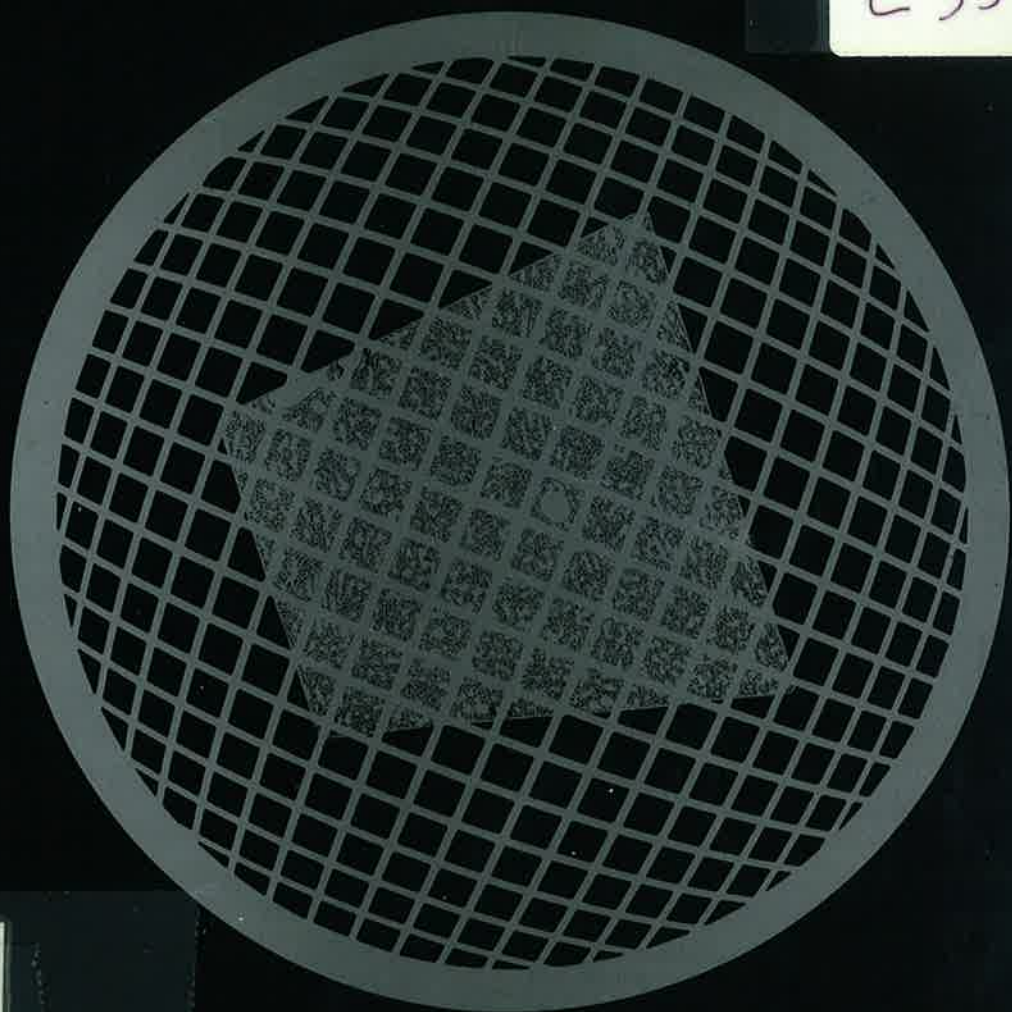


THE ULTRASTRUCTURE
OF SINUSOIDAL LIVER CELLS
IN AGING RATS IN RELATION TO FUNCTION

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PROEFSCHRIFT

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A.M. de Leeuw

1265

**THE ULTRASTRUCTURE
OF SINUSOIDAL LIVER CELLS OF
AGING RATS IN RELATION TO FUNCTION**

Drukkerij Van Dooren, B.V. - Vlaardingen

CIP

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STELLINGEN

1

Bij het gebruik van de "fat-storing" celindex (het aantal "fat-storing" cellen per 1000 hepatocyten) als maat voor veranderingen in het aantal "fat-storing" cellen per volume-eenheid van de lever, is het noodzakelijk om ook rekening te houden met veranderingen in het aantal hepatocyten per volume-eenheid van de lever.

2

De stelling van Irving et al., dat collageen type IV een specifieke marker is, waarmee in celkweek de sinusoidale leverendothelcellen kunnen worden gekarakteriseerd, is niet juist, aangezien dit type collageen ook in de "fat-storing" cellen van de lever kan worden aangetoond.

M.G. Irving, F.J. Roll, S. Huang, D.M. Bissell
(1984) Gastroenterol. 87, 1233-1247.
Dit proefschrift.

3

De conclusie van Blomhoff et al., dat in de lever het vitamine A direct vanuit het bloed in de parenchymcellen wordt opgenomen, is voorbarig.

R. Blomhoff, P. Helgerud, M. Rasmussen, T. Berg, K.R. Norum (1982) Proc.Natl.Acad.Sci. USA, 79, 7326-7330.

4

De opslag van vitamine A in de "fat-storing" cellen van de lever is een onvoldoende basis voor de veronderstelling, dat deze cellen wezenlijk verschillen van andere fibroblastachtige cellen, die zich elders in het lichaam onder het endotheel bevinden.

5

Een universeel toepasbaar systeem van celnomenclatuur dient primair gebaseerd te zijn op de relatie, die er gelegd kan worden tussen de ultrastructuur en de functie van een cel. Dit betekent voor de lever, dat de Kupffercel als levermacrofaag en de "fat-storing" cel als leverfibroblast herbenoemd zouden moeten worden.

6

Het is nog altijd noodzakelijk om de samenstelling van preparaten van geïsoleerde levercellen te karakteriseren op grond van hetzij morfologische hetzij celspecifieke biochemische parameters, teneinde te voorkomen, dat functies ten onrechte aan een bepaald celtype worden toegeschreven.

Zolang niet is aangetoond, dat in *vivo* ingespoten leupeptine een effectieve remmer is van de lysosomale afbraak in alle lichaamscellen, kan de leupeptine-afhankelijke stapeling van radioactiviteit in sommige weefsels niet gebruikt worden als een bewijs voor de specifieke betrokkenheid van deze weefsels in het catabolisme van radioactief gemerkte lipoproteïnen.

F.M. van 't Hooft, G.M. Dallinga-Thie, A. van Tol (1985) *Biochim.Biophys.Acta* 834, 75-84.

Bij ziekten, die gepaard gaan met een verminderde absorptie in de darm van vetten, dient er rekening mee gehouden te worden, dat sommige van de klinische symptomen veroorzaakt worden door een verhoogde absorptie in de darm van specifieke moleculen.

Daar waar het begrip omtrent de functionele betekenis van ultrastructurele celkenmerken te kort schiet, neigt de elektronenmicroscopist tot bloemig taalgebruik. De schollen in de gonadotrope cellen van de hypofyse ("irregular membrane bound masses"; Peute et al.) en de wormachtige structuren in de Kupffercellen van de lever ("worm-like structures"; Wisse) hebben dan ook niets van doen met de desbetreffende organismen.

J. Peute, R. de Leeuw, H.J.Th. Goos, P.G.W.J. van Oordt (1984) *Cell Tiss.Res.* 238, 95-103.
E. Wisse (1974) *J.Ultrastruct.Res.* 46, 393-426.

De stelling, dat: "Electron microscopy too often leaves us just as ignorant but at higher resolution", is met name van toepassing bij de diagnostiek van lichtmicroscopisch ongedifferentieerde tumoren.

G.I. Kaye (1981) In: *Progress in surgical pathology*, C. Fenoglio M. Wolff, eds., Masson, New York, p. 171-179.

In de immunocytochemie moeten positieve reacties kritisch doch positief gewaardeerd worden als een indicatie voor de aanwezigheid van een bepaald antigeen; negatieve reacties daarentegen zijn geen bewijs voor de afwezigheid van antigenen.

12

Gezien de hoge doses piracetam, die worden voorgeschreven aan demente patiënten lijkt het realistischer om te spreken van een dieet dan van een geneesmiddel.

13

De kwaliteit van een ondernemingsraad wordt hoofdzakelijk bepaald door de wil en het vermogen tot overleg van de zijde van het management.

14

Onderwijsbegeleidingsdiensten zouden zich eerder moeten bezighouden met de daadwerkelijke begeleiding van schoolteams bij het invoeren van nieuwe schoolsystemen, dan met het wegnemen van frustratiegevoelens binnen deze teams, die het gevolg zijn van steeds weer nieuwe richtlijnen uitgevaardigd door het Ministerie van Onderwijs en Wetenschappen.

15

De elektronenmicroscopist(e) in de experimentele gerontologie is op zoek naar rimpels in de cel.

Stellingen behorende bij het proefschrift "The ultrastructure of sinusoidal liver cells of aging rats in relation to function"

A.M. de Leeuw
4 juni 1985

**THE ULTRASTRUCTURE
OF SINUSOIDAL LIVER CELLS OF
AGING RATS IN RELATION TO FUNCTION**

A.M. de Leeuw

1985

This work represents a thesis for a doctoral degree at the University of
Utrecht (Promotores: Prof.Dr. D.L. Knook and Prof.Dr. C.F. Hollander)

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Rijswijk, The Netherlands**

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CHAPTER 1

GENERAL INTRODUCTION

1.1 THE LIVER: ORGANIZATIONAL PRINCIPLES

The structural organization of the cellular and vascular elements of the liver is adapted to its special function as a key organ interposed between the digestive tract and the rest of the body (Arias et al., 1982). Hepatic function includes the effective uptake from the blood of nutrient components absorbed in the intestine and their subsequent metabolism, storage and distribution to blood and bile as well as the biotransformation of xenobiotic components, drugs and endogenous metabolites. As the liver also contains a major part of the cells belonging to the reticulo-endothelial system (RES), hepatic function additionally involves the clearance from the circulation of a wide variety of materials such as bacterial endotoxins, effete red blood cells, circulating tumor cells, immune complexes and specific macromolecules. To meet these specific demands, the liver architecture guarantees optimal exchange between blood and liver cells. Accomplishment of specific functions is achieved by inter- and intracellular compartmentalization and separation of biliary and blood spaces.

Two primary models have been developed to describe the histological and/or functional units of which the liver is composed. (i) While studying the pig liver, Kiernan in 1833, introduced the concept of the hepatic lobule centered about a "central" vein and surrounded at its edges by "interlobular" portal canals. The portal canal was defined as the roughly cylindrical space which contains branches of the portal vein and the hepatic artery, bile ducts, lymph vessels and nerves in a coat of connective tissue. (ii) In most other mammalian species, including the rat, it was observed that lobules are centered around portal canals in such a manner that liver cell plates and sinusoids radiate from the portal canals. Rappaport et al. (1954) embraced this concept of the "portal lobule" and proposed the name "liver acinus" for an irregular mass of tissue arranged around a portal canal and lying between two or more central veins. The liver acinus is now generally considered as the smallest functional unit of liver tissue and the term will be used throughout this thesis.

Liver acini consist of both cellular elements and spaces through which the blood and bile are transported. The capillary bed of acini is formed by a number of sinusoids radiating from one terminal branch of the portal vein and draining into several central veins, the sinusoids being separated from each other by a one-layer parenchymal cord (Fig.1.1). The blood supply of liver acini is of a dual origin: (i) oxygen-rich blood entering the liver through branches of the hepatic

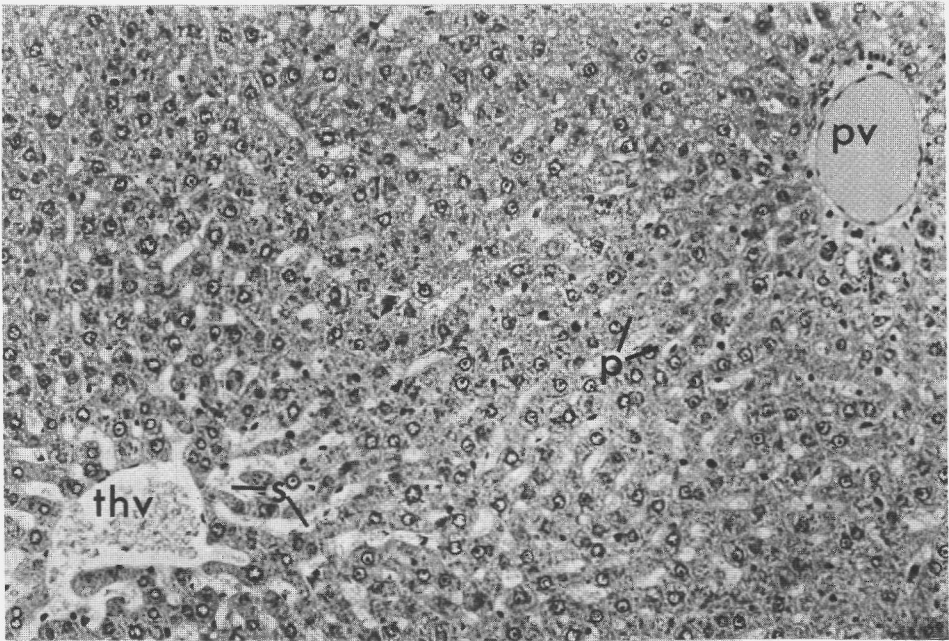


Figure 1.1 Part of a rat liver acinus.
The sinusoids (s) separated from each other by a one-layer parenchymal cord (p) radiate from a terminal branch of the portal vein (pv) and drain into a number of terminal hepatic veins (thv). Histological section; HE stain. 200 x.

artery; (ii) nutrient-rich blood originating from the intestinal microvascular system and entering the liver through branches of the hepatic portal vein. After passage through the liver sinusoids in which the two types of blood are mixed, the blood is drained into branches of the hepatic vein (Fig.1.2).

Completely separated from the blood space by tight junctional complexes between neighbouring hepatocytes, the biliary system traverses the liver acini, transporting bile produced in the hepatocytes. Biliary drainage commences as small spaces (the bile canaliculi) which are formed through local widening of the space between the lateral surfaces of two adjoining hepatocytes. Junctional complexes around these canaliculi prevent paracellular flow of bile to the sinusoidal surface of the hepatocytes. Through the transitional canals of Hering, bile canaliculi converge into bile ductules lined by squamous epithelial cells and then into larger ducts lined by cuboidal epithelial cells. The bile ductules and ducts form part of the portal canals mentioned above (Fig.1.2).

Liver acini are composed of various cell types. The main cell type is the liver parenchymal cell or hepatocyte, which in the rat accounts for about 70% of total liver volume (Blouin et al., 1977). Sinusoidal liver cells, a group of cells consis-

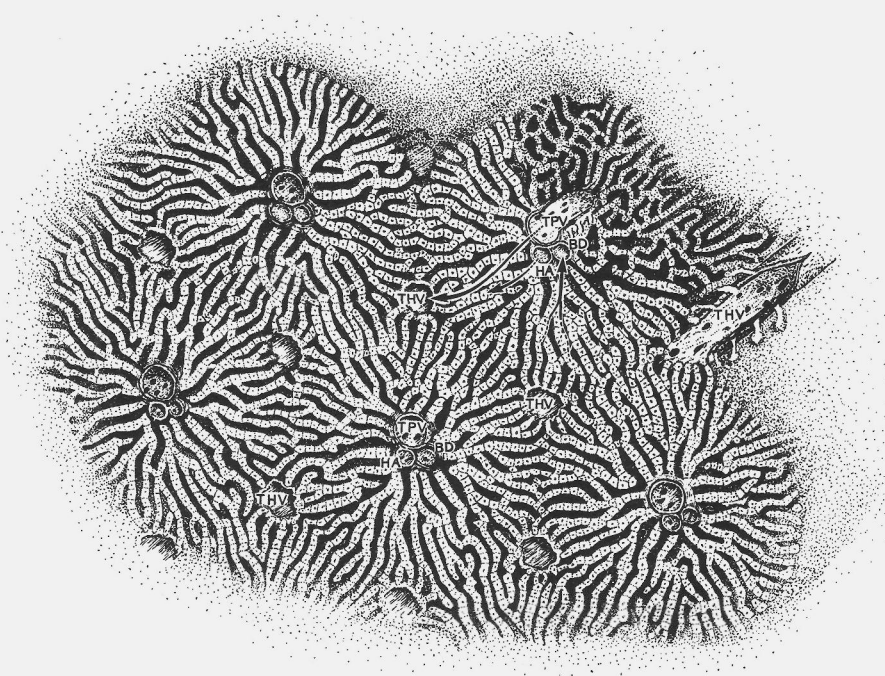


Figure 1.2 Diagram of a hepatic acinus. Nutrient-rich blood carried from the intestine by the hepatic portal vein (TPV: terminal branches of portal vein) and oxygen-rich blood from the hepatic artery (HA) are mixed in the sinusoids. After passage through the acinus, the blood is carried out of the liver via the hepatic veins (THV: terminal branches of hepatic vein). Bile produced in hepatocytes is excreted into bile canaliculi. The direction of transport of the bile is opposite to that of the blood. Transport of bile out of the liver to the intestine occurs via bile ductules (BD) and ducts.

ting of sinusoidal endothelial cells, Kupffer cells and fat-storing cells, account for only 6% of total rat liver volume, but contribute considerably to the total number of liver cells (30-35%)(Fabrikant, 1968; Greengard et al., 1972). Extracellular spaces consisting of the blood space, the biliary space and the space of Disse or perisinusoidal space which designates the lumen between sinusoidal endothelial cells and hepatocytes, form a large part of the remaining volume (15% of total rat liver volume). A minor proportion of total liver volume is occupied by epithelial cells lining bile ductules and ducts, connective tissue cells, smooth muscle cells and cells lining the lymphatic and the larger blood vessels, all of these found mainly outside the liver acinus in the portal canals.

The various cell types of liver acini are found at specific locations (Fig.1.3). An integral lining of sinusoidal endothelial cells separates the blood contained in the sinusoids from the parenchymal cells. The space of Disse between these cells and the hepatocytes contains numerous microvillous projections of the hepatocytes. Kupffer cells are located on or even form an integrated part of the endothelial lining and often bulge into the lumen of the sinusoids. Fat-storing cells are located under the endothelial lining, often in recesses between two hepatocytes. The cells have long cytoplasmic processes which run in the space of Disse parallel to the endothelial lining.

The maintenance of the structural organization and function of the liver is also determined by extracellular components (Rojkind and Ponce-Noyola, 1982). In general, the extracellular matrix consists of fibrous components, including collagens and noncollagenous glycoproteins, embedded in a homogeneous matrix of glycosaminoglycans and proteoglycans (Rojkind, 1982). The distribution pattern of the various extracellular matrix components within the liver varies considerably. In the liver capsule and in the stroma around the branches of the portal and central veins, extracellular matrix components reveal a densely packed arrangement (Grimaud et al., 1980; Martinez-Hernandez, 1984). In the liver acini, however, the various components form a loosely arranged network found exclusively in the space of Disse (Grimaud et al., 1980; Hahn et al., 1980). The cellular origin of the components present in liver acini under both normal and pathological conditions (as in various types of liver fibrosis) is a matter of debate, but most likely involves both parenchymal and sinusoidal liver cells (Martinez-Hernandez, 1984).

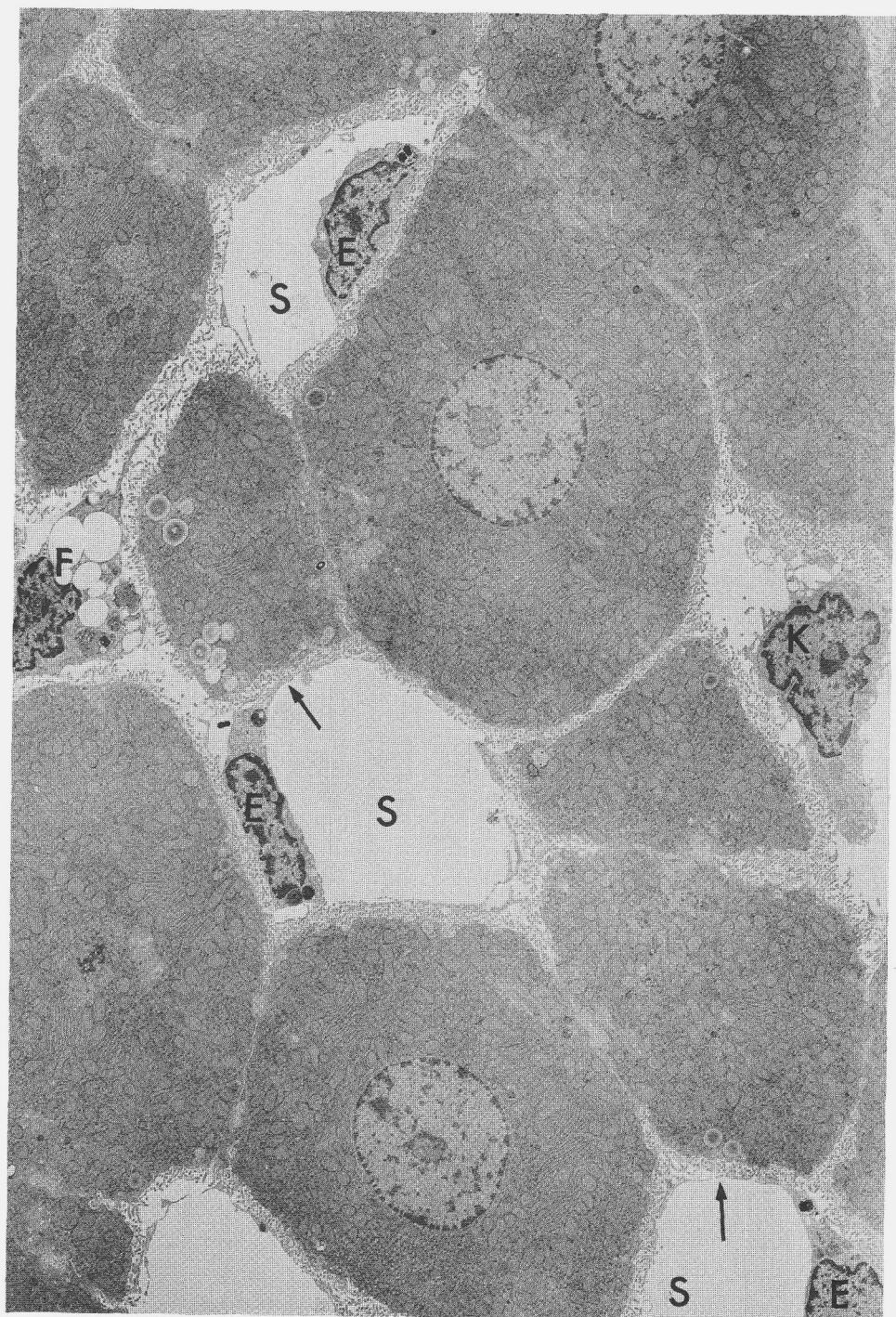
1.2. SINUSOIDAL LIVER CELLS

1.2.1 General introduction

The group of sinusoidal liver cells is formed by those three cell populations present in the liver acini besides parenchymal cells, i.e., sinusoidal endothelial cells, Kupffer cells and fat-storing cells.

These three cell populations are often considered as one group, the sinusoidal liver cells, although they are three distinct types of cells. In fetal liver, at an early stage of development, these three types of cells behave independently in all aspects and are self-proliferating (Naito and Wisse, 1977; Pino and Bankston, 1979; Bankston and Pino, 1980; Tatsumi and Fujita, 1983; Bartók et al., 1983). In adult liver, these three cell types also differ under normal conditions and in their

Figure 1.3 Transmission electron micrograph of part of a rat liver acinus (3-month-old rat). Liver acini are comprised of four cell types: the parenchymal cells (p), the sinusoidal endothelial cells (e), the Kupffer cells (k) and the fat-storing cells (f). Sinusoids (s); endothelial lining (†). 2950 x.



response to various types of stimulation. The sinusoidal endothelial cells of the liver are distributed homogeneously over the liver acini and, under normal conditions, form a cell population with a low proliferation rate. After certain stimuli such as partial hepatectomy or estrogen administration, sinusoidal endothelial cells exhibit self-proliferation (Widmann and Fahimi, 1975, 1976). Kupffer cells have a preferential localization around the branches of the portal vein (Wisse, 1977), a distribution pattern which is correlated with a morphological and functional heterogeneity of Kupffer cells (Sleyster and Knook, 1982). Upon stimulation such as partial hepatectomy, repopulation of the Kupffer cell compartment is achieved partly by self-proliferation and partly by bone marrow derived precursor cells (Van Furth, 1982; Bouwens et al., 1984). Kupffer cells have the capacity to migrate within the liver. Glucan-induced granulomas, for example, consist largely of Kupffer cells (Delmann and Fahimi, 1980). In rat liver, the distribution of fat-storing cells within the acini appears to be homogeneous (Hosemann et al., 1979). Determination of the distribution of vitamin A within acini by making use of the gold-chloride staining or the autofluorescence properties of vitamin A, demonstrates under normal conditions more positive cells in periportal than in perivenous areas (Wake, 1980). These data suggest that not the distribution of fat-storing cells within the liver acini is heterogeneous but only that the storage of vitamin A occurs preferentially in periportal fat-storing cells (Wake, 1980). Self-proliferation of fat-storing cells is observed after partial hepatectomy and in early stages during the development of liver fibrosis (Kent et al., 1976).

Although the group of sinusoidal liver cells comprises three different cell types, it is also obvious that these share several capacities which are important for the achievement of hepatic function. Endothelial and Kupffer cells cooperate intensively in the clearance function of the liver, revealing certain common cellular mechanisms such as an extensive system for uptake and degradation (Blouin et al., 1977). Also with regard to the turnover of the various components of the extracellular matrix present in the space of Disse, cooperation among the three sinusoidal liver cell types and the parenchymal cells seems to be a necessity. Both direct and indirect evidence indicates that parenchymal cells, fat-storing cells and probably also sinusoidal endothelial cells synthesize components of the extracellular matrix (Clement et al., 1984). Collagenases, a group of enzymes which participate in the catabolism of collagens, are synthesized by Kupffer cells (Fujiwara et al., 1973). Thus, the maintenance of the extracellular matrix in the liver acini apparently depends on a cooperation involving all four cell types.

Although sinusoidal liver cells form a major part of all nonparenchymal liver cells (Greengard et al., 1972; Blouin et al., 1977), the use of these two terms as synonyms should be avoided. Besides sinusoidal liver cells, nonparenchymal liver cells include epithelial cells lining bile ductules and ducts, cells lining lymphatic and true blood vessels, connective tissue cells and smooth muscle cells. Fractions of cells obtained by digestion of the liver with pronase, an enzyme preparation

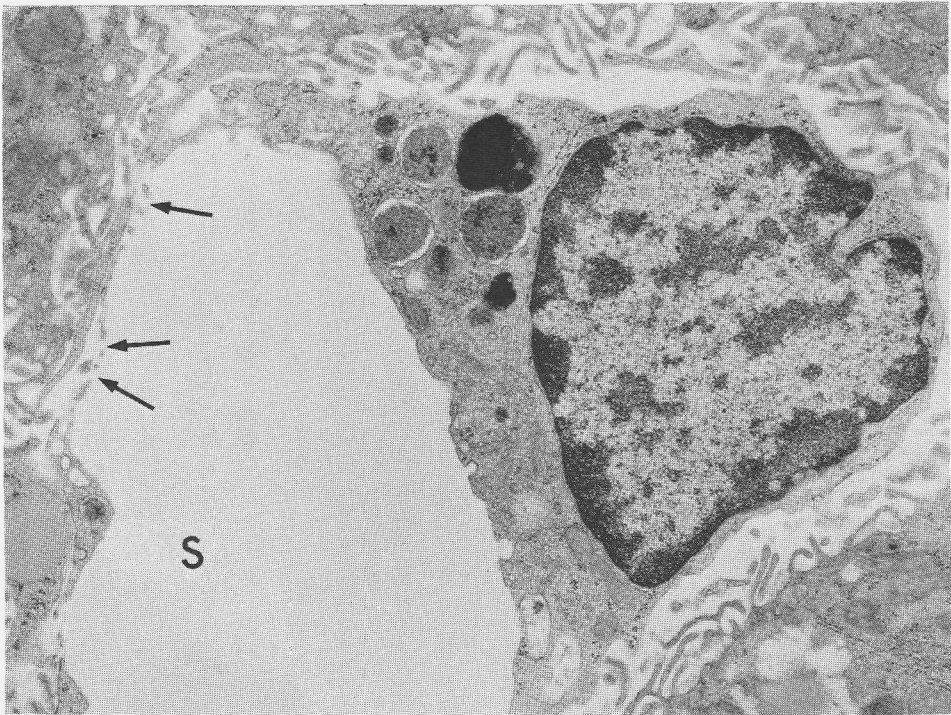


Figure 1.4 Sinusoidal endothelial cell (3-month-old rat). The endothelial cells form the continuous lining of the liver sinusoids (s). Passage of substances of up to $0.1\ \mu\text{m}$ from the sinusoids to the parenchymal cells and vice versa can occur through open fenestrations in the lining ($\dagger$). 11450 x.

which specifically destroys liver parenchymal cells, may contain in addition to non-parenchymal liver cells blood cells which were not flushed out from the liver sinusoids during perfusion. However, these fractions will be designated also as nonparenchymal liver cell fractions throughout this thesis.

1.2.2 Sinusoidal endothelial cells

Sinusoidal endothelial cells like endothelial cells elsewhere in the body form the continuous lining of the blood capillaries (Fig.1.4). The liver endothelial cells, however, differ from endothelial cells lining small capillaries in that the former lack a distinct basement membrane and that they contain open pores (or fenestrae) in the thin cytoplasmic projections which constitute the wall of the sinusoids. These fenestrae are grouped in so-called sieve plates and allow passage only of substances having a diameter of up to $0.1\ \mu\text{m}$ from the sinusoidal blood to the parenchymal cells and vice versa. Sinusoidal endothelial cells thereby have an important function as selective sieves (Fahimi, 1982).

There are various indications that sinusoidal endothelial cells also play an important role in the clearance function of the liver. In a morphometric study in the rat, Blouin et al. (1977) showed that sinusoidal endothelial cells contain relatively high proportions of the total liver volume of cell organelles which are involved in uptake, cellular transport and degradation processes, such as pinocytic vesicles, tubular systems and various lysosomal structures. Biochemical data obtained with purified liver cell suspensions show that the level of several lysosomal enzymes in endothelial cells of the rat liver is comparable to those in Kupffer cells and much higher than in parenchymal cells. (Knook et al., 1977; Knook and Sleyster, 1980). In addition, clearance studies performed both in vivo and in vitro suggest an important contribution of sinusoidal endothelial cells to the removal of various lipoproteins (Van Berkel, 1982), glycoproteins and inert colloids (Praaning-van Dalen et al., 1981, 1982) from the blood. These findings suggest that selective uptake especially of macromolecules, and degradation by lysosomal enzymes are important functions of liver endothelial cells.

1.2.3 Kupffer cells

Kupffer cells represent the largest population of fixed mononuclear phagocytes in the mammalian body and they form the major part of the reticuloendothelial system (RES). Kupffer cells are located on or even form part of the endothelial lining of the liver sinusoids (Fig.1.5). The localization of Kupffer cells along the sinusoidal blood stream reflects their most important function: the clearance of substances from the blood. Kupffer cells have been shown to be involved in the uptake and degradation of a variety of substances, including bacteria, endotoxins, viruses, tumor cells, cellular debris, clinical RES test substances and various specific macromolecules (Jones and Summerfield, 1982). Their function in uptake and degradation is reflected morphologically by the prominent presence of cellular constituents involved in these processes such as pseudopodia, endocytic vesicles and lysosomes. Biochemical data obtained with purified liver cell suspensions show high levels of various lysosomal enzymes in Kupffer cells (Knook and Sleyster, 1980). This indicates an important contribution for these cells in the degradation of lipoproteins and glycoproteins.

Kupffer cells as a part of the immune system of the organism were not considered until recently to participate in the generation of immune responses. However, studies with isolated Kupffer cells from mice and guinea pigs have shown that 15% and 50% of the cells, respectively, express Ia determinants on their cell membrane, which is a prerequisite for accessory cells presenting antigen in an antigen-specific T-cell proliferative response (Rogoff and Lipsky, 1981). It is still largely unknown, however, whether also Kupffer cells or only other types of macrophages are involved in the generation of in vivo immune responses. This probably depends, e.g., on the nature of the antigen, the type of immune response and the age of the host (Brouwer and Knook, 1983).

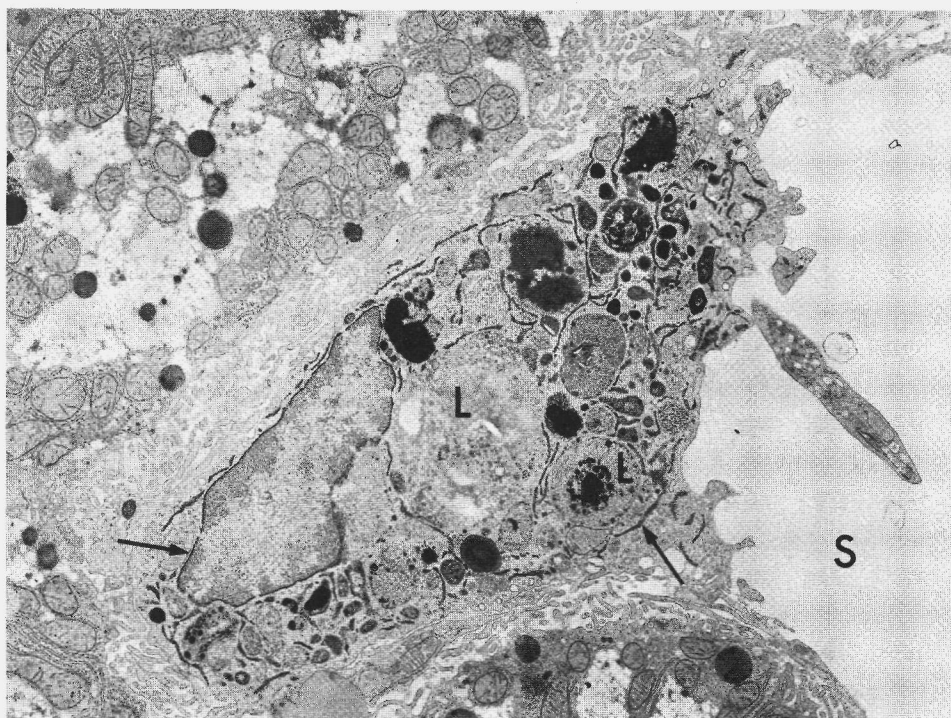


Figure 1.5 Kupffer cell (12-month-old rat).
The localization of Kupffer cells along the sinusoidal (s) blood stream and the prominent presence of lysosomes (l) in the cytoplasm reflect their most important function: the clearance of substances from the blood. Endogenous peroxidatic activity, a marker activity for rat Kupffer cells, is present in the cisternae of the rough endoplasmic reticulum and in the nuclear envelope (†). 7400 x.

An other function of Kupffer cells concerns the synthesis and excretion of a wide variety of monokines, i.e., active compounds in general secreted by cells of the mononuclear phagocytic system. These monokines include cellular modulators, such as prostaglandins, endogenous pyrogen, colony stimulating and fibroblast growth factors, immunomodulators such as interleukin 1, and enzymes such as lysosomal enzymes and collagenases (Page et al., 1978).

1.2.4 Fat-storing cells

In 1876, Kupffer described a cell population in the liver of various mammals which could be demonstrated by a gold chloride staining method. He indicated that these cells were located perisinusoidally and designated them as "Sternzellen" (stellate cells) because of their characteristic shape. In the same period, "phago-

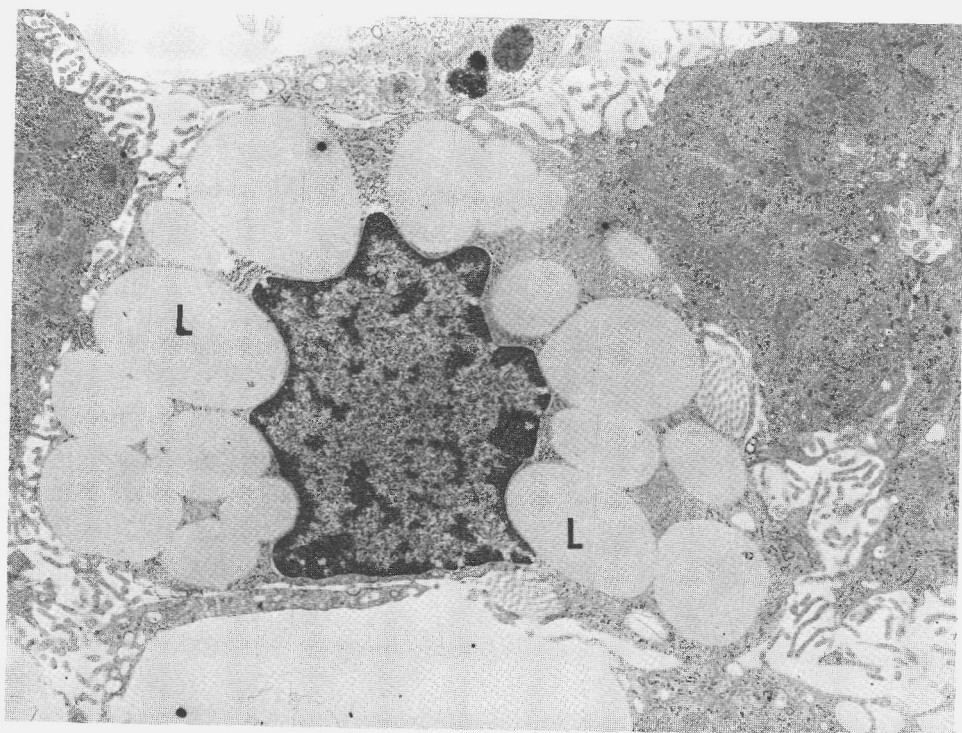


Figure 1.6 Fat-storing cell (3-month-old rat). Fat-storing cells are localized under the endothelial lining in the space of Disse. They are characterized mainly by the presence of lipid droplets (L) in their cytoplasm, in which a major part of the liver vitamin A is stored. 7700 x.

cytic" cells in the liver attracted attention and in 1898 Kupffer stated that: "the stellate cells are phagocytic in nature and are integral elements of the wall of liver sinusoids". The phagocytic cell of the liver became known under the name of "Kupffer cell". Around 1960, it was reported that vitamin A in the liver was not stored in the phagocytic Kupffer cells but in perisinusoidal cells of the liver (Nakane, 1963; Wake, 1964). Then, it became clear for the first time that the stellate cells first described by Kupffer belong to a separate cell population distinct from the endothelial cells and the phagocytic Kupffer cells. In 1971, Wake reported that the gold chloride staining in stellate cells originally described by Kupffer is based on a reduction of gold chloride by vitamin A, resulting in gold precipitation at those sites where vitamin A is present.

Since then, a number of investigators has studied the stellate cells in a variety of species by electron microscopy (for a review, see Wake, 1980)(Fig.1.6). It is clear from these studies that: (i) stellate cells form a separate cell population clearly distinguishable from endothelial and Kupffer cells on the basis of their

ultrastructural morphology and localization in the space of Disse; (ii) stellate cells are primarily characterized by the presence of lipid droplets; (iii) vitamin A is present in these lipid droplets and the number of lipid droplets per cell increases in conditions of high vitamin A administration. The characteristic presence of lipid droplets in stellate cells prompted Ito (1951) to call these cells "fat-storing cells". This name will also be used throughout this thesis because of its frequent use in the literature.

The functions now ascribed to fat-storing cells include a role in vitamin A metabolism, especially storage, and in the synthesis of extracellular matrix components of the liver acinus under normal and pathological conditions (Fahimi, 1982). The latter function is based mainly on morphological observations. Except for the presence of lipid droplets, fat-storing cells closely resemble fibroblasts and an increase in the number of fat-storing cells has been reported in experimentally induced liver fibrosis (Kent et al., 1976), a condition in which extracellular matrix components are increased.

1.3 LIVER AND AGING

The liver is widely used in morphological, biochemical, pharmacological and clinical studies to investigate its various functions, also with respect to the aging process. However, the cellular basis for the age-related decline in various hepatic functions is still poorly understood. The literature on the aging liver deals almost exclusively with functional studies at the organ level, structural studies of the parenchymal cells and biochemical changes determined in liver homogenates or comparable preparations. In such studies, it is difficult to determine whether only intrinsic cellular causes or also external factors contribute to age-related changes observed at the organ level. With respect to aging studies and external factors which may influence hepatic function, the multiple pathology which is highly characteristic for aging organisms should also be taken into account. In aging studies on specific organs such as the liver, those species or strains of experimental animals with as few pathological changes as possible that may influence hepatic functions should be chosen.

In order to be able to correlate findings at the organ level with those at the cellular level, model systems of isolated liver cells have been devised to study age-related changes in hepatic function in specific classes of liver cells. The availability of procedures for obtaining both parenchymal cells and each type of sinusoidal liver cell in isolation offers the possibility to study age-related changes in the very diverse functions of these cells independently from external influences as compared with the complex situation in whole liver (Berry and Friend, 1969; Knook and Sleyster, 1976; Knook et al., 1982b). Isolated endothelial and Kupffer cells have been extensively used to study the clearance capacity of these cells for a variety of substances (Praaning-van Dalen et al., 1981, 1982; Van Berkel, 1982) and recently isolated fat-storing cells were used to clarify their role in the storage

of vitamin A (De Leeuw et al., 1983; Hendriks et al., submitted).

Almost all liver cell isolation methods are based on perfusion of the liver with dissociating enzymes. After liver digestion with collagenase, a cell suspension is obtained which consists mainly of parenchymal with some sinusoidal cells (Van Berkel and Van Tol, 1978). Further purification of both parenchymal cells and sinusoidal cells can be achieved by various centrifugation techniques, including centrifugal elutriation.

Digestion of the liver with pronase results in selective breakdown of the parenchymal cells of the liver and nonparenchymal liver cell suspensions are obtained containing all three types of sinusoidal liver cells in higher yields than with the collagenase method (Sleyster et al., 1977; Knook et al., 1982b). Further purification of the three types of sinusoidal liver cells can be achieved by selective adherence during overnight culture for purification of Kupffer cells or by density centrifugation and centrifugal elutriation for purification of all three types of sinusoidal liver cells. Other isolation methods have been described, but digestion by collagenase or pronase are the most widely used.

The choice for a specific isolation method is greatly dependent on the type of experiment. Important criteria include cell yield, cell viability and intactness of the cell membrane, especially of the receptors. The advantages and disadvantages of various isolation methods have been recently discussed (Brouwer et al., 1982b).

In the following part, a brief overview will be given only of those results obtained in these model systems which can be correlated with hepatic function at the organ level with age. More details can be found in a series of recent reviews (Knook, 1982a; Zurcher et al., 1982; Brouwer and Knook, 1983; Van Bezooijen, 1984).

A pronounced age-related change in liver is the shift in hepatocytes to higher ploidy levels. Because of a concomitant increase in nuclear and cell volumes, this shift results in a decrease in the number of hepatocytes per unit of liver volume with age (Van Bezooijen, 1984). Whether this phenomenon at the organ level leads to a decrease in the total number of hepatocytes - an often quoted cause of functional decline with age - or is fully compensated for by an observed increase in liver volume cannot yet be decided.

In hepatocytes a marked increase occurs with age in the volume of secondary lysosomes, which is partly due to the accumulation of lipofuscin, the so-called aging pigment. Lysosomal enzyme activities in isolated parenchymal cells show changes in localization patterns as well as a decline in most enzyme activities with age. These observations may indicate an impairment in the autophagic degradation process in old parenchymal cells (Knook, 1982a).

Morphological changes in liver mitochondria with age indicate a possible decline in the energy supply of parenchymal cells. However, studies on age-related changes in respiration and energy supply using isolated parenchymal cells strongly suggest that cells isolated from old rats have an unaltered energy supply, which is

in agreement with the absence of a significant decrease in enzyme activities of liver mitochondria with age (Knook, 1982a).

One important aspect of hepatic function is the transfer of organic anions from the plasma via the parenchymal cells into the bile, a function which can be determined in vivo by the clinical bromsulphophthalein (BSP) retention test. An age-related decrease in the capacity of the liver for the removal of serum BSP is found in both humans and rats. Studies using isolated rat hepatocytes to determine which step in the process is altered with age reveal a sharp decrease in the storage capacity of hepatocytes for BSP at between 3 and 12 months of age and a less pronounced decline thereafter. Comparison of in vivo with in vitro data leads to the conclusion that a decrease in both storage capacity and maximal excretion rate of hepatocytes may contribute to age-related changes in blood removal rates of BSP and other organic anions (Van Bezooijen, 1984).

The capacity of the liver to metabolize drugs has generally been shown to decrease with advancing age both as determined in vivo and with isolated parenchymal cells. Where the use of isolated cells has distinct advantages by excluding all external influences, it should always be kept in mind that, especially with regard to drug metabolism, changes, for example with age, found in such experimental models may be strongly species and sex dependent (Van Bezooijen, 1984).

Another aspect of hepatic function is the synthesis of specific proteins such as albumin, a transport protein in serum. Studies on age-related changes in protein synthesis in isolated rat hepatocytes reveal that most changes appear to be strain and sex dependent, with the exception of the sharp increase in protein synthesis in hepatocytes isolated from old rats. This increase in protein synthesis, most clearly evident for albumin, is thought to compensate for an increased elimination of serum albumin, thereby keeping the serum concentration of albumin constant with age (Van Bezooijen, 1984).

Our knowledge on changes with age in characteristics and functional capacities of sinusoidal liver cells is very limited. Brouwer and Knook (1983) recently reviewed the literature on the reticuloendothelial system (RES) and aging. This review reveals that there are only few studies on those functional aspects of endothelial and Kupffer cells which determine them as cells of the RES. Studies on the uptake capacity of the liver for heat-denatured albumin revealed a progressive decrease with age for rat liver Kupffer cells, and no change for endothelial cells (Brouwer et al., 1985). Other functions attributed to sinusoidal liver cells have not at present been evaluated for their response to aging.

In this thesis, some aspects of sinusoidal cells of the rat liver will be described in relation to aging, with emphasis on the ultrastructural morphology of these cells in the intact liver, after cell isolation and in culture. The age-related ultrastructural morphology of sinusoidal liver cells is studied to detect a possible cellular substrate for age-related changes in sinusoidal cell functions. The results obtained on the number of sinusoidal cells isolated per rat liver are used to

calculate theoretically changes in the cellular composition of rat liver with age.

1.4 OUTLINE OF THE PRESENT STUDY

The experiments described in this thesis were performed with female BN/BiRij rats kept in our Institute under "clean conventional" conditions as described by Hollander (1976). The diet consisted of a pelleted rodent diet (AM II[®], Hope Farms). The animals were allowed free access to water. All investigations were generally performed with young rats (3-month-old), adult rats (12-month-old) and old rats (36-month-old). Some investigations included other age groups in between the ones above to enable the detection of patterns in age-related changes. The age groups used are indicated in each Chapter separately.

The ultrastructural morphology of sinusoidal liver cells in the intact liver of rats of various ages is described in Chapter 2. Special attention is paid to those cell organelles which directly reflect certain functional capacities of sinusoidal liver cells. The localization and staining characteristics of endogenous peroxidatic activity, which is used in young-adult rat liver as a specific marker for Kupffer cells, was studied in relation to aging using an ultrastructural enzyme cytochemical assay. Also the relative numbers of each type of sinusoidal liver cell were determined at various ages to ascertain possible variations in the composition of the sinusoidal cell population with age.

Chapter 3 describes general characteristics of various suspensions of sinusoidal liver cells obtained from rats of various ages isolated by digestion of the liver with pronase and collagenase. Attention is paid to cell yield and cellular composition as determined by both light and electron microscopic analyses in relation to the age of the donor rat. The ultrastructural morphology of isolated cells is compared with that of cells in the intact liver to evaluate the comparability of cells in vivo and in vitro.

In Chapters 4 to 7, various culture systems for sinusoidal liver cells are described in relation to the age of the donor animal. Chapter 4 characterizes cultures of nonparenchymal liver cells with regard to cellular composition in relation to culture time and to the effects of mutual cellular interactions on culture characteristics. In addition, a brief outline of major changes in the ultrastructural morphology of sinusoidal liver cells with increasing culture time is given.

Culture conditions which allow for large scale survival of sinusoidal endothelial cells in culture are described in Chapter 5. The ultrastructural morphology of these cells is discussed in relation to culture time and age of the donor animals and compared with that of endothelial cells in the intact liver. Reappearance in culture of cell membrane-specific proteins such as receptors was investigated by testing the ability of the cells to take up horseradish peroxidase.

In Chapter 6, the ultrastructural morphology, enzyme cytochemistry and endocytic capacity of Kupffer cells in culture are described in relation to culture time and age of the donor animal.

Chapter 7, includes descriptions of investigations on fat-storing cells with regard to changes in morphology during culture and to cell-specific vitamin A fluorescence. Cells in culture were further characterized by specific patterns of various cytoskeletal proteins as tested by indirect immunofluorescence. The culture system of fat-storing cells was also used to investigate the role of these cells in the synthesis of components of the extracellular matrix.

In the general discussion of Chapter 8, attention is paid to a possible correlation between age-related changes in the ultrastructural morphology of sinusoidal liver cells and cell function. Literature data on liver cell ratios and data obtained in the present study on the number of sinusoidal liver cells isolated per liver are compared to ascertain the validity of the use of such cell ratios in liver research. Characteristics of sinusoidal cells in the intact liver, after isolation and during culture are compared to evaluate the validity of in vitro studies for the investigation of cell-specific functions also in relation to aging.

CHAPTER 2

THE ULTRASTRUCTURE OF SINUSOIDAL LIVER CELLS IN THE INTACT RAT LIVER IN RELATION TO AGE

2.1 INTRODUCTION

From a series of papers dedicated to the ultrastructural aspects of cells present in rat liver acini, it was concluded that two different types of cells, namely endothelial and Kupffer cells, directly line the liver sinusoids (Wisse, 1970, 1972, 1974a,b; Fahimi, 1970; Widmann et al., 1972). These two cell types could be clearly discriminated on the basis of morphological characteristics and did not under any circumstances transform into each other. Endothelial cells were characterized by long cytoplasmic extensions forming the covering of the liver sinusoids and containing pores clustered in sieve plates. The endothelial cell body showed a high nuclear-cytoplasmic ratio and a smooth cell membrane (Wisse, 1970, 1972). Kupffer cells, on the other hand, revealed a low nuclear-cytoplasmic ratio, a ruffled cell membrane caused by the presence of lamellipodia and a large number of heterogeneous lysosomes in their cytoplasm (Fahimi, 1970; Widmann et al., 1972; Wisse, 1974a).

Descriptions of the ultrastructural morphology of Kupffer and endothelial cells in other species largely confirmed the observations in the rat (cat: Tanuma et al., 1981; rabbit: Tamaru and Fujita, 1978; teleost: Ferri and Sesso, 1981; lizard, snake and turtle: Taira and Mutoh, 1981; flat fish: Tanuma et al., 1982; human: Gendrault et al., 1982; monkey: Tanuma et al., 1983). In teleost liver, exceptions included the presence of true desmosomes not only between endothelial cells but also between endothelial cells and fat-storing cells and between endothelial cells and parenchymal cells; a low frequency or absence of Kupffer cells was also observed in the same species (Fujita et al., 1980; Tanuma et al., 1982).

Endogenous peroxidatic activity present in the cisternae of the rough endoplasmic reticulum and in the nuclear envelope of Kupffer cells was found to be an exclusive marker for this cell type in rat liver (Fahimi, 1970; Widmann et al., 1972; Wisse, 1974a). However, studies in mice have shown that in this species more than 50% of the endothelial cells also contain such activity (Stöhr et al., 1978; De Leeuw et al., 1982).

Fat-storing cells can be clearly discriminated from endothelial and Kupffer cells by their localization in the space of Disse and characteristic content of lipid droplets (Bartók et al., 1979; Wake, 1980).

The two main functions attributed to fat-storing cells are reflected in the large number of names proposed for this cell type. On the one hand, these stress the

supposed capacity of fat-storing cells to store lipid and lipid-soluble compounds such as vitamin A (fat-storing cells, lipocytes, lipid-storing cells, vitamin A-storing cells). On the other hand, these indicate a functional relationship of fat-storing cells with fibroblastic cells present in connective tissue surrounding larger blood vessels and blood capillaries (pericytes, interstitial cells, perisinusoidal cells, mesenchymal cells, adventitious connective tissue cells).

Investigations on the age-dependent ultrastructure of liver cells have been restricted to hepatocytes. These studies included morphometric analyses of parenchymal cell and nuclear volumes and cell organelle composition in relation to age (see for reviews, Knook, 1982; Van Bezooijen, 1984). In addition, age-related parenchymal enzyme patterns were studied in rat and mouse liver (Wilson et al., 1982; Van Manen et al., 1983).

The observations described in this chapter provide data on the ultrastructural morphology of sinusoidal liver cells in the intact rat liver with age. Endogenous peroxidatic activity in cells of the liver of rats of various ages was studied by means of an enzyme cytochemical assay. Also, the relative numbers of each type of sinusoidal liver cell were determined at various age levels in a search for possible variations in the composition of the sinusoidal cell population with age.

2.2 MATERIALS AND METHODS

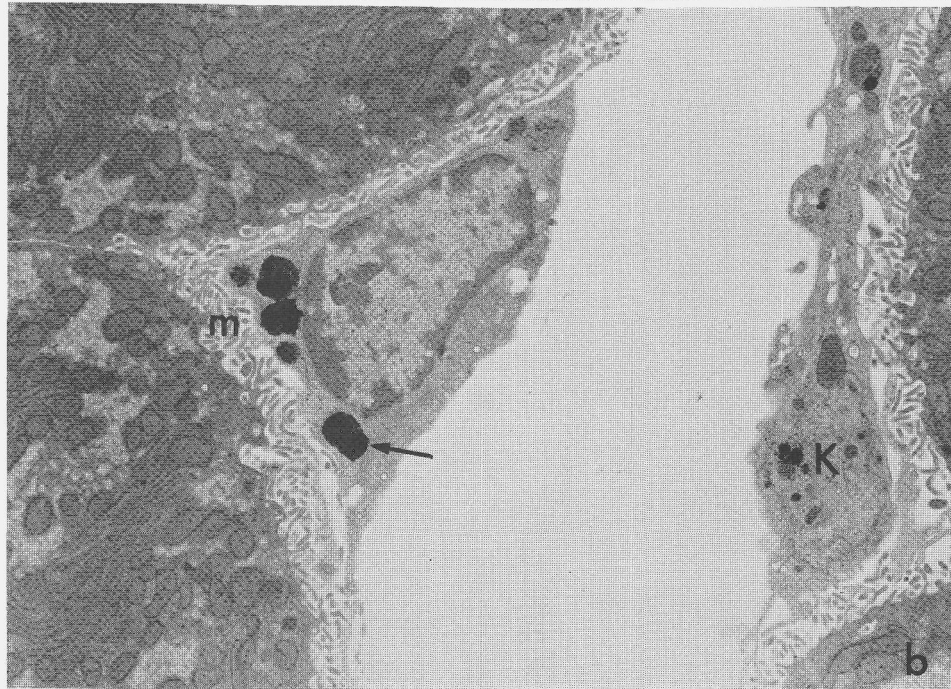
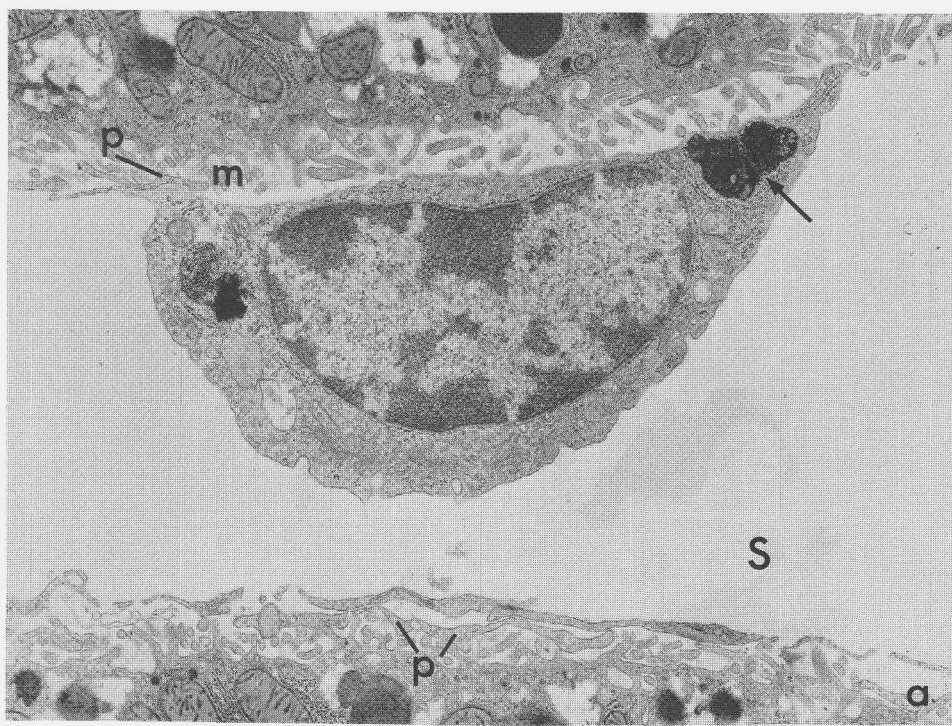
2.2.1 Perfusion-fixation of the liver and preparation for transmission electron microscopy

Experiments were performed with 3-, 6-, 12-, 24-, and 30- to 36-month-old rats. Perfusion-fixation of the liver was done in essence as previously described by Wisse (1970). Under aether anaesthesia, the abdomen was opened and the portal vein cannulated with a Braunüle 0.5-G-18 canula. The liver was first perfused with buffered saline for 2 min at 37°C, followed by 2% glutaraldehyde in 0.15 M sodium cacodylate buffer (pH 7.4) for 6 min at 4°C. Postperfusion was done with 0.05 M Tris-HCl buffer containing 7% sucrose for 6 min at room temperature. In all steps the flow rate was kept at 5 ml.min⁻¹ by use of a peristaltic pump (LKB, type 2115).

After dissection of the liver, 1 mm³ blocks of tissue were cut from the median

Figure 2.1 Transmission electron micrographs of sinusoidal endothelial cells from a 12- (a) and a 6-month-old rat (b).

- a. The cell body of the endothelial cell is bulging into the lumen of the sinusoid (s). Under the endothelial lining, parts of the meshwork of cytoplasmic processes (p) extending from fat-storing cells are present. 11850 x.
- b. The cell body of the endothelial cell is hidden in a recess between two hepatocytes. Part of a Kupffer cell (k). 6500 x. Lysosomes filled with ferritin particles (†); Microvilli of parenchymal cells (m).



lobe (Bivin et al., 1979). These were postfixed in 1% OsO_4 in 0.15 M sodium cacodylate buffer for 45 min, dehydrated in a graded series of ethanol and embedded in LX 112 (Ladd Research Industries). Ultrathin sections were cut with a Reichert OmU2 ultramicrotome and contrasted with uranyl acetate and lead citrate. Sections were examined in a Philips EM 300 and EM 410 HM.

2.2.2 Endogenous peroxidatic activity

After the perfusion procedure, 75 μm thick slices were cut from pieces of 5x5x1 mm of the median lobe by using a vibratome (Oxford, type G). These were incubated for the demonstration of endogenous peroxidatic activity following the procedure described by Wisse (1974a). Vibratome slices were washed three times in 0.05 M Tris-HCl buffer containing 7% sucrose. The slices were then incubated for 60 min at 27°C in a medium containing 15 mg 3,3-diaminobenzidine tetrachloride (Merck) in 0.05 M Tris-HCl buffer containing 7% sucrose and 0.02% H_2O_2 . The pH was adjusted to 7.4 with 1 N NaOH. Controls were incubated without H_2O_2 .

After incubation, the slices were washed three times in the Tris-HCl buffer and further processed for transmission electron microscopy as described above.

2.2.3 Relative numbers of sinusoidal liver cells

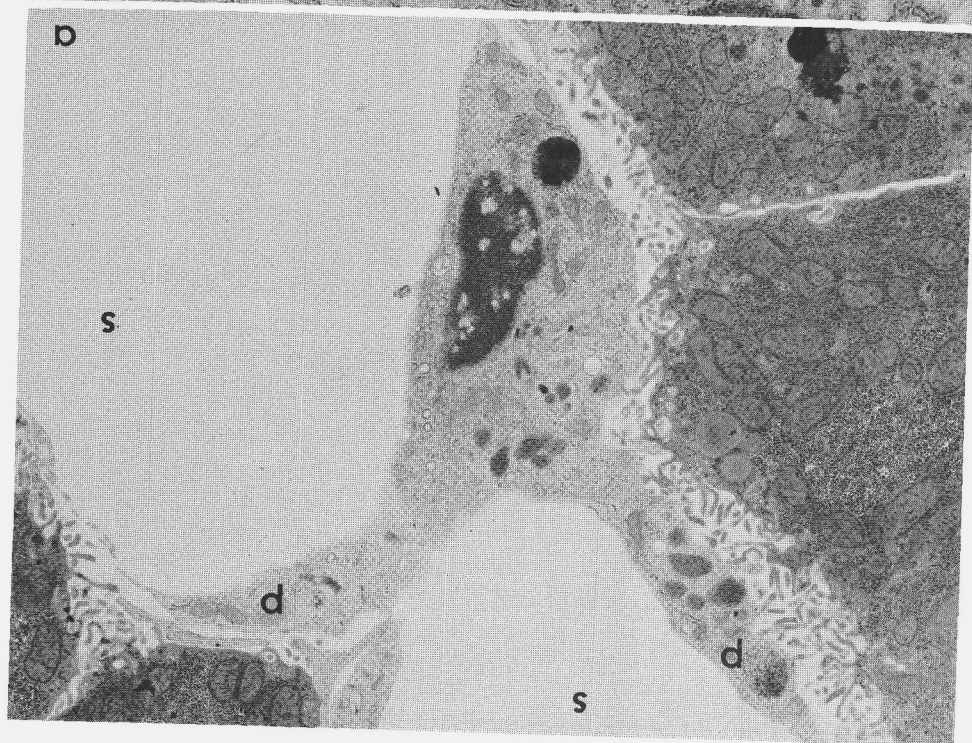
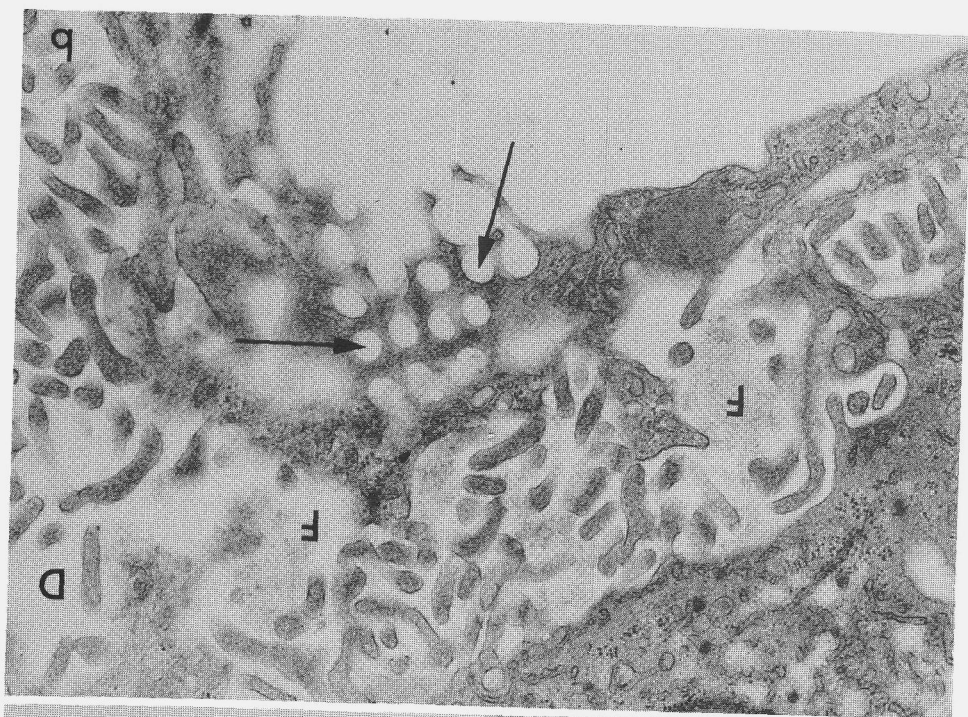
In ultrathin sections, the sinusoidal liver cells with cross-sectioned nuclei were characterized as endothelial, Kupffer or fat-storing cells on the basis of morphological criteria. The relative numbers of endothelial, Kupffer and fat-storing cells were expressed as a percentage of the total number of sinusoidal liver cells. Per animal, 150-200 sinusoidal liver cells were determined.

2.2.4 Demonstration of iron by X-ray microanalysis

The specimens were analyzed with a Jeol 200C electron microscope equipped with a SEM and STEM unit and a energy dispersive X-ray spectrometer (Link systems). The 200 to 400 nm thick sections were screened at 200 kV in order to localize the lysosomal structures in endothelial and Kupffer cells to be analyzed. Analysis was done at 100 kV to minimize background radiation. To avoid copper peaks in the spectrum, sections were spread over 1 mm one-hole grids covered with a carbon-coated formvar film.

Figure 2.2 Transmission electron micrographs of sinusoidal endothelial cells from a 6- (a) and a 24-month-old rat (b).

- a. Slender cytoplasmic processes (p) forming the lining of two sinusoids (s) extend from either side of the endothelial cell body. Due to cross-sectioning of the nucleus at the edge, the chromatin pattern appears differently. 7050 x.
- b. Clusters of open pores, the sieve plates (†), allow free passage of substances from the sinusoidal blood to the parenchymal cells and vice versa. In the space of Disse (d), some fine fibrils (f) are present. 26100 x.



2.3 RESULTS

2.3.1 Ultrastructure of sinusoidal endothelial cells in relation to age

Two types of endothelial cells which varied in cell shape were regularly observed. The first type was situated along the sinusoids, showing a cell body (or perikaryon) slightly bulging into the sinusoidal lumen or hidden in recesses between two parenchymal cells (cf. Figs.2.1a,b). The second type revealed a cell body apparently bridging the sinusoid with cytoplasmic projections extending from either side of the cell body, forming the lining of two sinusoids (Fig.2.2a). This type was thought to be located at sinusoidal ramifications.

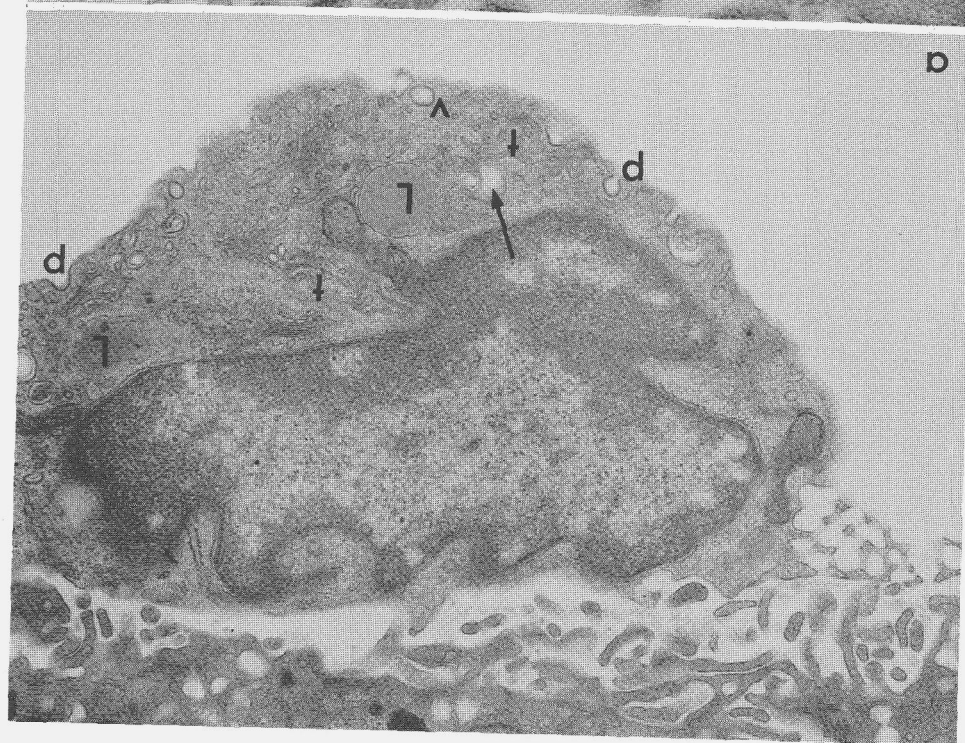
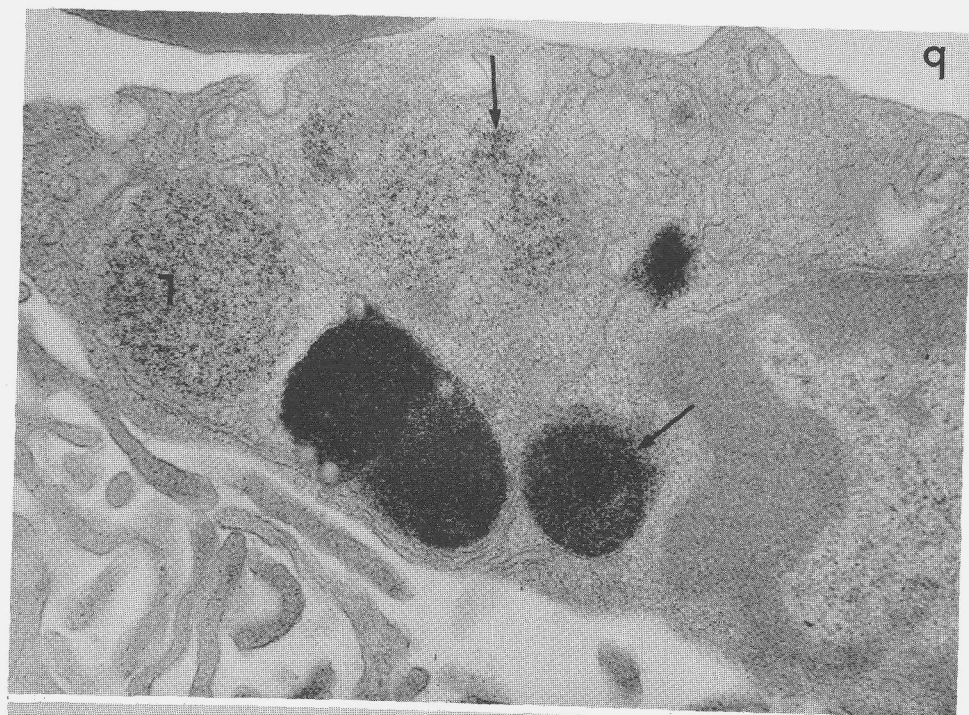
Endothelial cells were always separated from hepatocytes by the space of Disse. Numerous microvilli of the hepatocytes projected into this space (Figs.2.1a, b and 2.2a). Parts of the meshwork of cytoplasmic processes extending from fat-storing cells were regularly observed directly beneath the endothelial cells in the space of Disse (Fig.2.1a). No distinct basement membrane was observed at the base of the endothelial cells. However, cross-striated collagen type I and III fibers and finely fibrillar material (presumably collagen type IV or VI or laminin) were found in the space of Disse, sometimes in close association with endothelial cells (Fig.2.2b). Endothelial cell bodies were characterized by being in continuity with long cytoplasmic processes, which formed the covering of the sinusoids (Fig.2.2a). These processes contained clusters of open pores, the sieve plates, at irregular intervals (Fig.2.2b).

The nuclear-cytoplasmic ratio of the endothelial cell body was relatively high as compared with Kupffer cells. The regular nuclei were round to ovoid in shape, showing a coarse chromatin pattern with a broad rim of heterochromatin along the nuclear membrane and patches distributed throughout the nucleus (Fig.2.1b).

The perikaryon of endothelial cells contained a large number of different cell organelles such as mitochondria, a few cisternae of the rough endoplasmic reticulum and a well-developed Golgi system. In addition, a complex of vacuoles existed, which was indicated to be the morphological representation of different stages in the uptake, transport and degradation system of these endothelial cells (De Bruyn et al., 1977, 1983a,b). This complex included membrane invaginations, also called bristle-coated pits, bristle-coated micropinocytic vesicles ($\emptyset$ 100-150 nm), smooth-walled electron-lucent vesicles of various shapes ($\emptyset$ 200 nm), an ex-

Figure 2.3 Transmission electron micrographs of endothelial cells from 12-month-old rats.

- a. A complex of vacuoles including bristle-coated pits (p) and vesicles (v), smooth-walled electron-lucent vesicles ($\uparrow$), tubules (t) and lysosomes (l) are present in the cytoplasm of endothelial cells. 18550 x.
- b. Electron dense particles ($\uparrow$) resembling ferritin are found both in lysosomes (l) and free in the cytosol. 38800 x.



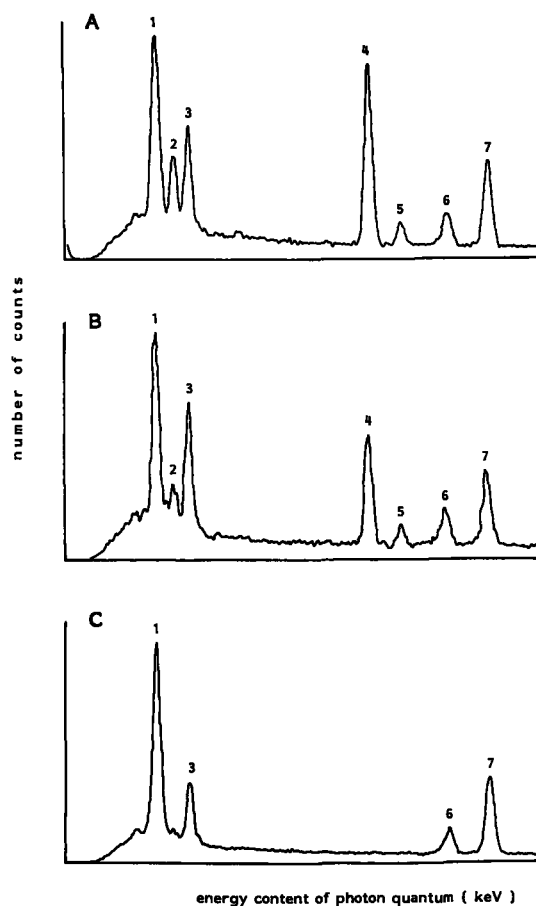
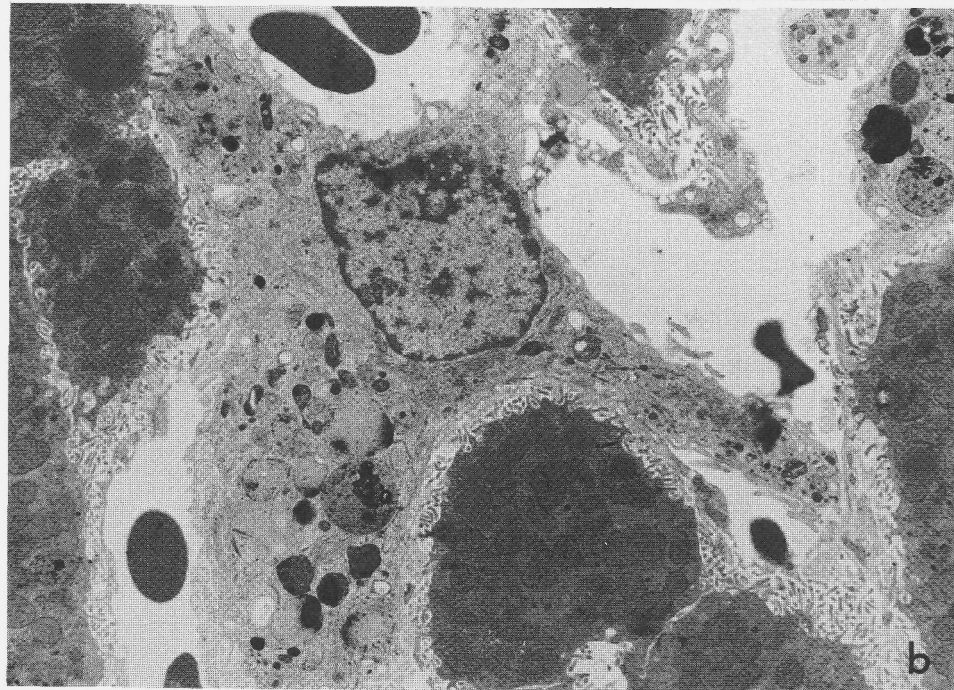
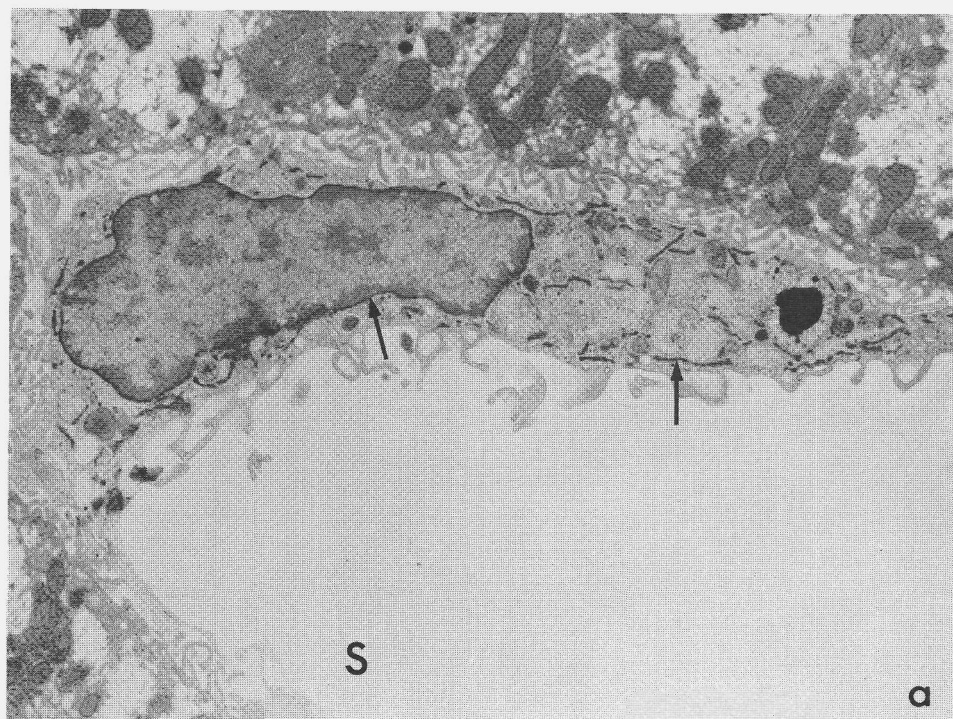


Figure 2.4 X-ray microanalysis of lysosomes in endothelial cells and Kupffer cells of a 24-month-old rat. A, endothelial cell; B, Kupffer cell; C, erythrocyte. Lysosomes of both endothelial and Kupffer cells contain large amounts of iron (peaks 4 and 5). Osmium (peaks 1, 6 and 7) is detected as a result of fixation, whereas sulfur (peak 2) and chlorine (peak 3) are part of the embedding medium. Copper (part of peak 6) from the grid as a source is detected.

Figure 2.5 Transmission electron micrographs of Kupffer cells from a 24- (a) and a 12-month-old rat (b).

- a. A Kupffer cell is partly integrated into the lining of the sinusoid (s). Endogenous peroxidatic activity is present in the cisternae of the RER and in the nuclear envelope (†). 7050 x.
- b. A large Kupffer cell projects cell parts in various directions. 4950 x.



tensive network of branching tubules ($\emptyset$ 30–40 nm) and larger smooth-walled vesicles ($\emptyset$ 700 nm) with contents of varying electron density (Fig.2.3a).

Sinusoidal endothelial cells exhibited a remarkably constant ultrastructural morphology at all age levels studied. No signs of a decrease in the intactness of the endothelial lining were observed, which indicated the preservation of their filtration function until late age.

However, with increasing animal age, a gradual increase was observed in the occurrence of small electron-dense particles, both in the large smooth-walled vesicles and free in the ground substance (cytosol) of the endothelial cells (Fig.2.3b). These closely resembled morphologically ferritin particles. X-ray microanalysis of smooth-walled vesicles in endothelial cells of a 24-month-old animal demonstrated high levels of iron to be present in these organelles (Fig.2.4).

2.3.2 Ultrastructure of Kupffer cells in relation to age

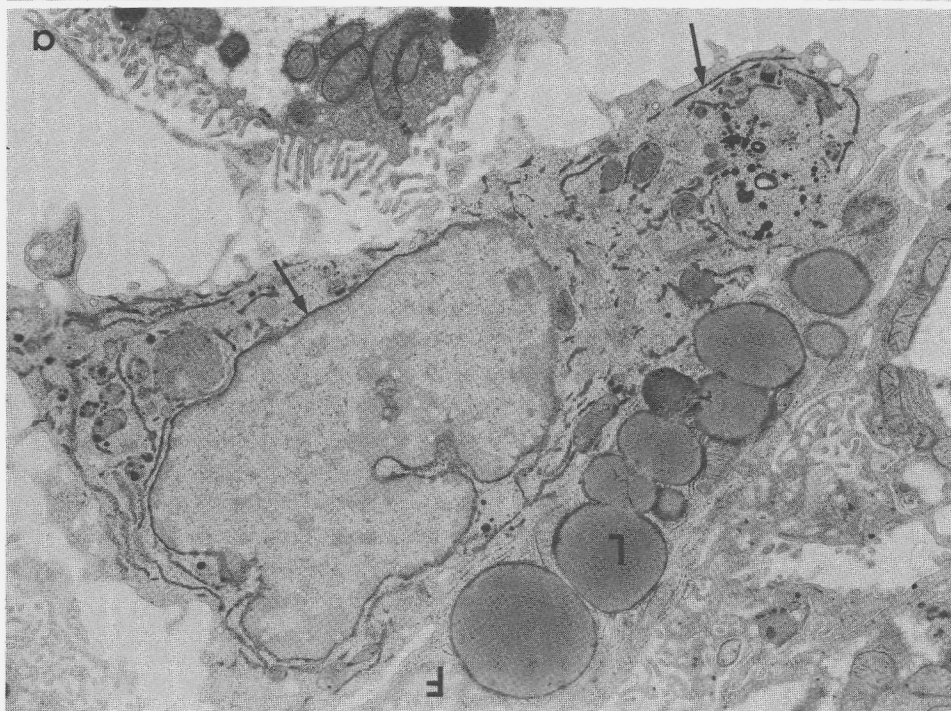
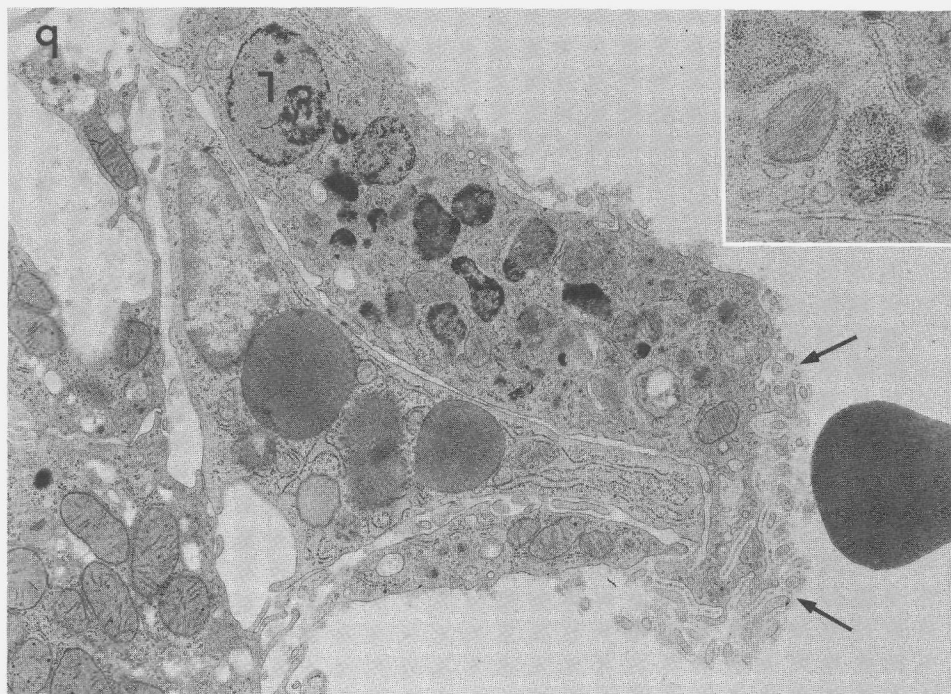
Kupffer cells were found at various places within the sinusoids. Some were partly integrated into the endothelial lining of the sinusoids and others were much more bulging into the sinusoidal lumen, projecting cell parts in several directions (cf. Figs.2.5a,b). At all age levels studied both "small" and "large" Kupffer cells were observed. Kupffer cells were frequently found in close association with fat-storing cells, the endothelial lining being absent between the two cell types (Fig.2.6a). This relationship was previously observed during high vitamin A intake (Wake, 1980), and may suggest a functional cooperation between the two cell types.

Kupffer cell nuclei generally had a more irregular appearance than those of endothelial cells due to various indentations (Fig.2.6a). The chromatin pattern had a less coarse appearance than in endothelial cells, with only a small rim of heterochromatin along the nuclear membrane and a few patches throughout the nucleus (Fig.2.5a).

The main characteristics of Kupffer cells were a highly ruffled cell membrane caused by the presence of lamellipodia, a relatively low nuclear–cytoplasmic ratio as compared with endothelial cells (Figs.2.5a,b and 2.6a) and the presence of a variable number of lysosomes ($\emptyset$ 0.2 to 2.3 μ m) filled with heterogeneous material

Figure 2.6 Transmission electron micrographs of Kupffer cells from a 6- (a) and a 35-month-old rat (b).

- a. A Kupffer cell is in close association with a fat-storing cell (f). Endogenous peroxidatic activity is present in the cisternae of the RER and in the nuclear envelope ($\uparrow$). Lipid droplets (l). 7800 x.
- b. Ferritin particles are present in lysosomes (l) and free in the cytosol (inset). The cell membrane is specialized to form worm-like structures ($\uparrow$). Vesicles of differing diameter are found throughout the cytoplasm. 9500 x; inset, 27050 x.



(Fig.2.5b). With increasing age of the animal, the lysosomes appeared more electron dense. This was partly due to an increased incidence of lipid-like material in the lysosomes and partly to the presence of ferritin-like particles (Fig.2.6b). These particles were also observed free in the ground substance of the cytoplasm (Fig.2.6b, inset). X-ray microanalysis of Kupffer cell lysosomes of a 24-month-old animal confirmed the presence of iron in these organelles (Fig.2.4).

Besides these prominent lysosomal structures, Kupffer cells contained a well-developed Golgi system, strands of rough endoplasmic reticulum, mitochondria and a number of coated and uncoated vesicles thought to be involved in the transport of endocytosed material (Wisse, 1974b)(see also Fig.2.6b). Worm-like structures described by Wisse (1974a) were only rarely observed in this rat strain, in all age groups (Fig.2.6b).

Endogenous peroxidatic activity was shown to be present in all Kupffer cells in the cisternae of the rough endoplasmic reticulum and in the nuclear envelope (Figs.2.5a and 2.6a). No differences were observed in the localization or in the staining intensity of the reaction product in Kupffer cells of animals of the various age groups.

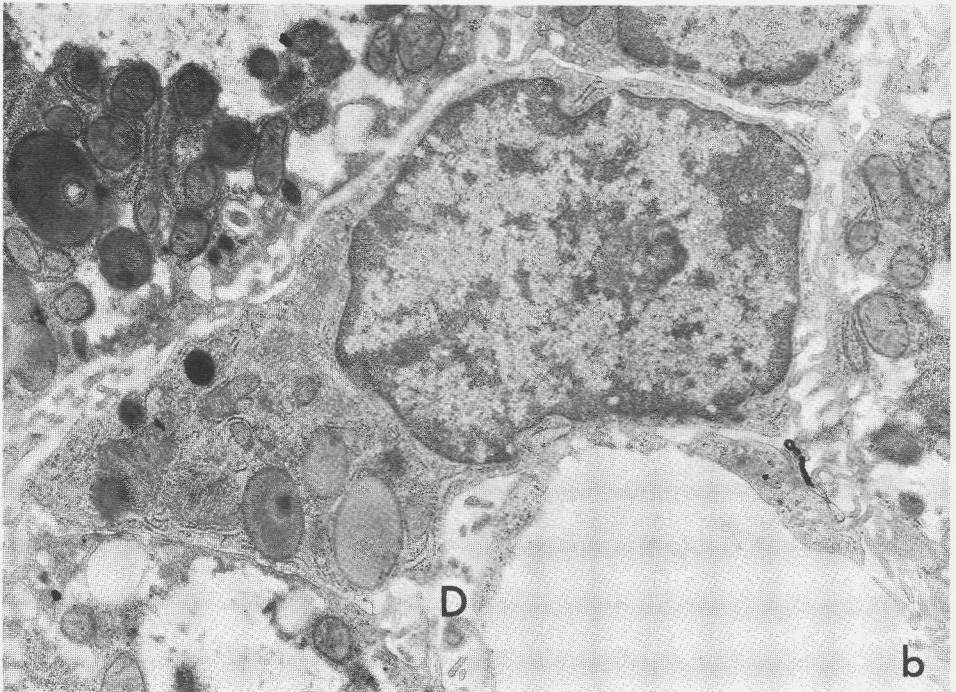
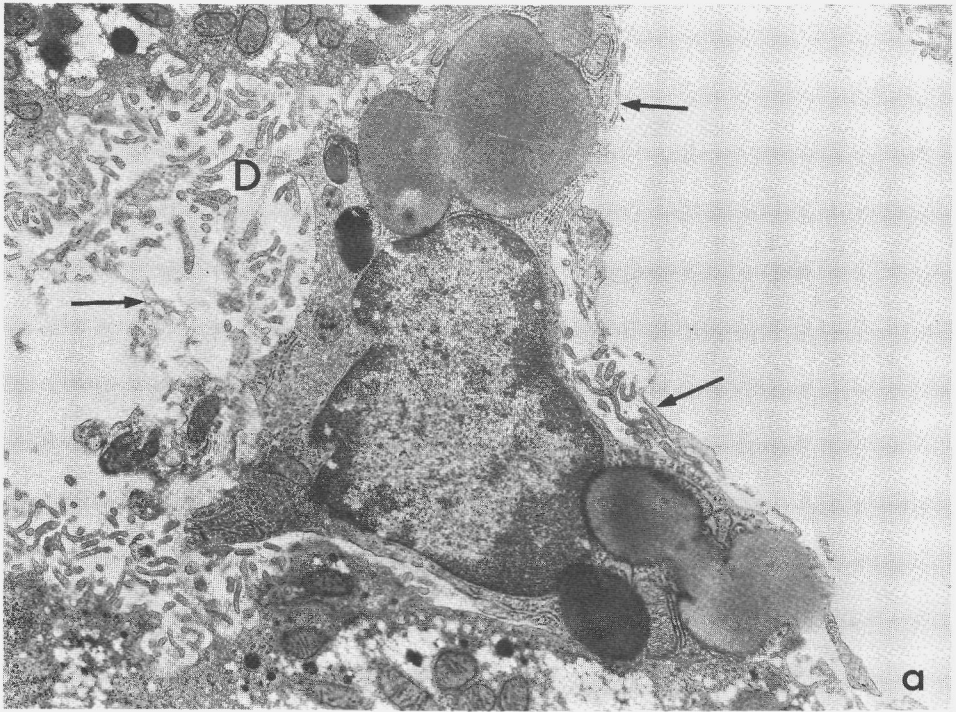
2.3.3 Ultrastructure of fat-storing cells in relation to age

Fat-storing cells were located in the space of Disse under the sinusoidal lining, often in recesses between two adjacent hepatocytes (Figs.2.7a,b). From the cell body, long cytoplasmic processes, penetrating between two hepatocytes or in the space of Disse, parallel to the endothelial lining extended (Fig.2.8a). The nuclei were round in shape, with a few small indentations and showing a coarse chromatin pattern with a broad rim of heterochromatin along the nuclear membrane and small patches distributed throughout the nucleus (Figs.2.7b and 2.8a).

The main characteristic of fat-storing cells was the occurrence of lipid droplets in their cytoplasm, which often indented the nucleus when the cytoplasm was full of lipid droplets (Fig.2.8b). All cells identified by their localization and further appearance as fat-storing cells contained lipid droplets; so-called empty fat-storing cells were not found in any of the livers studied. Fat-storing cells in the livers of 3-month-old animals showed the highest variability as compared with the other age groups with regard to the number of lipid droplets present per cell (3-12 droplets per cross-section of a cell). With increasing age of the animal, fat-storing cells

Figure 2.7 Transmission electron micrographs of fat-storing cells from a 12- (a) and a 3-month-old rat (b).

- a. Fat-storing cells are always located beneath the endothelial lining (↑) in the space of Disse (d). 9150 x.
- b. Fat-storing cells are often hidden in recesses between two hepatocytes. 10800 x.



appeared more homogeneous with regard to the number of lipid droplets per cell (10-15 droplets per cross-section of a cell). The increase in the area occupied by lipid droplets resulted in a reduction of the area of cytoplasm occupied by other cell organelles at the 6, 12, 24 and 36 months of age levels as compared with 3 months and also resulted in an apparent increase in cell volume as illustrated in Figures 2.7b and 2.8a.

Besides lipid droplets, some cell organelles appeared to be well developed. Dilated cisternae of the rough endoplasmic reticulum filled with finely flocculent material were distributed regularly in the cells and often closely associated spatially with large mitochondria (Fig.2.9a). One Golgi system, from which numerous small coated vesicles ($\emptyset$ 50-100 nm) were seen to pinch off, was localized close to the nucleus (Fig.2.9a). These features suggested active synthesis of proteins for external use to occur in these cells.

Occasionally, small electron dense "granules" were observed in fat-storing cells (Fig.2.9a, inset). These may be identical to microperoxisomes described by Fahimi et al. (1976) in fat-storing cells of the rat liver. Multivesicular bodies which in fat-storing cells are considered to be a precursor stage in the development of the lipid droplets (Wake, 1980), were found in cross-sections of some cells at all age levels (Fig.2.9a). Large lysosomal structures were observed in some fat-storing cells from 6 months onwards (Fig.2.9b). They contained dark, electron-dense lipid-like material, and indicate that degradation of lipids may occur in these cells. In some instances, ferritin-like particles were present in close association with lipid droplets (Fig.2.10a). Fat-storing cells revealing large clusters of smooth-walled membrane invaginations, sometimes containing a small electron-dense core such as described earlier by Dubuisson (1982), were occasionally observed (Fig.2.10b). These clusters are reminiscent of those seen in typical myofibroblasts and this may indicate a certain homology between fat-storing cells and myofibroblasts (see also Section 7.4).

2.3.4 Relative cell numbers

The results of the determinations of the relative numbers of the three types of sinusoidal liver cells in relation to age are given in Table 2.1. The relative numbers of endothelial, Kupffer and fat-storing cells expressed as a percentage of the total number of sinusoidal liver cells appeared to remain constant over the entire life span.

Figure 2.8 Transmission electron micrographs of fat-storing cells from a 6- (a) and a 33-month-old rat (b).

- a. Long cytoplasmic processes often also containing lipid droplets (1) extend from the cell body penetrating between two hepatocytes or in the space of Disse (d). 5450 x.
- b. Fat-storing cells filled with lipid droplets often show indented nuclei. Part of a Kupffer cell (k). 7000 x.

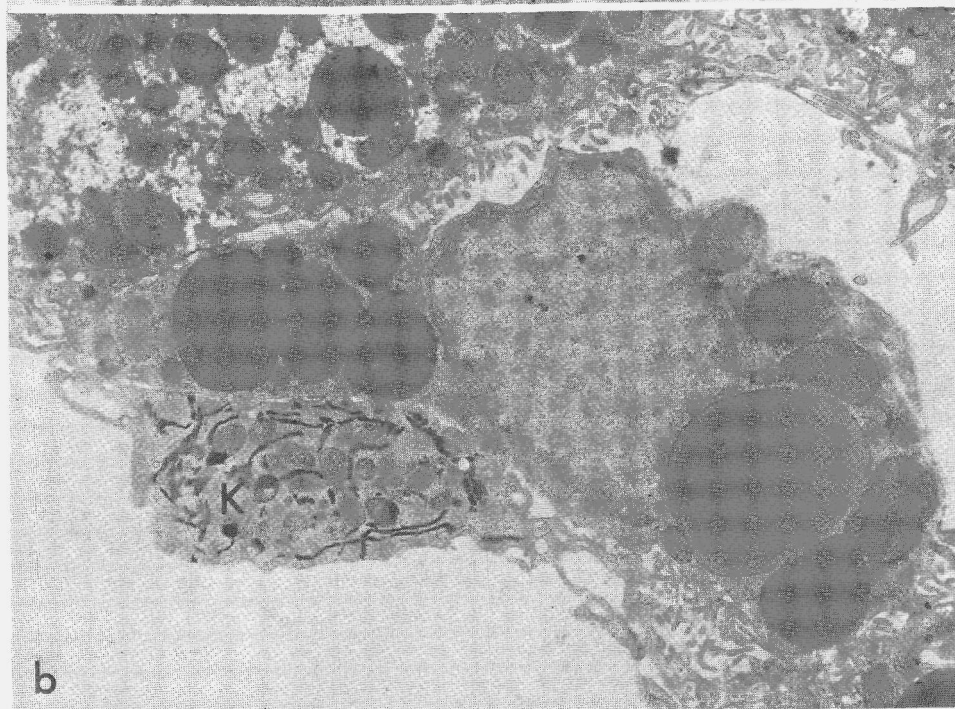
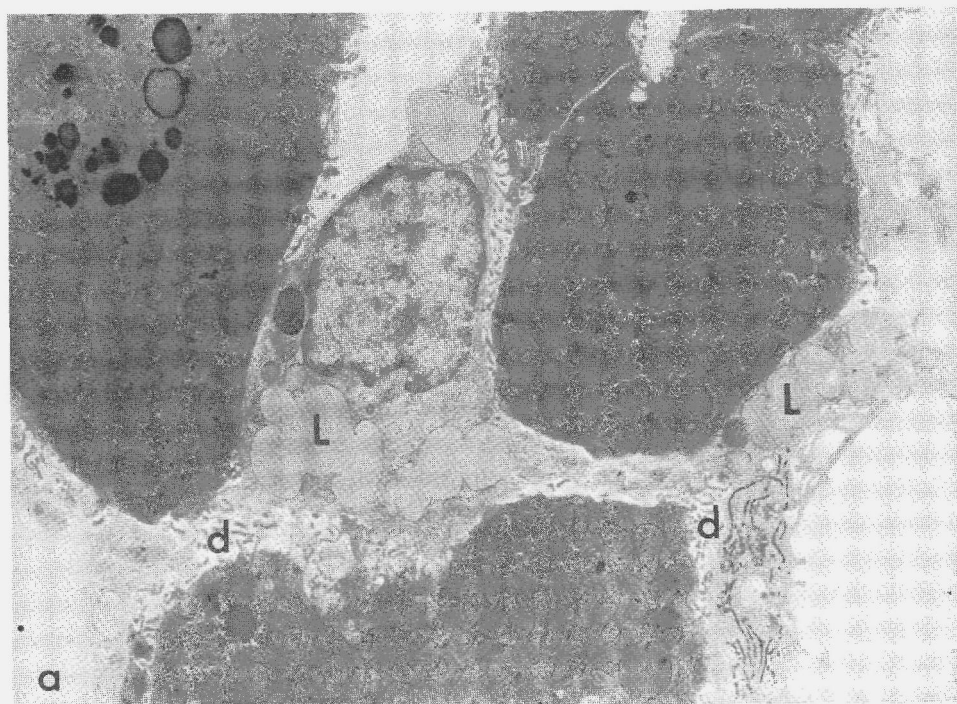


TABLE 2.1 RELATIVE NUMBERS OF ENDOTHELIAL, KUPFFER AND FAT-STORING CELLS IN LIVERS OF FEMALE BN/BIRij RATS OF DIFFERENT AGES

Age (mo)	Endothelial cells	Kupffer cells	Fat-storing cells
3	48.7 $\pm$ 6.1	19.9 $\pm$ 2.4	31.4 $\pm$ 5.2
6	44.1 $\pm$ 0.1	22.8 $\pm$ 0.4	33.1 $\pm$ 0.3
12	42.6 $\pm$ 4.4	21.1 $\pm$ 0.3	36.3 $\pm$ 4.3
24	44.2 $\pm$ 3.7	23.3 $\pm$ 3.5	32.5 $\pm$ 1.2
33	48.5 $\pm$ 0.3	22.7 $\pm$ 0.8	28.8 $\pm$ 1.1

The relative numbers are presented as a percentage of the total number of sinusoidal liver cells.

The values represent the mean $\pm$ standard deviation of 3 animals. Per animal, 150-200 sinusoidal cells were determined.

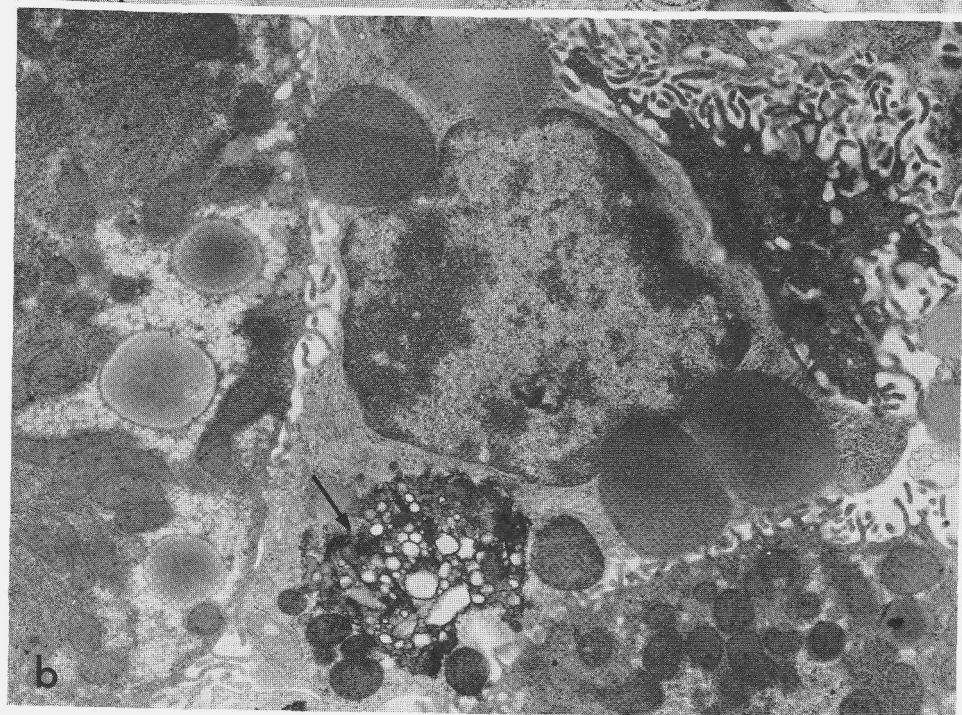
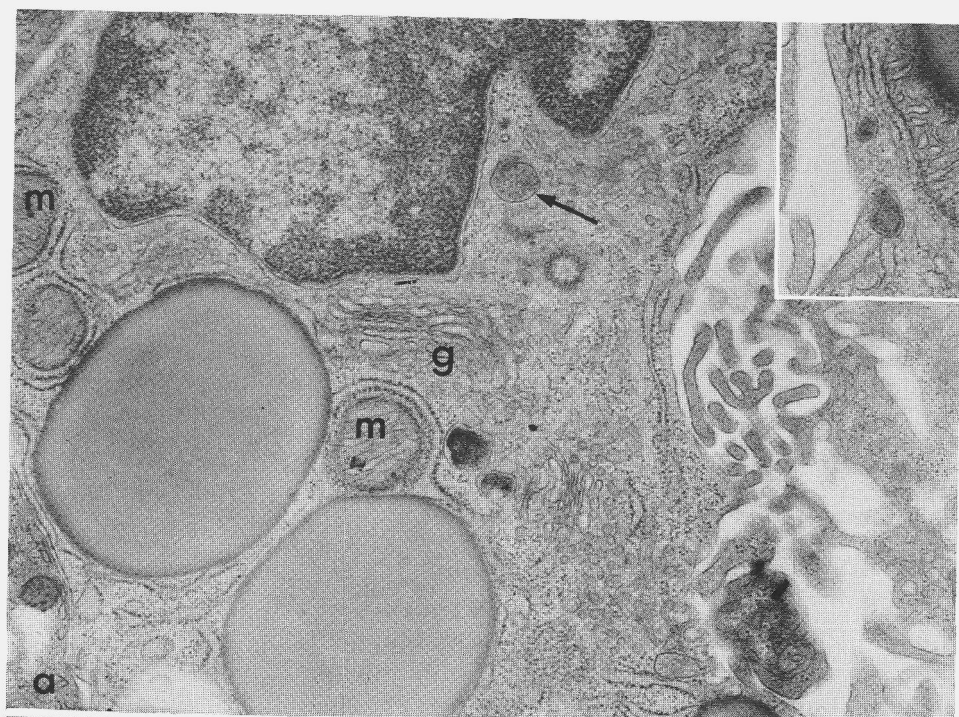
2.4 DISCUSSION

In the study described in this chapter on the ultrastructural aspects of sinusoidal liver cells in relation to the age of the animal, no changes in the cellular characteristics were found, which may have reflected an altered functional capacity of these cells with age. The changes observed with age involved mainly an increased storage of iron in both endothelial and Kupffer cells and of lipid droplets in fat-storing cells.

At all age levels studied both "small" and "large" Kupffer cells were observed. As was reported previously the incidence of Kupffer cells is higher in periportal than in perivenous areas (Hosemann et al., 1979). A functional and morphological heterogeneity of Kupffer cells related to their position in the liver acinus was shown already earlier. Larger Kupffer cells with a high endocytic activity, large and heterogeneous lysosomes and high lysosomal enzyme activity were found in the periportal zone (Sleyster and Knook, 1982). Although in the present study no

Figure 2.9 Transmission electron micrographs of fat-storing cells from a 12- (a) and a 24-month-old rat (b).

- Besides lipid droplets, fat-storing cells contain active mitochondria (m) associated spatially with cisternae of the RER, a Golgi system (g), multivesicular bodies (†), a pair of centrioles and some granules (see inset). 23650 x.
Inset: small granules, presumably microperoxisomes, are found in close association with the RER. 28300 x.
- A lipid containing lysosomal structure (†). 8300 x.



attention has been given to the localization of Kupffer cells within the liver acini, the observation of both "large" and "small" Kupffer cells at all age levels indicates Kupffer cell heterogeneity to persist.

When compared with literature data, the proportion of fat-storing cells in the sinusoidal cell population was higher and the proportion of Kupffer cells lower. In similar determinations, Widmann et al. (1972) found 48% endothelial cells, 39% Kupffer cells and 13% fat-storing cells in female albino rats and Stöhr and Fahimi (1978) found 49% endothelial, 42% Kupffer, and 9% fat-storing cells in both male and female Swiss albino mice (see also Table 2.2). Other data were obtained by light microscopic counting on semithin sections and are therefore not directly comparable with the electron microscopic determinations. Tamura and Fujita (1978) thus found a ratio between endothelial and Kupffer cells of about 6 to 4 in male albino rabbits (Table 2.2), whereas the present results give a ratio of almost 7 to 3 in female BN/BiRij rats. Podoprigora and Zaitsev (1979) determined the number of Kupffer cells per 1500 parenchymal cells and reported a consistently lower Kupffer cell content in livers of gnotobiotic rats as compared with conventional ones (Table 2.2). It may therefore be that the relatively low Kupffer cell content of female BN/BiRij rats found in the study presented here is due to the careful control of the health status and microbial flora of the animals used (Hollander, 1976).

In humans, the number of fat-storing cells per 1000 parenchymal cells (the Ito cell index) is often determined in relation to a possible development of liver fibrosis in light microscopic semithin sections of plastic embedded liver (Table 2.2) (Horvath et al., 1973). Large variations in the Ito cell index were described in control groups as well as in any individual pathological condition (Horvath et al., 1973; Hopwood and Nyfors, 1976; Koch et al., 1978, 1980; Kobayashi and Fujiyama, 1981; Giampieri et al., 1981). This indicates a large interindividual variability in humans. Such variability in relative cell numbers was not encountered in the inbred rat strain used in the present study. The large variability in the Ito cell index observed in humans may further be caused by an underestimation of the number of fat-storing cells under normal conditions due to the fact that identification of cells not containing many lipid droplets is rather difficult (Kent et al., 1976; Leo and Lieber, 1983).

However, the differences in the relative numbers of fat-storing cells between various electron microscopic studies are difficult to explain (cf. Tables 2.1, and

Figure 2.10 Transmission electron micrographs of fat-storing cells from a 12- (a) and a 24-month-old rat (b).
a. Ferritin particles are occasionally found in membrane-bound structures (†) also containing lipid droplets. 42400 x.
b. Fat-storing cell showing clusters of smooth-walled membrane invaginations (†). 15500 x.

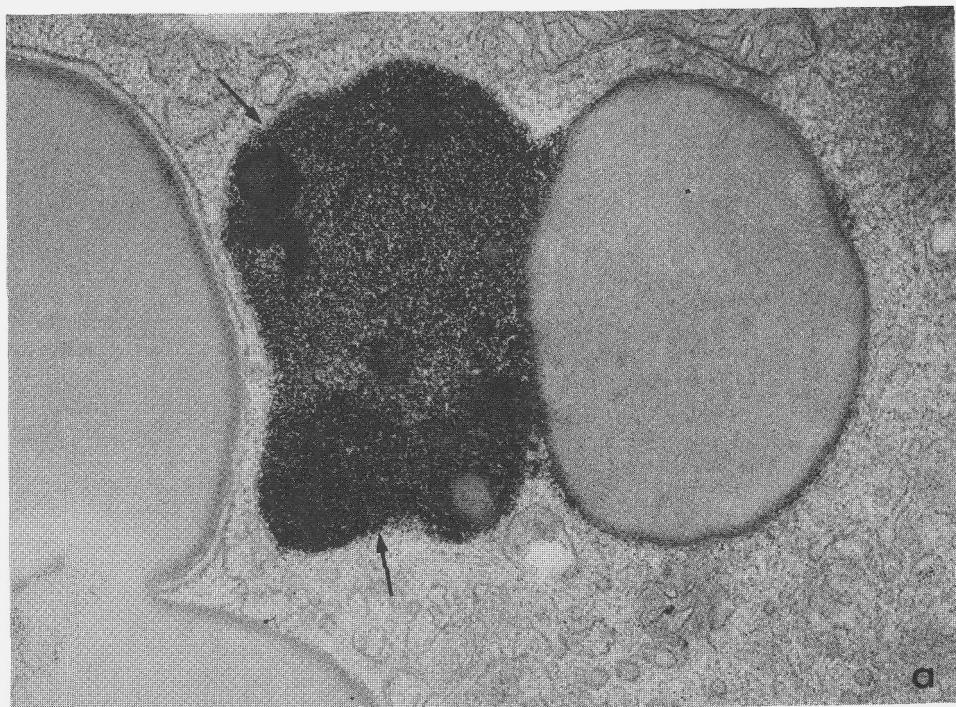


TABLE 2.2 LITERATURE DATA ON RELATIVE NUMBERS OF LIVER CELLS

<u>Species/Treatment</u>	<u>Type of data (A to D) and results</u>	<u>References</u>
A. Relative numbers (%) of endothelial, Kupffer and fat-storing cells		
1. Albino rats, ♀	E:K:FSC=48:39:13	Widmann et al., 1972
2. Albino mice, ♀ + ♂	E:K:FSC=49:42:9	Stöhr & Fahimi, 1978
B. Relative numbers (%) of endothelial and Kupffer cells		
3. Albino rabbits, ♂	E:K=60:40	Tamura & Fujita, 1978
C. Kupffer cell index (KCI) (number of Kupffer cells per 1500 hepatocytes)		
4. "rats" gnotobionts conventional	KCI=17.2±0.8 KCI=33.2±1.4	Podoprigora & Zaitsev, 1979
D. Ito cell index (ICI) (number of fat-storing cells per 1000 hepatocytes)		
5. humans, ♀ + ♂ "controls" psoriatics, post-MTX* alcoholic liver disease hyperlipoproteinemia	ICI=18 n=10 ICI=81 n= 9 ICI=13 n=10 ICI=24 n=10	Horvath et al., 1973
6. humans, ♀ + ♂ psoriatics, pre-MTX* psoriatics, post-MTX*	ICI=27 (6-63) n=24 ICI=67 (36-118) n=24	Hopwood & Nyfors, 1976
7. humans, ♀ + ♂ "controls" gall-stone patients pre-CDCA° short-CDCA° long-CDCA°	ICI=60-70 n= 8 ICI=67 (18-113) n=19 ICI=67 (17-112) n=19 ICI=83 (52-104) n= 6	Koch et al., 1978, 1980
8. humans, ♀ + ♂ "controls"	ICI=64 (41-91) n= 8	Giampieri et al., 1981
9. humans, ♀ + ♂ various liver diseases	ICI=166-200 n=51	Kobayashi & Fujiyama, 1981
10. Sprague-Dawley rats, ♂	ICI=124 n=12	Ballardini et al., 1983

Table 2.2 (cont'd)

11. Sprague-Dawley rats, ♂ high vitamin A diet				Leo and Lieber, 1983
+ ethanol	2 mo	ICI= 70	n= 7	
- ethanol		ICI=120	n= 7	
+ ethanol	9 mo	ICI=130	n= 9	
- ethanol		ICI=180	n= 9	
12. Baboons, ♀ + ♂		ICI=110	n=17	Mak et al., 1984

* MTX: methotrexate; ° CDCA: chenodeoxycholic acid

2.2). One reason may be species or strain differences, as Nopanitaya (1979) concluded from a comparative study of fat-storing cells in four species. He employed backscattered electron imaging modes to visualize these cells and reported that the number of fat-storing cells in rabbits was greater than in rats, mice and hamsters. Other factors may include the diet and the housing of the animals under conditions as specified by Hollander (1976). These may influence, for example, their microbial flora and possibly also their vitamin A status (Goodman, 1984). A strong indication for such factors is formed by the determination of relative sinusoidal cell numbers in two other rat strains kept in our laboratory (Sprague-Dawley and WAG/Rij) which gave comparable ratios (unpublished observation) to those reported in the present study.

The origin of the iron which accumulated with age in Kupffer cells and endothelial cells cannot be unequivocally explained. Accumulation of iron in rat livers with age has been previously described and it was thought to be localized in Kupffer cells (Burek, 1978). An increased degradation of red blood cells by Kupffer cells in the liver with age may explain such increasing storage of iron in Kupffer cells. However, as endothelial cells of the liver are not able to endocytose particles larger than 0.2 μm (Praaning-van Dalen et al., 1982), such as red blood cells, the accumulation of iron in endothelial cells cannot originate directly from red blood cell degradation.

In patients with idiopathic haemochromatosis and those with transfusional siderosis, storage of iron in both endothelial and Kupffer cells of the liver has been observed (Düllmann et al., 1982). In these cases, increased storage in the liver was explained by either a high affinity of the various liver cell types for iron in idiopathic haemochromatosis and by a redistribution of abundant macrophageal iron from the bone marrow and spleen to the liver in transfusional siderosis. Uptake of iron and its storage in apoferritin molecules by both endothelial and Kupffer cells of the rat liver have been shown also after intravenous injection of saccharated iron oxide and iron-dextran (Kuchta et al., 1982). Transport of iron from various sites in the body to the liver in all these cases is most probably mediated by transferrin, the main transport protein for iron in the blood. Cellular receptors for transferrin in the liver were described to be present

on hepatocytes (Unger and Hershko, 1974). Two recent reports dealt with the presence of transferrin receptors also on Kupffer and endothelial cells of the liver, respectively (Kumazawa et al., 1984; Soda and Tavassoli, 1984). If the latter is true, accumulation of iron in endothelial and Kupffer cells of the rat liver under various conditions, including aging, may be explained as removal from the circulation and storage of excess iron present in the blood in complex with transferrin.

Another possible source of iron is provided by ferritin circulating in the blood. In contrast to cellular ferritin, the storage form of cellular iron, it is unclear whether this serum ferritin contains appreciable amounts of iron. Preliminary experiments on the uptake of radioactively labeled iron-ferritin complexes isolated from rat liver showed uptake in the liver mainly by hepatocytes when this complex was injected intravenously (unpublished data). This suggests serum ferritin to be an unlikely source of the iron stored in endothelial and Kupffer cells. Caution must be exercised, however, since the identity of cellular and serum ferritin has not yet been proven (Zuyderhoudt, 1982).

The number of lipid droplets per fat-storing cell was found to increase with age. The age-related changes observed in fat-storing cells closely resembled the reactions in these cells in hypervitaminosis A in humans (Russell et al., 1974) and after high doses of vitamin A in experimental animals (Leo and Lieber, 1983). Determination of retinoids in purified populations of liver cells by high pressure liquid chromatography revealed a 3-fold increase in the vitamin A content of fat-storing cells of rats at between 6 and 36 months of age (H.F.J. Hendriks, personal communication). As so-called "empty" fat-storing cells (Wake, 1980) were not found in the present study at any age level, the vitamin A status of these rats may be considered as sufficient. It remains to be established whether the increased storage of vitamin A in rat liver with age is due solely to dietary intake of vitamin A in the pelleted rodent diet and whether therefore the amount of vitamin A in the diet is excessive. However, signs of vitamin A intoxication were not observed at any age level.

CHAPTER 3

ISOLATION AND PURIFICATION OF SINUSOIDAL LIVER CELLS FROM RATS OF VARIOUS AGES

3.1 INTRODUCTION

Investigation of specific functions of the major classes of liver cells with the use of isolated cells either in suspension or in monolayer cultures has become an important activity in liver research (Wisse and Knook, 1979; Van Berkel, 1982). These *in vitro* systems allow the study of the contribution of each specific cell type to one aspect of hepatic function under standardized conditions, unaffected by influences of other cells and cellular products such as occurs when studying whole intact liver (Grisham, 1983).

A number of methods is now available for the isolation and purification of all major classes of liver cells, i.e., parenchymal cells, sinusoidal endothelial cells, Kupffer cells and fat-storing cells. The two isolation procedures most applied are: (i) perfusion and incubation of liver with collagenase, to yield both parenchymal and some sinusoidal liver cells (Van Berkel and Van Tol, 1978); and (ii) perfusion and incubation of the liver with pronase, an enzyme which destroys parenchymal cells, to obtain sinusoidal liver cells in high yield but virtually without fat-storing cells (Knook and Sleyster, 1976; Sleyster et al., 1977). A procedure combining pronase and collagenase resulted in the isolation also of large numbers of fat-storing cells (Knook et al., 1982b).

Purification of several classes of liver cells from crude cell suspensions can be achieved by a variety of techniques including density centrifugation and centrifugal elutriation, procedures which have recently been extensively reviewed (Brouwer et al., 1984). In addition, a combination of velocity sedimentation and flow cytometry two-parameter analysis was introduced in order to obtain fractions enriched in lymphocytes, endothelial and Kupffer cells from nonparenchymal liver cell suspensions (Morin et al., 1984).

Fractions of liver cells obtained by these various isolation and purification methods differ significantly in yield, cellular composition and certain cell characteristics such as the preservation of cell membrane-specific proteins (e.g., receptors) (Brouwer et al., 1982b). A careful characterization of the cells in terms of both morphological and cell-specific biochemical parameters is therefore a prerequisite to determine the value of a given cell sample for investigation of various aspects of hepatic function (Brouwer et al., 1982b; Van Berkel, 1982).

In the determination of the contribution of each specific cell type to hepatic

function, it would be most advantageous to isolate all major liver cell classes in high yield and purity from one animal. With the present methodology, however, it is possible only to purify either parenchymal cells or the three classes of sinusoidal liver cells from one liver. Several aspects of hepatic function in relation with age have already been studied in detail by making use of isolated parenchymal cells in suspension (Knook, 1980; Van Bezooijen, 1984). Purified suspensions of endothelial and Kupffer cells isolated by pronase have been used to study the effect of aging on the endocytic capacity and levels of lysosomal enzymes in these cells (Knook and Sleyster, 1980; Brouwer et al., 1985). Although the isolation procedure combining pronase and collagenase has been applied to study the contributions of sinusoidal endothelial, Kupffer and fat-storing cells to vitamin A metabolism in the liver (De Leeuw et al., 1983; Hendriks et al., submitted), the value of this procedure for age-related research has not been investigated in detail up to now.

A description is given in this chapter of the isolation of sinusoidal liver cells from rats of various ages making use of pronase and collagenase treatment, followed by purification of the cells by density centrifugation and centrifugal elutriation. Special attention was given to cell yield and the ultrastructural morphology of the isolated cells in relation to the age of the donor.

3.2. MATERIALS AND METHODS

3.2.1 Isolation and purification of sinusoidal liver cells

Experiments were performed with 3-, 6-, 12-, 24-, 30- and 36-month-old rats.

Nonparenchymal liver cells were isolated by enzymatic perfusion and incubation of the liver with pronase and collagenase as described previously (Knook et al., 1982b). The liver was first perfused in situ through the portal vein with Gey's balanced salt solution (GBSS) for 5 min. This was followed by a postperfusion of 6 min with 0.2% pronase E (Merck) dissolved in GBSS. During this postperfusion process, the liver was carefully excised and placed on a sieve. It was then connected to a circulation perfusion system containing 0.05% collagenase type I (Sigma Chemical Co.) and 0.05% pronase E dissolved in GBSS and perfused for 30 min. All media were kept at 37°C and perfused at a flow rate of 10 ml.min⁻¹.

Following perfusion, Glisson's capsule was removed and the main vessels sectioned. The paste-like substance was then stirred in 100 ml GBSS containing 0.05% collagenase type I and 0.02% pronase E at 37°C for 30 min. The pH of this mixture was kept at 7.4 by the addition of 1 M NaOH. After incubation, the cell suspension was filtered through nylon gauze and the filtrate centrifuged at 450 x g for 10 min. The cell pellet was resuspended by gentle pipetting in GBSS to a final volume of 11.1 ml.

The first step in the purification procedure consisted of centrifugation in a

discontinuous two-layer Nycodenz[®] (Nyegaard & Co.) density gradient (Hendriks et al., submitted). The lower layer consisted of 10 ml of a 17.2% Nycodenz solution in GBSS, without NaCl. The upper layer consisted of a mixture of 11.1 ml of cell suspension and 7.4 ml of a 28.7% Nycodenz solution to yield a final concentration of 11.5% Nycodenz. Finally, 1 ml of GBSS was applied on top of the gradient. After centrifugation at 1500 x g for 17 min, two fractions were collected. Fraction A consisted of the GBSS layer and the cells at the interphase between this and the upper Nycodenz layer. Fraction B consisted of the upper Nycodenz layer and the cells at the interphase between this and the lower Nycodenz layer. After washing in GBSS, these fractions were analyzed for yield and cellular composition (Section 3.2.2).

Fraction B, which contained a mixture of lymphocytes, endothelial and Kupffer cells was submitted to centrifugal elutriation (Brouwer et al., 1984). A JE-6B elutriation rotor with a Sanderson separation chamber (Beckman Instruments) was used in a J-21 type Beckman centrifuge at a constant rotor speed of 3250 rpm. GBSS was used as the elutriation fluid. The complete system, including the collected samples, was kept at 4°C. Fractions were collected at the following flow rates: a first fraction of 100 ml (Fraction I) was collected at 17-18 ml.min⁻¹, a second fraction of 200 ml (Fraction II) at 32-34 ml.min⁻¹ and a third fraction of 150 ml (Fraction III) at 57-70 ml.min⁻¹. To ensure optimal separation of different cell types, the flow rate, especially of Fraction III, had to be increased with increasing age of the animals. At the end of the run, the pellet was removed from the separation chamber and diluted in GBSS. The fractions and the pellet were centrifuged at 400 x g for 10 min and resuspended in GBSS. Samples were taken from each fraction to determine yield and cellular composition (Section 3.2.2).

3.2.2 Determination of yield, viability and cellular composition

Cell counts were performed in a hemocytometer. The integrity of the cell membranes, further referred to as cell viability, was estimated by the capacity of the cells to exclude trypan blue and further confirmed by transmission electron microscopy.

Light microscopically, the number of Kupffer cells in the various samples was determined by positive cytochemical staining for endogenous peroxidatic activity as defined by Wisse et al. (1974). The cellular composition of the various samples was further determined on the basis of morphological cell characterization by both light and electron microscopy.

3.2.3 Fixation procedure for transmission electron microscopy

Cell suspensions were fixed by adding samples of at least 1×10^6 cells in GBSS to an excess of 2% glutaraldehyde in 0.15 M sodium cacodylate buffer (pH 7.4) for 15 min, followed by postfixation in 1% OsO₄ in the same buffer for 20 min, both at

4°C. After the first dehydration step in 70% ethanol, aliquots of the cell suspensions were put into microfuge tubes and pelleted at 7960 x g for 5 min in a microfuge B (Beckman Instruments). The cell pellets (Ø 1 mm) were further treated as tissue blocks (Section 2.2.1).

3.3 RESULTS

3.3.1 Yield and cellular composition of Fractions A and B

After centrifugation in the two-layer Nycodenz density gradient, most cells were recovered in Fractions A and B. The lower Nycodenz layer did not contain an appreciable number of cells. The pellet consisted mainly of cell debris and erythrocytes.

In Table 3.1, the cell yield per liver and the cellular composition as determined by electron microscopy are given for the two fractions in relation to the age of the animals.

In these series of experiments, fractions A can be considered as fat-storing cell fractions for animals of 12 and 24 months of age (% of fat-storing cells: 80 and 83, respectively). At 3 and 6 months of age, both the cell yield and the percentage of fat-storing cells in fraction A are lower as compared with 12 and 24 months. At 30 and 36 months, the cell yield in fraction A is considerably higher than in the younger ages, but the percentage of fat-storing cells is lower as compared with 12 and 24 months, due to a higher content of mainly endothelial cells.

Fractions B can be considered as intermediate fractions used later for further purification of sinusoidal endothelial cells and Kupffer cells by centrifugal elutriation (Section 3.3.3). These fractions are quite comparable with those obtained by centrifugation of crude liver cell suspensions in a single layer metrizamide or Nycodenz density gradient (cf. Brouwer et al., 1984). The cell yield in Fraction B is mostly comparable at all age levels, with somewhat higher values at 12 and 36 months of age. The cellular composition of fractions B is similar for those obtained from 3- to 24-month-old animals (lymphocytes: 19-20%; endothelial cells: 53-58%; Kupffer cells: 17-22%; bile duct epithelial cells: 1-3%; fat-storing cells: 0-7%). At 30 and 36 months of age, the percentage of endothelial cells in fraction B is somewhat lower and the percentage of bile duct epithelial cells significantly higher than in the other age groups. The increased number of bile duct epithelial cells isolated in late age may be related to the high incidence of biliary cysts in this rat strain (Burek, 1978).

The values for the percentages of fat-storing cells determined by electron microscopy were always higher than those determined by light microscopy. Light microscopic determination of the percentages of Kupffer cells making use of staining for peroxidatic activity gave results comparable to those of electron microscopic cell counting based on morphological characterization.

TABLE 3.1 YIELD AND CELLULAR COMPOSITION OF FRACTIONS A AND B RECOVERED AFTER CENTRIFUGATION IN A TWO-LAYER NYCODENZ DENSITY GRADIENT

Age (mo)	Fraction	Yield (10 ⁶ cells/liver)	Composition (% of total cell number)				
			FSC	L	E	K	BD
3	A	35.4 ± 10.6	17.8 ± 14.3	4.8 ± 1.5	73.2 ± 15.5	1.6 ± 0.7	2.4 ± 2.2
	B	186.0 ± 7.6	2.6 ± 1.7	20.3 ± 5.8	53.3 ± 7.2	20.9 ± 6.2	2.8 ± 1.6
6	A	16.3 ± 4.0	45.4 ± 14.2	9.4 ± 6.4	39.9 ± 10.6	4.8 ± 1.3	0.2 ± 0.2
	B	184.0 ± 45.2	7.0 ± 2.3	19.4 ± 4.8	55.3 ± 3.0	17.1 ± 4.8	1.3 ± 1.0
12	A	66.0 ± 3.4	79.6 ± 6.4	1.9 ± 1.4	16.5 ± 5.6	1.0 ± 0.7	0.9 ± 1.3
	B	224.0 ± 75.6	0.4 ± 0.3	19.7 ± 4.3	57.9 ± 6.1	20.3 ± 2.5	1.7 ± 1.2
24	A	62.8 ± 44.1	82.7 ± 8.0	3.1 ± 1.9	12.3 ± 7.5	1.3 ± 0.5	0.6 ± 0.8
	B	180.5 ± 25.5	1.3 ± 0.5	20.2 ± 0.9	54.3 ± 3.7	22.2 ± 3.8	1.9 ± 1.3
30	A	134.1 ± 19.7	51.7 ± 10.2	1.6 ± 0.4	38.5 ± 3.0	5.3 ± 4.5	2.5 ± 1.8
	B	159.3 ± 11.0	0.9 ± 0.5	22.8 ± 11.8	36.8 ± 10.7	26.3 ± 2.5	12.7 ± 2.1
36	A	222.7 ± 92.9	45.0 ± 8.7	4.5 ± 1.0	37.5 ± 15.1	5.8 ± 3.1	6.8 ± 6.1
	B	259.6 ± 67.8	0.5 ± 0.8	24.3 ± 7.3	36.0 ± 20.7	24.3 ± 11.1	14.5 ± 4.9

The values represent the mean ± SD of three experiments.
 FSC: fat-storing cells; L: lymphocytes; E: endothelial cells;
 K: Kupffer cells; BD: bile duct epithelial cells.

3.3.2 Total nonparenchymal cell yield and total yield of each type of sinusoidal liver cell

In Table 3.2, the nonparenchymal cell yield and that of each type of sinusoidal liver cell is expressed per liver and per gram of liver weight (in parentheses).

The total nonparenchymal cell yield per liver increased with age, with the exception of the value for 12-month-old animals. Per gram of liver weight, 40 to 50×10^6 cells were isolated at 3, 6, 24 and 30 months of age, with a consequently higher value at 12 months of age and a much higher value at 36 months of age.

Endothelial cells represented the most abundant cell type isolated from the rat liver; very similar numbers of fat-storing cells and Kupffer cells were obtained with the exception of 3- and 6-month-old animals. In these cases, the number of fat-storing cells isolated per gram of liver weight was much lower as compared with the other age groups.

TABLE 3.2 CELL YIELDS IN RELATION TO THE AGE OF
THE DONOR ANIMAL

Age (mo)	Cell yields (10^6 cells)				Average liver weight (g)*
	NPC	FSC	E	K	
3	221 (49)	11 (3)	125 (28)	50 (11)	4.5 (n=36)
6	200 (43)	20 (4)	108 (23)	32 (7)	4.7 (n= 9)
12	290 (64)	53 (12)	141 (31)	46 (10)	4.5 (n=14)
24	243 (42)	54 (9)	106 (18)	41 (7)	5.8 (n=13)
30	293 (46)	71 (11)	110 (17)	49 (8)	6.4 (n=18)
36	482 (74)	101(16)	177 (27)	76 (12)	6.5 (n= 8)

Cell yield is expressed per liver and per gram of liver weight (values in parentheses). These values represent the product of fractions A and B in relation to the percentages of specific cell types given in Table 3.1.

* The average liver weights of female BN/BiRij rats at various ages were calculated from literature data of Kitani et al. (1982) and Brouwer et al. (1985). In parentheses, the number of animals of which the average liver weights were calculated.

NPC: nonparenchymal liver cells; FSC: fat-storing cells; E: sinusoidal endothelial cells; K: Kupffer cells.

TABLE 3.3 YIELD AND CELLULAR COMPOSITION OF FRACTIONS I, II AND III
RECOVERED AFTER CENTRIFUGAL ELUTRIATION

Age (mo)	Fraction	Yield (10^6 cells/liver)	Composition (% of total cell number)				
			FSC	L	E	K	BD
3	I	45.9 $\pm$ 13.8	13.7 $\pm$ 5.9	67.5 $\pm$ 7.3	17.4 $\pm$ 3.2	0.0 $\pm$ 0.0	1.4 $\pm$ 1.7
	II	108.3 $\pm$ 31.2	8.3 $\pm$ 7.1	13.3 $\pm$ 3.8	73.5 $\pm$ 5.4	2.4 $\pm$ 1.4	2.5 $\pm$ 1.9
	III	37.5 $\pm$ 6.3	1.9 $\pm$ 2.1	2.4 $\pm$ 1.4	8.9 $\pm$ 4.1	80.2 $\pm$ 2.2	5.9 $\pm$ 2.8
6	I	41.4 $\pm$ 3.4	23.9 $\pm$ 12.1	66.4 $\pm$ 8.6	9.7 $\pm$ 3.5	0.0 $\pm$ 0.0	0.0 $\pm$ 0.0
	II	99.8 $\pm$ 27.1	4.3 $\pm$ 1.5	13.7 $\pm$ 1.8	80.8 $\pm$ 1.8	1.1 $\pm$ 1.5	0.2 $\pm$ 0.2
	III	33.0 $\pm$ 7.9	3.0 $\pm$ 0.8	5.6 $\pm$ 0.6	15.9 $\pm$ 5.8	72.1 $\pm$ 4.8	3.0 $\pm$ 2.2
12	I	52.3 $\pm$ 14.7	2.5 $\pm$ 0.2	55.9 $\pm$ 13.5	37.1 $\pm$ 16.1	0.5 $\pm$ 0.5	3.9 $\pm$ 2.9
	II	112.9 $\pm$ 40.9	0.6 $\pm$ 0.5	7.2 $\pm$ 1.9	85.9 $\pm$ 2.0	2.9 $\pm$ 0.3	3.4 $\pm$ 2.7
	III	33.5 $\pm$ 17.8	0.4 $\pm$ 0.3	2.9 $\pm$ 2.4	14.4 $\pm$ 8.6	74.8 $\pm$ 6.6	6.3 $\pm$ 6.3
24	I	33.5 $\pm$ 2.6	3.0 $\pm$ 2.5	94.8 $\pm$ 1.9	1.6 $\pm$ 1.3	0.0 $\pm$ 0.0	0.5 $\pm$ 0.7
	II	88.8 $\pm$ 25.7	0.6 $\pm$ 0.6	15.1 $\pm$ 6.5	79.3 $\pm$ 5.3	2.4 $\pm$ 1.4	2.5 $\pm$ 1.1
	III	22.7 $\pm$ 4.5	0.3 $\pm$ 0.3	1.3 $\pm$ 1.3	23.8 $\pm$ 8.4	71.0 $\pm$ 7.5	3.7 $\pm$ 0.2
30	I	39.3 $\pm$ 10.3	1.6 $\pm$ 1.8	77.6 $\pm$ 21.8	17.3 $\pm$ 17.9	2.9 $\pm$ 2.6	0.6 $\pm$ 0.5
	II	88.0 $\pm$ 5.9	0.7 $\pm$ 0.5	19.5 $\pm$ 5.6	67.2 $\pm$ 7.3	5.2 $\pm$ 1.6	7.1 $\pm$ 0.6
	III	18.7 $\pm$ 2.2	0.3 $\pm$ 0.4	3.4 $\pm$ 3.0	9.5 $\pm$ 10.4	78.4 $\pm$ 16.6	7.4 $\pm$ 4.5
36	I	107.4 $\pm$ 21.1	2.4 $\pm$ 2.5	85.8 $\pm$ 8.7	6.0 $\pm$ 7.6	0.4 $\pm$ 0.3	4.3 $\pm$ 5.6
	II	104.9 $\pm$ 19.0	0.5 $\pm$ 0.4	28.7 $\pm$ 15.7	57.4 $\pm$ 17.3	4.8 $\pm$ 1.9	8.4 $\pm$ 5.2
	III	35.2 $\pm$ 13.2	0.7 $\pm$ 0.6	6.5 $\pm$ 4.0	15.8 $\pm$ 12.2	62.1 $\pm$ 20.6	14.9 $\pm$ 7.1

The values represent the mean $\pm$ SD of three experiments.
FSC: fat-storing cells; L: lymphocytes; E: endothelial cells,
K: Kupfer cells; BD: bile duct epithelial cells.

3.3.3 Yield and cellular composition of fractions I, II and III

Fractions B were submitted to centrifugal elutriation to obtain pure suspensions of sinusoidal endothelial cells and Kupffer cells. Lymphocytes, endothelial and Kupffer cells were recovered in fractions I to III. The pellets consisted mainly of clusters of bile duct epithelial cells; however, a number of Kupffer cells was also sometimes found.

The yield and cellular composition of fractions I, II and III are given in relation to the age of the animal in Table 3.3.

The cell yield in fractions I, II and III was largely comparable for all age groups, with the exception of fraction I at 36 months of age, where much higher cell numbers were recovered.

Fractions I consisted mainly of blood cells, mostly small and intermediate lymphocytes. Sinusoidal endothelial cells were present in percentages of up to 37%. At 3 and 6 months of age, fraction I contained appreciable numbers of fat-storing cells.

Fractions II consisted mainly of sinusoidal endothelial cells. The major contamination was with blood cells (up to 30% at 36 months of age). The presence of some bile duct epithelial cells in fractions II at 30 and 36 months of age further decreased the purity of the endothelial cell fractions at these age levels.

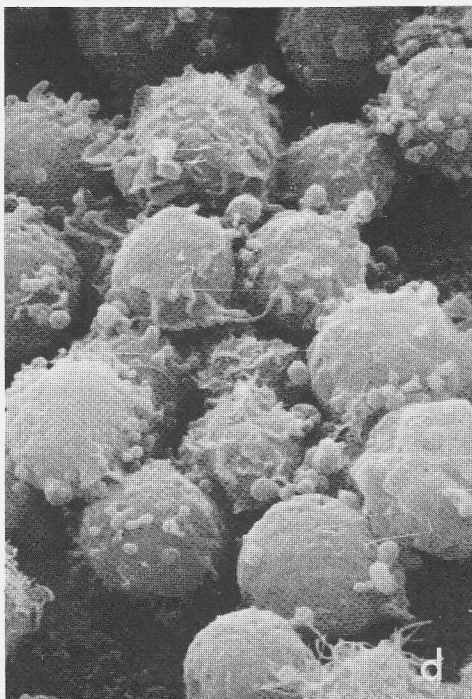
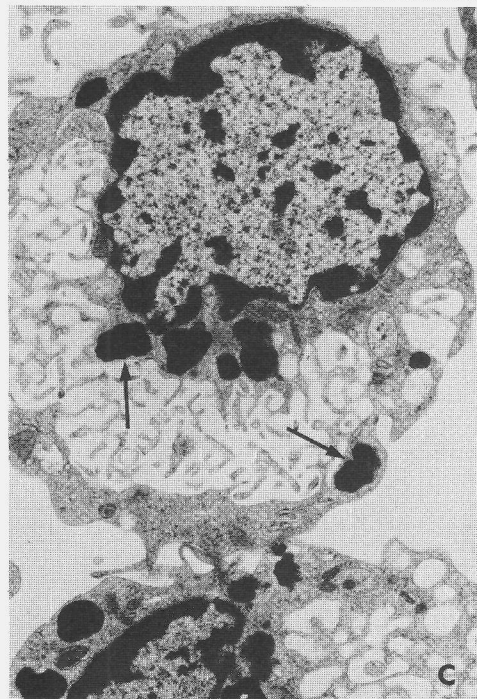
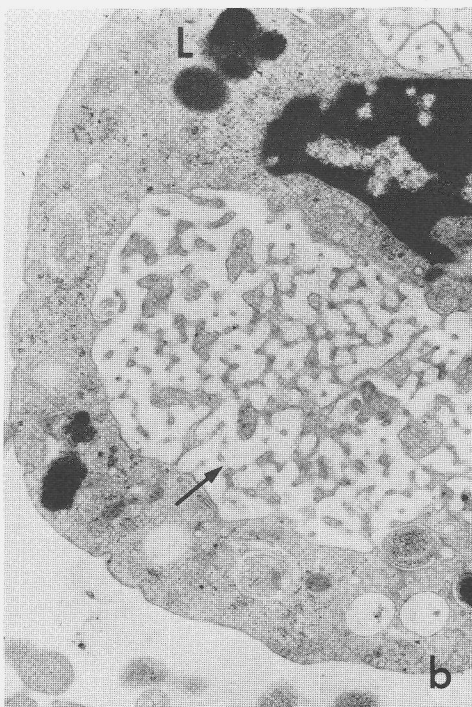
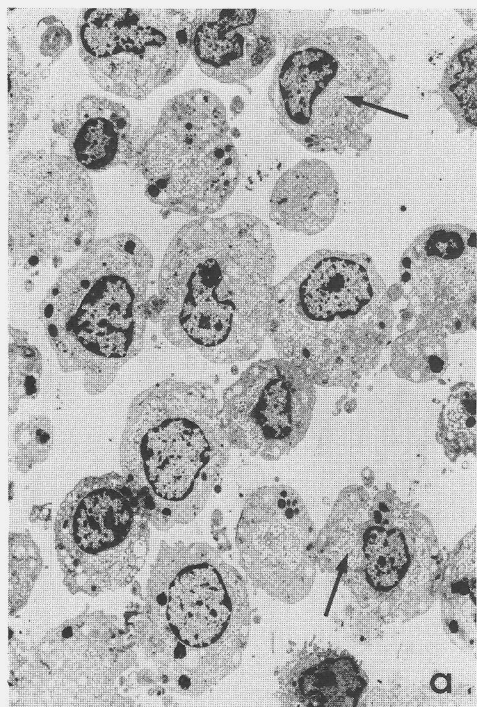
Fractions III contained high numbers of Kupffer cells. The purity of these Kupffer cell fractions was comparable at all age levels; only at 36 months of age did the high content of bile duct cells somewhat lower the purity of fractions III for Kupffer cells.

3.3.4 Ultrastructural morphology of freshly isolated sinusoidal liver cells

3.3.4.1 Sinusoidal endothelial cells

After isolation and purification, sinusoidal endothelial cells retained much of the characteristic appearance of endothelial cells in the intact liver, but the fenestrated lining was withdrawn and clustered close to the nucleus, leading to a

- Figure 3.1 Fractions of freshly isolated and purified endothelial cells.
- Low magnification transmission electron micrograph of a fraction II isolated from a 3-month-old rat. Retracted cell processes containing the sieve plates (†). 2000 x.
 - Detail: A broad rim of cytoplasm encircles the retracted cell processes. Fenestrae (†); Lysosomes (l). 12800 x.
 - Endothelial cell from a 24-month-old rat. The cell body shows a number of ferritin-containing lysosomes (†). 8000 x.
 - Scanning electron micrograph of a fraction II from a 3-month-old rat. Endothelial cells show a heterogeneous surface morphology. Smooth cells with only a few membrane blisters as well as cells with lamellipodia-like processes are present. 3000x. (Photograph by R. de Zanger; Free University of Brussels, Belgium).



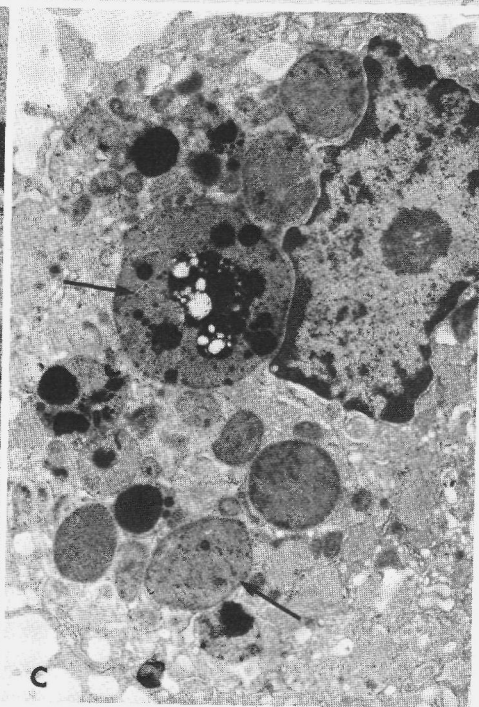
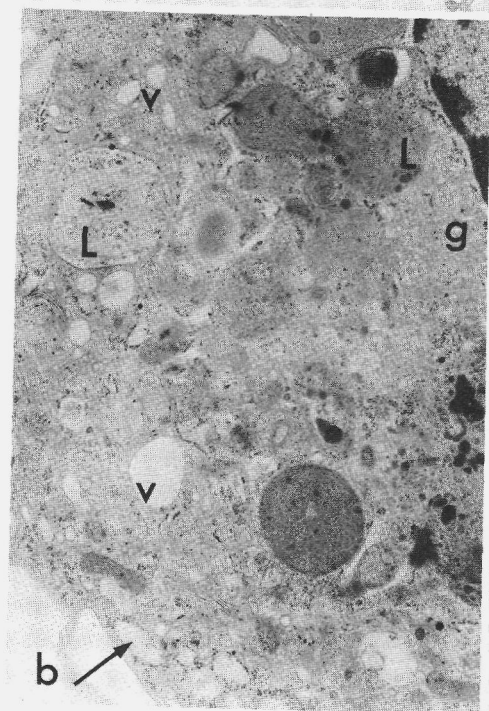
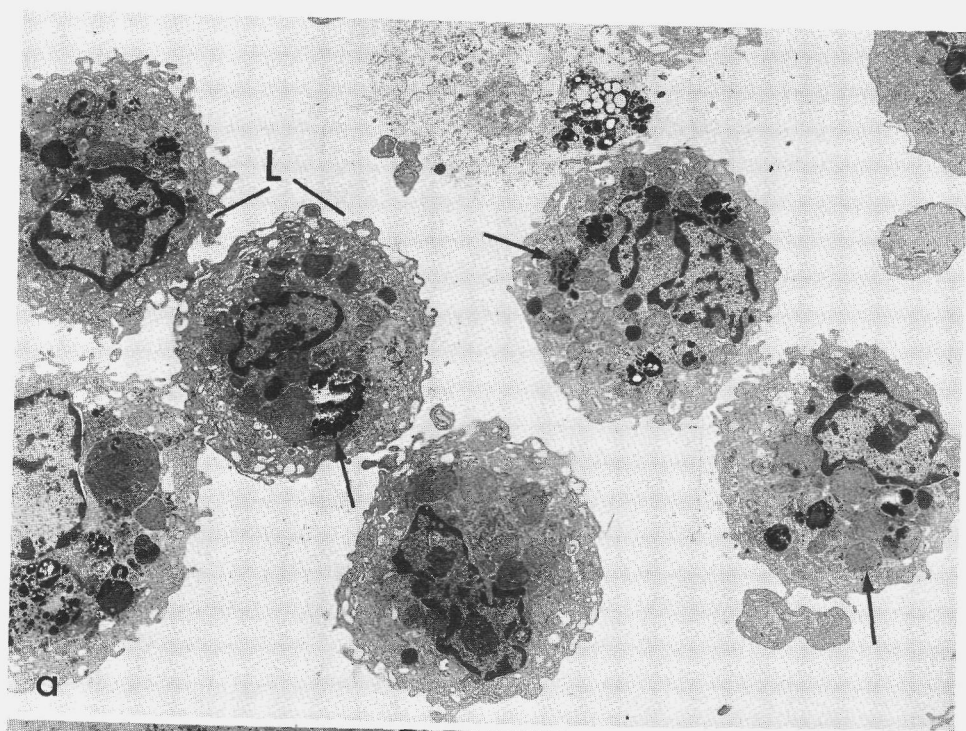
sponge-like appearance (Fig.3.1a). Despite this alteration, the pores in the sieve plates appeared to be well preserved (Fig.3.1b). A broad rim of cytoplasm containing some dense lysosomes, pinocytic vesicles, mitochondria and a Golgi system often enclosed the enfoldings of the thin cytoplasmic extensions containing the sieve plates (Fig.3.1b). The general ultrastructural morphology of isolated endothelial cells was similar at all age levels. However, with increasing age of the donor animal, sinusoidal endothelial cells contained in their cytoplasm an increasing number of very electron dense lysosomal structures (Fig.3.1c). These represent the ferritin-containing lysosomes described for sinusoidal endothelial cells in the intact liver (cf. Section 2.3.1).

The surface morphology of freshly isolated sinusoidal endothelial cells was analyzed by scanning electron microscopy (for Methodology, see Section 5.2.2). Most endothelial cells showed an almost smooth surface, except for a few small pits (indentations; $\emptyset$ 0.10–0.15 μ m) possibly representing fenestrae or pinocytic vesicles (Fig.3.1d). The cells also showed only a few microvillous structures and some rounded blebs or membrane blisters (Fig.3.1d). The surface characteristics were similar to those observed by transmission electron microscopy (cf. Figs.3.1.a,d). Some cells had a rougher surface which showed lamellipodia-like processes (Fig.3.1d). Whether these cells represented contaminating lymphocytes or Kupffer cells or whether this surface morphology was caused by differences in the fixation procedures for transmission and scanning electron microscopy could not be definitely established.

3.3.4.2 Kupffer cells

After isolation and purification, Kupffer cells were the most similar to their counterparts in intact liver of all three types of sinusoidal liver cells (Fig.3.2a). Isolated Kupffer cells were more rounded up and as in situ, were characterized by the presence of lamellipodia causing a ruffled cell membrane. In addition, they contained a large number of lysosomes which varied in size and structure (Fig.3.2a). The nucleus in isolated Kupffer cells was mostly located eccentrically, while the distribution of various cell organelles such as the Golgi system, mitochondria, strands of rough endoplasmic reticulum and various endocytic vesicles

- Figure 3.2 Fractions of freshly isolated and purified Kupffer cells.
- Low magnification transmission electron micrograph of a fraction III isolated from a 24-month-old rat. Kupffer cells are characterized by an eccentric nucleus, a high content of lysosomes ($\uparrow$) and lamellipodia (l). 3450 x.
 - Kupffer cell from a 3-month-old rat. The distribution pattern of various cell organelles is quite similar to that found in cells in the intact liver. Lysosomes (l); Golgi system (g); RER ($\uparrow$); endocytic vesicles (v). 12100 x.
 - Kupffer cell from a 36-month-old rat. The lysosomes ($\uparrow$) contain large amounts of ferritin. 8000 x.



had a similar appearance to that found in cells *in situ* (Fig.3.2b; cf. Section 2.3.2).

Figure 3.2c shows that Kupffer cells isolated from old animals contained, like cells in the intact liver (cf. Fig.2.6b, inset), a large amount of ferritin.

3.3.4.3 Fat-storing cells

At the ultrastructural level, freshly isolated and purified fat-storing cells exhibited a characteristic morphology. They had an irregular cellular outline due to numerous cellular extensions (Fig.3.3a), most probably representing subendothelial processes described for cells in the intact liver (Wake, 1980).

Lipid droplets occupied the largest part of the cytoplasm in the majority of isolated fat-storing cells (Fig.3.3a), but some cells with a small number of lipid droplets were also observed (Fig.3.3b). The latter may represent the "empty" fat-storing cells described in the intact liver (Wake, 1980).

Besides lipid droplets, all other cell organelles described as being present in fat-storing cells in the intact liver (Bartók et al., 1979; Wake, 1980; Section 2.3.3) were retained in isolated cells. These included the slightly dilated cisternae of the rough endoplasmic reticulum (RER) filled with flocculent material (Fig.3.3b), a well-developed Golgi system (Fig.3.3c), mitochondria, multivesicular bodies (Fig.3.4a) and a single cilium (Fig.3.4b).

The ultrastructural morphology of isolated fat-storing cells was similar at all age levels studied. In general, fat-storing cells isolated from 6- to 36-month-old animals were more heavily filled with lipid droplets than those from 3-month-old ones. Figure 3.4c shows that some organelles such as lipid-containing lysosomes occasionally observed in fat-storing cells in the intact liver (cf. Fig.2.9b), were also found in isolated cells.

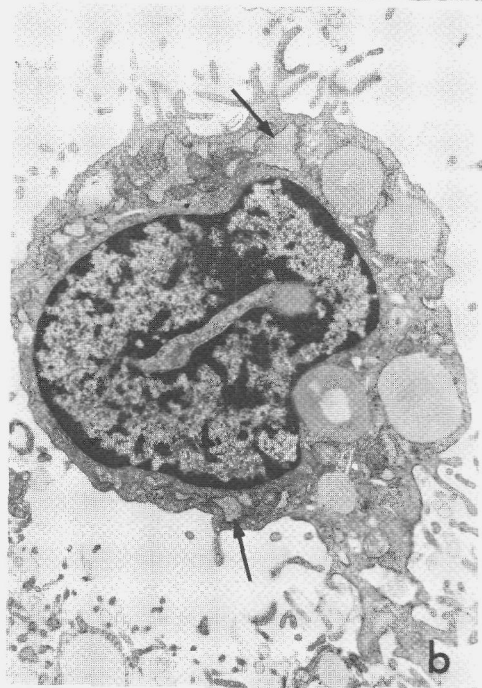
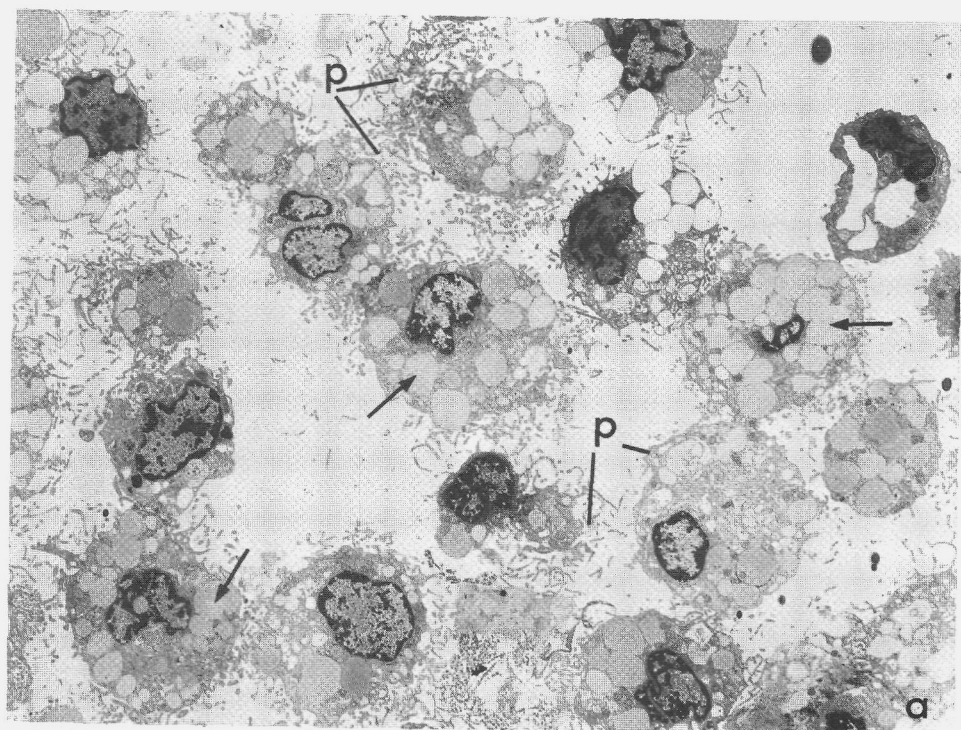
3.3.4.4 Other cells isolated from rat liver

Most other cells isolated from rat liver, recovered mainly in fractions B after centrifugation in Nycodenz, belonged to various populations of blood cells. Major representatives were small and intermediate lymphocytes (Fig.3.5) which were mainly recovered in fraction I after centrifugal elutriation.

Portions of fractions B (1-15%) and of the pellets remaining after centrifugal

Figure 3.3 Fractions of freshly isolated and purified fat-storing cells.

- a. Low magnification transmission electron micrograph of a fraction A isolated from a 24-month-old rat. Fat-storing cells contain lipid droplets (†). The cell membrane is highly irregular due to large numbers of thin cellular processes (p). 2400 x.
- b,c. Fat-storing cells from fraction A of 12-month-old rats (b). Besides some lipid droplets, the cytoplasm of this cell contains dilated cisternae of the RER (†). 7550 x. (c) A Golgi system can also be recognized. 61900 x.



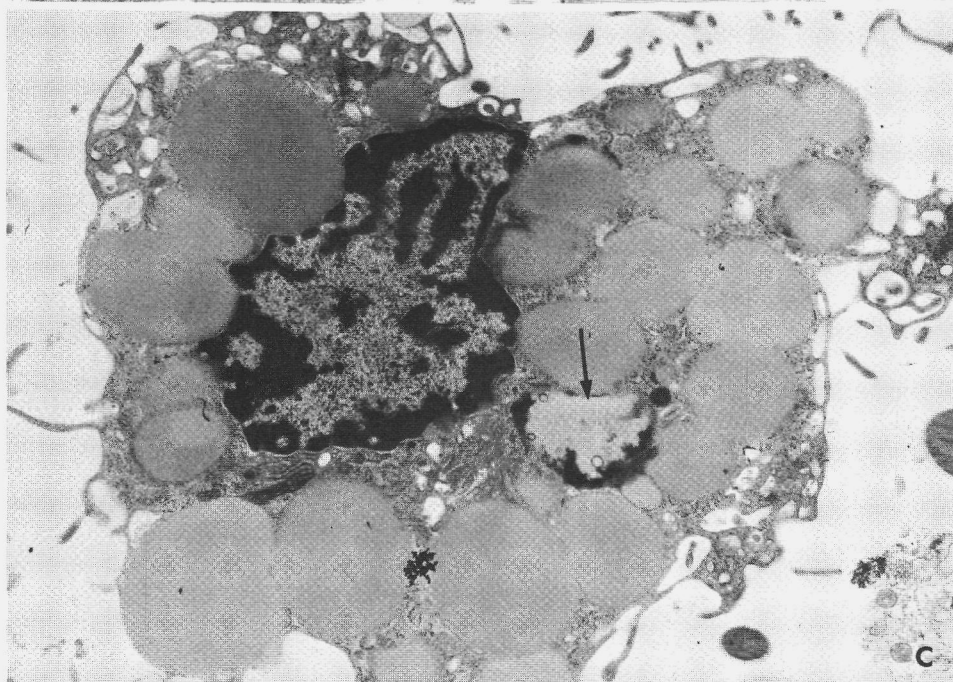
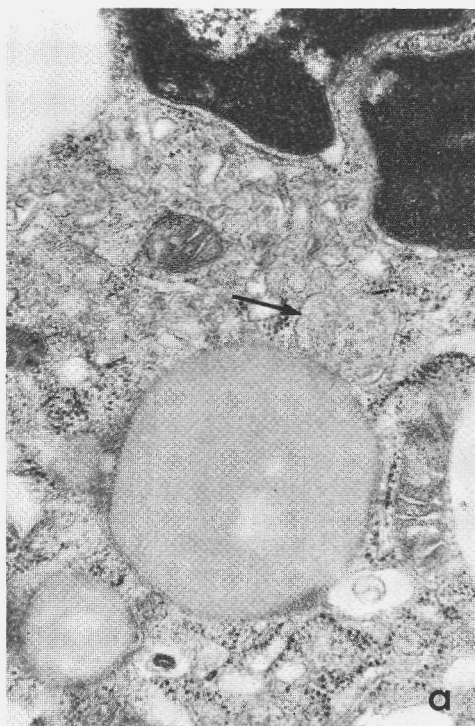
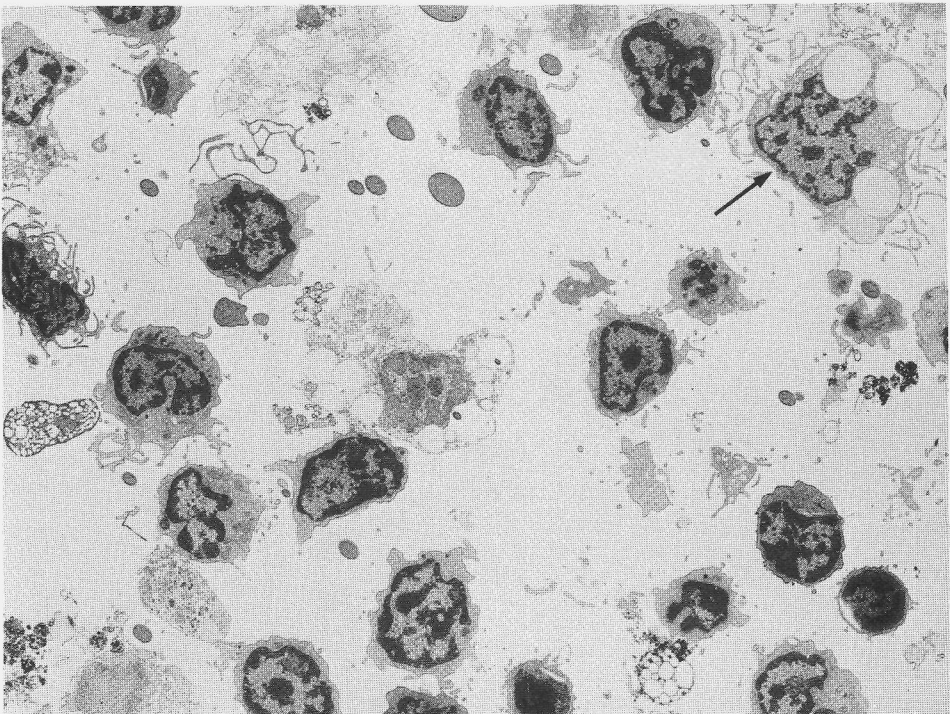
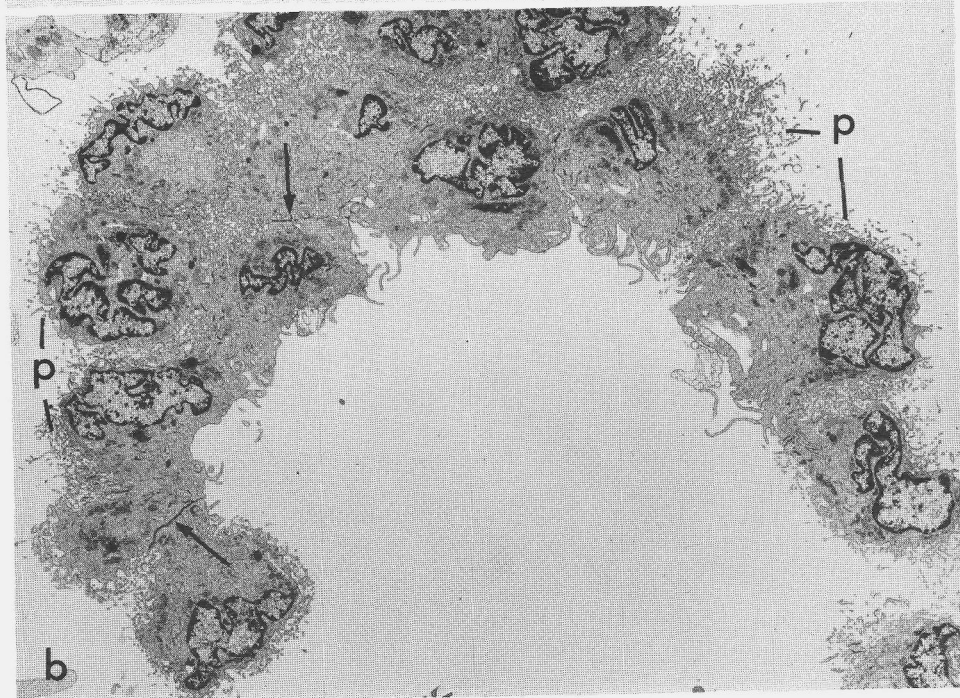
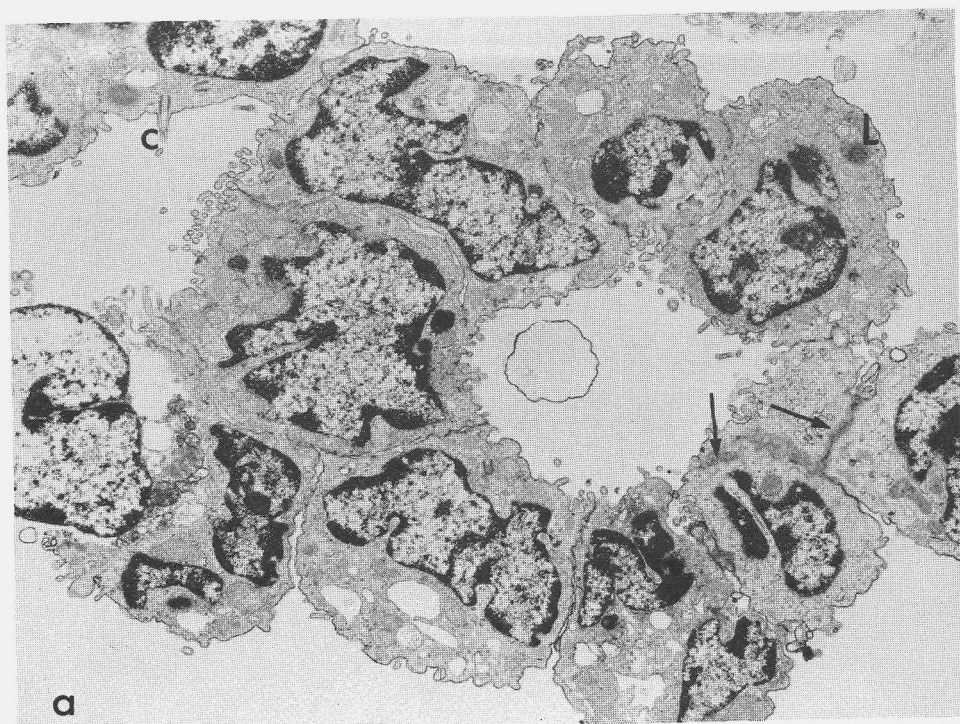


Figure 3.4 Fractions of freshly isolated and purified fat-storing cells.
 a,b. Fat-storing cells from fraction A of a 12-month-old rat. Multi-vesicular bodies (a;↑) and cilia (b;↑) are found also in cells immediately after isolation. (a) 35350 x and (b) 34800 x.
 c. Fat-storing cell from fraction A of a 36-month-old rat. The cell contains a large number of lipid droplets. A lysosome-like structure containing lipid (↑) can be recognized. 9450 x.

elutriation were formed by epithelial cells of bile ductules and ducts. Figures 3.6a and 3.6b reveal that two types of cells were found. These were often attached to each other by desmosomes, forming small groups of 5 to 10 cells. Single cells were regularly encountered in all elutriation fractions. Epithelial cells of bile ductules and ducts were characterized by irregular shaped nuclei showing deep indentations and the presence of several lipid droplets in their cytoplasm. One cell type showed large numbers of microvilli (Fig.3.6b).

Figure 3.5 Fraction I of a 24-month-old rat.
 This fraction contains mainly small and intermediate lymphocytes. A fat-storing cell can also be recognized (↑). 2650 x.





3.4 DISCUSSION

The values for total nonparenchymal cell yield per gram of liver weight (42 to 74 $\times 10^6$ cells; cf. Table 3.2) obtained in the series of experiments described in this chapter are higher than or similar to those reported in the literature (see also Table 3.4) (Bissell et al., 1972; Emeis and Planqué, 1976; Sleyster et al., 1977; Polliack and Herskho, 1978; Blomhoff et al., 1984). Comparison of fractions B with nonparenchymal liver cell suspensions obtained by digestion of the liver with pronase alone, shows that these are largely comparable both with regard to cell yield and cellular composition (Emeis and Planqué, 1976; Sleyster et al., 1977). The major advantage of the isolation procedure combining pronase and collagenase is the simultaneous isolation of fat-storing cells in relatively high yields. These cells are scarce in fractions obtained by other isolation procedures (Sleyster et al., 1977).

The ratio of the yields of the three sinusoidal liver cell types remained the same irrespective of total nonparenchymal cell yield for 12- to 36-month-old animals. The yield of fat-storing cells was significantly lower at 3 and 6 months of age as compared with the other age levels. However, from determinations of the relative numbers of sinusoidal endothelial, Kupffer and fat-storing cells in the intact liver (cf. Section 2.3.4), no indications of a change in the composition of the sinusoidal cell population at various age levels were found. It is unlikely therefore that the low yield of fat-storing cells at 3 and 6 months of age is due to a lower incidence of these cells per unit of liver as compared with the other age levels. It may be speculated that the lower yield of fat-storing cells at young age results from their firm attachment either to other cells or to components of the extracellular matrix.

The values calculated for sinusoidal liver cell yield per gram of liver tissue must be interpreted carefully. Firstly, the cell yield cannot be related to the liver weight of the same animal, due to the cell isolation methodology. Determination of the liver weights of animals of the same sex, strain and age, however, reveal variations if they relate to different animal cohorts (Kitani et al., 1982; Brouwer et al., 1985). Secondly, due to the prevalence of biliary cysts in female BN/BiRij

Figure 3.6 Transmission electron micrographs of pellets recovered after centrifugal elutriation of cells obtained from 6-month-old rats.

- a,b. Pellets consist mainly of clusters of cells kept together by junctional complexes ($\dagger$). Two types of cells are recognized:
 - a. Cells with indented nuclei and a relatively high nuclear-cytoplasmic ratio. The cytoplasm contains some lipid droplets (l) and cilia (c) are sometimes observed. 6650 x.
 - b. Cells with almost lobulated nuclei and a low nuclear-cytoplasmic ratio. The cytoplasm contains strands of RER, some dense mitochondria and lipid droplets. The cell membrane is highly irregular due to large numbers of microvillous processes (p). 3250 x.

TABLE 3.4 LITERATURE DATA ON CELL YIELD FROM RAT LIVER
USING DIFFERENT CELL ISOLATION PROCEDURES

<u>Species</u> ^o	<u>Cell yield</u> [*]	<u>References</u> ⁺
A. 10 ⁶ cells per 100 g body weight		
1. Sprague-Dawley, ♂ (225-400 g)	53 (range:23-129)	Bissell et al.,1972 ^a
2. Wistar, ♂ (180-250 g)	70 n=20 (range:40-120)	Emeis and Planqué,1976 ^a
3. Wistar, ♂ (±200 g)	56 n=8 120 n=8 126 n=8	Blomhoff et al.,1984 ^b " " ^c " " ^d
B. 10 ⁶ cells per g liver weight		
4. BN/BiRij, ♀ (140-170 g; 3 mo)	25 n= 3 19 n= 2 37 n=21	Knook et al, 1977 ^c " " ^b " " ^a
5. Wistar, ♀ (100-200 g)	50	Polliack and Hershko,1978 ^a
6. Sprague-Dawley, ♂ (250 g)	2 n=5 (range:2-3)	McPhie, 1979 ^a
7. Wistar, ♀ (180 g)	7 n=6	Ottenwalder and Bolt,1981 ^b
8. BN/BiRij, ♀ (3 mo)	44	Brouwer et al.,1984 ^e
BN/BiRij, ♀ (12 mo)	61	

^o When found in the references, strain, sex, body weight and age of the animals used, are given.

^{*} Cell yields are given per 100 g body weight (A) or per g liver weight (B). When found in the references, the range in cell yield and the number of experiments from which the average cell yields were calculated, are given.

⁺ The characters a to e relate to the different cell isolation procedures:

a : pronase perfusion

b : collagenase perfusion

c : collagenase perfusion followed by pronase incubation

d : collagenase perfusion followed by enterotoxin incubation

e : pronase-collagenase perfusion

rats the assessment of functional liver mass by determination of liver weight may prove to be difficult. Considering this, no firm conclusions can be drawn from the age-related values for sinusoidal liver cell yield per gram of liver weight. However, if the tendency would prove to be correct, that with age more nonparenchymal cells can be isolated per liver, this would indicate that the ratio between parenchymal and sinusoidal liver cells changes with age (see below).

The polyploidy level of rat liver parenchymal cells increases during development and aging (Van Bezooijen et al., 1984), a phenomenon which is accompanied by an increase in cell volume. It was, for example, calculated for female WAG/Rij rats (assuming that a $2n$ cell has half the volume of a $4n$ and of a $2 \times 2n$ cell, which have half the volume of a $8n$ and of a $2 \times 4n$ cell, etc.) that the mean cell volume of the hepatocytes increases significantly between 0.5 and 1.5 months of age, increases only slightly between 1.5 and 30 months of age, and somewhat more thereafter (Van Bezooijen et al., 1984). In rats of the same strain and sex, the liver weight and the protein content of hepatocytes, a good parameter for cell volume increases between 3 and 36 months of age in a pattern similar to that observed for the calculated increase in the mean cell volume of hepatocytes (Van Bezooijen, 1978; Horbach et al., 1984). Considering that hepatocytes constitute about 70% of total rat liver volume (Blouin et al., 1977), it appears that the age-related increase in liver weight is mainly due to an increase in the average volume of the hepatocytes. From the similarity in the increase in mean cell volume and in liver weight, it may even be speculated that the number of hepatocytes per whole liver does not change with age.

Changes in the average volume and the number of sinusoidal liver cells will remain unnoticed in the total liver volume, because of their small contribution (Blouin et al., 1977). In the present studies, indications were obtained that, with age, an increased number of sinusoidal liver cells can be isolated per liver (cf. Table 3.2) and that the number of sinusoidal cells per unit of liver tissue remains constant (cf. also Tables 2.1 and 6.1). If these data prove to be correct, it would indicate that the ratio between parenchymal cells and sinusoidal liver cells is not a constant with age (see footnote*).

In the study described in this chapter, there were several indications, that a procedure resulting in optimal isolation and purification of different liver cell classes at a given age (Knook et al., 1982b) cannot be directly applied to other age groups. Major variables which may influence separation characteristics include:

* For example, the liver weight of female WAG/Rij rats increases by a factor of 1.4 between 3 and 36 months of age (Horbach et al., 1984). The ratio between parenchymal and sinusoidal liver cells in 3-month-old NEDH rats approximates 64:36 (Greengard et al., 1972). Assuming that the number of parenchymal cells per total liver remains constant and the number of sinusoidal cells remains constant per unit of liver tissue with age, it can be calculated that the ratio between parenchymal and sinusoidal liver cells in female WAG/Rij rats approximates 56:44 at 36 months of age.

(i) variations in the cell number subjected to density centrifugation; (ii) changes in the cellular composition of the fractions; and (iii) changes in cell volume. Examples of these are: firstly, the shift of endothelial cells from fraction B to A at 30 and 36 months of age, which may be due to overloading of the density gradient (Brouwer et al., 1984); secondly, the increase in the number of bile duct epithelial cells isolated with increasing age and thirdly, the necessity to adjust the flow rate with increasing age for the purification of Kupffer cells in the centrifugal elutriation procedure. The last point indicates that the cell volume of Kupffer cells increases with age in agreement with the observation that "old" Kupffer cells have an increased protein content (Sleyster and Knook, 1980). It can be concluded from these observations that, to obtain optimal results in cell yield and purity with aging, experimental conditions for cell isolation and separation must be adjusted for any given situation.

The ultrastructural morphology of isolated cells with age was remarkably stable. With the exception of some fractions obtained from 36-month-old animals which contained damaged cells, there were no indications of increased lability of cells with age. As in the intact liver (cf. Section 2.3), sinusoidal endothelial cells and Kupffer cells were seen to contain increasing amounts of ferritin with age and similarly fat-storing cells contained increasing numbers of lipid droplets.

The value of a careful characterization of isolated cell suspensions with regard to cell viability and composition cannot be stressed enough. Any contamination with cell debris or viable cell parts (parenchymal cell "blebs": Nagelkerke et al., 1982) may significantly influence conclusions on the contribution of a certain cell type to one aspect of hepatic function. Determination of the cellular composition by electron microscopy and/or characterization of cell fractions according to cell-specific biochemical parameters seem to be the only two reliable methods for determining the value of isolated liver cell suspensions (Brouwer et al., 1982b; Van Berkel; 1982).

CHAPTER 4

CULTURES OF NONPARENCHYMAL LIVER CELLS

4.1 INTRODUCTION

Primary cultures and cell lines of liver parenchymal cells and primary culture systems of some nonparenchymal liver cells, have been used widely in hepatic research, such as in the field of pharmacology, toxicology, endocrinology and metabolism (Knook, 1985a).

The use of liver cell cultures offers several advantages over that of freshly isolated cells. Firstly, culturing makes possible the recovery of the cells from damage occurring during cell isolation, for example, to the metabolic system of the cells (Pogson et al., 1984) and to cell membrane receptors (Munthe-Kaas et al., 1975). Secondly, investigations can be conducted over the longer periods of time necessary, for example, for those processes which occur at low rate and frequency. Although the availability of epithelial cell lines derived from various types of liver cells (Grisham, 1979) may be useful in studying processes such as liver-specific carcinogenesis, data on normal hepatic function obtained in these in vitro systems must be evaluated critically due to rapid dedifferentiation and the occurrence of phenotypic drift in cells in culture (Tseng et al., 1982, 1983).

A general problem when using primary cultures of liver cells is contamination by cell types other than those under investigation. Although the contamination of isolated and purified cell suspensions of all major liver cell types (i.e., parenchymal, endothelial, Kupffer and fat-storing cells) can be reduced to less than 15% of the total number of cells (Sleyster et al., 1977; Van Bezooijen, 1978; Knook et al., 1982b), it cannot be excluded that in long-term cultures the initially major cell type is gradually replaced by one which is either favoured by the culture conditions (Munthe-Kaas et al., 1975) or has higher potential for replication.

Cocultures of two or more types of liver cells may be a useful model system for studying those aspects of hepatic function which require intensive cellular cooperation such as, for example, vitamin A metabolism (Hendriks et al., submitted). However, application of such liver cell cultures as model systems requires clearly defined culture conditions as well as an exact knowledge of the cellular composition of the cultures over the entire experimental period (Grisham, 1983).

In this chapter, cultures of nonparenchymal liver cells established under various culture conditions are described morphologically. An overview of the

cellular composition of various cultures as a function of culture time is given. In addition, changes in structural characteristics of cells in coculture are compared with those of purified cells in culture.

4.2 MATERIALS AND METHODS

4.2.1 Isolation procedure and culture conditions

The experiments were performed with 3-, 12-, 24- and 36-month-old rats. Non-parenchymal liver cells were isolated as described earlier (Section 3.2.1; Knook et al., 1982b). Erythrocytes and cell debris were removed by centrifugation in a discontinuous single layer Nycodenz[®] density gradient (Brouwer et al., 1984). Nonparenchymal liver cells were harvested from the top of the Nycodenz gradient and suspended in Dulbecco's modification of Eagle's (DME) medium with L-glutamine containing 20 mM HEPES and 10 mM NaHCO₃ (pH 7.4) and supplemented with penicillin (100 units.ml⁻¹) and streptomycin (100 µg.ml⁻¹). This medium will be further referred to as the basic culture medium. Sterile conditions were maintained during the entire isolation procedure.

Nonparenchymal liver cells were cultured in plastic 24-well culture plates (Falcon 3047) at a seeding density of 1×10^6 cells per 2.0 cm² well.

Four culture conditions were chosen: cells were cultured with or without a collagen substratum in basic culture medium supplemented with either 20% fetal calf serum (Gibco Biocult) or 30% homologous rat serum (prepared in our Institute). The culture plates were incubated at 37°C in a humidified atmosphere containing 3% CO₂ in air. The culture medium was refreshed every two days.

4.2.2 Preparation of collagen-coated culture dishes

Tendons were dissected from the tails of 4 female BN/BiRij rats. They were incubated in 200 ml of 0.1% acetic acid in H₂O for 24 h at 4°C to dissolve the collagen. The undissolved remnants were then centrifuged for 2 h (1000 x g; 4°C). The collagen remained in the supernatant. The collagen-containing solution was dialyzed against a 200-fold volume of distilled water at 4°C in the dark for 24 h prior to use. One drop of this solution was equally distributed on the bottom of the plastic culture dishes. The dishes were incubated at room temperature for 4 h in an atmosphere of ammonia. After this period, they were rinsed three times with distilled water and three times with DME medium. A visible film of collagen of about 20 µm thickness remained firmly attached to the bottom of the wells.

4.2.3 Morphological examinations

Sample cultures were prepared for transmission electron microscopy at 3, 6 and 18 h and 1, 2, 3, 5 and 7 days in culture. Cells were fixed *in situ* in 2% glutaraldehyde in 0.15 M sodium cacodylate buffer (pH 7.4) for at least 10 min,

followed by postfixation in 1% OsO_4 in the same buffer for 20 min. The cells were dehydrated in a graded series of ethanol and directly embedded in LX 112 (Ladd Research Industries). After polymerization of the embedding medium at 60°C for two days the plastic culture dishes were removed. Semithin sections (0.5 μm) and ultrathin sections (70–100 nm) were cut parallel to the bottom of the culture dishes. Semithin sections were stained with toluidine blue and ultrathin ones were contrasted with uranyl acetate and lead citrate.

4.2.4 Endogenous peroxidatic activity

Cells in culture were tested for endogenous peroxidatic activity at days 1, 2, 3, 5 and 7. For the demonstration of peroxidatic activity, they were fixed for 10 min in 2% glutaraldehyde in 0.15 M sodium cacodylate buffer (pH 7.4) and washed three times in 0.05 M Tris/HCl buffer (pH 7.4) containing 7% sucrose (w/v). They were then incubated at 27°C in a medium containing 0.05% 3,3-diaminobenzidine tetrachloride (Merck) and 0.02% H_2O_2 in 0.05 M Tris/HCl buffer with 7% sucrose for 1 h. Following rinsing in buffer, the cells were further processed for transmission electron microscopy (Section 4.2.3). Controls were incubated without H_2O_2 .

4.2.5 Endocytosis of latex beads

At days 1, 2, 3, 5 and 7 of culture cells were incubated for 30 min with latex particles (ϕ 0.65 μm ; Sigma Chemical Co.) at a final dilution of 1:41 in culture medium. Following washing, cells were further processed for transmission electron microscopy (Section 4.2.3).

4.3 RESULTS

4.3.1 Choice of optimal culture conditions

The morphological characteristics of isolated nonparenchymal liver cells described in Chapter 3 were used to differentiate between various types of cells in culture in both light and electron microscopic preparations. The main characteristics used for cell identification in toluidine blue-stained semithin sections were the sponge-like appearance of the sinusoidal endothelial cells with their retracted cell extensions, the ruffled cell membrane and the presence of a heterogeneous population of vacuoles (lysosomes) in Kupffer cells, the presence of lipid droplets in fat-storing cells and the duct-like structures representing clusters of bile duct epithelial cells.

Light microscopic investigation of semithin sections provided a rapid screening of the cellular composition of the cultures as a function of time. Similarly, evaluation of cells under the four culture conditions enabled the choice of an optimal condition for studying in detail the effects of coculturing on the three types of sinusoidal liver cells.

Sinusoidal endothelial cells in coculture with the other nonparenchymal liver cells exhibited light microscopically similar characteristics in the presence of both fetal calf and homologous rat serum when seeded on a collagen substratum. Without collagen, sinusoidal endothelial cells became attached to the plastic surface of the culture dishes and survived for some time in both fetal calf and homologous rat serum. However, cell spreading was much less pronounced than on collagen and most endothelial cells were removed by changing of the culture medium at 48 h.

The morphological characteristics of Kupffer cells in coculture were not otherwise influenced by culturing under different conditions, than by a gradual accumulation of large numbers of lipid droplets in Kupffer cells in cultures supplemented with homologous rat serum.

The behavior of fat-storing cells in coculture was influenced very little by the various culture conditions.

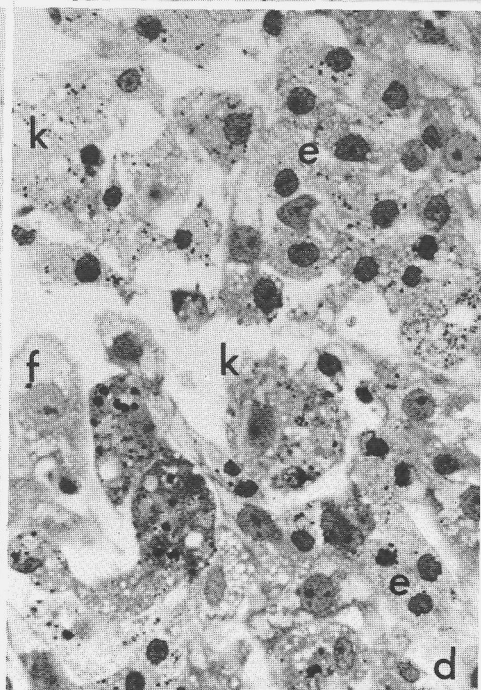
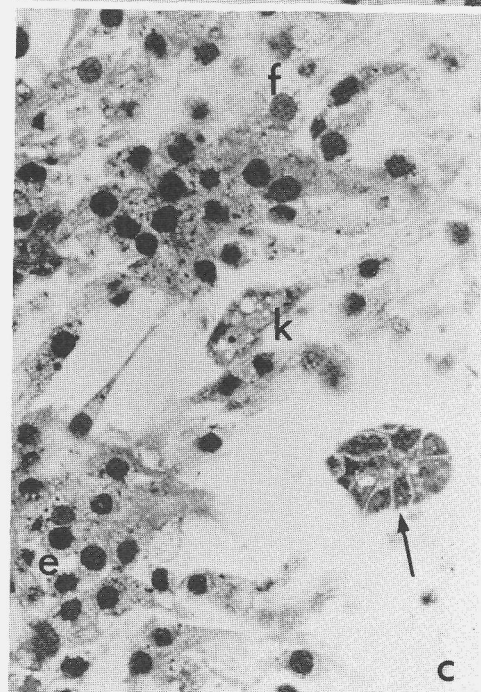
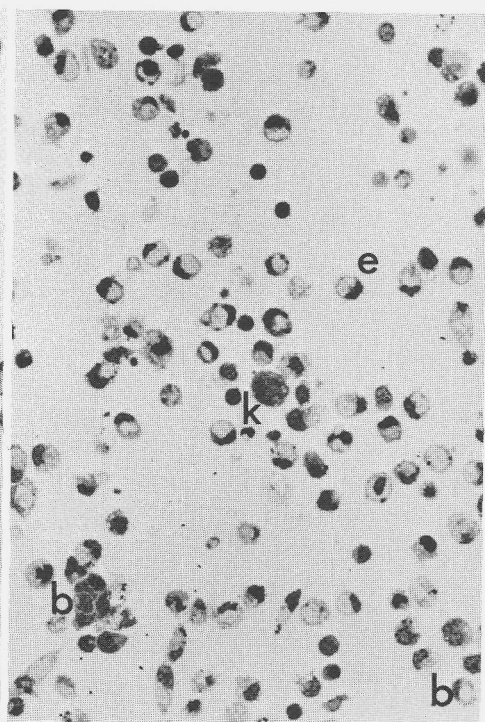
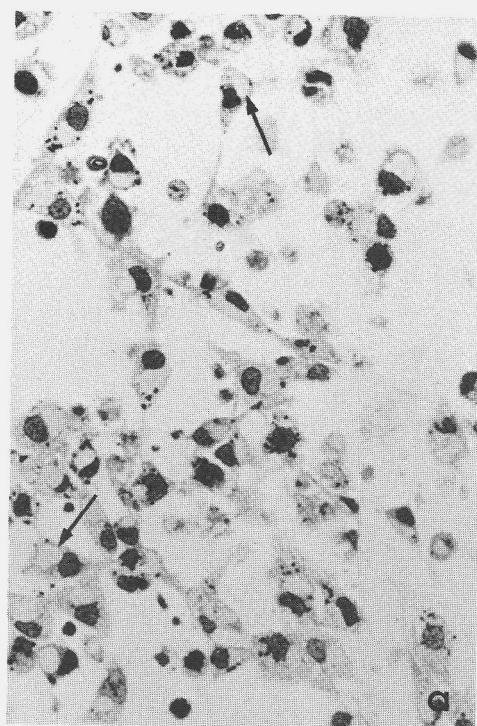
On the basis of these observations, cultures of nonparenchymal liver cells seeded on a collagen substratum in a medium supplemented with fetal calf serum were chosen to study in detail the effects of coculturing on the morphology and behavior in culture of the three types of sinusoidal liver cells.

4.3.2 Cellular composition of cocultures as a function of culture time

When sampled after 3 h of culture, the only cells adhering to the collagen substratum were endothelial cells (Fig.4.1a). Some of these cells already revealed spreading of their fenestrated cell processes, which became clustered close to the nucleus during the cell isolation procedure (cf. Section 3.3.4.1). In sections cut more above the collagen substratum, all three types of sinusoidal cells were observed to still exhibit the morphology of cells immediately after isolation (Fig.4.1b).

Figure 4.1 Light micrographs of semithin sections (toluidine blue staining) of cultures of nonparenchymal liver cells derived from rats of various ages.

- a. Cells cultured for 3 h; 12-month-old rat. Mainly endothelial cells are spread out on the collagen substratum. The retracted cell processes containing the sieve plates are seen as clear areas close to the nucleus (†). Note the dark spots in the cytoplasm representing iron-ferritin containing lysosomes. 440 x.
 - b. Cells cultured for 3 h; 12-month-old rat. Farther above the collagen substratum, cells still exhibit the morphology of isolated cells. 440 x.
 - c. Cells cultured for 24 h; 24-month-old rat. Mainly endothelial cells, at this stage characterized by vacuoles, are spread out on the collagen substratum. Note the bile duct-like structure (†). 440 x.
 - d. Cells cultured for 48 h; 24-month-old rat. Kupffer and fat-storing cells mingled with groups of endothelial cells are now observed. 440 x.
- Endothelial cells (e); Kupffer cells (k); fat-storing cells (f); bile duct epithelial cells (b).



At between 18 and 24 h all endothelial cells attached to the collagen layer exhibited a flattened appearance as a result of the respreading of cell processes (Fig.4.1c). A large proportion of the area available for cell attachment was thereby covered by endothelial cells. At these culture stages, some Kupffer and fat-storing cells were seen to be mingled with or present at the edges of groups of endothelial cells (Fig.4.1c); both cell types were more flattened due to spreading on the substratum as compared with 3 h of culture.

At between 24 and 48 h in culture, the number of Kupffer cells and fat-storing cells spreading on the collagen substratum between the groups of endothelial cells gradually increased (Figs.4.1d and 4.2a).

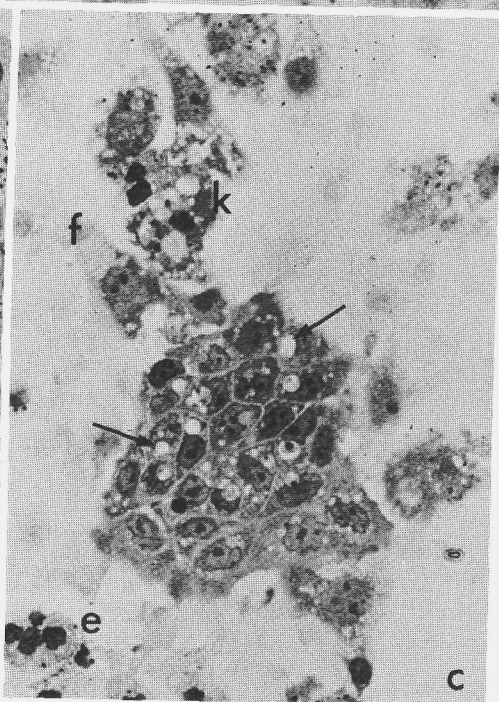
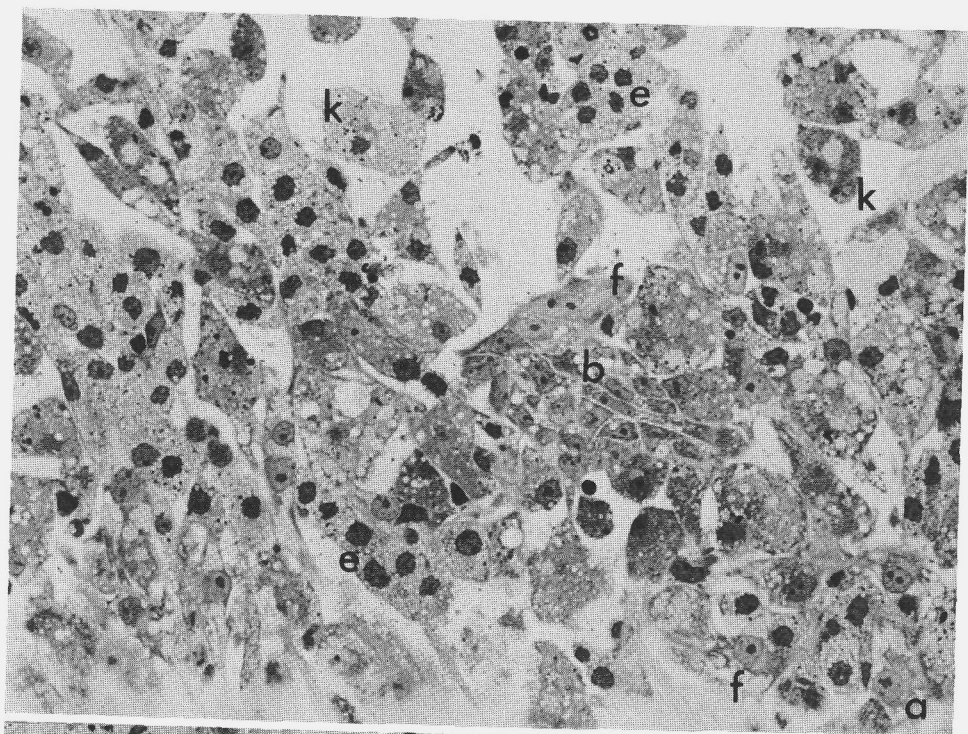
At 3 days in culture and thereafter, viable looking endothelial cells were only rarely observed (Fig.4.2b). From this stage onwards, Kupffer cells and fat-storing cells represented about 95% of the cells in culture. Mitoses were regularly observed in fat-storing cells in cultures from 48 h and thereafter (Fig.4.2b), resulting in a predominance of this cell type in cultures at day 7 (not shown).

Groups of epithelial-like cells were occasionally observed in cultures (Figs.4.1c and 4.2c). They appeared to be attached to each other and were thought to originate from groups of epithelial cells of bile ductules and ducts reported to be present in nonparenchymal liver cell suspensions (cf. Section 3.3.4.4).

It was discussed in Chapter 3, that nonparenchymal liver cell suspensions obtained from rats of various ages were not completely comparable with regard to their cellular composition. In suspensions obtained from 12-, 24- and 36-month-old animals, the percentage of fat-storing cells was higher than in those from 3-month-old rats (cf. Table 3.1). In cultures of nonparenchymal liver cell suspensions of 3-month-old rats, fat-storing cells were only rarely detected during the first 48 h, but more regularly in cultures obtained from animals of 12 months and older. Probably due to the high mitotic activity of this cell type, this difference in detectability was no longer obvious at culture stages from 72 h and thereafter. The culture characteristics of nonparenchymal liver cells were not otherwise influenced by the age of the donor animal.

Figure 4.2 Light micrographs of semithin sections (toluidine blue staining) of cultures of nonparenchymal liver cells derived from rats of various ages.

- a. Cells cultured for 48 h; 12-month-old rat. Endothelial cells and bile duct epithelial cells are seen mainly as groups, Kupffer and fat-storing cells as single cells. 410 x.
- b. Cells cultured for 5 days; 24-month-old rat. At this stage, cultures consist mainly of Kupffer and fat-storing cells. 440 x.
- c. Cells cultured for 48 h; 24-month-old rat. Groups of bile duct epithelial cells closely associated and characterized by lipid-like vacuoles (†) are regularly found. 440 x. Endothelial cells (e); Kupffer cells (k); fat-storing cells (f); bile duct epithelial cells (b).



4.3.3 Ultrastructural morphology of nonparenchymal liver cells in coculture

The ultrastructural morphology of sinusoidal cells in culture will be discussed in detail in Chapters 5 to 7, in which cultures of pure suspensions of, respectively, sinusoidal endothelial cells, Kupffer cells and fat-storing cells are described.

Sinusoidal endothelial cells in coculture with other nonparenchymal liver cells revealed morphological changes which were comparable with those observed in cultures of purified endothelial cells (Fig.4.3a)(cf. Section 5.3.2). The presence of other nonparenchymal liver cells did also not result in a prolongation of the survival time of endothelial cells in culture.

The ultrastructural morphology of Kupffer cells in coculture with the other nonparenchymal liver cells differed considerably from that of purified Kupffer cells in culture (Fig.4.3b)(cf. Section 6.3.4). The lysosomes of Kupffer cells in coculture were more heavily filled with substances such as cellular debris and lipid droplets than those of purified Kupffer cells in culture. In addition lipid droplets were observed free in the cytoplasm (Fig.4.3b).

The ultrastructural morphology of fat-storing cells in coculture with other nonparenchymal liver cells and their behavior in culture was not influenced as compared with purified fat-storing cells in culture (Fig.4.4a)(cf. Section 7.3.1).

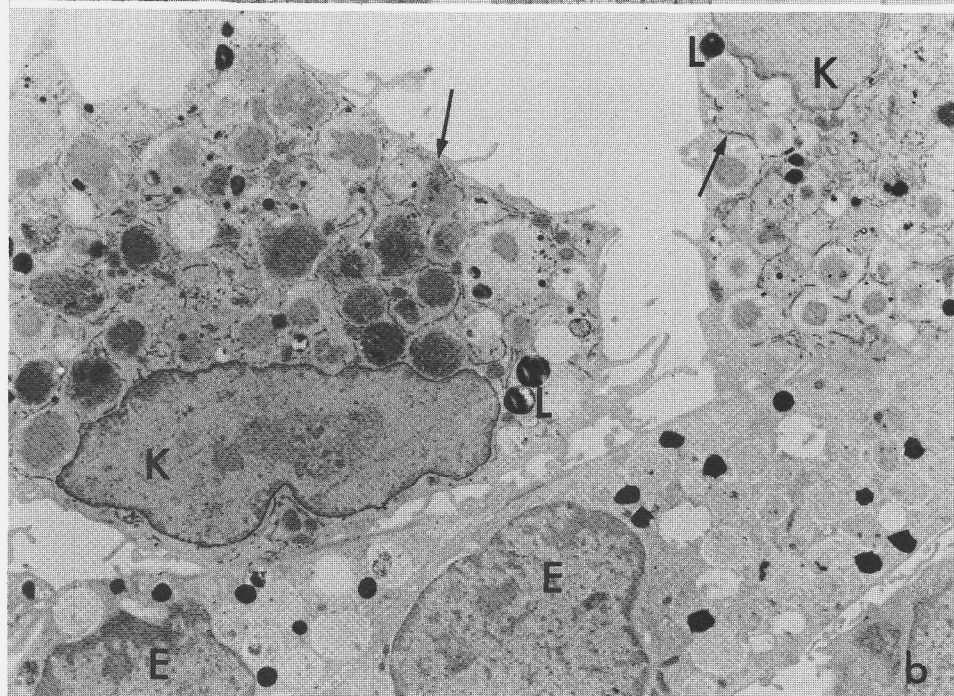
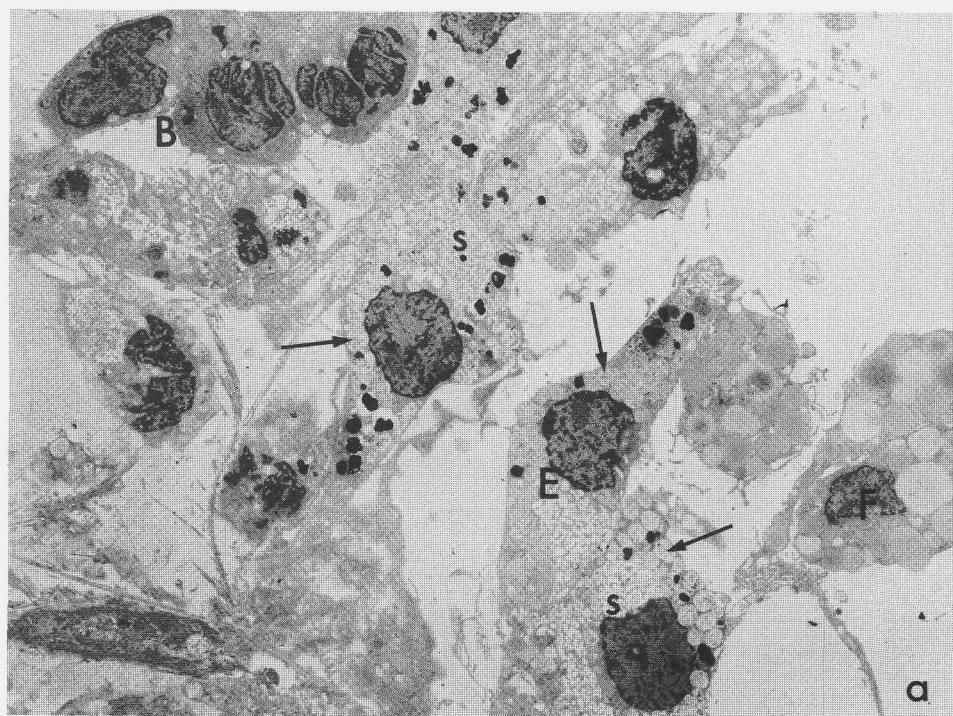
Groups of bile duct epithelial cells were occasionally encountered (Fig.4.4b). These were characterized as a number of cells attached to each other by large junctional complexes. Major characteristics were the highly indented nucleus and the inconspicuous cytoplasm containing only a few lipid droplets and lysosomal structures (Fig.4.4b). Lumen formation was sometimes observed, in which microvilli from the surrounding cells projected.

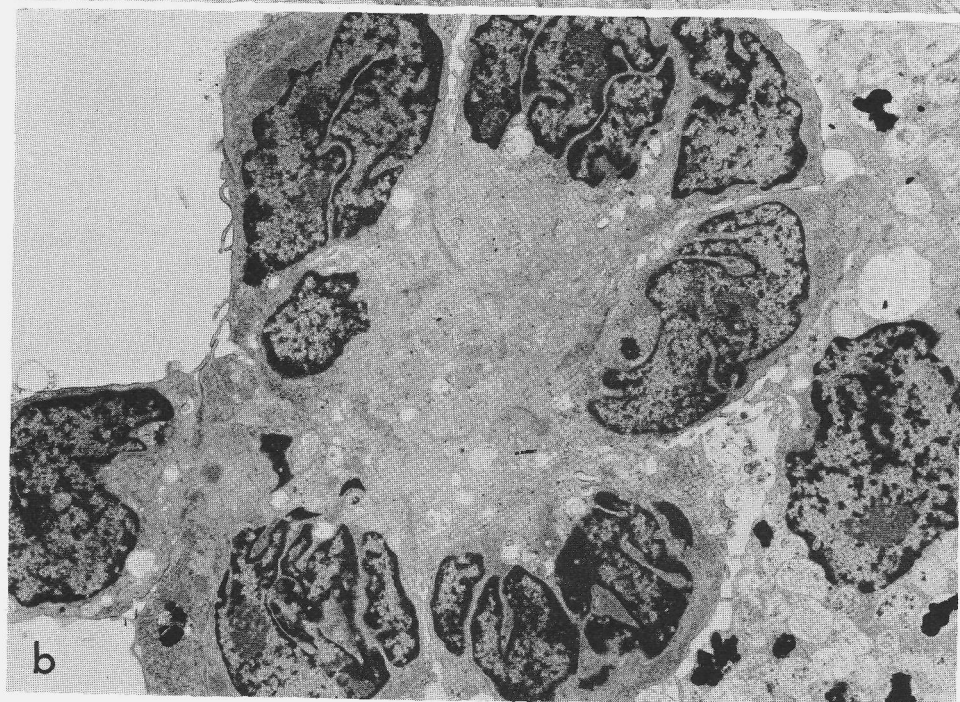
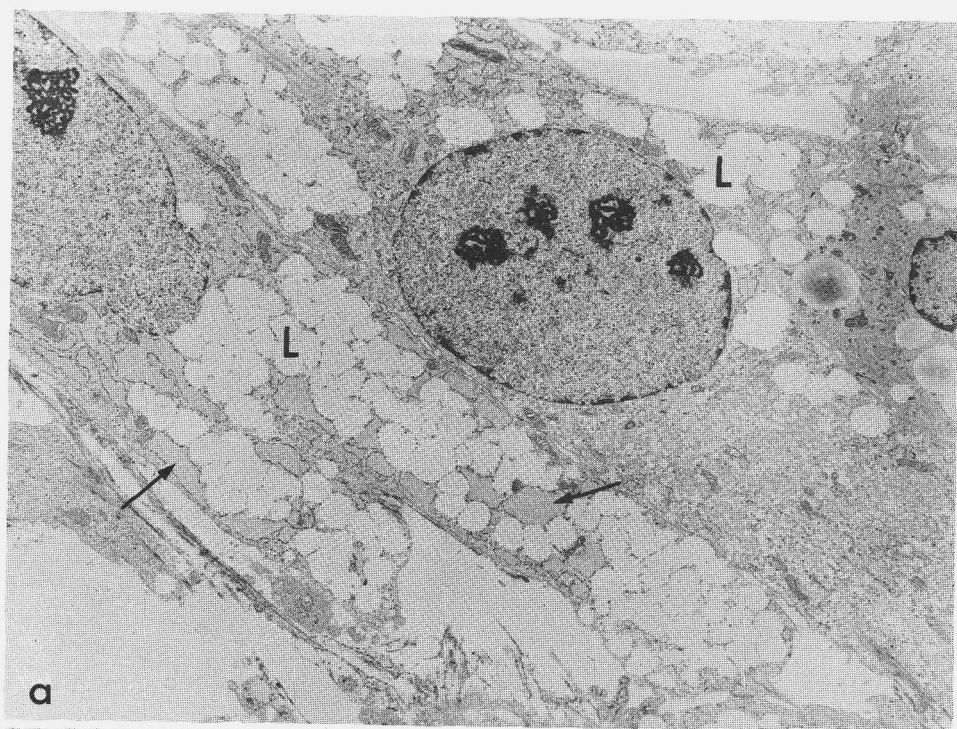
4.4 DISCUSSION

Culturing of nonparenchymal liver cell suspensions obtained by pronase digestion of the liver was originally applied to achieve selective enrichment of Kupffer cells by making use of the capacity of macrophages in general to attach to

Figure 4.3 Transmission electron micrographs of cultures of nonparenchymal liver cells derived from rats of various ages.

- a. Cells cultured for 18 h; 24-month-old rat. Endothelial cells are characterized by their sponge-like appearance (s) and the occurrence of vacuoles filled with flocculent material (†). 2050 x.
- b. Cells cultured for 24 h; 3-month-old rat. Endogenous peroxidatic activity (†) characterizes Kupffer cells containing lysosomes heavily filled with cellular debris and lipid-like material (l). 5100 x.
Endothelial cells (e); Kupffer cells (k); fat-storing cells (f); bile duct epithelial cells (b).





glass and plastic surfaces (Melly et al., 1972; Munthe-Kaas et al., 1975; Emeis and Planqué, 1976; Brouwer et al., 1980). An additional advantage of culturing was the reoccurrence of receptor-mediated endocytic activity in Kupffer cells, shown to be absent in freshly isolated cells. This reoccurrence allowed investigation of endocytic processes in cells *in vitro* (Munthe-Kaas et al., 1975; Munthe-Kaas, 1976; Kirn et al., 1982; Brouwer et al., 1985).

Survival of sinusoidal endothelial cells in culture is observed only when cells are not isolated by pronase at 37°C and when cultured under very rigorous conditions. Steffan et al. (1981) cultured purified suspensions of both rat and human sinusoidal endothelial cells isolated by collagenase on a collagen substratum in culture media supplemented with homologous serum. Smedsrød et al. (1982) and Roos et al. (1982) cultured collagenase-isolated purified rat endothelial cells on, respectively, fibronectin and serum-coated surfaces in culture media supplemented with fetal calf serum only. Praaning-van Dalen and Knook (1982) isolated sinusoidal liver cells by pronase at 8°C, a procedure which preserved membrane receptors. Cultures of purified rat liver endothelial cells obtained by pronase at 8°C (Praaning-van Dalen, 1983) or by combining pronase and collagenase (cf. Chapter 5) showed attachment and survival of these cells only when seeded on a collagen substratum in medium supplemented with homologous rat serum. Finally, in coculture with the other nonparenchymal liver cells, endothelial cells could also be maintained in culture on a collagen substratum in the presence of fetal calf serum (this chapter).

Considering these data, it can be concluded that certain requirements must be fulfilled for culturing liver sinusoidal endothelial cells as was also suggested for endothelial cells originating from other parts of the body (De Groot et al., 1982). On the one hand, culturing of liver endothelial cells seems to be strongly dependent on some degree of expression of cell surface membrane receptors required for interaction with attachment proteins such as fibronectin (Couchman et al., 1983). On the other the presence of an attachment protein, specific for liver endothelial cells also seems to be a prerequisite. As may be speculated from the literature data, either fibronectin or another factor which is either derived from homologous serum or (as in coculture) from other cells, may function as such an attachment protein. In addition, both fibronectin and collagen seem to mediate a second phase in adhesion, namely, cell spreading (Couchman et al., 1983).

The behavior of Kupffer and fat-storing cells, on the contrary, appeared to be

- Figure 4.4 Transmission electron micrographs of cultures of nonparenchymal liver cells derived from rats of various ages.
- Cells cultured for 6 days; 3-month-old rat. Fat-storing cells are characterized by the presence of lipid droplets (l) and dilated cisternae of the RER (†). 3200 x.
 - Cells cultured for 18 h; 24-month-old rat. Group of bile duct epithelial cells forming a duct-like structure. 4650 x.

relatively independent of cell isolation procedures and culture conditions. Kupffer cells isolated by either pronase, collagenase or a combination of the two behaved similarly (Melly et al., 1972; Munthe-Kaas et al., 1975; Emeis and Planqué, 1976; Brouwer et al., 1980; Kirn et al., 1982; Chapters 4 and 6). A disadvantage of Kupffer cell cultures derived from nonparenchymal liver cell suspensions is the large amount of cellular debris present in these cultures at various stages. As was indicated by Brouwer et al. (1980), the endocytic activity of Kupffer cells in coculture due to this cell debris may result in an increase in the activities of several lysosomal enzymes.

The presence of fat-storing cells in nonparenchymal liver cell suspensions resulted in an overgrowth of the cultures by this cell type after 5 to 7 days. Overgrowth of both parenchymal liver cell cultures and nonparenchymal liver cell cultures by fibroblastic cells has been occasionally reported (Bennett, 1966; Melly et al., 1972; Emeis and Planqué, 1976; Marceau et al., 1980). It may be postulated that at least some of these cells originated from a small number of fat-storing cells present in the isolated cell suspensions.

No effect of the donor age on the survival time in culture or on the proliferative capacity of nonparenchymal liver cells was observed.

The value of coculturing different types of liver cells has been rather limited up to now. It was shown that the survival time of parenchymal liver cells was significantly increased by coculturing with nonparenchymal liver cells (Wanson et al., 1979). In the present study, such cell-cell interactions were not observed both with regard to the survival time in culture of each cell type and to the culture characteristics. Coculturing of parenchymal cells with epithelial cell lines also derived from liver has proved to be a more useful model for studying parenchymal cell function in vitro, because of the highly differentiated phenotypic expression of parenchymal cells in these culture systems (Guguen-Guillouzo et al., 1983).

Coculturing of different types of liver cells would be useful only for those aspects of hepatic function in which two or more liver cell types closely cooperate, such as in vitamin A metabolism. However, cellular characteristics and culture conditions must then mimic the in vivo situation as closely as possible.

CHAPTER 5

MAINTENANCE CULTURES OF SINUSOIDAL ENDOTHELIAL CELLS

5.1 INTRODUCTION

Sinusoidal endothelial cells of the mammalian liver form the continuous lining of the liver sinusoids and constitute a barrier between the sinusoidal blood and the liver parenchymal cells (Wisse, 1970; Gendrault et al., 1982). In contrast to other capillary endothelial cells, the sinusoidal endothelial cells have no basement membrane and possess pores approximately 0.1 μm in diameter which are arranged in clusters, the sieve plates (Fahimi, 1982). Through these fenestrations, sinusoidal endothelial cells influence the filtration of particles from the blood to the parenchymal cells and vice versa (Naito and Wisse, 1978).

Several investigations on the uptake capacity of liver endothelial cells have shown that these cells play a considerable role in the clearance of a variety of substances from the blood (Hubbard et al., 1979; Praaning-van Dalen et al., 1981, 1982; Van Berkel, 1982).

Liver cell preparations which are obtained by enzymatic digestion with pronase have limited use in the study of cellular processes which require interaction with specific cell membrane proteins (Praaning-van Dalen et al., 1982). In culture, however, reconstitution of specific membrane proteins was indicated for Kupffer cells which allowed the investigation of receptor-mediated endocytosis by these cells (Munthe-Kaas et al., 1975; Brouwer and Knook, 1977; Munthe-Kaas, 1976; Wanson et al., 1979; Brouwer et al., 1982a).

Therefore, the aim of the study described in this chapter was to develop a maintenance culture system for purified rat liver sinusoidal endothelial cells. Such a system may provide a model for studies on endocytosis and for investigation of interactions of liver endothelial cells with blood constituents or other cells.

5.2 MATERIALS AND METHODS

5.2.1 Isolation procedure and culture conditions

The experiments were performed with 3-, 12-, 24-, 30- and 36-month-old rats. Nonparenchymal liver cells were isolated as earlier described (Section 3.2.1; Knook et al., 1982b). Fat-storing cells, erythrocytes and cell debris were removed from the nonparenchymal cell suspensions by centrifugation in a discontinuous two-layer Nycodenz[®] density gradient (Section 3.2.1).

Sinusoidal endothelial cells were purified from the nonparenchymal cell suspensions by centrifugal elutriation (Section 3.2.1). The endothelial cell fraction

was centrifuged at 400 x g for 10 min and the pelleted cells were used to establish maintenance cultures of sinusoidal endothelial cells. Sterile conditions were maintained during the entire isolation and purification procedures.

Cell viability and the purity of the endothelial cell fraction were determined as described in Section 3.2.2. Cell counts were performed in a hemocytometer.

Purified endothelial cells were suspended in basic culture medium supplemented with 30% homologous rat serum (prepared in our Institute) and cultured in plastic 24-well culture plates (Falcon 3047) coated with collagen at a seeding density of 1×10^6 cells per 2.0 cm^2 well (Sections 4.2.1 and 4.2.2). The cultures were incubated as described in Section 4.2.1.

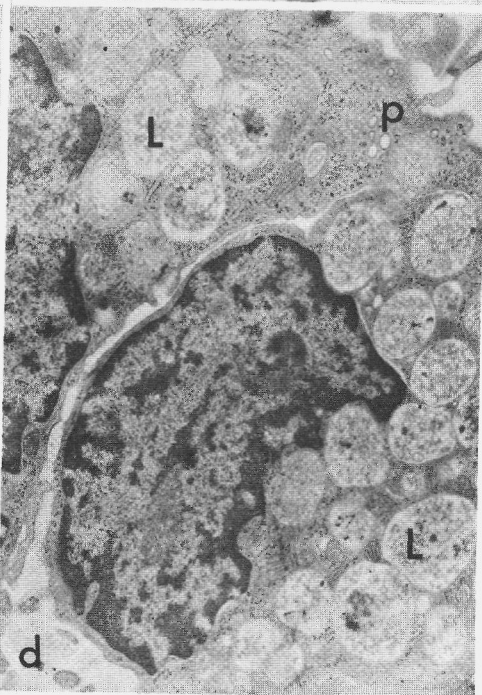
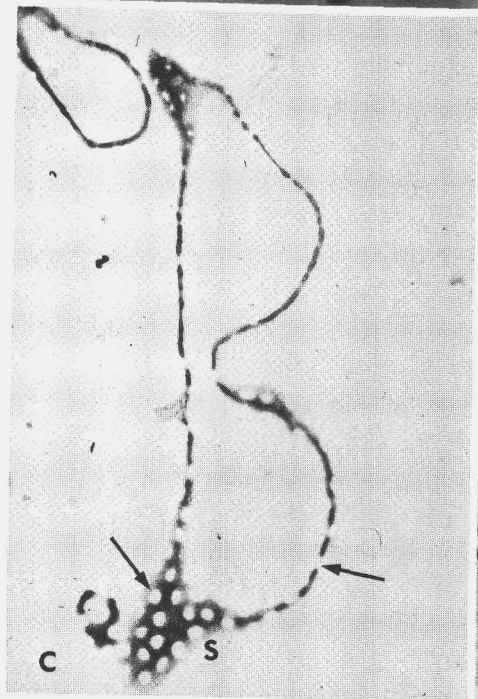
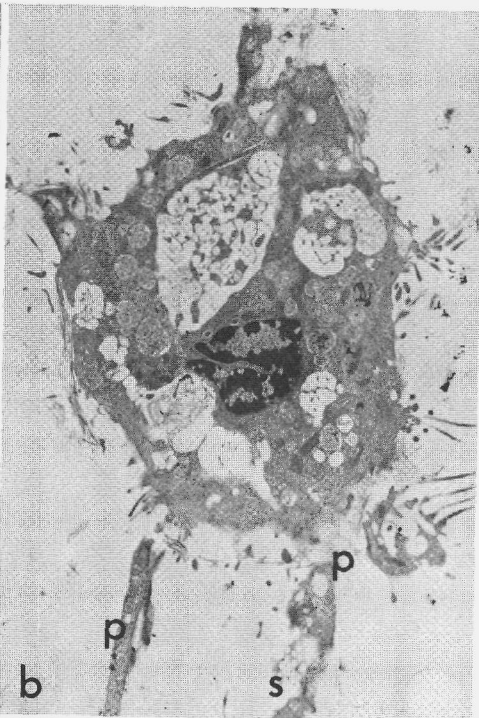
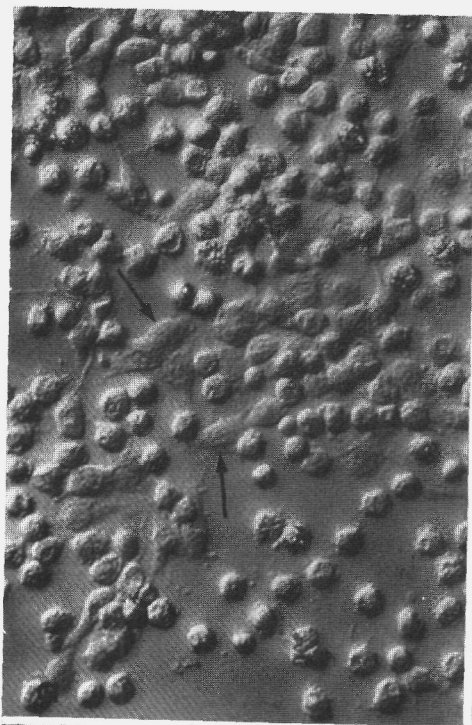
5.2.2 Morphological examinations

Sample cultures were taken at every 3 h during the first 24 h of culture and then at each 24 h. For light microscopic examinations by phase or Nomarski contrast, cells were grown on collagen-coated glass coverslips. At various time points, coverslips were removed from the culture wells, fixed for 30 min in 2% glutaraldehyde in 0.15 M sodium cacodylate buffer (pH 7.4), washed in the same buffer and mounted on microscope slides by placing the inverted coverslips on a drop of rapid mounting medium (Entellan, Merck).

For transmission electron microscopy, cells were processed as described earlier (Section 4.2.3).

Scanning electron microscopy of sinusoidal liver cells was performed in collaboration with R. de Zanger from the laboratory of Cell Biology and Histology, Free University of Brussels, Belgium. For scanning electron microscopy, freshly isolated cells were fixed for 15 min in 1.5% glutaraldehyde buffered with 0.1 M sodium cacodylate (pH 7.35) containing 2 mM CaCl_2 . Cells were collected by millipore (pore size, $0.45 \mu\text{m}$) filtration and postfixed in 1% OsO_4 for 1 h. Cells grown in culture on collagen-coated glass coverslips ($\emptyset$ 13 mm; $1.5\text{--}2.0 \times 10^6$ cells per coverslip) for 3 and 28 h were fixed for 1 h at room temperature by immersion in the 1.5% glutaraldehyde fixative. Some of the cultures were osmicated for 1 h immediately after glutaraldehyde fixation. Others were rinsed in 0.1 M phosphate buffer for 15 min and then treated with a mixture of 1% guanidine-HCl, 1% arginine-HCl, 1% glycine and 1% tannic acid (modified after Murakami, 1978). After a 1

Figure 5.1 Light micrograph (a) and transmission electron micrographs (b to d) of sinusoidal endothelial cells from 3-month-old rats cultured for 3 h.
a. Nomarski contrast. Spreading of some of the cells ($\dagger$). 400 x.
b. The thin cytoplasmic processes (p) containing the sieve plates (s) are respread. 4100 x.
c. Reformation of endothelial lining containing the sieve plates (s). Fenestrae ($\dagger$). 17000 x.
d. Occurrence of lysosome-like structures (l); pinocytic vesicles (p). 11000 x.



h treatment with this mixture, specimens were rinsed in distilled water for 1 h and stained with a half-saturated solution of uranyl acetate for 30 min and rinsed. For critical point drying, specimens were dehydrated in a graded series of ethanol and dried over carbon dioxide in a Polaron critical point drying apparatus. Finally, a relatively thin layer of gold was applied by sputtering. The specimens were viewed in a Philips 505 electron microscope at 20 kV.

5.2.3 Endogenous peroxidatic activity

Cells in culture were tested for the presence of endogenous peroxidatic activity as described in Section 4.2.4.

5.2.4 Endocytosis

Cells cultured for 24 h were incubated for 30 min with a mixture of latex particles ($\emptyset$ 0.460 and 0.806 μm ; Sigma Chemical Co.) at a final dilution of 1:41 in culture medium or for 15 min with culture medium containing 5 $\text{mg}\cdot\text{ml}^{-1}$ horseradish peroxidase (HRP)(Sigma Chemical Co.). In the latter case, the cells were fixed for 1 h in glutaraldehyde to destroy the endogenous peroxidatic activity (Fahimi, 1970). The endocytosed HRP was then demonstrated with the same procedure as described for the endogenous peroxidatic activity (Section 4.2.4).

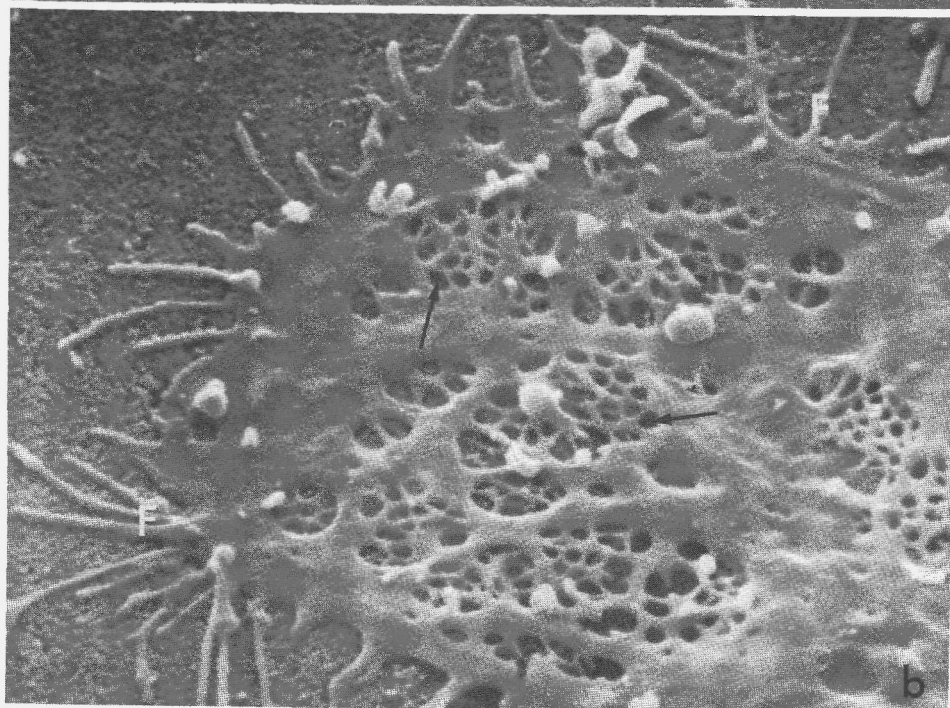
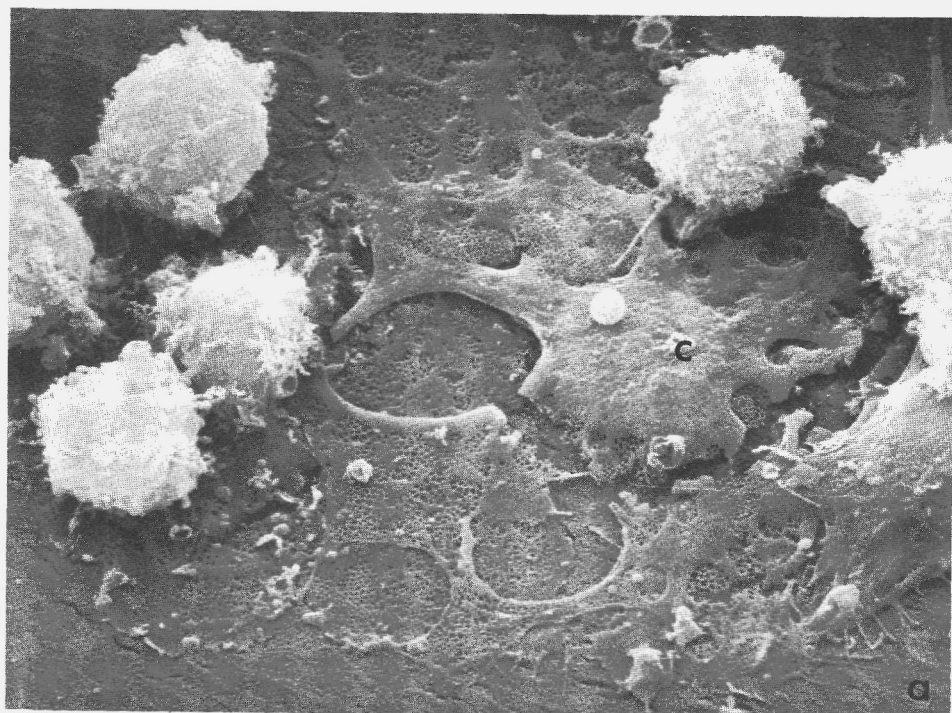
5.3 RESULTS

5.3.1 General culture conditions

Sinusoidal endothelial cells isolated from rat liver by enzymatic digestion with pronase and collagenase and purified by centrifugal elutriation (cf. Section 3.2.1) showed attachment and large scale cell survival only when cultured on a collagen matrix in medium containing homologous rat serum. Under the other experimental conditions as described in Section 4.2.1, sinusoidal endothelial cells remained spherical and were removed by changing the culture medium.

Figure 5.2 Scanning electron micrographs of sinusoidal endothelial cells from 3-month-old rats cultured for 3 h.

- a. One endothelial cell has already extended thin cytoplasmic processes containing the sieve plates (s). Other cells are still rounded up. Cell body (c). 3000 x.
- b. Detail of a cytoplasmic process showing well-preserved sieve plates and very thin anchoring fibers (f). Fenestrae (†). 12000 x.
(Photographs by R. de Zanger; Free University of Brussels, Belgium).



5.3.2 Morphological observations

5.3.2.1 Cells cultured for 3 hours

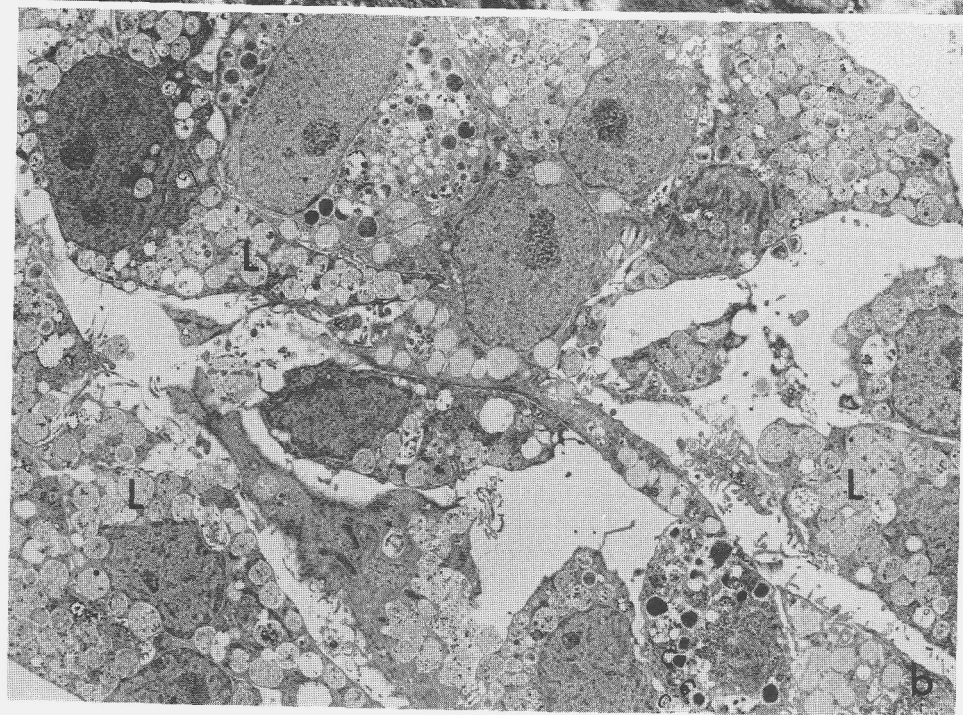
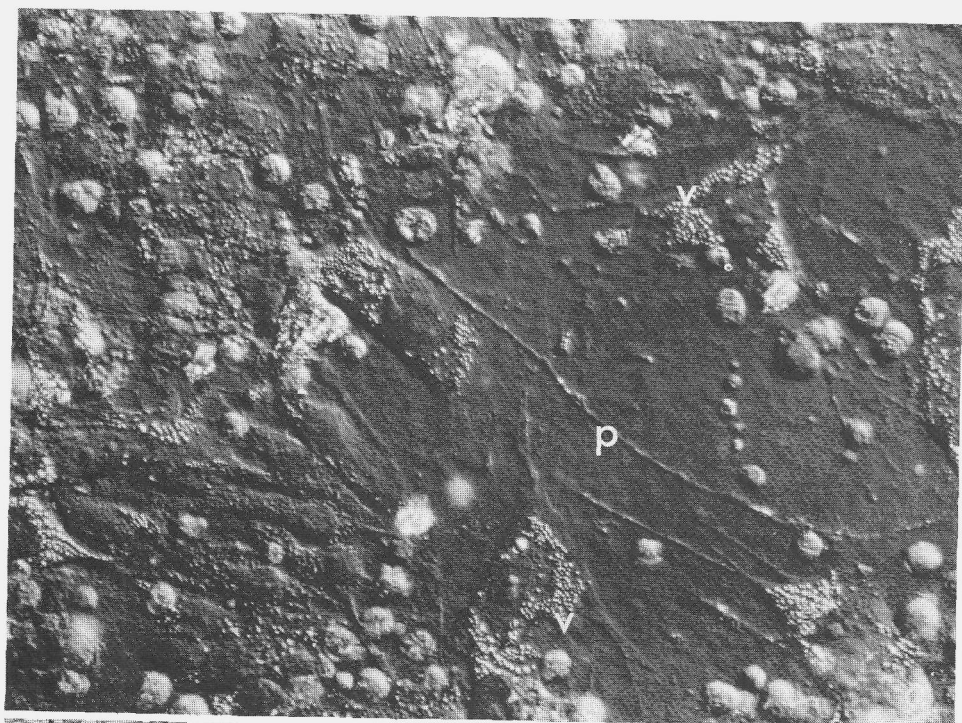
At 3 h in culture, some of the endothelial cells were already firmly attached to the collagen matrix (Fig.5.1a). This attachment was accompanied by a marked change in ultrastructure (Fig.5.1b). The smooth cell membrane characteristic of freshly isolated cells was lost (cf. Section 3.3.4.1). The cells seemed to reextend their fenestrated processes, as they showed long thin cytoplasmic projections, some of which had a characteristic sieve plate appearance (Fig.5.1c). Lysosome-like structures filled with flocculent material appeared in the cytoplasm (Fig.5.1d). Sinusoidal endothelial cells isolated from rats of various ages exhibited similar phenomena.

Scanning electron microscopy showed that some of the cells had spread on the collagen matrix. The spreading of some of these occurred in two more or less opposite directions, giving rise to the formation of a spindle-shaped cell (see also Fig.5.5a). The distal ends of the cells were usually more or less flattened and contained fenestrations. Small clusters of fenestrae were also sometimes seen in the thicker parts of the cell. Other cells had spread more symmetrically in all directions (Fig.5.2a). This resulted in the formation of a central cell body containing the nucleus surrounded by very thin parts of cytoplasm with fenestrae clustered in sieve plates and several somewhat thicker parts around the sieve plates radiating as short arms from the cell body (Fig.5.2a). Only a very small number of microvilli was seen on these cells. The fenestrae in the sieve plates appeared to be somewhat larger (about 0.15 μm) in size than seen in perfusion-fixed liver (about 0.10 μm) (De Zanger et al., 1982).

Besides fenestrae, cells also contained small pits on their surface, probably pinocytic vesicles in the process of pinching off. An interesting phenomenon not normally encountered in endothelial cells in the intact liver was the occasional occurrence of very thin processes. Like anchoring fibers, these processes radiated from the central body as well as from the edges of the thin cytoplasmic extensions (Fig.5.2b). Whether these threadlike processes serve some function in spreading is not clear at present.

Figure 5.3 Sinusoidal endothelial cells from 3-month-old rats cultured for 24 h.

- a. Nomarski contrast. Endothelial cells are well spread on the collagen substratum, some showing long cytoplasmic processes (p). Vacuoles (v) probably representing the lysosome-like structures observed by transmission electron microscopy (cf. Fig.5.1d) are visible in the cytoplasm. 500 x.
- b. Transmission electron micrograph at low magnification (staining only with lead citrate). All cells are filled with lysosome-like structures (l). 2600 x.



5.3.2.2 Cells cultured for 24 hours

At between 3 and 24 h of culture, an increasing number of endothelial cells was seen to attach to and spread on the collagen matrix. Cells tended to grow in clusters and some of them showed long cytoplasmic projections (Fig.5.3a).

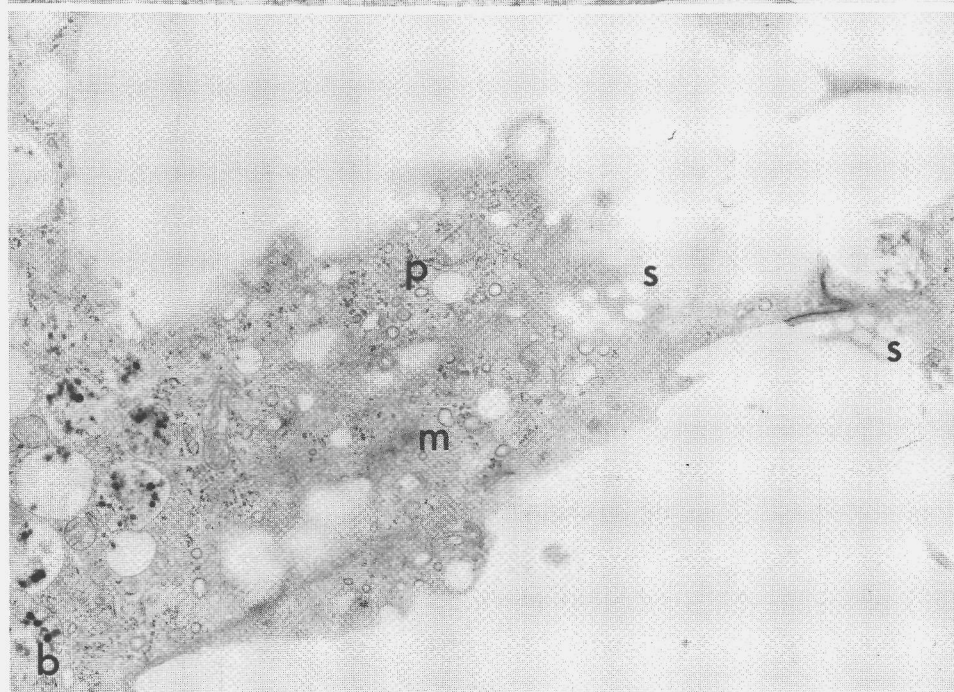
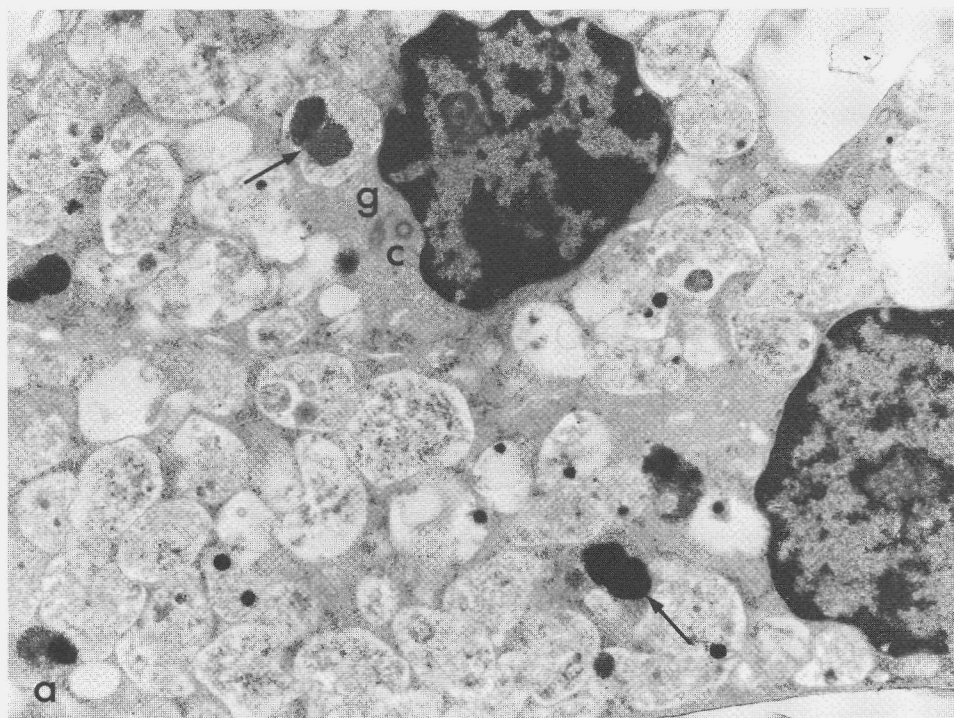
At 24 h of culture, endothelial cells exhibited the following characteristics at the ultrastructural level (Figs.5.3b and 5.4a,b). The cell membrane was smooth and the nucleus round to oval, sometimes showing indentations. Heterochromatin was distributed as a broad rim along the nuclear membrane and as patches throughout the nucleus. One, sometimes two, nucleoli were present (cf. Fig.5.3b). A well-developed Golgi system associated with many small vesicles some of them bristle-coated, was situated close to the nucleus (Fig.5.4a). A pair of centrioles was frequently seen in the same region. Lysosome-like structures which occupied a major portion of the cytoplasm were homogeneous in size ($\approx 1.5 \mu\text{m}$) and appeared to be empty or to contain flocculent material. A few small mitochondria were scattered in the cytoplasm. Short strands of rough endoplasmic reticulum were occasionally observed, the peripheral cytoplasm mainly filled with polyribosomes (Fig.5.4a). Microtubules and microfilaments were prominent, especially in the cytoplasmic processes (Fig.5.4b). Bristle-coated pinocytic vesicles were frequently observed (Fig.5.4b). Except for the presence of ferritin particles in some of the lysosome-like structures, cells isolated from rats of various ages did not differ in any of these morphological aspects.

Sieve plate-like structures with well-preserved fenestrae were observed at the periphery of several cells (Fig.5.4b), where the cytoplasm was extended in thin broad plates reminiscent of those cytoplasmic processes forming the endothelial lining in the intact liver. Scanning electron microscopy of cells at 28 h in culture showed that most of the cells which had attached to and spread out on the collagen matrix had clear endothelial cell characteristics, most prominently demonstrated by the presence of various sieve plates (Fig.5.5a). Small single pits were seen distributed over the surface of the cell body (Fig.5.5a).

5.3.2.3 Cells cultured for 48 hours

At this stage, significant changes with regard to the cellular composition of the cultures were noted. Some clusters of sinusoidal endothelial cells were still regularly encountered (Fig.5.5b). However, the number of Kupffer and fat-storing

Figure 5.4 Transmission electron micrographs of sinusoidal endothelial cells from a 30- (a) and a 3-month-old rat cultured for 24 h.
a. Detail of the cytoplasm. Some of the lysosome-like structures are filled with ferritin particles ($\uparrow$). Golgi system (g); a pair of centrioles (c). 10000 x.
b. Detail of a cytoplasmic process. Microtubules (m); pinocytic vesicles (p); sieve plate (s). 8500 x.



cells seemed significantly increased. At stages later than 48 h, cultures consisted primarily of Kupffer and fat-storing cells (Fig.5.6a).

5.3.3 Endocytosis of horseradish peroxidase and latex particles

Horseradish peroxidase (HRP) is taken up *in vivo* by both Kupffer and endothelial cells (Wisse et al., 1976). Endocytosis of HRP in the liver is supposed to be an adsorptive process, which would imply interaction most likely with a membrane receptor for mannose-terminated glycoproteins (Rodman et al., 1978). Freshly isolated endothelial cells obtained by enzymatic incubation with pronase and/or collagenase at 37°C take up only very small amounts of such substances which require for their endocytosis an interaction with specific membrane receptors (Praaning-van Dalen, 1983).

After 24 h of culture, endothelial cells clearly showed endocytosis of HRP. The reaction product was visible in micropinocytic vesicles close to the cell membrane, in small canaliculi (transfer tubules: De Bruyn et al., 1977, 1983a,b) and in larger lysosomes (Fig.5.6b). It was also sometimes observed in morphological structures resembling the macropinocytic vesicles described by Wisse (1972). Fusion of lysosomes containing HRP with the lysosomal structures filled with flocculent material was occasionally observed (Fig.5.6b).

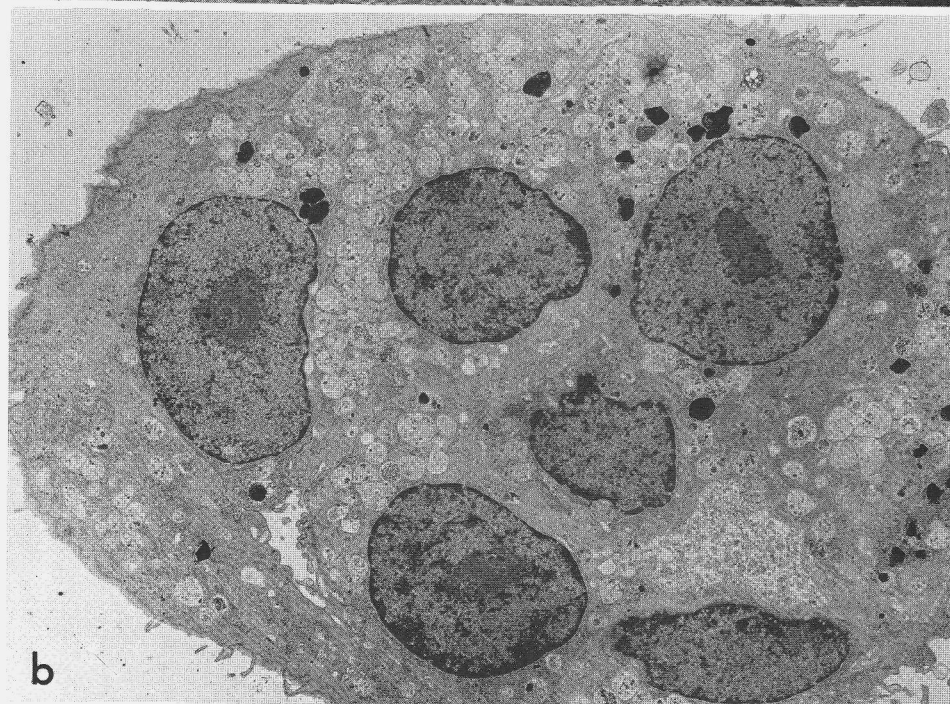
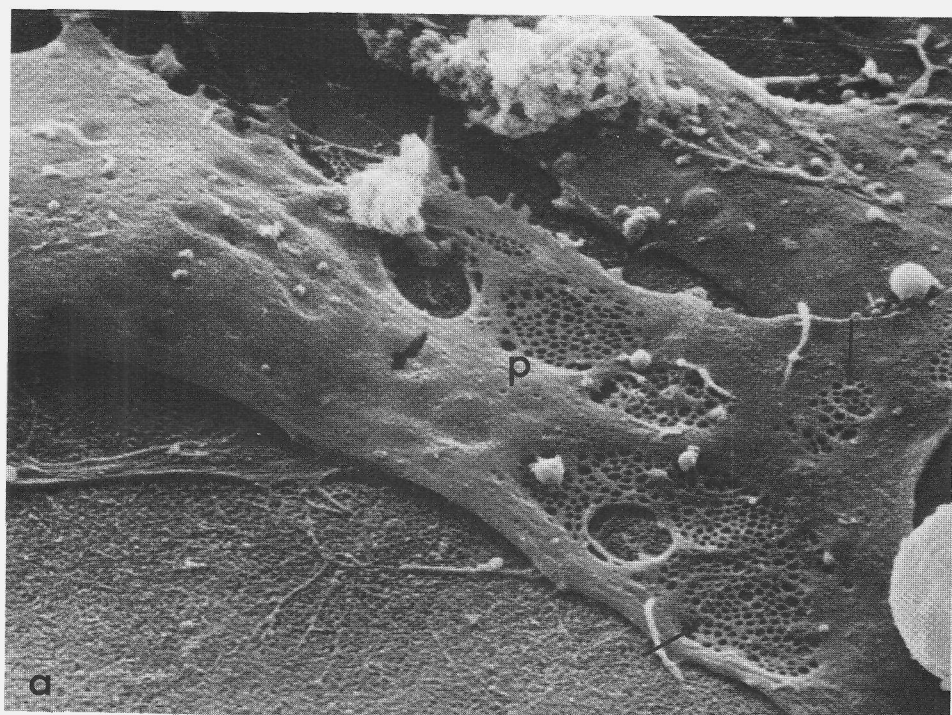
Endocytosis of latex particles was observed only in a few cells which could be clearly distinguished from endothelial cells on a morphological basis and which were identified as Kupffer cells. Endogenous peroxidatic activity was also only found in such cells (Results not shown).

5.4 DISCUSSION

Survival of rat liver endothelial cells in culture was first observed in co-cultures of parenchymal and nonparenchymal liver cells (Wanson et al., 1979). The number of surviving endothelial cells, however, was rather low and reconstitution of cytoplasmic extensions containing the sieve plates was not noted under these conditions. More recently, a number of investigators has described culture systems of purified sinusoidal endothelial cells from both rat and human liver (Steffan et al., 1981; Roos et al., 1982; Smedsrød et al., 1982; this chapter). The require-

Figure 5.5

- a. Scanning electron micrograph of sinusoidal endothelial cells from a 3-month-old rat cultured for 28 h. Sieve plates are noted mainly at the cell periphery (†). The surface of the cell body exhibits pinocytic vesicles (p). 6000 x.
(Photograph by R. de Zanger; Free University of Brussels, Belgium).
- b. Transmission electron micrograph of a group of sinusoidal endothelial cells isolated from a 12-month-old rat at 48 h of culture. 3300 x.



ments for culturing liver endothelial cells have been discussed extensively in Section 4.4.

All reports on liver endothelial cells in culture describe similar phenomena with regard to the behavior and the morphological changes of these cells (Steffan et al., 1981; Roos et al., 1982; Smedsrød et al., 1982; this chapter). Attachment and initiation of spreading of endothelial cells occurs within 3 h after plating. Reconstitution of cytoplasmic extensions containing sieve plates is observed and vacuoles increasing in number with culture time appear in the cytoplasm. The maximum survival time of endothelial cells in maintenance culture is 3 to 4 days at which detachment of cells occurs and fibroblast-like cells are often increased in number (Smedsrød et al., 1982; cf. Section 4.3.2). Recently, Irving et al. (1984) described continuous cell lines derived from rat liver endothelial cells. Such cell lines could be established by the addition of endothelial cell growth supplement and fibroblast growth factor to the culture medium.

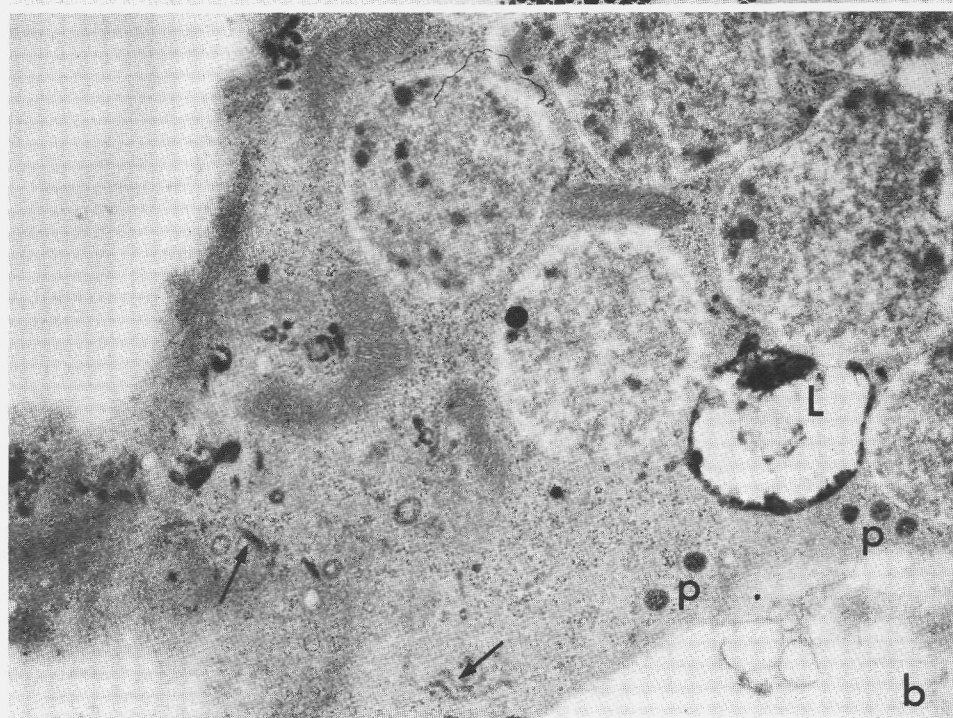
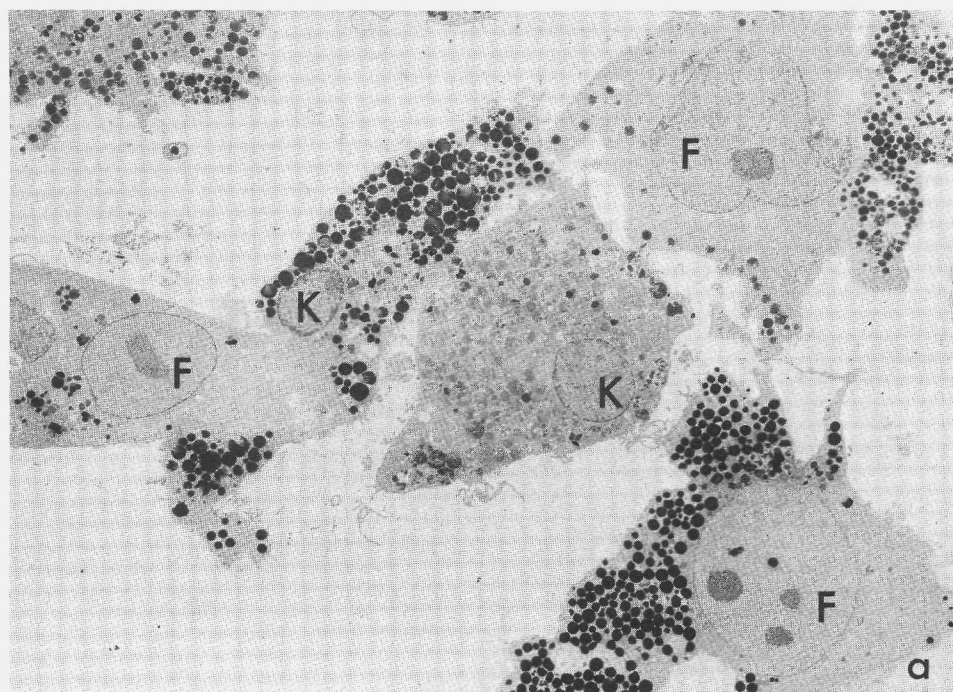
A major difference between endothelial cells in intact liver and in culture is the presence of lysosome-like structures in the cytoplasm of cultured endothelial cells. A possible explanation for the increase in lysosomal volume of endothelial cells in culture may be a metabolic disturbance which prevents normal degradation.

In this study, first indications have been obtained that the capacity of endothelial cells for adsorptive endocytosis, which is almost completely lost during the isolation procedure, is restored during culture. Horseradish peroxidase is taken up in vivo by both Kupffer and endothelial cells (Wisse et al., 1976). Cellular uptake of horseradish peroxidase requires interaction with a membrane receptor specific for mannose-terminating compounds (Rodman et al., 1978). Pronase can damage the protein moiety of receptors and therefore, endothelial and Kupffer cells isolated by pronase show very little endocytosis of, for example, horseradish peroxidase (Praaning-van Dalen, 1983). The uptake of horseradish peroxidase by liver endothelial cells in culture is an indication for the reappearance of specific receptors on the membrane of the cells. Maintenance cultures of endothelial cells can therefore be used to study mechanisms of endocytosis in a manner similar to cultures of Kupffer cells (Brouwer et al., 1982a).

Maintenance cultures of liver endothelial cells seem to be a promising system for studying the regulation of the pore size of the fenestrae by various factors. The sieve plates of liver endothelial cells play an important regulatory role in the

Figure 5.6

- a. Transmission electron micrograph of a five-day-old culture of cells isolated from a 24-month-old rat. Kupffer (k) and fat-storing cells (f) represent the major components. 1450 x.
- b. Transmission electron micrograph of sinusoidal endothelial cells at 24 h of culture incubated with horseradish peroxidase. Uptake is indicated by the presence of reaction product in pinocytic vesicles (p), tubular structures (†) and lysosomes (l). 25300 x.



exchange of substances between sinusoidal blood and parenchymal cells. Chronic ingestion of ethanol (Fraser et al., 1980) as well as certain hormones (Van Dierendonck et al., 1979) have been shown to increase the pore size of the fenestrae in the sieve plates. This increase in pore size has been postulated to affect lipid metabolism in parenchymal cells due to ingestion of larger triglyceride-enriched chylomicrons by these cells. This change in lipid metabolism may be a factor in the pathogenesis of fatty liver mediated by ethanol (Fraser et al., 1980).

Interactions of endothelial cells with other liver cells, blood cells, tumor cells, viruses or bacteria may also be investigated in the in vitro system of liver endothelial cells in culture (Roos et al., 1982; Kirn et al., 1983). Such studies may give insight in the role of endothelial cells as a barrier for parenchymal cells against pathogenic agents present in the blood.

CHAPTER 6

MAINTENANCE CULTURES OF KUPFFER CELLS

6.1 INTRODUCTION

Kupffer cells form the largest population of fixed mononuclear phagocytes in the mammalian body and they represent the major part of the reticuloendothelial system (RES). A decline in the functional capacity of the RES with age has been described in humans (Brouwer and Knook, 1983) and there are indications that specific functions of rat Kupffer cells diminish with increasing age (Knook et al., 1982a; Brouwer and Knook, 1983; Brouwer et al., 1985).

Methods for the isolation, purification and culture of Kupffer cells have greatly facilitated the study of specific functions (Wisse and Knook, 1979; Brouwer et al., 1982a). The value of such in vitro systems for studying physiological cell functions, however, is greatly dependent on the comparability of cells in vitro and in vivo.

The aim of the study described in this chapter was two-fold: (i) to compare the ultrastructure, enzyme cytochemistry and endocytic capacity of Kupffer cells in maintenance culture with these characteristics as described in perfusion-fixed liver (Wisse, 1974a,b, 1977; Praaning-van Dalen et al., 1981); (ii) to compare in maintenance culture Kupffer cells isolated from rats of different ages in a search for possible changes in the characteristics as described in (i), which may reflect age-related functional changes.

6.2 MATERIALS AND METHODS

6.2.1 Isolation procedure and culture conditions

The experiments were performed with 3-, 12-, 30- and 36-month-old rats. Nonparenchymal liver cells were isolated as described previously (Section 3.2.1; Knook et al., 1982b). Erythrocytes and cell debris were removed by centrifugation in a discontinuous single-layer metrizamide density gradient (Knook and Sleyster, 1980). Metrizamide and Nycodenz[®] (cf. Section 3.2.1) have similar properties (Brouwer et al., 1984).

Kupffer cells were separated from the nonparenchymal cell suspensions by centrifugal elutriation as described in Section 3.2.1 in a standard separation chamber (Knook and Sleyster, 1980). A fraction of 150 ml which consisted primarily of Kupffer cells (cf. Table 6.1) was collected at a flow rate of 40, 45 or 50 ml.min⁻¹ for 3-, 12- and 30- to 36-month-old rats, respectively. This fraction was centri-

TABLE 6.1 CHARACTERISTICS OF KUPFFER CELL FRACTIONS
OBTAINED FROM RATS OF VARIOUS AGES

age of rat (mo)	3	12	30	36
Kupffer cell yield (10 ⁶ cells per liver)	34.2 ± 9.5	41.1 ± 5.3	53.5 ± 2.7	61.1 ± 7.8
Kupffer cell yield (10 ⁶ cells per 100 g body weight)	24.3 ± 5.1	23.3 ± 2.6	24.7 ± 1.5	26.7 ± 2.5
Kupffer cell yield (10 ⁶ cells per g liver weight)*	7.6 ± 1.8	9.1 ± 1.0	8.4 ± 0.4	9.4 ± 1.0
purity (%)	78.5 ± 5.1	81.9 ± 2.9	83.9 ± 2.3	86.0 ± 2.1

The values represent the mean ± S.E.M. of 4 experiments.

The viability was more than 98% for all age groups.

* Average liver weights at the various age categories were calculated from Kitani et al. (1982) and Brouwer et al.(1985).

fuged at 400 x g for 10 min and the pelleted cells were used to establish maintenance cultures of Kupffer cells. Sterile conditions were maintained during the entire isolation and purification procedures.

Cell viability and purity of the Kupffer cell fraction were determined as described in Section 3.2.2. Cell counts were performed in a hemocytometer.

Purified Kupffer cells were suspended in basic culture medium (Section 4.2.1) supplemented with 20% (v/v) fetal calf serum (Gibco Biocult) and cultured in plastic culture dishes (Ø 3 cm; Corning) at a seeding density of 5×10^6 cells per dish. The cultures were incubated as described in Section 4.2.1.

6.2.2 Morphological examinations

Kupffer cell cultures were prepared for phase contrast, for scanning and transmission electron microscopy according to the methods described previously (Sections 4.2.3 and 5.2.2).

6.2.3 Enzyme cytochemistry

Kupffer cells in culture were tested for endogenous peroxidatic activity on days 1, 3, 5 and 7 as earlier described (Section 4.2.4) and for acid phosphatase activity on day 1. Acid phosphatase was demonstrated by means of a method described by Wilson et al. (1982) with p-nitrophenyl phosphate as the substrate.

6.2.4 Endocytosis

Kupffer cells in culture were tested for their endocytic capacity by use of colloidal carbon (Faber Castell; Higgins, drawing ink); 0.8 μ m latex beads (Sigma Chemical Co.) as a phagocytosis marker, horseradish peroxidase (Merck) as a marker for adsorptive endocytosis (Rodman et al., 1978) and *E. coli* endotoxin 0.26:B6W (Difco Laboratories) as an exclusive Kupffer cell marker (Praaning-van Dalen et al., 1981). The incubation procedures for the four substances tested are listed in Table 6.2.

TABLE 6.2 ENDOCYTOSIS BY CULTURED KUPFFER CELLS:
INCUBATION PROCEDURES

Marker	Concentration	Incubation time(min)	Visualization	Endocytic mechanism
Colloidal carbon	0.1% (v/v) ^a	30	direct	phagocytosis + bristle-coated pinocytosis ^b
Latex (0.8 μ m)	5% (v/v) ^a	30	direct	phagocytosis ^c
Horseradish peroxidase	125 μ g/ml	30	DAB+H ₂ O ₂ ^d	adsorptive endocytosis ^e
Endotoxin	3 mg/ml	5 and 30	direct	phagocytosis + bristle-coated pinocytosis (this chapter)

The endocytosis of colloidal carbon was studied by light microscopy and that of the other markers by electron microscopy.

a Latex and colloidal carbon were diluted to 1/20 and 1/1000 of the original suspensions, respectively.

b Widmann et al., 1972.

c Wisse, 1977.

d Horseradish peroxidase was visualized by incubation in a medium containing diaminobenzidine (DAB) and H₂O₂ (see Section 5.2.4).

e Rodman et al., 1978.

6.3 RESULTS

6.3.1 Characteristics of fractions of Kupffer cells

The yield, viability and purity of Kupffer cell fractions obtained by centrifugal elutriation of nonparenchymal liver cell suspensions prepared from rats of various ages are presented in Table 6.1. There was an increase with age in the number of Kupffer cells which could be isolated and purified from one rat liver. However, the number of isolated Kupffer cells expressed per 100 gram of body weight or per

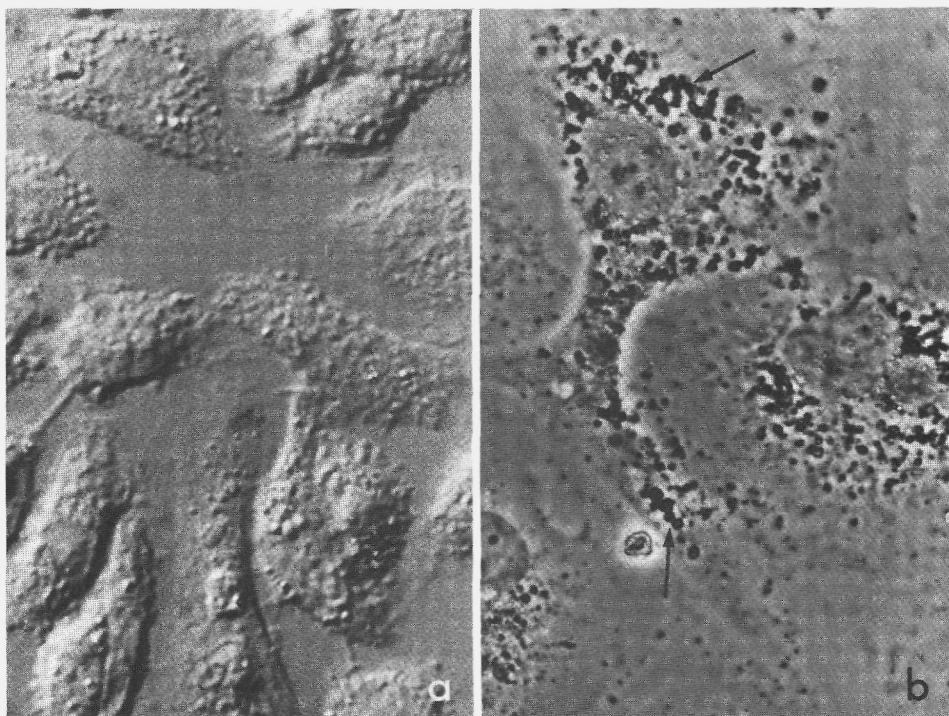


Figure 6.1 Light micrographs of Kupffer cells isolated from 3-month-old rats cultured for 24 h.

- a. Nomarski contrast. Cells are well spread out on the plastic surface and show a cytoplasm rich in vacuoles and an organelle-poor periphery representing the lamellipodia. 1100 x.
- b. Colloidal carbon (†) is endocytosed by 99% of the adhered cells. 1500x.

gram of liver weight was unchanged with age. The viability of the isolated cells was unaffected by the age of the donor rat. The increase in the purity of Kupffer cell fractions with age is probably a result of the increase in the total yield of Kupffer cells and the increase in size differences between endothelial and Kupffer cells with age (Sleyster and Knook, 1980).

6.3.2 Phase contrast microscopy

Figure 6.1a shows a light micrograph (Nomarski contrast) of cultured Kupffer cells obtained from a 3-month-old rat after 24 h of culture. Most of the cells had become firmly attached to the bottom of the petri dish. They were well spread out and many showed an irregular outline. Time-lapse video recording (Barelds et al.,

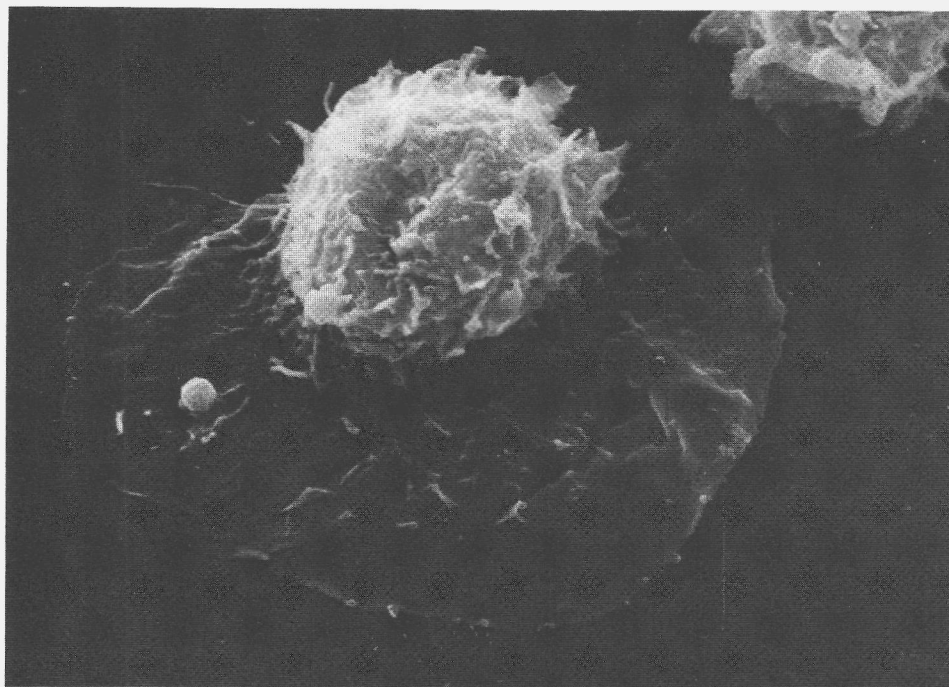


Figure 6.2 Scanning electron micrograph of a Kupffer cell from a 3-month-old rat cultured for 20 h in the process of spreading. The cell shows characteristic membrane ruffles: the lamellipodia. 3900 x.
(Photograph by R. de Zanger; Free University of Brussels, Belgium)

1982) showed continuous changing of the cell shape by extrusion and withdrawal of large lamellipodia. A large number of translucent vacuoles was observed in the cytoplasm. Only a few cells retained their spherical shape and remained unattached. These, which were probably not Kupffer cells, were removed when the medium was refreshed. Cultured Kupffer cells obtained from 30- to 36-month-old rats were indistinguishable from those from 3- and 12-month-old rats by light microscopy.

It was observed that the extent of Kupffer cell spreading gradually increased and the number of translucent vacuoles slowly decreased with increasing culture time. No mitoses were observed in Kupffer cells under the conditions established. After about 10 days in culture, rapidly dividing fibroblast-like cells were often seen and these represented the majority of cells in culture within a few days afterwards. They were most probably contaminating fat-storing cells (cf. Sections

4.3.2 and 7.3.2). In some cases, however, cultures consisting primarily of Kupffer cells could be retained for more than 3 weeks.

The capacity to endocytose latex particles ($0.8\ \mu\text{m}$) or colloidal carbon was evident in about 99% of the adherent cells at 24 h in culture (Fig.6.1b).

Culture conditions were shown to influence the morphological appearance of Kupffer cells. Concentrations of penicillin and streptomycin normally used in the cultivation of cells ($1000\ \text{units.ml}^{-1}$ and $1000\ \mu\text{g.ml}^{-1}$, respectively) inhibited Kupffer cell spreading; therefore ten times lower concentrations (see Materials and Methods) were used in our cultures. The use of rat serum instead of fetal calf serum had little influence on cell spreading, but it gave rise to the occasional appearance of giant cells with up to 14 nuclei and an increase in the number of lipid droplets in the cytoplasm of Kupffer cells (cf. Section 4.3.1).

6.3.3 Scanning electron microscopy

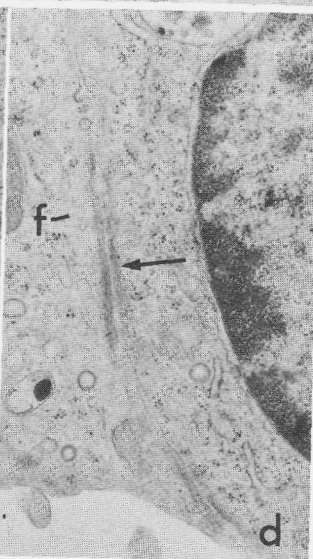
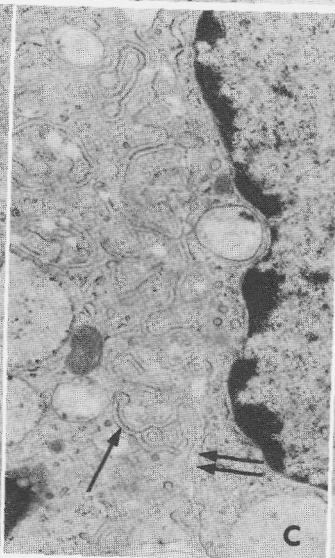
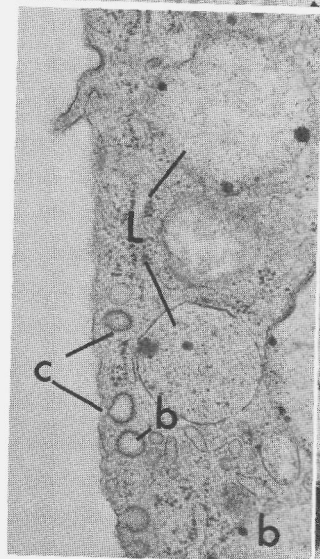
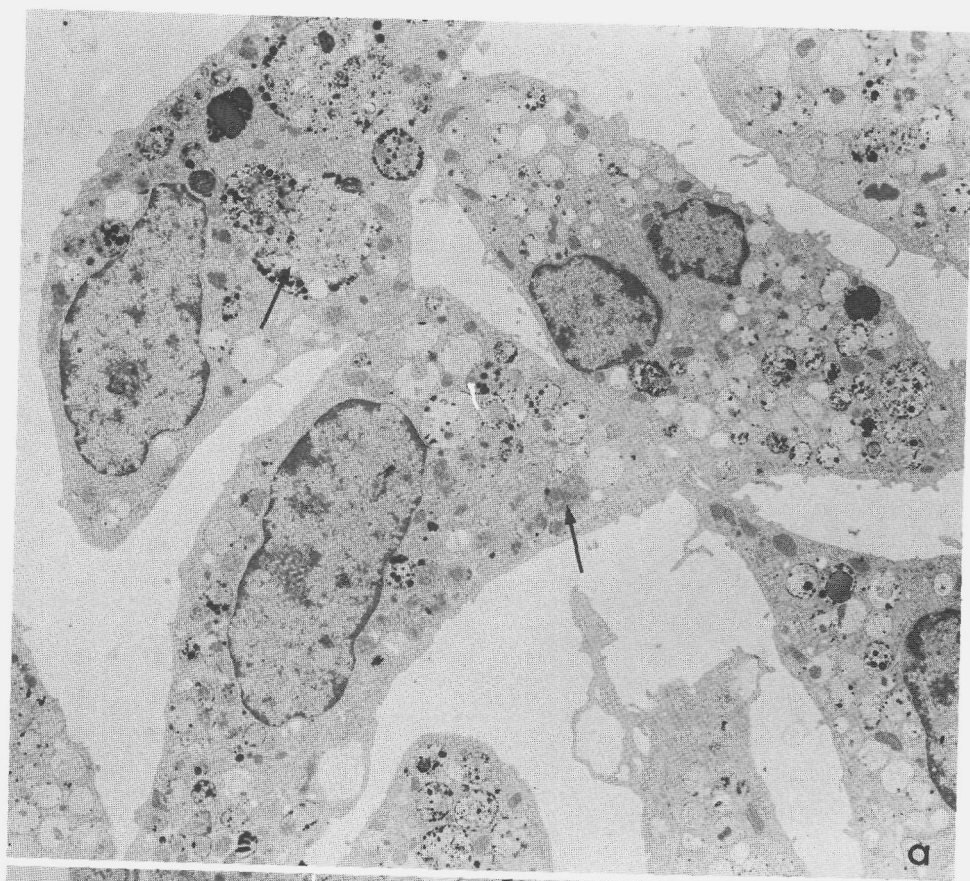
Figure 6.2 shows a scanning electron micrograph of purified Kupffer cells after 20 h of culture. Many lamellipodia comparable with those of Kupffer cells in perfusion-fixed liver (Motta, 1975) were observed on the cell surface.

6.3.4 Transmission electron microscopy

Figure 6.3a shows the typical ultrastructural features of Kupffer cells from a 3-month-old rat after 24 h of culture. The nucleus was generally of an oval shape and the heterochromatin formed a rim at the periphery, with only a few patches throughout the nucleus. Nucleoli were often present and showed a loose structure. The cell membrane generally appeared irregular and showed lamellipodia. Coated pits and bristle-coated micropinocytic vesicles were commonly observed (Fig.6.3b). Worm-like structures were consistently found in most cultures; however, in only a few cells (Fig.6.3c). These typical enfoldings of the cell membrane, which "display" the fuzzy coat, are considered to be highly characteristic of Kupffer

Figure 6.3 Transmission electron micrographs of Kupffer cells from 3-month-old rats cultured for 24 h.

- a. The most striking characteristic of such cells is the large number of lysosomes (†) of differing size which are filled with a variety of substances. 3250 x.
- b. At the cell membrane, several structures essential for the process of endocytosis can be readily observed: coated pit (c); bristle-coated micropinocytic vesicle (b); lysosomes (l). 27300 x.
- c. The fuzzy cell coat is preserved in the so-called worm-like structures (†) and between the closely apposed cell membranes (††). 15500 x.
- d. Cell contacts between Kupffer cells consisting of filaments (f) in close association with condensed material (†). 17900 x



cells (Wisse, 1974a). A fuzzy coat was also observed between closely apposed cells. No true junctions were seen between Kupffer cells, but specialized cell contacts consisting of microfilaments in association with condensed material were occasionally encountered (Fig.6.3d). Close contacts between Kupffer cells with heavily intertwined lamellipodia were frequently observed when the cells were seeded at high densities. Kupffer cells from 30- to 36-month-old rats showed considerably more lamellipodia (cf. Figs.6.3a and 6.4b).

Lysosomal structures were the most abundant cell organelles. They were present throughout the cytoplasm and contained residual material, electron dense lipid-like droplets and occasionally ferritin and fragments of erythrocytes or other cellular debris (Fig.6.4a). With increasing age of the donor rat, Kupffer cells contained more electron dense material inside lysosomes (Fig.6.4b). Some of this was due to an increase in lipid droplets and erythrocyte fragments, but the major part was the result of a marked increase in the amount of ferritin (Figs.6.4a, inset, c).

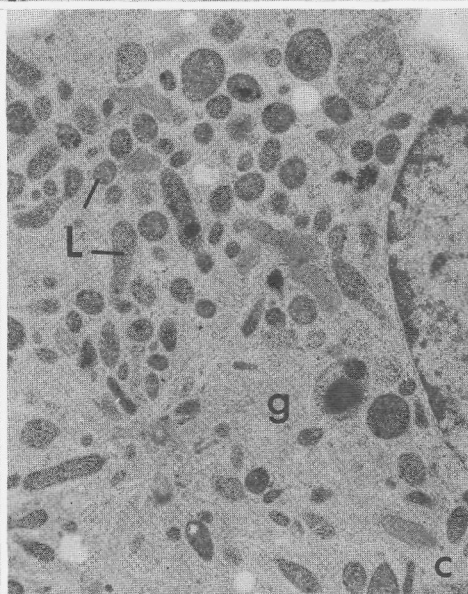
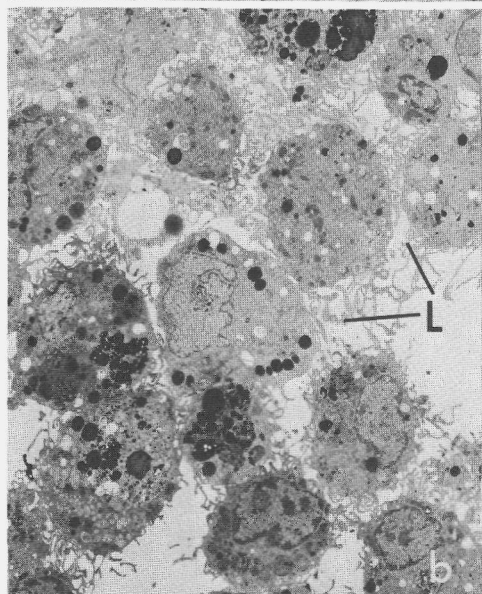
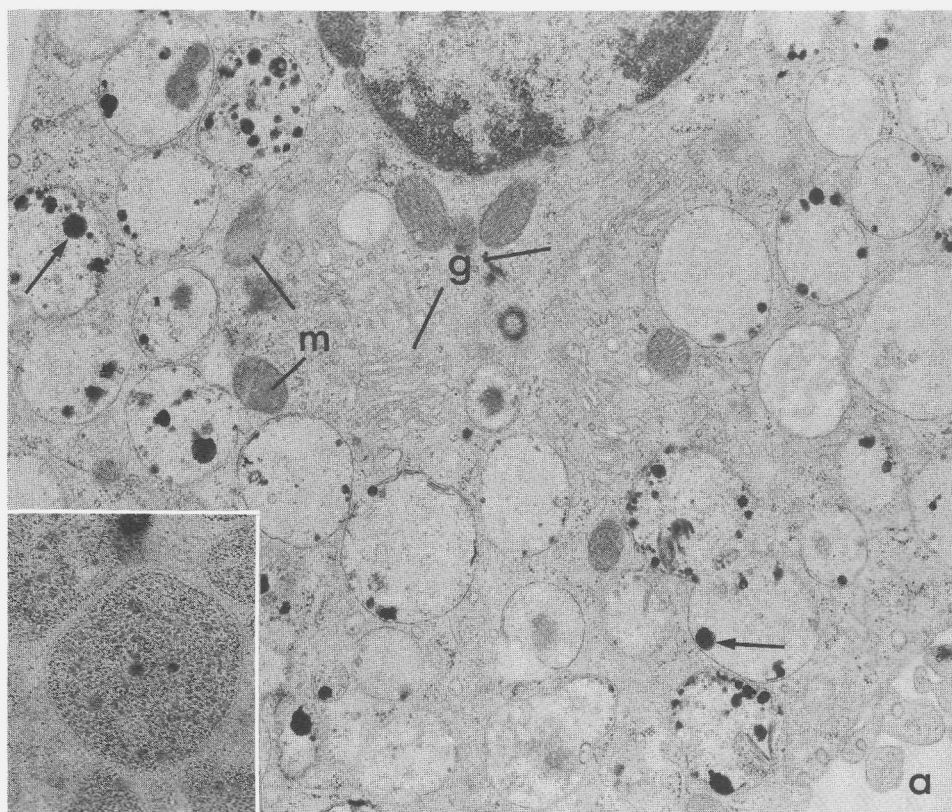
The rough endoplasmic reticulum (RER) appeared to be well developed. Mitochondria were present throughout the cytoplasm but not in large numbers; they were not condensed and had lamellar cristae. Kupffer cells generally showed an active Golgi apparatus located close to the nucleus, often in association with a pair of centrioles. In Kupffer cells derived from 30- to 36-month-old rats (Fig.6.4c), a more extensive Golgi system than that in cells derived from 3- or 12-month-old rats was often present. Annulate lamellae (Wisse, 1974a) were not observed in Kupffer cells in culture, which is in accordance with the absence of these structures in Kupffer cells of this rat strain in perfusion-fixed liver (cf. Section 2.3.2).

6.3.5 Enzyme cytochemistry

Figure 6.5a shows an electron micrograph of a Kupffer cell cultured for 24 h after incubation for endogenous peroxidatic activity. In all Kupffer cells, the reaction product was found exclusively in the nuclear envelope and in the cisternae of the RER. The staining pattern was identical to that described for Kupffer cells in perfusion-fixed liver (Wisse, 1974a; Section 2.3.2). At 3 days in culture

Figure 6.4 Transmission electron micrographs of Kupffer cells from 3-month-old (a) and 36-month-old (inset, b and c) rats cultured for 24 h.

- a. Cytoplasmic detail showing lysosomes, often with lipid-like droplets (†); golgi system (g); mitochondria (m). 16500 x. Inset: lysosome from a Kupffer cell from a 36-month-old rat filled with ferritin. 38400 x.
- b. A difference between Kupffer cells from young and old rats is observed in the increased incidence of electron dense material in the cytoplasm. Note the numerous lamellipodia (l). 2100 x.
- c. Cytoplasmic detail. Lysosomes (l) filled with ferritin seem to radiate from the extensive Golgi system (g). 10600 x.



and thereafter, both the number of positive cells and the staining intensity were seen to decrease. Reaction product could not be demonstrated in any cell at day 7 in culture.

Cytochemical testing for acid phosphatase showed this enzyme to be present in Kupffer cell lysosomes (Fig.6.5b). There were no differences in enzyme patterns in Kupffer cells of the various age groups.

6.3.6 Endocytosis

The phagocytic capacity of Kupffer cells after 24 h in culture was confirmed by incubating them with 0.8 μ m latex particles. Figure 6.6 shows that latex particles are phagocytosed (Fig.6.6a) and transported to lysosomes (Fig.6.6b).

When Kupffer cells after 24 h of culture were incubated with horseradish peroxidase (HRP), a glycoprotein with a mannose-terminating carbohydrate chain (Rodman et al., 1978), enzyme reaction product could be demonstrated at the cell membrane in coated pits and within bristle-coated micropinocytic vesicles. Ultimately, HRP accumulated within lysosomes (Fig.6.6c).

Incubation of Kupffer cells after 24 h of culture with endotoxin resulted in attachment of bacterial membrane fragments of different sizes to the cell membrane of all Kupffer cells, subsequent internalization through micropinocytic (Figs.6.7a,b) or phagocytic events (Fig.6.7c) and accumulation in large lysosomes (Fig.6.7d). Some Kupffer cells accumulated much more endotoxin in their lysosomes than did others. This may be related to the difference in endocytic capacity of Kupffer cells in periportal and perivenous areas of the intact liver (Sleyster and Knook, 1980) and the results indicate that this heterogeneity may persist in culture.

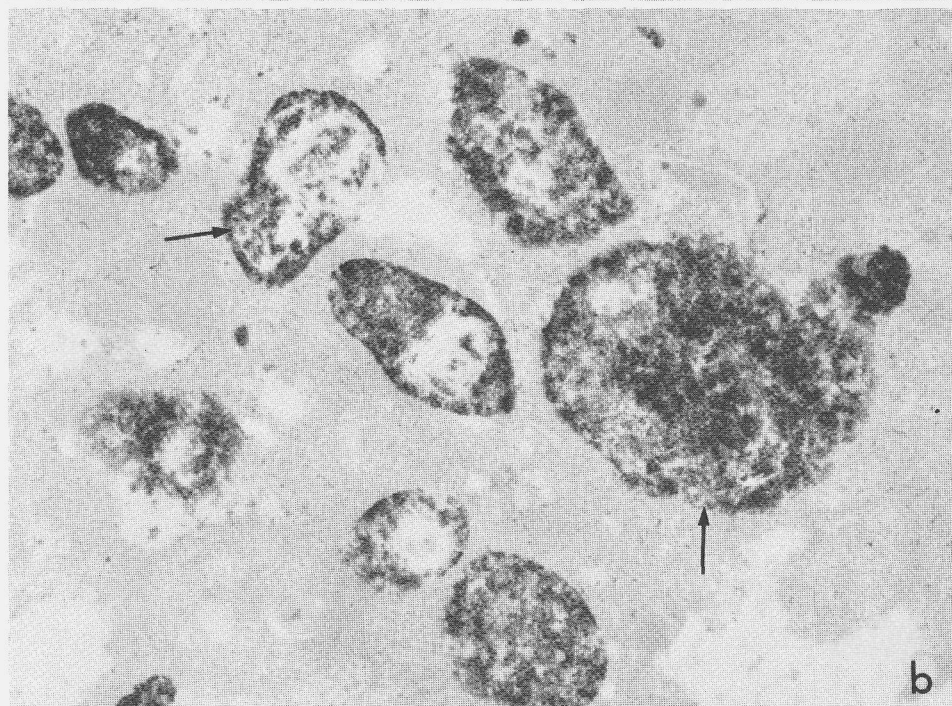
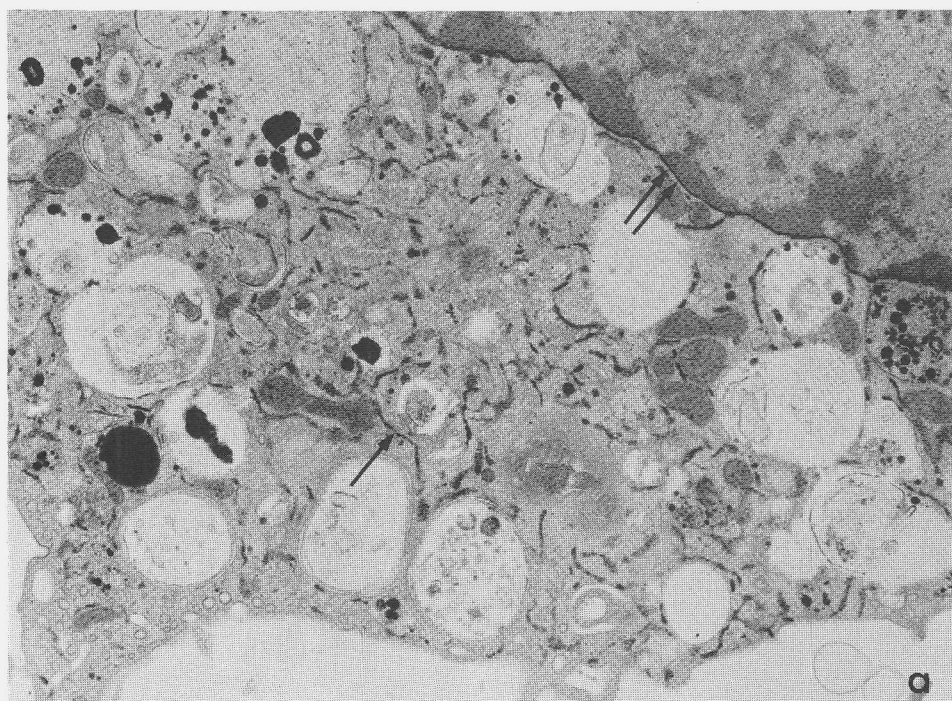
Kupffer cells from 3-, 12-, 30- or 36-month-old rats showed no qualitative differences in endocytic capacity.

6.4 DISCUSSION

The morphological and enzymatic characteristics of Kupffer cells in culture compare very well with the properties of such cells in perfusion-fixed liver (Wisse, 1974a, 1977; Section 2.3.2). The typical ultrastructural features of the cells preserved during culture include the so-called worm-like structures and the fuzzy coat. Both acid phosphatase and peroxidatic activity were shown to be present for

Figure 6.5 Transmission electron micrographs of Kupffer cells from a 3-month-old rat after incubation for endogenous peroxidatic activity (a) and for acid phosphatase (b).

- a. Endogenous peroxidatic activity is present in the cisternae of the RER ($\uparrow$) and in the nuclear envelope ($\uparrow\uparrow$). Controls incubated without H_2O_2 showed reaction product in only 1% of all Kupffer cells. 12300 x.
- b. Acid phosphatase is present in lysosomes ($\uparrow$). 50000 x.



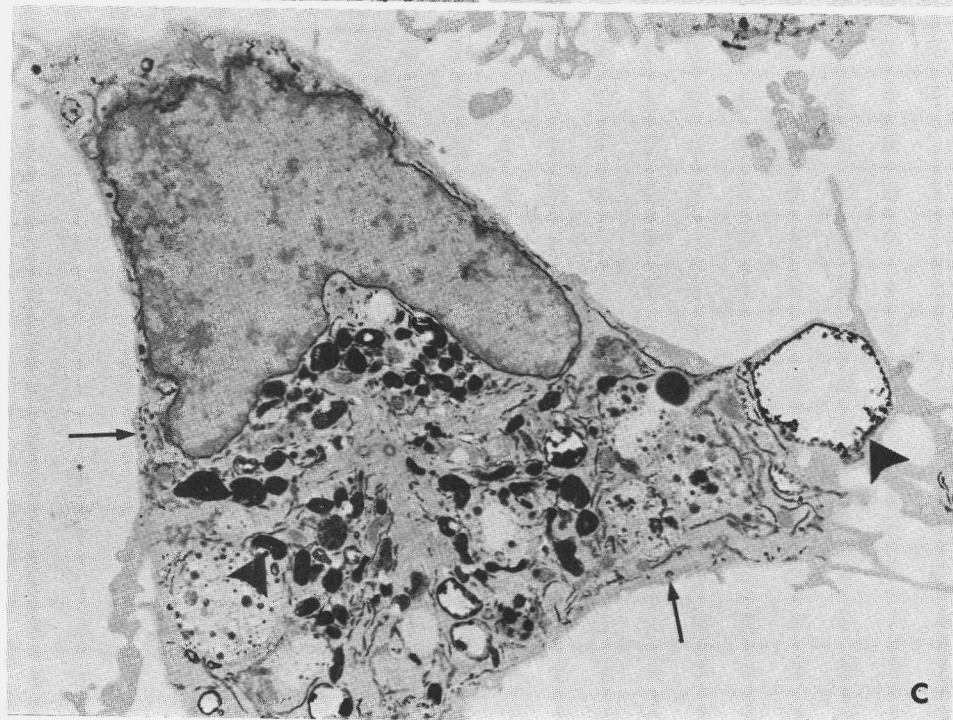
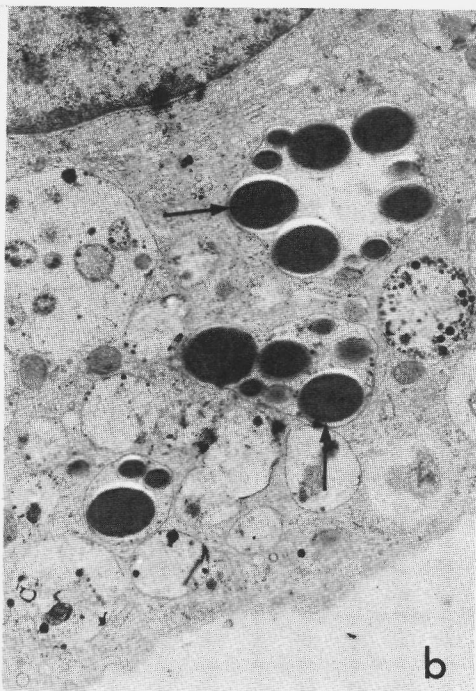
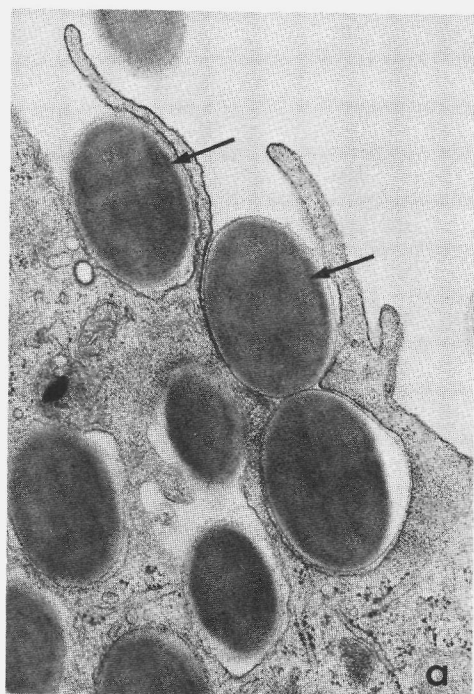
several days in Kupffer cells in maintenance culture, the localization of each of these enzymes being identical to that in perfusion-fixed liver (Wisse, 1974a, 1977).

One of the main functions of Kupffer cells is the endocytosis of foreign substances from the blood, a process in which several cell membrane components play an important role. The fuzzy cell coat of Kupffer cells is supposed to be involved in the attachment of substances to the cell. In addition, several types of specific membrane receptors are required for the endocytosis of bacteria (Tamaru and Fujita, 1978), endotoxins (Ruiter et al., 1981), viruses (Kirn et al., 1980), tumor cells (Roos, 1981), cellular debris after different types of trauma (Saba, 1970), clinical RES test substances and various specific macromolecules (Brouwer et al., 1982a; Praaning-van Dalen et al., 1981, 1982). It was described in the present study that specific membrane components such as the fuzzy coat were present during cell culture and, furthermore, that different types of endocytosis, i.e., phagocytosis and bristle-coated micropinocytosis occurred. This is of great importance because of the absence of the fuzzy coat and specific receptors immediately after Kupffer cell isolation at 37°C by use of pronase.

Most of the experimental work described in other publications was performed with Kupffer cells which were purified from nonparenchymal cell suspensions by selective attachment of the cells to plastic (Munthe-Kaas et al., 1975; Emeis and Planqué, 1976). In such cultures, uptake by Kupffer cells of cell debris, mainly derived from endothelial cells, significantly altered functional properties of Kupffer cells such as endocytic capacity and levels of lysosomal enzymes (Brouwer et al., 1980). In addition, several essential characteristics of Kupffer cells such as the fuzzy coat, worm-like structures and peroxidatic activity could not be demonstrated in such Kupffer cell cultures derived from nonparenchymal cell suspensions (Emeis and Planqué, 1976; Wisse, 1980). In the present study, findings have been discussed which show that Kupffer cells that are isolated under specified conditions behave in culture for at least 3 days in all aspects studied as do Kupffer cells *in vivo*. These observations and the ultrastructural, enzymatic and functional similarities of cells in culture to those in the intact liver suggest maintenance cultures of purified Kupffer cells to be a valuable model system for investigations on several aspects of Kupffer cell functions.

Figure 6.6 Transmission electron micrographs of Kupffer cells from a 3-month-old rat cultured for 24 h: endocytosis.

- a. Latex particles (†) are phagocytosed separately or in clusters. 27700 x.
- b. After phagocytosis, the particles (†) are transported to larger vacuoles. 10600 x.
- c. Horseradish peroxidase is taken up by bristle-coated micropinocytosis (†) and accumulates in larger lysosomes (▲). Note the presence of endogenous peroxidatic activity in the RER and the nuclear envelope (cf. Fig.6.5a). 7000 x.



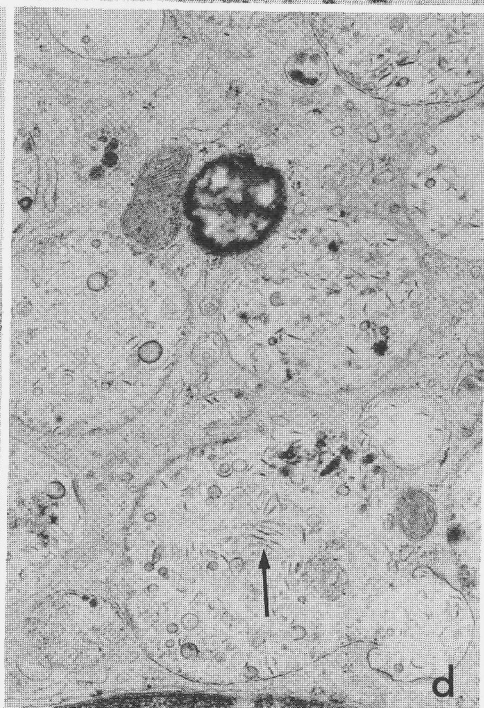
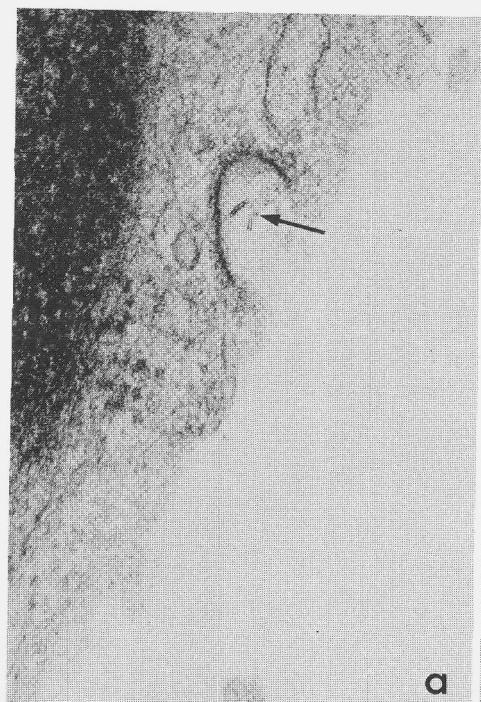
The in vitro system of cultured Kupffer cells described was employed to investigate a possible cellular basis for the functional decline with age observed for the reticuloendothelial system (Knook et al. 1982a; Brouwer and Knook, 1983), of which Kupffer cells represent the majority. Age-related changes in Kupffer cell yield or cell viability were not found. Changes observed in the ultrastructure of these cells in culture with age included an increase in the number of lamellipodia, in the area occupied by the Golgi apparatus and particularly in the amount of iron-ferritin complex in the lysosomes. The possible origin of the iron accumulating in Kupffer cells with age was already discussed in Section 2.4. However, the absence of gross overall changes in cellular morphology at very advanced age seems to be a highly significant finding in view of the marked physiological differences between young and old animals.

The enzyme patterns of endogenous peroxidatic activity and acid phosphatase in Kupffer cells in culture were qualitatively unaffected by the age of the donor rat. In a enzyme cytochemical assay, it was shown that freshly isolated Kupffer cells from both 3- and 24-month-old rats contained acid phosphatase in their lysosomes (Wilson et al., 1982). A quantitative biochemical assay revealed a small but significant decrease with age in the activity of one of the multiple forms of acid phosphatase (Sleyster and Knook, 1980), employing the same substrate as used in this study. Also, the endocytic properties of Kupffer cells in culture were not age-dependent. A biochemical study on the endocytic capacity of Kupffer cells showed a decrease with age in the endocytosis of radioactively labeled colloidal albumin by the cells both in vivo and in vitro (Brouwer and Knook, 1983; Brouwer et al., 1985). Although apparently the decrease in both acid phosphatase activity and endocytic capacity are not revealed in the qualitative assays used in the present study, the ultrastructural investigation gives additional information on intracellular enzyme localization and types of mechanisms involved in endocytosis. This study showed that both were unchanged with age and also in comparison with perfusion-fixed liver.

It was concluded from the close similarity between Kupffer cells in vitro and in the intact liver that the in vitro system described in this chapter may also be suitable for studying the role of Kupffer cells in the immune response (Rogoff and Lipsky, 1981; Nadler et al., 1979) and in processes such as the secretion of

Figure 6.7 Transmission electron micrographs of Kupffer cells from a 3-month-old rat cultured for 24 h: endocytosis.

- a,b,c. Endotoxin (†) is taken up by bristle-coated micropinocytosis (a and b) or by phagocytosis (c). 101700 x, 107100 x and 57600x, respectively.
- d. The vesicles containing the endotoxin are transported inside the cell and fuse with one another or with other preexisting vacuoles to form larger lysosomes. 8500 x.



enzymes, prostaglandins, and other monokines in response to specific stimuli (Page et al., 1978; Welscher et al., 1980) These are possible functions of Kupffer cells which have received less attention up to now.

CHAPTER 7

CULTURES OF FAT-STORING CELLS

7.1 INTRODUCTION

Functions ascribed to fat-storing cells include a role in vitamin A metabolism in the liver, especially storage, (Wake, 1980; Knook et al., 1982b) and in the synthesis of components of the extracellular matrix in the liver acini under both normal and pathological conditions (McGee and Patrick, 1972; Kent et al., 1976; Ballardini et al., 1983).

The development of a procedure for preparing pure suspensions of rat liver fat-storing cells (Knook et al., 1982b) permitted analytical and functional studies of these cells. The vitamin A content of fat-storing cells and other liver cells was quantified by high performance liquid chromatography (De Leeuw et al., 1983; Hendriks et al., submitted). From these experiments it was calculated that under normal physiological conditions a major part ($\pm 80\%$) of total liver vitamin A is stored in the fat-storing cells and not, as has been assumed, in hepatocytes (Goodman, 1980). In addition, it was shown that in the rat both total liver vitamin A and the vitamin A content of fat-storing cells increased significantly with increasing animal age (H.F.J. Hendriks, personal communication).

The aim of the study described in this chapter was to investigate the role of fat-storing cells in fibrogenesis. To enable this a well-defined system for culturing purified rat liver fat-storing cells was devised. Cells were characterized morphologically and by testing for vitamin A fluorescence and for their pattern of cytoskeletal proteins. This system was used for the determination of the presence of various components of the extracellular matrix in fat-storing cells in culture.

7.2. MATERIALS AND METHODS

7.2.1 Isolation procedure and culture conditions

The experiments were performed with 12-, 24- and 36-month-old rats. Immunocytochemistry investigations of the cytoskeleton and of the extracellular matrix (Section 7.2.5) were done only with cells derived from 12-month-old animals. The latter were performed in collaboration with A. Geerts, Laboratory for Cell Biology and Histology, Free University of Brussels, Belgium.

Nonparenchymal liver cells were isolated as previously described (Section 3.2.1; Knook et al., 1982b). Fat-storing cells were purified from the nonparenchymal cell suspensions by centrifugation in a discontinuous two-layer Nycodenz®

density gradient as described in Section 3.2.1.

Cell viability and the purity of the fat-storing cell fraction were determined as described in Section 3.2.2. Cell counts were performed in a hemocytometer.

Purified fat-storing cells were suspended in basic culture medium (Section 4.2.1) supplemented with either 20% (v/v) fetal calf serum (Gibco Biocult) or 20% (v/v) newborn calf serum (prepared in our Institute) and cultured in plastic 24-well culture plates (Falcon 3047) at a seeding density of 0.25×10^6 cells per 2.0 cm^2 well directly on the plastic or on glass coverslips ($\emptyset$ 12 mm, Chance Proper). The cultures were incubated as described in Section 4.2.1.

For passaging, cultures at confluency were washed in phosphate buffered saline (PBS) and this was followed by trypsinization for 5 min in PBS containing trypsin (2.5 g.l^{-1}) and EDTA (0.25 g.l^{-1}), pH 7.2. Trypsinization was stopped by adding an excess of newborn calf serum to the wells and washing the cells for 5 min at $450 \times g$. Cells were pooled and replated in a 1:2 split either in 24-well culture plates or in small plastic culture flasks (Falcon 3013).

7.2.2 Morphological examinations

Fat-storing cell cultures were prepared for phase contrast and transmission electron microscopy according to the methods described earlier (Sections 4.2.3 and 5.2.2).

7.2.3 Vitamin A fluorescence

The presence of vitamin A in fat-storing cells in culture was assessed by making use of the autofluorescence property of vitamin A when irradiated with light of 328 nm. Cells were isolated and cultured on glass coverslips in reduced light to avoid photoinactivation of vitamin A. Maintenance cultures of purified rat liver Kupffer cells (cf. Section 6.2.1) were used to compare fluorescence properties. At various time points during culture, coverslips were removed, washed in PBS and mounted on microscope slides by placing the inverted coverslips on a drop of 90% glycerol in PBS (pH 8-9) containing 1% o-phenyldiamine (o-PD). The o-PD was added to reduce fading of the fluorescence. Coverslips were sealed with coverslip varnish (Schmid and Co.) to prevent drying. Microscope slides were either examined immediately or stored at -20°C in the dark for reexamination at a later time.

7.2.4 Incorporation of ^3H -thymidine

First passage cells grown on glass coverslips for 24 h after trypsinization were washed in PBS and then incubated in Dulbecco's modification of Eagle's medium (DME, with 45 mM NaHCO_3 and supplemented with 10% fetal calf serum) containing ^3H -thymidine ($10 \text{ }\mu\text{Ci.ml}^{-1}$; specific activity 25 Ci.mmol^{-1}) for 3 h at 37°C in an

atmosphere containing 7% CO₂ in air. Incubations were stopped by washing in PBS containing CaCl₂.2H₂O and MgCl₂.6H₂O (0.1 g.l⁻¹ each). Cells were fixed in a graded series of ethanol-acetic acid (3:1) for 5 min in a 10% dilution of this fixative in DME, 5 min in a 50% dilution and 5 min in 100% fixative. They were finally washed in 70% ethanol in water. Autoradiography was performed according to the method of Ehman et al. (1978). After development of the slides, cells were counterstained with toluidine blue. The percentage of cells in S-phase was calculated by expressing the number of cells with heavily labeled nuclei as a percentage of the total number of cells.

7.2.5 Immunocytochemistry

7.2.5.1 Cytoskeleton

Elements of the cytoskeleton were detected by indirect immunofluorescence with antibodies against actin, tubulin, prekeratin and vimentin. Fat-storing cells were grown on glass coverslips and semiconfluent first passage cultures were used. The incubation procedures and the specifications of the antibodies are given in Table 7.1.

7.2.5.2 Extracellular matrix

Various components of the extracellular matrix were detected by indirect immunofluorescence. Two and four-day-old primary cultures and semiconfluent first and fourth passage cultures of fat-storing cells were tested. Cells grown on glass coverslips were washed three times in 0.1 M sodium cacodylate buffer (pH 7.4) containing 0.02% CaCl₂.2H₂O and 3% sucrose. They were then fixed with 0.5% formaldehyde in the same buffer for 1 h at room temperature, after which they were kept in buffer. Indirect immunofluorescence was performed as described earlier (Geerts et al., 1982). Details of the primary antisera are given in Table 7.2. GARa/coll.I was used at a dilution of 1:100 in PBS, the other antibodies at a dilution of 1:50 in PBS.

Fluorescein isothiocyanate labeled secondary antibodies were purchased from Miles Laboratories Inc. and Cappel Laboratories Inc. Control samples were incubated in nonimmune serum of the same species in which the primary antibodies were raised.

TABLE 7.1 IMMUNOFLUORESCENCE OF CYTOSKELETAL PROTEINS:
INCUBATION PROCEDURES

Cytoskeletal protein	Actin ^a	Tubulin ^b	Prekeratin ^c	Vimentin ^d
Washing (3x)	PBS	PBS	PBS	PBS
Fixation	3.7% Formaldehyde 20 min, 4°C 0.2% Triton X-100 10 min, 4°C	Methanol 4 min, -20°C Acetone 30 sec, -20°C	Acetone 10 min, -20°C	Methanol 4 min, -20°C Acetone 30 sec, -20°C
Washing (3x)	PBS	PBS	PBS	PBS
Primary antibody (moist atmosphere)	1:5 in PBS 1 h, 37°C	1:5 in PBS 1 h, 37°C	1:20 in PBS 45 min, 37°C	1:10 in PBS 1.5 h, 37°C
Washing (3x)	PBS	PBS	PBS	PBS
Secondary antibody* (moist atmosphere)	1:20 RAM-FITC in 3% BSA in PBS pH 7.4 1 h, 37°C	1:20 RAM-FITC in 3% BSA in PBS pH 7.4 1 h, 37°C	1:20 GAM-FITC in 3% BSA in PBS pH 7.4 45 min, 37°C	1:40 GAR-FITC in 3% BSA in PBS pH 7.4 30 min, 37°C
Washing (3x)	PBS	PBS	PBS	PBS
Mounting	o-PD in PBS	o-PD in PBS	o-PD in PBS	o-PD in PBS

^a Mouse monoclonal antibody to actin, isolated and purified from *Acanthamoeba castellanii*.

^b Mouse monoclonal antibody to tubulin, isolated and purified from rat brain.
^a and ^b were prepared by Dr. H. Amesz, Department of Molecular Biology, State University of Utrecht, The Netherlands. The specificity of the antibodies was tested by the sliced gel immunoprecipitation method as described by Van Raamsdonk et al. (1977) and modified by Oestreicher et al. (1978).

^c Mouse monoclonal antibody to mouse prekeratin, prepared by Dr. J. Taylor-Papadimitriou, Imperial Cancer Research Fund, London, U.K. (provided by Dr. J. Hilkens, Cancer Institute, Amsterdam, The Netherlands).

^d Rabbit antivimentin, purchased from Transformation Research.
Mounting medium: 1% o-phenyldiamine in PBS + 90% glycerol, pH 8-9, with sodium bicarbonate (see Materials and Methods: Vitamin A fluorescence).

Controls: In the primary antibody step serum from nonimmunized animals was used.

* RAM-FITC; GAM-FITC and GAR-FITC were purchased from Nordic Immunological Laboratory.

TABLE 7.2 SPECIFICATION OF ANTIBODIES USED FOR THE DETECTION OF COMPONENTS OF THE EXTRACELLULAR MATRIX

Abbreviations	Animal immunized	Antigen used for immunization	Source of antigen
GARa/coll.I ^a	goat	collagen type I	rat skin
RAB/coll.III ^a	rabbit	N-terminal propeptide of procollagen type III	bovine skin
ShAM/coll.IV ^b	sheep	collagen type IV	mouse EHS tumor ^o
RAHu/coll.V ^b	rabbit	collagen type V	human placenta
RAB/coll.VI* ^a	rabbit	collagen type VI	bovine placenta
RAM/laminin ^b	rabbit	laminin	mouse EHS tumor ^o
RAHu/fibronectin ^a	rabbit	fibronectin	human plasma

a Prepared by Drs. B. Voss and J. Rauterberg, Institut für Arterioscleroseforschung, Münster, Germany. The specificity of the antisera was tested by passive hemagglutination (Beil et al., 1972).

b Prepared by Dr. G.R. Martin and co-workers, NIH, Bethesda, USA. The specificity of the antisera was tested by an ELISA method (Rennard et al., 1980).

* Also called short chain collagen; ^o Orkin et al. (1977)

7.3. RESULTS

7.3.1 Morphological observations

During the first 24 h of primary culture, fat-storing cells attached to and slightly spread out on the culture dish (Fig.7.1a). Spreading was more pronounced during the second day. The nucleus containing 2 or 3 nucleoli was surrounded by lipid vacuoles and broad cellular processes (Fig.7.1b). The cellular cytoskeleton, revealed as bundles of filaments, became pronounced especially at the cell periphery (Fig.7.1b). During days 3 and 4 of primary culture, the cells assumed an extremely extended shape with broad, flat processes (Fig.7.1c) which gave the impression of a decreased nuclear-cytoplasmic ratio as compared with day 1 (cf. Fig.7.1a). At this stage of cultivation, an increase in cell number resulting in confluent cultures at day 4 was also observed (cf. Figs.7.1e,f). Fat-storing cells derived from 24- and 36-month-old animals contained larger numbers of lipid droplets in the cytoplasm than cells from 12-month-old rats (cf. Figs.7.1c,d). Cells of animals of various ages, however, showed no significant differences in their behavior in primary culture.

Contaminating Kupffer cells were occasionally present in primary culture. These cells spread much more rapidly than did fat-storing cells but never became as flattened. They contained lipid droplets most probably derived from damaged

fat-storing cells in their cytoplasm (Fig.7.2a).

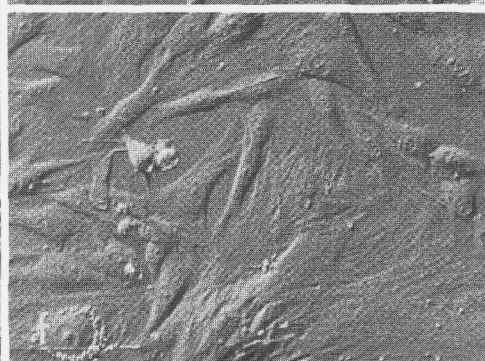
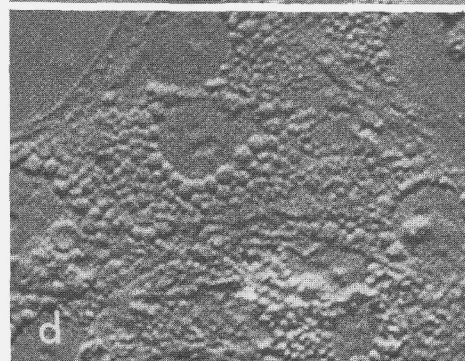
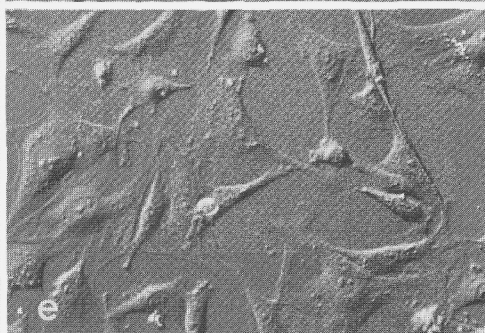
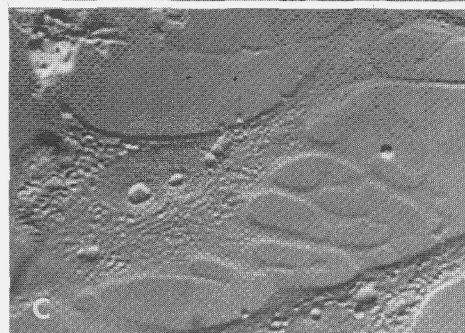
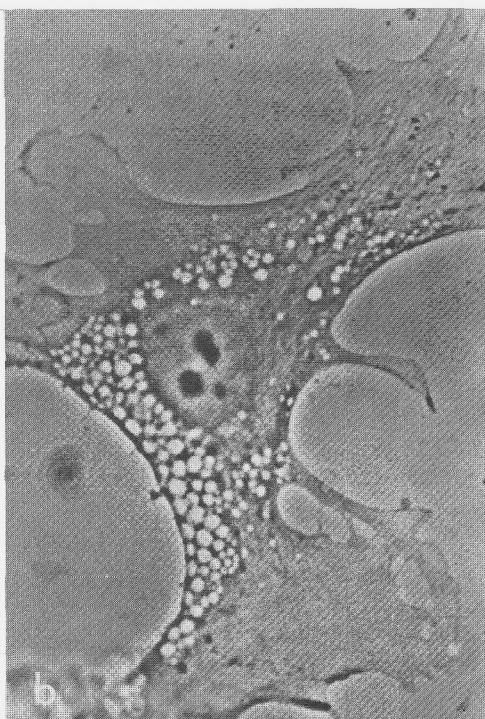
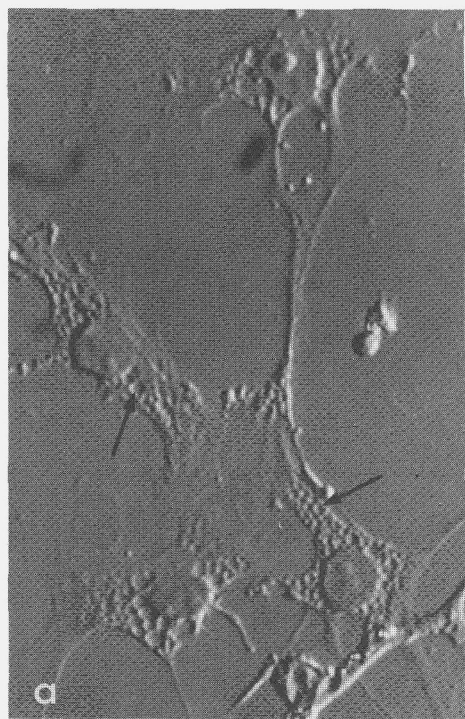
Structural changes in fat-storing cells during primary culture were studied in detail by electron microscopy. During the first day of primary culture, the cells attached to the substratum. Spreading occurred by extension of broad cellular processes; at this time the thin microvillous processes observed in freshly isolated cells had disappeared (Fig.7.2b). Lipid droplets remained present but became smaller as culture time increased (Fig.7.2c). The chromatin pattern showed marked changes by shifting from a clumped heterochromatin to a homogenous euchromatin pattern after two days of primary culture (cf. Figs.7.2b,c). Microtubules and bundles of microfilaments, the latter often showing focal condensations (Fig.7.3a) were pronounced after 3 to 4 days in primary culture. The number of cisternae of the rough endoplasmic reticulum increased in primary culture, but especially during cell passaging. These cisternae showed more dilatation as compared with those in fat-storing cells in perfusion-fixed liver (Fig.7.3b). Other cell organelles such as a Golgi system (Fig.7.3c), mitochondria, multivesicular bodies (Fig.7.3d) and cilia (Fig.7.3e) remained present during culture. During primary culture, but especially after passaging, fat-storing cells showed an increasing number of vacuoles in the Golgi area (Fig.7.4a); they were often filled with clumped granular material (Fig.7.4a, inset). These vacuoles are very reminiscent of those observed in fibroblasts which were shown to contain procollagen (Marchi and Leblond, 1984). Fat-storing cells isolated from 12-, 24- and 36-month-old animals were largely similar with regard to the time pattern and nature of the phenotypic changes described above.

7.3.2 Cell passages

Fat-storing cells in primary culture were seen to detach from the substratum during trypsinization within 5 min. Contaminating Kupffer cells remained attached

Figure 7.1 Light micrographs of fat-storing cells in primary culture.

- a. Nomarski contrast. Fat-storing cells from 12-month-old rats after 24 h of culture. Cells are spread out on the glass surface and show an abundance of lipid vacuoles in the perinuclear zone (†). Long cell extensions develop. 1100 x.
- b. Phase contrast. Fat-storing cell from 12-month-old rats at 2 days in culture. The cellular extensions have flattened out on the glass surface; the cytoskeleton has become pronounced, especially in the cell periphery. 1250 x.
- c,d. Nomarski contrast. Fat-storing cells from a 12- (c) and a 36-month-old rat (d) at 3 days in culture. The apparent increase in cell volume is very significant as compared with day 1 (cf. Fig.7.1a). Fat-storing cells from 36-month-old rats (d) contain more lipid droplets than those from 12-month-olds (c). c) 900 x and d) 1250 x.
- e,f. Nomarski contrast. Fat-storing cells from 12-month-old rats at 3 days (e) and 4 days (f) in culture. Cultures reaching confluency. 400 x.



and were not transferred. After replating, most cells reattached within 30 min, although the respreading process took approximately 4 h (Fig.7.4b). Thereafter, cell division again occurred and resulted in confluent cultures after 3 to 4 days if a 1:2 split was made. No differences were observed in the culture behavior of fat-storing cells isolated from animals of 12, 24, or 36 months of age. Determination of the number of cells with labeled nuclei in autoradiographs showed that on the average 10 to 20% of the cells were in S-phase (see also Fig.7.4c).

Spontaneous transformation was occasionally observed in later passages; this was evidenced by the loss of contact inhibition and the formation of clones of cells growing on top of each other. Transformed cells could be distinguished from nontransformed fat-storing cells, as they were smaller and more spindle-shaped. If transformation occurred, the spindle-shaped cells gradually represented the major portion of cells in culture and continuous cell lines were formed. Fat-storing cells could be transferred for at least two passages without the occurrence of transformed cells.

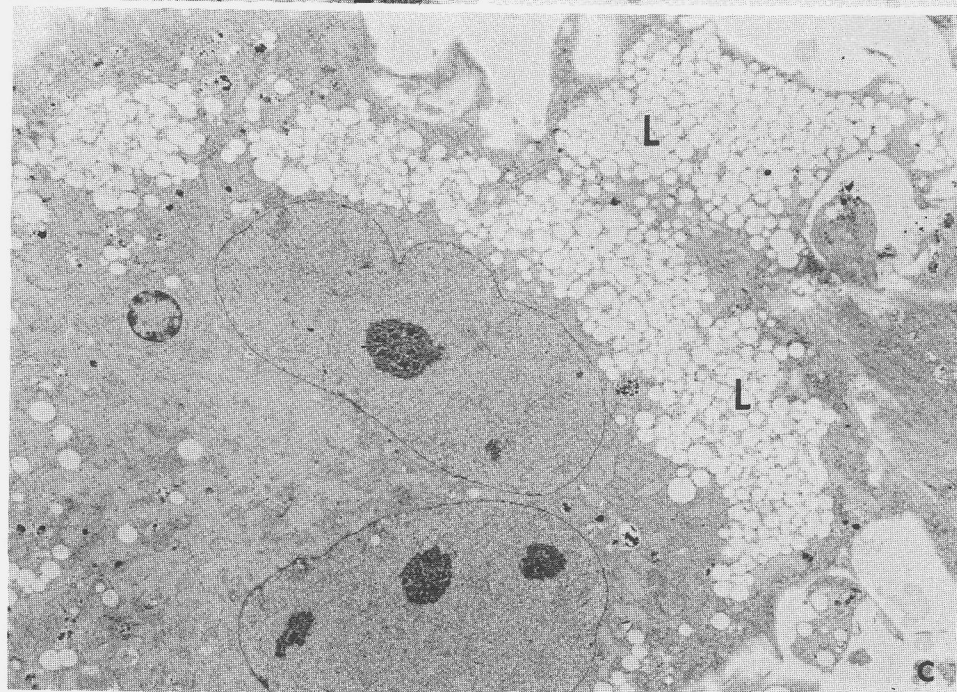
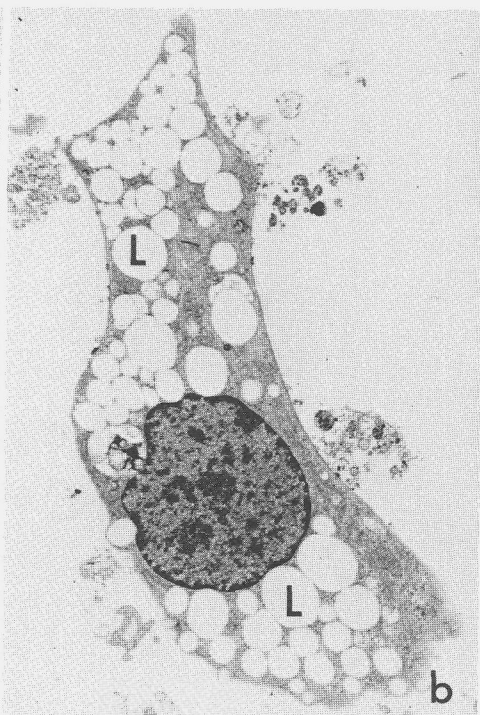
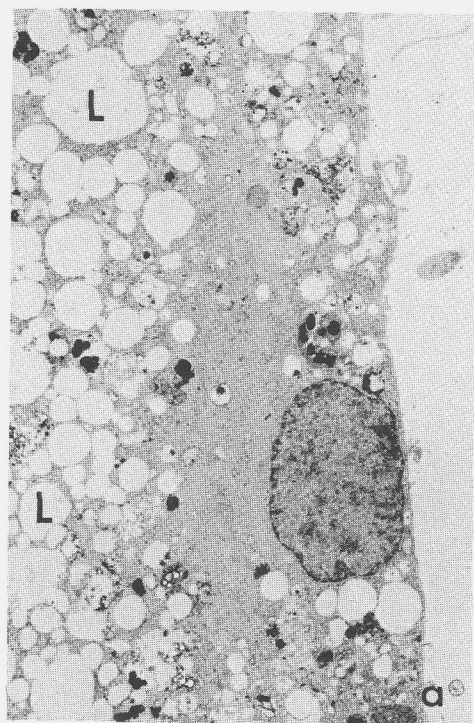
7.3.3 Vitamin A fluorescence

Characteristic vitamin A fluorescence was detected in fat-storing cells in culture up to the second passage. The rapidly fading, blue fluorescence was confined to lipid droplets present in the cytoplasm (Fig.7.4d). The intensity of the fluorescence per cell gradually decreased after two days of primary culture, probably due to dilution of vitamin A by cell division. Fat-storing cells from 36-month-old animals, which contain on the average a greater number of lipid droplets per cell directly after isolation (cf. Section 3.3.4.3), longer retained a well detectable vitamin A fluorescence than did cells from younger animals.

Contaminating Kupffer cells present during primary culture also showed some rapidly fading, blue fluorescence which most likely originated from the lipid droplets present in the cytoplasm (cf. Section 7.3.1). The Kupffer cells could be distinguished from fat-storing cells because the former also exhibited a lasting, bright yellow fluorescence probably derived from lipid material present in lysosomes. Only this latter fluorescence was observed in control cultures of purified Kupffer cells and also in Kupffer cells in the intact liver (unpublished observation).

Figure 7.2 Transmission electron micrographs of primary cultures of fat-storing cells from 12-month-old rats.

- a. A contaminating Kupffer cell during primary culture. The cell contains a large number of lipid droplets (1) probably derived from fat-storing cells. 2900 x.
- b. Fat-storing cell after 24 h of culture. The cell has a smooth cell membrane and contains almost exclusively lipid droplets (1). 3600 x.
- c. Fat-storing cell after 4 days of culture. Compared with day 1 (Fig.7.1b), the chromatin pattern has significantly changed; the lipid droplets (1) are smaller and the cytoplasm containing other organelles is more prominent. 2400 x.



7.3.4 Immunofluorescence

7.3.4.1 Cytoskeleton

Indirect immunofluorescence revealed the presence of vimentin, actin and tubulin in fat-storing cells in culture, whereas no prekeratin was present. Vimentin was diffusely present as filaments which seemed to radiate from the nucleus to the cell periphery (Fig.7.5a, upper part). High magnifications disclosed an interwoven network of fine filaments between the radiating ones (Fig.7.5a, lower part). In most cells, the diffuse staining was somewhat more intense around the nucleus. Tubulin was present as filaments extending from one pole of the cell to the other, often crossing over the nucleus (Fig.7.5b). No diffuse perinuclear staining was observed, but the number of fibers was greater around the nucleus. The distribution of actin showed a similar pattern to that observed for vimentin (Fig.7.5c). Stress-fibers were present in some cells (Fig.7.5d). In all cases, controls showed only a weak and diffuse staining of the cells.

7.3.4.2 Extracellular matrix

The presence or absence of various components of the extracellular matrix in fat-storing cells in primary culture and in first and fourth passage cultures are summarized in Table 7.3.

Type I collagen was the predominant collagen type present in fat-storing cells in culture (Figs.7.6a,b). Even after two weeks of culture (4 passages), cells were strongly positive for type I collagen, as illustrated in Figure 7.6b (Table 7.3). Both the large fat-storing cells with long flat processes and the transformed, spindle-shaped ones contained numerous small collagen type I positive granules. These granules were located perinuclearly and had a similar distribution to that of the vacuoles observed by electron microscopy (cf. Fig.7.4a). The flat cell processes contained only a few positive granules.

Type IV collagen (Fig.7.6c) and small amounts of laminin were present inside granules in fat-storing cells in primary culture and in the first passage; thereafter, cells were negative (Table 7.3).

Figure 7.3 Transmission electron micrographs of fat-storing cells from a 12- (a and c-e) and a 36-month-old rat (b) in primary culture. Details of the cytoplasm.

- a. Microtubules (m) and bundles of microfilaments (f), sometimes showing focal condensations (†), become very pronounced after 2 days of primary culture. 24250 x.
- b. The RER (†) filled with flocculent material has become more abundant and dilatated. 8000 x.
- c. The Golgi area is pronounced; its localization is always at one side, but close to the nucleus. 16650 x.
- d. Multivesicular bodies (†) are often seen in the Golgi area, where the centrioles (c) are also present. 12000 x.
- e. A cilium (†), growing out from one of the centrioles is sometimes observed. 26100 x.

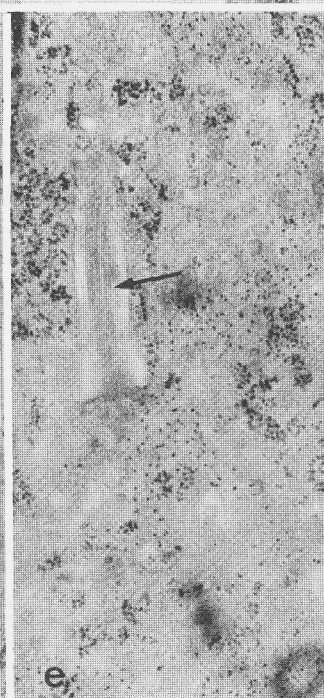
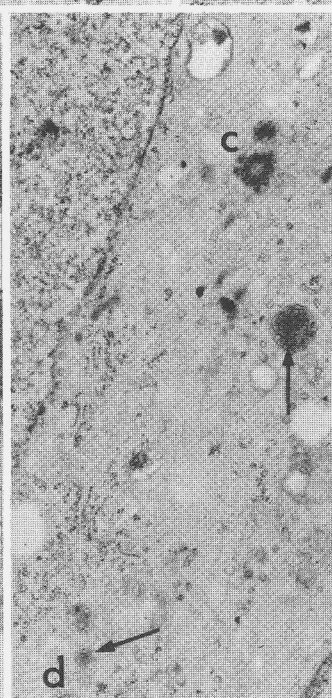
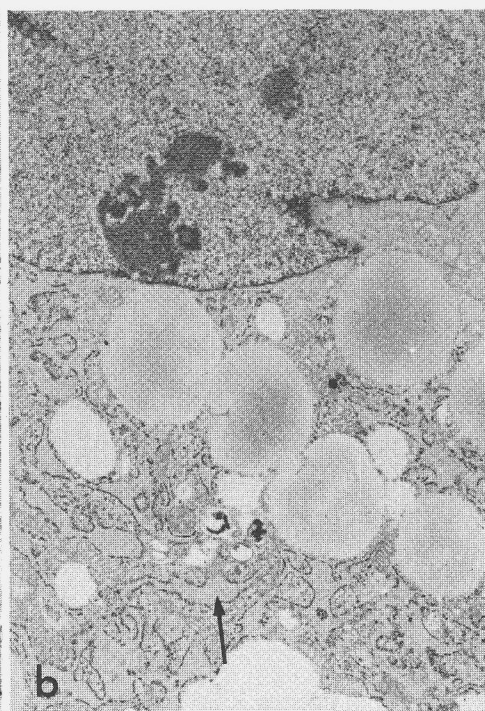
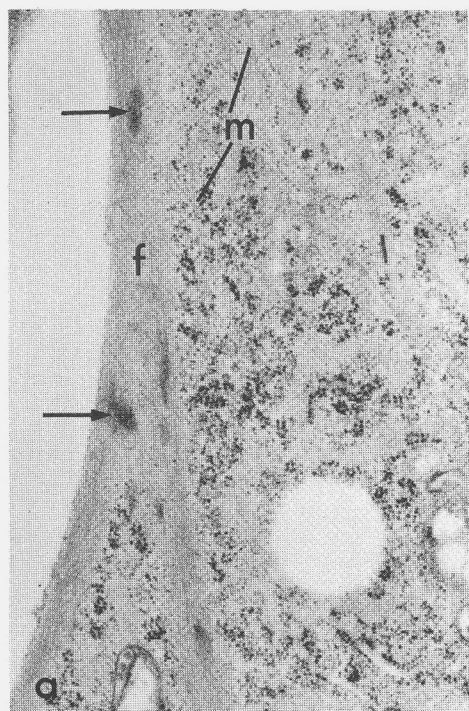


TABLE 7.3 PRESENCE OF COMPONENTS OF THE EXTRACELLULAR MATRIX IN FAT-STORING CELLS IN CULTURE

	primary culture		1st passage	4th passage
	2d	4d	8d	14d
collagen type I	±	+	+	++
procollagen type III	n.t.	-	-	-
collagen type IV	±	±	+	-
collagen type V	n.t.	-	-	-
collagen type VI	n.t.	-	-	-
laminin	-	±	±	-
fibronectin	+	+	+	+

++ strongly positive; + positive; ± weakly positive; - negative
n.t. not tested.

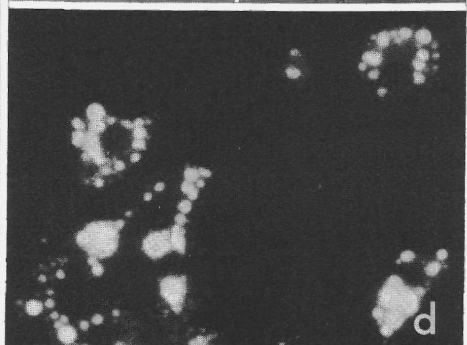
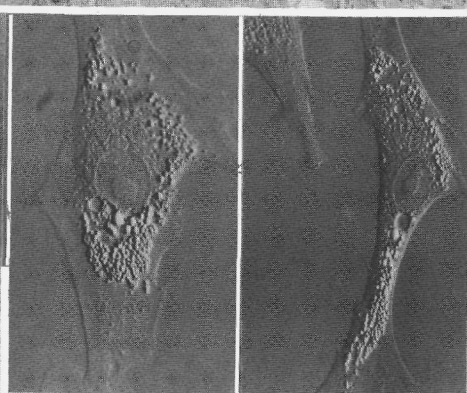
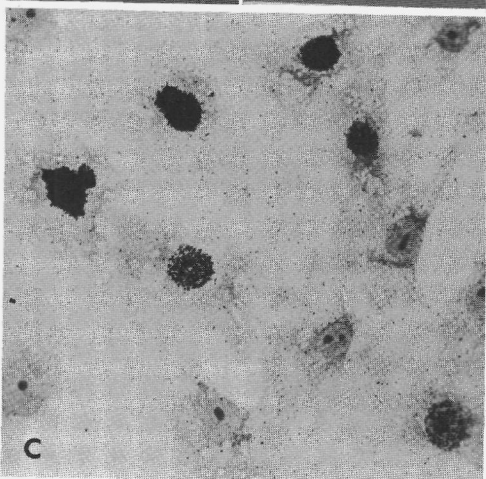
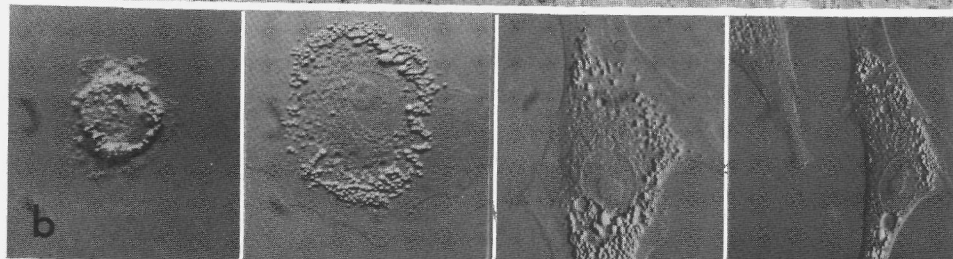
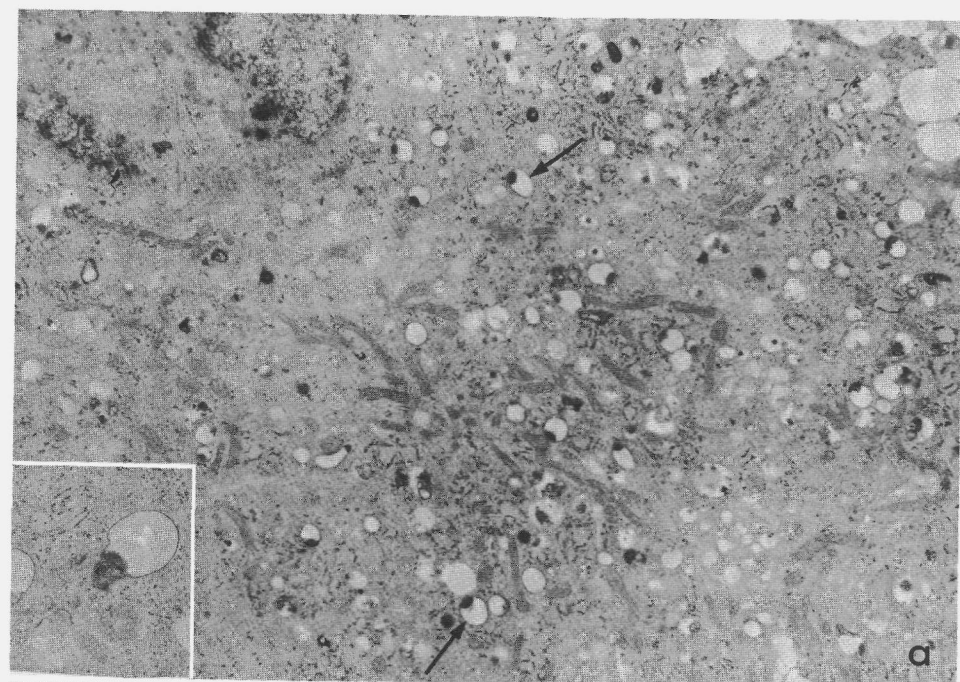
Fibronectin (Fig.7.6d) was found in close contact with the cell surface. In contrast to the collagens type I and IV and to laminin, fibronectin was not found intracellularly. In view of this, fat-storing cells may have adsorbed fibronectin, which is normally present in serum, onto their cell membranes. For all components tested, controls showed only weak and diffuse staining of the cells.

7.4 DISCUSSION

Primary cultures of fat-storing cells were established in a highly reproducible manner from pure suspensions of fat-storing cells prepared from the livers of rats of various ages. In culture, these cells could be transferred for a minimum of two passages without spontaneous transformation. Cells in culture were characterized by morphological investigation and vitamin A fluorescence. Morphological character-

Figure 7.4

- Late during primary culture, but especially in the cell passages, vacuoles (†), which may contain procollagens, develop in the Golgi area. 5800 x. Inset: high magnification of vacuoles containing clumped granular material. 9750 x.
- Cell spreading after trypsinization. From left to right: 30 min, 90 min, 4 h (900 x) and 7 h after trypsinization. 600 x.
- Autoradiogram of fat-storing cells in first passage: incubated for 3 h with ³H-thymidine. Heavily labeled and unlabeled nuclei are seen. 300 x.
- Vitamin A autofluorescence confined to the lipid droplets of fat-storing cells at 1 day in primary culture. 300 x.



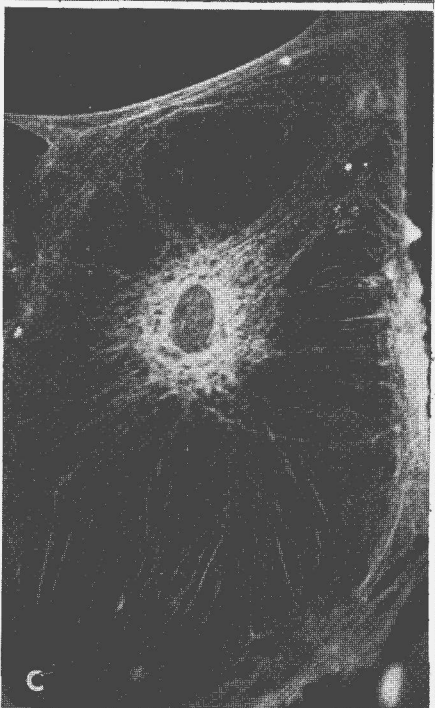
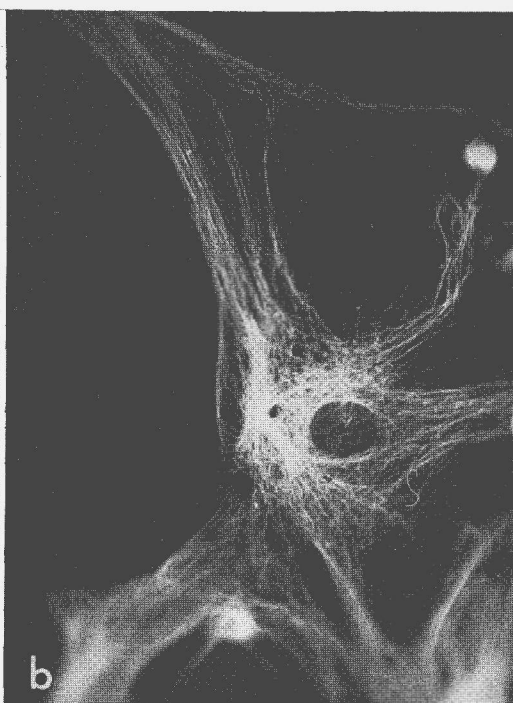
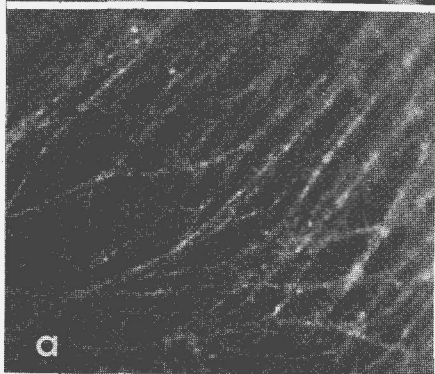
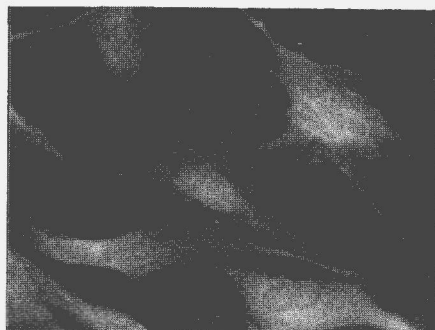
istics of fat-storing cells in vivo were retained during primary culture. Vitamin A, known to be stored in the lipid droplets of fat-storing cells in vivo (Wake, 1980; Knook et al., 1982b; Knook and De Leeuw, 1982), was detected in lipid droplets in cultured cells up to and including the second passage. Fat-storing cells derived from animals of the three age groups behaved similar in culture and the time pattern and nature of phenotypic changes were also largely comparable. The longer persistence of lipid droplets and lipid-associated vitamin A in cells derived from 36-month-old animals is probably a consequence of the accumulation of lipid droplets in fat-storing cells with age and the age-related increase in the amount of vitamin A (H.F.J. Hendriks, personal communication).

The presence of vimentin in fat-storing cells in culture indicates that these cells are of mesenchymal origin, probably fibroblastic. However, it has also been previously suggested that fat-storing cells may be myofibroblasts (Hruban et al., 1974; Farrell et al., 1977) based on the observation of bundles of microfilaments with focal condensations in such cells in vivo. Similar morphological observations were made in the present study for fat-storing cells in culture. The cytoskeletal protein desmin is suggested to be an exclusive marker for myofibroblasts (Osborn and Weber, 1982). It was shown recently that in the intact liver antibodies to desmin stain only fat-storing cells (Yokoi et al., 1984). These results strongly indicate a certain homology between fat-storing cells and myofibroblasts and suggest that desmin may be used as an exclusive marker for fat-storing cells.

The presence of various components of the extracellular matrix in fat-storing cells in culture suggests the involvement of these cells in fibrogenesis in the intact liver, although the capacity to synthesize components of the extracellular matrix has also been shown in culture for other cell types derived from normal and fibrotic liver (Hata and Nagai, 1980; Patrick et al., 1981; Guzelian et al., 1981; Tseng et al., 1982; Voss et al., 1982; Konomi et al., 1982; Irving et al., 1984). Fat-storing cells in vitro contained collagen types I and IV and laminin but no type III procollagen was found. However, fat-storing cells were thought to be involved in the synthesis of the type III rich "reticular" fibers present in the space of Disse (Kent et al., 1976). Recently, Clement et al. (1984) demonstrated

Figure 7.5 Indirect immunofluorescence of cytoskeletal proteins, detected in first passage cultures of fat-storing cells from 12-month-old rats.

- a. Vimentin. Upper part: antibodies diffusely stain the entire cytoplasm with a slight concentration around the nucleus. 400 x. Lower part: staining of individual fibers is revealed at higher magnification. 3500x.
- b. Tubulin. Antibodies are decorating thin intensely stained fibers often seen to cross over the nucleus. 1200 x.
- c,d. Actin. Antibodies are decorating fibers radiating out from the nucleus to the cell periphery. A dotted perinuclear staining is also seen (c) 1200 x). High magnifications reveal the fibrous pattern: thick stress-like fibers, sometimes with an interwoven network of thin fibers (d). 3500 x.



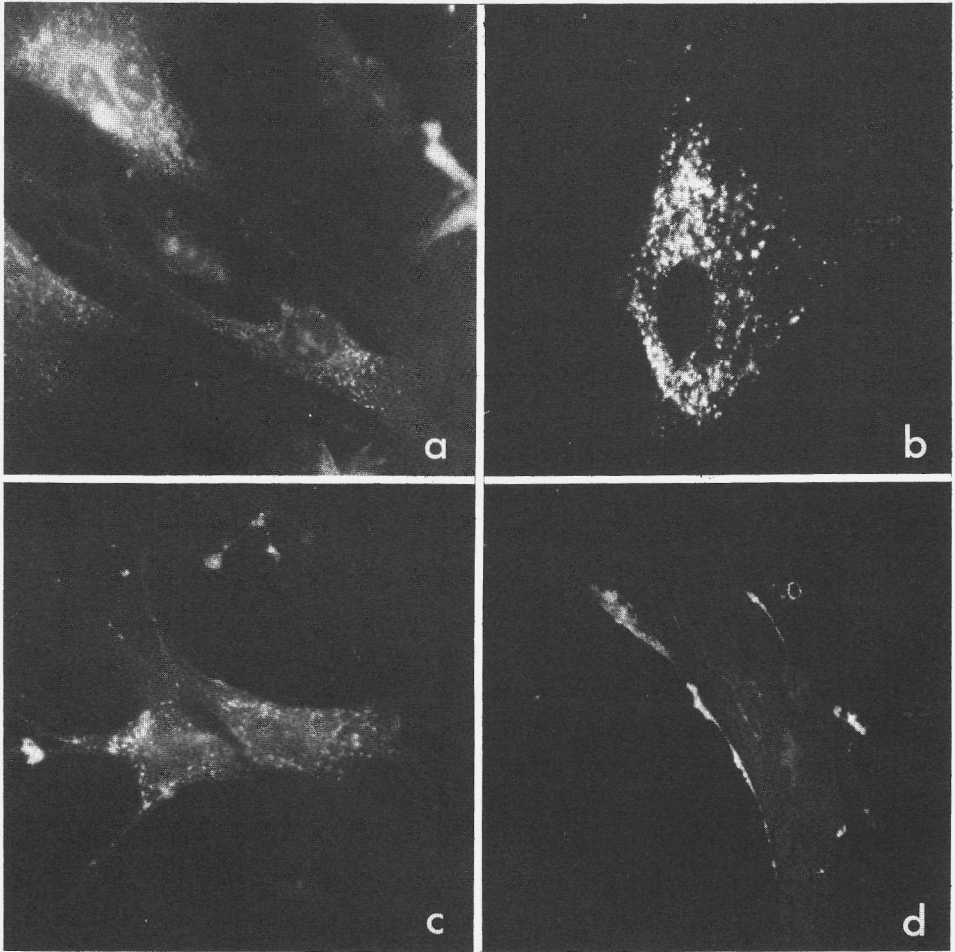


Figure 7.6 Indirect immunofluorescence of extracellular matrix components detected in fat-storing cells from 12-month-old rats.

- a,b. Collagen type I. a) Four-day-old primary culture: label is present in small granules which are primarily located around the nucleus. 400 x. b) Fourth passage culture: fat-storing cell containing type I collagen intracellularly. 600 x.
- c. Collagen type IV: first passage culture. Label is present in small granules similar to those as seen with type I collagen. 400 x.
- d. Fibronectin, first passage culture. Label is seen aligned parallel to the cell membrane. No label is detected inside the cell. 600 x.

(Photographs by A. Geerts; Free University of Brussels, Belgium)

the presence of procollagen type III in fat-storing cells in the intact liver. The discrepancy between these and the present results can be explained by the different nature of the antibodies used. The presence of type IV collagen and laminin suggests the involvement of fat-storing cells in the formation of the incomplete basal lamina known to be present between parenchymal cells and sinusoidal endothelial cells (Geerts et al., 1982; Voss et al., 1982). However, liver endothelial cells also seem to participate in this, because type IV collagen is demonstrated in such cells in culture (Irving et al., 1984).

Our results on fat-storing cells in culture show that these cells represent a population with a potentially high proliferative capacity. The number of fat-storing cells present in rat and human liver under normal physiological conditions is unknown. Estimations for these two species range from 5 to 12×10^6 cells per gram of liver (Bronfenmayer et al., 1966; Horvath et al., 1973; Giampieri et al., 1981; Knook et al., 1982b). Several investigators have reported an increase in the number of fat-storing cells in hypervitaminosis A in humans (Horvath et al., 1973; Hopwood and Nyfors, 1976), in psoriatics treated with methotrexate (Horvath et al., 1973; Hopwood and Nyfors, 1976) and in rats kept on a low magnesium diet (Rozycka et al., 1978). The only criterion used to identify fat-storing cells under the light microscope in these studies was the presence of lipid droplets, which could easily lead to an underestimation of the number of fat-storing cells per unit of liver. In our opinion, the increase in the number of fat-storing cells reported in these publications was most likely due to the fact that such cells which contain more lipid droplets than under normal conditions (due to the various abnormalities mentioned above) are better recognized under the light microscope, as was earlier suggested by Kent et al. (1976).

In experimental liver fibrosis, a true proliferation of cells, at least partly derived from fat-storing cells, seems to occur. In liver fibrosis induced by carbon tetrachloride (McGee and Patrick, 1972; Kent et al., 1976) or heterologous serum (Ballardini et al., 1983), transitions between cells with the morphological characteristics of fat-storing cells and those of fibroblasts have been observed. Leo and Lieber (1983), using long-term administration of ethanol combined with moderate vitamin A supplementation, reported the development of liver fibrosis in the rat, which is normally refractory to ethanol-induced liver fibrosis. This condition correlated with decreased levels of liver vitamin A and numbers of fat-storing cells in addition to the appearance of a large number of myofibroblasts associated with abundant collagen fibers.

In summary, there seems to be accumulating evidence that fat-storing cells play an important role in various stages of the development of liver fibrosis. In vivo data suggest that at least a part of the increasing population of (myo)-fibroblastic cells may be derived from fat-storing cells (McGee and Patrick, 1972; Kent et al., 1976; Ballardini et al., 1983; Leo and Lieber, 1983). Fat-storing cells in vitro proved to be a population of cells with a potentially high proliferative

capacity. Both in vivo and in vitro, division of fat-storing cells is accompanied by phenotypic changes and a decrease in the vitamin A content. This similarity in changes leads to the question of whether there is a common factor both in vivo and in vitro which induces these changes. Fat-storing cells in culture may prove to be a useful model system for studying such factors regulating the phenotypic expression of fat-storing cells.

CHAPTER 8

GENERAL DISCUSSION

The aim of the studies described in this thesis was to evaluate information obtained on age-related changes in the ultrastructural morphology of the three types of sinusoidal liver cells in the intact rat liver, after cell isolation and in culture. This was done to gain insight into a possible cellular substrate for age-related changes in cell functions. In addition, results obtained on the number of sinusoidal liver cells isolated per liver from rats of various age groups were employed to determine changes in the cellular composition of the rat liver with age.

8.1 AGE-RELATED ULTRASTRUCTURAL MORPHOLOGY OF RAT SINUSOIDAL LIVER CELLS AS A POSSIBLE INDICATOR OF FUNCTIONAL CHANGES:

A SUMMARY

Age-related studies on the ultrastructural morphology of liver cells have been limited to parenchymal cells. These included morphometric studies which revealed an increase with age mainly in cell size and lysosomal volume, the latter being partly due to accumulation of lipofuscin in these organelles (Pieri et al., 1975; Schmucker et al., 1978; Meihuizen and Blansjaar, 1980). Other changes reported were an age-related decrease in the total mitochondrial volume and there were contradictory results concerning age-related changes in the amounts of smooth endoplasmic reticulum (Pieri et al., 1975; Schmucker et al., 1978; Meihuizen and Blansjaar, 1980). A recent study combining enzyme cytochemistry of acid phosphatase with morphometry showed much greater increases in lysosomal volume density than found with morphometry alone (De Priester et al., 1984). This suggested that the use of the enzyme activity as a marker for lysosomes may yield more accurate values for lysosomal volume density than do morphological characteristics alone.

During the past few years, studies have indicated that some functions of sinusoidal liver cells change with age. In humans, functions of the reticuloendothelial system of which Kupffer cells represent a major part were found to diminish with age (for a review, see Brouwer and Knook, 1983). In rats, the capacity of Kupffer cells to endocytose heat-denatured albumin was found to decrease with age (Brouwer et al., 1985). Changes with age also occur in the lysosomal systems of both endothelial and Kupffer cells, as age-related changes in several lysosomal enzyme activities in these cells have been found (Knook, 1985b).

In general, the ultrastructural morphology of cells reflects their functional capacity. With respect to sinusoidal cells of the liver, no information was available on the age-related ultrastructural morphology. Therefore, in the study described

in this thesis the age-related ultrastructural morphology of sinusoidal cells of the rat liver was investigated in an attempt to find possible cellular substrates for age-related changes in sinusoidal cell function.

The ultrastructural morphology of sinusoidal cells in the intact liver was found to be quite similar for 3- to 36-month-old female BN/BiRij rats. However, there was an age-related increase in the iron content of endothelial and Kupffer cells. The iron was found in complex with apoferritin mainly in the lysosomal systems of both cell types. Endogenous peroxidatic activity in Kupffer cells appeared to be similar at all age levels as regarded both its staining intensity and intracellular location. The number of cytoplasmic lipid droplets per cross-sectioned fat-storing cell was greater and more homogeneous in 6- to 36-month-old animals than in 3-month-old ones (Chapter 2). In addition to the ultrastructural morphology of the cells, information was obtained on the age-related cellular composition of the sinusoidal cell population and the number of sinusoidal liver cells per unit of liver tissue. Both appeared to be unchanged with age (Chapters 2 and 3).

From the results obtained in these studies, it can be concluded that in healthy aging animals no great aberrations are to be expected in the functioning of the sinusoidal cells. Moreover, the stability of cell organelle systems such as the sieve plate structures in endothelial cells and those involved in synthesis of proteins in, for example, fat-storing cells, indicates that sinusoidal cell functions are well maintained in old age.

With the strict descriptive methods applied in the studies described here, it is not very likely that small changes in the volume densities of certain cell organelle systems in sinusoidal liver cells reflecting age-related functional changes will be detected. To study possible age-related changes in some specific functions of sinusoidal liver cells in more detail, further morphological investigations regarding these cells should include additional methods such as morphometry and enzyme cytochemistry which have also been applied in age-related studies of the liver parenchymal cells (Jones and Schmucker, 1977; Wilson, 1983). These studies may concern the following.

(i) The cellular composition of the liver with age. With age, parenchymal and sinusoidal liver cells are increased in functional cell mass by polyploidization and cell multiplication, respectively. Age-related morphometric studies of the densities of these two cell populations in the liver can give further information on the cellular composition of the liver in relation to age (see also Section 8.2).

(ii) The uptake and degradation capacity of endothelial and Kupffer cells. Some studies have indicated that changes may occur with age in parameters indicative for these processes. Brouwer et al. (1985) found that the uptake capacity of rat liver Kupffer cells for heat-denatured albumin progressively decreased with age, whereas no change was observed for endothelial cells. In addition, age-related changes in lysosomal enzyme activities were found in purified endothelial and Kupffer cells (Knook, 1985b). The storage of iron in the lysosomal systems of

Kupffer and endothelial cells may also affect the degradation capacity of these cells (Chapter 2), as has been postulated for the storage of lipofuscin in lysosomes of the parenchymal cells of the liver (Knook, 1985a).

Age-related morphometric studies of volume densities of those organelle systems in endothelial and Kupffer cells which are involved in uptake and degradation combined with clearance studies and morphological analysis of uptake and transport in liver of various compounds using detection methods such as immunocytochemistry and autoradiography may further clarify the capacity of these cells for uptake and degradation in relation to the aging process.

(iii) Fibrogenesis in the liver. Grasedijk et al. (1980) and Heck et al. (1981) reported that no age-related changes occur in the rat liver in a number of parameters indicative for fibrogenesis. However, it is well-documented that a variety of factors may disturb the balance between the synthesis and degradation of the extracellular matrix, often leading to the development of liver fibrosis and cirrhosis (Patrick, 1982). These observations, therefore, indicate only that in healthy individuals a delicate balance between synthesis and degradation of the extracellular matrix is maintained throughout the entire life span. Several types of injury to the liver can disturb this balance (Patrick, 1982) and start a complex process of regeneration, the course of which could quite possibly be dependent on the age of the individual. To study such processes, the rat liver may represent a good model system for the human situation with respect to the pathogenesis of liver fibrosis and cirrhosis caused by a variety of agents. The contribution of the various cell types and the primary mechanisms of the process can be determined accurately only in animal models such as the rat.

8.2 CELLULAR COMPOSITION OF THE LIVER WITH AGE

It was explained in Chapter 3 that the cellular composition of the liver may not be a constant with age. The phenomenon of an age-related increase in the ploidy status of hepatocytes results in a decrease in their number per unit of liver tissue. In studies described in this thesis, it was observed that the number of sinusoidal liver cells isolated per liver increased with age. As liver weight in rats also increases with age, this was an indication that the number of sinusoidal cells per unit of liver tissue remains constant with age. These two tendencies were postulated to result in an age-related shift in the ratio between parenchymal and sinusoidal liver cells (cf. Section 3.4). The significance of such a shift for liver research is exemplified below.

The contribution of each liver cell type to a specific liver function can be quantitatively determined by the use of isolated and purified cell preparations. This makes possible the determination of the parameter under investigation on a per cell basis. To determine the contribution of a given cell type to the activity of the whole liver, it is necessary to know the total number of each cell type present in the liver. In young rats, sinusoidal liver cells comprise about one-third of all liver

cells (Fabrikant, 1968; Greengard et al., 1972). However, from other literature data (cf. Table 3.4) and from studies described in this thesis, it appears that both the composition of the sinusoidal cell population and the ratio between parenchymal and sinusoidal liver cells may be dependent not only on species and husbandry conditions including diet but also on age (cf. Section 3.4). These factors considerably complicate reliable recovery calculations.

The use of a so-called Ito cell index (the number of fat-storing cells per 1000 hepatocytes) should be evaluated critically in this respect. This ratio is often used to determine the effect of various treatments on the number of fat-storing cells (cf. Table 3.4) in groups of individuals which, especially in humans, are not age-matched. This implies that the ratios determined may also be influenced by age-related changes in the cellular composition of the liver. This can be illustrated by a study of Leo and Lieber (1983), who determined Ito cell indices in rats maintained for 2 and 9 months, on an alcohol-containing liquid diet with moderate vitamin A supplementation. An increase in the Ito cell index was observed at between 2 and 9 months, which was explained as an increase in the number of fat-storing cells. However, the authors also reported that the number of hepatocytes per cm^3 of liver tissue was smaller when the animals were kept on the alcohol-containing liquid diet for 9 months than was found for 2 months. These observations indicate that the increase in the number of fat-storing cells as determined by the Ito cell index was overestimated due to the age-related decrease in the density of the hepatocytes in the liver.

It is clear from the literature and from the example given above that standard values for total numbers of each type of liver cell per whole liver and for cell ratios cannot be provided because of variations in these parameters related to species, age and diet.

8.3 THE USE OF ISOLATED AND PURIFIED SINUSOIDAL LIVER CELL SUSPENSIONS IN HEPATIC RESEARCH

Sinusoidal liver cells were isolated and purified from 3- to 36-month-old rats in high yield, purity and cell viability. The cellular composition of such cell preparations isolated from 12- to 36-month-old animals was similar, irrespective of total nonparenchymal cell yield. At 3 and 6 months of age, the yield of fat-storing cells was much lower. The numbers of sinusoidal liver cells isolated per liver increased with age. The cellular composition of the sinusoidal liver cell suspensions obtained from animals of different ages was slightly different due to variations in the number of bile duct epithelial cells isolated (which increased in late age) and to changes in some of the characteristics of sinusoidal liver cells such as cell volume and density (Chapter 3).

Generally, it was possible to isolate and purify from 3- to 36-month-old animals all three types of sinusoidal cells from one liver in high yield and sufficient viability and purity. It was concluded that minor modifications in the cell isolation

and purification procedures when applied to rats of different ages will result in cell preparations which are optimally comparable with respect to yield, composition and purity. The application of the isolation and purification procedure to rats of various ages, therefore, enables the investigation of specific hepatic functions in populations of cells isolated from one animal, also in relation to the aging process.

Cell preparations obtained by pronase and collagenase digestion of the liver now also include the fat-storing cells. Such preparations can be used to characterize biochemically all three types of sinusoidal liver cells with respect to their contribution to various metabolic processes occurring in the liver (for reviews, see Wisse and Knook, 1979; Van Berkel, 1982). Furthermore, the contributions of endothelial cells, Kupffer cells and fat-storing cells to the processing by the liver of a variety of compounds such as glycoproteins, lipoproteins, drugs and vitamins can be evaluated by the determination of labeled substances in pure liver cell preparations after their *in vivo* administration prior to cell isolation (Praaning-van Dalen et al., 1981; Praaning-van Dalen and Knook, 1982; Van Berkel et al., 1982; Brouwer et al., 1985; Hendriks et al., submitted).

No matter how useful determinations in experimental animal models may be, basic differences can exist in the hepatic functioning between rodent and human liver. These may now be further investigated by application of the isolation and purification procedures developed in rats to human liver (Kirn et al., 1982). Investigations with such human liver cell preparations may substantially increase our knowledge of the similarities and differences between human and rat liver as concerns a number of hepatic functions and may also give further information on the suitability of the rat liver as a model for the human situation.

8.4 CULTURES OF SINUSOIDAL LIVER CELLS

8.4.1 Mixed cultures of sinusoidal liver cells

Mixed cultures of sinusoidal liver cells may be of limited use because of the great changes in the cellular composition of the cultures with time (Chapter 4). However, certain aspects of hepatic function which involve close cooperation between different liver cell types such as vitamin A metabolism or the regulation of the delicate balance between the synthesis and degradation of the extracellular matrix may be better understood as a result of studies in mixed cultures of different types of liver cells.

It was found that sinusoidal liver cells in mixed cultures behaved only slightly differently from endothelial, Kupffer and fat-storing cells in pure cultures (Chapter 4). In contrast to endothelial cells in pure cultures which could be maintained only when the culture medium was supplemented with homologous rat serum (Chapter 5), endothelial cells in mixed cultures also survived in medium supplemented with fetal calf serum (Chapter 4). The lysosomal system of Kupffer cells in mixed cultures was more heavily filled with cell debris than that of cells in

cultures derived from pure Kupffer cell suspensions (cf. Chapters 4 and 6). No age-related changes in the culture characteristics of sinusoidal liver cells were found in either mixed or pure cultures (Chapters 4 to 7).

From the study described in Chapter 4, it appears that investigations on certain liver functions can be conducted in mixed cultures only when the cellular composition of the cultures is carefully considered. For such studies, it may be better to develop culture systems which are established by mixing pure cell suspensions of different types in ratios which are determined by factors such as the cell ratio in the intact liver.

8.4.2 Cultures of sinusoidal endothelial cells

A culture system for purified suspensions of rat liver endothelial cells was developed by use of a collagen substratum enabling cell attachment and a culture medium supplemented with homologous rat serum. Respreading of the cell processes containing intact sieve plates was observed as early as 3 hours in culture. The presence of receptor-mediated endocytic activity in cells in culture was indicated by the reappearance of the capacity to endocytose horseradish peroxidase. Endothelial cells could be maintained in culture for 3 to 4 days, after which cell detachment and cell death occurred. Endothelial cells isolated from rats of various ages were similar in all of these characteristics (Chapter 5).

Primary cultures of nondividing sinusoidal endothelial cells have been employed by several authors for the investigation of a number of endothelial cell functions (Steffan et al., 1981; Roos et al., 1982; Smedsrød et al., 1982; Chapter 5). Recently, Shaw et al. (1984) described a culture system for guinea pig sinusoidal endothelial cells (characterized and cultured for up to nine passages) employing a substratum of guinea pig liver collagen and gelatin. Irving et al. (1984) succeeded in establishing subcultures of rat liver endothelial cells by the addition of endothelial cell growth supplement and fibroblast growth factor to the culture medium. Such cultures of dividing endothelial cells may be important for defining factors which regulate the phenotypic expression of liver endothelial cells. However, for investigations of physiological cell functions, primary cultures of nondividing cells are more relevant.

Primary cultures of sinusoidal endothelial cells from rat and human liver may prove useful for investigation of various aspects of liver endothelial cell function, also with respect to the effect of aging on cell function. These aspects of endothelial cell function include the following.

(i) The filtration function of endothelial cells and its regulation. It has been postulated that the number and size of endothelial fenestrations can change in response to changes in the sinusoidal microenvironment such as alterations in sinusoidal blood flow, intraluminal pressure, O₂ content and the deposition of extracellular matrix components in the space of Disse (Mak and Lieber, 1984). In addition, injection of serotonin and noradrenalin into the portal vein of rats was

shown to transiently decrease the diameter of endothelial fenestrations (Wisse et al., 1980), whereas alcohol administration in both rats and baboons was shown to increase the diameter (Fraser et al., 1980; Mak and Lieber, 1984) but to decrease the number of fenestrae (Mak and Lieber, 1984). The alterations in the sinusoidal endothelium due to alcohol most likely also interfere with the exchange of particles and solutes between sinusoidal blood and parenchymal cells and may thereby contribute to the pathogenesis of alcoholic liver injury (Fraser et al., 1980; Mak and Lieber, 1984). For investigation of factors such as hormones which regulate the pore size and number of fenestrae, short-term culture of sinusoidal endothelial cells may be an adequate system for comparing regulators in an endothelial cell population derived from one animal.

(ii) The barrier function of endothelial cells. Endothelial cells in general protect underlying tissues from the damaging effects of factors present in the blood, such as viruses, bacterial endotoxins and tumor cells. Liver sinusoidal endothelial cells have been shown to protect parenchymal cells against viral penetration (Steffan and Kirn, 1979) and to interact with endotoxin (Frenzel et al., 1977) and tumor cells (Roos et al., 1982). Investigation of the interactions of liver endothelial cells with blood constituents may be facilitated by studies in short-term cultures of these cells in attempts to understand the role of endothelial cells in and contribution to a protective barrier for parenchymal cells.

(iii) Endocytosis. Liver endothelial cells have been shown to significantly contribute to the uptake of mainly macromolecules from the blood (Van Berkel et al., 1982; Praaning-van Dalen et al., 1981, 1982). It also appears that, in analogy with Kupffer cells in culture, receptor-mediated uptake is restored in cultured liver endothelial cells (Chapter 5). Short-term culture of purified endothelial cells may therefore be an adequate system for qualitatively analyzing the occurrence of various receptors on these cells. However, determination of the quantitative contribution of liver endothelial cells to hepatic uptake of various substances is possibly better investigated by *in vivo* clearance studies combined with quantitative determinations of labeled substances in isolated and purified liver cell populations (Praaning-van Dalen and Knook, 1982).

8.4.3 Cultures of Kupffer cells

A culture system for purified suspensions of Kupffer cells was developed. Cells could be maintained in culture for several weeks, but no mitoses in Kupffer cells were observed. Cells in culture were quite similar to Kupffer cells in the intact liver with respect to their ultrastructural morphology, endocytic activity and the intracellular localization of endogenous peroxidatic activity and acid phosphatase for 2 to 3 days in culture. A decline in enzymatic and endocytic activity was found thereafter. Kupffer cells isolated from rats of various ages behaved similarly in all of these aspects (Chapter 6).

Kupffer cells in culture have been used for some time to investigate their

various functions. In most of these studies, the capacity of Kupffer cells to attach to plastic or glass surfaces was used to enable the enrichment of isolated liver cell preparations in these cells (Munthe-Kaas et al., 1975; Emeis and Planqué, 1976). However, some functional properties of these Kupffer cells such as endocytic activity and levels of lysosomal enzymes were significantly altered as compared with freshly isolated Kupffer cells and such cells in maintenance cultures previously purified from the nonparenchymal cell suspension by centrifugal elutriation (Brouwer et al., 1980).

As described in Chapter 6, purified Kupffer cells in maintenance culture exhibit morphological and functional characteristics which are closely similar to those of cells in the intact liver for at least two days of culture. This similarity enables the use of culture systems of purified Kupffer cells for investigation of their various functions.

Kupffer cells in culture have been particularly useful for studying their participation in the liver clearance of various compounds, also in relation to age (Brouwer et al., 1985), their function in the synthesis and secretion of monokines such as pyrogen and prostaglandins in response to various stimuli (Haeseler et al., 1977; Decker et al., 1982), their participation in the immune response (Nadler et al., 1979) and in the detoxification of endotoxins (Brouwer et al., personal communication) and their role in the protection of the liver against viral infections (Kirn et al., 1982).

Future studies with Kupffer cells in culture may also result in the elucidation of their participation in the complex of processes occurring at the beginning of liver fibrosis and in the further clarification of the functional capacity of Kupffer cells at different age levels. This latter aspect is important in view of the decline observed in humans in the activity of the reticuloendothelial system (Brouwer and Knook, 1983) of which the Kupffer cells form a major part.

8.4.4 Cultures of fat-storing cells

Primary cultures and subcultures of fat-storing cells isolated from 12- to 36-month-old rats were established. Fat-storing cells in culture assumed a fibroblastic appearance and gradually lost their vitamin A content as determined by fluorescence microscopy. Cells in culture were shown to contain vimentin, actin and tubulin as elements of the cytoskeleton. Fat-storing cells in primary culture and in subculture contained collagen type I, whereas type IV collagen and laminin could be demonstrated only in cells in primary culture. The presence of collagen types III, V and VI could not be demonstrated. Fibronectin was seen to be extracellularly aligned along the cell membrane of cells in culture (Chapter 7).

Recently, Okabe et al. (1984) isolated and cultured a vitamin A storing cell type from rat lungs and described features of cells in culture largely comparable to those of liver fat-storing cells. The similarity in culture characteristics between lung and liver cells suggests that in a variety of organs, including the liver and

lungs, cells of a fibroblastic origin capable of storing vitamin A may be present, as was earlier suggested by Wake (1980). Whether these cells should be classified under a common denominator, for example, "vitamin A-storing cells" awaits a general opinion on whether vitamin A storage occurs exclusively in such cells.

Fat-storing cells in culture exhibited characteristics which are also observed in such cells in prefibrotic livers, such as in alcoholic liver diseases in humans (Nakano et al., 1982; Okanoue et al., 1983; Minato et al., 1983) and after experimental induction, e.g., by carbon tetrachloride, heterologous serum and ethanol (Kent et al., 1976; Ballardini et al., 1983; Leo and Lieber, 1983). These characteristics include cell multiplication, a phenotypic change to a more myofibroblastic or fibroblastic appearance and loss of stored vitamin A. These observations suggest that fat-storing cells in culture more closely resemble stimulated cells and not normal "resting" ones. Attempts to maintain fat-storing cells in culture in a phenotype more similar to their appearance in normal liver, for example by addition of different retinoids to the culture medium, have been unsuccessful up to now (H.F.J. Hendriks, personal communication). Comparison of fat-storing cells in the intact liver at the beginning of liver fibrosis and of cells in culture indicates that fat-storing cells in the normal liver are under a strong inhibitory control based on either cellular contacts or cell products and that upon liver injury, by ethanol for example, these regulatory mechanisms are disturbed.

Cultures of purified fat-storing cells offer potential means for clarifying the role of fat-storing cells in vitamin A metabolism and in the pathogenesis of liver fibrosis as well as for investigations into the possible correlation between these two processes. Studies involving possible pathogenic factors inducing liver fibrosis in culture systems of fat-storing cells as well as other liver cell types which can be the primary targets of such factors may lead to a better understanding of the role of various liver cell types in the complex of processes occurring at the beginning of fibrosis.

8.5 FUTURE PERSPECTIVE IN SINUSOIDAL LIVER CELL RESEARCH

During the last two decades, our knowledge of sinusoidal liver cells has been greatly increased by the application of advanced techniques with respect to biochemistry, morphology and cell isolation and culture procedures. In the coming years, major lines of investigation will focus on the contribution of endothelial and Kupffer cells to the liver clearance of glycoproteins and lipoproteins, the participation of fat-storing cells in the liver metabolism of vitamin A and other lipid-soluble vitamins and the role of all liver cell types in the pathogenesis of liver fibrosis. Morphological investigations, including morphometry, enzyme cytochemistry, immunocytochemistry and autoradiography may play a significant role in the elucidation of these processes as an integrated part of larger projects also including other methodologies.

With age, a decrease in some specific functions of both sinusoidal and paren-

chymal cells of the liver does occur, that may lead to a decreased functioning of the liver as a whole and that may cause specific problems in the performance of the total organism. The investigation of such functions as the capacity to detoxify endotoxins and to metabolize various drugs, may be rewarding with respect to the prevention of problems occurring in old age; however, it does not necessarily lead to a better understanding of underlying mechanisms of the aging process.

At our Institute, the liver was chosen as a model system to study aging phenomena at the cellular level because of the presence in liver of two cell populations showing different cell kinetics during the life span of the organism (Knook, 1980). The parenchymal cells are so-called reverting postmitotic cells. This means that in healthy organisms during their entire life span the same parenchymal cells remain present in the liver. In contrast, the sinusoidal liver cells are constantly renewed by cell multiplication and do, therefore, live much shorter than do parenchymal cells. It was hypothesized that by investigation of the main functional capacities of these two cell populations in relation to age, a general picture might develop correlating the functioning of the cell and its "age" (Knook, 1980). However, the results obtained in age-related liver cell research indicate that such a simple picture does not hold true. Within one cell population, specific functions may either remain the same, get worse or may even improve. As a consequence, no essential difference could be established between the long-lived parenchymal cells and the short-lived sinusoidal cells with respect to the pattern of changes in cell functioning with age. In addition, the complexity of the picture indicates that during aging not all cellular processes are affected in a similar manner and that the affection may be cell type-dependent.

In the unifying concept of cellular aging as the basis of aging of the organism (Knook, 1982b) more attention is drawn to the variety of factors which may cause damage to the cell. These include environmental factors such as radiation or components of the diet but also damaging agents produced inside the cell such as free radicals. In general, cells are quite capable of repairing damage incurred by environmental or intracellular factors. However, it must be clear that different cell types will not be exposed in a similar extent to the damaging effect of various factors. In addition, one cell type may have a different repair capacity as compared to others and "old" cells may reveal a diminished repair (Vijg et al., 1985).

Cell damage caused by mechanisms such as mentioned above may be the underlying causes of functional changes observed with age in various types of liver cells. As the liver is composed of different cell types, this organ may be a valuable model to study damage to specific cell structures and the efficiency of cellular repair under varying conditions in relation to the aging process. The possibility to isolate and purify all major liver cell types and detailed ultra-structural investigations may thereby contribute to a better understanding of factors which characterize and effect the aging process.

SUMMARY

The liver is a key organ interposed between the intestinal tract and the rest of the body. Hepatic function includes the effective uptake from the blood of nutrient components absorbed in the intestine and their subsequent metabolism, storage and distribution to blood and bile as well as the biotransformation of xenobiotic components, drugs and endogenous metabolites. The liver acinus is the smallest functional unit of the liver where the exchange between blood and liver cells of nutrients and various other compounds occurs. The liver acini are composed of two cell populations, the parenchymal cells or hepatocytes and the sinusoidal liver cells. Parenchymal cells account for 70% of total liver volume and for 60% of the total number of liver cells in young-adult rats. The sinusoidal liver cells contribute only 6% to the total liver volume. They play an important role, however, in a number of liver processes which can also be recognized from their contribution to the total number of liver cells (30%). The remaining portion of the total liver volume is accounted for by the spaces through which the blood and bile are transported. The remaining portion of the total number of liver cells is formed by cells which line the larger blood vessels and bile ducts. The latter vessels and ducts occur mainly outside the liver acini.

The sinusoidal liver cells are comprised of three cell populations, the sinusoidal endothelial cells, the Kupffer cells and the fat-storing cells. Each cell type has its own specific functions. Other functions of the liver are achieved by mutual collaboration between the sinusoidal and parenchymal liver cells. The functional capacities of families of cells and, therefore, also of the different types of liver cells, is reflected by the ultrastructural morphology characteristic for each cell type. The different liver cell types can, therefore, be clearly distinguished by their morphology and their localization within the liver acini.

The endothelial cells form a lining for the blood spaces of the liver known as hepatic sinusoids. In this way, these cells create a selective filter or barrier between blood contained in the sinusoid and the underlying liver parenchymal cell. In addition, these endothelial cells are involved in the uptake and metabolism of various macromolecules such as glycoproteins and lipoproteins.

The Kupffer cells are hepatic macrophages located in the sinusoidal spaces of the acinus. They play an important role in the protection of the organism against toxic compounds present in the blood such as components of bacteria. Kupffer cells also synthesize and secrete a number of compounds which may regulate certain physiological processes. In addition, these cells take part in the immunological response.

The fat-storing cells are located under the endothelial cell layer and between the parenchymal cells. They play an important role in the storage of vitamin A and other lipid soluble vitamins. Furthermore, these cells have recently been shown to

act as fibroblasts and thus play a role in the production of the extracellular matrix components in liver. These components are important for maintenance of the structural organization of the liver.

A number of liver functions decreases with increasing age of the organism. The cellular basis for such a change is not yet completely clear. Age-related research with respect to specific liver functions has been focussed mainly on parameters specific for liver parenchymal cells. In contrast, function of sinusoidal liver cells has been investigated only sporadically. The possibility for specifically evaluating the function of sinusoidal liver cells has been greatly enhanced by development of methods to isolate and purify different classes of liver cells. Specific liver functions can now be investigated using suspensions and cultures of purified liver cells which allows observation of cell processes independent of influences from outside the liver.

The study described in this thesis concerns the ultrastructural morphology of the sinusoidal liver cells of female BN/BiRij rats, in age groups from young-adult (3-month-old) to old (36-month-old). The purpose of the study was to investigate the morphology of these cells in both the intact liver, after cell isolation and also in culture in order to find a possible correlation between cellular morphology and age-related functional changes in the liver. In addition, the composition of the sinusoidal cell population and the number of cells isolated per total liver were correlated with age-related changes in the ratio between parenchymal cells and sinusoidal liver cells.

In the intact liver, the ultrastructural morphology of sinusoidal liver cells is quite similar in rats varying in age between 3 and 36 months old. However, clear changes were observed with respect to the content of the lysosomal systems of both endothelial and Kupffer cells. With increasing age, an increasing amount of iron is found in the lysosomes but also free in the cytosol. This increase in iron storage in endothelial and Kupffer cells may be due to a surplus of iron in the body of the rat. In addition, the number of lipid droplets in fat-storing cells increases with age. This increase is correlated with an increase in vitamin A which is stored in the lipid droplets of fat-storing cells. On the basis of these morphological observations, it is concluded that great changes in the functional capacities of sinusoidal liver cells are not to be expected until late in age.

Specific liver cell functions can be investigated clearly in suspensions of isolated and purified cells. At our Institute, a cell isolation method has recently been developed in which the three types of sinusoidal liver cells can be obtained from one liver in high yield, purity and viability. This method has now been applied to 3- to 36-month-old rats. By using this technique it is also possible to obtain the three types of sinusoidal liver cells from rats of various ages in suspensions which are similar with respect to their cellular composition and purity. These methods make possible the investigation of the correlation between ultrastructural morphology and specific functional capacities of sinusoidal liver cells in

relation to age.

A clear relationship exists in rats between the age-related increase in liver weight and the increase in the number of sinusoidal liver cells which can be isolated from one liver. This correlation indicates that the density of these cells in the liver, e.g. the number of cells per unit of liver tissue, does not change with age. It was shown before that the density of the parenchymal cells in the liver decreases with age due to an age-related increase in the mean volume of these cells. Therefore, the ratio between parenchymal cells and sinusoidal liver cells changes with age. A certain cell ratio, e.g. the Ito cell index (the number of fat-storing cells per 1000 hepatocytes) is often used to determine changes in the number of fat-storing cells as a result of various treatments. Because the ratio between parenchymal cells and sinusoidal liver cells changes with age, the increase in the number of fat-storing cells expressed as the Ito cell index reported after, for example, long-term vitamin A or methotrexate treatment may therefore be overestimated.

A third possibility to study liver cell function besides the intact liver and the use of freshly isolated cells is the maintenance of cells in culture. The use of cell cultures as a model system for functional research has some advantages as compared with methods which utilize freshly isolated cells. For example, in culture cells may repair damage incurred during the cell isolation procedure to cell membrane components such as receptors and to the metabolic apparatus.

Sinusoidal liver cells can be maintained in culture both from mixed cell suspensions and from purified ones. Each cell type, however, has its own specific culture conditions and shows different characteristics in culture. Sinusoidal liver cells in mixed cultures do not differ much from cells in cultures derived from purified cell suspensions. The advantage of mixed cultures is the possibility to study in such culture systems liver functions which are achieved by a collaboration between different liver cell types.

Sinusoidal endothelial cells can be maintained in culture only for three to four days. Strict culture conditions such as a collagen substratum for cell attachment and spreading and the addition of homologous rat serum to the culture medium are thereby essential. The long cell processes of the endothelial cells which in the intact liver cover the sinusoids and which contract close to the cell body during the cell isolation procedure respread after three hours in culture. The receptor-mediated uptake of horseradish peroxidase is also reestablished in culture. These observations indicate that short-term cultures of sinusoidal endothelial cells can be used to study various endothelial cell functions. These may concern (i) the regulation of the selective filtration by hormones, (ii) the function of endothelial cells as a barrier between compounds circulating in the blood and the parenchymal cells located under the endothelium and (iii) the endocytic capacity of these cells.

Kupffer cells can be maintained in culture quite easily for several weeks. Mitoses are not observed in Kupffer cells in cultures derived from purified cell

suspensions. The ultrastructural morphology and some functional aspects of Kupffer cells in culture in the first 2 to 3 days are similar to those of cells in the intact liver. This similarity enables the investigation of specific Kupffer cell functions in cells in culture. These may be their endocytic capacity, the synthesis of effector molecules in response to various stimuli and their role in the immunological response.

Fat-storing cells in culture behave as do fibroblasts. The ultrastructural morphology of these cells has much in common with that of fibroblasts since the lipid droplets containing stored vitamin A, disappear from these cells in culture. In general, the composition of the cytoskeleton of cells indicates something about the origin of cells. The characterization of the cytoskeleton of fat-storing cells in culture using specific antibodies suggests that fat-storing cells belong to the family of fibroblasts and possibly are myofibroblasts. It appears that fat-storing cells in culture contain various components of the extracellular matrix, thereby suggesting a role for these cells in production or maintenance of the liver skeleton. The behavior of fat-storing cells in culture and that of cells in the intact liver after the induction of liver fibrosis is quite similar. Fibrosis of the liver may occur after liver damage and is characterized by the presence of increased amounts of extracellular matrix components in the liver. The disturbance in the balance between the synthesis and degradation of these components is a complex process most probably involving various liver cell types. A better understanding of the role played by fat-storing cells and other liver cell types in the pathogenesis of liver fibrosis using liver cells in culture may give possibilities to prevent the disturbance in the balance.

At our Institute the liver has been chosen as a model system in which to investigate the cellular basis of the aging process. Normally, investigation of ultrastructural morphology is considered to play an important role in such research because changes in the functional capacity of cells is often reflected by changes in their morphology. However, the results from the research described in this thesis show that use of the classical descriptive methods for cell morphology are alone not detailed enough to detect small changes in the morphology of the cells which occur with aging. It is possible, however, to study the cellular morphology in greater detail by use of additional methods such as morphometry, immunocytochemistry and autoradiography. Such anatomical research integrated with projects investigating biochemical aspects of metabolic processes occurring in liver may lead to a better understanding of factors (both environmental and intra- and extracellular) which both characterize and effect the aging process.

SAMENVATTING

De lever neemt in het lichaam een sleutelpositie in als het orgaan, waarin stoffen, die het lichaam binnenkomen via het spijsverteringssysteem eerst bewerkt worden voor hun verdere verspreiding door het lichaam. Na bewerking door de levercellen worden de reststoffen, of weer in het bloed uitgescheiden of in de gal, die in de lever geproduceerd wordt. De kleinste functionele eenheden van de lever, waar de uitwisseling van stoffen tussen het bloed en de levercellen plaatsvindt, zijn de leveracini. Deze zijn opgebouwd uit twee groepen cellen, de leverparenchymcellen of hepatocyten en de sinusoidale levercellen. De leverparenchymcellen vormen 70% van het totale leverbolume en 60% van het totale aantal cellen in de jong-volwassen rat. De sinusoidale levercellen dragen slechts 6% bij tot het totale leverbolume. Toch spelen zij een wezenlijke rol bij tal van processen, die zich in de lever afspelen, hetgeen ook tot uitdrukking komt in hun bijdrage tot het totale aantal levercellen (30%). Het resterende deel van het totale leverbolume wordt grotendeels ingenomen door de ruimtes, waarin het bloed en de gal vervoerd worden en het resterende deel van het totale aantal cellen wordt gevormd door cellen, die de grotere bloedvaten en galgangen omgeven. Deze laatste bevinden zich grotendeels buiten de eigenlijke leveracini.

De sinusoidale levercellen bestaan uit drie verschillende celpopulaties: de sinusoidale endotheelcellen, de Kupffercellen en de "fat-storing" cellen. Ieder van deze celtypen heeft zijn eigen specifieke functies; andere leverfuncties komen tot stand door een nauwe samenwerking tussen de sinusoidale cellen onderling en met de leverparenchymcellen. De specifieke functionele capaciteit van families van cellen, en dus ook van de verschillende leverceltypen, wordt weerspiegeld in de voor ieder celtype karakteristieke ultrastructurele morfologie. De leverceltypen zijn op grond van deze morfologie en van hun ligging in de leveracini duidelijk van elkaar te onderscheiden.

De leverparenchymcellen spelen een grote rol bij o.a. de opname, de verwerking, de opslag en de uitscheiding naar het bloed en de gal m.b.t. voedselcomponenten, die opgenomen worden in de darm en daarnaast bij de biotransformatie van exogene en endogene stoffen, zoals respectievelijk geneesmiddelen en hormonen.

De sinusoidale endotheelcellen bekleden de bloedruimten, de sinusoiden, van de lever. Zij vormen een selectieve barrière tussen het bloed in de sinusoiden en de leverparenchymcellen, die zich onder het endotheel bevinden. De sinusoidale endotheelcellen zijn ook betrokken bij de opname en de verwerking van macromoleculen, zoals glycoproteïnen en lipoproteïnen.

De Kupffercellen of levermacrofagen bevinden zich in de sinusoiden en spelen een belangrijke rol bij de bescherming van het organisme tegen in het bloed circulerende toxische stoffen en deeltjes, waaronder bacteriële componenten. De Kupffercellen synthetiseren en secreteren verschillende stoffen, die als regulatoren

optreden in een groot aantal fysiologische processen. Verder zijn deze cellen ook betrokken bij de immunologische afweer.

De "fat-storing" cellen liggen tussen de parenchymcellen onder het endotheel. Zij spelen een grote rol bij de opslag van vitamine A en van andere in vet oplosbare vitaminen. Bovendien kunnen deze cellen waarschijnlijk beschouwd worden als de eigenlijke leverfibroblasten, die mede verantwoordelijk zijn voor de vorming van extracellulaire bindweefselcomponenten in de lever. Deze componenten vervullen een belangrijke functie bij de instandhouding van de structuur van dit orgaan.

Bij het ouder worden van het organisme neemt een aantal leverfuncties af. De cellulaire basis van deze veranderingen is niet altijd even duidelijk. Specifiek cellulair onderzoek, gerelateerd aan de leeftijd, is vooral gericht geweest op functies van de leverparenchymcellen. Onderzoek naar het functioneren van de sinusoidale levercellen heeft slechts sporadisch plaats gevonden. Nu er methoden ontwikkeld zijn, om levercellen te isoleren en vervolgens te zuiveren in verschillende klassen van cellen is de mogelijkheid om dit soort onderzoek te doen aanzienlijk verruimd. Specifieke leverfuncties kunnen dan in suspensies en kweken van gezuiverde leverceltypen bestudeerd worden, zodanig dat invloeden van buiten de lever geen rol spelen.

De studie, beschreven in dit proefschrift, heeft als onderwerp de ultrastructurele morfologie van de sinusoidale levercellen van de vrouwelijke BN/BiRij rat, in leeftijd variërend van jong-volwassen (3 maanden) tot oud (36 maanden). Het doel van deze studie is om de morfologie van de cellen te onderzoeken, zowel in de intacte lever als na isolatie en in kweek, teneinde een mogelijk cellulair substraat te vinden voor leeftijdsgerelateerde functionele veranderingen in de lever. De gegevens, die verkregen zijn omtrent de cellulaire samenstelling van de sinusoidale populatie en de aantallen cellen geïsoleerd uit de totale lever, beide in relatie tot de leeftijd, zijn gebruikt om aan de leeftijd gerelateerde veranderingen in de ratio tussen parenchymcellen en de sinusoidale levercellen te bepalen.

In de intacte lever is de ultrastructurele morfologie van de sinusoidale levercellen in ratten in de leeftijd van 3 tot 36 maanden grotendeels gelijk. Duidelijke veranderingen worden echter waargenomen in zowel de endotheel- als de Kupffercellen in die celorganellen, die betrokken zijn bij de afbraak van in de cellen opgenomen stoffen. Met het toenemen van de leeftijd wordt in deze celorganellen, de lysosomen, maar ook vrij in het celsap meer ijzer opgeslagen. Het is waarschijnlijk dat deze toename van de opslag van ijzer in endotheel- en Kupffercellen, het gevolg is van een ijzeroverschot in het lichaam van de rat. Voorts neemt met het vorderen van de leeftijd het aantal vetbollen in de "fat-storing" cellen toe, hetgeen parallel verloopt met een toename van vitamine A, dat in de vetbollen van deze cellen opgeslagen ligt. Op grond van deze waarnemingen wordt geconcludeerd dat grote veranderingen in de functionele capaciteit van de sinusoidale levercellen niet te verwachten zijn, tot op hoge leeftijd van het organisme.

Specifieke levercelfuncties zijn goed te bestuderen in suspensies van geïso-

leerde en gezuiverde cellen. In ons Instituut is recent een celisolatiemethode ontwikkeld, waarbij uit één lever de drie typen sinusoidale cellen verkregen kunnen worden in hoge opbrengst, grote zuiverheid van de celsuspensies en goede vitaliteit van de cellen. De isolatiemethode is nu toegepast op ratten van verschillende leeftijdsgroepen tussen de 3 en 36 maanden oud. Het bleek mogelijk om, uit ratten van verschillende leeftijden, de drie sinusoidale celtypen te isoleren in suspensies, die onderling vergelijkbaar zijn voor wat betreft de cellulaire samenstelling en zuiverheid. Dit maakt het mogelijk om gerelateerd aan de leeftijd, de specifieke functionele capaciteit van deze cellen te bestuderen.

Er bestaat een duidelijke correlatie tussen de toename van het levergewicht bij de rat en de toename van het aantal sinusoidale cellen, dat uit één lever geïsoleerd kan worden. Dit wijst erop, dat de dichtheid, d.w.z. het aantal cellen per volume-eenheid van de lever, van de sinusoidale cellen niet verandert bij het ouder worden. Aangezien als gevolg van een aan de leeftijd gerelateerde toename van het gemiddelde volume van de hepatocyten, de dichtheid van deze cellen in de lever wel afneemt, verandert de ratio tussen de parenchymcellen en de sinusoidale levercellen. Dit gegeven is van belang, gezien het feit dat een bepaalde celratio, namelijk de Itoel-index (het aantal "fat-storing" cellen per 1000 hepatocyten) veel gebruikt wordt als maat voor veranderingen in het aantal "fat-storing" cellen als gevolg van bepaalde behandelingen. De toename in het aantal "fat-storing" cellen, uitgedrukt als Itoel-index, die bijvoorbeeld na langdurige behandeling met vitamine A of methotrexaat gevonden wordt, wordt dan overschat.

Een derde mogelijkheid om levercelfuncties te bestuderen, naast de intacte lever en het gebruik van vers geïsoleerde cellen, is het kweken van cellen. Het gebruik van celkweken als modelsysteem voor functioneel onderzoek biedt bepaalde voordelen ten opzichte van andere preparaten, zoals vers geïsoleerde cellen. In kweek kan er bijvoorbeeld herstel optreden van schade, die de cellen oplopen tijdens de celisolatie-procedure, ondermeer aan bestanddelen van de celmembraan zoals receptoren en het metabole apparaat van de cellen.

De sinusoidale levercellen kunnen, zowel uitgaande van gemengde als van gezuiverde celsuspensies, in kweek gehouden worden. Ieder celtype vraagt echter specifieke kweekcondities en vertoont andere karakteristieken in kweek. Sinusoidale levercellen in gemengde kweken verschillen niet wezenlijk van cellen in kweken, die uitgaan van gezuiverde celsuspensies. Het voordeel van een gemengde kweek is de mogelijkheid om leverfuncties, die tot stand komen door de samenwerking tussen verschillende celtypen, in een dergelijk systeem te bestuderen.

De sinusoidale endotheelcellen kunnen slechts gedurende 3 tot 4 dagen in kweek gehouden worden. Stringente kweekcondities, waaronder een collageensubstraat voor celhechting en spreiding en een aanvulling van het kweekmedium met homoloog ratteserum, blijken daarvoor noodzakelijk. De lange celuitlopers van de endotheelcellen, die in de intacte lever de bekleding van de sinusoiden vormen en die zich tijdens de celisolatie dicht bij het cellichaam opvouwen, strekken zich

reeds na drie uur in kweek weer uit. Ook de opname van het mierikswortelperoxidase, die afhankelijk is van de aanwezigheid van receptoren op de celmembranen, herstelt zich in kweek. Voorgaande bevindingen geven aan, dat primaire kweken van sinusoidale endotheelcellen geschikt zijn om een aantal endotheelcelfuncties te bestuderen. Deze functies betreffen (i) de hormonale regulatie van de selectieve filtratie, (ii) de functie van de endotheelcellen als barrière tussen in het bloed circulerende stoffen en de onder het endotheel gelegen parenchymcellen en (iii) de endocytose capaciteit van deze cellen.

Kupffer cellen zijn relatief gemakkelijk in kweek te houden gedurende een periode van enkele weken. Celdelingen treden niet op in kweken van gezuiverde Kupffercelsuspensies. De ultrastructurele morfologie en enige functionele aspecten van deze cellen vertonen gedurende de eerste dagen in kweek veel overeenkomsten met die van Kupffercellen in de intacte lever. Deze overeenkomst biedt een goede basis om een aantal specifieke Kupffercelfuncties in kweek te bestuderen. Hierbij valt te denken aan hun endocytosecapaciteit, aan de synthese van effectormoleculen in reactie op bepaalde prikkels en aan de rol van Kupffercellen in de immunologische afweer.

"Fat-storing" cellen in kweek gedragen zich als fibroblasten. Ook ultrastructureel lijken de cellen veel op fibroblasten, te meer daar de vetbollen met het daarin opgeslagen vitamine A, verloren gaan tijdens de kweek. De samenstelling van het cellulaire cytoskelet zegt in het algemeen iets over de oorsprong van cellen. De karakterisering van het cytoskelet van "fat-storing" cellen in kweek, met behulp van antilichamen, geeft aan dat "fat-storing" cellen tot de familie van de fibroblasten behoren en mogelijk gladde spiercelachtige cellen zijn. Het blijkt, dat "fat-storing" cellen in kweek verschillende bindweefselcomponenten bevatten. Dit wijst er op, dat zij betrokken zijn bij de vorming van het leverskelet. Het gedrag van "fat-storing" cellen in kweek en van deze cellen in de intacte lever, bij de inductie van leverfibrose, vertoont veel overeenkomsten. Leverfibrose kan ontstaan na leverbeschadiging en wordt gekenmerkt door een verhoogde hoeveelheid bindweefselcomponenten in de lever. Deze verstoring van de balans in de aanmaak en afbraak van deze componenten is een ingewikkeld proces, waarbij waarschijnlijk verschillende leverceltypen betrokken zijn. Een beter begrip van de rol die "fat-storing" cellen, maar ook andere leverceltypen spelen in het ontstaan van leverfibrose, door onderzoek aan cellen in kweek, zal kunnen leiden tot mogelijkheden om de verstoring van de balans te voorkomen.

In ons Instituut is de lever als modelsysteem gekozen om onderzoek te verrichten naar de cellulaire basis van het verouderingsproces. Het elektronenmicroscopisch onderzoek speelt hierbij een rol, aangezien veranderingen in de functionele capaciteit van cellen zich zullen weerspiegelen in de ultrastructurele morfologie. Het is gebleken dat de hoofdzakelijk beschrijvende methode, die is toegepast bij het in dit proefschrift beschreven onderzoek, mogelijk te weinig nauwkeurig is om kleine, structurele veranderingen in de cellulaire morfologie te detecteren. Het is

echter mogelijk om met behulp van additionele methoden, zoals morfometrie, immunocytochemie en autoradiografie, de morfologie gedetailleerder te bestuderen. Dergelijk anatomisch onderzoek zal, in een geïntegreerde opzet samen met een biochemische benadering van de processen, die zich in de lever afspelen, kunnen leiden tot een beter inzicht in de factoren (zowel omgevingsinvloeden als intra- en extracellulaire processen), die het verouderingsproces karakteriseren en beïnvloeden.

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