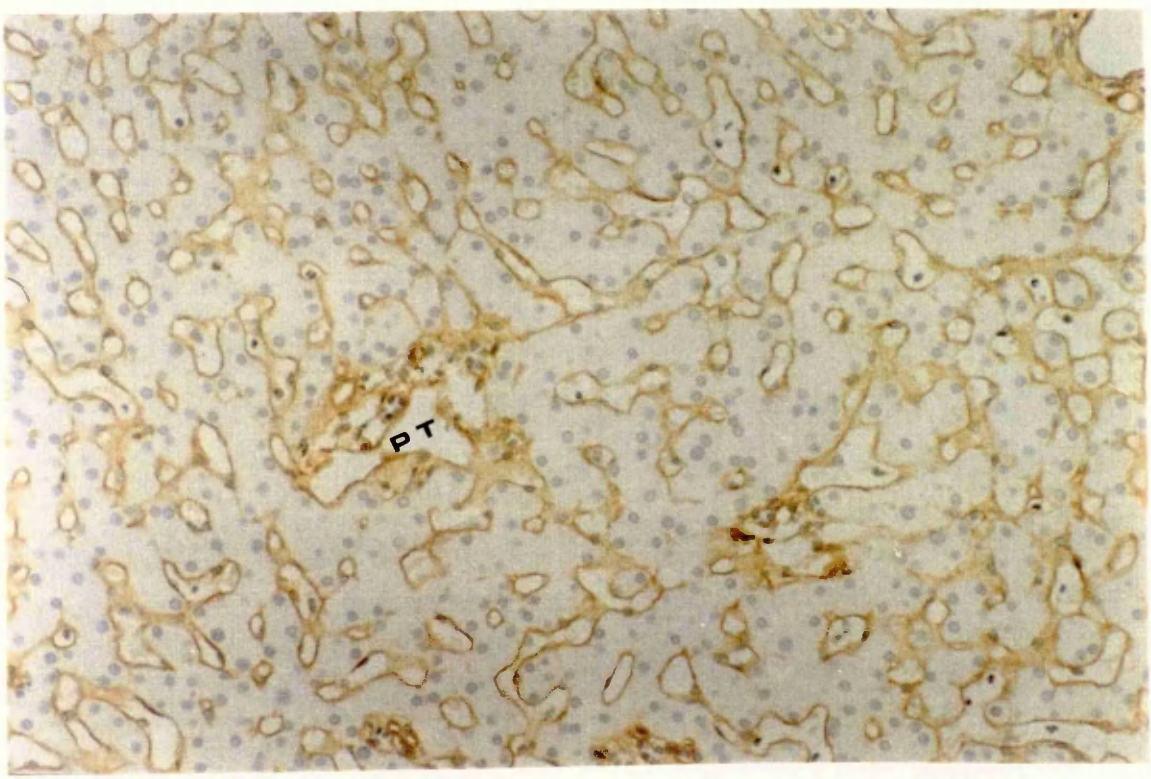

FIG 9.2

FIG 9.3

Distribution of type III collagen in normal liver. (peroxidase-antiperoxidase labelling).

There is no zonal heterogeneity for type III collagen within the liver. It is found in the space of Disse and around portal tracts (PT).

FIG 9.4

Distribution of type IV collagen in normal liver (peroxidase-antiperoxidase labelling).

There are small amounts of type IV collagen present within the space of Disse, but it is more intense around blood vessels. Weak labelling can be seen within portal tracts (PT).

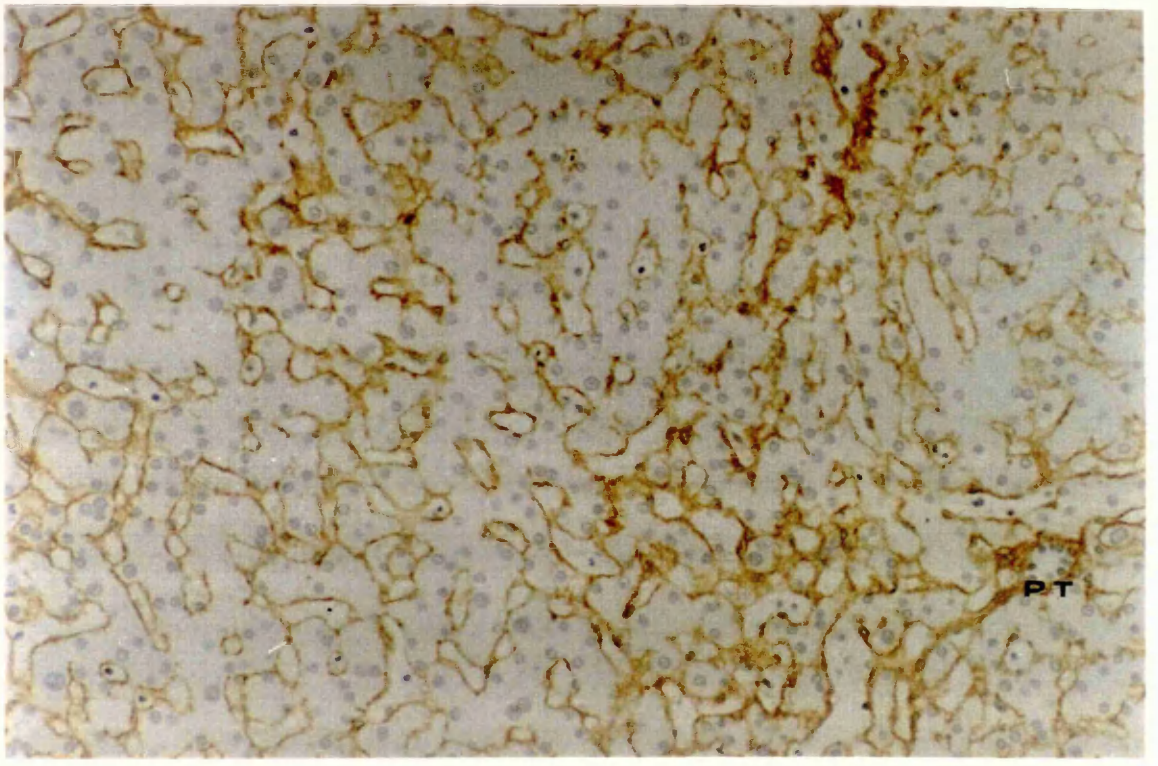

FIG 9.3

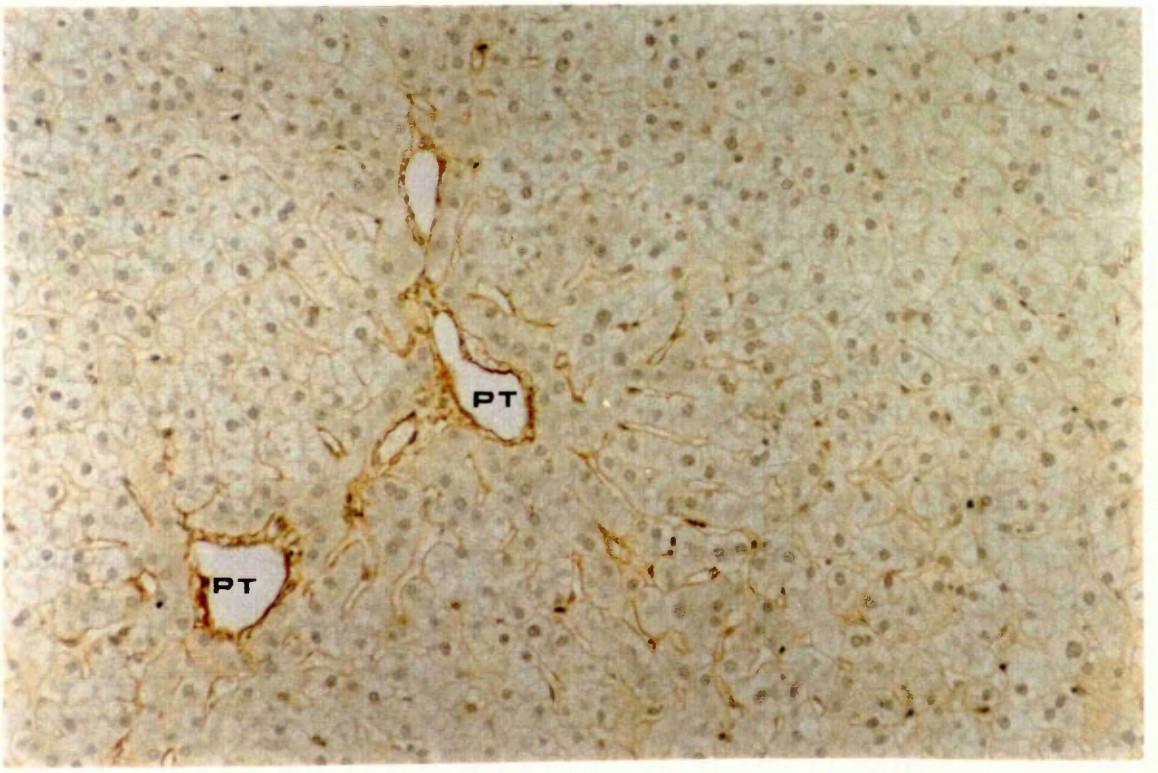

FIG 9.4

FIG 9.5

Distribution of type V collagen in normal liver (peroxidase-antiperoxidase labelling).

Type V collagen is found in the space of Disse and around the terminal hepatic vein branches. There is no zonal heterogeneity.

FIG 9.6

Distribution of type VI collagen in normal liver (peroxidase-antiperoxidase labelling).

Although type VI collagen is found in the space of Disse and around blood vessels, the overall distribution has a zonal pattern. The area of weakest staining was always the periportal zone.

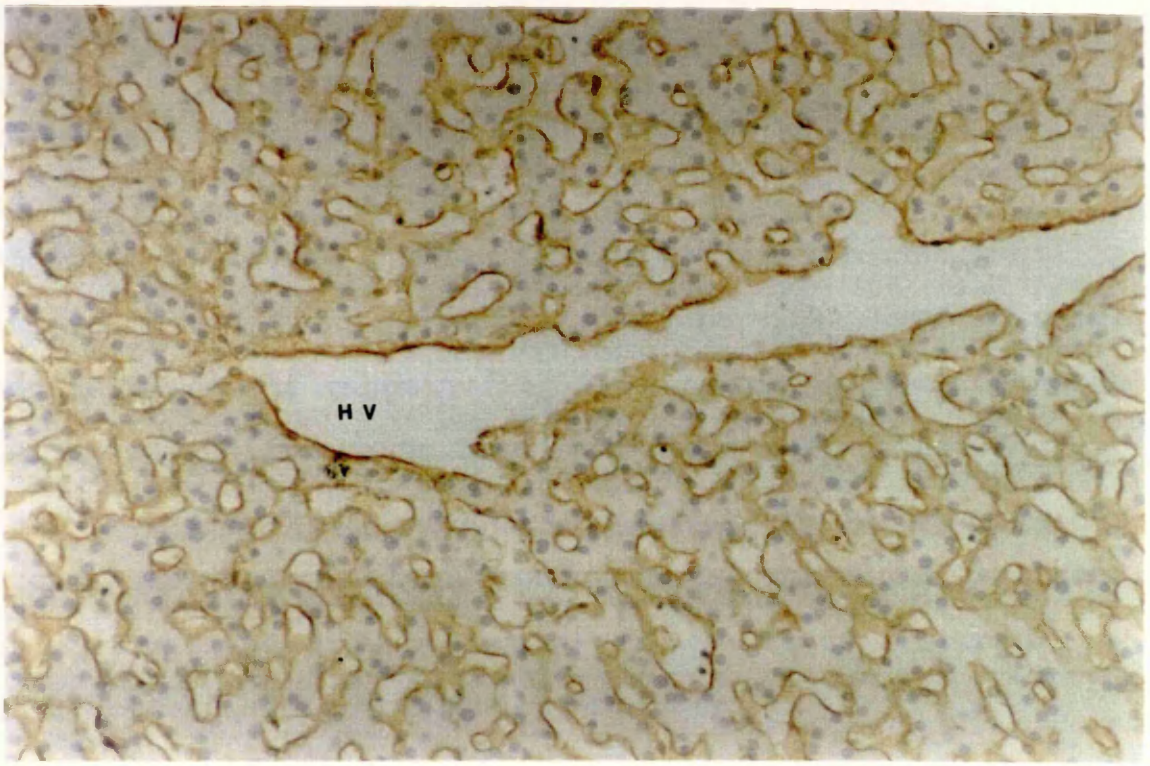

FIG 9.5

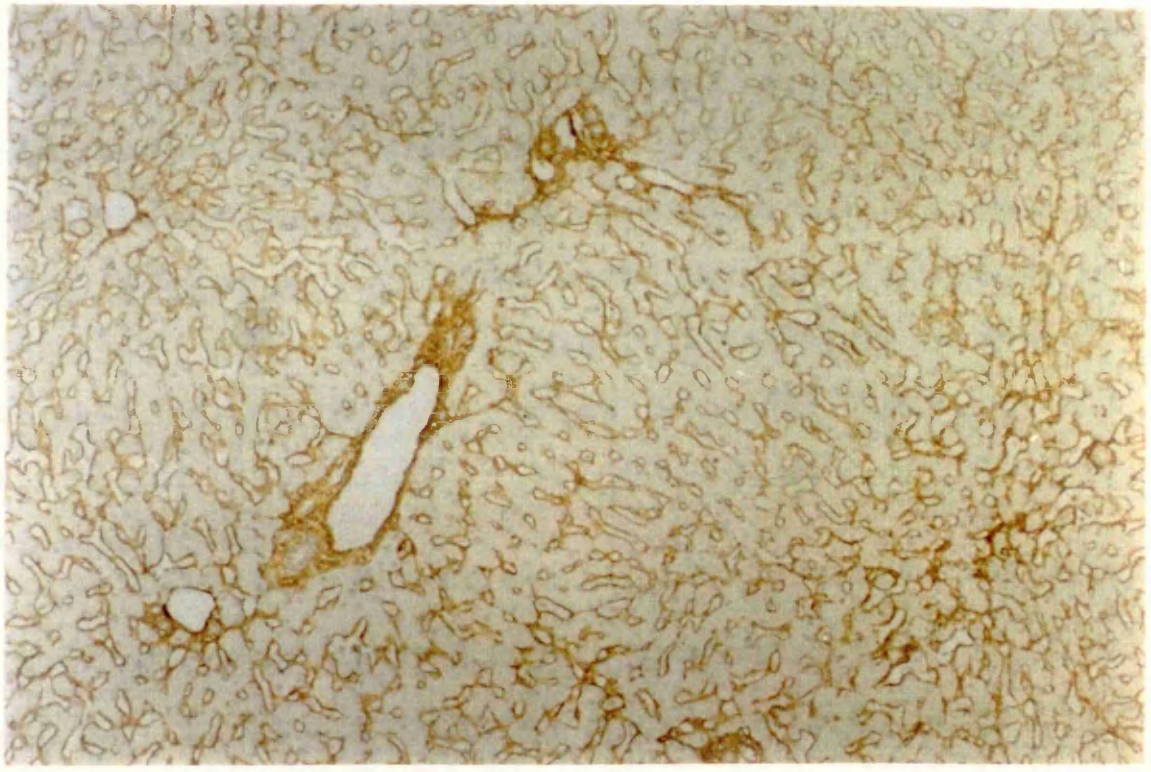

FIG 9.6

periportal zone (zone 1) when compared to zones 2 and 3. This zonal heterogeneous distribution was found in all sections of normal liver. There was also immunolabelling in the portal tract stroma, around terminal hepatic veins and in Glisson's capsule (Fig 9.6). This was seen in both perfusion and immersion fixed tissues, and was not thought to be a staining artifact. At this level, it was occasionally possible to detect some intracellular staining of sinusoidal cells with a stellate morphology. Hepatocytes were consistently negative.

9.3 Fibronectin. This was found in the space of Disse with a linear distribution and also in portal tracts and around terminal hepatic veins. The fibronectin was evenly distributed between the acinar zones. There was intracellular labelling in a number of hepatocytes and these were randomly distributed.

9.4 Vitronectin. This could only be identified in frozen sections. The distribution appeared to be similar to that of fibronectin, apart from the lack of any intracellular labelling. The antibody was not suitable for further studies.

9.5 Undulin. The staining obtained with this antibody were weak and inconsistent. Using Bouins fixed material it was possible to localise Undulin within the space of Disse and in portal tracts, but this antibody was not used for further studies.

9.6 Laminin. The ability to identify laminin was dependent on the fixative used (Table 10.1). The best results for all laminin antibodies were obtained with Bouins-fixed material. Laminin showed a similar distribution to type IV collagen, with strong labelling around blood vessels and bile ducts. It was also possible to localise laminin within the space of Disse, although this labelling was less intense than that found around the blood vessels and bile ducts. Occasional intracellular staining was found in cells with morphology typical of fat-storing cells.

9.7 Immunocytochemistry at electron microscopy level.

The majority of the tissue used in this study was obtained from needle biopsies. The initial protocol involved perfusing the biopsies with buffer via a very fine glass pipette in order to remove blood cells and to dilate the sinusoids before fixation. Although this gave small pieces of well perfused tissue, the damage caused at the immediate site of perfusion by both penetration of the glass pipette and pressure of the perfusate meant that only a small portion of the tissue was suitable for immunolabelling. As the biopsies were only a few millimetres in size to start with, this meant an unacceptably high proportion of the tissue was lost. As a result of this the biopsies were immersion fixed for the remainder of the study. The morphology was still

acceptable, and the pieces of tissue were small enough to allow even fixation throughout the entire biopsy.

In the preliminary stages of this study a number of different fixatives and embedding techniques were compared. Liver tissue which had been embedded in araldite resin gave excellent morphology but irrespective of the fixative used no positive staining was obtained. This suggested that the embedding procedure was either masking the antigenic sites or physically altering them, preventing recognition by the antibody. Etching the resin with periodic acid to remove the surface resin and prevent antigen masking did not result in any labelling. As a result of this, it was decided to use ultrathin frozen sections.

A range of sucrose concentrations from 1.3M to 3M were tested for use with liver biopsies (1.3M, 1.5M, 1.8M, 2M and 2.3M) and the most successful concentration was found to be 2.3M. This was a similar concentration to that used by other groups (Tokuyasu, 1986). It prevented any damage and allowed the tissue to retain plasticity during sectioning.

Positive immunostaining with good morphology could be obtained with either 2% paraformaldehyde alone or 2% paraformaldehyde with 0.1% glutaraldehyde. If the concentration of glutaraldehyde increased above this level the staining was rapidly lost. There was still an unacceptably high level of non-specific or background staining and a number of different blocking techniques were tested in order to reduce the background staining. These included incubating sections face down on gelatin plates,

incubating in 5% normal calf serum or incubating in 1% ovalbumin. The most effective agent was found to be the addition of 1% immunoglobulin free-bovine serum albumin (BSA). The sections were pre-incubated in this for 1 hour at room temperature or overnight at 4°C before immunolabelling and it was also used as the diluent for the antibody. The degree of background labelling was assessed by examining the control (diluent only, and diluent plus an appropriate serum) sections. Each individual staining run was only acceptable if the level of immunolabelling in these control sections was extremely low, and was evenly distributed over the section. If the amount of gold labelling was high, or if clusters of gold were visible, the run was discarded.

When the sections were examined using light microscopy methods the immunolabelling was improved by pre-treatment with trypsin. In tissues fixed for immunolabelling with electron microscopy, trypsin is too harsh a treatment and an alternative had to be found. As many of the antigenic sites of the extracellular matrix are masked by an abundance of "ground substance" or hyaluronic acid, the effect of pre-incubating sections with hyaluronidase before immunolabelling was examined. Using this enzyme helped to remove background staining and increased the amount of specific labelling. The morphology of the tissue did not appear to be affected and the only minor difference seen was a small reduction in the amount of amorphous material in the extracellular spaces.

The use of hyaluronidase only affected the labelling of extracellular areas and had no effect on intracellular labelling. In an attempt to improve intracellular labelling, the ultrathin sections were either pretreated with 0.1% saponin or saponin was added to the antibody and wash buffers. This did not increase the amount of intracellular labelling or improve the extracellular labelling and the use of saponin was discontinued.

When all of these different aspects of immunolabelling were examined, it was decided that the best fixative for use in this study was 2% paraformaldehyde with 0.1% glutaraldehyde. The morphology was poorer than that found in glutaraldehyde fixed material but the immunolabelling was far superior and this was the prime consideration. Unless otherwise stated all illustrations of ultrastructural immunocytochemistry are of tissue fixed in 2% paraformaldehyde/0.1% glutaraldehyde.

Figure 9.7 illustrates the preservation of morphology using the adapted ultracryomicrotomy protocol. Membraneous structures such as the endoplasmic reticulum are well preserved and can clearly be identified. The vacuoles seen in the hepatocytes are believed to be due to the loss of glycogen rosettes.

In comparison with Figure 9.7, Figure 9.8 illustrates the morphology obtained when normal human liver is resin embedded. The boundaries of the space of Disse are clear and the collagen bundles within the space of Disse are easily identified by their striated appearance.

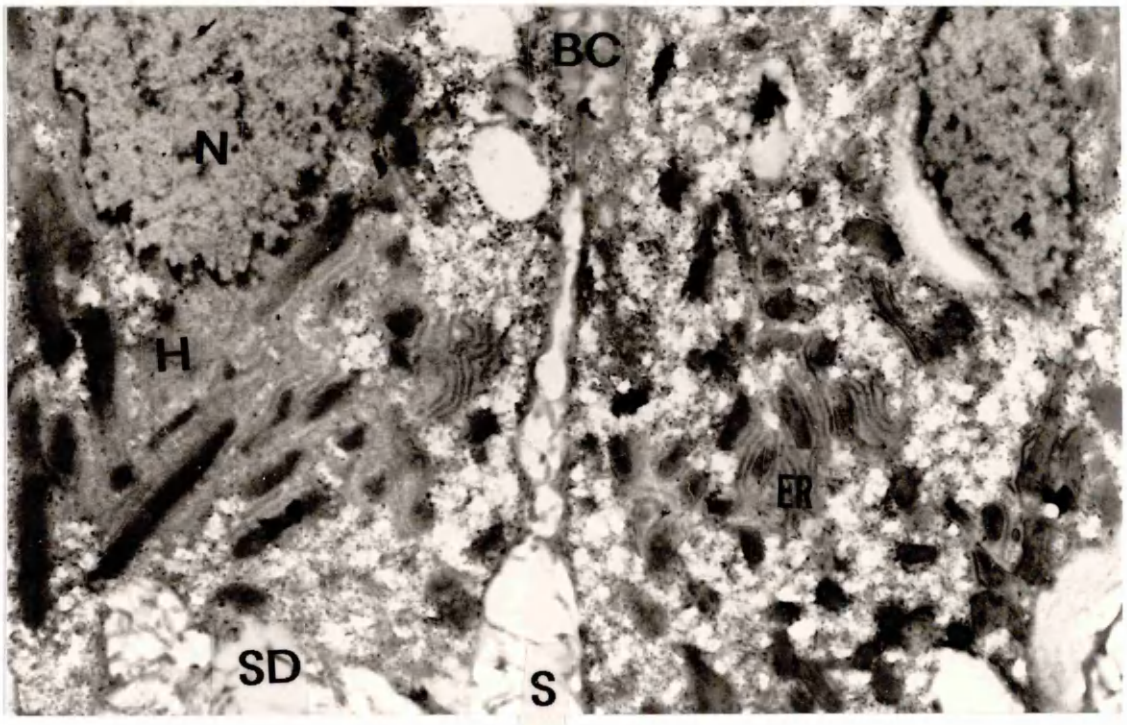

FIG 9.7

Ultracryosection of normal human liver.

This shows the degree of preservation of morphological detail typical of sections obtained by ultracryomicrotomy. The membraneous structures such as the endoplasmic reticulum are well preserved and easily identified, but some cytoplasmic components such as glycogen rosettes are lost. Mag x 7792

N - nucleus SD - space of Disse S - sinusoid

B.C. - bile canaliculus ER - endoplasmic reticulum

H - hepatocyte

FIG 9.8

Resin embedded ultrasection of normal human liver. In comparison with Fig 9.7 the glycogen rosettes can be seen in the cytoplasm. Collagen bundles can be seen within the space of Disse, and the fenestrated endothelial cells divide the sinusoid from the space of Disse. Mag x 32,000

G - glycogen

S - sinusoid

EC - endothelial cell

C - collagen

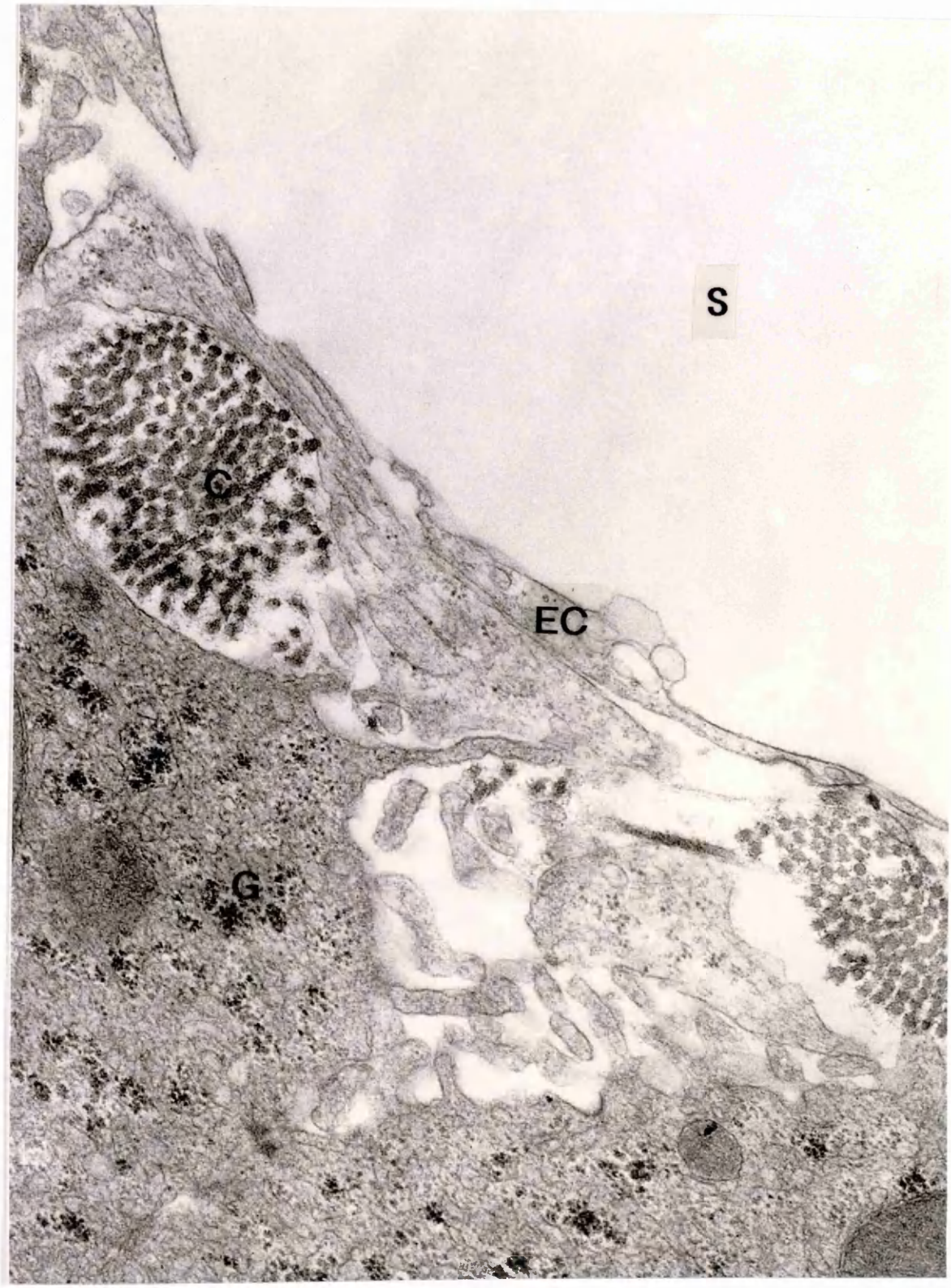

Figure 9.8

9.8 Collagen

Type I collagen. Labelling for type I collagen was identified on striated fibres within the space of Disse. This was most intense along the edges of bundles (Fig 9.9). The labelled bundles were often adjacent to fat-storing cells (Figure 9.11). There was also labelling within all portal tracts. As well as ordered bundles, there were also disorganised areas of collagen fibres; these may be newly synthesised fibres which have not yet formed bundles (Fig 9.10).

Type III collagen Type III collagen was also found within the space of Disse (Fig 9.12) but the labelling was often more intense than that found for type I collagen (Figs 9.13 and 9.14). The pattern of staining was even throughout the collagen bundles and was not concentrated at the margins as found with type I collagen (Figs 9.15, 9.16 and 9.17). When the tissue was examined with antibodies to procollagen types I and III it was apparent that a far higher proportion of the type III collagen fibres retained the propeptide portion of the molecule after fibril formation in comparison with type I.

When fat-storing cells were identified, they were often surrounded by type III collagen bundles (Figs 9.18 and 9.19). Despite the close proximity of the collagen bundles to the fat-storing cells intracellular labelling of the fat-storing cells was an extremely rare occurrence.

FIG 9.9

Ultracryosection of normal human liver.

Immunogold labelling (10nm) for type I collagen.

Heavy labelling for type I collagen can be seen on this collagen bundle. The labelling is concentrated on the single fibres at the outer edges of the bundles, although there is some labelling throughout the entire bundle. Mag x 25,186

SD - space of Disse H - hepatocyte

FIG 9.10

Ultracryosection of normal human liver.

Immunogold labelling (10nm) for type I collagen.

This area of partially disorganised striated collagen contains type I collagen. It is probably newly synthesised collagen which has not yet been organised into bundles as seen in the previous figure. The labelling is evenly distributed throughout. Mag x 22,050

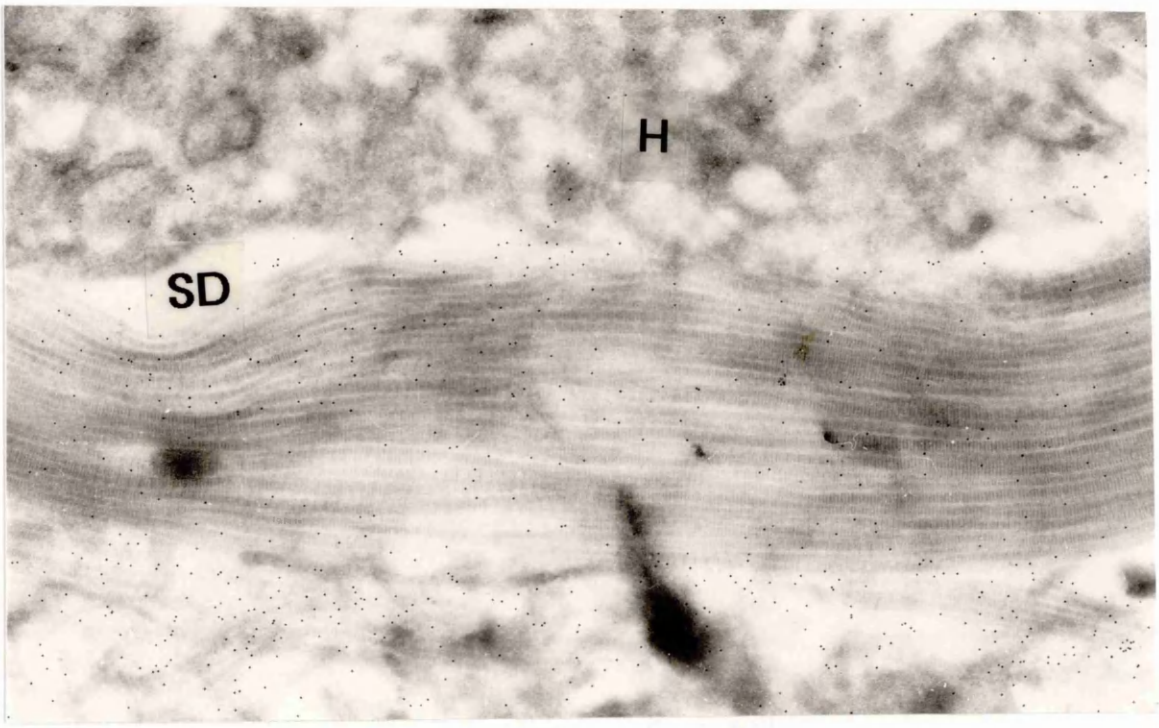

FIG 9.9

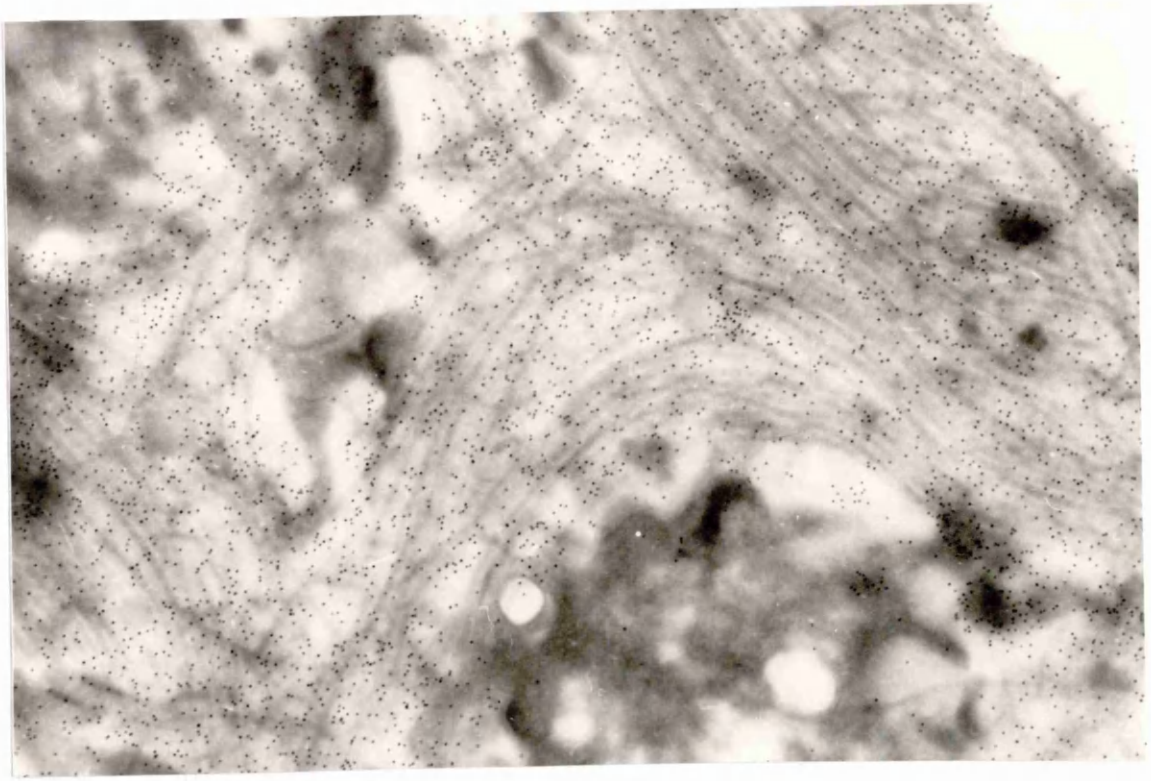

FIG 9.10

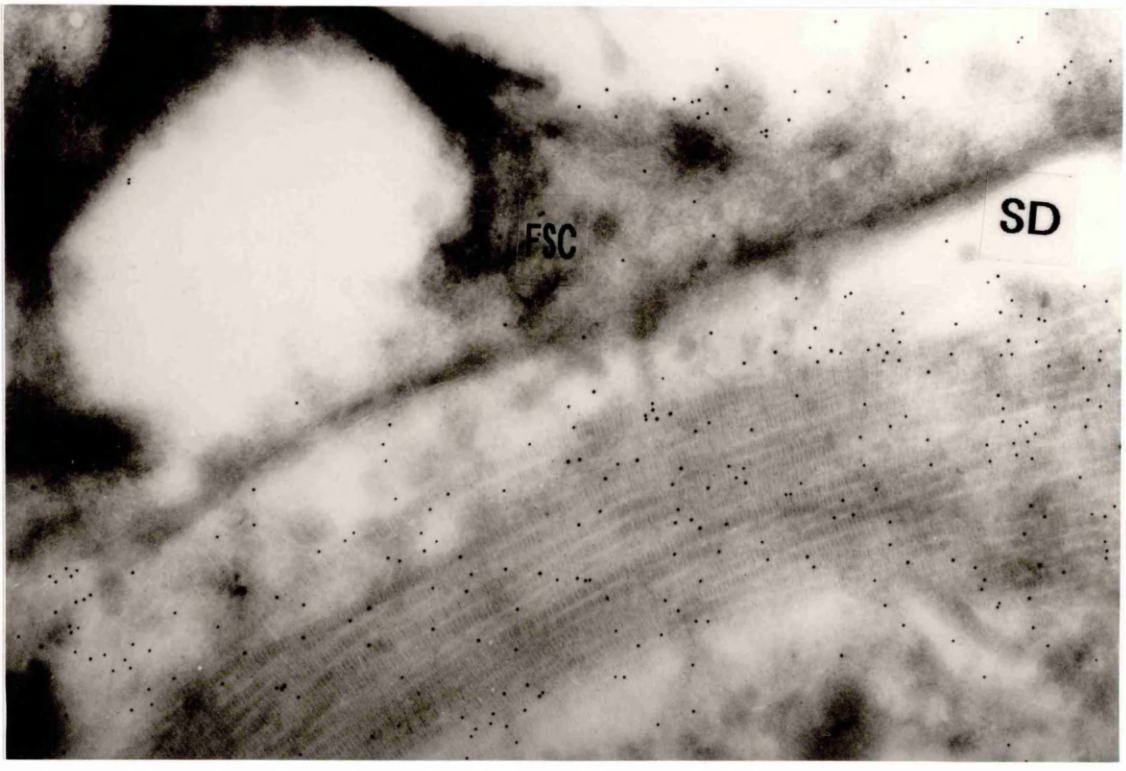

FIG 9.11

Ultracryosection of normal human liver.

Immunogold labelling (10nm) for type I collagen.

The collagen bundle adjacent to the fat-storing cell contains type I collagen. There is labelling around the cell and within the space of Disse, but there is no intracellular labelling in the fat-storing cell.

Mag x 19,500

FSC - Fat-storing cell

SD - space of Disse

FIG 9.12

Ultracryosection of normal human liver.

Immunogold labelling (10nm) for type III collagen.

This is a loosely associated bundle of collagen within the space of Disse. The labelling for type III collagen is evenly distributed across the bundle. There is no intracellular labelling in either the hepatocyte or the endothelial cell. Mag x 49,500

SD - space of Disse EC - endothelial cell

H - hepatocyte

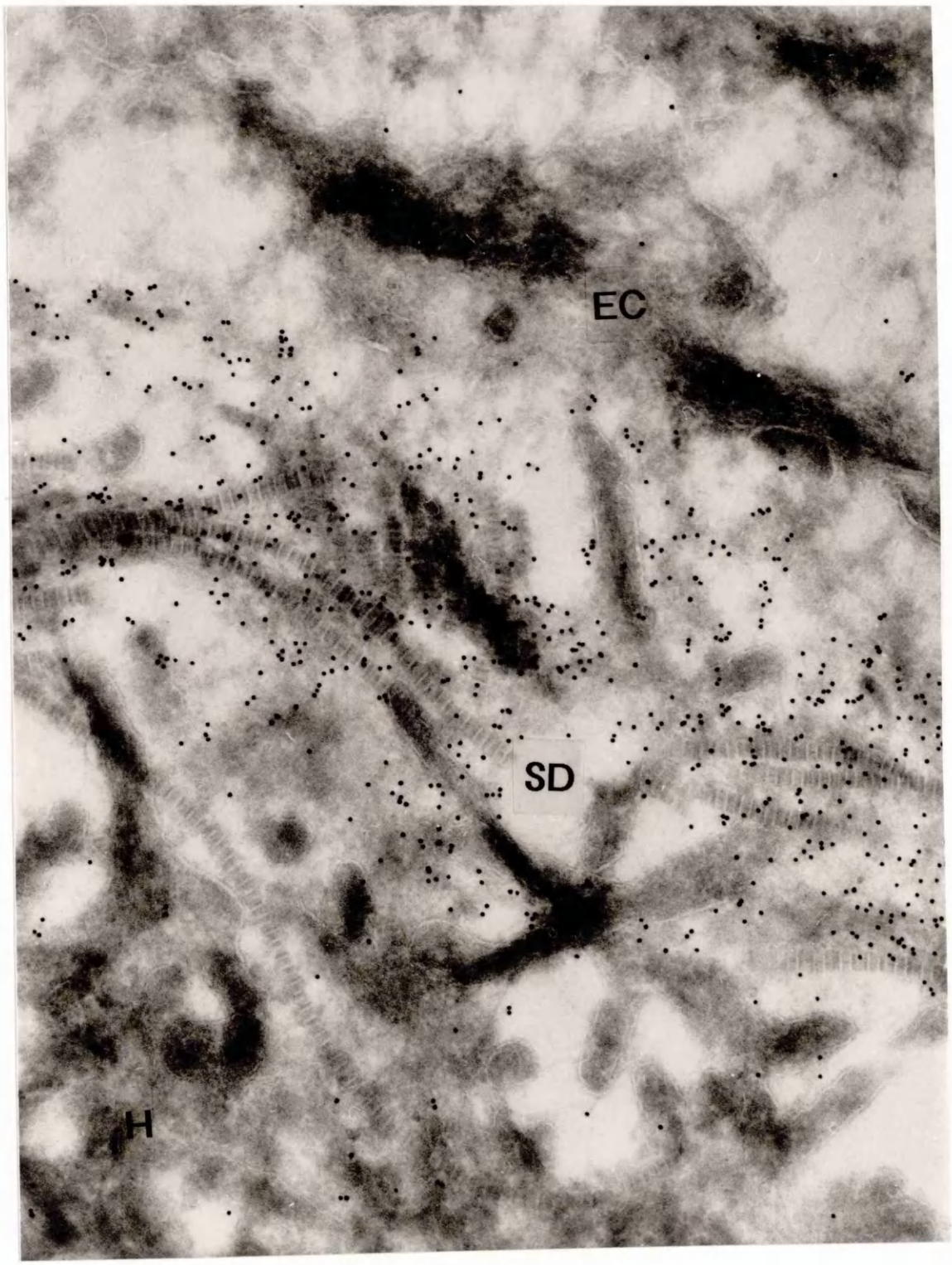

FIG 9.12

Fig 9.13

Ultracryosection of normal human liver.

Immunogold labelling (10nm) for type III collagen.

This view shows strong labelling for type III collagen which is confined to striated fibres in the space of Disse. Mag x 29,2500

H - hepatocyte

SD - space of Disse

FIG 9.14

Ultracryosection of normal human liver.

Immunogold labelling (10nm) for type III collagen.

The collagen fibres adjacent to the hepatocyte are strongly labelled for type III collagen, but the hepatocyte is completely negative. Mag x 49,500

H - hepatocyte

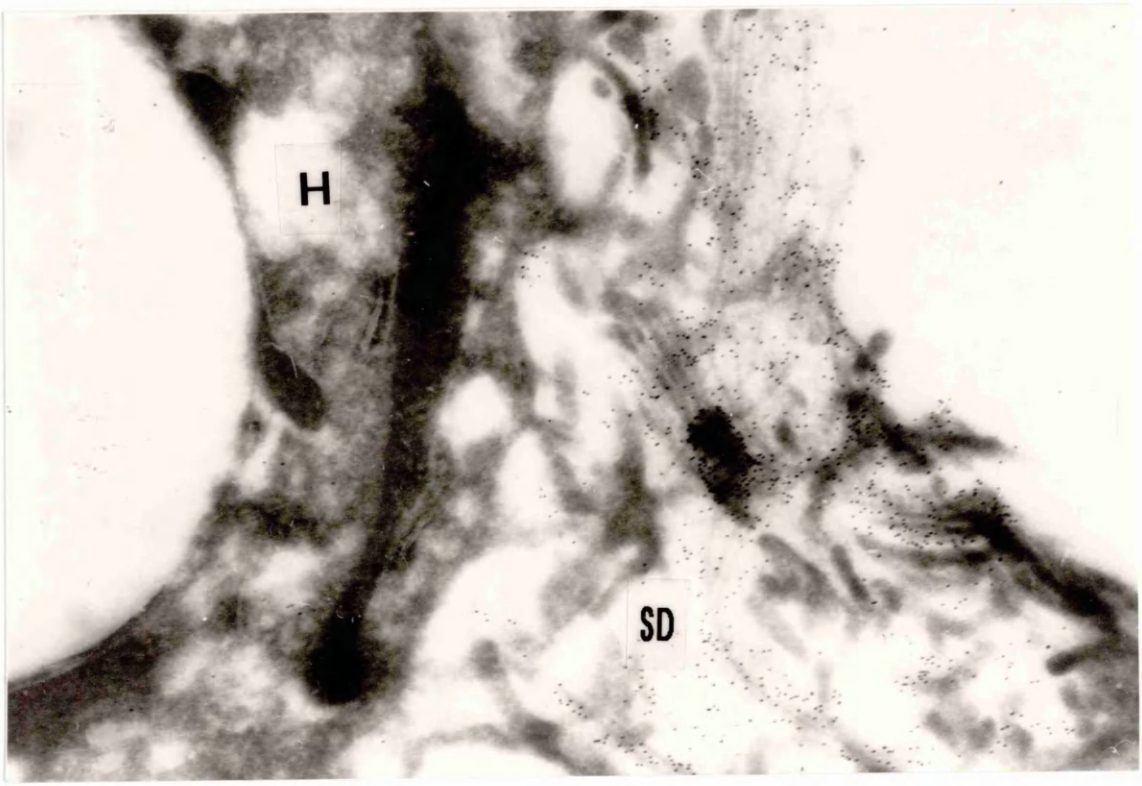

FIG 9.13

FIG 9.14

FIG 9.15

Ultracryosection of normal human liver.
Immunogold labelling (10nm) for type III collagen.
Labelling for type III collagen can be seen on collagen bundles in the space of Disse. Individual fibres and small aggregates are also labelled within the space of Disse. There is no intracellular labelling in this section.
Mag x 20,500

H - hepatocyte EC - endothelial cell

FSC - Fat-storing cell KC - Kupffer cell

FIG 9.16

Ultracryosection of normal human liver.
Immunogold labelling (10nm) for type III collagen.
At high magnification the type III collagen is seen to be evenly distributed throughout the bundle of striated collagen fibres. This is unlike the pattern found with type I collagen which is concentrated at the edges of the bundle. Mag x 135,200

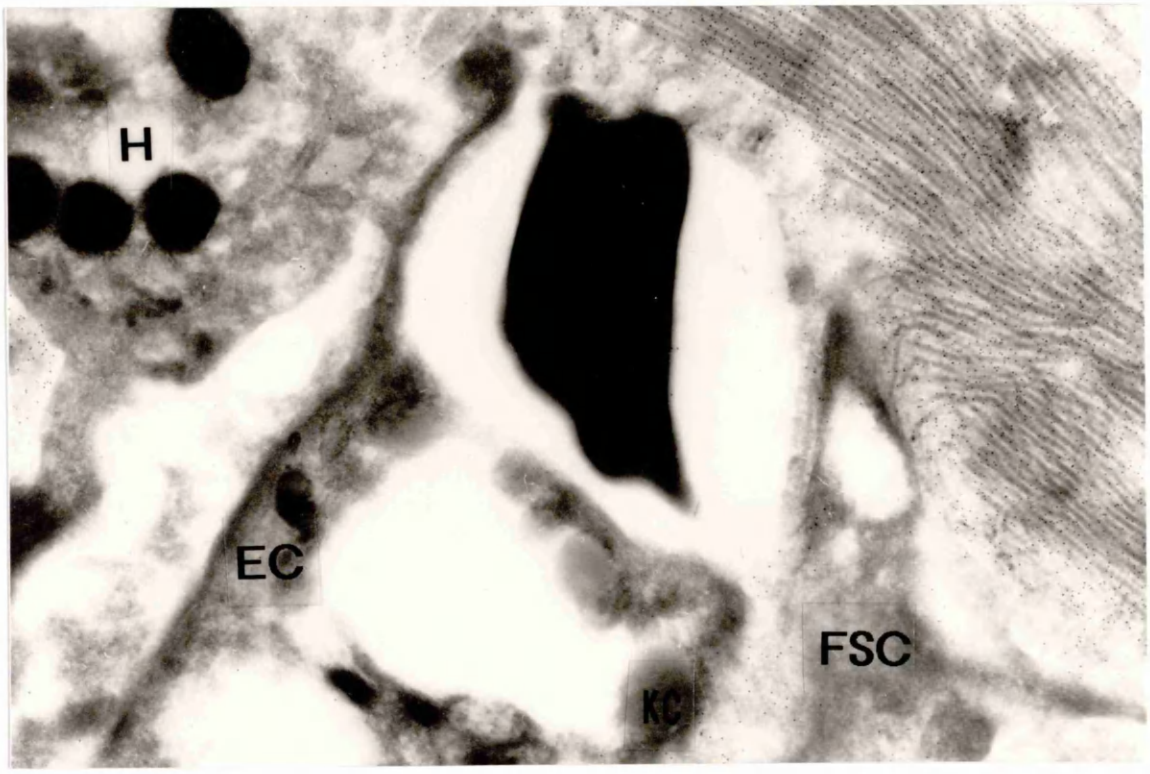

FIG 9.15

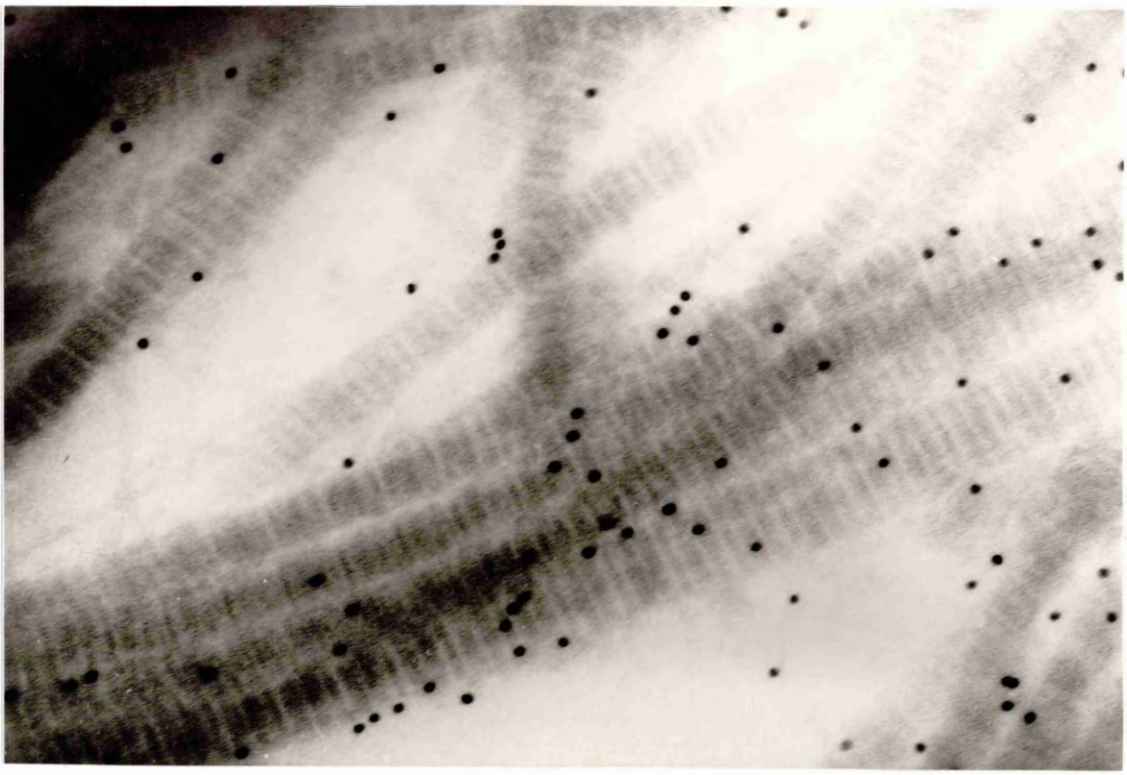

FIG 9.16

FIG 9.17

Ultracryosection of normal human liver.

Immunogold labelling (10nm) for type III collagen.

This bundle of striated collagen lying between two hepatocytes is heavily labelled for type III collagen. In comparison, there is no intracellular or pericellular labelling of the hepatocytes. Mag x 16,875

H - hepatocyte

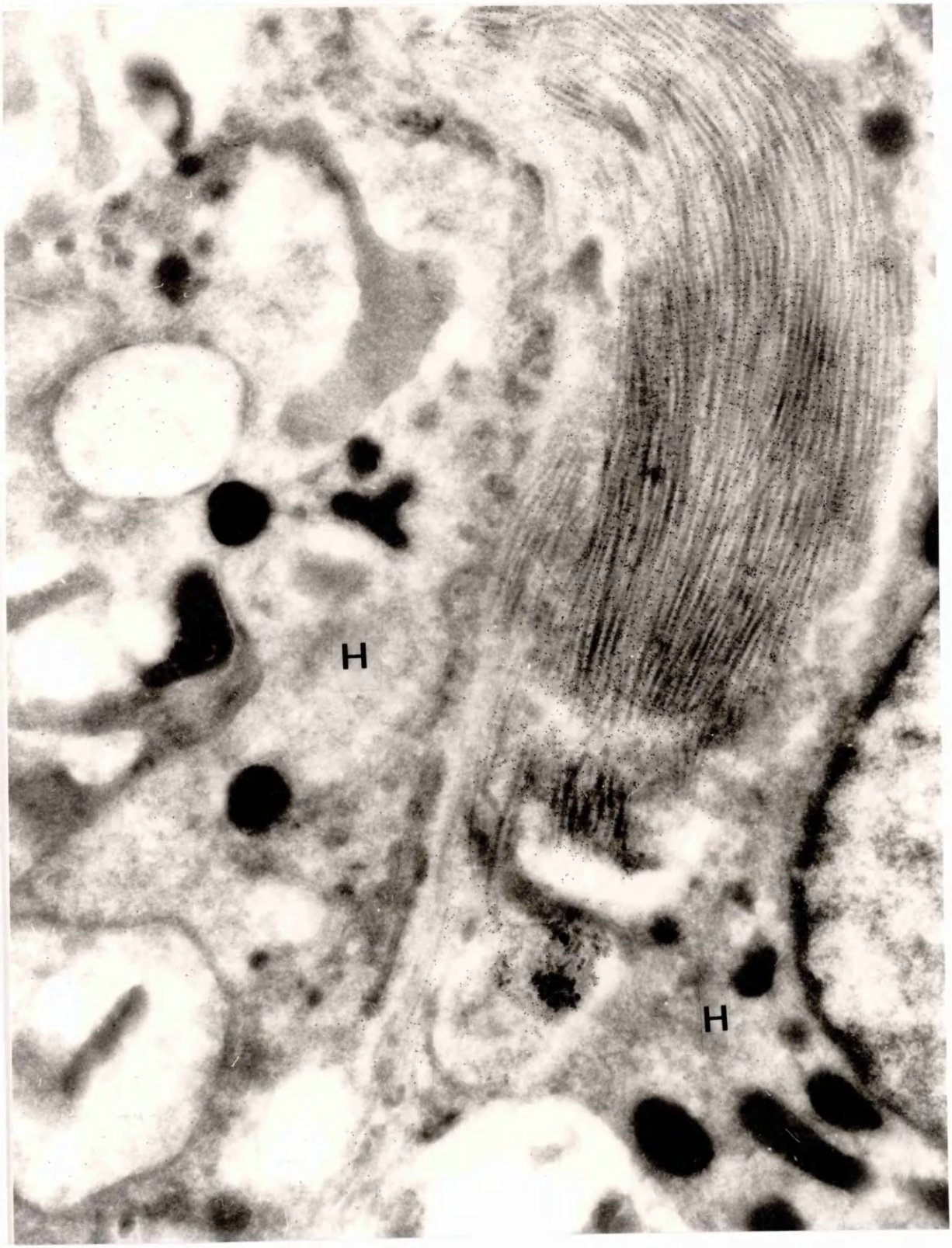

Figure 9.17

FIG 9.18

Ultracryosection of normal human liver.

Immunogold labelling (10nm) for type III collagen.

Fat-storing cells were often surrounded by bundles of collagen. In contrast to hepatocytes, the fat-storing cells were frequently labelled for type III collagen in a pericellular pattern in many instances. There was no intracellular labelling found in these areas.

Mag x 7,150

FSC - Fat-storing cell EC - endothelial cell

FIG 9.19

Ultracryosection of normal human liver.

Immunogold labelling (10nm) for type III collagen.

A higher magnification of Figure 9.18 demonstrates the type III collagen adjacent to the fat-storing cell.

Mag x 14,275

FSC - Fat-storing cell

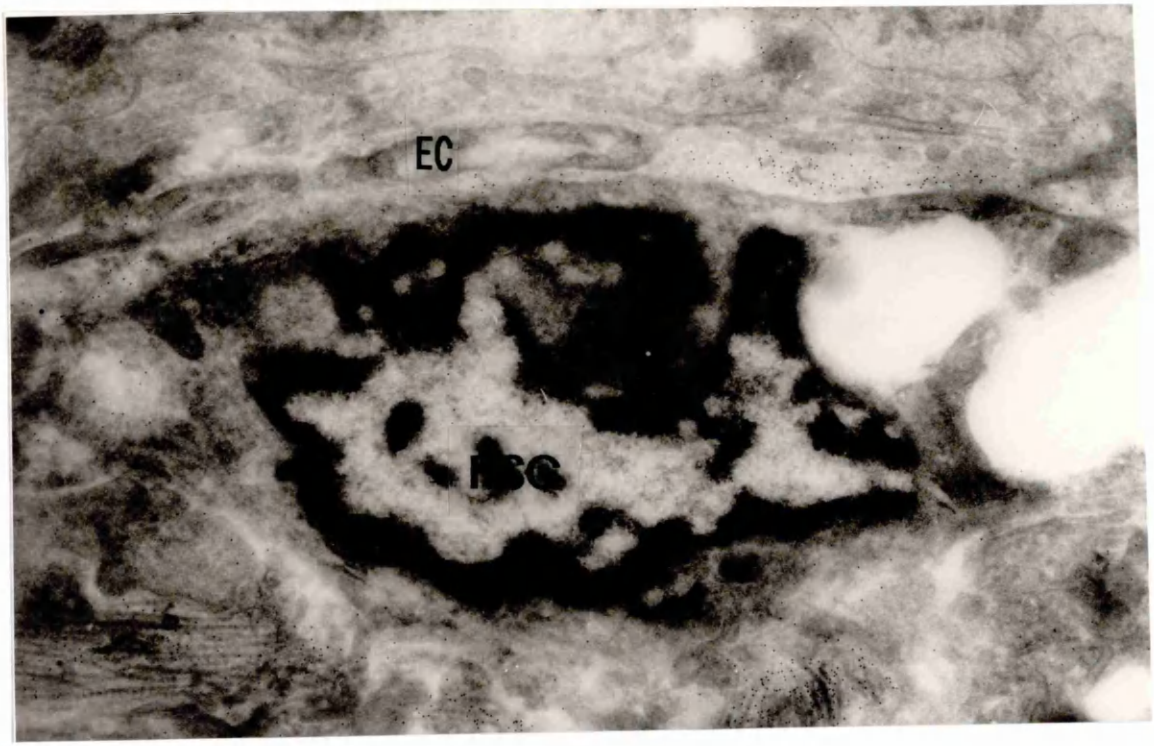

FIG 9.18

FIG 9.19

Type IV collagen Labelling for this component could be identified around blood vessels and bile ducts. Although a conventional basement membrane could not be identified by electron microscopy, type IV collagen was found within the space of Disse, often near bundles of striated collagen but not on the bundles or within hepatocytes (Fig 9.20), fat-storing cells (Fig 9.21) or Kupffer cells (Figs 9.22 and 9.23). Fig 9.24 shows a low power view of a hepatic sinusoid, and Fig 9.25 illustrates the labelling for type IV collagen within the space of Disse. It was found below the endothelial cells (Fig 9.26), and surrounding the fat-storing cells (Fig 9.27) but no intracellular labelling was seen. The subendothelial labelling was present along the space of Disse (Figs 9.28a and 9.28b). When this is examined at a higher magnification, the labelling is confined to a number of small linear areas which correspond to amorphous material within the space of Disse (Fig 9.29).

Type V collagen. Immunolabelling with anti-type V collagen was only effective in Bouins fixed tissue at light level microscopy, and gave no positive results when used for ultrastructural studies, irrespective of the use of the pre-treatments previously discussed.

Type VI collagen When this collagen was immunolocated at the ultrastructural level, labelling for type VI collagen was present within the space of Disse and portal tract stroma. It differed from the other collagen types in several ways. The gold particles were frequently found

FIG 9.20

Ultracryosection of normal human liver.

Immunogold labelling (10nm) for type IV collagen.

This shows type IV collagen within the space of Disse. Labelling is present but striated collagen bundles are not labelled and there is no intracellular labelling.

Mag x 26,500

C - collagen SD - space of Disse

H - hepatocyte

FIG 9.21

Ultracryosection of normal human liver.

Immunogold labelling (10nm) for type IV collagen.

Type IV collagen can be identified within the space of Disse in areas containing amorphous material rather than striated collagen bundles. The fat-storing cell is not labelled intracellularly . Mag x 31,500

FSC - Fat-storing cell

H - hepatocyte

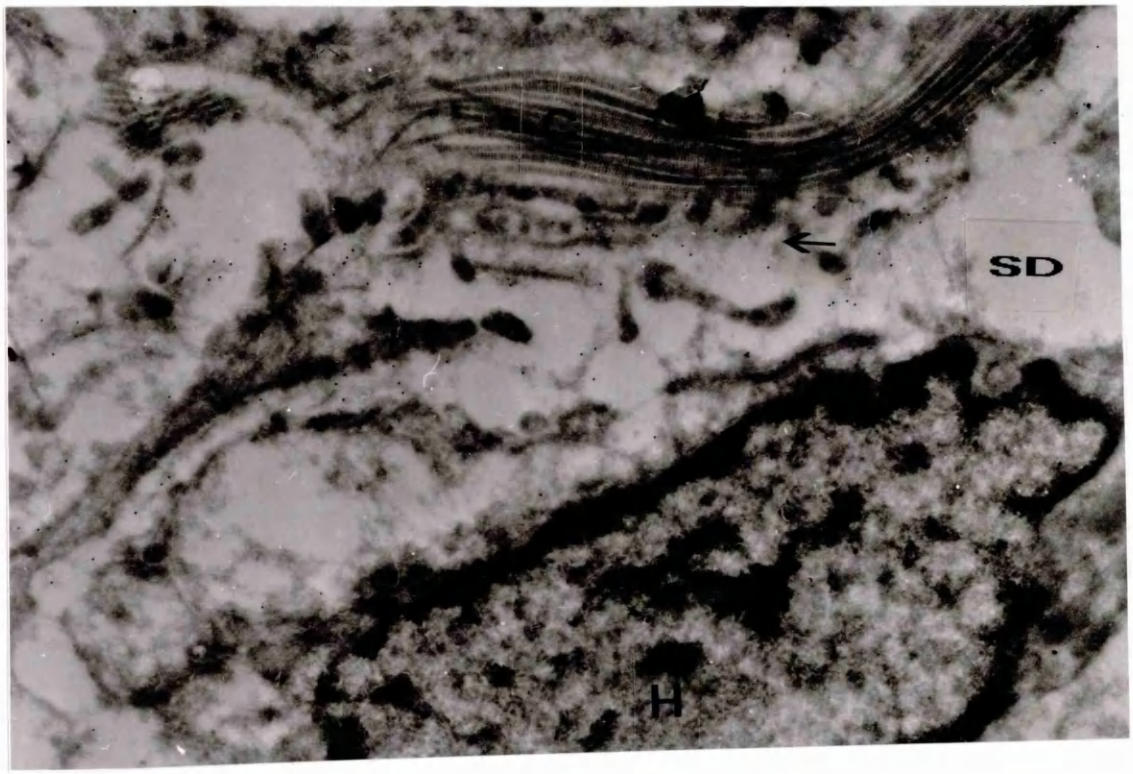

FIG 9.20

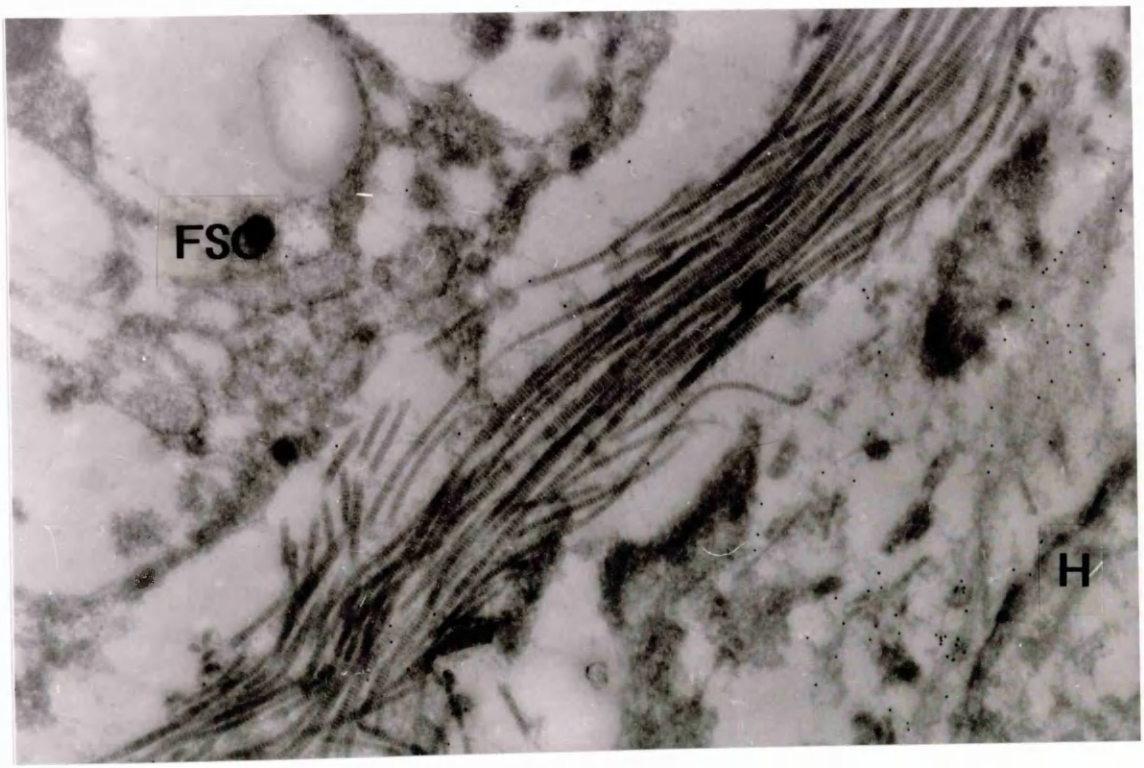

Fig 9.21

FIG 9.22

Ultracryosection of normal human liver.

Immunogold labelling (10nm) for type IV collagen.

Here the type IV collagen is deposited along the space of Disse. The gold particles are regularly distributed. Mag x 22,050

SD - space of Disse

KC - Kupffer cell

H - hepatocyte

FIG 9.23

Ultracryosection of normal human liver.

Immunogold labelling (10nm) for type IV collagen.

When the Kupffer cells were examined for type IV collagen there was no intracellular labelling, although there was extracellular labelling immediately adjacent. Mag x 16,750

KC - Kupffer cell

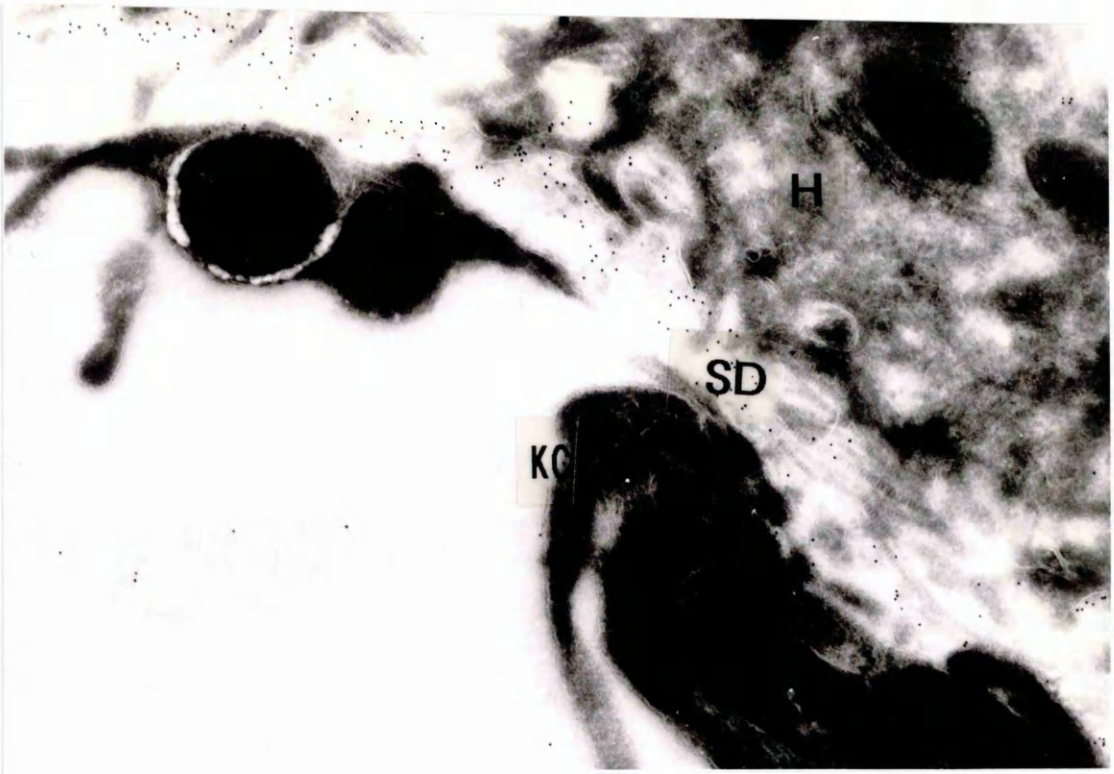

FIG 9.22

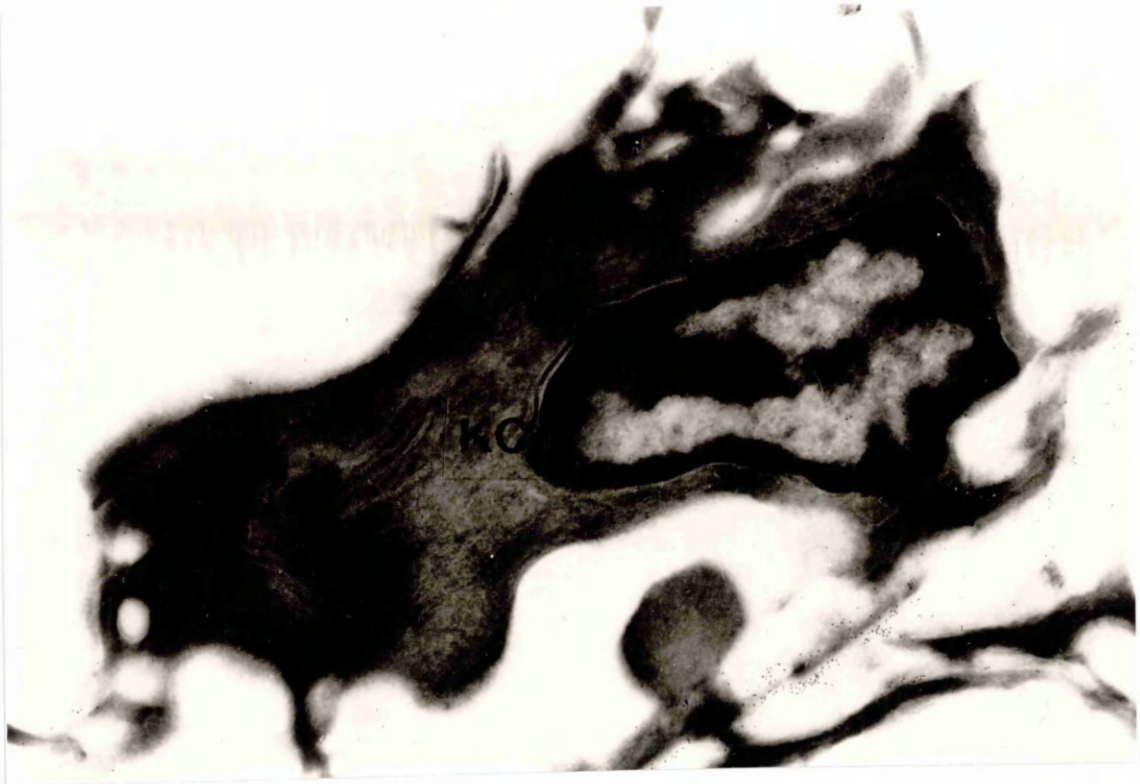

FIG 9.23

FIG 9.24

Ultracryosection of normal human liver.

This low power shows the hepatic sinusoid lined with endothelial cells. Fat-storing cells and hepatocytes can also be seen. The area marked * is enlarged in Fig 9.26. Mag x 6,300

S - sinusoid SD - space of Disse

EC - endothelial cell FSC - Fat-storing cell

H - hepatocyte

FIG 9.25

Ultracryosection of normal human liver.

Immunogold labelling (10nm) for type IV collagen.

The space of Disse contains linear deposits of type IV collagen, but no conventional basement membrane is visible. Mag x 9,675

FSC - Fat-storing cell H - hepatocyte

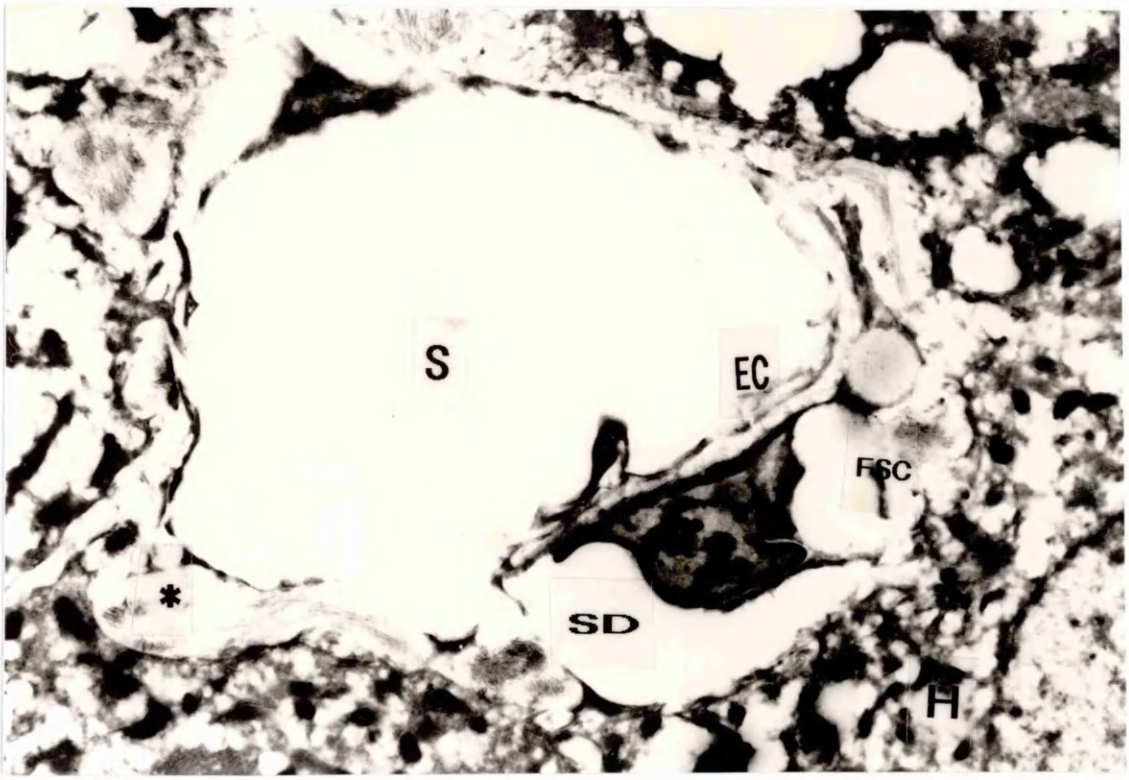

FIG 9.24

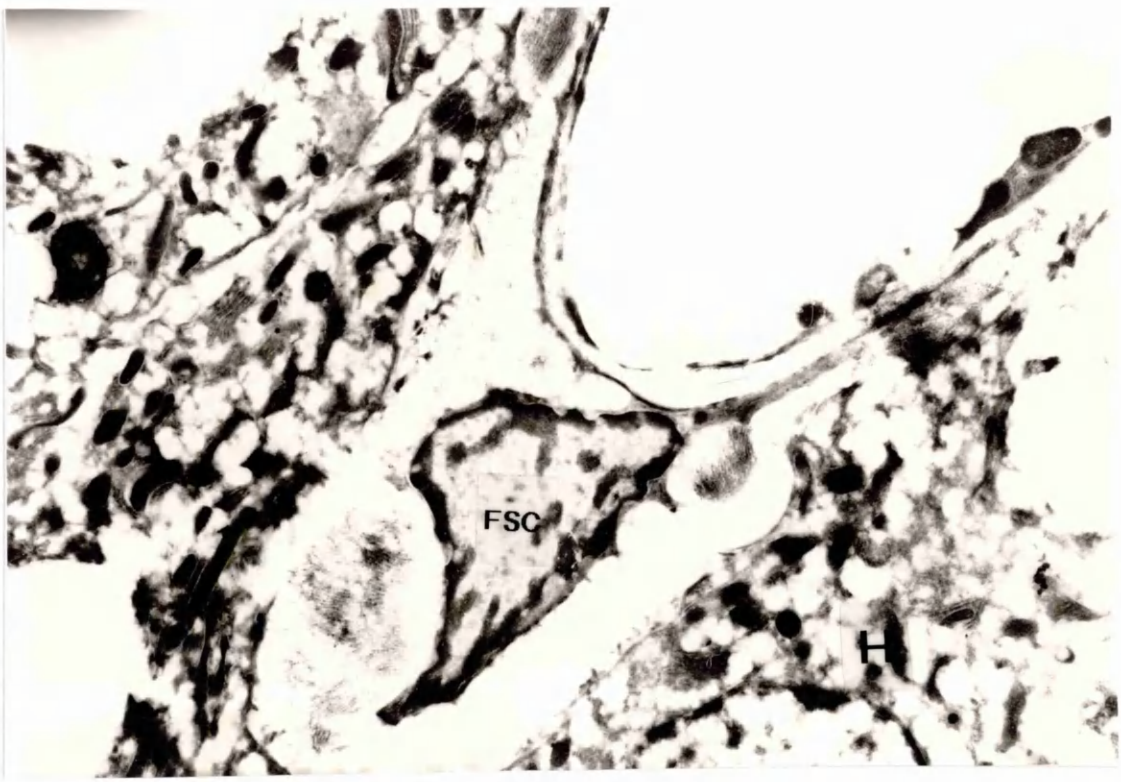

FIG 9.25

FIG 9.26

Ultracryosection of normal human liver.

Immunogold labelling (10nm) for type IV collagen.

Labelling for type IV collagen is seen here below the endothelial cells within the space of Disse. It is also present around collagen bundles and fat-storing cells. Mag x 15,500

FSC - Fat-storing cell C - collagen

EC - endothelial cell H - hepatocyte

FIG 9.27

Ultracryosection of normal human liver.

Immunogold labelling (10nm) for type IV collagen

At high power, the type IV collagen can be seen immediately adjacent to a fat-storing cell. No intracellular labelling is visible. Mag x 28,500

FSC - Fat-storing cell C - collagen

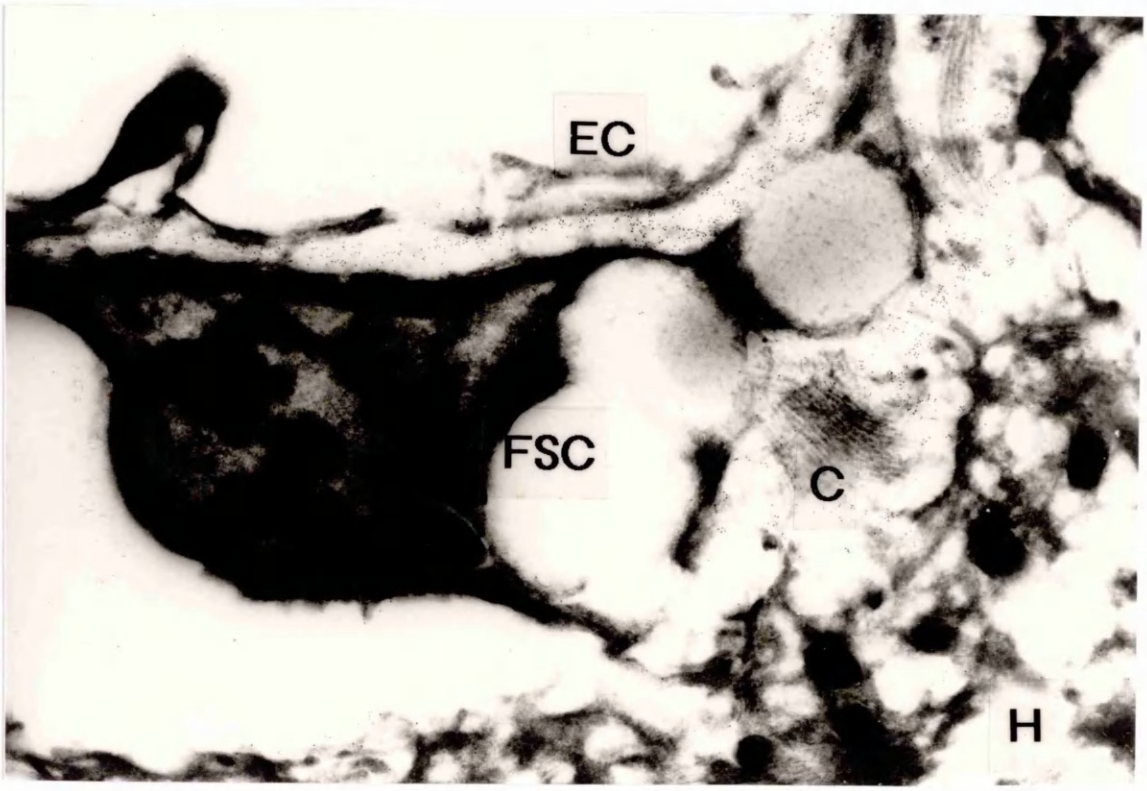

FIG 9.26

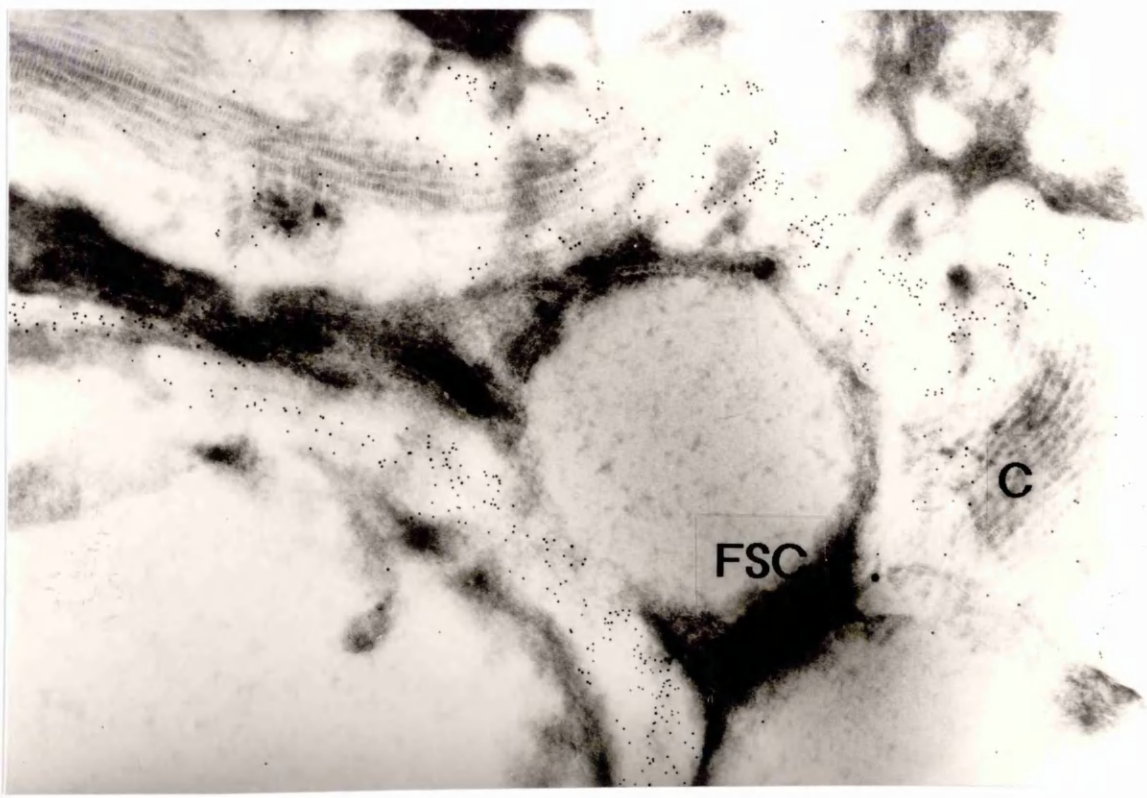

FIG 9.27

FIG 9.28a

Ultracryosection of normal human liver.

Immunogold labelling (10nm) for type IV collagen.

In this view of type IV collagen within the sinusoid, the endothelial cell can be seen lining the hepatic sinusoid. Mag x 26,500

EC - endothelial cell S - sinusoid

FIG 9.28b

Ultracryosection of normal human liver.

Immunogold labelling (10nm) for type IV collagen.

At higher power, the type IV collagen is seen deposited in a regular pattern along the space of Disse. Mag x30,450

EC - endothelial cell H - hepatocyte

SD - space of Disse

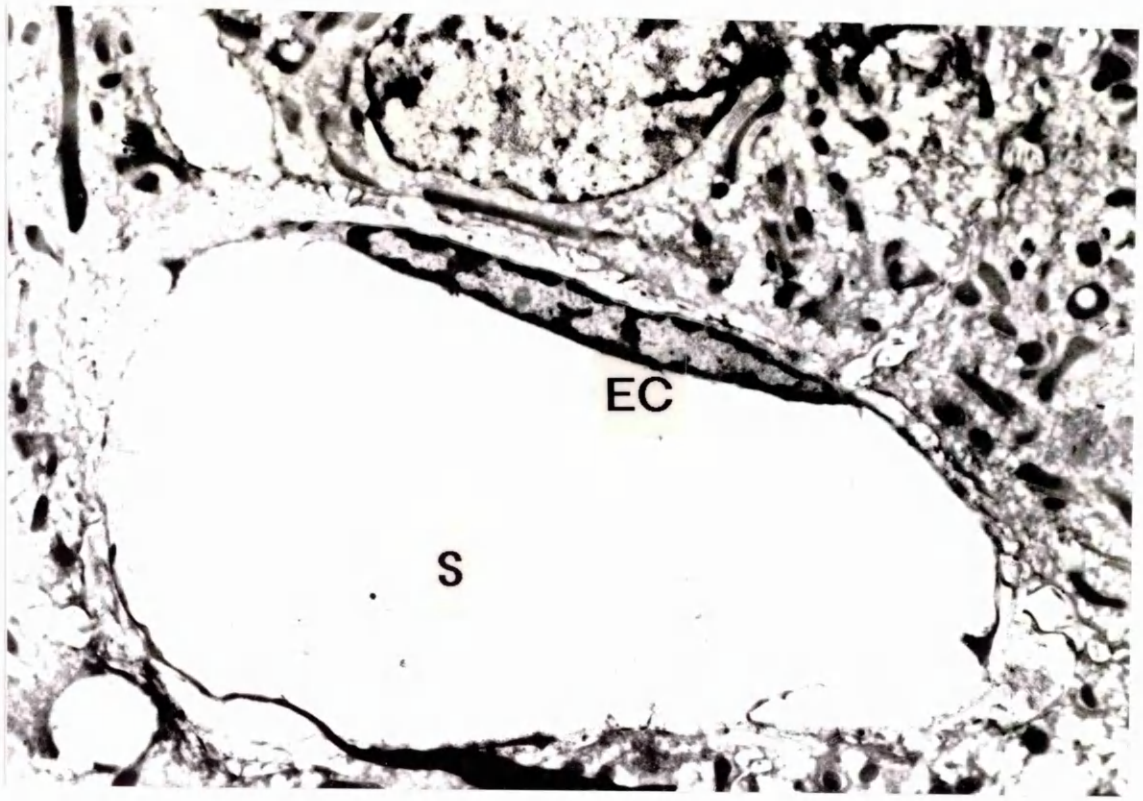

FIG 9.28a

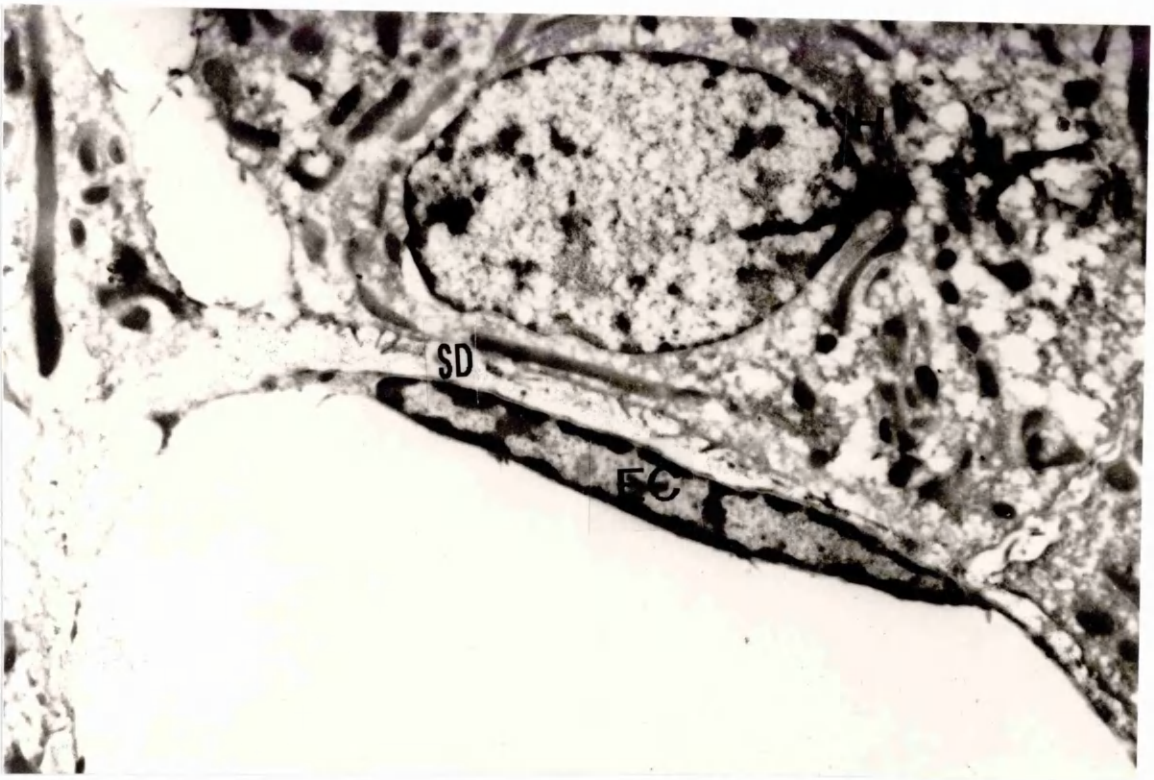

FIG 9.28b

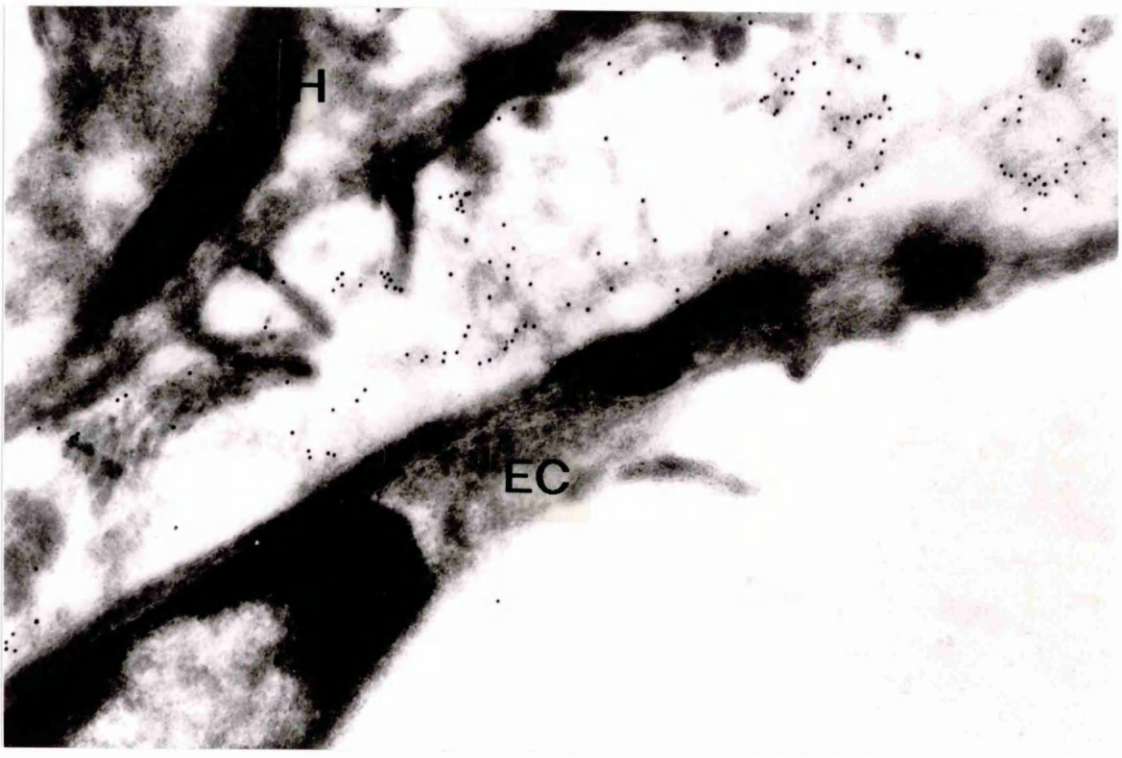

FIG 9.29

Ultracryosection of normal human liver.

Immunogold labelling (10nm) for type IV collagen

When Figure 9.28b is examined at a higher magnification, it can be seen that the labelling for type IV collagen is present on amorphous material within the space of Disse, but this does not have the same appearance as a conventional basement membrane. Mag x 49,500

EC - endothelial cell H - hepatocyte

FIG 9.30

Ultracryosection of normal human liver.

Immunogold labelling (10nm) for type VI collagen

The labelling for type VI collagen is present around the edges of striated collagen bundles and also between the individual striated fibres. It is less commonly found on the individual fibres. Mag x 47,250

FSC - Fat-storing cell

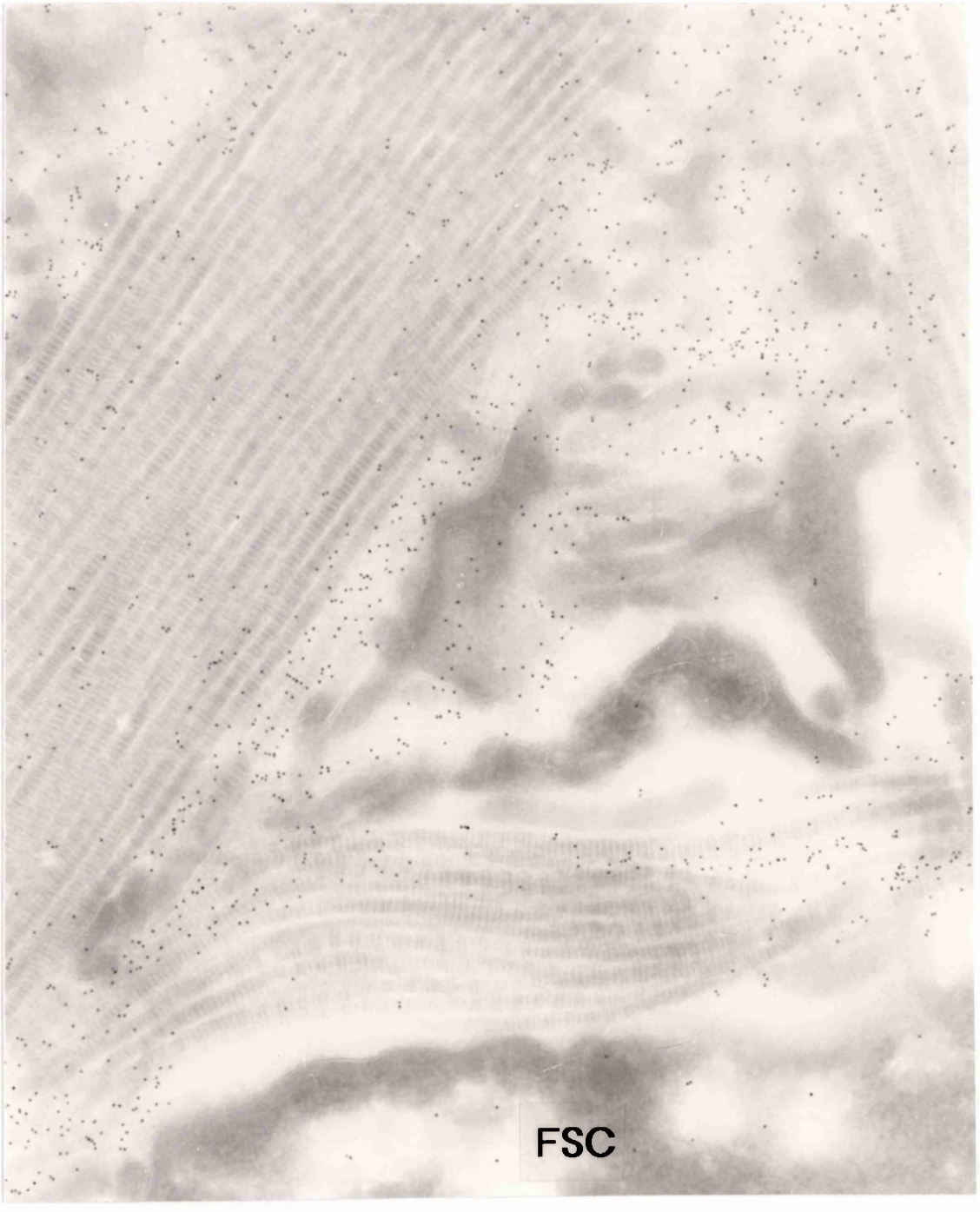

Figure 9.30

immediately adjacent to the bundles of striated collagen fibres, but were rarely found on the bundles. Instead, they could be found in between and surrounding the fibres (Fig 9.30). The type VI collagen structures which were labelled were rarely visible, but were occasional seen as fine unstriated filaments or amorphous material weaving between the collagen bundles. Intracellular labelling was restricted to the endoplasmic reticulum of occasional fat-storing cells and was of low intensity.

9.9 Fibronectin

This was immunolocated within the space of Disse (often adjacent to fat-storing cells (Fig 9.31), and in portal tracts. It was also found within several cell types. In some hepatocytes, it was localised to the endoplasmic reticulum and lysosomes (Fig 9.32). It was also seen in lysosomes within Kupffer cells. In many cases it was closely associated with bundles of striated collagen (Fig 9.33)

9.10 Vitronectin

When anti-vitronectin antibody was used for ultrastructural studies, the degree of labelling was so low that it could not be distinguished from the background labelling. None of the pre-treatments previously described improved the degree of labelling.

FIG 9.31

Ultracryosection of normal human liver.

Immunogold labelling (10nm) for fibronectin.

This shows fibronectin within the space of Disse. The labelling is present immediately adjacent to fat-storing cells, but not intracellularly. Mag x 25,875

FSC - Fat-storing cell

FIG 9.32

Ultracryosection of normal human liver.

Immunogold labelling (5nm) for fibronectin.

This shows fibronectin within the endoplasmic reticulum of a hepatocyte. The mitochondria of the cell are all negative. Mag x 49,500

M - mitochondria

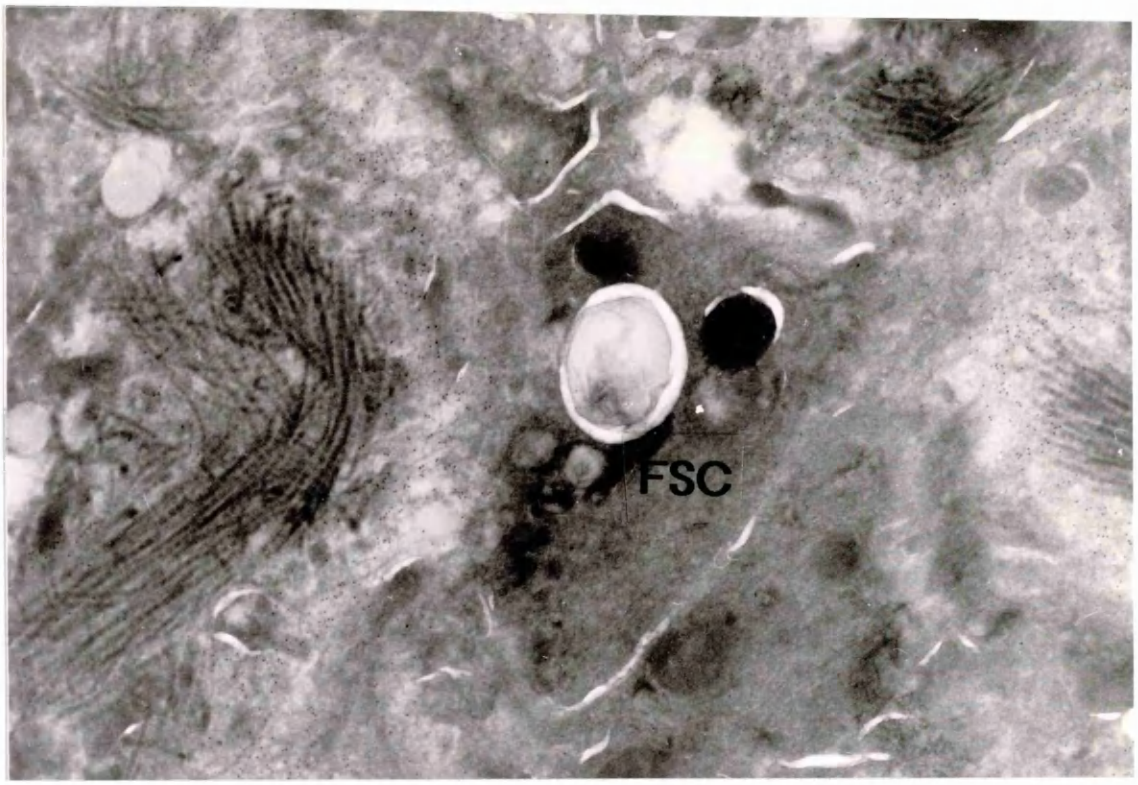

FIG 9.31

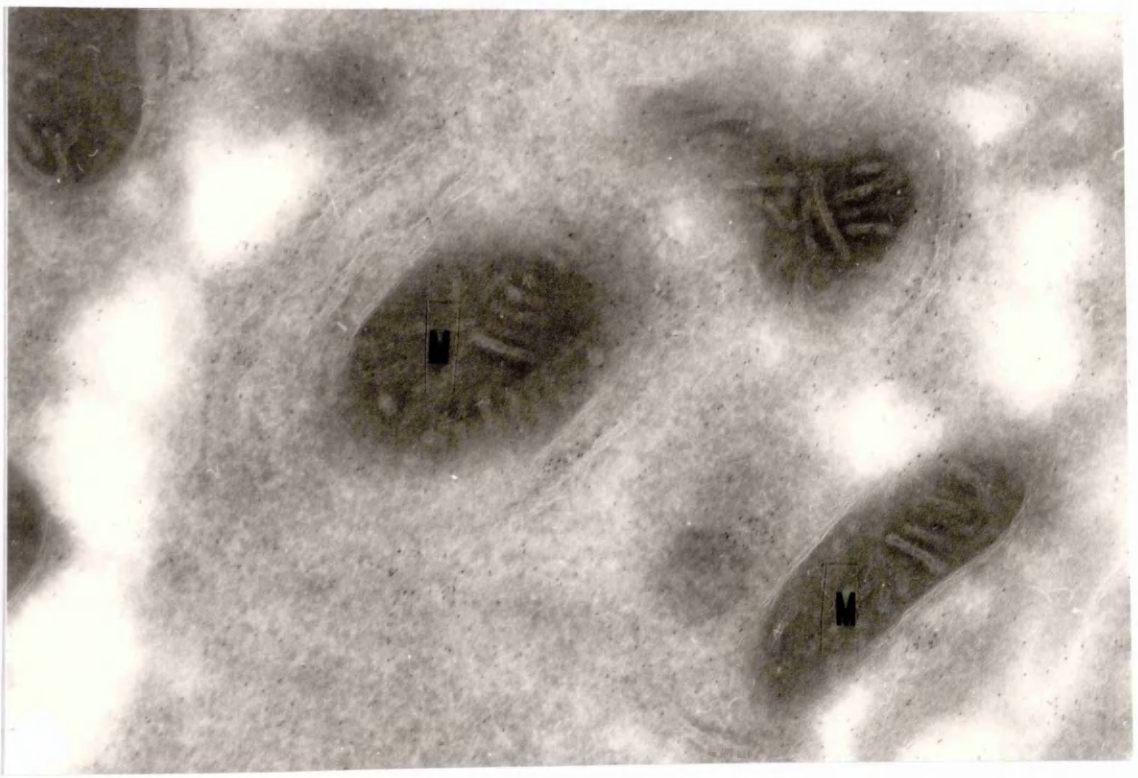

FIG 9.32

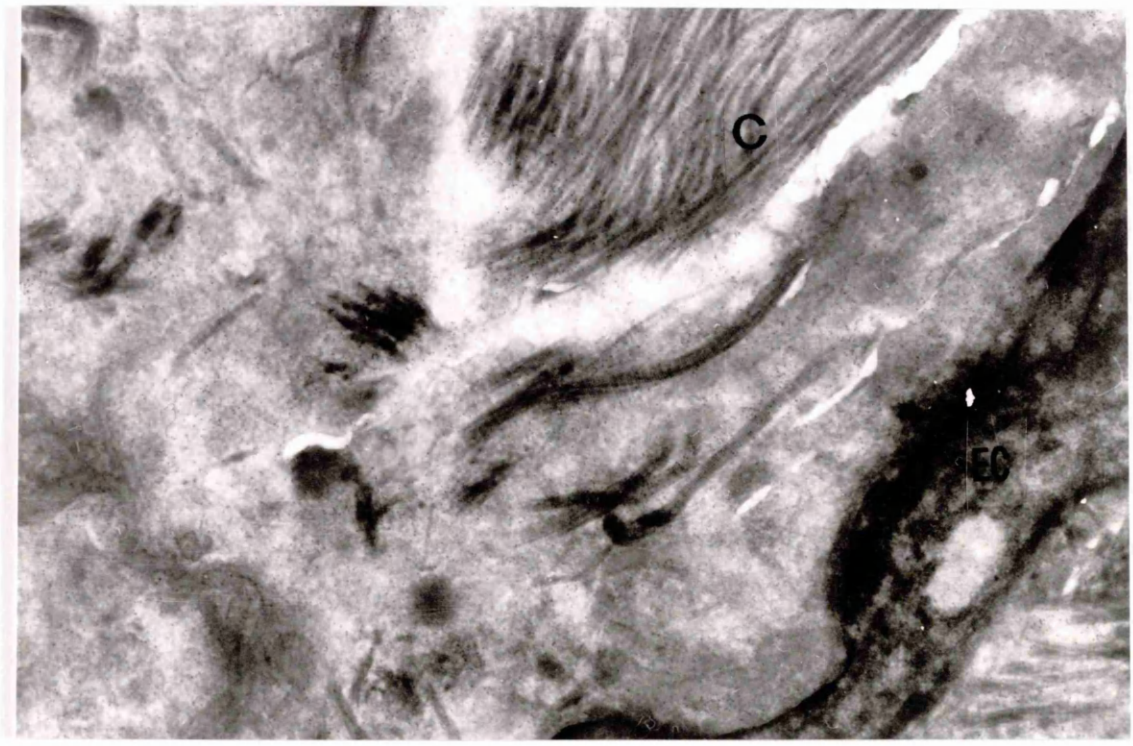

FIG 9.33

Ultracryosection of normal human liver.

Immunogold labelling (10nm) for fibronectin.

Fibronectin is present throughout the space of Disse, but the labelling is strongest when associated with the bundles of striated collagen fibres. Mag x 18,000

C - collagen

EC - endothelial cell

9.11 Laminin

The antibodies for laminin were the most fixation sensitive in this study. They were tested in a range of fixatives (see table 10.1) and the only positive results obtained were with tissue fixed in Bouins fixative or in 2% paraformaldehyde (± 0.1 glutaraldehyde). The only tissue samples large enough to be divided into a number of samples and fixed in a range of fixatives were those obtained from cadaveric renal transplant donors. Bouins fixed tissue was unsuitable for ultrastructural studies. The laminin 120kD antibody did give positive results when used for light microscopic immunolabelling studies on the paraformaldehyde fixed material, but the labelling obtained with ultrastructural techniques was far weaker. There was occasional labelling around the blood vessels and bile ducts, with faint discontinuous deposits in the space of Disse. This staining pattern was weak and in many cases there was no appreciable labelling.

9.12 Ultrastructural immunolabelling of normal liver.

Using the antibodies available for this study it was possible to immunolocalise collagen types I, III, IV and VI, and fibronectin with both light and ultrastructural microscopy. Antibodies for type V collagen, vitronectin and laminin were only suitable for light microscopy studies, and the antibody for undulin was unsuitable for all of the material used in this study.

The area where the majority of immunolabelling was found was within the space of Disse. This was true of all

the antibodies used. It was decided to concentrate on a smaller number of antibodies for the study of diseased liver tissue. These would be used for light microscopy and ultrastructural studies. The antibodies chosen were to collagen types I, III, IV and VI, and to fibronectin. Type V collagen was examined with light microscopy alone.

CHAPTER 10. EXTRACELLULAR MATRIX COMPONENTS IN DISEASED LIVER.

10.1 Immunocytochemistry at light microscopy level

10.2 Distribution of collagen

10.3 Fibronectin

10.4 Immunocytochemistry at electron microscopy level

10.5 Collagen

10.6 Fibronectin

10.1. Immunocytochemistry at light microscope level.

The immunocytochemical studies on diseased liver were limited by two factors. The first problem was the availability and size of material. The vast majority of biopsies used in this study were needle biopsies of liver. These are small cores of tissue and a substantial part of the material was required for diagnostic purposes. The second problem which arose from the use of archival material was the adverse effects of fixation used for routine biopsies on immunoreactivity. Buffered formalin or formol corrosive were the fixatives used routinely, and these were unsuitable for a number of antibodies including those against type IV collagen, laminin, type V collagen and vitronectin. For these reasons, the majority of the study of diseased liver was carried out with material which had been specifically prepared for electron microscopy. The exception to this was the study of type VI collagen. The localisation of this collagen within human liver was unknown, in both normal and diseased tissue. As anti-type VI collagen antibody was effective on formalin-fixed material, it was examined at both light microsopic and ultrastructural levels. The antibodies for types I and III collagen were also suitable for use with formalin-fixed material although the distribution of these collagens has already been documented by other groups using light microscopy (see chapter 6).

The most striking overall observation was the difference between acute and chronic liver diseases. There was no alteration of the distribution or intensity of

	Frozen	Bouins	2% paraform	2% paraform/ 0.1% glutaraldehyde
Type IV				
(7s)	+++	+++	++	++
(NC1)	+	+	+	-
pro IV	++	++	+	-
Laminin				
P1	++	++	-	-
120kD	+++	+++	++	+
60kD	++	+	-	-

Table 10.1 Effects of fixation on immunolabelling.

For all of the above antibodies, neither 2% glutaraldehyde or formol corrosive gave positive labelling

labelling for extracellular matrix proteins in acute hepatitis. In contrast , several alterations were found in chronic liver diseases. As there were only a small number of biopsies available for each of the different forms of liver disease, the alterations to the extracellular matrix described here may not be found in all cases and must be regarded as an indication of the changes in the hepatic extracellular matrix during the disease process.

10.2 Distribution of collagen

Types I and III collagen. In pre-cirrhotic primary biliary cirrhosis the pattern of collagen distribution was altered in the periportal zones. Elsewhere the pattern of distribution was similar to that in normal liver (Figs 10.1 and 10.2). Types I and III collagen were immunolocalised to fibrotic septa (Fig 10.5). In one of the sclerosing cholangitis cases, increased labelling for type I and III collagen was confined to the areas of damage around the bile ducts (Figs 10.3 and 10.4). In cases of acute liver disease such as Hepatitis A, there was no alteration in the pattern of collagen types I and III (Fig 10.6).

Type VI collagen. When biopsies from patients with primary biliary cirrhosis were examined, the pattern of type VI collagen expression altered as the disease progressed. There were no alterations in stage 1 biopsies, but in stages 2 and 3 intense staining was seen around proliferating bile ductules, which were located

within developing fibrotic septa (Fig 10.7). In stage 4 PBC with established cirrhosis, there was strong immunoreactivity within the fibrotic septa (Fig 10.10). Although there were no appreciable alterations in immunoreactivity along sinusoidal walls at any stage, the level of staining was always higher in zones 2 and 3 than in zone 1. This finding was unique to type VI collagen.

In other chronic liver diseases, the pattern of type VI collagen distribution was also altered. In alcoholic cirrhosis and in fibrosis there is strong staining within the septa (Fig 10.8 and Fig 10.11). In acute liver disease such as Hepatitis A, there was an increase in type VI collagen within the areas of inflammation (Fig 10.12).

Type V collagen. The distribution of type V collagen was similar to that of types I and III collagen. It was found within areas of fibrosis (Fig 10.13) but there was no increase in acute liver disease (Fig 10.14). Although type V collagen was always increased within areas of fibrosis, the remainder of the liver did not show any signs of increased type V collagen deposition (Figs 10.15 and 10.16)

10.3 Fibronectin. The intensity of labelling for fibronectin was increased in chronic liver disease, but the pattern of distribution was the same as in normal liver and it was not associated with any fibrotic septa present.

FIG 10.1

Distribution of type I collagen in primary biliary cirrhosis. (peroxidase-antiperoxidase labelling). In this example of primary biliary cirrhosis, the increased labelling for type I collagen can be seen in the periportal zone (zone 1). The rest of the parenchyma appears to have a normal pattern of type I collagen distribution.

FIG 10.2

Distribution of type III collagen in primary biliary cirrhosis. (peroxidase-antiperoxidase labelling). The deposition of type III collagen in this example of stage 3 primary biliary cirrhosis is the same as type I collagen.

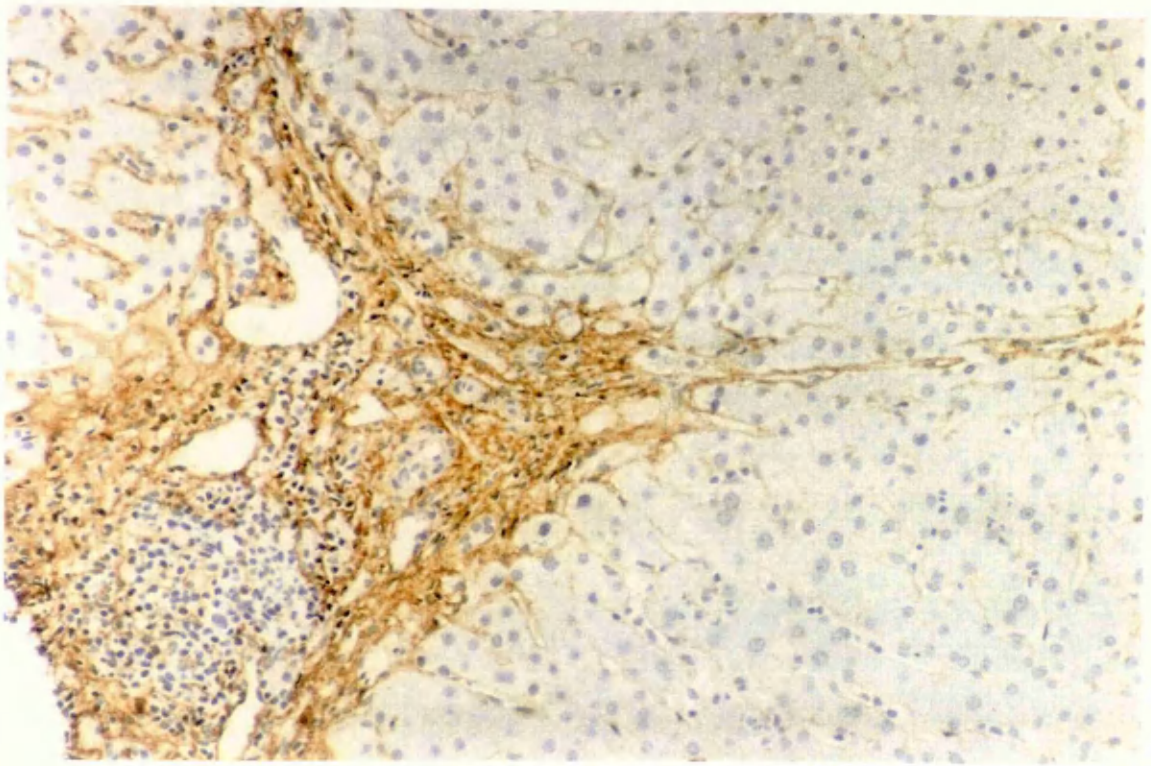

FIG 10.1

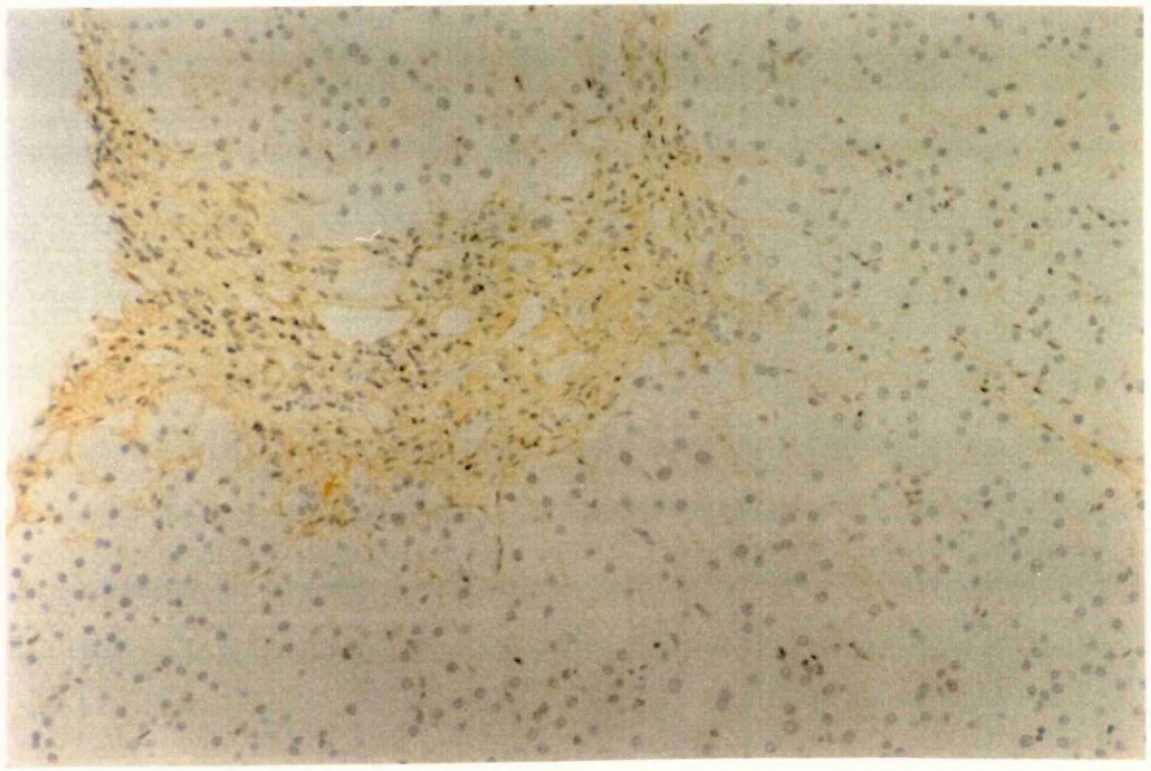

FIG 10.2

FIG 10.3

Distribution of type I collagen in primary sclerosing cholangitis. (peroxidase-antiperoxidase labelling).

In this case of primary sclerosing cholangitis, the labelling is seen surrounding the proliferating bile ductular structures. Increased labelling for type I collagen is also seen in the space of Disse.

FIG 10.4

Distribution of type III collagen in primary sclerosing cholangitis. (peroxidase-antiperoxidase labelling).

This low power illustration of type III collagen demonstrates the localised areas of increased collagen deposition. The labelling is prominent around the portal tract (PT) which has accompanying portal/periportal fibrosis. It is also seen in this case around an interlobular hepatic vein branch (HV)

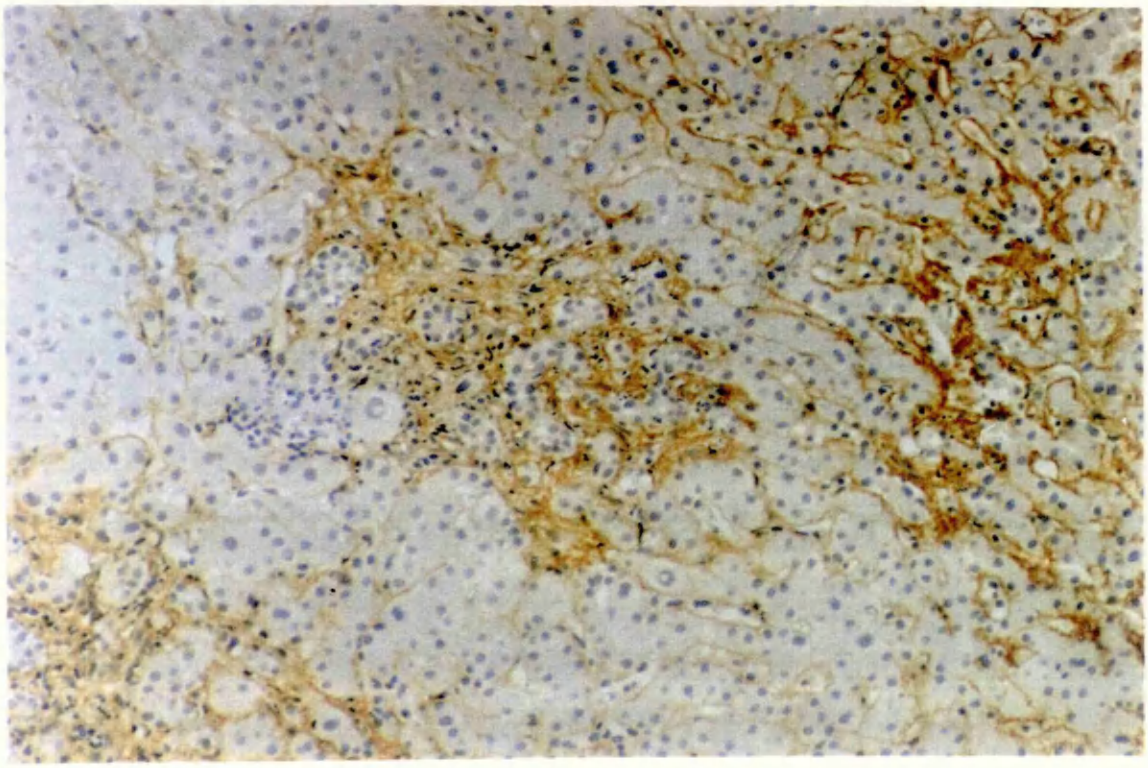

FIG 10.3

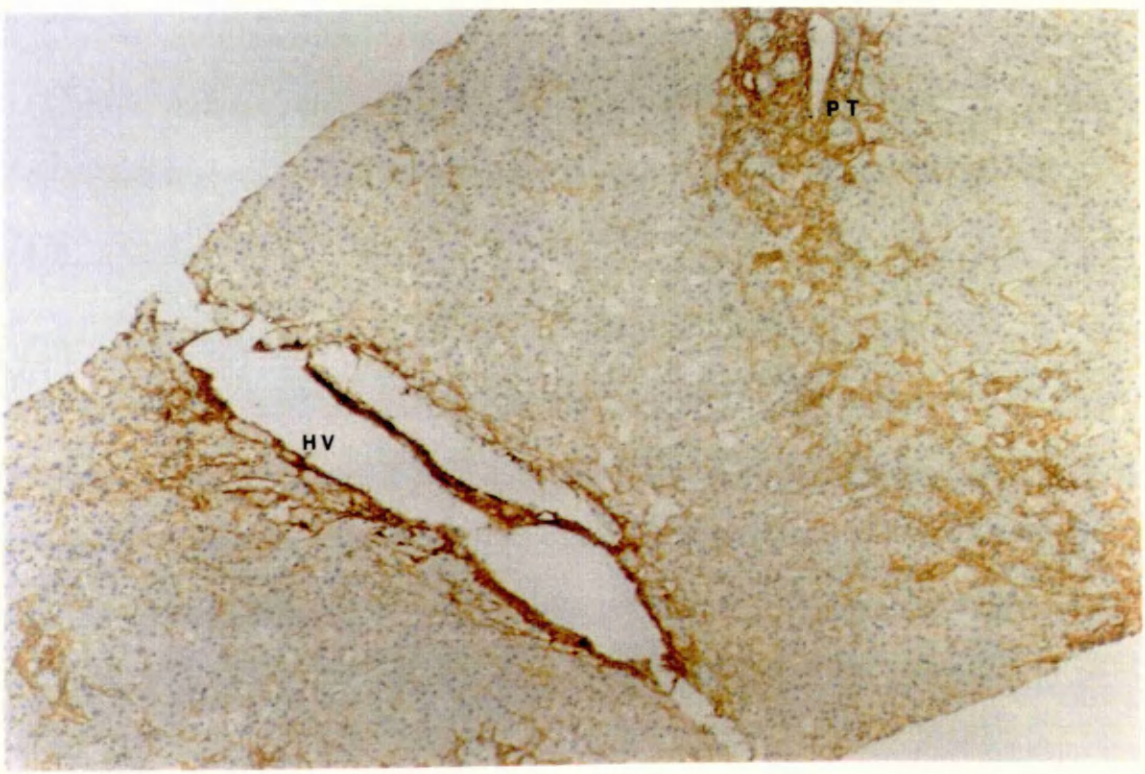

FIG 10.4

FIG 10.5

Distribution of type I collagen in primary biliary cirrhosis. (peroxidase-antiperoxidase labelling). This case of biliary cirrhosis is illustrative of a chronic fibrotic liver disease. Type I collagen is present in increased amounts in the areas of fibrotic tissue deposition, and these are starting to join together and form fibrotic septa.

FIG 10.6

Distribution of type I collagen in hepatitis A. (peroxidase-antiperoxidase labelling).

In contrast to Fig 10.5, this form of acute liver disease (Hepatitis A) shows little sign of increased type I collagen deposition and there are no fibrotic areas present.

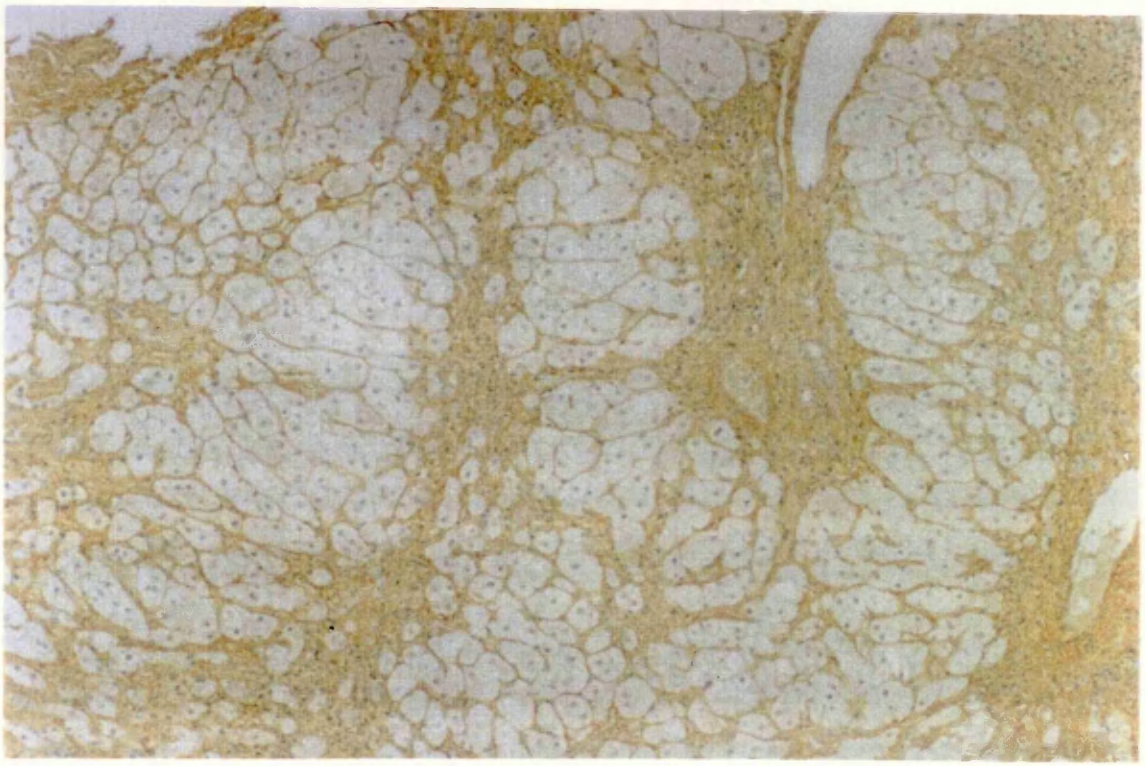

FIG 10.5

FIG 10.6

FIG 10.7

Distribution of type VI collagen in primary biliary cirrhosis. (peroxidase-antiperoxidase labelling). In this case of primary biliary cirrhosis, type VI collagen can be seen around proliferating bile ductules.

FIG 10.8

Distribution of type VI collagen in alcoholic cirrhosis. (peroxidase-antiperoxidase labelling). There is strong staining for type VI collagen in the fibrotic septa found in a case of alcoholic cirrhosis. The level of staining in the space of Disse within adjacent cirrhotic nodules is weaker.

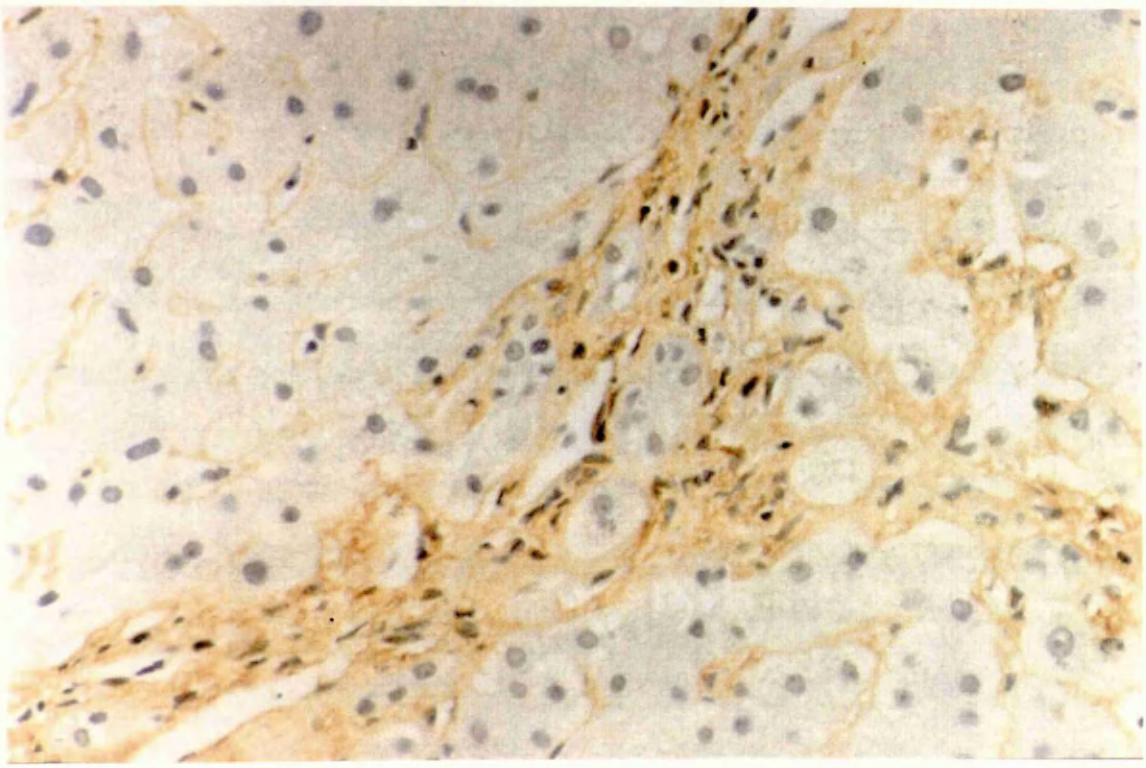

FIG 10.7

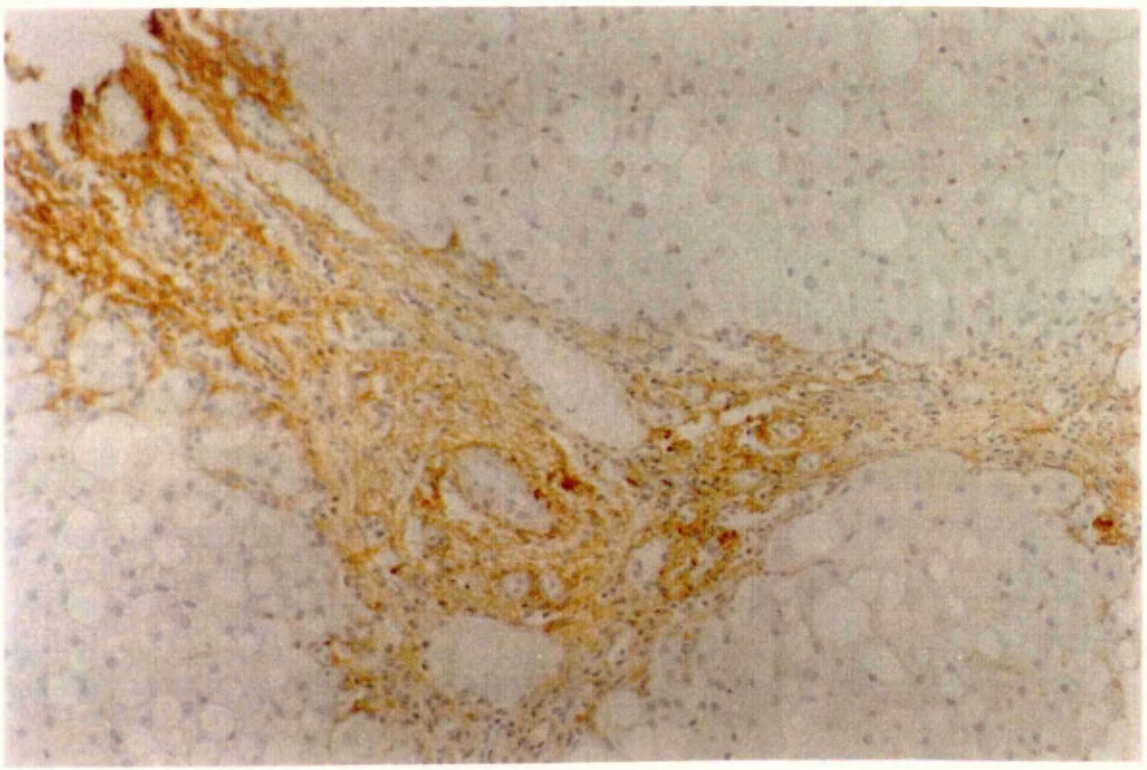

FIG 10.8

FIG 10.9

Distribution of type VI collagen in sclerosing cholangitis. (peroxidase-antiperoxidase labelling). In sclerosing cholangitis the areas of increased type VI deposition are the same as for types I and III collagen, i.e. around proliferating bile ductules and portal tracts.

FIG 10.10

Distribution of type VI collagen in primary biliary cirrhosis. (peroxidase-antiperoxidase labelling). Labelling for type VI collagen is present in greatly increased amounts throughout the areas of fibrosis in this case of primary biliary cirrhosis, and is not limited to areas immediately adjacent to the proliferating ductules.

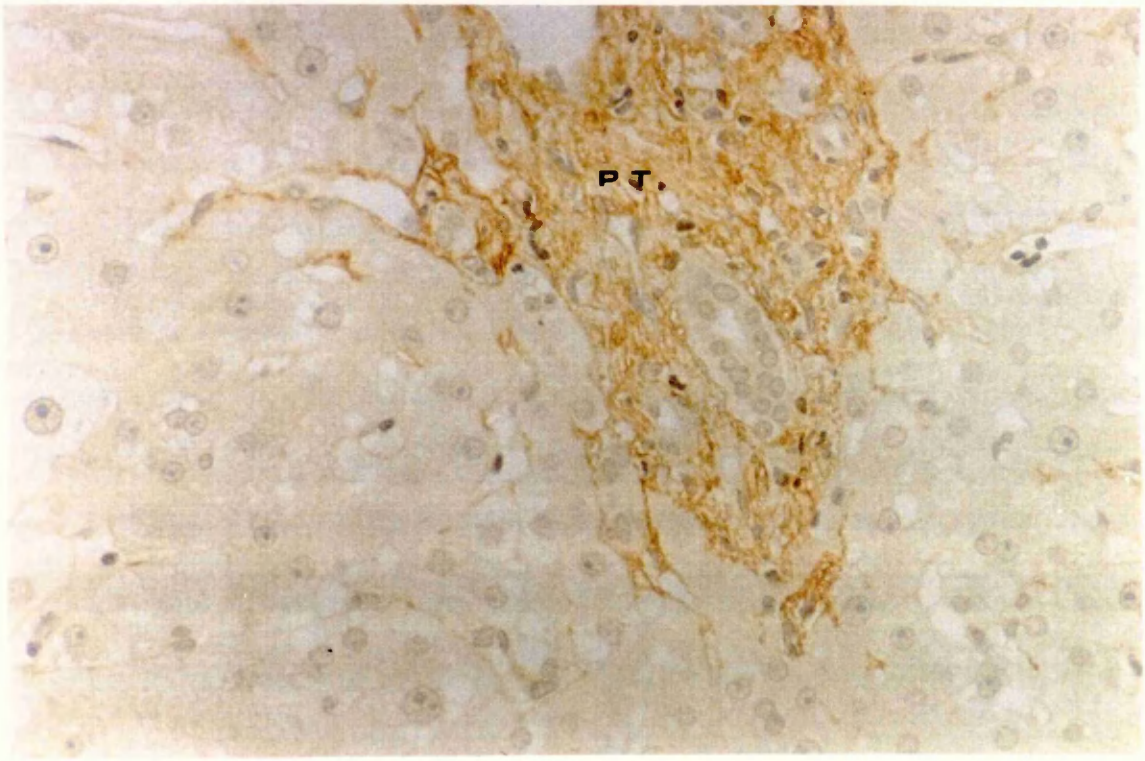

FIG 10.9

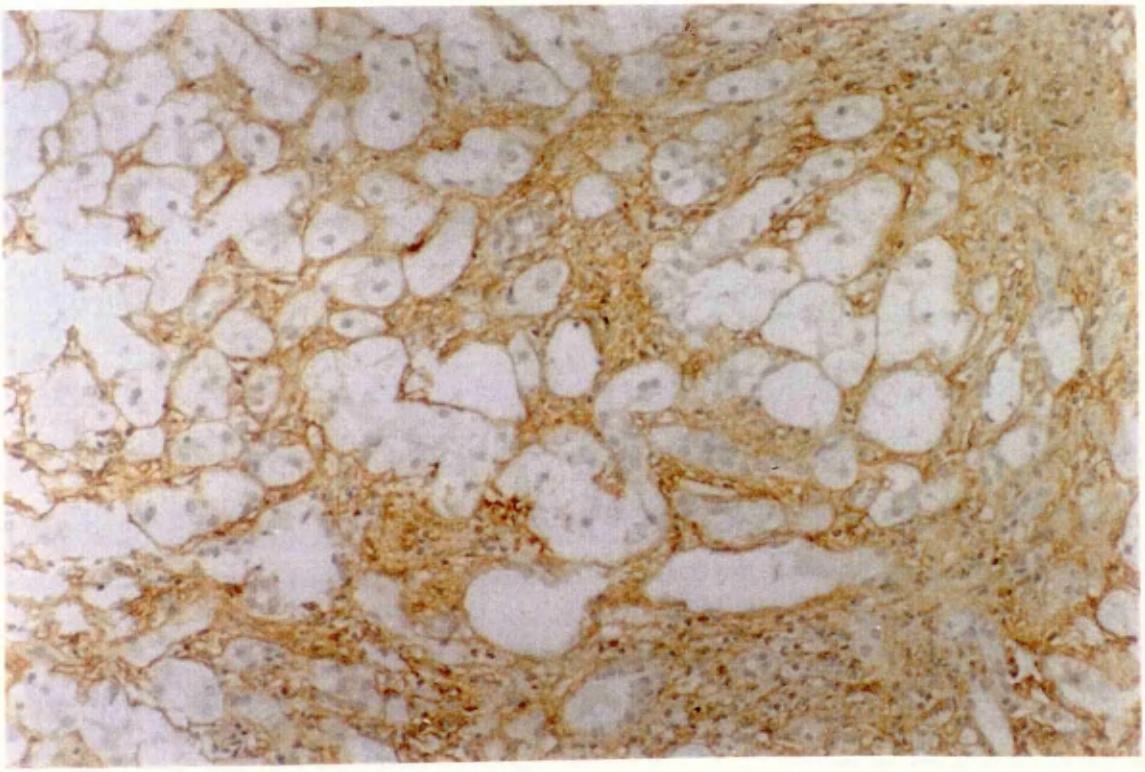

FIG 10.10

FIG 10.11

Distribution of type VI collagen in fibrotic liver. (peroxidase-antiperoxidase labelling).

This low power view of type VI collagen in fibrotic liver illustrates the similarity of type VI distribution to that of the other fibrillar collagens.

FIG 10.12

Distribution of type VI collagen in hepatitis A. (peroxidase-antiperoxidase labelling).

In acute liver disease such as hepatitis A, there is no increase in the amount of type VI collagen.

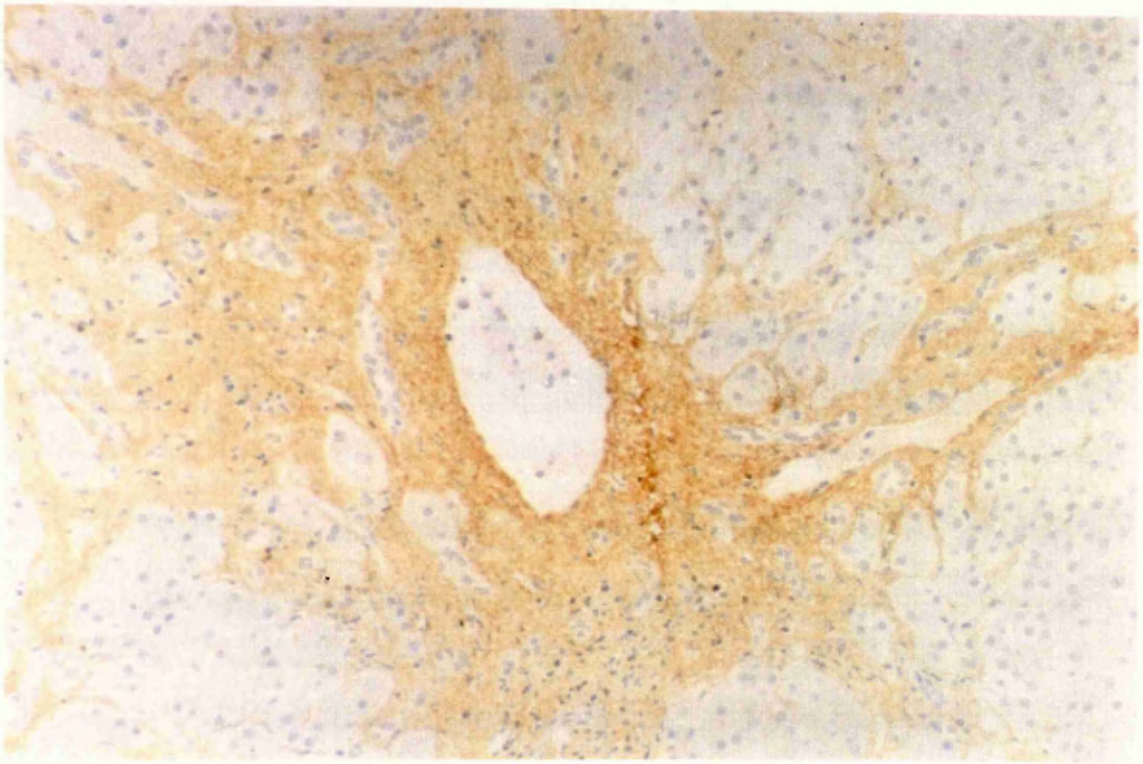

FIG 10.11

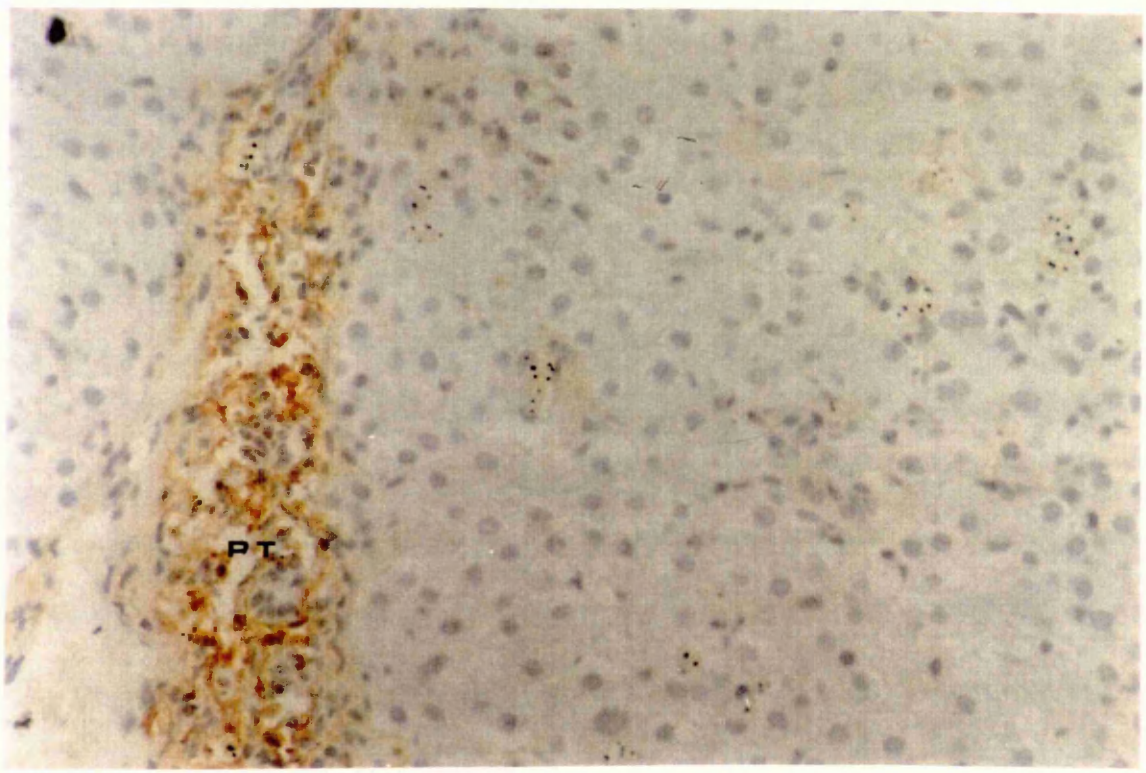

FIG 10.12

FIG 10.13

Distribution of type V collagen in primary sclerosing cholangitis. (peroxidase-antiperoxidase labelling).

Labelling of type V collagen can be seen within a fibrotic portal tract.

PT - portal tract BD - bile ductules

FIG 10.14

Distribution of type V collagen in hepatitis A. (peroxidase-antiperoxidase labelling).

In acute liver disease (Hepatitis A) there is no increase in type V collagen deposition.

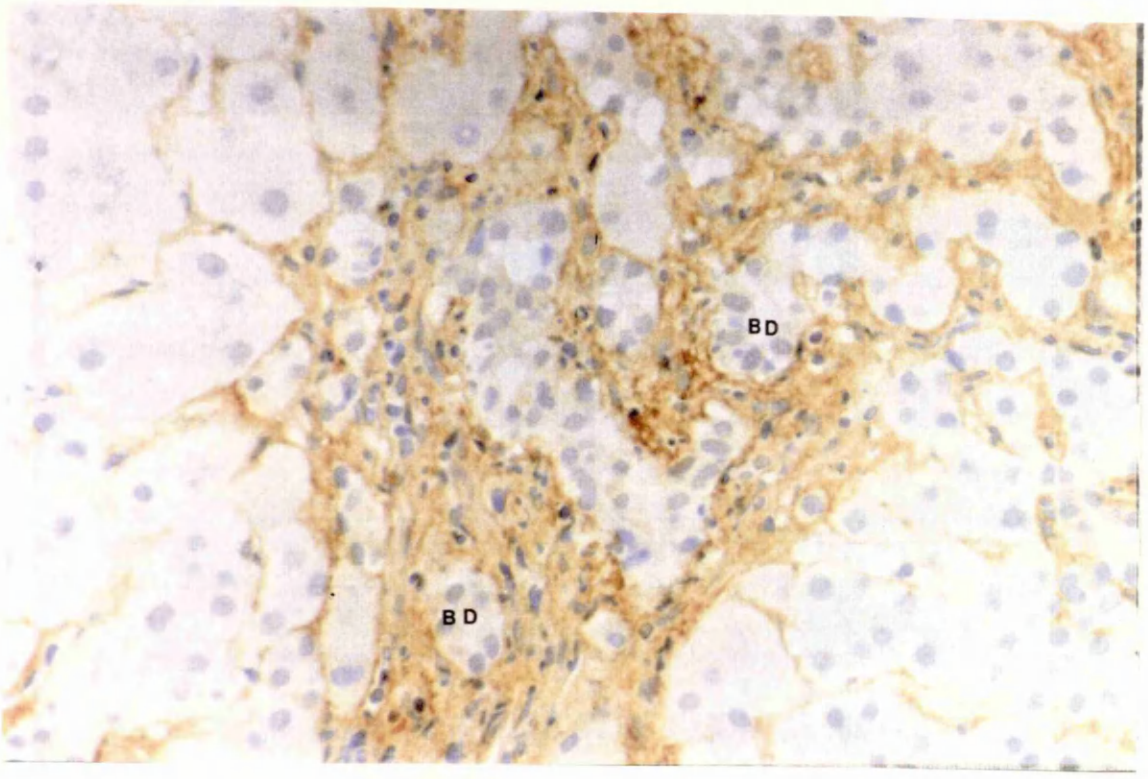

FIG 10.13

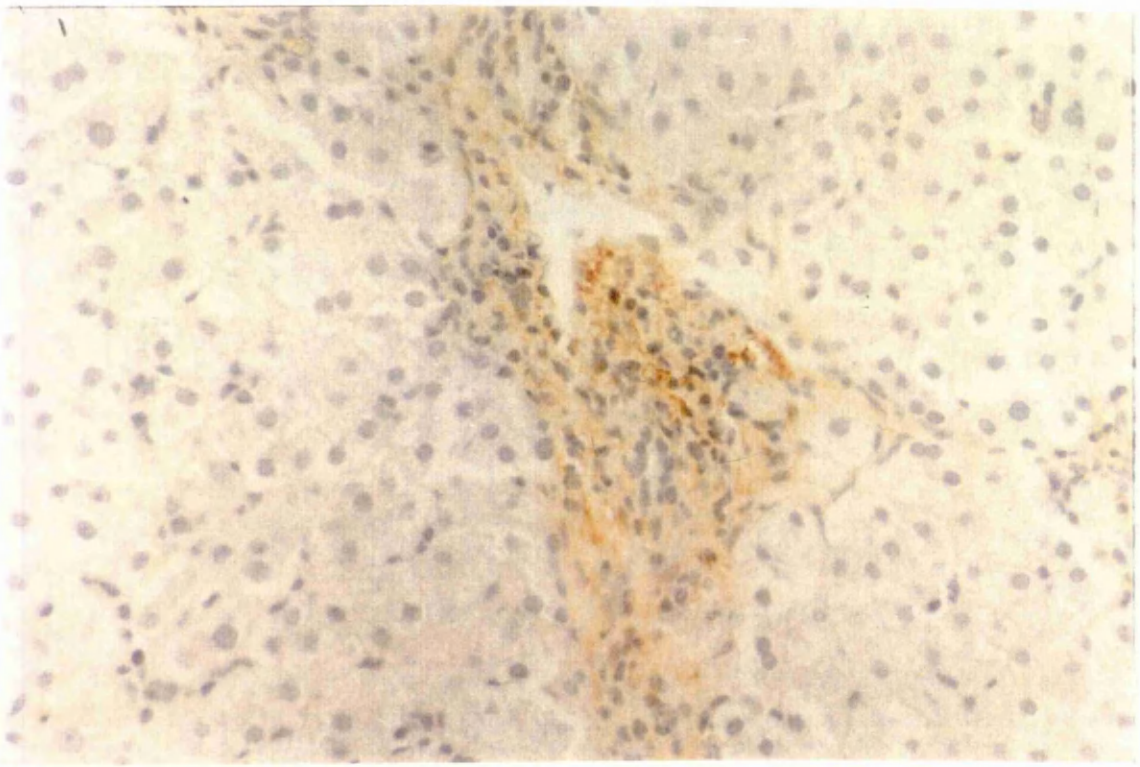

FIG 10.14

FIG 10.15

Distribution of type V collagen in alcoholic cirrhosis. (peroxidase-antiperoxidase labelling). The fibrotic septa found in alcoholic cirrhosis contain type V collagen in a similar distribution to that of other fibrillar collagens.

FIG 10.16

Distribution of type V collagen in alcoholic cirrhosis. (peroxidase-antiperoxidase labelling). There is no increased labelling of type V collagen in the space of Disse within cirrhotic nodules

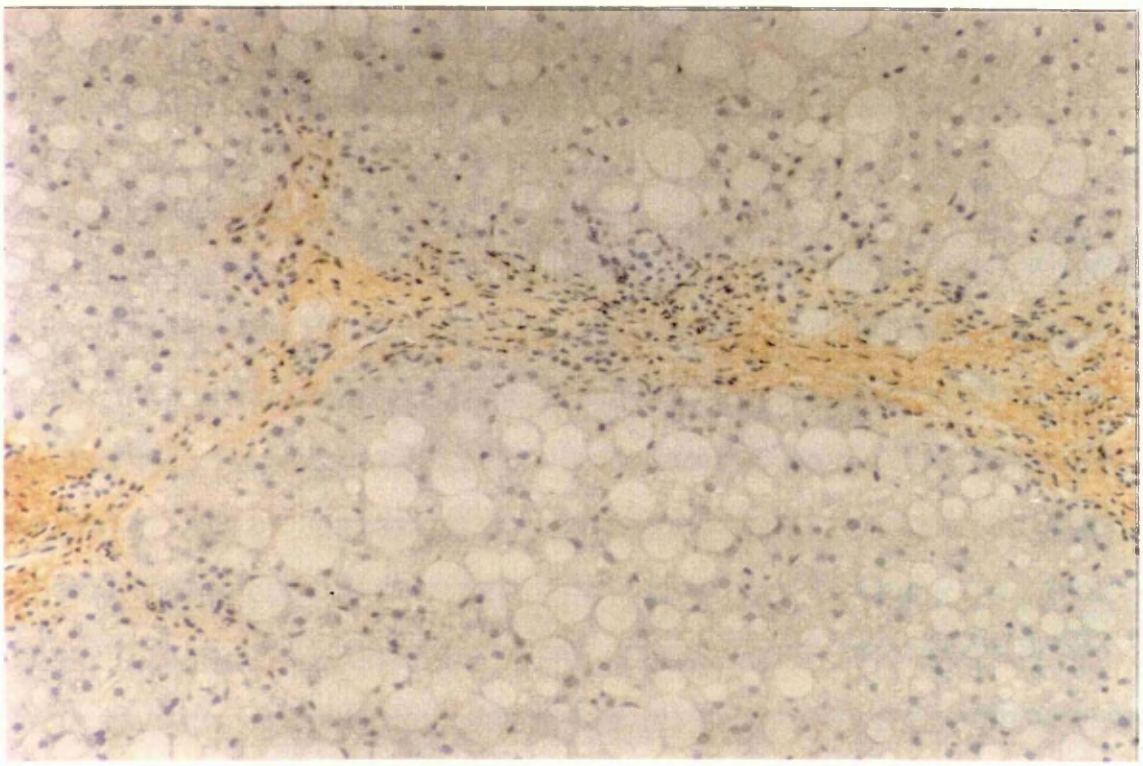

FIG 10.15

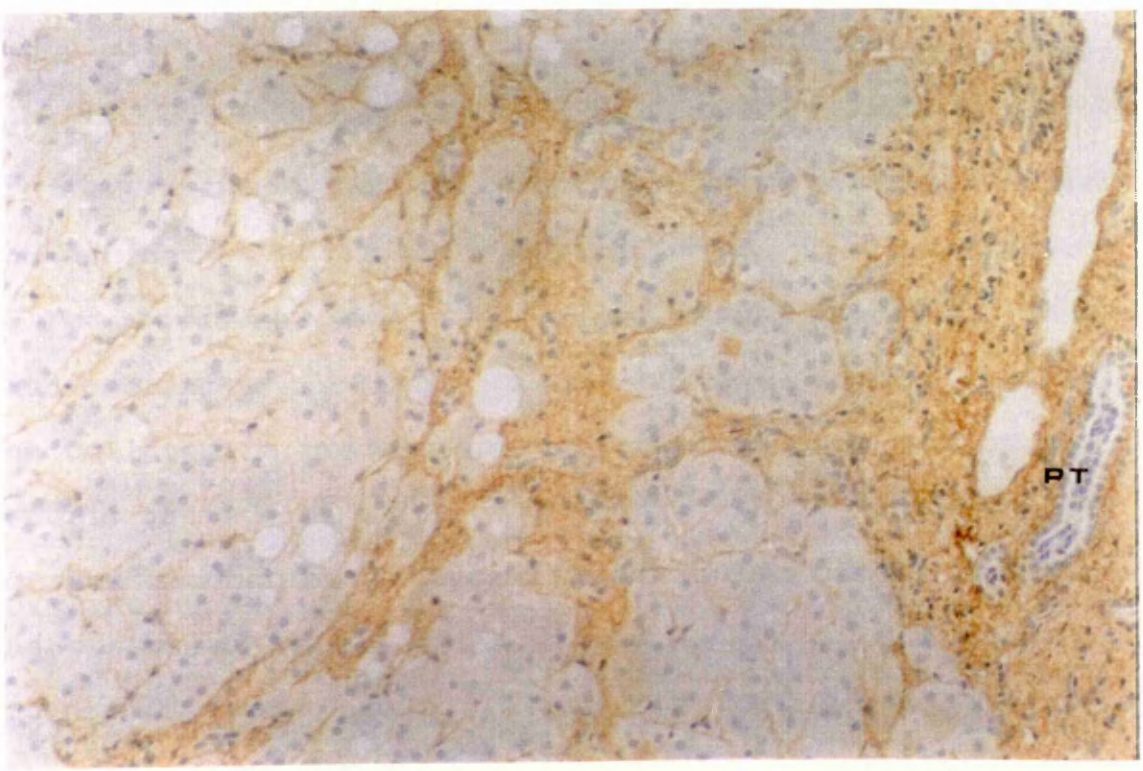

FIG 10.16

10.4 Immunocytochemistry at electron microscopy level.

10.5. Collagen

Type I collagen. In some conditions (nodular regenerative hyperplasia and intrahepatic cholestasis), there were no detectable alterations in either the distribution or intensity of immunolabelling. In comparison material from patients with primary biliary cirrhosis and primary sclerosing cholangitis had increased labelling for both types I and III in the portal tracts and space of Disse (Figs 10.17, 10.18). Patients with alcoholic hepatitis also had increased labelling for type I and III in the space of Disse. This increased labelling was found in all of the chronic diseases studied, but not in acute liver disease. When fibrotic septa were present, in chronic liver diseases such as primary biliary cirrhosis or hepatitis B, they also contained type I collagen.

All the labelling in these sections was extracellular. Tha antibodies which had been used were raised against the fully processed form of the collagen, which is found extracellularly. Within collagen-producing cells, much of the collagen is present in the partially processed or pro-peptide form. In order to determine the cells responsible for the production of type I collagen, antibodies to both the processed type I collagen and to the type I collagen pro-peptide were used. Only the procollagen antibody appeared to be effective, with positive labelling within some fat-storing cells (Fig 10.19, 10.20). All other cell types were negative. As discussed in 9.7, the

FIG 10.17

Ultracryosection of hepatitic liver.

Immunogold labelling (10nm) for type I collagen

These heavily labelled type I collagen fibres are immediately adjacent to a fat-storing cell.

Mag x 96,750

FSC - Fat-storing cells

FIG 10.18

Ultracryosection of a liver with primary sclerosing cholangitis.

Immunogold labelling (10nm) for type I collagen

Labelling for type I collagen can be seen surrounding the entire fat-storing cell, in both bundles and individual fibrils.

Mag x 16,000

FSC - Fat-storing cells

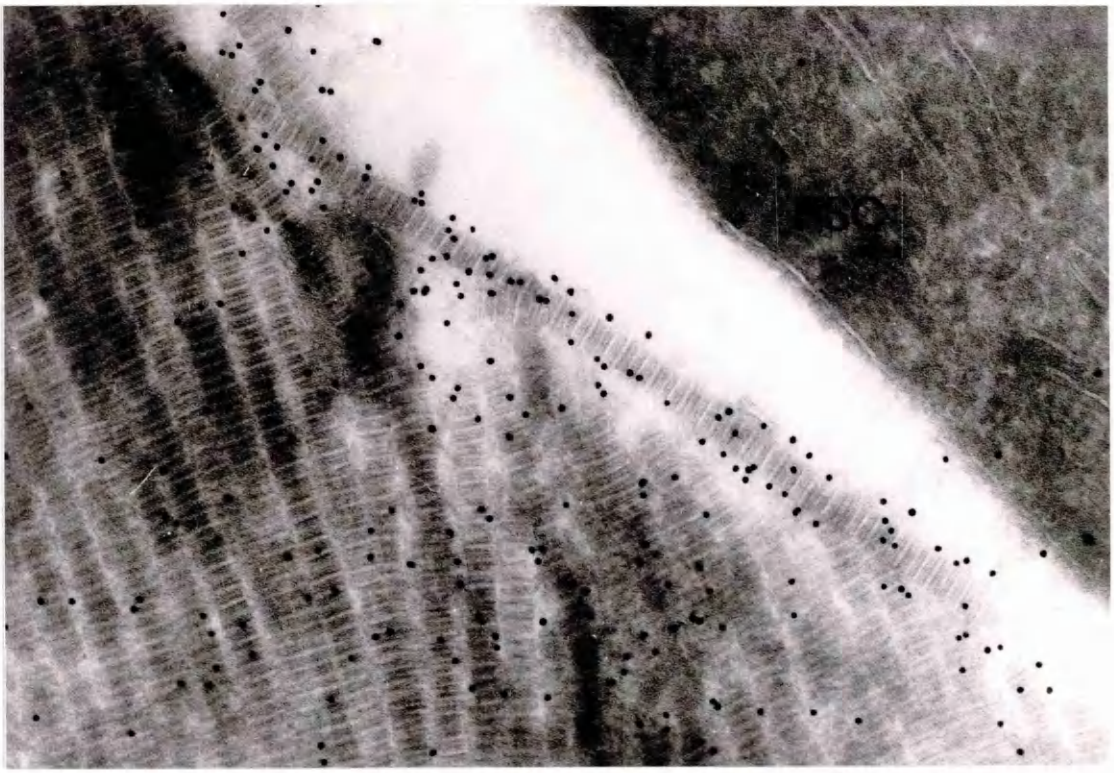

FIG 10.17

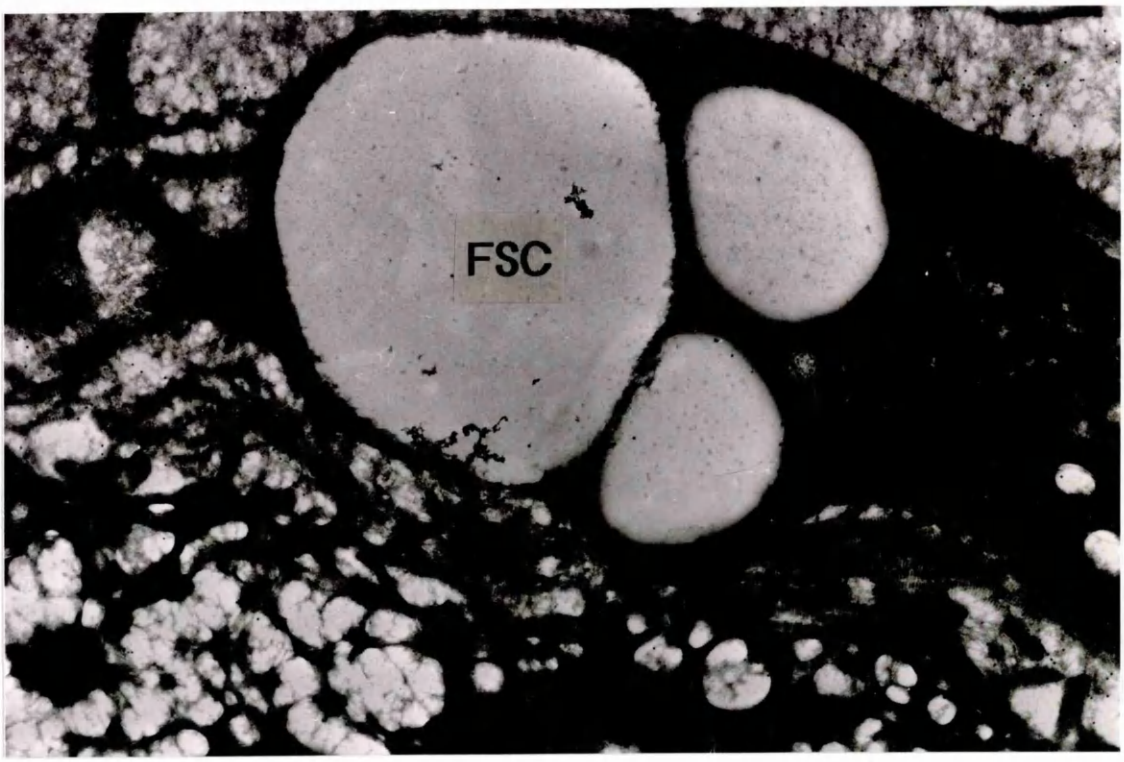

FIG 10.18

FIG 10.19

Ultracryosection of hepatitic liver

Immunogold labelling (10nm) for type I collagen

This fat-storing cell is surrounded with collagen bundles containing immunoreactive type I collagen. The endoplasmic reticulum is swollen, indicating active protein synthesis.

Mag x 15,500

FSC - Fat-storing cells

FIG 10.20

Ultracryosection of hepatitic liver

Immunogold labelling (10nm) for type I collagen

At higher magnification, the dilated endoplasmic reticulum can be seen to contain immunoreactive type I procollagen. Mag x 40,500

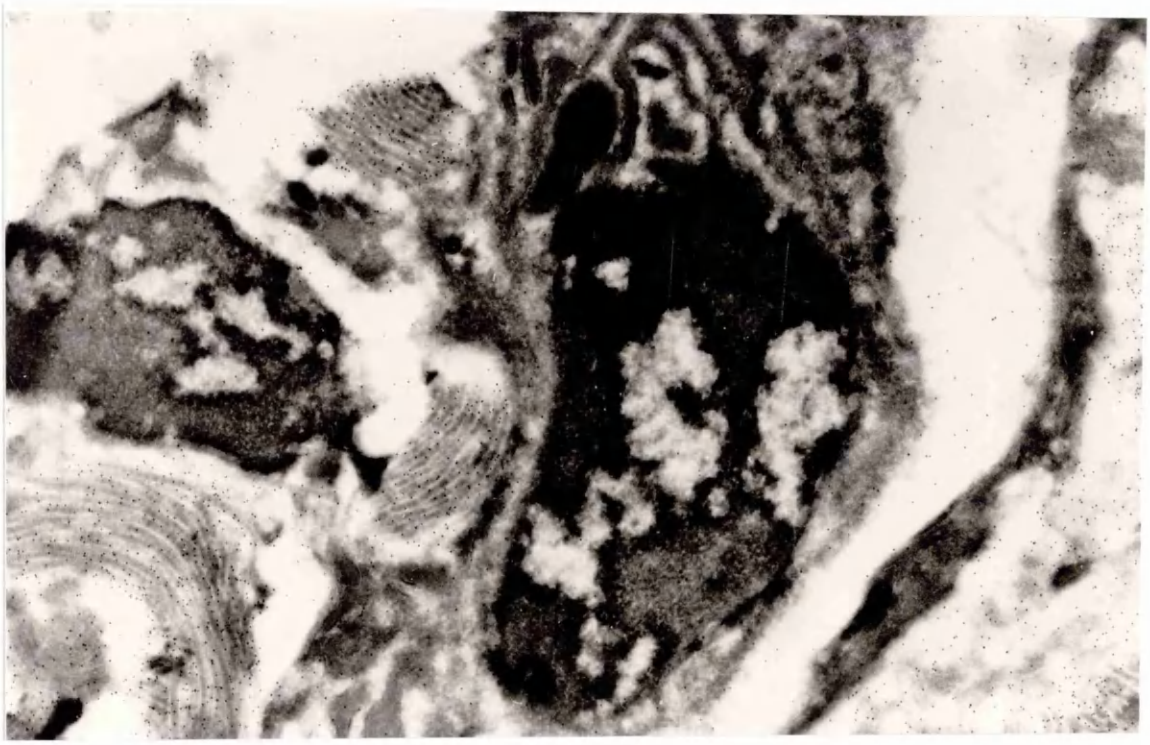

FIG 10.19

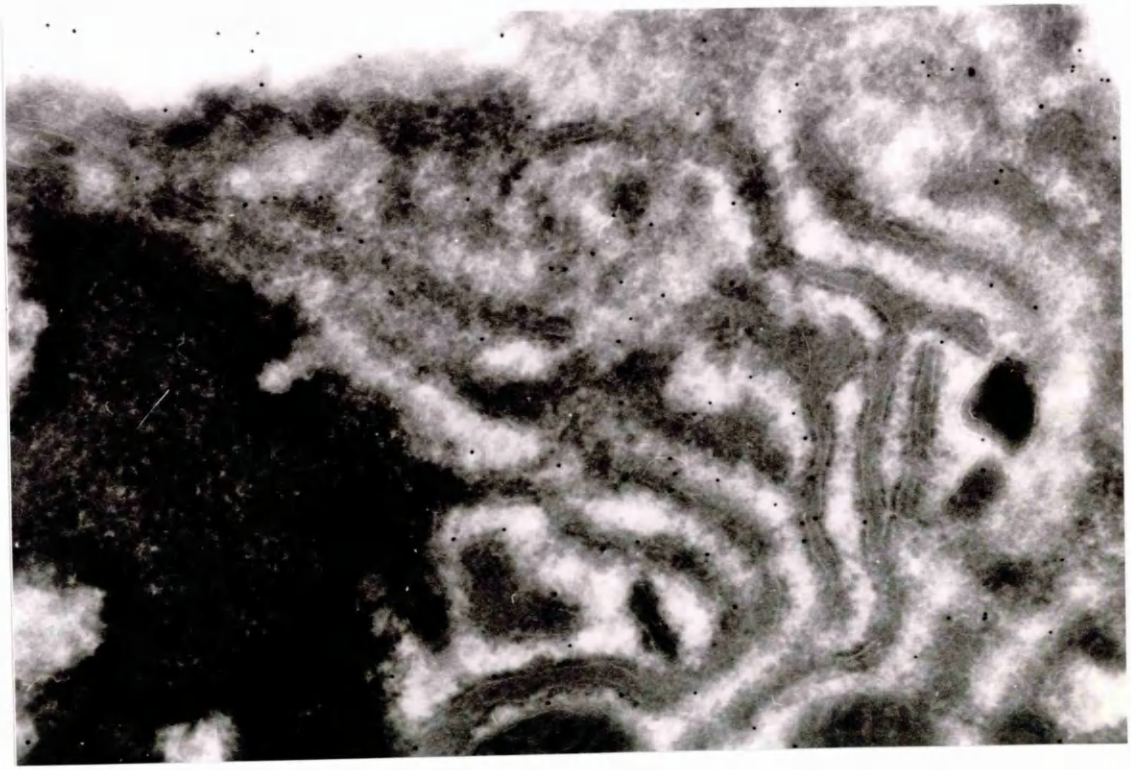

FIG 10.20

FIG 10.21

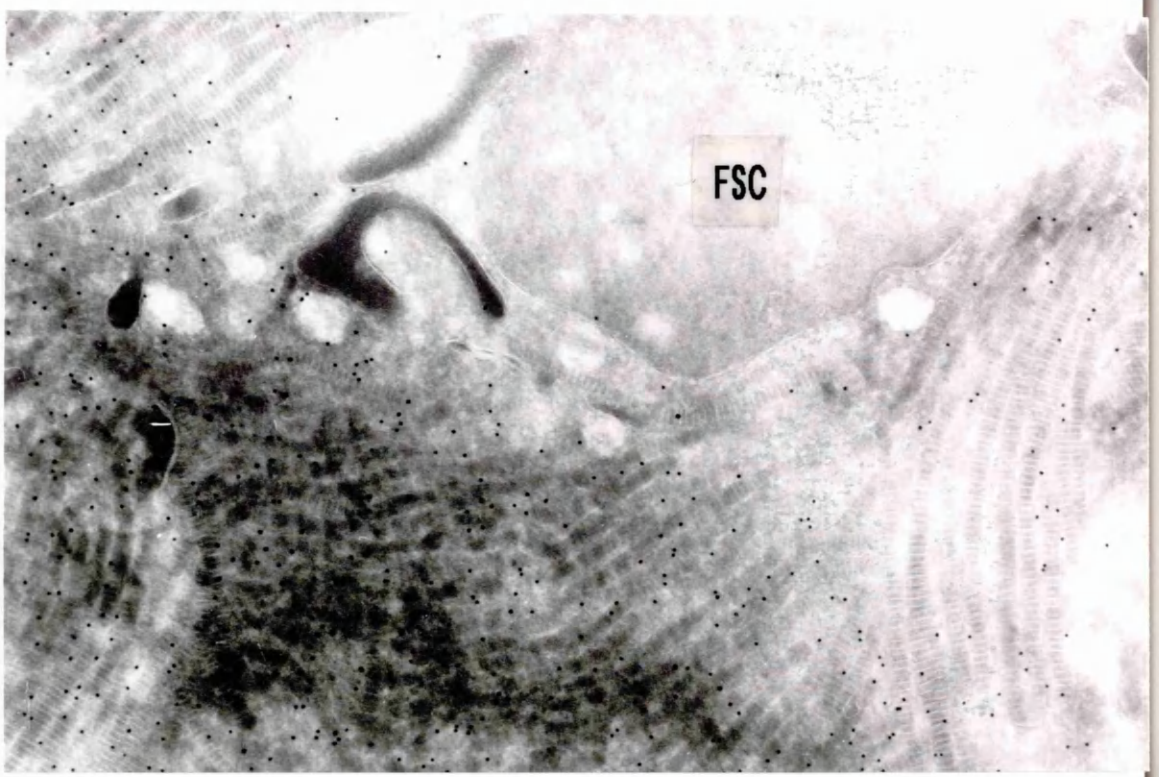

FIG 10.22

FIG 10.21

Ultracryosection of cholestatic liver

Immunogold labelling (10nm) for type III collagen

In this cholestatic liver, there is heavily labelled type III collagen deposited in the space of Disse.

Mag x 29,900

FIG 10.22

Ultracryosection of a liver with alcoholic cirrhosis.

Immunogold labelling (10nm) for type III collagen

Fat-storing cells in the space of Disse are surrounded by large amounts of striated collagen.

Mag x 47,250

FSC - Fat-storing cell

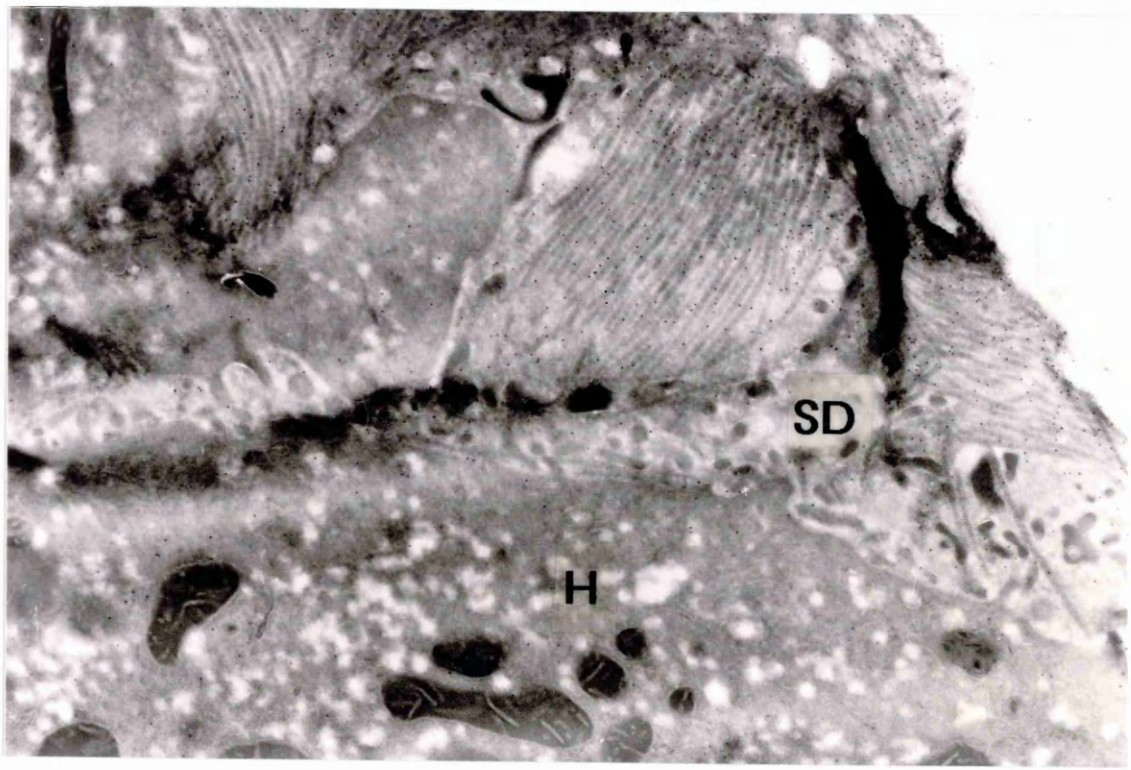

FIG 10.23

Ultracryosection of cirrhotic liver.

Immunogold labelling (10nm) for type III collagen

This biopsy was immunolabelled for type III collagen. The hepatocyte is not labelled intracellularly , although type III collagen can be seen immediately adjacent to the cell. The collagen bundles occupy most of the space of Disse, with labelled individual fibres also present.

Mag x 17,500

SD - space of Disse H - hepatocyte

FIG 10.24

Ultracryosection of a liver with alcoholic liver disease

Immunogold labelling (10nm) for type III collagen

In some cases of alcoholic liver disease, type III collagen fibres may encase the hepatocytes as seen here. These bundles form a meshwork which surrounds the cell. The hepatocytes are negative.

Mag x 15,000

H - hepatocyte

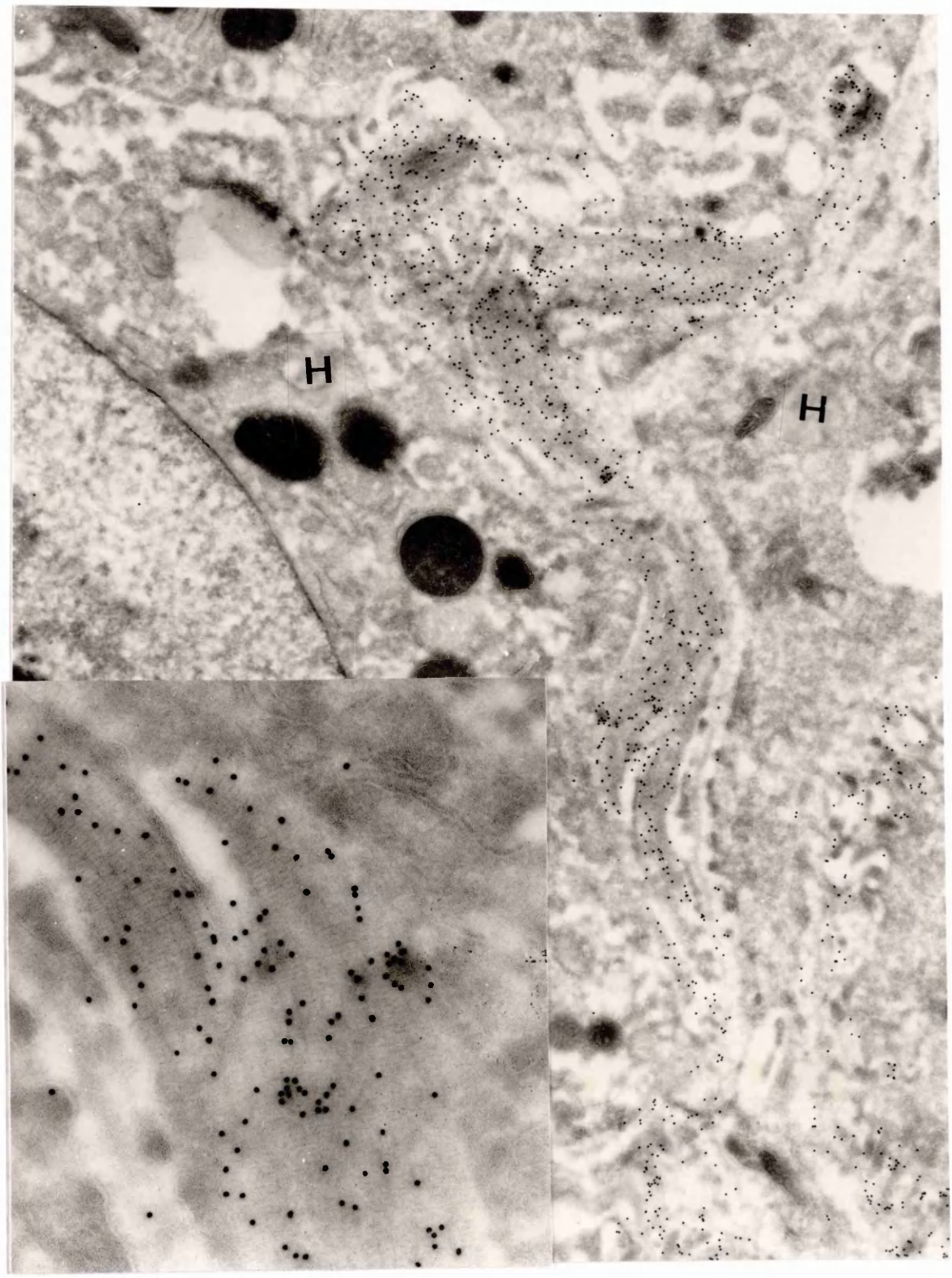

FIG 10.24

staining was marginally enhanced if the sections were pre-treated with hyaluronidase to remove the glycosaminoglycan component of the extracellular matrix, but the level of intensity remained low.

Type III collagen. The pattern of distribution was essentially the same as for type I collagen with increased amounts of collagen in chronic liver diseases (Fig 10.21, 10.22). In most fibrotic conditions, increased amounts of collagen were found in the space of Disse adjacent to the sinusoidal aspect of the hepatocyte (Fig 10.23). In a number of cases of alcoholic hepatitis,the extent of collagen deposition was increased and striated fibres could be seen around hepatocytes. Fig 10.24 shows an example of this pattern. The labelled collagen extends from the space of Disse around the perimeter of the hepatocyte.

Type IV collagen. There were several major alterations in the distribution of type IV collagen in diseased liver. In primary biliary cirrhosis, the areas around the bile ducts were far more strongly labelled than in normal liver (Fig 10.25). This labelling was adjacent to, but separate from, the striated fibres also present. There was also increased type IV collagen in the sub-endothelial region of the space of Disse (Fig 10.26). The increase in type IV collagen was often adjacent to fat storing cells (Fig 10.27), but there was no intracellular labelling in any of these sections.

FIG 10.25

Ultracryosection of a liver with primary biliary cirrhosis

Immunogold labelling (10nm) for type IV collagen

The labelling for type IV collagen is more intense than that present in normal liver.

Mag x 22,050

BD - bile duct epithelial cell

FIG 10.26

Ultracryosection of a liver with primary biliary cirrhosis.

Immunogold labelling (10nm) for type IV collagen

Increased labelling for type IV collagen is also found in the space of Disse, in a sub-endothelial distribution.

Mag x 17,550

C - collagen bundles EC - sinusoidal endothelial cell

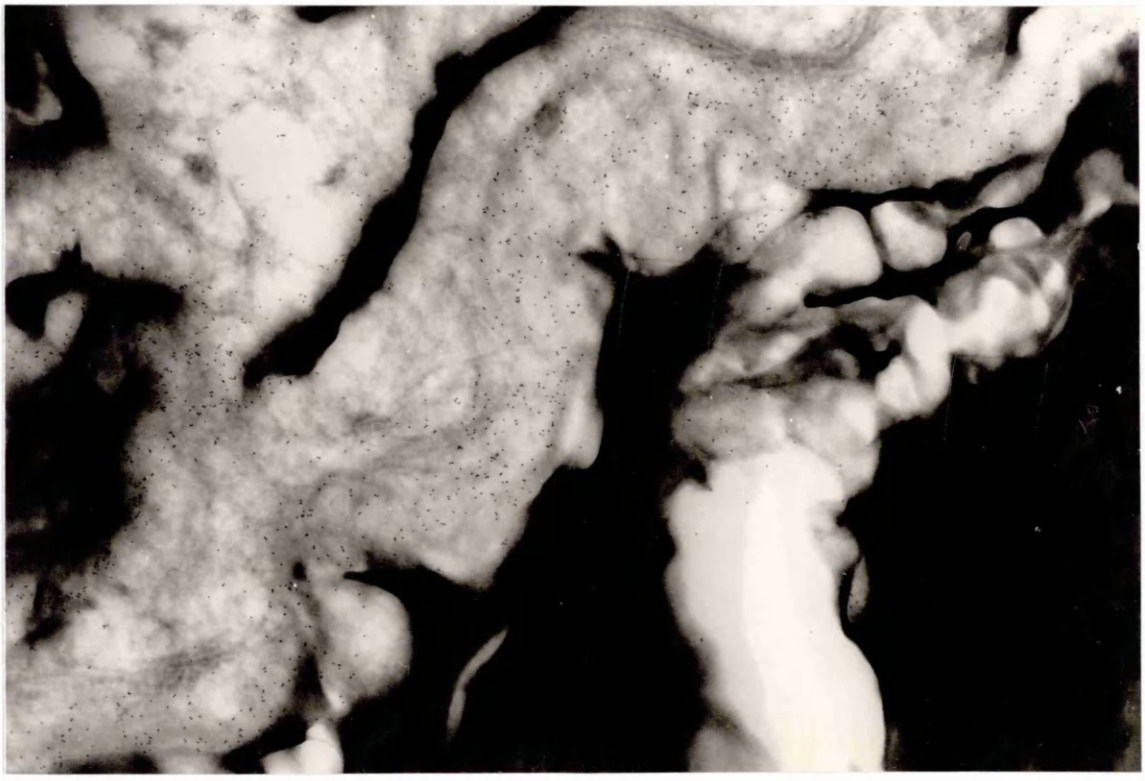

FIG 10.25

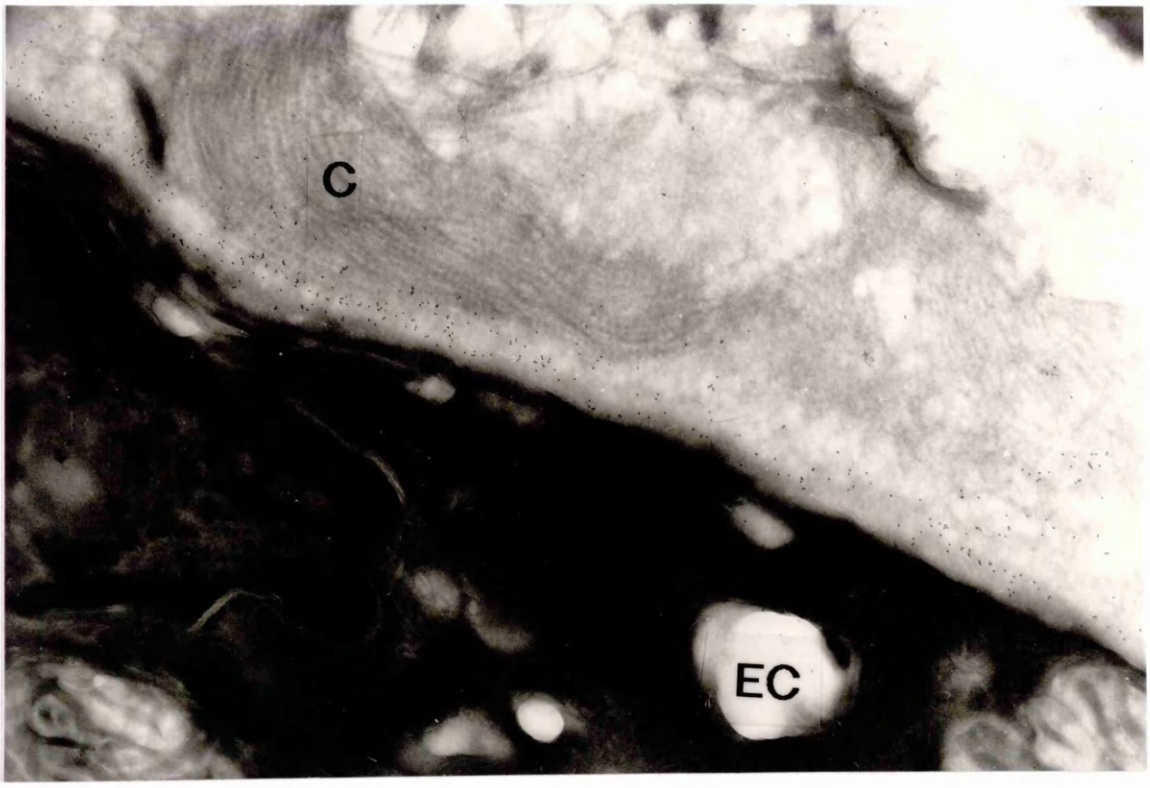

FIG 10.26

FIG 10.27

Ultracryosection of a liver with primary biliary cirrhosis.

Immunogold labelling (10nm) for type IV collagen

This fat-storing cell is surrounded by immunoreactive type IV collagen. No intracellular labelling is present.

Mag x 26,500

FSC - Fat-storing cells

FIG 10.28

Ultracryosection of a liver with primary sclerosing cholangitis.

Immunogold labelling (10nm) for type IV collagen

Type IV collagen has been deposited along the borders of the space of Disse, in a regular pattern.

Mag x 44,000

SD - space of Disse H - hepatocyte

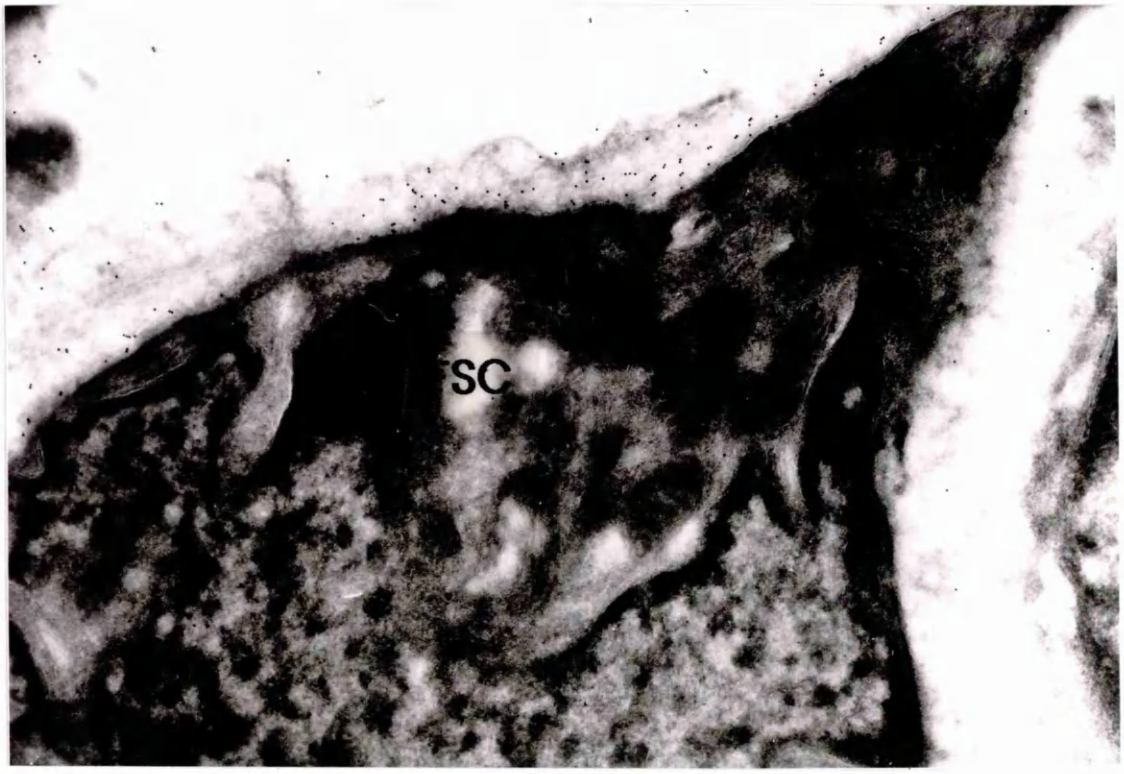

FIG 10.27

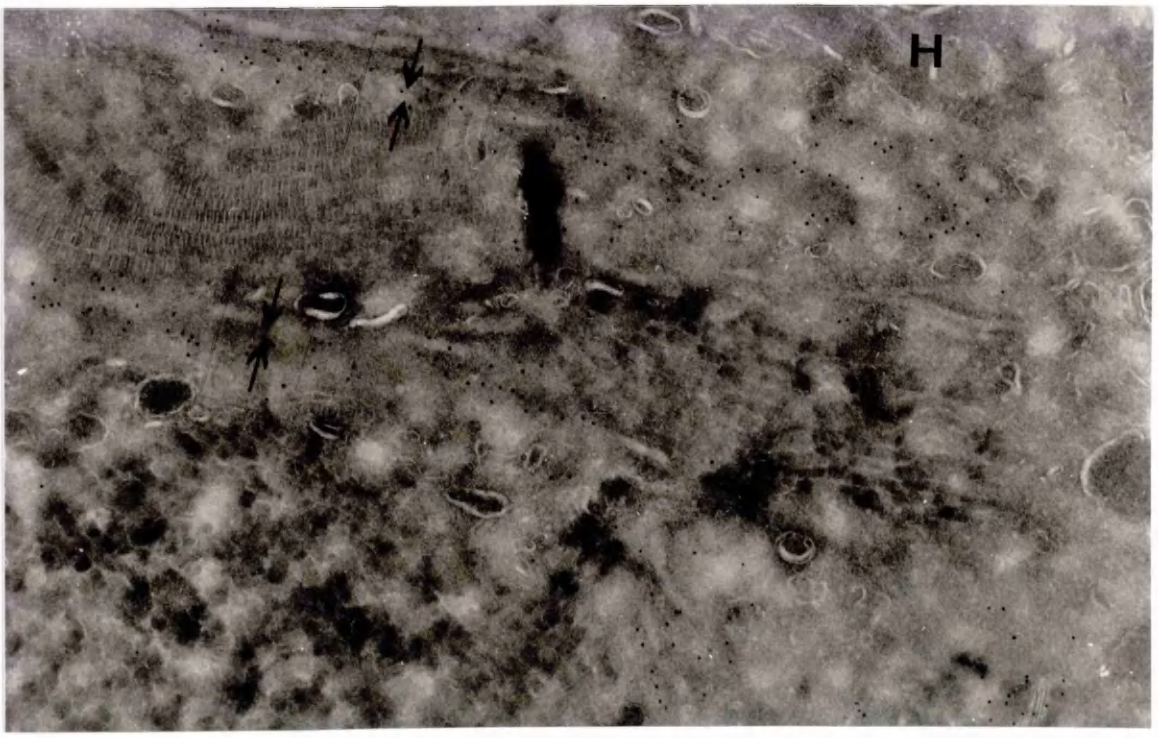

FIG 10.28

FIG 10.29

Ultracryosection of a liver with primary sclerosing cholangitis

Immunogold labelling (10nm) for type IV collagen

Type IV collagen can be seen around the edges of the hepatocytes. The striated collagen bundle are negative.

Mag x 22,050

C - collagen bundles H - hepatocyte

FIG 10.30

Ultracryosection of a liver with primary sclerosing cholangitis.

Immunogold labelling (10nm) for type IV collagen

This higher power illustrates labelling of type IV collagen around the hepatocytes. The labelling is localised to amorphous material.

Mag x 30,050

H - hepatocyte

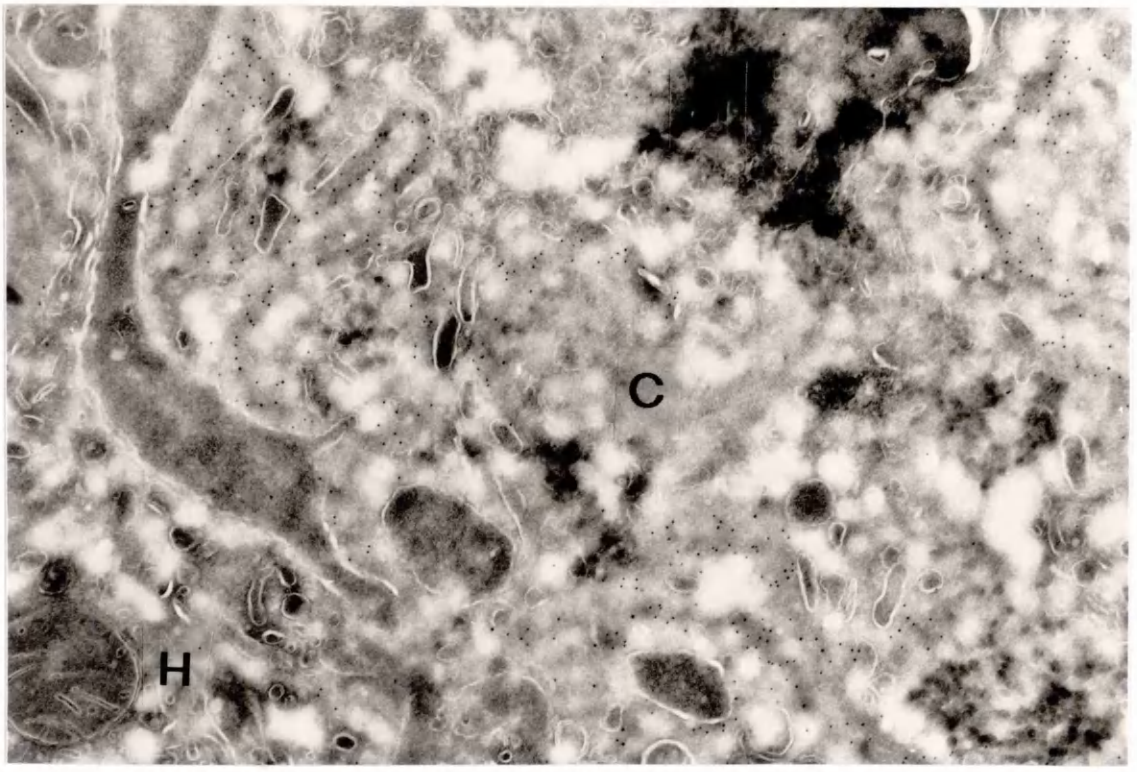

FIG 10.29

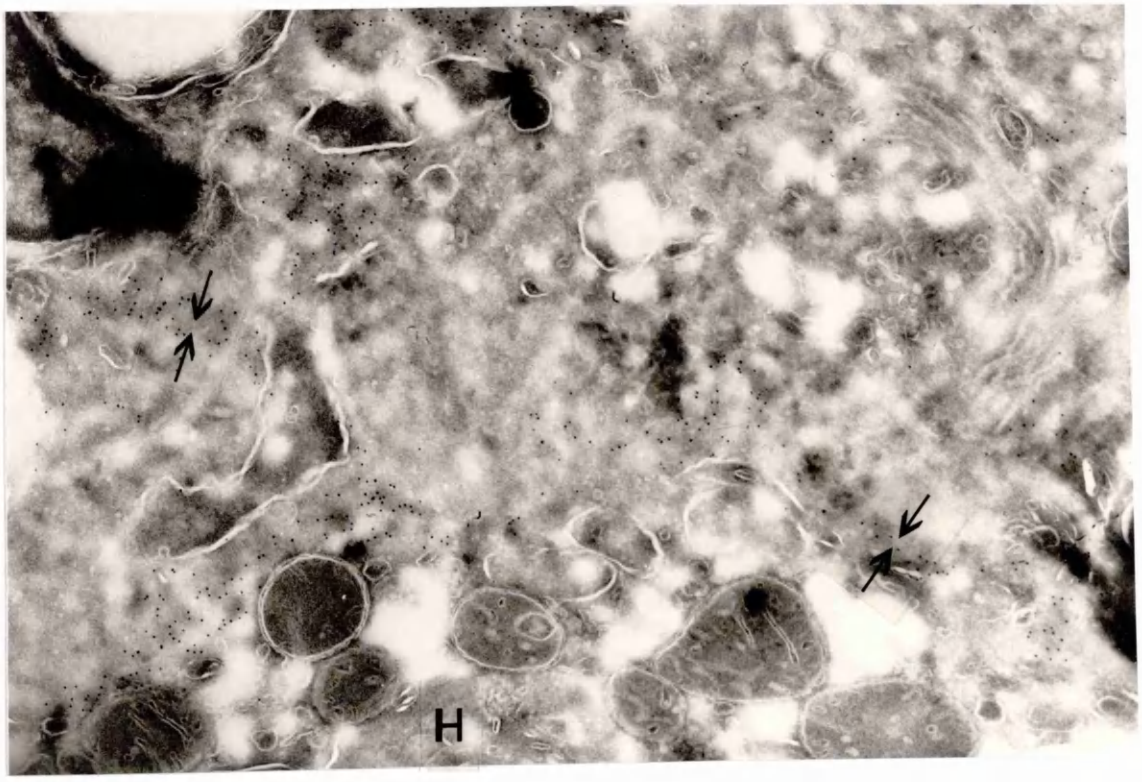

FIG 10.30

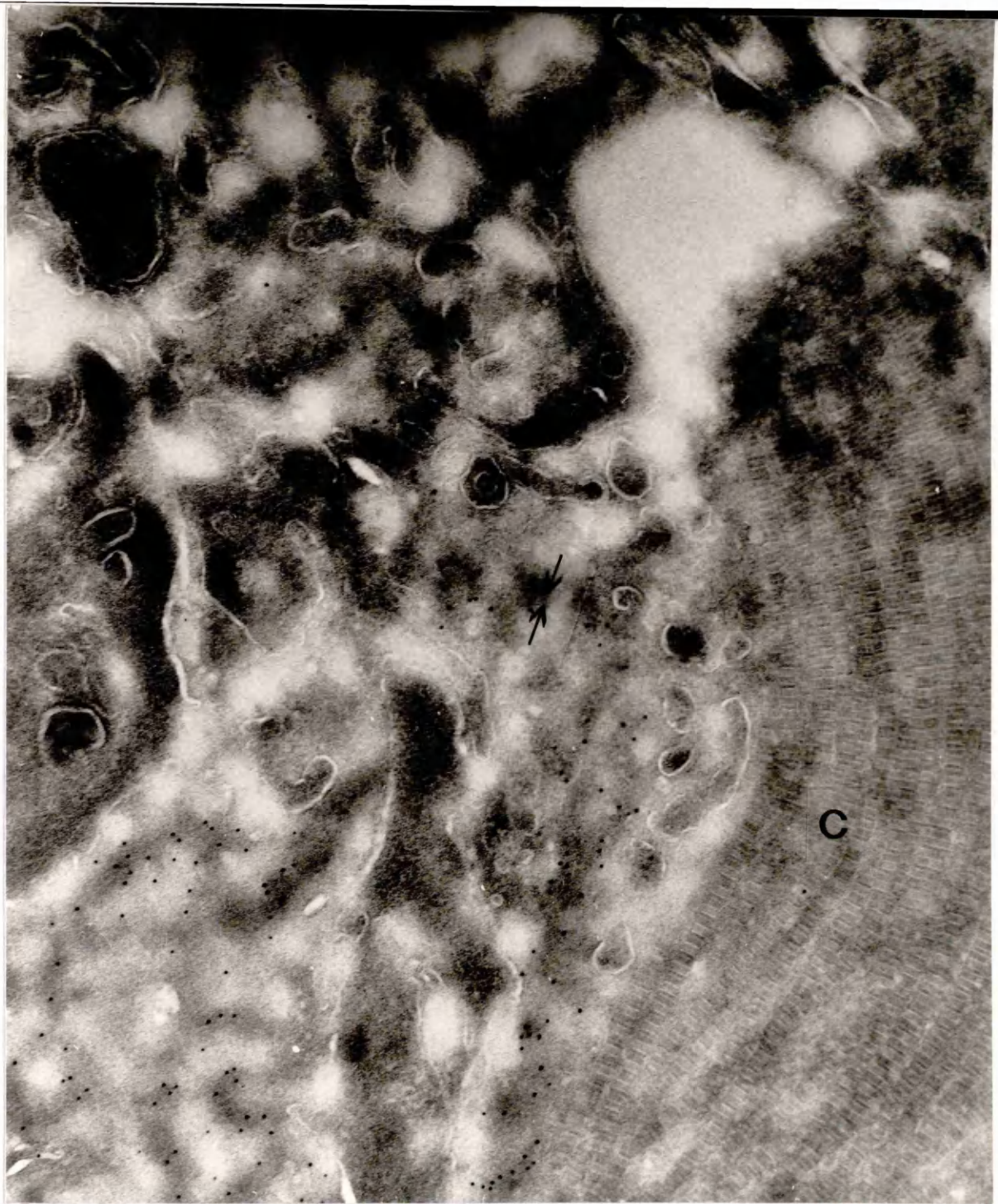

FIG 10.31

Ultracryosection of a liver with primary sclerosing cholangitis.

Immunogold labelling (10nm) for type IV collagen

The striated collagen in the space of Disse is negative for type IV collagen. The labelling is confined to the amorphous material surrounding the hepatocyte.

Mag x 49,500

C - collagen

The pattern of type IV collagen deposition in sclerosing cholangitis appeared to become more organised than in normal liver, where it is localised to discrete, amorphous clumps. Labelling forms a continous line along the space of Disse, with a few gaps (Fig 10.28). As well as an increase in the amount of type IV collagen in the space of Disse, it is distributed around the hepatocytes. A continuous undulating line which contains type IV collagen, surrounds individual cells (Figs 10.29, 10.30 and 10.31).

Type VI collagen. The intensity of labelling for type VI collagen was increased in a number of fibrotic conditions. It was also increased in one case of nodular regenerative hyperplasia, within the space of Disse and associated with amorphous material (Fig 10.39).

In cirrhotic livers, the type VI collagen was frequently seen adjacent to striated collagen bundles (Figs 10.32, 10.33). Intracellular labelling was also seen with labelling found in fat-storing cells (Fig 10.34), often adjacent to the collagen bundles. In some biopsies it could be clearly seen that the type VI collagen was concentrated at the edges of the striated collagen bundles within the space of Disse (Fig 10.35). Due to the size of the tissue pieces used for electron microscopy, it often was not possible to identify the zone present and it was therefore not possible to compare the intensity of type VI labelling within the acini at the ultrastructural level.

FIG 10.32

Ultracryosection of cirrhotic liver

Immunogold labelling (10nm) for type VI collagen

Type VI collagen within the space of Disse is frequently associated with striated fibres, and the majority of the gold particles are located on and around striated collagen fibres.

mag x 49,500

SD - space of Disse KC - Kupffer cell

FIG 10.33

Ultracryosection of cirrhotic liver

Immunogold labelling (10nm) for type VI collagen

When fig 10.32 is examined at higher magnification, it can be seen that the type VI collagen is located around (a), and between (b), the striated fibres.

mag x 96,750

C - collagen

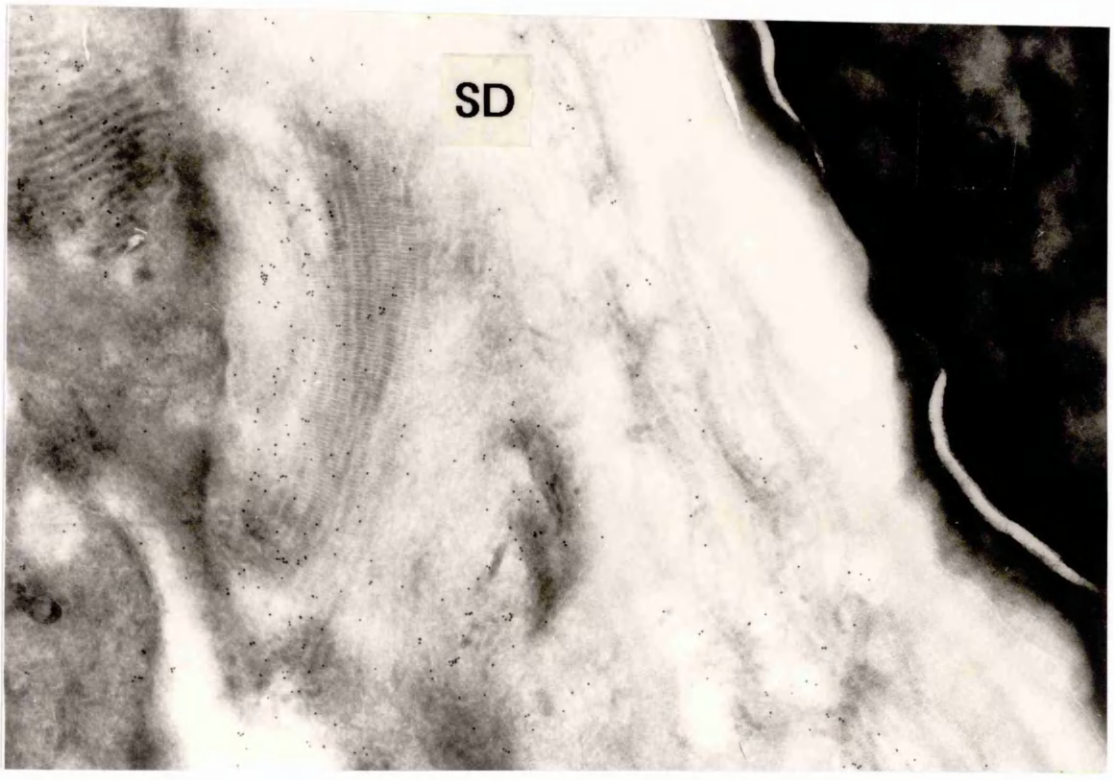

FIG 10.32

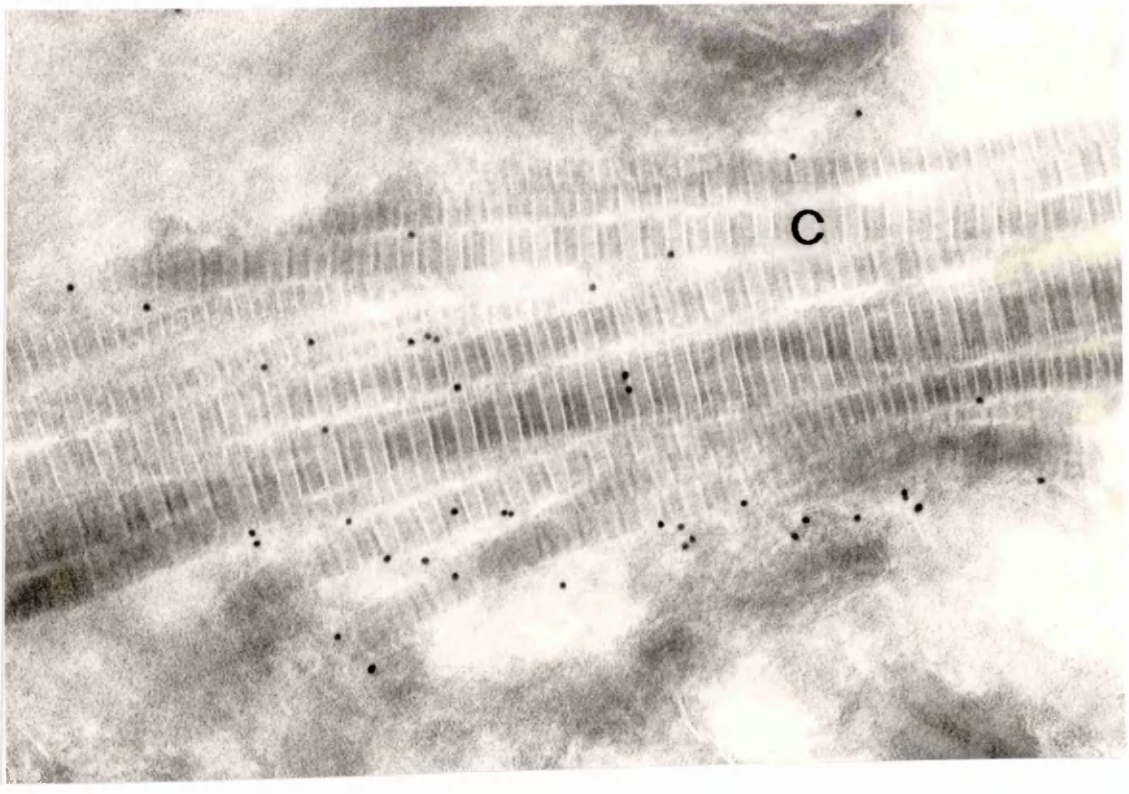

FIG 10.33

FIG 10.34

Ultracryosection of cirrhotic liver

Immunogold labelling (10nm) for type VI collagen

Labelling for type VI collagen is seen on the endoplasmic reticulum of this fat-storing cell, suggesting synthesis of the type VI collagen.

Mag x 44,000

ER - endoplasmic reticulum

FIG 10.35

Ultracryosection of a liver with primary biliary cirrhosis.

Immunogold labelling (10nm) for type VI collagen

The type VI collagen in the space of Disse is concentrated around the edges of the striated collagen bundle, with small amounts present within the bundle itself.

mag x 49,500

C - collagen bundle H - hepatocyte

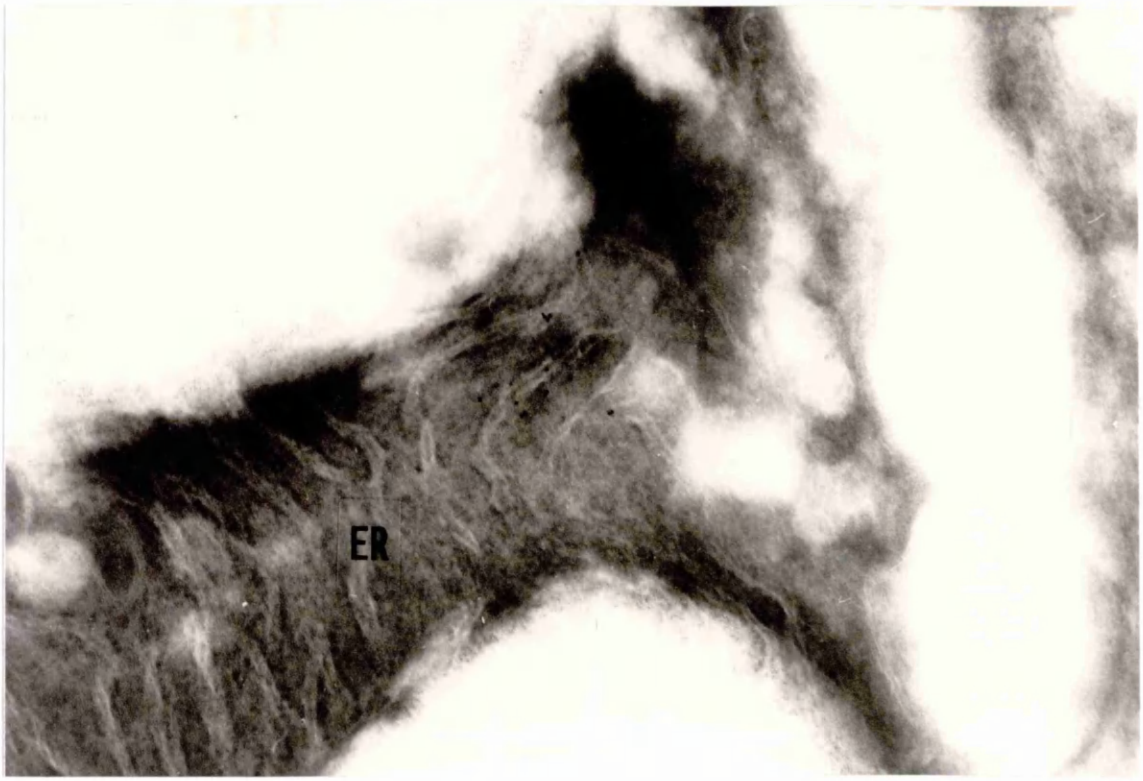

FIG 10.34

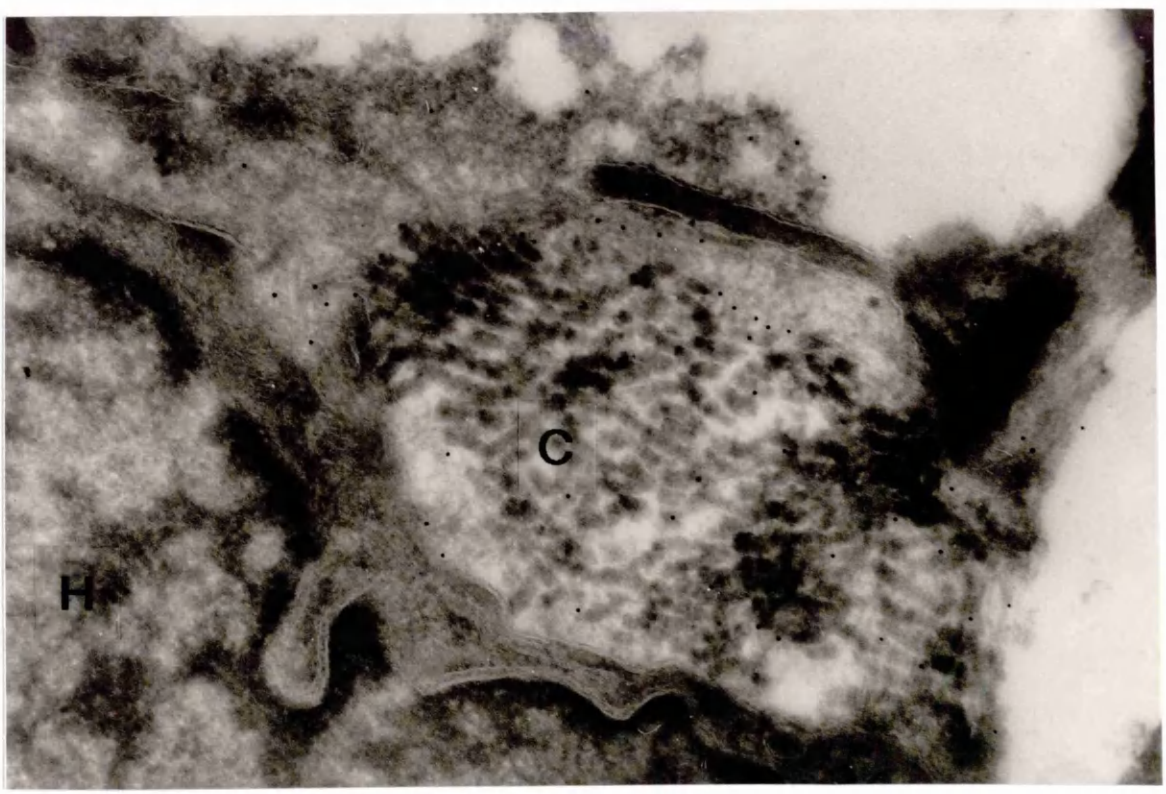

FIG 10.35

FIG 10.36

Ultracryosection of a liver with chronic active hepatitis

Immunogold labelling (10nm) for type VI collagen

The type VI collagen in this section is evenly distributed throughout the striated fibres. The labelling is far more intense than in normal liver.

mag x 60,000

EC - endothelial cell H - hepatocyte

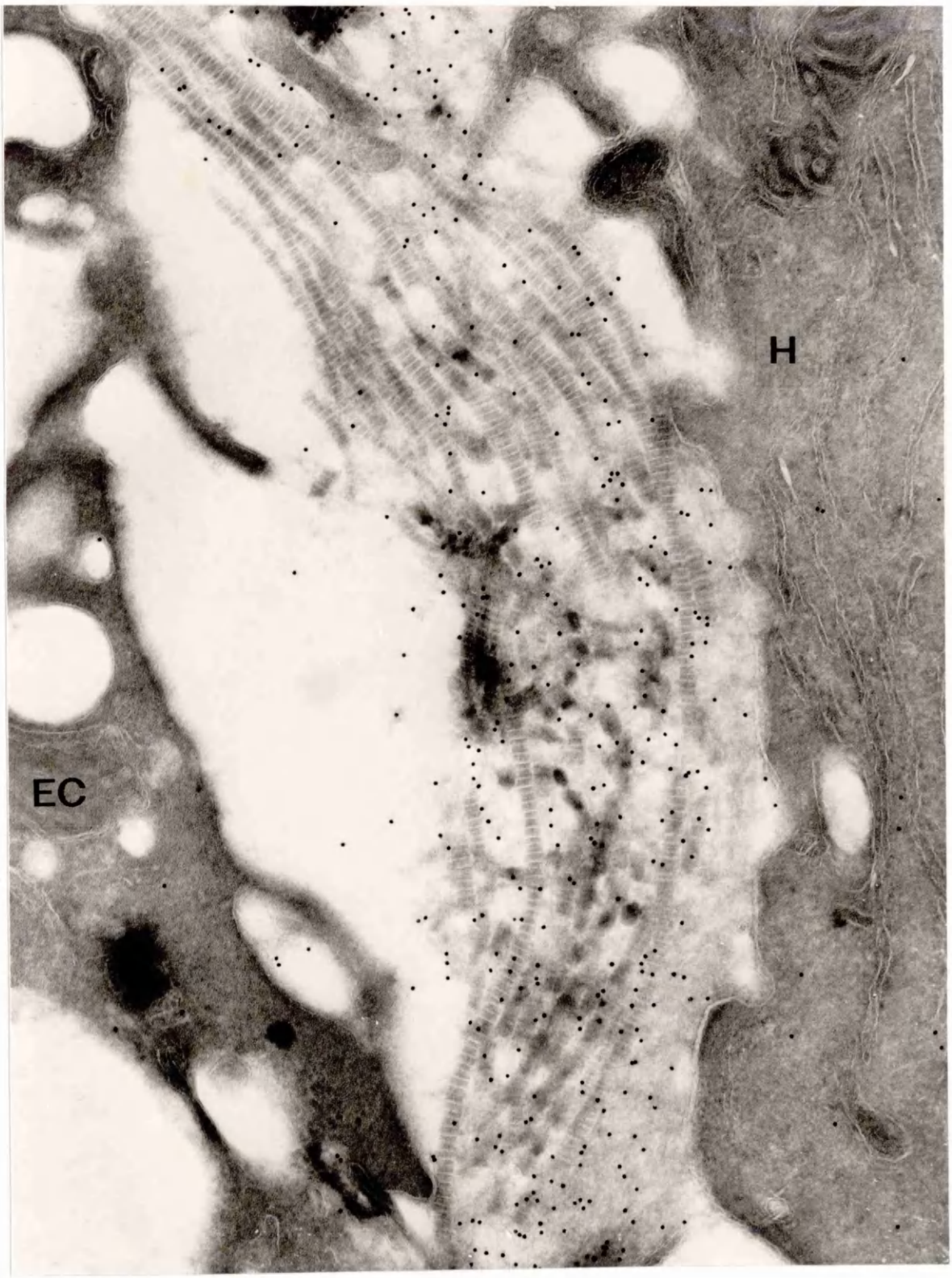

Figure 10.36

FIG 10.37

Ultracryosection of a liver with chronic active hepatitis

Immunogold labelling (10nm) for type VI collagen

There is heavy labelling for type VI collagen in this section, but the distribution is uneven and concentrated on the outer edges of the bundle.

mag x 40,500

C - collagen fibres EC - endothelial cell

FIG 10.38

Ultracryosection of a liver with chronic active hepatitis

Immunogold labelling (10nm) for type VI collagen

In this section, labelling for type VI collagen can be seen next to the striated fibres and also within an adjacent fat-storing cell.

mag x 55,000

EC - endothelial cell SD - space of Disse

FSC - Fat-storing cells

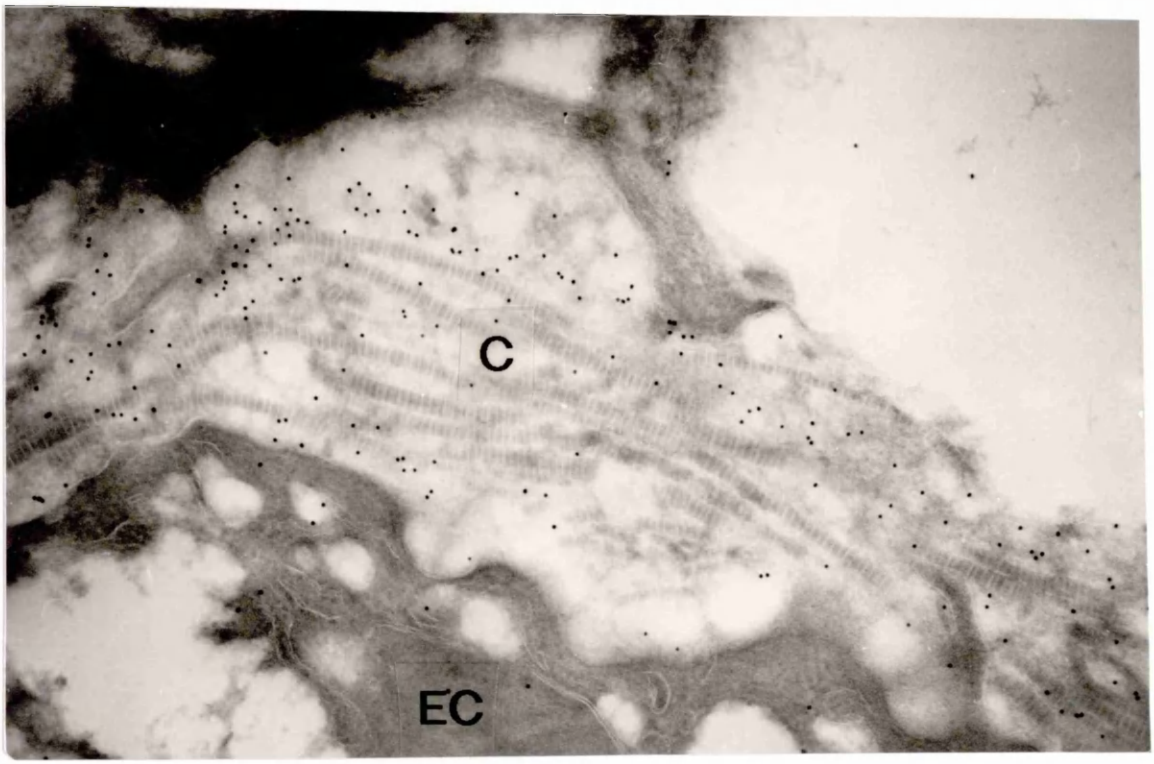

FIG 10.37

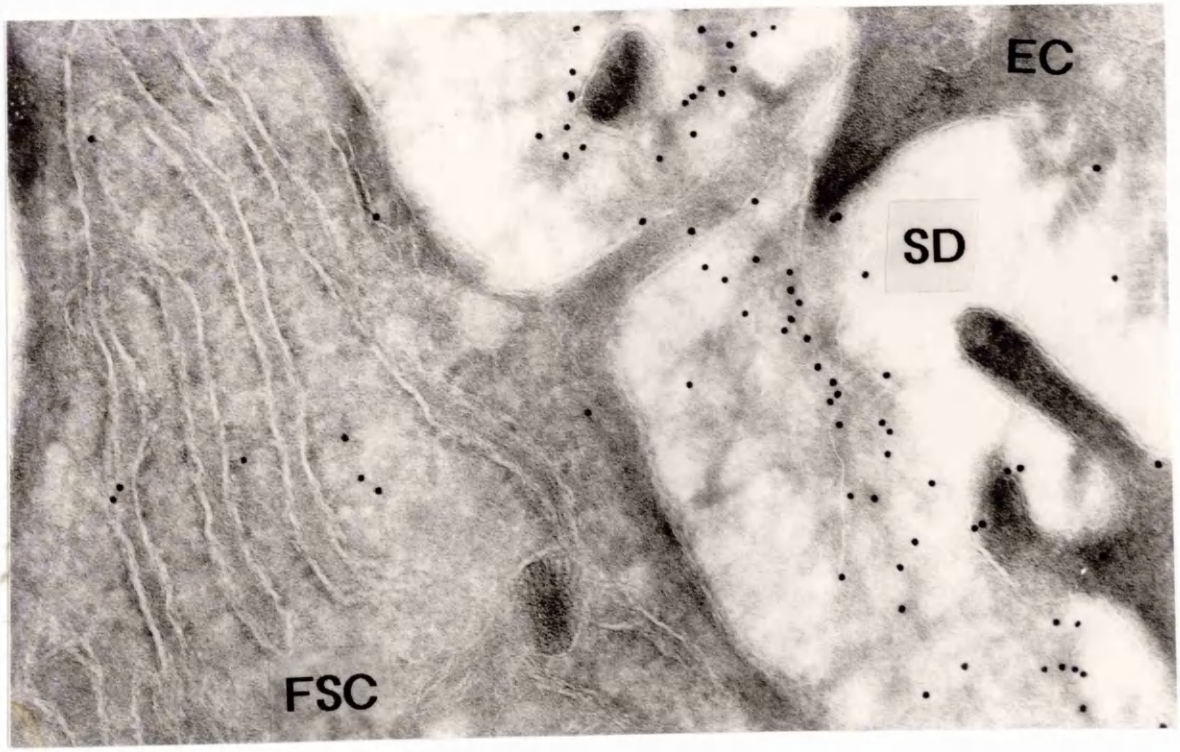

FIG 10.38

FIG 10.39

Ultracryosection of a liver with nodular regenerative hyperplasia

Immunogold labelling (10nm) for type VI collagen

The space of Disse is heavily labelled, both on amorphous material and adjacent to striated fibres. There is no intracellular labelling.

mag x 34,875

EC - endothelial cell SD - space of Disse

H - hepatocyte

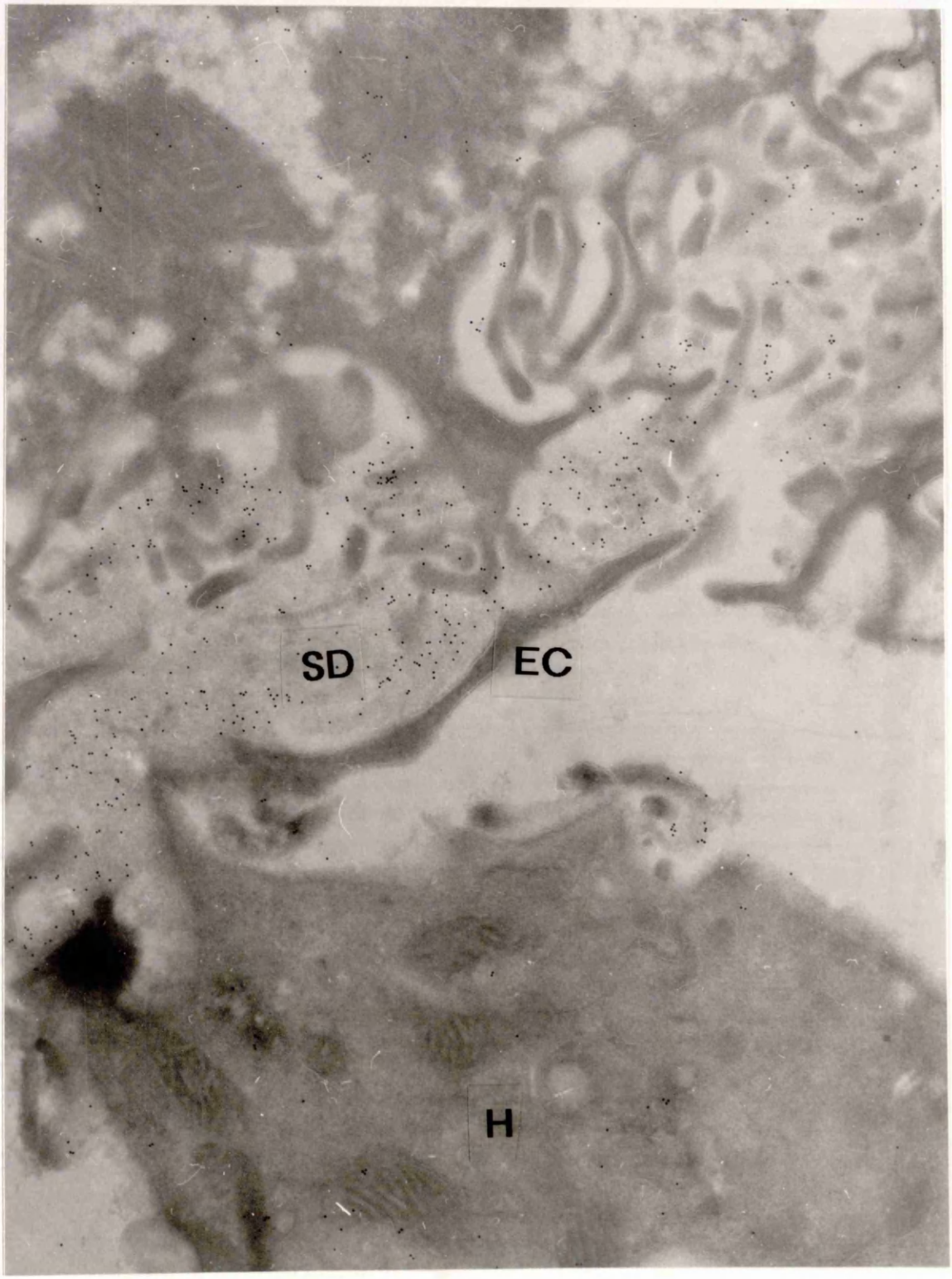

Figure 10.39

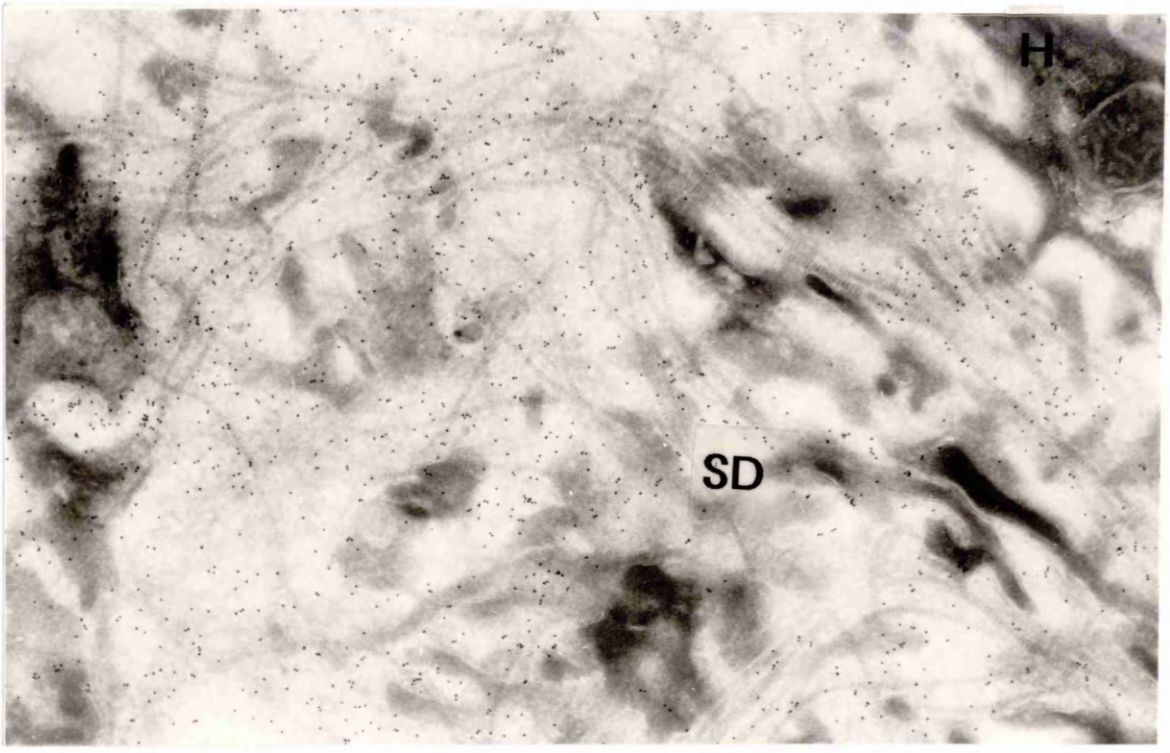

FIG 10.40

Ultracryosection of a liver with nodular regenerative hyperplasia

Immunogold labelling (10nm) for type VI collagen

This area of disordered collagen fibres within the space of Disse is heavily labelled for type VI collagen.

mag x 29,250

SD - space of Disse H - hepatocyte

In cases of chronic active hepatitis the type VI collagen was strongly labelled within the space of Disse (Figs 10.36 and 10.37). The fat-storing cells within the space of Disse were occasionally labelled intracellularly (Fig 10.38).

Some of the strongest labelling was found in the case of nodular regenerative hyperplasia. This was in the space of Disse, on amorphous material as well as near striated collagen (Fig 10.39). An unusual finding in this condition was the large amount of collagen which was disordered rather than in discrete bundles (Fig 10.40). This was rarely seen in other conditions. These disordered areas of collagen were very strongly labelled.

10.6 Fibronectin.

There was no alteration in the distribution of fibronectin, although the strength of labelling was increased. It was often found intracellularly in hepatocytes and fat-storing cells, and in lysosomes of Kupffer cells. It was found in association with most collagen bundles and throughout the space of Disse.

CHAPTER 11. DISCUSSION

11.1 The development of ultracryomicrotomy

11.2 The use of immunogold rather than immunoperoxidase labelling.

11.3 The effect of fixation

11.4 The problems of intracellular labelling

11.5 The confirmation of type I and III distribution

11.6 The novel description of type VI distribution

11.7 The presence of a basement membrane in the space of Disse

11.8 Cellular origin of extracellular matrix proteins

11.9 Liver as an ecosystem

11.1. The development of ultracryomicrotomy.

When immunocytochemical studies were first carried out at the ultrastructural level, most groups used resin-embedded material. The use of resin as an embedding material often rendered the tissue unsuitable or even unuseable for some immunocytochemical studies.

One possible solution to this problem was to immunolabel the tissue prior to processing and resin embedding (pre-embedding). Unfortunately this was not a suitable method for this study. In order to examine the tissue with a range of antibodies, a number of blocks would each have to be immunolabelled with a single antigen before embedding. Using post-embedding techniques, a number of individual sections from the same block can be immunolabelled with different antibodies.

A number of post-embedding techniques have been developed. The use of ultracryomicroscopy overcame many of the deficiencies discussed above. Many different fixation regimes are suitable. After fixation, the only substance which the tissue is exposed to during embedding is an aqueous solution of sucrose, and if the freezing proceedure has been carried out correctly, there is no ice crystal damage. Initially this method seemed to remove many of the problems associated with ultrastructural immunocytochemistry.

There are however, a number of novel drawbacks with this method. The tissue must be stored in liquid nitrogen after sucrose treatment, and the maximum storage time has not been determined. In comparison, resin embedded blocks

can be stored indefinitely. It is far more difficult to identify the area of the block face of interest and trim it for ultrasectioning in a frozen piece of tissue than in a resin embedded block, and if it is necessary for one particular aspect of the tissue to be studied, e.g portal tracts in liver, this may result in the waste of significant amounts of tissue and make it necessary to examine a number of individual blocks in order to obtain suitable material.

In this study, one of the first difficulties encountered in the use of ultracryomicrotomy sections was the identification of the different cell types and of the intracellular features. Some of the cells, such as Kupffer cells and fat-storing cells, did not differ much in appearance from those in resin embedded tissue, but the biggest difference was found in the appearance of hepatocytes. This however, did not interfere with their identification. Within the hepatocytes the best preserved structures were all membraneous, whereas the water soluble structures, such as glycogen, were poorly preserved (Burt et al, 1990). This gave the initial impression of a number of holes within the tissue. Although some of these "holes" were due to freezing damage (formation of ice crystals), the majority are probably due to the loss of the water-soluble glycogen particles. This is supported by the fact that the non-parenchymal cells which do not contain glycogen showed far fewer signs of damage.

This alteration in appearance was an important aspect to consider when using ultracryomicrotomy. If the

morphological appearance is critical then it may be advisable to consider other, more conventional, ultrastructural methods which allow easy identification of cell structure. In this study the immunocytochemical aspects were as least as important as the morphological ones, and this resulted in the use of ultracryomicrotomy.

11.2. The use of immunogold rather than immunoperoxidase labelling.

There are a number of different labelling systems which can be used for immunocytochemistry, but at the ultrastructural level the choice is more limited due to several factors. The two systems most commonly used are immunoperoxidase and immunogold. The advantage of the immunoperoxidase method is that a number of commercial antibodies are available ready conjugated to peroxidase (giving a one-step method), and an indirect method can also be used, especially if amplification of the signal is required. In this study it would have been necessary to use the indirect method, as the antibodies were only available in an unconjugated form. Although it was technically possible to conjugate our own antibodies, this could have introduced labelling variables (each antibody would bind differently to the gold particles, and there would also be labelling variation between batches).

The major drawback of the peroxidase method was that although the label was electron dense, it has a rather fuzzy appearance and can be difficult to identify in some tissues when they have been counterstained. In liver, it

may be confused with glycogen rosettes, which have a similar ultrastructural appearance. Another potential problem was that the size of the peroxidase particles cannot be altered, and this precludes the use of double labelling. As double labelling may be necessary to distinguish two different antigens which have similar but not identical distributions, the use of peroxidase labelling can limit experimental design.

The choice of an immunogold labelling method solved a number of these problems. It is unusual to use specific antibodies directly conjugated to gold particles. The gold conjugates which are used as the second layer are commercially available in a range of sizes from <1nm to 40nm in diameter. The most common sizes used are 5, 10 and 15nm in diameter, which are all commercially available. Anything larger than this has reduced labelling efficiency due to poor penetration into the tissue (Lackie, 1975). Secondary antibodies conjugated to gold particles which are smaller than 5nm are very sensitive and will penetrate tissue easily, but require silver enhancement to be identified. The silver enhancement technique utilises the ability of the gold particles to act as a nucleating agent with a silver suspension. This means that silver grains are deposited on the outside of the gold particles, thus giving a larger and more easily visualised label. If double labelling is necessary, the immunogold system is suitable as two different particle sizes (usually 5 and 15nm) can be used to label the two antigens (Beesley, 1989).

Ultracryomicrotomy was chosen for the electron microscopy part of this study in an attempt to minimise the damage to the tissue antigens, and the immunogold labelling method was believed to give the clearest results. Once this decision was made, the basic techniques had to be adapted and developed to suit the tissue and the antibodies which were to be used. There were a number of factors which had to be determined. For optimum preservation of tissue morphology and antigenicity, a range of freezing conditions and different counterstaining regimes were examined. As well as this, each antibody required a different combination of enzymatic pretreatments to remove other constituents of the extracellular matrix, blocking agents to prevent non-specific binding and antibody dilutions. These requirements varied between light microscopy and ultrastructural microscopy and have already been described in Chapter 9.

11.3. The effect of fixation.

One of the most important factors which determined the direction of this study was the effect of fixation on immunolabelling. While the tissue for the ultrastructural studies was collected and fixed specifically for this , the small size of tissue biopsies available meant that it was not possible to use a wide range of fixatives. As a result, the ultrastructural studies were limited to the antibodies which worked with paraformaldehyde or paraformaldehyde/glutaraldehyde-fixed material.

With the material to be used for light microscopy, a number of the antibodies available were found to be ineffective on tissue which had been fixed in formol corrosive, and this meant that it was not feasible to use archival material for antibodies such as anti-laminin and anti-type IV collagen. The use of these antibodies was restricted to tissue which was collected during this study and processed in a number of different fixatives. Archival material was used for the antibodies which were less "fixation-sensitive".

11.4. The problems of intracellular labelling.

When the different parameters for sectioning and immunolabelling had been optimised, the extracellular matrix of human liver was examined using a range of antibodies. It soon became obvious that although a number of the the antibodies were effective at labelling the extracellular spaces and pericellular areas, there was little or no labelling found intracellularly. Other groups have failed to demonstrate intracellular labelling (Grimaud et al, 1980, Martinez-Hernandez, 1984). Previous studies by other groups working on animal tissues (Geerts et al, 1986; Clement et al, 1984; Clement et al, 1985) gave contradictory evidence of intracellular labelling, with some of the positive labelling found in vesicles rather than in endoplasmic reticulum. Although these were described as Golgi vesicles, it was possible that they could be endocytotic vesicles. This meant that the labelling could indicate the uptake of extracellular matrix

proteins rather than their synthesis. Smedsrod et al (1986) have already demonstrated the hepatic endothelial cells are capable of endocytosing extracellular matrix components such as type I collagen. The amount of intracellular labelling in these studies was low in comparison to the extracellular labelling seen. This decrease may be due to masking of the antigens present within the cell. All reports of intracellular labelling were obtained with the ultrastructural immunoperoxidase method (Clement 1986, 1988)

Another possible explanation is that the collagen present within the cells is in the form of precursor collagen and the antibodies used are raised against the fully processed collagen. In order to test this theory, antibodies were used which had been raised against the propeptide portion of collagen molecules. These label extracellular collagen fibres which have not been fully processed, but no intracellular labelling was seen (Geerts et al, 1986).

The use of ultrathin cryosections meant that a lack of intracellular staining was unlikely to be due to an inability of the antibodies to penetrate the sections and bind to the antigen. This left two other possibilities. The first was that the fixation regime used had altered the antigen to such an extent that it was unrecognisable, and the second possibility was that the antibody was unable to access the antigen to which it was raised.

The first theory, that of the fixation effect, may be at least partially true in cases where neither

intracellular or extracellular labelling could be identified after the use of specific fixation regimes. This was the case with some of the antibodies used in this study, such as anti-laminin. When tissues which had been immunolabelled for laminin were examined using light microscopy, the presence or absence of staining in extracellular spaces was determined by the fixative used i.e. all the laminin antibodies gave positive extracellular staining in Bouins-fixed material, but negative results in formol corrosive-fixed tissue (Table 10.1). Intracellular staining was consistently negative, regardless of the fixative used. This theory does not explain why the antibodies which are capable of extracellular labelling in optimally fixed tissue are unable to identify any intracellular matrix proteins.

All antibodies bind to the antigenic site via a number of forces such as hydrophobic, hydrogen bonding, electrostatic and Van der Waals forces. As the distance between antigen and antibody increases, the bonds are weakened. When tissues are chemically fixed, the degree of cross-linking within the tissue increases. This can lead to weakening of the antibody-antigen bonding and a consequent loss of immunolabelling in the tissue.

In order to explain the absence of intracellular staining and presence of extracellular cellular staining in the same tissue it was necessary to examine the second possibility, i.e that the antibody was physically unable to recognise the antigen. There were two possible causes of this. The first was linked to the complex processing of

the extracellular matrix proteins. As described in chapter 4, the synthesis of collagen requires a number of processing steps including hydroxylation, glycosylation and triple helix formation within the cell, as well as the cleavage of extension peptides and assembly of microfibrils outside the cell. Much of the collagen within the cell (procollagen) was unprocessed and in the form of individual α-chains. In contrast to this, the majority of extracellular matrix antibodies were raised against collagen molecules which were isolated and purified in the fully processed state. It was possible that the partially-processed intracellular collagen molecules were not recognised by the antibodies which were used in this study, although the same antibodies were capable of identifying the fully processed collagen molecules which were present in the extracellular spaces. This theory is unlikely as demonstated by the pro-peptide labelling studies (Geerts et al, 1986) discussed above.

Although each of these explanations have been proposed as the sole cause for the lack of intracellular labelling, it is possible, if not probable, that it is a combination of two or three factors which results in the lack of intracellular labelling.

11.5. The confirmation of type I and III distribution.

There have been a number of previous descriptions of the distribution of type I and III in rat liver and in both normal and diseased human liver. The majority of these used light microscopy (Hahn et al, 1980, Voss et al, 1980

Grimaud et al, 1980), with a few resulting from ultrastructural studies (Geerts et al, 1986, 1990; Clement et al, 1985; Martinez-Hernandez, 1984).

The results obtained in this study were generally compatible with the findings of these papers, with some novel observations in the case of alcoholic hepatitis. The distribution of the types I and III collagen were similar in normal liver and in diseased liver. There were two exceptions to this. The first was the presence of types I and III in any fibrotic septa which had developed. The second difference was noted in cases of alcoholic hepatitis where some of the damaged hepatocytes were surrounded by type III collagen.

When the tissue was examined at the ultrastructural level, several differences between the two types of collagen were apparent. The first was the amount of each collagen type, with the majority of fibres positive for type III collagen rather than type I. The type III collagen appeared evenly distributed throughout the collagen bundles whereas the type I fibres were often more apparent towards the edges of the bundles. Each of the antibodies appeared to label a high proportion of the collagen fibres, and although double labelling experiments were not carried out, this pattern of distribution was consistent with previous suggestions that collagen fibres may contain both types I and III collagen (Geerts et al, 1990).

The high proportion of collagen type III propeptide retained on fibres was consistent with the suggestion that

cleavage of the propeptide fraction of type III collagen in a fibre is necessary before more type III molecules can attach and enlarge the fibre (Lapiere et al, 1977). The control mechanism behind this method of size control is not yet known. All of these differences in propeptide distribution were found in both normal and diseased liver.

11.6. The novel description of type VI distribution.

No previous studies had examined the detailed distribution of type VI collagen in the human liver. Von der Mark (1984) reported the presence of type VI collagen in the liver but not the pattern of distribution. It has previously been regarded as one of the minor collagen components due to the relatively small amounts found in tissue. This study found a zonal heterogeneity of type VI collagen unlike the distribution of collagen types I and III (Griffiths et al, 1991). As mentioned in chapter 10 this zonality was unlikely to be artefactual as other sections of the same tissue blocks were labelled for type I or III collagen and the distribution of these matrix components was homogeneous throughout the liver. Another possible explanation for the apparent zonal distribution was that type VI collagen was evenly distributed within the liver, but in zone 1 it was masked by other molecules such as glycosaminoglycans.

Evidence of functional heterogeneity within the liver has been demonstrated for a number of metabolic processes (Gumucio, 1989), and Martinez-Hernandez and Amenta have described alterations in the composition of the

extracellular matrix across the acinus. Reid et al (1992) hypothesised that there were hepatic gradients for a number of hepatic extracellular matrix components and the type VI collagen distribution described above is consistent with this. In the area of the portal triad the matrix consists of basement membrane components with fibrillar collagen. This alters as the central vein is approached, when the matrix consists of fibrillar collagens, fibronectin and proteoglycans. It is already acknowledged that the composition of the extracellular matrix can affect the function and gene expression of liver cells by binding to integrins in the cell membranes (Bucher et al, 1990), and the zonal distribution of the type VI collagen suggests a possible role for this extracellular matrix component in modulating the functional heterogeneity of the liver.

The ultrastructural study showed type VI collagen as a fine fibre which was intertwined between the larger striated fibres of the other interstitial collagens. This was consistent with Keene et al's description of type VI collagen in skin using stereopair electron microscopy, when it appeared as a branching filamentous network surrounding interstitial collagen fibres. Type VI collagen may therefore play an important role in organising the other components of the extracellular matrix. All striated collagen fibres are individually synthesised and secreted from their cell of origin, but within the tissue they form compact and parallel bundles. It is the type VI collagen which is at least partially responsible for this organisation. In 1992, Loreal et al also described the

presence of type VI collagen around bundles of striated collagen. They did not describe any zonal heterogeneity of distribution.

When the immunolabelling of type VI collagen was examined in acute liver injury or non-specific reactive changes the amount and distribution was essentially the same as in normal liver. In comparison, the amount was increased in fibrotic liver, even in precirrhotic conditions. The relatively large increase of this minor extracellular matrix component from the onset of fibrosis suggests that it may play an important role in fibrogenesis, particularly in the early stages. This is in agreement with the recent study by Loreal et al (1992), who also identified intracellular labelling for type VI collagen in fat-storing cells, using ultrastructural immunoperoxidase. In fibrotic liver increased amounts of type VI collagen were often associated with large areas of disordered striated collagen fibres. Such disordered areas are often an indication of newly synthesised collagen and type VI collagen may be synthesised in order to organise the fibres of collagen types I and III into their characteristic bundles, as seen in established cirrhosis.

11.7. The presence of a basement membrane in the space of Disse.

Basement membranes are found around the hepatic arteries, portal veins, lymphatic vessels, bile ducts and nerve axons (Vracko, 1982). Although in normal liver these basement membranes can be viewed by conventional

electron microscopy, the space of Disse is unusual, in that individual components of the basement membrane have been identified immunocytochemically but no basement membrane is visible by electron microscopy (Griffiths et al, 1991). The formation of the basement membrane in the space of Disse (capillarization), often occurs during hepatic fibrosis (Schaffner and Popper, 1963). These are two possible explanations for this. The first is that there is a functional membrane present at all times, but that the structure differs from other basement membranes i.e. it is more diffuse in normal tissue, and it thickens during capillarization. The second theory is that there is no membrane present in normal liver although the individual components are there, and that the membrane is only formed during capillarization. If this is the case, there may be one vital component of the membrane which is only produced during capillarization.

The results obtained from this study tend to support the second theory. The two major components of the baement membrane, type IV collagen and laminin, are both present in normal liver, although in small quantities when compared to fibrotic liver. As there is no evidence of either a structural or a functional basement menbrane in normal liver it seems likely that at least one component is missing, and that this is produced during fibrosis. One molecule which has been suggested for this role is nidogen, also known as entactin (Ramadori et al,1991). It is capable of binding to laminin and to type IV collagen, and it has been localised in the basement membrane of rat liver

around bile ducts and blood vessels. It is not present sub-endothelially in normal liver.

Irrespective of the origin of the basement membrane found in fibrotic liver, it has a major effect on hepatic function. In normal liver, there is a ready flow of plasma between sinusoids and hepatocytes. This is blocked in fibrotic liver and may contribute to the onset of portal hypertension, as well as contributing to disordered hepatocyte function by depriving the cells of oxygen and nutrients.

11.8. Cellular origin of extracellular matrix proteins

The identification of the cell types which actually synthesise the hepatic extracellular matrix has been the subject of debate for many years. Initial studies using cell culture techniques suggested that hepatocytes were a major source of collagen synthesis (Saber, Schafritz and Zern, 1983). This however was disputed by Maher et al (1988) who believed that this could be explained by contamination of the cultures by fat-storing cells. In all cell culture studies there is the problem in that results from in vitro studies are directly applied to an in vivo situation, where the factors controlling extracellular matrix synthesis may be totally different.

In this study we attempted to use intracellular labelling in order to identify the cells which were responsible for extracellular matrix synthesis. Irrespective of the anti-extracellular matrix protein antibody used, significant amounts of intracellular

staining were rarely observed. On the few occasions where labelling did occur, it was always within the fat-storing cells. This was consistent with the findings of other groups which identified collagen either within (Kent et al, 1976; Takahara et al, 1988 and Clement et al, 1988) or very closely associated with (Grimaud et al, 1980) fat-storing cells. The possible reasons for the failure to detect the matrix proteins in an intracellular pattern are discussed in section 11.4. The next approach was to look at the cells which were immediately adjacent to the different matrix proteins. These proteins are normally deposited in the immediate vicinity of their synthesis and are not exported far from the cell of origin. When the pericellular distribution of the individual matrix proteins were examined, they could all be identified around fat-storing cells. The only other occasion in which a cell was surrounded by matrix proteins was in some cases of alcoholic liver disease, where hepatocytes were surrounded by a layer of striated collagen (types I and III). This may indicate collagen synthesis by hepatocytes in fibrotic liver conditions. The other possible explanation is that the synthesis was due to nearby fat-storing cells. When thin sections of tissue are being examined, it is difficult to envisage the three-dimensional structure of the liver and it is always possible that the cell responsible for the synthesis of the extracellular matrix, and the matrix itself may not be present in the same section. This means that although the presence of collagen next to a particular cell indicates that there is a strong probability of that

cell being the source of the collagen, it cannot be regarded as an absolute fact. This must be borne in mind when interpreting results from histological sections.

A new technique which has been successfully developed since the start of this study is that of in-situ hybridisation. Instead of antibodies, this uses a molecular probe, which is a small piece of DNA or RNA complementary to the DNA or RNA of interest. The probe is labelled, (this can be with a radionucleotide, biotin or digoxygenin) and once the probe has bound and the label is developed, it can be detected on the section by light microscopy. This method identifies the cells which are producing the molecule. Preliminary work carried out in this department using digoxygenin-labelled RNA probes to type I collagen, indicated that it is the fat-storing cells which are responsible for type I collagen synthesis. This was true in both normal and CCl_4-treated rat liver. In contrast to similar work by Yamada et al examining type III collagen in liver (1989), we did not find any evidence of collagen production in hepatocytes.

11.9. Liver as an ecosystem.

When the pattern of hepatic extracellular matrix synthesis is studied, it cannot be regarded in isolation. If the ability of hepatocytes to produce liver specific proteins is affected by the substrate which they are attached to, alterations in the pattern of extracellular matrix protein synthesis will lead to alterations in liver function (Bissell,1990). One such example is the

capillarization of the space of Disse, in which the hepatocytes come into contact with newly synthesised basement membrane. This means that in addition to the consequences of isolating the hepatocytes from the plasma supply as discussed in chapter 11.7, the hepatocyte phenotype may be altered by the change in substrate.

This is one example of the balance within the liver, where cell-matrix and cell-cell interactions are equally important. In normal liver and during liver regeneration, there is a carefully maintained balance between all of the components of the extracellular matrix, and the cells within the liver. When this balance is lost i.e. during fibrosis, the liver is affected both structurally and functionally. It can be difficult, if not impossible to separate cause and effect, i.e. the formation of scar tissue may affect the function of adjacent cells which then alter their pattern of extracellular matrix synthesis and produce more fibrotic tissue. It can be difficult to distinguish this from scar tissue due to the primary injury of cells.

Another such example is the alteration during fibrosis of the ratio of type I to type III collagen, which decreases in the early stages of fibrosis, and increases later on. If each individual collagen type has a specific effect on parenchymal or non-parenchymal cells via a collagen specific receptor, then the altered ratio of the collagen types will have functional as well as structural implications. The receptors which could be

important in this respect are likely to be part of the integrin family.

When these complex relationships are taken into consideration, the deposition of collagen and other extracellular matrix proteins cannot be regarded as a purely structural change, with any functional abnormalities such as the development of portal hypertension due to mechanical blockages.

Caution must also be used when examining alterations to one component of the hepatic extracellular matrix in liver disease. In many cases there is an increase in all components of the matrix and it is important to consider the extent of any increase in one component in relation to the others i.e. how is the overall ratio of the components affected? One such example is in type VI collagen. As this is one of the minor collagens the increase in cirrhotic liver is low when measured in terms of total collagen (0.1% to 0.2% of total collagen), but this represents a 100% increase in the amount of type VI collagen during cirrhosis. It is likely that this collagen plays an important role during fibrosis, as the amount of type VI collagen is increased, even in pre-cirrhotic stages.

Although we can identify fibrotic liver and identify the components of the extracellular matrix which are present, the broad pattern is usually the same, irrespective of the original cause of injury. The only differences found are between chronic and acute liver injury, and in the area of the acinus where the injury

occurs. Irrespective of the cause of fibrosis, the alterations in the extracellular matrix are essentially the same, indicating a common mechanism of fibrosis.

This means that if there are any differences in the extracellular matrix they are most likely to be identifiable at the ultrastructural level, where the tissue can be examined in detail and the individual collagen types can be identified. The techniques developed as part of this study will allow further study of the ultrastructural changes in hepatic fibrosis. These results will be of most use when examined in conjunction with results obtained by a range of other methods such as in situ hybridisation.

The extracellular matrix cannot be understood by examining a small number of its many and complex components in isolation. It is also true that one individual technique cannot be used to reveal its secrets. The techniques which have been developed during this thesis, and the results which they have produced, may help to place a few more pieces in the complex (and three-dimensional) puzzle which is the hepatic extracellular matrix.

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APPENDIX A FIXATIVES

1. 0.2M phosphate buffer p.H. 7.4

800ml 0.23M $Na_2HPO_4 2H_2O$ solution A

250ml 0.2M KH_2PO_4 solution B

Add solution B to solution A until the pH. reaches 7.4

2. 4% paraformaldehyde

Add paraformaldehyde (Fluka, Germany) to 0.2M phosphate buffer (final paraformaldehyde concentration of 4%) and heat to dissolve.

3. 2% paraformaldehyde

Mix equal volumes of 4% paraformaldehyde and 4% sucrose/H_2O.

4. 2% paraformaldehyde / 0.1% glutaraldehyde.

Add 0.4ml of 25% glutaraldehyde (EM grade, Agar Aids, U.K.) to each 100 ml of 2% paraformaldehyde solution.

All fixatives were stored at -20°C after preparation, although glutaraldehyde was not added until fixative was required.

APPENDIX B RESIN EMBEDDING

1. Sorensens' phosphate buffer pH 7.4	5 min
2. Sorensens' buffer	5 min
3. 1% OsO_4 in phosphate buffer pH 7.4	45 min
4. Sorensens' buffer	5 min
5. Sorensens' buffer	5 min
6. 25% ethyl alcohol	10 min
7. 50% ethyl alcohol	10 min
8. 75% ethyl alcohol	20 min
9. 100% ethyl alcohol	20 min
10.100% ethyl alcohol	20 min
11.100% ethyl alcohol	20 min
12.100% ethyl alcohol	10 min
13.Propylene oxide	5 min
14.Propylene oxide	10 min
15.Araldite:propylene oxide 1:1	1 hr 40 min
16.Araldite:propylene oxide 3:1	2 hours
17.Araldite mix (60°C)	overnight

Araldite mix

Araldite resin (CY212)	10 ml
Dodecenylsuccinic anhydride (DDSA)	10 ml
DMP-30	0.5 ml
2,4,6-tri(dimethylaminomethyl phenol) dibutyl phthalate	1 ml

APPENDIX C

Staining resin embedded sections

1. Saturated uranyl acetate in 50% ethanol (in dark) — 10 min
2. Rinse with distilled H_2O
3. Blot dry
4. "Reynolds" lead citrate — 1-2 min
5. Rinse
6. Blot dry